CHAPTER 4

High-performance liquid chromatography in pharmaceutical bioanalysis

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4.1 *Introduction*

Since the mid-1980s the most frequently used technique in the bioanalysis of drugs has been high-performance liquid chromatography (HPLC). Prior to this the technique of choice was capillary gas–liquid chromatography (GLC). The reason for the rapid and dominant emergence of HPLC is fairly straightforward. In GLC it is a necessity that the analyte of interest can be volatilised as the separation is carried out in the gas phase, with the key factor for separation being the difference between analytes of their relative affinities for a gaseous mobile phase and a liquid stationary phase. Often, to facilitate this process, derivatisation of the analyte to a more volatile form is required. For example, acids would frequently need to be modified chemically to their more volatile ester forms prior to the chromatographic process. This requirement often led to assay procedures being complicated and not easy for the inexperienced operator to perform. In addition to this, GLC usually operates at

considerably elevated temperatures and a further consideration is therefore the thermal stability of the analyte(s) of interest.

HPLC on the other hand usually exhibits its resolving power at ambient or slightly raised temperatures in the liquid phase with the key requirements being that the analyte has some solubility in the liquid mobile phase and some affinity for the solid stationary phase. It is the relative strength of the analytes' affinity for each of these phases that gives the technique its separating capability.

Another factor in the emergence of HPLC in pharmaceutical applications has been the types of detectors that may be used 'generically' for wide varieties of drugs and which are compatible with HPLC. The most obvious example is the ultra-violet (UV) absorption detector, which has found extremely wide use as most drugs have a chromophore which will absorb UV light of the appropriate wavelength. Other detectors which are somewhat more selective but have found wide usage and are particularly suited to the liquid environment are fluorescence and electrochemical detectors. In recent years it has become routine to couple HPLC to mass spectrometric detectors and this has cemented the position of HPLC as the technique of first choice for the bioanalyst in today's pharmaceutical industry.

4.2 *A brief look at the theory of chromatographic separation in HPLC*

In HPLC, separation occurs due to partitioning between a stationary phase contained in a column and a liquid phase which is pumped under pressure through this column.

Let us consider a two-component mixture, A and B. Each of the components will have a certain affinity for the stationary phase and a certain affinity for the mobile phase. Provided there is sufficient difference between the analytes in their relative affinities for the two phases, i.e. in their partition coefficients, then in an HPLC system they will separate. For example, analyte A may have a stronger affinity for the mobile phase than it does for the stationary phase and will thus spend, relatively, greater time in the mobile phase than it does bound to the surface of the stationary phase. For analyte B the reverse may be true and the compound will spend a longer time bound to the stationary phase. In this case one would expect the two compounds to separate (to be resolved) and the compound with the highest affinity for the mobile phase, analyte A, to be eluted from the column first (Figure 4.1).

Analyte A would be said to exhibit a lower *retention time*, t_r, than analyte B. An analyte that had no affinity for the stationary phase and therefore moved through the column at the same velocity as the mobile phase travels through the column is said to be unretained. The time taken for this to occur is defined as t_0 and is equal to the column length divided by the velocity of the mobile phase flow. The properties

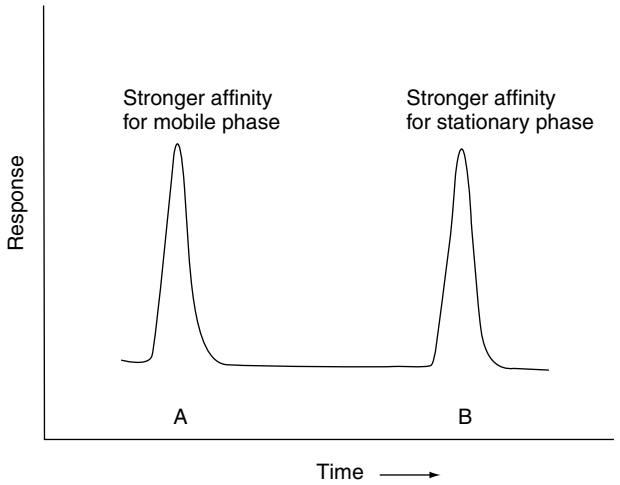

FIGURE 4.1 *Relative affinities of compounds A and B.*

t_r and t_0 can be related to each other to define one of the fundamental liquid chromatography parameters, *the capacity factor*, k', as follows:

$$k' = \frac{t_r - t_0}{t_0}$$

k' can be thought of as expressing the relative migration rate of an analyte.

One characteristic of any chromatographic separation is that each band is to a greater or lesser degree dispersed as it travels along the column. Therefore, we can consider each chromatographic peak as a band with a certain width. Each band is composed of a distribution of concentrations of the analyte around the retention time of that analyte. In an idealised situation this distribution takes the form of a symmetrical Gaussian, or standard error, curve. The width of this band is commonly expressed in HPLC in terms of the *theoretical plate number, N*, of the column. This parameter is usually calculated as

$$N = 5.54 \times \left(\frac{t_r}{w_{0.5}}\right)^2$$

where, $w_{0.5}$ is the width of the peak or band at half height. N can be viewed as a measure of column efficiency, that is, the ability of a column to produce narrow peaks. The higher the value of N, the more efficient the column is and the greater its ability to perform separations.

As has been said, the truly 'Gaussian distribution' represents an idealised case. More commonly this is not actually the case, and in fact it is usual that the decline of analyte concentration from the peak apex back to the baseline occurs over a greater period of time than does the rise in concentration from the peak beginning

to the apex. This gives rise to another important parameter in considering the quality of the chromatographic process, *the peak asymmetry factor*, *As*. This is defined in Figure 4.2.

The object of the chromatographic procedure is to perform a separation of a mixture of analytes, or frequently in the case of pharmaceutical bioanalysis, a separation between a compound which we wish to quantify and interferences which would compromise the quantification. In either case, it is necessary to have adequate separation between components. This can be measured quantitatively in terms of the *resolution, Rs*. *Rs* is equal to the distance in time between the apexes of two adjacent bands, divided by their average bandwidth.

$$Rs = \frac{(t_2 - t_1)}{0.5(w_1 + w_2)}$$

Rs is a measure of separation, but to use this as a parameter we can adjust to achieve a required separation, it has to be related to experimental characteristics such as N and k'. How these parameters are related is described in the relationship

$$Rs = \frac{1}{4}(\alpha - 1)\sqrt{N}\left[\frac{k'}{1 + k'}\right]$$

where α is the *separation factor* and is equal to k'_2/k'_1 the capacity factors of the two components to be resolved.

The above equation can be considered as being comprised of three terms: $\alpha - 1$ is a selectivity term which can be varied by altering the composition of the mobile and stationary phases; $\sqrt{N}$ is an efficiency term which can be affected by changes in the column length, column packing or the mobile phase velocity; and the third term involving k' which can be altered by changing the solvent strength.

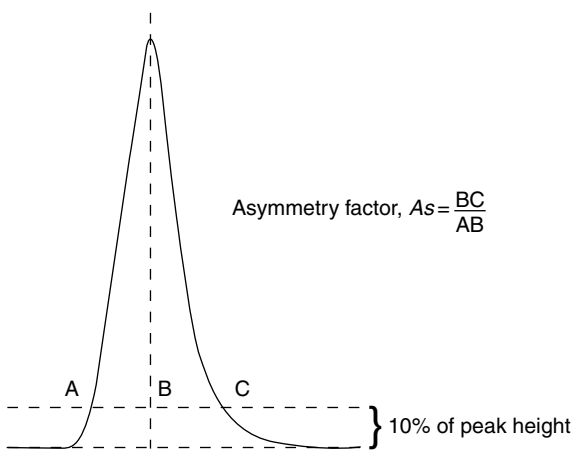

FIGURE 4.2 *Derivation of the peak asymmetry factor (As).*

These factors k', N, As, Rs and α may be considered the primary factors describing a chromatographic separation and it has been shown how they interact and need to be adjusted or controlled to affect the required separation. It is not the intention here to investigate these parameters in any greater depth, though more complex treatments have been undertaken.

4.3 *The basic equipment comprising a modern HPLC system*

Fundamentally, a liquid chromatograph consists of a high pressure pump to deliver mobile phase, an injector for the introduction of samples into the system, a column on which the analytes are resolved, a detector for measuring the response for each analyte and a means of displaying and handling the detector signal (Figure 4.3). In the modern case, frequently the pumps allow the mixing of different solvents at varying ratios over the time course of the separation to generate what is known as gradient chromatography; the injection systems are normally automated to allow the unattended injection of large numbers of samples e.g. overnight; and the resultant signals are handled by computerised peak integration systems which allow rapid data interpretation. Further, the column may be packed with one of many hundreds of different commercially available stationary phases. The detectors have become ever more powerful and sensitive and the most commonly used in pharmaceutical bioanalysis are UV absorption, fluorescence, electrochemical, radioactivity, mass spectrometric and NMR detectors.

In considering each of the parts of the chromatographic system it should be borne in mind that all parts of the system which come into contact with the mobile phase must be made of materials which are inert to the chemicals which make up the mobile phases. Also that all parts of the system from the pump outlet to the column end are under high pressure and must be made of appropriate materials and

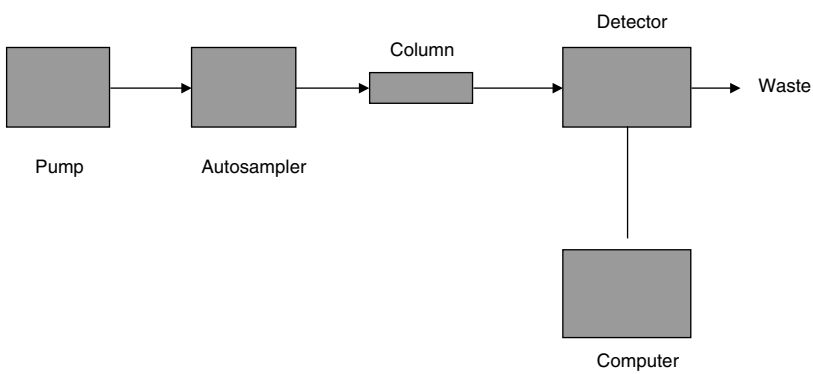

FIGURE 4.3 *Schematic representation of a modern HPLC system.*

designed in such a way to withstand this pressure, commonly up to 250–300 bar. A further fundamental design consideration is that all tubing from the point of sample introduction to the point of detection must be kept to a minimum to minimise 'dead volume'. Excess dead volume causes excessive extra-column band broadening and leads to drastic reductions in efficiency. Therefore, narrow bore tubing in short lengths should be used for all connections. It is worth noting here that in the case of gradient pumps the internal volume should also be as low as possible so that defined changes in mobile phase occur with the minimum of delay. Gradient HPLC will be discussed more fully later.

4.3.1 Pumps

In almost all modern HPLC systems the requirement of the pump is that it delivers mobile phase through the column at a constant flow rate. The pressure generated by this flow may vary with time, as the condition of the column changes with age, for example, but within limits this is allowed so long as the flow remains constant, as it is the flow rate rather than the pressure which is related to the velocity of the mobile phase and hence the retention time of the analytes of interest. The most commonly used type of pump is the reciprocating pump. These pumps use relatively low volume solvent chambers with reciprocating pistons to drive the flow through the pressurised system. Probably the most common type of these pumps is the dual reciprocating pump, wherein two identical piston pumping heads operate at 180° out of phase. This leads to a much smoother flow profile against time than is the case for a single-head pump. This is due to alternate filling of one head whilst the other is delivering solvent to the column and the rest of the chromatographic system. It is possible with such systems to largely remove pulsing from the flow profile. These pumping systems generally deliver reproducible flow, are fairly durable and are of moderate cost.

As previously stated, the parts of the pump which come into contact with the mobile phase need to be resilient to the chemicals typically used. However, even if this is adhered to, there are still conditions which should be avoided to maximise the performance of the pump. For example, mobile phases containing salts as buffers should not be allowed to stand in the pump for a long period as evaporation can lead to the formation of salt crystals which will scratch the piston and damage the high-pressure seal.

In the specific case of gradient HPLC pumps (pumps capable of delivering mixtures whose composition varies with time and discussed later), it is necessary that a pump be able to deliver a mixture of solvents, the composition of which changes with time. There are two approaches to this problem. First, the solvents may be mixed and then the required mixture is pumped through the system; this is called low-pressure mixing. The second option is to pump each solvent and then mix to the required composition under high pressure. There has been

much debate over the relative merits of these two types of pumping system and each has its proponents. Irrespective of this, the key requirement is that the system is able to deliver accurately and reproducibly mixtures of two or more solvents at different compositions with time. Most frequently the mixtures used as mobile phases comprise two components, binary systems, but on occasion three or even four components are mixed; these systems are called ternary or quaternary gradients.

4.3.2 Autosamplers

In order to obtain good column performance it is essential that the sample be introduced onto the column in an appropriate manner. Ideally, the sample should be injected in a small volume of solvent so that the analytes are contained in a narrow plug as they reach the top of the chromatographic column, i.e. they are not overly dispersed. Any system should be capable of reproducibly introducing the sample in a narrow band, be capable of operating at high pressure and should be easy to use. Modern automated sample injectors, autosamplers, incorporate valves for injection. In one position, the valve directs the flow of mobile phase through the column whilst the sample is introduced into a loop which is held out of the line of flow. The valve is then switched and the flow passes through the loop en route to the column thereby sweeping the sample into the mobile phase and onto the column. This is shown in Figure 4.4. It is now the norm that sample injectors are automated which allows the injection of large numbers of samples without operator intervention. Typically today, samples are prepared during the day, injected unattended overnight and the data processed the following morning.

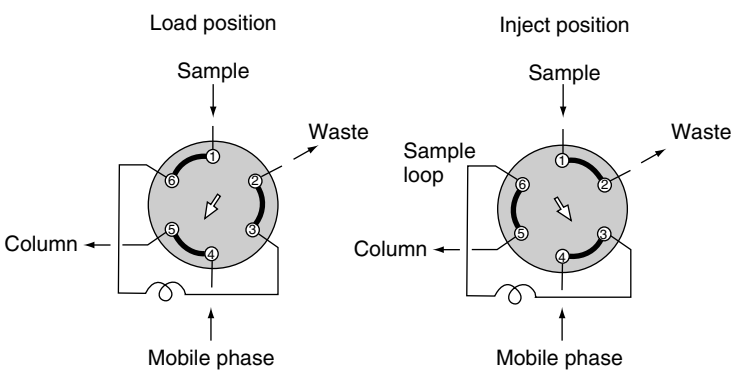

Figure 4.4 *Schematic representation of the two stages of the injection process.*

4.3.3 Columns

There are a myriad of different stationary phases which are today commercially available as HPLC columns. The most commonly used, however, are microparticulate, porous silica-based materials. Typically these are 3–5 μm spherical silica particles with internal pores of approximately 80–100 Å and offer efficiencies, N, in excess of 50,000 plates per metre. Frequently, the silica base has a surface-modifying chemical covalently bonded to it to alter the polarity and nature of the affinity by which the stationary phase binds analytes. The most common of these is an octadecyl (C18) alkyl chain in which case the surface is made very non-polar and separation occurs between this and a more polar mobile phase. This is known as reversed phase chromatography and will be discussed more fully later. To put in context the variety facing the chromatographer today, there are over 600 different reversed phase HPLC columns alone commercially available.

The most common format of the column is a stainless steel tube of 5–25 cm length with an internal diameter of 1–4.6 mm. The ends of the column contain stainless steel frits or meshes to retain the packing material. Low dead volume end fittings and short lengths of microbore tubing (i.d. 5,000–10,000 of an inch) are used to connect the column to the injector and the detector. It is worth noting that for spherical particles the stationary phase occupies only approximately 40 per cent of the volume of the column, so there is a degree of dead volume contributing to peak dispersion inherent in any column.

4.3.4 Detectors

There are a number of detectors which have found wide usage in HPLC, but the most commonly used in the pharmaceutical industry are UV absorbance, fluorescence, electrochemical, radiochemical and mass spectrometric detectors.

The most widely used detector of all is the UV absorbance detector. These detectors have high sensitivity for many drugs and metabolites but the analytes must absorb light in the UV/visible spectrum e.g. 190–600 nm. In modern UV absorbance detectors, the incident light is generated from a high intensity source such as a deuterium lamp and the wavelength of light required is then directed through the sample by the use of diffraction gratings, which deflect the light of the chosen wavelength according to the angle of the grating to the incident light. Light passes through the sample and a reference cell and is detected by a photomultiplier. The energy passing through each cell is compared electronically and the output is proportional to the sample concentration. The detector is, therefore, said to be concentration sensitive, that is, the detector response will be greater for a higher concentration of analyte in the flow cell and is governed by the Beer–Lambert law:

$$A = \epsilon c L$$

where, A is the absorbance, ϵ the molar absorptivity, c the concentration of the analyte in moles/L and L the cell pathlength. As can be seen, the longer the flow cell pathlength, the greater will be the detector sensitivity for any given concentration of analyte. Many cells have dimensions in the order of 1 mm i.d., 10 mm length with internal volumes of about 8 μL. Increasing the pathlength to further increase the response has an optimal point beyond which the increased volume of the cell becomes a factor adding to peak dispersion and thus lowering the sample concentration which has a detrimental effect on sensitivity.

A versatile extension of the applicability of UV absorbance detectors is the diode-array or rapid scanning detector. These detectors allow the collection of absorbance measurements at a range of wavelengths during the analysis of a sample. This offers the benefit of obtaining full spectral information for each analyte which can be of use qualitatively to determine whether a chromatographic peak is derived from the analyte or not. In the context of drug metabolism, very often the metabolism of a compound affects the functional groups which are part of the extremities of the molecule rather than the 'core' of the molecule which often includes the chromophore. Therefore, collection of UV spectra during the analytical run provides a means of determining whether a chromatographic peak results from a component which is drug related or not.

If the analyte of interest fluoresces there is the opportunity to use this characteristic as a means of detection. A particular molecule may absorb light at one wavelength and be energised to an excited state. The excited molecule may then release this energy as light at a different, longer wavelength in relaxing back to its ground state. This process is fluorescence. By exciting at one wavelength and monitoring the light emitted at the longer emission wavelength we have a very selective way of detecting analytes. The primary advantage of this technique is that it is not only very sensitive but also very selective, the compound has to not only excite at a specific wavelength but also emit at another specific wavelength. This is not only a key benefit but also its major drawback, as many analytes do not possess the ability to fluoresce. For fluorescence to be exhibited in a drug it is necessary that the drug molecule contains a fluorophore; this is usually a highly aromatic 'core' structure. As with the UV absorbance detector this detector is concentration sensitive. The fluorescence process is illustrated in Figure 4.5.

Another detection process which absolutely allows the determination of whether a peak is related to the drug of interest is radioactivity detection. Frequently, the study of a drug's metabolism involves the analysis of complex matrices such as plasma, urine, bile or faeces. These matrices contain many components, many of which exhibit substantial UV absorbance spectra of their own, making detection of one component alone, even after chromatographic separation, extremely difficult. By administering a radiolabelled version of the drug to the test species, a very easy way of deciding which chromatographic peaks relate to and/or are derived from the drug is available. Provided the radio-isotope is attached to the molecule in a metabolically stable position, one can reasonably assume that any peaks that are

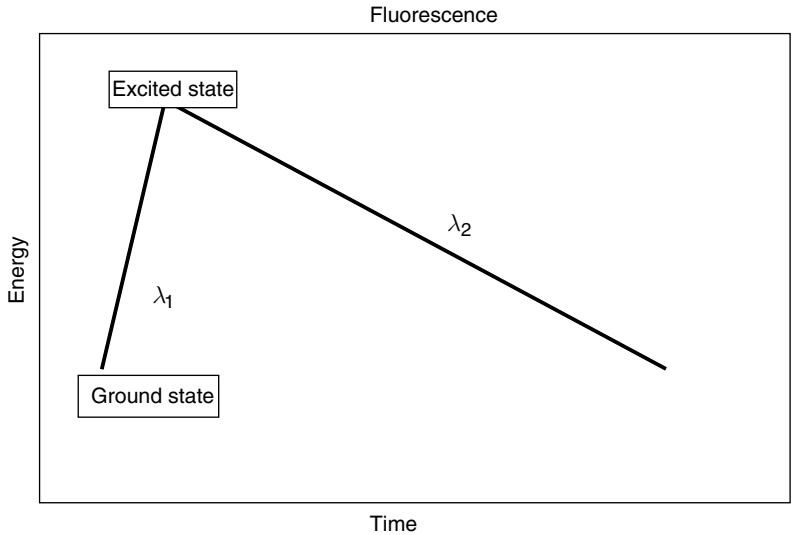

FIGURE 4.5 *Schematic representation of the fluorescence process, where λ_1 is the excitation wavelength and λ_2 the emission wavelength.*

detected by the radioactivity they exhibit are drug-related. Typically such detectors require a second pump which delivers scintillation fluids for mixing with the sample after the chromatographic separation has been performed on column.

Some drug structures contain moieties which are susceptible to oxidation or reduction. That is, the application of a voltage to the analyte as it passes through an electrochemical cell triggers a reaction to an oxidised or reduced species with the generation of an electrical current. This is the basis of electrochemical detection. Usually, either the current (amperometric detection) or the charge (coulometric detection) is detected. Not all molecules are susceptible to electrochemically induced reactions but some that are susceptible are listed in Table 4.1.

Often electrochemical detectors involve a graphitic carbon working electrode with a platinum auxiliary electrode and a reference electrode. Classes of drugs detected this way include the tricyclic antidepressants and the phenothiazines, many of which incidentally are also fluorescent.

The most widely applied use of electrochemical detection is in the oxidative rather than reductive mode as most modern HPLC is conducted in the reversed phase mode with largely aqueous mobile phases, which means that at substantial negative voltages a major problem is the reduction of oxygen in the mobile phase resulting in high background responses.

Recent years have seen the development of highly effective interfaces for coupling liquid chromatography to mass spectrometers. This has led to an explosion in the use of mass spectrometers as detectors for quantitative HPLC analyses as a result of the excellent sensitivity and selectivity of these instruments. A mass spectrometer can be

TABLE 4.1 *Molecules which are susceptible to electrochemically induced reactions*

Oxidation	Reduction
Phenolics	Ketones
Aromatic amines	Aldehydes
Dihydroxy (e.g. catechols)	Nitro compounds
Purines	Conjugated unsaturated systems
Heterocyclic rings	
Peroxides and hydroperoxides	

tuned to collect data from molecules or analytes of a specified molecular weight and furthermore, triple quadrupole mass spectrometers can be set up to fragment the analyte molecules and then detect a specific 'product' ion of a specified 'precursor' molecular mass. In practice, this means that the first quadrupole is used to select ions of the desired mass into the second quadrupole where the molecule is fragmented with a collision gas, then the third quadrupole selects only ions of a specified reaction product onto the detector. This is called selected reaction monitoring, SRM (or multiple reaction monitoring, MRM, if more than one analyte is involved such as a drug and an internal standard, a structurally similar compound added to samples at a constant amount to provide a reference for quantification). Although mass spectrometers are expensive, their extreme sensitivity and selectivity have seen them become often the detector of choice for quantitative bioanalysis (Figure 4.6). This topic

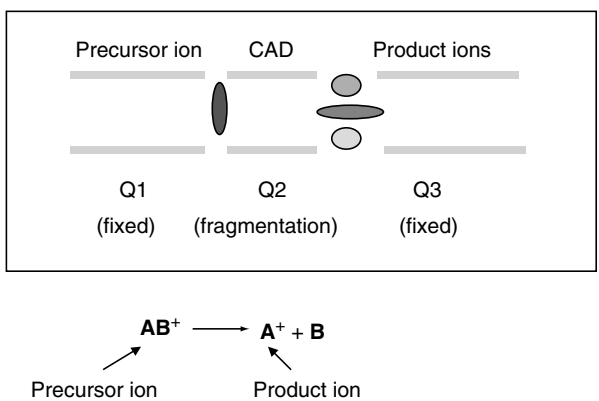

FIGURE 4.6 *Representation of the single reaction monitoring process in a triple quadrupole mass spectrometer. Q1 focuses only ions of a selected mass into Q2, the collision cell, where fragmentation is induced by collision with a gas, Q3 focuses only a specified fragment (product ion) through to the detector.*

is discussed in more detail in Chapter 5. The most widely used method of coupling HPLC to mass spectrometry for a wide range of typical drug analytes is atmospheric pressure ionisation using an electrospray mass spectrometer source. These systems can successfully cope with a limited volume of solvent; therefore, the eluent from the HPLC column may need to be split so that a flow rate of ~0.5 mL/min or less enters the mass spectrometer. As the solvent and the contained analytes enter the source, gases are mixed with the flow causing the formation of an aerosol. Voltage is applied to the stainless steel capillary through which the flow enters the source so that the droplets leaving the capillary are charged species. The addition of a nebulising gas encourages solvent evaporation and a decrease in droplet size – leading to a 'coulometric explosion' producing small ion droplets which enter the mass spectrometer. Obviously for this process, the key requirement of the analytes is that they are ionisable.

Another detection method which is increasingly being applied to liquid chromatography is nuclear magnetic resonance (NMR) spectroscopy. This technique is extremely information rich and is generally used for the determination of molecular structures rather than quantification. Although NMR can be directly coupled to the chromatograph the technique is insensitive compared to the other detectors discussed and is more often used off-line after collection of fractions from the column eluent. A further consideration is that NMR spectrometers, particularly the high resolution instruments contain very high field strength cryo-magnets and are not only very expensive but often need a specialised facility in which to house them. See Chapter 17 for a discussion of this technique.

4.3.5 Data handling systems

Historically, peaks arising from liquid chromatography detectors were displayed on pen recorders. The detector response was converted to a voltage output which moved the pens across a moving bed of paper in accordance to the size of the detector signal. However, in the modern pharmaceutical bioanalysis laboratory, most HPLC detector responses are captured electronically by computer-based systems. Frequently these systems are networked throughout a facility so that analysts can view their data from desks, remote from the actual chromatography laboratory. The programs contain algorithms which perform accurate integration of peaks, can produce calibration lines from the derived peak areas or heights and subsequently generate concentration results for the samples being analysed. Many of these systems are also capable of automatically calculating parameters such as k', N, As and Rs, which are useful for monitoring the performance of an assay. It is also a requirement that these systems maintain an audit trail, that is, modifications to the manner of data collection, integration or the contents of the

calibration file and derived results are logged so that the integrity of the data is maintained.

4.4 *Modes of liquid chromatography*

4.4.1 Normal phase liquid chromatography

In normal phase chromatography the stationary phase is more polar than the mobile phase. Thus, polar analytes are more strongly attracted to the stationary phase and will elute from the column later than less polar analytes (e.g. metabolites) which will have a greater affinity for the less polar mobile phase. The types of attraction by which analytes bind to the stationary phases are dipole–dipole interactions and hydrogen-bonding. Figure 4.7 shows the interactions commonly involved in normal phase HPLC.

Once it has been decided to attempt a separation by normal phase liquid chromatography using, say, a silica column one can consider the stationary phase to be fixed. The most obvious way of affecting a polarity-based separation, therefore, becomes the relative polarity of the mobile phase. Table 4.2 shows the polarity of some commonly used solvents together with their relative polarity and eluting power. It should be remembered that in normal phase the more polar the mobile phase the greater the eluting power (known as the eluotropic strength) of the solvent or combination of solvents used, the faster analytes will be eluted from the column into the detector.

There are further factors pertaining to specific properties of the mobile phase solvents which may affect the relative affinities of the analyte for the stationary or mobile phase to a greater extent than a relatively small difference in polarity. For example, Dichloromethane is a good proton donor and will interact strongly with basic solutes such as amines. On the other hand, isopropyl ether is a good proton acceptor and will interact better with acidic functions.

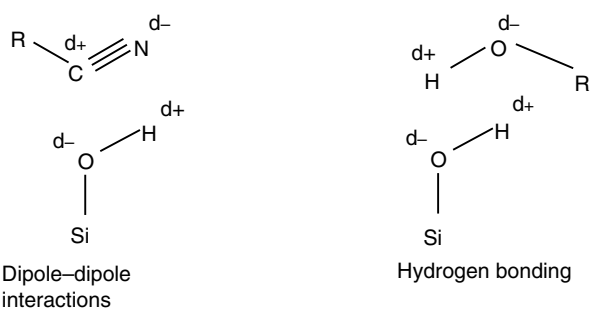

Figure 4.7 *Typical interactions in normal phase HPLC.*

TABLE 4.2 *The relative polarity and eluting power of polarity of some commonly used solvents*

Solvent	Increasing polarity	Increasing eluotropic strength
Hexane		
Toluene		
Iso-propyl ether		
Dichloromethane (DCM)		
Tetrahydrofuran (THF)		
Ethyl acetate		
Ethanol		
Acetone		
Acetonitrile		
Methanol		
Water		

4.4.2 Reversed phase liquid chromatography

Reversed phase chromatography is the term applied to the situation where the mobile phase is more polar than the stationary phase. This is the opposite of the case in normal phase HPLC, which was developed earlier, hence the terms normal and reversed phase chromatography. The use of reversed phase chromatography has a number of advantages:

1 Reversed phase HPLC allows the use of binding interactions ranging from hydrophobic bonding to ionic interactions, and a mixture of these and polar interactions in between, offering a large range of selectivities.
2 The use of a more polar mobile phase compared to the stationary phase often involves the use of primarily aqueous mobile phases which are safer to use and easier to dispose of than the primarily organic mobile phases of normal phase HPLC.
3 Frequently, reversed phase HPLC separations are performed on stationary phases which are modified silicas and as silica is fairly reactive this allows for many variations in the nature of the stationary phase and so a wide range of selectivities are available. A further consideration which is key to the value of reversed phase HPLC in the area of pharmaceutical bioanalysis is that the retention is reversed compared to that in normal phase HPLC (i.e. the more polar the analyte, the faster it is eluted). Most drug metabolites are more polar than the drugs they derive from, therefore, the metabolites elute first. This is beneficial as it allows, in many cases, the use of the retention time of the parent drug compound as a definer of the end of the chromatography of interest for any given analysis. This mode of liquid chromatography is by far the most commonly used today.

Typically, in reversed phase chromatography the silica particles that are the backbone of the stationary phase are chemically reacted with a hydrocarbon to produce a non-polar surface. The most common bonded structure is an octadecyl hydrocarbon (C18 surface modifier). To consider the retention mechanism in reversed phase HPLC let us consider the stationary phase. The surface of the silica is covalently bonded to a long chain hydrocarbon which provides a very non-polar surface characteristic. This attracts and binds non-polar molecules or sub-structures of molecules whilst more polar moieties are less well, or not all, bound. Typically mobile phases are primarily aqueous with a degree of lesser polarity introduced by an organic solvent such as methanol or acetonitrile. With regard to Table 4.2, the solvents may be viewed as having reversed eluotropic strength. Thus, more polar analytes have a greater affinity for the mobile phase than the stationary phase and are eluted earlier than non-polar analytes. However, no matter how good the bonding chemistry of the surface modifier, not every surface silanol (Si—OH) is attached to a non-polar group. Thus, there are always some silanol functions on the stationary phase surface through which polar or even ionic binding may occur. Indeed, the nature of the stationary phase may be altered depending on the composition of the mobile phase. If we consider the unmodified silanol groups on the surface, these exhibit a degree of acidity. If the mobile phase pH is high the silanols will be ionised ($Si—O^-$) and ionic interactions will play a large role in binding of analytes. If, on the other hand, the mobile phase is more acidic, the silanol groups will be un-ionised and hydrogen bonding or hydrophobic interactions will be more important. These polar/ionic interactions can occur at the same time, as the hydrophobic binding processes depending on the analyte's molecular structure and are called secondary interactions (Figure 4.8). Such interactions can lead to a mixed mode of retention which can be very selective and, thus, useful in terms of selectivity or can be detrimental to the chromatographic

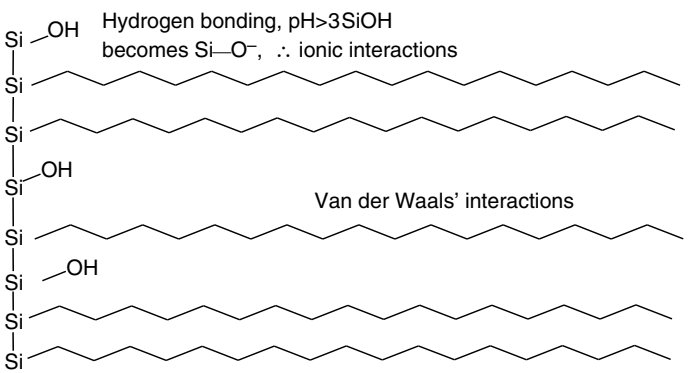

FIGURE 4.8 *Types of binding possible in reversed phase liquid chromatography on a bonded silica stationary phase.*

efficiency. For example, bases, as many drugs are, may be eluted from their hydrophobic binding sites only to experience a polar interaction with the silanol sites leading to additional binding and an appearance of *peak-tailing*, i.e. poor peak asymmetry.

As has been said, in reversed phase liquid chromatography, the more polar an analyte the earlier it will be eluted. However, very polar ionic molecules can be retained by use of an *ion-pair* reagent. For example, a basic drug structure that contains an amine may at the appropriate pH be positively charged. At the same pH an acidic reagent may be negatively charged. The two molecules will attract each other forming what is called an ion-pair. If the acidic moiety is attached to a hydrophobic structure (Figure 4.9) this will be attracted to the stationary phase. By this means very polar structures can be made to retain sufficiently in reversed phase HPLC to effect useful chromatography. Equally, an acidic drug can be ion-paired with a basic reagent in the mobile phase, such as tetrabutylammonium bromide.

4.4.3 Ion exchange chromatography

An alternative approach is to use ionic binding as the principal mode of retention by having an ionic stationary phase and ionic analytes and effecting elution by including a competitor ion to the analyte in the mobile phase. This is *ion exchange chromatography*. The most common retention mechanism involves a simple exchange of analyte ions and mobile phase ions with the charged groups on the stationary phase. Obviously, there are two modes, anion exchange and cation exchange (Figure 4.10). Analytes that interact weakly with the ion exchanger, the stationary phase, will be eluted more rapidly than those that interact strongly.

This type of chromatography is often used for the separation of organic acids and bases whose degree of dissociation into ionic forms is dependent on the pH. Higher pH will lead to greater ionisation of acids and less ionisation of bases which will lead to greater retention of the most acidic species.

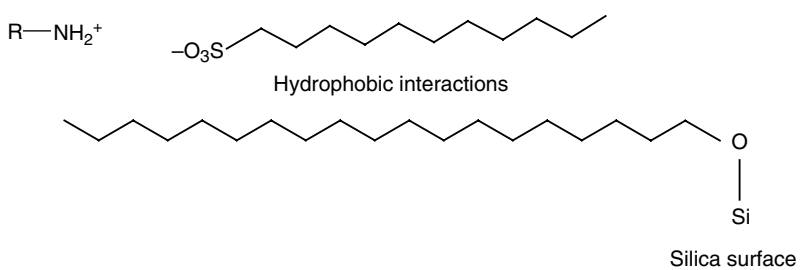

Figure 4.9 *Representation of the binding mechanism in ion-pair chromatography.*

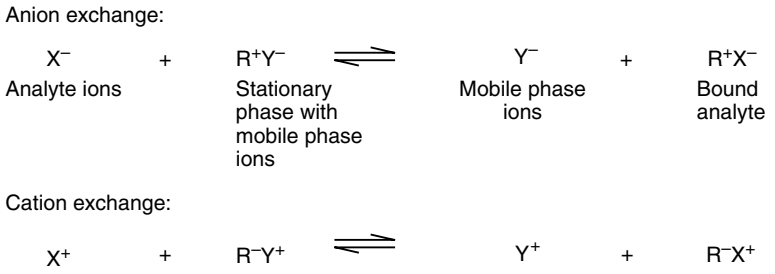

FIGURE 4.10 *Ion exchange.*

There are many other variations of HPLC, for example, using stationary phases with polarities intermediate between the very polar ionic or silica phases and the hydrophobic octadecyl phases; or size exclusion chromatography wherein only molecules below a certain size are able to penetrate the pores of the stationary phase and are retained. These all have their uses and full discussions on them are available elsewhere, but it should be remembered that the vast majority of pharmaceutical bioanalysis is carried out in the reversed phase mode on octadecyl bonded silicas.

4.4.4 GRADIENT HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Thus far the chromatography discussed has involved a mobile phase with a constant composition throughout the separation process. The separation is said to be performed under *isocratic* conditions. For some cases, where a complex mixture requires separation or where a system is required that will not need altering to successfully chromatograph a wide range of compounds (a *generic* system), it is advantageous to be able to change the composition of the mobile phase during the course of a separation. This is called *gradient HPLC*. In this discussion only gradient HPLC in the context of reversed phase HPLC, which is its most usually applied mode, will be considered. Furthermore, this discussion will concentrate on mixtures of two solvents (*binary gradients*) which are most common although ternary or even quaternary gradients have found uses. A weak solvent, typically water or an aqueous buffer, is mixed with an organic solvent, such as acetonitrile, so that the percentage of the organic solvent in the mobile phase increases with time. That is, the solvent is a weak eluent at the start of the separation, but a strong one at the end. This allows the retention of polar analytes under the initial conditions and the elution at the final conditions of even quite highly bound hydrophobic analytes. Under the initial conditions, a given analyte, X, will exhibit a high k' value, whereas under the final conditions

X will have a low k' value i.e. its k' value will decrease as the gradient progresses. At some point the k' of X will decrease to the point where it begins to migrate along the column. For a more highly retained analyte, Y, this point will not be reached until later in the gradient and hence the analytes are separated. For each analyte eluting from the column, the end of the peak will be eluting under conditions whereby it is travelling faster than the front of the peak. This leads to reduced tailing and narrow peaks, which aids both resolution and sensitivity. The overall result is an extremely powerful system for analysing compounds of considerably differing polarities in a single separation. Figure 4.11 shows a typical gradient separation of a test mix of 13 compounds chosen to cover a wide range of polarities. This approach can provide highly efficient separations of many compounds in a single chromatographic run.

A further significant development which has recently led to even greater usage of gradient chromatography is *fast gradient* HPLC. As can be seen from the chromatogram in Figure 4.11, gradient chromatography has traditionally involved the development of a gradient over a period of 30–40 minutes which has resulted in some very fine separations. However, in the modern pharmaceutical bioanalytical facility, more and more samples from studies investigating increasing numbers of compounds need to be analysed rapidly, and this timescale

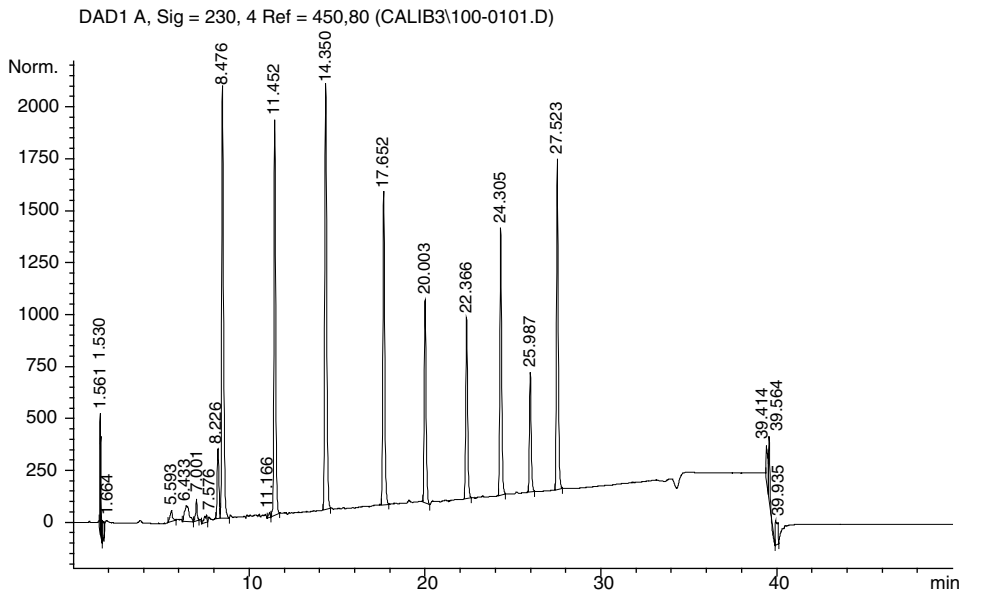

FIGURE 4.11 *25 μL injection of a test mix containing uracil, theophylline, acetofuran, acetanilide,* m-*cresol, acetopheneone, propiophenone, benzofuran, butyrophenone, valerophenone, hexanophenone, heptaphenone and octaphenone on a Supelco ABZ + 5 μm, 10 cm × 4.6 mm column at a flow rate of 1 mL/min. Gradient 0–95 per cent MeCN in 0.1 per cent aqueous formic acid over 35 min.*

is no longer acceptable for routine bioanalysis. Fortunately in gradient chromatography:

$$\text{Resolution} \propto \text{gradient time (i.e. the time to run the entire gradient)} \times \frac{\text{flow rate}}{\text{column length}}$$

This means that the column can be shortened and the flow rate increased (within pressure constraints) and the gradient time can then be reduced without loss of separating power. If we assume we have acceptable resolution and wish to maintain it, then the gradient time can be reduced 10-fold if the column length is reduced by a factor of 2.5 and the flow rate increased four-fold. These conditions do lead to higher operating system pressures but not higher than can be easily maintained by modern HPLC equipment. This approach has created the opportunity to do some very rapid separations of complex mixtures. The same mixture of analytes shown in Figure 4.11 is shown in Figure 4.12 separated under fast gradient conditions.

Obviously, this offers a lot of potential for higher throughput in modern pharmaceutical bioanalysis, whilst still offering the benefits of a method which is capable of application to a wide range of pharmaceuticals.

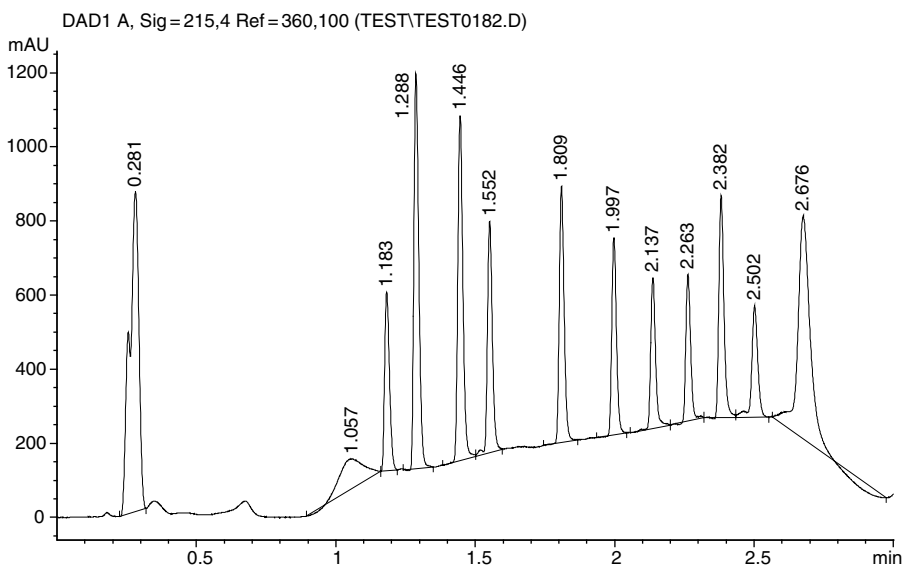

FIGURE 4.12 *An injection (1 μL) of an 11-component test mix onto a 50 × 2 mm Phenomenex Magellan C18. The column was maintained at 40 °C and eluted with a 0–95 per cent aqueous formic acid (0.1 per cent) and acetonitrile gradient over 2 min followed by a 1 min isocratic period of aqueous formic acid (0.1 per cent) at a flow rate of 800 μl/min. The column eluent was monitored by ultraviolet radiation at 215 nm.*

4.5 *High-throughput bioanalysis*

The advent of combinatorial chemistry approaches to drug synthesis has led to ever increasing numbers of candidate new pharmaceuticals entering the early stages of research and development. In consequence, there are increasing numbers of samples requiring bioanalysis per unit time. Not only is the number of samples increasing, but also the time available for method development is decreasing. Therefore, high throughput, generic strategies for bioanalysis are required to meet the demands of modern drug development.

In the Bioanalysis and Drug Metabolism Division of Glaxo Wellcome, we developed two key approaches to this problem. The first is the use of fast gradient chromatography as described above, following sample preparation by either solid phase extraction or protein precipitation. This is proving very successful for a large number of compounds of widely differing chemical structures.

The second approach we have developed is termed as ultra-high flow rate liquid chromatography and involves direct analysis of plasma samples without sample preparation. This is achieved by using large particle size (30–50 μm) stationary phases in contrast to conventional analytical columns, which are typically packed with 5 μm particles held in the column by 2 μm frits. Direct injection of plasma samples onto these columns quickly causes blocking. With the large particle sizes and the consequently larger (10–20 μm) column end frits this problem is removed. Also, with the large particle size stationary phases the back pressure generated is greatly reduced, so extremely high flow rates can be used. Typically, a standard system with a 1 mm i.d. column would be operated at 4 mL/min (for a conventional 5 μm stationary phase in a column of these dimensions, the normal flow rate would be 40–50 μL/min). This allows some very fast chromatography and using this approach a throughput of one sample every 1.2 minutes, equivalent to 50 samples per hour, can be achieved, with the only sample preparation being addition of internal standard solution. Indeed we have operated a system in which four such columns were operated in parallel, allowing a throughput approaching 200 samples per hour.

It should be noted that although the technique has some resolving power, it is not a high efficiency separation and plate counts, N, are low. As a result, a highly selective detector is required, normally a mass spectrometer operated in the MRM mode. Also, where there are metabolites which may breakdown back to the parent structure in the source of the mass spectrometer, such as *N*-oxides or glucuronides, it is necessary to check that these are chromatographically resolved from the parent under the conditions used before using this technique. However, within these limitations this technique offers the ability to analyse plasma samples for drug concentrations extremely rapidly and has found wide usage in our laboratories. Furthermore, a recent innovation has been the development of monolithic silica stationary phases, which allow high flow rates

combined with high chromatographic efficiencies for the direct analysis of drugs in plasma samples. These columns comprise a single piece of silica with a defined bi-modal pore structure. Large macropores provide low flow resistance and permit the direct injection of plasma samples coupled with high flow rates and low back pressures, and small mesopores provide excellent diffusion characteristics leading to high efficiencies. Standard conditions for ultra-high flow rate liquid chromatography for direct pharmaceutical bioanalysis:

Column: 50×1 mm, 50 µm Octadecylsilane (ODS) or 50×4.6 mm Chromolith^{TM} silica rod

Flow rate: 4 mL/min

Injection volume: 25–50 µL

Mobile phase: Solvent A: 0.1% aqueous formic acid
Solvent B: 5% 0.1% aqueous formic acid
95% acetonitrile

Gradient profile:

Time (mins)	%A	%B
0	100	0
0.2	100	0
0.8	0	100
0.9	0	100
1.0	100	0
1.2	100	0

Detector: Mass spectrometer in MRM mode with atmospheric pressure ionisation.

4.6 *Chiral HPLC*

Many pharmaceuticals are optically active. An optically active substance is one that rotates the plane of polarised light. Optically active drug molecules contain one or more *chiral* or *asymmetric* centres, carbon atoms to which are attached four different groups. For a structure containing a single chiral centre the molecule can exist as two non-superimposable forms which are mirror images of each other. These are called *enantiomers*. One enantiomer will rotate polarised light to the left, whereas the other will rotate it to the right. These are termed the *S*- and *R*-enantiomers, respectively. In many cases, only one of the enantiomers exhibits the desired pharmacological effect while the other may have no activity or worse, unwanted toxic activity. It is, therefore, necessary in drug development to be able to determine which stereochemical form the drug is in the body, how much of each form is present and whether one form inverts to the other. Analytical methods are, therefore, needed that are capable of resolving the enantiomers.

Enantiomers of the same molecule differ from each other only in the manner in which they rotate polarised light; all their other physicochemical properties are identical and conventional chromatography cannot separate them.

There have been two key approaches to this problem. The first has been to derivatise the drug, after extraction from the biological sample, with a stereochemically pure single enantiomer derivatising reagent. For example, amine-containing drugs can be derivatised with chiral acid reagents as shown below:

$$\text{S} - \text{reagent} + \text{S/R} - \text{analyte} \rightarrow \text{SS} - \text{product} + \text{SR} - \text{product (diastereoisomers)}$$

The products containing two chiral centres are called *diastereoisomers*. These diastereoisomers have differing physicochemical properties and can be resolved on conventional chromatographic columns. This approach has been very successful.

The second approach has been direct chromatographic resolution of the two enantiomers. Direct chromatographic resolution of the enantiomers can be achieved in two ways: either by the use of a chiral mobile phase or a chiral stationary phase. The former of these is not widely used, as separation factors achieved are rarely sufficiently high, but the latter is now a well-established and widespread means of performing chiral separations.

There are many different chiral stationary phases commercially available offering a number of different separation mechanisms. One of the most commonly used 'family' of chiral stationary phases is the cyclodextrins. Cyclodextrins are toroidal-shaped molecules formed of glucose units. The most common are the α-, β- and γ-cyclodextrins containing 6, 7 and 8 glucose units, respectively. The molecules are shaped like truncated cones with an inner cavity the size of which depends on the number of glucose units in the molecule. Due to the orientation of the glucose units the interior of the cavity is relatively hydrophobic whereas the surface is hydrophilic. For chiral resolution, the analyte must enter the cavity such that its asymmetric centre, or groups near to it, interact with the surface hydroxyl groups, forming an *inclusion complex*. The separation is then based on a difference in the stability of the inclusion complex formed by each enantiomer of the analyte (see Figure 4.13).

Other types of chiral stationary phases include immobilised proteins, celluloses, macrocyclic antibiotics and immobilised metal co-ordinating ligands. These have different mechanisms of separation and are well described elsewhere.

4.7 *Future trends in HPLC*

HPLC, like any vibrant technique, is still evolving. The last few years have seen many new variations explored. The drive in the late 80s and early 90s was towards

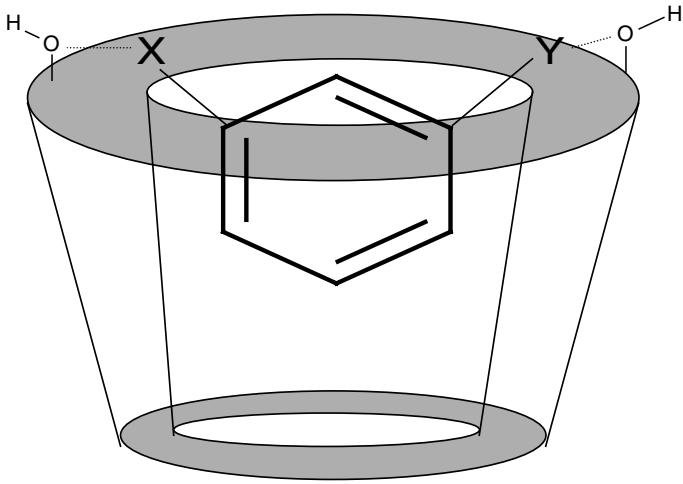

FIGURE 4.13 *Schematic representation of an inclusion complex in a toroidal cyclodextrin molecule. X and Y represent the groups at or near the chiral centre and interact with the surface hydroxyl groups of the cyclodextrin, whilst the hydrophobic part of the molecule sits within the cavity.*

ever more automation of the entire sample analysis process, including the introduction of robotic systems to automate all parts of the analytical procedure. Included in this has been a trend towards equipment which is compatible with the now widely used 96-well plate format for handling samples. Increased use of mass spectrometers has to some extent removed the need for extensive sample preparation and is also replacing many other detectors. However, the biggest trend which will affect HPLC in the pharmaceutical industry in the coming years is almost definitely, miniaturisation. The use of smaller scale HPLC has several distinct advantages.

First a typical, conventional HPLC column has an internal diameter of 4.6 mm and is operated at a mobile phase flow rate of approximately 1 mL/min. To maintain the same linear flow rate on a 1 mm i.d. column, the reduction in flow rate is proportional to the square of the radius of the column and the flow rate would, therefore, be reduced by a factor of approximately 21 to approximately 45–50 μL/min. Further reduction in the internal diameter of the column to the capillary scale (0.32 mm or less i.d. columns) results in flow rates of 5 μL/min. These days economic and environmental concerns regarding the disposal of waste solvents make such a significant reduction in the amount of HPLC waste mobile phase highly desirable.

Second, and more important, most detection systems are concentration-sensitive. That is, the size of response they exhibit is proportional to the concentration of analyte in solution reaching them. If a 0.32 mm column system is operated under conditions that show the same efficiency as a 4.6 mm column, then the peak width will be unchanged and the volume of mobile phase in which an eluting peak is contained will be also related to the square of the column radius. Changing from a 4.6 mm to a 0.32 mm i.d. column, therefore, offers a 207-fold increase ($4.6^2/0.32^2$)

in the concentration of an analyte in a peak as it reaches the detector. Even if the reduced column dimensions restrict the volume of sample that can be injected by 10–20-fold, there is obviously a significant gain in sensitivity that can be achieved this way. Alternatively, if the available sample volume is extremely small, then a miniaturised HPLC system offers the maximum sensitivity for chromatographic analysis of that sample.

A further benefit of miniaturised HPLC systems is that some mass spectrometer sources perform more efficiently at very low flow rates in terms of their ionisation rates, thus offering further gains in sensitivity when using these detectors. This includes techniques such as the rapidly emerging nano-spray LC–MS systems.

There are potential problems which need to be borne in mind when using micro-scale HPLC systems. The most important of these is that if the peak is eluting from the column in a volume of approximately 1 μL or less, it is vital that all extra-column volumes are minimised to avoid dispersion effects destroying the peak integrity and thus the sensitivity. This means that in the case of HPLC–MS, the column end needs to be as close to the source as possible and connected to it by no more than a short length of very narrow bore capillary tubing.

It should also be borne in mind that with UV absorbance detection systems the path length of the detector cell is proportional to the response and so minimising the volume puts constraints on the sensitivity of the detection system. The use of detector flow cells with narrow, long path length channels can help but is limited as too long a channel will lead to the danger of more than one peak entering the detector at any one time thereby destroying the resolution achieved by the chromatography.

Nevertheless, the potential gains in terms of environmental, economic and sensitivity factors, coupled with the wish for ever faster separations, are such that the future will see a swing away from columns of 1–5 mm i.d. and 10–25 cm length to columns of 50–320 μm diameter and 2–3 cm length and being operated with very fast micro-scale gradients. Indeed, many of the manufacturers of HPLC are already actively involved in the development of systems capable of meeting these requirements.

4.8 *Bibliography*

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