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Screening for diuretics in urine and blood serum by capillary zone electrophoresis

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ABSTRACT

Diuretics are therapeutic agents used to promote the excretion of bodily fluids and salts. They are also misused by some athletes to decrease body mass or to mask the use of anabolic steroids and other drugs. We have developed a method that screens for diuretics in urine and blood serum. Two successive runs were required because of the heterogeneity of this group of compounds. Screening for diuretics that contained sulphonamide and/or carboxylic groups was done at pH 10.6 with 3-(cyclohexylamino)-1-propanesulphonic acid (0.06 *M*) as buffer. Diuretics that contained primary, secondary or tertiary amine groups were investigated at pH 4.5 with an acetate (0.07 *M*)–betaine (0.5 *M*) buffer system. Hydrostatic injection mode for 5 s gave the best efficiency. Longer injection times were acceptable but efficiency was then somewhat reduced. Detection limits at the low femtomole level are achievable for most compounds with a UV–Vis detector operating at 220 and 215 nm. Temperature affected the separation, and 20°C proved best. All compounds were separated in less than 30 mins. A confirmation analysis of all compounds was done by GC–MS.

INTRODUCTION

Capillary zone electrophoresis (CZE) [1] is a powerful separation technique which is rapidly gaining popularity in a number of analytical fields, among them the determination of pharmaceuticals in biological fluids. The separation efficiency of CZE depends on the electrophoretic mobilities of the analytes, if there are no interactions between the analytes and the silanol groups of the capillary wall which may cause, *e.g.*, adsorption of the analytes on the wall. Silanols can work as cation exchangers [2]. All analytes are carried from the anode to the cathode by the electro-osmotic flow [3], whose magnitude is normally significantly greater than the electrophoretic mobilities of the analytes.

The adsorption of polyelectrolytes, such as proteins, can be prevented or diminished by several different methods, alone or in combination. For example, the pH can be adjusted so that the coulombic repulsion forces between the capillary wall and the adsorbing compound become strong enough to prevent adsorption [4]. The pH can be made so low that the negative charge of the capillary wall becomes weak [5], or so high that the polyelectrolytes become negatively charged [6]. The adsorption of polyelectrolytes can also be prevented by adding salt [7] or zwitterionic compounds [8] to the electrolyte solution. Yet a further method involves dynamic modification of the capillary wall by adding organic modifiers to the electrolyte solution [4,9–13]. An alternative approach is to add the modifier to the sample [14,15]. Adsorption can also be prevented by coating the capillary in a permanent fashion [16–22].

Diuretics are a heterogeneous group of compounds which promote excretion of bodily fluids and salts. They vary in mechanisms of operation and duration of their efficacy, and hence in their clinical use. Despite the total ban on them by the International Olympic Committee (IOC),

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diuretics are also misused by some athletes to decrease body weight or to mask the use of anabolic steroids. A number of screening methods have been published for diuretics, based on urine samples and HPLC [23,24]. Sample preparation is usually done by liquid–liquid extraction, but solid phase extraction has also been successfully employed [24,25]. No methods have been published in which blood serum is screened for diuretics.

We have developed a method for detecting diuretics in both urine and blood serum. Two consecutive runs at different pH values are required to separate all compounds.

EXPERIMENTAL

Apparatus

CZE was performed in a fused-silica column with 50 μm I.D. and 360 μm O.D. (Polymicro Technologies, Phoenix, AZ, USA). A detection window was placed 60 cm from the injection end of the column. The instrument was a Beckman 2000 P/ACE System 2000 capillary electrophoresis system with UV–VIS detector (Beckman Instruments, Fullerton, CA, USA). The system is temperature controlled as it employs liquid cooling. The pH of the solutions was adjusted using a Jenway 3030 pH meter and electrode (Jenway, Felsted, UK).

The GC–MS studies were carried out with a Hewlett-Packard Model 5989A single-stage quadrupole mass spectrometer and electron impact mode (EI, 70 eV) (Avondale, PA, USA). A Hewlett-Packard Model 5890A gas chromatograph, an HP 98785A monitor, an HP 6000 330S digital data storage system, an HP 9000 345 data system and an HP Laserjet III printer were used for analysis, data storage and reporting. The carrier gas (helium) was purified with a Supelco high-capacity carrier gas purifier (Supelco, Bellefonte, PA, USA). The compounds were separated on an HP ULTRA-I high-performance GC column (12.5 m × 0.20 mm I.D., 0.33 μm film thickness).

TABLE I

RELATIVE RETENTION TIMES, MAIN FRAGMENTS AND DETERMINATION LIMITS FOR THE METHYLATED DIURETICS

Compound and its group, (A or B)	Relative retention times	Main fragments	Determination limits (ng)	
			Serum	Urine
Diphenylamine (I.S.)	1.00	169		
Amiloride (B)	1.35	229, 204, 202, 171, 144, 116	38	50
Caffeine	1.46	194, 165, 137, 109, 98, 82, 55	14	16
Metyrapone (B)	1.51	226, 135, 120, 106, 92, 78	68	74
Acetazolamide (A)	1.78	264, 249, 142, 108, 83	36	42
Probenecid (A)	2.03	299, 270, 228, 199, 135, 76	20	19
Ethacrynic acid (A)	2.10	316, 281, 261, 243, 203, 109	21	34
Dichlorphenamide (A)	2.53	362, 360, 316, 255, 253, 108	53	51
Chlorothiazide (A)	2.81	355, 248, 220, 168, 140, 108	28	26
Benzthiazide (A)	2.95	353, 351, 259, 244, 56	39	35
Clopamide (A)	2.98	429, 358, 281, 207, 127, 111	36	26
Furosemide (A)	3.03	372, 357, 339, 96, 81, 53	20	13
Chlorthalidone (A)	3.13	363, 351, 255, 176	31	27
Bumetanide (A)	3.21	406, 363, 318, 254, 167, 77	11	19
Hydrochlorothiazide (A)	3.29	357, 355, 248, 220, 168, 140	21	31
Trichlormethiazide (A)	3.36	355, 341, 281, 207, 147, 73	73	98
Triamterene (B)	3.41	337, 323, 308, 294, 280, 265	58	57
Bendroflumethiazide (A)	3.75	386, 278, 145, 91	30	30

Materials

3-(Cyclohexylamino)-1-propanesulphonic acid (CAPS) and all diuretics (Table I) were purchased from Sigma (Poole, UK). Potassium dihydrogenphosphate, dipotassium hydrogenphosphate, morpholine, methanol, potassium hydroxide, hydrochloric acid, sodium acetate, potassium carbonate, diphenylamine and methyl iodide were purchased from Merck (Darmstadt, Germany), and betaine (trimethyl glycine) from Fluka (Buchs, Switzerland). Acetonitrile was purchased from Rathburn (Walkerburn, UK) and was of glass-distilled grade. All chemicals were used as received. Blood and urine were donated by the staff. Samples were taken with sterilized equipment and were frozen until used. Distilled water was further purified with a Water-I system from Gelman Sciences (Ann Arbor, MI, USA). All water needed for buffer or sample solutions was filtered through 0.45-μm membrane filters (Millipore, Molsheim, France). Solid phase extractions of urine and blood serum were carried out with Supelclean LC-18 tubes (3 ml) (Supelco). The calibration of the pH meter was done with standard buffer solutions purchased from Radiometer (Copenhagen, Denmark).

Methods

Separation of compounds by CZE

All injections were made on hydrostatic injection mode by applied pressure. Detection for group A compounds was at 220 nm because this gave the best average signal with minimum background noise. For the same reason group B compounds were detected at 215 nm. The capillary was rinsed with running buffer for 2 min prior to each run. The capillary was regenerated, as required, by treatment with 0.1 *M* potassium hydroxide (10 min) and water (15 min). Spiked sample solutions (water, blood serum and urine) were prepared by combining 10% (v/v) 0.1 *M* potassium hydroxide and 10% (v/v) methanol with 100 ppm of each diuretic and 80% (v/v) sample matrix. This gave 10 ppm as the concentration of each diuretic. The urine was diluted 1:10 (v/v) with water prior to spiking in order to simulate the real density of urine after the abuse of diuretic agents. The elution order of the compounds was determined via spiking of the samples.

Endogenous and the possible exogenous compounds were almost totally removed from the urine by solid-phase extraction (SPE). SPE columns were regenerated with 3 ml of methanol and water, prior to operation. The matrix compounds were removed by washing with 3 ml of water, and diuretics were then extracted with 3 ml of methanol. After this the methanol was evaporated and water, potassium hydroxide and methanol with 100 ppm of each diuretic were added to the sample to give the initial sample volume. This step can, of course, also be used to concentrate the sample if it is large enough. Proteins in blood serum were precipitated with methanol prior to the SPE procedure.

The pH of CAPS buffer solutions was adjusted with potassium hydroxide (0.1 *M*), and the pH of acetate and glycine buffer solutions with hydrochloric acid (0.1 *M*). The pH of phosphate buffers was adjusted by calculating the amounts of potassium dihydrogenphosphate and dipotassium hydrogenphosphate needed to give a solution of desired pH and ionic strength.

Identification of compounds by GC–MS

Solid-phase extraction. SPE columns were regenerated with methanol (3 ml) and distilled, deionized water (3 ml). The blank urine and serum samples each contained 100 ppm of each diuretic, were adsorbed on the C_{18} phase, washed with 1 ml of water, dried in a vacuum and eluted with 2 ml of water–methanol (10:90, v/v).

Preparation of methyl derivatives

After the SPE treatment the methanol extract was evaporated to dryness in a heating block under nitrogen. A 1.5-g amount of potassium carbonate, 200 μl of acetonitrile and 50 μl of methyl iodide were added to the dry residue. The resulting solution was incubated at 60°C for 60 min before the acetonitrile was evaporated under nitrogen and the residue was dissolved in 100 μl of methanol–toluene (4:96, v/v) mixture.

GC–MC procedure

GC–MS was carried out by injecting 1 μl of the derivatized solution. The temperature was programmed from 120 to 280°C at 15°C/min and from 280 to 310°C at 10°C/min. The temperatures of the injector, transfer line, source and quadrupole were 290, 290, 200 and 120°C, respectively. The carrier gas was helium (1.0 ml/min at 150°C). Injection was done by the solvent flush method (2 μl with the solvent plug) with methanol–toluene (4:96, v/v) as solvent. The full-scan mass spectra of the diuretics were scanned from 60 to 650 u at a rate of 1.09 ms/u.

RESULTS AND DISCUSSION

Diuretics are such a heterogeneous group of compounds that there is no pH within the operational range at which all of them carry a charge. Because of this, two consecutive runs at different pH values were required to screen all compounds. Compounds containing carboxylic (–COOH) and/or sulphonamide ($-SO_2NH_2$) groups (group A) were separated under basic conditions, at which all of them are anionic. Molecules containing primary, secondary or tertiary amine groups (group B) were separated at acidic pH as cations. All compounds are listed in Table I.

To find conditions giving a satisfactionary separation in reasonable time, we studied the effects of pH, ionic strength, organic modifier, temperature, running voltage and injection time on the separation.

The effect of pH was studied from pH 3.5 to 5.5 with acetate buffer, from 6.0 to 8.5 with phosphate buffer and from 9.0 to 11.2 with CAPS buffer. The pH was increased in steps of 0.5 units or less. The concentration of the buffer constituent was always 0.03 *M*. Best separations were achieved at pH 10.6 for group A and at pH 4.5 for group B. Caffeine, which is nearly always present in human urine and serum, was separated from the other compounds of interest. However, its detection at 215 and 220 nm was found to be difficult, especially at pH 4.5, when its presence only broadened the system peak. Its location was confirmed with detection at 260 nm.

The effect of concentration of the buffer was then studied at both pH values, from 0.01 to 0.08 *M*. The best separation at pH 10.6 was achieved with 0.06 *M* CAPS. All compounds from group A were separated in less than 20 min. Probenecid and ethacrynic acid were better resolved with 0.08 *M* CAPS, but the separation of caffeine, metyrapone, triamterene and amiloride from the system peak was worse than with 0.06 *M* CAPS. This last separation had to be maintained in order to achieve preliminary information about whether or not compounds from group B existed in the sample. The best separation at pH 4.5 (group B) was achieved with 0.07 *M* acetate. All three components were adequately separated in less than 6.5 min.

Organic modifier was added to the system in the hope of inhibiting the possible adsorption of the endogenous and exogenous compounds in urine and the residue of proteins in blood serum on the capillary wall. Morpholine was tested because it had been found to improve the separation and repeatability at pH 7.0–8.5. The pK_a value of morpholine is 8.5 [26] and over 99% of it is neutral at pH 10.6 so it cannot work as a masking agent for the silanol groups at such a high pH. Nevertheless, experiments were carried out at pH 10.6 to see if it might have some other effect. Addition of morpholine (0.01, 0.02 and 0.03 *M*) to 0.06 *M* CAPS buffer increased the time of analysis and resulted in overlapping peaks of chlorthalidone, ethacrynic acid and probenecid. At the highest concentration of morpholine the three compounds co-eluted as a single peak. However, the resolution of the group B compounds from the system peak was improved. Morpholine was not tested at pH 4.5 because we did not want to increase the analysis time excessively. Instead, the experiments were made with betaine. This zwitterionic compound is reported to be good at preventing adsorption on the capillary wall [27]. Addition of betaine to acetate buffer both improved the separation and gave better repeatability. This also yielded in faster time of analysis because the addition of betaine increased the time of analysis only a little, while it resulted in sharper peaks. Because of this we did not have to increase the concentration of acetate further to achieve these sharper peaks. This behaviour was best seen with

metyrapone. Also, the addition of betaine resulted in the absence of a negative peak prior to the caffeine peak. A combination of 0.07 *M* acetate and 0.5 *M* betaine was found to give a good and fast separation for the group B compounds.

Temperature is always a very important parameter in capillary electrophoresis because the viscosity (η) of the liquid is exponentially proportional to the absolute temperature (T), as can be seen from the equation [28]:

$$\eta = A \exp(E_\eta / RT)$$

where R is the universal gas coefficient, A is a constant and E_η is an effective activation energy for molecular displacement. The effect of temperature was studied from 20 to 30°C. Although the separation time was increased, the peaks were sharper at lower temperature. At pH 10.6 the elution order of trichlormethiazide and benzthiazide was reversed when the temperature was raised from 20 to 30°C. Some unknown components, probably generated by the breakdown of sample constituents when the sample was maintained at room temperature for few days while exposed to light, displayed similar behaviour. We do not yet fully understand this effect of temperature on elution order. Trichlormethiazide and benzthiazide co-eluted as one peak when the temperature was 28°C. The best of the investigated temperatures was 20°C. Lowering the temperature further was not studied because the operational temperature range of the P/ACE 2000 is ambient temperature ±5°C.

The running voltage was selected to yield fast times of analysis without causing a deterioration in the resolution. The effect of running voltage on the separation was studied from 10 to 30 kV, and 25 kV was found to be suitable for both runs.

The effect of injection time (hydrostatic injection with pressure) on the final peak width was studied from 1 to 30 s, with 5 s found to give the best result at pH 10.6 and 4.5. However, through sample stacking, much longer injection times could be exploited with only a slight loss of separation efficiency [29]. Without any off-line preconcentration the detection limit for hydrochlorothiazide at 220 nm was 0.1 ppm ($S/N = 3$) when the injection time was 30 s [29]. Very probably most of the other compounds can also be detected at the low femtomole range.

To summarize, suitable running conditions for group A were pH 10.6; CAPS 0.06 *M*; $T = 20$°C; voltage = 25 kV; hydrostatic injection for 5 s. The electropherograms for spiked urine and serum are reproduced in Fig. 1a and b. The samples were spiked with the diuretics so as to yield a final concentration of 10 ppm each. For group B, suitable running conditions were pH 4.5; acetate 0.07 *M*–betaine 0.5 *M*; $T = 20$°C; voltage = 25 kV; hydrostatic injection for 5 s. The electropherograms for spiked urine and serum at pH 4.5 are presented in Fig. 2. These samples were spiked only with group B compounds and caffeine (10 ppm) since it had been confirmed earlier that none of the compounds of group A elute before caffeine.

GC–MS IDENTIFICATION

The poor response of diuretics was attributed to the high boiling temperatures and decomposition of the compounds. Derivatization was carried out to facilitate the GC analysis. Three of the diuretics —amiloride, caffeine and metyrapone— did not react with methyl iodide. In addition, diphenylamine, the internal standard used in GC–MS studies, was not derivatized.

Total ion chromatograms for blood serum and urine are reproduced in Figs. 3 and 4, respectively. The extracted endogenous and exogenous compounds did not interfere with the identification of the methylated diuretics in either serum or urine samples owing to the good resolution of the components under optimized conditions.

The GC retention times increased as follows: diphenylamine < amiloride < caffeine < metyrapone < acetazolamide < probenecid < ethacrynic acid < dichlorphenamide < chlorothiazide < benzthiazide < clopamide < furosemide < chlorthalidone < bumetanide < hydrochlorothiazide < trichlormethiazide < triamterene < bendroflumethiazide. With the help of full-scan mass spectra a library was created for the derivatized diuretics. The main peaks in the total ion chromatograms of blank urine and

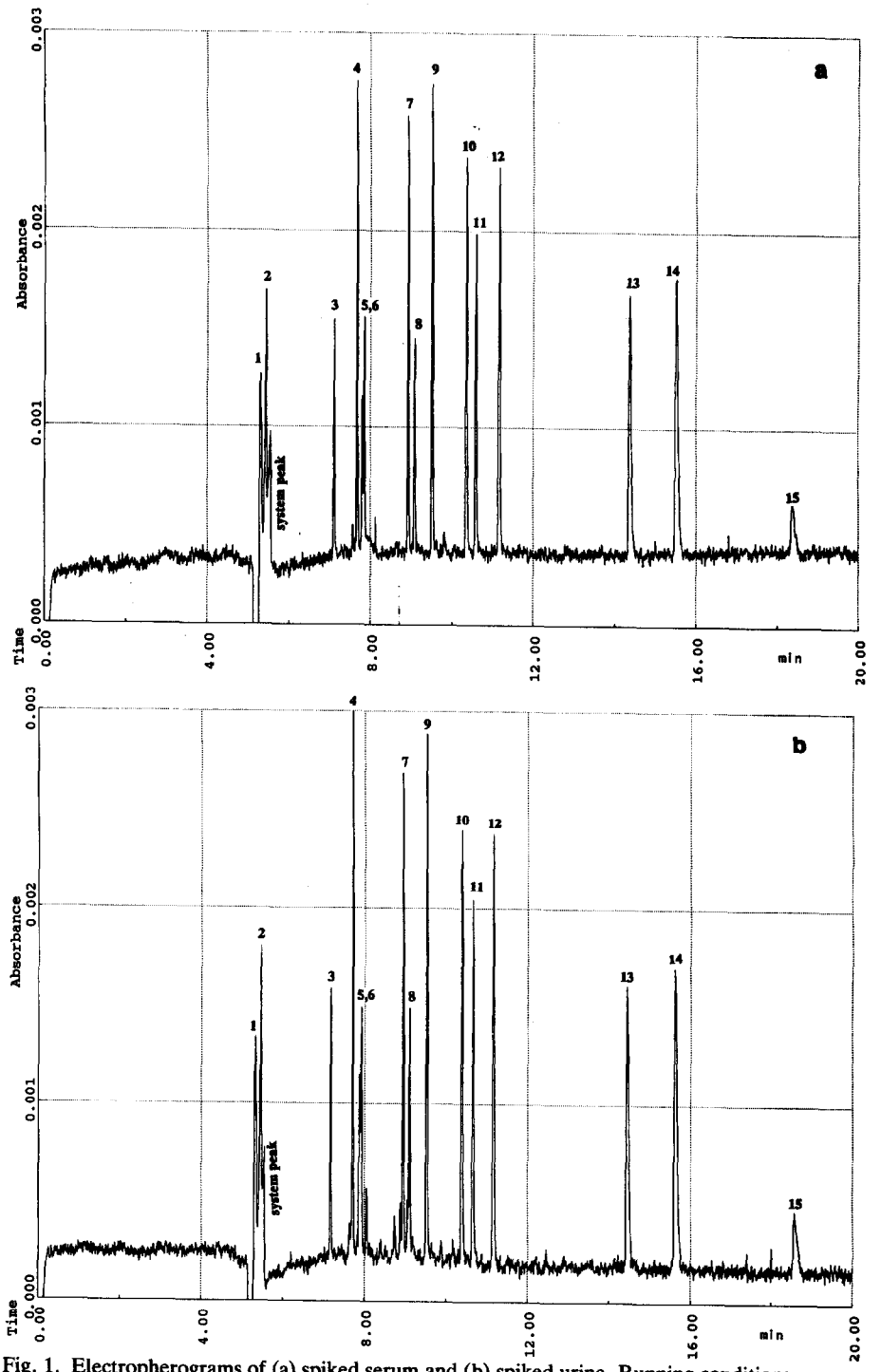

Fig. 1. Electropherograms of (a) spiked serum and (b) spiked urine. Running conditions were: uncoated silica capillary 67 cm (60 cm to the detector), 50 μm I.D., 360 μm O.D.; 220 nm; pH 10.6; CAPS 0.06 *M*; 20°C; 25 kV; hydrostatic injection by pressure 5 s. The spiked samples contained all of the diuretics (10 ppm each) (Table I). Elution order was: 1 = metyrapone and caffeine; 2 = triamterene and amiloride; 3 = clopamide; 4 = chlorthalidone; 5 = ethacrynic acid; 6 = probenecid; 7 = bumetadine; 8 = bendroflumethiazide; 9 = furosemide; 10 = trichlormethiazide; 11 = benzthiazide; 12 = hydrochlorothiazide; 13 = dichlorphenamide; 14 = chlorothiazide; and 15 = acetazolamide.

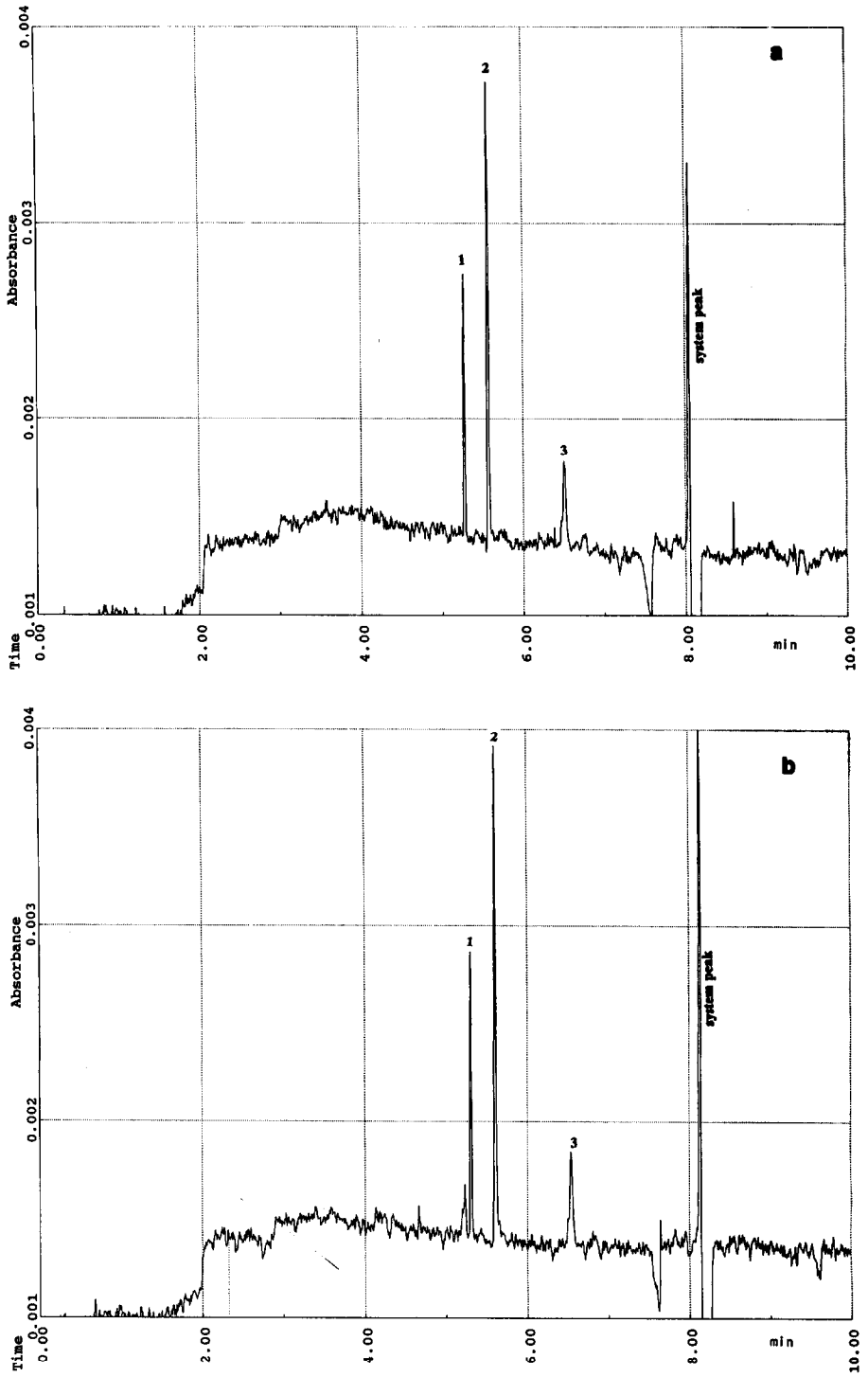

Fig. 2. Electropherograms of (a) spiked serum and (b) spiked urine. Running conditions were: uncoated silica capillary 67 cm (60 cm to the detector), 50 μm I.D., 360 μm O.D.; 215 nm; pH 4.5; acetate 0.07 *M*–betaine 0.5 *M*; 20°C; 25 kV; hydrostatic injection by pressure 5 s. The spiked samples contained the group B compounds and caffeine (10 ppm each) (Table I). The elution order was: 1 = amiloride; 2 = triamterene; 3 = metyrapone; and 4 = caffeine.

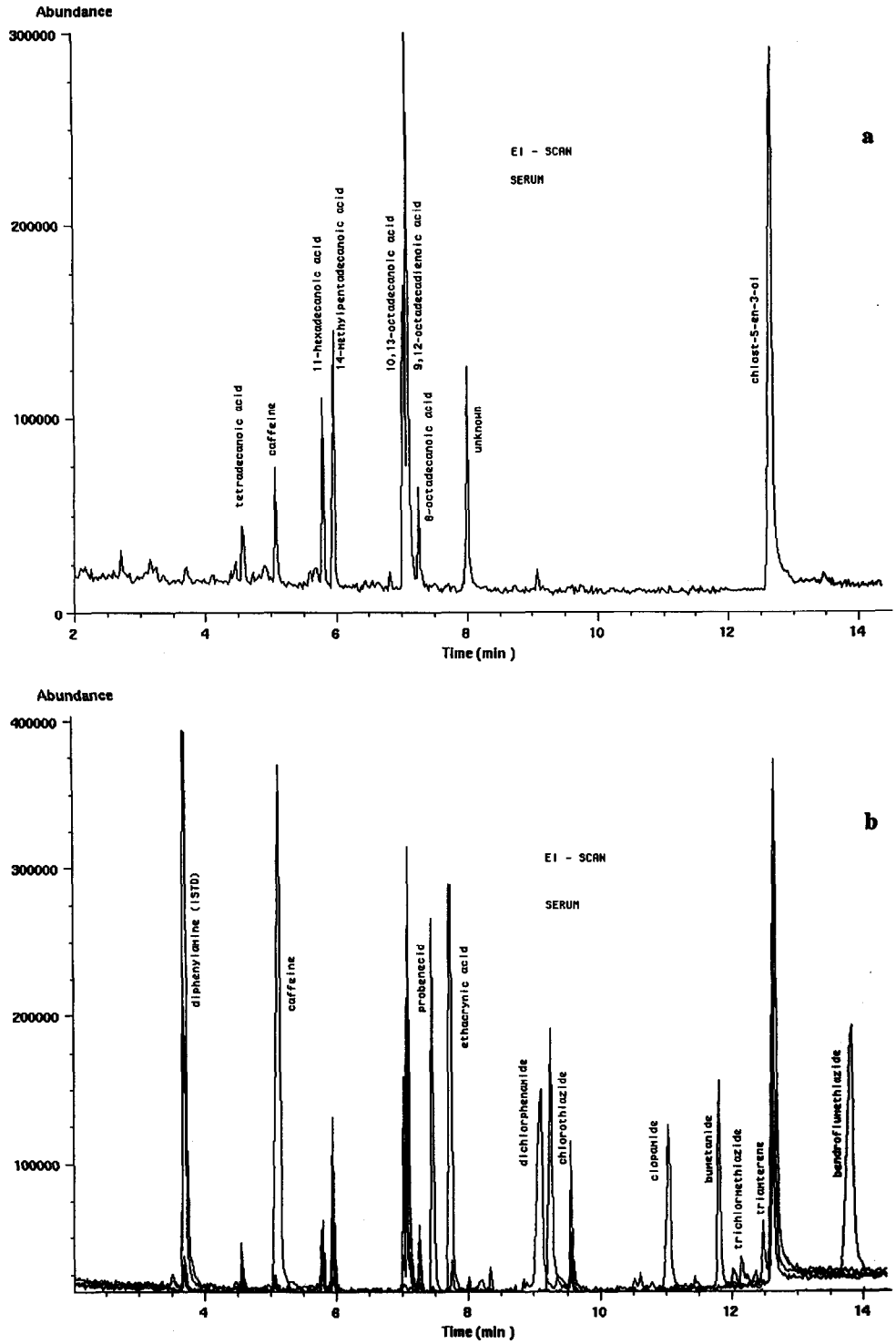

Fig. 3. Total ion chromatograms of (a) blank human serum and (b) spiked serum samples. Preparation by SPE. Running conditions are reported in the Experimental section.

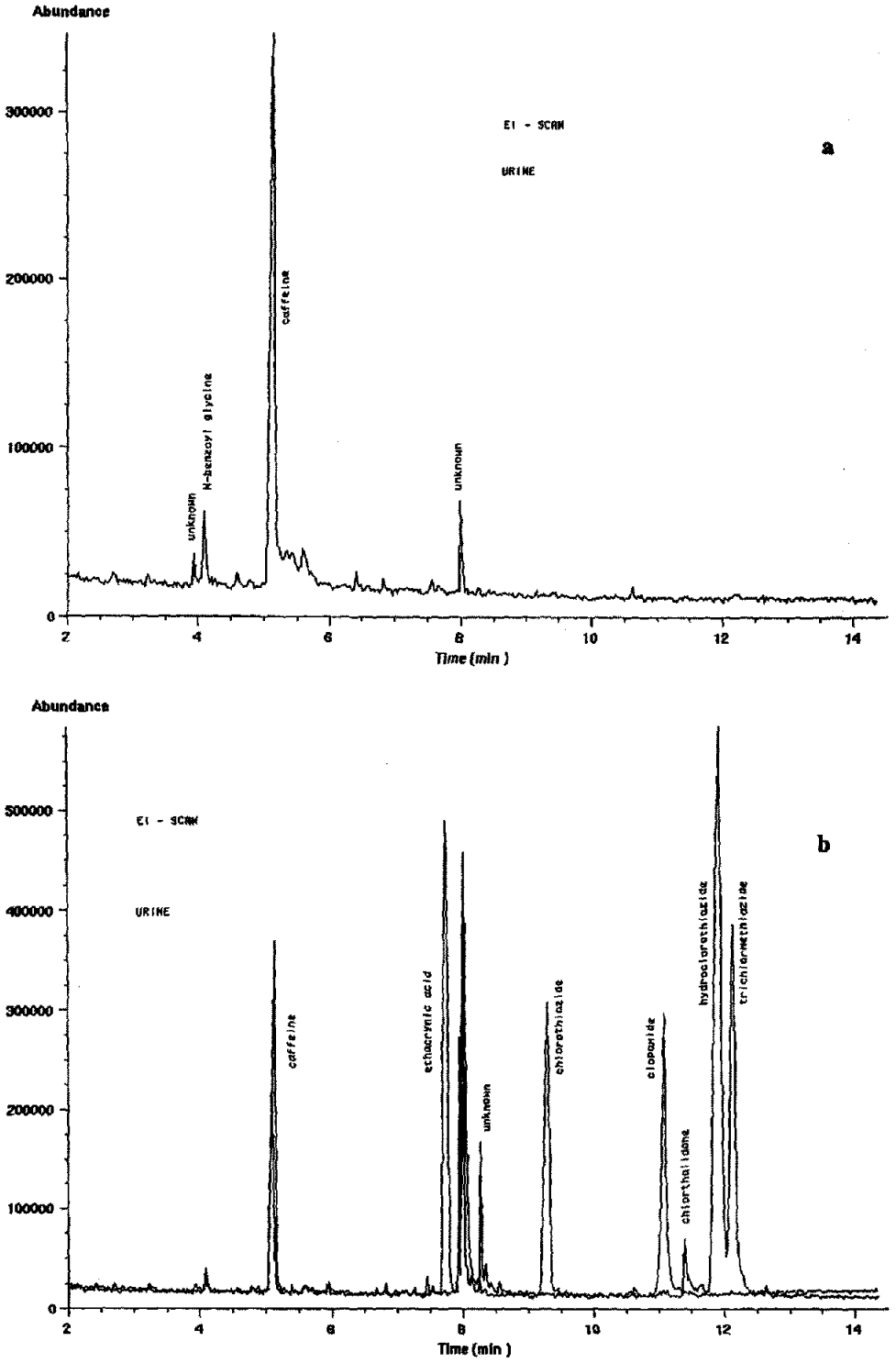

Fig. 4. Total ion chromatograms of (a) blank human urine containing caffeine and (b) spiked urine samples prepared by SPE. Running conditions are reported in the Experimental section.

serum were identified as methyl esters except cholest-5-en-3-ol, which was identified as the parent compound using the Wiley library. The relative retention times and determination limits are listed in Table I.

CONCLUSIONS

With our CZE method, urine and serum can be effectively screened for diuretics in less than 30 min. Although the heterogeneity of the diuretics made it impossible to separate them all in one run, all seventeen compounds that were investigated were well separated in two successive runs. CZE is a good alternative to HPLC since less sample is required and separation is as good or better and lower detection limits are achievable for most of the compounds.

Some problems may nevertheless arise when CZE is used to analyse biological fluids. If proteins are not completely removed from blood serum, they may destroy a good resolution of otherwise easily determined sample constituents by causing irregular electro-osmotic flow inside the capillary. Some of the endogenous compounds in urine behave in a similar way. In view of this it is advisable to design separation methods for pharmaceuticals that do not allow small amounts of disturbing compounds, such as proteins, left in the sample matrix to hinder the separation. Unfortunately, although the repeatability of the method may thus be improved, it may be at the cost of a slight decrease in separation efficiency. Matrix effects can be minimized by methods that have been reported for protein and peptide analyses. These methods can also be combined with each other, to solve even very difficult analytical problems.

Further research on the on-line concentration, electrolyte systems and the diffusion-related processes and their mathematical treatment is in progress.

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