Analysis of trantinterol enantiomers by pre-column derivatization UPLC-MS/MS and application to enantioselective drug protein binding study in rat plasma

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Running title: Enantioselective drug protein binding study of trantinterol enantiomers in rat plasma

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Abstract

Background: Trantinterol, a novel β2-adrenoceptor agonist, currently undergoing clinical trials for the treatment of asthma. As a chiral molecular, it has attracted lots of attention by analytical scientists

with respect of stereoselective pharmacological, stereoselective pharmacokinetic and metabolic studies. However, as an important factor for the difference in pharmacokinetic or pharmacodynamics properties of chiral drugs, stereoselectivity of trantinterol enantiomers in plasma protein binding study is an essential issue and it has not been conducted

Method: In this study, a reliable, selective and efficient ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method for the quantification of trantinterol enantiomers in rat plasma was developed. By pre-derivation, trantinterol enantiomers derivatives were well resolved on a UPLC BEH C18 column with a mobile phase consisting of 30mM ammonium acetate and acetonitrile. A Waters Quattro micro API Triple-Quadrupole Tandem Mass Spectrometer operating in positive electrospray ionization mode was used for detection.

Results: The developed method was fully validated in terms of selectivity, linearity, precision, accuracy, recovery, matrix effect, and stability, and met the requirements of every issue.

Conclusion: Subsequently, the developed method was well used in the stereoselectivity of trantinterol enantiomers in rat plasma protein binding study.

Keywords: trantinterol enantiomers, UPLC-MS/MS, derivatization, drug protein binding

1. Introduction

Binding of drugs to plasma proteins is one of many factors that influence drug ADME[1] and is an important factor, which determines the pharmacokinetics and pharmacological effects of drugs. Binding of a drug to plasma protein reduces free drug available to penetrate from the blood circulation

into tissues to reach the therapeutic target or the kidney for elimination. It is generally accepted that the effect of a drug is related to the exposure of a patient to the unbound concentration of the drug at its action site rather than its total concentration[2]. For chiral drugs, the stereoselectivity in plasma concentrations after administration can also be the result of stereoselective ADME, and a clear understanding of the plasma protein binding behavior of the enantiomers is therefore fundamental to their safe and rational use. Hence, it is necessary to stereoselectively detect and quantify each enantiomer in biological media [3,4].

Trantinterol, 2-(4-amino-3-chloro-5-trifluoromethylphenyl)-2-tbutylaminoethanol, a novel scaffold of 2-amino-2-phenylethanol instead of the classical scaffold of 2-amino-1-phenylethanol, has been proved that it has significant trachea relaxing effects in guinea pigs and rabbits as a β_2-adrenoceptor selective agonist [5,6]. Preclinical trials have revealed that trantinterol is a potent and highly selective β_2-adrenoceptor agonist with long duration of action and low cardiac side effects[5,7]. Trantinterol is a chiral molecule chemically, as shown by stereoselective pharmacological studies of trantinterol enantiomers, (-)-trantinterol exhibited more potent efficacy, higher affinity and better selectivity for β_2-adrenoceptor than (±)- and (+)-trantinterol[7]. Therefore, it is required to study the possibly different profiles of trantinterol enantiomers in pharmacokinetics and metabolic pathways. Over the last two decades, some analytical methods have been reported for the enantioselective determination of trantinterol enantiomers and the stereoselective difference investments of trantinterol enantiomers in pharmacology and pharmacokinetics[8,9,10]. However, none of these papers were focused on the stereoselectivity of trantinterol enantiomers in plasma protein binding study.

Since the enantiomers ADME profile can be modified by stereoselectivity in plasma protein binding, to fill this gap, a method of pre-column derivatization ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC– MS/MS) with multiple reaction monitoring (MRM) was presented. After method validation, the developed method was applied to quantify and study the plasma protein binding of trantinterol enantiomers in rat plasma.

2. MATERIALS AND METHODS

2.1. Chemical and Reagents

Racemic and (-)-and (+) trantinterol were synthesized at the Department of Pharmaceutical Chemistry, Shenyang Pharmaceutical University (Shenyang, China) with a purity higher than 99.4%.

Diphenhydramine as the internal standard (IS) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Blank rat plasma was purchased from Shanghai Yuduo Biotechnology Co., Ltd. (Shanghai, China). Semi-permeable membranes with a molecular weight cut-off of 8, 000–14,000 Da for equilibrium dialysis were purchased from Viskase (Darien, IL, USA). Diacetyl-L-tartaric anhydride (DATAAN) as the derivatization reagent was purchased from Fluka (Ronkonkoma, NY, USA). Acetonitrile and ammonium acetate of HPLC grade were purchased from Dikma Company (Richmond Hill, NY, USA). Other chemicals were all of the analytical grade.

2.2 Apparatus and Operation conditions

The analysis was performed on an ACQUITYTM UPLC system (Waters Corp., Milford, MA, USA) with cooling autosampler and column oven. An ACQUITY UPLC™ BEH C18 column (50 mm × 2.1 mm, 1.7 μm; Waters Corp., Milford, MA, USA) was employed with the column temperature maintained at 40 ℃. Chromatographic separation was achieved with isocratic elution using a mobile phase composed of acetonitrile-30 mmol/L ammonium acetate (32:68, *v*/*v*). The flow rate was set at 0.08 mL/min. The autosampler temperature was kept at 4 ℃ and 10 μL of sample solution was injected.

Mass spectrometric detection was carried out on a Micromass Quattro micro API mass spectrometer (Waters) with an electrospray ionization (ESI) interface. The ESI source was set in positive ionization mode with optimal operation parameters as follows: capillary 1.0 kV, cone 20 V, source temperature 105 ℃ and desolvation temperature 450 ℃. The quantification was performed using MRM of the transitions of *m/z* 527 → 454 for trantinterol derivative and *m/z* 256 → 167 for diphenhydramine, respectively, with a scan time of 0.10 s per transition. Nitrogen was used as the desolvation and cone gas with a flow rate of 600 and 30 L/h, respectively. Argon was used as the collision gas at a pressure of approximately 2.53×10^{-3} mbar. The optimized collision energy for trantinterol and diphenhydramine was 15 and 10 eV, respectively. All data collected in centroid mode were acquired and processed using MassLynx™ NT 4.1 software with QuanLynx™ program (Waters Corp., Milford, MA, USA).

2.3 Preparation of stock standards and quality control samples

Calibration standards for trantinterol enantiomers in a concentration range of 0.500-50.0 ng/mL were prepared by dilution of 100 μg/mL stock solution with methanol. A 50 ng/mL IS working solution was obtained by diluting the stock solution of diphenhydramine with methanol. All the solutions were stored at 4 ℃ and brought to room temperature before use. Calibration standards were prepared daily by spiking blank rat plasma at 0.500, 1.00, 2.50, 5.00, 10.0 and 25.0 ng/mL for each enantiomer. Quality control (QC) working solutions were prepared separately using another stock solution. QC samples, which were used in the validation and during the study, were prepared at the beginning of the experiment by independent dilution at three levels of plasma concentration for each enantiomer: 1.00, 5.00, 20.0 ng/mL. The standards and quality controls were extracted on each analysis day along with the unknown samples.

2.4 Plasma sample, dialysate sample preparation and derivatization

A 100 μL aliquot of IS solution (50 ng/mL) was pipetted into a 10 mL clean glass tube and evaporated to dryness. The residue was vortex-mixed with 500 μL sample (plasma or dialysate). After alkalifying with 100 μL of 0.1 % NaOH, the analytes were extracted into 3 mL ethyl acetate by vortex-mixing for 1min and centrifugating at 3500 rpm for 10 min. Then, the upper organic layer was transferred into another clean glass tube and evaporated to dryness at 40 ℃ under a gentle stream of nitrogen. The residue was derivatized by treating with 100 μL of DATAAN solution, 75 mmol/L in acetic aciddichloromethane (1:4, *v*/*v*) solution, and kept at 40 ℃ for 1 h. After that, the solution was evaporated to dryness under a gentle stream of nitrogen at 45 ℃. The residue was dissolved in 100 μL of acetonitrile-water (32:68, *v*/*v*), and an aliquot of 10 μL was injected into the UPLC-MS/MS system for analysis.

2.5 Method validation

The analytical methodology was validated according to the Food and Drug Administration or International Conference on Harmonization guidelines set by the United States Food and Drug Administration[11]. The contents to be verified were selectivity, linearity, precision, accuracy, recovery, matrix effect, and stability.

2.5.1 Selectivity

Selectivity was investigated by comparing chromatograms of blank sample (rat plasma or dialysate) with those of blank sample (rat plasma or dialysate) spiked with trantinterol and IS and plasma (dialysate) after the equilibrium dialysis.

2.5.2 Linearity and LLOQ

Calibration standards in plasma or dialysate at six concentration levels ranged 0.500-25.0 ng/mL for each trantinterol enantiomer were prepared and assayed respectively on 3 consecutive days. The calibration curves for trantinterol enantiomers in plasma or dialysate were generated by plotting the peak area ratio (y) of enantiomer derivatives to IS versus nominal concentrations (x) of trantinterol enantiomers by $1/x^2$ weighed least square linear regression. The LOQ was defined as the lowest concentration of trantinterol enantiomer for which an acceptable accuracy within ±20% was obtained.

2.5.3 Precision and accuracy

The accuracy and precision were assessed to determine QC samples at three concentration levels of trantinterol enantiomer (1.00, 8.00 and 20.0 ng/mL of each enantiomer) on 3 consecutive days. Precision was expressed as relative standard deviation (RSD) and accuracy as relative error (RE). Intra-day precision and accuracy were determined by six replicate analysis of QC samples on 1 day, while inter-day precision and accuracy were determined by six replicate analysis on 3 consecutive days, using standard curve prepared on the same day.

2.5.4 Extraction recovery and matrix effect

Extraction recoveries of (-)- and (+)-trantinterol were determined by comparing the peak areas obtained from blank plasma or dialysate samples spiked with analytes before extraction with those from blank plasma or dialysate samples to which analytes were added after extraction. This procedure was performed at three QC levels. The recovery of IS was determined similarly. To evaluate the matrix effect on the ionization of analytes, i.e. the potential ion suppression or enhancement due to the matrix components, three concentration levels of trantinterol enantiomers were added to the extract of 100 μL of blank plasma, derivatized as descried in Section 2.4, the corresponding peak areas (A) were compared with those of the trantinterol standard solutions derivatized directly (B). The ratio (A/B

*100) % was used to evaluate the matrix effect. The matrix effect of the internal standard was also evaluated using the same method.

2.5.5 Stability

The stability test was designed to cover the anticipated conditions that real samples may experience. The stability of trantinterol and IS stock solutions was evaluated after storage at room temperature for 4 h and at 4 ℃ for 30 days. QC plasma samples of three concentration levels were subjected to the conditions below. The stability of QC plasma samples kept at room temperature for 4 h was evaluated. This time exceeds the routine preparation time of samples. To estimate the stability of derivatized trantinterol enantiomers in processed extracts of rat plasma or dialysate samples, respectively, the pretreated QC samples were kept in an autosampler maintained at 4 ℃ for 12 h.

2.6 Application of the assay

In order to determine the plasma protein binding rates, the equilibrium dialysis method was used[12]. The dialysis membranes were pre-prepared according to the guidelines provided by the supplier. Briefly, the membranes were washed with distilled water, and then were soaked in phosphate buffer (PBS, pH 7.4) before being placed into the plasma for analysis. Firstly, 2 mL blank rat plasma was added into the semipermeable membrane bag. Then, the bag was placed in a flask with 20 mL PBS buffer containing 2.00, 10.0, 50.0 ng/mL of (-)- and (+)- trantinterol. Prior to analysis, the dialysis system was incubated at 4 ℃ for 48 h to achieve equilibrium between plasma and PBS buffer. The fluid outside and inside the dialysis bags was collected after the incubation. The concentration in the dialysis bag was determined by UPLC-MS/MS using standard curves as the total concentration, i.e., the unbound concentration plus the concentration of drug bound to protein, and the concentration of the fluid outside the dialysis bags was measured as the unbound fraction. The bounding ratio of (-)- and (+)-trantinterol in the equilibrium dialysis experiments was calculated using the following formula[12] : Fu (%)= (Dt - Df) /Dt × 100%, where Dt reprents the total compound concentration in the plasma compartment and Df is the concentration of the compound in free form in the phosphate buffer compartment.

3. RESULTS AND DISCUSSION

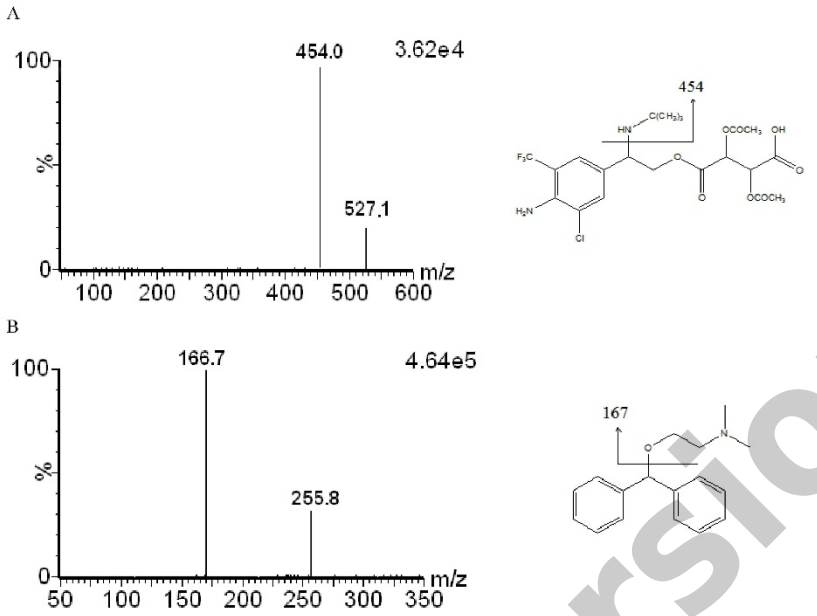
3.1 method development

Sample preparation plays an important role in analytical method development. Protein precipitation (PPT), solid phase extraction (SPE) and liquid–liquid extraction (LLE) are the most widely employed biological sample preparation techniques. PPT known as an easy and rapid procedure may introduce significant matrix effects due to its inability to remove many residual matrix components. In the present study, LLE and SPE were selected. Using LLE, several extraction solvents, diethyl ether, methyl tert-butyl ether, dichloromethane, ethyl acetate and mixed solvent, were tested. It was found that a consistent and reproducible response could be obtained with the extraction solvent of using ethyl acetate to the plasma. SPE using Oasis HLB cartridges (1 cc, 30 mg, Waters, Milford, USA) was tested. Truly SPE afforded clean extracts, but in the present investment the SPE strategy provided similar extraction recovery with LLE. Considering cost saving, we chose LLE as a sample preparation procedure.

To separate the trantinterol enantiomers, a simplified pre-column chiral derivatization method was developed[8]. Subsequently, the derivatized diastereomers could be resolved on an achiral C18 column using routine LC solvents as mobile phase which were easily adjusted and interfaced with high sensitive MS/MS detection. For the liquid chromatographic conditions, the organic phase (methanol and acetonitrile), column temperature (25-40℃) and mobile phase (different concentrations of ammonium acetate and formic acid) were optimized in order to enhance higher sensitivity, achieve better peak shape and avoid matrix effect. Furthermore, compared to methanol, the acetonitrile was chose as the organic phase of the mobile phase due to its strong elution effect and low background noise. After testing, 30 mM ammonium acetate and acetonitrile (68:32, *v*/*v*) could produce better peak shape and higher mass response to the trantinterol derivative and IS. Under the sufficient sensitivity and separation, 0.08 mL/min of flow rate and 40 ℃ of column temperature were adopted for rapid analysis of samples.

For the MS/MS determination, we applied the MRM in positive mode with trantinterol derivative to achieve the higher sensitivity and better specificity. The product ions mass spectra of trantinterol derivative and IS were shown Figure.1. Meanwhile, the parameters of mass spectrum conditions (capillary voltage, voltage and temperature of ion spray, nebulizer gas, heater gas, curtain gas and collision gas and so on) were optimized to acquire higher mass response.

Figure 1 Full scan product ion mass spectra of [M+H] + of trantinterol derivatives and proposed fragmentation (A); diphenhydramine and proposed fragmentation (B).



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231**3.2 Method validation**

232**3.2.1 Selectivity**

233 Comparing the chromatograms of six batches of blank plasmas or dialysates with the spiked 234plasmas demonstrated the good selectivity of the method. The elution order of derivatized trantinterol 235enantiomers by this method was (+)-trantinterol followed by (-)trantinterol. The peak confirmation was 236performed by comparing the retention times of pure (+)-trantinterol and (-)-trantinterol standard after 237derivatization. The representative chromatograms are shown in Figure 2 and 3. All plasma lots were 238found to be free of interference with the compounds of interest.

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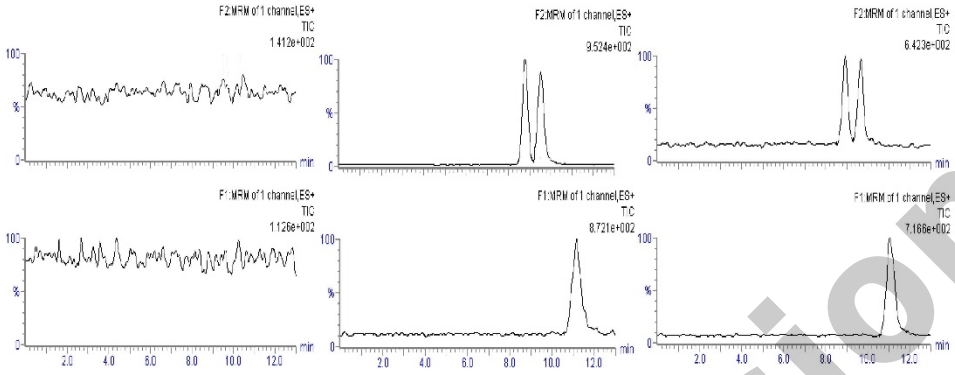
246**Figure 2 Representive MRM chromatograms of trantinterol enantiomers and diphenhydramine 247(IS) in rat plasma samples.**

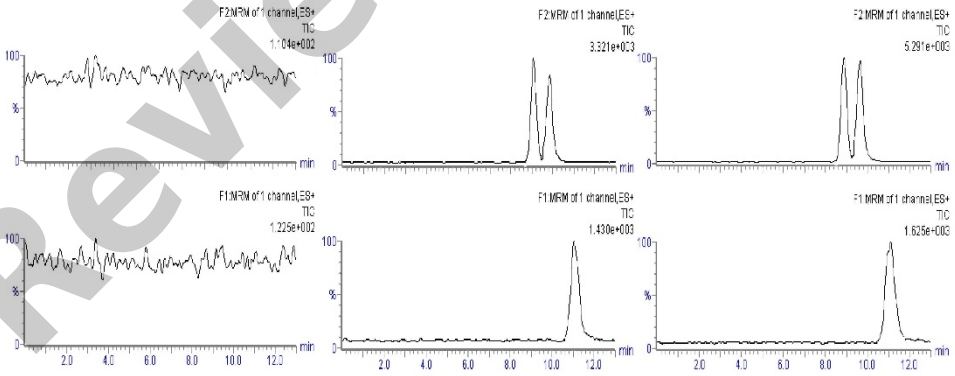
(A) Blank dialysate sample. (B) Blank dialysate sample spiked with trantinterol and IS. (C) The real sample.



A B C

Figure 3. Representive MRM chromatograms of trantinterol enantiomers and diphenhydramine (IS) in dialysate samples.

(A) Blank dialysate sample. (B) Blank dialysate sample spiked with trantinterol and IS. (C) The real sample.



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3.2.2 Linearity and LOQ

The calibration curves for trantinterol enantiomers were linear over the range of 0.500-25.0 ng/mL (r 0.99) by using weighted ($1/x^2$) least squares linear regression. Typical equations for the calibration curves of (+)-and (-)-trantinterol were as follows:

The plasma sample:

(+)-trantinterol: Y=0.152x+0.00759 (r =0.9903)

(-)-trantinterol: Y=0.157x+0.00562(r =0.9908)

The dialysate sample:

(+)-trantinterol: Y=0.144x+0.00768(r =0.9925)

(-)-trantinterol: Y=0.129x+0.00897(r =0.9912)

The limit of quantification for each enantiomer was 0.500 ng/mL with acceptable precision and accuracy presented in Table 1.

Table 1 Precision and accuracy for determination of (+)-trantinterol and (-)-trantinterol in rat plasma (intra-day: n=6; inter-day: n=6 series per day, 3 days).

	(+)-trantinterol				(-)-trantinterol			
	0.500	1.00	8.00	20.0	0.50	1.00	8.00	20.0
N	18	18	18	18	18	8	18	18
Mean (ng/mL)	0.522	0.96	8.63	18.9	0.51	0.934	8.27	19.3
SD	0.054	0.089	0.44	5.3	0.042	0.083	0.41	3.4
RE (%)	4.4	-4.0	7.9	-5.5	2.0	-6.1	3.4	-3.6
Intra-day *RSD* (%)	6.1	9.0	5.7	4.6	5.7	4.7	5.0	8.5
Inter-day *RSD* (%)	3.8	5.8	3.2	7.8	2.3	2.6	2.8	10

Table 2 Precision and accuracy for determination of (+)-trantinterol and (-)-trantinterol in dialysate (intra-day: *n*=6; inter-day: *n*=6 series per day, 3 days)

	(+)-trantinterol				(-)-trantinterol			
	0.500	1.00	8.00	20.0	0.500	1.00	8.00	20.0
N	18	18	18	18	18	18	18	18
Mean (ng/mL)	0.513	0.987	7.77	19.5	0.518	0.98	7.90	20.8
SD	0.053	0.058	0.29	2.6	0.039	0.046	0.48	2.8
RE (%)	2.6	-2.2	-2.9	-2.5	3.6	-2.0	-1.2	4.0
Intra-day *RSD* (%)	4.1	6.2	3.8	6.2	3.8	4.6	6.4	6.9
Inter-day *RSD* (%)	2.9	3.2	3.3	8.6	3.9	5.6	4.1	5.7

3.2.3 Precision and accuracy

The data of intra- and inter-day precision and accuracy for trantinterol enantiomers in plasma or dialysate are listed in Table 1 and 2. The intra- and inter-day precisions were less than 9.0% and 10%, while the corresponding accuracy was from -6.1 to 7.9%, indicating an acceptable accuracy and precision of the method.

3.2.4 Extraction recovery and matrix effect

In plasma, the extraction recovery values of each enantiomer at concentration levels of 1.00, 8.00, 20.0 ng/mL were 81.2±2.9%, 74.9±3.1%, 76.4±1.7% for (+)-trantinterol and 77.4±5.3%, 76.8±4.2%, 79.4±3.9% for (-)-trantinterol, respectively. Meanwhile, in dialysate, the extraction recovery values were 77.5±4.2%, 80.4±2.6%, 78.2±5.0% for (+)-trantinterol and 80.9±1.8%, 76.7±3.8%, 79.0±4.5% for (-)-trantinterol, respectively. While the recovery of the IS was 82.3±2.6% in plasma, and 77.3±4.1% in dialysate. These result indicated that the recovery of trantinterol enantiomers and the IS was consistent and not concentration dependent.

In terms of matrix effect, all the ratios (A/B*100)% defined as in Section 2.5.4 were between 85 and 115%, which means no significant matrix effect in this method.

3.2.5 Stability

The stock solutions of trantinterol and IS were found to be stable at room temperature for 4 h and 4 ℃ for 12 h. Table 3 and Table 4 summarize the results for stability in other terms, and all the results well met the criterion for stability measurements.

Table 3 Stability of (+)-trantinterol and (-)-trantinterol in rat plasma (*n*=3)

	RE%					
	(+)-trantinterol			(-)-trantinterol		
	1.00	8.00	40.0	1.00	8.00	40.0
Short term (room temperature for 4 h)	5.2	-3.8	6.3	-5.1	-4.1	5.7
	-3.8	2.3	5.4	8.3	6.8	4.1
	7.6	-9.2	-2.5	6.9	-3.7	-5.4
Three freeze-thaw cycles	-5.6	7.4	3.4	2.6	3.2	-3.8
	-7.2	-13	-5.7	-6.1	6.8	-4.2
	5.9	-11	6.1	-2.9	-4.9	-3.5
Long term (-20 ℃ for 30 days)	5.5	2.9	3.9	7.6	0.4	4.5
	-6.4	6.0	-3.0	-8.2	-5.9	-0.7
	4.5	4.8	2.8	-6.4	-6.8	5.0
Post-preparative (room temperature for 12h)	8.9	-4.3	4.7	1.8	9.0	5.8
	4.8	3.7	-5.1	-7.3	-4.7	2.5
	-3.7	5.2	3.5	-12	6.1	4.1

Table 4 Stability of (+)-trantinterol and (-)-trantinterol in dialysate (*n*=3)

	RE%					
	(+)-trantinterol			(-)-trantinterol		
	1.00	8.00	40.0	1.00	8.00	40.0
Short term (room temperature for 4 h)	-4.8	-5.4	-6.4	8.3	-5.7	-6.9
	3.6	12	-5.1	6.2	6.1	4.4
	5.2	-9.6	3.9	-4.0	4.8	5.9
Three freeze-thaw cycles	-2.9	6.0	4.3	0.9	5.4	-3.5
	-5.9	-8.3	5.6	-1.5	-3.9	-4.1
	12	-3.1	-8.9	3.6	-6.0	-3.9
Long term (-20 ℃ for 30 days)	-7.8	-7.0	4.1	-8.0	9.5	7.1
	-6.2	6.5	-1.9	13	-5.8	-0.9
	3.9	4.8	6.0	-9.7	1.3	1.2
Post-preparative (room temperature for 12 h)	6.9	-4.5	-5.8	-3.6	-13	4.4
	4.0	3.0	-3.0	5.2	-4.0	1.9
	-3.7	-2.6	6.1	-4.9	3.0	-7.6

3.3 Application of the method to enantiomers protein binding study

Equilibrium dialysis method is one of the most commonly used techniques to study plasma protein binding in drug discovery and drug development[13]. Due to its simplicity and general applicability to many different systems in vitro and ex vivo, it was selected in the present study of rat plasma protein binding of trantinterol enantiomers.

Before the analysis, a non-specific binding of a drug onto the filter membrane was tested, which was a limiting factor in membrane isolation technique and may lead to a loss of the analyte concentration[12]. The determination of non-specific binding of (+)-and (-)-trantinterol onto the dialysis membrane was processed according to section 2.6 Equilibrium dialysis, except for the inside of the semi-permeable membrane bag was blank phosphate buffer. After investigation, we found that the nonspecific binding of (+)-and (-)-trantinterol to the membrane was negligible.

The bound fraction of (+)-and (-)-trantinterol to the rat plasma are presented in Table 5 and 6. The results are presented as mean values ± standard deviation (mean ± SD). The paired Student's t-test was used to determine statistical significance when two groups of data were compared. All of the statistical analyses were performed using DAS 2.0 software. After statistical analyses, $p > 0.05$ was considered that there was no significant differences between (+)-and (-)-trantinterol binging to the rat plasma. This might be helpful in pharmacokinetic stereoselectivity study if there was some stereoselectivity pharmacokinetic difference phenomenons observed, the reason might be not due to the binding of plasma proteins.

Table 5 The binding ratio of (+)-trantinterol to rat plasma protein (*n*=6)

	Balance time	Concentration in dialysate (ng/mL)	Concentration in plasma (ng/mL)	Binding ratio(%)	Mean±SD(%)
1.00	48h	0.735	1.39	47.4	44.5±6.1
		0.683	1.34	49.2	
		0.844	1.29	34.8	
		0.753	1.47	48.9	
		0.818	1.38	41.3	
	72 h	0.697	1.23	42.5	41.7±5.3
		0.815	1.34	37.6	
		0.654	1.28	49.2	
		0.873	1.36	36.0	
		0.792	1.27	37.8	
		0.677	1.19	43.7	
		0.713	1.31	45.8	
5.00	48 h	3.78	5.75	34.2	38.9±7.2
		3.66	6.29	41.8	
		3.59	6.73	46.6	
		3.36	5.09	33.9	
		3.47	5.27	34.1	
	72 h	3.61	6.14	41.2	37.6±8.2
		3.41	5.97	42.8	
		3.56	6.14	42.0	
		4.23	6.15	31.5	
		3.98	5.83	31.7	
		3.17	5.69	44.2	
		4.09	6.14	33.3	
25.0	48 h	18.8	30.4	38.0	41.7±2.5
		15.9	27.8	42.8	
		17.2	31.4	45.1	
		16.7	28.1	40.4	
		15.2	25.3	40.5	
	72 h	15.1	26.5	42.9	42.8±5.4
		17.6	28.7	38.4	
		16.2	23.7	31.4	
		18.7	33.4	44.0	
		15.3	29.6	48.3	
		15.9	28.0	43.2	
		17.2	31.7	45.7	

Table 6 The binding ratio of (-)-trantinterol to rat plasma protein (*n*=6)

	Balance time	Concentration in dialysate (ng/mL)	Concentration in plasma (ng/mL)	Binding ratio(%)	Mean±SD (%)
1.00	48 h	0.691	1.29	46.5	42.4±5.0
		0.648	1.16	44.8	
		0.785	1.24	37.1	
		0.778	1.44	46.5	
		0.926	1.42	35.2	
	72 h	0.753	1.34	44.0	38.0±5.8
		0.834	1.24	33.0	
		0.687	1.31	48.0	
		0.772	1.29	40.3	
		0.876	1.28	32.0	
		0.674	1.08	37.9	
		0.747	1.17	36.7	
5.00	48 h	3.65	5.67	35.6	38.4±5.2
		3.58	6.21	42.3	
		3.52	6.59	46.5	
		3.48	5.43	35.9	
		3.62	5.34	32.2	
	72 h	3.87	6.21	37.6	39.9±6.8
		3.52	5.64	37.5	
		3.68	6.53	43.6	
		4.15	6.17	32.7	
		3.77	6.04	37.5	
		3.09	6.17	49.9	
		3.91	6.35	38.4	
25.0	48 h	19.7	31.3	37.0	42.5±5.6
		18.2	34.2	46.9	
		16.8	30.9	45.5	
		15.2	28.7	47.0	
		16.3	24.8	34.2	
	72 h	17.3	28.1	38.3	44.1±6.9
		19.1	30.1	36.5	
		16.8	25.6	34.3	
		17.0	34.8	51.1	
		16.3	31.2	47.7	
		15.6	30.5	48.8	
		18.1	33.4	45.8	

In general, as shown in Table 5 and 6, concentration dependents of plasma protein binding of (+)- and (-)-trantinterol were not observed over the selected concentration range (1–25 ng/ml) in rat plasma. With the increase of drug concentration, the bound fraction of (+)-and (-)-trantinterol was nearly the same around 40%. This phenomenon may be due to the saturability of the combination of drug and protein. Therefore, since the administration dosage was high but no significant rise of free drug concentration was obtained by the change of plasma protein binding rate, this should be taken into

consideration. Comprehensive study of two aspects of the pharmacokinetic data and the change of plasma protein binding rate should be crucial because they may affect the drug distribution, excretion, metabolism as well as the efficacy and toxicity.

4. CONCLUSION

A sensitive and simple pre-column derivatization UPLC–MS/MS method was developed for resolution of trantinterol enantiomers and applied to study the rat plasma protein binding of (+)-and (-)-trantinterol. The results showed that trantinterol enantiomers had high plasma protein binding in the physiological conditions of rat, and there were no significant enantioselective differences. Since the interactions between drug and protein can affect the pharmacokinetic or pharmacodynamic properties of the drug within the body, the method and the results will be proved to be potentially valuable for further study of trantinterol enantiomers.

Compliance with Ethical Standards

This study was not funded.

Conflict of interest

The authors have declared no conflict of interest.

Ethical approval

This article does not contain any studies with animals performed by any of the authors.

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Table 1 Precision and accuracy for determination of (+)-trantinterol and (-)-trantinterol in rat plasma (intra-day: n=6; inter-day: n=6 series per day, 3 days).

	(+)-trantinterol				(-)-trantinterol			
	0.500	1.00	8.00	20.0	0.50	1.00	8.00	20.0
N	18	18	18	18	18	8	18	18
Mean (ng/mL)	0.522	0.96	8.63	18.9	0.51	0.934	8.27	19.3
SD	0.054	0.089	0.44	5.3	0.042	0.083	0.41	3.4
RE (%)	4.4	-4.0	7.9	-5.5	2.0	-6.1	3.4	-3.6
Intra-day *RSD* (%)	6.1	9.0	5.7	4.6	5.7	4.7	5.0	8.5
Inter-day *RSD* (%)	3.8	5.8	3.2	7.8	2.3	2.6	2.8	10

Table 2 Precision and accuracy for determination of (+)-trantinterol and (-)-trantinterol in dialysate (intra-day: *n*=6; inter-day: *n*=6 series per day, 3 days)

	(+)-trantinterol				(-)-trantinterol			
	0.500	1.00	8.00	20.0	0.500	1.00	8.00	20.0
N	18	18	18	18	18	18	18	18
Mean (ng/mL)	0.513	0.987	7.77	19.5	0.518	0.98	7.90	20.8
SD	0.053	0.058	0.29	2.6	0.039	0.046	0.48	2.8
RE (%)	2.6	-2.2	-2.9	-2.5	3.6	-2.0	-1.2	4.0
Intra-day *RSD* (%)	4.1	6.2	3.8	6.2	3.8	4.6	6.4	6.9
Inter-day *RSD* (%)	2.9	3.2	3.3	8.6	3.9	5.6	4.1	5.7

Table 3 Stability of (+)-trantinterol and (-)-trantinterol in rat plasma (*n*=3)

	RE%					
	(+)-trantinterol			(-)-trantinterol		
	1.00	8.00	40.0	1.00	8.00	40.0
Short term (room temperature for 4 h)	5.2	-3.8	6.3	-5.1	-4.1	5.7
	-3.8	2.3	5.4	8.3	6.8	4.1
	7.6	-9.2	-2.5	6.9	-3.7	-5.4
Three freeze-thaw cycles	-5.6	7.4	3.4	2.6	3.2	-3.8
	-7.2	-13	-5.7	-6.1	6.8	-4.2
	5.9	-11	6.1	-2.9	-4.9	-3.5
Long term (-20 ℃ for 30 days)	5.5	2.9	3.9	7.6	0.4	4.5
	-6.4	6.0	-3.0	-8.2	-5.9	-0.7
	4.5	4.8	2.8	-6.4	-6.8	5.0
Post-preparative (room temperature for 12h)	8.9	-4.3	4.7	1.8	9.0	5.8
	4.8	3.7	-5.1	-7.3	-4.7	2.5
	-3.7	5.2	3.5	-12	6.1	4.1

Table 4 Stability of (+)-trantinterol and (-)-trantinterol in dialysate (*n*=3)

	RE%					
	(+)-trantinterol			(-)-trantinterol		
	1.00	8.00	40.0	1.00	8.00	40.0
Short term (room temperature for 4 h)	-4.8	-5.4	-6.4	8.3	-5.7	-6.9
	3.6	12	-5.1	6.2	6.1	4.4
	5.2	-9.6	3.9	-4.0	4.8	5.9
Three freeze-thaw cycles	-2.9	6.0	4.3	0.9	5.4	-3.5
	-5.9	-8.3	5.6	-1.5	-3.9	-4.1
	12	-3.1	-8.9	3.6	-6.0	-3.9
Long term (-20 ℃ for 30 days)	-7.8	-7.0	4.1	-8.0	9.5	7.1
	-6.2	6.5	-1.9	13	-5.8	-0.9
	3.9	4.8	6.0	-9.7	1.3	1.2
Post-preparative (room temperature for 12 h)	6.9	-4.5	-5.8	-3.6	-13	4.4
	4.0	3.0	-3.0	5.2	-4.0	1.9
	-3.7	-2.6	6.1	-4.9	3.0	-7.6

Table 5 The binding ratio of (+)-trantinterol to rat plasma protein (*n*=6)

	Balance time	Concentration in dialysate (ng/mL)	Concentration in plasma (ng/mL)	Binding ratio(%)	Mean±SD(%)
1.00	48h	0.735	1.39	47.4	44.5±6.1
		0.683	1.34	49.2	
		0.844	1.29	34.8	
		0.753	1.47	48.9	
		0.818	1.38	41.3	
	72 h	0.697	1.23	42.5	41.7±5.3
		0.815	1.34	37.6	
		0.654	1.28	49.2	
		0.873	1.36	36.0	
		0.792	1.27	37.8	
		0.677	1.19	43.7	
		0.713	1.31	45.8	
5.00	48 h	3.78	5.75	34.2	38.9±7.2
		3.66	6.29	41.8	
		3.59	6.73	46.6	
		3.36	5.09	33.9	
		3.47	5.27	34.1	
	72 h	3.61	6.14	41.2	37.6±8.2
		3.41	5.97	42.8	
		3.56	6.14	42.0	
		4.23	6.15	31.5	
		3.98	5.83	31.7	
		3.17	5.69	44.2	
		4.09	6.14	33.3	
25.0	48 h	18.8	30.4	38.0	41.7±2.5
		15.9	27.8	42.8	
		17.2	31.4	45.1	
		16.7	28.1	40.4	
		15.2	25.3	40.5	
	72 h	15.1	26.5	42.9	42.8±5.4
		17.6	28.7	38.4	
		16.2	23.7	31.4	
		18.7	33.4	44.0	
		15.3	29.6	48.3	
		15.9	28.0	43.2	
		17.2	31.7	45.7	

Table 6 The binding ratio of (-)-trantinterol to rat plasma protein (*n*=6)

	Balance time	Concentration in dialysate (ng/mL)	Concentration in plasma (ng/mL)	Binding ratio(%)	Mean±SD (%)
1.00	48 h	0.691	1.29	46.5	42.4±5.0
		0.648	1.16	44.8	
		0.785	1.24	37.1	
		0.778	1.44	46.5	
		0.926	1.42	35.2	
	72 h	0.753	1.34	44.0	38.0±5.8
		0.834	1.24	33.0	
		0.687	1.31	48.0	
		0.772	1.29	40.3	
		0.876	1.28	32.0	
		0.674	1.08	37.9	
		0.747	1.17	36.7	
5.00	48 h	3.65	5.67	35.6	38.4±5.2
		3.58	6.21	42.3	
		3.52	6.59	46.5	
		3.48	5.43	35.9	
		3.62	5.34	32.2	
	72 h	3.87	6.21	37.6	39.9±6.8
		3.52	5.64	37.5	
		3.68	6.53	43.6	
		4.15	6.17	32.7	
		3.77	6.04	37.5	
		3.09	6.17	49.9	
		3.91	6.35	38.4	
25.0	48 h	19.7	31.3	37.0	42.5±5.6
		18.2	34.2	46.9	
		16.8	30.9	45.5	
		15.2	28.7	47.0	
		16.3	24.8	34.2	
	72 h	17.3	28.1	38.3	44.1±6.9
		19.1	30.1	36.5	
		16.8	25.6	34.3	
		17.0	34.8	51.1	
		16.3	31.2	47.7	
		15.6	30.5	48.8	
		18.1	33.4	45.8	

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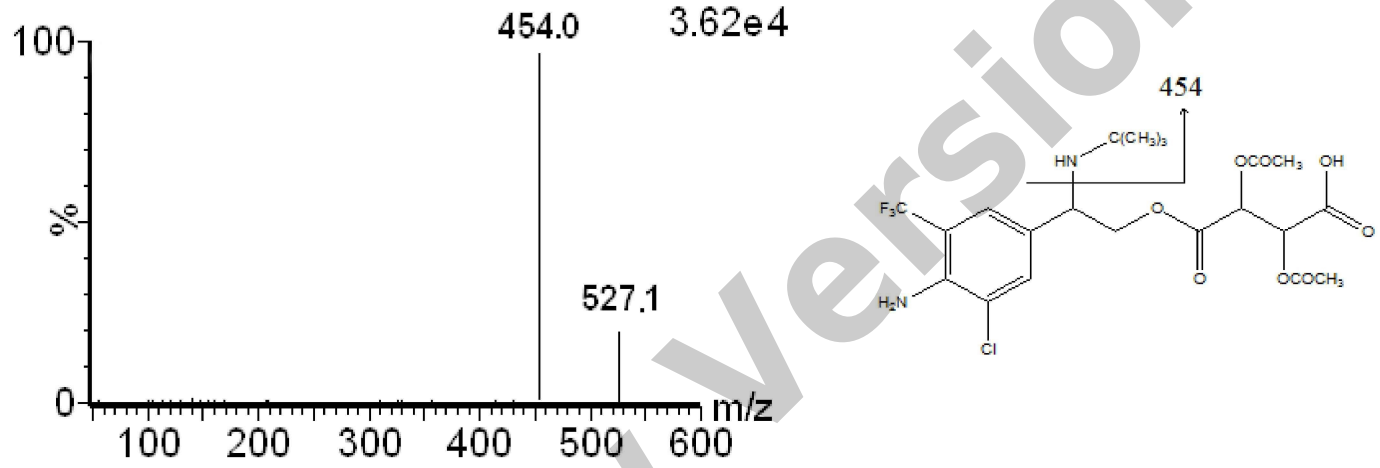

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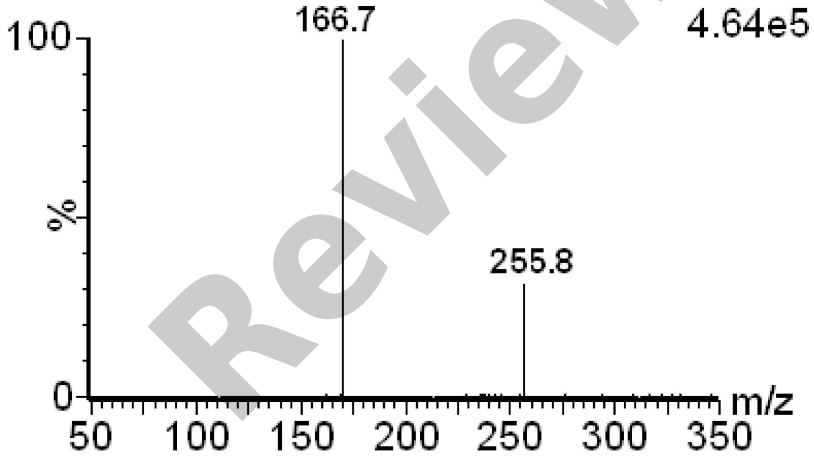

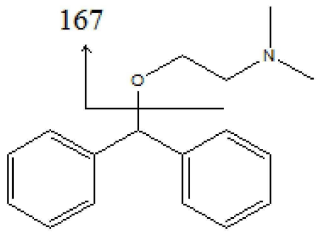

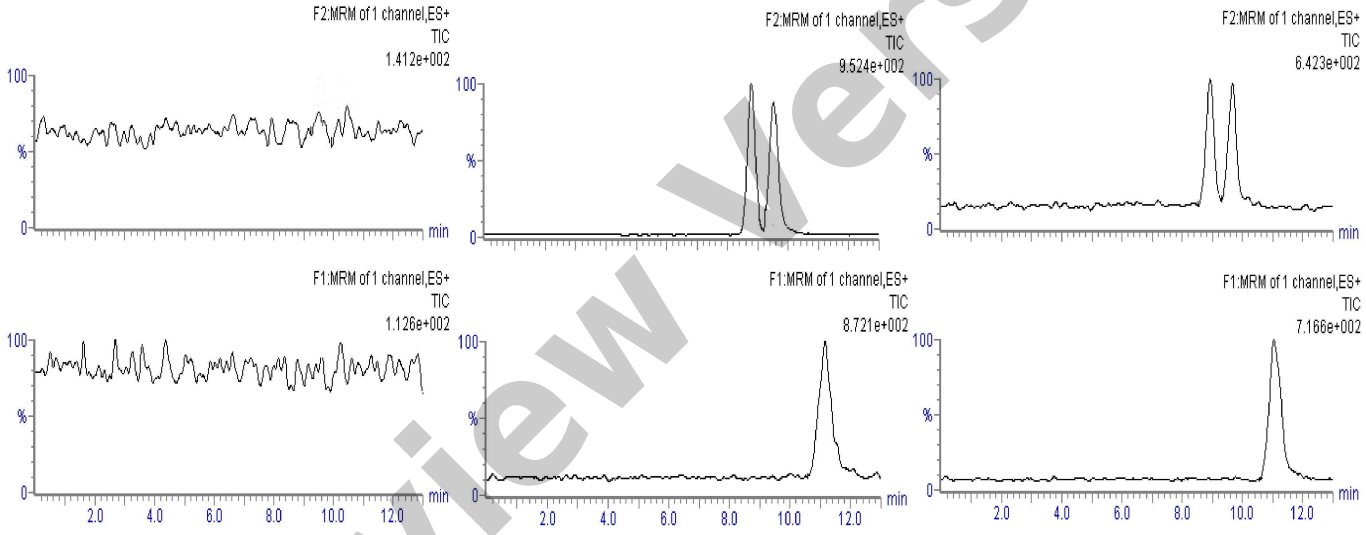

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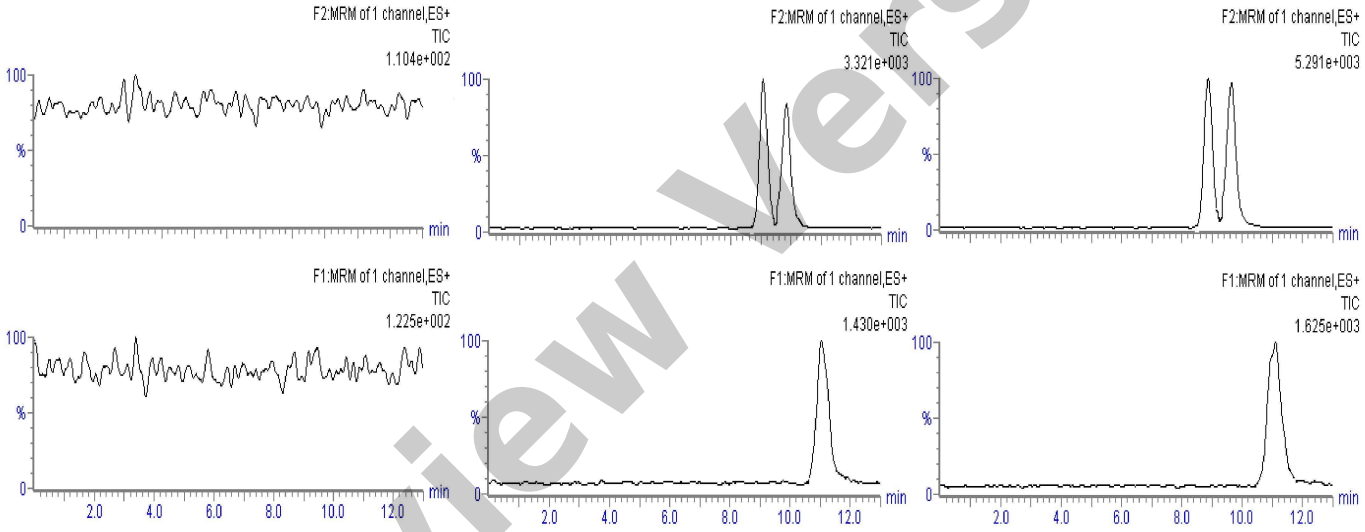

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