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Simultaneous determination of benazepril hydrochloride and benazeprilat in plasma by high-performance liquid chromatography/electrospray-mass spectrometry

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Abstract

An analytical method for simultaneous determination of benazepril and its active metabolite, benazeprilat, in human plasma by high-performance liquid chromatography/electrospray-mass spectrometry was developed and validated. Rutaecarpine was selected as the internal standard. The separation was achieved on a C_{18} column with acetonitrile and aqueous solution (0.1% formic acid) as mobile phase with a gradient mode. The quantification of target compounds was using a selective ionization recording at *m*/*z* 425.5 for benazepril, *m*/*z* 397.5 for benzeprilat and *m*/*z* 288.3 for rutaecarpine. The correlation coefficients of the calibration curves were better than 0.992 ($n = 6$), in the range of 6.67–666.67 ng/ml for benazepril and benazeprilat. The inter- and intra-day accuracy, precision, linear range had been investigated in detail. The method can be used to assess the bioavailability and pharmacokinetics of the drug.

Keywords: Benazepril; Benazeprilat; Rutaecarpine; HPLC–ESI-MS

1. Introduction

Benazepril hydro chloride, (3-[(1 -ethoxycarbonyl-3 -phenyl-(1*S*)-propyl)-amino]-2,3,4,5 -tetrahydro- 2 -oxo-1-(3*S*)-benazepine-1-acetic acid hydrochloride) [1], is a prodrug-type angiotensin-converting enzyme (ACE) inhibitor [2], which is proved effective in treating congestive heart failure and hypertension [3–5]. It is hydrolyzed in vivo to a pharmacologically active metabolite, the diacid benazeprilat, (3-[(1-carbonyl-3-phenyl-(1*S*)-propyl)-amino]-2,3,4,5-tetrahydro-2-oxo-1-(3*S*)-benazepine-1-acetic acid) [6,7].

For analysis of the compounds, a number of methods, such as high performance liquid chromatography (HPLC) [8–16], derivative spectrophotometry [10,17–18], capillary electrophoresis (CE) [19–20], and high performance thin layer chromatography-densitometry (HPTLC) [14], enzymatic method [21], etc. were developed. Among these methods, most of them were used to monitor the quality of the pharmaceutical dosage forms.

However, the analysis of the compounds in human body fluids is very important. The bioavailability and pharmacokinetics research must depend on the results of determination of the compounds in the body fluids, especially in plasma. To our knowledge, the general method for simultaneous analysis of benazepril and benazeprilat in human plasma was gas chromatography-mass spectrometry (GC–MS) [22–24]. This method involved not only solid phase extraction (SPE), but also sample derivatization, and hence seemed a bit complicated.

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In this paper, a HPLC–electrospray ionization mass spectrometry (ESI-MS) method for simultaneous analysis of benazepril and its active metabolite benazeprilat, in human plasma was developed. The sensitivity of the method had no significant difference comparing with GC–MS. Moreover, this method is also much simpler and reagent-saving, time-saving than GC–MS. Its specifications are suitable for assessing bioavailability and pharmacokinetics of the drug.

2. Experimental

2.1. Instrumentation

A Waters (Milford, MA, USA) Alliance 2695 liquid chromatographic system interfaced to a 2487 dual wavelength UV detector, a Micromass ZQ 2000 ESI mass spectrometer and a Masslynx^{TM} 3.5 data system was equipped with a Johnson Spherigel analytical column (250 mm × 4.6 mm) (Dalian, China) packed with 5 μm C_{18} silica. Acetronitrile (A) and 0.1% formic acid aqueous solution (B) were used as mobile phase. The gradient elution was programmed as follows: 0–5 min, 65% B; 5–5.5 min, 65–35% B; 5.5–12 min, 35% B; 12–12.5 min, 35–65% B, 12.5–15 min, 65% B. The column temperature was maintained at 30 °C. The flow rate was 0.8 ml/min. The outlet of the UV detector was split, and only 0.2 ml/min portion of the column eluent was delivered into the ion source of MS. ESI-MS was performed using a Micromass quadrupole mass spectrometer (Milford, MA, USA). The mass spectrometer was operated in the positive mode to generate $[M+H]^+$ ions at *m*/*z* 425.5 for benazepril, *m*/*z* 397.5 for benazeprilat and *m*/*z* 288.3 for rutaecarpine. Nitrogen was used as desolvation gas at a flow rate of 200 1/h and cone gas at a flow rate of 50 1/h. The desolvation temperature was 250 °C. Capillary and cone voltages were 4000 and 30 V, respectively. The ionization source was worked at 105 °C. Extractor voltage was set at 4 V, and 0.5 V for RF lens. An Eppendorf 5804R Multipurpose Centrifuge (Brinkmann, Westbury, NY) worked at 12 000 rpm to deprotein for samples.

2.2. Chemicals and reagents

Benazepril hydrochloride, benazeprilat and internal standard rutaecarpine were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purity for the standards was higher than 99%. Structures of the compounds are shown in Fig. 1.

HPLC-grade acetonitrile and methanol purchased from Tedia Company Inc. (Fairfield, OH, USA), and ultrapure water prepared by a Millipore Milli-Q purification system (Millipore Corp. Bedford, MA, USA) were used as mobile phase of HPLC–MS. Other reagents were of analytical-grade. Drug-free and drug-containing plasma was obtained from volunteers and patients, respectively. Plasma was stored below −40 °C until used for analysis.

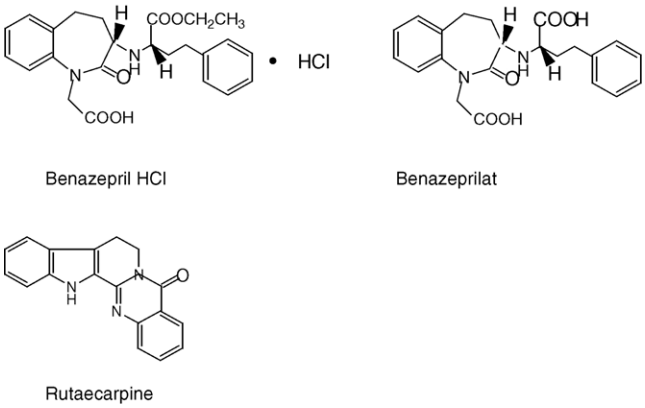

Fig. 1. Chemical structures of benazepril hydrochloride, benazeprilat and internal standard rutaecarpine.

2.3. Stock solutions

The stock solution of benazepril hydrochloride was prepared by dissolving 10.8 mg in 10 ml of methanol. The stock solution of benazeprilat was prepared by dissolving 10.4 mg in 6 ml of 1.2% ammonia solution, and then diluted to 10 ml by methanol. The stock solution of internal standard rutaecarpine was prepared by dissolving 1.2 mg in 10 ml of methanol. A series of mixed standard solutions, which were used for preparing calibration standards, were then gained by the appropriate dilutions of the above-mentioned stock solutions with methanol to reach concentration range of 20–2000 ng/ml for benazepril and benazeprilat. Further dilution of the stock solution with methanol resulted in the spiked internal standard solution (60 ng/ml).

2.4. Calibration standard solutions

For calibration standards, 0.5 ml mixed standard solutions together with 0.5 ml acetronitrile and 0.25 ml internal standard of the concentration of 60 ng/ml were added to 0.25 ml drug-free plasma to obtain calibration standards in the range of concentrations of 6.67–666.67 ng/ml for benazepril and benazeprilat in polypropylene tubes. After vortex mixed for 3–5 s, all the samples were then centrifuged at 12,000 rpm for 10 min at 4 °C. A 10 μL aliquot of the supernatant of each sample was then directly injected on the Spherigel analytical column.

2.5. Sample preparation

Plasma samples (0.25 ml) obtained from healthy volunteers, who had the administration of a single 10 mg benazepril capsule, together with 0.5 ml methanol and 0.5 ml acetronitrile, 0.25 ml of 60 ng/ml rutaecarpine internal standard stock solution were added to polypropylene tubes. After vortex mixed for 3–5 s, all the samples were then centrifuged at 12,000 rpm for 10 min at 4 °C. A 10 μL aliquot of the su-

pernatant of each sample was then directly injected on the Spherigel analytical column.

3. Results and discussion

3.1. HPLC and ESI-MS conditions

The comparison of C_8 and C_{18} HPLC columns (Spherisorb ODS, Spherigel ODS, respectively.) for separating the target compounds had been completed. However, C_8 column needed a longer analysis cycle ($t_R > 15$ min for benazepril) and badly broadened peak shapes, In contrast, the C_{18} column displayed a better separating ability for target compounds.

To obtain the optimal elution conditions for the simultaneous separation and quantification of benazepril and benazeprilat, various elution conditions of acetronitrile (A) and 0.1% formic acid aqueous solution (B) at a flow rate of 0.8 ml/min had been investigated. Three elution conditions and corresponding chromatograms are shown in Fig. 2. Under gradient condition of method ③, better resolution and peak shapes were achieved than under the other methods. This was probably due to the weak polarity of the target compounds, which only can be well eluted by a stronger mobile phase. A good separation of the target compounds could also be achieved by an isocratic of 58% methanol and 42% B, but in acetonitrile, benazepril and benazeprilat showed a better MS sensitivity, which was in accordance with Voyksner [25].

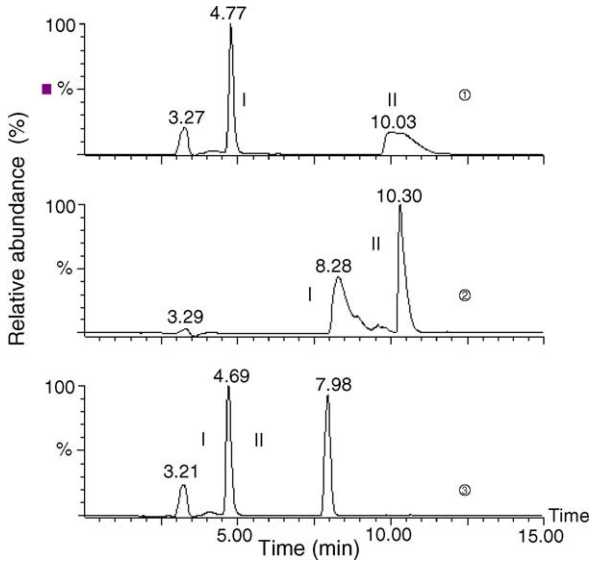

Fig. 2. The corresponding HPLC profiles of 0.5 mg/ml benazeprilat (I) and benazepril (II) under different elution conditions: A is acetronitrile, B is 0.1% formic acid aqueous solution, (①) 1–15 min, 65% B; (②) 0–5 min, 75% B; 5–5.5 min, 75–35% B; 5.5–12 min, 35% B; 12–12.5 min, 35–75% B; 12.5–15 min, 75% B; (③) 0–5 min, 65% B; 5–5.5 min, 65–35% B; 5.5–12 min, 35% B; 12–12.5 min, 35–65% B; 12.5–15 min, 65% B, respectively. A Johnson Spherigel C_{18} (250 mm × 4.6 mm, 5 μm) column was used at a 0.8 ml/min flow rate at 30 °C.

In order to obtain an optimum response of mass spectrometry, different concentrations of mobile phase modifiers (ammonium acetate, formic acid) were investigated. However, the addition of ammonium acetate had the disadvantage of restraining the ionization of benazeprilat, with no SIR response of benazeprilat in 15 min. In contrast, the addition of formic acid could not only improve the sensitivity by a factor of 2 and 3, but also shorten the retention time of the target compounds, than when no mobile phase modifier added. For further improvement, the effect of pH of mobile phase on chromatographic efficiency was examined by varying the concentration of formic acid in the aqueous solution to obtain pH between 3 and 7. At pH above 5, complete separation of target compounds could not be achieved within 15 min. The overly acidic condition would be disadvantageous for the lifetime of C_{18} column. Based on the above-mentioned comparison, 0.1% formic acid aqueous solution and acetonitrile were used as mobile phase.

Capillary and cone voltages of the ESI interface were optimized by flow injection analysis (FIA) with a microliter injector at 100 ng/ml of mixture of standard benazepril and benazeprilat to obtain a maximum generation of the protonated molecular ion $[M + H]^+$ of each analyte. The abundance of protonated molecular ions of benazepril (*m*/*z* 425.5), benazeprilat (*m*/*z* 397.5), enlarged with the increasing of the capillary voltage, and reached plateaus at 4000 V.

The intensity of the $[M + H]^+$ of compounds mainly depends on collision-induced dissociation (CID) fragmentation voltage, so the cone voltage is the key factor influencing ionization and sensitivity in this program. Cone voltage was optimized by switching from 15 to 75 V in scan mode. Benazepril and benazeprilat produced more abundant protonated molecular ions at lower cone voltage. At higher voltage, the intensity of $[M + H]^+$ of target compounds became weaker and produced more fragmentations, which were shown in Fig. 3. In selective ionization recording (SIR), the influence of cone and capillary voltages was represented by plotting the molecular-

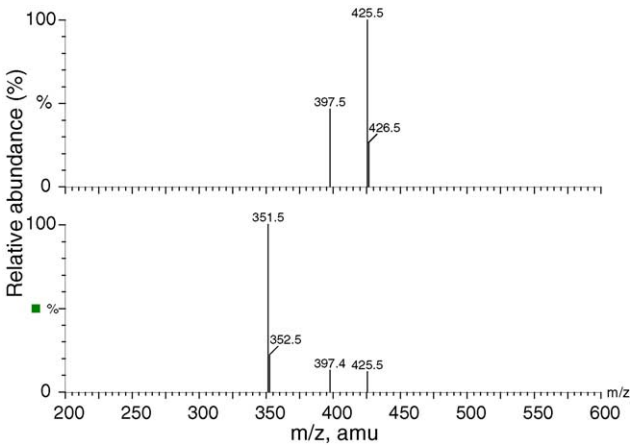

Fig. 3. The corresponding mass spectrum of 100 ng/ml of mixture of standard benazepril and benazeprilat under cone voltage 30 V (upper) and 55 V (lower), respectively. (*Note*: The capillary voltage was 3500 V.)

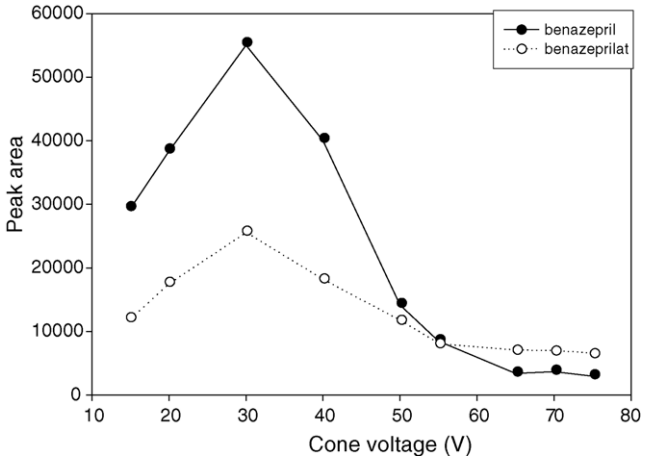

Fig. 4. The influence of cone voltage to the molecular-ion abundance of benazepril and benazeprilat. (*Note*: The capillary voltage was 3500 V.)

ion peak area of each analyte versus the cone and capillary voltages. These results were shown in Figs. 4 and 5. In our experiment, in order to achieve an optimum sensitivity, 30 and 4000 V were selected as the cone voltage, capillary voltage, respectively.

3.2. Specificity of the method

The specificity of the analytical method was checked by preparing and analyzing three different batches of blank plasma samples. The specificity was assessed by comparing the apparent signals for benazepril and benazeprilat in six blank plasma samples, which were spiked with standard solutions of different concentrations in the range of 6.67–666.67 ng/ml added, to the response of blank plasma samples. The blank plasma sample showed a clean baseline at *m*/*z* 397.5, 425.5 and 288.3 at the relevant retention time and no endogenous interferences were observed. Typical SIR

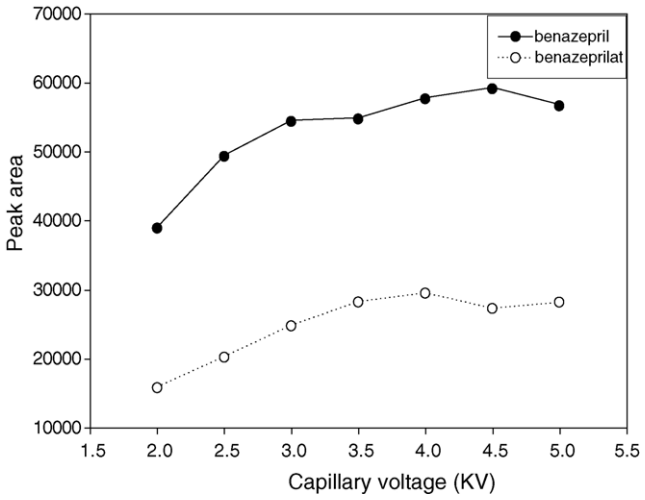

Fig. 5. The influence of capillary voltage to the molecular-ion abundance of benazepril and benazeprilat. (*Note*: The cone voltage was 30 V.)

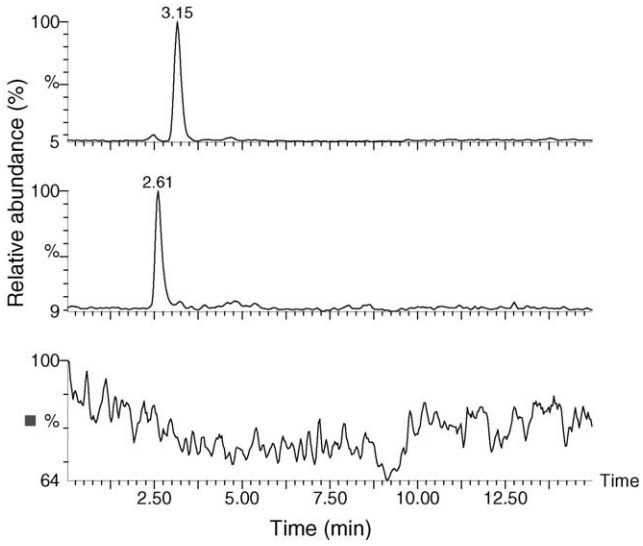

Fig. 6. Selected-ion current profiles at *m*/*z* 397.5, 425.5 and 288.3, respectively, of blank human plasma sample.

current profiles of the results were shown in Figs. 6 and 7. These profiles showed that the method adopted was applicable.

3.3. Calibration curves

Daily calibration standards were prepared at least six different concentrations, in the range from 6.67 to 666.67 ng/ml for benazepril and benazeprilat. Every calibration standard was injected in triples. Calibration curves ($y = ax + b$) were represented by plotting the peak area ratios (y) of the benazepril and benazeprilat to I.S. rutaecarpine versus the

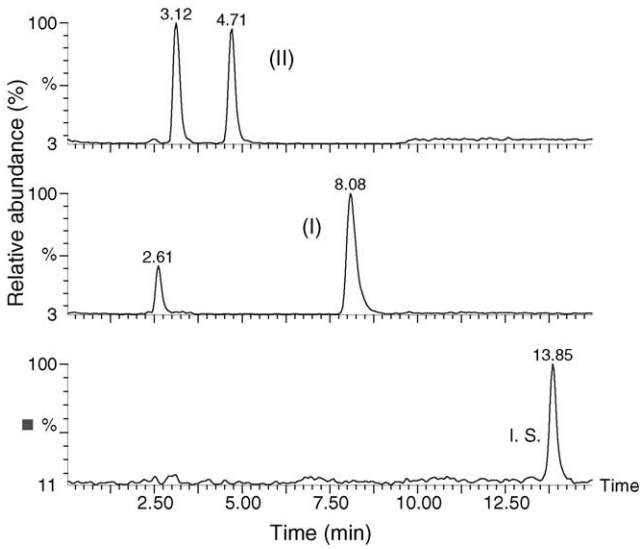

Fig. 7. Selected-ion current profiles at *m*/*z* 397.5, 425.5 and 288.3 of the same plasma sample, containing 333.33 ng/ml benazepril (I), benazeprilat (II) and 10 ng/ml rutaecarpine (I.S.), with the retention time at 8.08, 4.71, 13.85 min, respectively.

Table 1
Inter- and intra-day precision and accuracy of the method

Measurement	Added (ng/ml)	Benazepril		Benazeprilat	
		Precision[a] R.S.D. (%)	Mean accuracy[b] ($n = 6$)%	Precision R.S.D. (%)	Mean accuracy ($n = 6$)%
Inter-day	6.67	4.3	93.7	4.8	105.5
	300	3.5	103.5	4.6	100.6
	666.67	2.8	100.9	3.0	98.5
Intra-day	6.67	5.5	106.5	5.0	108.7
	300	4.0	102.1	4.2	96.5
	666.67	2.5	98.7	2.3	101.2

[a] Expressed as relative standard derivation.
[b] Expressed as [(mean observed concentrations/nominal concentrations) × 100] ($n = 6$).

concentrations (x) of the calibration standards. Calibration curves were obtained from weighted ($1/x^2$) least-squares linear regression analysis of the data. The linearity was also assessed for consecutive days for the standard solutions of the same range of concentrations prepared from the stock solutions. The R.S.D. for inter-day linearity is below 1.6%. The R.S.D. for intra-day linearity is below 5.2%. The regression equations for calibration curves at the range 6.67–666.67 ng/ml were $y = 0.01600x + 0.02662$ for benazepril and $y = 0.01040x - 0.001557$ for benazeprilat. The R.S.D. values for the intercept and slope are agreement with the international criteria [26]. The correlation coefficient was found to be more than 0.992, indicating a good linearity.

3.4. Precision and accuracy

The precision was evaluated by the inter- and intra-day ($n = 6$) assays at three different concentrations of benazepril and benazeprilat in the range of 6.67–666.67 ng/ml at levels corresponding to the lowest (6.67 ng/ml), near the middle (300 ng/ml) and the highest (666.67 ng/ml). The repeatability for inter-day and intra-day was below 4.8% R.S.D., 5.5% R.S.D., respectively.

The accuracy of the method was studied by calculating the mean recovery of the target compounds by adding standards known concentrations to the samples. The mean recovery was obtained by the determined concentrations as a percentage of the nominal concentrations. Every sample of the same concentration was injected at least six times. As a conclusion, the mean recovery for benazepril and benazeprilat was 93.7–108.7% at the concentrations at levels corresponding to the lowest (6.67 ng/ml), near the middle (300 ng/ml) and the highest (666.67 ng/ml). As listed in Table 1, these results about precision and accuracy met the acceptable criteria.

3.5. Lower limit of the quantitation (LLOQ)

The analyte response at the LLOQ should be at least five times the response of blank baseline. The LLOQ was defined as the lowest concentration on the standard calibration curves with acceptable repeatability, recovery. The LLOQ of benazepril and benazeprilat was 6.67 ng/ml. According to the international criteria, the analyte response at the limit of detection (LOD) should be at least three times the response of blank baseline. The LOD of benazepril and benazeprilat was 2.5 and 5 ng/ml, respectively.

3.6. Stability

These stock solutions were stored in the dark under refrigeration at −40 °C and were discovered to be stable for at least 1 year with the R.S.D. below 2%, which was in agreement with the report [22]. Because the ester linkage of benazepril could be cleaved chemically or enzymatically by hydrolases or proteins displaying an esterase-like activity [16], all the collected samples were immediately stored in the dark under refrigeration at −40 °C to deactivate the enzymes existing in the blood and the stability of samples after deproteinated were also investigated. The samples were found to be stable on the autosampler at room temperature for at least 24 h.

3.7. Application

The described method was applied to a clinical trial. Typical plasma concentration–time profiles of benazepril and benazeprilat obtained from one healthy subject after administration of a 10 mg benazepril capsule were shown in Fig. 8.

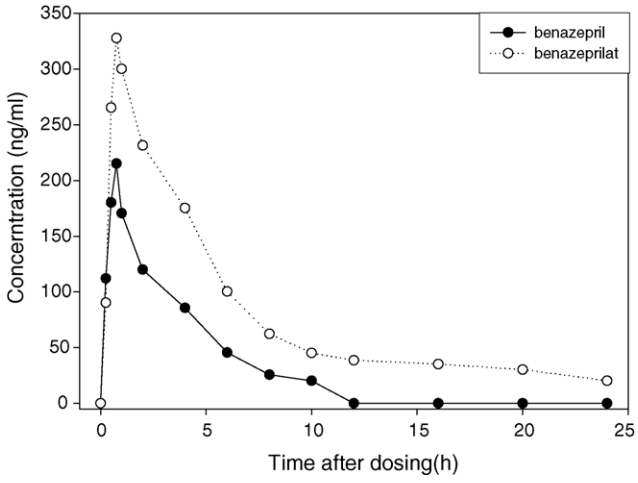

Fig. 8. Concentrations of benazepril and benazeprilat in plasma (ng/ml) of one healthy subject after oral administration of 10 mg of benazepril capsule.

The figure illustrated that the prodrug benazepril hydrochloride was rapidly absorbed and almost fully metabolized to the pharmacologically active metabolite benazeprilat in a short period.

4. Conclusion

A HPLC–MS method has been developed and validated for simultaneous analysis of benazepril and its active metabolite, benazeprilat, in human plasma at the range of 6.67–666.67 ng/ml, according to acceptable criteria. It is simple, reagent-saving and time-saving. The method is also suitable for assessing drug bioavailability and pharmacokinetics.

Acknowledgements

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