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Micellar electrokinetic chromatography as a fast screening method for the determination of the doping agents furosemide and piretanide in urine

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Abstract

The possibility of using micellar electrokinetic chromatography for the screening of the loop diuretics piretanide and furosemide in urine was studied. A fast and simple method with good repeatability is described. The method was applied to urine samples collected from a healthy volunteer after oral administration of therapeutic doses of each compound. Positive identification in the urine matrix was possible through recording diode array spectra.

Keywords: Furosemide; Piretanide

1. Introduction

Despite the application of rigorous and sophisticated sampling and analytical techniques, the fast and accurate determination of some performance-enhancing drugs in sports still remains a problem. The loop diuretics furosemide and piretanide, frequently used in the treatment of congestive heart failure, renal disease, liver cirrhoses, hypertension and some types of oedema [1,2], are considered as doping agents in sports.

The principal action of these compounds, as for most diuretics, is in the renal excretion of salts and water resulting in an as much as 40 times increased urine flow when compared to normal excretion [3]. As a result, these compounds can be abused in sports since they promote rapid urine excretion hereby concealing the presence and the misuse of other performance enhancing drugs. Hence they are referred to as masking agents and the use of diuretics can reduce the urinary concentrations of doping agents by a factor four- to five-fold.

Diuretics have also been applied to achieve rapid weight loss especially in sports where competition is based on weight classes, and also to control water retention, one of the adverse effects of anabolic steroids [4]. In addition, the intake of diuretics, belonging to the carbonic anhydrase inhibitor group, results in an alkaline urine and excretion of basic doping substances may be reduced, resulting in a negative determination [5]. Moreover the abuse of diuretics can result in abnormally low concentrations

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of potassium in the blood which can contribute to variations from the normal rhythm of the heart resulting in sudden death. Therefore diuretics have been banned since 1986 by the Medical Commission of the International Olympic Committee [6].

Several analytical methods have been described for monitoring furosemide and piretanide in the biological matrices plasma and urine, in which they are excreted mainly unchanged. They include liquid chromatography with photometric [7–13], fluorimetric [14–18] and electrochemical [19] detection, gas chromatography [20,21], radiochemical techniques [22] and voltamperometric methods [23].

Micellar electrokinetic chromatography (MEKC), a mode of capillary electrophoresis, was initially conceived for the electrokinetic analysis of neutral compounds [24]. By adding micelle-forming surfactants to the background electrolyte, separations resembling reversed-phase liquid chromatography are achieved with the benefits of high efficiency and speed of analysis. MEKC, however, can equally well be applied to the analysis of ionic compounds [25,26]. Moreover, it was shown that it is possible to perform sample stacking in MEKC of ionic compounds [27]. Injection of larger sample volumes is thus feasible hereby drastically improving detectability.

In this contribution the possibility of using MEKC for screening and quantitative analysis of furosemide and piretanide in urine samples was evaluated. A simple sample preparation step, involving liquid–liquid extraction, followed by fast MEKC analysis is described. The analytical parameters have been optimized to separate the target compounds from the urine matrix solutes.

2. Experimental

2.1. Chemicals and stock solutions

4-Phenoxy-3 - (1-pyrrolidinyl) - 5-sulphamoylbenzoic acid (piretanide) and 4-chloro-2-furfurylamino-5-sulphamoylbenzoic acid (furosemide) were obtained from Hoechst Ibérica (Barcelona, Spain).

Sodium dodecyl sulphate (SDS) was purchased from Sigma (St. Louis, MO, USA). Sodium dihydrogenphosphate, phosphoric acid, boric acid and sodium tetraborate were of analytical grade from Merck (Darmstadt, Germany). Chloroform was obtained from Rathburn (Walkerburn, UK) and water and from a Milli-Q system (Millipore, MI, USA).

Stock solutions of furosemide and piretanide were prepared in methanol (Rathburn) and stored in the dark under refrigeration to avoid possible decomposition.

2.2. Apparatus and analytical conditions

MEKC was performed on a HP^{3D} capillary electrophoresis system equipped with a diode array detector (Hewlett-Packard). The fused-silica capillaries were 56 cm×50 μm I.D., 375 μm external diameter with the detection window at 48 cm. The samples were introduced hydrodynamically for 10 s at 50 mbar injection pressure and the applied voltage was 30 kV. Detection was at 230 nm. The capillary temperature was set at 25°C. After each run the column was rinsed for 2 min with the separation buffer. The separation buffer was refreshed after each triplicate analysis. A PC workstation with the HP^{3D} software was used for instrumental control and data handling. All areas were corrected for differences in migration time.

2.3. Sample preparation

A 5-ml volume of human urine sample was spiked with the appropriate volume of a 100 μg/ml stock solution of piretanide and furosemide in 20% methanol–water (v/v) to give samples spiked at the 0.1, 0.3, 0.5, 1.0 and 1.2 μg/ml level.

The sample pH was adjusted to 2.5 by addition of 120 μl of 1 *M* phosphoric acid. A 10-ml volume of chloroform was added and the mixture shaken by hand for 2 min. The organic phase was removed and concentrated by a gentle stream of nitrogen at 45°C. The residue was taken up in 100 μl of a solution consisting of 20 m*M* SDS in deionized water. Before injection, the sample was sonicated for 1 min. All analyses were carried out in triplicate. The same procedure was applied for the real urine sample obtained after oral administration.

2.4. Buffer preparation

The separation buffers were prepared by mixing appropriate volumes of stock solutions of boric acid 50 m*M*, sodium tetraborate 50 m*M* and sodium dodecyl sulphate (SDS) 0.5 *M* as the surfactant to give the desired pH and SDS concentrations. The pH of the buffers was controlled through the borax/boric acid ratio in the mixture. The final appropriate running buffer was prepared as follows: sodium tetraborate 20 m*M* and boric acid 20 m*M* both prepared in SDS 150 m*M* were mixed to give a pH 9 solution.

2.4.1. Extraction recovery

Recoveries were evaluated at the different spiked levels by comparing the corrected peak areas with those obtained for direct injection of equivalent concentrations in 20 m*M* SDS aqueous solutions.

2.4.2. Repeatability of the extraction procedure

To evaluate the repeatability of the extraction procedure, five extractions were performed using a real urine sample collected from a healthy female volunteer after oral administration of a single therapeutic dose. All urine produced between 2–8 h was collected and pooled.

3. Results and discussion

The primary objective of this work was the determination of acidic diuretics, more especially furosemide and piretanide (Fig. 1) in urine samples at the therapeutic doses. With the aim to develop a highly sensitive method requiring no sample preparation step, initial studies included the derivatization of the amino functional group with the fluorescent derivatizing reagents, 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-fluoride), fluorescein isothiocyanate isomer 1 (FITC), and dichlorotriazinylaminofluorescein (DTAF) for laser induced fluorescence detection at 488 nm. All attempts failed because the amino groups did not react, most probably because of their low nucleophilic character by the presence of other functional groups.

UV detection, on the other hand, is far less sensitive and an enrichment step is needed. Some of

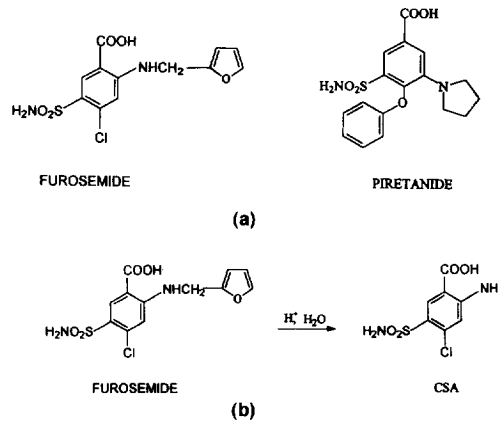

Fig. 1. (a) Molecular structures of the loop diuretics furosemide and piretanide. (b) Scheme of 4-chloro-5-sulphamoylanthranilic acid formation from furosemide by acid hydrolysis.

the urine matrix solutes will be extracted as well and the MEKC conditions had to be optimized to avoid overlap of the diuretics with urine matrix solutes.

In a recent article, the screening of some diuretics in urine and blood serum by capillary zone electrophoresis (CZE) was presented [28]. The authors described separations in spiked urine samples which were diluted ten times with water. This dilution reduced most of the endogenous compounds to concentration levels which were too low to interfere with the analyte peaks during the analytical run. Moreover, this does not simulate real urine samples after the abuse of diuretic agents as the authors suggested.

In fact, in the real case, the endogenous compounds are still present in very high concentrations in urine.

For MEKC optimization, both the pH and SDS concentration were varied between pH 7–9 and 80–150 m*M* respectively, while the borax/borate concentration was kept constant at 20 m*M*. The best separation of furosemide and piretanide from the urine matrix solutes was obtained at pH 9.0 and 150 m*M* surfactant concentration. The injected test mixtures were extracts from fresh urine samples (5 ml each) spiked with furosemide and piretanide at 100 ng/ml, 300 ng/ml, 500 ng/ml, 1 μg/ml and 1.2 μg/ml respectively.

Acidic extraction was performed using chloroform as extraction solvent as described in Section 2.3.

When the residue was redissolved in 100 μl of a 20 m*M* SDS solution, an enrichment factor of 50 was achieved. The calibration graphs obtained from the analysis under optimized conditions of the indicated concentrations were linear over the range tested (0.1–1.2 μg/ml) with the following equations:

Furosemide	peak area = 274.29*C* − 5.38	r^2 = 0.998
Piretanide	peak area = 269.15*C* + 1.78	r^2 = 0.998

where *C* is the concentration.

This is an indication that there is indeed no overlap between the two analytes and the urine matrix and, moreover, that the recovery is quite constant over the range investigated [(85±2)% for furosemide and (75±4)% for piretanide]. The limit of quantitation of the method, considered as the quantity of compound required for a signal-to-noise ratio of 5 is 25 ng/ml in urine. The sensitivity is sufficient for the determination of therapeutic doses but, if required, the detectability can be improved by using a larger sample volume instead of 5 ml or by utilizing extended light path capillaries instead of the straight capillaries [29]. Relative standard deviations obtained from triplicate analysis, for corrected migration times and areas, were under 5%.

The selection of the injection time was determined by measuring the peak heights at various injection times. From the injection times versus peak height data (Fig. 2) an optimal injection time of 10 s was selected. Peak-height saturation occurs when injection time is above 10 s.

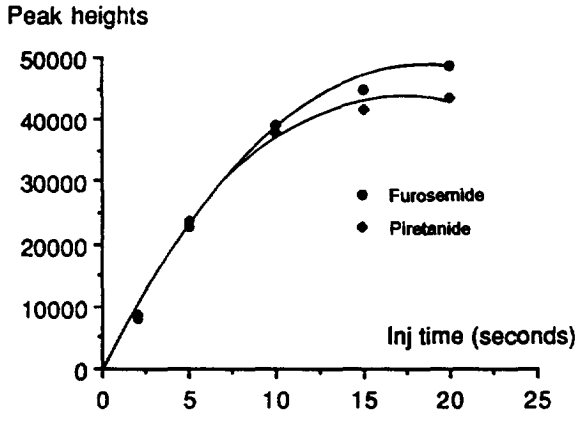

Fig. 2. Effect of the injection time on peak heights of furosemide and piretanide.

The method was applied to screen piretanide and furosemide in a urine sample collected from a healthy volunteer between 2–8 h after the oral administration of 40 mg furosemide (1 tablet of the pharmaceutical formulation Seguril) and 6 mg piretanide (1 tablet of the pharmaceutical formulation Perbilén). Fig. 3 shows the comparison of the electropherogram of the blank urine collected immediately before administration with an electropherogram of the excretion urine sample. Furosemide (F) was positively identified by co-injection and comparison of the diode array spectrum with a standard spectrum.

Diuretics belonging to thiazides are excreted in urine principally unchanged [13], but in the case of the loop diuretics a small amount can be metabolized in the liver. In the case of furosemide this results in the formation of defurfurylated derivative, 4-chloro-5-sulphamoylanthranilic acid (CSA), Fig. 1.

Both furosemide and its metabolite are rapidly excreted in urine by glomerular filtration and by secretion from the proximal tubule [30]. Due to this fact, it was suspected that one of the peaks appearing in the electropherogram close to furosemide (Fig. 3), could be this compound. To confirm this possibility, CSA was synthesized following the procedure proposed by P.C. Rowbotham et al. [31]: 0.35 g of furosemide in powder were weighted and then 10 ml glacial acetic acid and 2.5 ml water were added. The mixture was heated under reflux for 2 h. After cooling, the reaction mixture was poured onto ice (50 g) and the resultant precipitate was recrystallised from ethanol (m.p. 264–266°C). λ_{max} (0.1 *M* NaOH) 262 nm (ϵ = 11 100 mol cm^{-1} l) and 317 nm (ϵ = 3200 mol cm^{-1} l). Yield, 0.25 g (91%).

Standard solutions of CSA as well as blank and urine samples spiked with CSA were injected in the MEKC system. In all the cases it could be observed that the elution time for this compound is lower than for furosemide and its peak is overlapped by the endogenous urinary compounds eluting just before furosemide. Therefore the suspected peak can not be attributed to this metabolite.

Fig. 4 shows the electropherogram obtained from the analysis of a blank urine and a real urine sample that was collected between 2–8 h after the adminis-

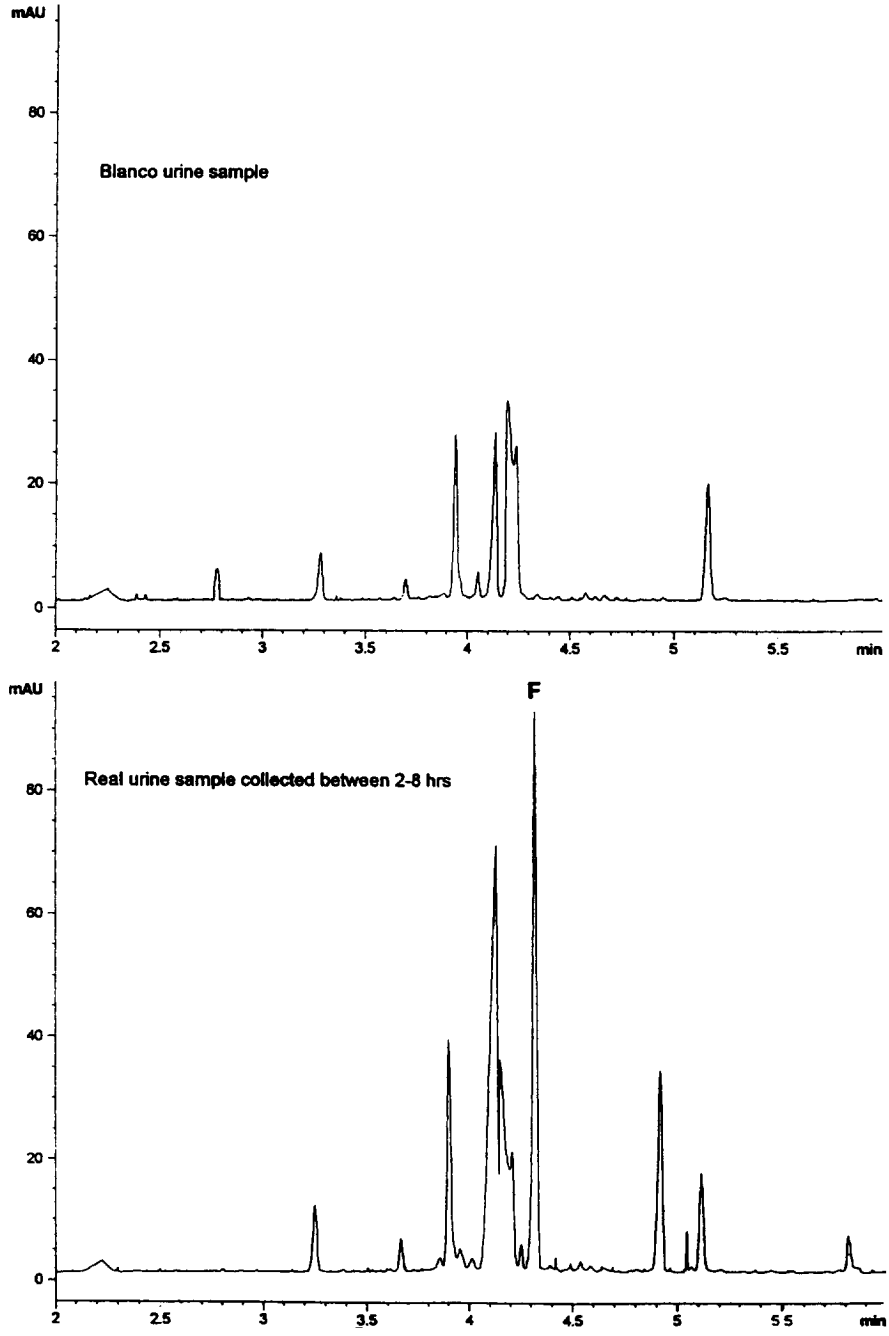

Fig. 3. Comparison of a blank urine sample with the real urine sample containing furosemide collected from a healthy volunteer at 2–8 h after a single dose of Seguril (furosemide 40 mg). Running buffer: 20 m*M* borax–borate, pH 9.0 and 150 m*M* SDS.

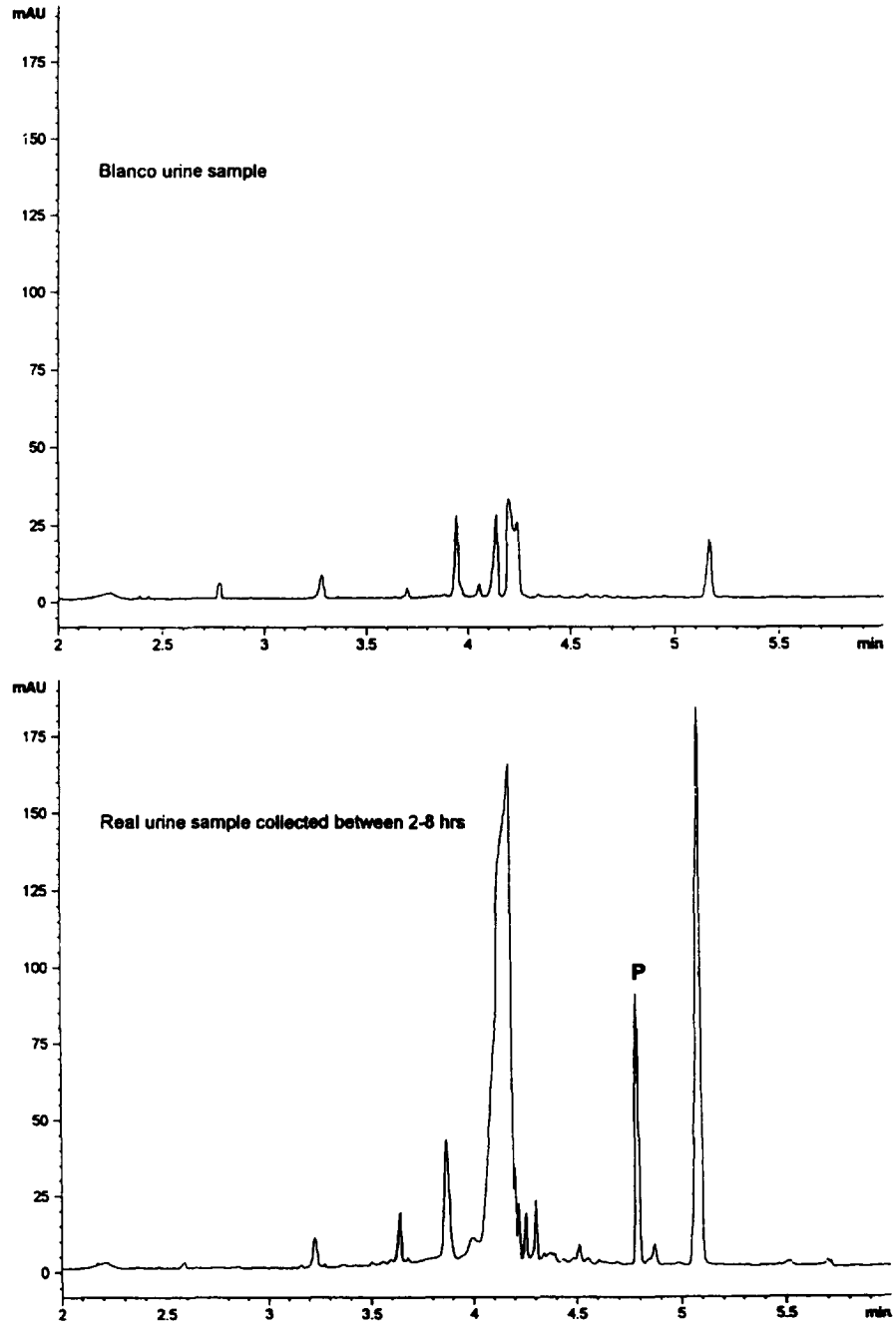

Fig. 4. Blank and urine sample collected from a healthy volunteer 2–8 h after the oral ingestion of a single dose of Perbilén (piretanide 6 mg). Analytical conditions as in Fig. 3.

Table 1
Reproducibility in terms of relative standard deviation (R.S.D.) of the method for within-day and between-day analysis of furosemide and piretanide for $n=5$

Diuretic	Within-day		Between-day	
	Migration time	Corrected area	Migration time	Corrected area
Furosemide	2.16%	3.60%	3.92%	4.20%
Piretanide	0.43%	0.40%	0.68%	0.62%

tration of piretanide 6 mg (one tablet of the pharmaceutical formulation Perbilén). Positive identification of piretanide (P) was possible. The nature and origin of the peak appearing at 5.2 min is unknown.

Repeatability of the extraction procedure and the analytical method was found to be good. As shown in Table 1, reproducibility of the corrected migration times and areas from five repeated extractions was satisfactory with a within-day R.S.D. in the range 0.4–3.6% and a between-day R.S.D. ranging from 0.62–4.20%.

4. Conclusion

This work demonstrates that micellar electrokinetic chromatography is a fast and cheap screening method for the determination of the loop diuretics furosemide and piretanide in biological samples and for doping control.

MEKC is a good alternative to other analytical techniques such as high-performance liquid chromatography (HPLC). The absence of packing material avoids problems with column contamination when working with complex matrices such as urine. The quantity of running buffer necessary to perform the analysis is very small compared to liquid chromatography where large volumes of mobile phase are consumed. This minimizes the problem of residual solvents and saves money.

Moreover, the fact that the small sample volume that is required is not consumed entirely during sampling, allows for several analyses to be made on each sample, thereby providing information on the reliability of the measurements.

Although the limits of detection are higher than those achieved by some HPLC methods, the sensitivity is enough for the usual concentration levels of these compounds in urine.

The potential of MEKC for the screening of other related diuretics can also be demonstrated. Some experiments were carried out in our laboratory on a standard mixture of four loop diuretics: furosemide, piretanide, torasemide and ethacrinic acid as well as one benzothiazide, clopamide. The five compounds could be resolved well in less than 7 min using the same conditions described for furosemide and piretanide. Urine spiked with 1 μg/ml of each of them gave good results using the same method, but conditions should be optimized to achieve the best results. The HPLC screening of these five compounds takes about 14 min to get the same degree of resolution. It shows that MEKC is a promising technique for screening purposes in drug analysis.

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