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Quantitative determination of the angiotensin-converting enzyme inhibitor cilazapril and its active metabolite cilazaprilat in pharmaceuticals and urine by high-performance liquid chromatography with amperometric detection

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Abstract

A rapid and simple high-performance liquid chromatographic method with amperometric detection has been developed for the quantitation of cilazapril and its active metabolite and degradation product cilazaprilat in urine and tablets. The chromatographic system consisted of a μBondapak C_{18} column, using a mixture of methanol–5 m*M* phosphoric acid (50:50, v/v) as mobile phase, which was pumped at a flow-rate of 1.0 ml/min. The column was kept at a constant temperature of (40±0.2)°C. Detection was performed using a glassy carbon electrode at a potential of 1350 mV. Sample preparation for urine consisted of a solid-phase extraction using C_8 cartridges. This procedure allowed recoveries greater than 85% for both compounds. The method proved to be accurate, precise and sensitive enough to be applied to pharmacokinetic studies and it has been applied to urine samples obtained from four hypertensive patients (detection limit of 50 ng/ml for cilazapril and 40 ng/ml for cilazaprilat in urine). Results were in good agreement with pharmacokinetic data.

Keywords: Cilazapril; Cilazaprilat; Angiotensin-converting enzyme

1. Introduction

Cilazapril, [1*S*-[1α,9α(*R**)]]- 9 -[[1-(ethoxycarbonyl) -3 - phenylpropyl]amino]octahydro - 10 - oxo - 6H - pyridazino[1,2a] [1,2]diazepine-1-carboxylic acid monohydrate is a potent and specific angiotensin-converting enzyme (ACE) inhibitor which lowers peripheral vascular resistance without affecting heart rate. It is used in the treatment of hypertension and congestive heart failure [1], and has been designed as a result of the knowledge of the binding functions for the active site of ACE [2,3]. The role of this kind of drug is to inhibit the last step of the biosynthesis of angiotensin II, a potent vasoconstrictor, and therefore, it causes a general vasodilatation and lowers blood pressure [3,4].

Following oral administration, cilazapril undergoes de-esterification in the liver to its active di-acid form, cilazaprilat. This metabolite is more polar than cilazapril, making it more difficult for the intestine to absorb, but cilazapril, although not active, is well absorbed orally [5]. Doses of cilazapril range from 0.5 to 5 mg per day. Maximum plasma concentration

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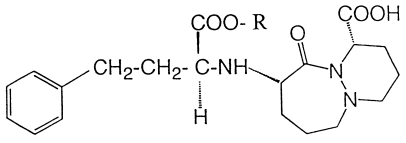
of unchanged cilazapril occurs within about 1 h, whereas peak plasma concentration of cilazaprilat occurs up to 3 h after oral administration. Around 57% of cilazapril is excreted in urine mainly as metabolised drug [6,7].



R=CH_2CH_3 Cilazapril
R=H Cilazaprilat

Analytical methods for the direct determination of this drug and its metabolite are very scarce. Analyses of the activity of these compounds have usually been carried out indirectly by measuring ACE inhibition, angiotensin II or renine levels [3,7,8]. Direct determination in plasma and serum has been carried out by enzyme immunoassay [9]. UV–Vis spectrophotometry has been applied to the determination of cilazapril in pharmaceutical formulations [10,11]. Electrochemical detection has only been applied to captopril, a thiol-containing ACE inhibitor and thus very easily oxidisable [12,13]. Other structurally similar ACE inhibitors are usually measured using GC–MS, but this involves two tedious derivatisation steps for the amine group and the carboxylic acid, that are very slow and can significantly affect the quality of the results [14–17].

The aim of this paper is to establish a validated chromatographic method with amperometric detection for the rapid separation and simultaneous quantitative determination of cilazapril and cilazaprilat in urine and dosage forms.

2. Experimental

2.1. Apparatus and column

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.

The electrochemical detector, EG&G Princeton Applied Research (Princeton, NJ, USA) Model 400 was equipped with a glassy carbon electrode, and operated at +1350 mV vs. a Ag/AgCl reference electrode and platinum auxiliary electrode in the DC mode, with a 5-s low-pass filter time constant. Chromatograms were recorded using Millennium Chromatography Manager (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 performed using Bond Elut 1 ml/100 mg C_8 cartridges (Varian, Harbor City, CA, USA) which were placed on a vacuum manifold system (Supelco, Bellefonte, PA, USA). The extracted urine samples were evaporated to dryness under a nitrogen stream using a Zymark Turbovap LV evaporator (Hopkinton, MS, USA).

Thin layer chromatography was performed using TLC aluminium sheets of silica gel 60 F_{254}, layer thickness 0.2 mm, with fluorescent indicator (Merck, Darmstadt, Germany).

^{1}H-NMR spectra were obtained using a Bruker (Rheinstetten-Forchheim, Germany) AC250 NMR spectrophotometer.

Elemental analysis was performed in a Perkin Elmer (Norwalk, CT, USA) 2400 CHN analyser.

2.2. Reagents and solutions

Cilazapril hydrate was kindly supplied by Roche Lab. (Barcelona, Spain), while cilazaprilat was prepared in our laboratory from cilazapril as described below. Solvents were HPLC grade (Lab-Scan, Dublin, Ireland). All reagents used were Merck Suprapur. Water was obtained from Milli-RO and Milli-Q Waters system.

Buffer solutions were made by preparing 0.1 *M* phosphoric acid and 0.5 *M* boric acid and the pH was adjusted to 2.0 and 9.0, respectively, using 3 *M* KOH.

A stock Britton–Robinson buffer solution, which contained 0.04 *M* of each of glacial acetic acid, phosphoric acid and boric acid was prepared. Buffer solutions were made by adding the necessary amount of 3 *M* KOH in order to achieve the appropriate pH value.

Stock solutions of cilazapril and cilazaprilat (200 μg/ml) were prepared in water and a mixture of

water–methanol (80:20, v/v), respectively, and stored at 4°C. Working solutions were prepared by appropriate dilution just before use.

2.3. Synthesis of cilazaprilat

Basic hydrolysis of cilazapril was carried out by heating 100 mg of cilazapril and 38 mg of KOH dissolved in 20 ml of methanol in reflux for 6 h. The course of the reaction was followed by thin-layer chromatography. The mobile phase consisted of chloroform–methanol–acetic acid (90:10:1, v/v/v). The corresponding R_F were 0.4 for cilazapril and 0.1 for cilazaprilat. Once the reaction finished, the solution was allowed to stay at room temperature for several minutes and drops of concentrated hydrochloric acid were added until complete precipitation of KCl was achieved. The solution was then filtered and the solvent was evaporated under a stream of nitrogen. A viscous yellowish liquid was obtained which was crystallised in water, yielding a white powder.

Characterisation of the synthesis product was carried out by ^{1}H-NMR and the spectrum compared with that of cilazapril, showing clearly the disappearance of the peaks corresponding to the ethyl group of the ester. Elemental analysis was also carried out, and good agreement with the expected results was obtained (calculated for $C_{20}H_{27}N_3O_5$: C 61.69, H 7.94, N 10.79, O 20.56; found: C 60.44, H 7.02, N 10.55, O 21.48). Purity was tested by the chromatographic method that will be described later, obtaining a single peak (t_R 5.12 min) both with amperometric and UV absorbance detection. In order to identify the compound obtained, the degradation conditions reported by Ezawa et al. [18] were assayed. A basic aqueous solution of cilazapril (pH 12) was allowed to stay at room temperature for one day, and then injected to the chromatographic system. One single peak was obtained at the same retention time than the synthesised compound, which Ezawa et al. [18] claimed to be cilazaprilat.

2.4. Procedure for tablets

Five different pharmaceutical formulations were analysed in this work: Inhibace (cilazapril 1 and 5 mg) and Inhibace plus (cilazapril 5 mg and hydrochlorothiazide 12.5 mg) commercialised by Andreu-Roche Lab. (Barcelona, Spain); Inocar (cilazapril 1 mg) and Inocar plus (cilazapril 5 mg and hydrochlorothiazide 12.5 mg) commercialised by Nezel (Barcelona, Spain).

Several tablets were weighed and then mixed and crushed into a fine powder in a mortar. A suitable amount of this powder was accurately weighed, dissolved in deionised water to a fixed volume and sonicated for 10 min. The solutions were centrifuged for 5 min at 800 *g*, decanted, and stored at 4°C. Aliquots of these concentrated solutions were diluted in water and measured under the selected chromatographic conditions. Calibration solutions were made by appropriate dilution in water of the concentrated standard solutions.

2.5. Clean-up procedure for urine samples

Urine samples were collected in plastic tubes and frozen until their analysis. Once thawed, two millilitres of human urine were alkalinised with 1 ml of 0.5 *M* borate buffer at pH 9. The mixture was shaken and centrifuged for 5 min at 800 *g*. The Bond Elut C_8 column was conditioned with 1 ml of methanol, 1 ml of water and 1 ml of 0.17 *M* borate buffer (dilution 1:3 of 0.5 *M* borate buffer). The column was not allowed to dry before the application of the sample (that consisted of 1.5 ml of alkalinised urine), which was slowly drawn through the column at very low vacuum or by gravity. The column was washed with 1 ml of 0.1 *M* phosphate buffer at pH 2 and 1 ml of hexane, drying at full vacuum (67 kPa) for 10 min. Another millilitre of hexane was then added, and the column completely dried at full vacuum for 5 min. A 0.5 ml volume of acetonitrile was then drawn through the column. The analytes were eluted with 1 ml of a mixture methanol–0.1 *M* phosphate buffer at pH 2.0 (60:40, v/v). The eluate was then evaporated to dryness at 60°C under a gentle stream of nitrogen. The remaining residue was dissolved in 1 ml of water and injected into the chromatographic system under the optimised chromatographic conditions. Standard solutions for calibration were made by spiking blank urine samples with different amounts of the stock solutions of cilazapril and cilazaprilat, and extracting them following this clean-up procedure.

2.6. Chromatographic conditions

The separation was performed using methanol–5 m*M* phosphoric acid (50:50, v/v) as a mobile phase, filtered through a 0.45-μm membrane and degassed by bubbling helium through. The column head pressure was maintained at 15 MPa at a flow-rate of 1.0 ml/min and the temperature was kept at 40±0.2°C. The injection loop volume was 20 μl.

2.7. Electrode maintenance

The working electrode was cleaned electrochemically every time that the sensitivity decreased, or a unstable baseline or drift was observed, by keeping it at −600 mV for 60 s and +1500 mV for 10 min, while passing methanol as mobile phase. If the problem persisted, the electrode was then hand-cleaned with a tissue wet with methanol to remove possible adsorbed molecules and rinsed with water to dissolve inorganic salts.

2.8. Quantitative determination

The reproducibility of the method was determined using two blank urine samples spiked with the standard solutions to obtain the concentrations of 0.5 and 4 μg/ml of both compounds. These two samples were extracted following the clean-up procedure developed. Three extraction replicates of each concentration level were performed during five different days. Reproducibility (inter-day) was calculated as the %R.S.D. of the average of the peak area for five days.

The extraction efficiency was estimated by measuring the peak areas obtained after extracting the spiked urine samples at two concentrations and comparing them with those obtained for standard solutions of the same concentration. Three replicates were done for each sample and the process was repeated for 5 days.

Accuracy was estimated at two concentrations by extracting spiked urine samples and comparing the concentrations obtained using the calibration curve with the spiked concentrations. Two replicates were made.

Three calibration curves in the range 50–1000 ng/ml were done in three different days. Noise was estimated from the standard deviation of the calibration. Detection and quantitation limits were calculated as the analyte concentrations giving rise to a signal-to-noise ratio of 3 and 10, respectively [19].

3. Results and discussion

Aqueous cilazapril and cilazaprilat solutions in Britton–Robinson buffers (pH 2–13) and 0.5 *M* KCl, did not show voltammetric peaks at a glassy carbon electrode using differential pulse voltammetry as electroanalytical technique. However, the application of amperometric detection coupled with the HPLC system to cilazapril and cilazaprilat, gave rise to chromatographic peaks with enough sensitivity for analytical purposes.

3.1. Optimisation of the chromatographic system

After choosing the adequate proportion of methanol–aqueous buffer to separate cilazapril and cilazaprilat in a short time (60:40, v/v), hydrodynamic voltammograms of both compounds at several pH values were carried out. The hydrodynamic voltammogram at pH 3.1 is shown in Fig. 1. A potential of 1350 mV was chosen, although it was not the most sensitive, gave rise to the best signal-to-noise ratio. Greater potentials caused peak deformation since a baseline drop was observed,

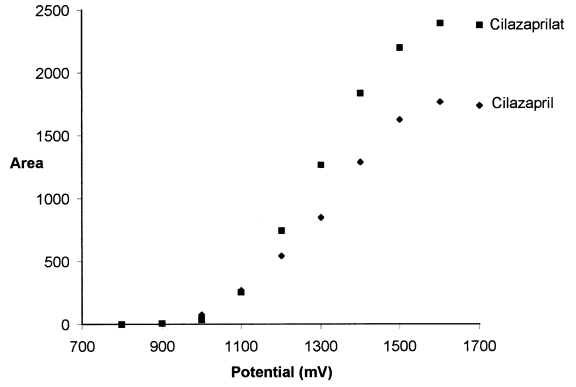


Fig. 1. Hydrodynamic voltammograms of cilazapril and cilazaprilat. Mobile phase: methanol–5 m*M* phosphoric acid (60:40, v/v) pH 3.1; flow-rate 1.0 ml/min; column temperature 40±0.2°C.

which made their correct integration difficult. The use of mobile phases containing acetonitrile as organic modifier, produced asymmetric and wider peaks than those obtained with methanol.

The influence of the pH on the retention time of both compounds was studied using 5 m*M* phosphate (pH 3–7.5) and 5 m*M* acetate buffer (pH 4.5–5.5). Acidic medium (pH 3.0) was found to be the most adequate for the separation of cilazapril and cilazaprilat, because the most polar compound, cilazaprilat, was separated from the injection peak, while the elution time of cilazapril was not too long (t_R 6.22 min), and a low baseline noise was observed.

Phosphoric acid was used as supporting electrolyte necessary for the amperometric detection. Different concentrations of the acid (1–10 m*M*) were tested which did not significantly affect the sensitivity and the resolution, but the baseline noise increased with concentration. A concentration of 5 m*M* was used throughout the work.

Influence of flow-rate and temperature was also studied. A flow-rate of 1.0 ml/min (pump pressure 15 MPa) was chosen. An increase of temperature caused a reduction of elution times and slightly better peak shapes maintaining adequate separation. 40°C was chosen as the optimal temperature.

This study was carried out with a mobile phase containing 60% of methanol. In order to test urine samples, it was necessary to change the proportion of methanol to 50%, to achieve a good separation of cilazaprilat from the endogenous compounds of urine which eluted in the first 4 min of the chromatogram and interfered with cilazaprilat.

During the study of optimisation of the chromatographic conditions, we have not observed peak tailing or splitting. Other ACE inhibitors show these phenomena under certain conditions due to the hindered rotation of the amide bound [20,21].

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

Due to the coexistence of a carboxylic acid and an amine group in the compounds, and knowing the pK_a values of cilazapril: 3.3 for carboxylic acid and 6.5 for the amino group [18], it is impossible to find a pH value at which the molecules are not charged, so liquid extraction was not retained as clean-up procedure. However, some assays were carried out using the methods reported to extract enalapril maleate, an ACE inhibitor that is extracted by forming a neutral ionic pair, without any interference of enalaprilat, its degradation product and metabolite [22]. The same behaviour was showed for cilazapril and cilazaprilat. Metabolites of these compounds were not extracted due to the additional negative charge, which do not allow them to reach neutrality.

Thus, as expected, solid-phase extraction resulted to be a more adequate clean-up procedure. Our first attempt was to use ion-exchange cartridges, since the molecule can be positively, negatively charged or zwitterion depending on the pH value. We found problems to elute both compounds once adsorbed on the cartridge surface, thus this procedure was not used.

Finally, the use of nonpolar cartridges was assayed (C_2, C_8, C_{18}, CN, PH, CH). C_8 cartridges were chosen since they completely retained both drugs with a minimum number of endogenous compounds. The optimal pH value for applying the sample was 9, since more acidic pH retained more endogenous compounds and pH values higher than 9 did not efficiently retain the drugs. An acidic washing of the cartridge was chosen to elute basic interferences. This washing was not sufficient to eliminate interfering compounds of cilazaprilat, and a more selective washing step had to be found. Acetonitrile was selected as washing solvent, but previously the cartridge had to be completely dried, if not, a considerable amount of the drugs was lost.

Different proportions of methanol–buffer mixtures were tested as elution solvents. The minimum proportion of methanol that completely eluted both compounds was 60%. The buffer pH selected was 2, as endogenous compounds tend to remain adsorbed on the surface at acidic pH values.

3.3. Quantitative determination

The relative standard deviation of the retention times was less than 2%, thus indicating high stability of the system. A calibration curve for both compounds using spiked blank urine samples was made. Peak areas were plotted against concentration obtaining a linear dependence for both compounds and recoveries greater than 85%. The quantitative and

Table 1
Quantitative parameters obtained for the analysis of cilazapril and cilazaprilat in urine

	Cilazapril	Cilazaprilat
Retention time (min)	5.12±0.02	8.44±0.04
Linear range	At least up to 8 μg/ml	At least up to 8 μg/ml
Calibration range (ng/ml)	0.2–2	0.5–8
Slope of the calibration curve ($n=6$)	2134±33	2554±35
Intercept ($n=6$)	55±38	129±156
Regression coefficient of the calibration curve (r^2)	0.999	0.999
% Recovery (mean value of 5 days) ±S.D.	89.4±4.0[a]	85.2±1.7[a]
	89.3±7.2[b]	86.1±3.5[b]
Reproducibility intra-day	4.0[a]	4.6[a]
(3 replicates, 5 days) (%R.S.D.)	1.6[b]	1.9[b]
Reproducibility inter-day	6.3[a]	4.3[a]
(5 days) (%R.S.D.)	7.7[a]	4.9[b]
Accuracy (spiked; found) (ng/ml); % relative	0.48; 0.46; −4.2	0.52; 0.49; −6.1
error 2 replicates	1.99; 1.97; −1.0	5.72; 5.81; 1.6
Detection limit (ng/ml) ($S/N=3$)	50	40
Quantitation limit (ng/ml) ($S/N=10$)	170	140

[a] Concentration of the spiked urine sample 0.5 μg/ml.
[b] Concentration of the spiked urine sample 4 μg/ml.

statistic parameters obtained are presented in Table 1. For tablets, the recovery for cilazapril was total and the chromatograms were as those obtained for standard solutions in water.

3.4. Analytical applications

The method was applied to the analysis of cilazapril and cilazaprilat in urine samples obtained from four hypertensive patients treated with a daily dose of 5 mg of cilazapril, and to pharmaceutical formulations containing cilazapril.

Urine samples of the four patients were collected at different time intervals after the administration of Inhibace, cilazapril 5 mg. Results are summarised in Table 2. Fig. 2 shows chromatograms of a blank urine sample and one obtained 4–8 h after the administration of Inhibace to a hypertensive patient. Values obtained were in accordance with pharmacokinetic studies. In general, cilazapril is excreted mainly in the first 4 h, disappearing progressively due to its transformation into cilazaprilat. This active antihypertensive agent remains being excreted in the last time interval studied (8–24 h).

Five different pharmaceuticals containing cilazapril were analysed. Values obtained were in accordance with those certified by the pharmaceutical company except for Inhibace, cilazapril 5 mg, because this formulation was already expired. In this case a considerable amount of cilazaprilat was found. Results are listed in Table 3. The chromatograms of the formulation Inhibace, cilazapril 1 mg, and the expired Inhibace, cilazapril 5 mg, are shown in Fig. 3.

4. Conclusions

The proposed method has proved to be useful for the quantitative determination of cilazapril and cilazaprilat in urine. It can be used for pharmacokinetic purposes, allowing the quantitation of the drugs at all time intervals studied.

To the best of our knowledge, this is the first time that an ACE inhibitor different from captopril has been quantified in urine by a chromatographic method. The high polarity and the ionic nature of this kind of compounds make the selective cleaning-up of the sample difficult, which probably has been the main obstacle to their determination in biological samples.

GC–MS is the usual technique for the determination of this kind of compound, but the method

Table 2
Determination of cilazapril and cilazaprilat in the urine of four hypertensive patients after the ingestion of one dose of the pharmaceutical formulation Inhibace: cilazapril 5 mg

Patient No.	Fraction (h)	Volume (ml)	[Cilazapril] (μg/ml)	[Cilazaprilat] (μg/ml)
1	0–1	310	0.47	1.93
	1–4	275	0.94	5.66
	4–8	275	0.19	3.74
	8–24	480	not detected	1.27
2	0–1	175	not detected	0.13
	1–4	120	0.41	0.95
	4–8	260	0.71	6.39
	8–24	850	not detected	0.33
3	0–1	250	0.08	not detected
	1–4	210	1.14	3.45
	4–8	140	0.25	3.71
	8–24	580	not detected	0.38
4	0–1	100	not detected	0.29
	1–4	270	0.54	5.13
	4–8	825	0.58	1.26
	8–24	1800	not detected	0.16

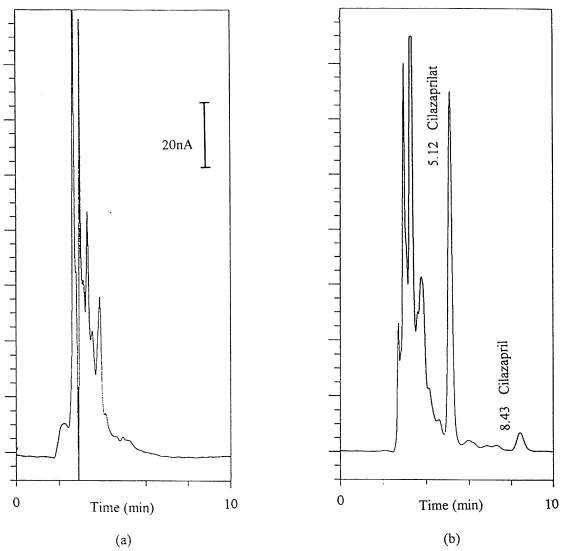


Fig. 2. Chromatograms of (a) blank urine sample, and (b) urine sample obtained from patient #2, 4–8 h after administration of one tablet of Inhibace 5 mg (concentration of cilazaprilat 6.39 μg/ml, concentration of cilazapril 0.71 μg/ml). Mobile phase: methanol–5 m*M* phosphoric acid (50:50, v/v); flow-rate 1 ml/min; column temperature 40°C; potential 1350 mV.

Table 3
Results of the analysis of five pharmaceutical formulations containing cilazapril

Pharmaceutical formulation	mg certified	mg found[a]
Inocar	1	1.00±0.01
Inocar plus	5	5.00±0.05
Inhibace	1	1.01±0.01
Inhibace plus	5	5.00±0.05
Inhibace (expired)	5	4.72±0.05

[a] Expressed as mean value±S.D., $n=3$.

developed is much shorter and easier, and no derivatisation step is required.

Several other ACE inhibitors have been tested during this work in the same chromatographic conditions, but all, except captopril, gave rise to peaks which could not be used for quantitative purposes in biological samples. The use of UV absorbance detection is now being investigated in order to determine other drugs of this family.

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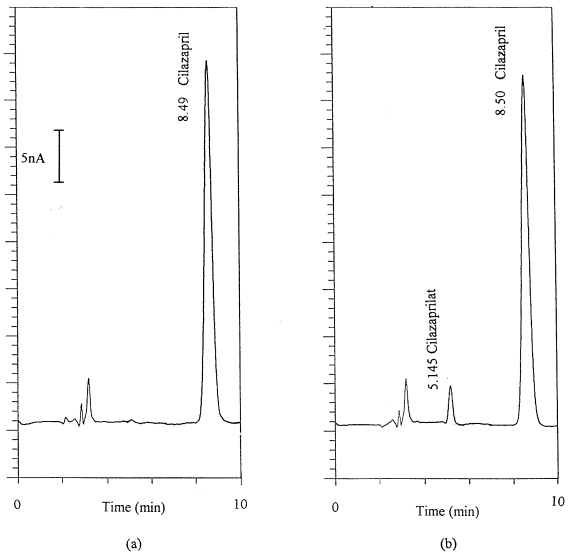

Fig. 3. Chromatograms of solutions: (a) the pharmaceutical formulation Inhibace, cilazapril 1 mg, and (b) the expired formulation Inhibace, cilazapril 5 mg. Final concentration of cilazapril 4 μg/ml. Mobile phase: methanol–5 m*M* phosphoric acid (50:50, v/v); flow-rate 1.0 ml/min; column temperature 40°C; potential 1350 mV.

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