minimised, e.g. by passing the pressurised solvent through a pulse-damping device. Tubing and connections are capable of withstanding the pressures developed by the pumping system.

Microprocessor controlled systems are capable of accurately delivering a mobile phase in either constant or varying conditions, according to a defined programme. In the case of gradient elution, pumping systems which deliver solvent(s) from several reservoirs are available and solvent mixing can be achieved on either the low or high-pressure side of the pump(s).

Injectors

Injection may be carried out directly at the head of the column using a valve.

Stationary phases

Stationary phases are contained in columns which have been described in the chapters on *Liquid chromatography* (*2.2.29*) (packed columns) and *Gas chromatography* (*2.2.28*) (capillary columns). A capillary column has a maximum internal diameter (Ø) of 100 µm.

Mobile phases

Usually the mobile phase is carbon-dioxide which may contain a polar modifier such as methanol, 2-propanol or acetonitrile. The composition, pressure (density), temperature and flow rate of the prescribed mobile phase may either be constant throughout the whole chromatographic procedure (isocratic, isodense, isothermic elution) or may vary according to a defined programme (gradient elution of the modifier, pressure (density), temperature or flow rate).

Detectors

Ultraviolet/visible (UV/Vis) spectrophotometers and flame ionisation detectors are the most commonly employed detectors. Light scattering detectors, infrared absorption spectrophotometers, thermal conductivity detectors or other special detectors may be used.

METHOD

Prepare the test solution(s) and the reference solution(s) as prescribed. The solutions must be free from solid particles.

Criteria for assessing the suitability of the system are described in the chapter on *Chromatographic separation techniques* (*2.2.46*). The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given in this chapter.

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2.2.46. CHROMATOGRAPHIC SEPARATION TECHNIQUES

Chromatographic separation techniques are multi-stage separation methods in which the components of a sample are distributed between 2 phases, one of which is stationary, while the other is mobile. The stationary phase may be a solid or a liquid supported on a solid or a gel. The stationary phase may be packed in a column, spread as a layer, or distributed as a film, etc. The mobile phase may be gaseous or liquid or supercritical fluid. The separation may be based on adsorption, mass distribution (partition), ion exchange, etc., or may be based on differences in the physico-chemical properties of the molecules such as size, mass, volume, etc.

This chapter contains definitions and calculations of common parameters and generally applicable requirements for system suitability. Principles of separation, apparatus and methods are given in the following general methods:

- paper chromatography (*2.2.26*),
- thin-layer chromatography (*2.2.27*),
- gas chromatography (*2.2.28*),
- liquid chromatography (*2.2.29*),
- size-exclusion chromatography (*2.2.30*),
- supercritical fluid chromatography (*2.2.45*).

DEFINITIONS

The following definitions have been used to calculate the limits in monographs.

With some equipment, certain parameters, such as the signal-to-noise ratio, can be calculated using software provided by the manufacturer. It is the responsibility of the user to ensure that the calculation methods used in the software are compatible with the requirements of the European Pharmacopoeia. If not, the necessary corrections must be made.

Chromatogram

A chromatogram is a graphical or other representation of detector response, effluent concentration or other quantity used as a measure of effluent concentration, versus time, volume or distance. Idealised chromatograms are represented as a sequence of gaussian peaks on a baseline.

RETENTION DATA

Retention time and retention volume

Retention measurements in elution chromatography may be given as the retention time (t_R) directly defined by the position of the maximum of the peak in the chromatogram. From the retention time, the retention volume (V_R) may be calculated.

$$V_R = v \times t_R$$

t_R = retention time or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to the component,

v = flow rate of the mobile phase.

Mass distribution ratio

The mass distribution ratio (D_m) (also known as the capacity factor *k'* or retention factor k) is defined as:

$$D_m = \frac{\text{amount of solute in stationary phase}}{\text{amount of solute in mobile phase}} = K_C \frac{V_S}{V_M}$$

K_C = equilibrium distribution coefficient (also known as distribution constant),

V_S = volume of the stationary phase,

V_M = volume of the mobile phase.

The mass distribution ratio of a component may be determined from the chromatogram using the expression:

$$D_m = \frac{t_R - t_M}{t_M}$$

t_R = retention time (or volume) or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to the component,

t_M = hold-up time (or volume): time (or volume) or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to an unretained component.

Distribution coefficient

The elution characteristics of a component in a particular column, in size-exclusion chromatography, may be given by the distribution coefficient (K_o) which is calculated from the expression:

$$K_o = \frac{t_R - t_o}{t_t - t_o}$$

t_R = retention time (or volume) or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to the component,

t_o = hold-up time (or volume): time (or volume) or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to an unretained component,

t_t = retention time (or volume) or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to a component which has full access to the pores of the stationary phase.

Retardation factor

The retardation factor (R_F) (also known as retention factor R_f), used in planar chromatography, is the ratio of the distance from the point of application to the centre of the spot and the distance travelled by the solvent front from the point of application.

$$R_F = \frac{b}{a}$$

b = migration distance of the analyte,

a = migration distance of the solvent front.

CHROMATOGRAPHIC DATA

The peak may be defined by the *peak area (A)* or the *peak height (h)* and the *peak width at half- height (w_h)* or the *peak height (h)* and the *peak width between the points of inflection (w_i).* In gaussian peaks (Figure 2.2.46.-1) there is the relationship:

$$w_h = 1.18 w_i$$

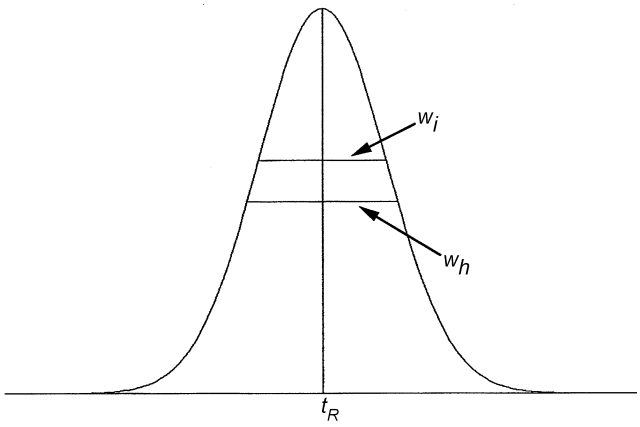

Figure 2.2.46.-1.

Symmetry factor

The symmetry factor (A_s) (or tailing factor) of a peak (Figure 2.2.46.-2) is calculated from the expression:

$$A_s = \frac{w_{0.05}}{2d}$$

$w_{0.05}$ = width of the peak at one-twentieth of the peak height,

d = distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

A value of 1.0 signifies complete (ideal) symmetry.

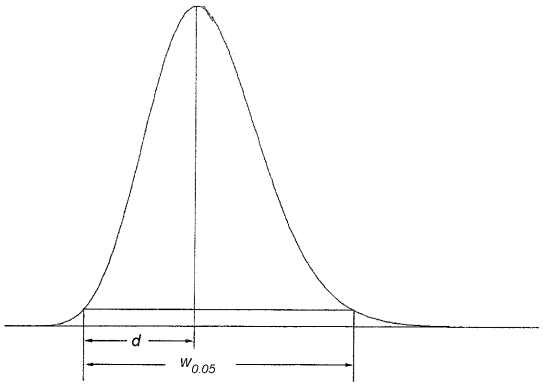

Figure 2.2.46.-2.

Column performance and apparent number of theoretical plates

The column performance (apparent efficiency) may be calculated from data obtained under either isothermal, isocratic or isodense conditions, depending on the technique, as the apparent number of theoretical plates (N) from the following expression, where the values of t_R and w_h have to be expressed in the same units (time, volume or distance).

$$N = 5.54 \left(\frac{t_R}{w_h} \right)^2$$

t_R = retention time (or volume) or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to the component,

w_h = width of the peak at half-height.

The apparent number of theoretical plates varies with the component as well as with the column and the retention time.

SEPARATION DATA

Resolution

The resolution (R_s) between peaks of 2 components may be calculated from the expression:

$$R_s = \frac{1.18\,(t_{R2} - t_{R1})}{w_{h1} + w_{h2}}$$

$$t_{R2} > t_{R1}$$

t_{R1} and t_{R2} = retention times or distances along the baseline from the point of injection to the perpendiculars dropped from the maxima of 2 adjacent peaks,

w_{h1} and w_{h2} = peak widths at half-height.

A resolution of greater than 1.5 corresponds to baseline separation.

The expression given above may not be applicable if the peaks are not baseline separated.

In quantitative planar chromatography, the migration distances are used instead of retention times and the resolution may be calculated using the expression:

$$R_s = \frac{1.18\,a\,(R_{F2} - R_{F1})}{w_{h1} + w_{h2}}$$

R_{F1} and R_{F2} = ratios of the distances from the point of application to the centres of the spots and the distance travelled by the solvent front from the point of application (retardation factor),

w_{h1} and w_{h2} = peak widths at half-height,

a = migration distance of the solvent front.

Peak-to-valley ratio

The peak-to-valley ratio (p/v) may be employed as a system suitability requirement in a test for related substances when baseline separation between 2 peaks is not reached (Figure 2.2.46.-3).

$$p/v = \frac{H_p}{H_v}$$

H_p = height above the extrapolated baseline of the minor peak,

H_v = height above the extrapolated baseline at the lowest point of the curve separating the minor and major peaks.

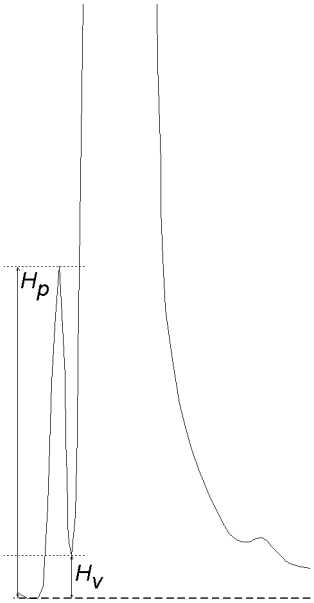

Figure 2.2.46.-3.

Relative retention

The relative retention (r) is calculated as an estimate from the expression:

$$r = \frac{t_{R2} - t_M}{t_{R1} - t_M}$$

t_{R2} = retention time of the peak of interest,

t_{R1} = retention time of the reference peak (usually the peak corresponding to the substance to be examined),

t_M = hold-up time: time or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to an unretained component.

The unadjusted relative retention (r_G) is calculated from the expression:

$$r_G = \frac{t_{R2}}{t_{R1}}$$

Unless otherwise indicated, values for relative retention stated in monographs correspond to unadjusted relative retention.

In planar chromatography, the retardation factors R_{F2} and R_{F1} are used instead of t_{R2} and t_{R1}.

PRECISION OF QUANTIFICATION

Signal-to-noise ratio

The signal-to-noise ratio (*S/N*) influences the precision of quantification and is calculated from the equation:

$$S/N = \frac{2H}{h}$$

H = height of the peak (Figure 2.2.46.-4) corresponding to the component concerned, in the chromatogram obtained with the prescribed reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to 20 times the width at half-height,

h = range of the background noise in a chromatogram obtained after injection or application of a blank, observed over a distance equal to 20 times the width at half-height of the peak in the chromatogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

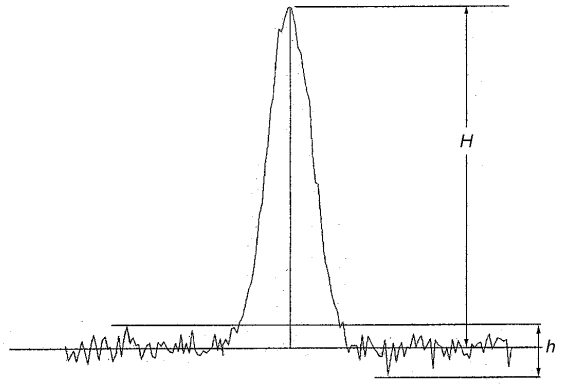

Figure 2.2.46.-4.

Repeatability

The repeatability of response is expressed as an estimated percentage relative standard deviation ($RSD_{\%}$) of a consecutive series of measurement of injections or applications of a reference solution and is calculated from the expression:

$$RSD_{\%} = \frac{100}{\bar{y}}\sqrt{\frac{\sum (y_i - \bar{y})^2}{n-1}}$$

y_i = individual values expressed as peak area, peak height, or ratio of areas by the internal standardisation method,

$\bar{y}$ = mean of individual values,

n = number of individual values.

The maximal permitted relative standard deviation (RSD_{max}) is calculated for a series of injections of the reference solution for defined limits using the following expression:

$$RSD_{\max} = \frac{K\mathrm{B}\sqrt{n}}{t_{90\%,n-1}}$$

K = constant (0.349), obtained from the expression $K = \frac{0.6}{\sqrt{2}} \times \frac{t_{90\%,5}}{\sqrt{6}}$ in which $\frac{0.6}{\sqrt{2}}$ represents the required *RSD* after 6 injections for B = 1.0,

B = upper limit given in the definition of the individual monograph minus 100 per cent,

n = number of replicate injections of the reference solution ($3 \leq n \leq 6$),

$t_{90\%,n-1}$ = Student's t at the 90 per cent probability level (double sided) with $n - 1$ degrees of freedom.

SYSTEM SUITABILITY

The system suitability tests represent an integral part of the method and are used to ensure adequate performance of the chromatographic system. Apparent efficiency, mass distribution ratio, resolution, relative retention and the symmetry factor are the parameters which are usually employed in assessing the performance of the column. Factors which may affect the chromatographic behaviour include the composition, ionic strength, temperature and apparent pH of the mobile phase, flow rate, column length, temperature and pressure, and stationary phase characteristics including porosity, particle size, type of particles, specific surface area and, in the case of reverse-phase supports, the extent of chemical modification (as expressed by end-capping, carbon loading etc.).

The various components of the equipment employed must be qualified and be capable of achieving the precision required to conduct the test or assay.

The following requirements are to be fulfilled unless otherwise stated in the monograph.

- The symmetry factor of the principal peak is to be between 0.8 and 1.5 unless otherwise stated in the monograph. This requirement has general applicability to tests or assays described in the monographs.
- Maximal permitted relative standard deviation for replicate injections of the prescribed reference solution do not exceed the values given in Table 2.2.46.-1. This requirement is applicable to assays for content only and does not apply to the test for related substances.
- The limit of detection of the peak (corresponding to a signal-to-noise ratio of 3) is below the disregard limit of the test for related substances.
- The limit of quantitation of the peak (corresponding to a signal-to-noise ratio of 10) is equal to or less than the disregard limit of the test for related substances.

Table 2.2.46.-1. – *Repeatability requirements*

	Number of individual injections			
	3	**4**	**5**	**6**
B (per cent)	**Maximal permitted relative standard deviation**			
2.0	0.41	0.59	0.73	0.85
2.5	0.52	0.74	0.92	1.06
3.0	0.62	0.89	1.10	1.27

ADJUSTMENT OF CHROMATOGRAPHIC CONDITIONS

The extent to which the various parameters of a chromatographic test may be adjusted to satisfy the system suitability criteria without fundamentally modifying the methods are listed below for information. The chromatographic conditions described have been validated during the elaboration of the monograph. The system suitability tests are included to ensure the separation required for satisfactory performance of the test or assay. Nonetheless, since the stationary phases are described in a general way and there is such a variety available

commercially, with differences in chromatographic behaviour, some adjustments of the chromatographic conditions may be necessary to achieve the prescribed system suitability requirements. With reverse-phase liquid chromatographic methods, in particular, adjustment of the various parameters will not always result in satisfactory chromatography. In that case, it may be necessary to replace the column with another of the same type (e.g. octadecylsilyl silica gel) which exhibits the desired chromatographic behaviour.

For critical parameters the adjustments are defined clearly in the monograph to ensure the system suitability.

Multiple adjustments which may have a cumulative effect in the performance of the system are to be avoided.

Thin-layer chromatography and paper chromatography

Composition of the mobile phase: the amount of the minor solvent component may be adjusted by ± 30 per cent relative or ± 2 per cent absolute, whichever is the larger; for a minor component at 10 per cent of the mobile phase, a 30 per cent relative adjustment allows a range of 7-13 per cent whereas a 2 per cent absolute adjustment allows a range of 8-12 per cent, the relative value being therefore the larger; for a minor component at 5 per cent of the mobile phase, a 30 per cent relative adjustment allows a range of 3.5-6.5 per cent whereas a 2 per cent absolute adjustment allows a range of 3-7 per cent, the absolute value being in this case the larger. No other component is altered by more than 10 per cent absolute.

pH of the aqueous component of the mobile phase: ± 0.2 pH, unless otherwise stated in the monograph, or ± 1.0 pH when neutral substances are to be examined.

Concentration of salts in the buffer component of a mobile phase: ± 10 per cent.

Application volume: 10-20 per cent of the prescribed volume if using fine particle size plates (2-10 µm).

Migration distance of the solvent front is to be not less than 50 mm or 30 mm on high-performance plates.

Liquid chromatography

Composition of the mobile phase: the amount of the minor solvent component may be adjusted by ± 30 per cent relative or ± 2 per cent absolute, whichever is the larger (see example above). No other component is altered by more than 10 per cent absolute.

pH of the aqueous component of the mobile phase: ± 0.2 pH, unless otherwise stated in the monograph, or ± 1.0 pH when neutral substances are to be examined.

Concentration of salts in the buffer component of a mobile phase: ± 10 per cent.

Detector wavelength: no adjustment permitted.

Stationary phase:

- *column length*: ± 70 per cent,
- *column internal diameter*: ± 25 per cent,
- *particle size*: maximal reduction of 50 per cent, no increase permitted.

Flow rate: ± 50 per cent. When in a monograph the retention time of the principle peak is indicated, the flow rate has to be adjusted if the column internal diameter has been changed. No decrease of flow rate is permitted if the monograph uses apparent number of theoretical plates in the qualification section.

Temperature: ± 10 per cent, to a maximum of 60 °C.

Injection volume: may be decreased, provided detection and repeatability of the peak(s) to be determined are satisfactory.

Gradient elution: the configuration of the equipment employed may significantly alter the resolution, retention time and relative retentions described in the method. Should this occur, it may be due to excessive dwell volume which is the volume between the point at which the 2 eluants meet and the top of the column.

Gas chromatography

Stationary phase:

- *column length*: ± 70 per cent,
- *column internal diameter*: ± 50 per cent,
- *particle size*: maximal reduction of 50 per cent, no increase permitted,
- *film thickness*: – 50 per cent to + 100 per cent.

Flow rate: ± 50 per cent.

Temperature: ± 10 per cent.

Injection volume: may be decreased, provided detection and repeatability are satisfactory.

Supercritical fluid chromatography

Composition of the mobile phase: for packed columns, the amount of the minor solvent component may be adjusted by ± 30 per cent relative or ± 2 per cent absolute, whichever is the larger. No adjustment is permitted for a capillary column system.

Detector wavelength: no adjustment permitted.

Stationary phase:

- *column length*: ± 70 per cent,
- *column internal diameter*:

  ± 25 per cent (packed columns),

  ± 50 per cent (capillary columns),
- *particle size*: maximal reduction of 50 per cent, no increase permitted (packed columns).

Flow rate: ± 50 per cent.

Temperature: ± 10 per cent.

Injection volume: may be decreased, provided detection and repeatability are satisfactory.

QUANTIFICATION

- *Detector response.* The detector sensitivity is the signal output per unit concentration or unit mass of a substance in the mobile phase entering the detector. The relative detector response factor, commonly referred to as *response factor*, expresses the sensitivity of a detector relative to a standard substance. The *correction factor* is the reciprocal of the response factor.
- *External standard method.* The concentration of the component(s) to be analysed is determined by comparing the response(s) (peak(s)) obtained with the test solution to the response(s) (peak(s)) obtained with a reference solution.
- *Internal standard method.* Equal amounts of a component that is resolved from the substance to be examined (the internal standard) is introduced into the test solution and a reference solution. The internal standard should not react with the substance to be examined; it must be stable and must not contain impurities with a retention time similar to that of the substance to be examined. The concentration of the substance to be examined is determined by comparing the ratio of the peak areas or peak heights due to the substance to be examined and the internal standard in the test solution with the ratio of the peak areas or peak heights due to the substance to be examined and the internal standard in the reference solution.

– *Normalisation procedure.* The percentage content of one or more components of the substance to be examined is calculated by determining the area of the peak or peaks as a percentage of the total area of all the peaks, excluding those due to solvents or any added reagents and those below the disregard limit.

– *Calibration procedure.* The relationship between the measured or evaluated signal *(y)* and the amount (concentration, mass, etc.) of substance *(x)* is determined and the calibration function is calculated. The analytical results are calculated from the measured signal or evaluated signal of the analyte by means of the inverse function.

For assays and for quantitative determination of components the external standard method, the internal standard method or the calibration procedure may be described in the monograph, and the normalisation procedure is not normally applied. In tests for related substances, either the external standard method with a single reference solution or the normalisation procedure is generally applied. However, with both the normalisation procedure or the external standard method, when a dilution of the test solution is used for comparison, the responses of the related substances are similar to the substance itself (response factor of 0.8 to 1.2), otherwise correction factors are included in the text.

When the related substances test prescribes the summation of impurities or there is quantitative determination of an impurity, it is important to choose an appropriate threshold setting and appropriate conditions for the integration of the peak areas. In such tests the *disregard limit*, e.g. the areas of peaks whose areas are below the limit are not taken into account, is generally 0.05 per cent. Thus, the threshold setting of the data collection system corresponds to, at least, half of the disregard limit. Integration of the peak areas of the impurities, which are not completely separated from the main peak, are preferably performed by valley-to-valley extrapolation (tangential skim). Peaks due to the solvent(s) used to dissolve the sample are also to be disregarded.

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2.2.47. CAPILLARY ELECTROPHORESIS

GENERAL PRINCIPLES

Capillary electrophoresis is a physical method of analysis based on the migration, inside a capillary, of charged analytes dissolved in an electrolyte solution, under the influence of a direct-current electric field.

The migration velocity of an analyte under an electric field of intensity *E*, is determined by the electrophoretic mobility of the analyte and the electro-osmotic mobility of the buffer inside the capillary. The electrophoretic mobility of a solute (μ_{ep}) depends on the characteristics of the solute (electric charge, molecular size and shape) and those of the buffer in which the migration takes place (type and ionic strength of the electrolyte, pH, viscosity and additives). The electrophoretic velocity (ν_{ep}) of a solute, assuming a spherical shape, is given by the equation:

$$\nu_{ep} = \mu_{ep} \times E = \left(\frac{q}{6\pi\eta r}\right) \times \left(\frac{V}{L}\right)$$

q = effective charge of the solute,

η = viscosity of the electrolyte solution,

r = Stoke's radius of the solute,

V = applied voltage,

L = total length of the capillary.

When an electric field is applied through the capillary filled with buffer, a flow of solvent is generated inside the capillary, called electro-osmotic flow. The velocity of the electro-osmotic flow depends on the electro-osmotic mobility (μ_{eo}) which in turn depends on the charge density on the capillary internal wall and the buffer characteristics. The electro-osmotic velocity (ν_{eo}) is given by the equation:

$$\nu_{eo} = \mu_{eo} \times E = \left(\frac{\varepsilon\zeta}{\eta}\right) \times \left(\frac{V}{L}\right)$$

ϵ = dielectric constant of the buffer,

ζ = zeta potential of the capillary surface.

The velocity of the solute (ν) is given by:

$$\nu = \nu_{ep} + \nu_{eo}$$

The electrophoretic mobility of the analyte and the electro-osmotic mobility may act in the same direction or in opposite directions, depending on the charge of the solute. In normal capillary electrophoresis, anions will migrate in the opposite direction to the electro-osmotic flow and their velocities will be smaller than the electro-osmotic velocity. Cations will migrate in the same direction as the electro-osmotic flow and their velocities will be greater than the electro-osmotic velocity. Under conditions in which there is a fast electro-osmotic velocity with respect to the electrophoretic velocity of the solutes, both cations and anions can be separated in the same run.

The time (t) taken by the solute to migrate the distance (l) from the injection end of the capillary to the detection point (capillary effective length) is given by the expression:

$$t = \frac{l}{\nu_{ep} + \nu_{eo}} = \frac{l \times L}{(\mu_{ep} + \mu_{eo}) \times V}$$

In general, uncoated fused-silica capillaries above pH 3 have negative charge due to ionised silanol groups in the inner wall. Consequently, the electro-osmotic flow is from anode to cathode. The electro-osmotic flow must remain constant from run to run if good reproducibility is to be obtained in the migration velocity of the solutes. For some applications, it may be necessary to reduce or suppress the electro-osmotic flow by modifying the inner wall of the capillary or by changing the concentration, composition and/or pH of the buffer solution.

After the introduction of the sample into the capillary, each analyte ion of the sample migrates within the background electrolyte as an independent zone, according to its electrophoretic mobility. Zone dispersion, that is the spreading of each solute band, results from different phenomena. Under ideal conditions the sole contribution to the solute-zone broadening is molecular diffusion of