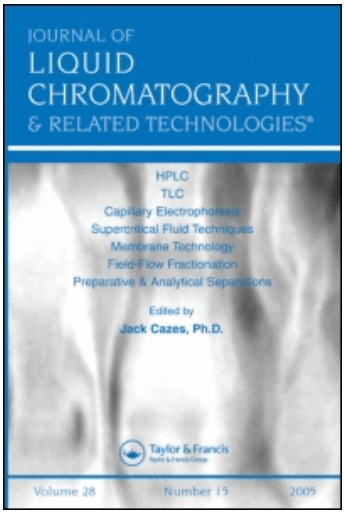

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M. Begoña Barroso[a]; Rosa M. Alonso[a]; Rosa M. Jiménez[a]
[a] Departamento de Química Analítica Facultad de Ciencias, Universidad del País Vasco/EHU, Bilbao, Spain

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SIMULTANEOUS DETERMINATION OF THE DIURETICS TRIAMTERENE AND FUROSEMIDE IN PHARMACEUTICAL FORMULATIONS AND URINE BY HPLC-EC

M. Begoña Barroso, Rosa M. Alonso, Rosa M. Jiménez

Departamento de Química Analítica
Facultad de Ciencias
Universidad del País Vasco/EHU
Apdo. 644, 48080 Bilbao, Spain.

ABSTRACT

A high performance liquid chromatographic method with amperometric detection has been developed for the simultaneous determination of two diuretics: triamterene and furosemide, using a μ-Bondapak C_{18} column. The mobile phase consisted of a mixture water:acetonitrile, 30:70, 5mM in KH_2PO_4/K_2HPO_4, pH 5.5 pumped at a flow rate of 1 mL/min. The amperometric detector, equipped with a glassy carbon electrode, was operated at +1300 mV.

The method was applied to the determination of both diuretics in the pharmaceutical formulation Salidur (triamterene 25 mg and furosemide-xanthinol 77.6 mg) and real urine samples obtained from a healthy volunteer after the ingestion of a single dose of Salidur. Using a simple liquid-liquid extraction procedure, good recovery and separation from interferences found in urine matrix is achieved and the simultaneous determination of both compounds is possible. Reproducibility is good with relative standard deviations lower than 1.5% intra-day

and 5% inter-day, and the method is accurate since errors obtained for tablets are lower than 1 %. The determination limits are 15 ng/mL for furosemide and 0.1 ng/mL for triamterene. Results obtained from urine samples are in accordance with those expected from pharmacokinetic studies.

INTRODUCTION

The combination of drugs with different mechanisms of action is a common therapeutic procedure for the treatment of hypertension, since sometimes the use of a single compound can lead to secondary effects in susceptible patients.

4-Chloro-2-furfurylamino-5-sulphamoylbenzoic acid (furosemide), an anthranilic acid derivative, is one of the most potent diuretics available. It inhibits the active reabsorption of chloride in the diluting segment of the loop of Henle, thus preventing the reabsorption of sodium which passively follows chloride.[1] This loop diuretic is commonly used for the treatment of renal disease, congestive heart failure and hypertension.[2] The continous administration of diuretics, especially furosemide, thiazides and related compounds, leads to a significant loss of potassium which can lead to hypokalemia and hypochloremic alkalosis.[3] Due to this fact, they must be given with a potassium replenisher or with potassium-sparing diuretics.[4] That is why pharmaceutical formulations containing more than one type of diuretic are used in the treatment of certain diseases.

6-Phenylpteridine-2,4,7-triamine (triamterene), is the most widely prescribed member of the group of potassium-sparing diuretics. It permits the reabsorption of potassium, but not sodium ions, in the tubules and it does not cause serious uric acid retention, which is a problem with some of the other diuretics.[5] Because of these properties, it is used mainly as an adjusment to thiazide and loop diuretics such as hydrochlorothiazide and furosemide in the treatment of edema and hypertension, to increase natriuresis and reduce kaliuresis.[6]

The pharmacokinetic and pharmacodynamics of the two diuretics are different. While furosemide is found unchanged in urine in a 60 %,[7] less than 10 % of the dose of triamterene is excreted unchanged.[8]

Some methods have been reported for the determination of triamterene in plasma and urine. Most of them use high performance liquid chromatography with photometric[9,10] and fluorimetric[11-13] detection. Gonzalez et al.[14] describe a method for the separation of three drugs, including triamterene, by capillary zone electrophoresis with pulsed-laser fluorescence detection, but only as an example to show the application of this type of detection and not focused on the determination of the compounds.

For furosemide determination, various methods are available, including mostly high performance liquid chromatographic separation followed by photometric[15-24] or fluorimetric[25-34] detection of the eluted fractions. Some simultaneous determination of furosemide with other compounds can be found in the literature. The determination of furosemide and metolazone in plasma and urine has been reported by Farthing et al.[35] and furosemide, phenylbutazone and oxyphenbutazone in plasma by Pinkerton et al.[19]

The aim of this work is the development of a simple and reliable HPLC-EC method for the simultaneous determination of triamterene and furosemide in pharmaceutical formulations and real urine samples obtained from a healthy volunteer after administration of tablets which contain both diuretics.

MATERIALS AND METHODS

Apparatus and Column

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

Electrochemical detection was carried out using an amperometric detector, PAR Model 400, equipped with a glassy carbon working electrode (EG&G Princeton Applied Research, Madrid, Spain). It was operated at +1300 mV vs a Ag/AgCl electrode, in the DC mode, 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, full scale.

The column was a μBondapak C_{18}, 30cm x 3.9mm I.D., 10-μm, 125 Å, (Waters Assoc.).

A precolumn module packed with μBondapak C_{18} (Waters Assoc.) was used to protect the column from degradation. All the measurements were made at room temperature.

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

Reagents and Chemicals

Triamterene was obtained from Aldrich (Milwaukee WI 53233, USA) and furosemide from Hoechst Ibérica (Barcelona, Spain). Solvents were Lab-Scan HPLC grade, and the water which was used was obtained from Milli-RO and Milli-Q Waters systems. All the reagents used, as well as the salts for the supporting electrolyte, were Merck Suprapur (Bilbao, Spain).

Standard Solutions

Stock solutions of furosemide and triamterene were prepared separately. 50 mg of furosemide were weighted into a volumetric flask, and made up to 100 mL with acetonitrile. The standard solution of triamterene was prepared by weighing 10 mg of the compound, making up to 100 mL with methanol and ultrasonicating for 5 min.

Chromatographic Conditions

The mobile phase was a mixture acetonitrile-water (30:70) containing 5 mM potassium dihydrogenphosphate/dipotassium hydrogenphosphate. The pH was adjusted to 5.5 and the buffer also served as the supporting electrolyte. This phase was filtered through Millipore membrane filters of 0.45 μm porosity, and the filtrate was degassed by bubbing helium through it.

The μBondapak C_{18} column head-pressure was 69 bar at a flow rate of 1.0 mL/min. The system was operated at room temperature and the injection volume was 20 μL.

Care of the Working Electrode

At the end of each working day, the electrode was cleaned electrochemically by keeping it at -800 mV for 2 min and, after that, at +1600 mV for 5 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 unstable or a drift could be observed, the glassy carbon electrode was removed from the cell compartment and rinsed with distilled water to remove any encrusted buffer salts from its surface. Once this was done, the electrode was cleaned with a tissue wet with methanol to remove possibly adsorbed compounds.

Procedure for Tablets

The pharmaceutical formulation analyzed in this work was presented as tablets. In order to perform the determinations, the tablets were pulverized and an adequate amount weighted out. Methanol was added and the sample shaken for 30 min. The mixture was then immersed for 5 min in an ultrasonic bath to facilitate dissolution, since triamterene is quite insoluble. Filtration of the dissolved sample through a filter paper, Albet 242, was necessary in order to avoid plugging the column. The residual solid was washed with more methanol to prevent the loss of analytes. The filtered solution was made up to 50 mL with methanol, and an aliquot of this one was diluted with mobile phase to provide the concentration required for the injection.

Different amounts of powder from each of the tablets were weighed and analyzed, and this operation was repeated for different tablets to calculate a mean value.

Procedure for Urine Samples

The clean-up procedure for urine samples was based on the method proposed for Ventura et al.[36]: 2 mL of urine were alkalinized with KOH 2M and adjusted to a pH value of 10.0. Then, 1.5 mg NaCl (s) were added. To this solution, 4 mL of ethyl acetate were added and it was shaken for 10 min. After that, the mixture was centrifugated at 2500 rpm for 5 min and the organic layer

was separated and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was dissolved in 2 mL of the mobile phase with ultrasonification, to facilitate dissolution, just before the analysis on the chromatograph, with a volume injected of 20 μL.

RESULTS AND DISCUSSION

Triamterene and furosemide are oxidized at a glassy carbon electrode.[37] Although triamterene gives rise to a unique voltammetric peak, furosemide produces two waves with a second peak potential which overlaps with the one of triamterene. This fact makes impossible the determination of triamterene in presence of furosemide, but this diuretic could easily be detected in presence of triamterene, using the first oxidation wave. The combined administration of these two diuretics in tablets, together with the impossibility of their simultaneous determination by voltammetric methods in static conditions, led us to the development of a chromatographic method to achieve their simultaneous determination.

Optimization of the Chromatographic System

In order to choose the optimum potential value to apply for the determination of these diuretics, a hydrodynamic voltammogram of each compound was obtained (Figure 1). An oxidative potential of 1300 mV was chosen as the working potential, since it was the lowest potential necessary to produce the oxidation of both diuretics, giving rise to reproducible peak areas. A higher potential would produce an increase of the background current together with a loss of reproducibility.

Because of the different acid-base characteristics and pK_a values of these drugs (triamterene 6.2[38] and furosemide 1.64, 3.97, 9.40[37]), their chromatographic behaviour was affected by the pH of the mobile phase. With an increase in the pH value of the mobile phase, an increase in the retention time of triamterene was observed, but it produced a large decrease in the retention time of furosemide. The study of the influence of pH gave an optimum value of 5.5, since the chromatograms of the two compounds were becoming too close when the pH was increased above this value while, with a lower pH, the retention time of furosemide was too large. This pH was also

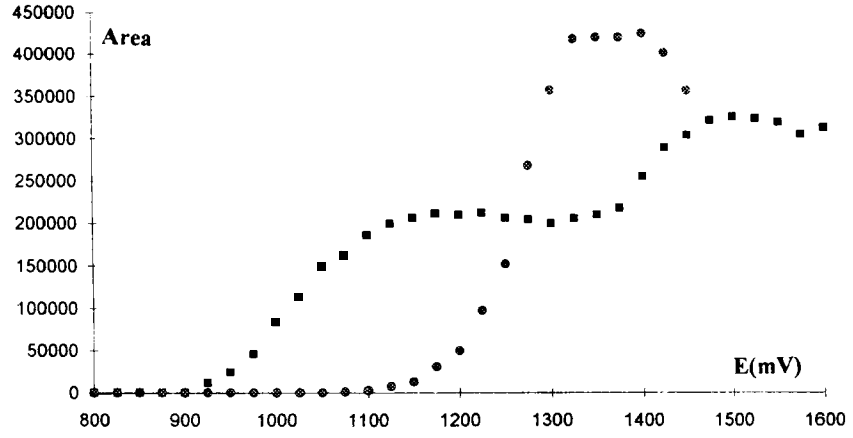

Figure 1. Hydrodynamic voltammograms of (●) triamterene and (■) furosemide. Amount of drug injected: triamterene 10 ng and furosemide 100ng in acetonitrile-water (30:70) containing 5mM KH_2PO_4/K_2HPO_4, pH 5.5 and flow rate 1 mL/min.

adequate for the separation of the electrooxidable interferences found in the urine matrix.

The supporting electrolyte used, which is necessary for the amperometric detection, was the buffer potassium hydrogen phosphate/dipotassium hydrogen phosphate. Other buffers, such as acetate were tried, but the area under the chromatographic peaks was higher with phosphate buffer. The best signal-to-noise ratio was achieved with an electrolyte concentration of 5 mM.

Different ratios of methanol-water and acetonitrile-water, containing 5 mM potassium dihydrogen phosphate/dipotassium hydrogen phosphate were tested as the mobile phase. Acetonitrile produced better defined chromatographic peaks than methanol, with less peak broadening. The ratio 60:40 (water-acetonitrile), used for the determination of furosemide in other work,[39] achieved a good resolution of both compounds (k'=1.56 for triamterene and k'=2.30 for furosemide), but it did not allow the separation from the urine interferences, so a ratio 70:30 water-acetonitrile had to be chosen as the best one, although the elution times were a little bit higher (k'= 1.81 for triamterene and 2.41 for furosemide); see Figure 2.

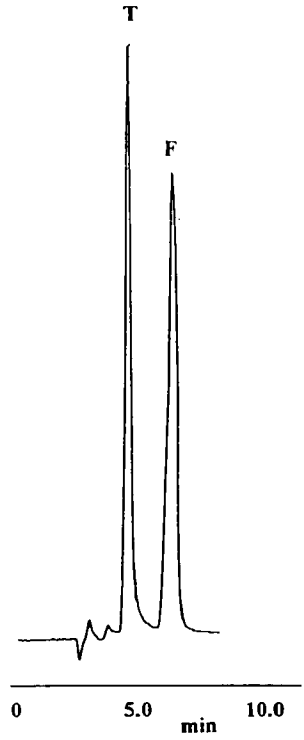

Figure 2. Separation of triamterene (T) and furosemide (F), using amperometric detection and a μBondapak C_{18} column; mobile phase acetonitrile-water (30:70) containing 5 mM KH_2PO_4/K_2HPO_4, pH 5.5 and flow rate 1mL/min; oxidation potential: 1.3 V vs Ag/AgCl/KCl. Amount of drug injected: triamterene 10 ng and furosemide 100 ng. Full scale current: 100 nA.

A study of the influence of the flow rate on the chromatographic separation was carried out. As was expected, the peak area decreased with an increase in flow rate, while the effect on k′ was practically negiglible. A value of 1mL/min was chosen as optimum. All the measurements were carried out at room temperature.

After establishing the optimum chromatographic conditions, a quantitative method for the simultaneous determination of both diuretics was developed. In Table 1 are collected the linear regresion for the calibration graphs, the intra-day and inter-day reproducibility studies made on n=10

Table 1

Determination of Triamterene and Furosemide.

Diuretic	Triamterene	Furosemide
Retention time (min)	5.01	6.70
Experimental determination limit (ng/mL)	0.1	15
Linear concentration range	Up to 5 mg/l	Up to 10 mg/l
Slopes of calibration graph	251454.9[a] (r^2=0.999)	26764.1[a] (r^2=0.999)
Reproducibility (%RSD)	1.4 (intra-day) 4.9 (inter-day)	0.85 (intra-day) 3.4 (inter-day)

[a] area/concentration (ppm)

solutions, as well as the experimental quantitation limit, defined as the minimum concentration which gives rise to a signal able to be quantified for the integrator.

Analytical Applications

In a first step, the method developed was applied to the determination of triamterene and furosemide in pharmaceutical formulations (Salidur: triamterene 25 mg and furosemide-xanthinol 77.6 mg) obtaining values in accordance with those certified, with relative errors lower than 1%. The results, collected in Table 2, show that accuracy is good for both diuretics. No interferences were noticed from the adjuncts used in the formulation with the present method, as can be observed in Figure 3.

Secondly, the method developed was applied to spiked urine samples of these diuretics in order to calculate the percentages of recovery, using the clean-up procedure described above. Quantitative recoveries, calculated from

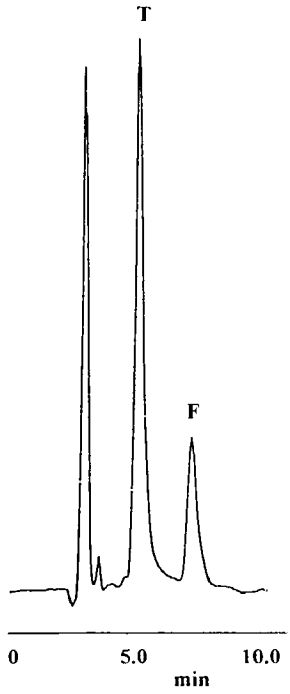

Figure 3. Chromatogram corresponding to a diluted solution of a tablet of Salidur (Furosemide-Xanthinol 77.6 mg and triamterene 25 mg). Full scale current: 100 nA. The same chromatographic conditions as in Figure 2.

Table 2

Determination of Triamterene and Furosemide in Pharmaceutical Formulations.

Formulation	Component	Found (μg)[a]	Nominal (μg)
Salidur	Triamterene	24.82 ± 0.91	25.00
Salidur	Furosemide-Xanthinol	77.02 ± 0.86	77.60

[a] amount ± ts/√n. n=3 different tablets and 3 replicates of each tablet.

urine samples spiked with 1μg/mL (triamterene) and 3 μg/mL (furosemide) were (98.7 ± 3.0)% for triamterene and (44.4 ± 3.5)% for furosemide. The high recovery for triamterene is in accordance with its basic character, which allows a good extraction from urine, while the acid characteristics of furosemide make it difficult to extract. This liquid-liquid extraction method, with these recoveries, allows the simultaneous determination of both compounds, given the different concentrations in which triamterene and furosemide are found.

The chromatographic method has been applied to the analysis of triamterene and furosemide in real urine samples obtained from a healthy female volunteer after a single dose of Salidur. Urine was collected at different time intervals for the quantitative determination of triamterene and furosemide: 0-2 hours, 2-8 hours and 8-24 hours. Following the clean-up procedure described in the experimental section, both compounds were easily detected at the different times. In Table 3 are collected the concentrations of unchanged drug found in urine (9.2 % for triamterene and 9.16 % for furosemide). Taking into account some reported works, triamterene concentrations are in accordance with the pharmacokinetic data,[7] while furosemide shows a lower percentage excreted,[8] perhaps due to the combination of both diuretics. The peak concentrations times for both drugs are in agreement with the literature data (2-8 hours for furosemide and 8-24 hours for triamterene).[7,8] Figure 4 shows the chromatograms corresponding to a blank urine and a real urine sample obtained from 2-8 hours after the administration of one tablet.

Table 3

Determination of Triamterene and Furosemide in Real Urine Collected at Different Time Intervals after the Ingestion of a Single Dose of Salidur (Triamterene, 25 mg and Furosemide-Xanthinol, 77.6 mg)

Time Intervals	Volume of Urine (mL)	Triamterene (mg/mL)	Furosemide (mg/mL)
0-2 hours	475	0.82	1.42
2-8 hours	560	1.45	3.58
8-24 hours	550	2.00	1.25

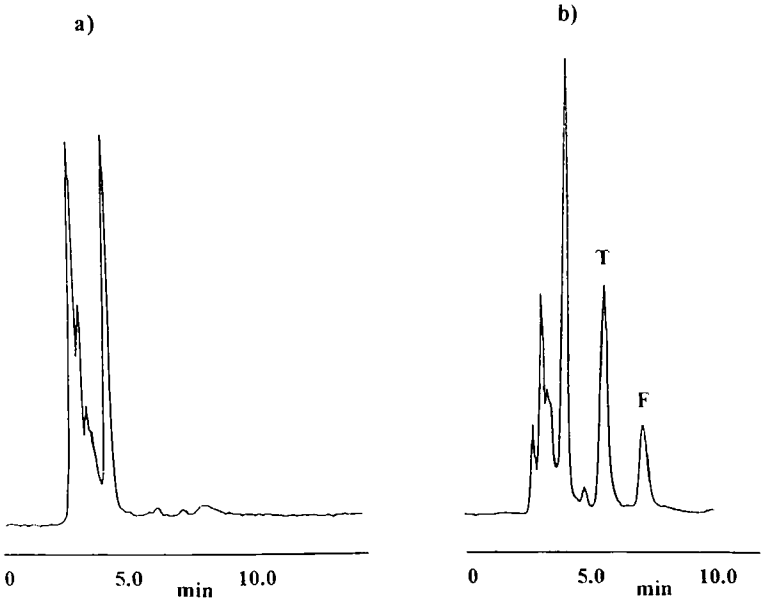

Figure 4. Chromatograms obtained from an extract of a) blank urine sample, and b) urine sample, 8 h after oral administration of 1 tablet of Salidur (Furosemide-Xanthinol 77.6 mg and triamterene 25 mg) to a healthy female volunteer. Full scale current: 100 nA. The same chromatographic conditions as in Figure 2.

High-performance liquid chromatography with amperometric detection has been shown to be a powerful method for the separation, identification and determination of the two diuretics triamterene and furosemide.

The main advantage of the method developed is the determination of triamterene in presence of furosemide, since it was impossible with static electrochemical methods. Moreover, as can be seen in the hydrodynamic voltammograms, it would be possible to choose a potential for the determination of furosemide without interferences from triamterene, for instance, 1100 mV.

The method shows a great sensitivity for the analysis of triamterene with a determination limit of 0.1 ppb, which is lower than the one reported by Sved et

al. using fluorimetric detection.[11]

The different chemical characteristics of these compounds makes it difficult to find an extraction procedure with good recoveries for both of them. On one hand, if an alkaline pH is used for the extraction, the recovery for triamterene is very high, but low for furosemide. On the other hand, if an acid pH is used, the recovery is high for furosemide but triamterene is almost not extracted. Taking into account the usual therapeutic doses and the percentage of unchanged drug excreted, a basic medium was chosen.

In our laboratory, new clean-up procedures based on the application of solid-liquid extraction are being evaluated in order to obtain good recoveries for both compounds without increasing the amount of interferences found in the chromatograms from the urine matrix.

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