10

Chromatographic theory

Introduction

Chromatography is the most frequently used analytical technique in pharmaceutical analysis. An understanding of the parameters which govern chromatographic performance has given rise to improvements in chromatography systems, so the ability to achieve high-resolution separations is continually increasing. The system suitability tests which are described at the end of this chapter are now routinely included in chromatographic software packages so that the chromatographic performance of a system can be monitored routinely. The factors determining chromatographic efficiency will be discussed first in relation to high-pressure liquid chromatography (HPLC).

Void volume and capacity factor

Figure 10.1 shows an HPLC column packed with a solid stationary phase with a liquid mobile phase flowing through it.

If a compound does not partition appreciably into the stationary phase, it will travel through the column at the same rate as the solvent. The length of time it takes an unretarded molecule to flow through the column is determined by the void volume of the column (V_o). The porous space within a silica gel packing is usually about 0.7 × the volume of the packing; a typical packing volume in a 0.46 × 15 cm column is *ca* 2.5 cm^3. Thus, in theory, it should take solvent or unretarded molecules, flowing at a rate of 1 ml/min, *ca* 1.8 min to pass through the void volume of such a column (the internal space is likely to be reduced where the silica gel has been surface coated with stationary phase). The length of time it takes a retarded compound to pass through the column depends on its capacity

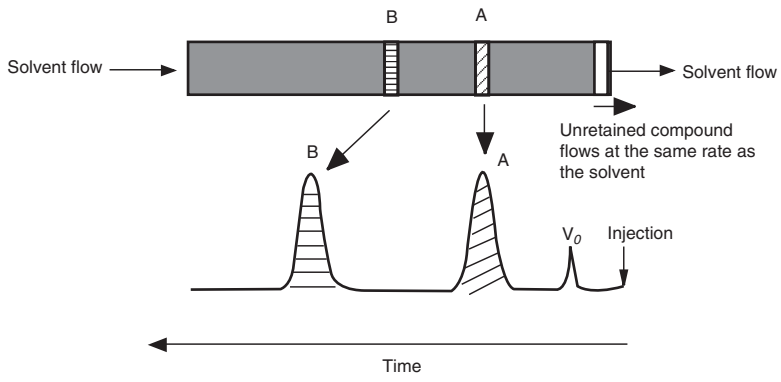

Fig. 10.1
Schematic diagram of bands for three different compounds travelling through an HPLC column. The compound with the largest capacity factor emerges last.

factor (K'), which is a measure of the degree to which it partitions (adsorbs) into the stationary phase from the mobile phase:

$$K' = \frac{V_r - V_o}{V_o} \text{ or } \frac{t_r - t_o}{t_o}$$

where V_o is the void volume of the column; V_r is the retention volume of the analyte; t_o is the time taken for an unretained molecule to pass through the void volume and t_r is the time taken for the analyte to pass through the column. In the example shown in Figure 10.1, compound B has a larger capacity factor than compound A. For example, if a compound had a K' of 4, the V_o of a column was 1 ml and the solvent was flowing through the column at 1 ml/min, the total time taken for the compound to pass through the column would be 5 min, i.e. for the 1 min required to pass through the void space in the column 4 min would be spent in the stationary phase. This is a simplification of the actual process, but it provides a readily understandable model. As can be seen in Figure 10.1 the peaks produced by chromatographic separation actually have width as well as a retention time, and the processes which give rise to this width will be considered later.

Self-test 10.1

Calculate the time taken for the following compounds to emerge from a chromatographic column under the specified conditions.

K' of compound	V_o of column (ml)	Solvent flow rate (ml/min)
6	1	1
10	1	2

Answers: 7 min and 5.5 min

Calculation of column efficiency

The broader a chromatographic peak is relative to its retention time, the less efficient the column it is eluting from. Figure 10.2 shows a chromatographic peak emerging at time t_r after injection; the efficiency of the column is most readily

See answer here

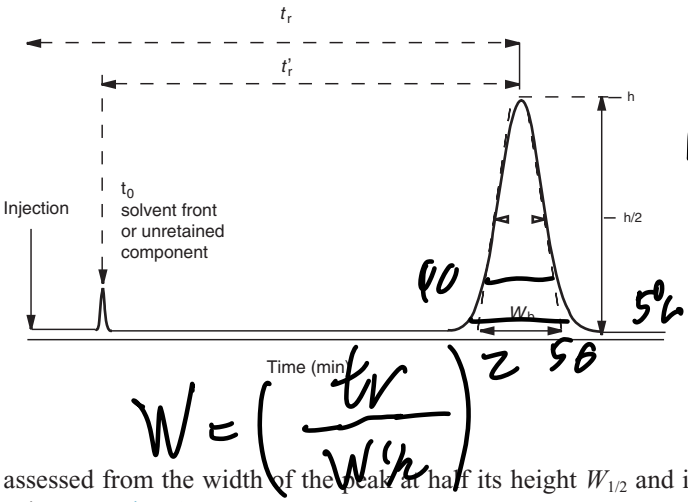

Fig. 10.2
Determination of retention time and peak width at half height.

assessed from the width of the peak at half its height $W_{1/2}$ and its retention time using Equation 1:

$$n = 5.54\,(t_r/W_{1/2})^2 \qquad \textbf{[Equation 1]}$$

where n is the number of theoretical plates.

Column efficiency is usually expressed in theoretical plates per metre:

$$n \times 100/L$$

where L is the column length in cm.

A stricter measure of column efficiency, especially if the retention time of the analyte is short, is given by Equation 2:

$$N\ eff = 5.54(t'_r/W_{1/2})^2 \qquad \textbf{[Equation 2]}$$

where $N\ eff$ is the number of effective plates and reflects the number of times the analyte partitions between the mobile phase and the stationary phase during its passage through the column and $t'_r = t_r - t_o$.

Another term which is used as a measure is H, the 'height of a theoretical plate':

$$H = L/N\ eff$$

where H is the length of column required for one partition step to occur.

Self-test 10.2

A standard operating procedure states that a column must have an efficiency > 30 000 theoretical plates/m. Which of these 15 cm columns meets the specification?

Retention time of analyte (min)	$W_{1/2}$ (min)
1. 6.4	0.2
2. 5.6	0.2
3. 10.6	0.6

Answer: Column 1

See answer here

Origins of band broadening in HPLC

Van Deemter equation in liquid chromatography

Chromatographic peaks have width and this means that molecules of a single compound, despite having the same capacity factor, take different lengths of time to travel through the column. The longer an analyte takes to travel through a column, the more the individual molecules making up the sample spread out and the broader the band becomes. The more rapidly a peak broadens the less efficient the column. Detailed mathematical modelling of the processes leading to band broadening is very complex.[1] The treatment below gives a basic introduction to the origins of band broadening. The causes of band broadening can be formalised in the Van Deemter equation (Equation 3) as applied to liquid chromatography:

$$H = \frac{A}{1 + C_m / u^{1/2}} + \frac{B}{u} + C_s u + C_m u^{1/2} \qquad \textbf{[Equation 3]}$$

H is the measure of the efficiency of the column (discussed above); the smaller the term, the more efficient the column.

u is the linear velocity of the mobile phase, simply how many cm/s an unretained molecule travels through the column, and A is the 'eddy' diffusion term; broadening occurs because some molecules take longer erratic paths while some, for instance, those travelling close to the walls of the column, take more direct paths, thus eluting first. As shown in Figure 10.3, for two molecules of the same compound, molecule X elutes before molecule Y. In liquid chromatography the eddy diffusion term also contains a contribution from streaming within the solvent volume itself, i.e. A (see the C_m term) is reduced if the diffusion coefficient of the molecule within the mobile phase is low because molecules take less erratic paths through not being able to diffuse out of the mainstream so easily.

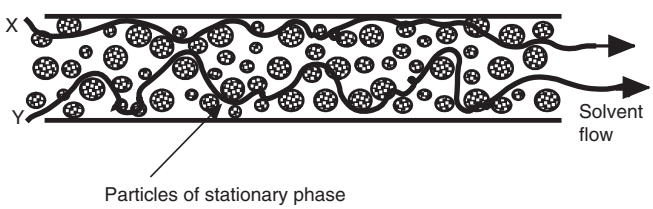

Fig. 10.3 Eddy diffusion around particles of stationary phase.

B is the rate of diffusion of the molecule in the liquid phase, which contributes to peak broadening through diffusion either with or against the flow of mobile phase; the contribution of this term is very small in liquid chromatography. Its contribution to band broadening decreases as flow rate increases, and it only becomes significant at very low flow rates.

C_s is the resistance to mass transfer of a molecule in the stationary phase and is dependent on its diffusion coefficient in the stationary phase and upon the thickness of the stationary phase coated onto silica gel:

$$C_s = \frac{d^2 \text{ thickness}}{D_s}$$

where d^2 thickness is the square of the stationary-phase film thickness and D_s is the diffusion coefficient of the analyte in the stationary phase.

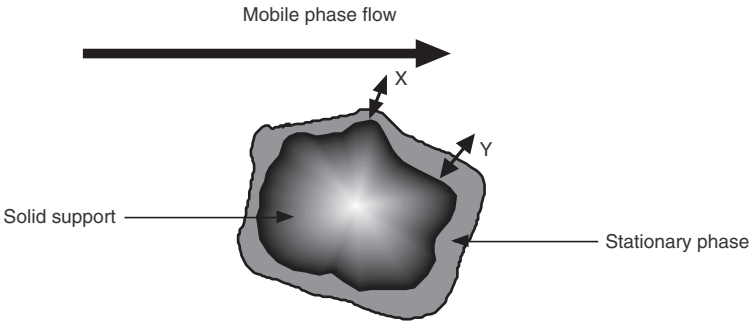

Fig. 10.4
Resistance to mass transfer of a molecule within a particle of stationary phase.

Obviously the thinner and more uniform the stationary-phase coating, the smaller the contribution to band broadening from this term. In the example shown in Figure 10.4, molecule Y is retarded more than molecule X. It could be argued that this effect evens out throughout the length of the column, but in practice the number of random partitionings during the time required for elution is not sufficient to eliminate it. As might be expected, C_s makes less contribution as u decreases.

C_m is resistance to mass transfer brought about by the diameter and shape of the particles of the stationary phase and the rate of diffusion of a molecule in the mobile phase.

$$C_m = \frac{d^2 \text{ packing}}{D_m}$$

where d^2 packing is the square of the stationary-phase particle diameter and D_m is the diffusion coefficient of the analyte in the mobile phase.

The smaller and more regular the shape of the particles of the stationary phase, the smaller the contribution to band broadening from this term. In Figure 10.5 molecule X is retarded more than molecule Y in terms of both pathlength (this really belongs to the eddy diffusion term) and contact with stagnant areas of solvent within the pore structure of the stationary phase. With regard to the latter effect, the smaller the rate of diffusion of the molecular species (D_m) in the mobile

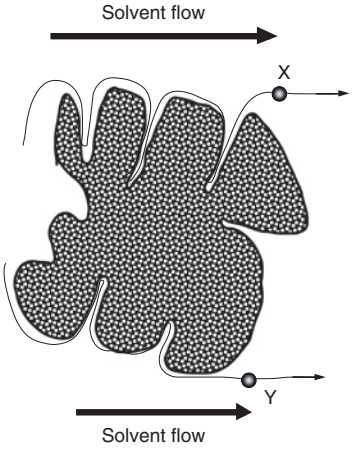

Fig. 10.5
Band broadening due to resistance to diffusion of a molecule within the mobile phase, contained within the pores of a stationary phase and due to irregularities in stationary-phase pore structure.

phase, the greater the retardation will be. There are an insufficient number of random partitionings during elution for these effects to be evened out.

Thus, a low diffusion coefficient for the analyte in the mobile phase increases efficiency with regard to the A term but decreases efficiency with respect to the C_m term. On balance, a higher diffusion coefficient is more favourable. Higher column temperatures reduce mass transfer effects because the rate of diffusion of a molecule in the mobile phase increases.

In practice the contributions of the A, $C_s u$ and $C_m u^{1/2}$ terms to band broadening are similar except at very high flow rates, where the $C_s u$ terms predominate. At very low flow rates, the B term makes more of a contribution. A compromise has to be reached between analysis time and flow rate. Advances in chromatographic techniques are based on the minimisation of the effects of the various terms in the Van Deemter equation, and it has provided the rationale for improvements in the design of stationary phases.

Self-test 10.3

Indicate which of the following parameters can decrease or increase column efficiency in liquid chromatography.

- Very low flow rate
- Large particle size of stationary phase
- Small particle size of stationary phase
- Thick stationary-phase coating
- Thin stationary-phase coating
- Regularly shaped particles of stationary phase
- Irregularly shaped particles of stationary phase
- High temperature
- Low temperature
- Uneven stationary-phase coating
- Even stationary-phase coating
- Uniform stationary-phase particle size
- Non-uniform stationary-phase particle size
- Low diffusion coefficient in the mobile phase
- High diffusion coefficient in the mobile phase
- Low diffusion coefficient in the stationary phase
- High diffusion coefficient in the stationary phase

Answers: Increases column efficiency: small particle size of stationary phase; thin stationary-phase coating; regularly shaped particles of stationary phase; high temperature; even stationary-phase coating; uniform stationary-phase particle size; high diffusion coefficient in the mobile phase; high diffusion coefficient in the stationary phase.

Decreases column efficiency: very low flow rate; large particle size of stationary phase; thick stationary-phase coating; irregularly shaped particles of stationary phase; low temperature; uneven stationary-phase coating; non-uniform stationary-phase particle size; low diffusion coefficient in the mobile phase; low diffusion coefficient in the stationary phase.

Van Deemter equation in gas chromatography

The Van Deemter equation can be applied to gas chromatography with a different emphasis on the relative importance of its terms. In fact, the interactions between an analyte and a stationary phase are much simpler in gas chromatography than those in liquid chromatography since the mobile phase does not modify the stationary phase in any way. The theoretical considerations are different for packed GC columns vs open tubular capillary columns.

See answer here

In gas chromatography the Van Deemter equation can be written as:

$$H = A + \frac{B}{u} + Cu$$

where H is the measure of column efficiency; A is the eddy diffusion coefficient; B is 2 × the diffusion coefficient of the analyte in the gas phase; C is composed of terms relating to the rate of diffusion of the analyte in the gas and liquid phases (mass transfer, see above) and u is the carrier gas velocity.

For an open tubular capillary column (Fig. 10.6), the eddy diffusion coefficient does not play a part in band broadening, and the C term is largely composed of the transverse diffusion coefficient in the gas phase since the liquid film coating of the capillary column wall is typically 0.1–0.2% of the internal diameter of the column. B/u is most favourable for nitrogen (diffusion coefficients of molecules are lower in nitrogen than in the other commonly used carrier gases hydrogen and helium). However, nitrogen only gives better efficiency where u is small, since the size of the term Cu is governed by the resistance to transverse diffusion, which is greatest for nitrogen, i.e. fast flow rates of nitrogen reduce the interaction of the analyte with the stationary phase. Most often helium is used as a carrier gas in capillary GC since it gives a good efficiency without having to reduce the flow rate, which would give long analysis times. Transverse diffusion effects are reduced by reducing the internal diameter of a capillary column, and thus the smaller the internal diameter of a column, the more efficient it is.

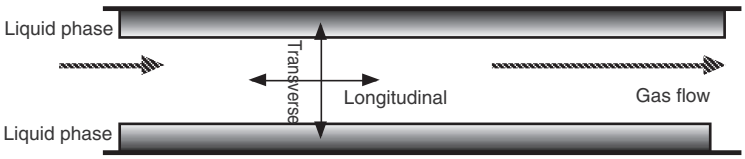

Fig. 10.6
Band broadening factors in open tubular columns.

With a packed GC column the separation efficiency is lower because, although the longitudinal diffusion coefficient is lower, the eddy diffusion coefficient (A) causes band broadening (Fig. 10.3). In addition, mass transfer effects are greater for a packed column because of the irregular structure of the particles of packing and the consequent uneven coating of a relatively thick liquid phase. However, whatever type of GC column is used, the C_m term is not as significant as that in liquid chromatography because of the high diffusion coefficient of molecules in the gas phase.

Parameters used in evaluating column performance

Having optimised the efficiency of a chromatographic separation, the quality of the chromatography can be controlled by applying certain system suitability tests. One of these is the calculation of theoretical plates for a column, and there are two other main parameters for assessing performance: peak symmetry and the resolution between critical pairs of peaks. A third performance test, the peak purity parameter, can be applied where two-dimensional detectors such as diode or coulometric array or mass spectrometry detectors are being used. The

reproducibility of peak retention times is also an important parameter for controlling performance.

Resolution

The more efficient a column, the greater degree of resolution it will produce between closely eluting peaks. The resolution between two peaks – A and B (Fig. 10.7) – is expressed in Equation 4:

$$R_s = 2(t_{r\mathrm{B}} - t_{r\mathrm{A}})/W_{b\mathrm{B}} + W_{b\mathrm{A}} \qquad \textbf{[Equation 4]}$$

where $t_{r\mathrm{B}}$ and $t_{r\mathrm{A}}$ are the retention times of peaks A and B and $W_{b\mathrm{B}}$ and $W_{b\mathrm{A}}$ are the widths of peaks A and B at baseline. An R_s of 1 indicates a separation of 4σ between the apices of two peaks. Complete separation is considered to be R_s = 1.2. The retention times of peaks A and B are 26.3 and 27.2 min respectively. The substitution of these values and the values obtained for peak widths at base for A and B into Equation 4 is as follows:

$$R_s = \frac{2(27.2 - 26.3)}{0.56 + 0.56} = 1.6$$

It is obvious without calculation that peaks A and B are well resolved. With incomplete separation, the determination of resolution is more difficult since the end and beginning of the two partially overlapping peaks has to be estimated; if the peak shape is good it is easiest to assume the same symmetry for the leading and tailing edges of the two peaks. If this is carried out for peaks B and C in Figure 10.7, their resolution is found to be 0.85, which is not an entirely satisfactory resolution. More is required of the integrator which is used to measure peak areas when peaks overlap since it must be able to decide where one peak ends and the other begins. Ideally peak overlap should be avoided for quantitative accuracy and precision.

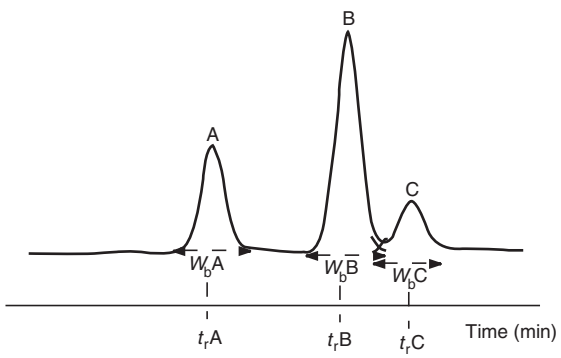

Fig. 10.7
Determination of the degree of resolution between chromatographic peaks.

The resolution equation may also be written as follows:

$$R_s = 1.18\frac{t_{r\mathrm{B}} - t_{r\mathrm{A}}}{W_{\mathrm{B}0.5} + W_{\mathrm{A}0.5}}$$

where $W_{B0.5}$ and $W_{A0.5}$ are the widths of peaks A and B at half height. This is the form of the equation that is used by the British and European Pharmacopoeias, whereas the American Pharmacopoeia uses Equation 4. The width of a peak at half height is easier to measure than its width at the base.

Self-test 10.4

The BP assay of betamethasone 17-valerate states that it must be resolved from betamethasone 21-valerate so that the resolution factor is > 1.0. Which of the following ODS columns meet the specification?

	Retention time of betamethasone 21-valerate (min)	Retention time of betamethasone 17-valerate (min)	Width at base of betamethasone 21-valerate (min)	Width at base of betamethasone 17-valerate (min)
1.	9.5	8.5	0.4	0.5
2.	9.3	8.6	0.4	0.4

Answer: 1 and 2

The resolution equation may be written in a more complex form for two peaks, A and B, in a chromatogram:

$$R_s = \left(\frac{1}{4}\right) N^{0.5} \left(\frac{\alpha - 1}{\alpha}\right) \left(\frac{K'_B}{1 + K'}\right)$$

N = efficiency

K'_A = capacity factor of peak A

K'_B = capacity factor of peak B **[Equation 5]**

$$\alpha = \frac{K'_A}{K'_B} = \text{selectivity}$$

$$K' = \frac{K'_A + K'_B}{2}$$

This equation is applicable if the calculated column efficiency is the same for both analytes. If the column efficiency is different for the two analytes then: $\sqrt{N}$ is replaced by $\sqrt[4]{N_A N_B}$ and K'_B is replaced by K'.

From Equation 5 it can be seen that column efficiency has less effect than might be expected on resolution and a 2 × increase in efficiency only results in a 1.41 × increase in resolution. An increase in capacity factor, however, does have a marked effect on resolution. Capacity factor exerts its strongest effect on separation via the α term, which can also be termed the relative capacity factor. The simplest way to increase capacity factor is to change the solvent composition of the mobile phase in liquid chromatography or the temperature in gas chromatography.

See answer here

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Calculation example 10.1

A column has an efficiency of 14 000 theoretical plates. It has a t_o value of 1.3 min. Two analytes have retention times of 10.4 and 12.2. Calculate their resolution factor.

$$K'_A = \frac{10.4 - 1.3}{1.3} = 9.4$$

$$K'_B = \frac{12.2 - 1.3}{1.3} = 11.2$$

$$\alpha = \frac{11.2}{9.4} = 1.2$$

$$R_s = \frac{1}{4} \times \sqrt{14000} \times \frac{1.21}{1.2} \times \frac{11.2}{1 + (9.4 + 11.2)/2} = 5.0$$

Figure 10.8 shows the effect of decreasing the % of organic solvent in a reverse-phase chromatographic separation of two analytes A and B (see Chapter 12). The column has a t_o of 1.2 min; thus the change in the composition has changed the capacity factor for analyte A from 6.4 to 8.1 (% change = 26) and that for B from 7.0 to 10.0 (% change = 43). If the rate of change of capacity factor for two analytes with change of mobile-phase composition is different, then this indicates some difference in their retention mechanism. Often a simple change in organic solvent composition will be sufficient to give adequate resolution between two analytes; however, for critical separations involving a number of components, the selectivity of a method may need to be changed by using ternary mixtures of solvents or by changing the chromatographic column in order to change the α term in Equation 5.

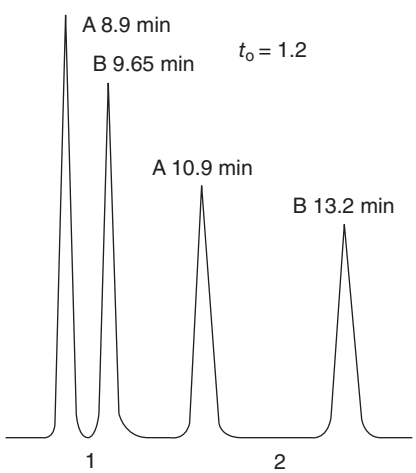


Fig. 10.8
Effect of changing solvent composition on the capacity factor of two analytes on a reverse-phase column.
1. Water/acetonitrile 50:50.
2. Water/acetonitrile 52:48.

Peak asymmetry

Another situation which may lead to poor integrator performance is where peaks are tailing and thus have a high element of asymmetry. The expression used to assess this is:

$$\text{Asymmetry factor } (AF) = b/a$$

where a is the leading half of the peak measured at 10% of the peak height and b is the trailing half of the peak measured at 10% of the peak height (Fig. 10.9). This value should fall, ideally, in the range 0.95–1.15. Poor symmetry may be caused through loading too much sample onto the column, sample decomposition, the analyte adsorbing strongly onto active sites in the stationary phase, poor trapping of the analyte when it is loaded onto the column or too much 'dead volume' in the chromatographic system.

The peak in Figure 10.9A has an asymmetry factor of 0.97, and this is due to its tailing slightly at the front edge; this may be due to inefficient trapping of the sample at the head of the column as it is loaded. The peak in Figure 10.9B has an asymmetry factor of 1.77 and is thus tailing quite badly; the most common cause of tailing is due to adsorption of the analyte onto active sites in the chromatographic column.

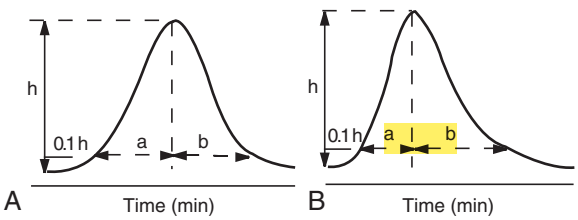


Fig. 10.9
Determination of peak asymmetry.

Data acquisition

An integrator, whether it is based on a microprocessor or PC software, simply measures the total amount of current which flows over the width of a chromatographic peak. To do this it measures the rate of increase of voltage approximately 30 times across the width of the peak. The parameter which indicates when measurement should start is the peak threshold, which determines the level that the voltage of the signal should rise to before accumulation of the signal will occur. To avoid storage of baseline drift the peak width parameter is linked to the peak threshold parameter, which indicates that if the signal rises above baseline the slope of the rise should have a certain steepness before it is regarded as a peak. A narrow peak width setting indicates that the expected slope should be steep and a wide peak width setting indicates that the expected slope should be relatively shallow. For good digital recording a peak should be sampled *ca* 30 times across its width. The setting relates to the estimated width at half-height of peaks in a chromatogram, e.g. a width setting of 0.4 min would cover many HPLC applications. There is quite a wide degree of tolerance in the peak width setting, although it should be set within ± 100% of the expected peak width at half-height to ensure accurate peak integration.

A factor which can cause a loss of precision in chromatographic quantification is the reproducibility of the way in which peaks are integrated. If peaks have good symmetry, are well resolved from neighbouring peaks and are well above baseline noise, integration is likely to be reproducible. The peak threshold (PT) setting has the greatest effect on peak area and it has to be set high enough for fluctuations in the baseline to be ignored. In the example shown in Figure 10.10, in the first case the threshold is set too low and a tail of baseline drift is attached to the peak during integration. In the second case, the threshold is set higher and the tail is ignored. The area of peak A determined with a peak threshold of 100 μV is only 94% of the area determined for peak A with tailing baseline included at a threshold of 10 μV. This could make a significant difference to the precision of the analysis, depending on how reproducibly the peak tail was integrated. This type of effect is only likely to be significant if the size of the peaks is low in relation to baseline fluctuations.

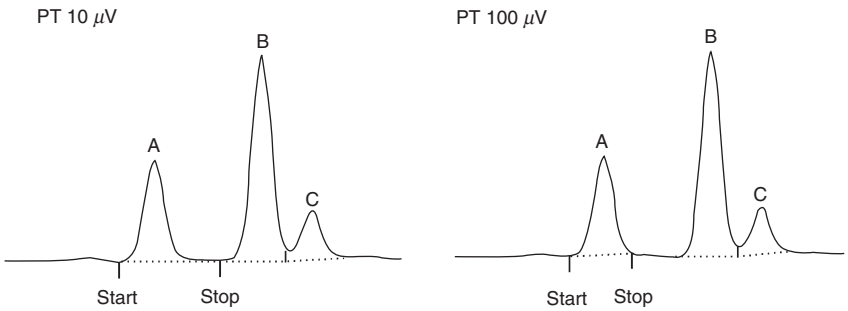


Fig. 10.10
The effect of the peak threshold setting.

The two fused peaks shown in Figure 10.10 are not as affected by a change in the peak threshold setting; however, their areas can only be approximated because of their overlap. It is possible, by setting the integrator to produce a tangent skim, to change the way in which these peaks are integrated, as shown in Figure 10.11. (see Animation 10.1, Animation 10.2 and Animation 10.3) In this instance, where the peaks are almost resolved and are not vastly different in height, the integration method used in Figure 10.10 probably gives a better approximation of the areas.

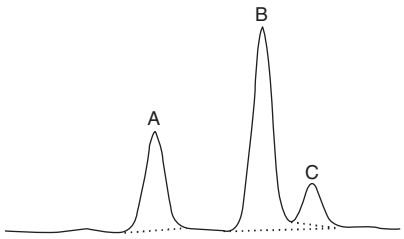


Fig. 10.11
Tangent skimming of a peak on a tailing edge.

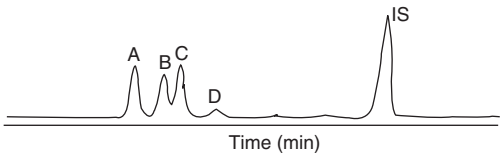


Fig. 10.12
Chromatographic trace with report including system suitability tests.

Report generation

Computerised data handling systems will generate reports including a number of system suitability parameters. Figure 10.12 shows a chromatogram with a report form appended. In order for the report to be generated, the computer has to be given some information, e.g. the expected retention times of peaks for which resolution factors have to be measured and the retention time of an unretained peak, in order to determine capacity factor. With increasing dependence on computers, it is important to be able to guesstimate whether the computer is generating sensible data; the ability to calculate the various efficiency parameters from first principles is an important check on the performance of the integrator.

Component	Retention time	Area %	*n* per column	*AF*	$W_{1/2}$ min	R_s	*K'*
A	20.1	16.3	50 166	0.96	0.2	–	18.3
B	20.8	13.2	65 229	0.87	0.2	1.4	18.9
C	21.2	15.5	81 189	1.13	0.17	0.81	19.2
D	22.0	2.21	44 397	0.99	0.23	1.8	20.0
IS	25.7	37.9	64 316	0.75	0.23	–	23.4

Reference

1. Giddings JC. *Unified Separation Science*. Chichester: Wiley Interscience; 1991.

11 Gas chromatography

KEYPOINTS

Principles

A gaseous mobile phase flows under pressure through a heated tube either coated with a liquid stationary phase or packed with liquid stationary phase coated onto a solid support. The analyte is loaded onto the head of the column via a heated injection port, where it evaporates. It then condenses at the head of the column, which is at a lower temperature. The oven temperature is then either held constant or programmed to rise gradually. Once on the column, separation of a mixture occurs according to the relative lengths of time spent by its components in the stationary phase. Monitoring of the column effluent can be carried out with a variety of detectors.

(Continued)

KEYPOINTS *(Continued)*

Applications

- The characterisation of some unformulated drugs, particularly with regard to detection of process impurities.
- Limit tests for solvent residues and other volatile impurities in drug substances.
- Sometimes used for quantification of drugs in formulations, particularly if the drug lacks a chromophore.
- Characterisation of some raw materials used in synthesis of drug molecules.
- Characterisation of volatile oils (which may be used as excipients in formulations), proprietary cough mixtures and tonics, and fatty acids in fixed oils.
- Measurement of drugs and their metabolites in biological fluids.

Strengths

- Capable of the same quantitative accuracy and precision as high-pressure liquid chromatography (HPLC), particularly when used in conjunction with an internal standard.
- Has much greater separating power than HPLC when used with capillary columns.
- Readily automated.
- Can be used to determine compounds which lack chromophores.
- The mobile phase does not vary and does not require disposal and, even if helium is used as a carrier gas, is cheap compared to the organic solvents used in HPLC.

Limitations

- Only thermally stable and volatile compounds can be analysed.
- The sample may require derivatisation to convert it to a volatile form, thus introducing an extra step in analysis and, potentially, interferants.
- Quantitative sample introduction is more difficult because of the small volumes of sample injected.
- Aqueous solutions and salts cannot be injected into the instrument.

Introduction (see Animation 11.1)

The use of gas chromatography (GC) as a quantitative technique in the analysis of drugs has declined in importance since the advent of high-pressure liquid chromatography (HPLC) and the increasing sophistication of this technique. However, it does still have a role in certain types of quantitative analyses and has broad application in qualitative analysis. Since HPLC currently dominates quantitative analyses in the pharmaceutical industry, the strengths of GC may be overlooked. Capillary GC is capable of performing much more efficient separations than HPLC, albeit with the limitation that the compounds being analysed must be volatile or must be rendered volatile by formation of a suitable derivative[1] and must also be thermally stable. GC is widely used in environmental science, brewing, the food industry, perfumery and flavourings analysis, the petrochemical industry, microbiological analyses and clinical biochemistry. Although packed column GC is still used in the pharmaceutical industry, this chapter will concentrate to a large extent on open tubular capillary GC, which is the more modern manifestation of GC.

Instrumentation

Figure 11.1 shows a schematic diagram of a GC system. The principles of the system are that:

(i) Injection of the sample may be made, manually or using an autosampler, through a re-sealable rubber septum.

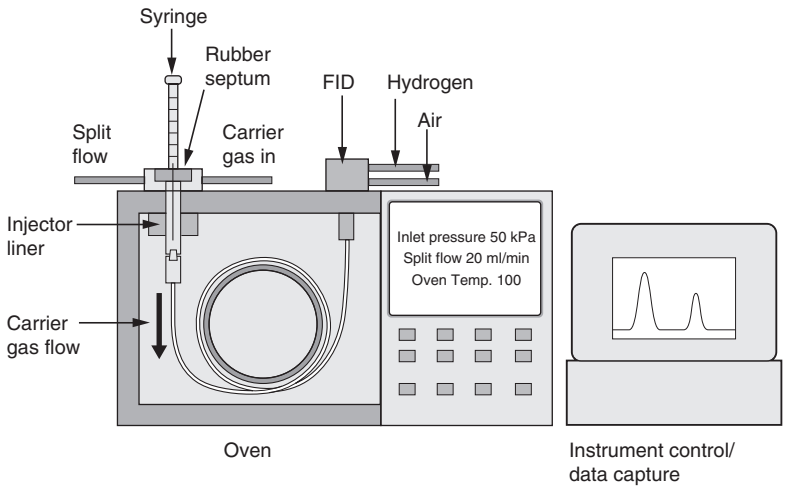


Fig. 11.1
Schematic diagram of a capillary gas chromatography (GC) system.

(ii) The sample is evaporated in the heated injection port area and condenses on the head of the column.

(iii) The column may be either a capillary or a packed column, which will be discussed in more detail later. The mobile phase used to carry the sample through the column is a gas – usually nitrogen or helium.

(iv) The column is enclosed in an oven which may be set at any temperature between ambient and *ca* 400°C.

(v) The most commonly used detector is the flame ionisation detector (FID).

Syringes

The volumes injected in GC are routinely in the range of 0.5–2 μl. The most commonly used type of syringe is shown in Figure 11.2; the usual syringe volumes are 5 and 10 μl. A recommended technique for injection into a capillary GC is to fill the syringe with about 0.5 μl of solvent and draw this solvent into the barrel slightly before filling with sample (see Animation 11.2). The sample is also drawn into the barrel to leave an air gap below it. The syringe needle can then be introduced into the injector and left for a couple of seconds to warm up before the plunger is depressed. The syringe is then withdrawn immediately from the injection port.

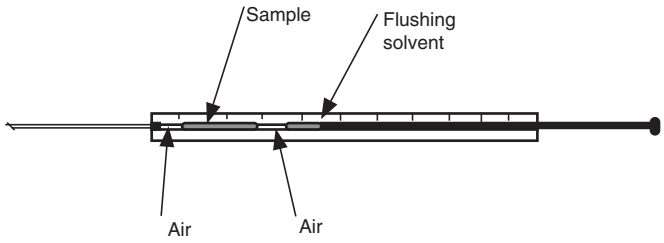


Fig. 11.2
10 μl sample in barrel syringe.

Injection systems

Packed column injections

Injection generally occurs through a re-sealable rubber septum. The injector port is held at 150–250° depending on the volatility of the sample and direct injection of 0.1–10 μl of sample is made onto the head of the column. The amount of sample injected onto a packed column is *ca* 1–2 μg per component. Injection into packed columns presents less of a problem than sample introduction into a capillary column, since all the sample is introduced into the packed column (Fig. 11.3). Thus, although packed columns do not produce high-resolution chromatography, this is their strength.

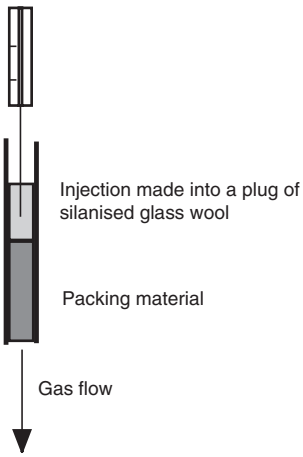


Fig. 11.3
Injection onto the head of a packed column.

Split/splitless injection

This type of injector is used in conjunction with capillary column GC. Capillary columns commonly have internal diameters between 0.2 and 0.5 mm and lengths between 12 and 50 m. Injection takes place into a heated glass or quartz liner rather than directly onto the column.

In the split mode, the sample is split into two unequal portions, the smaller of which goes onto the column. Split ratios range between 10 : 1 and 100 : 1, with the larger portion being vented in the higher flow out of the split vent. This technique is used with concentrated samples. Figure 11.4 shows a chromatogram of a 4 mg/ml solution of the pharmaceutical excipient cetostearyl alcohol injected in a GC split and splitless mode. In splitless mode the peak shapes are poor due to overloading of the column with too much sample. In the splitless mode, all the sample is introduced onto the column and the injector purge valve remains closed for 0.5–1 min after injection.

The difficulty faced with split/splitless injection onto a capillary column is in obtaining good injection precision.[2]

Attention has to be paid to certain points:

(i) Since injection is made at high temperatures into an injection port, a lack of precision resulting from decomposition of some of the components in a mixture before they reach the column has to be considered. Thus it

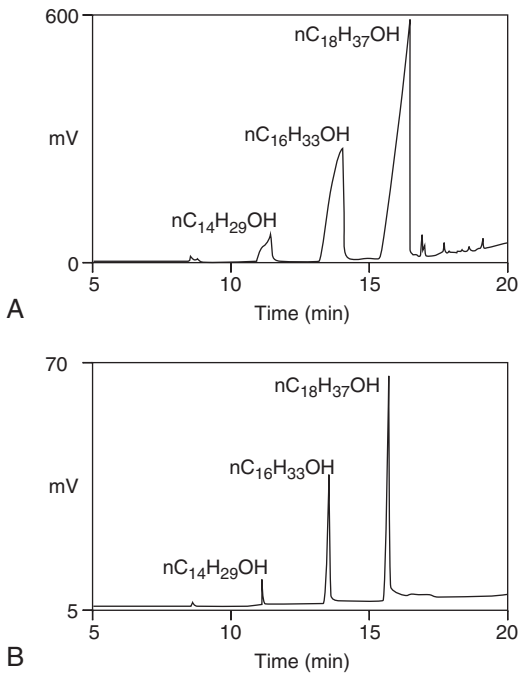


Fig. 11.4
Analysis of cetostearyl alcohol (4 mg/ml) in split and splitless mode A and splitless mode B 20:1 split (flow through column 1 ml/min flow out of split vent 20 ml/min). Column Rtx1 15 m × 0.32 mm i.d. × 0.5 μm film, programmed 100° (1 min) then 10°/min to 290°).

is important to ensure that the sample has minimal contact with metal surfaces during the injection process, since these can catalyse decomposition.

(ii) If a split injection is used, care has to be taken that there is no discrimination between more and less volatile components in a mixture in terms of the proportion lost through the split vent (see Animation 11.3).

(iii) If a splitless injection is made, volumes have to be kept below *ca* 2 μl in case the sample backflashes through rapid expansion of the solvent in which it is dissolved, into either the gas supply lines or the purge lines. Each 1 μl of solvent expands greatly upon vapourisation, e.g. methanol *ca* 0.66 ml/μl or ethyl acetate *ca* 0.23 ml/μl at atmospheric pressure.

(iv) Even if an internal standard (p. 287) is used to compensate for losses, the possibility of its being randomly discriminated against through differences in either volatility or decomposition compared with the sample has to be considered.

(v) In the splitless mode, the sample must be efficiently trapped at the head of the column. For this to occur, it must be sufficiently involatile, i.e. have a boiling point > *ca* 50°C higher than the column starting temperature. If the sample is relatively volatile, it has to be injected into the GC in a low-volatility solvent, which will condense at the head of the column, trapping the sample in the process. Figure 11.5 shows the effect of too high a starting temperature on the trapping of a volatile analyte resulting in misshapen peaks, which can be corrected by lowering the starting temperature and then programming the GC temperature to rise.

(vi) Sample transfer may be slow and it is important to take this into account when setting purge valve times, e.g. for a typical 1 ml/min flow of helium

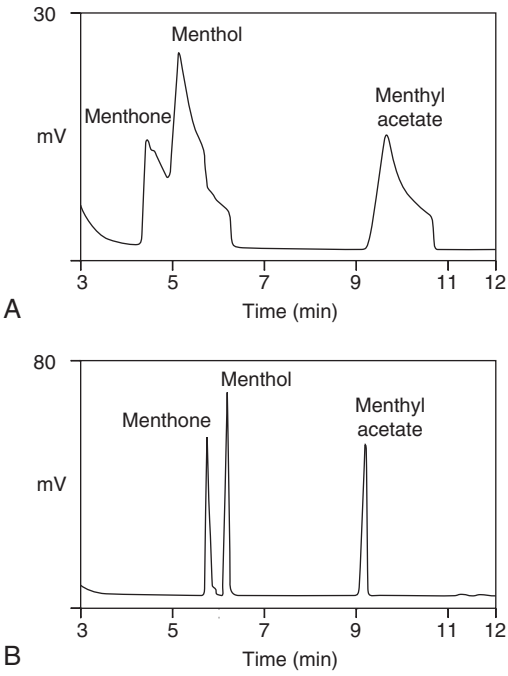


Fig. 11.5
(A) Gas chromatography (GC) trace for menthone, menthol and menthyl acetate under isothermal conditions, 110°C for 12 minutes. (B) 60°C (1 min) 30°C/min to 120°C for 9 minutes.

through a capillary column, about 0.5 min would be required to transfer a 2 μl injection volume of ethyl acetate onto the column.

(vii) Injection precision is greatly improved by the use of an autosampler to carry out injection since it can achieve much better precision in measuring volumes of *ca* 1 μl than a human operator.

Cool on-column injection

Direct on-column injection into the capillary column may be carried out in a manner analogous to injection into a packed column. This technique requires a syringe with a very fine fused-silica needle. The technique has the advantages of (1) reduced discrimination between components in mixtures, (2) no sample degradation in a hot injector and (3) no backflash, hence quantitative sample transfer. It also has the following disadvantages: (1) samples have to be clean otherwise residues will be deposited on the column; (2) the injector is mechanically more complex and requires more maintenance than a septum injection system and (3) the syringe needle may damage the head of the column.

Programmable temperature vapouriser (see Animation 11.4)

The programmable temperature vapouriser (PTV) is a recent innovation. This type of injector is designed to enable the injection of large volumes of sample onto capillary GC columns. Typically between 5 and 50 μl of sample are injected, with the injector being held at low temperature, e.g. 30°C. The solvent is then vented through a purge valve at high flow rate (e.g. 100 ml/min for 1 min). The less volatile, sample components are retained in the injection port; the purge valve is then closed and the injector temperature is ramped up rapidly (e.g. to 300°C

at 700°C/min). This is possible because the liner is of narrow diameter (1 mm) compared with those in other types of injectors and is made of Silicosteel, which provides an inert surface but conducts heat much more rapidly than glass. The boiling point of the lowest boiling component in the sample should be at least 100°C greater than that of the solvent for this injector to work well. This injector provides one option for use in conjunction with fast GC. Fast GC utilises the high efficiency of capillary GC by using very short columns so that separations of complex mixtures may be achieved in less than a minute.

GC oven

GC ovens incorporate a fan, which ensures uniform heat distribution throughout the oven. They can be programmed to produce a constant temperature, isothermal conditions or a gradual increase in temperature. Oven programming rates can range from 1°C/min to 40°C/min. Complex temperature programmes can be produced involving a number of temperature ramps interspersed with periods of isothermal conditions, e.g. 60°C (1 min)/5°C/min to 100°C (5 min)/10°C/min to 200°C (5 min). The advantages of temperature programmes are that materials of widely differing volatilities can be separated in a reasonable time and also injection of the sample can be carried out at low temperature, where it will be trapped at the head of the column and then the temperature can be raised until it elutes.

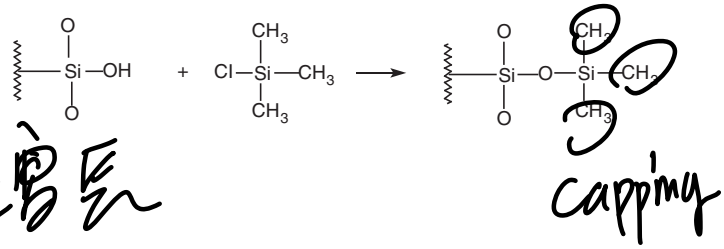
Types of columns

Packed columns

The columns are usually made from glass which is silanized to remove polar silanol Si–OH groups from its surface, which can contribute to the peak tailing of the peaks of polar analytes. These columns have internal diameters of 2–5 mm. The columns are packed with particles of a solid support which are coated with the liquid stationary phase. The most commonly used support is diatomaceous earth (mainly calcium silicate). This material is usually acid washed to remove mineral impurities and then silanized as shown in Figure 11.6 to remove the polar Si–OH groups on the surface of the support, which can lead to tailing of the analyte peak.

The support can then be mechanically coated with a variety of liquid stationary phases. The mobile phase most commonly used in packed column GC is nitrogen, with a flow rate of *ca* 20 ml/min. Packed column GC affords a relatively low degree of resolution compared to capillary GC; typically 4000–6000 plates for a 2 m column compared to > 100 000 plates for a 25 m capillary column. The high temperature limit of packed columns is *ca* 280°C; beyond this temperature the liquid stationary phase evaporates at a rate which creates a large background signal. However, for many routine quality-control operations, they are quite adequate.

Fig. 11.6
Silanisation of free silanol groups.



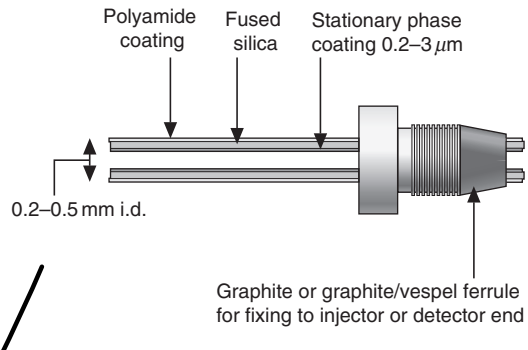


Fig. 11.7
Structure of a gas chromatography (GC) capillary column.

Capillary columns

Capillary columns are made from fused silica, usually coated on the outside with polyamide to give the column flexibility (Fig. 11.7). Coating on the outside with aluminium has also been used for high-temperature (> 400°C) work. The internal diameter of the columns ranges between 0.15 and 0.5 mm. The wall of the column is coated with the liquid stationary phase, which may have a thickness between 0.1 and 5 μm. The most common type of coating is based on organo silicone polymers, which are chemically bonded to the silanol groups on the wall of the column, and the chains of the polymers are further cross-linked. These types of phases have more or less replaced the wall-coated open tubular (WCOT) and support-coated open tubular (SCOT) columns, which are reported in earlier literature, for most routine applications. SCOT columns are sometimes encountered in very high-temperature work. The wall-bonded phases are stable to at least 325°C and some types of coating will withstand temperatures of 370°C. The non-silicone-based polymers, e.g. carbowax, cannot be bonded onto the wall of the column in the same way and columns with these coatings are less temperature stable. For instance, the temperature limit for a carbowax capillary column is *ca* 240°C. The most commonly used carrier gas in capillary GC is helium and the flow rates used are between 0.5 and 2 ml/min. Since the flow rate from the end of the capillary column is low compared to the internal space of some detectors, 'make up' gas often has to be added to the gas flow post column in order to sweep the sample through the internal volume of the detector at a reasonable rate. Typically *ca* 100 ng per component is loaded onto a capillary column.

Selectivity of liquid stationary phases

Kovats indices and column polarity

Kovats indices (*I*-values) are based on the retention time of an analyte compared to retention times of the series of *n*-alkanes. For a particular GC phase, *I*-values are very reproducible from one column or from one GC to another. However, they are slightly affected by GC programming conditions. *n*-Alkanes have most affinity for non-polar phases and tend to elute more quickly from polar phases. In contrast, a polar analyte will elute more slowly from a polar phase and thus, relative to the *n*-alkanes, its retention time, and thus its *I*-value, will increase as the polarity of the GC phase increases. A measure of the polarity of a stationary phase is given by its McReynold's constant (Table 11.1), which is based on the retention times of benzene, *n*-butanol, pentan-2-one, nitropropane and pyridine

Table 11.1 McReynold's constants

Phase	Chemical type	McReynold's constant
Squalane	Hydrocarbon	0
Silicone OV-1	Methylsilicone	222
Silicone SE-54	94% methyl, 5% phenyl, 1% vinyl	337
Silicone OV-17	50% methyl, 50% phenyl	886
Silicone OV-225	50% methyl, 25% cyanopropyl, 25% phenyl	1813
Carbowax	Polyethylene glycol	2318

on a particular phase. The higher the McReynold's constant, the more polar the phase. Many stationary phases are described by an OV-number. The higher the number after the OV, the more polar the phase.

I-values provide a useful method for characterising unknown compounds, and tables of *I*-values have been compiled for a large number of compounds.[3] Under temperature programming conditions, where the GC temperature rises at a uniform rate, e.g. 10°C/min, a plot of the carbon numbers of *n*-alkanes (where 1 carbon = 100) against their retention times is a straight line. Under isothermal conditions, where the column is maintained at the same temperature throughout the analysis, a plot of carbon number against the logarithm of the retention times of the *n*-alkanes is a straight line. Such calibration curves can be used to convert the retention time of a compound into an *I*-value.

Examples of the separation of mixtures by GC

Analysis of peppermint oil on two GC phases

Figure 11.8 shows the structures of some of the major components in peppermint oil. The use of the retention index system is illustrated in Figures 11.8 and 11.9 for peppermint oil run in comparison with *n*-alkane standards on both a weakly polar OV-5-type column and a polar carbowax column.

Figure 11.9 indicates approximate *I*-values for some of the components in peppermint oil on a BPX-5 column; this column selects mainly on the basis of molecular weight and shape. For example, ß-pinene has the same molecular weight as limonene but has a more compact shape and thus a lower *I*-value. Menthyl acetate has a higher *I*-value than menthol because of its higher molecular weight.

A carbowax column is highly selective for polar compounds. As can be seen in Figure 11.10, the group of polar compounds including menthol and menthone is resolved more extensively on a carbowax column, with the alcohol menthol

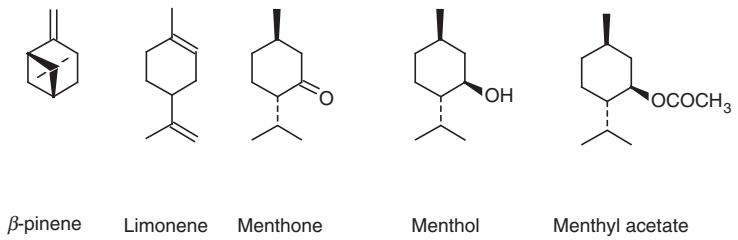


Fig. 11.8
The structures of some components in peppermint oil.

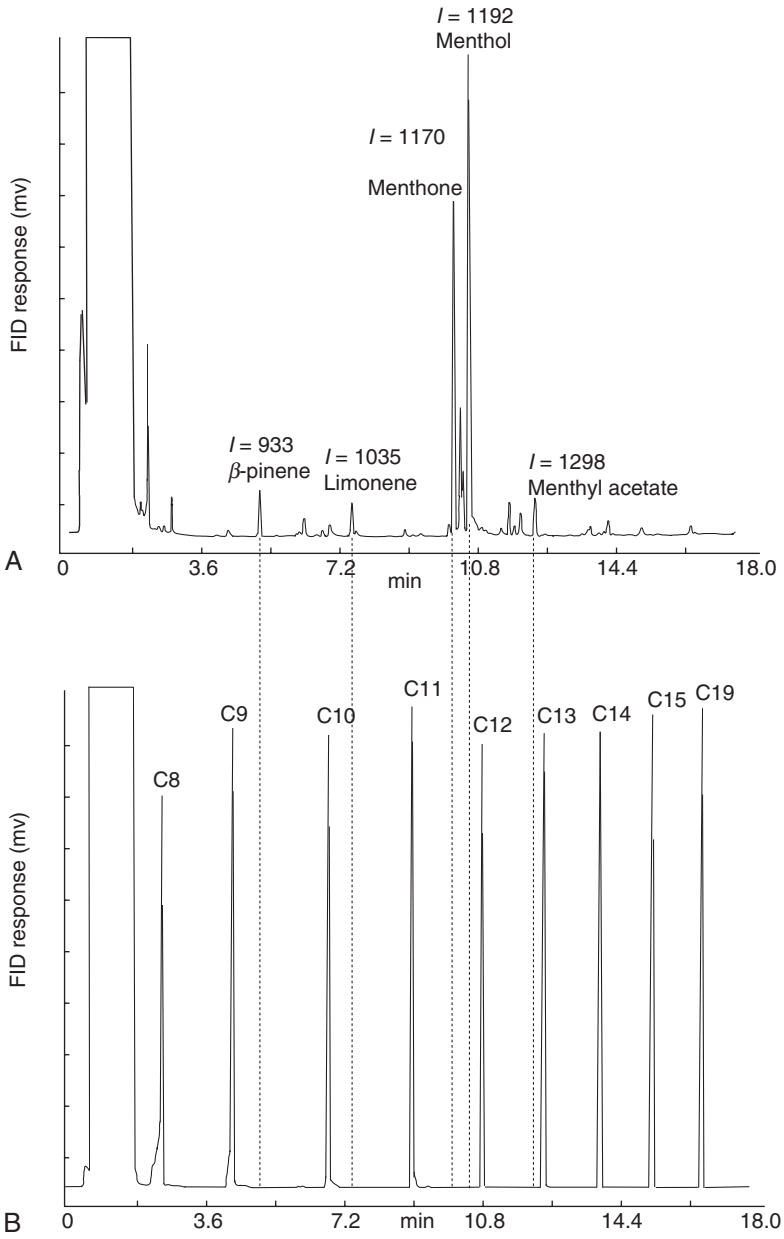
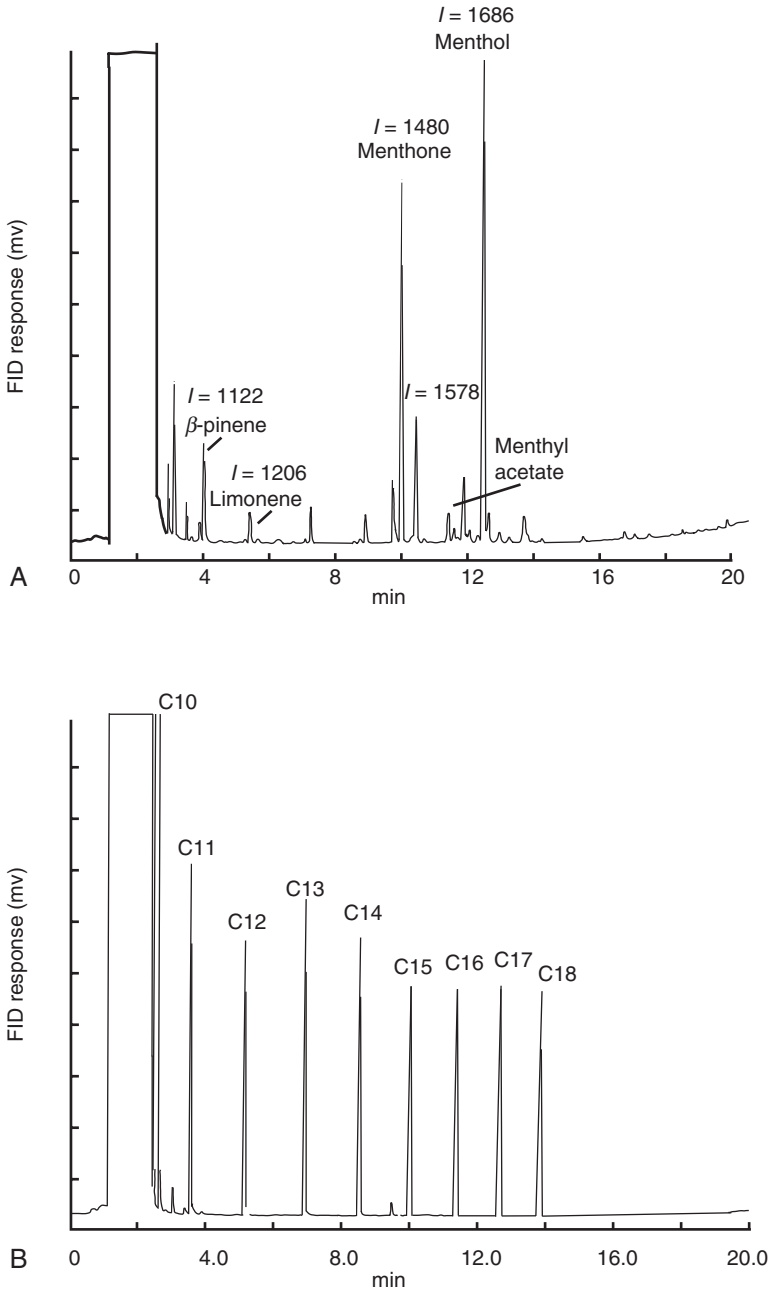


Fig. 11.9
(A) Peppermint oil analysed on a BPX-5 column (12 m × 0.25 mm i.d. × 0.25 μm film). Programmed 50°C (1 min), then 5°C/min to 70°C, then 10°C/min to 200°C. (B) *n*-alkanes C8-C16 chromatographed under the same conditions.

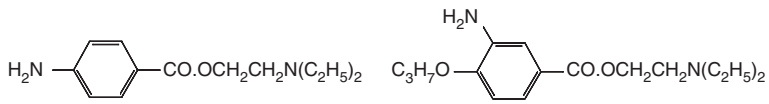
and a number of other minor alcohols eluting at around 12 min. In addition, the less polar ketone menthone and a number of minor ketones elute at around 10 min. Menthyl acetate, which on the non-polar BPX-5 column ran later than menthol, runs earlier than menthol on the carbowax column because its polar alcohol group is masked by the acetate and it thus has a lower polarity than menthol.

Fig. 11.10
(A) Peppermint oil analysed on a carbowax column (15 m × 0.25 mm i.d. × 0.5 μm film). Programmed 50°C (1 min), then 5°C/min to 70°C, then 10°C/min to 200°C. (B) *n*-alkanes C10-C18 chromatographed under the same conditions.



Self-test 11.1

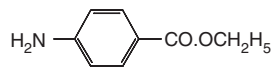
Associate the following *I*-values obtained on an OV-1-type column with structures of the local anaesthetics shown below. *I*-values: 1555, 2018, 2323 and 2457. Note: Oxygen in an ether linkage is equivalent to *ca* 1 CH_2 unit.



Procaine

Proxymetacaine

Butacaine



Benzocaine

Answers: Procaine 2018; Proxymetacaine 2323; Butacaine 2457; Benzocaine 1555

Analysis of the fatty acid composition of a fixed oil by GC

A very polar phase such as carbowax is generally only used for samples requiring a high degree of polar discrimination for adequate separation or retention. An example of this is in the analysis of fatty acids with differing degrees of unsaturation. On a non-polar column such as BPX-5, a series of C-18 acids such as stearic, oleic, linoleic and linolenic acids, which contain, respectively, 0, 1, 2 and 3 double bonds, overlaps extensively. However, on polar columns such as carbowax they are separated.

The BP monographs for many of the fixed oils contain a GC analysis to confirm the content of the fatty acids composing the triglycerides (fatty acid triesters of glycerol) present in the oil. The monograph for almond oil states the composition of the fatty acids making up the triglyceride should be:

- palmitic acid (16 : 0) 4.0–9.0%
- palmitoleic acid (16 : 1) < 0.6%
- margaric acid (17 : 0) < 0.2%
- stearic acid (18 : 0) 0.9–2.0%
- oleic acid (18 : 1) 62.0–86.0%
- linoleic acid (18 : 2) 7.0%–30.0%
- linolenic acid (18 : 3) < 0.2%
- arachidic acid (20 : 0) < 0.1%
- behenic acid (22 : 0) < 0.1%.

The first number in brackets, e.g. 16, refers to the number of carbon atoms in the fatty acid and the second number, e.g. 0, refers to the number of double bonds in the fatty acid. The percentage of each component is determined in relation to the sum of the areas of the chromatographic peaks of all the components listed above.

In order to determine the fatty acid composition of the triglycerides, they have to be first hydrolysed and the liberated fatty acids converted to their methyl esters, which have a good chromatographic peak shape compared to the free acids. A

See answer here

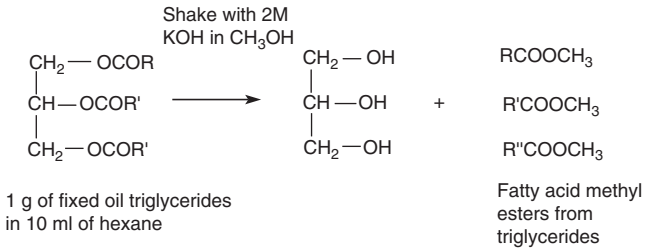


Fig. 11.11
Methanolysis of triglycerides prior to gas chromatography (GC) analysis.

convenient method for achieving hydrolysis and methylation in one step is shown in Figure 11.11.

A GC trace of methanolysed almond oil is shown in Figure 11.12. It can be seen that the methyl esters stearic, oleic and linoleic acid are incompletely resolved on a BPX-5 column. The esters of the minor C-20 and C-22 acids are also incompletely separated. When a carbowax column is used, complete separation of oleic (18 : 1), linoleic (18 : 2), stearic (18 : 0) and a small amount of linolenic acid (18 : 3) in the sample is achieved.

The chromatogram obtained on the carbowax column gave the percentage of areas of the peaks in this particular sample of almond oil as follows: 16 : 0 (7.0%), 16 : 1 (0.4%), 17 : 0 (0.12%), 18 : 0 (1.5%), 18 : 1 (62.8%), 18 : 2 (28.4%), 18 : 3 (0.16%), 20 : 0 (0.09%), 22 : 0 (0.09%). Thus the almond oil is within the BP specification given above.

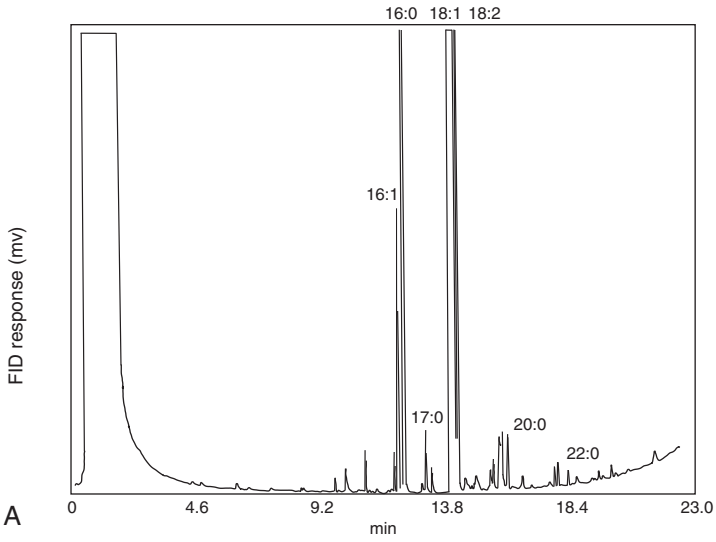
Chiral selectivity

An advanced type of column selectivity is chiral discrimination. Since enantiomers have identical physical properties, they are not separable on conventional GC columns. However, if chiral analytes are allowed to interact with a chiral environment, they will form transitory diastereomeric complexes, which results in their being retained by the column to a different extent. As increasing numbers of enantiomerically pure drugs are synthesised in order to reduce side effects, this type of separation will become increasingly important.

Chirasil Val was one of the first chiral GC phases; it has one chiral centre, as can be seen in its structure as shown in Figure 11.13.

A number of variations on this type of coating have been prepared and offer some improvement over the original phase. Figure 11.14 shows the volatile pentafluoropropionamide-trifluoroethyl ester (PFP-TFE) derivatives of *L* and *D* phenylalanine. Figure 11.15 shows the separation of PFP-TFE derivatives of the *D* and *L* enantiomers of the amino acids phenylalanine and *p*-tyrosine on a Chirasil Val column; the *D*(*R*)-enantiomers elute first. Chirasil Val generally performs best for the separation of enantiomers of amino acids; for many other compounds it is not as effective.

These phases are based on cyclodextrins, which are cyclic structures formed from 6, 7 or 8 glucose units. Alkylation of the hydroxyl groups in the structure of the cyclodextrins lowers their melting points and makes them suitable as GC phases. The cyclodextrins contain many chiral centres, and they separate enantiomers of drugs according to how well they fit into the chiral cavities of the cyclodextrin units (see Ch. 12, p. 351).



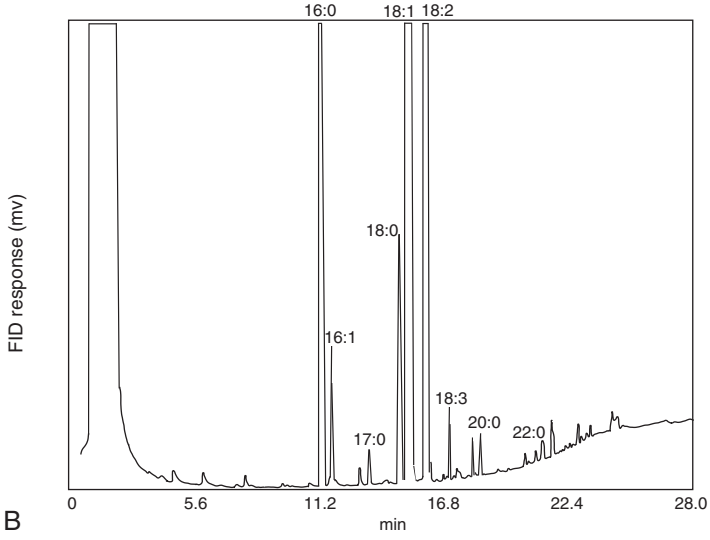


Fig. 11.12
(A) Separation of fatty acid methyl esters derived from almond oil on a BPX-5 column (12 m × 0.25 mm i.d. × 0.25 μm film). Programmed 100°C (1 min), then 10°C/min to 320°C. The major peaks are shown as offscale so that the minor peaks can be seen clearly. (B) The same sample separated on an HP Stabiliwax column (15 m × 0.25 mm × 0.5 μm film). Programmed 140°C (1 min), then 5°C/min to 250°C (5 min).

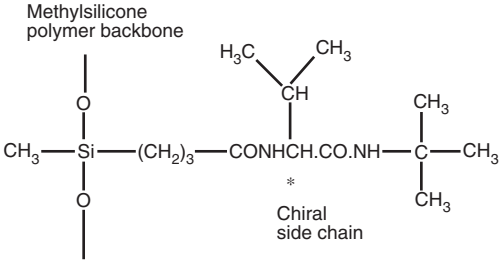
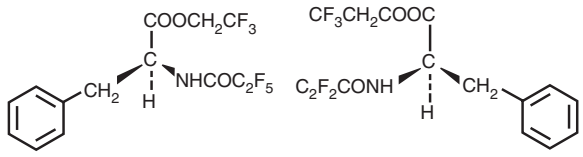


Fig. 11.13
The structure of Chirasil Val.

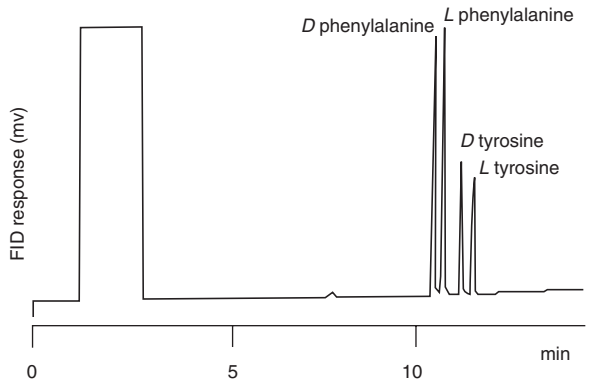
Fig. 11.14
Derivatives of *L* and *D* phenylalanine.



PFP–TFE derivative of *L* phenylalanine

PFP–TFE derivative of *D* phenylalanine

Fig. 11.15
Separation of the PFP-TFE derivatives of the *D* and *L* isomers of phenylalanine and *p*-tyrosine on a Chirasil Val column 25 m × 0.25 mm i.d. × 0.16 μm film. Programmed 120°C (1 min), then 3°C/min to 180°C.



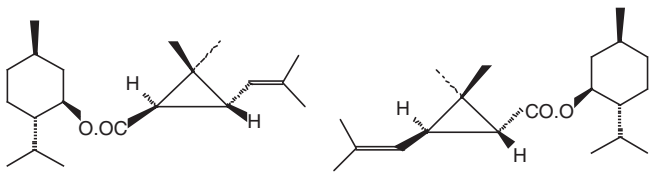
An alternative to buying expensive chiral columns in order to separate enantiomers is to use a chiral derivatisation agent. These reagents can be based on natural products which usually occur in an enantiomerically pure form. Chiral derivatising agents can often produce better separations than chiral columns, but if reaction conditions are too strong, there is a risk of small amounts of racemisation occurring in the analyte, i.e. chemical conversion of an enantiomer into its opposite. Reaction of an enantiomeric mixture with a chiral derivatising agent produces a pair of diastereoisomers which are separable by GC on non-chiral columns, e.g. the esters of menthol with (+) chrysanthemic acid.[4]

It can be seen in Figure 11.16 that, although the menthol portions of the esters are mirror images, addition of the chiral acylating reagent generates esters which are not mirror images but are diastereoisomers and thus have different physical properties.

Use of derivatisation in GC

Derivatisation has been mentioned above without fully indicating why it is necessary for conducting GC analysis. Derivatisation is generally required prior to GC

Fig. 11.16
(+) Chrysanthemyl esters of menthol.



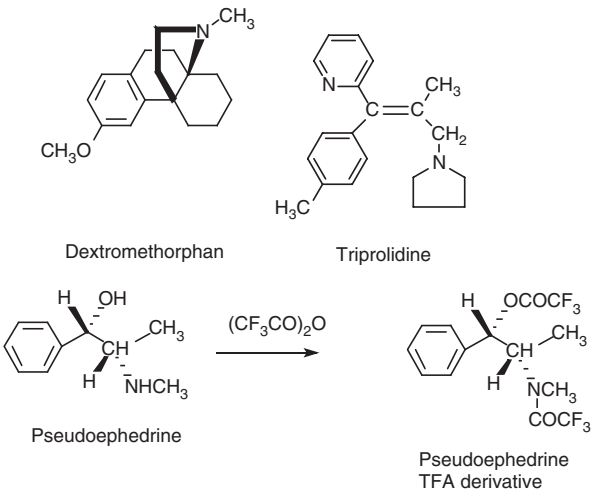


Fig. 11.17
The components in a decongestant + derivatisation of pseudoephedrine with trifluoroacetic anhydride (TFA).

if a compound is highly polar, so that good chromatographic peak shape can be achieved. A large number of derivatisation strategies are available.[2] In the following example, derivatisation is used to improve the peak shape of pseudoephedrine (Fig. 11.17).

A decongestant syrup was basified with ammonia and extracted into ethyl acetate, thus ensuring that the components extracted were in their free base forms rather than their salts, which is important for obtaining good chromatographic peak shape. Salts of bases will thermally dissociate in the GC injector port, but this process can cause a loss of peak shape and decomposition.

If the extract is run directly, the trace shown in Figure 11.18A is obtained. The free bases of triprolidine and dextromethorphan give good peak shape but pseudoephedrine, which is a stronger base and which has, in addition, a hydroxyl group in its structure, gives a poor peak shape. This can be remedied by masking the polar alcohol and amine groups of pseudoephedrine by reaction with trifluoroacetic anhydride TFA, resulting in the trace shown in Figure 11.18B. Treatment with TFA does not produce derivatives of the tertiary bases in the extract. This reagent is very useful because it is very reactive and boils at 40°C; thus excess reagent can be evaporated very easily prior to GC analysis and thus, unlike many reagents, it does not leave any residue. As outlined above, tertiary amines in their free base form give excellent GC peak shape.

Silylating reagents are another popular class of derivatisation reagents. Figure 11.19 shows the trimethylsilylation of glycerol and diethylene glycol (DEG) in order to improve GC peak shape by masking the hydroxyl groups in the structures and Figure 11.20 shows a GC trace of the separation of a mixture of glycerol and diethylene glycol in 20 : 1 ratio.

The pharmacopoeias utilise a GC test for DEG in glycerol. DEG is available in the form of anti-freeze, tastes sweet and has been used instead of glycerol to formulate syrups by unscrupulous manufacturers; it is, however, highly toxic. As can be seen in Figure 11.20 it is readily identified by using GC.

Fig. 11.18
Gas chromatography (GC) traces of an extract from a decongestant syrup. (A) Underivatised and (B) after treatment with trifluoroacetic anhydride.

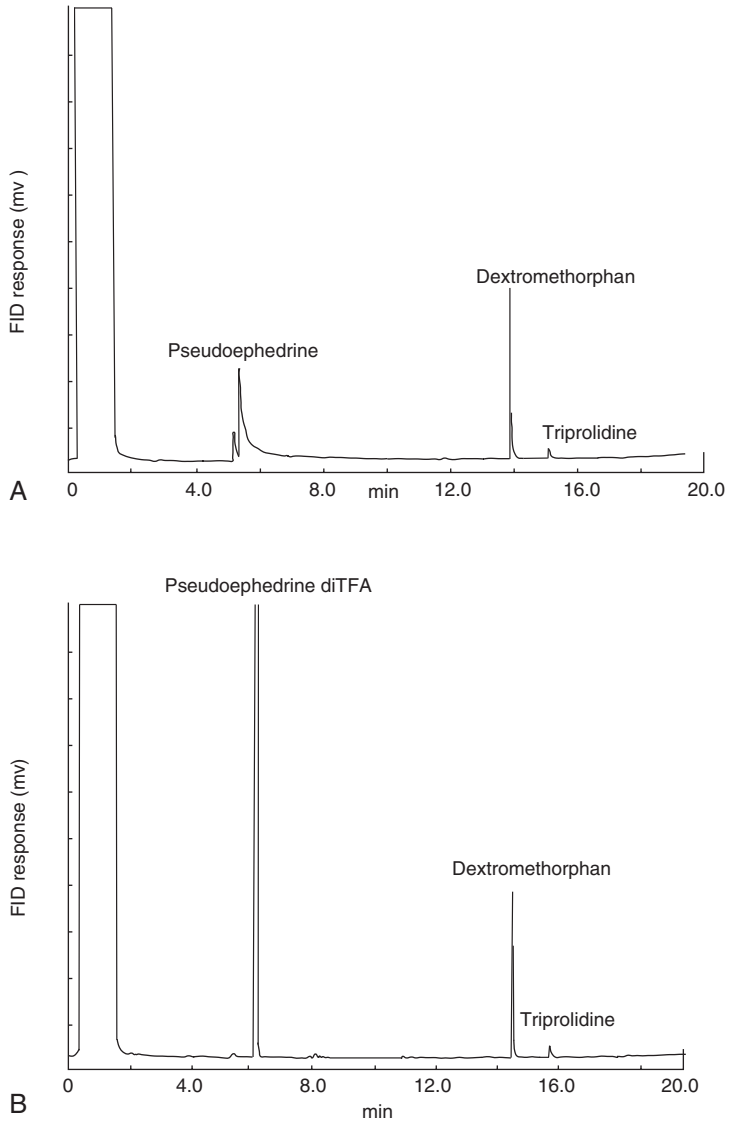
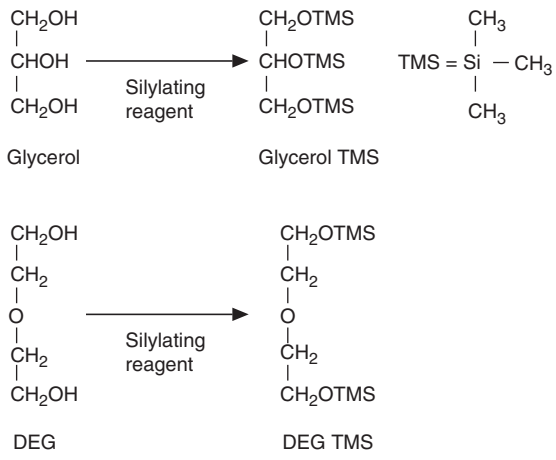


Fig. 11.19
Trimethylsilylation of glycerol and diethylene glycol in order to reduce their polarity prior to gas chromatography (GC) analysis.



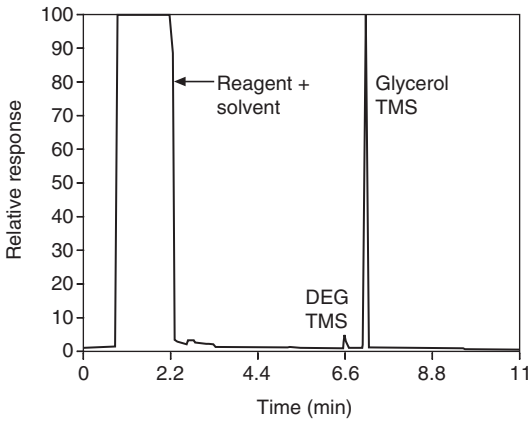
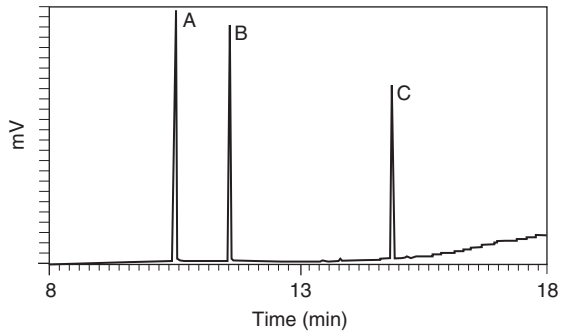
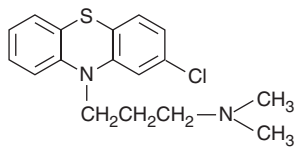


Fig. 11.20
Glycerol and diethylene glycol in 20:1 mixture separated on a 25 m × 0.32 mm i.d. × 0.5 μm film DB5 column programmed 100° (1 min), then 20°/min.

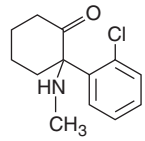
Self-test 11.2

A GC trace for bases A, B and C is shown below. Link the peaks to the structures.

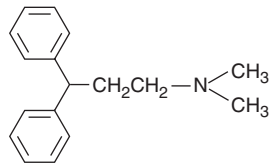




A Chlorpromazine



B Ketamine



C Chlorpheniramine

Answers: A ketamine, B chlorpheniramine, C chlorpromazine

See answer here

Summary of parameters governing capillary GC performance

Carrier gas type/flow

According to the Van Deemter equation, hydrogen and helium give higher efficiencies at high flow rates compared with nitrogen. For practical analysis times, hydrogen or helium is used in capillary GC and typical flow rates for hydrogen and helium are in the range of 30–50 cm/s; nitrogen has its optimum flow rate at 10–20 cm/s. Table 11.2 shows typical pressure settings to achieve optimal flow rate for three columns. The gas flow rate decreases with increasing temperature, and this may have an influence on column efficiency. Modern instruments have flow programming so that the flow can be set to remain constant as the temperature rises.

Table 11.2 Effect of temperature on flow rate at constant pressure

Column	Pressure	Temperature (°C)	T_o (s)	Flow rate	Temp (°C)	Flow rate	T_o (s)
25 m × 0.5 mm i.d.	22.2 KPa	100	83	30 cm/s	250	23.7 cm/s	106
25 m × 0.25 mm i.d.	91.1 KPa	100	83	30 cm/s	250	23.7 cm/s	106
12 m × 0.25 mm i.d.	42.8 KPa	100	40	30 cm/s	250	23.7 cm/s	51

Column temperature

As column temperature increases, the degree of resolution between two components decreases, because the degree of interaction with the stationary phase is reduced as the vapour pressure of the analytes increases. Lower temperatures produce better resolution.

Column length

The separating power of a column varies as the square root of its length. Thus, if a two-fold increase in resolution is required, a four-fold increase in column length would be required; this would result in a four-fold increase in analysis time. The increased resolution afforded by length can often be replaced with a decrease in temperature, ensuring that more interaction with the stationary phase occurs, especially if the stationary phase has characteristics that enable it to select one analyte more than another.

Film thickness phase loading

The greater the volume of stationary phase, the more a solute will partition into it. If the film thickness or loading of stationary phase doubles, then, in theory, the retention of an analyte should double. Thus thicker films are used for very volatile materials to increase their retention time and to increase resolution between analytes without increasing the column length.

Internal diameter

The smaller the internal diameter of a capillary column, the more efficient the column is for a given stationary-phase film thickness on the capillary wall. This is because the mass transfer characteristics of the column are improved, with the

analyte being able to diffuse in and out of the mobile phase more frequently because of the shorter distance for transverse diffusion (Ch. 10, p. 258).

Self-test 11.3

A fixed temperature is used and the head pressure is adjusted so that the linear velocity of a helium carrier gas through the following capillary columns is 20 cm/s: (i) 30 m × 0.25 mm i.d. × 0.25 μm OV-1 film; (ii) 15 m × 0.15 mm × 0.2 μm OV-1 film; (iii) 12 m × 0.5 mm i.d. × 1.0 μm OV-1 film.

a. List the columns in the order in which they would increasingly retain a *n*-hexadecane standard.
b. List the columns in order of increasing efficiency.

Answers: a. (ii) (i) (iii); b. (iii) (i) (ii)

GC detectors (see Animation 11.5)

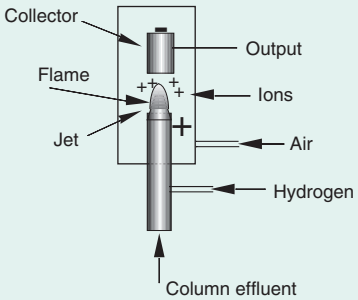
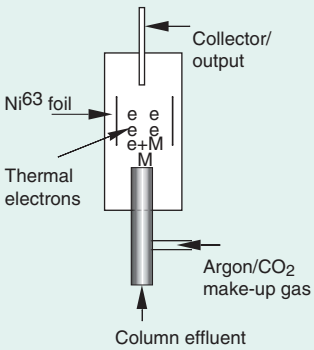
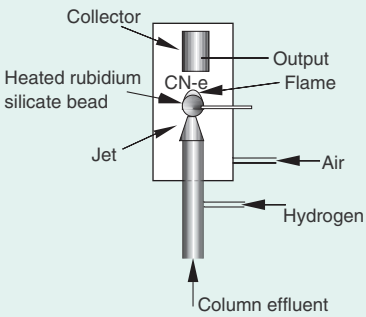
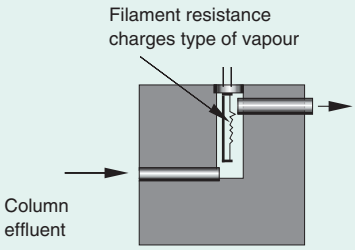
There are many GC detectors available, although the flame ionisation detector remains the most widely used and the most widely applicable to quality control of pharmaceutical products. However, newer detectors, such as the plasma emission detector for analysis of trace impurities or the GC-FTIR detector for the structural characterisation of components in mixtures, are becoming increasingly important. Selectivity in a detector is most often required for sensitive bioanalytical methods where trace amounts of compounds are being analysed in the presence of interferants, which are also present in the sample matrix. The properties of some commonly used detectors are summarised in Table 11.3.

Applications of GC in quantitative analysis

HPLC has more or less supplanted GC as a method for quantifying drugs in pharmaceutical preparations. Many of the literature references to quantitative GC assays are thus old, and the precision which is reported in these papers is difficult to evaluate based on the measurement of peak heights or manual integration. It is more difficult to achieve good precision in GC analysis than in HPLC analysis, and the main sources of imprecision are the mode of sample introduction, which is best controlled by an autosampler, and the small volume of sample injected. However, it is possible to achieve levels of precision similar to those achieved using HPLC methods. For certain compounds that lack chromophores, which are required for detection in commonly used HPLC methods, quantitative GC may be the method of choice for analysis of many amino acids, fatty acids and sugars. There are a number of assays in the BP, US Pharmacopoeia and European Pharmacopoeia, which are based on GC, but the selection of compounds analysed in this way appears to be rather random and many of the assays described could also be carried out by HPLC. The BP format for assays (for both HPLC and GC assays) is, most often, to run three samples. These are a calibration standard containing more or less equal amounts of a pure standard and an internal standard (Solution 1); an extract of the sample containing no internal standard to check for interference from the formulation matrix (Solution 2) and an extract from the sample containing the same amount of internal standard as Solution 1 (Solution 3). This is illustrated in Figure 11.21 for the analysis of methyltestosterone in a tablet formulation using testosterone as an internal standard (see p. 336).

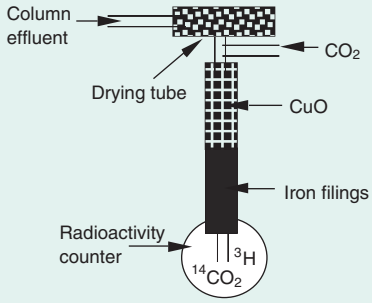
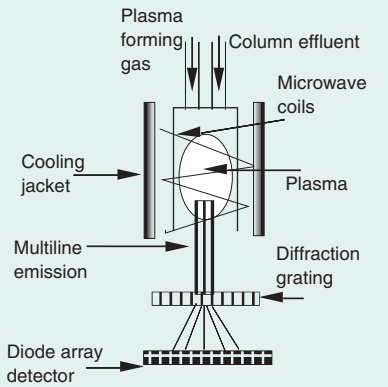
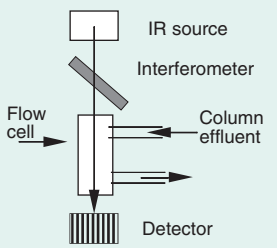
See answer here

Table 11.3 Commonly used gas chromatography (GC) detectors

Detector	Applications
Flame ionisation 	Compounds are burnt in the flame, producing ions and thus an increase in current between the jet and the collector. Detects carbon/hydrogen-containing compounds. Insensitive to carbon atoms attached to oxygen, nitrogen or chlorine. In combination with capillary GC it may detect as low as 100 pg–10 ng. Wide range of linear response *ca* 10^6
Electron capture 	Compounds with a high affinity for electrons enter the detector and capture the electrons produced by the radioactive source, thus reducing the current to the collector. Highly halogenated compounds can be detected at the 50 fg–1 pg level. Has a large internal volume, therefore, some chromatographic resolution may be lost. Linearity of response is not as great as flame ionisation detector (FID), e.g. 10^3. Mainly used for analysis of drugs in body fluids. Has wide application in environmental monitoring, e.g. chlorofluorocarbons in the atmosphere
Nitrogen phosphorus 	Nitrogen- and phosphorus-containing compounds react with the alkali metal salt in the detector to produce species such as CN^-, various phosphorus anions or electrons, all of which produce an increase in current which generates the signal. Detects phosphorus compounds at the pg level, nitrogen compounds at the low ng level. Highly selective for nitrogen- and phosphorus-containing compounds. Used mainly in the analysis of drugs and their metabolites in tissues and bodily fluids
Thermal conductivity (TCD) 	Responds to cooling effect of the analyte passing over the filament. Relatively insensitive to organic compounds in comparison to FID. It is a universal detector which can be used to determine water vapour. It is also nondestructive, so analytes can be collected after detection, if required. Used to determine water in some BP assays, e.g. water in the peptides menotrophin, gonadorelin and salcatonin

(*Continued*)

Table 11.3 *(Continued)*

Detector	Applications
Radiochemical detector 	^{14}C and ^3H present in the molecule are respectively converted to $^{14}CO_2$ and $^3H_2$. Its sensitivity depends on the degree of radioactivity of the analyte. Useful for metabolic labelling studies, making metabolites of drugs easy to detect. The detector tends to work better with packed columns, since it has a large internal space
Microwave-induced plasma atomic emission 	A hot plasma of argon is produced by heating to > 6000°C, which causes all the elements in the compound to produce emission spectra. The individual emission lines are passed through diffraction grating and detected by a diode array detector. Detects individual elements, e.g. chlorine in organochlorine pesticides and metals in organometallics. Sensitive to the pg level for some elements. Most widely used in environmental monitoring but also has useful potential for impurity profiling of drugs and metabolism studies
Fourier transform (FT) infrared (IR) detector 	Essentially just an FT-IR instrument coupled to a GC, thus allowing IR spectra of compounds eluting from the GC column to be obtained. More useful for structure elucidation rather than quantitative studies. The detector is sensitive to the 10 ng level. Used as a tool for qualitative identification. There are some examples of quantitative applications, e.g. determination of propandiol in acyclovir cream[5]

Analysis of methyltestosterone in tablets

A calibration solution containing *ca* 0.04% w/v of methyltestosterone and *ca* 0.04% w/v testosterone in ethanol is prepared (Solution 1). A weight of tablet powder containing *ca* 20 mg of methyltestosterone is extracted with 50 ml of ethanol to prepare Solution 2. Solution 3 is prepared by dissolving tablet powder containing *ca* 20 mg of methyltestosterone in *exactly* 50 ml of ethanol containing *exactly* the same concentration of testosterone as Solution 1. In this example, 0.5 μl amounts of the solutions were injected into the GC in the splitless mode.

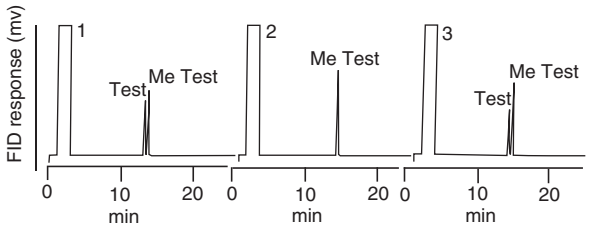


Fig. 11.21
Chromatograms of solutions 1, 2 and 3 prepared for the analysis of methyltestosterone tablets. RTX-1 column 15 m × 0.25 mm i.d. × 0.25 μm film. Programmed 150°C (1 min), then 10°C/min to 320°C (5 min).

Solution 1 gives a response factor for the calibration solution as follows:

$$\frac{\text{area of methyltestosterone peak in calibration solution}}{\text{area of testosterone peak in calibration solution}}$$

Solution 3 gives a response factor for the sample as follows:

$$\frac{\text{area of methyltestosterone peak in sample solution}}{\text{area of testosterone peak in sample solution}}$$

The amount of methyltestosterone in the tablet powder can be calculated as follows:

$$\text{amount of methyltestosterone} = \frac{\text{response factor for sample}}{\text{response factor for calibration solution}} \times \%\text{w/v of}$$

$$\text{methyltestosterone in calibration solution} \times \frac{\text{vol. sample solution}}{100}$$

Data from analysis of methyltestosterone tablets

- Weight of 5 tablets = 0.7496 g
- Stated content of methyltestosterone per tablet = 25 mg
- Weight of tablet powder taken for assay = 0.1713 g
- Solution 1 contains: 0.04% w/v methyltestosterone and 0.043% w/v testosterone
- Solution 3 contains: the methyltestosterone extracted from the powder taken for assay and 0.043% w/v testosterone
- Solution 1: Peak area testosterone = 216 268; Peak area methyltestosterone = 212 992
- Solution 3: Peak area testosterone = 191 146; Peak area methyltestosterone = 269 243.

Calculation example 11.1

$$\text{Response factor for Solution 1} = \frac{212\,992}{216\,268} = 0.9849$$

$$\text{Response factor for Solution 3} = \frac{269\,243}{191\,146} = 1.409$$

(Continued)

Calculation example 11.1 *(Continued)*

Amount of methyltestosterone in the tablet powder determined by analysis =

$$\frac{1.409}{0.9849} \times 0.04 \times \frac{50}{100} = 0.02861\,\text{g} = 28.61\,\text{mg}$$

Amount of methyltestosterone expected in tablet powder =

$$\frac{\text{weight of powder analysed}}{\text{weight of 5 tablets}} \times \text{stated content of 5 tablets} = \frac{0.1713}{0.7496} \times 5 \times 25$$

$$= 28.57\,\text{mg}$$

$$\text{Percentage of stated content} = \frac{28.61}{28.57} \times 100 = 100.1\%$$

A dilution factor may be incorporated into this calculation if the sample is first extracted and then diluted in order to bring it into the working range of the instrument. This approach to quantitation does not address the linearity of the method, but since the variation in the composition of formulations should be within ± 10% of the stated amount, there is some justification for using it. The precision of the method is readily addressed by carrying out repeat preparations of sample and calibration solutions.

Analysis of atropine in eyedrops

Another group which is used to mask polar groups in molecules in order to improve GC peak shape is the trimethylsilyl group. Atropine eyedrops BP are used to dilate the pupil prior to cataract surgery. The 1993 BP method for the analysis of atropine eyedrops BP uses derivatisation with a trimethylsilyl group to mask an alcohol group, as shown in Figure 11.22.

The method involves extraction of atropine and a homatropine internal standard from the aqueous phase, which is rendered alkaline by the addition of ammonia, followed by trimethylsilylation with *N*,*O*-bistrimethylsilyl acetamide (BSA).

In the calculation using the results of this experiment, it is better to use amount rather than concentration as a standard measure. The reason for this is, after the initial accurate volume, for the addition of the standard and internal standard to the calibration solution (Solution 1) and for the addition of the internal standard to a fixed volume of eyedrops, the volumes need only be measured approximately.

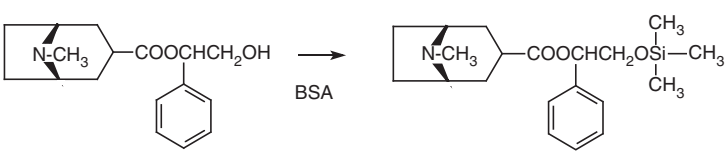


Fig. 11.22
Trimethylsilylation of atropine.

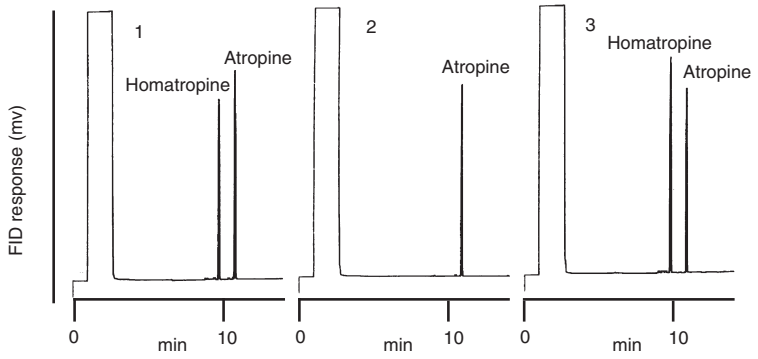


Fig. 11.23
Chromatograms of solutions 1, 2 and 3 prepared for the analysis of atropine in eyedrops. RTX-1 column 15 m × 0.25 mm i.d. × 0.25 μm film. Programmed 140°C (1 min), then 10°C/min to 320°C (5 min).

This is the advantage of using an internal standard (Fig. 11.23). The following formula is used:

$$\text{amount of atropine in the eyedrop sample} = \frac{\text{response factor for sample}}{\text{response factor for calibration solution}} \times \text{amount of atropine in calibration solution}$$

Brief description of the assay

Solution 1 is prepared from *exactly* 5 ml of 0.4092% w/v atropine sulphate solution and *exactly* 1 ml of 2.134% w/v homatropine hydrobromide solution. The solution is basified and extracted; the solvent is removed and the residue is treated with 2 ml of BSA and then diluted to 50 ml with ethyl acetate. Solution 3 is prepared from *exactly* 2 ml of eyedrops and *exactly* 1 ml of 2.134% w/v homatropine hydrobromide solution. The solution is basified and extracted; the solvent is removed and the residue is treated with 2 ml of BSA and then diluted to 50 ml with ethyl acetate.

Data from analysis of eyedrop formulation

- Volume of eyedrops analysed = 2.0 ml
- Stated content of eyedrops = 1.0% w/v
- Solution 1: Peak area homatropine TMS = 118 510; Peak area atropine TMS = 146 363
- Solution 3: Peak area homatropine TMS = 145 271; Peak area atropine TMS = 117 964.

Calculation example 11.2

Amount of atropine sulphate in Solution 1 = $0.4092 \times \frac{5}{100} = 0.02046$ g

Response factor for Solution 1 = $\frac{146\,363}{118\,510} = 1.2350$

(Continued)

Calculation example 11.2 *(Continued)*

$$\text{Response factor for Solution 3} = \frac{117\,964}{145\,271} = 0.8120$$

$$\text{Amount of atropine sulphate in Solution 3} = \frac{0.8120}{1.2350} \times 0.02046\ \text{g} = 0.01345\ \text{g}$$

This was the amount originally present in 2 ml of eyedrops; therefore, percentage of w/v of atropine sulphate in eyedrops

$$= 0.01345 \times \frac{100}{2} = 0.6725\%\ \text{w/v}$$

The amount determined in the eyedrops is well below the stated amount of 1% w/v, and this is because this sample of eyedrops was *ca* 10 years old and had probably suffered extensive degradation.

Self-test 11.4

Calculate the percentage of the stated content of hyoscine hydrobromide in travel sickness tablets from the following data. The assay is carried out in a manner similar to the eyedrop assay described above. The amount of atropine added as an internal standard does not enter into the calculation if we assume that the same amount is added to Solutions 1 and 3:

- Weight of 20 tablets = 2.1881 g
- Weight of tablet powder taken = 0.9563 g
- Stated content per tablet = 0.6 mg
- Concentration of hyoscine hydrobromide standard solution = 0.0341% w/v
- Volume of hyoscine hydrobromide solution added to Solution 1 = 15 ml
- Area of hyoscine peak in Solution 1 = 147 881
- Area of atropine peak in Solution 1 = 159 983
- Area of hyoscine peak in Solution 3 = 167 799
- Area of atropine peak in Solution 3 = 173 378.

Answer: 102.1%

Quantification of ethanol in a formulation

Gas chromatography provides a useful method for quantifying very volatile materials. In this case, columns are required which strongly retain volatile compounds. Ethanol is used in the preparation of tinctures and in disinfectant solutions. Typically ethanol may be quantified against a related alcohol. In the 1993 BP assay of chloroxylenol solution, ethanol is quantified against a propan-1-ol internal standard. The column used is packed with Porapak Q; Porapak is an example of a porous polymeric stationary phase which retains low-molecular-weight compounds strongly. These types of phases are also effective in separating gases such as CO_2, ammonia and acetylene. As an alternative to a Porapak column, a thick film (e.g. 5 μm film) GC capillary column may be used for this type of analysis.

See answer here

Determination of manufacturing and degradation residues by GC

Determination of pivalic acid in dipivefrin eyedrops

GC provides a useful technique for estimating volatile degradation products. For example, the pivalic acid release from the hydrolysis of dipivefrin in an eyedrop preparation (Fig. 11.24) used for treating glaucoma may be estimated by GC.[6] Isovaleric acid, which is an isomer of pivalic acid, provides a suitable internal standard. Breakdown products of esters are more likely to occur in aqueous formulations such as eyedrops or injections.

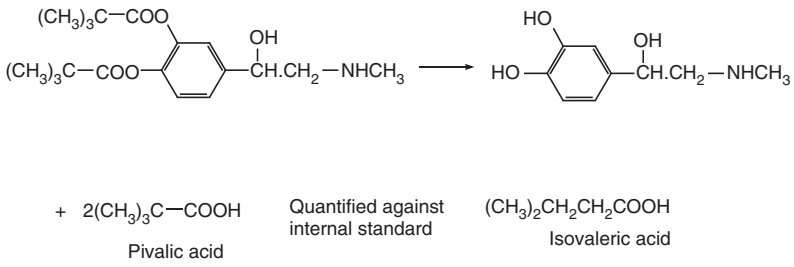


Fig. 11.24
Breakdown of dipivefrin resulting in formation of pivalic acid.

Determination of dimethylaniline in bupivacaine injection (Fig. 11.25)

Dimethylaniline is both a manufacturing impurity in bupivacaine and, since it is formulated in injections, a possible breakdown product, although hydrolysis of amides is much slower than hydrolysis of esters. The BP uses a spectrophotometric method to assay for this impurity, but GC provides a more sensitive and specific method for this determination.

The GC trace obtained from injection of a 10% w/v solution of bupivacaine free base extracted from an injection gave the trace shown in Figure 11.26. It is apparent from comparison with a standard for dimethylaniline that there is ≤ 0.1% of the impurity present, although a number of other peaks due to excipients or impurities can be seen in the GC trace.

Determination of N,N-dimethylaniline in penicillins

Determination of N,N-dimethylaniline in penicillins is carried out by GC. In this case the dimethylaniline is present in the sample since it is used as a counter ion in the purification of the penicillins by recrystallisation. The aniline counter ion

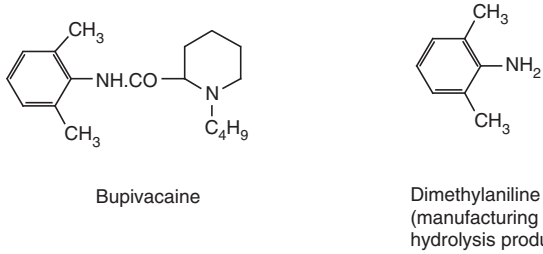


Fig. 11.25
Bupivacaine and its degradation product.

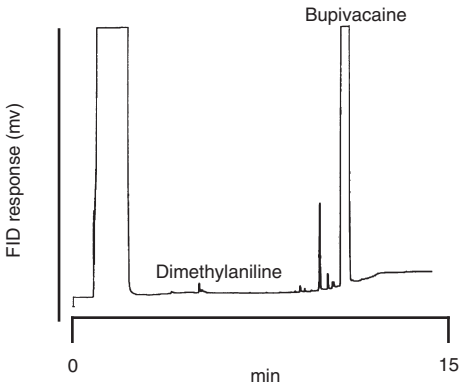


Fig. 11.26
Gas chromatography (GC) analysis of impurity residues in bupivacaine extracted from an injection. RTX-1 column 15 m × 0.25 mm i.d. × 0.25 μm film. Programmed 70°C (3 min), then 20°C/min to 320°C (5 min).

can be easily removed by mild basification and extraction. A pharmacopoeial limit test of 20 ppm is set for dimethylaniline using GC to analyse for the dimethylaniline residue. The penicillin is dissolved in 1 M NaOH, and the solution is extracted with cyclohexane containing naphthalene as an internal standard (Fig. 11.27). Then an aliquot of the cyclohexane layer is injected into the GC. Any peak obtained for N,N-dimethylaniline in the sample is compared with a peak obtained for a standard solution of dimethylaniline processed in the same way. The limit set by the BP for N,N-dimethylaniline in penicillins is 20 ppm.

Self-test 11.5

1.0512 g of ampicillin was dissolved in 5 ml of 1 M NaOH, and the solution was extracted into 1 ml of cyclohexane containing 50 μg/ml of naphthalene. 1 ml of a calibration sample containing approximately 20 μg/ml of N,N-dimethylaniline dissolved in 1 M HCL was mixed with 4 ml of 1 M NaOH and extracted into 1 ml of cyclohexane containing 50 μg/ml of naphthalene. The following data were obtained. Calculate the content of N,N-dimethylaniline in the sample in ppm:

Weight of N,N-dimethylaniline used to prepare calibration stock solution = 50.3 mg
Volume of calibration stock solution = 50 ml
Dilution of calibration stock solution= 5 ml to 250 ml
Peak area for dimethyl aniline obtained from calibration solution = 3521
Peak area obtained for naphthalene IS in calibration stock solution = 23616
Peak area for dimethyl aniline obtained from sample solution = 543
Peak area obtained for naphthalene IS in sample solution = 24773.

Answer: 2.81 ppm

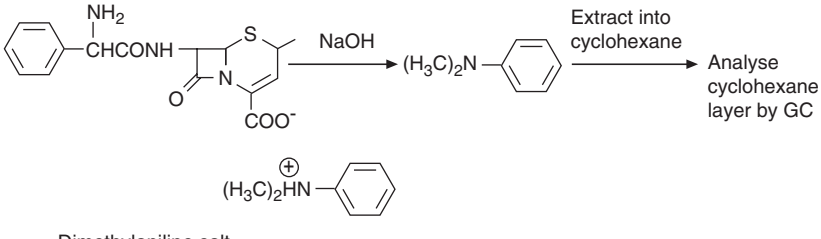


Fig. 11.27
Process used to analyse N,N-dimethylaniline residues in penicillins.

See answer here

Determination of a residual glutaraldehyde in a polymeric film

Sometimes derivatisation can provide a highly specific method of detecting impurities. In this example, the low-molecular-weight impurity glutaraldehyde, which is not stable to direct analysis by GC, is reacted with a high-molecular-weight derivatisating reagent pentafluorobenzyloxime; the reaction is shown in Figure 11.28. This reaction stabilises the analyte and increases its retention time into a region where it can be readily observed without interference from other components extracted from the sample matrix. The derivative is also highly electron capturing. In this example a GC method was found to be superior to an HPLC method using derivatisation with dinitrophenylhydrazine since the residues from the reagent produced less interference in the analysis.

The reaction is also useful for determining process residues such as formaldehyde and acetaldehyde, which occur as reaction residues in some plastic packaging materials.

The converse of this type of reaction has been used to determine hydrazine as a manufacturing impurity in the drug hydralazine by reaction of the hydrazine residue with benzaldehyde to form a volatile derivative for GC analysis.[7]

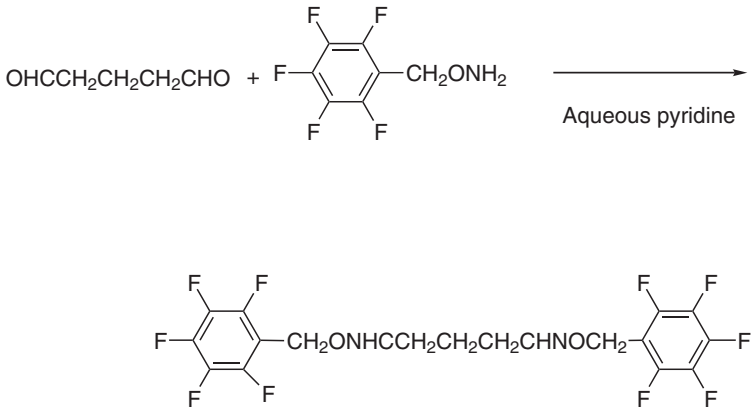


Fig. 11.28
Application of a selective derivatisation procedure used for the analysis of glutaraldehyde in a polymeric film.

Determination of residual solvents

Typical BP procedures

The current BP methods for determination of solvent residues remaining from the manufacturing process in pharmaceuticals rely on direct injection of the sample dissolved in a suitable solvent (often water) and are based on packed column GC. Some examples are given in Table 11.4.

Table 11.4 Some BP procedures for the analysis of residual solvents

Drug	Residues	Gas chromatography conditions
Ampicillin sodium	Dichloromethane	10% polyethylene glycol 60°C
Ampicillin sodium	Dimethylaniline	3% OV-17 80°C
Colchicine	Ethyl acetate and chloroform	10% polyethylene glycol 75°C
Gentamycin sulphate	Methanol	Porapak Q 120°C
Menotrophin	Water	Chromosorb 102, 114°C, TCD
Warfarin sodium	Propan-2-ol	10% polyethylene glycol 70°C

Determination of residual solvents and volatile impurities by headspace analysis (see Animation 11.6)

A more refined method for determining residual solvents and volatile impurities is based on headspace analysis. The simplest method of sampling is to put the sample into a sealed vial and heat it as shown in Figure 11.29. The sample, either in solution or slurried with a relatively involatile solvent with little potential for interference, e.g. water or dimethylacetamide, is put into a sealed vial fitted with a rubber septum and heated and agitated until equilibrium is achieved. Then a fixed volume of headspace, e.g. 0.25 ml, is withdrawn. The sample is then injected into a GC in the usual way. If capillary column GC is used, a split injection has to be used to facilitate sample injection; a flow of 10 : 1 out of the split vent would ensure that a 0.25 ml sample could be injected in about 1.5 s with the flow through the column being 1 ml/min.

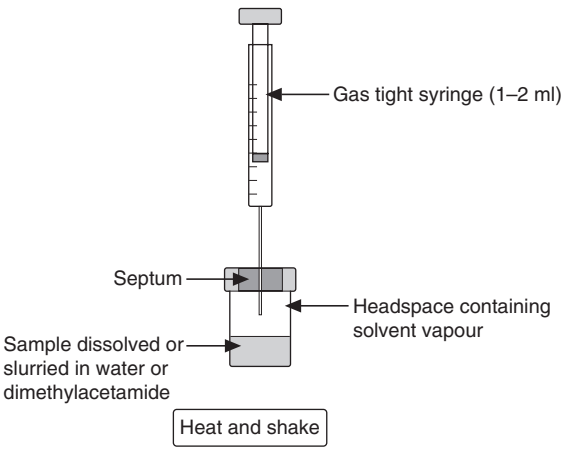


Fig. 11.29
Manual sampling of the headspace in a sealed vial with gas tight syringe.

Several points are important to note:

(i) Partition equilibrium must be established by heating for an appropriate length of time and at an appropriate temperature.

(ii) A clean room is required away from all other sources of volatiles such as laboratory solvents; potential interference from rubber septa needs to be checked; reactive contaminants may react with the sample matrix at high temperatures (e.g. dichloromethane is an efficient alkylating reagent and may react with amines).

(iii) If the sample is ground and mixed in preparation for the headspace analysis care has to be taken that no volatiles are lost.

(iv) For best reproducibility the process should be automated, an automated system for direct injection is shown in Figure 11.30, and for quantitative accuracy it might be best to use the method of standard additions (Ch. 6).

(v) N,N-dimethylacetamide or diethylene glycol do not interfere in the analysis since they are much less volatile than the common solvent residues. Water is the ideal solvent since it does not show up at all with FID detection; however, recently it has become less popular, since hot water vapour can damage GC capillary columns.

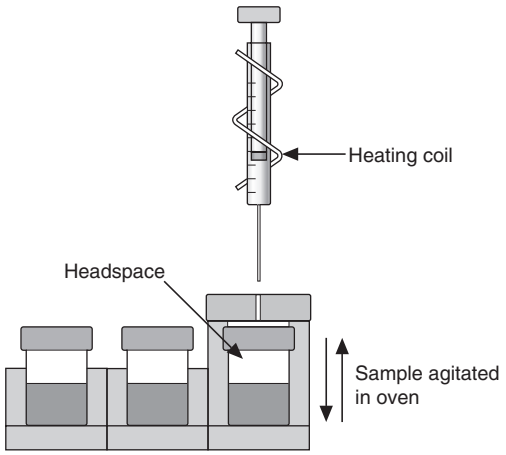


Fig. 11.30
Automated headspace sampling based on direct injection.

The European Pharmacopoeia has standardised on a phase composed of 6% cyanopropyl phenyl: 94% dimethylsiloxane (Fig. 11.31), e.g. DB-1301 or Rtx-1301. The film thickness used is generally 3 μm.

Figure 11.32 shows a chromatographic trace for some standard residual solvents dissolved in dimethyl acetamide, sampled using automated headspace sampling and separated on a Rtx-1303 column.

Self-test 11.6

Which of the following capillary columns would be most suitable for use in the determination of residual solvents by headspace analysis (consult Table 11.1)?

(i) OV-1 column 12 m × 0.2 mm i.d. × 0.25 μm film
(ii) OV-17 column 15 m × 0.33 mm i.d. × 0.5 μm film
(iii) OV-225 column 30 m × 0.5 mm i.d. × 3 μm film
(iv) OV-1 column 25 m × 0.5 mm i.d. × 1 μm film.

Answer: (iii)

Processes such as automated residual solvent analysis or determination of residual volatile monomers in packaging materials (e.g. ethylene oxide or vinyl chloride) may now be carried out during the manufacturing process. Such real-time monitoring has been aided by the development of fast GC. Since GC

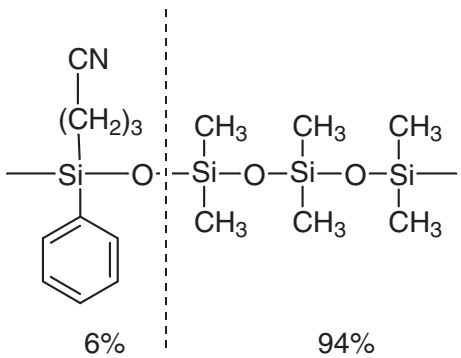


Fig. 11.31
The structure of Rtx-1301, a weakly polar phase.

See answer here

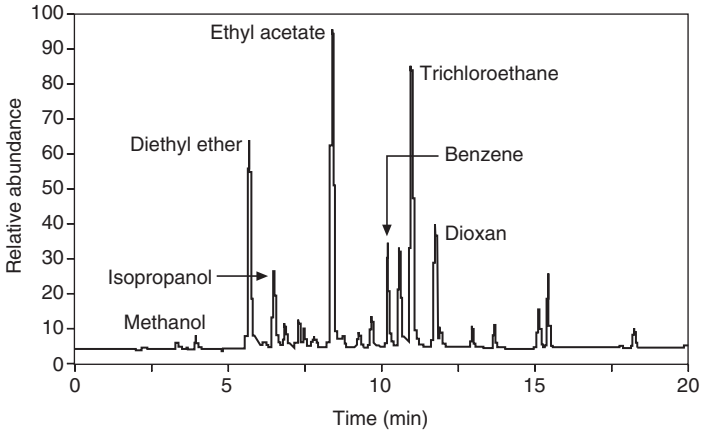


Fig. 11.32
Separation of a mixture of residual solvents on an Rtx 1303 column 30 m × 3 μm film. Sample dissolved in N,N-dimethylacetamide and headspace sampled. Flame ionisation detector.

columns have very high efficiencies, it is possible to reduce analysis times, in some cases to less than a minute, by using short columns with thin stationary-phase coatings in conjunction with rapid temperature programmes. Another advantage of short retention times is that detection limits are improved since the peaks produced are much narrower. In the case of a headspace analysis, improved detection limits mean that it is possible to inject a smaller volume of headspace in order to reduce injection time and make the injection compatible with a fast analysis time.

Purge trap analysis

Another form of headspace analysis uses a purge trapping device to trap volatile impurities. In this technique, a gas, e.g. helium, is bubbled through the sample, which is dissolved in suitable solvent (usually water), and the volatile impurities are thus 'stripped' from the solution and passed in the stream of gas through a polymeric adsorbant, where they become trapped and thus concentrated. The stream of gas is then switched so it passes in the reverse direction through the polymeric trap, which is heated to desorb the trapped volatiles, and the gas stream is then diverted into the GC. This type of procedure is used in environmental analysis to concentrate volatiles which are present at low levels in water.

Solid-phase microextraction (SPME) (see Animation 11.7)

Solid-phase microextraction (SPME) has developed rapidly over the past 10 years and has been applied quite widely in pharmaceutical analysis. It can be used to concentrate trace amounts of organic compounds either in the headspace of a sample or from an aqueous solution. SPME uses a fine fused-silica fibre coated with a polymer such as polydimethylsiloxane. The fibre is enclosed inside a metal needle so that it can pierce through a rubber septum into a vial. The fibre is then pushed out of the end of the metal needle and equilibrated with the sample while the sample is stirred and heated (Fig. 11.33). The fibre is then withdrawn into the needle and the needle is then pierced through the septum of a GC; the fibre is then pushed out of the metal needle again so that analytes can be thermally desorbed onto the GC column. Some GC autosamplers now offer the options of automatic injection from solution, headspace analysis and SPME all on the same instrument.

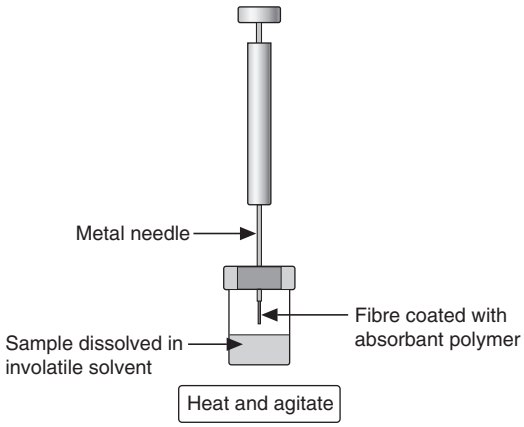
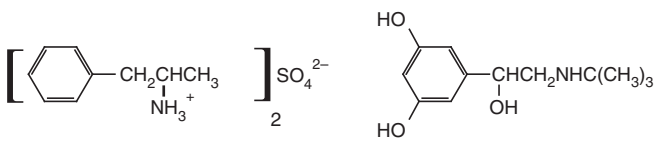


Fig. 11.33
Headspace sampling by solid phase microextraction.

Self-test 11.7

Suggest an analytical procedure based on gas chromatography for solving the following problems:

(i) Determination of residual solvents in a penicillin
(ii) Determination of the stability, in a formulation, of a drug with a propyl ester group
(iii) Determination of an amphetamine sulphate in tablet form
(iv) Determination of terbutaline in tablet form.



Amphetamine Terbutaline

Answers: (i) p289, (ii) p295, (iii) p280, (iv) p291

Applications of GC in bioanalysis

In order to determine an optimum dosage regimen for a drug and to determine its mode of metabolism, methods for analysis of the drug and its metabolites in blood, urine and tissues have to be developed. Analysis of drugs in biological fluids and tissues by GC is quite common, although GC-MS (see Ch. 9) has replaced many GC methods which are reliant on less selective types of detectors.

A typical application of GC to the determination of a drug in plasma is in the determination of the anti-epileptic drug valproic acid[8] after solid-phase extraction (see Ch. 15) by GC with flame ionisation detection. In this procedure, caprylic acid, which is isomeric with valproic acid, was used as an internal standard. The limit of detection for the drug was 1 μg/ml of plasma. The trace shown in Figure 11.34 indicates the more extensive interference that occurs in bioanalysis, from background peaks extracted from the biological matrix, compared with the quality control of bulk materials.

See answer here

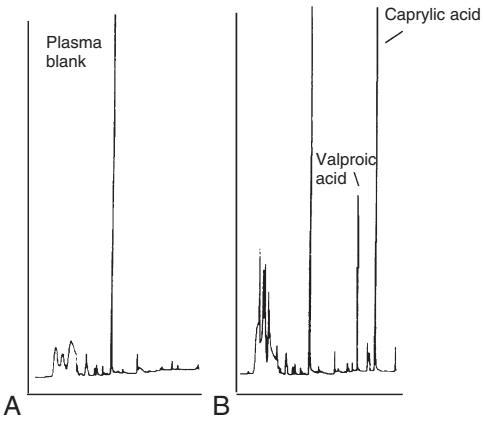


Fig. 11.34
(A) The gas chromatography (GC) trace of an extract of blank plasma obtained from a patient. (B) The GC trace of an extract of plasma obtained from the same patient after treatment with valproic acid (peak 1) to which caprylic acid (peak 2) has been added as an internal standard.

An example of the use of GC with nitrogen-selective detection is in the quantification of bupivacaine in plasma.[9] Bupivacaine contains two nitrogen atoms in its structure, which makes it a good candidate for this type of analysis. The limits of detection which can be achieved with a nitrogen-selective detector for this compound are much better than methods based on flame ionisation detection, which are much less selective.

Additional problems

1. Indicate the order of elution of the following compounds from an OV-1 column:
   1. Testosterone propionate
   2. Nandrolone
   3. Testosterone heptanoate
   4. Testosterone
   5. Methyltestosterone
   6. Nandrolone decanoate.

Answer: 2, 4, 5, 1, 3, 6

2. The manufacturing residue chloropropandiol was determined in iohexol; 1.0673 g of iohexol were dissolved in 2 ml of water. The solution was extracted four times with 2 ml of methyl acetate, and the extracts were combined and concentrated to 2 ml. A calibration solution was prepared by dissolving 0.5378 g of chloropropandiol in 100 ml of methyl acetate and then diluting 1 ml of this solution to 100 ml with methyl acetate. 2 μl of each solution were injected into the GC. Calculate the amount of chloroprandiol in ppm, in the sample of iohexol from the following data:
Peak area for chloroprandiol in sample = 3276
Peak area for chloroprandiol in standard= 123452

Answer: 2.67 ppm

3. A limit of 100 ppm methanol is set in ticlopidine. 0.2312 g of ticlopidine is dissolved in 1 ml of diethylene glycol (DEG) in a headspace vial. The calibration solution is prepared by dissolving 0.2023 g of methanol in 25 ml of DEG, then diluting 0.1 ml of this solution to 20 ml with DEG and finally transferring 0.5 ml of this dilution to a headspace vial and adding a further 0.5 ml of DEG. The solutions are then heated at 115°C for 15 minutes and then sampled and analysed by GC. Calculate the methanol content in the sample from the data below:
Peak area of methanol in ticlopidine sample = 1659
Peak area of methanol in calibration standard = 12432

Answer: 13.35 ppm

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Further reading

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www.separationsnow.com

This site is the separations equivalent of the spectroscopy site. Less comprehensive with regard to separation science but under development.

www.agilent.com

Agilent are one of the premier manufacturers of GC systems. The site contains many applications of gas chromatography in pharmaceutical quality control.

www.restek.com

Website of one of the premier manufacturers of gas chromatography columns.

www.chromatographyonline.com

The website of LCGC magazine has many practical tips on chromatography and the latest advances in chromatographic techniques.

12

High-performance liquid chromatography

KEYPOINTS

Principles

A liquid mobile phase is pumped under pressure through a stainless steel column containing particles of stationary phase with a diameter of 3–10 μm (1.7 μm in ultra-high-performance liquid chromatography (UPLC)). The analyte is loaded onto the head of the column via a loop valve, and separation of a mixture occurs according to the relative lengths of time spent by its components in the stationary phase. It should be noted that all components in a mixture spend more or less the same time in the mobile phase in order to exit the column. Monitoring of the column effluent can be carried out with a variety of detectors.

Applications

- The combination of high-performance liquid chromatography (HPLC) with monitoring by UV/visible detection provides an accurate, precise and robust method for quantitative analysis of pharmaceutical products and is the industry standard method for this purpose.
- Monitoring of the stability of pure drug substances and of drugs in formulations, with quantitation of any degradation products.
- Measurement of drugs and their metabolites in biological fluids.
- Determination of partition coefficients and p*K*a values of drugs and of drug protein binding.

Strengths

- Easily controlled and precise sample introduction ensures quantitative precision.
- HPLC is the chromatographic technique which has seen the most intensive development in recent years, leading to improved columns, detectors and software control.
- The variety of columns and detectors means that the selectivity of the method can be readily adjusted.
- Compared to gas chromatography (GC) there is less risk of sample degradation because heating is not required in the chromatographic process.
- It is readily automated.
- With the advent of UPLC methods can be very rapid.

Limitations

- There is still a requirement for reliable and inexpensive detectors which can monitor compounds that lack a chromophore.
- Drugs have to be extracted from their formulations prior to analysis.
- Large amounts of organic solvent waste are generated, which are expensive to dispose of.

Introduction

High-performance liquid chromatography (HPLC) is the technique most commonly used for the quantitation of drugs in formulations (Fig. 12.1) (see Animation 12.1). Pharmacopoeial assays still rely quite heavily on direct UV spectroscopy but, in industry, detection by UV spectrophotometry is usually combined with a preliminary separation by HPLC. The theoretical background of HPLC has been dealt with in Chapter 10. There are many comprehensive books on this technique.[1–5]

Instrumentation (see Animation 12.2 and Animation 12.3)

A standard instrumental system for isocratic elution consists of:

(i) a solvent reservoir

(ii) a pump capable of pumping solvent up to a pressure of 4000 psi and at flows of up to 10 ml/min

(iii) a loop injector, which may be fitted with a fixed-volume loop of between 1 and 200 μl (20 μl is often used as standard)

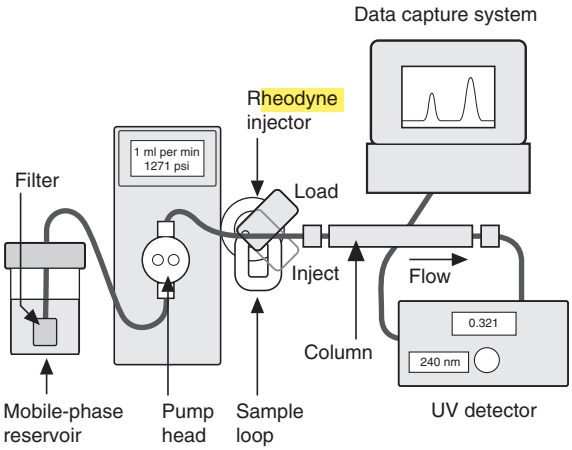


Fig. 12.1
Typical high-performance liquid chromatography (HPLC) system set to a flow rate of 1 ml/min producing a backpressure of 1271 psi and monitoring the column eluent at 240 nm.

(iv) a column, which is usually a stainless steel tube packed, usually, with octadecylsilane-coated (ODS-coated) silica gel with an average particle diameter (3, 5 or 10 μm)

(v) a detector, which is usually a UV/visible detector, although for specialist applications a wide range of detectors is available

(vi) a data capture system, which may be a computing integrator or a PC with software suitable for processing chromatographic data

(vii) the column is connected to the injector and detector with tubing of narrow internal diameter, *ca* 0.2 mm, in order to minimise 'dead volume', i.e. empty space in the system where chromatography is not occurring and band broadening can occur by longitudinal diffusion

(viii) more advanced instruments may have automatic sample injection and a column oven and are capable of mixing two or more solvents in varying proportions with time to produce a mobile-phase gradient.

Stationary and mobile phases

There are two principal mechanisms which produce retardation of a compound passing through a column. These are illustrated in Figure 12.2 for silica gel, which is a straight-phase packing, where the mechanism of retardation is by adsorption of the polar groups of a molecule onto the polar groups of the stationary phase and for an ODS-coated silica gel, which is a reverse-phase packing, where the mechanism of retardation is due to partitioning of the molecule into the stationary phase according to its lipophilicity.

Silica gel and ODS silica gel are two of the most commonly used packings for straight- and reverse-phase chromatography applications, respectively, but there is a variety of straight- and reverse-phase packings available, most of which are based on chemical modification of the silica gel surface, although in recent years stationary phases which are based on organic polymers have become available. The extent to which a compound is retained will depend primarily upon its polarity, in the case of silica gel, and primarily upon its lipophilicity in the case of a reverse-phase packing such as ODS silica gel. Most drug molecules have both lipophilic and polar groups. The other factor to consider with regard to the degree of retention of a particular compound, apart from the stationary phase, is the nature of the mobile phase. The more polar a mobile phase, the more

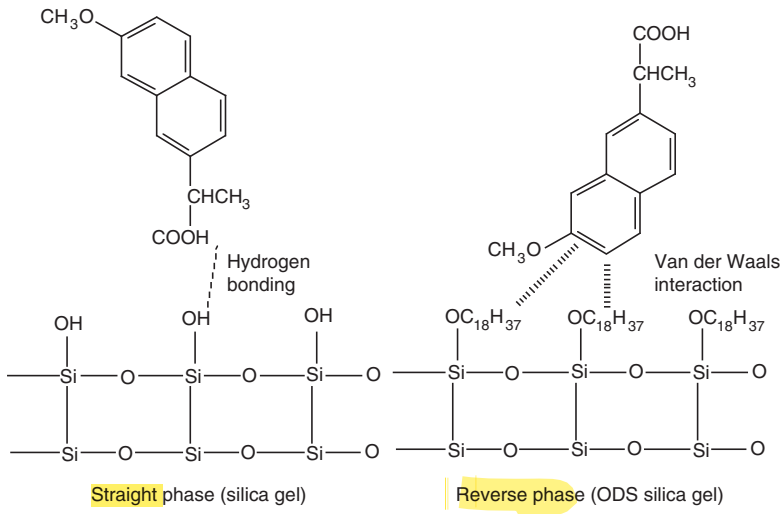


Fig. 12.2
Interaction of naproxen with the surfaces of silica gel and octadecylsilyl (ODS) silica gel high-performance liquid chromatography (HPLC) packings.

quickly it will elute a compound from a silica gel column, and the more lipophilic a mobile phase, the more quickly it will elute a compound from a reverse-phase column. Figure 12.3 shows the effect of increasing the % of organic solvent on the elution of a series of alkyl benzenes. In methanol/water mixture Dolan's rule of 3 applies where, in reverse-phase chromatography, a 10% decrease in methanol content produces a 3 times increase in capacity factor for an analyte. Taking the t_o value for both columns as 1.1 min then the capacity factors for propylbenzene can be calculated as follows:

In 80% methanol

$$K = \frac{5.6 - 1.1}{1.1} = 4.6$$

In 70% methanol

$$K = \frac{15.7 - 1.1}{1.1} = 13.3$$

thus supporting the rule of 3.

Self-test 12.1

Prednisolone (see Fig. 12.4 for the structure) is to be eluted from an ODS column. List the following solvent systems in order of decreasing rate at which they will elute prednisolone (i.e. in order of decreasing strength):

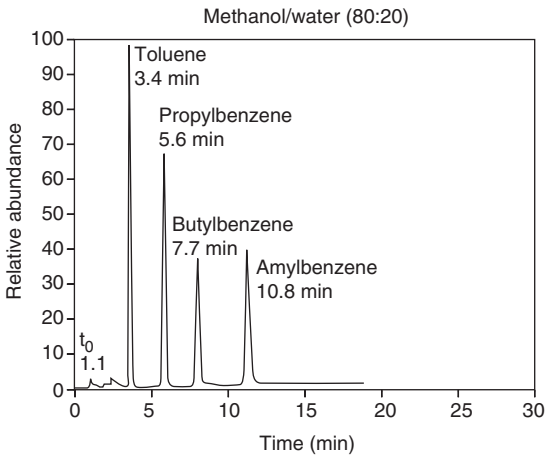
1. a. (i) methanol/water (20:80); (ii) methanol/water (80:20); (iii) methanol/water (50:50).
   b. (i) acetonitrile/water (50:50); (ii) methanol/water (50:50); (iii) acetonitrile/water/THF (50:40:10).

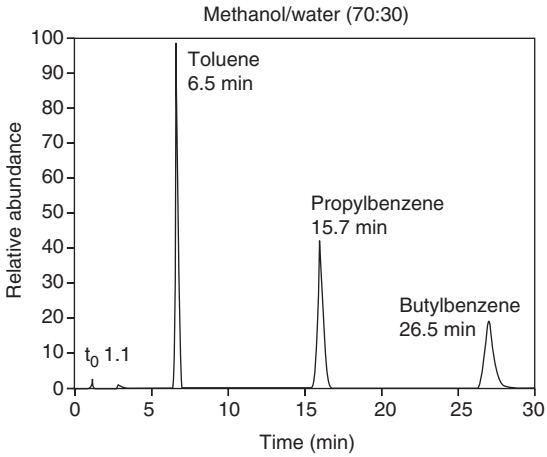
Prednisolone is to be eluted from a silica gel column.
List the following systems in order of decreasing rate at which they will elute prednisolone:

2. (i) hexane/isopropanol (90:10); (ii) hexane/dichloromethane (90:10); (iii) dichloromethane/methanol (90:10); (iv) dichloromethane/isopropanol (90:10); (v) dichloromethane/methanol (80:20).

Answers: 1. a. (ii), (iii), (i); b. (iii), (i), (ii); 2. (v), (iii), (iv), (i), (ii)

See answer here





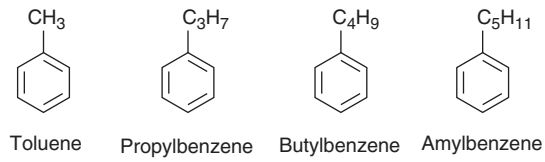


Fig. 12.3
Retention of a series of related compounds on a reversed phase high-performance liquid chromatography (HPLC) column (octadecylsilyl (ODS) 4.6 × 150 mm flow rate 1 ml/min) in methanol/water (80:20 v/v) and methanol/water (80:20). (see Animation 12.4, Animation 12.5 and Animation 12.6)

Structural factors which govern rate of elution of compounds from HPLC columns

Elution of neutral compounds

For a neutral compound it is the balance between its polarity and lipophilicity which will determine the time it takes for it to elute from an HPLC column; the pH of the mobile phase does not play a part. In the case of a reverse-phase column, the more lipophilic a compound is the more it will be retained. For a polar column such as a silica gel column, the more polar a compound is the more it will be retained. Polarity can often be related to the number and hydrogen-bonding strength of the hydroxyl groups present in the molecule; this is illustrated as follows for a series of corticosteroids shown in Figure 12.4. When these compounds are eluted from a reverse-phase column using a mobile phase containing

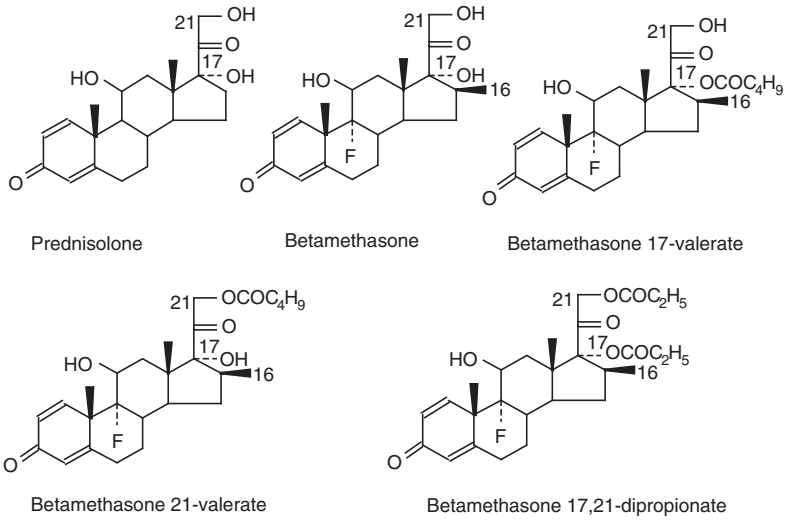


Fig. 12.4
The structures of some corticosteroids listed in order of their elution from an octadecylsilyl (ODS) column eluted with a methanol/water (75:25) mobile phase and below the chromatogram produced by the mixture.

methanol/water (75:25), the expected order of elution would be prednisolone, betamethasone, betamethasone 17-valerate, betamethasone 21-valerate and betamethasone dipropionate. Prednisolone should elute shortly before betamethasone since it lacks a lipophilic methyl group at position 16 (the fluorine group in betamethasone also contributes to its lipophilicity); the valerates both have large lipophilic ester groups masking one of their hydroxyl groups. The 21-hydroxyl group hydrogen bonds more strongly to the mobile phase, since it is an unhindered primary alcohol. Thus its conversion to an ester has a greater effect on the retention time of the molecule than esterification of the 17-hydroxyl group, which is a tertiary alcohol and is hindered with respect to hydrogen bonding to the mobile phase. Finally, the dipropionate of betamethasone has two lipophilic ester groups masking two hydroxyl groups, and this would mean that it would be most strongly retained by a lipophilic stationary phase. Figure 12.5 shows the chromatogram

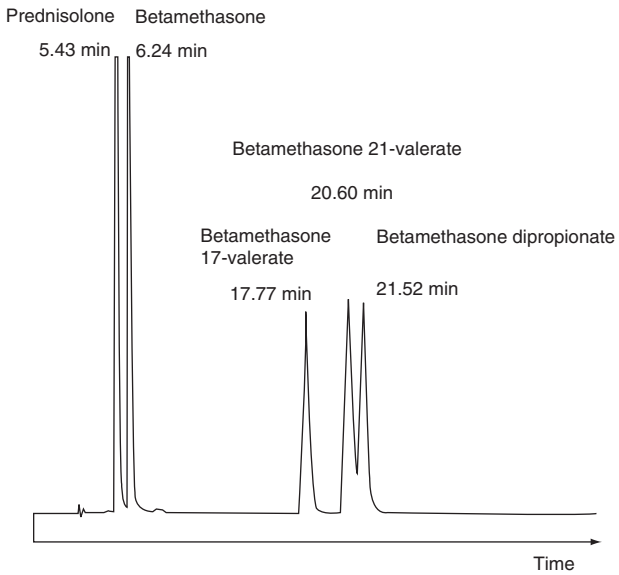
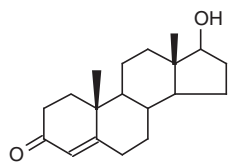


Fig. 12.5
Prednisolone and betamethasone and their esters eluted from an octadecylsilyl (ODS) column (25 cm × 4.6 mm) with methanol/water (75:25) as mobile phase, UV detection at 240 nm.

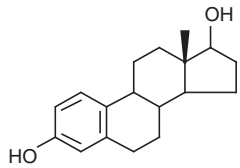
obtained from the mixture of corticosteroids using an ODS column with methanol/water (75 : 25) as the mobile phase, indicating that the order of elution fits prediction. The lipophilicity of the steroids reflects their pharmaceutical uses since the more lipophilic esters are used in creams and ointments for enhanced penetration through the lipophilic layers of the skin. The order of elution of these steroids would be more or less reversed on a polar silica gel column, although chromatographic behaviour is usually more predictable on reverse-phase columns. Considering the chromatogram shown in Figure 12.5 in more detail, the resolution between the betamethasone 21-valerate and the betamethasone dipropionate is incomplete. Increasing the water content of the mobile phase would result in longer retention times for these two components and better separation; however, increasing the water content would also give very long retention times. In the case of a formulation containing both the 21-valerate and 17,21-dipropionate, another type of column might be chosen to effect separation of these two components within a reasonable length of time, e.g. a silica gel column. If the betamethasone dipropionate were absent from this mixture, a different separation strategy could be adopted to bring the valerate esters closer to betamethasone and prednisolone. It would not be possible to add more methanol to the mobile phase without losing resolution between betamethasone and prednisolone, but, after these two compounds had eluted, if an HPLC system with a binary or ternary gradient system were used, the instrument could be programmed to gradually increase the methanol content in the mobile phase to expedite the elution of the later-running valerates (see Animation 12.7). For example, a suitable solvent programme might be as follows: methanol/water (75 : 25) for 7 min, then ramping the solvent composition to methanol/water (85 : 15) up to 17 min. This type of programme would greatly reduce the retention times of the valerates.

Self-test 12.2

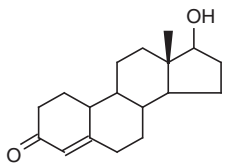
Predict the order of elution, from first to last, of the following steroids from an ODS column with methanol/water (70 : 30) as the mobile phase.



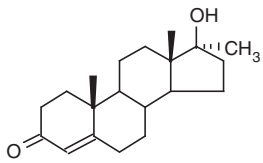
Testosterone



Estradiol



Nandrolone



Methyltestosterone

Answer: estradiol, nandrolone, testosterone, methyltestosterone

See answer here

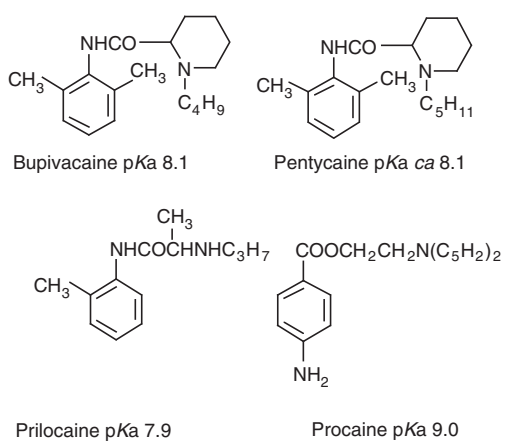
Control of elution rate of ionisable compounds by adjustment of pH of mobile phase

This area is not often considered in any detail in books on HPLC; however, pharmacists generally have a good grasp of the concept of p*K*a, and it is worth devoting some space to its effects in relation to HPLC. An additional factor which can be used to control the solvent strength of the mobile phase is pH; pH control is employed mainly in reverse-phase chromatography. However, mobile-phase conditions may be selected in straight-phase chromatography where the ionisation of the analytes is suppressed, and basic compounds are run in a basic mobile phase and acidic compounds are run with an acidic mobile phase. Control of the rate of elution via the pH of the mobile phase is, of course, only applicable to compounds in which the degree of ionisation is dependent on pH, but this covers a majority of commonly used drugs. The pH of the mobile phase can only be set within the range of *ca* 2–8.5 pH units because of the tendency for extremes of pH to dissolve silica gel and break the bonds between silane-coating agents and the silica gel support. This pH range is gradually being extended with the advent of stabler coatings. The effects of pH on retention time, surprisingly, are as yet not fully understood. The following examples give an approximation of the effect of the pH of the mobile phase on the retention time of drugs on a reverse-phase HPLC column, which provides a starting point for considering the effect of pH on retention time. In fact many drugs are still retained by lipophilic stationary phases to some degree even when they are fully ionised; in this case the drug is probably partitioning into the reverse phase as a lipophilic ion pair. The greatest effects of alteration of pH in the mobile phase are observed within 1 pH unit either side of the p*K*a value of the drug, i.e. where the partition coefficient of the partially ionised drug varies between 90% and 10% of the partition coefficient of the un-ionised drug (see Ch. 2, p. 31).

The same type of calculation as shown in Calculation example 12.1 can be carried out for basic drugs. Figure 12.6 shows the structures of some local anaesthetic drugs with their p*K*a values.

Figure 12.7 shows the effect of the pH of the mobile phase on the four local anaesthetics shown in Figure 12.6. The largest effects of pH are on bupivacaine

Fig. 12.6
The structures of some local anaesthetic bases.



Bupivacaine p*K*a 8.1

Pentycaine p*K*a *ca* 8.1

Prilocaine p*K*a 7.9

Procaine p*K*a 9.0

Calculation example 12.1

The effect of pH on the HPLC retention time of an ionisable acidic drug.

Ibuprofen, an acidic drug, which has a p*K*a of 4.4, is analysed by chromatography on ODS silica gel with a mobile phase consisting of acetonitrile/0.1 M acetate buffer pH 4.2 (40 : 60).

The t_o for the column at a mobile-phase flow rate of 1 ml/min is 2.3 min. The retention time of ibuprofen at pH 4.2 is 23.32. If K'app is the apparent capacity factor of the partially ionised drug, then:

$$K'\text{app at pH } 4.2 = 23.32 - 2.3/2.3 = 9.14$$

Using the expression introduced in Chapter 2 for the effect of pH on partition coefficient of an acid, it is possible to predict approximately the effect of pH on retention time, since the effect of pH on partition coefficient will reflect its effects on capacity factor and in theory:

$$K'\text{app} = K'/1+10^{\text{pH}-\text{p}Ka}$$

Using the observed K' app at pH 4.2: $9.14 = K'/1+10^{4.2-4.4} = K'/1.63$

$$K' = 9.14 \times 1.63 = 14.90$$

If ibuprofen is analysed using the same ODS column with the mobile phase now composed of acetonitrile/0.1 M acetate buffer at pH 5.2 (40 : 60), the partition coefficient will now be lowered as follows:

$$K'\text{app at pH } 5.2 = K'/1+10^{5.2-4.4} = 14.9/7.3$$

$$K'\text{app at pH } 5.2 = 2.04$$

$$\text{Retention time} = t_o + t_o \times K'\text{app} = 2.3 + 2.3 \times 2.04 = 7.0\,\text{min}$$

Experimentally, the retention time of ibuprofen was found in fact to be 12.23 min. This reflects the fact that the p*K*a of the drug may not be exactly as given in the literature under the conditions used for chromatography and the fact that the low dielectric constant of the mobile phase in comparison with water suppresses ionisation so that the drug is less ionised than predicted. However, the calculation gives a reasonable approximation of the behaviour of ibuprofen.

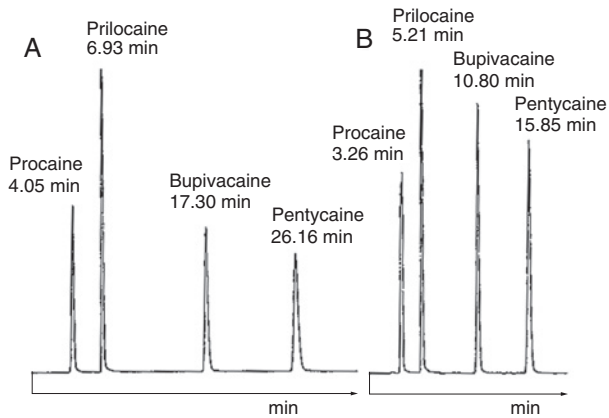


Fig. 12.7
Some local anaesthetics eluted from an octadecylsilyl (ODS) column with (A) acetonitrile/TRIS.HCl buffer pH 8 (40:40). (B) acetonitrile/TRIS.HCl buffer pH 7 (40:60).

Calculation example 12.2

The effect of pH on the HPLC retention time of an ionisable basic drug. Bupivacaine, which has a p*K*a of 8.1, is analysed by chromatography on ODS silica gel with a mobile phase consisting of acetonitrile/TRIS buffer pH 8.4 (40 : 60) at a flow rate of 1 ml/min. The t_o for the column at a mobile-phase flow rate of 1 ml/min is 2.3 min. The retention time of bupivacaine at pH 8.4 is 17.32. If K'app is the apparent capacity factor of the partially ionised drug, then for a base:

$$K'\text{app} = K'/1+10^{\text{p}Ka-\text{pH}}$$

$$\text{The } K'\text{app at pH } 8.4 = 17.82 - 2.3/2.3 = 6.75$$

$$6.75 = K'/1+10^{8.1-8.4} = K'/1.5$$

$$K' = 6.75 \times 1.5 = 10.13$$

If the drug were analysed using acetonitrile/TRIS buffer pH 7.4 (40 : 60) at a flow rate of 1 ml/min using the same column, the retention time can be estimated as follows:

$$K'\text{app at pH } 7.4 = K'/1+10^{8.1-7.4} = 10.13/6.01$$

$$K'\text{app at pH } 7.4 = 1.69$$

$$\text{Retention at pH } 7.4 \text{ time} = t_o + t_o \times K'\text{app} + 2.3 + 1.69 \times 2.3 = 6.18 \text{ min}$$

Experimentally, the retention time was found to be 10.80 min. The deviation from the theoretical value was probably due to the factors discussed earlier for ibuprofen.

and pentycaine, which are very close in structure; the pH adjustment made in the example is within ± 1 pH unit of their p*K*a values. The least effect is on procaine, which has a higher p*K*a (9.0) than the other drugs and is thus already 80% ionised at pH 8.4; for this reason, the lowering of the pH has a less marked effect on its retention time. The effect of pH on prilocaine might initially appear somewhat less than expected, but this is because it is closer to t_o than the other drugs; the decrease in its retention time observed at the lower pH is, in fact, in line with the decreases observed for bupivacaine and pentycaine. In chromatogram B, the procaine peak has lost some of its integrity due to its proximity to the solvent front; this results in poor trapping of the analyte at the head of the column. The effect of the organic content of the mobile phase on the p*K*a of analytes is given some additional consideration in Box 12.1.

Self-test 12.3

The retention time of the acidic drug naproxen on an ODS column with a t_o of 2.3 min in a mixture containing acetonitrile/0.05 M acetate buffer pH 5.2 (40 : 60) is 9.07 min. The p*K*a of naproxen is 4.2; what would be the effect of reducing the pH of the mobile phase to 4.2?

Answer: In theory, the retention time would be 39.47 min (in practice it was found to be 19.78 min. The p*K*a of this drug is probably lower than the literature value under the mobile-phase conditions used or it is less ionised at pH 5.2 than expected in the mobile phase, which has a lower dielectric constant than water)

Figure 12.8 shows the effect of pH on the retention of a series of acidic non-steroidal anti-inflammatory drugs shown in Figure 12.9.

See answer here

Box 12.1 Additional consideration of mobile-phase pH

A major factor that is often ignored in preparing mobile phases is the effect of the addition of organic solvent to the buffer. The effect of addition of acetonitrile on the pifa value of acetic acid has been calculated to be as follows:[6]

Percentage of w/w acetonitrile:	0	10	30	40	50
p*K*a value of acetic acid:	4.75	5.0	5.6	6.0	6.4

The addition of organic solvent thus suppresses the ionisation of the acid, reducing the $[H^+]$ in solution, and the overall effect is an increase in pH. The same effect can be observed for other buffers such as phosphate and citrate, and with 50% organic solvent the effective pH of the mobile phase may be 1–1.5 units higher than the measured pH of the buffer before mixing.

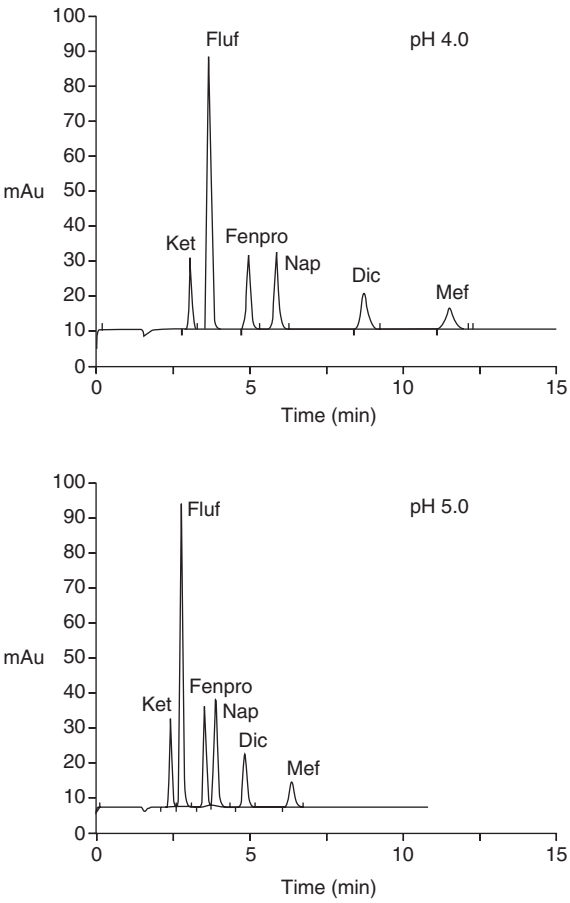


Fig. 12.8
Effect of pH on the retention of non-steroidal anti-inflammatory drugs (ketoprofen, flufenamic acid, fenoprofen, naproxen, diclofenac, mefenamic acid). Reversed phase column with methanol/0.05 M acetate buffer pH 5 (70:30) as the mobile phase 4 (60:40) and (B) acetonitrile/TRIS.HCl buffer pH 7.4 (60:40).

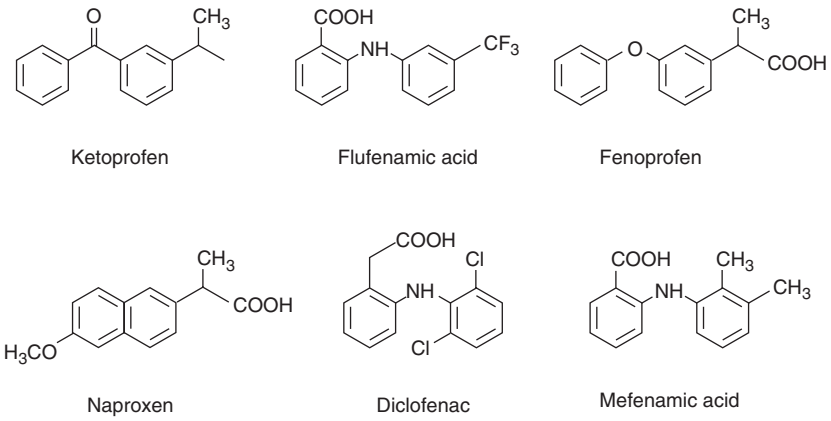


Fig. 12.9
Structures of fenoprofen, naproxen, diclofenac and mefenamic acid.

More advanced consideration of solvent selectivity in reverse-phase chromatography

The simplest selectivity factor that can be varied in reverse-phase chromatography is solvent composition. Lowering the amount of organic solvent in the mobile phase during reverse-phase chromatography increases the retention time. Dolan's rule of 3 states that a 10% decrease in the organic phase produces a threefold increase in capacity factor, this only really applies to methanol in the case of acetonitrile the increase in capacity factor is approximately 2 for a 10% reduction in organic component. The three common solvents used in reverse-phase chromatography are methanol, acetonitrile and tetrahydrofuran. Another approximate rule is that 40% methanol = 33% acetonitrile = 23% tetrahydrofuran. Apart from eluting power these three solvents also differ in the way that they interact with analytes. Selectivity is important in the impurity profiling of drug substances. Impurities closely related to the drug may elute with very similar retention times. The selection of an optimal solvent system is thus important, and in such cases mixtures of three or four solvents may be used. Figure 12.10 shows the effect of using mixtures of three solvents on the separation of hydrocortisone and the closely related steroid cortisone (Fig. 12.11). The cortisone is present in the mixture at 5% of the concentration of hydrocortisone. In chromatogram A, separation was obtained between the two peaks with acetonitrile/water (30 : 70). In chromatogram B, a mixture of acetonitrile/methanol/water (15 : 15 : 70) does not produce a separation, even though the introduction of methanol produces a longer retention time. In chromatogram C, a mixture of acetonitrile/THF/water (15 : 15 : 70) causes a reversal in the elution order of hydrocortisone and cortisone. The elution of the smaller, potential impurity, peak for cortisone earlier than the hydrocortisone is more desirable, since even slight tailing of the large peak for the major component can cause closely eluting later-running peaks to be obscured. Such a hit and miss approach for achieving an optimal solvent system for separating out minor impurities could be time consuming. A less hit and miss approach uses log K′ plots to predict retention times. Table 12.1 shows the data obtained

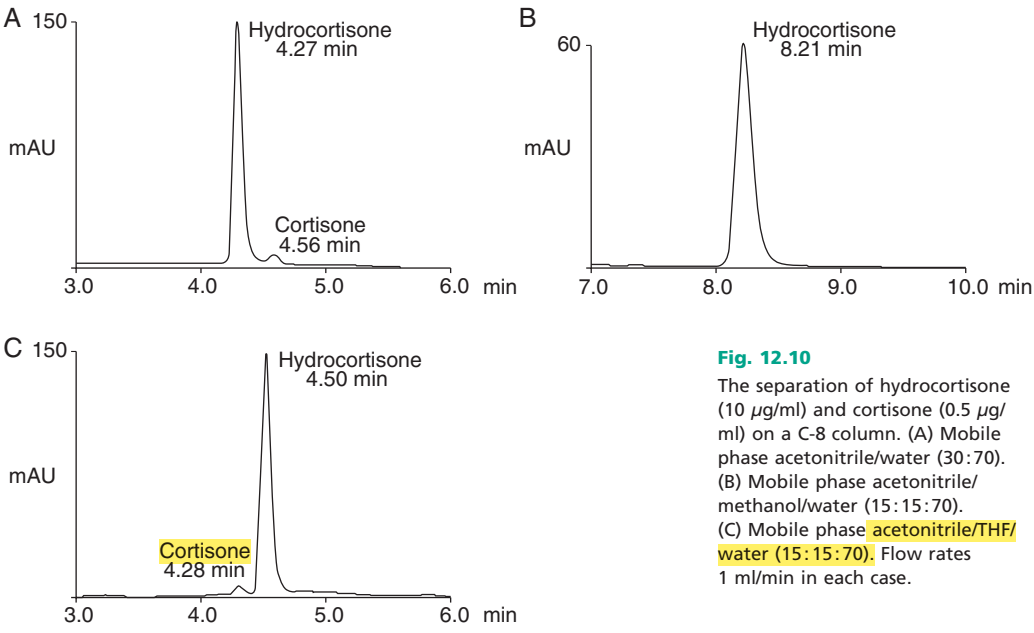


Fig. 12.10
The separation of hydrocortisone (10 μg/ml) and cortisone (0.5 μg/ml) on a C-8 column. (A) Mobile phase acetonitrile/water (30:70). (B) Mobile phase acetonitrile/methanol/water (15:15:70). (C) Mobile phase acetonitrile/THF/water (15:15:70). Flow rates 1 ml/min in each case.

Table 12.1 Data used plot log K′ against % MeOH (t_0 for column 1.8 min)

Compound	tr 60% MeOH min	K′ 60% MeOH	Log K′ 60% MeOH	tr 50% MeOH min	K′ 50% MeOH	Log K′ 50% MeOH
Cortisone	4.01	1.22	0.09	8.58	3.78	0.58
Hydrocortisone	5.03	1.77	0.25	11.01	5.11	0.71
Ketoprofen	7.95	3.44	0.53	21.49	10.9	1.04
Sulindac	8.28	3.61	0.56	27.57	14.3	1.16

for the analysis of two steroidal and two non-steroidal anti-inflammatory (Fig. 12.12) drugs on a reverse-phase column in mobile phase consisting of methanol and 0.1% v/v aqueous formic acid. The capacity factors for the four drugs can be calculated, and from just two runs log K′ plots can be constructed (Fig. 12.13) based on the assumption that plots of log K′ against percentage of organic solvent is linear where simple reverse-phase partitioning is occurring.

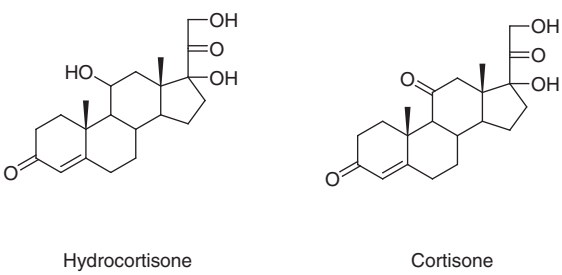
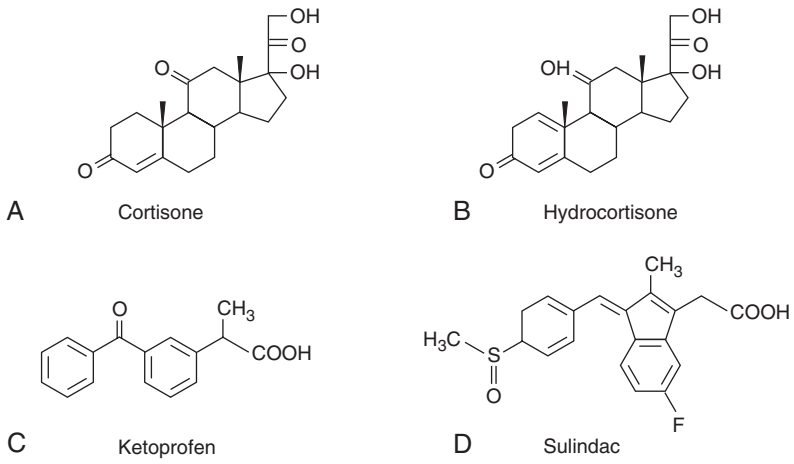


Fig. 12.11
Hydrocortisone and cortisone.

Fig. 12.12
Ketoprofen and sulindac.

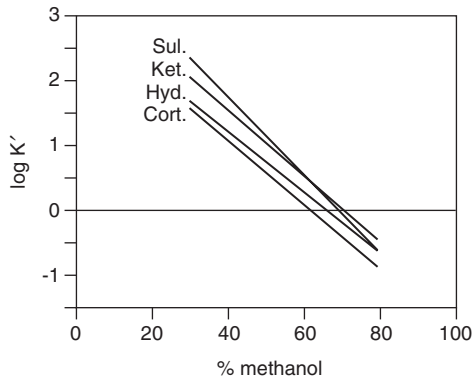


As can be seen in Figure 12.13, according to the log K′ plots there is an opportunity to produce a method with a short run time since the plots for sulindac and ketoprofen cross over when the percentage of methanol is high. Practical application of this information produced the chromatogram shown in Figure 12.14 where fast separation has been produced with sulindac eluting earlier than ketoprofen.

Software packages such as Drylab® can provide an automated approach to prediction of retention times based on log K′ plots and also model the effects of stationary phase particle size, column length and column diameter changes. A popular method for modelling retention times is based on a 2 gradient run. Figure 12.15 shows acebutolol and its impurities which are listed in the EP. Figure 12.16 shows the separation of acebutolol and its impurities using two different solvent gradient methods.

From the retention times obtained in the runs shown in Figure 12.16, Drylab® was asked to predict the mobile-phase conditions required for a rapid separation based on a 30 × 2.1 mm column. Figure 12.17 shows the rapid separation of acebutolol from its impurities spiked at the 0.1% w/w reporting level.

Fig. 12.13
Log K′ plots based on the data shown in Table 12.1.



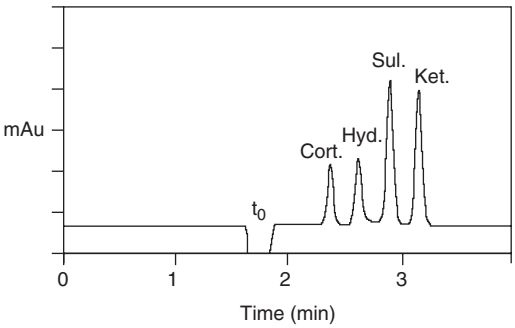
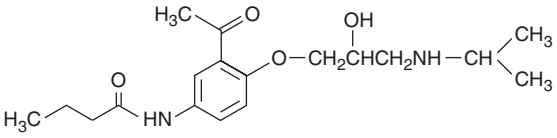


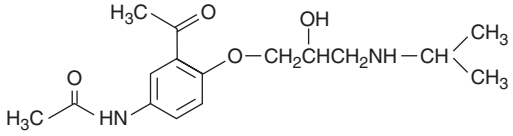
Fig. 12.14
Separation of cortisone, hydrocortisone, ketoprofen and sulindac in 0.1% formic acid/methanol (25:75) flow rate 1 ml/min on an ACE C18 5 μm column 150 × 4.6 mm (t_0 = 1.8 min.) with UV detection at 220 nm.

Effect of temperature on HPLC

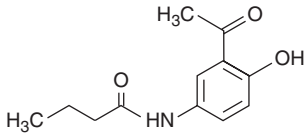
Temperature can also be used to change retention behaviour since capacity factor decreases with an increase in temperature according to the van't Hoff equation. In addition mass transfer effects are reduced at higher temperatures, and thus peak efficiency is better. Returning to the model mixture of cortisone, hydrocortisone, sulindac and ketoprofen, Figure 12.18 shows that increasing temperature both reduces retention times and changes selectivity. Drylab® can be used to model the effects of temperature as well.



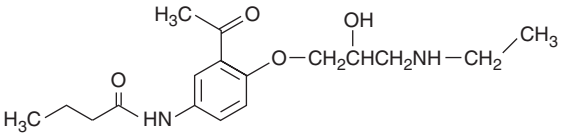
A Acebutolol



B Impurity B



C Impurity C



D Impurity I

Fig. 12.15
Acebutolol and its impurities.

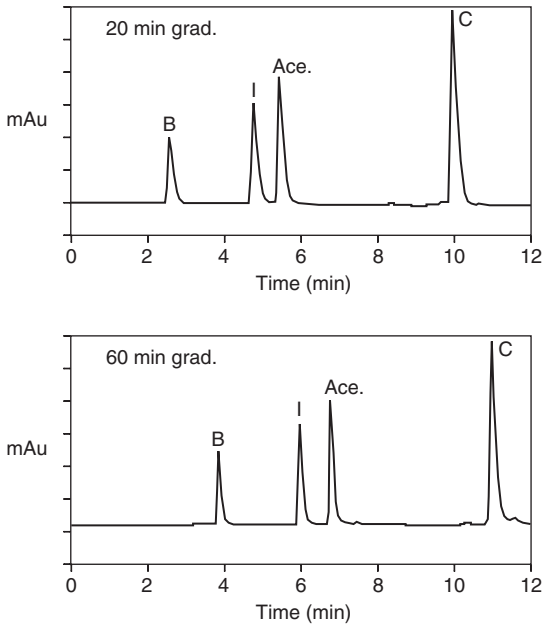


Fig. 12.16
Two gradient run for acebutolol (Ace) and its impurities (B, I and C). Gradient: 95% tris buffer pH 7.0:5% acetonitrile to 100% acetonitrile in 20 and 60 minutes on an ACE C18 column 4.6 × 150 mm × 5 μm particle size.

Summary of stationary phases used in HPLC

The intention of this book is to focus mainly on applications of techniques to pharmaceutical analysis. Detailed discussions of stationary phases and detectors can be found elsewhere.[1,2,4,5] Table 12.2 summarises some of the stationary phases which are used in HPLC. Currently, ODS silica gel or related phases such as octyl silica gel are used for > 80% of all pharmaceutical analyses, as judged from a comprehensive survey of the literature;[3] other phases are only used where special selectivity is required, such as for very water-soluble compounds or for bioanalytical separations which may be difficult because the sample matrix produces many interfering peaks. In recent years polymeric phases have become available for certain specialist applications; the surface chemistries of these phases are similar to those of the silica-gel-based phases. Advantages of the polymeric phases are stability to extremes of pH and the lack of secondary

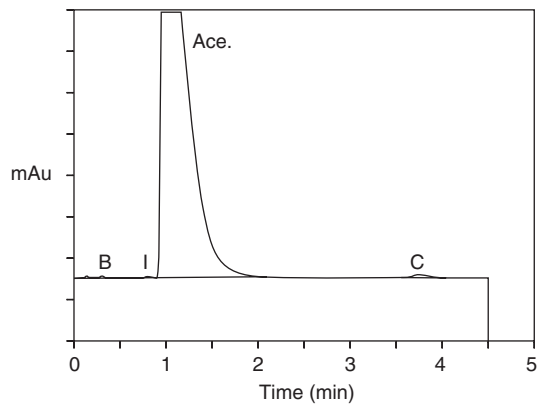


Fig. 12.17
Separation of acebutolol (1 mg/ml) from its manufacturing impurities at a concentration of 0.001 mg/ml using 87.5% tris buffer:12.5% acetonitrile at 1 ml/min on an ACE C18 column 2.1 mm × 30 mm × 5 μm particle size.

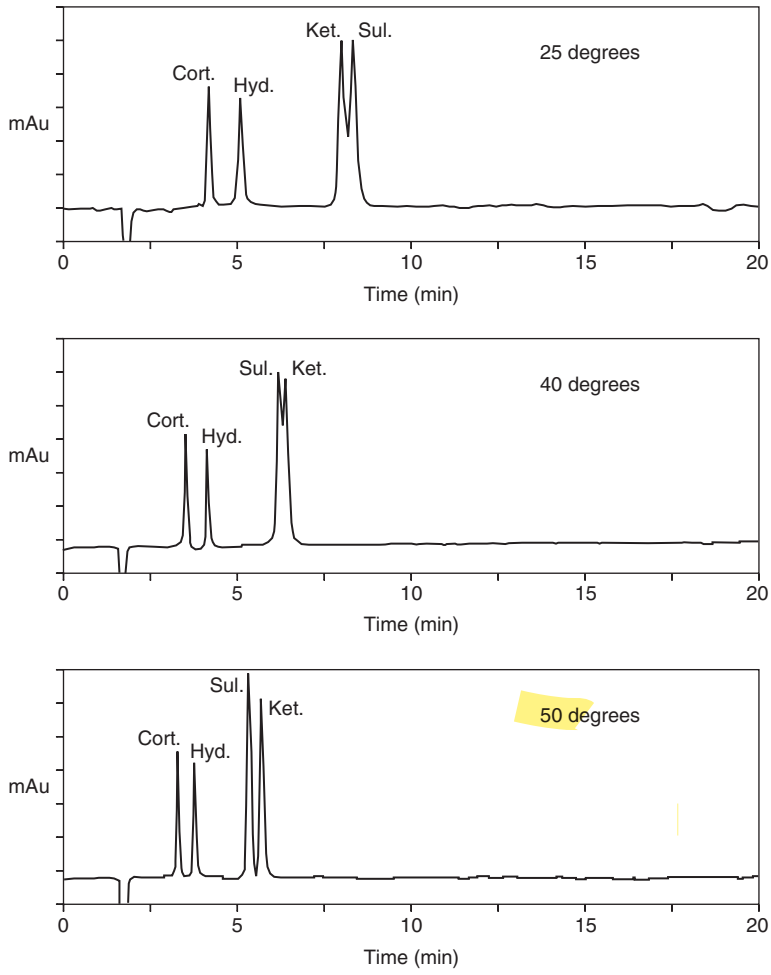


Fig. 12.18
Retention of cortisone, hydrocortisone, sulindac and ketoprofen eluted with 0.1% formic acid: methanol (40:60) at 1 ml/min on an ACE C18 column 4.6 × 150 mm × 5 μm particle size at different temperatures.

interactions of analytes with uncapped silanol groups. Disadvantages include expense and a tendency to swell when in contact with highly lipophilic mobile phases, which can destroy them. Such phases are best used with predominantly aqueous-based mobile phases. In the past 10 years the use of hydrophilic interaction chromatography (HILIC) has increased, and in the case of very polar molecules it provides an alternative to ion pair chromatography. However, there are still some doubts whether or not it is sufficiently robust for routine chromatographic methods.

A more advanced consideration of reverse-phase stationary phases

All reverse-phase chromatography columns are not equivalent. There are big differences between stationary phases obtained from different manufacturers. Recently, definitive work has been carried out on the classification of reverse-phase stationary phases.[8] Six variables that affected the performance of reverse-phase stationary phases were assessed:

Table 12.2 Some commonly used high-performance liquid chromatography (HPLC) stationary phases

Stationary phase	Applications/comments
Octadecylsilyl (ODS) silica gel	The most commonly used phase, applicable to most problems in analysis of pharmaceutical formulations. Early phases gave problems with strongly basic compounds because of low purity silica gels and incomplete endcapping of silanol (Si-OH) groups. Amines adsorb strongly onto free silanol groups not covered by the stationary phase. Fully endcapped phases and phases with low metal content are now available, which enable the analysis of strongly basic compounds that formerly tended to produce tailing peaks. ODS silica gel can even be applied to the analysis of peptides, where wide-pore packings are used to improve access of these bulky molecules to the internal surface of the packings
Octyl silane and butyl silane silica gels	Useful alternatives to ODS phases. The shorter hydrocarbon chains do not tend to lead to shorter retention times of analytes since the carbon loading on the surface of the silica gel may be higher for these phases and retention time is also dependent on how much of the stationary phase is accessible to partitioning by the analyte[7]
Phenyl silane silica gel	Useful for slightly more selective analyses of compounds containing large numbers of aromatic rings, e.g. propranolol and naproxen, where π-π interactions can occur with the phenyl groups on the stationary phase if the analyte is deficient π electrons, e.g. nitro compounds. These interactions are, however, very subtle
Silica gel	Often used in the past for problematical compounds but, with gradual improvement of reverse phases, increasingly less used. Useful for chromatography of very lipophilic compounds such as in the separation of different classes of lipids and in the analysis of surfactants, which tend to form micelles under the conditions used for reverse-phase chromatography
Aminopropyl silica gel	A moderately polar phase often used for the analysis of sugars and surfactants. In the case of sugars the chromatographic interactions are due to hydrophilic interactions (see below)
Cyanopropyl silica gel	A moderately polar phase applicable to the analysis of surfactants. Some of its selectivity is due to dipole–dipole interactions
Strong cation exchanger (SCX)	Usually based on ion pairing of the analyte with sulfonic acid groups on the surface of the stationary phase. Useful for analysis of very polar compounds such as aminoglycosides and other charged sugar molecules and polar bases such as catecholamines
Strong anion exchanger (SAX)	Usually based on ion pairing of the analyte with quaternary ammonium groups on the surface of the stationary phase. Useful for the separation of polar compounds with anionic groups such as nucleotides and anionic drug metabolites such as sulphates or glucuronides

(i) Retention factor for the lipophilic compound pentylbenzene, k_{PB}

This test provides a measure of how completely the surface of the silica gel is covered with reverse-phase coating and of the area of the surface that is available for interaction with the analyte. The higher K_{PB} for a column, the greater the surface coverage and available surface area of the stationary phase (mobile

phase methanol/water (80 : 20)). Different ODS columns give a wide range of K_{PB} values.

(ii) Hydrophobic selectivity $\alpha_{CH2} = k_{PB}/k_{BB}$

This is measured by the ratio of the capacity factors for pentylbenzene (K_{PB}) and butylbenzene (K_{BB}) and gives a measure of the surface coverage of the silica gel with the bonded phase. The higher α_{CH2}, the greater the surface coverage; this factor varies much less than K_{PB} between different reverse-phase columns (mobile phase methanol/water (80 : 20)).

(iii) Shape selectivity $\alpha_{T/O} = k_T/k_O$

This is based on the ratio of the capacity factors for triphenylene and *o*-terphenyl, which have very similar structures, but *o*-terphenyl is less rigid than triphenylene and thus has a more three-dimensional structure (Fig. 12.19). Retention of a molecule via lipophilic interactions depends on the size of molecular surface that interacts with the stationary phase. This means for a surface with large lipophilic groups less of the *o*-terphenyl molecule will be in contact with the surface, whereas the flat triphenylene molecule can contact a uniform surface more fully. Triphenylene, because of its flatness, is less sensitive to the surface of the stationary phase. Stationary phases with smaller alkyl chains attached to the surface, such as octyl, phenyl or butyl, often do not discriminate between *o*-terphenyl and triphenylene as strongly as octadecyl phases (mobile phase methanol/water (80 : 20)).

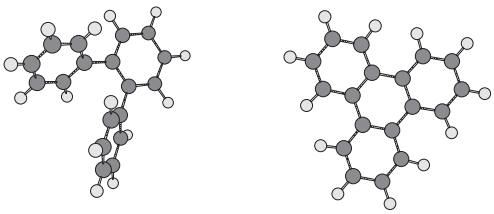


Fig. 12.19
The structures of *o*-terphenyl and triphenylene.

(iv) Hydrogen bonding capacity $\alpha_{C/P} = k_C/k_P$

Hydrogen bonding capacity may be measured as the ratio of the capacity factors for phenol (k_P) and caffeine (k_C). The smaller the value for $\alpha_{C/P}$ the higher the hydrogen bonding capacity, since phenol can hydrogen bond whereas caffeine cannot. The term measures the level of free silanol (Fig. 12.20), or other groups with hydrogen-bonding capacity, in the phase (mobile phase methanol/water (30 : 70)).

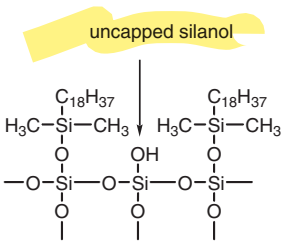


Fig. 12.20
Uncapped silanol group.

(v) Total ion exchange capacity $a_{B/P} = k_B/k_P$ (pH 7.6)

This term gives a measure of the total silanol group activity and is based on the ratio of the capacity factors for benzylamine (k_B) and phenol (k_P) when analysed using a mobile phase with a pH of 7.6, where most of the uncapped silanol groups (Fig. 12.17) in a silica-gel-based chromatographic phase will be ionised. The benzylamine interacts strongly with negatively charged silanol groups and is thus more strongly retained on phases where there are a high number of silanol groups. The higher the value of $\alpha_{B/P}$, the greater the ion exchange capacity of the phase.

(vi) The acidic ion exchange capacity $a_{B/P} = k_B/k_P$ (pH 2.7)

This term gives a measure of the activity of the most acidic silanol groups in the stationary phase and is based on the ratio of the capacity factors for benzylamine (k_B) and phenol (k_P) when analysed using a mobile phase with a pH of 2.7, where only the most acidic (isolated) uncapped silanol groups in a silica-gel-based chromatographic phase will be ionised.

Table 12.3 shows the specification details of some Hypersil columns taken from the manufacturer's literature along with some Tanaka test measurements.[8]

Table 12.3 Data for four different reverse-phase Hypersil columns

Column	%C	EC*	Silica**	K_{PB}	$\alpha_{B/P}$ pH 7.6	$\alpha_{B/P}$ pH 2.7
C18 HS 100	16	Y	UP	7.66	1.01	0.25
C18 BDS	11	Y	AW	4.5	0.19	0.17
C18 HyPurity	13	Y	HP	3.2	0.29	0.1
C8 HyPurity	8	Y	HP	1.59	0.3	0.11

*EC = endcapped
**UP = unpurified, AW = acid washed, HP = high purity

The C18 HS 100 column has a high carbon load and thus has the greatest retention factor of pentyl benzene. It is also made from unpurified silica gel, so that it strongly retains benzylamine relative to phenol due to strong ion exchange interactions. This column would not be useful for the analysis of many basic compounds. The C18 BDS column has a lower carbon load and so retains pentylbenzene less than the HS column; it does not show strong retention for benzylamine because the acid washing process has removed many of the trace metals from the silica gel used to prepare it, making it more suitable for the analysis of bases. The HyPurity C18 phase shows a lower retention factor for pentylbenzene despite having higher carbon load than the BDS phase; this is not immediately easy to explain but may relate to the surface areas of the phases since the HyPurity phase has a larger surface area per gram. The HyPurity C8 has a lower retention factor for pentylbenzene, which would be expected from its lower carbon load. This is not always the case for C8 phases; some have a carbon load closer to that of a C18 phase and thus a retention factor not much lower than the C18 phase. The use of column characterisation parameters enables selection of similar columns based on their performance according to the Tanaka tests. The data obtained by Euerby and Peterson[8] has been converted into column selection

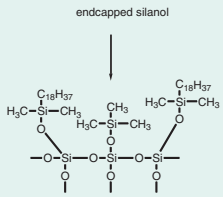
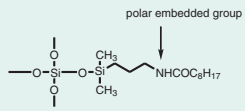
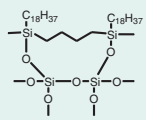
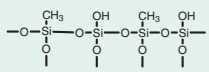
software which is available as a free download from ACD Labs at http://www.acdlabs.com/products/adh/chrom/chromproc/.

Table 12.4 shows some examples of recent developments in the chemistry of reverse-phase packings.

Self-test 12.4

Go to the ACD site and download column selector and associated software (this process takes about 30 minutes by the time the software is downloaded and installed, but it is useful software and free!!). Use the software to select the column most similar to the ACE C18 column. List the Tanaka parameters for the ACE C18 column and the most closely related column in the list.

Table 12.4 Some examples of modern reverse-phase chemistry

Stationary phase	Comments
Endcapped and high-purity phases 	The biggest source of variation in modified silica gel is the quality of the base silica supporting the alkyl coating. The latest generation of base deactivated phases is made from high-purity silica formed by the hydrolysis of tetramethoxysilane. Reaction of uncapped silanols with a non-polar end-capping group such as trimethylsilane also reduces unwanted interaction with basic compounds
	Polar embedded groups increase the penetration of water into the stationary phase, through its being able to hydrogen bond to a group such as an amide group. These phases give stronger retention of polar analytes
	Altered bonding such as bidentate attachment of ligands gives greater deactivation and stability so that the stationary phase can be used up to pH 11.0
	Base silica gel particles can be engineered to contain alkyl groups, making a more inert and stable gel

Summary of detectors used in HPLC

the majority of analyses of drugs in formulations, variable wavelength UV or diode array UV detectors are used. A typical UV detector has a narrow cell about 1 mm in diameter with a length of 10 mm, giving it an internal volume of about 8 μl. The linear range of such detectors is between 0.0001 and 2 absorbance units, and samples have to be diluted sufficiently to fall within the range. Although the exact concentration of a sample passing through the flow cell is not known, a suitable concentration can be approximated as shown in Calculation example 12.3.

Selective detectors tend to be employed where the analyte is present in small amounts in a complex matrix such as in bioanalytical procedures, where components extracted from the biological matrix along with the analyte can cause

Calculation example 12.3

A typical elution volume of chromatographic peak volume is 400 μl. If 20 μl (0.02 ml) of a solution containing paracetamol at a concentration of 1 mg/100 ml is injected into an HPLC system with a flow cell with a pathlength of 10 mm:

Amount of paracetamol injected = 1 mg × 0.02/100 = 0.0002 mg

Mean concentration of paracetamol in the peak volume = 0.0002 × 100/0.4 = 0.05 mg/100 ml

The A(1%, 1 cm) value for paracetamol at 245 nm is 668.

The absorbance of a 0.05 mg (0.00005 g) solution = 0.00005 × 668 = 0.0334.

The mean absorption across the peak would be 0.00334.

If the peak has a Gaussian shape, the maximum absorption for the peak would be *ca* 1.5 times the mean absorption, i.e. in this case 0.05 or 50 milliabsorbance units (mAU).

interference. Some formulated compounds have only very poor chromophores – these include sugars, lipids, surfactants, amino acids and some classes of drugs, e.g. a number of anticholinergic drugs lack chromophores. In these cases an alternative to UV detection has to be employed.

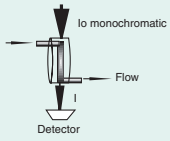
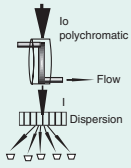
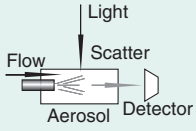
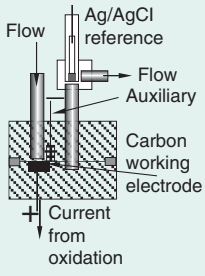
Performance of a diode array detector

Table 12.5 summarises the detectors commonly used in HPLC analyses (see also Animation 12.8). Sometimes it is not possible to be completely confident that an HPLC has chromatographically resolved all the compounds in a sample, and it might be suspected that a particular chromatographic peak might be due to more than one component. The DAD has developed into a tool of some sophistication for determining the purity of chromatographic peaks eluting from an HPLC column. Since a whole UV/visible spectrum is acquired several times across the width of a peak, this provides a means of checking the purity of the peak by checking for variations in the shape of the absorption spectrum across the chromatographic peak. Figure 12.21 illustrates four methods for looking at the purity of a peak using the information acquired by a DAD.

In the example illustrated in Figure 12.21, the spectrum of the apex of the peak (A) (where interference by impurities is likely to be the least) is compared with a spectrum from the leading edge of the peak (B). Comparison of individual spectra from anywhere across the width of the peak may also be made with a spectrum produced by combining each spectrum taken across the chromatographic peak to produce a composite spectrum for the peak. The four methods used are:

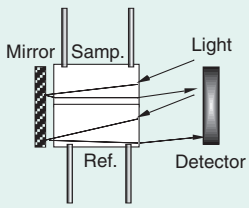
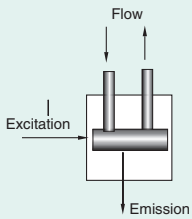
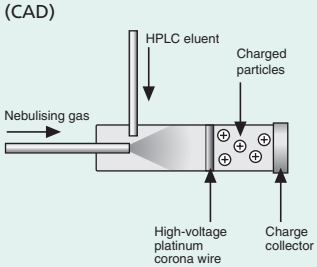
(i) Spectrum A and spectrum B are normalised to get the best possible overlay and are then correlated by plotting their absorbances at *ca* 1 nm intervals across the spectra against each other. The correlation coefficient of best-fit line through the resultant points can be determined (Ch. 1, p. 16). A good correlation between the spectra should give $r^2 > 0.995$, and the r^2 for such a plot is multiplied by 1000 to give a similarity factor, which is quoted as a measure of peak purity when the spectra of leading

Table 12.5 Some detectors commonly used in high-performance liquid chromatography (HPLC)

Detector	Applications
Variable wavelength UV detector 	Based on absorption of UV light by an analyte. A robust detector with good sensitivity works approximately in the range of 0.01–100 μg of a compound on-column. The sensitivity of the detector in part depends on the *A*(1%, 1 cm) value of the compound being analysed. The early detectors operated at a fixed wavelength (usually 254 nm); currently detectors are available which can be adjusted to operate at any wavelength over the full UV/visible range
Diode array detector (DAD) 	An advanced type of UV detector with the ability to monitor across the full UV range simultaneously, using an array of photodiodes which detect light dispersed by a fixed monochromator over a range of wavelengths, offering a resolution of *ca* 1 nm. Useful for complex mixtures containing compounds with widely different absorbance ranges and for mixtures where peaks overlap chromatographically but can be separated in terms of UV absorbance. The detector gives a full UV spectrum of each peak in the chromatogram, which aids in identification of unknowns
Evaporative light-scattering detector (ELSD) 	Detection is based on the scattering of a beam of light by particles of compound remaining after evaporation of the mobile phase. This detector is of growing importance; it is a universal detector and does not require a compound to have a chromophore for detection. Applications include the analysis of surfactants, lipids and sugars. Unlike the refractive index detector, which was formerly used for this analysis, it can be used with gradient elution and is robust enough to function under a wide range of operating conditions. However, it cannot be used with involatile materials such as buffers in the mobile phase or to detect very volatile analytes. Typical applications include analysis of chloride and sodium ions in pharmaceuticals, lipids used as components in formulations, sugars and sugar polymers. Sensitive to *ca* 10 ng of analyte
Electrochemical detector 	The electrochemical detector is usually used in the coulometric mode. A fixed potential is applied between the working and reference electrode. Detection is based on production of electrons when the analyte is oxidised, which is the more common mode of operation, or consumption of electrons in the reductive mode. The current flowing across the detector cell between the working and auxiliary electrodes is measured. The working electrode, which carries out the oxidation or reduction, is usually made from carbon paste. Most applicable to selective bioanalyses such as the analysis of drugs in plasma, e.g. catechols, such as adrenaline, and thiol drugs, such as the angiotensin-converting enzyme inhibitor captopril and the anti-rheumatic drug penicillamine
Pulsed amperometric detector	There is really no distinction between this detector and an electrochemical detector except that the detector has arisen largely as part of ion chromatography and tends to be used in the amperometric mode, where

(Continued)

Table 12.5 *(Continued)*

Detector	Applications
	conduction of current between two electrodes by an ionic analyte is measured rather than current changes resulting from oxidation or reduction of the analyte. The working electrode in this detector is usually gold rather than carbon paste. Highly sensitive to ionic compounds, the detector is used in ion chromatography for the analysis of inorganic ions such as phosphate and sulphate. Typical pharmaceutical applications include the analysis of cardenolides and aminoglycoside antibiotics which do not have chromophores. Sensitivity is typically down to 1 ng of analyte. Widely used in glycobiology for the analysis of sugar residues derived from glycoproteins. In the pulsed mode, the polarities of the electrodes are alternated in order to keep the electrode surfaces clean
Refractive index detector (RI)  	Detection is based on changes of refractive index when the analyte passes through the sample cell (Samp.) in the detector, the reference cell (Ref.) being filled with the mobile phase. Like the ELSD, the RI detector is a universal detector with even less selectivity than the ELSD. It is very sensitive to mobile-phase composition and temperature, making it non-robust. It is still used as a universal detector since it is cheaper than an ELSD. Sensitive to *ca* 1 μg of compound
Fluorescence detector  	Detection is based on fluorescent emission following excitation of a fluorescent compound at an appropriate wavelength. A robust and selective detector applicable to compounds exhibiting fluorescence and to fluorescent derivatives. Most useful for selective bioanalyses. Sensitive to below the ng level for highly fluorescent compounds. Normally uses a xenon lamp for excitation but instruments with high-intensity deuterium lamps are available for excitation of short-wavelength absorption bands
Corona® charged aerosol detector (CAD)  	The CAD detector is increasing in importance. It is a universal detector like RI and ELSD detectors but is much more sensitive than these detectors. Detection is based on nebulisation of the HPLC eluent, and then the nebulised analyte particles pass a platinum corona discharge pin which imparts a positive electrostatic charge to them. The charge is then finally collected and measured. The detector response is largely independent of the chemical structure of the analyte, unlike most other detectors, which makes it very useful for mass balance studies. The detector has a dynamic range > 10^4.

and tailing ends of the peak are compared to the spectrum of the apex. A perfect match is $r^2 = 1.000$.

(ii) Spectra can be correlated to the apex spectrum or to a composite spectrum at several points across the width of the chromatographic peak, giving rise to a similarity curve. The threshold curve gives an indication of the contribution from noise to spectral differences, which is greatest at the ends of the peak, where spectra are weak in comparison with background noise from the mobile phase, etc. An impurity is detected

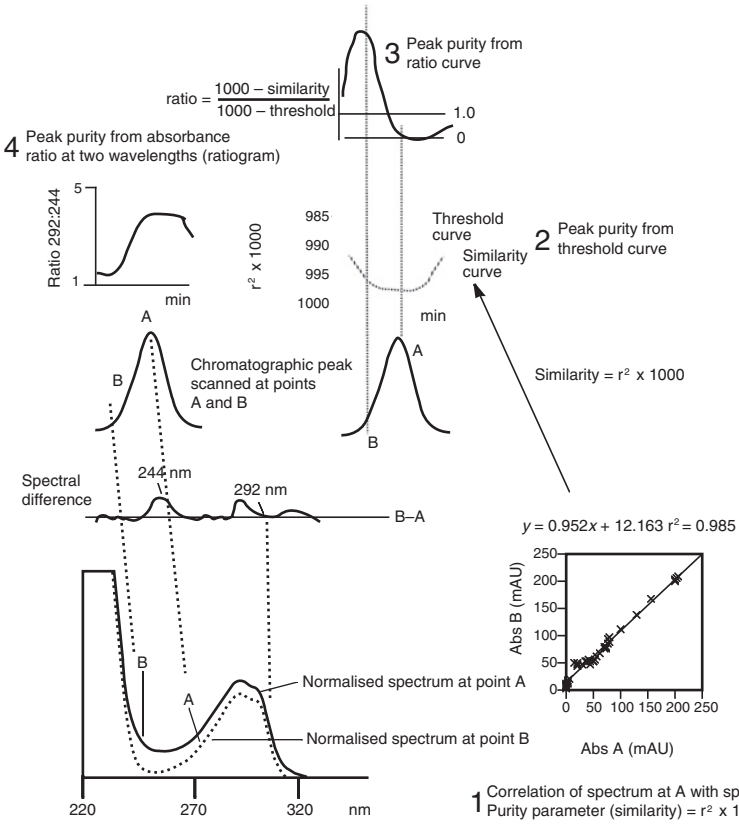


Fig. 12.21
Applications of diode array detection to peak purity determination.

Self-test 12.5

Rank the following detectors in order of decreasing: a. Selectivity b. Robustness c. Sensitivity:

(i) Variable wavelength UV detector
(ii) Evaporative light scattering detector (ELSD)
(iii) Refractive index (RI) detector
(iv) Electrochemical detector

Answers: Selectivity: electrochemical detector, variable wavelength UV detector, ELSD, RI detector. Robustness: variable wavelength UV detector, ELSD, electrochemical detector, RI detector. Sensitivity: electrochemical detector, variable wavelength UV detector, ELSD, RI detector

when the similarity curve rises above the threshold curve. In the example illustrated the major impurity in the peak is around point B.

(iii) For a very minor impurity, spectral differences across the peak can be amplified by plotting the values for 1000 – similarity/1000 – threshold across its width.

(iv) If it is possible to determine the wavelength where the impurity absorbs strongly relative to the analyte, a ratiogram can be constructed. This is obtained by plotting the ratio of a wavelength where the sample absorbs

See answer here

strongly and the impurity absorbs weakly against a wavelength where the impurity absorbs intensely. If the peak is impure, the ratio will fall around where the impurity elutes. A pure peak will exhibit a fairly constant ratio across the width of the peak.

Applications of HPLC to the quantitative analysis of drugs in formulations

The majority of applications of HPLC in pharmaceutical analysis are to the quantitative determinations of drugs in formulations. Such analyses usually do not require large amounts of time to be spent optimising mobile phases and selecting columns and detectors so that analyses of complex mixtures can be carried out. A standard joke is that most quality-control applications can be carried out with an ODS column and with methanol : water (1 : 1) as a mobile phase. Analyses of formulations are not quite that simple but, compared to analysis of drugs in biological fluids or elucidation of complex drug degradation pathways, they present fewer difficulties. The main potential interferants in analysis of a formulation are preservatives, colourants (see Ch. 15) and possible degradation products of the formulated drug. Some formulations contain more than one active ingredient, and these may present more of an analytical challenge since the different ingredients may have quite different chemical properties and elute at very different times from an HPLC column. In this case, achieving a short analysis time may be difficult. Since the emphasis in pharmaceutical analysis is on quantitative analysis of formulations, this will be considered first.

Analyses based on calibration with an external standard

HPLC assays of formulated drugs can often be carried out against an external standard for the drug being measured. The instrumentation itself is capable of high precision, and in many cases drugs are completely recovered from the formulation matrix. If complete recovery can be guaranteed, then the area of the chromatographic peak obtained from a known weight of formulation can be compared directly with a calibration curve constructed using a series of solutions containing varying concentrations of a pure standard of the analyte. The use of a single point of calibration can also be justified since, in quality control applications, the content of the formulation is unlikely to vary by $> \pm 10\%$ from the stated content. The Food and Drug Administration (FDA) have suggested that for an assay of the active ingredients in a formulation, calibration should be carried within a range of $\pm 20\%$ of the expected concentration in the sample extract. The steps required in a quantitative HPLC assay based on the use of an external standard are summarised as follows:

- Weigh accurately an analytical standard for the analyte, and dissolve it in a precise volume of solvent to prepare a stock solution.
- Prepare appropriate dilutions from the stock to produce a calibration series of solutions so that (1) appropriate amounts of analyte are injected into the instrument giving consideration to its operating range and (2) the concentration of analyte which is expected in a diluted extract from the sample is at

approximately the mid-point of the range of concentrations prepared in the calibration series.

- Inject the calibration solutions into the HPLC system starting with the lowest concentration and finishing with a blank injection of the mobile phase to check for carryover.
- Prepare the formulation for extraction, e.g. powder tablets, and weigh accurately portions of the prepared material.
- Extract the formulation with a solvent which is likely to give good extraction recovery and make up to a precise volume.
- Filter if necessary and take a precise aliquot of the sample extract and dilute this until its concentration falls at approximately the mid-point of the calibration series prepared using the analytical standard.
- Inject the diluted sample solution into the HPLC system. Replicates of the sample preparation and of the injection of the sample into the HPLC may be carried out; sample preparation procedures are more likely to give rise to imprecision than instrumental variation.
- Plot a calibration curve for the area of the peaks obtained in the calibration series against the concentrations of the solutions. The peak areas given by integrators are in arbitrary units and may be to seven or eight figures. Assays are not usually precise beyond four significant figures; thus it may be appropriate to only consider the first five figures from the integrator output to be of any significance, e.g. 78993866 might be better considered as being 78994000.
- Check the linearity of the calibration curve, i.e. $r > 0.99$. Determine the concentration of the diluted sample extract from the calibration curve by substituting the area of its chromatographic peak into the equation for the calibration line.

Analysis of paracetamol tablets using a calibration curve

Tablets

Tablets contain paracetamol 500 mg, phenylpropanolamine 5 mg.

Explanation of the assay

Even without chromatographic resolution the small amount of phenylpropanolamine present in the formulation could be disregarded since its A(1%, 1 cm) value at the wavelength 243 nm used for monitoring paracetamol is *ca* 4 compared to an A(1%, 1 cm) of 668 for paracetamol. An ODS column retains paracetamol adequately if the amount of water in the mobile phase is high. Thus the mobile phase used is 0.05 M acetic acid/acetonitrile (90 : 15); the weakly acidic mobile phase ensures there is no tendency for the phenol group in paracetamol (pKa 9.5) to ionise. The tablet extract has to be diluted sufficiently to bring it within the range of the UV detector. Figure 12.22 shows the chromatographic traces obtained for an extract from paracetamol tablets and a paracetamol standard (1.25 mg/100 ml) run using the system described above.

Assay

(i) Weigh out 125 ± 10 mg of the paracetamol standard, transfer it to a 250 ml volumetric flask made up to volume with acetic acid (0.05 M) and shake well (stock solution).

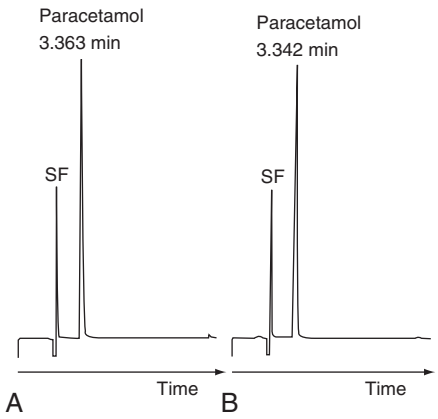


Fig. 12.22
(A) Extract from paracetamol tablets in comparison with (B) paracetamol standard at about the same concentration. Column: octadecylsilyl (ODS) 4.6 mm × 150 mm, mobile phase acetonitrile: 0.05 M acetic acid (85:15) flow rate 1 ml/min (SF = solvent front).

(ii) Prepare a series of solutions containing 0.5, 1.0, 1.5, 2.0 and 2.5 mg/100 ml of paracetamol from the stock solution.
(iii) Weigh and powder 20 tablets.
(iv) Weigh out tablet powder containing 125 mg ± 10 mg of paracetamol.
(v) Shake the tablet powder sample with *ca* 150 ml of acetic acid (0.05 M) for 5 min in a 250 ml volumetric flask, and then adjust the volume to 250 ml with more acetic acid (0.05 M).
(vi) Filter *ca* 50 ml of the solution into a conical flask, then transfer a 25 ml aliquot of the filtrate to 100 ml volumetric flasks and adjust the volume to 100 ml with acetic acid (0.05 M).
(vii) Take 10 ml of the diluted extract, transfer to a further 100 ml volumetric flask and make up to volume with 0.05 M acetic acid.
(viii) Analyse the standards and the extract using the chromatographic conditions specified earlier.

Data obtained

- Weight of 20 tablets = 12.1891 g
- Weight of tablet powder taken = 150.5 mg
- Weight of paracetamol calibration standard = 126.1 mg
- Area of paracetamol peak extracted from tablets = 45 205.

Calculate the percentage of the stated content of paracetamol in the tablet powder analysed.

The graph shown in Figure 12.23 is obtained from the data given in Table 12.6; it is linear with r = 1.000.

The equation of the line can be used to calculate the amount of paracetamol in the diluted extract of the tablet powder.

Assay of paracetamol and aspirin in tablets using a narrow-range calibration curve

Tablets

Tablets contain paracetamol 250 mg, aspirin 250 mg, codeine phosphate 6.8 mg.

Explanation of the assay

This problem is slightly more difficult than that posed by paracetamol tablets since there are two major active ingredients in the formulation. The codeine

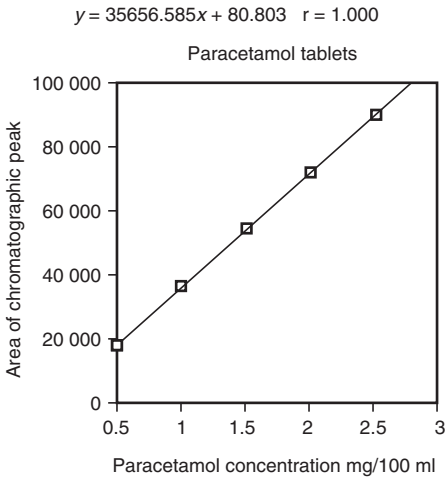


Fig. 12.23
Calibration curve for the determination of paracetamol in tablets obtained from high-performance liquid chromatography (HPLC) analysis of calibration standards.

Table 12.6 Data obtained from the analysis of paracetamol standard solutions by high-performance liquid chromatography (HPLC)

Concentration of paracetamol standard solution mg/100 ml	Area of chromatographic peak
0.5044	17 994
1.009	36 109
1.513	54 121
2.018	71 988
2.522	89 984

Calculation example 12.4

Substituting the area obtained for the paracetamol peak obtained from the analysis of the tablet powder extract into the equation for the line:

$$45205 = 35656x + 80$$

Solving for x gives the concentration of the extract in mg/100 ml.

$$\text{Concentration of paracetamol in diluted tablet extract} = \frac{45205 - 80}{35656} = 1.266 \text{ mg/100 ml}$$

Dilution steps

The dilution steps used were:

- 25 ml into 100 ml (× 4)
- 10 ml into 100 ml (× 10)
- Total = × 40.

Concentration of paracetamol in undiluted tablet extract

$$1.266 \text{ mg/100 ml} \times 40 = 50.64 \text{ mg/100 ml}$$

(Continued)

Calculation example 12.4 *(Continued)*

Amount of paracetamol in undiluted tablet extract

- The volume of the undiluted tablet extract = 250 ml
- Amount of paracetamol in 100 ml of the extract = 50.64 mg
- Amount of paracetamol in 250 ml of extract = 250/100 × 50.64 mg = 126.6 mg
- Amount of paracetamol found in the tablet powder assayed = 126.6 mg.

Amount of paracetamol expected in the tablet powder taken for assay

- Weight of 20 tablets = 12.1891 g
- Weight of one tablet = 12.1891/20 = 0.6094 g = 609.5 mg
- Stated content per tablet = 500 mg
- Amount of paracetamol expected in the weight of tablet powder taken for assay = 150.5/609.5 × 500 mg = 123.5 mg.

Percentage of stated content

- Percentage of stated content = 126.6/123.5 × 100 = 102.5%.

Self-test 12.6

Calculate the percentage of stated content in paracetamol tablets using the calibration curve given above and the following data:

Data

- Weight of 20 tablets = 12.2243 g
- Weight of tablet powder taken = 152.5 mg
- Stated content per tablet = 500 mg
- Initial extraction volume = 200 ml.

Dilution steps

- 20 ml into 100 ml
- 10 ml into 100 ml
- Area of chromatographic peak for paracetamol extracted from the tablets = 44519.

Answer: 99.8%

phosphate cannot be determined using the chromatographic system described here since it elutes from the column in the void volume and is obscured by the solvent front. Again an ODS column is quite suitable, and since aspirin is ionised extensively above pH 4.0, the pH of the mobile phase can be manipulated to move it to a region of the chromatogram where it can be run in the same mobile phase as paracetamol without its retention time being inconveniently long. Figure 12.24 shows the effect of mobile-phase pH on the elution time of aspirin; the p*K*a of paracetamol is much higher than that of aspirin, and it is unaffected by the adjustment in pH of the mobile phase. The mobile phase which resulted in chromatogram B is preferred for the analysis.

Brief outline of the assay

The assay is more or less the same as that described for the paracetamol tablets except that the tablets are extracted with 0.05 M sodium acetate buffer pH 4.4.

See answer here

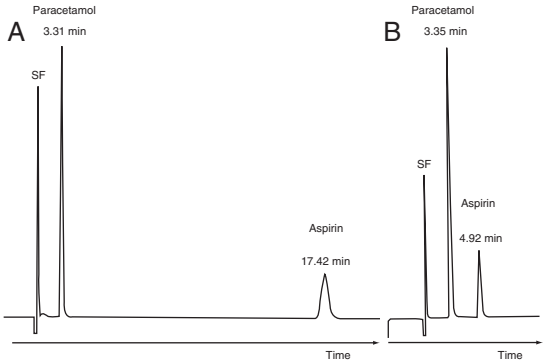


Fig. 12.24
(A) A tablet extract containing paracetamol and aspirin run at a pH of *ca* 3.7 in 0.05 M acetic acid/acetonitrile (85:15) (150 mm × 4.6 mm octadecylsilyl (ODS) column, flow rate 1 ml/min). (B) Shows the tablets extract run at pH 4.4 in 0.05 M sodium acetate buffer/acetonitrile (85.15). ODS column 150 mm × 4.6 mm, flow rate 1 ml/min. UV detection at 243 nm.

The calibration standard solutions are prepared so that they contain both aspirin and paracetamol in 0.05 M sodium acetate buffer pH 4.4 in the concentration range 1.0–1.5 mg/100 ml.

Data obtained

- Weight of 20 tablets = 11.2698 g
- Weight of tablet powder taken = 283.8 mg
- Weight of paracetamol standard = 125.5 mg
- Weight of aspirin standard = 127.3 mg.

Mean area of chromatographic peaks for a duplicate analysis of the tablet extract:

- Aspirin: 15 366
- Paracetamol: 44 535.

The equations for the calibration lines obtained were as follows:

- Aspirin: $y = 12\,136\ x + 139$
- Paracetamol: $y = 35\,374\ x - 35$.

Dilution of sample

- Initial volume in 250 ml.

Diluted:

- 25 to 100 ml
- 10 to 100 ml.

Self-test 12.7

Calculate the percentage of the stated content of aspirin and paracetamol in the tablet powder analysed using the data obtained above.

Answers: Paracetamol = 100.1% of stated content; aspirin = 99.7%

See answer here

Assay of active ingredients in an anaesthetic gel using a single-point calibration curve

Content per 100 g of gel

Lidocaine (lignocaine).HCl 2 g/100 g, chlorhexidine digluconate solution* 0.25% v/v and morphine sulphate 0.1 g/100 g. Specific gravity of gel = 1.03.

In addition to illustrating the use of ratios of chromatographic peaks in calculating content this example illustrates the importance of paying attention to the salt forms of a drug used in a formulation. In the case of lidocaine and morphine the salt forms used to calibrate the method are the same as those in the formulation, but in the case of chlorhexidine the standard is in the form of the free base. The salt form in the formulation is the gluconate which cannot be stored as a crystalline salt since it absorbs water so rapidly and thus is available as a 20% w/v solution.

Explanation of the assay

This assay is altogether more difficult since three active ingredients are involved and several excipients potentially could interfere in the analysis. In addition, the active ingredients are bases, which have a tendency to interact with any uncapped silanol groups in the stationary phase, and it is essential to use a column which is deactivated with respect to the analysis of basic compounds. The three active ingredients are all at different concentrations in the formulation so attention has to be paid to selection of a detection wavelength at which each component can be detected. In this particular assay a DAD would be useful.

Brief outline of the assay

One gram of the morphine sulphate in Instillagel admixture was removed from a syringe and weighed into a 50 ml volumetric flask; 10 ml of methanol was added and, finally, the volume was made up to 50 ml with water. Samples were then transferred to an auto-sampler vial, and 20 μl of the solution was injected.

A chromatogram obtained from the analysis of the sample is shown in Figure 12.25:

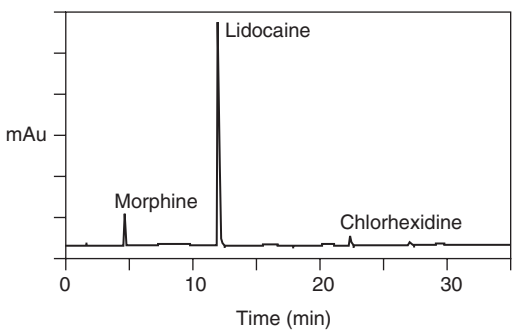


Fig. 12.25
Separation of components in Instillagel/Morphine Admixture. ACE C18 column (150 × 4.6 mm i.d.). Buffer pH 3 (A) and acetonitrile (B). The solvent programme used was as follows: 0 min, 95% A/5% B; 27 min, 55% A/45% B; 30 min, 95% A/5% B; 35 min, 95% A/5% B.

*Chlorhexidine digluconate solution contains 20% w/v.

- The concentrations of the calibration standards, the peak areas obtained for the calibration standards and the corresponding compounds in the sample are shown in Table 12.7.
- Weight of gel analysed = 1.0513 g
- Final volume of extract = 50 ml
- Calculate the percentage of stated content for the lidocaine. HCl in the formulation.

Table 12.7 Peak area data obtained for analysis of in Instillagel/morphine admixture

Standard + concentration	Area of peak in standard	Area of peak in sample
Morphine sulphate 2.132 mg/100 ml	15763	15967
Lidocaine (lignocaine).HCl 40.02 mg/100 ml	614757	633713
Chlorhexidine 0.5731 mg/100 ml	4107	4448

Calculation example 12.5

From a simple ratio:

$$\text{Concentration of morphine sulphate in extract} = 2.132 \times \frac{15967}{15763} = 2.160 \text{ mg/100ml.}$$

The sample was dissolved in 50 ml.

Amount of morphine sulphate in 50 ml = 1.080 mg

Thus, 1.080 mg was extracted from 1.0513 g of gel.

Morphine sulphate in formulation = 0.1 g/100 mg = 100 mg/100 g = 1 mg/g

$$\text{Expected content in 1.0513 g of gel} = 1 \times \frac{1.0513}{1.03} = 1.021 \text{ ml}$$

$$\text{Percentage of stated content} = \frac{1.080}{1.0513} \times 100 = 102.7\%$$

The calculation for the % of stated content of the chlorhexidine gluconate is more complicated. The gel is stated to contain 0.25% v/v (0.25 ml/100 ml) of chlorhexidine gluconate solution which contains 20% w/v chlorhexidine gluconate. Thus to be completely accurate the volume of gel analysed has to be calculated by dividing by its specific gravity.

$$\text{Volume of gel analysed} = \frac{1.0513}{1.03} = 1.021 \text{ ml}$$

There are 0.25 ml chlorhexidine gluconate solution per 100 ml of gel. Thus in 1.021 ml the

$$\text{volume of solution} = \frac{0.25}{100} \times 1.021 = 0.002553 \text{ ml.}$$

The chlorhexidine gluconate solution contains 20% w/v, or 20 g per 100 ml.

The expected content of chlorhexidine gluconate in the gel = 0.002553 × 0.2 = 0.0005105 g = 0.5105 mg.

(Continued)

Calculation example 12.5 *(Continued)*

From the HPLC data content of chlorhexidine =

$$0.5173 \times \frac{4448}{4107} = 0.5603 \text{ mg/100 ml} = 0.2801 \text{ mg/50 ml}.$$

Thus 0.2801 mg of chlorhexidine was extracted from 1.0513 g of gel.

However, the chlorhexidine content is stated in terms of its digluconate salt. The MW of chlorhexidine is 505.5 and the MW of its digluconate is 897.8.

Thus the content of chlorhexidine digluconate = $\frac{897.8}{505.5} \times 0.2801 = 0.4975$ mg

% of stated content = $\frac{0.4975}{0.5105} \times 100 = 97.5\%$.

Self-test 12.8

Calculate the percentage of stated content of lidocaine in the gel.

Answer: 98.1%

Assays using calibration against an internal standard

If the recovery in an assay is good and the instrumentation used for measurement of the sample is capable of high precision, the use of an internal standard (Box 12.2) is not necessary. HPLC instrumentation is usually capable of high precision, but for certain samples, recoveries prior to injection into the HPLC may not be accurate or precise. Examples of formulations in which recoveries may not be complete include ointments and creams, which require more extensive extraction prior to analysis. Problems of recovery are also typical of advanced drug delivery systems, which may be based on polymeric matrices in which a drug is dispersed. An internal standard is a compound related to the analyte (the properties required for an internal standard are summarised later), which is ideally added to the formulation being analysed prior to extraction. Quantification is achieved by establishing a response factor for the analyte relative to the internal standard, i.e. a ratio for the areas of the chromatographic peaks obtained for equal amounts of the analyte and internal standard; ideally this should be close to 1 for equal amounts of analyte and internal standard. The response factor may be based

Box 12.2 Properties of an internal standard

- Ideally should be closely related in structure to the analyte
- Should be stable
- Should be chromatographically resolved from the analyte and any excipients present in the chromatogram of the formulation extract
- Should elute as close as possible to the analyte with the restrictions above
- For a given weight should produce a detector response similar to that produced by the analyte

See answer here

on a single-point calibration or a full calibration curve may be constructed; all the BP assays of this type are based on single-point calibrations. Once a response factor has been established, the sample is extracted with a solution containing the *same* concentration of internal standard as was used in determining the response factor (or a solution which after dilution will yield an extract in which the internal standard is at the same concentration as in the calibration solution). Provided the solution containing the fixed concentration of internal standard is added to the sample in a precisely measured volume, any subsequent losses of sample are compensated for, since losses of the analyte will be mirrored by losses of the internal standard (see Animation 12.9). The example given in Box 12.3 is typical of a BP assay incorporating an internal standard.

Box 12.3 Response factors

Assays based on the use of an internal standard use response factors to compare the sample solution with the calibration solution. In this case a simple one-point calibration is used. The concentration of betamethasone can be ignored since it is the same in Solutions 1 and 3; it should usually be the case that the same concentration of internal standard is present in the calibration and sample solutions. If this is the case, then for the assay described above:

Response factor for Solution 1 (calibration solution)

$$= \frac{\text{area of hydrocortisone peak in Solution 1}}{\text{area of betamethasone peak in Solution 1}}$$

Response factor for Solution 3 (sample solution)

$$= \frac{\text{area of hydrocortisone peak in Solution 3}}{\text{area of betamethasone peak in Solution 3}}$$

The amount of hydrocortisone in the cream can be calculated as follows: Concentration of hydrocortisone in Solution 3 =

$$\frac{\text{Response factor for Solution 3}}{\text{Response factor for Solution 1}} \times \text{concentration of hydrocortisone in Solution 1}$$

$$\times \frac{\text{volume of Solution 3}}{100}$$

Assay of hydrocortisone cream with one-point calibration against an internal standard

Explanation of the assay

Excellent separations of corticosteroids can be achieved on an ODS column with a suitable ratio of methanol/water as an eluent. In this assay hydrocortisone is quantified using betamethasone as an internal standard. The structure of betamethasone is close to that of hydrocortisone but since it is more lipophilic it elutes from the ODS column after hydrocortisone (Fig. 12.26). The assay is a modification of the BP assay for hydrocortisone cream. In the assay described here, the internal standard is added at the first extraction step rather than after extraction has been carried out, in order to ensure that any losses in the course of sample preparation are fully compensated for. Extraction is necessary in the case of a cream because the large amount of oily excipients in the basis of the cream would soon clog up the column if no attempt was made to remove them. The corticosteroids are sufficiently polar to remain in the methanol/water layer as they have a low solubility in hexane, while the oily excipients are removed by

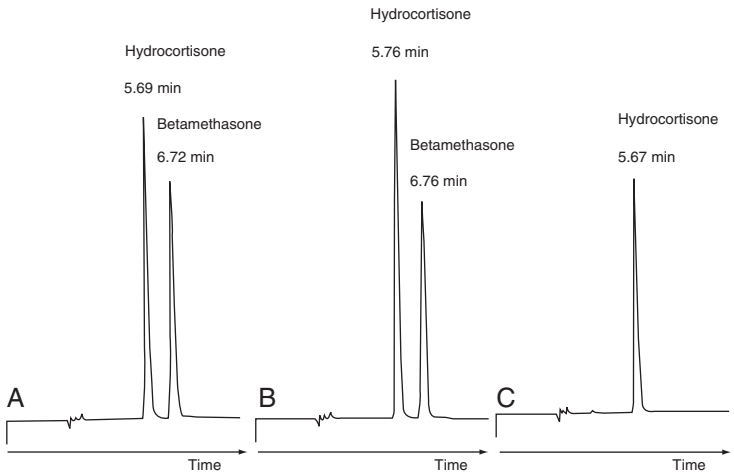


Fig. 12.26
Chromatogram from the analysis of hydrocortisone cream with betamethasone as an internal standard on an octadecylsilyl (ODS) column (25 cm × 4.6 mm) with methanol/water (70:30) as the mobile phase. (A) Calibration standard (Solution 1). (B) Cream extract + internal standard (Solution 3). (C) Cream extract without the addition of internal standard (Solution 2).

extraction into hexane. The sodium chloride (NaCl) is included in the sample extraction solution to prevent the formation of an emulsion when the extract is shaken with hexane. Solution 2, where the internal standard is omitted, is prepared in order to check that there are no excipients in the sample which would interfere with the peak due to the internal standard.

Brief outline of the assay

(i) Prepare a mixture of methanol/15% aqueous NaCl solution (2:1).

(ii) Prepare Solution 1 as follows:
- Mix together 10 ml of a 0.1% w/v solution of hydrocortisone and add 10 ml of a 0.1% w/v solution of betamethasone in methanol (internal standard solution).
- Add 20 ml of methanol and then add water to dilute the solution to 100 ml.

(iii) Prepare Solution 2 as follows:
- Disperse cream containing *ca* 10 mg of hydrocortisone in 30 ml of the methanol/NaCl solution + 10 ml of methanol.
- Extract the dispersed cream with warm hexane (50 ml).
- Remove the lower layer (methanol/water layer) and wash the hexane layer with 2 × 10 ml of the methanol/NaCl solution, combining the washings with the original extract.
- Dilute the extract to 100 ml with water.

(iv) Prepare Solution 3 as follows:
- Repeat the procedure used in preparing Solution 2 except, in the initial step, use 30 ml of methanol/NaCl solution + 10 ml of the betamethasone internal standard solution.
- Analyse the solutions using a mobile phase containing methanol/water (70:30) and an ODS column.
- Set the UV detector at 240 nm.

The calculation carried out from the data obtained in the assay described above uses response factors for the sample and standard (Box 12.3).

Data obtained

- Stated content of hydrocortisone cream = 1% w/w
- Weight of hydrocortisone cream used to prepare solution 3 = 1.173 g
- Area of hydrocortisone peak in Solution 1 = 103 026
- Area of betamethasone peak in Solution 1 = 92 449
- Area of hydrocortisone peak in Solution 3 = 113 628
- Area of betamethasone peak in Solution 3 = 82 920
- Concentration of hydrocortisone in the solution used in the preparation of Solution 1 = 0.1008% w/v
- Concentration of betamethasone used in preparation of Solutions 1 and 3 = 0.1003% w/v.

Calculation example 12.6

Solution 1 is prepared by diluting 10 ml of a 0.1008% w/v solution of hydrocortisone to 100 ml. Dilution × 10.

$$\text{Concentration of hydrocortisone in Solution 1} = \frac{0.1008}{10} = 0.01008\%\ \text{w/v}.$$

$$\text{Response factor for Solution 1} = \frac{103026}{92449} = 1.1144$$

$$\text{Response factor for Solution 3} = \frac{113628}{82920} = 1.3703$$

Concentration of hydrocortisone in Solution 3 =

$$\frac{1.3703}{1.1144} \times 0.01008 = 0.01239\%\ \text{w/v} = 0.01239\ \text{g/100 ml}$$

$$\text{Amount of hydrocortisone in Solution 3} = \frac{\text{volume of Solution 3}}{100} \times \text{weight of hydrocortisone/100 ml}$$

The volume of Solution 3 = 100 ml

$$\text{Amount of hydrocortisone in Solution 3} = \frac{100}{100} \times 0.01239 = 0.01239\ \text{g}$$

Weight of hydrocortisone cream analysed = 1.173 g

$$\text{Percentage of w/w of hydrocortisone in cream} = \frac{0.01239}{1.173} \times 100 = 1.056\%\ \text{w/w}$$

Stated content of hydrocortisone in the cream = 1% w/w

$$\text{Percentage of stated content} = \frac{1.056}{1} \times 100 = 105.6\%$$

The cream conforms to the BP requirement that it should contain between 90 and 110% of the stated content.

Assay of miconazole cream with calibration against an internal standard over a narrow concentration range

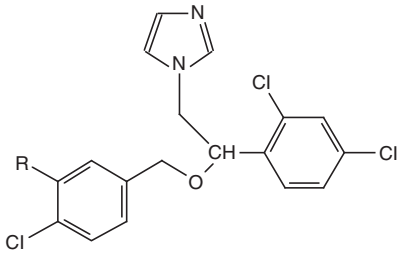
Explanation of the assay

In this case the selective extraction of oily excipients from the cream is made somewhat easier by the fact that the miconazole (p*K*a 6.5) is almost fully ionised at pH 4.0; the econazole internal standard used differs from miconazole by only one chlorine atom (Fig. 12.27). Thus a preliminary extraction can be made with hexane to remove much of the basis of the ointment, and then the sample can be simply diluted with mobile phase, filtered and analysed.

Brief outline of the assay

A chromatographic mobile phase consisting of acetonitrile/0.1 M sodium acetate buffer pH 4.0 (70 : 30) is prepared. Separate stock solutions in 250 ml of chromatographic mobile phase containing miconazole nitrate (200 ± 20 mg) and econazole nitrate (200 ± 20 mg) (internal standard) are prepared. 25 ml of econazole nitrate stock solution is transferred to five 100 ml volumetric flasks, and varying amounts of miconazole stock solution (15, 20, 25, 30 and 35 ml) are

Fig. 12.27
The structures of miconazole and econazole.



R = Cl miconazole R = H econazole

Self-test 12.9

Betamethasone valerate is analysed in a sample of ointment used for treating haemorrhoids; the related steroid beclomethasone dipropionate is used as an internal standard. The following data were produced:

- Stated content of betamethasone valerate in ointment = 0.05% w/w
- Weight of ointment analysed = 4.3668 g
- Area of betamethasone valerate peak in Solution 1 (calibration solution) = 89 467
- Area of beclomethasone dipropionate in Solution 1 = 91 888
- Area of betamethasone valerate peak in Solution 3 = 87 657
- Area of beclomethasone dipropionate peak in Solution 3 = 90 343
- Concentration of betamethasone valerate present in the calibration solution = 0.004481% w/v
- Concentration of beclomethasone dipropionate in the calibration solution and in the sample extract solution = 0.00731% w/v (Note: if this is the same in both the calibration and sample solutions, it can be ignored)
- Volume of sample extract = 50 ml.

Calculate the %w/w of betamethasone valerate in the cream.

Answer: 0.05113% w/w

See answer here

added to the five flasks. The flasks containing the calibration series are diluted to volume with mobile phase. A sample of cream containing 20 mg miconazole nitrate is shaken with 25 ml of the stock solution of econazole nitrate for 5 min. The sample is then extracted with 50 ml of hexane, and the hexane layer is removed and discarded. Nitrogen gas is then blown through the solution for a few minutes to remove residual hexane, and the solution is then transferred to a 100 ml volumetric flask, diluted to volume with mobile phase and a portion (20 ml) is filtered prior to analysis. The detection wavelength used is 220 nm since miconazole and econazole lack strong chromophores. On a 15 cm × 4.6 mm ODS column at a flow rate of 1 ml/min, econazole elutes at *ca* 6 min and miconazole elutes at *ca* 10 min; the extra chlorine atom in the structure of miconazole increases its lipophilicity considerably.

Data obtained

- Weight of miconazole used to prepare stock solution = 201.5 mg
- Weight of cream taken for assay = 1.0368 g
- Area of miconazole peak obtained from sample = 119 923
- Area of econazole peak obtained from sample = 124 118.

Table 12.8

Concentration of miconazole in calibration solution mg/100 ml	Area of miconazole peak	Area of econazole peak	In 80% methanol $K = \frac{5.6 - 1.1}{1.1} = 4.6$ In 70% methanol $K = \frac{15.7 - 1.1}{1.1} = 13.3$
12.09	70 655	123 563	0.5718
16.12	96 218	125 376	0.7674
20.15	119 793	126 783	0.9449
24.18	151 310	127 889	1.183
28.21	166 673	125 436	1.329

The equation of the line obtained from the data shown in Table 12.8 is $y = 0.048\,x - 0.006$; r = 0.998.

Self-test 12.10

Calculate the percentage of w/v of miconazole in the cream from the data obtained above.

Answer: 1.954% w/v

Assays involving more specialised HPLC techniques

Although more than 80% of all separations by HPLC utilise reverse-phase chromatography, there are certain analytes which require more specialised chromatographic methods. A few examples are given in the following section.

See answer here

Assay of adrenaline injection by chromatography with an anionic ion-pairing agent

Explanation of the assay

Injections of local anaesthetics often contain low concentrations of adrenaline in order to localise the anaesthetic for a time by constricting blood vessels in the vicinity of the injection. Adrenaline can be analysed by straight-phase chromatography, for instance on silica gel, but this generally requires strongly basic conditions, under which the catechol group in adrenaline is unstable. Adrenaline is not retained by reverse-phase columns and elutes in their void volume. A commonly used technique for the analysis of adrenaline and other highly water-soluble amines is ion pair chromatography. This can be viewed essentially as the generation of an ion exchange column in situ. The process is illustrated in Figure 12.28, where sodium octanesulphonic acid (SOSA) is added to the mobile phase (e.g. 0.1 M sodium phosphate buffer/methanol 9 : 1 containing 0.02% SOSA); the SOSA partitions into the lipophilic stationary phase and saturates it. The stationary phase is then able to retain adrenaline by electrostatic interaction. Elution occurs by a combination of displacement of adrenaline from its ion pair by sodium ions and by migration of the ion pair itself in the mobile phase. An additional benefit of using an ion-pairing reagent, rather than resorting to straight-phase chromatography, is that the organic solvent content in the mobile phase can be kept low, thus enabling the use of an electrochemical detector, which works best in mobile phases with a low content of organic solvent and which is highly selective for the readily oxidised catechol groups of adrenaline.

Assay of ascorbic acid by chromatography with a cationic ion-pairing agent and electrochemical detection

Ascorbic acid is highly polar and is not retained by reverse-phase columns. One technique for retaining it on a reverse-phase column is to use a cationic

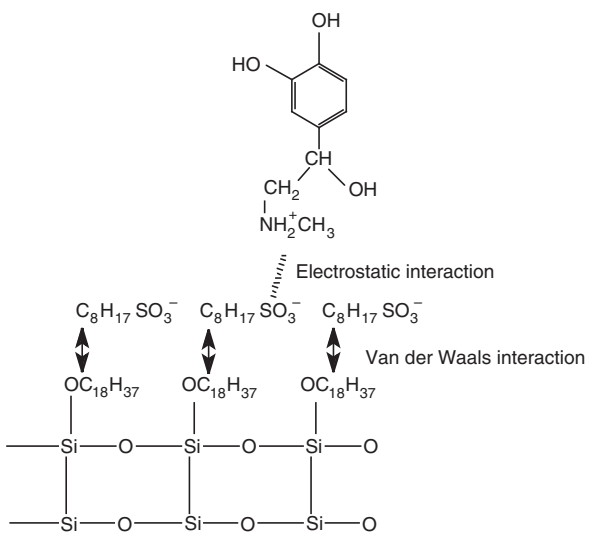


Fig. 12.28
Interaction of adrenaline with an ion-pairing agent coated onto an octadecylsilyl (ODS) stationary phase.

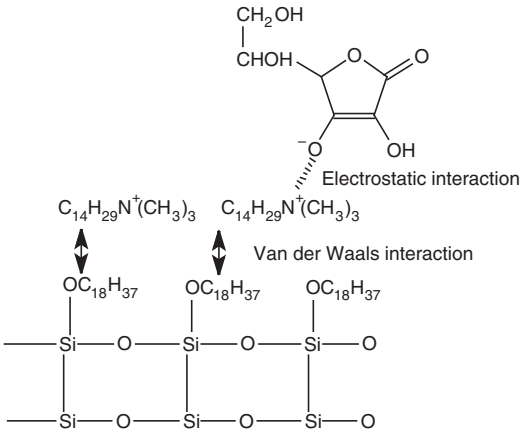


Fig. 12.29
Interaction of ascorbic acid with an ion-pairing agent coated onto the surface of an octadecylsilyl (ODS) column.

ion-pairing reagent. In the example given in Figure 12.29, cetrimide is used as the ion-pairing reagent in the mobile phase (e.g. 0.1 M sodium acetate buffer pH 4.2/acetonitrile 95 : 5 containing 0.03 M cetrimide). Again the low organic solvent content of the mobile phase enables monitoring with an electrochemical detector. Selectivity is important in the determination of ascorbic acid because it is often present in multivitamin formulations and as a preservative in pharmaceutical formulations containing other components in large amounts.

Assay of ascorbic acid by hydrophilic interaction chromatography

The hydrophilic interaction mechanism was first proposed by Alpert in 1990.[9] There are a number of HILIC phases. The simplest kinds are based on the use of bare silica, but the most popular is the ZIC-HILIC phase, the structure of which is shown in Figure 12.30. The mobile phase used is generally acetonitrile mixed with water or aqueous buffer. In this mode of chromatography the water associated with the surface of the stationary phase is regarded as acting as a pseudostationary phase. The phase thus works in the opposite way to a reverse phase. (1) The more polar or the lower the partition coefficient of a compound the more

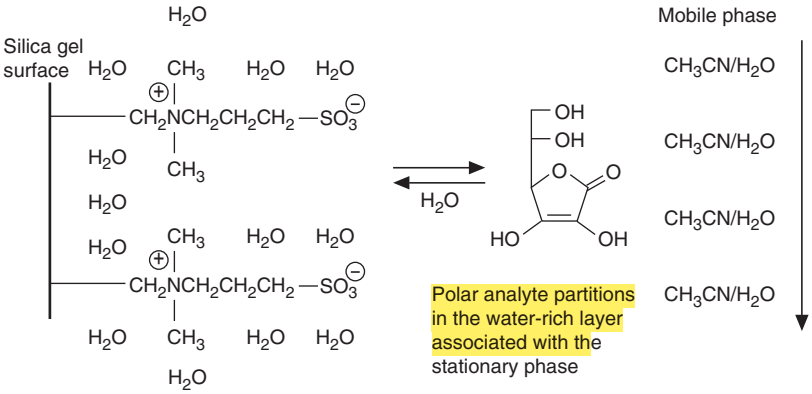


Fig. 12.30
The structure and proposed mode of action of the zwitterionic hydrophilic interaction liquid chromatography (ZIC-HILIC) stationary phase illustrated by its interaction with ascorbic acid.

strongly it is retained. (2) The higher the water content in the mobile phase, the more quickly compounds elute. There is also the possibility of ion exchange interactions which can occur in the case of strongly acidic and basic compounds.

Figure 12.31 shows chromatograms obtained on a ZICHILIC phase for two important physiological antioxidants, ascorbic acid and glutathione (GSH), and for oxidised glutathione (GSSG), which forms as a result of oxidative stress. The chromatographic peaks were detected using mass spectrometry. None of these molecules would be retained by a reverse-phase column even in 100% water.

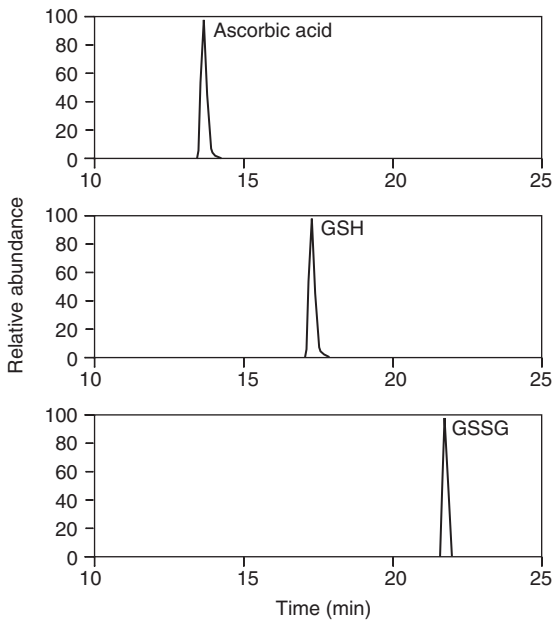


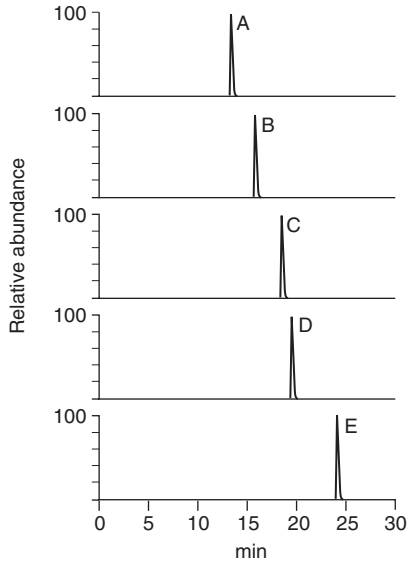
Fig. 12.31
Chromatograms for ascorbic acid, glutathione (GSH) and oxidized glutathione (GSSG) analysed on a zwitterionic hydrophilic interaction liquid chromatography (ZIC-HILIC) column 150 × 4.6 mm with a gradient between 0.1% formic acid/acetonitrile (10:90) and 0.1% formic acid/acetonitrile (80:20).

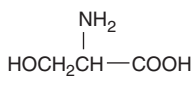
Assay of hyaluronic acid by size-exclusion chromatography (see Animation 12.10)

Polymeric materials have a number of pharmaceutical applications. Hyaluronic acid is a high-molecular-weight polymeric carbohydrate (Fig. 12.32) which has excited much interest in recent years because properties such as the promotion of wound healing are attributed to it. It is also used as a surgical aid during surgery to remove cataracts. In recent years, high-performance gel filtration columns containing rigid beads of porous polymers have become available for determination of high-molecular-weight analytes. The retention mechanism in size-exclusion or gel-permeation chromatography (GPC) is based on the extent to which an analyte enters pores within the stationary phase (Fig. 12.33). The largest molecules are completely excluded from the internal space of the column and elute from the column first. Columns with varying pore sizes are available, and for hyaluronic acid a large pore size is required since the polymer has a molecular weight > 10^6 Daltons. In order to determine molecular weights, such columns are

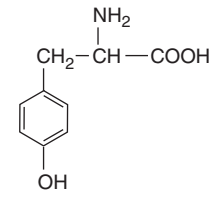
Self-test 12.11

The chromatograms below obtained on a ZICHILIC column are for various amino acids. Link the peaks to the amino acid structures shown below.

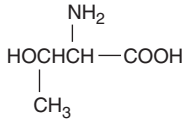




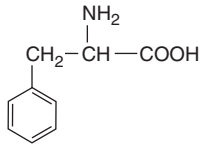
A Serine



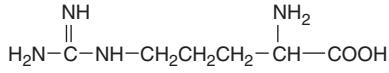
B Tyrosine



C Threonine



D Phenylalanine



E Arginine

Answers: A Phenylalanine, B Tyrosine, C Threonine, D Serine, E Arginine

See answer here

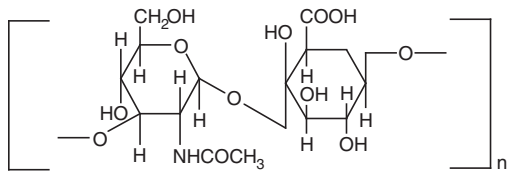


Fig. 12.32
The hyaluronic acid polymer.

calibrated with polymeric standards of known molecular weight. Although corrections related to the viscosity of the analyte are made when a column used for determining the molecular weight of one type of polymer is calibrated using a different type of polymer, because of differences in three-dimensional shape.

Typically such an assay can be carried out using a column packed with an aqueous compatible porous polymer with a mobile phase consisting of, for example, 0.05 M sodium sulphate solution. Hyaluronic acid exhibits some weak UV absorption due to its *N*-acetyl groups at short wavelengths, and UV monitoring of the eluent can be carried out at *ca* 215 nm. Alternatively a refractive index detector or an ELSD can be used to monitor the eluent for polymers exhibiting no UV absorption at all. GPC of lipophilic polymers can be conducted in the same way using polymeric phases which are compatible with organic solvents.

Methods used for the assay of proteins by HPLC

For large molecules such as peptides, chromatographic packings with wide pores have to be used to facilitate partitioning of the large structures into the stationary phase. Typically ODS packings with 0.0003 μm pores are used. The chromophores in proteins are usually not particularly strong so UV detectors are set at short wavelengths. The mobile phases used are similar to those used for chromatography of small molecules on ODS columns. The mobile phase used in the EP analysis of insulin is composed of a mixture of phosphate buffer pH 2.3 and acetonitrile, and detection is carried out with the wavelength of the UV detector

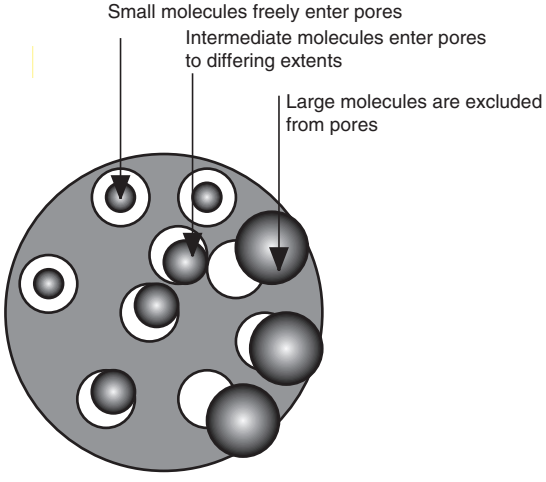


Fig. 12.33
The mechanism governing the analysis of high-molecular-weight analytes by gel permeation chromatography (GPC).

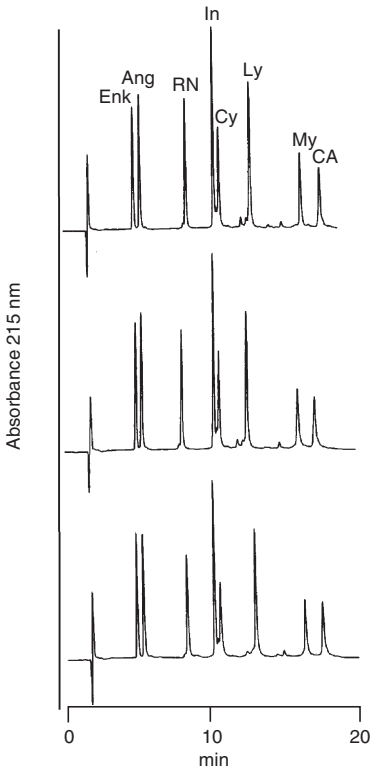


Fig. 12.34
Separation of a mixture of proteins on three lots of the same wide-pore (300 Å) octadecylsilyl (ODS) packing. Solvent A: H_2O/CH_3CN/trifluoroacetic acid (95:5:0.1 v/v/v). Solvent B: H_2O/CH_3CN/trifluoroacetic acid (5:95:0.085 v/v/v). Gradient 85% A + 15% B to 47% A + 53% B over 20 min. Proteins: Leucine enkephalin (Enk), angiotensin (Ang), Rnase (RN), insulin (In), cytochrome C (Cy), lysozyme (Ly), myoglobin (My) and carbonic anhydrase (CA). *(Reproduced with permission from Journal of Pharmaceutical and Biomedical Analysis.)*[10]

set at 214 nm. Peptide drugs may be contaminated with closely related peptides, which may differ by only one or two amino acids from the main peptide but may have high biological potency even when they are present in small amounts. The BP assay of human insulin includes a test for the presence of porcine insulin, which differs from human insulin by only one amino acid out of 30. The monograph stipulates that there should be a resolution of at least 1.2 between the peaks for human and porcine insulin when a test solution containing equal amounts of the two insulins is run.

Proteins may differ widely in lipophilicity depending on their amino acid composition. In the literature example shown in Figure 12.34, the reproducibility of three batches of a 300 Å ODS packing for the separation of a mixture of proteins was studied.[10] The mobile phase used was the popular system for protein analysis, utilising gradient elution with aqueous trifluoroacetic acid and acetonitrile with gradually increasing acetonitrile content. Under these conditions the most lipophilic proteins elute last.

With the increasing importance of drugs produced using biotechnology, which are mainly proteins, the pharmacopoeias have adopted a number of chromatographic methods specific to protein characterisation. To detail these methods in full would require a whole chapter; however, Table 12.9 summarises the main HPLC methods used in the quality control of protein drugs.

Table 12.9 The main high-performance liquid chromatography (HPLC) methods used in the quality control of protein drugs

Method	Description	Examples
Determination of protein impurities in bulk protein	Size exclusion chromatography using diol-modified silica gels. Proteins partition in the pores within the silica gel and high-molecular-weight (MW) species elute first	Control of high-MW proteins in somatropin, erythropoietin, aprotinin, alteplase, human and porcine insulins, size distribution in monoclonal antibodies and factor VIII. Control of low-MW heparins*
Analysis of tryptic and other proteolytic digests providing a unique fingerprint identity of the protein	Trypsin is the most widely used protease and breaks the protein down in low-MW peptides by cleaving at the C-terminus side of lysine or arginine residues, proteases with other specificities are used. The peptides are separated by reverse-phase chromatography	Fingerprint id of alteplase, somatropin, molgramostin, interferon-gamma 1b, erythropoietin, human, porcine and bovine insulins, insulin lispro, glucagon
Automated Edman degradation	The Edman degradation removes one amino acid at each cycle from the N-terminus. Separation of the amino acid derivatives is carried out by reverse-phase chromatography	Identification of the first 15 amino acids at the N-terminal of erythropoietin, alteplase and molgramostin

*Carbohydrate molecules.

Analysis of non-ionic surfactants with an evaporative light scattering detector and gradient elution

Non-ionic surfactants are used in formulations to solubilise drugs with poor water solubility; these compounds consist, in their simplest form, of an alkyl group attached to a polyethylene glycol chain. Non-ionic surfactants are usually mixtures, e.g. Cetomacrogol 1000, which has the general formula:

$$CH_3(CH_2)_m(OCH_2CH_2)_nOH$$

where m is 15 or 17 and n is 20 to 24. These compounds are amphiphilic and have affinity for water and organic solvents. Their analysis by HPLC requires a universal detector which does not require substances to have a chromophore in order to detect them. Formerly RI detectors were used for this type of analysis, but the ELSD allows gradient elution to be used, which is advantageous where complex mixtures contain compounds with widely different lipophilicities or polarities. For example, mixtures similar to Cetomacrogol 1000 have been separated on a polar aminopropyl column using a gradient between hexane/chloroform/methanol (76 : 19 : 5) and hexane/chloroform/methanol (56 : 14 : 30) over 30 min with ELSD monitoring of the eluent,[11] as shown in Figure 12.35. The methanol content of the mobile phase is gradually increased with time so that the more polar (longer chain) components elute within a reasonable time.

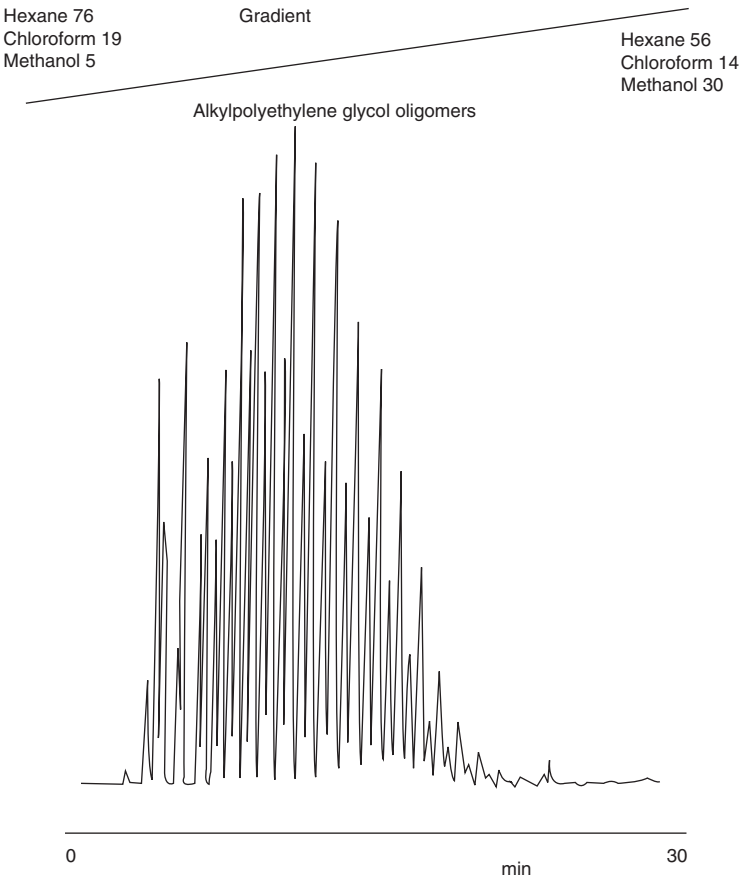


Fig. 12.35
Elution of non-ionic surfactants of varying chain lengths from an aminopropyl column with a hexane/chloroform/methanol gradient and evaporative light scattering detector (ELSD). *(Redrawn from Martin 1995.)*[11]

Derivatisation in HPLC analysis

Derivatisation in pharmaceutical analysis is most often used to improve the selectivity of bioanalytical methods. However, in some cases it is necessary to detect compounds which lack a chromophore. The analysis of aminoglycoside antibiotics is difficult because of complete absence of a chromophore and in addition the antibiotics are usually mixtures of several components. The BP assay of neomycin eyedrops carries out an identity check on the neomycin B and neomycin C components in the eyedrops by derivatising them so that they are detectable by UV monitoring (Fig. 12.36). The polarity of the highly polar amino sugars is reduced in some degree by the derivatisation so that they can be run on a silica gel column in a mobile phase composed of chloroform and ethanol. The advantage of using silica in this case is that the excess non-polar fluorodinitrobenzene derivatising agent will elute from the column well before the polar derivatised glycosides. Derivatisation reactions have also been extensively used in the analysis of amino acids. The literature on derivatisation for HPLC is extensive, but generally the use of a suitable detector would be preferred instead of resorting to derivative formation. In recent years pulsed amperometric detection has been increasingly applied to the analysis of aminoglycosides.

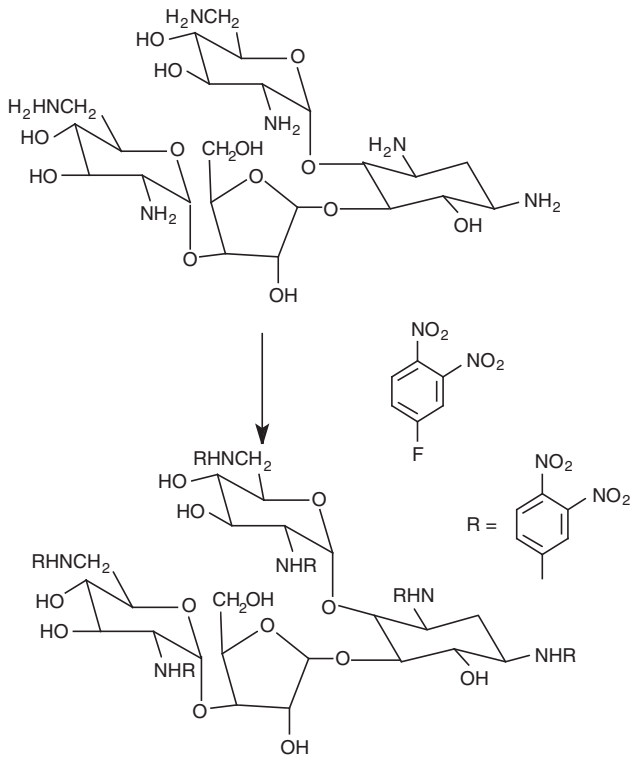


Fig. 12.36
Analysis of neomycin with derivative formation prior to chromatography.

Separation of enantiomers by chiral HPLC

Although about 40% of drugs are chiral compounds, only about 12% of drugs are administered as pure single enantiomers. This situation is gradually changing as a number of companies have now started to move toward producing enantiomerically pure forms of established drugs. Thus chromatographic separation of enantiomers is important from the point of view of quality control of enantiomerically pure drugs and also in bioanalytical studies, where the pharmacokinetics of two enantiomers may be monitored separately.

The basis of separation in chiral HPLC is the formation of temporary diastereomeric complexes within the chiral stationary phase. This causes enantiomers, which normally exhibit identical partitioning into a non-chiral stationary phase, to partition to a different extent into the stationary phase. Separation is due to the fact that enantiomers cannot interact with the three points of contact on a chiral surface in the same way. This is shown in Figure 12.37 where enantiomer 1 interacts with groups A, B and C. Its mirror image, enantiomer 2, is unable to interact in the same way with more than two of the groups on the chiral stationary phase, no matter how it is positioned.

There are numerous chiral stationary phases available commercially, which is a reflection of how difficult chiral separations can be, and there is no universal phase which will separate all types of enantiomeric pairs. Perhaps the most versatile phases are the Pirkle phases, which are based on an amino acid linked to aminopropyl silica gel via its carboxyl group and to (a-naphthyl)ethylamine via

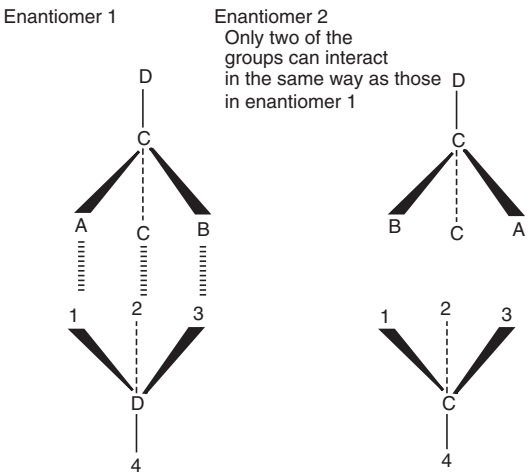


Fig. 12.37
The three points of attachment model for the separation of enantiomers on chiral stationary phase.

its amino group; in the process of the condensation, a substituted urea is generated. There is a range of these types of phases. As can be seen in Figure 12.38 the interactions with phase are complex but are essentially related to the three points of contact model. Figure 12.39 shows the separation of the two pairs of enantiomers (RR, SS, and RS, SR) present in labetalol (see Ch. 2, p. 47) on Chirex 3020.

Another popular chiral HPLC phase is based on cyclodextrins anchored onto the surface of silica gel. Cyclodextrins consist of 6, 7 or 8 glucose units linked together into a ring. They adopt a barrel-like shape, and the hydrophobic portion of an analyte fits into the cavity. For good separation, the chiral centre in the molecule must be level with the chiral 2 and 3 positions of the glucose units, which are arranged around the barrel rim, and which carry hydroxyl groups that can interact with the groups attached to the chiral centre through three-point contact. Figure 12.40 shows the β-blocker propranolol included within the cyclodextrin cavity.

Other chiral phases include those based on proteins, cellulose triacetate, amino acids complexed with copper and chiral crown ethers.

Two other strategies for producing separations of enantiomers involve the addition of chiral modifiers to the mobile phase (e.g. chiral ion-pairing reagents), which can bring about separation on, for instance, an ordinary ODS column and the formation of derivatives with chirally pure reagents that produce different

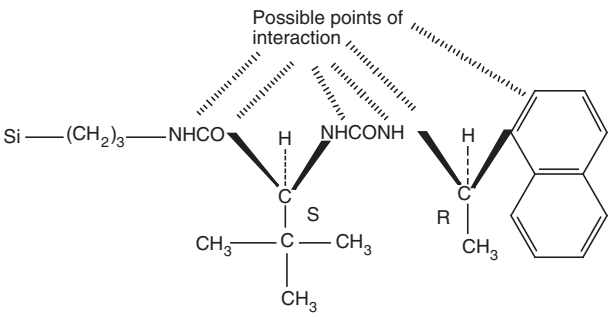


Fig. 12.38
Potential points for asymmetric interaction with a Pirkle-type chiral high-performance liquid chromatography (HPLC) phase. Pirkle type: Chirex 3020.

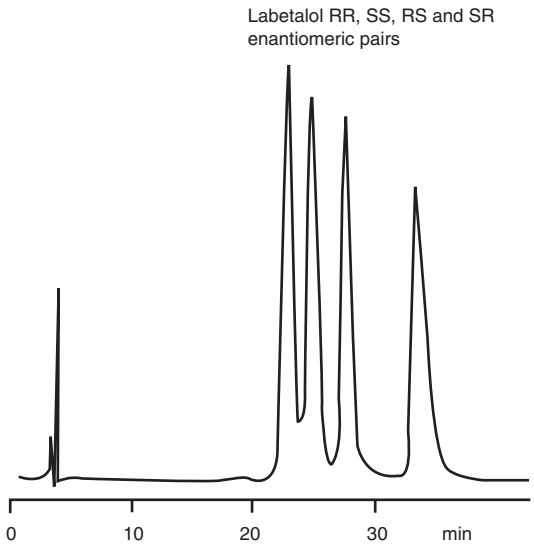


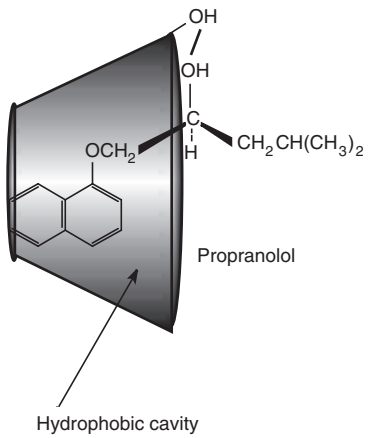
Fig. 12.39
'Separation of the four enantiomers of labetalol on Chirex 3020. Mobile phase hexane/1,2-dichloroethane/ethanol (containing one part in 20 of trifluoroacetic acid) (60:35:5). *(Reproduced with permission from Phenomenex Inc., technical document download TN-1015, file:///C:/Users/ConnF/Downloads/TN-1015%20Chiral%20HPLC%20of%20Antimalarials.pdf Fig 1.)*

diastereoisomers when reacted with opposite enantiomers of a particular compound (see GC example, Ch. 11, p. 280).

Ion chromatography

Pharmaceutical formulations usually contain a range of anions and cations in addition to the active ingredient. In order to ensure the quality of a product, tests should be carried out for these inactive components in the formulation. The columns used in this type of analysis are packed with either anion or cation exchange resins. The resins are polymeric with anionic or cationic groups on the surface that can interact with analyte ions. Typical interactions between ions and the surfaces of the resins are shown in Figure 12.41. Typical mobile phases consist of aqueous methane sulphonic acid for cation exchange and aqueous sodium hydroxide for anion exchange. Figures 12.42A and B show separations of anions and cations in two formulations. In example A, the anions are present in the

Fig. 12.40
Mechanism for chiral separation on a cyclodextrin phase.



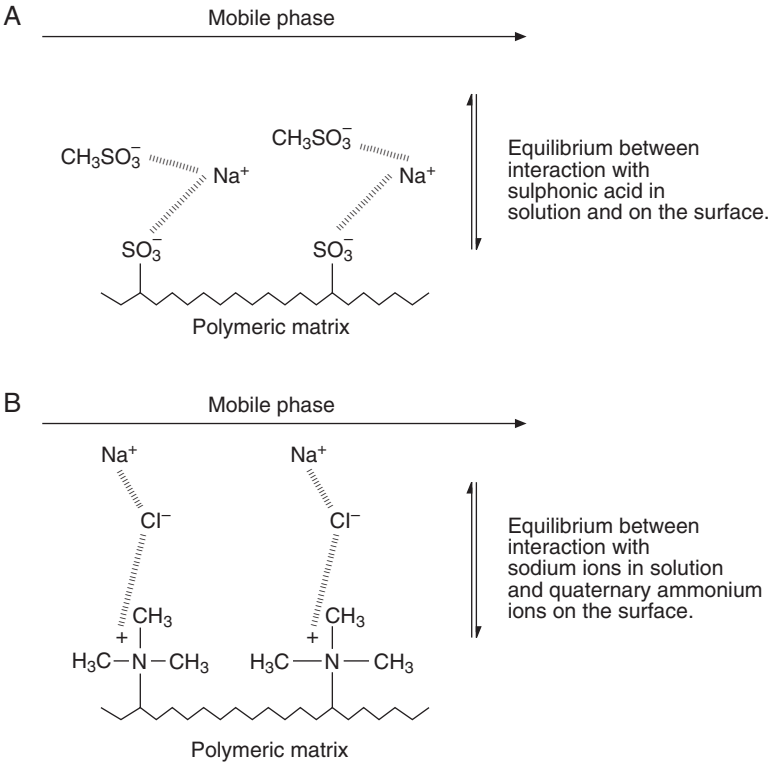


Fig. 12.41
Interactions in ion chromatography.

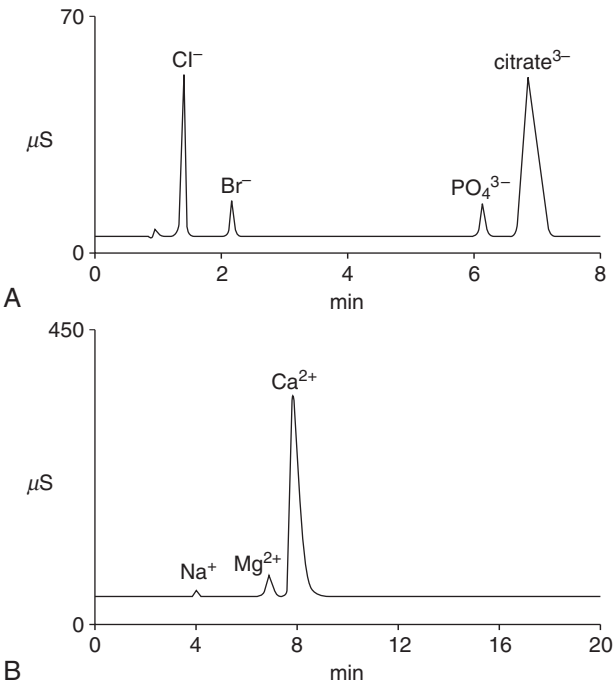


Fig. 12.42
(A) Analysis of anionic counter ions in a decongestant formulation, mobile phase sodium hydroxide gradient. (B) Analysis of cationic counter ions to excipients present in a decongestant/antihistamine tablet. μS = microsiemens (the units of conductivity are siemens). *(Reproduced with permission from © Dionex Ltd. from Ion Chromatography in the Pharmaceutical Industry Application Note 106, http://www.dionex.com/en-us/webdocs/4642-AN106_LPN0660.pdf fig 9 and fig 10.)*

formulation as counter ions for the bases in the mixture, and in the tablet formulation, in example B, the cations are counter ions to commonly used excipients such as calcium phosphate, which is used as a diluent, and magnesium stearate, which is used as a mould-release agent. The greater the charge of an anion or cation, the longer its elution time, e.g. Ca^{2+} elutes after Na^{+}. Also, the greater the ionic radius the longer the elution time, so the larger Ca^{2+} elutes after Mg^{2+}. A conductivity detector is used for detection of anions and cations. In this type of electrochemical detection, the conductivity of the mobile phase is measured as it passes through the detector. When an anion or cation passes through the detector, conductivity increases. In order for conductivity to be measured, the anions and cations used in the mobile phase (e.g. methane sulphonic acid and sodium) are removed, prior to detection, using a chemical suppressor device that replaces them with water. This leaves the anions or cations present in the sample as the main conducting species present in solution.

Ultra-high-performance liquid chromatography

As outlined in Chapter 10 (p. 256) the column efficiency is inversely proportional to the square root of the particle size of the stationary phase. The main factor that held back the use of very small particles was the very high pressures required to pump solvent through a column packed with such particles. In recent years ultra-high-pressure pumps which can pump up to 600 bar or more have been developed, and thus particle sizes down to 1.7 μm can be used. In Figure 12.43 a van Deemter plot is shown for propranolol run on a 5 μm and a 1.7 μm column. The reduction in the contribution for the mass transfer terms and the eddy diffusion terms resulting from lower particle size results in a plot where the value for H is less affected by flow rate and the column efficiency is higher. From the plot shown in Figure 12.43 the optimum point in the van Deemter plot for the 1.7 μm column is at 3.4 times the flow rate and twice the efficiency compared with the optimum for the 5 μm column. Thus UPLC can be used to produce comparable separations to HPLC but with much shorter run time.

UPLC also produces narrower peaks, meaning that the limits of detection are lower than with HPLC. Figure 12.44 shows a comparison between analysis of a steroid cream on a 5 μm C18 column and a 1.7 μm C18 column. It can be seen

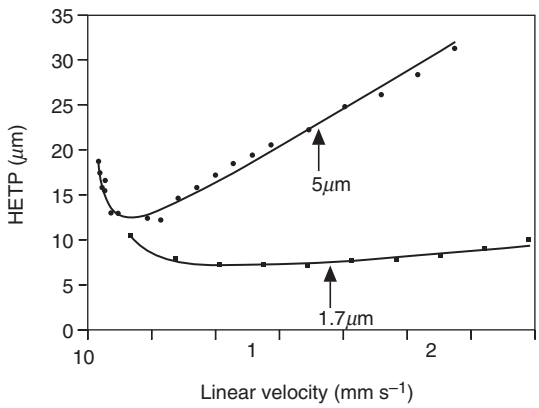


Fig. 12.43
Van Deemter plots for propranolol analysed on a 5 μm column and a 1.7 μm column. *(Redrawn with permission from Wren SAC, Tchelitcheff P. Use of ultra-performance liquid chromatography in pharmaceutical development.* J Chromatogr A *2006;**1119**:140–6.)*

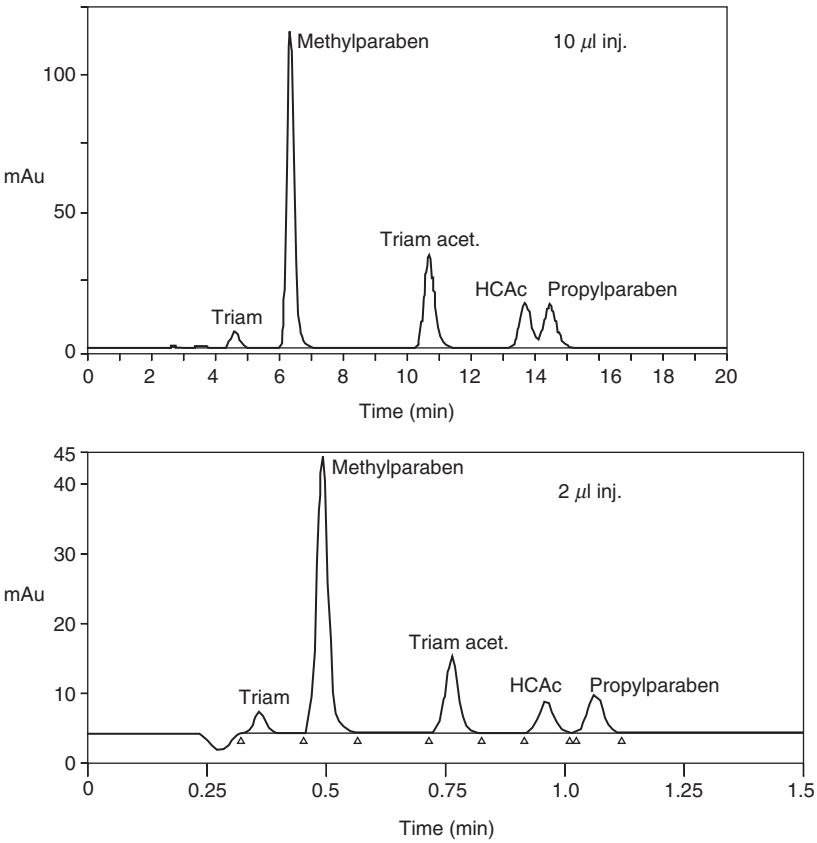


Fig. 12.44
Separation of triamcinolone acetonide in a cream from its excipients and related substances (Triam. = Triamcinolone, Triam. acet. = Triamcinolone acetonide, HCAc = hydrocortisone acetate–internal standard) by ultra-performance liquid chromatography (UPLC) and high-performance liquid chromatography (HPLC). *(Redrawn with permission from Wren SAC, Tchelitcheff P. Use of ultra-performance liquid chromatography in pharmaceutical development.* J Chromatogr A *2006;**1119**:140–6.)*

that separation of the drug and the excipients in the cream occurs in a much shorter time on the UPLC column. Thus for new registrations of drug products it is likely that UPLC methods will become standard.

Additional problems

1. Some non-steroidal anti-inflammatory drugs (NSAIDs) were found to have the following capacity factors in a particular mobile on a reverse-phase column: aspirin 0.4, naproxen 3.6, ibuprofen 14.5, diclofenac 10.4, paracetamol 0.2. Given that the column had a t_o of 2 min, determine the retention times of the NSAIDs.

Answers: aspirin 2.8 min; naproxen 9.2 min; ibuprofen 31 min; diclofenac 22.8 min; paracetamol 2.4 min

2. Predict the order of elution from first to last of the following steroids from an ODS column in methanol/water (60:40) as a mobile phase (Fig. 12.32).

Answers: triamcinolone, prednisolone, methylprednisolone, fluorometholone, fluorometholone acetate, progesterone

3. Predict the order of elution from first to last of the following morphinane compounds from an ODS column in an acetonitrile/buffer mixture pH 8.0 (10:90). Assume the p*K*a values of the bases are all similar (Fig. 12.33).

Answer: normorphine, morphine, codeine, ethylmorphine, thebaine, benzylmorphine

(Continued)

Additional problems *(Continued)*

4. An analysis is carried out on codeine linctus stated to contain 0.3% w/v of codeine phosphate. The mobile phase consists of 0.1 M acetic acid/methanol (40:60) and 0.01 M octane sulphonic acid, and chromatography is carried out on a reverse-phase column with UV monitoring at 285 nm. A one-point calibration was carried out against a calibration standard containing *ca* 0.06% w/v codeine phosphate. The following data were obtained:

- Weight of linctus analysed = 12.7063 g
- Density of linctus = 1.25 g/ml
- The linctus is diluted to 50 ml with water prior to analysis
- Area of codeine peak obtained by analysis of the linctus = 86 983
- Area of codeine phosphate calibration peak = 84 732
- Percentage of w/v of codeine phosphate in calibration standard = 0.06047.

Why is the octane sulphonic acid included in the mobile phase?
Calculate the percentage of w/v of codeine phosphate in the linctus.

Answer: 0.3053% w/v

5. Analysis is carried out on tablets containing naproxen 100 mg and aspirin 250 mg per tablet. A narrow-range calibration curve is constructed within ± 20% of the expected concentration of the diluted tablet extract. UV monitoring of the column effluent is carried out at 278 nm. Suggest a column and mobile phase for this analysis; both aspirin and naproxen are discussed earlier in this chapter. The following data were obtained for the analysis:

- Weight of 20 tablets = 10.3621 g
- Weight of tablet powder assayed = 257.1 mg
- Volume of initial extract = 250 ml.

Dilution steps:

- 10 to 100 ml
- 20 to 100 ml
- Calibration curve for naproxen $y = 174\,040\,x + 579$, r = 0.999
- Calibration curve for aspirin $y = 54\,285\,x + 1426$, r = 0.999 where x is in mg/100 ml
- Area of peak obtained for naproxen in diluted sample extract = 72 242
- Area of peak obtained for aspirin in diluted sample extract = 54 819.

Calculate the percentage of stated content for naproxen and aspirin.

Answers: naproxen 103.7%; aspirin 99.1%

6. Analysis is carried out on a cream stated to contain 2% w/w of both miconazole and hydrocortisone. An ODS column is used with a mobile phase consisting of acetonitrile/acetate buffer pH 4.0 (70:30) and the eluent is monitored at 220 nm. A narrow-range calibration curve, within ± 20% of the expected concentration of each analyte in the sample extract, was prepared for each analyte by plotting the ratio of the areas of the analyte peaks against fixed amounts of the internal standards for both analytes. The internal standards used were econazole and hydrocortisone 21-acetate, for miconazole and hydrocortisone, respectively.

How would the retention time of hydrocortisone compare in the mobile phase used in this assay with a mobile phase containing methanol/acetate buffer pH 4.0 (70:30), and why do you think hydrocortisone 21-acetate is used as an internal standard rather than the betamethasone used in the assay discussed earlier in this chapter?

Suggest a suitable extraction procedure for extracting the analytes from the cream and for removing oily excipients, and indicate any other preparation which might be required prior to analysis.

The following data were obtained:

- Weight of cream taken for assay = 1.0223 g
- Final volume of extract from cream = 100 ml
- Equation of line for miconazole $y = 0.044\,x - 0.013$, r = 0.999
- Equation of line for hydrocortisone $y = 0.048\,x - 0.024$, r = 0.999

where x is in mg/100 ml

- Area of hydrocortisone peak in sample extract = 62 114
- Area of hydrocortisone acetate peak in sample extract = 64 452
- Area of miconazole peak in sample extract = 35 557
- Area of econazole peak in sample extract = 38 385.

Calculate the percentage of w/v of miconazole and hydrocortisone in the cream.

Answers: hydrocortisone 2.013% w/w; miconazole 2.088% w/w

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www.agilent.com

One of the premier manufacturers of HPLC equipment. Many application notes on the use of HPLC in pharmaceutical analysis.

www.dionex.com

Contains a lot of information on ion chromatography.

www.separationsnow.com

A fairly new website containing much background material on various separation techniques.

www.chromatographyonline.findanalytichem.com.

13 Thin-layer chromatography

KEYPOINTS

Principles

- An analyte migrates up or across a layer of stationary phase (most commonly silica gel), under the influence of a mobile phase (usually a mixture of organic solvents), which moves through the stationary phase by capillary action. The distance moved by the analyte is determined by its relative affinity for the stationary vs the mobile phase.

Applications

- Used to determine impurities in pharmaceutical raw materials and formulated products.
- Often used as a basic identity check on pharmaceutical raw materials.
- Potentially useful in cleaning validation, which is part of the manufacture of pharmaceuticals.

Strengths

- Detection by chemical reaction with a visualisation reagent can be carried out, which means that more or less every type of compound can be detected if a suitable detection reagent is used.
- Robust and cheap.
- In conjunction with densitometric detection, it can be used as a quantitative technique for compounds which are difficult to analyse by other chromatographic methods because of the absence of a chromophore.
- Since all the components in the chromatographic system can be seen, there is no risk, as is the case in gas chromatography (GC) and HPLC analyses, that some components are not observed because they do not elute from the chromatographic system.
- Batch chromatography can be used to analyse many samples at once, increasing the speed of analysis, and can be automated.
- The method is flexible since thin-layer chromatography (TLC) plates can be simply treated with a variety of chemicals, thus imparting a wide range of properties to the stationary phase.

(Continued)

KEYPOINTS *(Continued)*

Limitations

- The number of theoretical plates available for separation is limited in routine TLC systems, although high-performance TLC (HPTLC) plates can offer nearly the same efficiency in a 10 cm distance as an HPLC column of the same length.
- Sensitivity is often limited.
- Not suitable for volatile compounds.
- Requires more operator skill for optimal use than HPLC.

Introduction

Thin-layer chromatography (TLC) has developed into a very sophisticated technique for identification of compounds and for determination of the presence of trace impurities. Since it was one of the earliest chromatographic techniques, a huge array of TLC-based tests is available and pharmacopoeial monographs reflect the extent to which this technique has been developed as a fundamental quality control technique for trace impurities. The reason for its prominence in this regard is due to its flexibility in being able to detect almost any compound, even some inorganic compounds. Following TLC, the entire chromatogram can be seen, and thus there is no doubt over whether or not components in a sample have failed to elute from a chromatographic system, as is the case with HPLC and GC and even capillary electrophoresis (CE). In this short chapter it would be impossible to outline all of the tests that can be used; comprehensive reviews of the technique have been written.[1,2] Even the most advanced form of TLC, high-performance TLC (HPTLC), remains essentially a simple technique. The sophistication in the application of the technique derives from the broad choice of stationary phases, mobile phases and the wide range of spray reagents which can be used for visualising the chromatogram.

The advances in the technology of the technique have been recently reviewed,[3] and these include the use of high-pressure TLC and interfacing with detection systems such as Raman spectroscopy and mass spectrometry.

Instrumentation

Figure 13.1 shows a simple thin-layer chromatography apparatus (see Animation 13.1). The most frequently used system is a glass or plastic plate coated with silica gel; for routine applications the silica gel particle size is in the range 2–25 μm. The method of use for this system is as follows:

(i) A few μl of sample solution are slowly spotted onto the plate at the origin. If more than *ca* 1 μl is applied at once, the spot will spread too far. The spot has to be allowed to dry between each application of 1 μl. Loadings of sample are typically 20 μg.

(ii) The bottom 0.5 cm of the plate is immersed in the mobile phase contained in a tank, and the liquid mobile phase is allowed to travel up the silica gel plate by capillary action.

(iii) The more polar a compound is the more it adsorbs (partitions into) the silica gel stationary phase, the less time it spends in the mobile phase as it

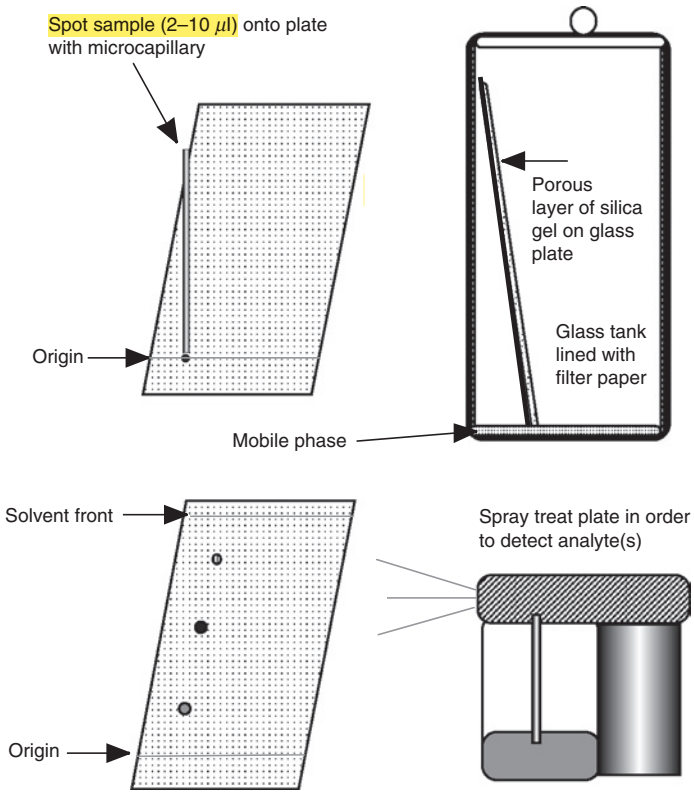


Fig. 13.1
Basic apparatus for thin-layer chromatography.

travels up the plate and thus the shorter the distance it travels up the plate in a given time. (see Animation 13.2)

TLC chromatogram

A diagram of a typical thin-layer chromatography plate after development and spraying to locate the analytes is shown in Figure 13.2.

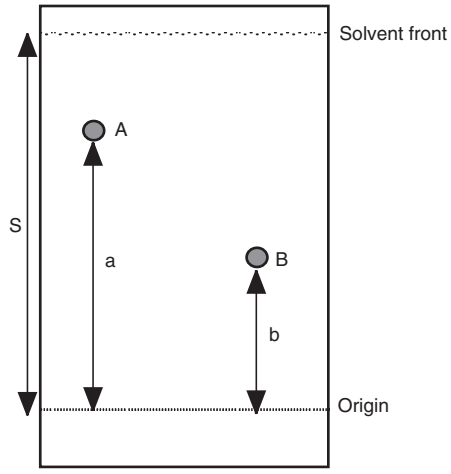


Fig. 13.2
Simple TLC chromatogram with silica gel used as the stationary phase.

In Figure 13.2, compound A is less polar than compound B since it travels further with the mobile phase in the same time. The distance travelled by the compound from the origin (where the compound is put onto the plate) divided by the distance travelled by the solvent from the origin is called the 'Rf value' of the compound. For example, for compound A, Rf = a/S; for compound B, Rf = b/S; the Rf is usually quoted as a Rf × 100 value. The area/intensity of a spot on a TLC plate is logarithmically related to the concentration of the analyte producing it.

Self-test 13.1

Solvent is allowed to move 10 cm from the origin up a TLC plate. The time taken to develop the plate for this distance is 15 min. On the basis of this information, complete the table below.

Distance moved by analyte	Rf × 100 value	Time spent in mobile phase	Time spent in stationary phase
(i) 8 cm			
(ii) 6 cm			
(iii) 4 cm			

Answers: (i) 80, 12, 3; (ii) 60, 9, 6; (iii) 40, 6, 9

Stationary phases

Silica gel (Fig. 13.3) is the most commonly used adsorbant for TLC. The rate at which compounds migrate up a silica gel plate depends on their polarity. In a given length of time, the most polar compounds move the least distance up the plate while the least polar move the farthest (Fig 13.4).

Although silica gel is used widely, some other polar stationary phases are also used in pharmacopoeial tests; silica gel may also be used in modified form. Some examples of stationary phases are given in Table 13.1.

Elutropic series and mobile phases

As described in Chapter 12, the strength of a mobile phase depends on the particular solvent mixture used. Table 13.2 lists common solvents in order of increasing polarity. The more polar a solvent or solvent mixture, the farther it will move a polar compound up a silica gel TLC plate. When non-polar compounds are

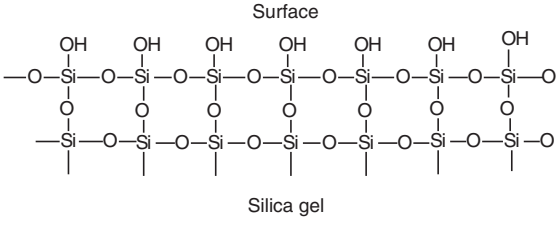


Fig. 13.3
The surface of silica gel.

See answer here

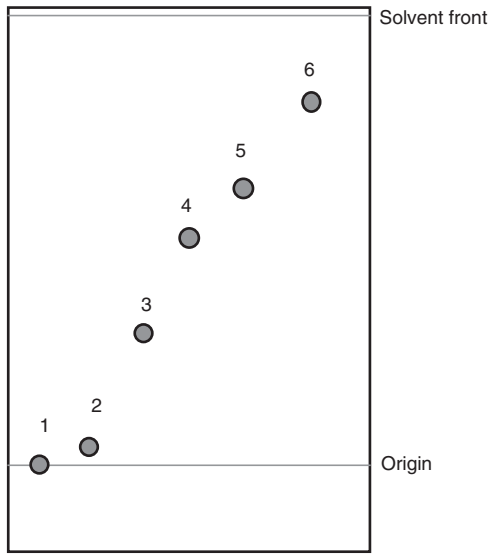


Fig. 13.4
Migration steroids on a silica gel TLC plate.

Table 13.1 Stationary phases which are commonly used in TLC

Stationary phase	Description	Applications
Silica gel G	Silica gel with average particle size 15 μm containing *ca* 13% calcium sulphate binding agent	Use in a wide range of pharmacopoeial tests. In practice commercial plates may be used which contain a different type of binder
Silica gel GF_{254}	Silica gel G with fluorescent agent added	The same types of applications as silica G where visualisation is to be carried out under UV light
Cellulose	Cellulose powder of less than 30 μm particle size	Identification of tetracyclines
Keiselguhr G	Diatomaceous earth containing calcium sulphate binder	Used as a solid support for stationary phases such as liquid paraffin used in analysis of fixed oils

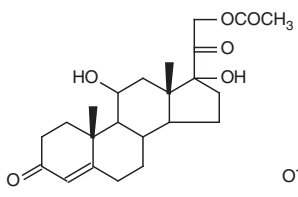
Table 13.2 Elutropic series

Solvent	Polarity index
Hexane (C_6H_{14})	0
Toluene (C_7H_8)	2.4
Diethylether ($C_4H_{10}O$)	2.8
Dichloromethane (CH_2Cl_2)	3.1
Butanol (C_4H_9OH)	3.9
Chloroform ($CHCl_3$)	4.1
Ethyl acetate ($C_2H_5COOCH_3$)	4.4
Acetone (CH_3COCH_3)	5.1
Methanol (CH_3OH)	5.1
Ethanol (C_2H_5OH)	5.2
Acetonitrile (CH_3CN)	5.8
Acetic acid (CH_3COOH)	6.2
Water (H_2O)	9.0

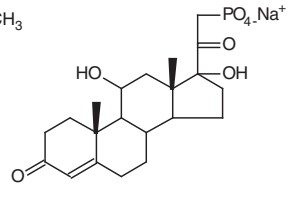
being analysed, there will not be a marked increase in the distance migrated with increasing polarity of the mobile phase since they migrate toward the solvent front under most conditions. Although water is polar, there are practical difficulties in using pure water as a solvent since many organic compounds are not very soluble in water; thus it is usually used in mobile phases containing a water-miscible organic solvent such as methanol. Quite subtle changes in separation can be achieved by using complex mixtures of solvents. Because of its simplicity, TLC is often used as a preliminary screen for identifying drugs, and thus mobile phases have been developed which ensure that a particular drug will have a quite different Rf value in one system compared with another.

Self-test 13.2

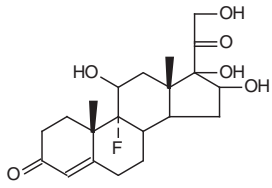
The steroids below are spotted onto a silica gel TLC plate. The plate is developed in methylene chloride/ether/methanol/water (77:15:8:1.2) and under UV light has the appearance shown in Figure 13.4. From your knowledge of the polarity of organic molecules, match the steroids to the spots on the TLC plate shown in Figure 13.4.



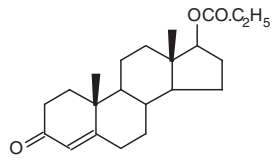
Hydrocortisone acetate



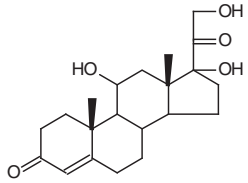
Hydrocortisone sodium phosphate



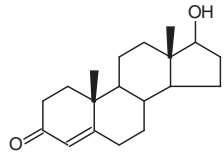
Triamcinolone



Testosterone propionate



Hydrocortisone



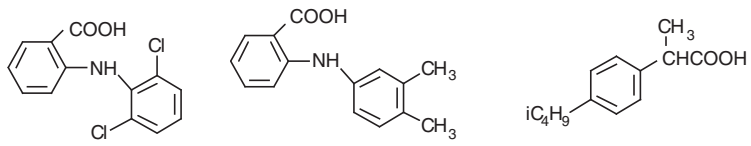
Testosterone

Answer: 1. hydrocortisone sodium phosphate; 2. triamcinolone; 3. hydrocortisone; 4. hydrocortisone acetate; 5. testosterone; 6. testosterone propionate

For example, in a general screen for acidic drugs, which includes most of the NSAIDs (Fig. 13.5), three mobile phases may be used.[1] Table 13.3 shows the Rf values obtained for three NSAIDs in three different mobile phases. It can be seen from the data in Table 13.3 that, even for closely related structures, slight differences in polarity and lipophilicity can be exploited to produce separation. For

See answer here

Fig. 13.5
Some NSAIDs.



Diclofenac | Mefenamic acid | Ibuprofen

Table 13.3

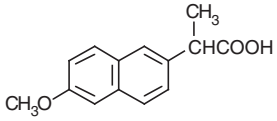
Mobile phase	Diclofenac Rf	Mefenamic acid Rf	Ibuprofen Rf
1. Chloroform/acetone (4:1)	25	41	46
2. Ethyl acetate	40	54	54
3. Ethyl acetate/methanol/ strong ammonia solution (80:10:10)	29	32	18

instance, ibuprofen is the least polar drug in system 1 but is the most polar drug in system 3, where the carboxyl groups in the structures will be ionised due to the ammonia in the mobile phase. It can also be seen that the polarity of a mobile phase containing a mixture of chloroform and acetone is similar to that of pure ethyl acetate.

The range of general TLC tests available for different classes of drugs has recently been reviewed.[4]

Self-test 13.3

Considering the three solvent systems (1, 2 and 3) given in Table 13.3, indicate which set of Rf values is most likely to apply to naproxen.



Naproxen

(i) 1. 18, 2. 25, 3. 24; (ii) 1. 40, 2. 25, 3. 14; (iii) 1. 33, 2. 45, 3. 14; (iv) 1. 20, 2. 28, 3. 10

Answer: (iii)

Modification of TLC adsorbant

Treatment of silica gel with KOH

For analysis of basic compounds, silica gel which has been sprayed with a solution of KOH in methanol may be used. Treating the plate with base ensures that basic compounds chromatograph as their free bases rather than as their salts. The salts of the amines have very low mobility in organic-solvent-based mobile phases since basic compounds tend to interact strongly with silanol groups on the surface of the silica; the presence of KOH in the stationary phase suppresses this interaction. The mobile phases used in these type of systems also typically

See answer here

contain a basic component. Examples of the mobile phases used for the analysis of basic drugs on KOH-impregnated silica gel include:

(i) Methanol/strong ammonia solution (100 : 1.5)
(ii) Cyclohexane/toluene/diethylamine (75 : 15 : 10)
(iii) Chloroform/methanol (90 : 10).

System 2 is quite non-polar and useful for discriminating between highly lipophilic bases, which include many of the antihistamines and narcotics, and sympathomimetic bases, which are often quite polar and move very little in mobile phase 2. The use of selective solvent systems is often combined with use of location agents that are selective for nitrogenous drugs.

Silanised silica gel

The surface of the silica gel can be rendered non-polar by reaction with dichlorodimethylsilane, as shown in Figure 13.6. A wide range of silanising reagents can be used in this type of reaction including octadecylsilanes, which produce ODS silica gel plates analogous to ODS HPLC columns. The BP uses silanised silica gel TLC plates in identity tests for penicillins. For example, a 0.25% w/v solution of ampicillin test material is applied to a silanised silica gel plate along with an ampicillin reference standard (0.25% w/v), Solution 2, and a mixture containing reference standards for ampicillin and amoxycillin trihydrate (0.25% w/v), Solution 3. The plate is developed with a mobile phase consisting of a solution of ammonium acetate adjusted to pH 5.0 with acetic acid and acetone (90 : 10). After development, the plate is stained with iodine vapour; the identity test specifies that the test substance should give a single spot with the same Rf as that seen for Solution 2 and that Solution 3 should show two clearly separated spots.

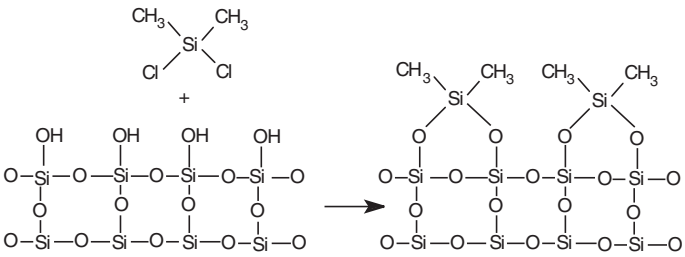


Fig. 13.6
Silanisation of silica gel with dichlorodimethylsilane.

This type of test could be carried out equally well with commercially produced ODS plates. Silica gel plates can be simply modified with reaction with organosilane reagents; the availability of a wide range of reactive organosilanes means that there is potential for producing a wide range of coated TLC plates for specific purposes.

Keiselguhr as an inert support

Keiselguhr in itself does not have strong absorptive properties, but it can be coated with a liquid or waxy stationary phase. The keiselguhr coated with liquid paraffin is used in a pharmacopoeial test for triglycerides and fatty acids in fixed oils. The keiselguhr plate is impregnated with a solution containing liquid paraffin in petroleum ether. This renders the surface hydrophobic. The samples of fixed

oil being examined are applied to the plate, and the plate is developed with acetic acid as the mobile phase. Acetic acid is a very polar solvent, and thus the liquid paraffin stationary phase does not dissolve in it appreciably. Furthermore, the triglycerides in the fixed oil are only weakly polar and will partition usefully between the liquid paraffin stationary phase and the acetic acid mobile phase. The longer the chain length of the fatty acids in the triglyceride, the lower the Rf of the triglyceride. The plate is visualised by staining with iodine and then permanently staining the iodine spots with starch solution. The BP shows the typical chromatograms that would be obtained from a number of fixed oils which are composed of mixtures of triglycerides in different proportions. The triglyceride composition of a particular fixed oil does not vary greatly and is very characteristic. A similar test to the one described above is carried out for the fatty acids composing the oil following hydrolysis of the triglycerides.

Other agents used to impregnate keiselguhr include formamide and propan-1,2-diol. In the case of these impregnating agents, the mobile phases used to develop the treated plates have to be of low polarity to avoid washing the agent off the plate.

Detection of compounds on TLC plates following development

A wide range of methods can be used to detect compounds on a TLC plate following its development with a mobile phase.

Ultraviolet light

In order to observe the absorption of UV light by an analyte, silica gel which has been impregnated with a fluorescent material is used to prepare the TLC plate. Light with a wavelength of 254 nm is used to illuminate the plate, and if the analyte absorbs UV light it can be seen as a black spot on a yellow background where it quenches the fluorescence of the background. This method of visualisation is used in many pharmacopoeial tests since most drugs possess chromophores. If a compound is naturally fluorescent, longer wavelength light at 365 nm may be used to visualise the plate. For example, the pharmacopoeial test for anthraquinones in aloes observes the fluorescence of these compounds under UV light at 365 nm.

Location reagents

There is a huge number of location reagents available, and these reagents range from those which are fairly specific for a particular type of analyte to those which will detect many different compounds.

Iodine vapour

The plate is put into a tank containing iodine crystals. This treatment will produce brown spots with many organic compounds; the staining is reversible, so if it is necessary to recover the compound once it has been located, the iodine may be allowed to evaporate by exposing the plate to air, and then the marked spot containing the compound of interest may be scraped off the plate. If a permanent record of the plate is required, it has to be covered to prevent the iodine evaporating, or the iodine spots may be sprayed with starch solution in order to stain them

permanently. Iodine is used as a location agent in pharmacopoeial TLC tests of fixed oils and of cetrimide.

Potassium permanganate

Potassium permanganate provides a method for the detection of sugars and sugar-like molecules and drugs with aliphatic double bonds. It is used in TLC identity checks for the antibacterial agents clindamycin and lincomycin and in a check for related substances in spectinomycin.

Ninhydrin solution

This reagent gives pink spots with primary amines and yellow spots with tertiary amines. It is used in pharmacopoeial identity tests for some of the aminoglycoside antibiotics such as gentamycin, in a limit test for aminobutanol in ethambutol and can be used as a general screen for nitrogen-containing drugs in conjunction with Dragendorff reagent. Dragendorff reagent will produce orange spots with tertiary amines and may be used to overspray plates which have been sprayed in the first instance with ninhydrin.

Alkaline tetrazolium blue

This reagent is quite specific for corticosteroids, producing blue spots on a white background. The tetrazolium spray is used in a test for related foreign steroids in fluclorolone acetonide.

Ethanol/sulphuric acid 20%

This reagent is used to produce fluorescent spots from corticosteroids such as dexamethasone or prednisolone by spraying the plate, heating to 120°C and then observing the plate under UV light at 365 nm.

Applications of TLC analysis

Qualitative identity tests

TLC is often used by BP monographs as part of a number of identity tests performed on pure substances. For extra confirmation of identity, more than one solvent system may be used and also different types of spray reagents may be used. Some examples of identity checks based on TLC have been mentioned earlier. Table 13.4 lists a few of the compounds which have their identity checked by TLC, and a variety of location reagents and mobile phases are used to illustrate the fact that there is much less uniformity about TLC methodology than there is in the case of HPLC or GLC methodology.

Limit tests

Where the structure of the impurity is known

TLC is used to perform limit tests for impurities in many pharmacopoeial monographs. A TLC limit test is based on comparison between a concentrated solution of an analyte and a dilute solution of an impurity. The intensities of the spots due to any impurities in the analyte are compared with the intensity of a spot or spots due to standards spotted separately onto the same plate. For the purposes of the examples illustrated as follows, intensity and size are regarded as being interchangeable, which they are to a large extent. For instance, a limit test might be

Table 13.4 Some examples of identity tests based on TLC described in pharmacopoeial monographs

Substance examined	Stationary phase	Mobile phase	Visualisation reagent	Comments
Framycetin sulphate	Silica gel + carbomer binder	10% w/v KH_2PO_4	Naphthalene diol/H_2SO_4	Rf and colour of the sample are compared with a pure standard. The resolution of the analyte from streptomycin is checked
Methyl prednisolone	Silica gel GF_{254}	Ether/toluene/ butan-1-ol saturated with water (85:10:5)	UV light 254 nm then ethanolic sulphuric acid (20%) + heat to 120°C	Rf and colours of the sample and standard are compared. Also Rf of an oxidation product is used as an additional check
Aprotinin	Silica gel	Acetate buffer	Ninhydrin spray	Rf and colour of the analyte spot is compared with standard
Levamisole	Silica gel H with fluorescent indicator	Toluene/ acetone/ 13.5 M ammonia (60:40:1)	UV light 254 nm	Rf and size of the spot obtained is matched to that of a standard.
Pentagastrin	Silica gel G	Analyte is examined by TLC in three different mobile phases	4-dimethyl-aminobenz-aldehyde in methanol/ HCl	The Rf of the analyte in three different mobile phases is determined and the colour of its spot is matched to that of the standard

conducted for hydrocortisone in hydrocortisone acetate as follows: 5 μl of 1% w/v solution of hydrocortisone acetate is spotted onto the origin of a TLC plate; at another position on the plate, 5 μl of a 0.01% w/v of hydrocortisone is spotted on. The TLC plate is developed in the solvent described in Self-test 13.2 and might appear as shown in Figure 13.7 when viewed under UV light. In the example shown, a small amount of hydrocortisone impurity can be seen running below the large spot due to hydrocortisone acetate, which is the main component in the sample. In line with the position where the hydrocortisone standard was spotted onto the plate, there is a very faint spot. In this case the spot for the hydrocortisone impurity in the sample can be seen to be more intense (larger)

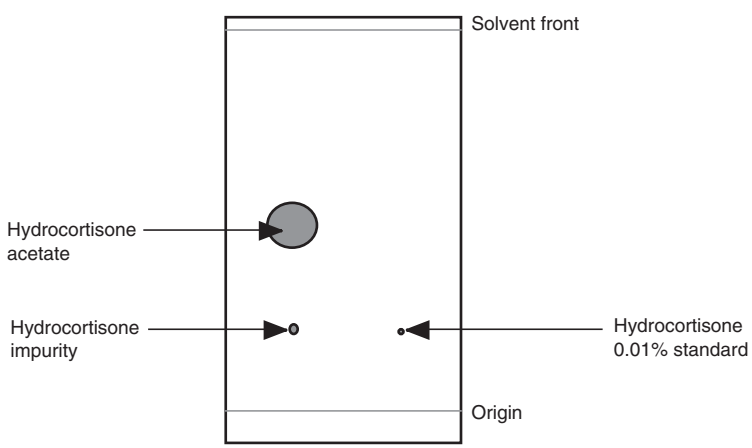


Fig. 13.7
TLC limit test for hydrocortisone in hydrocortisone acetate.

than the spot due to the 0.01% w/v hydrocortisone standard, and thus the sample has failed the limit test. This test is a 1% limit test since $0.01/1 \times 100 = 1\%$.

Self-test 13.4

A limit test is conducted for hydrocortisone in hydrocortisone sodium phosphate. 2 μl of a 1% w/v solution of hydrocortisone sodium phosphate is compared with 2 μl of a solution containing 0.02% w/v hydrocortisone standard using the solvent system given in Self-test 13.2.

(i) What is the percentage limit for hydrocortisone in hydrocortisone sodium phosphate set by this test?
(ii) Would the spot for the impurity appear above or below the substance being examined? (See Self-test 13.2.)

Answers: (i) 2%; (ii) Above

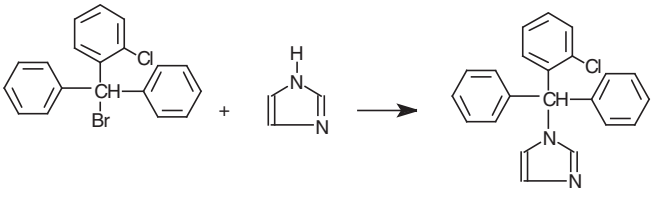
Table 13.5 shows some BP limit tests for known impurities used in pharmacopoeial monographs.

As in the case of hydrocortisone acetate, where hydrocortisone might be expected to occur as a result of hydrolysis of the acetate ester, tests for the presence of known impurities are based on the known manufacturing sequence or on likely degradation pathways. For example, the tests carried out on clotrimazole are based on the last step in its manufacture, as shown in Figure 13.8. Unreacted imidazole is an obvious impurity, and chlorotritanol would be readily formed from unreacted chlorotrityl bromide by hydrolysis, which would occur when the clotrimazole is extracted from the reaction mixture.

Most of the examples given in Table 13.5 have similar obvious origins in the drug manufacturing process.

Table 13.5 BP limit tests for known impurities in pharmaceutical raw materials

Test substance	Impurity	Limit set (%)
Clotrimazole	Chlorotritanol	0.2
Clotrimazole	Imidazole	0.2
Cyclizine	N-methylpiperazine	0.5
Dexpanthenol	3-aminopropanol	0.5
Ethinylestradiol	Estrone	1.0
Loprazolam mesylate	N-methylpiperazine	0.25
Mefenamic acid	2,3-dimethyl aniline	0.01
Mexiletine hydrochloride	2,6-dimethyl phenol	0.2
Phenoxymethylpenicillin	Phenylacetic acid	0.5



Chlorotrityl bromide Imidazole Clotrimazole

Fig. 13.8 Synthesis of clotrimazole.

See answer here

Where the structure of the impurity is unknown

A related type of TLC limit test is carried out where the identities of impurities are not completely certain. This type of test is used, for instance, on compounds of natural origin or partly natural origin, which may contain a range of compounds related in structure to the test substance which are co-extracted with the raw starting material. For example, the range of synthetic steroids originates from triterpenoids extracted from plants, which are extensively modified by fermentation and chemical synthesis.

The assumption which is made in the type of test described following is that the related unknown substances will produce a similar intensity of spot to the test substance itself at equal concentrations. For example, a limit test is conducted for related (foreign) alkaloids in codeine, which is extracted from the opium poppy, in which a range of alkaloids occurs: thus, the exact identity of the impurities may not be known. To conduct the test, 10 μl amounts of three solutions are applied separately to a TLC plate. The solutions contain 4.0% w/v codeine (Solution 1), 0.06% w/v codeine (Solution 2) and 0.04% w/v codeine (Solution 3). In the test, the dilute solutions of codeine are used as visual comparators for any impurities in the sample. The plate is developed in ethanol/cyclohexane/13.5 M ammonia (72 : 30 : 6), is dried and is then sprayed with iodobismuthate reagent, which is specific for nitrogenous drugs. After development and spraying, the plate might look like the diagram shown in Figure 13.9. The conditions set by the limit test are that:

(i) There should be no secondary spot in the chromatogram of Solution 1 which is more intense than the spot obtained with Solution 2.

(ii) There should be no more than one secondary spot with an Rf value higher than that of codeine which is more intense than the spot obtained with Solution 3.

In this test, two limits are being set: $0.06/4 \times 100 = 1.5\%$ and $0.04/4 \times 100 = 1.0\%$.

This type of test can be a little confusing at first since there are a number of permutations that can lead to the sample passing or failing of the test.

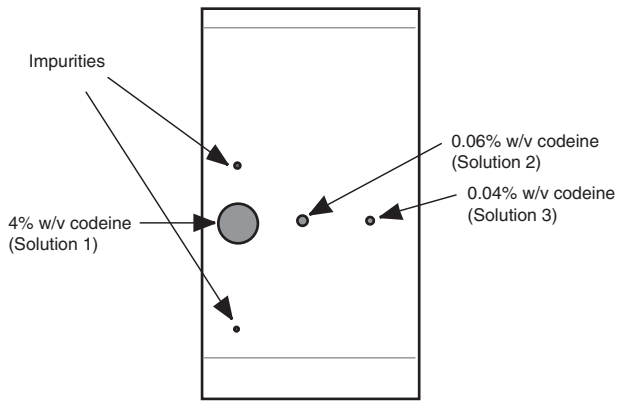
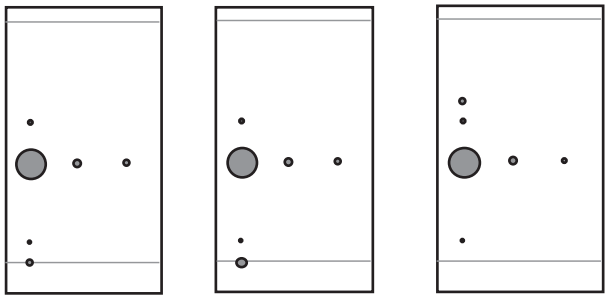


Fig. 13.9
TLC limit test for impurities in codeine.

Self-test 13.5

Three samples of codeine are analysed as described earlier. Indicate whether the TLC limit tests shown below pass or fail the samples. Solutions 1–3 appear in numerical order from left to right.



Answers: **(i)** Passes since there is no spot in the chromatogram of Solution 1 more intense than the spot due to Solution 2
(ii) Fails since the spot at origin is more intense than the spot due to Solution 2
(iii) Passes since, although one spot above codeine is more intense than the spot due to Solution 3, there is still no spot more intense than the spot due to Solution 2

Tests in which known and unknown standards are used

Table 13.6 shows some of the other limit tests set in pharmacopoeial monographs, ranging from a simple test for a known impurity to tests in which limits are set for more than one known impurity plus any unknown impurities which might be present.

Perhaps the most detailed pharmacopoeial limit test of this nature is carried out on tetracycline, where a 1% w/v solution is spotted onto the TLC plate with solutions of five structurally related tetracyclines ranging in concentration from 0.02 to 0.005% w/v.

Table 13.6 Some examples of pharmacopoeial limit tests

Analyte solution (Solution 1)	Limit test (Solution 2)	Limit test (Solution 3)	Limit test (Solution 4)
10% w/v procaine.HCl	No secondary spot > 0.005% w/v *p*-amino benzoic acid	–	–
1% w/v triamcinolone acetonide	No secondary spot > 0.02% w/v triamcinolone acetonide	No other spots > 0.01% w/v triamcinolone acetonide	–
2% w/v promethazine.HCl	No secondary spot > 0.02% w/v isopromethazine.HCl	No other spots > 0.01% w/v promethazine.HCl	–
1% w/v chloramphenicol palmitate	No spot with the same Rf > 0.02% w/v chloramphenicol palmitate isomer	No spot with the same Rf > 0.02% w/v chloramphenicol dipalmitate	No other spots > 0.005% w/v chloramphenicol palmitate

See answer here

High-performance TLC (HPTLC)

HPTLC is conducted on TLC plates which are coated with purified silica gel with a particle range of 2–10 μm, as opposed to 2–25 μm for standard commercial TLC plates. The narrower particle size range means that a greater number of theoretical plates are available for separation, and thus the spots on the TLC plate remain tighter. These type of plates may be run in a standard type of TLC tank, but optimal performance is obtained from horizontal development of the plates using apparatus of the type shown in Figure 13.10.

The advantages of horizontal development are:

(i) The mobile phase moves more quickly.

(ii) The proximity of the plate's surface to a saturating solution of mobile phase means that there is little evaporation of solvent from the surface of the plate, which in the case of vertical development can change the composition of the mobile phase as it moves up the plate.

(iii) In the vertical position, if the plate is not in a saturated atmosphere, solvent at the edge of the plate tends to evaporate, drawing solvent from the centre of the plate and causing the solvent at the edge of the plate to migrate more quickly. This does not occur when horizontal development is used.

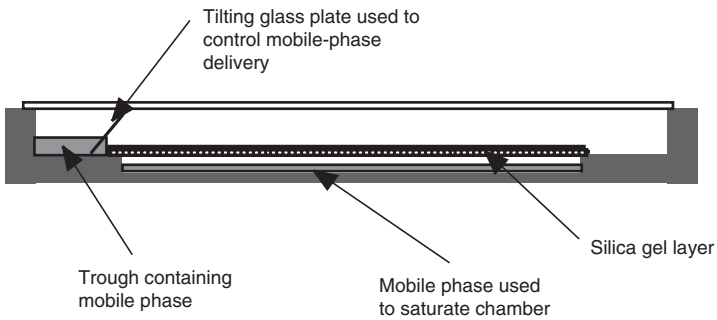


Fig. 13.10
Apparatus for HPTLC.

Applications of HPTLC

It is possible to use TLC as a quantitative method by using a densitometer to read spot intensity. Quantitative TLC is best carried out using high-performance systems. Densitometers can be used to quantify components in a sample on the basis of fluorescence or absorption of UV light. As discussed above, there are a number of advantages in using TLC and a major advantage is the ability to run batches of samples, which gives it an advantage over HPLC. HPTLC with fluorescence densitometry has been applied to the analysis of pharmacologically active thiols including the ACE inhibitor captopril.[5] Compounds such as captopril do not have a strong chromophore and thus require derivatisation to render them detectable, and this would be true whether HPLC or HPTLC were being used. In this example, the thiols were reacted with a thiol-specific reagent which produced fluorescent derivatives (Fig. 13.11) and were then analysed by TLC. Limits of detection for these compounds by this method were in the low picogram range.

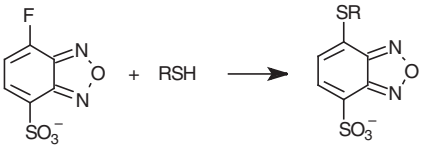
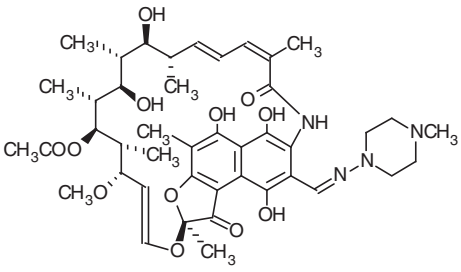
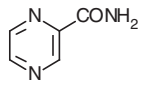


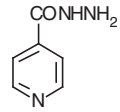
Fig. 13.11
Reaction of a thiol with reagent to form a fluorescent derivative.



Rifampicin (R)



Pyrazinamide (P)



Isoniazid (I)

Fig. 13.12

An HPTLC assay for rifampicin (R), isoniazid (I) and pyrazinamide (P) (Fig. 13.12) in a single dosage form was reported.[6] Pharmacopoeial methods only allow for the determination of each analyte in separate dosage forms. The HPTLC method was also able to resolve rifampicin from two of its named impurities. The analytes were quantified by densitometry by measuring absorbance at two different wavelengths. The precisions reported for quantification of the analytes were R ± 1.73, I ± 1.58 and P ± 1.07, which compared quite favourably with an HPLC method for the same dosage form. Figure 13.13 shows a densitogram for R, its related substances, desacetyl rifampicin (DAR) and rifampicin quinone (RQ), and I and P. Thus HPTLC can be used for the quantitative analysis of mixtures which include large and complex molecules such as rifampicin.

It would be possible to run many current pharmaceutical limit tests with a much higher degree of accuracy and precision if HPTLC methods were used.

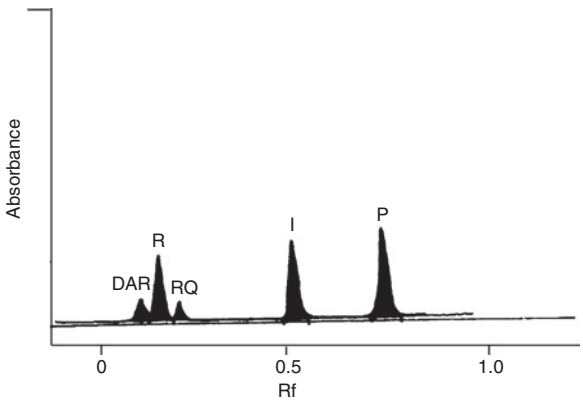


Fig. 13.13
Densitogram of the HPTLC separation of rifampicin (R), its related substances and isoniazid (I) and pyrazinamide (P). *(Reproduced with permission from Lin Ling B, Baeyens WRG, Del Castillo B, et al. J Pharm Biomed Anal 1989;7:1663–1670.)*

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