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ORIGINAL RESEARCH

Selective and sensitive determination of bis(7)-tacrine, a high erythrocyte binding acetylcholinesterase inhibitor, in rat plasma by high-performance liquid chromatography–tandem mass spectrometry

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ABSTRACT: The current study aims to develop a specific and sensitive LC-MS/MS method for determination of bis(7)-tacrine (B7T) in rat plasma. A 100 μL plasma sample was extracted with ethyl acetate. B7T and the internal standard (IS), pimozide, in the samples were then analyzed with LC-MS/MS in positive electrospray ionization condition. Chromatographic separation of B7T and IS was achieved in a C_{18} reversed-phase HPLC column (150 × 2.1 mm i.d.) by isocratic elution with a mobile phase consisting of 0.05% formic acid in water and acetonitrile (1:1, v/v) at a flow rate of 0.35 mL/min. Multiple-reaction monitoring (MRM) mode was employed to measure the ion transitions: *m/z* 247 to 197 for B7T and *m/z* 462 to *m/z* 328 for IS, respectively. The method was linear over the studied ranges of 100–5000 and 10–100 ng/mL. The intra-day and inter-day variations of the analysis were less than 6.8% with standard errors less than 9.0%. The detection limit of B7T in rat plasma was 1 ng/mL. The developed method was successfully applied to the pharmacokinetic study of B7T after intravenous administration of 1 mg/kg B7T and further proved to be readily utilized for determination of B7T in rat plasma samples. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: bis(7)-tacrine; Alzheimer's disease; LC-MS-MS; rat plasma

INTRODUCTION

Alzheimer's disease (AD) is a progressive neuro-degenerative disease characterized by deterioration of cognitive function, dementia, memory loss, and altered behavior (Tariot, 2001; Bird, 1998). Acetylcholinesterase inhibitors, which could increase the amount and residence time of acetylcholine at acetylcholine receptors within the brain, are the primary medications used to treat AD (Wilkinson *et al.*, 2004). Tacrine, an acetylcholinesterase inhibitor approved in the early 1990s, has been demonstrated to have hepatic adverse events and is no longer used extensively in clinical practice (Watkins *et al.*, 1994). Using a computer docking program, a dimeric tacrine analog linked with heptylene chain, bis(7)-tacrine (B7T), has been designed and synthesized. Pharmacological studies have demonstrated that B7T is much more potent and selective on acetylcholinesterase than tacrine (Wang *et al.*, 1999; Xiao *et al.*, 2000; Wu *et al.*, 2000). In addition, B7T was recently found to be able to protect against glutamate-induced neurotoxicity (Li *et al.*, 2005) and inhibit nitric oxide synthase (Li *et al.*, 2006).

The satisfactory biological effects of B7T encourage us to further investigate the pharmacokinetic properties of the compound. A sensitive and selective analytical method is essential for the pharmacokinetic study. Previous studies have reported the detection methods of B7T in phosphate buffer using HPLC/UV detection

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Abbreviations used: AD, Alzheimer's disease; B7T, bis(7)-tacrine; MRM, multiple-reaction monitoring.

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(Patani *et al.*, 2005) and in rat blood using ion trap mass (Yu *et al.*, 2007). The limit of quantification was 1.5 μg/mL for the HPLC/UV method, which may not be sensitive enough for the *in vivo* study. Although Thermo-Finnigan LCQ Advantage ion trap mass detector (Thermo, San Jose, CA, USA) was first tried in our research group for the analysis of B7T in blood, the selectivity may be limited by the detection method. Owing to the low fragmentation energy provided by the machine, no product ion of B7T was produced and the detection was only based on the molecular ion of B7T, resulting in a limit of quantitation (LOQ) of 42.3 ng/mL (Yu *et al.*, 2007). In addition, our preliminary study demonstrated that there might be very significant erythrocyte binding of B7T, implying that the *in vivo* plasma concentration of B7T would be expected to be much lower than its blood concentration. Since it is well known that drugs partitioning into the erythrocytes may not exert any pharmacological effects (except for those targeting the erythrocytes, such as antimalarials), it is necessary to establish a more sensitive and selective analytical method for the determination of plasma concentrations of B7T *in vivo*.

In the present study, we aim to develop a more selective and sensitive LC/MS/MS method by using Triple Quadrupole Q-TRAP to determine concentrations of B7T in rat plasma. Moreover, we plan to evaluate the extent of erythrocytes binding and pharmacokinetics of B7T in detail using the developed method to estimate the concentrations of B7T in plasma after intravenous administration to rats.

EXPERIMENTAL

Chemicals and materials. Bis(7)-tacrine dihydrochloride was synthesized as described previously (Pang *et al.*, 1996). Pimozide used as internal standard (IS) was purchased from Sigma Chemical Co. (St Louis, MO, USA; Fig. 1). Acetonitrile (HPLC grade) was obtained from Labscan (Labscan Asia, Thailand). All of the other reagents were of analytical grade or HPLC grade. Distilled and deionized water was used for the preparation of all solutions.

Chromatographic conditions. The HPLC system was composed of Perkin-Elmer PE-200 series micro-pumps and auto-sampler (Perkin-Elmer, Norwalk, CT, USA). The chromatographic separation of B7T, internal standard and potential endogenous interference from plasma was achieved by C_{18} revered-phase HPLC column (150 × 2.1 mm i.d., 5 μm particle size, Agilent), protected by a guard column (12.5 × 2.1 mm i.d., 5 μm particle size, Agilent). The HPLC column was eluted by a mixture of 0.05% formic acid in water and acetonitrile (1:1, v/v) at a flow rate of 0.35 mL/min.

Mass spectrometric conditions. B7T and internal standard were analyzed with a Perkin-Elmer Sciex API Q-Trap (Applied Biosystems, Toronto, Canada). The mass spectrometer was operated using electrospray ionization in positive mode with the following parameters. The ionspray voltage was 5500 V. The main ion optics parameters were: declustering potential (DP) 39 V, entrance potential (EP) 9 V and collision cell exit potential (CXP) 4 V. In MS/MS studies, the collision energy (CE) was set at 39 V. The flow rates of the curtain and nebulizer gases were 40 and 50 psi, respectively. Auxiliary gas was 80 psi, and the source temperature was 400°C. Quantitative determination of B7T was performed by using the instrument in the multiple reaction monitoring (MRM) mode to measure the following ion transitions: *m/z* 247 $[M + 2H]^{2+}$ to *m/z* 197 (product ion) for B7T and *m/z* 462 $[M + H]^{+}$ to *m/z* 328 (product ion) for IS.

Preparation of standards and quality control samples. Stock solution of B7T (1 mg/mL) was prepared in methanol. Working solutions of B7T were prepared by appropriate dilution with mobile phase to yield concentrations of 50, 20, 10, 5, 2, 1, 0.5, 0.2, 0.1 and 0.05 μg/mL, respectively. The IS was prepared in a mixture of 50% methanol in water (v/v) to reach a concentration of 30 μg/mL.

The samples for plasma calibration curves were prepared by spiking the blank rat plasma (100 μL) with 10 μL of the appropriate working solutions to yield concentrations of 5000, 2000, 1000, 500, 200, 100, 50, 20, 10 and 5 ng/mL. Calibration curves were plotted by the peak-area ratios of analyte/internal standard vs concentrations of analyte in plasma. In order to avoid undue bias to the low concentrations from the high concentrations in the standard curve, the calibration curves were plotted in two ranges: 5000–100 and 100–5 ng/mL. Specific quality control samples of B7T representing low, medium and high concentrations at 10, 100 and 2000 ng/mL as well as LOQ (5 ng/mL) were also prepared to validate the analytical method.

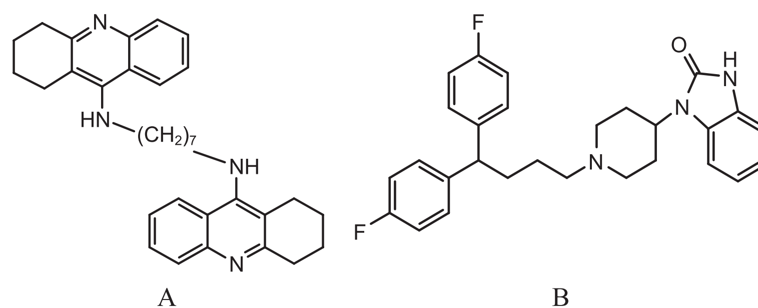

Figure 1. Chemical structures of bis(7)-tacrine (A) and internal standard pimozide (B).

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Sample preparation. A 100 μL aliquot of plasma sample was spiked with 10 μL mobile phase and 50 μL IS, followed by alkalization with 25 μL of 0.1 M NaOH. B7T and IS in the plasma were extracted with 1 mL ethyl acetate by vortexmixing for 1 min. After centrifuging at 16,000***g*** for 10 min, the organic supernatant of ethyl acetate was obtained. The ethyl acetate was evaporated to dryness in another 1.5 mL Eppendorf tube with a Centrivap concentrator. The residue was reconstituted with 100 μL mobile phase and centrifuged at 16,000***g*** for 10 min. An aliquot of 10 μL of the supernatant was injected into the HPLC column for analysis.

Method validation. The specificity of the method was investigated by comparing the chromatograms of blank plasma with the plasma samples collected after i.v. administration of B7T, or with that obtained from the blank plasma spiked with authentic standard of B7T.

The intra-day precision was determined within one day by analyzing five replicates of quality control (QC) samples at concentrations of 10, 100 and 2000 ng/mL. The inter-day precision was determined on five separate days for the QC samples. The intra-day and inter-day precision was defined as the relative standard deviation (RSD) and the accuracy was determined by calculating the relative error (RE).

The extraction recovery was estimated by comparing the peak areas of analyte of pre-spiking with those of post-spiking samples (Korfmacher, 2005). Pre-spiking the sample means spiking B7T and IS to yield the same concentrations as QC samples before sample preparation, while post-spiking means spiking neat solutions of B7T and IS at the same concentrations as QC samples into the extracted blank plasma samples. The matrix effect was estimated by comparing the peak areas of analyte of post-spiked samples with neat solutions of B7T and IS.

The limit of detection (LOD) was defined as the lowest concentration of the drug resulting in a signal-to-noise ratio of 3:1. The LOQ was defined as the lowest concentration of spiked plasma samples that can be determined with sufficient precision and accuracy, i.e. RSD less than 20% and relative error of –20–20% (Shah *et al.*, 2000).

Freeze–thaw stability of the plasma samples was evaluated by exposing quality control samples to three freeze–thaw cycles before sample preparation. The stability of the prepared samples in the auto-sampler was evaluated by analyzing extracted quality control samples after placing in the auto-sampler at room temperature for 24 h.

Erythrocyte binding of B7T. The fresh rat blood was incubated at 37°C with B7T at final concentrations of 1000, 500, 100 and 50 ng/mL for 30 min. Afterward, the blood samples were centrifuged at 16,000***g*** for 10 min to obtain the plasma. The concentrations of plasma were measured with LC-MS-MS. The binding of B7T with erythrocytes was calculated as follows:

$$\text{Fraction of drug in erythrocytes} = 1 - 0.55 \times C_p/C_{blood}$$

(Sun *et al.*, 1987)

where C_p and C_{blood} represent the plasma concentration and the blood concentration of the drug respectively.

Pharmacokinetic study. To confirm the further applicability of the method *in vivo*, the developed analytical method was applied to the pharmacokinetic study of B7T after intravenous administration to rats. Male Sprague–Dawley rats weighing 250–280 g were supplied by the Laboratory Animal Services Center at the Chinese University of Hong Kong. The rats were housed in an air-conditioned room under a 12:12 h light–dark cycle. The experiment was conducted after approval by the Animal Ethics Committee of the Chinese University of Hong Kong. One day before the experiment, the rats received surgery for jugular vein cannulation. The rats were first anesthetized with an intramuscular injection of a mixture containing 60 mg/kg ketamine and 6 mg/kg xylazine. The right jugular vein of the rat was exposed and cannulated with a polyethylene tubing (0.4 mm i.d., 0.8 mm o.d., Portex Ltd., Hythe, UK) that was guided subcutaneously out of the body at the back of the rat. After the surgery, the rats were allowed to recover overnight under fasting conditions with free access to water. On the day of the experiment, B7T was dissolved in a mixture of ethanol and saline (2:8, v/v) to reach a final concentration of 5 mg/mL. Six rats received a bolus dose of 1 mg/kg of B7T intravenously via their cannulated tubings. A 200 μL aliquot venous blood sample was withdrawn from the cannulated tubing at 1, 3, 5, 15, 30, 60, 120, 240, 360, 480 and 720 min, respectively. After each sampling, an equal volume of normal saline was replaced via the jugular vein. The collected blood samples were centrifuged at 16,000***g*** for 10 min and the plasma was collected and stored at –80°C until analysis. The plasma concentrations vs time profiles were analyzed with WinNonlin software (Pharsight corporation, Mountain View, CA, USA, Version 2.1). The two-compartmental model was used to estimate the pharmacokinetic parameters including distribution half-life ($t_{1/2\alpha}$), elimination half life ($t_{1/2\beta}$), area under the plasma concentration–time curve from time zero to infinity ($AUC_{0\rightarrow\infty}$), total plasma clearance (CL) and apparent volume of distribution (V_d).

RESULTS AND DISCUSSION

Analytical method development

LC-MS-MS. In the current study, the positive ionization mode was selected to provide a stronger signal intensity of B7T than that of negative mode. As shown in the full-scan spectrum (Fig. 2), the intensity of the protonated molecular ion of $[M + H]^+$ at *m/z* 493 was weak. On the other hand, B7T was preferentially double charged with the base peak $[M + 2H]^{2+}$ appearing at *m/z* 247. In order to maximize the signal intensity, $[M + 2H]^{2+}$ at 247 was selected for further fragmentation. The most abundant ion in the product ion mass spectrum was observed at *m/z* 197 due to the cleavage of tacrine moiety from B7T (Fig. 2). As for the internal standard, the molecular ion $[M + H]^+$ was observed at *m/z* 462 with a predominant production ion at *m/z* 328 (Fig. 2). Taking advantage of the selectivity of MRM over selected ion monitoring (SIM), the ion transitions from 247 to 197 for B7T and from 462 to 328 for the internal standard were selected for analysis. The representative chromatograms of blank plasma, blank plasma samples

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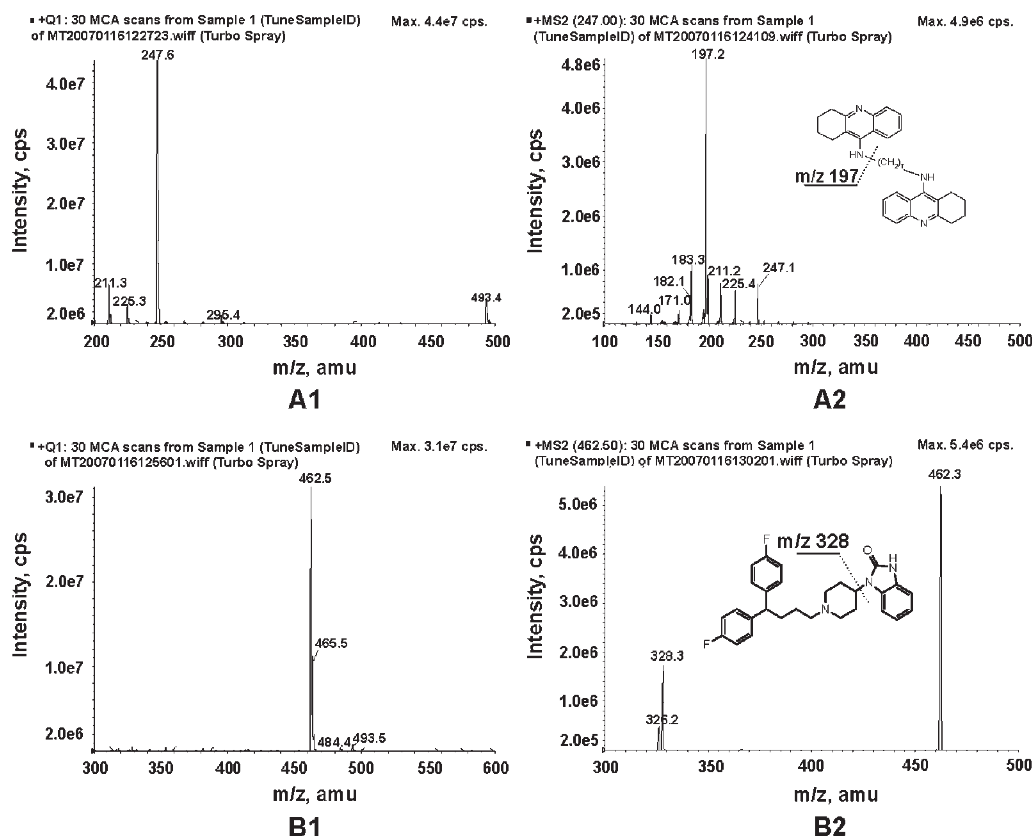

Figure 2. The full-scan mass spectra of B7T (A1) and IS (B1) as well as product ion mass spectra of B7T (A2) and IS (B2).

spiked with B7T, and a plasma sample obtained from a rat following i.v. administration of B7T (1 mg/kg) are shown in Fig. 3. Under the present assay conditions, no endogenous interference from plasma was observed at the retention times of both the analyte and the internal standard.

Precision, accuracy and linearity. The inter-day and intra-day precision and accuracy of the current developed assay are listed in Table 1. The inter- and intra-day RSD for B7T were below 6.8%. The accuracies calculated as the relative error inter- and intra-day at low, medium and high concentrations were within the range 0.64–9.0%. In addition, calibration curves for B7T were linear over both the low and high concentration ranges with regression coefficients (R^2) ranging from 0.9994 to 0.9997.

Extraction recovery, sensitivity, stability. The extraction recoveries of B7T in plasma at low, medium and high concentrations were 78.5, 81.1 and 85.1%, respectively, and the recovery of IS was 87.1%. The endogenous matrix in the extracted samples rendered about 15 and 5% reduction on the signal response of B7T and internal standard, respectively, which are acceptable for analysis.

The limit of quantification (LOQ) was 5 ng/mL and the limit of detection (LOD) was 1 ng/mL for the current assay.

Table 1. Linearity of calibration curve, intra- and inter-day accuracy and precision for determination of B7T in rat plasma

	Intra-day (n = 5)			Inter-day (n = 5)			Linearity	
Nominal concentration (ng/mL)	Determined concentration (mean ± SD, ng/mL)	RSD (%)	Accuracy RE (%)	Determined concentration (mean ± SD, ng/mL)	RSD (%)	Accuracy (%)	Range (ng/mL)	r^2
2000	2019 ± 72	3.54	0.97	1999 ± 91	4.57	−0.07	5000–100	0.9994
100	108.1 ± 6.7	6.18	8.14	103.8 ± 5.2	5.01	3.79		
10	10.4 ± 0.5	4.45	3.65	9.9 ± 0.7	7.19	−0.64	100–10	0.9997
5	5.2 ± 0.2	4.15	3.46	5.5 ± 0.4	6.82	9.01		

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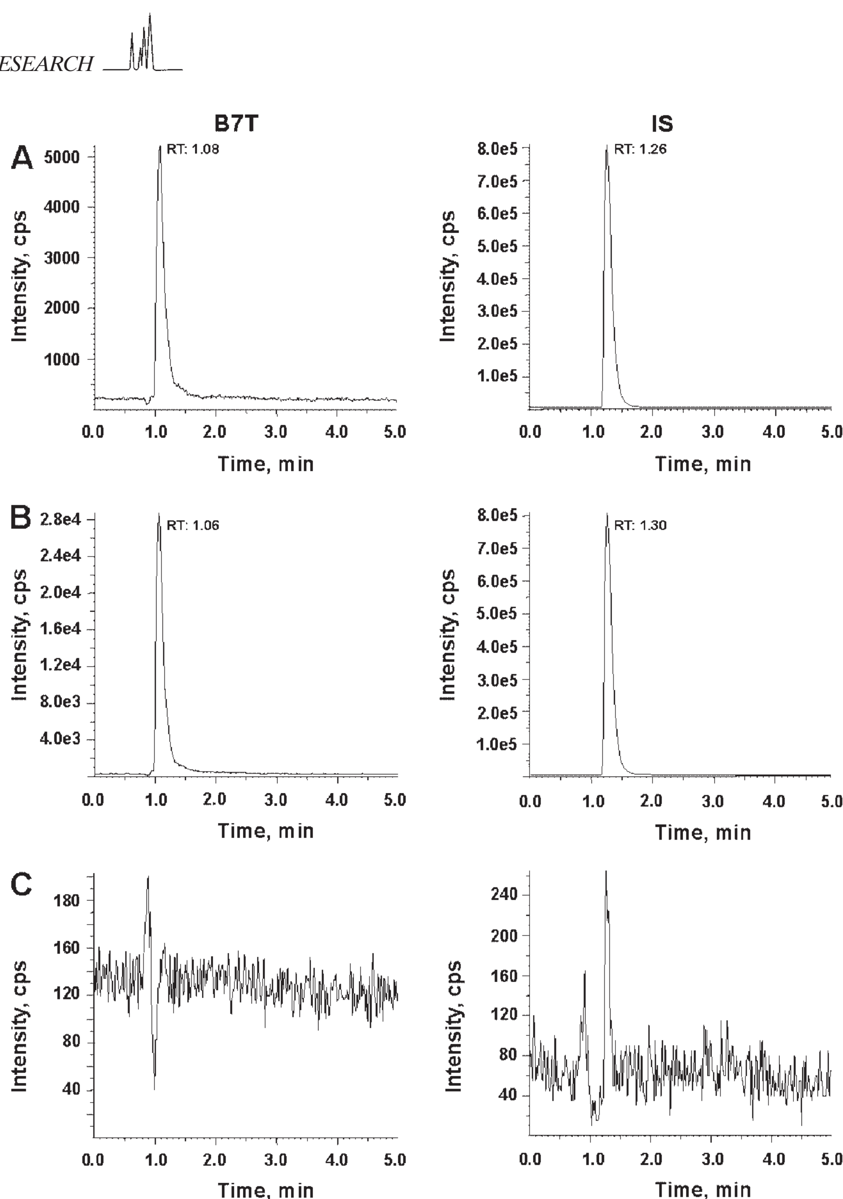

Figure 3. Representative LC-MS-MS chromatograms of QC sample (10 ng/mL of B7T) (A), plasma sample from rat 30 min after i.v. administration (B) and blank plasma sample (C).

The stability experiment indicated that B7T was stable for at least three freeze–thaw cycles (Table 2). In addition, B7T was also found to be stable in the prepared samples after placing in the auto-sampler at room temperature for 24 h (Table 2).

In summary, several improvements of our present analytical methods have been demonstrated over those reported previously. Using MRM mode in analysis, the LOQ (5 ng/mL) of the present method was significantly improved compared with that using HPLC/UV detection (1.5 μg/mL; Patani *et al.*, 2005) and that using previous LC-MS method with detection of only the molecular ion of B7T (42.3 ng/mL; Yu *et al.*, 2007). Moreover, the MRM mode for the current assay also provides better

Table 2. Stability of B7T in plasma after three freeze–thaw cycles and in prepared samples in the auto-sampler for 24 h

Nominal concentration (ng/mL)	After three freeze–thaw cycles Determined concentration (mean ± SD, ng/mL)	In auto-sampler for 24 h Determined Concentration (mean ± SD, ng/mL)
2000	2075 ± 155	2051 ± 56
100	98.6 ± 2.6	101.5 ± 11
10	9.7 ± 0.3	10.6 ± 1.2

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Table 3. Erythrocyte binding of B7T at different blood concentration levels ($n = 3$)

Blood concentrations (ng/mL)	Erythrocyte binding (%)
1000	87.4 ± 0.3
500	90.0 ± 0.5
100	88.6 ± 1.5
50	87.3 ± 1.0

selectivity, which enables a shorter analytical period (5 min) without interference from endogenous matrix in plasma.

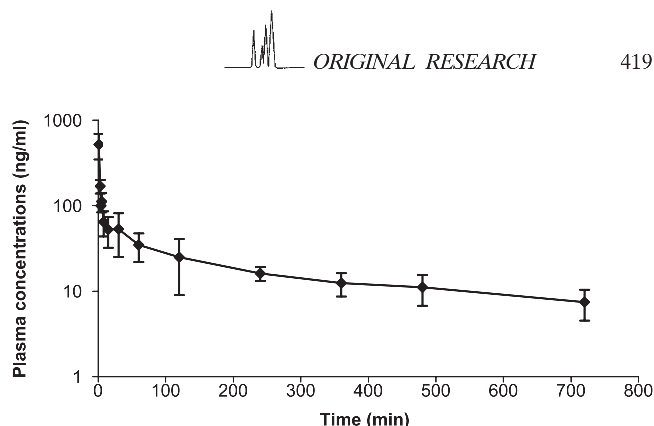

Figure 4. Mean (±SD) plasma concentration–time profile of B7T after i.v. administration at a dose of 1 mg/kg ($n = 6$).

Erythrocyte binding of B7T

As shown in Table 3, over 80% of B7T was bound to the erythrocytes at all the studied blood concentrations levels. Such extensive erythrocyte binding of B7T implied that there must be a significant difference between the blood concentrations and plasma concentrations of B7T. Since the *in vivo* plasma concentrations of B7T would be even lower than its *in vivo* blood concentrations, a more sensitive and selective analytical method for determination of B7T in plasma is essential for the further pharmacokinetic study of B7T.

Application to *in vivo* pharmacokinetic study

The developed analytical method has been successfully applied to determine the concentrations of B7T in plasma samples obtained after i.v. administration of 1 mg/kg B7T to rats. The pharmacokinetic parameters of B7T after i.v. administration of B7T were summarized in Table 4. The plasma concentrations of B7T declined biexponentially, implying that the plasma concentration vs time profile may follow a two-compartment model. As shown in Fig. 4, B7T is rapidly distributed in the body with a $t_{1/2\alpha}$ of 3.1 min, then gradually eliminated out of systemic circulation with a $t_{1/2\beta}$ of 289.3 min. The large volume distribution of B7T (27.1 L/kg) suggested its wide tissue distribution in the body of the rat, including its target tissues such as brain, which definitely needs further investigation. Compared with our previous pharmacokinetic profiles delineating from the blood concentrations of B7T, the distribution half-life of B7T in plasma was even shorter than that in blood (Yu *et al.*, 2007). This may result from ready distribution of B7T into the erythrocytes as proved above. The plasma and blood concentrations of B7T declined in parallel after the distribution phase with a similar elimination half-life of B7T in both blood and plasma. The resulting large volume distribution of B7T obtained from both blood and plasma concentrations consistently indicated the great tendency of B7T binding to various organ tissues *in vivo*. On the other hand, the large difference of the volume distribution of B7T obtained from the plasma concentrations (27.1 L/kg) vs that from the blood concentrations (1.43 L/kg) also implied a high level of erythrocyte binding of B7T.

Table 4. Pharmacokinetic parameters of B7T after i.v. administration to rats (1 mg/kg; $n = 6$)

Parameters	Mean ± SD
AUC (ng/mL · min)	14569.3 ± 4325.5
$t_{1/2\alpha}$ (min)	3.1 ± 1.2
$t_{1/2\beta}$ (min)	289.3 ± 39.5
CL (mL/min/kg)	75.3 ± 23.6
V_d (L/kg)	27.1 ± 7.0

CONCLUSION

Owing to the high erythrocyte binding of B7T, a specific and sensitive LC-MS-MS analytical method for determination of B7T in rat plasma has been developed and validated with satisfactory accuracy and adequate reproducibility. The method has also been successfully applied to estimate the plasma concentration–time profile of B7T in rat after i.v. administration of B7T, indicating that this analytical method can be readily utilized for the determination of B7T in rat plasma samples.

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