ChemComm FEATURE ARTICLE

Capillary electrophoresis: a major advancement in separation technology

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Capillary electrophoresis (CE) is becoming a powerful tool for the resolution of complex mixtures of analytes. It complements and is, in many ways, orthogonal to high-performance liquid chromatography (HPLC), the more traditional 'wet' separation technique, available in the majority of analytical laboratories. CE can be used to achieve rapid and efficient separations of mixtures of analytes of various sizes, hydrophobicity and states of ionization. The most common modes of operation of CE are open-tubular or free-zone, micellar electrokinetic capillary chromatography (MECC), gel, isotacophoresis and isoelectric focusing (IEF). These modes of operation can be used consecutively simply by changing buffer constituents. The ease of use and structural information obtained from CE is making it an important technique used not only by analysts but also by scientists from other disciplines, such as physical chemists and biochemists. In this article we have outlined the increasing role that CE is now playing in our laboratory and the contribution we have made in the development of CE methodology.

Introduction

Until recently high-performance liquid chromatography (HPLC) has been the most common 'wet' technique used to separate a variety of molecules of widely different physicochemical properties, especially size, hydrophobicity and ionic nature. Differentiation of analytes based on these molecular characteristics can be achieved by choosing a column containing the appropriate stationary phase. Size exclusion, normal and reverse phases and ion exchange are the most common modes of HPLC. On the other hand gel electrophoresis has been most useful in the resolution of biopolymers such as proteins, nucleic acids and oligosaccharides, and is widely used in biochemically orientated laboratories. The latter technique is not generally suitable for the resolution of small organic molecules as these will readily diffuse from the gel matrix during fixing and staining procedures.

It has been recognised[1,2] for almost half a century that the excellent heat-transfer properties of capillaries can lead to the rapid and efficient resolution of analytes. A major problem encountered in early studies on free-zone capillary electrophoresis was one of detection. This made the use of relatively wide-bore capillaries [internal diameter (i.d.) of the order of 200 μm] necessary; conductivity and UV transmission detectors were used with these capillaries.[3] The interest in capillary electrophoresis increased dramatically after Jorgenson and Lukacs published their work on the use of capillaries of i.d. < 100 μm coupled with sensitive detection technology in a simple instrumental set-up.[4,5] Separation efficiencies of the order of 4×10^5 were demonstrated by these authors, in their studies on the resolution of mixtures of fluorescamine and dansyl derivatised amino acids and peptides, using open-tubular silica-based capillaries and fluorescence detection. The immense popularity of CE over the past fifteen years is reflected in the large number of publications that have appeared following the studies of Jorgenson and Lukacs. Several books have also been published[6,7] about CE, together with at least a hundred reviews[8,9] appearing during the period 1993–1995. The introduction of commercial instrumentation in 1988 played a considerable role in the speed of development and application of capillary electrophoresis.

Capillary electrophoresis (CE) has now become an essential tool in an analytical laboratory. It is complementary to the other more traditional 'wet' separation techniques, such as HPLC and gel electrophoresis. We have used CE in our laboratory to solve a number of analytical problems of ever increasing complexity. In this article we introduce the various modes of operation of CE and outline some of the contributions that we have made to the development and application of this technique.

The main advantage of CE is the combination of high resolution and simplicity. Samples to be analysed are usually applied at the 'positive' or the anodic end of the capillary using pressure or electrokinetic injection (Fig. 1). On the application of a high voltage (in the range 5–30 kV) analytes travel along the capillary and are detected directly, most commonly by a UV absorbance detection system. Non-absorbing analytes can be detected either after derivatisation with an appropriate chromophore or fluorophore, or indirectly using an absorbing species in the separation buffer. Unlike the case of HPLC, where the change from one mode of operation to another necessitates a change in stationary phase, changing the mode of operation in CE very often only involves the introduction of different constituents in the separation buffer. The amount of sample used in CE is also at least 1000-fold lower than that used in HPLC. Thus applying CE to routine analysis can not only economise in the use of expensive HPLC reverse phases (columns) but may also be advantageous when the amount of sample to be analysed is in short supply.

A number of modes of operation of CE have been developed over the past fifteen years. The most common are free-zone capillary electrophoresis (CZE), micellar electrokinetic capillary chromatography (MECC), affinity electrophoresis, gel electrophoresis, isoelectric focusing (IEF) and isotacophoresis. CZE is one of the earliest CE modes and studies using this technique account for over 60% of the publications in the open literature. This technique has been used widely for the resolution of simple organic and inorganic analytes, peptides,

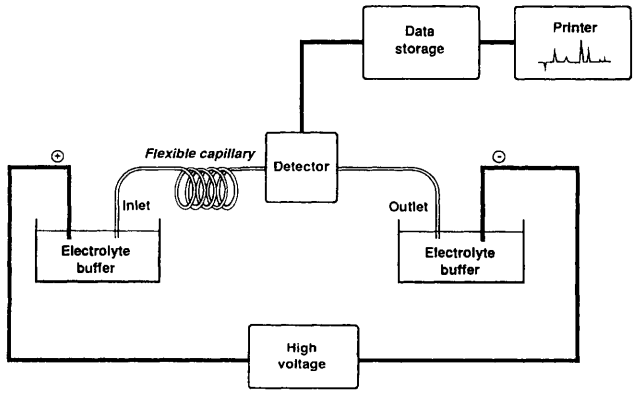


Fig. 1 Capillary electrophoresis instrument

proteins and oligosaccharides. The popularity of MECC is steadily increasing because, unlike the other modes of operation, this technique can be used for the separation of uncharged molecules.

Affinity capillary electrophoresis is becoming a powerful technique for the resolution of analytes and in the evaluation of binding interactions and in determining kinetic and thermodynamic parameters. Capillary gel electrophoresis has been found to be useful for the separation of mixtures of biopolymeric species, especially proteins and DNA strands; size is an important factor for the separation of these analytes. IEF is increasingly being used to focus proteins in relation to their isoelectric point (pI) values. Unlike slab-gel IEF, no staining and destaining procedures are required and analytes are dynamically pushed across the detection window by applying pressure and a low voltage simultaneously. Isotacophoresis is not very often used as a separation technique. However, this mode of operation has been found to be useful in the preconcentration of samples before analysis.

Basic principles of free-zone electrophoresis separations

In the case of free-zone electrophoresis separations occur as a result of the movement of ions in an applied electric field. The observed electrophoretic mobility (μ_{total}) is made up of the vector sum of the electrophoretic mobility (μ_{EP}) and the electroosmotic mobility (μ_{EO}). These two mobilities are expressed by eqns. (1) and (2).

$$\mu_{EP} = q/6\pi\eta r \quad (1)$$

$$\mu_{EO} = \varepsilon_0 - E\xi/4\pi\eta \quad (2)$$

where q is the charge of an ion, η is the viscosity of the buffer solution, r is the hydrodynamic volume of an ion, ε_0 is the relative permittivity, E is the electric field strength and ξ is the zeta potential.

In the above equations it is only in the case of electrophoretic mobility that charge (q) plays a role in determining the effective mobility and direction of migration of ions. Positively and negatively charged species will tend to move towards the cathode and anode, respectively. Neutral analytes will not have an electrophoretic mobility.

In contrast to electrophoretic mobility, electroosmotic mobility (EO) is the same for charged and neutral analytes. EO is thought to occur due to the presence of dissociated –SiOH groups on the surface of the silica capillary. The pK_a for the dissociation of silanol groups is about 6.5 so that the pH of the buffer plays a role in the degree of ionization of these groups. The negatively charged silanol groups attract positively charged ions from the buffer creating an electrical double layer. The application of an electric field across the capillary results in positively charged ions in the bulk solution flowing from anode to cathode under the influence of electroosmosis. Thus at neutral to alkaline pH the electrophoretic and electroosmotic mobilities of cations are both in the direction of the cathode, anions will only migrate towards this electrode if the electroosmotic flow is greater than their electrophoretic flow (in the direction of the anode) and all neutral species migrate at the same velocity as the electroosmotic front. To summarise, the order of migration of analytes towards the cathode in free-zone capillary electrophoresis is cations, neutrals, and anions.

Some examples of free-zone electrophoresis separations

An electropherogram of the separation of the anti-Parkinson drug Ropinirole **1** and closely related molecules **2–5** demonstrates clearly the mechanism of separation in free-zone electrophoresis [Fig. 2(*a*)]. At a pH of 7.82 this drug is positively charged. Although **2** is also expected to carry a positive charge under these conditions the small difference (<0.5) in the pK_a of a tertiary and a secondary amine leads to the excellent resolution shown. In the case of **3** the pK_a of the hydroxy group is about 10 so that this analyte is expected to be fractionally less positively charged than either **1** or **2** at the pH of this analysis, resulting in the observed longer migration time of **3**. The hydroxy group in **4** has a much lower pK_a value than that of a phenol so that this analyte is expected to be zwitterionic in character at pH 7.82. Compound **5** is the last to migrate as the electron withdrawing nature of the $-COCF_3$ group makes this amide negatively charged.

The mechanism of separation and the versatility of free-zone electrophoresis can again be demonstrated by the resolution of the mixture of the six simple carboxylic acids, shown in Fig. 2(*b*). As these analytes do not absorb much UV light at wavelengths above 200 nm, indirect photometric detection was used by including an absorbing compound, benzoic acid, to the background electrolyte. The order of migration of these acids is in relation to their size so that heptanoic acid migrates first and trifluoroacetic acid last. The electrophoresis of these organic acids was run at pH 8, which is well above the pK_a values of these molecules. The migration behaviour observed can be explained in terms of the charge density of the carboxylate solutes as follows: the electroosmotic velocity (bulk flow in the direction of the cathode) of all the carboxylate anions is the same; on the other hand the larger the dynamic radius of a negatively charged analyte the lower its charge density, and the slower it will tend to migrate electrophoretically in the opposite

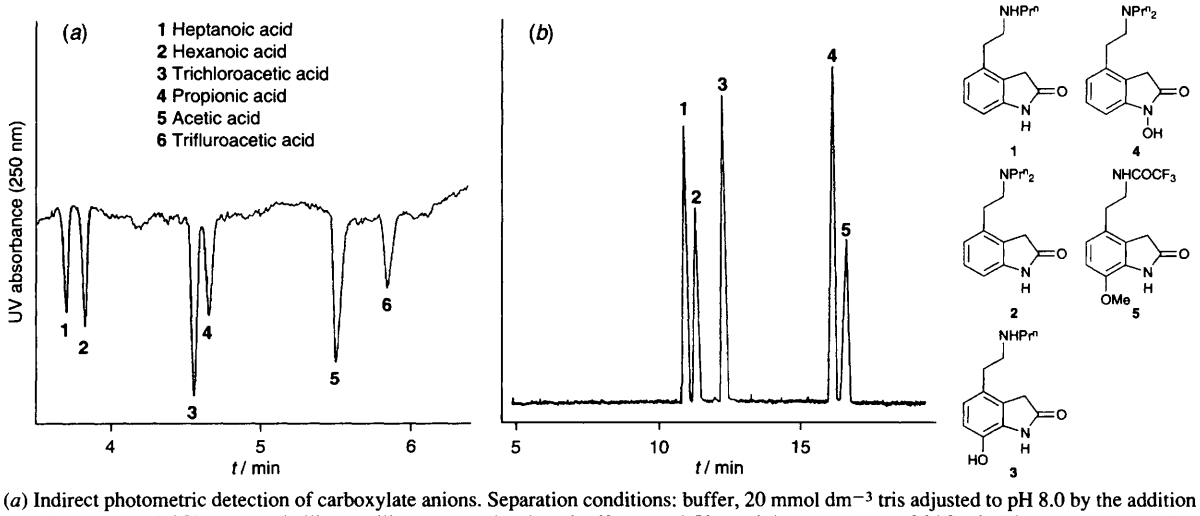


Fig. 2 (*a*) Indirect photometric detection of carboxylate anions. Separation conditions: buffer, 20 mmol dm^{-3} tris adjusted to pH 8.0 by the addition of solid benzoic acid; voltage, 10 kV; uncoated silica capillary, separation length, 60 cm and 50 μm i.d.; temperature, 25 °C. (*b*) Electropherogram showing the resolution of the drug ropinirole and a number of related compounds. Separation conditions: buffer, 20 mmol dm^{-3} sodium phosphate (pH 7.82); voltage, 10 kV; uncoated silica capillary, separation length, 50 cm and 50 μm i.d.; temperature, 25 °C.

direction towards the anode (the point of injection). As the observed mobility is the vector sum of the electroosmotic and electrophoretic mobilities this leads to the order of migration shown in Fig. 2(*b*).

Free-zone electrophoresis is also a suitable technique for the separation of mixtures of analytes considerably more complex than simple organic solutes. Eminase is an anisoylated plasminogen-streptokinase activator complex which is used as an intravenous thrombolytic agent for acute myocardial infarction. In commercial formulations of this drug serum albumin is added to stabilise the activator complex. Fig. 3 shows the electropherogram of the components of eminase; all peaks are well resolved, including the virtual separation of the two glycosylated forms of plasminogen. A low acidic pH was chosen for this separation to minimise electroosmotic flow and to ensure that all the proteins analysed are positively charged. The addition of hydroxypropylmethylcellulose improved resolution by further minimising electroosmotic flow so that separation was achieved predominantly by the electrophoretic mobility of the analytes. This CE assay has been shown[10] to be highly reproducible and allows the rapid and precise analysis of all the protein components of eminase.

The addition of chiral components to the separation buffer leads to chiral discrimination of racemic mixtures. Oligosaccharides,[11,12] crown ethers[13] and proteins[14] have been used as buffer additives in free-zone electrophoresis. As in the case of other separation techniques, resolution of enantiomers in a racemic mixture will take place (*i*) if transient (non-covalent) complexes are formed between these related 'guests' and the 'host' molecule and (*ii*) if the free energy of formation is different for the two complexes. Fig. 4 shows the resolution of the enantiomers from an anti-diabetes drug and the corresponding ester pro-drug. This enantiomeric discrimination was only possible when β-cyclodextrin, which has a number of chiral centres, was included in the separation buffer. The mechanism of chiral discrimination most probably involves inclusion of the aromatic moieties of these drugs in the cyclodextrin cavity and interaction of the rest of the 'guest' molecules with the chiral surface of the 'host'. The order of migration of the two pairs of enantiomers is also as predicted, that is the more positively charged pro-drug migrates faster than the drug itself.

Micro-preparative free-zone capillary electrophoresis

As in the case of most separation techniques peak matching in capillary electrophoresis very often is not enough to secure the identity of an analyte. The collection of the individual components of a mixture of solutes after separation may be necessary for spectroscopic or mass spectrometric verification of a chemical structure. Fraction collection in CE is more challenging than that from the other more traditional 'wet' separation techniques, HPLC and thin layer chromatography (TLC). The flow rate of buffer through the capillary is very low, 100 nl min^{-1} or less.

This leads to minute collection volumes. Moreover, diffusion of analytes has to be minimised when the current is unavoidably interrupted when the capillary is transferred from one collection vial to another. Collection in a vial containing 10 μl of buffer or more results in significant sample dilution. Despite these difficulties several successful attempts are recorded in the literature where samples have been collected for post-separation analysis.[15–17] Some time ago we reported[16] the separation and collection of peptide fragements resulting from the tryptic digestion of the calcium regulating drug elcatonin. Fig. 5 shows an electropherogram after a relatively high concentration (*ca.* 10^{-4} molar) of the tryptic mixture was dynamically injected in the silica capillary. Electrophoresis was carried out in deuteriated water which has a viscosity about 25% higher than that of water; this reduced electroosmotic flow and diffusion of the analytes during the collection period. The presence of one arginine and two lysine residues leads to the expected four peaks. The four collected fractions from this one experiment were subjected to Edman sequencing. Table 1 shows the yields obtained from three of the peptides. As expected no sequence was possible from fraction 4 (residues 1–11) which is a

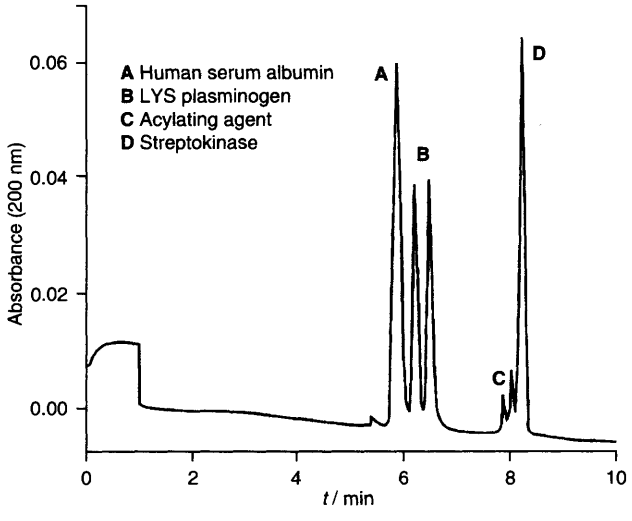


Fig. 3 Free-zone CE analysis of the components of eminase. Separation conditions: buffer, 100 mmol dm^{-3} sodium phosphate (containing 0.01% hydroxyprophymethyl cellulose) pH 2.5; voltage, 25 kV; uncoated capillary, separation length, 50 cm and 75 μm i.d.; temperature, 35 °C.

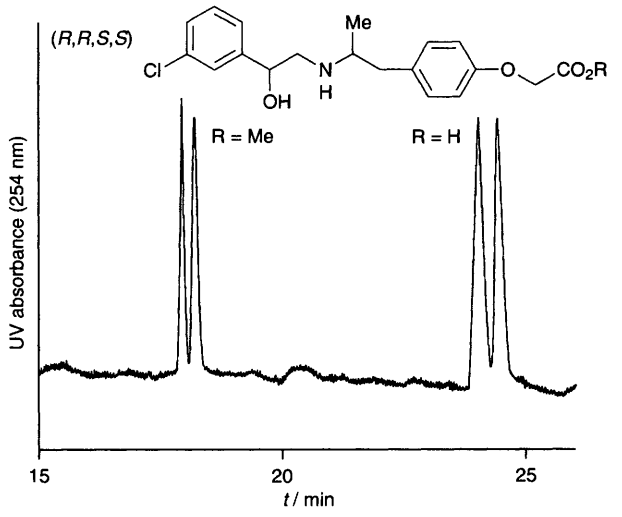


Fig. 4 Electropherogram of the enantiomers of an anti-diabetes drug and its ester pro-drug. Separation conditions: buffer, 100 mmol dm^{-3} sodium phosphate (containing 0.01% hydroxyprophymethyl cellulose) pH 2.5; voltage, 25 kV; uncoated capillary, separation length, 50 cm and 75 μm i.d.; temperature, 35 °C.

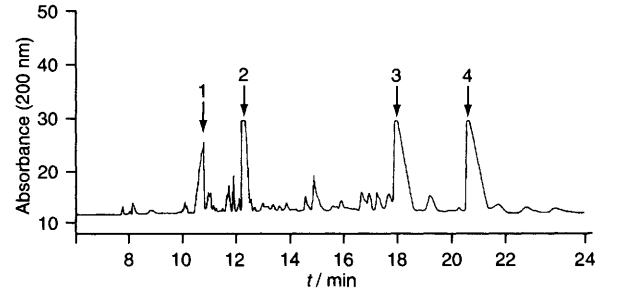


Fig. 5 Electropherogram of a relatively large injection (24 nl from a digest of about 5 mg ml^{-1} elcatonin) of the tryptic peptide fragments from elcatonin. Separation conditions: buffer, 150 mmol dm^{-3} sodium phosphate pD 2.93; voltage, 20 kV; uncoated capillary, separation length, 50 cm and 50 μm i.d.; temperature, 35 °C.

peptide derived from the derivatised (blocked) N-terminus of elcatonin.

The use of phytic acid in free-zone capillary electrophoresis

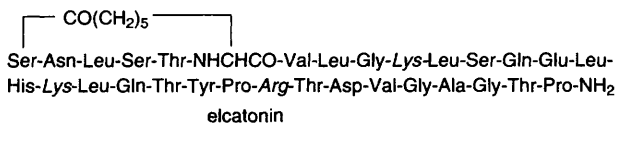
Basic proteins and peptides with pI >9 are usually found to interact with the negatively charged inner surface of the capillary when electrophoresis is carried out at a pH lower than or close to their pI value. This coulombic interaction leads to peak 'tailing' or total adsorption of the analyte to the capillary surface. Analysis at pH around 2 or 12 can overcome these problems either due to the complete suppression of the dissociation of –SiOH groups or because the capillary wall and the protein are both negatively charged.[18] Unfortunately these extremes of pH lead to the rapid deterioration of the silica surface, a high current and a loss of reproducibility from one run to another. Although coated capillaries[19] can be used effectively in the analysis of basic proteins and peptides, they can be expensive and may be unsuitable for use over a wide pH range.

Two years ago we made the discovery[20] that the addition of the sodium salt of myoinositol hexakis(dihydrogenphosphate) to the separation buffer at pH 6.0–9.5 can lead to improved separations. This polyphosphate is widely available commercially and is commonly known as phytic acid. The six phosphate groups of this naturally occurring molecule have pK_a values ranging from 1.9 to 9.5 so that it will exist as a polyanion over a wide pH range. Other advantages of the sodium salt of phytic acid are its high solubility in aqueous media and its low absorbance at wavelengths >200 nm.

We have used the sodium salt of phytic acid as a buffer additive in free-zone CE to improve the analysis of several basic peptides and proteins. This polyanion effectively ion-pairs with these positively charged analytes leading to transient complexes which are overall negatively charged and are therefore repelled from the likewise negatively charged silica surface.[21] Comparison of electropherograms in the presence and absence of this polyanion has allowed us to observe more clearly differences in the tryptic digests of two haemoglobin variants,[22] namely normal haemoglobin (HbA) and haemoglobin G-COUSHATTA B22 (HbG). In the latter variant an alanine residue replaces glutamic acid found at position 22 of the β-chain of normal haemoglobin. The total number of peptides from the tryptic digest of haemoglobin that absorb UV light at 214 nm is expected to be *ca.* 27. As shown in Fig. 6 less than half this number of peaks is observed in the electropherograms for both the digests of HbA and HbG in the absence of the sodium salt of phytic acid. In the presence of a 15 mmol dm^{-3} concentration of this polyanion in the separation buffer both the resolution and the number of signals have increased considerably.

Table 1 Sequencing yields (pmol) for peptide fractions 1–3

Cycle	Fraction 1 (Residues 12–18)	Fraction 2 (Residues 19–34)	Fraction 3 (Residues 25–32)
1	Leu (24)	Leu (13)	Thr (12)
2	Ser (8)	Gln (13)	Asp (13)
3	Gln (13)	Thr (5)	Val (9)
4	Glu (8)	Tyr (4)	Gly (20)
5	Leu (15)	Pro (9)	Ala (15)
6	His (2)	Arg (2)	Gly (16)
7	Lys (2)		Thr (5)
8			Pro (3)



elcatonin

Micellar electrokinetic capillary chromatography (MECC)

This technique, originally introduced[23,24] by Professor Shigeru Terabe, is sometimes referred to as MEKC, which stands for 'micellar electrokinetic chromatography'. As both names imply, this mode of operation of CE involves the use of micelles as buffer additives and allows the migration and resolution of analytes that are uncharged during electrophoresis. This method bears some similarity to reversed-phase HPLC in that the migration of a neutral analyte depends on the distribution coefficient between the micellar (hydrophobic) phase and the non-micellar (aqueous) phase. Negatively charged surfactants such as sodium dodecyl sulfate (SDS) and bile acids are commonly used in MECC. The polyanionic micelle migrates slowly in the direction of the cathode primarily under the influence of the electroosmotic velocity. The different free energies of partitioning of neutral analytes give rise to their resolution.

The migration of analytes in MECC is defined by the capacity factor k', which is the ratio of the amount of analyte incorporated within the micelle to that in the aqueous phase. The relation between k' and the corresponding migration time t is given by eqn. (3), where t_{mc} and t_0 are the migration times

$$k' = \frac{t_R - t_0}{t_0\{1 - (t_R/t_{mc})\}} \quad (3)$$

of the micelle and the bulk aqueous solvent (excluded from the micelle), respectively. The migration window stretches between these two migration times and hydrophilic solutes have faster migration times than ones which are relatively hydrophobic in nature. As t_{mc} approaches infinity the above equation reduces to the following simple relationship, which is analogous to the definition of capacity factor in chromatography [eqn. (4)].

$$k' = (t_R - t_0)/t_0 \quad (4)$$

Estimation of octanol–water partition coefficients by MECC

The dependence of the separation of analyte mixtures in MECC in relation to their lipophilicity has encouraged us to use this technique for the rapid estimation of octanol–water partition coefficients. The ease of partitioning of compounds through a variety of biological membranes very often has a considerable influence on their activity or efficacy. The logarithm of the partition coefficient of a biologically active compound between *n*-octanol and water (log *P*) often features prominently in quantitative structure–activity correlations. The measurement of log *P* by the 'shake-flask' method is rather laborious, especially when this physico-chemical parameter has to be determined for a number of compounds. For a number of years reversed-phase HPLC has been the only indirect method for the rapid estimation of log *P* for organic molecules.[25] In our experience the retention times or capacity factors of structurally unrelated solutes correlate poorly with log *P*.

The use of SDS in MECC for the rapid estimation of log *P* has been reported by us and by other authors.[26,27] Recently we have shown[28] that capacity factors of a number of structurally unrelated standard compounds and drug substances can be adequately related to log *P* when deoxycholic acid micelles are used in MECC. These micelles differ from those formed by SDS both in shape and in the number of monomers forming each micelle. In the case of SDS about 80 monomers form a sperical micelle compared to 3–4 forming cylindrical micelles in the case of deoxycholic acid. Fig. 7(*a*) shows an electropherogram obtained for the separation of a mixture of drugs using MECC. A linear plot of the logarithm of capacity factors of 32 analytes, including these drugs, against log *P* is given in Fig. 7(*b*). Capacity factors were measured in relation to the migration of halofantrine, an anti-malarial drug, which completely associates with the micelles. This methodology for estimating log *P* cannot be satisfactorily applied to negatively

charged molecules. Such compounds will be electrostatically repelled from the micelle and will migrate under the free-zone mode.

Using MECC in the analysis of complex mixtures of oligosaccharides

The importance of carbohydrates in the biological activity of glycoproteins is increasingly being appreciated. Oligosaccharide microheterogeneity can be involved in a variety of roles: in recognition processes, in protecting the protein from proteolytic degradation, and in the modulation of immunogenicity. A number of separation techniques, mainly HPLC and slab-gel electrophoresis have been used in the characterisation of oligosaccharides. More recently capillary electrophoresis has been playing an increasing role for the fingerprinting of oligosaccharides, as this technique can combine rapid analysis with high resolving power. Although some glycans can absorb UV light of wavelengths around 200 nm, high sensitivity detection requires labelling these analytes with a suitable chromophore or fluorophore. This is usually carried out by reductive amination at the reducing end of the carbohydrate. Aromatic amines, such as 2-aminopyridine[29] and aminonaphthalenes,[30] containing one or more sulfonate groups, have been used in a Schiff reaction, followed by reduction with sodium cyanoborohydride (Scheme 1). This is followed by CE analysis in the free-zone mode. However, it is often the case that excess of reagent has to be separated from the derivatised glycans as its early migration can interfere with the CE analysis.

The use of 2-aminoacridone as the derivatising agent avoids any cleaning procedure if CE analysis is carried out in the presence of micelles of taurodeoxycholate.[31] This reagent is hydrophobic and neutral over a wide pH range, so that excess is trapped by the micelle and its migration is well after that of the derivatised oligosaccharides. Another advantage of 2-aminoacridone is its intense fluorescence when excited either by an argon (λ_{ex} = 488 nm, λ_{em} = 525 nm) or a helium/cadmium (λ_{ex} = 442 nm, λ_{em} = 520 nm) laser. We have carried out a number of successful studies of glycan fingerprinting and identification using this methodology.[32,33] Fig. 8 shows the resolution of the components of a dextran 'ladder' (a linear polysaccharide) and those in the heterogeneous glycan pool from ribonuclease B (containing only high mannose-type dioligosaccharides). This methodology has been found to be also suitable for the resolution of hybrid and complex types of oligosaccharide structures.

Complexation of borate with the linear 1,2-diol of the alditol moiety appears to be essential in the mechanism of separation of 2-aminoacridone derivatised oligosaccharides. The order of migration of these analytes is related to their hydrodynamic volume: the larger this volume, the shorter the migration time. This migration behaviour is similar to that observed in size-exclusion chromatography and is the opposite to that obtained

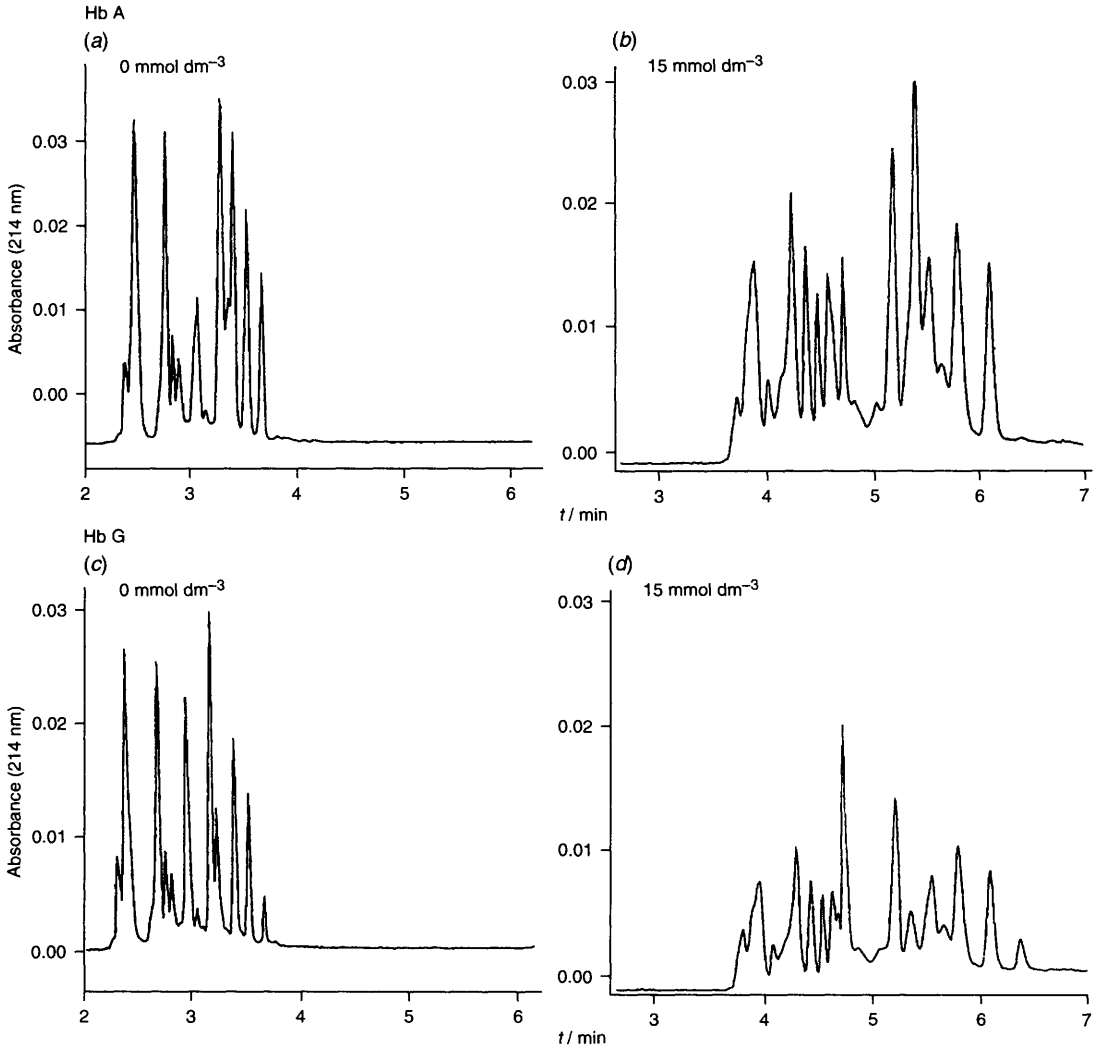


Fig. 6 CE separation of the tryptic fragements of the haemoglobin variants Hb A and Hb G in the absence and presence of the sodium salt of phytic acid (15 mmol dm^{-3}). Separation conditions: buffer, 150 mmol dm^{-3} sodium tetraborate pH 8.3; voltage, 10 kV; uncoated capillary, separation length, 50 cm and 50 μm i.d.; temperature, 30 °C.

in the CE analysis after derivatisation using charged reagents. The relationship of size to migration has been found to be very useful in the preliminary identification of the individual components of glycan mixtures. Moreover, derivatisation with 2-aminoacridone followed by MECC analysis is proving to be a fast method for the determination of the consistency in the glycan profile of batches of therapeutic glycoproteins produced in fermentation processes.

The role of chiral surfactants in enantiomeric resolution

It has already been mentioned that a number of naturally occurring 'pseudo-stationary' phases have been added to the separation buffer for the chiral resolution of racemic mixtures in free-zone CE. Proteins and oligosaccharides, mainly cyclodextrins, have been used extensively. Micelles produced by bile salts have also been applied for chiral discrimination in MECC.[34] Some time ago we reported[35,36] that mixtures of taurodeoxycholate and β-cyclodextrin in the ratio of *ca.* 3 : 1 can be used to resolve a variety of racemates. We have used this separation system to chirally resolve several complex molecules containing more than one chiral centre. We have also applied molecular modelling to obtain an insight as to the possible interaction between the bile salt and the cyclic oligosaccharide. These studies are at an early stage and presently it appears that a molecule of cyclodextrin is hydrogen bonded to each bile salt micelle, made up of three monomeric species. Independent experimental support for this transient complexation is being gathered from microcalorimetry studies in collabortion with Dr Alan Cooper at Glasgow University. Inclusion of aromatic moieties of analytes in the β-cyclodextrin cavity is essential for the resolution of racemates using this 'pseudo-stationary' phase.

The design and synthesis of chiral surfactants can offer advantages over separation phases made up of micelles from natural surfactants such as bile salts. Unlike the latter surfactants enantiomers bearing the opposite configuration (D and L) can usually be synthesised with the same ease. The availability of both surfactants may be analytically desirable especially when it is necessary to reverse the order of migration of two enantiomers to enable accurate estimation of a low-level antipode impurity. Mixed micelles made up of SDS and sodium dodecyl valinate[37] have been used for chiral discrimination in MECC. We have to date reported[38–40] the synthesis and application of glucopyranoside-, cysteine- and aminopenicillanic-based surfactants (structures shown on opposite page) for chiral selectivity in MECC. Physico-chemical advantages of these surfactants are good aqueous solubility and chemical stability, low critical micelle concentration and low absorption coefficients at wavelengths >200 nm. Micelles from these surfactants have been able to separate enantiomers of chiral

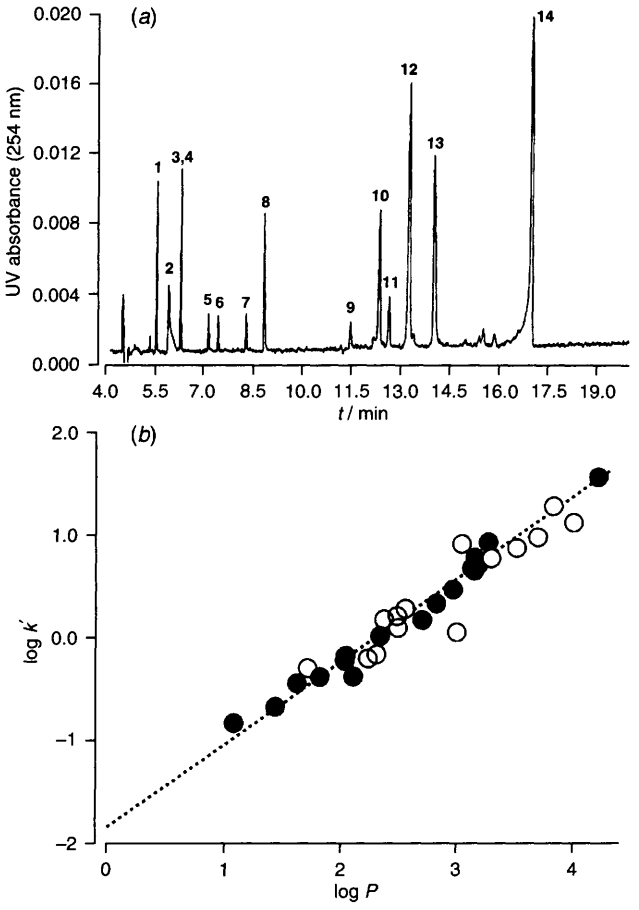
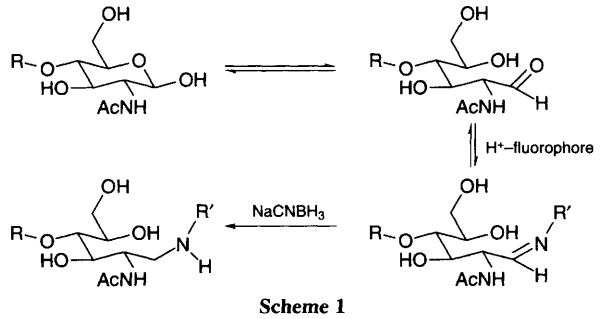


Fig. 7 (*a*) Electropherogram of 14 drugs using MECC. The last eluting analyte is halofantrine. Separation conditions: buffer, 50 mmol dm^{-3} boric acid adjusted to pH 9 with potassium hydroxide and containing 40 mmol dm^{-3} of the sodium salt of deoxycholic acid: voltage, 22 kV; uncoated capillary, separation length, 50 cm and 50 μm i.d.; temperature, 25 °C. (*b*) Correlation of log *P* with log *k'* for a number of standard compounds (■) and drug substances (○).



Scheme 1

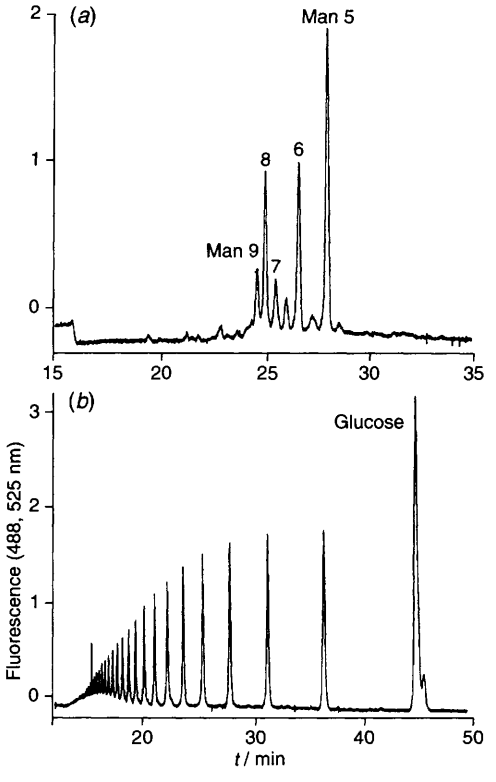
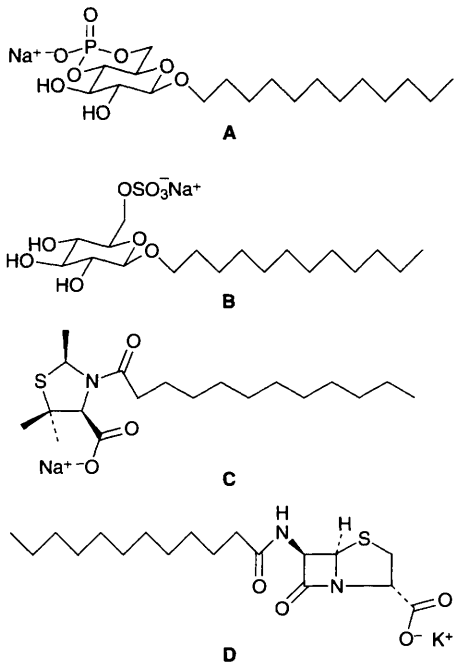


Fig. 8 Electropherograms of (*a*) N-linked oligosaccharides from ribonuclease B, and (*b*) linear oligosaccharides from dextran. Separation conditions: buffer, 500 mmol dm^{-3} sodium borate containing 80 mmol dm^{-3} sodium taurodeoxycholate, pH 8.9; voltage, 20 kV; uncoated capillary, separation length, 50 cm and 50 μm i.d.; temperature, 22 °C.



molecules that differ widely in chemical structure, hydrophobicity and acidity. Some examples of chiral separations are shown in Fig. 9. Presently we are devoting considerable effort to the design and synthesis of other novel surfactants for chiral discrimination in MECC. The general criteria for selection of the appropriate surfactants are the availability of starting materials, ease of synthesis and the extent of application. We are also carrying out studies on the suitability of mixtures of synthetic surfactants as chiral resolution agents.

Other applications of capillary electrophoresis

Capillary electrophoresis is still evolving as a separation technique for the qualitative and quantitative analysis of a variety of analytes of varying complexity. Its usefulness as a high-resolving technique is increasingly being recognised not only by analytical chemists but also by other scientists, in the areas of physical organic chemistry, biochemistry and biotechnology. In our laboratory we use CE routinely in the measurement of thermodynamic parameters such as the dissociation constants (pK_a) of acids and bases, and the inclusion constants of drugs in cyclodextrin related 'host' molecules. A knowledge of the latter parameters is useful especially when these cyclic oligosaccharides are used in the solubilisation of drug substances. We also pioneered CE studies to determine the binding constants between simple 'guest' molecules and structurally more complex 'hosts'. Four years ago we published[41] a communication in this Journal entitled '*A Study of the binding of Vancomycin to Dipeptides using Capillary Electrophoresis*'. In this study varying amounts of the 'guest' molecule, the mucopeptide mimic *N*-acetyl-D-Ala-D-Ala were added to the separation buffer and the electrophoretic behaviour of vancomycin was noted. Using a Scatchard plot the binding constants of the dipeptide with vancomycin by this CE method were found to be in good agreement with those obtained by spectrometric techniques. As expected the isomeric dipeptide *N*-acetyl-L-Ala-L-Ala was found not to bind to this broad spectrum antibiotic, first isolated from *Streptomyces orientalis*. A number of other publications by other authors have now appeared on the determination of binding constants by the technique now known as *Affinity Capillary Electrophoresis*.[42,43]

HPLC has often been used as the method of choice to study the kinetics of reactions in solution. The ability of CE to allow the simultaneous analysis of complex and simple analytes has made it an increasingly useful technique in the interpretation of the mechanism of the enzymic hydrolysis of drugs. The ability to measure the disappearance and formation of enzyme and drug related molecules makes CE unique in a biochemical laboratory. Recently we published a study on the hydrolysis of the β-lactamase inhibitor BRL 42715 by Tem-2-β-lactamase (Scheme 2).[44] BRL42715 acylates Ser 70 of the β-lactamase leading to the formation of a complex.

Differences in charge densities of this complex and that of the free enzyme allows resolution of these two protein species, using free-zone CE. The separation method developed also allowed monitoring of the formation of the seven-membered ring product. Time–response curves for an equimolar ratio of β-lactamase and BRL42715 showed that the disappearance of the enzyme–drug complex and the appearance of free enzyme and

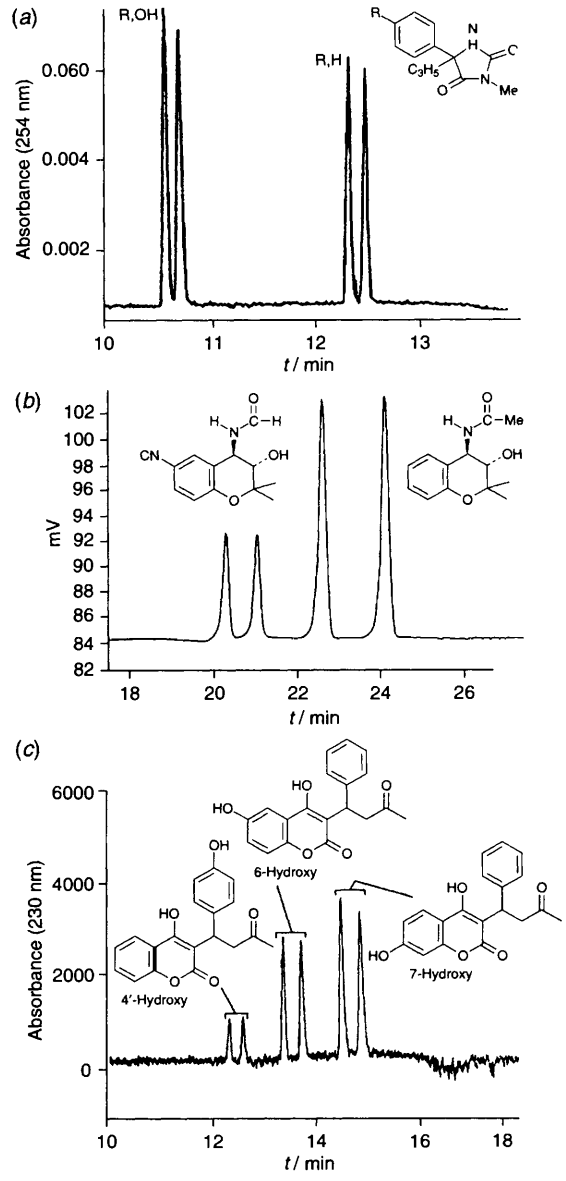
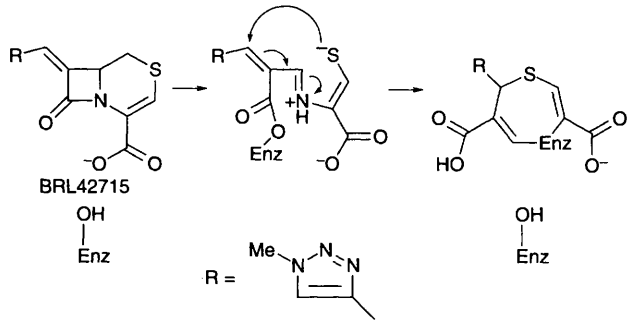


Fig. 9 Chiral separations of racemic mixtures using chiral surfactants. Separation conditions: (*a*) buffer, pH 8, phosphate–borate (containing 45 mmol dm^{-3} sodium salt of surfactant **A**); voltage, 20 kV; uncoated capillary, separation length, 50 cm and 50 μm i.d.; temperature, 25 °C. (*b*) Buffer, pH 7, borate–phosphate (containing 25 mmol dm^{-3} of the (2*R*,4*R*) diastereoisomer of surfactant **C**); voltage, 12.5 kV; uncoated capillary, separation length, 50 cm and 50 μm i.d.; temperature, 20 °C. (*c*) Buffer, pH 7, borate–phosphate (containing 25 mmol dm^{-3} of surfactant **D**); voltage, 12.5 kV; uncoated capillary, separation length, 50 cm and 50 μm i.d.; temperature, 20 °C.



Scheme 2

the seven-membered ring hydrolysis product were all close to 170 min, confirming the 1 : 1 stoichiometry of this reaction.

The future of capillary electrophoresis

Although CE has been available as an analytical tool over a relatively short period compared to the more traditional methods, it has made a substantial impact in the area of separation technology. Its complementarity to HPLC and slab-gel electrophoresis has given it a prominent and essential role in a modern analytical laboratory. As far as the future is concerned it is expected that CE will be a component in hyphenated techniques, in particular CE–mass spectrometry and HPLC–CE. These techniques will provide stuctural data and more sensitivity, respectively. Present indications suggest that capillary electrochromatography, using packed capillary columns, will be an important future development of CE.

Major advances are also expected to occur in the areas of sequencing of deoxyribonucleic acid (DNA) related to the human genome project and in microchip technology. The adaptation of multi-capillary systems in CE should accelerate the sequencing of DNA. Methodology being currently developed involves the simultaneous use of up to 25 gel-filled capillaries to sequence restriction fragments which are detected using up to four fluorescently labelled dye primers. The possibility of performing a number of the modes of operation of CE on a micromachined chip may open new opportunites for this technique. Important applications of CE microchip instrumentation may also be of use in drug design, especially in the rapid analysis of combinatorial libraries.

Acknowledgements

I thank my coworkers, whose names appear in the various publications cited. Without their help it would not have been possible to participate in the development of exciting technology. I also wish to thank SmithKline Beecham and Beckman Instruments (UK) for their support.

I was born in Malta where I received my Secondary and Graduate education. My Ph.D. in the area of gas kinetics was carried out at University College of Swansea under the supervision of the late Professor Howard Purnell. This was followed by a post-doctoral fellowship with Professor A. J. Kirby at Cambridge University. After lecturing for three years at the Royal University of Malta, I joined Shell Agrochemicals at Sittingbourne. I held this position for ten years after which I joined SmithKline Beecham in 1988, where I have been involved in the research and development of pharmaceuticals. In 1995 I was made Visiting Professor in the Chemistry Department of Imperial College of Science Technology and Medicine. This year I am the recipient of the Jubilee Silver Medal of the Chromatographic Society.

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Received, 9th April 1996; 6/02440H