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Pharmacokinetics of HZ08 in rats by liquid chromatography–tandem mass spectrometry

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Abstract

A selective and sensitive liquid chromatographic method coupled with ion spray tandem mass spectrometry detection (LC–MS/MS) was developed for the determination and pharmacokinetic study of *N*-cyano-1-[(3,4-dimethoxyphenyl)methyl]-3,4-dihydro-6,7-dimethoxy-*N*′-octyl-2(1H)-isoquinoline-carboximidamide (HZ08, a candidate reversing agent for multidrug resistance of cancer) liposome injection in rat plasma. The analyte was extracted from plasma using liquid–liquid extraction by methyl *tert*-butyl ether with drotaverine as internal standard. The chromatographic separation was performed on a Kromasil-C18 column (150 mm × 4.6 mm, i.d., 5 μm) with gradient elution. The tandem mass detection was made with electrospray ionization in positive ion selected reaction monitoring mode with argon collision-induced dissociation. The ion transitions were *m/z* 523.1 to 342.1 for HZ08 at 27 eV and *m/z* 398.1 to 326.1 at 35 eV for the internal standard, respectively. The determination was validated to be accurate and precise for the analysis in the concentration range of 5–10,000 ng/ml for HZ08 with the lower limit of detection (LOD) being 1 ng/ml, when 0.1 ml of rat plasma sample was processed. The main pharmacokinetic parameters found for HZ08 after intravenous (i.v.) administration of its liposome injection at doses of 2, 4 and 8 mg/kg were as follows: C_{max} (4511 ± 681), (5553 ± 1600) and (6444 ± 950) ng/ml, T_{max} (0.033 ± 0), (0.056 ± 0.048) and (0.033 ± 0) h, $t_{1/2}$ (1.75 ± 0.19), (1.63 ± 0.12) and (1.56 ± 0.18) h, AUC_{0-6} (899 ± 112), (1238 ± 190) and (1707 ± 307) h ng/ml, $AUC_{0-\infty}$ (917 ± 110), (1256 ± 189) and (1723 ± 306) h ng/ml, MRT (1.14 ± 0.21), (1.01 ± 0.13) and (1.16 ± 0.17) h, CL (2.90 ± 0.15), (3.01 ± 0.74) and (4.11 ± 0.59) l/h/kg, respectively. The plasma concentration–time profiles of HZ08 were best fitted with two-compartment models. Linear pharmacokinetics was found for HZ08 in rats after intravenous administration of the liposome injection.

Keywords: HZ08; Rat plasma; Pharmacokinetics; LC–tandem MS

1. Introduction

Drug resistance is a common problem in the treatment of cancers. Tumors may become resistant not only to the drugs administered initially, but also to those never have been exposed. It has been reported that, in process of cancer therapy, the resistance of human leukemic cell to anticancer drug, especially multidrug resistance, directly resulted in the failure of chemotherapy. Multidrug resistance (MDR) is a crucial problem in cancer chemotherapy [1]. *P*-glycoprotein, located in the apical membrane of cells as a member of normal physiological barrier, played an important role in the mechanism of cellular MDR [2,3]. These multidrug resistant cell lines have a distinct character of excessive expression of *P*-glycoprotein [4]. Therefore, the administration of active *P*-glycoprotein inhibitors is an effective strategy to enhance intracellular anticancer drug delivery to the tumor cells [5]. A number of non-cytotoxic *P*-glycoprotein inhibitors have been found that sensitize multidrug resistant cancer cells to oncolytic agents. The first-generation inhibitors are drugs originally used for other therapeutic indications including calcium channel blockers (such as verapamil [6]) and immunosuppressive agents (such as cyclosporin A [7]). The second-generation inhibitors developed from the first with reduced original pharmacological activity and higher affinity for *P*-glycoprotein includes valspodar (Psc833) [8] and biricodar (Vx710) [9]. However, an additional problem with most second-generation *P*-glycoprotein inhibitors is that they may significantly alter the plasma pharmacokinet-

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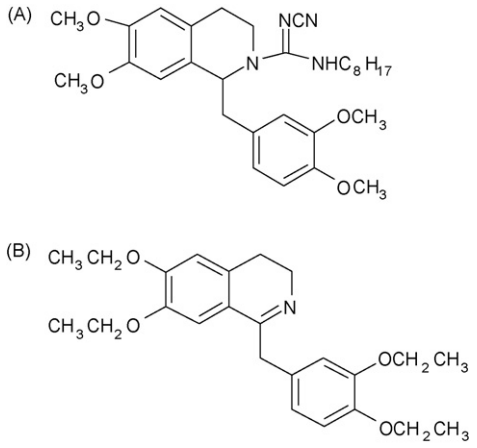

Fig. 1. Chemical structures of (A) HZ08 and (B) drotaverine (internal standard).

ics and/or metabolism of coadministered antitumor agents. The third-generation *P*-glycoprotein inhibitors (such as Zosuquidar (LY335979) [10] and Tariquidar (XR9576) [11]) have been reported without obvious interference with the metabolism of chemotherapy drugs.

Recently *N*-cyano-1-[(3,4-dimethoxyphenyl)methyl]-3,4-dihydro-6,7-dimethoxy-*N*′-octyl-2(1H)-isoquinoline-carboximidamide (HZ08, Fig. 1A), a hydroiso-quinoline, has attracted much attention due to its strong activity in reversing multidrug resistance of cancer cells in vitro[12]. HZ08 is structurally similar with verapamil but shows much lower calcium channel activity. Preliminary studies of our research have shown that HZ08 is more effective than verapamil in the activity of *P*-glycoprotein inhibition (IC50 < 0.4 μM) without obvious cardiovascular side effects which is common with other resistance reversal agents.

In order to explore the resistance reversal activity of HZ08 further, its ADME characteristics should be determined first. Therefore, the rat plasma pharmacokinetics of HZ08 was studied with a specific and sensitive LC–MS/MS method in this research following intravenous (i.v.) administration of an HZ08 liposome injection at doses of 2, 4 and 8 mg/kg, respectively. Although a HPLC-UV method with a limit of detection (LOD) of 20 ng/ml has been reported for the assay and related substances test of HZ08 [13], it was not sensitive and specific enough for this study.

2. Experimental

2.1. Chemicals and materials

The HZ08 liposome injection (HZ08 10 mg, lecithin 250 mg, cholesterol 30 mg, mannitol 700 mg, vitamin E 20 mg) in freeze–dried form for intravenous administration and the chemical reference substance of HZ08 were supplied by the drug research and development center of China Pharmaceutical University (Nanjing, China). The chemical reference substance of drotaverine (internal standard, Fig. 1B) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The HZ08 liposome injection was reconstituted in 5% glucose injection before intravenous administration with concentrations of 0.5, 1.0 and 2.0 mg/ml for 2, 4 and 8 mg/kg dosing groups, respectively.

HPLC grade methanol and methyl *tert*-butyl ether were obtained from Tedia Company Inc. (Fairfield, OH, USA). All other chemicals were of analytical reagent grade from Nanjing Chemical Reagent No.1 Factory (Nanjing, PR China). Water was double distilled before use.

Stock solution of HZ08 (1.0 mg/ml) was prepared in methanol, which was further diluted stepwise with methanol to give a series of standard solutions in the range from 10 to 20,000 ng/ml. The drotaverine solution was prepared in methanol with a concentration of 500 ng/ml. The stock and standard solutions were stable for at least 30 days. They were stored in a cool shaded place when not in use.

2.2. Pharmacokinetic study protocol

The pharmacokinetic studies were conducted on 24 Sprague-Dawley rats (12 male, 12 female, 8–9 weeks old) with mean ± SD body weights of 193 ± 17 g at the time of use. The rats were evenly allocated to one of three groups (A–C). Each rats in groups A–C was given a single intravenous dose of 2, 4 or 8 mg/kg of HZ08 liposome injection, respectively.

The rats were fasted overnight before drug administration with free access to water. Venous blood of about 0.3 ml was collected from the eye bottom in heparinized tubes at 0 (predose), 0.033, 0.083, 0.167, 0.25, 0.33, 0.50, 0.75, 1, 2, 4 and 6 h after the drug administration, respectively. The plasma samples were separated by centrifugation at 1500 × *g* force for 10 min and stored at −20 °C until analysis.

2.3. LC–MS/MS conditions

A Waters Alliance 2695 LC system accompanied with a column oven (Milford, MA, USA) was coupled with a Micromass Quattromicro tandem MS system (Micromass, Manchester, UK) which was equipped with an electrospray ion source and operated with MassLynx 4.0 software.

HPLC separation was performed on a Kromasil-C18 analytical column (150 mm × 4.6 mm, 5 μm) with a Security Guard C18 guard column (4 mm × 3.0 mm, 5 μm) maintained at 30 °C. Gradient elution at a flow-rate of 1.0 ml/min was employed with methanol as mobile phase A, a solution containing ammonium acetate (20 mmol/l) and formic acid (100:0.2, v/v) as mobile phase B, started with 55% A for 2 min, then linearly increased to 95% A by 2.5 min which was kept constant until 5.5 min before restored to 55% A by 5.7 min with a post run equilibration of 2 min. Split injection of 30% of the eluent was introduced into the inlet of the mass spectrometer.

The tandem MS detections were carried out with positive electrospray ionization and multiple reaction monitoring (MRM) of $[M+H]^+$ ions for both HZ08 and drotaverine with argon collision gas at 3.5×10^{-3} Pirani. The product ion scan spectra of $[M+H]^+$ ions for both HZ08 and drotaverine were shown in Fig. 2. The MRM transitions selected for determination were *m/z* 523.1 to 342.1 for HZ08 and *m/z* 398.1 to 326.1 for

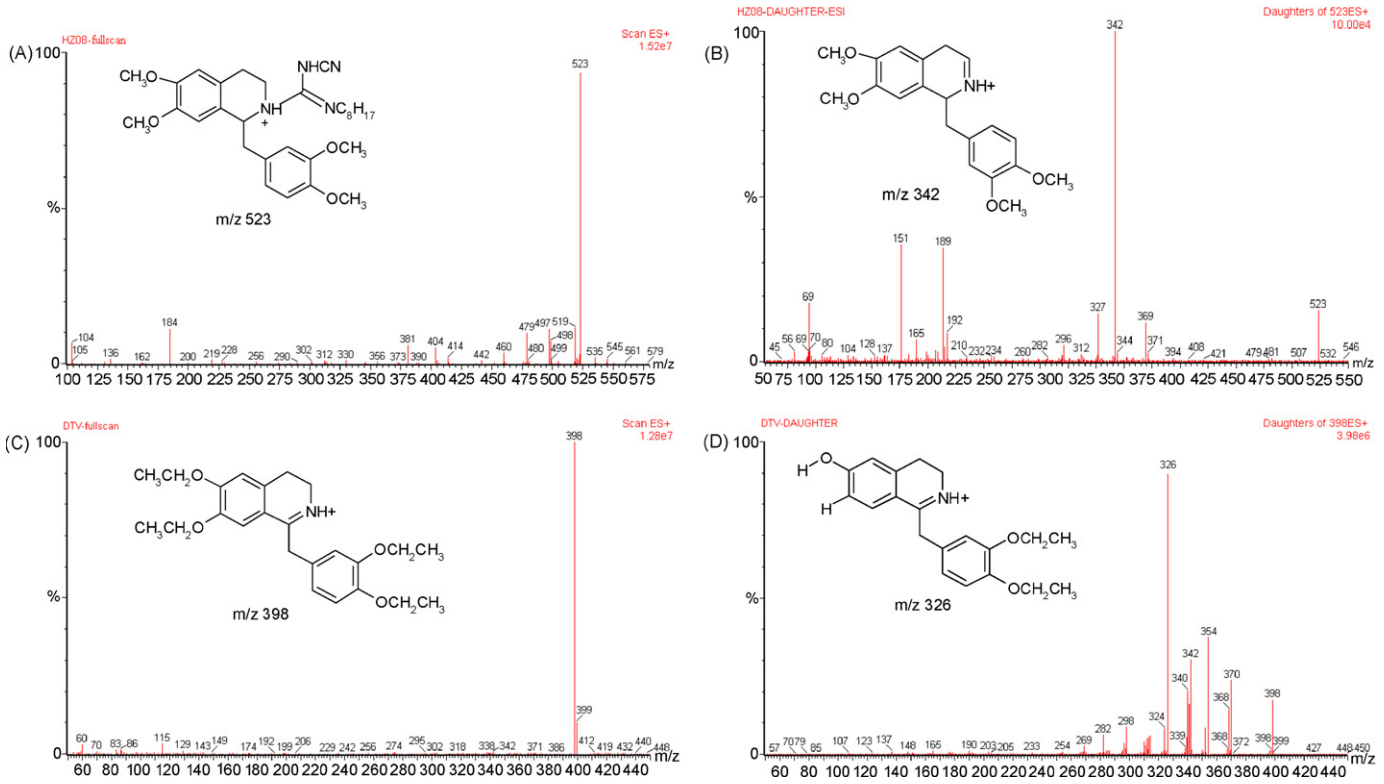

Fig. 2. Positive ion electrospray mass spectra of (A) protonated molecular ion at *m/z* 523.1 for HZ08 with major daughter ion (B) at *m/z* 342.1; (C) protonated molecular ion at *m/z* 398.1 for drotaverine with major daughter ion (D) at *m/z* 326.1, respectively.

drotaverine. The detection parameters were optimized as following: source temperature 100 °C, nitrogen desolvation gas 350 °C with a flow rate of 500 l/h, cone voltage 40 V for both HZ08 and drotaverine, the argon collision energy 27 eV for HZ08 and 35 eV for drotaverine.

2.4. Sample preparation

Plasma samples were thawed and vortex-mixed before use. To an aliquot of 0.1 ml plasma sample in a glass tube with stopper, 0.2 ml 0.9% sodium chloride and 50 μl internal standard solutions (500 ng/ml) were added and vortex-mixed briefly. The mixture was extracted with 3 ml methyl *tert*-butyl ether by vortex-mixing for 3 min. After centrifugation at 1500 × *g* force for 5 min the organic layer was separated and evaporated to dryness under a gentle stream of nitrogen at 40 °C. The residue was reconstituted in 150 μl of a mixture of mobile phases A and B (60:40) and centrifuged at 8000 × *g* force for 5 min. An aliquot of 20 μl of supernatant solution was injected into the LC–MS/MS system. The same sample handling processes were carried out for linearity, recovery, precision and stability tests.

2.5. Method validation

Specificity test was done by analyzing blank samples obtained from control group. To determine recovery and matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analytes ionization), extracted samples, unextracted reference samples (analytes prepared in methanol) and a set of post-extracted spiked samples all at four concentration levels (5, 50, 500 and 5000 ng/ml, $n = 5$) were analyzed in the same assay run. The recovery was determined by measuring an extracted sample against a post-extracted sample of the peak area ratio (HZ08/IS). The matrix effect was measured by comparing the peak response of the post-extracted spiked sample with that of the unextracted sample containing equivalent amounts of the analytes of interest [14]. The stability of HZ08 in rat plasma at room temperature, long-term storage (−20 °C) and freeze–thaw cycles were investigated with quality control samples of 50 ng/ml.

Calibration reference samples in the range from 5 to 10,000 ng/ml for HZ08 were prepared by mixing an appropriate volume of the standard solutions with 0.1 ml of blank rat plasma. The calibration curves were constructed by plotting the peak area ratios of HZ08 to drotaverine versus the concentrations.

Intra-day and inter-day validation studies for precision and accuracy were performed on four quality control levels (5, 50, 500 and 5000 ng/ml) each with five replicates. The accuracy of the method was expressed as the relative error (RE %) obtained by calculating the percentage difference between the measured and spiked concentration over that of the spiked value. The precision was denoted by the relative standard deviation (RSD%).

2.6. Pharmacokinetics

The maximum plasma concentrations (C_{max}) and their time of occurrence (T_{max}) of HZ08 were obtained directly from the observed data. The area under the plasma concentration–time

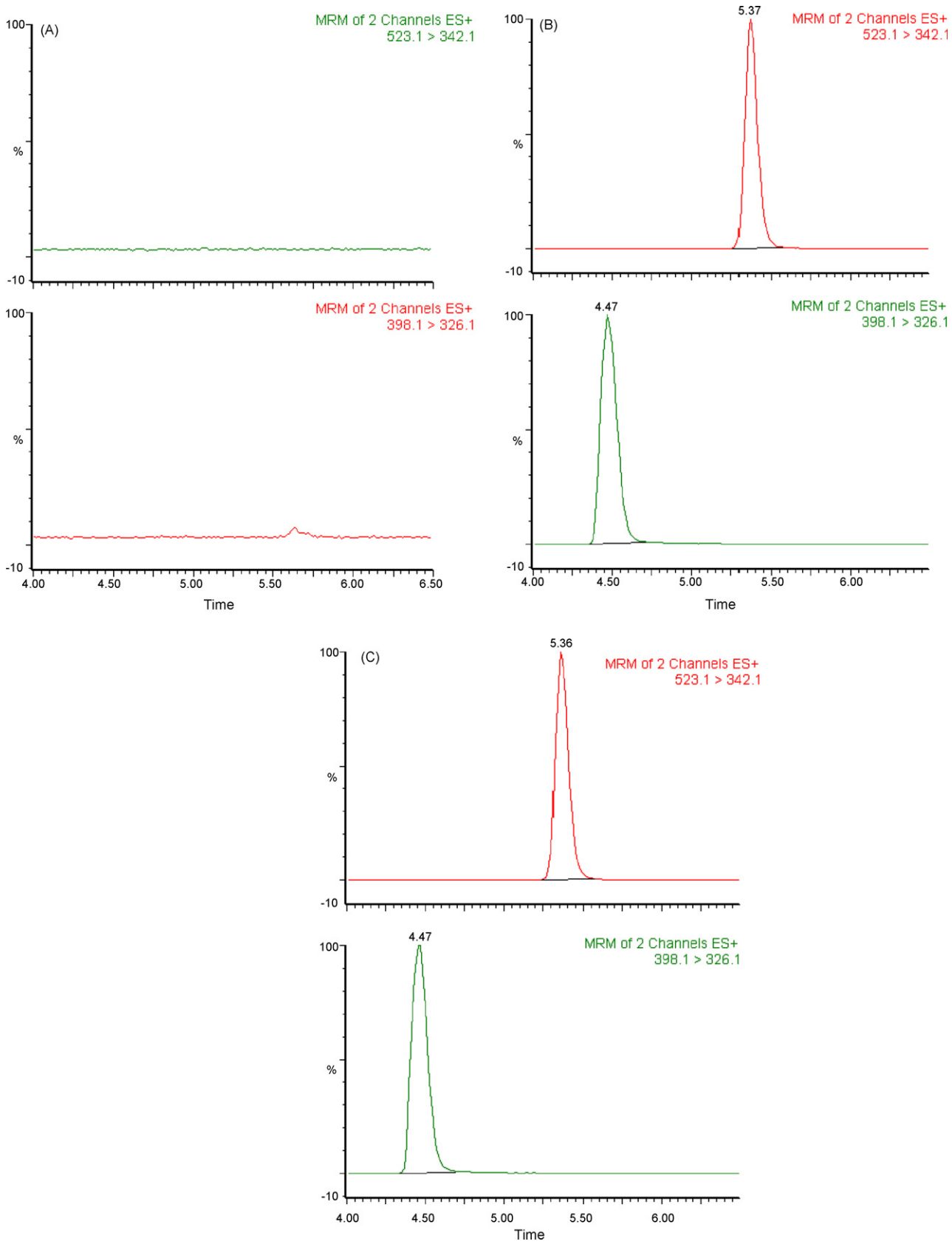

Fig. 3. Representative chromatograms for HZ08 in (A) blank rat plasma, (B) plasma spiked with 500 ng/ml HZ08 (t_R = 5.4 min) and 500 ng/ml drotaverine (t_R = 4.5 min), (C) plasma sample from a rat 0.5 h after i.v. administration of HZ08 liposome injection at a dose of 4 mg/kg (the concentrations of HZ08 was found to be 441 ng/ml).

Table 1
Intra-day ($n = 5$) and inter-day ($n = 3 \times 5$) precision and accuracy for the determination of HZ08 in rat plasma

Concentration (ng/ml)	Intra-day ($n = 5$)		Inter-day ($n = 3 \times 5$)	
	Precision (RSD%)	Accuracy (RE%)	Precision (RSD%)	Accuracy (RE%)
5.0	6.1	2.7	10.4	1.7
50	5.7	−4.7	6.4	−4.4
500	4.8	8.2	7.9	10.3
5000	2.8	1.2	7.0	3.8

curve (AUC) from the time zero to the last measured concentration ($AUC_{0–6}$) was calculated according to the linear trapezoidal rule. The terminal elimination rate constant (λ_Z) was calculated by least-square regression of the plot of logarithms of concentration against time for the last five measurable points, the terminal half-life was calculated with $t_{1/2} = 0.693/\lambda_Z$ accordingly, and the $AUC_{0–\infty}$ was the corresponding area extrapolated to infinity by $AUC_{0–6} + C_t/\lambda_Z$, where C_t was the last measurable drug concentration. The clearance (CL, l/h/kg) and the volume of distribution (V1, l/kg) were calculated with the statistical momentum method.

3. Results and discussion

3.1. LC–MS/MS determination

The MRM detections were made with $[M + H]^+$ ions for sodium and kalium adduct ions were observed with much less sensitivity (Fig. 2). Typical chromatograms were shown in Fig. 3. The retention times of HZ08 and drotaverine were approximately 5.4 and 4.5 min, respectively. Since the analytes were all well retained and separated from the major endogenous materials in the HPLC column, no obvious endogenous interferences and matrix effects on ionization were found. Drotaverine was selected as the internal standard for its similarity in the structure, the retention and ESI ionization conditions to those of HZ08.

Linear calibration curves were obtained for HZ08 ranging from 5 to 10,000 ng/ml with correlation coefficient values greater than 0.99. The limit of detection for HZ08 was 1 ng/ml and the lower limit of quantification was about 5 ng/ml. The intra- and inter-day precision for the analysis of HZ08 in rat plasma samples ranged from 2.8 to 10.4% at levels of 5, 50, 500 and 5000 ng/ml. The intra- and inter-day accuracy ranged from −4.7 to 10.3% (Table 1).

The extraction recoveries of HZ08 from spiked rat plasma determined at different concentration levels with as single-step methyl *tert*-butyl ether liquid–liquid extraction with proved to be simple, rapid and successful with an average recovery rate greater than 85%(Table 2).

Table 2
Recoveries of HZ08 from rat plasma ($n = 5$)

Spiked concentration (ng/ml)	Measured concentration (ng/ml)	Recovery (%)	RSD (%)
5.0	4.9	93.2	4.7
50.0	48.9	89.3	2.9
500	565	89.2	2.3
5000	5221	95.3	2.6

HZ08 in plasma was found to be stable for over 30 days kept at −20 °C, up to three freeze–thaw cycles and at least 12 h at room temperature since no obvious changes was found in the concentrations tested within the time period under the indicated storage conditions.

3.2. Pharmacokinetic studies

The mean plasma concentration–time curves of HZ08 after i.v. administration of the HZ08 liposome injection were presented in Fig. 4. The concentration time profiles conformed to two-compartmental pharmacokinetic models at all three

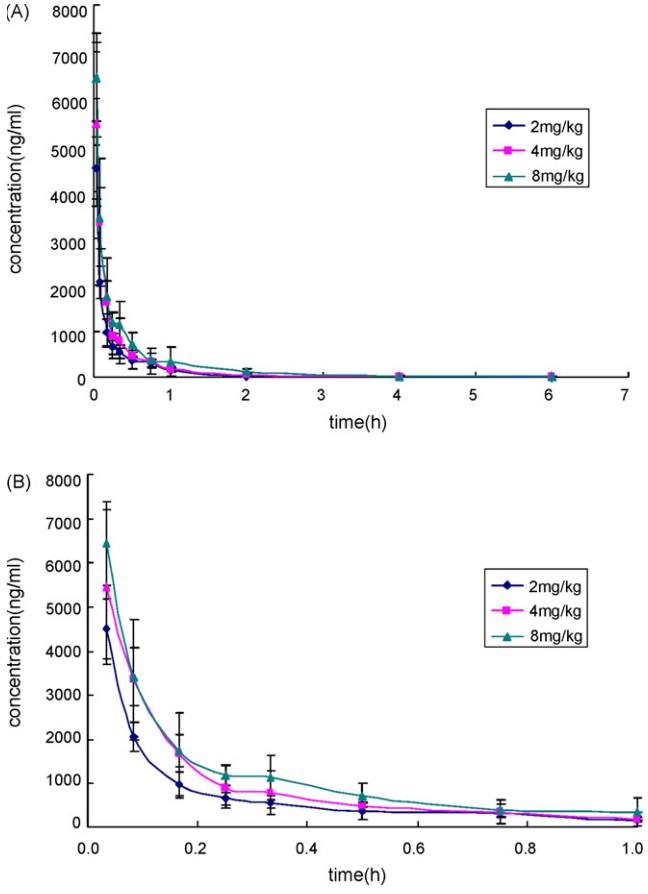

Fig. 4. Mean plasma concentration–time profiles of HZ08 following i.v. administration of HZ08 liposome injection at doses of 2, 4 or 8 mg/kg to rats, respectively. Each point represents the mean ± SD of eight subjects. (A) Full-scale view; (B) blow-up view.

Table 3
Pharmacokinetic parameters of HZ08 after i.v. administration of HZ08 liposome injection at doses of 2, 4 or 8 mg/kg to rats (mean ± SD, $n = 8$)

Parameter	2 mg/kg	4 mg/kg	8 mg/kg
C_{max} (ng/ml)	4511 ± 681	5553 ± 1600	6444 ± 950
T_{max} (h)	0.033 ± 0	0.056 ± 0.048	0.033 ± 0
MRT (h)	1.14 ± 0.21	1.01 ± 0.13	1.16 ± 0.17
$t_{1/2}$ (h)	1.75 ± 0.19	1.63 ± 0.12	1.56 ± 0.18
V1 (l/kg)	0.77 ± 0.61	0.71 ± 0.75	1.09 ± 0.33
CL (l/h/kg)	2.90 ± 0.15	3.01 ± 0.74	4.11 ± 0.59
AUC_{0-6} (h ng/ml)	899 ± 112	1238 ± 190	1707 ± 307
$AUC_{0-\infty}$ (h ng/ml)	917 ± 110	1256 ± 189	1723 ± 306
K_{10} (h^{-1})	4.52 ± 3.41	5.88 ± 4.71	4.26 ± 1.67
K_{12} (h^{-1})	7.19 ± 8.01	7.78 ± 8.11	8.17 ± 8.56
K_{21} (h^{-1})	2.03 ± 1.74	1.69 ± 1.04	2.51 ± 2.69

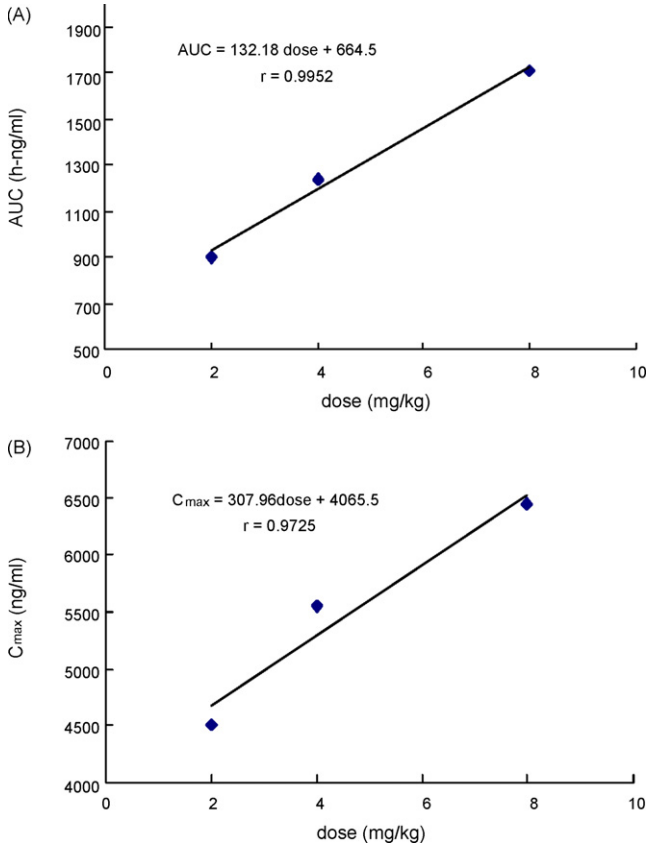

Fig. 5. Mean values of AUC-dose (A) and C_{max}-dose (B) profiles of HZ08 following i.v. administration of HZ08 liposome injection at doses of 2, 4 or 8 mg/kg to rats, respectively.

doses. The estimated pharmacokinetic parameters were shown in Table 3. There were good linear correlations between doses (2–8 mg/kg) and the mean values of AUC and C_{max}, respectively (Fig. 5). ANOVA analyses showed that all the other pharmacokinetic parameters did not have significant differences between doses. Therefore, linear pharmacokinetics was found for HZ08 in rats after intravenous administration of the liposome injection.

4. Conclusions

The pharmacokinetics of HZ08 liposome injection in rat plasma was studied with a validated selective and sensitive LC–MS/MS method. Linear pharmacokinetics of HZ08 in rats after intravenous administration of its liposome injection at doses of 2, 4 and 8 mg/kg was found.

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