Development of a high performance liquid chromatography-tandem mass method for determination of bis(7)-tacrine, a promising anti-Alzheimer's dimer, in rat blood

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Abstract

An analytical method using on-line high performance liquid chromatography-tandem mass spectrometry with electrospray ionization was developed and applied for the quantification of bis(7)-tacrine (B7T) in rat blood. B7T and pimozide (internal standard, IS) were extracted in a single step from $100\,\mu l$ of alkalized blood with ethyl acetate. Analytes were separated using an Extend C-18 column at $25\,^\circ C$. The elution was achieved isocratically with a mobile phase composed of 0.05% aqueous formic acid and acetonitrile (60:40, v/v) at a flow rate of 0.35 ml/min. Quantification was achieved by monitoring the selected ions at m/z 247 for B7T and m/z 462 → m/z 328 for pimozide. Retention times were 1.45 and 2.23 min for B7T and IS, respectively. Calibration curves were linear in the range from 86.4 to 2160.0 ng/ml. The established method is rapid, selective and sensitive for the identification and quantification of B7T in biological samples. The assay is accurate (bias <10%) and reproducible (intra- and inter-day variation <10%), with detection and quantification limit of 3.6 and 42.3 ng/ml, respectively. Furthermore, it was successfully applied for the pharmacokinetic measurement of B7T in rat with a single intravenous administration at 0.3 mg/kg.
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1. Introduction

First described by Alois Alzheimer in 1907, Alzheimer's disease (AD) is the most common type of dementia and the multifaceted neurodegenerative disorder, affecting millions of elderly people worldwide [1]. This disease ranks fourth as a leading cause of death in developed countries and has become a growing health problem as the population is aging. The current global prevalence of this type of disease is estimated to be 24 million and forecast to double every 20 years to 81 million in 2040 [2]. Although scientists are learning more about AD every day, the exact causes of AD remain unclear and there is no available cure. To date, the therapeutic strategy for AD focuses on addressing cholinergic and glutamatergic abnormalities through the use of acetylcholinesterase inhibitors

(AChEIs) and moderate-affinity *N*-methyl-D-aspartate (NMDA) receptor antagonists [3,4]. The use of AChEIs, such as tacrine (THA), donepezil, rivastigmine, galanthamine and huperizine A for treating AD is based on the link between cholinergic dysfunction and AD severity [5,6]. However, these AChEIs have had limited success in memory enhancements in moderate to severe AD patients and cannot stop the process of neurodegeneration [7,8]. Although memantine possesses neuroprotective effects, it has limited memory enhancement action. Encouragingly, well-controlled trials of memantine/donepezil dual therapy have shown superior efficacy in moderate to severe AD patient subgroups [9]. Therefore, developing novel anti-AD agents with efficacious properties is now very promising in AD treatment.

With the identification of the three-dimensional structure of AChE and the assistance of computer docking program, a novel class of THA analogs has been developed by linking two THA molecules with carbon chains of different lengths [10,11]. These dimeric agents are much easier to produce and have been shown to be more potent than THA. Bis(7)-tacrine (B7T) is one of the dimeric drugs which is developed in our research group and co-workers [10] and shows significant increases in ACh levels by inhibiting AChE in the hippocampus [12,13], cortex [14] and amygdale [15]. Our previous pharmacological studies indicate that B7T is more selective and potent on AChE inhibition than THA both in vitro and in vivo [16–19]. Recently, B7T has further been demonstrated to protect against glutamate-induced neurotoxicity via blocking NMDA receptors [20] and inhibiting nitric oxide synthase [21] and β-amyloid-induced apoptosis by regulating L-type voltage-dependent calcium channels [22]. The multi-target actions may synergistically increase the therapeutic effects of B7T in the treatment of AD [23].

Although B7T is a promising neuroprotective dimer for AD treatment with strong AChE inhibition, its pharmacokinetic fate has not been studied, and no suitable blood drug concentration monitoring method is available. To find a convenient, rapid, selective and sensitive method for the quantification of B7T in blood, in this study, a high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) was developed, validated and successfully applied to monitor the blood concentration of B7T after intravenous administration to rats.

2. Materials and methods

2.1. Chemicals

Bis(7)-tacrine dihydrochloride (B7T) was synthesized according to our previous publication [10], and the purity of the compound was up to 99.9% by HPLC analysis. Pimozide, sodium carbonate, ethyl acetate, acetonitrile and formic acid were all purchased from Sigma Chemicals Ltd. (St. Louis, MO, USA) as analytical grade. Methanol used was of analytical grade and purchased from Merck Company (Darmstadt, Germany). Double distilled water was prepared by an EASYpure UV system (model D7401; Barnstead Thermolyne Co. Dubuque, IA, USA).

2.2. Animals

Male Sprague–Dawley rats (6–8 weeks age) supplied by the Animal and Plant Care Facility at the Hong Kong University of Science and Technology were fed on a standard laboratory diet with free access to water under a controlled temperature at 20–22 °C and relative humidity of 50% with 12 h light/12 h dark cycle prior to the study. Before experiment, rats were surgically cannulated with polyethylene catheters on the right jugular veins under anesthesia with diethyl ether vapor. The animals recovered in individual metabolic cages and fasted but were allowed to have free access to water overnight.

2.3. HPLC-MS/MS

Chromatography was performed using a Finnigan P4000 quaternary pump with an AS3000 autosampler injector (all from Thermo Separation Products, Thermo, San Jose, CA, USA), and an Agilent Extend C-18 analytical column (150 mm × 2.1 mm i.d., 5 μm) coupled with an Agilent Extend C-18 guard column (12.5 mm × 2.1 mm i.d., 5 μm) maintained at 25 °C. The isocratic elution with a mobile phase of 0.05% aqueous formic acid and acetonitrile (60:40, v/v) was used at a flow rate of 0.35 ml/min.

The compounds were detected by an on-line Thermo-Finnigan LCQ Classic ion trap mass spectrometer (Thermo) equipped with an electrospray ionization (ESI) interface. The Thermo-Finnigan Xcalibur software was used for system control and data acquisition. Nitrogen was employed as the sheath and sweep gas at a pressure of 60 and 20 arbitrary units, respectively. The ESI source was operated in the positive ionization mode, and an ion-spray voltage of +4.5 kV was applied. The capillary temperature was set to 200 °C under a voltage of +40 V. The determination of B7T and pimozide (internal standard, IS) were performed by HPLC-MS/MS in full-scan mode (m/z 150–550) and MS/MS spectra were produced by collision-induced dissociation (CID) of each molecular ion interested, using a normalized collision energy of 55%. For the quantification, the selected reaction monitoring (SRM) mode was used for monitoring B7T (m/z 247) and pimozide (IS, m/z 462 → m/z 328), respectively.

2.4. Sample preparation and calibration curve

Concentrated stock solution of B7T was prepared by dissolving the appropriate amount of the sample in water at a concentration of 1.0 mg/ml and then further diluted with water to give appropriate working solutions used to prepare the calibration curve. About 60 μg/ml of pimozide (IS) solution was prepared by dissolving the appropriate amount of authentic sample in methanol. All prepared solutions were stored at 4 °C in amber glass tubes. Calibration curve was prepared by spiking B7T into blank rat blood (100 μl) to yield final concentrations of 86.4, 172.8, 259.2, 345.6, 432.0, 864.0, 1296.0 and 2160.0 ng/ml. The spiked blood samples were extracted with 1.0 ml of ethyl acetate after addition of 50 μl IS solution and 100 μl 0.2 M sodium carbonate solu-

tion (pH 12.6). The mixture was vortex-mixed for 1 min, and then centrifuged with a C0160-B Microcentrifuge (Labnet International Inc., NJ, USA) at 3000 rpm for 15 min. The organic layer (0.9 ml) was transferred into a 1.5 ml eppendorf tube and evaporated to dryness under a nitrogen stream at 25 °C. The residues were re-constituted into 180 μl of the chromatographic mobile phase (0.05% formic acid:acetonitrile, 60:40, v/v), and 20 μl were injected into the HPLC-MS/MS for analysis. The calibration curve was constructed by plotting the blood concentration of B7T as a function of peak area ratio of B7T to IS.

2.5. Method validation

2.5.1. Precision, accuracy and recovery

The intra-day precision was determined within one day by the B7T spiked blood samples with concentrations corresponding to low (86.4 ng/ml), medium (432.0 ng/ml) and high (2160.0 ng/ml) levels in calibration range ($n = 6$). The inter-day precision was determined on five separated days using the similarly prepared spiking samples. Absolute extraction recoveries from rat blood were evaluated at the corresponding low, medium and high concentration levels of the calibration curve ($n = 6$). All samples were extracted after addition of IS and then analyzed by HPLC-MS/MS as described above. The concentrations of individual samples were determined from the calibration curve. Precision was reported as the relative standard deviation (R.S.D.) and accuracy expressed as [(1 − (mean concentration measured—concentration spiked)/concentration spiked)] × 100%. For the recovery study, B7T water solutions containing IS at appropriate concentrations were also prepared and directly analyzed by HPLC-MS/MS for the control. Recovery was then calculated by comparing the peak areas of spiked blood samples with those of controls.

2.5.2. Sensitivity

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 limit of quantification (LOQ) was defined as the lowest concentration of spiked blood samples that could be determined with sufficient precision and accuracy, i.e. R.S.D. less than 10% and relative error of −10 to 10%.

2.5.3. Stability

The stability of B7T in the mobile phase was evaluated at 2, 4, 8, 12 and 24 h at 25 °C at low, medium and high concentrations of the calibration range ($n = 6$). At the same time, the stability of B7T spiked into rat blood (100 μl) at the same concentrations ($n = 6$) were stored at −20 °C for 7 days, and then extracted and analyzed as described above. The stability was expressed as [(mean concentration determined/concentration spiked) × 100].

2.6. Pharmacokinetics analysis

B7T was administrated to rats ($n = 5$) intravenously (IV) with a single dose of 0.3 mg/kg. Venous blood samples (0.15 ml) were collected at suitable time intervals up to 720 min after dose. At each blood sampling, an equivalent volume of heparinized normal saline (25% v/v, 0.15 ml) was injected into the animals to maintain a constant blood volume. The blood samples were stored at −20 °C, extracted and analyzed by HPLC-MS/MS as described above.

Pharmacokinetic parameters, including area under the blood concentration-time curve profile from zero to infinity ($AUC_{0 \to \infty}$), apparent blood clearance (CL), apparent volume of distribution (V_d), distribution half-life ($t_{1/2\alpha}$), and blood elimination half-life ($t_{1/2\beta}$) were calculated by WinNonlin™ Professional Version 5.0.1 software (Pharsight, Mountain View, CA, USA) with a non-dependent approach.

3. Results and discussion

It is necessary to use an IS to get high accuracy when a mass spectrometer is equipped with HPLC as the detector. As structural alike standard is unavailable, pimozide was adopted in the present study because of its similar physicochemical properties, such as both compounds are weak bases with similar ionization constants, lipophilicity and extraction efficiency. Furthermore, no other endogenous compounds in rat blood produced mass ions at m/z 462 and 328, leading to less endogenous interferences. The structures of B7T and pimozide are shown in Fig. 1.

The MS analysis of B7T was first investigated by direct introduction of the reference compound dissolved in the mixture containing acetonitrile and 0.05% formic acid (40:60, v/v) using electrospray ionization in positive ion mode. The tandem
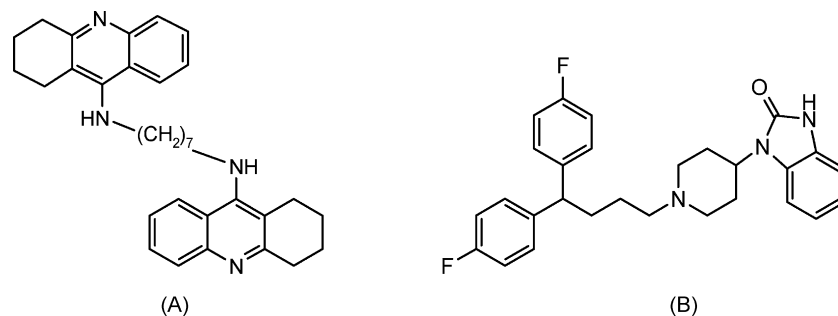

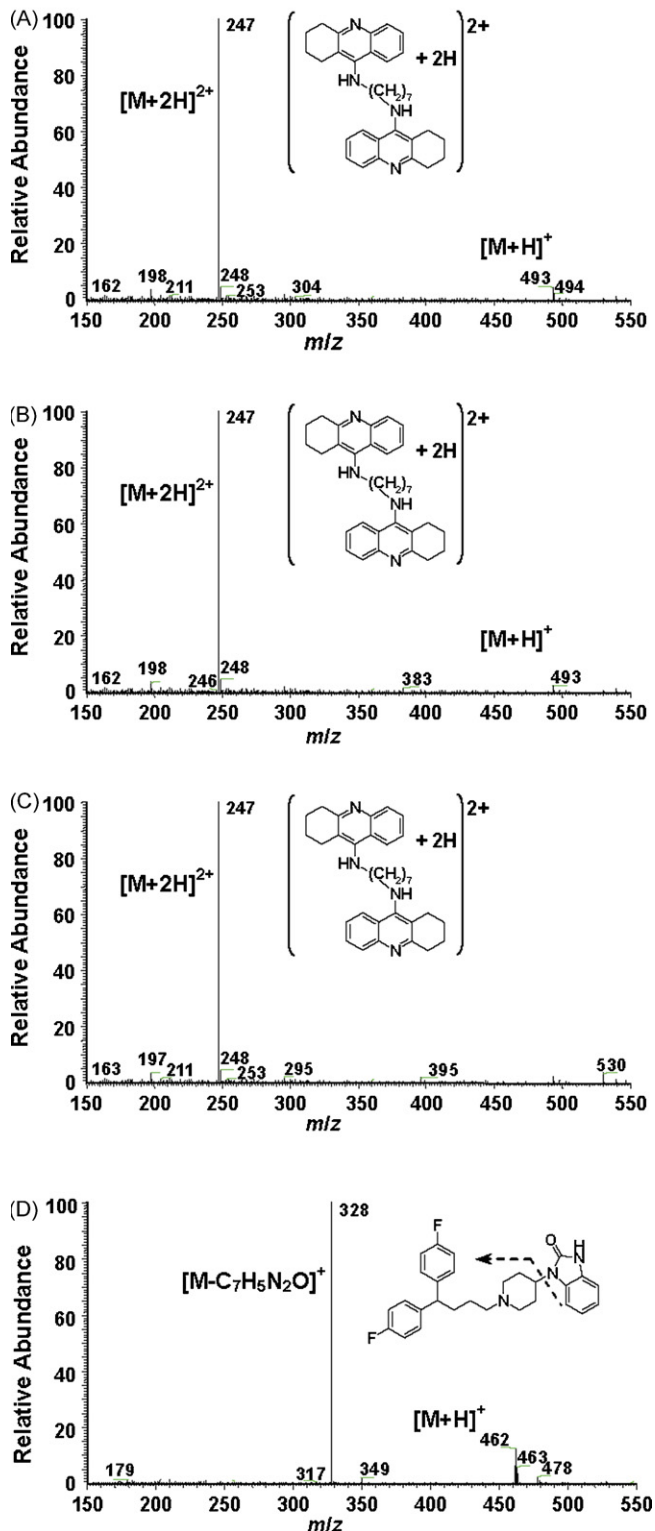Fig. 1. Structures of bis(7)-tacrine (A) and pimozide (B).

Fig. 2. The full-scan ESI-MS/MS spectra for B7T (A–C) and pimozide (D). A, B and C were obtained with different normalized collision energies of 55, 80 and 100%, respectively. D was obtained with normalized collision energy of 55%.

MS/MS spectra of B7T and IS were recorded in full-scan mode (m/z 150–550) and shown in Fig. 2. Only one peak (m/z 247) was found for B7T in the full-scan spectra (Fig. 2A–C) which corresponds to its doubly-charged protonated ion ([M+2H]$^{2+}$).

Interestingly, no product ions were observed in MS/MS spectrum of B7T even with a relative high normalized collision energy of 100% (Fig. 2C), indicating the molecular stability of B7T. However, the precursor ion of IS was found at m/z 462 ([M+H]$^{+}$) and the product ion observed in MS/MS mode was at m/z 328 (Fig. 2D) with a normalized collision energy of 55%. Therefore, the normalized collision energy of 55% was finally chosen for sample determination, and the observed ion m/z 247 for B7T and m/z 462 → m/z 328 for IS were chosen for the quantification in the present study. The HPLC-MS/MS chromatograms of B7T (86.4 ng/ml) and IS in the extract obtained from the spiked blood sample (Fig. 3A) and in the extract obtained from rat blood collected at 120 min after drug administration (Fig. 3B) are shown in Fig. 3. The retention times of B7T and IS were 1.45 and 2.23 min with no significant endogenous interferences (Fig. 3C) demonstrating both a short analysis and an adequate resolution between B7T and IS.

The calibration curve derived for B7T exhibited a good linear response within the range of concentrations from 86.4 to 2160.0 ng/ml ($y = 0.004x - 0.3351$, $r^2 = 0.996$; y: peak area ratio, x: concentrations). The data for the determination of accuracy and precision are presented in Table 1. The results demonstrated good overall intra-day ($\leq 6.97\%$) and inter-day variations ($\leq 6.24\%$). Adequate accuracy was obtained in a range of 95.2–106.0%. Furthermore, the analysis was highly sensitive with LOD of 3.6 ng/ml and LOQ of 43.2 ng/ml. The stability of B7T in mobile phase at 25 °C is shown in Fig. 4, the results indicate that B7T is very stable under this condition ($\geq 96.6\%$). Furthermore, no significant degradation of B7T in the blood samples was observed when the samples were store at −20 °C for 7 days ($\geq 91.2\%$).

A single-step liquid–liquid extraction was employed for reasons of speed and convenience. Results from the absolute recoveries were acceptable, ranging from 89.7 to 95.5% at concentration from 86.4 to 2160.0 ng/ml. At the same time, the effect of the species of the organic solvents and pH on recovery were also tested and compared. Ethyl acetate showed much higher recoveries for both B7T (more than 89.7%) and IS (more than 97.8%) than diethyl ether, 1-hexane, cyclo-hexane, petro-ether and chloroform. Furthermore, the effects of pH on the extraction, including with 0.01 M sodium hydroxide, 0.2 M sodium carbonate solution or direct extraction without adjusting pH were also examined in the initial tests. The results indicated that the established method with 0.2 M sodium carbonate (pH 12.6) was optimal for sample preparation with satisfactory extraction recoveries.

The blood concentration of B7T was quantified by the developed HPLC-MS/MS method. The blood concentration-time profile of B7T is shown in Fig. 5 and the determined pharmacokinetic parameters are summarized in Table 2. The results suggested a first-order kinetic process for B7T in rats with a two-compartment model after the bolus IV injection. The concentration of B7T was about 1664.6 ng/ml at the first sampling point (5 min), and the blood level declined biexponentially with a very rapid distribution ($t_{1/2\alpha}$ of 11.1 min). The mean blood clearance of 6.02 ml/min/kg and the elimination half-life ($t_{1/2\beta}$)
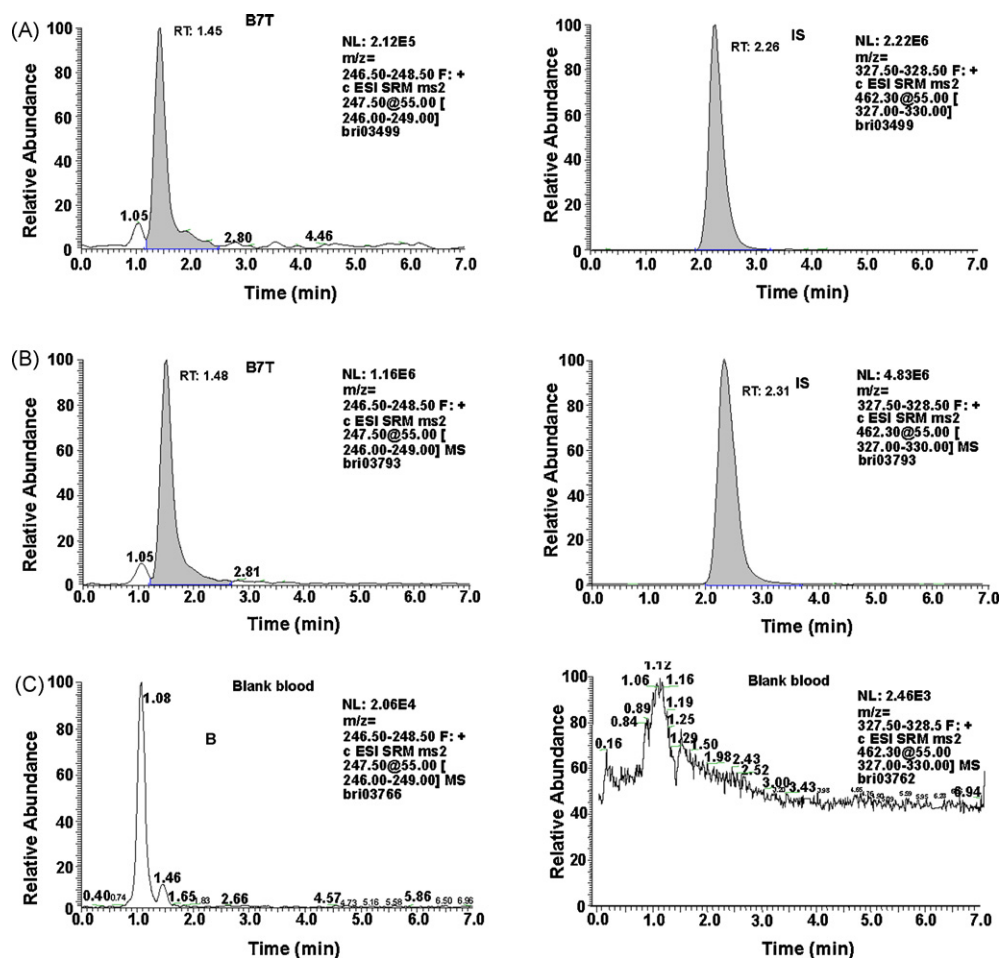
Fig. 3. HPLC-MS/MS chromatograms showing: (A) rat blood sample spiked with 86.4 ng/ml B7T and 50 μl of IS (60 μg/ml in methanol). B7T was monitored at m/z 247 and IS was monitored at m/z 462 → m/z 328, respectively; (B) rat blood sample obtained at 120 min after a single intravenous administration of B7T at 0.3 mg/kg and (C) rat blank blood sample.

of 264.0 min suggested a moderate elimination. Furthermore, a large volume of distribution (V_d, 1.42 l/kg) was observed, indicating that B7T, a lipophilic compound, distributed extensively into the extravascular tissues in rats.

In summary, a novel HPLC-MS/MS analytical method for quantification of B7T in the rat blood was established and successfully applied to determine the blood samples obtained from the dosed rats with no interference from endogenous substances. The developed assay is simple and accurate, and provides good reproducibility. The results of the pharmacokinetic study demonstrated that after IV administration, B7T had a rapid distribution ($t_{1/2\alpha}$ of ~11.1 min) and a moderate duration ($t_{1/2\beta}$ of ~264.0 min) of action in rats. To the best of our knowledge, this is the first report on the pharmacokinetic parameters of B7T. Further investigations into pharmacokinetics of B7T on dose-dependence and different administration routes are currently under progress in our research team.

Table 1
Intra-day ($n = 6$) and inter-day ($n = 5$) precision, and accuracy of the HPLC-MS/MS assay for bis(7)-tacrine in rat blood

	B7T added (ng/ml)	B7T detected (mean ± S.D., ng/ml)	R.S.D.[a] (%)	Accuracy[b] (%)
Intra-day	86.4	85.55 ± 2.91	3.40	101.0
	432	432.66 ± 29.57	6.83	99.8
	2160	2263.24 ± 157.64	6.97	95.2
Inter-day	86.4	86.55 ± 5.40	6.24	99.8
	432	405.92 ± 17.93	4.42	106.0
	2160	2191.19 ± 120.98	5.52	98.6

[a] R.S.D. (%) (relative standard deviation) = (S.D./mean) × 100%.

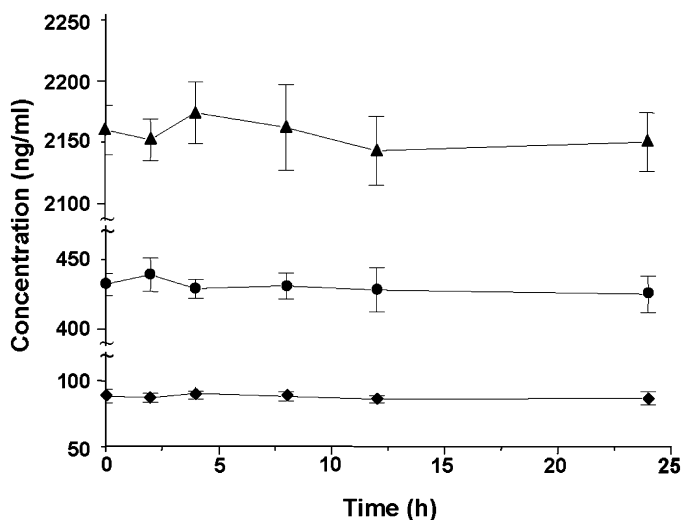[b] Accuracy (%) = [(1 − (mean concentration measured − concentration spiked)/concentration spiked)] × 100%.

Fig. 4. Stability of B7T (mean ± S.D.) in mobile phase at 25 °C (*n* = 6). Data are expressed as mean ± S.D. (♦): 86.4 ng/ml; (●): 432 ng/ml; (▲): 2160 ng/ml.
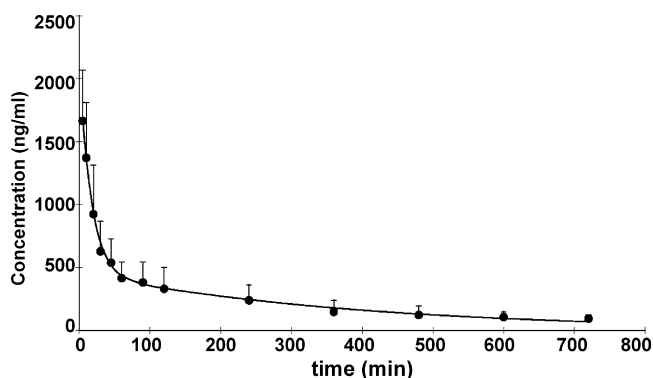

Fig. 5. Blood concentration-time profile of B7T (mean ± S.D.) after a single intravenous administration at 0.3 mg/kg to rat (*n* = 5). Data are expressed as mean ± S.D.

Table 2
Pharmacokinetic parameters following a single intravenous (0.3 mg/kg) administration of bis(7)-tacrine to rats (*n* = 5)

Parameters	Mean ± S.D.
$(t_{1/2\alpha})$ (min)	11.1 ± 0.7
$(t_{1/2\beta})$ (min)	264.0 ± 30.9
$AUC_{0 \to \infty}$ (μg/ml min)	199 ± 14
V_d (l/kg)	1.43 ± 0.12
CL (ml/min/kg)	6.02 ± 0.42

$(t_{1/2\alpha})$: α-Phase half-life; $(t_{1/2\beta})$: β-phase half-life; $AUC_{0 \to \infty}$: area under the blood drug concentration-time curve from zero to infinity; V_d: apparent distribution volume; CL: apparent clearance.

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