

1.14.4 High-performance liquid chromatography

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Introduction

High-performance liquid chromatography (HPLC) is a separation technique that can be used for the analysis of organic molecules and ions. HPLC is based on mechanisms of adsorption, partition and ion exchange, depending on the type of stationary phase used. HPLC involves a solid stationary phase, normally packed inside a stainless-steel column, and a liquid mobile phase. Separation of the components of a solution results from the difference in the relative distribution ratios of the solutes between the two phases.

HPLC can be used to assess the purity and/or determine the content of many pharmaceutical substances. It can also be used to determine enantiomeric composition, using suitably modified mobile phases or chiral stationary phases. Individual separation mechanisms of adsorption, partition and ion exchange rarely occur in isolation since several principles act to a certain degree simultaneously.

Apparatus

The apparatus consists of a pumping system, an injector, a chromatographic column, stationary and mobile phases, connecting tubing and fittings, a detector and a data collection device (computer, integrator or recorder).

Pumping system

HPLC pumping systems are required to deliver metered amounts of mobile phase at a constant flow rate. Pumping systems that deliver solvent from one or more reservoirs are available. Pressure fluctuations should be minimized, e.g. by passing the pressurized solvent through a pulse-dampening device. Tubing and connections should be capable of withstanding the pressures developed by the pumping system. Many HPLC pumps are fitted with a facility for "bleeding" the system of entrapped air bubbles.

Computer- or microprocessor-controlled pumping systems are capable of accurately delivering a mobile phase of either constant (isocratic elution) or varying (gradient elution) composition, according to a defined programme. In the case of gradient elution, solvent mixing can be achieved on either the low- or high-pressure side of the pump(s).

Depending on a number of factors including column dimensions, particle size of the stationary phase, the flow rate and composition of the mobile phase, operating pressures of up to 42 000 kPa (about 6000 psi) can be generated.

Injector

The sample solution is usually introduced into the flowing mobile phase at or near the head of the column using an injection system based on an injection valve design which can operate at high pressure. Such an injection system has a fixed-loop or a variable volume device which can be operated manually or by an auto-sampler. Partial filling of loops may lead to poorer injection volume precision.

Chromatographic column

Columns are usually made of polished stainless steel, are between 50 and 300 mm long and have an internal diameter of between 2 and 5 mm. They are commonly filled with a stationary phase with a particle size of 3–10 μm . Columns with internal diameters of less than 2 mm are often referred to as microbore columns. Ideally the temperature of the mobile phase and the column should be kept constant during an analysis. Most separations are performed at ambient temperature but columns may be heated using, for instance, a water-bath, a heating block or a column oven in order to achieve better efficiency.

Stationary phases

Separation of pharmaceuticals is usually achieved by partition of compounds in the test solution between the mobile and the stationary phases. HPLC systems consisting of polar stationary phases and non-polar mobile phases are described as normal-phase chromatography; those with non-polar stationary phases and polar mobile phases are called reversed-phase chromatography.

There are many types of stationary phases used in HPLC including:

- unmodified silica, alumina or porous graphite, used in normal-phase chromatography, where separation is based on differences in adsorption;
- a variety of chemically-modified supports prepared from polymers, silica or porous graphite, used in reversed-phase HPLC, where separation is based principally on partition of the molecules between the mobile phase and the stationary phase;
- resins or polymers with acid or basic groups, used in ion-exchange chromatography, where separation is based on competition between the ions to be separated and those in the mobile phase;
- porous silica or polymers.

Most separations are based on partition mechanisms using chemically-modified silica as the stationary phase and polar solvents

as the mobile phase (reversed-phase HPLC). The surface of the support, e.g. the silanol groups of silica, is reacted with various silane reagents to produce covalently bonded silyl derivatives covering a varying number of active sites on the surface of the support. The nature of the bonded phase is an important parameter for determining the separation properties of the chromatographic system.

Commonly used bonded phases are shown below.

Common bonded phases

octyl	$\text{Si}-(\text{CH}_2)_7-\text{CH}_3$	C_8
octadecyl	$\text{Si}-(\text{CH}_2)_{17}-\text{CH}_3$	C_{18}
phenyl	$\text{Si}-(\text{CH}_2)_3-\text{C}_6\text{H}_5$	C_6H_5
cyanopropyl	$\text{Si}-(\text{CH}_2)_3-\text{CN}$	CN
aminopropyl	$\text{Si}-(\text{CH}_2)_3-\text{NH}_2$	NH_2
diol	$\text{Si}-(\text{CH}_2)_3-\text{OCH}(\text{OH})-\text{CH}_2-\text{OH}$	

For the separation of enantiomers, special chemically-modified or coated stationary phases (chiral chromatography) are available, which contain usually one of the following structures as a chiral selector: polymeric structures including proteins, polysaccharide, crown ethers, cyclodextrins or macrocyclic glycopeptides. The enantiomers of a chiral test substance may differ in their affinity to the chiral selector and, therefore, may be retained differently by the stationary phase.

As a guide silica-based, reversed-phase columns are generally considered to be stable in mobile phases with an apparent pH in the range 2.0–8.0, but the column manufacturer's instructions should be consulted before using the column. Columns containing particles of polymeric materials such as styrene-divinylbenzene copolymer are stable over a wider pH range.

Analysis using normal-phase HPLC with unmodified silica, porous graphite or polar chemically-modified silica (e.g. cyanopropyl or diol) as the stationary phase and a non-polar mobile phase is employed in certain cases.

For analytical separations the particle size of the most commonly used stationary phases varies between 3 µm and 10 µm. The particle shape may be spherical or irregular, of different porosities and specific surface area. In the case of reversed phase, the extent of bonding of the stationary phase is expressed as the carbon-loading. Furthermore, stationary phases may be "end-capped", i.e. the number of residual silanol groups is reduced by methylation. These parameters contribute to the chromatographic behaviour of a particular stationary phase. Tailing of peaks, particularly for basic substances, can occur when residual silanol groups are present.

The selectivity of chromatographic tests may not only depend on the parameters mentioned in the methods' descriptions but also on the material of the used stationary phases. *The International Pharmacopoeia* thus provides on its web page (see <http://www.who.int/medicines/publications/pharmacopoeia>) a list of trade names of the stationary phases that were found to be suitable when the monographs were being developed. This information is provided solely for the convenience of users. The mentioned suppliers are neither endorsed nor recommended by *The International Pharmacopoeia* nor does the information imply any preference to other stationary phases of a similar nature which are not mentioned. The user should also be aware of the fact that some stationary phases can show significant batch-to-batch variations.

Mobile phases

The choice of mobile phases is based on the desired retention behaviour and the physicochemical properties of the analyte as well as the type of detector chosen.

For normal-phase HPLC using unmodified stationary phases lipophilic solvents should be employed. The presence of water in the mobile phase must be avoided as this will reduce the efficiency of the stationary phase. In reversed-phase HPLC aqueous mobile phases, with and without organic modifiers, are used.

The mobile phase should be filtered through suitable membrane-type filters to remove particles or undissolved material. Multicomponent mobile phases should be prepared by measuring the required volumes (unless masses are specified) of the individual components, followed by manual or mechanical mixing. Alternatively, the solvents may be delivered by the individual pumps or proportioning valves of the liquid chromatograph and mixed according to the desired proportion. Solvents are normally degassed by sparging with helium or by means of sonification before pumping to avoid the formation of gas bubbles in the detector cell.

If a spectrophotometric detector is employed the solvents used for the preparation of the mobile phase should be transparent at the wavelength of detection, especially when the test method prescribes a low measurement wavelength. The analyst has to make sure that the solvent grade used meets this requirement. Adjustment of the pH, if necessary, should be made in the

aqueous component of the mobile phase. If the adjustment is made on the mixture the resulting pH is called "apparent pH" and is far less reproducible. Buffers of high molarity should be avoided in the preparation of mobile phases. If buffers are used the system may be rinsed with an adequate mixture of water and the organic modifier of the mobile phase to prevent crystallization of salts.

Mobile phases may contain other components, e.g. a counter-ion for ion-pair chromatography or a chiral selector for chiral chromatography using an achiral stationary phase.

Connecting tubing and fittings

The full efficiency of an analytical column may never be achieved because of the design limitations of pumps, injectors and detectors. The connections between injector/column, column/detector and/or detector/detector may compromise the overall efficiency of the system. Any fittings should be of the "zero dead volume" (ZDV) type. It is recommended that minimum lengths of capillary tubing with a maximum internal diameter of 0.25 mm be used for these fittings to minimize band broadening.

Detectors

Ultraviolet/visible (UV/vis) absorption spectrophotometers are commonly used detectors in pharmaceutical analysis. In specific cases fluorescence spectrophotometers, differential refractometers (RI), electrochemical detectors, evaporative light-scattering detectors (ELSD), charged aerosol detectors (CAD), mass spectrometers (MS) or other special detectors may be used. Where an analyte possesses a chromophore that absorbs UV/vis radiation, the UV/vis detector is the first choice because of its favourable signal to noise ratio. Such a detector is not suitable for detecting analytes with very weak chromophores.

A variant on the UV/vis type of detector, which can furnish detailed spectral information, is the diode array spectrophotometer. This type of detector acquires absorbance data over a certain UV/vis range and can provide chromatograms at multiple, selectable wavelengths, together with spectra for the eluted peaks. In addition, the detector and accompanying computer programs can be used to assess the spectral homogeneity of peaks, which may provide information on the chromatographic purity of the peaks. This can be especially useful in method development and validation.

Enhanced sensitivity may be achieved in certain cases by using pre-column or post-column derivatization techniques. (These techniques are normally not used for the control of impurities.)

Data collection devices

Signals from the detector may be collected on chart recorders or electronic integrators that vary in complexity and in their ability to process, store and reprocess chromatographic data. The data storage capacity of these devices is usually limited.

Modern data stations are computer-based and have a large storage capacity to collect, process and store data for possible subsequent reprocessing.

Integration of peak areas and the setting of threshold levels are not normally problematic in an assay since the peak of the substance to be analysed should be free of interference. However, in a test for impurities, the selection of the peak area integrator parameters becomes very important, particularly when baseline separations are not always attainable. If baseline separations cannot be obtained, valley-to-valley integration should be employed for peaks of similar size, whereas for peaks of very different size tangent skimming is recommended.

HPLC allows limits to be set for individual impurities and for the sum of impurities, but there must be a threshold below which peaks should not be integrated. This "disregard level" or "reporting threshold" is set in relation to the area of the peak in the chromatogram of the prescribed reference solution and is usually equivalent to 0.1% or 0.05% of the substance being examined.

Dwell volume (gradient delay volume, D)

The equipment employed for gradient methods may significantly alter the resolution, retention time and relative retentions described in monographs. Should this occur it may be due to excessive dwell volume. The dwell volume is the volume between the point at which the eluents meet and the top of the column. It may vary markedly depending on the solvent mixing device employed and the connecting tubing and fittings used and should be taken into account when describing the elution conditions. It can be determined using the following procedure.

Replace the chromatographic column by an appropriate capillary tubing (e.g. 1 m × 0.12 mm).

Use the following conditions for gradient elution:

Mobile phase A: water R.

Mobile phase B: 1 volume of acetone R and 999 volumes of water R.

Time	Mobile phase A	Mobile phase B	Comments

(min)	(% v/v)	(% v/v)	
0–20	100 to 0	0 to 100	Linear gradient
20–30	0	100	Isocratic
30–5	0 to 100	100 to 0	Return to initial composition
35–45	100	0	Re-equilibration

Operate with a flow rate set to obtain sufficient back-pressure (e.g. 2 mL/min). As a detector use an ultraviolet spectrophotometer set at a wavelength of 265 nm.

Determine the time ($t_{0.5}$) in minutes when the absorbance has increased by 50% (see Figure 1).

$$D = t_D \times F$$

$t_D = t_{0.5} - 0.5t_G$ (in minutes);

t_G = pre-defined gradient time (= 20 minutes);

F = flow rate (in millilitres per minute).

Note: this measurement is usually performed with the injector in the *inject* position so as to include the injection loop volume in the dwell volume.

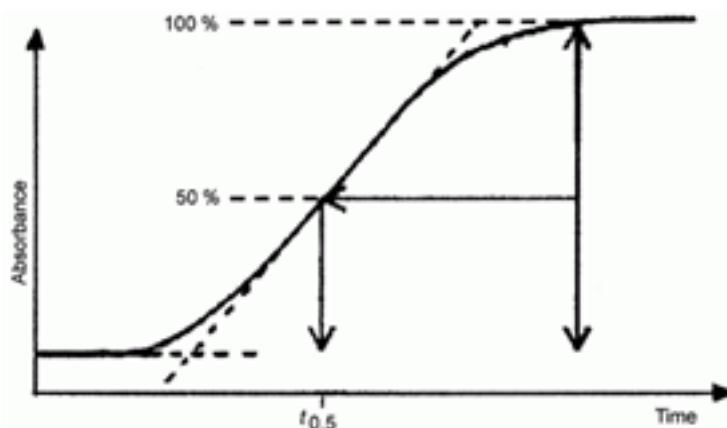


Figure 1. Determination of the dwell volume

System suitability

The system suitability test represents an integral part of the method and is used to ensure the adequate performance of the chosen chromatographic system.

Efficiency, capacity factor, peak-to-valley ratio, resolution factor, relative retention and symmetry factor are the parameters that are normally used in assessing the column performance; these terms are defined below. Factors that can affect chromatographic behaviour include mobile phase composition, temperature, ionic strength, apparent pH, flow rate and column length, and stationary-phase characteristics such as porosity, particle size and type, specific surface area and, in the case of reversed-phase supports, the type of chemical modification, carbon loading and end-capping.

Efficiency (N)

The efficiency of a chromatographic column is defined in terms of the number of theoretical plates (N) and can be calculated using the following formula:

$$N = 5.54 \frac{t_R^2}{W_h^2}$$

where

t_R = retention time or the baseline distance between the point of injection and the perpendicular dropped from the maximum of the

peak of interest.

W_h = the width of the peak of interest determined at half peak height, measured in the same units as t_R .

The number of theoretical plates can be expressed per metre (N'):

$$N' = \frac{N}{l}$$

where

l = length of column in metres.

Note: The efficiency should not be used as a system suitability criterion in gradient elution.

Capacity factor (mass distribution ratio, D_m)

The capacity factor or mass distribution ratio is defined as follows:

$$D_m = \frac{\text{amount of solute in stationary phase}}{\text{amount of solute in mobile phase}}$$

This factor determines the retention of a solute and can be calculated from the chromatogram using the following formula:

$$D_m = \frac{(t_R - t_M)}{t_M}$$

where

t_R = retention time of the solute

t_M = retention time of an unretained component

A low D_m value indicates that the peak elutes close to the solvent front which may compromise selectivity. A minimum D_m value of 1 is recommended for the peak of interest.

The retention time of the test substance can be varied, if necessary, by changing the relative proportion or composition of solvents in the mobile phase. Generally an increase in the proportion of a more polar solvent will lead to a shorter retention time on a normal-phase column and a longer retention time on a reversed-phase column.

Peak-to-valley ratio (p/v)

The peak-to-valley ratio between two partly overlapping peaks can be calculated using the following formula:

$$\frac{p}{v} = \frac{H_p}{H_v}$$

H_p = the height above the extrapolated baseline of the minor peak

H_v = the height above the extrapolated baseline at the lowest point of the curve separating the minor and the major peaks.

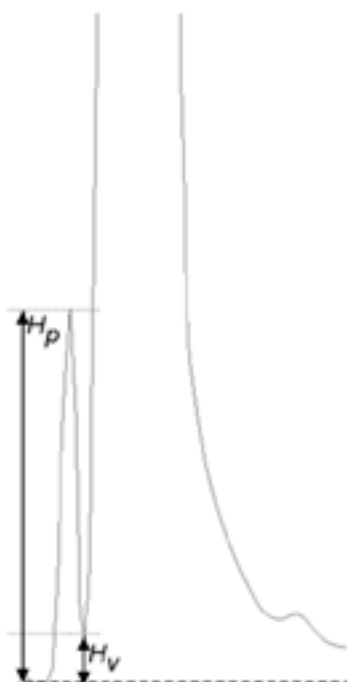


Figure 2. Determination of the peak-to-valley ratio

The peak-to-valley ratio may be employed as a system suitability criterion in a test for related substances when the baseline separation between two peaks is not achieved as illustrated in Figure 2.

Resolution factor (R_s)

The resolution between two peaks in a chromatogram can be calculated using the following formula:

$$R_s = \frac{1.18(t_{R2} - t_{R1})}{(W_{b1} + W_{b2})}$$

$$t_{R2} > t_{R1}$$

where

t_{R1} and t_{R2} = retention times or baseline distances between the point of injection and the perpendicular dropped from the maximum of each of the two peaks

W_{b1} and W_{b2} = the respective peak widths determined at half peak height, measured in the same units as t_{R1} and t_{R2}

The value of R_s which corresponds to a baseline separation between two symmetric peaks is greater or equal than 1.5.

Relative retention

The relative retention (r) is calculated as an estimate using the following formula:

$$r = \frac{t_{R2} - t_M}{t_{R1} - t_M}$$

where

t_{R2} = retention time of the peak of interest

t_{R1} = retention time of the reference peak

t_M = time required for elution of an unretained component (hold-up time).

The unadjusted relative retention (r_G) is calculated from the expression:

$$r_G = t_{R2}/t_{R1}$$

Unless otherwise indicated, values for relative retention stated in the monographs correspond to unadjusted relative retention.

Symmetry factor (tailing factor, A_s)

The symmetry factor for a peak can be calculated using the following formula:

$$A_s = \frac{W_x}{2d}$$

where

W_x = peak width at 5% of peak height, measured from the baseline

d = baseline distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at 5% of the peak height, measured in the same units as W_x .

A symmetry factor of 1.0 signifies complete symmetry. Values of A_s which are greater than 2 may lead to incorrect integration, resulting in erroneous quantitation. The main factors that influence peak symmetry depend upon retention, solvent effects, incompatibility of the solute with the mobile phase or development of an excessive void at the inlet of the column. In reversed-phase chromatography, adsorption phenomena due to the presence of residual silanol groups in the stationary phase may lead to tailing (poor peak symmetry).

Repeatability

Unless otherwise stated in the monograph under "Assay" the maximum permitted relative standard deviation of peak areas or peak heights for a series of injections of reference solutions, bracketing groups of test solutions, should not exceed the appropriate value given in Table 1.

Table 1. Repeatability requirements for the "Assay" of an active ingredient or a dosage form.

Number of individual injections			
3	4	5	6
Maximum permitted relative standard deviation			
0.41	0.59	0.73	0.85

In a "Related substances" test the relative standard deviation of peak areas or peak heights for three consecutive injections of the reference solution should not exceed 5.0%, unless otherwise stated in the individual monograph.

Adjustment of chromatographic conditions

The extent to which the various parameters of a chromatographic test may be adjusted to satisfy the system suitability criteria without fundamentally modifying the methods are described below for information. The chromatographic conditions described have been validated during the elaboration of the monograph. The system suitability tests are included to ensure the separation required for satisfactory performance of the test or assay. Nonetheless, since the stationary phases are described in a general way and there is such a variety available commercially, with differences in chromatographic behaviour, some adjustments of the chromatographic conditions may be necessary to achieve the prescribed system suitability requirements and to achieve the indicated retention time. The following adjustments are tolerated to the chromatographic conditions:

The retention time in gradient elution	Must be within $\pm 15.0\%$ of that indicated in the test and/or assay. With reverse-phase liquid chromatographic methods, in particular, adjustment of the various parameters will not always result in satisfactory chromatography. In that case it may be necessary to replace the column with another of the same general type (e.g. octadecylsilyl silica gel) that exhibits the desired chromatographic behaviour.
Composition of the mobile phase	The amount of the minor solvent component may be adjusted by $\pm 30\%$ relative or $\pm 2\%$ absolute, whichever is the larger. (Examples: For a minor component that is at 10% of the mobile phase a 30% relative adjustment allows a range of 7–13%, whereas a 2% absolute adjustment allows a range of 8–12%; in this case the relative value is the larger. For a minor component that is at 5% of the mobile phase a 30% relative adjustment

	<p>allows a range of 3.5–6.5%, whereas a 2% absolute adjustment allows a range of 3–7 %; in this case the absolute value is the larger.)</p> <p>No other component is altered by more than 10% absolute.</p>
pH of the aqueous component of the mobile phase	±0.2 pH, unless otherwise stated in the monograph.
Concentration of salts	In the buffer component of a mobile phase: ±10%.
Detector wavelength	No adjustment permitted.
Stationary phase	<p>Column length: ±70%, column internal diameter: ±25%.</p> <p>Particle size: maximal reduction of 50%, no increase permitted.</p>
Flow rate	<p>±50%. When the retention time of the principal peak is indicated in a monograph the flow rate has to be adjusted. No decrease in flow rate is permitted, however, if the monograph uses apparent number of theoretical plates in the qualification section.</p> <p>When column dimensions are changed the flow rate may be adjusted as necessary using the following equation:</p> $F_2 = F_1 \frac{l_2 d_2}{l_1 d_1}$ <p> F_1 = flow rate indicated in the monograph, in millilitres per minute; F_2 = adjusted flow rate, in millilitres per minute; l_1 = length of the column indicated in the monograph, in millimetres; l_2 = length of the column used, in millimetres; d_1 = internal diameter of the column indicated in the monograph, in millimetres; d_2 = internal diameter of the column used, in millimetres. </p> <p>The formula is applicable for changes in dimensions of the column but not for the particle size of the stationary phase.</p>
Temperature	When a temperature value is given, and unless otherwise prescribed, the column temperature is +/–5 °C for gradient and +/–10 °C for isocratic separations.
Injection volume	May be decreased, provided detection and repeatability of the peak(s) to be determined are satisfactory. However, no increase is permitted.
Gradient elution	<p>Adjustment of chromatographic conditions for gradient systems requires greater caution than for isocratic systems. Monographs preferably include an isocratic step before the start of the gradient programme so that an adaptation can be made to the gradient time points to take account of differences in dwell volume between the system used for method development and that actually used. It is the user's responsibility to adapt the length of the isocratic step to the analytical equipment used. If the dwell volume used during the elaboration of the monograph is given in the monograph the time points stated in the gradient table may be replaced by adapted time points (t_c min), calculated using the following equation:</p> $t_c = t - \frac{D - D_0}{F}$ <p> D = dwell volume, in millilitres; D_0 = dwell volume used for development of the method, in millilitres; </p>

	F = flow rate, in millilitres per minute.
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For critical parameters the adjustments are defined clearly in the monograph to ensure the system suitability.

Multiple adjustments which may have a cumulative effect on the performance of the system are to be avoided.

Procedure

To equilibrate the column allow the mobile phase to flow through the chromatographic system until the baseline and the retention times of the substances to be analysed are stable at the flow rate specified in the individual monograph (usually about 30 minutes). In case of ion-pair chromatography equilibration times may be significantly prolonged (up to 12 hours). Prepare the prescribed test and reference solutions as directed. Inject the prescribed reference solution and, if necessary, adjust the detector and/or recorder response to produce an adequate peak size. For chart recorders and integrators this should be at least 50% of the full-scale deflection of the principal peak in the chromatograms obtained with the reference solution. Ensure that the criteria for system suitability are met.

The reference solution should be injected at the start, at regular intervals during and at the end of a series of assays (e.g. every 2–4 samples). Both the reference and the test solutions should be injected in duplicate.

In determining the component composition of a complex mixture a "normalization" procedure, based on the calculation of individual peak areas as a percentage of the total area of all the peaks (excluding those due to solvents or reagents or arising from the mobile phase or the sample matrix and those at or below the limit at which they can be disregarded), may be used where the relative response factors of the individual components are similar and it has been demonstrated that the signals (responses) of the principal (major) and the minor peaks are within the linear range of the detector. The response factor is relative, being the response of the equal mass of one substance relative to that of another according to the conditions described in the test. For example, when an HPLC test with UV/vis detection is used for the control of impurities, the wavelength of detection should be such that the substance and its impurities have similar responses. If an impurity has a significantly different response (more than $\pm 20\%$) from that of the substance being examined, the preferred manner of limiting this impurity is to use a reference substance of the impurity. If a reference substance is not available the response factors of the potential impurities relative to those of the substance being examined are determined during method development. Subsequently the derived correction factors (i.e. the reciprocals of the response factors) are applied, if necessary, as described in the individual monograph.

If gradient elution is specified in the monograph an instrument equipped with a special pumping system, the dwell volume of which is known, capable of delivering a mobile phase of continuously varied composition, is needed. The mobile phase composition changes from the initial composition within a fixed period of time as specified in the monograph. Where the mobile phase composition is varied at a linear gradient elution perform a blank run to identify any interfering peaks by injecting the solvent specified for preparing the test solutions. Allow sufficient time for equilibration when the mobile phase is reset to the initial composition for the next injection.