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Capillary electrophoresis in the pharmaceutical industry: applications in discovery and chemical development

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Abstract

Over the last 10 to 15 years capillary electrophoresis (CE) has become an extensively used separation technique in the pharmaceutical industry. The attraction of the various modes of operation of CE to analysts is their complementarity to other more established methodology, in particular high-performance liquid chromatography. CE methods have been developed not only for the resolution of drug substances that vary widely in their structure, size and stereochemistry, but also for the determination of the physico–chemical constants of analytes, such as pK_a and isoelectric point (pI) values, binding and complexation constants, and octanol–water partition coefficients.

Keywords: Drug discovery; Chemical development; Pharmaceuticals

1. Introduction

Until a few years ago capillary electrophoresis (CE) was considered to be a tool only suitable for the discovery of analytical technology and without much use for ''serious'' analysis. However, nowadays the presence of CE instrumentation has become essential in a modern analytical laboratory, especially in the pharmaceutical industry. Thus, two of the most popular modes of operation of CE, namely capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) are used regularly alongside high-performance liquid chromatography (HPLC). The availability of the two separation techniques allows the successful resolution of the widest possible variety of analytes, differing in size, charge, hydrophobicity and stereochemistry [1].

CE offers a number of advantages. The experimental set-up is simple: separation of analytes take place when a high voltage (typically, 5 to 30 kV) is applied along a fused-silica capillary tube (20 to 100 μm I.D.), the two ends of which are dipped in two buffer solutions, each containing a platinum electrode. Both the optimisation of the separation process and the changing from one CE mode of operation to another can usually be carried out without much difficulty, simply by changing the constitution of the buffer. CE methodology is also especially useful if the amount of sample available is small (<5 μl). The utility of CE is such that it can be used for the analysis of a very large range of

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species from inorganic ions, to proteins, to whole cells.

The ease of use of CE has made it a powerful experimental tool not only for routine separations of complex mixtures of analytes, but also for the determination of physico–chemical and biochemical parameters. In this article we have outlined the contribution of our laboratories to the development of CE, and the application of CE methodology in the drug discovery and development process.

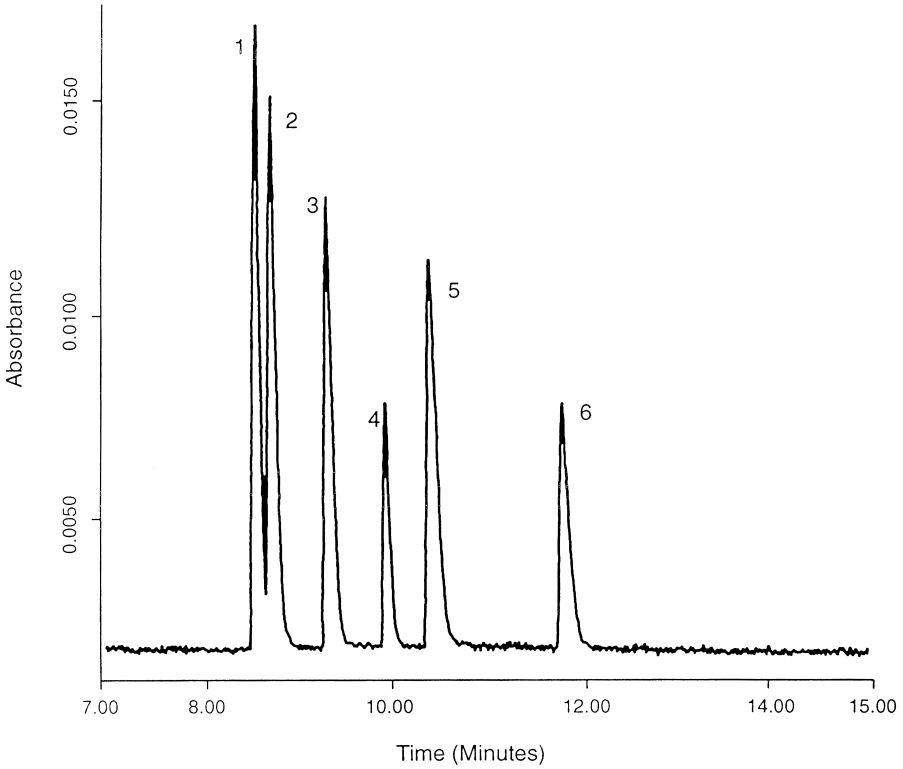
2. Materials and methods

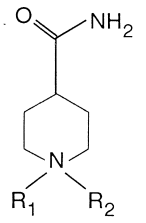
2.1. Chemicals and reagents

SB-238592-DB was supplied by Cortech (Denver, CO, USA). Brij 35, phytic acid and taurodeoxycholate were purchased from Sigma (Poole, UK). Orthophosphoric acid (85%), lithium hydroxide, sodium hydroxide and disodium tetraborate were purchased from Fisons (Loughborough, UK). Acetonitrile and dimethylsulphoxide were purchased from Romil (Waterbeach, UK). Methyl-β-cyclodextrin was purchased from Wacker (Egham, UK), Technicol (Stockport, UK) and Aldrich (Poole, UK). Hydroxyethylcellulose was purchased from Fluka (Gillingham, UK).

2.2. Apparatus

CE was performed using a Beckman P/ACE 5010 system (Palo Alto, CA, USA). Separations were performed using either 50 or 75 μm I.D. uncoated fused-silica capillary tubing manufactured by Polymicro Technologies (Phoenix, AZ, USA). Data acquisition and signal processing were performed using a Waters 860 data system (Milford, MA, USA).





Peak #	R_1	R_2
1	H	H
2	Me	H
3	Et	H
4	^{n}Bu	H
5	^{n}Pr	H
6	^{n}Bu	^{n}Bu

Fig. 1. Separation of cyclic amines by capillary zone electrophoresis. (Conditions: buffer, 50 m*M* sodium phosphate buffer, pH 2.5; voltage, 20 kV; uncoated silica capillary, 57 cm×50 μm I.D.; temperature, 25°C; wavelength, 198 nm).

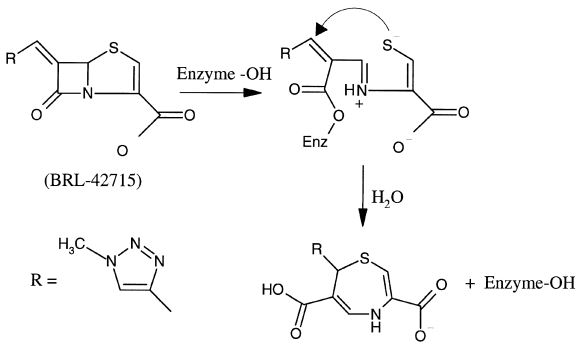


Fig. 2. Mechanism of reaction of BRL-42715 with the enzyme β-lactamase.

3. Results and discussion

3.1. Capillary zone electrophoresis separations

CZE is a technique that is most effective in the separation of molecules on the basis of differences in charge density. This mode of operation of CE is very useful in the pharmaceutical industry, especially as a large number of drug intermediates and drugs contain one or more ionisable amino and/or carboxylic groups. Fig. 1 shows the separation of a series of alicyclic amines under conditions where mobility is largely driven by electrophoresis rather than by electroosmosis. At the pH employed, all the amines examined were present in the form of singly charged cations. Increasing the size of the substituent on the amino group reduced charge density such that the bulkier the substituent present the slower the migration.

In cases of molecular interactions involving a change in charge density, CZE can be used to determine thermodynamic and kinetic parameters. In one of our studies we applied CZE to examine the binding of the broad-spectrum antibiotic vancomycin to the peptides *N*-acetyl-D-Ala-D-Ala and *N*-acetyl-L-Ala-L-Ala [2]. As vancomycin exerts its antibacterial activity by binding strongly to mucopeptides terminating with the dipeptide *N*-acetyl-D-Ala-D-Ala, the variation of the mobility of vancomycin was considerably greater when this peptide was introduced in the buffer solution. Binding constants obtained from these measurements were in good agreement with reported values using either differential UV spectroscopy or nuclear magnetic resonance. However, unlike the latter spectroscopic techniques, CZE also provided information on the net charge characteristics of the complex formed between vancomycin and the mucopeptide mimic [2].

The enzyme β-lactamase is involved in the metabolism of several antibiotics such as amoxycillin, thus decreasing their antibacterial efficacy. Much research has been carried out to inhibit this enzyme. In one of our studies we applied CZE to follow the interaction of a negatively charged drug, [(5*R*)-(*Z*)-6-(1-methyl-1,2,3-triazol-4-yl)methylene]penem-3-carboxylic acid, (BRL-42715), with the enzyme β-lactamase [3]. The mechanism of this reaction is shown in Fig. 2. The initial step is enzymatic attack of the β-lactam ring of the drug to form the intermediate species shown where the drug is covalently attached to the enzyme. Further rearrangement leads to the formation of a seven-membered ring compound. Using capillary electrophoresis it was possible to differen-

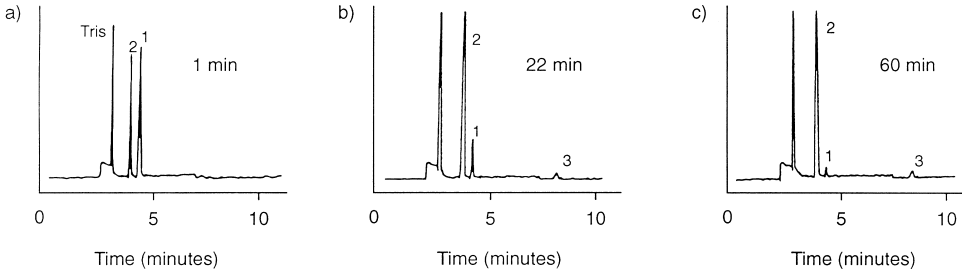


Fig. 3. Analysis of the decomposition of the β-lactamase–BRL-42715 complex (2) to give free enzyme (1) and a seven-membered ring product (3) after (a) 1, (b) 22 and (c) 60 min of reaction. (Conditions: 20 m*M* sodium phosphate buffer, pH 7.3; gradient voltage, 1 kV min^{-1} over 15 min; uncoated silica capillary, 27 cm×50 μm I.D.; temperature, 22°C; wavelength, 200 nm).

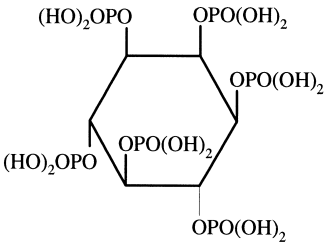


Fig. 4. Chemical structure of phytic acid.

tiate between β-lactamase and the enzyme intermediate, and to follow the formation of the seven-membered ring product. (Fig. 3). This particular application also illustrates one unique property of CZE, namely the ability to monitor concentrations of small and large molecules simultaneously.

The addition of the naturally occurring and abundant molecule phytic acid (inositol hexaphosphoric acid; Fig. 4) to the separation buffer can have beneficial effects in the resolution of complex mixtures of analytes. The presence of as many as 12 sodium ions per molecule of phytic acid increases ionic strength considerably when this polyanion is added to the separation buffer. This has the effect of increasing efficiency and allowing injection of relatively large quantities of water soluble analytes without loss of resolution. Fig. 5 shows a comparison of the analysis of the drug SB-202026 from a number of related components in the presence and absence of phytic acid in the separation buffer. It is of interest to note the improvement in peak shape on the addition of phytic acid, which is presumably due to more efficient shielding of the negatively charged silanols on the capillary wall, resulting in less interaction with the positively charged bicyclic nitrogen. The addition of phytic acid also facilitated the separation of component *v* from the electroosmotic flow. As expected, in the absence of phytic acid, this neutral component travels with the electroosmotic

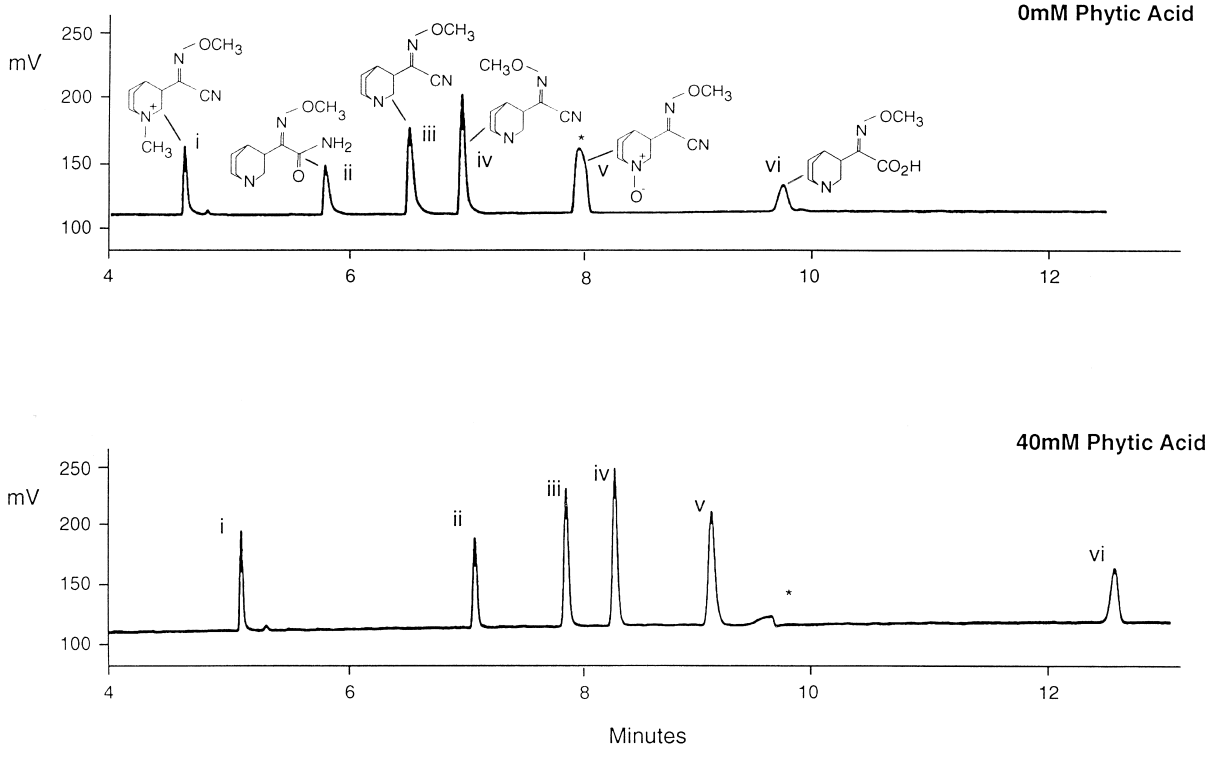


Fig. 5. Analysis of SB-202026 and related compounds using phytic acid in the separation buffer. * Represents the position of the electroosmotic flow. (Conditions: 100 m*M* sodium tetraborate buffer, pH 9.5; voltage, 20 kV; uncoated silica capillary, 77 cm×50 μm I.D.; temperature, 25°C; wavelength, 234 nm).

flow. The presence of the high ionic strength appears to induce a positive charge on the molecule, potentially due to an ion-pairing effect with sodium ions present. This methodology was successfully applied for the analysis of low level impurities in the analysis of samples of the drug substance injected at concentrations as high as 50 mg ml^{-1}. This was not possible in the absence of phytic acid (data not shown).

The reduction of electroosmotic mobility when phytic acid is added to the separation buffer is again due to more efficient shielding of the negative charges on the inner surface of the capillary wall. Thus, another effect of the high ionic strength, even at relatively low concentrations of the sodium salt of phytic acid, is to shield the negative charge on polyanionic molecules from the electrostatic field. The combined effects were found to lead to a considerable improvement in the resolution of short-chain synthetic oligonucleotides [4].

Phytic acid can also be used to improve the resolution of a number of basic peptides or proteins [5–8]. The six phosphate groups present have pK_a values over the range of 1.9 to 9.5, so that the number of negative charges can range from 1 at low pH to a maximum of about 11 at pH values close to 9. This excess of negative charge makes phytic acid a very effective ion-pairing agent. This ion-pairing effect has also been used to change the order of migration of basic peptides [5]. The methodology [6] was used to analyse samples of tryptic digests of haemoglobin A (HbA) and one of its variants, haemoglobin G (HbG), as shown in Fig. 6. In the absence of phytic acid, the total number of peptide fragments is about half that expected. Differences in the electropherograms of the tryptic peptide maps from the two types of haemoglobin could be related to the replacement of a glutamic acid residue by an alanine residue in position 22 of the β-chain of HbG.

The addition of phytic acid to the running buffer also facilitated analysis of basic proteins ($pI>9$), which under normal conditions, could not be analysed due to adsorption by the negatively charged silica surfaces of capillaries [7,8]. This major im-

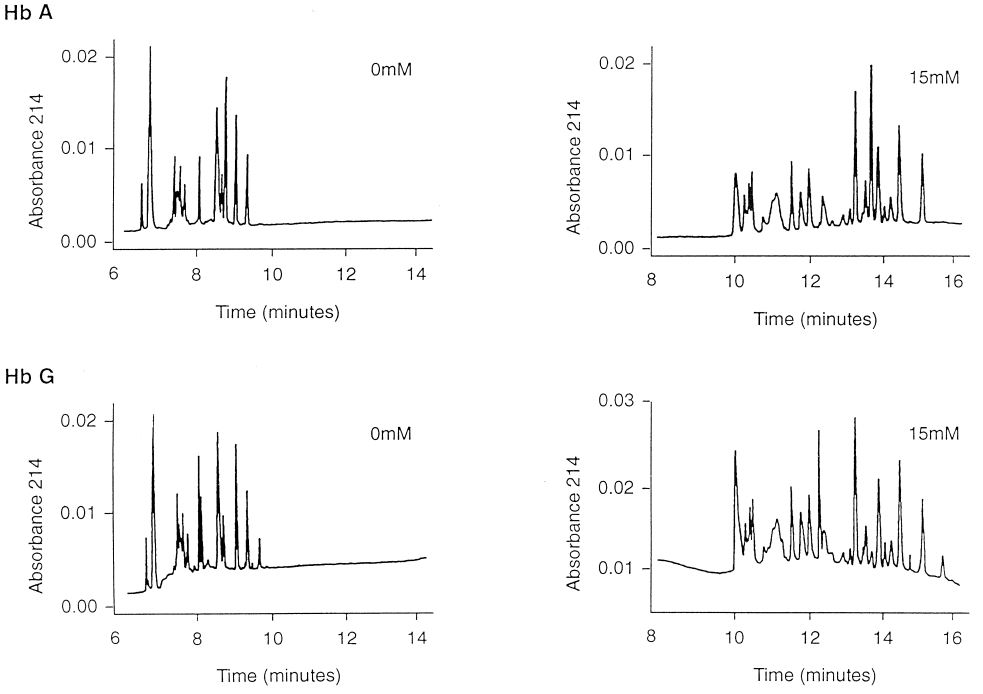


Fig. 6. CZE separations of tryptic digests of HbA and HbG in the presence and absence of phytic acid. (Conditions: 150 m*M* sodium tetraborate buffer, pH 8.3; voltage, 10 kV; uncoated silica capillary, 57 cm×50 μm I.D.; temperature, 30°C; wavelength, 214 nm).

provement in the analysis is again thought to arise from the suppression of coulombic interactions between the positively charged proteins (ion-paired with phytic acid) and the negatively charged silanol groups on the inner wall of the capillary.

3.2. Micellar electrokinetic chromatography separations

MEKC, unlike CZE, can be applied to the separation of neutral molecules. The presence of a surfactant above its critical micelle concentration (CMC) in the separation medium provides a pseudo-stationary phase with which neutral molecules can interact. This effect leads to separation of analytes due to differences in free energy of partitioning between the hydrophobic core of the micelles and the surrounding aqueous environment. We previously reported MEKC methodology for the analysis of simple and complex carbohydrates derivatised with the intense fluorophore 2-aminoacridone (2-AMAC) [9–11]. The sodium salt of taurodeoxycholate in borate buffer was used as the surfactant for this study and the 2-AMAC carbohydrate derivatives were analysed by laser induced fluorescence detection using a He–Cd laser. As the hydrophobicity of 2-AMAC is higher than that of carbohydrate analytes, it is retained by the micelles and migrates at a considerably longer time than the derivatised carbohydrates. The non-interference of excess fluorophore is a major advantage in this analytical procedure.

The separation of a 2-AMAC derivatised dextran ''ladder'' prepared from enzymatically hydrolysed dextran is shown in Fig. 7. The general structures of these derivatives are shown in Fig. 8, where one molecule differs from another by the number of glucose residues attached to the fluorophore. This MEKC method was used to analyse the profiles of a number of complex glycan mixtures. Combined with limited enzymatic digestions, such as sialidase and fucosidase treatment, much information can also be obtained rapidly about the composition of such mixtures.

We have also successfully applied MEKC for the separation of basic peptide diastereomers, present in the form of polycations [12]. Anionic surfactants such as sodium dodecyl sulphate (SDS) are typically used in MEKC separations, but the resulting pseudo-

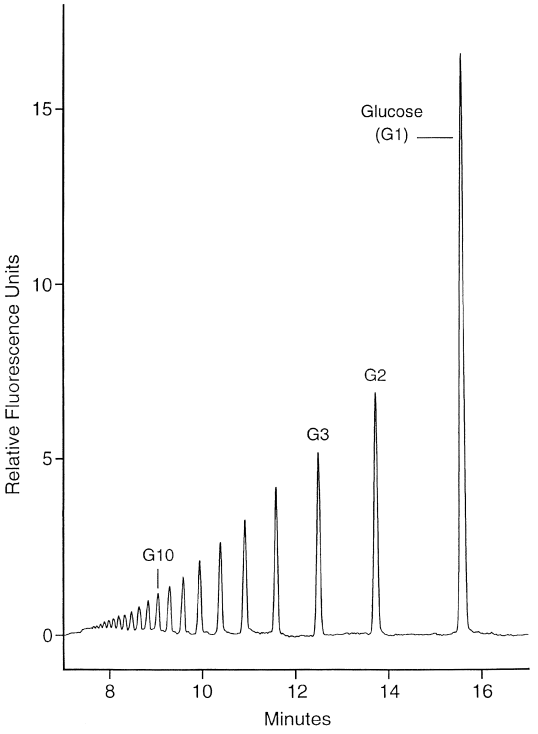


Fig. 7. MEKC analysis of a 2-AMAC derivatised dextran ''ladder''. (Conditions: 80 m*M* taurodeoxycholate in 300 m*M* borate buffer, pH 8.8; voltage, 25 kV; uncoated silica capillary, 57 cm×50 μm I.D.; temperature, 25°C; He–Cd laser, excitation wavelength 442 nm, emission wavelength, 525 nm).

stationary phases can sometimes interact too strongly with larger peptides and proteins such that separations are not possible [13]. An alternative approach is the use of more polar neutral surfactants as micelle forming agents which have weaker interactions with such species [14]. We recently reported the separation of the diasteromers of the synthetic *bis*-de-

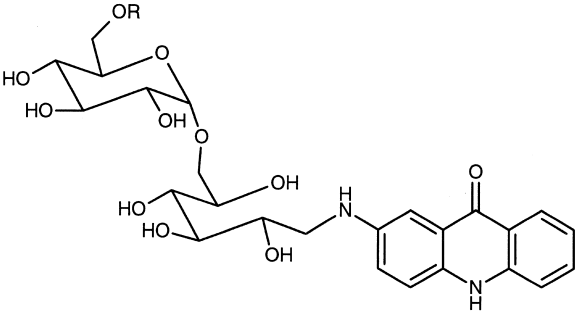


Fig. 8. General structures of 2-AMAC carbohydrate derivatives.

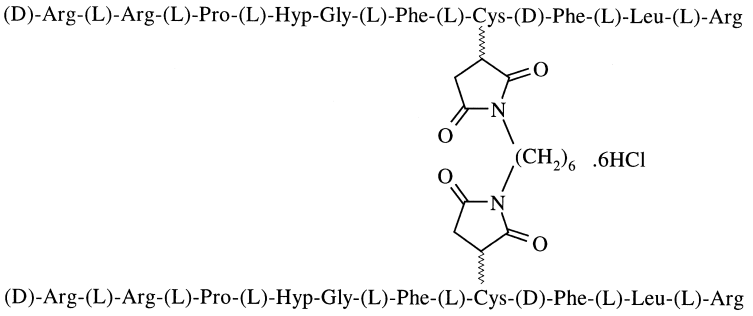


Fig. 9. Structure of the bradykinin B_2 inhibitor SB-238592-DB.

capeptide, SB-238592-DB (Fig. 9) using the non-ionic surfactant Brij 35 (main component: polyoxyethylene 23 lauryl ether) as a psuedostationary phase [12]. It should however be noted that separations based on neutral detergents cannot strictly be classed as MEKC as the micelles themselves do not actively migrate and analyte transport occurs by electrophoretic migration. Terabe et al. [15] correctly named this mode of CE capillary electrokinetic chromatography (CEKC). The bradykinin B_2 antagonist shown in Fig. 9 exists in three diastereomeric forms as the chirality about the C-3 atoms of the succinimide moieties is undefined. Under acidic conditions the peptide carries a net positive charge of 6 due to the presence of the arginine residues.

Separation of the three peptide diastereomers was not achieved by CZE under acidic conditions (Fig. 10A), or by use of MEKC employing SDS as a psuedostationary phase (data not shown). The stereoisomers were however separated in the presence of Brij 35 at low pH (Fig. 10B). Addition of 5% (v/v) acetonitrile to the optimised separation buffer resulted in a marked increase in the efficiency of the separation (Fig. 10C). The developed method was shown to be superior to an existing reversed-phase HPLC method in terms of analysis time and robustness. One major advantage of neutral surfactants such as Brij 35 is that they do not contribute to the overall ionic strength of the running buffer and therefore cause no additional Joule heating when present. The mechanism of the stereoisomeric separation may involve hydrogen bonding between the hydrophilic polyether chains of the Brij 35 and the peptides in addition to partitioning into the hydrophobic core of the micelles. The developed method was used routinely for the determination of the diastereomeric content of SB-238592-DB batches prepared during chemical development.

3.3. Chiral capillary electrophoresis separations

The addition of chiral additives to separation buffers allows the resolution of enantiomeric mixtures by CE. Chiral resolution by this method provides an analytical method which is complementary to the separation of enantiomers by chromatographic methods. Cyclodextrins (CDs) and their derivatives are the most commonly used chiral selectors in CE at the present time due mainly to factors such as solubility in aqueous buffers, UV transparency, commercial availability, low cost, stability and environmental compatibility [16]. This mode of CE is analogous to the use of neutral surfactants mentioned previously and should correctly be termed chiral capillary electrokinetic chromatography (CCEKC) [15].

Recently we developed and validated a chiral CE method for the determination of the enantiomeric purity of the anti-thrombotic agent SB-214857-A (Fig. 11). The specification limit for the distomer (SB-214856-A) is currently set at 0.5% peak area ratio (PAR). The method superceded a chiral HPLC method due to problems with the batch to batch reproducibility of the chiral stationary phase employed. The method uses a lithium phosphate buffer at pH 3.0 containing methyl-β-cyclodextrin as a chiral selector and hydroxyethylcellulose as a dynamic capillary coating agent to further suppress the electroosmotic flow. Under such conditions, a sensitive, robust chiral CE method was developed, with a

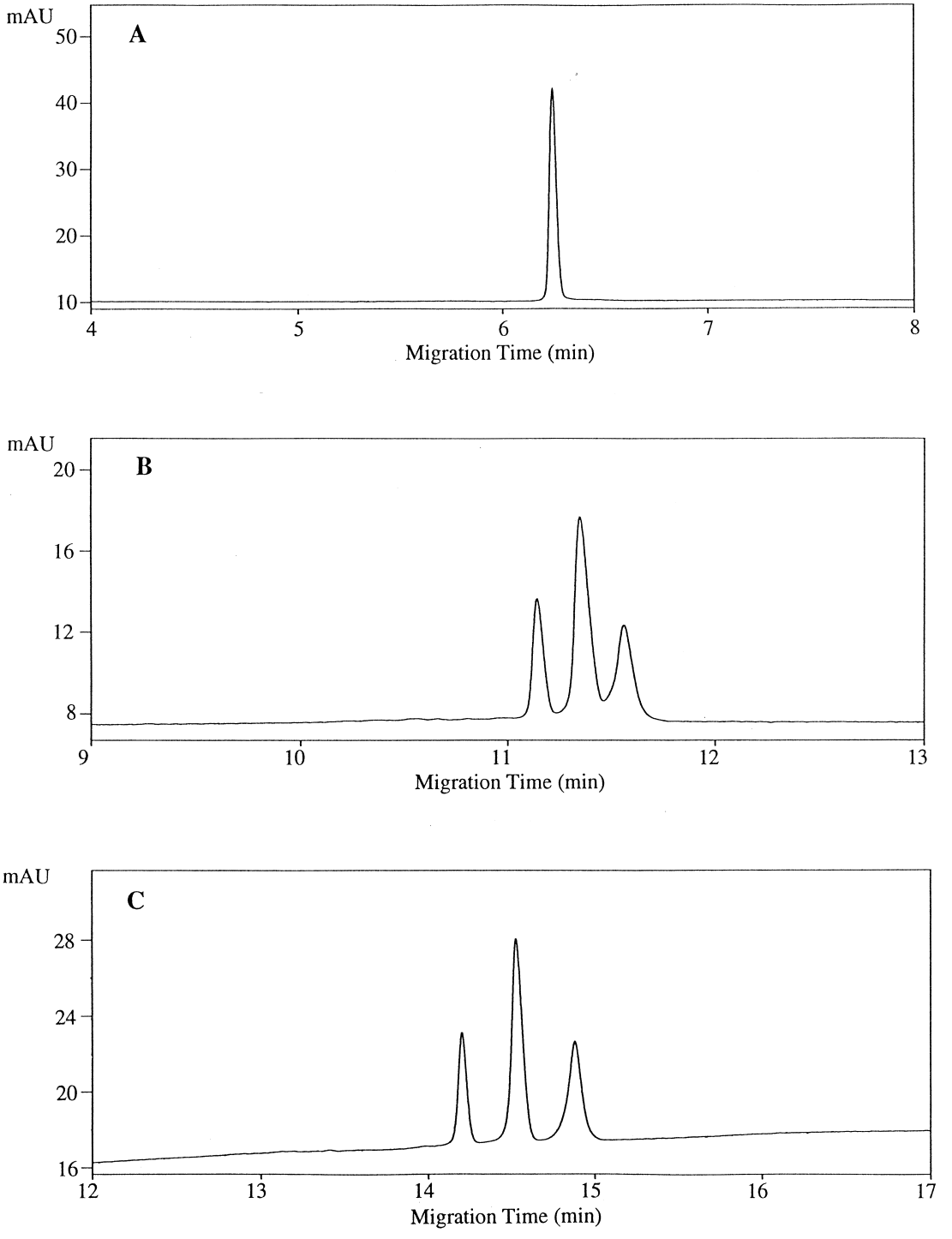


Fig. 10. Electropherograms of SB-238592-DB in the presence of (A) no additive, (B) 5 m*M* Brij 35 and (C) 15 m*M* Brij 35 with 5% (v/v) acetonitrile. (Conditions: 200 m*M* lithium phosphate buffer, pH 2.5; voltage, 30 kV; temperature, 20°C; uncoated silica capillary, 57 cm×50 μm I.D.; wavelength 200 nm).

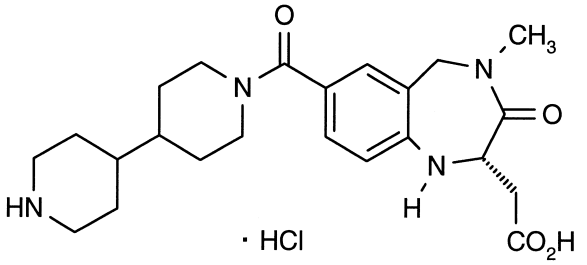


Fig. 11. Structure of the anti-thrombotic agent SB-214857-A.

limit of detection for SB-214856-A of 0.05% PAR. An electropherogram illustrating the separation of the racemate is shown in Fig. 12.

The method was validated with respect to specificity, linearity of response, recovery, enantiomeric stability in solution, repeatability, intermediate precision and robustness. The batch to batch variability of the methyl-β-cyclodextrin used as a chiral additive was also examined by selecting batches from three separate suppliers. Example electropherograms of the SB-214857 chiral system suitability standard containing ca. 1% PAR SB-214856 are shown in Fig. 13, illustrating only minor differences in migration times and resolutions between methyl-β-cyclodextrin purchased from different suppliers. The chiral CE method was chosen as the primary method for the determination of the enantiomeric purity of SB-214857-A final drug substance and has been included in recent regulatory submissions.

Recently we have also been involved in the design

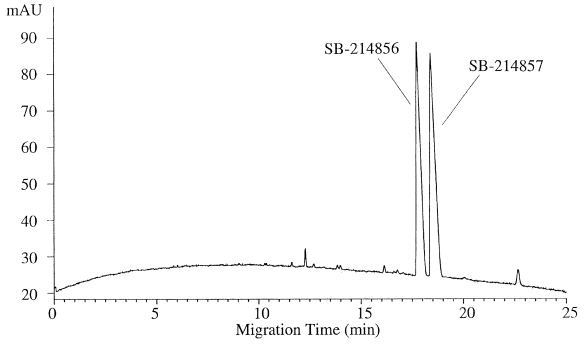


Fig. 12. Separation of a racemic sample of SB-214857-A. [Conditions: 100 m*M* lithium phosphate buffer, pH 3.0, containing 0.05% (w/v) hydroxyethylcellulose and 1.5 m*M* methyl-β-cyclodextrin; voltage, 30 kV; temperature, 20°C; uncoated silica capillary, 57 cm×75 μm I.D.; wavelength 200 nm].

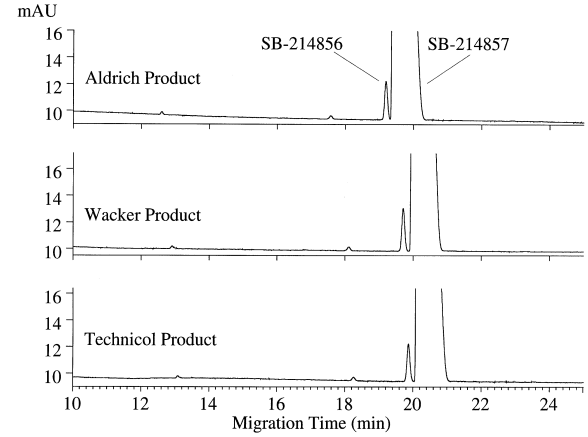


Fig. 13. Example electropherograms of the SB-214857 chiral system suitability standard resolved using methyl-β-cyclodextrin from different suppliers. (Conditions as in Fig. 13).

and synthesis of a number of anionic chiral surfactants, consisting of an amino acid [17,18]or a carbohydrate [19,20] moiety as the polar ‘‘head’’ group and an alkyl chain as the hydrophobic ‘‘tail’’.

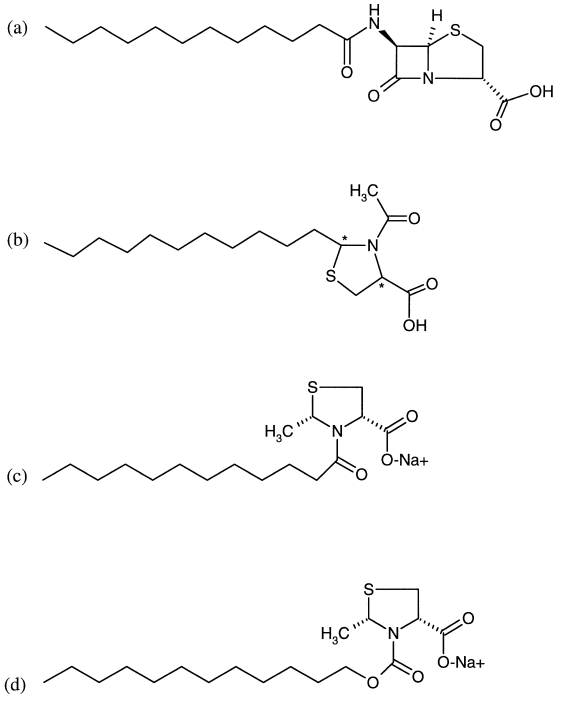


Fig. 14. Structures of the synthetic amino acid derived chiral surfactants.

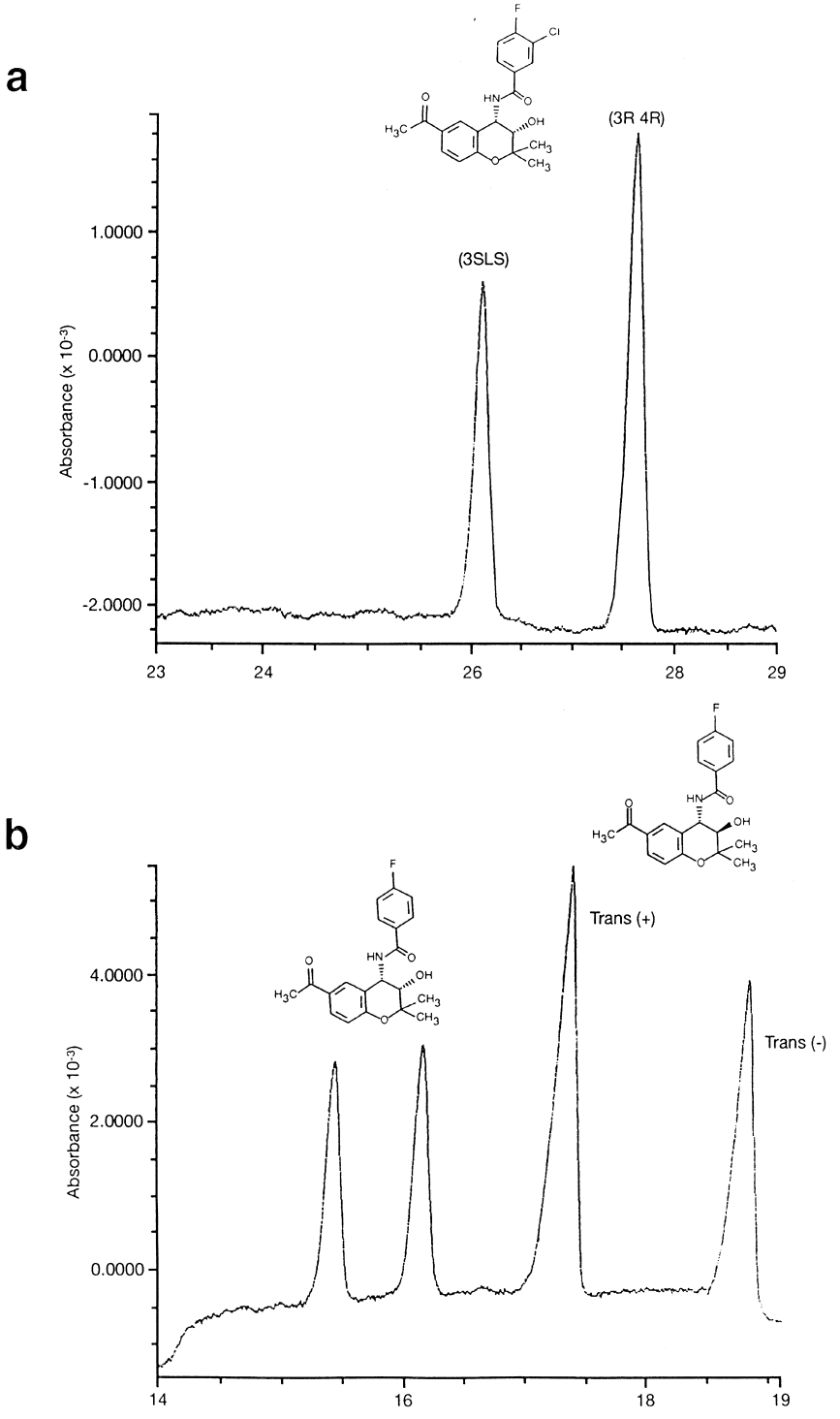


Fig. 15. Stereoisomeric separations of two novel anticonvulsant drugs. (Conditions: buffer, 50 m*M* sodium dihydrogenphosphate, 25 m*M* sodium tetraborate, pH 7.0; voltage, 12.5 kV; uncoated silica capillary, 50 cm×50 μm I.D.; temperature, 25°C; wavelength, 254 nm).

When these surfactants are added to the separation buffer at a concentration above the CMC, the corresponding micelles can discriminate between enantiomeric pairs due to the formation of transient (non-covalent) diastereoisomers, differing by their free energies of formation. One of the first amino acid derived surfactants synthesised was the dodecyl derivative of 6-aminopenicillanic acid (Fig. 14a). The starting material for this surfactant was 6-aminopenicillanic acid, which is an intermediate in the manufacture of the widely prescribed antibiotic amoxycillin and is therefore available in large quantities. Buffer solutions containing this selector were found to be excellent vehicles for the resolution of racemic mixtures of warfarin an a number of its hydroxy derivatives [17].

2-Undecyl-4-thiazoline carboxylic acid (Fig. 14b) was another anionic surfactant easily synthesised from either D- or L-cysteine, both of which are readily available commercially [18]. The ease of synthesis of the two stereoisomeric forms from either D- or L-cysteine meant that both enantiomeric surfactants were readily available. Thus in cases which involved resolution of two enantiomers, one of which was present in excess, reversal of the order of migration by a judicious choice of surfactant proved to be analytically advantageous. By varying the route for the synthesis of 2-undecyl-4-thiazoline carboxylic acid we prepared two other cysteine-based surfactants (Fig. 14c and d), one containing an amide and the other a carbamate linkage. These two surfactants in particular were found to afford chiral selectivity to a wide range of analytes. Fig. 15 shows the resolution of pairs of enantiomers from one or both of the diastereoisomers of two novel anticonvulsant drugs using the surfactant shown in Fig. 14d as a chiral surfactant.

4. Conclusions

In this article we have shown the broad-based application of CE to solve analytical problems typically encountered in the pharmaceutical industry. The simplicity in the use of this technique, for example the ease of changing from one mode of separation to another, makes CE a valuable complement to the more traditional ''wet'' separation methods, such as HPLC. Thus, one can use the same capillary for both achiral and chiral separations, simply by changing the buffer solutions and with relatively short periods of equilibration in between. Over the years we have also demonstrated that, besides analytical separations, CE can be useful in the measurement of physico–chemical parameters such as pK_a and CMC values, and estimates of 1-octanol–water partition coefficients. If the interaction or affinity between molecules involves a change in overall charge, then CE can also be applied to determine stoichiometry and binding constants, thus providing a means of interpreting the mechanism of interaction. Such a wide flexibility in the use of CE makes this technology an all round valuable tool in drug discovery and development.

References

[1] P. Camilleri (Ed.), Capillary Electrophoresis – Theory and Practice, CRC Press, Boca Raton, FL, 1998.
[2] J.L. Carpenter, P. Camilleri, D. Dhanak, D. Goodall, J. Chem. Soc., Chem. Commun. (1992) 804.
[3] G.N. Okafo, P. Cutler, D.J. Knowles, P. Camilleri, Anal. Chem. 67 (1995) 3697.
[4] G.B. Harland, G.N. Okafo, P. Camilleri, Anal. Chem. 68 (1996) 1982.
[5] G.N. Okafo, H.C. Birrell, M. Greenaway, M. Haran, P. Camilleri, Anal. Biochem. 219 (1994) 201.
[6] G.N. Okafo, D. Perrett, P. Camilleri, Biomed. Chromatogr. 8 (1994) 204.
[7] H.C. Birrell, P. Camilleri, G.N. Okafo, J. Chem. Soc., Chem. Commun. (1994) 434.
[8] G.N. Okafo, A. Vinter, T. Kornfelt, P. Camilleri, Electrophoresis 16 (1995) 1917.
[9] M. Greenaway, G.N. Okafo, P. Camilleri, D. Dhanak, J. Chem. Soc., Chem. Commun. (1994) 1691.
[10] P. Camilleri, G.B. Harland, G.N. Okafo, Anal. Biochem. 230 (1995) 115.
[11] G.N. Okafo, L.M. Burrow, W. Neville, A. Truneh, R.A.G. Smith, M. Reff, P. Camilleri, Anal. Biochem. 240 (1996) 68.
[12] M. Gilges, M. Hadley, Electrophoresis 18 (1997) 2944.
[13] T. Yashima, A. Tsuchiya, O. Morita, S. Terabe, Anal. Chem. 64 (1992) 2981.
[14] N. Matsubara, S. Terabe, Chromatographia 34 (1992) 493.
[15] S. Terabe, H. Ozaki, K. Otsuka, T. Ando, J. Chromatogr. 332 (1985) 211.
[16] B. Chankvetadze, Capillary Electrophoresis in Chiral Analysis, Wiley, Chichester, 1997.

[17] M. Bouzige, G.N. Okafo, D. Dhanak, P. Camilleri, J. Chem. Soc., Chem. Commun. (1996) 671.
[18] V. de Biasi, J. Senior, J.A. Zukowski, R. Curtis Haltiwanger, D.S. Eggleston, P. Camilleri, J. Chem. Soc., Chem. Commun. (1995) 1575.
[19] D. Tickle, R. Jones, G.N. Okafo, P. Camilleri, A.J. Kirby, Anal. Chem. 66 (1994) 4121.
[20] D. Tickle, A. George, K. Jennings, P. Camilleri, A.J. Kirby, J. Chem. Soc., Perkin II (1998) 467.