12 High-pressure 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. 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-pressure 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 in drugs in formulations with quantitation of any degradation products.
- Measurement of drugs and their metabolites in biological fluids.
- Determination of partition coefficients and p*Ka* 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 GC there is less risk of sample degradation because heating is not required in the chromatographic process.
- Readily automated.

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 is generated, which is expensive to dispose of.

Introduction

HPLC is the technique most commonly used for the quantitation of drugs in formulations (Fig. 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

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 between 1 and 200 μl (20 μl is often used as standard).

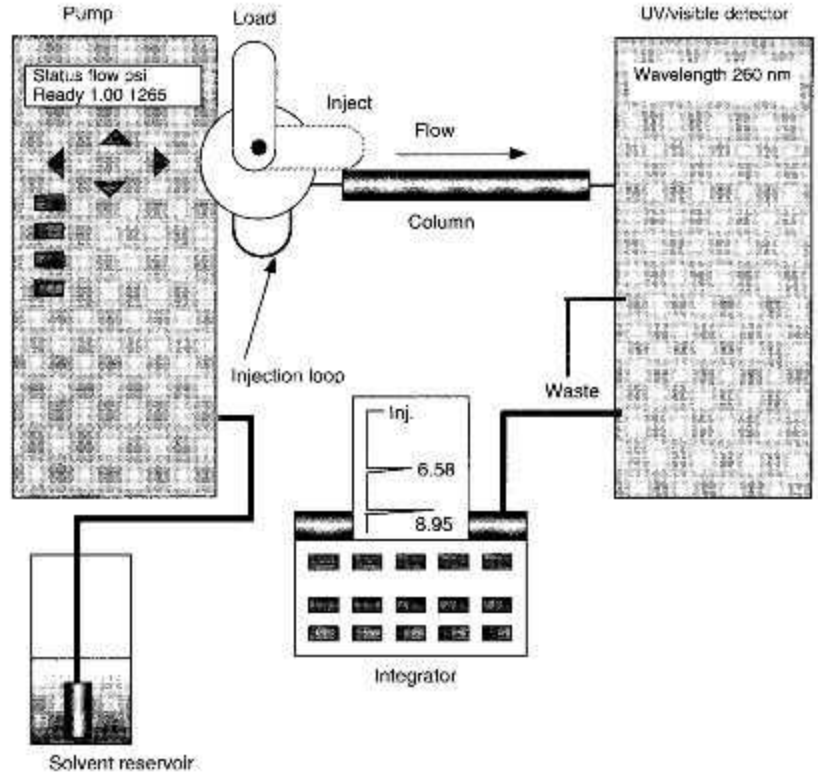


Fig. 12.1
A typical HPLC system set to a flow rate of 1 ml/min, indicating a column back pressure of 1265 psi and connected to a UV/visible detector which is set to monitor the column effluent at 260 nm.

(iv) A column, which is usually a stainless steel tube packed, usually, with octadecylsilane coated (ODS-coated) silica gel of 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. Also included in this figure is ODS-coated silica gel, which is a reverse-phase packing,

where the mechanism of retardation is due to partitioning of the lipophilic portion of a molecule into the stationary phase.

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 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.

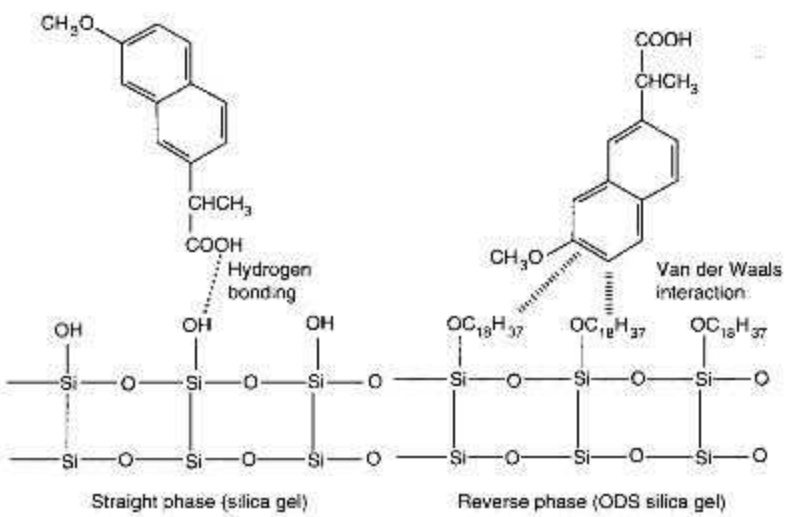


Fig. 12.2 Interaction of naproxen with the surfaces of silica gel and ODS silica gel HPLC packings.

In practice a restricted range of solvents is used with straight- and reverse-phase columns.

In straight-phase chromatography, four commonly used solvents are:

hexane; dichloromethane; isopropanol; methanol

Increasing strength →

In reverse-phase chromatography, four commonly used solvents are:

water; methanol; acetonitrile; tetrahydrofuran (THF)

Increasing strength →

The stronger a solvent or solvent mixture, the more quickly it will elute an organic compound from a particular column.

Self-test 12.1

Prednisolone (see Fig. 12.3 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. (ii), (i), (iii); 2. (v), (iii), (iv), (i), (ii)

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 12.3. When these compounds are eluted from a reverse-phase column using a mobile phase containing 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 12.4 shows the chromatogram obtained from the mixture of corticosteroids obtained 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.

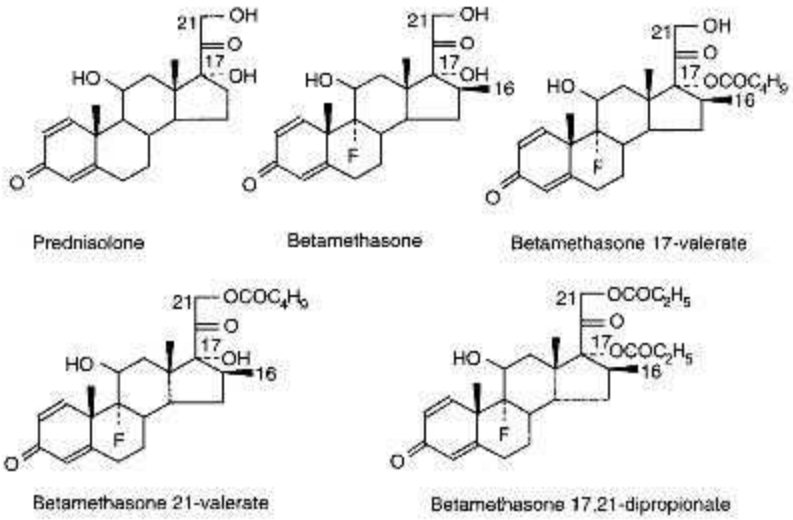


Fig. 12.3
The structures of some corticosteroids listed in order of their elution from an ODS column eluted with a methanol/water (75:25) mobile phase and below the chromatogram produced by the mixture.

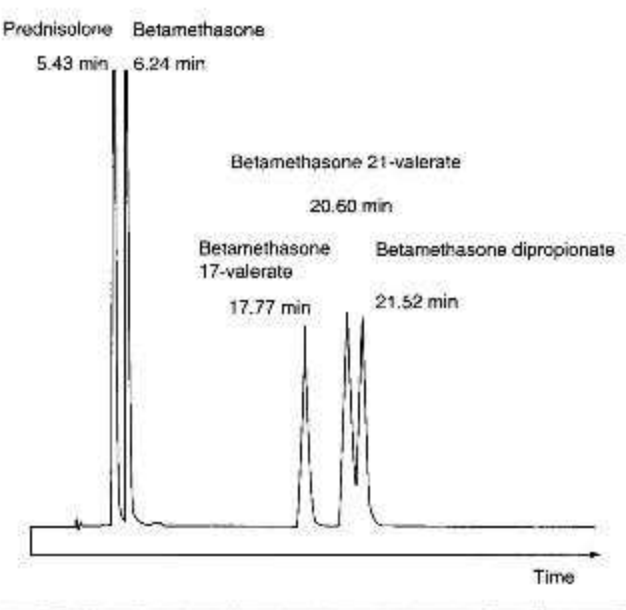


Fig. 12.4
Prednisolone and betamethasone and their esters eluted from an ODS column (25 cm × 4.6 mm) with methanol/water (75:25) as mobile phase, UV detection at 240 nm.

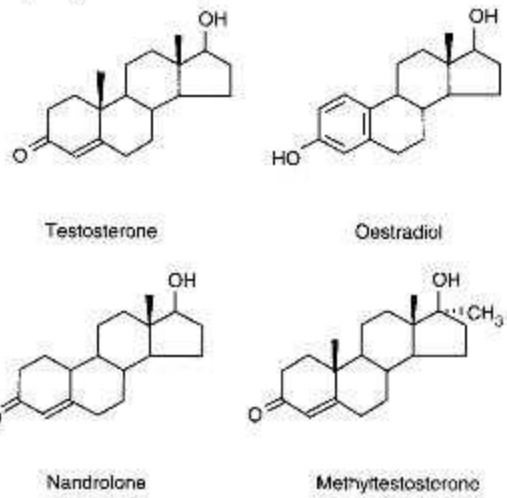
Considering the chromatogram shown in Figure 12.4 in more detail, the resolution between the betamethasone 21-valerate and the betamethasone dipropionate is incomplete, increasing the water content of the mobile phase would result in longer retention times for these two components and better separation, however, increasing the water content would also give very long retention times. If a formulation contained 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. 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.



Answer: oestradiol, nandrolone, testosterone, methyltestosterone

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 more stable coatings. The effects of pH on retention time, suprisingly, are as yet not fully understood. The following examples give an approximation of the effect of the pH 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. 29).

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'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.

The same type of calculation shown in Calculation example 12.1 can be carried out for basic drugs. Figure 12.5 shows the structures of some local anaesthetic drugs with their p*K*a values.

Figure 12.6 shows the effect of the pH of the mobile phase on the four local anaesthetics shown in Figure 12.5. The largest effects of pH are on bupivacaine 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

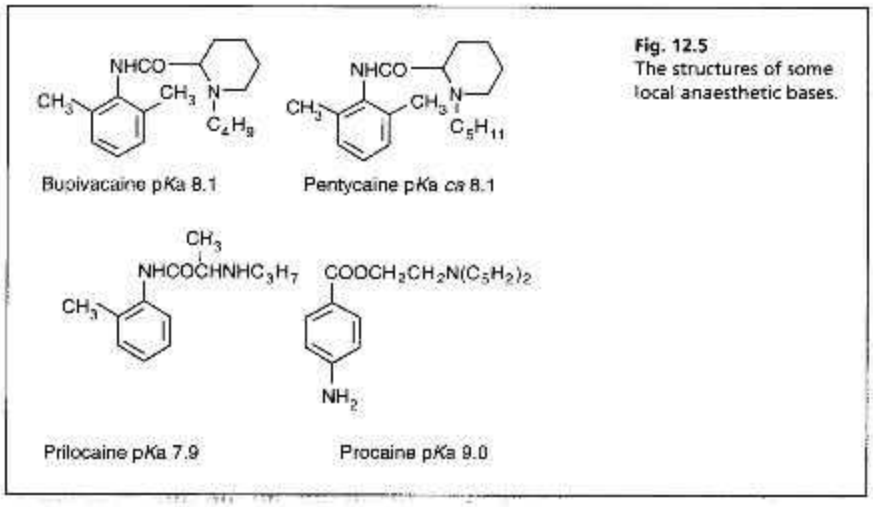


Fig. 12.5
The structures of some local anaesthetic bases.

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:

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

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

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

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

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:

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

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

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

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.

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.

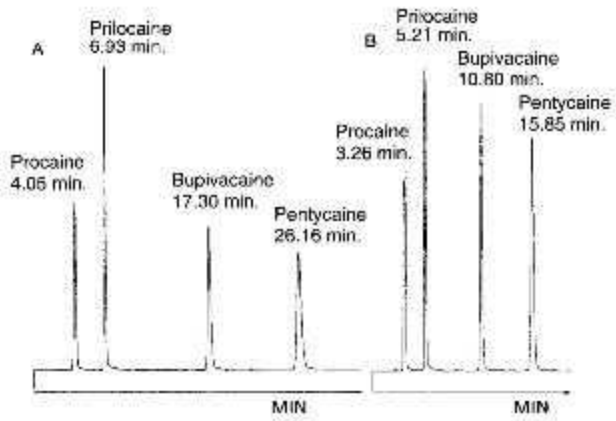


Fig. 12.6
Some local anaesthetics eluted from an ODS column with (A) acetonitrile/TRIS.HCl buffer pH 8.4 (60:40) and (B) acetonitrile/TRIS.HCl buffer pH 7.4 (60:40).

Self-test 12.3

The retention time of the acidic drug naproxen on an ODS column with a t_0 of 2.3 minutes in a mixture containing acetonitrile/ 0.05 M acetate buffer pH 5.2 (40:60) is 9.07 minutes. 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)

Box 12.1 Additional considerations of mobile phase pH

A major factor which 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 p*K*a 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.

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 12.1 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 critical 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 interactions of analytes with uncapped silanol groups. Disadvantages include expense and a tendency to swell when in contact with lipophilic mobile phases, which can destroy them. Such phases are best used with predominantly aqueous-based mobile phases.

Table 12.1 Some commonly used HPLC stationary phases

Stationary phase	Applications/comments
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 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 some additional interactions can occur with the phenyl groups on the stationary phase. 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
Cyanopropyl silica gel	A moderately polar phase applicable to the analysis of surfactants
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

Summary of detectors used in HPLC

For the majority of analyses of drugs in formulations, variable wavelength UV or diode array UV detectors are used. A typical UV dector 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.

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).

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 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.

Table 12.2 Some detectors commonly used in HPLC

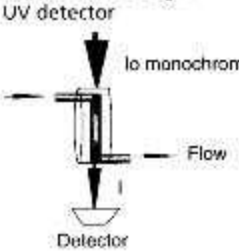
Detector	Applications
Variable wavelength UV detector 	Based on absorption of UV light by an analyte. A robust detector with good sensitivity works approximately in the range of 0.01–100 μg of a compound on-column. The sensitivity of the detector in part depends on the A(1%, 1 cm) value of the compound 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

Table 12.2 Some detectors commonly used in HPLC (*Cont.*)

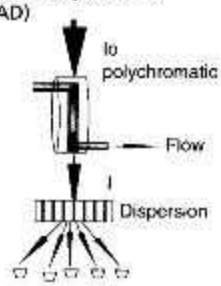
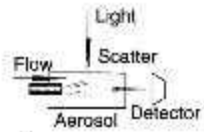
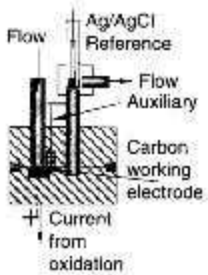
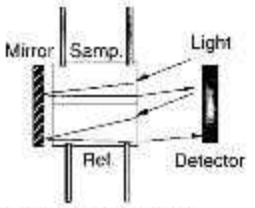
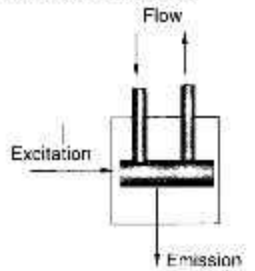
Detector	Applications
Diode array detector (DAD) 	An advanced type of UV detector with the ability to monitor across the full UV range simultaneously using an array of photodiodes which detect light dispersed by a fixed monochromator over a range of wavelengths offering a resolution of ca 1 nm. Useful 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 that 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 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

Table 12.2 Some detectors commonly used in HPLC (Cont.)

Detector	Applications
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 univeral 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

Self-test 12.4

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

(i) Variable wavelength UV detector.
(ii) ELSD.
(iii) 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

Performance of a diode array detector (DAD)

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 a HPLC column. Since a whole UV/visible spectrum is acquired several times across the width of a peak, this povides 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 12.7 illustrates four methods for looking at the purity of a peak using the information acquired by a DAD.

In the example illustrated in Figure 12.7, 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:

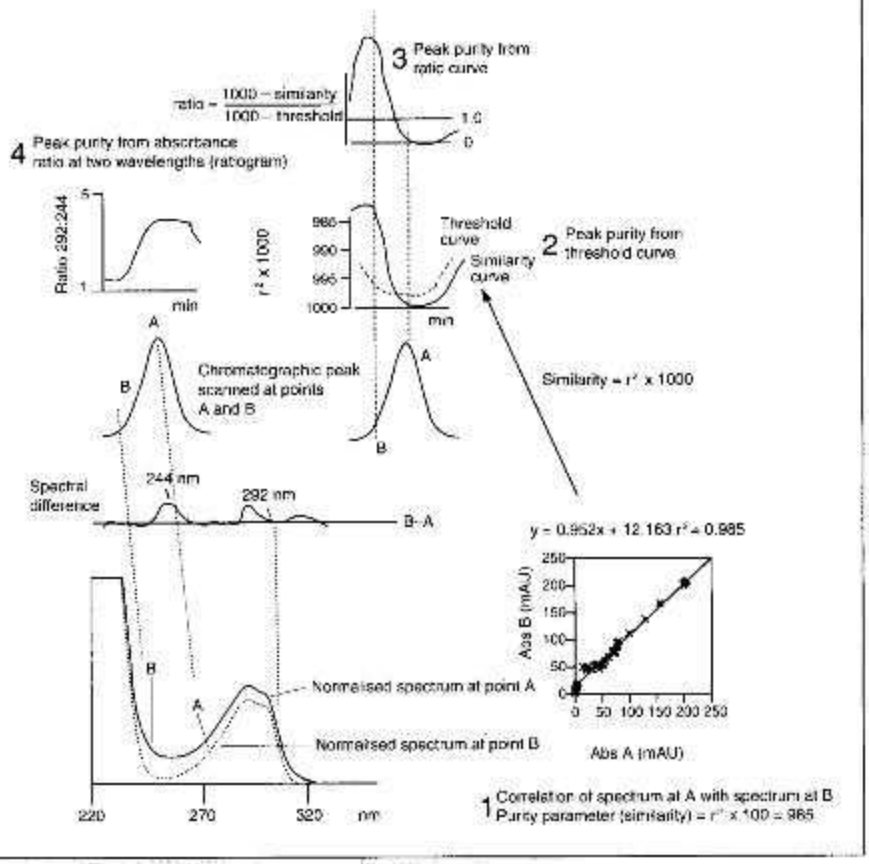


Fig. 12.7
Applications of diode array detection to peak purity determination.

(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. 12). 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 measure of peak purity when the spectra of leading 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 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 strongly and the impurity absorbs weakly against a wavelength where

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 in 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 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 $A(1\%, 1\text{ cm})$ value at the wavelength 243 nm used for monitoring paracetamol is *ca* 4 compared to an $A(1\%, 1\text{ cm})$ 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 12.8 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.

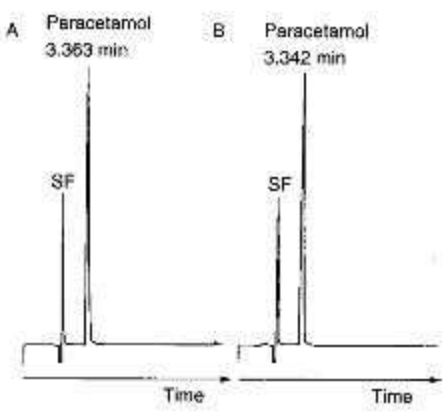


Fig. 12.8
(A) Extract from paracetamol tablets in comparison with (B) paracetamol standard at about the same concentration. Column: ODS 4.6 mm × 150 mm, mobile phase acetonitrile: 0.05 M acetic acid (85:15) flow rate 1 ml/min. (SF = solvent front).

Assay

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

(ii) Prepare a series of solutions from the stock solution containing 0.5, 1.0, 1.5, 2.0 and 2.5 mg/100 ml of paracetamol.

(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 and 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 and 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.

Table 12.3 Data obtained from the analysis of paracetamol standard solutions by 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

Area of paracetamol peak extracted from tablets = 45 205.

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

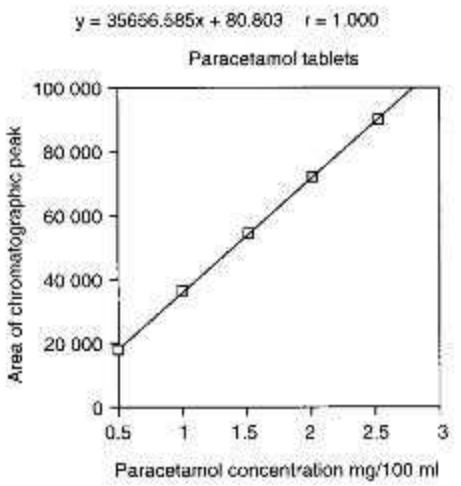


Fig. 12.9 Calibration curve for the determination of paracetamol in tablets obtained from HPLC analysis of calibration standards.

The graph shown in Figure 12.9 is obtained from the data given in Table 12.3; 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.

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:

$45\,205 = 35\,656x + 80$

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

$$\text{Concentration of paracetamol in diluted tablet extract} = \frac{45\,205 - 80}{35\,656} = 1.266 \text{ mg/100 ml}$$

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 mg/100 ml × 40 = 50.64 mg/100 ml.

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 12.5

Calculate the percentage of stated content in paracetamol tablets using the calbration 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 = 44 519.

Answer: 99.8%

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 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 12.10 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. 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.

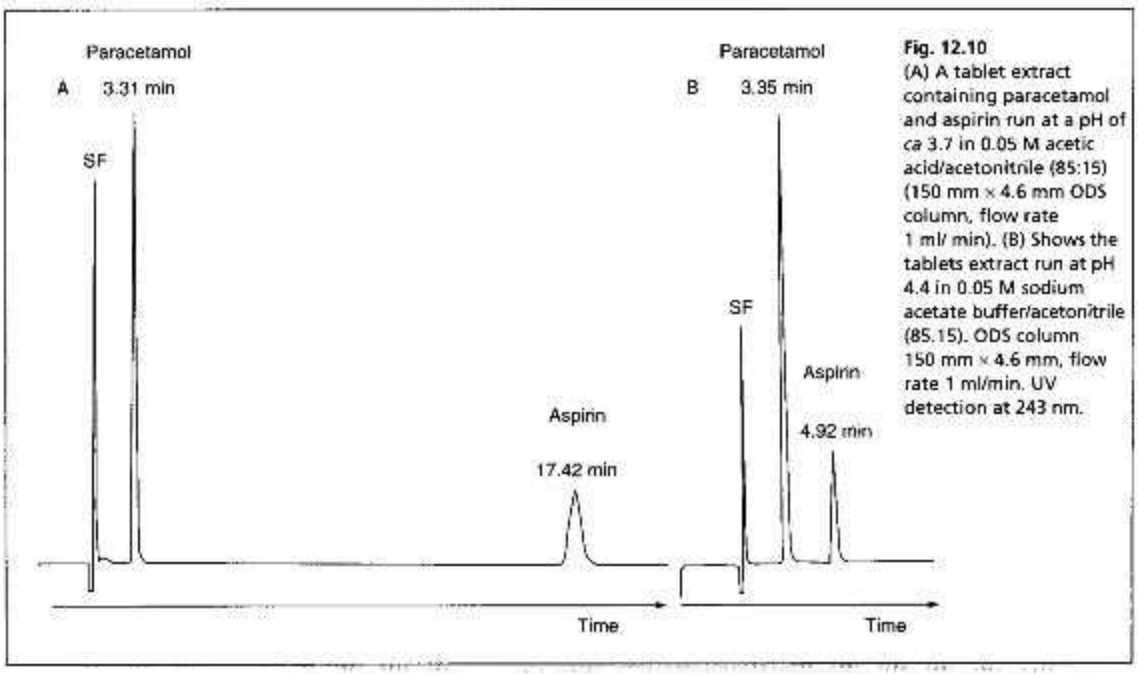


Fig. 12.10
(A) A tablet extract containing paracetamol and aspirin run at a pH of *ca* 3.7 in 0.05 M acetic acid/acetonitrile (85:15) (150 mm × 4.6 mm ODS column, flow rate 1 ml/ min). (B) Shows the tablets extract run at pH 4.4 in 0.05 M sodium acetate buffer/acetonitrile (85.15). ODS column 150 mm × 4.6 mm, flow rate 1 ml/min. UV detection at 243 nm.

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

Dilution of sample

- Initial volume in 250 ml.

Diluted:

- 25 to 100 ml
- 10 to 100 ml.

Self-test 12.6

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%

Assay of active ingredients in a linctus using a single point calibration for each analyte

Content per 5 ml of linctus

Pseudoephedrine hydrochloride 30 mg, triprolidine hydrochloride 1.25 mg, dextromethorphan bromide 10 mg and preservatives.

Explanation of the assay

This assay is altogether more difficult since three active ingredients are involved and several excipients interfere in the analysis, including one major excipient (methylparaben), which is not removed in the extraction process. 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 that 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

The linctus is sugar free so that it is sufficiently non-viscous to be measured with a pipette rather than weighed. An aliquot of the linctus (5 ml) is made basic by addition of 1 ml of 10 M ammonia solution. The aqueous layer is extracted with

2 × 10 ml of chloroform. The chloroform layers are combined and evaporated to dryness using a rotary evaporator. The residue is dissolved in *ca* 10 ml of methanol and transferred to a 100 ml volumetric flask and then diluted to volume with mobile phase. The areas of the peaks obtained from the linctus extract are compared with a solution containing pseudoephedrine.HCl, dextromethorphan.HBr and triprolidine.HCl (structures shown in Fig. 11.14) at the same concentrations as would be expected in the linctus extract.

Figure 12.11 shows the chromatogram of the linctus extraction; the eluent was monitored at 260 nm. This analysis illustrates some of the difficulties of analysing a more complex formulation and is by no means definitive. The main criticism of the chromatogram shown in Figure 12.11 is that the shape of the dextromethorphan peak is not perfect and it is incompletely resolved from the methylparaben. The pseudoephedrine peak elutes quite close to the void time of 2.3 min and although it is present in largest amount in the formulation (30 mg/5 ml) it produces a relatively small peak in terms of area because its $A(1\%, 1\text{ cm})$ value at 260 nm is only 12. Dextromethorphan produces a slightly tailing peak due to interaction with uncapped silanol groups; this interaction is less at lower pH values. The $A(1\%, 1\text{ cm})$ value of dextromethorphan at 260 nm is *ca* 30. Triprolidine produces a peak with a reasonable shape, probably because it is a weaker base (p*K*a 6.5) than dextromethorphan (p*K*a 8.5), and this also accounts for its longer retention time since it is less ionised at pH 6.8 than dextromethorphan. The $A(1\%, 1\text{ cm})$ value of triprolidine at 260 nm is *ca* 250 which accounts for the large area of its chromatographic peak despite the fact that it is the least abundant component in the formulation (the GC analysis of the active ingredients in this formulation is less problematical, see Ch. 13 p. 217). The data obtained are shown below.

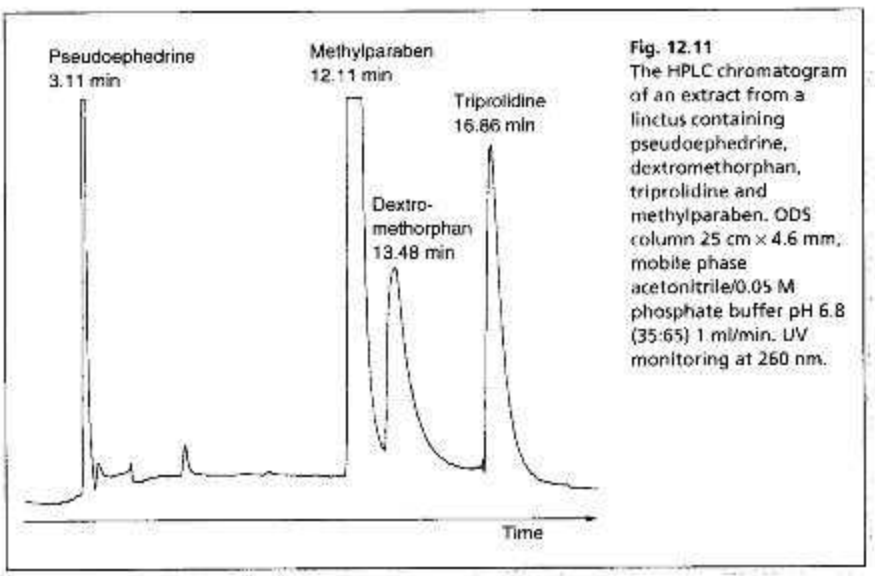


Fig. 12.11
The HPLC chromatogram of an extract from a linctus containing pseudoephedrine, dextromethorphan, triprolidine and methylparaben. ODS column 25 cm × 4.6 mm, mobile phase acetonitrile/0.05 M phosphate buffer pH 6.8 (35:65) 1 ml/min. UV monitoring at 260 nm.

Data obtained

- Volume of elixir extracted = 5 ml
- Final volume of extract = 100 ml
- Calculate the percentage of stated content for the triprolidine.HCl in the formulation.

Standard + concentration	Area of peak in standard	Area of peak in sample
Pseudoephedrine.HCl 31.23 mg/100 ml	325 178	318 915
Dextromethorphan.HBr 10.51mg/100 ml	479 918	469 293
Triprolidine.HCl 1.254 mg/100 ml	643 793	627 158

Calculation example 12.5

From simple ratio:

$$\text{Concentration of pseudoephedrine.HCl in extract} = 31.23 \times \frac{318\,915}{325\,178} = 30.63 \text{ mg/100 ml.}$$

5 ml of elixir were extracted to produce 100 ml of extract solution.

5 ml of elixir are stated to contain 30 mg of triprolidine.

$$\text{Percentage of stated content} = \frac{30.63}{30.0} \times 100 = 102.1\%.$$

Self-test 12.7

Calculate the percentage of stated content of: (i) triprolidine.HCl; and (ii) dextromethorphan.HBr in the elixir.

Answers: (i) 97.73%; (ii) 102.8%

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 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 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. The example given in Box 12.3 is typical of a BP assay incorporating an internal standard.

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

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. 12.12). 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 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

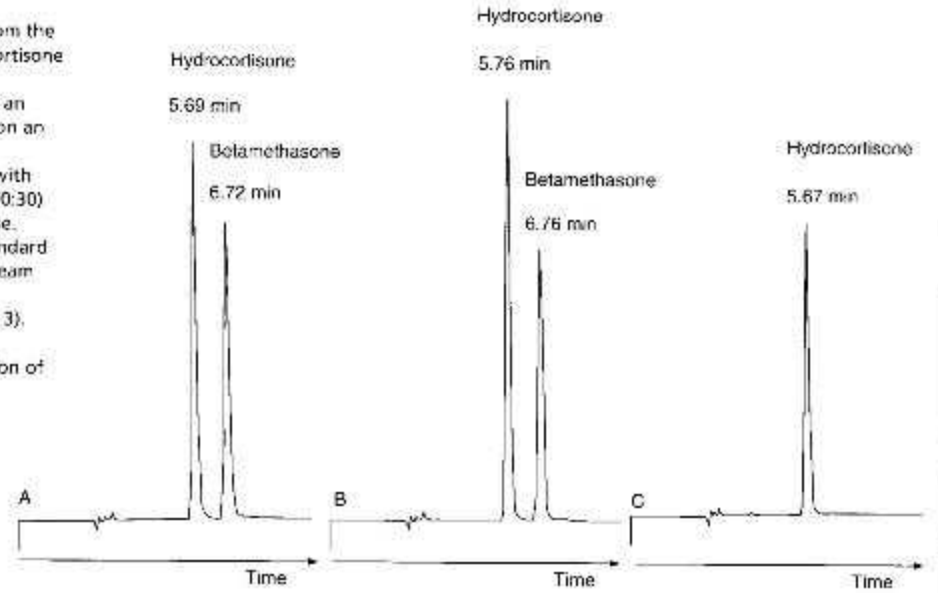


Fig. 12.12
Chromatogram from the analysis of hydrocortisone cream with betamethasone as an internal standard on an ODS column (25 cm × 4.6 mm) with methanol/water (70:30) as the mobile phase. (A) Calibration standard (Solution 1). (B) Cream extract + internal standard (Solution 3). (C) Cream extract without the addition of internal standard (Solution 2).

- 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.

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 =

$$\frac{\text{Response factor for Solution 3}}{\text{Response factor for Solution 1}} \times \text{concentration of hydrocortisone in Solution 1} \times \frac{\text{volume of Solution 3}}{100}$$

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.

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

Response factor for Solution 1 = $\frac{103\,026}{92\,449} = 1.1144$.

Response factor Solution 3 = $\frac{113\,628}{82\,920} = 1.3703$.

Concentration of hydrocortisone in Solution 3 = $\frac{1.3703}{1.1144} \times 0.01008 = 0.01239\%$ w/v = 0.01239 g/100 ml.

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

The volume of Solution 3 = 100 ml.

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

Weight of hydrocortisone cream analysed = 1.173 g.

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

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

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

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

Self-test 12.8

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

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. 12.13). 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.

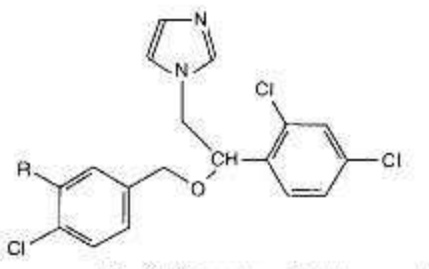


Fig. 12.13
The structures of miconazole and econazole.

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 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 12.4

Concentration of miconazole in calibration solution mg/100 ml	Area of miconazole peak	Area of econazole peak	Area miconazole / Area econazole
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 above data $y = 0.048\ x - 0.006$; $r = 0.998$.

Self-test 12.9

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.

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 12.14 where sodium octanesulphonic acid (SOSA) is added to the mobile phase (e.g. sodium phosphate buffer 0.1 M/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.

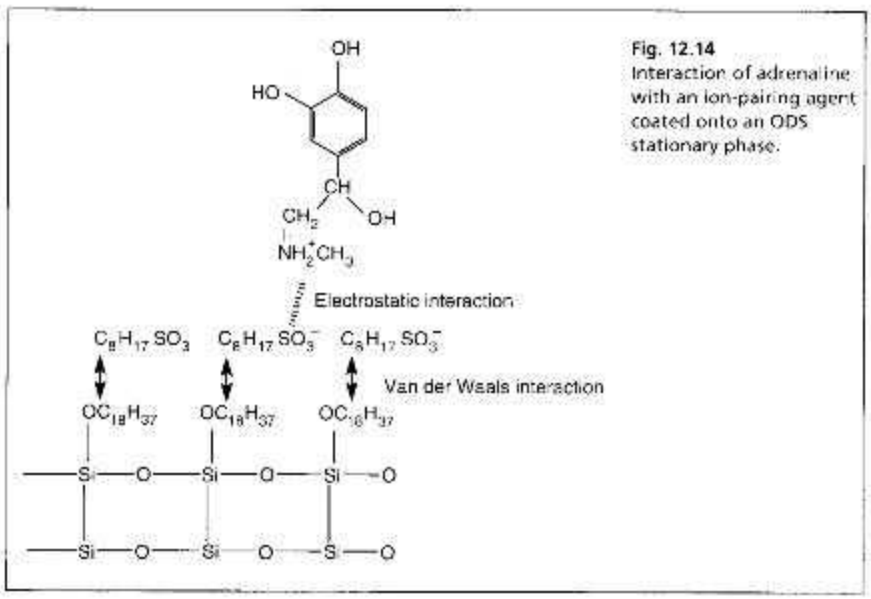


Fig. 12.14
Interaction of adrenaline with an ion-pairing agent coated onto an ODS stationary phase.

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 ion-pairing reagent. In the example given in Figure 12.15, 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.

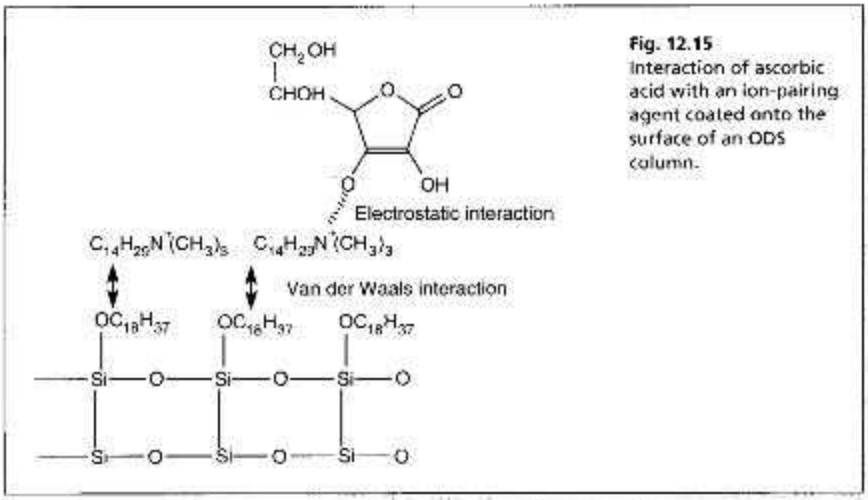


Fig. 12.15 Interaction of ascorbic acid with an ion-pairing agent coated onto the surface of an ODS column.

Assay of proteins with wide-pore HPLC packings

For large molecules such as peptides, chromatographic packings have to be used with wide pores to facilitate partitioning of the large structures into the stationary phase. Typically ODS packings with 0.0003 μm pores are used. The 1993 BP assay for human insulin is based on this type of packing. The chromophores in proteins are usually not particularly strong so that 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 BP 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 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 at 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 12.16, the reproducibility of three batches of a 300 Å ODS packing for the separation of a mixture of proteins was studied.[8] 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.

Another method used for eluting proteins from an ODS column is via the salting out effect, where mobile phase gradient is run from high to low salt concentration; again the most lipophilic proteins elute last.

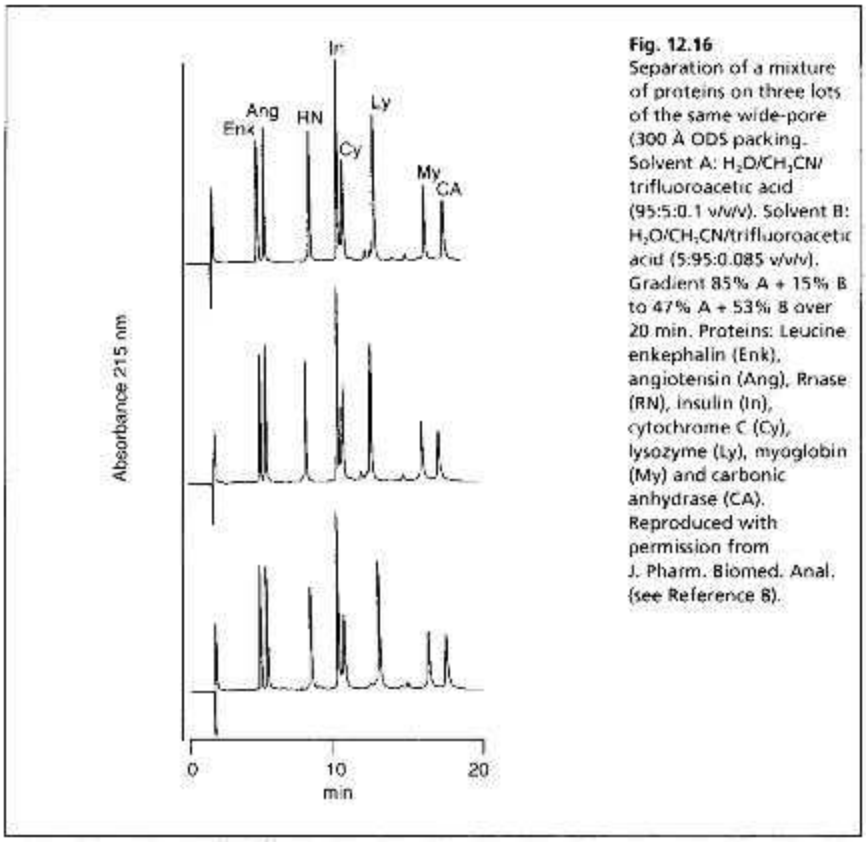


Fig. 12.16
Separation of a mixture of proteins on three lots of the same wide-pore (300 Å ODS packing. Solvent A: H_2O/CH_3CN/trifluoroacetic acid (95:5:0.1 v/v/v). Solvent B: H_2O/CH_3CN/trifluoroacetic acid (5:95:0.085 v/v/v). Gradient 85% A + 15% B to 47% A + 53% B over 20 min. Proteins: Leucine enkephalin (Enk), angiotensin (Ang), Rnase (RN), insulin (In), cytochrome C (Cy), lysozyme (Ly), myoglobin (My) and carbonic anhydrase (CA). Reproduced with permission from J. Pharm. Biomed. Anal. (see Reference 8).

Assay of hyaluronic acid by size exclusion chromatography

Polymeric materials have a number of pharmaceutical applications. Hyaluronic acid is a high molecular weight polymeric carbohydrate (Fig. 12.17) 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. 12.18). 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 calibrated with polymeric standards of known molecular weight, although corrections related to the viscosity of the analyte have to made when one type of polymer is used for calibrating a column used in order to determine the molecular weight of a different type of polymer because of differences in three-dimensional shape.

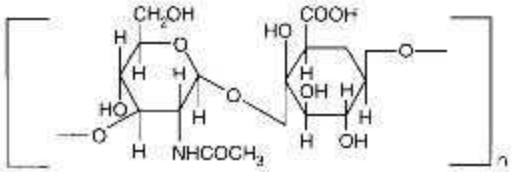


Fig. 12.17
The hyaluronic acid polymer.

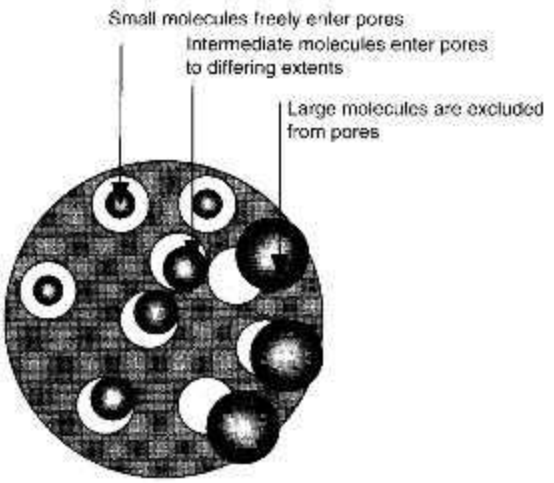


Fig. 12.18
The mechanism governing the analysis of high molecular weight analytes GPC.

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.

Analysis of non-ionic surfactants with an ELSD 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

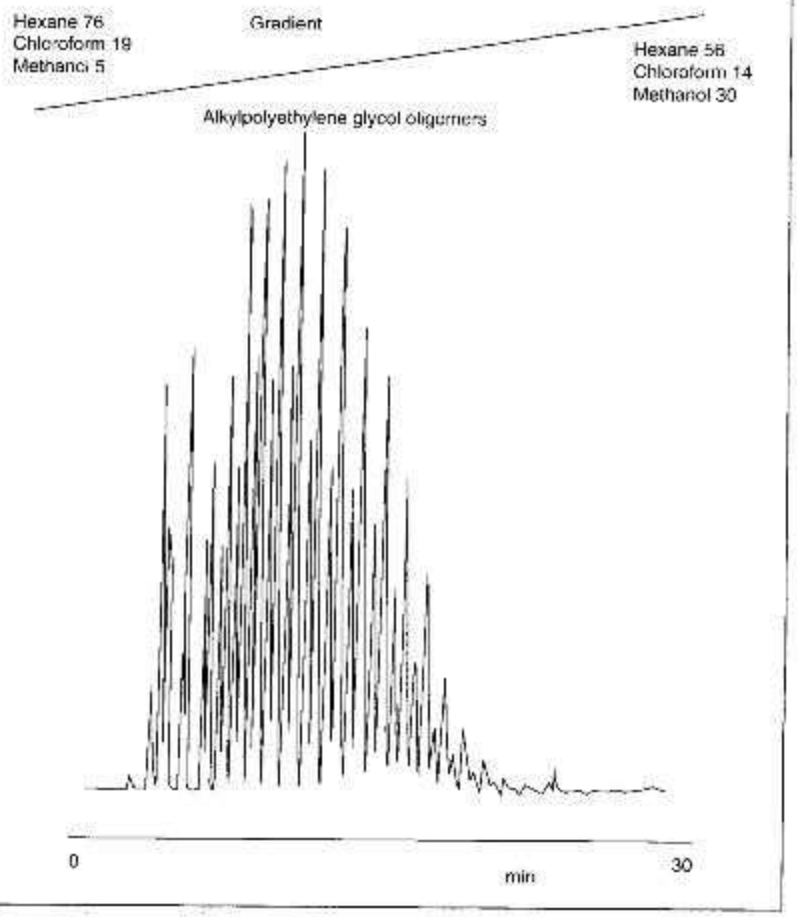


Fig. 12.19
Elution of non-ionic surfactants of varying chain lengths from an aminopropyl column with a hexane/chloroform/methanol gradient and ELSD detection.
Redrawn from Reference 9.

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[9] as shown in Figure 12.19. 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.

Assay of catecholamines in urine by ion exchange chromatography with electrochemical detection

The determination of drugs in biological matrices presents a particular analytical challenge. In the example given[10] an electrochemical detector is used because of its high selectivity for catecholamines which oxidise at a relatively low potential thus reducing interference by other less readily oxidised components in the urine. However, even with a selective detector there is still interference by the sample matrix. Figure 12.20A shows a chromatogram obtained using an ODS column with an ion-pairing agent which produces retention of the catecholamines as described earlier for assay of adrenaline in an injection (in the current example the ion-pairing

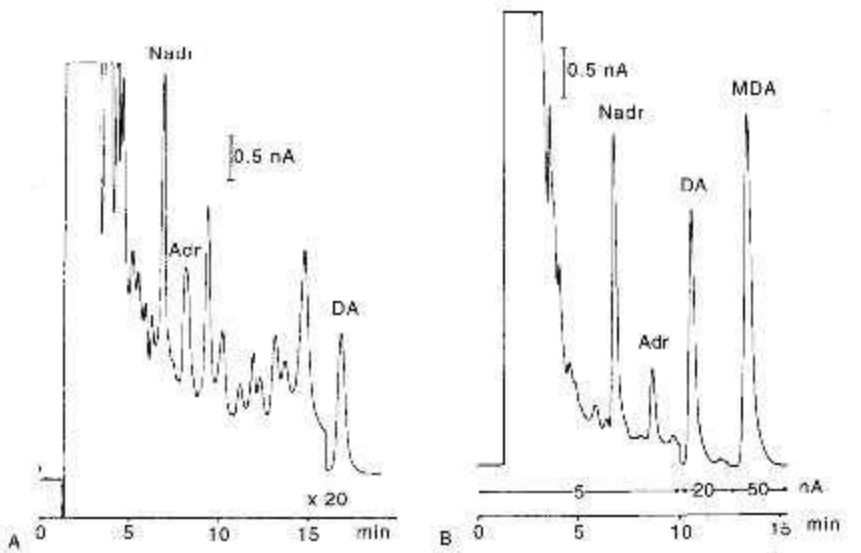


Fig. 12.20
Analysis of adrenaline (adr), noradrenaline (nadr) and dopamine (DA) in urine using: (A) An ODS column with ion-pairing agent in citrate buffer pH 5.0 with 2% tetrahydrofuran (THF) as the mobile phase; (B) a strong cation exchange column with citrate buffer pH 5.0 with 7% THF as the mobile phase. The eluent was monitored by electrochemical detection at a potential of 0.7 V. Methyldopamine (MDA) was used as an internal standard and added to the urine before extraction. Reproduced with permission from J. Chromatogr. Biomed. Apps. (see Reference 10).

agent is dimethylcyclohexyl sulphate). Figure 12.20B shows that selectivity for the catecholamines is increased when an ion exchange column is used in conjunction with electrochemical detection.

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. 12.21). 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.

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 towards producing enantiomerically

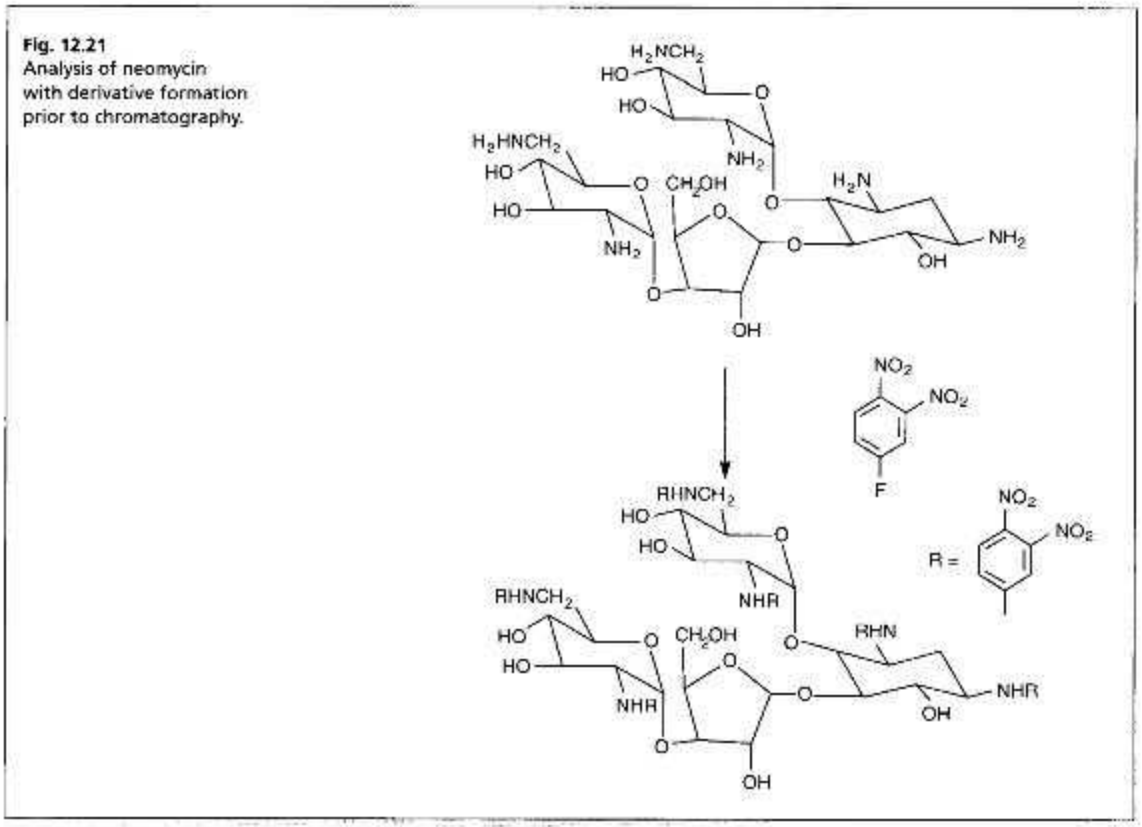


Fig. 12.21
Analysis of neomycin with derivative formation prior to chromatography.

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. In order for separation to occur, the enantiomers must have three points of contact with the stationary phase. This is shown in Figure 12.22, 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 pair. 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 via its amino group to (α-naphthyl)ethylamine; in the process of the condensation a substituted urea is generated. There is a range of these type of phases. As can be seen in Figure 12.23, the interactions with phase are complex but are essentially related to the three points of contact model. Figure 12.24 shows the separation of the two pairs of enantiomers (RR, SS, and RS, S,R) present in labetalol (see Ch. 2 p. 36) on Chirex 3020.

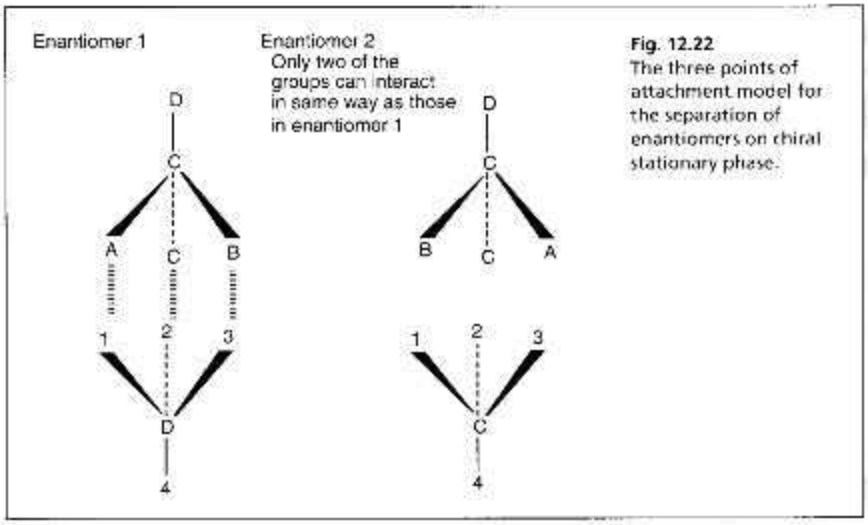


Fig. 12.22
The three points of attachment model for the separation of enantiomers on chiral stationary phase.

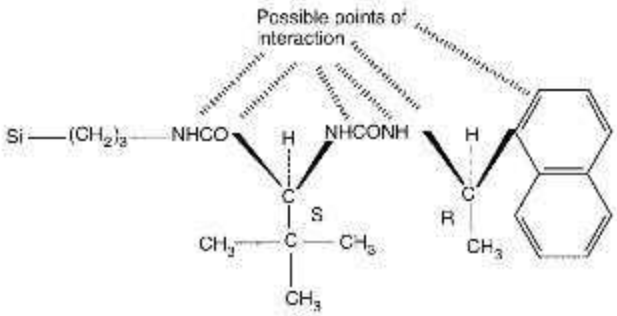


Fig. 12.23
Potential points for asymmetric interaction with a Pirkle-type chiral HPLC phase. Pirkle type: Chirex 3020.

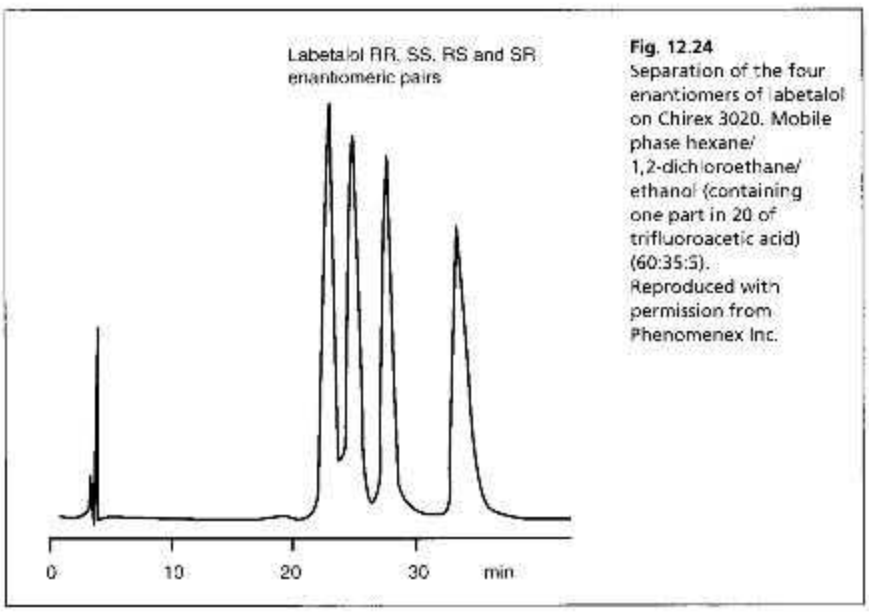


Fig. 12.24
Separation of the four enantiomers of labetalol on Chirex 3020. Mobile phase hexane/ 1,2-dichloroethane/ ethanol (containing one part in 20 of trifluoroacetic acid) (60:35:5). Reproduced with permission from Phenomenex Inc.

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 12.25 shows the β blocker propranolol included within the cyclodextrin cavity.

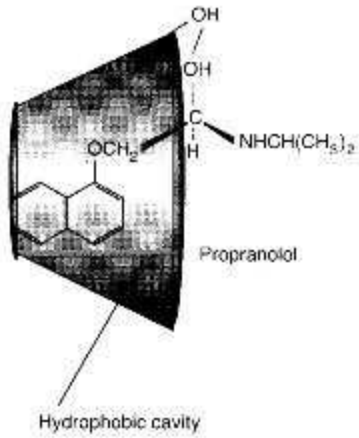


Fig. 12.25 Mechanism for chiral separation on a cyclodextrin phase.

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 diastereoisomers when reacted with opposite enantiomers of a particular compound (see GC example, Ch. 11 p. 219).

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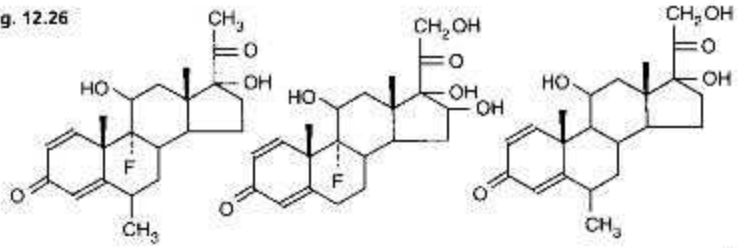
Additional problems

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_m of 2 min determine the retention times of the NSAIDs.

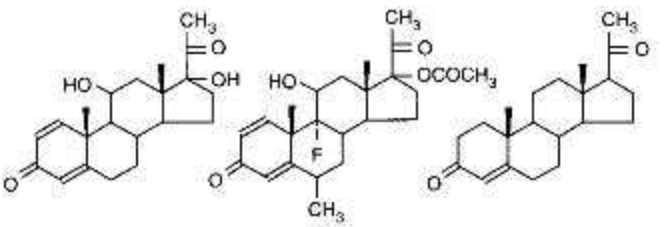
Answers: aspirin 2.8 min; naproxen 9.2 min; ibuprofen 31 min; diclofenac 22.8 min; paracetamol 2.4 min.

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. 12.26).

Fig. 12.26



Fluorometholone | Triamcinolone | Methylprednisolone

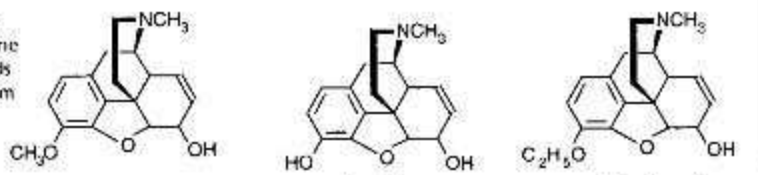


Prednisolone | Fluorometholone acetate | Progesterone

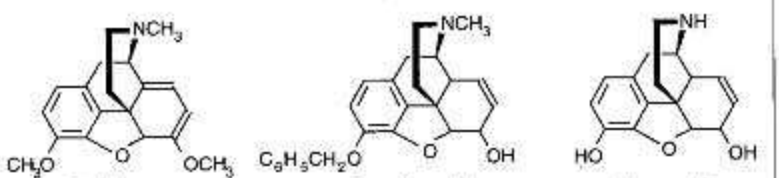
Answers: triamcinolone, prednisolone, methylprednisolone, fluorometholone, fluorometholone acetate, progesterone.

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 pKa values of the bases are all similar (Fig. 12.27)

Fig. 12.27 Morphinane compounds eluted from an ODA column.



Codeine | Morphine | Ethylmorphine



Thebaine | Benzylmorphine | Normorphine

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

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), contains 0.01 M octane sulphonic 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. Suggest a suitable column and mobile phase for this analysis. 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