Quantitative Determination of Indapamide in Pharmaceuticals and Urine by High-Performance Liquid Chromatography with Amperometric Detection

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Abstract

A high-performance liquid chromatographic method with amperometric detection for the determination of the diuretic indapamide using a µBondapak C_{18} column is developed. The mobile phase consists of an acetonitrile–water mixture (45:55, 5mM) in KH_2PO_4–K_2HPO_4 (pH 4.0). The compound is monitored at +1200 mV with an amperometric detector equipped with a glassy carbon working electrode. A liquid–liquid or solid–liquid extraction is performed prior to chromatographic analysis to avoid the interferences found in urine matrix. Percentages of recovery are 88.3 ± 5.6 and 82.9 ± 7.8 for liquid–liquid and solid–liquid extraction, respectively. The developed method has a linear concentration range from 25 to 315 ng/mL with a reproducibility in terms of relative standard deviation of 4% for a concentration level of 0.5 µg/mL and a quantitation limit of 1 ng/mL. The method is applied to the determination of indapamide in tablets and urine obtained from hypertensive patients after the ingestion of Tertensif (indapamide 2.5 mg).

Introduction

3-(Aminosulfonyl)-4-chloro-*N*-(2,3-dihydro-2-methyl-1H-indol-1-yl)benzamide (indapamide) is a drug endowed with both antihypertensive and diuretic activity (Figure 1). Indapamide differs from the majority of diuretics in that it has a comparatively high lipid solubility. It is also bound to blood proteins and elastin in vascular smooth muscle, and little is eliminated in the urine. It may be because of these reasons that the drug has less diuretic activity but a more pronounced effect on vascular smooth muscle than compounds of similar structure (1).

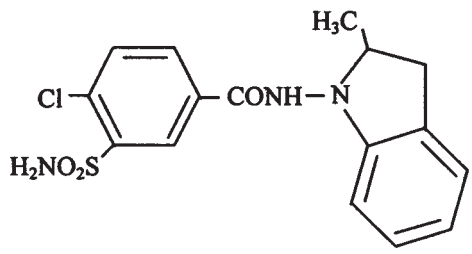
From a pharmacokinetic point of view, it has been documented that indapamide administered orally to humans is rapidly and entirely absorbed within 1 h with a maximum blood concentration of 140 ng/mL. Indapamide binds (79%) to plasma proteins; it is extensively metabolized and widely distributed throughout the tissues. Different metabolites (55%) and unchanged drug (only 5%) are excreted in the urine (2).

Indapamide belongs to the group of diuretics that have been considered banned substances in sports since 1986 (3). Diuretics have been used to reduce body weight in order to qualify for a lower weight class and to manipulate urine to avoid a positive result in doping tests.

Indapamide levels in biological fluids have been measured by thin-layer chromatography using both unlabeled and labeled drugs (4,5), fluorimetry (6,7), and high-performance liquid chromatography (HPLC) with photometric detection (8–10).

The oxidative properties of loop diuretics (11–13) and non-thiazide (14,15) have been studied in our laboratory, and based on these properties, chromatographic methods with amperometric detection have been developed for the analysis of torasemide (16), clopamide (17), and xipamide (18) and the simultaneous determination of furosemide and triamterene (19) and furosemide and piretanide (20).

Liquid–liquid extraction is the most commonly used procedure for the separation of diuretics from endogenous compounds of urine matrix (21–27). However, Park et al. (28) carried out a comparative study of the efficiency of solid–liquid and liquid–liquid extraction at different pH values for the analysis of these doping agents. Also, Campins et al. (29) made



Indapamide

Figure 1. Structure of indapamide.

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).

In the case of indapamide, Pietta et al. (8) used a liquid–liquid extraction with ethyl acetate in a clean-up procedure for the determination of this diuretic in urine, and Choi et al. (9) obtained recovery percentages of 87% for indapamide in urine samples using a double extraction with ethyl ether. The solid–liquid extraction has been only applied to the determination of indapamide in plasma (10), achieving recovery percentages greater than 95%.

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

Experimental

Reagents, chemicals, and standard solutions

Indapamide was supplied by Normón. S.A. (Madrid, Spain). HPLC-grade solvents were purchased from Lab-Scan (Bilbao, Spain), and water was obtained from the Waters (Barcelona, Spain) Milli-RO and Milli-Q systems. Potassium dihydrogenphosphate, dipotassium hydrogenphosphate, and all other reagents were from Merck Suprapur (Bilbao, Spain).

A stock solution of indapamide (1000 µg/mL) was prepared in methanol and stored in the dark under refrigeration. Working solutions were obtained by an appropiate dilution immediately prior to use.

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 made up to 100 mL with methanol, and an aliquot of this solution was diluted with the mobile phase to provide the concentration required for the injection. The procedure was repeated for different tablets, and the measurements were made in duplicate.

Procedure for urine samples

Solid-phase extraction

Waters C_{18} extraction cartridges (500 mg) were inserted into a vacuum manifold, activated by washing them with 15 mL methanol and 15 mL deionized water, and conditioned with 1 mL phosphate buffer (pH 9.0). Buffered urine samples (2 mL) of 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 and 1 mL of hexane and dried with air for 2 min. 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 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 was added. Tubes were 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 under a stream of nitrogen at 40°C. The residue was dissolved in 1 mL of mobile phase.

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 (EC) detector was a PAR model 400 equipped with a glassy carbon cell (EG&G Princeton Applied Research, Madrid, Spain). It was operated at +1200 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 125-Å µBondapak C_{18} column (30 cm × 3.9-mm i.d., 10-µm particle size) with a µBondapak C_{18} precolumn module (Waters) was used. To keep the column temperature constant, a Waters TMC temperature control system was used.

The mobile phase was a mixture of acetonitrile–water (45:55) containing 5mM potassium dihydrogenphosphate–dipotassium hydrogenphosphate. The pH was adjusted to 4.0 with a potassium dihydrogenphosphate–dipotassium hydrogenphosphate buffer, and this buffer was also used as a 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 was 1.0 mL/min, and the injection volume was 20 µL. The chromatographic separation was made at 30 ± 0.2°C.

The HPLC system used for validation assays consisted of a Hewlett-Packard (Palo Alto, CA) series II 1090 liquid chromatograph equipped with a diode-array detector (DAD). The instrument was linked to an HP 9000/300 workstation (Hewlett-Packard). A Beckman (San Ramon, CA) Ultrasphere ODS column (7.5 × 0.46-cm i.d., 3-µm particle size) was used. The chromatographic method was that applied in the screening of diuretics in the doping controls of the Olympic Games (27).

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 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 methanol on a tissue to remove any possible adsorbed compounds and rinsed with deionized water.

Results and Discussion

In order to choose the optimum potential value for the amperometric detection of indapamide, the hydrodynamic voltammogram of the compound was done (Figure 2). An oxidative potential of 1200 mV was chosen as the working potential, because it was the potential that provided the maximum sensitivity for indapamide.

Upon the basis of the studies carried out in our laboratories for the separation of other sulfonamidic diuretics (16–20), acetonitrile was used as an organic modifier in the mobile phase.

A study of the influence of the organic modifier proportion and pH of the mobile phase on the retention times and resolution of chromatographic peaks was carried out. As expected, an increase in the mobile phase polarity gave rise to an increase in retention time due to the low polarity and hydrophilicity of indapamide. A decrease in retention times is observed with the increase in pH. A ratio of 45:55 acetonitrile–water containing 5mM potassium dihydrogenphosphate–dipotassium hydrogenphosphate buffer (pH 4.0) was used throughout this work, because the retention time of indapamide was 7.1 min under these conditions, allowing it to be determined free from electrooxidizable interferences from the urine matrix.

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

A solid–liquid procedure was optimized for the treatment of urine samples. A study of the different stages of the procedure of extraction was carried out: the conditioning of the cartridge, introduction of the sample, elimination of interferences, and elution of the diuretic. The developed HPLC–EC method was utilized for the evaluation of each step of the procedure as a function of compound recovery. The pKa value of the diuretic (8.3) and the retention of the compound in C_{18} columns were kept in mind. The percentages of recovery of indapamide were calculated for the comparison of the chromatographic peak area obtained with the corresponding standard solution of similar concentration. Several volumes of methanol–water were assayed for the conditioning of cartridge; this variable does not considerably affect the recovery of this diuretic. The optimization of the adequate pH for the extraction of indapamide was carried out in the pH range 3.0–9.0 using urine samples spiked with 0.5 µg/mL of the diuretic. The pH of extraction does not affect the percentage of recovery of indapamide, although an increase in pH provides a better elimination of the endogenous compounds of the urine. For this reason, a value of pH 9.0 was chosen.

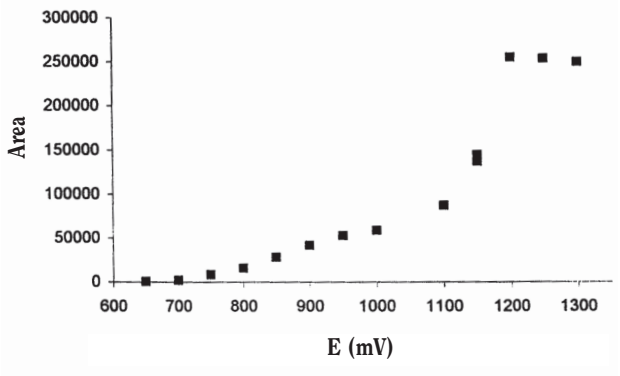


Figure 2. Hydrodynamic voltammogram of indapamide. Conditions: drug concentration, 1 µg/mL; mobile phase, acetonitrile–water (50:50) containing 5mM KH_2PO4–K_2HPO_4 (pH 4.0); flow rate, 1 mL/min; temperature, 30 ± 0.2°C.

Different elution solvents (diethyl ether, ethyl acetate, methanol, and acetonitrile) were assayed. Ethyl ether was chosen as the optimum eluent because an extract that was more free from interferences was obtained from urine. The possibility of reusing the cartridge after its regeneration with water and methanol was checked. The percentages of recovery kept practically constant after at least 5 different extraction assays.

Under optimal conditions found in the Experimental section, the percentage of recovery for urine samples spiked with 0.5 µg/mL of indapamide was 82.9 ± 7.8. The liquid–liquid procedure described by Fullinfaw et al. (22) for the screening of diuretics was also applied as a clean-up treatment for the urine samples. The percentage of recovery obtained was 88.3 ± 5.6.

A calibration curve was made for the urine solutions spiked with different concentrations of indapamide. The concentration range assayed for the determination of the diuretic was chosen upon the basis of its excretion percentages as unchanged form and the usual therapeutic dose of the antihypertensive agents (2). In Table I, the quantitative characteristics of the method are shown. The quantitation limit, defined as the minimum concentration of indapamide that gave rise to a signal able to be quantitated for the integrator, was 1 ng/mL.

The analytical method was applied to the determination of urine samples obtained from a healthy volunteer (female, 30 years old) and 2 hypertensive patients (a female, 58 years old, and a male, 45 years old) at different time intervals after the administration of Tertensif (indapamide, 2.5 mg). The results obtained are shown in Table II. As can be seen in Figure 3, the HPLC–EC method developed together with the solid–liquid clean-up procedure allows the determination of indapamide in

Table I: Quantitative Determination of Indapamide in Urine

Retention time	7.1 ± 0.6 min
Linear concentration range	25–315 ng/mL
Slope of calibration graph	468630 ± 26396*
Intercept	+24986 ± 5186
Correlation coefficient r^2	0.998
Reproducibility (%RSD)	4%†
Quantitation limit	1 ng/mL

* Area/concentration (ng/mL).
† 18 determinations at the 0.5-µg/mL level.

urine samples. Moreover, the obtained concentrations were in good agreement with those found by applying a liquid–liquid extraction procedure (Table II).

The validation of the method was carried out by comparing the HPLC–EC results with those obtained using the method applied in the doping controls in the Olympic Games (27), which used HPLC–DAD and a liquid–liquid extraction procedure. In Table III, concentrations of indapamide obtained using both methods are collected.

The chromatographic method was also applied to the determination of indapamide in tablets (Tertensif, 2.5 mg indapamide) following the procedure described in the Experimental section. A mean value of 2.49 ± 0.03 was obtained (in accordance with that certified by the pharmaceutical company) with relative errors lower than 0.5%. The chromatogram obtained for a solution of the pharmaceutical formulation was free from interferences (Figure 4). The quantitation of indapamide was made using the standard additions method.

Conclusion

HPLC with amperometric detection, together with a solid–liquid extraction procedure, has proved to be a potent method for the determination of indapamide in urine samples obtained from healthy volunteers and hypertensive patients. The developed chromatographic method has also demonstrated an applicability to the analysis of indapamide in pharmaceuticals. The quantitation limit (1 ng/mL in urine) was lower than that reported by Pietta et al. (8) and Choi et al. (9) (which was 25 ng/mL using HPLC with photometric detection and a liquid–liquid extraction procedure). The clean-up procedure for urine samples using solid–liquid extraction provides percentages of recovery for indapamide comparable to those obtained by means of liquid–liquid extraction, but it has the advantage of producing extracts more free from interferences in the urine matrix.

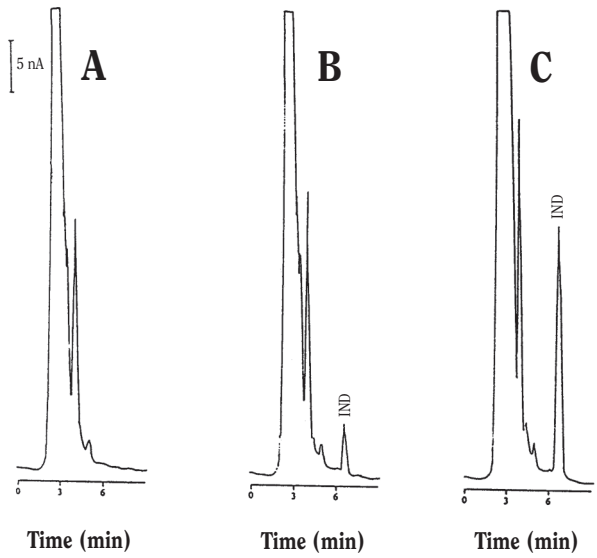


Figure 3. Chromatograms obtained from an extract of blank urine (A), a urine sample 2 h after oral administration of Tertensif (2.5 mg indapamide) to a healthy female volunteer (B), and a urine sample after the addition of 0.25 µg/mL of indapamide standard solution (C). Chromatographic conditions: mobile phase, acetonitrile–water (45:55) containing 5mM KH_2PO_4–K_2HPO_4 (pH 4.0); potential, 1200 mV; flow rate, 1 mL/min; temperature, 30 ± 0.2°C.

Table II: Concentrations of Indapamide (ng/mL) Obtained for Urine Samples from a Healthy Volunteer and 2 Hypertensive Patients After the Application of 2 Different Clean-Up Procedures

	Volunteer		Patient 1		Patient 2	
	0–8 h	8–24 h	0–2 h	2–6 h	0–2 h	2–8 h
Solid–liquid	220	150	140	180	8	76
Liquid–liquid	210	140	130	190	9	70

Table III: Concentrations of Indapamide (ng/ml) Obtained for Urine Samples from a Healthy Female Volunteer and Hypertensive Female Patient by Means of HPLC–DAD (Liquid–Liquid Extraction) and HPLC–EC (Solid–Liquid Extraction)

	Volunteer		Patient	
	0–8 h	8–24 h	0–2 h	2–6 h
HPLC–DAD (liquid–liquid)	200	260	100	140
HPLC–EC (solid–liquid)	220	250	140	180

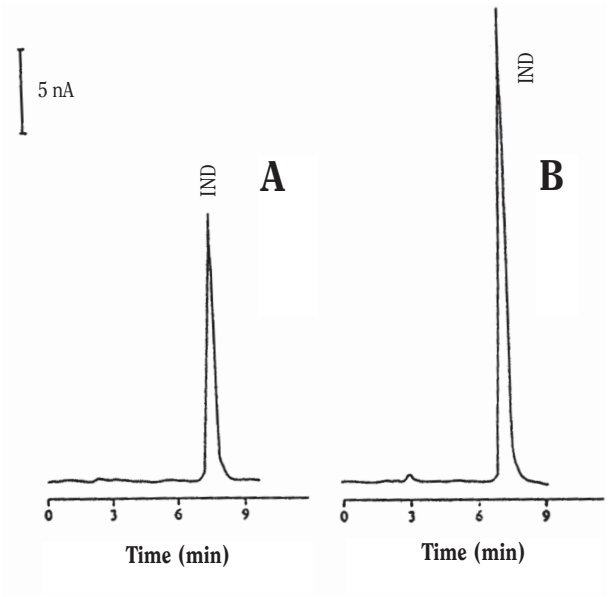


Figure 4. Chromatogram corresponding to a diluted solution of a tablet of Tertensif (2.5 mg) (A) and the same solution after the addition of 1 µg/mL of standard solution of indapamide (B). Chromatographic conditions: mobile phase, acetonitrile–water (45:55) containing 5mM KH_2PO_4–K_2HPO_4 (pH 4.0); potential, 1200 mV; flow rate, 1 mL/min; temperature, 30 ± 0.2°C.

Acknowledgments

The authors would like to thank Dr. Segura and R. Ventura for the facilities offered in the Institut Municipal d'Investigació Médica, Barcelona, and the collaboration in the part of the work corresponding to the HPLC–DAD system. The authors also wish to thank the Interministerial Commission of Science & Technology (project PB95-0316) and the Basque Government (project PI96/85) for financial support. Indapamide was kindly supplied by Normón S.A., Madrid, Spain.

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Manuscript accepted June 18, 1999.