Solid-Phase Extraction and High-Performance Liquid Chromatography Applied to the Determination of Quinapril and Its Metabolite Quinaprilat in Urine

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Abstract

Quinapril is an antihypertensive drug that belongs to the family of angiotensin-converting enzyme inhibitors. It is metabolized to quinaprilat, which is the compound that is really responsible for the therapeutic action. In this study, a rapid and simple liquid chromatographic method with photometric detection is described and applied to the determination of quinapril and quinaprilat in urine. The cleanup procedure for the urine samples consists of a solid–liquid extraction using C_8 cartridges. Under these conditions, both compounds and the internal standard (enalapril maleate) are separated in less than 9 min. Recoveries for quinapril and quinaprilat are greater than 80%. The method is sensitive enough (detection limit of 60 ng/mL for quinapril and 50 ng/mL for quinaprilat) to be applied for the determination of quinapril and quinaprilat in urine samples obtained from four hypertensive patients after the intake of a therapeutic dose.

Introduction

Quinapril HCl, also known as 2-{2-[[1-ethoxycarbonyl-3-phenylpropyl]amino]-1-oxopropyl}-1,2,3,4-tetrahydro-3-isoquinoline carboxylic acid hydrochloride, is a nonsulphydryl angiotensin-converting enzyme (ACE) inhibitor prodrug that is metabolized to quinaprilat, which is an active diacid metabolite (1) (Figure 1). ACE generates the potent vasoconstrictor substance angiotensin II by removing the carbon terminal dipeptide from the precursor decapeptide angiotensin I (2,3). This kind of drug surged in the 1970's with the introduction of the drug captopril (2,4). The side effects of captopril (such as loss of taste and cough) encouraged the development of new structures lacking the thiol function. Several ACE inhibitors have been designed with structural differences in order to obtain the optimal therapy (5,6). ACE inhibitors have not been shown to significantly affect the quality of life in contrast to the results reported with the use of diuretics, calcium antagonists, or thiazide diuretics (7). Recently, it has been reported that ACE inhibitors may protect against cancer (8).

One daily administration of quinapril (10 to 40 mg) provides blood-pressure control throughout the 24-h dosage interval. Approximately 60% of the dose is absorbed. Following oral administration, quinapril is converted by de-esterification in the liver to quinaprilat (the active diacid metabolite) and two minor inactive diketopiperazine metabolites. The mean elimination half-lives are approximately 1 and 2 h for quinapril and quinaprilat, respectively, although the strong tissue binding affinity offers long-term activity. Approximately 30% of a dose is recovered in urine as quinaprilat, and only 3% of a dose is recovered as unchanged quinapril (1,9,10).

The determination of this drug and its metabolite in urine and plasma has been carried out by gas chromatography (GC) with electron-capture detection (11) and mass spectrometry (MS) (12) after the derivatization of the polar groups. Precolumn fluorescent derivatization has been applied to the analysis of these compounds in plasma and urine using high-performance liquid chromatography (HPLC) (13). Kugler et al. (14) proposed an HPLC system with radiochemical detection that allows for the chromatographic separation of quinapril, quinaprilat, and two inactive diketopiperazine metabolites that

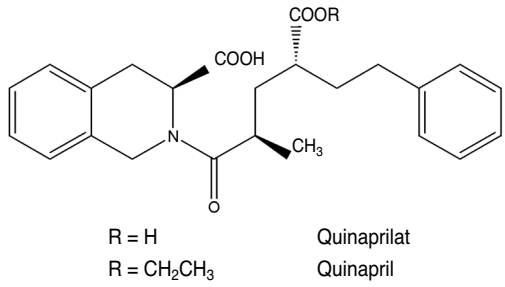

Figure 1. Chemical formula for quinaprilat and quinapril.

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have been applied to their determination in perfusate, urine, and perfusate ultrafiltrate.

Quinapril has also been included in other works related to the chromatographic behavior of several ACE inhibitors by HPLC with photometric detection (15) and the screening of this kind of drug in urine by GC–MS after extractive methylation (16).

The analysis of this antihypertensive agent in pharmaceutical dosage forms has been carried out by HPLC with photometric detection (17,18) and derivative UV spectroscopy (18).

The aim of this study is to establish a validated liquid chromatographic method with photometric detection for the rapid separation and quantitation of quinapril and quinaprilat in urine without the previous derivatization of the molecules.

Experimental

Apparatus and column

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

Detection was performed using a Waters 996 photodiode-array detector. Chromatograms and spectra were collected every second in the range of 190 to 300 nm using Millennium Chromatography Manager software (Waters). For quantitative purposes, detection was performed at 206 nm.

A µBondapak C_{18} HPLC column (300- × 3.9-mm i.d., 10-µm particle size) coupled to a µBondapak C_{18} Guard-pak HPLC precolumn insert (Waters) was used. The column was kept at constant temperature using a Waters TMC temperature-control system.

Solid-phase extractions were performed using Bond Elut 1-mL/100-mg C_8 cartridges (Varian, Harbor City, CA), which were placed on a vacuum manifold system (Supelco, Bellefonte, PA). The extracted urine samples were evaporated to dryness under a nitrogen stream using a Zymark (Hopkinton, MS) Turbovap LV evaporator.

The pH measurements were made by means of a Radiometer (Copenhagen, Denmark) PHM84 research pH meter equipped with a Crison (Alella, Spain) 52 09 pH electrode.

Reagents, chemicals, and solutions

Quinapril hydrochloride and quinaprilat hydrate were a kind gift from Parke-Davis (Barcelona, Spain). Enalapril maleate (used as the internal standard) was obtained from Sigma (Madrid, Spain). Solvents were of HPLC grade (Lab-Scan, Dublin, Ireland). All reagents used were obtained from Merck (Darmstadt, Germany) and were of pro analysis quality. Water was obtained from Milli-RO and Milli-Q systems (Waters).

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

Stock solutions of quinapril and enalapril maleate (200 µg/mL) were prepared in water, and the stock solution of quinaprilat (200 µg/mL) was prepared in methanol. All solutions were stored in darkness at 4°C, and working solutions were prepared by an appropriate dilution just before use.

Collection of urine samples

Blank urine samples were obtained from several healthy volunteers.

Urine produced in 24 h was completely collected in plastic bottles at four time intervals after the ingestion of a therapeutic dose, which were 0–1 h, 1–4 h, 4–8 h, and 8–24 h. The urine was transferred to 10-mL plastic tubes and immediately frozen and stored at –20°C.

The urine samples used in this study were obtained from four hypertensive patients that were under continuous treatment with the drug. Patients #1 and #2 (both males and 43 years old) were prescribed Ectren, which contained 20 mg of quinapril. Patients #3 and #4 were under treatment with Lidaltrin (5 mg of quinapril). Patient #3 was male (58 years old) and patient #4 female (84 years old). The latter was also under treatment with the cardiotonic digoxine, the diuretic chlortalidone, the laxative Plantaben, and the benzodiazepine lormetazepam. The patients were not asked to be on a special diet or modify their habits.

Cleanup procedure for urine samples

Once thawed, 2 mL of urine were buffered with 1 mL of a 0.5M borate buffer at pH 9, and 60 µL of the stock solution of the internal standard enalapril maleate was added before the extraction. Any precipitate present was centrifuged at 800 g for 5 min. A Bond Elut C_8 cartridge was conditioned with 1 mL of methanol, 1 mL of water, and 1 mL of 0.17M borate buffer at pH 9, which was prepared by diluting in water the 0.5M borate buffer (1:2, v/v). The column was not allowed to dry before the application of the sample consisting of 2 mL of the buffered sample, which was passed slowly through the cartridge (flow rate approximately 0.5 mL/min). The endogenous compounds that were retained were selectively washed in two steps. The first step consisted of passing 1 mL of 0.1M phosphate buffer (pH 2)–methanol (7:3, v/v), 3 mL of water, and then another milliliter of the 0.1M phosphate buffer (pH 2)–methanol mixture (7:3, v/v). The column was allowed to dry at full vacuum for 15 min. A second step was completed with the addition of 1 mL of acetonitrile. The analytes were eluted with 2 mL of a chloroform–isopropanol–water mixture (25:25:1, v/v/v). The eluate was evaporated to dryness under a stream of nitrogen, and the residue was dissolved in 0.5 mL of deionized water and injected in the chromatographic system. A concentration factor of 2.6 was achieved during the process.

Chromatographic conditions

The separation of the two compounds and the internal standard was performed with a mobile phase consisting of 1-propanol–acetonitrile–0.01M phosphoric acid (20:15:78, v/v/v). The mobile phase was previously filtered by a 0.45-µm pore membrane and degassed by passing helium through it. The column head pressure was 20 MPa at a flow rate of 1 mL/min. The column temperature was kept at 30°C ± 0.2°C. The injection loop volume was 20 µL and was totally filled with the sample. The detection wavelength was set at 206 nm.

Quantitative determination and validation

Standard solutions for the calibration curve were prepared by spiking blank urine samples with different amounts of quinapril, quinaprilat, and the internal standard enalapril maleate (the final concentration in the urine sample was 6 µg/mL). The solutions were extracted following the cleanup procedure described previously. The calibration curve was plotted by representing the ratio of the peak area of the urinary quinapril and quinaprilat with the peak area of the internal standard versus concentration. The interval of calibration was determined by the amounts of quinapril and quinaprilat that were expected in the urine samples, taking into account the reported percentages of excretion. The method of least-squares linear regression was applied and unknown concentrations calculated by interpolation.

The reproducibility of the method was determined at two concentration levels. Two spiked urine solutions were prepared—one containing 0.5 µg/mL of quinapril and quinaprilat and 6 µg/mL of enalapril maleate and the other containing 6 µg/mL of quinaprilat and enalapril maleate and 2 µg/mL of quinapril. The spiked solutions were treated as if they were unknown samples and frozen in 10-mL plastic tubes. Each day, one of the tubes of each concentration level was thawed, and three replicates (extractions) of each solution were performed. The process was repeated for 5 days. Data were collected as the ratio of the peak area of quinapril and quinaprilat versus the peak area of the internal standard. Intraday and interday reproducibility were calculated using the analysis of variance (ANOVA) method (19).

Recovery was calculated by comparing the peak areas of quinapril and quinaprilat that were obtained after extraction with the peak areas of standard solutions of the same concen-

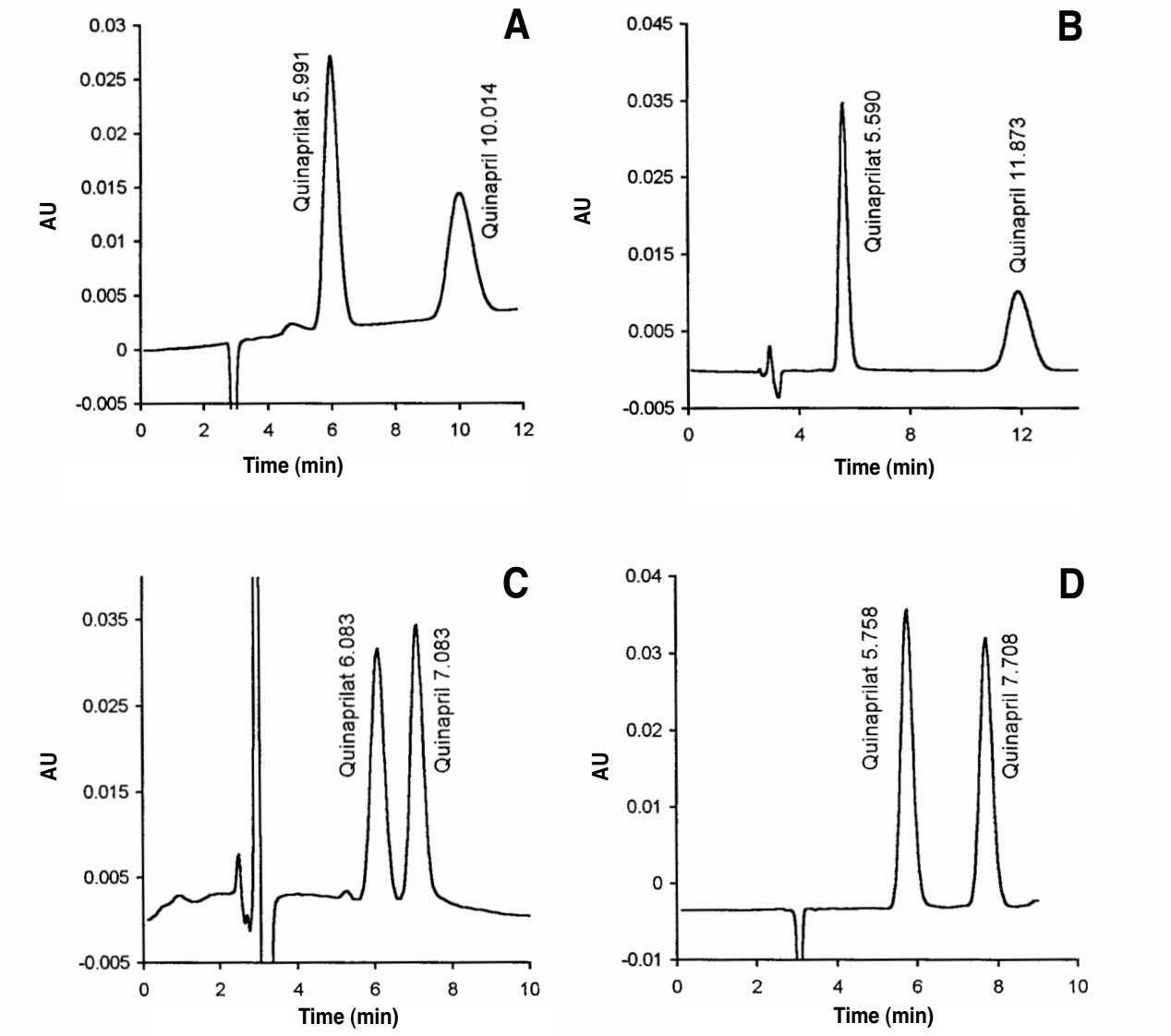

Figure 2. Influence of the organic modifier of the mobile phase on the chromatographic separation of quinapril and quinaprilat: (A) methanol–10mM phosphoric acid (5:4, v/v), (B) acetonitrile–10mM phosphoric acid (10:17, v/v), (C) *n*-propanol–10mM phosphoric acid (1:3, v/v), and (D) 1-propanol–acetonitrile–10mM phosphoric acid (20:15:78, v/v/v). The flow rate was 1 mL/min, column temperature 30°C ± 0.2°C, and detection wavelength 206 nm.

tration in water, taking into account that the extract was concentrated 2.6 times during its treatment.

Accuracy was estimated by extracting spiked urine samples at two concentration levels and comparing the spiked concentration with the one obtained using the calibration curve. Two replicates (extractions) were done.

Three calibration curves were made in the interval of 50 to 1000 ng/mL for each analyte for 3 days. Noise was estimated from the mean standard deviation of the regression during the 3 days. Detection and quantitation limits were obtained as the analyte concentrations giving rise to a signal-to-noise ratio of 3 and 10, respectively (19).

Results and Discussion

Optimization of the chromatographic system

The UV spectra of these drugs showed a maximum absorption band at 194.6 nm for quinaprilat and quinapril as well as a shoulder at 206 nm. Although the maximum sensitivity was not achieved, a better signal-to-noise ratio was obtained at the higher wavelength and thus was employed throughout this work.

ACE inhibitors are usually analyzed using acidic mobile phases. This is because of the proline residue present in the molecule. The relaxation time for *cis-trans* isomerization around the amide bound is of the same time-scale as the retention time in HPLC of these peptides and may show peak splitting. This effect is minimized at low pH values or at high temperature (15,20).

Our previous experience with cilazapril and its metabolite (21,22) showed that in order to avoid interfering peaks from endogenous compounds present in urine, the first peak (in this case quinaprilat) should elute at a retention time higher than 5 min at a flow rate of 1.0 mL/min. Different proportions of organic modifier–buffer were assayed in order to keep this requirement.

The first organic modifier tested was methanol mixed with phosphate or acetate buffers at different pH values (2.5 to 6) in a proportion of 5:4 (v/v). Quinaprilat was more retained at the most acidic pH value, and quinapril had its maximum retention at pH 3.7 (11 min). The acidic pH value provided by phosphoric acid was the best option because at this pH value quinaprilat was more strongly retained and the last peak (quinapril) eluted at a shorter retention time. The second organic modifier tested was acetonitrile in acetonitrile–buffer (10:17, v/v). It produced similar results in terms of a dependence of the retention time with the pH value; but in this case, a better peak shape was obtained for quinaprilat in comparison with the use of methanol as the organic modifier. However, very broad peaks were obtained for quinapril in both cases that were not suitable for their determination because of the low concentrations expected in urine samples.

Barbato et al. (15) have shown that the use of 1-propanol as an organic modifier in the HPLC screening of ACE inhibitors is convenient in terms of resolution and peak shape. The use of 1-propanol was tested as the organic modifier in a

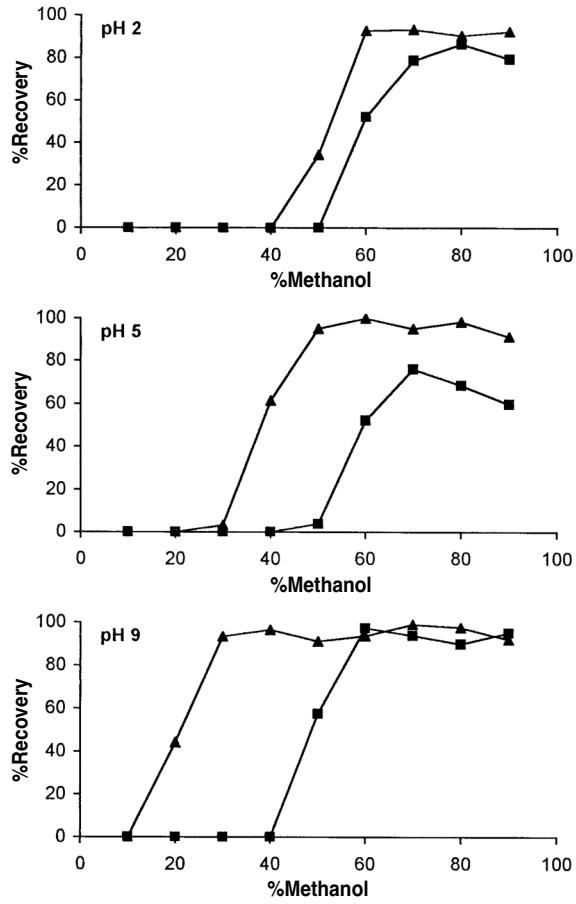

Figure 3. Elution profiles of quinapril (■) and quinaprilat (▲) at three pH values on C_8 cartridges. Recoveries of the drugs were plotted versus the percentage of methanol in the elution solvent.

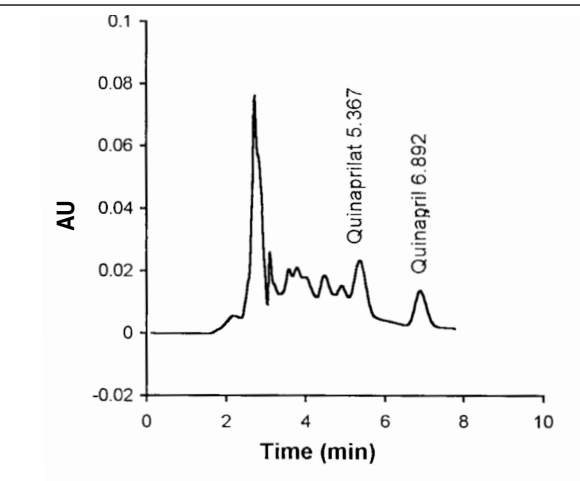

Figure 4. Chromatogram of a spiked urine sample after extraction with a C_8 cartridge using phosphoric acid–methanol (4:1, v/v) as the washing solvent and (3:7, v/v) as the elution solvent. The mobile phase was 1-propanol–acetonitrile–10mM phosphoric acid (20:15:78, v/v/v), flow rate 1 mL/min, column temperature 30°C ± 0.2°C, and detection wavelength 206 nm. The concentration of quinapril and quinaprilat was 2 µg/mL.

Table I. Quantitative and Statistical Parameters Obtained for the Analysis of Quinapril and Quinaprilat in Urine by HPLC–Photometric Detection

	Quinaprilat	Quinapril
Retention time (min), 6 injections	5.679 ± 0.002	7.604 ± 0.007
Calibration range (µg/mL)	0.5–20	0.5–4
Slope of the calibration curve (95%)	0.359 ± 0.008	0.342 ± 0.008
Intercept (95%)	–0.05 ± 0.08	–0.01 ± 0.03
Regression coefficient of the calibration curve	0.999	0.999
%Recovery (mean value of 5 days)	86.7 ± 2.3* 80.3 ± 2.5†	86.6 ± 4.6* 84.9 ± 1.5‡
Reproducibility intraday (3 replicates, 5 days) (%RSD)	3.0* 1.0†	3.4* 1.4‡
Reproducibility interday (5 days) (%RSD)	3.1* 4.7†	7.6* 3.6‡
Accuracy (%error)	9.9, 0.1, 0.3§	–2.3, –1.7, 0.1**
Detection limit (ng/mL) (S/N = 3)	50	60
Quantitation limit (ng/mL) (S/N = 10)	160	190

* Concentration of the 0.5-µg/mL spiked urine sample.
† Concentration of the 6-µg/mL spiked urine sample.
‡ Concentration of the 2-µg/mL spiked urine sample.
§ Concentrations of the respective 2-, 8-, and 20-µg/mL spiked urine samples.
** Concentrations of the respective 0.5-, 2-, and 4-µg/mL spiked urine samples.

Table II. Mean Value Determination of Quinapril and Quinaprilat for Two Extractions in the Urine of Four Hypertensive Patients at Different Time Intervals After the Ingestion of One Dose of the Pharmaceutical Formulation Ectren (20 mg of Quinapril HCl)* and Lidaltrin (5 mg of Quinapril HCl)†

Patient	Time interval (h)	Volume (mL)	Quinaprilat (µg/mL)	Quinapril (µg/mL)
1	0–1	260	14.6	0.71
	1–4	150	6.48	0.24
	4–8	600	1.22	not detected
	8–24	1600	0.39	not detected
2	0–1	275	1.62	not detected
	4–8	175	21.3	3.69
	8–24	450	16.7	0.26
3	0–1	470	0.33	not detected
	1–4	90	9.22	1.44
	4–8	100	3.85	not detected
	8–24	1020	0.34	not detected
4	0–1	400	2.52	not detected
	1–4	240	7.26	1.18
	4–8	240	7.09	0.15
	8–24	1060	1.86	not detected

* Administered to patients 1 and 2.
† Administered to patients 3 and 4.

1-propanol–phosphate buffer proportion of 1:3 (v/v). In this case, the retention time of quinaprilat did not decrease as quickly when increasing the pH value, and quinapril had its highest retention at a pH value of approximately 4. However, the resolution between both peaks was not good (6.1 min for quinaprilat and 7.1 min for quinapril at pH 2.5).

The use of the mixtures of 1-propanol and acetonitrile as the organic modifiers was investigated to increase the resolution between both peaks as well as keep a short analysis time and good peak shape for quinapril. A mixture of 1-propanol–acetonitrile–10mM phosphoric acid (20:15:78) was chosen as optimal. Figure 2 shows the chromatograms of a standard solution of quinapril and quinaprilat at pH 2.5 using methanol, acetonitrile, 1-propanol, and the finally selected mixture of solvents as organic modifiers.

Different concentrations of the buffer were also studied in the range of 2.5 to 20mM, but no significant influence in the chromatographic peaks was observed. A concentration of 10mM phosphoric acid was used. Changes in the flow rate produced proportional changes in the retention times as expected, and an increase in temperature did not significantly affect the peak shape. A flow rate of 1.0 mL/min and a column temperature of 30°C were used throughout this work.

Several ACE inhibitors and their metabolites were tested as internal standards (cilazapril, cilazaprilat, ramipril, ramiprilat, enalapril maleate, enalaprilat, fosinopril, fosinoprilat, lisinopril, and captopril). Most of them coeluted with one of the two analytes or eluted at very low retention times. Enalapril maleate was chosen because it did not interfere with any of the drugs studied and eluted just before quinaprilat.

Optimization of the cleanup procedure for urine samples

The low detection wavelength that must be used in the chromatographic system provides as a result very low selectivity; therefore, much effort was invested into finding a cleanup procedure to separate quinapril and quinaprilat from the endogenous compounds.

Because of the amphoteric properties of these amino acidic molecules, it was not

possible to isolate them from urine by means of a conventional liquid–liquid extraction technique. Solid–liquid extraction resulted in being a more adequate cleanup procedure. Our experience with cilazapril (21,22) showed that the use of ionic-change cartridges was not adequate and that among all of the nonpolar cartridges available, C_2, C_8, and C_{18} provided a fairly good range of polarities. C_8 was used because it effectively retained the drugs with the fewest amounts of endogenous compounds. In order to choose an appropriate washing solvent as well as an elution solvent, the elution profiles at three pH values (phosphate buffer at pH 2.0, acetate buffer at pH 5.0, and borate buffer at pH 9.0 at a concentration of 0.1M) were made as a preliminary step. Solutions of quinapril and quinaprilat were prepared in each buffer and applied to the activated cartridge. The adsorbed compounds were eluted with the use of different mixtures of buffer–methanol. Sigmoidal curves were obtained, and the recovery was higher when mixtures richer in methanol were used (Figure 3). Quinapril is always more retained than quinaprilat because a higher proportion of methanol is required for its total elution. Both compounds are more strongly retained at acidic pH values. From the curves, it can be deduced that the two washing solvents—0.1M phosphate buffer (pH 2)–methanol (7:3, v/v) and 0.1M borate buffer (pH 9)–methanol (9:1, v/v)—can be used. Two elution solvents—0.1M phosphate buffer (pH 2)–methanol (1:4, v/v) and 0.1M borate buffer (pH 9)–methanol (2:3, v/v)—were also selected. All four possible combinations of the sequence of washing and elution were assayed, and the best results were obtained when both steps were performed with the acidic mixtures, but the chromatographic peak of quinaprilat was not completely separated from some of the endogenous compounds. Figure 4 shows the chromatogram of a spiked urine sample after its extraction using this cleanup procedure.

In order to improve the extraction, different solvents were tested as an additional washing step. Water was very effective in removing endogenous compounds above all of those eluting during the first few minutes of the run. In addition, acetonitrile was found to be essential in eliminating some of the interfering compounds that eluted together with quinaprilat. Drying the cartridge before the application of acetonitrile was important. If this was not done, a high proportion of the drugs would be lost and low recoveries obtained.

Although the elution using the solvent 0.1M phosphate buffer (pH 2)–methanol (1:4, v/v) could be used, the presence of water in the mixture made its evaporation slow and expensive. The elution with 2 mL of a mixture of chloroform–isopropanol–water (25:25:1, v/v/v), which was a slight modification of the mixture proposed by Ferry et al. (11) used for the drug cilazapril (21), produced equally good recoveries and cleaner chromatograms than the initial mixture.

Quantitative determination and validation

The results of the application of the statistical tests that were explained previously are collected in Table I. Recoveries greater than 80% were obtained for both drugs. A calibration curve for quinapril and quinaprilat was found using spiked urine samples and extracting them following the cleanup procedure proposed in this study. The linearity of the calibration curve was excellent, and the behavior of the system in terms of reproducibility was favorably good.

Analytical applications

Urine samples obtained from four hypertensive patients taking the pharmaceutical formulation Ectren (20 mg of quinapril HCl) or Lidaltrin (5 mg of quinapril HCl) were analyzed. Patients were under continuous treatment with the formulations. Table II shows the results of the analysis of the urine samples obtained from the four hypertensive volunteers. The chromatogram of a urine sample from patient #2 that was taken in the 4- to 8-h interval after the intake of Ectren (20 mg of quinapril) is shown in Figure 5.

Conclusion

Because of the high polarity of this group of pharmaceuticals, the use of a direct HPLC method with photometric detection for their quantitation remained a challenge. Because of their low absorption wavelength, a very demanding cleanup procedure had to be found. This has probably been the main handicap for the analysis of this family of drugs in biological samples.

This procedure has proved to be suitable to determinate the concentration of quinapril and quinaprilat in urine samples at least during the 24-h interval after its oral administration. As expected from the pharmacokinetic data, most of the initial dose was excreted as quinaprilat and only a small amount was excreted as unchanged quinapril, which was not detected in the last time interval (8–24 h) in most samples. The percentage of the excretion of quinapril and quinaprilat was in accordance with the data published in the bibliography for patients #1 and #3, but the amount of quinaprilat excreted was clearly

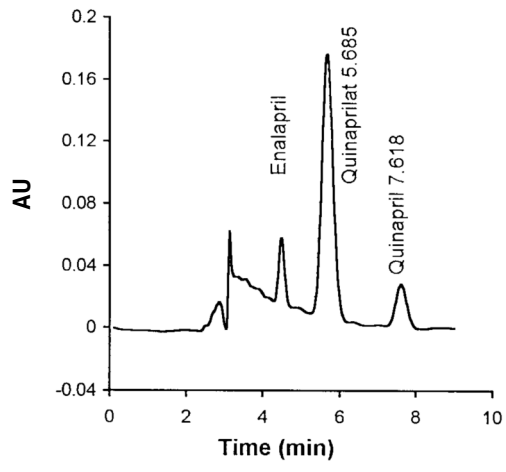

Figure 5. Chromatogram of a urine sample obtained from patient #2 at 4 to 8 h after intake of a therapeutic dose of Ectren (20 mg of quinapril). The mobile phase was 1-propanol–acetonitrile–10mM phosphoric acid (20:15:78, v/v/v), flow rate 1 mL/min, column temperature 30°C ± 0.2°C, and detection wavelength 206 nm. The concentration of quinaprilat was 21.3 µg/mL and for quinapril 3.69 µg/mL.

over 30% for patients #2 and #4. Wide interindividual variations have been reported (1). Patients were under continuous treatment with the formulation. Therefore, the results were not indicative of the excretion of a single dose. Besides, patient #4 was under treatment with other drugs that may have interacted with the elimination kinetics of quinapril and quinaprilat. It is worth mentioning that in this case, no interfering peak for the rest of the pharmaceuticals was observed, and in all cases, patients were not asked to change their diet.

In this work, no previous derivatization of the drugs was required, decreasing significantly the analysis time in comparison with previously reported methods. This also meant that fewer steps were used and thus fewer error sources were involved. Maurer et al. (16) have suggested that the derivatization reaction may cause the partial hydrolysis of the esters of the unchanged drug and produce the active metabolites (in this case quinaprilat) artificially.

The use of HPLC with photometric detection is potentially useful for the analysis of other members of this family, making possible their analysis with the use of inexpensive and easily available instrumentation.

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