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Chapter 1 Introduction

High performance liquid chromatography (HPLC) is probably the single most used analytical technique today, surpassing even gas chromatography in use for the separation and analysis of mixtures. This is due mainly to the extensive versatility of the technique which results from the fact that both stationary phase and mobile phase interactions may be utilised to alter the selectivity of the system. The art of eluent additive chromatography, whereby a small amount of an ionic or complexing species may be added to the eluent to interact specifically with certain types of solute, has been developed to the extent that it is now routine.

It is less than 20 years since the initial steps were taken to enable the evolution of classical liquid chromatography into the highly efficient analytical procedure that we have today (Horvath et al., 1967; Huber and Hulsman, 1967; Kirkland, 1968, 1969). The development of modern HPLC is inextricably linked to advances in silica technology. The important practical considerations which were necessary for the development of truly high performance liquid chromatography were the use of high operating pressures and small, uniform packing particle sizes, enabling rapid diffusion rates and fast analysis times. Diffusion problems were initially overcome by the development of pellicular packing materials (Horvath et al., 1967; Kirkland, 1969) which were comprised of a thin porous layer of material coated onto an impervious glass bead. These materials were, however, quickly superseded by totally porous microparticulate silicas (20–30 μm size) with carefully controlled characteristics such as surface area and pore volume. Reduction of the particle size to 10 μm and more efficient

fractionation of the materials to give narrow particle size ranges made it possible to pack very efficient columns ($h \approx 2$). Gradually even smaller particle sizes (3–5 μm) became available and, with the improvements in column packing technology, these have become established as the most commonly used packing materials.

At the same time procedures were being developed to chemically bond a variety of ligands to the surface silanols on the silica packing. The formation of stable (within the pH limit 2–8) siloxane bonds was recognised as the best approach and the chemistry of the preparation of bonded phases is now well established. Standard procedures now enable a wide variety of bonded stationary phases to be prepared. This is not usually necessary, however, because of the variations of selectivity which can be achieved by appropriate selection of the mobile phase. In consequence, the use of a few bonded stationary phases which might include a hydrocarbon bonded phase (reversed phase), an ion exchange material and a polar bonded phase in conjunction with the original silica material might well be expected to cover the full range of chromatographic modes.

In certain specialised areas more specific bonded phase interactions are required to effect resolution. One area of particular interest is the resolution of optical enantiomers. Several chiral bonded phases have been synthesised and excellent results have been achieved. This field is still in the development stage but many common racemic compounds have already been directly resolved into their enantiomers on specially designed chiral bonded phases.

Another recent and important development is the preparation of wide pore silica gels for the separation of macromolecules. Such materials may also be bonded, usually with short hydrocarbon chains, for the analysis of proteins using hydrophobic interaction chromatography. A reversed phase chromatographic technique is employed, since the bonded phase prevents denaturation of the protein on the silica surface. Short hydrocarbon chains are preferred, thus avoiding the use of high concentrations of organic modifiers in the eluent which may cause denaturation. There is much activity in this area of HPLC and many outstanding separations have been obtained.

Concurrent with the development of packing materials the necessary advancement of the chromatographic equipment has taken place. The design of the liquid chromatograph is very important, since the efficiency produced in the column can too easily be lost by the presence of dead space in poorly designed injectors, connectors or detectors. The band broadening processes in ancillary equipment have been examined both in theory and practice with resultant improvements in equipment design. The necessity of high pressure operation also places restrictions on equipment design, with respect to the choice of the materials used in the construction of the components as well as the means of coupling them together.

The standard column used in present day analytical HPLC consists of a 100–200 mm length of stainless steel tubing of about 4.6 mm bore, packed with 5 μm particles. Shorter columns (30–100 mm length) packed with 3 μm particles are becoming popular for the fast analysis of simple mixtures, while narrower

columns of 1 or 2 mm bore are being introduced for the analysis of more complex mixtures. At the other end of the scale large bore columns packed with materials of larger particle size can be used for the efficient separation and isolation of components by preparative HPLC.

The various aspects of current practice in HPLC are discussed in detail throughout the book, along with many operating hints and a wide variety of applications. It is, however, worthwhile at this point re-emphasising the versatility of the technique by illustrating the separation of widely differing compounds on similar packing materials. *Fig. 1.1* shows the rapid, efficient separation of several small aromatic molecules on a narrow bore column packed with ODS-silica, while *Fig. 1.2* illustrates the reversed phase separation of various insulins on a column packed with LiChrosorb RP-18.

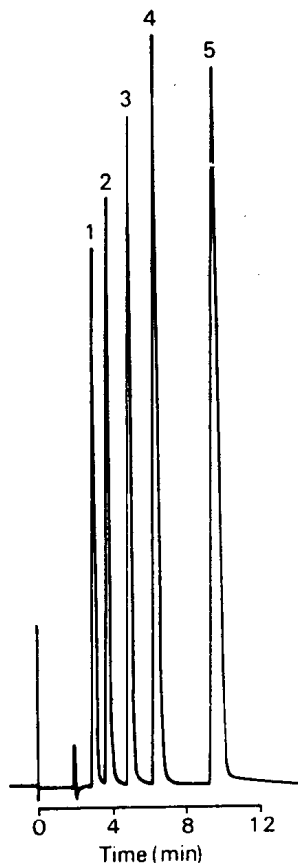


Fig. 1.1 Separation of components typically found in analgesic preparations by narrow bore HPLC. Column packing, Spherisorb ODS ($3\ \mu\text{m}$); column dimensions, $100 \times 1\ \text{mm}$ i.d.; eluent, methanol/water, 40:60, with H_3PO_4 added to pH 3.3; flow rate, $25\ \mu\text{l}\cdot\text{min}^{-1}$, detection, UV at 254 nm. Solutes, 1: theophylline; 2: caffeine; 3: 8-chlorotheophylline; 4: aspirin; 5: benzoic acid.

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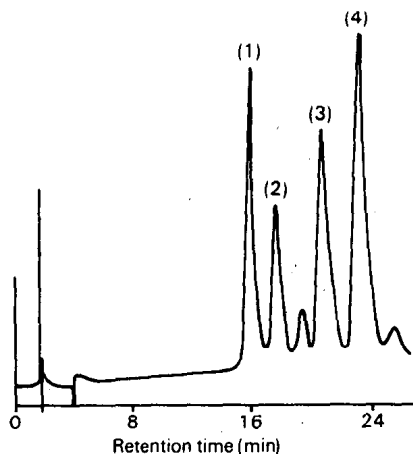


Fig. 1.2 Chromatogram of various insulins. Column packing, LiChrosorb RP-18 ($5\ \mu\text{m}$); column dimensions, $250 \times 4\ \text{mm}$ i.d.; eluent, acetonitrile/5 mM tartrate buffer, pH 3.0, 20:80 containing 0.57 M sodium sulphate; flow rate, $0.8\ \text{ml}\cdot\text{min}^{-1}$; column temperature, 40°C ; detection, UV at 280 nm. Solutes, 1: bovine insulin; 2: ovine insulin; 3: equine insulin; 4: porcine insulin.

Reproduced with permission from Ohta et al., 1983.

Because of the vast nature and diversity of modern HPLC there will undoubtedly be omissions in a monograph of this size. I have, however, tried to cover the basic topics which are needed to understand and practise the technique on a day-to-day basis. The emphasis is more towards practice than theory, although a certain amount of theory is necessary for complete understanding of the processes involved. The text is well illustrated with practical examples from the extensive, recent applications literature. Some particular areas of interest to myself, and hopefully the reader, have been discussed in more detail.

Chapter 2 Basic Chromatographic Theory

When a sample is injected onto a chromatographic column two basic processes are important, namely, the factors affecting retention (thermodynamic) and the factors which cause band dispersion within the column (kinetic). In combination these processes control the resolution of the components in the sample. The ideal approach maximises efficiency and resolution by minimising band dispersion within the column and with this end in view various parameters can be defined and their possible control evinced.

2.1 Column capacity ratio, k'

Solute retention is normally measured by the capacity ratio or k' of the solute. As indicated in Fig. 2.1, the time which elapses between the injection and the elution of a solute is called the *retention time* or *elution time*, t_R and similarly the volume of eluent which passes during this time is the *retention volume* or *elution volume*, V_R . These quantities are connected by the *volumetric flow rate*, f_v .

$$V_R = t_R f_v \quad (2.1)$$

By comparison the retention time and volume of an unretained (or solvent) peak are denoted t_o and V_m , respectively.

We can therefore define the column capacity ratio of a solute as

$$k' = \frac{t_R - t_o}{t_o} = \frac{V_R - V_m}{V_m} \quad (2.2)$$

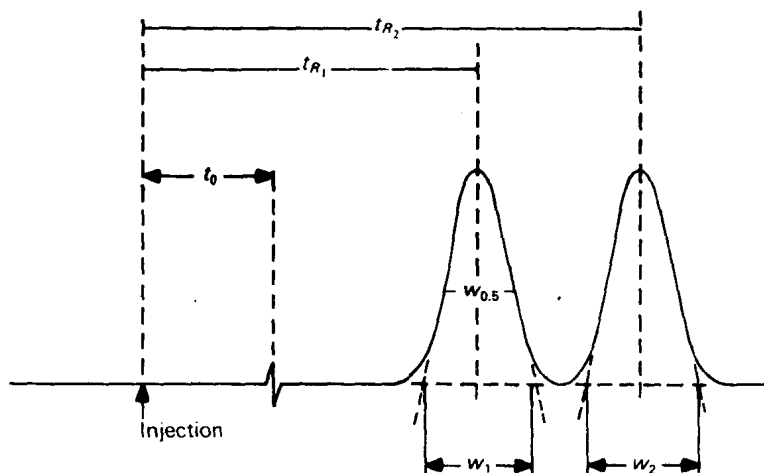


Fig. 2.1 Parameters for defining retention, peak width, column capacity ratio, column efficiency, and resolution.

Strictly speaking, retention volumes should be used rather than retention times, but in practice constant flow rates are normally assumed and it is more convenient to obtain the retention times directly from the chromatogram. It is, however, necessary to use k' values rather than simply retention times to ensure reproducibility from day to day, since chromatographic conditions such as flow rate or temperature can vary on successive days.

The essential feature of any chromatographic separation is the distribution of solutes between a mobile zone and a stationary zone. The mobile zone is the fluid outside the particles, but it is not necessarily identical to the eluent since part of the eluent will be held within the pores of the packing material. In most forms of chromatography, however, except exclusion chromatography, the rate of movement of a solute band is referred to the mean linear rate of movement of the eluent phase, u , where

$$u = L/t_0 \quad (2.3)$$

rather than to the mobile zone. Similarly, the stationary phase is normally used instead of the stationary zone which strictly should be the partitioning material inside the particles.

It is essential for good plate efficiency that molecules equilibrate rapidly between the two zones (thermodynamic effect). During chromatography the molecules will move at the mean linear eluent velocity, u , while in the mobile phase but not at all while in the stationary phase. It therefore follows that the speed of the band of solute molecules relative to the mobile phase is given by

$$\frac{u_{\text{band}}}{u} = \frac{q_m}{q_m + q_s} = \frac{1}{1 + (q_s/q_m)} = \frac{1}{1 + k'} \quad (2.4)$$

where q_m and q_s are the quantities of solute in the mobile and stationary phases, respectively and $k' = q_s/q_m$.

Because we assume equilibrium

$$q_s = C_s V_s, \quad q_m = C_m V_m \quad (2.5)$$

where C_s , C_m are the equilibrium concentrations of the solute in the stationary and mobile phases, while V_s , V_m are the volumes of the stationary and eluent phases. Thus

$$k' = \frac{C_s V_s}{C_m V_m} = D \frac{V_s}{V_m} = \phi D \quad (2.6)$$

where D is the equilibrium distribution coefficient for the solute between the two phases, and ϕ is the phase ratio. Since the retention volume of the solute is inversely proportional to the band velocity, it follows from Equation 2.4 that

$$\frac{u_{\text{band}}}{u} = \frac{1}{1 + k'} = \frac{V_m}{V_R}$$

$$\therefore V_R = V_m(1 + k') \quad (2.7)$$

Substitution for k' from Equation 2.6 then gives

$$V_R = V_m + D V_s \quad (2.8)$$

In adsorption chromatography it is difficult to envisage the 'volume of stationary phase' and it is appropriate to use the weight of adsorbent in the column, W , or the surface area of the adsorbent, A' . The appropriate equations for k' are then

$$k' = \frac{C_s' W}{C_m V_m} = K_{ads} \frac{W}{V_m} \quad (2.9)$$

and

$$k' = \frac{C_s'' A'}{C_m V_m} = K_a \frac{A'}{V_m} \quad (2.10)$$

where K_{ads} is the adsorption coefficient (ml.g^{-1}) and C_s' is the concentration of adsorbate in moles per unit weight; or K_a is the superficial adsorption coefficient (ml.m^{-2}) and C_s'' is the adsorbate concentration in moles per unit area of adsorbent surface.

The corresponding equations for retention volume are

$$V_R = V_m + K_{ads} W \quad (2.11)$$

and

$$V_R = V_m + K_a A' \quad (2.12)$$

2.2 Efficiency

The width of a band within a chromatographic column increases as the square root of the distance migrated. Martin and Synge in 1941 introduced the concept of plate height by considering the column to be divided into transverse slices of 'resolving power' (theoretical plates). It is possible to define the dispersion of a chromatographic band as the width of one of these slices or the height equivalent to a theoretical plate, H .

$$H = \frac{L}{16} \left(\frac{w}{t_R} \right)^2 \quad (2.13)$$

where L is the column length, and w the base width of the eluted peak (see Fig. 2.1). H has the dimensions of length.

The efficiency of a column is obtained from the number of theoretical plates, N

$$N = L/H = 16(t_R/w)^2 \quad (2.14)$$

N is dimensionless, since t_R and w are measured in the same units.

The higher N , the better the resolution that can be expected, and for a good column N will be between 2000 and 20 000.

It is also possible to calculate N from the width of the peak at half height, $w_{0.5}$, using

$$N = 5.54(t_R/w_{0.5})^2 \quad (2.15)$$

The plate height of a column varies with the linear velocity of the eluent, u . A plot of the dependence of H on u is known as a plate height curve, but since H depends on the particle size d_p of the packing material a series of columns packed with different sizes of material will give different plate height curves. As a result it will be difficult to directly compare the efficiencies of different columns. For this reason it is better to use the dimensionless parameters, reduced plate height, h and reduced velocity, γ conceived by Giddings (1963) and encouraged by Knox and co-workers (Knox and Saleem, 1969; Kennedy and Knox, 1972; Done and Knox, 1972). The reduced plate height is independent of particle diameter and measures the number of particles corresponding to H

$$h = H/d_p \quad (2.16)$$

The reduced velocity is a measure of the ratio of the rate of diffusion of a solute within the particle to the rate of flow outside the particle

$$v = u d_p / D_m \quad (2.17)$$

D_m is the diffusion coefficient of the solute in the mobile phase and may be

estimated from the Wilke-Chang equation (Wilke and Chang, 1955)

$$D_m = \frac{7.4 \times 10^{-12} T (\psi M_{\text{eluent}})^{1/2}}{\eta V_{\text{solute}}^{0.6}} \quad (\text{m}^2 \cdot \text{s}) \quad (2.18)$$

where T is the temperature ($^{\circ}\text{K}$); ψ is an eluent association factor (1 for non-polar solvents, 1.5 for ethanol; 1.9 for methanol and 2.6 for water); M_{eluent} is the eluent molecular weight (g); η is the eluent viscosity (mPa.s; 1 mPa.s = 1 cP = 1 mN.s.m $^{-2}$); and V_{solute} is the solute molar volume (ml), i.e. the solute molecular weight/density. The equation is accurate to about ± 20 per cent.

It is now possible, using these parameters, to obtain plate height curves which are independent of the particle size with any variations being due to differences in packing. A representative curve is shown in Fig. 2.2. The minimum obtained in the h versus v plot gives the reduced velocity required for maximum chromatographic efficiency.

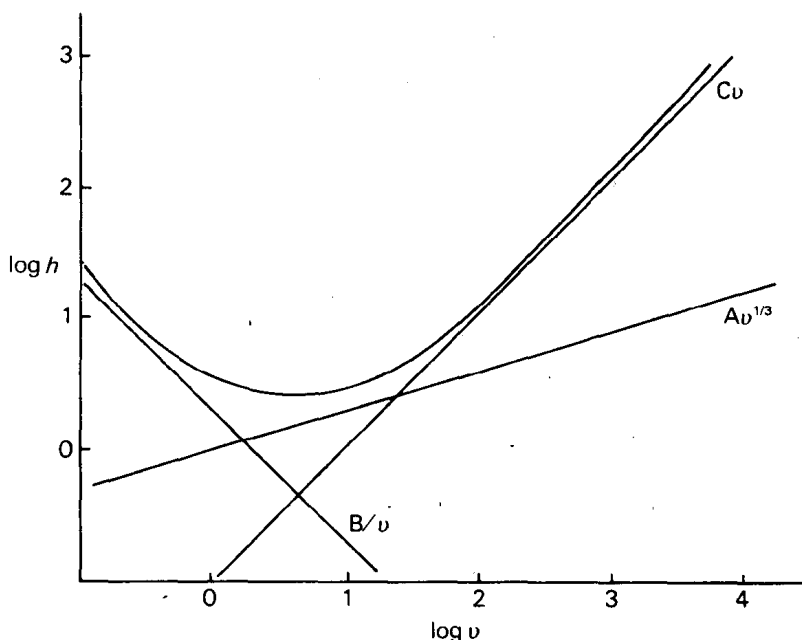


Fig. 2.2 Dependence of h upon v according to the equation $h = v^{0.33} + 2/v + 0.1v$.
Reproduced with permission from Knox, 1978.

The first mathematical expression for the relationship between plate height and fluid velocity was given by Van Deemter et al. (1956) but this has been superseded by the version using dimensionless terms produced by Knox and

co-workers (Kennedy and Knox, 1972; Done and Knox, 1972; Done et al., 1972; Grushka et al., 1975)

$$h = B/v + Av^{0.33} + Cv \quad (2.19)$$

The three terms arise from the three dispersive processes:

1. Dispersion due to axial molecular diffusion. This depends upon the residence time of the band in the column and hence is most important at low flow velocities. B has a value ≈ 2 .
2. Dispersion due to the tortuous nature of the flow through a packed bed. This is a measure of how well the column is packed and for good columns $A < 1$.
3. Dispersion due to slow mass transfer between the mobile and stationary zones. When equilibration between the two zones is slow molecules in the stationary zone tend to be left behind when the main band passes. This dispersion increases with flow velocity and C typically has a value < 0.1 .

This equation, although semi-empirical, will generally be found to give a good approximation to experimental data. More rigorous (and accurate) determinations of the band broadening processes, beyond the scope of this work, have been reported (Horvath and Lin, 1976, 1978; Chen and Weber, 1983). Examination of a plate height curve will give the best operating conditions to obtain maximum efficiency. Conditions can, however, only be varied within the limits set by the chromatographic equipment and an important equation in this respect connects the pressure drop across the column ΔP , with the column length and particle size of the packing material.

$$\Delta P = \frac{\phi' \eta}{t_o} \left(\frac{L}{d_p} \right)^2 \quad (2.20)$$

η is the eluent viscosity and ϕ' is the column resistance parameter. Values of ϕ' should be as low as possible and normally lie in the range 500–1000 for porous particles. Experimental determination of very large ϕ' values is an indication of column plugging and it is worthwhile calculating this parameter if high back-pressure is experienced.

2.3 Resolution

The ultimate purpose of chromatography is to effect resolution between solute bands. The resolution R_s of two bands is defined as the separation between the peaks divided by the mean peak width, i.e.

$$R_s = \frac{t_2 - t_1}{1/2(w_1 + w_2)} \quad (2.21)$$

For two gaussian peaks, effectively baseline resolution is obtained for $R_s = 1.5$.

By using the equations for N , t_o , w and k' it is possible to show that resolution can also be expressed by

$$R_s = 1/2 \frac{(\alpha - 1)}{(\alpha + 1)} \left(\frac{\bar{k}'}{1 + \bar{k}'} \right) N^{1/2} \quad (2.22)$$

where $\alpha = (k'_2/k'_1)$ and $\bar{k}' = 1/2(k'_1 + k'_2)$.

These three factors in the resolution equation indicate the obvious requirements for resolution that (a) the solutes must be retained to different extents, i.e. $\alpha \neq 1$; (b) the solutes must be retained, i.e. $\bar{k}' \neq 0$; and (c) the column must be equivalent to a minimum number of theoretical plates, N .

2.4 Asymmetry

Another important consideration is the symmetry of the peak. Tailed peaks may be produced by various factors such as dead volume in the column or as a result of secondary chemical equilibria (c.f. Section 4.4.3). The tailing factor, T_f is defined as

$$T_f = \frac{w_b}{w_f} \quad (2.23)$$

where w_b is the width of the back part of the peak at 5% of the peak height, and w_f is the distance from the peak maximum to the leading edge of the peak, as illustrated in Fig. 2.3. For a symmetrical peak, T_f is unity and the value of T_f increases as tailing becomes more pronounced. A value of T_f less than unity indicates peak fronting which may be a result of channelling within the column.

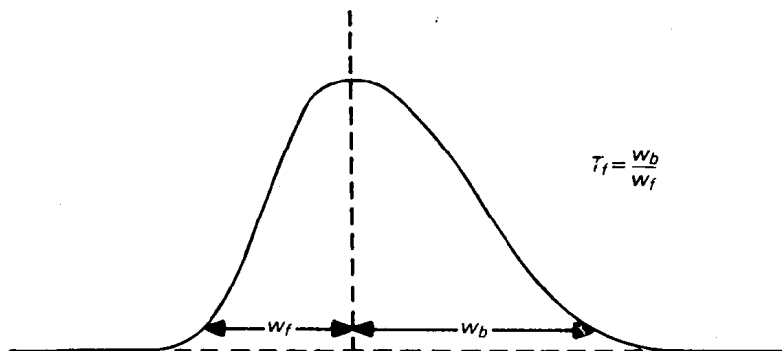


Fig. 2.3 Measurement of tailing factor for an asymmetric chromatographic peak.

Chapter 3 *Equipment*

Equipment for high performance liquid chromatography can range from a very simple home-built system to a highly sophisticated 'black-box'. The home-built or modular system is often cheaper and more versatile since components from various equipment sets may be interchanged. With these systems it is usually easier to diagnose faults, since it is possible to isolate each component; and down-time may be less since a faulty unit may be readily replaced by a spare piece of equipment in the laboratory. The user of a modular system also tends to learn more about the overall technique of HPLC and is, accordingly, more able to troubleshoot effectively. More sophisticated, integrated, micro-processor controlled systems, however, may be more suitable for routine analyses since they are designed to be less operator dependent. No matter the degree of sophistication of the apparatus, the basic system required to perform HPLC is illustrated in *Fig. 3.1*. Other components may be necessary for particular applications but this simple arrangement illustrates the basic equipment required for routine analytical work. Equipment required for preparative LC is described in Chapter 8.

The column is the most important component of any LC system, since this is where the separation is performed. For this reason a separate chapter is devoted to this subject (Chapter 4). The efficiency of the system, however, depends not only on maximising the plate count in the column but also on minimising any extra-column band broadening effects. This means that the peak width should not be significantly broadened by the injector or detector or by passage through long lengths of connecting tubing.

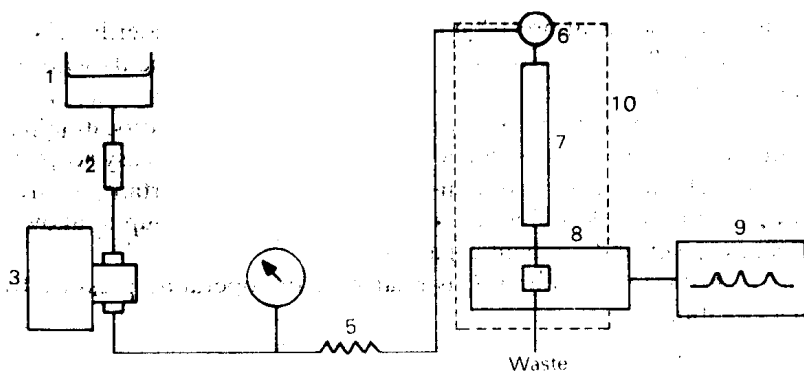


Fig. 3.1. Basic arrangement of equipment for high performance liquid chromatography. 1: eluent; 2: filter; 3: pump; 4: pressure gauge; 5: pulse damper; 6: injector; 7: column; 8: detector; 9: recorder; 10: oven.

The key relationship is

$$w_{\text{total}} = w_c + w_{\text{inj}} + w_{\text{con}} + w_{\text{det}} \quad (3.1)$$

$$= w_c^2 + w_{\text{app}}^2$$

where w_{total} is the total peak width measured by the recorder, w_c is the peak width produced by the column alone, w_{inj} the peak width produced by the injection alone, w_{con} the peak width produced by connectors and connecting tubing and w_{det} the peak width produced by the detector. The peak width produced by the combination of the last three terms, w_{app} , is the extra-column band broadening. This can be measured by omitting the column, connecting the injector directly to the detector and measuring the band width of the solute. For the best systems, additional peak spreading due to extra column dispersion should be less than 10% of total peak width. The effect of extra-column band broadening processes becomes more significant as the volume of the column is reduced. It is therefore a more significant problem with short 'fast LC' columns packed with 3 μm particles and for packed micro-bore capillary columns. These particular cases are discussed fully in Sections 4.2.1 and 4.6 respectively.

3.1 Pumps and gradient systems

Pumps for high performance liquid chromatography must be able to supply a constant, reproducible flow of eluent to the column under high pressure. The requirements of a good pump include (1) stable flow, without pulsations, to minimise detector noise; (2) capability of delivering a range of flow rates suitable for the various HPLC modes. This is normally 0.5–10 $\text{ml} \cdot \text{min}^{-1}$ for standard operation and 1 μl to 1 $\text{ml} \cdot \text{min}^{-1}$ with the use of micro-columns. (Higher flow rate capability is required for preparative work, but pressure limitations are not normally so stringent (see Section 8.1.)); (3) reproducibility of flow delivery to

ensure adequate analytical precision; (4) ability to operate under high pressure (usually up to 6000 psi); (5) compatibility of wetted components with a wide range of eluents. Pump parts which are exposed to the eluent are normally constructed of ceramic, stainless steel or PTFE. In some reciprocating pumps, pistons and check-valve balls are made of sapphire or ruby; (6) easy access to the high-pressure sliding seals and non-return valves is important for routine maintenance. It is also desirable to have a small volume hold-up to allow rapid solvent change-over and gradient capability.

The flow produced by a pump f_v is related to the operating pressure by the following equation:

$$\Delta P = f_v R \quad (3.2)$$

where R is the flow resistance of the system arising from the column, connecting tubing, etc.

Based on this relationship there are two main categories of pumps: 1) constant flow pumps where the pressure changes according to the resistance of the system; this includes all the mechanically driven pumps, and 2) constant pressure pumps where the flow rate is determined by the system resistance. This type includes coil pumps and pneumatic intensifiers.

3.1.1 Constant flow pumps

RECIPROCATING PUMPS (Fig. 3.2)

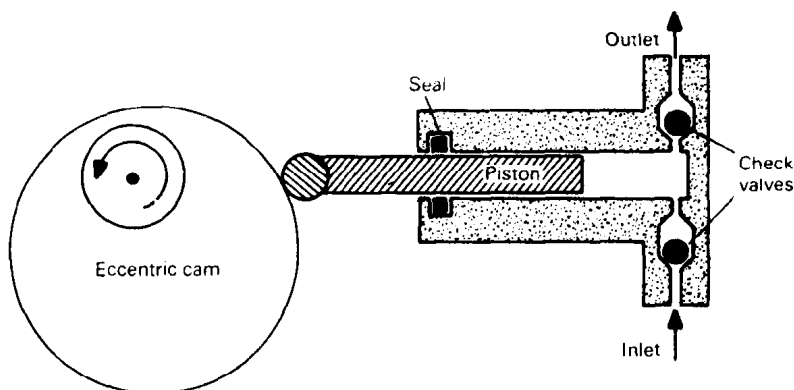


Fig 3.2. Reciprocating piston pump, displacement around 0.1 ml.
Reproduced with permission from Knox, 1978.

Reciprocating pumps are those most commonly used in HPLC since they permit delivery of a wide range of flow rates and are relatively inexpensive. These pumps function by means of a piston which is driven mechanically, with motors and gears, the more modern pumps being controlled by solid-state pulsing