# CHROMATOGRAPHY, LIQUID

## 1. Introduction

Liquid chromatography involves the separation of compounds by differential migration as a liquid mobile phase flows over a solid stationary phase. The mode of separation varies depending on the mobile and stationary phases. (see CHROMATOGRAPHY) In HPLC, small stationary-phase particle sizes and highly controlled conditions are used to achieve high resolutions. A representative separation might involve small (5 μm diameter) particles of chemically modified silica

uniformly packed into a 250 mm long by 4.6 mm i.d. stainless steel column. A mobile phase is pumped through the column at 1 mL/min at high pressure (several thousand psi). Sample injection occurs at one end of the column and the separated components are detected at the other end [eg, by ultraviolet (uv) absorbance].

For analytical applications very small amounts of material are generally added to the column. Preparative HPLC can also be used to isolate pure compounds from mixtures and this generally involves adding larger amounts of material to the column. For preparative separations columns of larger diameter (eg, 10 and 20 mm) are employed and for industrial-scale separations even larger columns are available. This article will, however, concentrate on analytical uses of HPLC.

## 2. Equipment

A representative HPLC instrument consists of a mobile-phase reservoir, a high pressure pump, an injection device, a separation column, a detector, and a data system (Fig. 1). The equipment can be modular, with parts from different manufacturers connected together, or an integrated system from one manufacturer.

Computers are almost universally employed with modern equipment and can be used to control the pump, detector, and robotic sample preparation equipment as well as interpret the output from the detector. In large enterprises, eg, pharmaceutical companies, the systems are highly automated and are coupled together by Laboratory Information Management Systems (LIMS). The source of each sample is recorded as are the chromatographic conditions and the

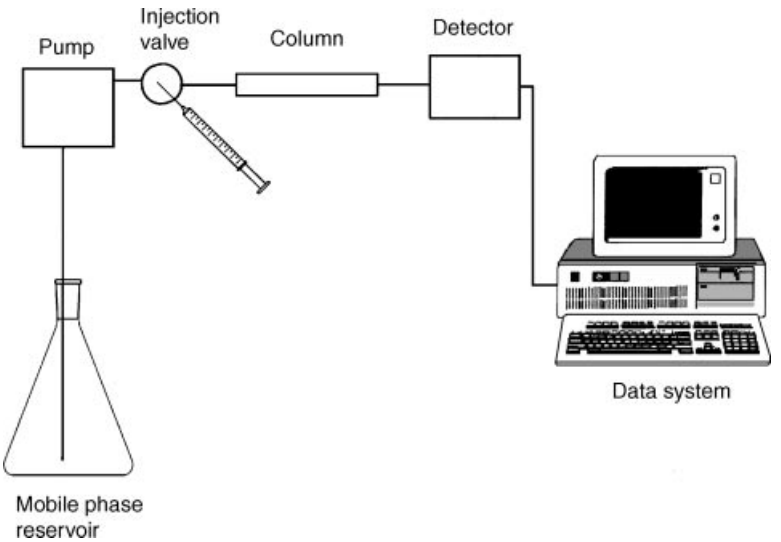


**Fig. 1.** Diagram of a basic HPLC instrument.

resultant chromatogram. At any time in the future, the chromatogram and related data can be retrieved for quality control purposes. The desired results are automatically calculated and presented to the operator.

The mobile-phase reservoir can be as simple as a conical flask. Some provision should be made for degassing the mobile phase by filtration, sonication, application of a vacuum, sparging with helium, online membrane degassing, or some combination of these methods. Degassing the mobile phase prevents bubbles forming in the system from outgassing and interfering with the separation. A filter should be used to prevent particulates being drawn into the pump.

The pump should provide a constant flow of mobile phase at ~0.5–2 mL/min for analytical separations (with higher flow rates being used for preparative separations) at high pressure (up to 6000 psi). Ideally, pressure variations caused by the action of the pump should be as low as possible to minimize baseline noise. Gradients, in which the relative proportions of, eg, the organic and aqueous components of the mobile phase are varied during the course of the run, are commonly used. The components of the mobile phase can be mixed either before the high pressure pump (low pressure mixing) or after passing through two high pressure pumps (high pressure mixing). Gradients are generally linear (ie, the mobile phase composition changes in a linear fashion with time) but the same separation may contain gradients with different slopes. In some cases, nonlinear gradients (eg, concave and convex) are used. Gradients are particularly useful for separating compounds of greatly different polarity but may be difficult to transfer between instruments from different manufacturers because of differences in dwell (mixing) volume. The need to reequilibrate the system after the separation adds to the total run time.

The injection device can be a high pressure switching valve with a loop of narrow-bore tubing that can be wholly or partially filled with the sample. Operation of the valve brings the sample loop into the circuit between the pump and the column and the sample is carried onto the column by the mobile phase. Automated sample injectors of various designs are also available.

The separation column and detector are the heart of the system. The separation column, in which the actual separation of the sample into its components takes place, is typically a stainless steel tube 50–250 mm in length and 1–4.6 mm in internal diameter packed with small particles of modified or unmodified silica through which the liquid is pumped at a typical rate and pressure of 0.8–2 ml/min and 1000–3000 psi, respectively. The properties of the stationary and mobile phase (ie, polarity, size of particles, charge, etc) determine the mechanism of separation; thus, these components should be specifically selected to suit the nature of the sample.

The detector produces a signal relative to the concentration of the analyte. The separated compounds are presented as a set of chromatographic peaks—the chromatogram—in which the elution time is typical of a specific compound, and hence allows for its identification, and the size of the peak is relative to the concentration of the compound. Each of the various available detectors measures a different physical property of the analyte, such as the uv absorbancy of a compound or the intensity of the light emitted by a fluorescent compound, and converts it to an electrical signal that can be further processed to obtain the chromatogram.

Chromatographic systems, ie, the combination of column and mobile phase, as well as various detectors are further discussed below.

Other components that are useful include a pressure gauge, flow meter, and column heater. Most separations take place in a satisfactory manner at ambient temperature but a thermostatted column compartment will remove a possible source of inconsistency. In addition, the reduced viscosity of the warmed mobile phase will reduce the required pressure. Generally, temperatures range from 30–70°C although subambient temperatures have been used. Temperature gradients are not used in HPLC.

Generally, all components in contact with the mobile phase are made of stainless steel but some compounds may adhere to stainless steel and so it may be necessary to use components made from inert materials such as titanium, glass, or polymer [eg, PEEK (polyetheretherketone)]. To prevent the dilution of the injected sample with mobile phase (which leads to broadened peaks) it is desirable to keep the dead volume after the injector to a minimum and so narrow bore tubing, eg, 1/16 in. o.d. × 0.010 in. i.d. (1.59 mm o.d. × 0.254 mm i.d.), should be used for all connections. For similar reasons, the volume of sample injected should be kept to a minimum ($\leq$25 μL). The best medium for the sample is generally the mobile phase or a weaker solvent. If a gradient system is used, the sample solvent should correspond to (or be weaker than) the initial composition of the mobile phase.

It is good practice to employ a filter before the analytical column. Guard columns, packed with a small amount of material similar, or identical, to that in the analytical column, are frequently used. Insoluble or highly retained contaminants will accumulate on the guard column rather than the main column and can be easily removed by changing the (cheaper) guard column. Sometimes aggressive mobile phases that may attack the stationary phase are passed through a column packed with low grade and less expensive chromatographic material (of the same type as in the analytical column) situated between the pump and the injector. The mobile phase attacks this sacrificial precolumn rather than the expensive analytical column. Since the precolumn is situated before the injector the sample never passes through it.

## 3. Sample Preparation

In some cases, eg, a solution of a bulk drug substance, sample preparation is as simple as filtering the sample before injection. However, in many cases complex sample preparation procedures are required to obtain reproducible results. Consider the analysis of a drug at a low concentration (in the ng/mL range) in blood plasma. Plasma is a complex mixture containing numerous compounds, such as proteins, and injection of an unprocessed sample would result in a large off-scale peak that would swamp any peaks attributable to the drug. Additionally, the HPLC column would rapidly become plugged and useless. To avoid such undesirable results, a variety of approaches have been developed and some of these are described below. All of these procedures can be automated using robotic equipment and this is desirable for long production runs. However, due to the effort involved in developing an automated procedure it is generally easier to use

manual procedures when the number of samples to be processed is not large. Because of the resources required to set up an automated procedure the Food and Drug Administration (FDA) in the United States prefers that manual methods be submitted for validation even though automated procedures will eventually be used by the drug company (1).

**3.1. Liquid–Liquid Extraction.** Liquid–liquid extraction generally involves the extraction of an aqueous phase (eg, urine, plasma, and serum) with an organic solvent (2). Separation, generally facilitated by the use of a centrifuge, results in an organic layer containing the drug and an aqueous layer containing most of the potentially interfering compounds. The extraction can be repeated several times. The layers can be separated using a pipet or the aqueous layer can be frozen using a dry ice–acetone bath and the organic layer (which does not freeze) poured off. Freezing the aqueous layer is particularly helpful in removing traces of water that may interfere with the subsequent processing. The combined organic layers are generally evaporated to dryness under a stream of nitrogen or under reduced pressure and the residue reconstituted with the HPLC mobile phase prior to injection.

More than one extraction can also be used to clean up the sample. For example, basic analytes can be reextracted from the organic layer with aqueous acid. Basification of the aqueous layer and extraction with an organic solvent will result in a cleaner sample.

An increase in sensitivity can be obtained by reconstitution with a volume of mobile phase less than that of the original matrix. For example, 1 mL of plasma can be extracted and the residue reconstituted with 100 μL of mobile phase, leading to a 10-fold concentration.

The usual organic solvents can be used for extraction, eg, dichloromethane or heptane. Since most of the organic phase will eventually be vaporized toxic solvents such as benzene or chloroform should be avoided. Hexane is surprisingly toxic and should not be used (3). Also, appropriate safety precautions should be taken, eg, all procedures should be carried out in a properly functioning chemical fume hood, not in a laminar flow cabinet or on the bench. An extraction solvent combination that is sometimes used is a mixture of a nonpolar hydrocarbon with a higher alcohol, eg, heptane:isoamyl alcohol 99:1 (v/v). A number of procedures can be used for agitating the extraction mixture such as using a vibrating mixer (vortex mixer), rotating the tube, gently shaking the tube using an orbital mixer, or shaking the tube using a wrist action shaker. Frequently, small polypropylene tubes of ∼1–2 mL are used for the vortex mixer and larger glass tubes (∼10 mL) for the other agitators. It goes without saying that all tubes should be securely capped! Although it might be thought that more is better when it comes to agitation, excessive agitation can lead to intractable emulsions. The most effective combination of extraction solvent and agitation method should be established by a close examination of the available literature (4) and by experimentation.

**3.2. Precipitation.** A variation of liquid–liquid extraction is to use a water-miscible organic solvent such as acetonitrile or methanol. For example, mixing acetonitrile with plasma causes the proteins in the plasma to precipitate. Centrifuging the mixture causes the proteins to collect in the bottom of the tube and the aqueous/organic mixture may be removed, evaporated, and reconstituted as described above.

**3.3. Ultrafiltration.** Plasma proteins may also be removed using a membrane filter having very small pores (eg, 0.2 μm) made of materials such as cellulose acetate, poly(tetrafluoroethylene) (PTFE), or polysulfone. The resulting filtrate is clean enough to be injected directly on an HPLC column. Low cost disposable equipment has been developed to make this procedure attractive. The plasma sample is placed in a plastic tube with a filter in the bottom and this tube is placed inside a plastic centrifuge tube. A centrifuge provides the force required to filter the plasma through the membrane and into the bottom of the tube. Small tubes of ~0.5-mL capacity are frequently used although larger tubes are also available.

**3.4. Solid-Phase Extraction.** Solid-phase extraction (SPE) is a technique whereby a crude chromatographic separation is used to effect an initial purification and produce a sample that is clean enough for injection onto the analytical HPLC column. Low cost disposable SPE cartridges are generally used although they can also be constructed by the analyst, eg, in a disposable Pasteur pipet. The ready-made SPE cartridges typically consist of 0.1–1 g of chromatographic material such as silica, C18, C8, phenyl, ion-exchange (see below) in a plastic body. The commercial cartridges may be cleaned and reused but this may prove to be a false economy if residual contaminants interfere with later separations. Note that there is no requirement that the same chromatographic material be used in the SPE cartridges and in the analytical HPLC column.

Immediately before use the SPE cartridges should be conditioned so as to remove manufacturing impurities and wet the chromatographic material. Reversed-phase cartridges (such as C18) are typically conditioned with methanol followed by water or buffer and normal phase cartridges may be conditioned with an organic solvent such as heptane. The sample (eg, plasma or urine) is added to the cartridge and, after it has been absorbed, the cartridge is washed. For example, a C18 cartridge may be washed with water and with aqueous solutions containing low percentages of methanol, and then the compounds of interest may be eluted with pure methanol. Interfering compounds such as plasma proteins are eliminated with the washes and the compounds of interest are obtained in a relatively clean methanol solution. The methanol eluate can be evaporated and reconstituted as described above. SPE cartridges of other types, such as silica or ion-exchange resin, would be washed and eluted with different solutions but the principle remains the same: Interfering compounds (in relatively large amounts) are removed with the washes and the compounds of interest (in relatively small amounts) are eluted in a relatively clean solution.

Generally, it is important not to let the SPE cartridge run dry between steps although some procedures call for all traces of liquid to be removed using a vacuum immediately before the final elution step. It is also important not to run liquid through the SPE cartridge at too fast a rate. Excessively fast flow rates will cause loss of resolution. In most applications, flow rates of 0.5–1 mL/min are satisfactory. Flow rates such as these can frequently be achieved using gravity alone. Otherwise a gentle vacuum may be applied.

Solid-phase extraction can also be used to concentrate large amounts of relatively dilute solutions. For example, a large volume (eg, 50 mL) of polluted wastewater may be run through a C18 cartridge and the water will pass straight through leaving the nonpolar pollutants trapped on the cartridge. The compounds

to be analyzed can then be eluted with a small amount (eg, 1 mL) of methanol. In this example, the compounds to be analyzed have been concentrated by a factor of 50, more if the methanol is evaporated and the residue reconstituted in a smaller volume.

**3.5. Column Switching.** Column-switching techniques are closely related to solid-phase extraction and in some cases the distinction may become blurred. There are many variations but the simplest involves two columns, two pumps, and a switching valve.

In the example shown in Fig. 2, with the switching valve in the "load" position, the solution to be analyzed (eg, plasma) is injected onto column A and eluted to waste with a "weak" mobile phase, eg, water. When the plasma proteins and other interfering species have been washed off column A the switching valve is turned to the "analyze" position (counterclockwise in Fig. 2) and a "strong" mobile phase, eg, methanol:water 50:50, is used to elute the compounds of interest from column A onto column B. Chromatographic separation takes place on column B and the compounds of interest, eg, drugs and metabolites, are detected in the effluent. Although not strictly necessary, automation is obviously very useful with column-switching techniques. The cleanup column A may require periodic replacement but the chromatographic column B should have a normal life. Many variations of this basic technique have been reported. Since the compounds of interest tend to stay at the top of column A it is common practice to reverse the flow of the "strong" mobile phase and backflush these compounds from column A onto column B. In this way, the compounds of interest are dissolved in the minimum amount of mobile phase as they pass onto column B. Once the analytes have moved from column A to column B, column A can be cleaned with a strong

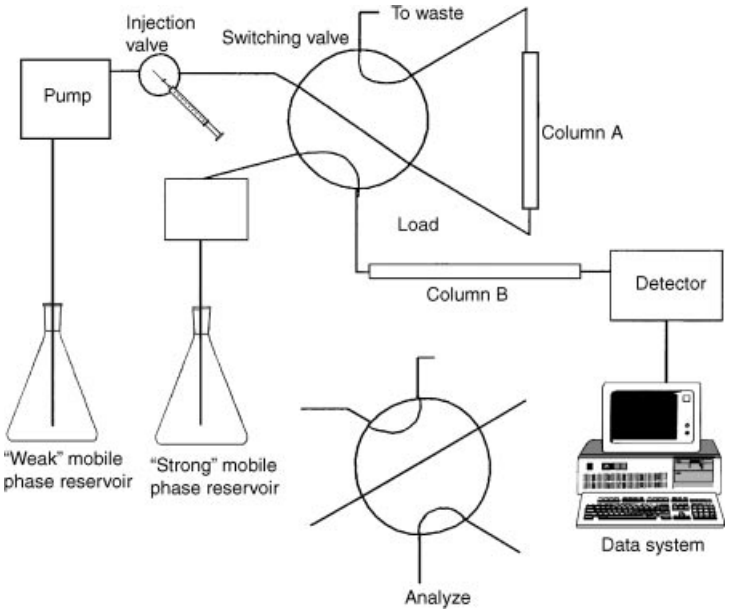


**Fig. 2.** Diagram of a column-switching HPLC arrangement.

solvent and reequilibrated. Different stationary phases can be used for columns A and B. Generally, the analytical column (B) is a conventional column. The initial column (A) can be a short analytical column, a commercial precolumn, or a specialized column developed for biological samples, such as an internal-surface reversed phase (ISRP) column. ISRP columns, also known as restricted internal access or Pinkerton columns, contain packing material that has narrow pores coated with reversed-phase material such as $C_{18}$. Large protein molecules cannot penetrate the pores and pass straight through the column. The small analyte molecules enter the pores, are separated in a conventional fashion, and elute later (5).

Column-switching techniques can be used to accomplish achiral separation of a racemic mixture of a drug from contaminants on one column, and then chiral separation of the two enantiomers on another column. For example, the enantiomers of nilvadipine can be separated in this way (6).

Just as solid-phase extraction can be used to concentrate large amounts of relatively dilute solutions so can column-switching techniques. For example, a large volume of polluted waste water may be run through a short reversed-phase column and the water will pass straight through leaving the nonpolar pollutants trapped on the cartridge. The compounds to be analyzed can then be eluted with the mobile phase onto the analytical column (7). Blood plasma can be dialyzed using a semipermeable membrane and the dialysate passed through a short column. The drug molecules pass through the membrane into the dialysate and are then trapped on the column. When dialysis is complete the compounds, eg, nonsteroidal anti-inflammatory drugs (8), are eluted from the trapping column onto the analytical column by the mobile phase and chromatographed in the usual manner.

In some cases, more than two columns are used in a column-switching setup. One group has described a complex method for the analysis of enprostil in plasma that involves solid-phase extraction, derivatization, and a column-switching apparatus with five separate columns (9).

Column-switching generally involves automation and complex plumbing and so is best suited to situations where many similar samples must be analyzed. Modern automation allows such systems to run with a minimum of attention once they have been set up.

**3.6. Derivatization.** In HPLC, derivatization is generally used to improve the detectability of the compounds of interest although it may also be used to improve their chromatographic properties (10,11). The derivatization reaction usually involves a reaction that is simple and irreversible, eg, reaction of an acyl halide with an alcohol or amine to give an ester or amide respectively. Thus alcohols or amines that do not have appreciable uv chromophores can be coupled to molecules with a high uv absorbance. Similarly, nonfluorescent analytes can be coupled to highly fluorescent molecules to achieve high sensitivity and selectivity. Although the chemistry of the derivatization reaction tends to be fairly simple the reagents can be quite sophisticated molecules and they can be tailored to have desirable properties, such as fluorescence at particular wavelengths.

Ideally, derivatization reactions should proceed rapidly, quantitatively, reproducibly, and irreversibly at room temperature but sometimes heating

may be necessary. In some cases, particularly with fluorescent reagents, it is advantageous to remove excess reagent with a washing step before injecting the sample into the chromatographic system. Chiral separations can be achieved by using chiral derivatization reagents (11,12).

Complexing agents can be used to make metal complexes that may be easily chromatographed and detected. (12).

The examples discussed above apply to the derivatization of molecules before they are chromatographed, ie, precolumn derivatization. However, postcolumn reactions can also be used to derivatize molecules after they have been chromatographed. This technique has its own advantages and disadvantages and will be discussed below. Generally, precolumn derivatization is used to improve the separation characteristics and the efficiency of detection of the analytes while postcolumn derivatization is only used to improve the efficiency of detection.

**3.7. Detectors.** Refractive index and evaporative light-scattering detectors are so-called universal detectors, ie, they can detect all analytes, but they have some limitations and suffer from limited sensitivity. Refractive index detectors monitor the refractive index of the eluting mobile phase. The presence of an analyte will change the refractive index and produce an output signal proportional to the amount of analyte. In an evaporative light-scattering detector, the eluting mobile phase is nebulized and evaporated as it passes through a drift tube. Particles of analyte are detected in a light scattering cell.

**3.8. UV Detectors.** The most commonly used detectors detect eluting compounds by their uv absorbance. These detectors are sensitive, robust, and of relatively low cost. Although uv detectors are not universal detectors most compounds of interest can be detected. Other compounds that have essentially no uv chromophore, such as aliphatic alcohols, can be detected after derivatization. Generally a wavelength between 200 and 500 nm is used. Normally, uv absorbance increases as the wavelength decreases and so greater sensitivity can be achieved at lower wavelengths but problems may arise due to noise and mobile phase absorbance. Although sensitivity is generally less at higher wavelengths selectivity may be increased because the absorbance of the interfering compounds may decrease more than the absorbance of the analyte as the wavelength increases.

Conventional uv detectors employ a uv source, typically a deuterium lamp for uv wavelengths ($<$400 nm) and a tungsten lamp for visible wavelengths ($>$400 nm), and a monochromator to make sure that light of only one wavelength shines through the flow cell. Generally, the wavelength can be varied over a wide range (eg, 190–700 nm) but some low cost single wavelength detectors are available, eg, operating at 254 nm. To minimize peak broadening it is desirable to keep the volume of the flow cell as small as possible. However, this will lead to reduced sensitivity. To counteract the loss of sensitivity flow cells are generally arranged so that the light shines along the column of liquid rather than across it. The light that emerges from the flow cell is detected by a diode or phototube and the signal is amplified and passed to the data handling system.

In diode array detectors (DADs) [also known as photodiode array detectors (PDAs)], wavelengths between, say, 190 and 600–900 nm are continuously

recorded. Light of all wavelengths passes through the flow cell and the light that emerges is dispersed by a prism or grating to provide an arc of different wavelengths. Photodiodes are situated at intervals along this arc. Each photodiode monitors light of a very narrow band of wavelengths (typically 1–4 nm). After a run a chromatogram recorded at any given wavelength can be displayed. The uv spectrum of any given peak can be obtained and this may help with compound identification. By comparing the ratio between two different wavelengths, peak purity can be assessed although this procedure is not necessarily conclusive. A pure peak will give a square wave because the ratio of the absorbance at the two wavelengths is always the same, regardless of the peak height. Two compounds eluting together will give a more complicated result because the ratio will vary if the compounds do not elute at exactly the same time.

**3.9. Fluorescence Detectors.** In a fluorescence detector, the mobile phase is illuminated with a beam of light and the light emitted by a fluorescent compound is picked up and quantitated by a detector placed at right angles to the light beam. Monochromators or filters may be used to set the excitation and emission wavelengths. Greater sensitivity may be obtained by using a laser as the excitation source but then the wavelength is not readily changed. When monochromators are used the excitation and emission wavelengths may be changed during the course of the run. Fluorescence detectors offer great sensitivity and selectivity. Some compounds exhibit native fluorescence but it is frequently necessary to make a fluorescent derivative

**3.10. Electrochemical Detectors.** Electrochemical detectors also offer great sensitivity and selectivity. However, only a limited range of compounds can be detected using this technique. Derivatization can be used to make the compounds of interest electroactive, ie, detectable using these detectors, but this is not commonly done. Generally, a working electrode is held at a fixed potential relative to a reference electrode and the current is monitored. When a compound that can be oxidized or reduced arrives at the electrode the change in current provides the signal. More generally (but not exclusively), oxidative conditions are used. Many different conditions have been reported for electrochemical detection. The most common electrode material is glassy carbon but many others, such as gold or platinum, have been reported. Electrode fouling and mobile-phase interference can be particular problems with this type of detector. Use of high purity solvents can help. Some methods use a guard cell before the injector to oxidize any electroactive compounds in the mobile phase. Recycling the mobile phase may also help maintain baseline stability.

One problem with electrochemical detectors is the fouling of the electrode surface by reaction products and intermediates. Fouled electrodes can be cleaned mechanically but this is a lengthy and tedious process. To get around this problem, pulsed electrochemical detectors have been developed. A series of rapid positive and negative pulses are used to clean the electrode. After the initial detection period at, eg, −50 to 200 mV a large positive potential (eg, +600 mV) is applied to the electrode. This potential causes a layer of oxide to form on the electrode and at the same time any fouling is desorbed. A large negative potential (eg, −600 mV) is then applied to remove the oxide layer. The “clean” electrode is then ready for the next detection cycle. Typically, one cycle will take <1 s. Numerous variations have been reported (13).

**3.11. Mass Spectrometric Detectors.** Historically, mass spectrometric (MS) detectors have been difficult to use and have lacked reliability. However, great advances have been made in recent years and LC–MS is now the method of choice for developmental pharmaceutical work, although production work and quality control generally use uv detectors. There are many different types of MS detectors and operating conditions tend to be instrument specific. Commonly used buffers, such as phosphate, that leave a residue on evaporation may not be used. Buffers that are used for LC–MS include ammonium formate and trifluoroacetic acid which evaporate completely. Gradients may be used for LC–MS.

In MS, detection ions are generated at the source where the eluate enters the detector. There are many different sources such as atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI), fast-atom bombardment (FAB), thermospray (TSP), and matrix-assisted laser desorption (MALDI). The ions are separated on the basis of their mass in the mass analyzer. Common mass analyzers are the quadrupole, ion-trap, and time-of-flight (TOF) instruments. The ions are detected by an electron multiplier.

Compounds that co-elute from the chromatographic column can be readily distinguished by monitoring different ions. One of the great attractions of LC–MS is that run times may be shortened since peaks that would be merged if a uv detector was used can readily be distinguished by monitoring the different ions. Some important applications will be discussed below.

**3.12. Other Detectors.** Many other detectors have been described in the literature but they are not in widespread use. Conductivity detectors are useful for detecting inorganic ions in ion-exchange chromatography. Other detectors using principles such as infrared (ir), neclear magnetic resonance (nmr), radioactivity, polarimetry, and viscometry have specialized applications. Chemiluminescence detectors have been reported in the literature with increasing frequency (14).

**3.13. Postcolumn Reaction Detection.** Postcolumn reaction detection involves the derivatization of compounds as they elute from the chromatographic column after separation. In many cases, the chemistry is the same as that used for precolumn derivatization. For example, putrescine [110-60-1] reacts with *o*-phthalaldehyde and 2-mercaptoethanol to form a fluorescent derivative. This can take place precolumn (15) or postcolumn (16).

Since chromatographic separation does not occur once the compound has eluted from the HPLC column, postcolumn reaction detection can involve the use of reactions that do not necessarily lead to a single well-defined derivative. For example, ampicillin [69-53-4] in the effluent from an HPLC column reacts with a solution of sodium hypochlorite pumped at 0.2 mL/min to give, probably, a mixture of compounds. This mixture flows through a reaction coil to the detector. However, since no chromatographic separation will take place in the reaction coil it is unimportant that a mixture of compounds is produced by the reaction. A single peak that is proportional to the amount of ampicillin is produced (17).

Another technique that may lead to a mixture of products is postcolumn photochemical derivatization. The column effluent flows through a length of (typically) narrow-bore PTFE tubing that is illuminated by uv light. The uv light causes photochemical reactions to occur that produce compounds with

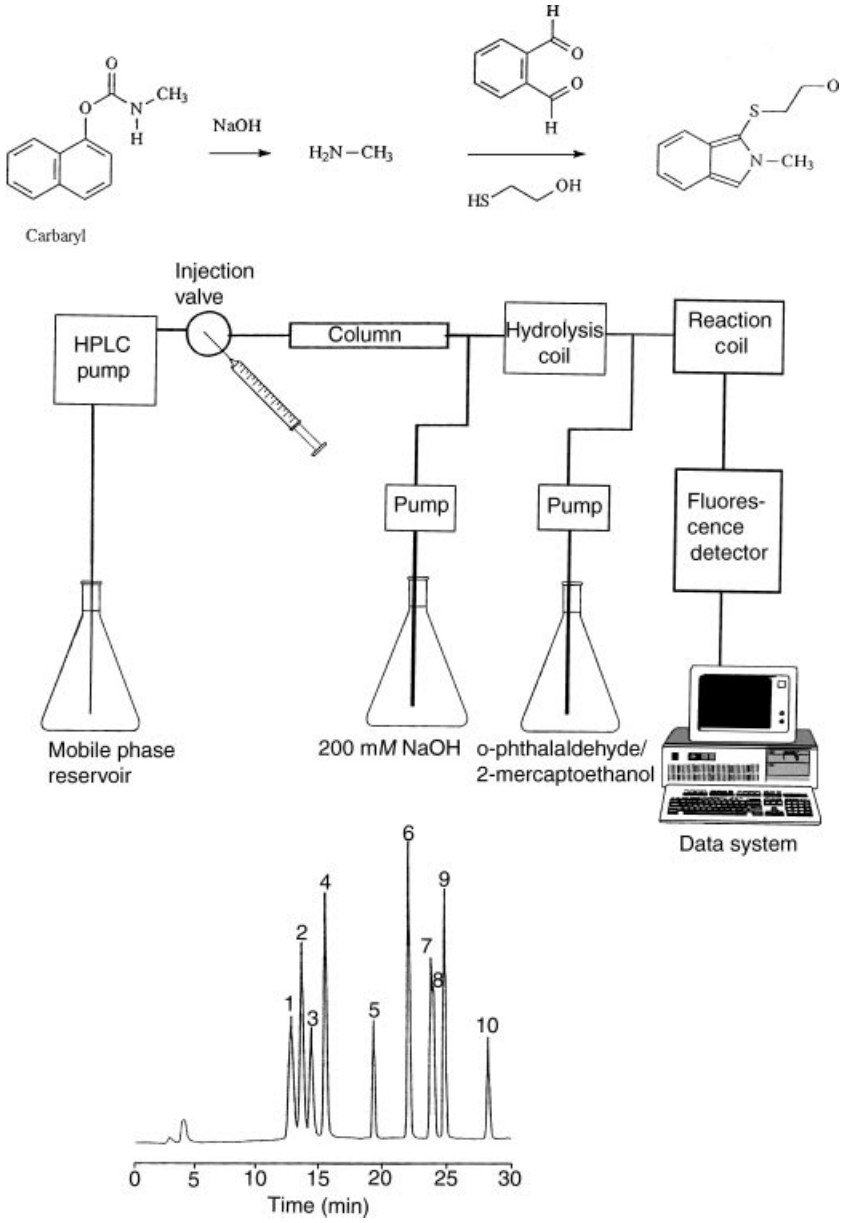


**Fig. 3.** Postcolumn reaction detection. Solution containing the insecticides aldicarb sulfoxide (*1*), aldicarb sulfone [1646-88-4] (*2*), oxamyl [23135-22-0] (*3*), methomyl [16752-77-5] (*4*), 3-hydroxycarbofuran [16655-82-6] (*5*), aldicarb [116-06-3] (*6*), propoxur [114-26-1] (*7*), carbofuran [1563-66-2] (*8*), carbaryl [63-25-2] (*9*), and methiocarb [2032-65-7] (*10*). Conditions: 50 × 4.6 mm i.d. Whatman pellicular ODS guard column, 250 × 4.6 mm i.d. 5 μm Apex ODS column; mobile phase methanol/water from 10:90 to 90:10 over 23 min, return to initial conditions over 4 min, reequilibrate at initial conditions for 10 min; flow rate 1.0 mL/min; detection as described in the text. Reproduced with permission from Ref. 20.

stronger uv chromophores or compounds that are fluorescent. Hence, sensitivity is increased (18). Knitting or crocheting the reaction coil leads to sharper peaks (19). Postcolumn photochemical derivatization is generally carried out empirically and the species responsible for the increased uv absorbance or fluorescence are not usually determined.

Quite sophisticated reactions can take place postcolumn. For example, *N*-methyl carbamate insecticides (eg, carbaryl [63-25-2]) can be separated on an HPLC column. The column effluent mixes with 200-m*M* NaOH pumped at 0.8 mL/min and this mixture flows through a 1 mL coil at 95°C. In the coil, the carbamate hydrolyses to methylamine and the corresponding alcohol. A reagent containing 500-mg/L *o*-phthalaldehyde and 1 mL/L 2-mercaptoethanol in 50-m*M* sodium tetraborate is added to the reaction stream at 0.8 mL/min. This mixture flows through a 0.5-mL coil at room temperature where the methylamine reacts to form a fluorescent derivative that is then detected by fluorescence (excitation 340 nm, emission 455 nm) (Fig. 3).

Postcolumn reaction procedures can take a lot of effort to set up and may require some maintenance but they can avoid the use of labor-intensive sample preparation and may provide superior sensitivity and selectivity.

## 4. Chromatographic Systems

**4.1. Normal Phase.** As originally developed, LC involved an unmodified polar solid stationary phase, such as alumina or silica, and a nonpolar liquid mobile phase, such as octane. Since this was the first type of LC to be developed it is referred to as "normal phase". Separation is achieved by the adsorption of analyte molecules on the surface of the stationary phase. Less polar (or hydrophobic) molecules are more weakly adsorbed, and hence elute quicker, than more polar (or hydrophilic) molecules.

As currently practiced normal-phase chromatography involving unmodified stationary phases uses silica particles of ~5–10 μm with a mobile phase consisting of organic solvents such as hexane, octane, dichloromethane, or methanol. Small amounts of acids or bases can be added to suppress tailing. It is important to control the amount of water in the mobile phase at a consistent level. Too little water will cause the stationary phase to dry out and separation efficiency will be poor. Too much water will saturate the stationary phase and the compounds will not be retained on the column.

Normal-phase chromatography may also take place using some kinds of modified silica stationary phases, eg, cyano, diol, or amino. Again, these polar stationary phases are used with relatively nonpolar mobile phases. For these modified stationary phases, control of the mobile phase water content is not critical. Confusingly, these modified stationary phases may also be used in a reversed-phase mode with polar mobile phases.

Currently normal-phase chromatography is not used extensively because of the expense of obtaining (and disposing of) high purity, possibly toxic organic solvents and the difficulty of maintaining the correct water level. However, normal-phase chromatography does have some advantages and in some cases separations may be obtained that are not feasible in any other way. Normal-

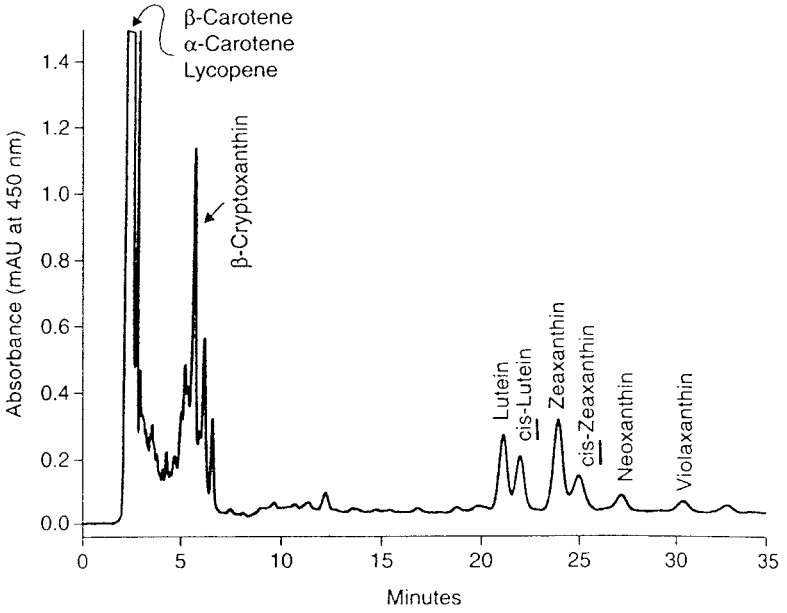


**Fig. 4.** A normal-phase separation. Food reference material carotenoids. Conditions: 250 × 4.6 mm i.d. 5-μm Lichrosorb Si60 column; mobile-phase hexane:dioxane/isopropanol/triethylamine 80:20:0.15:0.02; flow rate 1.0 mL/min; detector uv 450 nm. Reprinted with permission from Ref. 21.

phase chromatography is particularly useful for compounds that are unstable in aqueous solutions. It is also useful for preparative chromatography since the mobile phase is readily evaporated.

A number of good normal-phase methods using unmodified silica are found in the literature, eg, for the separation of vitamins, prostaglandins, and retinoids (Fig. 4).

**4.2. Reversed Phase.** By far, the most common HPLC technique is reversed-phase HPLC. In reversed-phase HPLC, stationary phases consisting of chemically modified silica are used with polar mobile phases, eg, methanol: water 50:50. Typically, these chemically modified stationary phases have nonpolar long-chain hydrocarbon groups (eg, an aliphatic chain containing 18 carbons, designated as $C_{18}$) bonded to the surface but many variations have been developed, particularly in recent years. Since these chemically modified stationary phases became available after unmodified silica normal-phase systems had become widely used these systems are called "reversed phase". The long chain organic groups behave like an organic liquid and molecules of the analytes partition between this nonpolar stationary phase and the polar mobile phase. Less polar (or hydrophobic) molecules spend more time in the stationary phase, and hence elute slower, than more polar (or hydrophilic) molecules. This is the reverse of the situation with normal-phase chromatography (Fig. 5).

A wide variety of stationary phases of this type is available, eg, $C_1$, $C_4$, $C_8$, $C_{18}$. Sample retention normally increases as the chain length increases.

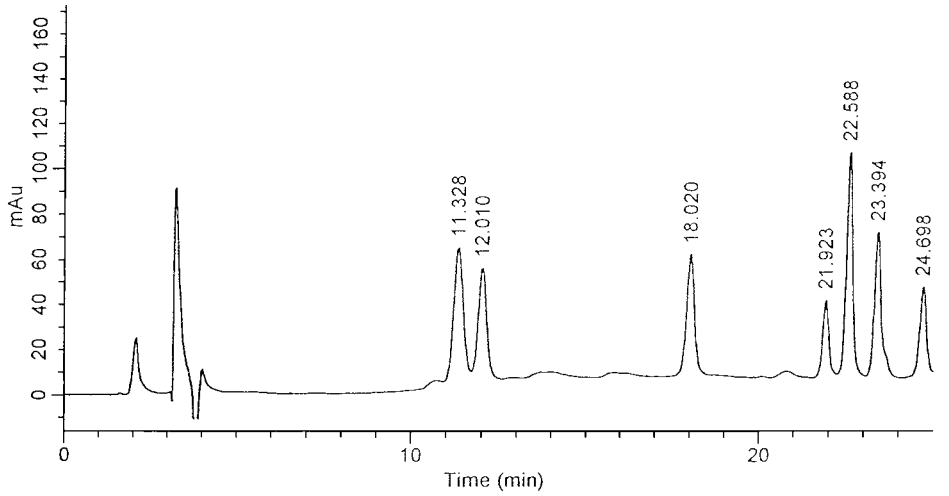


**Fig. 5.** A reversed-phase gradient separation. Determination of ginsenosides in ginseng root powder extract. Conditions: 250 × 4.6 mm 5 μm Phenomenex Hypersil phenyl; mobile-phase acetonitrile:water from 20:80 to 40:60 over 20 min, to 100:0 over 10 min, reequilibrate at initial conditions for 5 min; flow rate 1.0 mL/min; detector uv 202 nm.

However, there tends to be little difference between the longer chains, such as $C_8$ and $C_{18}$ (22). Different brands of the same type of stationary phase (eg, $C_{18}$) are not necessarily interchangeable and may exhibit, in fact, considerable differences.

As the polarity of the mobile phase decreases, ie, with increasing organic content, compounds are eluted more rapidly. The polarity of the mobile phase is adjusted so that compounds do not elute too rapidly, eg, with the unretained peak, or with inconveniently long retention times. If two compounds elute as overlapping peaks the polarity of the mobile phase may be increased and the peaks separated at longer retention times. Compounds of widely different polarity may conveniently be separated by using a mobile phase gradient. During the course of the run the polarity of the mobile phase is decreased, eg, from water: methanol 90:10 to water:methanol 10:90. In this way polar compounds are easily separated from the unretained peak and later on less-polar compounds elute at convenient retention times as the polarity of the mobile phase decreases. However, gradient elution requires a reequilibration period at the initial mobile phase composition when the separation has ended so gradient elution may result in a less efficient use of instrument time than nongradient (isocratic) elution.

Different types of columns, and even columns of the same class from different manufacturers, exhibit different selectivities and this can be used to achieve optimum resolution of all compounds of interest. A more convenient way to change the selectivity of the system may be to change the composition of the mobile phase (23). Retention times depend on the exact composition of the mobile phase and not just on its overall polarity (24) (Fig. 6).

**4.3. Ion-Pair Chromatography.** It is difficult to chromatograph ionic compounds by reversed-phase HPLC because the polar ionic compounds prefer to stay in the polar mobile phase. However, addition of an ion-pair reagent

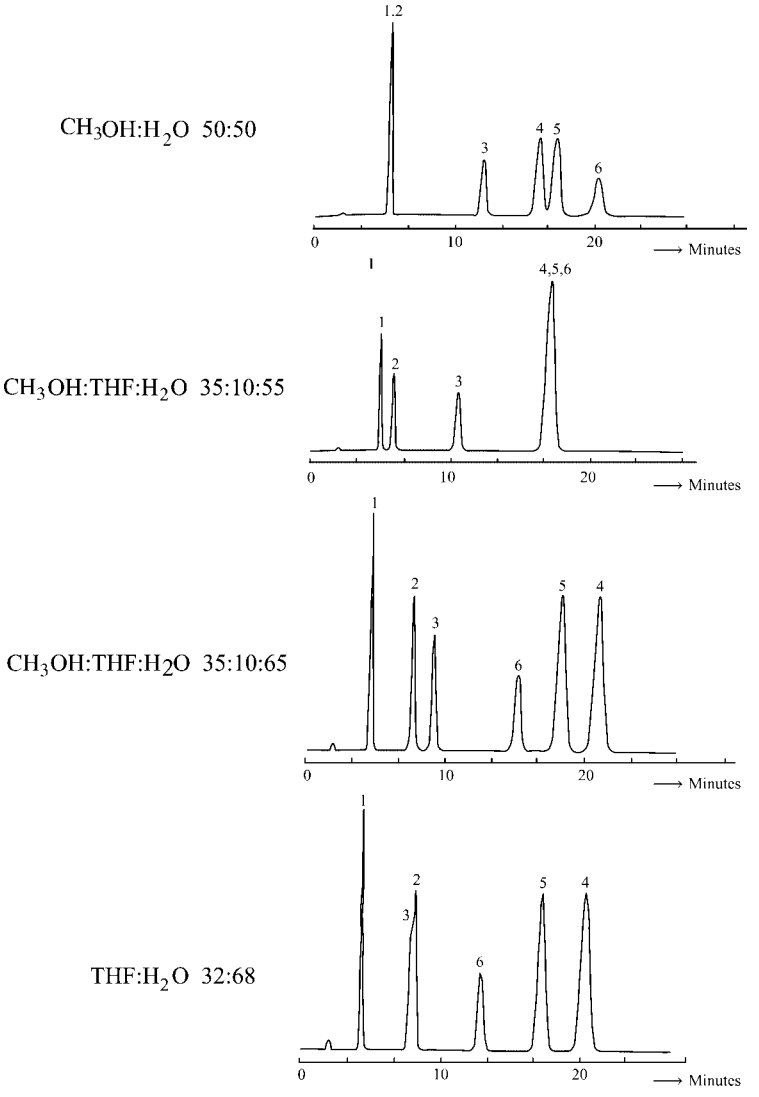


**Fig. 6.** An example of mobile-phase selectivity. A standard mixture of benzyl alcohol [100-51-6] (*1*), phenol [108-95-2] (*2*), 3-phenylpropanol [122-97-4] (*3*), 2,4-dimethylphenol [165-67-93] (*4*), benzene [71-41-2] (*5*), and dimethyl phthalate [131-11-33] (*6*) is separated using the mobile phases shown. The column was a 300 × 4.6-mm Nucleosil 10-RP18 column with a mobile phase flow rate of 1.5 mL/min. Reprinted with permission of Elsevier Science from Ref. 24.

such as a weak acid (eg, heptanesulfonic acid (as the sodium salt) or a weak base (eg, tetrabutylammonium phosphate) leads to the formation of neutral ion-pair between the analyte and the reagent. This neutral ion-pair exhibits the chromatographic behavior of a nonionic organic molecule and may be chromatographed by reversed-phase HPLC like neutral molecules (25). However, ion-exchange mechanisms may also be in effect (26).

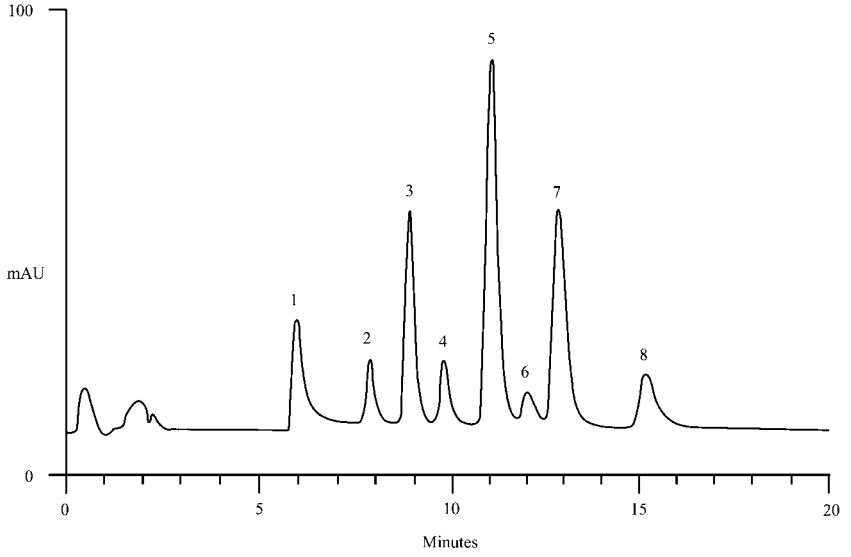


**Fig. 7.** An ion-exchange chromatogram. Solution containing $Fe^{3+}$ (*1*), $Cu^{2+}$ (*2*), $Ni^{2+}$ (*3*), $Zn^{2+}$ (*4*), $Co^{2+}$ (*5*), $Cd^{2+}$ (*6*), $Mn^{2+}$ (*7*), and $Fe^{2+}$ (*8*). Conditions: $250 \times 4$ mm i.d. IonPac CS5A column (Dionex) operated in anion-exchange mode; mobile phase pH 4.2 buffer containing 7 mM pyridine-2,6-dicarboxylic acid, 66 m*M* potassium hydroxide, 5.6 m*M* potassium sulfate, and 74 m*M* formic acid; flow rate 1.0 mL/min; detection at 530 nm following postcolumn reaction with a solution containing 4-(2-pyridylazo)resorcinol pumped at 0.6 mL/min. Reprinted with permission of Elsevier Science from Ref. 27.

**4.4. Ion-Exchange Chromatography.** In ion-exchange chromatography (Fig. 7), charged groups are covalently bound to the stationary phase. The charged ionic analyte is in competition with a counterion of the same charge in the mobile phase. Analytes that interact strongly with the stationary phase will be more retained (ie, elute later) than analytes that interact weakly.

In cation exchange, a positively charged species, $A^+$ (eg, a protonated base) interacts with a stationary phase containing a negatively charged group, $G^-$ (eg, sulfonate and carboxylate). The counterion in the mobile phase might be an alkali metal such as potassium. In anion exchange a negatively charged species, $B^-$ (eg, an ionized acid) interacts with a stationary-phase containing a positively charged group, $G^+$ (eg, quaternary ammonium). The counterion in the mobile phase might be a halogen such as chlorine. Thus (see Fig. 8).

**4.5. Size-Exclusion Chromatography.** Size-exclusion chromatography (SEC) is also known at gel-permeation chromatography (GPC) and involves

$$A^+ + G^-K^+ \rightleftharpoons G^-A^+ + K^+$$

$$B^- + G^+Cl^- \rightleftharpoons G^+B^- + Cl^-$$

**Fig. 8.** Ion-exchange chromatography.

the separation of analytes by size. The column is packed with material that contains pores of a tightly controlled size. Large molecules cannot penetrate the pores and elute first while very small molecules fully penetrate the pores and elute last. Molecules of an intermediate size partially penetrate the pores and elute somewhere in the middle. Columns with smaller pore sizes (eg, 125 Å) are useful for separating molecules of relatively small size while columns with larger pore sizes (eg, 1000 Å) are useful for larger molecules. SEC is particularly useful for synthetic polymers where the different molecules can be separated and for biological molecules such as proteins or oligonucleotides.

**4.6. Chiral Separations.** The two main ways of effecting chiral separations ie, separating the enantiomers of optically active compounds, are derivatization and the use of columns containing chiral stationary phases. Chiral mobile phase additives have been used but such techniques are less common, perhaps because large quantities of expensive additives may be required. In contrast, chiral additives are commonly used in capillary electrophoresis where the quantity of "mobile phase" used in the course of a day is much less.

Derivatization is an indirect method of chiral analysis. Reaction of a chiral reagent (consisting of only one enantiomer) with a racemic mixture of two enantiomers generates two diastereomers that have different physical properties and can be readily separated using reversed-phase HPLC. The advantages are that conventional HPLC equipment can be used. The disadvantages are increased sample preparation time and effort and the possibility that racemization or different reaction yields may occur during derivatization. This does not happen in every case but examples have been reported (28).

The use of a chiral column is a direct method of chiral analysis. A large variety of chiral columns are now available and many different types of compounds may be separated. In all cases, chiral molecules are bonded to the stationary phase. As the racemate moves down the column the enantiomers interact to different extents with the chiral stationary phase, and hence the enantiomers are separated. In some cases, stationary phases that are chemically identical but of opposite chirality are available. Changing the chirality of the stationary-phase changes the order of elution of the analyte enantiomers. This can be important in the analysis of trace amounts of one enantiomer in the presence of large amounts of the other enantiomer, eg, in pharmaceutical applications. The order of elution should be arranged so that the trace enantiomer elutes first since it is much easier to detect small peaks in the baseline before the large peak than in the tail of the large peak.

## 5. Applications

This section focuses on pharmaceutical applications of HPLC as the field in which this separation method is used most. However, other applications are mentioned and the reader is invited to look into the provided list of references to learn more about additional areas of aplication.

**5.1. Pharmaceutical Applications.** HPLC remains the most important chromatographic method for pharmaceutical applications. Immunoassays are also widely employed but they are specific for a single drug. Microbiological

methods are also used for antibiotics but have problems of specificity since active metabolites or other antibiotics will also give a response. Because of its specificity HPLC remains the "gold standard" for drug analysis (29). Although an increasing number of papers have been published on the use of capillary electrophoresis for the analysis of drugs these papers have, so far, been mostly confined to the chromatography literature. There are only a few descriptions of the use of capillary electrophoresis in clinical studies. Gas chromatography (GC) is used in some cases, particularly in forensic investigations and for volatile compounds.

Pharmaceutical applications can be divided into two broad categories: analysis of the drug substance or dosage form and the analysis of drugs in bodily fluids (eg, blood or urine). The drug substance is a chemical substance that contains the active ingredient (eg, aspirin or ibuprofen). The drug substance is often formulated with excipeitns (inactive ingredients) and made into a dosage form (eg, tablets or capsules) that can be consumed by the patient.

*Regulations.* In the United States a drug is required by the Federal Food, Drug, and Cosmetic Act and the drug regulations to meet its specifications by appropriate laboratory testing. The quality of a drug is typically controlled by a drug substance specification and a drug product specification, each of which may include test attributes such as appearance, assay, impurities, or water content. The number and type of these tests will vary for each drug and, in the case of a new drug, are established through the approval of a marketing application by the regulatory agency (ie, FDA).

In the United States the drug standards and analytical procedures found in the U.S. Pharmacopeia (30) are recognized as being official (31) and may be relied upon in legal proceedings. Additionally, analytical procedures, as well as standards, for many drugs are found in other pharmacopeias such as the European, Japanese, or British Pharmacopoeia.

In current practice, the specification for a particular aspect of the quality of a drug consists of three parts: attribute, analytical procedure, and acceptance criterion. For example, the attribute might be the assay, the procedure could be a fully described HPLC method, and the acceptance criterion could be a numerical value, eg, 90.0–110.0% of the amount stated on the label. In this case, the HPLC method should be fully validated. Validation is a complex testing process that ensures that the method gives accurate and reliable results and will continue to do so in the future.

*Drug Substance.* In the case of the drug substance, it is generally desirable to assay the amount of the drug that is present and determine the amounts of process impurities (compounds produced during the synthesis of the drug substance) and degradants (compounds produced as the drug ages). Impurities and degradants together are termed related substances (see Fig. 9). Frequently, the same analytical procedure is used to determine the drug and the related substances but separate procedures are sometimes used. If the related substances have widely different chromatographic properties a gradient or even two separate chromatographic systems may be used. The chiral purity of a drug is frequently determined using a different chromatographic system from that used to determine assay or related substances. Assay and impurities are determined when the drug substance is first manufactured. Selected batches are stored under controlled conditions and tested periodically to assess the stability of the

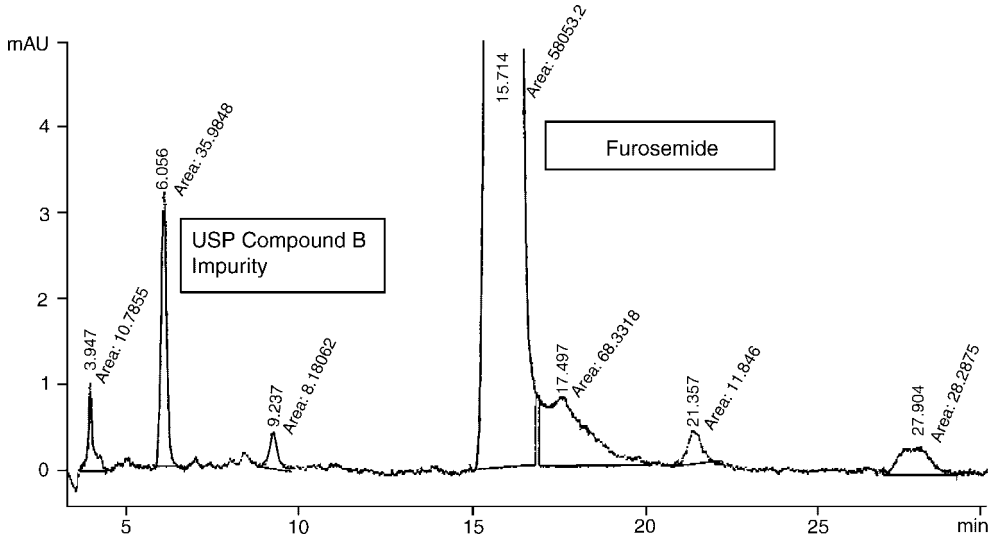


**Fig. 9.** Chromatogram of furosemide [54-31-9] and its impurities including USP Compound B. Conditions: 250 × 4.6 mm Phenomenex Luna (2) C-18 column; mobile phase THF:$H_2O$:acetic acid 35:65:0.1; flow rate 1.0 mL/min; detector uv 272 nm.

drug substance. If at all possible stability-indicating HPLC methods should be used. A stability-indicating assay is a validated quantitative analytical procedure that can detect the changes with time in the pertinent properties of the drug substance and drug product. A stability-indicating assay accurately measures the active ingredients, without interference from process impurities, degradation products, excipients, or other potential impurities. HPLC procedures can also be used to control the manufacturing process, eg, by determining when a reaction is complete. These tests are termed process tests or in-process controls.

*Drug Product.* HPLC methods developed for the analysis of the drug product tend to be similar to those developed for the analysis of the drug substance (see Fig. 10). Indeed, in many cases they are virtually identical. The main difference is that the drug product contains excipients such as microcrystalline cellulose, lactose, magnesium stearate, or gelatin. That contribute to various aspects of the drug product such as disintegration, dissolution, bioavailability, or stability. It is important that the HPLC procedures be such that the excipients do not produce interfering peaks in the chromatogram (32). It may be necessary to test for certain excipients such as preservatives, which may sometimes be done using the same HPLC procedure as used for the assay. The methods should be stability indicating.

Stability testing is also carried out for the drug product and HPLC may be used to determine assay and impurity levels as a function of time. Generally, the same HPLC procedure is used when the drug product is tested at the time of manufacture and when it is tested after storage under controlled conditions.

*Drugs in Biological Fluids.* Many studies involve the determination of drugs in biological fluids (4,33) (see Fig. 11). Although some procedures allow the

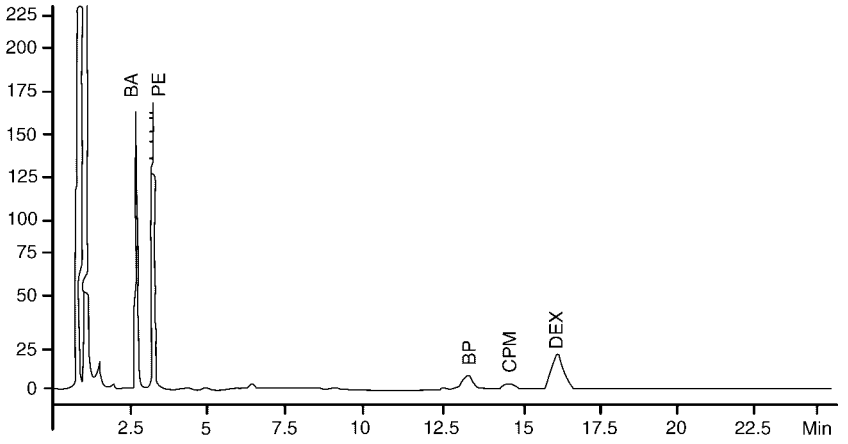


**Fig. 10.** Chromatogram of Children's Tylenol Flu suspension liquid. Peaks are benzoic acid (BA, a preservative) [65-85-0], pseudoephedrine [90-82-4] (PE, a nasal decongestant), butylparaben (BP, a preservative) [94-26-8], chlorpheniramine (CPM, an antihistamine) [132-22-9], and dextromethorphan [125-69-9] (DEX, an antitussive). Conditions: 75 × 4.6 mm 3-μm Phenomenex Luna C8(2) column; mobile-phase acetonitrile:methanol:THF: buffer 5:42:2:51, buffer was 100 m*M* pH 2.1 sodium phosphate buffer containing 50 m*M* sodium 1-octanesulfonate; flow rate 1 mL/min; detector UV 214 nm. Reprinted with permission of Elsevier Science from Ref. 32.

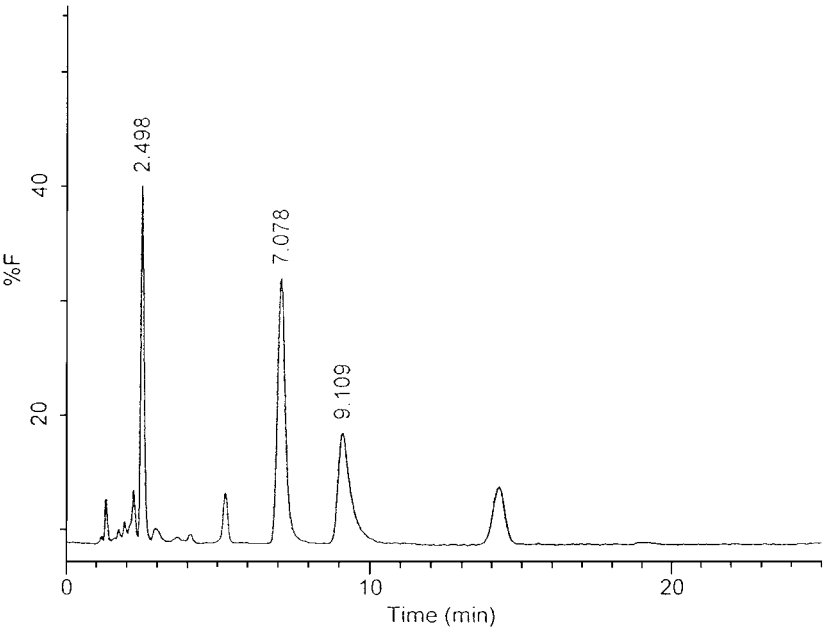


**Fig. 11.** Determination of metoprolol in human plasma after sample preparation using solid-phase extraction. Peaks are α-hydroxymetoprolol [563920-16-67] (2.498 min), metoprolol [37350-58-6] (7.7078 min), and dextrorphan [125-23-5] (9.109 min (internal standard)). Conditions: 150 × 4.6 mm Metachem C4 column; mobile phase acetonitrile: THF:20 m*M* pH 3.0 phosphate buffer 15:2:83; flow rate 1.75 mL/min; detector fluorescence 228 nm (excitation) 320 nm (emission).

direct injection of biological fluids, sample preparation is generally critical. The concentrations of drugs in biological fluids are generally very low, in the order of nanograms per milliliter or lower, so much effort has been expended in increasing the sensitivity and selectivity of HPLC detectors. Additionally, the sensitivity and selectivity of the analytical system can be increased by the use of a derivatization procedure and by use of an appropriate sample preparation procedure. These issues have been discussed above.

The main biological fluids that are investigated are blood and urine. Some procedures describe the analysis of whole blood but generally the blood is processed to produce plasma (by immediately centrifuging whole blood) or serum (by allowing the drug to clot then centrifuging). Generally, the plasma or serum still contain the drug and are easier to work with. Analyses of drugs in many other biological matrices have been reported, however. Some examples are tissue of various kinds, feces, hair, saliva, sweat, semen, cerebrospinal fluid, and nails. Although some matrices are "cleaner" than others a sample preparation step is generally required. For the same drug the procedure may vary depending on the matrix. Many drugs are excreted in the urine as a glucuronidate or sulfate. If desired, an enzyme preparation can be used to regenerate the parent drug prior to HPLC analysis.

Measurement of drug levels in biological fluids can be used to determine the pharmacokinetics of a drug, ie, the variation of drug level with time. This yields much important information such as how frequently the drug needs to be dosed or how drugs may interact with one another. Various metabolites may also be identified and the racemization of chiral drugs in the body may be investigated.

Drugs such as antibiotics are frequently administered to animals that are to be used for food. Procedures for the analysis of drugs in tissue can be used to make sure that the drug has completely disappeared before the animal is slaughtered for human consumption.

*Cassette Dosing.* Historically, drug discovery proceeded by the synthesis of one compound at a time. Compounds were then screened and analytical systems devised for those molecules that showed promise. The advent of combinatorial chemistry and high-throughput techniques has greatly increased the number of compounds that may be synthesized and screened. Now a bottleneck has become the difficulty of devising analytical methods to obtain pharmacokinetic and metabolite information for the greatly increased number of compounds. Additionally, companies are coming under increasing pressure to reduce the number of animals used in testing. To help overcome these problems, a technique called cassette dosing or "N-in-1" dosing has been developed. A number of compounds (eg, 10) are administered to one animal. Blood samples are taken, processed using standard automated techniques, and samples injected into an HPLC instrument equipped with an MS detector (39). A "universal" isocratic or gradient mobile phase is used that will chromatograph most compounds. Different compounds are distinguished by scanning for ions of different mass. Before conducting the experiment, computer programs are used to make sure that different compounds are not likely to give rise to metabolites that have the same mass. In this way, the pharmacokinetics of many compounds can be assessed rapidly using a smaller number of experimental animals and with shortened assay times (34–36). However, this technique is controversial because

drug–drug interactions can invalidate the results, eg, if one of the compounds is a potent inhibitor of a drug-metabolizing enzyme (37). A way to avoid the problems of drug–drug interactions is to dose one compound per animal then combine plasma samples from a number of animals and analyze the composite sample (38). However, this technique yields no reduction in the number of animals required and, as plasma samples are combined, the concentration of each drug decreases.

In a similar fashion, the power of HPLC–MS can also be used to eliminate bottlenecks in the investigation of the metabolites produced by isolated enzyme preparations acting on various substrates such as new drug candidates. In each reaction, a sample of the enzyme preparation (eg, liver microsomes) metabolizes only one compound but each reaction involves a different compound. Although it is easy to run many reactions at the same time, the expense of the analytical equipment usually causes analyses to proceed sequentially. However, when HPLC–MS is used a number of these reaction mixtures can be combined before analysis. The different compounds are then readily resolved and the extent of the metabolism of each substrate determined. Since only one compound is involved in each reaction there is no question of drug–drug interaction (40) (see Fig. 12).

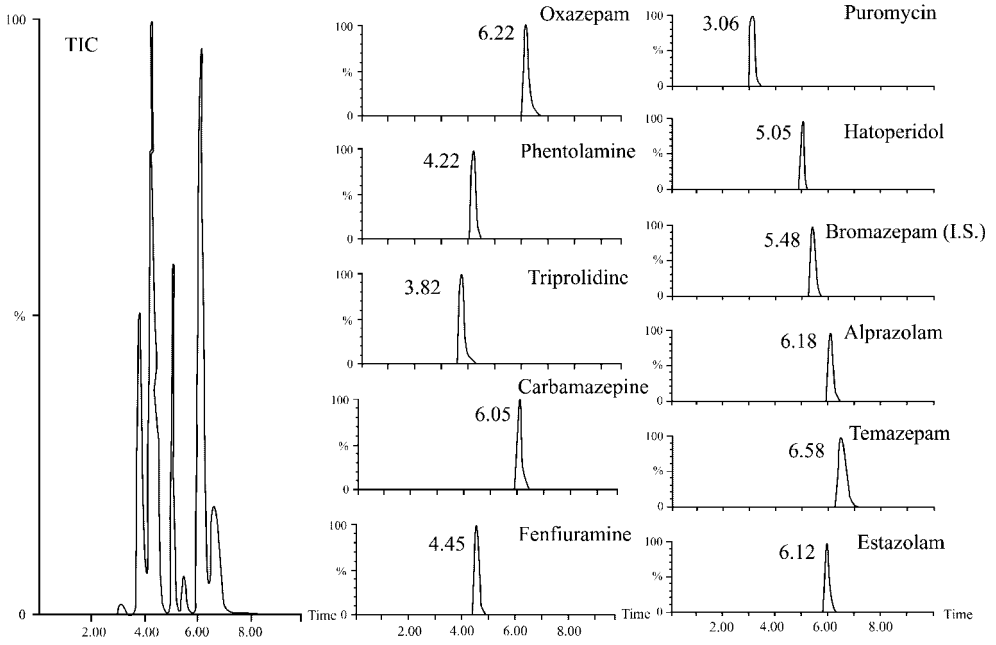


**Fig. 12.** Multiple compound determination. A sample of chimpanzee plasma was injected into a column-switching HPLC–MS–MS instrument. The column was a 150 × 2.0-mm Symmetry C18 (Waters) and the mobile phase was an acetonitrile/0.1% formic acid gradient pumped at 0.4 mL/min. The total ion chromatogram shows that the compounds overlap and would be hard to distinguish using a conventional (eg, uv) detector. However, the MS detector in multiple-reaction-monitoring mode can be used to monitor the distinctive ions of each compound and provide complete resolution as shown. Reprinted with permission of the American Chemical Society from Ref. 39.

Table 1. **Some Probe Compounds and the HPLC Conditions Associated with Them**[a,b]

| Enzyme | Substrate | CAS Registry number | Metablite | CAS Registry number | Column | Mobile phase | Detector |
|---|---|---|---|---|---|---|---|
| CYP1A1/2 | phenacetin | [62-44-2] | acetaminophen | [103-90-2] | Zorbax phenyl | $CH_3OH$:pH 3.5 buffer gradient | uv 254 |
| CYP2A6 | coumarin | [91-64-5] | 7-hydroxy-coumarin | [93-35-6] | NovaPak C18 | $CH_3OH$:1% acetic acid 35:65 | F ex 371 em 454uv |
| CYP2C8/9 | tolbutamide | [64-77-7] | 4-hydroxy-tolbutamide | [5719-85-7] | NovaPak C18 | $CH_3CN$:pH 4.3 buffer gradient | uv 237 |
| CYP2C19 | S-mephenytoin | [58-12-4] | 4-hydroxy-mephenytoin | [61837-65-8] | NovaPak C18 | $CH_3OH$/pH 5 buffer gradient | uv 204 |
| CYP2D6 | dextromethorphan | [125-69-9] | dextrorphan | [125-73-5] | Zorbax C18 | $CH_3CN$:1 mM perchloric acid 25:75 | F ex 270 em 312 |
| CYP2E1 | chlorzoxazone | [95-25-0] | 6-hydroxy-chlorzoxazone | [1750-45-4] | Zorbax C18 | $CH_3CN$:0.05% phosphoric acid gradient | uv 287 |
| CYP3A4 | testosterone | [58-22-0] | 6β-hydroxy-testosterone | [62-99-9] | Supelcosil LC18 | $CH_3OH$:$CH_3CN$:$H_2O$ gradient | uv 254 |

[a] Ref. 42.

[b] The flow rate was 1 mL/min except for phenacetin [62-44-2] (1.5 mL/min) and tolbutamide [64-77-7] (2 mL/min). Abbreviations:$CH_3OH$, methanol; uv, ultraviolet detector (with wavelength in nm); F, fluorescence detector (with excitation and emission wavelengths in nm); and $CH_3CN$, acetonitrile.

*Hepatic Drug Metabolism.* Many drugs and other xenobiotic compounds (such as industrial compounds and nonmedicinal natural products) are metabolized by enzymes in the liver. Enzyme activity may be measured by using probe compounds that are predominantly metabolized by a single enzyme (41). HPLC is used to measure the extent of metabolism of these probe compounds, and hence the extent and type of enzyme activity (see Table 1).

Much useful information can be derived from the use of these probe compounds. For example, inhibitors of various enzymes can be determined because they will slow the metabolism of the probe compound. Drug interactions can then be predicted since a drug that inhibits a particular enzyme will lead to increased blood levels of other drugs that are metabolized by that enzyme. Equally, an inducer of a particular enzyme will lead to decreased levels of compounds that are metabolized by that enzyme.

In an interesting extension of the cassette-dosing strategy five probe compounds (coumarin [91-64-5], midazolam [59467-70-83], tolbutamide [64-77-7], dextromethorphan [125-69-9], and chlorzoxazone [95-25-0]) and a test compound were added to a human liver microsomal preparation. Injection of the reaction mixture into an LC–MS instrument using a short $4 \times 2$-mm Phenomenex C18 column with an acetonitrile/water/formic acid gradient led to the determination of the level of the metabolite of each probe compounds and hence the extent of the inhibitory effect of the test compound on each enzyme. Since the probe compounds do not produce metabolites with interfering ions the high resolving power of the LC–MS can generate this information in one short (2.5 min) run rather than five longer individual HPLC assays (43).

**5.2. Nonpharmaceutical Clinical Applications.** It is frequently desirable to measure compounds other than drugs in biological fluids. Generally, the procedures are similar to those used to measure drugs in biological fluids. In some cases, these compounds may indicate the presence of certain diseases. For example, measurement of cysteine in plasma by HPLC (after derivatization) can show the metabolic disorder cystinuria (44). HPLC methods have been developed for many other clinically relevant analytes such as amino acids, lipids, or proteins (45).

**5.3. Environmental Applications.** Many methods have been published in the literature for the analysis of pollutants in the environment (46). Generally, the main problem is that of sensitivity and so solid-phase extraction is used extensively. The U.S. Environmental Protection Agency (EPA) has published a number of methods. A list of all EPA test methods is available at http://www.epa.gov/epahome/index/ and methods for organic chemical analysis are found at http://www.epa.gov/ostwater/Tools/guide/methods.html.

**5.4. Other Applications.** HPLC has found applications in many other areas. Some examples are shown below

| | |
|---|---|
| Art conservation | S. L. Vallance, *Analyst* **122**, 75R (1997) |
| Color additives | W. Horwitz, ed., Official Methods of Analysis of AOAC International, 17th ed., AOAC International, Arlington, Va., 2000 |
| Explosives | W. Horwitz, ed., Official Methods of Analysis of AOAC International, 17th ed., AOAC International, Arlington, Va., 2000 |

| | |
|---|---|
| Fertilizers | W. Horwitz, ed., Official Methods of Analysis of AOAC International, 17th ed., AOAC International, Arlington, Va., 2000 |
| Food | Current Protocols in Food Analytical Chemistry, John Wiley & Sons, Inc., New York |
| Food | W. Horwitz, ed., Official Methods of Analysis of AOAC International, 17th ed., AOAC International, Arlington, Va., 2000 |
| Food additives | W. Horwitz, ed., Official Methods of Analysis of AOAC International, 17th ed., AOAC International, Arlington, Va., 2000 |
| Forensic science | T.A. Brettell, K. Inman, N. Rudin, and R. Saferstein, *Anal. Chem*., **73**, 2735 (2001) |
| Industrial processes | J. Workman, Jr., D. J. Veltkamp, S. Doherty, B. B. Anderson, K. E. Creasy, M. Koch, J. F. Tatera, A. L. Robinson, L. Bond, L. W. Burgess, G. N. Bokerman, A. H. Ullman, G. P. Darsey, F. Mozayeni, J. A. Bamberger, and M. S. Greenwood, *Anal. Chem*., **71**, 121R (1999) |
| Infant formula | W. Horwitz, ed., Official Methods of Analysis of AOAC International, 17th ed., AOAC International, Arlington, Va., 2000 |
| Molecular biology | Current Protocols in Molecular Biology, John Wiley & Sons, Inc., New York |
| Natural toxins | W. Horwitz, ed., Official Methods of Analysis of AOAC International, 17th ed., AOAC International, Arlington, Va., 2000 |
| Pesticides | W. Horwitz, ed., Official Methods of Analysis of AOAC International, 17th ed., AOAC International, Arlington, Va., 2000 |
| Petroleum | C. T. Mansfield, B. N. Barman, J. V. Thomas, A. K. Mehrota, and J. M. McCann, *Anal. Chem*. **71**, 81R (1999) |
| Polymers | P. B. Smith, A. J. Pasztor, Jr., M. L. McKelvy, D. M. Meunier, S. W. Froelicher, and F. C.-Y. Wang, *Anal. Chem*. **71**, 61R (1999) |
| Vitamins | W. Horwitz, ed., Official Methods of Analysis of AOAC International, 17th ed., AOAC International, Arlington, Va., 2000 |
| Water quality | S. D. Richardson, *Anal. Chem*. **73**, 2719 (2001) |

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The opinions expressed in this article are those of the author and do not necessarily reflect the views or policies of the FDA.

## BIBLIOGRAPHY

1. *FDA Guideline for Submitting Samples and Analytical Data for Methods Validation*, Appendix C, February 1987.

2. L. R. Snyder, J. J. Kirkland, and J. L. Glajch. *Practical HPLC Method Development*, 2nd ed., John Wiley & Sons, Inc., New York, 1997, pp. 110–119.
3. V. Meyer, *Anal. Chem.* **69**, 18A (1997).
4. G. Lunn and N. R. Schmuff, *HPLC Methods for Pharmaceutical Analysis*, Vols. 1–4, John Wiley & Sons, Inc., New York, 1997–2000.
5. T. C. Pinkerton, *J.Chromatogr.* **544**, 13 (1991).
6. A. Shibukawa, C. Nakao, T. Sawada, A. Terakita, N. Morokoshi, and T. Nakagawa, *J. Pharm. Sci.* **83**, 868 (1994).
7. E. Pocurull, R. M. Marcé, and F. Borrull, *Chromatographia* **41**, 521 (1995).
8. R. Herráez-Hernández, N. C. Van der Merbel, and U. A. T. Brinkman, *J. Chromatogr. B* **666**, 127 (1995).
9. C. H. Kiang, T. Nolan, B. L. Huang, and C. P. Lee, *J. Chromatogr.* **567**, 195 (1991).
10. K. Blau and J. Halket, eds., *Handbook of Derivatives for Chromatography*, 2nd ed., John Wiley & Sons, Inc., Chichester, U.K., 1993.
11. G. Lunn and L. C. Hellwig, *Handbook of Derivatization Reactions for HPLC*, John Wiley & Sons, Inc., New York, 1998.
12. M. Y. Khuhawar and S. N. Lanjwani. *J. Chromatogr. A* **740**, 296 (1996).
13. W. R. LaCourse and C. O. Dasenbrock, *Adv.Chromatogr.* **38**, 189 (1998).
14. A. C. Calokerinos, N. T. Deftereos, and W. R. G. Baeyens, *J. Pharm. Biomed. Anal.* **13**, 1063 (1995).
15. O. Busto, J. Guasch, and F. Borrull, *J. Chromatogr. A* **718**, 309 (1995).
16. T. Takagi, T. G. Chung, and A. Saito, *J. Chromatogr.* **272**, 279 (1983).
17. J. Haginaka, J. Wakai, H. Yasuda, T. Uno, K. Takahashi, and T. Katagi, *J. Chromatogr.* **400**, 101 (1987).
18. A. M. Di Pietra, R. Gatti, V. Andrisano, and V. Cavrini, *J. Chromatogr. A* **729**, 355 (1996).
19. M. Uihlein and E. Schwab, *Chromatographia* **15**, 140 (1982).
20. D. Chaput, *J. Assoc. Off. Anal. Chem.* 71, 542 (1988).
21. R. E. Wrolstad, ed., *Current Protocols in Food Analytical Chemistry*, John Wiley & Sons, Inc., New York, 2001, p. F2.3.12.
22. L. R. Snyder, J. J. Kirkland, and J. L. Glajch. *Practical HPLC Method Development*, 2nd ed., John Wiley & Sons, Inc., New York, 1997, p. 193.
23. P. C. Sadek, *The HPLC Solvent Guide*, John Wiley & Sons, Inc., New York, 1996.
24. P. J. Schoenmakers, H. A. H. Billiet, and L. de Galan. *J. Chromatogr.* **218**, 261 (1981).
25. V. R. Meyer, *Practical High-Performance liquid Chromatography*, 3rd ed., John Wiley & Sons, Inc., Chichester, U.K., 1998, pp. 184–190.
26. L. R. Snyder, J. J. Kirkland, and J. L. Glajch, *Practical HPLC Method Development*, 2nd ed., John Wiley & Sons, Inc., New York, 1997, pp. 317–341.
27. N. Cardellicchio, P. Ragone, S. Cavalli, and J. Riviello, *J. Chromatogr. A* **770**, 185 (1997).
28. M. Vakily and F. Jamali, *J. Pharm. Sci.* **85**, 638 (1996).
29. P. M. Jones and K. Brune, *Clin. Chem.* **39**, 168 (1993).
30. *United States Pharmacopeia*, 24th Revision/National Formulary XVIII, United States Pharmacopeial Convention, Inc., Rockville, Md., 2000.
31. Federal Food, Drug, and Cosmetic Act, Section 501 (b); Title 21 Code of Federal Regulations 211.194(a)(2) and 314.50(d)(1).
32. M. D. Paciolla, S. A. Jansen, S. A. Martellucci, and A. A. Osei, *J. Pharm. Biomed. Anal.* **26**, 143 (2001).
33. R. S. Plumb, G. J. Dear, D. N. Mallett, D. M. Higton, S. Pleasance, and R. A. Biddlecombe, *Xenobiotica* **31**, 599 (2001).
34. J. Berman, K. Halm, K. Adkison, and J. Shaffer. *J. Med. Chem.* **40**, 827 (1997).

35. J.-T. Wu, H. Zeng, M. Qian, B. L. Brogdon, and S. E. Unger. *Anal. Chem.* **72**, 61 (2000).
36. J. E. Shaffer, K. K. Adkison, K. Halm, K. Hedeen, and J. Berman, *J. Pharm. Sci.* **88**, 313, (1999).
37. R. E. White and P. Manitpisitkul., *Drug Metab. Dispos.* **29**, 957 (2001).
38. Z. Cai, C. Han, S. Harrelson, E. Fung, and A. K. Sinhababu, *Rapid Commun. Mass Spectrom.* **15**, 546 (2001).
39. J.-T. Wu, H. Zeng, M. Qian, B. L. Brogdon, and S. E. Unger. *Anal. Chem.* **72**, 61 (2000).
40. Z. Cai, A. K. Sinhababu, and S. Harrelson, *Rapid Commun. Mass Spectrom.* **14**, 1637 (2000).
41. S. A. Wrighton, M. Vandenbranden, J. C. Stevens, L. A. Shipley, and B. J. Ring, *Drug Metab. Rev.* **25**, 453 (1993).
42. N. Chauret, A. Gauthier, J. Martin, and D. A. Nicoll-Griffith, *Drug Metab. Dispos.* **25**, 1130 (1997).
43. H.-Z. Bu, L. Magis, K. Knuth, and P. Teitelbaum, *Rapid Commun. Mass Spectrom.* **15**, 741 (2001).
44. A. Pastore, R. Massoud, C. Motti, A. Lo Russo, G. Fucci, C. Cortese, and G. Federici, *Clin.Chem.* **44**, 825 (1998).
45. D. J. Anderson, *Anal. Chem.* **71**, 314R (1999).
46. R. E. Clement and P. W. Yang, *Anal. Chem.* **73**, 2761 (2001).

## GENERAL REFERENCES

R. L. Cunico, K. M. Gooding, and T. Wehr, *Basic HPLC and CE of Biomolecules*, Bay Bioanalytical Laboratory, Hercules, Calif., 1998.
V. R. Meyer, *Practical High-Performance Liquid Chromatography*, 3rd ed., John Wiley & Sons, Inc., Chichester, U.K., 1998.
U. D. Neue, *HPLC Columns : Theory, Technology, and Practice*, John Wiley & Sons, Inc., New York, 1997.
P. C. Sadek, *Troubleshooting HPLC Systems: A Bench Manual*, John Wiley & Sons, Inc., New York, 2000.
L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, 2nd ed., John Wiley & Sons, Inc., New York, 1979.
L. R. Snyder, J. J. Kirkland, and J. L. Glajch, *Practical HPLC Method Development*, 2nd ed., John Wiley & Sons, Inc., New York, 1997.
J. Swadesh, ed., *HPLC: Practical and Industry Chromatography*, 2nd ed., CRC Press, Boca Raton, Fla., 2000.
Q. A. Xu and L. A. Trissel, *Stability-Indicating HPLC Methods for Drug Analysis*, American Pharmaceutical Association, Washington, D.C., 1999.
*Analyst* http://www.rsc.org
*Analytica Chimica Acta* http://www.elsevier.nl
*Analytical Chemistry* http://pubs.acs.org
*Analytical Letters* http://www.dekker.com
*Biomedical Chromatography* http://www.wiley.com
*Chromatographia* http://www.elsevier.nl
*Journal of AOAC International* http://www.aoac.org
*Journal of Analytical Toxicology* http://www.jatox.com/
*Journal of Chromatographic Science* http://www.j-chrom-sci.com/
*Journal of Chromatography A* http://www.elsevier.nl

*Journal of Chromatography B Biomedical Sciences and Applications* http://www.elsevier.nl
*Journal of Liquid Chromatography and Related Technologies* http://www.dekker.com
*Journal of Pharmaceutical and Biomedical Analysis* http://www.elsevier.nl
*Journal of Pharmaceutical Sciences* http://www.wiley.com
*LC.GC Magazine* http://www.lcgcmag.com/
*Pharmaceutical Research* http://www.aapspharmaceutica.com
*Therapeutic Drug Monitoring* http://www.drug-monitoring.com

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