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Determination of risperidone in human plasma by HPLC-MS/MS and its application to a pharmacokinetic study in Chinese volunteers

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Abstract: This study presents a rapid, specific and sensitive liquid chromatography/tandem mass spectrometry (LC-MS/MS) assay for determination of risperidone (RIS) in human serum using paroxetine as an internal standard (IS). An Alltima-C18 column (2.1 mm×100 mm, 3 μm) and a mobile phase consisting of 0.1% formic acid-acetonitrile (40:60, v/v) were used for separation. The analysis was performed by selected reaction monitoring (SRM) method, and the peak area of the *m*/*z* 411.3→191.1 transition for RIS was measured versus that of the *m*/*z* 330.1→192.1 transition for IS to generate the standard curves. The assay linearity of RIS was confirmed over the range 0.25~50.00 ng/ml and the limit of quantitation was 0.05 ng/ml. The linear range corresponds well with the serum concentrations of the analytes obtained in clinical pharmacokinetic studies. Intraday and interday relative standard deviations were 1.85%~9.09% and 1.56%~4.38%, respectively. The recovery of RIS from serum was in the range of 70.20%~84.50%. The method was successfully applied to investigate the bioequivalence between two kinds of tablets (test versus reference products) in 18 healthy male Chinese volunteers. The result suggests that two formulations are bioequivalent.

Key words: Risperidone, High performance liquid chromatography-mass spectrometry (HPLC-MS), Pharmacokinetics
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INTRODUCTION

Risperidone (RIS) (3-[2-[4-(6-fluoro-1,2-benzisoxazol-3-yl)-1-piperidinyl] ethyl]-6,7,8,9-tetrahydro-2-methyl 4H-pyrido[1,2-a] pyrimidin-4-one) is a benzisoxazole antipsychotagent, used to treat schizophrenia and other psychoses. It exerts its effect by blocking serotonin ($5\text{-}HT_2$) and dopamine (D_2) receptors and causes a lower incidence of extrapyramidal side-effects than standard neuroleptic drugs (Huang *et al*., 1993; Mannens *et al*., 1994). It is effective in the treatment of schizophrenia and other psychiatric illnesses in adults and children including pervasive developmental disorders, autism and attention-deficit disorder (McCracken *et al*., 2002; McDougle *et al*., 2005). Usually, oral doses of RIS in the treatment of chronic schizophrenia are 2~6 mg/d. After oral administration, RIS is rapidly and completely absorbed from the gastro-intestinal tract and mainly metabolised via hydroxylation and *N*-dealkylation (Huang *et al*., 1993; Mannens *et al*., 1994). The hydroxylation of RIS is catalysed by the cytochrome P450 isoenzyme CYP2D6, which is subject to genetic polymorphism (Remmerie *et al*., 2003).

For adequate support of clinical studies with RIS, an analytical method is required for the determination of plasma levels of RIS. It is necessary to establish a simple, accurate and specific analytical technique, which permits measurements of RIS in biological specimens at different therapeutic levels. Early methods for the quantification of RIS have mostly used high performance liquid chromatography (HPLC) with ultraviolet (UV) or electrochemical detection (Aravagiri *et al*., 1998; Balant-Gorgia *et al*., 1999). Although these methods may be suitable for therapeutic drug monitoring of target concentrations in the range of 4~30 ng/ml for RIS, their narrow range is not particularly suitable for pharmacokinetic stud-

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ies. The reported liquid chromatography-mass spectrometry (LC-MS) analytical method requires laborious extraction procedures like liquid-liquid extraction (Aravagiri and Marder, 2000) or solid-phase extraction (Remmerie *et al*., 2003), involving drying and reconstitution, long run time and high quantification limits (Flarakos *et al*., 2004; McClean *et al*., 2000; Zhou *et al*., 2004). In recent years, several mass spectrometric methods have been developed and all have much improved sensitivity and specificity (Bhatt *et al*., 2006; Cabovska *et al*., 2007; Kousoulos *et al*., 2007; Moody *et al*., 2004).

In this paper, we describe the simultaneous determination of RIS and paroxetine as an internal standard (IS) in human serum using modified LC–MS/MS method, and this method has been successfully applied to a bioequivalence study of test RIS tablet vs reference tablet (Risperdal®) in 18 healthy male Chinese volunteers.

MATERIALS AND METHODS

Chemicals and reagents

The RIS tablet (batch No. 040403, 1 mg/pill) is prepared for the test. The reference preparation (trade name Risperdal®, batch No. 040207366, 1 mg/pill) was produced by Xi'an Janssen Pharmaceutical (Xi'an, China). RIS pure drug (purity 99.9%) and paroxetine pure drug (IS, purity 99.8%) were kindly provided by Zhejiang Provincial Laboratory for the Control of Drugs (Hangzhou, China). Formic acid and acetonitrile were purchased from Merck (Darmstadt, Germany). Water was purified by a Milli-Q system from Millipore Corp. (Milford, MA, USA). Other chemicals were of analytical grade.

Instrumentation

Chromatographic separations were carried out on a Surveyor HPLC with an Alltima-C18 column (2.1 mm×100 mm, 3.0 μm). A mobile phase consisting of 0.1% formic acid-acetonitrile (40:60, v/v) was used with a flow rate of 0.2 ml/min. The mobile phase was filtered by passing through a 0.22 μm membrane filter. Measurements were made at 30 °C, and sample volume was 2 μl.

Mass spectra were obtained using a Finnigan TSQ Quantum discovery mass spectrometer (USA) and operated using electrospray ionization (ESI) condition. The positive-ion selected reaction monitoring (SRM) mode was chosen for quantification. The electrospray voltage: 3500 eV, nebulizer N_2 gas temperature: 350 °C, drying N_2 gas flow: 200 ml/min, sheath gas pressure: 30 Arb, and auxiliary gas pressure: 5 Arb. The peak area of transition from *m*/*z* 411.3, $[M+H]^+$ ion, to *m*/*z* 191.1, a product ion, with collision energy of 30 eV was measured for RIS. Paroxetine was monitored using the transitions from *m*/*z* 330.1 to *m*/*z* 192.1 with collision energy of 27 eV. The data acquisition was ascertained by Xcalibur software.

Standard solutions

RIS stock solutions were prepared in acetonitrile (1.0 μg/ml) and stored at 4 °C. Working standards from the concentrated stock solution were prepared with acetonitrile to yield the final concentrations of 2.5, 5.0, 10.0, 25.0, 50.0, 100.0, 200.0 and 500.0 ng/ml. The stock solution of IS (paroxetine, 12 μg/ml) was stored at 4 °C. It was diluted with acetonitrile to give a final concentration of 60 ng/ml before added to the samples.

The working standards, 30 μl, were dried under nitrogen at 40 °C. The dry residues were vortex-mixed with 300 μl blank serum to make a series of serum samples, which were added with 0.9 ml 60 ng/ml of IS. Then the samples were vortex-mixed up vigorously for 5 min and centrifuged at 15000×*g* (2 °C) for 15 min. Its supernatants (2 μl) were injected into the LC-MS/MS system.

Sample preparation

Blood samples drawn from 18 healthy volunteers after a single oral administration of 2 mg RIS were centrifuged at 15000×*g* for 15 min. Serum samples were then stored in polypropylene tubes at −20 °C until analyzed. The preparation of serum samples was treated as described in subsection "standard solutions".

Validation

1. Selectivity

The selectivity study was performed on the human serum from individual healthy donors receiving no medication for assessment of potential interferences with endogenous substances at the retention

time of RIS and IS.

2. Linearity

Serum samples spiked with RIS and IS working solutions were processed according to the procedure described above for the construction of calibration curves. The eight-point (0.25, 0.50, 1.00, 2.50, 5.00, 10.00, 20.00, 50.00 ng/ml) calibration curves were obtained by plotting the peak area ratio (y) of RIS to IS against the concentration (x) of RIS. The concentrations of calibration standards were analyzed for five replicates and the goodness-of-fit was determined by linear regression.

3. Precision and accuracy

The intraday precision and accuracy for the analytical method were evaluated by analysis of five replicates of serum samples containing RIS at three different concentrations of 0.25, 5.00 and 50.00 ng/ml on the same day. The interday precision and accuracy were evaluated at the above concentration levels on five separate days. The precision was estimated by calculating the relative standard deviation [*RSD* (%)] which is expressed as: *RSD* (%)=(standard derivation)/(mean observed concentration)×100%, and the RSD value must be within 15%. The accuracy was calculated for each batch as the mean concentration found for that batch relative to the actual concentration. The accuracy must be between 85% and 115%, and the range nearly LLOQ (the lowest limit of quantification) was 80%~120%.

4. Recovery

The recovery of RIS was evaluated in quintuplicate at three concentration levels of 0.25, 5.00 and 50.00 ng/ml from peak area ratios (R) of assayed samples compared to the reference standard prepared in acetonitrile. Each of the samples was also spiked with IS at working concentration of 60 ng/ml. The recovery was calculated using the formula:

$$Recovery\ (\%)=(R_{serum})/(R_{acetonitril})\times 100\%,$$

where R_{serum} was peak area ratios of serum samples, and $R_{acetonitril}$ was peak area ratios of standard solutions.

5. Stability

The processed serum samples (0.25, 5.00 and 50.00 ng/ml) were kept at room temperature for 24 h and then the stability was determined. The freeze-thaw stability was determined after three repeated freezing and thawing cycles on Days 0, 10 and 15.

Pharmacokinetic studies

Eighteen healthy male Chinese volunteers [median age: 20.5 years (18~22 years), median weight: 58.5 kg (54.5~75 kg), median height: 170.5 cm (166~185 cm)] were selected as subjects. All subjects gave written consent to their participation after informed by the medical supervisor about the aim, course and possible risks of the study. The study protocols were approved by the relevant Ethical Review Committee in accordance with the principles of the Declaration of Helsinki, and the recommendations of the State Food and Drug Administration of China.

The volunteers participated in a single dose fasting crossover bioequivalence study with a one-week interval between each administration. Subjects were admitted to the study clinical unit at evening before the day of each administration and remained under close medical supervision during the study. Each subject received two RIS test or reference tablets in a randomized order. Subjects were fasted for 12 h before drug administration. About 3 ml of venous blood samples were collected at 0.0, 0.33, 0.67, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0 and 16.0 h postdose administration.

Serum samples were prepared for analysis. Pharmaceutical Kinetics Software (PKS) 1.0.2 software package (Shanghai Hongneng Software Co., Ltd., China) was used for calculation of pharmacokinetic parameters. The peak concentration (C_{max}) and peak time (t_{max}) were observed values. The elimination rate constant (K_e) was estimated from the terminal linear segment of the log serum concentration/time data. The elimination half-life ($t_{1/2}(k_e)$) was calculated from $\ln 2/K_e$. The area under the curve ($AUC_{0\to t}$) was estimated by trapezoidal rule with extrapolation.

RESULTS AND DISCUSSION

Validation of LC-MS/MS method

1. Selectivity

Serum samples from different sources were found to be free from interfering molecular ions at the retention time of RIS and IS. Observed retention time was about 1.56 and 1.97 min for RIS and IS, respectively (Fig.1).

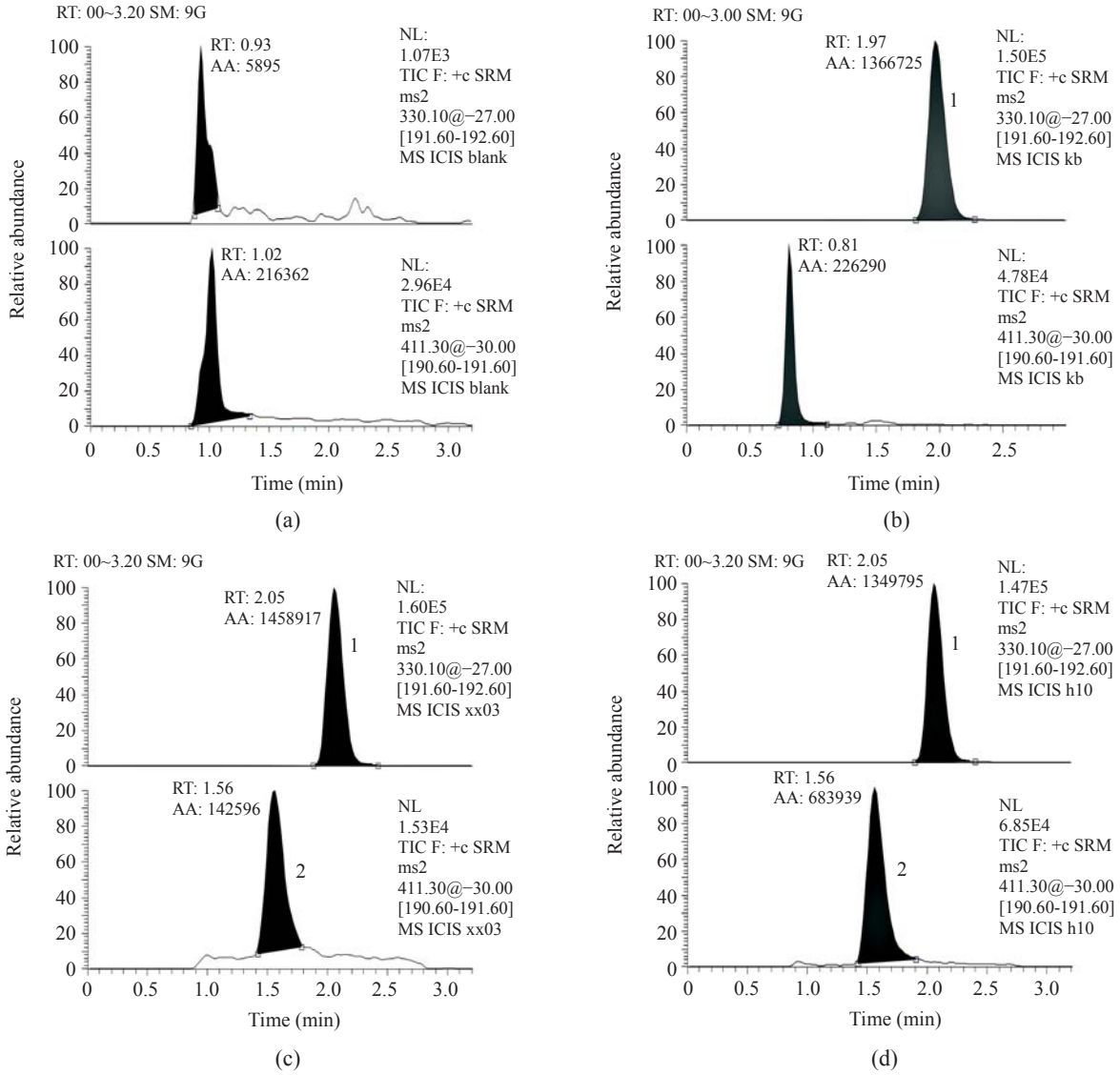

Fig.1 Representative LC-MS/MS chromatograms from human serum. (a) Blank serum; (b) Blank serum spiked with 60 ng/ml of IS; (c) Blank serum added with 1.0 ng/ml of RIS and 60 ng/ml of IS; (d) Serum sample from a subject 8 h after receiving 2 mg RIS tablets, and added with 60 ng/ml of IS (the assayed concentration of RIS was 4.97 ng/ml)

1: 60 ng/ml of IS; 2: RIS. RT: Retention time; AA: Area of peak

2. Linearity

The calibration curve of RIS in serum was linear in the range of 0.25~50.00 ng/ml. The average regression equation of calibration curve was:

y=(0.101±0.0039)x+(0.005±0.0007) (n=5, r=0.9999).

Its correlation coefficient showed good linearity. The accuracy of each point on the standard curve was greater than 96.45%. This result shows the usefulness of the present LC-MS/MS method in the assay of RIS from low to high serum levels. The limit of quantification (LOQ) of RIS (signal-to-noise ratio of 3) in serum was determined to be approximately 0.25 ng/ml.

3. Precision and accuracy

The intraday precision showed a *RSD* (%) of 1.85%~9.09% for RIS. The interday *RSD* (%) was 1.56%~4.34%. The intraday and interday accuracies for RIS were 88.80%~106.84% and 91.20%~103.76%, respectively. The data proved good precision of the developed LC-MS/MS method (Tables 1 and 2).

Table 1 Intraday and interday precision of LC-MS/MS for determination of risperidone in serum

	Nominal concentration (ng/ml)	Calculated concentration (ng/ml)					Mean±*SD* (ng/ml)	*RSD* (%)
		1	2	3	4	5		
Intraday	0.25	0.25	0.22	0.22	0.20	0.22	0.22±0.02	9.09
	5.00	5.31	5.29	5.43	5.22	5.46	5.34±0.10	1.88
	50.00	50.89	53.37	52.48	52.82	53.07	52.53±0.97	1.85
Interday	0.25	0.22	0.22	0.22	0.24	0.24	0.23±0.01	4.34
	5.00	5.34	5.43	4.97	5.18	5.11	5.21±0.18	3.45
	50.00	52.53	51.22	50.32	51.02	51.34	51.28±0.80	1.56

Table 2 Intraday and interday accuracies of LC-MS/MS for determination of risperidone in serum

	Nominal concentration (ng/ml)	Relative recovery (%)					Mean±*SD* (%)	*RSD* (%)
		1	2	3	4	5		
Intraday	0.25	100.00	88.00	88.00	80.00	88.00	88.80±7.16	8.06
	5.00	106.20	105.80	108.60	104.40	109.20	106.84±2.01	1.88
	50.00	101.78	106.74	104.96	105.96	106.14	105.12±1.97	1.87
Interday	0.25	88.00	88.00	88.00	96.00	96.00	91.20±4.38	4.80
	5.00	106.80	106.80	99.40	103.60	102.20	103.76±3.16	3.04
	50.00	105.06	102.44	100.64	102.04	102.68	102.57±1.60	1.56

4. Recovery

The mean recovery of serum samples from low to high concentrations after treatment was (70.20±3.21)% (0.25 ng/ml), (84.50±2.90)% (5.00 ng/ml), and (82.60±2.74)% (50.00 ng/ml) for RIS. These results suggest that there were no relevant differences in serum treatment recoveries at different concentration levels of RIS.

5. Stability

No significant loss of RIS (*RSD*≤7.50%) was observed after storage of the processed serum samples at room temperature for at least 24 h (Table 3). Serum samples were stable over at least three freeze-thaw cycles (Table 4), indicating that the serum samples can be frozen and thawed at least three times prior to analysis.

Table 3 The stability of risperidone in processed serum (*n*=5)

Nominal concentration (ng/ml)	Found concentration (ng/ml)*			*RSD* (%)
	0 h	10 h	24 h	
0.25	0.22±0.018	0.25±0.009	0.22±0.012	7.20
5.00	5.34±0.096	5.18±0.075	5.01±0.095	3.10
50.00	52.53±0.971	52.51±1.361	50.82±2.517	1.90

*Mean±*SD*

Table 4 Freeze-thaw stability of risperidone in human serum (*n*=5)

Nominal concentration (ng/ml)	Found concentration (ng/ml)*			*RSD* (%)
	Day 0	Day 10	Day 15	
0.25	0.22±0.017	0.24±0.009	0.26±0.012	7.50
5.00	5.34±0.096	4.97±0.064	5.19±0.087	3.60
50.00	52.53±0.971	48.65±0.487	51.15±0.822	3.90

*Mean±*SD*

Clinical application

The developed method was applied to a bioequivalence study of two RIS formulations. Fig.2 shows mean serum concentration-time profiles of RIS after a single administration of 2 mg of either formulation to 18 healthy male Chinese volunteers. Pharmacokinetic analysis of the serum concentration data using PKS gave comparable results for all volunteers. The mean values of the major pharmacokinetic parameters of C_{max}, $AUC_{0\to16}$, $AUC_{0\to\infty}$, t_{max} and $t_{1/2}(k_e)$ of the two RIS formulations in healthy male Chinese volunteers were shown in Table 5. There was no significant difference between the two formulations on the basis of assessment by a two one-sided *t*-test of the data obtained with logarithmically transformed C_{max} and *AUC*. The 95% confidence intervals of test to reference ratio of the $AUC_{0\to16}$, were within the bioequivalence criteria range of 80%~125%, and that of C_{max} was within 70%~143%. Therefore, two products were concluded to be bioequivalent.

Table 5 Pharmacokinetic parameters of risperidone after oral administration (2 mg) of two formulations to 18 subjects*

	t_{max} (h)	C_{max} (ng/ml)	$AUC_{0\to16}$ ((ng·h)/ml)	$AUC_{0\to\infty}$ ((ng·h)/ml)	$t_{1/2}(k_e)$ (h)	K_e (h^{-1})	K_a (h^{-1})	F (%)
Reference	1.28±0.26	16.94±2.31	62.43±16.23	64.65±16.85	3.23±1.05	0.2294±0.0522	4.4992±3.7885	100
Test	1.31±0.30	17.24±2.40	60.37±15.54	62.03±16.16	3.11±0.65	0.2320±0.0496	3.9294±3.4050	96.93±9.27

*Mean±*SD*; K_a: Absorption rate constant; *F*: Relative bioavailability

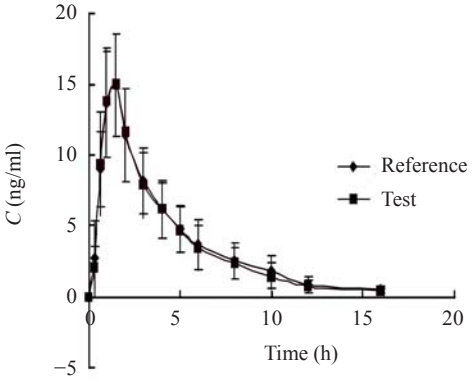

Fig.2 Mean serum risperidone concentration-time profiles following administration of test or reference containing 2 mg of risperidone to 18 healthy subjects
Error bars represent *SD*

CONCLUSION

The LC-MS/MS method for quantifying RIS in human serum has been developed in the present study. Method validation has been demonstrated for selectivity, linearity, sensitivity, precision, recovery and stability. The developed LC-MS/MS method has several advantages compared to the previously reported methods, as it provides simpler sample pretreatment and requires small mobile phase and sample volume and short chromatographic run. The LC-MS/MS method was successfully applied to a pharmacokinetic analysis of RIS in Chinese volunteers.

The results of statistical analysis show that the two formulations of RIS were bioequivalent in 95% confidential limit, and that the relative bioavailability of the test tablets was (96.93±9.27)%. The major pharmacokinetic parameters of RIS obtained in Chinese volunteers are very important for its optimal clinical usage.

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