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REVIEW ARTICLE

High-Performance Liquid Chromatography: An Overview

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ABSTRACT

Chromatography is considered extremely powerful separation technique for variety of samples. The common feature of all these techniques is that the different components in a sample mixture are distributed between two phases, one of which remains stationary while the other the mobile phase, runs through the interstices or over the surface of the fixed phase. The movement of the mobile phase results in differential migration of the sample components. High performance thin layer chromatography is an invaluable quality assessment tool for the evaluation of botanical materials and the equally suitable for qualitative and quantitative analysis of biological samples which are of particular interest to biologists, biochemists, hematologists, immunologists, medical diagnosticians, and molecular biologists. It allows for the analysis of a broad number of compounds efficiently and cost effectively. Over three decades of development in HPLC have given us a well understood separation tool capable of very reliable results. Refinements in HPLC column technology have lead to high resolution separations, fast analysis, and high sensitivity. However, to obtain all the benefits that HPLC can offer, careful attention must be paid to that part of the liquid flow system that carries the sample.

KEYWORDS

HPLC, Sample, Chromatography, Instrumentation technique.

HISTORICAL BACKGROUND

Prior to the 1970's, few reliable chromatographic methods were commercially available to the laboratory scientist. During the 1970's, most chemical separations were carried out using a variety of techniques including open-column chromatography, paper chromatography, and thin-layer chromatography. However, these chromatographic techniques were inadequate for quantification of compounds and did not achieve sufficiently high resolution to distinguish between similar compounds.

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During this time, pressure liquid chromatography began to be used to decrease flow through time, thus reducing purification times of compounds being isolated by column chromatography. However, flow rates were inconsistent, and the question of whether it was better to have constant flow rate or constant pressure was debated. (Analytical Chem. Vol 62, No 19, Oct 1, 1990). High pressure liquid chromatography was developed in the mid-1970's and quickly improved with the development of column packing materials and the additional convenience of on-line detectors. In the late 1970's, new methods including reverse phase liquid chromatography allowed for improved separation between very similar compounds. By the 1980's HPLC was

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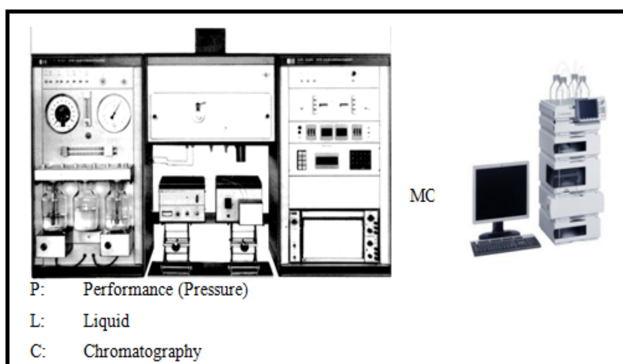
commonly used for the separation of chemical compounds. New techniques improved separation, identification, purification and quantification far above those obtained using previous techniques. Computers and automation added to the convenience of HPLC. Additional column types giving better reproducibility were introduced and such terms as micro-column, affinity columns, and Fast HPLC began to immerge. The past decade has seen a vast undertaking in the development of micro-columns, and other specialized columns. The dimensions of the typical HPLC column are: XXX mm in length with an internal diameter between 3-5 mm. The usual diameter of micro-columns, or capillary columns, ranges from 3µm to 200µm. Fast HPLC utilizes a column that is shorter than the typical column. A Fast HPLC column is about 3 mm long and is packed with smaller particles. Currently, one has the option of selecting from a lot of columns for the separation of compounds, as well as a variety of detectors to interface with the HPLC in order to obtain optimal analysis of the compound. Although HPLC is widely considered to be a technique mainly for biotechnological, biomedical, and biochemical research as well as for the pharmaceutical industry, in actual fact these fields currently comprise only about 50% of HPLC users(AnalyticalChem.vol62, no.19, Oct 1, 1990). Currently HPLC is used in a variety of fields and industries including the cosmetics, energy, food, and environmental industries.



P: Performance (Pressure)
L: Liquid
C: Chromatography

Definition

High-performance Liquid Chromatography (or High-pressure Liquid Chromatography, HPLC) is a chromatographic technique that can separate a mixture of compounds and is used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of the mixture.

INTRODUCTION

High Performance Liquid Chromatography (HPLC) is one mode of chromatography; the most widely used analytical technique. Chromatographic processes can be defined as separation techniques involving mass-transfer between stationary and mobile phases. HPLC utilizes a liquid mobile phase to separate the components of a mixture. These components (or analytes) are first dissolved in a solvent, and then forced to flow through a chromatographic column under high pressure. In the column, the mixture 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 in the column. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases. As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems and has the ability to easily separate a wide variety of chemical mixtures.

HPLC typically utilizes different types of stationary phases, a pump that moves the mobile phase(s) and analyte through the column, and a detector that provides a characteristic retention time for the analyte. The detector may also provide other characteristic information (i.e. UV/Vis spectroscopic data for analyte if so equipped). Analyte retention time varies depending on the strength of its interactions with the stationary phase, the ratio/composition of solvent(s) used, and the flow rate of the mobile phase. With HPLC, a pump (rather than gravity) provides the higher pressure required to propel the mobile phase and analyte through the densely packed column. The increased density arises from smaller particle sizes. This allows for a better separation on columns of shorter

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length when compared to ordinary column chromatography.

What Does a High Pressure LC Look Like?

Describing the 5 major HPLC components and their functions

Pump

- ✓ The role of the pump is to force a liquid (called the mobile phase) through the liquid chromatograph at a specific flow rate, expressed in milliliters per min (mL/min).
- ✓ Normal flow rates in HPLC are in the 1-to 2-mL/min range.
- ✓ Typical pumps can reach pressures in the range of 6000-9000 psi (400-to 600-bar).
- ✓ During the chromatographic experiment, a pump can deliver a constant mobile phase composition (isocratic) or an increasing mobile phase composition (gradient).

Injector

- ✓ The injector serves to introduce the liquid sample into the flow stream of the mobile phase.
- ✓ Typical sample volumes are 5-to 20-microliters (µL).
- ✓ The injector must also be able to withstand the high pressures of the liquid system.
- ✓ An autosampler is the automatic version for when the user has many samples to analyze or when manual injection is not practical.

Column

- ✓ Considered the "heart of the chromatograph" the column's stationary phase separates the sample components of interest using various physical and chemical parameters.
- ✓ The small particles inside the column are what cause the high back pressure at normal flow rates.
- ✓ The pump must push hard to move the mobile phase through the column and this resistance causes a high pressure within the chromatograph.

Types of Columns in HPLC

- ✓ Analytical [internal diameter (i.d.) 1.0 -4.6-mm; lengths 15 –250 mm]
- ✓ Preparative (i.d. > 4.6 mm; lengths 50 –250 mm)
- ✓ Capillary (i.d. 0.1 -1.0 mm; various lengths)
- ✓ Nano (i.d. < 0.1 mm, or sometimes stated as < 100 µm)

Detector

- ✓ The detector can see (detect) the individual molecules that come out (elute) from the column.
- ✓ A detector serves to measure the amount of those molecules so that the chemist can quantitatively analyze the sample components.
- ✓ The detector provides an output to a recorder or computer that result in the liquid chromatogram (i.e., the graph of the detector response).

Computer

- ✓ Frequently called the data system, the computer not only controls all the modules of the HPLC instrument but it takes the signal from the detector and uses it to determine the time of elution (retention time) of the sample components (qualitative analysis) and the amount of sample (quantitative analysis).

Types of Chromatography

1. Partition chromatography
2. Normal-phase chromatography
3. Displacement chromatography
4. Reversed-phase chromatography (RPC)

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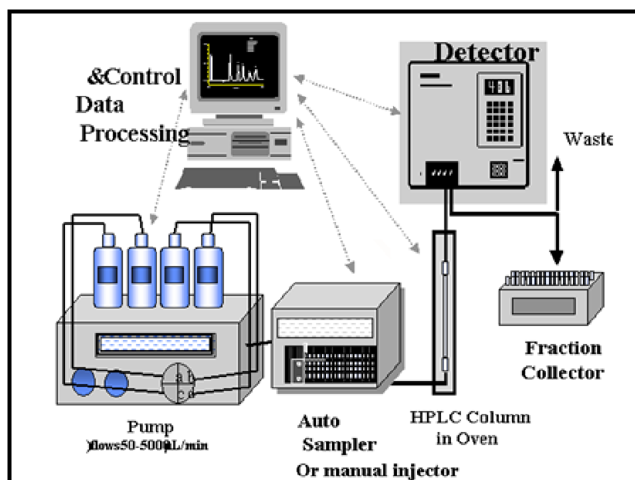
5. Size-exclusion chromatography
6. Ion-exchange chromatography
7. Bio-affinity chromatography
8. Aqueous normal-phase chromatography



Partition Chromatography

Partition chromatography was the first kind of chromatography that chemists developed. The partition coefficient principle has been applied in paper chromatography, thin layer chromatography, gas phase and liquid-liquid applications. The 1952 Nobel Prize in chemistry was earned by Archer John Porter Martin and Richard Laurence Millington Synge for their development of the technique, which was used for their separation of amino acids. Partition chromatography uses a retained solvent, on the surface or within the grains or fibres of an "inert" solid supporting matrix as with paper chromatography; or takes advantage of some additional coulombic and/or hydrogen donor interaction with the solid support. Molecules equilibrate (partition) between a liquid stationary phase and the eluent. Known as Hydrophilic Interaction Chromatography (HILIC) in HPLC, this method separates analytes based on polar differences. HILIC most often uses a bonded polar stationary phase and a non-polar, water miscible, mobile phase. Partition HPLC has been used historically on unbonded silica or alumina supports. Each works effectively for separating analytes by relative polar differences, however, HILIC has the advantage of separating acidic, basic and neutral solutes in a single chromatogram.

The polar analytes diffuse into a stationary water layer associated with the polar stationary phase and are thus retained. Retention strengths increase with increased analyte polarity, and the interaction between the polar analyte and the polar stationary phase (relative to the mobile phase) increases the elution time. The interaction strength depends on the functional groups in the analyte molecule which promote partitioning but can also include coulombic (electrostatic) interaction and hydrogen donor capability. Use of more polar solvents in the mobile phase will decrease the retention time of the analytes, whereas more hydrophobic solvents tend to increase retention times.

Normal-Phase Chromatography

Also known as normal-phase HPLC (NP-HPLC), or adsorption chromatography, this method separates analytes based on adsorption to a stationary surface chemistry and by polarity. It was one of the first kinds of HPLC that chemists developed. NP-HPLC uses a polar stationary phase and a non-polar, non-aqueous mobile phase, and works effectively for separating analytes readily soluble in non-polar solvents. The analyte associates with and is retained by the polar stationary phase. Adsorption strengths increase with increased analyte polarity, and the interaction between the polar analyte and the polar stationary phase (relative to the mobile phase) increases the elution time. The interaction strength depends not only on the functional groups in the analyte molecule, but also on steric factors. The effect of sterics on interaction strength allows this method to resolve (separate) structural isomers.

The use of more polar solvents in the mobile phase will decrease the retention time of the analytes, whereas more hydrophobic solvents tend to increase retention times. Very polar solvents in a mixture tend to deactivate the stationary phase by creating a stationary bound water layer on the stationary phase surface. This behavior is somewhat peculiar to normal phase because it is most purely an adsorptive

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mechanism (the interactions are with a hard surface rather than a soft layer on a surface).Partition and NP-HPLC fell out of favor in the 1970s with the development of reversed-phase HPLC because of a lack of reproducibility of retention times as water or protic organic solvents changed the hydration state of the silica or alumina chromatographic media. Recently it has become useful again with the development of HILIC bonded phases which improve reproducibility.

Displacement Chromatography

The basic principle of displacement chromatography is: A molecule with a high affinity for the chromatography matrix (the displacer) will compete effectively for binding sites, and thus displace all molecules with lesser affinities. There are distinct differences between displacement and elution chromatography. In elution mode, substances typically emerge from a column in narrow, Gaussian peaks. Wide separation of peaks, preferably to baseline, is desired in order to achieve maximum purification. The speed at which any component of a mixture travels down the column in elution mode depends on many factors. But for two substances to travel at different speeds, and thereby be resolved, there must be substantial differences in some interaction between the biomolecules and the chromatography matrix. Operating parameters are adjusted to maximize the effect of this difference. In many cases, baseline separation of the peaks can be achieved only with gradient elution and low column loadings. Thus, two drawbacks to elution mode chromatography, especially at the preparative scale, are operational complexity, due to gradient solvent pumping, and low throughput, due to low column loadings. Displacement chromatography has advantages over elution chromatography in that components are resolved into consecutive zones of pure substances rather than “peaks”. Because the process takes advantage of the nonlinearity of the isotherms, a larger column feed can be separated on a given column with the purified components recovered at significantly higher concentrations.

Reversed-Phase Chromatography (RPC)

Reversed phase HPLC (RP-HPLC or RPC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is silica which has been treated with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. With these stationary phases, retention time is longer for molecules which are more non-polar, while polar molecules elute more readily. An investigator can increase retention time by adding more water to the mobile phase; thereby making the affinity of the hydrophobic analyte for the hydrophobic stationary phase stronger relative to the now more hydrophilic mobile phase. Similarly, an investigator can decrease retention time by adding more organic solvent to the eluent. RPC is so commonly used that it is often incorrectly referred to as "HPLC" without further specification. The pharmaceutical industry regularly employs RPC to qualify drugs before their release.

RPC operates on the principle of hydrophobic forces, which originate from the high symmetry in the dipolar water structure and play the most important role in all processes in life science. RPC allows the measurement of these interactive forces. The binding of the analyte to the stationary phase is proportional to the contact surface area around the non-polar segment of the analyte molecule upon association with the ligand in the aqueous eluent. This solvophobic effect is dominated by the force of water for "cavity-reduction" around the analyte and the C_{18}-chain versus the complex of both. The energy released in this process is proportional to the surface tension of the eluent (water: 7.3×10^{-6} J/cm², methanol: 2.2×10^{-6} J/cm²) and to the hydrophobic surface of the analyte and the ligand respectively. The retention can be decreased by adding a less polar solvent (methanol, acetonitrile) into the mobile phase to reduce the surface tension of water. Gradient elution uses this effect by automatically reducing the polarity and the surface tension of the aqueous mobile phase during the course of the analysis.

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Structural properties of the analyte molecule play an important role in its retention characteristics. In general, an analyte with a larger hydrophobic surface area (C-H, C-C, and generally non-polar atomic bonds, such as S-S and others) results in a longer retention time because it increases the molecule's non-polar surface area, which is non-interacting with the water structure. On the other hand, polar groups, such as -OH, $-NH_2$, COO^- or $-NH_3^+$ reduce retention as they are well integrated into water. Very large molecules, however, can result in an incomplete interaction between the large analyte surface and the ligand's alkyl chains and can have problems entering the pores of the stationary phase.

Retention time increases with hydrophobic (non-polar) surface area. Branched chain compounds elute more rapidly than their corresponding linear isomers because the overall surface area is decreased. Similarly organic compounds with single C-C-bonds elute later than those with a C=C or C-C-triple bond, as the double or triple bond is shorter than a single C-C-bond. Aside from mobile phase surface tension (organizational strength in eluent structure), other mobile phase modifiers can affect analyte retention. For example, the addition of inorganic salts causes a moderate linear increase in the surface tension of aqueous solutions (ca. 1.5×10^{-7} J/cm² per Mol for NaCl, 2.5×10^{-7} J/cm² per Mol for $(NH_4)_2SO_4$), and because the entropy of the analyte-solvent interface is controlled by surface tension, the addition of salts tend to increase the retention time. This technique is used for mild separation and recovery of proteins and protection of their biological activity in protein analysis (hydrophobic interaction chromatography, HIC).

Another important component is the influence of the pH since this can change the hydrophobicity of the analyte. For this reason most methods use a buffering agent, such as sodium phosphate, to control the pH. The buffers serve multiple purposes: they control pH, neutralize the charge on any residual exposed silica on the stationary phase and act as ion pairing agents to neutralize charge on the analyte. Ammonium formate is commonly added in mass spectrometry to improve detection of certain analytes by the formation of ammonium adducts. A volatile organic acid such as acetic acid, or most commonly formic acid, is often added to the mobile phase if mass spectrometry is used to analyze the column eluent. Trifluoroacetic acid is used infrequently in mass spectrometry applications due to its persistence in the detector and solvent delivery system, but can be effective in improving retention of analytes such as carboxylic acids in applications utilizing other detectors, as it is one of the strongest organic acids. The effects of acids and buffers vary by application but generally improve the chromatography.

Reversed phase columns are quite difficult to damage compared with normal silica columns; however, many reversed phase columns consist of alkyl derivatized silica particles and should never be used with aqueous bases as these will destroy the underlying silica particle. They can be used with aqueous acid, but the column should not be exposed to the acid for too long, as it can corrode the metal parts of the HPLC equipment. RP-HPLC columns should be flushed with clean solvent after use to remove residual acids or buffers, and stored in an appropriate composition of solvent. The metal content of HPLC columns must be kept low if the best possible ability to separate substances is to be retained. A good test for the metal content of a column is to inject a sample which is a mixture of 2, 2'- and 4, 4'- bipyridine. Because the 2, 2'-bipy can chelate the metal, the shape of the peak for the 2, 2'-bipy will be distorted (tailed) when metal ions are present on the surface of the silica.

Size-Exclusion Chromatography

Size-exclusion chromatography (SEC), also known as gel permeation chromatography or gel filtration chromatography, separates particles on the basis of size. It is generally a low resolution chromatography and thus it is often reserved for the final, "polishing" step of purification. It is also useful for determining the tertiary structure

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and quaternary structure of purified proteins. SEC is used primarily for the analysis of large molecules such as proteins or polymers. SEC works by trapping these smaller molecules in the pores of a particle. The larger molecules simply pass by the pores as they are too large to enter the pores. Larger molecules therefore flow through the column quicker than smaller molecules, that is, the smaller the molecule, the longer the retention time.

This technique is widely used for the molecular weight determination of polysaccharides. SEC is the official technique (suggested by European pharmacopeia) for the molecular weight comparison of different commercially available low-molecular weight heparins.

Ion-Exchange Chromatography

In ion-exchange chromatography, retention is based on the attraction between solute ions and charged sites bound to the stationary phase. Ions of the same charge are excluded. Types of ion exchangers include:

- ✓ Polystyrene resins – These allow cross linkage which increases the stability of the chain. Higher cross linkage reduces swerving, which increases the equilibration time and ultimately improves selectivity.
- ✓ Cellulose and dextran ion exchangers (gels) – These possess larger pore sizes and low charge densities making them suitable for protein separation.
- ✓ Controlled-pore glass or porous silica

In general, ion exchangers favor the binding of ions of higher charge and smaller radius.

An increase in counter ion (with respect to the functional groups in resins) concentration reduces the retention time. An increase in pH reduces the retention time in cation exchange while a decrease in pH reduces the retention time in anion exchange. This form of chromatography is widely used in the following applications: water purification, preconcentration of trace components, ligand-exchange chromatography, ion-exchange chromatography of proteins, high-pH anion-exchange chromatography of carbohydrates and oligosaccharides, and others.

Bioaffinity Chromatography

This chromatographic process relies on the property of biologically active substances to form stable, specific, and reversible complexes. The formation of these complexes involves the participation of common molecular forces such as the Van der Waals interaction, electrostatic interaction, dipole-dipole interaction, hydrophobic interaction, and the hydrogen bond. An efficient, biospecific bond is formed by a simultaneous and concerted action of several of these forces in the complementary binding sites.

Aqueous Normal-Phase Chromatography

Aqueous normal-phase chromatography (ANP) is a chromatographic technique which encompasses the mobile phase region between reversed-phase chromatography (RP) and organic normal phase chromatography (ONP). This technique is used to achieve unique selectivity for hydrophilic compounds, showing normal phase elution using reverse-phase solvents.

Operation of HPLC

The sample to be analyzed is introduced in small volume to the stream of mobile phase. The solution movement through the column is slowed by specific chemical or physical interactions with the stationary phase present within the column. The velocity of the solution moves depends on the nature of the sample and on the compositions of the stationary (column) phase. The time at which a specific sample elutes (comes out of the end of the column) is called the retention time; the retention time under particular conditions is considered an identifying characteristic of a given sample. The use of smaller particle size column packing (which creates higher backpressure) increases the linear velocity giving the components less time to diffuse within the column, improving the chromatogram resolution. Common solvents used include any miscible combination of water or various organic liquids (the most common are

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methanol and acetonitrile). Water may contain buffers or salts to assist in the separation of the sample components, or compounds such as trifluoroacetic acid which acts as an ion pairing agent.

A further refinement of HPLC is to vary the mobile phase composition during the analysis; gradient elution. A normal gradient for reversed phase chromatography might start at 5% methanol and progress linearly to 50% methanol over 25 minutes; the gradient depends on how hydrophobic the sample is. The gradient separates the sample mixtures as a function of the affinity. This partitioning process is similar to that which occurs during a liquid-liquid extraction but is continuous, not step-wise. In this example, using a water/methanol gradient, more hydrophobic components will elute (come off the column) when the mobile phase consists mostly of methanol (giving a relatively hydrophobic mobile phase).

The choice of solvents, additives and gradient depend on the nature of the column and sample. Often a series of tests are performed on the sample together with a number of trial runs in order to find the HPLC method which gives the best peak separation.

Isocratic Flow and Gradient Elution

A separation in which the mobile phase composition remains constant throughout the procedure is termed isocratic (meaning constant composition). The word was coined by Csaba Horvath who was one of the pioneers of HPLC.

The mobile phase composition does not have to remain constant. A separation in which the mobile phase composition is changed during the separation process is described as a gradient elution. One example is a gradient starting at 10% methanol and ending at 90% methanol after 20 minutes. The two components of the mobile phase are typically termed "A" and "B"; A is the "weak" solvent which allows the solute to elute only slowly, while B is the "strong" solvent which rapidly elutes the solutes from the column. Solvent A is often water, while B is an organic solvent miscible with water, such as acetonitrile, methanol, THF, or isopropanol.

In isocratic elution, peak width increases with retention time linearly according to the equation for N, the number of theoretical plates. This leads to the disadvantage that late-eluting peaks get very flat and broad. Their shape and width may keep them from being recognized as peaks.

Gradient elution decreases the retention of the later-eluting components so that they elute faster, giving narrower (and taller) peaks for most components. This also improves the peak shape for tailed peaks, as the increasing concentration of the organic eluent pushes the tailing part of a peak forward. This also increases the peak height (the peak looks "sharper"), which is important in trace analysis. The gradient program may include sudden "step" increases in the percentage of the organic component, or different slopes at different times – all according to the desire for optimum separation in minimum time.

In isocratic elution, the selectivity does not change if the column dimensions (length and inner diameter) change – that is, the peaks elute in the same order. In gradient elution, the elution order may change as the dimensions or flow rate change.

The driving force in reversed phase chromatography originates in the high order of the water structure. The role of the organic component of the mobile phase is to reduce this high order and thus reduce the retarding strength of the aqueous component.

Parameters

Internal Diameter

The internal diameter (ID) of an HPLC column is an important parameter that influences the detection sensitivity and separation selectivity in gradient elution. It also determines the quantity of analyte that can be loaded onto the column. Larger columns are usually seen in industrial applications, such as the purification of a drug product for later use. Low-ID columns have improved sensitivity and lower solvent consumption at the expense of loading capacity.

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- ✓ Larger ID columns (over 10 mm) are used to purify usable amounts of material because of their large loading capacity.
- ✓ Analytical scale columns (4.6 mm) have been the most common type of columns, though smaller columns are rapidly gaining in popularity. They are used in traditional quantitative analysis of samples and often use a UV-Vis absorbance detector.
- ✓ Narrow-bore columns (1–2 mm) are used for applications when more sensitivity is desired either with special UV-vis detectors, fluorescence detection or with other detection methods like liquid chromatography-mass spectrometry
- ✓ Capillary columns (under 0.3 mm) are used almost exclusively with alternative detection means such as mass spectrometry. They are usually made from fused silica capillaries, rather than the stainless steel tubing that larger columns employ.

Particle Size

Most traditional HPLC is performed with the stationary phase attached to the outside of small spherical silica particles (very small beads). These particles come in a variety of sizes with 5 µm beads being the most common. Smaller particles generally provide more surface area and better separations, but the pressure required for optimum linear velocity increases by the inverse of the particle diameter squared.

This means that changing to particles that are half as big, keeping the size of the column the same, will double the performance, but increase the required pressure by a factor of four. Larger particles are used in preparative HPLC (column diameters 5 cm up to >30 cm) and for non-HPLC applications such as solid-phase extraction.

Pore Size

Many stationary phases are porous to provide greater surface area. Small pores provide greater surface area while larger pore size has better kinetics, especially for larger analytes. For example, a protein which is only slightly smaller than a pore might enter the pore but does not easily leave once inside.

Pump Pressure

Pumps vary in pressure capacity, but their performance is measured on their ability to yield a consistent and reproducible flow rate. Pressure may reach as high as 40 MPa (6000 lbf/in^2), or about 400 atmospheres. Modern HPLC systems have been improved to work at much higher pressures, and therefore are able to use much smaller particle sizes in the columns (<2 µm). These "Ultra High Performance Liquid Chromatography" systems or RSLC/UHPLCs can work at up to 100 MPa (15,000 lbf/in^2), or about 1000 atmospheres. The term "UPLC" is a trademark of the Waters Corporation, but is sometimes used to refer to the more general technique.

Modes of HPLC

There are various modes of operation of HPLC. The mechanism of interaction of the solutes with the stationary phases determines the classification of the mode of liquid chromatography (Table 1).

Modern High Performance Liquid Chromatography (HPLC)

Introduction

Although the subject of chromatography is covered comprehensively in the several volumes of the Chrom-Ed Chromatography Series this page presents some of the system requirements for modern High Performance Liquid Chromatography (HPLC).

HPLC is liquid chromatography which has been optimized to provide rapid high resolution separations. It evolved over nearly a century from the early work of Tswett in the late 1900s to the highly sophisticated reliable and fast liquid chromatography (LC) techniques in common use today. Early LC used gravity fed open tubular columns with particles 100s of microns in size; the human eye was used for a detector and separations often took hours (days?) to develop. Today's HPLC requires very special apparatus which includes the following.

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1. Extremely precise gradient mixers (optional).
2. HPLC high pressure pumps with very constant flow.
3. Unique high accuracy, low dispersion, HPLC sample valves.
4. Very high efficiency HPLC columns with inert packing materials.
5. High sensitivity low dispersion HPLC detectors.
6. High speed data acquisition systems.
7. Low dispersion connecting tubes for valve to column and column to detector.

- ✓ The modern form of liquid chromatography is now referred to as "flash chromatography".
- ✓ In principle, LC and HPLC work the same way except the speed, efficiency, sensitivity and ease of operation of HPLC is vastly superior.

HPLC Gradient Mixers

HPLC gradient mixers must provide a very precise control of solvent composition to maintain a reproducible gradient profile. This can be complicated in HPLC by the small elution volumes required by many systems. It is much more difficult to produce a constant gradient when mixing small volumes then when mixing large volumes. For low pressure systems this requires great precision in the operation of the miniature mixing valves used and low dispersion flows throughout the mixer. For multi-pump high pressure systems it requires a very precise control of the flow rate while making very small changes of the flow rate.

HPLC Pumps

Because of the small particles used in modern HPLC, modern LC pumps need to operate reliably and precisely at pressures of 10,000 p.s.i. or at least 6,000 p.s.i. To operate at these pressures and remain sensibly inert to the wide variety of solvents used HPLC pumps usually

Table: 1 Summarizes the variety of modes of liquid chromatography, of which Reversed Phase stands out as the most widely used mode in HPLC

Mode	**Normal Phase**	**Reversed Phase**	**Ion exchange**	**Chiral**	**Affinity**	**Size Exclusion**
Stationary Phases chemistry	Polar-hydrophilic	Non-polar-lipophilic	Ion-bonding	Chiral recognition	Bioaffinity	Sieving by size
Typical Stationary Phases	Silica, Alumina	Alkylated silica, mostly C18	Ionic functional groups on silica or polymer	Chiral groups on silica surfaces	Either substrates or biomolecules,	Gel type polymers
Typical mobile phase	Hexane; isopropanol; methylene chloride	Water; methanol; acetonitril; buffers; ion pairing agents	Water; buffers; acid; base	Two modes: aqueous and non-aqueous	Water; buffers	Two modes: aqueous and non-aqueous
Typical solutes	Fatty and oily	Almost all organic compounds	Any ion-charged compounds	Enantiomers small and large molecules	Biomolecules or their substrates	Polymers: synthetic or biological

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have sapphire pistons, stainless steel cylinders and return valves fitted with sapphire balls and stainless steel seats. For analytical proposes HPLC pumps should have flow rates that range from 0 to 10 ml/min., but for preparative HPLC, flow rates in excess of 100 ml/min may be required. It is extremely difficult to provide a very constant flow rate at very low flow rates. If .1% is considered acceptable then for 100uls/min a flow variation of less then .1ul/min is required. This level of constancy is required because most HPLC detectors are flow sensitive and errors in quantization will result from changes in flow rate.

HPLC Sample Valves

Since sample valves come between the pump and the column it follows that HPLC sample valves must also tolerate pressures up to 10,000 p.s.i. For analytical HPLC, the sample volume should be selectable from sub- micro liter to a few micro liters, whereas in preparative HPLC the sample volume may be even greater than 10 ml. To maintain system efficiency the sample valve must be designed to have very low dispersion characteristics, this is true not only for flow dispersion but also for the less obvious problems of dispersion caused by sample adsorption/desorption on valve surfaces and diffusion of sample into and out of the mating surfaces between valve moving parts. It goes without saying that the valves must deliver a very constant sample size but this is usually attained by the use of a constant size sample loop.

HPLC Columns

HPLC columns are packed with very fine particles (usually a few microns in diameter). The very fine particles are required to attain the low dispersion that give the high plate counts expected of modern HPLC. Plate counts in excess of 25,000 plates per column are possible with modern columns, however, these very high efficiencies are very rarely found with real samples because of the dispersion associated with injection valves, detectors, data acquisition systems and the dispersion due to the higher molecular weight of real samples as opposed to the common test samples. Packing these small particles into the column is a difficult technical problem but even with good packing a great amount of care must be given to the column end fittings and the inlet and outlet connection to keep dispersion to a minimum. Some state of the art systems are now 'chip' based and may use no particles at all. Some limited use has been made of HPLC for preparative purposes using half inch to one inch diameter columns. LC columns, in general, achieve their separation by exploiting the different intermolecular forces between the solute and the stationary phase and those between the solute and the mobile phase. The column will retain those substances that interact more strongly with the stationary phase than those that interact more strongly with the mobile phase. The basic intermolecular forces that are exploited in the HPLC are the same as those discussed in The Mechanism of Chromatographic Retention and The Thermodynamics of Chromatography of the Chrom-Ed series. The main consideration with HPLC is the much wider variety of solvents and packing materials that can be utilized as because of the much lower quantities of both which are required. In particular very expensive optically pure compounds can be used to make Chiral HPLC stationary phases and may even be used as (disposable) HPLC solvents.

HPLC Detectors

LC detectors have been extensively discussed in Liquid Chromatography Detectors and HPLC detectors use the same detection principals with extra care being given to the small solute elution volumes that result from the combination of high column efficiencies with small volumes. In order to give an accurate chromatographic profile the detector sampling (cell) volume must be a small fraction of the solute elution volume. If the detector volume were larger then the elution volume then you would have peaks that appeared with flat tops as the whole peak would be resident in the detector at the same time. This means that as column volumes decrease and system efficiencies increase the volume of the detector cell volume must also decrease. This is of course at odds for the requirement for

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detector to maintain high sensitivity as this is usually dependant on having a larger cell volume. Again, this requires the very careful design of modern detectors.

HPLC Data Acquisition

Data acquisition was discussed in Liquid Chromatography Detectors and the only extra consideration required for HPLC is the higher sampling rate needed for the rapidly eluting narrow peaks of the HPLC chromatogram. Although the theoretical number of samples needed for good quantization are actually quite small, for real systems a hundred samples or more per peak is recommended; thus, for a 4 sec wide peak, a rate of 25 samples per second may be required. The same data analysis and reporting software can be used as in ordinary LC.

Major HPLC Systems Suppliers:

- Waters Corporation
- Varian Corporation
- Agilent Corporation
- Shemadzu Corporation

Common Specific Applications

- ✓ Quantitative/qualitative analyses of amino acids, nucleic acids, proteins in physiological samples
- ✓ Measuring levels of active drugs, synthetic byproducts, degradation products in pharmaceuticals
- ✓ Measuring levels of hazardous compounds such as pesticides and insecticides
- ✓ Monitoring environmental samples
- ✓ Purifying compounds from mixtures
- ✓ Chemistry and biochemistry research analyzing complex mixtures
- ✓ Developing processes for synthesizing chemical compounds
- ✓ Isolating natural products, or predicting physical properties.
- ✓ Quality control to ensure the purity of raw materials
- ✓ To control and improve process yields
- ✓ To quantify assays of final products, or to evaluate product stability and monitor degradation.
- ✓ It is used for analyzing air and water pollutants
- ✓ For monitoring materials that may jeopardize occupational safety or health
- ✓ For monitoring pesticide levels in the environment.
- ✓ Federal and state regulatory agencies use HPLC to survey food and drug products,
- ✓ For identifying confiscated narcotics or to check for adherence to label claims.

Limitations

- ✓ Qualitative analysis may be limited unless HPLC is interfaced with mass spectrometry.
- ✓ Resolution is limited with very complex samples

CONCLUSIONS

HPLC is probably the most universal type of analytical procedure; its application areas include quality control, process control, forensic analysis, environmental monitoring and clinical testing. In addition HPLC also ranks as one of the most sensitive analytical procedures and is unique in that it easily copes with multi-component mixtures. It has achieved this position as a result of the constant evolution of the equipment used in LC to provide higher and higher efficiencies at faster and faster analysis times with a constant incorporation of new highly selective column packing.

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