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Development and validation of a liquid chromatography–tandem mass spectrometry method for the determination of zofenopril and its active metabolite zofenoprilat in human plasma

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ABSTRACT

A novel, sensitive and rapid liquid chromatographic-electrospray ionization mass spectrometric method was developed and validated for the determination of zofenopril and its active metabolite zofenoprilat in human plasma. The method was based on a single extraction step using methyl tert-butyl ether and did not require chemical derivatization. The chromatographic conditions were optimized; separation was performed on a phenyl–hexyl column (5 μm, 250 mm × 4.6 mm i.d.) with a mobile phase consisting of a solution of methanol and water (95:5, v/v) that also contained 0.1% of formic acid. A flow rate of 1.0 mL/min was used. Zofenopril, zofenoprilat and the internal standard (IS) fosinopril sodium were measured using an electrospray ion source in a positive reaction monitoring mode. Linear calibration curves were generated for zofenopril concentrations between 0.1052 and 1052 ng/mL and for zofenoprilat concentrations between 0.2508 and 2508 ng/mL. In both cases, the coefficients of determination were greater than 0.995. The extraction recovery for zofenopril was 93.5% on average. It was 92.5% for zofenoprilat. The inter- and intra-batch precision and accuracy for both zofenopril and zofenoprilat were higher than 14%. The method was applied to measure the concentrations of zofenopril and zofenoprilat in plasma samples.

1. Introduction

Zofenopril is a pro-drug designed to undergo metabolic hydrolysis and yield the active free sulfhydryl compound zofenoprilat, an angiotensin converting enzyme (ACE) inhibitor [1,2]. In a number of in vitro and in vivo models of ischemic myocardial injury, zofenopril was found to exert a remarkable cardioprotective effect [3,4]. Indeed, the early administration of zofenopril to patients with myocardial infarction improved their long-term survival [5,6].

Various analytical methods have been used for the immediate determination of zofenopril and its active metabolite zofenoprilat in plasma. Examples of such analytical methods include enzymatic techniques, radioimmunoassay (RIA) [1], GC–mass spectrometry [7] and HPLC–mass spectrometry [8,9]. These methods are rather troublesome. Indeed, they involve derivatization and/or specialized and expensive equipment because of the presence of a sulfhydryl group in the molecular structure of zofenoprilat and the polarity difference between zofenopril and zofenoprilat. The structures of the analytes are shown in Fig. 1.

A derivatization method based on a procedure described by Wu et al. [8] was evaluated for the determination of zofenoprilat in plasma samples. We found that the derivatization efficiency was poor and that the preparation of the plasma sample required a complex derivatization procedure, which was affected by a variety of experimental factors.

The goal of our study was to develop and validate a simple and reproducible reversed-phase LC–MS/MS method for the determination of zofenopril and zofenoprilat in human plasma without chemical derivatization. In our method, a proper chemical stabilizer was used to transform the converted disulfide dimers (or conjugates) into zofenoprilat and prevent the formation of disulfide dimers during sample preparation and analysis.

2. Experimental

2.1. Reagents and chemicals

Zofenopril, zofenoprilat (purity >99.5%, HPLC) and fosinopril sodium (purity >99.5%, HPLC) reference standards were purchased from the National Institute for the Control of Pharmaceutical and

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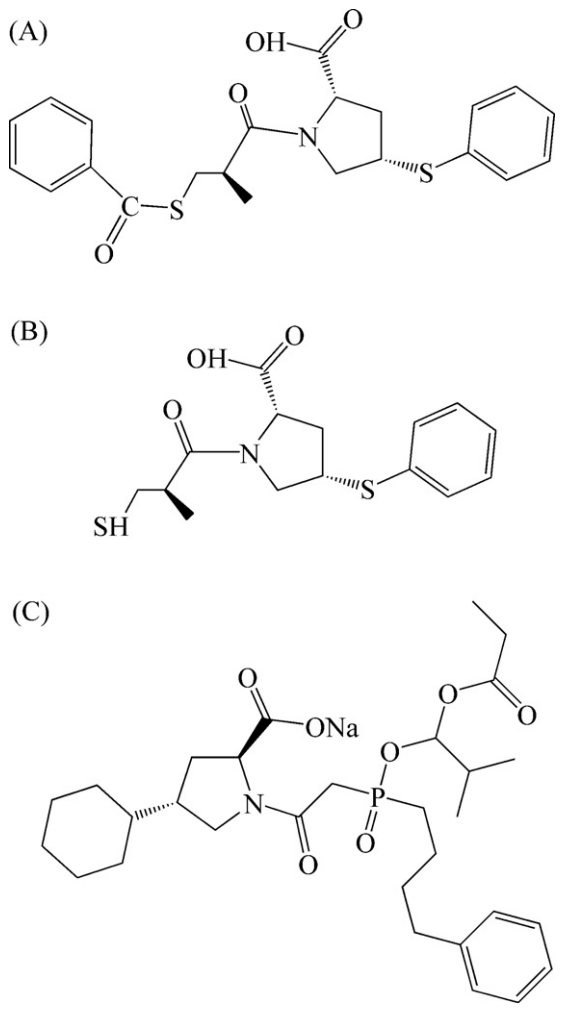

Fig. 1. Chemical structures for zofenopril (A), zofenoprilat (B) and fosinopril sodium (IS) (C).

Biological Products of China (Beijing, PR China). Methanol (HPLC grade) and methyl tert-butyl ether (HPLC grade) were obtained from the TEDIA company, Inc. (Fairfield, OH, USA). Mercaptoethanol was of analytical grade purity and purchased from Shanghai Bioengineer Co. Ltd. (Shanghai, PR China). Formic acid, KH_2PO_4, L-cysteine and EDTA–2Na were of analytical grade purity and purchased from Nanjing Chemical Reagent Co. Ltd. (Nanjing, PR China). Deionized water was purified using PL5242 Purelab Classic UV (PALL Co. Ltd., USA) before use. Blank plasma was supplied by the Nanjing Branch of the Red Cross Society of China.

2.2. Instrumentation

A Thermo-Finnigan TSQ quantum ultra tandem mass spectrometer equipped with an electrospray ionization (ESI) source, a Finnigan surveyor LC pump and an autosampler were used for LC–MS/MS analyses. Data acquisition was performed with Xcalibur 1.4 software (Thermo-Finnigan, San Jose, CA, USA).

2.3. LC–MS/MS conditions

Chromatographic separations were performed using a phenyl–hexyl analytical column (5 μm, 250 mm × 4.6 mm i.d., Phenomenex, Torrance, CA, USA). A mixture of methanol and water with 0.1% formic acid (95:5, v/v) was used as the mobile phase at a flow rate of 1.0 mL/min. The total elution time for one sample was about 6.0 min. A 30 μL sample was injected into the column and 30% of the eluent was split into the inlet of the mass spectrometer using an ESI source. The column temperature was maintained at 35 °C and the autosampler was set at 10 °C. The mass spectrometer was operated in the positive ion detection mode with a spray voltage of 5000 V. The heated capillary temperature was 350 °C. The nitrogen sheath gas and the auxiliary gas were set at 40 and 10 psi, respectively. Quantification was performed using selected reaction monitoring (SRM) with argon at a pressure of 1.0 mTorr for collision-induced dissociations (CIDs) of the following transitions: zofenopril m/z 429.70 → 280.18 with a collision energy of 15 eV, zofenoprilat m/z 325.89 → 177.97 with a collision energy of 22 eV and fosinopril sodium m/z 435.88 → 389.92 with a collision energy of 22 eV; the dwell time was 0.50 s per transition. The positive parent ion mass spectra and product ion mass spectra of zofenopril, zofenoprilat and the internal standard (IS) are shown in Fig. 2.

2.4. Analytical procedure

2.4.1. Preparation of stock solutions, calibration standard and quality control sample solutions

A stock solution of zofenopril calcium in a mixture of methanol and water (9:1, v/v) at a concentration of 263.0 μg/mL (based on zofenopril) was prepared. A stock solution of zofenoprilat in methanol at a concentration of 627.0 μg/mL was also prepared. The IS (fosinopril sodium) that was used for all analyses was prepared as a stock solution (522.8 μg/mL) in methanol and diluted with methanol to a concentration of 41.82 ng/mL.

Subsequently, 1 mL of the zofenopril stock solution and 1 mL of the zofenoprilat stock solution were then accurately transferred into a 10-mL brown volumetric flask and diluted with methanol to 10 mL.

The working solution was further diluted with methanol to obtain zofenopril calibration standard solutions with the concentrations as follows: 10,520, 2630, 657.5, 263.0, 65.75, 26.30, 7.890 and 2.630 ng/mL which also has zofenoprilat with the concentrations as follows: 25,080, 6270, 1567.5, 627.0, 156.75, 62.70, 18.81 and 6.270 ng/mL. Before adjusting the volume of the various solutions, 100 μL of a 0.3 mol/L L-cysteine solutions were added into the volumetric flasks to obtain stable stock solutions. The zofenopril and zofenoprilat stock solutions were found to be stable in a refrigerator (4 °C) for 15 days. All stock solutions were stored at 4 °C and brought to room temperature before use.

Quality control (QC) working solutions of zofenopril and zofenoprilat were prepared following the same procedure as that used for the preparation of zofenopril and zofenoprilat standard solutions in methanol. Specifically, the stock solutions were further diluted to obtain three levels of QC standard working solutions (zofenopril 7.890, 263.0 and 21,040 ng/mL in methanol; zofenoprilat 18.81, 627.0 and 50,160 ng/mL in methanol). The QC samples, which were used in both the pre-study validation and the pharmacokinetic study, were prepared by spiking 20 μL of one of the QC standard working solutions in 0.5 mL of blank human plasma that contained 35 μL of a 0.3 mol/L L-cysteine solution and 15 μL of a 0.1 mol/L EDTA–2Na solution. The concentrations of the zofenopril QC samples were 0.3156 (low), 10.52 (medium) and 841.6 ng/mL (high); those of the zofenoprilat were 0.7524 (low), 25.08 (medium) and 2006 ng/mL (high).

2.4.2. Collection and preparation of the samples

Precautions were taken to avoid sunlight. Blood samples (3 mL each) were collected in tubes, mixed with 100 μL of a 0.3 mol/L L-cysteine solution and 40 μL of a 0.1 mol/L EDTA–2Na solution and dried before use. The samples were then centrifuged for 10 min at 3200 × g (at 4 °C) and 1 mL of separated plasma was transferred to

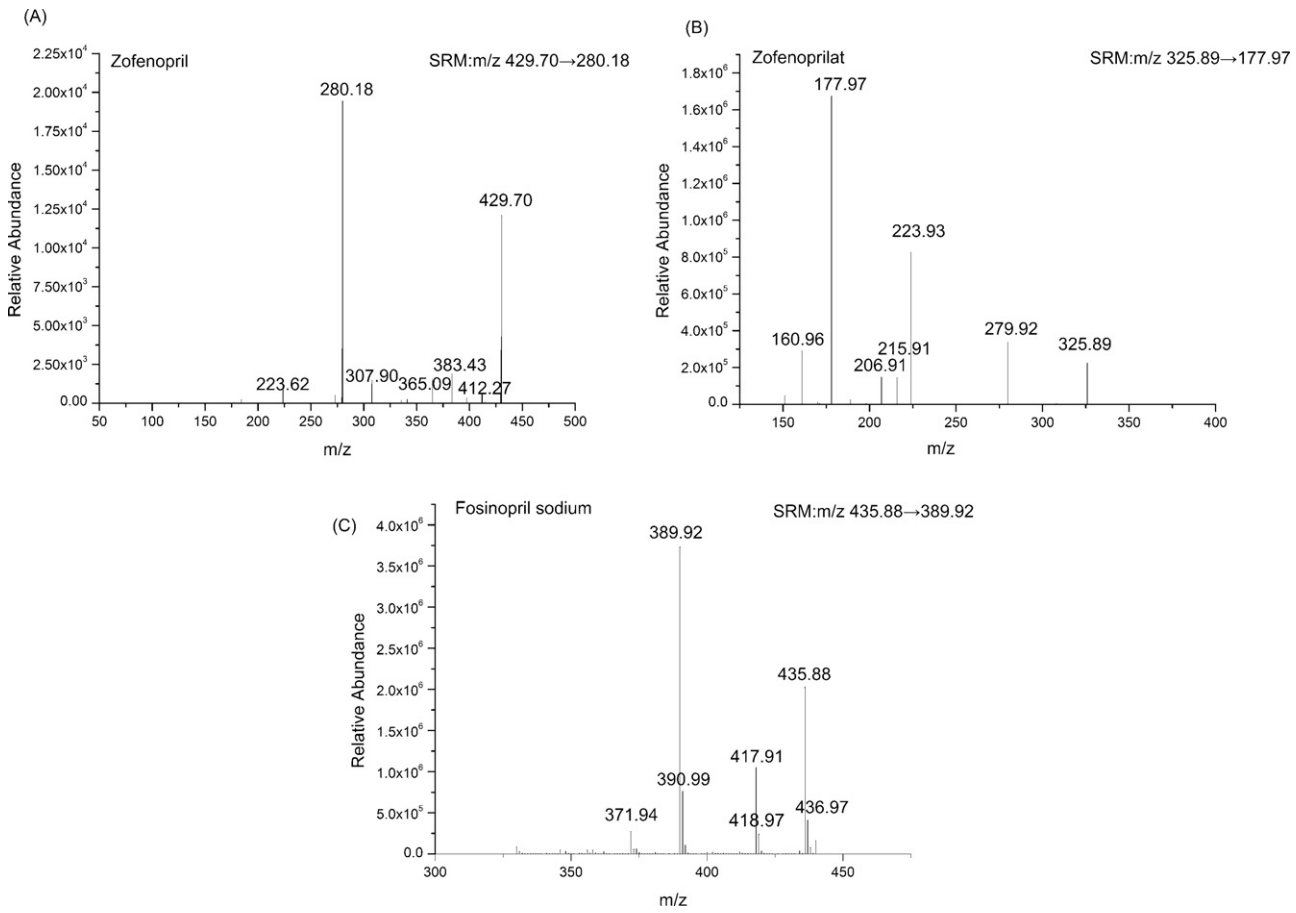

Fig. 2. Parent and product ion scan mass spectra of zofenopril (A), zofenoprilat (B) and IS (C).

Eppendorf tubes. The obtained samples were spiked with 70 μL of a 0.3 mol/L L-cysteine solution and 30 μL of a 0.1 mol/L EDTA–2Na solution, vortexed and stored at −80 °C until the day of analysis.

A plasma sample (0.5 mL) was placed in a 5-mL Eppendorf tube. After addition of 30 μL of a 41.82 ng/mL IS solution and 200 μL of a 0.5 mol/L of KH_2PO_4 solution, the tube was briefly vortexed and 2.5 mL of methyl tert-butyl ether (containing 0.5% mercaptoethanol) was added into the tube. After vortexing for 3 min, the tube was centrifuged at 12,000 rpm for 5 min at room temperature and 1.8 mL of supernatant was transferred into centrifuge tubes and evaporated to dryness under a gentle stream of nitrogen at 37 °C. The residue was dissolved with 150 μL of a mixture of methanol and water (95:5 v/v), transferred into an autosampler vial and 30 μL of the obtained solution was injected into the analytical column.

2.5. Assay validation

2.5.1. Selectivity

The selectivity was investigated by preparing and randomly analyzing six samples of human blank plasma samples at the random. Each blank plasma sample was tested using the above-described extraction procedure and LC–MS/MS chromatographic conditions to ensure no interferences of IS, zofenopril and zofenoprilat from plasma.

2.5.2. Linearity of calibration curves and lower limit of quantitation

Quantitation was achieved from nine point calibration curves covering a range between 0.1052 and 1052 ng/mL for zofenopril and a range between 0.2508 and 2508 ng/mL for zofenoprilat. To evaluate the linearity of the method, calibration curves were prepared and assayed on five different days. The calibration curves were obtained by plotting the peak-area ratios of zofenopril and zofenoprilat to the IS versus the concentrations of zofenopril and zofenoprilat, using a weighted least-squares linear regression (the weighting factor used was $1/C^2$). The LLOQ was established using five samples that were independent of the standards.

2.5.3. Precision and accuracy

The validation samples were prepared and analyzed on three different days (one batch per day) to evaluate the accuracy and the intra-batch and inter-batch precision of the analytical method. The QC samples for the determination of the intra- and inter-batch precision and accuracy were prepared at three different concentrations for both zofenopril and zofenoprilat. The concentrations were 0.3156, 10.52 and 841.6 ng/mL for zofenopril and 0.7524, 25.08 and 2006 ng/mL for zofenoprilat.

2.5.4. Stability

The stability of zofenopril and zofenoprilat in QC samples was investigated after storage for 5 h at room temperature, after three freeze–thaw cycles and in reconstituted samples left for at least 12 h at 10 °C on an autosampler. A long-term stability test was also performed at −80 °C in plasma for 30 days. The samples were analyzed against calibration curves obtained from newly prepared standards, and the results were compared with those obtained for samples immediately processed.

2.5.5. Extraction recovery

The recovery values of zofenopril, zofenoprilat and the IS were calculated by comparing the analytical results of extracted QC samples with those of samples at the same concentrations obtained by spiking extracted blank plasma samples with analytes of the working standard solutions.

2.5.6. Matrix effects

The matrix effects were measured by comparing the peak responses of the analytes resolved in the blank plasma with those of the analytes resolved in the mobile phase containing equivalent amounts of the analytes. The blank plasma samples used in this study were obtained from five different healthy volunteers. An ME value that is not in the range between 85 and 115% indicates an exogenous matrix effect.

3. Results and discussion

3.1. Conditions for ESI–MS/MS

Operation parameters, such as the sheath gas, auxiliary gas, CID and collision energy, were adjusted to achieve the detection sensitivity of zofenopril and zofenoprilat. The optimum MS conditions are listed in Section 2.3. Zofenopril, zofenoprilat and IS were separately scanned under the Q1 MS full scan mode to determine the parent ions and the Q1/Q3 (MS/MS) product ion scan mode to locate the parent/product ion pairs. $[M+H]^+$ was the predominant ion in the Q1 spectra and was used as the parent ion to obtain the product ion spectra. The most sensitive mass transition was from m/z 429.70 to 280.18 for zofenopril, from m/z 325.89 to 177.97 for zofenoprilat and from m/z 435.88 to 389.92 for IS. To achieve a desired sensitive mass transition for the analytes and the IS, the collision energy was set at different levels and the response intensity of zofenopril, zofenoprilat and the IS was maintained constant. Because of the presence of a carboxyl group in the chemical structures of zofenopril and zofenoprilat, the negative ion $[M-H]^-$ was also tested. The response intensity was lower in the negative ion mode than in the positive ion mode.

3.2. Optimization of chromatographic conditions

The retention behaviors of the analytes were evaluated on different types of columns. The retention times of zofenopril and zofenoprilat were extremely different on Lichrospher C_{18}, Lichrospher CN and Lichrospher C_8 columns because of their polarity. Long periods of analysis were necessary. However, the retention times of zofenopril and zofenoprilat on a phenyl–hexyl column (5 μm, 250 mm × 4.6 mm i.d.) with a flow rate of 1 mL/min were reasonable and the peak shapes were symmetric, which could be due to interactions between the benzyl groups of the column and those of zofenopril and zofenoprilat.

Several mobile phases were compared and it was found that a system of water and acetonitrile could slightly inhibit the response of samples in the MS detection. The percentage of methanol was optimized to obtain a retention time of zofenopril as short as 5.2 min. The experimental results showed that acidifying the mobile phase with formic acid could not only improve the retention of zofenopril and zofenoprilat but also increase the MS sensitivity. Concentrations of formic acid of 0.05%, 0.1% and 0.2% in the mobile phase were evaluated. Based on the obtained results, a concentration of 0.1% of formic acid in the mobile phase was used in the experiments.

3.3. IS selection

A proper IS should be structurally and chemically similar to the analytes. It should also have a retention time similar to that of the analytes and be well resolved from the analytes and other peaks. Captopril, enalapril and fosinopril sodium, which are structural analogues of the analytes, were thus evaluated. Fosinopril sodium was found to be the proper IS because it had a structure, retention time and ESI ionization conditions that were similar to those of the analytes. Additionally, captopril and enalapril did not lead to good extraction recoveries.

3.4. Sample preparation

Due to the presence of a free sulfhydryl group in the molecular structure of zofenoprilat, some analytical methods for the immediate determination of zofenopril and zofenoprilat in plasma involved either derivatization or specialized and expensive equipment. Dal Bo et al. [9] protected the free sulfhydryl groups of zofenoprilat by reaction with N-ethylmaleimide (NEM) to obtain the corresponding succinimide derivative. The compound was then extracted from the plasma with toluene. This method was relatively complex and the LLOQs were 1 ng/mL for zofenopril and 2 ng/mL for zofenoprilat.

A solution of L-cysteine was added into the plasma samples of zofenoprilat to prevent its oxidative degradation by protecting the free sulfhydryl groups. Concentrations of L-cysteine of 0.1 mol/L, 0.2 mol/L and 0.3 mol/L were studied, and 0.3 mol/L L-cysteine solution prevented the oxidation of the free sulfhydryl groups. Three different reagents (ethyl acetate, ether, and methyl tert-butyl ether) were tested as extraction solvents. Methyl tert-butyl ether led to the best extraction of zofenopril and zofenoprilat. Additionally, ethyl acetate interacted with the analytes and ether, which led to poor extraction yields. Mercaptoethanol was added into the extraction solvent to protect the free sulfhydryl groups of zofenoprilat from oxidation during the evaporation to dryness under a gentle stream of nitrogen at 37 °C. The sample preparation procedure without any chemical derivatization simplified the sample preparation and obtain stable recoveries of the analytes. LLOQs as low as 0.10 ng/mL and 0.25 ng/mL were achieved for zofenopril and zofenoprilat, respectively.

3.5. Assay validation

3.5.1. Selectivity

The selectivity of the method was investigated by preparing and analyzing six individual human blank plasma and plasma samples (zofenopril: 10.52 ng/mL and zofenoprilat: 25.08 ng/mL). Representative chromatograms of blank human plasma samples, samples including the IS and samples of a volunteer's plasma are shown in Fig. 3. Good selectivities for the analytes were obtained for the analytes as evidenced by the symmetrical resolution of the peaks. There was no significant chromatographic interference close to the retention times of the analytes and IS in the blank human plasma samples. The typical retention times for zofenopril, zofenoprilat and IS were 4.7 ± 0.05 min, 4.0 ±0.05 min and 5.2 ± 0.05 min, respectively. The total run time was about 6.0 min. The blank human plasma samples that were collected from 6 subjects were run for up to 18 min. Because of the high selectivity of the SRM mode, no late-eluting interfering peaks were observed for up to 18 min. In addition, for all plasma samples analyzed, there was no peak with an intensity higher than 20% of the analyte LLOQs. There was also no signal close to the retention time of the IS.

3.5.2. Calibration curves and LLOQ

Table 1 shows the back-calculated concentrations of zofenopril (range: 0.1052–1052 ng/mL) and zofenoprilat (range:

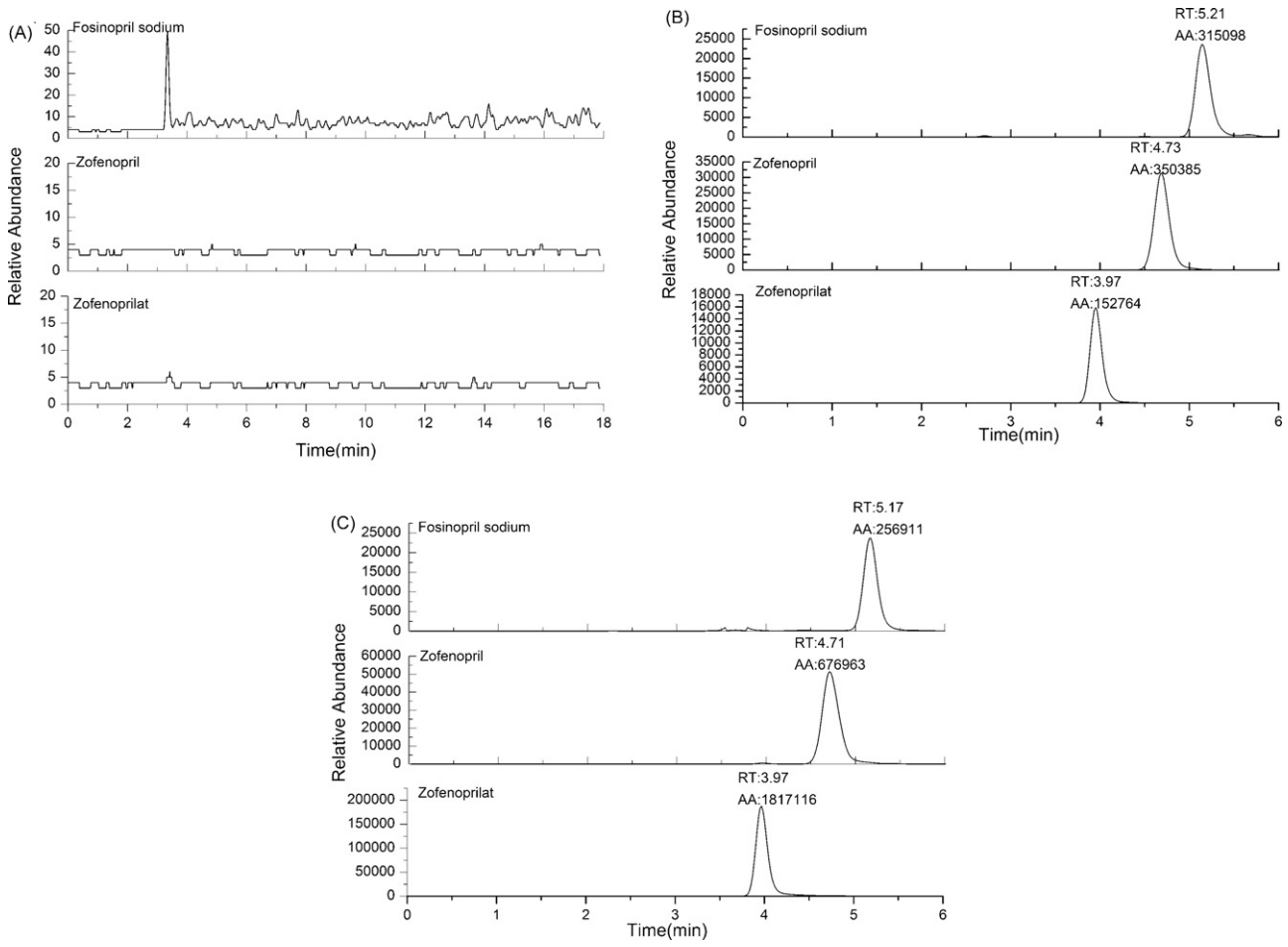

Fig. 3. Chromatograms by selected reaction monitoring (SRM) scan mode: (A) blank plasma (drugs and IS free); (B) blank plasma spiked with 10.52 ng/mL zofenopril, 25.08 ng/mL zofenoprilat and IS; (C) plasma sample of a subject 2.5 h post-oral administration of 30 mg zofenopril calcium tablet.

Table 1
Standard curve statistics of zofenopril and zofenoprilat mean values of five findings.

Zofenopril	Nominal concentrations (ng/mL)									Calibration curve parameters		
	1052	420.8	105.2	26.30	10.52	2.630	1.052	0.3156	0.1052			
	Back-calculated concentrations (ng/mL)									Slope	Intercept	*r*
Mean	954.6	434.1	108.9	25.02	11.41	2.529	1.051	0.3012	0.1069	8.49	−0.0314	0.9958
SD	22.7	16.4	7.8	2.0	0.4	0.2	0.2	0.04	0.003	0.5	–	–
Precision (% CV)	2.4	3.8	7.1	8.0	2.9	6.5	11.5	11.2	2.6	5.8	–	–
RE (%)	−9.3	+3.2	+3.5	−4.9	+8.5	−3.9	−0.1	−4.6	+1.6	–	–	–
Zofenoprilat	Nominal concentrations (ng/mL)									Calibration curve parameters		
	2508	1003	250.8	62.70	25.08	6.270	2.508	0.7524	0.2508			
	Back-calculated concentrations (ng/mL)									Slope	Intercept	*r*
Mean	2430	1023	271.7	60.04	25.08	5.599	2.565	0.7368	0.2531	43.3	0.0002	0.9952
SD	132.8	75.5	19.5	6.4	2.5	0.3	0.06	0.1	0.02	3.5	–	–
Precision (% CV)	5.5	7.4	7.2	10.6	9.7	5.3	2.1	13.4	4.5	8.1	–	–
RE (%)	−3.1	+1.9	+8.3	−4.3	−0.06	−10.7	+2.3	−2.1	+0.9	–	–	–

0.2508–2508 ng/mL) and the parameters obtained from the corresponding calibration curves. For zofenopril, the mean accuracy (%) of back-calculated concentrations ranged from −9.3% to +8.5% and the precision was below 11.5%. For zofenoprilat, the mean accuracy ranged from −10.7% to +8.3% and the precision was below 13.4%. The mean coefficient of correlation (*r*) was 0.9958 for zofenopril and 0.9952 for zofenoprilat.

The LLOQ was as low as 0.1052 ng/mL for zofenopril and 0.2508 ng/mL for zofenoprilat. At these concentrations, the precision and mean accuracy values were 15.1% and +1.7% for zofenopril and 14.0% and −11.4% for zofenoprilat, respectively.

3.5.3. Intra- and inter-batch precision and accuracy

The intra-batch precision was below 8.2% for zofenopril and below 14.0% for zofenoprilat. The value for the method accuracy ranged from +5.0% to +8.0% for zofenopril and from −10.8% to +6.3% for zofenoprilat.

Table 2
Intra- and inter-batch precision and accuracy of zofenopril and zofenoprilat in human plasma.

Quality control	Nominal concentrations	Intra-batch assay ($n = 5$)			Inter-batch assay (batch = 3, $n = 5$)		
	(ng/mL)	Mean (ng/mL)	Precision (% C.V.)	Accuracy (%RE)	Mean (ng/mL)	Precision (% C.V.)	Accuracy (%RE)
Zofenopril							
QC-low	0.3156	0.3408	4.7	+8.0	0.3041	11.0	−3.7
QC-medium	10.52	11.12	8.2	+5.7	11.15	5.9	+6.0
QC-high	841.6	883.7	6.9	+5.0	846.6	6.3	+0.6
Zofenoprilat							
QC-low	0.7524	0.7480	14.0	−0.6	0.7271	13.1	−3.4
QC-medium	25.08	26.66	8.4	+6.3	25.09	10.0	0.0
QC-high	2006	1791	1.9	−10.8	1891	8.0	−5.8

Table 3
Stability datas of freeze–thaw, short-term and long-term stability.

	Zofenopril	Zofenoprilat	Zofenopril	Zofenoprilat	Zofenopril	Zofenoprilat
Nominal concentrations (ng/mL)	0.3156	0.7524	10.52	25.08	841.6	2006
Processed immediately (0 h) ($n = 5$)	0.2975	0.7405	11.07	27.78	788.3	1939
Short-term for 5 h ($n = 5$)	0.2931	0.6264	10.37	27.33	798.1	1965
Freeze–thaw 3 cycles ($n = 5$)	0.3566	0.6752	11.60	27.24	936.4	1735
Long-term at −80 °C for 30 days ($n = 5$)	0.3180	0.6975	10.21	24.27	894.7	1934

For zofenopril, the inter-batch precision was below 11.0% and the accuracy value ranged from −3.7% to +6.0%. For zofenoprilat, the inter-batch precision was below 13.1% and the accuracy value ranged from −5.8% to +0.0%. Table 2 summarizes the intra- and inter-batch precision and accuracy for zofenopril and zofenoprilat evaluated by assaying the QC samples.

3.5.4. Stability

No significant change in the concentration of zofenopril and zofenoprilat was detected for the plasma samples that were maintained in solution at 10 °C for at least 12 h in autosampler vials prior to injection. The concentrations in zofenopril and zofenoprilat also remained unchanged in the plasma samples that were either stored at room temperature for 5 h, subjected to three freeze–thaw cycles or stored at −80 °C for 30 days.

The freeze–thaw, short-term and long-term storage stabilities are shown in Table 3.

3.5.5. Recovery

The recovery values of zofenopril from human plasma samples after extraction at concentrations of 0.3156, 10.52 and 841.6 ng/mL were 92.58%, 102.1% and 85.79%, respectively (with respective % CV values of 12.0, 5.2 and 8.6). For zofenoprilat concentrations of 0.7524, 25.08 and 2006 ng/mL, the recovery values were 100.5%, 86.20% and 90.69%, respectively (with respective % CV values of 18.9, 6.1 and 2.0). The extraction recovery value for the IS was 95.47% (with a % CV value of 11.8). The absolute recovery values were consistent, precise and reproducible.

3.5.6. Matrix effects

Matrix effects are generally problematic during LC–MS/MS analyses of biological samples. In our experiments, the ratio values of peak responses for zofenopril concentrations of 0.3156, 10.52 and 841.6 ng/mL were 94.08%, 99.34% and 93.07%, respectively (with respective % CV values of 6.7, 6.8 and 3.5). For zofenoprilat concentrations of 0.7524, 25.08 and 2006 ng/mL, the ratio values were 99.63%, 102.0% and 98.35%, respectively (with respective % CV values of 3.0, 3.2 and 1.1). These results suggest that no co-eluting endogenous substances interfered with the ionization of zofenopril and zofenoprilat under the experimental conditions used. The peak area value for the IS was 93.7% of that obtained for the reference solution (with a % CV value of 5.4).

4. Conclusions

A rapid and reproducible LC–MS/MS method with high selectivity was developed and validated for the simultaneous determination of zofenopril and its active metabolite zofenoprilat in human plasma. Compared with previous methods, the present method, which does not require any chemical derivatization, has a lower limit of detection, higher sensitivity, more satisfactory selectivity, and a shorter run time of 6.0 min. The method is therefore particularly suitable for routine assays. The analyses of validation parameter tests indicated that our method could be used to study the pharmacokinetics of zofenopril and other related substances. The obtained results also suggested that our method could be used for clinical drug monitoring.

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