10 Chromatographic theory

Introduction

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

Void volume and capacity factor

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

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

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

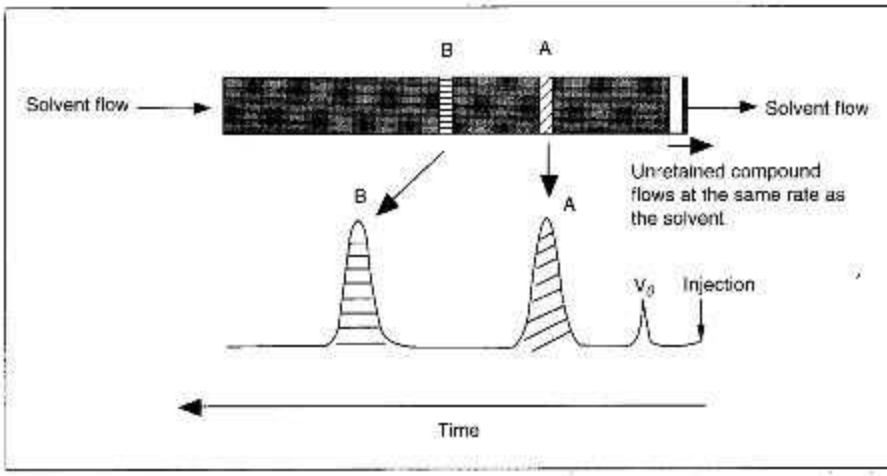

Fig. 10.1
Schematic diagram of bands for three different compounds travelling through a HPLC column. The compound with the largest capacity factor emerges last.

where V_0 is the void volume of the column, V_r is the retention volume of the analyte, t_0 is the time taken for an unretained molecule to pass through the void volume and t_r is the time taken for the analyte to pass through the column. In the example shown in Figure 10.1, compound B has a larger capacity factor than compound A. For example, if a compound had a K' of 4, the V_0 of a column was 1 ml and the solvent was flowing through the column at 1 ml/min, the total time taken for the compound to pass through the column would be 5 min, i.e. for the 1 min required to pass through the void space in the column 4 min would be spent in the stationary phase. This is a simplification of the actual process but it provides a readily understandable model. As can be seen in Figure 10.1 the peaks produced by chromatographic separation actually have width as well as a retention time and the processes which give rise to this width will be considered later.

Self-test 10.1

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

K' of compound	V_0 of column (ml)	Solvent flow rate (ml/min)
6	1	1
10	1	2

Answers: 7 min and 5.5 min

Calculation of column efficiency

The broader a chromatographic peak is relative to its retention time the less efficient the column it is eluting from. Figure 10.2 shows a chromatographic peak emerging at time t_r after injection; the efficiency of the column is most readily assessed from the width of the peak at half its height $W_{1/2}$ and its retention time using Equation 1:

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

where n is the number of theoretical plates.

Column efficiency is usually expressed in theoretical plates per metre:

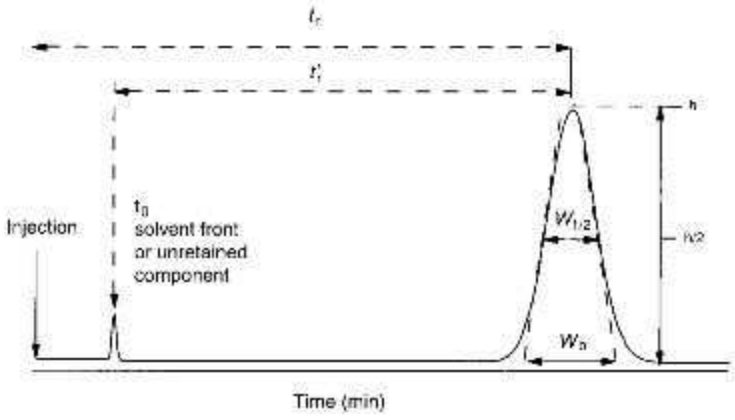

Fig. 10.2
Determination of retention time and peak width at half height.

$$n \times 100/L$$

where L is the column length in cm.

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

Equation 2 $$N\,eff = 5.54 \times t'_r/W_{h/2})^2$$

where $N\,eff$ is the number of effective plates and reflects the number of times the analyte partitions between the mobile phase and the stationary phase during its passage through the column and $t'_r = t_r - t_0$.

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

$$H = L/N\,eff$$

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

Self-test 10.2

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

Retention time of analyte (min)	W_b (min)
1. 6.4	0.2
2. 5.6	0.2
3. 10.6	0.6

Answer: Column 1

Origins of band broadening in HPLC

Van Deemter equation in liquid chromatography

Chromatographic peaks have width and this means that molecules of a single compound, despite having the same capacity factor, take different lengths of time to travel through the column. The longer an analyte takes to travel through a column, the more the individual molecules making up the sample spread out and the broader

the band becomes. The more rapidly a peak broadens the less efficient the column. Detailed mathematical modelling of the processes leading to band broadening is very complex.[1] The treatment below gives a basic introduction to the origins of band broadening. The causes of band broadening can be formalised in the Van Deemter equation (Equation 3) as applied to liquid chromatography:

Equation 3

$$H = \frac{A}{1 + C_m/u^{1/2}} + \frac{B}{u} + C_s u + C_m u^{1/2}$$

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

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

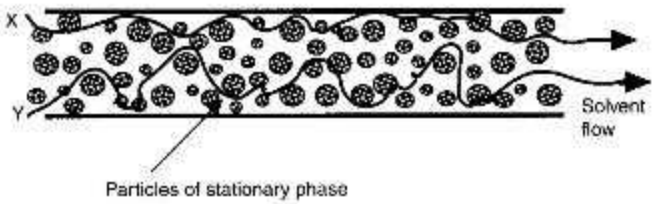

Fig. 10.3
Eddy diffusion around particles of stationary phase.

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

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

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

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

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

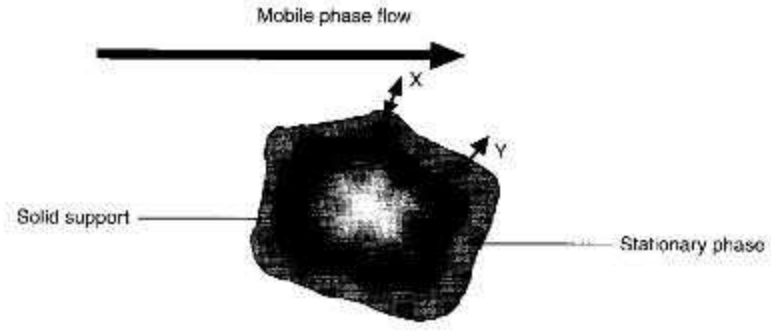

Fig. 10.4
Resistance to mass transfer of a molecule within a particle of stationary phase.

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

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

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

The smaller and more regular the shape of the particles of stationary phase, the smaller the contribution to band broadening from this term. In Figure 10.5 molecule X is retarded more than molecule Y both in terms of pathlength (this really belongs to the eddy diffusion term) and contact with stagnant areas of solvent within the pore structure of the stationary phase. With regard to the latter effect, the smaller the rate of diffusion of the molecular species (D_m) in the mobile phase, the greater the retardation will be. There are an insufficient number of random partitionings during elution for these effects to be evened out.

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

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

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

Self-test 10.3

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

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

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

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

Van Deemter equation in gas chromatography

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

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

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

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

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

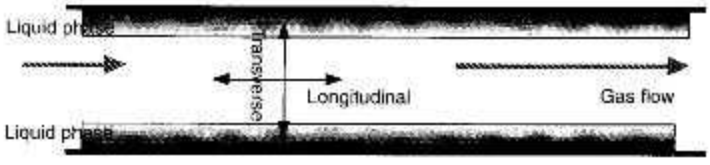

Fig. 10.6
Band broadening factors in open tubular columns.

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

Parameters used in evaluating column performance

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

Resolution

The more efficient a column the greater degree of resolution it will produce between

closely eluting peaks. The resolution beween two peaks – A and B (Fig. 10.7) is expressed in Equation 4:

Equation 4 $$R_s = 2\,(t_{rB} - t_{rA})/W_{bB} + W_{bA}$$

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

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

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

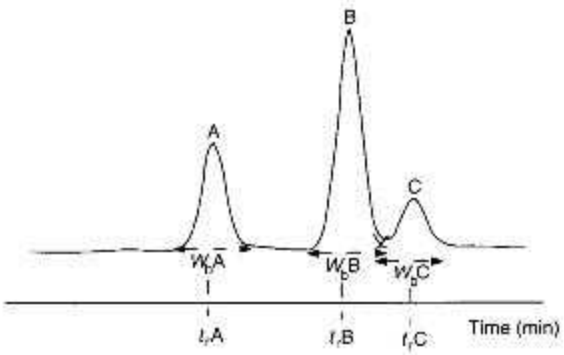

Fig. 10.7 Determination of the degree of resolution between chromatographic peaks.

Self-test 10.4

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

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

Answer 1 and 2

Peak asymmetry

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

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

where a is the leading half of the peak measured at 10% of the peak height and b is the trailing half of the peak measured at 10% of the peak height (Fig. 10.8). This value should fall, ideally, in the range 0.95–1.15. Poor symmetry may be caused through: loading too much sample onto the column, sample decomposition, the analyte adsorbing strongly onto active sites in the stationary phase, poor trapping of the analyte when it is loaded onto the column or too much 'dead volume' in the chromatographic system.

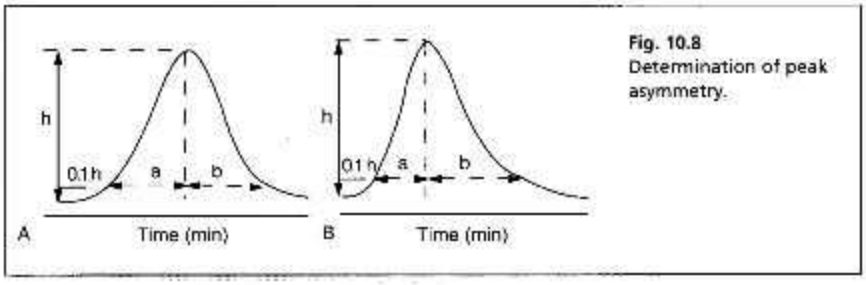

Fig. 10.8
Determination of peak asymmetry.

The peak in Figure 10.8A has an asymmetry factor of 0.97 and this is due to its tailing slightly at the front edge; this may be due to inefficient trapping of the sample at the head of the column as it is loaded. The peak in Figure 10.8B has an asymmetry factor of 1.77 and is thus tailing quite badly; the most common cause of tailing is due to adsorption of the analyte onto active sites in the chromatographic column.

Data acquisition

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

A factor which can cause a loss of precision in chromatographic quantification is the reproducibility of the way in which peaks are integrated. If peaks have good symmetry, are well resolved from neighbouring peaks and are well above baseline noise, integration is likely to be reproducible. The peak threshold (PT) setting has the greatest effect on peak area and it has to be set high enough for fluctuations in the baseline to be ignored. In the example shown in Figure 10.9, in the first case the threshold is set too low and a tail of baseline drift is attached to the peak during integration. In the second case, the threshold is set higher and the tail is ignored. The area of peak A determined with a peak threshold of 100 μV is only 94% of the area determined for peak A with tailing baseline included at a threshold of 10 μV. This could make a significant difference to the precision of the analysis depending on how reproducibly the peak tail was integrated. This type of effect is only likely to be significant if the size of the peaks is low in relation to baseline fluctuations.

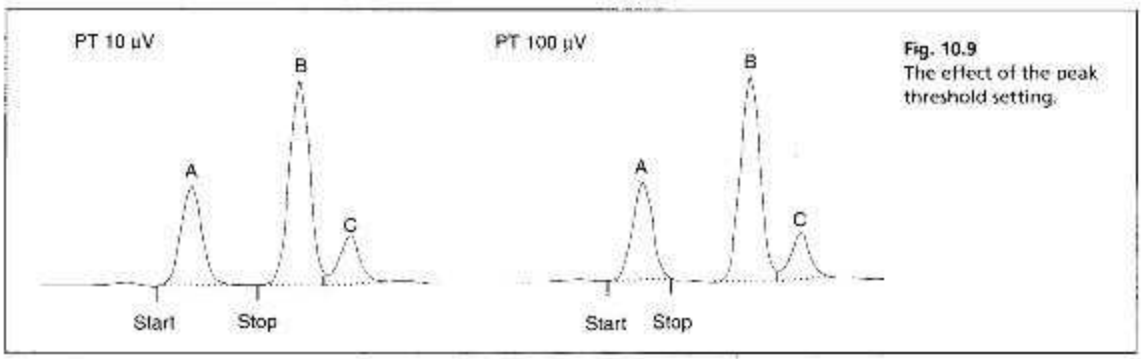

Fig. 10.9
The effect of the peak threshold setting.

The two fused peaks shown in Figure 10.9 are not as affected by a change in the peak threshold setting, however, their areas can only be approximated because of their overlap. It is possible by setting the integrator to produce a tangent skim to change the way in which these peaks are integrated as shown in Figure 10.10. In this instance where the peaks are almost resolved and are not vastly different in height, the integration method used in Figure 10.9 probably gives a better approximation of the areas.

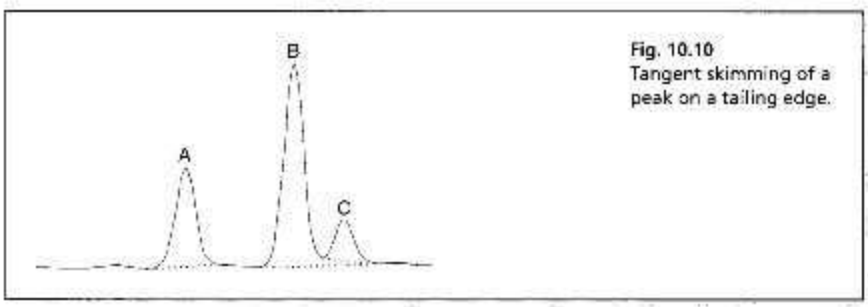

Fig. 10.10
Tangent skimming of a peak on a tailing edge.

Report generation

Computerised data handling systems will generate reports including a number of system suitability parameters. Figure 10.11 shows a chromatogram with a report form appended. In order for the report to be generated, the computer has to be given

some information, e.g. the expected retention times of peaks for which resolution factors have to be measured and the retention time of an unretained peak in order to determine capacity factor. With increasing dependence on computers, it is important to be able to guesstimate whether the computer is generating sensible data; the ability to calculate the various efficiency parameters from first principles is an important check on the performance of the integrator.

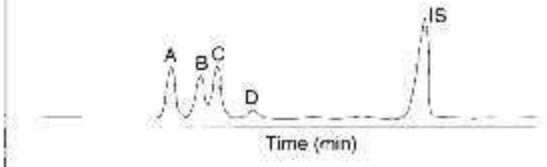

Fig. 10.11
Chromatographic trace with report including system suitability tests.

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

Reference

1. J.C. Giddings. Unified separation science. Wiley Interscience, Chichester (1991).