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Review

# Capillary electrophoresis of diuretics

Marja-Liisa Riekkola[a,*], Juho Hermanni Jumppanen[a,b]

[a]*Laboratory of Analytical Chemistry, Department of Chemistry, P.O. Box 55, University of Helsinki, FIN-00014 Helsinki, Finland*

[b]*Cultor Ltd., Finnsugar Development, FIN-02460 Kantvik, Finland*

**Abstract**

The review surveys the application of capillary electrophoresis to the screening, identification and determination of diuretics and probenecid. The number of publications is still limited, but the studies already published clearly show that capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography are excellent alternatives for the investigation of diuretics. High accuracy identifications of diuretics and probenecid, even in urine samples, can be obtained when CZE is used with the marker techniques. This review paper has been written from the viewpoint of practical use and some hints are given for future CE studies on diuretics

*Keywords:* Reviews; Diuretics; Probenecid

## Contents



## 1. Introduction

Diuretics are widely used in the treatment of congestive heart failure and hypertension. The major indications of diuretics are the enhancement of renal excretion of salt and water and lowering of blood pressure. In addition to sodium cations and chloride anions they may also affect the renal readsorption and excretion of potassium, calcium, magnesium and other ions. Some diuretics increase urinary potassium excretion and can cause hypokalemia in patients treated with these medications for prolonged times.

*Corresponding author.

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Probenecid is a uricosuric agent with a weak diuretic activity.

Diuretics and probenecid have been misused in sports in recent years, for two reasons. (1) In sports where mass categories are involved, athletes may wish to reduce their body weight quickly in order to qualify for a lower mass class. (2) Athletes may wish to dilute their urine to avoid a positive doping result.

Diuretics can affect the excretion of drug substances in several ways [1]. (1) Increase in the urine flow reduces the concentration of other doping agents in urine by producing a more plentiful excretion of the urine. The lower concentration may then be under the detection limit of the analytical method, and the drug abuse may escape detection. (2) Carbonic anhydrase inhibitor diuretics produce an alkaline urine with reduced excretion of basic doping agents. This may also result in a negative doping result.

Probenecid has been misused in sport because it decreases the urinary excretion of anabolic steroids. Health risks are always involved in misuse because of the possible serious side-effects. The Medical Commission of the International Olympic Committee decided in 1988 to include both diuretics and probenecid on its list of banned drugs.

The specific identification of diuretics and probenecid is very important in clinical laboratories and doping control. Although some diuretics are extensively metabolized most of them are excreted to variable extents unchanged in urine. Hence, the screening procedures for diuretics and probenecid in human urine can be designed to detect the suspected parent compound.

Several chromatographic methods [high-performance liquid chromatography (HPLC), gas chromatography (GC), thin-layer chromatography] are reported for the separation, detection and determination of individual diuretics including some screening methods as well. The most common method described for the separation of diuretics and probenecid is reversed-phase liquid chromatography with UV detection, with samples prepared by liquid–liquid extraction or solid-phase extraction (SPE). Fluorescence and electrochemical detections have also been used with HPLC [2]. Gas chromatography is another method of separation, but because of their low volatility and low thermal stability, the diuretics must first be derivatized, e.g. with methyl iodide. Hyphenated methods like GC–MS and GC–MS–MS are frequently used, and positive results in doping control are confirmed by GC–MS.

Capillary electrophoretic (CE) techniques are already fairly widely used in compound separations, and offer a useful assistance to conventional chromatographic methods. The advantages of CE methods in bioanalysis are extremely high resolution with short analysis times, small sample requirement, low cost of disposables and minimal solvent usage. In addition, polar, nonvolatile and thermally labile compounds can all be analysed by CE. Depending on the sample, the analysis of pharmaceuticals is often very straightforward. Only dissolution, dilution, filtering or centrifugatiion is needed before the analysis. It is also possible to inject biofluids such as urine and serum directly into the CE instrument.

In this paper, we describe some representative separations of diuretics and probenecid by CE, focusing on practical application and reliability of identification.

## 2. Diuretics and probenecid

Diuretics are a heterogeneous group of compounds with different physicochemical properties. They can be classified according to their pharmacological properties into four different main groups which are carbonic anhydrase inhibitors, loop diuretics, thiazide type diuretics and potassium-sparing diuretics. In their review paper on diuretics in 1992, Herráez-Hernández et al. [2] estimated that 18% of the diuretics studied by HPLC during the last decade were carbonic anhydrase inhibitors, 31% were loop diuretics, 34% were thiazide type diuretics, and 17% were potassium-sparing diuretics.

The enzyme carbonic anhydrase catalyses the production of bicarbonate from carbon dioxide and water. The production of protons in this reaction is necessary for a normal resorption of bicarbonate, followed by an excretion of bicarbonate into the urine. Carbonic anhydrase inhibitors have less effect on the urine flow than do loop and thiazide type diuretics. Acetazolamide is a carbonic anhydrase inhibitor.

Almost all loop diuretics are carboxylic acids, which strongly increase the urine flow by inhibiting

the resorption of cloride and sodium ions in the Henle's loop of the kidney. This enhances the excretion of sodium and chloride ions into the urine. Furosemide, bumetanide, ethacrynic acid and etozolin are members of this group.

Most of the diuretics are of thiazide type diuretics, which all contain a sulphonamide group. The pharmacological effect is an inhibition of the resorption of chloride and sodium ions in the distal renal tubuli. The diuretic effect is not as strong as with loop diuretics. Some examples are hydroflumethiazide, hydrochlorothiazide, polythiazide, benzthiazide, chlorthalidone, cyclopentathiazide, etc.

Potassium-sparing diuretics are clinically used in cases of potassium deficiency. Their pharmacological effect is an inhibition of potassium excretion. Their effect on the increase of urine flow is weak. Triamtere, amiloride and canrenone are potassium-sparing diuretics.

Osmotic diuretics like mannitol can be considered a fifth group of diuretics.

The physical and chemical properties of the diuretics are noticeably different, although little information can be found in the literature. Some of the properties are listed in Table 1 [3]. As can be seen, due to the different molecular structures [4] (Fig. 1) the pKa values and molar masses vary widely. These characteristics complicate sample preparation and screening of diuretics.

Both the metabolism and pharmacokinetics of diuretics have to be considered in a doping control test. Pharmacokinetic studies of urine samples after a single dose (see Table 2 [8,9]) have shown that most diuretics are primarily excreted unchanged. Exceptionally, etozolin and mefruside are nearly completely metabolized; the hydrolysed product ozolin being the main metabolite of etozolin, and lactone and open acid metabolite being the main metabolites of mefruside [1]. Probenecid is also metabolized in the liver to form an acyl glucuronide and other metabolites, which are excreted in the urine together with the parent compound [9]; because of the large dose administered (Table 2), detection is not difficult.

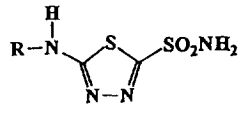
Table 1
Physicochemical properties of diuretics [3,4]

| Compound | Molecular mass | $pK_a$ | Ref. |
|---|---|---|---|
| Amiloride | 229.7 | 8.7 | [5][a] |
| Acetazolamide | 222.3 | 7.2; 9.0 | |
| Bendroflumethiazide | 421.4 | 8.5 | |
| Benzthiazide | 432.0 | | |
| Bumetanide | 364.4 | 5.2 | |
| Dichlorphenamide | 305.2 | 8.2 | |
| Ethacrynic acid | 303.2 | 3.5 | |
| Furosemide | 330.8 | 3.9 | [6] |
| Hydrochlorothiazide | 297.7 | 7.9; 9.2 | |
| Canrenone | 340.4 | | |
| Chlormerodrin | 367.2 | | |
| Chlortalidone | 338.8 | 9.3 | [6] |
| Chlorothiazide | 295.7 | 6.7; 9.5 | |
| Clopamide | 345.9 | 9.1 | [7] |
| Mannitol (D-) | 182.2 | | |
| Mefruside | 382.9 | | |
| Mercalyl | 505.9 | | |
| Metolazone | 365.8 | | |
| Metyrapone | 226.3 | | |
| Probenecid | 285.4 | 3.4 | |
| Spironolactone | 416.6 | | |
| Cyclothiazide | 389.9 | | |
| Triamterene | 253.3 | 6.2 | [6] |
| Trichlormethiazide | 380.7 | 8.6 | |

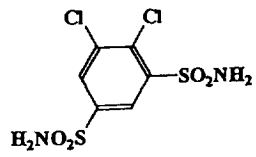
Table 2
Concentration of the unchanged compounds detected in urines after administration of single therapeutic doses of the drugs to healthy volunteers [8]

| Compound | Administered dose (mg) | Mean concentration in urine ($\mu$g/ml) | | |
|---|---|---|---|---|
| | | 0–8 h | 8–24 h | 0–24 h |
| Amiloride | 5 | 1.45 | 0.54 | NS[b] |
| Triamterene | 100 | 0.97 | 0.15 | NS |
| Acetazolamide | 250 | 185.56 | 20.83 | NS |
| | 250 | | 15.1[c] | 6.9[d] |
| | 250 | | | 4.3[e] |
| Diclorphenamide | 50 | 9.02 | 4.22 | NS |
| Chlorthalidone | 25 | 4.08 | 3.36 | NS |
| | 25 | 1.18 | 1.09 | NS |
| Hydrochloro- | 25 | 10.77 | 2.81 | NS |
| thiazide | 50 | 23.02 | 7.87 | NS |
| Bumetanide | 1 | 0.13 | 0.15 | NS |
| | 1 | 0.21 | 0.06 | NS |
| Ethacrynic acid | 50 | 1.57 | 0.61 | NS |
| | 50 | 3.56 | 0.19 | NS |
| | 50 | 2.91 | 0.33 | NS |
| Furosemide | 40[a] | 5.49 | 0.82 | NS |
| Piretanide | 6 | 1.10 | 0.10 | NS |
| Probenecid | 250 | NS | NS | 26.83 |
| | 250 | NS | NS | 23.93 |

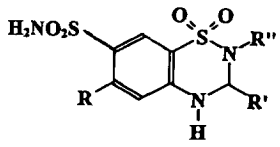
[a] Administered as furosemide xanthynol.
[b] NS = not studied.
[c] From Ref. [9] after 16 h.
[d] From Ref. [9] after 32 h.
[e] From Ref. [9] after 44 h.



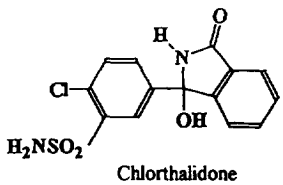
Acetazolamide, R = $OCCH_3$
Benzolamide, R = benzene sulfonyl



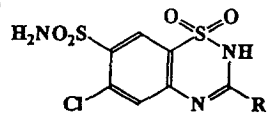
Dichlorphenamide



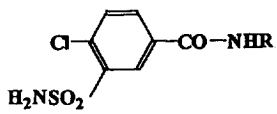
Hydroflumethiazide, R = $CF_3$, R′ = H, R″ = H
Hydrochlorothiazide, R = Cl, R′ = H, R″ = H
Methylchlothiazide, R = Cl, R′ = $CH_2Cl$, R″ = $CH_3$
Cyclopenthiazide, R = Cl, R′ = $CH_2C_5H_9$, R″ = H
Cyclothiazide, R = Cl, R′ = 3-bicyclo-(2,2,1)-hept-5-en-2-yl
Bendroflumethiazide, R = $CF_3$, R′ = $CH_2C_6H_5$, R″ = H
Buthiazide, R = Cl, R′ = $CH_2CH(CH_3)_2$, R″ = H
Polythiazide, R = Cl, R′ = $CH_2SCH_2CF_3$, R″ = $CH_3$
Trichlormethiazide, R = Cl, R′ = $CHCl_2$, R″ = H



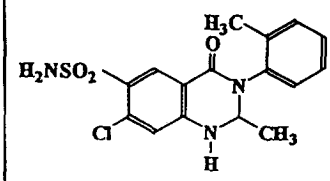
Chlorthalidone



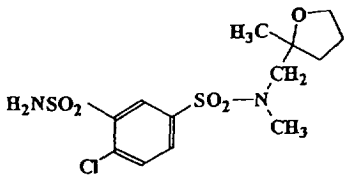
Chlorothiazide, R = H
Benzthiazide, R = $CH_2SCH_2C_6H_5$



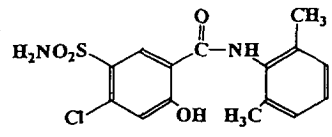
Clopamide, R = 2,6-dimethyl-1-piperidinyl
Indapamide, R = 2-methyl-1-indolinyl



Metolazone



Mefruside



Xipamide

Fig. 1. (*Continued on p. 155*)

### 2.1. Physicochemical properties of diuretics

The physicochemical properties are always relevant whether an efficient analytical method is being developed for one or a group of drug substances. Diuretics form a large and heterogeneous group of compounds, covering a wide spectrum of physicochemical properties which most affect the separation in capillary zone electrophoresis (CZE).

The diuretics can be classified as basic, neutral,

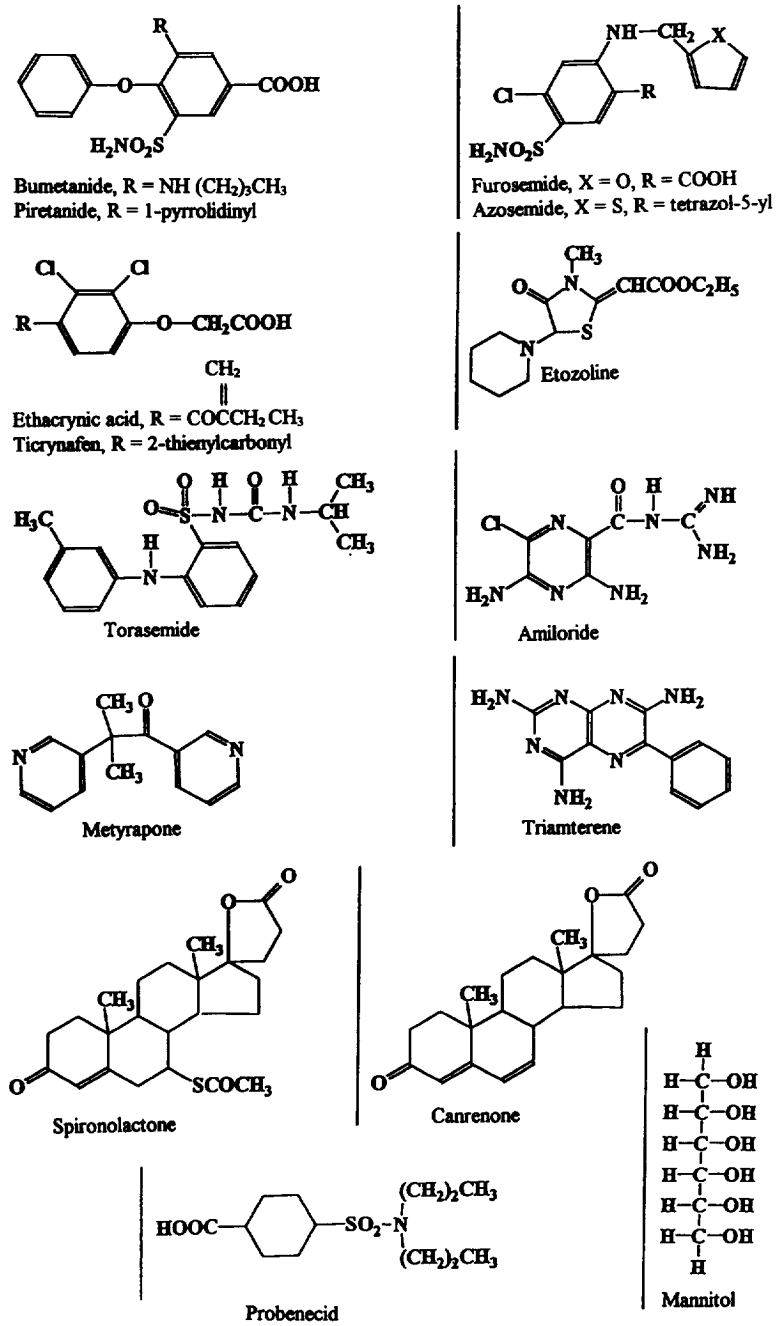



Fig. 1. Molecular structures of diuretics and probenecid [4].

weakly acidic, or strongly acidic [8]. This classification is made according to the functional groups attached to the molecules and their p$K_a$ values. The usual functional groups are primary, secondary, and tertiary amine groups, carboxylic acid groups and sulphonamide groups. Table 3 presents the most widely used diuretics according to this classification. Mannitol, which is a sugar alcohol, does not fall into any of these groups. Furthermore, the classification may be somewhat arbitrary for compounds exhib-

Table 3
Basic, neutral, weakly acidic and strongly acidic diuretics

| Group | Compound |
|---|---|
| Basic | Amiloride |
| | Triamterene |
| | Metyrapone |
| | Etozoline |
| Neutral | Spironolactone |
| | Canrenone |
| Weakly acidic | Hydroflumethiazide |
| | Hydrochlorothiazide |
| | Metychlorothiazide |
| | Cyclopentathiazide |
| | Cyclothiazide |
| | Bendroflumethiazide |
| | Buthiazide |
| | Polythiazide |
| | Trichlormethiazide |
| | Chlorothiazide |
| | Benzthiazide |
| | Torasemide |
| | Acetazolamide |
| | Benzolamide |
| | Dichlorphenamide |
| | Chlorthalidone |
| | Clopamide |
| | Indapamide |
| | Mefruside |
| | Metolazone |
| | Xipamide |
| Strongly acidic | Ethacrynic acid |
| | Ticrynafen |
| | Furosemide |
| | Bumetanide |
| | Piretanide |
| | Azosemide |
| Sugar alcohols | Mannitol |

iting more than one functional group of those groups used in the classification.

The molecular mass of the compounds varies from 182.2 (mannitol) up to 432 (benzthiazide). Thus the size of the compounds varies widely as well.

Size and charge of the compound determine the electrophoretic mobility of a compound in CZE, the hydrophobicity, while size and shape of the molecule are important parameters in micellar electrokinetic chromatography (MEKC). The heterogeneity of the diuretic compounds means that they also possess a wide range of these properties, which can be utilized in MEKC analysis.

In general, the diuretics absorb in the low UV region, and UV detectors can be used in monitoring them. Again, mannitol is an exception to the rule. The detection wavelength from 215 to 220 nm is usually a good choice. Diode array detectors, which are powerful for distinguishing partially overlapping peaks, will most probably become more popular in capillary electrophoresis, and especially in the screening for the diuretics.

## 3. Capillary electrophoretic separation strategy for diuretics

The primary factor determining the separation strategy is the objective of the analyst. CE is well suited for qualitative and/or quantitative analysis, rapid screening methods, and even for the determination of physicochemical properties of the compounds. If CE is used to monitor just one compound for quality control or clinical analysis, the analyst can choose to apply either CZE or MEKC. Although CZE is easier to use for charged compounds, MEKC may have some advantages over CZE if the sample matrix is problematic. For the determination of physicochemical properties of the compounds, such as $pK_a$ values or electrophoretic mobilities, CZE is the method of choice.

For a large screening operation such as doping control, the analyst must consider different alternatives. Since diuretics form a heterogeneous group, it is impossible to separate them all in a single CZE run. However, it is probably extremely difficult to separate them fully by MEKC as well. Spironolactone and canrenone cannot be separated by CZE, but presumably can be separated by MEKC. Although MEKC can provide more selectivity for the separation, the analyst must then deal with a multifaceted optimization scheme, which may be difficult to solve. CZE has the benefit of simplicity and good applicability to qualitative analysis. It is easier to study a CZE system, since there are fewer factors causing uncertainty than in MEKC. If other doping agents are to be screened simultaneously with diuretics, the situation changes again. The selection of CZE or MEKC is not straightforward, and therefore depends greatly on the final objective of the analyst.

### 3.1. Capillary electrophoresis for the analysis of diuretic drugs

Diuretics have traditionally been separated by conventional HPLC and GC techniques. While HPLC and GC have proven efficient for the separation of diuretics, the rapid development of CE techniques has made CE highly competitive. The high separation efficiency and ease of operation provided by CE challenges the performance provided by HPLC, although problems related to the qualitative and quantitative analysis remain to be solved. A few separation strategies applying to both CZE and MEKC have been reported in the literature. Evenson and Wiktorowicz have applied MEKC to the separation of hydrochlorothiazide, furosemide, trichlormethiazide, and chlorothiazide [11], while Gonzales et al. have employed CZE with pulsed-laser fluorescence in the determination of triamterene and bendroflumethiazide in urine [12]. Because of wide differences in molecular structures, we required two separate runs by CZE with two pH values to separate fifteen diuretics and probenecid in both serum and urine [13]. However, with MEKC we were able to separate the same compounds and caffeine in a single run in under 20 min [14]. Most studies reported in the literature describe either the separation of specific diuretics or the separation system itself [11–20]. Thomas et al. have introduced a validated method for the quality control of hydrochlorothiazide and chlorothiazide [21]. The operational conditions for the separation systems reported in the literature are summarized in Table 4.

## 4. CZE for the separation of diuretics

The separation of diuretics in CZE is based on the size and charge of the compounds. Thus, spironolactone and canrenone cannot be separated by CZE

Table 4
Operational conditions for various approaches on separation of diuretics by CZE and MEKC. The total length of the capillary is reported

| Buffer | pH | *U* [kV] | Injection | Capillary | No. of diuretics | Detection [nm] | *T* [°C] | Ref. |
|---|---|---|---|---|---|---|---|---|
| 30 m*M* borate–30 m*M* SDS–100 ml/l ACN in $H_2O$ | 9.3 | 30 | hydr[a] 1 s | Fused-silica 72 cm, 50 μm I.D. | 4 | UV 200 | 30 | [11] |
| Tris | 8 | up to 30 | 10 kV 10 s | Polyamide coating 100 cm, 75 μm I.D. | 2 | LIF 337 | amb | [12] |
| CAPS 60 m*M* | 10.6 | 25 | Hydr. 5 s | Fused-silica 67 cm, 50 μm I.D. | 12 | UV 220 | 20 | [13] |
| Acetate 70 m*M*–betaine 0.5 *M* | 4.5 | 25 | Hydr. 5 s | Fused-silica 67 cm, 50 μm I.D. | 3 | UV 215 | 20 | [13] |
| Glycine 50 m*M*–SDS 42 m*M* | 10.5 | 25 | Hydr. 7 s | Fused-silica 67 cm, 50 μm I.D. | 15 | UV 220 | 20 | [14] |
| CAPS 60 m*M* | 10.6 | 13 | – | PAG coating PP 44 cm, 85 μm I.D. | 2 | UV 215 | ambient | [20] |
| 20 m*M* borate–30 m*M* SDS | 9.5 | 17.5 to 20 | Hydr. 2.5 s | Fused-silica 70 cm, 100 μm I.D. | 2 | UV 225 | 15–25 | [21] |

[a]Hydr.: hydrostatic injection by pressure.

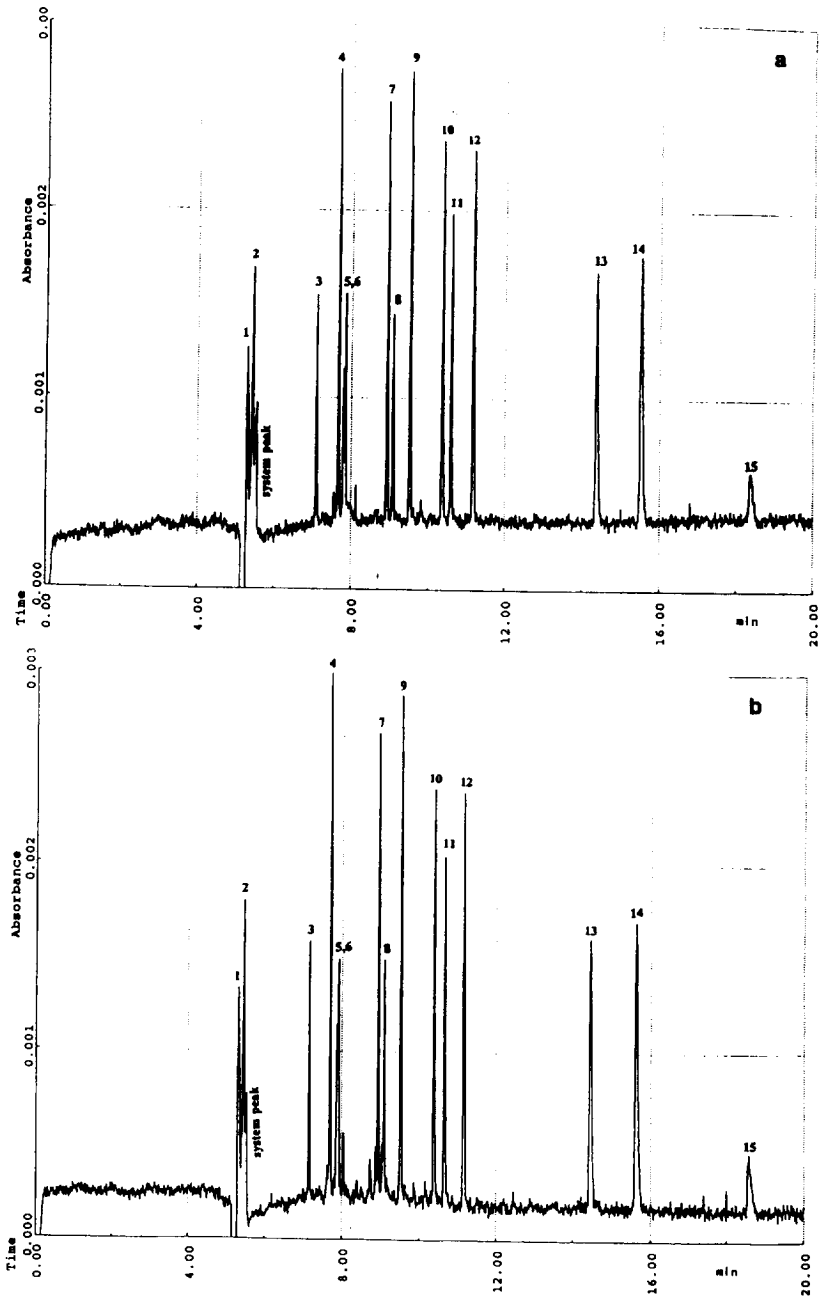



Fig. 2. Electropherograms of (a) spiked serum and (b) spiked urine. The operational conditions: [CAPS]=0.06 $M$; pH=10.6; $T$=20°C; $U$=25 kV; hydrostatic injection by pressure 5 s; $L_{tot}$=67 cm; $L_{det}$=60 cm; capillary I.D.=50 $\mu$m; capillary O.D.=360 $\mu$m; UV detection at 220 nm. The spiked samples contained all of the diuretics (10 ppm each). Migration order: 1=metyrapone and caffeine; 2=triamterene and amiloride; 3=clopamide; 4=chlorthalidone; 5=probenecid; 6=ethacrynic acid; 7=bumetanide; 8=bendroflumethiazide; 9=furosemide; 10=trichlormethiazide; 11=benzthiazide; 12=hydrochlorothiazide; 13=dichlorphenamide; 14=chlorothiazide; and 15=acetazolamide [13].

because they are neutral under the applicable pH range. Spironolactone is also excreted as canrenone and other metabolic products [10]. The separation strategy for the screening of a variety of diuretics includes two separate runs under basic and acidic conditions. Basic conditions are required to separate efficiently both the weakly and strongly acidic diuretics. Most of the known diuretic drugs fall into these groups. Acidic conditions, in turn, are used to separate the basic diuretics. The use of biological buffers such as 3-(cyclolhexylamino)-1-propanesulphonic acid (CAPS) has proven important as a means of minimizing Joule heating effects and excessive band asymmetry due to differences between the analyte and buffer mobility. Fig. 2 shows the successful separation of twelve weakly or strongly acidic diuretics and probenecid in spiked SPE-treated serum and urine, achieved in 60 m*M* CAPS buffer at pH 10.6 [13]. An interesting feature of this separation system is that three basic diuretics also included in the samples—metyrapone co-migrated with caffeine, and amiloride together with triamterene as one zone, even though these compounds should have been neutral under these separation conditions. Furthermore, these compounds emerge at the "negative" edge of the sample zone just before the system peak, which would indicate that, at some stage of the separation, they possess negative charge. This phenomenon is more pronounced at higher pH (11.5), but we have not yet been able to establish the mechanism through which this occurs.

The three basic diuretics just mentioned, spiked in SPE-treated serum and urine, have separated in 70 m*M* acetate–0.5 *M* betaine buffer at pH 4.5 (Fig. 3 [13]). In screening, it often is possible to screen for compounds from other groups of interest as well. For example, CZE can be used to screen for basic diuretics and $\beta$-blockers simultaneously at pH 2.9. This difficult separation system requires a long analysis time and high field strength, and hence the electroosmotic flow must be artificially suppressed through dynamic modification of the capillary wall [22].

Detailed study of the separation method for weakly and strongly acidic diuretics has shown the separation to be based on the charge and size of the compounds. This has been verified by studying the correlation between the self-diffusion coefficients and $pK_a$ values of certain diuretics and the separation obtained by CZE with marker techniques [15]. Marker techniques employ compounds whose electrophoretic mobilities are known, as a means of determining the physical parameters during an electrophoretic run. These parameters are the effective electric field strength ($E_{eff}$) [23] and the electroosmotic flow velocity ($v_{eo}$). With more than two markers it is possible to approximate the changes in $v_{eo}$ within a single run. A full description of the marker compounds is given in [18].

The diffusion coefficients of the diuretics vary from $5.8 \cdot 10^{-10}$ to $9.1 \cdot 10^{-10}$ $m^2 s^{-1}$ and the electrophoretic mobilities from $-1.7 \cdot 10^{-8}$ to $-4.4 \cdot 10^{-10}$ $m^2 V^{-1} s^{-1}$ in 60 m*M* CAPS at pH 10.6 when $T$=20°C [16]. Under these conditions, the choice of counter ion, as an alkali metal or ammonium ion, does not influence the selectivity of the separation system [17]. Metal ammonia complexes can be used to tune the electroosmotic flow velocity, however [16,17], making it possible to optimize the resolution of the compounds by varying the capillary length and metal ammonia content of the buffer, while at the same time maintaining high voltage [16]. Such a procedure may be important where adequate separation of all compounds is difficult to achieve as is often the case in screening procedures.

### 4.1. Sample pretreatment in CZE

Polyelectrolytes, such as proteins and large peptides, are known to adsorb onto the surface of the silica capillary wall when the pH is above 2 and less than the isoelectric point (p*I*) of the protein or the peptide. In some cases proteins may show strong adsorption, even when the pH of the buffer is somewhat higher than the p*I* of the protein, if such protein still has a large local positive charge somewhere on its surface.

Diuretics are usually dosed orally. As a rule they are analysed in patients' urine, but blood serum can be used as a sample matrix as well. Although direct injection of urine is feasible with CZE, the urine may sometimes contain protein or other polyelectrolytes that can ruin the separation. The use of sample pretreatment is recommended in those cases to improve the reliability of CZE separation, although it may cause problems in quantification and certainly

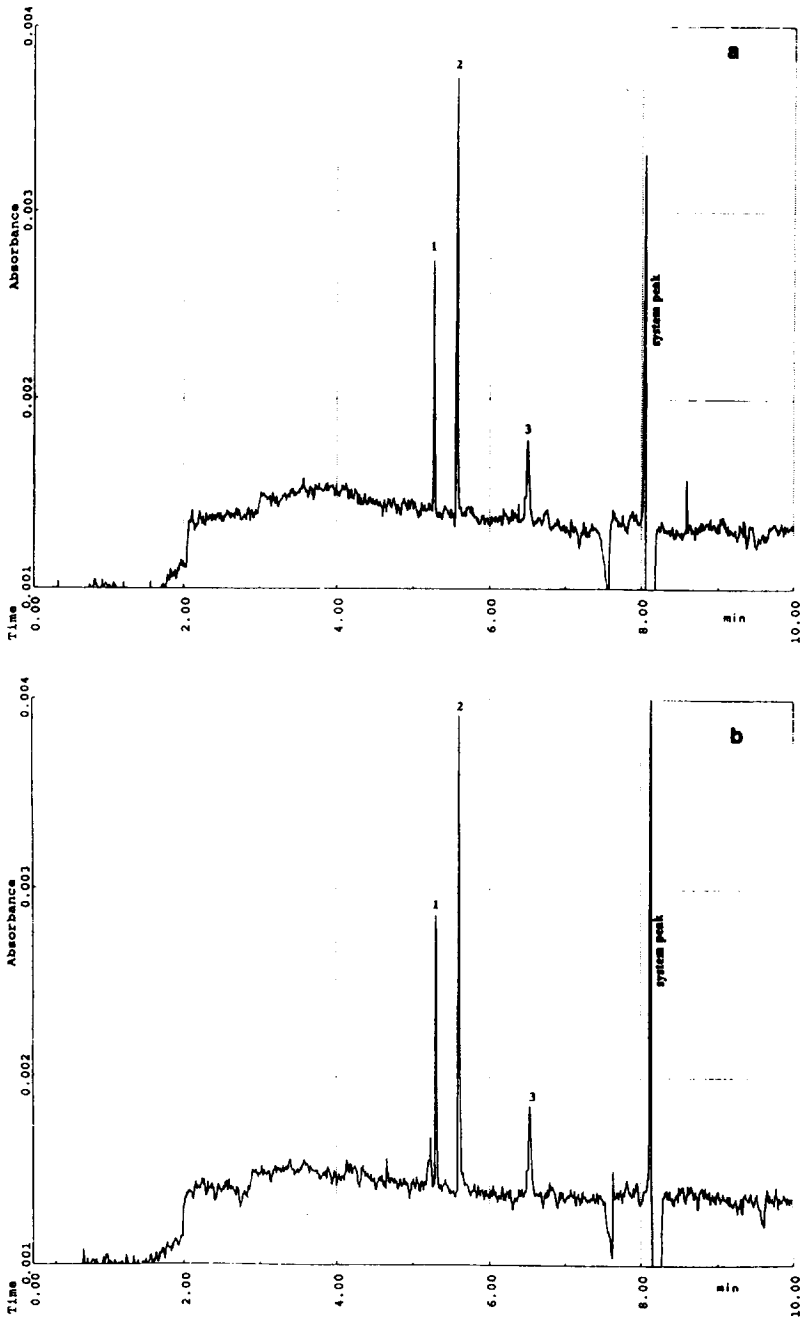



Fig. 3. Electropherograms of (a) spiked serum and (b) spiked urine. The operational conditions: 0.07 *M* acetate—0.5 *M* betaine; pH=4.5; $T$=20°C; UV=25 kV; hydrostatic injection by pressure 5 s; $L_{tot}$=67 cm; $L_{det}$=60 cm; capillary I.D.=50 $\mu$m; capillary O.D.=360 $\mu$m; UV detection at 215 nm. The spiked samples contained three basic diuretics and caffeine (10 ppm each). The migration order: 1=amiloride; 2=triamterene; 3=metyrapone; and 4=caffeine [13].

will increase the total analysis time and cost of the analysis. In the case of blood serum, the proteins must be removed from the sample unless extreme pH conditions are used in the analysis. The use of a hollow polypropylene fibre coated with linear polyacrylamide [20] may also prove helpful in improving the reliability of the CZE operation when serum or urine is directly injected into the capillary.

### 4.2. Identification of diuretics by employing marker techniques in CZE

When marker techniques are employed, as many as twelve diuretics can be identified [19]. The use of the three marker technique where $v_{eo}$ is approximated to be linearly accelerating, enables the estimation of the time dependency of $v_{eo}$ during each run. Table 5 shows the average values and the relative standard deviations for the analyte absolute migration times and the electrophoretic mobilities of the analytes determined by the three marker technique (3m) ($n=6$) when SPE-treated urine was used as a sample matrix (Fig. 4). Our group has introduced a new concept, the coefficient for identification, $Q_{id}$, to express the reliability of the identification between two compounds [18]. From Table 6 it can be seen that the $Q_{id}$ values calculated by 3m for the subsequent pairs of twelve diuretics and probenecid of Table 5 can be even 50 times greater than those calculated with absolute migration times.

In our labaratory, we have identified diuretics by using the absolute migration times and the electrophoretic mobilities determined by the four marker technique (4m) in samples some of which also included other compounds with very similar migration times to those of the diuretics. The reliability of the identifications was 100% successful when electrophoretic mobilities determined by the 4m were used when the confidence level from the calibration runs ($n=9$) was set to $3\delta$. It is important to notice that ethacrynic acid and probenecid can be easily identified by using 4m, even though the difference in the migration times of the two compounds is approximately 3.5 s when using 80 m*M* CAPS at pH 10.6 and 20 kV in a 77-cm long capillary with 50 $\mu$m I.D. (detection at 70 cm) [18]. The $Q_{id}$ values obtained for the pair ethacrynic acid and probenecid was 350 times greater compared to that calculated with absolute migration times.

## 5. MEKC for the separation of diuretics

The addition of micelles to the electrolyte solution frequently improve the selectivity of an electrophoretic separation markedly. The methods proposed by Evenson and Wiktorowicz [11], Thomas et al. [21]

Table 5
Average values and relative standard deviations for analyte absolute migration times and for the electrophoretic mobilities determined by the three marker technique (3m) ($n=6$).

| Analyte | $t_{abs}$ (min) | R.S.D. (%) ($t_{abs}$) | $\mu_{ep}$ $10^{-8}$ m$^2$ V$^{-1}$ s$^{-1}$ | R.S.D. (%) ($\mu_{ep}$) |
|---|---|---|---|---|
| Clopamide | 5.52 | 0.70 | −1.697 | 0.171 |
| Chlorthalidone | 5.86 | 0.70 | −2.015 | 0.086 |
| Probenecid | 6.04 | 0.70 | −2.176 | 0.026 |
| Ethacrynic acid | 6.08 | 0.69 | −2.210 | 0.037 |
| Bumetanide | 6.70 | 0.74 | −2.669 | 0.072 |
| Bendroflumethiazide | 6.81 | 0.76 | −2.745 | 0.056 |
| Furosemide | 7.07 | 0.80 | −2.909 | 0.070 |
| Trichlormethiazide | 7.68 | 0.82 | −3.252 | 0.052 |
| Benzthiazide | 7.91 | 0.85 | −3.364 | 0.051 |
| Hydrochlorothiazide | 8.09 | 0.86 | −3.449 | 0.078 |
| Dichlorphenamide | 10.37 | 1.08 | −4.268 | 0.034 |
| Chlorothiazide | 11.10 | 1.12 | −4.454 | 0.030 |
| Acetazolamide | 12.95 | 1.27 | −4.832 | 0.019 |

Triphenylacetic acid ($\mu_{ep}=-2.056\cdot10^{-8}$ m$^2$ V$^{-1}$ s$^{-1}$), *meso*-2,3-diphenylsuccinic acid ($\mu_{ep}=-3.910\cdot10^{-8}$ m$^2$ V$^{-1}$ s$^{-1}$), and *o*-phthalic acid ($\mu_{ep}=-5.105\cdot10^{-8}$ m$^2$ V$^{-1}$ s$^{-1}$ have been used as marker compounds. Experimental conditions; as in Fig. 4

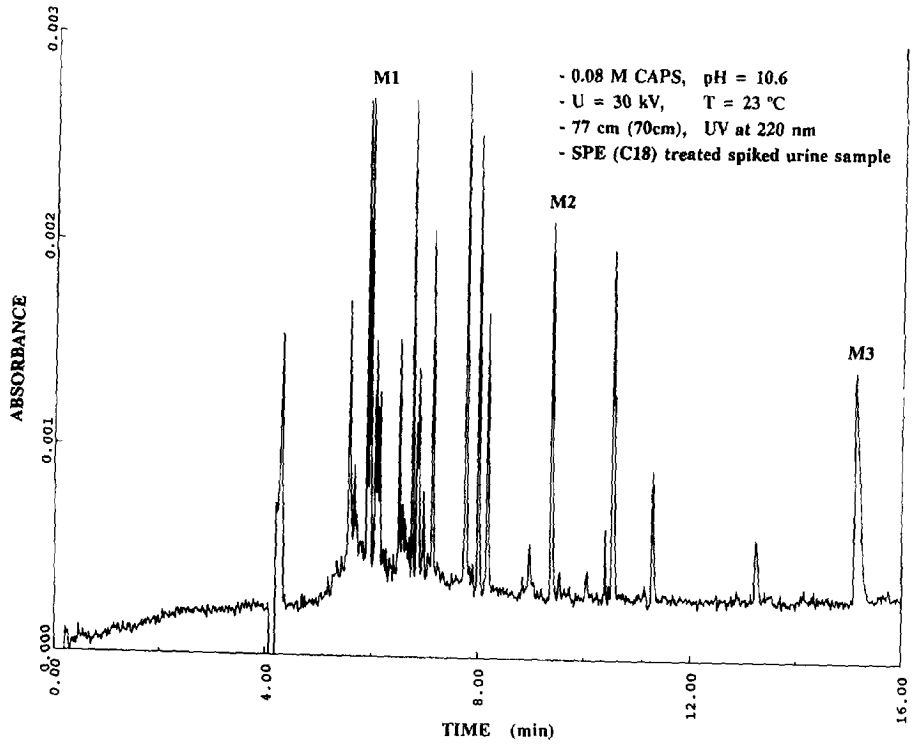



Fig. 4. The separation of twelve diuretics and probenecid in spiked urine (migration order in Table 5) by CZE. Triphenylacetic acid ($M_1$), *meso*-2,3-diphenylsuccinic acid ($M_2$) and *o*-phthalic acid ($M_3$) are as marker compounds [19].

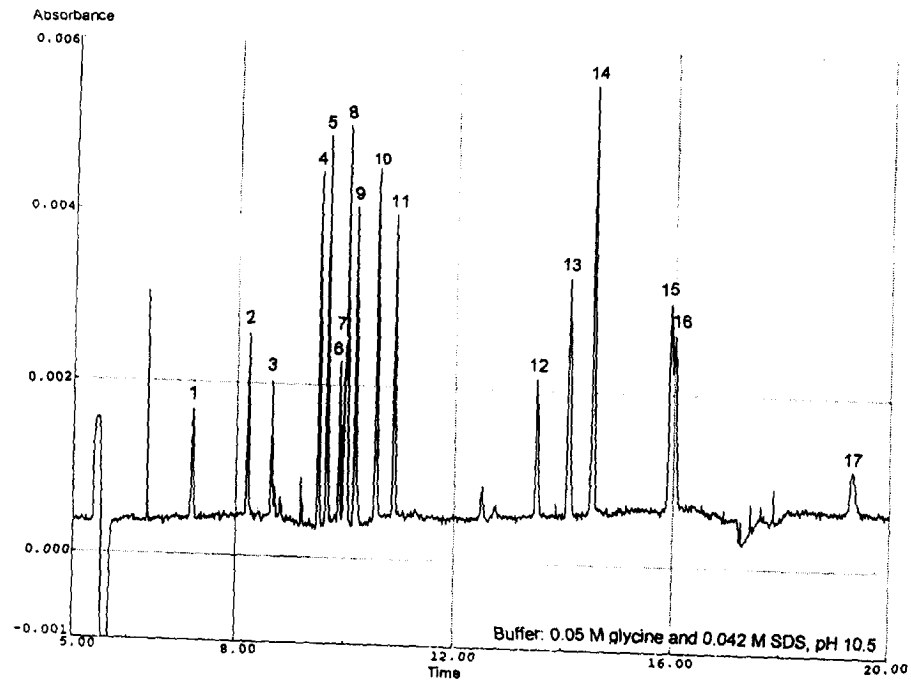



Fig. 5. The separation of caffeine, probenecid and fifteen diuretics by MEKC. $U$=25 kV, $L_{tot}$=67 cm; $L_{det}$=60 cm; capillary I.D.=50 $\mu$m; capillary O.D.=360 $\mu$m; UV detection at 220 nm [14]. 1=caffeine; 2=clopamide; 3=probenecid; 4=bumetanide; 5=furosemide; 6=bendroflumethiazide; 7=ethacrynic acid; 8=chlorthalidone; 9=trichlormethiazide; 10=hydrochlorothiazide; 11=benzthiazide; 12=metyrapone; 13=dichlorophenamide; 14=triamterene; 15=chlorthalidone; 16=amiloride; and 17=acetazolamide [13].

Table 6
The values of coefficient for identification ($Q_{id}$) obtained for successive pairs of twelve diuretics and probenecid, when analyte absolute migration times and electrophoretic mobilities determined by the three marker technique (3 m) are used

| Analyte pair | $Q_{id}$ ($t_{abs}$) | $Q_{id}$ ($\mu_{ep}$) | Ratio $Q_{id}(\mu_{ep})/Q_{id}(t_{abs})$ |
|---|---|---|---|
| Clopamide–chlorthalidone | 4.27 | 68.6 | 16 |
| Chlorthalidone–probenecid | 2.16 | 70.0 | 32 |
| Probenecid–ethacrynic acid | 0.48 | 24.6 | 51 |
| Ethacrynic acid–bumetanide | 6.77 | 168 | 25 |
| Bumetanide–bendroflumethiazide | 1.09 | 22.0 | 20 |
| Bendroflumethiazide–furosemide | 2.40 | 45.9 | 19 |
| Furosemide–trichlormethiazide | 5.10 | 92.0 | 18 |
| Trichlormethiazide–benzthiazide | 1.77 | 32.9 | 19 |
| Benzthiazide–hydrochlorothiazide | 1.32 | 19.3 | 15 |
| Hydrochlorothiazide–dichlorphenamide | 12.6 | 198 | 16 |
| Dichlorphenamide–chlorothiazide | 3.09 | 66.7 | 22 |
| Chlorothiazide–acetazolamide | 6.41 | 168 | 26 |

and our laboratory [14] all employ sodium dodecyl sulphate (SDS) as surfactant. Thus, no information is presently available on the suitability of other micelles or inclusion forming compounds, such as cyclodextrins and crown ethers, for the separation of diuretics. The use of other micelles or even a mixed micellar system might be efficient for the separation of this group as a whole. In addition, the separation in nonaqueous media is still a totally unexplored area. Fig. 5 shows the separation of fifteen diuretics, probenecid and caffeine obtained by using 50 m*M* glycine–42 m*M* SDS buffer at pH 10.5 [14].

While efficient separation methods are available for screening or even for identifying certain diuretics, the quantitative analysis has been neglected to some extent. Thomas et al. [21] have validated a quantitative method for the quality control of hydrochlorothiazide and chlorothiazide. They were able to obtain precision and repeatability in the range of 1% by employing careful injection procedure to minimize the time in which the capillary ends were not immersed in buffer or sample during the injection process, and by adjusting the ionic strength of the sample close to that of the buffer in order to diminish stacking, which caused poorer repeatability of peak areas.

Sample pretreatment is an essential part of any analytical technique. MEKC is preferable to CZE because it allows serum, plasma or urine to be injected directly into the capillary without creating wall adsorption problems. A surfactant such as SDS added to the electrolyte solution binds to proteins giving them an overall negative charge so that their interactions with the capillary wall surface are minimized.

## 6. Conclusions

Capillary electrophoresis has many advantages over HPLC for the screening and identification of diuretics and probenecid: very high resolution, which is particularly useful with complex samples; low cost of disposables and solvents; limited sample requirement; and the possibility to separate also very hydrophilic analytes and to reduce sample preparation steps. Both CZE and MEKC have proven to be efficient in the separation of diuretics and probenecid. Choice of the method depends on the objective of the analysis. CZE is simple and gives good results for the separation of charged diuretics. Furthermore, it can effectively be used to identify compounds when marker techniques are advantageous and to determine physicochemical properties of the analytes. MEKC, on the other hand, can be used to separate all diuretics simultaneously and is applicable to the analysis of neutral diuretics. Although the marker techniques can also be used in MEKC, they have been primarily developed for CZE. With MEKC, the sample pretreatment can be simplified, because MEKC allows direct injection of urine, plasma and serum samples. However, no one has yet attempted the direct injection of biological fluids containing diuretics. The separation of diuretics in nonaqueous media is also still a totally unexplored area.

UV detection, which is universal and is readily

available in commercial instruments, has proven efficient for the diuretics. The use of pulsed laser fluorescence detection has proven to be efficient for the determination of triamterene and bendroflumethiazide in urine. Fluorescence detection at 337 nm is highly sensitive and has the additional advantage that endogenous compounds in urine show no fluorescence at that wavelength. Use of fluorescence detection should increase the sensitivity and improve the identification and quantification by CE. The coupling of mass spectrometry with capillary electrophoresis can be expected to provide even more reliability in the future for the confirmation of diuretics in biological fluids.

## 7. Abbreviations and symbols

| | |
|---|---|
| HPLC | High-performance liquid chromatography |
| GC | Gas chromatography |
| CE | Capillary electrophoresis |
| CZE | Capillary zone electrophoresis |
| MEKC | Micellar electrokinetic chromatography |
| EKC | Electrokinetic chromatography |
| SPE | Solid-phase extraction |
| CMC | Critical micelle formation concentration |
| $v_{eo}$ | Electroosmotic flow velocity (m $s^{-1}$) |
| $E_{eff}$ | Effective electric field strength (V $m^{-1}$) |
| $\mu_{ep}$ | Electrophoretic mobility ($m^2$ $V^{-1}$ $s^{-1}$) |
| $U$ | Voltage (V) |
| $L_{det}$ | Capillary length to the detector |
| $L_{tot}$ | Total capillary length |
| $Q_{id}$ | Coefficient for identification |
| SDS | Sodium dodecyl sulphate |
| CAPS | 3-(cyclohexylamino)-1-propanesulphonic acid |
| ACN | acetonitrile |

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