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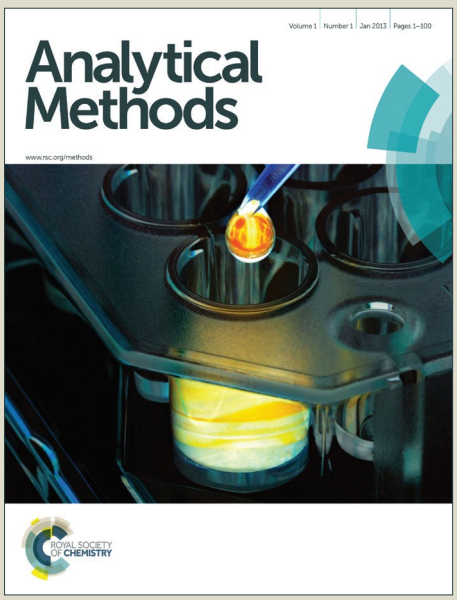

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Simultaneous determination of rabeprazole enantiomers and their four metabolites after intravenous administration in beagle dogs by a stereoselective HPLC-MS/MS method and its application to pharmacokinetic study

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Abstract

A sensitive, rapid and stable HPLC-MS/MS method has been developed and validated for the determination of rabeprazole enantiomers and their four metabolites, namely rabeprazole thioether, rabeprazole sulfone and desmethyl rabeprazole enantiomers, in beagle dog plasma using esomeprazole as the internal standard. Analytes and the internal standard were extracted from plasma samples by liquid-liquid extraction and separated on a Chiral-HSA column using acetonitrile-10mmol/L ammonium acetate as mobile phase by gradient elution. The method was validated with respect to sensitivity, specificity, linearity, precision, accuracy and especially the stability of analytes under various conditions, and was successfully applied in evaluating the pharmacokinetic profiles of racemic rabeprazole, the pure enantiomers and their metabolites in beagle dogs after single intravenous administrations of (R)-rabeprazole sodium injection (at 0.33, 1 and 3 mg/kg), (S)-rabeprazole sodium injection(at 1 mg/kg) and racemic rabeprazole sodium injection(at 2 mg/kg). The two enantiomers showed different profiles in the pharmacokinetic parameters. The $AUC_{0\sim t}$ and $t_{1/2}$ values of (R)-rabeprazole were higher and the clearance (CL) value of (R)-rabeprazole was lower than that of (S)-rabeprazole. Compared to (S)-rabeprazole, the higher absorption and slower elimination of (R)-rabeprazole explain that why (R)-rabeprazole is more effective than the racemate.

Keywords: rabeprazole, enantiomer, metabolite, HPLC-MS/MS, pharmacokinetic

Abbreviations: RA, rabeprazole; RT, rabeprazole thioether; RS, rabeprazole sulfone; DRA, desmethyl rabeprazole; IS, internal standard; HPLC-MS/MS, high performance liquid chromatography-tandem mass spectrometry; ESI, electrospray ionization; MRM, multiple reaction monitoring; LLOQ, lower limit of quantification; QC, quality control; LQC, low quality control; MQC, medium quality control; HQC, high quality control; RSD, relative standard deviation; DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision exit potential.

Introduction

Rabeprazole (RA), 2-{[4-(3-methoxypropoxy)-3-methyl-2-pyridinyl]-methyl sulfinyl}-1H-benzimidazole, structurally related to omeprazole, is a newly developed proton pump inhibitor (PPI) that suppresses gastric acid secretion through an interaction with (H+/K+)-ATPase in gastric parietal cells. RA is effective in the treatment of various peptic diseases, including gastric and duodenal ulcer, gastroesophageal reflux disease (GERD), and Zollinger–Ellison syndrome. This drug contains an asymmetric sulfur in its chemical structure and is clinically administered as a racemic mixture of (R)-RA and (S)-RA (**1a** and **1b**，respectively). While RA is primarily metabolized non-enzymatically to rabeprazole thioether (RT, **2**), which is pharmacologically active, some is oxidized to rabeprazole sulfone (RS, **3**) and desmethyl rabeprazole enantiomers ((R)-DRA and (S)-DRA, **4a** and **4b**) by CYP3A4 and CYP2C19, respectively (Fig.1)[1,2].

Drug enantiomers may have different pharmacokinetic, toxicological and pharmacodynamic properties due to biological stereoselectivity and potential inversion. Recently, a study demonstrated that half the dose of the racemate with the (R)-RA was more effective than (S)-RA in aspirin-induced ulcers of animal studies[3]. In addition, another study indicated that in the treatment of GERD, 10mg (R)-RA works better than 20mg racemic RA in order to improve the healing of endoscopic lesions and relief from symptoms of regurgitation[4]. So the efficacy of (R)-RA was obvious. Several methods involving enantioselective assay for the determination of RA concentration have been previously published. Gao *et al.*[5] developed an HPLC and an HPLC-MS/MS to study the chiral bioconversion and preclinical pharmacokinetic of (R)-RA without determination of metabolites in dog plasma. Moreover, new guidance for drug safety metabolite testing was issued by the US Food and Drug Administration and the International Conference on Harmonisation[6]. The metabolites accumulated in plasma for long term would produce toxic substances and lead to health damage. Moreover, the experimental data of metabolites provid theoretical basis for further safety evaluation and mass balance study. Consequently

the lack of steady-state metabolite data demanded the development of quantitative assays for RA metabolites. Simpemba *et al.*[7] and Uno *et al.*[8] reported methods for determination of racemic RA and only two metabolites without chiral resolution. Miura *et al.*[9] have reported an HPLC method for simultaneous determination of RA enantiomers and their metabolites, extracted with costly solid-phase extraction with large amounts (1mL) of plasma sample and required a very long run time (>60min) with high quantification limits for each compound.

In this study, a sensitive, simple and highly stable HPLC-MS/MS method was developed for simultaneous determination of (R)-RA, (S)-RA and their four metabolites, RT, (R)-DRA, (S)-DRA and RS in beagle dog plasma. The method was successfully applied to study the stereoselective pharmacokinetic profiles of racemic RA, and the pure enantiomers after intravenous administrations in beagle dogs.

Experimental

Chemicals and reagents

RA enantiomers, RT, RS, (R)-RA, (S)-RA and racemic RA for injection were provided by Nanjing Tech University. (R)-DRA and (S)-DRA (purity>99%) were purchased from TLC PharmaChem., Inc (Canada). Esomeprazole (internal standard, IS, purity>99%) was provided by National Institute for Food and Drug Control. Acetonitrile and methanol were HPLC grade and obtained from Merck (Darmstadt, Germany). Ammonium acetate (HPLC grade) was purchased from Aladdin (Shanghai, China). Purified water was produced by a Milli-Q Academic System (Millipore Corporate, Billerica, MA, USA). All other chemicals and solvents such as ethyl acetate were analytical grade and purchased from Nanjing Chemical Reagent (Nanjing, China).

Instrumentations

The LC-MS/MS system consisting of Agilent 1100 liquid chromatography (Agilent Technologies, Palo Alto, CA, USA) and an API4000 triplequadruple mass spectrometer (AB Sciex, Toronto, Canada) equipped with electrospray ionization (ESI) interface using positive ion mode. The chromatographic data acquisition and processing were performed using Analyst version 1.5.2 from AB Sciex.

HPLC-MS/MS conditions

Chromatographic separation of RA enantiomers and its four metabolites was achieved on a Chiral-HSA (150×4mm i.d., 5μm,Chrom Tech. Inc., UK) with a mobile phase consisting of 10mmol/L ammonium acetate (mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 0.8mL/min. The linear gradient profile was as follows: (a) 0min, 92% A; (b) 14min, 92% A; (c) 15min, 85% A; (d) 28min, 85% A; (e) 29min, 92% A; (f) 34min, 92% A. The total run time was 34min. 10μL of sample was injected into the system by autosampler set at 4°C.

The mass spectrometer was operated in an ESI positive ion mode. The multiple reaction monitoring (MRM) transitions were performed at m/z 360.2→242.1 for RA, m/z 346.5→228.2 for DRA, m/z 344.2→226.4 for RT, m/z 376.4→119.2 for RS and

m/z 346.5→198.3 for IS. Figure 2 shows the typical mass spectra of RA, DRA, RT, RS and IS. Optimized values for declustering potential (DP), entrance potential (EP), collision energy (CE) and collision exit potential (CXP) were 51V, 10V, 25eV, 18V for RA; 51V, 10V, 19eV, 16V for DRA; 101V, 10V, 27eV, 14V for RT; 86V, 10V, 39eV, 12V for RS; 51V, 10V, 17eV, 12V for IS. Other ion sourse conditions were as follows: Collision Gas was 7 psi, Curtion Gas was 25 psi, Ion source Gas 1 was 40 psi, Ion source Gas 2 was 60 psi, Nebulizer Current was 3μA, IonSpary Voltage was 5000 V and source temperature was 500°C.

Preparation of stock solutions, calibration standard and quality control samples

The stock solutions of (R)-RA (1mg/mL), (S)-RA (1mg/mL), (R)-DRA (1mg/mL), (S)-DRA (1mg/mL), RT (1mg/mL), RS (1mg/mL) and IS (1mg/mL) were prepared in methanol. A series of working standard solutions were prepared by dilutions of these stock solutions with methanol to several concentration levels: 10, 5, 1, 0.8, 0.5, 0.1, 0.05 and 0.01μg/mL for (R)-RA and (S)-RA; 2.5, 1.25, 0.25, 0.2, 0.125, 0.025, 0.0125 and 0.005μg/mL for (R)-DRA, (S)-DRA and RS; 50, 25, 5, 4, 2.5, 0.5, 0.25 and 0.05μg/mL for RT and 2.5μg/mL for IS. All the working solutions were kept at -20°C. Calibration standard plasma samples were prepared as follows: 20μL each working standard solution was mixed with 180μL blank dog plasma to obtain the concentration of 1, 5, 10, 50, 80, 100, 500 and 1000ng/mL for (R)-RA and (S)-RA; 0.25, 1.25, 2.5, 12.5, 20, 25, 125 and 250ng/mL for (R)-DRA, (S)-DRA and RS; 0.5, 2.5, 5, 25, 40, 50, 250 and 500ng/mL for RT. Quality control (QC) samples were prepared at concentration levels of 2, 20, 200 and 800ng/mL for (R)-RA and (S)-RA; 0.5, 5, 50 and 200ng/mL for (R)-DRA, (S)-DRA and RS; 1, 10, 100 and 400ng/mL for RT;

Plasma sample preparation

Plasma samples were extracted employing a liquid-liquid extraction technique. 200μL sample plasma was dispensed into 2mL polyethylene tubes followed by 20μL of IS (2.5μg/mL) and vortexed for 1 min. Then 800μL of ethyl acetate was added and vortexed for another 2 min. The mixture was centrifuged at 10000 rpm for 10min. Aliquots of 700μL supernatant were transferred and evaporated to dryness by nitrogen flushing at 40°C. The residues were reconstituted with 100μL of methanol and mixed

by vortexing for 30s, and centrifuged 14000rpm for 10min. 10ul of supernatant was injected into the HPLC-MS/MS system.

Method validation

Validation procedures of the method were carried out according to US FDA guidelines as follows[10].

Specificity and selectivity

The specificity of the method was evaluated by comparing the chromatograms of six different batches of blank dog plasma obtained from six different dogs spiked with standard solutions and dog plasma samples after intravenous administrations of (R)-RA, (S)-RA and racemic RA injection.

Linearity and lower limit of quantification

Standard curves (consisting of 8 concentration levels) were extracted and assayed with weighted ($1/x^2$) linear regression. Linearity was considered satisfactory if the correlation coefficient (r) of 0.99 or better. The acceptance criterion for each back-calculated standard concentration should be within 15% of the nominal value, except it should not exceed 20% at the LLOQ (the S/N ratio >10).

Precision and accuracy

The intra-day accuracy and precision were determined by analyzing six replicates of each quality control (LLOQ, LQC, M_1QC, M_2QC and HQC samples) of analytes within one day. The inter-day accuracy and precision were determined on three separate days by analysis of three batches of quality control samples at each level (LLOQ, LQC, M_1QC, M_2QC and HQC samples). The accuracy and precision were calculated and expressed as the percentage value of observed concentration to theoretical concentration and the relative standard deviation (RSD), respectively. For precision and accuracy, the acceptance criteria should be within 15% RSD and 85%-115% of nominal concentration, respectively.

Extraction recovery

Recovery was measured at LQC, M_1QC and HQC levels in three replicates, and was calculated by comparing the peak area of the analyte (A) added into blank plasma followed by sample extraction with that of the analyte (B) spiked to the already

extracted blank plasma at the same nominal concentrations. The ratio (A/B×100) % was defined as the extraction efficiency.

Matrix effect

The matrix effects of analytes and IS were evaluated by the ratios of the mean peak areas of the analytes spiked in post-treatment blank plasma to those of the mean peak areas of the pure standard solution at corresponding concentrations (LQC and HQC).

Stability

Stability of analytes was established by analysis of three replicates of QC samples (LQC and HQC) under the following conditions: freeze-thaw stability through three freeze-thaw cycles (-80°C to 21°C); short-term stability after storage at room temperature for 4h; long-term stability of the extracted plasma samples after keeping the processed samples frozen at -80°C for 20 days; autosampler stability of analytes in reconstitution after storage at 4°C for 48h.

Stability of standard solution was evaluated at one concentration (1μg/mL) in six replicates by analyzing samples that were diluted by different solvents (methanol and mobile phase), kept at room temperature for 4h, and stored at -20°C for 20 days. Samples were considered stable if the values were within the acceptable limits of accuracy (85-115% from fresh QC samples) and precision (15% RSD).

Dilution integrity and carryover

Dilution integrity was performed to extend the upper concentration limit with acceptable precision and accuracy. To assess carryover effects, blanks were injected immediately after the highest calibration standard, and the response of any interfering peak had to be <10% of the response of an LLOQ sample.

Pharmacokinetic study

Twelve adult Beagle dogs (6 males and 6 females) weighing 10.0±2kg, were obtained from the Agricultural College, Shanghai Jiao Tong University (Shanghai, China). Considering that the clinical dosage of recommendation for (R)-RA sodium injection was 10 mg/day (10mg/60kg), the equivalent dose was 0.3 mg/kg in beagle calculated by surface area conversion. Based on this, the administration doses in this assay were identified as 0.33mg/kg, 1mg/kg and 3mg/kg. The dogs randomly divided into two

groups (A_1 and A_2).Six dogs (A_1) were given single intravenous routes (R)-RA at 1mg/kg, (S)-RA at 1mg/kg and racemic RA at 2mg/kg through a set of 3 × 3 Latin square design. Another group of six dogs (A_2) received 0.33 and 3mg/kg (R)-RA by intravenous administration through a set of 2 × 2 Latin square design. The serial number of dogs and experimental groups were showed in **Table 1**. The use of dogs in the study was approved by the Institutional Animal Ethics Committee of Nanjing Tech University and all experiments were performed in compliance with the guide for the Care and Use of Laboratory Animals. Approximately 2ml of blood samples was collected into Eppendorff tubes with potassium EDTA at 0, 2, 5, 10, 20, 30, 40, 60, 90, 120, 150, 180 and 240min after single intravenous administrations of (R)-RA injection (at 0.33, 1 and 3mg/kg), (S)-RA injection (at 1 mg/kg) and racemic RA injection (at 2 mg/kg). Plasma samples were then obtained by centrifugation at 5000rpm for 10 min and kept at -80°C until analysis. The pharmacokinetic parameters were calculated using Drug and Statistics Software version 2.0 (DAS 2.0, Mathematical Pharmacology Professional Committee of China, Shanghai, China). Statistical analysis was performed using SPSS Statistics 19 (SPSS Inc., Chicago, IL, USA). Bivariate correlations analysis was used to evaluate the correlation and a p value < 0.01 indicated significant correlation. Student's t test was used to analyze the significance and a p value < 0.05 indicated significant differences.

Results and discussion

Optimization of HPLC-MS/MS condition

We aimed at the development and validation of a simple, rapid, sensitive and stable HPLC-MS/MS method for the simultaneous determination of RA enantiomers and their four metabolites in dog plasma. A viable method was consequently in need to separate chiral compounds (R)-RA and (S)-RA, (R)-DRA and (S)-DRA, respectively. Several trials were carried out to expect good resolution on Chiral-IC, AGP or HSA columns. It's revealed that better separation and suitable retention times can be achieved on the Chiral-HSA column. The ratio of organic modifier that the HSA column can afford is within 15%, because the filler of HSA column is human serum albumin immobilized on 5μm silica-gel. The proportion of organic modifier (acetonitrile or methanol) was optimized by changing ratio of the binary systems, i.e. acetonitrile-water or methanol-water. Additionally, gradient elution was selected to achieve shorter retention time. Ultimately, the qualities of peak shape, sensitivity and resolution were obtained by using 10mmol/L ammonium acetate in the aqueous phase-acetonitrile system.

In order to optimize ESI conditions for RA, RT, RS, DRA and IS, quadrupole full scans were carried out in positive ion detection mode. The precursor-product ion transitions were determined by Q1 MS full scan and product ion scan: RA (m/z 360.2→242.1); RT (m/z 344.2→226.4); RS (m/z 376.4→119.2); DRA (m/z 346.5→228.2); IS (m/z 346.5→198.3).

Selection of reconstituted solvent

Liquid-liquid extraction was adopted for sample pretreatment. When water-acetonitrile binary mixture with different ratios (80:20, 50:50 and 20:80, *v*/*v*) was tested primarily as reconstituted solvent, RA was surprisingly metabolized non-enzymatically to RT rapidly. Consequently pure organic solvents (methanol and acetonitrile) were estimated as reconstituted solvent instead of water-comprising solvent. Considering the serious solvent effect of Chiral-HSA column, methanol was finally selected as reconstituted solvent.

HPLC-MS/MS method validation

Specificity

Fig.3.1 and Fig.3.2 shows representative MRM chromatograms of blank plasma (A), blank plasma spiked with (R)-RA, (S)-RA, their metabolites at LLOQ with IS (B), plasma sample obtained at 5min after single intravenous administrations of (R)-RA injection (S)-RA injection and racemic RA injection at 2mg/kg (C , D and E, respectively). No interfering peak in the blank plasma was observed at the retention times of (R)-RA, (S)-RA, their metabolites, and IS.

Linearity and lower limit of quantification.

All calibration curves were linear over the concentration range of 1-1000ng/mL (r=0.9990, n=6) for (R)-RA, 1-1000ng/mL (r=0.9991, n=6) for (S)-RA, 0.25-250ng/mL (r=0.9989, n=6) for (R)-DRA, 0.25-250ng/mL (r=0.9986, n=6) for (S)-DRA, 0.5-500ng/mL (r=0.9981, n=6) for RT and 0.25-250ng/mL (r=0.9976, n=6) for RS. Table 2 shows the accuracy and precision (% RSD) for (R)-RA, (S)-RA and their metabolites at the LLOQ. LLOQ concentration levels (R)-RA, (S)-RA and their metabolites were follows: (R)-RA and (S)-RA, 1ng/mL; (R)-DRA, (S)-DRA and RS, 0.25ng/mL; RT, 0.5ng/mL.

Precision and accuracy

The intra- and inter-day accuracy and precision results of QC samples are listed in **Table 2**. At each concentration level, the RSD values of intra-day and inter-day precision were lower than 10% and the accuracy results (RE) ranged from -8 to 8 for all analytes.

Extraction recovery and matrix effect

The mean extraction recoveries and matrix effect of (R)-RA, (S)-RA and their metabolites from dog plasma at different concentration levels are presented in **Table 3**. The mean recoveries for (R)-RA, (S)-RA and their metabolites ranged from around 60% to 80% at three concentration levels. The matrix effects evaluated for QC standards at two concentration levels were in the range of 91-109%. The results indicated that the matrix effect on the ionization of analytes and IS was not obvious under these conditions.

Stability

Table 4 summarizes the stability of three freeze-thaw cycles at 21℃ and -80℃， short-term stability at room temperature for 4h, long-term stability at -80℃ for 20 days and storage in the autosampler for 48h at 4℃. Good stability of analytes in dog plasma was demonstrated under all the conditions.

The stability study of standard solution revealed that RA enantiomers and their metabolites were stable at room temperature for 4h and stored at -20℃ for 20 days when diluted by methanol. Under different conditions, the maximum relative errors (RE) between fresh and stored samples were 6.8, 5.4, 3.5, 3.2, 4.5 and -3.6 for (R)-RA, (S)-RA, (R)-DRA, (S)-DRA, RT and RS, respectively. However, when analytes were diluted in mobile phase, the maximum RE between fresh and stored samples exceed -20 after 4h at room temperature. The stability test results show that methanol can be used as solvent.

Dilution integrity and carryover

The carryover effect was not detectable for all the analytes. The upper concentration limits can be extended to HOQ×10 level by a 10-fold dilution with dog plasma. The results of the dilution integrity experiments indicate that the accuracy of the method was within ±10%, whereas the precision was less than 9.5%.

Pharmacokinetic study

The validated HPLC-MS/MS method was successfully used to measure the plasma concentration of RA and their metabolites after intravenous administration of racemate and individual enantiomers in dogs. After administration of individual enantiomers in dogs, the other antipode was not detected at any time point post-dose. Inversions between RA enantiomers or between DRA enantiomers were not observed in dogs.

The dog plasma concentration-time profiles of (R)-RA, (S)-RA and their metabolites, RT, RS, (R)-DRA and (S)-DRA are shown in Fig.4. The results showed that the plasma concentration of (R)-RA and (S)-RA after intravenous administration reached C_{max} at 2min, then decreased to below LLOQ at 240min quickly. Meanwhile, the plasma concentration of RT, RS, (R)-DRA and (S)-DRA was gently increased to

C_{max} at around 20min, and reduced to below LLOQ at 240min quickly. The concentration of RT was the highest of all metabolites in dog plasma, which meant RT was the main metabolite. The dog plasma concentration-time profiles of (R)-RA, (S)-RA and their metabolites showed that the concentration of all metabolites dropped to below LLOQ at 240min after single intravenous administrations, which indicated that there was no accumulation in dogs for (R)-RA, (S)-RA and their metabolites.

The main pharmacokinetic parameters of (R)-RA, (S)-RA and their metabolites are listed in **Table 5**. The C_{max} and AUC_{0-t} values of (R)-RA increased in proportion to the dose with the linear regression of C_{max}=1764Dose+475 (R^2=0.9965) and AUC_{0-t}=84081Dose-7462 (R^2=0.9906). In addition, the parameters of $t_{1/2}$ and T_{max} were apparently independent of dose. When equal available (R)-RA was administrated, the $t_{1/2,}$ C_{max} and AUC_{0-t} of (R)-RA in (R)-RA group and racemic group were similar (p>0.05). However, the AUC_{0-t} and $t_{1/2}$ values of (R)-RA were higher (p<0.05) and the clearance (CL) value of (R)-RA (p<0.05) was lower than that of (S)-RA when administrated as individual enantiomers or administrated of racemic RA. The higher absorption and slower elimination make enantiopure (R)-RA a better therapeutic agent that could efficiently reduce clinical dosage and decrease toxicology risks.

Conclusion

A sensitive, simple and specific HPLC-MS/MS method has been developed and validated for simultaneous quantification of (R)-RA, (S)-RA and their metabolites, RT, RS, (R)-DRA and (S)-DRA in dog plasma using a Chiral-HSA column for the first time. The method was successfully applied to study the pharmacokinetic of racemic RA and the pure enantiomers in dogs after intravenous administration. The pharmacokinetic results indicate that RA enantiomers and DRA enantiomers were not chiral-inverse in dogs. The (R)-RA, (S)-RA and their metabolites were quickly eliminated and no significant accumulation was observed in plasma in dogs. The finding of higher absorption and slower elimination of (R)-RA compared to (S)-RA provides future insights into the disposition of racemic RA and its pure enantiomers *in vivo* and may facilitate the development of (R)-RA in the future. These results provide the experimental data and theoretical basis for further pre-clinical research and clinical applications of (R)-RA.

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Fig.1. Metabolic pathways of enantiomers of RA.

Fig.2. The product ion spectra of RA (A), RT (B), RS (C), DRA (D) and IS (E).

Fig.3.1 Representative MRM chromatograms of (R)-RA (Ⅰ), (S)-RA (Ⅱ), (R)-DRA (Ⅲ), (S)-DRA (Ⅳ), RT (Ⅴ), RS (Ⅵ) and IS (Ⅶ) in dog plasma samples: (A) blank plasma; (B) a plasma sample spiked with (R)-RA, (S)-RA, RT, RS, (R)-DRA , (S)-DRA at LLOQ with IS.

Fig.3.2 Representative MRM chromatograms of (R)-RA (Ⅰ), (S)-RA (Ⅱ), (R)-DRA (Ⅲ), (S)-DRA (Ⅳ), RT (Ⅴ), RS (Ⅵ) and IS (Ⅶ) in dog plasma samples: (C) a plasma sample obtained at 5min after intravenous administration of 1mg/kg (R)-RA, (D) 1mg/kg (S)-RA, and (E) 2mg/kg racemic RA.

Fig.4. Plasma concentration-time profile of (R)-RA (A), (S)-RA (B), (R)-DRA (C), (S)-DRA (D), RT (E) and RS (F) after intravenous administration of 0.33mg/kg (L), 1mg/kg (M), and 3mg/kg (H) (R)-RA, intravenous administration of 2mg/kg racemic RA (X), intravenous administration of 1mg/kg (S)-RA (S) to beagle dogs; G: plasma concentration-time profile of (R)-RA and (S)-RA after intravenous administration of 2mg/kg racemic RA (n=6).

Table 1

The serial number of dogs and experimental groups

Period	serial number of dogs					
	01	02	03	04	05	06
Ⅰ	A	A	B	B	C	C
Ⅱ	C	C	A	A	B	B
Ⅲ	B	B	C	C	A	A
	07	08	09	10	11	12
Ⅳ	D	D	D	E	E	E
Ⅴ	E	E	E	D	D	D

A, single intravenous administrations of (R)-RA injection at 1mg/kg;
B, single intravenous administrations of (S)-RA injection at 1mg/kg;
C, single intravenous administrations of racemic RA injection at 2 mg/kg;
D, single intravenous administrations of (R)-RA injection at 0.33mg/kg;
E, single intravenous administrations of (R)-RA injection at 3mg/kg.

Table 2

Intra- and inter-day precision and accuracy for RA enantiomers and the metabolites.

Analytes	Nominal Con. (ng/mL)	Measured con. (mean±SD, ng/mL)	Intra-day RSD (%)（n=6）	Inter-day RSD (%)（n=18）	RE (%)（n=18）
(R)-RA	1	1.02±0.07	0.8	7.1	2.3
	2	2.03±0.19	2.4	9.1	1.3
	20	21.01±0.62	1.7	3.0	5.1
	200	213.56±6.94	2.1	3.3	6.8
	800	777.72±16.21	1.4	2.1	-2.8
(S)-RA	1	1.00±0.08	5.6	7.8	0.1
	2	1.98±0.17	3.8	8.9	-1.2
	20	20.92±0.70	1.7	3.3	4.6
	200	215.06±8.03	1.8	3.7	7.5
	800	803.89±20.14	1.0	2.5	0.5
RT	0.5	0.50±0.04	6.4	8.2	0.2
	1	1.08±0.06	5.2	5.2	7.9
	10	10.59±0.53	3.9	5.0	5.9
	100	105.66±4.19	2.6	4.0	5.7
	400	379.12±16.80	2.6	4.4	-5.2
RS	0.25	0.24±0.02	6.7	9.6	-3.4
	0.5	0.54±0.03	6.5	5.3	7.4
	5	5.38±0.19	2.8	3.6	7.6
	50	51.49±1.71	2.7	3.3	3.0
	200	183.98±8.80	2.8	4.8	-8.0
(R)-DRA	0.25	0.26±0.02	2.0	6.6	5.8
	0.5	0.51±0.04	1.6	8.4	1.2
	5	5.13±0.22	2.7	4.2	2.5
	50	53.02±2.13	3.4	4.0	6.0
	200	203.39±6.36	1.9	3.1	1.7
(S)-DRA	0.25	0.25±0.02	2.3	9.2	0.7
	0.5	0.49±0.04	3.8	7.9	-2.8
	5	5.19±0.19	2.7	3.8	3.9
	50	53.44±2.18	2.6	4.1	6.9
	200	206.22±7.42	1.6	3.6	3.1

RSD, residual standard deviation; RE, relative error

Table 3

Extraction recovery and matrix effect of RA enantiomers and the metabolites in dog plasma.

Analyte	Nominal Con. (ng/mL)	Extraction recovery (n=9) (mean±SD, %)	Matrix effects (n=21) (mean±SD, %)
(R)-RA	2	69.81±3.05	98.74±3.54
	20	75.34±3.40	-
	800	78.56±2.15	100.63±0.94
(S)-RA	2	69.55±3.81	98.13±3.87
	20	73.40±3.30	-
	800	77.18±3.56	100.07±1.00
RT	1	70.13±3.53	100.33±3.04
	10	74.37±4.50	-
	400	77.18±4.12	99.26±2.75
RS	0.5	71.25±3.68	102.25±2.68
	5	75.03±3.40	-
	200	76.12±4.12	100.15±2.22
(R)-DRA	0.5	64.38±2.17	105.21±3.37
	5	68.98±2.78	-
	200	73.36±3.45	98.18±0.96
(S)-DRA	0.5	65.19±4.20	107.00±3.48
	5	69.10±3.35	-
	200	71.63±2.68	99.27±0.65

SD, standard deviation

Table 4

Stability analysis of RA enantiomers and the metabolites in dog plasma under various conditions (n=3).

Analyte	Nominal Con. (ng/mL)	4h, Room temperature		48h, post-preparative		Three cylces, freeze/thaw		20 days, -80℃	
		RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)
(R)-RA	2	-9.3	2.8	5.2	8.4	-6.7	8.0	10.7	4.1
	800	-0.4	0.6	0.3	0.8	-1.0	1.0	-0.4	0.3
(S)-RA	2	-8.0	3.3	4.3	7.0	-7.5	4.9	6.3	3.8
	800	3.8	0.7	4.5	0.2	3.2	0.9	2.6	0.6
RT	1	9.7	3.5	5.7	1.1	12.0	1.8	-2.2	1.9
	400	-3.2	0.6	5.3	2.4	0.7	1.7	-4.3	1.2
RS	0.5	9.1	2.0	3.9	3.1	9.5	3.5	-6.3	1.3
	200	-6.4	0.5	0.5	2.3	-3.8	1.3	-1.4	1.5
(R)-DRA	0.5	-9.2	3.2	-10.1	2.0	-12.8	0.6	7.5	3.1
	200	6.7	1.9	3.8	1.7	1.8	1.4	5.5	1.3
(S)-DRA	0.5	-7.8	4.7	-10.6	2.1	-9.2	1.3	5.7	5.5
	200	11.2	1.8	6.2	1.9	5.7	1.1	4.3	0.6

RSD, residual standard deviation; RE, relative error

Table 5

Plasma pharmacokinetic parameters of RA enantiomers and the metabolites after intravenous administration of the individual enantiomers and racemate in beagle dogs (n=6).

Analyte	parameters	Individual enantiomers (mean±SD)				Racemic RA (mean±SD)	
		0.33mg/kg	1mg/kg	3mg/kg	1mg/kg	2mg/kg	
		(R)-	(R)-	(R)-	(S)-	(R)-	(S)-
RA	T_{max} (min)[a]	2	2	2	2	2	2
	C_{max} (μg/L)[b]	936.2±173.2	2400±542.6[*]	5726.7±833.0	2338.3±264.4	2435±471.8	2090±402.1
	$t_{1/2}$ (min)[c]	22.9±5.3	20.4±1.1[*,#]	25.2±1.7	16.2±2.9	21.8±1.4[**]	14.1±1.3
	$AUC_{0\rightarrow t}$ (min μg/L)[d]	29732±9348	64006±14482[*,#]	247946±46009	46703±6851	87139±22281[**]	45527±10222
	Cl (L/min/kg)[e]	0.012±0.005	0.016±0.003[#]	0.013±0.002	0.022±0.004	0.012±0.002[**]	0.023±0.005
DRA	C_{max} (μg/L)[b]	22.8±4.9	65.5±12.7	144.8±24.8	68.0±14.4	81.0±23.0	85.5±29.7
	$t_{1/2}$ (min)[c]	29.9±5.5	23.8±1.2	27.8±3.2	25.1±4.3	26.1±2.5	21.0±2.2
	$AUC_{0\rightarrow t}$ (min μg/L)[d]	1348±113	4225±779	11122±2003	3879±562	5322±1727	4104±1374
RT	C_{max} (μg/L)[b]	77.4±33.1	117.2±22.2	386.2±109.9	76.5±17.5	282.3±45.6	
	$t_{1/2}$ (min)[c]	40.0±15.1	50.2±11.4	33.5±4.1	59.3±16.3	37.1±7.3	
	$AUC_{0\rightarrow t}$ (min μg/L)[d]	3253±1545	6073±1490	26152±5839	3387±681	13678±1925	
RS	C_{max} (μg/L)[b]	8.2±2.8	11.0±2.3	26.8±5.9	27.0±13.0	65.7±15.8	
	$t_{1/2}$ (min)[c]	32.1±10.3	25.9±5.0	28.2±3.9	29.4±8.3	26.1±5.4	
	$AUC_{0\rightarrow t}$ (min μg/L)[d]	531±247	554±168	2255±551	1379±642	3553±1057	

*p>0.05 no significant difference between (R)-RA administrated as individual enantiomers and as a part of corresponding racemic RA.

421 [#] $p<0.05$ significant difference between 1mg/kg (R)-RA and 1mg/kg (S)-RA administrated as individual enantiomers.
422 [**] $p<0.05$ significant difference between (R)-RA and (S)-RA administrated of racemic RA.
423 [a]The time to maximum plasma concentration.
424 [b]Maximum plasma concentration.
425 [c]Half time.
426 [d]The area under the plasma concentration time curve from 0 to time.
427 [e]Plasma clearance/absorbed fraction.
428

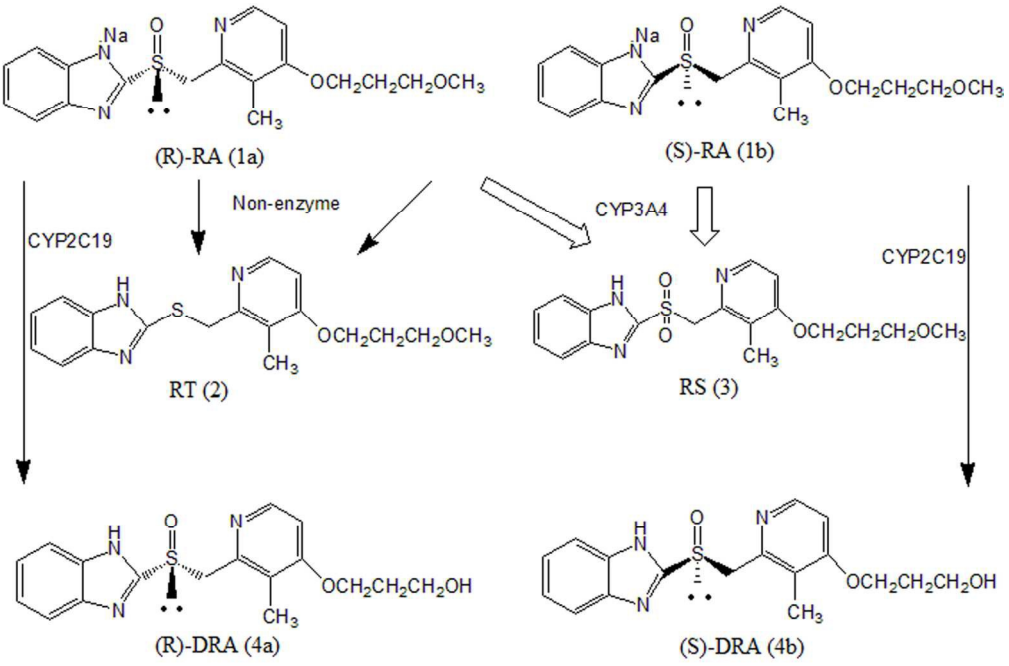

Fig.1. Metabolic pathways of enantiomers of RA.

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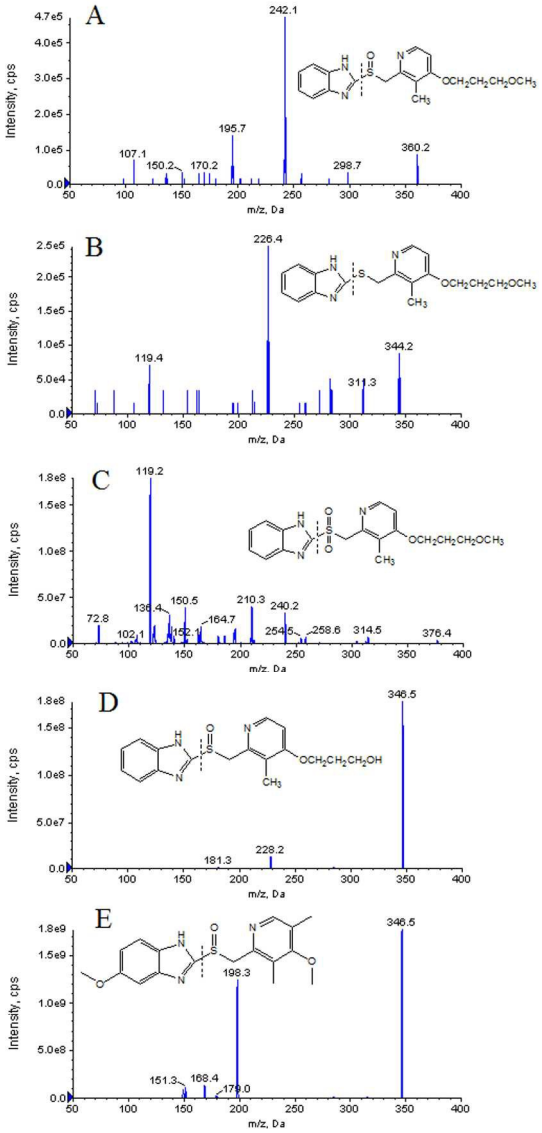

Fig.2. The product ion spectra of RA (A), RT (B), RS (C), DRA (D) and IS (E).

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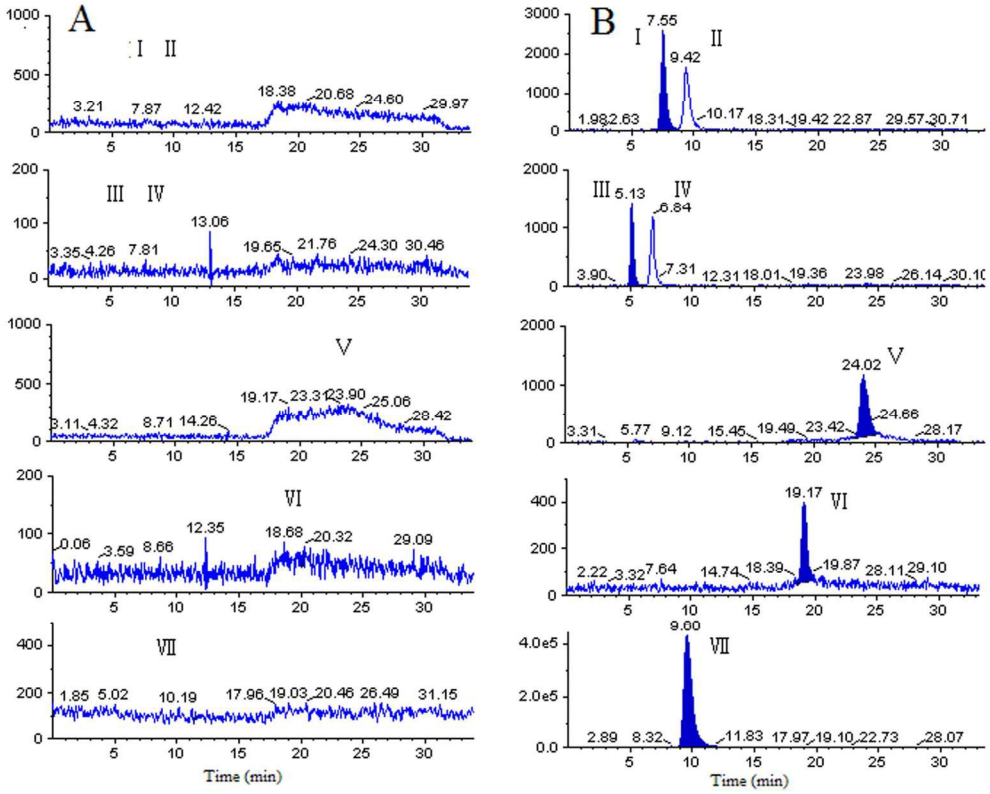

Fig.3.1 Representative MRM chromatograms of (R)-RA (Ⅰ), (S)-RA (Ⅱ), (R)-DRA (Ⅲ), (S)-DRA (Ⅳ), RT (Ⅴ), RS (Ⅵ) and IS (Ⅶ) in dog plasma samples: (A) blank plasma; (B) a plasma sample spiked with (R)-RA, (S)-RA, RT, RS, (R)-DRA , (S)-DRA at LLOQ with IS.

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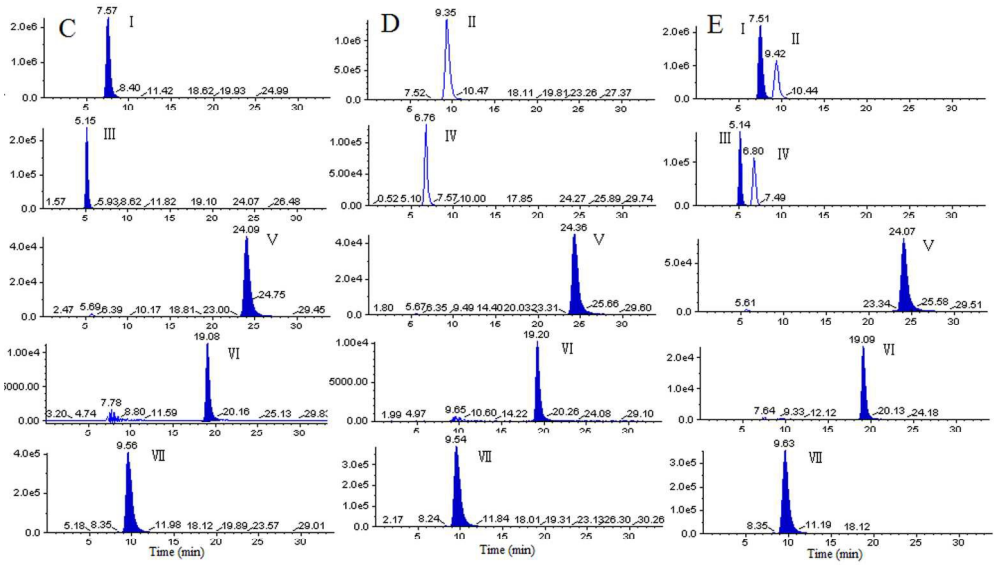

Fig.3.2 Representative MRM chromatograms of (R)-RA (Ⅰ), (S)-RA (Ⅱ), (R)-DRA (Ⅲ), (S)-DRA (Ⅳ), RT (Ⅴ), RS (Ⅵ) and IS (Ⅶ) in dog plasma samples: (C) a plasma sample obtained at 5min after intravenous administration of 1mg/kg (R)-RA, (D) 1mg/kg (S)-RA, and (E) 2mg/kg racemic RA.

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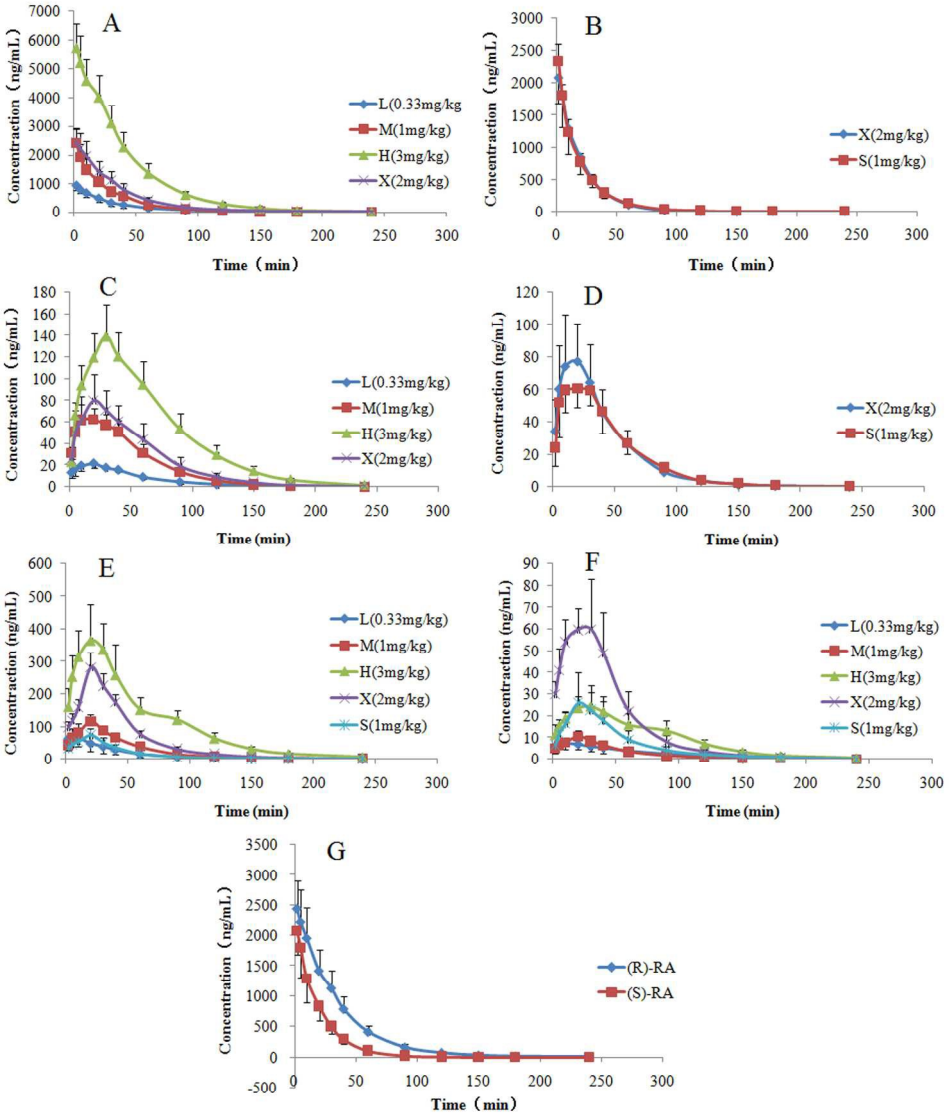

Fig.4. Plasma concentration-time profile of (R)-RA (A), (S)-RA (B), (R)-DRA (C), (S)-DRA (D), RT (E) and RS (F) after intravenous administration of 0.33mg/kg (L), 1mg/kg (M), and 3mg/kg (H) (R)-RA, intravenous administration of 2mg/kg racemic RA (X), intravenous administration of 1mg/kg (S)-RA (S) to beagle dogs; G: plasma concentration-time profile of (R)-RA and (S)-RA after intravenous administration of 2mg/kg racemic RA (n=6).

251x317mm (300 x 300 DPI)