High-Performance Liquid Chromatography: From Discovery to New Advancements

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Abstract

In the last few years, high-performance liquid chromatography has emerged as one the most commonly used instrument in analytical chemistry for wide range of applications. This instrument is used in almost every sector for different purposes. During this phase, the advancement in the technique also resulted in the discovery of advanced chromatographic instruments such as Ultra-high pressure liquid chromatography. This paper summarizes the principle, instrumentation and advancements in HPLC.

Keyword: Chromatography; HPLC, UHPLC.

Introduction

Conventional liquid chromatography primarily involves large columns with relatively large particle size of stationary phase, through which the mobile phase passes under gravity and the fractions are collected manually for measurement in a spectrophotometer [1, 2]. Although a century old technique, it still find applications in synthetic chemistry and natural product laboratories. However, in 1964, Calvin Giddings predicted the improved performance of liquid chromatography by decreasing the particle size of stationary phase which ultimately increases the theoretical plate number [3]. A couple of years later, first high-pressure liquid chromatograph was developed by Lipsky and Horvath at Yale University (US) [4]. In 1970, the improved efficacy and performance of the systems by using small size silica particles were reported, and this improved technology now a days is referred as high-performance liquid chromatography (HPLC). This technology is still being explored and has resulted in some of the advanced systems such as ultra-high-pressure liquid chromatography (UHPLC) capable of pumping mobile phase at 15000-19000 psi [5].

Principle

In HPLC, differential affinity of components towards stationary phase and mobile phase is the key principle. The different components in a mixture travel through the column depending upon their affinity towards solid stationary phase. The component which has more affinity towards adsorbent, travel slowly and eluted from the column in the end. On the other hand, the component having less stationary phase affinity travels faster and is eluted early. As the different components have different chemical structures, hence they have different physiochemical properties. Affinity, which is one of the physical property, is also different for different components and based on their affinity, they are separated in the column. The kinetics of distribution of solutes is largely diffusion-controlled and diffusion coefficient is 1000 to 10,000 times slower in liquid as compared to gases [6].

HPLC system

The basic components of HPLC system and a modern HPLC system have been shown in figures 1 and 2.

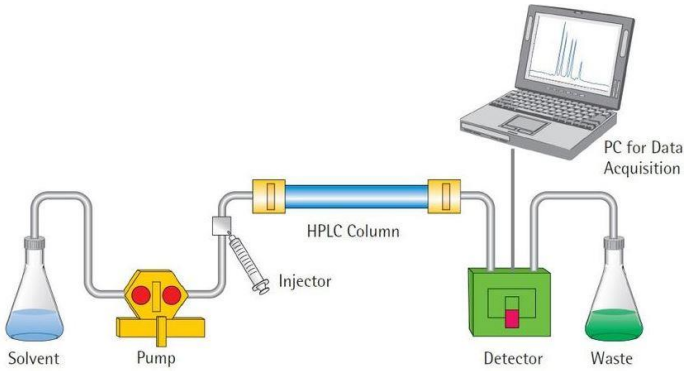


Figure 1: Basic components of HPLC

Figure 2: High-performance liquid chromatograph (Courtesy of Waters Corporation)

The detailed working of HPLC has been shown in figure 3.

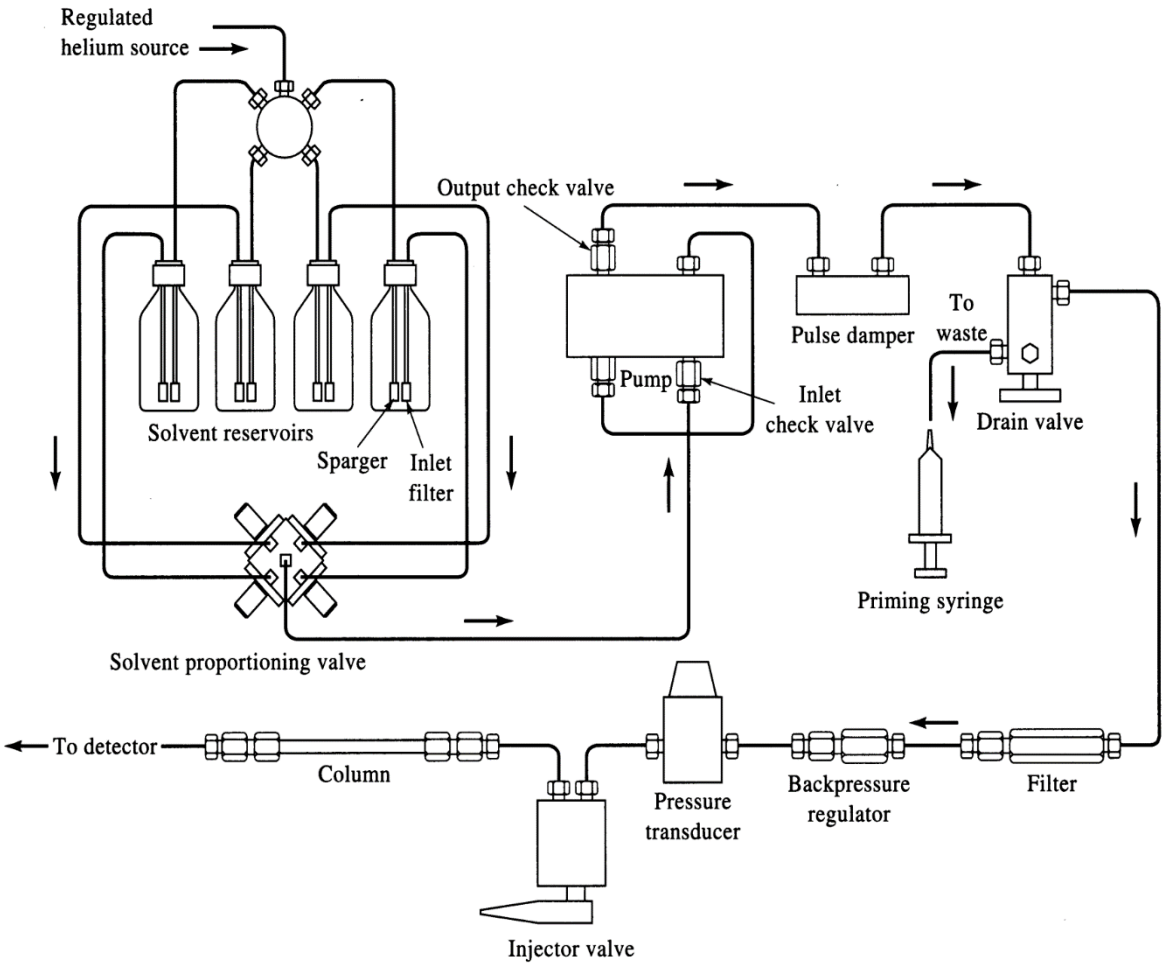


Figure 3: Detailed working of HPLC

The basic components of HPLC system have been discussed briefly in the following section.

Mobile phase supply system:

This tripartite system consists of mobile phase reservoirs, pump and degasser system. Glass bottles of variable capacity are the most common type of solvent reservoirs used in HPLC. A wide range of solvents with different polarities are used in HPLC and are filled in solvent reservoirs. The most commonly used organic phase is acetonitrile and methanol while aqueous phase consists of different buffers having a particular pH [7, 8]. The solvent must be pure (HPLC grade; pre-filtered through membrane filter of 0.22 µ or 0.45 µ pore size) and degassed to avoid formation of gas bubbles which prevent proper functioning of check valve, piston chamber and generate spurious peaks [9]. This problem is more serious during mixing of aqueous phase with organic phase as the solubility of dissolved air is less in mixture as compared to pure solvent. The most common methods of degassing are sparging with helium gas (removes 80% of air) or vacuum degassing (removes 80% of air) [10]. Solubility of helium in mobile phase is such that outgassing is not a problem after helium sparging. Sparging with helium gas also evaporates some of the volatile mobile phase (acetonitrile and methanol), due to this reason the commercial available systems incorporate pressure reservoirs to minimize this loss [11]. Many manufacturers incorporate an online degassing system in which the solvent is passed through a thin walled porous polymer tube such as PTFEor Teflon AF. Generally the mobile phase is first briefly sparged with helium, followed by degassing with vacuum degasser system.

In HPLC, typical flow rates for columns of internal diameter 4.6 mm are usually 1-2 mL/ min. As in case of UHPLC the back pressure is more, the commonly used flow rate is 0.3-0.5 mL/ min [5].

Due to small size of stationary phase as compared to conventional column chromatography, an efficient pump is required to force solvents through HPLC columns. The most commonly used pump for HPLC is

reciprocating pump. It consists of cylindrical piston chamber that is filled with the mobile phase and emptied via back-and-forth movement of the piston. Each pump is equipped with two check valves which allow the flow of mobile phase in single direction only. There are number of advantages of reciprocating pump such as small internal volume, high output pressure, can be readily used for gradient elution and provide constant flow rate. Other pumps used are motor-driven syringe pumps and pneumatic pumps [12].

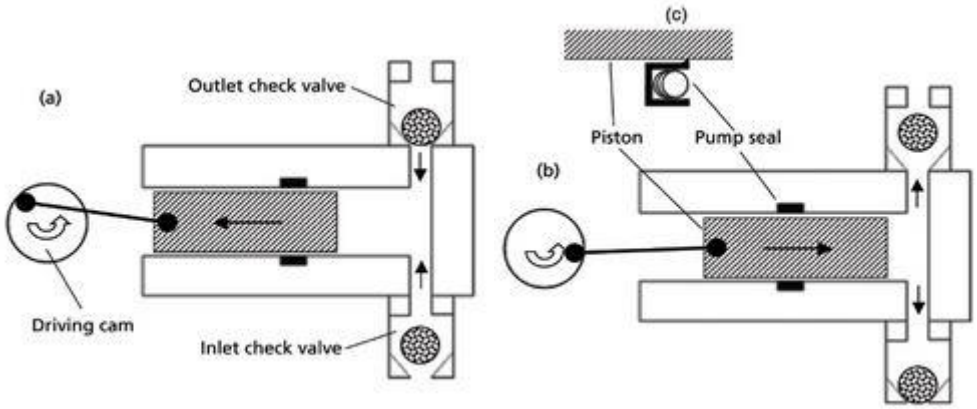


Figure 4: Reciprocating pump of HPLC

Sample injection system:

Multiple options are available for introduction of sample into HPLC system. Some of these are:

Septum injectors: The sample is introduced through rubber septum. This type of injector is rarely used now due to chances of leakage as a high pressure can damage the septum.

Stop flow (on line) injector: In this type of injector, the flow of the mobile phase is stopped and then the sample is introduced.

Rheodyne injector (Loop valve type): most commonly used; fixed loop of variable capacity like 20 µL or 50 µL; has two positions-load (sample is loaded) and inject (sample is injected). A rheodyne injector has been shown in figure 5 [13].

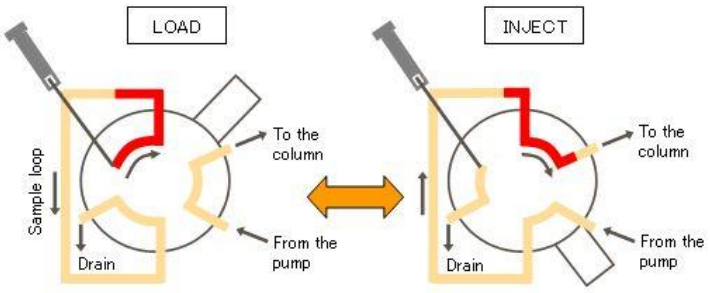
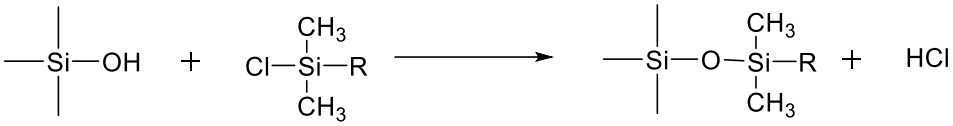


Figure 5: Sample loop injector

Column:

Generally stainless steel columns are used as they have to withstand a huge pressure. They are available in different sizes, and the selection of appropriate sized column depends upon the sample size. The column length varies from 5-30 cm while internal diameter ranges from 2-4.6 mm. Preparative column diameter usually varies from 19 mm to 50 mm.

Most commonly used column packing is based on siloxanes (Si-O-Si-C) which is prepared by reaction between silanol group of silica and organosilane reagents having different reactive groups such as chloro, alkoxy, alkylamino or other groups. The nature of final R group depends upon the groups present on organosilane reagents [14, 15]. For example, the reaction between monochlorosilane $R(CH_3)2SiCl$ (where $R = CH_3(CH_2)CH_2$-) will give octadecyl silica, commonly known as ODS or C18 silica. The other stationary phases are phenyl, C8, cyanopropyl, diol, amino and dimethyl amino silica derivatives [15].



A small guard column or pre-column (3-10 cm in length) is placed before the column and after the injector and generally contain the same packing as the analytical column. The purpose of the guard column is to prevent clogging of analytical column and hence improve the life of the HPLC column. An ideal guard column does not have any effect on the retention time of the component and also does not contribute to any resolution.

Detectors:

In HPLC, high sensitive detector (with sensitivity within micro to nano gram range) are required. The various detectors used are ultraviolet (UV), refractometer, photo diode array (PDA), fluorescence, electrochemical, evaporative light scattering detector (ELSD) and mass spectrometric [16, 17]. Refractive index detector is universal but also the least sensitive. Fluorescence and electrochemical detectors are quite sensitive but also quite selective. The most commonly used detector is PDA detector. Both UV and PDA detectors are used for analysis of compounds having chromophore groups. PDA detector is preferred over UV detector as it can be used to evaluate the co-eluting peaks also [18]. The compounds which lack the chromophore group can be analysed by ELSD detector [19]. Mass spectrometric detector is most powerful one but it is most expensive and complicated detector [20].

Types of HPLC

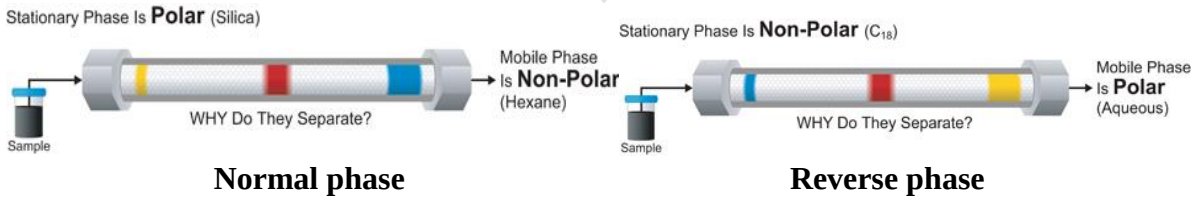
HPLC can be classified based on mode of chromatography, principle of separation, elution technique, operation scale and analysis type.

A. Based on mode of chromatography

Chromatography can be classified as normal phase and reverse phase chromatography depending upon the polarity of stationary phase and mobile phase. The salient features of both these chromatography have been summarized in table 1 [21, 22].

Table 1: Features of normal and reverse phase chromatography

Features	**Normal phase**	**Reverse phase**
Stationary phase	Polar	Non-polar
Mobile phase	Non-polar	Polar
Compound eluted first	Non-polar component	Polar component
Longer retention in column	Polar component	Non-polar component



Normal phase **Reverse phase**

Figure 6: Difference between normal and reverse phase chromatography

B. Based on principle of separation

HPLC can be classified as adsorption, ion exchange, ion pair, size exclusion/gel, affinity and chiral phase chromatography. All these techniques have been discussed briefly in table 2.

Table 2: Salient features of chromatography techniques classified on the basis of principle of separation

Chromatography	Features	Reference
Adsorption	➢ Principle of separation: adsorption ➢ Components are separated based on differential affinity towards mobile and stationary phase	[23, 24]
Ion exchange	➢ Separation principle: ion exchange ➢ Used for separation of similar charged ions in a mixture	[25]
Ion pair	➢ Temporarily conversion of reverse phase column into ion exchange by ion pair agents ➢ Alkane sulphonic acid sodium salt, tetramethyl or tetraethyl ammonium hydroxide are used as ion pair agents	[26]
Size exclusion/Gel	➢ Mixture of components are separated using gels ➢ Gels act as molecular sieves, hence mixture of components are separated based on their sizes ➢ Soft gels include dextran, agarose or polyacrylamide while semi-rigid gels include polystyrene, alkyl dextran etc.	[27]
Affinity	➢ Based on affinity of sample with specific stationary phase particularly liquid ➢ Rarely used as most of the time solid stationary phase is used, hence principle is adsorption chromatography	[28]
Chiral	➢ Separation of optical isomers using chiral stationary phase	[29]

C. Based on elution technique

Isocratic separation: involve same mobile phase composition through the process of analysis

Gradient separation: involve gradual change in mobile phase composition, usually starting from low elution strength followed by gradual increase in elution strength [30]

D. Based on scale of operation

Analytical HPLC: only sample analysis is done; sample size is in microgram

Preparative HPLC: individual fractions of pure compounds are collected; sample size is in mg to g depending upon column size [31]

E. Based on type of analysis

Qualitative analysis: used to identify the components, and detection of impurities/degradation products

Quantitative analysis: used to determine the proportion of individual components in a mixture [32]

HPLC descriptors

Retention time (R_t): It is the time interval between the introduction of sample in the system to the elution of component from the column and its subsequent detection. Retention time is dependent upon many factors such as flow rate, mobile phase composition, column chemistry and dimensions etc [33].

Void volume (V_0): is the volume of the liquid phase in the column. It is also named as "dead volume", "hold-up volume", and sometimes "retention volume of non-retained component" in the literature [34].

Retention factor/capacity factor (k): It is a measure of the retention of the peak and is a dimensionless chromatographic descriptor. It is independent on the mobile phase flow rate and column dimensions [35]. It is calculated by following equation:

$$k = \frac{V_R - V_0}{V_0} = \frac{V'_R}{V_0} = \frac{t_R - t_0}{t_0}$$

where, V_R: retention volume; V_0: void volume; t_R: retention time; t_0: void time; V'_R: reduced retention time

Selectivity (α): is the ability of the chromatographic system to discriminate different analytes. It is the ratio of retention factors or reduced retention times of two analytes [36].

$$\alpha = \frac{k_2}{k_1} = \frac{t_{R_2} - t_0}{t_{R_1} - t_0}$$

Resolution: is the measure of separation of two peaks appearing at different retention times. This descriptor encompasses both the efficiency and selectivity.

Ultra-high pressure liquid chromatography (UHPLC)

In 1997, the proof-of-concept study by Professor James Jorgenson brought the revolution in the field of liquid chromatography which resulted in the development of first commercial UHPLC in 2004 [37, 38]. Table 3 summarizes the prominent characteristics and benefits of UHPLC.

Table 3: Prominent Characteristics and benefits of UHPLC

System characteristics of UHPLC	**Range and Comment**
High pressure limit	15,000-19,000 psi (1000-1250 bar)
Low system dispersion	Instrumental bandwidth 5-20 µL
Low gradient dwell volume	100 to 400 µL
Benefits of UHPLC	**Comment**
High throughput	3-10 folds higher than HPLC
Rapid method development	Fast analysis with shorter column
High resolution	3 fold more than HPLC, Peak capacity 400-600 as compared to 200 in HPLC
Solvent saving	5-15 folds reduction than HPLC
High sensitivity	3-10 folds higher than HPLC
High precision	Significant higher than HPLC

Conclusion

In summary, HPLC remains a highly dynamic field with numerous innovations in instruments, column technologies, and approaches in recent years. Pharmaceutical scientists are early adopters and beneficiaries of these newer technologies for research, development and quality control. UHPLC is becoming the standard HPLC platform with rapid adoption by research & development, albeit slower implementation in QC labs. Newer column technologies allow faster and more efficient analysis of complex samples, chiral molecules and biomolecules.

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