ELSEVIER

Journal of Chromatography A, 735 (1996) 29–42

JOURNAL OF CHROMATOGRAPHY A

Review

Capillary electrophoretic analyses of drugs in body fluids: sample pretreatment and methods for direct injection of biofluids

David K. Lloyd

Meakins-Christie Laboratories, McGill University, 3626 St. Urbain, Montreal, Quebec, H2X 2P2, Canada

Abstract

A variety of strategies for the analysis of biological samples by capillary electrophoresis (CE) are described, with particular emphasis on the determination of drugs and metabolites. Analytical methods involving extensive sample pretreatment before CE analysis are considered, as well as strategies for directly injecting untreated biofluids. The application in CE of techniques common in liquid chromatography is first described, e.g. protein precipitation, liquid–liquid extraction and solid-phase extraction. On-capillary methods of sample concentration are considered. Approaches to performing CE assays of urine and plasma, without prior sample treatment, are described. The use of both capillary zone electrophoresis and micellar electrokinetic chromatography for direct-injection assays is compared for both urine and plasma analyses, and capillary washing strategies are discussed. Finally, direct-injection microanalyses are mentioned.

Keywords: Reviews; Injection methods; Sample preparation; Drugs

Contents

0021-9673/96/$32.00
SSDI 0021-9673(95)01350-4

1. Introduction

Capillary electrophoretic methods are beginning to find routine application for the analysis of drugs and metabolites as well as endogenous compounds in body fluids. Review articles appear in this special issue, as well as elsewhere [1–4]. The analysis of body fluids presents a variety of problems which are common to capillary electrophoresis (CE) and other analytical techniques. These include: a large number of individual compounds in the mixture, leading to difficulty in resolving the analytes of interest; the presence of components, such as proteins, that can modify the chromatographic or CE column; low concentrations of drugs or metabolites, leading to detection difficulties and conjugation of drugs to form much more hydrophilic metabolites. Considering the above list, it is not surprising that the majority of bioanalytical methods do not use just one simple chromatographic or electrophoretic separation step, but rather include one or more sample pretreatment steps [5], which progressively remove dissimilar components from the analyte(s) of interest. This sort of approach results in a relatively purified material being injected into the final separation process, making this separation simpler and, usually, more robust. The disadvantages are also obvious; multiple steps usually require more lengthy or more sophisticated sample handling and there is more chance for variability and errors to creep in.

In CE there has been considerable interest in performing single-step analyses, with direct injection of body fluids on-column. This is quite often feasible in CE because the open capillary columns are less prone to irreversible modification by sample matrix components than a packed HPLC column, and even if adsorption of some compounds does occur, there are few limits on the use of aggressive cleaning steps which can be taken (at least with uncoated fused-silica capillaries). Furthermore, CE often offers high resolution which is necessary in the analysis of complex mixtures. One advantage of cutting out sample preparation steps is the saving of time and effort. There is also another very important stimulus to the development of CE methodology for direct injection analyses. CE is essentially a microanalytical technique, capable of separating components in nanoliter or sub-nanoliter volumes. With direct injection from microcompartments within living systems, CE offers new possibilities for developing a detailed understanding of a variety of life processes that could not be studied previously because of the lack of a suitable microanalytical technique. In this review article, some of the applications of conventional-scale sample preparation techniques, microscale sample preparation and direct-injection methods will be described. Although most of the examples concern drug or metabolite determinations, some are included which touch on the analysis of endogenous compounds. These illustrate the use of interesting sample preparation or direct-injection techniques which are likely to be applied in drug and metabolite analyses in the near future.

2. CE bioanalysis with conventional sample preparation techniques

The sample preparation techniques dealt with in this section, precipitation and liquid–liquid extraction (LLE) or solid-phase extraction (SPE), are well established and account for the vast majority of sample preparation steps used in current HPLC assays. They also feature widely in the bioanalytical CE literature. A relatively large biofluid sample is needed, ranging from a few hundred microlitres to a few millilitres. After preparation, a few microlitres to a few millilitres are available for analysis. In such a case, the sample could be analyzed by HPLC or CE — the microanalytical abilities of CE are not utilized. The justification for choosing CE as the final separation step may be because of its excellent resolving capabilities for analytes which are often tricky to separate by HPLC, such as some diastereoisomers or enantiomers, but there may also be other good arguments for choosing CE, e.g. minimization of solvent usage.

2.1. Precipitation methods

Proteins are the principal components of biological matrices which can cause capillary modification [6], and so their removal is an important part of the sample preparation process. A variety of reagents can be added to plasma or serum to precipitate the proteins which are present [7]. After precipitation,

the sample is centrifuged and the clear supernatant is taken either to be injected onto the CE system, or for further preparation steps. Denaturation of the proteins during precipitation also results in release of bound compounds, so total drug is measured. Of the precipitation agents that are commonly used in HPLC sample preparation (acids, salts and organic solvents), only organic solvents (and in particular acetonitrile) have found widespread use in CE. There are a couple of reasons for this: 1. Efficiency, expressed as the volume of additive needed to precipitate a given proportion of proteins in a given sample. Typically four or five volumes of a concentrated salt solution (e.g. ammonium sulphate) are required to adequately deproteinize one volume of plasma. Thus, considerable sample dilution occurs. Trichloroacetic acid deproteinization results in only a small degree of dilution, while organic solvents have intermediate efficiencies. 2. Properties of the residual supernatant. Salt or acid additives leave the analyte in a high-conductivity matrix. This is not favourable, since the result is a lowered field strength in the sample zone which can lead to band-broadening [8]. In contrast, the supernatant after precipitation with organic solvent has a low conductivity, and so some of the dilution during the precipitation step can be compensated for by using large injections and relatively high conductivity separation buffers. The elevated field strength in the sample zone, which results if the sample zone has a lower conductivity than the background electrolyte (BGE), leads to a stacking or concentration of the charged sample components [9]. Precipitation using 1.5 volumes of acetonitrile to one volume of plasma has been recommended as the optimum system for CE use [10]. The benefits of precipitation methods using organic solvents can be summarized as: removal of proteins; release of bound drug and solubilization of hydrophobic analytes. The primary disadvantage is dilution of the sample.

Protein precipitation has been used for sample preparation for quite a number of CE assays. Some representative examples are given in Table 1 [11–15]. Simple precipitation with small-volume sample injections generally result in rather high limits of detection (LODs) [13]. Large injections under stacking conditions can be used to improve LODs [14]. Alternatively, the supernatant can be evaporated and the residue then reconstituted in a smaller volume to achieve concentration before CE analysis [12].

A simple precipitation step can be an excellent method for sample preparation involving minimal effort. Removal of proteins in this way can produce a matrix which is suitable for injection into the CE system. There are a few limitations on the content of the separation electrolyte. Higher buffer concentrations are favoured, to improve efficiency [10], although in micellar electrokinetic chromatography (MEKC) separations, the amount of on-capillary concentration may be limited when using organic solvents as precipitation agents, because of disruption of the micellar pseudophase [16]. Precipitation methods are likely to find application when speed and simplicity are important and when analyte

Table 1
Some representative CE assays using protein precipitation

Analyte	Precipitation method	LOD/μg ml^{-1} (detection method)	Reference
Gabapentin	Acetonitrile:human serum, 4:1 (v/v), with fluorescamine derivatization	1 (UV)	[11]
Antipyrene	Acetonitrile:rat serum, 4:1 (v/v)	0.25[a] (UV)	[12]
Iohexol	Acetonitrile:human serum, 1.5:1 (v/v)	10[b] (UV)	[13]
Theophylline	Acetonitrile:human plasma, 2:1 (v/v)	1.8 (UV)	[14]
Fosfomycin	Acetonitrile:human plasma, 1:1 (v/v); removal of excess acetonitrile with dichloromethane, then ultrafiltration	1 (UV)	[15]

UV = UV absorbance detection.
[a] Post-precipitation concentration used to obtain low LOD.
[b] LOD not given; this value is the lowest point on the calibration curve.

concentrations are high, such as in some therapeutic drug monitoring applications or in the determination of intoxication.

2.2. Liquid–liquid and solid-phase extraction

Both LLE [5] and SPE [17] have proved popular as sample preparation techniques for CE. In LLE, an immiscible organic phase is added to the biofluid and these are then shaken together, resulting in the more hydrophobic sample components being extracted into the organic phase. In SPE, the sample is passed over a particulate material (e.g. silica) which is covered with a bonded adsorbent phase and which is usually packed into small disposable columns. Some sample components are retained on the column. Weakly adsorbed components may be removed in a rinsing step and then strongly retained compounds are eluted, usually with an organic solvent. In both LLE and SPE the organic phase can be evaporated away, and the residue then redissolved in a small volume of liquid.

SPE and LLE can be used to extract the analyte from a biological matrix with some degree of specificity. Selectivity is obtained by adjusting the nature of the solid-phase (SPE), or the composition of the extracting or eluting solvents. Thus, not only is protein removal accomplished, but also many small molecules are removed from the analyte, possibly making the final separation step simpler. The inorganic salts present at high concentrations in most biofluids are absent from the extract, which is very useful from a CE viewpoint. Finally, a significant degree of analyte concentration can be achieved, often from 10 to 100 times. This helps to make up for the lack of concentration sensitivity when using UV absorbance detection. Thus, for all of the above reasons, LLE and SPE are excellent methods for CE sample preparation. Some of the main criticisms of these techniques are the time and effort which they require. For CE purposes, a further disadvantage in some applications is that SPE and LLE are not really capable of dealing with microvolume samples.

Quite a variety of CE analyses using SPE or LLE have been reported and some of these are summarized in Table 2 [18–25]. One point that is immediately obvious by comparison with Table 1 is that LODs are generally lower than with simple precipitation sample preparations, and that, for many compounds, LODs of around 0.1 μg ml^{-1} can be achieved even using UV absorbance detection. Usually sample concentration is achieved both in the extraction step and also by on- capillary sample stacking (e.g. refs. [18,25]).

The choice of a suitable internal standard can sometimes be problematic if orthogonal extraction

Table 2
CE assays using solid-phase extraction (SPE) or liquid–liquid extraction (LLE)

Analyte	Extraction (detection method)	LOD/μg ml^{-1}	Reference
Cytosine-β-D-arabinoside	SPE of plasma, C_{18} phase	0.1 (UV)	[18]
Cimetidine	SPE of plasma, C_{18} phase	0.1 (UV)	[19]
Theophylline and metabolites	SPE of urine, C_{18} phase	1 (UV)	[20]
Opioids and other drugs of abuse	SPE of urine, Bond-Elut Certify	0.1 (UV)	[21]
Nitrazepam and metabolites	SPE of urine, C_{18} phase	0.1–0.2 (UV)	[22]
7-Hydroxycoumarin	LLE of urine or serum using diethyl ether	1 (UV)	[23]
Theophylline	LLE of plasma using ethyl acetate	0.6 (UV)	[24]
Doxorubicin, epirubicin	LLE of plasma using chloroform, back extraction with phosphoric acid	0.00025 (LIF)	[25]

UV = UV absorbance detection; LIF = laser-induced fluorescence.

and CE separation principles are chosen (e.g. SPE based on analyte hydrophobicity, followed by capillary zone electrophoresis (CZE), separating on the basis of analyte mobility) [18]. An internal standard closely analogous to the analyte, with similar hydrophobicity and pK_a values is desired.

3. Microscale and on-capillary sample preparation and concentration

There are opportunities for loss of analyte at each step in an off-line sample preparation procedure. Furthermore, the difficulties of sample handling become greater as the sample size decreases. This has lead a number of workers to look at performing part, or all, of their clean-up and concentration procedures for CE either on-capillary, or using a miniaturized system coupled to the CE capillary. A variety of techniques have been used including chromatography, electrophoresis and microdialysis or ultrafiltration.

3.1. Chromatographic approaches

The chromatographic approach involves adapting the ideas of SPE to the capillary format. Essentially, a chromatographic sorbent is immobilized at the head of the CE capillary. Sample is loaded onto this and the phase is then washed with a solvent which will not elute the analytes of interest but which removes many of the unwanted sample components. Usually a considerable volume of sample is passed through the sorbent, so that a significant degree of concentration may be obtained. A small plug of eluting solvent is then introduced, after which electrophoresis in the BGE commences. A variety of approaches to on-line chromatographic clean-up and preconcentration have been used. A small amount of stationary phase (e.g. 5 μm diameter silica particles with a C_{18} bonded phase) can be immobilized for a few millimeters length of capillary between two frits (made by heating borosilicate glass or silica particles) at the head of a separation capillary [26]. Simple frit-making and packing procedures have been published for the preparation of electrochromatography capillaries [27], and can easily be adapted to making preconcentration capillaries. Alternatively, the packing material can be contained in a microcartridge, e.g. within a small-diameter PTFE tube [28], which is attached to the end of the separation capillary. By using large-size particles which do not fit into the separation capillary, the need for frits is avoided. The packed-concentrator approach has the disadvantage that the frits and packing limit pressure-driven flow through the capillary, and thus, hydrodynamic sample loading may not be possible using the levels of pressure available in some commercial CE machines. Furthermore, variations between packed concentrators can be quite large, so use of an internal standard is mandatory for quantitative work.

Hydrophobic adsorbent membranes, which act rather like a reversed-phase chromatographic packing, seem to offer an attractive alternative to concentrators that are packed in capillaries [29,30]. A typical configuration would have the membrane sandwiched between the ends of two capillaries, with a sheath tubing placed over all of this to form a mechanically stable structure. The assembly can then be butt-joined to the separation capillary, forming a removable concentrator assembly.

Because of the limited amount of stationary phase in such microconcentrators, overloading would quickly occur if neat biological fluids were applied. Furthermore, irreversible adsorption of some endogenous components could shorten the life of the preconcentrator — unlike commercial SPE cartridges, these are not throw-away devices. Therefore, some form of pretreatment for biofluids may be necessary before application onto the preconcentration capillary. For example, in their study of haloperidol metabolism, Tomlinson et al. [30] used $ZnSO_4$ precipitation of urinary proteins before injection of 10 μl of supernatant onto a preconcentrator. The present need to perform preliminary sample treatment steps rather limits the interest in such devices for microanalytical purposes, but the promising developments recently seen using membranes suggest that a throw-away system, similar to traditional SPE cartridges but capable of concentrating a microlitre or nanolitres of fluid with minimal handling losses, could be a commercial reality within a few years.

3.2. Electrophoretic approaches

On-column sample concentration of charged analytes can be achieved by electrophoretic processes under certain conditions. Stacking of extracts after desalting, which leaves the sample in a low-conductivity matrix, was mentioned above. Coupled-column isotachophoresis–CE (ITP–CE) has been used for enrichment of previously deproteinized plasma samples [31] and on-capillary transient isotachophoretic preconcentration [32,33] has been noted in the analysis of some endogenous compounds in urine [34], ultrafiltered serum [35] and in shellfish extracts [36]. Transient ITP can be performed in a variety of formats [32,33]. A leading and terminating electrolyte can be introduced before and after the sample, respectively. ITP concentration is allowed to take place, after which the terminator is replaced by the leading electrolyte and a zone electrophoretic separation occurs. Alternatively, a BGE with a low-mobility co-ion can be chosen, and a high mobility co-ion may either be added to the sample [33], or may already be present in the sample (e.g. chloride in ultrafiltered serum [35]); when the field is applied the analyte ions undergo an initial ITP concentration and then proceed to separate as discrete zones after they detach from the sample zone.

3.3. Capillary ultrafiltration and microdialysis

Capillaries made from microporous, hydrophilic membranes can be implanted into living animals, after which, suction is applied to draw an ultrafiltered fluid sample into the capillary [37]. On-line microdialysis sampling [38] has some similarities; a dialysis fiber is implanted into the tissue of interest and is slowly perfused with a fluid similar in electrolyte composition to the extracellular medium. Small molecules can pass across the membrane and are carried out by the flow of perfusion fluid. With both techniques, the fluid that is collected is free of proteins and is suitable for direct injection onto the CE system. Since these methods involve no degree of concentration, severe demands are placed on the CE detection system, unless the analyte of interest is present at high concentrations. The great attraction for coupling ultrafiltration and microdialysis with CE is the small sample volume required by CE. Because of the small sample volumes involved, the on-line coupling of the microdialysis system and the CE system is favoured, for example using low volume switching valves [39]. Fig. 1 shows a coupled system. Pump 1 provides a flow of liquid (≈ 1 μl min^{-1}) through the dialysis probe and into a commercial microchromatography switching valve with a 60-nl loop. The dialysate normally passes through the loop to waste. When switched briefly to the inject position, the flow of liquid from pump 2 flushes the loop, via the transfer line, into the injection interface. At this interface, the end of the transfer capillary is positioned in front of the CE capillary, with about a 50–100-μm gap. The interface is bathed in separation buffer, via which electrical contact is made.

In microdialysis and ultrafiltration, the absolute amount of analyte that can be recovered per unit time is low and, in the case of microdialysis, the recovery of analyte is dependant on the flow-rate through the capillary, with the best recoveries at flow-rates <1 μl min^{-1}. With the nanolitre volumes required in CE, enough sample for analysis can be obtained after a few minutes, or less, allowing high time resolution, for example in pharmacokinetic studies. With HPLC requiring typically a few microliters of sample, time resolution of microdialysis sampling is limited by the time taken to acquire enough sample. An example of the utility of this technique is in the determination of rapid drug pharmacokinetics. Hogan et al. [39], using the CE–microdialysis system illustrated in Fig. 1, investigated the pharmacokinetics of the antineoplastic agent 3-amino- 1,2,4-benzotriazine-1,3-di-N-oxide (SR4233) in anaesthetized rats, with sampling

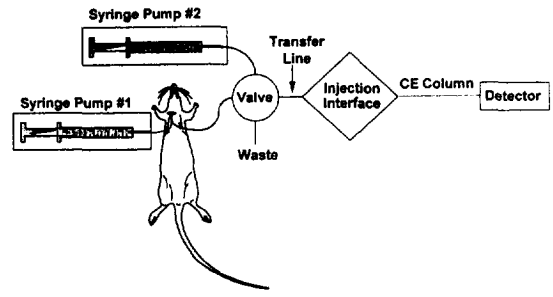

Fig. 1. Schematic of an on-line microdialysis–capillary electrophoresis system. Reprinted from [39], copyright 1994, American Chemical Society.

Table 3
Some representative CE assays using ultrafiltration (UF) or microdialysis (MD) sampling

Technique	Analyte and matrix	LOD/μg ml^{-1} (detection method)	Reference
UF	Theophylline measured subcutaneously	(UV)	[40]
MD	L-Dopa in blood	0.004 (EC)	[41]
MD	Phenobarbital in brain	2 (UV)	[42]
MD	Tryptophan (TRP) and kynurinine (KYN) in brain	4.8 n*M*, TRP 3.1 n*M*, KYN (EC)	[43]
MD	SR 4233 and SR 4317	<1 (LIF)	[39]

EC = oxidative electrochemical; LIF = laser-induced fluorescence; UV = UV absorbance detection.

every 90 s. Such rapid sampling permitted accurate estimation of the elimination half-life of 15 min and the distribution half-life of only 1.1 min. Other examples are shown in Table 3 [39–43].

4. Direct injection of biological materials

Direct injection of biological fluids seems a daunting prospect. Urine and plasma/serum, the most commonly analyzed biofluids, both present different challenges. Urine contains very many components, and pushes the resolving power of CE to the limits. Plasma may contain fewer individual components at high concentrations, but the high concentrations of the proteins that are present pose problems. Both matrices have high concentrations of electrolytes. Finally, adequate detection sensitivity must be available in the CE system to look at pharmacologically relevant concentrations of drugs and metabolites, without any large degree of pre-concentration. One point to note with routine direct-injection assays is that it is worthwhile, if possible, to filter the biofluid through a 0.45-μm membrane to remove any particulates capable of blocking the capillary.

4.1. Urine

Drug analyses in urine with direct injection have been published by many groups. The obvious advantage of direct injection is that the time and effort of extraction procedures are eliminated. However, acid or enzymatic hydrolysis of conjugated metabolites may still be necessary in many cases. Provided that the analyte concentration is high enough such that the analyte can be adequately quantitated by whatever detection system is available, there is quite a free choice of BGE. Both CZE and electrokinetic chromatography (EKC) with micellar or other pseudophases have been used, and the main problem is to provide a robust method with adequate resolution of the analyte(s) from other components. Basic drugs can often be analyzed successfully by CZE with moderately alkaline BGEs, e.g. borate at pH$\approx$9. Under such conditions, there are few endogenous compounds in urine which migrate in front of the electroosmotic flow marker and which give a significant response in a UV detector. One problem with urine analysis is the variable, but generally high, concentration of electrolytes in the sample. To minimize effects on resolution due to variations in the matrix composition, and if limits of detection are adequate, the urine can be diluted first and then analyzed in a moderately concentrated buffer [44]. Alternatively, high buffer concentrations can be used, with direct injection of neat urine [45]. This usually involves some compromise in terms of analytical speed, since the applied field strength may need to be limited in non-liquid-cooled CE systems, but the big advantage is that there is no loss of sensitivity. Indeed, relatively low detection limits may be obtained in this way, for example, 80 ng ml^{-1} in the case of dextromethorphan and its metabolite dextrorphan with a 15-nl direct injection of enzymatically deconjugated urine [45]. This injection represents approximately 10% of the entire column volume, but with a 0.175 *M* sodium tetraborate BGE, there was adequate peak stacking to achieve analyte efficiencies of >300 000 plates.

CZE of weakly acidic drugs and metabolites in urine poses some more problems because of the large number of potential interferants. MEKC is often

used, since it offers a greater potential for fine-tuning the separation to adequately resolve all the components of interest, by alteration of both the electrophoretic and chromatographic migration parameters for the analytes. An example of this approach is the determination of two caffeine metabolites in urine, 5-acetylamino-6-formylamino-3-methyluracil (AFMU) and 1-methylxanthine (1X), using MEKC with sodium dodecyl sulphate (SDS) [46]. In this assay, AFMU mobility changes little over the pH range 7–9, whilst that of 1X steadily increases, with the result that the pH adjustment proved adequate to resolve 1X from endogenous components. Since neither compound associates strongly with the SDS micelles, a change in SDS concentration could be used to fine-tune the separation to resolve an interferant from the AFMU peak. This assay was used for assigning metabolic phenotypes, by measuring the rate of production of different metabolites and several similar applications have since appeared for N-acetylation [47,48] and oxidative phenotypes [45,48]. CE is particularly attractive in this field since innocuous probe compounds are chosen which can be given to the test subject in relatively high concentrations, such that the metabolite concentrations are easily detectable by UV absorbance with direct urine injection.

One difficulty in the direct analysis of urine by MEKC is peak identification, especially when a relatively non-specific detection method such as UV absorbance is employed. Thormann and co-workers have illustrated that multi-wavelength UV detection is particularly useful for identifying analytes in urine [47,48]. Fig. 2 shows electropherograms of directly injected plain urine (panels A–C) and hydrolysed urine (panels D–F) from a subject who had been administered caffeine and mephenytoin. The caffeine metabolites, 1X, 5-acetylamino-6-amino-3-methyl-

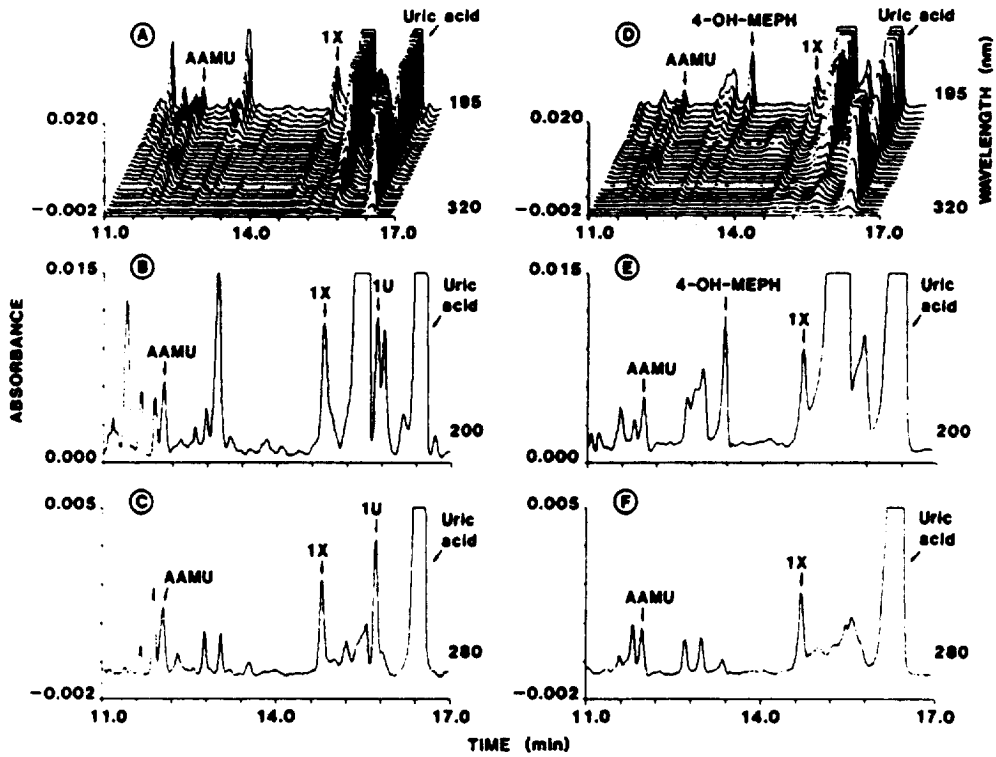

Fig. 2. Simultaneous screening for caffeine and mephenytoin metabolites in human urine by MEKC. Buffer, phosphate–borate, pH 8.6, with 70 m*M* SDS; capillary, 100 cm (70 cm effective length), 75 μm I.D.; applied field, ≈200 V cm^{-1}. Electropherograms using multiwavelength (panels A and D), 200 nm (panels B and E) and 280 nm (panels C and F) UV detection are shown. For abbreviations, see Section 7. Reprinted from Ref. [48], with the permission of Elsevier.

uracil (AAMU) and 1-methyl uric acid (1U), and the mephenytoin metabolite, 4-hydroxy mephenytoin (4-OH-MEPH), are seen. The data in the multi-wavelength scans shown in the upper panels can be used for peak identification and estimation of peak purity, while the single-wavelength traces at 200 and 280 nm shown below reveal the separation more clearly. A further modification of the BGE allowed analysis of the dextromethorphan metabolite, dextrorphan, thereby allowing the assessment of three metabolic phenotypes in only one analytical run; in this case the spectral scanning detection was absolutely necessary, since some of the caffeine metabolites were not completely resolved from other compounds in the separation step.

As noted above, LODs in CZE analysis of directly injected urine can be improved by using high ionic strength BGEs [45]. In MEKC there appears to be less opportunity for on-capillary sample concentration, although this can be viewed in a favourable light by noting that sample matrix effects seem to be relatively minor in MEKC [16].

4.2. Plasma

The main challenge in the direct analysis of plasma is dealing with the high concentrations of protein present, in the range of 70 g l^{-1}. The problems that the proteins cause are two-fold. First, they are capable of adhering to the surface of a fused-silica capillary. This leads to variability of electroosmosis and possibly to peak-broadening, due to analyte binding to the surface-adsorbed protein. Secondly, the broad protein peaks may interfere with detection of the desired analyte peaks. Nevertheless, a couple of approaches to direct injection of plasma are possible.

Direct injection of diluted serum onto fused-silica capillaries followed by CZE analysis is possible. There is little loss of efficiency or change in migration time compared to injections from water, provided the degree of dilution is fairly large, e.g. ten times, and, if the run buffer is at a fairly high concentration (e.g. 0.1 *M* borate) [49]. Unfortunately, such a large degree of dilution limits the usefulness of this approach to analytes present at high concentrations [13]. If undiluted plasma is used, the large quantity of adsorbed plasma proteins can cause a huge change in electroosmosis (e.g. a 50% reduction) after a single plasma injection [50] and vigorous washing between runs is needed even with diluted plasma [13,49]. In many bioassays, NaOH in the range 0.1–1 *M*, or a strong acid at similar concentrations is used as a between-run wash step. A 0.1-*M* solution of NaOH is not adequate for good capillary washing after direct injection of plasma in CZE, although it may be suitable for washing after analysis of urine, or of pre-treated samples. A 1-*M* solution of NaOH, maybe in combination with an acid washing step, is likely to provide adequate washing with directly injected plasma. However, considerable re-equilibration time is needed between runs to achieve good electroosmotic flow reproducibility [51]. This can lead to the unfortunate situation when trying to develop rapid analyses that the capillary washing and re-equilibration steps may take as long as the separation itself [52]. As an alternative to acid/alkali washing, SDS dissolved in run buffer can provide excellent removal of adsorbed proteins and short re-equilibration times [50]. Fig. 3 shows a plasma protein separation with an optimized wash procedure using 30 s of 200 m*M* SDS solution in run buffer, followed by a further 30 s with BGE. With this wash the C.V. (coefficient of variation) in migration time for the human serum albumin (HSA) peak was 0.4% ($n=17$). Alternatively, a 1 min wash with 0.1 *M* NaOH, followed by a 4 min rinse with BGE, resulted in a migration time C.V. of 1.6% for the HSA peak. For all the other peaks, the migration time C.V.s with the SDS wash were equal to or better than with the NaOH wash. With a shorter re-equilibration period after the NaOH wash, the C.V.s became even worse. Although little-used at present, CZE with direct plasma injection may turn out to be useful for the analysis of some basic compounds, which would migrate clear of the protein interferences. One point to note is that if the analysis is done under non-denaturing conditions, only free drug will be measured.

Micellar liquid chromatography has been shown to be useful for direct analysis of unextracted plasma [53], since the surfactant additive (often SDS) binds to the plasma proteins and solubilizes them, stopping them from irreversibly adsorbing to the stationary phase. In CE there is only the capillary wall to adsorb the proteins, and since a surfactant is present

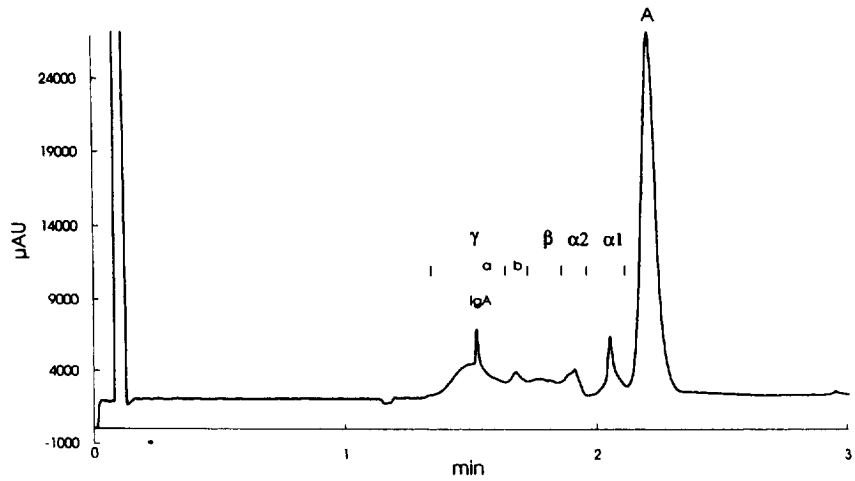

Fig. 3. CZE separation of proteins in human plasma after a 1:20 dilution with water. Separation conditions: instrument, ABI 270A-HT; capillary, 43 cm long (22 cm effective length), 50 μm I.D.; BGE, 60 m*M* borate, pH 10; field, 465 V cm^{-1}; detection, UV at 200 nm; washing, 30 s, 200 m*M* SDS in BGE followed by 30 s with BGE. A = albumin. α, β, etc, globulins.

in MEKC by definition, it is not surprising that direct injection of plasma is possible. Nakagawa and co-workers [54] first took advantage of MEKC to develop a CE direct injection assay, and since then there have been quite a number of methods reported using this approach. Examples where assay validation data have been reported are the analysis of phenobarbital, ethosuximide and primidone in human serum [55], and of diastereoisomers of L-buthionine-(*R,S*)-sulfoximine in human plasma [56]. Another potentially problematic biofluid, saliva, has been analyzed using MEKC with direct sample injection, for the quantitative determination of antipyrene [57]. An outstanding feature of this assay was the speed of analysis, <1 min separation and <2.5 min including all conditioning steps. With no sample extraction, the throughput is very high. These methods have been shown to be reliable enough to be put into routine use as the favoured approach for their particular applications.

Three recent articles have described the effects of various operating parameters and analyte properties on direct injection MEKC methods [58–60]. In a typical MEKC plasma analysis with direct sample injection, the surfactant (typically SDS; cationic surfactants such as cetyltrimethylammonium bromide cause protein precipitation) is strongly bound to the protein at a fairly constant ratio due to the hydrophobic effect [61]. This gives the proteins an overall strong negative charge, which reduces interactions with the capillary wall and gives them quite strong mobilities against the electroosmotic flow. Thus, the proteins migrate as a series of rather broad peaks late in the electropherogram, and there is a "separation window" (Fig. 4) between the electroosmotic flow point and the first of the plasma proteins, where small molecules can be analyzed [60]. Comparison of the protein peaks in Fig. 3 (CZE) and Fig. 4 (MEKC) reveals how the plasma protein peaks become bunched together in the SDS-containing system. Wätzig and Lloyd [60] have suggested the following starting conditions for development of an MEKC direct-injection assay: hydrophilic or moderately hydrophobic uncharged species, pH 7, with low SDS concentration (e.g. 25 m*M*); low mobility anions, pH 7, with 25–50 m*M* SDS, or 100 m*M* SDS for high mobility anions. A pH of 10 is quite useful since the proteins become highly negatively charged and can be used with low SDS concentrations for rather hydrophobic species, where a large degree of partitioning into the micellar phase is unwelcome; alternatively, pH 10 with high SDS concentrations

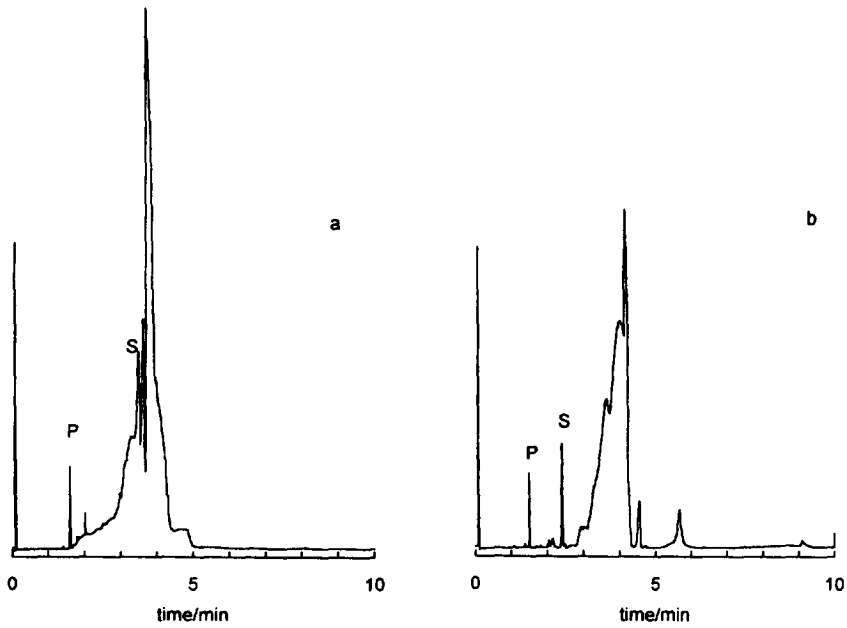

Fig. 4. MEKC electropherograms of directly injected human plasma spiked with acetaminophen (P) and salicylate (S) and analyzed using a borate BGE (pH 9) with (a) 10 m*M* SDS and (b) 200 m*M* SDS. Capillary, 43 cm (22 cm to detector), 50 μm I.D.; field strength, 465 V cm^{-1}; detection, UV at 200 nm. The separation window between the electroosmotic flow point at about 1.2 min and the plasma protein peak increases with increasing SDS concentration. Reprinted from [60], with the premission of VCH Verlagsgellschaft.

and 6 *M* urea (to limit incorporation of the analyte into the micellar phase) may be suitable for hydrophobic non-ions [58]. Cations may be difficult to analyze in this way, because of strong electrostatic interactions with the micelles. Relatively small injections (e.g. <2% of the column volume) were found to be advisable [58], although, if there is good resolution of the analytes of interest this can be pushed higher to reduce detection limits somewhat [56]. Release of bound drug due to denaturation of the plasma proteins by SDS is an important feature of MEKC with direct plasma injection. Schmutz and Thormann [59] found that ethosuximide (no plasma protein binding), pentobarbital (≈50% protein bound) and naproxen (>99% protein bound) were quantitatively analyzed by direct plasma injection, ie. that even highly bound compounds were completely released by the protein denaturation. They also showed that basic compounds or those with low protein binding eluted as sharp peaks. Only in the case of acidic drugs with high protein binding were broadened peaks observed and even in these cases quantitative analysis could be performed provided that adequate resolution was achieved.

5. Direct injection analyses and microanalysis

Handling and preparation steps become more difficult as one moves towards the analysis of submicrolitre volumes of sample. With CE requiring only nanolitres or picolitres of sample on column, the obvious way to exploit its microanalytical abilities is to actually take the end of the capillary and introduce it into the biological compartment which one wants to study. The ability of the CE separation method to handle directly injected biofluids is obviously important for this type of application. Since much CE microbioanalysis involves trying to find answers to questions beyond the scope

of previous analytical technology, the ability even to perform a few (maybe only semi-quantitative) analyses is valuable, in contrast to the emphasis on development of robust, validated methods, as described in Section 4, for drug analysis in plasma or urine. At present these studies tend towards the determination of endogenous species, but it is easy to see that drugs and metabolites may soon be measured, even at the level of a single cell.

Ewing's group have provided some elegant examples of direct microanalysis of biofluids, using CE with amperometric detection for the estimation of free dopamine concentrations in the cytoplasm of the giant dopamine cell of *Planorbis corneus* [62,63], or in vesicles within a single cell [64]. For these studies, small-diameter pulled tips on the end of the fused-silica separation capillaries were inserted directly into the cell and the cytoplasm was injected electrokinetically. Shear et al. [65] have demonstrated the CE analysis of 5-hydroxytryptamine in a single *Xenopus* oocyte, using a single cell biosensor as a detector. Excellent detection selectivity is one of the keys to the success of these methods, and clearly the choice of separation conditions may be dictated by the demands of the detection system. In the analysis of single human erythrocytes, Hogan and Yeung [66] took the very direct approach of using deactivated wall-coated capillaries, to avoid modification of the capillary surface. It is perhaps somewhat surprising that there are a wide variety of capillary coatings (for examples see refs. [67,68]) which have been developed to reduce protein adhesion, but which seem to be hardly considered at all for general drug or metabolite analysis.

Moving up somewhat from the sub-cellular or cellular scale, CE can be used for probing body compartments that have previously been difficult to explore due to problems of size and/or accessibility. Quantitative analyses by CE of inorganic ions in rat airway surface fluid (ASF) have recently been demonstrated, using one capillary inserted into the lung of a living rat to collect around 100 nl of liquid from the typically 5–30 μm thick ASF layer [69]. The sampling capillary is then interfaced to the CE separation capillary to inject 1–2 nl of the liquid contained therein, allowing multiple analyses for different components to be made. Occasional capillary modification (presumably due to ASF proteins) could be dealt with by washing with SDS [50].

6. Conclusions

A wide variety of sample preparation techniques for CE have been described, based mainly on chromatographic and electrophoretic interactions. Minimal pretreatment by precipitation of proteins is suitable for the analysis of biofluids, provided that the analyte concentration is within the useful range of the detection system in use. Extraction methods are usually employed to obtain sample clean-up and concentration, both during the extraction step and afterwards on- capillary is often important. CE following extensive sample clean-up involves no less effort than performing a HPLC separation, but for certain applications, such as chiral separations, there are clearly attractions in using CE. On-column transient ITP offers an alternative concentration strategy, particularly for urine samples.

If analyte concentrations are high enough, complex biofluids, even those containing high proportions of macromolecules, can be directly injected onto a CE system for analysis. Urine analyses can be very quick and simple, while plasma requires somewhat more care, for example using SDS additives to solubilize proteins. Nevertheless, there are several examples of validated bioanalytical methods using direct injection of both urine and plasma. The savings in time and effort of such an approach are obvious, but the great advantage of direct-injection CE is in its microanalytical abilities.

7. Abbreviations

AAMU	5-Acetylamino-6-amino-3-methyluracil
AFMU	5-Acetylamino-6-formylamino-3-methyluracil
ASF	Airway surface fluid
BGE	Background electrolyte
CE	Capillary electrophoresis
C.V.	Coefficient of variation (%)
EC	Electrochemical (detection)

ITP	Isotachophoresis
KYN	Kynurenin
LIF	Laser-induced fluorescence
LLE	Liquid-liquid extraction
LOD	Limit of detection
MEKC	Micellar electrokinetic chromatography
4-OH-MEPH	4-hydroxy mephenytoin
PTFE	Polytetrafluorethylene
SDS	Sodium dodecyl sulphate
SPE	Solid-phase extraction
TRP	Tryptophan
1U	1-Methyluric acid
1X	1-Methylxanthine

Acknowledgments

I would like to acknowledge the work of Dr. habil. Hermann Wätzig (presented in Fig. 3) and to thank the Fonds de la Recherche en Santé du Québec for a chercheur-boursier scholarship.

References

[1] D. Perrett, in P. Camilleri (Editor), Capillary Electrophoresis: Theory and Practice, CRC Press, Boca Raton, FL, 1993, p. 371.
[2] N.W. Smith and M.B. Evans, J. Pharm. Biomed. Anal., 12 (1994) 579.
[3] D.K. Lloyd, in C.M. Riley, W.J. Lough and I.W. Wainer (Editors), Pharmaceutical and Biomedical Applications of Liquid Chromatography, Pergamon, Oxford, 1994, p. 3.
[4] W. Thormann, S. Molteni, J. Caslavska and A. Schmutz, Electrophoresis, 15 (1994) 3.
[5] H. Lingeman and U.R. Tjaden, in H. Lingeman and W.J.M. Underberg (Editors), Detection Oriented Derivatization Techniques in Liquid Chromatography, Dekker, New York, 1990, p. 85.
[6] S. Hjerten, J. Chromatogr., 347 (1985) 191.
[7] J. Blanchard, J. Chromatogr., 226 (1981) 455.
[8] F.E. Mikkers, F.M. Everaerts and T.P. Verheggen, J. Chromatogr., 169 (1979) 1.
[9] D.S. Burgi and R.-L. Chien, Anal. Chem., 63 (1991) 2042.
[10] Z.K. Shihabi, J. Chromatogr. A, 652 (1993) 471.
[11] L.L. Garcia, Z.K. Shihabi and K. Oles, J. Chromatogr. B, 669 (1995) 157.
[12] L.J. Brunner, J.T. DiPiro and S. Feldman, J. Chromatogr., 622 (1993) 98.
[13] Z.K. Shihabi and M.S. Constantinescu, Clin. Chem., 38 (1992) 2117.
[14] I.M. Johansson, M.-B. Bron-Rydberg and B. Schmekel, J. Chromatogr. A, 652 (1993) 487.
[15] D. Leveque, C. Gallion, E. Tarral, H. Monteil and F. Jehl, J. Chromatogr. B, 655 (1994) 320.
[16] Z.K. Shihabi and M.E. Hinsdale, J. Chromatogr. B, 669 (1995) 75.
[17] M. Zief and S.V. Kakodar, in C.M. Riley, W.J. Lough and I.W. Wainer (Editors), Pharmaceutical and Biomedical Applications of Liquid Chromatography, Pergamon, Oxford, 1994, p. 127.
[18] D.K. Lloyd, A.M. Cypess and I.W. Wainer, J. Chromatogr., 568 (1991) 117.
[19] J. Luksa and Dj. Josic, J. Chromatogr. B, 667 (1995) 321.
[20] Z.-Y. Zhang, M.J. Fasco and L.S. Kaminsky, J. Chromatogr. B, 665 (1995) 201.
[21] P. Wernly and W. Thormann, Anal. Chem., 64 (1992) 2155.
[22] M. Tomita, T. Okuyama, S. Sato and H. Ishizu, J. Chromatogr. B, 621 (1993) 249.
[23] D.P. Bogan, B. Deasy, R. O'Kennedy, M.R. Smyth and U. Fuhr, J. Chromatogr. B, 663 (1995) 371.
[24] K.-J. Lee, G.S. Heo, N.J. Kim and D.-C. Moon, J. Chromatogr., 577 (1992) 135.
[25] N.J. Reinhoud, U.R. Tjaden, H. Irth and J. van der Greef, J. Chromatogr., 574 (1992) 327.
[26] M.E. Swartz and M. Merion, J. Chromatogr., 632 (1993) 209.
[27] S. Li and D.K. Lloyd, Anal. Chem., 65 (1993) 3684.
[28] A.J. Tomlinson, W.D. Braddock, L.M. Benson, R.P. Oda and S. Naylor, J. Chromatogr. B, 669 (1995) 67.
[29] A.J. Tomlinson, L.M. Benson, W.D. Braddock, R.P. Oda and S. Naylor, J. High Resolut. Chromatogr., 18 (1995) 381.
[30] A.J. Tomlinson, L.M. Benson, R.P. Oda, W.D. Braddock, B.L. Riggs, J.A. Katzmann and S. Naylor, J. Capillary Electrophoresis, 2 (1995) 97.
[31] D.S. Stegehuis, U.R. Tjaden and J. van der Greef, J. Chromatogr., 591 (1992) 341.
[32] P. Gebauer, W. Thormann and P. Boček, J. Chromatogr., 608 (1992) 47.
[33] F. Foret, E. Szökő, B.L. Karger, Electrophoresis, 14 (1993) 417.
[34] R. Weinberger, E. Sapp and S.E. Moring, J. Chromatogr., 516 (1990) 271.
[35] A.C. Schoots, T.P.E.M. Verheggen, P.M.J.M. De Vries and F.M. Everaerts, Clin. Chem., 36 (1990) 435.
[36] S.J. Locke and P. Thibault, Anal. Chem., 66 (1994) 3436.
[37] M.C. Linhares and P.T. Kissinger, Anal. Chem., 64 (1992) 2831.
[38] C.E. Lunte, D.O. Scott and P.T. Kissinger, Anal. Chem., 63 (1991) 773A.
[39] B.L. Hogan, S.M. Lunte, J.F. Stobaugh and C.E. Lunte, Anal. Chem., 66 (1994) 596.
[40] M.C. Linhares and P.T. Kissinger, J. Chromatogr., 615 (1993) 327.
[41] T.J. O'Shea, R.D. Greenhagen, S.M. Lunte, C.E. Lunte and M.R. Smyth, Electroanalysis, 4 (1992) 463.

[42] S. Tellez, N. Forges, N. Roussin and L. Hernandez, J. Chromatogr., 581 (1992) 257.
[43] M.A. Malone, H. Zuo, S.M. Lunte and M.R. Smyth, J. Chromatogr. A, 700 (1995) 73.
[44] M. Chicharro, Z. Zapardiel, E. Bermejo, J.A. Perez and L. Hernández, J. Chromatogr., 622 (1993) 103.
[45] S. Li, K. Fried, I.W. Wainer and D.K. Lloyd, Chromatographia, 35 (1993) 216.
[46] D.K. Lloyd, K. Fried and I.W. Wainer, J. Chromatogr., 578 (1992) 283.
[47] R. Guo and W. Thormann, Electrophoresis, 14 (1993) 547.
[48] J. Caslavska, E. Hufschmid, R. Theurillat, C. Desiderio, H. Wolfisberg and W. Thormann, J. Chromatogr. B, 656 (1994) 219.
[49] L.L. Garcia and Z.K. Shihabi, J. Chromatogr. A, 652 (1993) 465.
[50] D.K. Lloyd and H. Wätzig, J. Chromatogr. B, 663 (1995) 400.
[51] D.K. Lloyd, in E. Reid, H.M. Hill and I.D. Wilson (Editors), Biofluid and Tissue Analysis for Drugs, including Hypolipidaemics: Methodological Surveys in Bioanalysis of Drugs, Vol. 23, Royal Society of Chemistry, Cambridge, 1994, p. 41.
[52] F.-T.A. Chen and J.C. Sternberg, Electrophoresis, 15 (1994) 13.
[53] M.J. Koenigbauer, J. Chromatogr., 531 (1990) 79.
[54] T. Nakagawa, Y. Oda, A. Shibukawa, H. Fukada and H. Tanaka, Chem. Pharm. Bull., 37 (1989) 707.
[55] A. Schmutz and W. Thormann, Therap. Drug Monit., 15 (1993) 310.
[56] V. Sandor, T. Flarakos, G. Batist, I.W. Wainer and D.K. Lloyd, J. Chromatogr. B, 673 (1995) 123.
[57] D. Perrett and G.A. Ross, J. Chromatogr. A, 700 (1995) 179.
[58] A. Schmutz and W. Thormann, Electrophoresis, 15 (1994) 51.
[59] A. Schmutz and W. Thormann, Electrophoresis, 15 (1994) 1295.
[60] H. Wätzig and D.K. Lloyd, Electrophoresis, 16 (1995) 57.
[61] C. Tanford, The Hydrophobic Effect: Formation of Micelles and Biological Membranes, Wiley, New York, 1980.
[62] R.A. Wallingford and A.G. Ewing, Anal. Chem., 60 (1988) 1972.
[63] J.B. Chien, R.A. Wallingford and A.G. Ewing, J. Neurochem., 60 (1990) 633.
[64] H.R. Kirstensen, Y.Y. Lan and A.G. Ewing, J. Neurosci. Methods, 51 (1994) 183.
[65] J.B. Shear, H.A. Fishman, N.L. Allbritton, D. Garignan, R.N. Zare and R.H. Scheller, Science, 267 (1995) 74.
[66] B.L. Hogan and E.S. Yeung, Anal. Chem., 64 (1992) 2841.
[67] S. Hjerten, J. Chromatogr., 385 (1985) 347.
[68] S. Hjerten and K. Kubo, Electrophoresis, 14 (1993) 390.
[69] J.C. Transfiguracion, C. Dolman, D.H. Eidelman and D.K. Lloyd, Anal. Chem., 67 (1995) 2937.