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Quantitative analysis of racecadotril metabolite in human plasma using a liquid chromatography/tandem mass spectrometry

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Abstract

Orally administered racecadotril is rapidly hydrolyzed to the more potent enkephalinase inhibitor thiorphan *in vivo*. A sensitive and specific liquid chromatography/tandem mass spectrometry method was developed and validated to quantify thiorphan in human plasma using lisinopril as the internal standard. After a simple protein precipitation with methanol, the post-treatment samples were analyzed on a CN column interfaced with a tripe-quadruple tandem mass spectrometer using negative electrospray ionization. The method was validated to demonstrate the specificity, lower limit of quantification, accuracy, and precision of measurements. The assay was linear over the concentration range 9.38–600 ng/mL using a 5 μL aliquot of plasma. The correlation coefficients for the calibration curves ranged from 0.9985 to 0.9995. The intra- and inter-day precisions over the entire concentration were not more than 6.33%. Methanol and water (35:65, v/v) is used as the isocratic mobile phase, with 0.1% of formic acid in water. The method was successfully applied for pharmacokinetic study after a single oral administration of 200 mg racecadotril to 20 healthy volunteers.

Keywords: Racecadotril; Liquid chromatography/tandem mass spectrometry; Thiorphan

1. Introduction

Racecadotril, *N*-[(*R*,*S*)-3-acetylmercapto-2-benzylpropanoyl] glycine, benzyl ester, is a lipophilic diesterified prodrug of the enkephalinase inhibitor thiorphan. It is the first and only enkephalinase inhibitor in its class [1]. It has shown efficacy in patients with acute diarrhoea in well controlled clinical trials and was as effective as other antidiarrhoeal drugs. It was better tolerated than loperamide in both adults and children [2]. In peripheral tissues, orally administered racecadotril is rapidly hydrolyzed to the more potent enkephalinase inhibitor thiorphan, *N*-[(*R*,*S*)-1-oxo-2-mercaptomethyl-3-benzylpropanoyl] glycine [1,3,4]. (Fig. 1) The two enantiomers of thiorphan have a strong and equipotent *in vitro* affinity on the target enzyme. The corresponding prodrugs display different pharmaceutical profiles [5,6]. Indeed, the *R*-enantiomer is under clinical evaluation as an intestinal antisecretatory agent while the *S*-enantiomer is useful in the cardiovascular field [7].

To our best of knowledge, only few analytical methods have been reported for the determination of thiorphan in human plasma [8,9]. In the present work, a simple and sensitive HPLC/MS/MS method for the quantification of (±) thiorphan in human plasma, with lisinopril as the internal standard (IS), is described. Herein, the plasma sample preparation procedure was simple and rapid, requiring only precipitation of proteins with methanol which was suitable for the analysis of large batches of samples. This method was successfully applied to a pharmacokinetic study of racecadotril after an administration of 200 mg racecadotril to 20 healthy volunteers.

2. Experimental

2.1. Chemicals and reagents

Racecadotril was obtained from Jiangsu Yangtze River Pharmacy Group Company (Taizhou, China) and thiorphan and lisinopril (internal standard (IS)) from National Institute for the

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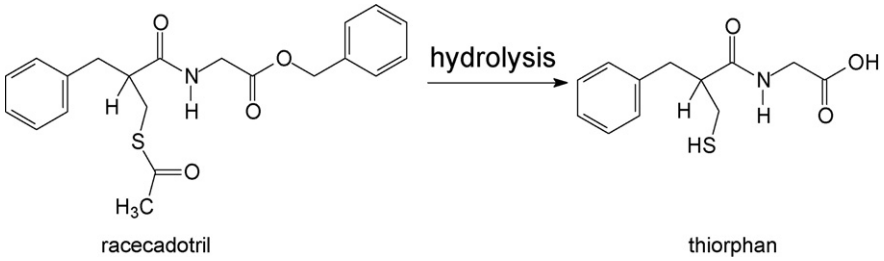

Fig. 1. Racecadotril is rapidly hydrolyzed to thiorphan in peripheral tissues *in vivo* [1,3,4].

Control of Pharmaceutical and Biological Products (Beijing, China). Methanol of HPLC-grade was obtained from Merck (Darmstadt, Germany). Formic acid of HPLC-grade was purchased from TEDIA (Fairfield, USA). Distilled water, prepared from demineralized water, was used throughout the study. Blank plasma was provided by The First Affiliated Hospital of Anhui Medical University (Hefei, China).

2.2. Instruments

A TSQ Quantum Ultra AM triple stage-quadruple tandem mass spectrometer (ThermoFinnigan), coupled with an electrospray ionization (ESI) source, a Finnigan Surveyor LC pump and Finnigan Surveyor autosampler, was used for LC/MS/MS analysis. Data acquisition was performed with Xcalibur 1.4 software (Finnigan).

2.3. LC/MS/MS conditions

Chromatographic analysis was performed using a Phenomenex Luna CN column (100 mm × 2.0 mm I.D., 3 μm; Phenomenex Company, Guangzhou, China) operating at 20 °C. A mixture of water (containing 0.1% formic acid) and methanol (65:35, v/v) was used as the isocratic mobile phase at a flow-rate of 0.2 mL/min. MS detection with the ESI source was performed in negative ion mode, using selected reaction monitoring (SRM). The precursor-fragment ion reaction for thiorphan was *m/z* 251.93 → 217.99, and for lisinopril was *m/z* 404.15 → 113.99. The product ion spectra of $[M–H]^-$ ions of thiorphan and lisinopril were shown in Fig. 2(A) and (B). In order to optimize the MS/MS parameters, standard solutions of the analyte (1000 ng/mL) and IS (6.67 μg/mL) were infused into the mass spectrometer. For both thiorphan and lisinopril, the following optimized parameters were obtained: source CID 9 V, temperature of heated capillary 340 °C electrospray voltage 3800 V; collision energy of 20 eV was used for thiorphan, and 25 eV for the IS. Argon was used as collision gas at a pressure of 1.3 mTorr. Nitrogen was used as sheath gas and auxiliary gas at the pressures (arbitrary units) of 20 and 6, respectively. Scan width for SRM is *m/z* 0.1; and scan time is 0.5 s. Peak width of Q1 and Q3 are both 0.7 FWHM.

2.4. Sample preparation

Ten microliter of cysteine saturation solution in methanol and 10 μL of IS solution (6.67 μg/mL lisinopril in methanol) were added to a 100 μL aliquot of plasma sample. The samples were briefly mixed. Then 300 μL of methanol was added to precipitate proteins. The mixture was vortex-mixed for 3 min and centrifuged for 10 min at 13,400 × *g*. The upper clean solution layer was collected, and a 5 μL aliquot of solution was injected into the LC–MS/MS system for analysis.

2.5. Calibration standards and quality control samples

A stock solution of thiorphan was prepared in methanol at the concentration of 60 μg/mL, and a stock solution of IS was prepared in methanol at the concentration of 6.67 μg/mL. Calibration curves for thiorphan were prepared by spiking blank plasma at concentrations of 9.38, 18.75, 37.5, 75, 150.0, 300.0, and 600.0 ng/mL, and the analyses were performed in triplicate for each concentration. The stock and diluted solutions of thiorphan and IS were stored at −20 °C.

The quality control (QC) samples were prepared in quintuplicate using a different stock solution of thiorphan, to obtain plasma concentrations of 18.75, 75.0, and 300.0 ng/mL, representing low, medium, and high concentration QC samples, respectively. The spiked plasma samples (standards and quality controls) were prepared fresh for each analytical batch along with the unknown samples.

2.6. Method validation

The method was validated for linearity, lower limit of quantification (LLOQ), accuracy and precision, recovery. Plasma samples were quantified using the ratio of the peak area of thiorphan to that of IS as the assay parameter. For the calibration standards, peak area ratios were plotted against thiorphan plasma concentrations, and linear standard curves were calculated using weighted least-squares ($1/x^2$) linear regression.

The LLOQ, defined as the lowest concentration at which both precision and accuracy were not more than 20%, was evaluated by analyzing samples which were prepared in quintuplicates.

To evaluate linearity, plasma calibration curves were prepared and assayed in triplicate on three separate days. Accuracy and precision were also assessed by determining QC samples using 15 quintuplicate ($n = 5$) preparations of plasma samples at three concentration levels on three separate days. The accuracy, i.e., percentage concentration deviation, was expressed by (mean observed concentration − spiked concentration)/(spiked concentration) × 100%, and the precision by relative standard deviation (RSD).

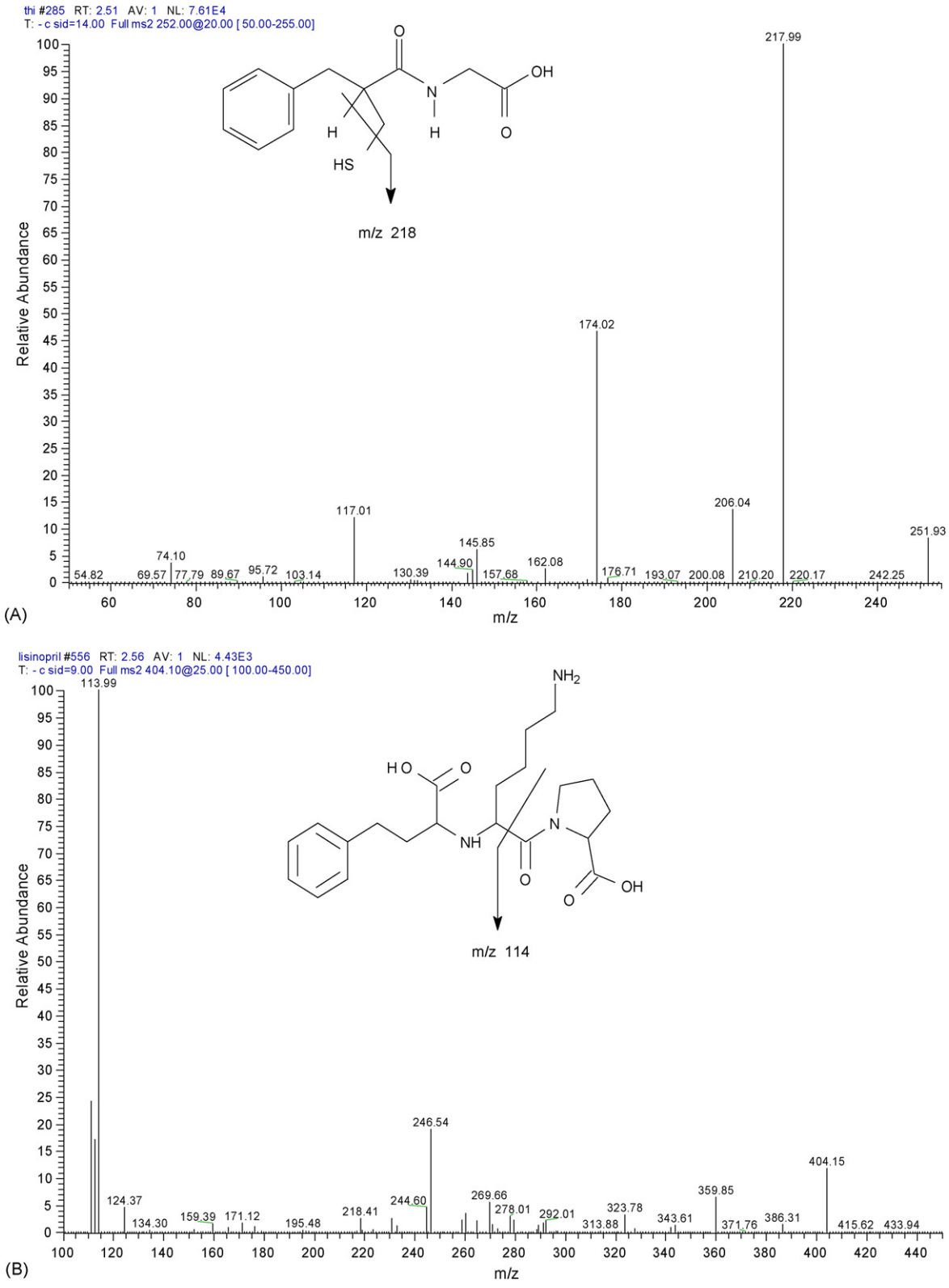

Fig. 2. (A) Product ion scan of $[M–H]^-$ ion of thiorphan. (B) Product ion scan of $[M–H]^-$ ion of lisinopril.

For the determination of recovery, blank human plasma was processed according to the sample preparation procedure described above. The supernatant were as solvent with addition of appropriate standards at concentrations corresponding to the final concentration of the pre-treated plasma samples. These spike-after-precipitation samples represented 100% recovery. The precipitation recoveries for thiorphan were determined by comparing the mean peak areas of nine precipitated low, medium

and high QC samples to mean peak areas of nine spike-after-precipitation samples at the same concentrations.

To evaluate the matrix effect in the experiment, chromatographic peak areas of each analyte from the spike-after-precipitation samples, at low and high concentration levels, were compared to those for the clean standard solutions at the same concentrations.

Thiorphan stability in plasma was assessed by analyzing QC samples at concentrations of 18.75, 75, and 300.0 ng/mL, respectively, in triplicate ($n = 3$), after exposure to different time conditions. The results were compared with those for freshly prepared QC samples, and the percentage concentration deviation was calculated. The short-term stability was evaluated after exposure of the plasma samples to room temperature (20 °C) for 4 and 12 h. The stability was also evaluated after storage of the plasma samples at −20 °C for 7 days.

2.7. Pharmacokinetic study

The developed method was applied to investigate the concentrations of thiorphan in human plasma from healthy volunteers. Twenty healthy male volunteers were selected for the study with an age range of 18–23 (20.7 ± 1.3) years and weight range of 60–71 (63.6 ± 4.8) kg. Before enrollment and at the end of the study, each volunteer underwent a physical examination and clinical laboratory tests, including bellows auscultation, palpation of liver and spleen, blood pressure, electrocardiogram, heart rate, liver function, and renal function, etc. After an overnight (more than 10 h) fast, volunteers received an oral dosage racecadotril tablets (containing 100 × 2 mg racecadotril, Jiangsu Yangtze River Pharmacy Group, China). Blood (3 ml) was removed by venepuncture into heparinized evacuated glass tubes prior to dosage and serially at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0, 16.0, and 24.0 h thereafter. Following standing for 30 min and centrifugation (3000 × g for 10 min) the plasma was removed and stored at −20 °C until analysis.

3. Results and discussion

3.1. Sample preparation

Thiorphan has hydrosulfide group which was unstable and oxidated easily in human plasma. The different concentration and volume of vitamin C and cysteine solution were tried as antioxidant. It was found that 10 μL cysteine saturation solutions as antioxidant can slower the rate of thiorphan oxidation effectively.

Protein precipitation was chosen for a simple and rapid sample preparation method. The method can save much time and simplify the operating process. Different reagents were evaluated for efficiency of protein precipitation; it was found that three times methanol of the plasma volume can precipitate the plasma proteins completely, and the chromatographic behavior of the analytes was not deteriorated by this procedure.

3.2. LC/MS/MS conditions

Thiorphan has both hydrosulfide and carboxyl group in its structure. The carboxyl group readily loses a proton to produce a negative ion. The signal intensities obtained in negative ion mode were much higher than those in positive ion mode. Because of thiorphan strong polarity, ESI yielded higher signals for m/z 251.93 compared to APCI. The collision-induced dissociation of the $[M–H]^-$ precursor ion of thiorphan at m/z 251.93 produced an abundant product ion at m/z 217.99 at the optimum collision energy of 20 eV. The product ion mass spectrum of thiorphan is shown in Fig. 2(A). Similarly, the collision-induced dissociation of the $[M–H]^-$ precursor ion of lisinopril at m/z 404.15 produced an abundant product ion at m/z 113.99 at the optimum collision energy of 25 eV, and its product ion mass spectrum is shown in Fig. 2(B). And source CID collision energy of 9 eV further improved the sensitivity.

In optimizing the LC system, four C18 columns (150 mm × 2.1 mm I.D. 5 μm) were tried: Hypersil Gold, BetaBasic-18, Cosmosil, and Luna. Chromatographic analysis was performed using a Phenomenex Luna CN column. To achieve maximum peak responses and symmetrical chromatographic peaks, mobile phases containing varying percentages of organic phase were tested. As a result, methanol and water (35:65, v/v) was chosen as the optimized mobile phase, with 0.1% of formic acid in the water.

A typical chromatogram for blank human plasma is shown in Fig. 3(A); no endogenous materials interfere directly in the SRM channels for thiorphan and IS. A fast liquid chromatographic approach was used to elute thiorphan and lisinopril, giving retention times of 2.90 and 2.25 min. Fig. 3(B) shows the chromatogram of blank human plasma spiked with 300 ng/mL thiorphan and 667 ng/mL lisinopril. Fig. 3(C) showed the chromatogram for a volunteer plasma sample 1.0 h after an oral dose of 200 mg racecadotril.

3.3. Linearity, precision, and accuracy

Correlation coefficients >0.99 confirmed that the calibration curves were linear over the concentration range 9.38–600 ng/mL for the analyte. A typical standard curve was $y = 0.0095x–0.0196$. The correlation coefficients for the calibration curves ranged from 0.9985 to 0.9995. Both precision and deviations from nominal concentration (accuracy) were not more than 6.06% for the lower QC concentration (18.75 ng/mL). Table 1 shows the intra- and inter-assay precision and accuracy for thiorphan from QC samples. The intra- and inter-assay relative standard deviations were both measured to be not more than 6.33%. These results demonstrated that the values were within the acceptable range and the assay was accurate and precise.

The lower limit of quantification (LLOQ) was confirmed to be 0.15 ng/mL and the calculated accuracy and precision were below 20%.

3.4. Recovery, matrix effect, and storage stability

A simple one-step protein precipitation procedure was introduced to extract analytes from plasma. The mean recov-

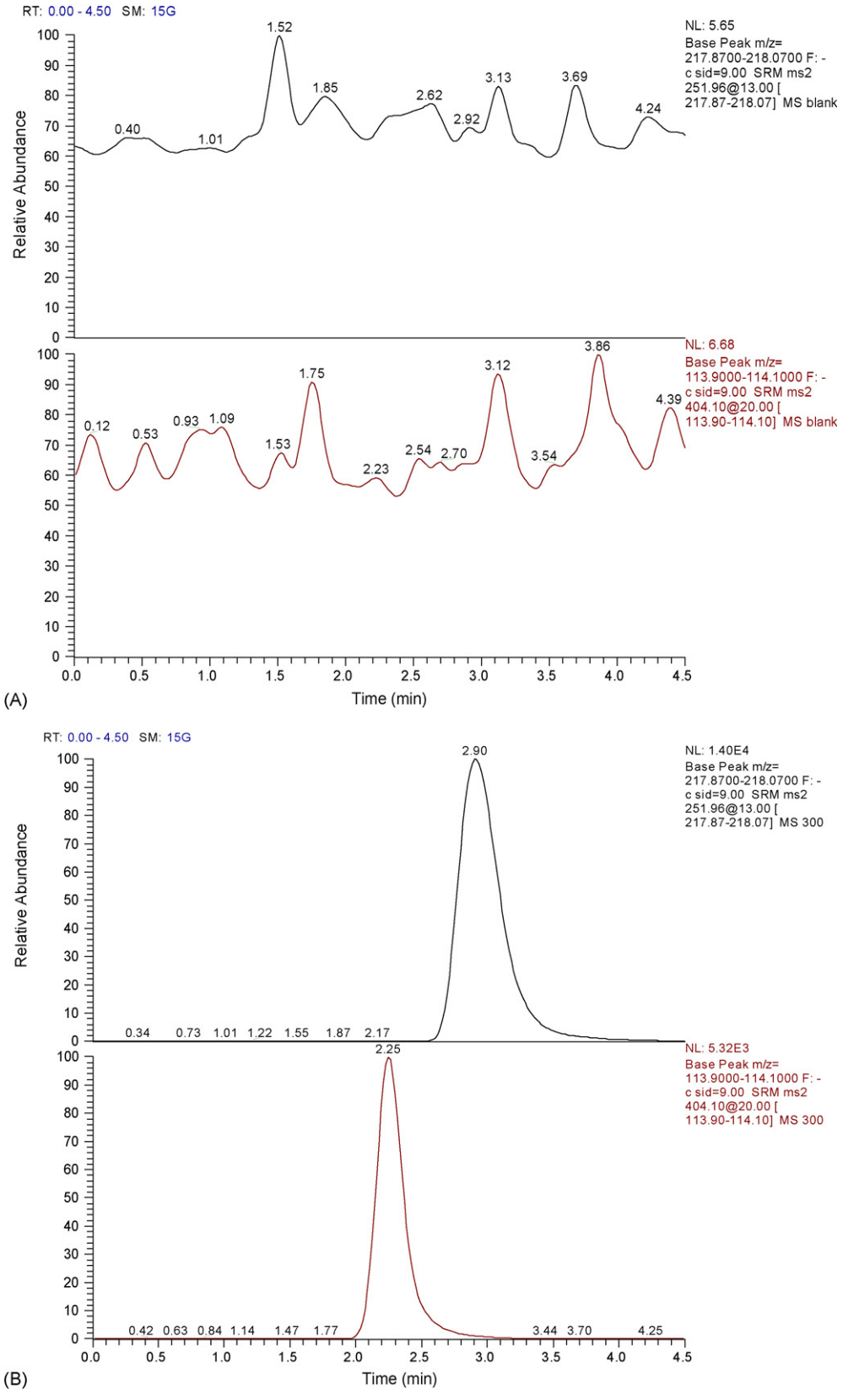

Fig. 3. SRM chromatograms for thiorphan (top) and lisinopril (IS, bottom) in human plasma samples. (A) Blank plasma; (B) plasma sample spiked with thiorphan (300 ng/mL) and IS (667 ng/mL); (C) a volunteer plasma sample 1.0 h after oral dose of 200 mg racecadotril.

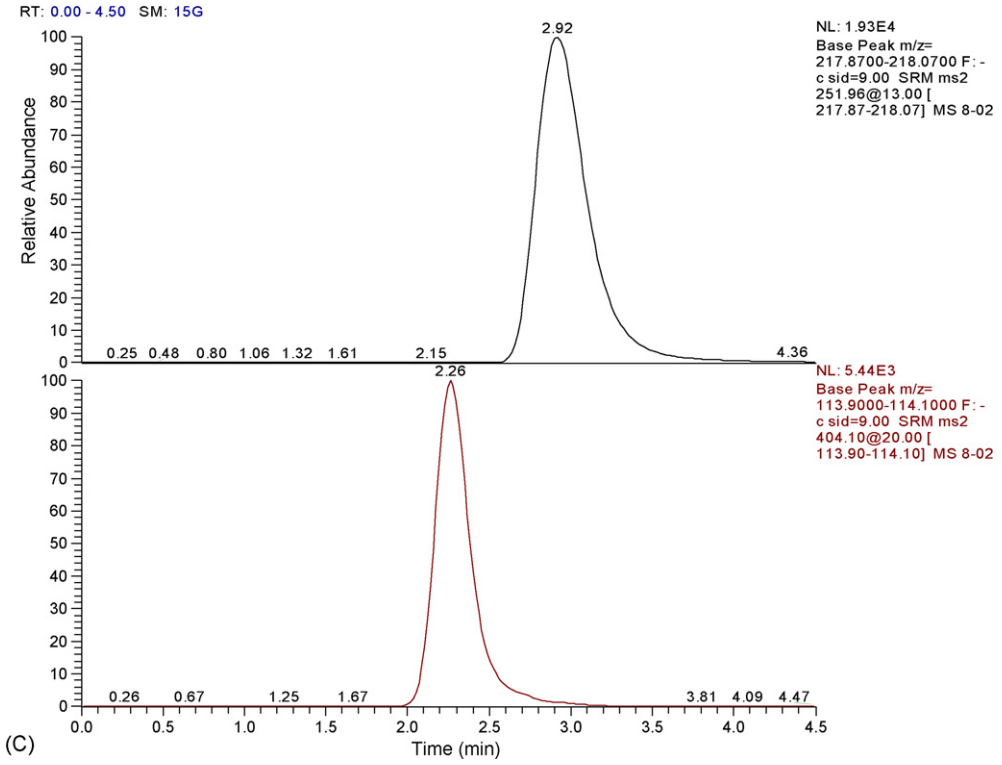

Fig. 3. (*Continued*).

Table 1
Accuracy and precision for the analysis of thiorphan in human plasma (in prestudy validation, 3 days, 15 quintuplicates per day, $n = 5$)

Added C (ng/mL)	Found C (ng/mL)	Intra-run RSD (%)	Inter-run RSD (%)	RE (%)
18.75	19.77 ± 1.20	4.16	6.06	5.44
75.00	76.80 ± 3.99	6.33	5.19	2.4
300.00	307.21 ± 13.20	5.53	4.30	2.4

eries of thiorphan were (76.40 ± 4.56)%, (79.27 ± 4.04)% and (78.37 ± 3.05)% at concentrations of 18.75, 75, and 300 ng/mL, respectively ($n = 5$).

Matrix effects were investigated by analysis of spike-after-precipitation samples with pure standard solutions at the same concentrations. The results were 92.8–103.2% for thiorphan and 90.6–101.5% for lisinopril.

The stability of thiorphan prepared sample in the Finnigan Surveyor autosampler (20 °C) was determined for 4 and 12 h. The sample concentrations following the stability test period were compared with freshly prepared QC samples. The prepared sample was stable for 4 h in room temperature (20 °C) and the coefficient of variation was within 3.6%. But the sample wasn't stable for 12 h in room temperature (20 °C) and the coefficient of variation was more than 15%, especially for low concentration sample which coefficient of variation was 24.65%. The methanol solution of IS (6.67 μg/mL) proved stable at room temperature (20 °C) for 12 h. So the prepared sample was suggested to be detected within 4 h at room temperature (20 °C). The plasma sample was also stable for 7 days at −20 °C.

3.5. Pharmacokinetic study

The method was applied to determine the plasma concentrations of thiorphan after oral administration of 200 mg racecadotril to 20 volunteers. The mean plasma concentration–time curve of thiorphan is shown in Fig. 4. The PK parameters

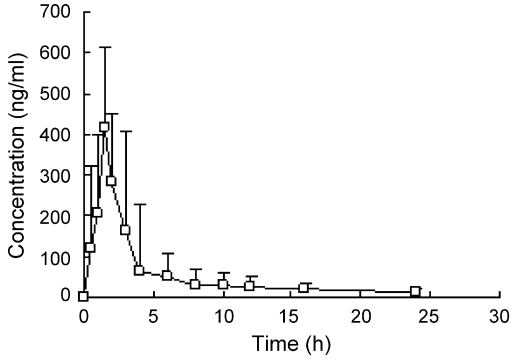

Fig. 4. Mean plasma concentration–time curve for thiorphan after an oral administration of 200 mg racecadotril to 20 healthy volunteers ($n = 20$, mean value and SD are plotted).

were received as follows: the maximum plasma concentration (C_{max}) 519.77 ± 201.97 ng/mL; area under the curve ($AUC_{0–24}$) 2113.70 ± 878.81 ng h/mL; the time to maximum plasma concentration (T_{max}) 1.35 ± 0.92 h; half-life ($t_{1/2}$) 6.14 ± 2.55 h. The biological half-life of enkephalinase activity is 3 h in the literature [2]. It is different from the result reported in our study. The following reasons can cause this difference: race, additive materials except racecadotril in the proceeding of medical manufacture, diet and etc. Because of the relatively short chromatographic run time (4.5 min) and simple sample preparation procedure, a sample throughput of 100 per day was routinely achieved. This simple and selective method for the determination of thiorphan in human plasma was readily applicable to the clinical pharmacokinetic study for racecadotril.

4. Conclusions

A simple and sensitive LC/MS/MS method for the quantification of racecadotril metabolite-thiorphan in human plasma was developed and validated with respect to linearity, precision and accuracy, and analysis of real samples was demonstrated. The method needed only a simple protein precipitation procedure, which reduced the preparation time and allowed the quantification of thiorphan in plasma for concentrations ranging from 9.38 to 600 ng/mL using 0.1 mL of plasma. The method satisfied the requirements of high sensitivity, selectivity, and high throughput for pharmacokinetic studies.

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