Journal of Chromatography B, 878 (2010) 2415–2420

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Enantioselective determination of doxazosin in human plasma by liquid chromatography–tandem mass spectrometry using ovomucoid chiral stationary phase

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ARTICLE INFO

Article history:
Received 2 June 2010
Accepted 30 July 2010
Available online 7 August 2010

Keywords:
Doxazosin
Enantiomers
Enantioselective LC–MS/MS
Stereoselective pharmacokinetics

ABSTRACT

An enantioselective and sensitive method was developed and validated for determination of doxazosin enantiomers in human plasma by liquid chromatography–tandem mass spectrometry. The enantiomers of doxazosin were extracted from plasma using ethyl ether/dichloromethane (3/2, v/v) under alkaline conditions. Baseline chiral separation was obtained within 9 min on an ovomucoid column using an isocratic mobile phase of methanol/5 mM ammonium acetate/formic acid (20/80/0.016, v/v/v) at a flow rate of 0.60 mL/min. Acquisition of mass spectrometric data was performed in multiple reaction monitoring mode, using the transitions of m/z 452 → 344 for doxazosin enantiomers, and m/z 384 → 247 for prazosin (internal standard). The method was linear in the concentration range of 0.100–50.0 ng/mL for each enantiomer using 200 μL of plasma. The lower limit of quantification (LLOQ) for each enantiomer was 0.100 ng/mL. The intra- and inter-assay precision was 5.0–11.1% and 5.7–7.6% for *R*-(−)-doxazosin and *S*-(+)-doxazosin, respectively. The accuracy was 97.4–99.5% for *R*-(−)-doxazosin and 96.8–102.8% for *S*-(+)-doxazosin. No chiral inversion was observed during the plasma storage, preparation and analysis. The method proved adequate for enantioselective pharmacokinetic studies of doxazosin after oral administration of therapeutic doses of racemic doxazosin.

1. Introduction

Doxazosin (Fig. 1A) is a long-acting selective α1-adrenergic blocker that is effective in the treatment of benign prostatic hyperplasia (BPH) and hypertension [1–3]. According to IOWA PDL Marketshare data, doxazosin accounted for 36% of the BPH market in the first half of 2010 in USA [4]. Doxazosin is marketed as a racemic mixture of *R*-(−) and *S*-(+) enantiomers. *In vitro* study showed that both enantiomers were approximately equipotent on human prostate α1-adrenoceptors [5]. However, compared with *R*-(−)-doxazosin, *S*-(+)-doxazosin could offer reduced adverse effect including orthostatic hypotension, nausea, lethargy, fatigue and dizziness [6–8]. And the synthesis or biosynthesis of *S*-(+)-doxazosin and its intermediate have also been reported [9,10]. Stereoselective pharmacokinetics of the two enantiomers are still unclear.

A number of achiral methods for determination of doxazosin in human plasma have been reported, including HPLC utilizing UV [11] or fluorescence detection [12,13], LC–MS [14], LC–MS/MS [15] and UPLC–MS/MS [16]. To answer whether the enantiomers of doxazosin have different pharmacokinetic profiles, it is necessary to develop and validate a highly sensitive and enantioselective method for determination of the enantiomers in human plasma. Alebic-Kolbah and Zavitsanos [17] developed a chiral LC–MS/MS technique using a Chiralpak AD column for the bioanalysis of four chiral drugs, including doxazosin. However, the method has not been recreated on a modern mass spectrometer and has not been validated.

Although chiral LC–MS/MS has been widely used for determination of enantiomers of drugs and/or their metabolites in biological matrices [18–20], there are still compatibility issues when coupling chiral chromatography with MS/MS detection. Firstly, non-volatile salts (e.g. perchlorate, phosphate) which are widely used in chiral separation to offer better resolution of enantiomers, could not be injected into mass spectrometer. Secondly, many developed HPLC methods for separation of enantiomers of drugs used hexane-based normal phase systems, which can cause a possible explosion hazard in ion source. One approach to avoid this problem is post-column addition of aqueous make-up solvent prior to the ion source. A principle drawback of this approach is that the dilution of analyte will decrease the method sensitivity. In addition, the enantiomeric resolution might be sacrificed due to the extra column band broadening effect [20]. The development of chiral LC–MS/MS methods for bioanalysis of drug enantiomers is still a challenge. The present

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doi:10.1016/j.jchromb.2010.07.027

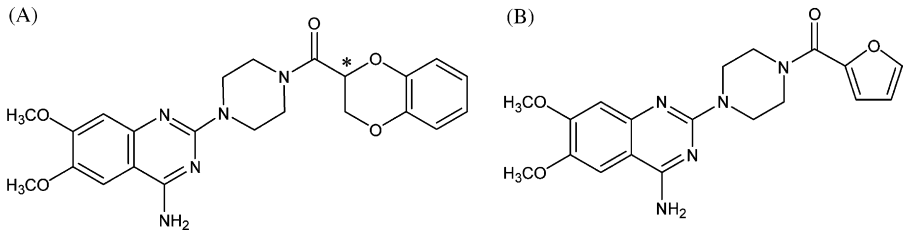

Fig. 1. Structures of doxazosin (A) and internal standard prazosin (B). The asymmetric carbon is marked with asterisk.

study is to develop and validate a sensitive chiral LC–MS/MS assay for determination of individual doxazosin enantiomers in human plasma after oral administration of therapeutic doses of racemic doxazosin.

2. Experimental

2.1. Chemicals and reagents

R-(−)-doxazsoin mesylate (chemical purity 99.7%) and *S*-(+)-doxazosin mesylate (chemical purity 99.7%) were kindly supplied from North China Pharmaceutical Group Co. Ltd. (Hebei, China). Prazosin (purity 98.0%, internal standard, IS), methanol and acetonitrile of HPLC grade were purchased from Sigma (St. Louis, MO, USA). Ammonium acetate, acetic acid and formic acid of HPLC grade were purchased from Tedia (Fairfeild, OH, USA). Deionized water was obtained from a Millipore Milli-Q Gradient Water Purification System (Molsheim, France). Other chemical reagents (analytical grade) were purchased from Sinopharm Group Chemical Reagent Co. Ltd. (Shanghai, China).

2.2. Instrumentation and analytical conditions

The HPLC system consisted of a LC-20AD pump and a SIL-HT_A autosampler (Shimadzu, Kyoto, Japan). The chromatographic separation of enantiomers was performed on an Ultron ES-OVM column (150 mm × 4.6 mm, 5 μm) with an Ultron ES-OVM cartridge (10 mm × 4.0 mm, 5 μm) (Agilent, Wilmington, DE, USA). A mixture of methanol/5 mM ammonium acetate/formic acid (20/80/0.016, v/v/v) was used as mobile phase at a flow rate of 0.60 mL/min for isocratic elution. The column temperature was maintained at 20 °C.

An API 4000 triple quadrupole mass spectrometer equipped with a TurboIonSpray (ESI) source was used for LC–MS/MS analysis (Applied Biosystems, Concord, Ontario, Canada). Data processing was performed on Analyst 1.4.1 software. The major working parameters are summarized in Table 1. The corresponding product ion scan spectra of doxazosin and prazosin (IS) are shown in Fig. 2.

Table 1
Major working parameters of API 4000 tandem mass spectrometer.

Parameters	Values
Ionization mode	Positive
Spray voltage (V)	4200
Source temperature (°C)	400
Ion source gas 1 (psi)	50
Ion source gas 2 (psi)	50
Curtain gas (psi)	10
CAD gas (psi)	4
Declustering potential (V)	100
Entrance potential (V)	10
Collision cell exit potential (V)	23
Collision energy (eV)	41 (enantiomers) and 38 (IS)
Transition for doxazosin enantiomers	*m/z* 452 → *m/z* 344
Transition for prazosin (IS)	*m/z* 384 → *m/z* 247
Dwell time per transition (ms)	200

2.3. Preparation of calibration standards and quality control (QC) samples

Stock solutions of *R*-(−)-doxazosin or *S*-(+)-doxazosin at a concentration of 400 μg/mL (calculated as free base) were prepared by dissolving the accurately weighed reference substance of each enantiomer in methanol/water (50/50, v/v). A standard working solution containing 4.00 μg/mL of each enantiomer was prepared by appropriate mixing of the two 400 μg/mL stock solutions and then dilution with methanol/water (50/50, v/v). The obtained solution was then serially diluted with blank plasma to give calibration standard samples at the concentrations of 0.100, 0.300, 1.00, 3.00, 10.0, 25.0 and 50.0 ng/mL for each enantiomer. The quality control (QC) samples were similarly prepared at concentrations of 0.250, 3.00 and 45.0 ng/mL for each enantiomer, by separate weighing of the reference substances. A 100 ng/mL of IS working solution was prepared by diluting the 400 μg/mL stock solution of prazosin with methanol/water (50/50, v/v). All the solutions were kept

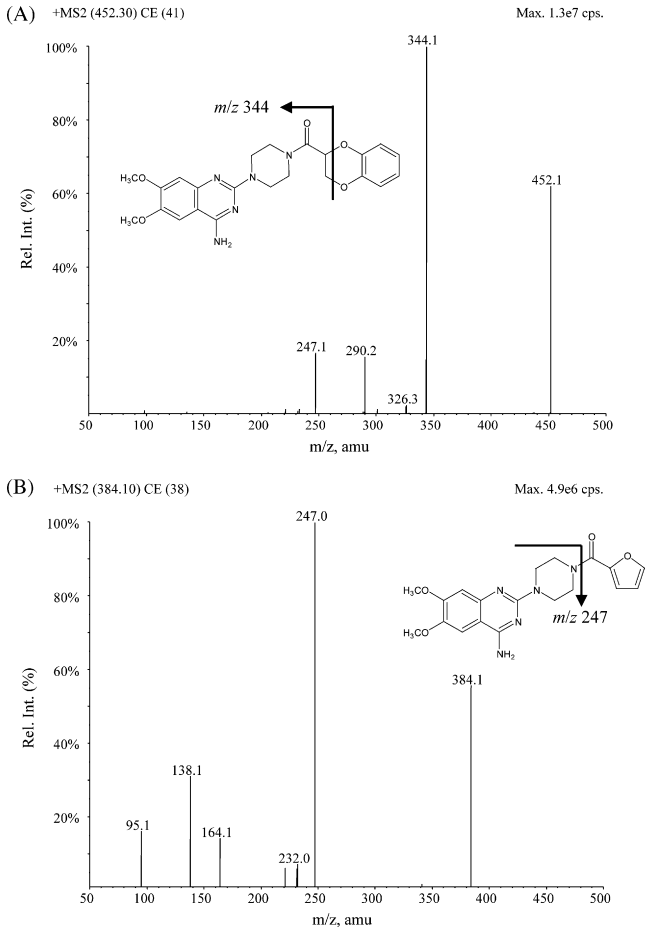

Fig. 2. Product ion spectra of $[M+H]^+$ of doxazosin (A) and prazosin (B).

refrigerated (2–8 °C) and were brought to room temperature before use. Calibration standards and QC samples were prepared and dispatched in 500 μL aliquots and stored in plastic tubes at −20 °C until analysis.

2.4. Sample preparation

A 50 μL aliquot of the IS solution (prazosin, 100 ng/mL) and 200 μL of water were added to 200 μL of plasma samples. The sample was vortex-mixed and extracted with 3 mL of ethyl ether/dichloromethane (3/2, v/v) by shaking for 10 min. The organic and aqueous phases were separated by centrifugation at 2000 × *g* for 5 min. The upper organic phase was transferred to another tube and evaporated to dryness at 40 °C under a stream of nitrogen in the TurboVap evaporator (Zymark, Hopkinton, MA, USA). The residue was reconstituted in 150 μL of the mobile phase. A 20 μL aliquot of the reconstituted extract was injected for the enantioselective LC–MS/MS analysis.

2.5. Method validation

The developed method was validated for selectivity, linearity, precision and accuracy, recovery, matrix effect and stability [21,22]. Selectivity was performed using six different sources of blank plasma. They were extracted and analyzed, and the responses at the retention times of the enantiomers and IS were assessed. Calibration standards were prepared and analyzed in duplicate in three independent runs. Calibration curves (peak area ratio of each enantiomer to IS versus nominal enantiomer concentration) were fitted by least square linear regression using $1/x^2$ as weighting factors. To assess linearity, the coefficient of correlation (r^2) should be more than 0.99 and deviations of the calculated concentrations should be within ±15% from nominal concentrations except for the LLOQ level, at which a deviation of ±20% is permitted. Accuracy and precision were determined by assaying six replicates of QC samples at the low, middle and high levels on 3 separate days. The accuracy and precision were calculated using one-way ANOVA. Accuracy and precision were calculated in terms of relative error (%RE) and relative standard deviation (%RSD), respectively. The LLOQ, defined as the lowest concentration at which both precision and accuracy were less than or equal to 20%, was evaluated by analyzing samples prepared in six replicates. The recovery of each enantiomer was determined by comparing the mean peak areas of the regularly pretreated QC samples at three concentration levels (six samples each) to those of spike-after-extraction samples. To evaluate the matrix effect (ME) of each enantiomer and IS, two sets of samples at LQC and HQC levels were prepared: *A*—plasma extracts from six different lots spiked with standards after extraction; *B*—standards in mobile phase at same concentration levels. The ratio ($A/B \times 100$) is defined as the matrix factor (MF). The variability in matrix factors should be less than 15% [22]. The stabilities of each enantiomer in human plasma were evaluated by analyzing replicates ($n = 3$) of plasma samples at LQC and HQC level, which were exposed to different conditions (time and temperature). Stability of extracts in the autosampler at 25 °C was assessed for at 24 h.

To assess the accuracy and precision of the method to determine doxazosin enantiomeric ratios, doxazosin enantiomers were combined to obtain three mixtures of known *S*-(+)/*R*-(−) ratios at 2/1, 3/1 and 4/1, containing *R*-(−)-doxazosin and *S*-(+)-doxazosin at total concentration of 24.0 ng/mL. The mixed standard solutions were spiked to the blank plasma (total doxazosin plasma concentration of 6.00 ng/mL) and were analyzed with the enantioselective LC–MS/MS system in six replicates on 3 consecutive days.

To evaluate the enantiomeric stability of *R*-(−)-doxazosin or *S*-(+)-doxazosin in human plasma, plasma samples at concentration of 40.0 ng/mL for each enantiomer were incubated in triplicate for 12 h at 37 °C, then extracted and analyzed as described.

2.6. Stereoselective pharmacokinetic studies

Two healthy male Chinese volunteers who provided written informed consent took part in the study. The study was approved by the Ethics Committee. Venous blood samples of about 5 mL were collected in heparin-containing tubes pre-dose (0 h) and 1, 3, 5, 8, 10, 12, 14, 24, 36, 48, and 72 h after an oral administration of 4 mg racemic doxazosin (Cardura XL®, Pfizer, Brooklyn, New York, USA). Plasma samples were obtained by centrifugation at 2000 × *g* for 10 min and were frozen at −20 °C until analysis.

3. Results and discussion

3.1. Enantioselective chromatographic conditions

When chiral columns are coupled to MS/MS detection for quantification of enantiomers in biological samples, mobile-phase composition is a decision factor and key to success or failure of a chiral resolution and detection. In our prestudy, several chiral stationary phases compatible with MS detection including macrocyclic glycopeptide-based phases (Chirobiotic V2 and T), protein-based phases (Chiral AGP and Ultron ES-OVM) were attempted. To Chirobiotic V2 and T columns, polar ionic and reversed-phase modes were tested. Partial resolution of doxazosin enantiomers ($R_s = 1.25$) was observed on Chirobiotic V2 column using methanol/acetic acid/ammonia water (100/0.1/0.1, v/v/v) as mobile phase in a 29 min run time, while no separation was obtained on Chirobiotic T column. For Chiral AGP column, although a various percentage of acetonitrile, methanol and acid/base modifier were tested, no resolution was observed. Baseline separation ($R_s = 1.9$) was observed on an ES-OVM column using a mobile phase of acetonitrile/10 mM ammonium acetate/acetic acid (10/90/0.027, v/v/v). However, the MS response of doxazosin enantiomers was poor under this chromatographic system. It has been reported that the plasma concentrations of racemic doxazosin were relatively low (less than 50 ng/mL) after oral administration of therapeutic doses. As a result, the study of stereoselective pharmacokinetics requires a highly sensitive analytical method. To improve the sensitivity, further optimization of the mobile phase system was performed using methanol instead of acetonitrile to increase the proportion of organic modifier for better spray in ion source, and using formic acid instead of acetic acid for better MS response. Finally, the mobile phase of methanol/5 mM ammonium acetate/formic acid (20/80/0.016, v/v/v) at a flow rate of 0.6 mL/min was used, and the MS signal intensity of doxazosin enantiomers was increased about 100-fold. The R_s value of doxazosin enantiomers under this mobile phase system was 2.1.

Prazosin, an achiral analog of doxazosin, was chosen as the internal standard. It produced good peak shape and suitable retention time (3.2 min) on the Ultron ES-OVM column.

3.2. Method validation

3.2.1. Assay selectivity

Selectivity was assessed by comparing the chromatograms of six different lots of blank human plasma with the corresponding spiked plasma. Fig. 3 shows the typical chromatograms of a blank plasma sample, blank plasma sample spiked with *R*-(−)-doxazosin and *S*-(+)-doxazosin at the LLOQ and IS, and a plasma sample obtained 8 h after oral administration of 4 mg racemic doxazosin to a healthy volunteer. No significant interference from endogenous substances was observed at the retention times of the enantiomers and IS.

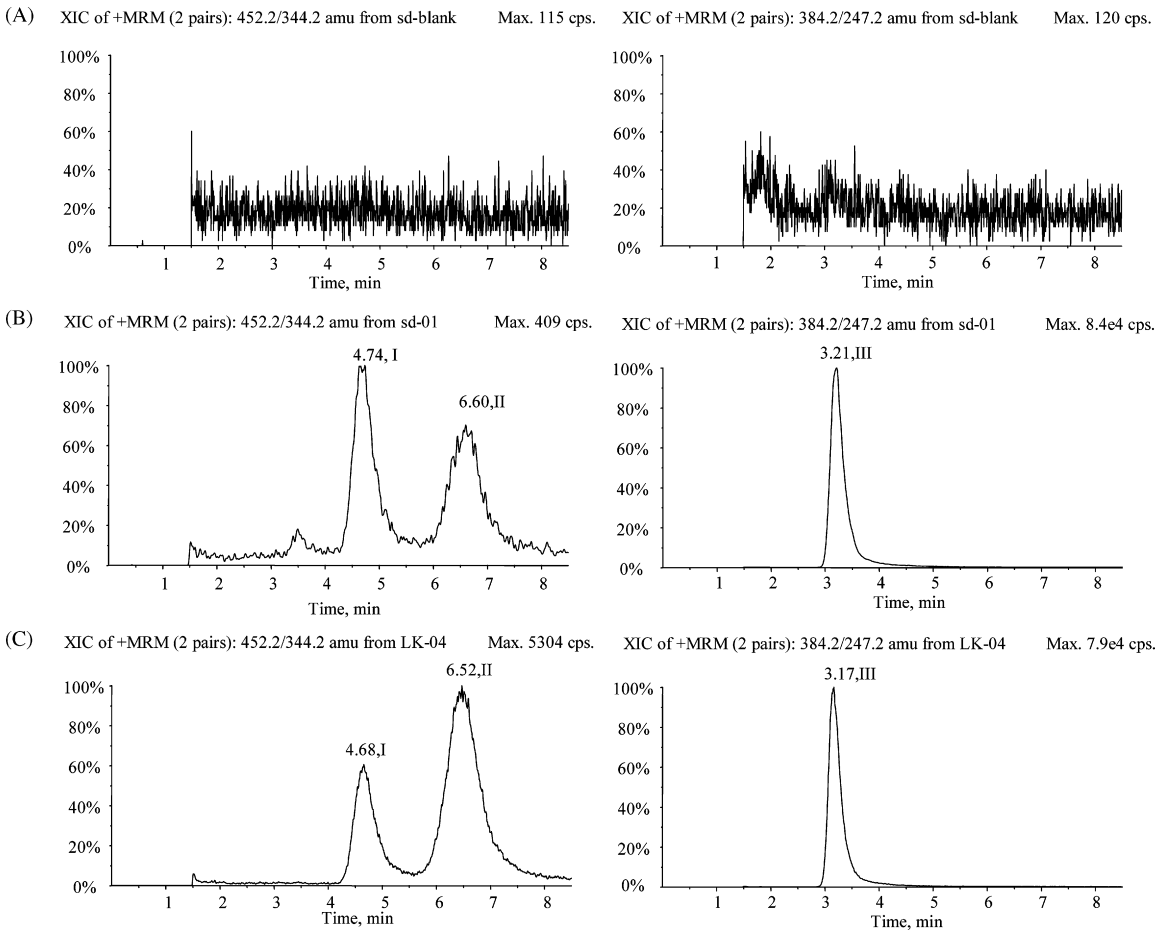

Fig. 3. Typical enentioseletive MRM chromatograms of *R*-(−)-doxazosin, *S*-(+)-doxazosin and prazosin(IS) in human plasma. (A) Blank plasma sample; (B) plasma spiked with 0.10 ng/mL *R*-(−)-doxazosin, 0.10 ng/mL *S*-(+)-doxazosin and 25 ng/mL IS; (C) plasma sample 8 h after oral administratoin of 4 mg racemic doxazosin mesylate controlled release tablet to a healthy volunteer. Peaks I, II, III refer to *R*-(−)-doxazosin, *S*-(+)-doxazosin and IS, respectively.

3.2.2. Linearity and LLOQ

The calculated peak-area ratios of doxazosin enantiomers to the IS versus the concentrations displayed a good linear relationship over the concentration range from 0.100 to 50.0 ng/mL (each enantiomer) in human plasma. The mean (±SD) regression equation from replicate calibration curves on different days was:

$$R-(-)-\text{doxazosin}: y = (0.0431 \pm 0.0025)x + (0.0023 \pm 0.0010),\ r = 0.9961$$

$$S-(+)-\text{doxazosin}: y = (0.0470 \pm 0.0011)x + (0.0012 \pm 0.0011),\ r = 0.9983$$

where y represents the peak-area ratio of each analyte to IS and x represents the plasma concentration of each enantiomer.

The lower limit of quantification was 0.100 ng/mL for determination of doxazosin enantiomers in plasma. The intra- and inter-day precision and accuracy at LLOQ are shown in Table 2. The precision and accuracy at this concentration level were acceptable, with RSD values below 14.7% and RE values within ±2.1%.

3.2.3. Precision and accuracy

Table 2 also summarizes the intra- and inter-day precision and accuracy for doxazosin enantiomers from QC samples. The intra-day and inter-day precisions were <9.9% and <11.1%, for *R*-(−)-doxazosin, respectively; <7.6% and <6.7% for *S*-(+)-doxazosin, respectively. The accuracy ranged from −2.6% to −0.5% (RE) for *R*-(−)-doxazosin and from −3.2% to 2.8% for *S*-(+)-doxazosin.

Table 2
Precision and accuracy of the chiral LC-MS/MS method to determine doxazosin enantiomers in human plasma (in pre-study validation, $n = 3$ days, six replicates per day).

Analyte	Concentration (ng/mL)		RSD (%)		Relative error (%)
	Added	Found	Intra-day	Inter-day	
R-(−)-doxazosin	0.100 (LLOQ)	0.102	8.0	5.0	2.1
	0.250 (L)	0.249	8.5	10.2	−0.5
	3.00 (M)	2.93	7.6	11.1	−2.5
	45.0 (H)	43.8	9.9	5.0	−2.6
S-(+)-doxazosin	0.100 (LLOQ)	0.101	7.3	14.7	1.4
	0.250 (L)	0.257	7.0	6.7	2.8
	3.00 (M)	2.90	7.6	6.0	−3.2
	45.0 (H)	44.8	5.7	6.7	−0.5

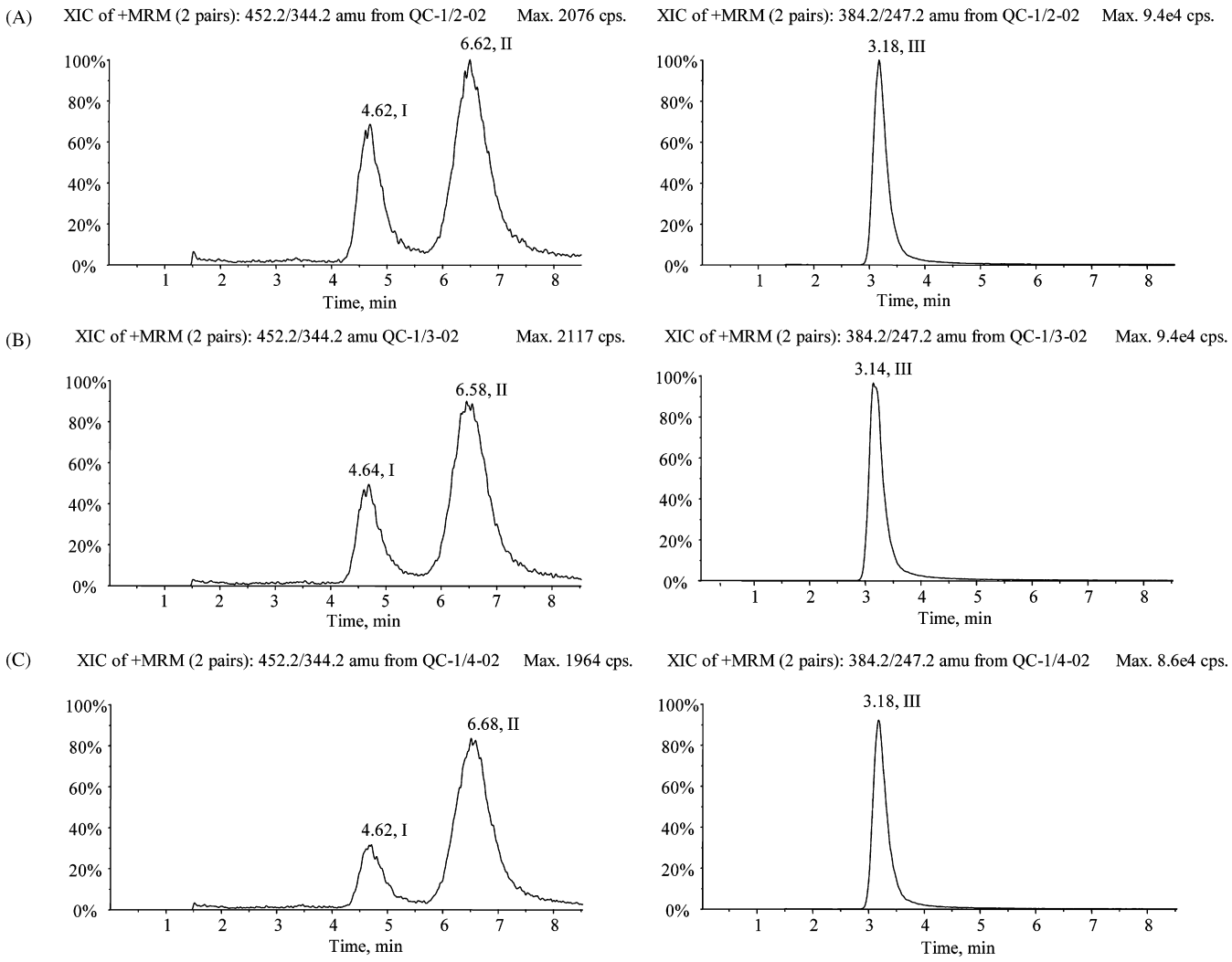

Fig. 4. Typical chromatograms of *R*-(−)-doxazosin and *S*-(+)-doxazosin from different enantiomeric ratios. (A) *S*-(+)/*R*-(−) = 2/1 (B) *S*-(+)/*R*-(−) = 3/1 (C) *S*-(+)/*R*-(−) = 4/1. Peaks I, II, III refer to *R*-(−)-doxazosin, *S*-(+)-doxazosin and IS, respectively.

The precision and accuracy for doxazosin enantiomeric ratios are presented in Table 3. The intra-day and inter-day precisions were <5.9% and 8.0%. The accuracy ranged from 3.1% to 5.2% (RE). Fig. 4 shows the typical chromatograms of *R*-(−)-doxazosin and S-(+)-doxazosin from different enantiomeric ratios QC samples at total doxazosin plasma concentration of 6.0 ng/mL.

3.2.4. Extraction recovery and matrix effect

Mean extraction recoveries of *R*-(−)-doxazosin were 87.0 ± 3.6%, 89.7 ± 3.5% and 84.0% ± 2.1% at the concentrations of 0.25, 3.00 and 45.0 ng/mL, respectively. The recoveries of *S*-(+)-doxazosin were 89.8 ± 5.3%, 88.4% ± 10.2% and 85.2 ± 3.0% at the concentrations of 0.25, 3.00 and 45.0 ng/mL, respectively. The recovery of IS (25.0 ng/mL) was 84.2% ± 1.7%. The peak area ratios (analyte/IS) in extracted blank matrix spiked with solutions of 0.25 and 45.0 ng/mL were in the range of 94.8–97.6% compared to those in the same nominal solutions prepared with the mobile phase, and the CV values from six lots of plasma were less than 4.6%. Obviously, ion suppression or enhancement from plasma matrix was negligible in present conditions.

Table 3
Precision and accuracy of the chiral LC–MS/MS method to determine doxazosin enantiomeric ratios in human plasma (total plasma concentration at 6.00 ng/mL, *n* = 3 days, six replicates per day).

S-(+)/*R*-(−) ratios		RSD (%)		Relative error (%)
Added	Found	Intra-day	Inter-day	
2.00	2.10	5.9	7.6	5.2
3.00	3.09	5.5	5.8	3.1
4.00	4.2	1.9	8.0	4.6

3.2.5. Stability

The enantiomers were found to be stable in following conditions: in plasma at 25 °C for 2 h, in the mobile phase at 25 °C for 24 h, three freeze/thaw cycles, or in plasma at −20 °C for 45 days. All RE values between post-storage and initial QC samples were within ±15%. The peak of another enantiomer was not observed after incubation of plasma samples containing one enantiomer at 37 °C for 12 h. As a result, no chiral inversion was observed between *R*-(−)- and *S*-(+)-doxazosin during the storage, processing and analysis.

3.3. Method application

The present enantioselective LC–MS/MS method provided the LLOQ down to 0.100 ng/mL for each enantiomer, which satisfied the demand of evaluating stereoselective pharmacokinetics of doxazosin. Under the present LLOQ, the doxazosin enantiomers concentration could be determined in plasma samples up to 72 h after an oral administration of 4 mg racemic doxazosin. After an

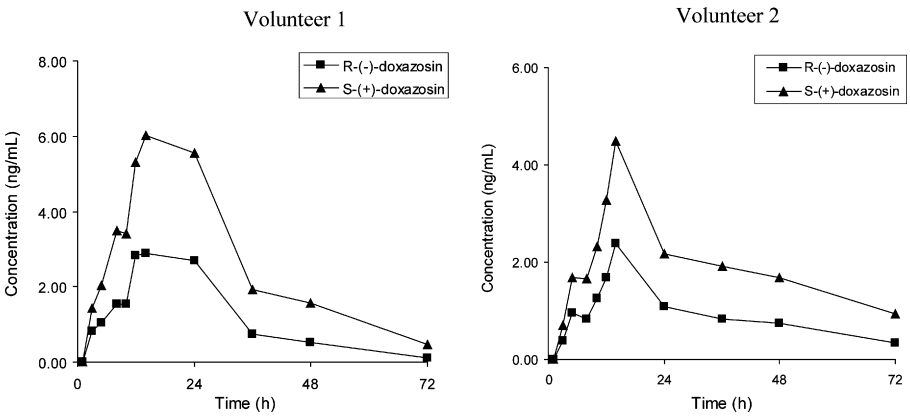

Fig. 5. Plasma concentration curves of *R*-(−)-doxazosin and *S*-(+)-doxazosin after an oral administration of 4 mg racemic doxazosin mesylate control release tablet to two healthy volunteers.

oral administration of 4 mg racemic doxazosin to two healthy male volunteers, profiles of the plasma concentrations of doxazosin enantiomers versus time are shown in Fig. 5. The plasma concentration of *S*-(+)-doxazosin for all the time points was higher than those of the *R*-(−)-enantiomer, with the *S*-(+)/*R*-(−) ratio ranged from 1.8 to 4.8. The $AUC_{0-\infty}$ of *S*-(+)-enantiomer was 2.3 times higher than that of *R*-(−)-enantiomer, and the S-(+)/*R*-(−) ratio of the total body clearance (CL/F) was 0.43. For the first time, these results indicated that the pharmacokinetics of doxazosin enantiomers was stereoselective. *S*-(+)-doxazosin provides higher system exposure than *R*-(−)-enantiomer, and it offered lower adverse effect. Our findings are very helpful for the development of individual enantiomer of doxazosin.

4. Conclusions

An enantioselective and sensitive method was developed and validated to determine doxazosin enantiomers in human plasma by combining separation via an ovomucoid chiral stationary phase column with tandem mass spectrometry. The lower limit of quantification was 0.100 ng/mL for *R*-(−)-doxazosin and *S*-(+)-doxazosin using a 0.2 mL aliquot of the plasma sample. This method was successfully applied to characterize the pharmacokinetic profiles of doxazosin enantiomers in healthy volunteers after an oral administration of therapeutic doses of doxazosin.

Acknowledgement

This work was supported by the National Basic Research Program of China (No. 2009CB930300) and Science and Technology Commission of Shanghai Municipality (No. 08DZ1980200).

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