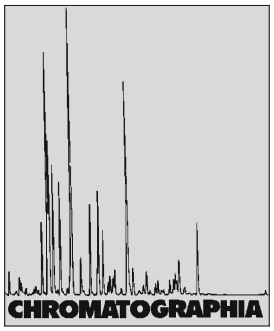
Simultaneous Analysis of Zofenopril and Its Active Metabolite Zofenoprilat in Human Plasma by LC–ESI-MS Using Pre-Column Derivatization with *p*-Bromophenacyl Bromide



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Abstract

Zofenoprilat is an active metabolite of zofenopril, which is very unstable in plasma because of oxidative degradation of its thiol group. In this method, *p*-bromophenacyl bromide was used as derivatization reagent, immediately after plasma separation, to react with the free thiol group of zofenoprilat and form the derivative zofenoprilat-*p*-BPB. After acidification with 50% acetic acid, the derivatized plasma samples were extracted with methyl *tert*-butyl ether and separated on a C_{18} column with 40:60 (*v*/*v*) 10 mM ammonium acetate buffer solution containing 0.1% formic acid–acetonitrile as mobile phase. Calibration plots were linear over the concentration range 1–500 ng mL^{-1} for zofenopril and 2–1,800 ng mL^{-1} for zofenoprilat. The method was successfully used to study the bioavailability of zofenopril calcium capsules relative to that of zofenopril calcium tablets in healthy Chinese volunteers.

Keywords

Column liquid chromatography
Mass spectrometry
Zofenopril
Zofenoprilat
p-Bromophenacyl bromide
Derivatization
Pharmacokinetics

Introduction

Zofenopril calcium, (4*S*)-1-[(2*S*)-3-(benzoylthio)-2-methylpropanoyl]-4-(phenylthio)-l-proline calcium salt (Fig. 1a) is a specific and long-lasting angiotensin-converting enzyme (ACE) inhibitor with antihypertensive, remarkable antioxidant, and cardioprotective properties, including the ability to improve endothelial function and protect against ischemia [1–4]. These peculiar characteristics are mainly because of the presence of a sulfhydryl group and the highly lipophilic nature of the compound. ACE inhibitors with a sulfhydryl group have the advantage of pharmacological action compared with non-sulfhydryl-containing ACE inhibitors [3, 4]. Like most other ACE inhibitors, zofenopril is a prodrug. After oral administration, zofenopril is completely and rapidly converted into its pharmacologically active metabolite zofenoprilat (Fig. 1b) by hydrolysis by esterases in vivo (Fig. 1).

The active free thiol group, which is exposed by enzymolysis, is prone to oxidative degradation in the presence of light and oxygen, so it is difficult to determine zofenoprilat directly in human plasma. Measurement of the concentration of unchanged zofenoprilat in biological samples must be preceded by addition of chemical stabilizer or by derivatization of the molecule to prevent the oxidative degradation of the thiol group. Two methods [5, 6] have been reported for simultaneous analysis of zofenopril and zofenoprilat with *N*-ethylmaleimide (NEM) as derivatization reagent. Jemal et al. [5] used solid-phase extraction (SPE) after liquid–liquid extraction (LLE) for isolation and purification, followed by methylation

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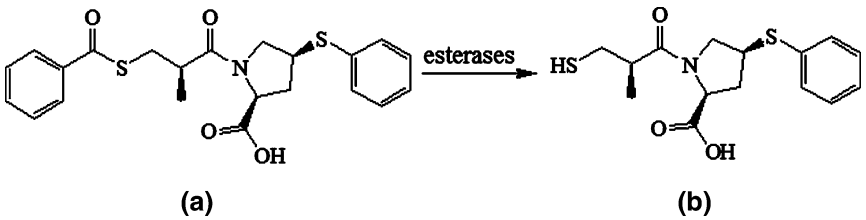

Fig. 1. The chemical structures of zofenopril (**a**) and its active metabolite zofenoprilat (**b**)

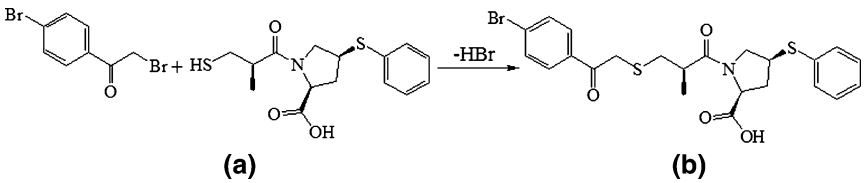

Fig. 2. Derivatization reaction of zofenoprilat with *p*-BPB

and reconstitution with tetramethylbenzene before GC–MS analysis. Dal Bo et al. [6] improved the sample-preparation procedure reported by Jemal et al. [5]. A new derivatization method developed in our laboratory has been mentioned in a pharmacokinetic report in Chinese [7], which was a preliminary publication without details of method development and validation, optimization of the derivatization procedure, or spectroscopic proof of the identity of the derivatization product. This paper reports the development and validation of this new, simple and sensitive LC–ESI-MS method with *p*-bromophenacyl bromide (*p*-BPB; Fig. 2a) as pre-column derivatization reagent for simultaneous analysis of zofenopril and zofenoprilat in human plasma, and its application to a study of the relative bioavailability of two zofenopril calcium dosage forms.

Experimental

Materials, Reagents, and Solutions

Zofenopril calcium capsules (the test formulation, each capsule containing 15 mg zofenopril calcium), zofenopril calcium tablets (the reference formulation, each tablet containing 15 mg zofenopril calcium), and the reference substances zofenopril (99.1% purity) and zofenoprilat (96.0% purity) were obtained from Jiangsu Kanion Pharmaceutical (Lianyungang, China). Bezafibrate, the internal standard (I.S.), was supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

The derivatization reagent, *p*-bromophenacyl bromide of HPLC grade, was provided by Sigma-Aldrich (Shanghai, China). The zofenoprilat derivative (zofenoprilat-*p*-BPB, Fig. 2b) was synthesized as described below in the Sect. 2.2.

Methanol and acetonitrile of HPLC grade were purchased from Merck (Darmstadt, Germany). Ammonium acetate, acetic acid, and formic acid were of analytical grade purity and purchased from Nanjing Chemical Reagent (Nanjing, China). Methyl *tert*-butyl ether and EDTA-2Na of analytical grade purity were purchased from Sinopharm Chemical Reagent (Shanghai, China). Distilled water was used throughout the study.

A stock solution (1.0 mg mL^{-1}) of zofenopril was prepared by dissolving zofenopril calcium in 50% methanol. Working standard solutions of concentration 100, 10, and 1 μg mL^{-1}, and 100 ng mL^{-1} were obtained by further dilution of the stock solution with 50% methanol. A stock solution (1.0 mg mL^{-1}) of zofenoprilat was prepared by dissolving zofenoprilat in methanol. Working standard solutions of concentration 100, 10, and 1 μg mL^{-1} and 100 ng mL^{-1} were obtained by further dilution of the stock solution with methanol. Because of the photodegradation of zofenoprilat, stock solutions, calibration standards, quality-control samples, and clinical plasma samples were stored in amber glass containers and protected from light.

A stock solution (1.0 mg mL^{-1}) of zofenoprilat-*p*-BPB was prepared by dissolving zofenoprilat-*p*-BPB in methanol. Working standard solutions of concentration 100, 10, and 1 μg mL^{-1} and 100 ng mL^{-1} were obtained by further dilution of the stock solution with methanol.

I.S. working solution (10 μg mL^{-1}) was prepared by diluting bezafibrate stock solution with methanol.

All solutions were stored at −20 °C and were brought to room temperature before use.

Preparation of the Zofenoprilat Derivative Zofenoprilat-*p*-BPB

To a solution of zofenoprilat (0.13 g) and *p*-BPB (0.16 g) in 10 mL methanol, 1 mol L^{-1} sodium hydroxide was added dropwise to adjust the pH to 7.0 at room temperature. After reflux in a water bath at 70 °C for 1 h, the solution was evaporated to dryness under a stream of nitrogen. A solution of the residue in phosphate buffer (pH 7.0; 10 mL) was washed with 10 mL methyl *tert*-butyl ether, adjusted to pH 2.0 by addition of 2 mol L^{-1} hydrochloric acid, and extracted with 10 mL methyl *tert*-butyl ether. The mixed organic phase was evaporated to dryness under a stream of nitrogen to give the zofenoprilat derivative zofenoprilat-*p*-BPB. The identity of the product was confirmed by MS (Fig. 3b) and NMR spectroscopy (in CD_3OD) ppm: 1.13 (d, 3H, CH_3), 1.93 (m, 1H, O = C–CH), 2.54 and 2.84 (dd, 2H, CH_2), 2.69 and 2.91 (m, 2H, S–CH_2–), 3.43 and 4.18 (dd, 2H, N–CH_2–), 3.78 (m, 1H, Ar–S–CH–), 3.86 and 3.92 (m, 2H, O = C–CH_2–S), 4.40 (t, 1H, N–CH–COOH), 7.28 (d, 1H, Ar–H), 7.35 (t, 2H, Ar–H), 7.47 (d, 2H, Ar–H), 7.66 (d, 2H, Br–Ar–H), 7.88 (d, 2H, Br–Ar–H).

Sample Preparation

Plasma (1 mL) was transferred into a 2-mL plastic tube containing 40 μL

EDTA-2Na (0.1 mol L^{-1}) and 150 µL *p*-BPB methanol solution (1 mg mL^{-1}). The tube was vortex mixed for 10 s and then kept in a water bath of 45 °C for 30 min to complete the process of derivatization. The reaction of *p*-BPB with zofenoprilat is shown in Fig. 2. After cooling to room temperature, the mixture was stored at −20 °C until LC–ESI-MS analysis.

The derivatized plasma samples were extracted with 5 mL methyl *tert*-butyl ether after addition of 20 µL I.S. (10 µg mL^{-1}) solution and 50 µL 50% acetic acid. After centrifugation and separation, the organic phase was evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 200 µL mobile phase and vortex mixed for 2 min. The supernatant was then transferred to an amber glass autosampler vial and 5 µL was injected for LC–ESI-MS analysis. Throughout the process samples were protected from light.

LC–ESI-MS

HPLC–ESI-MS was performed with an Agilent Technologies (Palo Alto, CA, USA) Series 1100 LC/MSD SL system comprising G1312A binary pump, G1322A vacuum degasser, G1316A injection temperature-controlled column compartment, G1313A autosampler, and G1956B MSD single-quadrupole mass spectrometer equipped with an electrospray source. Signal acquisition, peak integration, and concentration determination were performed by use of the ChemStation software (10.02 A) supplied by Agilent Technologies.

Compounds were separated on a 150 mm × 2.1 mm I.D., 5-µm particle, LiChrospher C_{18} column (Jiangsu Hanbon Science and Technology, Huaian, China) with 40:60 (*v*/*v*) 10 mM ammonium acetate buffer solution containing 0.1% formic acid–acetonitrile as mobile phase at a flow rate of 0.2 mL min^{-1}. The column temperature was maintained at 30 °C. LC–ESI-MS was performed using nitrogen to assist nebulization. The quadrupole mass spectrometer equipped with an ESI source was operated with the drying gas (N_2) flow at 10 L min^{-1}, nebulizer pressure 40 psig, drying gas temperature 350 °C, and capillary potential 3.5 kV in negative-ion mode. The fragmentor potential was 100 V. ESI-MS was performed in SIM mode using target $[M - H]^-$ ions at *m*/*z* 428.0, 520.0, and 360.0 for zofenopril, zofenoprilat-*p*-BPB, and the I.S., respectively. Figure 3 shows typical full-scan ESI mass spectra of zofenopril, zofenoprilat-*p*-BPB and the I.S.

Preparation of Calibration Plots and Analysis of Quality-Control Samples

Calibration standards were prepared by spiking 1.0 mL blank plasma with appropriate amounts of working standard solutions. The calibration plot for zofenopril was prepared after analysis of solutions of concentration 1, 3, 10, 30, 60, 150, 300, and 500 ng mL^{-1}; that for zofenoprilat after analysis of solutions of concentration 2, 6, 20, 60, 200, 600, 1,200 and 1,800 ng mL^{-1}. Calibration plots were prepared during analysis of quality-control (QC) samples and each batch of clinical plasma samples. QC samples were prepared in 1.0 mL blank plasma at concentrations of 2.5, 40, and 450 ng mL^{-1} for zofenopril and 5, 100 and 1,600 ng mL^{-1} for zofenoprilat. QC samples were assayed with clinical samples to monitor the performance of the assay and to assess the integrity and validity of the results obtained for unknown clinical samples. All standard solutions in plasma (calibration standards and quality-control samples) were analyzed as described in the sample preparation.

Assay Validation

Selectivity

Selectivity was assessed by comparing the chromatograms obtained from blank human plasma from six different sources with the corresponding spiked plasma. Each blank plasma sample was tested using the proposed extraction procedure and LC–ESI-MS conditions to ensure there was no interference with zofenopril, zofenoprilat-*p*-BPB, and the I.S. from the plasma.

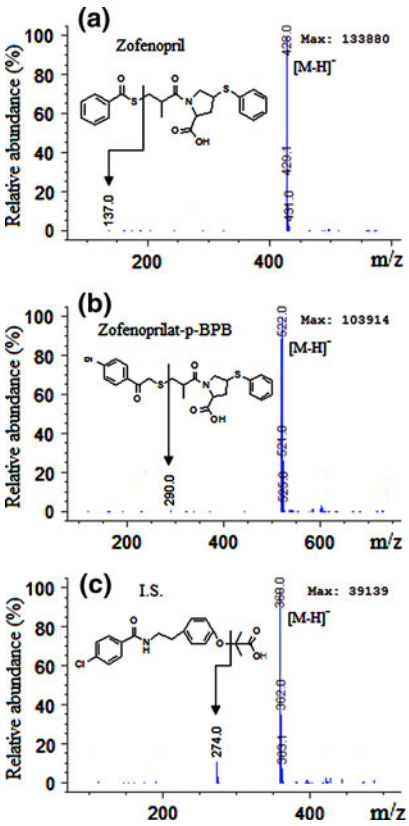

Fig. 3. Negative-ion mass spectra of zofenopril (**a**), zofenoprilat-*p*-BPB (**b**) and the I.S. (**c**) at a fragmentor potential of 100 V

Linearity of Calibration Plots, and Lower Limit of Quantification

Linearity was assessed by preparing calibration plots after chromatography of calibration solutions prepared in human plasma. Solutions at eight concentrations from 1 to 500 ng mL^{-1} for zofenopril and from 2 to 1,800 ng mL^{-1} for zofenoprilat were extracted and assayed. Calibration plots were constructed by plotting zofenopril-to-I.S. and zofenoprilat-*p*-BPB-to-I.S. peak-area ratios against the concentrations of zofenopril and zofenoprilat, respectively. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value.

The lower limit of quantification (LLOQ) is the lowest concentration on the calibration plot that can be measured with acceptable accuracy and precision.

It was verified by five replicates. The precision should be equal to or less than 20% and accuracy between 80 and 120% of the nominal concentration.

Precision and Accuracy

Validation samples were prepared and analyzed in three separate analytical batches to evaluate the accuracy and intra-batch and inter-batch precision of the method. The accuracy and intra-batch and inter-batch precision of the method were determined by analysis of five replicates of the QC samples and construction of one calibration plot for each of the three batches. Assay precision was calculated as the relative standard deviation (RSD, %) by use of one-way analysis of variance. The accuracy is the degree of closeness of the determined value to the nominal true value under the prescribed conditions. Accuracy is defined as the relative deviation of the value (E) calculated for a standard from the true value (T), expressed as a percentage (RE, %). It was calculated by use of the formula:

$$\mathrm{RE}(\%) = [(E - T)/T] \times 100$$

Intra-day and inter-day precision were required to be less than 15%, and the accuracy to be within ±15%.

Extraction Recovery

The extraction recovery was estimated by comparing the peak areas obtained from QC samples with the peak areas obtained from samples of concentration equal to those of the QC samples, prepared by spiking the supernatant from processed blank plasma samples.

Matrix Effects

The potential for a matrix effect (M.E.) was evaluated by comparing the peak areas obtained from the analytes added to the reconstituted solutions obtained from blank plasma samples, which originated from five different donors and were submitted to the sample-preparation process, with those obtained from the analytes dissolved in the mobile phase. QC samples were evaluated by analyzing five samples at each level. The M.E. value of the I.S. (200 ng mL^{-1}) was also evaluated. The matrix effect at every concentration level should be less than 15%.

Stability

Stability tests were designed to assess the stability of the analytes under the conditions expected during handling of clinical samples. The stability of zofenopril and zofenoprilat-*p*-BPB in plasma was studied under a variety of storage and handling conditions by analysis of replicates ($n = 3$) of QC samples. The short-term temperature stability was tested by storing the QC samples at ambient temperature for 14 h. Freeze–thaw stability (−20 °C in plasma) was checked through three freeze and thaw cycles. Autosampler stability (after extraction, in the mobile phase) was assessed by reanalysis of post-preparative QC samples kept under autosampler conditions (8 °C) for 18 h. Long-term stability was assessed after storage at −20 °C in plasma for 8 weeks. All the plasma samples were stored and handled with protection from light.

After these treatments, samples were analyzed by use of calibration plots obtained from freshly prepared standards and the results were compared with those of obtained from samples immediately processed after 0 h. The analytes were regarded as stable when values for precision were less than 15% and the accuracy was within the range 85–115% for all QC levels.

Application

The method described above was used to study the relative bioavailability of two dosage forms. The test formulation was zofenopril calcium capsule and the reference formulation was zofenopril calcium tablet; each formulation contained 15 mg zofenopril calcium. The clinical study protocol was approved by the Ethics Committee of the Dermatology Hospital Affiliated to the Chinese Academy of Medical Sciences. All volunteers gave written informed consent to participate in the study according to the principles of the Declaration of Helsinki.

Eighteen healthy young male Chinese volunteers participated in the study. The mean age of the volunteers was 21.9 ± 2.0 (range 18–26), and their mean body weight was 63.3 ± 4.7 kg (range 52–70 kg). After overnight fast, each volunteer received a dose of 30 mg of the test or reference formulations, which were swallowed with 250 mL water. Standard meals were provided 4 h post-dose. Blood samples were collected pre-dose and 0.25, 0.50, 0.75, 1.0, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 11, 14, and 24 h post-dose. The plasma-separation process should be completed within 10 min to protect zofenoprilat from oxidative degradation. Plasma samples (1 mL) were immediately transferred to 2-mL plastic tubes containing 40 µL EDTA-2Na (0.1 mol L^{-1}) and 150 µL *p*-BPB methanol solution (1 mg mL^{-1}). Tubes were vortex mixed for 10 s and kept in a water bath of 45 °C for 30 min. After cooling to room temperature, the mixtures obtained were stored at −20 °C until analysis. During these procedures the samples were protected from light.

Model-independent pharmacokinetic data were calculated. The maximum plasma concentration (C_{max}) and the time at which this occurred (T_{max}) were noted directly. The elimination rate constant (k_{el}) was calculated by linear regression of the terminal points of the semi-log plot of plasma concentration against time. Elimination half-life ($t_{1/2}$) was calculated by use of the formula: $t_{1/2} = 0.693/k_{el}$. The area under the plasma concentration–time curve to the last measurable plasma concentration (AUC_{0-24}) was calculated by use of the linear trapezoidal rule.

Results and Discussion

Optimization of the Derivatization Conditions

To determine the concentration of zofenoprilat accurately, oxidation of its thiol group must be prevented. During the early stage of method development, addition of appropriate antioxidants (for example 2-hydroxy-1-ethanethiol, cysteine, etc.) to plasma samples was tested. The experiment results showed,

however, that such methods could not prevent oxidation of zofenoprilat in plasma. Derivatization of the thiol group was therefore essential to protect it from oxidation. Finally, a new derivatization reagent, *p*-BPB, was introduced to react with the free active thiol group of zofenoprilat. To achieve the highest sensitivity and accuracy for zofenoprilat-*p*-BPB, the effects of the reaction conditions used for derivatization (reaction temperature, time, and the amount of *p*-BPB) were investigated in aqueous medium and blank plasma.

Effect of Temperature

Derivatization was performed at 25, 35, 45, and 55 °C. The experimental results showed that derivative peak areas were larger and relatively stable at 45 and 55 °C. Derivatization at 45 °C was eventually selected. A higher temperature had no significant effect on accelerating the reaction.

Effect of Time

The derivatization was monitored after 15, 30, 60, and 90 min. The results showed that the derivatization reagent was highly reactive and reaction was rapid. The derivatization reaction reached equilibrium in a short time, and the peak area of the derivative decreased as reaction time was increased. A derivatization time of 30 min was eventually selected.

The MS response to the derivative was compared for different reaction temperatures and time for three concentrations of zofenoprilat—4, 50, and 900 ng mL^{-1}. Maximum and constant peak areas were achieved when zofenoprilat in plasma were derivatized at 45 °C for 30 min. This indicated that *p*-BPB reacted rapidly and smoothly with zofenoprilat under mild conditions.

Effect of the Amount of Derivatization Reagent Added

Excess *p*-BPB was required for the derivatization reaction to proceed efficiently and completely. Because of the unknown concentration of clinical samples, complete derivatization had to be

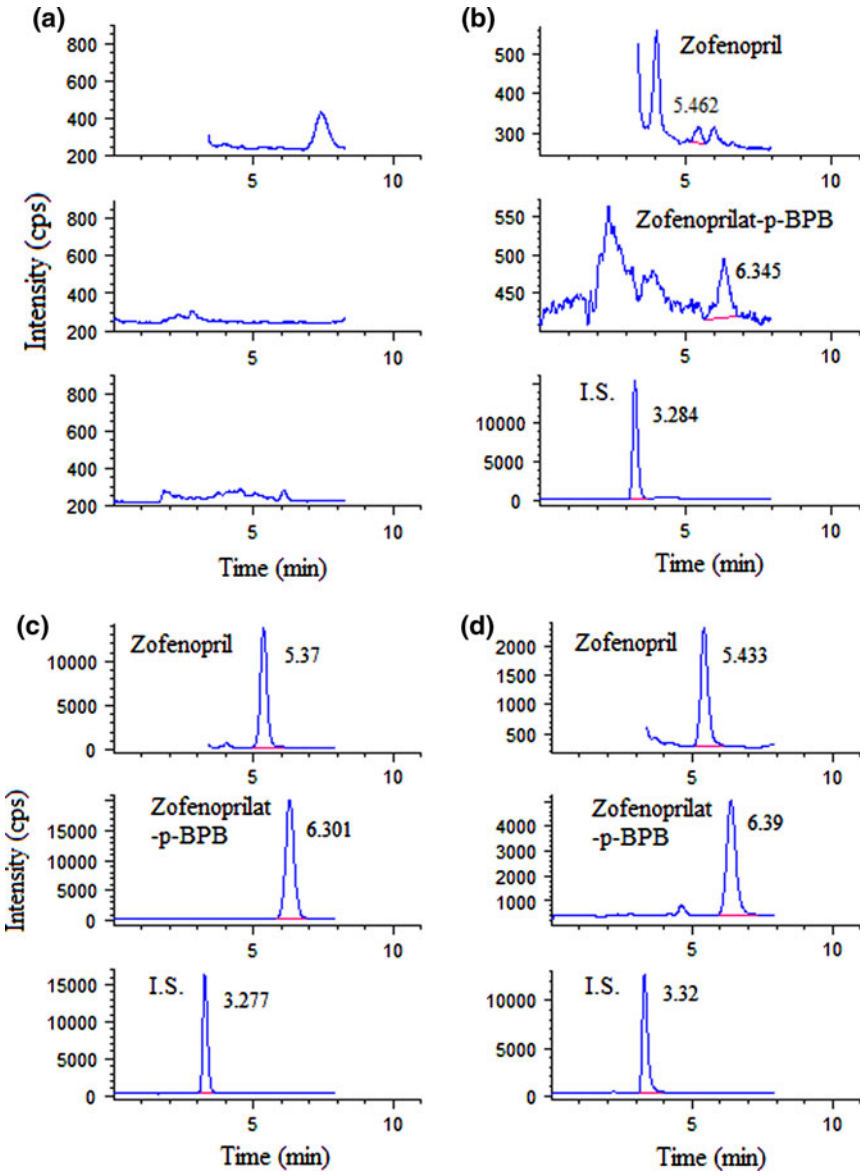

Fig. 4. Typical SIM chromatograms obtained from blank plasma (**a**); from plasma spiked at the LLOQ level with zofenopril (2.5 ng mL^{-1}) and zofenoprilat (5 ng mL^{-1}) and with the I.S. (**b**); plasma spiked with 450 ng mL^{-1} zofenopril, 900 ng mL^{-1} zofenoprilat, and the I.S. (**c**); and plasma obtained from a volunteer 0.25 h after oral administration of 30 mg zofenopril calcium; plasma concentrations were estimated to be 71.29 ng mL^{-1} for zofenopril and 217.8 ng mL^{-1} for zofenoprilat (**d**)

Table 1. Precision and accuracy of the assay for analysis of zofenopril and zofenoprilat in human plasma ($n = 3$ batches, five replicates per batch)

Analyte	Concentration (ng mL^{-1})		RSD (%)		RE (%)
	Added	Found	Intra-batch	Inter-batch	
Zofenopril	2.550	2.430	4.8	8.8	−4.7
	40.80	40.01	3.7	4.4	−1.9
	459.0	454.1	3.8	11.8	−1.1
Zofenoprilat	5.130	5.048	5.6	9.5	−1.6
	102.6	106.3	4.6	12.3	3.6
	1,642	1,633	1.8	1.1	−0.5

Table 2. Results from study of the stability of zofenopril and zofenoprilat-*p*-BPB in human plasma under different storage conditions ($n = 3$)

Storage conditions	Zofenopril				Zofenoprilat-*p*-BPB			
	Concn added (ng mL^{-1})	Concn found (ng mL^{-1})	RSD (%)	RE (%)	Concn added (ng mL^{-1})	Concn found (ng mL^{-1})	RSD (%)	RE (%)
Room temperature for 14 h	2.550	2.700	4.1	5.9	5.130	5.067	6.2	−1.2
	40.80	38.96	0.5	−4.5	102.6	103.9	1.0	1.3
	459.0	454.0	2.8	−1.1	1642	1567	2.8	−4.6
Three freeze–thaw cycles	2.550	2.542	10.7	−0.3	5.130	4.759	8.8	−7.2
	40.80	37.32	1.3	−8.5	102.6	97.01	6.6	−5.4
	459.0	469.3	3.0	2.2	1642	1,575	2.0	−4.1
Freezing for 8 weeks at −20 °C	2.550	2.557	3.8	0.3	5.130	5.330	0.8	3.9
	40.80	40.84	1.0	0.1	102.6	102.8	1.0	0.2
	459.0	479.1	1.6	4.4	1642	1,700	1.7	3.5
Autosampler for 18 h at 8 °C	2.550	2.526	5.2	−0.9	5.130	5.082	0.9	−0.9
	40.80	39.38	3.6	−3.5	102.6	102.3	7.7	−0.3
	459.0	477.7	3.6	4.1	1642	1,646	0.4	0.2

guaranteed by use of excess *p*-BPB until detector response (peak area) was constant. Calibration samples, especially the highest concentration point of the calibration plot, were tested to demonstrate the amount of *p*-BPB added in the assay was sufficient for derivatization of zofenoprilat, and that a greater amount did not increase the response to the derivatives. Comparison of the results obtained by use of 100, 150, and 200 µL (1 mg mL^{-1} solution) showed that 150 µL *p*-BPB was sufficient to achieve good linearity.

For fresh blood obtained from the volunteers, plasma separation should be complete within 10 min, followed by derivatization in a water bath at 45 °C for 30 min.

Sample Preparation

The sample extraction procedure reported by Jemal et al. [5] included LLE and SPE. It was time-consuming and laborious. Although Dal Bo et al. [6] improved this extraction procedure, the harmful, toxic toluene was still used as the extraction reagent. In our method, a simpler extraction procedure was developed. The plasma samples were prepared by liquid–liquid extraction with methyl *tert*-butyl ether as extraction solvent. Because the analytes and the I.S. are all acidic compounds, their extraction recovery can be improved by adjusting the pH of the plasma samples with acetic acid, so 50 µL 50% acetic acid was added to 1 mL plasma sample before extraction. Further tests proved that use of methyl *tert*-butyl ether resulted in cleaner chromatograms from blank plasma samples and higher extraction recovery of the analytes from the plasma than ethyl acetate and diethyl ether.

LC–MS

The mass spectrometric conditions were optimized to achieve maximum sensitivity. Because of the presence of carboxyl in the molecules, good sensitivity was achieved for both zofenopril and zofenopirlat-*p*-BPB in negative-ion mode. Electrospray ionization (ESI) was found to be more sensitive than atmospheric-pressure chemical ionization. To select the target ion for monitoring of zofenopril and zofenopirlat-*p*-BPB, full-scan ESI mass spectra obtained at different fragmentor voltages were investigated. Zofenoprilat-*p*-BPB contains bromine, which generates $[M - H]^-$ isotope peaks at m/z 520.0 and 522.0. Initially, m/z 522.0 was selected as the monitoring ion, but there was endogenous interference from plasma with zofenoprilat-*p*-BPB. Further test results showed there was no significant interference from endogenous impurities when m/z 520.0 was selected as the target ion. Finally, the negative $[M - H]^-$ ions at m/z 428.0 for zofenopril and m/z 520.0 for zofenoprilat-*p*-BPB were selected as the target ions. Comparison of the intensities of the negative ions obtained from zofenopril and zofenopirlat-*p*-BPB at different fragmentor potentials in SIM mode revealed that a fragmentor potential of 100 V resulted in the highest assay sensitivity for the analytes. At this potential the most abundant ion in the mass spectrum of the I.S. was $[M - H]^-$ at m/z 360.0.

Bezafibrate was chosen as the I.S. because it has similar functional groups to zofenopril and zofenoprilat-*p*-BPB, and its retention is similar to that of the analytes. Selection of appropriate mobile-phase components was critical in achieving good chromatography behavior. Methanol and acetonitrile were tested as organic mobile-phase components. Acetonitrile resulted in narrower peaks and better sensitivity than methanol. Further experiments indicated that addition of formic acid to the mobile phase enabled adjustment of the retention time and enhanced the MS response to the analytes. Use of an appropriate amount of ammonium acetate buffer solution in the mobile phase may ameliorate chromatographic peak shapes. After comparison of the effects of adding 10, 20, and 30 mM, 10 mM ammonium acetate buffer solution was chosen. Finally, acceptable retention and good separation of the target compounds was achieved by using 40:60 (v/v) 10 mM ammonium acetate buffer solution containing 0.1% formic acid–acetonitrile as mobile phase.

Assay Validation

Selectivity

No endogenous interferences were observed at the retention times of the analytes and the I.S. Figure 4 shows typical chromatograms obtained from blank plasma, plasma spiked with zofenopril and zofenoprilat at the LLOQ level and with the I.S., plasma spiked with 450 ng mL^{-1} zofenopril and 900 ng mL^{-1} zofenoprilat and with the I.S., and plasma from a healthy volunteer 0.25 h after oral administration of 30 mg zofenopril calcium. Typical retention times for zofenopril, zofenoprilat-*p*-BPB, and the I.S. were 5.4, 6.3, and 3.3 min, respectively.

Linearity of Calibration Plots, and Lower Limit of Quantification

The calibration plots were linear over the ranges 1–500 ng mL^{-1} and 2–1,800 ng mL^{-1} for zofenopril and zofenoprilat, respectively, in human plasma. A typical regression equation for zofenopril was:

$$f = 0.001992 + 0.003113 \times C, r = 0.9986$$

where f represents the zofenopril-to-I.S. peak-area ratio and C represents the plasma concentration of zofenopril. A typical regression equation for zofenoprilat was:

$$f = 0.002856 + 0.003620 \times C, r = 0.9968$$

where f represents the zofenoprilat-*p*-BPB-to-I.S. peak-area ratio and C represents the plasma concentration of zofenoprilat.

The LLOQ was 1 ng mL^{-1} for zofenopril and 2 ng mL^{-1} for zofenoprilat, so the sensitivity of this LC–MS method is more than that of a reported GC–MS method [5] and the same as that of a reported LC–MS–MS method [6]. At the LLOQ level, the RSD was 7.8% ($n = 5$) for zofenopril and 3.1% ($n = 5$) for zofenoprilat, and the RE ranged from −12.1 to 7.5% for zofenopril and from −1.7 to 11.8% for zofenoprilat.

Assay Precision and Accuracy

The results obtained from determination of intra-batch and inter-batch precision and accuracy are summarized in Table 1. These results show that precision and accuracy are within the acceptable range and the method is accurate and precise.

Extraction Recovery

Extraction recovery at low (2.5 ng mL^{-1} for zofenopril and 5 ng mL^{-1} for zofenoprilat-*p*-BPB), middle (40 ng mL^{-1} for zofenopril and 100 ng mL^{-1} for zofenoprilat-*p*-BPB), and high (450 ng mL^{-1} for zofenopril and 1,600 ng mL^{-1} for zofenoprilat-*p*-BPB) concentrations was 83.9, 81.9, and 74.9% ($n = 5$), respectively, for zofenopril, and 82.1, 82.2, and 75.1% ($n = 5$), respectively, for zofenoprilat-*p*-BPB.

Matrix Effects

Matrix effects at low, middle, and high concentrations were 101.5 ± 5.7%, 96.4 ± 2.7%, and 99.8 ± 1.6% ($n = 5$), respectively, for zofenopril and 98.9 ± 7.1%, 99.5 ± 12.4%, and 110.3 ± 2.0% ($n = 5$), respectively, for zofenoprilat-*p*-BPB. Matrix effects for the I.S. were 100.3 ± 8.9%. These results showed that no matrix effects on the analytes and I.S. were observed in this study.

Stability

The results obtained from determination of stability (Table 2) showed no significant degradation occurred under the conditions tested. Zofenopril and zofenoprilat-*p*-BPB were stable in plasma at ambient temperature for 14 h, during three freeze–thaw cycles, and at −20 °C for 8 weeks. Post-preparative samples were stable in the autosampler for 18 h.

Application

The method was successfully used to determine concentrations of zofenopril and its active metabolite zofenoprilat in plasma from healthy Chinese volunteers after single oral administration of test and the reference formulations. Figure 5 shows the mean plasma concentration–time curves for zofenopril and zofenoprilat. The main pharmacokinetic data for zofenopril and zofenoprilat calculated for 18 volunteers are summarized in Table 3. The relative bioavailability of the test formulation was 112.0% for zofenopril and 102.9% for zofenoprilat, based on the test-to-reference ratio of AUC. There were no remarkable differences between the bioavailability of the test and reference formulations. The experimental results showed that the C_{max} and AUC for zofenopril and zofenoprilat for Chinese volunteers were higher than for Caucasian volunteers [8, 9], possibly because of genetic differences. In this study, the pharmacokinetic data were essentially consistent with those obtained from a different group of volunteers in an previous, preliminary, study [7].

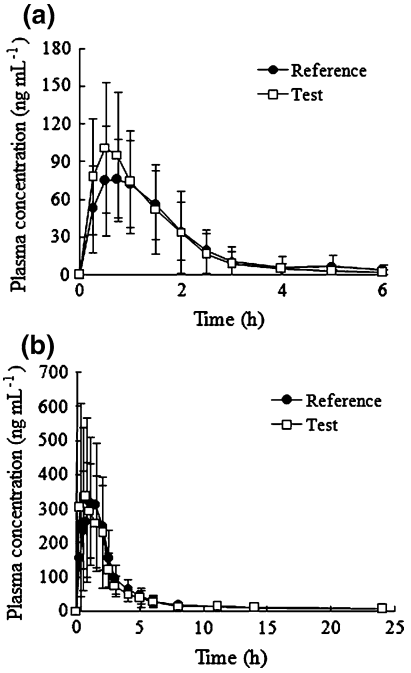

Fig. 5. Mean plasma concentration–time profiles for zofenopril (**a**) and zofenoprilat (**b**) after oral administration of 30 mg zofenopril calcium to 18 healthy volunteers. Each point represents the mean ± SD ($n = 18$)

Conclusion

A new, simple LC–ESI-MS method for simultaneous quantification of zofenopril and its active metabolite zofenoprilat in human plasma has been developed and validated. *p*-BPB was used as

Table 3. Mean pharmacokinetic data for zofenopril and zofenoprilat after oral administration of test and reference zofenopril calcium formulations to 18 volunteers ($n = 18$, mean ± SD)

	Test capsule		Reference tablet	
	Zofenopril	Zofenoprilat	Zofenopril	Zofenoprilat
C_{max} (ng mL^{-1})	112.7 ± 57.9	541.3 ± 265.1	92.4 ± 34.8	466.9 ± 174.6
AUC (ng mL^{-1} h)	157.5 ± 85.2	971.8 ± 230.6	145.0 ± 79.6	981.3 ± 244.3
T_{max} (h)	0.6 ± 0.2	0.9 ± 0.7	0.8 ± 0.4	1.2 ± 0.6
k_{el} (h^{-1})	1.43 ± 0.70	0.12 ± 0.04	0.98 ± 0.01	0.15 ± 0.05
$t_{1/2}$ (h)	0.7 ± 0.5	6.6 ± 2.4	0.6 ± 0.3	5.4 ± 2.6

derivatization reagent for the thiol group of zofenoprilat. There were no significant interferences from endogenous compounds. The method is simple, and suitable for pharmacokinetic study and evaluation of the bioavailability of zofenopril calcium formulations in human subjects.

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