Pergamon

0731-7085(93)E0012-C

Journal of Pharmaceutical & Biomedical Analysis, Vol. 12, No. 5, pp. 579–611, 1994

0731-7085/94 $7.00 + 0.00

Review

Capillary zone electrophoresis in pharmaceutical and biomedical analysis

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Abstract: Capillary zone electrophoresis is a technique that is rapidly gaining popularity in the pharmaceutical industry because of the wide range of compounds applicable to the technique. These range from small biologically active molecules through to large nucleotides and proteins. This review outlines the historical development of the technique and some of the basic theory of capillary zone electrophoresis. This is followed by an explanation of some of the adaptations now being used to enable the separation of neutral molecules as well as charged species, and ways of achieving chiral selectivity.

Keywords: *Capillary zone electrophoresis (CZE); electroosmotic flow (eof); micellar electrokinetic chromatography (MEKC); electrochromatography; chiral recognition.*

Historical Background

Electrophoresis has made many advances since its introduction in 1931 [1], but has always lacked the ability of being automated, and was generally considered inefficient and lacking sensitivity. Problems associated with conventional electrophoresis have been well documented [2]. Moving-boundary electrophoresis involves the migration of ionized components with a sharp boundary front where each solute is migrating at different rates. However, this technique is inherently inefficient due to problems associated with convection. During electrophoresis in free buffer solution, heat produced by the electric field (Joule heating) causes the electrolyte density to lower, and thus causes convection resulting in loss of resolution. Attempts to minimize the effects of convection led to the introduction of zone electrophoresis, and the use of stabilisers to suppress the convection process. Stabilisers included polymer gels, powdered cellulose and plastic, and all have been used successfully to limit convection due to the effect of Joule heating. As a result of this it was possible to use higher potentials resulting in increased velocities (shorter analysis time) and higher efficiencies due to reduced zone dispersion. However, these stabilisers have their own problems such as solute–matrix interactions, and eddy migration along the capillary channels formed by the stabilisers. In some instances, the stabilisers themselves are unstable, causing a breakdown in the electrophoresis process. Hjerten [3] described the use of free zone electrophoresis with high electric fields where he attempted to eliminate the effects of convection by conducting the electrophoresis in a 3 mm i.d. tube, that he then rotated about its longitudinal axis. However, the complexity of the equipment was a major stumbling block to its development. Kolin [4] also studied the effect that rotating small tubes had on convection caused within a liquid by temperature gradients. He found that if tubes containing liquid are rotated at a rate at which the centripetal acceleration is small compared to the acceleration due to gravity, then thermal convection in the presence of steep tempera-

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ture gradients is suppressed. Other workers [5, 6] subsequently were able to demonstrate zone electrophoresis in small capillaries (~200 μm i.d.) but the high efficiencies predicted were still not realised. It was the pioneering work of Jorgenson and Lukacs [7–9] that finally demonstrated the enormous impact that free solution electrophoresis (performed in small diameter capillaries) was to have in the analytical laboratory. In their papers, Jorgenson and Lukacs predicted theoretically that analysis time is proportional to the square of the tube length, and inversely proportional to the applied voltage, namely,

$$t = \frac{L^2}{\mu V}, \qquad (1)$$

where t = time required for a zone to migrate the length of tube L, μ = electrophoretic mobility and V = total applied voltage.

It is important to note that there are two capillary lengths worth considering, L_d and L_t, where: L_d = the length of capillary from the inlet to the detector and L_t = the total length of capillary.

Equation (1) can therefore be expressed as follows:

$$t = \frac{L_t L_d}{\mu V}. \qquad (2)$$

Although measured separations only occur in the capillary length L_d, the field strength is measured by dividing the voltage by the total length of capillary L_t. Since in most instances the differences between L_d and L_t are negligible, $L_t L_d$ reduces to L^2.

Efficiency, defined as a plate value, is given by an equation developed from a theory by Giddings [10], namely,

$$N = \frac{\mu V}{2D}, \qquad (3)$$

where N = separation efficiency of a solute (plate number), V = applied voltage and D = diffusion coefficient of the solute.

It follows from equations (1) and (2) that the highest efficiencies and shortest analysis times will be achieved by applying high voltages. Although undesirable temperature gradients might be expected arising from an inability to dissipate the heat generated as a result of these high applied voltages, Jorgenson and Lukacs demonstrated that this phenomenon could be reduced to acceptable levels simply by reducing the capillary diameter even further than that used by Mikkers and coworkers [5]. Using capillaries of 75 μm internal diameter, they were able to work at 30 kV resulting in high efficiency separations in very short analysis times. An important feature of the separations revealed by Jorgenson and Lukacs, is the effect of electroosmosis. Electroosmosis has been widely discussed [11–13] and is described by Pretorious [11] as 'the flow of liquid in contact with a solid surface under the influence of a tangentially applied electric field'. This phenomenon will be discussed further under the heading of theory. Jorgenson and Lukacs [7] gave examples of the separation of dansylated amino acids and also fluorescamine derivatives of some dipeptides. It was pointed out that although under the operating conditions used (pH 7.0) all analytes were negatively charged, they were detected at the cathode, and this is as a result of a strong electroosmotic flow in the direction of the negative electrode. Also as a result of this electroosmotic flow, the elution order for analytes detected at the cathode, will be cations, followed by neutral molecules, followed by anions. However, since all neutral compounds will travel towards the cathode with the same velocity, determined by the electroosmotic flow, they will not be separated.

As high-performance capillary zone electrophoresis developed, Terabe [14] introduced the technique of micellar electrokinetic chromatography (MEKC), which extended capillary zone electrophoresis to include the separation of neutral species via the inclusion of micelles into the carrier electrolyte. A further extension to this principle saw the introduction of chiral separations by means of chiral additives [15–20], and examples of these will be given later in this review. Because of the small sample requirements, on-column detection, and the fact that stabilisers are not necessary, high performance capillary zone electrophoresis has proved to be a technique which is very amenable to automation. Thus in the late 1980s a number of instrument manufacturers have produced equipment enabling the automated analysis of a diverse range of analytes from simple anions, cations, small pharmaceutical molecules through to biopolymers.

Basic Theory of Capillary Zone Electrophoresis

The basic theory of CZE has been adequately described by Jorgenson and Lukacs [7–9] and Jorgenson [21]. When an electric field is applied across a capillary filled with an ionic solution, anions are found to migrate towards the anode, whilst cations move towards the cathode. The velocity of migration, V_{ep}, is related to the potential field strength, E, by the equation:

$$V_{ep} = \mu_{ep}E, \tag{4}$$

where μ_{ep} is the electrophoretic mobility of a solute.

This equation can also be expressed as:

$$V_{ep} = \frac{\mu_{ep}V}{L}, \tag{5}$$

where L is the effective length of the capillary.

The time taken for a solute to migrate through the capillary under an applied voltage is given by:

$$t = \frac{L}{V_{ep}} = \frac{L^2}{\mu_{ep}V}. \tag{6}$$

This equation predicts that short columns and high voltages will lead to fast analysis times. As a solute migrates through a capillary, molecular diffusion occurs, and assuming that this is the only source of band broadening taking place, then the peak dispersion σ^2, is given by the following equation:

$$\sigma^2 = 2Dt = \frac{2DL^2}{\mu_{ep}V}, \tag{7}$$

where D = diffusion coefficient of the solute.

Using the equation derived by Giddings [10] for plate efficiency, the number of theoretical plates is given by:

$$N = \frac{L^2}{\sigma^2} = \frac{\mu_{ep}V}{2D}. \tag{8}$$

Equations (5) and (7) predict that operation at high voltages will yield highly efficient analyses within very short run times. One interesting feature of the plate number equation is that the efficiency appears to be independent of the capillary length. However, if large voltages are applied to very short capillaries, zone broadening can occur due to the formation of temperature gradients arising from Joule heating effects. Virtanen [6] showed that there is an optimum voltage for the maximum plate number, and this is dependent on the conductivity of the carrier electrolyte and the diameter of the capillary. The dissipation of heat from small diameter capillaries is found to be efficient due to the high surface-to-volume ratio. It is also worth noting from equation (7) that CZE is particularly suited to the analysis of large molecules such as biopolymers since they have low diffusion coefficients leading to large plate numbers. This is in contrast to HPLC where low diffusion coefficients lead to low efficiencies.

Electroosmosis

The equations previously used to describe the efficiency and time of analysis, are only valid in the absence of electroosmosis. This fundamental process occurs during electrophoresis due to the surface charge at the walls of the capillary, called the zeta potential. This results from the ionization of the free silanol groups present on the surface of fused-silica capillaries. The degree of ionization of these groups is dependent upon the pH of the electrolyte. The effect of the zeta potential on the electroosmotic flow (eof) was first described by Pretorius [11] and is given by the equation:

$$V_{eo} = \frac{\epsilon\zeta E}{4\pi\eta}, \tag{9}$$

where ϵ = the dielectric constant of the electrolyte, ζ = the viscosity of the electrolyte, and η = the zeta potential.

As a result of the negatively charged ions, an electrical double layer is formed and when a voltage is applied across the capillary, hydrated cations in the diffuse layer migrate towards the cathode. This bulk flow of liquid is called electroosmosis or electroomsotic flow, and at pH values at or above neutral, is sufficiently great to cause the movement of cations, neutral species and anions towards the negatively charged electrode (see Fig. 1).

One striking feature of electroosmotic flow in a capillary is that it is plug-like and does not have the characteristic parabolic flow profile associated with pressure driven liquids, as illustrated in Fig. 2.

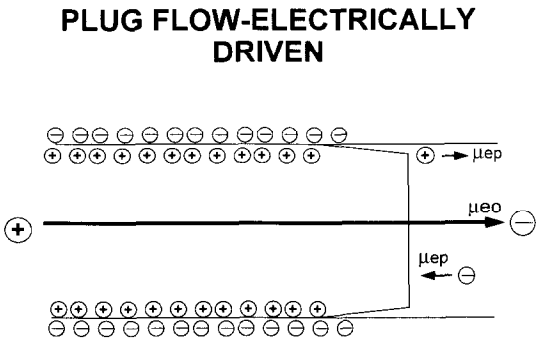


Figure 1
Source of electroendosmotic flow.

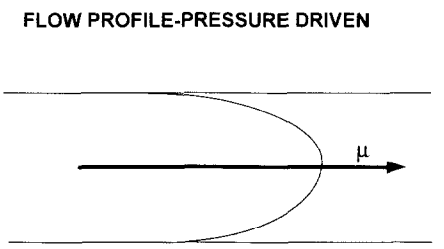


Figure 2
Flow profile in a pressure driven system.

When electroosmotic flow is taken into account, the equation for the migration time becomes:

$$t = \frac{L^2}{(\mu + \mu_{osm})V} \tag{10}$$

and that for the separation efficiency becomes:

$$N = \frac{(\mu + \mu_{osm})V}{2D}, \tag{11}$$

where μ_{osm} = electroosmotic flow coefficient.

Jorgenson and Lukacs [8, 21, 22] went on to derive an equation describing the resolution of two zones in capillary zone electrophoresis, based on an approach by Giddings [10], namely,

$$R_s = 0.177\ (\mu_1 - \mu_2)[V/D\ (\bar{\mu} + \mu_{osm})], \tag{12}$$

where R_s = resolution, μ_1 and μ_2 = electrophoretic mobilities of the two solutes, and $\bar{\mu}$ = average mobility of the two solutes.

From equations (9) and (10) it can be seen that lower values of μ_{osm} will give rise to high separation efficiencies with short analysis times, however, as pointed out by Wallingford and Ewing [23], the longer ionic species spend in the capillary, the greater the separation power of the system. Large values of electroosmotic flow result in all solutes being swept through the capillary very rapidly, with very little chance of resolution. Equation (11) predicts that for good resolution, μ_{osm} should in fact be small and in order to exploit small differences in the mobilities of solutes, the electroosmotic flow should be equal and opposite to the electrophoretic mobility, i.e.

$$\mu_{osm} = -\mu. \tag{13}$$

However, it is apparent when studying equation (9) that the trade off for increased resolution under these circumstances is a marked increase in analysis time.

Manipulation of electroosmotic flow

Electroosmotic flow can be manipulated by a variety of means, the vast majority of which involve the use of additives to the electrolyte in order to alter the zeta potential between the capillary wall and the bulk liquid interface. Since the zeta potential determines the electroosmotic flow and the direction of electroosmosis, manipulation of the sign and density of net charge at the capillary–liquid interface will have a bearing on the electroosmotic flow. Yoshida *et al.* [24] studied the effect of decyltrimethylammonium bromide (DeTAB), dodecyltrimethylammonium bromide (DoTAB) and tetradecyltrimethylammonium bromide (TTAB) on electroosmotic flow. They reported that the electroosmotic flow could be manipulated by the use of long chain surfactants and noted that a reversal of electroosmotic flow was possible with DoTAB and TTAB at concentrations below their critical micelle concentration (cmc). Long chain cationic surfactants have the added advantage of suppressing the electroosmotic flow at low concentrations and therefore result in much lower conductivity of the background electrolyte. Landers *et al.* [25] used diamino alkanes (chain lengths between 3 and 8) to modify electroosmotic flow, primarily as a result of a wall-coating effect. Belder and Schomburg [26] used low concentrations of polyvinyl alcohol and hydroxyethylcellulose to coat the surface of capillaries to manipulate the electroosmotic flow and in addition suppress solute–capillary surface interactions. By covalently bonding a quaternary ammonium compound to a fixed silica surface, and then further modifying the

surface with a hydrophilic layer of polyether chains, Smith and Rassi [27] were able to produce a surface capable of having both negative and positive charged groups, which by varying the pH of the electrolyte it was possible to manipulate both the size and direction of electroosmotic flow. These were referred to as switchable flow capillaries. Additionally, the long polyether chains were effective in reducing interactions between solutes and free silanol groups, and the positively charged quaternary ammonium sub-layer. Schomburg [28, 29] studied the effect of surface coatings on electroosmotic flow and the subsequent resolution of purine derivatives and nucleobases. Under CZE conditions the hydrophobic polymethylsiloxane phase, and to a lesser extent the hydrophilic polyoxyethylene glycol phase, will mask the capillary wall charge and reduce the electroosmotic flow. However, under MEKC conditions, adsorbtion of SDS onto the hydrophobic phase will result in an increase in the zeta potential and a corresponding increase in the electroosmotic flow with a reduction in the analysis time. The more polar polyoxyethylene glycol phase under the same conditions decreased the electroosmotic flow leading to longer analysis times with greater resolution. Yeung *et al.* [30] coated 10 μm fused silica capillaries with a cross-linked polymer in order to provide a non-extractable reversed-phase surface, and then induced a strong electroosmotic flow by using the surfactant CTAB in the electrolyte at a concentration less than the critical micelle concentration. Because CTAB adsorbs very effectively to the polymer surface (much more so than onto bare silica) the direction of electroosmotic flow was shown to reverse at concentrations as low as 1 μM. Regnier [31] studied electroosmotic flow in capillaries that were firstly derivatised with an octadecylsilane group, and then coated with a hydrophilic absorbed layer of non-ionic surfactant. This hydrophilic surface is capable of shielding proteins from the negatively charged silanol groups, resulting in very low electroosmotic flow rates that change very little over the pH range 4–11. This allows pH to be used to optimize selectivity without greatly affecting the electroosmotic flow. Lunte *et al.* [32] modified the capillary surface with a sulphonic acid functionality producing a capillary in which the eof is both constant and high even at low pH values. This allows separation of analytes based on their relative pK_as without the effect of pH on eof. Another way of altering electroosmotic flow is by the addition of organic modifiers [33–35]. Basically organic modifiers used in MEKC will lower the eof by producing changes in the zeta potential, as well as changing the viscosity and dielectric constant of the buffer. The net effect of this is to extend the elution range of the system.

External control of electroosmotic flow

Means by which the electroosmotic flow can be manipulated 'internally' have already been described, for instance, coating the inner walls of the capillary, adding surfactants, and altering the pH. Of these methods pH has the most dramatic effect on eof, and also plays a considerable part in the optimization process. With some analytes it is desirable to perform analysis at a low pH, however because eof is very low and non-reproducible at pH <4.0, unreliable analyses with long run times can be encountered. The ability to externally control eof might be expected to lead to faster, more reliable analysis. By applying an external radial field it is possible to control the size and magnitude of the zeta potential and therefore the direction and rate of electroosmotic flow. The use of external applied fields to control electroosmotic flow have been reported by many workers [36–44]. Lee *et al.* [36] achieved their external field by sheathing the working capillary with an outer capillary, and filling the annular space with an electrolyte across which an electric potential was applied, thus regulating the zeta potential. Ewing and workers [41] sheathed their capillary with Naflon and applied a radial voltage across the capillary in order to manipulate the eof. Because not all of the capillary is sheathed, parts of the capillary would be expected to be unaffected by the radial voltage, and this could possibly lead to a difference in flow at the capillary wall between the sheathed and unsheathed sections of the capillary. A laminar flow profile might be expected (as against plug-flow) with corresponding band broadening. However, Ewing and co-workers found that to the contrary the control of eof using a conductive sheath, which does not cover the whole of the capillary, appeared to slightly improve the efficiency, suggesting that plug-flow is in fact still maintained even in the region where the capillary is not covered by the Naflon sheath. More recently Ewing and workers [43] demonstrated that it was possible to control eof simply by

applying a voltage across a 4 cm steel plate attached to the capillary. Contrary to previous expectations, this was enough to induce a radial field along the whole length of the capillary. Although Belder and Schomburg [26] were able to demonstrate the modification of eof both by surface modification and by the use of an external radial field, for the analysis of tocainide type aromatic amines, the chemically modified silica surfaces gave rise to much higher efficiencies since in addition to suppressing electroosmotic flow, they also suppressed any undesirable surface adsorption of the analytes.

Micellar electrokinetic chromatography (MEKC)

During free solution capillary zone electrophoresis all analytes are swept towards the cathode as a result of the electroosmotic flow and can remain unresolved. To overcome this problem, Terabe [14] introduced the concept of micellar electrokinetic chromatography (MEKC) where he used a surfactant, in this instance sodium dodecyl sulphate, above its critical micelle concentration in order to form what he described as a 'pseudo-stationary phase' analogous to the stationary phase in HPLC, see Fig. 3.

In this form of MEKC, the micelle is negatively charged and will migrate towards the anode. However, the electroosmotic flow in such a system is towards the cathode, and because this flow is predominant, the micelles will also travel towards the cathode at a velocity lower than the velocity of the bulk liquid. A solute that is totally solubilized by the micelle will travel with the same velocity as the micelle and will be eluted at a time t_{mc}, whereas a solute which is not incorporated into the micelle at all will be transported to the cathode by the eof and will elute at a time t_o. All other neutral analytes will elute between

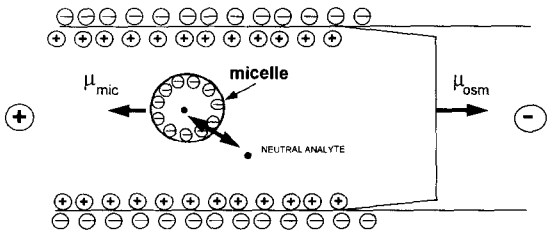


Figure 3
Principle of micellar electrokinetic chromatography.

times t_o and t_{mc} depending on their relative hydrophobicities, the more hydrophobic species being retained more than hydrophilic species.

It is possible to measure t_o with a marker such as methanol or mesityl oxide, whereas t_{mc} can be measured with Sudan III or Sudan IV. Terabe and workers derived the following equation for the capacity factor in MEKC:

$$k' = \frac{t_r - t_o}{t_o(1 - t_r/t_{mc})}, \qquad (14)$$

where t_r = retention time of analyte.

The resolution of two closely related peaks was described by Otsuka [45] as

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'}{1 + k'}\right)\left(\frac{1 - (t_o/t_{mc})}{1 + (t_o/t_{mc})k'}\right), \qquad (15)$$

where N = number of theoretical plates, and α = separation factor.

If t_{mc} becomes very large and approaches infinity, then the fourth part of this equation becomes unity and the resolution equation for MEKC is identical to that for conventional chromatography.

Whilst Lukacs and Jorgenson [46] studied the effect of pH on electroosmotic velocity in CZE, it was Otsuka and Terabe [45] who studied the corresponding effects in the presence of micelles. A solute which is not solubilised by the micelle will migrate at the electroosmotic flow velocity V_{eo}, whereas a solute completely solubilized by the micelle with travel at the same velocity as the micelle V_{mc}. If the electrophoretic velocity of the micelle is V_{ep}, then the net migration velocity of the micelle V_{mc} is given by

$$V_{mc} = V_{eo} + V_{ep}. \qquad (16)$$

Note that the sign of the migration direction is such that species migrating towards the negative electrode are defined as positive, and those migrating towards the positive electrode are negative. In their studies Otsuka and Terabe noted that in the pH range 5.5–7.0, the electroosmotic velocity V_{eo} changed very little, however below pH 5.0 the value of V_{eo} dropped sharply. In contrast, the electrophoretic velocity of the negatively charged micelle V_{ep} was almost constant over the pH range

3.0–7.0. Therefore as the pH is lowered below 5.0, V_{mc} turns from positive to negative and this denotes a change in migration direction towards the positive electrode. Rasmussen and McNair [47] demonstrated the effect of pH on elution order using a series of Parabens. They demonstrated a total reversal of elution order on lowering the pH from 7.0 to 3.37. Although Terabe introduced the concept of using a micelle in CZE in order to resolve neutral species, their presence can have an effect on the selectivity of charged as well as uncharged species.

Nishi *et al.* [48] described the separation of penicillin and cephalosporin antibiotics using MEKC with SDS and sodium N-lauroyl-N-methyl taurate (LMT). Retention time behaviour when using SDS was explained by, among other things, the number of negatively charged groups on the solute, the lipophilicity of the solute, and any ion-pair formation between the anionic surfactant and the cationic groups of the solute. Similar results were observed using LMT as surfactant. However, Otsuka [49] and workers using chlorinated phenols, noted that the capacity factor decreased with an increase of pH, and this was attributed to an increase in repulsion between the partly ionised phenols and the SDS micelle. In addition to using micelles in order to resolve neutral species it is possible to impart a charge on to a neutral analyte by solvophobic association. Jorgenson [50] demonstrated this by using tetrahexylammonium perchlorate in order to resolve five aromatic hydrocarbons. It was shown that the longer, more non-polar solutes formed the strongest association with the tetraalkylammonium ion and consequently eluted first. A similar effect was demonstrated by Ewing [51] who complexed neutral catechols with borate ion to form charged species that could now be resolved due to the different electrophoretic mobilities of the charged borate complexes. Nishi *et al.* [52] noted the effect of adding tetraalkylammonium salts to SDS, and found that certain ionic compounds that could not be resolved by either CZE or classical MEKC, could be resolved when TAA and SDS were used together.

Although in MEKC SDS is probably the most commonly used surfactant, applications including cationic surfactants, zwitterionic surfactants, and bile salts, as well as chiral micelles and complexing agents have been cited in the literature and will be discussed later in this article.

Instrumentation and Principles of Operation

There are many instruments that are available commercially, all of which can be represented by the schematic diagram shown in Fig. 4.

Power supply

This should be a reversible polarity unit capable of producing 30 kV. All commercial instruments have safety interlocks that prevent the user coming into contact with this power.

Injection methods

There are two basic modes of injection.

(i) Hydrodynamic. In this method, the capillary is placed in the sample solution which is then raised to a certain height for a given time, whereby the sample flows into the capillary. The amount of sample entering the capillary depends upon the viscosity of the liquid and is generally considered the least reproducible of the injection methods. Most commercial instruments use either a pressure or a vacuum as a means of introducing sample into the capillary, and this is much more controllable. In the case of vacuum injection, the capillary is dipped into the sample solution, and the buffer reservoir at the detector end is evacuated and the sample is then sucked into the capillary for a carefully controlled period at a regulated vacuum. With the pressure injection system, the sample is forced into the capillary by use of a precisely controlled external pressure device. Both of these systems are capable of introducing precisely reproducible volumes of sample into the capillary.

(ii) Electrokinetic injection. Here the analytical capillary is placed in the sample solution and a small voltage applied to cause the sample to flow into the capillary. Following this sample introduction, the capillary inlet is returned to the running buffer and electrophoresis is commenced. The only problem with this type of injection is sample discrimination, since the components of the samples with the highest mobility will enter the capillary first.

Capillaries

Capillaries generally consist of fused-silica

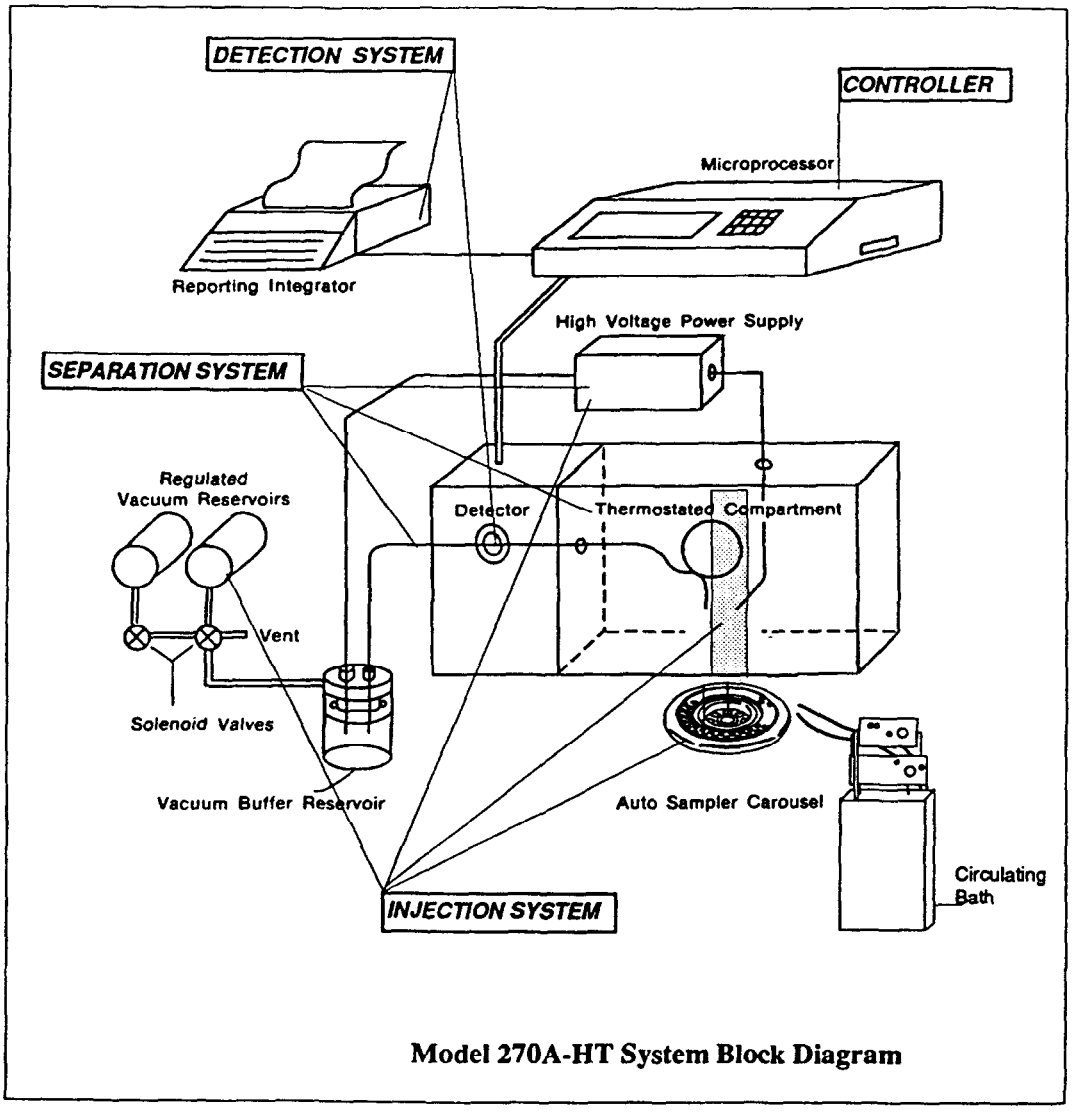


Figure 4
Block diagram of commercial CZE equipment (by permission of Applied Biosystems Ltd).

protected by a polyimide coating. The most common internal diameters are in the range 50–100 μm. In order to perform on-line solute detection, a small window is burnt at the appropriate position, usually by cautiously placing the capillary into a small flame.

Temperature control

Temperature control is very important in CZE in order to achieve reproducibility. The viscosity of electrolyte solutions change with temperature and this can have a dramatic affect upon retention time reproducibility. Apart from controlling the temperature of the capillary, usually between 30 and 90°C, it is possible with some manufacturers' instruments to control the temperature of the autosampler; this is particularly important if the thermal stability of samples is in doubt. There are some instruments that allow control of the capillary temperature down to approximately 15°C, and this could have implications in the area of chiral analysis where resolution often increases with a decrease in temperature [53, 54].

Detection

Although there are other detectors available for CZE — as in HPLC — by far the most common detector is the variable wavelength UV detector. However, because the cell path length is in effect the diameter of the capillary, this is typically only 50–100 μm, resulting in a lack of sensitivity. However, because the path length is so small, the lack of sensitivity can

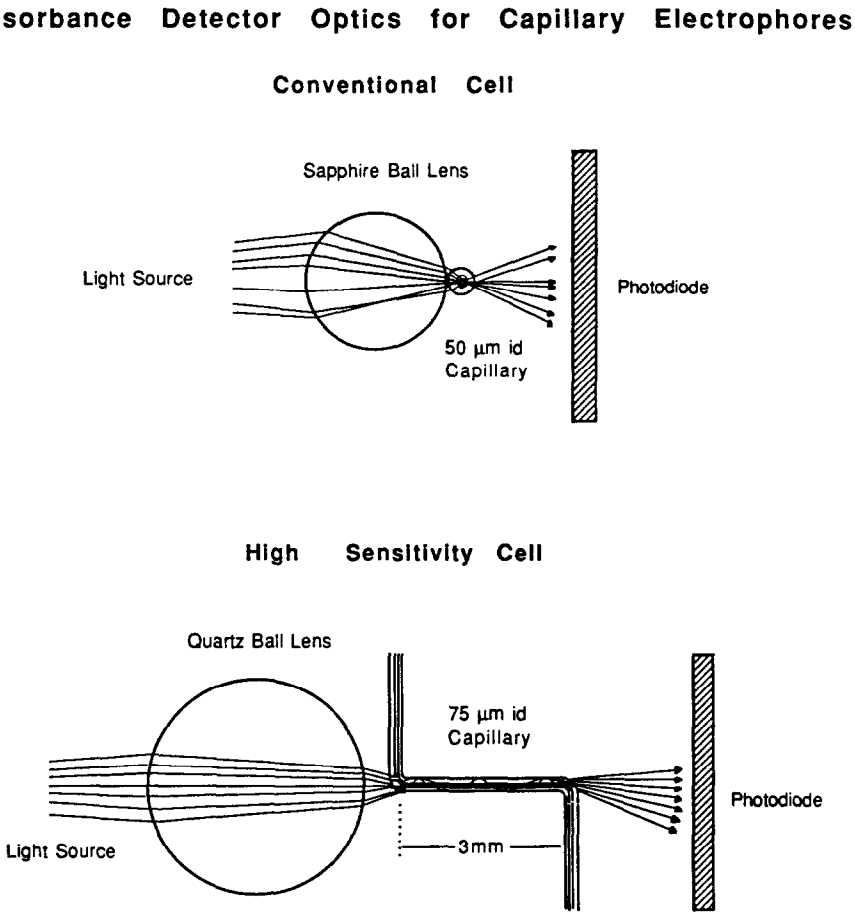


Figure 5
High sensitivity Z-cell (by permission of Applied Biosystems Ltd).

often be offset by detecting at low wavelength in the region of 190–200 nm. Another approach to increasing sensitivity is by the use of a Z-cell (see Fig. 5). This effectively increases the path length many times and as a result the sensitivity is increased by between 10 and 15 times. However some of the expected efficiency is lost as a result of dispersion within the cell.

Fluorescence and laser induced detectors are available but the latter tend to be expensive. They do have the advantage however of being many times more sensitive than UV detectors, but since not all compounds fluoresce naturally, it is necessary to derivatize inactive solutes with a fluorescent reagent and this can also produce problems.

Ease of operation

All major commercial instruments are microprocessor controlled, although some are more sophisticated than others. Some have small autosamplers (or none at all) whereas others are capable of running up to 100 samples unattended. Also it is possible with some instruments to replenish the electrolyte at both the sample and detector side of the capillary at regular intervals during a long unattended run. The ability to change the electrolyte in the capillary regularly, and in the case of MEKC, also to undertake regular caustic washes, is very important for reproducible analyses and most instruments are capable of being programmed to do this automatically. Examples of the parameters usually entered into a run file are as follows:

(i) wavelength, rise time, sensitivity;
(ii) caustic rinse time (important in MEKC);
(iii) electrolyte rinse time;
(iv) Sample injection type, e.g. vacuum or electro-injection, time of injection;
(v) voltage (include polarity);
(vi) temperature;
(vii) analysis run time;

(viii) sample numbers (if using an autosampler).

Once these parameters have been entered into the control system, it is then possible to run the samples unattended. Where the instrument is connected to a modern data handling system, electropherograms can be captured and presented with the corrected analytical data.

Pharmaceutical and Biochemical Applications

Capillary electrophoresis has already found application across the full spectrum of pharmaceutical and biomedical analysis. In this section the major areas of application will be discussed.

(a) Peptides and proteins

The equation for separation efficiency given in section 1 is as follows:

$$N = \frac{\mu_{ep} V}{2D}, \qquad (17)$$

where N = separation efficiency, measured as a plate number for a particular solute, V = applied voltage, μ_{ep} = electrophoretic mobility of the solute and D = diffusion coefficient of the solute.

With large molecules such as high molecular weight proteins, D is small which means that very high efficiency separations can be achieved. Although classical electrophoresis has been the traditional separation method for large biological molecules, difficulties are encountered because proteins can adsorb onto the surface of fused silica. As a consequence, a considerable effort has gone into the quest for ways of overcoming this problem. One way of tackling the problem is to use a running buffer at a pH higher than that of the iso-electric point of the protein, whereby both the capillary surface and the protein carry negative charges, so that coulombic repulsion will take place thus minimising adsorption of the protein onto the capillary wall. Another approach is to deactivate the capillary surface. Hjerten [55] reported the bonding of α-methacryloxypropyltrimethoxysilane to the surface of fused silica in order to eliminate electroosmosis. This in turn sterically prevented analytes from interacting with the capillary wall, reducing adsorption. Since the work reported by Hjerten, there have been many papers describing variants of this approach. Bruin and coworkers [56] modified the capillary surface by first coating with α-glycidoxypropyltrimethoxysilane and then opening the resulting epoxide group by reacting with polyethylene glycol-600 to give highly reproducible and stable capillaries. These capillaries were then used to separate mixtures of proteins including lysozyme, trypsin and chymotrypsinogen with elution orders as predicted on the basis of their individual pI (isoelectric point) values. However, although efficiencies were as high as 150,000 plates, they were well below those predicted by theory and this is assumed to be as a result of residual adsorption, despite the surface coating. When the effect of pH was studied, it was found that as the pH of the carrier was increased, so the net positive charge on the proteins decreased, resulting in longer retention times and badly tailing peaks. The conclusion drawn from this observation was that the particular PEG coated capillary was satisfactory for the analysis of proteins at pH values <5, but at the more useful pH values (5–8) a significant amount of adsorption still took place. Cobb *et al.* [57] produced a highly stable coated capillary in which polyacrylamide was bonded to the surface by the direct formation of stable Si—C bonds produced via a Grignard reaction. Because of the stability of this phase, it was possible to perform separations of proteins at both high and low pH. Using high pH buffers with this coated capillary, seven model proteins (which were all negatively charged) were separated using the cathode at the injection end of the capillary, since with the vinyl bound polyacrylamide coating, there was no electroosmotic flow. The protein test mixture included insulin A, ovalbumin and β-casein. Quite apart from a marked improvement in peak shape and efficiency compared with uncoated capillaries there was a marked improvement in the reproducibility of migration times. With uncoated capillaries, proteins bound to the capillary surface result in a change in the zeta potential which in turn effects the electroosmotic flow. The vinyl bound polyacrylamide coated capillaries successfully reduced both electroosmotic flow and peptide adsorption thereby leading to highly reproducible retention times. However, once again efficiency calculations lead to the conclusion that protein–capillary coating interactions still occur. Low pH separations were carried out on a protein mixture at pH

2.7, which included the basic proteins cytochrome C, lysozyme, trypsin and trypsinogen. To try and analyse these proteins as their anions would necessitate the use of very high pH buffers, conditions under which the bonded capillaries are not stable. All five components of the protein test sample were positively charged at pH 2.7 and therefore migrated towards the cathode. Once again there was a marked increase in efficiency, peak shape and reproducibility of migration time compared to the results obtained with an uncoated capillary. However, although there is no measurable electroosmotic flow at this pH due to the surface coating, plate numbers were below those predicted by theory, and this is once again indicative of protein–capillary coating interactions.

Towns and Regnier [58] reported the separation of proteins using modified capillaries which were first derivatized with octadecyltrichlorosilane, and then coated with a non-ionic surfactant such as Tween or Brij. They pointed out that it should be possible to prepare a surface whereby the electroosmotic flow has not been entirely eliminated, and yet there is very little protein adsorption. Although the octadecylsilane surface was shown to reduce eof by half, proteins still adsorbed onto the surface of the capillary. Both surfactants investigated were capable of forming hydrophilic networks that form barriers preventing protein adsorption at the capillary surface. In order to produce a stable surface coating, the concentration of surfactant must exceed the critical micelle concentration which in the case of Tween and Brij surfactants is very low. A comparison was made of each surfactant to study the role of the head group size and the length of the hydrocarbon chain in influencing electroosmotic flow, separation efficiency, and peak symmetry. Using the Tween series of surfactants, which have a constant head group size but different hydrocarbon chain lengths, they found little effect on the performance of the coated capillary. However, the Brij series of surfactants which do have variations in the head group size and structure, gave rise to a large decrease in electroosmotic flow, although not all of the Brij range of surfactants gave a corresponding increase in efficiency. Because these surface modified capillaries showed only a minor increase in the eof in the pH range 4–11, it is possible to fully utilize the effect of pH to optimize separations. Separations were shown of five basic and six acidic proteins using capillaries coated with Brij 35 neutral surfactant. Dougherty *et al.* [59] reported on the use of two hydrophobic phases (C8 and C18) and one hydrophilic phase for the separation of proteins. Of the two hydrophobic phases, the C8 phase was more effective at lower eof and this is probably a function of surface coverage due to the smaller silylating agent being less sterically hindered during bonding. The polar surface coated material however showed a dependence of eof upon pH not too dissimilar to untreated silica. The effect of pH on the separation of four acidic proteins (myoglobin, conalbumin and β-lactoglobulin A and B) was demonstrated using the C8 coated capillary where the separation was optimized without inducing large changes in protein migration times as a result of changes to the electroosmotic flow. Smith and Rassi [27] described the surface modification of capillary walls, first with a quaternary ammonium function, followed by the attachment of hydrophilic polyether chains. This surface was such that at different pHs and a given applied voltage, a reversal of the eof takes place, and were thus termed 'switchable'. A plot of pH versus eof showed that at pH <5.7 the eof was anodal but changed to cathodal at pH >5.7. An example of the usefulness of switchable capillaries was illustrated by the separation of four basic proteins which was carried out at pH 4 and at pH 7. Both sets of conditions yielded high separation efficiencies suggesting very little solute–capillary surface interaction. At pHs <5, because the eof increased, this resulted in a reduction in the net mobility of the proteins whose direction of migration is opposite to that of the electroosmotic flow. This results in longer migration times for the proteins. Because the eof and the electrophoretic mobility of the proteins are in the same direction at high pH values, the migration times are longer. Yashima *et al.* [60] demonstrated the separation of closely related large peptides using MEKC with organic modifiers. The peptides separated differed only slightly in their structures and therefore have very similar mobilities. This eliminates the use of CZE for their separation. Using CTAB and acetonitrile as an organic modifier, it was possible to resolve the two closely related motilins [Met^{13}] motillin and [Leu^{13}] motillin. The optimum acetonitrile content was found to be 13% v/v. Four very closely related insulins (porcine, equine,

bovine and sheep) all with the same net charge, were separated using both CTAB and SDS micelles. The optimum separation using CTAB was with 5% acetonitrile as organic modifier. Using SDS at high pH, the optimum acetonitrile concentration was found to be 15% v/v. Differences in the elution order of the insulins using the different surfactants were explained by changes in both charge and selectivity in going from pH 3.6 (CTAB) to pH 8.5 (SDS). A study of the effect on elution order of insulins was carried out using the SDS system at pH 8.0, 8.5 and 9.0. As the pH increased the net charge on the insulins increased, leading to an increase in the mobility towards the +ve electrode. However, because the eof is towards the −ve electrode, this results in an incrase in the migration time. However, as the pH increases away from the pI value of the insulins, the hydrophobicity decreases leading to a reduction in the interaction with the micelle, and therefore a reduction in migration time. Similarly, the increase in negative charge with an increase of pH leads to a strong coulombic repulsion between the insulin and micelles, also resulting in a faster migration time. These last two effects outweigh that of an increase in the intrinsic mobility of the insulins and so they migrate faster with an increase in pH. Matsubara and Terabe [61] also described the separation of motilins and angiotensin II compounds using the neutral surfactant Tween 20. Although Terabe initially introduced the concept of MEKC for the analysis of neutral species, the addition of a micellar compound when trying to separate ionic species introduces a separation mechanism in addition to simple electrophoretic mobility. The problem with using ionic surfactants is the fact that as the peptide chain length increases, so the interaction with micelles increases, resulting in a loss of separation. This interaction is usually reduced by the addition of organic modifiers [60]. However, in this report, Tween 20 is shown to have a weak interaction with peptides and the effect of Tween 20 concentration on the separation of [val^5]-angiotensin II and human angiotensin were shown. The optimum concentration was found to be 80 mM, but at this concentration there was only partial separation between the compounds [sar^1, gly^8]-angiotensin II and [sar^1, ala^8]-angiotensin II, however, at a concentration of 250 mM Tween 20, baseline resolution was achieved. Although human angiotensin II and [val^5]-angiotensin II have been separated using hexadecyltrimethylammonium bromide (HTAB) [62] and SDS [63], when these surfactants were used to separate large peptides, like the motilins, interactions between the micelles and the peptides became too large to allow separation. On the other hand, interactions between large peptides and Tween 20 were shown to be weak. The two motilins [met^{13}] and [leu^{13}] were readily separated using a carrier containing 120 mM Tween 20. Idei *et al.* [64] demonstrated the separation of new somatostatin analog peptides using capillary electrophoresis with a buffer containing triethylammonium phosphate and organic modifiers. A mixture of six S-analogue peptides were separated using CH_3CN/TEAP in less than 6 min. A study of the migration time versus organic modifier concentration was performed and it was shown that the effect of alcohol concentration was quite different to that of acetonitrile. Whereas the migration time increased with alcohol concentration, it actually decreased with acetonitrile. Also it was noted when using alcohol that migration time increased as the chain length of the peptide increased. The effect of organic modifier on migration was explained by the fact that alcohols increase the viscosity of buffers, whereas acetonitrile decreases the viscosity and also diminishes the ionic strength. This study also revealed that the addition of organic modifiers can alter the elution order as well as the migration time. Liu *et al.* [62] used anionic and cationic surfactants in order to resolve mixtures of derivatized and underivatized peptides. Although CZE is a very powerful technique capable of resolving closely related mixtures of peptides, complex mixtures of tryptic digest are best separated using a combination of CZE and hydrophobic interaction, i.e. MEKC. For example the separation of underivatized angiotensin analogues using HTAB, and of OPA derivatized peptides using SDS. The elution order of the derivatized peptides is explained in terms of net charge and hydrophobicity. Also, using β-cyclodextrin as a buffer additive, the separation of nine fluorescamine derivatized peptides was achieved with very high efficiency accompanied by an increase in fluorescence intensity. The increase in efficiency and selectivity is suggested to result from a match between the solute size and shape and the hydrophobic cavity of the β-cyclodextrin mol-

ecules. Cohen and Karger [65] demonstrated the high performance SDS–PAGE of peptides and proteins using narrow fused-silica capillaries containing a cross-linked polyacrylamide gel that is covalently bound to the walls of the capillary. Gels are an anti-convective media which, because of their structure, behave as sieves through which the smaller molecules migrate faster than larger species. In the case of SDS–PAGE, the denatured proteins are reacted with SDS and a reducing agent giving rise to SDS–protein complexes with identical charge-to-mass ratio. Separation of these complexes is based purely on size or molecular weight differences and can therefore be used to determine molecular weights. Tsuji [66] used ethylene glycol in the acrylamide buffer solution and in the running buffer in order to prevent bubble formation during electrophoresis. This greatly improved the life of the capillaries which were used to separate a range of recombinant biotechnology derived proteins. The molecular weight reference standards showed a linear response with respect to peak migration time, allowing the determination of apparent molecular weights of proteins ranging from 10,000–100,000 and 40,000–200,000 depending on the $T\%$ of the individual gel. $T\%$ is described as follows:

$$T\% = \text{acrylamide (g)} + \text{bisacrylamide (g) per 100 ml of solvent.} \quad (18)$$

Widhalm *et al.* [67] described the separation of proteins using a linear, non-cross-linked polyacrylamide gel. It was shown that the four test proteins myoglobin, ovalbumin, bovine serum albumin and conalbumin eluted as their SDS complexes in order of increasing molecular mass. At the pH chosen (5.5) the electroosmotic flow is reduced so much that the anionic proteins are detected at the anode. Guttman and workers [68] studied the effect of temperature on the sieving effect of two different polymers, branched dextran and linear polyethylene oxide. The sieving characteristics of these polymers are a function of the network structure, whereas cross-linked polyacrylamide gels have a well defined pore structure which accounts for its sieving characteristics. A series of SDS-protein complexes ranging from lysozyme (MW 14,400) through to phosphorylase B (MW 97,400) were studied between the temperatures of 20 and 50°C. Using a 15% solution of dextran polymer (MW 72,000) it was shown that the migration times of the SDS–protein complexes decreased with an increase in temperature, and the resulting peaks became sharper. Bovine serum albumin and phosphorylase B which co-eluted below 40°C, were resolved at 50°C. These improvements were explained in terms of improved polymer chain flexibility and mobility as the temperature increases, which helps the formation of organized structures. When a 3% polyethylene oxide gel is used with the same SDS–protein test mixture, it was found as with the dextran gel, that although the migration time decreases with an increase in temperature, the separation efficiency is much higher at lower temperatures with all six components resolved at 20°C. This decrease in plate number as the temperature increases with the polyethylene oxide gel is probably caused by the disorientation of the channel like structures in the matrix.

(b) Nucleic acids, nucleobases, nucleotides, nucleosides and oligonucleotides

Smith and Rassi [27] described the use of surface modified fused silica capillaries for the separation of oligosaccharides and nucleotides. The nature of the surface modification was such that the electroosmotic flow could be switched from anodal to cathodal and the mechanics of this have been described in an earlier section. Because the modified capillary surface is so well shielded, it is possible to achieve very high separation efficiencies with a whole range of biomolecules, including nucleotides. The applicability of these modified capillaries was demonstrated with a series of mono and diphosphate nucleosides run using an electrolyte at pH 2.0 and 3.5. These acidic analytes were eluted with very high efficiencies even though the capillary wall at this pH is positively charged. However, as the pH was increased, the overall increase in net negative charge resulted in electrostatic interaction between the acidic solutes and the positively charged quaternary ammonium group on the modified capillary surface, resulting in excessive band broadening. Although this band broadening could be overcome by increasing the electrolyte concentration, the resulting increase in Joule heating necessitated lowering the applied voltage from −18 kV to −15 kV with a resulting increase in analysis time.

Row and workers [69] described the separation of 14 normal and modified deoxyribo-

nucleosides, deoxyribonucleotides, a ribonucleoside and a pyrimidine using MEKC with SDS. The observed elution orders of pyrimidine before purine, normal before modified deoxyribonucleotides, and deoxyribonucleosides before deoxyribonucleotides were explained by a combination of separation mechanisms. For example, the increased retention of the purine compounds compared to the pyrimidines is explained by their greater solubility in the SDS micelle which is migrating against the eof. However, the deoxyribonucleotides are retained more than the deoxyribonucleosides since at the operating pH of 8.4, the former have a double negative charge resulting from the ionization of the acidic phosphate group, and consequently migrate against the electroosmotic flow. Other parameters studied were the effect of SDS concentration on retention times, the effect of operating voltage on efficiency and also the effect of injection voltage on efficiency. Generally the retention time of analytes increased with an increase in SDS concentration, although with differing degrees resulting in some instances to a change in elution order. The maximum efficiency at the optimum SDS concentration appeared to occur between 8 and 10 kV but this was shown to be very dependent on solute concentrations. Using the optimum SDS concentration and operating voltage, the effect of injection time and voltage on efficiency also was noted. Although the amount of solute introduced increased with injection time, the increase in peak height was not linear. The greatest efficiency was observed at 10 kV with the shortest injection time, and at a constant injection time at low voltages. In both instances this is attributed to a small sample plug-volume. Also it was noted that at lower injection voltages, there is less sample discrimination due to differences in electrophoretic mobilities.

Liu *et al.* [70] described the separation of mono, di and triphosphorylated ribonucleosides using MEKC, with one anionic and two cationic surfactants. A study of the effect of surfactant concentration on electroosmotic flow showed that SDS had little effect over the range studied, whereas with DTAB (dodecyltrimethylammonium bromide) there is a reversal in eof with an increase in concentration. Although a switching effect was expected with the cationic surfactant HTAB (hexadecyltrimethylammonium bromide) this occurred outside the concentration range of the study. A study of the effect of surfactant type on the peak capacity (t_o/t_{mc}) showed that SDS produced the widest effective elution range and therefore should possibly produce the best separation. This should be followed by DTAB then HTAB. However, when 12 common ribonucleotides (the mono, di and triphosphate nucleosides of cytidine, uridine, guanosine and adenosine) were injected, the best separation was achieved using DTAB. This discrepancy was explained in terms of the electrostatic repulsion of the negatively charged analytes by SDS and an electrostatic attraction with the cationic DTBA. Since DTAB gave the best separation of the three micelles, the effect of concentration on resolution was also studied. The resolution of the phosphorylated nucleosides was found to improve with an increase in surfactant concentration beyond the cmc, this resulting from a greater solute interaction with the pseudo stationary phase. Because the eof changes direction as the concentration of the DTAB increases, the polarity of the voltage has to be reversed at a concentration of approximately 2×20^{-4} M to ensure that all analytes are eluted in the original direction. The separation of 12 common phosphorylated nucleosides was optimized by increasing the length of the capillary and reducing the applied voltage, whereby all twelve components were almost baseline resolved within 4 min.

Cohen *et al.* [71] separated bases, nucleosides and oligonucleotides using a combination of MEKC and metal additives. While it is necessary to use micelles in order to resolve uncharged bases and nucleosides under the operating conditions used (pH 7), the oligonucleotides are negatively charged and can therefore be separated in free solution. However, the separation of complex mixtures is limited since in free solution, the elution window is small. The addition of micelles and divalent metals such as Mg, Cu and Zn, greatly expands the time window, leading to the separation of complex mixtures with very high efficiencies. The separation mechanism involves the electrostatic attraction of the divalent metal ions onto the surface of the negatively charged micelle, leading to differential metal complexation of the oligonucleotides with the surface of the micelle. Using SDS only, baseline separation of four bases and five nucleotides was achieved in a single run in

under 40 min. The separation order for both the bases and nucleotides is related to the hydrophobicity of the analytes, the most hydrophobic species eluting last. Because the nucleosides all have the ribose ring present making them more hydrophilic than their corresponding base they all elute earlier than these bases. The separation of oligonucleotides at neutral pH was demonstrated with the addition of urea to prevent sample aggregation. However, since in free solution separation is achieved as a result of differences in electrophoretic mobility, because these differences are very small and become even smaller as the base number increases, separation is poor for mixtures of 12–18 bases. Attempts to improve the separation by the inclusion of SDS into the carrier failed, probably due to the increase in electroosmotic flow and subsequent reduction in retention time. The addition of metal ions to a buffer system without the presence of SDS, gave a marked decrease in eof but significantly poorer peak shapes, and it is only when metal ions are used in the presence of SDS that there is a significant increase in eof accompanied by high efficiency separations. The separation of eight polythymidines was achieved using a mixture of SDS and 5 mM Mg (II) whereas it was possible to resolve up to 18 bases of polythymidine using the same system with Cu (II) instead of Mg (II). Although the peaks appeared slightly sharper in the Mg (II) system, because the electrophoretic velocity for Cu (II) is smaller than for Mg (II), this results in a wider time window which explains the increase in resolving power of that system. Baba and workers [72] described the preparation of polyacrylamide gel-filled capillaries for the analysis of polynucleotides. The performance of the gel was tested using a mixture of poly $(dA)_{12-18,40-60}$ and the single base resolution was achieved in less than 23 min with an efficiency of 300,000 plates per peak, which is equivalent to 1 million plates per metre. Ultra high resolution was demonstrated by the resolution of poly (A) which had been digested with nuclease P1. Here, 250 bands of poly (A) were baseline resolved within 60 min, with efficiencies in the range 1–5 million plates per metre. Assuming that single base resolution was achieved, the poly (A) in the chain length of 6 mer to 255 mer was completely resolved. These gels were prepared using a low concentration of both ammonium persulphate and TEMED, and introduced into the capillary under vacuum. There was no surface pretreatment before filling the capillaries. These simplified gel-filled capillaries were stable for over 60 analyses without an appreciable decrease in performance.

Yin *et al.* [73] described the production of stable polyacrylamide gel-filled capillaries that were used to separate a series of oligonucleotides with very high efficiencies. Attempts by other workers to produce stable gel-filled capillaries usually involved pretreatment of the capillary with a bifunctional reagent such as 3-methacryloxypropyltrimethoxysilane, followed by co-polymerization with an acrylamide/bis acrylamide mixture to form a gel. However it is shrinkage of this mixture during gel formation that produces bubbles in the capillary. In their work, Yin *et al.* used a non-cross-linked linear polyacrylamide bonded to the capillary walls in order to prevent bubble formation during the generation of the gel. This is because the structure of the non-cross-linked polyacrylamide layer does not restrict the shrinking of the gel, thus preventing bubble formation. Similarly, because surface silanol groups in the capillary are now shielded, electroosmotic flow is virtually eliminated resulting in gels that do not extrude and are stable for several hundred routine separations. Gel-filled capillaries produced in this way also give rise to better peak symmetry than those produced without surface pre-treatment. This was demonstrated by the separation of polythymidine ~30-mer, where baseline resolution was achieved with the pre-treated capillary, and only partial separation with the untreated capillary. A sample of synthesized oligonucleotides containing the series of homologous 40–60-mer polydeoxyadenosines $(Pd(A)_{40-60})$, each differing by only one base unit, was completely baseline resolved in under 40 min. An example of ultra-high resolution was demonstrated by the complete separation of poly (uridine 5'-phosphate) into 431 peaks, each differing by one nucleotide unit. Bevan *et al.* [74] described the resolution using MEKC of the diasterioisomers of oligonucleotides possessing several chiral phosphoramidate bridges. Of the oligonucleotides studied, modification of the internucleotide phosphate bridge by the introduction of a substituted amine, gave rise to a chiral centre at the phosphorus atom. Since the synthesis is not stereoselective a large number of diastereo-

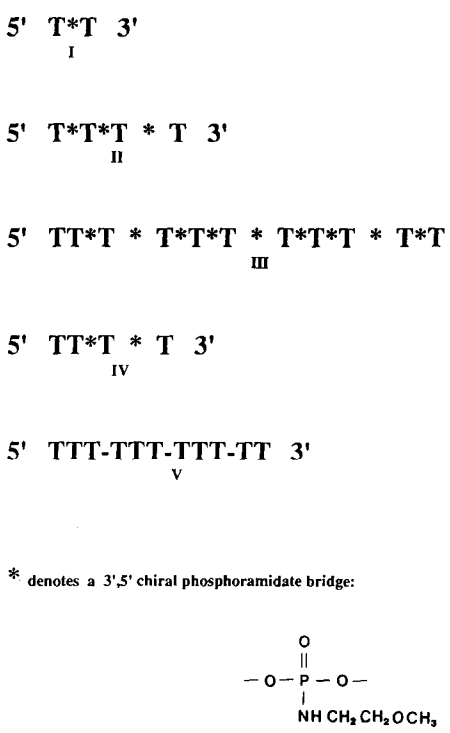


Figure 6
Structure of chiral phosphoramidates.

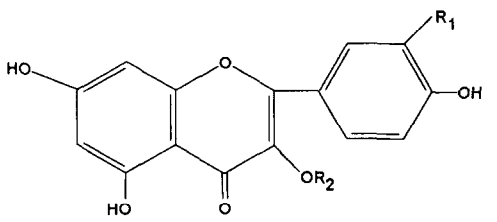
isomers can arise following modification of longer synthetic oligonucleotides. The structures of the compounds studied are shown in Fig. 6.

Using the five test compounds illustrated in Fig. 6, the effect on the resolution of the diastereoisomers of SDS and urea concentrations were studied, as was the effect of organic modifier. Using SDS and urea only, Bevan *et al.* were able to completely resolve the two diastereoisomers of compound I, and partially resolve the eight diastereomers of compound II. Although compound III can exist in 512 diastereomeric forms, resolution was observed for approximately 35 components using the same electrolyte as for I and II. Compound V which is a non-diastereomeric analogue gave the expected single peak under the same conditions. The effect of SDS and urea concentration was studied for the separation of the diastereoisomers of compound I, and here retention was found to increase with SDS concentration, whereas the addition of urea greatly reduced the capacity factor but enhanced the efficiency. However, although a similar dependence on SDS and urea concentration was observed for compounds II and III the accompanying increase in efficiency following the addition of urea was not observed. Compound IV was shown to be only slightly retained in an SDS system, even without the addition of urea, and this was attributed to electrostatic repulsion from the negatively charged micelle. In order to overcome this phenomenon, a cationic surfactant (DTAB) was chosen and excellent resolution of the four diastereoisomers was then achieved.

(c) Small molecule pharmaceutical compounds — achiral

Capillary zone electrophoresis now plays an important role within the analytical laboratory of most pharmaceutical companies because it is complementary to the most popular analytical technique of all, high-performance liquid chromatography. There is now an abundance of applications in the literature describing the analysis of small pharmaceutical compounds and some of these will be described in this section. Several workers [75, 76] have described the analysis of vitamins using MEKC. Burton *et al.* [75] resolved pyridoxine (B6) from five of its metabolites using an SDS, phosphate–borate micellar system. Pyridoxic acid, a metabolite of B6, was separated and determined in human urine using a laser induced fluorescence detector, with a limit of detection of <1 picogram injected. Fujiwara *et al.* [76] were able to resolve seven water soluble vitamins in approximately 25 min using 0.02 M phosphate pH 9.0–0.05 M SDS as the electrolyte. Here detection was by UV absorption at 254 nm. The MEKC method employed was used to determine the contents of a vitamin injection, and was found to be highly accurate and reproducible and was much simpler than the exiting gradient HPLC method. Pietha [77] reported the separation of flavonal-3-O-glycoside drugs using MEKC. Although the flavonoid drugs are weak acids, they are capable of separation by reversed-phase HPLC where they separate on the basis of hydrophobic interactions. This separation mode was easily transferred to MEKC where SDS was added to the carrier electrolyte to provide the hydrophobic interior for separation. The flavonoids have the general structure shown in Fig. 7.

A method was developed that allowed the baseline resolution of nine glycosides although the selectivity was slightly different to that found by HPLC. This is because unlike HPLC, where only hydrophobic interactions are responsible for separation, in CZE the electro-



Where R_1=OH,H or OMe
and R_2= Rutinose, Glucose, Galactose,Rhamnose or Arabinose

Figure 7
General structure of flavonoids.

phoretic mechanism also plays a role. Ackermans *et al.* [78] described the determination of aminoglycoside antibiotics in pharmaceuticals using a combination of MEKC with indirect UV detection. In order to detect the non-UV absorbing compounds, a background electrolyte consisting of imidazole or benzylamine was used, with the pH adjusted with either formic acid, 2-(N-morpholino) ethane sulphonic acid (MES), or acetic acid. Initial attempts to analyse the aminoglycosides using CZE were unsuccessful due to very poor peak shapes resulting from the interaction of the highly positively charged analytes and the capillary surface. The addition of the surfactant FC135 greatly reduced these interactions leading to much improved peak shapes, with a reversal of the eof due to shielding of the negatively charged silanol groups. An example of the separation of six aminoglycosides was demonstrated using a background electrolyte consisting of 0.1 M imidazole acetate at pH 5.0 with 50 μl ml^{-1} of the surfactant FC135. In order to resolve neutral analytes at the same time as the aminoglycoside antibiotics, it was necessary to add the cationic surfactant CTAB above its critical micelle concentration. Using the same carrier as before with the addition of 100 mM CTAB, it was now possible to resolve a variety of UV absorbing neutral compounds including paracetamol, dapsone and dexamethasone, from the non-UV absorbing aminoglycosides. This method was used to determine neomycin and hydrocortisone in eardrops, the values found being in good agreement with the labelled values. Soini *et al.* [79] reported the use of MEKC to perform the solid-phase extraction of cimetidine in serum. Samples were pre-concentrated on the reversed-phase packing and this was followed by a conditioning procedure. During the elution procedure, an external field was applied across the extraction cartridge which was placed in the horizontal position. Apart from the advantage of the pre-concentration that takes place, an additional advantage of the electrically driven system over pressure driven is the considerable lowering of an unidentified serum component in the resulting electropherogram. Wainwright [80] reviewed the separation of pharmaceutical compounds using CZE with both coated and uncoated capillaries. Coated capillaries used for these studies were prepared by coating with a linear polyacrylamide.

A study of the effect of using coated and uncoated capillaries was undertaken using naproxen, ibuprofen and tolmetin as test compounds. These are water soluble anti-inflammatory drugs each containing a carboxylic acid function. As expected with the coated capillary the drugs were separated with an increase in retention time as the pH of the electrolyte was reduced. This is because the separation is a result of the differences in electrophoretic mobilities of the analytes only, since electroosmosis in a coated capillary is negligible.

When an uncoated capillary was used, the samples eluted in the reverse order to that observed with the coated capillary, this is because elution is now a function of both the electrophoretic mobilities of the anions and the eof. However, although the retention time also increased with a decrease in pH, at pH 6.1 there was no elution of analytes within 30 min, since the eof and net mobilities of the analytes were almost equal and opposite. Because it was possible to elute positive and negative analytes in one run using the uncoated capillary, often with higher efficiencies, all subsequent studies were carried out using an uncoated capillary. A whole range of antibacterial compounds including sulphonamides, cephalosporins and penicillins were then resolved using capillary zone electrophoresis on an uncoated capillary. Using CZE, no separation was obtainable for a range of peptides that differed in structure by one neutral amino acid, at least in the pH range of 7–9. With the addition of SDS however, total separation of the peptides was achieved. This same electrolyte was used to successfully resolve a series of a barbiturates. CZE was then used to analyse a range of OTC drugs including those used to treat pain, colds and certain allergies. Terabe *et al.* [81] described the effect of the addition of urea on separation

by MEKC. He also pointed out that in MEKC the optimum capacity factor is approximately two for the maximum resolution, however k' often exceeds 10 for hydrophobic compounds because they are strongly retained by micelles due to partition. Plots of ln k' versus urea concentration showed that ln k' decreased linearly with an increase in urea concentration. This was explained by the fact that urea increases the water solubility of most solutes and this in turn effects the distribution coefficient in the micelle. It was also found that the migration time of the SDS micelle t_{mc} increased with urea concentration, whereas the eof remained almost unchanged. This results in an expanded migration range between t_o and t_{mc}. Examples of the beneficial effects to be gained from the addition of urea were given. Firstly, the addition of urea to a 100 mM solution of SDS resolved all 23 PTH amino acids, whereas without the urea, five pairs of amino acids were poorly resolved. A dramatic example of the effect of urea was demonstrated using a mixture of eight corticosteroids, all of which are poorly soluble in water. Because of this, the solutes are mostly incorporated inside the micelle giving rise to a migration time close to t_{mc} with a capacity factor $k' > 10$. The addition of 6 M urea to the carrier resulted in total resolution of the eight steroids in approximately 30 min. Nishi and Matsuo [82] also reported the separation of corticosteroids using MEKC. Here, resolution of the corticosteroids was achieved by the addition of γ-cyclodextrin and urea to the electrolyte. The effect of adding urea was to increase the solubility of the γ-cyclodextrin in the aqueous phase. This leads to a decrease in migration time and a different elution order to the SDS carrier without the cyclodextrin. All eight corticosteroids in the test mixture were resolved in under 20 min. Nishi *et al.* [83] had previously reported the separation of corticosteroids and benzothiazepin analogues by MEKC using bile salts. As previously described, these lipophilic analytes are solubilized by the micelle to such a degree that they migrate with the same velocity as the micelle and are therefore not separated. Bile salts were found to solubilize corticosteroids and benzothiazepin analogues to a much lesser extent than SDS and separations were consequently achieved. Attempts at resolving these compounds by MEKC with SDS only were unsuccessful. Eight corticosteroids were successfully resolved using the bile salt sodium cholate whilst 12 benzothoazepin analogues were resolved using sodium taurocholate. Samples of diltiazem tablets and fluocinonide cream were successfully assayed using internal standards. Other applications of MEKC using bile salts included the separation of 14 ingredients of a cold medicine, reported by Nishi [84], and the separation of aflatoxins by Cole [85]. In their work Nishi and workers [84] found that the resolution of 14 active ingredients in cold medicine was not possible using SDS, since five of the constituents migrated with a velocity very similar to that of the micelle. However, the use of sodium cholate or sodium deoxycholate produced a significant improvement in resolution of the five previously unresolved peaks, with a marked improvement in efficiency. Cole *et al.* [85] were able to demonstrate the baseline resolution of the three aflatoxins namely G_2, G_1 and B_3 using sodium deoxycholate as the carrier. Smith [author, unpublished results] used MEKC to analyse samples of the anti-inflammatory steroid fluticasone propionate (see Fig. 8) but was unable to resolve the potential impurity, the des-6α-fluoro analogue.

However, using the same carrier but with the addition of 20% methanol, it was now possible to resolve the des fluoro analogue and another unknown impurity as shown in Fig. 9.

Chee and Wan [86] reported the high speed separation of 17 basic drugs by CZE. Although there have been reports on the analysis of basic drugs by MEKC, GLC and HPLC, none covered the 17 described in their report. The buffer, pH, ionic strength and applied voltage were all optimized and of these the pH was found to be the most critical. A pH change of only 0.05 of a unit could lead to a deterioration in the resolution. The ionic strength and applied voltage were found to be much less

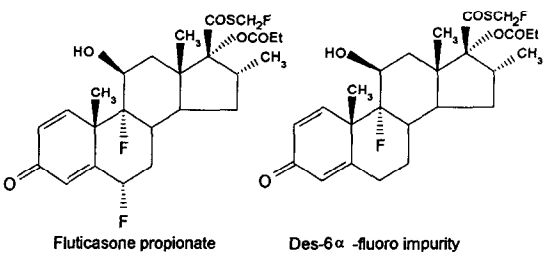


Figure 8
Structure of fluticasone propionate and its des-6α-fluoro impurity.

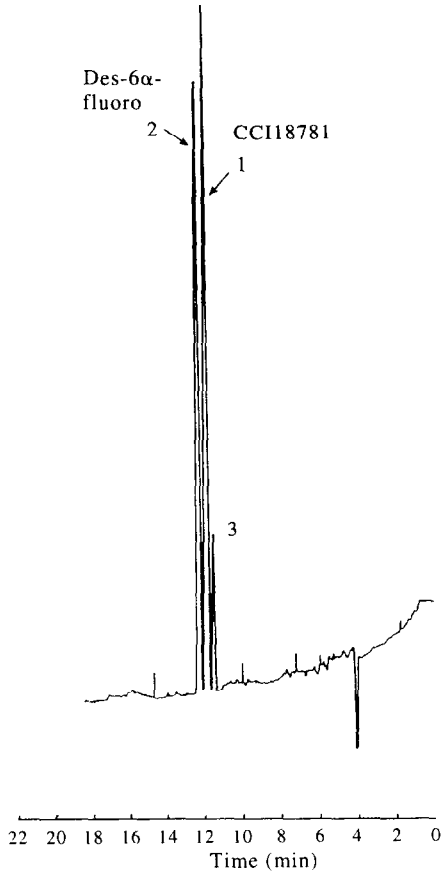


Figure 9
Separation of fluticasone propionate (1) from its des-6α-fluoro impurity (2) and another unknown (3). Conditions: instrument: ABI 270A, capillary 72 cm × 50 μm i.d., applied voltage 30 kV; detection at 238 nm, range 0.02 aufs; vacuum injection for 1.0 s, temp 60°C. Carrier: 20% MeOH–0.01 M Na_2HPO_4. 0.006 M $Na_2B_4O_7$. $10H_2O$. 0.05 M SDS.

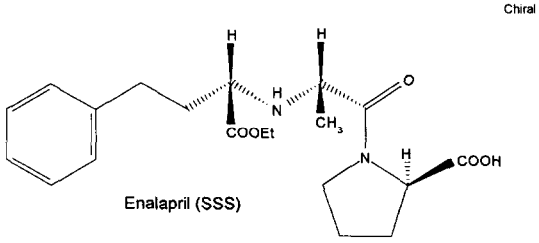


Figure 10
Structure of enalapril maleate (SSS).

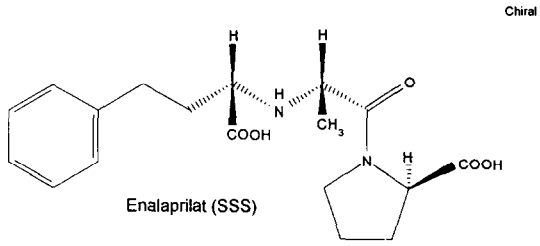
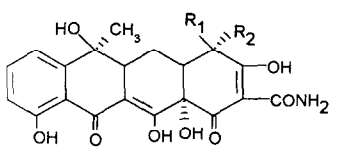


Figure 11
Structure of enalprilat (SSS).

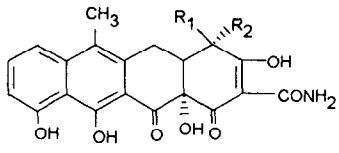
critical. Attempts to resolve the 17 basic drugs using MEKC based on methods reported by other workers were unsuccessful. In order to illustrate the usefulness of the method for the analysis of the drugs in biological samples, the 17 drugs were spiked into human urine or plasma, extracted and then analysed. Most of the drugs could be analysed, although meclizine was a problem since it was not extracted. Qin and co-workers [87] reported the separation of the rotamers of enalapril by MEKC. Enalapril maleate (Fig. 10) is an orally active inhibitor of angiotensin converting enzyme, widely used for the treatment of hypertension.

It was pointed out by Qin that enalapril is found only in the *trans* configuration around the amide bond in the solid form, but can exist as the *cis–trans* rotamers in solution. The *cis–trans* interconversion in solution is complete in the order of minutes, and consequently analysis of HPLC at room temperature where the elution times are comparable leads to poor peak shapes. Traditionally, higher temperatures are used in HPLC in order to overcome tailing peaks, but this is not conducive to the resolution of the rotamers. Attempts to resolve the rotamers of enalapril by CZE were unsuccessful since the *cis* and *trans* isomers have the same charge-to-mass ratio and will therefore migrate with the same electrophoretic mobility. CZE was successful in resolving the hydrolysis degradation product, enalaprilat (Fig. 11) which has two carboxyl groups and a different charge-to-mass ratio than that of enalapril.

Using MEKC with SDS as the micellar additive it was possible to resolve the enalapril rotamers at room temperature. Since only the pure (SSS) isomer was used for the study, the two peaks observed in the electropherogram were assigned to the two rotamers. The effect of temperature on the separation showed a marked deterioration as the temperature increased above 25°C, adding further support to the rotamer assignments. Zhang *et al.* [88] described the separation of tetracyclines and degradation products by CZE using electrolytes containing EDTA. Tetracyclines (Fig. 12) are broad spectrum antibiotics that in acidic media can degrade to give anhydro-tetra-



R_1=N(CH_3)$_2$ and R_2=H Tetracycline (TC)
or R_1=H and R_2=N(CH_3)$_2$ 4-Epitetracycline (ETC)



R_1=N(CH_3)$_2$ and R_2=H Anhydrotetracycline (ATC)
or R_1=H and R_2=N(CH_3)$_2$ 4-Epianhydrotetracycline (EATC)

Figure 12
Structure of tetracycline and its degradation products.

cyclines which in turn can epimerize to form other degradants.

Because these degradation products have widely differing biological activity, it is important to separate them from the parent tetracycline. The effect of pH on the separation of tetracycline from its degradants showed that the optimum separation was achieved at pH 3.9. However, a dramatic improvement in resolution and efficiency was observed when EDTA was added to the phosphate buffer. The CZE method was shown to be superior to the HPLC method reported in the US Pharmacopeia, with better resolution and shorter analysis times. A study of the effect of EDTA concentration on resolution, lead to the splitting of the ATC and EATC peaks at concentrations of about 0.0075 M. The results show that each of the ATC and EATC samples were separated into two large peaks at EDTA concentrations of 0.0075 M, and these have not previously been reported. Tricyclic antidepressants are notoriously difficult compounds to analyse by HPLC, due to solute–silanol group interactions [89]. Smith [author, unpublished results] successfully resolved the anti-depressant GR50360A (Fig. 13) from five closely related potential impurities including the *cis*-isomer, the des-5-fluoro analogue and several N-alkyl derivatives.

Attempts to resolve these compounds by CZE at low pH were unsuccessful and only with the addition of SDS with organic modifier was it possible to resolve all six compounds (see Fig. 14). The resolution of these compounds was found to be very dependent on sample and organic solvent concentration.

(d) Chiral compounds including biologically active drugs

Because many chiral drugs have displayed stereo selectivity in their pharmacological behaviour, pressure has been placed on the

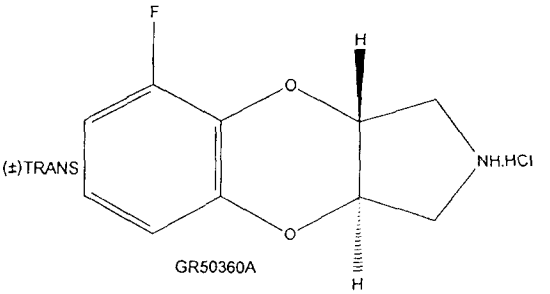


Figure 13
Structure of GR50360A.

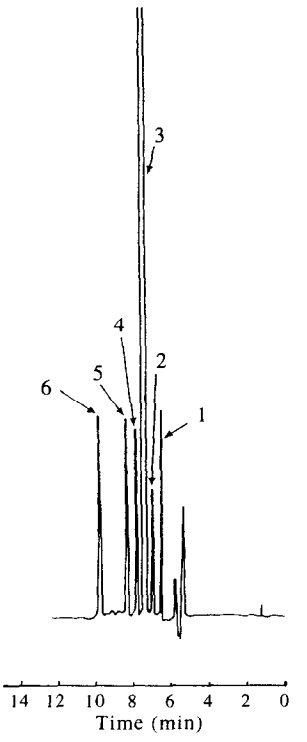


Figure 14
Separation of GR50360A from five closely related impurities; (1) *cis* isomer; (2) des-5-fluoro impurity; (3) GR50360A; (4) N-Me analogue; (5) N-Et analogue; (6) N-Pr analogue. Conditions; instrument ABI 270A, capillary 72 cm × 50 μm i.d., voltage 30 kV; detection 210 nm, range 0.03 aufs; vacuum injection for 1.0, temp. 60°C. Carrier: 20% IPA–0.01 M Na_2HPO_4. 0.006 M $Na_2B_4O_7.10H_2O$. 0.05 M SDS. Overall pH 7.0.

pharmaceutical industry by the regulatory authorities to produce single enantiomer products, and to have available chiral separation methods that provide reliable measurement of enantiomeric purity. Although gas chromatography and particularly HPLC have been used successfully in the past, CZE is now beginning to play an important role in the analysis of chiral molecules because of the extraordinary efficiencies achievable. Chiral recognition is achieved by the addition of a chiral selector to the electrolyte and examples of the types commonly used together with applications, will now be discussed.

(i) Cyclodextrins. These are by far the most common of all chiral additives used in CZE. Cyclodextrins are capable of forming inclusion complexes in aqueous solution, and consist of cyclic oligosaccharides containing either 6, 7 or 8 glucose units and are termed α, β or γ, respectively. They form a truncated cone containing secondary hydroxyl groups at the entrance of the cavity, with a relatively hydrophobic interior. The cyclodextrins are water soluble with solubilities of 14.5, 1.85 and 23.2 g/100 ml for α, β and γ-cyclodextrin, respectively. β-Cyclodextrin with a cavity diameter of 6.0–6.5 Å has proved to be the most useful additive, however it has the lowest water solubility of the three forms. Derivatized cyclodextrins tend to be much more soluble in water and organic solvents and also can exhibit different chromatographic selectivities.

Terabe [17] reported the use of carboxylated cyclodextrins to resolve a range of neutral isomeric substituted benzenes. Using a carboxylated methylethyl β-cyclodextrin, Smith [author, unpublished results] was able to resolve the isomers of *o*-, *m*- and *p*-nitrobenzyl alcohol as demonstrated in Fig. 15.

Terabe [16] reported the separation of Dns amino acids using the modified β-cyclodextrin mono-(6-β-aminoethylamino-6-deoxy)-β-cyclodextrin (CDen). The positively charged CDen migrates towards the cathode, thus the strongly included Dns amino acids are eluted first, whilst the weakly included analytes have longer retentions. Using CDen, Smith [author, unpublished results] was able to partially resolve the enantiomers of the anti-emetic drug, ondansetron (Fig. 16).

Best results were achieved using tetrabutylammonium bromide and a temperature of 20°C as illustrated by the electropherogram in Fig. 17.

Fanali [90] was able to resolve the enantiomers of five sympathomimetic drugs including ephedrine, norephedrine and isoproterenol using a solution of the derivatized cyclodextrin heptakis-(2,6-di-O-methyl-β-cyclodextrin). Attempts to resolve the enantiomers using underivatized β-cyclodextrin were unsuccessful even at the relatively high concentration of 20 mM. Nishi and co-workers [91] studied the effect of adding different cyclodextrins to SDS in order to separate uncharged or neutral compounds and found that the solutes were

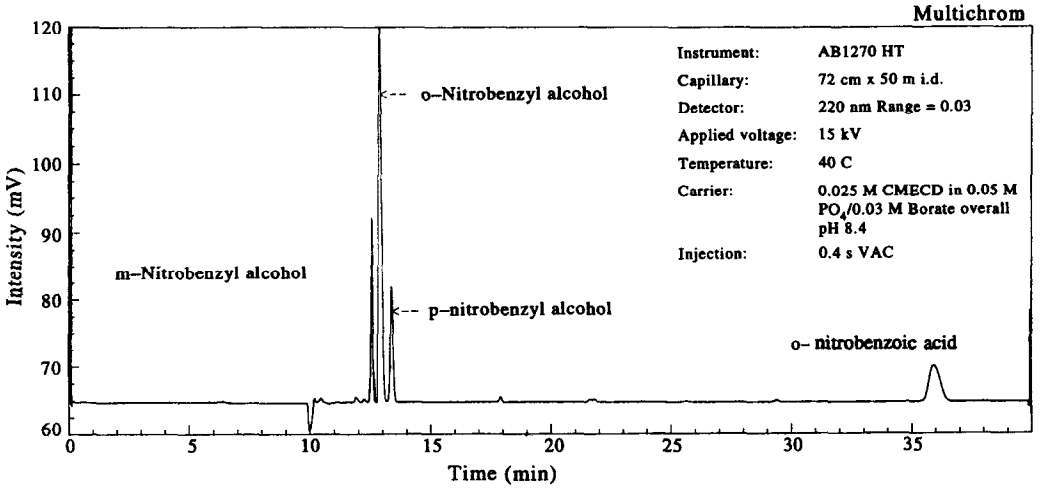


Figure 15
Resolution of *o*-, *m*- and *p*-isomers of nitrobenzyl alcohol. Conditions: instrument ABI 270HT, capillary 72 cm × 50 μm i.d., applied voltage 15 kV; detection at 220 nm, range 0.03 aufs; vacuum injection for 0.4 s; temp. 40°C. Carrier: 0.025 M carboxymethylethyl-β-cyclodextrin in 0.05 M PO_4–0.03 M borate. Overall pH 8.4.

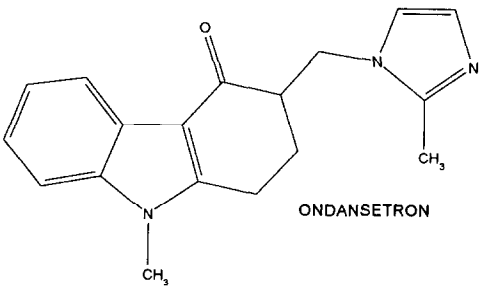


Figure 16
Structure of ondansetron (GR38032F).

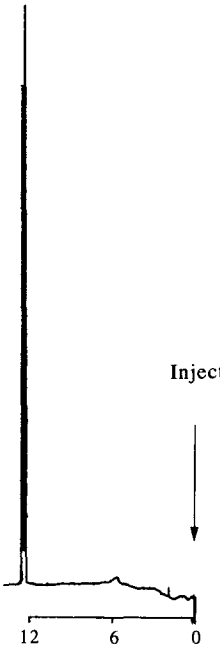


Figure 17
Separation of the enantiomers of ondansetron (GR38032F). Conditions: instrument ABI 270A; capillary: 72 cm × 50 μm i.d., voltage 30 kV; detection at 254 nm, range 0.02 aufs; vacuum injection for 1.0 s; temp. 20°C. Carrier: 0.025 M CDen–0.1 M tetrabutylammonium bromide (TBAB) in 0.01 M TRIS buffer pH 2.5.

distributed between three phases; an aqueous phase, the micelles, and the cyclodextrin. It was shown for the five racemic drugs studied that the cavity size in CD-MEKC was important and only γ-cyclodextrin was capable of including both the solute and the micelle monomer. The resulting cyclodextrin-surfactant monomer–enantiomer inclusion complex formation, provides diasteromeric pairs capable of resolving the neutral analytes. Also studied were the effects of adding additional chiral additives to the γ-CD-MEKC system, and with the two examples tried (Na D-camphor-10-sulphonate and L-menthoxyacetic acid) improvements were made to the separation of the test solutes. This was attributed to the fact that γ-CD was capable of including each of the chiral selectors thus enhancing the chiral properties of the CD cavity. Kuhn *et al.* [92] achieved excellent resolution of the cations of Trogers base (Fig. 18) at pH 2.5 and the anions of three Dns amino acids at pH 9.0.

Using propranolol and methyl β-cyclodextrin as models Wren and Crowe [93] showed that the degree of separation was dependent on the concentration of the chiral selector and that there was an optimum. When similar studies were made with β-cyclodextrin, the optimum concentration was significantly higher than with methyl β-cyclodextrin. Propranolol was shown to have a lower affinity for β-cyclodextrin where the difference between the stabilities of the two complexes is lower. This resulted in poorer resolution with β-cyclodextrin than with methyl β-cyclodextrin. Fanali [94] studied the effect of concentration of cyclodextrin modifiers on the resolution of the enantiomers of terbutaline and propranolol (Fig. 19).

Terbutaline enantiomers were readily resolved in the presence of either 5 mM di-O-

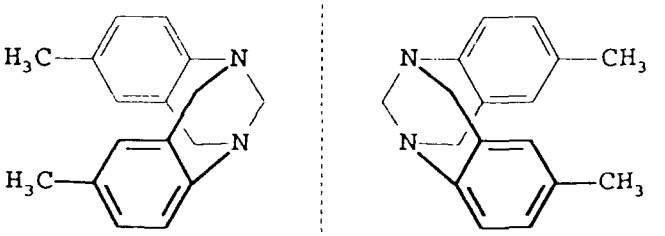


Figure 18
Enantiomers of Trogers base.

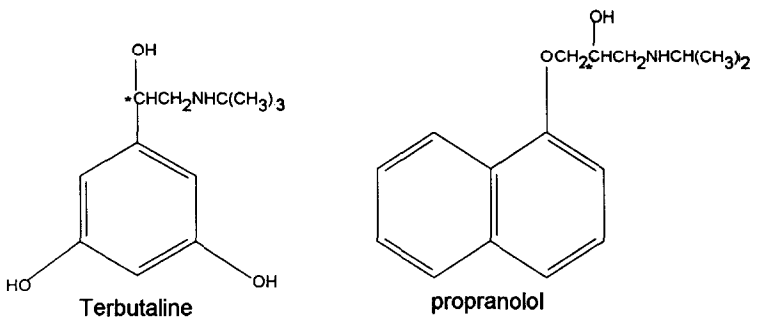


Figure 19
Structures of terbutaline and propranolol.

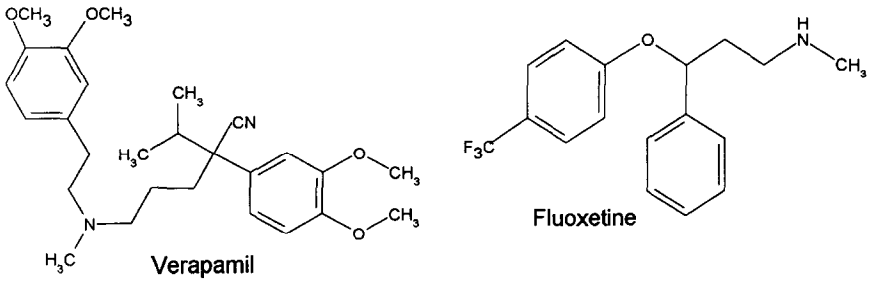


Figure 20
Structures of verapamil and fluoxetine.

methyl-β-cyclodextrin or 15 mM β-cyclodextrin. Resolution was shown to increase with cyclodextrin concentration. However, the background electrolyte used successfully for the resolution of terbutaline enantiomers (except for tri-O-methyl-β-cyclodextrin) failed to resolve the enantiomers of propranolol. However, because propranolol had been successfully resolved using a β-cyclodextrin HPLC column [95], Fanali *et al.* increased the effective concentration of β-cyclodextrin in the background electrolyte by the addition of urea, which acts as a solubilizing agent, and then found that by varying the methanol content the enantiomers of propranolol could be resolved. Soini *et al.* [96] studied the separation of the enantiomers of basic drugs, as well as the enantiomers of bupivacaine in serum. They showed that the use of cationic surfactants such as HTAB and cetyl pyridinium chloride (CTPC) were effective in overcoming the interference from endogenous substances in serum. Verapamil and fluoxetine (Fig. 20) were resolved using trimethyl β-cyclodextrin with HTAB and methylhydroxycellulose (MHEC) as additives.

Using dimethyl β-cyclodextrin with similar additives, it was possible to resolve the enantiomers of mepivacaine, bupivacaine, pindolol and carvedilol (see Fig. 21).

Novotny *et al.* [97] demonstrated that by adding certain alkylhydroxyalkylcellulose derivatives to cyclodextrins, that it is sometimes possible to enhance enantioselectivity and separation efficiency in CZE. The resolution of chloramphenicol was found to be improved by the addition of 0.1% methylhydroxypropyl cellulose (MHEC). Similar effects were reported for the separation of the positional isomers of methoxyphenylacetic acids. Using γ-cyclodextrin (without the addition of MHEC) Novotny demonstrated the separation of ketotifen (Fig. 22) from its synthesis intermediate K-I, and the structural related compound K-R whereas the N-oxide, K-NO, could only be resolved using β-cyclodextrin, also without the use of MHEC.

Schomburg *et al.* [26] demonstrated the effect of adding polyvinylalcohol and hydroxyethyl cellulose to electrolytes containing γ-cyclodextrin on the separation of chiral compounds. The addition of low concentrations of non-ionic polymers was a very effective way of suppressing solute-surface adsorption, and also eof, leading to longer migration times. It was shown that the enantiomers of a tocainide

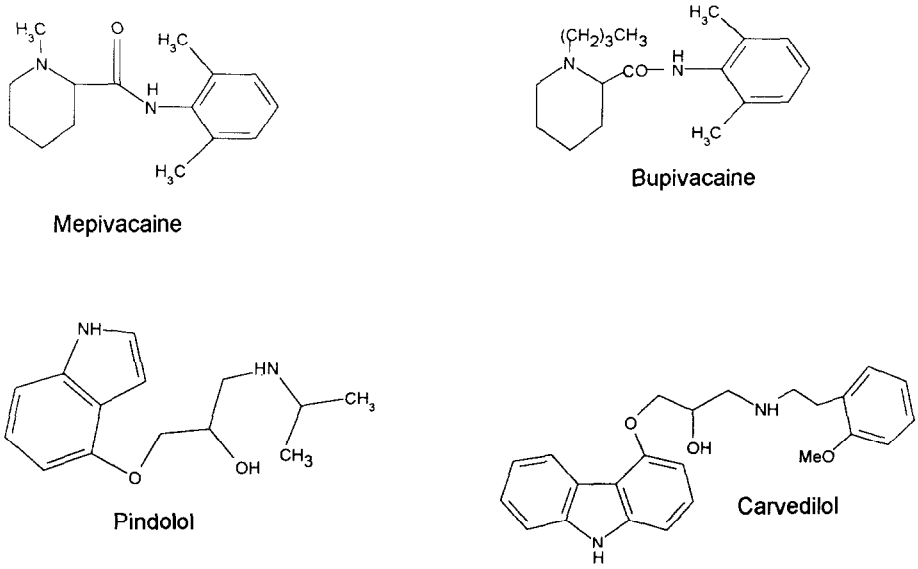


Figure 21
Structures of mepivacaine, bupivacaine, pindolol and carvedilol.

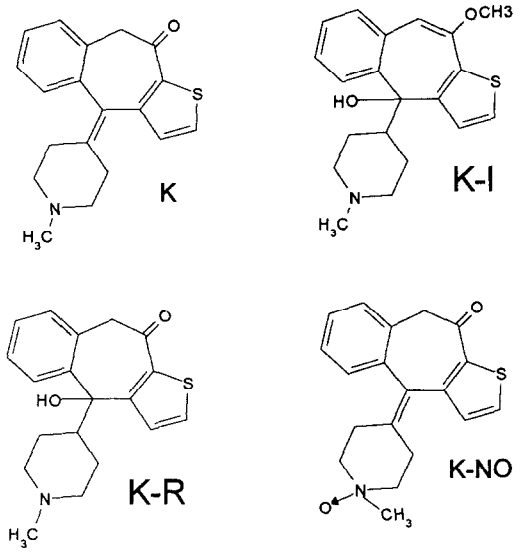


Figure 22
Structure of ketotifen (K), its synthesis intermediate K-I and the structurally related compounds K-R and the N-oxide, K-NO.

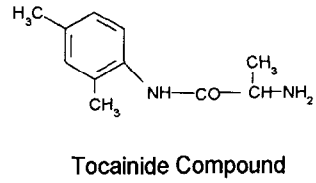


Figure 23
Structure of tocainide compound.

compound (Fig. 23) co-elute using a background electrolyte containing 50 mM γ-cyclodextrin and yet are baseline resolved when just 0.05% w/w of PVA is added to the same carrier.

Using a similar system but this time with 0.05%, w/w of hydroxyethylcellulose, it was possible to resolve the enantiomers of four tocainide compounds. Similar improvements in resolution (but not chiral) were observed when the same tocainides were run with and without PVA in the absence of chiral modifiers. Stalberg and co-workers [98] demonstrated that by adding an amphiphile such as tetrabutylammonium hydroxide to a background electrolyte containing hydroxypropyl β-cyclodextrin, and using a C_{18} coated capillary, then it was possible to baseline resolve the enantiomers of four local anaesthetic analogues including salbutamol and clenbuterol. The amphiphile adsorbed onto the C_{18}-coated capillary, eliminating any analyte adsorption resulting in excellent peak shapes and efficiencies. Tait *et al.* [99] used an anionic β-cyclodextrin, the sulphobutyl ether of β-cyclodextrin (β-CD-SBE) to resolve the enantiomers of small drug molecules containing one and two chiral centres. The enantiomers of salbutamol and terbutaline with one chiral centre were baseline resolved and it was shown that in the case of salbutamol it was possible to measure one enantiomer as an impurity at a level of 0.1%. The four enantiomers of ephedrine, with two chiral centres, were also resolved using the β-CD-SBE additive. Sepaniak and workers [100]

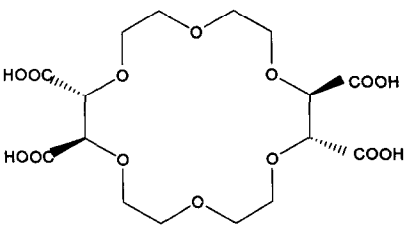
described the use of different cyclodextrin types on enantiomeric separations. Two charged binaphthyl enantiomers were analysed using α- and β-cyclodextrin but neither compound was able to resolve all enantiomers. Binaphthyl phosphate was resolved using β-cyclodextrin whereas the di-acid was unresolved. The opposite was found to be the case using α-cyclodextrin and if the α- and β-cyclodextrins were mixed in equal amounts, partial resolution of both compounds was possible. When the α-cyclodextrin exterior is modified by glycosylation, then it was possible to resolve all four enantiomers of the binaphthyl phosphate and di-acid. Nardi *et al.* [101] studied the effect of using different cyclodextrins on the resolution of a series of racemic tryptophan derivatives. Of the cyclodextrins examined, α-cyclodextrin and heptakis (2,6-di-O-methyl) β-cyclodextrin showed the best resolution of the test compounds. A resolution of 6.6 was attained for the separation of the enantiomers of 6-methyl tryptophan when using α-cyclodextrin.

(ii) Cyclodextrins incorporated into liquid gels. Guttman *et al.* [102] demonstrated the separation of a range of Dns amino acids using a polyacrylamide gel, $T = 5\%$, $C = 3.5\%$ containing 75 mM α, β or γ-cyclodextrin. β-cyclodextrin was shown to give high-performance chiral separations of the Dns amino acids tested. Swartz [103] reported the use of derivatized cyclodextrins incorporated into polyacrylamide gels for the resolution of both acidic and basic small pharmaceutical compounds. Vigh and co-workers [104] described the use of cyclodextrin-containing liquid polyacrylamide gels and drew particular attention to the role of pH, the acrylamide concentration, the cyclodextrin concentration and the organic modifier concentration of background electrolyte. Cruzado and Vigh [105] synthesized allyl carbamoylated β-cyclodextrin and then copolymerized this with acrylamide to form cyclodextrin containing gels. It was possible to provide both solid and liquid gels by adjusting the concentration of acrylamide, bis-acrylamide and the allyl cyclodextrin. The liquid gels produced in this way did not suffer from the usual problems of bubble formation, short lifetime and poor reproducibility. Baseline separations of the enantiomers of homatropine and various Dns amino acids were achieved using these liquid gel filled capillaries.

(iii) Other gels. Birnbaum and Nilsson [106] described the separation of the enantiomers of tryptophan using a capillary filled with a BSA gel. The gels were prepared by mixing bovine serum albumin (BSA) in a phosphate–acetate buffer with glutaraldehyde also in a phosphate–acetate buffer, and then pumping the mixture into the capillary. The gels were formed within approximately 10 min and then conditioned overnight at a potential of 2 kV. These gels consisted of BSA cross-linked with glutaraldehyde, and separated the enantiomers of tryptophan with a plate number N for the D-isomer of 85,000.

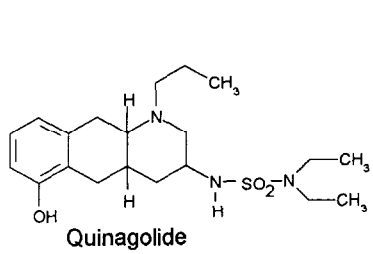
(iv) Host–guest complexion with crown ethers. Crown ethers are macrocyclic polyether rings that form cavities that are capable of forming stable complexes particularly with potassium, ammonium and protonated amines. The structure of the chiral selector [18]-crown-6-tetracarboxylic acid is shown in Fig. 24.

The mechanism responsible for chiral recognition has been proposed by several workers. Kuhn *et al.* [107] demonstrated the separation of D,L-tryptophan and D,L-dopa in a single run using 30 mM [18]-crown-6-tetracarboxylic acid in water. It was noted that the best separation was achieved without the addition of buffer to the electrolyte, the reason being that K^+ or NH_4^+ ions will compete with the sample for interaction with the receptor. Kuhn *et al.* [92, 109] demonstrated the synergistic effect of using a mixture of α-cyclodextrin and the crown ether 18C6. Using 20 mM α-cyclodextrin Kuhn *et al.* [92] were able to resolve the enantiomers of D,L-tryptophan with a resolution factor of 1.29. This increased to 5.67 when 10 mM crown ether 18C6 was used and when an electrolyte containing 20 mM α-cyclodextrin and 10 mM crown 18C6 was used, the resolution increased to 7.37. The effect of mixed chiral receptors on the separation of



18-Crown-6-tetracarboxylic acid

Figure 24
Structure of 18-crown-6-tetracarboxylic acid.



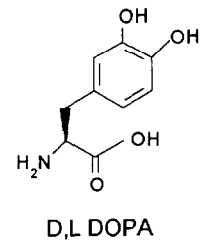
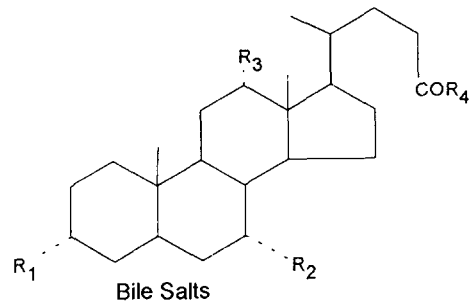


Figure 25
Structures of quinagolide and D,L-dopa.



Bile Salts	Symbol	R_1	R_2	R_3	R_4
Sodium cholate	SC	OH	OH	OH	ONa
Sodium taurocholate	STC	OH	OH	OH	$NHCH_2CH_2SO_3Na$
Sodium deoxycholate	SDC	OH	H	OH	ONa
Sodium tauro-deoxychloate	STDC	OH	H	OH	$NHCH_2CH_2SO_3Na$

Figure 26
General structure of bile salts.

quinagolide and D,L-dopa [Fig. 25] also were reported.

Quinagolide was shown to form complexes with α-cyclodextrin only, and D,L-dopa with crown 18C6. However, both could be baseline resolved using a mixture of α-cyclodextrin and crown 18C6. Kuhn *et al.* [108] demonstrated the ability of crown ethers to form complexes with compounds containing primary amino groups by screening 25 chiral amino compounds. Baseline resolution was achieved for 60% of the compounds tested, and 90% of the total showed some chiral recognition.

(v) Chiral surfactants — bile salts. Bile salts have the general structure shown in Fig. 26. They form micelles with approximately 10 monomers, and these micelles possess a helical structure with the hydrophilic region facing the interior of the micelle. Although bile salt surfactants can be used to separate a whole host of compounds, some of which will be discussed later in this review, because they are in fact inherently chiral they have been used successfully to resolve many chiral compounds. Cole *et al.* [110] demonstrated the resolution of the enantiomers of 1,1′ bi-2-naphthyl and 1,1′ binaphthyl diyl hydrogen phosphate. Using the bile salt sodium deoxycholate (SDC), a study was made of the effect of bile salt and methanol concentration on the separation. Optimum separations were achieved using 0.05 M SDC with 12% methanol. Also it was observed during this study that although bile

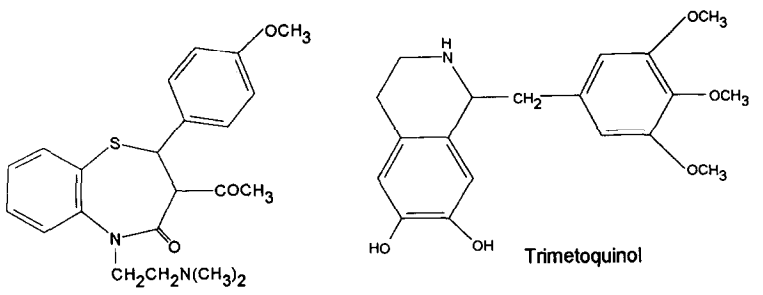


Figure 27
Structure of diltiazem and trimetoquinol.

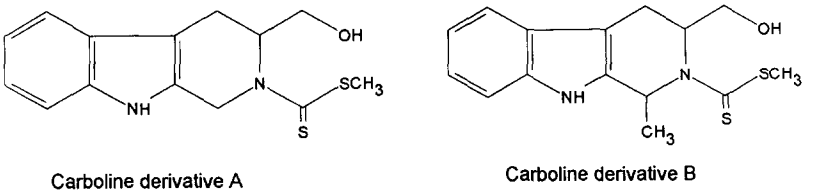


Figure 28
Structure of carboline derivatives A and B.

salts are able to interact best with compounds that have a rigid, planar structure, if they possess a charge under the operating conditions, repulsion between the bile salt micelle and the solute can lead to a lowering of interaction and consequently poor chiral recognition. Terabe and co-workers [111] studied the resolution of racemic Dns amino acids using a range of bile salts, noting that the best results were obtained using sodium taurodeoxycholate at pH 3.0. Resolution at pH 7.0 using sodium cholate as the micelle was unsuccessful; not only were the enantiomers unresolved, but in most cases the Dns amino acids were not resolved from one another. Nishi *et al.* [112] described the use of bile salts to resolve the enantiomers of diltiazem hydrochloride, trimetoquinol hydrochloride and other drugs shown in Fig. 27.

Of the bile salts studied only sodium taurodeoxycholate was successful in resolving the enantiomers of diltiazem hydrochloride and trimetoquinol hydrochloride and these were both carried out at pH 7.0. Nishi *et al.* [113] were able to resolve all four enantiomers of carboline derivatives A and B (see Fig. 28) and 2,2'-dihydroxy-1,1'-naphthyl (Fig. 29) with all of the bile salts tested, although sodium taurocholate proved to be the best bile salt for the separation of the four carboline enantiomers at neutral pH. However, the dinaphthyl derivative was found to be best resolved by the deoxy bile salts and this was thought to be due to the inclusion of the naphthalene skeleton into the micelle due to the lack of the hydroxyl group in the deoxy derivative, thus enhancing enantiomeric interaction.

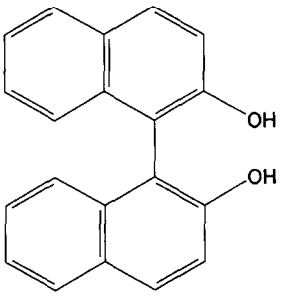


Figure 29
Structure of 2,2'-dihydroxy-1,1'-dinaphthyl.

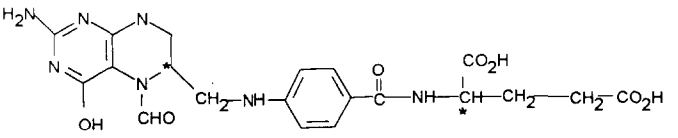
(vi) Chiral surfactants — sodium N-dodecanoyl-L-valinate (SDVal). Using SDVal, Dobachi *et al.* [114, 115] were able to resolve the enantiomers of N-acetylated amino acid esters, particularly the N-(3,5-dinitrobenzoyl) isopropyl esters. Chiral recognition resulted from hydrophobic entanglement of the chiral amide unit into the chiral micelle core. Using a comicellar solution of SDVal and SDS, it was possible to resolve the enantiomers of N-(3,5-

dinitrobenzoyl) *o*-isopropyl esters of alanine, valine, leucine and phenylalanine. The alanine derivative being the least hydrophobic, was eluted first, and the most hydrophobic, phenylalanine last. Resolution of the mixture was shown to be improved by the addition of between 5 and 10% methanol with a corresponding increase in the micellar elution range. Dobachi [114] pointed out that without esterification, no resolution of the enantiomers of the corresponding 3,5-dinitrobenzoyl amino acids was possible. Studies made with sodium N-dodecanoyl-L-alaninate (SDAIa) produced smaller separation factors for the solutes used with SDVal when used at the same concentration. Otsuka and Terabe [116] reported on the effects of the addition of both methanol and urea on the resolution of PTH amino acid enantiomers using SDVal. Although it was possible to resolve the enantiomers of some PTH amino acids, it was only possible to resolve the enantiomers of PTH-DL-methionine using between 5 and 10% methanol. However, peak shapes were very poor even with the addition of methanol. Much of the tailing was attributed to adsorption of the solute onto the surface of the capillary wall, and this was found to be greatly reduced by the addition of urea.

(vii) Chiral resolution via complexation. Gassmann *et al.* [117] reported on the use of Cu(II) complexes formed with L-histidine in order to resolve the enantiomers of the Dns amino acids. Separation is achieved by diastereomeric interaction between the enantiomers of the amino acid and the chiral Cu(II)–L-histidine complex. Because this complex carries a positive charge under the operating conditions, those amino acids most strongly bound to the complex are eluted fastest. Gozel and co-workers [118] also studied the resolution of Dns amino acids, this time using a Cu(II)–aspartame complex. The separation mechanism proposed involves the formation of a six membered ring consisting of Cu(II), the α-amino and β-carboxyl groups of the aspartyl residue of the aspartame molecule. Because this six membered ring is less stable than the five membered ring involving the α-amino and α-carboxyl groups of an amino acid, an amino acid added to the electrolyte containing the Cu(II)–aspartame complex is able to replace one aspartame ligand and form a ternary complex. Although it was possible to resolve up to 14 Dns amino acids, including the enantiomers of neutral amino acids, it was not possible to resolve them from each other. This was overcome by the addition of sodium tetradecyl sulphate in order to carry out MEKC. Under these conditions the neutral amino acids partition into the micellar phase and are fully resolved. Similar work was carried out by Cohen *et al.* [119] who used a mixed micelle consisting of SDS and N,N-didecyl-L-alanine in the presence of Cu(II), to resolve the enantiomers of Dns amino acids. Although this is referred to as a mixed micelle system, the L-alanine derivative itself does not form a micelle, but is incorporated into the SDS micelle in such a way that hydrophilic centres of the L-alanine are on the surface, while the hydrophobic tails are in the interior. Separation of enantiomers is based on differential metal chelate complexation at the surface of the micelle.

(viii) Miscellaneous chiral additives. D'Hulst and Verbeke [120] described the use of maltodextrins to resolve the enantiomers of 2-arylpropionic acid non-steroidal anti-inflammatory drugs, coumarinic anticoagulants as well as the diastereoisomers of cephalosporin antibiotics. Maltodextrins are described as saccharide polymers having D-glucose units linked primarily by α (1–4) bonds. Baseline resolution of the enantiomers of ibuprofen was readily achieved using a complex maltooligosaccharide mixture as chiral additive. The diastereoisomers of the cephalosporin antibiotics DL-cephalexin and DL-cephadroxyl also were baseline resolved using an electrolyte containing 1% of the maltodextrin Glucidex 2. Using the same modifier at a concentration of 2.5%, it was possible to resolve the enantiomers of four coumarinic anticoagulants including warfarin. Barker *et al.* [121] used an electrolyte containing the chiral discriminator bovine serum albumin in order to resolve the enantiomers of leucovorin (Fig. 30).

Although it is possible to achieve baseline resolution of leucovorin using BSA in uncoated capillaries, because of protein interactions with the capillary surface, migration time reproducibility is poor and capillaries had to be changed every 10–15 runs to obtain reliable assays. However, the problem was overcome by permanent coating of the capillary using a 20% solution of polyethylene glycol.



Leucovorin

Figure 30
Structure of leucovorin.

(ix) Derivatization. Tran and co-workers [122] were able to resolve amino acid enantiomers and peptides using either D- or L-Marfey's reagent to form diastereomeric pairs capable of resolution by free solution capillary electrophoresis at low pH. At high pH (8.5) no resolution of the enantiomers was observed. However, when the analysis is carried out by MEKC at pH 8.5, there is a dramatic improvement in the separation and peak shapes were vastly improved. Similar high efficiency results were observed for a range of di- and tripeptides.

Electrochromatography

Electrochromatography is a term coined to describe narrow bore packed column separations where the liquid mobile phase is driven by an electrical phenomenon rather than hydraulic pressure as in conventional liquid chromatography. The driving force in electrochromatography results from the electrical double layer at the liquid–solid interface as illustrated in Fig. 31. It is possible to manipulate the resulting electroosmotic flow by adjusting among other things, the pH and ionic strength off the mobile phase, and the type and concentration of the organic modifier.

Jorgenson and Lukacs [123] described the separation of 9-methylanthracene and perylene on a 10 μm reversed-phase packing using acetonitrile as the mobile phase and a voltage of 30 kV to drive the liquid through the packing. The relationship between the flow through a packed capillary and the applied field is given by the expresion:

$$V_{eo} = \frac{\epsilon \zeta E}{4\pi\eta}, \qquad (19)$$

where ϵ = dielectric constant, ζ = zeta potential and η = viscosity of the solvent.

Jorgenson and Lukacs pointed out from this equation that the velocity of flow is independent of the geometry and size of the channels in the packing. Therefore packing irregularities would be expected to cause little peak broadening and therefore a narrow particle size distribution may not be as important as in HPLC in order to produce efficient chromatography. Knox and Grant [124] pointed out that for electrically driven chromatography the velocity u is proportioned to d^2, where d = the average particle diameter of the packing. However, since d does not enter the flow equation given below, it would appear that the velocity is independent of the particle diameter, namely,

$$u = \frac{\epsilon_o \epsilon_r \zeta E}{\eta}, \qquad (20)$$

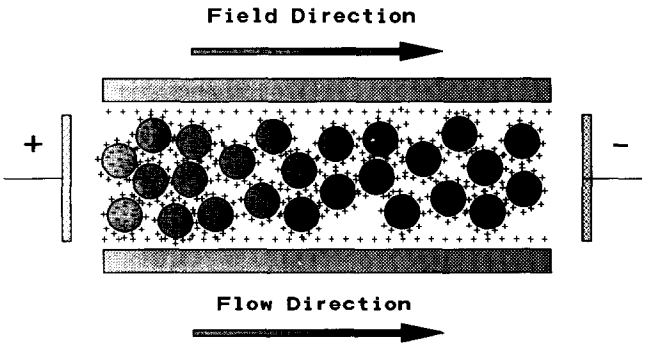


Figure 31
The origin of electroosmotic flow in a packed capillary, (by permission of Capital HPLC Specialists).

where ϵ_o and ϵ_r are the relative permitivity and vacuum permitivity, respectively and ζ = zeta potential.

Knox pointed out that the consequences of this are that it should be possible to electrically pump liquids through columns packed with very small diameter particles with no increase in the potential gradient across the packed capillary. Theory also predicts that the use of small particles should lead to small values of H, i.e. large values of N. The combination of small diameter particles together with the maintenance of plug-flow resulting from electroosmosis, should give rise to highly efficient chromatography, if packing such columns can be perfected. Whereas Jorgenson and Lukacs [123] have achieved reasonable efficiencies using 10 μm ODS packing, Knox and Grant [124] using 5 μm Hypersil ODS were able to separate six polynuclear aromatic hydrocarbons with efficiencies at least as good as could be achieved using a pressure driven system, with reduced plate heights below two. In a later report, Knox and Grant [125] studied the effect of particle size on electroosmotic velocity and showed that it remains unaffected by particle size down to at least 1.5 μm. Using drawn packed capillaries with both 3 μm and 5 μm Hypersil, which they subsequently derivatized with an octadecyl silyl group, they were able to compare pressure and electrically driven chromatography. In both instances the electrically driven system gave higher efficiencies than the pressure driven system. Detection in electrochromatography was traditionally carried out using fluorescence detection through the packed bed, but this would be technically impossible using UV detection. In order to overcome this problem several approaches have been adopted. Van Soest *et al.* [126] connected a capillary packed with 3 μm c_{18} packing material to a flow cell using a Teflon sheath. They were then able to resolve six aromatic hydrocarbons with efficiencies of 178,000 plates m^{-1}. By far the most elegant methods of on-line detection are those described by Yamamoto *et al.* [127] and Grant [private communication] involving the production of capillaries with small retaining frits within the capillary allowing detection using conventional CZE equipment with the absolute minimum of extra-column broadening as illustrated in Fig. 32.

Yamamoto *et al.* [127] demonstrated examples of electrokinetic reversed-phase chromatography using 3 μm Hypersil ODS, resulting in efficiencies of approximately 50,000 plates for benzyl alcohol with a reduced plate height of 1.8. The nine component mixture of benzene derivatives were resolved in under 10 min using a 285 mm long capillary and an applied voltage of 45 kV. Using 1.6 μm ODS packing, it was possible to push up the efficiency to more than 240,000 plates on a 680 mm long capillary giving a reduced plate height of 1.9 for the unretained thiourea peak. However, a dramatic decrease in plate number was observed for retained peaks and consequently toluene ($k' = 0.05$) had a plate number of only 47,000 or approximately 1/5th of that for an unretained peak. Grant [private communication] using a 3.0 μm octyl material has successfully packed capillaries that have efficiencies in excess of 200,000 plates m^{-1}. When tested using pressure driven capillary LC this same column had an efficiency of only 140,000 plates m^{-1}, illustrating the advantage of electrochromatography over pressure driven chromatography. More recently using 1.5 μm porous ODS, Grant has achieved efficiencies of 140,000 plates per 50 cm capillary with a reduced plate height of 2.36.

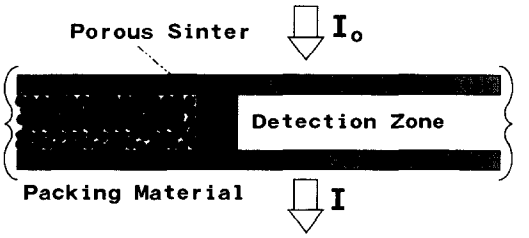


Figure 32
Illustration of on-column detection in packed capillary electrophoresis, (by permission of Dr I. Grant, Capital HPLC Specialists).

An example of an electrochromatogram obtained using 1.5 μm ODS is shown in Fig. 33.

Conclusion

Capillary chromatography is experiencing an almost exponential rate of growth and even during the period this review was being written many new applications have been reported. To have attempted to include the very latest work would have meant that this article would never have been completed. In preparing this review it has been the author's intention to show that capillary electrophoresis is now an established

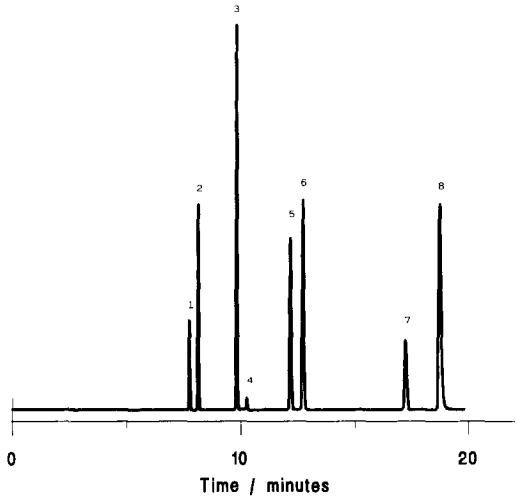


Figure 33
Chromatogram of test solutes using electrically driven chromatography. Test solutes: (1) acetone; (2) benzyl alcohol; (3) dimethyl phthalate; (4) acetophenone; (5) anisole; (6) methyl benzoate; (7) toluene; (8) phenyl benzoate. Conditions: column: 50 cm × 50 μm i.d., packed with 1.5 μm ODS; voltage 30 kV; detection at 195 nm; room temperature, mobile phase — 70% CH_3CN–H_2O (salt free), (by permission of Dr I. Grant, Capital HPLC Specialists).

technique for pharmaceutical and biomedical analysis with the potential of becoming the premium analytical method.

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[Received for review 5 July 1993; revised manuscript received 11 August 1993]

Appendix — Abbreviations

CZE	capillary zone electrophoresis
D	diffusion coefficient
E	potential field strength
eof	electroosmotic flow
MEKC	micellar electrokinetic chromatography
N	separation efficiency — (plate number)
R_s	resolution
t	time required for a zone to migrate the length of a tube L
t_{mc}	micelle migration time
t_r	retention time in MEKC
V	applied voltage
μ	electrophoretic mobility
μ_{osm}	electroosmotic flow coefficient
V_{eo}	electroosmotic velocity
V_{ep}	velocity of migration
σ^2	peak dispersion
ϵ	dielectric constant
ϵ_o	relative permitivity
ϵ_v	vacuum permitivity
ζ	zeta potential
η	viscosity of the electrolyte
SDS	sodium dodecyl sulphate
PVA	polyvinyl alcohol
BSA	bovine serum albumin