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Original Research Article

Separation and determination of acetyl-glutamine enantiomers by HPLC–MS and its application in pharmacokinetic study

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ABSTRACT

A high-performance liquid chromatography coupled with mass spectrometry (HPLC–MS) method was established for the separation and determination of acetyl-glutamine enantiomers (acetyl-L-glutamine and acetyl-D-glutamine) simultaneously. Baseline separation was achieved on Chiralpak AD-H column (250 mm × 4.6 mm, 5 μm). *n*-Hexane (containing 0.1% acetic acid) and ethanol (75:25, v/v) were used as mobile phase at a flow rate of 0.6 mL/min. The detection was operated in the negative ion mode with an ESI source. $[M-H]^-$ *m/z* 187.0540 for enantiomers and $[M-H]^-$ *m/z* 179.0240 for aspirin (IS) were selected as detecting ions. The linear range of the calibration curve for each enantiomer was 0.05–40 μg/mL. The precision of this method at concentrations of 0.5–20 μg/mL was within 7.23%, and the accuracy was 99.81%–107.81%. The precision at LOQ (0.05 μg/mL) was between 16.28% and 17.56%, which was poor than that at QC levels. The average extraction recovery was higher than 85% for both enantiomers at QC levels. The pharmacokinetics of enantiomers was found to be stereoselective. There was not chiral inversion in vivo or in vitro between enantiomers.

1. Introduction

Acetyl-glutamine (A-Gln) is a central nervous system stimulant which can directly pass through the blood-brain barrier. A-Gln is considered as glutamine (Gln) substitution and becoming indispensable in some diseases and injury treatment [1–3]. Although Gln serves as a direct building block for protein synthesis and supports the tricarboxylic acid cycle, it can undergo degradation into ammonia which induces cell toxicity [3,4]. Previous studies indicate that A-Gln seems to have a similar pharmacological function to Gln, while it does not produce toxic degradation product in solution. A-Gln has a chiral center, and the structures of enantiomers are given in Fig. 1. Great interests have been focused on A-Gln injection related substances detection [5–7]; however, the separation and determination of the enantiomers have not been studied. The U.S. Food and Drug Administration (FDA) now requires the evaluation of enantiomers as well as the racemic mixtures of a chiral drug before clinical use. A-Gln was approved in clinical use about 50 years ago, but the techniques for enantiomers separation were not well developed, and the selective pharmacokinetic study was almost nonexistent. Pharmacokinetics of enantiomers may be stereoselective [8–12], and chiral inversion may occur [13–16] in vivo or in vitro; hence the pharmacokinetic and chiral inversion studies of enantiomers become necessary.

The high-performance liquid chromatography coupled with mass spectrometry (HPLC–MS) method based on the chiral stationary phase (CSPs) was established to separate and determine enantiomers. Based on the optimization of operation parameters, a rapid, selective and sensitive method was developed and validated. The method was used for pharmacokinetic studies of enantiomers in rat plasma after caudal intravenous administration.

2. Materials and methods

2.1. Reagents and chemicals

HPLC grade acetonitrile, methanol and *n*-hexane were purchased from Merck KGA (Darmstadt, Germany). Ethanol (HPLC grade) was purchased from Tedia Company (Fairfield, USA). Acetic acid, formic acid, ammonium hydroxide, ethyl acetate, diethyl ether and *n*-butyl alcohol were of analytical grade and purchased from Nanjing Chemical Reagents Co., Ltd. (Nanjing, China). Perchloric acid was purchased from JinLu Chemical Co., Ltd. (Nanjing, China).

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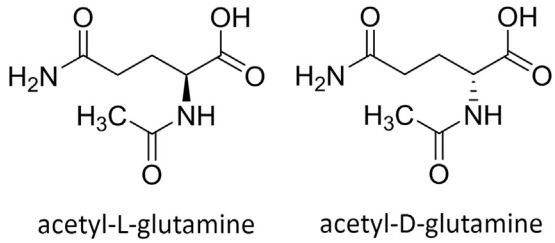

Fig. 1. Chemical structures of acetyl-glutamine enantiomers.

The pure compound of acetyl-L-glutamine (L, batch number: 100859-200501, purity: 99.9%) was obtained from National Institutes for Food and Drug Control (Beijing, China); the pure compound of acetyl-D-glutamine (D, batch number: 20150122, purity ≥ 99.0%) was obtained from Shenzhen Boxing Synthesis Technology Co., Ltd. (Shenzhen, China); the pure compound of aspirin (internal standard, IS, purity: 99.9%) was obtained from National Institutes for Food and Drug Control (Beijing, China).

2.2. Instrumentation and chromatographic condition

2.2.1. Liquid chromatography

Liquid chromatography was performed using a Shimadzu HPLC-IT-TOF/MS system consisting of a controller (CBM-20A), two LC-30AD pumps, a SIL-30AC auto-sampler and a CTO-20AC column heater. The separation was operated on Chiralpak AD-H column (250 mm × 4.6 mm, 5 μm) using *n*-hexane (containing 0.1% acetic acid):ethanol (75:25, v/v) as mobile phase. The column temperature was maintained at 30 °C; the flow rate was 0.6 mL/min; the injection volume was 5 μL; the auto-sampler temperature was 10 °C. The LCMSsolutionVer3 LC-MS software (Shimadzu, Kyoto, Japan) was used to control instruments and process the data.

2.2.2. Mass spectrometry

Enantiomers and IS were analyzed in the negative-ion with an electrospray ionization (ESI) source. The $[M-H]^-$ 187.0540 for enantiomers and $[M-H]^-$ 179.0240 for IS were selected as detecting ions. Nitrogen (99.9% purity) and argon (99.9% purity) were used as cone and collision gases, respectively. The main operation parameters were set as follows: nebulizer gas flow rate, 1.5 L/min; spray voltage, −3 kV for negative ion; curved desorption line (CDL) temperature, 200 °C heat block temperature, 200 °C drying gas (nitrogen, 99.9% purity), 100 kPa; detector voltage, 1.85 kV; and ion accumulated time, 30 ms.

2.3. Preparation of standard solution

Standard stock solutions of enantiomers and IS were prepared by weighting approptiate amount of enantiomers and IS and dissolving in methanol to obtain the concentration of 1 mg/mL, respectively. Working solutions of enantiomers and IS (10 μg/mL) were prepared by diluting stock solutions with methanol, respectively. All solutions were stored at 4 °C and brought out to room temperature before analysis.

2.4. Sample preparation

Sample preparation was performed by protein precipitation. Aliquots of 50 μL plasma and 5 μL IS working solution were put into a centrifuge tube, and 350 μL acetonitrile (containing 5% ammonium hydroxide) was added, and then vortex-mixed for 5 min. After centrifuged at 16,000 rpm under 4 °C for 10 min, the supernatant was transferred into another tube and evaporated till dry under a gentle stream of nitrogen at 37 °C in a water bath. The residue was redissolved in 100 μL methanol, then vortex-mixed for 2 min and centrifuged at 16,000 rpm under 4 °C for 10 min. The supernatant was injected into the HPLC–MS system for analysis.

2.5. Calibration curve and quality control (QC) samples

Calibration curves were prepared by spiking blank rat plasma with appropriate amount of enantiomers working solutions, and the final plasma concentration levels were 0.05, 0.1, 0.5, 5, 10, 20, and 40 μg/mL. Then samples were extracted as described in sample preparation. QC samples used in the method validation and pharmacokinetic studies were prepared by diluting corresponding working solutions with blank rat plasma, producing enantiomers concentration equivalent to 0.5, 5 and 20 μg/mL.

2.6. Method validation

The method was validated according to the bio-analytical method validation guidance accepted by FDA [17].

2.6.1. Specificity

Blank plasma used to assay the specificity of the method was obtained from six different rats. It was assessed by comparing the chromatograms obtained from blank plasma with those from the sample spiked with enantiomers and IS.

2.6.2. Linearity, limit of quantitation (LOQ) and limit of determination (LOD)

Linearity was achieved at concentration levels of 0.05, 0.1, 0.5, 5, 10, 20, and 40 μg/mL. Then the sample processing was the same as described in sample preparation. Calibration curves were fitted to the equation y=bx+a where x represents the plasma concentration of enantiomer, and y refers to the peak area ratio of enantiomer to IS dependent on the plasma concentration.

The LOQ was assessed by repeatedly analyzing the spiked rat plasma samples in five replicates and determined as the concentration with a signal-to-noise ratio of 10. Standard calibrators at LOQ should not deviate by more than 20%. The LOD was measured based on signal-to-noise ratio at 3.

2.6.3. Precision and accuracy

The intra-batch precision and accuracy were measured by analyzing five replicates plasma samples spiked with enantiomers at each QC level in a batch, and the inter-batch precision and accuracy were determined by analyzing three replicate plasma samples spiked with enantiomers at each QC level in three consecutive batches on different days. The precision (expressed as relative standard deviation (RSD)) and accuracy (expressed as the percentage of the actual concentration over the theoretical concentration) should not exceed 15%.

2.6.4. Extraction recovery

The extraction recovery of enantiomers was determined by comparing corresponding peak area ratios of enantiomer to IS obtained from extracted plasma samples (As-r, n=5) with those from blank plasma extracts spiked with parallel concentration solutions (Ar-r, n=2). This was investigated at three different concentration levels (0.5, 5 and 20 μg/mL).

The extraction recovery of IS was determined by comparing the peak area of IS obtained from extracted plasma samples (As-r, n=5) with that of blank plasma extracts spiked with the IS (Ar-r, n=2).

2.6.5. Stability

The stability of enantiomers in plasma sample during storage and sample processing was investigated by analyzing triplicate at QC levels. All analyte concentrations in the stability study samples were measured using freshly prepared stock solutions and calibrators. Freshly pre-

pared sample was measured immediately after sample extraction. The short-time stability was evaluated at room temperature for 12 h. The residue stability was conducted by analyzing re-dissolved residue after placed at 4 °C for 12 h. The post-preparative stability was determined by analyzing extracted samples placed in the auto-sampler at 10 °C for 12 h. The freeze-thaw stability was assessed by analyzing sample undergoing freeze (−20 °C, 12 h) and thaw (room temperature) cycles for two times. The accuracy should be in the range of 80%–120% and the precision should be less than 15% for all samples at QC levels.

2.6.6. Matrix effect

The matrix effect was carried out to assess ionization interference from co-eluted endogenous compounds with the analyte. It was evaluated by comparing the peak areas of enantiomers or IS spiked with the post-extraction residue of the blank plasma with those obtained by injection of working solutions at the same concentration. This procedure was conducted in five replicates at QC levels. If the ratio was between 115% and 85%, the matrix effect was negligible.

2.6.7. Dilution integrity

The dilution of plasma sample was required if enantiomer concentration in plasma was higher than the upper limit of quantification (ULOQ). Dilution integrity experiments were carried out by a 10-fold dilution of plasma sample (300 μg/mL) with blank plasma. Dilution of high concentration samples was considered acceptable if precision and accuracy of replicate (n=5) values varied by less than 15%.

2.7. Method applications

2.7.1. Pharmacokinetic studies

The pharmacokinetic studies of enantiomers were evaluated by using twelve healthy male SD (Sprague-Dawley) rats (200–220 g), obtained from the Qinglongshan Experimental Animal Center (Nanjing, China), the license number: SCXK (Shanghai) 2013-0016. The Animal Ethic Committee of China Pharmaceutical University approved all animal studies. After a 12 h overnight fasting, each rat received caudal intravenous administration of 60 mg/kg of enantiomer normal saline solution (20 mg/mL, L or D). Blood samples (0.3 mL) were collected from the orbital venous plexus into heparinized tubes at 0 h (pre-dose), and 0.03, 0.083, 0.25, 0.50, 1.00, 1.50, 2.00, 3.00, 4.00, 5.00, and 6.00 h after drug administration. The plasma was obtained by centrifugation of blood at 10,000 rpm, 4 °C for 10 min, and then stored at − 80 °C until analysis.

2.7.2. Liver microsomal incubation experiments

Liver microsomal incubation experiments were to study whether there was chiral inversion in vitro [13,14]. The method was validated using incubation medium and all data were met the requirements of biological sample testing.

10 μL enantiomer working solution was taken in a centrifuge tube, and evaporated till dry at 37 °C under a gentle stream of nitrogen. Aliquots of 20 μL liver microsomal (1 mg/mL) and 140 μL phosphate buffer solutions (pH 7.4) were added. After pre-incubated for 5 min, 40 μL NADPH enzymes (consisting of 10 mM glucose-6-phosphate, 0.5 mM $NADP^+$, 1 U/mL glucose-6-phosphate dehydrogenase, and 10 mM magnesium chloride) was added. The reaction was terminated by adding 1.4 mL acetonitrile (5% ammonium hydroxide) after incubated for 3 h and vortex-mixed for 5 min. Control samples were prepared as described above without liver microsomal. All incubations were performed at 37 °C in a water bath.

2.8. Pharmacokinetic and statistical analysis

Pharmacokinetic analysis was performed by using Drug and Statistics (DAS) 2.0 (Mathematical Pharmacology Professional Committee of China, Shanghai, China) as described. The statistical significance of the results was validated by GraphPad Prism 6.0. Pharmacokinetic parameters of enantiomers were estimated using the non-parametric model analysis. The one-way ANOVA was used for statistical test.

3. Results and discussion

3.1. HPLC–MS method development

In order to develop a rapid and sensitive analytical method for separation and determination of enantiomers, the chromatography separation conditions and MS detection parameters were optimized.

3.1.1. Chromatographic condition optimization

In this study, the Chiralpak AD (Chiralpak AD-RH and Chiralpak AD-H column), which was based on amylose tris (3, 5-dimethylphenylcarbamate), was investigated. Enantiomers separation was achieved by using n-

Table 1
Resolution (R) and relative retention time of enantiomers on Chiralpak AD-H under different conditions.

Flow rate (mL/min)	Ethanol (%)	Acetic acid (%)	Column temperature (°C)	Resolution (R)	Relative retention time
0.6	15	0.1	30	4.288	1.40
0.8	15	0.1	30	4.520	1.40
1	15	0.1	30	5.282	1.36
1.2	15	0.1	30	3.664	1.40
1	10	0.1	30	7.131	1.43
1	20	0.1	30	4.327	1.30
1	30	0.1	30	3.504	1.19
1	15	0.2	30	4.108	1.40
1	15	0.1	20	4.702	1.59
1	15	0.1	40	5.282	1.36

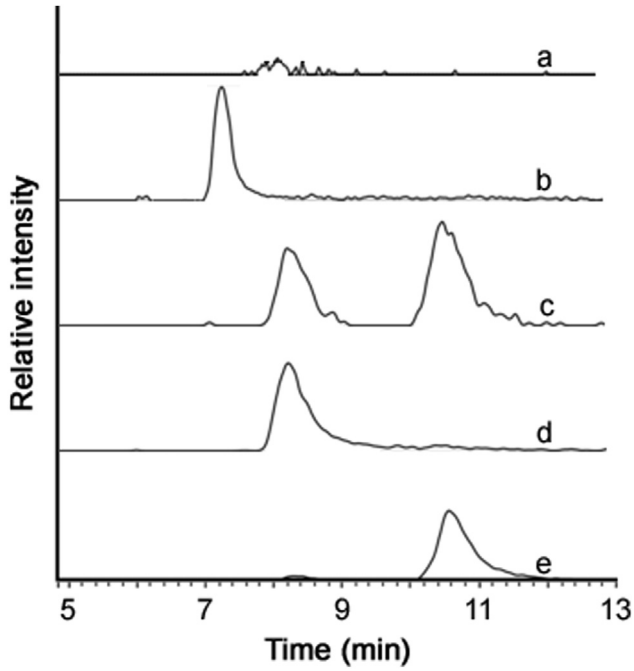

Fig. 2. Representative chromatograms of enantiomers and the IS resulting from analysis of (a) blank rat plasma, (b) blank rat plasma spiked with IS, (c) blank rat plasma sample spiked with enantiomers at LOQ, (d) rat plasma sample obtained after intravenously administration of acetyl-L-glutamine and (e) rat plasma sample obtained after administration of acetyl-D-glutamine.

Table 2
The inter- and intra-batch accuracy and precision of the method for the determination of enantiomers.

Compound	Concentration (μg/mL)	Intra-batch (n=5)		Inter-batch (n=9)	
		Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
Acetyl-L-glutamine	0.5	103.91	7.23	103.48	6.29
	5	99.81	4.03	100.15	3.72
	20	107.81	6.96	107.30	6.07
	LOQ	97.11	16.28		
Acetyl-D-glutamine	0.5	103.26	4.02	101.28	4.95
	5	102.16	6.16	101.86	5.60
	20	101.44	5.37	100.17	5.89
	LOQ	89.33	17.56		

hexane (containing 0.1% acetic acid):ethanol as mobile phase. The separation mechanism of Chiralpak AD-H is to form temporary diastereomeric analyte-CSP complexes through hydrogen bonding using C=O and NH groups, and dipole-dipole interaction using C=O moiety [18–20].

3.1.2. Robustness

Robustness was determined to evaluate the small influence in the chromatographic conditions by changing flow rate, mobile phase composition, and column temperature. The retention and resolution between two enantiomers were used to evaluate the separation under modified conditions. The resolution of enantiomers was higher than 3.5 under all conditions (Table 1).

3.1.3. Mass spectrometric condition optimization

In order to improve the sensitivity and reduce the retention time, HPLC–MS method was optimized by changing the proportion of mobile phase and flow rate. A better separation was achieved within 13 min; the retention time of L and D was 8.47 and 10.83 min, respectively.

Mass spectrometry conditions were optimized in order to achieve stable response. An ESI ionization source was selected. Both the positive and negative ionization modes were investigated. Better response was obtained in negative ion mode. Detector voltage, spray voltage, and ion accumulated time were also optimized.

3.2. Extraction optimization

Liquid-liquid extraction (LLE) and protein precipitation were both tried for sample preparation. For LLE, solvents such as ethyl acetate, diethyl ether and n-butyl alcohol were investigated. The extraction recovery was below 50% for enantiomers. Methanol and acetonitrile were all attempted as precipitating reagents. Acetonitrile was selected for higher extraction recovery. Formic acid, acetic acid and ammonium hydroxide (1%, 5% or 10%) in acetonitrile were tried. Ammonium hydroxide (5%) was chosen for higher extraction recovery. The influence of extraction reagent dosage and vortex time was studied. Under the normal phase conditions, water should be strictly prohibited. The supernatant was evaporated till dry under a steady stream of nitrogen in a water bath at 37 °C. The residues re-dissolved in methanol, acetonitrile, ethanol and mobile phase were tested, and methanol was selected due to the maximum response.

3.3. Method validation

3.3.1. Specificity

The specificity of the method was investigated by analyzing blank rat plasma samples, and the plasma spiked with enantiomers and IS. The endogenous substances of blank plasma samples were not interferences in the analyte (Fig. 2).

Table 3
The extraction recovery of enantiomers at different concentration levels (n=5).

Compound	Concentration (μg/mL)	Mean (%)	RSD (%)
Acetyl-L-glutamine	0.5	89.08	7.50
	5	88.20	8.49
	20	92.69	3.35
Acetyl-D-glutamine	0.5	85.73	7.93
	5	95.59	7.78
	20	88.62	4.94
IS	10	80.84	3.81

3.3.2. Linearity and LOQ

The calibration curves were prepared by assaying standard rat plasma samples at seven concentration levels of 0.05, 0.1, 0.5, 5, 10, 20, and 40 μg/mL. The calibration curves for enantiomers are described by the equation: C=6.9794A–0.2933 (r^2=0.9908, L and C=5.7852A–0.0296 (r^2=0.9926, D), where A corresponds to the peak area ratio of enantiomer to IS, and C refers to the concentration of enantiomer in plasma.

The LOQ of enantiomers was 0.05 μg/mL. Fig. 2 shows the chromatogram of LOQ for enantiomers with the signal-to-noise ratio exceeding 10. The deviation was within 20% at LOQ (Table 2). The LOD of enantiomer was 0.025 μg/mL.

3.3.3. Precision and accuracy

The precision and accuracy data of the method are listed in Table 2. The precision for enantiomers was within 7.23% at all QC levels. The mean accuracy for enantiomers was within the range of 99.81%–107.81%. These results indicated that this method had good accuracy and precision.

3.3.4. Extraction recovery

The extraction recovery of enantiomers and IS in plasma was consistent and reproducible. All data are listed in Table 3. Results showed that the mean extraction recovery for enantiomers and IS was more than 80.84%.

3.3.5. Stability

The results of stability tests of freshly prepared samples, short-time, post-preparation, residue and freeze-thaw are shown in Table 4. The results indicated that enantiomers were stable in plasma during storage and sample processing.

3.3.6. Matrix effect

The mean matrix effects for enantiomers at low, medium and high levels were between 10.71% and 19.02% (Table 5). Data suggested that endogenous compounds co-eluted could not produce obvious ion suppression or enhancement.

Table 4
Stability of enantiomers under different conditions (n=3).

Stability condition	Concentration (μg/mL)	Acetyl-L-glutamine			Acetyl-D-glutamine		
		Accuracy (%)	SD	RSD (%)	Accuracy (%)	SD	RSD (%)
Freshly prepared	0.5	105.35	4.51	4.28	101.46	3.55	3.50
	5	102.17	2.74	2.68	102.81	6.74	6.55
	20	108.24	6.33	5.85	100.40	7.15	7.12
Post-preparative stability (10 °C, 12 h)	0.5	98.17	4.57	4.65	94.15	4.11	4.36
	5	94.83	2.52	2.66	98.43	3.31	3.36
	20	96.24	3.91	4.06	96.98	4.22	4.35
Short-term stability (room temperature, 12 h)	0.5	91.51	4.82	5.27	99.40	7.52	7.57
	5	92.05	4.03	4.38	97.00	3.21	3.31
	20	94.87	9.83	10.36	91.13	6.17	6.77
Residue stability (4 °C, 12 h)	0.5	100.27	5.42	5.41	97.51	9.83	10.08
	5	100.18	3.53	3.52	99.49	5.79	5.82
	20	100.20	8.26	8.35	102.24	3.15	3.11
Freeze-thaw stability (two cycles)	0.5	105.88	3.70	3.49	103.62	3.43	3.31
	5	102.07	5.19	5.08	105.68	5.30	5.02
	20	105.96	7.00	6.61	105.63	8.68	8.22

Table 5
The matrix effect of enantiomers at different concentration levels (n=5).

Compound	Concentration (μg/mL)	Mean (%)	RSD (%)
Acetyl-L-glutamine	0.5	15.67	5.13
	5	17.71	5.45
	20	11.38	4.94
Acetyl-D-glutamine	0.5	19.02	7.25
	5	10.71	7.26
	20	12.37	5.66
IS	10	14.19	4.20

3.3.7. Dilution integrity

For diluted samples, the accuracy was 102.65% ± 2.70% (L) and 96.92% ± 3.56% (D), and the precision was within 4.34% for both enantiomers (Table 6). The results suggested that plasma samples whose concentrations exceed the ULOQ can be determined by an appropriate dilution.

3.4. Method applications

3.4.1. Pharmacokinetic studies

The mean concentration of enantiomers in plasma sample versus time plot is shown in Fig. 3. The main pharmacokinetic parameters of enantiomers are presented in Table 7. After administration of one enantiomer, another enantiomer could not be detected in plasma. This indicated that chiral inversion did not occur in vivo. The $AUC_{0\text{-}t}$ and $AUC_{0\text{-}\infty}$ of L were significantly larger than that of D (P=0.0127 and P=0.0104). The results showed that the pharmacokinetics of enantiomers after intravenous administration was stereoselective.

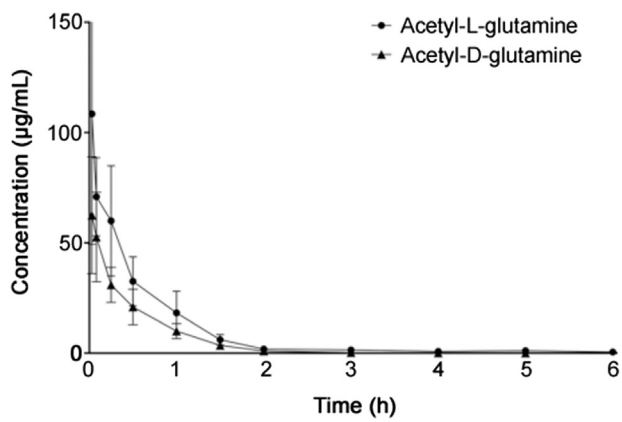

Fig. 3. Plasma concentration-time curves of enantiomers in rat plasma after caudal intravenous administration of enantiomer at a dose of 60 mg/kg.

Table 6
The dilution integrity of the method for the determination of enantiomers (10-fold dilution).

Compound	Measured concentration (μg/mL)	Theoretical concentration (μg/mL)	Accuracy (%)	Mean±SD (%)	RSD (%)
Acetyl-L-glutamine	29.58	295.77	98.59	102.65 ± 2.70	3.27
	30.87	308.72	102.91		
	31.90	318.96	106.32		
	29.99	299.94	99.98		
	31.64	316.40	105.47		
Acetyl-D-glutamine	30.25	302.53	100.84	96.92 ± 3.56	4.34
	28.06	280.64	93.55		
	27.42	274.18	91.39		
	29.61	296.06	98.69		
	30.04	300.36	100.12		

Table 7
Pharmacokinetic parameters of enantiomers in rats (mean ± SD).

Parameters	Acetyl-L-glutamine	Acetyl-D-glutamine
$t_{1/2}$ (h)	0.33 ± 0.16	0.34 ± 0.16
K_e (1/h)	2.91 ± 1.91	2.34 ± 0.86
V_e (L/kg)	0.54 ± 0.19	1.00 ± 0.60
CL (L/h/kg)	1.38 ± 0.83	1.96 ± 0.47
$AUC_{0\text{-}t}$ (μg/mL·h)	49.90 ± 21.13	30.11 ± 7.33
$AUC_{0\text{-}\infty}$ (μg/mL·h)	53.07 ± 21.41	32.14 ± 7.94
C_{max} (μg/mL)	108.45 ± 59.08	62.64 ± 26.30
$MRT_{0\text{-}t}$ (h)	0.71 ± 0.09	0.51 ± 0.13
$MRT_{0\text{-}\infty}$ (h)	0.92 ± 0.19	0.52 ± 0.13

3.4.2. Liver microsomal incubation experiments

After liver microsomal incubation for 3 h with one enantiomer, another enantiomer could not be detected. The results demonstrated that chiral inversion did not occur in the liver microsomal.

4. Conclusion

The HPLC–MS method for the separation and quantification of enantiomers in rat plasma was developed and validated for the first time. The method was also successfully applied to the pharmacokinetic studies and the liver microsomal incubation experiments. The AUC of enantiomers had significant difference; therefore, the pharmacokinetics was stereoselective. Enantiomers chiral inversion did not occur in vivo and in vitro studies. Furthermore, it would be interesting to study the stereoselective pharmacodynamics and the profile of enantiomers metabolites.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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