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Simultaneous Determination of the β-Blocker Atenolol and Several Complementary Antihypertensive Agents in Pharmaceutical Formulations and Urine by Capillary Zone Electrophoresis

M.I. Maguregui, R.M. Jiménez, and **R.M. Alonso**
Departamento de Química Analítica, Facultad de Ciencias, Universidad del PaísVasco/EHU, Apdo. 644, 48080 Bilbao, Spain

Abstract

A simple capillary zone electrophoresis method is developed for the quantitation of the β-blocker atenolol and the complementary antihypertensive agents bendroflumethiazide, amiloride, and hydrochlorothiazide in human urine samples. The electrophoretic separation is performed using a 78-cm × 75-μm-i.d. (70-cm effective length) fused-silica capillary. A borate buffer (pH 9) is used as running electrolyte. The sample is hydrostatically introduced for 20 s, and the running voltage is 25 kV at the injector end of the capillary. The analysis of urine samples requires the optimization of solid-phase extraction methods, achieving recoveries ≥ 61% for all the drugs and good separation from the urine matrix. The method is successfully applied to the determination of these compounds in pharmaceutical formulations and in urine samples collected after the intake of Neatenol Diu (100 mg atenolol–5 mg bendroflumethiazide) and Kalten (50 mg atenolol–25 mg hydrochlorothiazide–2.5 mg amiloride). The method is validated in terms of reproducibility, linearity, and accuracy.

Introduction

4-[2-Hydroxy-3-[(1-methylethyl)amino]propoxy]benzeneacetamide (atenolol) is a β-adrenolytic cardioselective drug. β-Receptor-blocking drugs were introduced in 1966 to treat cardiovascular disorders, and these drugs are especially efficient in cases of coronary failure (angina pectoris), arterial hypertension, and cardiac arrhythmia (1).

For those patients with mild or moderate hypertension who do not respond to monotherapy, the addition of a second antihypertensive drug with a different mechanism of action frequently causes an additional lowering of blood pressure without provoking substantial decreases in tolerability. Atenolol is usually combined with bendroflumethiazide, bendroflumethiazide–hydralazine, amiloride–hydrochlorothiazide, and chlortalidone. The pharmaceutical combinations "Neatenol Diu" (100 mg atenolol–5 mg bendroflumethiazide) and "Kalten" (50 mg atenolol–25 mg hydrochlorothiazide–2.5 mg amiloride) have been studied.

Atenolol is one of the most hydrophilic β-blockers and is cleared almost exclusively by the kidneys with minimal (< 10%) metabolism. After oral administration, atenolol is excreted in the urine to the extent of about 40% of the dose (2). 3-Benzyl-6-trifluoromethyl-7-sulfamoyl-3,4-dihydro-2H,1,2,4-benzothiadiazine 1,1-dioxide (bendroflumethiazide) is a diuretic of the benzothiadiazines group which is completely absorbed after oral administration, and approximately 30% of the drug is excreted unchanged in the urine within 48 h (3). 3,5-Diamino-*N*-aminoiminomethyl-6-chloro-pyrazinecarboxamide (amiloride) has a mild diuretic and antihypertensive activity, and between 20 and 50% of an oral dose is excreted unchanged in the urine in the first 24–72 h (4). 6-Chloro-7-sulfamyl-3,4-dihydro-1,2,4-benzothiadiazine 1,1-dioxide (hydrochlorothiazide) is a widely prescribed diuretic representative of the benzothiadiazine class. It is not metabolized in humans and is excreted almost entirely (> 95%) in the urine. The structure of these compounds is shown in Figure 1.

Determination of β-blockers in biological samples is required in many areas, including doping control, forensic analysis, and toxicology. Several methods have been reported for the determination of atenolol in plasma and urine, including high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection (5–13), HPLC with fluorimetric detection (14–19), liquid micellar chromatography with laser-induced fluorescence (20), ion pair chromatography (21), and gas chromatography–mass spectrometry (22–24).

There is a publication reporting the determination of atenolol combined with the diuretics hydrochlorothiazide and chlortalidone in tablet formulations by reversed-phase HPLC (25), but no simultaneous determinations of atenolol and the diuretics bendroflumethiazide, amiloride, and hydrochlorothiazide in biological fluids have been reported.

Micellar electrokinetic chromatography (MEKC) (21,26,27) and capillary zone electrophoresis (CZE) (28–30) have also been used for the qualitative analysis of β-blockers and diuretics

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independently, but only a paper (31) showing one quantitative application of this technique for the analysis of β-blockers in biological fluids exists. This work described the single determination of atenolol in urine.

The small sample volume requirement makes capillary electrophoresis (CE) very suitable for analyses of biological fluids. However, the inadequate detection sensitivity of CE, in addition to possible matrix effects from biological fluids, makes quantitative analysis in biological fluids a challenge. We have already developed a simple quantitative CZE method for the single determination of atenolol in urine (31). However, this method is not useful for the simultaneous determination of atenolol and the complementary antihypertensive agents studied in this work.

The aim of this work was to develop a simple and reliable CZE method for the simultaneous quantitative determination of atenolol–bendroflumethiazide and atenolol–amiloride–hydrochlorothiazide in pharmaceutical formulations and urine samples obtained from healthy volunteers after the administration of the corresponding pharmaceutical associations.

Experimental

Reagents and solutions

Solvents were Lab-Scan HPLC grade (Dublin, Ireland). The water was obtained from the Milli-RO and Milli-Q Waters systems (Barcelona, Spain). All reagents used were Merck proanalysis (Bilbao, Spain). Atenolol, amiloride, bendroflumethiazide, and hydrochlorothiazide were provided by Sigma (Barcelona, Spain). Bond-Elut Certify LRC and Bond-Elut C_{18} columns were supplied by Varian (Barcelona, Spain).

Apparatus and electrophoretic conditions

This work was performed on a 78-cm × 75-µm-i.d. fused-silica capillary tube (Composite Metal Services, Worcester, England). The effective separation distance was 70 cm. The CE system was a Waters Quanta 4000. Data were collected with the help of a personal computer and treated with Millennium 2010 software (Waters Chromatography Division, Barcelona, Spain). The wavelength used for photometric detection was 214 nm. Based on the previous work done at our laboratories (31), samples were injected hydrostatically for 20 s. The running voltage was 25 kV. The temperature was kept constant at 25°C.

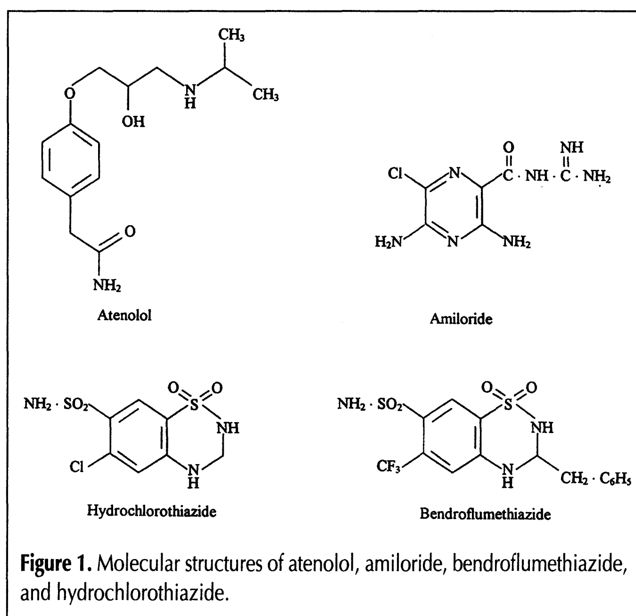

Figure 1. Molecular structures of atenolol, amiloride, bendroflumethiazide, and hydrochlorothiazide.

The study of the influence of the pH and composition of the electrolyte gave an optimum electrolyte consisting of 0.05M borate buffer (pH 9), inducing a 20-µA current across the capillary and providing good reproducibilities and resolution from the endogenous compounds in the urine matrix.

The capillary was conditioned every day with an initial wash cycle consisting of 1M NaOH for 20 min and deionized water for 20 min. The wash cycles before each injection were 0.1M NaOH for 2 min and running buffer for 3 min in order to reduce fouling. Daily wash cycles after finishing experiments were 1M NaOH for 5 min and deionized water for 5 min.

Procedure for tablets

The pharmaceutical formulations analyzed in this work were Neatenol Diu in commercialized tablet dosage form (Fides-Rottapharm S.A., Valencia, Spain) and Kalten in commercialized capsule dosage form (Zeneca Farma, Pontevedra, Spain). In order to perform the determinations, the tablets were weighed and then pulverized with a mortar. The capsules were opened and emptied, and an adequate amount was weighed out.

Procedure for Neatenol Diu

An adequate amount of powdered tablet (n = 5; equivalent to 2 mg bendroflumethiazide and 40 mg atenolol) was weighed out, extracted with 25 mL methanol under stirring for 10 min at room temperature, ultrasonicated for 5 min, and filtered through an Albet 242 filter paper. The residual solid was washed with 25 mL methanol and 25 mL water to prevent the loss of analytes. After filtration, the volume of the resulting solution was brought to 250 mL by adding water, and an aliquot of this solution was diluted with running buffer to provide the concentrations required for injection. This procedure was repeated three times.

Procedure for Kalten

Each capsule was emptied, and the contents were extracted with 25 mL methanol and stirring. The procedure was the same as previously described for Neatenol Diu, except that the solution was brought to 100 mL instead of 250 mL. The procedure was repeated for five capsules. The drug content in each formulation was calculated under calibration conditions with appropriate drug standard solutions.

Procedure for urine samples

Procedure I: Urine samples after the intake of Neatenol Diu. The pH of the urine samples (2.5 mL) was adjusted to 9 with 1M borate buffer (500 µL), vortex mixed for 5 s, and centrifuged at 734 × g.

Bond-Elut Certify LRC solid-phase extraction (SPE) columns were activated before use by washing with 2 × 2 mL of methanol followed by 2 mL of 0.1M borate buffer (pH 9). Buffered urine aliquots (2.5 mL) were drawn slowly through

the extraction columns placed in a Luer fitting in the top of a Vac-Elut cover. The columns were then washed with 2 mL distilled water and 1 mL hexane and left in full vacuum for 5 min. Elution of the analyte was performed with 2 mL of a mixture of chloroform–isopropyl alcohol (80:20, v/v) containing a 2%-ammonia solution. The eluate was evaporated to dryness at 50°C under a stream of nitrogen using a Zymark (Barcelona, Spain) Turbo Vap LV evaporator. The residue was dissolved in 100 µL of electrolyte containing 30% MeOH and measured under calibration conditions.

Procedure II: Urine samples after the intake of Kalten. The pH of the urine samples (2 mL) was adjusted to 6 with 1M phosphate buffer (400 µL), vortex mixed for 5 s, and centrifuged.

Several extraction columns were assayed (Bond-Elut Certify LRC, Bond-Elut C_2, and Bond-Elut C_{18}). Bond-Elut C_{18} extraction columns were chosen as optimal, activated with 4 × 1 mL methanol followed by 2 × 1 mL 0.1M phosphate buffer, and washed with water and hexane. The elution was performed using 2 mL of a mixture of ethyl acetate–isopropyl alcohol (80:20, v/v) containing 2% ammonia. The eluate was evaporated to dryness at 50°C under a stream of nitrogen and redissolved in 100 µL of electrolyte containing 30% MeOH.

The reproducibility and efficiency of the extraction procedures were determined by extracting replicate spiked urine samples. The urine samples were spiked with concentrations similar to the ones usually found in urine. The recoveries were estimated by comparing the peak areas of non-extracted standard solutions with the areas of extracted urine samples

Table I. Extraction Recoveries Obtained from Spiked Urine Samples

	Compound	Recovery (%)	%RSD
Procedure I	atenolol	79.23*	3.92
	bendroflumethiazide	63.58*	4.75
		63.61†	5.66
Procedure II	atenolol	79.16*	4.94
	amiloride	65.61*	6.92
	hydrochlorothiazide	65.12*	5.66

* 4-µg/mL level.
† 0.4-µg/mL level.

Table II. Determination of Atenolol, Bendroflumethiazide, Amiloride, and Hydrochlorothiazide*

	Atenolol	Bendroflumethiazide	Amiloride	Hydrochlorothiazide
Migration time ± SD (min)	4.57 ± 0.03	6.50 ± 0.06	5.32 ± 0.01	7.13 ± 0.02
Linear range (µg/mL)	0.6–10	0.1–10	0.5–10	0.5–10
Slope of calibration graph (area/concentration)	5690	7021	9936	14828
Correlation coefficient r^2	0.996	0.994	0.990	0.997
Intraday area repeatability (RSD%)	2.80†	2.74‡	3.76†	4.78†
Interday area repeatability (RSD%)	4.16†	5.28‡	4.35†	5.51†
Quantitation limit (µg/mL in urine)	0.6	0.1	0.5	0.5

* The optimization of the electrophoretic conditions and estimation of the given parameters were done with spiked urine samples.
† Five determinations at the 100-ppm level.
‡ Five determinations at the 10-ppm level.

Results and Discussion

In order to choose the optimum extraction procedures and electrophoretic conditions, several parameters were optimized.

Optimization of the extraction procedures

There are several works that report SPE methods for β-blockers that provide recoveries ranging from 50 to 100% for atenolol (13,27,32,33). However, no previous works that report a simultaneous SPE method for the β-blocker atenolol (or any other β-blocker) and the diuretics studied in this report were found.

Special attention was paid to the optimization of the extraction procedures because the studied compounds had great differences in molecular structures and physicochemical properties. For instance, atenolol and amiloride have basic characteristics, whereas hydrochlorothiazide and bendroflumethiazide are weakly acidic. Therefore, the two combinations required different SPE cartridges, pH levels at which the urine was buffered, and eluant compositions (as shown in the Experimental section).

The pH levels at which the urine samples were buffered was different for each pharmaceutical formulation because hydrochlorothiazide could not be retained in the extraction cartridges at pH values above 6 (atenolol, amiloride, and bendroflumethiazide were well retained at all pH values in this study). On the other hand, buffering the urine at pH values lower than 9 resulted in the retention and coelution of many interfering substances.

Using MeOH or any MeOH–H_2O proportion greater than 20:80 (v/v) in the cleaning step induced the complete elution of hydrochlorothiazide and bendroflumethiazide and the partial elution of amiloride in the cleaning step. Several eluants were studied in the elution step: chloroform, ethyl acetate, diethyl ether, acetonitrile, dichloromethane, and hexane. Different proportions of these organic solvents with isopropyl alcohol or MeOH were also studied in order

Table III. Determination of Atenolol, Bendroflumethiazide, Amiloride, and Hydrochlorothiazide in Pharmaceutical Formulations

Formulation	Component	Found (mg)	Nominal (mg)	Error (%)
Neatenol Diu	atenolol	100.22	100	0.22
	bendroflumethiazide	5.01	5	0.20
Kalten	atenolol	50.19	50	0.38
	amiloride	2.55	2.5	2.00
	hydrochlorothiazide	25.16	25	0.64

to provide more polarity to the eluant, because atenolol is a very polar compound (for optimal extraction conditions, refer to the Experimental section). Finally, the dry residues were redissolved in 30% MeOH to assure redissolution of all compounds.

Quantitative recoveries calculated from spiked urine samples are provided in Table I and expressed as the mean value and its percent relative standard deviation (%RSD = [standard deviation / mean value] × 100). Once the optimum electrophoretic conditions had been established, quantitative methods for the simultaneous determination of atenolol and the complementary antihypertensive agents present in both formulations were developed.

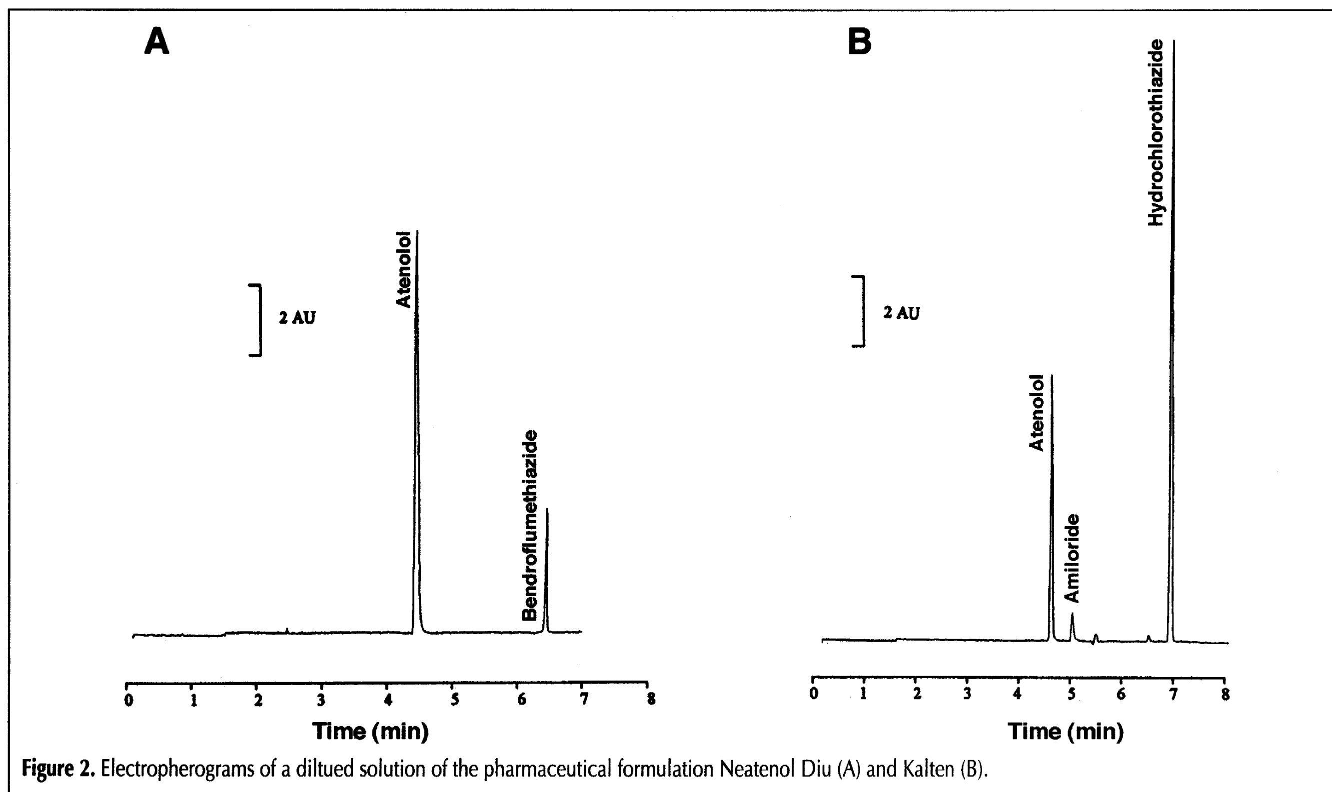

Figure 2. Electropherograms of a diltued solution of the pharmaceutical formulation Neatenol Diu (A) and Kalten (B).

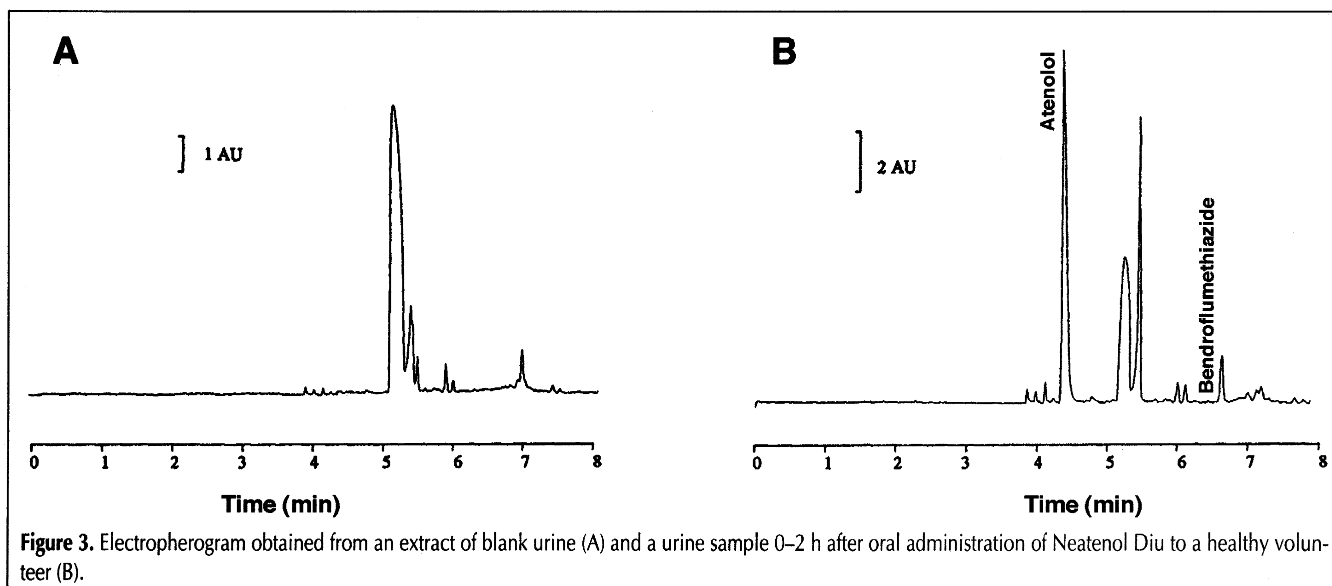

Figure 3. Electropherogram obtained from an extract of blank urine (A) and a urine sample 0–2 h after oral administration of Neatenol Diu to a healthy volunteer (B).

Linearity, repeatability, and accuracy

The %RSD of the retention times was less than 1% over the course of 10 injections when wash cycles were performed with 0.1M NaOH before each injection, indicating high stability for the system. This deviation increased considerably when no wash cycles were carried out, and a delay in the retention times between runs was observed.

Calibration was performed by analyzing 2.5-mL aliquots of blank urine containing the proper amount of each drug. Seven concentrations were used for each calibration graph. Linearity was obtained from at least the limit of quantitation to concentrations slightly above those expected in the urine. Higher concentrations were not assayed because the studied range was considered wide enough for analytical applications.

The within-day and day-to-day area repeatabilities (expressed as %RSD) were determined by injecting replicate samples of each compound listed in Table II. The limit of quantitation, defined as the concentration that gives a signal 10 times the standard deviation of the baseline noise, was obtained from the calibration curves of spiked urine samples (after extraction of 2.5 mL of urine for Procedure I, 2 mL for Procedure II, and a final preconcentration to 100 µL). Limits of quantitation were low enough to allow the quantitation of the compounds at all studied time intervals (Table II). These limits of quantitation were also experimentally tested by extracting urine samples spiked with these concentrations.

The accuracy of the method was determined by the analysis of five control urine samples spiked with 4 µg/mL of each diuretic. Acceptable accuracy, defined as mean ([found concentration / actual concentration] × 100) ± standard deviation (%), was obtained: atenolol (100.78 ± 4.34) (Procedure I), bendroflumethiazide (99.64 ± 1.07) (Procedure I); atenolol (101.77 ± 1.12) (Procedure II), amiloride (100.93 ± 0.98) (Procedure II) and hydrochlorothiazide (100.33 ± 0.92) (Procedure II).

Analytical applications

First, the developed method was applied to the determination of atenolol–bendroflumethiazide–amiloride and atenolol–hydrochlorothiazide in pharmaceutical formulations. Values were obtained in accordance with those certified by the pharmaceutical companies with relative errors less than 1% for most of the compounds, as shown in Table III. In Figure 2, electropherograms of both pharmaceutical formulations are shown.

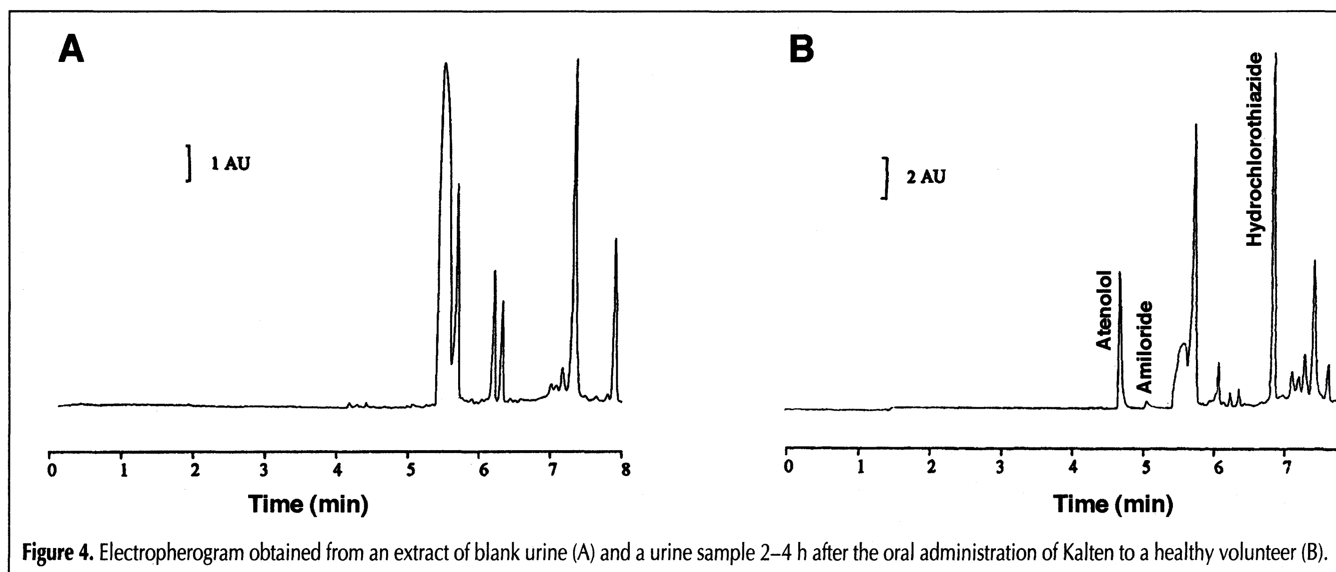

Figure 4. Electropherogram obtained from an extract of blank urine (A) and a urine sample 2–4 h after the oral administration of Kalten to a healthy volunteer (B).

Table IV. Determination of Atenolol, Bendroflumethiazide, Amiloride, and Hydrochlorotiazide in Human Urine Samples Collected at Different Time Intervals After the Ingestion of a Single Dose of Neatenol Diu and Kalten

Pharmaceutical formulation	Time interval (h)	Volume collected (mL)	Atenolol (mg)	Bendroflumethiazide (mg)	Amiloride (mg)	Hydrochlorotiazide (mg)
Neatenol Diu	0–2	120	2.50	0.06		
	2–4	530	5.86	0.20		
	4–8	900	1.93	0.08		
	8–12	300	4.67	0.06		
	12–24	480	6.08	0.02		
Kalten	0–2	600	–		–	–
	2–4	460	4.54		0.56	5.58
	4–8	860	4.15		0.94	3.23
	8–20	590	4.51		0.75	2.20

The method was also applied to the determination of these compounds in urine samples obtained from a healthy volunteer after the intake of a single dose of Neatenol Diu and after the intake of a single dose of Kalten. Urine was collected at different time intervals, and the samples were treated following Procedure I for Neatenol Diu and Procedure II for Kalten, as described in the Experimental section.

Figures 3 and 4 show the electropherograms of a blank urine sample and a sample collected after the intake of the pharmaceutical formulations Neatenol Diu and Kalten, respectively. The distortion of the baseline observed at approximately 5.2 min is caused by MeOH and does not interfere with the compounds of interest. The remainder of the peaks eluting at times greater than 5 min do not interfere and are probably caused by UV-absorbing components, such as uric acid, creatinine, and urea, present in the urine in high concentrations.

The compounds of interest were easily detected at the different time intervals, and the concentrations are provided in Table IV. The obtained results are reasonable and in good agreement with the data in the literature (2) and the data previously obtained at our laboratories when testing atenolol alone at these doses (31), showing peak urine concentrations 4 h after the intake of the therapeutic dose.

Conclusion

A thoroughly validated CE assay of the β-blocker atenolol and the complementary antihypertensive agents bendroflumethiazide, amiloride, and hydrochlorothiazide in human urine has been reported.

In CZE, the mass/charge ratio governs the elution pattern, and the compounds have been easily separated because of the wide differences in their chemical structure, size, and charge. Atenolol is the first eluting compound, followed by amiloride, bendroflumethiazide, and hydrochlorothiazide, which show a similar migration behavior because they are also structurally similar.

In spite of the matrix interferences, acceptable %RSD values were obtained for peak areas and migration times when wash cycles were carried out between runs. If these wash cycles are not carried out, compounds from the urine matrix could also be absorbed on the capillary surface, affecting electroosmotic flow and inducing irreproducibilities between runs.

SPE of the tested drugs using Bond-Elut Certify LRC and Bond-Elut C_{18} cartridges has been shown to provide extracts that are sufficiently clean, allowing preconcentration of the sample. The quantitation limits could be improved if a larger urine volume was extracted or the residue was dissolved in a final volume smaller than 100 µL.

The method was applied to the quantitation of these compounds in pharmaceutical formulations, and the obtained values were in accordance with those certified by the pharmaceutical companies. The method was also applied to the quantitation of urine samples obtained from a healthy volunteer after the ingestion of the pharmaceutical formulations Neatenol Diu and Kalten, and the results are in good agreement with those expected from the pharmacokinetic data.

CZE has proved to be a useful technique for the simultaneous quantitative analysis of atenolol and the antihypertensive agents, despite urine matrix effects.

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