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Short communication

Validation of a chiral liquid chromatography–tandem mass spectrometry method for the determination of pantoprazole in dog plasma

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ABSTRACT

Pantoprazole (PAN), a selective proton pump inhibitor, is used clinically as a racemic mixture for the treatment of acid-related gastrointestinal disorders. To investigate its stereoselective pharmacokinetics, a chiral liquid chromatography–tandem mass spectrometry method was developed and validated to determine the pantoprazole enantiomers in dog plasma. After liquid–liquid extraction, a baseline resolution of enantiomers was achieved on an ovomucoid column using the mobile phase of methanol:acetonitrile:10 mM ammonium formate (pH 7) (10.4:2.6:87, v/v/v) at 30 °C within 10 min. Stable isotopically labeled (+)-d_3-pantoprazole and (−)-d_3-pantoprazole were used as internal standards. Acquisition of mass spectrometric data was performed in multiple reaction monitoring mode via positive atmospheric pressure chemical ionization. The method was linear in the concentration range of 20.0–10,000 ng/mL for each enantiomer using 25 μL of dog plasma. The lower limit of quantification (LLOQ) for each enantiomer was 20.0 ng/mL. Intra- and inter-day precision ranged from 3.2% to 10.3% for (+)-pantoprazole and 3.7–10.0% for (−)-pantoprazole. Accuracy varied from −1.4% to −0.2% for (+)-pantoprazole and −1.6% to 0.8% for (−)-pantoprazole. The validated method was applied successfully for stereoselective pharmacokinetic studies of racemic pantoprazole.

1. Introduction

Proton pump inhibitors (PPIs) are a group of drugs with pronounced and long-lasting reduction of gastric acid production; they act by blocking the H^+/K^+ ATPase enzyme system of the gastric parietal cells covalently [1]. All PPIs, including omeprazole, pantoprazole, lansoprazole, and rebaprazole, have a chiral benzimidazole sulfoxide structure and are administered to humans as racemic mixtures of stereoisomers.

The enantiomers of racemic drugs may exhibit potentially different pharmacokinetic, pharmacodynamic, and toxicological profiles. Omeprazole is the first of the PPIs, and both enantiomers show the same potential for decreasing gastric acid formation. However, due to the slower metabolism and relatively small individual difference of the (−)-enantiomer in humans, it has been approved by the Food and Drug Administration (FDA) as a new PPI and marketed under the commercial name Nexium® [2,3].

Pantoprazole is a selective and long-acting PPI in the management of upper gastrointestinal disease. Similar to other PPIs, pantoprazole is a chiral benzimidazole sulfoxide and has two optically active forms. Clinical studies have proven that the serum concentration of (−)-pantoprazole was slightly higher than that of (+)-pantoprazole [4–7].

Several chiral methods have been reported for the enantioselective determination of pantoprazole in serum or plasma, including HPLC-UV using a chiral-AGP column [8] and column-switching HPLC-UV using a Chiralcel OJ-R column [9,10] or a chiral polysaccharide column [11]. All published works had a long chromatographic run time exceeding 23 min and generally required complex extraction procedures to remove interferences, which were clearly impractical for high-throughput analysis. Therefore, it was necessary to develop a completely new method to quantify the enantiomers of pantoprazole in plasma.

Recently, mass spectrometry combined with chiral HPLC applications has been used increasingly in stereoselective pharmacokinetic studies due to its specificity, short run time, and high sensitivity [12,13]. However, the development of enantioselective liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods for bioanalysis of drug enantiomers remains a research problem to this day in terms of compatibility. The normal-phase HPLC systems are generally considered to be incompatible with electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) techniques due to the poor ionization or potential explosion of the mobile phase. For reversed-phase HPLC systems, the percentage of organic solvents and the types of additives could affect the ionization efficiency significantly, which would limit the selection of solvents. To date, no enantioselective LC–MS/MS

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method has been applied for the determination of pantoprazole enantiomers in biological samples.

The objective of this study was to establish and validate a chiral LC–MS/MS method with high sensitivity and specificity for the determination of pantoprazole enantiomers in dog plasma and to support stereoselective pharmacokinetic studies of pantoprazole.

2. Experimental

2.1. Chemicals and reagents

(±)-Pantoprazole sodium (99.5% purity), (+)-pantoprazole sodium (99.1% purity), and (−)-pantoprazole sodium (99.9% purity) were supplied by Jiangsu Chia-Tai Tianqing Pharmaceutical Co., Ltd. (Jiangsu, China). (±)-d_3-Pantoprazole (99.2% purity) was purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada). HPLC-grade methanol, acetonitrile, ammonium formate, and ammonium hydroxide were obtained from Sigma–Aldrich Corp. (St. Louis, MO, USA). All other reagents were of analytical grade and obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Instrumentation

An Agilent 1200 liquid chromatography system consisting of a G1322A vacuum degasser, a G1312B binary pump, a G1316B column oven, and a G1367D autosampler (Agilent, Waldbronn, Germany) was used for solvent and sample delivery. Mass spectrometric detection was performed on an Agilent 6460 triple quadrupole instrument (Agilent, Waldbronn, Germany) equipped with an APCI source. Data processing was performed using Agilent MassHunter software (version B.03.02, Agilent).

2.3. Analytical conditions

Pantoprazole enantiomers were separated on an Ultron ES-OVM column (150 mm × 4.6 mm, 5 μm) protected with an Ultron ES-OVM cartridge (10 mm × 4 mm, 5 μm) (Agilent, Wilmington, DE, USA). The mobile phase consisting of methanol:acetonitrile:10 mM ammonium formate buffer (pH 7, adjusted by 10% ammonium hydroxide) (10.4:2.6:87, v/v/v) was delivered at a flow rate of 1.3 mL/min in the first 5 min, then shifted to 0.6 mL/min in 0.1 min and held constant until the end of the run. Column temperature was maintained at 30 °C. Stable isotopically labeled (+)- and (−)-d_3-pantoprazole were used as international standards (ISs) for (+)- and (−)-pantoprazole, respectively.

The mass spectrometer was operated in positive ion mode using an APCI source. The mass spectrometer conditions were set as follows: gas temperature 325 °C, vaporizer temperature 350 °C, gas flow 4 L/min, nebulizer 60 psi, capillary 3500 V, and corona current 4 μA. Fragmentor voltage was set at 100 V for both analytes and ISs. Optimized multiple reaction monitoring (MRM) fragmentation transitions were *m/z* 384 → 200 for pantoprazole enantiomers and *m/z* 387 → 203 for d_3-pantoprazole enantiomers. Collision energy was 10 V. Dwell time for each transition was 120 ms.

2.4. Preparation of standards and quality control samples

Stock solutions of (±)-pantoprazole were prepared by dissolving the test compounds in methanol to obtain 500 μg/mL concentration for each enantiomer. Calibration standards at concentrations of 20.0, 50.0, 150, 500, 1000, 2500, 5000, and 10,000 ng/mL for each enantiomer were prepared through serial dilution of (±)-pantoprazole stock solution with blank plasma. Quality control (QC) samples were independently prepared in blank plasma at four different concentrations (20.0, 60.0, 1000, and 8000 ng/mL for each enantiomer, respectively). Stock solutions of (+)-pantoprazole or (−)-pantoprazole at a concentration of 1.00 mg/mL were prepared by dissolving the reference substance of each enantiomer in methanol, which were then used to prepare the mixtures with different pantoprazole enantiomeric ratios (2:1 and 1:2). All solutions were stored at 4 °C and brought to room temperature before use. The QC samples were stored at −20 °C.

2.5. Sample preparation

A 25 μL aliquot of IS solution (250 ng/mL (+)-d_3-pantoprazole and 250 ng/mL (−)-d_3-pantoprazole) and 400 μL of phosphate buffer solution (PBS, pH 7.4) were added to 25 μL of plasma sample. The mixture was vortex-mixed and extracted with 3 mL of n-hexane:dichloromethane:isopropanol (200:100:10, v/v/v) by vortexing for 5 min, followed by centrifugation at 2000 × *g* for 5 min. The organic phase was transferred to another tube and evaporated to dryness at 40 °C under a stream of air in the TurboVap evaporator (Zymark, Hopkinton, MA, USA). The residue was reconstituted in 100 μL of the mobile phase, and an aliquot of 5 μL was injected into the LC–MS/MS system.

2.6. Method validation

Validation experiments of the method were carried out according to US FDA guidelines [14].

2.6.1. Selectivity

The selectivity of the method was evaluated by analyzing six different sources of dog blank plasma and spiked plasma samples at LLOQ level. The analyte responses of LLOQ should be >5 times that of blank plasma.

2.6.2. Linearity

Linearity was assessed by analyzing calibration curves with eight levels in duplicate on three consecutive days. The curves were constructed from a linearly weighed ($1/x^2$) least squares regression obtained by plotting peak area ratios of the analyte to IS against the nominal concentration of each enantiomer.

2.6.3. Precision and accuracy

Precision and accuracy were assessed by analyzing six replicates of QC samples at 60.0, 1000, and 8000 ng/mL for each enantiomer on three separate days. LLOQ was established by analyzing six blank plasma samples spiked with 20.0 ng/mL of each enantiomer. Accuracy and precision were expressed as relative error (RE) and relative standard deviation (RSD), respectively. Intra- and inter-day precisions were required not to exceed 15%, whereas accuracy was required to be within ±15%. For LLOQ, both precision and accuracy were less than or equal to 20%.

To assess the accuracy and precision of different pantoprazole enantiomeric ratios, pantoprazole enantiomers were combined and diluted with dog blank plasma to obtain two mixtures of enantiomeric ratios at 2:1 and 1:2 [(+)/(−)], with total concentration of 1500 ng/mL.

2.6.4. Matrix effect

To evaluate the matrix effect in the study, six different lots of blank plasma were extracted and spiked with enantiomers at low or high QC levels. The matrix effect was estimated as IS-normalized matrix factor by dividing the corresponding peak area ratios of each analyte to IS in spiked plasma post-extraction with those of the solution standards in mobile phase. The variability of the IS-normalized matrix factor should not be greater than 15%.

2.6.5. Recovery

The extraction recoveries of pantoprazole enantiomers at three QC levels ($n = 6$) were calculated by comparing the peak area ratios of each analyte to IS in samples spiked with analytes prior to extraction ($n = 6$) with samples to which the analytes were added post-extraction. The extraction recoveries of ISs were also determined using the QC samples at medium concentration as a reference.

2.6.6. Stability

Inversion of chiral compounds may occur during storage and extraction procedure, especially for the sulphoxides. To evaluate the potential inversion and stability of each pantoprazole enantiomer in dog plasma subjected to different conditions, plasma samples at concentrations of 125 and 8000 ng/mL were prepared individually by diluting (+)-pantoprazole or (−)-pantoprazole stock solutions. Analytes were considered stable when accuracy was within ±15%. The enantiomers of pantoprazole were monitored simultaneously as described.

2.7. Applications to stereoselective pharmacokinetic studies

The validated enantioselective LC–MS/MS method was applied to the quantification of pantoprazole enantiomers in stereoselective pharmacokinetic studies of pantoprazole. The studies were performed following an animal protocol approved by the Institutional Animal Care and Use Committee at Shanghai Institute of Materia Medica. Following the continuous intravenous infusion of 2 mg/kg of each pantoprazole enantiomer or 4 mg/kg racemic pantoprazole to six beagle dogs, venous blood samples were collected in heparinized tubes before treatment and at 0.25, 0.5 (end of infusion), 0.583, 0.75, 1, 1.5, 2.5, and 3.5 h after the initiation of the infusion. Plasma samples were obtained immediately by centrifugation at 2000 × *g* for 10 min and stored at −20 °C until analysis.

3. Results and discussion

3.1. Enantioselective chromatographic conditions

To resolve pantoprazole enantiomers from their racemic mixture, several types of chiral stationary phase columns were evaluated, including macrocyclic glycopeptide-based (Chirobiotic T column and Chirobiotic V2 column) and protein-based columns (AGP column and Ultron ES-OVM column). There was no obvious resolution for pantoprazole enantiomers on the macrocyclic glycopeptide-based columns, although various organics (acetonitrile, methanol, and ethanol) and acid/base modifiers were tested. Higher resolution was achieved on protein-based columns, and as the resolution achieved on AGP column was not as good as OVM column, the Ultron ES-OVM column, immobilized with ovomucoid, was chosen for further optimization.

When protein-based chiral column is coupled to MS/MS detection, the main problem is the compatibility of the mobile phase with MS. On the one hand, this type of columns could only tolerate low concentration of organic solvent in the mobile phase (usually <30%) [15,16], which may reduce the sensitivity of MS. On the other hand, the most commonly used buffer of ovomucoid column is 20 mM phosphate buffer, which is non-volatile and should be avoided in MS detection. In this study, further optimization of the mobile phase was performed in terms of buffers, pH values, and percentage of organic modifier. The preferred buffers were ammonium acetate and ammonium formate, and the pH was adjusted by formic acid or ammonium hydroxide. It was found that ammonium formate could provide better chiral resolution and peak efficiency for pantoprazole enantiomers than ammonium acetate. The resolution factor (R_s) increased from 2.36 to 2.84 when the pH of 10 mM ammonium formate was raised from 5.5 to 7.3, the retention time changed from 11.8 to 12.6 min for (+)-pantoprazole, and from 14.4 to 16.3 min for (−)-pantoprazole. Although baseline separation could be obtained using acetonitrile:10 mM ammonium formate (pH 7.3) (5:95, v/v) at 1 mL/min, the MS response of pantoprazole enantiomers was poor. As a result, a mixture of methanol and acetonitrile was used as a substitute for acetonitrile in the experiment. Baseline separation ($R_s = 1.75$) was achieved using methanol:acetonitrile:10 mM ammonium formate (pH 7) (10.4:2.6:87, v/v/v) as the mobile phase. The total run time was shortened from 30 min to 10 min by setting a flow gradient.

The elution order of pantoprazole enantiomers on the ovomucoid column was determined by injecting each reference substance at 8000 ng/mL under the same chromatographic conditions. The first elution peak was identified as (+)-pantoprazole and the second elution peak was (−)-pantoprazole, with retention time at 6.3 and 8.3 min, respectively. For (+)-pantoprazole and (−)-pantoprazole, enantiomeric excess were 97.4% and 100%, respectively.

3.2. Sample preparation and IS selection

The separation of pantoprazole enantiomers was operated on an Ultron ES-OVM with a low percentage of organic phase and the coeluent contaminants may cause matrix effects. To reduce the matrix effects and enhance reproducibility, liquid–liquid extraction was firstly considered for relatively clean extracts could be obtained with good sensitivity. The application of stable isotopically labeled ISs for each enantiomer is critical to counterbalance the matrix effects due to its similarity to pantoprazole in terms of chromatographic behavior and ionization property. It was reported that signal suppression in ESI was significantly more intense than that occurring in APCI [17]. In our study, an APCI source was used to minimize potential matrix effects.

3.3. Method validation

3.3.1. Assay selectivity

In the positive APCI, protonated molecules at *m/z* 384 and *m/z* 387 were observed as the most abundant ions for (±)-pantoprazole and (±)-d_3-pantoprazole, respectively. The transitions of *m/z* 384 → 200 for pantoprazole enantiomers and *m/z* 387 → 203 for d_3-pantoprazole enantiomers were chosen in MRM mode. The product ion spectra of $[M+H]^+$ ions of pantoprazole enantiomers and d_3-pantoprazole enantiomers are shown in Fig. 1.

Selectivity of the method was assessed by comparing the chromatograms of blank plasma from six different sources with the corresponding spiked plasma. Typical chromatograms of a blank plasma sample, a blank plasma sample spiked with (+)-pantoprazole and (−)-pantoprazole at LLOQ and IS, and a plasma sample obtained from a beagle dog 1 h after initiation of intravenous infusion of 4 mg/kg racemic pantoprazole are shown in Fig. 2. No interfering peaks from endogenous compounds were observed co-eluting with analytes and ISs in dog plasma.

3.3.2. Linearity of calibration curves and LLOQ

Linear regression curves were obtained over the concentration range of 20.0–10,000 ng/mL for each pantoprazole enantiomer in dog plasma with a coefficient of correlation ($r^2 > 0.99$). Typical regression equations of calibration curves are as follows:

(+)-Pantoprazole: $y = 3.40 \times 10^{-3}x + 6.30 \times 10^{-3}$ ($r^2 = 0.9953$);
(−)-Pantoprazole: $y = 3.20 \times 10^{-3}x + 6.00 \times 10^{-3}$ ($r^2 = 0.9965$).

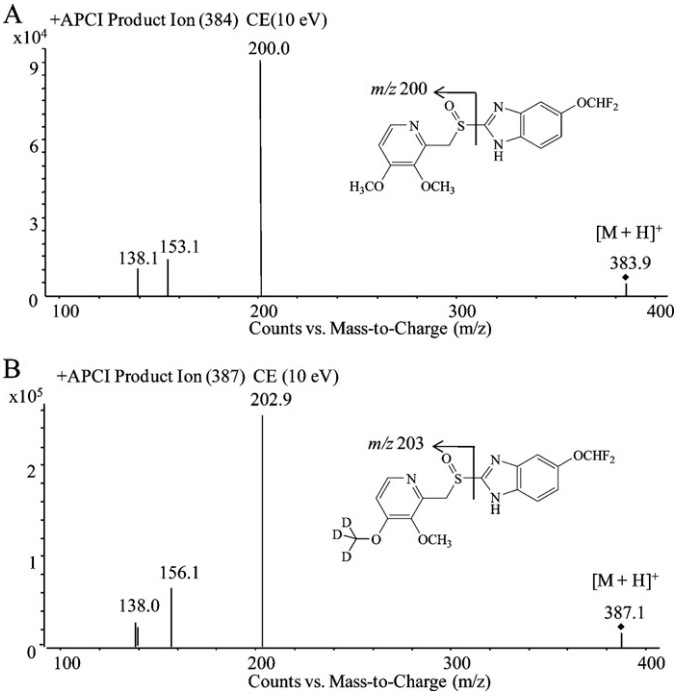

Fig. 1. Product ion spectra of $[M+H]^+$ of (A) pantoprazole enantiomers and (B) d_3-pantoprazole enantiomers.

where y is the peak area ratio of analyte to IS and x is the plasma concentration of the analyte. In this assay, the LLOQ of each enantiomer was 20.0 ng/mL.

3.3.3. Precision and accuracy

The intra- and inter-day precision and accuracy were calculated based on replicates (three days, three concentrations, each $n = 6$) of QC samples. Results are summarized in Table 1. These data were in accordance with recommended guidelines for assay precision and accuracy.

The intra- and inter-day precision and accuracy of different pantoprazole enantiomeric ratios in dog plasma at total plasma concentration of 1500 ng/mL are summarized in Table 1. Typical chromatograms of (+)-pantoprazole and (−)-pantoprazole from different enantiomeric ratios are shown in Fig. 3.

3.3.4. Matrix effect and recovery

Matrix effects for (+)-pantoprazole at concentrations of 60.0 and 8000 ng/mL were 95.3% and 96.8%, respectively. Matrix effects for (−)-pantoprazole at concentrations of 60.0 and 8000 ng/mL were 98.4% and 93.9%, respectively. The variability of IS-normalized matrix factors for both enantiomers from six different lots of plasma was less than 10.8%. Matrix effects for (+)- and (−)-d_3-pantoprazole were 105% and 103%, respectively, with a variability less than 11.2%. These data indicated the absence of significant ion suppression or enhancement from the blank plasma.

Recoveries of (+)-pantoprazole were 98.7%, 103%, and 98.6% at concentrations of 60.0, 1000, and 8000 ng/mL, respectively. Recoveries of (−)-pantoprazole were 90.9%, 108%, and 97.5% at concentrations of 60.0, 1000, and 8000 ng/mL, respectively. The recoveries of ISs were 97.6% and 95.0% for (+)-pantoprazole and (−)-pantoprazole, respectively.

3.3.5. Stability

In this study, plasma samples of pantoprazole enantiomers were stable under investigated conditions, including being on a bench top for 6 h (RSD ≤ 5.1%, RE in the range of −9.1% to 7.8% for each enantiomer), through three freeze–thaw cycles from −20 °C to ambient temperature (RSD ≤ 4.6%, RE in the range of −2.9% to 12.4% for each enantiomer), and a long period of storage (90 days) at −20 °C (RSD ≤ 7.9%, RE in the range of −6.9% to 10.0% for each enantiomer). In the stability study of (+)-pantoprazole in dog plasma, a trace amount of (−)-pantoprazole could be detected, which was no larger than 1.3% of the total pantoprazole, consistent with the enantiomeric excess result. On the other hand, the peak of (+)-pantoprazole was not observed in (−)-pantoprazole stability samples. The results indicated that no chiral inversion between (+)-pantoprazole and (−)-pantoprazole occurred during storage, handling, and analysis.

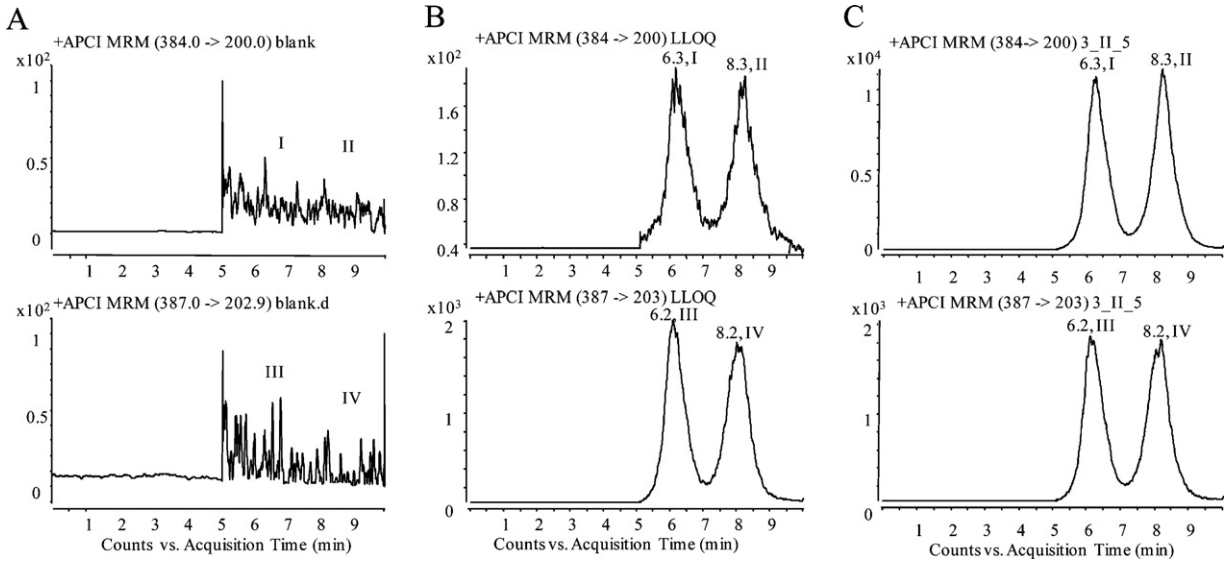

Fig. 2. Representative MRM chromatograms for (+)-pantoprazole (I), (−)-pantoprazole (II), (+)-d_3-pantoprazole (IS, III), and (−)-d_3-pantoprazole (IS, IV) in dog plasma: (A) blank plasma sample; (B) blank plasma sample spiked with (+)-pantoprazole (20.0 ng/mL), (−)-pantoprazole (20.0 ng/mL), (+)-d_3-pantoprazole (250 ng/mL), and (−)-d_3-pantoprazole (250 ng/mL); and (C) plasma sample obtained at 1.0 h after the initiation of intravenous infusion of 4 mg/kg racemic pantoprazole.

Table 1
Precision and accuracy for analysis of (+)-pantoprazole, (−)-pantoprazole, and different pantoprazole enantiomeric ratios (total plasma concentration at 1500 ng/mL) in beagle dog plasma (three days, six replicates per day).

Analyte	Concentration (ng/mL)		RSD (%)		RE (%)
	Added	Found	Intra-day	Inter-day	
(+)-PAN	20.0	19.6	6.5	7.0	−2.1
	60.0	59.2	6.1	7.5	−1.4
	1000	998	3.2	5.0	−0.2
	8000	7952	10.3	6.6	−0.6
(−)-PAN	20.0	20.1	7.7	7.5	0.6
	60.0	60.5	8.3	8.5	0.8
	1000	990	3.7	6.8	−1.0
	8000	7874	10.0	6.3	−1.6
(+)/(−) ratios	2.00	2.11	2.6	5.3	5.3
(+)/(−) ratios	0.500	0.477	4.7	3.7	−10.7

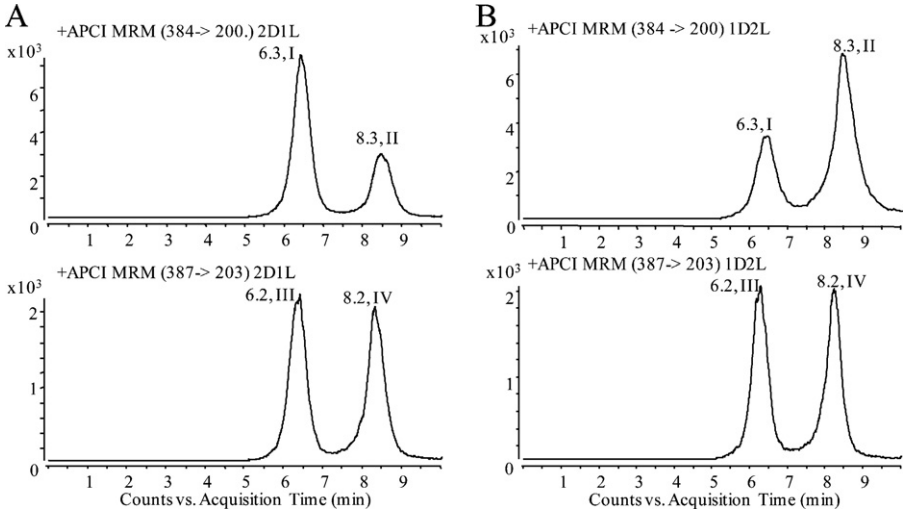

Fig. 3. Representative MRM chromatograms of (+)-pantoprazole (I) and (−)-pantoprazole (II) from different enantiomeric ratios (total plasma concentration at 1500 ng/mL): (A) (+)/(−) = 2/1; (B) (+)/(−) = 1/2.

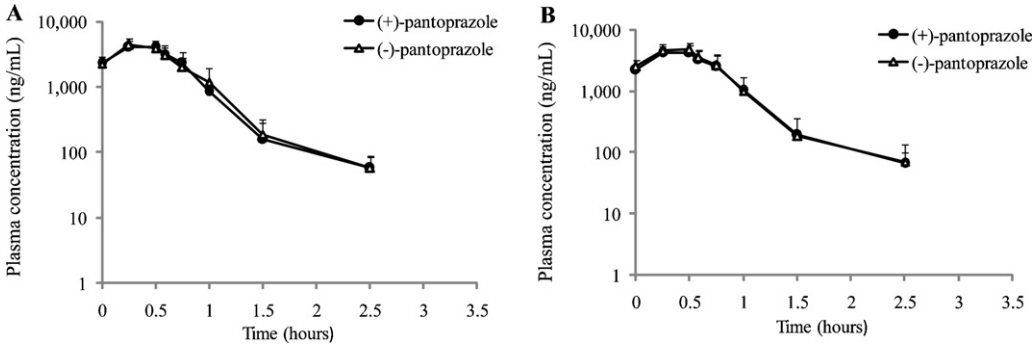

Fig. 4. Mean plasma concentration–time profiles of (+)-pantoprazole and (−)-pantoprazole after continuous intravenous infusion of (A) 2 mg/kg of each pantoprazole enantiomer or (B) 4 mg/kg racemic pantoprazole to beagle dogs.

3.4. Application

The validated chiral LC–MS/MS method was successfully applied in the determination of plasma concentrations of pantoprazole enantiomers in beagle dogs after intravenous infusion of 2 mg/kg of each enantiomer or 4 mg/kg of racemic mixture. Mean plasma concentration–time profiles of (+)-pantoprazole and (−)-pantoprazole are presented in Fig. 4.

4. Conclusion

A sensitive and enantioselective LC–MS/MS method was developed and validated for the quantification of pantoprazole enantiomers in dog plasma. Liquid chromatography conditions were developed on an ovomucoid protein column, using mass spectrometric compatible mobile phases. Baseline separation (R_s 1.75) was achieved within 10 min, increasing the sample

throughput. Stable isotopically labeled d_3-pantoprazole enantiomers were used as ISs to decrease the matrix effect. The method was successfully applied to evaluate the stereoselective pharmacokinetics of racemic pantoprazole in beagle dogs after intravenous infusion. The LLOQ of the method was 20.0 ng/mL only using 25 μL of plasma, which was adequate for the stereoselective pharmacokinetics of racemic pantoprazole in beagle dogs after intravenous infusion. And it is clear that the LLOQ could be easily improved using more amount of plasma or more sensitive MS detector.

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