Determination of Zofenopril and Its Active Metabolite in Human Plasma Using High-Performance Liquid Chromatography Combined With a Triple-Quadruple Tandem Mass Spectrometer

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A simple, selective and sensitive LC–MS-MS method has been developed and validated to simultaneously quantify zofenopril and its active metabolite zofenoprilat in human plasma, using diazepam as internal standard. 1,4-Dithiothreitol was used as a reducer to release and stabilize the thiol group of zofenoprilat from dimer and mixed forms with endogenous thiols in the treatment of plasma samples. After a liquid–liquid extraction with methyl tert-butyl ether under acidic conditions, the post-treatment samples were analyzed on an Agilent ZORBAX Eclipse XDB-C8 column interfaced with a triple-quadrupole tandem mass spectrometer using positive electrospray ionization. A solution of methanol and 0.1% formic acid solution (85 : 15, v/v) was used as the isocratic mobile phase with a flow rate of 0.2 mL/min. The method was validated to demonstrate the specificity, lower limit of quantitation, accuracy and precision of measurements. The validated LC–MS-MS method has been successfully applied to study the pharmacokinetics of zofenopril calcium in healthy Chinese volunteers.

Introduction

Zofenopril calcium, [1(5), 4(5)]-1(3-mercaptop-2-methyl-1-oxopropyl)-4-phenyl-thio- L-proline-3-benzoylester, is a new sulfhydryl-group-containing ACE inhibitor with antihypertensive, remarkable antioxidant and cardioprotective properties, including the ability to improve endothelial function and protect against ischemia (1, 2). These peculiar characteristics are mainly due to the presence of a sulfhydryl group and the highly lipophilic nature of the compound. Zofenopril (Figures 1 and 2), as most compounds of this class, is a prodrug that is deesterified to the active metabolite, the sulfhydryl-group-containing ACE inhibitor with antihypertensive, remarkable antioxidant and cardioprotective properties, in including the ability to improve endothelial function and protect against ischemia (1, 2). These peculiar characteristics are mainly due to the presence of a sulfhydryl group and the highly lipophilic nature of the compound. Zofenopril (Figures 1 and 2), as most compounds of this class, is a prodrug that is deesterified to the active metabolite, the sulfhydryl group containing compound, zofenoprilat (Figure 1).

Thiol compounds can be easily oxidized to disulfides either as dimer or mixed forms with endogenous thiols in biological matrices (3). So, it is difficult to determine zofenoprilat directly in human plasma because of its active thiol group. The measurement of free or unchanged zofenoprilat concentration needs to be preceded by the addition of chemical stabilizer or by molecule derivatization of biological samples in order to prevent zofenoprilat disulfide formation. Several analytical methods have been reported with complicated sample preparation. Jemal et al. (4) used solid-phase extraction after liquid–liquid extraction for isolation and purification, followed by methylation and reconstitution with tetramethylbenzene before GC–MS analysis. Dal Bo et al. (5) described an LC–MS–MS method for simultaneous analysis of zofenopril and zofenoprilat with N-ethylmaleimide as derivatization reagent. Gao et al. (2) used p-bromophenacyl bromide as a derivatization reagent for the simultaneous determination of zofenopril and zofenoprilat in human plasma by LC–MS–MS. These methods involved derivatization and/or specialized and expensive equipment, which appeared to show low reproducibility and time-consuming sample preparation. Although Jiang et al. (6) proposed a relatively simple and fast method for the simultaneous determination of zofenopril and zofenoprilat by LC–MS–MS; 2-mercaptoethanol was used as the stabilization agent which carried the disadvantage of being toxic. Therefore, it was considered necessary to use a safer derivatization reagent.

In this paper, a relatively simple, fast and sensitive HPLC–MS–MS method for direct simultaneous determination of zofenopril and zofenoprilat in human plasma is proposed. In the sample preparation process, 1,4-dithiothreitol (DTT) was used for the stabilization to revert the converted disulfide dimers (or conjugates) into zofenoprilat as well as to prevent the formation of the disulfide dimers in plasma. In this investigation, the validated HPLC–MS–MS method has been successfully applied to a clinical pharmacokinetic study of zofenopril calcium tablets in healthy Chinese volunteers.

Experimental

Instrumentation and reagents
Liquid chromatography was performed using the Finnigan™ TSQ Quantum Discovery MAX™ LC–MS–MS system consisting of a Finnigan™ Surveyor LC pump, a Finnigan™ Surveyor autosampler and combined with a triple-quadrupole TSQ Quantum mass spectrometer (Thermo Electron Corporation).

The zofenopril calcium reference standard (Batch No.: 20090110, 99.15% purity) was identified and supplied by Hefei Xinfeng Co. Ltd (Hefei, P. R. China); the zofenoprilat reference standard (Batch No.: 080701, 99.2% purity) was obtained from Shanghai Haini Pharmaceutical Co. Ltd (Shanghai, P. R. China); the diazepam reference standard (Batch No.: 1230-9601, 99.0% purity) was purchased from National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, P. R. China). HPLC grade methanol was purchased from Merck (Merck Company, Germany). DTT was of analytical grade purity.

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and purchased from Aladdin Chemistry Co. Ltd. (Shanghai, P. R. China); other reagents of analytical grade such as methyl tert-butyl ether, formic acid, hydrochloric acid, EDTA-2Na and vitamin C were purchased from Nanjing Chemical Reagent No. 1 Factory (Nanjing, P. R. China). The mobile phase was filtered through a 0.22 μm film before use. Water was distilled twice before use.

**LC–MS-MS instrumentation and conditions**

The chromatographic separation was performed on an Agilent ZORBAX Eclipse XDB-C8 (150 × 2.1 mm I.D., 3.5 μm) column at 30°C. The mobile phase consisting of methanol–0.1% formic acid solution (85:15, v/v) was set at a flow rate of 0.2 mL/min. The tandem MS system was equipped with an ESI source, and run with the Xcalibur 2.0 software (Thermo Electron Corporation). The mass spectrometer was operated in positive ion and selective reaction monitoring (SRM) mode with a precursor to product transition m/z 430 → 280 for zofenopril, m/z 326 → 178 for zofenoprilat and m/z 285 → 193 for internal standard (IS). Spray voltage was optimized at 4,200 V, transfer capillary temperature at 300°C, sheath gas and auxiliary gas (nitrogen) pressure at 30 arbitrary units (52.5 × 10^5 Pa) and 5 arbitrary units (8.75 × 10^5 Pa), (set by the LCQ software, Thermo Electron Corporation). Argon was used as collision gas at a pressure of 1.5 mTorr (0.200 Pa) and the optimized collision energy was set as follows: 15 V for zofenopril, 21 V for zofenoprilat and 30 V for IS. The scan width for SRM was 0.01 m/z and scan time was 0.05 s. The peak width settings (FWHM) for both Q1 and Q3 were 0.70 m/z.

**Methods**

**Preparation of stock and working solutions**

Standard stock solutions of zofenopril calcium were prepared in a mixture of methanol–water (9:1, v/v) at a concentration of 200 μg/mL (based on zofenopril). Standard stock solutions of zofenoprilat were prepared in methanol at a concentration of 500 μg/mL. Subsequently, 1 mL of zofenopril stock solution and 1 mL of zofenoprilat stock solution were accurately transferred into a 5 mL brown volumetric flask and diluted with methanol to 5 mL. Before adjusting the volume, 100 μL of a 200 mM DTT solution were added to the flask to obtain stable stock solutions. The IS stock solutions were also prepared in methanol at a concentration of 1.0 mg/mL. All stock solutions were stored at 4°C.

Working solutions of zofenopril and zofenoprilat were prepared daily in methanol: 0.1% formic acid solution (85:15, v/v) by appropriate dilution at 0.01, 0.02, 0.04, 0.1, 0.4, 1, 4, 10 and 40 μg/mL for zofenopril and 0.025, 0.05, 0.1, 0.25, 1, 2.5, 10, 25 and 100 μg/mL for zofenoprilat. The IS working solution was prepared by diluting the stock solution to 500 ng/mL.

**Sample preparation**

Precaution was taken to avoid sunlight. Blood samples (3 mL each) were collected in tubes, mixed with 100 μL of 0.1 M vitamin C solution and 50 μL of 0.1 M EDTA-2Na solutions, and dried before use. The samples were then centrifuged for 8 min at 4,000 rpm and 1 mL of separated plasma was transferred to Eppendorf tubes. The obtained samples were spiked with 200 μL of 0.1 M vitamin C solution, vortexed and stored at −26°C until the day of analysis. An aliquot (500 μL) of plasma samples was transferred to polypropylene tubes. After adding 10 μL of IS working solution (500 ng/mL) and 100 μL of 200 mM DTT solution, the samples were vortex-mixed for 30 s and left for 20 min at room temperature. Then, 100 μL of 0.5 M hydrochloric acid solution and 4 mL of methyl tert-butyl ether were added to the plasma samples. Finally, the mixtures were vortex-mixed for 3 min and centrifuged for 10 min at 4,000 rpm. The 3 mL organic layer was transferred to another clean glass tube and evaporated under a steady stream of nitrogen to dryness in a water bath at 25°C. The residue was reconstituted in 200 μL of mobile phase and 5 μL were injected into the LC–MS-MS system. The chromatographic eluent was diverted to waste for 4.0 min after each sample injection in order to keep the ion source as clean as possible.

**Calibration standards and quality control samples**

Calibration curves were prepared on five different days by spiking blank plasma with 10 μL of one of the working solutions mentioned above to produce the standard curve points equivalent to 0.2, 0.4, 0.8, 2, 8, 20, 80, 200 and 800 ng/mL for zofenopril and 0.5, 1, 2.5, 5, 20, 50, 200 and 2,000 ng/mL for zofenoprilat. The following assay procedures were the same as those described above. In each run, a blank plasma sample (processed without IS) was analyzed to confirm the absence of interferences, but not used for construction of the calibration function.

Quality control (QC) samples were prepared by spiking blank plasma with 10 μL of one of the working solutions mentioned above to produce a final concentration equivalent to 0.4 ng/mL (low level), 20 ng/mL (middle level), 640 ng/mL (high level) for zofenopril and 1 ng/mL (low level), 50 ng/mL (middle level), 1,600 ng/mL (high level) for zofenoprilat. The following procedures were the same as those described above.
Validation of the bioanalytical method

The method was validated following the currently accepted United States Food and Drug Administration (FDA) bioanalytical method validation guidance (7).

Specificity

Specificity was assessed by analyzing the blank plasma samples from six different sources to ensure that no visible interferences were present at the retention time of zofenopril, zofenoprilat and IS.

Linearity

Linearity was determined at five replicates at concentration levels of 0.2, 0.4, 0.8, 2, 8, 20, 80, 200 and 800 ng/mL for zofenopril and 0.5, 1, 2, 5, 20, 50, 200, 500 and 2,000 ng/mL for zofenoprilat. Calibration curves were generated by using the ratios of the analyte peak area to the IS peak area versus concentration, and fitted to the equation $y = bx + a$ by weighted least-squares linear regression, respectively.

The limit of detection (LOD) was determined as the concentrations with a signal-to-noise ratio of 3. The lower limit of quantification (LLOQ) should be at least five times the response compared with blank response. The LLOQ should be established using at least five samples and determining the CV, and/or appropriate confidence interval should be determined.

Precision and accuracy

The intra-batch accuracy and precision were determined by analyzing five sets of spiked plasma samples at QC levels in a batch. The inter-batch precision and accuracy were measured on three
matrix effect of internal standard (10 ng/L concentration levels were evaluated by analyzing five samples of those of standard solutions at the same concentration. Three QC (external in plasma) was evaluated in the same way.

**Extraction recovery**

The absolute extraction recoveries of zofenopril and zofenoprilat were evaluated by comparing the peak areas resulting from extracted samples to those from samples containing the same amount of analyte that was added after the extraction step, respectively (on the extraction regents). The procedure was performed at three QC concentration levels for five replicates.

**Matrix effects**

Matrix effects were thoroughly evaluated using blank plasma from different sources. A quantitative estimate of the matrix effects was obtained by comparing the peak areas of the analytes dissolved in the supernatant of the processed blank plasma with those of standard solutions at the same concentration. Three QC concentration levels were evaluated by analyzing five samples of each set. The matrix effect of internal standard (10 ng/mL of internal in plasma) was evaluated in the same way.

**Stability**

Stability was evaluated under different conditions that occurred during sample analysis. The short-term stability was evaluated by keeping QC samples at room temperature for 8 h. Long-term stability was assessed by keeping QC plasma samples at low temperature (−20°C) for 15 days. Post-preparative stability was assessed by re-analyzing the extracted QC samples kept under the auto-sampler conditions (10°C) for 48 h. Freeze–thaw stability was tested by analyzing QC samples undergoing three freeze (−20°C)–thaw (room temperature) cycles on consecutive days. Stability of zofenopril and zofenoprilat in human plasma under different conditions were evaluated by fresh calibration curve. The working solutions and stock solutions of zofenopril, zofenoprilat and the IS were also evaluated for stability at room temperature for 24 h and at 4°C for 15 days, respectively.

**Pharmacokinetic study**

The above-validated method was successfully applied to a pharmacokinetic study of zofenopril calcium tablets in healthy Chinese volunteers. The clinical protocol was approved by the Medical Ethics Committee of the Institute of Dermatology, Chinese Academy of Medical Sciences and Peking Union Medical College (Xuanwu District, Nanjing City, Jiangsu Province, China). Clinical Trial Approval Number of our study was 2009-50, and authorization holds good until 9 June 2011. All volunteers written informed consent to participate in the study according to the principles of the Declaration of Helsinki. After assessment of the clinical history, physical examination, electrocardiogram and standard laboratory biochemical examination (blood cell count, biochemical profile and urine analysis), 12 healthy volunteers (6 males and 6 females) were selected to participate in the single-dose, food effect and multiple-dose experiment. The age of the 12 healthy volunteers ranged from 21 to 33 years old and the weight ranged from 50 to 75 kg. All volunteers gave written informed consent to participate in the study. The volunteers were asked to stop taking any medication at least 2 weeks before the study.

An open-label, randomized study was conducted among 12 healthy volunteers (6 males and 6 females). In the single-dose study, each subject was administered the following doses: one tablet (15 mg), two tablets (30 mg), four tablets (60 mg) of zofenopril calcium under fasting conditions in the first, fourth, and second period, respectively. Then, serial blood samples (3.0 mL) were collected from vein at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 12 and 24 h. To study the effect of food, two tablets of zofenopril calcium (30 mg) was given under fasting and nonfasting conditions with a washout interval of 1 week in the third and fourth period. For the nonfasting group, a glass of milk, two pieces of bread, two eggs, two slices of meat and one scoop of green salad were given, and the tablets were administered 30 min after meals. Time collection points of blood sample were the same as those of the single-dose study. The multiple-dose study started after the food effect study with a week washout. In the multiple-dose study, the same 12 volunteers received an oral administration of 30 mg of zofenopril calcium once a day (at 7:00 h) until the morning of the 6th day. Venous blood (3.0 mL) was drawn at the 4th day (−48 h), 5th day (−24 h) and 6th day (0 h) morning before administration to ensure that the steady state has appeared. On the 6th day (0 h) blood was drawn again following the pre-specified time points after the last dose. Venous blood samples were obtained at −48, −24, 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 12 and 24 h. Each plasma sample was collected into tubes, mixed with 100 μL of 0.1 M vitamin C solution and 50 μL of 0.1 M EDTA-2Na solution. The samples were then centrifuged for 8 min at 4,000 rpm and 1 mL of separated plasma was transferred to Eppendorf tubes. The obtained samples were spiked with 200 μL of 0.1 M vitamin C solutions, vortexed and stored at −26°C until the day of analysis.

Pharmacokinetic parameters were calculated by the program package (Drug and Statistics v 2.0) software. The maximum plasma concentration after a single-dose (Cmax), maximum plasma concentration observed at steady state (Cmax,ss), minimum plasma concentration observed at steady state (Cmin,ss), time corresponding to Cmax (Tmax) and time corresponding to Cmax,ss (tmax,ss) were determined from the plasma concentration–time curves. The elimination rate constant (k1) was obtained as the slope of the linear regression of the log-transformed concentration values versus time data in the terminal phase. The elimination half-life (t1/2) was calculated as 0.693/k1. The area under the plasma concentration–time curve (AUC) from time 0 to the last data point (AUC0−t) after the single-dose study was estimated by the linear trapezoidal rule, while the AUC from time 0 to infinity (AUC0−∞) was calculated as AUC0–t + C∞/k1, where C∞ was the last measurable concentration. The AUC0−t was the steady-state AUC after the multiple-dose administration, and was also calculated by the linear trapezoidal rule. The mean residence time (MRT) was calculated as AUMC0−∞/AUC0−∞. The steady-state average plasma concentration (Cav) was calculated as AUCav/τ, where τ represents the dosing interval, while the steady-state
average plasma concentration (DF) was calculated as \((C_{\text{max}} - C_{\text{min}})/C_{\text{max}} \times 100\%\).

Statistical comparisons of the pharmacokinetic parameters (MRT, \(t_{1/2}\) and \(C_{\text{max}}\)) of zofenoprilat and zofenopril were assessed utilizing the \(t\)-test on the ln-transformed data. A nonparametric test (Wilcoxon signed rank test) was applied to assess the effect of the administration conditions on \(t_{\text{max}}\) values. A value of \(P < 0.05\) was considered to be statistically significant.

**Results**

Due to the presence of a free sulphydryl group in the molecular structure, zofenoprilat was readily oxidized to disulfides either as dimer or mixed forms with endogenous thiols. For this reason, unless the thiol group can be stabilized in the biological sample, quantitative analysis of the zofenoprilat can be very problematic. Therefore, reduction of the disulfide bonds is required to revert the converted disulfide dimer as well as to prevent the formation of the disulfide dimers during the sample preparation and analysis. The most commonly used reducing agents for thiol compound are 2-ME, DTT, sodium borohydride (NaBH₄), tributylphosphine (TBP). NaBH₄ needs high concentration or long time for complete reaction and TBP is anmytic and explosive. Dithiothreitol (DTT) displays lower toxicity and was considered to be a safer alternative to 2-mercaptoethanol. Therefore, DTT was selected as the competitive reducer to protect and stabilize the zofenoprilat activity.

A solution of 200 mM DTT was added to the plasma samples of zofenoprilat to prevent its oxidative degradation by protecting the free sulphydryl group. Different volumes of 200 mM DTT solution, including 25, 50 and 100 \(\mu\)L, were studied, and the relationship between the deoxidizing time and yield of the free zofenoprilat in the pretreatment step was investigated. From the results, 20 min of deoxidizing time with 50 \(\mu\)L of 200 mM DTT solution was enough in the pretreatment process. Furthermore, according to previous studies, the oxidation reaction forming disulfide can also be delayed by adding chelating agents or incorporating antioxidants and anti-irritants (8). Therefore, in our experiment, we adopted EDTA-2Na as an anticoagulant agent and selected vitamin C as an antioxidant to reduce the formation of disulfide bonds in the preparation of blood samples.

Liquid–liquid extraction was necessary and important as this technique could not only purify but also concentrate the sample. Ethyl acetate, diethyl ether and methylene chloride–ethyl acetate (20 : 80, v/v), methyl tert-buty1 ether were all tried and methyl tert-buty1 ether was finally adopted because of high extraction efficiency, and the extracted endogenous compounds did not interfere with the determination of zofenopril, zofenoprilat and IS. One hundred microliters of 0.5 M hydrochloric acid solution were added to the plasma to enhance the extraction efficiency.

**Specificity**

The representative chromatograms of blank plasma (Figure 3A), blank plasma spiked with zofenopril and zofenoprilat at the LLOQ of 0.2 ng/mL for zofenopril and 0.5 ng/mL for zofenoprilat (Figure 3B) and volunteer plasma sample at 1 h after a single oral administration of 15 mg zofenopril calcium (Figure 3C) meant that no endogenous interferences were present at the retention times of 6.5 min (zofenoprilat), 7.7 min (zofenopril) and 7.1 min (IS), respectively.

**Linearity and LLOQ**

The assay exhibited good linear response over the concentration range of 0.2–800 ng/mL for zofenopril and 0.5–2,000 ng/mL for zofenoprilat by weighted (1/\(x^2\)) least-squares linear regression analysis. The mean standard curve was typically described by the equation: \(y = 37.71x + 0.06719\), \(r = 0.9985\) for zofenopril and \(y = 68.25x + 0.1415\), \(r = 0.9985\) for zofenoprilat, where \(y\) corresponds to the peak area ratio of analytes to IS and \(x\) refers to the concentration of analytes added to plasma. The results of five representative standard curves for LC–MS-MS determination of zofenopril and zofenoprilat are given in Table I.

The lower limit of quantitation for zofenopril proved to be 0.2 ng/mL, and the LOD was 0.1 ng/mL. The lower limit of quantitation for zofenoprilat proved to be 0.5 ng/mL, and the LOD was 0.25 ng/mL.

**Precision and accuracy**

An assessment of intra- and inter-batch precision was conducted by analyzing QC samples at three levels. Data of precisions are presented in Table II. The QC concentrations were calculated from calibration curve, and the intra- and inter-batch precision deviation values were <15% and the accuracy deviation values for intra- and inter-batch were all within (100 ± 15)% of actual values at each QC level. The results revealed good precision and accuracy.

**Extraction recovery**

The data of extraction efficiency measured for zofenopril and zofenoprilat in human plasma were consistent, precise and reproducible. The mean absolute extraction recovery of zofenoprilat at each QC level (0.4, 20 and 640 ng/mL) was (87.67 ± 5.63), (91.61 ± 4.58) and (88.86 ± 4.85)% respectively. The mean absolute extraction recovery of zofenopril at each QC level (1, 50 and 1,600 ng/mL) was (86.73 ± 6.20), (82.96 ± 4.41) and (84.55 ± 5.04)% respectively.

**Matrix effect**

Matrix effects are generally problematic during LC–MS-MS analyses of biological samples. In our experiments, the average matrix effect values were 104.10, 99.01 and 100.53% for zofenopril at three QC concentration levels, and the relative standard deviation (RSD) values were 10.18, 1.26 and 2.43% for each QC level. The average matrix effect values were 95.14, 100.19 and 99.91% for zofenoprilat at three QC concentration levels, and the RSD values were 10.62, 1.37 and 1.90% of each QC level. The average matrix effect for IS was 99.43% with RSD of 4.62%. These results indicated that the extracts had little or no undetectable endogenous substances that could influence the ionization of the analytes and IS.

**Stability**

Stability results are shown in Table III. The accuracy values at three QC levels of zofenopril and zofenoprilat were both within ±15%. No significant degradation of the analytes in human plasma occurred after short-term storage for 8 h at room temperature, long-term storage for 15 days at −20°C, three freeze–thaw...
cycles or post-preparative storage for 48 h at 10°C. The stock and working solutions of zofenopril, zofenoprilat and IS were stable for 15 days at 4°C and for 24 h at room temperature, respectively.

**Application**

**Single-dose study**

The mean plasma concentration–time curves of zofenoprilat after single oral doses of 15, 30 and 60 mg of zofenopril calcium in 12 healthy volunteers are shown in Figure 4A. Kinetic parameters are listed in Table IV. The mean plasma concentration–time curves of zofenopril after single oral doses of 15, 30 and 60 mg of zofenopril calcium in 12 healthy volunteers are shown in Figure 4B. Kinetic parameters are listed in Table V.

In our study, the \( C_{\text{max}} \) and \( \text{AUC}_{0-t} \) increased linearly with the increase from 15 to 60 mg. No significant differences were found in \( t_{1/2} \), \( T_{\text{max}} \) and MRT (\( P > 0.05 \)) between doses. The finding of dose proportionality suggests that the processes responsible...
for the absorption and disposition of zofenoprilat and zofenopril are not saturated over the dose range of 15–60 mg.

**Food effect study**
The mean plasma concentration–time curves of zofenoprilat following an oral dose of 30 mg under nonfasting and fasting conditions are shown in Figure 5A. The pharmacokinetic parameters are listed in Table IV. The mean plasma concentration–time curves of zofenopril following an oral dose of 30 mg under nonfasting and fasting conditions are shown in Figure 5B. The pharmacokinetic parameters are listed in Table V.

### Table I
The Results of Five Calibration Curves for the Simultaneous Determination of Zofenopril and Zofenoprilat in Human Plasma (n = 5)

| Analyte | Nominal concentration (ng/mL) | Back-calculated concentration (mean ± SD) (ng/mL) | Mean accuracy (%) | RSD (%)
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>Zofenopril</td>
<td>0.2</td>
<td>0.20 ± 0.00</td>
<td>102.37 ± 1.66</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.40 ± 0.01</td>
<td>99.76 ± 2.41</td>
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<tr>
<td></td>
<td>0.8</td>
<td>0.76 ± 0.03</td>
<td>94.55 ± 3.74</td>
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<tr>
<td></td>
<td>2</td>
<td>1.84 ± 0.08</td>
<td>91.80 ± 4.42</td>
<td></td>
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<tr>
<td></td>
<td>8</td>
<td>7.84 ± 0.31</td>
<td>97.95 ± 3.97</td>
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<tr>
<td></td>
<td>20</td>
<td>19.53 ± 0.60</td>
<td>97.63 ± 3.09</td>
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<tr>
<td></td>
<td>80</td>
<td>80.33 ± 4.08</td>
<td>100.41 ± 5.08</td>
<td></td>
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<tr>
<td></td>
<td>200</td>
<td>211.45 ± 7.13</td>
<td>105.73 ± 3.37</td>
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<tr>
<td>Zofenoprilat</td>
<td>0.5</td>
<td>0.52 ± 0.01</td>
<td>104.13 ± 2.20</td>
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<td></td>
<td>1</td>
<td>0.97 ± 0.06</td>
<td>96.57 ± 6.04</td>
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<tr>
<td></td>
<td>2</td>
<td>1.85 ± 0.03</td>
<td>92.64 ± 1.88</td>
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<tr>
<td></td>
<td>5</td>
<td>4.61 ± 0.22</td>
<td>92.21 ± 4.83</td>
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<td></td>
<td>20</td>
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<td>99.53 ± 4.20</td>
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<tr>
<td></td>
<td>50</td>
<td>50.28 ± 1.58</td>
<td>100.57 ± 3.14</td>
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</tr>
<tr>
<td></td>
<td>200</td>
<td>205.09 ± 11.16</td>
<td>102.54 ± 5.44</td>
<td></td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>529.10 ± 15.38</td>
<td>105.82 ± 2.91</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>2041.66 ± 173.58</td>
<td>102.08 ± 8.50</td>
<td></td>
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</table>

### Table II
The Inter- and Intra-batch Precision, Accuracy of the Method for Simultaneous Determination of Zofenopril and Zofenoprilat

<table>
<thead>
<tr>
<th>Quality control</th>
<th>Nominal concentration (ng/mL)</th>
<th>Intra-batch assay (n = 5)</th>
<th>Inter-batch assay (batch = 3, n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (ng/mL)</td>
<td>Precision (% CV)</td>
<td>Accuracy (%RE)</td>
</tr>
<tr>
<td>Zofenopril</td>
<td>0.4</td>
<td>0.43</td>
<td>6.06</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>19.79</td>
<td>2.44</td>
</tr>
<tr>
<td></td>
<td>640</td>
<td>676.89</td>
<td>1.00</td>
</tr>
<tr>
<td>Zofenoprilat</td>
<td>1</td>
<td>1.04</td>
<td>7.05</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>51.30</td>
<td>1.56</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>529.10</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>1,600</td>
<td>2,041.66</td>
<td>0.91</td>
</tr>
</tbody>
</table>

### Table III
Data Showing Stability of Zofenopril and Zofenoprilat in Human Plasma at Different QC Levels (n = 5)

<table>
<thead>
<tr>
<th>Stability</th>
<th>Concentration found (mean ± SD) (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zofenopril</td>
<td>Zofenoprilat</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td>Short-term stability (8 h, room temperature)</td>
<td>0.42 ± 0.03</td>
</tr>
<tr>
<td>Freeze and thaw stability (Three cycles, −20°C, room temperature)</td>
<td>0.43 ± 0.01</td>
</tr>
<tr>
<td>Long-term stability (15 d, −20°C)</td>
<td>0.41 ± 0.03</td>
</tr>
<tr>
<td>Post-preparative stability (48 h, 10°C)</td>
<td>0.40 ± 0.02</td>
</tr>
</tbody>
</table>

Figure 4. The mean drug plasma concentration–time curves of zofenoprilat in 12 volunteers after oral administration of zofenopril calcium in single-dose study; 1 tablet: 15 mg; 2 tablets: 30 mg; 4 tablets: 60 mg [B]; the mean drug plasma concentration–time curves of zofenopril in 12 volunteers after oral administration of zofenopril calcium in single-dose study; 1 tablet: 15 mg; 2 tablets: 30 mg; 4 tablets: 60 mg [A].
The statistical comparisons of the pharmacokinetic parameters for zofenoprilat showed that there were significant differences between fasting and nonfasting conditions in terms of \( C_{\text{max}} \) values. The statistical comparisons of the pharmacokinetic parameters for zofenopril showed that there were significant differences between fasting and nonfasting conditions in terms of AUC\( _{0-24} \), \( C_{\text{max}} \), and \( T_{\text{max}} \) values. The 90% confidence interval of AUC\( _{0-24} \) was 0.481–0.843 for zofenoprilat, and 1.016–1.116 for zofenopril. Therefore, the food affects the pharmacokinetics of zofenoprilat and zofenopril in healthy Chinese volunteers.

### Multiple-dose study

The mean plasma concentration–time curve of zofenopril obtained at steady state after multiple oral doses of zofenopril calcium in the same 12 healthy volunteers are depicted in Figure 6A. Kinetic parameters are listed in Table IV. The mean plasma concentration–time curve of zofenopril obtained at steady state after multiple oral doses of zofenopril calcium in the same 12 healthy volunteers are depicted in Figure 6B. Kinetic parameters are listed in Table V.

For zofenoprilat, the pharmacokinetic parameters, such as \( T_{\text{max}} \), \( T_{\text{1/2}} \), \( C_{\text{max}} (P > 0.05) \) at steady state had no significant differences to those obtained after a single dose of 30 mg. For zofenopril, the pharmacokinetic parameters, such as \( T_{\text{max}} \) and \( C_{\text{max}} (P > 0.05) \) at steady state, had no significant differences, while \( T_{\text{1/2}} \) had significant differences \((P < 0.05)\) to those obtained after a single-dose of 30 mg.

### Conclusion

A simple, sensitive and reproducible LC–MS-MS method was developed and validated for the simultaneous quantification of zofenopril and its active metabolite zofenoprilat in human plasma, in which DTT was chosen as a reducing agent to release and stabilize the thiol group of zofenoprilat from dimer and mixed forms with endogenous thiols in the treatment of plasma samples. The assay method provided sufficient sensitivity with accuracy and precision for all the analytes and offered a high linearity over the range 0.02–500 ng/mL for zofenopril and 0.5–2,000 ng/mL for zofenoprilat. It proved to be superior when compared with the previously reported LC–MS-MS method (9, 10), because DTT is a lower toxic and safer reducing agent for sample preparation. Furthermore, the new analytical conditions (lower flow rate, column temperature setting) were developed to improve the performance of the method and also to use less organic solvents during sample preparation and analysis. Hence, our method is more suitable for supporting...
environmental responsiveness and, altogether, very appropriate for quantitative high-throughput analysis, such as pharmacokinetic studies at therapeutic drug concentrations in human plasma.

Acknowledgments
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References
