

# Quality-Control Analytical Methods: High-Performance Liquid Chromatography

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## Introduction

Chromatography is an analytical technique based on the separation of molecules due to differences in their structure and/or composition. In general, chromatography involves moving a sample through the system over a stationary phase. The molecules in the sample will have different affinities and interactions with the stationary support, leading to separation of molecules. Sample components that display stronger interactions with the stationary phase will move more slowly through the column than components with weaker interactions. Different compounds can be separated from each other as they move through the column. Chromatographic separations can be carried out using a variety of stationary phases, including immobilized silica on glass plates (thin-layer chromatography), volatile gases (gas chromatography), paper (paper chromatography) and liquids (liquid chromatography).

High-performance liquid chromatography (HPLC) is a type

of liquid chromatography used to separate and quantify compounds that have been dissolved in solution. HPLC is used to determine the amount of a specific compound in a solution. For example, HPLC can be used to determine the amount of morphine in a compounded solution. In HPLC and liquid chromatography, where the sample solution is in contact with a second solid or liquid phase, the different solutes in the sample solution will interact with the stationary phase as described. The differences in interaction with the column can help separate different sample components from each other.

## Practical Aspects of HPLC Theory

In order to understand HPLC and to utilize its practical applications effectively, some basic concepts of chromatographic theory are presented here.

### Chromatographic Principles

**Retention.** The retention of a drug with a given packing material and eluent can be expressed as a retention time or retention volume. Retention or elution volume is the quantity of the mobile phase required to pull the sample through the column. Retention time is defined as how long a component is retained in the column by the stationary phase relative to the time it resides in the mobile phase. The retention is best described as a column capacity ratio ( $k'$ ), which can be used to evaluate the efficiency of columns. The longer a component is retained by the column, the greater is the capacity factor. The column capacity ratio of a compound (A) is defined by the following equation:

$$k' = \frac{T_A - T_0}{T_0} = \frac{V_A - V_0}{V_0}$$

where  $V_A$  is the elution volume of component A and  $V_0$  is the elution volume of a nonretained compound. At constant flow rate, retention times ( $T_A$  and  $T_0$ ) can be used instead of retention or elution volumes.

**Resolution.** Resolution is the ability of the column to separate peaks on the chromatograph. Resolution (R) is expressed as the ratio of the distance between two peak maxima to the mean value of the peak width at the base line

$$R = \frac{(T_B - T_A)2}{w_A + w_B}$$

where  $T_B$  is the retention time of component B,  $T_A$  is the retention time of component A,  $w_A$  is the peak width of component A and  $w_B$  is the peak width of component B. If R is equal to or more than 1, then components are completely separated, but if R is less than 1, then components overlap.

**Sensitivity.** Sensitivity is a measure of the smallest detectable level of a component in a chromatographic separation and is dependent on the signal-to-noise ratio in a given detector.

### Continuing Education

#### GOALS AND OBJECTIVES

“Quality-Control Analytical Methods: High-Performance Liquid Chromatography”

**Goal:** The goal of this presentation is to provide compounding pharmacists with auxiliary information concerning the use of high-performance liquid chromatography for quality control of compounded preparations.

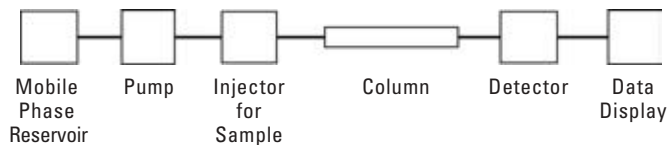
**Objectives:** After reading and studying the article, the reader will be able to:

1. Discuss basic theory behind chromatographic separation.
2. Discuss basic instrumentation and components of high-performance liquid chromatography.
3. Discuss different analysis methods for high-performance liquid chromatography.
4. Discuss validation and maintenance of high-performance liquid chromatography.

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**Figure 1. Schematic of a High-Performance Liquid Chromatograph.**



Sensitivity can be increased by derivatization of the compound of interest, optimization of chromatographic system or miniaturization of the system.

### Chromatographic Mechanisms

Systems used in chromatography are often categorized into one of four types based on the mechanism of action, adsorption, partition, ion-exchange and size exclusion. Adsorption chromatography arises from interactions between solutes and the surface of the solid stationary phase. Partition chromatography involves a liquid stationary phase that is immiscible with

the eluent and is coated on an inert support. Ion exchange chromatography has a stationary phase with an ionically charged surface that is different from the charge of the sample. The technique is based on the ionization of the sample. The stronger the charge of the sample, the stronger the attraction to the stationary phase; therefore, it will take longer to elute off the column. Size exclusion is as simple as screening samples by molecular size. The stationary phase consists of material with precisely controlled pore size. Smaller particles get caught up in the column material and will elute later than larger particles. Several other types of chromatographic separation have been described, including ion-pair chromatography, which is used as an alternative to ion-exchange chromatography and chiral chromatography (to separate enantiomers).

### Instrumentation

As shown in the schematic diagram in Figure 1, HPLC instrumentation includes a pump, injector, column, detector and integrator or acquisition and display system. The heart of the system is the column where separation occurs. Since the stationary phase may be composed of micron-sized porous particles, a high-pressure pump is required to move the mobile phase through the column. The chromatographic process begins by injecting the solute into the injector at the end of the column. Separation of components occurs as the analytes and mobile phase are pumped through the column. Eventually, each component elutes from the column as a peak on the data display. Detection of the eluting components is important, and the method used for detection is dependent upon the detector used. The response of the detector to each component is displayed on a chart recorder or computer screen and is known as a chromatogram. To collect, store and analyze the chromatographic data, integrators and other data-processing equipment are frequently used.

### Mobile Phase and Reservoir

The type and composition of the mobile phase affects the separation of the components. Different solvents are used for different types of HPLC. For normal-phase HPLC, the solvent is usually nonpolar, and, in reverse-phase HPLC, the solvent is normally a mixture of water and a polar organic solvent. The purity of solvents and inorganic salts used to make the mobile phase is paramount. A general rule of thumb is to use the highest purity of solvent that is available and practical depending on the particular application. The most common solvent reservoirs are as simple as glass bottles with tubing connecting them to the pump inlet.

### Pumps

High-pressure pumps are needed to push the mobile phase through the packed stationary phase. A steady pump pressure (usually about 1000–2000 psi) is needed to ensure reproducibility and accuracy. Pumps are typically known to be robust, but adequate maintenance must be performed to maintain that characteristic. Inability to build pressure, high pressures or leakage could indicate that the pump is not functioning correctly. Proper maintenance of the pump system will minimize down time.

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## Injectors

The injector can be a single injection or an automated injection system. An injector for an HPLC system should provide injection of the liquid sample within the range of 0.1-100 mL of volume with high reproducibility and under high pressure (up to 4000 psi). For liquid chromatography, liquid samples can be directly injected and solid samples need only to be diluted in the appropriate solvent.

## Detectors

There are many different types of detectors that can be used for HPLC. The detector is used to sense the presence of a compound passing through and to provide an electronic signal to a data-acquisition device. The main types of detectors used in HPLC are refractive index (RI), ultraviolet (UV-Vis) and fluorescence, but there are also diode array, electrochemical and conductivity detectors. Each detector has its assets, limitations and sample types for which it is most effective. Most applications in drug analysis use detectors that respond to the absorption of ultraviolet radiation (or visible light) by the solute as it passes through the flow-cell inside the detector.

The recent development of the so-called hyphenated techniques has improved the ability to separate and identify multiple entities within a mixture. These techniques include liquid chromatography-mass spectrometry (LC-MS), liquid chromatography-mass spectrometry-mass spectrometry (LC-MS-MS), liquid chromatography-infrared spectroscopy (LC-IR) and liquid chromatography-nuclear magnetic resonance (LC-NMR). These techniques usually involve chromatographic separation followed by peak identification with a traditional detector such as UV, combined with further identification of the compound with the MS, IR or NMR.

## Data Acquisition/Display Systems

Since the detector signal is electronic, use of modern data-acquisition techniques can aid in the signal analysis. The data-acquisition system of most HPLC systems is a computer. The computer integrates the response of the detector to each component and places it into a chromatograph that is easy to read and interpret. Other more advanced features can also be applied to a chromatographic system. These features include computer-controlled automatic injectors, multi-pump gradient controllers and sample fraction collectors.

## Columns

The column or stationary phase is the core of any chromatographic system. Columns are commercially available in different lengths, bore sizes and packing materials. The use of the correct combination of length and packing material in correlation with the appropriate mobile phase can assist in the most effective separation of a sample compound. A variety of column dimensions are available including preparative, normal-bore, micro- and mini-bore and capillary columns. Different column dimensions can be used for different types of separations and can utilize different packing materials and flow rates. The most widely used packing materials for HPLC separations are silica-based. The most popular material is octadecyl-silica (ODS-silica), which contains C<sub>18</sub> coating, but

materials with C<sub>1</sub>, C<sub>2</sub>, C<sub>4</sub>, C<sub>6</sub>, C<sub>8</sub> and C<sub>22</sub> coatings are also available. Miscellaneous chemical moieties bound to silica, as well as polymeric packing, are designed for purification of specific compounds. Other types of column packing materials include zirconia, polymer-based and monolithic columns.

Theoretical plates relate chromatographic separation to the theory of distillation and are a measure of column efficiency. The number of theoretical plates (n) can be determined by the following equation

$$n = 16 \left( \frac{t_{R1}}{w} \right)^2$$

where  $t_{R1}$  is the total retention time and w is the band width of the peak.

In general, LC columns are fairly durable with a long service life unless they are used in some manner that is intrinsically destructive—for example, with highly acidic or basic eluents or with continual injections of “dirty” biological or crude samples. Column degradation is inevitable, but column life can be prolonged with proper maintenance. Flushing a column with mobile phase of high elution strength following sample runs is essential. When a column is not in use, it is capped to prevent it from drying out. Particulate samples need to be filtered and when possible a guard column should be utilized. Column regeneration could instill some life into a column, but preventive maintenance is the key to preventing premature degradation.

## Separation Techniques

### Isocratic versus Gradient Elution

Elution techniques are methods of pumping mobile phase through a column. In the isocratic method, the composition of the mobile phase remains constant, whereas in the gradient method the composition changes during the separation process. The isocratic method is the simplest technique and should be the first choice when developing a separation. Eluent gradients are usually generated by combining the pressurized flows from two pumps and changing their individual flow rates with an electronic controller or data system while maintaining the overall flow rate constant.

### Derivatization

Derivatization of samples involves a chemical reaction that alters the molecular structure of the analyte of interest to improve detection. In HPLC, derivatization of a drug is usually unnecessary to achieve satisfactory chromatography. Derivatization is used to enhance the sensitivity and selectivity of detection when available detectors are not satisfactory for the underivatized compounds.

## Quantitative Analysis

The quantification methods incorporated in HPLC are borrowed mostly from gas chromatography methods. The basic theory for quantitation involves the measurement of peak height or peak area. To determine the concentration (conc.) of a compound, the peak area or height is plotted versus the concentration of the substance. For peaks that are well resolved, both peak height and area are proportional to the concentration. Three different calibration methods, each with its

own benefits and limitations, can be utilized in quantitative analysis: external standard (std.), internal standard and the standard addition method.

### External Standard

The external standard method (see Figures 2 and 3) is the simplest of the three methods. The accuracy of this method is dependent on the reproducibility of the injection volume. To perform this method, standard solutions of known concentrations of the compound of interest are prepared with one standard that is similar in concentration to the unknown. A fixed amount of sample is injected. Peak height or area is then plotted versus the concentration for each compound. The plot should be linear and go through the origin. The concentration of the unknown is then determined according to the following formula.

$$\text{Conc.}_{\text{unknown}} = \left( \frac{\text{Area}_{\text{unknown}}}{\text{Area}_{\text{known}}} \right) \text{conc.}_{\text{known}}$$

### Internal Standard

Although each method is effective, the internal standard method tends to yield the most accurate and precise results. In

this method, an equal amount of an internal standard, a component that is not present in the sample, is added to both the sample and standard solutions. The internal standard selected should be chemically similar to, have similar retention time and derivatize similarly to the analyte. Additionally, it is important to ensure that the internal standard is stable and does not interfere with any of the sample components. The internal standard should be added before any preparation of the sample so that extraction efficiency can be evaluated. Quantification is achieved by using ratios of peak height or area of the component to the internal standard.

$$\text{Conc.}_{\text{unknown}} = \left( \frac{\text{Area}_{\text{InternalStd.in known}}}{\text{Area}_{\text{InternalStd.in unknown}}} \right) \times \left( \frac{\text{Area}_{\text{unknown}}}{\text{Area}_{\text{known}}} \right) \times (\text{Conc.}_{\text{known}})$$

### Validation

It is important to utilize a validated LC method when performing analysis. Typical analytical characteristics evaluated in an LC validation include but are not limited to precision, accuracy, specificity, limit of detection, limit of quantitation, linearity and range. It is important to consider the US Food and Drug Administration (FDA) and *United States*

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*Pharmacopeia-National Formulary (USP-NF)* guidelines when validating LC methods used for pharmaceutical samples. *USP 27-NF 22*, section <1225> provides guidance on validation of compendial methods, including definitions and determination. The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines provide suggestions concerning validation of pharmaceuticals. Valuable sources of information providing regulatory guidance may be found on the FDA website at <http://www.fda.gov/cder/guidance>.

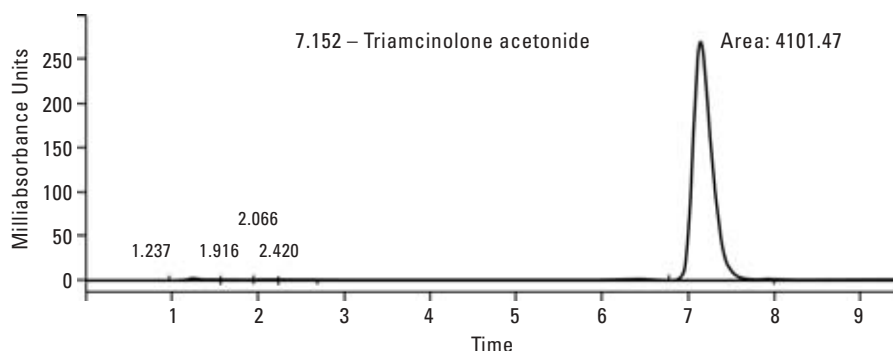
System suitability tests provide an evaluation for the function of the overall LC system. This includes all components that make up a system, such as the instrument, reagents, column packing material, details of the procedure and even the analyst. These tests imply that all of the components of a system constitute a single system in which the overall function can be tested. System suitability tests are valuable and have been accepted in general application because reliable and reproducible chromatographic results are based on a wide range of specific parameters.

In most laboratories there is a standard operating procedure that outlines the specifications for running a system's suitability test. For the test, at least five replicate injections are made of a single solution that contains 100% of the expected active and excipient ingredients level. The peak response is measured and the standard deviation of that response should not exceed the limit set by the testing monograph or 2%, whichever of the two is the lowest. Using the *USP* method, the tailing factors of the analytes should be determined. The values should not exceed 2. Peak-to-peak resolutions are also determined by using the *USP* calculations, and the value should not be lower than 1.5. The system test is used to ensure the quality of the data and of the analysis.

## Summary

HPLC is currently the most widely used method of quantitative analysis in the pharmaceutical industry and in pharmaceutical analysis laboratories.

**Figure 2. Chromatogram of Reference Standard.**



Note: This was a 200 ppm (mcg/mL) solution, so the amount (200 ppm) is divided by the area (4101.47 mAU), resulting in amt/area ratio (4.8763e-2) used for sample determination.

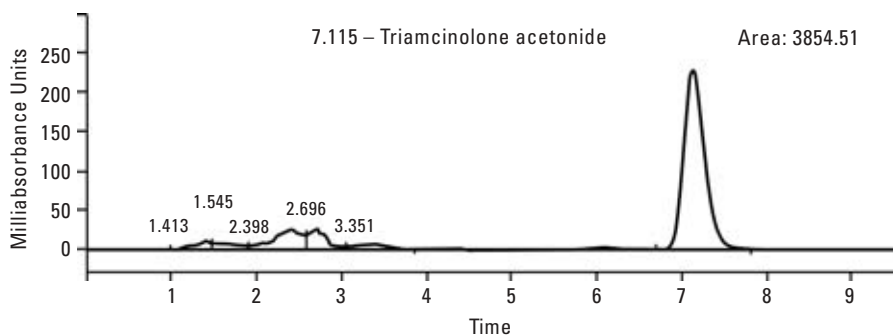
mAU = milliabsorbance units

mcg = microgram

mL = milliliters

ppm = parts per million

**Figure 3. Chromatogram of Sample.**



Note: This was a 200 ppm (mcg/mL) solution, so the amount (200 ppm) is divided by the area (4101.47 mAU), resulting in amt/area ratio (4.8763e-2) used for sample determination.

mAU = milliabsorbance units

mcg = microgram

mL = milliliters

ppm = parts per million

## Resources

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