

Quantitative Determination of the Loop Diuretic Bumetanide in Urine and Pharmaceuticals by High-Performance Liquid Chromatography with Amperometric Detection

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Abstract

A high-performance liquid chromatographic method with amperometric detection has been developed for the determination of the diuretic bumetanide using a μ Bondapak C₁₈ column. The mobile phase consists of a 50:50 acetonitrile–water mixture containing 5mM KH₂PO₄–K₂HPO₄ (pH 4.0). The compound is monitored at +1350 mV with an amperometric detector equipped with a glassy carbon working electrode. A liquid–liquid or solid–liquid extraction is done prior to chromatographic analysis in order to avoid the interferences found in the urine matrix. The percentages of recovery obtained are 71% \pm 1% for liquid–liquid extraction and 84.2% \pm 0.7% for solid–liquid extraction. The method developed has a linear concentration range from 50 to 499 ng/mL with a reproducibility in terms of relative standard deviation of 1.73% and 3.85% for a concentration level of 70 ng/mL and 237 ng/mL, respectively, and a detection limit of 0.25 ng/mL (3:1 signal-to-noise ratio). The method is applied to the determination of bumetanide in pharmaceutical formulations and urine obtained from hypertensive patients and healthy volunteers after the ingestion of a therapeutic dose of Furosemide (1 mg bumetanide).

Introduction

3-(Aminosulfonyl)-5-(butylamino)-4-phenoxy-benzoic acid (bumetanide) is a potent high-ceiling loop diuretic that has an efficiency 40 to 60 times greater than furosemide (1) (Figure 1). This compound belongs to the sulfonamide family, although its structure differs considerably from furosemide and others of its class. It has a phenoxy group in the position in which other sulfonamidic diuretics usually have a halogen or pseudohalogen.

The excretion of bumetanide is maximum at 1 to 4 h after its oral administration. The urine recovery from a dose is generally

approximately 40% to 80% in a time period of 24 to 48 h. The excretion percentage of unchanged bumetanide reported by different authors varies from 34% to 75% (1–7).

Bumetanide belongs to the groups of diuretics that have been a banned substance in sports since 1986 (8). Diuretics have been used to reduce body weight in order to qualify for a lower weight class and manipulate urine in order to avoid a positive result in a doping test.

Different galenic forms in which bumetanide is commercialized have been analyzed by volumetric titration and colorimetric (9), fluorimetric (10), voltammetric (11), coulometric (12), and high-performance liquid chromatographic (HPLC) (13) methods. Bumetanide levels in biological fluids have been measured by radioimmunoassay (1,14), potentiometry (15), fluorimetry (16), paper electrophoresis (1), gas chromatography (GC) (2,17), GC–mass spectrometry (18), and HPLC with photometric detection (19–27).

Oxidative properties of loop (11,28,29) and nonthiazide diuretics (30,31) have been studied in our laboratory, and based on these properties, chromatographic methods with amperometric detection have been developed for the analysis of torasemide (32), clopamide (33), xipamide (34), indapamide (35), and the simultaneous determination of furosemide and triamterene (36) and furosemide and piretanide (37).

Liquid–liquid extraction is the most commonly used procedure

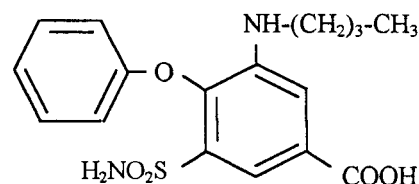


Figure 1. Structure of bumetanide.

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for the separation of diuretics from the endogenous compounds of the urine matrix (38–44). However, Park et al. (45) carried out a comparative study of the efficiency of solid–liquid extraction and liquid–liquid extraction at different pH values for the analysis of these doping agents. However, Campins et al. (46) made a most exhaustive study on the possibility of solid–liquid extraction for the separation of acidic, basic, and neutral diuretics using different extraction columns (C_{18} , C_8 , C_2 , cyclohexyl, phenyl, and cyanopropyl).

Bumetanide has been included in the majority of reports related to the screening of diuretics, thus liquid–liquid extraction has been the most widely used method as a clean procedure for the urine matrix. Also, Marcantonio et al. (19), Howlett et al. (22), and Gradeen (26) have described liquid–liquid extraction procedures for urine samples only containing the diuretic bumetanide. Solid–liquid extraction has been scarcely used for the extraction of this diuretic from human urine, and it is worthwhile to mention the reports made by Wells et al. (27), which combined liquid–liquid and solid–liquid extractions, and Ameer et al. (25), which described a solid–liquid procedure for this drug.

The aim of this study is the development of a chromatographic method with amperometric detection for the separation and determination of bumetanide in urine samples obtained from healthy volunteers and hypertensive patients. Also, a solid–liquid extraction procedure has been optimized as a clean-up treatment for urine. A comparative study of liquid–liquid and solid–liquid extraction procedures has also been carried out.

Experimental

Reagents, chemicals, and standard solutions

Bumetanide was kindly supplied by Boehringer Ingelheim S.A. (Barcelona, Spain). HPLC-grade solvents were purchased from Lab-Scan (Bilbao, Spain), and water was obtained from Waters (Barcelona, Spain) Milli-RO and Milli-Q systems. Potassium dihydrogenphosphate and dipotassium hydrogenphosphate were obtained from Merck Suprapur (Bilbao, Spain). The remainder of the reagents used were also from Merck Suprapur.

A stock solution of bumetanide (1000 $\mu\text{g}/\text{mL}$) was prepared in methanol and stored in the dark under refrigeration to avoid degradation. Working solutions were obtained by an appropriate dilution immediately prior to use.

Apparatus and chromatographic conditions

The HPLC system consisted of a Pharmacia (Barcelona, Spain) Model 2150-LKB HPLC pump and a Rheodyne (Pharmacia) Model 7125 injector with a 20- μL sample loop. The electrochemical detector (ED) was a PAR Model 400 equipped with a glassy carbon cell (EG&G Princeton Applied Research, Madrid, Spain). It was operated at +1350 mV versus an 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. A Waters 125Å $\mu\text{Bondapak } C_{18}$ column (30-cm \times 3.9-mm i.d., 10- μm particle size) with a $\mu\text{Bondapak } C_{18}$ precolumn module (Waters) was used. In order to

keep the column temperature constant, a Waters TMC temperature control system was used.

The mobile phase was a mixture of acetonitrile–water (50:50) containing 5mM potassium dihydrogenphosphate–dipotassium hydrogenphosphate (pH 4.0). This buffer was also used as the supporting electrolyte. The phase was filtered through a 0.45- μm membrane, and the air was removed from the phase by sparging with helium. The flow rate used was 1.0 mL/min and the injection volume was 20 μL . The chromatographic separation was made at $30^\circ\text{C} \pm 0.2^\circ\text{C}$.

Electrode maintenance

The electrode was cleaned electrochemically at the end of each working day by keeping it at -800 mV for 2 min and then at $+1.6$ V for 5 min. This operation was carried out using pure methanol as the mobile phase at a flow rate of 1.5 mL/min.

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

Procedure for tablets

Five tablets were pulverized in a mortar. An adequate amount of the powder was weighed and treated with methanol. After shaking for 5 min, the mixture was centrifuged at 1800 g for 5 min and the supernatant was filtered with Albet 242 paper in order to avoid plugging the column. The precipitate was washed several times with the solvent. The filtered solution was diluted to 100 mL with methanol, and an aliquot of this solution was diluted with mobile phase to provide the concentration required for the injection. The procedure was repeated for different tablets, and the measurements were made by duplicate. The quantitation of the bumetanide content was made using the standard addition method.

Urine samples

Drug-free urine samples were collected from healthy volunteers—two women (29–30 years old) and one man (24 years old)—and stored at -20°C without additives. Before analysis they were thawed to room temperature.

Spiked urine samples were obtained from aliquots of drug-free urine doped with known concentrations of bumetanide. Human urine specimens were obtained from one healthy volunteer (female, 25 years old) after the ingestion of a single therapeutic dose and one hypertensive patient (female, 51 years old) under medical treatment with Fordiuran (1 mg/day). Samples were collected at different times after the ingestion.

Clean-up procedure for urine samples

Solid-phase extraction

Waters C_{18} extraction cartridges (500 mg) were inserted into a vacuum manifold and activated by washing with 15 mL methanol and 15 mL deionized water and conditioned with 1 mL of a phosphate buffer (pH 4.0). Buffered urine samples (2 mL) at the same pH were poured into each cartridge reservoir and drawn slowly through the cartridge. The cartridges were washed with 5 mL of deionized water, 1 mL of hexane, and dried with air for 2 min. The elution of the analyte was performed with 2 mL of ethyl ether. The

eluate was evaporated to dryness at 40°C under a stream of nitrogen using a Zymark (Barcelona, Spain) Turbo Vap evaporator. The residue was dissolved in 1 mL of the mobile phase.

Liquid-liquid extraction

Urine samples (4 mL) were acidified with 4 mL of 1M KH_2PO_4 (pH 4.3), and 8 mL of ethyl acetate were added. Tubes were mechanically shaken for 20 min and centrifuged at 1800 g for 5 min. The organic phase was transferred to a second tube containing 8 mL of 0.1M KH_2PO_4 - K_2HPO_4 (pH 7.5) and shaken for 20 min. Then, the mixture was centrifuged and the organic layer was separated and evaporated to dryness at 40°C under a stream of nitrogen. The residue was dissolved in 1 mL of mobile phase.

Reproducibility and extraction efficiency

The percentages of recovery of bumetanide were calculated by comparing the areas of the chromatographic peaks of urine extracts to those obtained by direct injection onto the column of the same amount of bumetanide in the mobile phase. Each measurement was made by triplicate. Reproducibility was expressed as the relative standard deviation.

The reproducibility and extraction efficiency were determined by extracting replicate ($n = 5$) spiked urine samples. The samples were spiked with 70 ng/mL and 237 ng/mL of bumetanide for reproducibility studies.

Results and Discussion

Upon the basis of the oxidation of bumetanide (11) in the pH range of 1.0 to 9.5 on a glassy carbon electrode, a chromatographic method with ED has been developed.

In order to choose the optimum potential value for the amperometric detection of bumetanide, the hydrodynamic voltammogram of the compound was done (Figure 2). An oxidative potential of 1350 mV was chosen as optimum, because it was the one that provided the maximum sensitivity for the analysis of bumetanide.

The chromatographic conditions used in our laboratories for the separation of other sulfonamidic diuretics (37) were initially assayed for the analysis of bumetanide.

A study of the influence of the organic modifier proportion and pH of the mobile phase on the retention times and resolution of

the chromatographic peaks was carried out. As was expected, an increase of the mobile phase polarity gave rise to an increase of retention time because of the low polarity and hydrophilicity of bumetanide. A decrease in retention times was observed with the increase of pH. A 50:50 ratio of acetonitrile-water containing a 5mM potassium dihydrogenphosphate-dipotassium hydrogenphosphate buffer (pH 4.0) was used throughout this work because the retention time of bumetanide was 8.3 min under these conditions, which allows its determination to be free from the electro-oxidable interferences of the urine matrix.

Once the optimum chromatographic conditions had been established, a quantitative method for the determination of bumetanide in urine samples was developed.

A solid-liquid procedure was optimized for the clean-up of urine samples. A study of the different stages of the procedure of extraction was carried out (conditioning of the cartridge, introduction of the sample, elimination of interferences, and elution of the diuretic). The developed HPLC-ED method was used for the evaluation of each step of the procedure as a function of compound recovery. The $\text{p}K_a$ value of the diuretic was kept in mind ($\text{p}K_a = 3.6$ and 7.7) (47) as well as the retention of the compound in the C_{18} columns. Several volumes of methanol-water were assayed for the conditioning of the cartridge. This variable does not considerably affect the recovery of this diuretic. The optimization of the adequate pH for the extraction of bumetanide was carried out in the pH range of 3.0 to 7.0 using urine samples spiked with 2 $\mu\text{g/mL}$ of the diuretic. A pH value of 4.0 was chosen as optimal, taking into account the compound recovery and the amount of endogenous compounds existing in the extract.

Different elution solvents (diethyl ether, ethyl acetate, methanol, and acetonitrile) were assayed. Ethyl ether was chosen as the optimum eluent because the obtained extract contained a lesser amount of interferences from the urine matrix. The possibility of reusing the cartridge after its regeneration with water and methanol was checked. The percentages of recovery were kept practically constant after at least five different extraction assays.

In optimal conditions (collected in the Experimental section) the percentage of recovery for urine samples spiked with 2.0 $\mu\text{g/mL}$ of bumetanide was $84.2\% \pm 0.7\%$. The liquid-liquid procedure described in the Experimental section and commonly used in our laboratory for other sulfonamidic diuretics was also applied as the clean-up treatment for the urine samples. The percentage of recovery obtained was $71\% \pm 1\%$.

A calibration curve was made from urine solutions spiked with

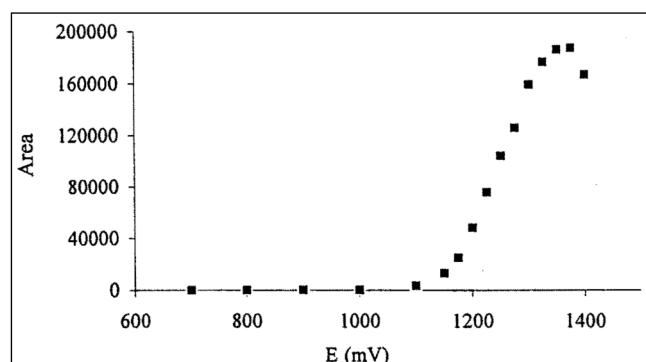


Figure 2. Hydrodynamic voltammogram of bumetanide.

Table I. Quantitative Determination of Bumetanide in Urine

Retention time	8.3 ± 0.1 min
Linear concentration range	50–499 ng/mL
Slope of calibration graph*	267338 ± 8036
Intercept	2462 ± 2450
Correlation coefficient	0.998
Reproducibility (%RSD)	$173\%^{\dagger}, 3.85\%^{\ddagger}$
Detection limit	0.25 ng/mL

* Area/concentration in nanograms per milliliter.

[†] Five determinations at the 70- $\mu\text{g/mL}$ level.

[‡] Five determinations at the 237- $\mu\text{g/mL}$ level.

different concentrations of bumetanide. The concentration range assayed for the determination of the diuretic was chosen upon the basis of its excretion percentages as an unchanged form and the usual therapeutic dose of this antihypertensive agent (1). In Table I, the quantitative characteristics of the method are collected. The detection limit (0.25 ng/mL) was defined as the minimum concentration of bumetanide, which gave rise to a 3:1 signal-to-noise ratio.

The analytical method was applied to the determination of urine samples obtained from one healthy volunteer (female, 25

Table II. Concentrations of Bumetanide* Obtained for Urine Samples from One Healthy Female Volunteer and One Hypertensive Female Patient Collected at Different Time Intervals After the Ingestion of 1 mg Fordiuran Treated with Two Different Cleanup Procedures

	Volunteer		Patient		
	0-2 h	2-8 h	0-2 h	2-8 h	8-24 h
Solid-liquid	240	450	13	56	70
Liquid-liquid	170	380	13	54	64

* ng/mL.

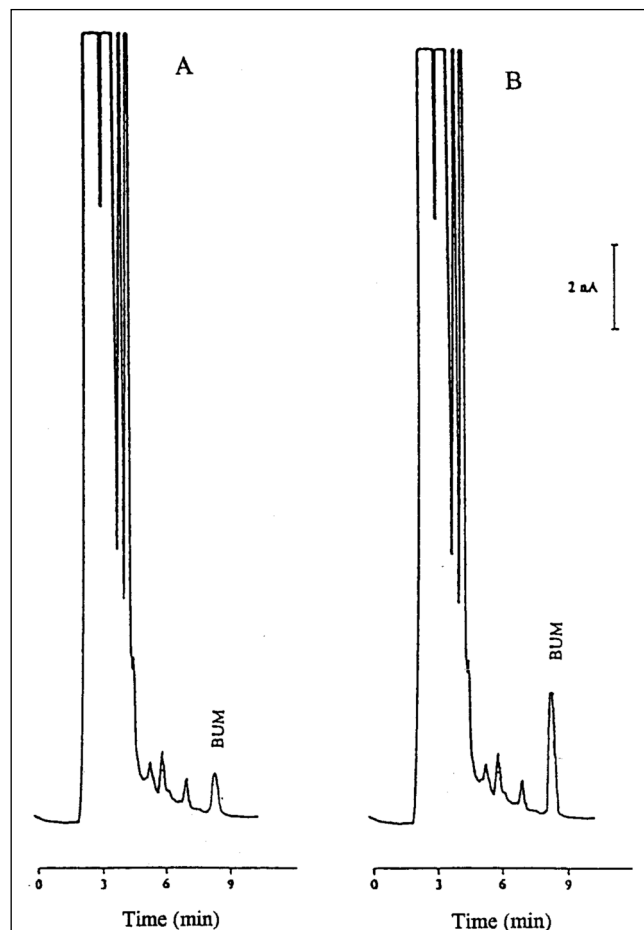


Figure 3. Chromatograms obtained from an extract of urine sample obtained 2-8 h after oral administration of Fordiuran (1 mg bumetanide) to (A) a hypertensive patient and (B) a urine sample after the addition of 50 ng/mL of the bumetanide standard solution.

years old) and one hypertensive patient (female, 51 years old) at different time intervals after the administration of a single dose of Fordiuran (1 mg bumetanide). The results obtained are collected in Table II. As can be seen in Figure 3, the HPLC-ED method that was developed together with the solid-liquid clean-up procedure allowed the determination of bumetanide in urine samples without interferences from the endogenous compounds of urine. Also, the concentrations of the diuretic obtained were in good agreement with those found applying a liquid-liquid extraction procedure previous to the determination by HPLC-ED (Table II).

The chromatographic method developed was also applied to the determination of bumetanide in tablets (Fordiuran, 1 mg bumetanide) following the procedure described in the Experimental section. A mean value of 0.97 ± 0.03 was obtained, which was in accordance with that certified by Boehringer Ingelheim S.A. The chromatogram obtained for a solution of the pharmaceutical formulation is shown in Figure 4. The quantitation of bumetanide was made using the standard additions method.

Conclusion

HPLC with amperometric detection, together with a solid-liquid extraction procedure, was proved to be a potent method for the determination of bumetanide in urine samples obtained from healthy volunteers and hypertensive patients at nanogram-per-milliliter levels. The chromatographic method developed has also demonstrated its applicability to the analysis of bumetanide in pharmaceuticals.

The solid-liquid extraction procedure gave rise to higher recovery percentages of bumetanide than those obtained by liquid-liquid extraction.

The detection limit obtained (0.25 ng/mL) showed the high sensitivity of the HPLC-ED method developed for bumetanide determination, which was lower than those reported by Ameer et al. (10 ng/mL) (25) and Gradeen et al. (10 ng/mL) (26) using HPLC with fluorimetric and diode-array detection, respectively.

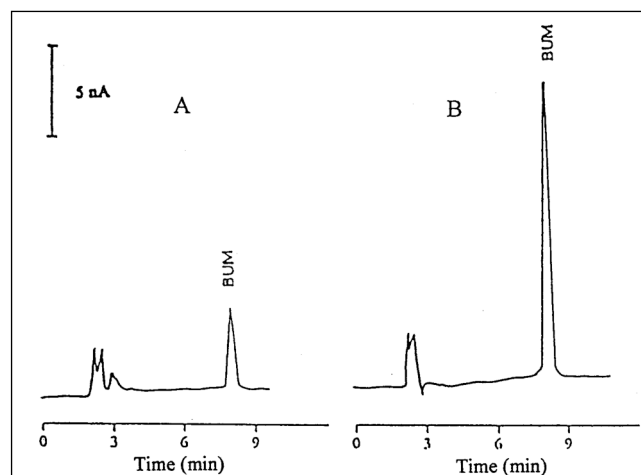


Figure 4. Chromatogram corresponding to (A) a diluted solution of a tablet of 1 mg Fordiuran and (B) the same solution after the addition of 0.5 µg/mL of the standard solution of indapamide.

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