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Determination of piretanide and furosemide in pharmaceuticals and human urine by high-performance liquid chromatography with amperometric detection

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Abstract

A high-performance liquid chromatographic method with electrochemical detection (ED) has been developed for the determination of two diuretics: 4-phenoxy-3-(1-pyrrolidinyl)-5-sulfamoylbenzoic acid (piretanide) and 4-chloro-2-furfurylamino-5-sulfamoylbenzoic acid (furosemide). The chromatographic separation was performed on a μ Bondapak C₁₈ column with a mobile phase of acetonitrile–water (40:60) containing 5 mM KH₂PO₄/K₂HPO₄ and with a flow-rate of 1 ml/min (69 bar). The temperature was optimized at 30 ± 0.2°C. The amperometric detector equipped with a glassy carbon electrode was operated at +1200 mV versus Ag/AgCl in the direct current mode. The method was applied to the determination of these compounds in two concentration ranges (ppm and ppb), obtaining a reproducibility in terms of relative standard deviations lower than 1% for within-day and 4% for day-to-day and determination limits of 15 ppb for both compounds. Recoveries greater than 90% were obtained for spiked urine samples, using a liquid–liquid extraction method in the sample clean-up procedure. The LC–ED method was applied to commercially available pharmaceuticals (Seguril, furosemide 40 mg, and Perbilén, piretanide 6 mg) and urine samples obtained from healthy volunteers and hypertensive patients.

Keywords: Piretanide; Furosemide

1. Introduction

4-Phenoxy-3-(1-pyrrolidinyl)-5-sulphamoylbenzoic acid (piretanide) and 4-chloro-2-furfurylamino-5-sulphamoylbenzoic acid (furosemide) are two compounds belonging to the high ceiling or loop diuretics group. Their pharmacological activity produces an increase in electrolyte urin-

ary excretion and urine flow. They can increase it by a factor of 30 compared with normal excretion. These drugs are clinically used mainly in the treatment of renal disease, liver cirrhosos, oedema and hypertension [1–3].

The loop diuretics studied, piretanide and furosemide, have their principal site of action in the thick ascending limb of the loop of Henle [4]. Piretanide has been found to be 5–7 times more potent than furosemide, but only one-tenth as

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potent as bumetanide, another loop diuretic [5]. Piretanide has been reported to have a half-life of about 1 h [6], and its binding to plasmatic proteins is around 90% [7]. After the oral administration of 3–12 mg of piretanide, 45% of the dose is recovered in urine in 24 h [8]. Furosemide is absorbed rather rapidly after oral administration, and almost 60% is excreted in the urine, part of it in the form of glucuronide [9].

Diuretics are substances forbidden in sports since 1986. Because of the increase in urine flow, diuretics can be used to reduce body weight in order to qualify for a lower weight category. Because of their diluting effects, the intake of this kind of compounds could result in a suppression of other doping substances below their detection limits, so diuretics can be used as masking agents to decrease urinary excretion of other banned drugs [10]. For instance, the potent diuretic effect of furosemide can reduce the concentration of some respiratory stimulants, such as dimeflin below the detection limit of 10 ng/ml [11].

Numerous methods have been reported for the quantitative determination of furosemide, while the literature on diuretic piretanide is scarce. Among the methods used for the analysis of furosemide, colorimetric [12], spectrophotometric [13], fluorimetric [14,15] and electroanalytical methods [16,17] are included. An assay for the quantitation of piretanide in biological fluids has been reported by Spahn-Langguth et al. [5], using HPLC with fluorimetric detection. Piretanide analysis is also found in some papers dealing with screening of diuretics, which preferably use high-performance liquid chromatography (HPLC) with photometric detection [18], gas chromatography (GC)–mass spectrometry [19] and HPLC–mass spectrometry [20].

Chromatographic methods reported for the separation and determination of furosemide include HPLC with photometric [21–30] and fluorimetric detection [31–41], micellar liquid chromatography [42–45], thin layer chromatography [46], high-speed liquid chromatography [47] and gas chromatography–mass spectrometry [48–50] as the confirmation method.

The aim of this work is the application of amperometric detection joined to HPLC for the separation and determination of the loop diuretics piretanide and furosemide in pharmaceuticals and real urine samples obtained from healthy volunteers and hypertensive patients, after oral ingestion of the compounds.

2. Experimental

2.1. Apparatus and column

The HPLC system consisted of a Model 2150-LKB (Pharmacia, Barcelona, Spain) pump and a Rheodyne (Pharmacia) Model 7125 injector with a 20- μ l loop.

The electrochemical detector was a PAR Model 400 with a glassy carbon cell (EG&G Princeton Applied Research, Madrid, Spain). It was operated in the d.c. mode (applying a steady direct current potential to the LC cell and measuring the resulting current versus time) at +1200 mV vs. an Ag/AgCl electrode, with a 5-s low-pass filter time constant, and a current range between 0.2 and 100 nA. Chromatograms were recorded using an LKB Model 2221 integrator. The chart speed was 0.5 cm/min, and the attenuation was 8 mV FS (8 mV for a full scale deflection).

The column was a 30 cm \times 3.9 mm I.D., 10- μ m, 125 Å μ Bondapak C₁₈ (Waters). A μ Bondapak C₁₈ guard column module (Waters) was used to prevent column degradation. The column was kept at a constant temperature using a Waters TMC temperature control system.

The extracted urine samples were evaporated to dryness under a nitrogen stream using a Zymark TurboVap LV evaporator (Barcelona, Spain).

2.2. Reagents and solutions

Piretanide and furosemide were obtained from Hoechst Ibérica (Barcelona, Spain). Stock solutions were prepared by weighing 10 mg of each compound into a volumetric flask, and making up to 100 ml with acetonitrile. Solvents were

Lab-Scan HPLC grade, and the water used was obtained by the Milli-RO and Milli-Q Waters systems. All the reagents used were Merck Suprapur (Bilbao, Spain).

2.3. Chromatographic conditions

Acetonitrile–water (40:60) containing 5 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ was used as the mobile phase. The buffer served as the supporting electrolyte. This phase was filtered through a 0.45- μm membrane, and the air was removed from the phase by sparging with helium.

The $\mu\text{Bondapak C}_{18}$ column head-pressure was 69 bar at a flow-rate of 1.0 ml/min. The injection volume was 20 μl . The temperature was kept constant at $30.0 \pm 0.2^\circ\text{C}$.

2.4. Electrode maintenance

The electrode was cleaned electrochemically at the end of each working day by keeping it at -800 mV for 120 s and after that at $+1.6$ V for 15 min. This operation was carried out using a mobile phase of pure methanol at a flow-rate of 1.5 ml/min.

When the baseline was noisy or there was a baseline drift, the glassy carbon electrode was cleaned with a tissue with methanol to remove any possibly adsorbed compounds, and rinsed with deionized water.

2.5. Procedure for tablets

Tablets were reduced to a homogeneous fine powder in a mortar. A suitable amount of this powder was weighed, and methanol was added. After shaking for 20 min, the mixture was filtered, and the precipitate washed with the solvent. Solutions obtained after this procedure were made up with methanol. Aliquots of these concentrated solutions were diluted with the mobile phase and injected into the chromatographic system. Different amounts of initial solid sample were assayed in order to obtain a mean value.

2.6. Clean-up procedure for urine samples

The method used was a liquid–liquid extraction procedure based on the one proposed by Fullinaw et al. [51] with some modifications. Fullinaw's method uses 4 ml of urine, 8 ml of ethyl acetate, pH 4.1, and after extraction the organic layer was washed with Na_2HPO_4 0.1 mol/l (pH 7.5), while in our method, urine samples (2 ml) were acidified with 2 ml of KH_2PO_4 1 mol/l (pH 3.25), and 4 ml of ethyl acetate were added. The tubes were vortex-mixed for 20 min and centrifuged for 5 min at 734 g. The organic phase was separated and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was dissolved in 2 ml of mobile phase, and 20 μl of this solution were injected into the chromatographic system.

3. Results

Under static conditions, piretanide and furosemide are oxidized at the glassy carbon electrode, giving rise to voltammetric peaks [17] whose potential and current are pH-dependent. The impossibility of electrochemically determining a mixture of both diuretics because their voltammetric peaks overlap led to the development of a chromatographic system with amperometric detection.

3.1. Optimization of the chromatographic system

Hydrodynamic voltammetry of each compound was carried out to choose the optimum potential value (Fig. 1). For this purpose a potential scan in the range 500–1600 mV was made by applying different potential values to standard solutions of 5 $\mu\text{g}/\text{ml}$ of each of the compounds. An increase in the potential produced an increase in the peak current of both drugs in accordance with their oxidative behaviour under static conditions. Potential values higher than 1300 mV produced a lack of reproducibility in the peak current of piretanide, probably due to an adsorptive process, and an oxidative potential of $+1200$ mV was chosen as

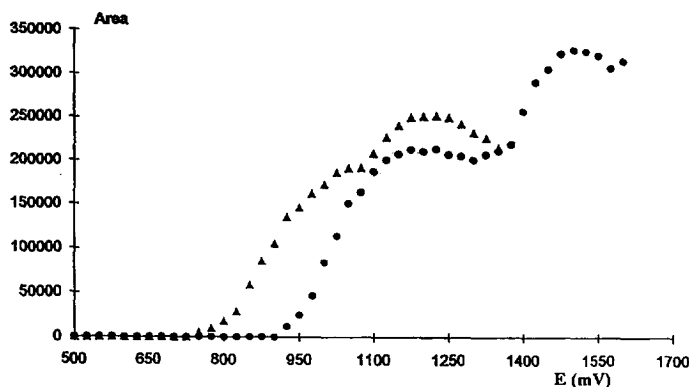


Fig. 1. Hydrodynamic voltammograms of (●) furosemide and (▲) piretanide. Amount of drug injected: 100 ng in acetonitrile–water (40:60) containing 5 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$.

the working potential, since it was the lowest potential able to produce a reproducible area with the maximum sensitivity for both diuretics without a high increase of the background current.

Because of the different $\text{p}K_a$ values of these diuretics (piretanide 1.32, 3.75, 10.23, and furosemide 1.64, 3.97, 9.40 [17]), their chromatographic behaviour was greatly affected by the pH of the mobile phase: an increase produced a large decrease in the retention times of piretanide and furosemide. The study of the influence of pH gave an optimum pH value of 4.25.

The supporting electrolyte used, which is necessary for amperometric detection, was the buffer $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$. Assays were also made with acetate buffer, but the peak areas were higher using phosphate buffer. The effect of the electrolyte concentration on the signal-to-noise ratio was studied. Concentrations ranging from 1 to 8 mmol/l were assayed, and an increase in the background signal was observed when the electrolyte concentration was higher than 5 mmol/l. The capacity factor values (k') increased slightly with the change of buffer concentration. A concentration of 5 mmol/l was chosen as optimal.

Different ratios of methanol–water and acetonitrile–water containing 5 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ were used as the mobile phase. The ratio 60:40 (water–acetonitrile) was chosen as the

most adequate one, since a good resolution in terms of k' (piretanide 3.45; furosemide 1.61) as well as a good elution time (lower than 15 min) was achieved for both diuretics (Fig. 2).

A study of the influence of the flow-rate on the

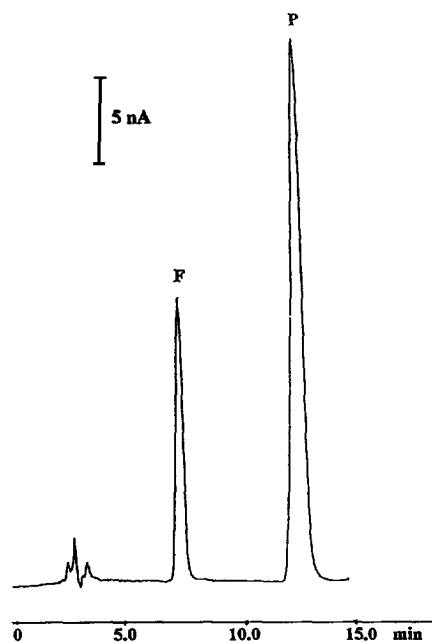


Fig. 2. Separation of furosemide (F) and piretanide (P), using amperometric detection and a $\mu\text{Bondapak C}_{18}$ column; mobile phase acetonitrile–water (40:60) containing 5 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$; flow-rate: 1 ml/min; oxidative potential: +1200 mV vs. $\text{Ag}/\text{AgCl}/\text{KCl}$. Amount of drug injected: 100 ng of furosemide and 100 ng of piretanide. Full current scale: 50 nA.

chromatographic separation was carried out. As expected, the peak area decreased with the increase of flow-rate, while the effect on k' was practically negligible. A value of 1 ml/min was chosen as optimal.

A variation in temperature from 26 to 55°C produced small variations on the peak area of chromatograms. A linear relationship between k' and $\log 1/T$ was obtained. A temperature of $30 \pm 0.2^\circ\text{C}$ was used throughout the study.

When optimal chromatographic conditions had been established, a quantitative method for the determination of both diuretics was developed in two concentration ranges: $\mu\text{g/ml}$ and ng/ml . In Table 1, the linear regression for both concentration levels, reproducibility studies and the experimental quantitation limit defined as the lowest concentration which gives rise to a signal able to be quantified for the integrator (signal-to-noise ratio ≥ 3) are collected.

3.2. Linearity, repeatability and accuracy

The relative standard deviation of the retention times was less than 1%, thus indicating high stability for the system. This deviation, higher for piretanide than for furosemide, was due to its longer retention and peak width producing slight variations in the computing integrator.

Linearity was noted at least from the limits of quantitation to 10 mg/ml , and the correlation was good (Table 1). Higher concentrations were not assayed because we considered that the range was wide enough for the applications commonly carried out.

The within-day and day-to-day repeatabilities were determined by injecting replicate samples ($n = 10$) of each diuretic at 300 ng/ml and 4 $\mu\text{g/ml}$ levels. It is expressed as the relative standard deviation (R.S.D.) listed in Table 1 and calculated by the formula $\% \text{R.S.D.} = (\text{standard deviation/mean of the peak areas}) \times 100$.

The accuracy of the method was determined by the analysis of six control urine samples spiked with 0.5 mg/ml of each diuretic. Acceptable accuracy, defined as mean (found concentration/actual concentration) $\times 100$, was demonstrated in the assay: furosemide 100.05% and piretanide 99.98%.

3.3. Analytical applications

Firstly, the method developed was applied to the determination of piretanide and furosemide in pharmaceutical formulations, obtaining values in accordance with those certified by the pharmaceutical company, with relative errors better than 1%, as shown in Table 2; in Fig. 3 chromato-

Table 1
Determination of piretanide and furosemide at two concentration ranges: $\mu\text{g/ml}$ and ng/ml

Diuretic	Furosemide	Piretanide
Retention time \pm S.D. (min)	7.7 ± 0.07	12.3 ± 0.11
Experimental quantitation limit (ng/ml)	15	15
Linear range	15–500 ng/ml 0.5–10 $\mu\text{g/ml}$	15–500 ng/ml 0.5–10 $\mu\text{g/ml}$
Slopes of calibration graphs	26764.1 ^a ($r^2 = 0.999$) 571.40 ^b ($r^2 = 0.999$)	40343.8 ^a ($r^2 = 0.999$) 1348.10 ^b ($r^2 = 0.999$)
Repeatability in the $\mu\text{g/ml}$ range R.S.D. (%)	0.85 ^c (within-day) 3.4 ^c (day-to-day)	0.39 ^c (within-day) 1.72 ^c (day-to-day)
Repeatability in the ng/ml range R.S.D. (%)	0.83 ^d (within-day) 3.5 ^d (day-to-day)	0.90 ^d (within-day) 3.43 ^d (day-to-day)

^a Area/concentration ($\mu\text{g/ml}$).

^b Area/concentration (ng/ml).

^c Ten determinations at the 4 $\mu\text{g/ml}$ level.

^d Ten determinations at the 300 ng/ml level.

Table 2
Determination of piretanide and furosemide in pharmaceutical formulations

Formulation	Component	Found (mg) ^a	Nominal (mg)
Perbilén	Piretanide	5.96 ± 0.01	6.00
Seguril	Furosemide	39.96 ± 0.01	40.00
Salidur	Furosemide + triamterene	77.39 ± 0.02	77.60

^a Amount ± ts/\sqrt{n} , where t is student parameter, s is the standard deviation and n is the number of replicates; $n = 3$ different samples and 3 replicates of each sample.

grams of both pharmaceutical formulations are observed.

Secondly, the method developed was applied to spiked urine samples of diuretics in order to calculate the percentages of recovery, using the clean-up procedure described above. The recoveries were estimated by measuring the peak areas of non-extracted standard solutions and comparing them with the peak areas obtained from extracted spiked urine samples of the same concentration. Quantitative recoveries calculated

from urine samples spiked with 2 µg/ml (piretanide) and 6 µg/ml (furosemide) were 92.7% ± 3.0% for furosemide and 94.4% ± 3.5% for piretanide.

The chromatographic method has been applied to the analysis of piretanide and furosemide in urine samples obtained from a healthy female volunteer after a single dose of Seguril (furosemide 40 mg) and Perbilén (piretanide 6 mg) and hypertensive patients administered Seguril and Perbilén. Urine was collected at

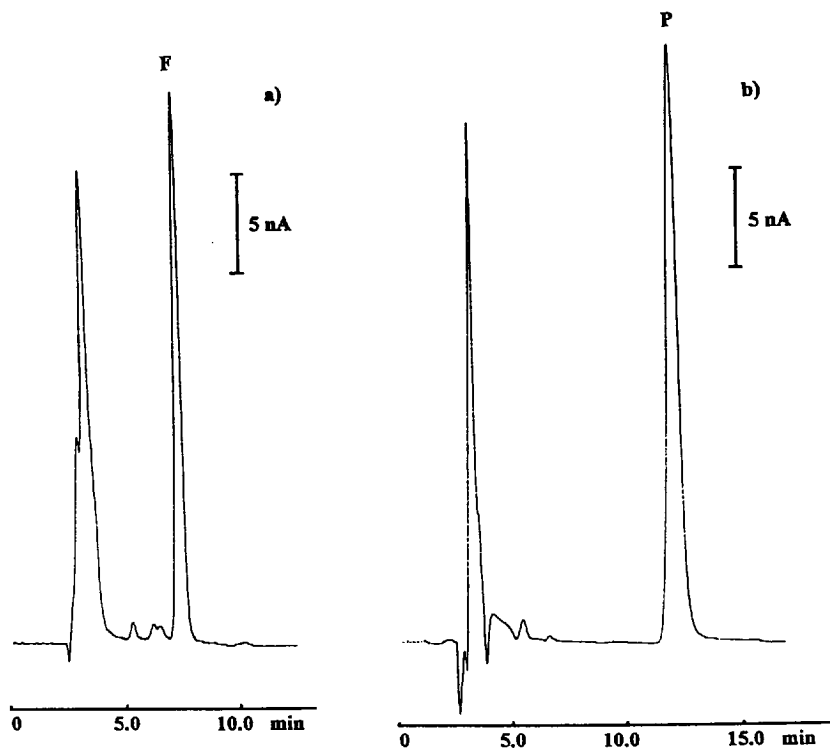


Fig. 3. Chromatograms of the pharmaceutical formulations: (a) diluted solution of Seguril (furosemide 40 mg) and (b) diluted solution of Perbilén (piretanide 6 mg).

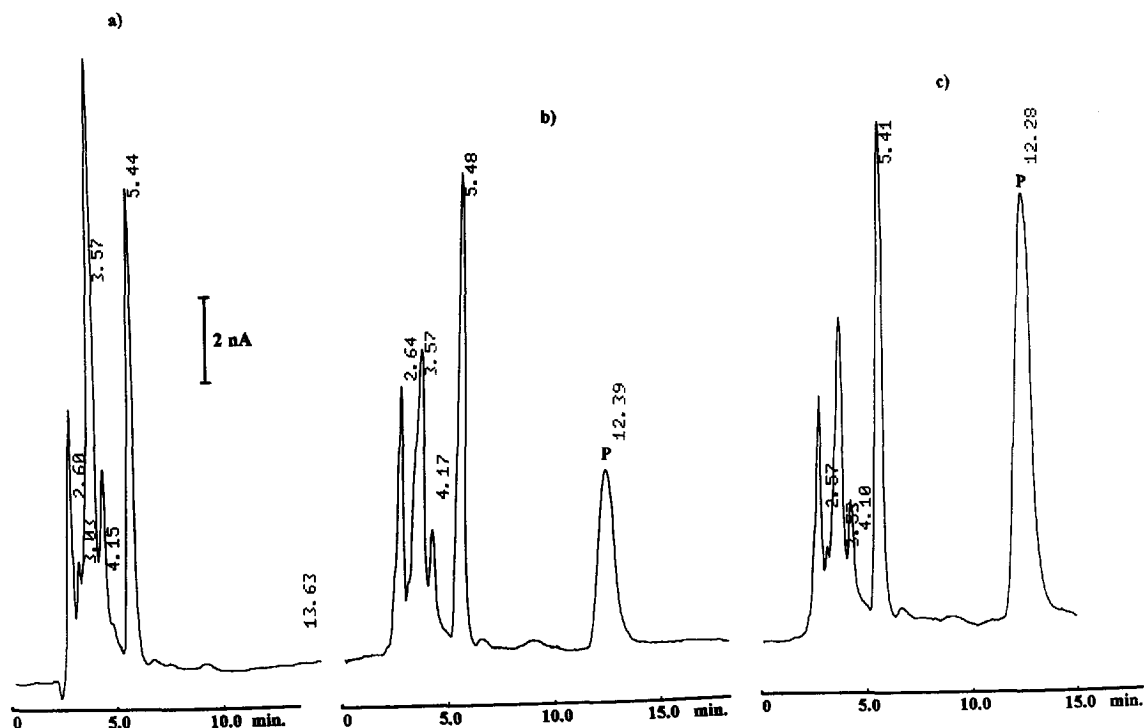


Fig. 4. Chromatograms obtained from an extract of (a) blank urine sample, (b) urine sample 8 h after oral administration of 6 mg of pirtanide (Perbilén 6 mg) to a healthy volunteer, and (c) urine sample after the addition of 5 μg of standard solution of pirtanide. Full current scale: 20 nA.

different time intervals for the quantitative determination of pirtanide and furosemide: 0–2 h, 2–8 h and 8–24 h. Urine samples were treated following the clean-up procedure described in Section 2, and 20- μl aliquots were injected into the chromatograph. Figs. 4 and 5 show the chromatograms corresponding to the urine samples and the one obtained after addition of 5 μg of standard solution of pirtanide and 5 μg of standard solution of furosemide. Both compounds were easily detected at the different times, and the concentrations found are collected in Table 3.

4. Discussion

High-performance liquid chromatography with amperometric detection has proved to be a

potent method for the separation, identification and determination of the two loop diuretics pirtanide and furosemide.

Although in this work a clean-up step prior to injection in the chromatographic system was used, pirtanide and furosemide can be determined directly in urine samples, since no interference of other electroactive compounds present in urine has been observed. This fact simplifies and shortens the analysis time, saving money and avoiding the lack of analyte during the extraction procedure. However, this shortens the column life, and in the case of low concentrations of the analytes (below 0.5 $\mu\text{g}/\text{ml}$), direct injection presents some troubles with interferences, making necessary some changes in the mobile-phase composition.

The clean-up procedure used achieves percentages of recovery greater than 90%, which is higher than those obtained by Fullinfa's meth-

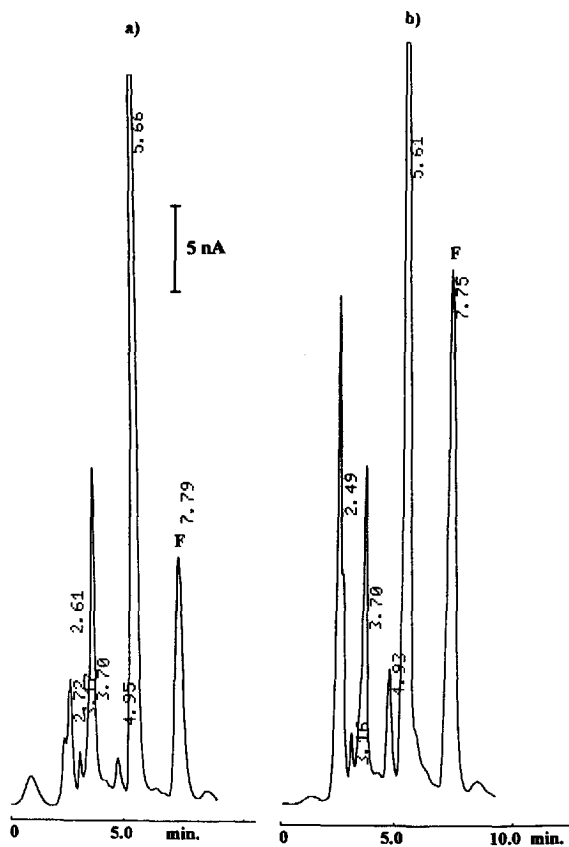


Fig. 5. Chromatograms obtained from an extract of (a) urine sample 2 h after oral administration of 40 mg of furosemide (Seguril 40 mg) to an hypertensive patient and (b) urine sample after the addition of 5 μg of standard solution of furosemide. Full current scale: 50 nA.

od [51] for furosemide and also using C_{18} , C_2 , CH, CN and PH cartridges described by Campins-Falcó et al. [28]. The procedure described has the

advantage that only a small volume of urine is required (2 ml).

The quantitation limit obtained for each of the diuretics was lower than that achieved by differential pulse and square wave voltammetry, and also the reproducibility in terms of relative standard deviations was better than the one obtained with static methods [17]. This determination limit achieved (0.3 ng) with amperometric detection is better than the ones reported by several authors using HPLC with photometric [24] and fluorimetric [37,39,40] detection and also micellar HPLC [45] and slightly inferior to the one reported by Farthing et al. [41] using HPLC with fluorimetric detection (0.12 ng).

The analytical characteristics of the developed method allow its application to pharmacokinetic studies of both diuretics. The results obtained for piretanide and furosemide at different time intervals after the ingestion of the pharmaceutical are in good agreement with those expected from the pharmacokinetics of piretanide and furosemide, with the peak concentration found at the time interval 2–8 h.

This work is part of a broad research project on the application of electroanalysis to the screening of diuretics which is first focused preferentially on loop diuretics. Assays done in our laboratory for the screening of nine diuretics – loop (piretanide, furosemide, torasemide, bumetanide), thiazidic (hydrochlorothiazide), benzothiazidic (clopamide, indapamide, xipamide) and potassium-sparing (triamterene) in pure solutions – showed the necessity of future research on alternative clean-up procedures, liq-

Table 3

Determination of piretanide and furosemide concentrations ($\mu\text{g}/\text{ml}$) in human urine samples collected at different time intervals after the ingestion of a single dose of Perbilén (piretanide 6 mg) and Seguril (furosemide 40 mg)

Pharmaceutical formulation	0–2 (h)	2–8 (h)	8–24 (h)
Perbilén	0.80 ^a 0.65 ^b	1.10 ^a 1.02 ^b	0.32 ^a 0.47 ^b
Seguril	1.50 ^a 1.10 ^b	4.10 ^a 3.87 ^b	0.99 ^a 1.05 ^b

^a Healthy volunteer.

^b Hypertensive patient.

uid–liquid and solid–liquid extraction methods in order to avoid the interferences found in the urine matrix which make it impossible to determine the diuretics eluting before 5 min.

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