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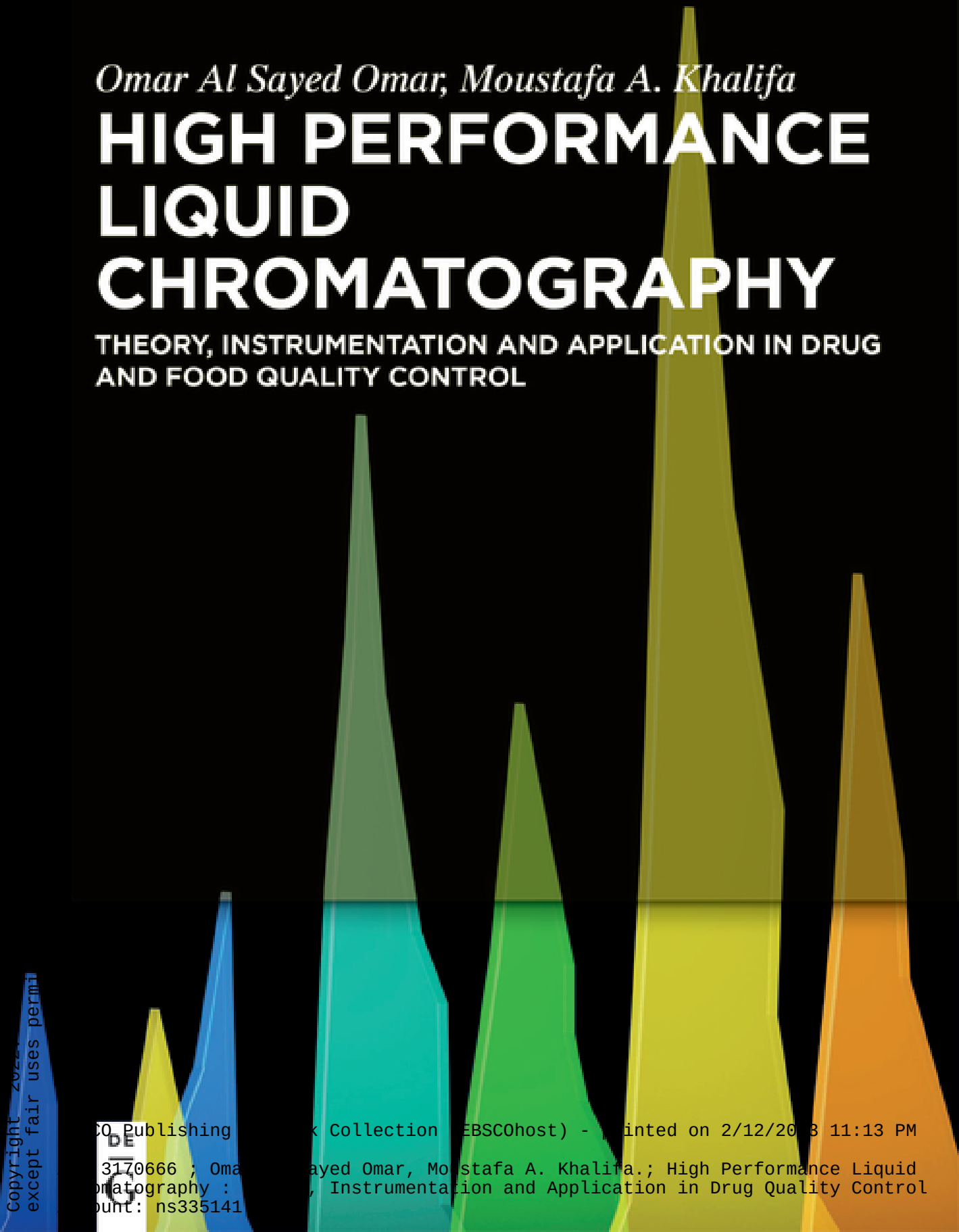

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**High Performance Liquid Chromatography**

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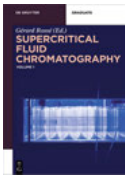


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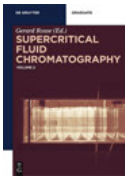


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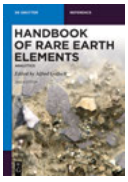


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Omar Al Sayed Omar, Moustafa A. Khalifa

# High Performance Liquid Chromatography

Theory, Instrumentation and Application in Drug Quality Control

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**Authors**
Dr. Omar Al Sayed Omar
Ministry of Health
Drug and Food Quality
Control Laboratories
PO Box 4575
13046 Safat
Kuwait
yorkd2@yahoo.com

Prof. Dr. Moustafa A. Khalifa
Ministry of Health
Drug and Food Quality
Control Laboratories
PO Box 4575
13046 Safat
Kuwait
drma.moustfa@gmail.com

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# Preface

HPLC is the premier analytical technique in pharmaceutical analysis, which is predominantly used in the Pharmaceutical Industry and Drug Quality Control Laboratories for a large variety of samples. It is the method of choice for checking the purity of new drug candidates, monitoring changes or scale up of synthetic procedures, evaluating new formulations, and scrutinizing quality control/assurance of final drug products (DPs). To support each new drug application or commercial product, tens of thousands of HPLC tests are conducted by a host of dedicated scientists to assure the potency and quality of the new drug product.

Our goal was to provide the pharmaceutical analysts who constitute a significant fraction of all HPLC users in Drug Quality Control Laboratories with an updated view (new trends) of the concepts and practices application of modern HPLC, illustrated with many figures and case studies. Note that this basic book for practitioners was written at both an introductory and intermediate level.

The main focus of this book is on small drug molecules and pharmaceutical dosage forms. This book provides practical guidelines using case studies on sample preparation, column and instrument selection. It captures the essence of major pharmaceutical applications in drug quality control laboratories (assays, content uniformity and dissolution testing). In addition the book highlights novel approaches in HPLC and the latest developments in hyphenated techniques, such as LC-MS, and data handling.

Each of the 11 chapters (see table of contents), provides the reader with an in-depth understanding of HPLC theory, hardware, methodologies, regulations, applications, and new developments.

This book can be broadly classified into 5 major sections:

1. Overview, theory, instrumentation, and columns including ion chromatography and size exclusion (Chapters 1–5). The HPLC-column is the heart of the HPLC instrument and essential to its success. The book provides an extensive collection for technical information about HPLC-columns (physico-chemical properties and chromatographic characteristics) from various manufacturers, and help the analyst for decide on the ideal approach for their analysis according to the requirements of drug manufacturers specifications or/and the desired Pharmacopeia. In addition the authors give practical advices on how to prepare mobile phases, choose a suitable detector and set up an HPLC analysis.

2. HPLC methods applications and practices, including sample preparation and assays for Active Pharmaceutical Ingredients (API) in drug substances (DS) and drug products (DP), content uniformity and dissolution testing for DPs pharmaceutical dosage forms (Chapters 6–8). Also Chapter 8 highlights the regulatory aspects of ICH and USP guidelines for selection which analytical method can be use by the analyst in Drug Quality Control Laboratories, instrumental calibration, HPLC system qualifications,

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analytical method validation and checking for suitability of HPLC system before its using in analysis.

3. HPLC–Mass Spectrometry (HPLC–MS) and its applications in Drug Quality Control Laboratories (Chapter 9). In this Chapter and based on HPLC–MS we have presented result from study the adulteration of some natural herbal products with undeclared some synthetic PDE-5 inhibitors (Sildenafil, Tadalafil and Varddenafil).

4. Safety in HPLC laboratories (Chapter 10). This Chapter addresses the OSHA and EPA concerns about safety laboratory protocol when using HPLC equipment, solvents, samples and waste disposal in HPLC laboratories. Also we presents in this Chapter preventive measures and procedures which are related to safety in HPLC laboratories.

5. Glossary (Chapter 11)
Furthermore the book will serve as a definitive reference source for laboratory analysts, researchers, managers, and executives in industry, academe, and government agencies, who are engaged in various phases of using HPLC as analytical tool.

The book was written to be self sufficient interims of the needs of the average professional or technicians who plans to work with modern HPLC. We believe this book will prove its useful in most Drug Quality Control Laboratories where modern HPLC is practiced.

Following a hands-on approach, the book gives insight into the key pharmaceutical applications of HPLC and the latest requirements of the major regulatory agencies such as ICH, FDA, USP.

It is with a great deal of pleasure that I would like thank those who have contributed in so many ways to be completion of this book. First and foremost the authors wishes thank De Gruyter for giving chance and publishing this book. Most important, we would like to acknowledge the professionalism of my editor Stella Mueller, De Gruyter, Germany, whose enthusiasm and support made this a happy project, for her encouragement and keeping track of all the paper work. We also owe much to the reviewer (reading for proof), who has given me many insights and valuable advice. Special thanks go to Ulla Schmidt, Data Group / De Gruyter, Germany, for well organization of my book draft during the production and for her fast response for my questions.

The authors also are indebted to thank Professor Dr Moustafa Abbassy, Damanhur University, Professor Dr Ahmed Massoud, Kafer Elsheikh University, Egypt for given me many insights and valuable advice during the preparation of the book and Eng. Rana Marwan, Al Sharhan Industries, Kuwait for her technical support during the preparation of the manuscript.

Numerous manufacturers of analytical instrumentation and other products and services related to HPLC have contributed to the writing of this book by providing diagrams, applications notes and photos of their products. We are especially grateful

to Waters Co. USA; S.C.A.T. Europe, Germany; Shimadzu, Japan; The Royal Society of Chemistry, UK; De Gruyter publisher, Germany and Mourn Training Service, Onoa Mcpolin, UK.

Finally, I am professor Dr Moustafa A. Khalifa (2nd author) and I would like to thank my wife Howida, my sons Dr Mohammed and Dr Amr, my grandsons Hesham, Moustafa and Hady and my granddaughter Howida for their continue to put up with the inconveniences brought about by rushed deadline for the book project science 2 years ago. To them, I pledge more quality time to come after 2021 for playing and enjoying.

The Authors
10-12-2021
Kuwait

# Contents

# 1 High-performance liquid chromatography (HPLC): theoretical principles

## 1.1 Introduction

High-performance liquid chromatography (HPLC, formerly referred to high-pressure liquid chromatography) is, for many scientists, an essential piece of apparatus for the separation, identification, purification and quantification of various compounds, especially very polar (organic) in their complex mixtures. Users of HPLC work in a variety of fields, including analysis of pharmaceuticals, foods, waters, cosmetics, biochemicals and environmental samples [1–6]. For HPLC analysis, mixtures from interested compounds are first dissolved in a liquid solvent and then forced to flow with mobile phase through a stationary phase in the form of column (usually stainless steel column) under high pressure. On this stationary phase (column), the mixture of chemical compounds is resolved into its components. The amount of resolution is important and is dependent upon the extent of interaction between the solute components and the stationary phase. The stationary phase is defined as the immobile packing material of the column. The moving part of the system is the mobile phase, which is a liquid. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of solvents and columns. As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems. Thus, HPLC has the ability to easily separate a wide variety of chemical mixtures, especially which contains thermally labile or easily oxidized compounds.

In the opposite, gas chromatography (GC) is limited in its applications, and the analysis of compounds which are thermally labile or easily oxidized is not possible. So, HPLC can fill this gap and has thus become an essential addition to GC in every analytical laboratory. Advances in HPLC technology have been assisted by the continual development of new stationary phases, improvements in instrumentation and the facility the method offers for the application of computer technology and developments in automation techniques.

It is, therefore, not surprising that the number of publications which are now dealing with HPLC exceeded than GC [7].

**In this chapter we will discuss the following topics:**

- **What is HPLC in relation to other chromatographic techniques?**
- **Components of an HPLC system**
- **History of HPLC**
- **Types of separation in HPLC**
- **Mechanism of separation in HPLC**

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- **Where HPLC can be used as an analytical tool?**
- **Advantages of modern HPLC**

In this part, we will discuss the previously mentioned topics in detail.

## 1.2 What is HPLC in relation to other chromatographic techniques?

In chromatographic technique, the components of a chemical mixture are separated based upon the rates at which they are carried through a stationary phase (solid or liquid on solid support) by gaseous or liquid phases (mobile phase). Chromatographic method is categorized into three types based on the nature of the mobile phase [7]. The three types of phases include liquid, gases, and supercritical fluids as shown in Fig.1.1. Liquid chromatography (LC) can be performed in columns and on planar surfaces, but GC and supercritical fluid chromatography are restricted to column procedures. Column chromatography is often described as elution chromatography in which solutes are washed through stationary phase by the movement of the mobile phase (eluent).

The chromatographic separation of a mixture of various compounds depends primarily on the fact that each of them is physicochemically different from two nonmiscible phases (the stationary phase and the mobile phase). Thus, thermodynamic *effects such as partition and absorption are mostly involved. Depending on the* physical state of the mobile phase, a distinction is made between GC and LC (as shown in Fig.1.1). A further distinction depends on the form of stationary phase, layer or column, which leads to other classifications, thin-layer chromatography and column chromatography. The stationary phase may be solid, porous chemically modified or liquid film on solid support.

## 1.3 Components of an HPLC system

Schematic representation of the essential components of an HPLC system is found in Fig.1.2. We shall discuss briefly these following components.

### 1.3.1 Mobile phase pumping system (mobile phase supply unit)

It consists of high-pressure pump to force the mobile phase through the HPLC system and provides flow rates from 100 μL/min for small bore columns and 1–3 mL/min for other columns. Suitable pressure gauge (manometer) over pressure protection and flow meter is placed in pump to measure the system pressure and flow rate of the

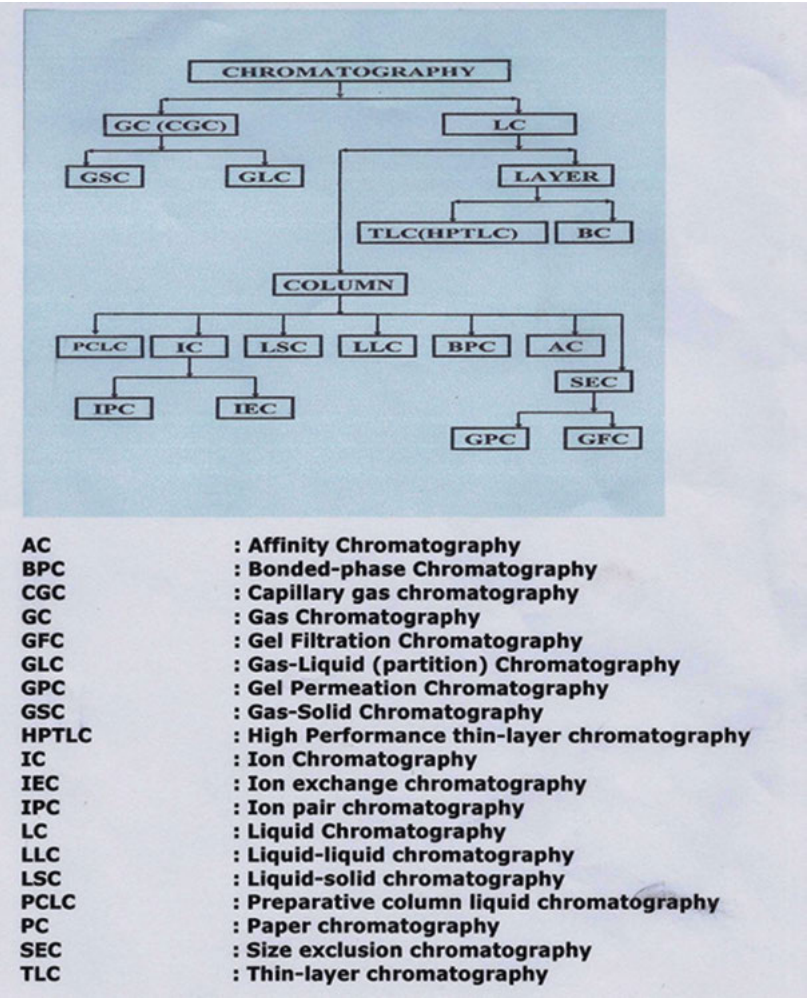


**Fig.1.1:** Categories of chromatography and their relationship to each other.

mobile phase. Mobile phase reservoir presents a part of the system and it holds the mobile phase that will be pumped into the HPLC system through mobile phase inlet line (Teflon tubing) which is supported by metal frit (mobile phase inlet filter) for in-line filtration of mobile phase. Two basic elution modes for the mobile phase are used in HPLC analysis for pharmaceutical products in drug quality control laboratories. The first is called isocratic elution. In this mode, the mobile phase, either a pure solvent or a mixture, *remains the same during the separation and sample run.* The second type is called gradient elution, wherein, as its name implies, the mobile phase composition changes during the separation and sample run (for details, see Chapters 3 and 6).

### 1.3.2 Injection system (injector)

Sampling valves and loops injectors to inject the sample into the mobile phase just at the head of separation column (see Chapter 4 for details).

### 1.3.3 Separating system (HPLC column)

HPLC column (Fig.1.3) consists of two components: the column packing materials (stationary phase) and the hardware (container) in which the stationary phase is found (as shown in Fig.1.4). On separation column, the sample component can be separated to their individuals. Since the stationary phase inside the column is composed of micro-size particle that is irregular or spherical (as shown in Fig.1.5), a high-pressure pump is required to move the mobile phase through the column. In fact the heart of the HPLC is considered to be the column (for details, see Chapters 2 and 8).

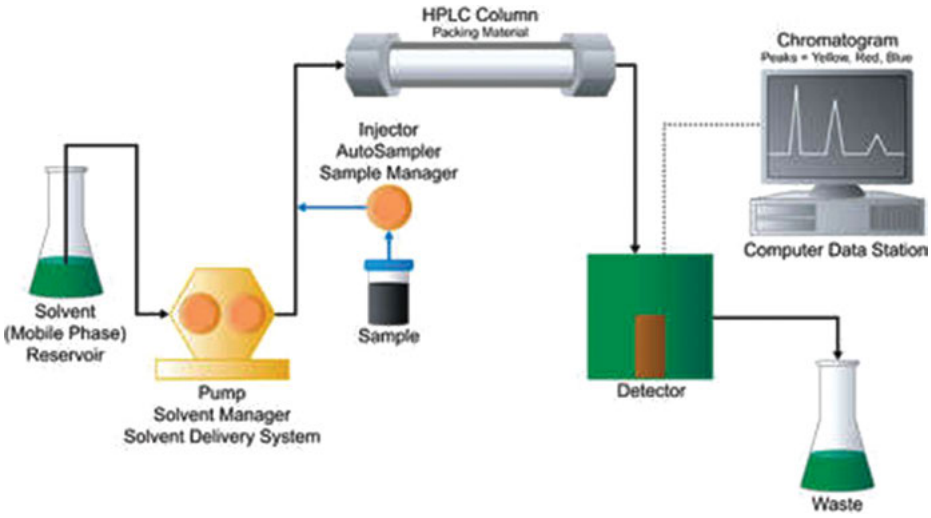


**Fig.1.2:** Schematic diagram of an HPLC system (permission granted to reproduce by Waters Corporation, www.waters.com).


**Fig.1.3:** Hardware of an HPLC column.

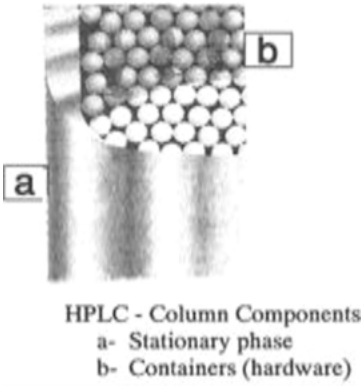


**Fig.1.4:** Stationary phase (a) inside the hardware and (b) of an HPLC column.

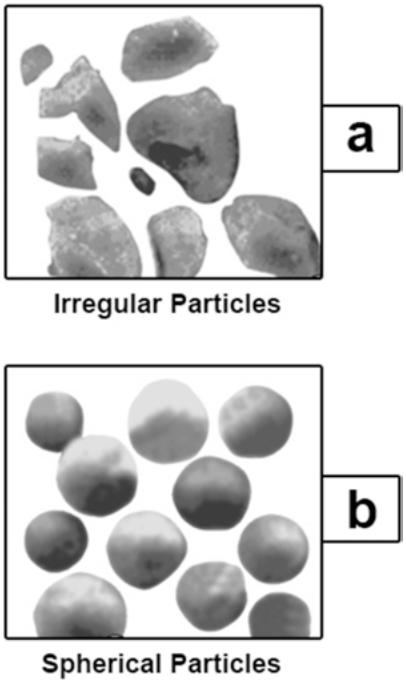


**Fig.1.5:** Stationary phase particles: irregular (a) or spherical (b).

### 1.3.4 Detection system (detector)

For detection, the presence of solutes which have been separated by column and eluted out in mobile phase (for details, see Chapter 5).

### 1.3.5 Data acquisition and analysis (computer)

To collect, store and analyze the chromatographic data resulting from HPLC, nowadays computers and other data processing equipments are being used more frequently.

### 1.3.6 Fraction Collector

HPLC can also be used to purify and collect desired amounts of each compound separated using a fraction collector downstream of the detector flow cell. This process is called preparative chromatography (see Fig.1.11).

### 1.3.7 Connection tubing

In HPLC, there is connection tubing for joining the parts of the instrument together (for details, see Chapter 6).

HPLC systems can be either modular or integrated as shown in Fig.1.6 and 1.7, respectively. In practice, both types are popular in drug quality control laboratories [3].

## 1.4 History of HPLC

The term *chromatography* means "color writing" (Chroma is the Greek word meaning color combined with graphy means writing). This was first discovered by Mikhail Tswett (1903) [8, 9], a Russian botanist who separated plant pigments, particularly the carotenoids and the chlorophylls on chalk ($CaCO_3$) packed in glass columns and eluted by organic solvent, petroleum ether. The result of this process was that the plant pigments were separated into a series of discrete colored bands on the column, divided by regions entirely free of pigments as they passed through the column (stationary phase).

Since the 1930s, chemists used gravity-fed silica columns (Fig.1.8) to purify organic materials and ion-exchange resin columns to separate ionic compounds and radionuclides [8, 9]. Lacking both speed and resolution, the technique of chromatography languished many years until the field was revolutionized by the Nobel Prize winning work of Martyn and Synge in 1941 [8, 9]. This work was important because it established a firm theoretical basis for the separation mechanism.

The first generation of HPLC was developed by researchers in the 1960s, including Horvath, Kirkland and Huber [8, 9]. With continued advances in performance during this time (smaller particles for stationary phase), the term of HPLC remained the same but the name was changed to HPLC. Commercial development of in-line

**Fig.1.6:** Examples of modular HPLC systems (Agilent 1100 Series System) (permission granted to reproduce by Agilent Technologies, www.agilent.com).


**Fig.1.7:** Examples of integrated HPLC (Waters Alliance System) (permission granted to reproduce by Waters Corporation, www.waters.com).

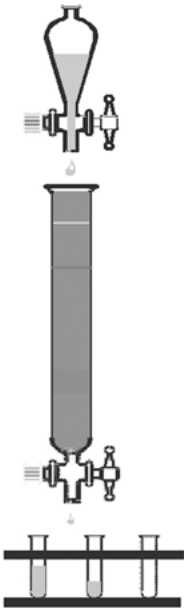

**Fig.1.8:** Illustrative diagram showing the traditional technique of low-pressure liquid chromatography using a glass column and gravity-fed solvent with manual fraction collection.

detectors and reliable injectors allowed HPLC to become a sensitive and quantitative technique leading to an explosive growth of applications.

The science of HPLC was revolutionized a few years ago [10–12] with the advent of ultra-HPLC (UHPLC) (such as Waters Co. Acquity UHPLC Systems [12]), which made it possible for researchers to analyze samples with greater speed, resolution and sensitivity. Today, HPLC continues to evolve rapidly toward higher speed, efficiency and sensitivity, driven by the emerging needs of life sciences and pharmaceutical applications.

The annual worldwide sales of HPLC systems and accessories approached US $3 billion in 2002 [13].

## 1.5 Types of separation in HPLC

According to many scientists [3, 4] and on the bases of the chromatographic modes of separation, HPLC stationary phase for HPLC columns can be categorized into four major types: normal phase (NP), reversed phase (RP), ion-exchange chromatography and size-exclusion chromatography (SEC). In addition, there are other specialized modes too (e.g., affinity, chiral or specified applications) [3, 4]. NP-HPLC means that the polarity of the stationary phase is higher than that of the mobile phase, for example, when using silica in adsorption chromatography. RP means that the polarity of the stationary phase is less than that of the mobile phase, for example, when using hydrocarbon-type bonded phases and polar mobile phase. With both modes, solutes

are eluted in order of polarity, *with normal phase least polar elute first and with reverse phase most polar elute first*. Details will be given in Chapter 2.

These previously mentioned modes of HPLC present most important ones in the field of drug quality control. In this book, we shall discuss all of these modes and their applications for pharmaceutical products analysis in drug quality control laboratories.

## 1.6 Mechanism of separation in HPLC

In HPLC analysis, the chromatographic process begins by injecting the sample to be analyzed (a mixture of some components A, B and C as shown in Fig.1.9) on top of the column. Components in the mixture distribute between two immiscible phases: one phase is mobile phase and the other is the stationary phase. The rate of migration of each species is determined by its distribution coefficient: species which are distributed mainly into the mobile phase move rapidly (component A in Fig.1.9(3)), and species which are distributed mainly in the stationary phase move slowly (component C in Fig.1.9(3)). Solutes that move in stationary phase will form bands (Fig.1.9(3)). Solute bands grow border as they pass through the column. As the bands of solutes emerge from the column it can be detected using selective or universal detectors depending upon the property of the component being measured. The response of the detector to the presence of each component is displayed on chart recorder or computer.

The HPLC detector's output signal should normally produce bell-shaped Gaussian peak (Fig.1.9(4)) representing concentration profile for the eluting components. Concentration profile is called a peak, and a series of peaks from the sample mixture is called chromatogram (Fig.1.9(4)). The time required to elute a peak of the chromatogram is called retention time and it is given the symbol $t_R$. The amount of time required for a solute that does not interact with the stationary phase is known as the *void time*, $t_0$. No compound can be eluted in less than the void time. Retention time is dependent on flow rate (F) and retention volume ($V_r$) of the mobile phase. Retention volume ($V_r$) is the volume of mobile phase that was passed through the column at $t_R$ and it is simply product of the retention time and flow rate F according to the following equation: $V_r = F \times t_R$. So ($t_R \times F$) is a constant, thus changing flow rate will change $t_R$. Retention of solutes in HPLC system can be controlled by varying the composition of the mobile phase (mobile phase is usually a mixture of strong and weak solvents). Solvents that give low retention are called strong solvents.

Tentative identification using HPLC for a compound can be made by comparing its retention time to the retention time of known compound suspected to be present in sample under analysis ($t_R$A, $t_R$B and $t_R$C of components A, B and C on chromatogram of sample (Fig.1.9(4)) can be compared with $t_R$A, $t_R$B and $t_R$C on chromatogram of known compounds A, B and C produced at the same HPLC conditions has been used

for the analysis of sample). The most frequent application of HPLC is quantitative analysis. The area (or height) under a chromatographic peak is proportional to the amount of material present. Peak area is usually measured with an electronic integrator or computer data system. Comparing peak area in the sample and standard, the quantity of unknown compound can be determined (for details, see Chapter 8).

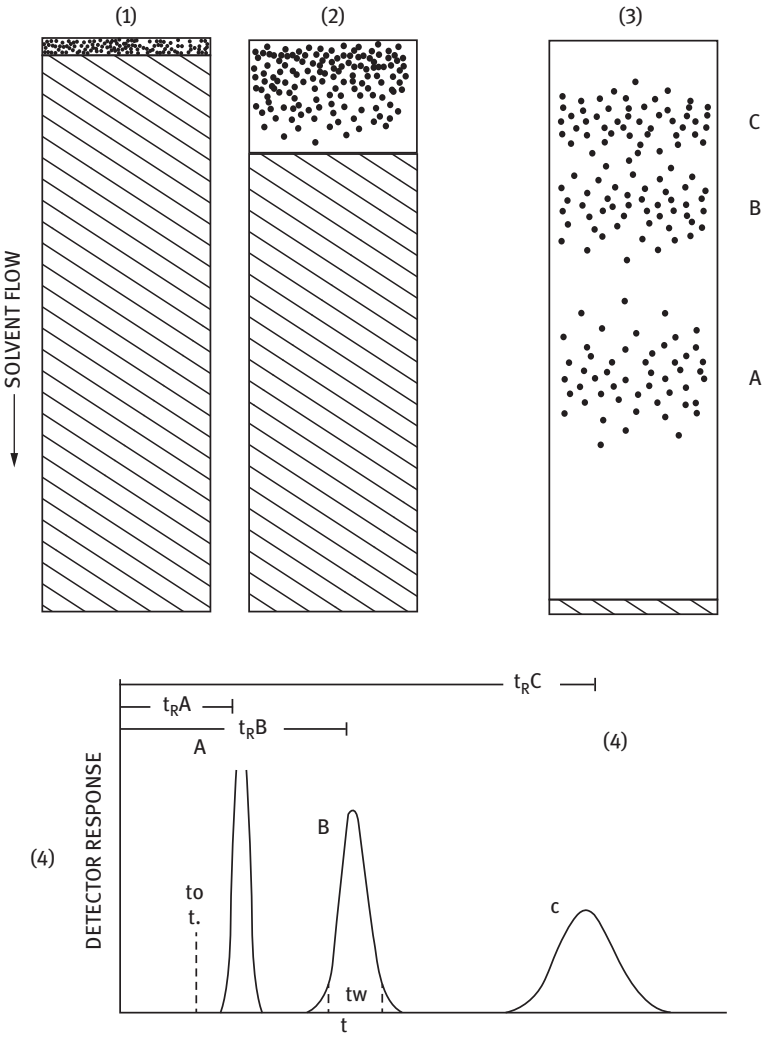


**Fig.1.9:** Separation of hypothetical chemical components (A + B + C) in a sample by HPLC: (1–3) separation inside the column, and (4) the resulting chromatogram contains three peaks: peak A, peak B and peak C.

The objective of using HPLC in analysis is the separation of a mixture of components, so resolution is a term used quantitatively to describe how well the objective was met. Resolution (Rs) is defined as the differences in retention time for the two adjacent peaks on the HPLC chromatogram divided by the average width of their peaks (Fig.1.10) as follows:

$$Rs = \frac{t_R B - t_R A}{1/2\,(w_B + w_A)}$$

$w_B$ is the width of peak B and $t_R B$ is the $t_R$ for peak B;

$w_A$ is the width of peak A and $t_R A$ is the $t_R$ for peak A

(as shown in Fig.1.10).

The amount of resolution (high, moderate and low as shown in Fig.1.10) is important and is dependent upon the extent of interaction between the solute component and the stationary phase and mobile phase. Thus, HPLC has the ability to separate a wide variety of chemical mixtures. The primary decision of separation is made with the selection of the stationary phase, and the separation procedure is finally tuned through mobile phase selection and manipulation. Frequently, these require changing the mobile phase composition during chromatography. When this process involves more than one solvent, it is called gradient programming and requires some specialized equipment called *HPLC gradient* (for details, see Chapters 3 and 6).

If there is no change in the composition of the mobile phase during the HPLC analysis, it is *called isocratic elution* for mobile phase, isocratic – HPLC. No matter what the mode is, a careful selection of operating parameters based upon the knowledge of the solute chemistry will usually allow separation of very similar molecular species.

## 1.7 Where HPLC can be used as an analytical tool?

The use of various chromatographic separation techniques such as GC, LC and SEC **is primarily limited by the molecular weight of the sample as shown in** Tab.1.1 **[7].** In comparison, HPLC is more suitable than GC for the analysis of thermally unstable, easily oxidizable and nonvolatile compounds [14–20].

In addition, advances in instrumentation design and performance and the use of smaller, more porous particles as stationary have resulted in an improvement in the theoretical plate height of the LC column.

In general, HPLC can be used whenever the sample is a liquid or can be dissolved in a liquid. Given a particular problem, the extreme versality of the HPLC technique often makes it the separation technique of first choice. Some of the more common uses are listed in Tab.1.2 [20–30]. One of the most attractive uses of HPLC

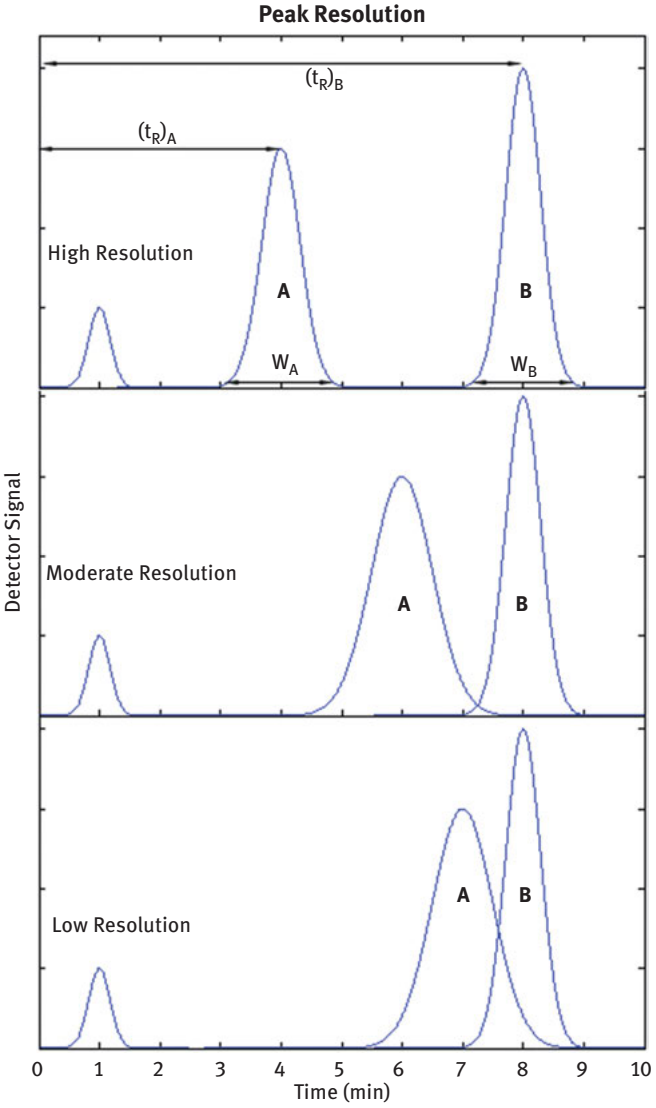


**Fig.1.10:** HPLC chromatogram showing two peaks and $t_R$ for each peak and the amount of resolution (high, moderate and low).

is in the analysis of trace components [21–22], especially related substances and degradation products in pharmaceutical products [23–30]. There are a variety of sophisticated approaches, ranging from preconcentration to selective detection, that aid in trace analysis. Two applications of HPLC, which have benefitted significantly from advances in this area, are clinical chemistry and trace pollution analysis (pesticide

**Tab.1.1:** Applications of chromatographic separation techniques according to the molecular weight of the solute.

| Chromatographic method | Molecular weight range (Da) | | |
|---|---|---|---|
| GC | 2 | to | 400 |
| HPLC | 100 | to | 2,000 |
| SEC | 500 | to | 5,000,000 |

residue analysis in food and water). The clinical measurement of both endogenous and exogenous compounds in body fluids is becoming increasingly practical using HPLC. The inherent sensitivity of the HPLC technique allows detection of extremely low levels of carcinogenic pollutants.

**Tab.1.2:** Common HPLC uses.

| Life science | Industrial | Pharmaceutical |
|---|---|---|
| Drug monitoring | Polymers | Drug analysis |
| Toxicology | Oil products | Formulation testing |
| Amino acids | Pesticides | Trace analysis for impurities and degradation products |
| Proteins | Antioxidants | Quality control |
| Lipids | Surfactants | Raw materials |
| Carbohydrates | Food | Drug screening in human fluids |

It was in the year 1980 HPLC methods appeared for the first time for the assay of bulk drug material [31]. HPLC has become the principal method in USP XXVII [32] and, to a lesser extent, one of the most widely used methods is also in Ph. Eur. [33].

Today, a very specialized HPLC field has developed a well-characterized approach and highly sophisticated equipment. According to most chromatographic theories, the optimum limits of separation potential are now being approached. These limits are quite impressive, and because of this, HPLC enjoys an unprecedented position of deserved popularity.

HPLC can also be used to purify and collect desired amounts of each compound separated using a fraction collector downstream of the detector flow cell. This process is called preparative chromatography (see Fig.1.11).

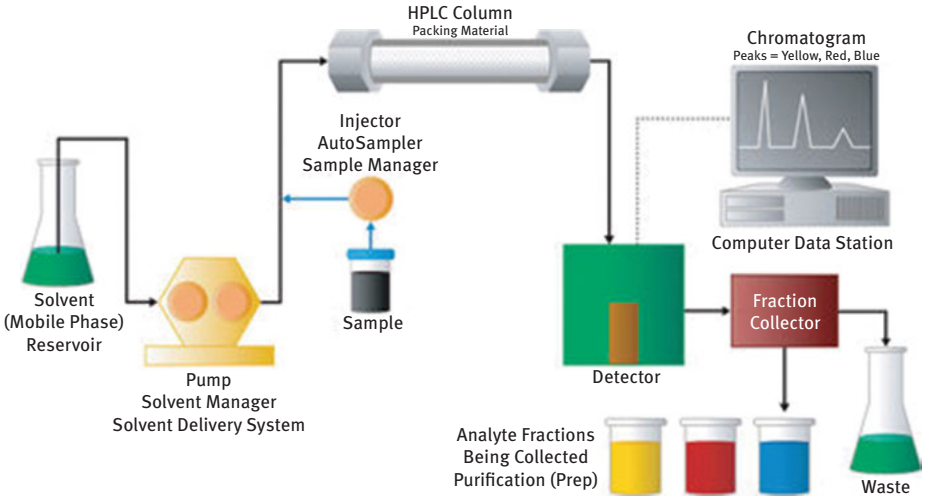


**Fig.1.11:** HPLC system for purification: preparative chromatography (permission granted to reproduce by Waters Corporation, www.waters.com).

## 1.8 Advantages of modern HPLC

The major advantages of HPLC over the other chromatographic techniques are speed, resolution and high sensitivity Rapid resolution of even difficult problems is often routine. In part, this is due to stationary phase improvements which have increased column efficiency so much that shorter columns are becoming increasingly practical. Much work is being done in this area, and the final goal is to increase efficiency so that even shorter columns can be used in HPLC analysis.

Resolution is maintained with these shorter columns by better exploiting the inherent selectivity of different interaction modes. In addition to the increased speed and resolution, standard detectors are now capable of detecting very minute component concentrations. This makes HPLC one of the best techniques for trace chemical analysis, especially analysis for degradation and related substances of pharmaceutical products in drug quality control laboratories.

GC is limited in its applications, and the analysis of compounds that are thermally labile or easily oxidized is not possible. HPLC fills this gap and has thus become an essential addition to GC in drug quality control laboratories.

In addition to the abovementioned advantages, HPLC offers further attractions. From an economic viewpoint, the stability of the equipment and the columns is important. These are now so well designed that they maintain a trouble-free existence for extended periods of time. This is in contrast to other chromatographic techniques,

where column degradation often occurs. Also important is growing automation trend in HPLC, which frees the user from some of the more mundane aspects of chromatography. In addition, automation streamlines HPLC methods development, thereby yielding better results in less time.

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# 2 HPLC columns for pharmaceutical analysis

## 2.1 Normal- and reversed-phase columns

### 2.1.1 Introduction

HPLC column *is called the separating system of HPLC*. It consists of two components (Fig.2.1): the column packing materials (stationary phase) and the hardware (container) in which the stationary phase is found (as shown in Fig.2.1). On separation column, the sample component can be separated to their individuals. Because of the fact that the inside phase of the column is made up of spherical or irregular micro-sized particles (Fig.2.1b1 and Fig.2.1b2), it is important that a high-pressure pump is used. This will move the mobile phase by pressure inside the column. In fact it has been mentioned by many scientists [1–11] that the heart of the HPLC is considered to be the column. In this chapter, there are details about these two components of *HPLC* columns: the column packing materials (stationary phase) and the hardware (container). In addition, this chapter discusses HPLC column specifications and selection for pharmaceutical analysis in drug quality control (QC) laboratories. In addition, column operation, handling, care, maintenance and trouble shooting are taken into consideration.

### 2.1.2 Packing materials or stationary phase for HPLC columns

Packing materials or the stationary phase of the HPLC columns are based either on the organic polymer or on inorganic ceramic substances [1–3]. Out of the inorganic ceramics used, alumina and silica are the most common ones. The inorganic packing is high in rigidity and cannot swell in any sort of solvent. Methacrylates and cross-linked styrene divinylbenzene make the basis for polymeric HPLC-grade packing. The best thing about it is that polymeric packing is not rigid like the inorganic ones. It is compressible. Analytes and solvents can easily enter the polymer matrix. This can cause the particles to swell. As a result, this will reduce the transfer of mass and decrease the efficiency of the column.

Based on the chromatographic modes of separation and according to many scientists [1–11], the stationary phase of HPLC columns is classified into four classes: NP-HPLC (normal phase), IEC (ion-exchange chromatography)-HPLC (ion-exchange phase), RP (reversed phase)-HPLC and SEC (size-exclusion chromatography)-HPLC (size-exclusion phase). In addition, other specialized modes like chiral and affinity exist, and other applications specified [1]. The NP in HPLC means that the stationary phase has a higher polarity when compared to the mobile phase, for instance, when using silica in adsorption chromatography (Fig.2.2). In the RP, the stationary

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phase has a lower polarity when compared to the mobile phase, for example, when using hydrocarbon-type bonded phases and polar mobile phase (Fig.2.3). In both the modes, the order of polarity is used to elute the solutes. In NP, the least polar elute comes first and in the RP the most polar elute comes first. A comparison is shown in Table 2.1, and diagramed in Fig.2.2–2.4 for the general characteristics of NP and RP mode chromatography.

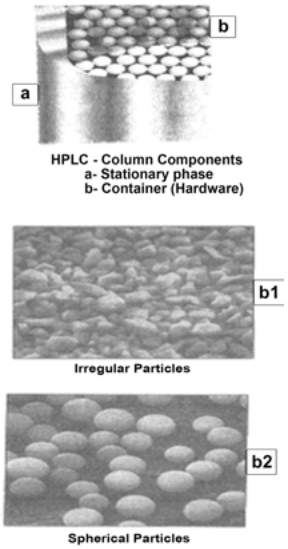



**Fig.2.1:** HPLC column components: (a) stationary phase and (b) container (hardware).

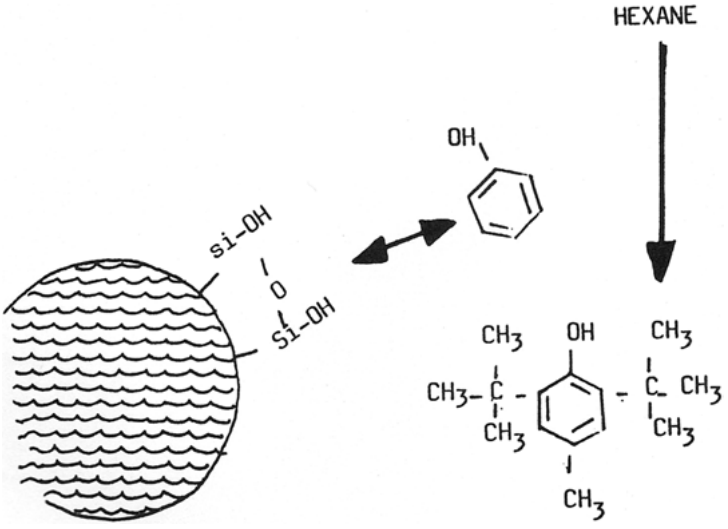



**Fig.2.2:** Mechanism of interaction between the solute and the stationary phase or normal-phase HPLC (NP-HPLC).

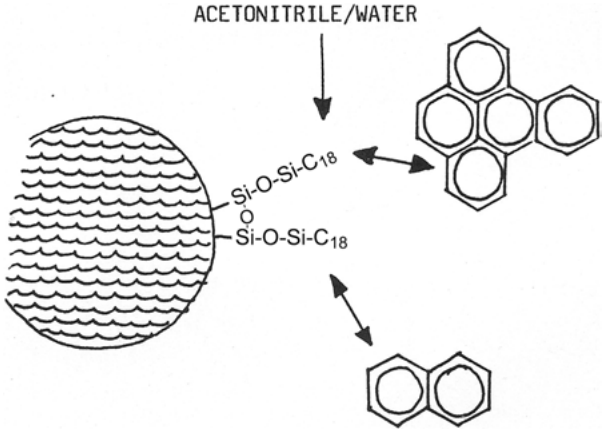



**Fig.2.3:** Mechanism of contact between both the stationary phase and the solute of the reversed-phase HPLC (RP-HPLC).

**Tab.2.1:** General properties of normal- and reversed-phase stationary phases for HPLC columns.

| Properties | Normal phase | Reversed phase |
|---|---|---|
| 1. Packing polarity | High to medium | Low to medium |
| 2. Solvent polarity | Low polarity to medium | Medium polarity to high |
| 3. Order of sample elution | Least polar first | Most polar first |
| 4. Effect of increasing solvent polarity on elution time | Elution time is reduced | Elution time is increased |

Silica-based RP-HPLC columns have gained wide popularity use in drug QC laboratories because of the following advantages:

- More reproducible and quicker as compared to different HPLC modes.
- Generally easier to perform experiments.
- Broader application scope allowing samples that have an extensive polarity range to be divided and separated.
- Makes use of the mobile phase which is inexpensive.
- The application includes separating ionizable or ionic organic compounds. This is done by using ion pairing technique ion pairing mode = IPC) (see Chapter 3).
- Used in 70–80% of all HPLC applications.

In this chapter, we focus on RP-HPLC columns. The reason for this is that more than 70–80% of the applications of HPLC use RP-HPLC. In addition, NP-HPLC packing for HPLC columns is considered. Followed by this, we shall give enough information about packing materials for HPLC columns (NP- and RP-HPLC columns).

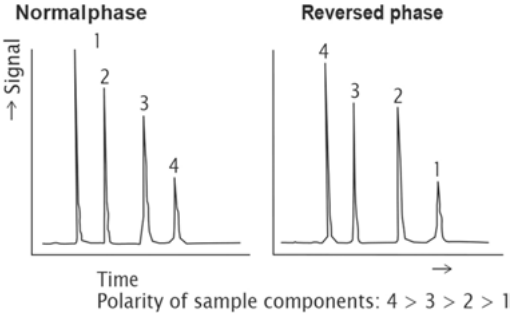



**Fig.2.4:** Elution of analytes with varying polarity from reversed- and normal-phase HPLC column matrices.

### 2.1.3 Silica as a packing material for NP-HPLC columns

Silica is the most popular material for HPLC stationary phase (packing materials for HPLC column, and it is called NP-HPLC.

NP chromatography (NPC) started with silica packing, and the name reflects that this was the first type. Silica has a high affinity for water and since chromatographic characteristics change after hydration, the reproducibility of analysis is not as easy as with other packings. NP presents a polar surface, and solvents used are nonpolar and nonaqueous; to increase the elution strength, the polarity of the eluent has to be increased.

Silica packing for HPLC columns is small porous silica particles with spherical or irregular shape (Fig.2.5) having nominal diameter of 3, 5 or 10 μm. They are manufactured so as to have a narrow particle size and pore size distribution. Silica packing is also available as porous-layer beads (Fig.2.6). They consist of an inert spherical core of glass, 30–40 μm in diameter, with a thin outer coating of silica. Silica is very soluble in solutions with a pH value over 7.5 and under 2.6.

During continuous use of silica, small quantities of the packing are washed out of the column, leading to the formation of voids over a period of time. Silica-packed columns quickly change their chromatographic behavior in storage and must be thoroughly conditioned by the mobile phase before being used in analysis.

In addition, alumina can be used as a matrix in NPC [1–3].

Also silica is the most popular base material for other HPLC stationary phases such as silica-based RP (bonded silica, RP), symmetry bonded phase (SRP), symmetry shield bonded phase (symmetry shield reversed phase, SSRP) and XTerra

packing (see Section 2.1.5). Also silica is the most popular base materials for IEC and SEC packing materials for HPLC columns (see Section 2.2).

For the previously mentioned disadvantages of using silica as packing materials for HPLC columns, it is advisable to transfer separations with this case to modified silica (RP silica, Sections 2.1.5 and 2.1.6).

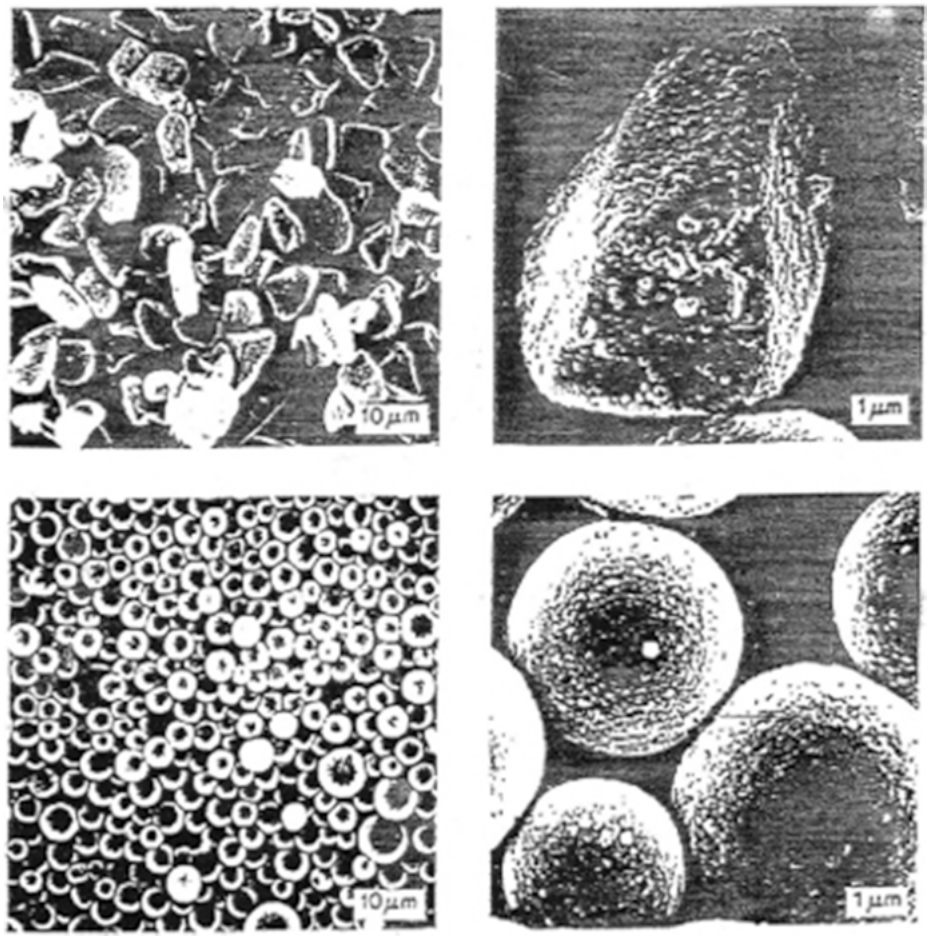



**Fig.2.5:** Scanning electron micrograph showing irregular (mean particle diameter 5 µm) (above) and spherical (mean particle diameter 5 µm) (below) silica particles.

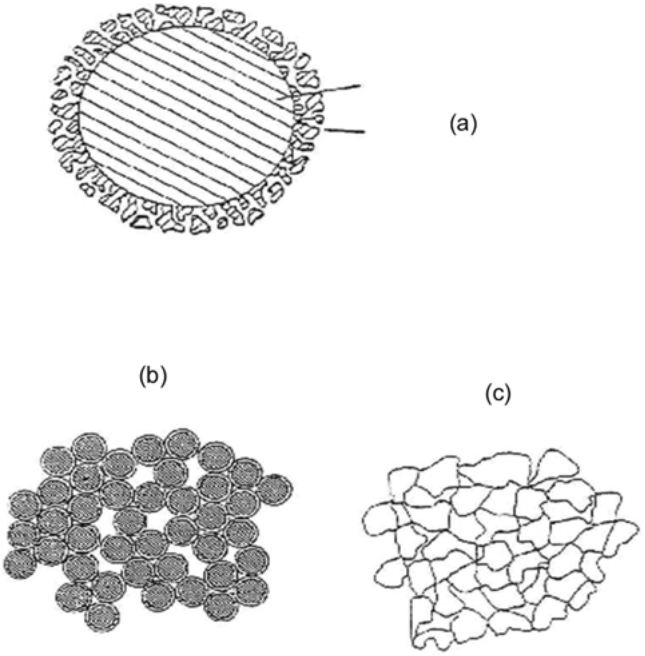



**Fig.2.6:** Silica particles in different forms.
(a) Porous layer, (b) Spherical, (c) Irregular

### 2.1.4 Silica as a base material for other HPLC packing materials

#### 2.1.4.1 Silica gel structure

The structure of silica gel used for chromatography is in amorphous form (i.e., say, it has no regularity) and is also the *silicon dioxide* (SiO2) porous form. It is made up of unbalanced tridimensional framework of changing both the *oxygen* and silicon atoms (Fig.2.7) with the voids and pores that are *nanometer* sized. There is no hard-and-fast rule for filling the voids; they can either contain a gas, water or liquid. One predominant feature of the surface of the silica is silanol (SiOH) groups (Fig.2.8). The surface of silica is usually strongly hydrated, and the extent of hydration depends upon the thermal history of the silica. In general, there is reversible hydrolysis–dehydration process at the surface siloxane bonds, where, according to the prevailing conditions, the siloxane links can open to form silanol groups or two adjacent silanol groups can condense to form a siloxane link. Further, the degree to which the adsorbed water molecules are bound can vary and the adsorption can be very strong, and is so much difficult to distinguish between the adsorbed water and water derived from silanol condensation. It is, therefore, difficult to be precise about

the structure of silica, since it can be prescribed as a living polymer rather than as a material of fixed structure. A representation of the structure is shown in Fig.2.7. The main features of such surfaces are the silanol groups and the iloxane backbone, which, being mildly hydrophobic, has little to do with the separations.

There are three important types of silanol groups, which have been identified on the surface of silica particles: free silanol, the hydrogen bonded silanol and the gem – silanol (Fig.2.8). The hydrogen bonded silanols make up the most part of the surface, and silanols with the gem and free hydroxyls comprise around 30% of the total. The silanol groups (bound and free) contribute to about 8 μmol/$m^2$ of total concentration. Out of all of these, the free group of silanol makes up for the reaction sites and premier adsorption sites. In case of the process of adsorption, bound silanol groups have a secondary role to play.

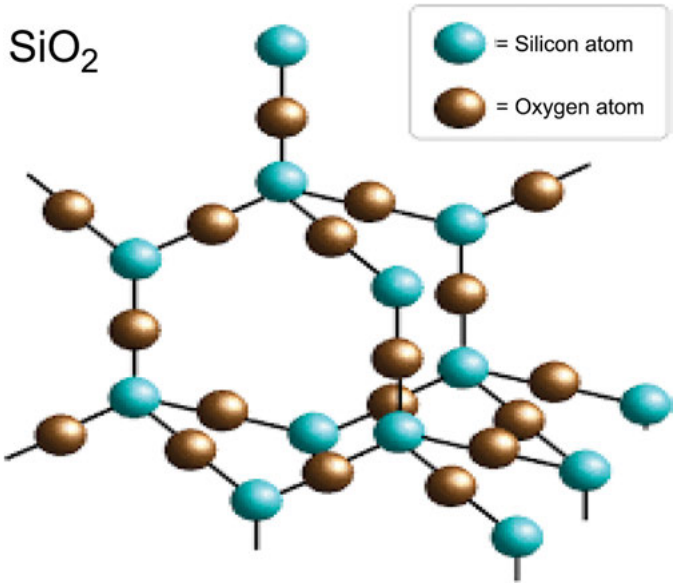
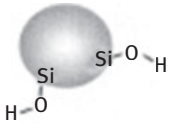
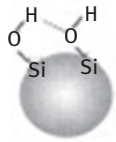



**Fig.2.7:** Network of silicon–oxygen–silicon linkages in silica structure.

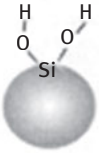
If silica is heated, a number of changes take place. Between ambient temperature and around 120 °C, there is a reversible loss of surface adsorbed water. Workers studying the silica surface often use silica which has been heated under vacuum at 120 °C as a standard, dry but fully hydroxylated surface. As the temperature is increased, the loss in the weight is due to the condensation of hydrogen bonded silanols to form siloxane bonds. This loss in hydroxyls is reversible at temperature up to around 500 °C. As temperature increased from 500 to 850 °C, the changes begin



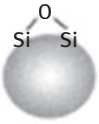

**Isolated Silanol**



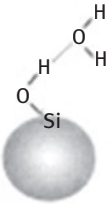

**Vicinal Silanol - Hydrogen Bonded**




**Geminal Silanol**




**Siloxane**




**Hydrogen Bonded Water**

**Fig.2.8:** Silanol species on silica particles.

become irreversible, with the less accessible silanols condensing. At this particular point, the structure of the silica starts to change. These changes include the decrease in surface area and an increase in pore diameter. Above 850 °C, the silica structure begins to sinter and eventually, depending upon the level of impurities, it begins to melt. Pure silica can withstand temperatures above 1,000 °C but relatively small amounts of impurities lead to fusing at much lower temperature.

If the silica is to form reproducible packing, it is essential that the surface is as fully hydroxylated as possible. This is because in use there will be a slow rehydroxylation of the surface as it equilibrates with the mobile phase to the point of maximum hydroxylation. This process is catalyzed by acid and accelerated by heating, so silica is usually acid washed at high temperatures to ensure full hydroxylation. If there are significant numbers of metal ions present in the structure or if the packing has been heated to excessive temperatures, the silica surface will not be fully hydroxylated by such procedures. Use of hydrogen fluoride or ammonium bifluoride to clean the silica surface has been suggested.

The silanol groups are the main sites of adsorption in NPC (silica is used as stationary phase) and are also the points of bonding for most bonded phase packing. Besides the adsorptive properties of the surface, silanol groups are weakly acidic, and silica can be used as a weak, low capacity ion exchanger under certain circumstances. Silica is stable to acid pH, remaining essentially, but not completely insoluble at pH values below 7.5. Above pH 8.0, silica dissolves more rapidly and should not be used other than in very exceptional circumstances.

#### 2.1.4.2 Important properties of silica

Important properties of silica to be considered are size, shape, pore width, surface area, surface reaction and purity:

- *Size*: μm is the measurement that shows the particle size. This is the approximate diameter of the particular silica particles under consideration. The relative standard deviation expresses the particle size distribution that is of importance to the efficiency of packing and to the pack pressure during pushing the mobile phase through it.
- *Shape*: Shape is either spherical or irregular. Spherical silica gel is more expensive than the irregularly shaped type. Although no significant differences in performance have been reported, irregular silica gel will require higher pressure for the mobile phase flow.
- Pore width: This should be in excess of 5 nm except for the separation of macromolecules that have wide pore size of 30 nm. In silica gel, pore widths have about 10 μm particle size and can measure anywhere near 400 nm; hence, this has been used in SEC.
- *Specific surface area*: Both the specific surface area and pore width are inversely proportional to each other. The greater the specific area, the better the separation will be.
- *Surface reaction*: Silica gel may react as an acid, base or neutral substance depending on the manufacturing conditions.
- *Purity of silica*: It has been noted for many years that some compounds, most notably basic drugs, do not give chromatographic peaks of good shape on all silica. This behavior is now generally believed because different metal

impurities are present and are fixed to the silica structure. Silica specially treated with basic compounds or media prepared from highly pure starting materials are available, which result in packings with good properties. The most usual impurities in silica result from its preparation, and their origins lie in either sand or sodium hydroxide used for the process. These impurities are usually aluminum, iron, nickel, calcium and sodium, although others have been found to be present in some silica. Heat treatment of the silica during its production binds the metal into the silica surface, thus affecting the acidity of the adjacent silanol groups. These more acidic groups, although present in small numbers, have sufficiently poor properties for separation of basic compounds that the properties of a silica can be very seriously affected by their presence. The effects of impurities can be avoided by the use of silica gel derived from the hydrolysis of tetraethylchlorosilicate (Fig.2.9), which can be distilled clear of any impurities prior to use or from the highly pure fume silica. Silica particles have strength and excellent mass transfer properties.

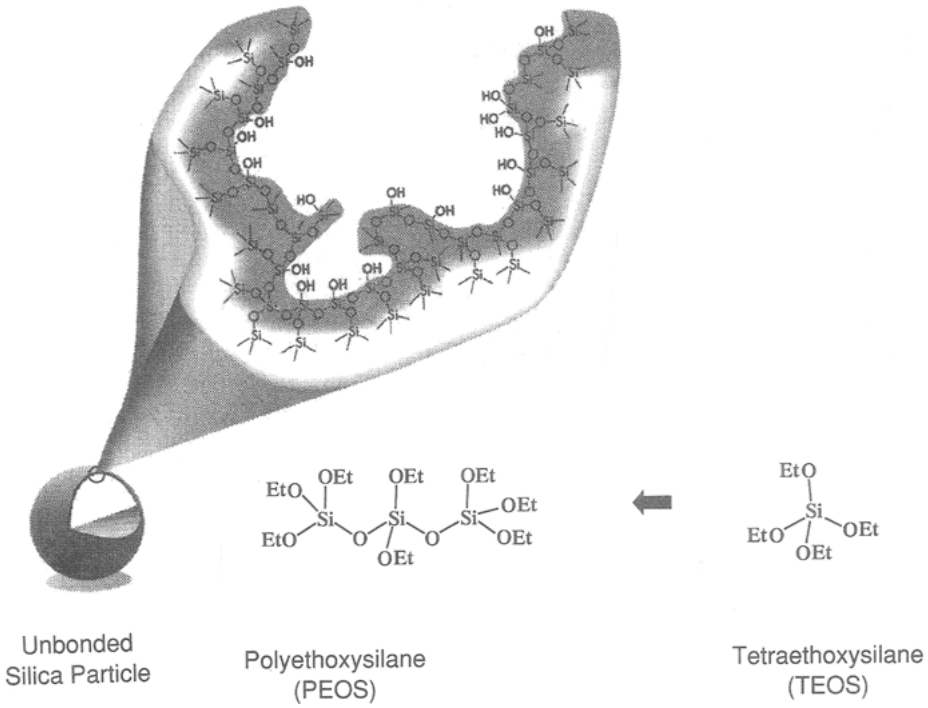



**Fig.2.9:** Diagram illustrating the traditional manufacturing process for pure silica. Reproduced with permission from Waters Co. [14].

With silica particles, a surface is provided to the ligands. This allows a variety of ligands to attach with it through the use of a well-stabilized silanization reaction (Fig.2.10). So silica-based RP can be prepared by modification of silica particles as in the following part of this text.

### 2.1.5 Silica-based RP-HPLC (bonded silica) as a packing material for HPLC columns

In bonded silica packing (RP packing, RP-HPLC) for HPLC columns, sorption properties of silica have been modified. The term "RPC" or "reversed-phase chromatography" indicated a phase where the stationary phase has a lower polarity as compared to the mobile phase. In contrast to NPC, the opposite is the case. RP-HPLC packing is produced by reacting the silica particles (it has polar surface) (Fig.2.10) with organochlorosilane which is in the general state $Si(CH_3)_2RCl$, where R represents hydrophobic functional groups having different polarities like alkyl chains with 1, 2, 3, 6, 8 or 18 carbon atoms, and phenyl-, cyano-, diol-, nitro-, amino- and aminopropyl groups onto the silica surfaces (Tab.2.2). The stationary phase has a number of properties that depend on the organosilane's alkyl group. The stationary phase will end up being polar if R belongs to the functional group that is polar. Some examples of the polar stationary phase are those in which R is made up of $-C_3H_6OCH_2CHOHCH_2OH$ (diol), $-C_2H_4CN$ (a cyano) and $-C_3H_6NH_2$ (amino) functional groups. Most commonly, the nonpolar stationary phases make use of an organochlorosilane. In this, R group is either $C_{18}$ (*n*-octyldecyl) or $C_8$ (*n*-octyl) hydrocarbon chain.

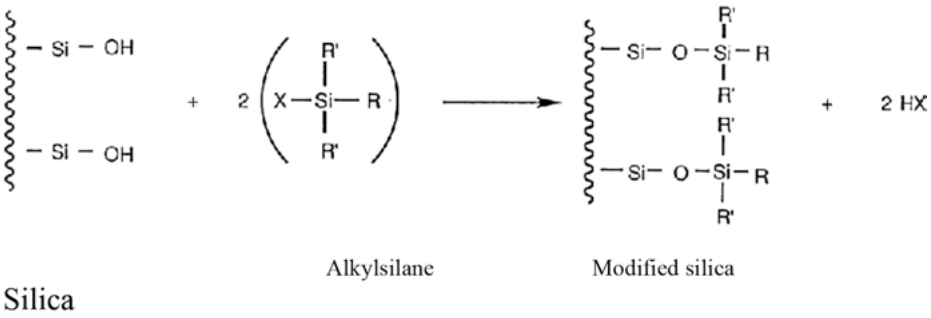



**Fig.2.10:** Generalized bonding reaction for derivatization of silica surface by alkylsilanes.
X = leaving group. R and R are any desired functionalities; R' is typically methyl, and R is C8, C18, etc. as shown in Tab.2.2.

**Tab.2.2:** Organic functional groups can be used for the synthesis of modified silica (silica-based reversed phase, RP-HPLC) for HPLC columns.

| Group | Formula | Group | Formula |
|---|---|---|---|
| Octadecyl | $-(CH_2)_{17}CH_3$ | Amino | $-NH_2$ |
| Octyl | $-(CH_2)_7CH_3$ | Aminopropyl | $-CH_2CH_2CH_2NH_2$ |
| Hexyl | $-(CH_2)_5CH_3$ | Alkylamino | $-(CH_2)_{11}NH_2$ |
| Dimethyl | $CH_3$<br>Si<br>$CH_3$ | Nitro<br><br>Nitrile | $-NO_2$<br><br>$-C\underline{=}N$ |
| Trimethyl | $-Si(CH_3)_3$ | Alkylnitrile | $-(CH_2)_{11}C\underline{=}N$ |
| Cyclohexyl | $-C_6H_{11}$ | Propionitrile | $-CH_2CH_2C\underline{=}N$ |
| Phenyl | $-C_6H_5$ | Oxypropionitrile | $-OCH_2CH_2C\underline{=}N$<br>$-CH-CH_2$ |
| Diphenyl | $-C_6H_5)_2$ | *vic*.-Hydroxyl (diol) | |

Since 1970 till the present time, 18-bonded silica is the most popular and common technique that is used for packing the HPLC columns. After this, the second popular one is the C8-bonded silica which is followed by phenyl-bonded silica. Depending on the conditions of both the mobile phase and the analyte, silica columns that are cyano-bonded can also be used when the RP is conducted. Solvents and mixtures that are polar organic or water can be used as an option for mobile phase during the RP-HPLC packing.

HPLC column manufacturers employ various techniques for producing RP materials. The silanol groups that are present on the surface of the silica gel are able to be chemically modified to nonpolar (e.g., C-18, phenyl, C8), polar ($-NH_2$, -CN) or ionizable (sulfonic acid, $SO_3$, quaternary ammonium, N+ $(CH_3)_3$) according to the silanization reaction (Fig.2.10). On the basis of this reaction, the available functional groups (R) are shown in Tab.2.2. Fig.2.11–2.13 show the resultant RP. After this reaction, the surface of silica particles has became hydrophobic and the resulting materials are called silica-based RP packing or bonded phase packing. The chemical structures of the most popular HPLC silica-based RP are shown in Fig.2.11–2.13 and Tab.2.2 and are classified by many researchers [12, 13] as old packing materials for HPLC columns as shown in Fig.2.14.

It is well known that during the salinization reaction, less than 40% of the silanol groups of silica particles can be reacted with the silane reagents. To ensure that there is no interaction without our consent among the solutes and whatever is remaining of the -SiOH groups, we add $Si(CH_3)_3Cl$ to it. This helps in the conversion of the unreacted sites to something else, that is, $SiOSi(CH_3)_3$. These types of columns are the end-capped ones. **End-capping** is based on the use of smaller or

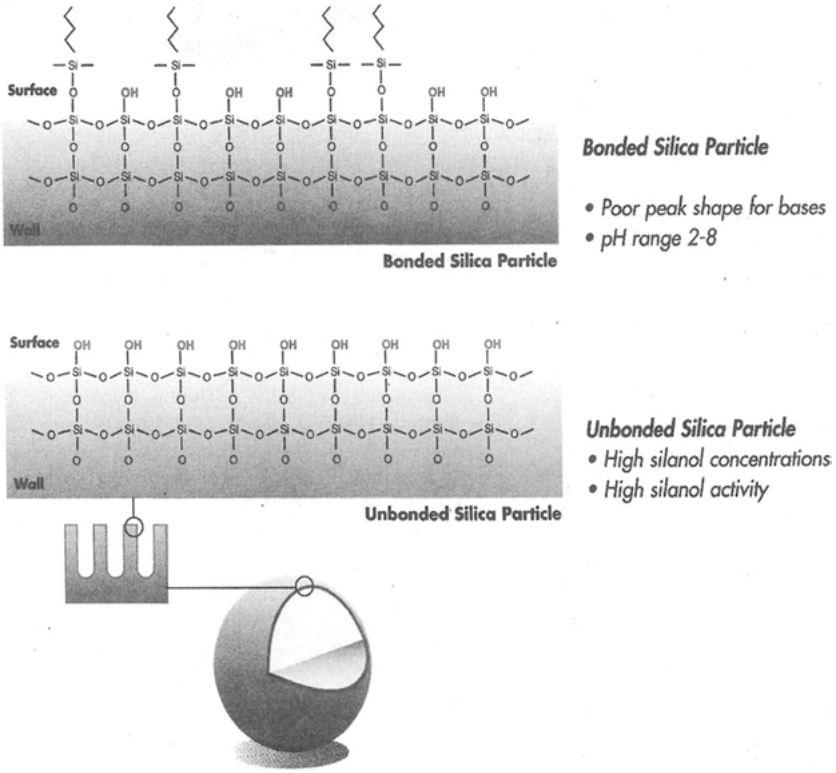



**Fig.2.11:** Diagram illustrating the chemical structure for the surface of unbounded and bonded (nonmodified and modified) silica particles after silanization). Reproduced with permission from Waters Co. [14].

less satirically hindered silane reagent such as trimethylsilane (Fig.2.15). The residual silanol population through the use of end-capping results in performance improvements of the RP packing. However, the population of residual silanols still approaches 60%. At present, the majority of work and studies done in the analytical HPLC is carried out either with bonded silica or silica that has been chemically modified, and till date the most important one is the nonpolar C-18 type. Even for the C-18 stationary phase or packing, there is a large selection available from HPLC column manufacturers such as Waters, Agilent, Alltech Dionex, Hamilton, Shimadzu, Macherey-Nagel, Merck, Phenomenex, Restek, Sigma-Aldrich, Thermo, Varian, Tosoh, and Vydac. Refer to references [14–28] to obtain the online addresses of the companies. Even though all HPLC columns designed so far are for the same purpose, you can still find differences in the C-18 packing that is manufactured by different manufacturers, as shown in Tab.2.4.

These differ in the shape of silica particles, size of the pores, carbon content of the bonded phase and the extent of end-capping.

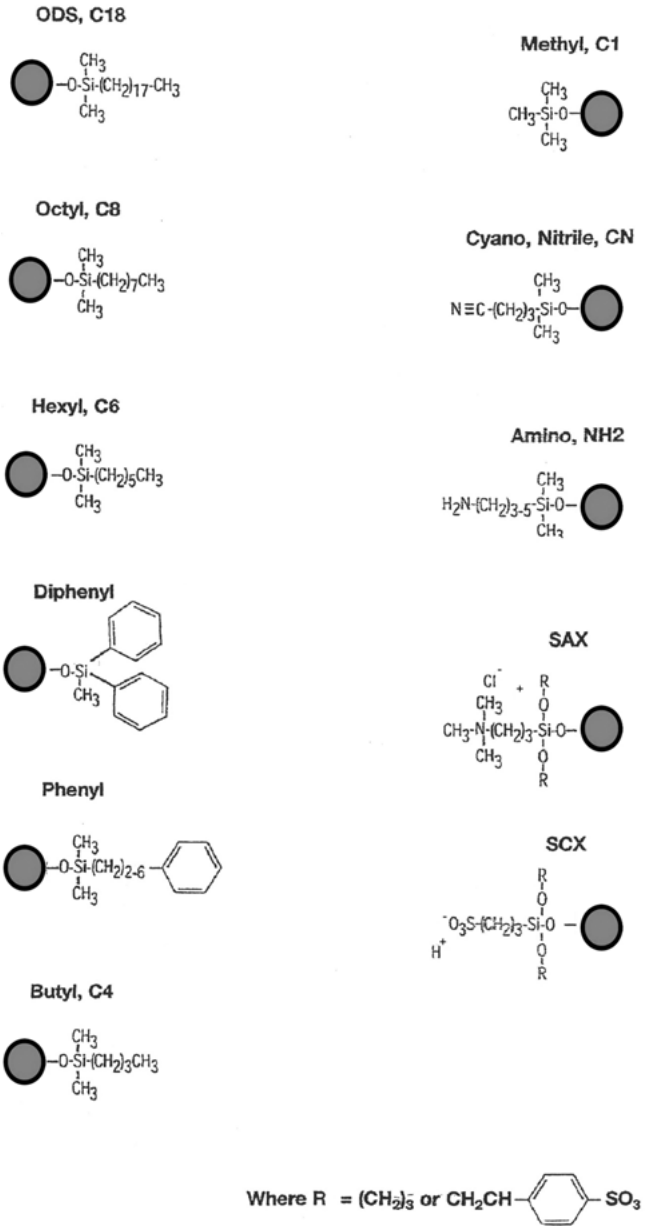



**Fig.2.12:** The chemical structures of the most popular HPLC silica-based reversed-phase packing materials for HPLC columns which are being used to analyze the different pharmaceutical products in the laboratories for drug quality control. Reproduced with permission from Waters Co. [14].

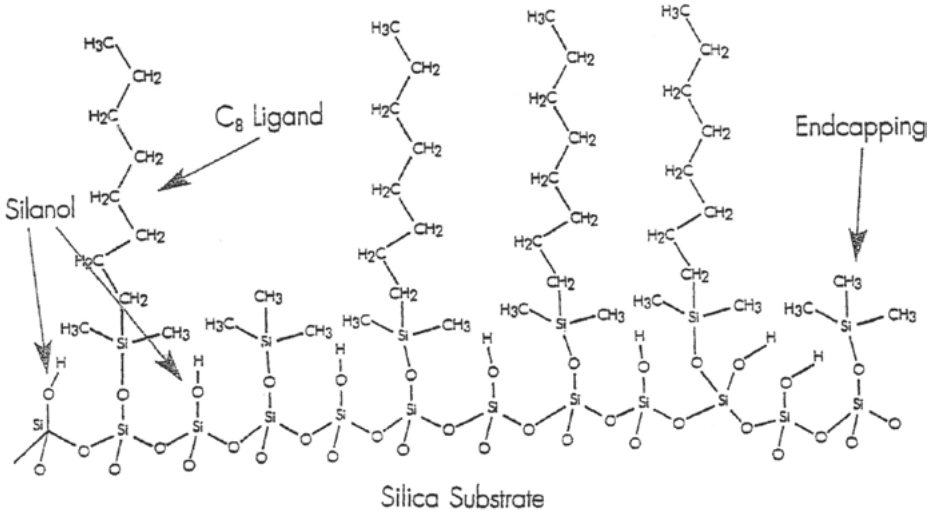



**Fig.2.13:** Chemical structure for surface of a typical reversed-phase packing C8 (RP8). Reproduced with permission from Waters Co. [14].

**Tab.2.3:** Names and abbreviations of the commonly used HPLC packings (normal and reversed phases, NP-HPLC and RP-HPLC) for analysis of pharmaceutical products in drug quality control laboratories.

| Packing material | Abbreviations | Other abbreviations |
|---|---|---|
| Octadecylsilyl-bonded silica | ODS+ | C18, RP18 |
| Octylsilyl-bonded silica | OSB++ | C8, RP8 |
| Phenyl-bonded silica | PBS | |
| Cyanopropyl-bonded silica | CPB+++ | CN |
| Diol-bonded silica | DBS | |
| Aminopropyl-bonded silica | APS++++ | $NH_2$ |
| Microparticulate silica | SIL | |

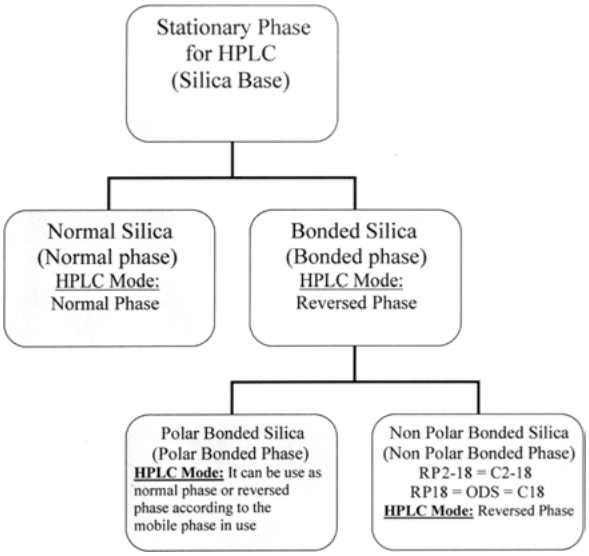



**Fig.2.14:** Classifications of old packing materials for HPLC columns.

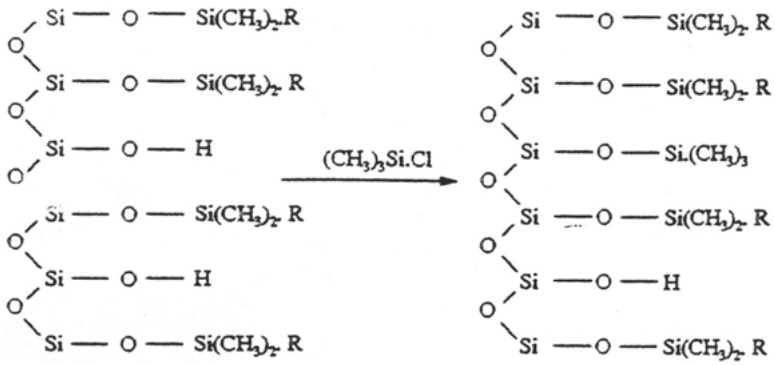



**Fig.2.15:** End-capping reaction.

**Tab.2.4:** Listing of some commercial HPLC packing materials, their manufacturers and some of their physical properties.

| Manufacturer | Sorbent | Surface area ($m^2/g$) | Pore size (Å) |
|---|---|---|---|
| Waters [14] | Symmetry 300 | 110 | 300 |
| Waters [14] | m-Bondapak | 330 | 125 |
| Waters [14] | Spherisorb | 220 | 80 |

**Tab.2.4** (continued)

| Manufacturer | Sorbent | Surface area ($m^2/g$) | Pore size (Å) |
|---|---|---|---|
| Waters [14] | Atlantis | 340 | 90 |
| Agilent [15] | Zorbax-Rx | 180 | 80 |
| Macherey Nagel [20] | Nucleosil 100 | 350 | 100 |
| Macherey Nagel [20] | Nucleosil 120 | 200 | 120 |
| Merck [21] | LiChrospher | 350 | 100 |
| Phenomenex [22] | Prodigy | 310 | 150 |
| Phenomenex [22] | Synergi | 475 | 80 |
| Restek [23] | Allure | 500 | 60 |
| Restek [23] | Ultra | 300 | 100 |
| Thermo [25] | Hypersil | 170 | 120 |
| Thermo [25] | Hypersil Gold | 220 | 175 |
| Varian [27] | Polaris | 180 | 180 |

### 2.1.6 Modern packing materials for HPLC columns

As mentioned previously, in RP-HPLC, the silanol groups remaining after modification with nonpolar or polar groups (see Fig.2.11–2.14) are responsible for many undesirable effects in this type of separation. The undesirable effects are:

***First***: In basic mobile phase (contact with basic mobile phase), RP skeleton is hydrolyzed. Next, the OH anion, which is present in the mobile phase goes ahead and attacks the surface silica. This is what causes the silica to dissolve. As more and more silanol sites are exposed, the process is accelerated (the RP materials just dissolves slowly). This behavior constitutes a serious limitation on the free choice of mobile phase. For that reason, use of pH values above 8 is generally considered impractical.

***Second:*** HPLC peak tailing: Basic compounds, such as amines, interact strongly with the unreacted surface of silanols on conventional C18 and this results in severe tailing of the peak. How much the peak tails depend largely on the mobile phase's pH level and the analytes' p$K_a$ level? As shown in Fig.2.16 at low pH ≤3, the surface silanols are in the associated form (Si-O-H) and do not interact strongly with higher basic compounds (p$K_a$ > 9) as in scopolamine bromide (hyoscine *N*-butylbromide) (Buscopan; Fig.2.17A). As shown in Fig.2.16 at pH > 3, the surface silanols begin to deprotonate and interact strongly with basic drug resulting from severe tailing.

From all these disadvantages, successful efforts have been devoted for development of other packing materials for HPLC columns (nowadays, it is called modern HPLC columns) such as ***symmetry (Section 2.2.4.1), symmetry shield (Section 2.2.4.2) and XTerra (Section 2.2.4.3) packing***. We shall give enough information about the advantages for these previously mentioned packing materials for HPLC columns.

### 2.1.6.1 Symmetry packing

Nowadays, numerous base-deactivated high-purity silica have become available as mentioned in Section 2.2.3. These silica have very low trace metal content [Fe, Al, Na] (≤1 ppm [14]). Trivalent metal ions in the silica matrix are known to increase the ion-exchange activity of the surface of silanols. These base-deactivated silica are also designed to have a fully hydroxylated surface (silanol concentration of 8 μmol/$m^2$), which significantly reduces the concentration of the highly acidic ionic silanol population in the starting silica. Acidic ionic silanols have been documented as a primary cause for severe peak tailing. Nowadays, most state-of-the-art RP columns combine the use of high-purity base-deactivated silica with high surface coverage to achieve the lowest degree of possible silanolic interaction with basic compounds and deliver sharp symmetrical peaks (Fig.2.16 and 2.18). So, these packing materials are called *symmetry packing*.

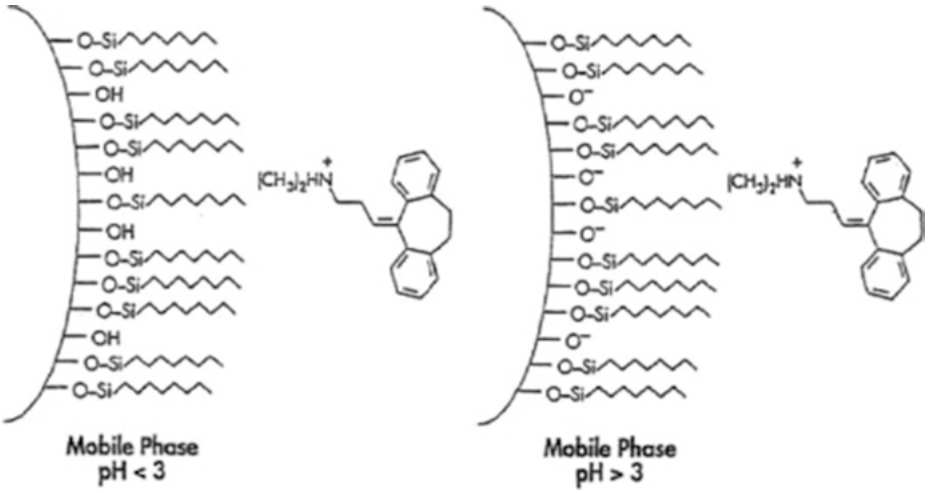



**Fig.2.16:** The mechanism of tailing peak for one of the basic compounds (amitriptyline) when it separated using mobile phase pH > 3 and pH ≤ 3 using conventional C18. Reproduced with permission from Waters Co. [14].

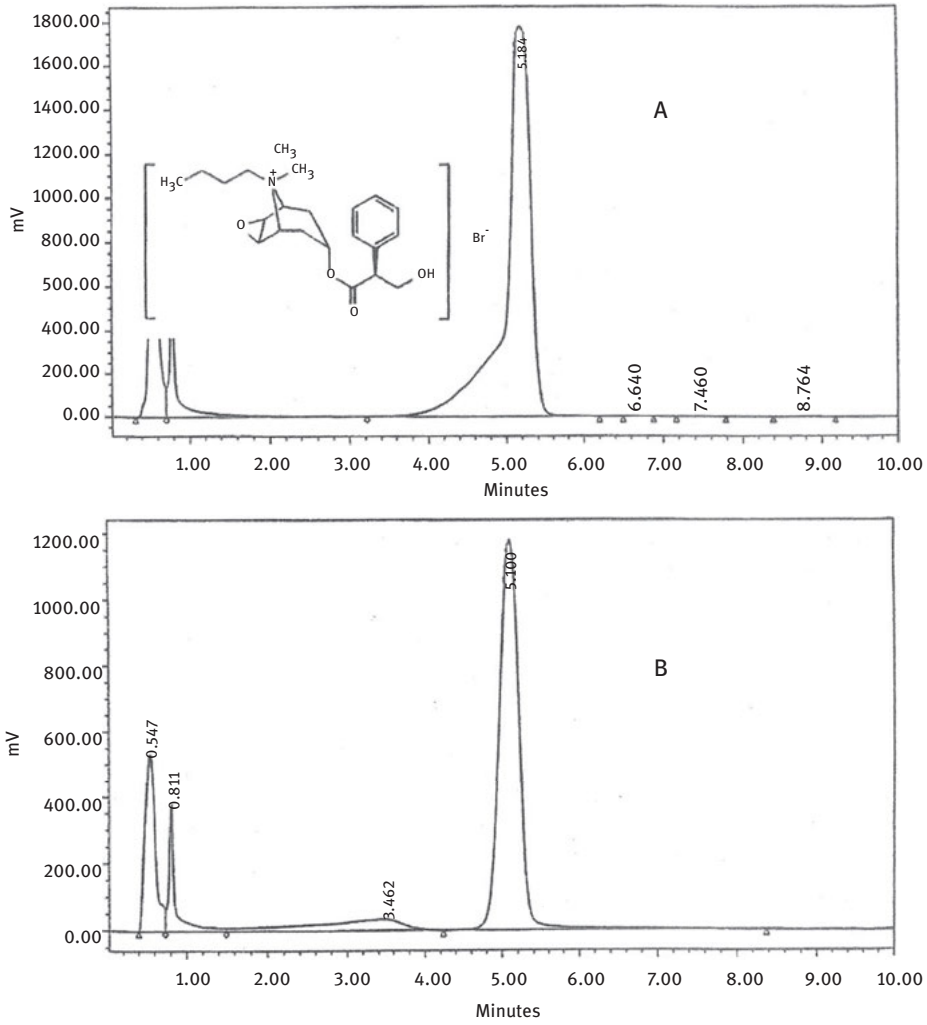



**Fig.2.17:** The peak shape improvement of scopolamine bromide (Buscopan) (hyoscinebutylbromide) using symmetry column C18 in comparison with conventional C18.
A. Using conventional C18
B. Using symmetry C18

Mobile phase:

Purified water 56 volumes
Acetonitrile 24 volumes
Methanol 14 volumes
Glacial acetic acid 4 volumes
SHS* solution 2 volumes

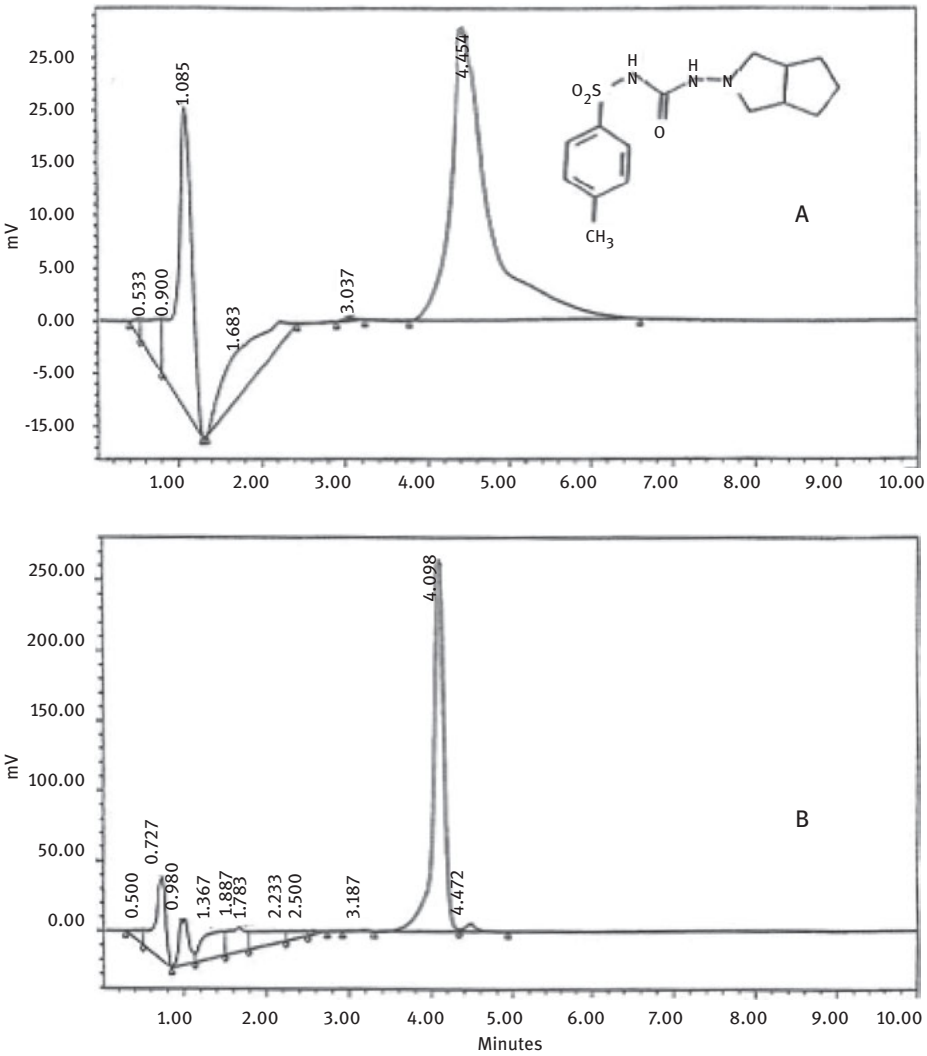



**Fig.2.18:** Illustrative diagram showing the HPLC peak shape improvement of gliclazide (diamicron, anti-diabetic drug) using symmetry column C18 in comparison with conventional C18.
(A) Using conventional C18
(B) Using symmetry C18

*SHS solution consist of sodium 1-heptanesulfonate 5.5 g + 3 mL glacial acetic acid to 100 mL using purified water.

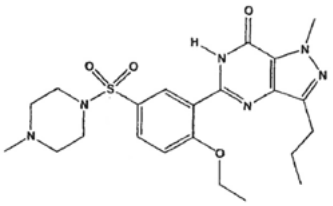
Depending on the HPLC PDA (photodiode array detector), an analytical procedure has been developed and validated according to the ICH and UNODC [29–30] by Omar and Khalifa [31–35] to do screening for checking whether three of the synthetic PDE-5 inhibitors such as V (vardenafil), T (tadalafil) and S (sildenafil) are present or

not (see Fig.2.19) for their structure formula). These have been adulterated illegally in the herbal natural products and have reached the KUFDA (Kuwait Drug and Food Quality Control Administration) to be registered as herbal products that will be used to increase the sexual performance of men. This was done from 2003 to 2012 in Kuwait. Extraction of sildenafil, tadalafil and vardenafil from samples were accomplished on a symmetry 300 analytical column packed with 5 µm C18 (150 mm × 2.1 mm, 5 µm, 120 Å) column (Waters Co. [14]). The mobile phase in this case was made up of acetonitrile:methanol:1% acetic acid (20:20:60, v/v/v) at 0.4 mL/min flow rate when the conditions were ambient. PDA was used to detect the analytes. Tab.2.5 summarizes the chromatographic data about sildenafil, tadalafil and vardenafil {average retention times $t_R$ for each compound during the course of this study, resolution (R), peak tailing factor ($T_f$) and % RSD of $t_R$}. From the data in Tab.2.5 we can conclude that sildenafil, tadalafil and vardenafil made use of the mobile phase that was eluted isocratically to resolve at the baseline. Resolution values of 2.8 and 16.1 between V and S and among S and T are shown in Fig.2.20 respectively. It is clear from Fig.2.19 that the difference in the molecular weight between vardenafil and sildenafil is $CH_3$ group, 489−475 = 14 amu, which means high molecular similarity between sildenafil and vardenafil (this indicates the separation power of the used symmetry HPLC column). The peaks separated on the PDA chromatograms are symmetrical in shape and the $T_f$ (tailing factors) for vardenafil, tadalafil and sildenafil are 1.01, 1.02, 1.05, respectively (Fig.2.20). The tailing factor has >1.5 as the % RSD values for V, T and S. This shows that the chromatographic system is stable. According to the requirements of USP [36], the chromatographic data which we have obtained from tadalafil, sildenafil and vardenafil are of high quality and meet the acceptance criteria. This indicates that the chromatographic system is stable and effective for both the quantitative and qualitative purposes of study for this course.

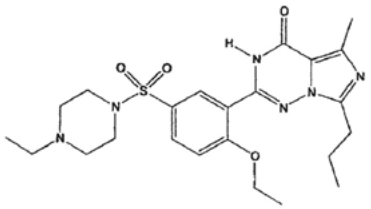
### 2.1.6.2 Symmetry shield reversed-phase (SSRP) packing materials for HPLC columns (embedded polar group technology)

Embedded polar group technology(Waters Co. Patent [14]) can be considered as a second approach toward further deactivation of the surface silanols of silica particles and is based on the design of new RP ligands which contains embedded polar group. Results for a new stationary phase are called *symmetry shield reversed-phase* packing. *SSRP* packing is a bonded phase in which a hydrophilic group (polar group) is incorporated (embedded) into the bonded phase structure. It lies between the RP chain and the surface of the silica as shown in Fig.2.21. The embedded polar groups for carrying out these functions are sulfonamide or urea groups, carbamate, amide and all groups that have strong hydrogen bonding characteristics.

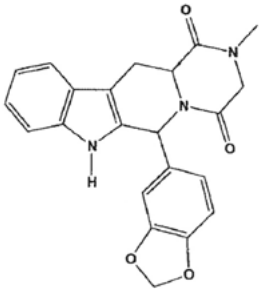
SS HPLC columns have set the standard of performance for peak shape of basic compound (Fig.2.22–2.23). This acts like a literal shield and “shields” the surface



Sildenafil, VIAGRA
Mol. Wt. 474.59
$C_{22}H_{30}N_6O_4S$



Vardenafil, LEVITRA
Mol. Wt. 488.60
$C_{23}H_{32}N_6O_4S$



Tadalafil, CIALIS
Mol. Wt. 389.40
$C_{22}H_{19}N_3O_4$

**Fig.2.19:** Chemical structure of three synthetic therapeutic drugs such as *vardenafil* (MW = 490 amu), *sildenafil* (MW = 475 amu)and *tadalafil* (MW = 390 amu) were used as adulterants for natural herbal products (dietary supplements and herbal medicine) [31–35].

**Tab.2.5:** Chromatographic data obtained from the analysis of standard mixture of sildenafil, vardenafil and tadalafil injected through HPLC–PDA system.

| Compound | Chromatographic data | | | |
|---|---|---|---|---|
| | $t_R$ (min*) | %RSD of $t_R$** | Resolution*** factor (R) | Tailing*** factor (As) |
| Sildenafil | 5.73 | 1.1 | V–S = 2.8 | 1.05 |
| Vardenafil | 4.85 | 1.2 | S–T = 16.1 | 1.02 |
| Tadalafil | 14.17 | 1.1 | | 1.01 |

*Reproducibility of $t_R$ was evaluated during 2 months with minimum of 30 injection of reference standard mixture solution and the %RSDs were determined.
**$t_R$ on the PDA chromatogram.
***Determined according to USP [36].

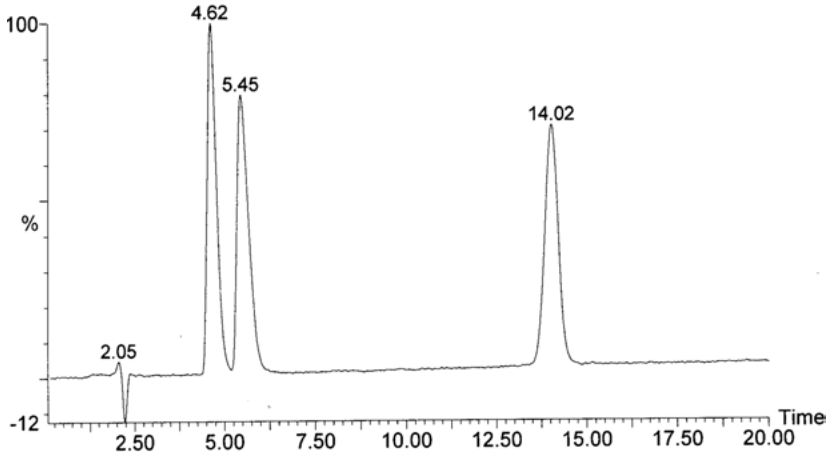



**Fig.2.20:** UV chromatogram showing separation of three synthetic therapeutic drugs were used as adulterants for natural herbal products (dietary supplements and herbal medicine): *vardenafil* (V, $t_R$ = 4.82 min, MW = 490 amu), *sildenafil* (S, $t_R$ = 5.45 min, MW = 475 amu) and *tadalafil* (T, $t_R$ = 14.0 2 min, MW = 390 amu) in the mixture of standards on *symmetry 300* (150 mm × 2.1 mm, 5 µm, 120 Å) column (Waters Co. [14]); *mobile phase*: 1% acetic acid:acetonitrile:methanol (20:20:60) at a 0.4 mL/min flow rate and photodiode array as a detector (PDA).

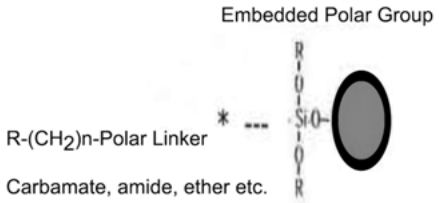
silanols on the residual surface of the silica for high basic analytes. The RP column is dependent upon the patent *embedded polar group technology* by Waters Co. [14]

SS columns are the perfect choice when applications that require very aqueous mobile or low organic conditions are under consideration. For better sample and particle interaction, particles of the SSRP are best. The reason is that they are water-wettable. This results in stable retention levels when the mobile phase is highly aqueous and the peak shape of it is superior (Figure 2.23).

The activity of the silanols in the symmetry shield packing materials on the surface is further reduced when the polar group embedded is closer to the silica surface. Hence, the advantages of SSRP packing are as follows:

- Significant improved peak shape, no tailing and resolution over a broad pH range between pH 2 and 8.
- Basic compounds are retained less as compared to the conventional C8 and C18 columns. Tab.2.6 gives the $T_f$ at a neutral pH level of the analyte amitriptyline. A number of different commercial packages are considered. Difference is clearly visible between the packaging that is old like the Nova-Pak C18, Bondapak C18 and the newer packing SS C18 which is based on high-purity silica.
- The presence of SSRP columns enables the analysts to come up with improved and faster ways to improve the analytical laboratories productivity.
- Most of the drugs (analytes) found today are largely polar. Therefore, it is desirable to go ahead and use 100% water-based mobile phases to ensure that maximum retention occurs. This is not truly possible when dealing with the totally

hydrophobic C18 packing's older packing. This is because these hydrophobic surfaces have an unfavorable wetting angle of the water. If a mobile phase is fully aqueous, on pressure, the mobile phase itself will be pushed out from the pores as it cannot deal with the pressure. This will be a failure as there will be zero retention and it will not return unless the packing is wetted again. This time with either acetonitrile or methanol or any other organic solvent. This is not possible when dealing with stationary phases that have a fixed polar group. Hence, if the fully aqueous conditions exist, the stationary phase can be used. In the recent past, different phases have come to surface that claim that they are using the procedure of polar end-capping to increase the wettability of the C18 packing.



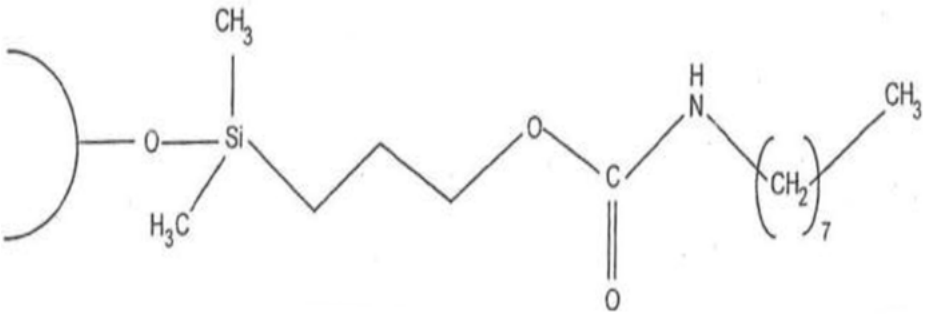



**Fig.2.21:** Chemical structure of a symmetry shield packing materials for HPLC column (C8). Reproduced with permission from Waters Co. [14].

### 2.1.6.3 XTerra packing (hybrid particle technology)

The recent years have seen an advance in the technology related to the base materials. This involved combining polymer and silica like the hybrid particles (XTerra brand hybrid particle technology manufacturing process as per Waters Co. [14]. XTerra particles are organic/inorganic hybrid materials developed and synthesized by Waters Co. [14]. The technology makes use of $CH_3$ (methyl) groups and uses it as a

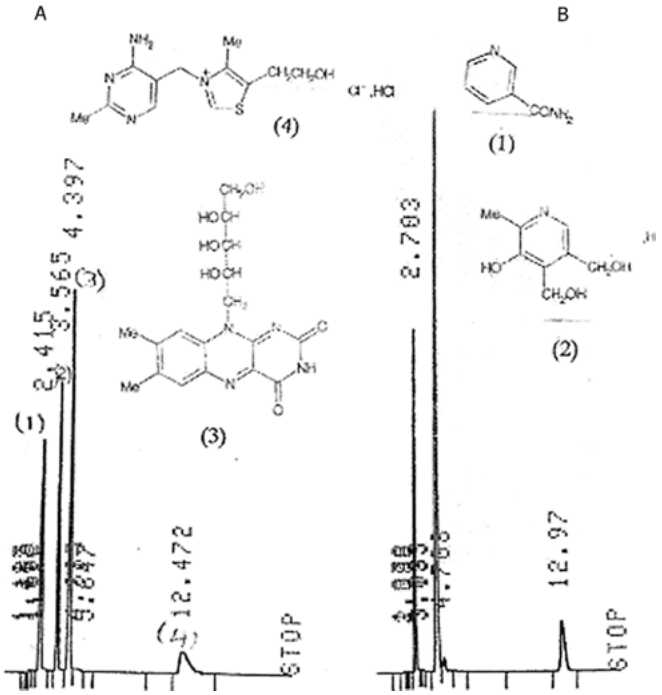



**Fig.2.22:** Separation of four water-soluble vitamins such as niacinamide (1), pyridoxine hydrochloride (2), riboflavin (3) and thiamine hydrochloride (4) using symmetry shield C18 HPLC column (A) in comparison with using a conventional C18(B).

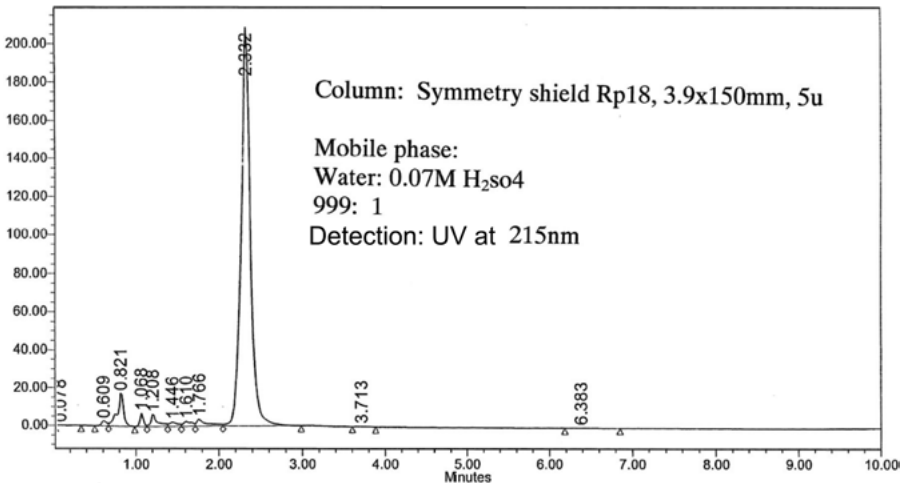



**Fig.2.23:** Separation of sodium citrate in *Kufdrin* syrup (*cough syrup*). Each 5 mL contains ammonium chloride (131.5 mg), sodium citrate (57.0 mg) and diphenhydramine HCl (13.5 mg) using symmetry shield column C18.

**Tab.2.6:** Peak tailing for amitriptyline for various packings at pH 7.0.

| Packing | USP* tailing factor |
|---|---|
| μBondapak C18[1] | 4.60 |
| Nova-Pak C18 | 4.47 |
| **Symmetry shield C18** | **1.14** |

[1]Column dimensions: 4.6 mm × 150 mm.
*For calculation of USP tailing factor, see Chapter 8.

portion of the base material structure of the silica lattice (Fig.2.24–2.26). As shown in Fig.2.24–2.26 during the process of synthesis, every one out of the three silanols has an element from the methyl group replacing it. This means that the methyl groups replace 33% less of the surface silanol groups before the process of end-capping and ligand bonding occurs. This reduces the total number of silanol groups that are present on the silica particles surface, which allows an improved and better shape of the peak for the analytes at a pH level of 7 (Fig.2.27). Furthermore, surface modification for XTerra particles through the bonding of ligands as shown in Tab.2.1 provides materials with excellent RP properties (Fig.2.25–2.27). As shown in Fig.2.28, when the pH level is high enough, the hydroxyl anion in mobile phases goes ahead and attacks the surface silica, which initiated the silica's dissolution process. As more and more silanol sites are exposed, the process is accelerated. In case of XTerra particle, methylsiloxane bond is present and poses high resistance toward the dissolution process when the pH level is high enough in the mobile phase. Additionally, the particles are protected against higher pH values because of the presence of methyl groups (8–12). These extend the column life as shown in Fig.2.29. This happens because the XTerra particles are rugged. Their molecular structure is such that greater resistance is presented during the dissolution process when the pH is high and when it is compared to the particles that are traditionally silica based.

It is well known that the tool which is most impactful when it comes to manipulating the selectivity for the compounds that are ionizable is the pH. For compounds that are acidic, the retention increases when the pH value is below the p$K_a$ value of the compound. On the other hand, the retention becomes longer for basic compounds when the pH value is more than the value of p$K_a$ of the compound. As most of the active compounds found pharmaceutically are able to be ionizable, a column that has a wide pH range allows to have greater retention time manipulation when the method development is occurring. In case of XTerra columns, they have a pH range of 1–12. This is more than twice that of the columns based on silica. This in turn makes the process of method development 2 times as fast and as easy. For more details about symmetry, SS and XTerra packing materials for HPLC columns [37–40].

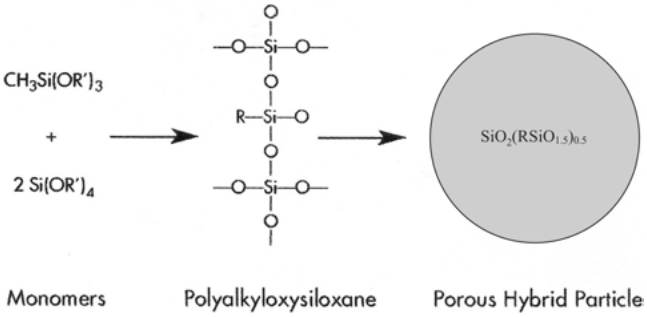



**Fig.2.24:** Illustrative diagram showing manufacturing process of hybrid particle technology] (built-in methylsiloxane groups, XTerra particles) as per Waters Co. [14]. You can replace one out of three silanols during the process of synthesis with a methyl group, 33% less of the surface silanol groups are replaced with methyl group prior to ligand bonding and end-capping. Reproduced with permission from Waters Co. [14].

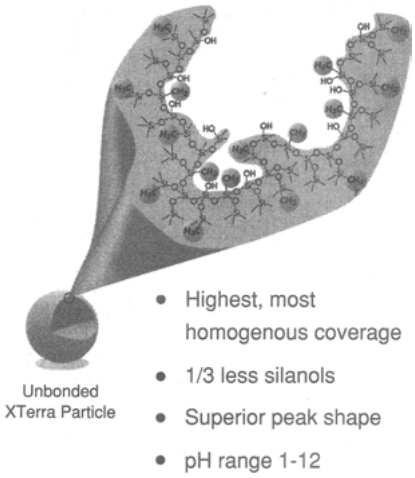



**Fig.2.25:** Diagram illustrating the chemical structure for surface of unbonded Waters XTerra hybrid silica particles. Reproduced with permission from Waters Co. [14].

### 2.1.7 HPLC column hardware (container)

HPLC columns are usually made of stainless steel and are highly pressure resistant up to about 6,000 psi (55 MPa). Usually, the materials of column are HPLC pressure resistant. Additionally, they are resistant to chemical corrosion too. It is important that the inner part of the column is as smooth as possible, also extremely flat tube ends ensure a tight. Mostly, a tube made of stainless steel of grade 316 is used to make the containers of the HPLC column (Fig.2.30–4) with conventional end fittings (end fitting body, male/female union, 316 stainless steel compression style; Fig.2.30–2). The column packing material (stationary phase) is held in the column

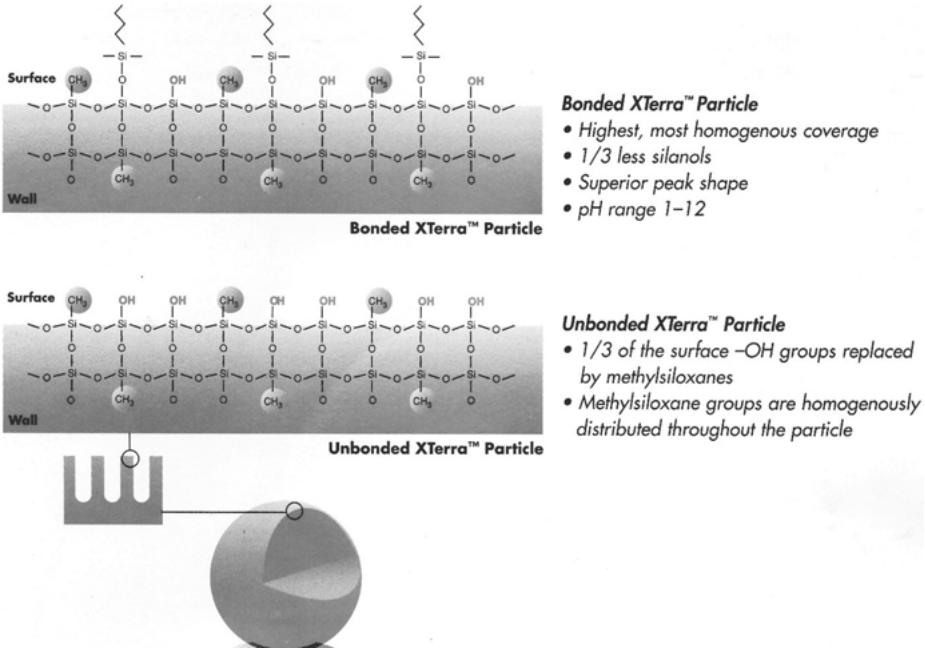



**Fig.2.26:** Diagram illustrating the chemical structure for surface of bonded and unbounded Waters XTerra hybrid silica particles. Reproduced with permission from Waters Co. [14].

tubing (Fig.2.30–4) by 1/16″ thick, 0.5 µm pore size of 316 stainless steel frits (Fig.2.30–5) at the bottom of the tube. At the top column, there is a stainless steel frit on top of the packing materials (stationary phase). At the lower and upper ends, there is a reducing union (inlet compression screw fitting, male nut, Fig.2.30–1) to connect the column to HPLC system. Fig.2.30 shows HPLC column hardware as described previously. As shown in Tab.2.7 and Fig.2.31 columns of internal diameter (i.d.) of 0.1–10 mm (often 4.6 mm) and length of 5–25 cm are generally used for analytical purpose; and columns that are wider and have an i.d. of around 10–50 mm may be used for preparative work. HPLC columns can be packed with 10, 5, 4 or 3 µm diameter of particles.

The i.d. and the size of the matrix particles, which make up the analytical column dimensions, greatly affect the separation efficiency and the tempo of the analysis process. Column lengths that are short, that is, around 3–5 cm, have lower back pressure and shorter run times. On the other hand, columns that are longer in size from around 25 to 30 cm have better resolution of the analytes separated but take more time in analysis, require more solvents and hence have higher costs.

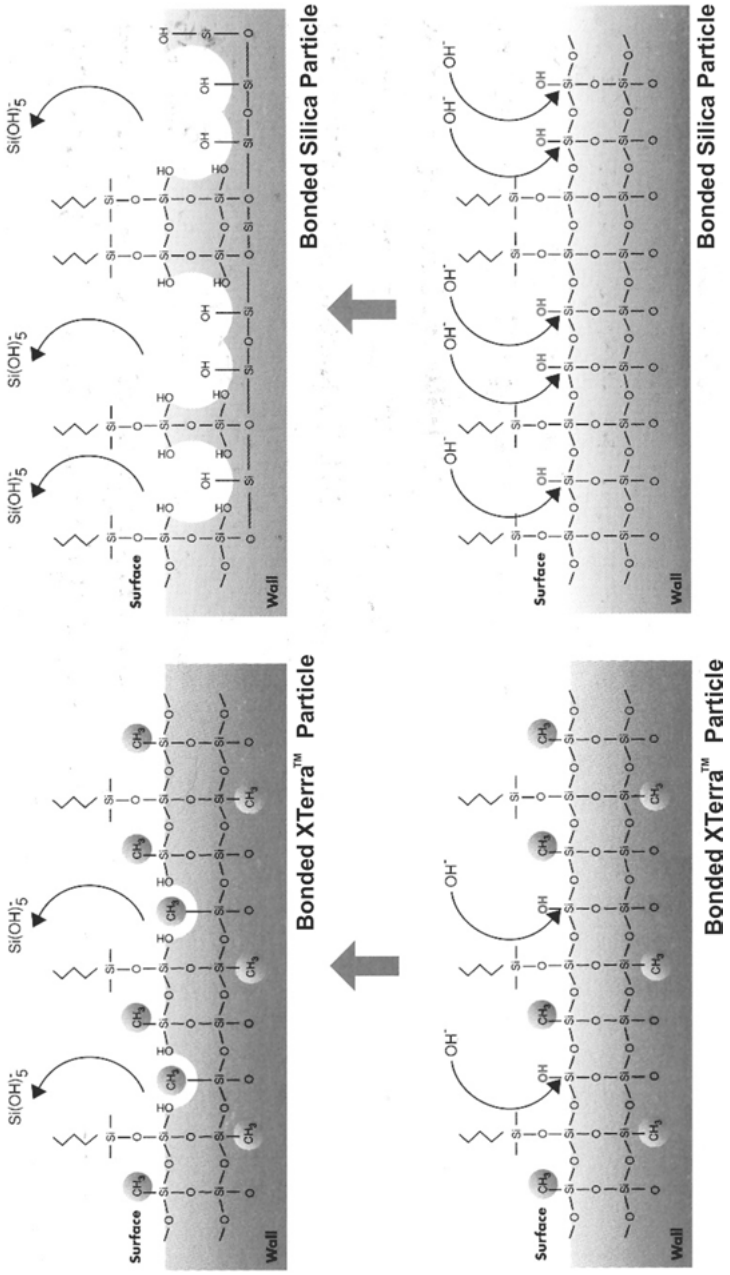



**Fig.2.27:** XTerra column exhibiting superior peak symmetry for strong basic drug nortriptyline was separated using neutral mobile phase (pH 7) in comparison with some benchmark columns (bonded silica C18). Mobile phase: 20 mM $K_2HPO_4$ pH 7/acetonitrile, 65/35, 25 °C column temperature; flow rate: 1.4 mL/min; and the detector: 254 nm UV. Reproduced with permission from Waters Co. [14].

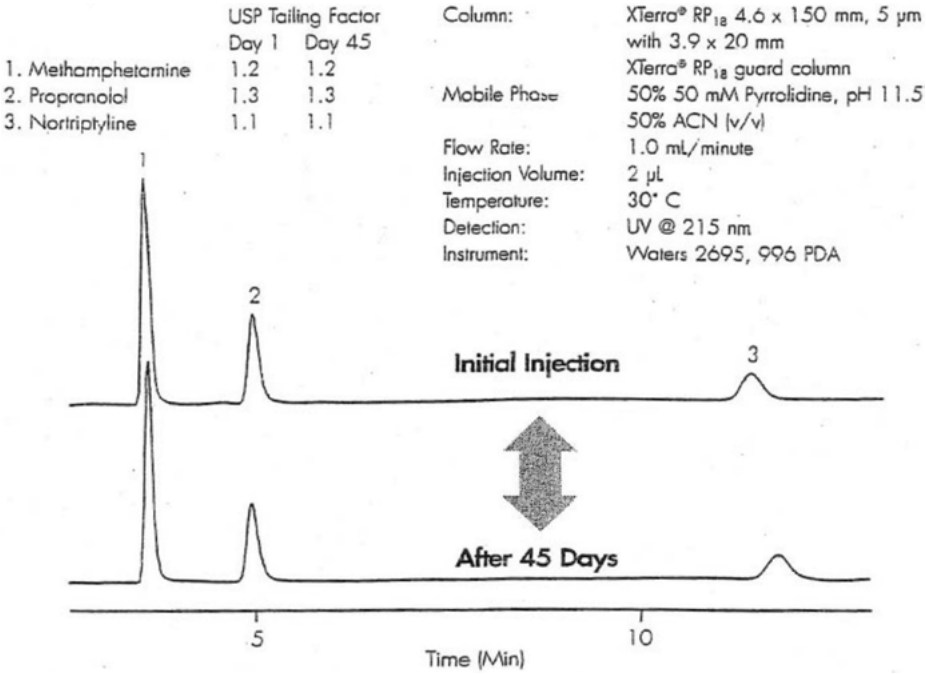



**Fig.2.28:** Diagram illustrating improved stability of the XTerra stationary phase for HPLC columns against high pH of mobile phase. At high pH, the hydroxyl anion in the mobile phase attacks the silica surface, thereby causing dissolution of the silica. This is accelerated as more silanol sites became exposed. Reproduced with permission from Waters Co. [14].

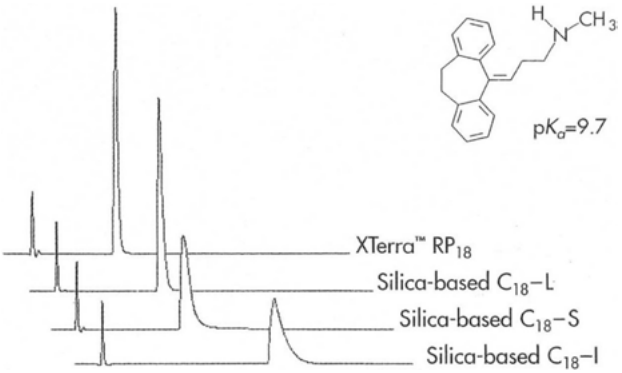



**Fig.2.29:** XTerra column exhibiting superior stability at high pH (pH 11.5) for separating basic drugs with no deterioration of peak symmetry (no change in USP tailing factor; for calculation of this factor, see Chapter 8) within 45 days. Reproduced with permission from Waters Co. [14].

**Tab.2.7:** Column dimensions and flow rates of HPLC systems.

| Type | Column inner diameter (mm) | Flow rate (mL/min) |
|---|---|---|
| Preparative HPLC | 10–50 | 5–100 |
| Traditional (“analytical”) HPLC | 1–10 (often 4.6) | ≥1 |
| Microbore HPLC | 0.5–1 | 0.1–0.5 |
| Capillary HPLC | 0.1–0.5 | 0.001–0.025 |
| Nano-HPLC | <0.1 | <0.001 |

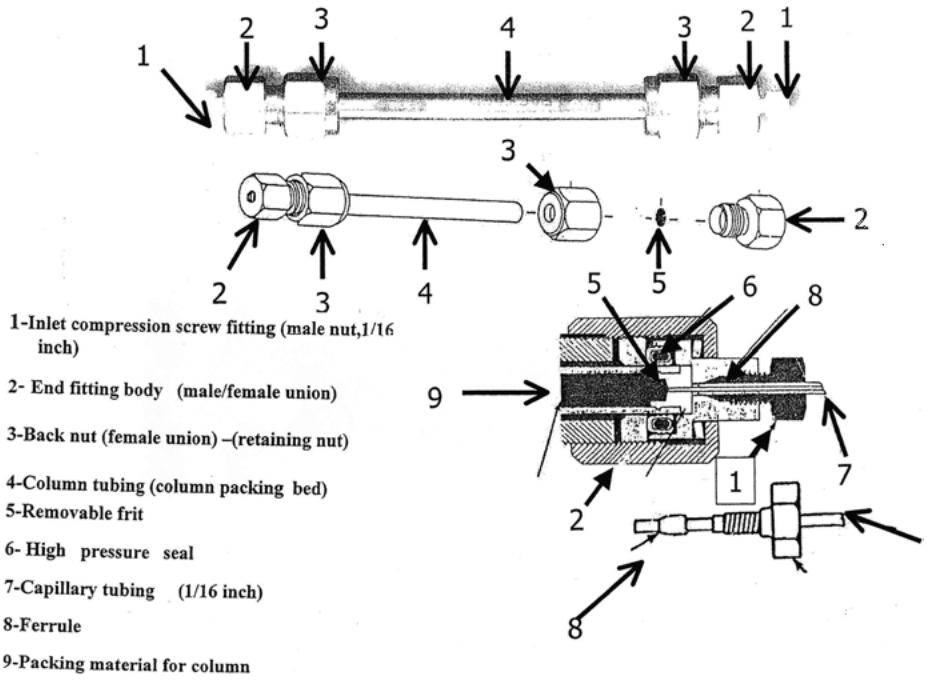



**Fig.2.30:** A typical conventional HPLC column hardware.

### 2.1.8 HPLC column efficiency

Extensive quality testing is done to ensure that the quality of the HPLC columns is up to the mark [41,43,57–62]. This is done by the manufacturers as they ensure that the columns produced give long-term results. Every column manufactured comes with a “QC certificate of analysis” This shows the results of analyzing a standard


**Fig.2.31:** Analytical and preparative HPLC column dimensions. Reproduced with permission from Waters Co. [14].

test mixture by using the particular column. Every column needs to be able to meet the conditions specified for selectivity and resolution, peak symmetry, capacity factor, operating back pressure and theoretical plates. An example of a QC certificate of analysis of HPLC column is shown in Figure 2.32. This certificate illustrates the type of QC analysis and quality for a column produced by Waters Co. Each column comes with a data sheet that has all these specifications or a handful of them.

– *Test conditions*:

Mobile phase: 60/40 acetonitrile/water

Sample: Acetone 6 µL/mL + acenaphthene 0.5 mg/mL

1.4 mL/min flow rate

254 nm detection rate

Ambient temperature

4 µL volume of injection

– *Results*:

USP tangent efficiency for acenaphthene: 23,091

USP tailing for acenaphthene: 0.98

$t_R$: 15.75 min

Pressure (Psig): 1,845

Theoretical plate number is a very important parameter which describes the column efficiency. We will further describe this parameter and its roles for measurement of column efficiency.

There are several parameters that influence the determination of column efficiency:

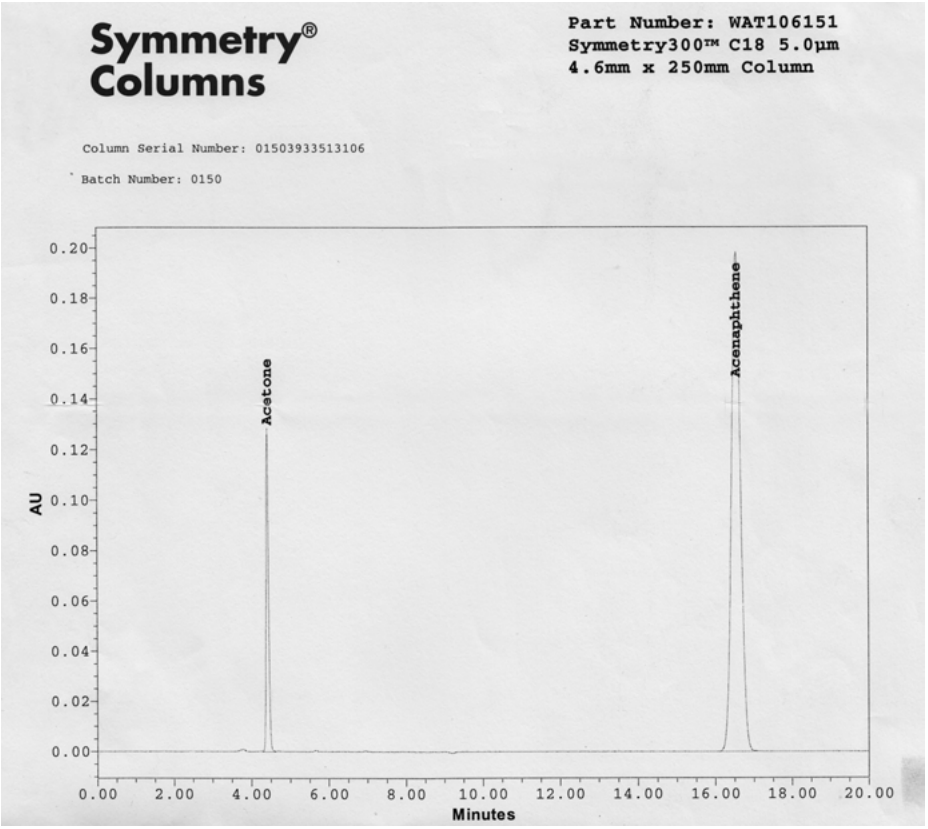



**Fig.2.32:** HPLC column performance test report. Reproduced with permission from Waters Co. [14].

- The composition of mobile phase and its flow rate
- Which solute was used for measurement
- Method selected for calculation and measurement
- The temperature

Therefore, it is imperative that all the efficiency values are accompanied by something that states under what conditions and plate count these values were obtained (see Fig.2.32).

### 2.1.9 HPLC column back pressure

HPLC column back pressure is the pressure that is needed to pump and send the mobile phase from the column. It is possible to set the flow rate of the mobile phase when it passes through the column and should be set. The measurement for this is

done in mL/min. Depending on the conditions of separation and the flow rate, there is a pressure drop that happens all through the column. This is the pressure that is indicated when you take a pressure reading at the beginning of the column and is shown to be the pressure gauge or the HPLC controller. Even though it is possible to operate a HPLC column at a pressure of 6,000 psi, 1 MPa = 40 atm = 145 psi = 10 bar (generally, it works on anything between 1,000 and 6,000 psi) the column pressure should be below the highest value. This is why predicting pressure changes when the conditions change is imperative. This relationship is given as follows:

$$P(\text{MPa}) = 2.1 \times f \times L\,(\text{mm})/(d_p\ \mu\text{m})^2 \times (d_c\,\text{mm})^2 \tag{2.1}$$

where P is the HPLC column back pressure (MPa), using water as mobile phase at a flow rate of 1 mL/min; L is the column length (mm); $d_p$ is the particle diameter of packing materials (μm); $d_c$ is the column internal diameter (mm); f = 10 for steel column (constant).

The results from this equation should then be multiplied by the viscosity (η) of the mobile phase in centipoises (cP) (see Table 2.9) to obtain the typical operating back pressure (MPa) at 1 mL/min. Table 2.9 summarizes viscosity values for some popular HPLC solvents. For calculation of column pressure operated at a flow rate more than 1, the previous results must be multiplied by the amount of the flow rate of mobile phase which have been used.

In the RP-HPLC (which is the commonest method of LC (liquid chromatography) used), the total value of *η falls in the range of 0.5–1.5, with a particle size of 5 μm, and the length of the column is almost 15,025 cm*. Thus, when the flow rate of the column is 1 mL/min, the pressure of the column is between 500 and 2,000 psi.

When the flow rate is kept at a constant, the HPLC's pressure changes because of the following reasons:

- The mobile phase's composition or temperature changes (higher temperature leads to lower pressure, see Section 2.1.11)
- The column changes (particle size or length)
- There is a blockage in the column because of the particulate matter (it is normal but the pressure in the phase is increased)
- The back pressure may rise significantly during the course of a gradient elution of mobile phase. Mostly seen in the case of methanol/water gradient.
  - Each HPLC column has its own maximum recommended pressure as an example in Tab.2.8. If the pressure is high even after using the column at normal rate of flow, this is probably because of some sort of contamination that has deposited itself on the packing material. Hence, the frit will be unplugged first. Next, reverse the column and use a proper solvent to flush down the contamination while the instrument is disconnected.

**Table 2.8:** Typical operating back pressure for some HPLC column using methanol or water at 1 mL/min as mobile phase.

| Column name and its dimensions | MPa[1*] Methanol (water) | Psi[2**] Methanol (water) |
|---|---|---|
| Resolve, 3.9 mm × 150 mm × 5 µm | 5.0 (8.5) | 700.0 (1,200.0) |
| Resolve, 3.9 mm × 300 mm ×5 µm | 10.0 (17.0) | 1,500.0 (2,500.0) |

[1]Megapascal (MPa)
[2]Pound square inch (psi)
*To convert to MPa from psi, multiply by 0.00690.
**To convert to psi from MPa, multiply by 145.04.

**Tab.2.9:** Viscosity (η) for some HPLC solvents, Centipoise (cP) at 20 °C·

| **Mobile phase** | **Viscosity, η (20 °C)** | **Mobile phase** | **Viscosity, η (20 °C).** |
|---|---|---|---|
| n-Pentane | 0.235 | Acetone | 0.32 |
| n-Hexane | 0.33 | Dioxane | 1.54 |
| n-Heptane | 0.42 | Nitromethane | 0.65 |
| ISO-octane | 0.50 | Acetonitrile | 0.37 |
| n-Propylchloride | 0.35 | n-Propanol | 2.3 |
| Benzene | 0.65 | Ethanol | 1.2 |
| Methylene chloride | 0.44 | Methanol | 0.6 |
| Tetrahydrofuran | 0.46 | Water | 1.00 |
| Methyl ethyl ketone | 0.4 | | |

### 2.1.10 HPLC column storage

Leaving the column unused for less than 72 h does not require specialized storage procedures. For longer storage, use the following guidelines:

- Never let columns dry out. Allowing them to dry out may result in poor chromatographic performance.
- RP columns should be kept in a 10% solution of isopropanol if bacterial growth is to be prevented or in 70% methanol. The solution should be aqueous. Store NP column in hexane. Store polar bonded phase in NP or RP or NP solvent according to the use of the column (see Tab.3.6 Chapter 3).

- Do not store the column in buffered solutions, as salts may precipitate.
- Once used, the column should be put back inside the box by firmly putting the end plugs in place for storing it.
- A column should not be present at high temperatures in the absence of a mobile phase.

### 2.1.11 Column temperature

All HPLC columns can be used at ambient temperature and up to 70 °C. In general, high temperature results in reduced sample retention, higher separation efficiency and lower column pressure. As the sample retention is influenced by the temperature of the column, controlling it carefully is required to get repeated results from it.

In many applications, closely controlling the temperature of the column is not a requirement. In this case, operating the column at ambient temperatures suffices. Even though this is true, at times, by keeping the temperature of the column to a consistent degree centigrade (preferably a few tenths), the results of the chromatograms vary. Mostly, the modern instruments available commercially have a column heater in them (Fig.2.32); which controls the temperature of the column from almost ambient to a confirmed 60–70%. To keep the temperature constant, the columns are sometimes fitted with water jackets. These come from the temperature bath.

To improve the precision of retention time, a column oven is fixed. About 60 °C and temperatures greater then this are used to increase column and flow efficiency. To increase the selectivity during the chiral separations, sub-ambient operations can be used. The oven in the column can work either by directly coming in contact with it or by throwing in circulated hot air. For solvent preheating, the mobile phase should be passed through coiled long tube that is fixed inside the heating element before the column.

Because both the viscosity of the mobile phase and the partitioning process are dependent on the temperature, the column temperature should be maintained. Generally, a 1 °C increase in the temperature will cause a 1–2% decrease in the retention time of the analyte [10] (see Fig.2.34). A viscosity decrease of the mobile phase while the temperature is increasing will give you lower pressures in HPLC columns and that will lead to better diffusion leading to narrower peaks. All this happens in the chromatographic phase. Likewise, narrower peaks arise because of the shorter analyte retention time; hence, detection limits are lower.

This shows that the partitioning of a solute that happens between the mobile and stationary phase phases is driven thermodynamically, and the partitioning is affected by temperature changes. As a result, peak height and retention times change, and there is a change of misidentifying the peaks of the solutes.

Sisco and Gilpin [45] carefully studied the results of how temperature affects the accuracy of the measurement of retention time for both the RP- and NP-HPLC.

By increasing the temperature to 10 °C, there was a change of a 5% in the total time of retention in the RP-HPLC system and for the NP system, and also a change of almost 25%. The solute selectivity changes are also an outcome of the change in the temperature. Poppe et al. [46, 47] have discussed about this phenomenon of temperature gradients that are a result of the viscous heat dissipation and how this affects the modern LC column efficiency. Wilder and Perchalski [48] also saw how important the concept of preheating was in the mobile phase. Heating it to the column temperature prior to sending it to the column is necessary.

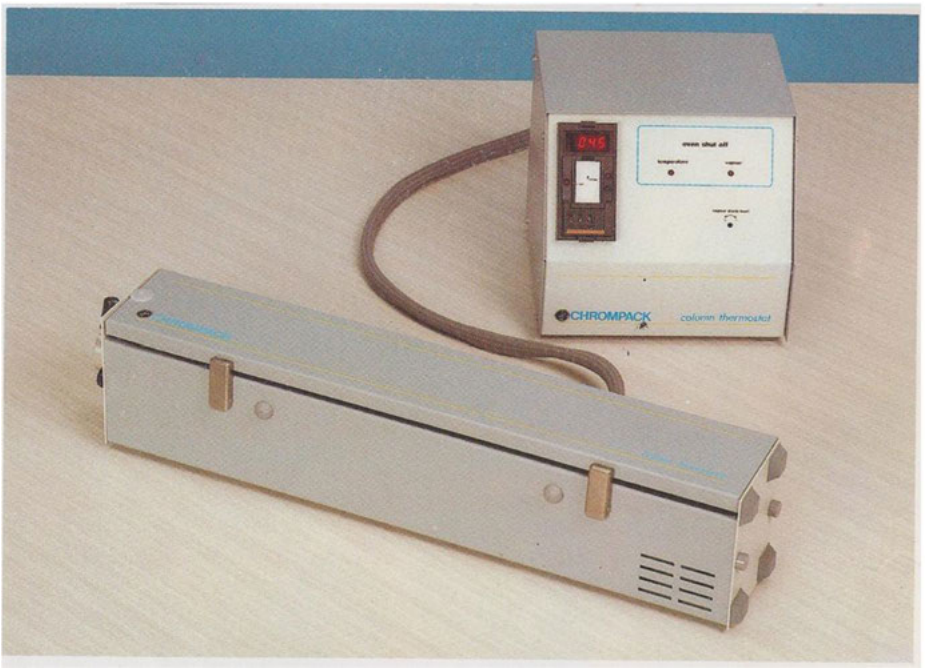



**Fig.2.33:** HPLC column heater.

How thermostating of the column is done also holds special interest. Nowadays, air ovens have been popular with all HPLC manufacturers as shown in Fig.2.33.

### 2.1.12 Column protection

Column life depends on mobile phase composition and pH, sample matrix, contaminants and other factors. You can make approximately 1,000–1,500 injections onto a properly protected RP column. NP columns have slightly shorter life span but should accept about 1,000 injections. Where subjected to extreme conditions,

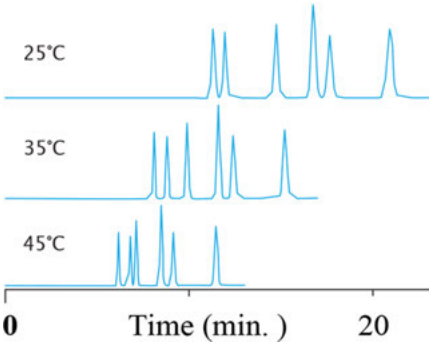



**Fig.2.34:** Effect of temperature on the elution of analytes in HPLC analysis.

columns will be stable for only a few hundred injections. Protect your analytical column with an in-line mobile phase filter as shown in Fig.2.35 and 2.36 for in-line filtration of mobile phase to remove particulate matter from mobile phase that might otherwise clog the column inlet frit.

With columns of different diameters, the issue is with the problem of contamination. This mostly occurs at the top of the column and is because there is a presence of retained compounds while the sample is undergoing analysis. When this contaminated sample is injected again and again in the column, the performance is affected. This is done by the capacity factor, when the plate counts change or sometimes due to solute selectivity. This requires the usage of a "guard" column when you are dealing with actual samples. Typically, it is a short column that is hardly 1–5 cm long, has the same packing as one of the analytical columns and is present in the middle of the analytical column and the injector Fig.2.35 and 2.36. Once done, you can discard the guard column. It can be used again depending on how much contamination is in the sample which is being analyzed. The degradation of the analytical column is used and represents a site for further sample dispersion and dilution. Kirkland et al. [49] show that the variance experiments and calculation should be likely to make use of pellicular packing for the guard column. This will not affect degradation of the separation significantly from the analytical column and will happen only when the sample is at the exact same or lesser on the guard column when compared to the analytical column.

Another problem that occurs is that the modern siliceous stationary phases can slow the silica's dissolution when the mobile phase is predominantly aqueous. This occurs in the RP LC. Using a "saturator" column will suffice to overcome this problem. It is found between both the injector and the pump [50]. Silica is filled in it, and it helps to saturate the dissolved silicates in the mobile phase. It can have any diameter.

The analytical columns' dissolution is greatly slowed down by it. This happens because of the placement of the column. Best of all, it does not add any extra

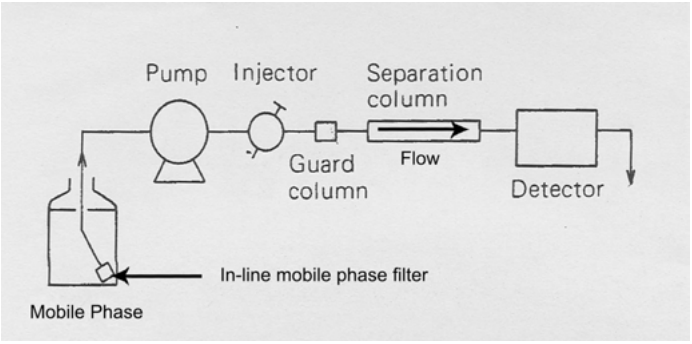



**Fig.2.35:** Illustrative diagram showing the position of an in-line mobile phase filter for filtration of mobile phase and guard column just before the analytical column for its protection from contaminants of sample matrix and particulate matter of mobile phase.

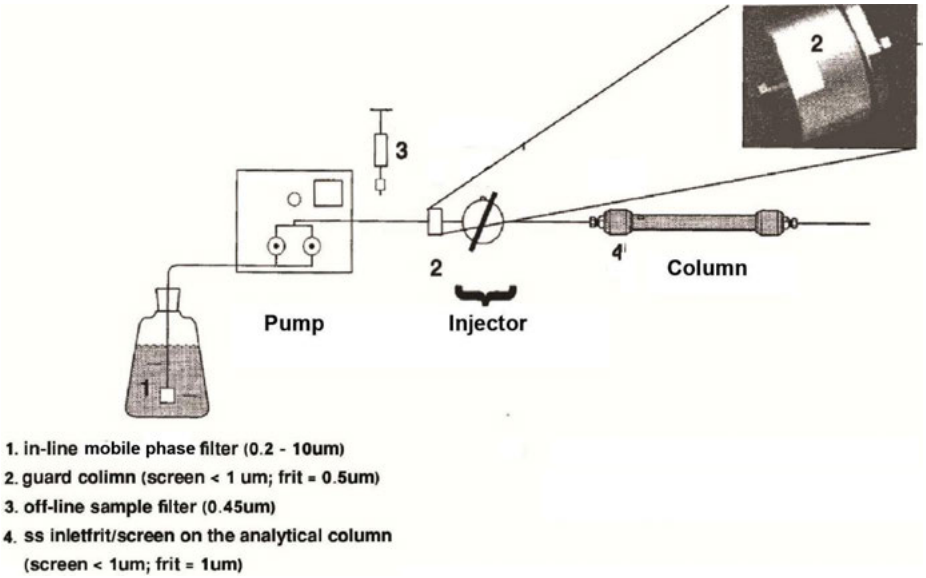



**Fig.2.36:** Illustrative diagram showing in-line mobile phase filter for filtration of mobile phase, off-line sample filter for sample filtration and in-line guard column for protection of HPLC columns from contaminants. Reprinted with permission from Millipore Co., Waters Chromatography Division, 43 Maple Street, Milford, MA 01757, USA [22].

column volume to the separation system. The saturator and guard columns along with the dimensions, expected lifetimes, packing and the characteristics of dispersion have been discussed by Dong et al. [51].

It is important to change the guard columns frequently to make sure the performance of the column is enhanced to its lifetime extent. It is imperative when we are dealing with biological samples or when beverage analysis is conducted.

Properly prepared samples for off-line sample preparation (see also in detail for sample preparation of pharmaceutical in drug QC laboratories, Chapter 9) will extend column's lifetime. There should be nothing introduced in the column that has oils, pertinacious materials, fats and particulates that arise from either the sample or the mobile phase. Ultimately, these will increase the operating pressure of the columns and make it impossible or difficult to remove. The literature contains many methods to conduct sample purification [52–56]. If a scheme is absent, the centrifugation followed by membrane filtration of the sample is sufficient (see Chapter 9). Before injection, the biological samples need to be deproteinized. For deproteinization, sulfosalicylic acid is the preferred agent.

Solvents that have been used for preparation of sample should match the mobile phase composition whenever possible (for details, see Chapter 9 about sample preparation and column protection).

### 2.1.13 HPLC column, cleaning and regeneration

The problem with different diameter columns is common. It is the contamination that occurs on the top of the column because of the presence of retained compounds that are in the sample being analyzed. Repeatedly injecting the samples in the column affects the performance because of the change in either capacity factor, plate count or sometimes the selectivity if the solute. For this, it is going to be beneficial to use a "guard" column when real samples are being dealt with. Typically, it is a short column that is hardly 1–5 cm long, has the same packing as one of the analytical columns and is present in the middle of the analytical column and the injector. After usage, the guard column can be discarded and can also be replaced after intervals if required. It makes use of the slow degradation of the analytical column and offers an extra sample dispersion and dilution. Using both experiments and calculation, Kirkland et al. have shown that it is possible to use pellicular packing for the guard column. This does not degrade the separation of the analytical column without affecting it significantly. This happens if the sample is retained to a lower or the same extent on the guard column when it is compared to the analytical columns [49]. Another problem that occurs with the siliceous stationary phase is the fact that dissolution is very slow in the mobile phase which is aqueous. All this is done in the RP LC. The problem is simple to rectify. Only a "saturator" column is used which is placed in between the injector and pump [50]. Bare silica is used to fill up the saturator. It can be of any diameter and can serve the mobile phase with the dissolved silicates. As a result, the process of dissolution in the analytical column is greatly

slowed down. It adds no extra column value to the separation system. Scavenger and guard columns along with the dimensions, expected lifetimes and packings and the dispersion characteristics have been thoroughly discussed by Dong et al. [51].

The mobile phase and sample impurities can adsorb the inlet of the column and can make changes in the peak splitting or selectivity. Mostly, the dirty columns can be regenerated and cleaned.

Flushing RP columns (phenyl, C18, C4, C1, CN and C8 are RP packing) with a miscible pure organic solvent (methanol, tetrahydrofuran or acetonitrile) will remove a contaminant from a column. Monitoring the UV absorbance during this procedure may permit observation of contaminants being flushed out of the column (Fig.2.37). If you suspect a particular contaminant is building up on the column, use solvents that are known to dissolve the contaminant materials. If the problem is not solved by inverting the column, it can be washed with a sequence of progressively more nonpolar solvents, always making sure that the solvent in the column and the washing solvent are miscible (see Chapter 3 for solvent miscibility). Return the column to the standard RP conditions by reversing the wash sequence. Wash sequence example for regeneration of a contaminated (impurities from the sample and mobile phase) RP packing is found in Fig.2.38. In Fig.2.39, there are wash sequence examples for regenerating a polluted NP packing (silica, $NH_2$, diol and nitro are NP packing). Return the column to the standard NP conditions by reversing the wash sequence. A sequence example for regeneration of a contaminated (impurities from the sample and mobile phase) RP packing is found in Fig.2.38.

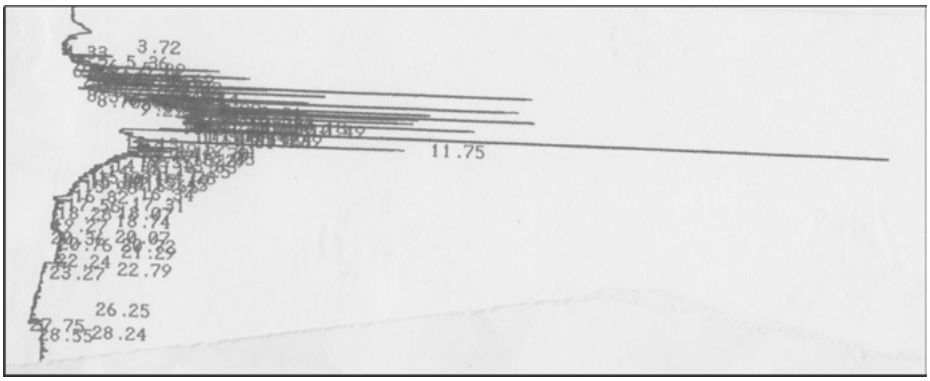



**Fig.2.37:** Monitoring the UV absorbance for an HPLC detector during the cleaning procedures of an HPLC column.

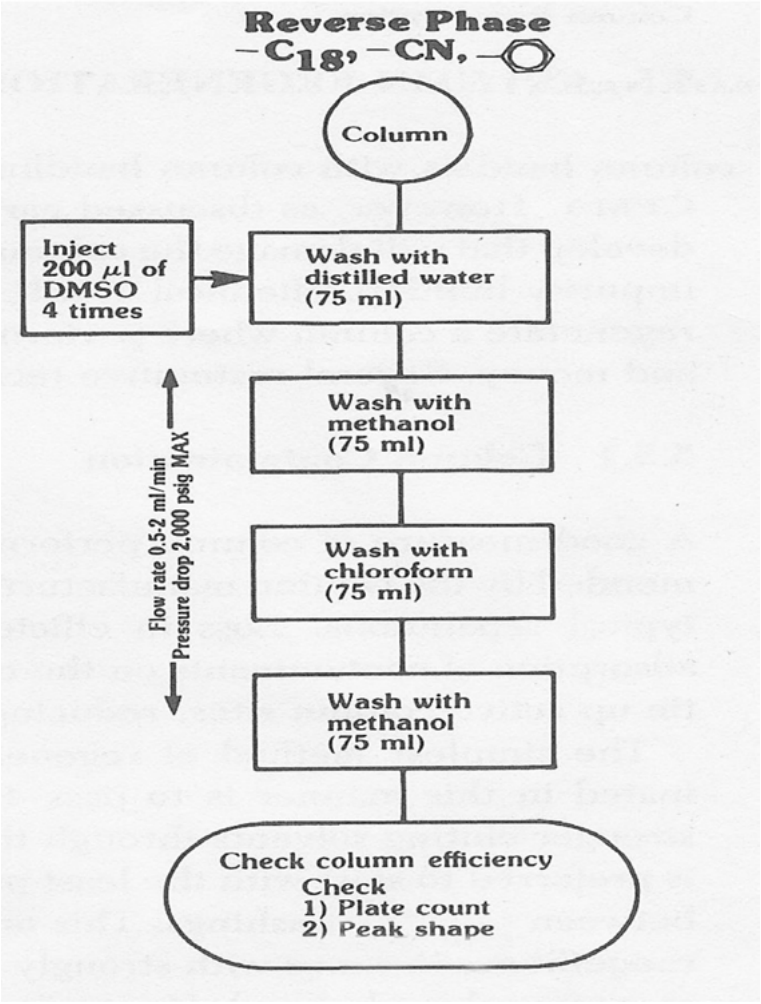



**Fig.2.38:** Example procedure for regeneration of RP-HPLC columns.

**Caution**

During washing:

- Be sure to remain within the limits of the mobile phase pH which is compatible with the column.
- Use only miscible solvents in sequence. This makes sure that there is no precipitate form when the washing solution is mixed with the solvent present in the column (for solvent miscibility, see Chapter 3).
- Monitor the pressure to protect the packed bed.
- Never flush amino columns with ketones or aldehydes which may react with the stationary phase.

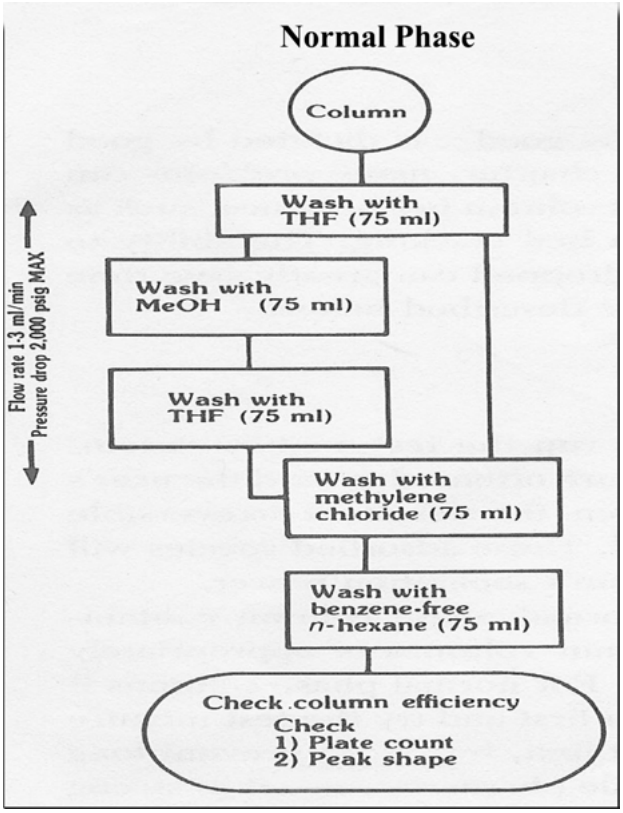



**Fig2.39:** Example procedure for regeneration of NP-HPLC column.

### 2.1.14 Specification of HPLC packings

Each packing materials for HPLC columns can be characterized by means of physicochemical and chromatographic properties Tab.2.10 as follows:

#### 2.1.14.1 Physicochemical properties

The stationary phase is characterized using column dimensions [length (L) and i.d.], particle size ($d_p$), pore size ($r_p$), surface area (A), ligand density (Γ) and carbon content (% C) as shown in Tab.2.10.

- Column dimensions: the separation efficiency is proportional to the length of the column (L), if the HPLC column is longer, and the efficiency will be better. For instance, a double column length will increase the resolution by about 1.4 factors. The i.d. (Fig.2.40) of the HPLC column is an imperative parameter that affects the sensitivity of the detection. It also shows what quantity of the analyte

**Tab.2.10:** Physicochemical and chromatographic characteristics of some HPLC packing (Waters Co. [14]).

| Brand | Chemistry | Particle shape | Particle size (µm) | Pore size (Å) | Surface area (m2/g) | % Carbon load | End-capped |
|---|---|---|---|---|---|---|---|
| µ BondapakTM | C19 | Irregular | 10 | 125 | 330 | 9.8 | Yes |
| µ BondapakTM | Phenyl | Irregular | 10 | 125 | 330 | 9.3 | Yes |
| µ BondapakTM | CN | Irregular | 10p | 125 | 330 | 6.0 | Yes |
| µ BondapakTM | $NH_z$ | Irregular | 10 | 125 | 330 | 4.0 | No |
| Delta-PakTM | $C_4$ | Spherical | 5,15 | 100 | 300 | 3.0 | Yes |
| Delta-PakTM | C18 | Spherical | 5,15 | 100 | 300 | 10. 0 | Yes |
| Delta-PakTM | $C_4$ | Spherical | 5,15 | 300 | 125 | 2.6 | Yes |
| Delta-PakTM | C18 | Spherical | 5,15 | 300 | 125 | 6.8 | Yes |
| Nova-PakTM | C18 | Spherical | 4,6 | 60 | 120 | 3.0 | Yes |
| Nova-PakTM | $C_8$ | Spherical | 4 | 60 | 120 | 4.0 | Yes |
| Nova-PakTM | Phenyl | Spherical | 4 | 60 | 120 | 4.6 | Yes |
| Nova-PakTM | CN HP | Spherical | 4 | 60 | 120 | 3.0 | Yes |
| Nova-PakTM | Silica | Spherical | 4, 6 | 60 | 120 | n a | No |
| µPorasilTM | Silica | Irregular | 10 | 125 | 330 | n a | No |
| ResolveTM | C18 | Spherical | 5 10 | 90 | 200 | 10.2 | No |
| ResolveTM | $C_8$ | Spherical | 5 | 90 | 200 | 5.1 | No |
| ResolveTM | CN | Spherical | 10 | 90 | 200 | 3.0 | No |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| ResolveTM | Silica | Spherical | 5, 10 | 90 | 200 | n a | No |
| Symmetry | C18 | Spherical | 3.5, 5 | 100 | 335 | 19.1 | Yes |
| Symmetry | $C_8$ | Spherical | 3.5, 5 | 100 | 335 | 11.7 | Yes |
| Symmetry ShieldTM | $RP_8$ | Spherical | 3.5, 5 | 100 | 335 | 15.0 | Yes |
| Symmetry ShieldTM | $RP_{18}$ | Spherical | 5 | 100 | 335 | 17.0 | Yes |
| Waters Spherisorb™ | $ODS_2$ | Spherical | 3, 5, 10 | 80 | 220 | 11.5 | Yes |
| Waters Spherisorb™ | ODS | Spherical | 3, 5, 10 | 80 | 220 | 6.2 | No |
| Waters Spherisorb™ | ODSB | Spherical | 5 | 80 | 220 | 11.5 | No |
| Waters Spherisorb™ | $C_8$ | Spherical | 3, 5, 10 | 80 | 220 | 5.8 | No |
| Waters Spherisorb™ | $C_6$ | Spherical | 3, 5, 10 | 80 | 220 | 4.7 | No |
| Waters Spherisorb™ | $C_1$ | Spherical | 3, 5, 10 | 80 | 220 | 2.2 | No |
| Waters Spherisorb™ | Nitrile | Spherical | 3, 5, 10 | 80 | 220 | 3.1 | No |
| Waters Spherisorb™ | Amino | Spherical | 3, 5, 10 | 80 | 220 | 1.9 | No |
| Waters Spherisorb™ | Phenyl | Spherical | 3, 5, 10 | 80 | 220 | 2.5 | No |
| Waters Spherisorb™ | OD/CN | Spherical | 5 | 80 | 220 | 5.0 | No |
| Waters Spherisorb™ | SAX, | Spherical | 5, 10 | 80 | 220 | 4.0 | No |
| XTerraTM RP | $RP_{18}$ | Spherical | 2.5, 3.5, 5, 7 | 125 | 175 | 15.0 | Yes |
| XTerraTM RP | $RP_8$ | Spherical | 2.5, 3.5, 5, 7 | 125 | 175 | 13.5 | Yes |
| XTerraTM RP | C18 | Spherical | 2.5, 3.5, 5, 7 | 125 | 175 | 15.5 | Yes |
| XTerraTM RP | $C_8$ | Spherical | 2.5, 3.5, 5, 7 | 125 | 175 | 12.0 | yes |

needs to be loaded inside the column. When the columns' i.d. is reduced, the back pressure increases. Preparative and larger columns have lower resolving power and sensitivity when compared to the analytical columns that are smaller.

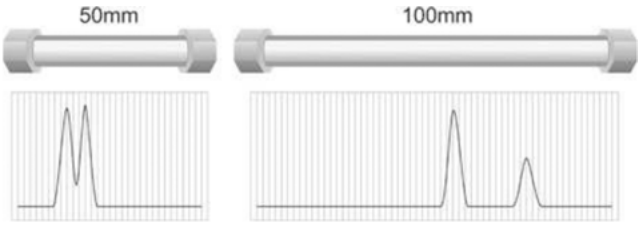



**Fig.2.40:** Effect of column length on its separation power.

- **Surface area of the phase (A) $m^2/g$:** In adsorption chromatography (NP), retention is directly proportional to the surface area. Also the sample loading is related to the surface area.
- **Carbon content (% C):** This expresses the molar percentage of carbon in the bonded phase.
- **Ligand density (Γ) $\mu mol/m^2$:** This represents the amount of silanols (total about 8 $\mu mol/m^2$) that has reacted with the ligand. So the higher the number, the less residual groups remain on the surface.
- **Particle size ($d_p$) µm:** The relative standard deviation expresses the particle size distribution that is of importance to the efficiency of packing and to the back pressure. Fig.2.41 shows the effect of **particle size of stationary phase on the** separation power of an HPLC column.

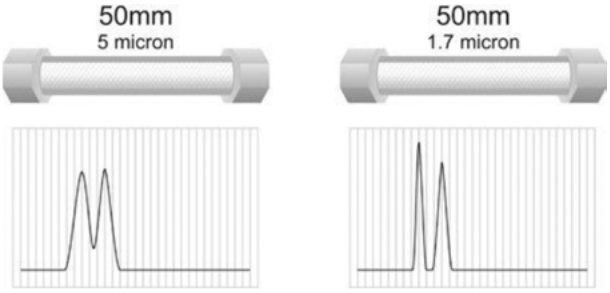



**Fig.2.41:** Effect of **particle size of stationary phase on the** separation power of HPLC column.

- **Pore size ($r_p$) nm or Å (1 nm = 10 Å):** The pore size or even more the pore size distribution is of importance for the diffusion of components.

#### 2.1.14.2 Chromatographic characteristics

The use of dimensionless parameters such as retention, selectivity, efficiency, peak **asymmetry** and flow resistance gives a total view of each column, regardless of dimensions. Also, between different kinds of stationary phases, comparisons can be made (for details about this subject, see Chapter 8).

### 2.1.15 HPLC column troubleshooting

Changes in retention time, resolution or back pressure are often due to column contaminations. Also poor chromatography is often the result of a system problem. HPLC system manufacturers usually published a guide to successful operation for their equipment and troubleshooting for any problems as with Waters Co. [14] and Phenomenex [21]. At local level, the experienced team of chromatographic specialists of these manufacturers can provide a customer support for corrective, preventive and quality maintenance. Information about HPLC column troubleshooting is given in Tab.2.11.

**Tab.2.11:** HPLC column troubleshooting.

| Symptom | Cause | Corrective action | Prevention action |
|---|---|---|---|
| Buildup pressure in the system | Inlet filter plugged with particulates | Replace the inlet filter, or cleaning it in ultrasonic bath | Usually filter the mobile phase using the filtration system (see Chapter 3) |
| | Sample precipitate on the column (sample not soluble in the mobile phase) | Wash the column using a solvent that will dissolve the sample | Use mobile phase that is compatible with sample |
| | Clogged tubing | Replace the tubing | Identify and eliminate the source of the material clogging the tubing |
| | Poor sample preparation | Clean up the sample | Use guard column and good sample preparation (see Chapter 9) |
| | Contaminated column | Column regeneration according to Fig.2.38 and 2.39 | Use guard column to clean up the sample and protect the column. Use HPLC-grade solvent |

**Tab.2.11** (continued)

| Symptom | Cause | Corrective action | Prevention action |
|---|---|---|---|
| Loss of resolution, low plate count | Sample solvent is incompatible with, or stronger than, the mobile phase | Dissolve sample in another solvent | Dissolve sample in mobile phase |
| | Insufficient equilibration | Continue equilibration | Dedicate each column to a single application |
| Loss of resolution, low plate count (continued) | Incorrect connecting tubing | Replace with 0.25 mm (0.009″) internal diameter tubing | All tubing from injector to the detector should be 0.25 mm internal diameter |
| | Frit partially blocked | Replace or clean the filter | Use an in-line filter between the column and injector<br>Filter the sample or use a Guard-Pak holder and insert |

## 2.2 Ion-exchange (IC, IEC or IEX) and size-exclusion (SEC) columns for HPLC

### 2.2.1 Introduction

The importance of stationary and mobile phase selections in HPLC cannot be overemphasized, since variations in these parameters are used to exploit different types of solute interactions (different types or modes of HPLC separation; different modules of HPLC separations). These interactions give rise to the following separation modes which are familiar in use in drug QC laboratories Tab.2.12. The most simple interactions involves separation of solute molecules based on their charge. This approach is known as IC (ion chromatography) or IEC [10, 44, 64–68, 70–72]. Very complex interactions can be accessed by this technique, and it is quite sensitive to even minute ionic compounds. Another means of separating molecules is based on size or shape differences. This is called SEC [10, 44, 64–70] and is widely used in the comparison of complex formulations of pharmaceutical products.

We shall try to describe these two modules in some detail further.

**Tab.2.12:** General specifications for IC and SEC packings for HPLC columns.

| Mode or type | Description |
|---|---|
| Ion-exchange chromatography (IC or IEC) | Uses packings that have functional groups that are charged and bind together ions that have opposite charges, and mobile phases that have a mixture of salt, buffer and water. |
| Size-exclusion chromatography | Uses inert packings with pores of a defined diameter, and mobile phases can be aqueous or organic; used for separating compounds by molecular weight, especially macromo molecules |

### 2.2.2 Ion Chromatography (IC, IEC and IEX)

This is a type of HPLC where modifications in the system are done to carry out the analysis of inorganic anions ($Cl^-$, $I^-$, $NO_3^-$, etc.), cations ($Na^+$, $K^+$, $Mg^{2+}$, etc.) and ionized organic compounds (amines, amino acids and organic acids) [10, 44, 64–68, 70–72]. For the system, an exclusive ion-exchange analytical column is required which is only specific for the analytes that are of interest, a very simple detector to check for conductivity [10, 64–68, 70–72], along with ion suppressor column that is used to remove ions apart from the analytes that will produce the electrical conductivity in the flow cell detector [10, 44, 64–68, 70–72]. Tubing and columns are mostly composed of PEEK (Poly Ether Ether Ketone) to make sure there are no metal surfaces involved.

This chromatographic technique is used to pull apart and determine ions on the columns that have a lower ion-exchange capability. This is based on the ion-exchange equilibrium that occurs in the solution and the counter ions to pair with oppositely charged ions that fix to the stationary phase. It is going to have either a negative and positive functional groups fixed to it. Usually it is a quaternary amine ($-N(CH_3)_3^+$), or a sulfonate ($-SO_3^-$).

IC is a very important tool to conduct pharmaceutical analysis, which is being used by the chemists who are working in the labs to carry out QC for drugs. It is used to analyze cations, anions and the ionized active ingredients of pharmaceuticals including organic acids, amino acids and amines [10, 44, 64–68, 70–72]. The technique's high sensitivity along with the dynamic and wide operating range is only made possible because of the modern stationary phases that are higher in capacity; this makes the technique ideal for analyzing the ions present in the pharmaceutical applications. Both the suppressed conductivity detection and the mobile phase's gradient elution combine to provide a very powerful and useful tool for screening of the drug substance ions that are present in the pharmaceutical formulations. This offers the base for analyzing the counter additives, manufactured by-products and the counter ions. The IC technique has already been expanded to a number of different fronts and now covers many of the analytes, and there is a

great improvement in the stationary phase design, suppressor design, detection sensitivity, chromatographic performance and electrolytic generation of eluents. Now that there has been so much improvement, IC is not restricted to only ion exchange but is so much more. Fig.2.42 and Fig.2.43 present a chromatogram for analysis of eight cations and eight anions using IC technique. It is apparent that using IC technique, the analysis time takes about 15–25 min. Complete analysis of the same set of these anions or cations by a combination of potentiometry and spectrophotometry requires 1–2 days.

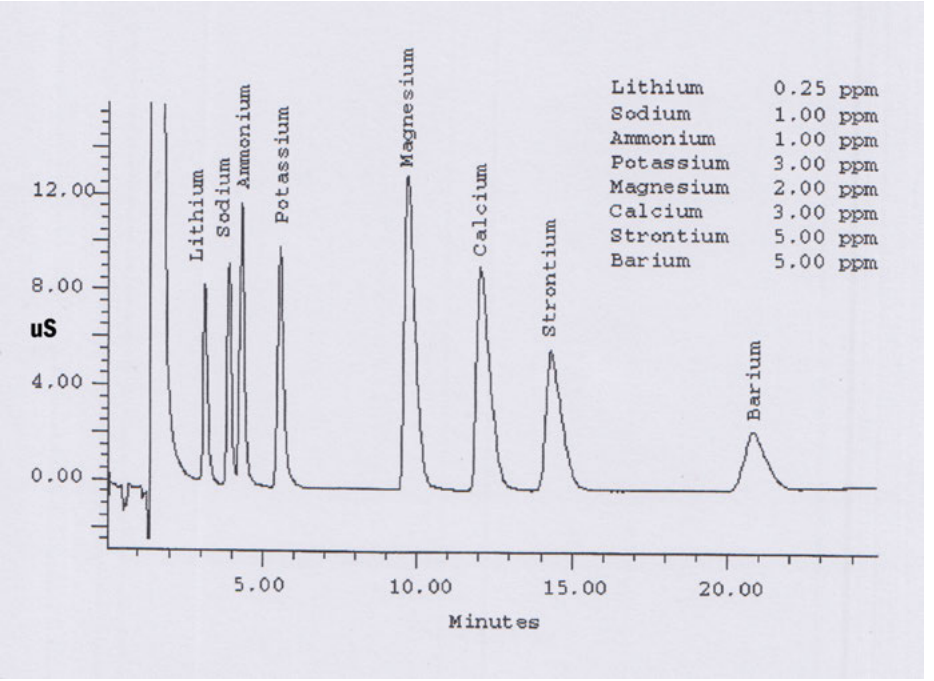



**Fig.2.42:** A representative chromatogram of the eight cation standards run on an IC-Pac C M/D column with mM EDTA/3 mM $HNO_3$ eluent at 1 mL/min flow rate and conductivity detection (reproduced with permission from Waters Co. [14]).

The stationary phase in this mode of chromatography is capable of ion exchange, that is, the surface has electric charges on the surface. The ionic groups like $[N(CH_3)_4]^+$, $[SO_3]^-$, $[N\,HR_2]^+$ and $[COO]^-$ have been included in the gel or resin of the stationary phase. The counter ions of the mobile phase are used to carry out neutralization. Ionic sample molecules and ions are present in the mobile phase and they fight for getting a place on the surface of stationary phases (columns with charged functional groups that bind ions of opposite charge).

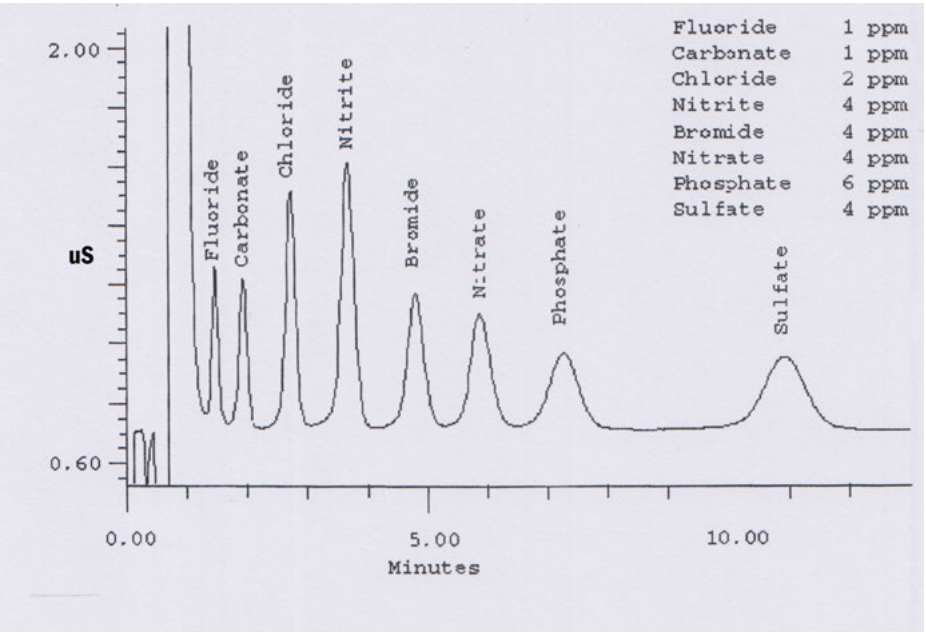



**Fig.2.43:** A representative chromatogram of the eight anion standards run on an IC-Pac column with sodium borate/gluconate eluent at 1.2 mL/min flow rate and conductivity detection (reproduced with permission from Waters Co. [14]).

As mentioned in Chapter 3 and in Section 2.1, the mobile phase of the IEC needs to have the same characteristics of other chromatography methods. This means that the sample needs to be dissolved, the solvent strength should be enough to lead to good time of retention and have the right values of K, and the interaction of it with solutes should be such that it leads to selectivity. The IEC has aqueous mobile phases that can have a moderate amount of methanol or something else.

– SI unit for measuring the electrical conductivity [Siemens/m (S/m) or μS/cm].

*SI unit for measuring the electrical conductivity [Siemens/m (S/m) or μS/cm].

### 2.2.2.1 Principles of ion chromatography

In IC, separations between ions are dependent on the electrical charges. The rule is simple: opposites attract while similar repel. Therefore, the IC's stationary phase is characterized through the strength and the nature of the basic or acidic functions group on the surface. It also depends on the ions that are retained and attracted. Cation exchanger stationary phase is used for separating and retaining the positively charged ions known as cations on the surface that is negative (Fig.2.44A). On the other hand, when the negatively charged ions known as anions are to be retained

and separated on a positive surface, the anion exchanger stationary phase is used see Fig.2.44B. Whenever an ion exchange takes place, at a minimum, two approaches are generally used to separate and elute the investigation of the ions.

- Strong ion exchangers. These ion exchangers contain a functional group like sulfonic acids or quaternary amines (Fig.245a,c) that are almost always ionized. They are usually used as a means to separate and retain the ions that are weak. One method to carry out the elute process of these weaker ions is through displacement with the mobile phase that has stronger ions that will strongly attract to the sites of the stationary phase. The alternate method to use is to retain the weak ions in the column and end up neutralizing them in a site changing of the pH level of the mobile phase. This causes a loss of attraction resulting in being an elute.
- Weak ion exchangers. These ion exchangers contain a functional group, e.g. carboxylic-acid or secondary amine (Fig.2.45b,d) might be neutralized below or above the pH value defined and lose their ability to use charge to retain the ions. Once they are charged, they can be used to separate and retain the stronger ions. If these ions are not able to be displaced, then it is required to neutralize the stationary phase exchange, the ionic attraction is shut off and elution of the charged analytes occurs. When the weak ion exchange neutralizes, they may retain and separate species by hydrophobic (RP) or hydrophilic (NP) interactions; in these cases, elution strength is determined by the polarity of the mobile phase. Thus, weak ion exchangers may be used for mixed-mode separations (separations based on both polarity and charge).

Analytical columns for IC are mainly divided into columns for anions and cations and have the same diameter like the standard HPLC columns but it is a little longer when it comes to length. Cationic-exchange columns contain particles that have on its surface weak sites for acid like $-COO^- H^+$ carboxylic acid groups (Fig.2.45b) or sites with strong acids like $-SO_3^- H^+$ sulfonic acid (Fig.2.45a). These change preferentially with the analyte cations. As presented in Fig.2.44A, these cation exchange particles have negative charge on their surface.

Common anionic exchange sites include the strongly basic tertiary amines ($-N(CH_3)_3^+OH^-$) (Fig.2.45c and the weakly basic primary amine group ($-NH_{R2}^+OH^-$) (Fig.2.45d). As presented in Fig.2.44B these cation-exchange particles have negative charge on their surface. Both of these exchange groups are placed on the porous microparticles of silica (SAX and SCX columns, see Fig.2.12) or polymer resin (Fig.2.46). As the sample injected goes through the analytical column, the following reaction occurs on the surfaces of the cation and anion exchangers:

- Cation exchanger

$R\text{-}SO_3^- H^+$ (solid) + (metal cation)$^+$ in solution to be analyzed $\leftrightarrow$ R-SO3$^-$ metal cation$^+$ (solid) (metal retained on the surface of the cation exchanger) + $H^+$ in solution.

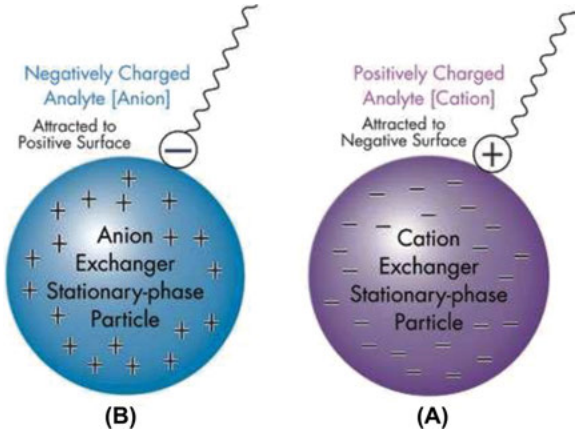



**Fig.2.44:** Illustrative diagram showing anion (positive charged surface) and cation (negative charged surface) exchanger stationary phase particles.

Similarly for anion analysis, the reaction would be

– Anion exchanger

$NHR_2^+OH^-$ (solid) + (anion ion)$^-$ in solution to be analyzed ↔ -$NHR_2^+$ anion ion$^-$ (solid) anion retained on the surface of the anion exchanger + OH in solution.

At the correct rate of flow, each analyte that passes through the column is set up through equilibrium migration. Each of the analytes have different affinities for its ion exchange, and each analyte will elute from the column at different times. As in all types, chromatographic identification is largely based on the retention time of the eluted ions.

In Tab.13 there are guidelines which were proposed by Waters Co. [14] for the principal categories of ion exchangers. For example, to retain a strongly basic analyte (always positively charged), use a weak-cation-exchange stationary phase particle at pH > 7; this assures a negatively charged particle surface. To release or elute the strong base, lower the pH of the mobile phase below 3; this removes the surface charge and shuts off the ion-exchange retention mechanism (as it is seen in the last column in Tab.13).

To assure an essentially neutral, or a fully charged, analyte or particle surface, the pH must be adjusted to a value to at least 2 units beyond the p$K_a$ (note that a p$K_a$ is the pH value at which 50% of the functional group is ionized and 50% is neutral).

As appropriate (indicated in Tab.2.13). to be delete do not use a strong cation exchanger to retain a strong base; both remain charged and strongly attracted to each other, making the base nearly impossible to elute. The stationary phase in this mode is capable of ion exchange, that is, it has a number of electric charges on the surface. Ionic groups such as $SO3^-$, $COO^-$, $NH_3^+$ or $NR_3^+$ are some of the ionic groups that are used in the resin or gel of the stationary phase. Through counter

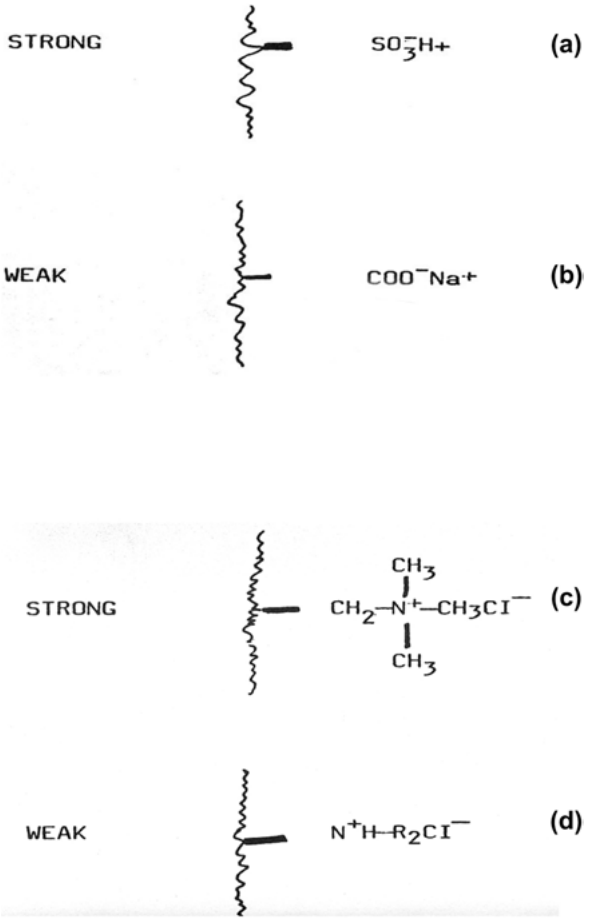



**Fig.2.45:** Generalized structure for strong and weak ion exchangers.

ions that are present in the mobile phase, the charges can be neutralized. There are ionic sample molecules and ions present in the stationary phase that fight each other for a place on the surface. The question is: how stimulation of the competition of the ions on the sample and the mobile phase can happen? The answer is: by ensuring optimum conditions through careful selection of the following:

- What types of ions are in the ion exchanger?
- The mobile phase's pH
- The mobile phase's concentration
- The type of counter ions in the mobile phase

Note: One or all of them can vary, if required.

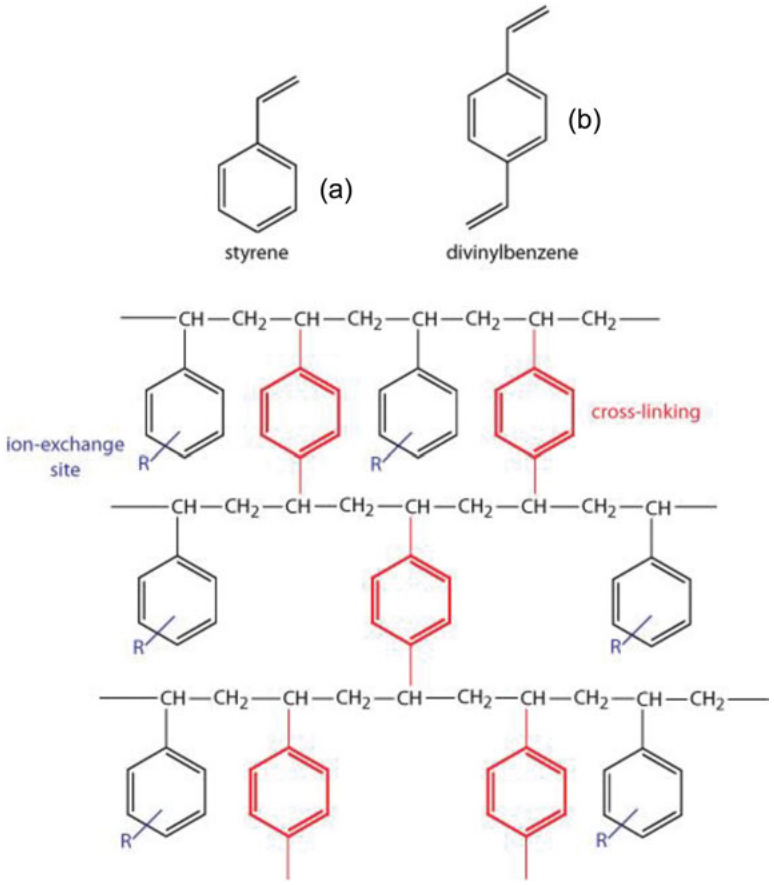



**Fig.2.46:** Structure of a cross-linked polystyrene ion-exchange resin. Different packings can be prepared in which the following chemical groups $[N(CH_3)_4] + Cl^-$, $[SO_3]^- H+$, $[N\ HR_2]^+Cl^-$ and $COO^- Na^+$ can be incorporated as R.

### 2.2.2.2 Packing materials (stationary phase) for IC columns

Generally, the IC is conducted on porous, small beads that are formed in the emulsion copolymerization process of divinylbenzene Fig.2.46b and styrene (Fig.2.46a). The reason for cross-linking is the fact that divinylbenzene is present in the sample (Fig.2.46). But the quantity should be around 8%, which gives the beads their mechanical stability. To ensure that the polymer is active when it comes to acids, ions and other functional groups are chemically bonded to the structure. Mostly, quaternary amines and sulfonic acid are used. You can see strong acid resin structure in Fig.2.47 (note the cross-linking that holds the linear polystyrene molecules together). Apart from the functional groups, all the resins have structures similar to each other. When it comes to chromatographic packings, particles that are porous

**Tab.2.13:** Guidelines for selection of ion exchangers according to Waters Co. [14].

| Analyte Type | Weak ACID e.g PK a -5 | | Strong ACID | Weak BASE e.g -Pk a -10 | | Strong BASE |
|---|---|---|---|---|---|---|
| Charge State vs. pH | No Charge at PH < 3 | - (anion) at pH >7 | - ( anion) Always Charged | + Cation at pH <8 | No Charge at PH > 12 | + Cation Always Charged |
| Stationary Phase Particle | Strong Anion Exchanger | | Weak Anion Exchanger e.g -Pk a -10 | Strong Cation Exchanger | | Weak Cation Exchanger e.g PK a -5 |
| Charge State vs. pH | + Always Charged | | + at pH < 8 | No Charge at PH > 12 | - Always Charged | No Charge at PH < 3 | - at pH > 7 |
| Mobile Phase pH Range | | | | | | |
| To Retain analyte (capture ) | pH >7 | | pH < 8 | | pH < 8 | pH >7 |
| To Retain analyte (elute ) | pH <3 | | pH > 12 | | pH >12 | pH < 3 |

polymeric are not good. The reason is that the diffusion rate is very slow. The molecules of the analyte pass through the polymer matrix's pores because the matrix has compressibility. This problem has been overcome after the production of two new packings that are generally used more than this type of porous polymer. One of the two techniques is pellicular bed packing. This has a larger surface area of around 30 and 40 µm, is made of glass, is spherical and nonporous, and the polymer bed is covered with a synthetic ion-exchange resin. The second packing uses a porous coating of the silica microparticles. The ones that can be used in absorption chromatography have a thin exchanger film. Whichever type is used, efficiency is increased as the diffusion process becomes faster because of the polymer film. On the contrary, these particles have lower sample capacity, especially when the pellicular type is considered.

For the selection of the type for ion exchanger, a resin that has $SO_3^-$ groups is a strong cation exchanger and a $COO^-$ resin is a weak exchanger + is a strong exchanger while the one with $NR_2H^+$ considered to be a weak ion exchanger. The exchange capacity may also vary by altering the pH of the eluent. A weak cation exchanger is not dissociated if the pH is below 4, as its ionic groups are weak acids. This cation exchanger is fully dissociated if the pH rises above 8 when its ions are able to interact with the sample ions resulting in maximum exchange capacity. Therefore, in the pH range 4–8, weak cation exchanger is only partly dissociated. Anion exchangers follow a similar pattern. In general, strong ion exchangers are preferred. If the molecules of the samples are either strong bases or acids, the retention times are hardly affected by the changes that occur in the mobile phase of the pH. In this case, it may be difficult to elute sample molecules, so a weak exchanger may be chosen instead.

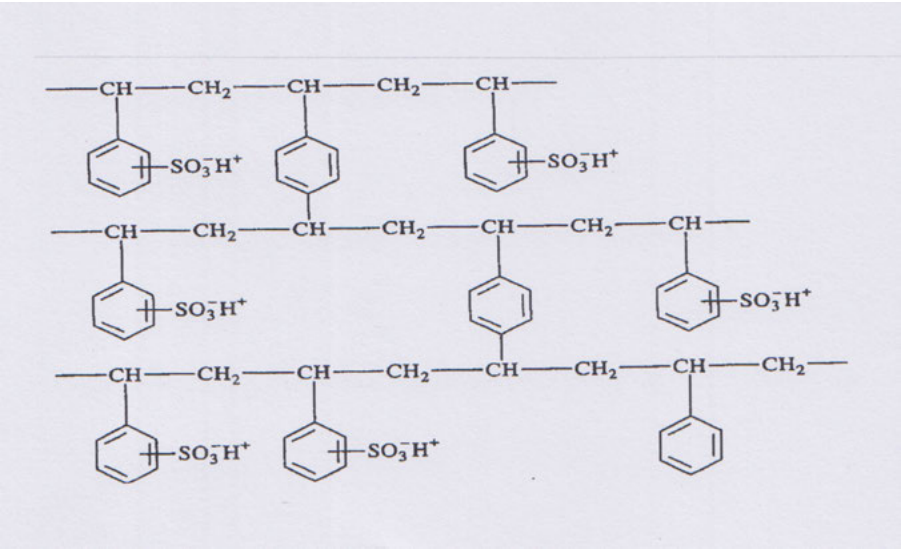



**Fig.2.47:** Illustrative diagram showing the structure of a cation exchanger resin with strong acid group (note the cross-linking that holds the linear polystyrene molecules together).

### 2.2.2.3 Mobile phase for IC

IC has aqueous solutions in the mobile phase that has ionic species. Mostly, these are in a buffer form. The eluent which is aqueous and has sodium bicarbonate of the nM of sodium carbonate, this can be used to separate a number of inorganic ions. The relative and absolute concentrations of $NaHCO_3$ and $Na_2CO_3$ define the eluent strength. With about 1 mM hydrochloric acid as eluent, the alkali metal ions $Li^+$, $Cs^+$, $NH_4^+$, and the ions of small aliphatic amines can be eluted. The mobile phase for IC need to have similar characteristics that other chromatography types require (see Chapter 3). This means that they have to dissolve the samples and have enough strength of the solvent that allows for good retention times or the right values of K, and their interaction with solutes should be as such that gives rise to selectivity.

### 2.2.2.4 IC instrumentation

IC is a type of HPLC which needs modification of the system for carrying our analysis of the inorganic anions ($Cl^-$, $I^-$, $NO_3^-$, etc.), cations ($Na^+$, $K^+$, $Mg^{2+}$, etc.) and ionized organic compounds (amines, amino acids and organic acids [10, 44, 64–68, 70–72]. There needs to be a dedicated ion-exchange special analytical column that is only used for the analytes that are of interest, a simple detector to check the conductivity [10, 64–68, 70–72] and an exclusive column for ion suppressor that helps in removing the ions and not the analytes that will lead to generation of the

electrical conductivity in the flow cell of the detector [10, 44, 64–68, 70–72] (the principles of ion suppressors will be discussed in Section 2.2.2.5).

The instrument that is typically required for IC can be seen in Figure 5.10. Sample injection is the usual, that is, 100 µL is injected into the mobile phase, for example, $NaHCO_3/Na_2CO_3$ solution for anion separation, and approximately 1 mM of hydrochloric acid is used as an eluent. The alkali metal ions $Li^+$, $Cs^+$, $NH_4^+$, and the ions of small aliphatic amines that can be eluted [10, 44, 64–68, 70–72] is pumped into the system through a reservoir, filtered as well as degassed to the column for separation. Once the injector is done, the sample enters into the guard column first (which is full of gel-like phase that is size excluding) for removing the matter that can end up blocking the analytical column over a period of time. The process of separation is done in the analytical column that consists of either a cation exchange when the cations are to be separated or the cation exchanger when the cations are to be separated. Depending on the molecular dimensions and the charge, for mobile analytes, the time of residence is different. Afterward, a so-called suppressor column follows filled with a cation exchanger for analysis of anions and anion exchanger for analysis of cations (the ion suppressor) will reduce the background conductivity and relatively increase the conductivity of the analyte (the principles of ion suppressors will be discussed in Section 2.2.2.5).

The most widely used detection system in IC is the conductometric detector (see Chapter 5) where two electrodes have a potential placed in between them. When there are no ions in the solution that is passed through the detector, no to minimum current is generated by the electronic instruments. As either of the cations, anions and analytes pass in the detector's flow cell, the current is generated. This is proportional to the analyte concentration. The detector is used to measure conductivity, which means the total mobile phase in the flow cell as a bulk property (see Chapter5). Inorganic ions or ionizable, charged analytes and the pharmaceutical product's active ingredients like the amino acids and amines are measurable only if the conductivity they have is different than the conductivity of the eluents. In solvents that have lower conductivity, the charged analyte separation can pose a problem. On the contrary, the eluents that are buffered have better characteristics for separation, higher basal conductivity and differences in the conductivity when they are difficult to detect the analytes present. Under these conditions, to compensate for the buffering component charges, an ion suppressor is used between the detector and the chromatographic column as can shown in Figure 5.10 (the principles of ion suppressors will be discussed in Section 2.2.2.5).

A suitable flow cell having two electrodes placed inside it makes up the conductivity detector (Figure 5.10). For the cells that come into contact, electrodes that are made of gold, platinum or stainless steel are positioned right in the eluent. Two electrodes make up the electric circuit. They are arranged in the shape of the arm of the Wheatstone Bridge. As the sensor cell has movement of the ions, the electrical impedance of the electrodes is modified and the signal goes into the electronic unit that is suitable

from the bridge. The final outcome comes from the amplifier and is either directly passed to the potentiometric recorder or digitized to be used in computer form.

#### 2.2.2.5 Principles of ion suppressors

As mentioned previously, the conductivity detector measures the total conductivity of the mobile phase that is present in the flow cell in the form of a bulk property. Inorganic ions, charged analytes or organic chemicals that can be ionizable like amino acids and amines are measurable if they have a different conductivity than that of the eluent. In solvents that have lower conductivity, separating charged analytes will pose difficulty. On the contrary, buffered eluents with higher and better characteristics for separation, higher basal conductivity and when a difficult to detect analyte is present, differences in the level of conductivity arise. If this is the case, an ion suppressor placed between the detector and the chromatography column needs to be used to compensate for the buffering components. Because of the ion suppressor, the background conductivity will be reduced and the analyte conductivity will increase relatively. To explain this principle, chloride anion (NaCl) in a $NaHCO_3$ containing eluent is analyzed. A strong cation exchanger is present in the ion suppressor column and that too in the protonated form ($R\text{-}SO^{-}\ 3H^{+}$). The following exchange reactions will occur:

$$(R\text{-}SO_3^{-}\ H^{+}) + NaHCO_3 \text{ ---------- } R\text{-}SO_3^{-}\ Na^{+} + H_2O + CO_2 \tag{2.2}$$

$$R\text{-}SO_3^{-}\ H^{+} + Na^{+} + Cl\,(\text{anion to be analyzed}) - R\text{-}SO_3^{-}\ Na^{+} + H^{+} + Cl^{-} \tag{2.3}$$

Neutralization of the bicarbonate of the eluent occurs (eq. (2.2)) to significantly lower the eluents conductivity. Even though chloride as an analyte does not have any effect on it, the counter ion $Na^{+}$ gets exchanged with a proton (eq. (2.3)). Overall, the sensitivity on the whole is increased significantly because the conductivity detector can analyze both the counter ion and the analyte, $Na^{+}$ and $Cl^{-}$, respectively.

To make use of conductivity detection in the analysis of cations, the high background conductivity of the HCl (for cation analysis using IC HCl can be used as mobile phase) has to be eliminated. For this, a suppressor that has a strong basic anion exchanger that is in the $OH^{-}$ form is used, and this converts the $H^{+}$ to $H_2O$ and the ions of interest to their hydroxides as follows:

$$R\text{-}N(CH_3)^{+}\ 3OH^{-} + HCl\,(\text{from mobile phase}) \text{ ---------- } R\text{-}N(CH_3)3^{+}Cl^{-} + OH \tag{2.4}$$

Nowadays, some companies (Water Cor.[14]) have developed an electronic technique for removal of the electrolyte background in the mobile phase passing through the conductivity detector (no need for ion suppressors). Other companies use very low capacity ion exchanger that allows the dilute electrolyte solutions as the mobile

phase. For these systems, they will not exhibit good detection limits that the suppressed methods show.

#### 2.2.2.6 IC applications for ion analysis

As we have mentioned previously, this separation principle has its basis on the repulsion or attraction of the species that are charged and it follows Coulomb's law. Most of the molecules that are organic have different functional groups. These are easily chargeable and can be done by simple changing the pH value when they are dissolved in the aqueous phase. The sulfonic acids, carboxylic acids, amino acids and amines can be separated successfully by IC [10, 44, 64–68, 70–72]. The larger molecules which include nucleotides and proteins carry these groups and can be separated and charged too. This is because mostly these analytes are in the aqueous solution. This allows introducing them directly in the IC. This cannot be done for LC and gas chromatography.

The nature of the applied ion exchange allows one to divide the application into two chromatographic forms: anion exchange and cation exchange.

The exchange equilibrium for IC is

$$R-X-C^{+} + M^{+}B^{-} \text{----------} R-X-M^{+} + C^{+} + B^{-} \tag{2.5}$$

for cation-exchange chromatography, or

$$R-X+A^{-} + M^{+}B^{-} \text{----------} R-X+B^{-} + M^{+} + A^{-} \tag{2.6}$$

for anion-exchange chromatography.

R–X is the ion-exchanging backbone (e.g., for anion exchange, the ion-exchanging backbone is ion-exchange resin containing positively charged groups, such as diethylaminoethyl groups) and is applied as stationary phases; for cation exchange, the ion-exchanging backbone is ion-exchange resin containing negatively charged groups such as $SO_3^{-}$. The resins are packed as small sphere particles into a tubular column. For mobile phase, weak salt or buffer solutions of appropriate pH and ion strength are prepared.

##### 2.2.2.6.1 IC applications for anion analysis

Before the modern-day IC came into existence, determining and separating the anions was a very difficult and laborious process. An analyst who has ever tried analyzing $SCN^{-}$, $CN^{-}$, $Cl^{-}$ and $I^{-}$ knows about the difficulties and uncertainties that can occur. It can be either quantitative or qualitative. Now that modern IC is here, it only takes some minutes to answer "how much" and "what."

For anionic analysis using IC, the stationary phase consists of a highly efficient ion-exchange resin with a lower capacity of the R–N $(CH_3) + 3$ type. The process of elution is isotactically carried out. The retention of ions to be separated is determined by their respective affinity to the ion-exchange active sites on the stationary

phase and the strength of the eluent. The higher affinity the exchange sites have, the higher the respective ionic solutes' retention. To increase the transfer of mass quickly and decrease the great peak broadening, a number of exchange resins that are tailor-made are developed commercially. Mostly, these are pellicular in type.

Multiple inorganic anions are separated easily when you use one or more than one anion exchanger along with an eluent that is aqueous in nature and has a little quantity of mM of either sodium bicarbonate of carbonate. In the end, the eluent strength depends on the relative and absolute concentrations of $Na_2CO_3$ and $NaHCO_3$.

Removal of $NaHCO_3$ and $Na_2CO_3$'s high conductivity in the background is essential if conductivity detection of anions is to be analyzed. For this, a suppressor that has a strong acidic cation exchanger with H form converts the $H^+$ to $H_2O$ and the ions of interest to their hydroxides.

A typical application of IC in our laboratories for analysis of $Cl^-$ in MOVICOL sachets (for effective relief from constipation, each sachet contains NaCl 350.7 mg, $NaHCO_3$ 178.5 mg and KCl 46.6 mg; each sachet provides $Na^+$ 65 mmol/L, $K^+$ 5.4 mmol/L, $Cl^-$ 53 mmol/L and $HCO_3^-$ 17 mmol/L when reconstitution in 125 mL of water is done) on anion exchange column (IC-Pak Anion, 150 × 4.6 mm, Waters Co. [14]); mobile phase: 80 mg sodium bicarbonate + 380 mg anhydrous sodium carbonate in 1 L of water and detection using conductivity detector without ion suppressor (Waters 432 conductivity detector, Waters Co. [14]) is shown in Figure 5.12.

An alternative separation mechanism for anions can be done by ion-pair chromatography (IPC; see Section 2.2.2.8).

#### 2.2.2.6.2 IC applications for cation analysis

Inert polymer resins that are superficially sulfonated have been used to separate cations. The strong hydrophobic core, pellicular structure of stationary phase and layer of thin sulfonate all make up for a quick transfer of solute. With about 1 mM hydrochloric acid as eluent, the alkali metal ions $Li^+$, $Cs^+$, $NH_4^+$ and the ions of small aliphatic amines can be eluted. If you want to use conductivity detection to analyze the cations, HCl's high conductivity in the background needs to be eliminated. For this, a suppressor that has a strong basic anion exchanger in the $OH^-$ form is used. This helps in the conversion of $H^+$ to $H_2O$ and the ions of interest to their hydroxides. The alkaline earth metal methods show high affinity to the cation exchanger. Thus, elution with 1 mM hydrochloric acid is not possible. Using higher concentration of HCI is not always preferable. This is because the suppressor column exhausts very fast. A good eluent for $Mg^{2+}$, $Ca^{2+}$, $Sr^{2+}$ and $Ba^{2+}$ is 2 mM *m*-phenylene-diamine dihydrochloride. Typical application of IC in our laboratories for analysis of $Na^+$, $K^+$, $Mg^{2+}$ and $Ca^{2+}$ in balanced salt solution $(BSS)^+$ and analysis of $Na^+$, $K^+$ in MOVICOL on IC-Pak cation column (150 × 3.9 mm, Waters Co. [14]), 10 mM methanesulfonic acid as mobile phase and using conductivity detector from Waters Co. [14] is found in Figures 5.11 and Fig.2.48 respectively.

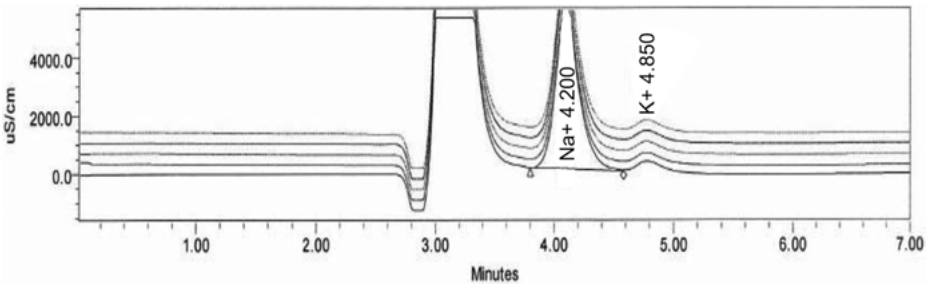



**Fig.2.48:** Typical application of ion chromatography for analysis $Na^+$ and K in Movicol$^+$ sachets on cation-exchange column and detection using conductivity detector.

- Column: IC-Pak cation, 150 × 4.6 mm (Waters Co.)
- Mobile phase: 10 mM methanesulfonic acid
- Detector: conductivity detector (Waters 432 conductivity detector, Waters Co.[14])

MOVICOL$^+$: Each sachet contains NaCl 350.7 mg, $NaHCO_3$ 178.5 mg and KCl 46.6 mg. Each sachet provides $Na^+$ 65 mmol/L, $K^+$ 5.4 mmol/L, $Cl^-$ 53 mmol/L and $HCO_3^-$ 17 mmol/L when reconstitution is done in 125 mL of water.

### 2.2.3 Ion-pair chromatography (IPC)

Just like detergent molecules, ion pairing reagents are included in the HPLC mobile phase to give retention to basic and acidic analytes [2, 11–13, 68, 71]. So, IPC is just an alternative method to separate ionic species. You can increase the sample ion retention just by putting another ion-pair reagent in the mobile phase. This forms an association between the ion pairs and the sample ions that are charged. The sample's charge is shielded with the help of the ion pair, and the interaction of it with the stationary phase is increased (usually nonpolar, such as C18). It is mostly practiced with RPLC as we have mentioned previously in Section 2.1. IPC is employed when the charged sample molecules are not sufficiently retained by the standard method, or when the support is not stable at a mobile phase pH under which the sample is not ionized, or when the charged sample components interact with the packing materials to cause asymmetrical peaks. IPC is applicable for samples containing only weak acids or only weak bases in addition to natural compounds present in undissociated form. An alkyl sulfonate (IP reagent) must be added to cationic samples and tetrabutyl ammonium phosphate (IP reagent) to anionic samples. Sulfonates with long-chain alkyl like C5 to C12 easily combine along with the basic solutes when the acidic pH conditions make neutral "ion pairs" that the RP retains. Retention is proportional to the length of the hydrophobic chain of the ion-pairing agent and its concentration. Hexane sulfonate binds with the basic

analytes that are thiamine and pyridoxine and they make a neutral ion pair. Fig.2.49 shows separation of three vitamins (pyridoxine, riboflavin and thiamine) using mobile phase containing IP reagent (heptane sulfonic acid and pentane sulfonic acid as IP reagents) when compared to the protonated amines that are unassociated. These ion pairs that are now neutral are more hydrophobic and give much higher times of retention. For acidic analytes, reagents that are ion-paired like tetra-alkylammonium salts are commonly used. It is clear that the reagent that ion-pairs gives a parameter that is added to help resolve the basic analytes from the sample.

It is important to note that the TFA (Trifluro Acetic Acid) is capable of some ion-pairing features and is very useful when it comes to RPLC of peptides and proteins. Heptafluorobutyric acid is a very useful ion-pairing reagent that is violated and can be compatible with a number of mass spectrometers. Waters Corporation [14] offers two types of ion-pairing reagents (IP reagents): regular (A and B series) and low UV (low UV A and low UV B series).

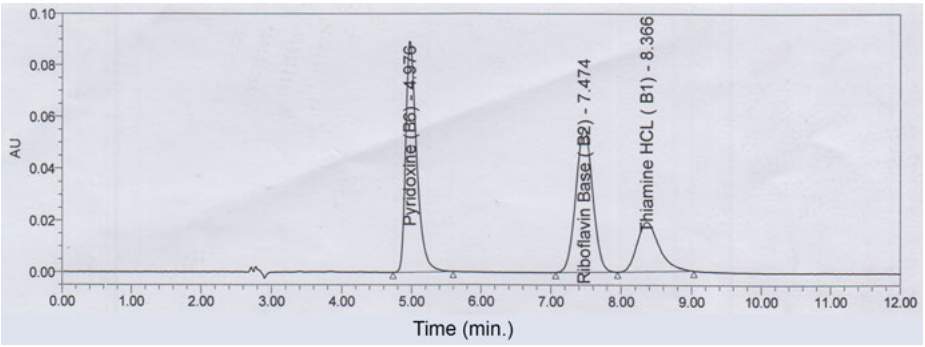



**Fig.2.49:** Chromatogram illustrating separation of three vitamins (pyridoxine, riboflavin and thiamine) using mobile phase containing IP reagent (heptane sulfonic acid and pentane sulfonic acid as IP reagents).

### 2.2.4 Ion-exclusion chromatography (IEXC)

#### 2.2 4.1 Introduction

IEXC or ion-exclusion chromatography makes use of a cation-exchange resin or the anion resin to conduct separation of ionic solutes from the weakly ionized ones like the neutral solutes and carboxylic acids [67, 68, 73, 74]. This chromatographic mode, and the same charge size for both the ion-exchange resin and the solutes were weakly ionized solutes. Here, partially negative charges are divided on the cation-exchange resin that has anionic sulfonate functional groups. On the other hand, anion-exchange resin is used to separate the positively charged solutes. It

has cationic quaternary ammonium functional groups. This is the opposite situation to that occurring in IEXC as we have mentioned previously.

IEXC finds application for separating a large range of partially ionized, neutral and small molecules. The first time it was performed was done on high-capacity, large particle size and fully functionalized polystyrene-divinylbenzene polymers. The stationary phases in the modern age are mostly the same and are made of similar material, and the difference lies in some respects. The different parameters that change the retention of the solute are as follows: resin structure, resin cross-linking degree, capacity for ion exchange and the size of the particle. We will further discuss the principles of IEXC and its application in drug QC laboratories for analysis of pharmaceutical products.

#### 2.2.4.2 Principles of ion-exclusion chromatography (IEXC)

Just like the other modes used in high-performance chromatography, IEC is used. It gets the name because of the dominant separation or retention mechanism that is exploited which in this case is the exclusion of ions. Mostly used to separate the weaker acids, especially carboxylic acids, it is not confined to this. It is also used to separate phenols, amino acids and carbohydrates for weaker bases [67, 68]. The commonly accepted retention mechanism at play within IEXC involves the formation of a pseudo-semipermeable membrane around the resin stationary phase. Commonly, it is called the Donnan membrane equilibrium as it uses accumulation of the water molecules with a dense hydrated layer which forms on the surface and even seeps inside the structure of the pore of an ion-exchange resin that has a high and strong capacity. This gives rise to an occluded phase. The solutes that are ionic and have charges similar to the ones of the stationary phase (sulfonated cation exchangers that are mostly used to separate the weaker acid analytes) are met with repulsion from the surface of the resin. On the other hand, the species that are neutral can easily enter the pores of the resins and the occluded stationary phase, hence, gives rise to retention. The selectivity of separation in this system is dependent on the solute. The IEXC makes use of simple systems of eluents, for example, diluted sulfuric acid (in case of acid solutes that are weak) and the exchange groups on the resin surface have no interaction with solutes that are charged similarly. Hence, the solute charge is the thing that governs the potential of the solutes to break away from the mobile phase and go to the stationary phase. (this does not include the hydrophobic secondary interactions, which can actually be very significant). In case the acid is weak, it totally depends on the constants for acid dissociation or the $pK_a$ value. Hence, the elution volume retention inside the IEXC is directly correlated with the values of $pK_a$ for basic solutes and weaker acids.

Anions of weaker acids are generally separated on a cation exchanger that is highly acidic. It is in the hydrogen form and can be eluted as partially ionized or neutral acids. Protonated cations make use of an anion exchanger to separate when they are in the hydroxide form and end up being eluted as corresponding bases.

Water is the common mobile phase, even though it can lead to peak fronting in excess when compounds that are hydrophobic are involved. For this, going for acid solutions in the diluted form is a better option (e.g., 1–10 mM $H_2SO_4$, HCl and tartaric acid) or mixtures of organic solvent and water can be used. Mobile phases that are made up of weaker acids allow for unsuppressed conductivity detection to be used [67, 68, 73, 74]. Both a conductivity detector and a membrane suppressor are used for stronger acids. Indirect and direct UV absorbance detection is made use of as a selective or general detector for neutral or ionic compounds [67, 68, 73, 74]. The applications include separating the inorganic ions like phosphates, fluoride, borate, nitrite, bicarbonate, silicates, aromatic and aliphatic carboxylic acids, ammonium and other alkylammonium ions and amines and sugars. The separation of low-molecular-weight carboxylic acids from complex matrices is the most important practical application.

Traditionally, low cross-linked porous polymers modified by sulfonic or carboxylic acid groups (quaternary amines for the separation of cations) were the most widely used stationary phases. In recent years, silica-based chemically bonded or surface-modified (e.g., alumina treated) ion exchangers have found increasing use [67, 68]. The trend toward increased use of modern porous polymer and silica-based materials is due to their higher performance and greater dimensional stability with different mobile phase compositions.

At low pH, weak organic acids are in the neutral or partially ionized form and can diffuse into the stationary phase pores largely uninhibited by the ionic groups on the stationary phase. Their retention depends primarily on the extent of acid dissociation (they generally elute in order of their p$K_a$ values) and the extent of sorptive (nonpolar and polar) interactions of the acid with the stationary phase [67, 68]. It is generally found that dibasic acids are less strongly retained than monobasic acids, branched chain aliphatic carboxylic acids are less retained than straight chain acids with the same carbon number and aromatic (also unsaturated aliphatic) acids are more strongly retained than saturated aliphatic acids.

#### 2.2.4.3 Application of IEXC for analysis of pharmaceutical products

The theory and applications of IEXC are conveniently illustrated by the separation of simple carboxylic acids shown by the chromatogram in Fig.2.50. Here the stationary phase was a cation-exchange resin in its acidic form, and elution was accomplished with a dilute solution of HCl acid. The analytical column was followed by a suppressor column that was packed with a cation-exchange resin in the silver form. The hydrogen ions of the eluent were exchanged for silver ions, which then precipitated the chloride ions, thus removing the ions contributed by the eluent. The undissociated form of the analyte acids was distributed between the mobile phase in the column and the immobilized liquid held in the pores of the packing. The distribution constants for various acids are primarily related to the inverse of their dissociation constants, although other factors also play a part in the extent to which various

species are distributed between the two phases. It is apparent from Fig.2.50 that $SO_4^{2-}$ was excluded because its charge sign is similar to that found on the ion-exchange resin being in use. So this ion goes out from the column early.

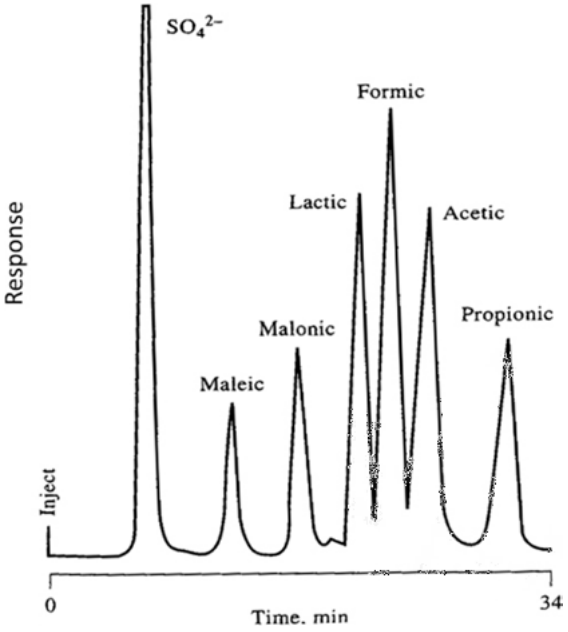



**Fig.2.50:** An ion-exclusion chromatogram for a mixture of six weak acids.

There are a number of applications when it comes to ion exclusion and it is done for determining and identifying the acidic species as shown in Fig.2.51 in materials like milk, wine, pharmaceuticals, and other commercial products.

The weak acid salts can also be analyzed once they have been converted to an acid by the exchanger. Separation and determination of acetate and citrate anions in BSS# on cation-exchange column (IEXC mode; column: Spherisorb 5 SSC × 4.0 × 250 mm; mobile phase: 0.03 N $H_2SO_4$ and UV detection at 220 nm) was accomplished in our drug QC laboratories [75] as shown in Fig.2.52.

Both the salts and their weak bases are easily determined through IEXC. For these applications, anion-exchange columns that are in the hydroxide form are used.

In IEXC mode, the conductometric detector is given preference over the others. Before the eluate enters inside the detector, it is important to remove the hydrochloric acid from the IEXC eluent. A suppressor is used for this purpose and the best way to do so is to use the one that is silver-loaded. $Cl^-$ is eliminated by the $Ag^+$ and the $H^+$ replaces it. Consequently, the weak acids are able to go in the conductivity detection, and many other detectors that are more common and famous are less successful.

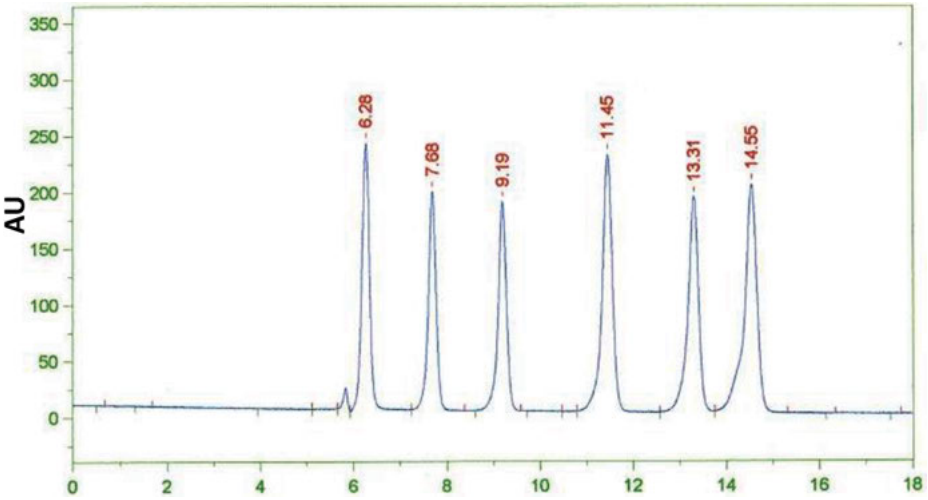



**Fig.2.51:** Separation of citric acid (6.28 min), oxalic acid(7.68 min.), malic acid (9.19 min), succinic acid (11.45 min), formic acid (13.31 min) and acetic acid (14.55 min) in a mixture on ion-exclusion column (Aminex HPX-87 H,300 mm × 7.8 mm, 9 µm, cat. no. 125-0140, BIO-RAD), mobile phase 0.008 N sulfuric acid and UV detection at 210 nm.

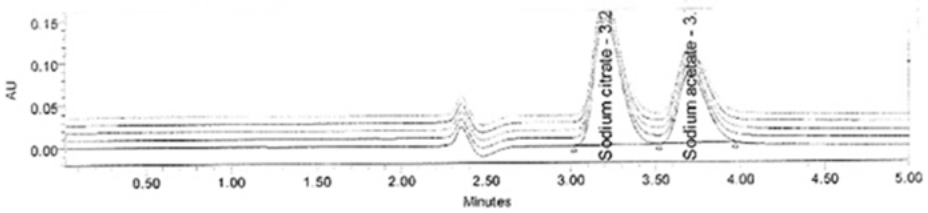



**Fig.2.52:** Separation of acetate and citrate in balanced salt solution (BSS)[#] on cation exchange column (IEXC mode) [75].

Column: Spherisorb 5 SSC × 4.0 × 250 mm
Mobile phase: 0.03 N $H_2SO_4$
Detector: UV detector at 220 nm
#BSS solution contains:

- Sodium chloride 6.4 mg/mL
- Sodium citrate dihydrate 1.7 mg/mL
- Sodium acetate trihydrate 3.9 mg/mL
- Potassium chloride 0.75 mg/mL
- Calcium chloride dihydrate 0.45 mg/mL
- Magnesium chloride hexahydrate 0.39 mg/mL

In our drug QC laboratories [75], separation of bicarbonate ion in renal dialysis solution (HOMOSOL) has been conducted on ion-exclusion column (Aminex HPX-87H, 300 mm × 7.8 mm, 9 µm) (BIO-RAD), mobile phase 0.005 N sulfuric acid and RI detector, polarity (–) as shown in Fig.2.53.

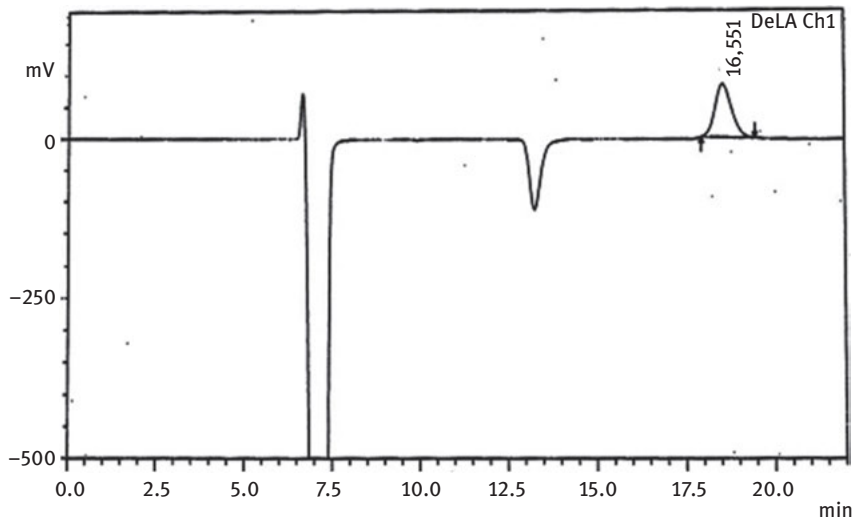



**Fig.2.53:** Separation of bicarbonate ion in renal dialysis solution (HOMOSOL) on ion-exclusion column (Aminex HPX-87H, 300 mm × 7.8 mm, 9 µm) (BIO-RAD), mobile phase 0.005 N sulfuric acid and RI detector, polarity (–) [75].

One of the main applications of IEXC in inorganic chemistry is the analysis of carbonate in aqueous solution. The eluent, in this case, is pure water, $CO_3^-$ and $HCO_3$ anions elute well behind all other anions.

## 2.2.5 Size-exclusion chromatography (SEC)

### 2.2.5.1 Introduction

SEC is a method that involves separation of solute molecules based on size or shape differences in solutions and is widely used in the comparison of complex formulations of pharmaceutical products [75–79]. SEC can grip large molecular load models in a short time with simple technique development. SEC presents one of the greatest simple interactions. It is unlike all other modes of chromatography in a sense that a meek molecule mass classification process is used rather than interaction phenomenon on the foundation of separation. SEC uses permeable particles with different diameter sizes to separate molecules. The biological molecules are separated, and it is

also used to decide molecular weights and determine the distributions of molecular weight of polymers. Its application is usually on huge molecules or macromolecular complexes like proteins and industrialized polymers. Gel filtration chromatography is a technique used when sample is transported in a column using a liquid solution. Gel-permeation chromatography (GPC) is a method in which a carbon-based solvent is taken as a mobile phase. Fractionation occurs when the molecules are separated. The exclusion limit is determined by the pore size in stationary phase (what goes through the beads and what goes around the beads, Figure 2.54).

Size-exclusion chromatography is basically the easiest mode of chromatography to comprehend and perform and is related to a large range of constituents covering both large and small molecular weights. It is exclusive amid all of the LC methods in which separation is solely from purely entropic powers. Contribution to retention should not be enthalpic. In other cases, links such as adsorption, exchange of ions and partitioning must not present in the best mass-exclusion system.

#### 2.2.5.2 Principles of SEC

A combination of molecules mixed in liquid (the mobile phase) is smeared to a chromatography pole which contains a dense sustenance in the form of tiny spheres, or "beads" (the nonmoving phase). The column bed is the size of the beads inside the column. The beads perform as "traps" or else as "sieves" and function to screen tiny molecules that become momentarily stuck within the pores.

Bigger molecules go around or are "excluded" from being a part of the beads. The small size molecules can penetrate most of or all the pores while the bigger sized molecules cannot or only some can go in the pores. Therefore, small-sized molecules elute later, whereas the bigger molecules elute first while the lastly eluting molecules are the ones that can go in all the pores that elute at the end from the column. Different sized particles will end up eluting (filter) through a motionless phase at variable rates.

Packing for mass-exclusion chromatography comprises small (10 μm) sized polymer or silica particles comprising a network of homogeneous pores in which diffusion takes place between the molecules of the solute and solvent molecules. When inside the pores, molecules are successfully trapped and detached from the course of the stationary phase. The normal dwelling time spent in the pores is dependent upon the actual mass of the analyte particles. As we can see in Fig.2.54, the species that are among the first to be eluded are the particles that are bigger than the normal pore size of the stuffing and thus undergo essentially no retaining Fig.2.54A. The particles that are last to elute from the column are the particles having diameters that are considerably smaller than the pores can enter or fill throughout the pore and are thus trapped for the longest time Fig.2.54B. The intermediate-sized particles come between these two extremes whose regular dissemination into the pores within the packing is dependent on their widths. Fractionation occurs within this group which is related to the particle size and to the particle

shape, to some extent, also. Hence, the bigger particles elute first, while the little molecules move slower (because of their movement in and out of the pores) and elute at a later stage, in reducing order of their mass in solution. They follow the simple method that the larger ones come outside first.

Note that mass exclusion differs from the other methods that we have taken into consideration, that is, no physical or chemical interaction between analytes and the nonmoving phase is involved. They lead to reduced column efficiencies, and every effort is made to prevent such incursions. It is also noted that in contrasting other forms of chromatography, there is a higher limit to retaining time because no analyte type is retained for a longer time than those that penetrate the stationary phase.

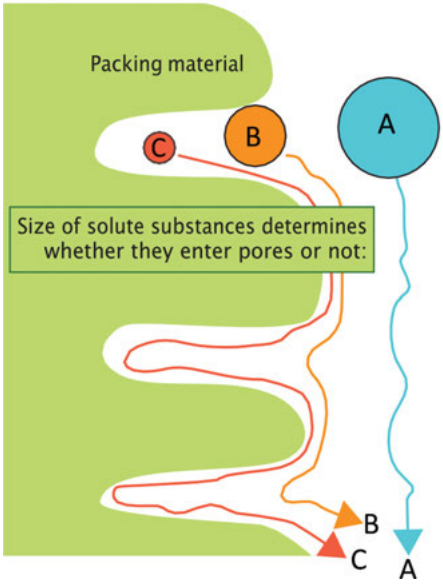



**Fig.2.54:** Principle of size-exclusion separation.

Mobile phases for SEC are chosen for two reasons: firstly, they are amazing solvents for the analytes; and, secondly, they may stop any interactions (based on charge or polarity) between the analytes and the nonmovement phase surface.

### 2.2.5.3 Packing materials for SEC columns

Two types of packing for SEC are encountered as shown in Tab.2.14 silica and polymer bead-based molecules, both having diameters of 5–10 µm. The latter have the advantages of higher solidness, which results in easier packing and allows the usage of larger pressures, better stability, which allows the use of a large range of diluters, consisting of water, faster equilibration with new diluters and

higher stability at maximum temperatures. The drawbacks of particles consisting of silica include their propensity to hold solutes by adsorption and their prospective for catalyzing the dilapidation of solute molecules. Most early SEC work was done on styrene-RS cross-linked in a like structure (except that the sulfonic acid group is absent). The amount of divinyl benzene present during manufacture controls the pore size of these polymers to the extent of cross-linking. As a consequence, packing by polymers having multiple different regular pore sizes is promoted. Originally, hydrophobic gels were styrene divinylbenzene gels and thus could be used only with nonaqueous movable phases. However, hydrophilic gels are accessible, making the possible use of aqueous thinners for the parting of large, water-soluble particles like sugars. Sulfonated divinylbenzenes or polyacrylamides constitute these hydrophilic gels.

Absorbent glass and silica elements, having regular pore size fluctuating from 40 Å to 2,500 Å, are now available commercially. To decrease adsorption, the exteriors of these units are often altered by reaction with carbon-based substituents. For example, the surface of one hydrophilic packing has this structure.

### 2.2.5.4 Applicatuon of SEC for analysis of pharmaceutical products in drug quality control laboratories

- Size-exclusion methods are sectioned into gel separation and gel infiltration chromatography. The former uses hydrophilic packing and aqueous solvents. The last is founded upon nonpolar carbon-based diluters and hydrophobic stuffing. The methods are balancing in the sense that the one is functional to water-soluble samples and the other is functional to materials soluble in less polar carbon-based diluters.
- One useful application of the size-exclusion procedure is the parting of great molecular mass, natural product particles from low-molecular-weight types and salts. For sample, a gel with an elimination limit of several thousands can clearly isolate proteins from amino acids and low molecular mass peptides.
- Another useful presentation of GPC is the parting of homologue and low-molecular-weight polymer. These applications are illustrated by the example shown in Fig.2.55. The chromatogram presents the separation of polymer albumin, dimer albumin and monomer albumin in pharmaceutical products in our laboratories [80].
- As a supplement to other HPLC modes, it is observed as an operative means of unscrambling small-sized molecules as for preparative work for the parting of multifaceted mixture to substitute the cleaning or the processing period. For example, the elimination of salts and other low-molecular-weight components from biological samples.

**Tab.2.14:** Properties of typical commercial packing for size-exclusion chromatography [74].

| Type | Particle size (µm) | Average pore size (Å) | Molecular weight exclusion limit[1] |
|---|---|---|---|
| Polystyrene-divinylbenzene | 10 | $10^2$ | 700 |
| | | $10^3$ | (0.1 to 20) × $10^4$ |
| | | 10 | (1 to 20) × $10^4$ |
| | | 10 | (1 to 20) × $10^5$ |
| | | 10 | (5 to >10) × $10^6$ |
| Silica | 10 | 125 | (0.2 to 5) × $10^4$ |
| | | 300 | (0.03 to 1) × $10^5$ |
| | | 500 | (0.05 to 5) × $10^5$ |
| | | 1,000 | (5 to 20) × $10^5$ |

[1]No retention occurs above this molecular weight [73, 74].

- For the determination of molecular mass distribution, this plays a vital role in polymer production and QC.
- Classification of models of biological origin (analysis by qualitative and quantitative aspects, with preparative separation of discrete proteins).
- Another major application of SEC is the rapid determination of molecular weight or molecular weight distribution of larger polymers or natural products. Here, the elution volumes of the sample are compared with elution volumes for a series of standard compounds that possess the same chemical characteristics.
- The most important advantages of size-exclusion procedures include (1) short and well-defined separation times, (2) narrow band which leads to good sensitivity, (3) freedom from sample loss because solutes do not interact with the stationary phase and (4) absence of column deactivation brought about by the interaction of solute with the packing.

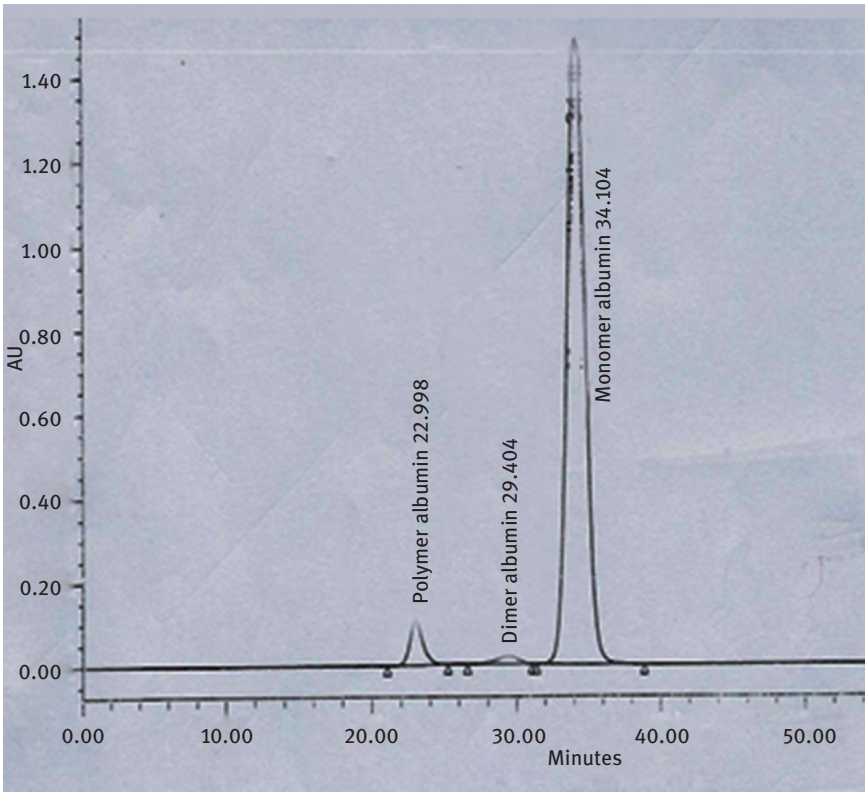



**Fig.2.55:** Application of size-exclusion technique for separation of polymer albumin, dimer albumin and monomer albumin in a pharmaceutical product (albumin 20%) and determination of molecular size distribution as a quality control test in drug quality control laboratories. Column: TSK-gel, G3000 SW 300 × 7.5 mm, particle size 10 µm, mean pore size 250 Å (TOSOH Bioscience [25]). Exclusion limit for globular proteins is $5 \times 10^5$ Da. *Detector*: UV 280 nm *Mobile phase*: 4.873 g $Na_2HPO_4 \cdot 2H_2O$ + 1.741 g $NaH_2PO_4 \cdot H_2O$ + 11.688 g NaCl in 1,000 mL HPLC-grade water.

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# 3 HPLC mobile phase and HPLC pumping system

## 3.1 Introduction

Mobile phase is the liquid phase of a chromatography separation that carries the dissolved sample through the high-performance liquid chromatography (HPLC) column (stationary phase). It may be a single solvent or a homogeneous mixture of several solvents. There is an option of keeping the composition of the mobile phase constant (isocratic elution) or varied (gradient elution)

The solvents which constitute the mobile phase play an essential role in the HPLC separation [1–12]. Therefore, the solvent to be used for preparation of mobile phase must be selected carefully. Also preparation and handling of the mobile phase must be done carefully to maintain optimum chromatographic performance, especially reproducible results and operation with minimal maintenance. So, in this chapter, considerations concerning solvent selection, mobile phase preparation and handling for HPLC will be discussed as well as the components of mobile phase pumping system and a guide for its operation will be in our considerations. So this chapter covers the following topics:

- Solvent selection for mobile phase preparation
- Mobile phase preparation and handling
- Mobile phase pumping system, component and operating guide

## 3.2 Solvent selection for mobile phase preparation

It is true that separation of the compounds loaded on the HPLC column is usually achieved by eluting with an appropriate mobile phase. Obviously, the success of the elution process depends on the ability of the solvent in the mobile phase to selectively desorb the components retained by the stationary phase. The choice of the solvent is a relatively complex task and requires an understanding of the mechanism of retention, separation and elution if a similar system has not been recorded in the literature for the same or similar compounds.

For the purpose of quality control in drug quality control laboratories, preparation of the mobile phase must be according to the drug manufacturer specifications or specified pharmacopoeia. The name of the solvents to be used as a mobile phase and the composition of mobile phase are generally given in the manufacturer specifications or specified pharmacopoeia within the described analytical method and this method was already validated. Examples for preparation of mobile phases according to the United States Pharmacopoeia and the European Pharmacopoeia are present in [13–15]. So, solvent selection to be used as a mobile phase according to the manufacturer specifications or specified pharmacopoeia has became mandatory.

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Generally, a wide range of solvents have a potential of solving any particular problem, so the selection of these solvents needs to be based on different criteria.

### 3.2.1 Polarity

Polarity is a term used in chromatography, which is used as an index of the ability of compounds to interact with water. Polar solvents are hydrophilic, and nonpolar solvents are hydrophobic. For example, it is well known that sodium chloride and sucrose (polar compounds) dissolve in polar solvents such as water, kerosene dissolves in hexane (both nonpolar solvents), in other words, like dissolves like. The term is also applied to solutes, and stationary phases in addition to mobile phases. If the polarities of stationary phase and mobile phase are similar, then it is likely that the interaction of solutes with each phase may be similar, leading to poor separations. Thus, we need a polar mobile phase for a nonpolar stationary phase such as a hydrocarbon type, whereas unmodified silica which is highly polar needs a mobile phase with relatively low polarity. The retention of solutes is usually altered by changing the mobile phase polarity. Tab.3.1 provides values of the polarity index, *P*, for several common HPLC solvents, where greater values of *P* correspond to more polar solvents. Mixing together more than one solvent, thinking they are miscible, creates a mobile phase of intermediate polarity. For example, a binary mobile phase made by combining solvents A and B has a polarity index $P_{AB}$. A mobile phase of 60:40 water–methanol has polarity 8.2, which can be calculated as follows: 60/100.10.2 + 40/100.5.1 = 8.16 = 8.2, where 10.2 and 5.1 are the polarities of water and methanol, respectively, as shown in Tab.3.1. As a general rule, a two-unit change in the polarity index corresponds to approximately a 10-fold change in a solute's retention factor. In a reversed-phase (RP) separation, the retention factor for any solute becomes smaller when the composition of the mobile phase switches from a more polar to a less polar one.

**Tab.3.1:** Solvent polarity index [16].

| Solvent | Solvent polarity index, *P* |
|---|---|
| Hexane | 0.1 |
| Carbon tetrachloride | 1.56 |
| Isopropyl ether | 1.83 |
| Toluene | 2.4 |
| Methyl-*t*-butyl ether | 2.4 |
| Chloroform | 2.7 |
| Diethyl ether | 2.8 |

**Tab.3.1** (continued)

| Solvent | Solvent polarity index, *P* |
|---|---|
| Dichloromethane | 3.1 |
| Isopropanol | 3.92 |
| Tetrahydrofuran | 4.0 |
| Ethyl acetate | 4.4 |
| Methanol | 5.1 |
| Acetone | 5.1 |
| Dioxane | 5.27 |
| Acetonitrile | 5.8 |
| Water | 10.2 |

### 3.2.2 Viscosity

A low viscosity solvent produces a lower pressure drop than a solvent with high viscosity for a specific flow rate. The number of the theoretical plate of the column is also increased with the low viscosity eluent as mass transfer takes place faster. High viscosity is a draw back in many separations science it reduces solute diffusion coefficients, thus reducing mass trnafere between solvents and the stationary phase. In general the mobile phase viscosity should be 0.5–1.5Cp.

### 3.2.3 UV transparency

Using ultraviolet (UV) detector as a detection tool for HPLC, at the wavelength required, the mobile phase needs to be totally transparent. For example, carbon tetrachloride (Tab.3.2) is not suitable at a detection level of 254 nm. The reason is: it is not optically transparent enough until 265 nm. Solvent compatibility with the detector is also important, and this is particularly true for UV and fluorescence detectors.

### 3.2.4 Refractive index

Using refractive index (RI) as a detection tool for HPLC, it is important to observe the difference between the refractive indices of the sample and the solvent. The greater the difference, the better. The highest sensitivity for these detectors will be

found with the solvent that has a widely different refractive index from that of the solute components (Tab.3.2).

### 3.2.5 Boiling point

It is well known that solvents with a high vapor pressure (low boiling point)can produce vapor bubble in the detector at the operating temperature (presence of bubble in detector makes disturbance for it). On the other hand, to be able to recover and further process the eluate, there should be a low boiling point in the mobile phase. This will require less laborious work while evaporation of the heat-sensitive components in the mobile phase occurs.

### 3.2.6 Inertness to the sample

There should be no reaction of the mobile phase and the sample. Any such reaction may alter sample constituents.

**Tab.3.2:** Properties of some solvents [17–21].

| Solvent | Viscosity[1] | UV cut off[2] | RI[3] | BP[4] |
|---|---|---|---|---|
| Acetone | 0.30(25) | 330 | 1.359 | 56.3 |
| Acetonitrile | 0.34(25) | 190 | 1.344 | 81.6 |
| Benzene | 0.65 | 278 | 1.501 | 80.1 |
| 1-Butanol | 2.95 | 215 | 1.399 | 117.7 |
| 2-Butanol | 4.21 | 260 | 1.397 | 99.6 |
| n-Butyl acetate | 0.73 | 254 | 1.394 | 126.1 |
| Carbon tetrachloride | 0.97 | 263 | 1.460 | 78.8 |
| Chlorobenzene | 0.80 | 287 | 1.525 | 131.7 |
| Chloroform | 0.58 | 245 | 1.446 | 61.2 |
| Cyclohexane | 0.98 | 200 | 1.426 | 80.7 |
| Cyclopentane | 0.40 | 200 | 1.406 | 49.3 |
| *o*-Dichlorobenzene | 1.32 (25) | 295 | 1.551 | 180.5 |
| Dimethyl sulfoxide | 2.20 | 268 | 1.478 | 189.0 |
| Dioxane | 1.44 (15) | 215 | 1.422 | 101.3 |
| 2-Ethoxyethanol | 2.05 | 210 | 1.408 | 135.6 |

**Tab.3.2** (continued)

| Solvent | Viscosity[1] | UV cut off[2] | RI[3] | BP[4] |
|---|---|---|---|---|
| Ethylene dichloride | 0.79 | 228 | 1.445 | 83.5 |
| Methanol | 0.55 | 205 | 1.328 | 64.7 |
| Methylene chloride | 0.45 (15) | 233 | 1.424 | 39.8 |
| Pentane | 0.24 | 200 | 1.357 | 36.1 |
| Petroleum ether | 0.30 | 226 | 30–60 | |
| 1-Propanol | 2.26 | 210 | 1.386 | 97.2 |
| 2-Propanol | 2.86 (15) | 205 | 1.377 | 82.3 |
| Pyridine | 0.95 | 330 | 1.510 | 115.3 |
| Tetrahydrofuran | 0.55 | 212 | 1.407 | 66 |
| Toluene | 0.59 | 284 | 1.497 | 110.6 |
| 2.2.4-Trimethyl pentane | 0.50 | 215 | 1.391 | 99.2 |
| Water | 1.00 | 190 | 1.333 | 100.0 |
| Xylene | 0.81 | 288 | 1.505 | 144.4 |

[1] Viscosity in cP at 20 °C, unless otherwise noted, for example, 0.3 (25) would have viscosity at 25 °C.
[2] The UV cutoff is here defined as the wavelength at which the solvent absorbance in a 1 cm path length cell is equal to 1 AU (absorbance unit) using water in the reference cell.
[3] RI is refractive index at 20 °C unless otherwise noted; for example, 1.391 (25) would have RI at 25 °C.
[4] BP is the boiling point at a pressure of 760 mm of mercury.

### 3.2.7 Corrosion resistant

Mobile phase, which contains chlorinated solvents such as carbon tetrachloride, tends to release HCl upon exposure to light. At that time, the presence of any traces of water in the system may join together and combine with HCl to make hydrochloric acid that can attack the stainless steel (SS) tubing connectors in HPLC system and make it corroded. Corrosion for tubing connectors in HPLC system is not accepted.

### 3.2.8 Toxicity

It is a good practice to avoid toxic solvents, as possible, when selecting a mobile phase. Toluene should always replace benzene (a highly carcinogenic solvent).

In addition, care should be exerted when purchasing solvents to use as mobile phases. Only HPLC-grade solvents are to be selected. Some solvents may require a preliminary purification process before use. Stabilizers added to certain solvents may alter solvent polarity completely, for example, chloroform stabilized with ethanol is much more polar than the pure compound. Additives may also reduce the UV transparency or may accumulate at the top of the column reducing its lifetime.

## 3.3 Mobile phase preparation and handling

It is true that the mobile phase plays an essential role in separation during HPLC analysis; therefore, the mobile phase must be prepared and handled carefully to maintain optimum chromatographic performance, especially reproducible results and operation with minimal maintenance.

### 3.3.1 Mobile phase preparation

Preparation of the mobile phase in drug quality control laboratories must be according to the drug manufacturer's specification. The name of the solvents to be used as a mobile phase and the composition of mobile phase are generally given in the manufacturer specifications or specified pharmacopoeia within the described analytical method, and this method was already validated. According to Dong and Ahuja [23, 24], when mixing organic solvents with water, measure the volume of each solvent separately and combine them in a suitable volumetric flask. Do vacuum filtration for mobile phase using the MilliSolve clarification system as described in Section 3.3.3.1 and degassing as described in Section 3.3.4.

### 3.3.2 Buffered mobile phase

A lot of pharmaceutical products have either acidic or basic functional group. So these products can exist in solution as ionized or nonionized forms. According to Dong [12], the ionic state and degree of ionization are highly affected by the chromatographic retention. Typically, it is seen that the ionic form has a significantly lower k' when compared with the unionized, neutral form. The reason is that it is not partitioned well in the hydrophobic stationary phase [12, 25, 26]. The significance of pH on the separation of basic drugs can be understood from Fig.3.1. At pH level 2.0, both the ionized drugs are not eluted and retained as one single peak. At pH level 8, the drugs end up separated well and partially ionized. At pH level 10, both the unionized drugs are highly resolved and retained.

To control the pH of the mobile phase, buffers are used. These affect the retention of basic or acidic analytes greatly. Tab.3.3 summarizes the common buffers for HPLC along with their respective UV and p$K_a$ cutoffs. Buffers are only effective within a range of ±1.5 pH unit from their p$K_a$ [12, 25, 26]. Although 50 mM buffers have been specified in many previously older methods, now lower ones are used in modern times.

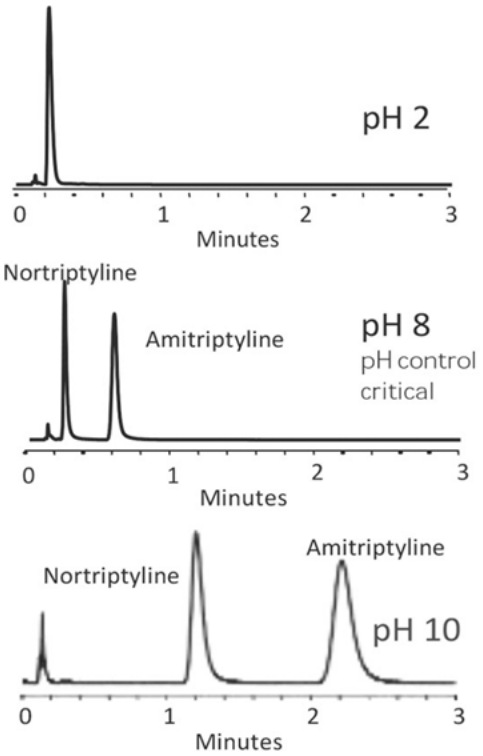


**Fig.3.1:** Illustrative diagram showing three chromatograms of two basic antidepressant drugs were separated on an RPLC column using mobile phases at various pH with percentage of organic solvent unchanged. Reprinted with permission from Millipore Co., Waters Chromatography Division, 43 Maple Street, Milford, MA 01757, USA [22].

**Tab.3.3:** Common HPLC buffers and their respective p$K_a$ and UV cutoff.

| Buffer | p$K_a$ | UV cutoff (nm) |
|---|---|---|
| Trifluoroacetic acid* | 0.3 | 210 |
| Ammonia* | 9.2 | 200 |
| Tris(hydroxymethyl) | 8.3 | 210 |

**Tab.3.3** (continued)

| Buffer | p$K_a$ | UV cutoff (nm) |
|---|---|---|
| Phosphate | 2.1, 7.2, 12.3 | 190 |
| Formate* | 3.8 | 200 |
| Acetate* | 4.8 | 205 |
| Carbonate* | 6.4, 10.3 | 200 |
| aminomethane | | |
| Borate | 9.2 | 190 |
| Diethylamine | 10.5 | 235 |

*Volatile buffer systems, which are MS-compatible.
Data extracted from [2].

Buffer strengths are typically in the range of 10–20 mM [12, 23–26].

According to the authors in [1–3, 24], *follow these guidelines* when using buffered mobile phase:

a. Buffered mobile phase older than 10 days cannot be used. The reason is the high risk of bacterial growth, which can end up damaging the HPLC column.
b. Never let buffered mobile phases sit in the HPLC system.
c. Before switching of the HPLC system, it is important to go ahead and use water to flush the buffers from the system (or 10% acetonitrile in water).
d. Always check the pH of the mobile phase before use.
e. Pay great attention to the measurement of pH values in the mobile phases. Bad handling of the pH meters, particularly in organic solvents, can cause the potassium chloride dissolved in the reference electrode to precipitate.
f. Using silica-bonded packing, the mobile phase should not exceed a pH value of 7.5 because of the increased solubility of the packings. In the same way, the C-bonding in modified silica pickings are broken at pH values below 2.5. Recently, HPLC column manufacturers have introduced polymer-coated silica phases with good stability at a wider pH range.

#### 3.3.2.1 Acidic mobile phases

As mentioned in [2, 3, 12, 28], in RP liquid chromatography (RPLC), the low pH suppresses the ionization of weakly acidic API in pharmaceutical products, leading to higher retention on the stationary phase of the column. So using RPLC in the presence of acidic mobile phase (pH 2.5–3) results in many applications for analysis of pharmaceutical products in drug quality control laboratories [12]. Surface silanols are not ionized at low pH, lessening tailing with basic solutes. Most silica-based bonded phases are not stable below pH 2 due to acid-catalyzed hydrolytic cleavage of the bonded groups [2, 3, 12]. Common acids used for mobile phase preparations are phosphoric acid, trifluoroacetic acid, formic acid and acetic acid. However,

basic analytes are ionized at low pH and might not be retained. For most of the pharmaceutical applications, the acidic pH level of 2.5–3 is going to be a good initial point because the relatively low pH suppresses the ionization process of most of the acidic analytes (this provides a much higher retention) and reduces the interaction of surface silanols and the basic analytes which are present on the packing of the silica (reason: it is not possible for the silanols to ionize at this pH level).

#### 3.3.2.2 Ion-paired mobile phase

Long-chain alkyl sulfonates ($C_5$–$C_{12}$) as ion-pairing reagents can combine with basic drugs such as pyridoxine and thiamine. This allows the formation of neutral "ion pairs" which happens under acidic pH conditions. These are retained in RP column [1, 2, 6, 12, 23, 25, 26]. Analyte retention is relative to the entire length of only the hydrophobic chain of the ion-pairing agent as well as its concentration.

For acidic analytes, mostly we use *tetra*-alkyl ammonium salts because these are ion-pairing reagents. Heptafluorobutyric acid is a volatile and useful ion-pairing reagent because it is also compatible with the mass spectrometers available. Waters Corporation [22] offers two types of ion-pairing reagent (PIC reagents) (Waters PIC): regular (A and B series) and low UV (low UV A and low UV B series). PIC reagents are specifically formulated for detection of high sensitivity UV at wavelengths to 200 nm [22].

#### 3.3.2.3 Basic mobile phase

Prior to 1990, the use of a high-pH mobile phase was not feasible with silica-base columns due to the dissolution of the silica support at pH > 8. The development of improved bonding chemistries and hybrid particles now extends the useful pH range from 2 to 10 or, in many cases, from 1 to 12 [3, 5 and 12] (see Chapter 2). This offers an important alternative approach for the separation of basic analytes and, in particular, for impurity testing of water-soluble basic drugs and their impurities. [3, 12 and 29]. Fig.3.1 illustrates the basis of this approach in the separation of two closely related basic drugs, amitriptyline and nortriptyline. At low pH, both analytes are ionized and coelute with the solvent front. At pH close to the pKa of the analytes, the partially ionized solutes are well separated with a large selectivity value. At high pH, the non-ionized solutes are well retained and resolved.

### 3.3.3 Mobile phase filtration

The most important preparation consideration is for all mobile phases to be free of particulates before they enter the chromatographic system (logging of filters and column frits by particulates could disable the HPLC system). The pump and stationary phase in the column are also potentially sensitive to particulates.

Two approaches are recommended for particulate removal from mobile phase before its use: first, using off-line filtration and second using IN-LINE filtration for mobile phase.

#### 3.3.3.1 Off-line filtration (vacuum filtration)

It is acceptable for off-line filtration for mobile phase to use vacuum filtration for clarification of HPLC mobile phase. The filtration apparatus should be made of all-glass with 95 or 47 mm membranes with a vacuum-grade, thick-walled filtration flask.

The system uses a 0.45 μm filter (membrane filter) under vacuum operation to remove particles longer than 0.45 μm. Filtration through this filter clears the chromatography column inlet of closing particles. In addition, vacuum filtration removes a large portion of dissolved gases from the mobile phase which reduces the risk of air bubbles that enter the detector during analysis.

Recently, a clarification system for vacuum filtration, degassing and storage (the MilliSolve filtration and storage system; Millipore Co., Waters Chromatography Division, 43 Maple Street, Milford, MA 01757, USA) [22] has became available in the laboratories as shown in Fig.3.2, Fig.3.3, Fig.3.4 and 3.5. The system consists of the following items (Fig.3.2):

- Suitable filter (47 mm) according to Tab.3.4 (A)
- A glass vacuum cover that fits on top of the glass base and tabulated cap (B)
- Aluminum clamp (C)
- All glass vacuum base and tabulated cap, 47 mm diameter (H) (on its top the suitable membrane filter can be placed) with side arm (D) to the vacuum source (E)
- Vacuum outlet of the glass base and tabulated cap (E) to the vacuum pump
- Teflon tubing, 70 cm (G) for connecting the mobile phase into the filtration unit through the unit (B)
- A 2 L flask with a conical bottom (F) for collection of the filtrated mobile phase
- A plastic plug with two holes for inserting two small tubing (Fig.3.5)

The following steps explain how to set up the MilliSolve system. So you can filter any mobile phase:

a. Connect one end of the vacuum tubing (E) to the vacuum outlet of glass base and tubulated cap (H). Push gently to secure the tubing, wet the glass vacuum cover fitting with water, if necessary.
b. One end of the vacuum tubing should be connected to the vacuum source.
c. The Teflon tubing (G) should be connected to the inlet of the glass vacuum cover (B). Slowly push the cover inlet inside the tubing. Note: If you have difficulty in connecting the tubing, carefully immense the end of the tubing (G) approximately

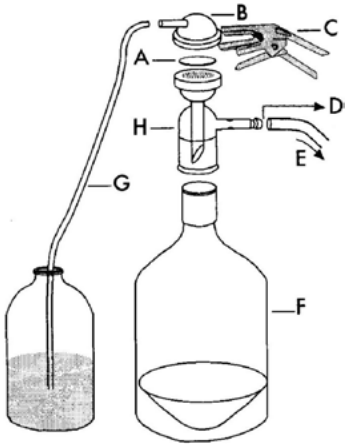


**Fig.3.2:** All glass vacuum filtration, degassing and storage system for off-line filtration of HPLC mobile phase. Reprinted with permission from Millipore Co., Waters Chromatography Division, 43 Maple Street, Milford, MA 01757, USA, Waters Corporation [22].

**Fig.3.3:** The MilliSolve vacuum filtration degassing and storage system for off-line filtration of HPLC mobile. Reprinted with permission from Millipore Co., Waters Chromatography Division, 43 Maple Street, Milford, MA 01757, USA, Waters Corporation [22].

1 cm in hot water before inserting it into the vacuum cover inlet. This softens the tubing so it is easier to insert.

d. Place the glass base and tubulated cap (which is connected to the vacuum source) on top of the 2.0 L flask.
e. Using the forceps (Fig.3.4) place the desired membrane filter (according to Tab.3.2) (according to Tab.3.4) on the glass base and tubulated cap.

f. Place the vacuum cover (with the Teflon tubing attached to it) on the top of the filter.
g. Using the aluminum clamp (C), clamp the cover to the base to close the system. The system is now ready to operate for filtration of the mobile phase.

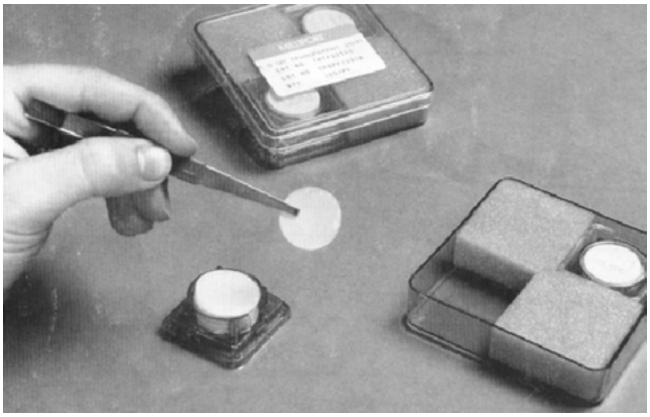

**Fig.3.4:** Handling membrane filter to filtration apparatus. Reprinted with permission from Millipore Co., Waters Chromatography Division, 43 Maple Street, Milford, MA 01757, USA [22].

**Tab.3.4:** Membrane filter selection according to the type of mobile phase [22].

| Type of solvent and buffer | Filter to be used |
|---|---|
| Water, buffers | Mixed esters of cellulose, 0.45 µm |
| Water, buffers, alcohol or mixtures | Durane, hydrophilic, 0.45 µm |
| All organic solvents (methylene chloride, hexane, tetrahydrofuran) | Fluoropore (PTFE), 0.45 µm |

Millipore Corporation offers a wide variety of the membrane filter to meet drug analysis application [22].

#### 3.3.3.1.1 Filtration procedures

a. Prepare the mobile phase you want to filter in a beaker, an Erlenmeyer flask, or any other suitable laboratory-grade container before filtration.
   Caution: Make sure the volume you want to filter does not exceed 2,000 mL (2.0 L).
b. Immerse the end of the Teflon tubing (G) into the mobile phase you want to filter. Make sure that the end of the tubing reaches the bottom of the beaker (Fig.3.2).

c. Start the vacuum source (pump or other). The liquid flows through the Teflon tubing and into the vacuum cover and wets the filter. It then passes through the filter and is collected in the 2.0 L vacuum flask (F). Once the filtration process begins, you can leave the system unattended until filtration is complete.
d. After the total volume of the filtrate was reached, *turn off the vacuum*.
e. Remove the vacuum cover and place it on a clean surface.
f. Using the forceps, discard the filter.
g. Remove the vacuum base and let it dry on a clean surface or in a laboratory oven at 40–60 °C.

#### 3.3.3.1.2 Use the filtered mobile phase

a. Use the 2.0 L vacuum flask to store the mobile phase as it is pumped through the HPLC.
b. Pass as much of the HPLC mobile phase tubing through the two-hole plug as needed (Fig.3.5).
c. Close the 2.0 L vacuum flask by inserting the two-hole plug into the flask (Fig.3.5).
d. Connect the HP LC mobile tubing to the HPLC system, and helium source (as degassing gas) and so on.
e. You can now pump the prepared mobile phase through your HPLC system.

The Millisolve system has been designed to meet the requirements of modern mobile phase preparation for chromatographic use because:

a. The system provides automatic and continuous filtration of mobile phase. Contrary to conventional filtration systems (Fig.3.6), there is no need to continuously pour liquid into a funnel as filtration progresses.
b. The large volume receiving flask has a capacity of 2 L instead of 1 L as in conventional systems. This is especially useful when filtering mobile phase containing buffers that are difficult to prepare.
c. A plug with two holes for mobile phase tubing (which is directly connected to the pump of your HPLC system) and helium tubing is supplied with the 2 L flask. This plug also allows the flask to be used for storing the mobile phase.
d. Eliminating the need to transfer the filtered mobile phase from one flask to another reduces labor and the risk of contamination.
e. The 2 L flask has a conical bottom for complete removal of all filtered mobile phase (the conical bottom of the vacuum flask allows you to completely empty the flask).
f. The 100% borosilicate glass construction of the system assures resistance to even the most aggressive solvents.

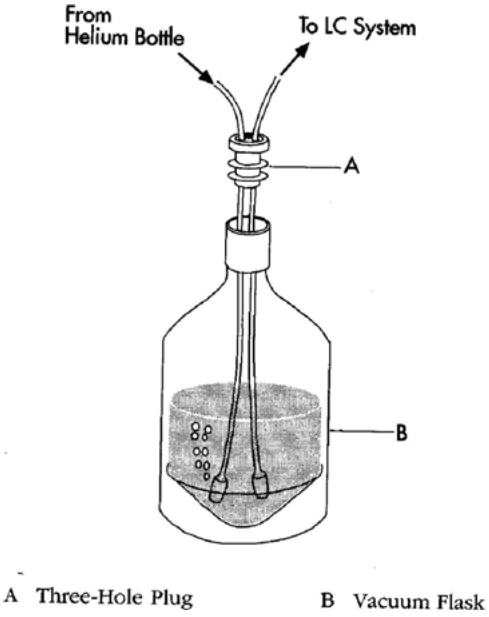


**Fig.3.5:** The MilliSolve vacuum flask for use as a mobile phase reservoir and/or storage container of filtered mobile phase. Additional degassing with helium is possible, while mobile phase is directly dispensed to the HPLC system. Reprinted with permission from Millipore Co., Waters Chromatography Division, 43 Maple Street, Milford, MA 01757, USA [22].

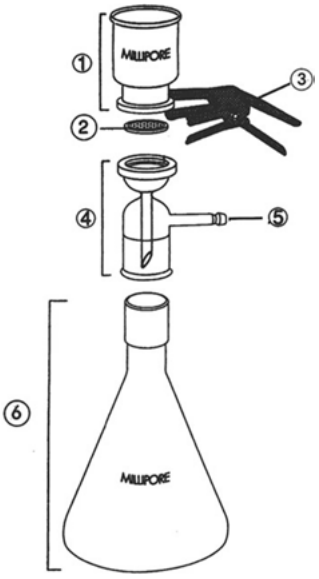


**Fig.3.6:** A conventional vacuum filtration and degassing system for off-line filtration of HPLC mobile phase.
(1) Borosilicate glass funnel, 300 mL, ground glass seal
(2) Suitable filter (47 mm) according to Tab.3.4
(3) Aluminum clamp
(4) All glass base and tubulated cap, 47 mm diameter with side arm to vacuum. On its top, the suitable membrane filter can put
(5) Vacuum outlet of the glass base and tubulated cap (6 mm OD)
(6) Ground joint flask, 1 L
Reprinted with permission from Millipore Co., Waters Chromatography Division, 43 Maple Street, Milford, MA 01757, USA, Waters Corporation [22]

**Fig.3.7:** The Millipore conventional vacuum filtration and degassing system. Reprinted with permission from Millipore Co., Waters Chromatography Division, 43 Maple Street, Milford, MA 01757, USA [22].

#### 3.3.3.1.3 Maintenance and storage of the Millisolve system

The following steps are required to maintain and store the Millisolve system:

a. Clean the glass parts of the Millisolve system by flushing them with a filtered, volatile solvent (methanol or acetone).
b. Dry all parts of the Millisolve system at room temperature or in a suitable laboratory oven. Do not use laboratory paper towels to clean the parts. The paper towels may release fibers and other particulates.
c. Make sure that all of the parts of the Millisolve system are clean and dry before storage.

### 3.3.3.2 In-line mobile phase filtration

Inlet mobile phase filter and in-line mobile phase filter are usually employed for in-line filtration of mobile phase as shown in Fig.3.8. Mobile phase inlet filter is a cylindrical filter (Fig.3.9) design to maximize the surface area. The filter was made from SS and has SS stem on tip to accommodate 1/16 in. i.d. tubing. Its position in the mobile phase inlet and under mobile phase surface in mobile phase reservoir is shown in Fig.3.8. The porosity of the filter is 0.2–10 µm. Replacing the filter is easy, and no tools are needed. Filter should be changed periodically based on the usage and mobile phase. HPLC in line filter(2), its position just before the injector as shown in Fig.3.8. It consists of SS frit (2.1–4.6 mm × 0.2 µm porosity of the frit) in between the tapered edges of inserts inside SS holder as shown in Fig.3.8. Frit

contamination can increase column back pressure and reduce the column efficiency. To avoid this problem, periodically change the frit.

In addition, some HPLC column manufacturers recommend placing "guard" column just before the analytical column as shown in Fig.3.8. The guard column not only further filters any particulates but also acts to retain highly reactive species that might otherwise enter and degrade the analytical column. Thus, the guard column serves a dual purpose in protecting the analytical column. Following these procedures will help maintain high performance and reduce system downtime. Additionally, in the RP chromatography, the stationary phase and the mobile phase are saturated because of the guard column. So the losses that occurred because of this stationary phase are minimized due to the analytical column.

The guard column should have a very similar composition to the analytical column. Usually, to reduce the pressure drop, a larger particle size should be there. Once the guard column is contaminated, it should be discarded, repacked and replaced with a similar one. Thus, it is sacrificed, and the analytical column, which is much more expensive is protected (for details, see Chapter 2).

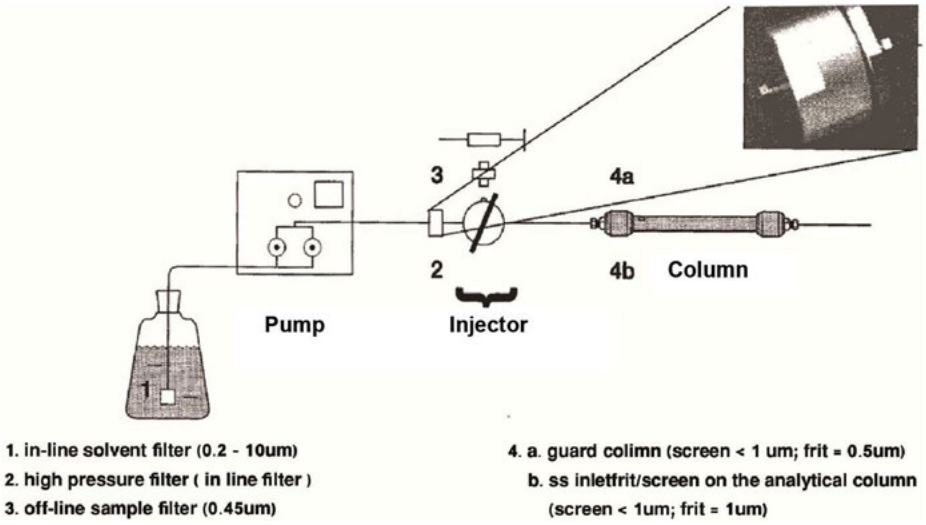


**Fig.3.8:** In-line filtration of mobile phase. Reprinted with permission from Millipore Co., Waters Chromatography Division, 43 Maple Street, Milford, MA 01757, USA [22].

### 3.3.4 Mobile phase degassing

One other important consideration regarding the mobile phase preparation is the removal of dissolved gasses. It is well known under high pressure (normal HPLC conditions) that gases present minimal problems since they remain dissolved.

**Fig.3.9:** Inlet mobile phase filter for in-line filtration of mobile phase. Reprinted with permission from Millipore Co., Waters Chromatography Division, 43 Maple Street, Milford, MA 01757, USA [22].


**Fig.3.10:** In-line filter for in-line filtration of mobile phase. Reprinted with permission from Millipore Co., Waters Chromatography Division, 43 Maple Street, Milford, MA 01757, USA [22].

However, when exiting the column, the pressure drops dramatically, frequently allowing dissolved gasses to form tiny bubbles. When these bubbles pass through the detector cell, signals to the recorder will become quite erratic. Oxygen dissolved in solvents affects not only flow stability but also UV absorption. This is caused by the complex interaction between the oxygen and the solvent molecules. Free, noncomplexed oxygen does not change the absorbance to any great degree. Oxygen dissolved in organic solvents or water changes the actual absorbance depending on the complex formed. As shown in Tab.3.5, the presence of oxygen (air) in mobile phases particularly influences detection at wavelengths lower than 230 nm causing baseline drift and higher noise levels and reducing the energy throughput of the detectors. Other detection techniques like fluorescence or electrochemical measurements at negative potentials are also affected. In fluorescence measurements, the excitation is reduced by quenching effects. The oxygen quenching varies with different types of compounds, and aromatic hydrocarbons, aliphatic aldehydes and ketones are very susceptible.

**Tab.3.5:** UV absorbance of methanol saturated with various gases at 20 °C.

| Gas | 220 nm | 230 nm | 280 nm |
|---|---|---|---|
| Helium | 0.202 | 0.179 | 0.074 |
| Nitrogen | 0.195 | 0.178 | 0.074 |
| Air | 0.429 | 0.288 | 0.076 |
| $CO_2$ | 0.278 | 0.229 | 0.073 |

If, for any reason, the characteristics of the solvent change, it can adversely affect the accuracy and precision of the entire solvent delivery system. This might lead to changes in both the peak time of retention and, to some extent, peak area or height. Therefore, any change in dissolved $O_2$ content may affect a UV baseline considerably. This phenomenon is particularly evident in the solvent tetrahydrofuran. Dissolved $O_2$ does not seem to affect the absolute sensitivity of UV system but primarily causes baseline drift.

The four main methods used to degas the mobile phase are as follows:

- Sparging with a less soluble gas such as $N_2$ or He,
- Sonication
- Reducing pressure by vacuum and
- Heating

These methods may be used singly or in combination.

#### 3.3.4.1 Sparging

Sparging or bubbling a gas through solvent reduces the pressure partially of the unwanted gas that is present on the top of the solvent. This will remove unwanted gas from solution but it will saturate the solvent with the second gas. Sparging with either $N_2$ or He will remove background absorbance on a UV detector and the quenching phenomenon caused by dissolved $O_2$ on a fluorescence detector. Helium sparging tends to give a more stable baseline on an RI detector than the air-equilibrated solvents, and the absolute mass of dissolved gas is considerably lower than with air-equilibrated solvents. Somewhat better pumping reproducibility is obtained from the degassed solvents in a piston pump. There is no significant difference in absolute mass between $N_2$ equilibration and air equilibration, which results in no significant improvement in pump stability. Sparingly will the solvent come in the equilibrium state because of gas. It can only be maintained if there is success in covering the solvent with a blanket of gas completely.

It has been found that helium and nitrogen are most effective in replacing oxygen in solvents, and proton donors like water and methanol are more difficult to degas than alkanes like hexane. In this context, helium sparging is less efficient than nitrogen sparging. This may be explained by the rapid rediffusion of air into the solvent, as helium is specifically lighter than air [10]. The use of nitrogen is very effective in removing oxygen from solvent but does tend to form bubbles in the system. Fig.3.11 illustrates a suggested method of preventing a rediffusion of air during helium sparging (using compensating reservoir). After 1 min of sparging at a flow rate of 100 mL helium/min, the gas stream is reduced to 5–10 mL/min. Sparging helium is the best degassing technique you can opt for to prevent the absorbance of the mobile phase.

#### 3.3.4.2 Heat

Heat alone accomplishes one, and possibly two, thing(s) for mobile phase. The first is to prevent further absorption of gas into solution. Secondly, heat may reduce the solubility of a gas in solution. It does not necessarily hold true for most of the organic solvents but is true for most of the gases present in $H_2O$.

#### 3.3.4.3 Vacuum

The surface pressure of the solvent is reducible through a vacuum, so as the pressure is reduced and the mass of gas in solution is reduced.

#### 3.3.4.4 Sonication

Energy is driven inside the solvent through sonication with the help of very high-energy sound waves. This seems to cause aggregation of the submicron-sized “particles” of gas. With the aggregation of gas, the bubbles increase and become so big

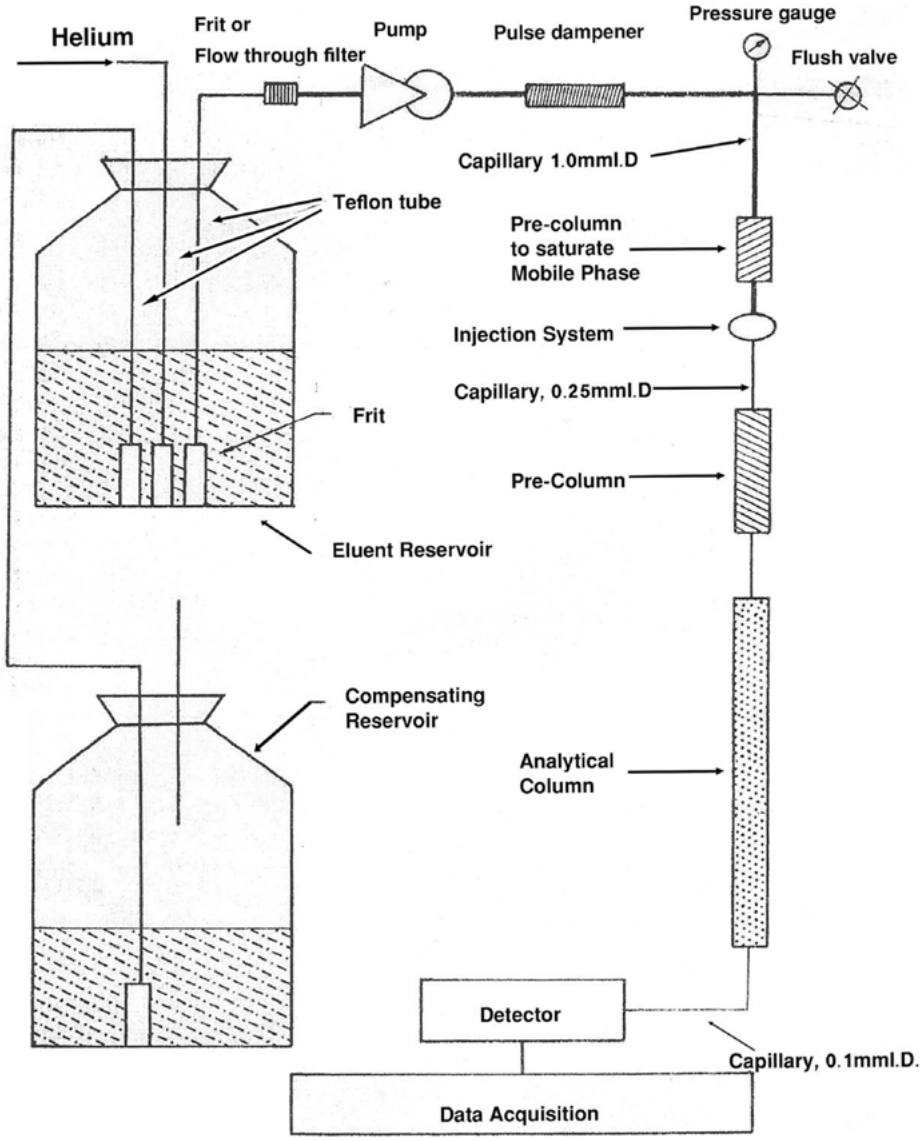


**Fig.3.11:** Schematic diagram of an HPLC system using sparging technique with He gas for degassing the mobile phase. Permission granted to reproduce by copyright holder [10].

that they easily come out and dissipate from the solvent. If only sonication happens, it alone takes almost 20 min to degas a gallon of the solvent.

The degassing operation should be as efficient as possible. If the gas needs to be removed in the least possible time, one should be aware of the following considerations:

a. In a fluorescent detector, using helium sparging will give a better sensitivity and stable baselines. It will also prevent the reabsorption of the atmospheric gases.
b. If one has to use heat for degassing the solvent, it needs to be boiled. This is not practical in the case of mixed solvents. Even though it is not practical, you can increase the partial pressure of the solvent by low health levels, thereby reducing the rate of resolubilization of the gas.
c. If used alone, vacuum cannot be accepted as an efficient method to degas a solvent because it is very slow. However, if vacuum is used in conjunction with a 0.45 µm sintered glass Millipore vacuum filter (Tab.3.4) with a 300 mm pressure differential across the bed, a gallon of solvent can be filtered and totally degassed in approximately 8 min. However, use of vacuum from degassing might end up changing the mixed solvent composition.
d. Vacuum along with sonication can quickly degas a 1 L solvent. It will not change the composition of mixed solvents appreciably. Fig.3.12 provides comparison between using ultrasonic agitation of the mobile phase and helium sparging for removing air. It is clear that helium sparging presents a good method [10].

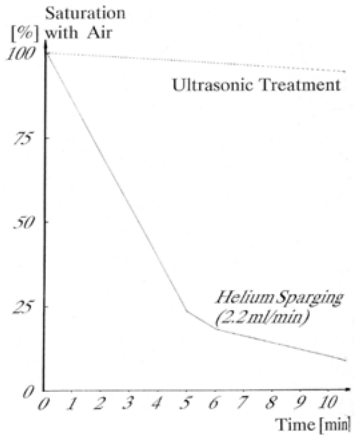


**Fig.3.12:** Illustrative diagram showing the efficiency of helium sparging and ultrasonic agitation for removing air from mobile phase. Permission granted to reproduce by copyright holder [10].

*According to the previously mentioned information*, a ranking of the effectiveness methods for degassing a mobile phase would be:

a. Vacuum combined with ultrasonication for a few minutes
b. Sparging
c. Ultrasonication for 20 min
d. Vacuum alone
e. Heating

Note: In all of the above techniques (except sparging), the solvent will reequilibrate to air saturation in 12–24 h dependent on the solvent in the mobile phase.

### 3.3.4.5 Mobile phase degasser (in-line degassing of mobile phase)

Just a while ago, a common technique to degas is the in-line degassing method for the HPLC mobile phase [12]. Most HPLC systems have this as a standard component. As shown in the figure, a membrane of tube that allows gas to pass through and a vacuum chamber are main components of the degasser as shown in Fig.3.13. The in-line degasser comes into function when vacuum is applied. It is done on the outer layer of the membrane. From here, the membrane pore works in two ways. Firstly, it allows the gas that has been dissolved to go out. Secondly, it ensures that the remaining mobile liquid phase should stay inside. The degasser will be able to remove sufficient dissolved gas only when the combination of vacuum, residence time and membrane porosity is perfect. Once this is done, it will ensure outgassing so that the detector and LC pump operate accurately and reliably.

When it comes to these degassers, they are reliable and accurate. This allows them to operate with very little or zero maintenance for many months and years at a time. To avoid ending up with any microbial growth, it is preferable not to store it near any water or buffer inside the tubing. This can lead to the pored becoming contaminated generally or blocked because of the growth. Some of the contamination,

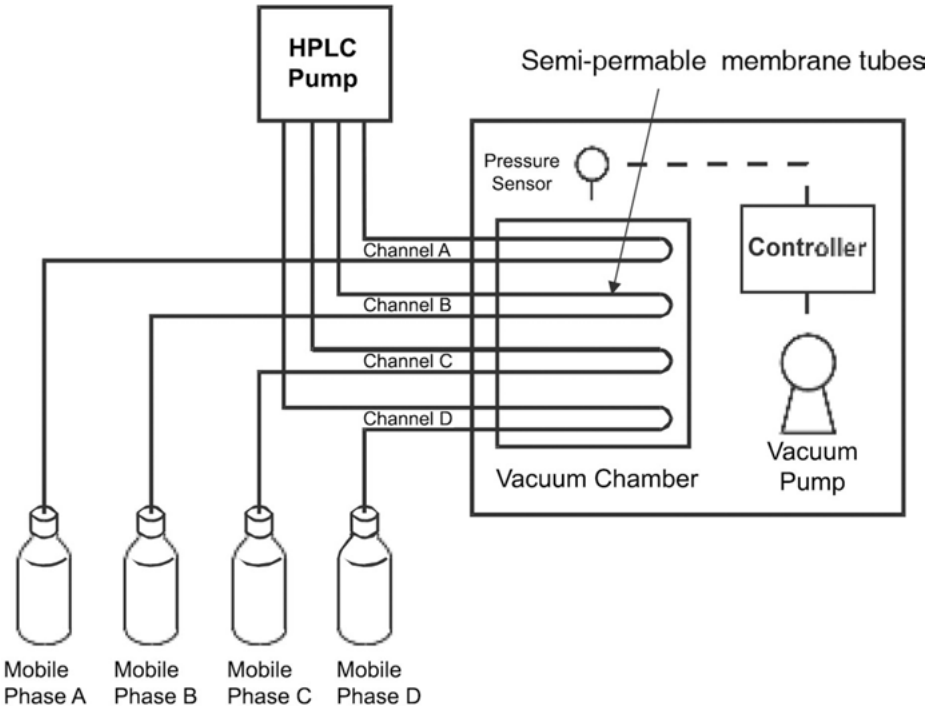


**Fig.3.13:** A schematic diagram showing an online vacuum degasser with four solvents. Reprinted with permission from Millipore Co., Waters Chromatography Division, 43 Maple Street, Milford, MA 01757, USA, Waters Corporation [22].

the biological one, can be removed with cleaning the pathways of the mobile phase with 30% phosphoric acid. After flushing with that, rinse it with water. Sometimes, the entire membrane needs to be replaced and removed because of the level of contamination. This can end up being very expensive. The best way here is to try not to end up with contamination by removing the water phases when it is not being used. There are other causes like damaging, blockage or loosening of the waste and pump or the tubing in between the vacuum or failing or the pump of the vacuum. Because of the universal application and presence of the in-line degasser and the fact that it hardly requires any maintenance, most people do not even know about the problems that can arise due to the bubbles present in the mobile phase.

### 3.3.5 Water for mobile phase (water HPLC grade; ultra pure water)

Water presents more important solvent for preparation of mobile phase. Specification of HPLC-grade water must be as follows:

1. Resistivity = 18.2 MΩ cm at 25 °C
2. Total organic compounds (TOC) ≤ 5 ppb
3. Conductivity = 0.055 μS/cm

This type of water is called HPLC-grade water or ultra pure water. There are commercial apparatus for production of HPLC-grade water from tap water (*conductivity μS/cm < 20 and TOC < 50 ppb*) such as Milli-Q-Ultra pure water purification systems (Millipore Co., Waters Chromatography Division, 43 Maple Street, Milford, MA 01757, USA [22]) according to the diagram shown in Fig.3.14.

If you do not have Milli-Q-Ultra pure water purification system, you can use trace organic removal cartridges (NORGANIC; Millipore Co., Waters Chromatography Division, 43 Maple Street, Milford, MA 01757, USA [22]; Fig.3.14) for preparation of HPLC-grade water. As shown in Fig.3.15 the NORGANIC cartridge is a disposable cartridge designed to remove organic contaminants from pretreated water (deionized water, reverse osmosis and distilled water).

It contains a proprietary resin that scavenges organic contaminants from the feed water. A suitable 0.65 μm low extractable membrane filter is intended to be used downstream of the NORGANIC cartridge to remove all particulates larger than 0.65 μm.

The Norganic cartridge housing and end caps are made of a low extractable plastic with male luer slip connections on each end. The luer slip comes sealed with end caps, which may be reused for storing the cartridge. The Norganic cartridge produces HPLC-grade water, that is, 99% free of organic contaminants from pretreated feed water. The resulting water from NORGANIC cartridge must be free from the ionic contaminants to trace levels using anionic and cationic exchangers.

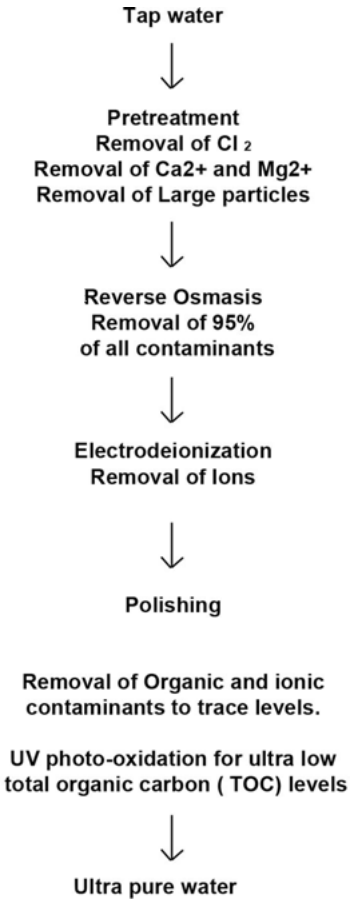


**Fig.3.14:** Diagram showing steps for production of ultrapure water from tap water in the laboratory using Milli-Q-Ultra pure water purification system. Reprinted with permission from Millipore Co., Waters Chromatography Division, 43 Maple Street, Milford, MA 01757, USA [22].

#### 3.3.5.1 *Directions for use of* Norganic cartridge (trace organic removal cartridge)

a. Place the glass base and tabulated cap (which is connected to the vacuum source) on top of the 2.0 L flask.
b. Place one suitable membrane filter on the SS support, screen, attaché cartridge holder and connect vacuum tubing.
c. Remove cartridge from package and twist off the inlet end cap.
d. With the tubing provided with the system, establish feed water flow and then attach the tubings to be the inlet end of the cartridge.
e. Remove the other end cap from the outlet end and place cartridge into cartridge holder, making sure a tight connection is made.
f. Apply vacuum, 16 Hg draining through and discarding the first two 250 mL volumes of water.
g. Process the required amount of water. The membrane may become darkened by sorbent fines.

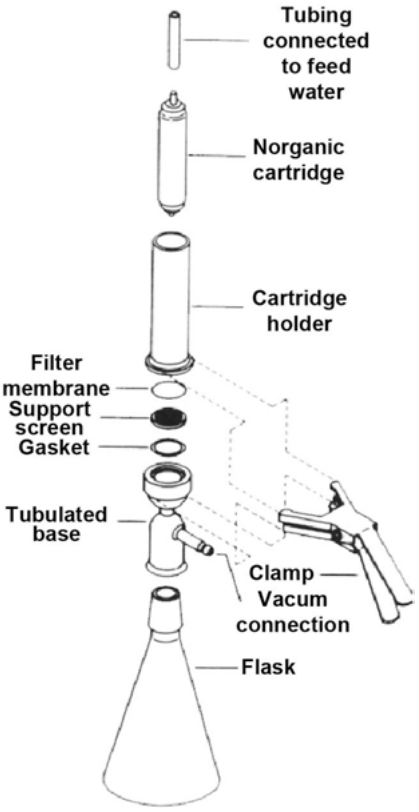


**Fig.3.15:** System for preparation of HPLC-grade water in the laboratory using Norganic cartridge (trace organic removal cartridge). Reprinted with permission from Millipore Co., Waters Chromatography Division, 43 Maple Street, Milford, MA 01757, USA [22].

h. For store the cartridge between uses, replace the end caps on both ends. The cartridge should be purged and the receiver flask should be rinsed with two 75 mL volumes of Norganic-grade water.

### 3.3.6 Important notices about mobile phase selection and preparation

a. Analysts are now fortunate, in that manufacturers supply HPLC solvents of adequate purity. Nevertheless, care must be taken when using them as, during their production, often only UV-absorbing contaminants are eliminated, and small particles and other interfering substances can remain. Often organic solvents contain residues of small quantities of water, causing problems with absorption chromatography (normal phase chromatography). It is advisable to dry all solvents used in normal phase chromatography with silica by storage over a molecular sieve. To prevent damage to valves and pump seals, the dried solvent should be purified by passing through a membrane filter or by distillation. Usually after purification by

passing through ion-exchange resins or distillation, a lot of interfering organic materials like phthalates still remain.

b. Mobile phases are mixtures of volumes which are separately volumetrically measured and finally combined. The final volumetric construction has to be considered, for instance, a mixture of 50 mL of methanol and 50 mL of added water has a total volume of 96 mL rather than 100 mL.
c. After preparation, all mobile phases should be filtered before use. A suitable mobile phase membrane filter of 0.20–0.45 μm is selected in accordance with the nature of the organic solvent being used. Examination of the stability of the mobile phase filter materials in the organic solvents being in use in preparation of the mobile phase is recommended.
d. During filtration, the initial few milliliters drawn off should be discarded, as they are often contaminated by organic stabilizing agent or other unwanted elements.
e. A porous SS frit (0.2–10 μm) is placed at the very end of the Teflon tubing through which the mobile phase is to be drawn. At least once a week, this frit must be washed in nitric acid (6 N) and finally in water using an ultrasonic bath.
f. Water-containing buffer solutions tend to precipitate when left standing for any length of time. These precipitations are not retained by the mobile phase filter in the solvent reservoir and may cause column blockage.
g. The solubility of buffer solutions and ion-pairing agents is limited in organic solvents. If the proportion of organic solvent increases during a gradient elution the column could be irreversibly blocked by insoluble salts and therefore it is necessary to purge water throughout the system before and after analysis using buffered eluents. Before carrying out the gradient elution, the solubility of all solvents used should be examined to prevent buffer salt precipitations or nonmixed eluents during the chromatographic separation. As a precaution a further in-line mobile phase filter can be placed between the mixing chamber and the injection valve.
h. For compatibility of the stationary and mobile phases, use mobile phase that is compatible with stationary phase as shown in Tab.3.6.
i. Practical considerations show that mobile phase should not degrade the column packing or either the equipment.
j. Strong acids (particularly halide acids), halide solutions and bases should not be used. These can corrode many system components and cause spurious results.

To extend column lifetime, keep in mind the following information about HPLC – mobile phase must be put in your consideration:

a. All mobile phases should be freshly prepared, filtered and degassed.
b. Use recommended guard column and in-line precolumn filter.
c. To maintain the pressure of the column under 4000 psi, the flow rate of the mobile phase needs to be adjusted.
d. Filter samples through 0.2–0.45 μm membrane before injection.

e. Use analytical grade or better reagents' HPIC-grade solvents and water HPLC grade for all work.
f. Discard any solutions that show evidence of bacterial growth.
g. Another important element is the chemical purity of solvents. This is an important consideration because a large quantity of the solvents comes through the column. Because of the large solvent quantity, it is possible that trace solvent impurities concentrate inside the column. Consequently, these end in detrimental analytical results. Spectrograde or HPLC-grade solvents are recommended.

**Tab.3.6:** Column/solvent compatibility chart.

| Column | Useful solvent | Storage solvents | Risky solvent |
|---|---|---|---|
| Normal phase | Hexane Methylene chloride Isopropanol Tetrahydrofuran | Hexane Heptane | Water Methanol |

| Reversed phase | Water Methanol | Methanol | Solvents with pH <2 or >7.5 |
|---|---|---|---|
| Polar-bonded phase | Normal or reverse-phase solvents | Hexane Heptane | Solvents with pH <2 or >7.5 |

## 3.4 HPLC mobile phase pumping system

### 3.4.1 HPLC mobile phase pumping system components and operating guides

A typical HPLC **mobile phase pumping system** includes between 1 and 4 reservoirs for mobile phase. Mobile phases are stored in 500 or 1,000 mL glass vessels. To prevent contamination of the working environment, the mobile phase reservoirs have to be sealed with a safety cap (Fig.3.16; for details, see Chapter 10). The mobile phase comes up from the reservoirs by pumping of one or more of the pumps.

The majority of HPLC pumps are reciprocating single, dual or triple piston pumps, with or without diaphragm delivering continuous constant flow (Fig.3.17 and 3.18). They all operate on the principle that the spring-loaded piston delivers the mobile phase by a continuous backward and forward movement, sucking the mobile phase from the reservoir through a sapphire or ruby ball check valve into the pump chamber on the input stroke and expelling in through another ball check valve into the column feed tubing on its reverse stroke [3,4,6,7,9,12, 23].

In direct piston pumps, the eluent is displaced out of the pump chamber by the piston itself, driven by a cam linked to the pump motor (Fig.3.18a). In the case of the diaphragm-type pump, the piston reciprocating movement takes place in an oil-filled chamber, causing corresponding movement of a diaphragm forming the far wall of the

chamber, and this diaphragm movement performs the intake and expulsion of the eluent through a second chamber remote from the pump piston itself which thus does not come into contact with the eluent use (Fig.3.18b). For precision pumping, the pistons must operate through effective seals, and as in any mechanical device these seals suffer wear over a period and have to be replaced from time to time. Dual piston pumps are the most common, delivering constant flow with virtually no pulsation, even at great differences in the mobile phase viscosities constant flow is achieved by measures such as automatically monitoring the pressure at the pump inlet and outlet and adjusting the pump drive to maintain balance. The air bubbles present in the inlet or outlet valves cause the pressure pulsation. Purging the pump with isopropanol can often eliminate this condition.

Lower mobile phase pulsation (pulseless flow) can be obtained from using a dual-head reciprocating pump Fig.3.17. These pumps are expensive and have two pistons filled and pump is 180° out of phase [6]. These pumps have either two or four check valves.

A detailed discussion of the mechanics of these and other pumping systems is provided in [30].

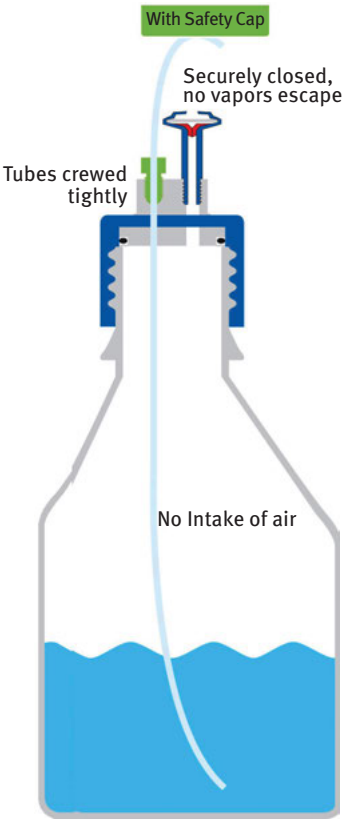


**Fig.3.16:** Illustrative diagram showing a mobile phase reservoir equipped with safety cap.

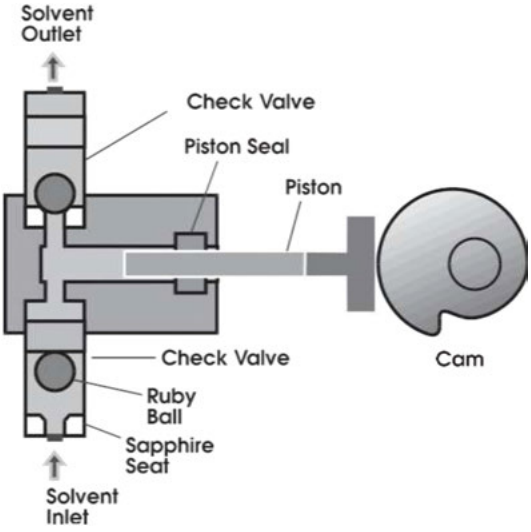


**Fig.3.17:** Illustrative diagram showing a single piston reciprocating pump for HPLC.

*The following standard operating procedures according to Dong [12, 23] are recommended for HPLC pump operation:*

a. Place (10 µm filters) solvent line into the reservoirs of the intermediary solvent.
b. Turn on the in-line vacuum degasser and the seal wash pump system (if available).
c. Set the upper pressure limit (typically at 3,500–4,000 psi).
d. By drawing out 10 mL of every solvent from the solvent line present by opening the purge/prime valve performs the "dry" prime. This is done if the system has not been used for quite a long time or the pump is dry.
e. A wet prime should be performed on all lines while you change the solvent reservoir. Remember, many of the old vacuum degassers have a 10 mL or internal volume. So, this or a higher quantity should be purged with new solvents.
f. Program the pump to automatically shut down after purging out the sample sequence.

### 3.4.2 Mobile phase pressure and flow relationships

An arrow may appear on the HPLC column body (see Fig.1.2). This arrow is for reference purpose only and indicates the flow direction used during testing of the column. The column can be operated with mobile phase flowing in either direction.

The flow rate is the value on which you can set the mobile phase flow. Generally, it is measured in mL per minute. A pressure drop along the column occurs after the flow rate is determined along with separation conditions. This is accountable for a

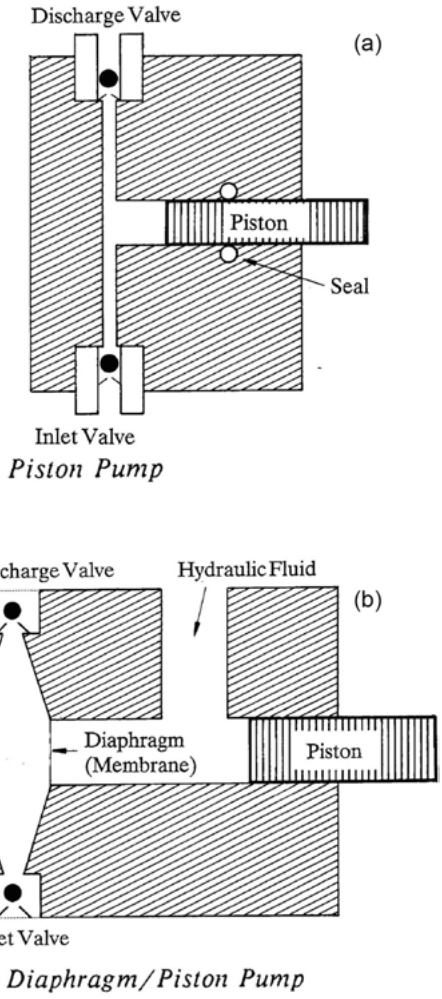


**Fig.3.18:** Illustrative diagram showing a single piston reciprocating pump for HPLC.
a. Direct piston pump
b. Diaphragm-type pump
Permission granted to reproduce by copyright holder [10].

majority of the pressure reading that comes in measurement at the beginning of the column (it is shown in the pressure gauge or LC controller). Even though mostly the LC operation shows pressures around 1,000–6,000 psi, the pressure of the system needs to be maintained at a value lower than the maximum. Hence, it is pivotal to predict the pressure changes and its variations. The relations can be expressed as follows:

$$P = 250\,L\,r\,F/d_p2\,d_c2$$
$$= 1,200\,L\,r\,F/d_p2\,(0.46\text{ cm i.d. columns})$$

L stands for column length (cm), r stands for mobile phase viscosity in cP, the flow rate is denoted by F (mL/min), $d_c$ stands for the internal diameter of the column (cm) and $d_p$ represents the diameter of the particles of column packing inside the column. In RP HPLC (which is the most commonest LC method), values of r range falling between 0.5 and 1.5, usually the particles are 5 μm in size, and 15–25 cm columns are common. If the flow rate is 1 mL/min, the column pressure will usually be in between 500 and 2,000 psi.

The following reasons can be responsible for a varied pressure (with constant flow rate):

a. A change in the temperature (higher temperature with less pressure) or composition of the mobile phase
b. Change in either the particle size or length of the column
c. Blockage in the column by some particulate matter or substance (this is a very normal occurrence that ends in a small and consistent rise in the pressure, Tab.2.9 Chapter 2) summarizes the viscosity values that are used for RP HPLC in the mobile phase.

### 3.4.3 HPLC mobile phase flow control and programming systems

#### 3.4.3.1 Gradient elution

As a part of HPLC pumping system, many commercial instruments are equipped with computer-controlled devices for measuring the flow rate by determining the pressure drop across a restrictor located at the pump outlet. Any differences in signal from a preset value is then used to incrrease or decrease the speed of the pump motor.

Nowadays, most of the HPLC instruments also have a means for changing the composition of the solvent involved either in one go or step by step (gradient elution). For example, the instrument shown in Fig.3.19 contains a proportioning valve that permits mixing up of four solvents in a preprogrammed and continuously variable way. In Fig.3.20, there is an instrument using two pumps for gradient elution of mobile phase.

In the following example (Fig.3.21), a mixture of six components is to be analyzed using mobile phase A, mobile phase B or a gradient of both. As shown in chromatogram, the solvent A is optimum for the first two weakly retained components 1 and 2, which are well separated and quickly eluted in narrow peaks. However, components 3 and 4 are eluted much later as very broad peaks.

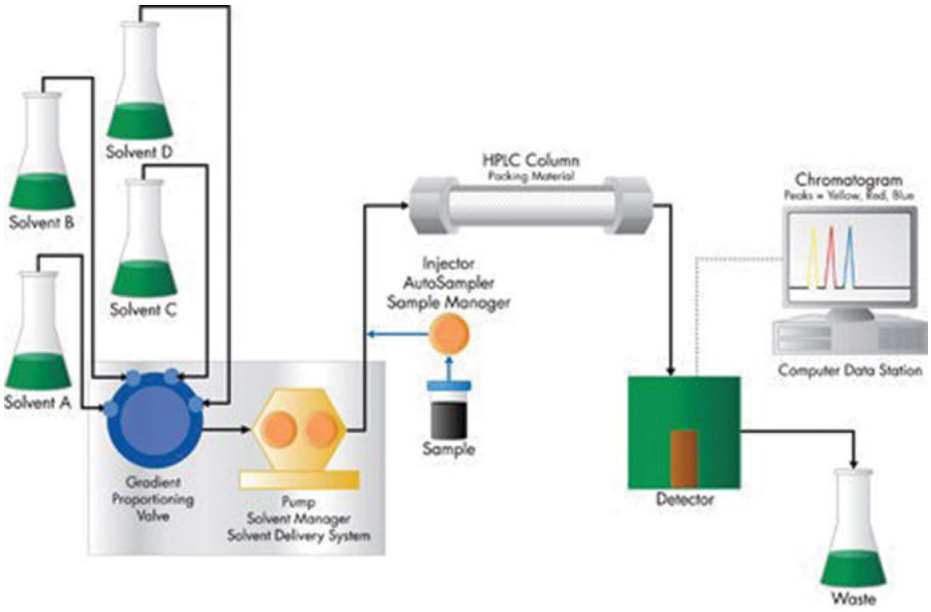


**Fig.3.19:** HPLC gradient elution system for mobile phase using two pumps. Reprinted with permission from Millipore Co., Waters Chromatography Division, 43 Maple Street, Milford, MA 01757, USA [22].

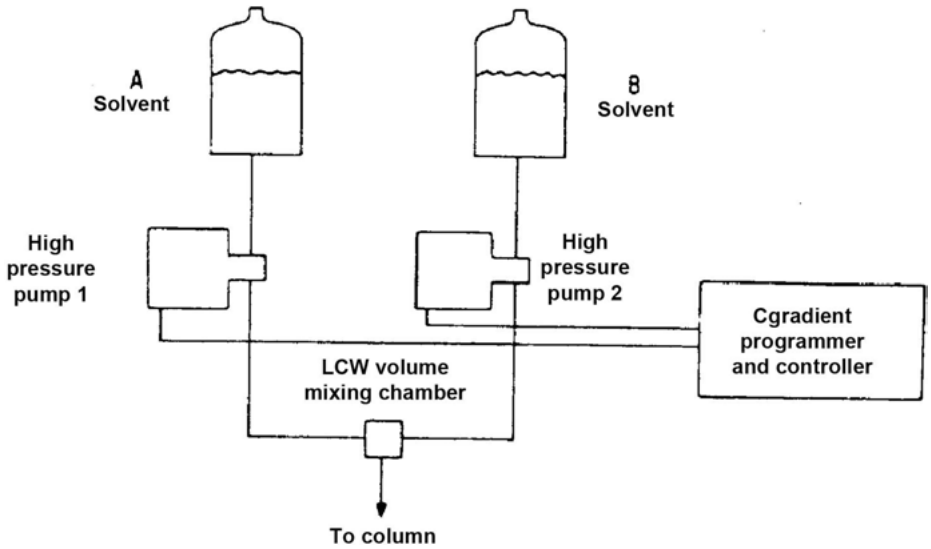


**Fig.3.20:** HPLC gradient elution system for mobile phase using four pumps.

In chromatogram b, solvent B is optimum for the separation and elution of the more strongly retained components 5 and 6, while components 1 to 4 are eluted very fast and are not well separated. A certain mixture of solvents A and B may have improved separation and elution of 3 and 4, but again 1 and 2 are not well separated while 5 and 6 have rather long retention time and appear as broad peaks (chromatogram c).

A convenient strategy that can be employed is to start elution with mobile phase A, allow 1 and 2 to elute one after the other, switch to an appropriate mixture of A and B to separate 3 and 4 and finally elute 5 and 6 using solvent B (chromatogram d).

In a special case of gradient elution, solvent composition is changed continuously, that is, from 100% solvent A to 100% solvent B (chromatogram e).

The advantage of this type of elution lies mainly in the increased speed of analysis through faster elution and increased sensitivity resulting from the sharper peaks.

The fact that gradient analysis requires polished skills and technical instruments in method development, and difficulties in method transfer are a big disadvantage.

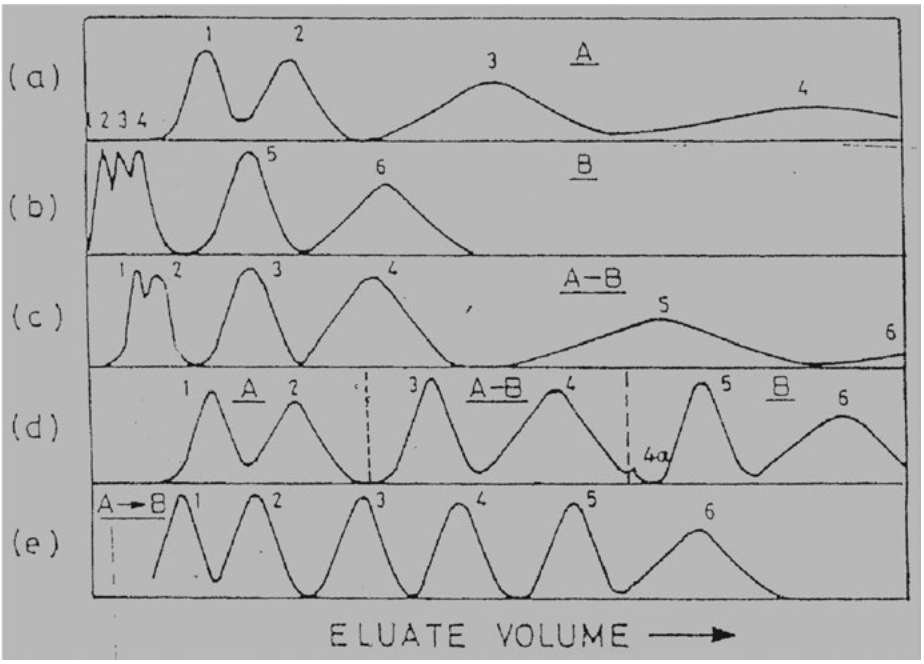


**Fig.3.21:** A mobile phase elution strategy for separation of a mixture of six chemical compounds by HPLC gradient.

#### 3.4.3.2 Isocratic elution

An isocratic elution occurs if the composition of the mobile phase is constant in the process of separation of the analytes. It is a Greek word, which means that the strength of the elution will remain constant. Equilibration of the column matrix all the time is the biggest advantage of the isocratic elution. This is why most of the pharmaceutical assays have always been isocratic analysis. These employed one mobile phase in the entire sample elution, and are frequently found in applications of quality control. The reason is that mobile phases are premixed and the equipment for HPLC is simpler.

The most obvious disadvantage of it is the very limited and restricted capacity of peaks that occur. Peak capacity is the most number of peaks that the chromatogram can accommodate. Samples that have analytes of diverse polarities can show problems too. In case samples are complex, gradient analysis is used. In this, the mobile phase strength increases consistently during the sample elution. Gradient analysis is better for samples that have wider polarity analytes.

Gradient chromatography is amenable for impurity testing of drugs in drug quality control laboratories and applications with high-throughput screening.

## 3.5 Solvent miscibility

Before changing from one solvent to another, refer to Fig.3.22 to come to the miscibility of the solvents that are going to be used. The following considerations are applied when changing solvents.

a. You can directly make changes that involve two miscible solvents. When it comes to changes comprising two solvents which are not totally miscible, for example, from water and chloroform, an intermediate solvent is required (like methanol).
b. Temperature operations affect the solvent miscibility; if operating at an elevated temperature, keep in mind that solvent solubility is affected by higher temperature.
c. When dissolved in water, buffers can precipitate when you mix them with the organic solvents.

## 3.6 Optimization of the mobile phase

The chromatographic method works to achieve some degree of the separation of the components involved in the sample.

When we develop the HPLC method, we may want to separate the different components with a minimum resolution. Or you can also work to get a maximum resolution in a preset time and may also try to resolve either one or a number of components from each other quickly without wasting time. Parameter operating on it like flow rate, column specifications and temperature can influence the separation

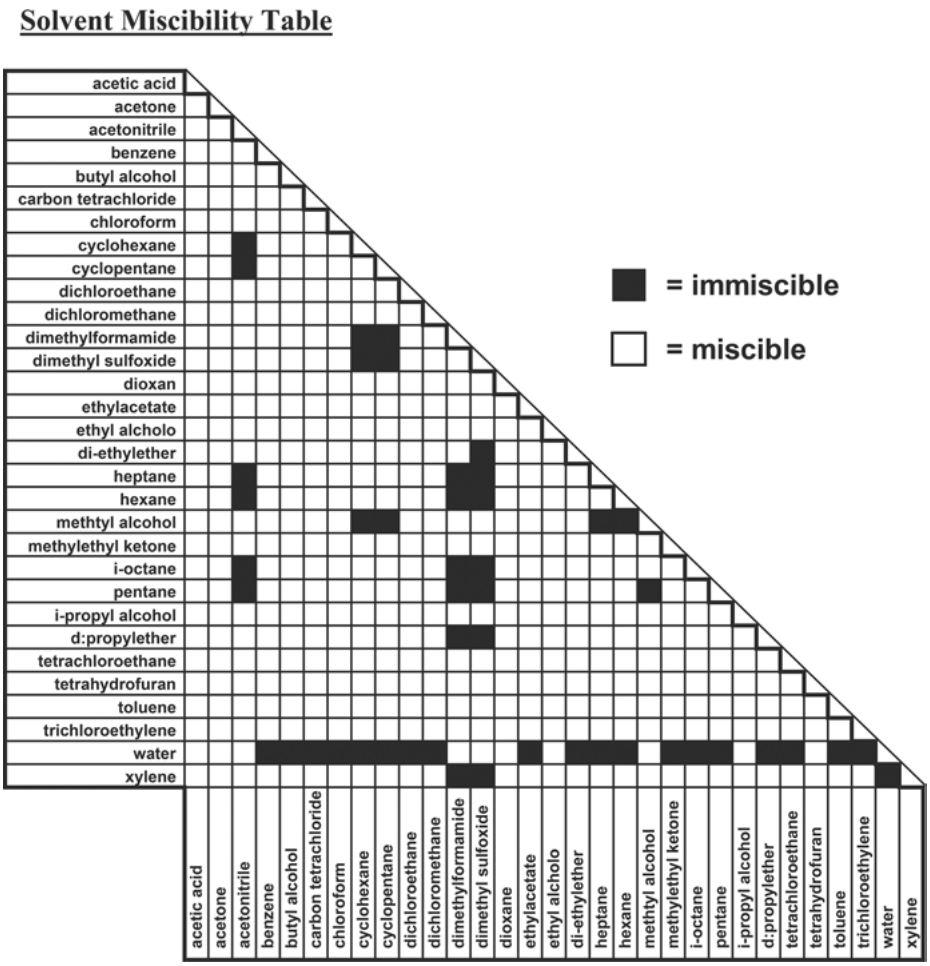


**Fig.3.22:** Miscibility for some HPLC solvents.

process. Of course, it depends if the choices are made sensibly, and the most influential factor controlling the separation is still the composition of the mobile phase.

In the examples given earlier, for the mobile phase we used a combination of two different solvents to achieve separation. This is called binary solvent mixture or system. A combination of three solvents or four solvents may also be used if separation was not achieved. These are called ternary or quaternary mixture, respectively.

A scientist named L.R. Snyder has developed a systematic scheme or approach for the selection of the optimum mobile phase composition using what is know as *Snyder Solvent Selectivity Triangle* [2].

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# 4 HPLC injection system (injector)

## 4.1 Introduction

The sample is introduced in the high-performance liquid chromatography (HPLC) column with the help of the HPLC injector. As mentioned in Chapter 1 (Fig. 1.2) and as shown in Fig.4.1, the injection system in the HPLC instrument is positioned at the high-pressure side, after the pump head, in the solvent delivery system. When we inject the sample inside the HPLC system, it is done at atmospheric pressure and is a critical and important step in the entire chromatographic procedure. The fact that the mobile phase is delivered in the HPLC system under pressure demanded a suitable sample injection device. This job can be done by loop injection system (injection valve) which is located between the analytical column and the high-pressure pump of mobile phase. Loop injection system (injection valve) by far is the best and is also the most widely used for HPLC injection system(this was the method followed to avoid any changes in the flow or pressure of the HPLC eluent stream. For this, reproducible amounts of the sample were introduced. In this chapter, we discuss the valve injection system in HPLC as a manual injector for HPLC equipment. Also we discuss the roles of HPLC autosampler injector as a more advanced technique [1–5] for injection of pharmaceutical product samples during their analysis in drug quality control laboratories.

## 4.2 Loop injector for HPLC

A common injector for HPLC Fig.4.1 and Fig.4.2 is the manual injection valve (loop injector) such as Rheodyne model 7125 or 7725 injector (Rheodyne Inc., P.O. Box 996, CA 94931, USA [6]); for the past years, the industry standard was the Rheodyne 7125 injector but the Rheodyne 7725 injector was then used as a replacement in the 1990s. This was possible without any disruptions to the monetary flow [7]. It consists of a sample loop (a), a rotor that has a six-port valve (b), an injection needle port for sample injection (c), and a handle assembly (d) (Fig.4.2). Practically, a loop injector in HPLC requires a multiple-port rotary switching valve in which this loop can be filled manually (using a syringe) by the sample at the LOAD position (low pressure). As shown in Fig.4.3, the injection valve has six ports including a loop of specified volume (5 µL to 2 mL) which is filled by a sample using a normal low-pressure syringe. The valve can be moved manually between LOAD and INJECT positions (Fig.4.2–Fig.4.5). In this load position, you can directly lead the mobile phase through the pump to the column. This allows you to directly inject the sample inside the loop. During the INJECT position, the mobile phase passes through the sample loop. By doing so, it flushed all the sample contents on top of the column.

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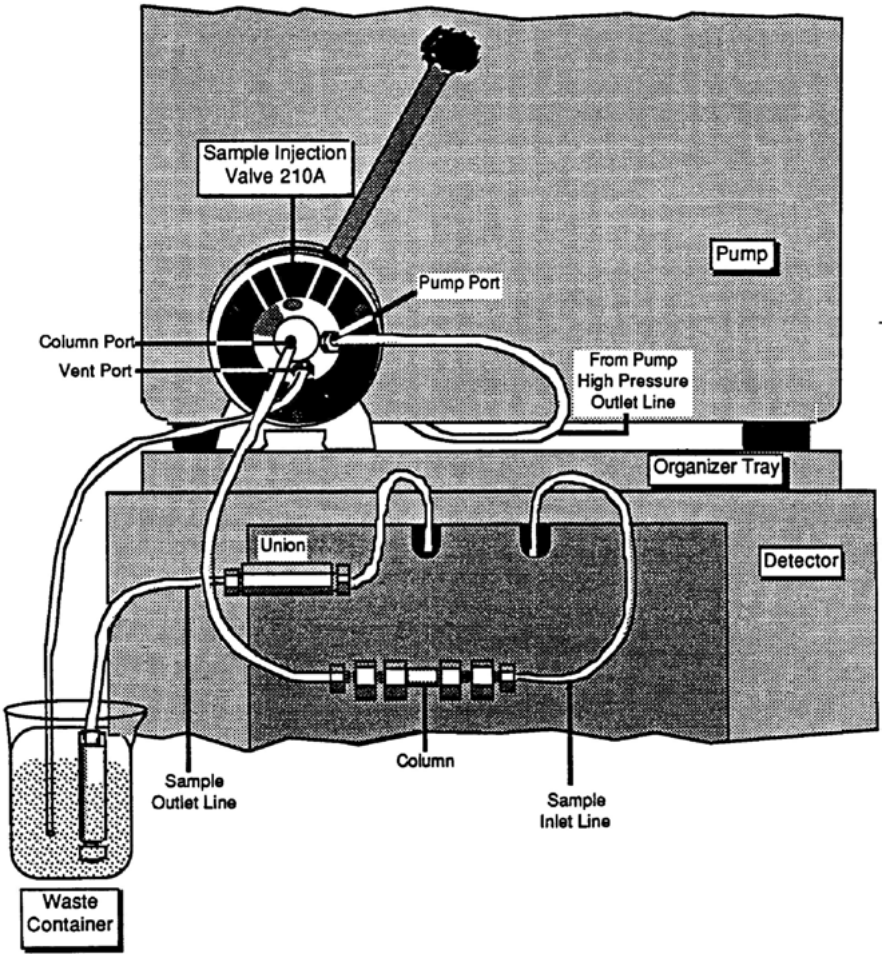


**Fig.4.1:** The diagram shows the location of the loop injector of an HPLC (injection valve) in relation to the other ports and parts of the valve injector. These include pump port, column port, vent port and the back view of the injector.

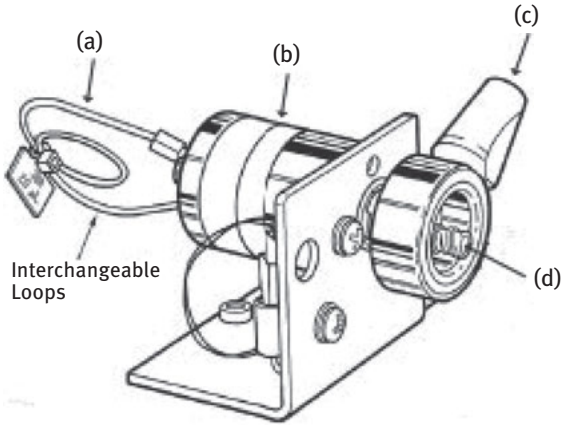


**Fig.4.2:** The diagram of the loop injector of HPLC (manual injector valve) consists of (a) sample loop, (b) rotor with a six-port valve, (c) handle assembly can be moved between INJECT and LOAD positions and (d) an injection needle port for sample injection.

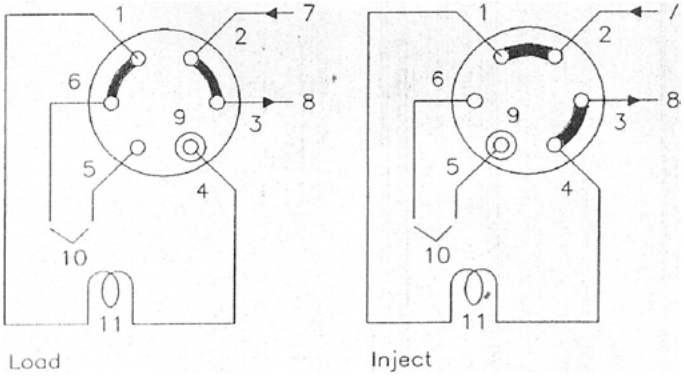


**Fig.4.3: Diagram of a six-port valve of an HPLC injection valve and the injection valve** positions (LOAD and INJECT positions).
1–6, valve ports; 7, mobile phase inlet from pump; 8, mobile phase outlet to column; 9, needle port; and 10, vents.

## 4.3 Manual injection

There are two modes in which the injector valve can be operated. One is the fixed-loop mode and the second one is the partial-loop mode [1, 3]. In the fixed-loop mode, you fill the sample more than the volume of the loop. It is overfilled with the sample to almost 2–4 times the actual volume of the loop. On the contrary, exactly <50% of the total volume of the loop is filled with a syringe with the sample aliquot. For manual injection, remove the loading plug from the injector port. Set the LOAD/INJECT handle

to LOAD position Fig.4.2–Fig.4.4. Load the syringe (it should have a 22-gauge and should support a blunt-tip type needle, (Fig.4.6) with the solution to be analyzed. Insert the needle of syringe into sample loading port Fig.4.7a. Engage the needle in the needle port sleeve until the needle is fully seated (Fig.4.7a and Fig.4.7b), and load the desired volume of standard or sample solution by pushing the plunger of the syringe. Just by turning the valve to the INJECT position, the sample can be delivered to the column Fig.4.2, Fig.4.3 and Fig.4.5. All you need to do is to take out the syringe and keep the valve in the INJECT position. Once you are done and the sample run time has ended, switch the valve back to the LOAD position for start second injection. To avoid cross-contamination, you need to flush the valve passages and the needle port between the runs. This has to be done when the valve is in the LOAD position.

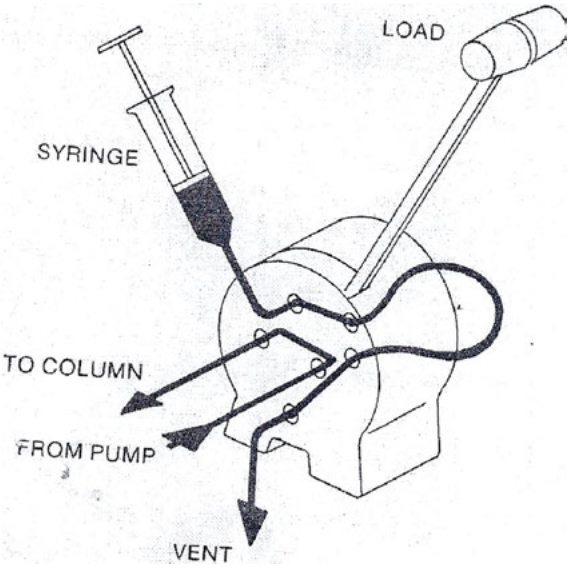


**Fig.4.4:** A flow diagram of the HPLC injection valve in the LOAD position.

As shown in Fig.4.6; syringe for manual injection valve has a blunt point style needle to prevent damaging the valve's internal parts. Fig.4.7b goes ahead to show the port geometry of the needle when it is fully inside. The flat front tip of the needle goes ahead to touch the surface of the stator or its flat face. By doing so, the sample that comes out of the syringe completely goes into the stator passage. This is an integral component of the sample loop. Hence, there is zero sample loss. The entire sample is discharged and injected inside the column through the syringe. The capacity of columns (in particular in micro-HPLC) is limited, and overloading the column with sample materials reduces the resolution of neighboring peaks in the

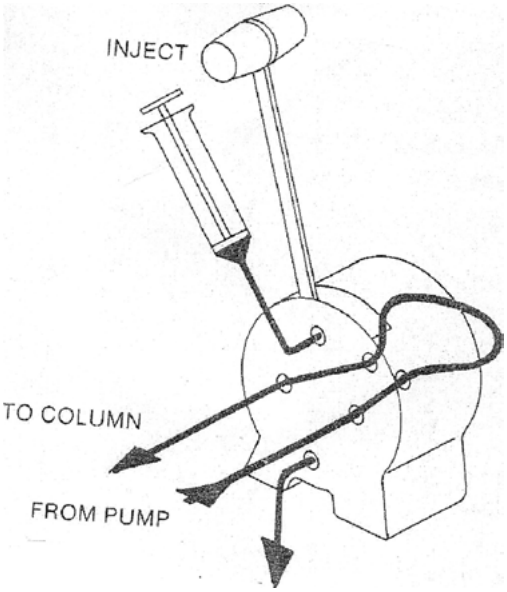


**Fig.4.5:** Flow diagram of a typical HPLC injection valve (INJECT position).


**Fig.4.6:** Manual syringe for injecting the sample manually in the HPLC injection valve which is fitted in the plunger.

resultant chromatogram [11]. Be sure that the sample aliquot is not more than half the loop volume of the sample. This is necessary for proper quantitative analysis [1, 3]. In some cases, one can adjust the injected volume by partially filling the sample loop by means of handheld syringe. To ensure minimized band dispersion, the sample solution needs to be introduced right at the end of the sample loop. Next, it is back flushed to ensure minimum band dispersion. To be sure that productivity levels are maintained, you will hardly come across manual injectors being used in pharmaceutical laboratories. The only exceptions are preparative applications nothing else. Autosamplers have became popular in drug quality control laboratories and in the following we will give some details.

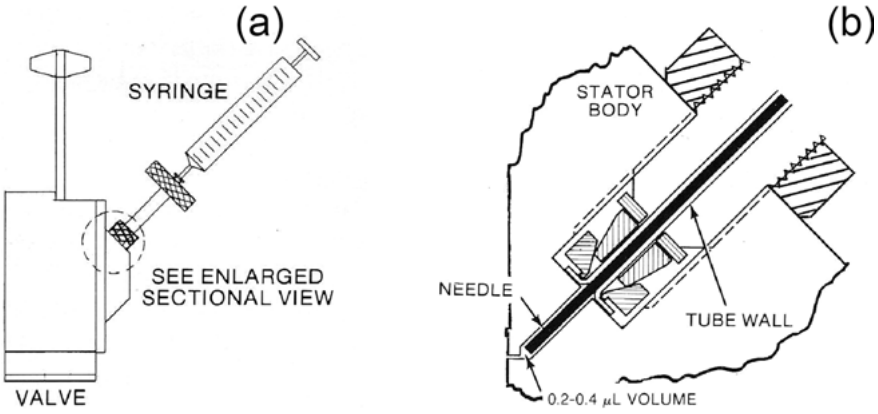


**Fig.4.7:** Descriptive diagram for the sample injection with needle port and needle of sample injection for an HPLC. (a) Sample injection through needle port and syringe. (b) A sectional view of a needle of manual injection valve in the needle port.

## 4.4 Automation of HPLC injection valve (autosamplers)

The autosamplers are being used widely, especially in the analytical laboratories. Doing this ensures better injection precision, increases sample throughput and allows for unsupervised operation. This allows decreasing labor costs that are related to manual injection [1–5]. Generally, most of the autosamplers use a loop injection valve that has six ports. This helps in delivering the sample inside the analytical column. In the old models of autosamplers, the rotor used compressed air to run it. But, in the modern ones, an electric motor does the trick. All autosamplers have similar basic parts. For instance, the Shimadzu SIL-HT [9], Waters Alliance 2790 [8], Agilent 1100 Series micro [10] and the Bechman [10] all have a loop (it can either be adjustable or fixed), sampling needle or syringe, injection valve, the injection port that helps pushing the sample inside the loop and the metering pimp which aspirated the sample from the vial. The manufacturers of HPLC **autosamplers** have come up with different strategies and principles for the autosamplers. Even though there are different techniques, all of them have the same basic four components. These allow the manual injection system to be mechanically automated effectively as follows:

1. Only standard vials are used to hold the samples. Furthermore, each of the vial is then sealed with the help of septum. This can either be an internal component of the cap of the vial or can be held in place because of the cap. This is done to avoid the selective evaporation of sample solvents. If this happens, there can be concentration changes to the sample solvent.
2. To permit the random injection or serial order, the sample vials are held inside trays. For injecting smaller volumes, mixed autosamplers or well plates are

used. The good thing about them is that they are thermostat controlled; this helps to prevent any sort of degradation of the thermally affected samples. Sampling can be done from either well plates or vials or from a mixture of both of them.

3. With the help of an accurate and precise metering device or a small analytical pump head, you can draw a specified sample volume to be injected inside the septum. For doing this, the injection needle is allowed to go inside the septum. The needle can either be fixed or movable. This totally depends on the type of autosampler being used.
4. Most autoinjectors are microprocessor controlled and this allows variable programming to include, for example, repeat injection of the same sample or a number of wash procedures between injections to ensure complete removal of the previous sample. These automated systems are quite sophisticated and allow operator-free analyses to be performed. They are particularly suited to overnight operation of routine assays, especially when combined with a data handling system.

## 4.5 Operating principles the HPLC autosampler works on

*To automatically inject samples from vials, an autosampler is used. Mostly, the labs make use of an autosampler to increase their productivity and accuracy along with precision and to reduce the total cost of labor* [1–5]. Autosamplers commonly come in two types: the push loop autosampler and the integrated loop autosampler design [1, 3]. Typically, the injection sequence copies the functioning of the manual injection. Firstly, you clean the needle by using the flush solvent into the drain port of the flush system to waste. Secondly, the sample aliquot is withdrawn for the sample vial with the needle. It moves the sample aliquot to the injector needle port by moving and entering inside it to deliver it. Lastly, sample analysis starts after the motor moves the valve (injection) to INJECT. Recently, the integrated loop design is the more popular of the two [1, 3].

### 4.5.1 Advantages of using HPLC autosamplers

The important features that make the autosampler stand out are the minimum requirements or sample volume and the programmability of it. The programmability includes variable volume injections and the number of injections/vial. The biggest performance parameter on which the performance is evaluated is the carryover and sampling precision. Over the years, these autosamplers have only improved. Number such as precision values of 0.2% with a relative standard deviation and low carryover values (<0.05%) are easily and frequently achievable [1, 3–5]. Carryover is

the percentage of the sample that was previously used and can be carried or transferred over to the sample that is going to be used next. For some critical applications like bioanalytical analysis, this needs to be minimized. There are autosamplers that have added capability for handling liquids for standard addition, derivatization and even dilution [8]. Most of the HPLC autosamplers also come with different options to cool samples [8]. There is even one design in the Waters 2690D range that allows for dissolution interface [8]. This lets the autosampler to act as a dual-purpose machine, acting like a fraction collector used to collect aliquots from the dissolution baths. This is done at preset time points (see for details in Chapter 7). There are other HPLC autosamplers that have a number of sampling injectors and probes. These let you carry out parallel injection of eight or four samples at the same time. As a familiar autosampler in a drug quality control laboratories, we will show the claimed features of the 2695 Waters Sample management (autosampler) [8] as follows:

- There are reproducible injections starting from one to hundreds of microliters. These are compatible with any analytical-scale LC column chemistry.
- The volume injections are variable and you do not need to change loops to carry out any analytical measurements.
- An active needle wash solution and a needle wash cycle that can be programmed are used for carryover management. It does not include the wash vials.
- You can program the inject-needle height. This can accommodate different vial geometries. Moreover, it accounts for thickness of the vial bottom.
- The syringe draw rate is programmable for viscous solvents/samples.
- It has independent carousels. These are 5 in number. You can run the samples in one and simultaneously prepare the next one. This allows the next carousels/samples to go in the system. The best thing is that there is no disturbance and the queue keeps going on.
- Sampling routines can be conducted for automated derivatization procedures of the pre-column and automated reference peak addition.
- There are no tools required for replacing the injection syringe or lowering the seal-wash frit. Hence, maintenance is very easy.

### 4.5.2 Practical guidelines for operation of HPLC autosampler

The recommended guidelines for *HPLC autosampler operation* are as follows:

- It is better to use acetonitrile or methanol with water to make a mixture for flushing solvent. Remember, it should be buffer-free. These make the perfect autosampler flush solvents. It is important to eliminate any sort of bubbles. For doing this, degassing of the flush solvent is required.
- The injector should be purged on a daily basis. Apart from that, it should also be purged before conducting the sample analysis. This is done to remove all the bubbles and is integral if you want sampling precision.

- There is a side port needle in some autosamplers. This further requires a minimum of 0.5 mL volume of the sample if the vial is 2 mL. Example is the Waters Alliance [8].
- There is an option of rotor seal and post- and pre-injection flush. This can be used if there is a large carryover.
- In case of large volume injections, you need to use a larger sampling loop or syringe.
- The peak width is determined largely by the injection process. The reason is that it is an important source for extra column variance (for details, see Colin et al. [11]).

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# 5 HPLC detectors

## 5.1 Introduction

An HPLC detector is a device which senses the presence of a chemical component, emerging from the column and converts that information to an electrical signal. Not only the detector monitor a chemical component elution, but the electrical signals also provides quantization. Choice of detector depends on the chemical structure of the sample and the requirement of the analytical method.

Detectors for modern HPLC are of several distinct types [1–10]. There are those that rely on the difference in a bulk property of the solute-plus mobile phase and the mobile phase itself. Thus a physical characteristic of the effluent is monitored, and the change that occurs in that characteristics during the elution of a solute produces the detector response. The most commonly used detector of this type is the refractive index detector. A second type of detector is defined as the solute detector. In this case a physical properly of the solute is utilized for detection. This same property of course, must be absent or minimal in the mobile phase itself. The UV absorption detector by far the most common detector of this type, other include florescence, radiochemical, and electrochemical detectors. The use of these detectors necessitates the choice of *mobile phase solvents that will not interfere with detection* of the solute [11–14]. This is normally of no great concern since the variety of solvents for HPLC is very large.

## 5.2 Types of HPLC detectors

The following types of detectors are the most widely used in current HPLC practice in drug and food quality control laboratories [1–2, 9, 15–16]:

UV absorbance detectors (UV detectors).
UV–visible (VIS) absorbance detectors (UV/UV–VIS detectors).
Diode array detector (DAD) and photodiode array (PDA) detectors
Conductivity detectors
Electrochemical (EC) detectors
Fluorescence (FL) detectors
RI detectors
Mass spectrometry (MS) detectors

In the following, there are details about the previously mentioned HPLC detectors:

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### 5.2.1 UV absorbance detectors (spectrophotometric detectors)

UV absorbance detectors (UV spectrophotometric detectors) are widely used as a detector for HPLC since UV absorbing species are more common in organic chemistry. Absorption of UV energy by organic molecule depends on very specific chemical features as well as the location of these features with respect to the rest of the molecule. Different components absorb different quantities of light. The strongly absorbing chemicals are considered *chromophores (chromophores are unsaturated organic functional groups that absorb in the UV or VIS light) [12]. Some strongly absorbing chemical chromophores and their absorption wavelengths ($\lambda_{max}$) are listed in Tab.5.1. Because not all organic molecules possess these chromophores, UV detections is specific. Because certain solvents are UV absorbers, they cannot be sued with a UV detector since their use would cut off the detector response. A list of common HPLC solvents and their transmittance cutoffs is given in Tab.5.2 [12] and in Tab.3.1. Of course, useful HPLC solvent must have cutoff below the detector absorption wavelength. UV detector operations at a wavelength near or below the cutoff reduce its ability to sense the compounds and increase the baseline noise. The design in Fig.5.1 shows a UV absorbance detector. All of them are based on a similar design. Basically, all of them consist of a flow cell, light sensor and lamp. Moreover, to achieve a particular light wavelength, a grating monochromator or filter can be added. The detector that has a filter attached to it is known as a fixed wavelength detector. The reason for this is that only a single narrow band of wavelength is used. Some UV absorbance detectors are designed to permit simultaneous monitoring at two wavelengths (254 and 280 nm). When it is coupled with grating, it uses different wavelengths. This is why this type of detector is known as the variable wavelength detector. For getting light, a D2 lamp or deuterium discharge lamp is used. It has a light range of 380 nm starting from 190 nm. The detectors that are UV absorbent make use of UV-enhanced photodiodes. These are the sensor elements of the UV absorbance detector. For both the variable and fixed wavelength detectors, two photodiodes are used. One is the reference cell and the other is the sample cell.

Many of the solute components are absorbed in the VIS or UV region. Even though this is the case, you need to know that the different components do not have the same spectrum, rather a different spectrum. The components that have greater coefficient of molar extinction have large peaks when the amount is small. Hence, we cannot determine the concentration from the size of the peak. Generally, a certain wavelength is fixed to perform the measurements. In the analysis, if all the sample components are going to use high sensitivity for detection, we can use the function of time programming to calculate every component and also the maximum absorption wavelength.

**Tab.5.1:** UV absorption characteristics of some organic chromospheres [17, 18].

| Chromophore | Example | Solvent | $\lambda_{max}$ (nm) |
|---|---|---|---|
| Alkene | $C_6H_{13}CH = CH_2$ | n-Heptane | 177 |
| Conjugated alkene | $CH_2 = CHCH = CH_2$ | n-Heptane | 217 |
| Alkyne | $C_5H_{11}C \equiv C{-}CH_3$ | n-Heptane | 178 |
| | | | 196 |
| | | | 225 |
| Carbonyl | $CH_3\overset{\overset{O}{\|}}{C}CH_3$ | n-Hexane | 186 |
| | | | 280 |
| | $CH_3\overset{\overset{O}{\|}}{C}H$ | n-Hexane | 180 |
| | | | 293 |
| Carboxyl | $CH_3\overset{\overset{O}{\|}}{C}OH$ | Ethanol | 204 |
| Amido | $CH_3\overset{\overset{O}{\|}}{C}NH_2$ | Water | 214 |
| Azo | $CH_3N = = NCH_3$ | Ethanol | 339 |
| Nitroso | $C_4H_9NO$ | Ethyl ether | 300 |
| Nitro | $CH_3NO_2$ | Isooctane | 280 |
| | | | 665 |
| Nitrate | $C_2H_5ONO_2$ | Dioxane | 270 |
| Aromatic | Benzene | n-Hexane | 204 |
| | | | 256 |

**Tab.5.2:** UV transmittance cutoffs for typical HPLC solvents [12][1].

| Solvent | UV cutoff[2] (nm) |
|---|---|
| Acetone | 330 |
| Acetonitrile | 190 |
| n-Butyl alcohol | 215 |
| Chloroform | 245 |
| Cyclohexane | 200 |
| *o*-Dichlorobenzene | 295 |
| Dichloromethane | 233 |
| 1,4-Dioxane | 215 |
| Ethyl acetate | 256 |
| Ethyl alcohol | 210 |
| Ethyl ether | 215 |
| Heptane | 200 |
| Hexane | 195 |
| Isopropyl alcohol | 205 |
| Methanol | 205 |
| Pentane | 190 |
| Tetrahydrofuran | 212 |
| Toluene | 284 |
| Water | 190 |

[1]Permission granted to reproduce by the copyright holder.
[2]Wavelength at which solvents become opaque in the UV.

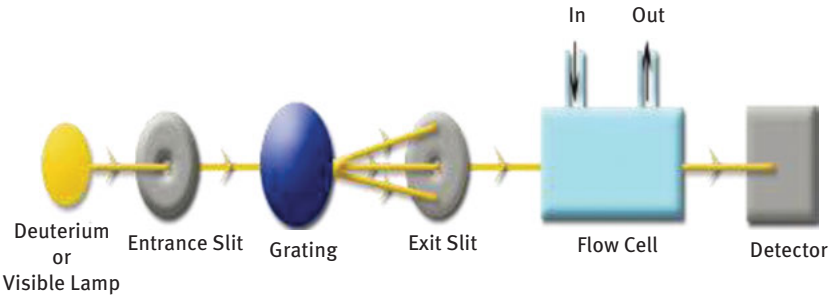


**Fig.5.1:** Diagrammatic presentation of an UV detector optical system.

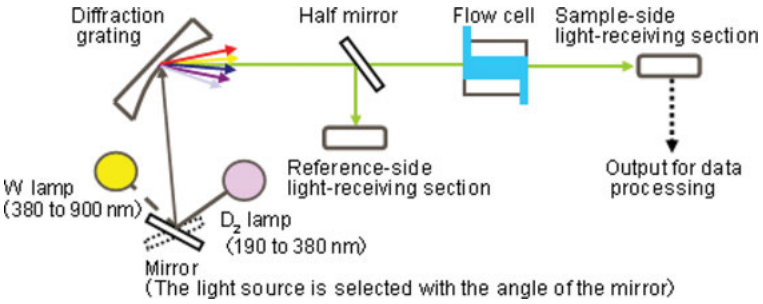


**Fig.5.2:** Diagrammatic illustration of a UV–VIS detector optical system.

### 5.2.1.1 Principles of UV and UV/VIS absorbance detection

When UV or VIS light is transmitted through a mobile phase containing an UV absorbing compound, the compound absorbs some of the light as shown in Fig.5.3. The amount of light absorbed (A) is relative to the total concentration (C) of the compound that can be found in the solution as shown in Fig.5.4 and depends on:

- The wavelength of the light and the absorption spectrum which is characteristic of that compound) (molar absorptivity).
- The distance that the light travels through the solution (path length, i).

The Lambert–Beer law (commonly called Beer's law) can be used to describe the relationship between the sample concentration and the total quantity of the light of a specific wavelength that comes at the photodiode.

Absorbance (A) = molar absorptivity (ę) × path length (b, cm) × concentration (C mol/L):

$$A = Cbe = -\log I/I_0$$

where $I_0$ is the intensity of source light and I is the sample cell's light intensity.

For well-equilibrated dilute solutions, the detector response is linear with increasing concentration (Fig.5.4) assuming the sample RI is constantly the same, and the stray light can reach the detector and it is monochromatic.

In case of higher concentration, the instrumental and chemical requirements of the law can result in violation. This will ultimately result in deviation from the initial linearity (see Fig.5.4).

### 5.2.1.2 UV/UV–VIS detector flow cell

The detector flow cell is a small cuvette that allows the sample stream to flow through and pass. The flow cell is mounted inside the optical path which is visible in Fig.5.1 and Fig.5.2. There are two common cell designs for detectors which are mostly used for HPLC–VIS/UV/UV detectors. One of them makes use of a cylindrical cell (Fig.5.5A). The second one makes use of the tapered cell (Fig.5.5B). Other flow

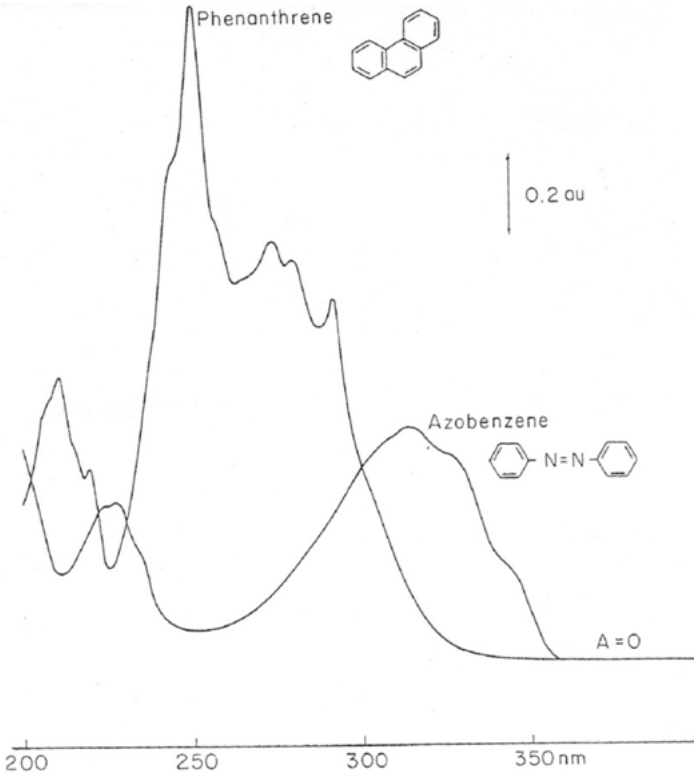


**Fig.5.3:** Typical ultraviolet absorption spectra by azobenzene and phenanthrene (UV spectrum).

cells like the Z path or conventional/cylindrical cell (Fig.5.5A) are made up of solid block of Teflon that has two holes drilled right through it or only stainless steel. The sample cell is in one hole and the reference cell goes through the other. The cell body is covered on both sides by quartz windows and further sealed inside with the help of a Teflon gasket.

A Z-shaped pattern is followed to ensure the best flow characteristic. This is used to make the sample go through the cell (Fig.5.6A). The cell configuration you can find the most is 1 mm i.d. × 10 mm in length. This gives a total of 8 µL volume. The cells with less volume result in lower band broadening. But they also have a low signal-to-noise level. The second cell design that you come across is the stepped flow or tapered cell (Fig.5.5B). A novel feature of the Waters Corporations flow cell detector design is the taper cell [19]. This design is for the reference and sample cells. Apparently, the cell is used to keep the false signals arising because of the changes in the RI to a bare minimum. These changes inside the cell further result in making some of the light that passes through to end up bending. This then strikes the walls of the cell (Fig.5.6B). Here, these can end up being absorbed. Hence, the photodetector records

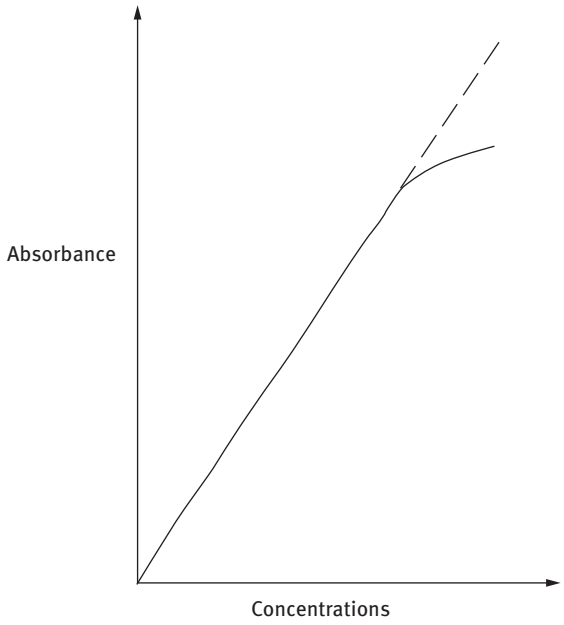


**Fig.5.4:** UV or visible light absorbance versus concentration of an UV/UV–VIS HPLC detector.

this loss of energy and shows it as absorption. The changes in the RI are dependent on many things like changes in flow rate, solvent composition and even the solute itself.

Only one passage is used to direct the sample stream from the column to the flow cell. The next of the two passages is used as the reference cell. It can either be filled with a solvent or used as an empty air reference, when you are dealing with very absorbent mobile phases. Mostly, air references are used. This is why most of the detectors do not come with a reference.

#### 5.2.1.3 Characteristics of a UV/UV–VIS detectors

- It works by measuring the absorption of either VIS or UV light through the column effluent.
- Less versatile but much less expensive.
- UV absorption is widely used since UV absorption species are more common in organic chemistry. Different compounds absorb different quantities of light, and the strongly absorbing chemicals are *chromophores.
- UV detectors for HPLC are the most reliable and easier to use and should be considered first.

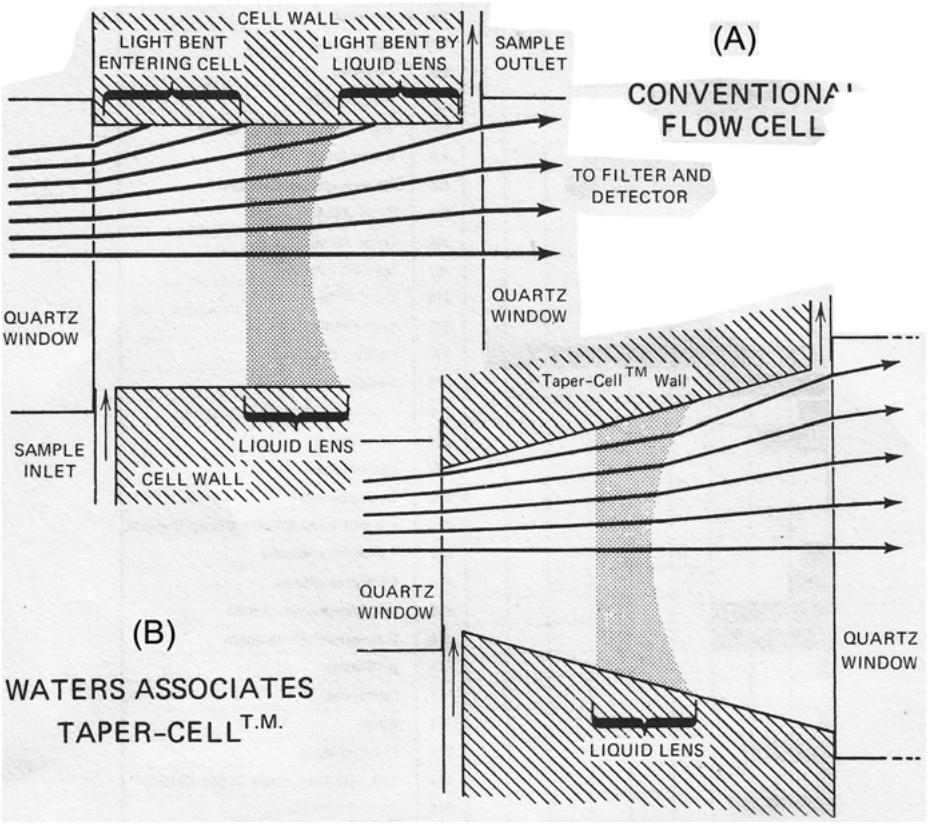


**Fig.5.5:** HPLC UV/UV/VIS detector flow cell designs [19] (permission granted to reproduce by copyright holder).
(A) Cylindrical (conventional cell) and (B) tapered cell.

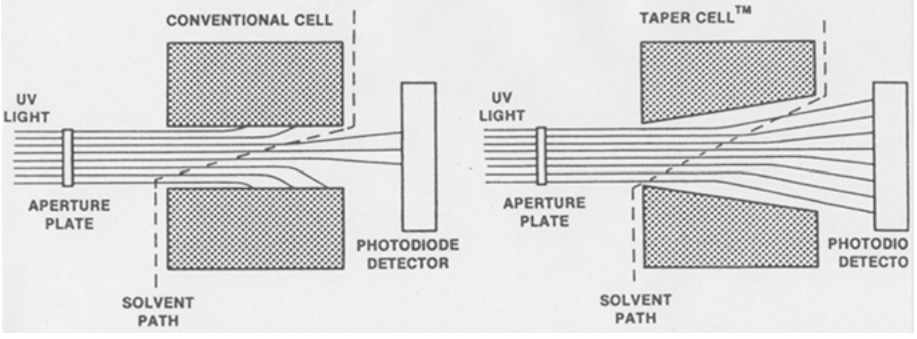


**Fig.5.6:** The light path through the (conventional) cylindrical and the tapered flow cell [19] (permission granted to reproduce by copyright holder).

- Ability to optimize sensitivity or for each analyte by favorable wavelength (using variable wavelength detectors).
- Wavelength (190–850 nm) changes can be programmed along the run time.

Problem:
- Air bubbles in UV flow cell
- Improperly prepared mobile phase
- Temperature fluctuation
- Bad source lamp

Maintenance:
- Degassed mobile phase
- Back pressure regulator
- Restructure tubing
- Clean the cell or change the lamp

### 5.2.2 Diode array detector (DAD) or photodiode array detector (PDA)

As shown in Fig.5.7, the DAD is different from the VIS–UV detectors. Hence, light is directly shone on the flow cell. The diffraction grating is used to spread the light that is passing from the flow cell. In the photodiode, the light dispersed for each wavelength can be calculated. In the detector, the semiconductor devices (PDAs) are used. To see the absorption from the UV region to the VIS region, a DAD detector is used. In comparison to the UV–VIS detector, the DAD detector has a number of PDAs (32–1,024) instead of just one sample-side section which receives light. These are used to get information from a number of different wavelengths simultaneously. The idea is that while the elute delivery is continuous, you can measure the spectra through the HPLC at an interval of 1 s or even less during the separation process. If the wavelength is kept fixed, the measurement and identification of the components is dependent on the retention time. Therefore, even a small deviation when it comes to the retention time can end up, making the identification process of the components difficult. In this case, to identify the components by the comparison of the spectrum, a DAD is used. In Fig.5.7, an optical system of the DAD is shown along with the UV–VIS detector. There are disadvantages of the DAD. These include higher noise ratio because of the low amount of light. It is highly susceptible to many changes like fluctuation of the lamp because in this case it cannot receive the reference light. In the recent past, it has improved immensely to lower the performance difference ratio in relation to the UV–VIS detectors. Fig.5.8 shows the results when a DAD is used for measurement. Most of the functions are present; these include library searches, peak purity checks and even quantitative analysis.

All this is for a specified chromatogram. Science 2000 and in our Drug and Food Quality Control Laboratories in Kuwait, HPLC-PDA-MS have been used successfully as a highly sensitive and highly confirmatory analytical tool to conduct screening studies that are used to find and study the presence of three synthetic PDE-5 inhibitors (vardenafil, tadalafil and sildenafil) that were illegally adulterated inside herbal natural products. These were made to be used to improve the sexual potency of males [20, 21].

Also Gafour et al. [22] and Khalifa et al. [23, 24] have used HPLC-PDA-MS as a highly sensitive and highly confirmatory analytical tool for analysis of vitamins A, E, D and C in raw, pasteurized and ultra-high-temperature cow's milk. PDA detectors must be used in conjunction with a computerized data station and identity confirmation by comparison of spectra with library of standard spectra (disk storage).

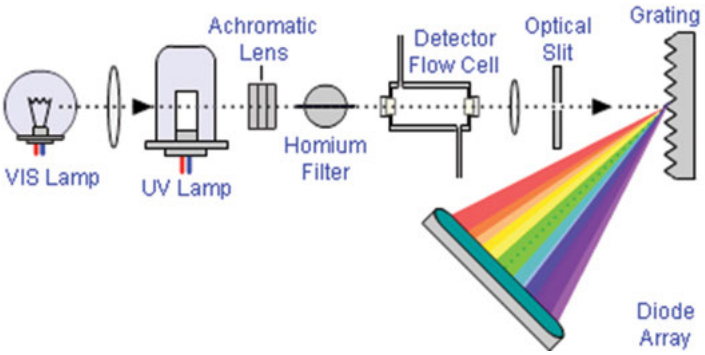


**Fig.5.7:** A graphic presentation of the photodiode array detector.

In the HPLC, if you find DAD spectra quickly available at different points in the chromatogram while the peak evolution is taking place, you can use these to see if there is one co-eluting component or more than one. This is possible only if the components have different spectra for absorption.

To get the best sensitivity level, you need a measurement of the right wavelength that measures how much the analyte is absorbed. You can achieve this either by examining the spectra of the standards or by analyzing the analytes by the help of a DAD inside the spectrum. pH level, sample solvent and temperature of the solution can change the intensity and position of the molecule's absorption.

### 5.2.3 Conductivity detectors

The conductivity detectors are used to quantitatively and continuously measure the electrical conductivity inside the HPLC column effluent that passed in the flow cells.

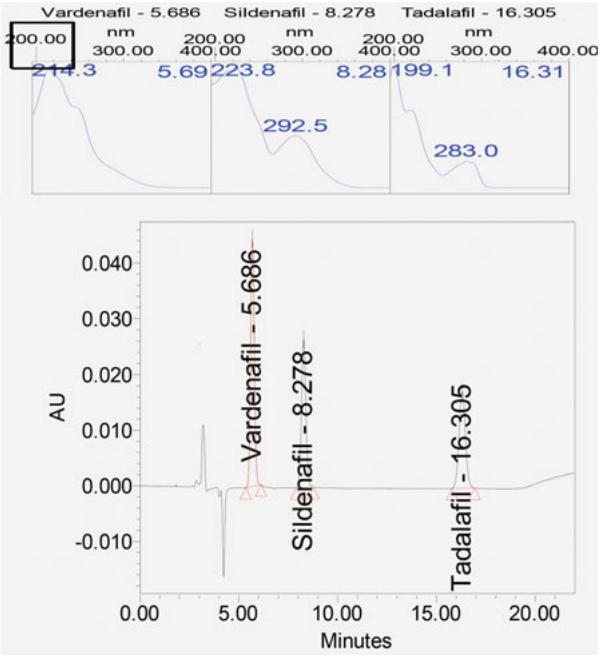


**Fig.5.8:** HPLC chromatogram (chromatogram for specified wavelength, 214.3 nm for vardenafil, 292.5 nm for sildenafil and 283.0 nm for tadalafil) and UV spectra (a spectrum during a specified $t_R$, 5.68 min for vardenafil, 8.278 min for sildenafil and 16.305 min for tadalafil) resulting from using PDA detector for identification of vardenafil, sildenafil and tadalafil reference standard in their mixture [20, 21, 23, 24].

Conductivity detectors have proved to be extremely valuable for analyzing both inorganic and organic ionic substances in aqueous mobile phases. It can be seen in Fig.5.9 that one of the two electrodes are present in the conductivity detector that is inside a flow cell suitable for the electrode (volume of the cell is only 2.5 µL). The contact cells have electrodes placed in the eluent. These are made of gold, platinum or stainless steel. The contactless design does have electrodes that are kept separate from the effluent with the help of insulators. They are capacity coupled with the sample. Inside an electric circuit, two electrodes are used. These are positioned in a shape that makes the impedance component inside one of the arms of the Wheatstone bridge. When the ions shift inside the sensor cells, a signal is fed from the bridge to the suitable electronic circuit. This signal comes from the change in electrical impedance of the electrodes. There are two ways for the results from an amplifier can be used. Either it can directly pass to the potentiometric recorder or it can be digitized for treatment by the computer. Actually, the detector is used to calculate the electrical resistance that arises from the electrodes. Through nonlinear amplification, it can provide suitable output which is linearly associated with the

concentration of the solute. To avoid the occurrence of electrode polarization, an alternating current voltage should be used along the electrodes. This is done to measure the total cell impedance. The final readings are measured in μS/cm (specific conductivity units). They can be a result of differential or absolute modes. The differential mode shows very low conductivity differences. Separating the charged analytes can become and pose a problem when dealing with lower conductivity eluents. On the contrary, when dealing with the better separation properties in buffered eluents because of the high basal conductivity, it becomes very difficult to detect the difference in the conductivity while an analyte is present. This is when the ion suppressor needs to be used to compensate the charges of the buffering components.

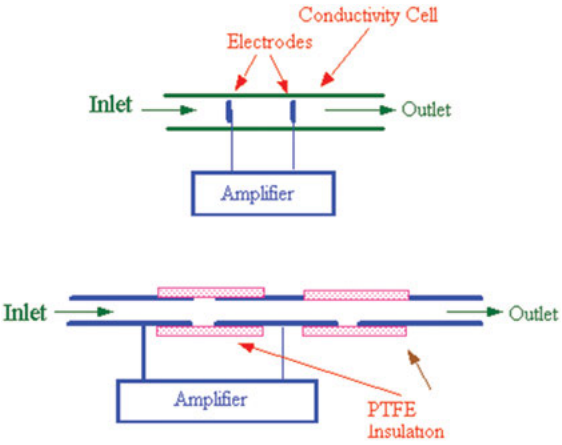


**Fig.5.9:** Diagram of a conductivity detector (top is the contact cell and the bottom is a contactless cell) [12] (permission granted to reproduce by copyright holder).

In between the detector and the chromatography column (Fig.5.10), to decrease the background conductivity, you can use the ion suppressor. This will increase the overall relativity of the analyte. This principle can be easily explained by conducting a simple analysis of chloride anion (NaCl) in an eluent with $NaHCO_3$. You can find a strong cation exchanger in the ion suppressor column. The exchanger is in the form of ($R\text{-}SO_3\ H^+$). This is a protonated situation.

When this happens, two reactions that will take place are:

$$R - SO_3^-\ H^+ + Na^+ + HCO_3^- \leftrightarrow R - SO_3^-\ Na^+ + H_2O + CO_2 \tag{5.1}$$

$$R - SO_3^-\ H^+ + Na^+ + Cl^- \leftrightarrow R - SO_3^-\ Na^+ + H^+ + Cl^- \tag{5.2}$$

In the first equation, you can see neutralization of the bicarbonate of the particular eluent (eq. (5.1)).

Hence, the conductivity level of the eluent lowers significantly. Even though there is no effect on the analyte chloride, $Na^+$, the counter ion is exchanged in eq. (5.2) for a proton. The presence of both the counter ion ($Na^+$) and analyte ($Cl^-$) are analyzed by the conductivity detector. Hence, the overall sensitivity is increased significantly [12].

### 5.2.3.1 Characteristics of conductivity detectors

- There are detectors that are suitable for use with the mobile phases. These vary from concentrated salt solutions to distilled water.
- It can be used to detect organic and inorganic ions after separation by ion exchange or ion chromatography (Fig.5.11 and Fig.5.12) [25].
- If the conductivity of the inorganic ions, ionizable organic chemicals and charged analytes such as amino acids or amines is different from the conductivity of the sample, you can measure it.

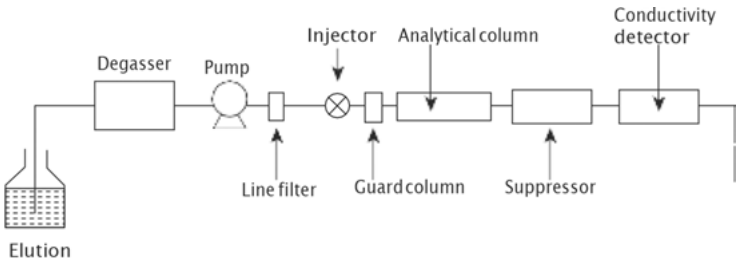


**Fig.5.10:** Schematic diagram for an ion chromatography system showing suppressor [12] (permission granted to reproduce by copyright holder).

### 5.2.4 Electrochemical detector (ED)

There is another group of HPLC detectors. These are categorized according to the electrochemical measurements [26–31] and these include coulometry, voltammetry and amperometry. In Fig.5.13, you can see an example in the form of the amperometric flow cell. The flow cell has the effluent present in the column. It then goes to the working electrode. This is kept at a constant potential in relation to the downstream reference electrode. This electrode either decreases the analytes or entirely oxidizes them. The analytical signal comes from the current that flows between the auxiliary and working electrode. This type of detector is mostly employed in either ion separations or used in the reverse phase. These have comparatively polar eluents and make use of the electrochemical properties of the analytes to determine them. Some of the popular examples of appropriate analytes are indole, aromatic amines, phenols, quinones, nitro derivatives and thiols (Fig.5.14 and Fig.5.15). To increase the conductivity of eluents, at

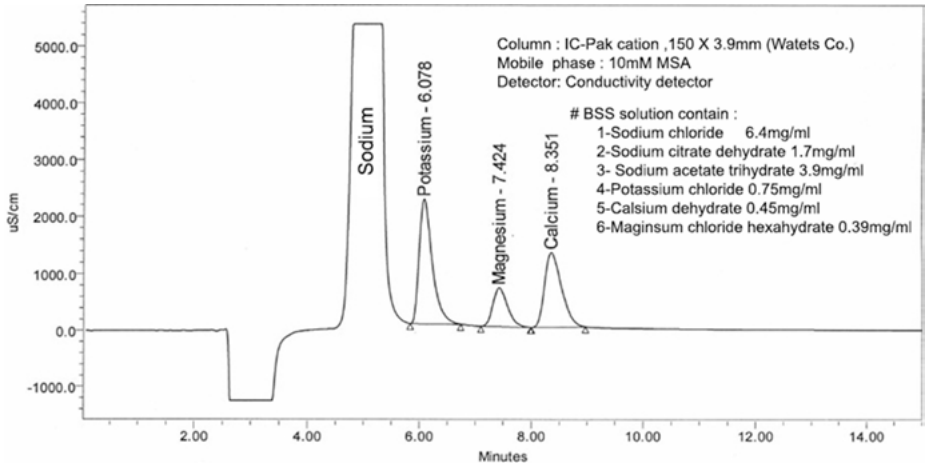


**Fig.5.11:** Separation and conductivity detection of $Na^+$, $K^+$, $Mg^{2+}$ and $Ca^{2+}$ in balanced salt solution (BSS) on cation-exchange column [25].

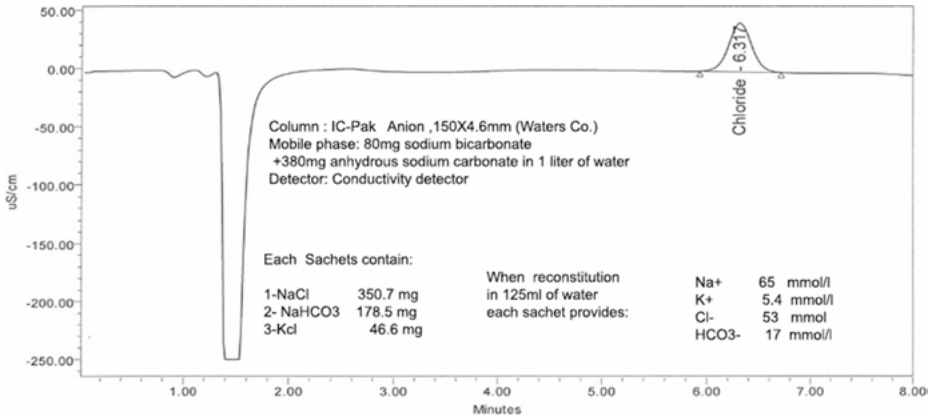


**Fig.5.12:** Separation of $Cl^-$ ion in MOVICOL on anion-exchange column and detection using conductivity detector [25].

times, an inert electrolyte can be used. For the most sensitive analytes, electrochemical detectors are used. In the most favorable cases, they can detect femtomolar (10–15 M) concentrations. Additionally, they are very selective. The reason is that only reducible and oxidizable analytes are detected. These have the same construction as the contact conductivity detectors. In them, there is direct contact of the eluent and the electrodes. The underlying principle is built on the electron flow measurements at the surface of the electrode when oxidization or reduction of the analyte occurs. When the process of oxidization occurs, the electrons get free from the analyte and go to the counter electrode (Fig.5.16).

In case reduction happens, the analyte gets the electrons from the counter electrode. In case of coulometric detection, the generated charge is totally proportional to the analyte that is being transformed. On the other hand, in the case of amperometric detection, the analyte moves with the eluent with the electrodes producing current. This is dependent on the concentration of the analyte.

Metals like nickel, copper, platinum and gold along with carbon are used to make solid electrodes. These are used in oxidative detection. To go for reductive applications, mercury is the best used material as shown in Fig.5.17. This requires three electrodes: number 1, a working one (where the process of reductions and oxidization occurs); number 2, the auxiliary one; and number 3, an electrode for reference, that is, reference electrode that is used to compensate for the changes on the eluent's conductivity (Fig.5.17). To make things work, the working electrode needs to be kept at a constant potential relative to the reference electrode. This is shown in Tab.5.3 and Fig.5.18.

In areas of neurotransmitters like serotonin and dopamine, the HPLC can perform sensitive analysis by making use of electrochemical detectors [26].

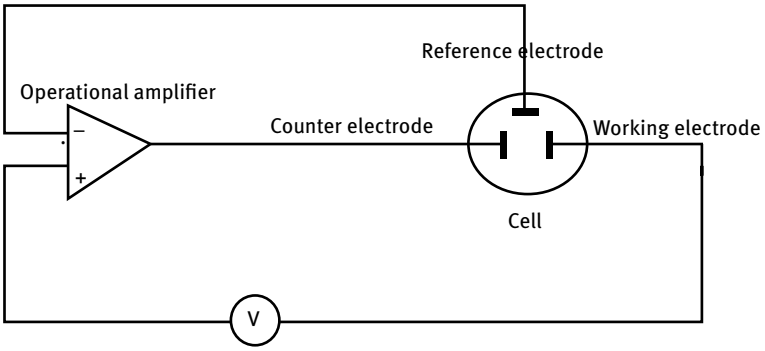


**Fig.5.13:** Generalized scheme of an electrochemical detector.

### 5.2.5 Fluorescence detector

A type of photoluminescence called fluorescence or fluorescent is a process in which chemicals that are excited by using light at a particular wavelength produce light. This is done at a much longer wavelength. In reality, you observe a redshift. If the fluorescent molecule absorbs a photon emitted by the exciting light, the electronic ground state of the valence electrons increases to a greater energetic vacant state. From here on, the relaxation process of the electron rapidly increases (10–11–10–14 s) and it reached the lowest excited state of energy. Eventually, it falls into the ground electronic state. Now, there is emission of a photon, that is, fluorescence.

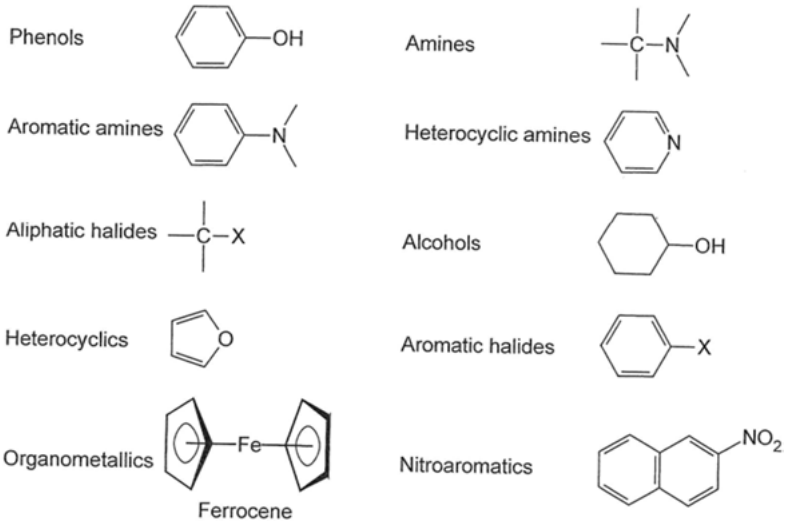


**Fig.5.14:** Some electro-oxidizable organic functional groups [26] (permission granted to reproduce by copyright holder).

When compared to the UV–VIS detectors, the fluorescence ones have a greater selectivity and sensitivity. This allows them to calculate the analytes at the trace concentration level. Only the analytes that host fluorescence properties can be seen or are detected by the detector. There can be no derivatization of the fluorescence analytes that are with the fluorescent functional groups [31].

It is nothing like the UV–VIS detector. In that type of detector, the absorption method is different. The transmittance in the light coming in is lowered to an extent if there is a scattering and absorbing analyte inside the flow cell. This is responsible for producing the detector's signal. In this type of detector, to deliver a signal, the analyte from the emitted light is used. The fluorescence signal is going to have a zero photodiode output if the analyte concentration is zero. Additionally, the signal and strength of the excitation light are relative. Hence, it can be made better by using lasers as they are a stronger light source.

The fluorescence light is calculated off-axis or at an angle of 90° with respect to the emission light as shown in Fig.5.19. Therefore, it makes highly sensitive to detect the separation of it from the excitation light.

As shown in Fig.5.19 and Fig.5.20, the fluorescence detectors are made on the common right angle plan. The same lamps are used in it as are used with the UV detectors. The commonly used light sensor (photo detector) in the fluorescence detectors is a photomultiplier tube (the UV detectors have photodiodes). To restrict the maximum excitation wavelength, cutoff filter with a short pass is placed between the cell and the lamp. To act as an emission filter amid the photomultiplier tube and cell, a long pass cutoff or band pass filter is used. Keep in mind that the

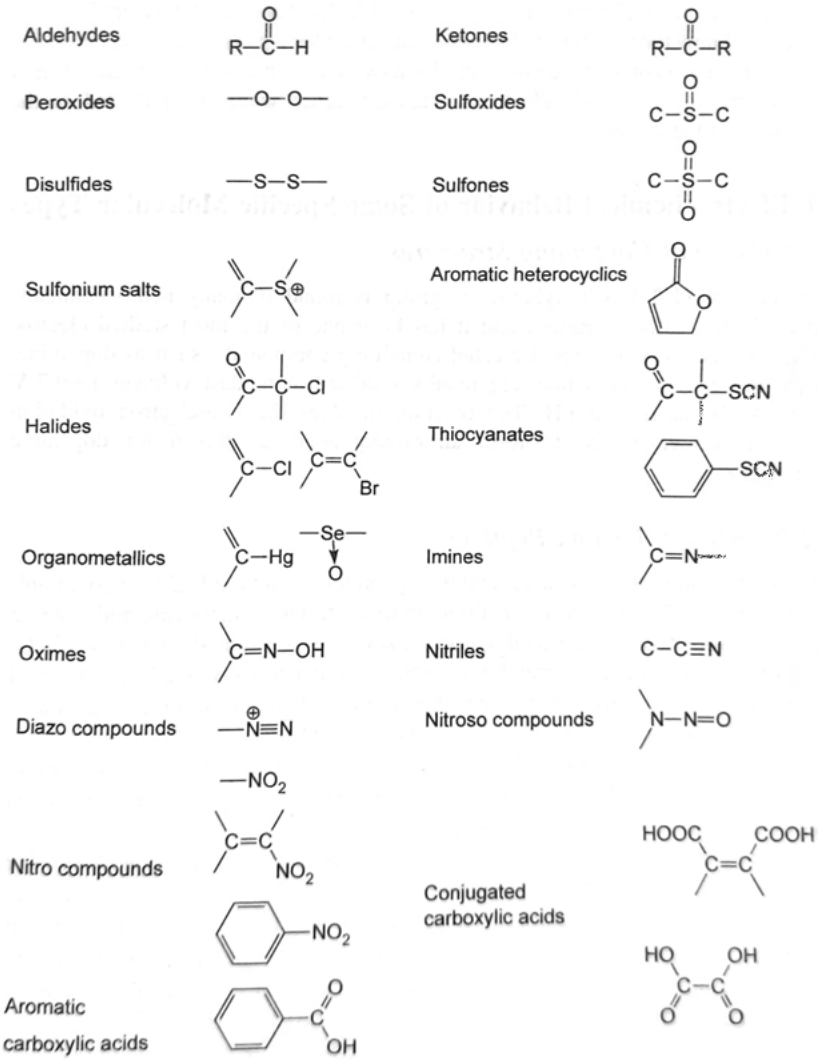


**Fig.5.15:** Some electro-reducible organic functional groups [26] (permission granted to reproduce by copyright holder).

lower perimeter of the wavelength that passed through the emission filter needs to be more than the upper limit of the excitation filter.

Filter fluorometers are those types of fluorescence detectors that make use of filters to choose the emission wavelength and excitation. Another type of fluorescence detectors, the spectrofluorometers, make use of a diffraction grating to choose the emission wavelengths of the excitation. In the scanning spectrofluorometer,

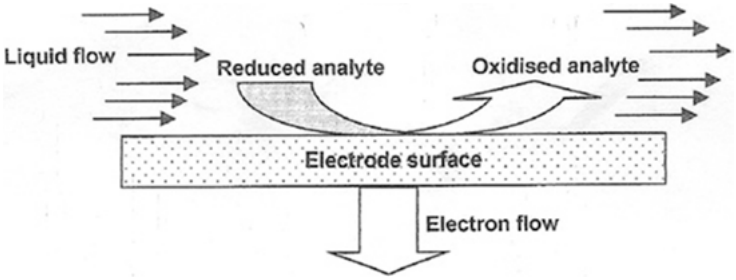


**Fig.5.16:** Schematic diagram of the electrode processes occurring when, in this case, a reduced analyte flows over static electrode held at an appropriate voltage according to Tab.2.5 and Fig.5.19.

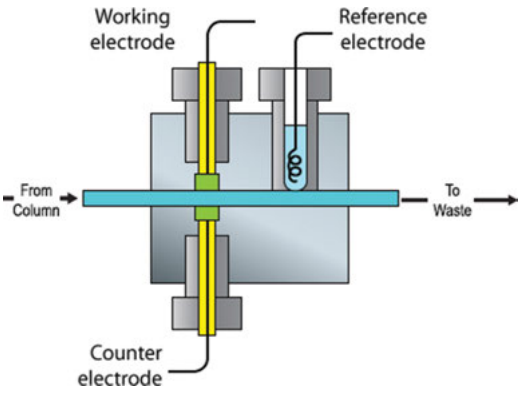


**Fig.5.17:** Thin-film cell configuration for EC detector.

grating(s) is used during the scanning mode. The right angle fluorometers used a flow cell of cylindrical shape which is made from quartz tubing (Fig.5.19 and Fig.5.20).

#### 5.2.5.1 Major application for FL detectors

- Environmental analysis of polyaromatic hydrocarbons in food, water and soil [32].
- Food quality, analysis of mycotoxins, vitamins and amino acid (after derivatization, see HPLC amino acid analysis in Chapter 11).
- Pesticide residues in food, soil and water.
- Drug analysis of fluorescence drugs.
- Analysis of quinines and phenols in biological matrix.

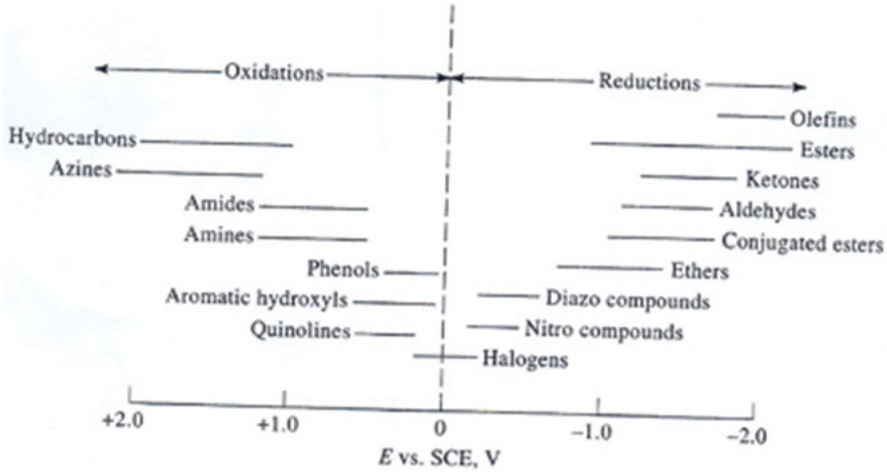


**Fig.5.18:** Potentially detectable organic functional groups by amperometric measurements. The horizontal lines show the range of oxidation or reduction potentials wherein compounds containing the indicated functional groups are electroactive [17, 18].

**Tab.5.3:** Electrochemically detectable functional groups and their oxidative or reductive potential [30].

| Functional group or compound | Potential (V) |
|---|---|
| Amines | +1.6 to +1.0 |
| Phenothiazines | +1.5 to +0.5 |
| Phenols | +1.2 to +0.3 |
| Quinolones | +0.6 to +0.2 |
| Catecholamines | +0.9 to +0.3 |
| Halogens | +0.2 to −0.3 |
| Nitro | −0.1 to −0.5 |
| Diazo | −0.2 to −0.6 |
| Ethers | −0.8 to −1.5 |
| Olefin esters | −1.1 to −1.7 |
| Aldehydes | −1.2 to −1.8 |
| Ketones | −1.2 to −1.9 |
| Esters | −0.9 to −2.4 |
| Olefins | −1.8 to −2.4 |

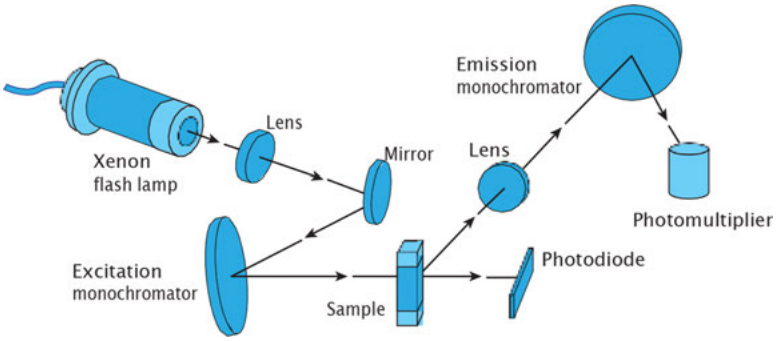


**Fig.5.19:** Diagram of a fluorescent detector used in HPLC system [12] (permission granted to reproduce by the copyright holder).

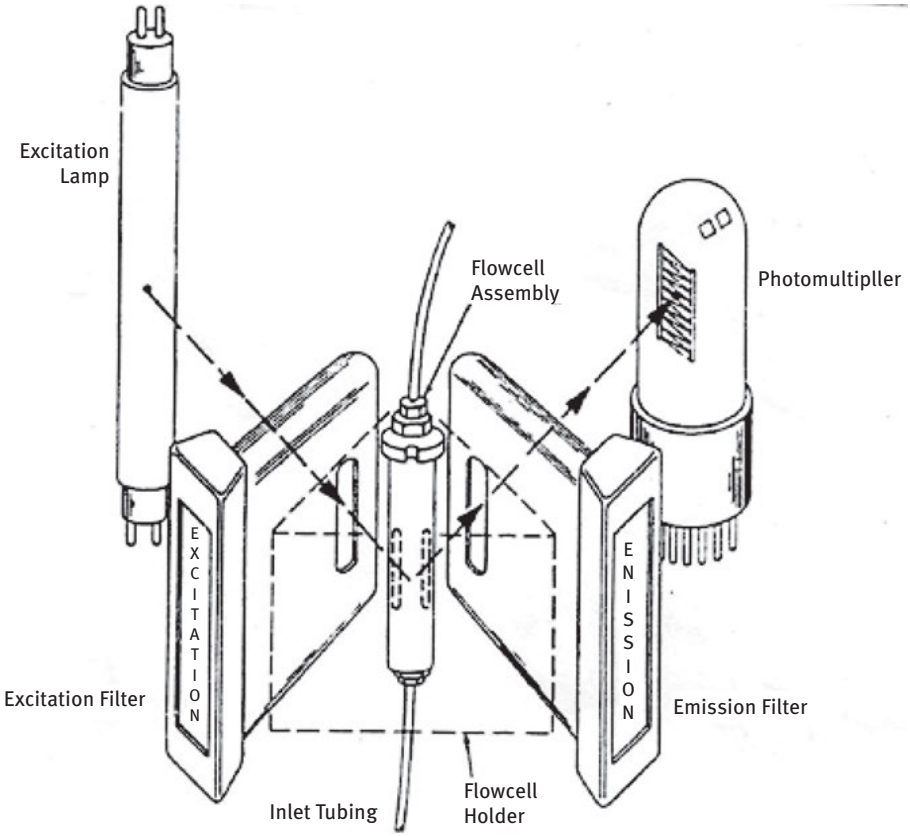


**Fig.5.20:** Optical layout for right angle filter of HPLC fluorescence detector.

#### 5.2.5.2 Characteristics of fluorescence detector

- Fluorescence detector is 100–1,000 times more sensitive and selective than UV/VIS absorbance detection.
- Applicable to compounds with natural fluorescence or derivatized analytes with a derivatization reagent (pre- or post-column derivatization) [29].
- The main difference between the UV–VIS absorption detection and the fluorometric one is that in the fluorescence one, the wavelength of the light emitted which gets to the photodetectors is not the same from the incident light that is turned toward the sample stream. To block the incident light, a suitable filter is used. Therefore, only the fluorescence emission from the sample goes to the detector. As a result of this, the errors that can occur because of the reflection or transmission of the incident radiation decrease to a great extent.
- When compared to the UV/VIS techniques, the fluorescent ones are more direct. As the quantity of the emitting species is present, the light values that are calculated are positive and increase constantly. They are directly proportional. If the fluorescent components are absent from the sample stream, you cannot obtain any emission signal. However, when done practically, there are always some particles of fluorescent impurities present in the mobile phase. Therefore, you can obtain a small background signal. Most of the fluorometers have a detector noise. This is because of the signal implication circuit.
- It offers great sensitivity and selectivity.
- Very attractive for environmental, biochemical and medical applications.
- It responds only to fluorescent compounds, which means analyte must fluoresce or be converted to fluorescent derivative before detector.
- Fluorescence occurs when a compound absorbs light at a shorter wavelength (excitation) and re-emits it at a longer wavelength (emission).
- Most fluorescent compounds absorb light at or less than 400 nm and re-emit at wavelength less than 700 nm.
- Molecules that have highly conjugated double bonds or that contain aromatic, carbony1 or other functional groups are more likely to be naturally fluorescent (steroids, catecholamines, vitamins, plant pigments and fused ring aromatic molecules).
- Generally affected by mobile phase pH, temperature and dissolved $O_2$ (see Chapter 3).

### 5.2.6 Refractive index detector

In the HPLC systems, the RI detectors present are being used to examine the samples that do not have strong chromospheres in the VIS or UV regions such as sugars, triglycerides and organic acids and are not fluorescent and have no electrical

activity (oxidation–reduction). Being a universal detector (to a specific extent, all the analytes do deflect light), the RI detector has restricted sensitivity. As compared to the UV–VIS detector, the RI detectors has almost two to three orders less than it. As shown in Fig.5.21, the detector calculates the capability of the analyte to redirect the light coming from the eluent for HPLC applications. This phenomenon occurs in a flow-through cell that is relative to the reference cell (Fig.5.21). In this case, it is not possible to use an RI detector. The reason is that the solvent's RI changes very strongly in the gradient elution. Apart from this, the flow rate and pressure also affect the RI that needs to be as constant as can be. Moreover, the solvent and analyte have RI themselves.

A major concern is the temperature stability in RI detectors. The detector noise is very prominent even with tiny fluctuations. Hence, most of the commercial detectors are thermostated especially to decrease this problem. Another step taken is that all detectors, irrespective of type, are being operated in a differential mode. Thus, the difference in the RIs of the mobile phase plus solute and the mobile phase is calculated. This is done by letting the mobile phase directly into the reference cell before it enters the column. This is done by passing it through the pump. Once it exits the column, it goes over to the sample cell and passes from it. Gradient elution and flow programming are nearly impossible because the RI detectors are responsive to the flow changes.

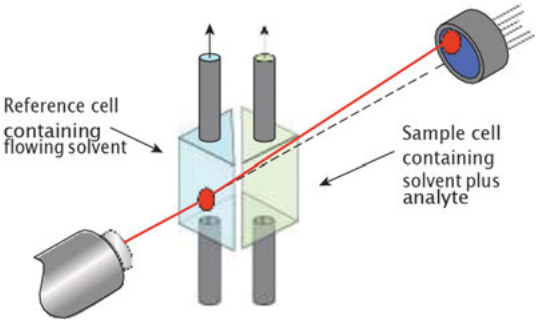


**Fig.5.21:** Schematic diagram of an HPLC refractive index detector deflection type and two flow cells (reference and sample flow cell) [12] (permission granted to reproduce by the copyright holder).

RI detectors lack both selectivity and sensitivity. This is why they are not suitable to trace analysis. Because of the sensitivity mentioned earlier, they are also limited to solvent and flow composition.

#### 5.2.6.1 Characteristic of RI detectors

- The RI detector calculates the differentiation in the RI between the sample cell and the reference cell (containing pure eluent).
- RI detection is universal (nonspecific).
- RI detectors require pulse-free isocratic pumps, degassed solvents and column oven, and pump blending is not recommended.
- RI detectors are truly universal detectors and can work with any solute.
- Severe limitation is lack of absolute sensitivity in comparison to other detectors.
- RI detectors sense both the analyte and the mobile phase.
- You can see the difference in the RI of the sample flow and reference cell by the output.
- Very affected by pressure and temperature of the mobile phase.
- Commonly used for sugar analysis, cereal beverages, soft drinks, dates and honey.

### 5.2.7 Mass Spectrometric Detectors

A fundamental problem in coupling liquid chromatography with mass spectrometry is the enormous mismatch between the relatively large solvent volumes from the former and the vacuum requirements of the later. Several interfaces have been developed for solving this problem. In one, which is available commercially, the eluent from the column is split, with only a tiny fraction being introduced directly into the mass spectrometer. Direct liquid introduction systems appear to hold considerable promise when used in conjunction with the new micro bore columns, which typically have flow rates of 10 to 50ul/min. In a second type of interface, which is also sold commercially, the effluent is deposited on a continuous, moving belt or moving-wire that transports the solvent and analyte to a heated chamber for removal of the former by evaporation. Following solvent evaporation, the analyte residues on the belt or wire pass into ion source area, where desorption ionization occurs.

A promising interface which has been available commercially, is called a thermos pray. A thermos pray interface permits direct introduction of the total effluent from a column at flow rates as high as 2mlL/min. with this interface, the liquid is vaporized as it passes through a stainless steel, heated capillary tube to form an aerosol of solvent and analyte molecules. In the spray, the analyte is ionized through a charge exchange mechanism with a salt, such as ammonium acetate, which is incorporated in the eluent. Thus, the thermos pray is not only an interface but also an ionization source. The resulting spectra are generally simple and provide molecular weight data but lack the details that make electron impact spectra so useful for identification purposes. Furthermore, the thrmospray interface is application only to polar analyte molecules and polar mobile phases that will dissolve

a salt such as ammonium acetate. With these limitations, the thermospary interface provides spectra for a wide range of nonvolatile and thermally stable compounds such as peptides and nucleotides. Detection limits down to 1 to 10 picograms have been reported.

Recently [33–34] a new interface has been introduced commercially that makes it possible to obtain either electron impact or chemical ionization spectra. In this device, thermal nebulization and desolvation occur simultaneously to produce a mixture of particulate solute molecules and gaseous mobile phase molecules. This aerosol is accelerated through a nozzle into a vacuum away. The particulate analyte molecules are then ionized in an electron beam or chemically. Computer control and data storage is generally used with mass spectrometric detectors. At this date instruments for HPLC/MS are fully developed and presents significant importance in drug quality control laboratories with many applications [20–22] (for details see Chapter 9).

## 5.3 HPLC detector performance

HPLC detector performance features include sensitivity, detection method, signal range and linearity [17–18]. The sensitivity indicates the minimum detectable concentration of a given component to which the detector will respond. High sensitivity is essential for trace work. The main determinant of sensitivity is the detection principle. The detection principle relates to which type of chemical and/or physical characteristics are detected. For example, a universal detector would measure a characteristic displayed by all molecules. More selective detectors measure a specific characteristic possessed by fewer molecules. Fluorescence is an example of a highly selective detection method.

In general, as the detector type becomes increasingly specific, the sensitivity also increases. Range refers to the upper and lower limits of detection through which the detector response remains linear to the effluent concentration. Universal detectors often have the highest linearity ranges. This favorable characteristic tends to offset their lack of sensitivity.

The detector sample flow cell design is most important in determining detector performance. Minimal cell volumes avoid losses in resolution or chromatographic "bandspreading."* Detector flow cells should also be designed to reduce or eliminate air bubble formation as well as to provide a clear flow path. State-of-the-art detectors are usually designed to avoid these problems, often having cell volumes under 10 μL [17, 18].

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# 6 HPLC as analytical tool in drug quality control laboratories: getting started

## 6.1 Introduction

In Chapters 2–5, we investigated individual components for the HPLC system (columns and stationary phase for columns, Chapter 2; mobile phase and mobile phase pumping system, Chapter 3; injectors and autosamplers, Chapter 4; and detectors, Chapter 5). Assuming that all of the individual components are operational, we would then connect it together (this is the main aim of this chapter). By other means, this chapter details the particular steps in the process of starting up an HPLC system and using it as analytical tool in drug quality control laboratories. These steps can be summarized as follows:

- HPLC hardware component selection
- HPLC component connection (tubing and fitting) (HPLC instillation)
- HPLC column connection (HPLC column instillation)
- HPLC instillation quality

In addition, this chapter provides an overview about the modern HPLC equipment and the most important performance characteristics of its components including mobile phase pumping system (pumps), sample introduction system (manual injectors or autosamplers), detectors and data system (data acquisition, processing and handling) before it is purchased for the drug quality control laboratories and used as an analytical tool. The most important performance characteristics are mobile flow precision and mobile phase compositional accuracy for the pump, sample volume precision and carry over for the injector (autosampler), and sensitivity, selectivity and liner dynamic range for the detector. Also, manufacturers and selection criteria for an HPLC equipment to be used as analytical tool in drug quality control laboratories were reviewed.

After successful instillation of an HPLC system in drug quality control laboratories, the mobile phase and solutions from the standard of active ingredient (Active Pharmaceutical Ingredient API ) of the pharmaceutical products and samples of the pharmaceutical substance under analysis would be prepared, followed by selection of separation conditions based on the API composition. Finally, the system is turned ON, allowed to stabilize, solutions from the standard of (Active Pharmaceutical Ingredient API ) of the pharmaceutical substance and samples of the pharmaceutical products under analysis would be injected, and analysis started as will be explained in detail in Chapter 8. So, this chapter is presented as an introduction to Chapter 8.

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## 6.2 HPLC hardware component selection

An HPLC system is usually purchased for drug quality control laboratories as analytical tool for separation, identification and quantification of a specific drug or family of drug mixtures. Since the user may not initially be aware of all of its potential uses, it becomes important to "build" the system from the standpoint of upward compatibility. As mentioned previously in Chapter 1, it is well known that an HPLC system has six major components: pumping system for mobile phase (pumps), injector, column, detector, computer and printer and fraction collector. In order to support the operation of these components, additional parts are necessary, such as in-line solvent filter, pressure relief valve, pulse dampener, pressure gauge, guard column, column end frits, steel tubing, Teflon tubing, unions and glassware for mobile phase. We will treat each section of equipment separately as follows.

### 6.2.1 HPLC pumping system for mobile phase (pumps)

Pump is the first item we will discuss here. The most distinguishing factor among pump types is pressure limit [1–13]. The higher this pressure limit is, the more expensive and complex a pump will be. For this reason, the user must carefully consider his overall separation needs. High-pressure pumps are needed to force solvents through packed stationary phase beds inside the HPLC column. Smaller bed particles require higher pressure to force solvent through the column. There are many advantages to using smaller particles, but they may not be essential for all separations. Many separation problems can be resolved with larger particle packings that require less pressure. Thus, if the user has only moderate needs and a restricted budget, his money need not essentially be spent on a maximum pressure pump.

Maximum flow rate is another important pump feature [1–13] that varies between pumps. High flow rates are usually not essential for analytical chromatography. In fact, efficiency decreases with higher flow rates.

An additional pump feature found on the more elaborate pumps is external electronic control [1–13]. Although it adds to the expense of the pump, external electronic control is a very desirable feature when automation or electronically controlled gradients are to be run. Alternatively, this becomes an undesirable feature, since it is an unnecessary expense when using isocratic elution methods for mobile phase during sample analysis.

The degree of flow control also varies with pump expense [1–13]. More expensive pumps include state-of-the-art technology as complex electronic feedback and multiheaded configurations. These features provide greatly enhanced flow rate stability. For an user who does not operate at the highest sensitivity, it may be wise to purchase a more basic pump and add a pulse dampener. These pump systems work very well, are relatively inexpensive and provide good performance in spite of their simplicity.

In general, the pump choice depends on the needed versatility and performance criteria. The user may find his money better spent on two or three less expensive lower pressure pumps, rather than on one top-line model. The user can upgrade the system by buying a more suitable pump, keeping the original pump available for a variety of other tasks.

#### 6.2.1.1 Isocratic and gradient HPLC system

For HPLC analysis of pharmaceutical products in drug quality control laboratories there are two basic elution modes that can be used for mobile phase. The first is called isocratic elution. In this mode, the mobile phase composition remains the same during the separation and sample run. The second type is called gradient elution the mobile phase composition changes during the separation and sample run (for details, see Chapter 3) with the increase the mobile phase strength in relation to time. For example, in reversed-phase chromatography, the mobile-phase composition at the start of the run is polar (for instance, high amounts of water added to acetonitrile) and the percentage of the organic solvent (such as methanol or acetonitrile) is increased with time, thereby raising the elution strength (Fig.6.1). In normal-phase chromatography, the initial mobile-phase composition is usually nonpolar such as hexane and then a more polar organic solvent is added, such as chloroform, tetrahydrofuran (THF), ethanol or isopropanol. Also, the ionic strength or the pH may be changed during chromatography by adding a suitable second solvent. This mode is useful for samples that contain compounds that span a wide range of chromatographic polarity. It is often found that isocratic solvent conditions cannot effectively separate all of the components in a mixture. When this is the case, either a gradient separation or change in chromatographic method is indicated.

Nowadays, often HPLC runs are based on a gradient of the mobile phase during elution, that is, the solvent strength is increased with time (Fig.6.1). The advantage of gradient runs is that the run time for separation of the analytes in mixture can be significantly improved and shortened compared to isocratic runs as shown in Fig.6.1b.

Isocratic analyses are particularly common in quality control applications for routine analysis of pharmaceutical products in drug quality control laboratories since they use simpler HPLC equipment and premixed mobile phases as usually mentioned in Unites States Pharmacopeia, Ph.Eur [14–16] and drugs manufacturers specifications. Notable disadvantages of isocratic analysis are limited, such as peak capacity (the maximum number of peaks that can be accommodated in the chromatogram), and problems with samples containing active pharmaceutical ingredient of diverse polarities. In contrast, gradient (Fig.6.1) is suited for complex samples and those containing analytes of wide polarities. Gradient chromatography is amenable for screening applications for routine drug analysis and for impurity testing in drug quality control laboratories [9–11].

Mobile phasem for HPLC analysis shouldm be degassed in order to prevent bubble formation upon mixing under pressure. Nowadays, degassing stations are included in commercial HPLC systems (see Chapter 3).

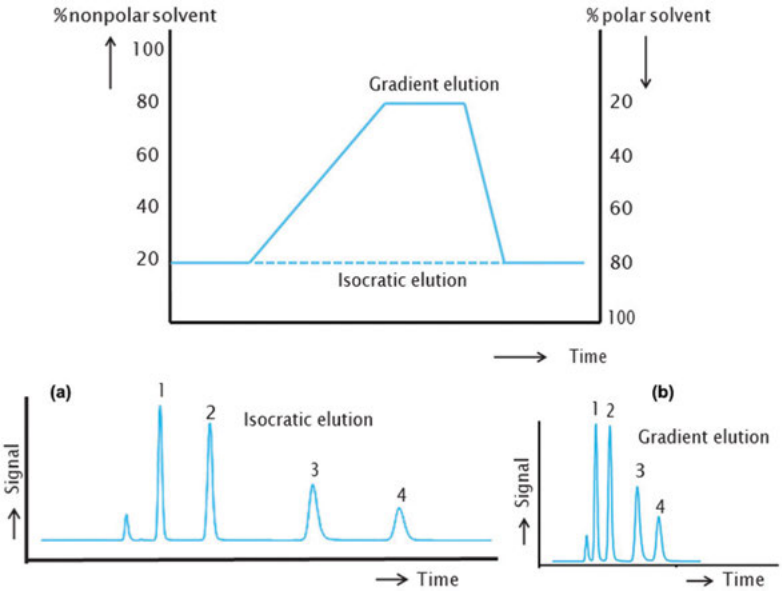


**Fig.6.1:** Exampleforan isocratic elution (a ) and a gradientelution (b ) under reversed-phase conditions for seperarions a mixture of four analytes (1,2,3 and 4).

### 6.2.1.2 Types of HPLC pumping systems

The mobile phases are introduced into the HPLC system by a suitable pumps applying pressures. Such pressure is necessary to overcome the resistance of the narrow stainless steel capillaries in which the solvents reach the column which is filled with small-sized matrix particles (3–10 μm). No solvent flow could be observed without such high pressure. A typical HPLC *mobile phase pumping system* includes between one and four reservoirs for mobile phase (must be equipped with safety caps (see Chapter 10). The majority of HPLC pumps are reciprocating single, dual or triple piston pumps with or without diaphragm delivering continuous constant flow [1], (see Chapter 3). They all operate on the principle that the spring loaded piston delivers the mobile phase by a continuous backward and forward movement, sucking the mobile phase from the reservoir through a ruby ball check valve into the pump chamber on the input stroke and expelling in through another ball check valve into the column feed tubing on its reverse stroke [1–13]. In direct piston pumps, the eluent is displaced out of the pump chamber by the piston itself, driven by a cam linked to the pump motor (see Chapter 3 and [1]). In the case of the diaphragm type

pump, the pistons reciprocating movement takes place in an oil-filled chamber, causing corresponding movement of a diaphragm forming the far wall of the chamber, and this diaphragm movement performs the intake and expulsion of the eluent through a second chamber remote from the pump piston itself which thus does not come into contact with the eluent sequentuse (see Chapter 3). For precision pumping, the pistons must operate through effective seals, and as in any mechanical device, these seals have to be replaced from time to time. Pumps are the most common, delivering constant flow with virtually no pulsation, even at great differences in the mobile viscosities. Constant flow is achieved by measures such as automatically monitoring the pressure at the pump inlet and outlet and adjusting the pump drive to maintain balance. Pressure pulsations are often caused by air bubbles in the outlet or inlet valves. Purging the pump with mobile phase or 70% methanol can often eliminate this condition.

Dual-head reciprocating pumps offer lower solvent pulsation at the expense of mechanical complexity. Here, two pistons fill and pump 180° out of phase and in theory provide "pulseless" flow [1, 5]. Dual-head pumps are more expensive and have either two check valves (series heads) or four check valves (parallel heads). Both designs generally provide for some type of pressure or flow feedback control to further compensate for minor flow variations during switching from one head to the next. A detailed discussion of the mechanics of these and other pumping systems is provided in [1–13].

### 6.2.2 HPLC – sample introduction system (HPLC injectors/autosamplers)

The injector is the next part of HPLC equipment. An HPLC injector is used to introduce the sample on the top of the HPLC column. As mentioned previously in Chapter 1 (Fig.1.2) and as shown in Fig.4.1, the injection system in HPLC instrument is positioned after the pump head, and is therefore located on the high-pressure side of the mobile-phase delivery system. The injection of a sample at atmospheric pressure into the HPLC system, at high pressure, represents a critical step in the chromatographic process. The fact that the mobile phase is delivered in the HPLC system under pressure demanded a suitable sample injection device. This job can be done by loop injection system (injection valve; for details, see Chapter4) which is located between the analytical column and the high pressure pump of mobile phase. Loop injection system (injection valve) by far is the best and also is the most widely used for HPLC injection system (was used to introduce reproducible amounts of sample into the HPLC eluent stream without causing changes in pressure or flow). A common injector for HPLC (Fig.4.1 and 4.2, Chapter 4) is the manual injection valve (loop injector) such as Rheodyne model 7125 or 7725 injector (Rheodyne Inc., P.O. Box 996, California 94931, USA [17]) (for many years, the Rheodyne 7125 injector was the industry standard, and in the early 1990s, it was replaced by the Rheodyne 7725m injector, which injects samples

without momentary flow disruptions [18]). HPLC autosampler injector, as a more advanced technique for injection of pharmaceutical products samples during their analysis in drug quality control laboratories, has become widely in use [4, 6, 7, 9, 10].

### 6.2.3 Columns

The HPLC column is considered the heart of the system. No separations can occur without it. Nowadays, stainless steel (SS) high-pressure columns can be used up to 10,000 psi. In general, it is advisable to maintain minimal pressure in all systems since the higher pressures cause increased HPLC component wear and fatigue.

Pre-packed columns are available with a variety of stationary phase packings [19–32]. These provide most demanding needs for the users in drug quality control laboratories. In drug quality control laboratories, for using HPLC as analytical tool, select the desired column as it is given in the current method of analysis or the equivalent one (for details about column selections, see Chapter 2), use log book of columns and use HPLC column vendor manual as a guide for column handling which you can find with each column in its box. Consider the direction of arrow on the column and all the information written on it, such as, column length, column inner diameter (i.d.), column packing materials, particle size of packing materials and column materials. In Chapter 2 [19–32], there is an excellent review about all these previously mentioned items about HPLC columns.

### 6.2.4 Detectors

In HPLC analysis, an electronic signal from the detector is used for qualitative and quantitative analyses of the analytes eluting from the chromatographic column. The detector should provide a linear response related to a certain concentration range of the solute, a wide linear dynamic range and high sensitivity (high signal-to-noise ratio) are required for quantitative analysis of the pharmaceutical products in drug quality control laboratories. A wide variety of detector types can be integrated in an HPLC system. The most common detector types are based on ultraviolet (UV)–visible light absorption, fluorescence, refractive index, evaporative light scattering and mass spectrometry. In this book and as previously mentioned in Chapter 5, we have focused on UV–visible light absorption detectors because this detector type is used in more than two-thirds of all HPLC systems [1–13].

The detectors are the one item of equipment where quality rather than price should be the determining factor, since the detector qualities directly influence the separation results. Most people start with a simple fixed wavelength UV detector. Originally, this type of detector was available with wavelengths of 254 and 280 nm. Almost all UV absorbing compounds can be detected with one or the other of these

wavelengths. Now, more sophisticated fixed wavelength detectors are available with a multitude of wavelength selection in the UV or visible region of 190–850 nm (see Chapter 5).

Different types of detectors are commercially available, each with certain properties, working principles and selectivities, for instance, fluorescence and electrochemical detectors are selective for analytes, whereas UV absorption detectors are nonselective and universal (for all absorbing analytes at certain wavelengths). We also distinguish between destructive and nondestructive detectors; UV-visible light absorption, fluorescence and refractive index detectors are nondestructive. The compounds passing through the detection cell remain intact and can be recovered. In contrast, detectors based on evaporative light scattering and mass spectrometry are destructive because the compounds are destroyed during the detection process.

The most widely used detector type in modern HPLC and UHPLC is based on absorption of UV and visible light (UV-Vis detectors). This detector type exhibits high sensitivity for many a.i. in pharmaceutical products. Nevertheless, a compound must absorb light in the UV or visible region of 190–850 nm to be detected. The basis of quantification in UV-Vis absorption photometry is Lambert–Beer's law (see Chapter5). Absorbance, A, is the logarithmic ratio of the light intensities before and after passing through the eluting medium, and is proportional to the product of the concentration of the absorbing compound, the length of the light path, and the molar coefficient of absorbance, (ę). Two optical techniques were developed in absorption detectors:

(1) With direct optics, light of the wavelength of interest (190–850 nm) passes through the detector flow cell and a single photodiode captures the emerging light. Absorption detectors with direct optics are called fixed wavelength detectors.
(2) With reversed optics, light of all wavelengths from 190 to 850 nm pass through the flow cell. The emerging light is separated by a grating into its constituent wavelengths, and an array of photodiodes acquire the resulting spectral information. Absorption detectors with reversed optics and photodiode arrays (PDA) are called PDA detectors (see Chapter 5). Diode array detectors not only facilitate detection at multiple wavelengths but also generate UV spectra for unambiguous identification of specific compounds [33–41].

#### 6.2.4.1 HPLC detector performance

**As mentioned by Colin et al. [**1**],** HPLC detector performance features include selectivity, sensitivity and dynamic range/linear dynamic range. In the following, we will discuss these items:

– **Selectivity**

**Selectivity** determines how specific a detector can be used for a certain compound. **More selective detectors measure a specific characteristic possessed by fewer molecules eluted from HPLC column. A nonselective (universal) detector would**

**measure a characteristic displayed by all molecules. Fluorescence detector is an example of a highly selective detection method.**

– **Sensitivity**

**The detector sensitivity indicates the minimum detectable concentration of a given component to which the, detector will respond. It is expressed by the detection limit** or limit of detection (LOD). Detector sensitivity is equal to the LOD which is the amount of solute necessary to obtain a detector signal equal to a given number of times, usually 3, the base line of the detector noise [1], see Fig.6.2 [$S_{Signal} = 3\ N_{Noise}$**(Signal/Noise = 3, S/N = 3)**].

When using calibration solutions of an analyte in a calibration diagram, signal versus concentration, the sensitivity corresponds to the slope of the calibration curve in its linear range. In UV detection, signal height depends on the compound-specific absorption coefficient (ę), path length, peak dispersion and detection wavelength. Detector noise depends on many different factors, for example, light intensity, pump pulsation, electronic noise, data rate, temperature, flow rates and flow cell design.

The main determinant of an HPLC detector sensitivity is the detection principle. The detection principle relates to the type of chemical and/or physical characteristics detected. In general, as the detector type becomes increasingly specific, the sensitivity also increases. Highest sensitivity can be achieved by increasing the path length without increasing the internal volume of the flow cell, and by keeping the level of detector noise as low as possible. In contrast to older flow cells, where the flow path of the detection cells often has a conical design, modern detectors have flow cells that facilitate total internal reflection and increase the detector sensitivity (see Chapter 5).

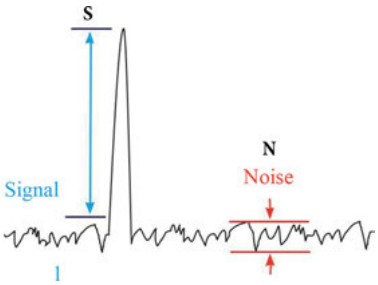


**Fig.6.2:** Illustrative diagram showing the measurements of an HPLC – UV–Vis detector sensitivity.

In the field of pharmaceuticals analysis in drug quality control laboratories, high sensitivity detector is essential for trace work especially for analysis of their related substances and degradation products**.**

– **Dynamic range/linear dynamic range**

Detector response can be expressed both as dynamic range and as linear dynamic range. Dynamic range is the ratio of the maximum and minimum concentration

over which the measured property (i.e., UV absorbance) can be recorded. However, in practice, linear dynamic range, the range of solute concentration over which detector response is linear, is more commonly used. Plotting the response of injections of different analyte concentrations against nominal concentration should give a straight line over a large concentration range. In the other words, **the linear *dynamic range* of an HPLC detector means the change of the electronic output** of that detector in relation to the analyte concentration in between the LOD and the highest concentration at which a signal change is observed (**it refers to the upper and lower LODs through which the detector response remains linear to the HPLC column effluent concentration)** as shown in Fig.6.3.

For quantification, the linear relation of analyte concentration versus detector signal should be used. Most detectors eventually become nonlinear as sample size is increased. The chromatographer should know where this occurs to avoid errors in quantification.

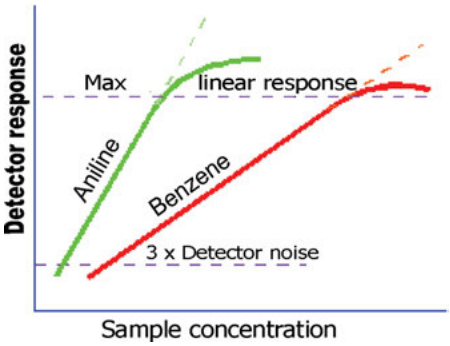


**Fig.6.3:** Illustrative diagram showing the linear dynamic range of an HPLC UV detector.

The linear dynamic range of a detector is important when quantification across a wide range of concentrations is required. Linear dynamic range is also important when the compounds of interest have significantly different concentrations and UV responses. This situation occurs in samples containing a predominant main component and minor impurities. In such samples, quantitative information must be acquired for all compounds at the same time.

Current UV detectors for HPLC have a linear dynamic range of 4 to 5 orders of magnitude. This can be significantly extended using detection solutions that quantify low and high concentrations in a single analysis. This approach facilitates simultaneous quantification of major and minor components, and their impurities, such as in fixed-dose combinations in some pharmaceutical products. This kind of detectors is significantly time and cost saving.

– **HPLC detector data rate**

HPLC detector data rate is the number of data points per second that a detector can acquire. Whereas conventional UV detectors with data rates up to 20 Hz are sufficient

for standard HPLC analysis, UHPLC demands much higher data rates for precise quantification during fast runs with narrow peaks.

The detector sample flow cell design is most important in determining detector performance. The detector flow cell should be small enough to minimize back diffusion and peak broadening, but still provide enough analyte to receive a significant signal output. Detector flow cells should also be designed to reduce or eliminate air bubble formation as well as provide a clear flow path. State-of-the-art detectors are usually designed to avoid these problems, often having cell volumes under 10 μL. For instance, for UV absorption detectors, this is accomplished by special constructions of the flow cell in HPLC, that is, the Z cell (for details, see Chapter 5).

#### 6.2.4.2 HPLC-UV/Vis/PDA detector operating guides

The following operating guides are recommended for UV/Vis absorbance or PDA detectors:

- Turn the lamp on for at least 15 min to warm up before analysis.
- Set to the appropriate wavelength and detector response time (i.e., 1–2 s).
- Set scanning range of the PDA (e.g., 200–400 nm or 190–400 nm) for method development.
- Lamp should be replaced after 12 months or over 1,000 h.
- Switch off lamps when not in use to increase lifetime.

References [1–13] and information in Chapters 5 and 12 provide an excellent operating guide for using other HPLC detectors (fluorescence, FL; refractive index, RI; electrochemical, EC; conductivity, CD and mass spectrometry, MS)

Computers and printers are the last essential items of the modern HPLC equipment's. Computers are used for the collection of the resulting chromatographic data, storage, handling and processing these data. In addition, computers doing control on the instrument parameters, such as flow rate of mobile phase, column heater temperature and autosampler. Nowadays, computers and printers are included in commercial HPLC systems [19–32].

### 6.2.5 Miscellaneous items

Some miscellaneous items may also be essential in assembling a user-built for an HPLC system in drug quality control laboratories such as: (1) In-line mobile phase filters are highly recommended to provide additional filtering. Two types of these filters are available. One type attaches to the end of the mobile phase intake line. In addition to filtering, this unit acts as a weight and holds the intake line below the surface of the mobile phase in its reservoir. The second filter type is an in-line filter located near the pump inlet. The use of at least one in-line filter (5–10 μm) is highly

recommended. (2) A pressure relief valve is another highly recommended item for a low-cost user system. This item fits between the pump and the injector and operates by venting pressure in excess of present value. These relieve valves are not necessary with the more expensive pumping systems, because they generally include some sort of pressure-relieving system internal to the pump, either electrically or mechanically. Positioned between the pump and injector, there might be other miscellaneous items, such as a pulse dampener and pressure sensor. In simpler models, the pressure sensor is merely a gauge. In more complex HPLC, the pressure sensor is an electronic transducer that feeds a digital readout module. Pulse dampeners vary in size and design, depending on the pump type, operating pressure and flow rate.

Guard columns are primarily used in chromatographic setups to protect the chromatographic column. The guard column can be placed in two different positions. When placed between the pump and the injector, it helps clean the solvent of potential chemical impurities. When placed between the injector and the column, it helps clean the samples of potential impurities. The use of a guard column may be desirable in applications involving dirty samples such as encountered in clinical or environmental situations. Some other miscellaneous items are also essential for HPLC set up such as column end frits, tubing and unions (see Section 6.4). In addition, a small amount of glassware to purify and hold the mobile phase are needed. Besides glassware and connectors, an array of small tools will be needed to connect and troubleshoot the equipment (screw drivers, wrenches, hex key set (1/16″ through ¼″), plastic tube flaring tool for preparation of Teflon fittings, SS and plastic tubes cutter, triangular file, emery cloth, different sizes and lengths from plastic and SS tubes, pump seal insertion tool, knife and flashlight).

## 6.3 HPLC manufacturers and equipment selection

Major manufacturers of HPLC instruments includes Waters [19], Agilent [20], Dionex [21], Shimadzu [22], Varian [23] and Thermo [24].The Internet addresses of these companies can be found in the reference section. HPLC is a mature technology and most manufacturers have highly reliable products with sufficient performance and feature sets to be competitive in the market place. However, there are still be significant differences between the vendors on these performance characteristics of the systems (instrument band spreading or instrument band width, IBW), pumps (low flow, seal life), autosamplers (carryover, speed, sample capacity, minimum sample volume), and detectors (sensitivity, gradient base line shift, selectivity, linear dynamic range). So, analysts in drug quality control laboratories must select the manufacturers who provide and fulfil their requirements.

## 6.4 HPLC – components (units) connection (tubing, fittings and instillation of an HPLC system) without internal dead volume and leak free

### 6.4.1 HPLC tubing

Tubing connects the parts of HPLC system and provides a path for mobile phase from the reservoir through the HPLC and finally to the waste, Fig.6.4. The connection between components for high-pressure fluid flow usually required the use of small bore SS tubing, as shown in Fig.6.5. Plastic tubing [Teflon (fluorocarbon) and PEEK (polyether ether ketone)] can also be used (Fig.6.5), but it is not good for trace work. Teflon tubing does have positive features, such as ease of handling and flexibility. Teflon

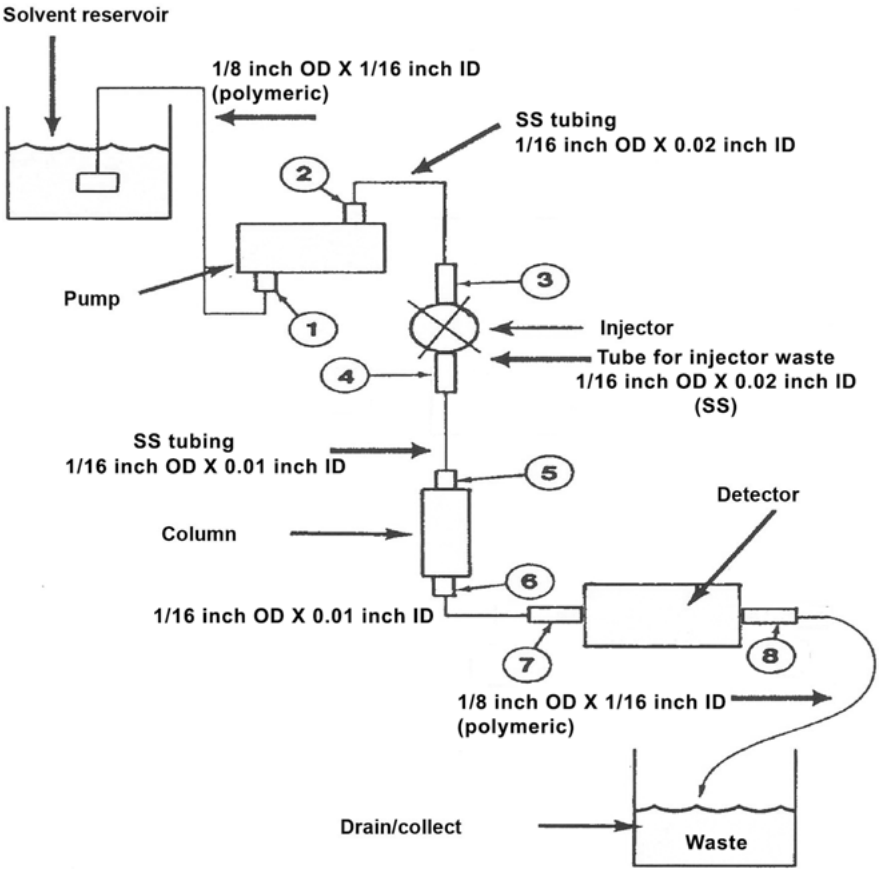


**Fig.6.4:** A minimum of eight fittings (compression fittings) are required for the connection of an HPLC system.

tubing is limited in its use to supply line that connect the mobile phase reservoirs to pump, to the outlet of the detector and to lines such as the injector waste.

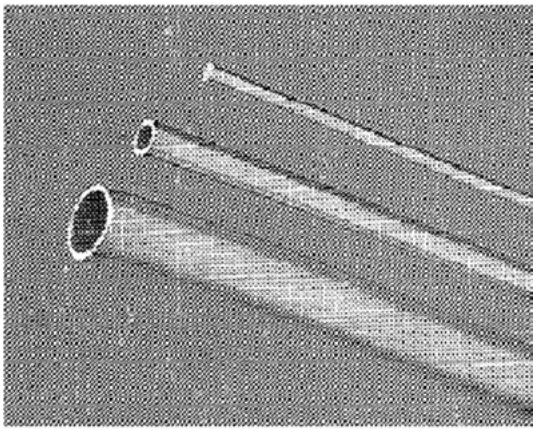

**Fig.6.5:** Stainless steel tubes for connections between HPLC components (from outlet of the pump to the inlet of the detector).

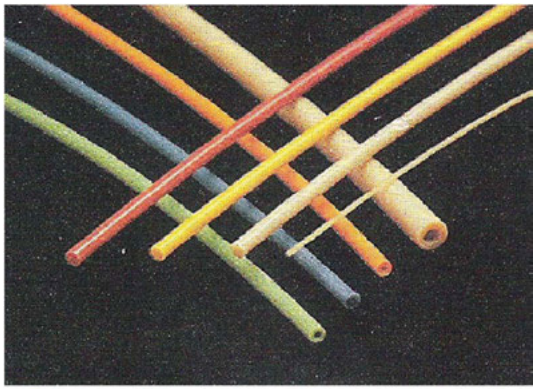

**Fig.6.6:** Polymeric tubes for connection of HPLC parts in low-pressure region (before pump and after detector).

PEEK tubing has recently been introduced as a substitute for SS tubing for some HPLC applications. It is available in the popular 1/16″ o.d. × 0.01″ i.d. size, has pressure limit in excess of 5,000 psi and is more inert to biomolecules than SS tubing. Flexibility, inertness and ease of handling make polymeric tubing a viable alternative to SS tubing for many HPLC applications.

Polymeric tubing is commonly used when SS is not required, because it does not have as high a pressure resistance as stain less steel. Polymeric tubing is limited in its use to (a) supply lines connecting the mobile phase reservoirs and the pump, (b) the outlet side of the detector and (c) other low-pressure lines such as the injector waste line. Teflon tubing is usually the best choice in plastic tubing, because it is inert to the reagents used in HPLC work.

SS tubing must be used in regions subject to high pressure, that is, between the pump outlet and the in-let of the detector. Connecting tubing between injectors and the column and the column and the detector (for the most part, tubing that contacts the samples [2]) must be of minimum bore and length (0.01″ i.d. × 1/16″ o.d.) and 100 mm length between components, to minimize dead space (volume) in the HPLC system (Fig.6.4). It is well known that the band broadening for resulting peak from an HPLC system due to the connection tubings increase rapidly as tubing internal diameter (ID) is increased [2]. Longer narrower bore tubing may appear satisfactory but problems may arise from blockage. SS tubing (HPLC-grade SS316) provides chemically inert connection (is more corrosion resistant, because it contains Mo as part of the alloy).

The tubing length and internal diameter must be selected with care so that the system performance is not degraded. Incorrect tubing choice can cause band broadening or high pressure (flow resistance). Most part of tubing that comes in contact with the sample should be as short as possible and of small internal diameter [2].

## 6.4.2 HPLC fittings

### 6.4.2.1 Compression fittings

Fittings are working with the tubing to connect the various modules of an HPLC. The fittings must be inert and leak free, they also must add no unnecessary volumes to the system. The minimal number of fittings needed for standard HPLC setup is eight as shown in Fig.6.4 (point 1 for inlet pump, point 2 for outlet pump, point 3 for inlet injector, point 4 for outlet injector, point 5 for inlet column, point 6 for outlet column, point 7 for inlet detector and point 8 for outlet detector). Two additional fittings are needed on the injector if a loop injector is used. These fittings commonly utilize a compression fitting (male – ferrule fittings) as shown in Fig.6.7 and Fig.6.8. The ferrule type of fittings is essential because it provides a high pressure seal (pressure up to 6,000 psi; its beveled lip tightly fits into the opening). As the fitting is tightened, the ferrule body compresses and seals against the steel tubing. Connections made with this way are very reliable, and can be disconnected and rejoined many times without loss of integrity.

As shown in Fig.6.9, the tip of steel tubing is prepared for the compression fitting as follows: **first**, an indentation is scored around the tubing using a tubing cutter. By bending the tubing back and forth about this indentation, the tubing should break leaving an open inner hole or lumen. Cutting through the tubing in any other way will probably close the lumen and is, therefore, not recommended. Once the tubing is cut, the tips are smoothed and made flat by sanding the tip with emery cloth (this is particularly important if the tubing is to butt against similar end inside of the union or connector (see Section 6.4.2.2).

The **next** step is to place the nut on the tubing with the threaded opening facing the fitting body (union), as shown in Fig.6.9. The ferrules are then placed on the tubing with the flat end toward the nut. The tubing is inserted back into the end of the fitting body (union) until it hits the bottom and tighten the nut to secure the ferrule. At that moment, the tip of the ferrule seals around the tube, and the tapered portion seals against the taper of the fitting body to form a leak-free assembly of a compression fitting (Fig.6.10). The amount of tightening depends on the size of the fitting body and the type of ferrule. Most manufacturers specify tightening the nut 3/4 turn past finger – tight for a seal good to at least 6,000 psi. Once the fittings have been assembled, it can be taken out (disconnect) from the fitting body and used as often as necessary for connection at any point (1–8), which is indicated in Fig.6.4. Always follow the manufacturer's recommendation in this tightening process [2]. The user is cautioned not to overtighten fittings, since overtightening is the major cause for connection failure. Connection made this way are very reliable, having no internal dead volume and can be disconnected and rejoined many times without loss of integrity.

– Note:

1. Mismatched or improperly assembled fittings components will result in leaks or/ and broadened peaks.
2. Compression nuts and ferrules from Parker, Swagelok or Rheodyne are compatible and interchangeable with each other. Use Waters fittings for Waters HPLC and Waters columns.

#### 6.4.2.2 HPLC tubing connectors

When two tubes are connected, they must be carefully butt-fitted and coupled with zero-dead volume connector (female union) (Fig.6.11) to prevent the dead volume in the HPLC system.

#### 6.4.2.3 Teflon fittings

Teflon fittings (connections) (Fig.6.12) can be used for low-pressure chromatography. They are treated differently from steel connections in several ways. First, the union is female, and the Teflon ends are guided through a bushing as pictured in Fig.6.12. Flat flare on each tubing end is made with a special apparatus, plastic tube flaring tool [2] that has a small heated nipple fitting into the tip of the tubing end. The flat flares are butted together in the female union, and the bushings tightened. Practice is usually needed to make perfect Teflon flares. When carried out correctly, however, they are usually leak proof and add no dead volume.

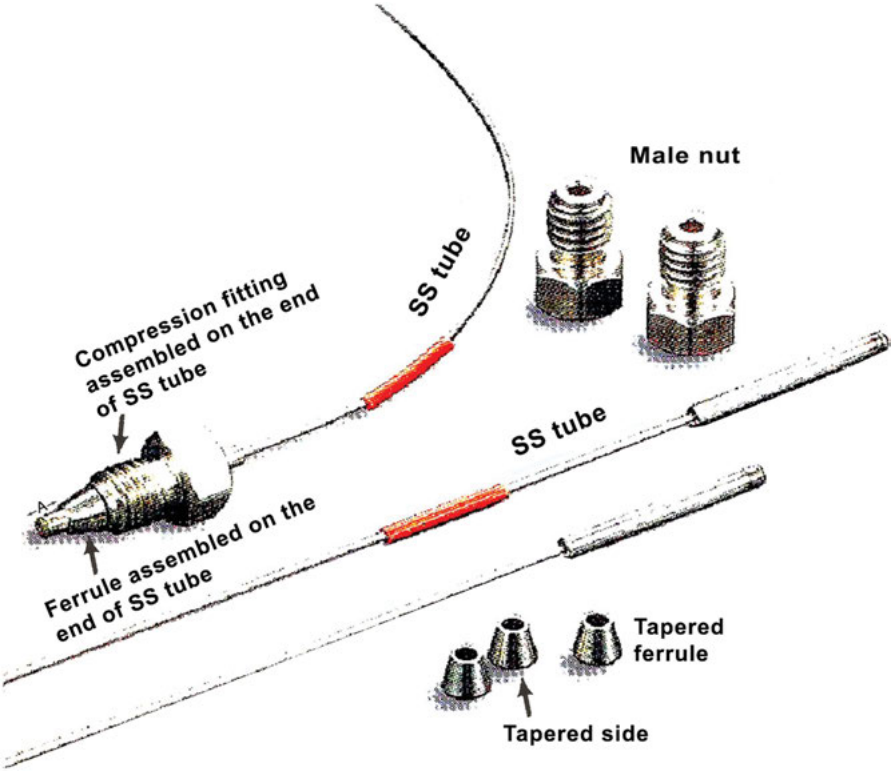


**Fig.6.7:** Male nut (size 10 with thread 30″), tapered ferrule and SS tube (the end, 0.01″ i.d. × 1/16″ o.d.) parts needed for preparation a compression fitting.

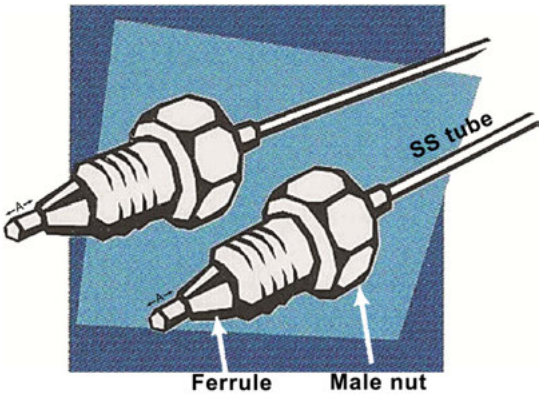


**Fig.6.8:** Assembled compression fitting (ready to be used as a fitting for connection of an HPLC system units).

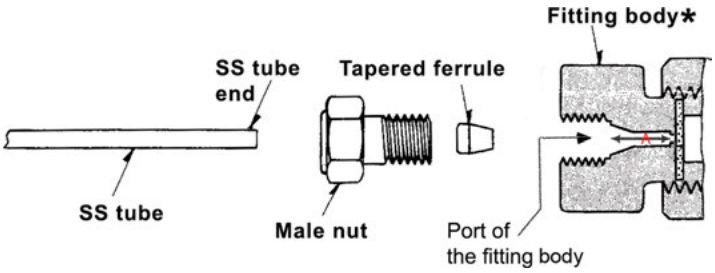


**Fig.6.9:** Illustrative diagram showing how we can use a fitting body* for the preparation of a compression fitting from a male nut, a tapered ferrule and the end of SS tube.
*Fitting body is an union with a thread, usually present in fitting kits which contains a variety of nuts, ferrules, pre-cut tubing and tubing cutter. Fitting body usually comes with a purchased HPLC as spare parts and provided with HPLC manufacturers and suppliers.

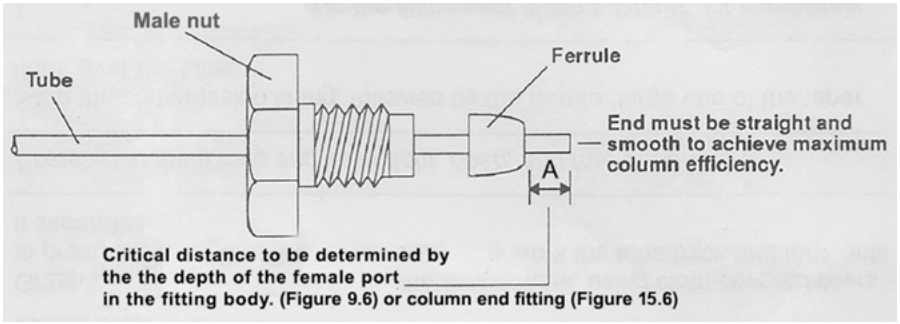


**Fig.6.10:** Assembled compression fitting (male nut/ferrule type) to be used for connection of HPLC units.

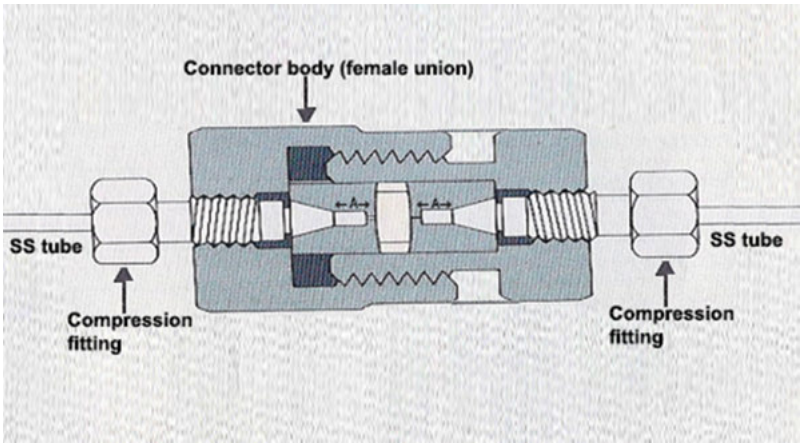


**Fig.6.11:** Illustrative diagram showing a connector (female union) for HPLC tubing connection.

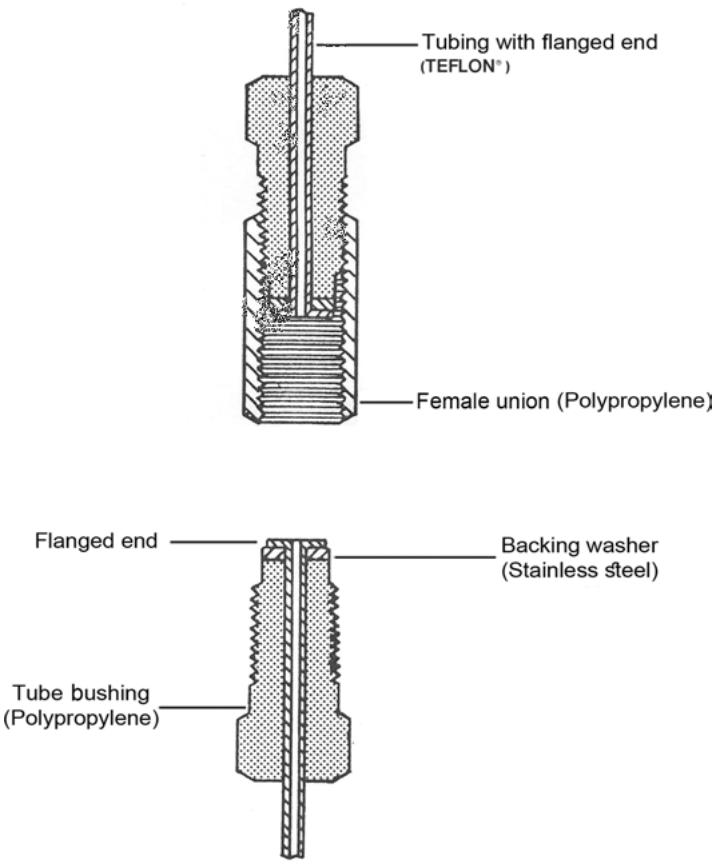


**Fig.6.12:** Illustrative diagram of a Teflon fitting.

#### 6.4.2.4 Finger-tightened fittings (polyether ether ketone)

An alternative to SS compression fittings is to use polymeric fittings. These are available as a single piece fitting as shown in Fig.6.13a. This fittings have the advantage that the ferrule is flexible and grips the tubing when the fittings is tightened; however, the ferrule does not bite into the tubing and become permanently seated. Thus, the ferrule can be easily moved to allow for a longer or shorter tubing extension when used with another manufacturers fitting body.

This one-piece PEEK **finger-tightened fittings** are designed for the connection of a column to the 1/16″ (1.6 mm) o.d., inlet line to the detector flow cell. To install the fitting, refer to Fig.6.13b for orientation. Push the fitting onto the detector inlet tubing and position it about ½″ from the end. Insert the tubing as far as possible into the column end fitting. While keeping the tubing inserted into the end fitting, tighten the fitting onto the tubing (this fitting is designed to be hand tightened). If leaks occur,

gradually tighten the fitting until the leakage stops. The fitting can be reused many times. Although it is designed for the column-to-detector connection, it can be used as general-purpose fittings for all 1/16″ (1.6 mm) o.d. tubing. **PEEK – finger-tightened fittings** are **convenient, inert and bio-compatible. Use these fittings with 1/16″ o.d. such as SS and PTFE tubing. PEEK fittings are compatible with all HPLC solvents (avoid concentrated sulfuric and nitric acids)** (the user should be aware of the solvent compatibility of **PEEK – finger-tightened fittings**) **and can be used at temperatures up to 150 °C. Unlike SS ferrules, PEEK – finger-tightened fittings ferrules do not permanently lock into a place on the tubing. This allows you to interchange fittings with tubing and columns, from manufacturer to manufacturer (e.g., from Agilent to Waters), and still form a zero dead volume, leak-free connection.**

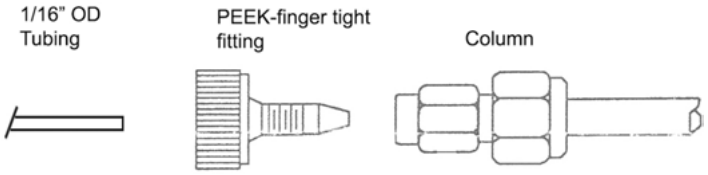


**Fig.6.13:** Illustrative diagram showing PEEK (polyether ether ketone) finger-tightened fitting.

## 6.5 HPLC column installation (column connection)

HPLC column design is shown in Fig.6.14 and Fig.2.30 (see Chapter 2). Usually the tag attached to the column indicates packing type, particle size, frit size, shipping solvent composition and column number. These important information must be keep with the column at all times. All these information are very useful for column instillation and care. Also flow direction during the column packing process is indicated by (flow→) on one of the reducing union or tube (Fig.6.14). For installing the column with stainless tube, prepare the tubes which will be working as inlet and outlet of the column (Fig.6.5, points 5 and 6, respectively) for compression fittings as we mentioned previously. The following connection parts are required (2 male nut + 2 ferrules) as shown in Fig.6.15. As previously mentioned (Section 6.4.2.1) from these parts, prepare two compression fittings (ferrule type compression fitting, one for column inlet and the other one for column outlet,) which are in need for column connection to HPLC system (two proper and assembled compression fittings in good conditions are required for HPLC column connection to an HPLC system (Fig.6.15).

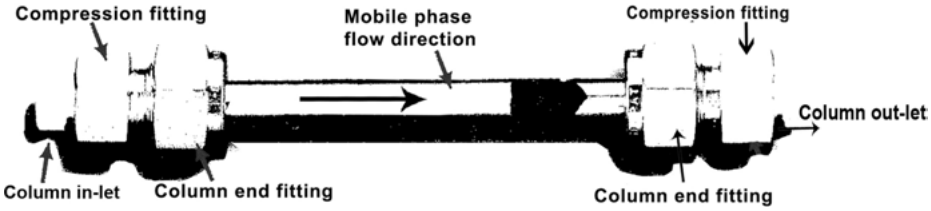


**Fig.6.14:** An arrow on the body of HPLC column showing flow direction of mobile phase.

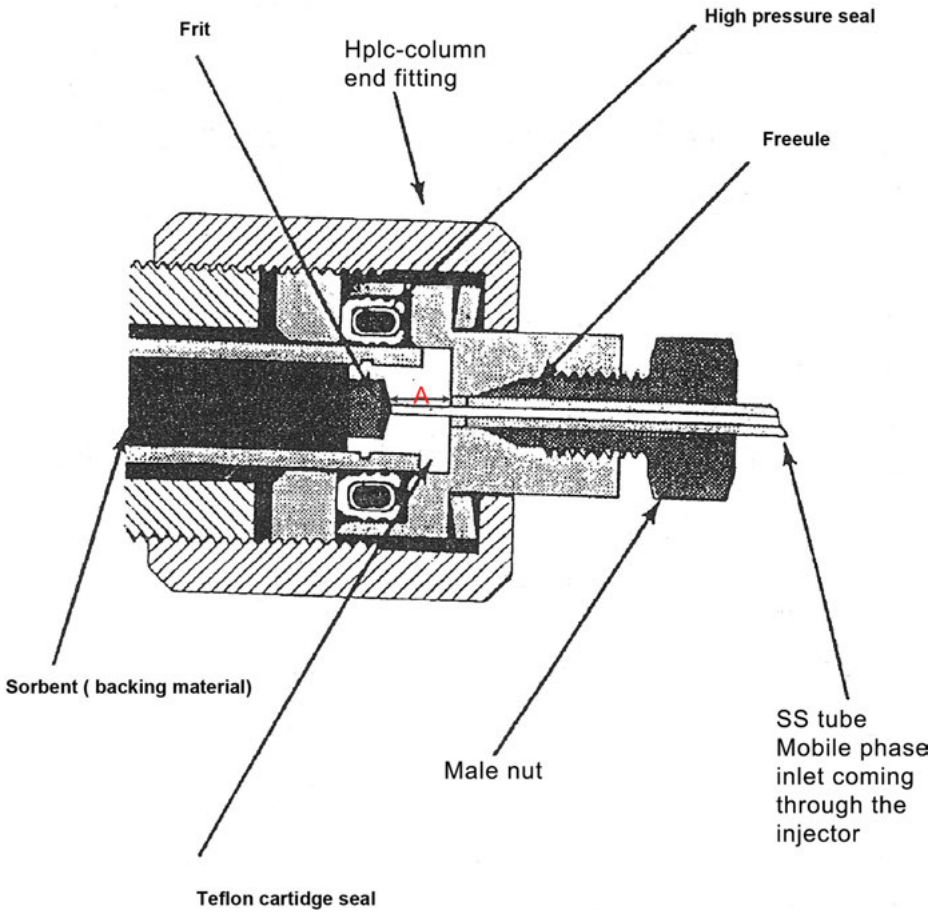


**Fig.6.15:** Illustrated diagram showing a compression fitting assembled with the column end fitting for connection of the column to HPLC system. Note: column end fitting typically is reducing union by 1/16″ o.d.

### 6.5.1 Use the following procedure to install your column

a. Make sure that all connection lines are well purged with mobile phase before attaching column to HPLC system. For that purpose, directly connect the HPLC system injector to the detector by replacing the old column with a zero dead volume union and flush the system to remove any particulates and old solvent.
b. Remove the end of the plugs from your column and save them for use when you store the column.
c. Put the column in its place as shown in Fig.6.16 and make sure that the solvent flow is in the direction shown by the arrow on the column label.
d. Thread the inlet and outlet compression fittings to the end fitting of the column as shown in Fig.6.15 using finger tight, and then tighten the fittings ¼ to ½ turn using the tool. Do not overtighten the fittings, overtightening will damage the connection.
e. At the required flow rate and when solvent is following freely from the outlet end of the column, attach the column to the detector. This procedure minimizes the likelihood of air entering the system and therefore facilitates quick column equilibration.
f. In the case of disconnecting (replacing) the column, reduce the flow rate of mobile phase to zero (decrease of flow rate must always take place in small steps to prevent packing bed disturbance) and wait until no mobile phase is coming out of the column (2 min). Removing the column without reducing the pressure to zero will damage the column.

Once all of the parts of the equipment are connected together for fluid flow, the users can begin the next phase. This involves preparing the mobile phase and the solute standard and sample solution for starting analysis (details are in Chapter 8).

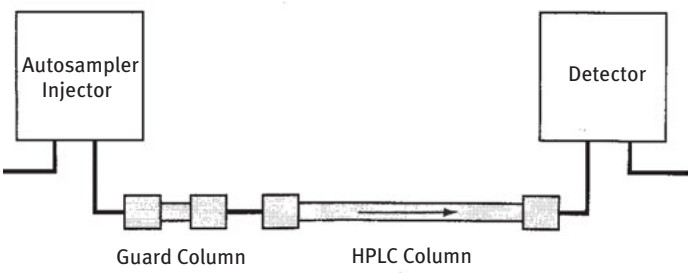


**Fig.6.16:** HPLC column position in-between injector and detector.

In order to avoid dead volume and leakage in the HPLC system related to the column instillation, the HPLC column must be correctly fitted with compression fitting compatible with its end fittings. It must be the extension of the tubing past the ferrule, once the ferrule is swaged on the tubing in the compression compatible with the depth of the port (Fig.6.9) of the end fitting of the column. We designated the extension of the tubing past the ferrule in compression fitting as distance A, as shown in Fig.6.7, Fig.6.8, Fig.6.10, Fig.6.15 and Fig.6.17. Assembling a compression fitting for HPLC column with improper extension distance A will cause problems. If the distance A is too short, a dead volume will be created in the HPLC system (the dead volume will contribute to increase peak broadening and poor system wash out; Fig.6.17a). If the distance is too long, the ferrule will not seat correctly in the column end fitting and the connection will leak (Fig.6.17b).

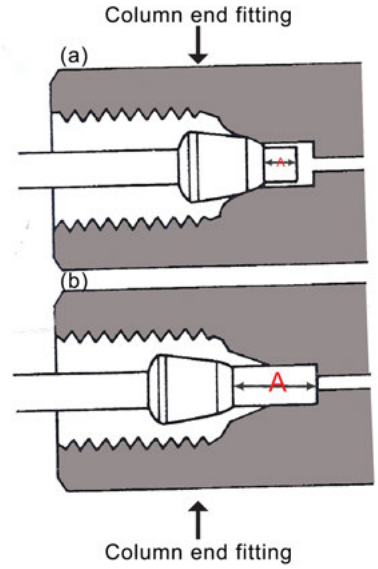


**Fig.6.17:** Illustrative diagram showing improperly assembled compression fitting for connection of HPLC column to HPLC system. (a) Tube extension (distance A) is too short. (b) Tube extension (distance A) is too long.

It is important to avoid mixing fittings from different manufacturers [2]. These may look similar and couplings may be ostensibly compatible but once a ferrule has been swaged onto a piece of tubing, it may be incompatible with another type of fittings.

As mentioned by Dolan and Snyder [2], the differing length of the extension of tubing past the ferrule, once the ferule is swaged on the tubing, is caused by differences in the depth of the female port in the fitting body because different HPLC manufacturers use different port depths. So, using compression fittings for the HPLC, from the same manufacturer, means the interchangeability between HPLC brands is difficult [2].

**Notes:**
- **Fitting problems can be avoided by using fittings parts that are designed to work together (same manufacturers).**
- **Assembling and tightening the fittings must be according to the manufacturers' directions; taking care to avoid overtightening is very important.**

## 6.6 Running of an HPLC system

The separation procedure can begin after the mobile phase, standard for a.i. and sample solutions of the pharmaceutical product under analysis have been properly prepared. The first step is to start the mobile phase running through the system. The mobile phase is placed in the mobile phase reservoir, which is usually located above the pump. A feed line connects the reservoir to the pump through an in-line filter. A priming procedure is used to fill the pump heads with mobile phase and the pump is then turned ON. The pumping rate should be less than, or equal 1.0 mL/min at this point. After mobile phase has passed out of the pump, into the column for defined time, a stable, non-fluctuating baseline should soon develop, indicating that the column has equilibrated with the mobile phase (see Chapter 2 for column equilibration). Solute injections can now be made. For loop injectors, the solution is loaded with the injector in the load position. Turning the injector to inject places is putting the loop in the path of the mobile phase flow which is carrying the sample. If all of the equipment is working correctly, and the proper separation conditions used, a typical bell-shaped, well-separated peaks should be observed on the screen of the computer of the HPLC. However, if this does not occur, the user must carefully troubleshoot the system for the source of the problems. Depending on the chromatographic method, components will elute from the column in 2–30 min, nominally. In Chapter 8, there are details about the standard test procedures which are related to HPLC when using it as analytical tool for qualitative and quantitative analysis of pharmaceutical products in drug quality control laboratories.

## 6.7 HPLC – instillation quality

Chromatography is a band-broadening process in which the sample aliquot broadens and differentiates into analyte bands as they travel through the HPLC column. This innate broadening process is reduced by using tightly packed columns filled with small particles. Broadening also occurs outside the column (extra-column band-broadening), in connection tubing and other devices in the fluidic path such as the injector valve and detector flow cell. This extra-column band-broadening or dispersion in the HPLC system is detrimental to column resolution and must be controlled by using small diameter connection tubing, a low-dispersion injector and a

small detector flow cell. This instrumental broadening effect is particularly damaging to applications of small diameter or short columns which produce smaller peak volumes. So, for test of the HPLC –instillation quality before using it for analysis, measurement of HPLC instrument band spreading (IBW) has become mandatory. In the following, we will discuss measurements of HPLC instrument band spreading (IBW) as indicator for **HPLC – instillation quality.**

### 6.7.1 Measurement of HPLC instrument band spreading (IBW)

Some band spreading is inherent in any chromatographic system due to the fittings (connections), tubing, and equipment. It is important to know the degree of instrument band spreading in your system. So, before installing your column, measure the instrument band spreading. The band spreading of a properly operating system should be less than 100 µL (100 ± 20 µL). To determine the band spreading of your system, use the following procedures:

- Directly connect the system injector to the detector by replacing the old column with a zero dead volume union.
- Flush the system to remove the old mobile phase.
- Inject a sample, 2 µL of a 0.5% caffeine solution into the HPLC system under investigation. In our case, it is Waters Alliance system [29].
- Operate the HPLC system using mobile phase ratio of water:acetonitrile (30:70) at 0.5 mL/min (isocratic) and detection at 254 nm (you may need to adjust the detector sensitivity to keep the peak on scale).
- From the resulting chromatogram (Fig.6.18) and using 5-sigma method (see Chapter 2), measure the peak base width in min (time unit).
- For this system, calculate IBW = the peak base width in min (time unit) × flow rate (mL/min) (0.5 mL/min) = 0.35 min × 0.5 mL/min × 1,000 = 175 µL. The limit is 100 ± 25 µL [29]. Science IBW for this system is 175, which means that the system is not good and cannot be used for analysis.

**Note:**

- Modern HPLC systems having a lower IBW of less than 80 µL.

The IBW of most HPLC systems can be reduced by the following modifications according to

  - Replace connection tubing with shorter lengths of 0.005–0.007″i.d. tubing.
  - Replace the detector flow cell with an optional semi-micro-flow cell volume **under 10 µL.**
  - Reduce the sample injector volume <20 µL for isocratic analysis.
- Further reduction of IBW might involve the replacement of the existing injector with a low-dispersion microinjector or an autosampler designed for micro LC.

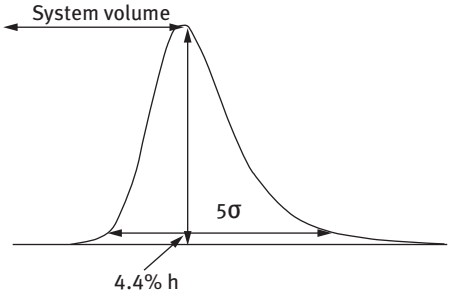


**Fig.6.18:** The chromatogram of injection of a Caffeine solution without the column showing the instrumental bandwidth of a Waters Alliance HPLC system connected with 966 PDA detector and a standard flow cell.

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# 7 Preparation of pharmaceutical samples for HPLC analysis in drug quality control laboratories

## 7.1 Introduction

To analyze pharmaceutical dosage forms in drug quality control laboratories, an *important* step is sample preparation. In this process, isolation of the components of interest (active ingredients in pharmaceutical dosage form, API (active pharmaceutical ingredient)) can be done and greatly simplifies the task of analysis. In Fig.7.1, you can see main stages of the entire analytical process. As far as the successful development of an application is concerned, choosing the proper measurement technique is one of the steps required. All the rest of the steps that are used to lead to the measurement are just as important. The reason for all the sample preparation is to give a sample aliquot that (a) will not hurt the HPLC (high-performance liquid chromatography) instrument or the column and (b) has minimum interferences and is highly compatible with the analytical method in mind. When talking about the HPLC analysis, it is important that the sample solvent dissolves in the HPLC mobile phase with no effect on the sample resolution or retention or even the stationary phase. It should also not interfere with the detection. Furthermore, the best way is to concentrate or derivatize the analytes for better separation or better detection.

Bad preparation of the sample shortens the life span of HPLC columns, increasing quantities of contaminants at the column head continuously build up the column back pressure. The purpose of good sample preparation which will be analyzed using HPLC can be summarized as follows:

- To improve the limits of detection
- To enhance selectivity of analyte identification
- To improve precision and accuracy for quantitative analysis of active ingredients in the sample of drug under analysis
- To protect HPLC columns from contamination

The process for sample preparation and sampling starts as soon as collection occurs and ends to the measurement phase. Primary sampling, that is, collecting the sample properly in the sampling process is the first contact one has with the sample. Hence, it is important that the sample taken is a true representation of the sample that is going to be investigated. The purpose of sampling is a volume or mass reduction of the sample from the original batch. The batch can be heterogeneous or homogeneous. The process of collecting and selecting the sample which needs to be analyzed is called primary sampling. There are some valuable books about the sampling process and its practical and theoretical aspects [1–7]. It also talks about collecting a sample that is statistically representative of the entire sample before conducting an analysis. This chapter does not include primary sampling methodology or theory

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as it is not required. But it is enough to say that one of the most underrated sources of an analysis error is sample preparation. The best way is to develop a well-drafted.

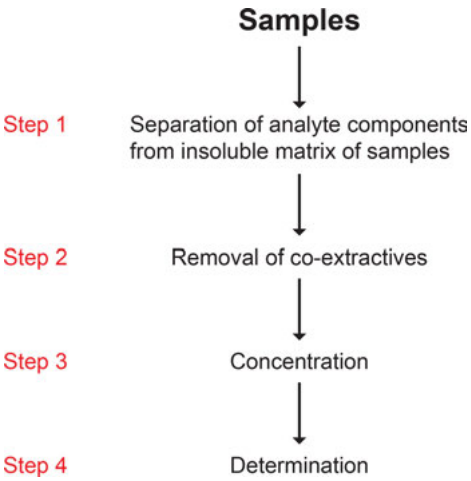


**Fig.7.1:** Schematic illustration of the steps involved in pharmaceutical sample preparation prior to HPLC analysis.

sampling plan to overall analyze different quality control labs for drugs. Just as important is to realize that both sample information and flow are parallel to each other during the analytical process. This is the case from the collection of sample till the report is generated. For instance, the sample tracking process starts right at the beginning when collection occurs. This is why it is thought of to be a part of the entire analysis process. To be sure that in the further stages, it will be easy to trace the sample taken to the parent sample, and proper methods of identification need to be carried out in the primary collection phase. These can be bar code application for automatic reading, writing labels by hand, using inedible ink to write on the containers, using RFI (Radio Frequency Identification) (Using RIF reader = RIFD) technique and incorporating the other documentation methods. Similarly, to make sure all the good laboratory practices) are followed, there is a need for proper tracking of not only the sample but also the subsample. This needs to be done at all stages of the analytical process. After the initial sample is taken, transporting it to an analytical laboratory should be done properly without causing a change in the chemical or physical characteristics of it. In case the sample has unstable, reactive or volatile materials and the laboratory is far away from the point of collection, the transportation can be challenging. The changes caused by the transportation process in the representative sample can also pose difficulties for the secondary data sampling process. There are many preservation techniques that are being used to reduce the chance of changes from point of collection till the point of analysis. Through these techniques, chemical changes like microbiological

degradation and oxidation along with physical changes like diffusion, volatilization and adsorption are highly reduced through the preservation techniques. Some examples of these techniques that should be used for avoiding these changes are:

- Choosing the right container for sampling
- Using additional chemical stabilizers like antioxidants along with antibacterial agents
- Freezing or putting the sample in a refrigerator to eliminate the change of thermal degradation

After the sample reached the laboratory, before analysis is done, it is very important to have proper storage conditions to ensure the integrity of the sample. For example, volatile and thermally labile samples have to be stored in the freezer or refrigerator inside sealed containers. Samples that are sensitive to light need to be stored in a dark, cool place without any exposure to sunlight till the time they are ready for the analysis process. Samples that have a chance of any chemical reaction or oxidation need to be kept in vacuum desiccators before handled or analyzed.

The sample preparation is the most complicated step in the development process of pharmaceutical analysis. The main aim of the sample preparation process is to make sure 100% of the analyte in a ready-to-use solution for analysis. In drug quality control laboratories, methods of analysis for API (Active Pharmaceutical Ingredient) in pharmaceutical products must be a validated method by the product manufacturer or the defined pharmacopeia. The aim of the validation experiments is to see if the method being used is appropriate or not for the purpose it is being used. This is done by running the method. Once it is done, compare the results to preset criteria. This will evaluate the precisions, specificity, accuracy, linearity, ruggedness and range. The criteria for validation and the experiments are occasionally suggested by different regulatory authorities like the International Committee on Harmonization, U.S. Pharmacopeia for drug substance and drug substance methods and the U.S. Food and Drug Administration [8]. Validation of accuracy (% recovery of API during sample preparation) is carried out by using the samples for which the total API amount is known off and applying the method to it. To do this, not one but series of samples are used, and the API of which are already known. These are then added to the particular pharmaceutical placebo (the developed method needs to have some tough experiments that test the variables of the sample preparation). For instance, in case of studies on extraction time studies, they should be continued till maximum recovery happens. So sample preparation procedure in drug quality control laboratories will be followed according to the manufacturer of pharmaceutical product or according to the defined pharmacopeia methods. Even though many protocols for sample collection are being in use in drug quality control laboratories according to the manufacturer of pharmaceutical product or according to the defined pharmacopeia, the book will not include all the topics but will be limited to only the popular methods for preparing the sample for the chromatographic analysis using HPLC, ultra-HPLC (UHPLC) and HPLC-MS.

## 7.2 Methods for preparations of pharmaceutical dosage forms for analysis using HPLC

### 7.2.1 Extraction

Solvent extraction method can be applied for the following types of *pharmaceutical* dosage forms [9–17]:

*Liquid forms*: Syrups, lotions, eye drops, ear drops and nasal drops
*Vaporized forms*: Inhalers and sprays
*Solid forms*: Tablet, capsules and powder

Generally, the approach of "dilute and shoot" is used for most of the drug substances which are in the liquid form and for the parenteral products [9]. The most common approach that is being used for solid dosages like capsules or tablets use a "grind extract dilute filter" approach. Lotions, suppositories, physiological samples like plasma and other complex forms of dosage may need added cleanup along with additional extraction techniques like SPE (solid-phase extraction) and LLE (liquid–liquid extraction) [11, 12]. The result of the pharmaceutical preparation of the sample is generally an HPLC vial that contains the final solution to be tested along with the analytes that have been extracted and are ready for the HPLC analysis. To get more details on the preparation of the sample for pharmaceutical products is not included here and can be found through other sources [17]. In the following, we shall discus different types of extraction techniques being in use for extraction of the desired API in pharmaceutical products.

#### 7.2.1.1 Liquid–liquid extraction

LLE is used to extract active ingredients in liquid (aqueous) pharmaceutical dosage form (syrup, eye drops, dissolution media of dissolved drugs, syrup, lotion, suspensions, ear drops, nasal drops, etc.) by using an immiscible organic solvent as a second phase (organic solvent examples to be used for extracting aqueous samples are hexane or ether, both their density is ca. 0.7 kg/L; or dichloromethane, its density = 1.3 kg/L; and chloroform, its density = 1.5 kg/L). In LLE of API depending on $K$ (the distribution coefficient, which acts as a partition between both the phases of the API), concentrations in the aqueous sample and the organic solvent will reach an equilibrium, often obtained by shaking the two phases for a certain period of time. Separation funnels (Fig.7.2) are commonly used for LLE (in which depending on the densities of the solvent, either the lower or upper phase contains the extracted API of drug).

To do LLE add the two liquids – the sample and the extracting solvent – to the funnel (Figure 9.2), insert the stopper, shake vigorously to get maximum contact between the liquids, let the funnel stand while the liquids separate and carefully draw off the desired layer. If a single extraction does not reveal a good extraction of


**Fig.7.2:** A separator funnel for LLE which depends on the density of the immiscible organic solvent. It will separate after shaking either to the upper (1) or lower (2) phase.

the analyte, repeated extractions may be performed by using the same solvent volume in each of the successive extraction steps.

Shaking it too vigorously can result in an emulsion being formed. This is the reason why you should try not to do so. Just in case it happens and is unavoidable, there are two ways to go about it. The first way is to centrifuge both phases. The second way is to use an immiscible organic solvent. Another technique to break the emulsion is to add a methanol (only a few drops) or add salt ($Na_2SO_4$ or NaCl) to make sure to make both phases separate.

One of the common sample preparation techniques is the "dilute and shoot." This is used for liquid samples of the pharmaceutical products that are simple in nature. These include syrups, lotions, eye drops, ear drops and nasal drops to avoid HPLC column overload (avoid excess peak broadening or distortion), for reducing the strength of the solvent, or to get the output signal in the linear range of the HPLC detector.

#### 7.2.1.2 Solid–liquid extraction

Solid–liquid extraction technique is sometimes called "shake/filter" technique. In solid–liquid extraction technique, sample (solid samples such as tablets and capsules) is put in a container that is totally sealed and then the solvent is added in it (water, methanol, acetonitrile, etc.). Then sample (with an appropriate solvent) can be shaken manually or mechanically for a set period of time using appropriate shaker Fig.7.3a–Fig.7.3c. The solution is then separated from insoluble solid through the process of centrifugation and filtration. The filtrate can then be analyzed by HPLC after cleanup. For improving the efficiency of the extraction process, sample must be in the finely divided state (see Section 7.2.5).

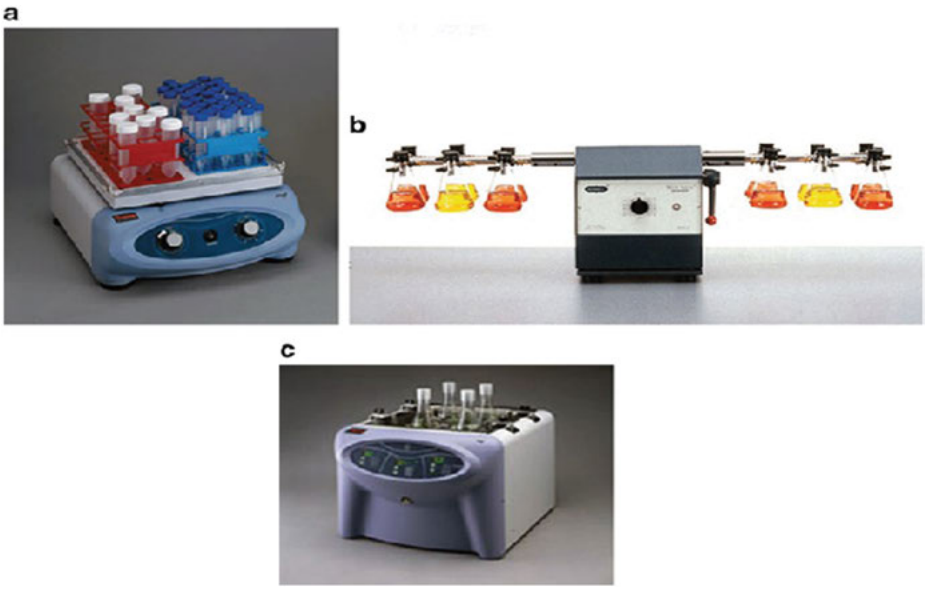


**Fig.7.3:** Examples of (a) reciprocating shaker, (b) wrist-action shaker and (c) water bath orbital shaker).

### 7.2.1.3 Soxhlet extraction

Soxhlet extractors had been around for150 years, the first time they were invented. But despite this, they are still widely used across laboratories. Fig.7.4 shows a Soxhlet extractor. It was made for extracting a lipid from the solid material. They are often used for liquid–solid extraction. The sample, in a thimble or porous holder (Fig.7.4), is covered in a hot and fresh solvent. Periodically, the solvent is drained off by a siphon and replaced. The insoluble matrix remains in the thimble, while the analytes are transferred to the Soxhlet extractor's solvent reservoir. The Soxhlet extraction is used mostly when the compound can only have solubility to a limited extent in the solvent, and also when the impurity is highly insoluble in the solvent involved. To use Soxhlet extractors for solid–liquid extraction, a sample from the pharmaceutical dosage (solid sample) needs to be mixed mostly with drying agents like sodium sulfate because wet samples may repel lipophilic solvents, and loaded in a porous extraction sleeve made of thick filter paper (thimble; Figure 7.4). In Fig.7.4, a reservoir is used to distill a solvent. Once that is done, a water-cooled condenser is used to liquefy it. The hot solvent then drips inside the thimble or sample sleeve. The construction of the apparatus is unique. It is made as such that all the solvent is collected. But if the solvent goes to the siphon or drain pipe, it goes back to the reservoir of the sample where the entire solvent cycling process starts from the beginning. The hot solvent flux continuously flows through the sample and that results in efficient results of the extraction process.

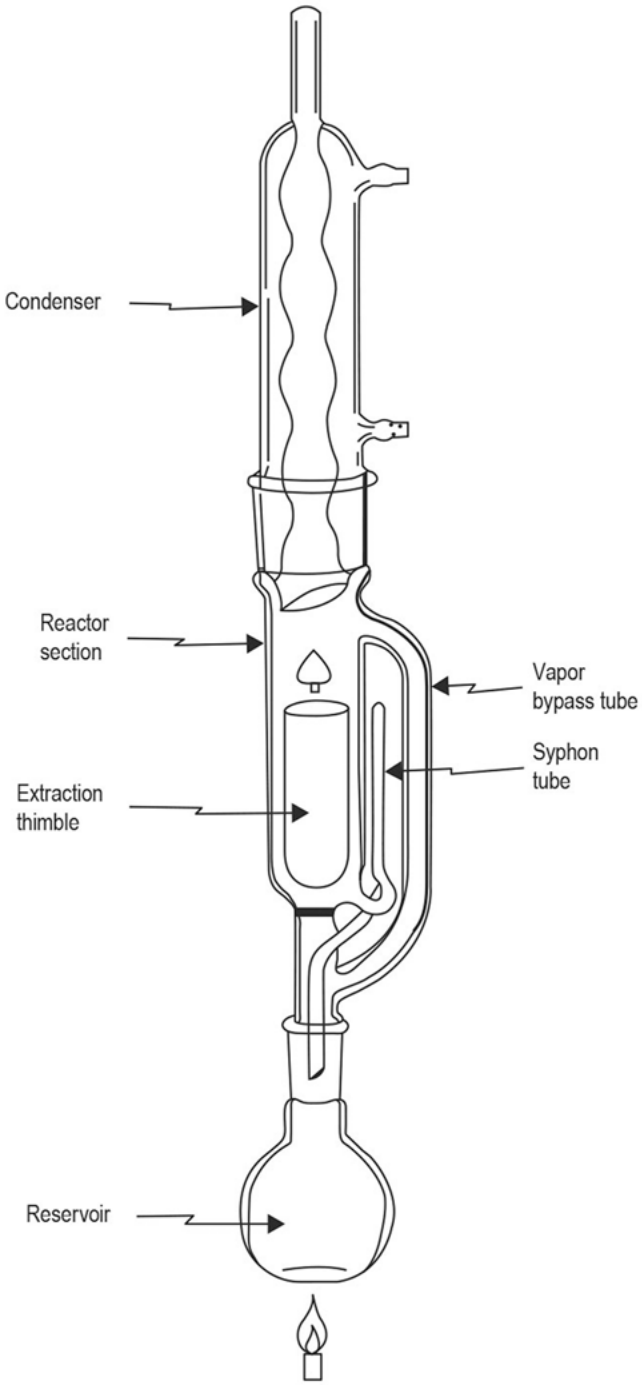


**Fig.7.4:** Diagram of the Soxhlet extractor.

In all of the above, the API ends up in an organic solvent of reservoir of Soxhlet extractors and became ready for analysis. Soxhlet extraction apparatus is suitable for continuous extraction of the solvent of semivolatile analytes from the solid matrices. To ensure that the sample does not lose any semivolatile analyte, proper care needs to be taken. Another thing to consider is that the extraction process that spans over several hours leads to a chance of thermal degradation. This is why Soxhlet apparatus is very rarely used for preparing samples of the pharmaceutical products.

#### 7.2.1.4 Ultrasound-assisted extraction (sonication)

This extraction technique is all about making use of ultrasound to make vigorous agitation on just the surface of the solid material that is finely divided. One of the most popular extraction approaches is sonication, which is carried out through the ultrasonic cleaner baths especially for products that have a controlled release. Extractions that are ultrasound assisted are done with an ultrasound disrupter finger or the ultrasonic bath. These extractions are done at room temperature. They combine the physical and chemical extraction together of the analytes from solid matrices. Ultrasonication produces cavitation bubbles that might attain significant internal pressure and temperature that eventually end in a collapse that causes the solvent extraction to spread in an outward direction with significant velocity and leads on toward the matrix particles on a collision course. As a result of collisions, the matrix is broken down into smaller particles. In this way, a greater surface area is exposed to the particular extraction solvent. Extraction is helped by the ultrasonic process. The heart helps to increase the rate at which extraction occurs.

The ultrasound equipment should have a minimum power of 300 W, with pulsing capability. Extraction supported by ultrasonication, therefore, is an effective method to extract API from solid matrices of pharmaceuticals when compared to the other frequently used methods like Soxhlet, LLE and solid–liquid extraction techniques. Ultrasound-assisted extraction (sonication) technique is rapid, is safe and is the best for granular, coarse materials.

Remember that different parameters like the presence of a perforate tray, the total wattage power of the sonicator, bath temperature, water-level depth and the total amount of sample flasks that are being sonicated affected the rate of extraction. In case of laboratory extraction, 0.5–1″ of water is filled in the bath to maximize efficiency of the extraction. Both the flask volume and extraction time are optimized empirically with a particular sonicator in the method development. Under some circumstances, sonication ends up producing enough heat to result in degradation and/or oxidation of the API and produces artifact peaks in impurity testing of the pharmaceutical products.

### 7.2.1.5 Solid-phase extraction (SPE)

A sample preparation technique, the SPE method, comes from the technology of liquid chromatography. It is extensively used for analyzing the biological samples and along with that also pharmaceutical product [11, 12]. The SPE method follows a step-gradient process. First, the analyte is dissolved in a solvent (weak) and is kept in the stationary phase. Subsequently, additional moving phases are added to increase the strength of the solvent. This results in controlled and selective elution of the analytes and interferences. In its most common mode, a small (about 0.1–1 g) open-bed column of octadecyl-bonded silica is used to retain an analyte from an aqueous sample while allowing interferences to pass through. With the help of the correct individual mixture or solvent, the interferences are properly washed from the column, as a result only the analyte is left there. An organic, common HPLC solvent is used to elute the analyte. For this, methanol or acetonitrile is used. Finally, the eluate left is analyzed either directly or is further treated with either derivatization or evaporation to help concentrate the analyte. On the other hand, you can use reverse-mode SPE too. This is done to filter or retain the interference while the sample passes from the column.

According to Niessner and Schaffer [11], SPE consists of five steps (Fig.7.5): firstly, methanol or a similar solvent is taken to solvate the solid matrix. Next, a buffer or water is used to wash the matrix. In the third step, the sample of the pharmaceutical dosage forms (such as dissolution media of drug) is put inside. A solvent, buffer or water is used to wash the matrix that has the sorbed AI. Doing this removes all the co-contaminants and impurities present in the sample. Air is drawn out in the last step to dry the sorbent, and then elutes the analyte from the matrix by using a solvent. By these steps, the analyte from the water sample will not only be extracted but the volume will be reduced manifold. Not only this, the volume becomes much smaller and that too at a rapid pace. A few seconds are all that are needed to reduce a few hundred milliliters of sample to only a few 100 μL. This is a concentrated solution that is present in the organic solvent. This can be analyzed directly by HPLC.

Trace analytical's general SPE sizes are anywhere from 50 mg to 10 g sorbent mass and column volume of 1 mL to 60 mL, respectively. Solid phases like the ones present in liquid chromatography are there for SPE. Moreover, depending on the hydrophobic interactions, ion-exchange and electrostatic forces, hydrogen bridging all play a role in the process of partitioning. Different cartridges are available commercially in the market [11] with varying fillings (particle sizes ca. 30–70 m) and sizes. Moreover, these are also recommended for carrying out the SPE work by the manufacturers [18–25]. Depending on the analytes, different sorbents are selected. Using the normal phase silica, polar compounds are extracted. The silica include cyano-, Florisil**-,** ethyl- and phenyl-, or silica that have been modified/alumina or slightly moderate polar substances or by cyclohexyl-modified silica, unpolar substances by $C_8$-bonded silica in the reversed phase or $C_{18}$ and ionic compounds by

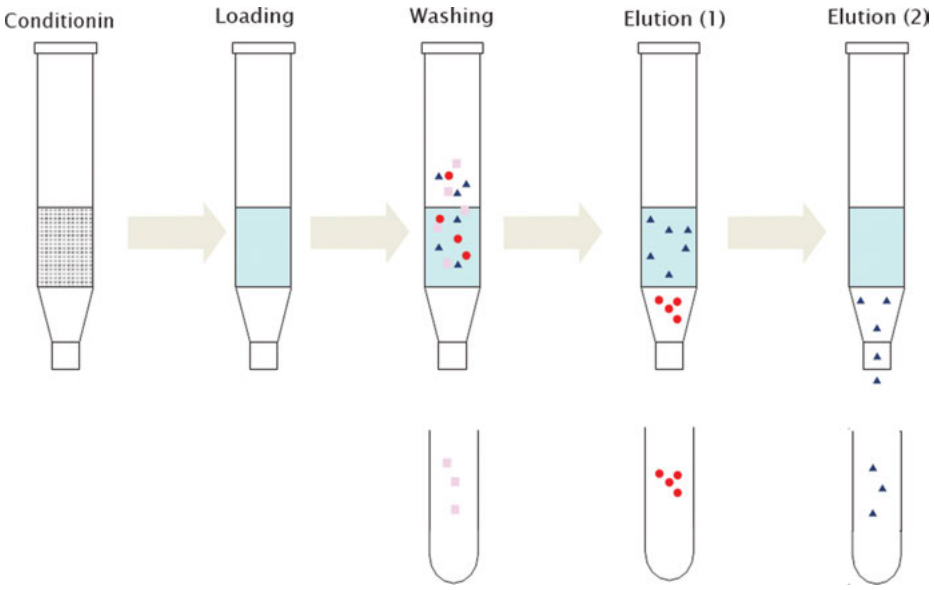


**Fig.7.5:** Steps in a generic SPE procedure.

ion-exchange phases. The best eluents for absorbing compounds on the reversed-phase beds are ethyl acetate, methanol and acetonitrile. The ionic compounds adsorbed to ion-exchanger beds are eluted by adjusting the pH well below or above the p$K_a$ or by buffers with high ionic strength.

To get the best results for SPE, the combination of washing solution, elution solvent and sorbent needs to be perfect. When compared with LLE, SPE produces less solvent waste, is simple to operate and gives rapid results. Using it off-line is also possible. This means the solution of analyte is independently and subsequently analyzed by using a simple technique like HPLC. The other way is to directly use it by coupling it to an analytical device like HPLC. SPE has some potential drawbacks too. This includes the nonefficient analyte binding to the sorbent (this can be overcome by using different packing) and the analyte not being completely eluted from the sorbent (this can be overcome by using some other eluting solvent). To establish quantitative recovery, a reference compound (as external or internal standard) should be present for conducting corresponding analysis. Problems that co-contaminants have a similar affinity to the sorbent as the analyte may be overcome by changing the composition of the solvent used for the partitioning, that is, by changing the selectivity of the sorbent.

The SPE offers an important advantage when compared to LLEs which have the potential to automate a normal, routine process. Adding the samples and solvent together in a small column is going to be much easier to automate when compared to the LLE. In that, mixing the tube contents and removing the solvent are required. Additionally, transferring the eluate from the solid phase to the chromatographic

phase is also easy to automate. Many vendors already offer off-the-shelf instruments to automate the SPE process [24]. Another benefit is that the SPE method works with low organic solvent volumes as compared to the liquid–liquid method and other conventional methods. To limit the cost of the SPE process, the SPE columns can be reused.

Another technique that is related to this is the solid disk extraction method. It resembles the solid phase geometry. In this technique, silica that is octadecyl-bonded is embedded in a network of polymer fibers. The disk shape is totally flat and that gives rise to amazing flow properties. Therefore, majority of the applications are involved in isolation of trace organics. The technique needs tight and quick retention of the particular drug in the solid phase. This is because the solid phase has a very short sample path. This offers the advantage of easy removal of the drug with only a small amount of elution solvent.

### 7.2.2 Distillation

Distillation is suitable for samples that can easily volatilized. To bring the solvent to a boiling point, heat is provided to the sample and the AI volatile in the vapor phase are both connected. They are then collected and sent for analysis. Vacuum distillation might be used when compounds with low vapor pressure are involved. Reaching the temperature of only 100 °C, stem distillation is gentler.

### 7.2.3 Dissolution

To measure the API released in standardized conditions, dissolution testing is done. These conditions are the ones United States Pharmacopoeia has specified while using the paddle method or type II or the basket method or type I and other types of apparatus [8]. In vitro evaluation, it can be correlated and compared with the in vivo bioavailability of the formulation of solid dosage from different clinical studies. To check the product consistency, dissolution testing is done in the product releases, stability studies and formulation development. **At the end of dissolution test, the API of the drug in the dissolution media can be directly (or after concentration, Section 9.2.7) determined by injecting a desired volume in HPLC system.**

### 7.2.4 HPLC column switching (online SPE) as a sample preparation for pharmaceutical products for HPLC analysis

Extracts to be analyzed must be cleaned up (i.e., removing interfering co-extractives). The common approach is to use pre-column, venting as shown in Fig.7.6. This is

done using a pre-column that is short and is identically packaged to the one in the key HPLC column [26–29]. The underlying fact is that in many of the HPLC assays, the studied compounds elute at values of 2–10 K. The rest of the sample elutes near the void volume as a front peak (see Chapter 2), especially in the case of trace analysis, the original peak tails and results in an overlap between the peaks of interest. Because of the pre-column venting in HPLC, it becomes easy to elute the disturbing materials into waste from column I as shown in Fig.7.6. Once the elution process is complete in pre-column I, the elution is allowed to go on the separation column by switching the HPLC injection valve. Therefore, the total amount of the interfering material that enters the separation system only consists of the compound of interest required. This allows for a good level of separation.

HPLC pre-columns or guard columns are a good option to automate as they are able to endure the high pressures that occur during HPLC separation. This allows for online analyses. A schematic of online extraction in the HPLC analysis is shown in Figure 9.6 when it is applied to any aqueous sample, for instance, drugs dissolved in the dissolution media. In the diagram on the top, pump A sends a predefined volume of the sample in the reversed-phase pre-column, where AI's have been retained through the injection value. Whatever water is left, it goes to waste through the column. Depending on the organic compounds' infinity in case of the reverse-phase materials, the sample volume is passed. In case too much samples enter, a part of the analyte can be washed away from the column. Even in the case of larger volumes of the sample (more than 100 mL or even more), the aqueous solution should have very high values of K. This will make sure the analyte is retained properly in the pre-column. As shown in Figure 9.6, during the separation phase, the pumps stop and the injection valve turns. This allows the mobile phase in pump B to elute the sample to the analytical column from the pre-column. Here, API or the separation of the organics is done either through the isocratic HPLC system or gradient elution.

(1) *Preconcentration step* in which the valve position is for absorption of analyte(s) on pre-column.
(2) *Separation step* in which the valve position for transfer is of analyte(s) to the analytical column.

### 7.2.5 Sample size reduction

It is preferable to go ahead and render the solid samples of the pharmaceutical products before they are analyzed into a well-divided state. This is done for the reasons mentioned below:

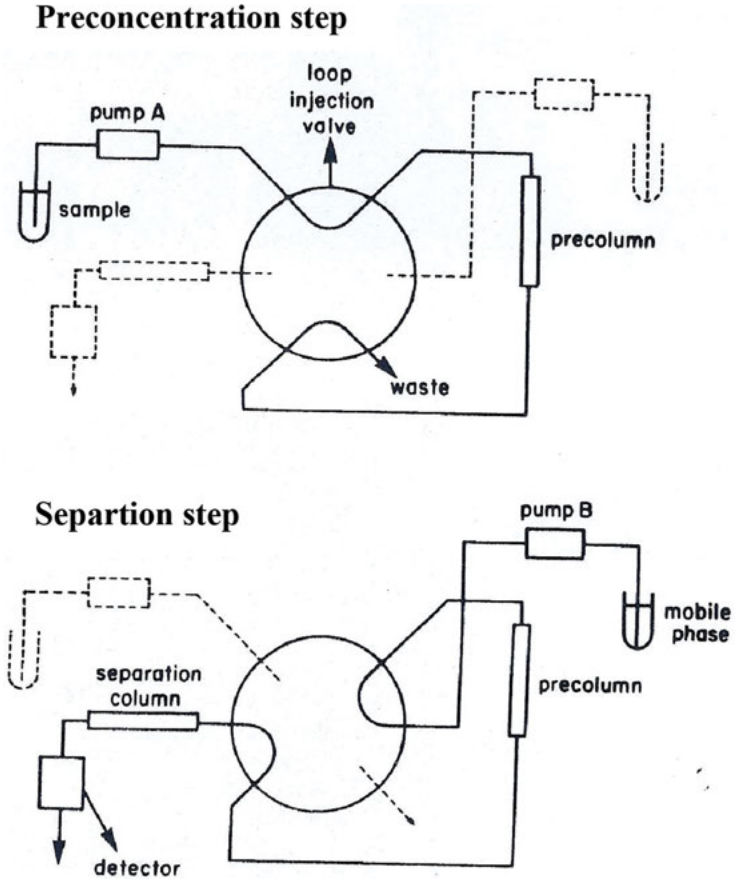


**Fig.7.6:** Schematic diagram for pre-column trace enrichment as applied to aqueous samples such as dissolution media of a pharmaceutical product.

- These samples are highly homogeneous. In this way, more representative subsampling is possible with higher accuracy and precision if they are mixed carefully.
- They are easier to dissolve and extract. This is because they have greater surface area.

Generally, the methods that are used for grinding the solid samples or reducing the size of the particles are mentioned below:

Grinding: As shown in Fig.7.7, automated pestles and motors as well as manual grinding are popular. Both dry and wet grinding are being used.

Blending: To bring a semisoft substance into smaller pieces, they are chopped with a mechanical blender. It can also be used for the process in which a heterogeneous sample is blended into a uniform and consistent sample.

Homogenizing: The process of breaking down the sample into little pieces and blending it to make it consistent and uniform in texture.

Milling: Sub-μm fineness is achieved by grinding the material with the help of ball mills. This is done by achieving higher grinding energy through planetary actions like tungsten, polytetrafluoroethylene (PTFE)-coated steel balls, agate or through centrifugal.

Sieving: The process in which the sample is passed through a plastic or metal mesh with a uniform cross-sectional area (with square opening from 3 to 123 μm). This is done to separate the particles.

**Fig.7.7:** The common sequence of sample preparation steps for solid drug products: grinding, extraction, dilution and finally filtration.

Mostly, techniques that are used to reduce the size are done during the dry state. For some samples like the ones that can agglomerate in the grinding process, or those that during the heating effect alter the crystalline structure. For these, slurry or wet grinding is the alternative.

To discuss about the different equipment available to help particle size reduction is not in the scope of this particular section. To read about the literature regarding this subject, the readers can refer to [30–32] along with the manufacturer's literature. The companies that are active in this subject are Spex SamplePrep (Metuchen 65 Liberty St., NJ 08840, USA) and Retsch Technology (Haan, Germany [33]).

In case the sample has volatile or thermally labile compounds, you need to make sure to reduce the heat when the grinding process is taking place. To ensure that the sample does not become too hot and remains cool in the grinding process, adding dry ice to the ball mill or mortar is done. It is important to ensure that the ice being used should be made from pure carbon dioxide. The dry ice should not have any impurities to refrain it from contaminating the sample. To keep the sample cool, a cooling block with circulating cool liquid can also be fitted in some ball mills when grinding is being done. As already mentioned, a freezer mill can be used to pulverize the sample with liquid nitrogen to solidify it to reach lower temperatures. Furthermore, the material can be sieved to allow further breakage to get a homogeneous sample.

There are grinding mills that have an enclosure fitted in them that allows inert gases to enter while the grinding process is going on. This is done for samples that are sensitive to air. Mills where vacuums are used to carry out the grinding process are also available, in case the material calls for the condition.

After the sample is broken into smaller pieces through grinding and crushing, the need may arise to classify the particles further into the ground sample. Mechanical sieves are most popular when it comes to particle sizing, and are classified according to the size of the square opening in mm or the mesh size. To separate the particles, a chain of sieves is used. These sieves are known but have different mesh numbers.

### 7.2.6 Clean up of sample extracts

Extracts to be analyzed must be clean up (i.e., removing interfering co-extractives). Centrifugation, freezing followed by centrifugation and filtration are effective techniques for cleanup of sample extracts. We shall discuss all of them further.

#### 7.2.6.1 Freezing and centrifugation

Fatty and protein's samples cause a lot of trouble in liquid chromatography. Filtration and careful sample preparation do not always remove all disturbing substances which may lead to precipitation or deterioration of the sample during injection. The increase of contaminants on the column changes the chromatographic behavior of the stationary phase. Treatment with dry ice is a good aid in sample cleanup. The dissolved sample material is put into a bath filled with dry ice and acetone for about 30 min and is then immediately centrifuged at 10,000 rpm for 10–20 min to separate insoluble particles.

#### 7.2.6.2 Filtration or centrifugation

Removal of particulate matter in the sample extract is critical for HPLC stability. Both column frit and top of column packing can become clogged (becomes narrow) by particles, leading to increase in back pressure and adverse effects on chromatographic results, decreased column efficiency, production of split peak, tailing peaks and so on. The particulates can go ahead and affect the hardware of HPLC like the rotary injection valves, flow lines and the inlet columns, frits and the detectors. Mostly, the last step in sample preparation is filtration. This is used to separate the solid particles and the analyte solution. Mostly, syringe filters are used to directly filter table extracts (Fig.7.8) into the HPLC sample vial. The syringe membrane filters come in different dimensions and media (25, 13 or 4 mm) and different pore sizes like 0.45 or 0.2 mm. Polyvinylidene fluoride, NYL (25 mm nylon) and PTFE are some of the popular ones. Whatman, Pall, Millipore, Gelman and many other suppliers provide the 0.45 mm filters. Sedimentation, centrifugation and

filtration are the most commonly used methods to remove the particulates from the given sample. Cellulose or filter paper can be used to remove particles larger than 40 µm. Also in-line filters placed ahead of the column can be used for sample clarification before entering the analytical column (for details, see Chapters 2 and 3). Removal of particulate matter in the sample extract can be done using centrifugation.

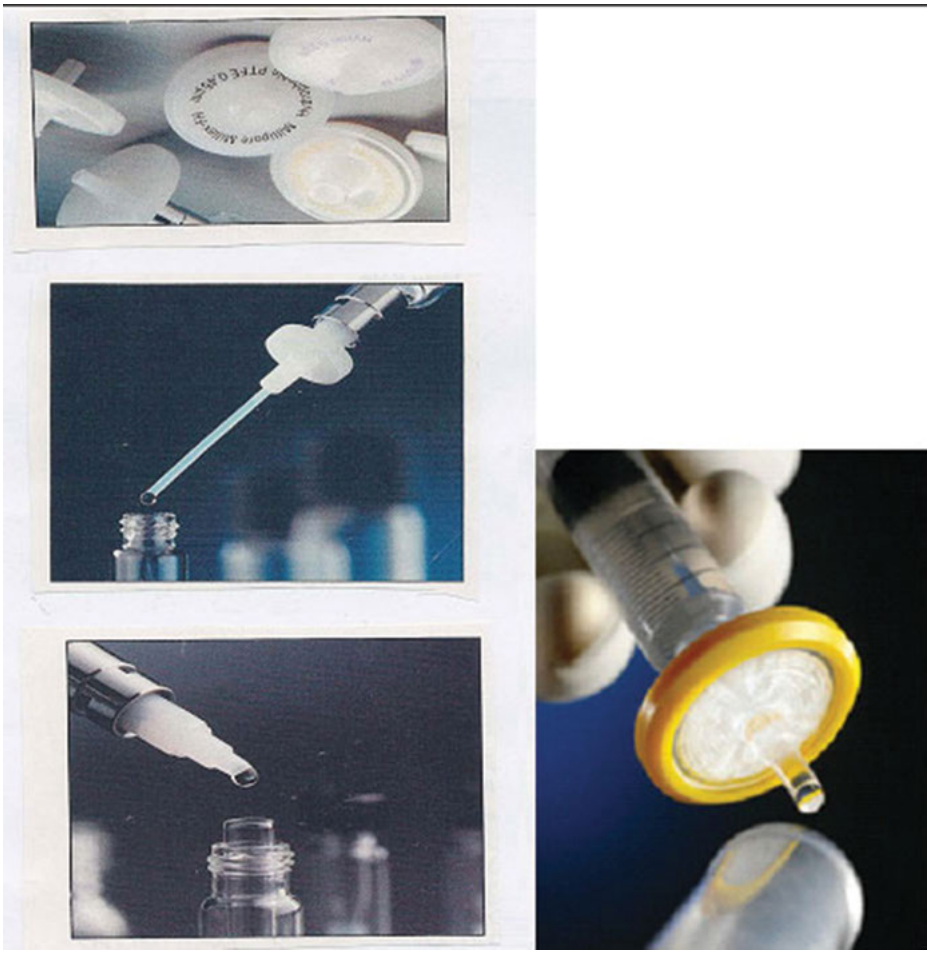

**Fig.7.8:** Millex-HV syringe filter unit (Millex-HV, pore size = 0.45 µm, hydrophilic polyvinylidene fluoride = PVDF) for clarification of aqueous and mild organic solution before injection in HPLC (Merck).

### 7.2.7 Preconcentration of sample extract before HPLC analysis

To conduct the analytical method, the extraction process (solid extraction, SPE or LLE) needs to reduce the organic solvent volume to such an extent that the analyte's

concentration is high enough. Freeze-drying or rotary evaporation technique is used to reduce the total extract volume. For many decades, the rotary evaporation technique is being used in the laboratories for preparative and analytical purposes as shown in Fig.7.9. Because of the reduced pressure of the system, gentle heating is used to evaporate the solvent. This is another advantage in case the analyte is heat sensitive. Inside the rotating flask, the dissolved solvent is rotated. This is done to decrease the chance of bumping and to ensure that the temperature in the sample remains homogeneous to give rise to a solvent film that has a large surface. To evaporate solvents that boil at temperatures higher than 100 °C are possible to evaporate at manageable temperatures provided that the pressure is very low. A cold trap is used to condense the evaporated solvents. After this, generally only a few milliliters of sample remains. This can be reduced by allowing nitrogen to pass gently over the surface of the solvent till you reach the volume required.

Riding a sample of solvents while it is in a frozen state with lower pressures is known as lyophilization or freeze-drying. This is a direct phase transfer of solid to gaseous state, also known as sublimation. Freeze-drying is generally done with samples in which the major liquid element is water. On the other hand, the ones that have organic solvents as the major liquid component are dried without freezing and using the vacuum concentration method. The liquid sample is firstly frozen at very low temperatures, below triple points of the phase diagram. At this point, the liquid and solid phases coexist. This is done by taking a bath and rotating the flask in it. It is first filled with an organic solvent and ice, or ice and liquid nitrogen. After it is fully frozen, reduced pressure is applied to sublimate it at a low temperature. The free water (primary drying) and eventually that bounds to the dissolved sample, for example, a protein (secondary drying) is recompensed at a cold trap installed in the freeze-drying apparatus.

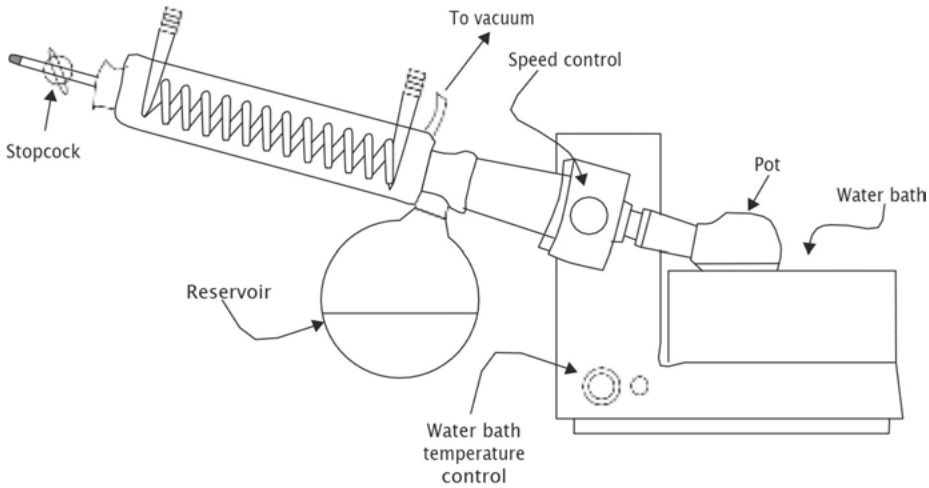


**Fig.7.9:** A rotary vacuum evaporator.

## 7.3 Extraction efficiency (API recovery)

The initial concern while selecting the solvent for sample preparation is to ensure optimized recovery of AI. The sample preparation recovery needs to be assessed properly as it will determine how accurate the analysis is going to be.

Percentage recovery of an API during sample preparation can be done by applying the method of extraction to a series of samples where the API is added to a pharmaceutical placebo. For drug substance and drug product analysis, recovery of 100% is generally required to maintain required levels of accuracy and precision.

## 7.4 Sample stability

*Once the sample is completely* prepared, it is important that the analyte remains stable for quite some time before the instrumental analysis phase. In this case, sometimes the sample needs to be adjusted for pH to make it stay stable. This is an important step for degradant methods and trace impurity. If the method is being used to report degradants, the sample needs to be degradation resistant.

## 7.5 Choice of sample solvent

The most important thing to consider is how the sample solvent will affect the entire analysis. In case of HPLC as the technique for carrying out the analysis, this statement holds true. This effect of the solvent can be because of the error that occurs in the HPLC analysis, and the sample solvent that is used for injection can change the overall absorptivity of the analyte to an extent that it changed the accuracy and precision of the analysis [34, 35]. This issue can arise when the slug of the injection solvent does not dissolute before the analyte reaches the UV detector. It is possible to control it by using the same solvents for measurement and calibration but it is preferable to ensure that the sample stream of the HPLC is consistent during the entire analysis. This is done by making the sample to dissolve in the mobile phase. Or, it can be done by ensuring that the HPLC separation is long enough to dissolve the analyte when it reached the detector in the mobile phase. To ensure stability and solubility, it becomes difficult to use the mobile phase as the injection solvent.

Chromatographic effects are more important to consider when selecting the HPLC injection solvent. It is extensively reported that distortion of the chromatographic peaks that occur because of the sample solvent is common in most of the method development laboratories [34, 35]. This can be seen clearly when higher volumes of analytes that have been dissolved in strong solvents are injected, for example, acetonitrile for the reverse-phase system. Unless there is an almost fully organic mobile

phase, the shape of the peak will be sharp when it is dissolved in the mobile phase compared to when it is dissolved in a strong solvent. The same is the case when there is a very strong injection solvent in the normal phase. This changes the retention volume and also results in peak distortion like splitting, fronting and tailing (43 and 35). Using a weak sample solvent is the better option as it will only lag until the mobile phase reached it from behind. Once they meet, the separation process will occur as designed.

To reduce the effects of the injection, the sample can be dissolved in a weak solvent. Even if there are no effects of the injection solvent, the volume of injection should be optimized depending on the objectives and goals of the assay. Bristow [36] recommended an injection volume less than one-tenth of the peak volume to maintain resolution and injection volume up to one-fourth of the peak volume if sensitivity concerns override resolution concerns.

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# 8 HPLC as analytical tool in drug quality control laboratories: qualitative and quantitative analysis

## 8.1 Introduction

In the pharmaceutical quality control (QC) laboratories,it is well known that the pharmaceutical QC is an important task. According to WHO 2010 [1], QC *(pharmaceutical drug quality control)* is the process in which the quality of a product is assessed. This is done by comparing the quality of a product in its final form to the specifications of the product release. To ensure the clinical performance, safety, efficacy and quality of a pharmaceutical product, competent regulatory authorities of the concerned country approve and fix certain specifications. These are done in the countries where the drug is going to be marketed. Through analytical testing, it is seen whether the specifications are being met or not. It is done by checking the purity, content uniformity, assay for potency and identity of the drugs before they are released into the market to be used commercially. To make sure the bioavailability is reliable, you need to conduct performance testing of the drug product (DP). This can be done either through disintegration tests or dissolution.

In today's pharmaceutical QC laboratories, one of the most important analytical techniques to conduct DP and drug substance (DS) is HPLC. This is widely used in the drug QC laboratories [2–7].

This chapter reviews how HPLC is used in the pharmaceutical analysis (routine QC) of DP or DS in pharmaceutical QC laboratories. Impurity and assay testing is the key focus when conducting HPLC testing for DS. For DP, HPLC is used for the same, that is, impurity and assay testing. It can also be used for content uniformity (to make sure that the total contents of the drug are uniform and the same in one entire batch) and dissolution testing (this indicates the bioavailability).

## 8.2 HPLC safety in drug quality control laboratories

Usually the practice of HPLC is carried out primarily in chemistry or analytical instrumentation laboratories. These laboratories are designed for safe and efficient use of their facilities. But no matter how well designed the laboratory is, personal safety is ultimately the responsibility of each analyst as well as the management directory for the lab. This requires well-informed and well-trained laboratory users. In Chapter 10, there is an excellent review about elements of good, safe laboratory practice pertinent to the HPLC user. The fact is that the majority of an user's time is

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spent with instrumentation and there is no excuse for neglecting safe laboratory practices. There are many occurrences of lost time accidents when performing HPLC. Many of these potential accidents and methods to avoid them are presented in Chapter 10. So, it is advisable for analysts to read Chapter 10 before using HPLC as analytical tool in drug QC laboratories. Generally, it is important to say that safety handling for solvents, mobile phase preparation and its supply for HPLC equipment during analysis, safe management for liquid disposal of HPLC and safety complete set for HPLC in laboratories need to be according to the national and international standards that are applied all over [8–10].

## 8.3 What analytical methods can be used by analysts in drug quality control laboratories

Analysts in drug QC laboratories use HPLC validated analytical methods for routinely analyzing the DP and DS . The validation of these methods is the responsibility of the drug manufacturers during pharmaceutical development cycle in the industry and have been done so according to the one of these rules: Center for Drug Evaluation and Research (CDER) [11], ICH [12–14] or United States Pharmacopeia (USP) [15], as shown in Fig.8.1. The purpose of validation of an HPLC analytical method is to show that the method used is in accordance with the acceptance criteria that is already predefined, such as accuracy, sensitivity, precision, linearity, robust/rugged as well as detection and quantization limit. It is not the purpose here to discuss in details the HPLC analytical validation process during pharmaceutical development cycle in industry. On the other hand, you can find further details in some texts and reviews [16–25].

According to ICH, FDA and USP rules, method validation experiments and the resulting data about the validation parameters in the relation of required criteria must be a part of drug manufacturer specification (in good description and documentation) ready to be used in laboratories carrying out QC of drugs by experienced analysts.

On the other hand, validated analytical methods for analysis of many common DS and DP were published by analytical development organizations such as the USP [15], European Pharmacopoeia (EP) [26] and British Pharmacopoeia (BP) [27]. These methods can be used by analysts in drug QC laboratories for analysis of specific drug.

In addition, analyst in drug QC laboratories can develop and validate specific analytical method according to CDER [11], ICH [12–14] or USP [15] rules for solving specific problem in his laboratory. As an example, based on the HPLC–photodiode array detector (PDA), a unique analytical procedure has been developed and validated according to ICH [12–14] and UNODC guidelines [28] in regard to precision, linearity, robustness, accuracy, lowest limit of quantification and specificity by

Omar and Khalifa [29–33]. This validated method was used to conduct screening to check whether any of the three synthetic PDE-5 inhibitors were adulterated illegally in the herbal products or not. These include vardenafil (V), sildenafil (S) and tadalafil (T) (see Chapter 2). These are the herbal products that are registered to improve male sexual performance. These were registered by the Kuwait Drug and Food Quality Control Administration from 2003 to 2012. Separation of sildenafil, tadalafil and vardenafil in extracts of samples were accomplished on a symmetry 300 analytical column packed with 5 µm C18 (150 mm × 2.1 mm, 5 µm, 120 Å) column (Waters Co.; for details, see Chapter 2) and mobile phase had acetonitrile:methanol:1% acetic acid (20:20:60, v/v/v) at a flow rate of 0.4 mL/min under ambient conditions. PDA was used to detect for the analytes. From the obtained chromatographic data about sildenafil, tadalafil and vardenafil (average retention times $t_R$ for each compound during the course of this study, resolution ($R_s$), peak tailing factor ($T_f$) and %relative standard deviation (RSD) of $t_R$); it is safe to conclude that sildenafil, tadalafil, and vardenafil were resolved at the baseline with the help of the mobile phase eluted isocratically with resolution values, R = 2.8 and 16.1 between vardenafil and sildenafil and between sildenafil and tadalafil, respectively (see Fig.2.20, Chapter 2). The peaks that were separated on PDA chromatograms were symmetrical in shape and had a $T_f$ of 1.01, 1.02

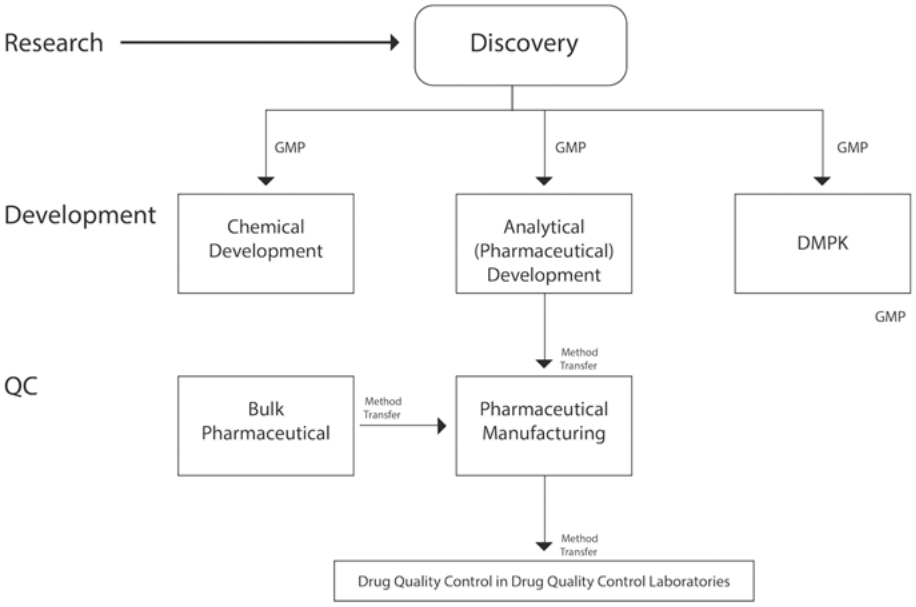



**Fig.8.1:** Schematic diagram showing the different departments in pharmaceutical research, pharmaceutical manufacturing and their relations to the analytical work in drug quality control laboratories. Drug metabolism and pharmacokinetics (DMPK).

and 1.05 for vardenafil, tadalafil and sildenafil, respectively (Fig.2.20). The %RSD values for the tailing factor for V, S and T were lower than 1.5. This shows that the chromatographic system is stable. According to the requirements of USP [15], the data quality achieved by vardenafil, tadalafil and sildenafil meets the acceptable criteria limit. All this shows that the chromatographic system is effective for both quantitative and qualitative purposes and is suitable to be used for this study.

In the scope's original method, if the concentration range or the procedure for sample preparation is changed, the analytical methods should be revalidated again to ensure their validity. Actually, the most common reason for carrying our revalidation is that the method of sample preparation changes. For example, the method that was used to validate a drug's table form is not okay to use for validating the encapsulated dosage of that particular drug without carrying our revalidation. The reason for this is the matrix of the sample changes. This is because the original diluent of the sample might not be enough to get the API from the formulation back.

## 8.4 Qualitative and quantitative analysis of pharmaceutical products (drug products) and drug substances in drug quality control laboratories

Standard test procedures need to be followed in the drug QC laboratories when conducting quantitative and qualitative analysis of the pharmaceutical products. They are described as follows:

- Stationary phase (packing materials) and their selection for HPLC columns to be used in drug QC laboratories for analysis of DP and DS
- Column connection to the HPLC instrument
- Mobile phase preparation and connection to HPLC
- Set up parameters for HPLC instrument
- HPLC column equilibration
- Checking for HPLC system qualifications
- Preparation of working standard solution
- Checking for suitability of HPLC system
- Qualitative (identification) and quantitative analysis for pharmaceutical products
- Data reporting and evaluation

We will discuss all of these procedures in details below:

### 8.4.1 Stationary phase (packing materials) and their selection for HPLC columns to be used in drug quality control laboratories for analysis of DP and DS

#### 8.4.1.1 Conventional stationary phase (packing materials) for HPLC columns to be used in drug quality control laboratories for analysis of DP and DS

HPLC column is called the separating system of the HPLC, no separations of an active pharmaceutical ingredient (API) from its DP or DS can occur without the column. In fact, and as mentioned by many scientists [2, 34–43], the heart of the HPLC is considered to be the column.

HPLC column consists of two components (Fig.2.1), the column packing materials (stationary phase) and the hardware (container) in which the stationary phase is found (as shown in Fig.2.1). On separation column, the sample component can be separated to its individuals. In Chapter 2, there are details about these two components of HPLC columns, which are the column packing materials (stationary phase) and the hardware (container). In addition, Chapter 2 discussed in details the physicochemical characteristics and specifications of the stationary phase to be used as a packing materials for HPLC column in drug QC laboratories. Also, details about HPLC columns operation, handling, care, maintenance and troubleshooting have been included in Chapter 2. In the following paragraph, we will summarize some aspects of the HPLC columns which can be useful to the analyst in these laboratories.

Stationary phase or packing materials for HPLC columns can be used in drug QC laboratories for the analysis of pharmaceutical products (DP) and DSs (DS can either be based on organic polymeric or inorganic ceramic substances) [2, 34–36]. The predominant inorganic ceramics are aluminum and silica. The inorganic packing does not swell in a solvent and has higher rigidity. The polymeric HPLC-grade packaging is based on methyacrylates or divinyl benzene. These are both crosslinked styrene. Unlike inorganic packaging, they are not rigid enough and can easily be compressed. Analytes and solvents can easily enter the polymer matrix. This in turn causes the particles to swell. Resultantly, the mass transfer reduces and you get low column efficiency.

According to many scientists [2, 34–43] and on the bases of the chromatographic modes of separation, HPLC stationary phase for HPLC columns can be divided into four major categories: reversed phase (RP-HPLC), normal phase (NP-HPLC), ion exchange (IEC-HPLC) and size exclusion (SEC-HPLC). In addition, other specialized modes also exist like chiral, affinity or other applications [34]. NP-HPLC means that stationary phase has higher polarity as compared to the mobile phase, for example, when using silica in adsorption chromatography (Fig.2.2). RP is the one in which the stationary phase's polarity is lesser when compared to the mobile phase, for example, when using hydrocarbon-type bonded phases and polar mobile phase (Fig.2.3). With both modes, solutes are eluted in order of polarity, with NP least polar elute first and with RP most polar elute first.

In science, from 1970 to present day, C18-bonded silica is still the most preferred and popular form of RP packaging when it comes to HPLC columns. Next in line are the C8-bonded and phenyl-bonded silica. Another option to use in the RP mode is cyano-bonded silica columns. The usage depends on the conditions of the mobile phase and the analyte. The mobile phase used in RP-HPLC packing is water, polar organic solvents or a mixture of both.

HPLC columns manufactures employ various techniques for producing RP materials. Silanol groups on the silica gel surface can be chemically modified to nonpolar (e.g., C-18, phenyl, $C_8$), polar ($-NH_2$, $-CN$) or ionizable (sulfonic acid, $SO_3$, quaternary ammonium, $N+ (CH_3)_3$) according to the salinization reaction (Fig.2.10). On the basis of this reaction and the available functional groups (R) which are listed in Tab.2.2, the resulting RP are pictured in Fig.2.11–2.13. After this reaction, the surface of silica particles has become hydrophobic and the resulting materials are called silica-based RP packing (RP) or bonded phase packing. The chemical structures of the most popular HPLC silica-based RP can be seen in Fig.2.11–2.13 and Tab.2.3 and classified by many researchers [44, 45] as old or conventional packing materials for HPLC columns as shown in Fig.2.14.

It is well known that during the salinization reaction, less than 40% of the silanol groups of silica particles can be reacted with the silane reagents. To make sure that there is no unnecessary interaction among the remaining –SiOH groups and the solutes, $Si(CH_3)_3Cl$ is added. This works as it converts the unreacted sites into $-SiOSi(CH_3)_3$. These columns are termed end-capped. End-capping is based on the use of smaller or less satirically hindered silane reagents such as trimethyl silane (Fig.2.15). The residual silanol population through the use of end-capping results in performance improvements of the RP packing. However, the population of residual silanols still approaches 60%.

At present, most HPLC analytical work in drug QC laboratories can be done with chemically modified or bonded silica, and by far the most important one is the nonpolar C-18 type. Even for the C-18 stationary phase or packing, there is a large selection available from HPLC columns manufacturers, which include Waters, Agilent, Alltech, Dionex, Hamilton, Macherey-Nagel, Phenomenex, Restek, Thermo, Tosoh, Varian, Vydac and Shimadzu. You can see the references section to know the Internet addresses of the abovementioned companies [46–58]. As shown in Tab.2.4, although all the HPLC columns are generally designed to carry out standards jobs, there are certain differences. For instance, different manufacturers have different packing for C-18. They differ on the basis of silica particles' pore size and shape, size, end-capping extent and the bonded phase's carbon content (see Section 2.4 for details).

#### 8.4.1.2 Modern packing materials for HPLC columns

As mentioned above, in RP-HPLC, the sialons groups remaining after modification with nonpolar or polar groups are responsible for many undesirable effects in this type of separation. The undesirable effects are:

***First:*** **Using the conventional packing materials for HPLC columns with basic mobile phase (contact with basic mobile phase, pH > 8), RP skeleton is hydrolyzed**. In the mobile phase, the OH anion goes ahead and attacks the surface of the silica, resulting in dissolution (as more and more silanol sites are exposed, the process accelerates). This behavior constitutes a serious limitation on the free choice of mobile phase. For that reason, use of pH values above 8 is generally considered impractical.

***Second:*** **HPLC peak tailing for basic drugs**
Basic compounds, such as amines, interact strongly with the unreacted surface silanols on conventional C-18 resulting in severe peak tailing. The severity of the peak tailing is highly dependent upon the analyte's p$K_a$ and the mobile phase's pH. At low pH ≤ 3, the surface silanols are in the associated form (Si–O–H) and do not interact strongly with higher basic compounds (p$K_a$ > 9) as in scopolamine bromide (hyoscine *N*-butyl bromide; Buscopan)). At pH > 3, the surface silanols begin deprotonating and interact strongly with basic drug resulting for severe tailing.

From all these disadvantageous, successful efforts have been devoted for development of other packing materials for HPLC columns (nowadays, it is called modern HPLC columns) such as symmetry, symmetry shield and XTerra packing (for details, see Sections 2.2.4.1, 2.2.4.2 and 2.2.4.3). In the following, we shall give brief information about the advantages for these previously mentioned packing materials for HPLC columns.

#### 8.4.1.2.1 Symmetry packing

Nowadays, numerous base-deactivated high-purity silica have become available as mentioned previously in Chapter 2. These silica have very low trace metal content [Fe, Al, Na]; ≤50 ppm) [34, 46]. Trivalent metal ions in the silica matrix are known to increase the IEC activity of the surface silanols. The base deactivated silica are also designed to have a fully hydroxylated surface (silanol concentration of 8 μmol/$m^2$) which significantly reduces the concentration of the highly acidic ions of silanol population in the starting silica. Acidic ionic silanols have been documented as a primary cause for severe peak tailing for basic drugs such as amitriptyline, scopolamine bromide and gliclazide.

Many of the old silica materials are highly acidic and have larger contents of metals like Spherisorb in them. If this is the case, during the mobile phase, it is important to add amine additives like triethylamine. This prevents any sort of adsorptive interaction between the silica materials and the basic analytes [2]. The reason there is inconsistency of the acidic silica materials in different batches is because of the inbuilt differences in these acidic and active silanols [2].

Nowadays, most state-of-the-art RP columns combine the use of high-purity base-deactivated silica with high surface coverage in order to obtain the lowest possible degree of silanolic interaction with basic compounds and deliver sharp symmetrical

peaks. So, these packing materials are called *symmetry packing*. In Tab.8.1, you can find some of the most frequently used RP columns today which are the most popular. Most of the products are based on high-purity silica.

#### 8.4.1.2.2 Symmetry shield reversed-phase packing materials for HPLC columns (embedded polar group technology)

Embedded polar group technology (Waters Co. Patent [46]) can be considered as a second approach toward further deactivation of the surface silanols of silica particles and is based on the design of new RP ligands which contains embedded polar group which results in a new stationary phase called *symmetry shield reversed phase* packing (SSRP). SSRP packing is a bonded phase in which a hydrophilic group (polar group) is incorporated (embedded) into the structure of the bonded phase between the silica surface and the RP chain (see Chapter 2). The embedded polar groups used are carbamate, urea, amide or other sulfonamide groups – all the groups that have stronger hydrogen bonding properties.

The performance standards for the peak shape of basic compounds have been set by the symmetry shield HPLC columns. These actually "shield" the silica residual surface silanols for high basic analytes. This type of RP column has its foundation on the patented *embedded polar group technology* put forth by Waters Co. [46].

For high aqueous and low organic mobile condition applications, the best column to go for is the symmetry shield one. The particles in the SSRP allow superior interaction of the sample and the particles. This is because they are water wettable. As a result, retention is stable with superior peak shape in the mobile phase which is highly aqueous.

In the symmetry shield packaging materials, the silanols on the surface have decreased activity due to the embedded polar group that is close to the surface of the silica. So, the advantages of using SSRP packing are:

- Significant improvement in peak shape, no tailing and resolution over pH range between pH 2 and pH 8.
- As compared to C-8 and C-18 conventional columns, it has lesser retention of the basic compound. Because of these SSRP columns, new methods will be developed by the analysts to quickly increase the analytical labs productivity.

The phases are easily usable in different conditions such as fully aqueous mobile phases. A few of the modern day polar-embedded columns are: Zorbax Bonus-RP (amide), Agilent, Waters RP18 (carbamate), Varian Polaris C18-ether and Dionex Acclaim PA (sulfonamide). Check the reference section to find the web addresses of the abovementioned companies [46–58].

**Tab.8.1:** Listing of some commercial packing materials for HPLC columns, their manufacturers and some of their physical properties.

| Manufacturer | Stationary phase | Surface area ($m^2/g$) | Pore size ( Å) |
|---|---|---|---|
| Agilent [47] | Zorbax | 300 | 70 |
| Macherey-Nagel [51] | Nucleosil 100 | 350 | 100 |
| Phenomenex [52] | Luna(*) | 400 | 100 |
| Phenomenex [52] | Prodigy | 310 | 150 |
| Phenomenex [52] | Synergi(*) | 475 | 80 |
| Thermo [54] | Hypersil | 170 | 120 |
| Thermo [54] | Hypersil Gold(*) | 220 | 175 |
| Thermo [54] | HyPURITY | 200 | 180 |
| Varian [56] | Polaris(*) | 180 | 180 |
| Waters [46] | Atlantis | 340 | 90 |
| Waters [46] | U-Bondapak | 330 | 125 |
| Waters [46] | NovaPak | 120 | 60 |
| Waters [46] | Spherisorb | 220 | 80 |
| Waters [46] | SunFire | 340 | 100 |
| Waters [46] | Symmetry | 335 | 100 |
| Waters [46] | Symmetry 300 | 110 | 300 |
| Waters [46] | XTerra*[a] | 175 | 125 |

– The underlined columns are the ones with high-purity silica.
– Phases stable in high pH are marked with *.
– [a]Columns that have hybrid particles.

#### 8.4.1.2.3 XTerra packing (hybrid particle technology)

In the past few years, advances in base materials technology have been made by combining tetraethoxy silane (the traditional monomer as in preparation of conventional RP particles) and methyl triethoxy silane together. This results in a new silica support that is hybrid and has 1/3 less surface silanols [2, 34–37] (XTerra Brand hybrid particle technology manufacturing process as per Waters Co. [46]) (see Chapter 2 for details). Hence, for the basic analytes, hybrid particles have lower peak tailing and lesser residual silanol activity.

Furthermore, surface modification for XTerra particles through the bonding of ligands provide materials with excellent RP properties (see Chapter 2 for more details).

When the methyl siloxane bond is present in the XTerra particle, the resistance to dissolution of the particle increases at high pH mobile phase. In addition to that methyl groups help protect the particles at high pH values (8–12) providing extended column life times. This happens because of the XTerra particles' ruggedness. In comparison to the traditional silica particles, the XTerra particles' molecular structure has higher resistance when it comes to the dissolution process at a higher pH level. The most popularly used RP columns can be seen in Tab.8.1. Most of the new products are based on hybrid particle.

#### 8.4.1.2.4 Sterically hindered group and polyfunctional silane chemistry

As already mentioned in Chapter 2, in the conventional bonding chemistry that uses monofunctional silane,, the maximum efficiency in bonding can go up to ~50%. Or, it can achieve ~4 mmol/$m^2$ legend density. There are many disadvantages of these types of bonded phases. These are mentioned in Section 8.4.1.2.

Another innovation in bonding chemistries [2, 34] have been made to help get rid of the problem areas such as:

- Sterically hindered group: To ensure that the labile Si–O bonds are protected against the process of acidic hydrolysis, bulky di-isobutyl or di-isopropyl groups are added in the bonding reagents [2].
- Polyfunctional silane chemistry. As mentioned previously, to prepare for the monomeric bonded stationary phase, the common synthetic scheme used is to create a reaction among the functionalized silane and the active silanol group which is present on the surface of silica particles. There has to be a single leaving group in the functionalized silane. If tri or di-active silane is used during the synthetic scheme, a result with higher complex surface chemistry is achieved. This type of phase is called polymeric phase and generally it gives lower bleed for LC/MS and higher stability when in both high and low pH [2, 34–37].
- HPLC column bleed is a major source of background signal in LC-MS analysis. This phase bleed occurs when the bonded phase elute from the column during the analysis. The bleed may originate from acid hydrolysis of the bonded phase at low mobile phase PH values or from dissolution of the silica substrate under more basic conditions. As mentioned by [2, 34–37] polyfunctional saline stationary phase (polymeric stationary phase) exhibit higher stability when in both high and low PH and less intense mass spectral bleed response when compared to other commercially available monomeric bonded stationary phase.

Note: Polyfunctional silane chemistry is more difficult to control.

#### 8.4.1.2.5 Modern HPLC columns trends

Because column technologies are evolving at a rapid pace, it is important to understand the column trends in modern days. Newer products with higher consistency

and better performance are coming on the forefront [2, 34]. New innovations are being made to overcome the problematic areas of the traditional silica-based RP columns. They are:

- Lower column life
- Inconsistency among batches
- Basic analytes peak tailing factor
- pH limitations (2–8)

Most of the problems mentioned above have already been minimized or resolved through the new developments that occurred in the last 20 years. Hybrid particles, novel bonding chemistries and high-purity silica are the modern trends that will be discussed briefly in this chapter (already discussed in Chapter 2).

Column chemistry as well as silica-based stationary phases have already been discussed in detail in Chapter 2. We have also discussed newer developments like stationary phases that are water wettable and polar groups with embedded packages. The advantages of modern packaging were emphasized. These were related to hybrid packing and high-purity silica that had embedded polar groups. In the part where we talked about column configuration, we focused on looking at the performance of the column depending on the column length and particle size ratio. If this ratio is constant, generally the column's performance characteristics will also be constant.

Without a doubt, pH is one of the most powerful tools when it comes to manipulating the selectivity for the compounds that are ionizable. If the pH value is below the p$K_a$ value of acidic compounds, the retention is increased. For pH values higher than the p$K_a$ value of the basic compound, the retention is increased. As most of the active compounds found pharmaceutically are easily ionizable, a column that has a wide usable pH range is important. This results in higher retention manipulation time during the process of method development. As shown in Tab.8.2, XTerra columns as a modern one in comparison with an conventional one Hypersil BDS C 18, it has a PH range that is usable (pH 1–12). Silica columns are only half as wide as they were when compared together. This ensures thatthe method development is twice as fast and easy as compared to silica columns. For more details about symmetry, symmetry shield and XTerra packing materials for HPLC columns see Omar and Khalifa [60–62] and Khalifa and Omar [63].

### 8.4.2 HPLC column hardware

HPLC column design is shown in Fig.2.30 and 6.14.

Mostly constructed with stainless steel, HPLC columns can withstand high pressures of up to 8000 PSI. The column materials are usually resistant to the normal pressure in HPLC and are also quite inert when it comes to chemical corrosion. The column interior needs to be as smooth as possible, also extremely flat tube ends

**Tab.8.2:** Specifications for some modern HPLC columns.

| Particle size column (µm) | Length (mm) | i.d. (mm) | Pore size (Å) | Surface area ($m^2/g$) | Carbon load (%) | End-capping | pH range |
|---|---|---|---|---|---|---|---|
| Zorbax Bonus RP 3.5 | 100 | 4.6 | 80 | 180 | 9.5 | Triple | 2.0–9.0 [47] |
| Luna phenyl–Hexyl 3 | 100 | 4.6 | 100 | 400 | 17.5 | Yes | 1.5–10.0 [52] |
| Zorbax Extend C18 3.5 | 100 | 4.6 | 80 | 180 | 12.5 | Double | 2.0–11.5 [47] |
| Waters XTerra RP 3.5 | 100 | 4.6 | 125 | 180 | 15.0 | Yes/ polar | 2.0–12.0 [46] |
| Waters Symmetry | | | | | | | |
| Shield RP 18 3.5 | 100 | 4.6 | 90 | 330 | 17.5 | Polar shielded | 2.0–8.0 [46] |
| [46] Waters XTerra MS 3.5 | 100 | 4.6 | 125 | 180 | 15.5 | Yes | 1.0–12.0 |
| Hypersil BDS C 18 3 | 10 | 4.6 | 130 | 170 | 11.0 | Yes | 2.0–7.5 [46] |

ensure a tight fit. Mostly stainless steel tubes (316 grade) are used to make the containers of the HPLC columns. These come with conventional end fittings (end fitting body, male/female union, 316 stainless steel compression style). The column packing material (stationary phase) is held in the column tubing by 1/16″ thick, 0.5 µm pore size of 316 stainless steel frits (Fig.2.30–2.35) that are present at the bottom of the tube. The column top has a stainless steel frit on top of the packing materials (stationary phase). At the lower and upper end, there is a reducing union (inlet compression screw fitting, male nut, Fig.2.30) to connect the column to HPLC system. Fig.2.30 shows HPLC column hardware as described previously. Columns of internal diameter (i.d.) of 0.1–10 mm (often 4.6 mm) and length 5–25 cm are generally used for analytical purpose as shown in Tab.2.7 and Fig.2.31. Wider columns of i.d. between 10 and 50 mm may be used for preparative work as shown in Tab.2.7 and Fig.2.31. HPLC columns can be packed with 10, 5, 4 or 3 µm diameter of particles.

The analytical columns dimensions, namely i.d., or the inner diameter and the particle size of the stationary phase affects the total speed of the process of analysis and also the effectiveness of the separation. Shorter column lengths (3–5 cm) allow short run times and low back pressure, whereas longer columns (25–30 cm) perform better in terms of resolution of separated analytes but with longer analysis time, it requires more solvents and increased costs.

Nowadays, stainless steel high-pressure columns can be used up to 10,000 psi. In general, it is advisable to maintain minimal pressure in all systems since higher pressures cause increased HPLC component wear and fatigue. Pre-packed columns are available from many HPLC column vendors with different varieties of stationary phase pickings [46–58]. These provide for the most demanding needs of analysts in drug QC laboratories.

### 8.4.3 HPLC columns selection for analysis of pharmaceutical products and drug substances in drug quality control laboratories

For the purpose of QC in control laboratories for checking drug quality, selection of the packing materials for HPLC columns must be according to drug manufacturer specifications or specified pharmacopeia. The name of the packing materials and their physical and chemical characteristics (carbon load, particle size, surface area or particle shape) to be used as a stationary phase is generally given in the drug manufacturer specifications or specified pharmacopeia within the described analytical method and this method was already validated. So, it is mandatory for HPLC packing materials to be used as a stationary phase according to manufacturer specifications or specified pharmacopeia. Generally, most of the HPLC columns found on the market can easily passing analysis conducted in the laboratories and a lot of these columns or the equivalents/alternative can be supplied by many vendors include: Waters, Agilent, Alltech and so on [46–58]. Therefore, the selection criteria, which you have to follow, depends on the specifications of the drug manufacturer and also on making sure that a high quality, reliable column vendor is used.

#### 8.4.3.1 HPLC columns selection for United States Pharmacopeia analytical methods

It is well known that for many years, the USP specified the HPLC columns which are needed for their analytical method by packing material rather than by manufacturers/vendor [59]. In addition, the HPLC columns needed for USP analytical methods are designated by USP as L1, L2,. . ., L84, L85. Looking for the USP column specifications, we can find that they are quite broad. For instance, when there is specifically an L1 involved, a column that has some ceramic packing or silica in it is required. It should be either 10 or 5 μm in diameter and bonded with C18, that is, octadecylsilane. Many of the columns offered commercially are available in the market through different HPLC column suppliers and manufactures [46–58] to meet the basic specifications for USP analytical methods. However, the reality is that not all of the C18 columns are the same. In reality, only a few C18 columns available in the market are able to perform the process of separation efficiently.

In the review prepared by Omar and Khalifa [60–62] and Khalifa and Omar [63], there are commercial columns that are equivalent to USP specification and it can be used successfully for USP-HPLC method for analysis of pharmaceutical products in drug QC laboratories according to USP. It can be said that the USP specifically gives analytical chemists to decide themselves which is the best column they want to work with in the control laboratories that handle drug quality. You can find the list of vendors and manufacturers in the USP. These are alternative or equivalent columns that will give better or even equal quantitation and resolution which is acceptable. Depending on the requirements, the analysts working in the drug quality laboratories can choose a column of their choice which will give the best results.

Also, HPLC column manufactures or suppliers give a guide from their sides for choosing a specific HPLC column that meets all the specifications which are set by the USP. This can be seen in Tab.8.3.

**Tab.8.3:** Displays HPLC columns recommended by Varian [56] and Agilent [47] to fulfill the requirements for USP methods which is using L1 column (octadecylsilanethat is chemically bonded to either ceramic microparticles or porous silica (5–10 μm in diameter).

| **1– HPLC columns recommended by Varian [56]:** |
|---|
| Pursuit ™XRs C18,MetaSil ™AQ C18,Polaris C18-Ether,Microsorb™100 C18,OmniSpher™C18, MicroSpher™C18, MonoChrom C18,Polaris™C18-A and Pursuit C18 |
| **2– HPLC columns recommended by Agilent [47]** |
| Polaris C18-Ether,Polaris C18-A, Pursuit C18,Pursuit XRs C18,ZORBAX ODS ZORBAX Eclipse XDB-C18,ZORBAX Eclipse Plus C18,ZORBAX Extend-C18, ZORBAX ODS classic,ZORBAX Rx-C18,ZORBAX SB-C18,Poroshell 120 SB-C18, Poroshell 120 EC-C18. |

Currently, the USP established database [64] for column selection. In this database, all the information about USP-HPLC column specifications and the equivalents or alternatives from many HPLC vendors are available for analysts in drug QC laboratories.

Another way to select the column that will be appropriate for conducting the separation process is to go ahead and consult a vendor who specializes in columnsor technical help desks for a specific column such as in Varian Company (helpdesk.eu@varianinc.com, helpdesk.us@varianinc.com). These specialists mostly provide you with chromatographic examples on the type of separation you want to work with.

Finally, analysts who work in the laboratories for drug QC need to choose a column that will help achieve best results in the analysis process they are conducting.

From our experiences, to increase efficiency and resolution, we recommend spherical particles of 5 μm diameter or less. Another thing that improves the load capacity of the HPLC column is when the surface area is higher. 25 and 30 cm columns are generally used for providing the maximum resolving power.

### 8.4.4 Column installation (column connection) to an HPLC instrument

Usually, the tag attached to the column indicates packing type, particle size, frit size, shipping solvent composition, column length, column inner diameter (I.D), column packing materials, particle size of packing materials, column materials and so on. These important information must be kept with the column at all times. All these information are very useful for column instillation and care. Also, flow direction during the column packing process is indicated by (flow→) on one of the reducing union or tube (Fig.6.14, Chapter 6). Use logbook of columns and use HPLC column vendor manual as a guide for column handling which you can find with each column in its box. In Chapters 2 and 6, there is an excellent review about all these previously mentioned items about HPLC columns.

Use the previously mentioned procedure in Chapter 6 (Section 6.5, HPLC column installation, column connection) to install your column.

For installing the column with stainless tube, prepare the tubes which will working as inlet and outlet of the column for compression fittings as we mentioned previously in Chapter 6 (proper and assembled two compression fittings in good conditions are required for HPLC column connection to an HPLC system.

### 8.4.5 Recommended operating guides for RP-HPLC and NP-HPLC columns

The following operating guides are for good operating HPLC-RP and HPLC-NP columns. Consult other references [2, 34–45] or other vendor's column instructions [46–58] for special precautions or column regeneration guides.

- It must be using mobile phase (solvents) compatible with the stationary phase (see Tab.3.6, Chapter 3).
- Sometimes, for eliminating any highly retained analyte from the column, flush it with a strong solvent such as methanol.
- If you have "dirty" samples, using an off-line or in-line sample filter or a guard column will be effective.
- RP columns should be stored either in methanol or acetonitrile. They can also be stored in a mixture of organic solvents and water (see Tab.3.6, Chapter 3).
- To stop the columns from drying, cap unused columns with "closed" fittings.
- When dealing with the silica columns, the mobile phase's pH range should fall in the range of 2.5–8. If the pH is greater than this, the silica is going to dissolve.

When it dissolves, the column will have a void. In case of a pH level lower than this range, the bonded phase is going to be stripped away. These problems can cause loss of resolution or even change the retention time. Most of the silica-based HPLC columns today have the ability to be used at higher ranges of pH like 1.5–12 [34–45] (see Tab.8.2)

- Generally, the HPLC column has a temperature limit of 60–80 °C; do not use temperature more than this limit.
- Never let the mobile phase containing buffers sitting for long a time inside the column.
- The temperature of the column should be changed only when it is pressurized.
- A cutting wheel should be used to cut the stainless steel tubing in a square cut. It is important to polish or debar the cut end. A readymade tubing or precut one is also available with different HPLC manufacturers [46–58].
- While attaching the new ferrule, it is important that the tubing goes ahead and is "bottoms out" when it is inside the fitting (see Chapter 6).
- Plastic finger-tight fittings are user-friendly and convenient to use. In case you are using columns that come from multiple vendors, this is going to be excellent. But, there is a chance that at a high pressure, it can slip (see Chapter 6).
- The most flexible and convenient tubing is the PEEK tubing. The pressure rating it has is lower and is not compatible with THF, DMSO and other solvents like methylene chloride.
- Depending on the sample being injected and the type of mobile phase being used, the column lifetime is typically anywhere from 1,000 to 3,000 injections.
- The efficiency of the column goes down with time. This is because of the increased peak width.
- The performance efficiency and backpressure of the column needs to be monitored and action needs to be taken to put things right instantaneously without waiting. You do not need to even wait to plug in the column. If pressure is abnormally high, try back flushing the column as soon as possible with a strong solvent. In some columns, the inlet frits can be replaced if plugged.
- Regenerate RP and NP columns according to the HPLC vendor's instructions whenever it is possible [46–58].
- You can get to know about any contamination just by looking for shift in resolution or retention time. It is possible to remove the contamination from the column just by flushing it with an organic solvent only.
- Strong bases and acids should be used in smaller amounts if you want to adjust the mobile phase's pH.
- Each HPLC columns are made to operate at pressure up to 6,000 psi = 40 Mpa = 400 atm). On the other hand, there is a gradual increase in back pressure columns with age increase. If the results show high pressure from use of the column at normal flow rates, this usually indicates that some contaminants have become

deposited on the frit of packing material. Reversing and flushing the column with the appropriate solvent will help plug the frit. But, make sure that the column and the detector are disconnected. If, for instance, the back pressure of the HPLC system increases while being used, the guard column needs replacement. If the guard column is not being used, the column's inlet frit will be replaced. Even after this, if the issue persists, there are chances that the column has been plugged with a contamination that needs a solvent to dissolve it. Monitoring of UV absorbance of the detector during this procedure may permit observation of contaminants being flushed.

### 8.4.6 Mobile phase preparation and connection to HPLC instrument

Put in your mind the following points about mobile preparations:

- Prepare the mobile phase as is given in the current method of analysis and according to the drug manufacturer's specifications or a specified pharmacopeia.
- Weight all solids separately.
- The composition of mobile phase is generally given in the manufacturer's specifications in two different ways, composition in volume percent or dilution of a given quantity of component A to the mark with component B.
- Use HPLC-grade solvents in the required composition and filter through the specified filter by vacuum pump as outlined in Chapter 3.
- Place the mobile phase in its reservoir and degas by keeping the bottle in the ultrasonic bath for about 5–10 min (keep the cap of the bottle loose during sonification) as outlined in Chapter 3.
- Transfer the mobile phase reservoir to its place and connect it to the HPLC instrument by dipping the reservoir filter in the mobile phase (reservoir filter is perforated metallic components having very fine pores through which mobile phase is socked under influence of pressure created by the pump). Remove the air bubbles (if any) from inlet connecting tube.

### 8.4.7 Set up parameters for HPLC instruments

Before setting up parameters for HPLC, be sure that all fittings are good as mentioned previously in Chapter 6. Then, turn ON the main power supply for all units of HPLC instrument (pump, detector and integrator or computer) as mentioned in instrument manual (follow the instrument manufacturer's recommendation). Setting up parameters for HPLC instruments and using it as analytical tool in drug QC laboratories must be done according to drug manufacturer's specifications or specified pharmacopeia. Select the specified wave length in the detector by wave length dial,

select and introduce the specified flow rate mobile phase using pump flow meter, always adjust the flow rate in symmetrical order (flow rate should be 0.0 mL/min before the pump on). Setting up parameters for HPLC instruments may be slightly modified as a function of the column and apparatus in use.

In the following are example parameters for HPLC instruments:

- Column: LiChrospher RP select B
- Mobile phase: A (methanol:$KH_2PO_4$ buffer (30:70) : B (methanol:$KH_2PO_4$ buffer (70:30); column temperature: ambient
- Flow rate: 1.0 mL/min; detection: UV at 272 nm; injection volume: 10 µL
- Gradient program:

| Time | %A | %B |
|---|---|---|
| 0–6 min | 85 | 15 |
| 6–13 linear to | 10 | 90 |
| 13–30 | 10 | 90 |

### 8.4.8 HPLC column equilibration

Before using any HPLC column for analysis, it must be equilibrated (equilibration of an HPLC columns is necessary before using it for analysis). For this, a stable baseline needs to be achieved. This can be done by purging at a minimum of 10 times the column volume of the mobile phase recommended until it is achieved. In case of the presence of additives (for instance, ion-paring reagents, at 9 to 10 mmol/L), 10 to 30 column volumes are needed to achieve complete equilibration.

For the calculation of required equilibration time for any HPLC column, we need to know the column void volume ($V_m$). With the help of Eq.(8.1), this void volume can easily be calculated.

$$\text{Void volume, } V_m = F \times t_0 \tag{8.1}$$

For that purpose, inject an unretained solute (such as uracil) into your HPLC system to obtain $t_0$, the column dead time (minutes) which is equal to.1.05 min as shown in Fig.8.2. Then, multiply this by the flow rate (1 mL/min) to get the void volume ($V_m$) = 1.05 min × 1 mL/min = 1.05 mL. Hence, the equilibration time is the multiplication of dead column time with the number of column volumes required. Divide this by the flow rate and you get equilibration time (1 mL/min in this case).

So, the required equilibration time for this HPLC column = $V_m$ (1.05 mL) × 10 column volumes/F (1 mL/min) = 10.0 5 min.

As we previously know, for stationary phases that are chemically bonded with organic solvent and water mixture, accurately determining $t_0$ is now becoming

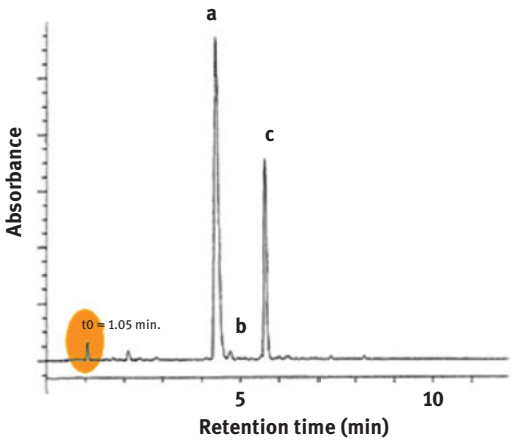



**Fig.8.2:** Chromatogram showing method for determination $t_0$ ($t_0$ = the time for unretained component of analyzed sample). Column length (L) = 10 cm, flow rate = 1 mL/min. Reproduced with permission from Mourne Training Service Ltd. (MTS) [66].

complex. In such cases, it can be estimated by regarding the theoretical equation according to Engelhardt [65]):

$$t0 = LEr^2 3.14/F \tag{8.2}$$

where L is the column length (cm), as shown in Fig.8.3, E is the total porosity for packing materials of the column and it is equal to 0.75 for RP-HPLC; r is the column radius (cm) = i.d. of column (cm)/2, as shown in Fig.8.3. F is the flow rate of mobile phase (mL/min)

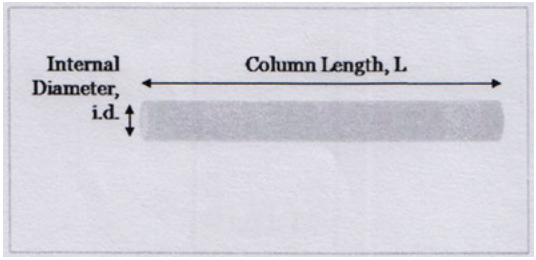



**Fig.8.3:** Illustrated diagram shows the measuring column length and column internal diameter.

On the other hand, recently, Mourne Training Service Ltd. (MTS) [66] paid successful efforts for making these calculation very easy by innovating a software we can use called MTS calculator for HPLC.

In summary, we can say that:

Before starting up the analysis using HPLC, the column must be properly equilibrated (a column not properly equilibrated may cause problems like bad performance, changing separation and so on.

- Equilibrate the column when you instill it and when you use it after it has been stored.
- For equilibrating your column, set the monitoring wavelength, temperature and set the pump at 0.0 mL/min, then turn the pump ON. Gradually increase the flow rate by increments of 0.1 mL/min till the desired flow rate is achieved can be seen in the specifications of the manufacturer.
- The column should be purged with the mobile phase that you want. Keep going until the detector base is stable and the pressure of the column stabilizes.
- A stable baseline should be obtained prior to beginning using HPLC in analysis, it takes 30 min for RP column and 30–45 min for silica column.
- After having established a stable baseline, verify that a blank injection with mobile phase medium does not gives rise any peaks on the resulting chromatogram.
- Begin injections of the standard solution until two successive injections have $t_R$ values which agree within 2% relative.

If the results are unsatisfactory, continue the equilibration process. If a stable baseline cannot be produced, determined the cause of the problem and correct it before proceeding with the analysis.

Notes: Column conditioning under *Use of normal and reversed phase condition.*

- HPLC column can be conditioned before its equilibration by pre-flush at low flow (0.5 mL/min). The pre-flush volume should be approximately 50 mL for 25 cm column and 30 mL for 15 cm column. In RP column (methanol/water) or acetonitrile/water (the water content should not be lower than 50%, preferred 70%) are preferred flushing solvent and preferably contains no buffer or other additives. In the last part of the pre-flush period the column can be flushed at normal flow rate for another 20 mL.

In NP column, flushing with dried dichloromethane or isoproponol is preferred.

Column conditioning under *use of polar bonded condition.*

The fact is that these columns are easy to use for both NP and RP conditions; before conditioning, always first check if the solvent in the column is miscible with the flushing solvent or eluent (see Chapter 3 for solvent miscibility). If the solvent is not immiscible, an intermediate flush with an appropriate solvent is necessary.

### 8.4.9 Checking for HPLC system qualifications and calibration

HPLC system qualification and calibration are two of the most important issues in the standards set by current good manufacturing practice that can be seen in the Code Federal Registration-FDA, 21CFR part 210 and part 211 [67–68], and USP [69]. To ensure the reliability of resulting data from an HPLC instrument, checking for HPLC system qualification must be first followed by checking for HPLC system calibration [70–78].

#### 8.4.9.1 Checking for HPLC system qualifications

HPLC qualification is a proper and formal process that helps in gathering documented evidences about an instrument and on how it should be used. According to Reuter [72], the process is made up of four parts: (1) design qualification (DQ), (2) installation qualification (IQ), (3) operational qualification (OQ) and (4) performance qualification (PQ) as mentioned by Reuter [72]. In the following, we will discuss these items. In Section 8.4.9.6, we will discuss checking HPLC system calibration.

#### 8.4.9.2 Design qualification (DQ)

DQ is a description of the requirements of the users and it defines how the operational and functional specifications of the instruments should be, which are being used for OQ (OQ testing, 8.4.9.4). In Chapter 6, we have discussed this subject in details.

#### 8.4.9.3 Installation qualification (IQ)

IQ shows that the instrument under consideration came in good order just as the manufacturer specified and designed it and is also installed properly in the location of the user. All the steps recommended for IQ have already been listed by Reuter [72]. In the IQ process, another step should be taken to analyze a test sample. This will show whether the installation process is correct or not. This should also include fluidis, data collections and electrical collections. Once IQ is a success, the system is kept in a "qualified state" or a state of service. This is done by conducting a program of periodic calibration. Additionally, the specific applications performance also gets verified during the system suitability testing (SST) (Section 8.4.11).

#### 8.4.9.4 Operational qualification (OQ)

This process shows that the performance of the instrument and the functions it has are in accordance with what the manufacturer specified. To be more specific, the functions should be in well in limits of the functions put forth in the DQ of the user. Mostly, this test is carried out in the laboratories by a service specialist who is appointed by the manufacturer. The OQ protocol is given for a specific model of the equipment by the manufacturer.

#### 8.4.9.5 Performance qualification (PQ)

The process in which it is shown that an instrument performs the application it was intended for consistently with some predefined acceptance criteria. In reality, the SST and PQ are synonymous when carried out with specific columns, test compounds and mobile phases. This is performed in the initial stages of either the system qualification stage or once the system has been relocated. On the other hand, SST is done daily. This is done before initial usage or before conducting regulatory analysis. It often combines with IQ/OQ protocol in the initial installation process. Section 8.4.11 further discusses the SST.

#### 8.4.9.6 HPLC system calibration

In most drug QC laboratories, periodically checking the operational qualification of the HPLC system is known as system calibration. This is done after every 6 to 12 months. This is combined with an annual preventative measure (PM). The HPLC system calibration is done right after the PM process is done. Once completed, the calibration sticker is given to the instrument and pasted on it. This shows what the status of calibration is and how readiness for the GMP works. The strategies and principles which are the base for the HPLC calibration, the calibration procedures and calibration criteria were published [28, 67–70, 78]. According to these publications, there are various calibration parameters for HPLC which can be summarized as the following:

- HPLC detector (UV-Vis) wave length accuracy
- HPLC injector (autosampler) accuracy and precision
- Mobile phase flow rate accuracy and precision
- Column oven temperature accuracy
- Injector carryover or autosampler carryover
- HPLC detector linearity (linearity response of the HPLC UV-Vis detector)
- HPLC injector (autosampler)linearity

In the following, we will discuss in details each HPLC calibration parameter mentioned above.

##### 8.4.9.6.1 Wave length accuracy testing of UV-Vis detector in HPLC

It is well known that the HPLC-UV/Vis variable wavelength detector has become one of the most popular analytical tool for analysis of DS and DP in drug QC laboratories. In these instruments, wave length selection is dependent on many different things like the transparency of the mobile phase, the compound's band position absorption and the sensitivity requirements. In the relation to the HPLC-UV/Vis detector, one of the most important parameters is the accuracy of the wavelength. This can have extremely serious effects on the analytical results, response of the detector and the inter lab reproducibility. After the repairing or services of

the UV/VIS detector, the accuracy of the wavelength can change or be affected adversely. Moving, bumping, physical shocks, extreme temperature changes, change in any optical component, lamp, flow cell or even because of natural wear and tear or because of contamination. All the applicable detectors like DAD, UV, UV/Vis, PDA and Vis should have the wavelength accuracy checked on a regular basis according to the "good laboratory practices" (GLP). Even though it depends on the particular guidelines or regulations being applied, mostly an accuracy of +/−2 is acceptable.

Following is a representation of how the accuracy of the wavelength detector of the HPLC UV*/*Vis module needs to be measured.

***First*:** Built-in test method: You can find a built-in accuracy check in most of the detectors. This is done by the manufacturers and is good for checking the wavelength accuracy. This helps to save time by automatically giving accurate and quick measurements of the accuracy of the wavelength of the detector. Most of the instruments are made of holmium oxide as an in-built filter. These are chemically treated to ensure repeated wavelength spectra that helps in determining the detector's accuracy (and adjust it to given specification in most cases). If the instrument you have has any sort of built-in filter for testing, it is important to check the instructions provided by the manufacturer.

***Second*:** The best way is to use a solution of caffeine which is high in purity and mix it in the grade water of the HPLC. The reason for this is that it has two important lambda maximum (maximum absorbance) that can be used for checking the accuracy of the wavelength in the UV region, 205 nm and 273 nm. Measure maximum absorbance of caffeine solution (1 µg/mL water). For people with a scanning PDA or DAD or UV/Vis detector, it is possible to scan all the wavelengths between 205nm and 273 nm.

Acceptance criteria: 205 ± 2 nm and 273 ± 2 nm

Complete documentation of the wavelength checks needs to be described properly for their application and specificity; purpose and detailed procedures need to be reviewed.

#### 8.4.9.6.2 HPLC injector (autosampler) accuracy

Determine gravimetrically the average volume of water per injection withdrawn from a tared vial after six 50-µL injections.

**Procedure:**

- For the HPLC under test, both the inlet of the detector and the outlet of the pump should be connected with the union.
- Use of a mixture of methanol and water (30:70 /v) in the mobile phase.
- A 1 min run time and a 0.5 mL/min flow rate needs to be set.
- At 25 ± 2 °C, set the column temperature.

- A standard, normal HPLC vial needs to be only filled 2/3 of the total quantity with Milli-Q Water. It should be properly sealed with a cap.
- Once done, the weight of the vial should be taken and denoted with W1. The weight should be in grams.
- Six injections with a volume of 50 µL should be injected from the vial when it is placed inside the chromatographic system.
- After this, the vial needs to be weighed again and the weight needs to be calculated in grams and denoted as W2.
- The formula to calculate the mean injected volume/injections is as follows:

$$\text{Mean injected volume } (\mu L) = (W1 - W2)/6.$$

Acceptance criteria: the volume should fall between a range of 50.0 ± 2.0 µL.

#### 8.4.9.6.3 HPLC injector (autosampler) precision (sampling precision)

**Procedure:**

Note: Before using HPLC, be sure that the column has been conditioned and equilibrated as outlined in Section 8.4.8 and the mobile phase has been prepared according to the procedure mentioned in Section 8.4.6.

- Accurately weigh about 60 mg of caffeine and transfer it into a flask with a volume of 100 mL.
- It needs to be diluted and dissolved to the mobile phase volume, methanol: water (30:70 v/v; which is the reference standard solution).
- From this solution, transfer 10 mL into a volumetric flask of 100 mL and dilute it to the volume with mobile phase (this is working standard solution).
- Inject the mobile phase as a blank injection, followed by working standard solution in 10 replicates into the HPLC under test and operate under below conditions.
- It is important to see the retention time and the areas and to compute the RSD in percentages for the peak areas and total retention time. This should be done for 10 replica injections.

HPLC conditions:

- Column: C18,150 mm × 4.6 mm, 5 µm
- Detection: UV at 272 nm
- Mobile phase flow rate: 1.0 mL/min
- Column temperature: 25 °C ± 2 °C
- Run time: 15 min
- Injection volume: 20 µL

Acceptance criteria: Both the peak area and retention time should have a %RSD of <0.5%.

#### 8.4.9.6.4 Mobile phase flow rate accuracy and precision

- While the HPLC system is running on the test of injector precision (Section 8.4.9.5.3), in a 10 mL volumetric flask, collect the amount of mobile phase at the detector outlet and start a stopwatch simultaneously to calculate the time.
- Stop the stopwatch when you can see that the level of the collected mobile phase has reached the flasks 10 mL mark.
- See and record how much time passes when the collection of 10 mL mobile phase is completed.
- Similarly check for the mobile phase flow rates of 0.5 mL/min and 2.0 mL/min.
- Repeat the test 10 times and %RSD is calculated to determine mobile phase flow precision.

Acceptance criteria:

- Mobile phase flow rate accuracy. The total time it takes to gather 10 mL of the mobile phase should not exceed ±2.0% of the original value, that is, 0.5 mL/min = 20.0 min, 1 mL/min = 10.0 min and 2 mL/min = 5 min.
- Mobile phase flow rate precision should have %RSD of < 0.5%.

#### 8.4.9.6.5 Column oven temperature accuracy

Note: You can refer to Section 8.4.9.5.3 which states the injector precision test to see the chromatographic conditions, mobile phase and accuracy of oven temperature.

- At 30 °C and 60 °C, the accuracy of the columns' oven temperature can be calculated with the help of a digital, calibrated thermometer. The thermometer needs to be placed inside the column. Next, set the oven temperature at exactly 30 °C. Until the temperature is stabilized, wait.
- When the temperature stabilizes, check what the temperature is. Repeat the same process but set the temperature at 60 °C.

Acceptance criteria:

- The oven temperature should be in the range of ±2 °C of the set temperature.

#### 8.4.9.6.6 Injector or autosampler carryover

During HPLC analysis, when the sample peak shows in a blank chromatogram, a sample carryover comes into play. This comes from injecting blank after the sample. From a quantitative point of view, this carryover is a hindrance only if the peak of the carryover is big enough that the results of the HPLC method are affected. For instance, a 1% carryover on content uniformity assay or potency with a 100% concentration of the analyte of the label claim for a pharmaceutical product. According to the manufacturer, the tolerance value should fall ±2% of this value. Hence, all the samples should fall in a range of 98–102% of the label claim. If the true content of a table is 101.5%, then it will be approved as it falls in the specified limit. Now, if

there was even a 1% carryover, the value would go up to 102.5%. Eventually, the sample is going to fail. Such a flawed result is not only going to cost a lot of money to correct, a lot of time is also going to go waste in tracking down what the problem actually is. This is actually because of the analysis and not the manufacturer's production process.

So, the question that arises is what is the acceptable limit of carryover? There is no definite answer and it depends on the situation. Desirably, 0% carryover is going to be the best, but practically, it is not possible. Apart from all this, a carryover of anything more than 0.05–0.1% is not acceptable.

The biggest reason for carryover occurring is that there is residue of the sample that remains in the autosampler of the injector. By adjusting the fittings and tubing, choosing the best wash solvent or adding more wash cycles, the majority of the problems related to carryover can be finished. In case none of these work, the injection sequence needs to be altered. Another thing you can do is to perform a number of injections of every sample to get acceptable results.

Carryover is determined by measuring the carryover peak area of an 80 µL injection of a mobile phase blank sample immediately after an 80 µL injection of the ethyl paraben standard.

For estimation of the injector or autosampler carryover for the HPLC instrument, under test use the chromatographic conditions as mentioned in system precision test (Section 8.4.9.5.3. Into this HPLC, inject 80 µL of mobile phase (water: methanol,70:30) as a blank after injecting 80 µL of working standard solution of caffeine. Note down the area on blank chromatogram (carryover peak area) corresponding to the $t_R$ of caffeine. Calculate the %carryover according to Eq.(8.3) as follows:

$$\%\,\text{Carryover} = \text{A x } 100/B \tag{8.3}$$

where

Ais the area or height of the respective caffeine peak in the chromatogram of the blank, B is the mean area or height of the system precision injection.

Acceptance criteria:
Height carryover is ≤0.2
Area carryover is <0.1%

#### 8.4.9.6.7 Detector linearity (linearity response of the HPLC UV-Vis detector)

**Standard preparation:**

– Accurately and exactly weigh about 60 mg of caffeine and transfer it into a 100 mL flask. Dilute and dissolve till the volume of the mobile phase is reached. This is known as the standard preparation solution.

- Detector linearity working standard solution at concentrations 0.012, 0.03, 0.048 and 0.06 mg/ml were prepared by transferring a defined volume from the standard preparation solution into 100 mL of mobile phase.
- Note: Before using HPLC, be sure that the column has been conditioned and equilibrated as outlined in Section 8.4.9 and the mobile phase has been prepared according to the procedure mentioned in Section 8.4.6.
- In the HPLC under test, inject20 µL of mobile phase in three replicates as a blank, followed by 20 µL in three replicates from detector linearity working solution. The caffeine's average peak areas need to be recorded and plotted against the concentration.
- The relation between the peak areas and $V_s$ needs to be plotted.
- Acceptance criteria: $R_2$ or regression coefficient should not be lower than 0.999 and the plot needs to be linear.

#### 8.4.9.6.8 Injector linearity

- Accurately weigh about 60 mg of caffeine reference standard and transfer it into a 100 mL flask.
- Dilute and dissolve to the mobile phase's volume.
- 10 mL of the solution should be transferred into a 100-mL volumetric flask and dilute to the volume with mobile phase; this is the working standard caffeine solution.

**Procedure:**
Note: Before using HPLC, be sure that the column has been conditioned and equilibrated as outlined in Section 8.4.8 and the mobile phase has been prepared according to the procedure mentioned in Section 8.4.6.
- Into the HPLC under test, inject 5 µL of the mobile phase in three replicates as a blank injection followed by 5 µL, 10 µL, 20 µL, 50 µL and 80 µL in three replicates of the working standard caffeine solution.
- The average peak areas of the caffeine should be recorded and plotted against the corresponding concentration.

**HPLC conditions**
- Column: C18, 150 mm × 4.6 mm, 5 µm
- Run time: 15 min
- Column temperature: 25 ± 2 °C
- Mobile phase flow rate: 1.0 mL/min
- Detection: UV at 272 nm

Acceptance criteria: $R_2$ or regression coefficient should not be lower than 999 ($R^2 \geq 0.999$) and the graph should be linear.

Note: Additional test for measuring of HPLC instrument band spreading (instrument bandwidth (IBW)) presents very important parameter for evaluation of its qualification. Details about IBW measuring procedure and the acceptance criteria are found in Chapter 6 Section 6.7.1.

#### 8.4.9.7 Documentation

Once the qualification of the equipment is done, proper documentation for the following needs to be made available: IQ document, PQ test procedure and the results, OQ testing procedure, DQ document, OQ test report and the results of these tests.

**Calibration results record:**

- Record calibration results in HPLC calibration work sheet as shown in Tab.8.4.
- Stick calibration label which shall include date of calibration, due date and sign.
- Frequency of calibration every 6 months and/or after instrument service.
- Nonconformity of calibration results: If calibration results do not conform, refer maintenance engineer and stick Out-of-Service label, do not carry out any test with the equipment till repair and recalibration is done and the results of calibration conform.

### 8.4.10 Preparation of reference standard solution

The use of HPLC as an analytical tool for qualitative and quantitative analysis in the pharmaceutical substances (DS) and pharmaceutical products (DP) in the drug control laboratories relies on the reference standards. To ensure that only accurate data is collected, it is critical to check the purity and quality of the reference standards being used. The materials used as a reference standard need to be highly characterized and well purified for accurate results. According to legal definition of the materials used as reference standards in the USP/NF, all reference standards are "pure" materials until a purity level apart from 100% is assigned. No other characterizations are required before you can use it.

A non-compendial primary reference standard maybe certified for use in drug QC laboratories if USP reference standards are not available. These need to be well characterized and should be highly pure. This will ensure that they are pure and the quality of material used is high. Methods Purity correction factor must incorporate into the calculations if the analytical method is using non-compendial reference standards. It is imperative to document the reference materials that are being used in method validation. This is necessary for each and every reference material

**Tab.8.4:** HPLC calibration worksheet* (Date: . . .. Instrument lab no. . . .. Batch/serial no. Instrument location: . . . . . Used chemicals and instruments).

| Test | Acceptance criteria | Results | Comment (pass/failed) |
|---|---|---|---|
| Mobile phase flow accuracy and precision | Flow accuracy (%) <5% | ——— | ———— |
| | Flow precision (%RSD) <5% | ——— | ———— |
| Temperature accuracy of column oven | Accuracy at left [C] difference <2 nm | ——— | ———— |
| | Accuracy at left [C] difference <2 nm | ——— | ———— |
| Injector precision. Carryover | Area precision (%RSD) <1% | ——— | ————— |
| | Height precision (%RSD) <2% | ——— | —————————— |
| | Area carryover <0.2% | ——— | —————————— |
| | Height carryover <0.4% | —— | |
| Detector response linearity | $r_2$ not less than 0.999 | —— | ———— |

* This document is valid for 6 months.

being used and it should be documented well when you make the report of validation. A working reference standard is any material that had its impurity established and is characterized against a compendial reference standard. You can use these when it is better in terms of cost to certify the lot in-house rather than go ahead and buy USP reference materials to undergo routinely analysis. As outlined in drug specifications or desired pharmacopeia, prepare the working reference standard solutions. Use certified reference standards (primary or secondary reference standard). The recommended weights of the standard or aliquot size and dilution volumes may be modified, provided that its concentrations are the same as those stated in the drug specifications (look for validation method range and you must fulfill these requirements according to manufacturer's specification).

### 8.4.11 Checking for suitability of HPLC system (system suitability test) (SST)

In today's drug QC laboratories, analytical testing for the most part is conducted by HPLC. One of the most reason drug analysis is conducted in the laboratories is to ensure that all the data being produced by the HPLC are accurate and reliable. If the HPLC system is used continuously, its performance will change, or during their transfer from side of industry after validation to the analyst in drug QC laboratories (this problem might arise because of the difference in the mobile phase variability, temperature fluctuations, column variance, chromatographic systems and many more), which can affect reliability of the analytical results (the changes of HPLC system performance raise the uncertainty factor while taking the chromatographic measurements). This might result for the systematic error that can affect the consistency of

the analytical results during the routine analysis of DS and DP. For this reasons different parameters according to FDA, USP and ICH rules [11–15] which are associated with the running of the entire HPLC system, such as injection reproducibility of the HPLC system (injection precision), column efficiency, system detection limit, asymmetry factor for the resulting chromatographic peak corresponding to the tested analyte and the resolution power of the HPLC system to be sure that it is possible to separate the closely eluting compounds and they can end up being resolved from one another, capacity factor and the retention factor or (and selectivity or separation factor

These activities are called System Suitability Test SSTs [11–15]. After these parameters are checked, the HPLC being tested is declared suitable only if the responses occur within a permitted limit [11–15].

According to FDA, USP and ICH rules [11–15], the SST is a very important and essential part in the HPLC method development. This is usually carried out to check the effectiveness and suitability of the complete chromatographic system. This is done during and before the analysis is carried out. In addition, the SST is also a fundamental part of the analytical procedure. It is widely used in the method of internal QC. For this reason, the criterion needs to be established before, and in the method validation part, during the pharmaceutical development cycle in the pharmaceutical industry. This helps in finding out the reliability and integrity of the entire HPLC system.

The objective of this part is to stress on the significance of the SST for using the HPLC as analytical tool in drug QC laboratories. Additionally, the calculations and measurements have already been discussed in relation to the different SST parameters. These are done in a very simple way. The respective formulae for calculating these parameters are in our considerations.

According the ICH, USP and FDA guidelines [11–15], the parameters that are related to the whole HPLC system are mentioned below:

- Injection reproducibility of the HPLC system (injection precision).
- Theoretical plate value (N) (column efficiency) measures the efficiency of the chromatographic column and must be taken as requirements for system suitability. Column efficiency is actually a measurement of peak sharpness. This is integral for detecting trace components (drug degradation products).
- Asymmetry factor for chromatographic peak ($A_s$)
- Tailing factor for the chromatographic peak ($T_f$). To measure the peak symmetry, the $T_f$ is used. The $T_f$ is in harmony for perfectly balanced peaks and the value increases just as the tailing becomes more obvious. But, is some situations, values lesser than the unity level can be seen. As the peak asymmetry keeps on increasing, integration and then the precision becomes less and less reliable.

- Capacity factor (*k'*) and retention factor
- Selectivity(α) or separation factor

To make sure that the closely eluting compounds in the HPLC system can end up being resolved from one another (the chromatographic system's revolving power), $R_s$, a function of N, is specified to make sure that the eluting components are able to be resolved from one another. To establish the resolving power of the HPLC system and to make sure that all the internal standards (ISs) are resolved from the API in the products of the pharmaceutical products.

To conduct the tests, data is collected from five injections of either the standard or any other solutions that are specifically listed in each monograph or drug manufacturer specifications into the HPLC under test. After HPLC has become equilibrated according to the previously mentioned procedures in Section 8.4.8, check for SST according to the following procedures in Sections 8.4.11.

The SST results must meet the manufacturer's specification limits (e.g., as shown in Tab.8.5) or international limits prior to injection of sample as shown in Tab.8.6.

The analyst should immediately put a stop to the sequence of the sample analysis if the preliminary SST ends up in failure. Next, you are supposed to see what the problem is and make all the important adjustments to solve those problems. Once these steps are done, you can perform the test again. The final analysis of the samples should be done only after all the conditions and limitations of SST test are achieved, not on the failed criteria. If even one of the SST injections ends up failing, all the data samples even if they have passed the SST are deemed invalid and should be repeated to get the correct results.

An important thing for the analyst to remember is that by using only a single component in the SST solution to check the SST is not at all practical. The reason is that the separation capability of the system cannot be demonstrated with it. In this case, using resolution test mixtures or system suitability samples (SSS) that contain both the expected impurities and the major components is necessary (Fig.8.4A, Fig.8.4C and Fig.8.4D). In case of impurity testing, it is required to add either one of the many impurities in the SSS to show the system sensitivity and resolution. Fig.8.4 depicts the impurity methods of SST chromatogram.

SSS are analyzed both before and during the testing phase. This means that for the initial SST testing, five replicate injections of SSS are required. After the initial injections, one SSS injection for every 10 dissolution or assay samples is needed.

In summary, we can say that:

- SSTs are necessary to check the performance of the HPLC chromatographic system prior to acceptance for sample analysis.
- A system and especially the column meet certain criteria which are calculated from a standard or sample chromatogram.

**Tab.8.5:** System suitability parameters for one of the validated method for analysis of pharmaceutical product containing two PAIs.

| | PAI (1) | PAI (2) |
|---|---|---|
| Retention time (min) | 3.37 | 3.21 |
| Capacity factor (*k'*) | 2.32 | 1.52 |
| Efficiency (N) | 7,997 | 6,369 |
| Peak symmetry | 1.18 | 1.39 |
| Resolution | 8.9 | |

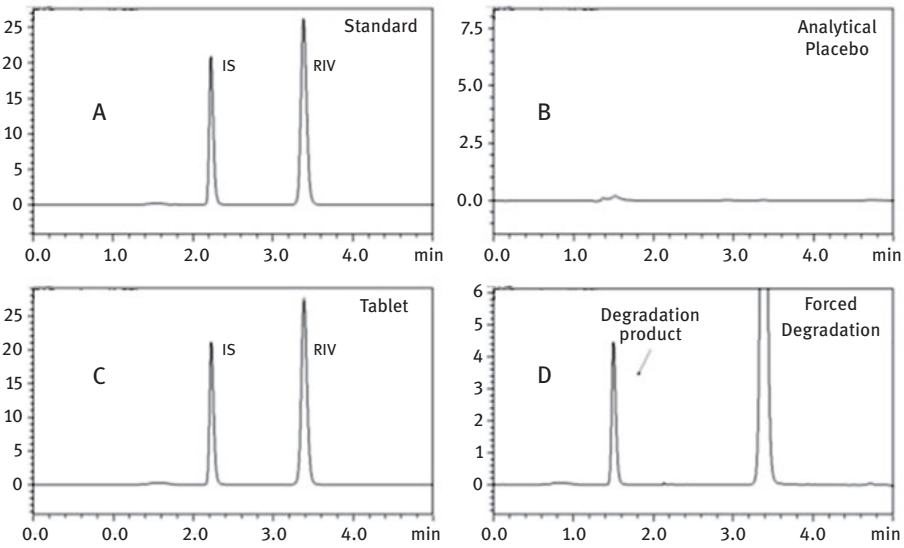



**Fig.8.4:** HPLC chromatogram illustrating injection of different SST composition of SST solution.
(A) API standard + IS
(B) Blank
(C) API sample + IS
(D) API standard + standard degradation product

- SST is an integral part of HPLC methods of analysis.
- It is used for verifying whether the reproducibility and resolution of chromatographic system is adequate to conduct the analysis or not.
- It relies on the idea that the electronics, equipment, analytical samples and operations that need to be studied make a very important part of the system. It can be evaluated just like that.

It is a true fact that the results of SST will depend on sample preparation, mobile phase composition, column condition, column composition and the HPLC hardware. If the SST yields unacceptable results, the above factors shall be examined, modified if necessary and the test repeated. If the SST yields acceptable results, you can start your analysis using this HPLC.

#### 8.4.11.1 SST acceptance limit (acceptance criteria)

A practical and useful approach is to fix the limit of SST and base it on the historical performance data of the particular method used. SST acceptance limits are predefined in most official analytical methods (USP, BP and EP). Different tests give different limits of SST. They are typically less severe for the trace and biological impurities.

Tab.8.6 summarizes the guidelines you need to follow to see the SST limits from the U.S. FDA's CDER [11].

Several studies have recommended using statistical analysis (like any factional designs of Plackett and Burman) on data that are generated from method robustness test results during method validation experiments in pharmaceutical industry [79–87]. All of this is in accordance with the guidelines set out by ICH. According to them, SST is an important step for method validation. Eventually, replicated experiments were executed in such worst conditions and SST limits were determined.

**Tab.8.6:** SST criteria in accordance to the FDA's guidelines (U.S. FDA's Center for Drug Evaluation and Research (CDER) [11].

| | |
|---|---|
| Repeatability(RSD) | ≤1.0% for five replicates |
| Resolution (R) | >2.0 in general |
| Tailing factor ($T_f$) | ≤2.0 |
| Capacity factor (k) | >2 |
| Plate count | >2,000 |

Practically, parameters for SST are generally calculated for HPLC system after the column has been conditioned and equilibrated with mobile phase as previously mentioned in Section 8.4.8; five injections of reference standard are injected and the respective chromatogram is recorded. From these data, you can calculate the following parameters (according the ICH, USP and FDA guidelines [11–15], the parameters that are related to the whole HPLC system and mentioned previously).

#### 8.4.11.2 Reproducibility injection of the HPLC system (injection precision)

The area responses for the standard peaks should have a 1% or lower RSD (unless it is specified otherwise in the individual monograph, data collected from five replicate injections of the particular analyte is used to estimate what the RSD is going to be, data from six replicate injection is used in case a RSD of greater than 2% is required).

#### 8.4.11.3 Column efficiency and plate number (theoretical plates number), height equivalent to a theoretical plate, plates per meter (N/m) and reduced plate height ($h$) = H/dp = L (μm)/N × dp (μm)

You can measure the column's efficiency with the help of the place number (N) of the theoretical plate numbers (this is the total theoretical plates that an HPLC column contains). The final packed column's quality is usually characterized by measurement of a column efficiency (N), or theoretical plate value. The chromatographic peak is understood to be a result of sample spreading. This sample spreading has a Gaussian distribution with sample concentrations in the stationary as well as the mobile phase. For this reason, the Gaussian model for a peak shape is used as a base to build the theoretical plate calculation. A column is known to be efficient if it can create sharp peaks along with separating a number of sample components in a very short time. Generally, chromatographic peaks on an HPLC chromatogram tend to be in the Gaussian shape. The shape further broadens over time. When this happens, the peak width at baseline (wb) tends to widen with a longer tR. Inside a column, the broadening of the band is essential to all the chromatographic processes [34, 39].

Theoretical plate number is a very important parameter which describes the column efficiency. In the following, we will describes this parameter and its roles for measurement of a column efficiency.

One index that is used to decide the effectiveness and performance of the column is the number of the theoretical plates, N. This can be calculated with the help of Eq.(8.4).

$$N = 16(tR/Wb) \tag{8.4}$$

Retention time is denoted by tR, and the peak width at baseline is denoted by Wb (Fig.8.5) shows how wb is measured. For that purpose, two tangent lines are drawn from the steepest points of inflection of the peak. Wb is the distance between those two points on which the two tangents cut off with the baseline. A point to note is that the peak area is estimated to be equal to half of (wb × h) [1, 6]. This method is used by the USP [15]. Because of this, as the peak overlap increases, you will get smaller N values. In case of peak distortion, they can present a big problem (this method is called tangent line method.

Another method is using peak width at ½ of the peak height ($W_{1/2h}$). Peak width (wb) at half peak height ($W_{1/2h}$) can be measured by the dropping a line from

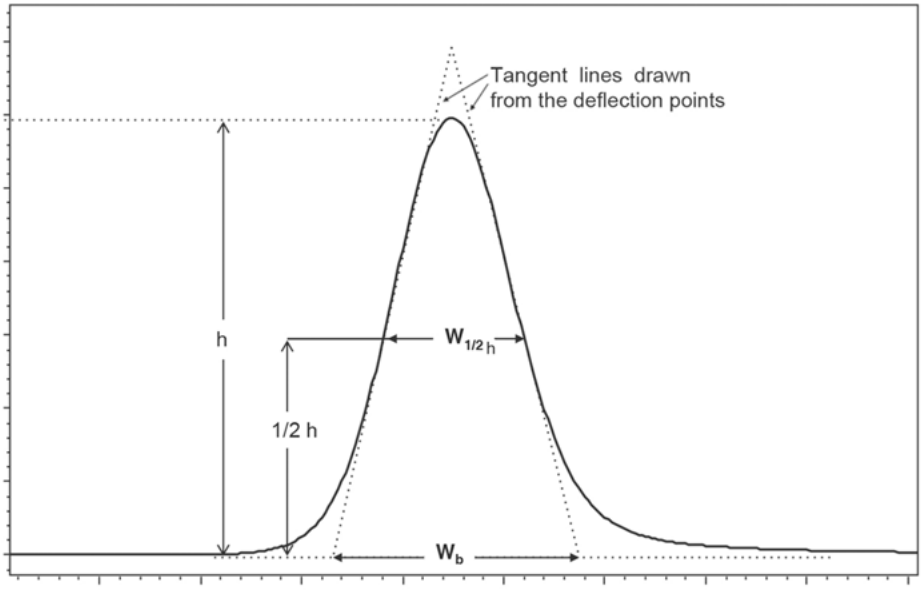



**Fig.8.5:** Diagram illustrating how peak width (wb) and peak width at half height (w1/2) are measured.

peak maximum perpendicular to the baseline followed by dividing this distance in half's, finally drawing a line parallel to the baseline halfway between the top and bottom of the peak as shown in Fig.8.5.

The most common method to calculate width is by hand. The reason is that it is the easiest method to do so and the simplest. This makes it the most popular method used by many entities like BP. Especially, for these cases, it is possible to calculate N through Eq.(8.5).

$$N = 5.54(tR/W1/2h) \tag{8.5}$$

The Japanese Pharmacopoeia issued 15th revision in April 2006 and changed the coefficient from 5.55 to 5.54.

When broader peaks are considered, as compared to the other methods for calculation, the half peak height method gives you larger N values.

If there is poor resolution between peaks on an HPLC chromatogram, the half-height method enables the determination of the number of theoretical plates per column (N).

For automatic determination of data systems, the most common method used is the half-height measurement.

Note: As the number of theoretical plates increases per column, the peak gets sharper.

To see how to calculate N by using Eq.(8.5), refer to Fig.8.6.

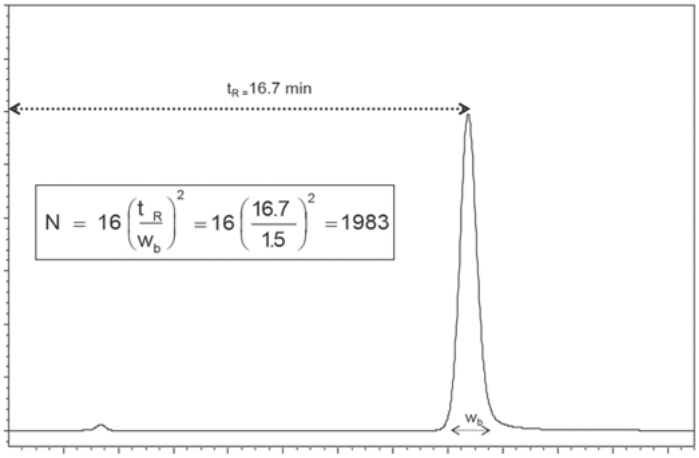



**Fig.8.6:** A chromatogram presenting a peak from a column with N = 1,983.

#### 8.4.11.3.1 Height equivalent to a theoretical plate or plate height

The height equivalent to a theoretical plate (HETP or H) is equal to the length of the column (L) divided by the plate number (N) [4] according to Eq.(8.6)

$$\text{HETP, } H = L/N \tag{8.6}$$

When it comes to the HPLC, the particular diameter of the (dp) stationary phase packing is the major controlling factor. For a well-packed column, H is roughly equal to 2.5 dp.

A 15 cm column that is packed with materials of 5 μm must have N = L/H = 150,000 (μm)/(2.5 × 5 (μm). This means that there should be almost 12,000 plates. Likewise, if we take the same column, that is, 15 cm long, but it is packed with 3 μm materials, it should have N = L/H = 150,000 (μm)/2.5 × 3 (μm), or about 20,000 plates. Thus, columns packed with smaller particles generally have more number of plates and are more efficient. For a typical well-packed HPLC column with 5 μm, H values are usually between 0.01 and 0.03 mm. HETP should be approximately 2–3 $d_p$ for 5 μm particles with a typical well-packed HPLC column, HETP (or H) values usually are in the range of 0.01–0.03 mm.

#### 8.4.11.3.2 Plates per meter (N/m)

Another parameter which defines the efficiency range of columns. If you want to calculate how many theoretical plates are there in a meter, refer Eq.(8.7).

$$\text{Number of theoretical plates per column} \times 100/\text{length of HPLC column(cm)} = \text{Number of theoretical plates perm} \tag{8.7}$$

#### 8.4.11.3.3 Reduced plate height

Reduced plate height ($h$), compares the efficiency of each column, regardless of length (L) and diameter of particle (dp). It can be calculated using Eq.(8.8)

$$(h) = \mathrm{H}/\mathrm{dp} = \mathrm{L}(\mu\,\mathrm{m})/\mathrm{N} \times \mathrm{dp}(\mu\,\mathrm{m}) \tag{8.8}$$

The lower the number (with 2.0 as optimal), the higher the efficiency (range 2–5).

Notice:

- The chromatograms with (small tR) early peaks are known as poor chromatograms. Because of the early peaks and broad (large) Wb, it results in smaller N values. On the other hand, excellent chromatograms are the ones in which the peaks are late-appearing (large tR) that remain quite narrow (small Wb); hence, they produce a larger value of N.
- The column "goodness" can be measured by the number of theoretical plates.
- Depending on the column length, the number of plates falls in the range of 100 to $10^6$.
- A better parameter to measure the efficiency is plate height of the HETP (H). This is measured as L/N. L denotes the column length.

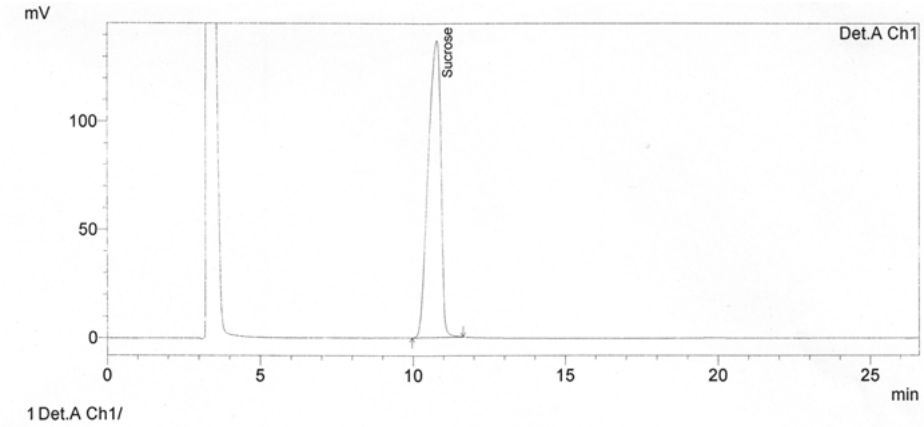
There are several parameters which influence the determination of column efficiency. These are:

- The flow rate and mobile phase composition.
- The solute used in the measurement.
- The process selected for calculations and measurements.
- The temperature

Hence, it is vital that all the efficiency values should have a statement of the situations in which the value of the plate count was attained, as shown in Fig.2.32 (Chapter 2).

Nowadays, computer data stations being found in each HPLC systems are capable of delineating the peaks which have been resolved and can estimate the corresponding plate number. This can be seen in Fig.8.7.

In Fig.8.7, there is a recording for the amount of N (3,157.07) as an indication for column efficiency along with analytical results about the analyte under analysis, such as sucrose, using Shimadzu's LC workstation software [58].




1 Det.A Ch1/

<Results>

Detector A

| ID# | Name | Ret. Time | Area | Height | Conc. | Units | Mark |
|---|---|---|---|---|---|---|---|
| 1 | Sucrose | 10.785 | 3980617 | 137254 | 339.880 | mg/L | |

<Column Performance>

Detector A

| ID# | Name | Theoretical Plates | Resolution | Tailing Factor |
|---|---|---|---|---|
| 1 | Sucrose | 3157.070 | 0.000 | 0.746 |

**Fig.8.7:** Illustrated diagram showing how the plate number was calculated automatically using computer data stations of a Shimadzu's HPLC [58].

### 8.4.11.4 Peak asymmetry factor

Often $A_s$ is used to represent peak asymmetry factors. Additionally, it is estimated from the chromatographic peak using the Eq.(8.9). As shown in Fig.8.8, by dropping a perpendicular at the peak apex and a horizontal line at 10% of the peak height, at the intersection, the distance to the tail of the peak along the horizontal line is b. The distance along the horizontal line to the front of the peak is a. For the symmetrical peak, the ratio will come out to be 1. For the fronting peak, it is lower than 1, and for the trailing peak, there will be a ratio greater than one. As a general rule, as the value increases, the symmetrical peak is less. If the value comes out to be higher than 2, it is not accepted. If $A_s > 1$: tailing, if $A_s < 1$: fronting

$$A_s = b/a \tag{8.9}$$

### 8.4.11.5 Tailing factor ($T_f$)

The tailing factor ($T_f$) is actually the USP [15] coefficient of the peak symmetry. $T_f$ is calculated using Eq.(8.10). In the equation, the total distance from the leading edge to the midpoint of the peak is known as a. It is perpendicular from the highest point

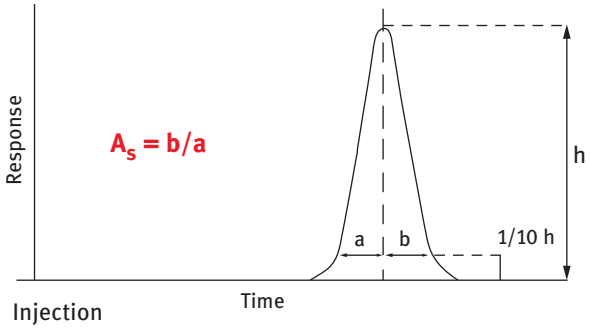



**Fig.8.8:** A diagram showing the calculation of the peak asymmetry factor ($A_s$) from peak width at 10% height (W0.1 h = W1/10 h).

of the peak and is calculated at 5% of the peak height. The distance from the midpoint to the trailing edge of the peak is known as b and is calculated at 5% of the total height of the peak. This is shown in the Fig.8.9.

$$T_f = (a + b)/2a \tag{8.10}$$

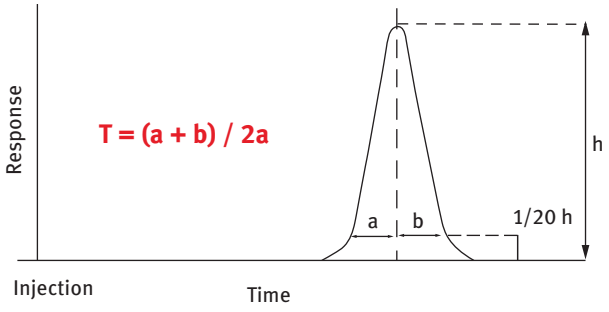



**Fig.8.9:** A diagram showing the calculation of tailing factor ($T_f$) from peak width at 5% height (W0.05 = W1/20 h) according to the USP.

Just as tailing becomes more prominent, the value and unity of the $T_f$ or perfectly symmetrical peaks increases. There are some cases in which the values lesser than unity can be seen. Data precision and integrations become less accurate and reliable just as the peak asymmetry gradually increases.

If the conditions are perfect, the chromatographic peaks will emerge with the Gaussian shape having perfect symmetry. But, this does not happen in reality. Generally, the peaks will be tailing or fronting.

For the majority of the HPLC peaks, the value of $T_f$ should fall somewhere between 0.9 and 1.4. If the value is 1.0, it is known to be a perfect symmetrical peak. Peak tailing occurs when a strong interaction or absorption of the analyte takes

place in the stationary phase. For example, in silica-based bonds, many of the amines or basic analytes show some to little peak tailing. The reason for this is the powerful interaction of the residual silanol groups. Peak fronting can occur because of isomerization or chemical reaction and the column overloading during the chromatographic process phases.

In the HPLC column technology today, low silanophilic activity takes place with the development of silica with high impurity. Majority of the HPLC columns nowadays are filled with these silica materials after derivitization. These then show a great reduction of the peak trailing in the base of the basic analytes (for details, see Chapter 2).

#### 8.4.11.6 Capacity factor (*k*') or retention factor

In HPLC columns, the resides time for a salute in the stationary phase (tR) when compared to the time the salute resides is in the mobile phase (tm) is called the retention factor (*k*'). It is also an IUPAC term and is used to refer the capacity factor in many of the references. In most analyses, analytes go ahead and elute with the retention factor between a range of 1–20. This allows them to have enough chance to interact with the stationary phase. This results in differential separation and migration. When the peak has k = 0, it is a component that is not retained by the phase. Additionally, it elutes with the solvent front. In case the retention factor is less than 1, it shows that it is a highly retained component. Analytes that elute with retention factor equal to 20 are not able to be detected due to excessive band bonding. In Fig.8.10, there is an example how can *k*' be calculated using Eq.(8.11)

$$k' = \mathrm{tR} - \mathrm{t0}/\mathrm{t0} \tag{8.11}$$

where $t_0$ is the retention time of non-retained component and is equal to 1, as shown in Fig.8.10.

As an example, we present here the results for calculation of retention factor (capacity factor; *k*') for compounds A, B and C, Fig.8.10 according to Eq.(8.11).

$$k'\mathrm{B} = (4.6 - 1)/1 = 3.6$$

$$k'\mathrm{C} = (6.2 - 1)/1 = 5.2$$

Note: A peak having retention factor equal to zero is known as an unretained component by the stationary phase. And elutes with the solvent front. Also, its tR = t0; in this case, t0 = 1

#### 8.4.11.7 Separation factor (selectivity)

The separation factor ($\alpha$) in HPLC separations is a calculation of the differential retention which occurs between two analytes. In other words, it is also known as the ratio

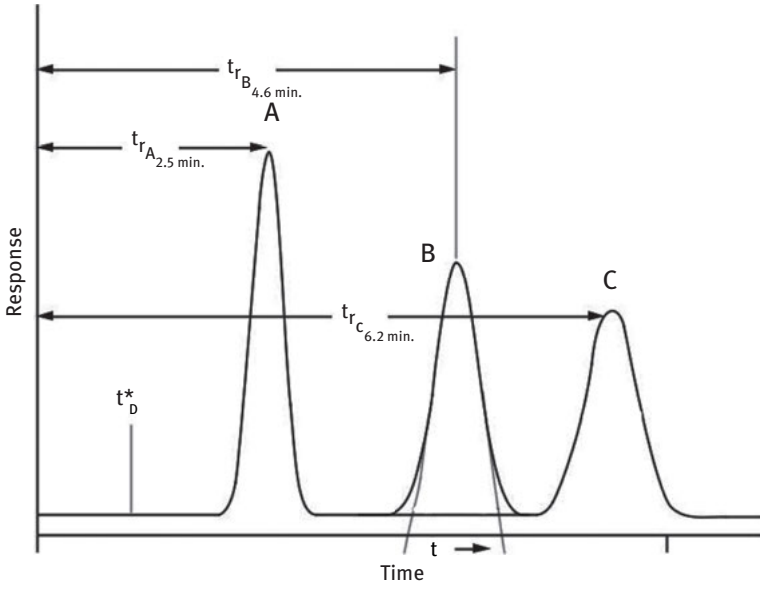



**Fig.8.10:** An HPLC chromatogram of three components A, B and C is used for the calculation of capacity factor(retention factor; *k*') and selectivity (α) according to Eq.(8. 11) and Eq.(8.12).

of the capacity factors of two peaks *k*'2 /*k*'1, as shown in Eq.(8.12). Separation between two peaks of two components is only possible if α > 1.0. A number of factors affect *k*'. These include the stationary phases' nature (i.e., C8, C18, cyano, phenyl); the properties and composition of the mobile phase solutes can influence the process of selectivity. The more experienced and skilled they are, chromatographers can easily make use of the selectivity effects in the method development phase. By doing this, they can help to increase the separation process of the sample's key elements.

Separation factor (selectivity; α) can be calculated according Eq.(8.12)

$$(\alpha) = k'2/k'1 \tag{8.12}$$

As an example, we present here results for calculation selectivity (α) from the HPLC chromatogram of compounds A, B and C (shown in Fig.8.10; according to Eq.(8.11))

- Selectivity (α) (C–B)

$$\alpha = k\text{'C}k\text{'B} = 5.2/3.6 = 1.44$$

- Selectivity (B–A)

$$\alpha = k\text{'B}/k\text{'A} = 3.6/1.5 = 2.4$$
$$\alpha = 2.4$$

See the difficulty of the separation depending on the value of selectivity.
In case the selectivity is:
Here: a > 2: Easy to separate.
Possible separation*: 1.5–2
Difficult to separate: 1.2–1.5
1.2<: Very difficult to separate**
* Method adjustment was not required
** You may require selectivity optimization

#### 8.4.11.8 Resolution

In addition to selectivity (α), another indicator used to see the separation of two peaks is the resolution ($R_s$). It is also a measurement of the quality of separation. To check the resolution of two peaks, measuring the retention time of the peaks studied is important. These are $t_{R2}$ and $t_{R1.}$ Also, the width of the two peaks at the baseline between the tangents drawn on the peaks sides is measured ($wb_1$ and $wb_2$). Normally, we calculate this with the help of the Eq.(8.13) and (8.14), as shown in Fig.8.11.

$$Rs = tR2 - tR1/1/2(Wb1 + Wb2) \tag{8.13}$$

$$Rs = 1.18(tR2 - tR1)/(W1/2h1 + W1/2h2) \tag{8.14}$$

where $W_1/2h_1$ and $W_1/2h_2$ stand for the peak widths that are measures at half the height of the peak as can be seen in Fig.8.11. For the simplicity of the technique, it is used with most of the data systems for measuring resolution. As compared to the baseline width method, it is easier.

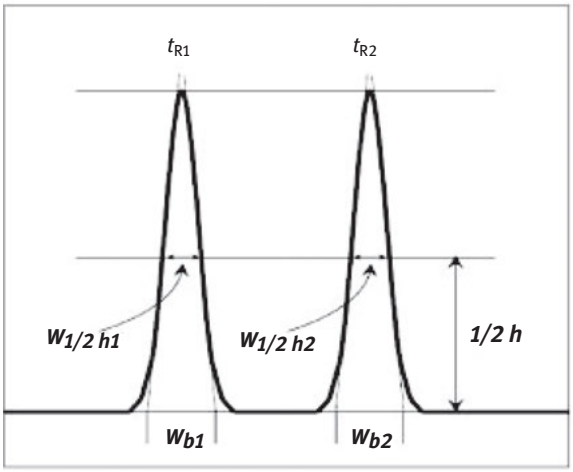
When it comes to tailing, almost every peak shows some signs of it. So, to get the proper resolution between two interest peaks, it is imperative to allow a little tailing and still keep the baseline flat between the peaks of interest. Generally, $R_s \geq 1.5$ is the perfect value for the right resolution.

To go for excellent results for the calculation for peak resolution, go for Eq.(8.13). Remember, it is useful only when the peaks resolve at the baseline level. But in reality, most of the situations have peaks which are slightly separated. Because of the fact that the peaks are overlapping at the base, it is not possible to measure the peak width.For the simplicity of the technique, it is used with most of the data systems for measuring resolution. As compared to the baseline width method, it is easier."

If this is the case, we can use the same approach taken to measure the column efficiency at the mid-peak height; you can use Eq.(8.13) for calculating resolution.

#### 8.4.11.9 Resolution and peak separation

To measure resolution, numerical values are used. For instance, 1.0, 1.5 or 0.8 can be used. But, the point to ponder is what the relationship between the actual peak




$t_{R1}$. $t_{R2}$ : Retention time for each peak ( $t_{R1} < t_{R2}$ )
$W_{1/2\ h1}$.$W_{1/2\ h2}$ : Full width at half maximum (FWHM) of each peak
$W_{b1}$ . $W_{b2}$ : Width of each peak

**Fig.8.11:** An HPLC chromatogram of two adjacent peaks for components A and B is used for the calculation of resolution ($R_s$) Eq.(8.13) and (8.14).

separation and the number which represents the resolution is? As shown in Fig.8.12A, at a 1.0 resolution, if we assume that the two peaks have a Gaussian distribution with the same peak width and height, almost 2.3% of the peak goes into the second peak from a perpendicular line that is drawn inside the trough. Likewise, a 1.5 resolution indicated an overlap of almost 0.15%, as shown in Fig.8.12B and as mentioned in Shimadzu website [58].

Note: Baseline separation of peaks is achieved with R ≥ 1.5 and is ideal for quantitative analysis.

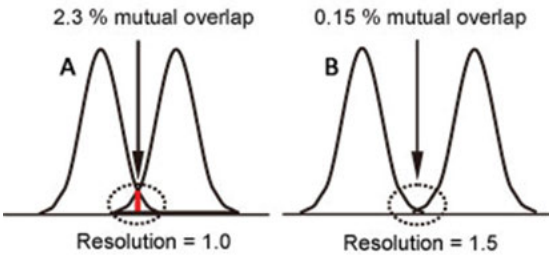



**Fig.8.12:** Illustrated diagram showing the relationship between resolution and peak separation according to Shimadzu [58]. Reproduced by permission from [58] (http://www.Shimadzu.com).

Finally, $R_s$ can be expressed in terms of the number of theoretical plates, separation factor and retention factor, as shown in Eq.(8.15)

$$\mathrm{Rs} = 1/4\mathrm{N} \times \alpha - 1/\alpha \times K2'/1 + K2' \tag{8.15}$$

where N is the of theoretical plates,

α is the selectivity and

$K2'$ is the retention factor of product #2

Truly, the results of SST depend on sample preparation, mobile phase composition, column condition, column composition and the HPLC hardware. If the SST yields unacceptable results, the above factors shall be examined, modified if necessary and the tested again. If the SST yields acceptable results, you can start your analysis using this HPLC.

Nowadays, computer data stations (HPLC LC workstation software) which are found in each HPLC system such as Shimadzu's LC workstation software [58] and Waters HPLC software [46] can automatically delineate each resolved peak and calculate SST parameters, such as N, $A_s/T_f$, $R_s$, and output SST results are reported along with analytical results of the sample.

### 8.4.12 Sampling and sample preparation

Three independent functions are used to analyze the pharmaceutical dosage forms. These functions are (1) collecting the sample and preparing it, (2) analysis of the sample and (3) analyzing the data and generating results.

We shall discuss all of them in detail below:

#### 8.4.12.1 Sampling

In drug QC laboratories, the process of sampling and preparation starts right when the sample collection starts and goes all the way up to the point of measurement. The first contact that happens with the sample is the primary sampling or the process of actually collecting the sample in the sampling process. Hence, the sample should be a representation of the total lot that needs to be investigated. The underlying reason for sampling is to reduce the volume or mass of the sample from the batch it is being taken from, that is, the parent batch. In the primary sampling, the sample that is going to be analyzed is selected and collected. Many important books [88–94] have been written on the practical and theoretical aspects of sampling. These also state on how to go ahead and collect a sample for analysis that is a statistical representative of the pharmaceutical drug which is collected in the drug laboratories for QC. This chapter does not cover the methodology and theory

on primary sampling as it is not in its scope. But, it can be said with confidence that the preparation of the sample is one of the biggest and often overlooked reasons for errors occurring in the analysis process. Preferably, the drug QC labs should have their sampling plans that are a major part of the analytical procure. Another important thing to consider is that the information of the sample flows parallel to the sample flow in the analytical process from start to end, that is, from the point of collection to the final report generation process. For instance, the sample can be tracked from the starting point that is the point of collection and is an integral part of the analysis process. Proper identification methods need to be present to ensure that primary sample collection is effective. These include labels written by hand, labeling them with inedible ink, bar code application to allow automatic reading, including RFI (Radio Frequency Identification) (Using RIF reader = RIFD) devices or any other means of certification needs to be present. This will ensure that at the end of the analysis process, you can trace the sample unequivocally to the sample it originated from or the primary sample. To make sure that the GLPs are being achieved, it is important to have proper sample tracking methods during the sample and sub-sample stage.

After the primary sampling process is complete, it needs to be transported without any chemical or physical change in the properties of the sample to the lab where the analytical process will be carried out. For samples that have unstable, reactive or volatile materials, the transportation can become challenging. This is problem if the lab is far away from where the sample was collected from. In case there is any change in the primary sample because of the transportation process, the secondary sampling process can become difficult. To ensure changes are minimized between the analysis and collection process, a number of prevention techniques can be done. The physical changes like diffusion, volatilization or adsorption and also the chemical changes like microbiological degradation and oxidation can be minimized if preserved properly. Some of the preservation techniques you can use from point of collection to the point of sample preparation are as follows:

- Adding a chemical stabilizers like antibacterial and antioxidant agents.
- Choosing the proper container for sampling
- Freezing or keeping the sample in a refrigerator to stay away from thermal degradation

Once the sample reaches the laboratory and before the analysis process is carried out, it is very important to maintain the integrity of the sample. For this, it is very important to check the conditions in which it is stored. For samples that are volatile or thermally labile, it is imperative to keep them in a freezer, fridge or in a container that is well sealed. Light sensitive samples need to be stored in a dark, cool area that has no exposure to sunlight till they are ready for conducting analysis. The samples that are prone to chemical reactions or oxidation need to be placed in a vacuum desiccator. They are to be kept there until analysis or further sampling.

#### 8.4.12.2 Sample preparation

Preparing the sample is an integral part when it comes to conducting analysis of the dosage form of pharmaceutical drugs in the drug QC labs, in which isolation of the components of interest (active ingredients in pharmaceutical dosage form, API) can be done and greatly simplifies the task of analysis. The aim of sample preparation is to provide a sample aliquot that (a) is relatively free of interferences, (b) will not damage the HPLC column or the HPLC instrument and (c) is compatible with the intended analytical method. In HPLC analysis, the sample solvent should dissolve in the HPLC mobile phase without affecting sample retention or resolution, the stationary phase itself and with no interference with the detection process. Preferably, concentrating the analytes or/and derivatizing them is a good idea for aiding in the separation process and for higher chances of detection. Bad sample preparation shortens the life span of HPLC columns, when the quantity of the contaminants is constantly increased at the head or the beginning of the columns, a back pressure is created. Purpose of good sample preparation which will be analyzed using HPLC can be summarized in the following:

- To improve the limits of detection
- To enhance selectivity of analyte identification
- To improve precision and accuracy for quantitative analysis of active ingredients in the sample of drug under analysis.
- To protect HPLC columns from contamination
- As we have mentioned previously in Section 8.3, the analysts in drug QC laboratories are using HPLC-validated analytical methods for the routine analysis of DP and DS. Validation for these methods is the responsibility of the drug manufacturers during pharmaceuticals' development cycle in the industry and have been done according to the one of these rules CDER [11], ICH [12–14] or USP [15] as shown in Fig.8.1. The objective of the validation experiments is to determine if the method is suitable for its intended purpose by running the method and comparing the results to predetermined criteria which will assess accuracy, precision, linearity, specificity, linearity range and ruggedness. Validation of accuracy (accuracy representative of API recovery from manufactured product matrices) is done by applying the method to samples for which we know what the API is. This is done by making use of a series of samples where the API is added to a pharmaceutical placebo (spiked placebo experiment). To ensure that the procedure for sample preparation is authentic, the method for validation should include some robust experiments which test the sample preparation variables. For instance, studies regarding mixing time should be carried out in a way that the time is increased until recovery is maximized. According to CDER [11], ICH [12–14] or USP [15] rules, method validation of experiments for extraction and the resulting data about the validation parameters in the relation of required criteria must be a part of drug manufacturer's specification

(in good description and documentation) ready to be uses by the analyst in drug QC laboratories.

A practical example for drug samples preparation method development have been discussed by Choic and Dong [93] for assay of water soluble vitamins in multivitamins tablets.

A validated analytical method should be revalidated if there is any change in the procedure for sample preparation. Generally, a major reason for revalidation is when there is any change during the sample preparation process. For instance, if a method has been validated for being used for a DP in the tablet form, it should not be used for the reformulated encapsulated form of the same dug. The method needs to be revalidated. The reason for this is the change in the matrix of the sample. This is because that the solvent used for recovery/sample diluents might not be enough to derive API from the second formulation.

On the basis of the previously mentioned information, preparation of the a pharmaceutical product as outlined in drug specifications or desired pharmacopeia has become mandatory. The recommended weights of sample or aliquot size and dilution volumes must be the same as those stated in the drug specifications (look for validation method range and you must fulfill these requirements according to manufacturer specification).

It is well known that there is a wide variety of samples that are present in the laboratories for drug QC. Depending on the different chemical mixtures of the different samples, different problems arise in the sample preparation process. This is because of the varied forms of dosage and the type of sample being prepared. On the other hand, pharmaceutical products are necessarily formulated with an inactive ingredients (pharmaceutical excipients, such as starch, artificial flavors, sweeteners, gelatin, oils and magnesium stearate), as chemical mixtures to achieve the desired physiological effect. So, the presence of the inactive ingredients is an important consideration when developing sample preparation methods.

Generally, the "dilute and shoot" approach is the most widely used in the parenteral products and DSs. For capsules, tables and other solid forms of dosage, the most common process to use is the "grind ▷ extract ▷ dilute ▷ filter." As the dosage gets complex, like creams, lotions, suppositories and so on, additional extractions and sample cleanup is required. These include the liquid–liquid extraction method or the SFE, SPME, ASE and SPE [92–100].

Details about methods for sample preparation of pharmaceutical products in drug QC laboratories are found in Chapter 7. This chapter reviews the common sample preparation techniques focusing largely on capsules and tablets, for instance shaking, dissolution, vortexing, sonication, liquid–liquid extraction, filtration, centrifugation, evaporation. In addition, Chapter 7 elaborately addressed sample preparation technique such as SPE, SPME, SFE and ASE for extraction of specific API from a complex sample matrices such as cream, lotion, suppositories,

and creams. Additional details on the pharmaceutical products or sample preparation can be read from external sources provided in reference [92–100]. In the process of sample preparation, the product steps must produce a solution with the following properties:

- The analyte concentration should be in the measurable range of the HPLC instrument. The sample prepared must be rightly concentrated so that it falls in the instrumental method's working range of concentration. This determines the samples dilution volume. For instance, if it is very dilute, the sample needs to be concentrated. Hence, the development of the instrumental method should be a step before the method of sample preparation.
- The recovery percentage for the PAI from the sample matrices must be quantitative or reproducible from sample to sample. The exactness of the analysis process depends on the recovery of the sample preparation process. This is why it should be assessed. For the DP and DS analysis, the preferable recovery percent required is 100%. This ensures that precision and accuracy are achieved.
- The extracted API must be stable until it is analyzed. Once the sample preparation is done and before the instrumental analysis is done, it is necessary that the API be stable for some time. If needed, the sample might require one additional step, such as pH adjustment. This is usually imperative when degradant methods or trace impurities exist. In case of reporting degradants, the sample must resist against degradation.
- During sample preparation, the method should not be restricted to deliver the preferable amount of API, but the analyte's compounds should not pose any interference with the sample. A selective sample preparation method should be used.
- Sample extract must be compatible with the HPLC system.

Finally, the result of the sample preparation for pharmaceutical drugs is mostly an HPLC vial. This contains the solution that is in its final form and ready to be tested with the right amount of API to carry out the HPLC analysis.

### 8.4.12.3 Sample analysis

#### 8.4.12.3.1 Sample solution injection

Before injecting sample solution , note the following:

- The necessary step before using HPLC in an analysis is the initial solvation and equilibration of the column with the actual mobile phase. For that purpose, purge the column with the desired mobile phase until you obtain a stable detector baseline (see Fig.8.13) and column pressure. For equilibrating your column, set the monitoring wavelength, temperature and set the pump at 0.0 mL/min, then turn the pump ON. Increase the flow rate gradually, in 0.1 min increments to the

desired flow rate as outlined in manufacturer specification of the drug (details about HPLC column equilibration you can found in Section 8.4.8).
- Stable baseline should be obtained prior to using HPLC analysis (see Fig.8.13); Xit takes 30 min for RP column and 30–45 min for silica column.
- After having established a stable baseline, verify that a blank injection with mobile phase medium does not give rise any peaks on the resulting chromatogram.
- Begin injections of the standard solution until two successive injections have $t_R$ values which agree within 2% relative.
- If a stable baseline cannot be produced, evaluate the cause of the problem and correct it and continue the equilibration process before proceeding with the analysis.

Particle-free samples should be injected during the HPLC analysis in the system. That can be achieved by filtering or, if the filter retains part of the analyte, by centrifugation. Ideally, samples should be applied in a small volume to optimize the resolving power. Manually injecting the samples if possible with the help of a syringes or a syringe through a sample loop with a defined volume (typically few µL–100 µL) by use of a six-port valve injection system (for details, see Chapter 4). Some of the advantages are good reproducibility and pressure resistance of the injection volumes during the delivery of the sample loop. The disadvantage is loss of parts of the sample when filling the loop. Nowadays, injections can be programmed using automated systems (autosampler). This allows the user to withdraw the preferable volumes from a large sample vial set. This is done by using pressure-driven syringes that can take up to several hundred and inject the samples anytime, for example, even overnight.

Removal of particulate matter in the sample extract is critical for HPLC stability. Both column frit and top of column packing can become clogged (becomes narrow) by particles, leading to increase in back pressure and adverse effects on chromatographic results, decreased column efficiency, production of split peak, tailing peaks and so on. In addition, particulates can also affect HPLC hardware (e.g., flow lines, rotary injection valves and inlet frits columns and detectors). Filtration is the final step in most sample preparation to separate the final analyte solution from the solid particles. In most cases, the tablet extracts are filtered through syringe filters (Fig.7.8, Chapter 7) directly into the HPLC sample vial. Syringe membrane filters are made in various pore sizes (0.2 or 0.45 mm), media and dimensions (4, 13 or 25 mm). The most popular are the polytetrafluoroethylene, 25-mm nylon, polyvinylidene fluoride and 0.45-mm filters that you can get from Pall, Gelman, Milipore, Whatman and many other suppliers. Some of the widely used and common means for helping in removal of the sample particulates are centrifugation, sedimentation and filtration. Filter paper (cellulose) can be used for removal of larger particles (>40 µm). Also, in-line filters placed ahead of the column can be used for sample clarification

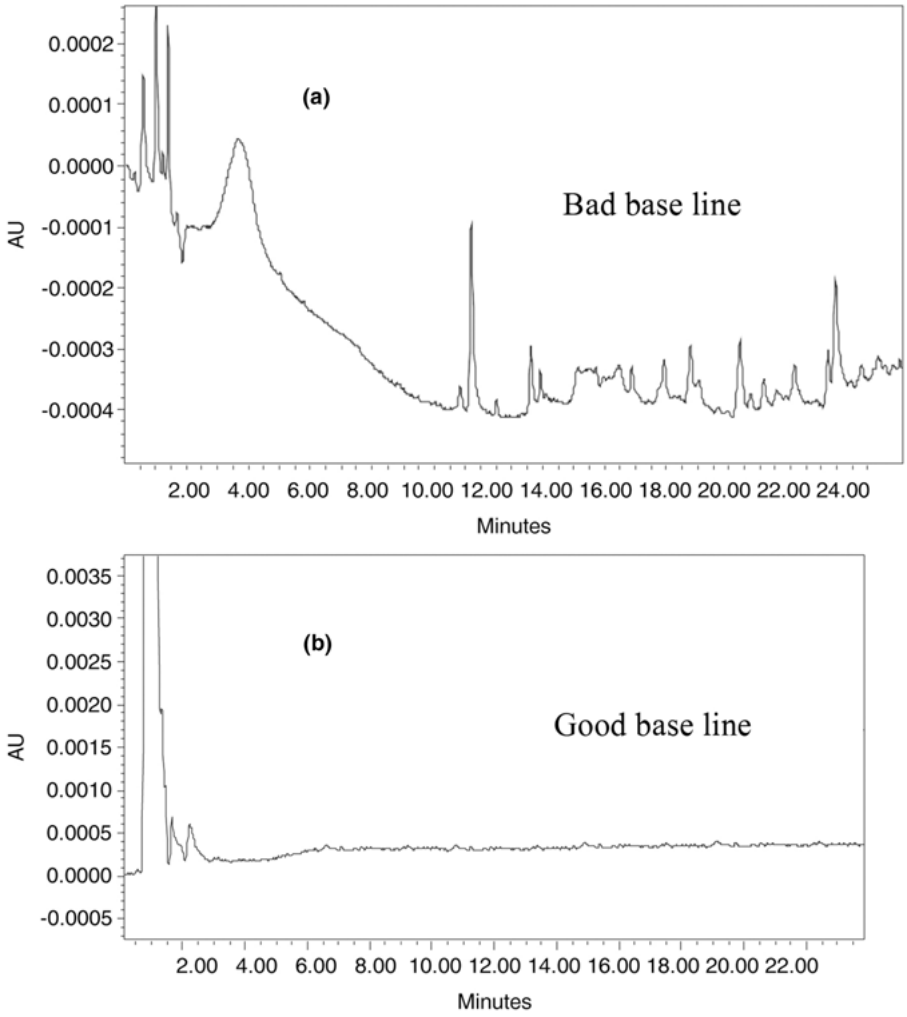



**Fig.8.13:** HPLC baseline monitoring resulting from non-equilibrated (a) and equilibrated HPLC system.

before interring the analytical column (for details, see Chapters 2 and 3). Also, removal of particulate matter in the sample extract can be done using centrifugation.

After HPLC system has become conditioned and equilibrated, the SST criteria were met according to CDER [11] or according to the manufacturer's criteria, and the standard and sample were prepared, then proceeded with standard and sample injection, set two injections per standard working solution and two injections per sample

working solution (inject appropriate volume). If all parts of the HPLC are working correctly and the proper separation conditions has been used as mentioned in drug specification, a well-separated peak or peaks should be observed on the chromatogram during the specified run time as outlined in drug manufacturer's specification. Each time after completion of analysis, computer/printer will print the chromatogram with the baseline, after this it prints the analysis report (tR, peak area, etc.)

#### 8.4.12.3.2 Qualitative analysis (identification)

Qualitative analysis or identification testing is used to confirm the presence of the API in samples of DSs or DPs. In most cases, two independent tests need to be conducted. These two are spectroscopic test and the HPLC retention time match test. These can be MS spectral match, UV, IR and are done against a standard reference point in case of critical and complex tasks like stability evaluation, product release or even after-market support like analyzing the complaints of the customers or in case of fake samples. Usually, the HPLC assay procedure provides positive identification, when retention times of API peaks on the chromatogram of the tested sample the same as the retion time of the API in reference standard as shown in Fig.8.14.

Also, identification of API in the pharmaceutical products can be established by determination of the percent ratio of the retention times of API in the sample and standard solutions chromatograms using Eq.(8.16) as follows:

$$\%\mathrm{R\,tR} = \mathrm{tR1}/\mathrm{tR2} \times 100 \tag{8.16}$$

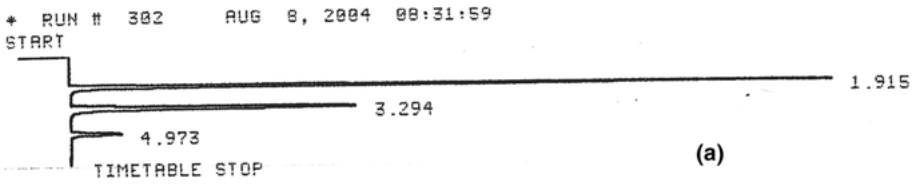
where $tR_1$ is the retention time in minutes for the API peak in the sample chromatogram and $tR_2$ is the retention time in minutes for API peak in the standard chromatogram.

%t RtR should be within 98–102%.

Also, it must be:

- Elution characteristics for the chromatogram of sample are essentially the same as in reference standard solution containing all the constituents. Usually, the chromatogram obtained on the investigated sample and API in drug QC laboratory must meet the requirements for the manufacturer specification or specified pharmacopeia.
- The chromatogram of the working sample solution and reference standard solution should be qualitatively similar to those attached to the manufacturer's specification with respect to peak shape and baseline quality (as shown in Fig.8.14).

Due to differences in columns and instrumentations, it may be necessary to adjust the mobile phase composition and/or instrument parameters for achieving the identification.

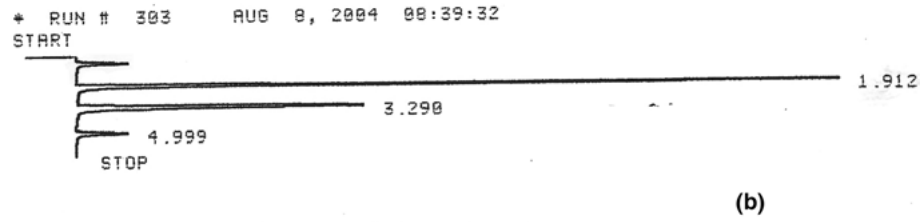



RUN# 302 AUG 8, 2004 08:31:59

AREA%

| RT | AREA | TYPE | WIDTH | AREA% |
|---|---|---|---|---|
| 1.915 | 886358 | PB | .106 | 57.90597 |
| 3.294 | 549506 | PB | .191 | 35.89936 |
| 4.973 | 94821 | PB | .181 | 6.19468 |

TOTAL AREA=1530685
MUL FACTOR=1.0000E+00




RUN# 303 AUG 8, 2004 08:39:32

AREA%

| RT | AREA | TYPE | WIDTH | AREA% |
|---|---|---|---|---|
| 1.912 | 879738 | PB | .105 | 57.54302 |
| 3.290 | 553209 | PB | .191 | 36.18499 |
| 4.999 | 95888 | BB | .181 | 6.27196 |

TOTAL AREA=1528835
MUL FACTOR=1.0000E+00

**Fig.8.14:** HPLC identification for sample contains three compounds by comparing their (a) retention times $t_R$ on the standard chromatogram (b) against their $t_R$ on the sample chromatogram.

- Although retention time comparisons using a single group of HPLC parameters are not an absolute method of identification, using a second group of parameters, for example, different column, solvents and flow will prove adequate for many situations. Since other compounds can have the same retention times, the chromatographer should be constantly alert to the presence of possible interfering substances.
- There are many reasons why the $t_R$ may vary over time. For instance, degradation of column performance, change of column, differences between batches of the mobile phase, variation of ambient temperature. For this reason, it is impossible to give an absolute retention time, rather an acceptable range of retention time is

given. The reproducibility of relative retention time (RRT) is better than that of $t_R$, it is defined as the ratio of the retention times of any component (Eq.(8.17)) to the retention times of reference compound (relatively stable).

$$tR(i)/tR(ref) = RRT \tag{8.17}$$

Here, tR(ref) and tR(i) denote the retention times of component i and the reference compound, respectively.

– **Spiking technique**. Another method for identification of a particular peak is by the technique called spiking. This technique can be used when the identity of some specific peak is questioned and pure compounds are available. For example, Fig.8.15, consider a vitamin separation for which the location of vitamin B1 and vitamin B6 have become confused. By spiking the original sample with additional vitamin B6, we can say that the vitamin B6 has come on the resulting chromatogram before vitamin B1.

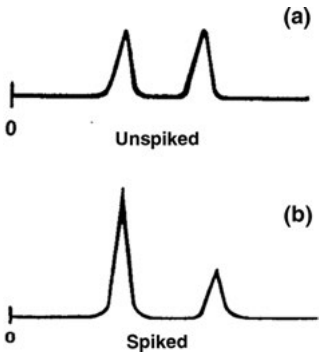



**Fig.8.15:** Confirmation of HPLC peak identification by spiking.

#### 8.4.12.3.3 Quantitative analysis

Once the identity for the API in a pharmaceutical product or in pharmaceutical substance is established as previously mentioned, then another important information to look at is the quantity of compound that exists in the sample. HPLC quantization involves the comparison of standards and samples, their areas (or heights) and retention time. Two methods that can be used are the peak height and peak area, peak area being the popular one. Both the data collected from the detector and the chromatogram help us in calculating the amount of concentration of every compound. The detector works as a response to the compound band's concentration as the band crosses the flow cell. The higher the concentration, the stronger the signal becomes. This is represented as an increase in the height of the peak you can see above the baseline or the area of the peak that lies beneath the peak. As the Fig.8.16 shows, API A's peak in the chromatogram sample has a peak area 10 times more than that for a standard API A. From this data, the amount of API A in sample can be determined as 10 µg/mL.

In Fig.8.16, there are two chromatograms, 1 and 2 obtained from injecting 10 µL of standard solution containing 1µg/mL of API A and 10 µL of a pharmaceutical product sample solution contains unknown amount from API A into an HPLC system operated under the same conditions respectively. On the time scale, both the chromatograms are placed one on top of the other. As we can see, the peak of both chromatograms is displayed at the same $t_R$ or retention time, that is, 2.85 min, indicating that the sample contains the API A.

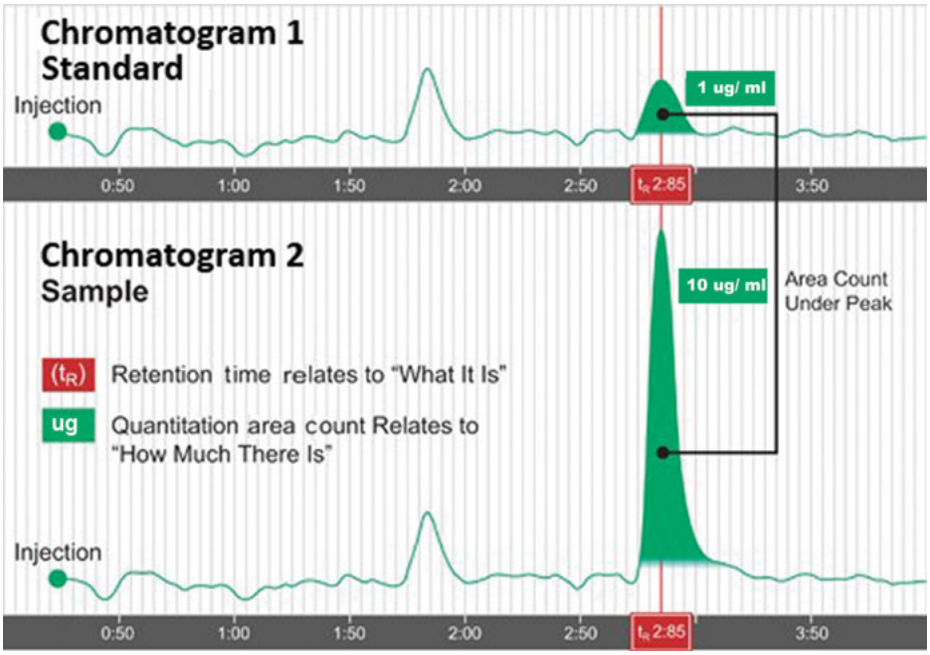



**Fig.8.16:** Identification using $t_R$ and quantification using peak area for pharmaceutical products using HPLC.

It is well known that HPLC quantitation is based upon two requirements:

- Reproducible chromatography.
- The detector should give a linear response when dealing with compounds under observation

When these criteria have been met, and the chromatogram produced, quantitation of the peaks (measuring peak height or peak area) can proceed. Quantitation of the peaks is determined through measurements which come from the peak area/height. The drawbacks to peak height usage are because of the different chromatographic conditions like the flow rate or the sensitivity of the peak height. These errors can

be because of the lack of resolution, extremely small peaks or a drifting baseline. All these lead to very poor measurements.

Nowadays, computers and printers are the most essential items of the modern HPLC equipment. Computers are used for collecting the resulting chromatographic data, storing, handling and processing these data. In addition, computers control the instrument parameters, such as, flow rate of mobile phase, column heater temperature and autosampler.

The major difference between a microcomputer (as used in LC) and a microprocessor is the presence of a user-accessible language that is usually build in the computer – this is mostly BASIC. Just like a computing integrator, the microcomputer performs data reduction and acquisition in the same manner. Similar commands and parameters regarding data handling used on the integrator are used in the microcomputer.

The biggest difference in terms of performance between an integrator and microcomputer is that the microcomputer can process extensive data calculations when compared with the computing integrator. For example, programs written in BASIC can do linear regressions, print tables of data from a series of runs, standard deviation or any calculation that the user wants to carry out.

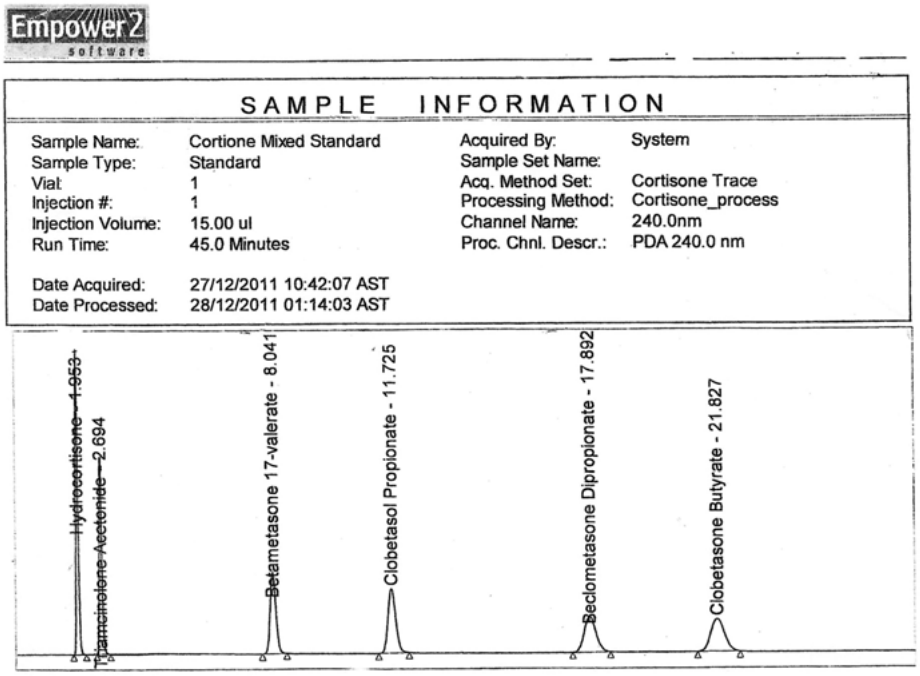
Microcomputers further allow interfacing and control of the peripheral equipment which other systems do not allow. Imagination is generally the limiting factor on utilization of the microcomputer. Increased cost and technical demands on the operators are the major drawbacks of this type of integrator.

In today's laboratories, analysts have typical key processes or functions (quantization, calibration and integration) to change the sample chromatograms, which is the data collected from the detector in its raw form to chromatographic reports that are useful for us. Predefined user-specified methods already present in the data system are used to control these processes. Integration is the process of converting digital chromatography raw data into peak data (series of peak retention times and peak areas). In the traditional algorithm of integration, the components and baseline peaks are achieved by monitoring the raw data's slope and then these are compared with a set "threshold" to see what the "peak start" is.

A series of integration events in the processing method allow for customizing the process of integration. A modern algorithm of the integration process that makes use of the 2nd derivative taken from the raw data to determine the peak is present in some of the available data systems on the market. These include Shimadzu Class VP [58] and Waters EmPower [46]. This is seen to be much superior to the traditional methods when dealing with complex chromatograms that have a sloping baseline.

Once the peaks on the HPLC chromatogram have been quantized as shown in Fig.8.17, the user now has numbers representing peak heights or areas. What one needs to know, however, is what these numbers mean in real or absolute measurement terms. There are four different mathematical manipulations (calibrations = construction of a calibration curve, calibration is the process of establishing a calibration

curve of the specified analyte from a different concentration set of injected calibration standard solutions) which can be used to translate these numbers (raw data) to valuable means (calculate the amount of the API or impurities in pharmaceutical products where this is the main aim of analyst in drug QC laboratories).




| | Peak Name | RT | Area | % Area |
|---|---|---|---|---|
| 1 | Hydrocortisone | 1.953 | 672527 | 21.79 |
| 2 | Triamcinolone Acetonide | 2.694 | 525219 | 17.02 |
| 3 | Betametasone 17-valerate | 8.041 | 475569 | 15.41 |
| 4 | Clobetasol Propionate | 11.725 | 516613 | 16.74 |
| 5 | Beclometasone Dipropionate | 17.892 | 425746 | 13.80 |
| 6 | Clobetasone Butyrate | 21.827 | 470505 | 15.25 |

**Fig.8.17:** An HPLC report documenting sample/standard information (tR, peak areas and peak height) and the chromatogram for six compounds were separated.

In the following, we will discuss the calibrating methods which they are familiar in drug QC laboratories. These methods of calibration can be concluded as:

- Peak area normalization.
- External standard calibration
- IS calibration
- Standard addition calibration

In the following, we shall discuss these methods of calibrations in details:

#### 8.4.12.3.3.1 Peak area normalization

There are two basic forms of peak area normalization:

**First**: Area normalization for compounds with similar detector response and for which 100% of the components of the solute elute. For four components, A, B, C and D, a simple normalization calculation would be made as follows using Eq.(8.18):

$$\%A = \text{area of peak A}/\text{Total peak areas}\,(A + B + C + D \times 100) \tag{8.18}$$

**Second**: Area normalization using the response factors to correct the detector response's variation for different components.

Area normalization using response factors to correct variations in detector response for the different components.

For calculating the response factors, we use a calibration curve using reference standards (minimum three different concentrations are used) for D, C, B and A individually. Reference standards concentrations versus peak area are plotted and the best straight line is drawn through the points as shown in Fig.8.18. The slope of this curve is the response factor (Rf) for that particular component, as seen in Fig.8.18.

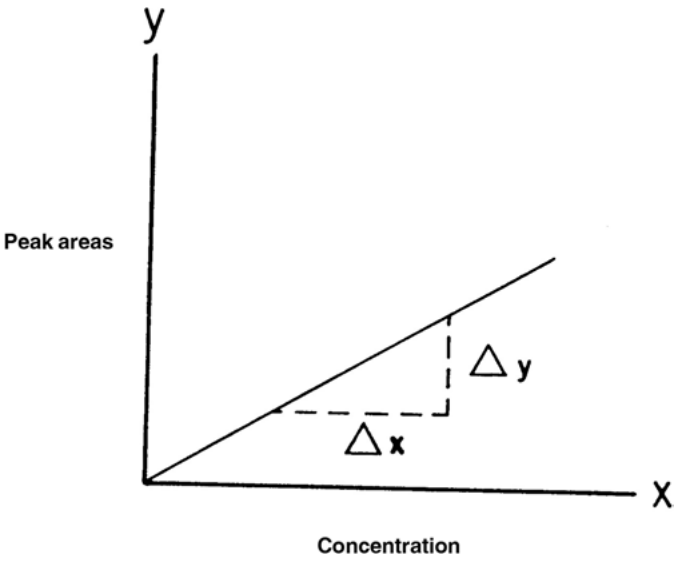



**Fig.8.18:** A typical calibration curve resulting from plotting the peak area of the analyte against the amount injected in the HPLC. It should be noted that the curve's slope is equal to the Rf (response factor) of the pharmaceutical product's API.

$$\text{Response factor} = \text{Area standard}/\text{Amount of standard} = \text{Area sample}/\text{Amount sample} \tag{8.19}$$

To calculate the correct area of every single component, the peak area needs to be divided by the response factor as shown in Eq.(8.19).

So, the corrected area for component

$$\text{B} = \text{Area peak B} / \text{Response factor (Rf)} \tag{8.20}$$

#### 8.4.12.3.3.2 External standard calibration method

This method is the most simple calibration method and is less complicated than the IS method, but it extremely operator dependent, and demands good analytical technique. First, the calibration curves of the varying concentrations are prepared. These are the pure standard ones. This is essential to get accurate measurements that the volumes injected always be identical for the measurement that injection volumes always be identical for the components of interest, as well as the pure standards. The curve is plotted with peak height (area) versus concentration, and the unknown concentration is then read from the curve, Fig.8.19.

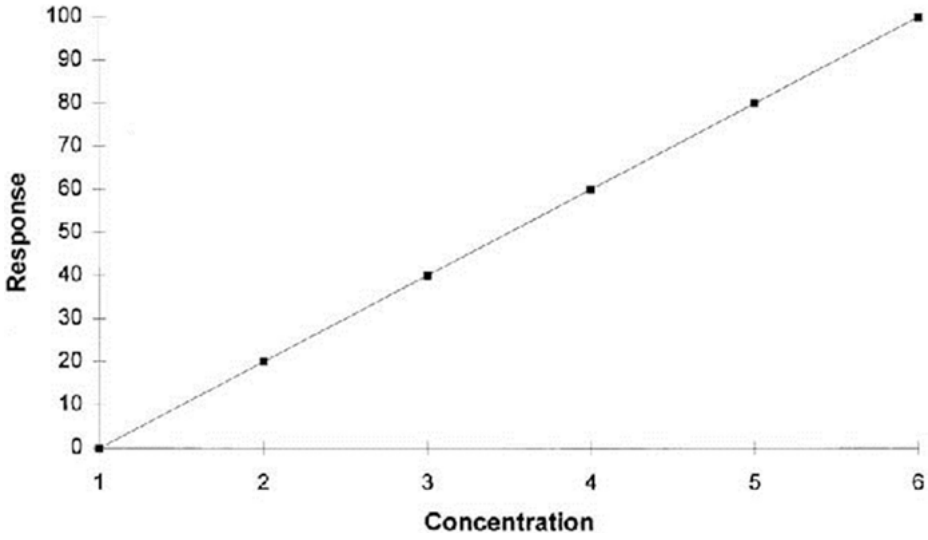



**Fig.8.19:** A calibration curve used by carrying out external calibration.

This method is used if there is 100% chance of the analyte's recovery from the sample components. To get a consistent sample-to-sample recovery, you need to have samples whose matrices are predictable and constant. There can be DPs and DSs.

This calibration method also requires reproducible dilution volumes. In case the chromatograph's injection system is not able to provide reliability, you should go for internal calibration.

#### 8.4.12.3.3.3 Internal standard calibration method

Just like the external method for calibration, the internal method also requires preparing external standard solution; the difference between the two is the addition of the second compounds constant concentration. This is added to the standard working solution and the sample. The analyte's ratio to the IS and the concentration of the sample are both directly proportional to each other. Use this method only for analyzing difficult or biological samples.

Just as it is done in the normalization process, you can easily calculate the Rf by mixing together a concentration of pure IS that is known with different concentrations of the pure component standard. Once this is done, the chromatogram is run for an individual standard, and the peak area or heights are measured for both the IS peaks and the component. The ratio of standard component to the ISs is made for each of the runs and plotted versus concentration, as shown in Fig.8.20. In calculation, the curve's slope is the same of the PAI or the analyte's repose factor. To determine the concentration of samples unknown, the following method is used:

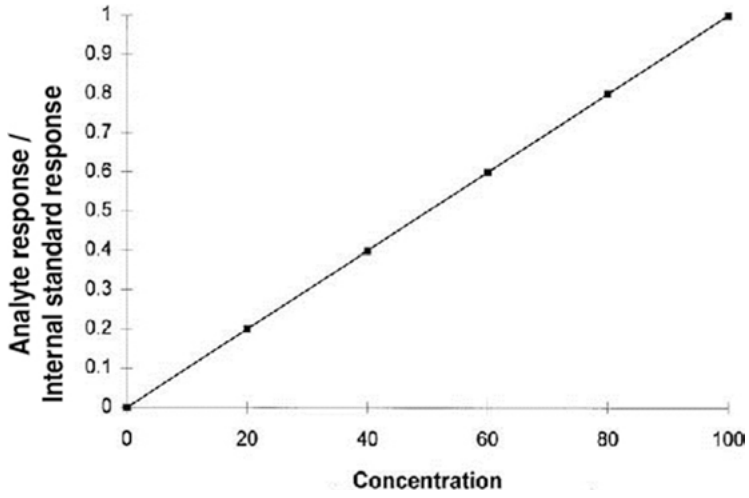



**Fig.8.20:** A typical calibration curve using internal calibration.

Peak area or height of component in the sample chromatogram divided by the Peak height (or area) of IS in the sample chromatogram divided by its respective response factor

The ISneeds to meet the criteria mentioned below:

- Never be found as normal component of the sample.
- Completely resolved from sample component peaks.
- Added at a concentration similar to that of the components of interest.
- Available in pure form
- Non-reactive with any of the sample components.

We can perform many functions with the help of the ISs. The most widely used and common application is to correct it for the standard injection volume and sample. Every standard and sample has a constant amount of ISs; hence, the amount injected needs to be proportional to the IS concentration. Furthermore, the analyte concentration is proportional to the ratio of the analyte to IS signals. Similarly, the standard dilution and sample volume can be replaced by an IS.

If the IS has been selected properly, it allows to correct and increase the extraction efficiency. The trick is to choose an IS that has a structure similar to the analytes analog. This allows partition coefficients that are similar in liquid extractions and also similar to the solid-phase extraction processes extraction efficiencies. Preferably, a carbon-14 analog or a deuterate of the analyte is an optimal standard. This is because both have the same chemical properties. As a result, we get the same extraction efficiencies and they are also similarly reactive in case of derivatization. Because of the fact that the deuterated analogs co-elute chromatographically with the analyte, they are used with mass-selective detectors. The reason for this is that the mass spectrometers allow for the mass discrimination of the co-eluting compounds.

#### 8.4.12.3.3.4 A typical calibration curve using standard addition calibration

A method that is useful for calibration when there is no chance of reproducing the sample matrix in enough quantity that the standard solution can be prepared. The calibration standards are used to prepare the sample when known amounts of the analyte are added to three, four or greater aliquots of the sample. The total quantity of added standard should cover a range of about 10–100% of the sample concentration. Sample concentration can be calculated when we plot the instrument response against the total concentration added and then the absolute value of the xi intercept (see Fig.8.21). This technique is often used in conjunction with ISs, where the instrument response is then the ratio of the analyte to IS response. This is a very good reference method to crosscheck a primary method. Rarely do we use if for normal analysis of many samples because each test article requires multiple sample preparations and analyses.

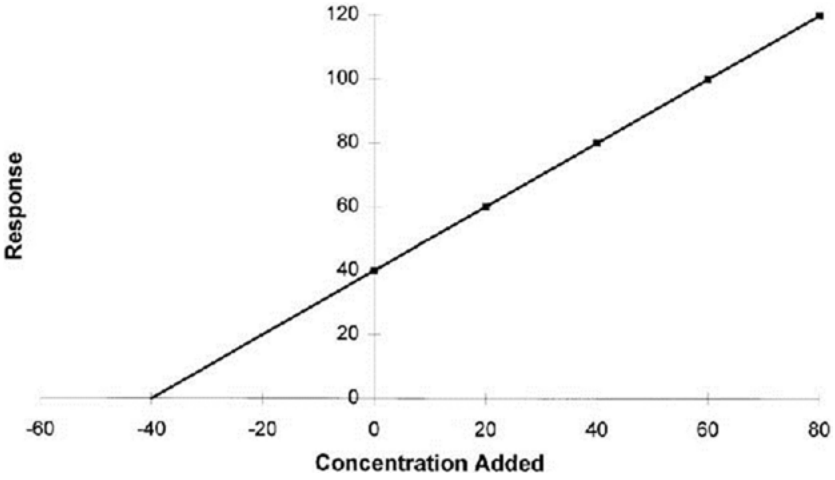



**Fig.8.21:** Schematic representation for the plot of the standard addition calibration method.

For HPLC quantization, the guidelines are mentioned below:

- Use peak area for quantization which is less susceptible to flow variations.
- Use normalized area percent analysis for impurities and related substances testing.
- Use "external standardization" for assays and dissolution. Bracketed standards are preferred for improved accuracy.

### 8.4.13 Application for HPLC as analytical tool for analysis of pharmaceutical substances and pharmaceutical products in drug quality control laboratories

In this part, we will give a general idea about how modern HPLC can be applied as an analytical tool to conduct pharmaceutical analysis in drug QC laboratories. The applications which we will be discussing in this chapter consist of content uniformity and assays for potency, dissolution performance and testing the purity of the DP and DS. Of course, this kind of work needs analytical methods and analytical tools.

As we have mentioned previously in Sections 8.3, 8.4.12.2 and 8.4.12.3, the analysts in drug QC laboratories must use validated analytical methods for routine analysis, assays for potency and content uniformity, testing for purity and dissolution performance for DP and DS. Validation for these methods is the responsibility of the drug manufacturers during pharmaceutical development cycle in the industry and have been done according to the one of these rules: CDER [11], ICH [12–14] or USP [15]. According to ICH, FDA and USP rules, method validation experiments and the resulting data about the validation parameters in the relation of required criteria must be a part of drug

manufacturer specification (in good description and documentation) ready to be used by the analyst in drug QC laboratories.

In case the original scope of the analytical method changes, for instance if the concentration range changes, it is important to revalidate the method. A change in the preparation method of the sample also calls for revalidation. In reality, modifying the way in which a sample is prepared is one of the most common reasons that lead to revalidation. For instance, a method that has already been validated for a drugs table form cannot be used for the reformulated encapsulated form of the same drug without revalidating it. The reason is simple, the sample matrix of the drug changes. If this is the case, the diluents of the original sample might not be enough to get the API from the formulation.

As we have seen previously, the presence of inactive ingredients in the mixture is very important when you are considering what sample preparation method to go for.

To conduct analysis of the DPs and DSs in the laboratories working for QC of the drugs, the most common analytical tool used is HPLC [2–7]. In modern day laboratories, most of the tests are done using HPLC. Actually, methods of wet chemistry are now obsolete. In case of DS, the focus of HPLC testing is on impurity and assay testing. For DP dissolution, tests that indicate bioavailability and techniques for content uniformity are carried out. Content uniformity indicates that the content in the drug substance across batches is the same. Both the technology used in the HPLC instruments and the software that are used have become advanced. It can be seen by the fact that in most of the modern laboratories, analysis can run unattended for 24 h a day and even overnight.

As we have mentioned previously in this book, HPLC separation can fall in either of the five mentioned categories: NP, ion pair, IEC, SEC or RP. The most commonly method used is the RP. For detection principles, the most commonly used one is UV [2–7].

Before running the HPLC analysis, the system needs to have the right mobile phase, column, method for detection and an accurate wavelength. The testing directions will show how to set up and prepare the mobile phase to achieve the correct separation as it affects the process of separation. Depending on the process of preparation of the mobile phase, the API's retention times can be changed. Generally, only a slight adjustment to the mobile phase allows for the correct range of retention time to be achieved.

According to FDA, USP and ICH rules [11–15], SST testing is conducted to conclude the effectiveness and suitability of the HPLC system before using it and throughout all regulated assays on a daily basis (making assumptions about the proper functioning of the system is not enough once the first phase of the experiment has passed) as analytical tool in drug QC laboratories. SST is the fundamental part of any analytical procedure being conducted. Moreover, it is used for checking the internal QC for the method being used. Hence, the criteria of the method needs to be established in the method validation during pharmaceuticals developments

cycle in the pharmaceutical industry to find out the integrity and reliability of the whole HPLC systems.

In case SST fails in the initial stages, the sequence needs to be stopped immediately by the analyst. He should then see what the problem is, repair or adjust the system as required and perform the SST again. Actual samples can only be analyzed once all the SST limits are passed, not only the criteria that had failed before. Mostly, issues in the mobile phase, pump, autosampler or column give rise to these failures.

Parameters which are related to the operation of the whole HPLC system established by the USP, ICH and FDA guidelines [11–15] are found in Section 8.4.11.

Quantization of the interested component is achieved when it is compared with an external or IS for reference. Other methods for standardization (100% standardization or normalization) are not given very much importance when it comes to the laboratories conducting QC of pharmaceutical products. For external standards, separate tests are used to analyze the sample. On the other hand, ISs are added in the sample and that is when they appear on the same chromatogram. In drug quality laboratories, external standardization methods using one point calibration are used for most quantitative assays [2–7].

The HPLC methods heavily rely in the different reference standards if you want to get accurate and correct data. The reference standards need to be highly pure, high quality and well characterized if you want proper results. The NF/USP reference standards are deemed to be "pure" legally is they have a 100% purity level assigned to them by the USP. Other than this, there is no other characterization required to use them. If non-compendial reference standards are being used, it is important to add any correction factors used to treat the impurity before reaching a final calculation. The validation report needs to have proper documentation of all the materials used as reference for method validation. Working references are those materials that already have their purity recognized and are characterized against a well-established standard for reference. The best situation in which to use this is using in-house lot is cost effective when compared to purchasing the reference materials provided by USP to conduct the routinely analysis (Section 8.4.10 has further details).

Prepare the working reference standard solutions as outlined in drug specifications or desired pharmacopeia. Use certified reference standards (primary or secondary reference standard). The recommended weights of the standard or aliquot size and dilution volumes may be modified, provided that its concentrations are the same as those stated in the drug specifications (look for validation method range and you must fill full these requirements according to manufacturer specification).

In this part, the applications for HPLC as analytical tool in drug QC laboratories will be discussed, including the following tests for DS and DP:

- Assays potency test
- Content uniformity test
- Dissolution test
- Purity test

In the following, we will discuss Assay potency, Content uniformity test and Dissolution test in details. For getting information about Purity test which it is will not include here in this part and present one of the most important test in drug quality control laboratories, the authors are recommended the following sources [2, 3, 6 and 102] for reading by the readers.

#### 8.4.13.1 Assay for potency

For determining the analyte's weight fraction (known as API) in a sample batch of the formulated pharmaceutical product, assay potency is the common analysis technique performed by the laboratories for conducting quality drug control checks. Some other contents are the impurities and precursors from the synthetic route, both the water and organic solvents, excipients and the residual metals of the formulated products. Typically, an assay is used when we need to check for the conformance toward a label claim (usually 90% to 110% label claim for DPs) [101]. The assay for potency is also performed in other conditions like stability evaluation or product release.

Because most of the drugs are chromophoric, that is, they absorb UV, the UV detection HPLC method is most widely used when it comes to the assays for potency of the DS or DP. The EP and the USP have the assay methodologies published for most of the DP and DS [15, 26, 27].

Capsules or tablets and other solid dosage forms require a composite assay that falls in a range of 10 to 20 units. This minimized the variation you can find from tablet to tablet. The process of extraction is done in a volumetric flask. This process is sped and helped by mechanical shaking, ultrasonication and vortexing. In the products that have a control–release feature, a two-step process for extracting the API needs to be taken. Only then will be the API be extracted from the polymer matrix.

Both the extraction solvents nature (pH, % organic solvent, etc.) and the time for extraction can be optimized while method development is occurring in the industry [11–15]. A syringe membrane that directly filters and sends the extracts to the HPLC vial is used (for details, see Chapter 7).

According to external standard and one-point calibration method for quantization, the following equation shows how the %label claim is calculated

$$\%\mathrm{LC} = \frac{\mathrm{AS} \times \mathrm{StWt} \times \mathrm{DfSt} \times \mathrm{Stpurity} \times \mathrm{CF} \times 100}{\mathrm{ASt} \times \mathrm{N} \times \mathrm{DfS} \times \mathrm{LC}} \tag{8.21}$$

where % LC is the percentage label claim of an API per capsule or tablet, AS is peak area responses from the API of sample assay preparation solution, StWt is weight of

standard (mg), Df St is dilution factor for standard preparation, StPurity is purity of the reference standard after correction for residual solvents, compound's actual purity and moisture (Ex.99.9% = 99.9÷ 100 = 0. 999), CF is conversion factor from salt to base = MW of drug base/MW of salt, ASt is peak area responses from the API of standard preparation solution, DfS is dilution factor for sample preparation, Ntable/caps is number of tablets or capsules tested, LC is label claim of API per capsule or tablet and 100 is conversion to %.

#### 8.4.13.1.1 Assay potency for risperdal tablets (LC = 2 mg/tablet)

**Example:**

A psychotropic agent, risperidone, or risperdal (RIS) is used for the treatment of schizophrenia. It works by bringing into action a mixture of both ($5HT_2$) serotonin Type 2 and ($D_2$) dopamine Type 2 receptor antagonism. It has a very high likeness for $D_2$, H1 and $5HT_2$ histaminergic receptors and is a selective monoaminergic antagonist. It comes from benzisoxazole derivatives chemical class and is 3-[2-[4-[6-fluoro-1, 2-benzisoxazol-3-yl)-1-piperidinyl] ethyl]-6,7,8,9-tetrahydro-2-methyl-4H-pyrido-[1,2-a]-pyrimidin-4-one with molecular formula of $C_{23}H_{27}FN_4O_2$ and molecular weight of 410.4

- For using HPLC as analytical tool for potency assay of RIS in tablet dosage forms as a test for QC in our drug QC laboratories, we prepared a stock solution of the drug. This was done by using a volumetric flask of 250 mL. 10 tablets were transferred in it and almost 200 mL of methanol was added to it. Overall, it was occasionally shaken to help in the process of dissolution. Mobile phase was used to adjust the volume of the solution according to mark. From this data, we can calculate sample dilution factor,

$$\mathrm{DfS} = 1/250 = 0.004 \tag{8.22}$$

- A stock solution of the reference standard of RIS was prepared in a volumetric flask of 50 mL by transferring 49.7 mg (standard purity 98.75%) into the flask. Almost 20 mL of methanol was then added. Occasional shaking helped dissolve the RIS in the solution and the volume was then adjusted to reach the mark through the mobile phase. Again, mobile phase was made use of by diluting an estimate of 4 mL aliquot to almost 50 mL. Resultant solution was filtered through 0.45 Um membrane filter . . . . . .. From this data, we can calculate reference standard dilution factor,

$$\mathrm{DfSt} = 1/50 \times 4/50 = 0.0016 \tag{8.23}$$

- RP C-18 column equilibrated with mobile phase methanol:acetonitrile:50 mM potassium dihydrogen orthophosphate (80:10:10,v/v) was used. The flow rate in the mobile phase was kept at a constant of 1.3 mL/min, and 234 nm was the rate at which monitoring of the effluents took place. Sample injection happened with a fixed loop 20 µL. It was run for a total of only 10 min. This is depicted in Fig.8.22.

Data resulting from HPLC analysis of sample and standard are as follows:

- The sample assay preparation solution's peak API area response = 3,353,160
- ASt = Peak area responses from the API of the reference standard preparation solution = 3,374,971

From the previous data about RIS tablet and application for Eq.(8.20) on these data, %LC for RIS tablet can be calculated as shown in Eq.(8.23)

$$\%LC = \frac{3,353,160 \times 49.7 \times 0.0016 \times 0.991 \times 100}{3,374,971 \times 0.004 \times 10 \times 2} = 97.86 \tag{8.23}$$

Comparing the resulting data about %LC RIS tablet (96.5%) with the specified limit in the manufacturer specification (limit 90–110%), it can be concluded that assay for potency of the pharmaceutical product complies with manufacturer's specification and is suitable for use by patients.

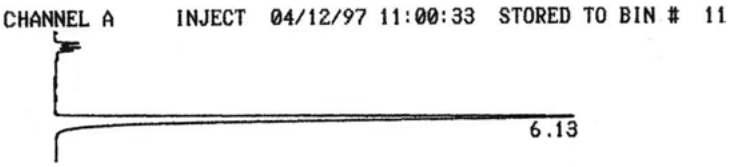



**Fig.8.22:** HPLC chromatogram obtained from assay for potency (%LC) of risperdal tablet.

#### 8.4.13.1.2 Assay potency based on a portion of the composite ground powder taken from tablet or capsules equal to the average tablet weight or average capsule weight

Even though extracting of the intact dosages can easily be done by the methods mentioned earlier, prior grinding in most of the products is required to ensure quantitative extraction is done. For doing this, a segment of the powder collected from the capsule or tablet that is equal to the average weight of the table or average weight of the capsule and might need to be assayed and extracted. These reduce the need for using a lot of extraction solvents and reduce its number. The following equation shows the %label claim

$$\%LC = \frac{AS \times StWt \times DfSt \times Stpurity \times CF \times 100}{ASt \times DfS \times LC \times SWt} \tag{8.24}$$

where %LC is percentage label claim of API per tablet or capsule,

- AS is peak area responses from the API of sample assay preparation solution, StWt is weight of standard (mg), Df St is dilution factor for standard preparation, StPurity is purity of the reference standard after correction for moisture residual solvents, and actual purity of the compound (Ex.99.9% = 99.9÷ 100 = 0.999), CF is conversion factor from salt to base = MW of drug base/MW of salt, Avg.Wt tab/caps is

average weight of tablets or capsules under analysis (average weight using 20 units), ASt is peak area responses from the API of standard preparation solution, DfS is dilution factor for sample preparation, SWt is sample weight as a powder after taking it out of 10 capsules and mixed (g); equal to the average capsules weight (in the case of tablet), LC is label claim of API per tablet or capsule, 100 is conversion to %.

**Example: Assay potency for Eulexin capsule (250 mg flutamide capsule)**
Eulexin capsules (Schering–Plough) contain nonsteroidal flutamide and nonsteroidal antiandrogen that is orally active and has the chemical name: 2-methyl-*N*-[4-nitro-3 (trifluoromethyl) phenyl] propanamide with molecular formula $C_{11}H_{11}F_3N_2O_3$ and MW = 276.21. Each capsule contains 125 or 250 mg flutamide.

Eulexin is used to treat advanced levels of prostate cancer which has reached stage D2 in men. It is a hormone therapy and can be used when there is proof that it has reached metastases, that is, the cancer has spread all over the body. It is known as an "anti-androgen." Flutamide can be eaten orally as it is a capsule. If not taken orally, the capsule can be opened and you can mix it with softer foods like applesauce. Mixing with liquids is not recommended. The daily dosage of the drug should be properly distributed in the entire day. For instance, three doses a day should be given after every 8 h.

Standard solution preparation: Into a 20 mL volumetric flask, weight accurately 25.4 mg of flutamide reference standard (purity 99.1%), dissolve and make up to *volume using mobile phase*. So, the dilution factor(DfSt) = 1 ÷ 20 = 0.05 (8.25)

Sample solution preparation: Take 20 capsules, empitted, mix the resulting powder, *a portion of the composite powder approximately equal to the average capsule weight* (ACW)(0.8 g) was transferred to 200 mL volumetric flask, dissolve and make up to *volume using mobile phase*.So, dilution factor, DfS = 1 ÷ 200 = 0.005 (8.26)

HPLC analysis for standard and samples resulted in the following data:
- Peak area responses from the API of sample assay preparation solution =6, 493, 921
- Peak area responses from the API of the reference standard preparation solution =6, 033, 927

We have applied the previously obtained equations eqs. (8.24–8.26) on the data obtained from HPLC assay for Eulexin capsules; %LC can be calculated as present in Eq.(8.27)

$$\%LC = \frac{6,493,921 \times 25.4 \times 0.05 \times 0.991 \times 0.760 \times 100}{6,033,927 \times 0.005 \times 250 \times 0.8} = 102.94 \tag{8.27}$$

By comparing the resulting data of %LC Eulexin capsules (102.94%) with the specified limit in the manufacturer specification (limit 90–110%), it can be concluded that assay for potency of the pharmaceutical product complies with the manufacturer's specification and is suitable for use by patients.

#### 8.4.13.1.3 Assay potency for APIs in liquid form of pharmaceutical products

**Example 1: Assay for chlorhexidine gluconate in KWIK surgical scrub skin cleaner 4% (W/V) solution (manufacturing by Al Sharhan Industries, Kuwait)**

Surgical scrub skin cleaner 4% (W/V**)** solution (manufacturing by Al Sharhan Industries, Kuwait) is part of a medicine group called antiseptics. These drugs prevent any sort of infections by killing the germs of the infection which are present on the human skin. This is also known for removing the skin's surface bacteria. The solution is red, clear and slightly sticky and can be found in white bottles of either 5 L, 500 mL or 250 mL of liquid. It is used as a hand disinfectant by the surgical medical staff at the Ministry of Health. KWIK can also be used by the patients for skin antisepsis before operation. The method of using it is simple. The patient bathes in it two times a day before the operation and on the day of the operation.

We have five batches from KWIK surgical scrub skin cleaner 4% (W/V) under QC in our laboratories. For that purpose assay for chlorhexidine gluconate (g/100 mL) in our consideration. For that purpose, the following solutions were prepared as follows:

**Reference standard solution preparation:**

– 14.4mg chlorohexidine acetate (96.3%) was dissolved in 100 mL methanol. So, dilution factor for the standard solution preparation

$$(\text{Df St}) = 1/100 = 0.01 \ldots.. \tag{8.28}$$

**Sample solution preparation**

– Weight of sample as shown in Tab 8.6 was taken from each batch and dissolved in 25 mL methanol. So, dilution factor for sample solution preparation is

$$(\text{DfS}) = 1/25 = 0.04 \ldots\ldots\ldots\ldots \tag{8.29}$$

From the following equations Eq.(8.30) and Eq.(8.31), we can calculate the amount of:

Chlorohexidine gluconate (g/mL)

$$= \frac{\text{AS} \times \text{StWt} \times \text{DfSt} \times \text{D} \times \text{Stpurity} \times \text{CF}}{\text{ASt} \times \text{DfS} \times \text{SWt} \times 1,000} \tag{8.30}$$

$$\text{Chlorohexidine gluconate}\,(\text{g}/100\,\text{mL}) = \text{Chlorohexidinegluconate}\,(\text{g}/\text{mL}) \times 100 \ldots\ldots \quad (8.31)$$

**Tab 8.7:** HPLC analytical data for five batches of KWIK surgical scrub skin cleaner 4% (W/V).

| Batch No. | Average area of sample (AS) (three replicates) | Weight of sample (g) (WS) | Density | %Chlorhexidine gluconate (w/v) found in the finished product | Results according to the specified limit of the manufacturer (3. 6–4.4%, w/v) |
|---|---|---|---|---|---|
| 1 | 4,585,456 | 0.1055 | 1.034 | 4.24 | Amount of Ch. gluconate is in agreement with labeled |
| 2 | 3,931,752 | 0.09 | 1.033 | 4.26 | Amount of Ch. gluconate is in agreement with labeled |
| 3 | 4,398,472 | 0.1010 | 1.034 | 4.25 | Amount of Ch. gluconate is in agreement with labeled |
| 4 | 4,728,966 | 0.1085 | 1.032 | 4.24 | Amount of Ch. gluconate is in agreement with labeled |
| 5 | 4,155,795 | 0.1000 | 1.0 33 | 4.05 | Amount of Ch. gluconate is in agreement with labeled |
| Average standard peak area (ASt) (three replicates) | 5,267,177 | | | | |

HPLC chromatogram obtained from assay for potency (%LC) chlorhexidine gluconate is found in Fig.8.23.

where AS is peak area responses from the sample assay preparation, StWt is weight of standard (mg), Df St is dilution factor for standard preparation = 1 ÷ 100 = 0.01

- Stpurity is 96.3 ÷ 100 = 0.963, SWt is sample weight (g), according to Tab.8.7, ASt is peak area responses from the standard preparation, DfS is dilution factor for sample preparation = 1 ÷ 25 = 0.04, D is sample density, according to Tab.8.7, and was determined by the analyst for sample according BP, CF is conversion factor from salt to base = MW of drug base/MW of salt = molecular weight of chlorhexidine gluconate÷ molecular weight of chlorhexidine acetate = 898.1÷ 625.6, 1 ÷ 1,000 is for transformation of mg to g and 100 is conversion to %.

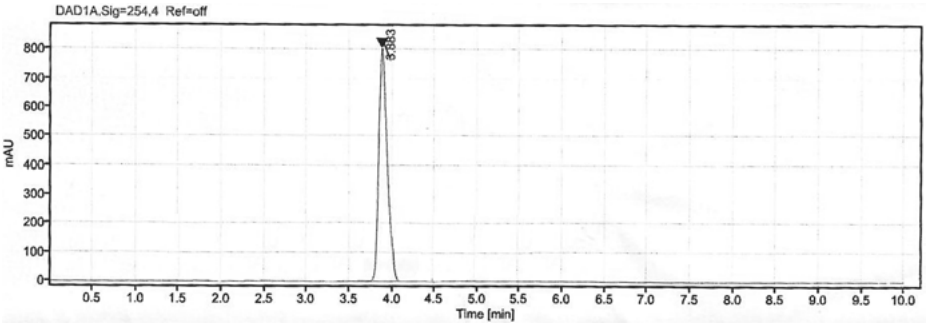



**Fig.8.23:** HPLC chromatogram obtained from assay for potency (%LC) chlorhexidine gluconate.

Applying Eq.(8.30) and Eq.(8.31) on the data of batch No. 5 (Tab 8.6), the results obtained can be found as following

Chlorohexidine gluconate (g/100 mL) (in Batch No.5) =

$$\frac{4155795 \times 14.4 \times 0.01 \times 0.963 \times 1.033 \times 100}{5,267,177 \times 0.04 \times 0.1 \times 1,000} \times \frac{898}{625.6} = 4.05\% \qquad (8.32)$$

Similarly, we have applied Eq.(8.30) and (8.31) on the data found in Tab.8.7 regarding Batches 1, 2, 3 and 4;%chlorhexidine gluconate can be calculated as found in Tab.8.6. Comparing results of %chlorhexidine gluconate for each batch with the specified limit of the manufacturer (4.5–5.5%, w/v), we can say that the determined amount of chlorhexidine gluconate is in agreement with labeled five batches of KWIK surgical scrub skin cleaner and the product can be used according its purpose.

**Example 2: Assay potency for APIs in tripofed DM syrup**

Tripofed DM Syrup (Saudi Pharmaceutical Industries Co., Kuwait) contains dextromethorphan, pseudoephedrine and triprolidine as active ingredients. This is a combination medicine and is mostly used to give relief to the symptoms that occur because of allergies, common cold and flu. It also works for breathing issues like bronchitis and sinusitis. To get rid of itchy eyes/throat/nose, watery eyes and sneezing, antihistamines are used. For symptoms related to ear congestion and stuffy nose, decongestants are used. Tripofed DM syrup narrows the blood vessels of the nasal passage and also reduces swelling. This does not allow histamine to act. As a result, the activity in the brain that causes coughing decreases.

Tab.8.8 summarized specifications about tripofed DM syrup. The product is under QC in our laboratories. For that purpose, the following solutions were prepared.

**Tab 8.8:** Active ingredient, LC and limit of assay according to the manufacturer's specification for tripofed syrup.

| No. | Pharmaceutical active ingredient in tripofed syrup | LC | Limit according to the manufacturer specification |
|---|---|---|---|
| 1 | Triprolidine HCl | 1.125 mg Triprolidine HCl/5 mL | **1.125–1.375 mg** Triprolidine HCl/5 mL |
| 2 | Pseudoephedrine HCl | 30 mg Pseudoephedrine HCl/5 mL | 27–33 mg/5 mL Pseudoephedrine HCl/5 mL |
| 3 | Dextromethorphan HBr | 10 mg Dextromethorphan HBr/5 mL | 9–11 mg Dextromethorphan HBr/5 mL |

**Reference standard solution preparation.**

Take a volumetric flask of 50 mL, weigh accurately 25 mg of triprolidine HCl reference standard (USPRS), dissolve and make up to volume using 0.01 NHCl. Next, 5 mL of the total solution should be transferred into a volumetric flask of 50 mL containing 60 mg of pseudoephedrine HCl (USPRS) and 20 mg of dextromethorphan HBr (USPRS) accurately weighted. Dissolve in 0.01 N HCl and make up to volume with the same solvent. From these data, the dilution factor (DfSt) for the standards solution of triprolidine HCl, pseudoephedrine HCl and dextromethorphan HBr can be calculated as follows:

$$\text{Dilution factor for the standards solution of triprolidine HCl} = 1 \div 50 \times 5 \div 50 = 0.002 \ldots\ldots\ldots \tag{8.33}$$

$$\text{Dilution factor for the standards solution of pseudoephedrine HCl} = 1 \div 50 = 0.02 \ldots\ldots\ldots\ldots \tag{8.34}$$

$$\text{Dilution factor for the standards solution of dextromethorphan HBr} = 1 \div 50 = 0.02 \ldots\ldots\ldots \tag{8.35}$$

**Sample solution preparation**

10.1 g of the finished product is dissolved in 50 mL of 0.01N HCl. From this data, we can calculate dilution factor for sample.

$$\text{Dilution factor for assay solution} = 1 \div 50 = 0.02 \ldots\ldots \tag{8.36}$$

**Method**

Equal volumes of both the assay preparation and standard solution for preparation need to be injected into the HPLC separately. About 20 µL of both should be taken. The chromatogram needs to be recoded and measurements for the peak area of HCl, dextromethorphan HBr, pseudoephedrine HC and triprolidine need to be taken and recorded.

Using Eq.(8.37), we can calculate %LC for each API as follows:

$$\%\mathrm{LC} = \frac{\mathrm{AS} \times \mathrm{StWt\,(mg)} \times \mathrm{DfSt} \times \mathrm{Stpurity} \times \mathrm{Wtper\,mL} \times 5 \times 100}{\mathrm{ASt} \times \mathrm{Wt\,S\,(g)} \times \mathrm{DfS} \times \mathrm{LC}} \tag{8.37}$$

where %LC is percentage label claim of API per tablet or capsule, AS is peak area responses from the API of sample assay preparation solution, StWt is weight of standard (mg), Df St is dilution factor for standard preparation, StPurity is the reference standards purity once it has been corrected for moisture residual solvents, and actual purity of the compound (Ex.99.1% = 99.1÷ 100 = 0.991), ASt is peak area responses from the API of standard preparation solution, DfS is dilution factor for sample preparation, WtS is weight of sample (g), LC is label claim of API per capsule or tablet and 100 is conversion to %

HPLC data obtained from analysis of tripofed syrup are found in Tab 8.8. Applying Eq.(8.24) on these data, we can calculate mg/mL and %LC for the three APIs triprolidine HCl, pseudoephedrine HCl and dextromethorphan HBr in tripofed syrup as shown in Eq.(8.28)–(8.30). The resulting data are found in Tab.8.9.

**Tab 8.9:** HPLC data obtained from analysis of tripofed MD syrup.

| No. | Name of the APIs | Peak area sample (average three replicates) | Peak area standard (average three replicates) | mg/5 mL (limit, mg/5 mL) | % LC (limit as 100) | Results |
|---|---|---|---|---|---|---|
| 1 | Triprolidine HCl | 196,901 | 229,791 | 1.250 (1. 125–1.3750 | 90–110 | Pass |
| 2 | Pseudoephedrine HCl | 373,444 | 432,581 | 29.52 (27–33 mg/5 mL) | 90–110 | Pass |
| 3 | Dextromethorphan HBr | 68,271 | 73,570 | 10.576 (9–11 mg/mL) | 90–110 | Pass |

HPLC chromatogram obtained from assay for potency (%LC) of tripofed MD syrup is found in Fig.8.24.

$$\%\mathrm{LC\ triprolidine\ HCl} = \frac{196,901 \times 25 \times 0.002 \times 0.991 \times 1.15 \times 5 \times 100}{229,791 \times 10.1 \times 0.02 \times 1.25} = 96.9 \tag{8.38}$$

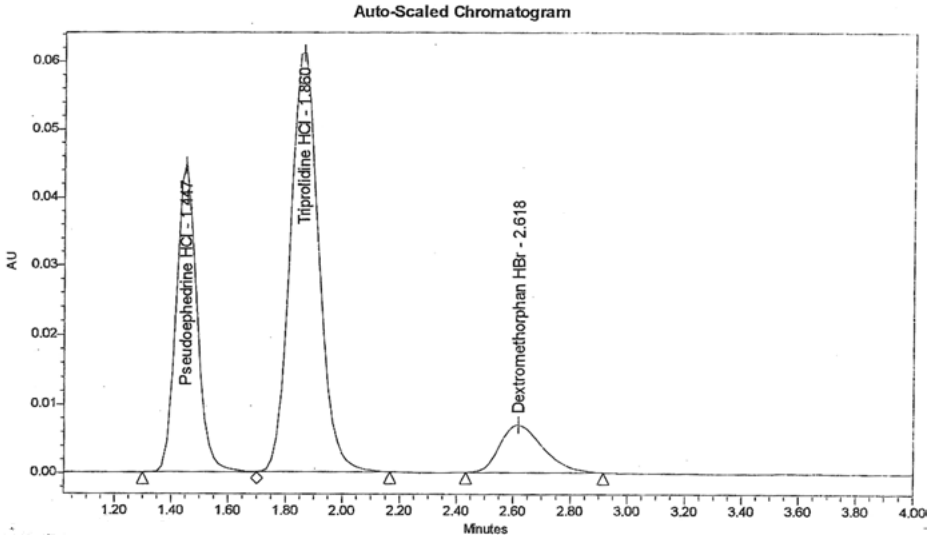



**Fig.8.24:** HPLC chromatogram obtained from assay for potency (%LC) of tripofed MD syrup.

$$\%\text{LC pseudoephedrine HCl} = \frac{373,444 \times 60 \times 0.02 \times 1.15 \times 0.991 \times 5 \text{ X } 100}{432,581 \times 10.1 \times 0.02 \times 30} = 97.4 \tag{8.39}$$

$$\%\text{LC dextromethorphan HBr} = \frac{68,271 \times 20 \times 0.02 \times 1.15 \times 5 \times 0.991 \times 100}{73,570 \times 10.1 \times 0.02 \times 10} = 104.7 \tag{8.40}$$

Comparing the resulting data about %LC triprolidine HCl (96.9%), %LC pseudoephedrine HCl (97.4%) and %LC dextromethorphan HBr (104.7%; 102.94%) with the specified limit in the manufacturer specification (limit 90–110%), it can be concluded that assay for potency of the pharmaceutical product complies with manufacturer's specification and it is suitable for use by patients

### 8.4.13.2 Assay of preservatives

A substance that can be used to prolong the shelf life of drugs is known as a preservative. It works by stopping the process of oxidation and also by preventing the microbial growth [102].

For semisolid and liquid dosage forms of pharmaceutical drugs, it is important to add preservatives in the formulation of the drug dosage. The most common forms of preservatives used are sorbic acid, sodium benzoate, EDTA, parabens and acid [103]. Butylated hydroxytoluene, an antioxidant, usually is used as a preservative for solid dosage forms.

As preservatives are active components, their percentage in the products needs to be monitored properly. Testing the preservatives is also very important if a percentage of the particular preservative is added in the DP to ensure that the shelf life of it is increased.

Using a universal and generic method HPLC is recommended for analyzing the preservatives that are used in the solid and liquid formulations of the pharmaceutical drugs. This is done for routine monitoring to make sure that the preservatives are stable and need to be validated for the form of dosage they are being used [102].

Typically, the assay specifications for the additive components is almost 85–115% of label claim.

**Example: Assay for benzalkonium chloride in a FML eye drops (FML is Medicine Brand Name manufactured by NPS Medicine Wise , Australia ,https://www.nps.org.au )**

Benzalkonium chloride has antiseptic properties and is a ammonium quaternary salt, similar to other cationic surfactants. The general formula for it is $[C_6H_5CH_2N(CH_3)_2R]Cl$, where R represents *n*-$C_8H_{17}$ to *n*-$C_{18}H_{37}$. The mode of action of quaternary ammonium compounds seem to be connected with the effect on the cytoplasmic membrane. This is what controls the permeability of the cells. There is extensive data reported for the effective concentration (0.01%–0.1%) and the bacterial species affected. The homologs n-C12 and n-C14 in benzalkonium chloride products comprise a major portion of the alkyl group. In general, the homolog n-C12 is most effective against yeast and fungi, and the homolog n-C14 against gram-positive bacteria. Benzalkonium chloride is not effective against gram-negative bacteria, and is not recommended for treatment when sanitization is critical. The CN stationary phase feature is effectively deactivates the ionic contact between the cationic surfactants and the silica surface, resulting in excellent peak shapes for cationic benzalkonium chloride. In the following, there is an example showing that the total amount of benzalkonium chloride in a FML liquid film eye drops was determined using HPLC as analytical tool. According to the manufacturer's specifications, the following solutions were prepared:

**Reference standard solution preparation**

Measure 4 mL of BAC standard (50% in water) and transfer to 25 mL volumetric flask and dilute to mark using water. From this solution, dilute 1 mL in 20 mL volumetric flask using water. So, the dilution factor

$$\text{DfSt} = \frac{1}{25} \times \frac{1}{20} = 0.002 \ldots\ldots \tag{8.41}$$

HPLC chromatogram obtained from assay for potency (%LC) of benzalkonium chloride, as preservative for FML eye drops, is found in Fig.8.25.

Sample solution preparation. From the sample, 2 mL was diluted to 20 mL using water in volumetric flask. So, dilution factor is

$$\text{DfS} = \frac{1}{20} = 0.05 \tag{8.42}$$

$$\%\text{BZCl} = \frac{\text{As}(\text{C12} + \text{C14} + \text{C16}) \times \text{St Wt} \times \text{Stpurity} \times \text{DfSt} \times 100}{\text{ASt}(\text{C12} + \text{C14} + \text{C16}) \times \text{VS} \times \text{DfS} \times 1,000} \tag{8.43}$$

$$\%\text{BZCl} = \frac{742,229 \times 4 \times 50 \times 0.002 \times 100}{735,552 \times 2 \times 0.05 \times 100 \times 1,000} = 0.0040 \tag{8.44}$$

Limit %BZCl = 0.0036–0.0056

The determined amount of BZCl in the FML eye drops is in agreement with the labeled value.

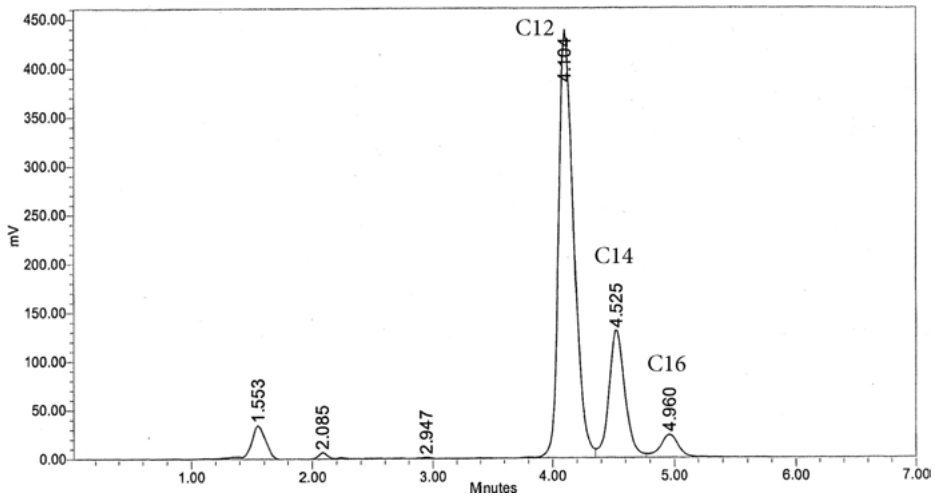



**Fig.8.25:** HPLC chromatogram obtained from assay for potency (%LC) of benzalkonium chloride as preservative for FML eye drops.

### 8.4.13.3 Content uniformity test

The common method to assay capsules and tablets individually is to perform content uniformity (i.e., formulate DP). This is done to make sure that the processing of manufacturing the drugs is consistent. The content uniformity of the final dosage form of the finished dosage is a major consideration when it comes to quality testing for the product release and development. In accordance with USP [101, 104],

both composite assay and uniformity test are same, except assay of an individual tablet/capsule is conducted to ensure processing consistency in industry. Generally, 10 single-tablet assays are required. The minimum criteria for acceptance of the dosage unit uniformity must lie between the range of 85.0% and 115.0% of the label claim. A RSD of only 6% is acceptable. In case this criteria is not fulfilled, additional testing of more tablets is needed according to the guidelines of USP (USP General Chapter < 905 > Uniformity of Dosage Units USP [59]. Looking for specific techniques to prepare samples for content uniformity do not exist. Recently, HPLC analysis is the most popular technique to quickly conduct uniformity tests. In the following, we will present an example of the content uniformity test for one solid dosage form, Sinemet tablet.

**Example: Content uniformity for Norvasc tablets 10 mg.**
In 1982, a patent was approved for Norvasc tablet (amlodipine). It was later approved to be used medically in 1990. Now, it has made to the list of essential medicines recommended by the World Health Organization (World Health Organization's List of Essential Medicines) [105]. By 2017 [105], it was the fifth most prescribed medicine in the US, having been prescribed for more than 72 million times. It is used in the treatment of high blood pressure. By lowering the blood pressure, many problems like kidney problems and strokes can be prevented. Amlodipine is the member of the drug class "calcium channel blockers." It relax the blood vessels to allow the blood to flow through the vessels.

Norvasc tablet, 10 mg/tablet, is under QC in our laboratories. According to the manufacturer's specification, a validated analytical method for assay was used for study content uniformity for Norvasc tablets. For that purpose, the following solutions were prepared:

**Reference standard solution preparation**
10.7 mg of amlodipine besylate (purity 99.1%) needs to be mixed in a volumetric flask of 100 mL, dissolved and made up to volume using mobile phase. 5 mL of the solution needs to be transferred to a volumetric flask of 20 mL and should be brought up to the mark by making use of the mobile phase. So, the dilution factor calculated is

$$\mathrm{DfSt} = \frac{1}{100} \times \frac{5}{20} = 0.0025 \ldots . \tag{8.45}$$

**Sample solution preparation**
In an individual 10 volumetric flasks capacity 100 mL, add one Norvasc tablet, 10 mg for each, dissolve and make up to volume using mobile phase. 5 mL of the solution needs to be transferred to a volumetric flask of 20 mL and should be brought up to the mark by making use of the mobile phase. So, the dilution factor calculated is

$$\mathrm{DfS} = \frac{1}{100} \times \frac{5}{20} = 0.0025 \tag{8.46}$$

Inject 10 μL from sample and standard solutions into the recommended HPLC operated at the conditions mentioned in the manufacturer's specification.

Data in Tab.8.10 show HPLC content uniformity assay of Norvasc tablet, 10 mg.

**Tab 8.10:** HPLC analysis for 10 individual tablet of Norvasc, 10mg, for measuring content uniformity.

| No. of tablet | HPLC peak area for sample | %LC found | 85–115% of the average content |
|---|---|---|---|
| 1 | 2,968,219 | 98.12 | Yes |
| 2 | 2,952,253 | 97.6 | Yes |
| 3 | 2,963,196 | 97.95 | Yes |
| 4 | 3,188,895 | 105.41 | Yes |
| 5 | 2,977,245 | 98.42 | Yes |
| 6 | 2,962,197 | 97.92 | Yes |
| 7 | 2,965,198 | 96.35 | Yes |
| 8 | 2,967,298 | 97.65 | Yes |
| 9 | 2,759,219 | 91.21 | Yes |
| 10 | 2,967,970 | 98.11 | Yes |
| Average | 2,967,168 | – | Yes |
| %RSD | 3.422 | – | – |
| Average HPLC peak area for reference standard (three replicates) | 2,312,989 | – | – |
| Result | | | Pass |

Using external standard and one-point calibration method, the following equation can be used for the calculation of %LC of Norvasc tablet

$$\%\mathrm{LC} = \frac{\mathrm{AS} \times \mathrm{StWt} \times \mathrm{DfSt} \times \mathrm{Stpurity} \times \mathrm{CF} \times 100}{\mathrm{ASt} \times \mathrm{N} \times \mathrm{DfS} \times \mathrm{LC}} \tag{8.47}$$

where %LC is percentage label claim of API per capsule or tablet, AS is peak area responses from the API of sample preparation solution, StWt is weight of standard (mg), Df St is dilution factor for standard preparation, StPurity is the reference standard of the purity after correcting for residual solvents, moisture and actual purity of the compound (Ex.99.9% = 99.9 ÷ 100 = 0. 999), CF is conversion factor from salt

to base = MW of drug base/MW of salt. In this case, the API is amlodipine besylate in the reference standard (MW = 567.1) and the API in the label claim of the product is amlodipine (MW = 408.9), CF = 408.9÷ 567.1).

- ASt is peak area responses from the API of standard preparation solution, DfS is dilution factor for sample preparation, Ntable/caps is number of tablets or capsules tested = 1, LC is label claim of API per tablet or capsule and
- 100 is conversion to %

**Content uniformity test procedures**

1. Nothing less than a total of 30 units should be selected.
2. According to the specification of the manufacturer, 10 units assays need to be used individually.
3. The contents of the active ingredients can be calculated from the assay for each unit.
4. Eq.(8.48) and Eq.(8.47) can be used to calculate the %LC.
5. Calculate $X^- = \sum Xi/n$ (no. of tablets tested)
6. Calculate standard deviation $(S) = [(Xi–X^-)2/(n-1)]^{1/2}$
7. Calculate RSD = $(S/X^-)$*100

Application of Eq.(8.47) and Eq.(8.48) on the data in Tab.8.9 related to tablet no. 1, %LC for Norvasc 10 mg tablet can calculated as shown in Eq.(8.48)

$$\%\mathbf{LC} = \frac{2,968,219 \times 10.7 \times 0.0025 \times 0.991 \times \ 408.9 \times 100}{2,312,989 \times 1 \times 0.0025 \times 567.1 \times 10} = \mathbf{98.12} \tag{8.48}$$

Similarly, we have applied Eq.(8.48) on the data found in Tab 8.9 regarding tablets 2–10, %LC of amlodipine can be calculated as found in Tab 8.9.

**Criteria:**

Results of content uniformity test are accepted, if the %LC of the 10 tablets lies within the range of 85–115% and %RSD of the 10 tablets is less than or equal to 6%. Suspected if one %LC is outside the range 85–115% but not outside the range of 75– 125% or RSD is greater than 6% or both conditions, test another 20 tablets. Not more than one %LC of 30 tablets are outside the range of 85–115%. None outside the range 75–125%. RSD of the 30 tablets is less than or equal to 7.8%.

### 8.4.13.4 Dissolution testing

Any solid drug that enters the human body through the stomach must first be disintegrated into small aggregates and eventually dissolved. So, measuring DP solubility has become mandatory. To test the solubility of the drug, dissolution testing is used. It is used to compute the APIs being released from the pharmaceutical dosages of the drugs like tablets/capsules when they are in standard conditions. These

conditions are the ones that have been specified by the USP. Paddle method (Type II) and basket method (Type I) or other forms of apparatuses are used [101].

The commonly used apparatus of dissolution tests are USP Type I (basket method) and Type II (paddle method) in vessel, which is more suitable for immediate release tablets, where sampling typically occurs a t5–15 min intervals for a total of 1–2 h. The medium selected for the dissolution test must consider the solubility of the tested drug. Aqueous media with a typical pH range between 1 and 7 to mimic the human gastrointestinal tract are preferred over organic solvents. The operating parameters of the dissolution setting should be optimized to ensure complete dissolution.

Dissolution as in vitro evaluation for drug solubility can both be correlated and compared to the in vivo bioavailability of the solid dosage formulation that is found in different clinical studies. To check the consistency of the product in the formulation development phase, product release phase and the stability studies, dissolution testing is conducted [5].

Most of the drugs found are chromophoric. This is why the analytical technique used for conducting dissolution testing is HPLC and UV spectrometry. HPLC has advantages when compared to UV and these are higher sensitivity and specificity, stability indication and an applicability to analyze the formulations with low doses and many APIs [26]. Generally, the isocratic HPLC assay methods for potency determination are validated and adopted for dissolution testing [5].

#### 8.4.13.4a Choice of the dissolution apparatus

Preferably, the apparatus that allows observing the pharmaceutical product that is being examined and the stirrer while the test is being performed. Depending on the physicochemical properties of the form of drug dosage, the apparatus should be selected. The portion of the apparatus that will be coming into direct contact with either the dissolution medium or the preparation needs to be inert, so it does not interfere, and chemically react or adsorb with the sample being tested. The metallic parts of the selected apparatus that have a chance of contacting the dissolution medium or the preparation need to be made of stainless steel. If not, they should be coated with a material suitable to make sure that they do not interfere or cause a reaction with the dissolution medium or the preparation. Going for an apparatus that allows the analyst to observe both the stirrer and the preparation is best.

– **Paddle apparatus**

According to the European Pharmacopeia [26] (Fig.8.26), the apparatus consists of:
A vessel in the shape of a cylinder and made of borosilicate glass or any other material that is suitable and transparent, having a 100 mL nominal capacity and a hemispherical bottom. There is a cover fitted on it to retard the evaporation process; in the center of the cover, there is a hole that allows the stirrers shaft to probe inside along with holes for the thermometer and other things that are needed to take out

liquid. The stirrer is made of a vertical shaft that has the lower end attached to a blade. It is in the form of a circle that is subtended with two parallel chords. To make the bottom of the shaft and blade's bottom flush, it is passed through the shaft's diameter. The placement of the shaft is such that the axis is within 2 mm of

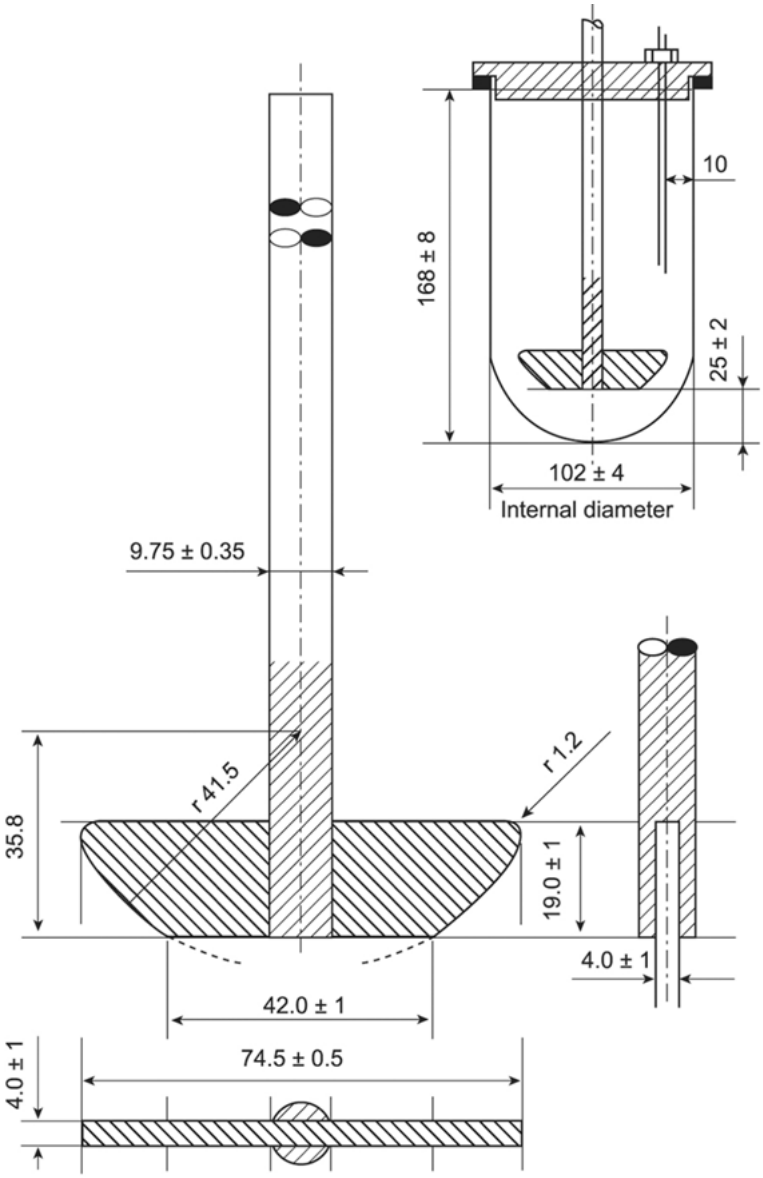



**Fig.8.26:** Schematic diagram showing dissolution apparatus (paddle type; dimensions are in millimeters).

the axis of the vessel and the blades bottom is around 25 ± 2 mm from the inner bottom of the vessel; a motor is connected to the top of the shaft and has a speed regulator with it. All of this allows the stirrer to move and rotate in a smooth motion with no wobble; the water bath is used to maintain the dissolution medium at a constant 37 ± 0.5 °C. In Fig.8.27, there is a diagram for a commercial dissolution apparatus (paddle type, manufactured by DISTEK company, USA, sales@distekinc.com).


**Fig.8.27:** Commercial dissolution apparatus (paddle type, manufactured by DiSTEK company, USA, sales@distekinc.com).

– **Basket apparatus.**

According to the European Pharmacopeia [26] (Fig.8.28), the apparatus consists of:
The vessel is identical to the one used in the paddle apparatus; a stirrer that is made up of a vertical shaft, the end part has a cylindrical basket attached to it. It is further divided into two portions: the portion at the beginning that has a 2 mm vent and is welded firmly on the shaft instrument that allows to remove the bottom part

of the basked. Mostly, spring clips are used and three should be enough. The bottom of the basket needs to be removed to introduce the preparation that has been prepared to undergo the examination process. It holds the bottom of the concentric firmly with the axis to ensure that the vessel rotates properly. The bottom of the basket is made of seam cloth that is welded and forms into a cylinder and has a slender rim of metal that is wrapped around it at the bottom and beginning, unless it has been prescribed otherwise. The diameter of the cloth is 0.254 mm in diameter and has a square opening of 0.381 mm for tests that need to be carried out in a medium of dilute acid, 2.5 µm thick baskets may be used, the bottom of which should be 25 ± 2 mm from the inner bottom of the vessel during the test. A motor is connected to the top of the shaft and has a speed regulator with it. All of this allows the stirrer to move and rotate in a smooth motion with no wobble; the water bath is used to maintain the dissolution medium at a constant 37 ± 0.5 °C.

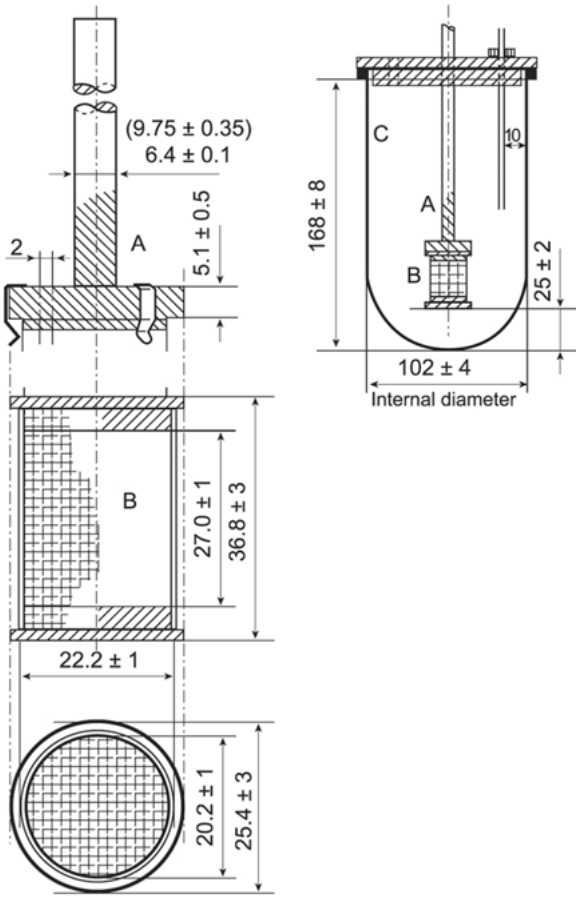



**Fig.8.28:** Schematic diagram showing dissolution apparatus (basket type; dimensions in millimeters).

#### 8.4.13.4b Dissolution medium

Consider the solubility of the drug before selecting the medium to conduct the dissolution test. Typically, the pH range for aqueous media is anywhere between 1 and 7. This is the range on which the human gastrointestinal tract is mimicked and this is why it is better than organic solvents. To ensure that dissolution is complete, the operating parameters need to be optimized.

In case the medium for dissolution is buffered, it can be adjusted. For this, you need to adjust the pH level to ±0.05 units of the level prescribed. Any sort of dissolution gases needs to be removed before the test is conducted. The dissolution medium should not have any gas as it can negatively affect the outcome by causing bubbles to form.

When the basket apparatus is being used, you need to put the preparation in a clean dry basket and then lower it before the rotation process starts. There should be no air bubbles on the preparations surface. Without wasting time, the rotation should be started at a rate of ±4%, which has been prescribed.

#### 8.4.13.4c Dissolution procedures

- **Paddle and basket apparatus**

After the dissolution medium's stated volume is put into the vessel, the apparatus needs to be assembled. With the help of a thermometer, warm the dissolution medium till it reaches a temperature of 37 ± 0.5 °C. Once done, you can take out the thermometer. First, one unit of the pharmaceutical drug that is going to be examined should be placed in the apparatus. In case the paddle apparatus is being used, as mentioned earlier, the product goes in the vessels bottom before rotation. If the drug dosage form is floating, it needs to be put horizontally in the vessels bottom like the glass helix or wire. When the basket apparatus is being used, the product should be placed and lowered in the dry basket prior to rotation. It is imperative to avoid air bubbles on the preparations surface. Once all is done, immediately start the apparatus at the speed prescribed. At the end of dissolution test, the API of the drug in the dissolution media can be directly (or after concentration, Section 7.2.7) determined by injecting a desired volume in HPLC system.

#### 8.4.13.4d Sampling and sample analysis

For both the basket apparatus and the paddle apparatus, it is important to withdraw them at the time prescribed, or the intervals prescribed or continuously, the volume(s) prescribed from a position between the top of the blade/basket to the dissolution medium and not anything less than a value of 10 mm from the wall of the vessel. An inert filter that has the right pore size should be used for filtering the liquid that was removed. It should not lead to a significant level of adsorption of the ingredients active in the solution. Moreover, it should not have any substance

that can easily be extracted from the dissolution medium as it will interfere with the analytical method prescribed. Filtrate analysis should be done as has been prescribed. The denoted dissolution quantity of the active ingredient in a given time is defined as a percent of the content that is specified on the label.

Most of the drugs found are chromophoric. This is why the analytical technique used for conducting dissolution testing is HPLC-UV and UV spectrometry. HPLC-UV has the advantage of higher sensitivity and specificity, stability indication and the ability to analyze formulation with many API doses [5]. Generally, assay methods involving isocratic HPLC for potency determination are validated and adopted to conduct dissolution testing [5, 101].

At the end of dissolution test, the API of the drug in the dissolution media can be directly (or after concentration, Section 7.2.7 Chapter 7) determined by injecting a desired volume in HPLC system.

In the following, we will present an example of the dissolution test of one solid dosage form, Sinemet tablet.

**Example: Dissolution test for Sinemet tablets (carbidopa and levodopa tablets, 25 + 250 mg/tablet)**

Sinemet is a tablet that comes in three strength forms: Sinemet 10–100 that contains 100 mg of levodopa and 10 mg of carbidopa, Sinemet 25–250, which has levodopa 250 mg and carbidopa 25 mg and sinemet 25–100, which has 100 mg levodopa and 25 mg of cabidopa. Combination of levodopa and carbidopa is mostly used to treat diseases like Parkinson's. It is sometimes called paralysis agitans or shaking palsy. It is a central nervous system disorder (spinal cord and brain). A naturally occurring brain substance, dopamine, helps with controlling the activities and movements of the body like talking and walking. Patients who are suffering with Parkinson's lack the presence of dopamine in some of the sections of the brain. Levadopa enters inside the brain and changes the dopamine presence in the human brain. When the quantity of dopamine is increased, the levodopa in the brain gives you control over the normal symptoms which you perform daily like handling utensils, walking and dressing. Carbidopa stops the breakdown of the levodopa that is present in the bloodstream; this allows for a higher quantity of the levodopa to come inside the brain. Carbidopa reduces the side effects like nausea and vomiting caused by levodopa.

**Dissolution test for Sinemet tablets (carbidopa and levodopa tablets, 25 + 250 mg/tablet)**

One of the most important parameters for QC in drug QC laboratories is undertaken in our laboratories. For that purpose, the following solutions were prepared:

- **Reference standard solution preparation.** In 50 mL volumetric flask, add 55.2 mg carbidopa reference standard (92.6%), dissolve and complete to the volume using 0.1 N HCl. Transfer 5 mL from this solution to 100 mL volumetric

flask which contains 33.5 mg of levodopa reference standard (99.1%), dissolve and complete to the volume using 0.1 N HCl. Dilution factor for

$$\text{Carbidopa} = \frac{1}{50} \times \frac{5}{100} = 0.001 \ldots\ldots \tag{8.49}$$

$$\text{Dilution factor for levodopa} = \frac{1}{100} = 0.01 \ldots\ldots\ldots \tag{8.50}$$

– **Sample solution preparation:** Six individual tablets are dissolved in 750 mL of dissolution media (0.1 N HCl). Dilution factor,

$$\text{DfS} = \frac{1}{750} = 0.0013 \ldots\ldots \tag{8.51}$$

Using external standard and one-point calibration method, the following equations can be used for calculation of %APIs (carbidopa and levodopa) dissolved in relation to the LC of Sinemet tablet as shown in the following equations:

$$\%\text{Carbidopa dissolved} = \frac{\text{AS} \times \text{StWt} \times \text{DfSt} \times \text{Stpurity} \times \text{CF} \times 100}{\mathbf{ASt} \times \mathbf{N} \times \mathbf{DfS} \times \mathbf{LC}} \tag{8.52}$$

where

%LC is percentage label claim of API per tablet or capsule, AS is peak area responses from the API of sample preparation solution, StWt is weight of standard (mg), Df St is dilution factor for standard preparation, StPurity is purity of the reference standard after correction for moisture, residual solvents, and actual purity of the compound (e.g., 99.9% = 99.9÷ 100 = 0. 999), CF is 1, ASt is peak area responses from the API of standard preparation solution, DfS is dilution factor for sample preparation, Ntable/caps is number of tablets or capsules tested = 1, LC is label claim of API per tablet or capsule and

100 is conversion to %.

%levodopa dissolved

$$= \frac{AS \times StWt \times DfSt \times Stpurity \times CF \times 100}{ASt \times N \times DfS \times LC} \tag{8.53}$$

where

%LC is percentage label claim of API per tablet or capsule, AS is peak area responses from the API of sample preparation solution, StWt is weight of standard (mg), Df St is dilution factor for standard preparation, StPurity is purity of the reference standard after correction for moisture,residual solvents, and actual purity of the compound(Ex.99.9% = 99.9 ÷ 100 = 0. 999), CF is 1, ASt is peak area responses from the API of standard preparation solution, DfS is dilution factor for sample

preparation, Ntable/caps is number of tablets or capsules tested = 1, LC is label claim of API per tablet or capsule and 100 is conversion to %.

Application of Eq.(8.52) and Eq.(8.53) on the data found in Tab 8.10 and 8.11 in relation to Tablet No.1, %dissolved carbidopa and %dissolved of levodopa from Sinemet tablet can be calculated as follows:

%Carbidopa dissolved =

$$\frac{205,261 \times 36.1 \times 0.001 \times 0.926 \times 1 \times 100}{204,537 \times 1 \times 0.0013 \times 25} = \mathbf{102.824} \tag{8.54}$$

%Levodopa dissolved =

$$\frac{2,998,200 \times 33.5 \times 0.01 \times 0.991 \times 1 \times 100}{2,978,512 \times 1 \times 0.0013 \times 250} = 100.68 \tag{8.55}$$

Similarly, we have applied Eq.(8.45) and Eq.(8.55) on the data found in Tab.8.11 and Tab.8.12 regarding to tablets No. 2–6, %levodopa and % carbidopa dissolved from Sinemet tablet

as found in Tab.8.11 and Tab.8.12.

**Tab 8.11:** HPLC analysis for six individual tablet of **Sinemet (carbidopa and levodopa 25 + 250 mg/ tablet) for** measuring %dissolved of carbidopa as indictor for dissolution.

| Tablet no. | Peak area responses of carbidopa in sample preparation solution | *% carbidopa dissolved | Complies with the manufacturer specification (not less than 80%) |
|---|---|---|---|
| 1 | 205,261 | 103.22 | Yes |
| 2 | 204,352 | 103.45 | Yes |
| 3 | 208,846 | 99.8 | Yes |
| 4 | 209,984 | 106.3 | Yes |
| 5 | 200,876 | 101.69 | Yes |
| 6 | 200,728 | 101.62 | Yes |
| Peak area responses from carbidopa reference standard preparation solution (average three replicates) | 204,537 | | |

* %Levodopa dissolved in a specified time is expressed as a percentage of the content stated on the label.

**Tab 8.12:** HPLC analysis for six individual tablet of **Sinemet (carbidopa and levodopa 25 + 250 mg/ tablet) for** measuring %dissolved of levodopa as indictor for dissolution.

| Tablet no. | Peak area responses of levodopa in sample preparation solution | *% Levodopa dissolved | Complies with the manufacturer specification (not less than 80%) |
|---|---|---|---|
| 1 | 2,998,200 | 100.68 | Yes |
| 2 | 2,999,068 | 100.71 | Yes |
| 3 | 2,973,019 | 99.835 | Yes |
| 4 | 3,097,911 | 103.86 | Yes |
| 5 | 2,999,849 | 100.73 | Yes |
| 6 | 3,006,687 | 100.96 | Yes |
| Peak area responses from levodopa reference standard preparation solution (average three replicates) | 2,978,512 | | |

* %Levodopa dissolved in a specified time is expressed as a percentage of the content stated on the label.

### 8.4.13.4e Dissolution Criteria

According to USP [101], in the dissolution test, Q is denoted as the quantity of the API dissolved. For different manufacturers and monograph specifications, Q's limit is going to be different. It depends on the nature of the formulation and what active ingredients are present in it. For example, Q for Sinemet tablet = 75%. In the first stage S1 of the dissolution experiments, six drug units or dosage forms were tested under previously mentioned conditions and the amount of dissolved API for each unit can be calculated. This amount should not less than Q + 5. In case units below this limit are found, six more units need to be analyzed for the dissolved content in the stage S2. The estimate of all 12 units cannot be less than the value of Q, and none of the units should be lower than Q-15%. If any unit is found below the Q-15 or average of all units is less than Q, then the sample is analyzed in the S3 stage. In this stage, another 12 more units are also analyzed for the dissolved active content. At this stage, the average of all 24 units should not be less than Q, only two units may be below the Q-15% and no unit should be less than Q-25%.

From the application for the previous criteria on the data obtained (Tables 8.10 and 8.11) on dissolution study of Sinemet tablets, it can be concluded that drug

dissolution test complies with the manufacturer's specification in S1 stage. The average %dissolved of levodopa ranged between 99.835% and 100.96% and the average %dissolved of carbidopa ranged between 99.8% and 106.3% ≥ Q + 5% ≥ 80%.

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# 9 HPLC–Mass Spectrometry (HPLC–MS) and its applications in drug quality control laboratories

## 9.1 Introduction

The goal of this chapter is to get more and faster information on samples under analysis by combining high-performance liquid chromatographic (HPLC) data (as we have mentioned in Chapters 1–8, such as retention time and ultraviolet (UV) information) with mass spectrometry (MS) information (mass spectrum, molecular fragmentation and very specific detection) in the form of HPLC–MS. Also the goal is not to describe or even mention all the different aspects of MS but it is simply a quick outline of some commonly used approaches in the field of HPLC–MS, designed to make chromatographers more comfortable with a technique which is already to revolutionizing traditional HPLC. So we will discuss first the principles of MS and then the principles of HPLC–MS and its applications in drug quality control laboratories. Nowadays, the application of MS combined with chromatography as a hyphenated technique (combining the separation power of chromatography with MS detection power) has became numerous in pharmaceutical, environmental (organic trace), food, biochemical, natural products and analysis [1–13].

## 9.2 Principles of mass spectrometry

The mass spectrometer is an instrument designed to separate gas phase ions according to their m/z (mass-to-charge ratio) value [3–8]. The "heart" of the mass spectrometer is the analyzer. This element separates the gas phase ions. The analyzer uses electrical or magnetic fields, or combination of both, to move the ions from the region where they are produced, to a detector, where they produce a signal which is amplified. Since the motion and separation of ions is based on electrical or magnetic fields, it is the mass-to-charge ratio, and not only the mass, which is of importance. The analyzer is operated under high vacuum so that the ions can travel to the detector with a sufficient yield. In addition to the analyzer, the mass spectrometer also includes the following parts as shown in Fig.9.1:

a. Tools that will help in introducing the sample (HPLC in HPLC–MS and gas chromatography (GC) in GC-MS)
b. A vacuum system
c. Essential tools required to make and fragment the gas phase ions from the sample molecule (ion source), in order to obtain structural information or to get more selective detection
d. A detection system
e. Computing and software (data system)

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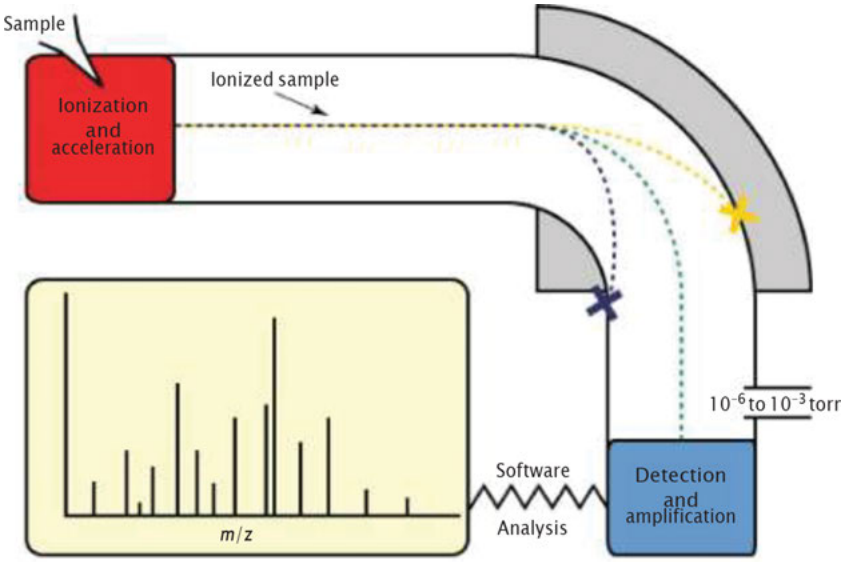


**Fig.9.1:** Basic components of mass spectrometer. Reproduced from Ref. [4] by permission from De Gruyter, Berlin.

In analytical MS, the sample is subjected to ionization in the ion source. To do so, the sample is usually in the gaseous state,as it is already the case when leaving a GC unit. Liquid samples as obtained from HPLC separation need to become transformed into the gaseous state. There are several ways of achieving ionization. By principle, they can be divided into hard and soft ionization techniques, which describes the amount of energy transferred to the analyte molecule. *Hard ionization* means bombardment with highly energetic particles like accelerated thermal electrons (*electron impact ionization*, EI) [3–8]. This is done by crossing the analyte stream with an electron beam under vacuum conditions. Depending on he kinetic energy of the colliding electrons (50–100eV), analyte molecules not only become charged but also fragmented. As shown in Fig.9.2 the simplified process is:

$$A + e^- \rightarrow A^+ + 2e^- \tag{9.1}$$

where A is the analyte molecule, $e^-$ is the colliding electron and $A^+$ is the resulting molecular ion. The high energy transferred by the electrons leads to ionization and after bond dissociation to fragmentation (X, Y, Z . . . (Fig.9.2) . . . After charging and rearrangement of the fragments, the stable ion clusters reach the separator stage and are recorded by the subsequent ion detector resulting in mass spectrum (the relation between ions abuancen and m/z as shown in (Fig.9.1 and Fig.9.2). From the data on the spectrum the structure of compoenet A can be known from the fragments $X^+$, $Y^+$, $Z^+$ and $A^+$ (can be identified) and this is the benfit of the MS, good method for identification of unknown chemical compounds. Typically, 70 eV is applied for EI fragmentation in MS.

EI often creates a variety of typical fragments, which can be used for structural elucidation of unknown molecules. A complex sample ionized by EI would provide a tremendous mixture of fragments; therefore, a pre-separation (purification) is needed. This is the reason why the combination MS with GC or HPLC is a powerfull analytical technique (combining the separation power of HPLC, with the detection power of mass spectrometry). *Soft ionization* means application of far less energy in order to achieve only a few and possibly selective bond breakings. Use of a lower ionization energy reduces fragmentation and increases the intensity of the molecular ion. So, for analytical purposes, soft ionization with 14 or 16 eV is favorable(reduced fragmentation leads to an increase of the molecular ion peak $M^+$). Usually *Soft ionization use Chemical ionization* technique (CI). *Chemical ionization* (CI) happens when reactant gases, like methane, Nitrogen, water, higher alkanes or ammonia, become admixed to the gaseous analyte molecules within an electron beam (150 eV), causing plasma conditions with ionic adduct formation [3–8] and [14]. Often, CI is performed under atmospheric pressure conditions then it called Atospheric Pressure Chemical Ionization (APCI) [15–18], [19–28]. Many applications are reported [19–24], especially after LC separations of analytes which need a gentle fragmentation, for example, lipids and drugs [9].

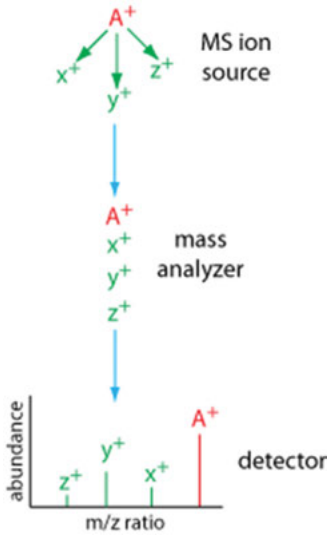


**Fig.9.2:** Block diagram illustrating a typical analyte ionization in ion source, ion separations in analyzer and ions detected in the detector in MS instrument.

In the following pages we will discuss the working principle of the *atmospheric pressure chemical ionization* (APCI) as an ionization tool (interface) in HPLC–MS.

## 9.3 High Performance Liquid Chromatography – Mass Spectrometry (HPLC–MS)

HPLC–MS is a hyphenated technique, which combines MS's detection power with HPLC's separation power. Even if there is already a highly sophisticated and advanced MS instrument, using HPLC is still very important to help in removing interferences of the sample which would otherwise affect ionization.

### 9.3.1 Principles of High Performance Liquid Chromatography – Mass Spectrometry (HPLC–MS)

Fig.9.3 and Fig.9.4 show a typical HPLC–MS instrument which incorporates the following parts:

a. Sample introduction device: In HPLC–MS, HPLC is used as a device for introduction of an analyte which have been separated by the analytical column to the HPLC–MS (in Chapters 1–8, we have discussed what is HPLC).
b. Mass spectrometer: This consists of:
   - An ion source for producing ions from gas phase of an analyte (HPLC–MS interfacing, in the following pages we will discuss this part in detail).
   - An analyzer: Helps in separating the ions of the analyte.
   - A detector: To detect which ions have been separated because of the analyzer.
   - A vacuum system: The analyzer must be operated under high vacuum.
c. Software and computing system (data system) (in the following pages, we shall discuss all these components).

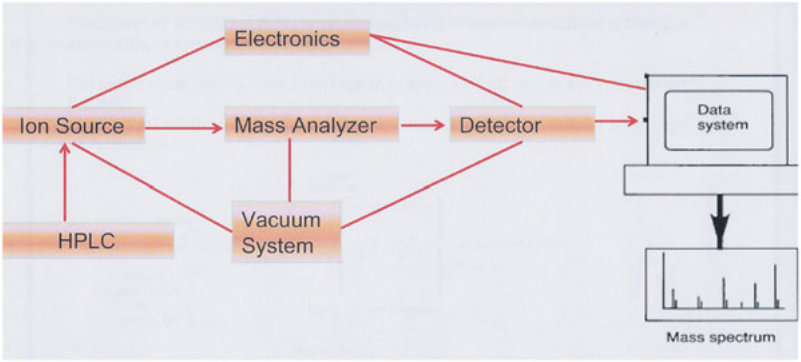


**Fig.9.3:** Generalized diagram showing basic components of an HPLC–MS.

In all cases, there is the need for an interface that will eliminate the solvent and generate gas phase ions, then transfer to the optics of the mass spectrometer. (The benefit


**Fig.9.4:** Configuration showing a combination of Waters HPLC system (Waters Alliance 2695 Separation Module) (1), photodiode diode array detector (Waters 996) (2) and Waters – micromass ZQ detector (3). Reproduced with permission from Waters Corporation (www.waters.com) [16].

of HPLC–MS interfacing part in HPLC–MS system is to convert the analytes separated by the HPLC analytical column and found in the mobile phase to gas phase ions for MS experiments.) A fundamental problem in coupling HPLC with MS is the enormous mismatch between the relatively large solvent volumes from the former and the vacuum requirements of the later. Nowadays, most of the HPLC–MS instruments use atmospheric pressure ionization (API) technique as an interface for HPLC, where solvent (mobile phase) elimination and ionization steps are combined in the interface and take place at atmospheric pressure as shown in Figure 9.5.

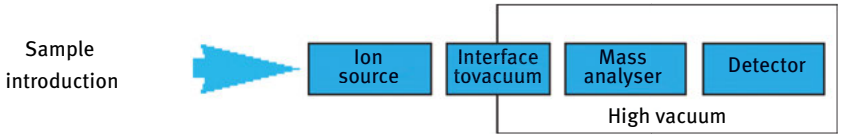


**Fig.9.5:** Diagram showing atmospheric pressure ionization (API) as an interface in HPLC–MS instruments.

For API approaches, facilitation of the eluent evaporation and breaking of the HPLC flow is important and the spray formation is best for it. The spray formation needs energy to operate. That can come from gas flow and/or heat. For a very low flow rate, the electrical field is sufficient to generate the spray in the electrospray interface.

The term API covers two techniques:

ESI or electrospray ionization

APCI

The common thing in these techniques is:
- To ensure ionization to occur, atmospheric pressure should be maintained.
- The methods used for ionization should be soft.
- There is no fragmentation, and the predominant ion obtained is the deprotonated molecule (M–H) or the protonated molecule $(M + H)^+$ or some species related to this ion (adducts with sodium, potassium, solvent molecules, dimers, etc.).
- The ionization can be made in positive or in negative mode.

**These techniques differ by**
- The probe design
- The ionization process
- The application field

### 9.3.2 Interfacing MS and HPLC

The benfit of interfacing part in HPLC MS system, is to convert the analytes seperated by the HPLC analytical column and found in the mobile phase to gas phase ions for mass spectrometry experiments and eliminate the mobile phase. A fundamental problem in coupling HPLC with MS is the enormous mismatch between the relatively large solvent (mobile phase) volumes from the former and the vacuum requirements of the later. Since the early seventies, a number of approaches have been applied for using some interfaces for coupling HPLC and MS, such as Thermo Spray and the Particle Beam. At that time, HPLC–MS has became really popular with the introduction of the Thermo Spray and the Particle beam interface. The next big improvement was the introduction of the Atomsphiric Pressure Ionization (API), [Electro Spray Ionization (ESI) and Atomsphiric Chemical Ionization (APCI)] [3–8], [15–18], [19–28]. Actually nowdays, the large majority of applications are done with ESI and APCI ionisation. In the next pages, we will cover the principle and application of ESI and APCI as the most common interfaces used for HPLC–MS. In these interfaces, ionisation takes place at atmospheric pressure and both are considered to be soft ionisation method. The mass spectrum provides mainly the molecular weight information, unless fragmentation techniques are used as in HPLC–MS–MS [22–24].

#### 9.3.2.1 Atmospheric pressure chemical ionization (HPLC–MS–APCI) interface for MS

A common ionization method, APCI , is useful in MS. This makes use of the gas phase ion-molecule reactions when done at atmospheric pressure [6–7, 20]. This is not done in isolation and is done with HPLC [3–9]. Just like CI, APCI is also a soft method for ionization. In both, a solvent spray has ions produced on it [3–9]. Compounds that are

stable thermally and less polar having an MW of 1,500 Da or less can be used in APCI along with polar compounds. HPLC and APCI together are gaining popularity when discussing about trace analysis detection. For instance, pesticides and steroids are even used for drug metabolites in pharmacology. Fig.9.6 shows the main three components of an APCI. These are the nebulizer probe that can tolerate heat of 350–500 °C, the second part is the region where ionization takes place and has a corona discharge needle [16], and lastly it is made up of the ion-transfer area that comes under the intermediate pressure. A stable flow rate of 0.2–2.0 mL/min is used to introduce the HPLC analyte elute to the pneumatic nebulizer. The nebulizer is already heated and this causes the analyte to flow coaxially along with $N_2$ (99.999% grade) nebulizer gas. As a result, fine droplets of mist are formed. Both the gas and heat flow combine together and force the mist emerging out to convert into the gas stream. After this, it goes on to the ionization area. The atmospheric pressure here allows the molecules to ionize at a corona discharge. This has 2–3 kV potential difference to exit the counter-electrode (because the eluent vapors have been ionized due to the corona effect, they are able to react chemically with the molecules of the analyte that are in the gas phase). A little orifice skimmer allows the ions of the sample to move ahead into the ion-transfer area. For mass analysis, the ions need to be passed further through an ion-focusing length or another skimmer that leads to the mass analyzer. As shown in Figure 9.6, the molecules of the solvent(s) are protonated by the corona ones to produce $[SH]^+$. They go on to react with the analyte molecule or M and result in $[MH]^+$, the protonated form. If the mode is positive, the eluent's proton affinity needs to be lower than the analyte's proton affinity (or to put it another way, it is possible that the analyte can easily catch the proton coming from the protonated solvent). This can be seen in the following equation:

$$[SH]^+ + M - - - - - - - - - S + . [MH]^+ . \tag{9.2}$$

If the mode is negative, the eluent's gas phase acidity must be higher than the analyte's gas phase acidity (or to put it another way, it is possible for a deprotonated solvent to get a proton from the analyte). This is shown in the following equation:

$$[S - H]^{- -} + M - - - - - - - - - S + [M - H]^+ \tag{9.3}$$

#### 9.3.2.1a Operating conditions for atmospheric pressure chemical ionization (HPLC–MS–APCI) interface for MS

The working conditions of the APCI are as follows [16]:

- Analyte: The analyte needs to be ready to be volatile under operation conditions and thermally stable.
- **Eluent:** It is necessary that ionization is possible in the mobile phase as it is suitable. If not, to get the solvent gas phase ions, a little modifier must be added.

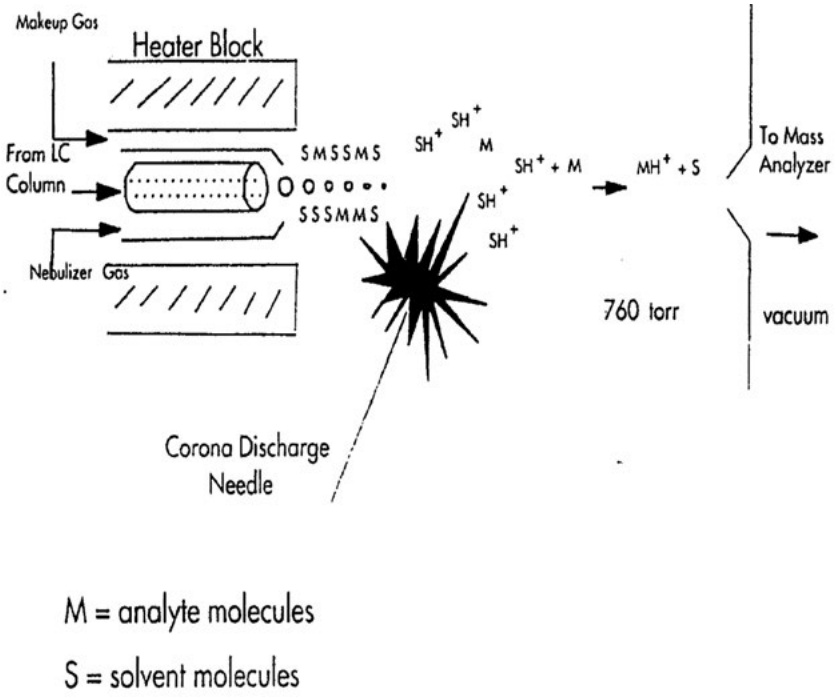


**Fig.9.6:** Scheme of an atmospheric chemical ionization HPLC–MS interface and mechanism of charged ion formation from analyte separated by HPLC column. Reproduced from Ref. [16] by permission from Waters Corporation, 34 Maple Street, Milford, MA 01757c (www.waters.com).

- **Flow rate:** To get the best conditions, the flow rate should be between 0.2 and 2.0 mL/min. ESI is used at much lower flow rates when compared to APCI.
- **Buffers:** It is important that they are volatile.

#### 9.3.2.2 Electrospray ionization

As illustrated in Fig.9.7, the HPLC line is connected to the electrospray probe, which consists of a metallic capillary surrounded with a nitrogen flow. A voltage is applied between the probe tip and the sampling cone. In most instruments, the voltage is applied on the capillary (3–5 kV), while the sampling cone is held at low voltage. First step is to create a spray. At very flow rate (a few µL/min), the differences in potential are sufficient to create spray. At higher flow rate, a nitrogen flow is necessary to maintain a stable spray.

As illustrated in Fig.9.7 the ESI interface includes a heating device, in order to speed up solvent evaporation. It is a mandatory condition to work with electrospray is that the compound of interest must be ionized in solution.

If it is not compatible with the HPLC conditions (i.e., in case of normal phase chromatography), it is possible to use post column addition to get appropriate conditions.

In the electrical field, at the tip of the capillary, and as mentioned by Niessner and Schaffer [4], the eluent is nebulized at the end of the capillary forming a fine spray of charged droplets (either positively or negatively, depending on the voltage polarity) in the nm range. Since the charge of the droplets has the same polarity as the needle, they are repelled. The vapor pressure of the solvent increases exponentially in the small droplets with the result of fast shrinking sizes. Once the droplet size becomes so small that the intrinsic charges exceed the so-called Rayleigh limit, a Coulombic explosion happens producing many ultrafine charged particles or molecules (Fig.9.7). Under the influence of vacuum conditions, finally individual charged analyte molecules enter the separation stage of the MS (Fig.9.7). Usually, no fragmentation happens during this process. The ion source and subsequent ion optics can be operated to detect either positive or negative ions. Multiply charged ions can be obtained depending on the chemical structure of the analyte.

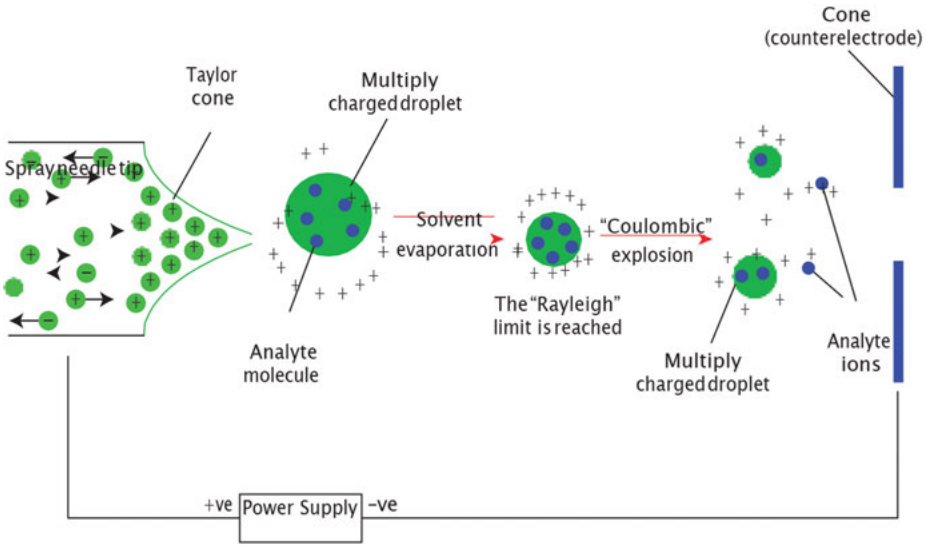


**Fig.9.7:** Scheme of an electrospray ionization HPLC–MS interface and mechanism of charged ion formation from analyte separated by HPLC column. Reproduced from Ref. [4] by permission from De Gruyter, Berlin.

ESI is a rather soft ionization method, that is, little fragmentation of the analyte ion will occur. By increasing voltages within the source, fragmentation in ESI can be increased. Coupling ESI with tandem MS may also overcome the problem of rather little structural information due to limited fragmentation [10, 11, 13]. Other

fragmentation methods, such as collision-induced dissociation (CID), are used to produce stronger fragmentation of the analyte for structural studies.

Small molecules with a single functional group capable of carrying an electrical charge give predominantly singly charged ions by addition of a proton $[M + H]^+$ when the ion source is operated in positive ion mode or by the loss of a proton when in negative ion mode $[M - H]^-$. Larger molecules with several charge-carrying functional groups, such as proteins and peptides, can exhibit multiple charging resulting in masses $[M + 2H]^{2+}$, $[M + 3H]^{3+}$ and $[M + nH]^{n+}$.

Multiply charged ions can be obtained depending on the chemical structure of the analyte. This is why ESI is the technique of choice for analyzing proteins and other biopolymers on quadrupole or ion trap analyzers [13, 15, 23, 24].

*Typical ions produced by ESI are as follows:*

Positive mode: $[M + H]^+$ protonated molecule
$[M + Na]^+$, $[M + K]^+$ adducts (see Fig.9.16 and 9.20)
$[M+CH_3CN+H]^+$ protonated + solvent adducts

Negative mode: $[M - H]^-$ deprotonated molecule
$[M + HCOO^-]^-$ adducts

#### 9.3.2.2a Operating conditions for electrospray ionization interface of HPLC–MS

A mandatory conditions to work with ESI *interface of HPLC–MS* is that the compound of interest must be ionized in solution. (It is not compatible with the HPLC conditions of normal-phase chromatography.) The following conditions are required for ESI to work:

1. Mobile phase: Typical mobile phase for ESI must contain volatile organic solvents, for example, methanol or acetonitrile mixed with some water. Compounds that increase the conductivity, for example, acid or ammonium salts, are often added to the mobile phase.
2. Flow rate: The best sensitivity is achieved at low flow rate. Working at higher flow rate is technically possible but may cause a reduction in the signal-to-noise ratio.
3. Eluent pH: The mobile phase should have a pH such that the analytes will be ionized. An acidic mobile phase is suitable for the analysis of basic compounds, using positive ESI, while a basic pH will be chosen for analyzing acidic molecules. However, some exceptions exist to this general rule:
4. Buffers
   - Volatile buffers are to be preferred for routine use.
   - Operating the instrument with nonvolatile buffers such as phosphate is technically possible but the salt deposit in the source will have to be removed periodically.
   - The concentration of the buffer, or acid or base used to adjust/control the pH should be as low as possible.

5. Ion-pairing agents (sodium octane sulfonates): These molecules have surfactant properties. The presence of surfactants in the mobile phase impacts the spray formation and droplet evaporation. There is also a surface competition mechanism phenomenon.
6. Matrix effects: When the sample contains high concentration of salts, or an excess of another analyte that can ionize in the operating condition, there might be a competition effect in the ionization. This is called "ion suppression." The chromatographic separation must be developed to remove this effect, at least when doing quantitative analysis.

## 9.4 Mobile phase selection for HPLC–MS

Using a mass detector for HPLC brings some constraints upon the eluent selection. These constraints are different from what is to be considered with other detectors. For example, the eluent's UV absorbance, which is of high importance with UV detection, is irrelevant with MS detection. The following items about mobile phase must be in the analyst consideration when they use HPLC–MS:

**Ionization:** The eluent must be suitable for the ionization. Thus, the eluent must be selected depending on the Ionization mode (ESI or APCI, positive or negative mode) and the analyte (p$K_a$, gas phase acidity).

**Eluent MW:** It is not possible to analyze compounds in which MW is lower than the one of the eluent (or eluent additives).

**Volatility:** For routine operation, it is easier to use volatile buffers.

**Acids:** HCl, $H_2SO_4$, methane sulfonic acid and so on might damage the instrument and should not be used. They must be replaced by volatile organic acids (TFA, formic, acetic).

**Adduct formation:** Ions (Na, $NH_4$, acetate, etc.) from the eluent will trend to form adducts. In the case of phosphate, multiple adducts are observed, which produce complicated mass spectra. The formation of adduct is usually not a reason for avoiding an eluent, and at the opposite, adduct formation might be forced for analytical reasons.

**Ion-pairing reagents and surfactants:** Impact the spray formation and the droplet evaporation, and compete in terms of ion formation.

**Buffer concentration:** Must be kept as low as possible (mM range). If the buffer concentration is too high, ion suppression occurs.

**Common eluents for LC/MS:** Methanol/water, acetonitrile/water (methanol usually gives a better sensitivity than acetonitrile). pH modifiers for the eluent such as

formic acid, acetic acids, TFA, $NH_4$, TEA, DEA, carbonates, ammonium formate, ammonium acetate and ammonium carbonates can be used for preparation of the mobile phase for HPLC–MS.

**HPLC column:** The column must give a good separation without using high concentration of buffers, nor ion-pairing reagents. The bonding must be stable so that the column will not "bleed." Special MS versions of columns are available from suppliers.

## 9.5 Design of API interface for HPLC–MS (ion source design)

All HPLC–MS suppliers are making a continuous effort to improve the design of the API sources, particularly as the source is a key element of the HPLC–MS instrument. A good source is essential for:
- clean MS spectra
- sensitive detection
- robustness and reliable operation
- easy use and maintenance

As mentioned previously, an API source always comprises:
- the probe (ESI or APCI)
- the corona electrode (for APCI operation)
- gas flows for nebulization, evaporation and desolvation
- sampling cone
- transfer optics to the MS analyzer

Those improvement can be found in MK II ZSpray ion source patented by Waters – Micromass HPLC–MS (Fig.9.8) [16]. In this design, the probe is perpendicular to the sampling cone, which is protected by a "cone gas" flow. The second extraction cone is also perpendicular to the ion beam, the so-called ZSpray geometry. This design protects very efficiently the optics against nonvolatile material and nonionized molecules. Consequently, cone orifices can be larger than in previous designs. The combination of larger orifices and noise reduction largely compensates for transmission losses due to the orthogonal geometry (Fig.9.8) giving a large gain in sensitivity. Efficient desolvation is provided by a heated nitrogen flow close to the probe and by the cone gas. The source block is also heated for final desolvation. For easy maintenance, an isolation valve is installed, so that it is possible to disassemble the sampling cone for cleaning without having to vent the system. A glass window gives the operator the possibility to observe the say and the interior of the source during operation.

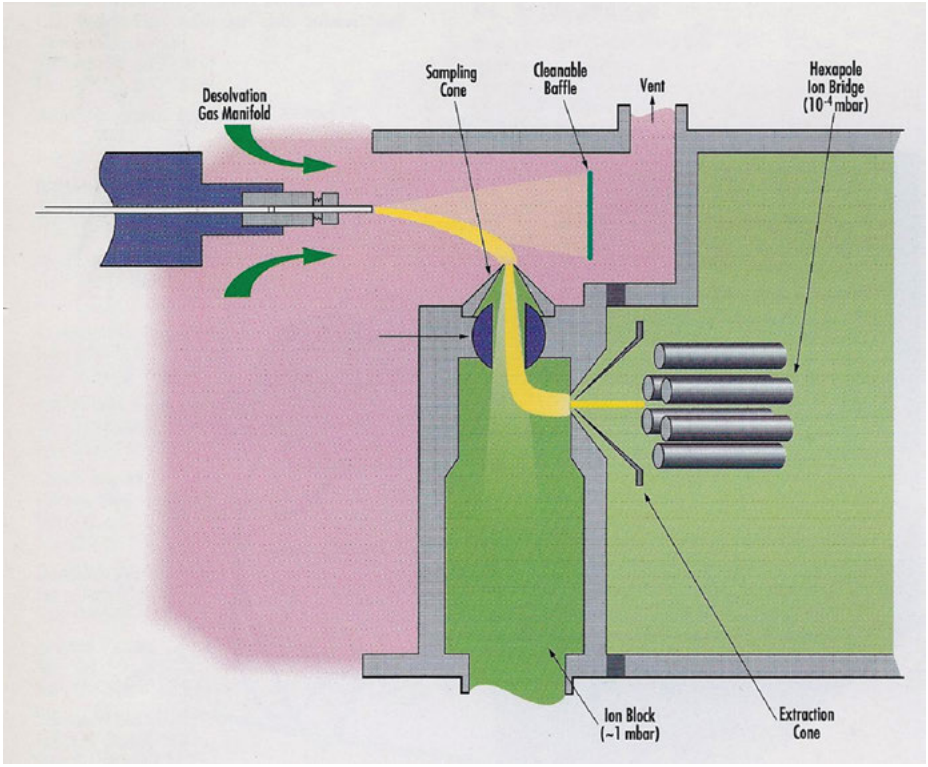


**Fig.9.8:** Schematic layout of ZSpray design for API interface used in HPLC–MS manufactured by Waters–Micromass company. Reproduced from Ref. [16] by permission from Waters Corporation, 34 Maple Street, Milford, MA 01757c (www.waters.com).

## 9.6 The analyzer

The analyzers used in HPLC–MS instruments were quadrupole [1–6], ion trap [18] and [28], time of flight and combinations such as triple quadrupoles [13, 22, 24], time-of-flight quadrupole (QTOF) [27]. In this book, we will cover instruments that are using quadrupole and triple quadrupole analyzers. These analyzers are commonly used in the HPLC–MS application in drug quality control laboratories.

### 9.6.1 The quadrupole mass analyzer

The quadrupole mass analyzer is actually the most widely used analyzer for HPLC–MS applications due to their mass range coverage, resolution, easy and simple usage, mass spectra quality and good linearity for quantitative work. All these features are

present for a very affordable price. As for every MS analyzer, ions are not analyzed for just the mass but are analyzed for the mass-to-charge ratio or m/z. The gas phase ions got into the quad and reach the detection phase with the help of electrical fields. As Figure 9.9 shows, one rod set is at the negative potential and the other is at the positive potential. Each set has a combination of radio frequency (rf) and dc applied to it. The negative rod pairs act as the low mass filter while the positive rod pairs act as a high mass filter. The dc value is responsible for the applicable resolution. The operation of the quads is at a constant resolution, that is, the ratio of rf/dc is kept constant. At a given amplitude of the voltages and dc, not all ions but only ions with a particular m/z ratio are going to resonate and pass forward through the quadrupole to end up being detected. The ions that are left will collide with the rods as they are destabilized. The ability to pull apart two masses adjacent to each other on the range or performance depends on a number of factors. These include the geometry of the quad, voltage setting, electronic and what the manufacturer's quality was. A higher resolution will reduce the number of ions reaching the detectors. As a result, the sensitivity of the process will be impacted.

There are two modes to use the quadrupole: scan or the single ion recording or monitoring (SIM). The parameters like the amplitude of the rf and dc voltages in the SIM mode are set to see a selection or just a specific mass. If a user shows interest in fragments or specific ions, this mode is the best as it has the greatest sensitivity. During scan mode, amplitudes of both the rf and dc voltages are ramped with (the ratio of rf/dc is constant). This is done to get the value of mass spectrum over the mass range required. In scan mode, the signal can be acquired in different ways:

- *Continuous*: This is the acquisition, storage and display of every acquisition point, as defined in the instrument method. This mode presents the advantage that every point is retrievable but gives large data files.
- *Centroid*: The different acquisition points are processed to obtain a bare, located at the maximum of the mass peak. Both the line height and the abundance of ions are directly proportional to each other. In this case, the mass spectrum appears as a series of lines as shown in Fig.9.16, Fig.9.18 and Fig.9.20. A centroid representation can be computerized from a continuous acquisition but reverse is not possible.
- *Multichannel acquisition*: To adopt this mode, it is important to infuse the sample with the help of a syringe pump in the MS. During the time periods defined by the user, the software collects mass spectra. This is one way to increase the signal quality.

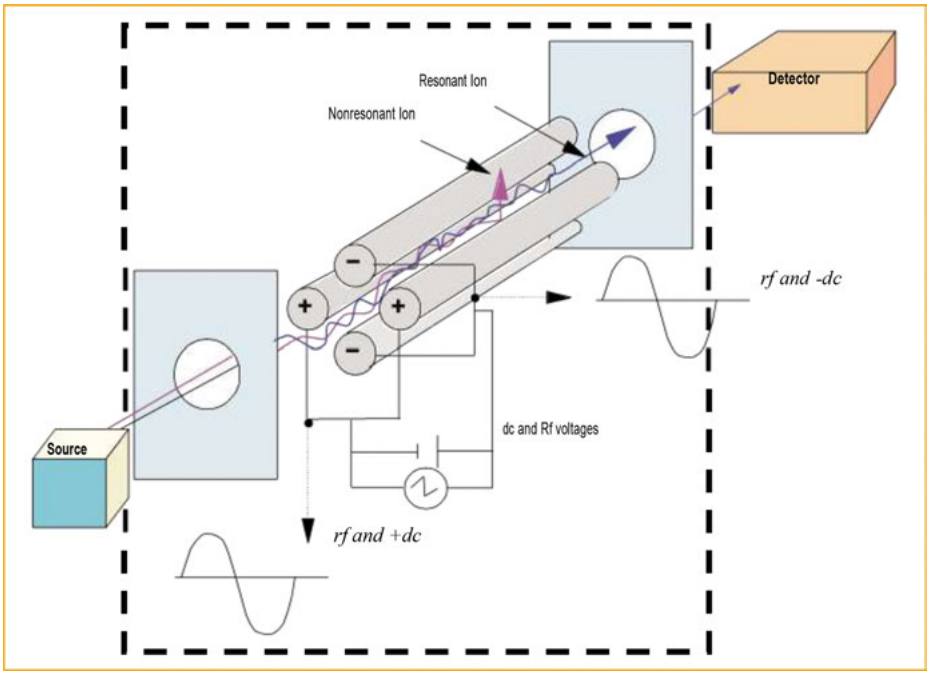


**Fig.9.9:** Schematic diagram showing a quadrupole analyzer for HPLC–MS. Reproduced with permission from Waters Corporation, 34 Maple Street, Milford, MA 01757c (www.waters.com) [16].

*A few important points must be mentioned about quadrupoles and must be in considerations of HPLC–MS analyst:*

- *Mass range*: This is determined by the physical dimension of the quad and by the possibilities of the RF generator. Extending the mass range while maintaining the resolution over the full mass range is rapidly increasing the instrument cost and dimensions. Maximum mass range achievable with quads is in the 6,000 amu.
- *Sensitivity: The number of ions that make it to the detector and signal is directly proportional.* As the quadruple is the mass filter, the amount of time spent on observing each resolution and ion and the signal/noise ratio are related to each other. To get higher sensitivity, a low scan speed will allow better scanning on the narrower range. Higher resolution will lead to better sensitivity as few ions will go to the detector.
- *Scan time*: When doing HPLC/MS, the scan time must be set also according to the chromatographic peak width, keeping in mind that a minimum of nine acquisition points is necessary to define a chromatographic peak.
- *Construction*: Quads are very high precision devices. Rods are made of metal (stainless steel, molybdenum, etc.) or of metallized ceramic bars. Rods are mounted on ceramic supports. Quality of the construction and quality of the electronics are key factors for performances (ion transmission, resolution and mass stability).

### 9.6.2 The triple quadrupole analyzer (HPLC–MS–MS technique)

Combining two MS experiments or more is known as MS/MS. The aim of which is to make the sensitivity and selection better when doing quantitative analysis and to undergo fragmentation to isolate the ions to get information about the structure during the initial experiment. It is done by

- Using an iron trap and conducting all the experiments in that trap
- Either coupling multiple analyzers (of the same or different kind)
- Or coupling same or different analyzers together

As Fig.9.10 shows, a "triple quad" instrument's analyzer has two quadrupoles which are kept apart with a collision cell. This type of design is called "tandem in space" instrument. Product ions and precursor ions are analyzed and also created in various physical spaces. The ions need to be shifted to an analyzer from the source (different physical regions). Once this is done, here different functions are performed on the source.

The whole point of the process is either to get better sensitivity or selectivity to conduct quantitative analysis or to obtain information about the structure of the fragmented ions that have already been isolated in the initial experiments. In this analyzer, the ions comprising a peak are made to undergo further fragmentations usually by a technique known as collision-induced fragmentation (CID).

The precursor (first ion) is selected in quadrupole number 1. This is the ion that goes to the collision cell for fragmentation. This is done by using helium, argon or other types of collision gases to speed up the acceleration of the ions. For different fragmentation degrees, the energy of the collision and the gas can be changed. The fragments that are formed in the first quadrupole go to the second quadrupole where they are analyzed. This is done by one of the two modes, that is, the scan or SIM mode. Structural information can be obtained by studying the mass spectral fragments. If the instrument only has a single quadrupole, you can conduct in-source CID to get fragmentation. Before the ions are introduced in the optics section of the mass spectrometer, fragmentation is done. Only if there is zero chromatographic interference does this technique work. In case the system is triple quad, the first quadrupole is used as a separation device that is required to lessen the need to accurate chromatographic separation. This system is also used for quantitation. The parent ion is selected through the first analyzer in the SIM mode. The SIM mode makes use of the second analyzer too to keep an eye on a particular fragment. The efficiency of the selectivity process is increased by two analyzers. Overall, the signal-to-noise ratio is largely improved as the signal of the ion is lessened in the transmission. The biggest limitation in complex samples, the chemical noise, is also reduced manifold. For this reason, quantitative analysis of complex samples has become possible. Samples with zero separation at very little chromatographic separation like serums are made possible. In pharmacology studies, this technique is applied.

But a point to be kept in mind is that during quantitation, ionization is a crucial step. This happens in the source. Unexpected and sudden effects like "ion suppression" may arise in the source due to the interfering compounds there. Irrespective of the MS analyzer being used, quantitation is affected by these. The MS/MS system does decrease the problem but it also does not fully eliminate it.

Depending on different types of instruments, the CID can take place after the first analyzer in an MS–MS configuration as shown in Figure 9.10. In an MS–MS configuration, a collision gas (i.e., argon, helium, neon, etc.) is introduced into the CID region (Figure 9.10). Helium ions collide with the gas and fragment. A particular ion which is isolated in the first quad can be fragmented in the SIM mode. Analysis of the direct mixture is possible.

In these types of instrument, a voltage is applied on the sampling cone. Fragmentation occurs when the charged ions are speeded up and bump the gas molecules.

As we have mentioned previously, the API soft ionization techniques produce mainly the protonated molecular ion that allows to determine the weight of the molecule.

Although MW information is a valuable piece of information, it is necessary to produce some fragmentation to get information on the structures, to conform the identification, or simply to fragment some adducts.

The "in-source" fragmentation increases with the voltage. In this configuration, all the ions present in the source are fragmented. In the case of mixtures, the obtained spectra could be difficult to elucidate without prior HPLC separation.

## 9.7 The detector

No information could be gained from sources or analyzers without a suitable detector being available. Most detectors are ion collection type. All types of detectors require a surface on which the ions impinge, and the charge is neutralized either by collection or donation of electrons. Hence, electron transfer occurs and an electric current flows that may be amplified and ultimately converted into a signal recorded on a chart or processed by a computer. The TIC or total ion current is the addition of all the currents carried by all the ions.

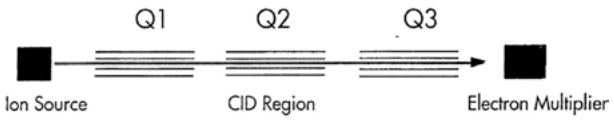


**Fig.9.10:** Schematic diagram showing collision-induced dissociation (CID) region in HPLC–MS–MS configuration (triple quad).

### 9.7.1 Electron multiplier

To convert the positive or negative ions into electrons, a conversion dynode is used. A device with the shape of a horn is used to amplify the electrons by producing a cascade effect Fig.9.11. This leads to the production of the current. Mostly used in the quadrupole analyzer, it is called a channeltron.

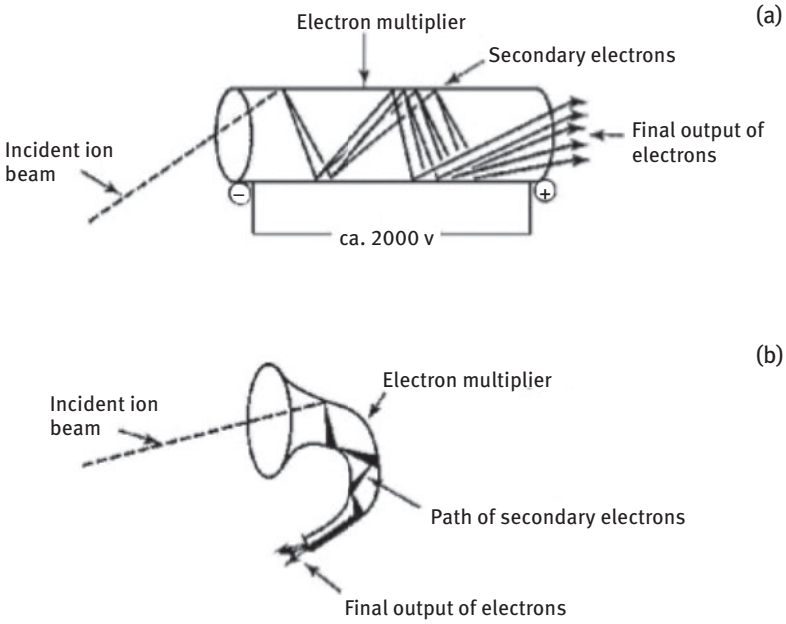


**Fig.9.11:** Schematic diagram of an electron multiplier as a detector for detection of ions separated by the analyzer of HPLC–MS.

### 9.7.2 Dynolyte photomultiplier

A conversion dynode is used to convert the detector ions that are in the quadrupole into electrons (Fig.9.12). The electrons get excited when they hit a phosphor and then emit photons. There is a photocathode at the beginning of the photomultiplier; when the photons strike on it, electrons are producers and the signal of them are amplified by it. It is held beneath a vacuum and glass is used to seal it. Hence, there is no contamination and the detector can perform well for a longer duration.

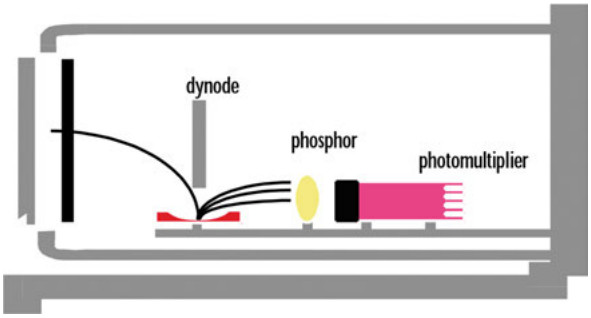


**Fig.9.12:** Schematic diagram of a dynolyte photomultiplier as a detector for detection of ions separated by the analyzer of an HPLC–MS.

## 9.8 Performance criteria for HPLC–MS system

When performing HPLC, and especially when doing HPLC–MS, each constituent of the analysis system is important. In the technical side, the following elements are all important for obtaining the best performance in HPLC–MS.

– **The sample preparation**

This step is the highest importance, since many of the analytes are in a matrix making HPLC analysis difficult. Direct injection of such samples may rapidly degrade the column, lead to some interference in the analysis or contaminate the MS source (cleaning the sample will give cleaner information). For pre-concentration of the analytes to check trace determination, preparing the sample is necessary.

In both situations, an extraction is necessary using liquid–liquid or liquid–solid protocols (see Chapter 6).

– **Sample management**

Large series of samples can be injected on an HPLC–MS instrument. Even if the number of samples is less, an automatic injector is convenient, especially for injecting low volumes on narrow bore columns.

– **The HPLC column**

A good HPLC separation is still necessary to get the best information on analytes, especially with “detector-type” mass spectrometers. A good performance column, adapted to the application, is therefore necessary.

The column also plays a role in many analysis performance measures.

## 9.9 Combination between HPLC–MS and PDA detectors (HPLC–PDA–MS)

Although mass detection usually offers a better sensitivity than UV detection, having an UV detector in the HPLC–MS configuration presents many advantages for the chromatographer. It helps in developing *separation* and completes MS detection for the characterization of compounds. Two kinds of UV detectors can be connected on the LC/MS: the tunable UV–visible detector (UV/VIS) or the photodiode array detector (PDA [16]). That last one gives a signal which is acquired in parallel with the signal of the mass detector as shown in Fig.9.15, Fig.9.17 and Fig.9.19.

### 9.9.1 HPLC–PDA–ESI(+)–MS as analytical tool in drug quality control laboratories (screening for presence of some approved synthetic drugs—PDE5 inhibitors, such as Sildenafil, Tadalafil and Vardenafil—as illegal adulterants for natural hebbal products–herbal medicines and dietary supplementary food

Over the last few decades, there has been an exponential growth in the field of using "natural" herbal products as herbal medicine or dietary supplementary food, and nowadays plays an active part in human health care. These products are regarded by many consumers as being harmless because of their natural origin, and helpful to the treatment of some chronic diseases such as erectile dysfunction (ED). On the other hand, there are currently three synthetic phosphodiesterase-type 5 (PDE-5) inhibitors Fig.9.13, such as sildenafil citrate (brand name Viagra) (S), vardenafil hydrochloride (brand name Levitra) (V) and tadalafil (T, brand name Cialis) (T), which have been evaluated as orally effective drugs for the treatment of ED [29–32]. Adulterations of "natural" herbal products with at least one of the previously mentioned synthetic PDE-5 inhibitors or their structural analogue has been reported since 2001 [33–40]. It is true that adulteration of "natural" herbal products with undeclared synthetic PDE-5 inhibitors, or their structural analogues, shows a growing trend and poses a health threat to consumers. It is important to note that synthetic PDE-5 inhibitor drugs should be taken only under the supervision of a physician because of their side effects such as headache flash, dyspepsia, rhinitis, back pain and color visual disturbances. In addition, it is well known that the analogues of commercial PDE-5 inhibitors are not FDA-approved drugs and no report on their biological activities can be found in published literatures. Based on their structural similarities with their synthetic PDE-5 inhibitors, similar biological activities might be expected. More importantly, the toxicity profile of these analogues is unknown. In Japan, one case of liver function impairment was reported that might be due to the use of herbal product containing hydroxyhomosildenafil (a structural analogue of (S)) [32]. So it is dangerous to consume these analogues in adulterated herbal products [32 and 37]. In order to prohibit the sale of those illegally adulterated

"natural" herbal products and save human health in Kuwait, the Kuwait Food and Drug Administration (KUFDA) has been conducting thorough ongoing inspection on "natural" herbal products for the presence of S, T and vardenafil (V) in these products and their analogues. For that purpose, an analytical procedure based on liquid chromatography-PDA detector combined with ESI–MS (HPLC–PDA–ESI(+)–MS) has been developed and validated by Omar and Khalifa [40] according to the USP and ICH rules [41–42] for screening the presence of three synthetic PDE-5 inhibitors, such as S, T and V (see Figure 9.13 for their structure formula), which are illegally adulterated in natural herbal products. These herbal products have been a subject for registration by Kuwait Drug and Food Quality Control Administration (KUFDA) as a natural herbal product for improving sexual performance for men in the period from 2003 to 2012 in Kuwait.

A four-step analytical strategy based on HPLC-PDA-ESI (+)-MS as an analytical tool was suggested and elaborated by Omar and Khalifa [43–48] *for analysis of three synthetic PDE-5 inhibitors such as S, T and V and*/or their structural analogues as follows:

a. Extraction of targeted compounds
b. Conditional optimization for HPLC and MS
c. Method validation according to the international rules [41, 42]
d. Application of the developed and validated method for analysis of herbal products

Extraction of targeted analytes was performed by a mixture of methanol:acetonitrile:water (1:3:2, v/v/v). After sonification for 10 min and centrifugation at 3,000 rpm for 10 min, 10 µL from the clear supernatant was injected on symmetry 300 C18 column. Analyte separation was carried out by isocratic elution using mobile phase consisting of methanol:acetonitrile:1% acetic acid (20:20:60 v/v/v) at a flow rate of 0.4 mL/min under ambient conditions. Detection was done simultaneously by PDA detector combined with a single quadruple mass analyzer interfaced with ESI operated in positive ion mode[HPLC-PDA –––ESI [40, 43–48].

In this study, qualitative and quantitative analyses have been done using an HPLC-PDA coupled to a single quadruple mass spectrometer (MS). The HPLC-PDA system comprised a Waters HPLC Model Alliance 2695 equipped with an autosampler, a column oven and a Waters 996 PDA detector (34 Maple Street, Milford, MA, USA). The HPLC-PDA system was coupled to a single quadruple mass spectrometer equipped with an ESI source (Waters–Micromass ZQ, 34 Maple Street, Milford, MA, USA). Analytes detection were done simultaneously by PDA and MS. The electrospray was operated in positive ion mode and the voltage of capillary, extractor, RF lens and cone was set at 4 kV, 6 V, 0.5 V and 30 V, respectively. The temperature was maintained at 140 and 420 °C for source and desolvation, respectively. The gas ($N_2$ purity = 99.999%) flow rate was set at 430 L/h. Complete system control, data acquisition and data processing were performed using a MassLynx software. This software has the ability to view and compare, and print MS data and PDA data

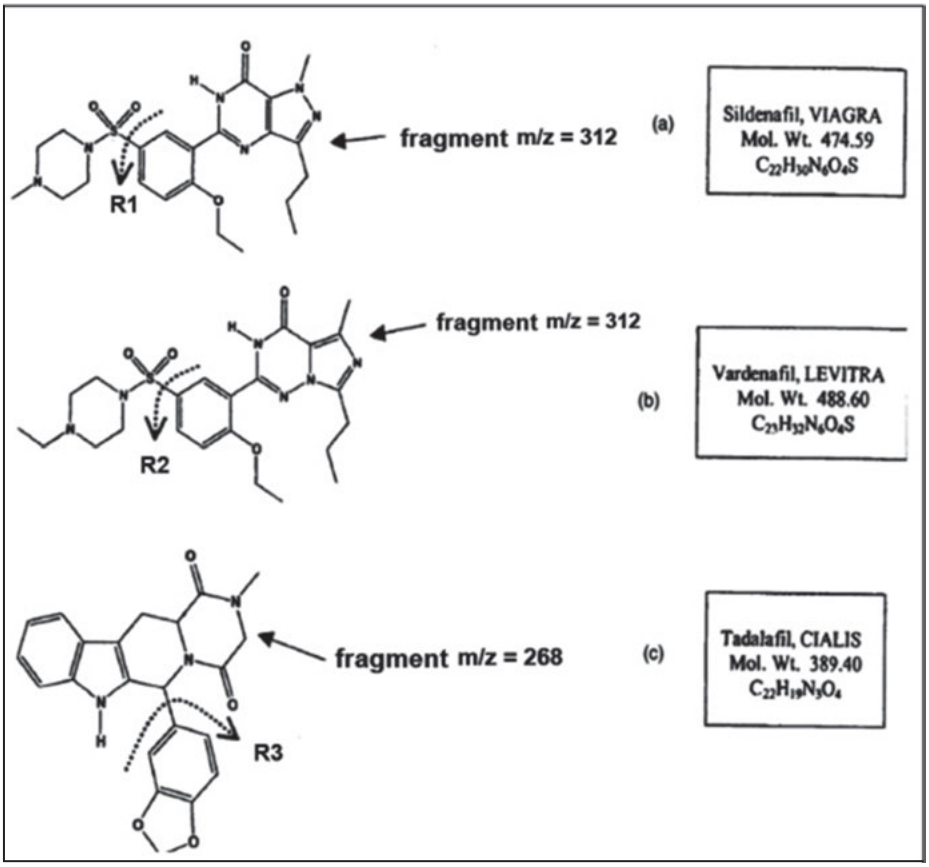


**Fig.9.13:** Structural formula and predicated cleavage positions for S(a), V(b) and T(c) in MS.

simultaneously. The full scan mass spectra (TIC chromatogram) and PDA–UV spectra were acquired over a range of m/z 100–550 amu and 200–400 nm, respectively. ESI parameters were optimized using flow injection analysis mode (FIA). For that purpose, individual solution from S, T and V was directly infused to MS. Retention times of separated compounds as well as on the PDA chromatogram as on the TIC of the MS detector of reference standards and samples were used for preliminary identification of the investigated adulterants. Confirmatory identification was carried out using UV and MS spectral data obtained from reference standards and samples at the same retention time of ±5%. In addition, selective ion monitoring technique was used for more confirmation. Also UV and MS data, which have been published in the corresponding literatures, were used for comparison. For quantitative analysis, LC-PDA-ESI(+)-MS (PDA mode) was used, and calibration curves for S, T and V were constructed.

The chromatographic conditions, especially the composition of mobile phase, was optimized through several trials to achieve high sensitivity, high resolution between analytes (especially between S and V) and symmetrical peak shapes for analytes as well as short chromatographic time for analysis using HPLC-PDA-ESI(+)–MS. In this study, separation of S, T and V were accomplished on a Symmetry 300 analytical column packed with 5 μm C18 and mobile phase consisted of methanol:acetonitrile:1% acetic acid (20:20:60, v/v/v) at a flow rate of 0.4 mL/min under ambient conditions. Detection of the analytes was done using PDA and MS detectors simultaneously.

Data in Tab.9.1 and in Fig.9.13 summarize the results related to the optimized conditions for HPLC and MS. Using the FIA mode, the ESI parameters, such as capillary voltage, the desolvation gas flow rate and cone voltage, were optimized for producing maximum ion intensities of S, T and V in MS. It can be seen that the mass spectrum of S, T and V obtained from FIA experiments exhibits the intensive protonated molecule $[M + H]^+$ of S at m/z = 475 amu, and ion at m/z = 312 amu is the fragment ion of S resulting from losing R1 group from protonated molecular ion $[M + H]^+$. The presence of ion at m/z 489 amu represents the protonated molecular ion of V $[M + H]^+$ and ion at m/z = 312 amu is the fragment ion resulting from losing R2 group from protonated molecular ion of V. Ion at m/z = 390 amu is the protonated molecular ion $[M + H]^+$ of T, while ion at m/z =268 amu resulting from losing R3 group from protonated molecular ion of T. It can be seen from Fig.9.13 and Tab.9.1 that S and V produce the same fragment ions because they possess the very similar structure. The same spectral data about S, V and T were obtained in [47, 48]. From these spectral data, it is apparent that in addition to the ion at $[M + H]^+$ there are structurally useful fragments $[M–R1]^+$, $[M–R2]^+$ and $[M\text{-}R3]^+$ for S, V and T, respectively. In this study, the previously mentioned ions were selected when using SIM technique for identification of S, T and V in investigated samples.

Tab.9.1 summarizes the chromatographic data about S, T and V (average retention times $t_R$ for each compound during the course of this study, resolution (R), peak symmetry factor (As) and % RSD of $t_R$). From the data in Table 9.1, it can be concluded that S, T and V were resolved at the baseline using the mobile phase eluted isocratically with resolution values, R = 2.8 and 16.1 between V and S and between S and T, respectively. The separated peaks on TIC and PDA chromatograms have symmetrical shapes with symmetry factor (As), 1.05, 1.02 and 1.01 for S, T and V, respectively. The % RSD values for the $t_R$ of S, T and V were less than 1.5, indicating the stability of the chromatographic system. In accordance with USP [41] requirements, the quality of chromatographic data obtained for S, T and V met the acceptable criteria. These demonstrate the suitability of the chromatographic system and its effectiveness for qualitative and quantitative purposes during the course of this study.

Figure 9.14 shows the UV spectrum obtained from PDA chromatogram corresponding to S, T and V peaks. It is apparent that the UV spectra of S, T and V are significantly different and these three compounds can be easily differentiated. S showed $\lambda_{max}$ at 293 nm, T showed $\lambda_{max}$ at 284 nm and V showed $\lambda_{max}$ at 225 nm.

From these data, it can be said that UV data obtained from PDA can provide preliminary identification of S, T and V. Further confirmation can be carried out using the previously mentioned MS data. Additionally, the developed method was validated according to USP and ICH [41, 42].

**Tab.9.1:** Mass spectral and chromatographic data obtained from the analysis of individual reference standard solutions of S, T and V infused directly to MS using the FIA technique and from analysis of standard mixture injected through LC-PDA-ESI(+)-MS system.

| Compound | Mass spectral data | | Chromatographic data | | | |
|---|---|---|---|---|---|---|
| | $[M + H]^+$ ion | Fragments $[M–R]^+$ | $t_R$ (min*) | %RSD of $t_R$** | Resolution factor (R)*** | Symmetry factor (As)*** |
| Sildenafil | m/z = 475 amu | m/z = 312 amu $[M–R1]^+$ | 5.73 | 1.1 | V–S = 2.8 | 1.05 |
| Vardenafil | m/z = 489 amu | m/z = 312 amu $[M–R2]^+$ | 4.85 | 1.2 | S–T = 16.1 | 1.02 |
| Tadalafil | m/z = 390 amu | m/z = 312 amu $[M–R3]^+$ | 14.17 | 1.1 | | 1.01 |

* Reproducibility of $t_R$ was evaluated during 2 months with a minimum of 30 injections of reference standard mixture solution and the %RSDs were determined.

** $t_R$ on the PDA chromatogram.

***Determined according to USP [41].

From the previous data, the analytical procedure that depends on LC–PDA–EI (+)-MS is specific, sensitive, accurate and relatively simple in both sample preparation and equipment. The procedure providing a very useful tool for rapidly screening (inspection studies) of large amount of natural herbal product samples. Using this analytical procedure, the time from sample receipt to structural identification and determination of the adulterant was about 20 min.

The applicability of the method has been demonstrated for analyzing the extracts of 485 natural herbal product samples. These samples (a premarket samples) have been a subject for inspection by KUFDA for the presence of previously mentioned synthetic PDE-5 inhibitors in the period from 2003 to 2012. The obtained data revealed that the percentage of adulterated samples with at least one of known synthetic PDE-5 inhibitors ranged between 20.5% and 84.4% within 10 years. T followed by S presents the main adulterants in the investigated samples. Quantification of S, T and V revealed that the doses are sufficiently high to be therapeutic. On the other hand, 2–4% of positive samples for adulteration were found to contain over therapeutic dose. One of S analogues, hydroxyhomosildenafil, or one of T analogues, aminotadalafil, were found

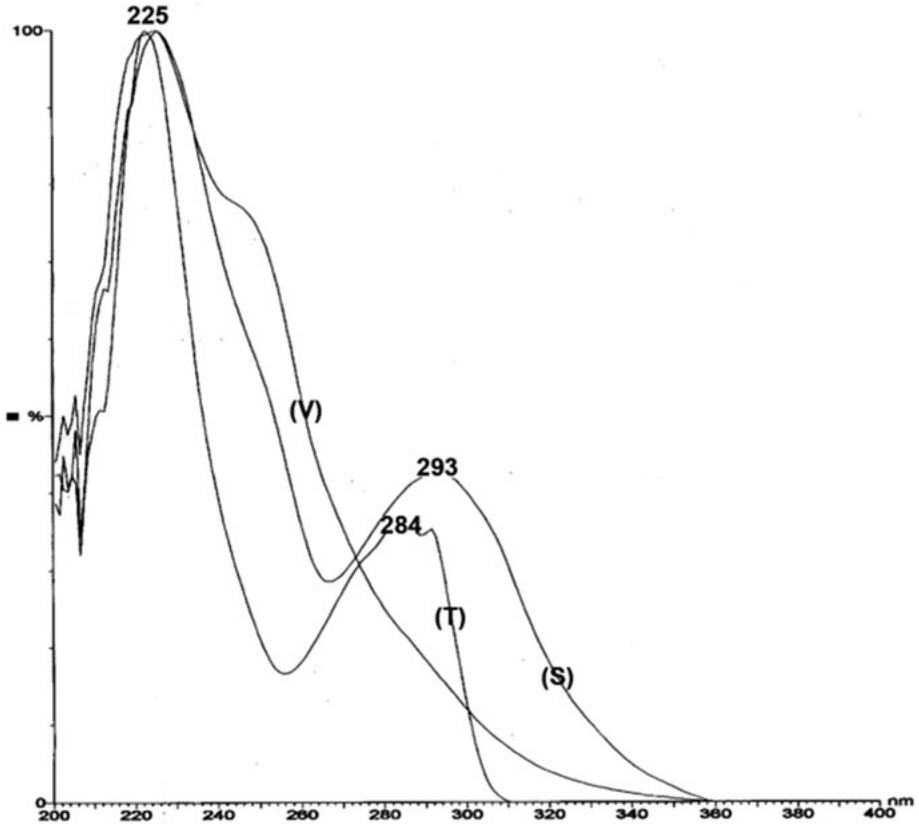


**Fig.9.14:** Overlaid UV spectrum obtained from scanning peaks on PDA chromatogram of reference standard mixture of S, V and T.

as adulterants in some investigated samples with 2–4%. According to the KUFDA rules, all adulterated herbal products have been canceled from registration and banned to be used by men in Kuwait. Finally, it can be concluded that during the last 10 years (2003–2012), the KUFDA laboratories have rendered great services for protection of human health in Kuwait against the risk associated with consuming herbal products that were illegally adulterated by well-known three synthetic PDE-5 inhibitors such as S, T and V or their analogues.

In the following pages, we will discuss a representative sample of herbal products that were found positive for the presence of T, one S analogue, hydroxyhomosildenafil, and one T analogue, aminotadalafil, as adulterants and the rules of HPLC-PDA–ESI (+)-MS for identification and confirmation.

### Sample 1

As shown in Fig.9.15, the PDA chromatogram of sample has a peak at a retention time of 7.52 min. The UV spectrum of sample Fig.9.16a is similar to the UV spectrum of T reference standard Fig.9.14. The most abundant peak in the MS spectrum of sample Fig.9.16b at that retention time is m/z 390 amu, indicating the ion $(M + H)^+$ for a compound with a molecular mass of 389 amu. Other ions observed in the MS spectrum of

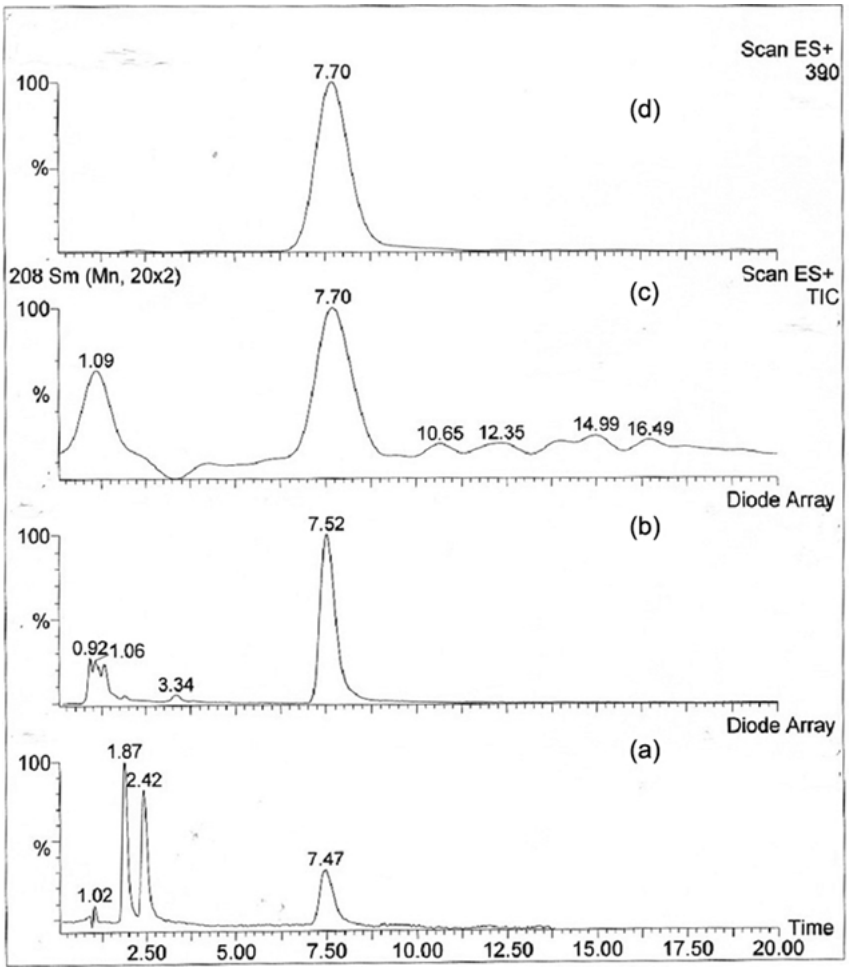


**Fig.9.15:** HPLC-PDA-ESI(+)-MS analysis for extract obtained from sample of herbal products illegally adulterated by T (synthetic and approved phosphodiesterase-type 5 (PDE-5) inhibitors are orally effective drugs for the treatment of erectile dysfunction, MW = 389 amu):
(a) PDA chromatogram for standard mixture (V ($t_R$ 1.87 min), S ($t_R$ 2.42 min) and T ($t_R$ 7.47 min));
(b) PDA chromatogram of sample; (c) TIC chromatogram of sample; and
(d) extracted ion chromatogram for ion m/z (M + H) = 390 amu of sample.

sample such as m/z 268 amu are observed in the MS spectrum of T reference standard (Fig.9.13 and Tab.9.1). On the basis of these data and publications in [35–40], the compound adulterated in sample 1 was identified to be T.

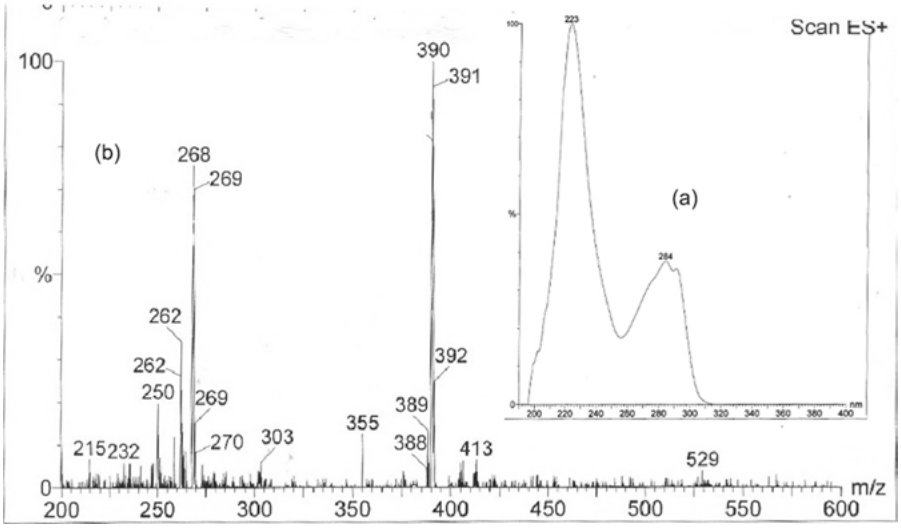


**Fig.9.16:** PDA spectrum (a) and mass spectrum (b) of chromatographic peaks at $t_R$ = 7.52 min and 7.7 min found on PDA chromatogram and TIC chromatogram (see Fig.9.15), respectively, for sample of herbal products illegally adulterated by T (synthetic and approved phosphodiesterase-type 5 (PDE-5) inhibitors are orally effective drugs for the treatment of erectile dysfunction, MW = 389 amu).

### Sample 2

Sample 2 was analyzed for the presence of synthetic PDE-5 inhibitors S, T and V by LC-PDA-ESI(+)-MS, and the data are presented in Fig.9.17 as TIC and PDA chromatograms. Although peaks related to S, T and V are absent, a peak was eluted before the established retention time of T at 9.88 min on the PDA as well as on the TIC chromatograms at 10.05 min. The UV spectrum for this peak (Fig.9.18) is very similar for UV spectrum of T reference standard (Fig.9.14). The most abundant peak on the TIC chromatogram for that compound (Fig.9.18) is at m/z = 391 amu, indicating the ion is $[M + H]^+$ for a compound with a molecular mass of 390 amu. The only difference was that the unknown compound has a molecular ion greater than the T molecular ion by 1 mass unit. Based on these data, the compound adulterated in sample could be aminotadalafil. This proposal was supported by the data which have been published in [35].

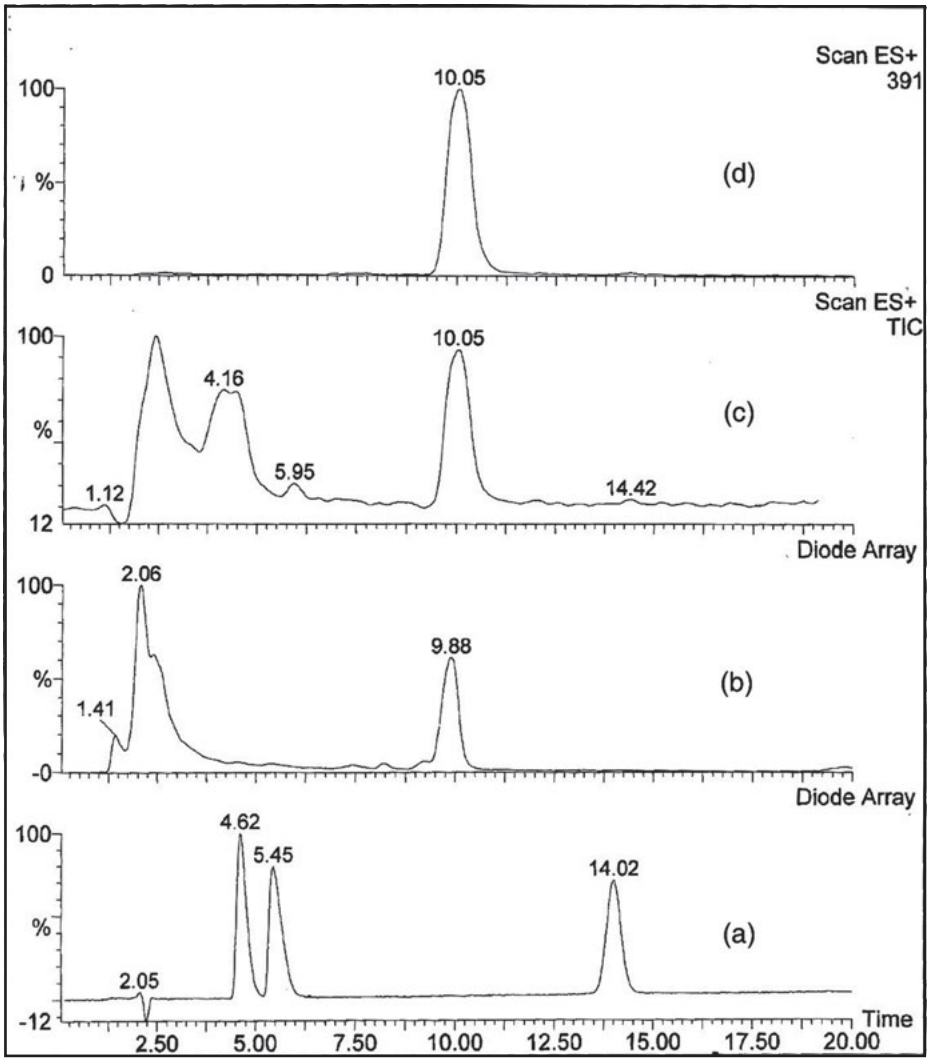


**Fig.9.17:** HPLC-PDA-ESI(+)-MS analysis for extract obtained from sample of herbal products illegally adulterated by tadalafil analogue (aminotadalafil, MW = 390, a synthetic organic compound has structural similarities with tadalafil): (a) PDA chromatogram for standard mixture (V ($t_R$ = 4.62 min), S ($t_R$ = 5.45 min) and T ($t_R$ =14.02 min)); (b) PDA chromatogram of sample; (c) TIC chromatogram of sample; and (d) extracted ion chromatogram for ion $[M + H]^+$ at m/z = 391 amu of sample.

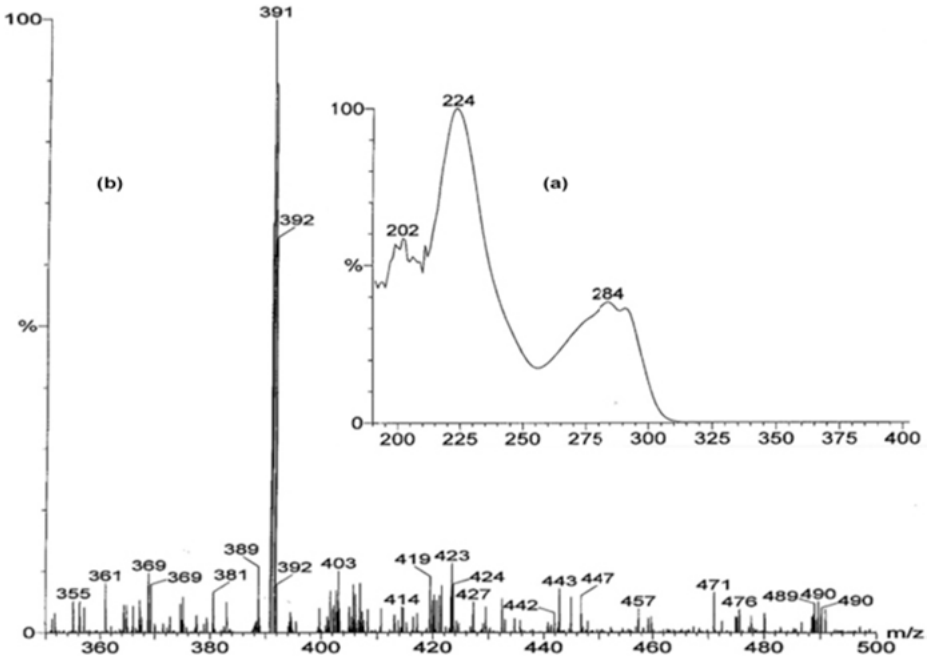


**Fig.9.18:** PDA spectrum (a) and mass spectrum (b) of chromatographic peaks at $t_R$ = 9.88 min and 10.05 min found on PDA chromatogram and TIC chromatogram (see Fig.12.18), respectively, for sample of herbal products illegally adulterated by the T analogue (aminotadalafil, MW = 390, a synthetic organic compound has structural similarities with T).

### Sample 3

TIC and PDA chromatograms resulting from LC-PDA-ESI(+)-MS analysis for sample 3 is shown in Fig.9.19. As shown in Fig.9.19, there is peak with a retention time at 0.7 min relative to S on the PDA and TIC chromatograms. The UV spectrum for this peak (Fig.9.20a) is comparable with standard S UV spectra (Fig.9.14). In the MS spectrum of this compound (Fig.9.20b), there are peaks at m/z = 505, 528 and 543 amu indicating for ions $[M + H]^+$, $[M + Na]^+$ and $[M + K]^+$ for a compound with a molecular mass of 504 amu. Based on these data, the compound adulterated in this sample could be identified as hydroxylhomosildenafil. This proposal was supported by the data which have been published in [38].

To the best of our knowledge we have achieved for the first time in Kuwait, a screening study for the presence of three synthetic PDE-5 inhibitors S, T, V and two of their analogues in investigated herbal products.

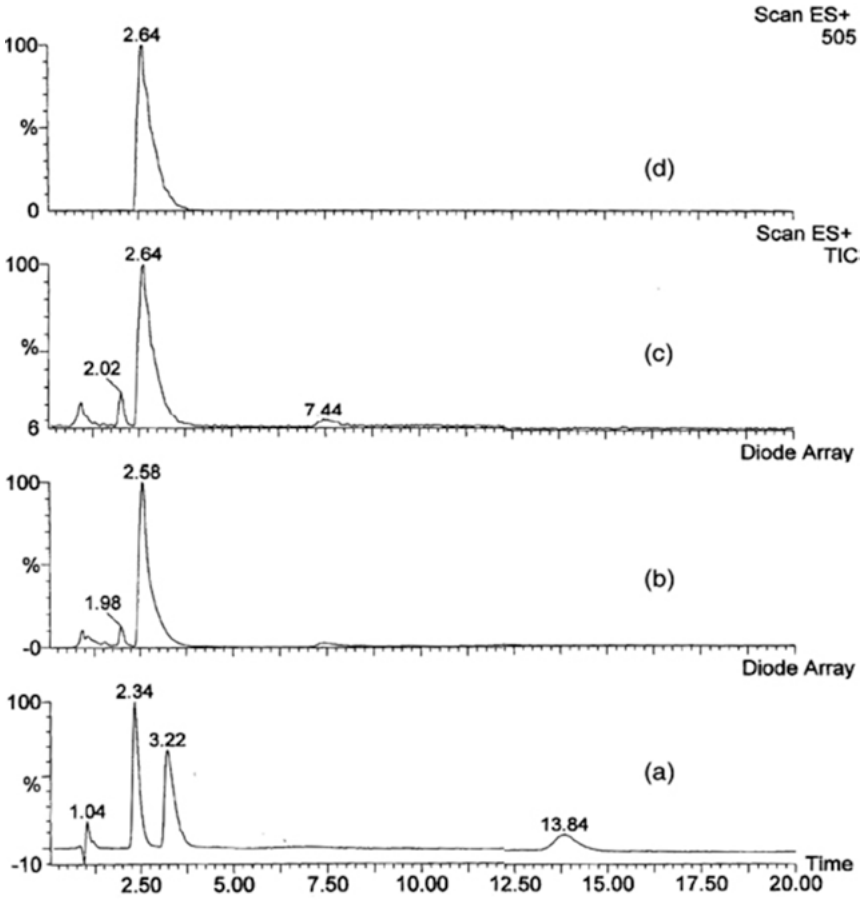


**Fig.9.19:** HPLC-PDA-ESI(+)-MS analysis for extract obtained from sample of herbal products illegally adulterated by one of sildenafil analogues (a synthetic organic compound has structural similarities with S, MW = 504 amu): (a) PDA chromatogram for a standard mixture (V ($t_R$ = 2.34 min), S ($t_R$ = 3.22 min) and T ($t_R$ = 13.84 min)); (b) PDA chromatogram of sample; (c) TIC chromatogram of sample; and (d) extracted ion chromatogram for ion $[M+H]^+$ at m/z = 505 amu of sample.

According to KUFDA rules, all adulterated herbal products have been canceled from registration and banned to be used by men in Kuwait. Finally, it can be concluded that during the last 10 years (2003–2012), the KUFDA laboratories have rendered a great service for protection of human health in Kuwait against the risk associated with consuming herbal products that were illegally adulterated by these adulterants.

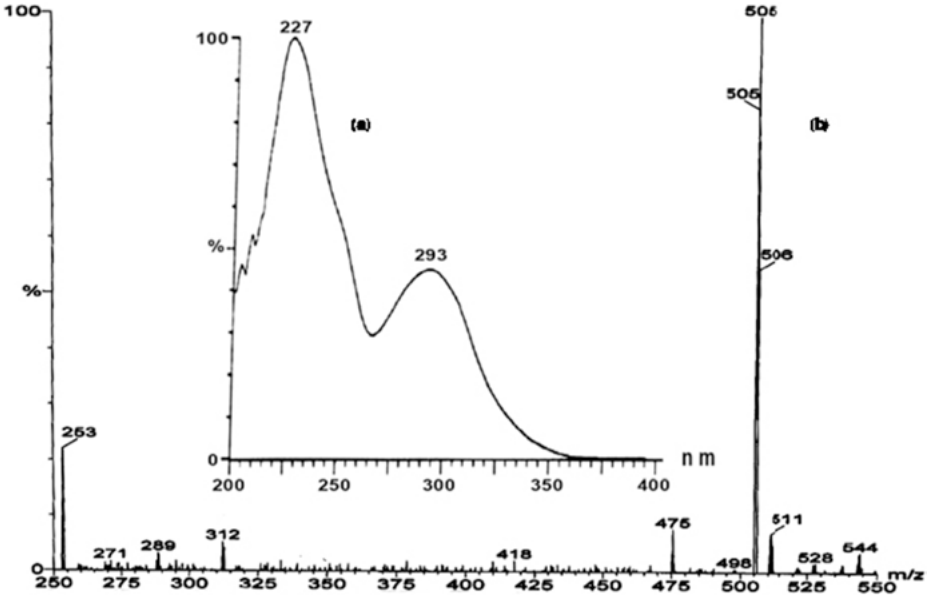


**Fig.9.20:** PDA spectrum (a) and mass spectrum (b) of chromatographic peaks at $t_R$ = 2.58 min and 2.64 min found on a TIC chromatogram (Fig.9.19), respectively, for sample of herbal products illegally adulterated by one of S analogues (a synthetic organic compound has structural similarities with S, MW = 504 amu).

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# 10 Safety in HPLC laboratories

## 10.1 Introduction

The practice of high-performance liquid chromatography (HPLC) is carried out primarily in *chemistry or analytical instrumentation laboratories*. These laboratories are designed for safe and efficient use of their facilities. But no matter how well designed the laboratory is, personal safety is ultimately the responsibility of each analyst as well as management. This requires well-informed and trained laboratory users. This chapter discusses elements of good, safe laboratory practice pertinent to the HPLC user. The fact that majority of the user's time is spent with instrumentation is no excuse for neglecting safe laboratory practices. Many other lost time accidents are possible when performing HPLC. Many of these potential accidents, and methods to avoid them are presented below. Generally, it is important to say that handling solvents, preparation mobile phase, managing mobile supply for HPLC equipotent and managing of HPLC waste disposal in HPLC laboratories must be in accordance with well-known applicable international and national regulations [1–10].SCAT Europe GmbH [11] is a German company, www.scat-europe.com,that has principal activity concerning the protection of users of analytical laboratories, especially HPLC users in front of substances harmful to their health. For that purpose, SCAT Europe GmbH has developed more than 600 products, related to safety in analytical laboratories and saving significant cost via its range of solutions. About 80% of HPLC users in Europe place their trust in SCAT Europe GmbH as a market leader and developer of safety caps. In more than 150 countries (of 192 worldwide, equivalent to 78%), SCAT Europe products are contributing to increased safety in laboratories and production. In the area of HPLC safety, their activities are directed to the following points:

- Safety caps for covering HPLC – mobile phase reservoir (safe management for mobile phase supply to HPLC equipment during analysis)
- Safety waste caps for waste container (safe management for liquid disposal of HPLC)
- Safety funnels to fill your liquids safely in waste container.
- Fill level control and monitoring devices for HPLC liquid waste in waste container.
- Safety complete set for HPLC
- Simple and safe pouring of liquid waste of HPLC into collecting tanks from waste container

In this chapter, we shall discus in details the previously mentioned topics.

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## 10.2 Safety handling for solvents, mobile phase preparation and its supply for HPLC equipment during analysis

### 10.2.1 Safety handling for solvent and mobile phase preparation

The most hazardous materials when an HPLC user is frequently in contact are the volatile and flammable solvents (such as acetonitrile or methanol, and these are often mixed with acidic components such as formic or trifluoroacetic acid) used for mobile phases and sample preparation, flushing and cleaning. The volume of these solvents will vary from a few milliliters to many liters at a time. The dangers involve toxic fumes, either inhaled or absorbed through the skin, and fires.

**Using dangerous or volatile substances with leaky or uptight containers leads to:**

- **Health hazards**
- **Contamination of mobile phase**
- **Shrinkage of mobile phase from evaporation 18**
- **Air and environmental pollution in laboratory**

The following list highlights many precautions that HPLC users should follow when working with solvents and preparation of mobile phase:

1. The toxicity of organic solvents used in the mobile phase needs to be reviewed and integrated into safe laboratory practices and procedures. The Material Safety Data Sheets of each solvent should be consulted prior to use, preferably with a safety and health professional who is knowledgeable about workplace chemical exposures and fire safety. The two common RPLC solvents, acetonitrile and methanol, can be used safely in the lab if good engineering control and appropriate personal protective equipment are being employed. Other solvents used in NPC or GPC like tetrahydrofuran (THF), methylene chloride, dimethyl sulfoxide (DMSO) and dimethyl formamide need to be handled with a higher level of work place safety and environmental control. In the case of methylene chloride, OSHA [1] has a specific set of regulations for workplace monitoring and control.

2. Store all flammable solvents in OSHA [1] approved flammable storage cabinets. Most HPLC laboratories use and store large volumes of flammable solvents even if large volume central storage and stock areas are provided. The quantities usually range between 20 and 60 gal. Since liter quantities are usually prepared daily, large gallon bottles are generally stocked; portable approved storage cabinets are allowed to hold up to 60 gal. Storing solvent bottles under laboratory benches, on top of the benches and in hoods only produces clutter and increases the hazard of breakage and spillage. This is particularly important for HPLC users who store their solvents in glass rather than metal or plastic.

3. Most HPLC-grade solvents are available in 1-gal glass bottles and should be transported in an appropriate secondary container such as a rubberized carrier. Many common solvents are available in stainless steel "kegs" or cycletainers (Fig.10.1), which are pressurized with nitrogen for convenient dispensing in a laboratory and must use local exhaust ventilation, such as a laboratory fume hood. A common cycletainer size is 19 L or 5 gal. These should be transported with the use of safety carts. Be sure that all safety storage cans or containers are OSHA [1] approved and compatible with the solvent to avoid contamination.

4. Ground any metal drums used to store bulk solvents for future transfer; grounding drums will prevent static electricity from building up which could spark between the drum and the transfer container and cause an explosion or fire.

5. Prepare all mobile phases in a well-ventilated, non-cluttered laboratory hood. The chromatographer typically prepares liter quantities of mobile phase. Toxic vapors are always present when pouring, mixing or degassing solvents. For details about safety preparation and handling of mobile phase inside HPLC laboratories, see Chapter 3 of this book [12–16]. The potential danger for flask implosion during vacuum filtration and degassing of mobile phase should be noted [13]. This danger can be substantial when vacuum filtering larger volumes (i.e., 4 L) or if non-vacuum grade glassware is mistakenly used. Coated glass equipment and additional safety shielding need to be considered for this operation.

### 10.2.2 Safety caps for safe handling of mobile phase supply to HPLC equipment during analysis

During your work with HPLC systems, toxic vapors' are leaked from solvent reservoirs if they are not accurately closed. It is well known that the permanent hazards for environment and health of operators are a breaking issue [12], especially while considering today's safety regulations. In order to comply with OSHA [1] requirements for safe handling of organic solvents, the mobile phase reservoirs must be covered. This will prevent concentration changes from occurring in the mobile phase where more than one solvent is present. Also covering the mobile phase will prevent introduction of solvents vapors into the laboratory. This can easily be handled by using a safety cap [11], as seen in Fig.10.2 and 10.3. Safety cap, as shown in Fig.10.2 and according to [11], is consisted of a screw cap, PTFE body is 360° freely rotatable, connectors for fitting capillaries which supply mobile phase from mobile phase reservoir to HPLC equipment and air valve. The air valve in the safety cap works as a check valve and includes a membrane filter for the incoming air (Fig.13.3). The valve allows air to flow into the interior of the mobile phase reservoir to compensate for the pressure during mobile phase withdrawal. The membrane in the interior of the air valve also prevents the escape of solvent vapors and thus protects the user

**Fig.10.1:** Containers for storage HPLC solvent during handling inside the laboratory.

from potentially toxic solvents vapors. Since there no escape of solvent vapors, the solvent concentration stays constant [11, 17]. This solvent is also protected from contamination, which enables reliable and reproducible measurement results [11, 18]. Harmful solvent vapors are blocked so that they stay inside the mobile phase **reservoir** and your staff is prevented from breathing contaminated air. At the same time, the filter membrane keeps air-borne particles from entering your mobile phase **reservoir** (Fig.13.3) and changing the composition of your mobile phase. *Since the filter membrane absorbs contaminants from the surrounding air, it is recommended that the valve be replaced every 6 months in order to ensure flawless operation.*The regular replacement of air valve allows optimal use of your safety caps, ensuring not only safety at work, but also provides reliable and reproducible analysis results by avoiding contamination in the solvent or the mobile phase. The air valve is delivered with a change-advice-label (Fig.10.4). Just sign-in the current date at the installation and you are safe in observing of the service life.

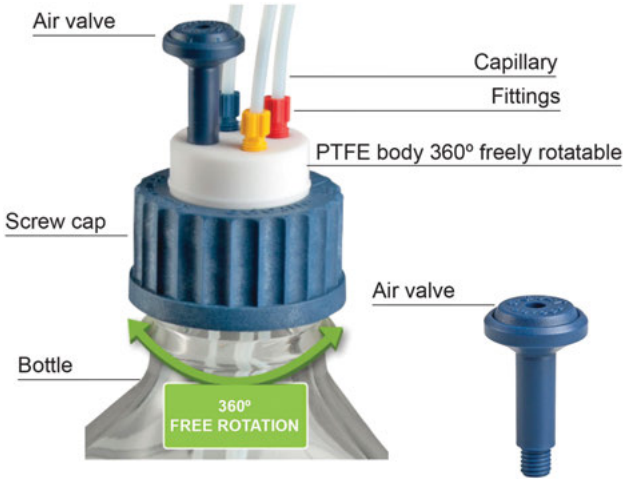


**Fig.10.2:** Safety cap with three connectors for capillaries and air valve for the safe supply of mobile phase to HPLC equipment. *Image source:* www.scat-europe.com.

Others benefits of safety caps are as follows:

- Safety caps have become indispensable for the improvement of safety in these laboratories.
- Increased safety for your laboratory and the health of your employees
- Prevention of the escape of harmful gases or vapors
- Protection of the solvent and the mobile phase
- Many safety and environmental directives are already regulated by law [1], and safety caps help you safely use these toxic or flammable organic substances without pollution.
- In cooperation with safety inspectors, HPLC operators and synthetics specialists, safety caps were developed as a cost-efficient and reliable solution for those well-known problems.
- Made of chemically inert materials
- No contamination of the solvent and the mobile phase
- Safety caps for the laboratory not only bring many benefits but also decrease your cost of analysis.
- Safety caps provide secure working without contamination and ensure the basis for reliable and reproducible measurement results.
- The connectors in the safety cap tightly secure the capillary tubes so that they do not slip out of place. No capillary slippage, so no accidental intake of air into the HPLC system. No interruption of analytical and work process due to air pockets in the capillary (Fig.10.5).

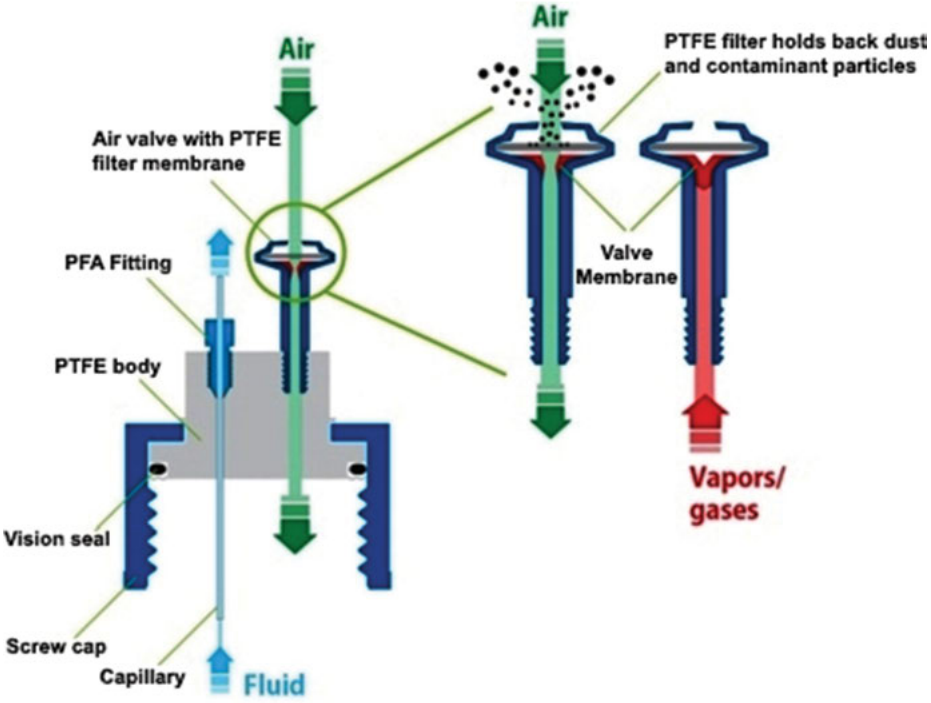


**Fig.10.3:** Diagram showing air valve functionality in safety cap.*Image source:* www.scat-europe.com.

Note: Some improper practice in handling of mobile phase inside the laboratory.

In some analytical laboratories, it is a common improper practice to "seal" bottles of HPLC solvents simply with a perforated aluminum foil (Fig.10.6A) or parafilm cover (Fig.10.6B). Connecting tubes are inserted into the bottles through the holes in the aluminum foil/parafilm/cap without any additional sealing. However, such basic covers do not seal the solvent containers efficiently. This uncontrolled evaporation of solvents is not only a potential health hazard but also the loss of solvent by evaporation which interferes with the quality and reproducibility of HPLC runs.

## 10.3 Safety waste caps for safe disposal of HPLC wastes (safety handling for waste in HPLC laboratories)

Operation of HPLC equipment often creates hazardous liquid waste. This process frequently results in wastes containing flammable and toxic solvents, such as acetonitrile, methanol and THF, which are often mixed with acidic components such


**Fig.10.4:** Change advice label on air valve.*Image source:* www.scat-europe.com.

as formic or trifluoroacetic acid. Presence of a toxic or volatile substance in leaky or untight containers leads to:

- Air and environmental pollution
- The toxic solvents in the waste containers often form an uncontrolled poisonous mixture for lab employees (health hazards)

So, safe disposal of HPLC waste is an important process. The following list highlights many of the precautions that HPLC users should take when working with HPLC waste:

1. In order to comply with OSHA [1] hazardous waste management requirements, all HPLC system drain containers (this includes sample injector waste containers) must be covered with waste safety caps (Fig.10.7 and Fig.10.8). These containers must

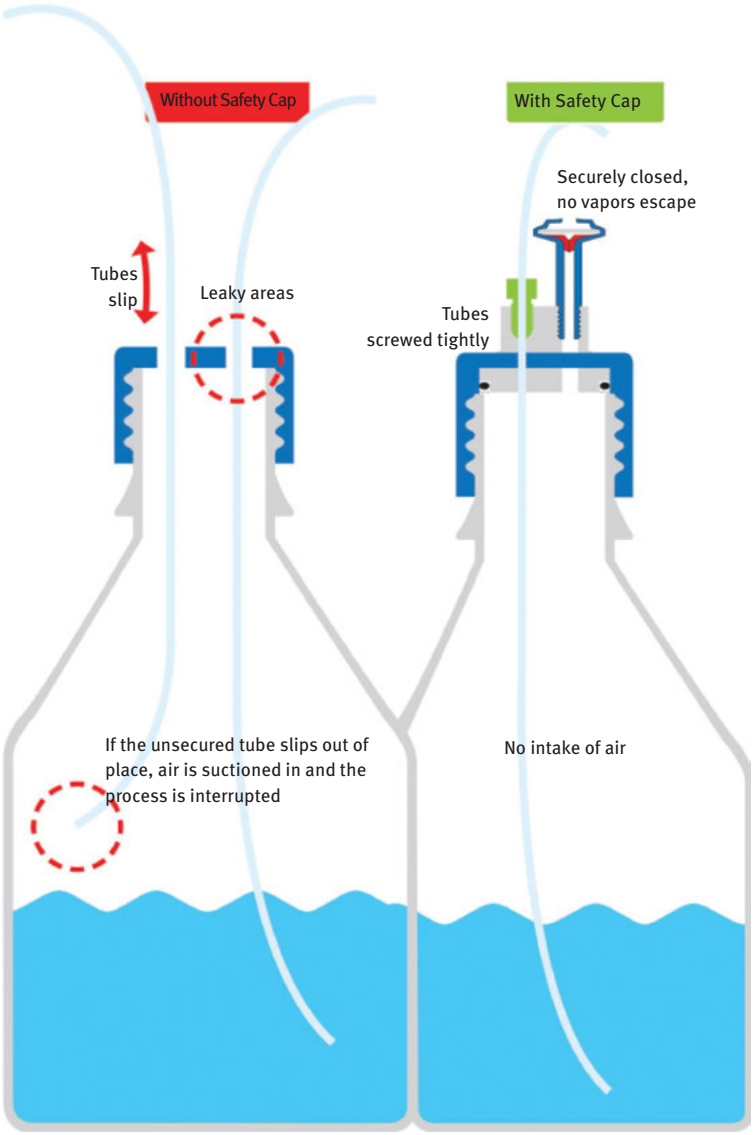


**Fig.10.5:** Illustrative diagram showing capillary tube slippage and leaky area in the case where no safety cap is used (left), and the pros of using safety cap (right).*Image source:* www.scat-europe.com.

properly be labeled as hazardous with a yellow label. The safety cap was equipped with exhausted air filter (Fig.10.8 and Fig.10.9) which has active charcoal that cleans the exhausted air by adsorbing the toxic solvent vapors, and makes the lab a healthier place to work. The exhaust filter is an essential component of the SCAT –

**Fig.10.6:** Improper HPLC mobile phase reservoir covering practices. (A) Cover with aluminum foil and (B) cover with parafilm.

Europe safety system [11]. It cleans solvent vapors from the exhaust filter and is available in different sizes. Fig.10.8 presents illustrated diagram showing the functionality of the exhausted air in safety waste cap. Its functionality is based on its content of the active charcoal (with a specific surface of 1,200 $m^2/g$) and contains additional components which prevent sticking or clumping and thus prevents restriction of the filter performance [11]. The safety waste caps are delivered ready for connection and fit capillaries with an outer diameter of 2.3 and 3.2 mm (Fig.10.7) and tube (the standard connector design is for flexible tubes with 6. 4–9.0 mm inner diameter) (Fig.10.10). Those drainage tubes are prevalent at HPLC-MS systems.

2. These waste containers should be properly labeled and must be stored in secondary containment as shown in Fig.10.11. Filled waste containers must be transported safely to a hazardous waste accumulation area.

Improper HPLC waste collection practices using open bottle, unlabeled and the bottle is not found in secondary containment as shown in Fig.10.12.


**Fig.10.7:** Direct collection for effluent of HPLC equipment (three connectors for fitting three capillaries for mobile phase drainage) in closed containers (canister) covered with safety waste cap equipped with exhaust filter.*Image source:*www.scat-europe.com.

## 10.4 Safety funnels for filling the liquid waste safely in waste container

SCAT Europe GmbH [11] developed two types of safety funnels: first one is safety funnel with ball valve and the second one is safety funnel with hinged lid.

### 10.4.1 Safety funnel with ball valve

Safety funnel with ball valve (Fig.10.13) is:
- Made of PE-HD plastic, so no corrosion by acids
- Suitable for all types of solvents
- The ball floats and automatically closes after filling (Fig.10.14).
- The screw cap rotates freely, making it easier to unscrew the funnel (Fig.10.13 and 10.14).
- Splash protection and sieve to catch dirt and larger contaminant particles.

### 10.4.2 Safety funnels with hinged lid

Safety funnels with hinged led (Fig.10.14) is:
- Made of PE-HD
- Suitable for all types of solvents
- Properly closed, thanks to the hinged lid
- Removable sieve for easy clean up

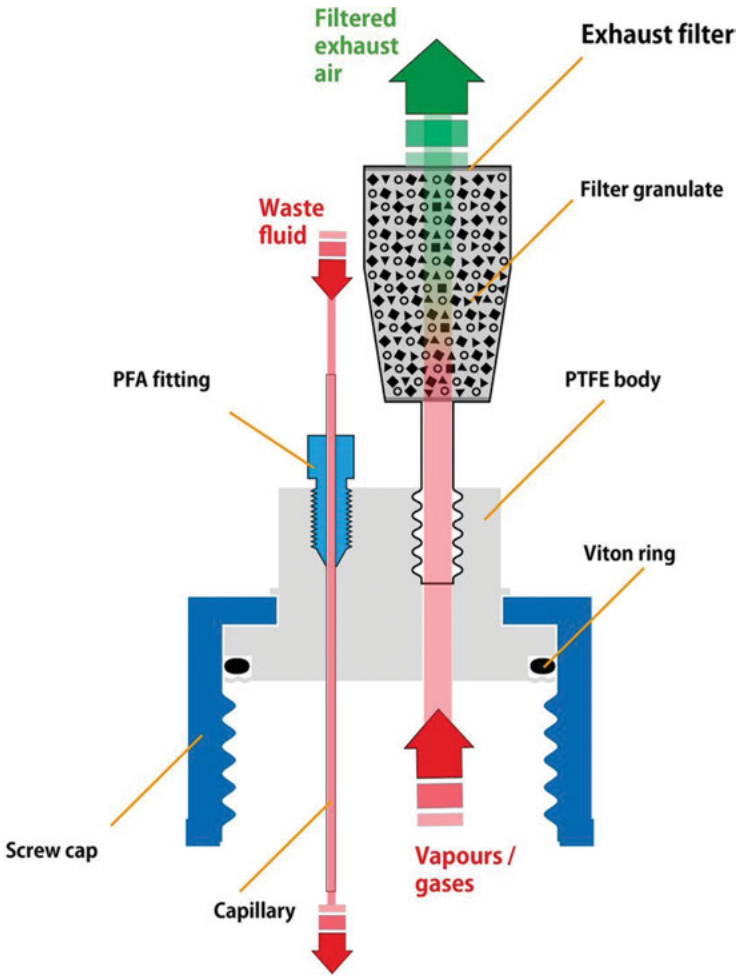


**Fig.10.8:** Illustrated diagram showing the functionality of exhaust filter in safety waste cap.*Image source:* www.scat-europe.com.

### 10.4.3 Safety waste caps with included funnel

– In this case, the **safety waste caps are equipped with a funnel as shown in** Fig.10.15. **This funnel is** practical for disposal of small volumes of liquid waste, for example, emptying autosampler vials to waste container (filling small amount of liquid waste into the closed waste container without inconveniently unscrewing it). This funnels come either with automatic closure (Fig.10.15A) or shutoff (Fig.10.15B).


**Fig.10.9:** Exhaust filter with change indicator.
*Image source:* www.scat-europe.com.


**Fig.10.10:** Safety waste cap ready for connection and fits two capillaries with an outer diameter of 2.3 and 3.2 mm for mobile phase drainage, one tube (the standard connector design is for flexible tubes with 6.4–9.0 mm inner diameter) and exhaust filter.
*Image source:* www.scat-europe.com.


**Fig.10.11:** Proper HPLC waste collection using a waste canister covered with safety waste cap and stored in secondary containment (collection tray). The canister was properly labeled that contain waste.

**Fig.10.12:** Improper HPLC waste collection practices using open bottle, unlabeled and the bottle not found in secondary containment.

## 10.5 Waste container with filling level control and monitoring devices to avoid any risk of overflow

Overflowing waste containers in laboratories can be dangerous when work is done with critical substances and solvents (toxic, flammable). Dangerous fumes can spread fast. This is not only dangerous for individual health, but under certain circumstances can also lead to an explosion. Fig.10.16 shows a waste container provided with optical signal (through waste safety cap) for controlling filling levels. The red floater is immediately visible when the container has reached the critical filling level. No overflow or interruption occurs due to drained containers.

Fig.10.17 shows a waste container provided with electronic levelling control (through safety waste cap) for controlling filling levels of liquid waste inside waste container. If the critical filling level has reached, you will be informed about the status of the container by an acoustic signal (beep) and a LED warning light (*light signal and audio warning*).


**Fig.10.13:** Safety funnel with ball valve: (A) general diagram. (B) Illustrative diagram showing the functionality of ball valve in safety funnel. *Image source:* www.scat-europe.com.

## 10.6 Waste container with filling level control and safety funnel for waste collection in HPLC laboratories (disposal unit)

In order to get your waste fluid collection site ready for operation quickly, you can put the most popular combinations together in a complete set (**disposal unit),** as shown in Fig.10.18. No time consuming compilation for a configuration. The delivery is made completely by SCAT [11] **and it is immediately ready for use.** In order to prevent static discharge of fluids in plastic containers (waste container), the contents of the waste container are secured with the ground connection (grounding cables) **to prevent electrostatic charging and sparking.** This allows you to establish an additional safety factor when collecting combustible fluids.


**Fig.10.14:** Safety funnels with hinged lid.Image source: www.scat-europe.com.

**For simple and safe pouring of liquid waste from the waste containers, you can use safety spouts as shown in** Fig.10.19.

## 10.7 Complete safety set for HPLC

Recently, safety complete set can be used with any HPLC system for managing mobile phase supply using safety caps and managing waste using safety canister equipped with safety waste caps. As shown in Fig.10.20 and according to SCAT Europe [11], this complete safety set for HPLC is consist of:

- Safety caps for the safe withdrawal of mobile phase
- Mobile phase bottles
- Fittings and connectors for a wide range of tube diameter
- Safety waste cap provided with exhaust filter filled with special active charcoal
- Waste canister


**Fig.10.15:** Safety funnels for safe filling of liquid waste into waste container. (A) Safety funnels with automatic closure and (B) safety funnels with shutoff. ***Image source:*** www.scat-europe.com.

## 10.8 Pressured liquids safety in HPLC laboratories

Constant volume pumps pose very little danger. However, constant pressure pumps can be a hazard as they will continue to pump, releasing the solvent with some force when disconnected from the rest of the HPLC system. Care must be taken when cracking fittings on the pump HPLC unit is in use. Reciprocating constant volume pumps often employ restrictors and/or dampening units between the pump and the sample injector. If the system becomes clogged and blocked, it is possible for the pump to continue pumping until the restrictor or dampener explodes. The break in the system will occur at the weakest section, and if the pumps pressure relief system is set above the pressure limit of these devices, they can blow open. A plug of this type can come from a piece of septum logged in some tubing or an injection valve stuck between ports. Although it cannot prevent all pressure related


**Fig.10.16:** Safety waste cape equipped with floater level control: (1) safety waste cap, (2) exhaust filter, (3) level control (floated level control), (4) connectors for fitting cappliers and (5) waste canister.*Image source:* www.scat-europe.com.

problems, the user should consider purchasing pumps or system devices with pressure limit relief systems which can minimize system component damage and personal harm.

## 10.9 Electrical safety in HPLC laboratories

Nearly every component or accessory of the HPLC system uses electricity. This requires that the HPLC laboratory have enough electrical circuits to handle all the instruments and sufficient grounding. When repairing any piece of equipment, be sure that the power is off and the system is preferably unplugged. This will avoid shocks and physical harm from moving parts. If power is needed to test the equipment, use insulated tools and caution. Never poke into circuit boards with tools such as screwdrivers and awls. Capacitors, especially in power supplied, store charges that can dissipate through the user with a good jolt. Always ground them first. Finally, have your qualified electrical engineers or service team examine the electrical schematics for any hazards or difficult repairs.


**Fig.10.17:** Safety waste cap equipped with electronic filling level control.
*Image source:* www.scat-europe.com.


**Fig.10.18:** Disposal unit for collection of HPLC solvent waste.
*Image source:* www.scat-europe.com.

**Fig.10.19:** Safety spouts for pouring liquid waste from waste containers.
*Image source:* www.scat-europe.com.

### 10.9.1 Electrical fire precautions

- Switch off power and disconnect line cord before performing maintenance.
- Do not restrict air intake or exhaust at the back of the instrument.

## 10.10 Physical injuries and safety in HPLC laboratories

Next to solvent-related accidents, physical injuries and accidents are most common. These usually occur through carelessness and when no attention is paid to what is going on. The first area of concern involves physically moving HPLC equipment. It is very common for laboratories to relocate or to move the equipment for maintenance and repair work. What users often forget is that the weight of HPLC instruments and individual components is particularly deceiving, and usually the equipment is not in a position for the safest leverage for the user's back. Furthermore, many users have not kept up with their physical fitness programs and may easily strain and pull muscles when moving equipment. It is recommended that the user have experience with help when unpacking, setting up and moving HPLC equipment. The appearance of herculean efforts prove nothing when an injury results. Also, safety shoes should always be worn when this equipment is moved. Some equipment may have spring loaded parts or heavy doors and lids which can cause an injury. If the lid is not


**Fig.10.20:** Safety complete set for HPLC system for managing mobile phase supply using safety caps and managing waste using safety waste cap and safety canister.
*Image source:* www.scat-europe.com.

properly secured when opened as the manufacturer has provided, it can drop on the user's arm, hand or head, causing an injury. Any compartment on a piece of equipment which opens and closes should be checked periodically for safe operation.

Finger cuts are a very common hazard. They can occur while cutting stainless steel tubing, repairing a broken sapphire pump plunger, being stabbed with a syringe needle and cleaning up glass from broken solvent bottles, sample vials and so on. Syringes should always be kept in a locked drawer or cabinet and never be placed in a drawer of miscellaneous items for an unsuspecting user to find while rummaging through the drawer.

## 10.11 HPLC detectors handling and safety in HPLC laboratories

The major problem with detectors is eye damage from the UV source lamps. The user should never look directly at these lamps and should wear appropriate protective eye cover. Some detectors still use UV source lamps that produce ozone. Ozone in the laboratory atmosphere can cause respiratory problems. These detectors should be operated with a source vacuum directly above the lamp. A simple technique to accomplish this is to place an inverted funnel over the lamp, connected to a hose and the source of a house vacuum or an aspirator.

### 10.11.1 UV precautions

Wear UV blocking eye protection when aligning or changing UV lamps.

## 10.12 General safety rules in HPLC laboratories

- The regular replacement of consumables such as activated carbon filters for waste safety cap and air valve for safety cap allows optimal use of safety caps, ensuring not only safety at work, but also providing reliable and reproducible analysis results by avoiding contamination in the solvent or the mobile phase.
- Flush and purge pump heads carefully and in a well-ventilated area. This usually occurs when a solvent like methanol is used to eliminate an air bubble from the pump; during this process, solvent can be running over and down the face of the pump. One method of minimizing the buildup of fumes is to use an elephant hose or some other suitable form of vacuum or aspiration directly above the pump to withdraw the fumes from the user. Also, a drain pan should be used to prevent solvent from dripping on the floor.
- Keep solvents off the skin. When checking for leaks or getting solvents on the hands or other parts of the body. Quickly rinse and/or flush these areas with

plenty of water. Organic solvents can be absorbed through the skin. Most of the time, a user will be unaware of this absorption. However, when cleaning columns with DMSO (see Chapter 2), the smallest amount of this solvent on the skin will be absorbed. This can be detected quickly by the taste of DMSO in the mouth, which can last for days. Therefore, it is not recommended to use the thumb or finger to increase the back pressure on a detector when eliminating air bubbles.

- Do not squirt solvents from injection syringes on the floor. This is a common habit of most GC and HPLC users. Although it may only be a few microliters, it will add dangerous fumes to the laboratory air, spot the floor and, if aimed wrong, can injure another person.
- Do not operate HPLC systems in laboratories or areas where open flames, sparks or excessive heat may be present. This includes Bunsen burners, muffle furnaces, ovens, non-explosion proof refrigerators and gas chromatographs, especially those equipped with flame ionization detectors.
- Always dispose of waste solvents, effluents, samples and so on, in suitable waste solvent disposal cans. These should be emptied into bulk waste storage for the entire laboratory and picked up by an authorized solvent disposal company. Most organic solvents can be mixed in the same waste containers.
- The halogenated solvents such as $CHCl_3$ and $CH_2Cl_2$ should always be stored separately from the other organic wastes. Halogenated solvents will react violently, for example, in the presence of acetone. Solvents like dioxane, either and THF can form explosive peroxides. These containers should be kept covered and neutralized with aqueous sodium sulfite or a solution of ferrous salt. These solutions can also be diluted and washed down the drain.
- Have adequate spill control materials or kits in the HPLC laboratory. The following typical materials can be used for spills, sodium bicarbonate for acids, citric acid, boric acid or 6 N hydrochloric acid for bases.
- Properly label every solvents and sample container.
- Never work alone.
- Never eat, drink or smoke in the laboratory.
- Always remove jewelry and watches when working with the equipment and when carrying out any maintenance or trouble shooting practices.
- Always wear OSHA [1] approved safety glasses
- Wear proper laboratory clothing and safety shoes.
- Use proper tools for any repairs.
- Keep informed and alert to safety practices and regularly attend scheduled safety meetings and seminars.
- Know where all the exits and the safety equipment are located.
- Know the telephone numbers of all emergency personnel in your area, including local police, fire and hospitals.
- When working in a chemical laboratory, contact with acids and caustic solute at low concentrations may cause long-term damage to the cornea. There is also the

risk of dust contact with the eyes. As a result, according to safety requirements, all chemical laboratories must be equipped with high quality eye showers (one or two spray heads) and body safety showers (Fig.10.21).

**Fig.10.21:** Eye shower and body safety showers.

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# 11 Glossary

**Table 11.1:** Glossary of frequent abbreviations and terms used in HPLC.

| | |
|---|---|
| AUFS<br>(absorbance units full scale) | When being detected by visible light or ultraviolet absorption, the highest value of absorbance can be measured at a specific attenuation of the detector. AUFS represents absorbance units (per) full-scale deflection. |
| Adsorption chromatography | Separations that occur due to differences in adsorption characteristics of the components of interest on the stationary phase. |
| Affinity chromatography | A specialized type of chromatography in which bonded phases that have the property of absorbing only a particular kind of molecule are used. |
| Ambient temperature | It is the standard temperature of air around the media surrounding the equipment. |
| Band of chemical components on separation column | The area (or volume) in the column in which either the chemical components or the chemical component (solute) is present and that too at any point in time. |
| Band spreading | The diffusion of the band into a greater area (or volume) due to column effects or fittings. |
| Bonded phase | A separation technique based upon the ability of components to adsorb to a particular stationary phase as a constant flow of liquid moves across the stationary phase. |
| Baseline | The portion of a chromatogram recorded when only the mobile phase is flowing through the HPLC system. |
| Beam splitter | The optical component in an HPLC which accepts the light emitted from the grating and splits the beam into two separate light beams. It directs the beams into the reference and sample side of the flow cell. |
| Calibration | Process of determining the HPLC by comparing or measuring it against a standard, of the value (area) corrected of the injected sample. |
| Column | In HPLC, a steel or plastic tube packed with a particular stationary phase separates similar molecular groups from a common matrix or sample. |

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**Table 11.1** (continued)

| | |
|---|---|
| Chromatographic peak | The peak in a recorder trace shows how the detector responds to the chromatography band exiting the mobile phase in the form of a chromatographic column. The ideal shape it should have should be Gaussian. |
| Chromatography | A method of separating and analyzing mixtures of chemical substances. |
| Connector | A physical device (such as a plug, socket or jack) used to connect one hardware component of an HPLC system to another. |
| Column capacity factor | It is the total solute moles in the stationary phase/the total solute moles in the mobile phase – retention of the component compared to the solvent front (or dead volume). |
| Column volume | A volume of solvent that is identical to the particular void value stated in the system. See void volume. |
| Degassing | The process of separating gas from the mobile phase. This is done to ensure that there is no formation of bubbles in either the fluid system or the pump. |
| Detector | A device for assaying the solutes that are present in the effluent in the HPLC column. This assessment is both quantitatively and qualitatively done. Measurement of the mobile phase's solute property is taken. Common means to do so are: when light is absorbed by the solute in the visible or ultraviolet, the solute's fluorescence, contrast in the refractive index of the solute and amperometric (electrochemical) solute sensing. |
| Detector response | The detector's signal (electric output) is the eluting components concentration function; it can be compared and measured as the peak area or peak height. The calibration curve is made when a known concentration of compound is given as the detector response and is done through chromatography of amounts that are known of a standard over the range of interest, and the detector response (peak area and peak height) is then plotted versus the amount being injected. This plot slope is known as the *response factor*. |
| Deuterium lamp | The optical source for HPLC detector. It provides the continuous light intensity in the ultraviolet and visible range. |

**Table 11.1** (continued)

| | |
|---|---|
| Diffraction grating | Optical component of an HPLC detector. When light strikes the grating, it separates the light into its individual wavelengths. Each wavelength then travels to next optical component in the light path by movement to the grating to the proper angle. |
| Dead volume | Any volume between the injector and detector where separation does not occur. |
| Elution chromatography | The removal of the components of interest from the column with an eluent. |
| Electrical ground | The conducting path that is between the earth and the equipment or electric circuit. It can also be a huge conducting body that is used as a reference for voltage instead of using the earth. |
| Exclusion limit | The molecular size limitation for a particular exclusion method or column |
| Eluent | Mobile phase which passes through the column (stationary phase) and removes the sample components from the stationary phase. |
| Flow cell | An optical component of an HPLC system allows the sample and solvent to pass in front of the detector. Any absorbance detected by the photodiode is transferred to the electronics. |
| Gel filtration | A form of size-exclusion chromatography utilizes a soft gel as the stationary phase. |
| Gradient elution program for the mobile phase 12 | Altering the mobile phase's composition at the rate per time specified during the chromatographic process. |
| HPLC equilibrations | The process of bringing an HPLC system (solvent, column and interactive surfaces) to a state at which forward and reverse reactions occur at equal rates so the balance of the system does not change during the run. Usually indicated by a stable, drift-free baseline resulting from proper conditioning of the mobile phase (assuming the rest of the system is clean and functioning properly). |
| HPLC column plate number (theoretical plate number) (N) or column efficiency | Measurement of the number of plates in an HPLC column. It is desirable that this number is large. The larger the number of plates per meter, the greater the column efficiency (N). |

**Table 11.1** (continued)

| | |
|---|---|
| Hardware | The physical components of the HPLC system: detector, pumps, and so on. |
| HPLC peak fronting | A lack of symmetry on the upside of the HPLC peak (opposite of tailing). |
| Ion-exchange chromatography | A mode of chromatography based upon ionic charge affinities. |
| Liquid chromatography (LC) and HPLC | A method used to break down different mixtures into separate components for the following purposes: identification, quantification or isolation. A liquid is used to dissolve a sample that goes through the column (tube) and has the stationary phase (very fine solid materials) and is passed on by the mobile phase (flowing liquid). While the components are passing through the column, they have varied movement because of the physical and chemical interaction of the component with the stationary phase. At the end, they leave the column reparatory. The separating power is directly related to the stationary phase. If the separating power is high, it is because of a larger surface area. Because of the inadequate gravity, proper liquid flow rates are not achieved. Hence, the modern microparticulate column does not get proper flow rate. For this, a pump is used. The pressure of which can go to several 1,000 psi. Thus, the origin of the designation “high-pressure liquid chromatography” along with high-speed liquid chromatography and performance liquid chromatography are not being used and the name liquid chromatography replaces them. |
| Linearity of the calibration curve | How close the calibration curve and the straight line specified beforehand is known as linearity of the calibration curve. It is the degree proportion of the HPLC peak area or output to its input (analyte concentration) in a linear device. |
| Low dead volume connection | A fitting allows two pieces of an HPLC tubing to be joined in such a manner that there is minimal volume between their ends. |

**Table 11.1** (continued)

| | |
|---|---|
| Mobile phase | The liquid phase of an HPLC separation carries the dissolved sample through the stationary phase in liquid chromatography/HPLC. It can either be a single solvent or it can be a homogeneous combination of a number of different solvents. Keeping the configuration of the mobile phase constant during the elution process happens in isocratic elution, and when it is varied, it is gradient elution. |
| Mobile phase flow rate | The rate at which a volume of the mobile phase is flowing under pumping pressure per unit of time (milliliters/min, mL/min) through an HPLC system. Milliliters of mobile phase are pumped during a given time frame. |
| Mobile phase flow rate programming | Altering the flow rate during the elution process in HPLC. |
| Paired ion chromatography (PIC) | The technique by which the functionality of the mobile phase is altered by adding ionic reagents to the mobile phase to improve selectivity of separation of RP columns. |
| Peak height | The distance (on a chromatogram) from the base of the peak to the highest point of the same peak. |
| Photodiode | A component of an HPLC detector accepts light energy in the form of photons and converts it to electrical current for the signal processing. |
| Partition chromatography | Chromatographic technique based on the variation of solubilities in the middle of the stationary and the solute phases. |
| HETP or plate height | Height equivalent to a theoretical plate measures the efficiency of the column and should be very small. HETP indicates the length of a plate in which the stationary and mobile phases are in equilibrium. |
| Pulse damper | The device used to smooth flow pulsations produced by the pump. |
| Sample | A representative fraction of material tested or analyzed in order to determine the nature of composition and percentage of specified constituents. |
| System | An organized collection of parts that are interacting and interrelated to carry out a specific purpose or function. |

**Table 11.1** (continued)

| | |
|---|---|
| System interface (SI) | The unit that services as a link for the transfer of data and control commands between the liquid chromatography system and the computer. |
| Resolution | The degree of separation of a solute mixture into its individual components. |
| Variable | A quantity that can assume any set of values. |
| *UHPLC* | Known as the ultra-high-pressure liquid chromatography. It is mostly used when the process of separation cannot be performed, that is, for pressures over (400 bar) the capacity of the conventional pumps. |

**Table 11.2:** Glossary of frequent abbreviations and terms used in HPLC–MS.

| Term | Signification |
|---|---|
| A.M.U (amu) | Atomic mass unit |
| API | Atmospheric pressure ionization |
| APCI | Atmospheric pressure chemical ionization |
| CID | Collision-induced dissociation |
| DLI | Direct liquid introduction |
| DC | Direct current |
| EI | Electron impact ionization |
| ESI | Electrospray ionization |
| eV | Electron volt |
| FAB | Fast atom bombardment |
| FTMS | Fourier-transformed MS |
| ITMS | Ion trap MS |
| MALDI | Matrix-assisted laser description ionization |
| MALDI TOF | Time of flight with MALDI ionization |
| MS | Mass spectrometry |
| PB | Particle beam |
| RF | Radio frequency |

**Table 11.2** (continued)

| Term | Signification |
|---|---|
| SIM | Selected ion monitoring |
| SIR | Selected ion recording |
| SRM | Selected reaction monitoring |
| TIC | Total ion current |
| TOF | Time of flight |

# Index



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