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Determination of the antihypertensive drug cilazapril and its active metabolite cilazaprilat in pharmaceuticals and urine by solid-phase extraction and high-performance liquid chromatography with photometric detection

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Abstract

A liquid chromatographic method with photometric detection for the determination of cilazapril and its active metabolite and degradation product cilazaprilat in urine and pharmaceuticals has been developed. The chromatographic method consisted of a μBondapak C_{18} column maintained at 30±0.2°C, using a mixture of methanol-10 m*M* phosphoric acid (50:50 v/v) as mobile phase at a flow-rate of 1.0 ml/min. Enalapril maleate was used as internal standard. The detection was performed at a wavelength of 206 nm. A study of the retention of cilazapril and cilazaprilat using solid–liquid extraction has been carried out in order to optimise the clean-up procedure for urine samples, which consisted of a solid–liquid extraction using C_8 cartridges. Recoveries greater than 85% are obtained for both compounds. The method was sensitive, precise and accurate enough to be applied to the determination of urine samples obtained from three hypertensive patients up to 24 h after intake of a therapeutic dose (detection limit of 70 ng/ml for cilazapril and cilazaprilat in urine). A comparison of the method developed using photometric and amperometric detection has been carried out.

Keywords: Cilazapril; Cilazaprilat

1. Introduction

Cilazapril, [1*S*-[1α,9α(*R**)]]-9-[[1-ethoxycarbonyl)-3-phenylpropyl]amino] octahydro-10-*oxo*-6*H*-pyridazino[1,2*a*][1,2]diazepine-1-carboxylic acid monohydrate is the monoethyl ester prodrug form of a potent, specific and long-acting antihypertensive inhibitor of angiotensin-converting enzyme (ACE) [1,2]. It is used in the treatment of hypertension and congestive heart failure and after absorption, it is hydrolysed in the liver to the active ACE inhibitor cilazaprilat. Cilazapril is the prodrug form administrated orally due to the low absorption of cilazaprilat [3]. Cilazaprilat has a long terminal phase elimination half-life allowing once daily administration (Scheme 1). As a result of the inhibition of the enzyme that transforms angiotensin I into angiotensin II, cilazaprilat causes an increase in plasma renin activity and a decrease in plasma angiotensin II and

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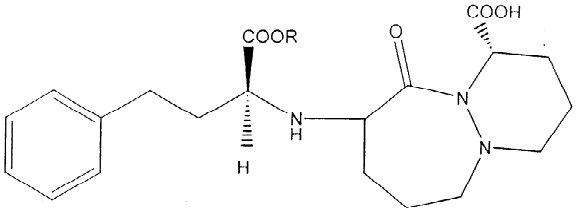

Scheme 1. Molecular structures of cilazapril and cilazaprilat.

aldosterone concentrations. Reduction of angiotensin II levels produces dilatation of peripheral vessels and reduction of vascular resistance [4,5].

Structure-activity studies of these inhibitors established a model of the molecular features that were important for binding the inhibitors to the active centre of ACE. Cilazaprilat is one of the conformationally restricted chemical structures that allow optimal binding to the ACE [1,6,7].

Doses of cilazapril range from 0.5 to 5 mg per day. Bioavailability of cilazaprilat is 57% and it is excreted almost exclusively by the kidney and no further metabolism occurs. The elimination kinetic of cilazaprilat is biphasic, the distribution half-life is 1.5–2 h and the terminal elimination half-life is 30–50 h [4,8,9].

Analytical methods for the direct determination of this drug and its active metabolite are very scarce. The activity and efficacy of these compounds have been usually assayed indirectly by measuring ACE inhibition, angiotensin II or renin levels [1,7,8,10]. Enzyme immunoassay has been used for the direct determination in plasma and serum [11], and HPLC with amperometric detection for its analysis in urine [12]. Methods applied to the analysis of cilazapril in tablets include UV spectrophotometry [13], and HPLC with photometric [14] and amperometric detection [12]. HPLC with photometric detection has been also applied to the determination of the ACE inhibitors lisinopril in urine [15] and captopril in blood and urine after derivatization of its thiol group [16,17]. Other ACE inhibitors have been analysed in biological fluids by GC–MS, but this involves two derivatization steps for the amine group and the carboxylic acid, that are very slow and can affect significantly the results [18–22].

In this work, a validated quantitative chromatographic method with photometric detection for the rapid determination of cilazapril and cilazaprilat in urine and its dosage forms is developed. A study of the extractive properties of the compounds on C_8 cartridges is carried out and a comparison of the proposed method with the previously reported using amperometric detection [12] is discussed.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of a Waters 510 (Milford, MA, USA) pump and a Rheodyne (Cotati, CA, USA) Model 7125 injector fitted with a 20-μl loop.

Photometric detection was performed using a Waters 996 photodiode array detector. For amperometric measurements an EG&G Princeton Applied Research (Princeton, NJ, USA) detector model 400 was used. This detector was equipped with a glassy carbon electrode, a platinum auxiliary electrode and a Ag/AgCl reference electrode and operated at 1350 mV in the DC mode, with a 5-s low-pass filter time constant. Chromatograms were recorded at 206 nm using the Millennium Chromatography Manager software (Waters).

A μBondapak C_{18}, 300×3.9 mm I.D. (10 μm) HPLC column with a μBondapak C_{18} Guard-pak precolumn insert (Waters) was used. The column was kept at constant temperature using a Waters TCM temperature control system.

Solid phase extraction was carried out using Bond Elut 1 ml/100 mg C8 cartridges (Varian, Harbor City, CA, USA). Extractions were performed on a vacuum manifold system (Supelco, Bellefonte, PA, USA). The extracts of urine samples were evaporated to dryness under a nitrogen stream using a Zymark Turbovap LV evaporator (Hopkinton, MS, USA).

2.2. Reagents and solutions

Cilazapril hydrate was kindly supplied by Roche Laboratories (Barcelona, Spain), while cilazaprilat was prepared from cilazapril by alkaline hydrolysis and crystallisation in water [12]. Enalapril maleate

was purchased from Sigma (Madrid, Spain). Solvents were HPLC grade (Lab Scan, Dublin, Ireland). Reagents used were Merck, p.a. Water was obtained from Milli-RO and Milli-Q systems (Waters).

Buffer solutions were prepared from 0.1 *M* phosphoric acid, 0.1 *M* acetic acid and 0.5 *M* boric acid, adjusting the pH value to 2, 5 and 9 respectively by adding drops of 3 *M* KOH.

Stock solutions of cilazapril hydrate and enalapril maleate (200 μg/ml each) were prepared in water, whereas cilazaprilat (200 μg/ml) was dissolved in a mixture water–methanol (80:20 v/v). All solutions were kept in dark and stored at 4°C. Working solutions were prepared by appropriate dilution of the standard just before use.

2.3. Collection of urine samples

Urine samples were obtained from three hypertensive patients under treatment with Inhibace, cilazapril 5 mg. All urine produced was collected in plastic bottles at four time intervals after the ingestion of the therapeutic dose: 0–1, 1–4, 4–8 and 8–24 h. The samples were transferred to 10 ml plastic tubes and immediately frozen at −20°C.

The hypertensive volunteers used in this work had the following features: #1 female, 57 years; #2 female, 79 years and #3 male, 44 years. Patient number 2 was also under treatment with the cardiotonic digoxine, with the diuretic torasemide and the benzodiazepine lormetazepam. Patients were not asked to be on a special diet.

2.4. Procedure for tablets

Five pharmaceutical formulations have been analysed using the HPLC method developed in this work: Inhibace (cilazapril 1 and 5 mg) and Inhibace Plus (cilazapril 5 mg and hydrochlorothiazide 12.5 mg) commercialised by Andreu-Roche Laboratory (Barcelona, Spain); Inocar (Cilazapril 1 mg) and Inocar Plus (cilazapril 5 mg and hydrochlorothiazide 12.5 mg), commercialised by Nezel (Barcelona, Spain).

The same procedure was used for all formulations. Four tablets were weighed and then mixed and crushed in a mortar. A suitable amount of the powder was accurately weighed and dissolved in water. The mixture was sonicated for 10 min and centrifuged at 800 *g* for 5 min. The clear solution was transferred into a volumetric flask, and the solid washed twice with water in order to dissolve completely the active component. The clear solutions were also added into the volumetric flask and finally made up to a fixed volume with water. Aliquots of these solutions were diluted in water and injected three times in the chromatographic system. Calibration standards in the range from 2 to 20 μg/ml were made by appropriate dilution in water of the stock solutions of cilazapril and cilazaprilat. No internal standard was used.

2.5. Clean-up procedure for urine samples

Urine samples contained in 10 ml tubes were thawed. A total of 2 ml of human urine were alkalinised with 1 ml of 0.5 *M* borate buffer at pH 9 and 40 μl of the stock solution of the internal standard enalapril maleate were added. The mixture was shaken and centrifuged at 800 *g* for 5 min.

A total of 2 ml of this mixture was applied to a C_8 cartridge previously activated with 1 ml of methanol, 1 ml of water and 1 ml of 0.17 *M* borate buffer at pH 9 (dilution in water 1:2 v/v of the 0.5 *M* buffer). The cartridge was not allowed to dry before the application of the sample. The sample was slowly drawn through the column using very low vacuum at an approximate flow-rate of 0.5 ml/min. The cartridge was washed in two steps, firstly with 1 ml of 0.1 *M* phosphate buffer at pH 2–methanol (80:20 v/v), 3 ml of water and 1 ml of the same mixture phosphate–methanol. A measure of 1 ml of hexane was then added and the cartridge dried at full vacuum (70 KPa) for 10 min. Another millilitre of hexane was passed in order to ensure the complete dryness of the column, drying the cartridge for 5 min. Secondly, 1 ml of acetonitrile is added to complete the washing. The compounds were eluted with 2 ml of a mixture 2-propanol–chloroform (1:1 v/v) containing 2% of water. The eluate was evaporated to dryness at 50°C under a gentle stream of nitrogen. The remaining residue was dissolved in 0.5 ml of water and injected to the chromatographic system under the optimized chromatographic conditions. A concentration factor of 2.6 times is achieved during the process.

Standard solutions for the calibration were made

by spiking blank urine samples with known amounts of cilazapril and cilazaprilat, and extracting them using the same clean-up procedure.

2.6. Chromatographic conditions

The mobile phase for the separation of cilazapril and cilazaprilat consisted of methanol-10 m*M* phosphoric acid (50:50 v/v), previously filtered through a 0.45-μm pore diameter membrane and degassed by bubbling helium through. The column head pressure was maintained at 17 MPa at a flow-rate of 1.0 ml/min. The temperature was kept at 30±0.2°C. The 20 μl volume of the injector loop was completely filled with the sample. The detector operated at a wavelength of 206 nm.

For comparative studies, the amperometric detector was connected to the outlet of the photometric detector and both detectors worked on-line. The selected working potential was 1350 mV [12].

2.7. Method validation

The calibration curves for cilazapril and cilazaprilat were constructed in the range 0.2–2 and 0.5–20 μg/ml, respectively, taking into account the concentrations expected in urine samples.

Detection and quantitation limits were calculated as the analyte concentrations giving rise to a signal-to-noise ratio of 3 and 10 respectively [23]. Noise was estimated from the standard deviation of the calibration. In this case, a calibration plot in the range 50–1000 ng/ml was used. The value quoted in Table 1 is the mean value of three consecutive days. In order to assure that the accuracy and precision at the quantitation limit were lower than 20% [24], a spiked urine sample with the obtained concentrations was prepared and extracted six times.

An internal standard, enalapril maleate, was used for calibration equations, which were obtained by the least-squares linear regression of the ratio peak area of urinary cilazapril and cilazaprilat/peak area of enalapril maleate versus concentration. Unknown concentrations were calculated by interpolation. Calibration range was selected to cover the expected concentrations of the drugs, taking into account their pharmacokinetic profile.

The reproducibility of the method was determined at three concentration levels: 0.5, 4 and 10 μg/ml for cilazaprilat, and 0.5, 1 and 2 μg/ml for cilazapril. The solutions were made by spiking blank urine samples, which were frozen in 10 ml plastic tubes

Table 1
Quantitative parameters obtained for the analysis of cilazapril and cilazaprilat in urine by HPLC–photometric detection

	Cilazaprilat	Cilazapril
Retention time (min) (mean±SD)	5.13±0.01	8.57±0.01
Calibration range (μg/ml)	0.5–10	0.2–2
Slope of the calibration curve (n=6)	0.279±0.001	0.267±0.004
Intercept (n=6)	0.000±0.006	−0.001±0.004
Regression coefficient of the calibration curve (r^2)	0.999	0.999
Reproducibility intra-day (%RSD)[c]	2.7; 1.6; 1.8[a]	2.1; 2.3; 3.2[b]
Reproducibility inter-day (%RSD)[c]	5.9; 3.5; 2.9[a]	3.3; 3.3; 3.7[b]
Accuracy (Spiked; % relative error)[d]	0.51; −0.5	0.48; 0
	4.03; 0.24	0.96; 0.38
	8.07; 0.40	1.92; 0.50
Detection limit (ng/ml)	70	70
Quantitation limit (ng/ml)	220	220

[a] Concentrations of cilazaprilat 0.5, 4 and 10 μg/ml respectively.
[b] Concentrations of cilazapril 0.5, 1 and 2 μg/ml respectively.
[c] Five days, three replicates each day.
[d] Concentrations expressed in μg/ml. Found value is the mean of two replicates.

until their use. Each day, one tube of each concentration was thawed and three extractions of each were done. The process was repeated during 5 days. The data collected during the 5 days (the ratio obtained dividing the areas of the drugs/area of the internal standard) were treated using the ANOVA method (analysis of variance) [23].

Accuracy was calculated at three concentration levels, extracting blank urine samples spiked with cilazapril and cilazaprilat and comparing the concentration obtained using the calibration equations with the initially added concentrations. Two replicates were made.

The recovery was calculated during the 5 days by comparing the peak areas of cilazapril and cilazaprilat obtained after their extraction with those corresponding to standard solutions of the same concentration in water (taking into account that the extract is concentrated 2.6 times).

3. Results

3.1. Optimisation of the chromatographic system

The chromatographic conditions selected are a modification of the previously published using amperometric detection [12]. Due to the low selectivity of the photometric detection at the short wavelength employed (206 nm), some endogenous compounds were found to elute very close to the peak of cilazaprilat and it needed to be delayed. The retention of cilazaprilat was enhanced by decreasing the column temperature from 40 to 30°C. The modification of other variables, such as a change of pH value or the proportion of organic modifier in the mobile phase, meant an increase in analysis time due to an important delay in the elution time of cilazapril.

The maximum absorbance of the drugs is located at 194.6 nm for cilazaprilat and 195.8 nm for cilazapril. Both spectra have also a shoulder at 206 nm. Although the maximum sensitivity is not achieved, the selectivity and signal-to-noise ratio is improved at the latter wavelength and thus was used throughout this work.

3.2. Optimisation of the clean-up procedure for urine samples

The lack of chromophores in cilazapril and cilazaprilat meant that detection had to be performed at a very low wavelength where endogenous substances gave numerous interfering peaks in unprocessed urine samples. Accordingly, much effort was invested in developing an extensive clean-up procedure that eliminated as much of these intrinsic interferences as possible.

The aminoacidic character of this kind of compounds makes their liquid–liquid extraction impossible since they are charged at any pH value (pK_a 3.3 and 6.5) [25]. Sample preparation has probably been the main difficulty for their analysis and explains the lack of easy and direct analytical HPLC methods for this family of antihypertensives. Although enalapril, other ACE inhibitor, forms ion-pairs with sulphophthalein dyes such as bromothymol blue [26], attempts to extract cilazaprilat with this reagent were unsuccessful. The additional negative charge of the diacid does not allow it to reach the neutrality required for its extraction towards the organic phase.

Although the use of the same clean-up procedure than the optimised in the previous work [12] was attempted, the lower sensitivity and specially the lower selectivity of the photometric detection made inappropriate its use due to the presence of absorbent endogenous compounds. Fig. 1 shows the chromatograms corresponding to a spiked urine sample containing 2 μg/ml of cilazapril, and cilazaprilat and 4 μg/ml of enalapril maleate following the previously reported clean-up procedure and using both kind of detection, amperometric (a) and photometric (b). Although the separation is sufficient for the amperometric detection, it is not entirely satisfactory for the photometric detection, making necessary a new treatment of the urine sample.

Upon the basis of the previous results, we used C_8 cartridges for the extraction of urine samples, and an exhaustive study of the retention of these drugs on C_8 cartridges was carried out. The cartridges were conditioned with 1 ml of methanol, 1 ml of water and 1 ml of buffer solution in which the sample was diluted. A standard solution of cilazapril and cilazaprilat in 0.1 *M* buffer at three pH values (2, 5 and 9)

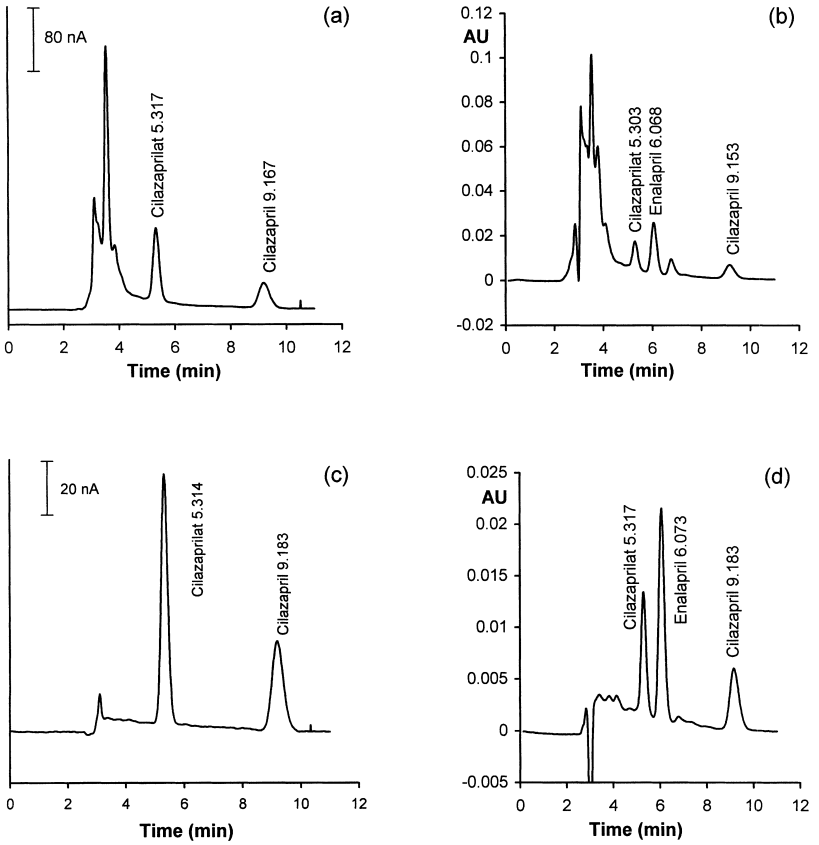

Fig. 1. Chromatograms of blank urine samples spiked with 2 μg/ml of cilazapril and cilazaprilat and 4 μg/ml of enalapril maleate obtained after applying the extraction procedure published in [12] (a) with amperometric detection, (b) with photometric detection; and using the clean-up procedure proposed in this paper (c) with amperometric detection and (d) with photometric detection. Mobile phase: methanol-10 m*M* phosphoric acid (50:50 v/v); flow-rate 1.0 ml/min; column temperature 30°C; detection wavelength 206 nm, detection potential 1350 mV vs. Ag/AgCl.

was applied to the cartridge and then eluted with different proportions of methanol-0.1 *M* buffer mixtures at the same pH values. These eluates were evaporated to dryness, dissolved in water and injected in the chromatographic system. The peak areas were compared with the obtained from standard solutions of the same concentration in water.

The effect of the proportion of methanol and pH on the recovery of the drugs is shown in Fig. 2. Three sigmoidal curves were obtained. It can be observed that cilazapril is more retained than cilazaprilat at all pH values, due to the additional carboxilic moiety of the latter, and that the drugs are more strongly retained when the elution solvent is buffered at pH 2, since a greater proportion of methanol is required for their elution. Cilazapril is not completely eluted at pH 5. This behaviour seems to suggest that the mechanism of adsorption of the drugs on the cartridge surface is not a purely hydrophobic interaction, that there is also an ionic interaction with the charged residual silanols [27]. This component is weaker at a high pH value when both, the molecules and the silanols, are negatively charged. From the study of the curves, two washing solutions were selected: 0.1 *M* phosphate buffer at pH 2–methanol (80:20 v/v), or 0.1 *M* borate buffer at pH 9; and two elution mixtures: 0.1 *M* phosphate buffer at pH 2–methanol (40:60 v/v), or 0.1 *M* borate buffer at pH 9–methanol (50:50 v/v). All four possible combinations of the sequence of washing and elution

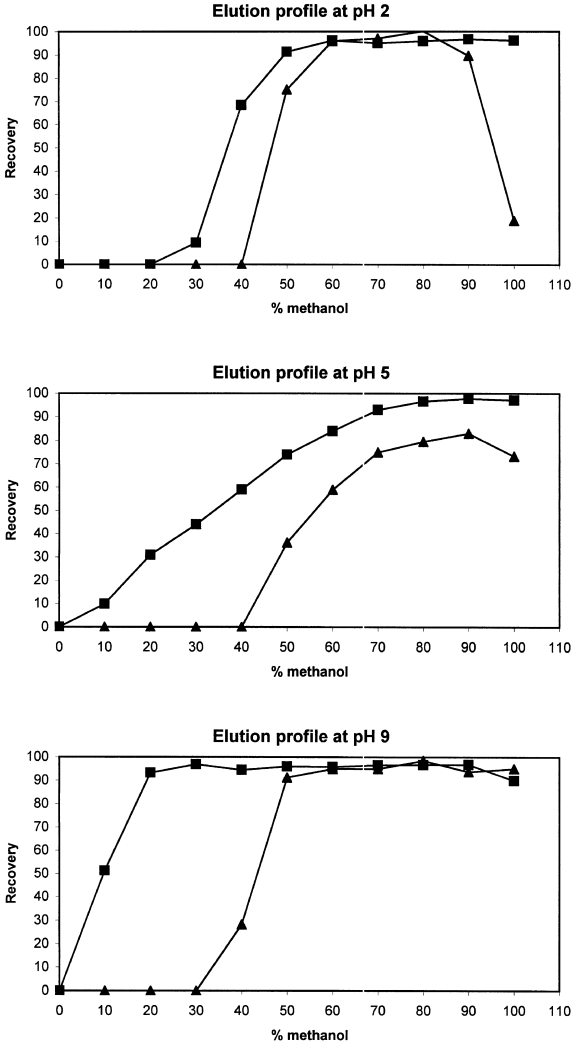

Fig. 2. Elution profiles at three pH values of cilazapril (▲) and cilazaprilat (■) adsorbed on C_8 cartridges. Recoveries of the drugs are plotted vs. the percentage of methanol in the elution solvent.

were tested with urine samples and it was concluded that the washing, as well as the elution using mixtures buffered at pH 2, produced the cleanest extracts. However, this procedure was not still good enough since the region of the chromatogram where cilazaprilat eluted was not completely free from endogenous compounds.

It was observed that fewer compounds of the matrix were retained on the cartridge when the sample was applied at pH 9, thus this value was used. In addition, when the urine is mixed with this buffer, many endogenous compounds are precipitated and separated.

In order to improve the clean-up procedure, it was observed that water was a very efficient solvent to remove some interfering compounds, especially those appearing during the first minutes of the chromatogram. A measure of 3 ml of water was optimal.

Several organic solvents and mixtures were tested as an additional washing step prior to the elution with the mixture of methanol–buffer at pH 2. The use of non-polar solvents such as hexane, ether or ethyl acetate did not elute the compounds of interest, but few improvements were achieved in the chromatogram. However, the most polar solvents, such as acetone, methanol or acetonitrile, produced the partial elution of the analytes, producing a loss of recovery, as well as of a great number of endogenous compounds. Nevertheless, it was found that if the cartridge was dried before the washing with acetonitrile, this was still able to elute interfering compounds and, however, it did not elute cilazapril and cilazaprilat. The drying was performed passing air at full vacuum through the column for at least 30 min. In order to accelerate the process, 2 ml of hexane were added meanwhile, and 15 min of drying were enough. Fig. 3 shows the influence on drying time of the recovery of quinapril and quinaprilat.

These facts also seem to confirm the ionic component of the interaction between the analytes and the surface of the column, since the compounds are not eluted with the use of a non-polar solvent. C_8

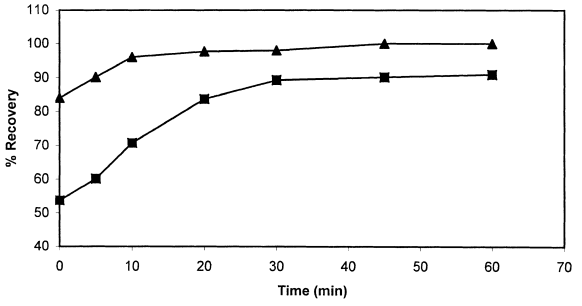

Fig. 3. Influence of the drying time of the extraction cartridge on the recovery of cilazapril (▲) and cilazaprilat (■).

cartridges show significant ionic interactions because the shorter hydrocarbon chain does not mask the silica surface as effectively as in C_{18} cartridges [27].

Finally, a different elution solvent was investigated, mainly because the high proportion of aqueous solvent (40% of phosphate buffer) made the evaporation of the eluate slow and expensive. Solvents reported to elute the ACE inhibitor quinapril [28,29] from the cartridge are mixtures of chloroform and an alcohol (butanol and methanol). Mixtures chloroform–methanol were assayed, but produced the elution of many endogenous compounds due to the use of a very polar solvent, methanol. Previous research in our laboratory with β-blockers led us to use 2-propanol as alcoholic solvent [30]. Different proportions of chloroform and 2-propanol were tested, and the mixture one-to-one in volume produced the greatest recoveries, although it did not get a good recovery for cilazapril that remained partially adsorbed on the cartridge. The addition of a small amount of water to the mixture produced improved recoveries. Moreover, this eluent gave rise to chromatograms with a lower number of interferences than the mixture methanol–phosphate buffer previously used, and had the advantage of its rapid evaporation. Fig. 4 shows a chromatogram of a spiked urine sample using the phosphate–methanol mixture and the finally selected elution solvent.

Due to the structural similarities among ACE inhibitors, several drugs and metabolites of this group were tested as internal standards (quinapril, quinaprilat, enalapril maleate, enalaprilat, fosinopril, fosinoprilat, ramipril, ramiprilat, lisinopril, captopril). Enalapril maleate was found to be the best, because it was the only one that gave rise to a narrow peak separated from the peaks of the analytes. In addition, a very similar behaviour in terms of recovery and reproducibility was found. Maleic acid was lost during the clean-up step and did not interfere.

This improved sample preparation has been also applied to the chromatographic system using amperometric detection [12], yielding excellent results and chromatograms free of interferences. Fig. 1 also shows the chromatograms obtained from the same spiked blank urine sample using amperometric (c) and photometric detection (d) after being extracted with the method described in this paper.

3.3. Quantitative determination

The relative standard deviation of the peak retention times was less than 1%, thus indicating the stability of the system. A calibration curve for both drugs in urine was done, using spiked blank urine samples, extracting them using the method developed and injecting the resulting solutions in the chromatographic system. The ratios of peak areas of the analytes divided by the area of the internal standard were plotted against the spiked concen-

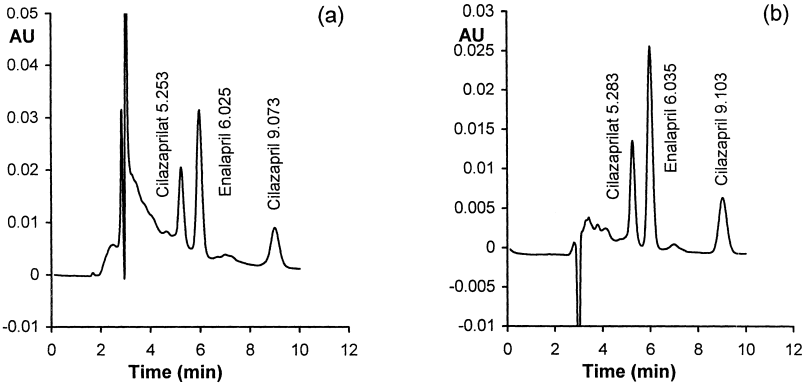

Fig. 4. Chromatograms of a urine sample spiked with 2 μg/ml of cilazapril and cilazaprilat and 4 μg/ml of enalapril maleate eluted with a mixture 0.1 *M* phosphate buffer at pH 2–methanol (20:80 v/v) (a), and with a mixture 2-propanol–chloroform (1:1 v/v) containing 2% of water (b). Mobile phase: methanol-10 m*M* phosphoric acid (50:50 v/v); flow-rate 1.0 ml/min; column temperature 30°C; detection wavelength 206 nm.

Table 2
Recovery percentages of the solid–liquid extraction of cilazapril and cilazaprilat at three concentration values[a]

Cilazaprilat		Cilazapril	
Concentration (μg/ml)	% Recovery	Concentration (μg/ml)	% Recovery
0.5	87.8±2.6	0.5	104.5±3.2
4	93.2±2.4	1	95.9±3.0
10	91.8±1.5	2	98.5±4.1

[a] Mean value±SD of 5 days.

tration. At the quantitation limit the relative error was 18.6 and 1.5% and the %RSD of six determinations was 5.5 and 8.8 for cilazaprilat and cilazapril respectively. The quantitative parameters obtained for the determination of cilazapril and cilazaprilat in urine are collected in Table 1.

Good recoveries were obtained for both drugs, especially for cilazapril (nearly 100%) at all concentrations tested. Data are collected in Table 2.

3.4. Analytical applications

The method was applied to the determination of cilazapril and cilazaprilat in urine samples obtained from three hypertensive patients at four time intervals after intake of a daily therapeutic dose of Inhibace, cilazapril 5 mg.

Table 4
Total amounts (mg) of cilazapril and cilazaprilat excreted in the urine of three hypertensive patients under continuous treatment with Inhibace: cilazapril 5 mg[a]

Patient	Cilazaprilat		Cilazapril	
	PD	ED	PD	ED
1	4.54	4.50	0.65	0.65
2	2.72	2.69	0.50[b]	0.13
3	3.12	3.28	0.45	0.51

[a] The urine was collected during 24 h after ingestion of one therapeutic dose.
[b] Presence of an interfering compound. PD: photometric detection. ED: electrochemical detection.

Results obtained using both detectors are collected in Table 3. Generally cilazaprilat is detected at all time intervals, and its maximum excretion occurs 1–4 h after the intake for patients ≠1 and ≠2 and 4–8 h for patient ≠3. Cilazapril is totally metabolised and its concentration is below the detection limit in the last time interval of urine collected. Great variations in terms of concentration and excretion of the drugs have been found among the different patients. In Table 4 the total amounts of cilazapril and cilazaprilat excreted in urine are collected. For

Table 3
Determination of cilazapril and cilazaprilat using photometric detection (PD) and electrochemical detection (ED) in the urine of three hypertensive patients at different time intervals after the ingestion of one dose of the pharmaceutical formulation Inhibace: cilazapril 5 mg[a]

Patient	Fraction (h)	Urine volume (ml)	[Cilazaprilat] (μg/ml)		[Cilazapril] (μg/ml)	
			PD	ED	PD	ED
1	0–1	210	1.78	1.96	0.40	0.45
	1–4	450	6.65	6.63	1.11	1.11
	4–8	330	1.66	1.65	0.22	0.19
	8–24	840	0.76	0.68	n.d.	n.d.
2	0–1	220	n.d.	n.d.	n.d.	n.d.
	1–4	180	10.7	10.7	2.56[b]	0.72
	4–8	280	2.20	2.26	0.15[b]	n.d.
	8–24	600	0.28	0.22	n.d.	n.d.
3	0–1	520	n.d.	n.d.	n.d.	n.d.
	1–4	600	n.d.	n.d.	n.d.	n.d.
	4–8	320	6.34	6.75	1.40	1.61
	8–24	600	1.81	1.87	n.d.	n.d.

[a] Mean value of two extractions. (n.d.=not detected).
[b] Presence of an interfering compound.

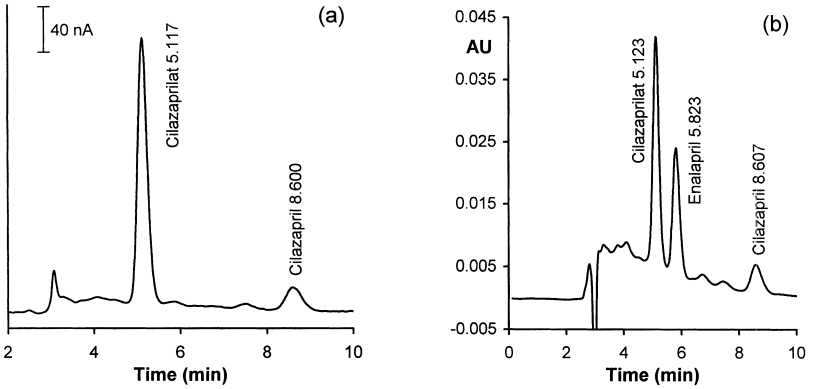

Fig. 5. Chromatograms of a urine sample obtained from patient # 3, 4–8 h after oral intake of Inhibace, cilazapril 5 mg, using amperometric (a) and photometric (b) detection. Mobile phase: methanol-10 m*M* phosphoric acid (50:50 v/v); flow-rate 1.0 ml/min; column temperature 30°C; detection wavelength 206 nm, detection potential 1350 mV vs. Ag/AgCl.

patients 2 and 3 the amount excreted is lower than the therapeutic dose whereas in the case of patient 1, the amount is slightly higher than 5 mg.

In order to compare both kinds of detection, the amperometric detector was connected to the outlet of the photometric detector and worked simultaneously. Fig. 5 shows the chromatogram using both types of detection of the sample that corresponds to patient #3, 4–8 h after the intake of the tablet. The quantitative results were similar using both kinds of detection, with differences of less than 5% in the case of cilazaprilat. The presence of an interfering compound for cilazapril was found in the photometric chromatogram of patient #2, 1–4 h and 4–8 h after the intake. This interference could be noticed by comparing the spectra of cilazapril and the unknown compound, which do not exhibit a shoulder at 206 nm. This interfering compound was not electroactive. Since the volunteer was under treatment with other drugs, this peak might be attributed to any of them, but no further identification was tried. Several other blank urine samples were also extracted, but no peak was found at that retention time.

Five different pharmaceutical formulations containing cilazapril were analysed. Values found were in accordance with the labelled by the pharmaceutical company except for Inhibace, cilazapril 5 mg. This formulation had expired 3 years ago and a considerable amount of cilazaprilat is found. Table 5 shows the results of the analysis for two different groups of tablets.

4. Discussion

This chromatographic method has been capable to determine cilazapril and cilazaprilat during at least 24 h after the intake of the drug. It has also been applied to pharmaceutical formulations without any

Table 5
Results of the analysis of five pharmaceutical formulations containing cilazapril[a]

Pharmaceutical formulation	mg certified	mg found[b]
Inocar	1	0.99±0.02; 0.98±0.02
Inocar plus	5	5.03±0.06; 5.03±0.06
Inhibace	1	0.99±0.02; 1.00±0.02
Inhibace plus	5	4.99±0.06; 5.00±0.06
Inhibace (expired)	5	4.69±0.06; 4.61±0.06

[a] Two different samples, three injections of each sample.
[b] Expressed as mean value±SD. $n=3$.

interference of their excipients or hydrochlorothiazide in the case of Inhibace Plus and Inocar Plus. The simultaneous determination of cilazapril and hydrochlorothiazide has not been possible due to the low retention of the latter using these chromatographic conditions.

Comparing both types of detectors, amperometric and photometric, it can be concluded that the advantages of the photometric detection are derived of its easier use and operation, whereas electrochemical detection is more sensitive and selective for the analysis of urine samples, as can be observed in Fig. 1.

Amperometric detection can be significantly affected by substances adsorbed on the electrode surface that can strongly affect the inter-day reproducibility if periodic cleanses are not carried out. The high potentials used in the amperometric method (1350 mV) require long equilibration periods before achieving a stable baseline and thus fewer samples can be measured during a working session. On the other hand chromatograms obtained using this detection are much cleaner and allow the quantitation of slightly lower concentrations of the analytes (reported detection limits 50 ng/ml for cilazaprilat and 40 ng/ml for cilazapril) [12]. These detection limits could probably be significantly lowered using the improved clean-up procedure described in this paper. It can also be observed that the electrochemical detector is ''blind'' to enalapril maleate. A suitable internal standard for this detection could not be found among the rest of available ACE inhibitors.

Photometric detection permits a quicker method development since not many variables affect the UV spectra of these compounds. The detector is reliable and easy to use, and a stable baseline is achieved quickly. In addition, the high number of photometric detectors available in the analytical laboratories, clearly much more spread than the amperometric, encouraged us to find a method using this kind of detection. The low detection wavelength of the proposed method implies that a great number of endogenous compounds present in the sample will be detected. Moreover, the absorptivity of the drugs studied is not very high and thus the sample must be preconcentrated before its injection.

The comparison of the statistic parameters and test with those published in [12] do not show significant differences and they do not help decide which method is more appropriate.

It is worth mentioning the difficulties found to find a suitable clean-up procedure for these polar and ionic compounds. This has been, doubtless, the part of the analytical method that has taken more time to develop. It has probably been the main cause for the lack of suitable and direct analytical methods for this kind of compounds.

To sum up, the use of photometric detection is enough for the analysis of cilazapril in its pharmaceutical formulations, whereas amperometric detection seems to be advantageous for the analysis of urine samples, mainly due to its higher selectivity, although the use of this detector is usually more problematic and difficult.

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References

[1] I.L. Natoff, J.S. Nixon, R.J. Francis, L.R. Klevans, M. Brewster, J. Budd, A.T. Patel, J. Wenger, E. Worth, J. Cardiovasc. Pharmacol. 7 (1985) 569.
[2] J.R. Rosenthal, U. Osowski, Cardiology 87 (1996) 54.
[3] B.G. Reuben, H.A. Wittcoff, Pharmaceutical chemicals in perspective, John Wiley and Sons, New York, 1989.
[4] F. Deget, R.N. Brogden, Drugs 41 (1991) 799.
[5] M. Litter, Farmacología Experimental y Clínica, El Ateneo, Buenos Aires, 1986.
[6] M.R. Attwood, C.H. Hassal, A. Kröhn, G. Lawton, S. Redshaw, J. Chem. Soc. Perkin Trans. 1 (1986) 1011.
[7] I.L. Natoff, S. Redshaw, Drugs Future 12 (1987) 475.
[8] T. Szucs, Drugs 41 (Suppl. 1) (1991) 18.
[9] J. Essig, G.G. Belz, A. Wellstein, Br. J. Clin. Pharmac. 27 (1989) 217S.
[10] A.A. Ajayi, H.L. Elliott, J.L. Reid, Br. J. Clin. Pharmac. 22 (1986) 167.
[11] H. Tanaka, Y. Yoneyama, M. Sugawara, Y. Ohta, J. Pharm. Sci. 76 (1987) 224.
[12] J.A. Prieto, R.M. Jiménez, R.M. Alonso, J. Chromatogr. B 714 (1998) 285.

[13] N. Erk, F. Onur, Anal. Lett. 29 (1996) 1963.
[14] A. Gumieniczek, L. Przyborowski, Liq. J. Chromatogr. & Rel. Technol. 20 (1997) 2135.
[15] Y.C. Wong, B.G. Charles, J. Chromatogr. B 673 (1995) 306.
[16] G. Shen, T. Weirong, W. Shixiang, J. Chromatogr. 582 (1992) 258.
[17] K. Hayashi, M. Miyamoto, Y. Sekine, J. Chromatogr. 338 (1985) 161.
[18] A. Sioufi, F. Pommier, J. Chromatogr. 434 (1988) 239.
[19] H.J. Leis, M. Leis, W. Welz, J. Chromatogr. 529 (1990) 299.
[20] N. Goto, T. Kamata, K. Ikegami, J. Chromatogr. 578 (1992) 195.
[21] H. Shioya, M. Shimojo, Y. Kawahara, Biomed. Chromatogr. 6 (1992) 59.
[22] H.H. Maurer, T.K. Kraemer, J.W. Arlt, Ther. Drug Monit. 20 (1998) 706.
[23] J.C. Miller, J.N. Miller, Statistics for analytical chemistry, Ellis Horwood, London, 1984.
[24] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilberay, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, J. Pharm. Sci. 81 (1992) 309.
[25] T. Ezawa, M. Hashimoto, M. Abe, M. Nishigaya, Y. Tsuchiya, K. Sasai, Iyakuhin Kenkyu 20 (1989) 602.
[26] D.A. Ip, G.S. Brenner, Anal. Profiles Drug Substances 16 (1987) 207.
[27] K.C. Van Horne (Ed.), Sorbent extraction technology, Analytichem International, Harbor City, USA, 1990.
[28] J.J. Ferry, A.M. Horvath, M. Easton-Taylor, R.D. Toothaker, W.A. Colburn, J. Chromatogr. 421 (1987) 187.
[29] H. Hengy, M. Most, J. Liq. Chromatogr. 11 (1988) 517.
[30] C. Ceniceros, M.I. Maguregui, R.M. Jiménez, R.M. Alonso, J. Chromatogr. B 705 (1998) 97.