PHARMACOPOEIAL DISCUSSION GROUP
SIGN-OFF DOCUMENT

CODE: B-02
NAME: Capillary Electrophoresis

REVISION 1

It is understood that sign-off covers the technical content of the draft and each party will adapt it as necessary to conform to the usual presentation of the pharmacopoeia in question; such adaptation includes stipulation of the particular pharmacopoeia's reference materials and general chapters.

Harmonised provisions:

Provision	EP	IP	JP	USP
General Principles	+	+	+	+
Apparatus	+	+	+	+
Capillary Zone Electrophoresis	+	+	+	+
Capillary Gel Electrophoresis	+	+	+	+
Capillary Isoelectric Focusing	+	+	+	+
Micellar Electrokinetic Chromatography (MEKC)	+	+	+	+
Quantitation	+	+	+	+
System suitability	+	+	+	+
Adjustments of Operating Conditions for Capillary Electrophoresis	+	+	+	+

Legend
+ will adopt and implement
- will not stipulate

Non-harmonised parts:

EP	None
IP	None
JP	None
USP	None

European Pharmacopoeia

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B-02 CAPILLARY ELECTROPHORESIS

GENERAL PRINCIPLES

Capillary electrophoresis is a physical technique 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:

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

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 (EOF). The velocity of the EOF 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:

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

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 EOF and their velocities will be smaller than the electro-osmotic velocity. Cations will migrate in the same direction as the EOF 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:

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

In general, uncoated fused silica capillaries above pH 3 have negative charge due to ionized silanol groups in the inner wall. Consequently, the EOF is from anode to cathode. It is recommended that the EOF is maintained 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 EOF 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 the solute along the capillary (longitudinal diffusion).
In this ideal case the efficiency of the zone, expressed as the parameter N corresponding to the apparent number of theoretical plates, is given by:

$$N = ((\mu_{ep} + \mu_{eo}) \times V \times l) / (2 \times D \times L)$$

D = molecular diffusion coefficient of the solute in the buffer.

In practice, other phenomena such as heat dissipation, sample adsorption onto the capillary wall, mismatched conductivity between sample and buffer, length of the injection plug, detector cell size and unlevelled buffer reservoirs can also significantly contribute to band dispersion.

Separation between two bands (expressed as the resolution, R_s) can be obtained by modifying the electrophoretic mobility of the analytes, the electro-osmotic mobility induced in the capillary and by increasing the efficiency for the band of each analyte, according to the equation:

$$R_s = (\sqrt{N}\ (\mu_{epb} - \mu_{epa})) / (4(\bar{\mu}_{ep} + \mu_{eo}))$$

μ_{epa} and μ_{epb} = electrophoretic mobilities of the two analytes separated,
$\bar{\mu}_{ep}$ = mean electrophoretic mobility of the two analytes.

$$\bar{\mu}_{ep} = ½\ (\mu_{epb} + \mu_{epa})$$

APPARATUS

An apparatus for capillary electrophoresis is composed of :
— a high-voltage, controllable direct-current power supply,
— two buffer reservoirs, held at the same level, containing the prescribed anodic and cathodic solutions,
— two electrode assemblies (the cathode and the anode), immersed in the buffer reservoirs and connected to the power supply,
— a separation capillary (usually made of fused silica) which, when used with some specific types of detectors, has an optical viewing window aligned with the detector. The ends of the capillary are placed in the buffer reservoirs. The capillary is filled with the solution prescribed in the monograph,
— a suitable injection system,
— a detector able to monitor the amount of substances of interest passing through a segment of the separation capillary at a given time. It is usually based on absorption spectrophotometry (UV and visible) or fluorimetry, but conductimetric, amperometric as well as mass spectrometric detection can be useful for specific applications. Indirect detection is an alternative method used to detect non-UV-absorbing and non-fluorescent compounds,
— a thermostatic system able to maintain a constant temperature inside the capillary is recommended to obtain a good separation reproducibility,
— a data acquisition system.

The definition of the injection process and its automation are critical for precise quantitative analysis. Modes of injection include hydrodynamic injection and electrokinetic injection. The amount of each sample component introduced electrokinetically depends on its electrophoretic mobility, leading to possible discrimination using this injection mode.

Hydrodynamic injection
The sample is injected into the capillary by applying a pressure difference (Δp) between the ends of the capillary. The volume of sample injected can be calculated by the Hagen-Poiseuille equation:

$$V_{inj} = (\Delta p \, d_i^4 \, \pi \, t_{inj})/(128 \, \eta L)$$

t_{inj} = injection time,
η = dynamic viscosity of the buffer,
d_i = internal diameter of the capillary,
L = total length of the capillary.

According to the equation, under constant pressure, the injection volume decreases when the length of the capillary is increased. However, the internal diameter of the capillary (d_i) has a dominant influence on the injection volume, since doubling the capillary internal diameter allows the injection of 4 times as much sample while keeping the plug length (l_{inj}) constant:

$$l_{inj} = (\Delta p\ d_i^2 t_{inj})/(32\ \eta L)$$

Electrokinetic injection

In this mode of injection, analytes are injected into the capillary by applying an electrical field (E) across the capillary. Neutral analytes migrate into the capillary with the EOF whereas the charged analytes move according to their own electrophoretic mobilities and to the EOF ($\mu_{ep} + \mu_{eo}$). The amount of each analyte injected depends on its apparent mobility. In contrast to the hydrodynamic mode of injection, this is therefore a discriminative mode and consequently, the analyte sample vial may only be used for one injection, since the concentration of the analytes in the remaining sample will change after injection.

The injection amount (Q_{inj}) of each analyte can be calculated from the following equation:

$$Q_{inj} = (E\ k_b\ \mu_{app}\ t_{inj}\ \pi\ d_i^2\ c_s) / 4\ k_s$$

μ_{app} = apparent electrophoretic mobility (=μ_{ep}+μ_{eo})
t_{inj} = injection time,
k_b/k_s = ratio of conductivities of the background electrolyte and sample,
c_s = concentration of the analyte in the sample.

The plug length (l_{inj}) is not dependent on the capillary internal diameter and can be estimated from the following equation:

$$l_{inj} = E\ (k_b/k_s)\ \mu_{app}\ t_{inj}$$

This injection mode is mainly used for capillary gel electrophoresis using viscous gel or polymer solution.

For fused silica capillaries, the following internal diameter tolerance is recommended:

Capillary internal diameter	Recommended tolerance
25 µm	± 2 µm
50 µm	± 3 µm
75 µm	± 3 µm

Use the capillary, the buffer solutions, the preconditioning method, the sample solution and the migration conditions prescribed in the monograph of the considered substance.
The electrolyte solution employed is filtered to remove particles and degassed to avoid bubble formation that could interfere with the detection system or interrupt the electrical contact in the capillary during the separation run. A rigorous rinsing procedure should be developed for each analytical method to achieve reproducible migration times of the solutes.

CAPILLARY ZONE ELECTROPHORESIS

PRINCIPLE

In capillary zone electrophoresis, analytes are separated in a capillary containing only buffer without any anticonvective medium. With this technique, separation takes place because the different components of the sample migrate as discrete bands with different velocities. The velocity of each band depends on the electrophoretic mobility of the solute and the EOF in the capillary (see General Principles).

Coated capillaries can be used to increase the separation capacity of those substances adsorbing on fused silica surfaces.

Using this mode of capillary electrophoresis, the analysis of both small (relative molecular mass (M_r) < 2000) and large molecules (M_r > 2000) can be accomplished. Due to the high efficiency achieved in capillary zone electrophoresis, separation of molecules having only minute differences in their charge-to-size ratio can be effected; size referring to hydrodynamic size or hydrodynamic volume. This separation mode also allows the separation of chiral compounds by addition of chiral selectors to the separation buffer.

OPTIMISATION

Optimisation of the separation is a complex process where several separation parameters can play a major role. The main factors to be considered in the development of separations are instrumental and electrolyte solution parameters.

Instrumental parameters

Voltage. A Joule heating plot is useful in optimizing the applied voltage and capillary temperature. Separation time is inversely proportional to applied voltage. However, an increase in the voltage used can cause excessive heat production, giving rise to temperature and, as a result thereof, viscosity gradients in the buffer inside the capillary. This effect causes band broadening and decreases resolution.

Polarity. Electrode polarity can be normal (anode at the inlet and cathode at the outlet) and the EOF will move toward the cathode. If the electrode polarity is reversed, the EOF is away from the outlet and only charged analytes with electrophoretic mobilities greater than the EOF will pass to the outlet.

Temperature. The main effect of temperature is observed on buffer viscosity and electrical conductivity, and therefore on migration velocity. In some cases, an increase in capillary temperature can cause a conformational change in proteins, modifying their migration time and the efficiency of the separation.

Capillary. The dimensions of the capillary (length and internal diameter) contribute to analysis time, efficiency of separations and load capacity. Increasing both effective length and total length decreases the electric field (working at constant voltage) which increases migration time. For a given buffer and electric field, heat dissipation, and

hence sample band-broadening, depends on the internal diameter of the capillary. The latter also affects the detection limit, depending on the sample volume injected and the detection system employed.

Since the adsorption of the sample components on the capillary wall limits efficiency, methods to avoid these interactions should be considered in the development of a separation method. In the specific case of proteins, several strategies have been devised to avoid adsorption on the capillary wall. Some of these strategies (use of extreme pH and adsorption of positively charged buffer additives) only require modification of the buffer composition to prevent protein adsorption. In other strategies, the internal wall of the capillary is coated with a polymer, covalently bonded to the silica, that prevents interaction between the proteins and the negatively charged silica surface. For this purpose, ready-to-use capillaries with coatings consisting of neutral-hydrophilic, cationic and anionic polymers are available.

Electrolyte solution parameters

Buffer type and concentration. Suitable buffers for capillary electrophoresis have an appropriate buffer capacity in the pH range of choice and low mobility to minimise current generation.

Matching buffer-ion mobility to solute mobility, whenever possible, is important for minimising band distortion. The type of sample solvent used is also important to achieve on-column sample focusing, which increases separation efficiency and improves detection.

An increase in buffer concentration (for a given pH) decreases EOF and solute velocity.

Buffer pH. The pH of the buffer can affect separation by modifying the charge of the analyte or additives, and by changing the EOF. In protein and peptide separation, changing the pH of the buffer from above to below the isoelectric point (pI) changes the net charge of the solute from negative to positive. An increase in the buffer pH generally increases the EOF.

Organic solvents. Organic modifiers (methanol, acetonitrile, etc.) may be added to the aqueous buffer to increase the solubility of the solute or other additives and/or to affect the degree of ionisation of the sample components. The addition of these organic modifiers to the buffer generally causes a decrease in the EOF.

Additives for chiral separations. For the separation of enantiomers, a chiral selector is added to the separation buffer. The most commonly used chiral selectors are cyclodextrins, but crown ethers, polysaccharides and proteins may also be used. Since chiral recognition is governed by the different interactions between the chiral selector and each of the enantiomers, the resolution achieved for the chiral compounds depends largely on the type of chiral selector used. Other factors controlling the resolution in chiral separations are concentration of chiral selector, composition and pH of the buffer and temperature. The use of organic additives, such as methanol or urea can also

modify the resolution achieved. For the development of a given separation it may be useful to test cyclodextrins having a different cavity size (α-, β-, or γ-cyclodextrin) or modified cyclodextrins with neutral (methyl, ethyl, hydroxyalkyl, etc.) or ionic/ionisable (aminomethyl, carboxymethyl, sulfobutyl ether, etc.) groups. When using modified cyclodextrins, batch-to-batch variations in the degree of substitution of the cyclodextrins need to be taken into account since it will influence the selectivity.

CAPILLARY GEL ELECTROPHORESIS

PRINCIPLE

In capillary gel electrophoresis, separation takes place inside a capillary filled with a gel that acts as a molecular sieve, under conditions where the EOF is suppressed. Molecules with similar charge-to-size ratios are separated according to molecular size since smaller molecules move more freely through the network of the gel and therefore migrate faster than larger molecules. Different biological macromolecules (for example, DNA fragments and SDS-treated proteins), which often have similar charge-to-size ratios, can thus be separated according to their molecular hydrodynamic size by capillary gel electrophoresis.

CHARACTERISTICS OF GELS

Two types of gel are used in capillary electrophoresis: cross-linked gels and entangled linear polymer solutions.

Cross-linked gels are prepared inside the capillary by polymerisation of the monomers. They are usually bonded to the fused silica wall and cannot be removed without destroying the capillary. If the gels are used for protein analysis under reducing conditions, the separation buffer usually contains sodium dodecyl sulfate and the samples are denatured by heating in a mixture of sodium dodecyl sulfate and 2-mercaptoethanol or dithiothreitol before injection. When non-reducing conditions are used (for example analysis of an intact antibody), 2-mercaptoethanol and dithiothreitol are not used. Separation in cross-linked gels can be optimised by modifying the separation buffer (as indicated in the capillary zone electrophoresis section) and controlling the gel pore size during the gel preparation. For cross-linked polyacrylamide gels, the pore size can be modified by changing the concentration of acrylamide and/or the proportion of cross-linker. As a rule, a decrease in the pore size of the gel leads to a decrease in the mobility of the solutes. Due to the rigidity of these gels, only electrokinetic injection can be used.

Entangled linear polymer solutions contain hydrophilic polymers, such as linear polyacrylamide, cellulose derivatives, dextran, etc., which can be dissolved in aqueous separation buffers giving rise to a separation medium that also acts as a molecular sieve. These separation media are easier to prepare than cross-linked polymers. They can be prepared in a vial and filled by pressure in a capillary. Replacing the gel before every injection generally improves the separation reproducibility. The dynamic pore size of the gels can be increased by using polymers of higher molecular mass (at a given

polymer concentration) or by decreasing the polymer concentration (for a given polymer molecular mass). A reduction in the gel dynamic pore size leads to a decrease in the mobility of the solute for the same buffer. Since the dissolution of these polymers in the buffer gives low viscosity solutions, both hydrodynamic and electrokinetic injection techniques can be used.

CAPILLARY ISOELECTRIC FOCUSING

PRINCIPLE

In isoelectric focusing, the molecules migrate under the influence of the electric field until they reach their isoelectric point, in a pH gradient generated by ampholytes having pI values in a wide range (poly-aminocarboxylic acids), dissolved in the separation buffer.

The three basic steps of isoelectric focusing are loading, focusing and, if needed, mobilisation.

Loading step. Two methods may be employed:

— loading in one step: the sample is mixed with ampholytes and introduced into the capillary either by pressure or vacuum;
— sequential loading: a leading buffer, then the ampholytes, then the sample mixed with ampholytes, again ampholytes alone and finally the terminating buffer are introduced into the capillary. The volume of the sample is kept small enough not to modify the pH gradient.

Focusing step. When the voltage is applied, ampholytes migrate toward the cathode or the anode, according to their net charge, thus creating a pH gradient from anode (lower pH) to cathode (higher pH). During this step the components to be separated migrate until they reach a pH corresponding to their isoelectric point (pI) and the current drops to very low values.

Mobilisation step. Unless imaging is used, mobilisation is required for detection, using one of the following three methods:

— in the first method, mobilisation is accomplished during the focusing step under the effect of the EOF; the EOF needs to be small enough to allow the focusing of the components ;
— in the second method, mobilisation is accomplished by applying positive pressure after the focusing step;
— in the third method, mobilisation is achieved after the focusing step by adding salts to the cathode reservoir or the anode reservoir (depending on the direction chosen for mobilisation) in order to alter the pH in the capillary when the voltage is applied. As the pH changes, the proteins and ampholytes are mobilised in the direction of the reservoir

which contains the added salts and pass the detector.

The separation achieved, expressed as ΔpI, depends on the pH gradient (dpH / dx), the number of ampholytes having different pI values, the molecular diffusion coefficient (D), the intensity of the electric field (E) and the variation of the electrophoretic mobility of the analyte with the pH ($-d\mu / dpH$):

$$\Delta pI = 3 \times \sqrt{((D\ (dpH / dx)) / (E\ (-d\mu / dpH)))}$$

OPTIMISATION

The main factors to be considered in the development of separations are:

Voltage. Capillary isoelectric focusing uses very high electric fields, 300 V/cm to 1000 V/cm in the focusing step.

Capillary. The EOF needs to be reduced or suppressed depending on the mobilisation strategy (see above). Coated capillaries tend to reduce the EOF.

Solutions. The anode buffer reservoir is filled with a solution with a pH lower than the pI of the most acidic ampholyte and the cathode reservoir is filled with a solution with a pH higher than the pI of the most basic ampholyte. Phosphoric acid for the anode and sodium hydroxide for the cathode are frequently used.

Addition of a polymer, such as methylcellulose, in the ampholyte solution tends to suppress convective forces (if any) and EOF by increasing the viscosity. Commercial ampholytes are available covering many pH ranges and may be mixed if necessary to obtain an expanded pH range. Broad pH ranges are used to estimate the isoelectric point whereas narrower ranges are employed to improve accuracy. Calibration can be done by correlating migration time with isoelectric point for a series of protein markers.

During the focusing step precipitation of proteins at their isoelectric point can be prevented, if necessary, using buffer additives such as glycerol, surfactants, urea or zwitterionic buffers. However, depending on the concentration, urea denatures proteins.

MICELLAR ELECTROKINETIC CHROMATOGRAPHY (MEKC)

PRINCIPLE

In micellar electrokinetic chromatography, separation takes place in an electrolyte solution which contains a surfactant at a concentration above the critical micellar concentration (*cmc*). The solute molecules are distributed between the aqueous buffer and the pseudostationary phase composed of micelles, according to the partition coefficient of the solute. The technique can therefore be considered as a hybrid of electrophoresis and chromatography. It is a technique that can be used for the separation of both neutral and charged solutes, maintaining the efficiency, speed and instrumental suitability of capillary electrophoresis. One of the most widely used

surfactants in MEKC is the anionic surfactant sodium dodecyl sulfate, although other surfactants, for example cationic surfactants such as cetyltrimethylammonium salts, are also used.

The separation mechanism is as follows. At neutral and alkaline pH, a strong EOF is generated and moves the separation buffer ions in the direction of the cathode. If sodium dodecyl sulfate is employed as the surfactant, the electrophoretic migration of the anionic micelle is in the opposite direction, towards the anode. As a result, the overall micelle migration velocity is slowed down compared to the bulk flow of the electrolyte solution. In the case of neutral solutes, since the analyte can partition between the micelle and the aqueous buffer, and has no electrophoretic mobility, the analyte migration velocity will depend only on the partition coefficient between the micelle and the aqueous buffer. In the electropherogram, the peaks corresponding to each uncharged solute are always between that of the EOF marker and that of the micelle (the time elapsed between these two peaks is called the separation window). For electrically charged solutes, the migration velocity depends on both the partition coefficient of the solute between the micelle and the aqueous buffer, and on the electrophoretic mobility of the solute in the absence of micelle.

Since the separation mechanism in MEKC of neutral and weakly ionised solutes is essentially chromatographic, migration of the solute and resolution can be rationalised in terms of the retention factor of the solute (k), also referred to as mass distribution ratio (D_m), which is the ratio of the number of moles of solute in the micelle to those in the mobile phase. For a neutral compound, k is given by:

$$k = (t - t_0) / (t_0 \times (1 - (t / t_{mc}))) = K (V_S / V_M)$$

t = migration time of the solute,
t_0 = migration time of an unretained solute (determined by injecting an EOF marker which does not enter the micelle, for instance methanol),
t_{mc} = micelle migration time (measured by injecting a micelle marker, such as Sudan III, which migrates while continuously associated in the micelle),
K = partition coefficient of the solute,
V_S = volume of the micellar phase,
V_M = volume of the mobile phase.

Likewise, the resolution between two closely-migrating solutes (R_s) is given by:

$$R_s = (\sqrt{N} / 4) \times ((\alpha - 1) / \alpha) \times (k_b / (k_b + 1)) \times ((1 - (t_0 / t_{mc})) / (1 + (t_0 / t_{mc}) \times k_a))$$

N = apparent number of theoretical plates for one of the solutes,
α = selectivity,
k_a and k_b = retention factors for both solutes, respectively ($k_b > k_a$).

Similar, but not identical, equations give k and R_s values for electrically charged solutes.

OPTIMISATION

The main factors to be considered in the development of separations by MEKC are instrumental and electrolyte solution parameters.

Instrumental parameters

Voltage. Separation time is inversely proportional to applied voltage. However, an increase in voltage can cause excessive heat production that gives rise to temperature gradients and viscosity gradients of the buffer in the cross-section of the capillary. This effect can be significant with high conductivity buffers such as those containing micelles. Poor heat dissipation causes band broadening and decreases resolution.

Temperature. Variations in capillary temperature affect the partition coefficient of the solute between the buffer and the micelles, the critical micellar concentration and the viscosity of the buffer. These parameters contribute to the migration time of the solutes. The use of a good cooling system improves the reproducibility of the migration time for the solutes.

Capillary. As in capillary zone electrophoresis, the dimensions of the capillary (length and internal diameter) contribute to analysis time and efficiency of separations. Increasing both effective length and total length decreases the electric field (working at constant voltage), increases migration time and can improve the separation efficiency. The internal diameter controls heat dissipation (for a given buffer and electric field) and consequently the sample band broadening.

Electrolyte solution parameters

Surfactant type and concentration. The type of surfactant, in the same way as the stationary phase in chromatography, affects the resolution since it modifies separation selectivity. Also, the log k of a neutral compound increases linearly with the concentration of surfactant in the mobile phase. Since resolution in MEKC reaches a maximum when k approaches the value of $\sqrt{(t_{mc} / t_0)}$, modifying the concentration of surfactant in the mobile phase changes the resolution obtained.

Buffer pH. Although pH does not modify the partition coefficient of non-ionised solutes, it can modify the EOF in uncoated capillaries. A decrease in the buffer pH decreases the EOF and therefore increases the resolution of the neutral solutes in MEKC, resulting in a longer analysis time.

Organic solvents. To improve MEKC separation of hydrophobic compounds, organic modifiers (methanol, propanol, acetonitrile, etc.) can be added to the electrolyte solution. The addition of these modifiers can influence the migration time and the selectivity of the separation. Since the addition of organic modifiers affects the critical micellar concentration, a given surfactant concentration can be used only within a certain percentage of organic modifier before the micellisation is inhibited or adversely affected,

resulting in the absence of micelles and, therefore, in the absence of partition. The dissociation of micelles in the presence of a high content of organic solvent does not always mean that the separation will no longer be possible; in some cases the hydrophobic interaction between the ionic surfactant monomer and the neutral solutes forms solvophobic complexes that can be separated electrophoretically.

Additives for chiral separations. For the separation of enantiomers using MEKC, a chiral selector is included in the micellar system, either covalently bound to the surfactant or added to the micellar separation electrolyte. Micelles that have a moiety with chiral discrimination properties include salts of *N*-dodecanoyl-L-amino acids, bile salts, etc. Chiral resolution can also be achieved using chiral discriminators, such as cyclodextrins, added to the electrolyte solutions which contain micellised achiral surfactants.

Other additives. Several strategies can be carried out to modify selectivity, by adding chemicals to the buffer. The addition of several types of cyclodextrins to the buffer can also be used to reduce the interaction of hydrophobic solutes with the micelle, thus increasing the selectivity for this type of compound.

The addition of substances able to modify solute-micelle interactions by adsorption on the latter, is used to improve the selectivity of the separations in MEKC. These additives may be a second surfactant (ionic or non-ionic) which gives rise to mixed micelles or metallic cations which dissolve in the micelle and form co-ordination complexes with the solutes.

QUANTITATION

With the exception of capillary isoelectric focusing, peak areas are typically divided by the corresponding migration time to give the corrected area in order to:

— compensate for the shift in migration time from run to run, thus reducing the variation of the response,
— compensate for the different responses of sample constituents with different migration times.

Where an internal standard is used, verify that no peak of the substance to be examined is masked by that of the internal standard.

CALCULATIONS

From the values obtained, calculate the content of the component or components being examined. When prescribed, the percentage content of one or more components of the sample to be examined is calculated by determining the corrected area(s) of the peak(s) as a percentage of the total of the corrected areas of all peaks, excluding those due to solvents or any added reagents (normalisation procedure). The use of an automatic integration system (integrator or data acquisition and processing system) is recommended.

SYSTEM SUITABILITY

In order to check the behaviour of the capillary electrophoresis system, system suitability parameters are used. The choice of these parameters depends on the mode of capillary electrophoresis used. They can include: retention factor (k) (only for micellar electrokinetic chromatography), apparent number of theoretical plates (N), symmetry factor (A_s) and resolution (R_s). In previous sections, the theoretical expressions for N and R_s have been described, but more practical equations that allow these parameters to be calculated from the electropherograms are given below.

APPARENT NUMBER OF THEORETICAL PLATES

The apparent number of theoretical plates (N) may be calculated using the expression:

$$N = 5.54\ (t / w_h)^2$$

t = migration 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,
w_h = width of the peak at half-height.

RESOLUTION

The resolution (R_s) between peaks of similar height of two components may be calculated using the expression:

$$R_s = ((1.18\ (t_2 - t_1)) / (w_{h1} + w_{h2}))$$

$$t_2 > t_1$$

t_1 and t_2 = migration times or distances along the baseline from the point of injection to the perpendiculars dropped from the maxima of two adjacent peaks,
w_{h1} and w_{h2} = peak widths at half-height.

When appropriate, the resolution may be calculated by measuring the height of the valley (H_v) between two partly resolved peaks in a standard preparation and the height of the smaller peak (H_p) and calculating the peak-to-valley ratio:

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

SYMMETRY FACTOR

The symmetry factor (A_s) of a peak may be calculated using the expression:

$$A_s = w_{0.05} / 2d$$

$w_{0.05}$ = 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.

REPEATABILITY

Tests for area repeatability (standard deviation of areas or of the area/migration-time ratio) and for migration time repeatability (standard deviation of migration time) are introduced as suitability parameters. Migration time repeatability provides a test for the suitability of the capillary washing procedures. An alternative practice to compensate for the lack of repeatability of the migration time is to use migration time relative to an internal standard.

SIGNAL-TO-NOISE RATIO

The detection limit and quantitation limit can be estimated using signal-to-noise ratios of 3 and 10 respectively. The signal-to-noise ratio (*S*/*N*) is calculated using the expression:

$$S / N = 2H / h$$

H = height of the peak corresponding to the component concerned, in the electropherogram 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 twenty times the width at half-height unless otherwise justified,
h = range of the background in an electropherogram obtained after injection of a blank, observed over a distance equal to twenty times the width at the half-height of the peak unless otherwise justified, in the electropherogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

A test for the verification of the signal-to-noise ratio for a standard preparation (or the determination of the limit of quantitation) may also be useful for the determination of related substances.

ADJUSTMENTS OF OPERATING CONDITIONS FOR CAPILLARY ELECTROPHORESIS

The operating conditions described in the pharmacopoeial procedures were validated during the elaboration of the monograph. Compliance with the system suitability criteria is required to verify that conditions for satisfactory performance of the test or assay are achieved.
Capillary length may be adjusted to suit the individual dimensions of the capillary electrophoresis instrument used. If a capillary with a different length is used, its suitability for use should be verified.

If the intended analytical performance is not met either due to the instrument or to different capillary lengths, then:
- the voltage,
- rinsing conditions,
- temperature settings,
- refreshment frequency of the electrolyte solution at the inlet and outlet,
- and injection conditions

may be adjusted to satisfy the analytical performance, provided these adjustments are properly evaluated by the users and do not fundamentally modify the pharmacopoeial procedures. Additional verification tests and/or revalidation for any adjustments made may be required.
No further changes are permitted, unless otherwise prescribed in the individual monograph or justified.