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Determination of triamterene and its main metabolite hydroxytriamterene sulfate in human urine by capillary electrophoresis using ultraviolet absorbance and laser-induced fluorescence detection

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Abstract

Two capillary electrophoresis methods have been developed for the direct determination of triamterene and its main metabolite hydroxytriamterene sulfate in human urine. Analytes were detected using conventional UV detection as well as laser-induced fluorescence (LIF) detection with an HeCd-laser operating at a wavelength of 325 nm. The results of both detection techniques were compared. Indeed, the limit of quantification was eightfold lower using LIF detection (50 ng/ml) in comparison to UV detection (400 ng/ml). As no interference due to endogenous urine compounds was observed, direct urine analysis was feasible. Analysis was very simple and fast—one run could be performed within less than 10 min (CE–UV method) and 2.5 min (CE–LIF method), respectively. Both assays were fully validated and applied to urine samples from a human volunteer. The results of the application of the CE–LIF method to human urine samples are presented in this publication.

Keywords: Triamterene; Hydroxytriamterene sulfate

1. Introduction

Triamterene (TA; Fig. 1A) is a potassium-sparing diuretic used mainly in combination with other diuretics, such as hydrochlorothiazide and furosemide, to reduce their potassium-wasting effects [1–3]. It inhibits the reabsorption of sodium, which exchanges for potassium and hydrogen ions in the distal renal tubule and the collecting duct [4]. After oral administration the drug is rapidly absorbed at a rate of about 80% [5]. It is hepatically metabolized by hydroxylation and subsequent immediate conjugation with sulfuric acid to its main metabolite hydroxytriamterene sulfate (STA; Fig. 1C) [6–8]. The intermediate phase-I metabolite hydroxytriamterene (Fig. 1B) was either not detectable in urine or

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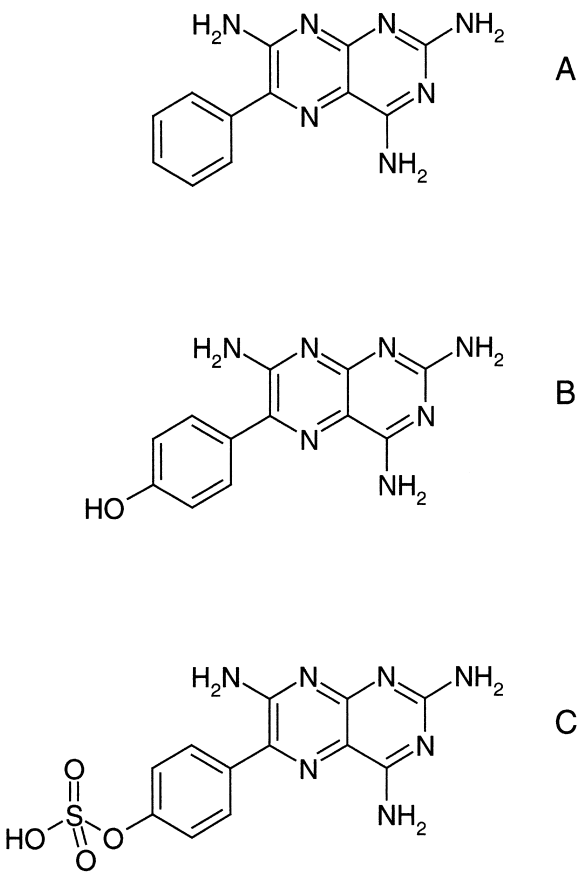

Fig. 1. Chemical structures of triamterene (A) and its metabolites hydroxytriamterene (B) and hydroxytriamterene sulfate (C).

plasma [9] or was only identified in trace amounts below 1 ng/ml [5]. Further metabolic pathways lead to the formation of very small amounts of other metabolites, such as *N*-glucuronides and others [6,10,11]. TA and its metabolite STA are excreted in urine as well as in feces. Urinary concentrations of STA exceeded those of TA by 4- to 13-fold [9]. Indeed, the phase-II metabolite STA is still pharmacologically active and therefore contributes to the pharmacological profile of TA [12,13]. As metabolism of TA takes place in the liver, the formation and excretion of STA decreases in patients with liver cirrhosis and ascites, consequently the rate of the drug excreted unchanged increases [14]. Determination of the STA/TA ratio in plasma and urine has been proposed as a simple diagnostic test for hepatic function [10].

Capillary electrophoresis is becoming a very useful technique for the determination of drugs in biological fluids because of its high resolution, mass sensitivity and speed. However, using conventional UV detection, concentration sensitivity and detection limit are frequently insufficient owing to the short optical path length in the capillary. In order to improve sensitivity, laser-induced fluorescence (LIF) detection can be used, if the analytes can be excited to natural fluorescence at the emission wavelength of the engaged laser.

CE has been used previously for the screening of diuretics and other banned drugs in sport (including TA) in plasma or urine using UV detection [15,16] as well as fluorescence detection with a xenon arc lamp [17] or a pulsed nitrogen laser [18] as light source. However, none of these methods considered the metabolite STA, and beyond this, they were only used for a qualitative identification in doping control and not for quantitative pharmacokinetic investigations.

In this publication, we present two validated CE methods for the simultaneous quantification of TA and STA in human urine. In the method presented first, conventional UV detection was used. As the limit of detection obtained by UV detection was insufficient for some of the urine samples, an advanced method using LIF detection with a HeCd-laser emitting at 325 nm was established.

2. Experimental

2.1. Chemicals and reagents

Triamterene (6-phenyl-pteridine-2,4,7-triamine) and hydroxytriamterene sulfate (sulfuric acid mono-[4-(2,4,7-triamino-pteridine-6-yl)-phenyl] ester) as well as Dytide® H tablets (containing 50 mg triamterene and 25 mg hydrochlorothiazide) were provided by Procter and Gamble Pharmaceuticals (Weiterstadt, Germany).

RPh 3040b (6-[2-(methyl-*p*-tolyl-amino)-ethyl]-pteridine-2,4,7-triamine), a synthetic triamterene derivative used as internal standard (I.S.) for UV detection was synthesized by Röhm (Darmstadt, Germany; now: Procter and Gamble, Weiterstadt, Germany). Quinine sulfate (I.S. for LIF detection)

was supplied by Caesar and Loretz (Hilden, Germany).

Sodium dihydrogen phosphate monohydrate, sodium hydrogen phosphate dihydrate, methanol, dimethyl sulfoxide and sodium hydroxide solutions (0.1 and 1 *M*) were obtained from E. Merck (Darmstadt, Germany). Triethanolamine was supplied by Fluka (Buchs, Switzerland), and orthophosphoric acid 85% by J.T. Baker B.V. (Deventer, Holland). Water was purified by bidistillation in our own laboratory. Nitrogen was purchased from Messer Griesheim (Krefeld, Germany). All reagents were of analytical grade unless otherwise specified.

Different lots of blank urine were obtained from healthy volunteers and stored at −18 °C in a freezer until use.

2.2. Capillary electrophoresis with UV detection

A Spectra Phoresis 500 instrument (Thermo Separation Products, Darmstadt, Germany) was set at 18 kV and the UV–Vis detector at the optimal wavelength of 353 nm. An internally uncoated fused-silica capillary (Thermo Separation Products, Darmstadt, Germany) with a length of 44 cm (34.5 cm detection length) and an I.D. of 75 μm was used in a Spectra Physics cassette. The samples were injected hydrodynamically for 6 s. The electrophoresis buffer was a 75 m*M* phosphate buffer (sodium hydrogen phosphate and sodium dihydrogen phosphate) adjusted to a pH of 6.0. The run time was 10 min. During analysis the temperature of the capillary cartridge was kept at 20 °C. The data were processed by Borwin™ Software (Jasco, Groß-Umstadt, Germany).

At the start and the end of a day the capillary was flushed with 1 *M* NaOH, 0.1 *M* NaOH and double-distilled water at 60 °C for 10 min each. For equilibration the capillary was rinsed at the beginning with the electrophoresis buffer at 20 °C for 10 min. Between single runs, the capillary was rinsed with 0.1 *M* NaOH and water for 3 min and afterwards with phosphate buffer for 5 min.

2.3. Capillary electrophoresis with LIF detection

A Beckman P/ACE System 2100 equipped with a laser-induced fluorescence (LIF) detector (Beckman Instruments, Fullerton, CA, USA) was used with an untreated fused-silica capillary of 50 μm I.D., 20 cm effective length and 27 cm total length (Polymicro Technologies, Phoenix, AZ, USA).

The electrophoresis buffer was prepared from 0.1 *M* sodium dihydrogen phosphate solution adjusted to pH 6.2 by dropwise addition of triethanolamine.

Separation was carried out by applying a voltage of 14 kV (≈519 V/cm) resulting in a current of approximately 100 μA. The temperature of the capillary cartridge was maintained at 20 °C. Detection was performed by laser-induced fluorescence using a HeCd-laser (Omnichrome, Series 74, Model No. 3074-EOS-A01, Laser 2000, Wessling, Germany) operating at 325 nm, that was connected to the LIF-detector by an optical fiber (Omnichrome POS FDS-A$\frac{1}{2}$, Laser 2000, Wessling, Germany). Emission was measured at 450 nm using an interference filter with a half bandwidth of 10 nm.

Data collection and processing were performed using System Gold™ software 7.11 (Beckman Instruments, Fullerton, CA, USA).

To determine the fluorescence maxima of TA and STA, in a previous investigation fluorescence emission spectra (λ_{ex} = 325 nm) of TA and STA were recorded on a Shimadzu Spectrofluorometer RF-540 equipped with a Shimadzu Data Recorder DR-3 (Shimadzu, Kyoto, Japan) (Fig. 2).

At the beginning of a series, the capillary was rinsed with 0.1 *M* NaOH for 30 min and with a freshly prepared running buffer for at least 15 min. Rinsing procedures were always performed at a pressure of 20 p.s.i. (= 1379 mbar). In order to equilibrate the system and to obtain reproducible migration times, three pre-runs under assay conditions were performed afterwards. Samples were introduced into the capillary by pressure injection with 0.5 p.s.i. (= 34.5 mbar) for 4 s. This was equivalent to an injected sample volume of 7.83 nl calculated using the equation of Hagen-Poiseuille

$$V = \frac{\Delta p \cdot \pi \cdot r^4 \cdot t}{8 \cdot \eta \cdot l}$$

where V is the injected volume; Δp, pressure difference; r, radius of the capillary lumen; t, injection time; η, viscosity of the buffer; l, total capillary length. After each run, the capillary was washed with

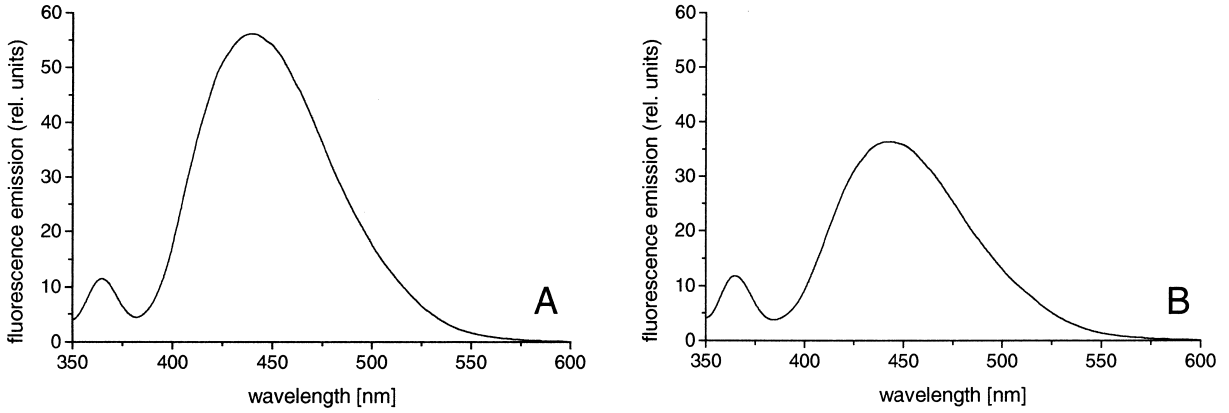

Fig. 2. Fluorescence emission spectra (λ_{ex} = 325 nm) of triamterene (A) and hydroxytriamterene sulfate (B) dissolved in 100 m*M* phosphate/triethanolamine buffer pH 6.2 at a concentration of 10 ng/ml.

0.1 *M* NaOH for 1.5 min and with double-distilled water for 1 min before reequilibrating with running buffer for 2 min. At the end of a day the capillary was always rinsed with 0.1 *M* NaOH and double-distilled water for 5 min each, and was dried afterwards with nitrogen/air for 5 min.

2.4. Preparation of stock and reference solutions

2.4.1. CE–UV method

As triamterene (TA) and hydroxytriamterene sulfate (STA, as free acid) are almost insoluble in water, stock solutions were prepared in methanol at an exactly known concentration of about 100 μg/ml. For the dissolution of STA the addition of a few drops of 1 *M* NaOH was necessary to transform the free acid into the sodium salt. Stock solutions were spiked in 500 μl blank urine at the concentration levels needed for method calibration and validation. The internal standard RPh 3040b was also dissolved in methanol and spiked to the urine samples to yield a final concentration of approximately 20 μg/ml.

2.4.2. CE–LIF method

For the CE–LIF method a different procedure of preparing the stock solutions was chosen. About 1 mg of TA or STA, exactly weighed, was dissolved in 500 μl dimethyl sulfoxide by ultrasonic treatment. This solution was then diluted with 0.02 *M* phosphate buffer pH 6.0 (made from 0.02 *M* sodium dihydrogen phosphate solution and 0.02 *M* sodium hydrogen phosphate solution) to a final volume of 10.0 ml. These stock solutions proved to be stable for at least 2 h, afterwards as a consequence of supersaturation a precipitation of yellow crystals was frequently observed.

The internal standard (I.S.) quinine sulfate was dissolved in 0.02 *M* phosphoric acid at a concentration of 1 mg/ml.

The stock solutions of TA and STA were diluted with blank urine up to the concentration levels required for calibration and validation of the method. Due to their poor stability, this dilution had to be done within 2 h after preparation. To 5 ml of every dilution, 100 μl of I.S. solution were added before analysis.

2.5. Calibration and validation

Validation was performed according to the FDA guideline for validation of bioanalytical assays in human biomatrices [19].

2.5.1. CE–UV method

The CE–UV assay was calibrated by analyzing seven different spiked standard solutions in the concentration range between 400 ng/ml and approximately 10 μg/ml. Calibration curves were calculated in an upper concentration range from 1.14 to 9.12 μg/ml for TA and 1.18 to 11.82 μg/ml for STA and in a lower concentration range from 400 ng/ml to

1.5 μg/ml for both analytes by unweighted linear regression.

To determine accuracy and precision of the method, spiked urine samples at three concentration levels (0.6, 1.5 and 8.0 μg/ml for both analytes) were prepared and analyzed six times within 1 day. Inter-day reproducibility was evaluated by repetition of the procedure on three different days.

Specificity of the assay was assessed by analysis of different lots of blank urine.

2.5.2. CE–LIF method

For the calibration of the CE–LIF assay, spiked standard solutions at eight different concentrations in the range between 50 ng/ml and 10 μg/ml (0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 μg/ml) for both TA and STA were prepared as described in Section 2.4.2. Every calibration sample was analyzed three times, and the mean corrected peak areas (=peak area×effective capillary length/migration time) were calculated. Calibration curves were obtained by plotting the concentration on the *x*-axis against corrected peak area ratios (corrected peak area of the analyte/corrected peak area of the I.S.) on the *y*-axis. The optimal calibration functions were calculated by $1/x^2$-weighted linear regression using the following equations [20–22]:

$$w_i = \frac{x_i^{-2}}{\sum_i x_i^{-2}/n} \quad (1)$$

$$m_w = \frac{\sum_i w_i \sum_i w_i x_i y_i - \sum_i w_i x_i \sum_i w_i y_i}{\sum_i w_i \sum_i w_i x_i^2 - (\sum_i w_i x_i)^2} \quad (2)$$

$$b_w = \frac{\sum_i w_i y_i - m_w \sum_i w_i x_i}{\sum_i w_i} \quad (3)$$

where x_i is the *x*-coordinate of a single data point; y_i, *y*-coordinate of a single data point; w_i, weights; n, number of data points; m_w, slope of the weighted calibration line; b_w, intercept of the weighted calibration line.

To determine accuracy and precision of the method, four spiked urine samples at four concentration levels spreading over the entire working range (0.05, 0.5, 2.0 and 10.0 μg/ml for both analytes) were prepared as described in Section 2.4.2. using separately weighed stock solutions. Each of these samples was analyzed six times within 1 day to assess intra-day accuracy and precision. The procedure was repeated on three different days to investigate inter-day variability.

Investigations concerning sample stability under freeze–thaw conditions were performed with original urine samples from a human volunteer. The procedure is described in Section 2.6.

A total absence of matrix interference was confirmed through analysis of three different lots of blank urine.

2.6. Collection and analysis of urine samples

The CE–LIF method was applied to urine samples from a human volunteer. These urine samples were collected at certain time intervals (0–1, 1–3, 3–4, 4–6, 6–10, 10–14, 14–24 h) after oral administration of a commercially available tablet (Dytide® H) containing 50 mg of triamterene and 25 mg of hydrochlorothiazide. Blank urine was collected from the same volunteer directly before administration of the tablet. The volume of urine was measured directly after collection.

Each urine sample was divided into two fractions, the first one was stored at room temperature and analyzed within 48 h after collection, and the second one was stored in a freezer at −18 °C and investigated after one and two freeze–thaw cycles, respectively. Directly before analysis all samples were filtered through a folded filter, and to 5 ml of each sample 100 μl of I.S. solution was added.

Each sample was analyzed twice and the mean value of the corrected peak area ratios was calculated to determine the concentrations of the analytes. Samples whose content was higher than the upper limit of the working range, were adequately diluted with blank urine before analysis.

To ensure that there were no interferences of the assay with the co-administered hydrochlorothiazide, the specificity of the assay was reinvestigated by analysis of a urine sample spiked with hydrochlorothiazide.

3. Results and discussion

3.1. Assay conditions (CE–UV method)

Triamterene and its phase-II metabolite, the sulfate conjugate, show a maximum of light absorption at 353 nm, thus first of all UV detection was used for the development of a CE method with an automated instrument. Different conditions were applied using varying buffers, ionic strengths, pH ranges, buffer additives like organic solvents, SDS or cyclodextrines and instrumental conditions like different voltages and temperatures. The optimum conditions for the separation were obtained with a capillary zone electrophoresis (CZE) method using a simple phosphate buffer 75 m*M*, pH 6.0. The internal standard (RPh 3040b) was a synthetic triamterene derivative with a basic side chain, implying its movement before TA and no prolongation of the separation time. At pH 6.0, TA ($pK_a = 6.2$) and the I.S. were positively charged and migrated before the electroosmotic flow (EOF) with migration times of 3.3 and 2.6 min, respectively, whereas STA was negatively charged at its sulfate moiety and moved with a migration time of 6.4 min after the EOF. The total run time was 10 min. In Fig. 3A a typical electropherogram of blank urine spiked with TA, STA and I.S. is shown.

As there were no interferences with urine components, urine samples were injected directly without prior extraction steps.

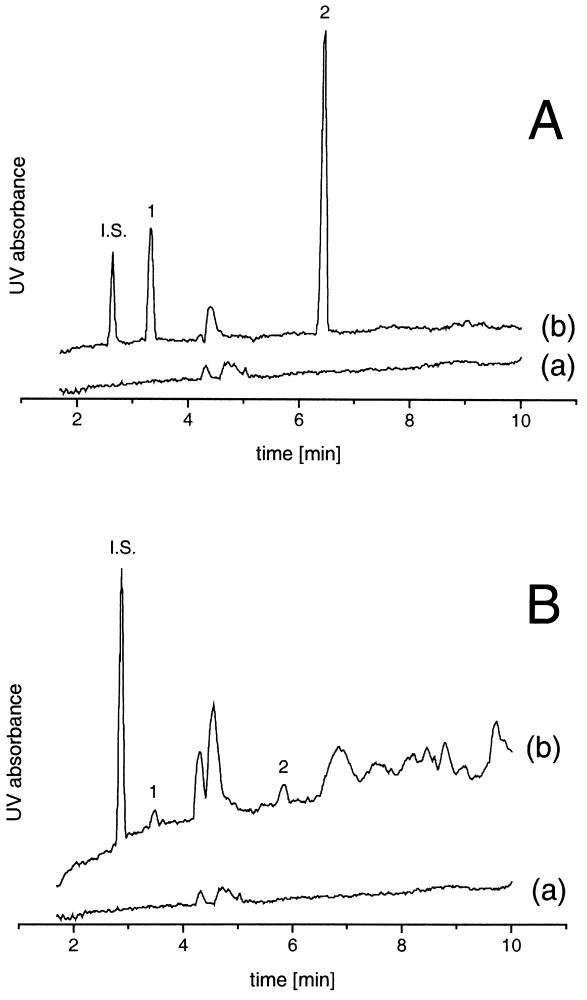

Fig. 3. Electropherograms of (A) blank urine (a) and a urine sample spiked with TA, STA and I.S. (b) and (B) blank urine (a) and a spiked urine sample (b) containing 400 ng/ml of TA and STA (LOQ) (I.S. = RPh 3040b, 1 = TA, 2 = STA); electrophoretic conditions: 34.5/44 cm capillary (I.D. = 75 μm), applied voltage: 18 kV, running buffer: 75 m*M* phosphate buffer pH 6.0, UV detection ($\lambda = 353$ nm).

3.2. Assay validation (CE–UV method)

The CE–UV method was validated in a concentration range from 0.4 to 10 μg/ml urine for TA and STA. It proved specific, linear ($r = 0.9994$–0.9999), precise (RSD 2.9–12.6%; 9.6–17.2% at the LOQ) and accurate (relative error: −2.4 to +6.5%; −7.2 to 3.1% at the LOQ) for the diuretic and its phase-II metabolite. The limit of quantification was 400 ng/ml (Fig. 3B). Validation data of the UV method in detail can be found in Ref. [10].

As urinary concentrations of TA and STA were mostly higher than 400 ng/ml within the 24 h collecting interval after oral intake of 50 mg triamterene, the assay was applicable for the quantification of TA and STA in human urine.

The limiting factors are certainly given by the available Spectra Phoresis instrument with a fixed capillary length and UV detection, resulting in a relatively high limit of quantification. The method is therefore not suitable for urinary concentrations below 400 ng/ml and it may not be transferred to plasma analysis which requires detection limits of 1 ng/ml TA. Because of a high fluorescence of TA and STA, a fluorescence detection method was

developed and seemed more appropriate for lower limits of detection.

3.3. Assay conditions (CE–LIF method)

Assay conditions have been optimized not only with regard to an improvement of the detection limit using LIF detection, but also in order to obtain shorter migration times and an increased sample throughput. Therefore, the capillary length was chosen as short as possible (20/27 cm). In addition, the I.D. of the capillary was changed from 75 to 50 μm. With regard to these changed capillary dimensions, other parameters of the assay also had to be adapted. Thus, not only injection time and separation voltage were decreased, but also duration of rinsing steps, which additionally resulted in shorter analysis times.

In addition, with quinine sulfate, a different I.S. was used, which required a slight increase in the pH value of the running buffer from 6.0 to 6.2 in order to achieve sufficient separation of I.S. and TA. A further increase in pH, however, led to an overlapping of the peaks of STA and an unidentified metabolite (Y, Fig. 6), that could also be detected in this assay.

In order to increase peak symmetry and avoid tailing effects, different organic bases like triethylamine, triethanolamine, trometamole and hexamethylene tetramine were investigated as additives for pH adjustment instead of sodium hydrogen phosphate, and triethanolamine was found to be the best for this purpose. Triethylamine and trometamole did not cause any improvement, and hexamethylene tetramine even decreased peak symmetry.

Under the conditions described, the migration times were 1.38 min for TA, 2.12 min for STA and 1.26 min for the I.S. with an EOF of about 1.7 min (Fig. 4A). Thus, separation was achieved in less than 2.5 min. Migration time stability was excellent with relative standard deviations of less than 1% (intra-day) and 2% (inter-day), respectively. At the corresponding migration times of the analytes, no interfering peaks from blank urine were observed.

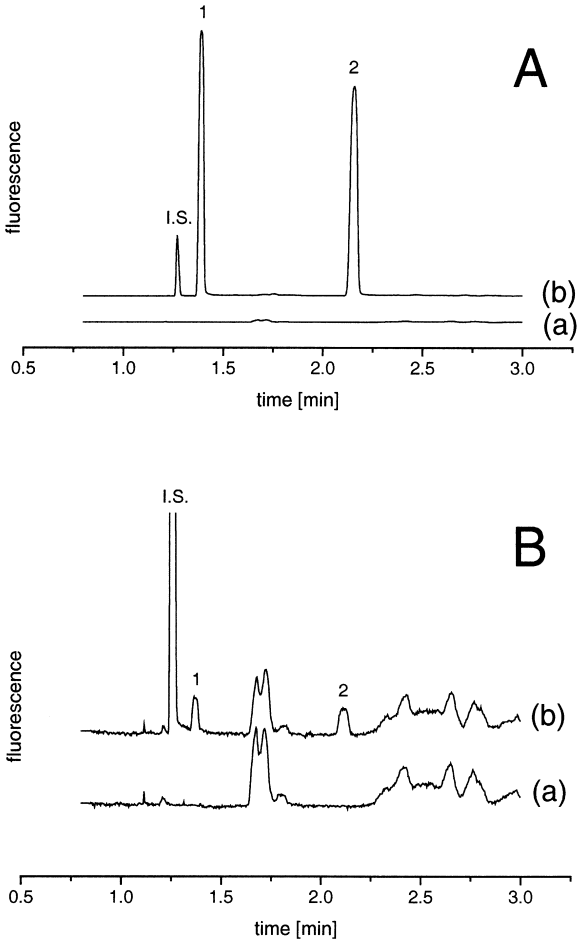

Fig. 4. Electropherograms of (A) blank urine (a) and a spiked urine sample (b) containing 10 μg/ml of TA and STA and (B) blank urine (a) and a spiked urine sample at the LOQ (b) with 50 ng/ml of each analyte (I.S. = quinine, 1 = TA, 2 = STA); electrophoretic conditions: 20/27 cm capillary (I.D. = 50 μm), applied voltage: 14 kV, running buffer: 100 m*M* phosphate/triethanolamine pH 6.2, LIF detection (λ_{ex} = 325 nm, λ_{em} = 450 nm).

3.4. Assay validation (CE–LIF method)

The method was calibrated in the concentration range from 0.05 to 10.0 μg/ml for TA and STA. The limit of quantification (LOQ) was 50 ng/ml for both analytes. To illustrate this, an electropherogram of a spiked urine sample (with blank) at the LOQ is presented in Fig. 4B. Calibration functions calculated by $1/x^2$-weighted linear regression were

$$y = 0.7508x - 0.0161 \text{ for TA}$$

and

$$y = 0.5424x - 0.0071 \text{ for STA.}$$

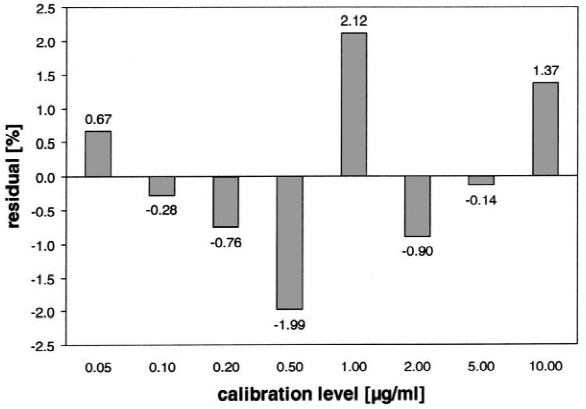

Fig. 5. Residuals of the calibration of the CE–LIF method.

Residuals of less than 2.2% for all calibration levels (Fig. 5) and correlation coefficients of $r = 0.99996$ for TA and $r = 0.99954$ for STA demonstrate excellent linearity of the assay. Comparing the slope values of the two calibration functions, it appears that the slope and consequently the sensitivity is higher for TA than for its metabolite. This observation corresponds to the different fluorescence intensities, which can be seen in the fluorescence spectra presented in Fig. 2.

Weighted linear regression was preferred to unweighted regression due to the heteroscedasticity of the data, i.e. that the absolute *y*-direction errors were not constant over the entire calibration range, but increased with increasing analyte concentration. In such cases, it is commonly recommended to use a weighted linear regression method, as it is more important for the calculated regression line to pass close to the points with small error bars than to those representing higher concentrations with the largest errors [20]. The most common procedure is that each calibration point is weighted according to the reciprocal of its variance, but it has been reported that $1/x$, $1/y$ and $1/x^2$ are valid approximations of this variance [23,24]. Thus, in order to obtain the best results for the accuracy, i.e. to minimize the deviations of the found concentrations from the nominal concentrations, we applied an $1/x^2$-weighting to the calibration data.

The results of intra- and inter-day reproducibility experiments are summarized in Tables 1 and 2. For the determination in biological fluids, precision (RSD) and accuracy (percentage deviation of the found concentration from the nominal concentration) should always be within ±15% except at the limit of quantification (LOQ), where they should not exceed

Table 1
Intra- and inter-day accuracy and precision data for the determination of triamterene (CE–LIF method)

	Nominal concentrations (μg/ml) of triamterene			
	0.05	0.5	2	10
Concentration found (arithmetic mean value) (μg/ml)				
Day 1 ($n=6$)	0.057	0.486	2.00	9.69
Day 2 ($n=6$)	0.052	0.474	2.10	9.30
Day 3 ($n=6$)	0.050	0.487	1.98	10.23
Inter-day ($n=18$)	0.053	0.483	2.03	9.74
Accuracy (arithmetic mean value) (%)				
Day 1 ($n=6$)	113.1	97.3	99.9	96.9
Day 2 ($n=6$)	103.3	94.9	105.2	93.0
Day 3 ($n=6$)	101.0	97.4	99.1	102.3
Inter-day ($n=18$)	105.8	96.5	101.4	97.4
Precision (arithmetic mean value) (%)				
Day 1 ($n=6$)	5.17	0.98	0.87	1.59
Day 2 ($n=6$)	3.16	0.58	2.10	0.82
Day 3 ($n=6$)	6.61	0.52	0.55	1.71
Inter-day ($n=18$)	9.84	1.45	3.05	4.27

Table 2
Intra- and inter-day accuracy and precision data for the determination of hydroxytriamterene sulfate (CE–LIF method)

	Nominal concentrations (μg/ml) of hydroxytriamterene sulfate			
	0.05	0.5	2	10
Concentration found (arithmetic mean value) (μg/ml)				
Day 1 ($n=6$)	0.049	0.475	1.89	9.40
Day 2 ($n=6$)	0.051	0.499	1.94	9.48
Day 3 ($n=6$)	0.047	0.462	1.87	9.86
Inter-day ($n=18$)	0.049	0.479	1.90	9.58
Accuracy (arithmetic mean value) (%)				
Day 1 ($n=6$)	99.0	94.9	94.3	94.0
Day 2 ($n=6$)	102.0	99.9	96.8	94.8
Day 3 ($n=6$)	94.9	92.5	93.4	98.6
Inter-day ($n=18$)	98.6	95.8	94.9	95.8
Precision (arithmetic mean value) (%)				
Day 1 ($n=6$)	6.64	0.88	1.29	1.53
Day 2 ($n=6$)	2.99	1.39	3.81	1.25
Day 3 ($n=6$)	6.24	1.02	0.82	1.33
Inter-day ($n=18$)	6.56	3.58	2.76	2.51

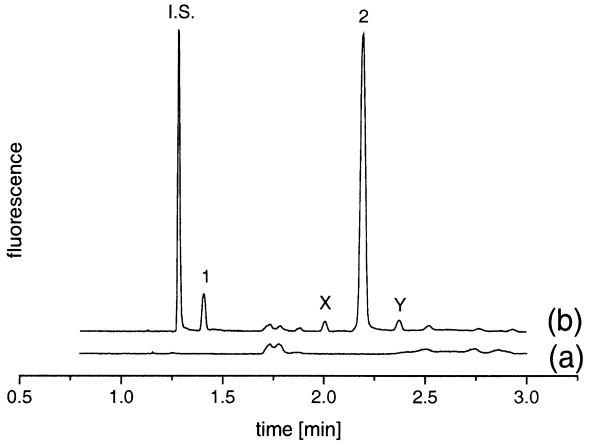

Fig. 6. Electropherograms of blank urine (a) and a human urine sample collected 14 h after oral administration of 50 mg triamterene (b) (I.S. = quinine, 1 = TA, 2 = STA, X and Y = unidentified metabolites); for electrophoretic conditions, see Fig. 4.

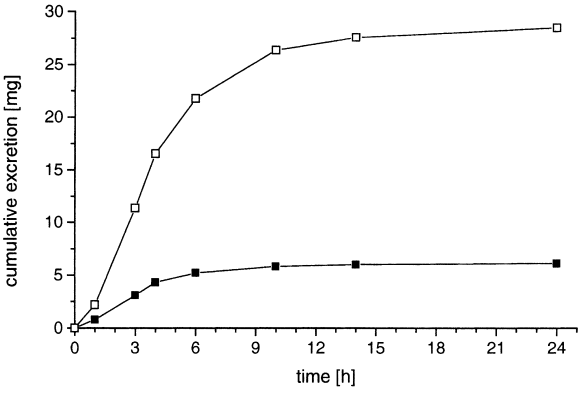

Fig. 7. Cumulative urinary excretion of TA and STA (calculated as TA) after oral administration of 50 mg triamterene (■ = TA; □ = STA).

20% [19]. In our experiments, precision was usually lower than 4% (10% at the LOQ) and the maximum deviation from the nominal concentration was 7.5% (13.1% at the LOQ, respectively). Thus, the method fulfills the requirements for bioassays and was suitable for our purpose.

3.5. In vivo analysis

The assay described in Section 2.3 was applied to urine samples collected from a human volunteer after oral administration of 50 mg triamterene (in combination with 25 mg hydrochlorothiazide as described in Section 2.6).

In a first series, all urine samples stored at room temperature after collection were analyzed. A typical electropherogram is shown in Fig. 6. Compared with blank urine, in addition to TA and STA, two further metabolites (named X and Y) were observed in the sample. They have not been identified, but as they migrate more slowly than the EOF, they should be negatively charged (possibly *N*-glucuronides). We can exclude the possibility that one of these peaks corresponds to the co-administered hydrochlorothiazide, because in a spiking experiment, hydrochlorothiazide could not be detected under the described conditions.

Urinary concentrations of TA and STA and concentration ratios (STA/TA) in the time intervals 0–1, 1–3, 3–4, 4–6, 6–10, 10–14 and 14–24 h after the intake of the drug are summarized in Table 3. As expected for human beings with normal hepatic functions, STA/TA ratios ranged between 3.8 and 10.4 [9]. Cumulative excretion of TA and STA is presented in Fig. 7. About 70% of the dose was excreted in urine within 24 h (6.1 mg in the form of the unchanged drug and 28.5 mg in the form of its main metabolite).

In a second step, the stability of TA and STA in

Table 3
Concentrations of TA and its main metabolite STA and concentration ratios (STA/TA) in human urine after oral administration of 50 mg triamterene (results obtained by CE–LIF method)

	Time interval						
	0–1 h	1–3 h	3–4 h	4–6 h	6–10 h	10–14 h	14–24 h
[TA] (μg/ml)	6.07	3.50	2.89	4.09	1.36	0.25	0.35
[STA] (μg/ml)	23.14	19.29	17.06	32.79	14.09	2.39	3.59
[STA]/[TA]	3.81	5.51	5.90	8.02	10.35	9.48	10.36

human urine under freeze–thaw conditions was evaluated. Therefore all urine samples were analyzed again after one and two freeze–thaw cycles, respectively. The results of this investigation are presented in Fig. 8. It could be demonstrated that storage of urine at −18 °C led to an enormous decrease in the concentration (partly more than 50%) of TA as well as of STA. This effect has been previously described as a consequence of formation of insoluble complexes between analytes and urinary matrix compounds [25]. In order to avoid this, it is very important either to analyze urine samples directly after collection, which entails the problem of low microbiological stability of urine at room temperature, or to follow a previously published suggestion to dilute urine samples with methanol (1:2) before storing at −18 °C [25].

4. Conclusion

Capillary electrophoresis proved to be a very simple and fast separation method for the determination of triamterene and its major metabolite hydroxytriamterene sulfate in human urine. Direct urine analysis without any sample preparation was feasible, and under optimized conditions a run time of less than 2.5 min could be achieved. Both assays presented in this publication, the CE–UV assay as well as the CE–LIF assay, showed excellent linearity, accuracy, precision and selectivity. Sensitivity, however, was rather low when using UV detection. Switching from UV to HeCd-LIF detection, sensitivity was improved resulting in an eightfold lower limit of detection of 50 ng/ml.

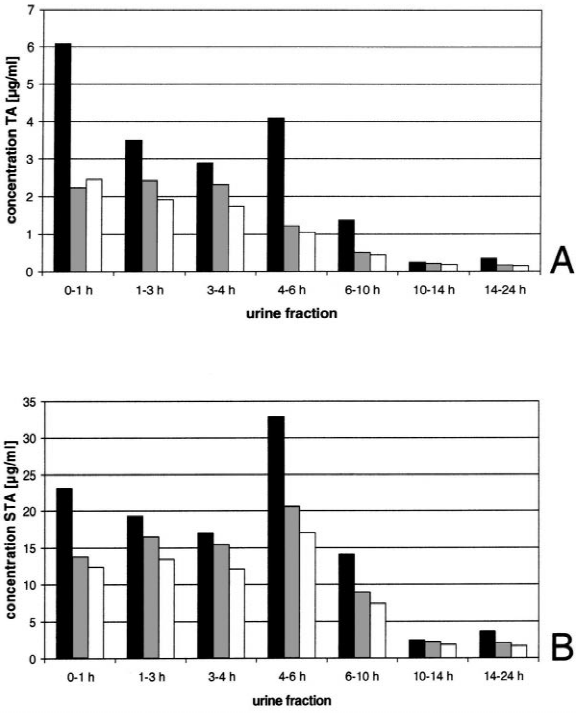

Fig. 8. Sample stability under freeze–thaw conditions: concentrations of TA (A) and STA (B) in human urine measured directly after collection (black columns) and after one (grey columns) and two (white columns) freeze–thaw cycles, respectively.

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References

[1] E.J. Thompson, E. Torres, S.J. Grosberg, M. Martinez-Maldonado, Clin. Pharmacol. Ther. 21 (1977) 392.
[2] H. Knauf, W. Möhrke, E. Mutschler, K.-D. Völger, Arzneim.-Forsch. 30 (1980) 1001.
[3] K.B. Hansen, A.D. Bender, Clin. Pharmacol. Ther. 8 (1967) 392.
[4] H. Knauf, E. Mutschler, Therapiewoche 35 (1985) 2214.
[5] H.J. Gilfrich, G. Kremer, W. Möhrke, E. Mutschler, K.-D. Völger, Eur. J. Clin. Pharmacol. 25 (1983) 237.
[6] K. Lehmann, Arzneim.-Forsch. 15 (1965) 812.
[7] B. Grebian, H.E. Geißler, E. Mutschler, Arzneim.-Forsch. 26 (1976) 2125.
[8] U. Gundert-Remy, D. von Kenne, E. Weber, H.E. Geißler, B. Grebian, E. Mutschler, Eur. J. Clin. Pharmacol. 16 (1979) 39.
[9] F. Sörgel, J. Hasegawa, E.T. Lin, R.L. Williams, Clin. Pharmacol. Ther. 38 (1985) 306.
[10] S. Kober, Triamteren als potentielles Leberfunktionsdiagnostikum—Neue pharmakokinetische Aspekte, Shaker, Aachen, 1999, ISBN 3-8265-4870-1.
[11] S. Kober, R. Lotz, M. Dahms, H. Spahn-Langguth, B. Scriba, W. Rösch, E. Bayer, E. Mutschler, (2002) in preparation.
[12] G. Leilich, H. Knauf, E. Mutschler, K.-D. Völger, Arzneim.-Forsch. 30 (1980) 949.
[13] H. Knauf, E. Mutschler, K.-D. Völger, U. Wais, Arzneim.-Forsch. 28 (1978) 1417.
[14] J.P. Villeneuve, F. Rocheleau, G. Raymond, Clin. Pharmacol. Ther. 35 (1984) 831.

[15] J. Jumppanen, H. Sirén, M.-L. Riekkola, J. Chromatogr. A 652 (1993) 441.
[16] E. Gonzalez, J.J. Laserna, Electrophoresis 15 (1994) 240.
[17] E. Gonzalez, A. Becerra, J.J. Laserna, J. Chromatogr. B 687 (1996) 145.
[18] E. Gonzalez, R. Montes, J.J. Laserna, Anal. Chim. Acta 282 (1993) 687.
[19] U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM): Guidance for Industry, Bioanalytical Method Validation, May 2001, Internet at http://www.fda.gov/cder/guidance/index.htm
[20] J.N. Miller, J.C. Miller, Statistics and Chemometrics for Analytical Chemistry, Prentice-Hall, Harlow, England, 2000, ISBN 0-130-22888-5.
[21] S.M. Gort, R. Hoogerbrugge, Chemometr. Intell. Lab. Syst. 28 (1995) 193.
[22] K. Baumann, H. Wätzig, J. Chromatogr. A 700 (1995) 9.
[23] H.T. Karnes, G. Shiu, V.P. Shah, Pharm. Res. 8 (1991) 421.
[24] H.G. Boxenbaum, S. Riegelman, R.M. Elashoff, J. Pharmacokinet. Biopharm. 2 (1974) 123.
[25] K.J. Swart, H. Botha, J. Chromatogr. 413 (1987) 315.