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Capillary Electrochromatography – Challenges and Opportunities for Coupling with Mass Spectrometry

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Abstract. Capillary electrochromatography (CEC) has attracted considerable interest within recent years because of its potential to generate very high efficiencies within relatively short analysis time. Since CEC combines the attributes of both capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC), neutral as well as charged analytes can be separated. Usually, CEC is performed with UV detection, but mass spectrometry (MS) is becoming a more common detection method because additional information about the molecular weight and the structure of an analyte can be obtained. Due to the low flow rates in the packed capillary and the small sample amounts that are required, CEC is ideally suited for the implementation into miniaturized systems and for coupling with MS. While numerous advantages have been made in CEC/MS, the coupling technique is still in a development and growth stage. So far, the development of the technique seems to be limited by the lack of robust and automated specially designed CEC instruments and CEC interfaces. As soon as these practical constraints have been solved, CEC/MS will be a powerful separation/detection technique with unrivaled sensitivity and specifity. This article aims at highlighting the potential of CEC as coupling technique with mass spectrometry.

Introduction

Capillary electrochromatography (CEC) is a merger of capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC) and combines advantageous features of both techniques, namely the high efficiency of CE and the outstanding selectivity of HPLC [1]. It has been invented in the early seventies by *Pretorius et al.* [2] but has not attracted considerable interest until the last few years. Usually, CEC is performed on a modified CE equipment with fused-silica capillaries packed with 3 µm reversed-phase material or mixed-mode phases. Eluents comprise aqueous buffers mixed with organic modifiers such as acetonitrile and methanol and should have an adequate dielectrical constant to stabilize the electroosmotic flow (EOF). The mobile phase is driven through the capillary by the EOF. The EOF has its origin in the electrical double layers that exist at the solid-liquid interfaces of the fused-silica capillary and the mobile phase, and, additionally, of the packing material and the mobile phase. Studies on the influence of the packing material on the EOF showed that the contribution of the capillary wall to the EOF is negligible and that the flow is almost exclusively generated by the silica beads [3]. The magnitude of the EOF depends on the surface charges of the particles as well as on the properties of the eluent, *i.e.*, the dielectric constant and viscosity of the mobile phase, the temperature, and the concentration of the electrolyte. The ideal electroosmotic-flow profile is plug-like so that the flow velocity is the same across the inner diameter of the capillary. In CEC, separation can be achieved by adsorptive solute-surface interactions of the analyte with the mobile and the stationary phase, as well as by electromigration. Depending on their charge and their size, ionized compounds can elute ahead or after the EOF marker. Due to the small dimensions of the capillary and the buffer/sample vials, CEC is advantageous where only small sample volumes are available, *i.e.*, biological and combinatorial library samples.

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Advantages of CEC *vs.* HPLC

A characteristic that distinguishes electroosmotic flow from pressure-driven flow is the difference in the flow profile. Because EOF is generated by the electrical field action on mobile-phase counterions at the solid-liquid interface, the flow velocity is independent of the diameter of the flow channels between adjacent stationary-phase particles, and between particles and the capillary wall. This holds as long as the channels are large enough to prevent overlap of the oppositely charged ions of the electrical double layer [4]. The near-constant flow velocity across the capillary diameter produces a flat flow profile. In contrast, the flow profile in HPLC is parabolic reflecting the flow inequalities among flow channels of different diameter. The differences in flow increase the degree of solute dispersion, *i.e.*, the eddy diffusion, resulting in significantly greater band broadening compared to CEC. As no pressure limitation exists in CEC, porous and nonporous micron and submicron silica beads with an average particle diameter dp < 3 µm have been investigated as stationary phases [5]. It has been found that the EOF is independent of the particle diameter dp in the range between 0.2 and

3.0 µm using the same *n*-alkyl-bonded phase and identical experimental conditions. This result encourages the application of submicron silica beads since small particles enable high separation efficiencies and short analysis times [6]. According to the modified *van Deemter* equation, the influence of the A- and the C-term will diminish when submicron silica beads are applied leaving the B-term (axial diffusion) as the only factor contributing to band spreading. This will result in extremely sharp peaks of the order of almost 1 million plates per meter.

Equation. Modified *van Deemter* equation

$$H = A\, dpu^{1/3} + \frac{BD_m}{u} + \frac{Cdp^2}{D_m} u$$

A is the eddy-diffusion term, B is the axial-diffusion term, C is the mass-transfer term, D_m the diffusion coefficient of the analyte in the mobile phase, and dp the particle diameter. Calculations of theoretical H *vs.* u curves based on the modified *van Deemter* equation for 1.0 µm and 0.5 µm particles clearly indicate that at moderate EOF velocities (~3 mm/s) the plate height H is indeed controlled by the axial diffusion whereas the influence of the A- and the C-term can be neglected [7]. Since the H *vs.* u curve is still descending at these velocities, a further decrease in analysis time can be attained by increasing the flow velocity, *i.e.*, increasing the electrical field strength, without compromising the efficiency. Unfortunately, this cannot be realized using current commercial instruments because they only generate field strengths up to 1000 V/cm. Therefore, the potential of submicron silica beads cannot yet be fully exploited with modified CE instruments.

Advantages of CEC *vs.* CE

Since the EOF is more or less exclusively generated by the packing material, a more reproducable flow is established across the entire diameter of the capillary. This allows the use of much wider capillaries leading to greater sensitivity due to increased sample loading and, concerning optical detection, increased path length. Unlike CE, CEC enables the separation of neutral and charged compounds due to the hydrophobic stationary phase in the packed capillary. In CE, the separation of neutral analytes can only be achieved when micelles are used (MECC). The micelles consist of anionic or cationic surfactants and form a *pseudo*-stationary phase within the capillary. Unfortunately, micelles lack selectivity, cause high background, particularly with laser-induced fluorescence, as well as high currents at high voltages and cannot be used in a mass spectrometric system as they would clog up the ion source. As there is no stationary phase in CE, the selectivity of a separation is entirely controlled and adjusted by the mobile-phase composition.

Advantageous Features of CEC with Respect to Coupling with MS

Mass spectrometry has become established as an extremely powerful adjunct to chromatography techniques. The sensitivity that mass spectrometry brings to separation techniques is well established when combined with gas chromatography, high-performance liquid chromatography, supercritical fluid chromatography, and capillary electrophoresis. Numerous applications of HPLC/MS as well as GC/MS and CE/MS have been described. Despite the progresses that have been made within the last years, it is sometimes still necessary to split typical HPLC flow rates of about 1 ml/min to those compatible with electrospray mass spectrometry (ESI/MS) devices. The advent of capillary separation techniques, such as capillary electrophoresis and capillary electrochromatography, offer more compatible flow rates, though still not being ideal. Conversely, the flow rates are too low for the existing MS devices and require a supplementary sheath liquid. The invention of microelectrospray (µESI/MS) and nanoelectrospray (nESI/MS) mass spectrometry, with flow rates of of 20–100 nl/min for nanospray and several hundred nanolitres per minute for microspray, facilitates the coupling with CEC. CEC/MS has successfully been applied by a number of groups using various types of ionization techniques. In 1991, *Verheij et al.* [8] reported on the coupling of *pseudo*-capillary electrochromatography, a combination of both pressure-driven and electroosmotically driven chromatography, with mass spectrometry. In 1994 and 1995, *Gordon et al.* reported on CEC combined with coupling to both continuous-flow fast atom bombardement (CF-FAB) [9] and ESI mass spectrometry [10]. All work published since has dealt with CEC interfaced to ESI-MS. Several classes of substances have been investigated, *e.g.*, peptides [11], DNA-adduct mixtures [12], corticosteroids [13], and surrogate tags [14]. *Wu et al.* [15] analyzed protein-digest samples by ion-trap storage/reflection time-of-flight mass spectrometry (IT/reTOF). So far, CEC has not been coupled with atmospheric-pressure chemical ionization (APCI) which has already been successfully interfaced to capillary electrophoresis [16]. CEC has attracted reasonable interest within recent years because it is ideally suited for implementation into miniaturized systems. In CEC, the EOF moves through the packed capillary at a flow rate of about several hundred nl/min depending on the type of packing material, the composition of the mobile phase and the electrical field strength. This low flow rate is directly compatible with nanospray emitters without the need for either splitting the flow or supplementing it with a sheath liquid. Therefore, no dilution of the sample occurs which is beneficial as the mass spectrometer is a concentration-sensitive detector. A significant increase of sensitivity of several orders in magnitude can be attained compared to LC/MS and regular CEC/electrospray mass spectrometry.

Due to the reduced band-spreading effects and the high efficiencies that can be attained in CEC, narrow peaks are obtained. They are quite beneficial because narrower chromatographic peaks produces higher mass flux leading to an increased sensitivity of the MS. The full electrophoretic integrity of the separation is maintained in the mass spectrometer. In CEC as

Table. *Characteristic Data of Packed CEC Capillaries*

dp [µm]	L [mm]	i.d. [mm]	V_m [µl]	EOF [mm/s]	F_c [µl/min]	V_s [nl]
3	100–295	0.05–0.1	0.16–1.86	1.60	0.6	0.5–3.3
1	100–295	0.05–0.1	0.16–1.86	1.60	0.15	0.3–2.0

dp particle size
L Lenght
i.d. internal diameter
V_m volume mobile phase
EOF electroosmotic flow
F_c flow mobile phase
V_s volume stationary phase

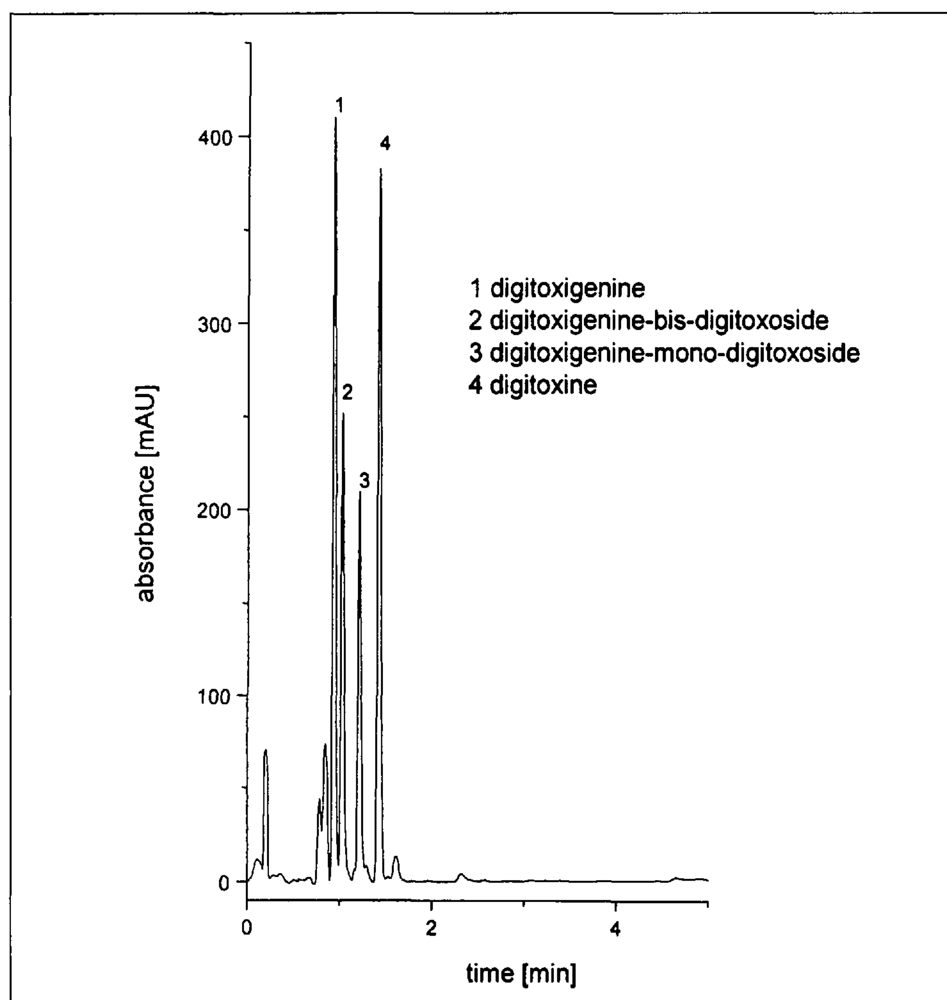

Fig. *Separation of four cardioactive substances.* Capillary: 8.5 (38) cm × 100 µm, packed with 1 µm C8; Mobile phase: 60/40 ACN/TRIS-HCl 12 mM, pH 6, 30°, −790 V/cm, detection: 254 nm UV.

well as in CEC/MS, short capillaries and high voltages are desirable for rapid separations. Several authors have already performed fast CEC separations using capillaries with a packed bed <10 cm at a field strength of about 800 V/cm. The *Figure* displays the separation of four cardioactive substances. The separation has been achieved packing the outlet side of the capillary up to the detection window (using an $HP^{3D}CE$ cartridge holder). The analysis was carried out in less than two minutes.

To avoid deplenishment of the mobile phase, the zwitterionic buffer TRIS (tris-(hydroxymethyl)aminomethane) has been used as electrolyte. It is well established that zwitterionic buffers tend to give a high background signal which can reduce mass spectrometric detection sensitivity. Some authors have already used involatile buffer systems which have not been problematic if operated at nanolitre flow rates [17].

To improve the efficiency of the separation, the capillary was packed with porous *n*-octyl-bonded submicron silica beads of dp = 1 µm. Most of the capillaries that are commercially available are either packed with a 3-µm C18 material or a mixed-mode phase. Mixed-mode phases have both C18 alkyl chains and strong cation-exchange groups attached to the surface of the silica. Since strong cation-exchange groups have a permanent negative charge even at low pH values, they maintain a relatively stable EOF [18]. As low-pH buffer systems are typically used with mass spectromeric detection, the mixed-mode phases are advantageous. The application of small particles, short capillaries, and high field strengths in CEC/MS will offer the potential for a rapid and efficient separation/detection technique approaching the efficiency of GC/MS but without its limitations regarding volatility and thermal lability of the samples.

The column length is one of the major restrictions in CEC/MS, especially when commercial CE instruments are used. As demonstrated, there is no need for CEC capillaries with a packed bed longer than 10 cm, but most of the instruments that are commercially available require a total length of ≥25 cm. Furthermore, most of the CEC interfaces are sub-optimal. They require even longer capillaries or additional transfer capillaries. Transfer capillaries often cause postcolumn dispersion reducing the performance of the separation capillary, whereas long separation capillaries cause longer analysis times due to the diluted field strength. The development of CEC/MS has been limitited by the lack of purpose-built automated CEC instruments, CEC/MS interfaces, and special CEC capillaries. In-house constructed CEC systems often lack robustness and automation capabilities, although their potential has clearly been demonstrated. The coupling of commercial CE instruments with mass spectrometry is not optimal due to the reasons outlined above. To implement CEC/MS as routine method in chemical and pharmaceutical industry, these problems have to be overcome in future.

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