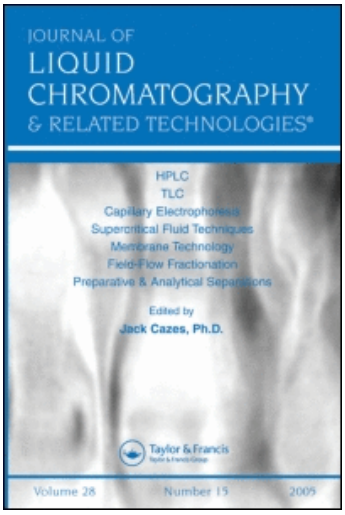

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SELECTED APPLICATIONS OF CAPILLARY ELECTROCHROMATOGRAPHY IN THE PHARMACEUTICAL INDUSTRY: TO BUY OR NOT TO BUY?

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SELECTED APPLICATIONS OF CAPILLARY ELECTROCHROMATOGRAPHY IN THE PHARMACEUTICAL INDUSTRY: TO BUY OR NOT TO BUY?

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ABSTRACT

The general state of the art of capillary electrochromatography (CEC) is presented. Emphasis is placed on its relevance to the pharmaceutical industry. Its progress in development areas such as column technology, detection, stationary phases, chiral separations, and instrumentation is presented. Problems and possible solutions are identified. Also, "lab-on-a-chip" development, related to CEC is briefly described.

Key Words: CEC; Pharmaceuticals; Polar compounds; MS detector; Chiral separations

INTRODUCTION

Capillary electrochromatography (CEC) is often described as a hybrid of high performance liquid chromatography (HPLC) and capillary electrophoresis

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(CE), but it can be also regarded as a combination of ion exchange chromatography (IEC) and CE if ion exchange stationary phases are applied. Nevertheless, a description of CEC as micro-HPLC with electroosomotic flow (EOF) as a driving force is the most appropriate so far.

CEC equipment is practically identical to that used in CE, except that the capillary column is usually packed with a stationary phase, and often pressure is applied at both capillary ends to prevent bubble formation. However, this additional pressure is not mandatory if lower voltage, zwitterion buffer, and low ionic strength are applied. Similarly, packing is not imperative if the stationary phase attached to capillary walls can provide sufficient adsorption sites. CEC is also similar to micellar electrokinetic chromatography (MEKC), the major difference being that in MEKC a pseudo stationary phase is created, as micelles are present in the mobile phase and move with EOF.

CE capillaries, typically 40–90 cm in length and 50 μm in diameter, are usually packed with 1.5–5 μm reversed phase particles. In the not so distant past, and even often today, exactly the same silica based stationary phases are used as in HPLC. The most common detection technique, UV/VIS, is facilitated by making a window in the capillary just below the packed bed by burning the imide coating exactly as in CE. The analytes are injected electrokinetically to overcome the high backpressure caused by the column packing. The cations of the electrolyte present in the mobile phase form an electrical double layer on residual silanol groups of the silica support. Solvated cations move towards the negative electrode, pulling along most of the surrounding solvent and other molecules present, when a typical CE voltage of 10–30 kV is applied. This phenomenon is known as electroosmotic flow (EOF). Neutral compounds separate in CEC due to interactions with the stationary phase as in reversed phase chromatography, while the separation of charged molecules depends mostly on differences in their electrophoretic mobilities.

Victor Pretorius is acknowledged as the father of CEC because of his 1974 paper.[1] Jorgensen and Lukacs[2] developed the CEC idea further. However, only after the works of Knox and Grant[3–5] were published, did CEC start to draw serious attention from academia and industry.

The purpose of this article is to find an answer to the following question: Is it justifiable for a medium size, fast growing bio-pharmaceutical company to invest in capillary electrochromatography?

This review covers the last decade of CEC development and focuses predominantly, but not solely, on pharmaceutical applications. The author made the utmost effort to search for any articles relevant to the above question. This paper is not an extensive review of all publications related to pharmaceutical applications—for this purpose the reader is referred to the excellent reviews of Sandra et al.[6,7] which discuss a broad spectrum of CEC applications and de Jong et al.[8] dealing with electrokinetic separation techniques for drugs and

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related products. Many recently published papers deal with various aspects of CEC: chiral separations,[9–16] stationary phases and column technology,[17–19] gradient elution techniques,[20] instrumentation,[21] use of mass spectrometric detection,[22] theory,[23] general reviews.[24,25] The reader is also referred to the review of Issaq[26] who mostly deals with capillary electrophoresis, but in a small paragraph devoted to capillary electrochromatography also looks at this technique from a more critical perspective.

WHY CEC?

Why use CEC when HPLC is adequate to solve most separation problems encountered in the pharmaceutical industry? The simplified answer is—because CEC is capable of giving 2–4 times higher efficiency than HPLC, and when small ≤ 1.5 μm nonporous particles are used, efficiencies can be up to 10 times greater than HPLC. For example, Kraak et al.[27] achieved 600,000 theoretical plates per meter (N/m) for a mixture of steroids. This is considered typical. However, Smith and Evans[28] have reported values of (N/m) as high as eight million, when separating basic drugs on a strong cation exchanger (SCX) stationary phase (Fig. 1). Unfortunately, at present, these extremely high N/m values are not reproducible enough for practical implementation.

High efficiencies are possible in CEC, because the applied voltage causes a flat plug-like EOF flow profile as in CE and there is no backpressure unlike in HPLC and in μLC. The only restrictions in terms of particle size stem from practical difficulties with packing columns and overlapping electric double layer for very small particles, as discussed theoretically by Knox and Grant.[5] However, Unger et al.[29] showed that particles with diameters as small as 0.2 μm can still be used.

Although, at present, CE is capable of higher efficiencies than CEC, CEC has two significant advantages over CE.

Since CEC columns are packed with particles while CE utilizes empty capillary, therefore, loading capacity in CEC is of one to two orders of magnitude higher than in CE.

The CEC is compatible with mass spectroscopy (MS) even when neutral compounds are separated. The MEKC technique that has to be used in CE to separate neutral compounds is incompatible with MS detection.

CEC IN THE PHARMACEUTICAL INDUSTRY

The pharmaceutical industry has recognized the benefits of CEC when high efficiency and compatibility with mass spectrometric detection are of paramount

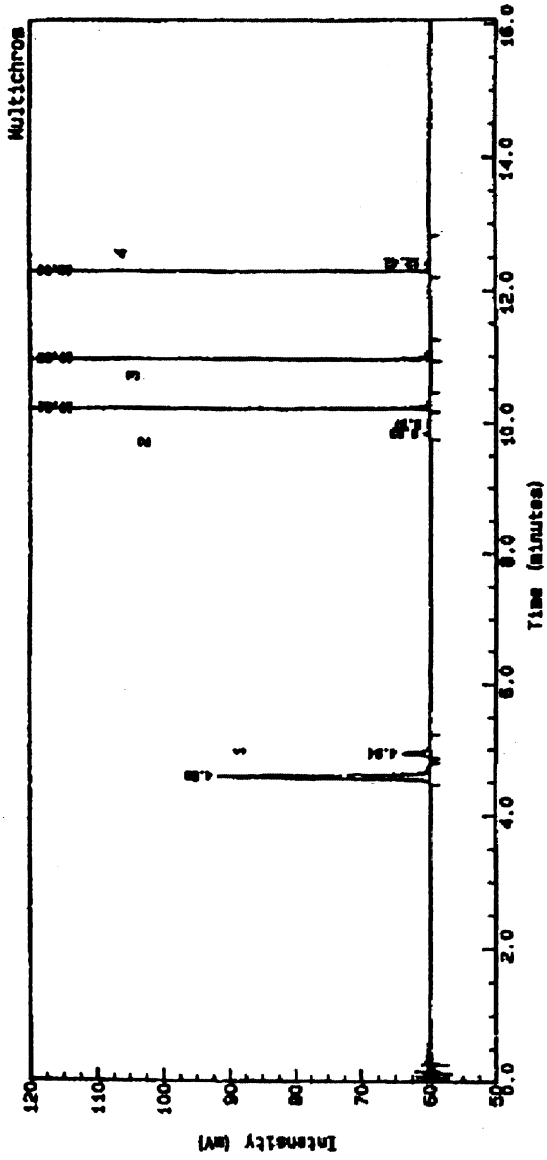

Figure 1. The separation of bendroflumethiazide from nortriptyline, clomipramine, and methdilazine. Instrument: Modified ABI 270A; Capillary: 26 cm packed, 50 cm total × 50 μm i.d.; Packing: 3 μm Spherisorb SCX; Detection: $\lambda = 210$ nm, 0.04 absorbace units full scale; Rise time: 0.2 s; Applied voltage: 30 kV; Temperature: 30°C; Mobile phase: 70% CH3CN/30% 0.05 mol/L NaH2PO4 apparent pH = 3.5: Injection: 2 kV for 0.5 min. **1** = bendroflumethiazide, **2** = nortriptyline, **3** = clomipramine, **4** = methdilazine. Data captured using V.G. Multichrom system. (Reprinted with permission from Ref. 28).

importance. The early stages of CEC development were spurred by both industry and academia where analytical chemists had extensive experience in HPLC, CE, and also MS. It was found to be relatively simple to adapt existing CE systems to CEC applications, such as by pressurizing both sides of the column HP^{3D}.[30,31]

However, CEC was slow to be accepted by the pharmaceutical industry. One of the first papers was published in 1992 by Erni et al.[32] from Sandoz Pharma Ltd., who demonstrated CEC separation of isradipin and its six different by-products. The authors used 3.0 μm and 1.8 μm Monospher ODS, and achieved very high efficiency (790 theoretical plates/s) for unretained thiourea. For retained compounds, like benzyl alcohol and isradipin, the numbers of theoretical plates were lower (710 and 230 N/s, respectively). Nevertheless, it was quite an achievement.

Two years later, Smith (Glaxo Research and Development Limited) and Evans (University of Herfordshire) published important work on separation of a steroid from its impurities, diastereoisomers of antibiotics, prostaglandins and their intermediates, on 3.0 μm and 1.8 μm ODS stationary phases with efficiencies higher than 300,000 N/m.[33]

In 1996, Euerby et al.[34] from Astra Charnwood, showed that, in principle, the rules of RP–HPLC are applicable to RP–CEC for neutral compounds: the logarithm of the capacity factor k was proportional to the percentage of acetonitrile in the mobile phase. At the same time, however, they pointed out that there are small but important differences between CEC and HPLC in selectivity, as shown by different elution orders of diastereoisomers of tipredane.

In the following years there were more and more papers published by scientists affiliated with most major pharmaceutical companies: Miyawa et al.[35] from DuPont Merck Pharmaceutical Company, presented a CEC method for the separation of an antibacterial drug from its related S-oxidation products. Reilly, from Eli Lilly & Co, and Saeed (LGC: Teddington Ltd.) reported validation of a CEC method for determination of drug-related impurities in Lilly compound LY300164, using CEC as an alternative technique orthogonal to HPLC and CE.[36] Wang et al.[37] from R. W. Johnson Research Institute, presented partial validation for a CEC method for analysis of norgestimate and its possible degradation products, achieving a quantitation limit of 0.1% for impurities. Bruin et al.[38] from Novartis Pharma AG, showed separation of steroids on fritless CEC with nonporous 1.5 μm reverse-phase particles with 500,000 N/m efficiency, although addition of SDS was necessary. Dovletoglou and Hoffman,[39] from Merck Research Laboratories, reported the first application of nickel ion complexation in CEC in separating MK-991 (semisynthetic dimethyl myristoylated cyclic hexapeptide) and its isomer. Two years later, the same lab even provided the opportunity for a summer intern program on the separation of positional isomers of trifluoroacetophenone and bromobenzonitrile by CEC.[40]

These examples almost entirely relate to reversed phase type separations of neutral drugs, but Owens et al.[41] from AstraZeneca R&D in Molndal, decided to take full advantage of the uniquely high efficiency of CEC and validated a chiral CEC method for determination of enantiomeric purity of metoprolol on a teicoplanin stationary phase, obtaining adequate accuracy, linearity, robustness, and repeatability. The accuracy of this method was evaluated by calculating recovery of known spikes and, also, by comparing the results to a validated LC method. The sensitivity was sufficient to detect the minor enantiomer with LOQ smaller than 0.1% of the peak area of the major optical isomer.

The pharmaceutical industry also made significant contributions in new instrument development. For example, the idea of utilizing pressure on both ends to prevent bubble formation as first suggested by Knox and Grant[5] was practically implemented by Boughtflower et al.[30] from Glaxo Research and Development, UK, and later a similar solution was applied by Hewlett-Packard in their modified HP^{3D} CE system. Glaxo Research Centre, UK was particularly active by contributing to a new design of mass spectrometer/CEC interface (Lane et al.[42]), sharing very practical advice on the production (packing, preparing of the 1st and 2nd frit) and handling and restoration of CEC columns (Boughtflower et al.[30,31]).

ANALYSIS OF POLAR COMPOUNDS BY CEC

With the exception of steroids, most pharmaceutical compounds display multifunctional polarity. Therefore, almost from the beginning, applications of CEC in the pharmaceutical industry for the separation of polar compounds were in high demand. Finding appropriate conditions for the separation of acidic species was relatively easy by working in an ion suppression mode, i.e., low pH, where acids are in their non-dissociated forms. Euerby et al. showed good separation of six substituted barbiturates[43,44] and several diuretic compounds[45] at pH 2.5 in reversed phase mode. The initial concern that EOF would not be present at such low pH was shown to be incorrect in that case. However, Euerby et al.[44] demonstrated that a higher EOF could be obtained by using mixed mode stationary phases containing C18 and a strong cation exchanger (SCX).

The situation is much more complicated in the case of basic compounds, which are the most important pharmaceutical class. Initially, the outlook was very optimistic, because by using SCX as a stationary phase Smith and Evans[28] achieved enormous separations (Fig. 1). Although, it was confirmed by the others,[44,45] nevertheless, this phenomenon was highly irreproducible. To this day it is not understood, and apart from vague descriptions attributing those high efficiencies to stacking effects, only Moffatt et al.[46] and Stahlberg[47,48] seriously attempted to explain it. The former, with a concept of pulsed gradient under

nonequilibrium conditions, and the latter by a special case in his theoretical model where a peculiar combination of electric field and adsorption effects could create this phenomenon.[47] But since samples of steroids in the work of Frame et al.[49] were dissolved in mobile phase, the unusually high and reproducible efficiency for betamethasone-17-valerate ($N/m > 1$ million) on reversed phase type stationary phase, can be explained neither by focusing effect nor by Moffatt's theory. Fortunately, this often-irreproducible phenomenon is not always observed and, therefore, SCX type phases are usable.

Recent publications by Enlund et al.[50] describing the successful and reproducible separation of tricyclic antidepressant drugs on various strong cation exchangers connected to porous silica particles, and by Westerlund et al.[51] who studied the CEC of hydrophobic amines on SCX in form of monolithic continuous beds based on acrylamide polymers confirmed this possibility. In both cases, a respectable efficiency of 200,000 N/m was achieved and no anomalies were observed (Fig. 2). The possibility of using SCX for reproducible separations of bases without any mobile phase additions, which can be incompatible with mass spectrometry, is very beneficial even though the efficiency is much lower than in case of the above-mentioned anomaly. Of course, if the described phenomena can be properly controlled in future that would be even better. This approach is further supported by Klampfl et al.[52] who separated pyrimidine derivatives on mixed-mode stationary phases, which exhibit both strong ion-exchange (either SCX or SAX) and reversed-phase chromatographic characteristics.

However, the latest work of Hindocha and Smith[53] contradicts the above-mentioned results on using SCX for separation of basic compounds. They showed that monolith columns, based on negatively charged monomer of 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS), interact so strongly with base analytes (e.g., amytriptyline, nortriptyline, caffeine, and niacinamide) that peaks are either highly distorted or are permanently retained on the column. However, the same type monolith columns, but based on positively charged monomer of *N*,*N*-dimethylaminoethyl acrylamide (DMAA), provide good peak shapes for all basic compounds when operated using negative polarity voltage. The authors presented data showing good chromatography for acidic and neutral compounds with AMPS columns and also good chromatography for basic and neutral compounds with DMAA columns. In conclusion, they postulated that SCX monoliths are good for separation of neutral and acidic compounds, while SAX monoliths are suitable for separation of neutral and basic compounds.

Such contradictory conclusions might lead to total confusion. However, in the author's opinion, the above results show that successful application of SCX to separation of basic compounds, depends on characteristics of individual SCX stationary phase and has to be further investigated.

The use of a monolith column[51,53] is of special importance because it avoids many pitfalls of packed capillaries, like bubble formation, difficulty in

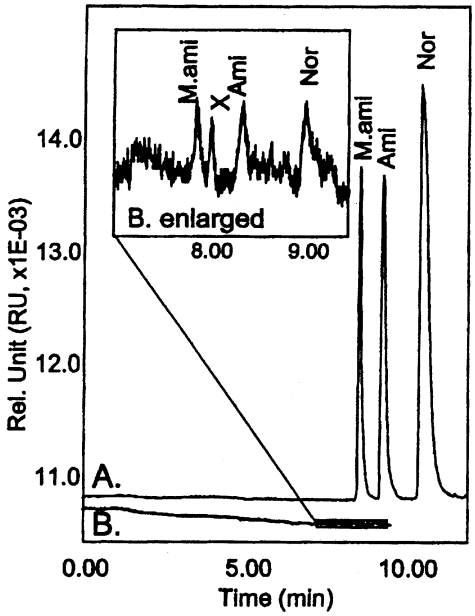

Figure 2. Electrochromatograms showing the separation when using samples of very low conductivity and long injection times on column 3B. (A) 122 ng/mL *N*-methylamitriptyline, 253 ng/mL amitriptyline and 369 ng/mL nortriptyline, (B) 24 pg/mL *N*-methylamitriptyline, 52 pg/mL amitriptyline, and 74 pg/mL nortriptyline. Mobile phase, 10% phosphate buffer stock solution, pH 2.75, 50% ACN and 40% water (0.012 M ionic strength); sample, 0.08% buffer, 1.92% water, 2% ACN, and 96% 2-propanol (0.000092 M ionic strength, excluding the contribution from the analytes); injection, 5.0 kV for 3.0 min, V = 20.4 kV, Leff = 28 cm, Ltot = 45.5 cm. (x) = a peak of unknown origin that did not grow with increased concentration of the analytes, i.e., may be a system peak. (Reprinted with permission from Ref. 51).

packing, and other problems, particularly their fragility at the frit points, which makes their handling in real laboratory-life very demanding. It is worth noticing that the authors were able to lower the concentration limit of detection (CLOD) from 1.3 μg/mL to 50 pg/mL by cleverly applying a stacking effect and injecting samples dissolved in solution containing 96% of 2-propanol that had much lower conductivity than the mobile phase.

Another way to separate basic compounds by CEC is to use a competing base as part of the mobile phase, as in HPLC. Independently, Euerby et al.[54] and Lurie et al.[55] separated different acidic, basic, and neutral drugs in one run on reversed phase type stationary phases. Both groups worked at low pH:

Lurie et al.[55] used mixtures of acetonitrile and phosphate buffer pH 2.5 containing hexylamine as a mobile phase, while Euerby et al.[54] showed that triethylamine or triethanolamine added to a low pH buffer provides very good CEC results. That approach was further developed by others: Enlund and Westerlund[56] investigated the influence of different amines on separations; de Jong et al.[57] studied the influence of amine concentration and pH of the mobile phase on CEC performance, demonstrating unique selectivity of this technique in comparison with CE and much higher efficiency than in LC. Rozing et al.[58] showed that different basic drugs could be well separated on CEC in comparison to μLC and CE (Fig. 3).

Slightly different procedures were proposed by Euerby et al.[59] who used bare silica as a stationary phase instead of reversed phase and by Zou et al.[60] who applied a strong cation exchanger, but in both cases a competing base was added to the mobile phase.

Luo et al.[61] published an interesting paper on the feasibility of performing CEC on a bare silica stationary phase using a reversed phase type mobile phase consisting of acetonitrile and trishydroxymethylaminomethane buffer for the separation of strongly basic compounds, and obtained better selectivity than with CE.

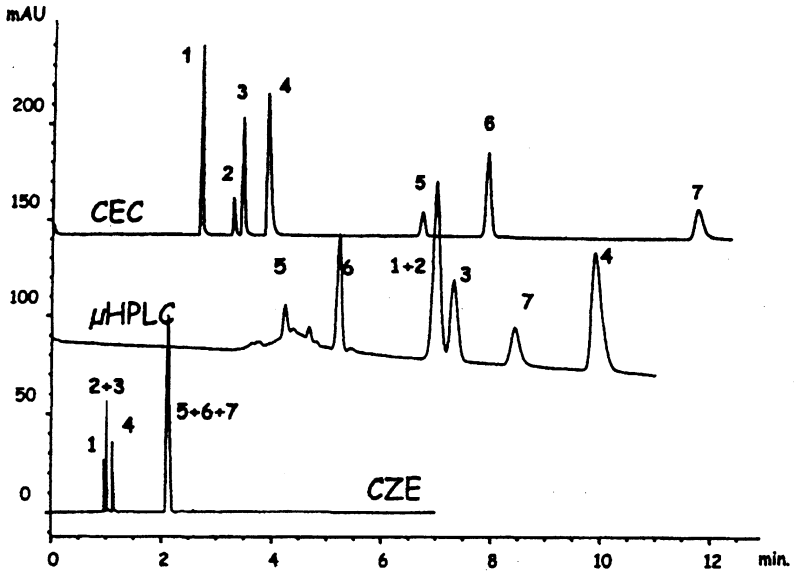

Figure 3. Comparison of drug mix by CEC, HPLC, and CZE. Column, Spherisorb ODS-I, 3 μm 250(335) × 0.1 mm. Eluent: ACN-25 mM phosphate, 0.2% hexylamine, pH 2.5 (80 : 20). Voltage: 25 kV, pressure (HPLC), 200 bar, CZE, uncoated fused-silica capillary 250(335) × 0.075 mm. (Reprinted with permission from Ref. 58).

These results were supported by work of Euerby et al.[62] who demonstrated a separation of acidic, basic, and neutral compounds, also on unbonded silica stationary phase, in relatively high pH of 7.8. As with Luo et al.[61] they demonstrated that silica exhibits small, but substantial reversed phase character.

Zou et al. separated acidic and neutral compounds on strong anion exchangers[63] and acidic, neutral, and basic compounds on uncharged monolithic columns;[64] in both cases SDS or CTAB were added to mobile phases.

The last approach for the separation of basic compounds is to select an appropriate HPLC stationary phase with protected silanol residual groups and use high pH at a range of about 9.0, as demonstrated by Moffatt et al.,[65] Smith et al.,[66,67] and Fitzpatrick et al.[68]

Thus, it may be stated with confidence, that the problem of separating polar compounds by CEC is solved. Methods where no mobile phase additives are necessary are of particular interest, because of its possible application into CEC–Mass Spectrometry (CEC-MS).

INTERFACING CEC WITH MS AND OTHER DETECTORS

MS Detector

The first CEC–MS interfacing was accomplished by a group from University of Leiden and reported in the paper of Tjaden et al.[69] where pressure assisted CEC (pCEC) was connected to a continuous-flow fast atom bombardment mass spectrometer. Then, in 1995 Tjaden et al.[70] reported the connection of μLC and pCEC to an electrospray mass spectrometer. By 2000 they abandoned pCEC and developed nanoelectrospray MS without a sheath liquid for characterization of peptides.[71] However, in 2001 they reported that despite the 20–40-fold loss in sensitivity, the sheath flow assisted interface was superior compared to the nanoelectrospray-sheathless approach in terms of ruggedness.[72] In their recent publication (Walhagen et al.[73]) they addressed the problem of using short columns. Luedtke and Unger[74] had theorized that short columns, not longer than 10 cm, would be beneficial. Walhagen et al.[73] designed and constructed an injection valve (Fig. 4) connecting a 15 cm long capillary column to a MS detector, using a nano-spray interface with a sheath flow. The valve allowed operation under pressure-assisted mode, which prevents bubble formation and also leads to faster analysis. Although, the necessity of manual operation was a serious disadvantage of the valve, yet its good performance was demonstrated by the analysis of peptide mixtures.

Boughtflower and Lane of the Glaxo Group, Stevenage UK, through liaisons with the Universities of Edinburgh, Wale, and Leiden made significant progress in developing interfaces to MS.[41,75–81] Starting from innovative design

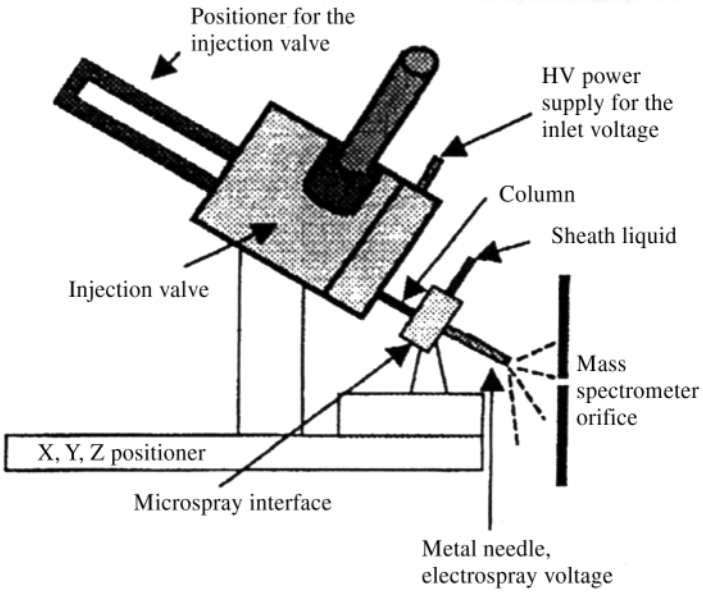

Figure 4. Schematic of the CEC valve interfaced with the mass spectrometer. (Reprinted with permission from Ref. 73).

of home built devices in 1995–1997,[41,75,76] they reached final achievement in 1998;[77,78] an automatic injector with capacity for 10-position sample carousel with nano- and micro-spray interfaces. Figures 5–6 show the design of interfaces and, Fig. 7, a comparison of nano-spray and microspray sensitivity . The methods developed by this group were applied to proprietary drug candidates,[76] tags-used in combinatorial chemistry[79] and for analyzing drugs in blood plasma.[80]

Boughtflower/Lane and Leiden Group abandoned the nano-spray interface in favour of the micro-spray interface with sheath flow.[79] They found that that the loss of sensitivity was compensated by the ruggedness of the micro-spray interface. This lead to the development of a robust CEC-MS system[79] comprising of an automatic injection system connected to a relatively short column (25–35 cm) and a micro-spray interface. Columns with mixed stationary phases (C18/SCX or C6/SCX) proved to be most efficient for separating basic compounds (N/m up to 400,000).[79,80]

Boughtflower et al.[81] investigated various column-tube connection systems and concluded, that in systems with both UV and MS detection, the UV window must be as close as possible to CEC column's frit end. They reduced dispersion occurring in the tube connecting CEC to the MS by keeping the

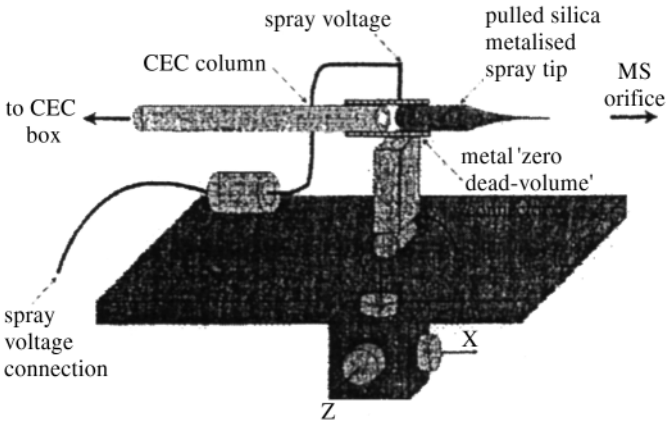

Figure 5. Schematic of the CEC/nanospray interface, showing the column butted up to the metalized pulled silica spray tip inside a metal "zero-dead volume" connector, to which the spray voltage is connected. All of this assembly is mounted onto an xyz positioning stage. (Reprinted with permission from Ref. 77).

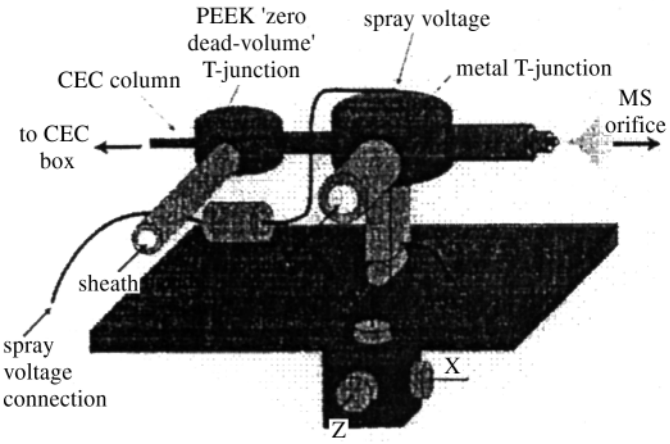

Figure 6. Schematic of the CEC/microspray interface, showing the triaxial probe arrangement, with the CEC column at the center, surrounded by the sheath liquid needle with liquid added via a PEEK T-junction. The sheath gas is added via a metal T-junction into the outermost needle of the arrangement. The spray voltage connection is also made at this metal T-junction. A xyz stage is also used for positioning this interface. (Reprinted with permission from Ref. 77).

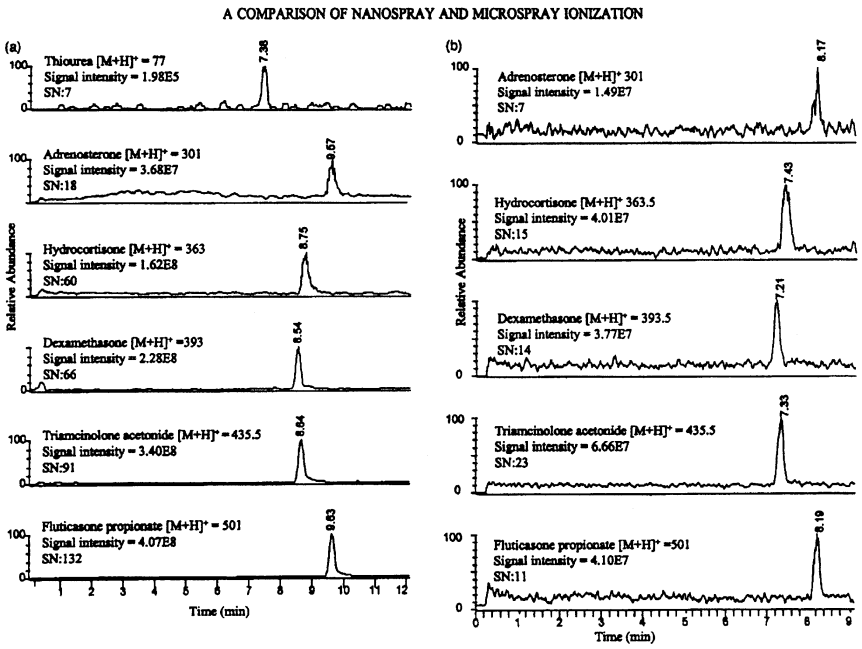

Figure 7. (a) CEC/nanospray-MS analysis of 5 pg each of the steroids and thiourea. Full scan (m/z 55–550). (b) CEC/microspray-MS analysis of 5 pg each of the 5 steroids. Full scan (m/z 55–550). (Reprinted with permission from Ref. 77).

connector as short as practically possible, and its diameter should be no larger than 0.25, that of the CEC column.

A group from Manchester, after their initial interest in fast-atom bombardment (Gordon et al.[82]), presented an innovative work (Lord et al.[83]) on using different tapers and restrictors in CEC for connecting to electrospray MS. In both publications, separations of steroids were presented.

Bayer et al.[84] were one of the first to demonstrate coupling CEC to electrospray MS via placing the CEC column directly into the inner steel needle of the ion source of Sciex API III. They created an interface without the sheath flow by applying supplementary pressure to stabilize EOF. Such a pCEC–MS system has been used to identify the components of peptide mixture. Four years later, they presented a novel approach by connecting pCEC (80 bars) to coordination ion spray MS (CIS-MS). In order to supply silver ions necessary for this ionization technique, they used a coaxial sheath flow interface. They demonstrated separation and mass spectra of unsaturated fatty acid esters, vitamins D2 and D3, and estrogenic compounds.[85]

Horvath et al.[86] presented an interesting report on separation of PTH-amino acids within 5 min, by combining a gradient home built CEC system with time of flight MS (TOF-MS), using short (15 cm) columns with an internal taper on outlet side. TOF allowed for fast scanning (0.25 s/spectrum) and obtaining MS data without peak distortion.

Novotny et al.[87] presented separation of bile acids and their conjugates on two types of macroporous monolithic CEC columns (hydrophobic-C12 and hydrophylic-amine) coupled to ion trap MS through nanospray interface. Since monolithic columns do not require frits, there was no need for the electrode reservoir pressurization to prevent gas bubble formation, which simplified interfacing to the mass spectrometer. They achieved efficiency of separations up to 610,000 N/m.

Blaschke et al.[88] separated and quantified the non-steroidal anti-inflammatory drug etodolac and its metabolites in different matrices, including biological samples, by coupling CEC with an ion trap MS via a laboratory-made interface. Initially, an additional pressure of 12 bars was applied. Later, this was found to be unnecessary, as sufficient EOF was present even at low pH values.[89]

Fanali and Desiderio[90] separated ten non-steroidal anti-inflammatory drugs using CEC coupled to an ion trap MS\ and using "short-end injection" method, as first suggested by Euerby et al.[91] Novotny et al., reported that his group undertook a series of projects on separation of different oligosaccharides from various protein-glycans by CEC-ESI/FTMS.[92]

Fluorescence Detector

Fluorescence detectors can be used instead of MS if the compounds of interest can readily be converted to fluorescent analogues.

Novotny et al.[93] showed that dansylated ketosteroids were detectable through laser-induced fluorescence (LIF) at the attomole level, three orders of magnitude better than underivatized ketosteroids by MS. Similarly Nagaraj and Karnes[94] achieved LOD of 1.7 ng/mL of doxorubicin in plasma after derivatisation with Cy5.29.Osu, and by using a visible diode laser induced fluorescence detector. Dovichi et al.[95] presented separation of PTH-amino acids with LOD $< 0.5\ \mu M$ with LIF detection.

NMR Detector

Coupling of CEC to NMR is a relatively novel and difficult task. A collaboration of English and German scientists from industry and academia produced notable results.[96–99] They designed and built a detection cell and

interface that allowed them to investigate drugs and their metabolites in biofluids[96,97] by μLC, CE, CEC, and pCEC, both in continuous and stopped flow mode. Later, CE was abandoned due to its low sensitivity. In a recent paper, Bayer et al.[99] postulated that pressure assisted CEC (pCEC), due to its better efficiency compared to μLC and shorter retention time compared both to CEC and μLC, appear to be most promising. Moreover, in this pCEC type design, a gradient was also applied.[98] Relatively low pressure (up to 80 bars) helped not only to reduce time of analysis but also to stabilize the system. No significant efficiency loss compared to pure CEC was observed, most likely due to low efficiency of pure CEC itself in this case, because of the length of empty capillary connection (1 m) between packed column and NMR detection window. Nevertheless, any micro capillary technique coupled to NMR has one important advantage against HPLC—their low consumption of eluent allows for using fully deuterated solvents. There is definitely a strong trend to link μLC and CEC, or pCEC, with NMR detection.

CEC IN CHIRAL SEPARATIONS

Since enantiomers of active ingredients in drugs often exhibit different pharmacokinetic activities and toxicity, chirality has become an important issue for the pharmaceutical industry in new drug discovery. Regulatory requirements made it obligatory to either produce a drug in its pure optical form, or to provide all relevant data for its enantiomeric forms, if in the form of racemate. Therefore, analytical methods for determination of chiral purity became of paramount importance. Any strategy, which provides additional information in this aspect, is of vital interest for the industry.

One of the most popular methods of separating enantiomers is the application of cyclodextrins and their derivatives, either as additives to mobile phase and/or as part of the stationary phase. CEC evolving from both HPLC and CE was naturally inclined to use similar procedures. Some examples of this approach can be found in numerous publications.[100–109] The work by Schurig et al. is particularly noteworthy.[101,102,104] They attached permethylated cyclodextrin (CHIRASIL-DEX) to the capillary wall and separated different enantiomers in the mode of open tubular liquid chromatography (OTLC) or open tubular electrochromatography (OTEC), with or without pressure assistance. Although, results were often spectacular, they suffered one major disadvantage-low stationary to mobile phase ratio and, hence, low sensitivity. By increasing film thickness, that phase ratio can be improved but mass transfer problems immediately become evident in the form of poor performance. Therefore, classical CE, with addition of cyclodextrins to mobile phase, was superior.[102] Recently, Schurig started to use different cyclodextrins for coating of silica

particles packed to CEC columns.[106,108] Also, Zou et al.[109] described interesting work on modifying SAX stationary phase by dynamically adsorbed sulfated β-cyclodextrin. Although, the overall mechanism is quite complex, nevertheless, separations of several drugs were achieved with efficiency reaching 400,000 N/m.

Another important group of stationary phases is based on derivatives of cellulose. Here, two groups were particularly active: Otsuka et al.[110,111] and Blaschke/Chankvetadze et al.[112–116] Initially, both groups reported rather poor separations of different chiral drugs on silica based particles coated with cellulose tris(3,5-dimethylphenylcarbamate).[110,112–114] However, recently Otsuka et al.[111] presented excellent efficiency (200,000 N/m) when 3 μm particles were used instead of 5 μm previously studied.[110] Good ruggedness, repeatability in migration time, peak height, and corrected peak areas, together with sensitivity of 0.1% content of first and 1% of second migrated enantiomer, were reported. In the case of cellulose tris(3,5-dichlorophenylcarbamate), a strong effect of its loading ratio on silica base particles on efficiency was observed.[115,116] This was explained by slow mass kinetics. This may also account for poor results obtained using silica coated with cellulose tris(3,5-dimethylphenylcarbamate) reported previously. Not only was smaller particle diameter responsible for better efficiency, but also probably the load ratio of the coating was different. Unfortunately, that information was not given for cellulose tris(3,5-dimethyl-phenylcarbamate). Recently chiral CEC[116] was shown to be superior to μLC in resolving the enatiomers of 2-(benzysulfinyl)benzamide (Fig. 8) and etozolin (Fig. 9) This was accomplished by using a low coating ratio of silica particles with cellulose tris(3,5-dichlorophenylcarbamate) to minimize the slow kinetics of mass transfer.

Other popular types of chiral stationary phases are based on macrocyclic antibiotics. The most often used in CEC are teicoplanin[41,117–119] and vancomycin[120–123] in a form of either in-house made stationary phases or commercially available Chirobiotic T and Chirobiotic V. Although, as initially reported, they are characterized by slow mass transfer,[120] nevertheless, high efficiency of 135,000 N/m was achieved with teicoplanin,[119] and 190,000 N/m with vancomycin.[121] Their selectivity and efficiency depends on type of solute, temperature, quality of column packing, and many other factors. No general dependency was established—even reports on the influence of temperature on efficiency are contradictory. It seems that measuring efficiency and selectivity as a function of the rate of particle coating with macrocyclic antibiotic, as it was done in case of cellulose tris(3,5-dichlorophenylcarbamate), might be very beneficial. Nevertheless, these stationary phases showed remarkable chiral separation power to many different drugs. Methods for enantiomeric separation of metoprolol on teicoplanin stationary phase,[38] as well as for simultaneous baseline separation of enantiomers of venlafaxine and its main active metabolite in human plasma,[123]

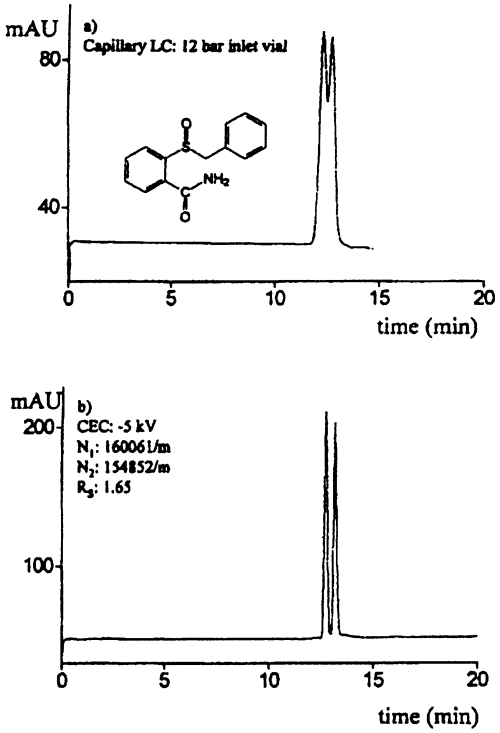

Figure 8. Enantioseparation of 2-(benzylsulfinyl)benzamide in capillary LC (a) and CEC (b) mode. Capillary was packed with aminopropylsilanized Daisogel (5 μm, 2000Å), which was coated with 0.5% (*w*/*w*) cellulose tris(3,5-dichlorophenylcarbamate). Applied pressure: 12 bar (a); applied voltage:-5 kV (b). (Reprinted with permission from Ref. 116).

were both validated. In the latter method, the limit of quantitation was 0.05 μg/mL.

The use of protein coated stationary phases for chiral separations by CEC has been unsuccessful so far.[124,125] In this case, CEC was inferior to both HPLC and CE.

Lammerhofer et al.[126] reported the separation of various chiral acids on a weak anion-exchange chiral stationary phase under non-aqueous conditions (acetonitrile–methanol with addition of acetic acid and triethylamine). The stationary phase was prepared by coating 3 μm silica with *tert*-butylcarbamoylquinine. Efficiency was of the order of 100,000 N/m and run times of about around 10 min.

Pirkle et al.[127] prepared brush-type chiral stationary phases (CSP) based on (S)-Naproxen and (3R, 4S)-Whelk-O immobilized on 3 μm silica supports in

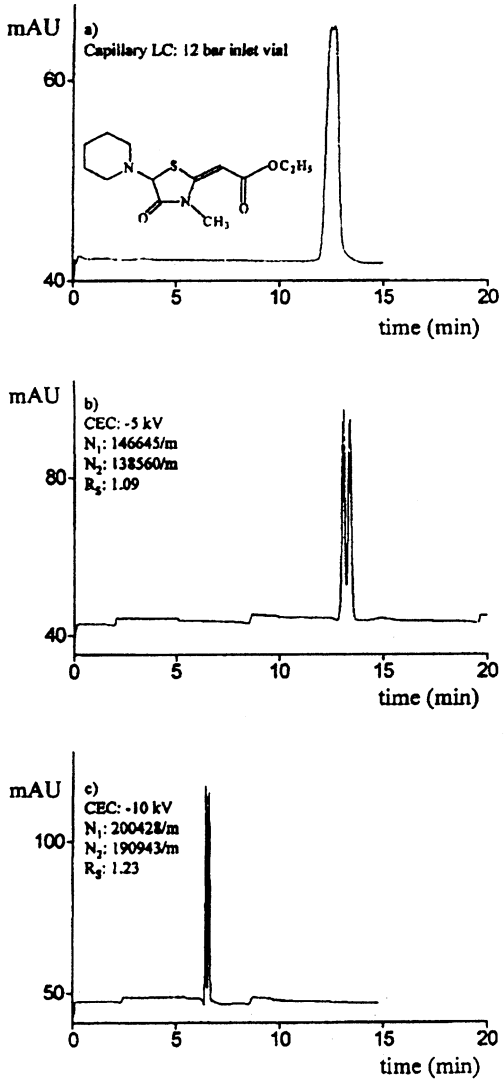

Figure 9. Enantioseparation of etozolin in capillary LC (a) and CEC (b, c) mode. Capillary was the same as indicated in the experiment shown in Fig. 8. Applied pressure: 12 bar (a); applied voltage: −5 kV (b) and −10 kV (c). (Reprinted with permission from Ref. 116).

order to improve mass-transfer kinetics. Ten test chiral analytes, representing a variety of classes of compounds, have been separated into their respective enantiomers with high selectivity and high efficiency (up to 200,000 N/m).

The fragility of CEC columns, susceptibility to drying out and bubble formation are some of the serious disadvantages of CEC based on particle packed columns. Therefore, capillary columns with continuous beds, i.e., monoliths, where such problems are absent, are attractive alternatives to packed columns in CEC. Several papers report chiral separations on various monolith type CSPs.[128–132] Cyclodextrin derivatives, either copolymerized[131] or bonded[132] to monoliths, provided acceptable chiral separations although, the profound rise of baseline reported by Vegvari et al.[131] needs to be eliminated in the future.

Frechet et al.[128] used a valine-based chiral selector in production of a "moulded" rigid monolithic CEC column, which initially did not give very good results. But in later papers,[129,130] the copolymerization of a monomer with quinidine functionality proved to be an excellent choice. (The same group used tert-butylcarbamoylquinine attached to silica,[126] also with excellent results). For numerous enatiomers, but mostly amino acid derivatives, high selectivity and high efficiency were obtained (250,000 N/m). Moreover, by applying a "short-end" injection technique, full separations were recorded in a short time (5–10 min).

Another way of achieving selectivity in separation science and in chiral CEC, in particular, is molecular imprinting methodology. It is based on synthesis of organic polymer in the presence of a template/imprint molecule. Subsequent removal of the imprint molecules leaves recognition cavities with affinity for the original imprint species. Thus, the choice of imprint molecule determines what compounds would be separated. The most widely used approach to create molecular imprint polymers (MIP) is through non-covalent synthesis, as shown in Fig. 10.

For short but concise reviews on MIP and their applications, readers are recommended to look through other papers.[133,134] Particularly, the group of Nilsson et al.[135,141] has been outstanding in this field. Alas, despite some spectacular achievements and high selectivity, the MIP approach suffers from high tailing and very slow mass transfer kinetics, which translates into very poor efficiency. Nevertheless, in a recent paper, Nilsson et al.[141] reported separation of propranolol enantiomers in less than 1 min using a short (8.5 cm) "super-porous" monolith column. Here, efficiency was also low, but because of enormous selectivity power it is conceivable that this methodology could be used in "lab-on-a-chip" technology for fast and very efficient screening of combinatorial libraries. Although, still in the form of capillary columns, this concept was already tested by Nilsson et al.[138] who screened 10 amino alcohols and tryptophane using an R-propranolol MIP as a target model. Later, Vallano

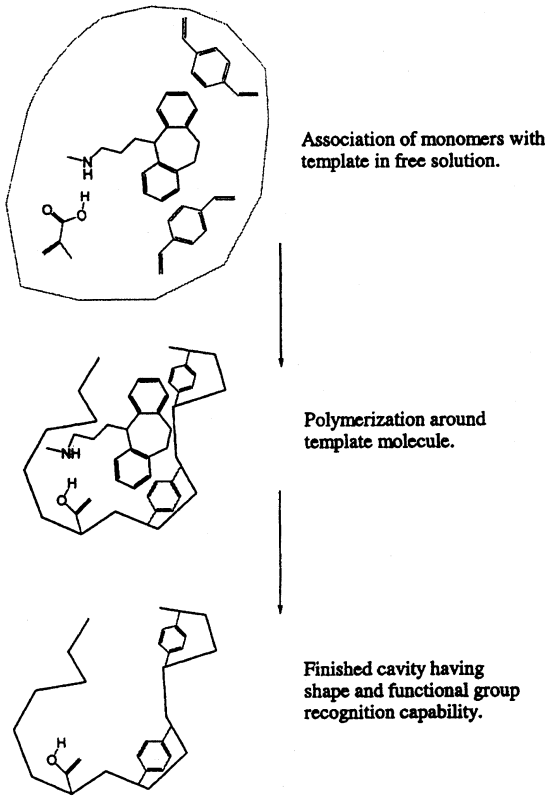

Figure 10. A simplified representation of the synthesis of a noncovalent molecular imprint polymer. (Reprinted with permission from Ref. 133).

and Remcho[133] used this approach for screening a small simulated combinatorial library consisting of tricyclic antidepressant drugs, using one of them (nortryptiline) as the template (Fig. 11).

According to Andersson,[134] MIP might find an important application as highly selective phase tailor-made extraction sorbents for sample preconcentration, and as alternatives to antibodies used in immunoassays, offering similar molecular recognition. The very important advantage of micro techniques for such task is that only minute amounts of compounds have to be used, and it is only a matter of time before MIPs in the form of CEC, μLC, or "lab-on-a-chip" will be used for screening big combinatorial libraries and/or used in sample preparations for extraction and concentration from complex media, like for example bio-fluids.

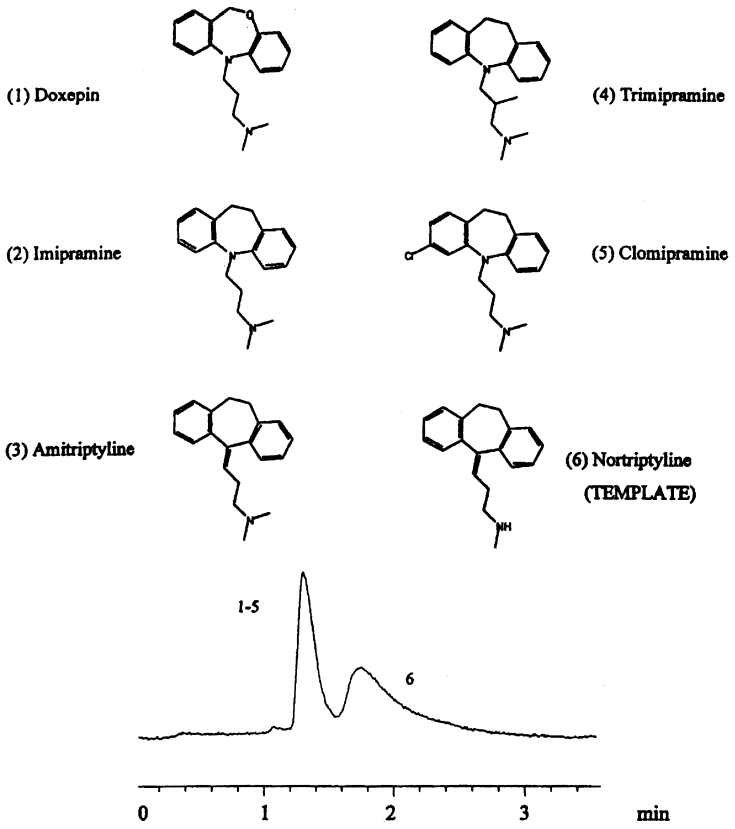

Figure 11. MIP-CEC separation of a simulated combinatorial library consisting of several tricyclic antidepressants. Conditions: capillary I.D. 100 μm; L_{tot}: 33 cm; L_{bed}: 22.5 cm; eluent: acetonitrile: 10 mM Na acetate pH 3.0 (98 : 2) with 0.02% trifluoracetic acid and 0.015% triethylamine (v/v); voltage +30 kV constant; injection: +2 kV, 2 s; column temperature: 50°C. (Reprinted with permission from Ref. 133).

CEC IN ANALYSIS OF BIOPOLYMERS AND BIOFLUIDS

Biopolymers

Since many present drugs are based on biopolymers, e.g., sandostatin, and/or interact with them, therefore, their analysis is important for the industry. Readers particularly interested in this subject are referred to a recent review of Krull et al.[142] devoted to this subject.

Examples of separation of peptides by CEC coupled with MS were reported by Gucek et al.[71] Proteins and peptides were also successfully resolved on etched chemically modified open tubular CEC by Pesek et al.[143,144]

Horvath et al.[145] showed the superiority of CEC with porous-layer open-tubular columns over CE in separation of basic proteins. Very interesting work on the application of DNA aptamers for separation of bovine β-lactoglobulin variants A and B was presented by Rehder and McGown,[146] who accomplished that by covalently bonding aptamer to a capillary surface of open-tubular CEC. It was established, that the G-quartet 4 plane structure of the aptamer was essential, since separation did not occur on stationary phase with similar oligonucleotide, which did not form a G-quartet structure. It was also not possible to separate β-lactoglobulin variants by CE. McGown et al. reported that aptamer phenomenon of reversible structure melting at 35°C (below they have G-quartet 4 plane structure and above this temperature it disappears) might be utilized for selective trapping of proteins (Pittcon 2). They showed that at temperatures below 35°C, only one peak is obtained from injection of proteins, while at 45°C another peak appears. These are very preliminary results but, nevertheless, suggest possibility of using aptamers for selective retention of proteins that can be switched "on" or "off". Taking into account that aptamers are stable and easy to handle, it might open very promising new possibilities.[147]

Rassi et al.[148] came to a similar conclusion on the superiority of CEC over CE. They separated small and large nucleic acid fragments by using a mixed type stationary phase (octadecyl-sulfonated-silica) and addition of tetrabutylammonium bromide to the eluent, whereas CE failed to resolve these fragments. Helboe and Hansen[149] separated six important nucleotides in 13 min, twice as fast as by HPLC, and successfully validated that method; however, it was necessary to use thymidine as internal standard to be able to achieve acceptable area repeatability. Horvath et al.[150] demonstrated certain advantages (selectivity, peak capacity) of CEC over HPLC in separations of conalbumin and hemoglobin variants by using an anion exchanger type column. In the next paper,[151] they presented the unique selectivity of CEC based on anion exchange type monolith columns for separation of peptides, proteins, and tryptic digest of cytochrome *c*. It was shown that CEC is as good as CE or better.

Singh et al.[152] demonstrated good separation of amino acids and bioactive peptides by using both negatively- and positively-charged polymer monoliths, either in form of capillaries for CEC or cast in microchannels of glass chips. In the latter instance, peptides were labeled with naphthalene-2, 3-dicarboxyaldehyde for laser-induced fluorescence detection. Rassi et al.[153] presented a novel application of CEC in separating neutral and acidic glycosphingolipids on octadecyl sulfonated silica. Novotny et al.[92] showed very encouraging results on the separation of glycobiologically significant oligosaccharide mixtures. All these

examples clearly indicate that CEC is an important supplementary technique to CE and HPLC in the separation of biopolymers.

Bio-Fluids

Analysis of drugs and their metabolites in biofluids (urine, plasma, etc.) is an integral part of new drugs development for assessing pharmacodynamics, pharmacokinetics, and toxicology. Analysis of bio-fluids from a patient is essential in proper diagnosis as well. Also, other types of analysis (e.g., forensic, nutritional, etc.) deal with similar sample matrixes. Usually different types of extractions, filtration, dialysis, etc., are required prior to analysis; this, however, pertains to a separate domain of analytical science. Nevertheless, even after careful and meticulous sample preparation, there is often still a substantial amount of interferences where CEC, with its high efficiency separating power, is all-important.

Some applications of CEC-MS in analysis of drugs in biological matrices,[76,80,88,89] as well as CEC linked to other detectors, such as visible diode laser induced fluorescence[94] or even NMR[96,97] have been discussed earlier.

However, work of Desiderio et al.[123] is worth mentioning again because that was the first successful chiral separation of a drug and its metabolite in real administration samples i.e., in biological fluids from a patient under therapy; and also, the method was partially validated. Determination of enantiomeric content of venlafaxine and its metabolite *O*-desmethylvenlafaxine in plasma, from patients suffering from depression, was important for understanding the mechanism of action of each enantiomer and their pharmacokinetic and pharmacodynamic relations, since the drug is administered in the form of a racemic mixture.

As shown in Fig. 12, the enantiomeric ratio varied between patients and further studies were required. Nevertheless, CEC proved to be an analytical tool for correlating enantiomeric ratios with effectiveness, gender, age, and other parameters important for physicians.

Tjaden et al.[154] provided another interesting example of CEC application for detection of drugs in biofluids. On line isotachophoresis (IT) for focusing of analytes prior to separation by CEC linked to a single quadrupole MS, achieved a limit of detection for neostigmine, salbutamol, and fenoterol spiked in human plasma and urine in the low nmol/L range!

Taylor et al.[155] were first to apply a gradient CEC method for the quantitative analysis of corticosteroids content in equine urine provided. The method was partially validated.

Choudhary et al.[156] presented applications of an "integrated analytical instruument" (it was a prototype of HP (now Agilent) instrument as a combination of CE HP^{3D} and HP 1100 pumps) for analysis of clinical samples from cancer patients. Here, CE, μLC, pCEC, and CEC were applied as orthogonal

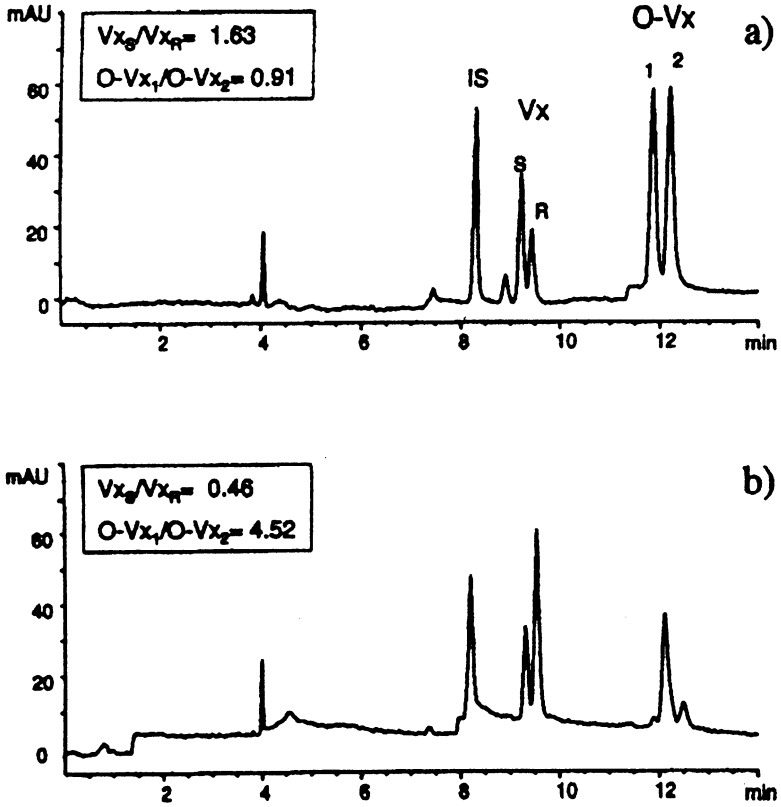

Figure 12. Analysis of extracted plasma samples of (a) and (b) from different patients under depression therapy with venlafaxine. Mobile phase composition: 100 mM ammonium acetate buffer pH 6.0/water/acetonitrile (5 : 5 : 90, v/v); applied voltage, 27 kV. Capillary: 75 μm I.D. × 35 cm total length, packed as following: 11 cm diol/silica, 23 cm vancomycin CSP/silica, and 1 cm diol/silica. Both the inlet and the outlet ends were pressurized at 10 bar. Capillary temperature 20°C. Sample injection: 10 kV for 30 s followed by 12 bar for 0.2 min of mobile phase injection plug. (Reprinted with permission from Ref. 123).

techniques for detection of cachectic factors (sulfated glycoprotein) in urine samples. CE proved to be the least suitable because of its low loading capacity and, hence, low detectability. The remaining techniques gave comparable results as shown in Fig. 13.

Warner et al.[157] applied CEC for analysis of cholesterol, cholesterol linoleate, and oleate retrieved from atherosclerotic plaque deposits on arterial walls of human aortas. Roed et al.[158] analyzed the retinyl esters content in liver

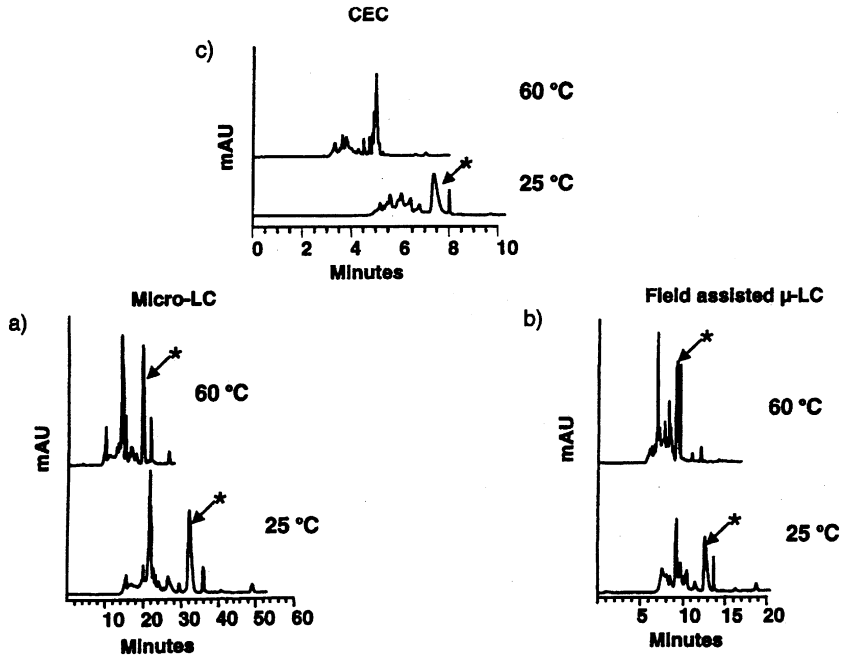

Figure 13. Effect of temperature on the separation of a urine sample from cachectic patient by capillary liquid chromatography, field assisted capillary liquid chromatography, and capillary electrochromatography. Column, 33.5 cm (effective length 25 cm) × 75 μm packed with 3 μm Hypersil ODS particles; starting eluent (A), 10 mM borate buffer, pH 8.5; gradient former (B), 10 mM borate in a water–acetonitrile (1 : 4, v/v) mixture; gradient, isocratic at 20% B; electrokinetic injection, 5 kV, 5 s. Modes, (a) CEC, 15 kV, 0 bar, (b) electric field assisted μLC, 5 kV, 250–180 bar, (c) μLC, 160–110 bar. (Reprinted with permission from Ref. 156).

samples of seals by CEC, on continuous bed columns of sol-gel bonded large pore C18 material.

These are only but a few examples of possible applications of CEC in analysis biopolymers and bio-fluids.

DISADVANTAGES OF CEC AND POSSIBLE COUNTERMEASURES

After more than a decade of development, there are still theoretical aspects of CEC, which are debatable. For example, Horvath et al.[159] postulated that

pressure assisted CEC (pCEC) was providing more benefits (gain in speed of analysis) than losses in efficiency. He also postulated that intraparticulate electroosmotic convection could assist mass transfer inside macroporous particles and, hence, increase the efficiency. That was found to be true for neutral molecules, but for charged molecules the chromatographic mechanism is blurred and difficult to describe. Even the effect of porosity of particle packing on CEC efficiency is not so obvious. For example, Poppe et al. in one paper[27] show very high efficiency on non-porous particles and in the next article[160] also very high plate numbers, but this time on particles with 500Å–4000Å pore diameter. However, it is impossible to draw a clear conclusion, as there is variability in other parameters, except for pore size. Therefore, any new model capable to describe separation of both charged and neutral compounds by CEC is always in high demand. The recent paper of Horvath and Xiang[161] is such an example. Their model is based on conditions of ideal/linear chromatography with "a simple random walk" approach. They assumed, that in a sufficiently high electric field, ionized samples could migrate in their adsorbed state. This is most probably responsible for gradient like elution visible in electrochromatograms of proteins obtained under isocratic CEC conditions. Again, it was postulated that the combination of pressure-driven and voltage-driven mode is the most beneficial in terms of selectivity and time of separations. Similar conclusion was made by Colon et al.[162] who showed separations of different PAHs to demonstrate benefits of using high pressure (10,000 psi) assisted CEC on submicron particle columns in comparison to pure μHPLC or CEC.

Columns

One of the notorious problems associated with CEC are columns. Classically, they are made of fused silica with internal diameter of around 75 μm and up to 100 cm length, packed with 3–5 μm particles based on silica; and the current tendency is to pack even smaller size particles. The packing process itself and preparation of frits are tedious and difficult and the columns are extremely delicate. The frits are particularly fragile and cause bubbles, loss of efficiency, etc. The smaller the particle size, which is beneficial in terms of number of theoretical plates, the more difficult it is to prepare and handle columns.

There were many attempts to address these problems, which are difficult to evaluate. Here are a few examples of overcoming these difficulties. Bruin et al.[38] recommended a specific fritless approach, which was further developed by Rapp and Bayer[163] using internal tapers and PTFE-FEP shrink tube connectors. Tjaden et al.[164] proposed an easy pseudo-electrokinetic packing procedure, utilizing a high electric field in conjunction with a hydrodynamic flow. It took only 15 min by using a basic commercially available CE instrument to pack

a column. No additional pressure was necessary to prevent formation of bubbles, and columns were stable for at least a hundred injections. Saevels et al.[165] provided tips how to pack columns in-house, using simple equipment consisting of an ordinary LC pump and materials available in any LC/CE orientated laboratory. Nevertheless, it seems that practical problems related to packed columns are still far from over.

Therefore, it is no wonder that open-tubular capillary electrochromatography (OTCEC) is still under investigation despite its major drawbacks of low loading capacity and low retention. Jinnno and Sawada[166] recently presented a good review on this subject. In principle, the difference between OTCEC and CE is that OTCEC column is a small diameter ($< 25\,\mu m$) fused capillary, coated with an appropriate stationary phase that has an ability to retain solutes. OTCEC attracts scientists because of the relative ease of preparation and handling. In order to increase sample capacity, two approaches are used: either creating polymeric porous-layer as described by Horvath et al.[145] used successfully for separation of proteins and peptides, or by etching the inner surface of the fused silica tube to increase the surface area and then attaching different types of compounds as a stationary phase, as in Pesek and Matyska's method.[144,167] The major problem is that increasing the capacity of wall surface results in a higher mass resistance and, hence, decreases efficiency. Therefore, a compromise is necessary between loadability and mass transfer kinetics while maintaining high selectivity of stationary phase. A partial solution to this can be found in the work of Malik and Hayes,[168] where sol-gel chemistry-based method was developed for preparation of C18 coated OTCEC. Furthermore, by applying quaternary ammonium moiety in the process, they created a wall surface with an isoelectric point of c.a. 8.5 and were able to reverse the direction of EOF by using an appropriate pH of the mobile phase buffer. Various types of model compounds were separated on these columns, with very high efficiency reaching 400,000 N/m for thiourea, 383,000 N/m for fluorine, and always above 200,000 N/m for other compounds.

The most promising column technology so far is the monolith type described by Majors.[169] It is worth noting, that a similar approach is slowly gaining momentum in HPLC column technology.[170] There are many different approaches, which will not be discussed here, and more interested readers are referred to other reviews.[17,24] No loose/unconnected particles are present and frits are unnecessary in columns of this type. There are many papers, which describe this technology, for example by Novotny et al.[87] Singh et al.[152] and others.[171–174]

In terms of efficiency, monoliths are often inferior to packed columns, but this situation is changing. Lee and Tang[172] achieved 180,000 N/m with good run to run and column-to-column retention time reproducibility. In this particular case, sol-gel bonded 3 μm silica particles with mixed ODS/SCX coatings were tested with mixtures of aromatic compounds. Freitag and Hoegger[174] obtained an RSD of 5% for batch-to-batch EOF reproducibility, using a macroporous

acrylamide-based polymer as stationary phase. The ability to prepare very long monolith columns might be useful if proper stationary phases with high charges are developed and/or a very high voltage could be applied. This would provide sufficient EOF for separation in a reasonable time. Horvath and Xiang[161] have suggested applying additional pressure, as in most cases, monoliths have a lower backpressure than classical columns. This is analogous to progressing from a 2 m packed GC column to a 50 m capillary columns.

This brings us to development of new stationary phases and, particularly, to mixed-mode stationary phases, which exhibit both strong ion-exchange and reversed-phase chromatographic characteristics (SCX/RP or SAX/RP) or even switchable.[168] All are being developed in the form of classical packed columns[52,80,175] and monoliths.[51,60,172,173] As shown by Steiner and Scherer,[175] EOF as high as 1.7 mm/s might be achieved with very little loss in efficiency. This allows the unretained analyte to pass a one long column in 10 min.

The concept of physically and dynamically modified columns, which has found applications in a few areas like for example, chiral separations,[109] should also be mentioned. An excellent review on this subject has been recently published.[176]

CEC exhibits different mechanisms for the separations of charged and neutral molecules due to the electric field. These are dependant on differences in absorptivity on the stationary phase and on differences in electrophoretic mobility in the mobile phase, or even in the stationary phase if the model of Horvath and Xiang[161] is correct. It is demonstrated by separation of sulfonamides by Smith and Dube.[177] However, neutral molecules display different separation profiles in CEC and HPLC when reversed phase conditions from HPLC are applied to CEC. Euerby et al.[45] reported that it was impossible to separate steroid tipredane from its C-17 diastereoisomer on any typical stationary phase by HPLC, but separation was achieved without any problems by CEC using ODS 1 packed capillary. This phenomenon is even more noticeable in the work of Miyawa et al.[178] who observed that two neutral substances related to DuPont Merck proprietary anti-inflammatory drug DUP 654 had a different order of elution in CEC than on HPLC under the same conditions, and concluded that adaptation of ODS based separations from HPLC to CEC requires more than simple transfer of conditions. In this work, method development for CEC was also presented. Therefore, it can be concluded that CEC often exhibits unique separation characteristics different to these in LC and CE.

Sensitivity

UV/VIS detection in CEC is more sensitive than CE, as CEC can take more samples than CE but is not as sensitive as HPLC due to the diminished optical path. MS detection[71,72,78] is more compatible with CEC, since the whole

eluent flow can be analyzed, whereas in HPLC only a small fraction of the eluent flow is diverted for the MS detector. Both HPLC-MS and CEC-MS have similar sensitivities. LIF is one way of increasing sensitivity of CEC.[93,94] The sensitivity of CEC can also be increased by using specific preconcentration techniques.[51,154,179] Preconcentration can be achieved by dissolving the components in a weaker solvent[179] or in lower conductivity diluent[51] than the mobile phase. Then, by increasing the sample loading time it is possible to inject more samples without the loss of efficiency. In the first mode, LOD might be easily improved by factor of 10 and, in the latter, even by factor of 10,000! This was accomplished by using a high content (96%) of 2-propanol in the sample as shown in Fig. 2. However, if the sample is not soluble in 2-propanol, a more universal but also more complicated approach of concentration, is to use of isotachophoretic (ITP) on-line sample focusing prior to CEC separations.[154] Here, LOD was lowered by factor of 100–1000. The ITP-CEC procedure is illustrated schematically in Fig. 14. The only limitation of this combined technique is that IT applies exclusively to charged compounds.

In conclusion, CEC offers advantages over CE and its sensitivity is approaching that of HPLC.

Instrumentation

The problem of generating gradient elution using commercially available CE/CEC instruments is one of the weaknesses of CEC. Readers are encouraged to look through reviews totally devoted to instrumental aspects in general,[21] or only to gradient techniques.[20]

The simplest, but very effective solution, was demonstrated by Euerby et al.[180] who applied a step-gradient. It is based on the following principle: the run is started with the initial mobile phase conditions (weak eluent), then at a predefined time (by instrument programming) the inlet and outlet vials are replaced with stronger mobile phase. This might consist of many cycles if necessary. The achieved reproducibility of retention times showed an RSD below 1% and the chromatography was not compromised. By applying a one step-gradient, it was possible to decrease run times of six diuretics from 37 min to 17 min. There were, however, two setbacks in this method, namely: (a) short stops in analysis, which were expressed as disturbances in baseline and (b) lack of smoothness of continual gradient profile, so popular in chromatography.

The second approach is by so called electroosmotically driven solvent gradients demonstrated by Zare et al.[181] Here, dynamic gradients were generated by merging two electroosmotic flows. Their ratio was regulated by computer-controlled voltages. The drawback was that each time samples were injected, the inlet end of capillary had to be disconnected from T-connector and placed into sample vial and

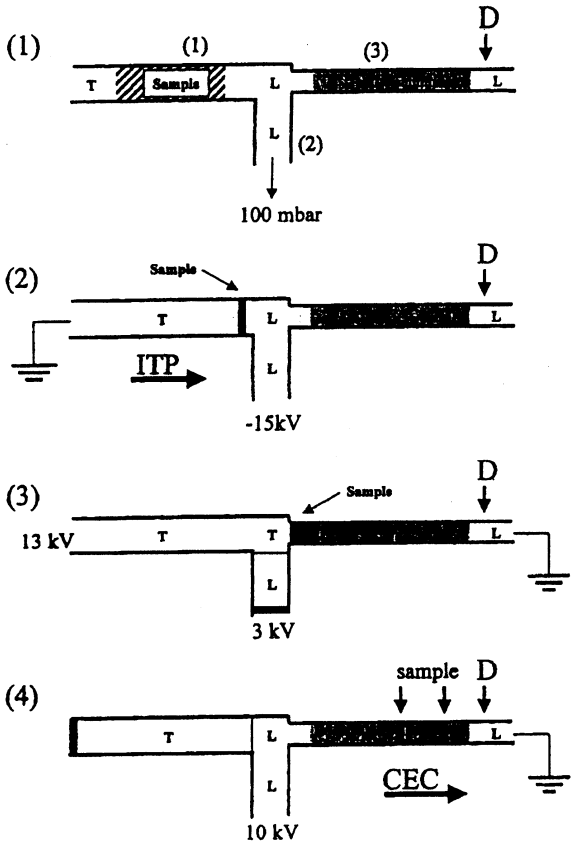

Figure 14. Schematic representation of the ITP-CEC procedure. The sample loading, ITP focusing step, sample zone transfer and CEC separation are shown in step 1, 2, 3, and 4, respectively. The set-up contains a (D) UV-VIS absorbance or MS detection, (T) terminator buffer and (L) leading buffer. Untreated fused-silica capillaries of 220 μm I.D. (1 and 2) and 75 μm (3) are used. (Reprinted with permission from Ref. 154).

then re-connected again. This can cause easy damage to fragile column and is not easy to automate. The system must be carefully calibrated in order to know the exact composition of eluent, since EOF is very sensitive even to small changes in ionic strength, viscosity, and other parameters, which influence zeta potential.

Another solution was proposed by Novotny et al.[182] where a gradient was generated through the difference in the linear flow velocity of two mobile phases, in a gradient generator device consisting of a short fused silica capillary connected with a piece of glass capillary with a bigger internal diameter. The

glass capillary was manually filled with a stronger solvent, while the inlet buffer reservoir contained a weaker solvent. Since the volumetric flow rate was kept constant, the linear flows in both capillaries were different and this caused the inflowing weaker mobile phase to mix with the stronger phase in the capillary, thus, generating a gradient. Similar problems as in previous cases might occur.

These difficulties can be minimized or completely eliminated if the gradient is generated by pressure driven delivery of different strength eluents. The easiest solution was to attach normal HPLC pump or pumps via different connectors. The simplest strategy was to connect an HPLC pump through a splitter to CEC and, thus, create pressure assisted CEC.[183] Another approach was more complicated and required a different interface device, which allowed an HPLC pump to generate a gradient to the CEC column but without additional pressure flow.[86,98,155,184,185] In both cases, however, long dwell times of 9 min[155] or even 17 min[184] were reported.

Therefore, applying a micro pump with even more complicated interfaces might be a better option. Dorsey et al.[186] used a micro-LC system and simple flow-injection analysis-capillary electrochromatography interface. Although the dwell time was only 2 min, serious peak broadening occurred.

Lee et al.[187] demonstrated a much more complicated interface device. A syringe pump delivered pure organic solvents to the initial buffer vial, where they were mixed with a micro stirring bar.

It is worth to mention, that temperature gradient was also tried. Djordjevic et al.[188] applied temperature gradients from 25°C to 60°C at a rate of 3°C/minute for successful separation of 11 steroids. A 50% decrease in run time was achieved compared to a run at 20°C.

To summarize, the simplest and the most reliable approach is through pump generated mobile phase gradients. However, adding another costly HPLC or micro-LC unit to an expensive CE/CEC system only for gradient generation does not seem to be justifiable. This matter looks, however, completely different if this additional pump can be used for creation of so called *triskelion*, named so by Horvath et al.[189] or in other words, a unified system for performing CE/CEC/pCEC/μLC.[156,189,190] So far, to the author's best knowledge, almost all these instruments were either in-house assembled/constructed/modified[189,190] or delivered to the lab as a prototype.[156] The only commercially available unified system seems to be Ultra-Plus II Capillary LC-CE/CEC System from Micro-Tech Scientific, Inc.[191]

REGULATORY

In the pharmaceutical industry, any new technique sooner or later has to meet scrutinized Regulatory Authority requirements. A new non validated technique

might be used in the early stage of drug development, but from the moment it proceeds into clinical trials it has to be validated and, hence, pass through quality assurance requirements and finally through national authority like e.g., FDA, which is bound by International Conference on Harmonization (ICH) requirements. Therefore, it is important that a new technique has to pass validation tests or other more suitable techniques, such as HPLC, must replace it in a later stage. This has been an issue in CEC for some time and there are many papers dealing with at least partial validations. The examples can be found in many of aforementioned publications.[35–37,41,76,149,155,192–198] As Reilly and Saeed[36] stated in their paper, "regulatory bodies prefer that purity assays for pharmaceuticals be complemented with another technique", which guarantee that no "unknown" impurity is present and not counted. A very good agreement between CE, HPLC, and CEC on total and individual impurity quantitation has been shown in this paper, although, LOD in HPLC is still better than in CE and CEC.

For example Smith and Hindocha[197] compared nano-HPLC, GC, and CEC in the determination of two positional isomers of a potential drug. The results obtained by these three techniques, which might be considered as complementary to each other, were similar. In the next paper, Hindocha et al.[198] described a full validation of proprietary drug substance assay according to ICH guidelines, demonstrating sufficient specificity, linearity, and range, accuracy, precision, limit of quantification (LOQ), and robustness. The CEC method met all criteria and the overall results showed that CEC could be used in a highly regulated environment. The only disadvantage was that LOQ was just below 0.1% by peak area and was higher than for HPLC.

A very important similar paper was published by Owens et al.[41] who fully validated a method for the determination of optical purity for both enantiomers of metoprolol. Again the CEC method passed all criteria, however, the authors complained about column fragility and susceptibility to drying.

Obviously, CEC can pass regulatory requirements although LOQ/LOD and precision should still be improved. However, CE was in a similar situation in early 90s and now it is an officially recognized technique by current United States Pharmacopoeia.

"LAB-ON-A-CHIP"

This new technique, also called micro total analysis system (μTAS) was "born" almost ten years ago,[196] and its principle is based on using electrophoretic pumping through microchannels created on silica based chips. Most applications were developed from the canons of capillary zone electrophoresis and it can be best imagined as CE, where capillary columns were replaced with microchannels. This technology has undergone very fast

development and already achieved a stage of commercial implementation, especially in genomic and proteomic areas. A special web site[200] is solely dedicated to commercially available instruments and technology. The detectors most used are LIF, MS, or electrochemical. Papers by Cowen[201] and Dolnik et al.[212] are recommended for readers who are less familiar with this technology. As was in the case of normal CEC, here also electrochromatography was introduced mainly to enhance loadability of sample and to diversify the mechanism of separations. For example, Harrison et al.[203] trapped ODS coated silica beads into weirs type cavities within chip channels, and used this device to increase concentration of two analytes by a factor of 500 and then separate them within 20 seconds, achieving a plate height of 2 μm.

Regnier[204] presented a completely different novel concept of monoliths and chip type chromatography. He microfabricated monolith columns by ablation of quartz chips. Multiple crisscrossing channels (1.5 μm wide and 10 μm deep) were sculpted by etching. This way, cubic support structures mimicking particles in classical columns were created. Then, walls were derivatized with octadecylsilane (ODS), and such 4.5 cm long columns were able to separate a tryptic digest of ovalbumin. The efficiency for Rhodamine 123 reached 777,000 N/m.

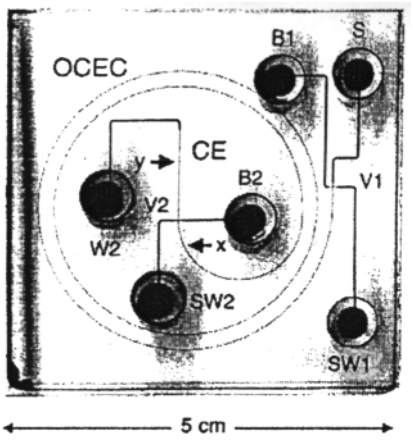

Figure 15. Image of the microchip used for 2D separations. The separation channel for the first dimension (OCEC) extends from the first valve V1 to the second valve V2. The second dimension (CE) extends from the second valve V2 to the detection point y. Sample (S), buffer 1 and 2 (B1, B2), sample waste 1 and 2 (SW1, SW2), and waste (W) reservoirs are positioned at the terminals of each channel. The arrows indicate the detection points in the OCEC channel (x) and CE channel (y). The channels and reservoirs are filled with black ink for contrast. (Reprinted with permission from Ref. 205).

A more classical and less complicated strategy for separation of low molecular weight analytes and peptides was proposed by Ramsey et al.[205] Here, two types of separation mechanisms were employed: first step of separation occurred in open-channel electrochromatography (OCEC) performed in 25 cm long spiral channel modified with ODS and the second, in a 1.2 cm long straight CE channel coupled with it. An image of the microchip used for this two-dimensional separation is shown in Fig. 15. The peptides were labeled by tetramethylrhodamine isothiocyanate (TRITC) to facilitate laser-induced fluorescence (LIF) detection. An example of 2D separation of TRITC-peptides is presented in Fig. 16. The only disadvantage of this method is still relatively low capacity of OCEC.

Frechet et al.[206] and Shepodd et al.[207] created UV-cured polymers for monolith type CEC columns with potential application to microchips. This possibility was explored by demonstrating a separation of three PAHs on fused-silica chips an with 25 μm deep, 50 μm wide, and 8.6 cm long channel, achieving at least 150,000 N/m.[207] This was further developed and improved with surpassing efficiency in plate numbers (600,000 N/m) by Throckmorton, Shepodd,

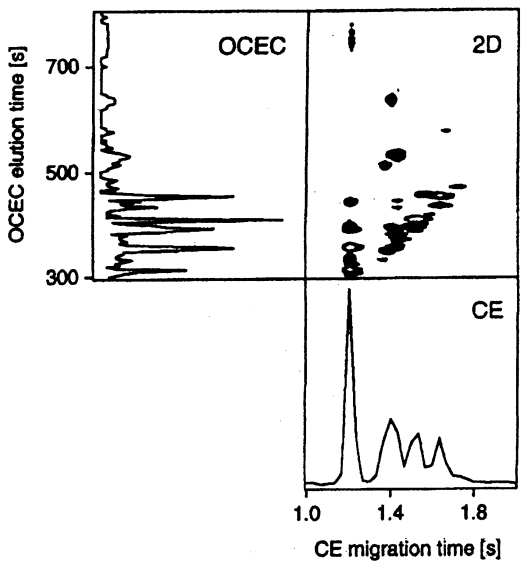

Figure 16. 2D separation of TRITC-labeled tryptic peptides of β-casein. The projections of the 2D separation into the first dimension (OCEC) and second dimension (CE) are shown to the left and below the 2D contour plot, respectively. The field strengths were 220 V/cm in the OCEC channel and 1890 V/cm in the CE channel. The buffer was 10 mM sodium borate with 30% (v/v) acetonitrile. The detection point y in Fig. 15 was 0.8 cm past valve V2 in the CE channel. (Reprinted with permission from Ref. 205).

and Singh.[208] Six peptides labeled with naphthalene-2, 3-dicarboxaldehyde (NDA) were separated in less than 45 seconds. The channels were 25 or 40 μm deep, 90 to 130 μm wide, and with 5 cm separation length, LIF was used for detection. The authors also separated five NDA labeled amino acids in less than 2 min.

All these examples show that "lab-on-a-chip" is a reality. This technique has already found many applications for bio-analysis, and its future development might be even exponential.

CONCLUSIONS

The papers discussed demonstrate that CEC might find its important place in the pharmaceutical industry. The present situation is similar to the history of development of CE. Nevertheless, there are some problems, which seriously undermine the perspectives of CEC, mainly column technology. The other aspect is lack of qualified scientists able to properly utilize CEC. Therefore, for a medium size bio-pharmaceutical company, it is not justifiable to make a considerable investment into instruments and manpower if it is only for development of CEC. However, it might appear completely different if a more universal approach is considered (to utilize all micro techniques, μLC, CE, CEC, and pCEC in one instrument-*triskelion*), particularly, if offering the possibility of interfacing to MS or NMR. In such cases, investments in these techniques might prove very beneficial in the longer term (4–5 years). Later it might add a necessary edge to company competitiveness. If, at the same time, the specialist keeps abreast with the "lab-on-a-chip" developments, then the benefits are undeniable, even if only in the form of knowledge, which provides necessary competence in dealing with different contracted-out research laboratories. Therefore, an answer to the original question: "is it justifiable for medium size, fast growing bio-pharmaceutical company to invest into capillary electrochromatography?" is YES, but only in all three microtechniques at once.

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