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Development and validation of an HPLC-DAD method for *bis*(12)-*hupyridone* and its application to a pharmacokinetic study

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ABSTRACT

A rapid and simple method of high performance liquid chromatography with UV detection for the quantification of bis(12)-hupyridone in rat blood has been developed and validated. Chromatographic separation was carried out in an Agilent Extend C_{18} 5 μm column (length, 250 mm; inner diameter, 4.6 mm) using a mixture of water–acetonitrile–trifluoroacetic acid (81:19:0.04, v/v/v) as the mobile phase at a flow rate of 1 mL/min, with detection at 229 nm. The method used for the bis(12)-hupyridone quantification showed linearity for concentration range of 0.1–7.5 μg/mL with $r^2 = 0.9991$. The limit of detection and quantification of this method were 0.05 μg/mL and 0.1 μg/mL, respectively. The intra- and inter-day variations of the analysis were less than 4.22% with standard errors less than 13.3%. The developed method was successfully applied to the pharmacokinetic study of bis(12)-hupyridone after intravenous administration of 5 mg/kg and intraperitoneal administration of 10 and 20 mg/kg in rats.

1. Introduction

The whole plant of *Huperzia serrata* (*Thumb.*) *Trev.*, also called "*Qian Ceng Ta*" in China, is a traditional herbal medicine which is widely used in China for centuries in the treatment of conditions such as contusions, strains, swelling, and schizophrenia [1]. As the peripheral cholinergic effect were often observed as the main side-effect for this herb in clinic practice, it was suggested that the active component from *H. serrata* might be developed as a new drug for the treatment of neurodegenerative disorder such as Alzheimer's disease (AD).

In the early 1980s, two sesquiterpene alkaloids named huperzine A (Hup A) and huperzine B (Hup B) were first purified from the crude extract of *H. serrata* and identified by the Chinese phytochemists [2]. Among the two identified compounds, Hup A was found to act as a cholinesterase (ChE) inhibitor both *in vitro* and *in vivo* [3–5]. Based on the 50% inhibitory concentration (IC_{50}), Hup A was demonstrated to have greater specificity on acetylcholinesterase (AChE) (IC_{50}: 0.082 μM) than butyrylcholinesterase (BuChE) (IC_{50}: 74.43 μM). Its potency on AChE inhibition is even higher than tacrine (IC_{50}: 0.093 μM), rivastigmine (IC_{50}: 181.39 μM) and galatamine (IC_{50}: 1.995 μM) [2,6]. Moreover, Hup A has demonstrated its neuroprotective effect by preventing glutamate toxicity [7], hydrogen peroxide induced oxidative stress [8] and transient focal cerebral ischemia-induced brain injury [9]. All the above-mentioned activities of Hup A may be beneficial for the treatment of AD. Currently, Phase II clinical trial of Hup A for the evaluation of its safety and efficiency in the treatment of AD and on the improvement of cognitive function in the US has finished [10]. Although Hup A is generally considered as a promising AChE inhibitor for the treatment of AD, its limited natural resource and costly synthesis are the major barriers for its further development [11]. The current focus of many investigators is to develop more effective Hup A derivates or analogs to search for novel anti-AD drugs for further clinical usage [12–15].

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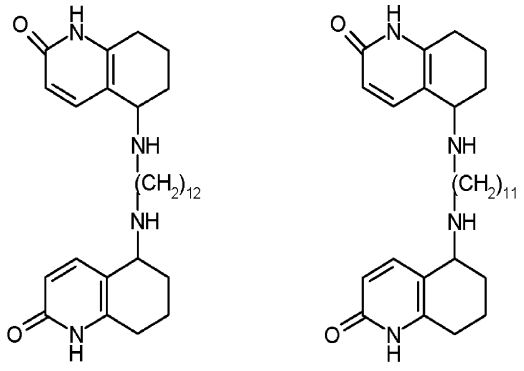
Recently, a series of Hup A analogs has been designed and reported by our research group and co-workers based on the theory of the acetylcholinesterase dual-binding hypothesis [16]. Among these Hup A analogs, bis(12)-hupyridone (B12H), a homo-dimer by linking two hupyridone fragments derived from Hup A with a 12-methylene tether, has demonstrated with twice potency on AChE inhibition (IC_{50}: 52 nM) than Hup A *in vitro* and 185-fold greater selectivity in terms of inhibition on AChE than BuChE (IC_{50}: 9600 nM) [12]. Comparing with tacrine and Hup A, B12H is 4.4 or 2.2 times more potent in inhibiting rat brain AChE, respectively [12,13]. B12H is 616 times more selective than tacrine [13] but with a relatively less selectivity than Hup A [17]. Moreover, the *in vivo* AChE inhibitory effect test indicated that B12H could be quickly absorbed and well permeated into brain after an intraperitoneal injection (3 mg/kg) to mice [18]. The maximum AChE inhibition was reached at 15 min after dosing and lasted for more than 4 h. In addition, as a more potent and selective anti-AChE agent, chemically synthesized B12H can be more easily prepared for its further commercialization and clinical use.

In order to further develop B12H into as a potent neuroprotective drug for the treatment of AD, its pharmacokinetics study is essential. However, the effective bio-analytical method for B12H concentration determination in biological fluid is still absent. Our preliminary of B12H demonstrated that its high binding to blood cells (>68%) may lead to very low plasma concentration *in vivo*. In addition, a satisfied correlation could be well established by the blood concentration of B12H to its AChE inhibition in rat brain. Therefore, in this study, the whole blood was selected as the sample to evaluate the pharmacokinetic property of B12H in rats. To effectively monitor the blood concentration thus well understanding the pharmacokinetic properties of B12H *in vivo*, in this study, a high performance liquid chromatographic (HPLC-DAD) method was developed and validated for the quantitative determination of B12H in rat blood. With this established bio-analytical method, the pharmacokinetic profiles of B12H were evaluated following intravenous (5 mg/kg) or intraperitoneal (10 or 20 mg/kg) administration to rats.

2. Experimental

2.1. Chemicals and reagents

Bis(12)-hupyridone (B12H, Fig. 1A) and bis(11)-hupyridone (B11H, Fig. 1B), the internal standard, were synthesized according to our previous publication [12,13]. Their structures were identified by HPLC, NMR and MS analysis and the purities were greater than 99.9% by HPLC determination. Acetonitrile (ACN) was purchased from Merck Company (Darmstadt, Germany) as HPLC grade. Heparin, sodium hydroxide, ethyl acetate and trifluoroacetic acid (TFA) were purchased from Sigma Chemicals Ltd. (St. Louis, MO, USA) as analytical grade. Water was prepared by an EASYpure UV system (Model D7401; Barnstead Thermolyne Co. Dubuque, IA, USA).



(A) bis(12)-hupyridone (B12H) (B) bis(11)-hupyridone (IS)

Fig. 1. Structures of bis(12)-hupyridone (B12H) (A) and bis(11)-hupyridone (IS) (B).

2.2. Liquid chromatography

Separation of B12H from B11H, the IS, was achieved by an Agilent HP1100 system (Hewlett Packard, Agilent, USA) coupled with an Agilent Extend C-18 analytical column (250 mm × 4.6 mm I.D., 5 μm) and protected with an Agilent Extend C-18 guard-column (12.5 mm × 4.6 mm I.D., 5 μm) maintained at 25 °C. The iso-cratical elution with a mobile phase of water–ACN–TFA (81:19:0.04, v/v/v) was used at a flow rate of 1.0 mL/min for the separation of analytes. The analytes were monitored at the UV wavelength of 229 nm.

2.3. Standard and working solutions

Individual standard stock solutions of B12H (1 mg/mL) and the internal standard (10.0 μg/mL) were prepared by accurately weighting the required amounts into volumetric flasks and dissolving in water. Further dilutions were made from this stock solution with water to yield the working concentrations from 1.0 μg/mL to 75 μg/mL. All prepared solutions were stored at −20 °C in amber glass tubes and brought to room temperature before use.

The calibration curve for B12H was prepared by spiking 200 μL of blank rat blood with 20 μL of the working solutions to achieve concentrations in the range of 0.1–7.5 μg/mL. Quality control (QC) samples at three different concentrations of 0.1, 1.0, and 7.5 μg/mL were prepared separately. The QC samples were used to assess the accuracy and precision of the assay method. All the calibration and QC samples were then extracted by the method described in the subsequent section and analyzed. The QC samples were stored along with the test samples at −20 °C until analysis.

2.4. Sample preparation

The rat blood containing B12H (0.1–7.5 μg/mL) was extracted by 1.0 mL ethyl acetate after addition of 20 μL IS solution (10.0 μg/mL) and 20 μL 1.0 M sodium hydroxide solution. The mixture was vortex-mixed for 1 min, and then centrifuged with a C0160-B Microcentrifuge (Labnet International Inc., 31 Mayfield Ave., NJ, USA) at 16,000 × g for 5 min. 0.9 mL of the organic layer was transferred into a 1.5 mL Eppendorf tube and followed to dryness by evaporation under a nitrogen stream at 25 °C. The residues were re-constituted with 90 μL of the chromatographic mobile phase (water:ACN:TFA/81:19:0.04, v/v/v), and 20 μL of which were injected into the HPLC for analysis. The calibration curve was constructed by plotting the concentration of B12H in blood as a function of peak area ratio of B12H to IS.

2.5. Method validation

The specificity of the method was investigated by comparing the chromatograms of blank blood with blood samples collected after intravenous (i.v.) administration of B12H, or with that obtained from the blank blood spiked with authentic standard of B12H.

2.5.1. Precision, accuracy and recovery

The intra-day precision was determined within 1 day by analyzing six replicates of QC samples at concentrations of 0.1, 1.0, and 7.5 μg/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 (R.S.D.) and the accuracy was defined by calculating the relative error (R.E.).

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 can be determined with sufficient precision and accuracy, i.e. R.S.D. less than 20% and R.E. of −20 to 20% [19].

2.5.3. Stability

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

2.5.4. Recovery

The extraction recovery was estimated by comparing the peak area of analyte of pre-spiking samples with those of post-spiking samples [20]. Pre-spiking sample means spiking B12H and IS to yield the same concentrations as QC samples before sample preparation, while post-spiking sample means spiking neat solutions of B12H and IS at the same concentrations as QC samples into the extracted blank blood samples. The matrix effect was estimated by comparing the peak areas of analyte of post-spiked samples with neat solutions of B12H and IS.

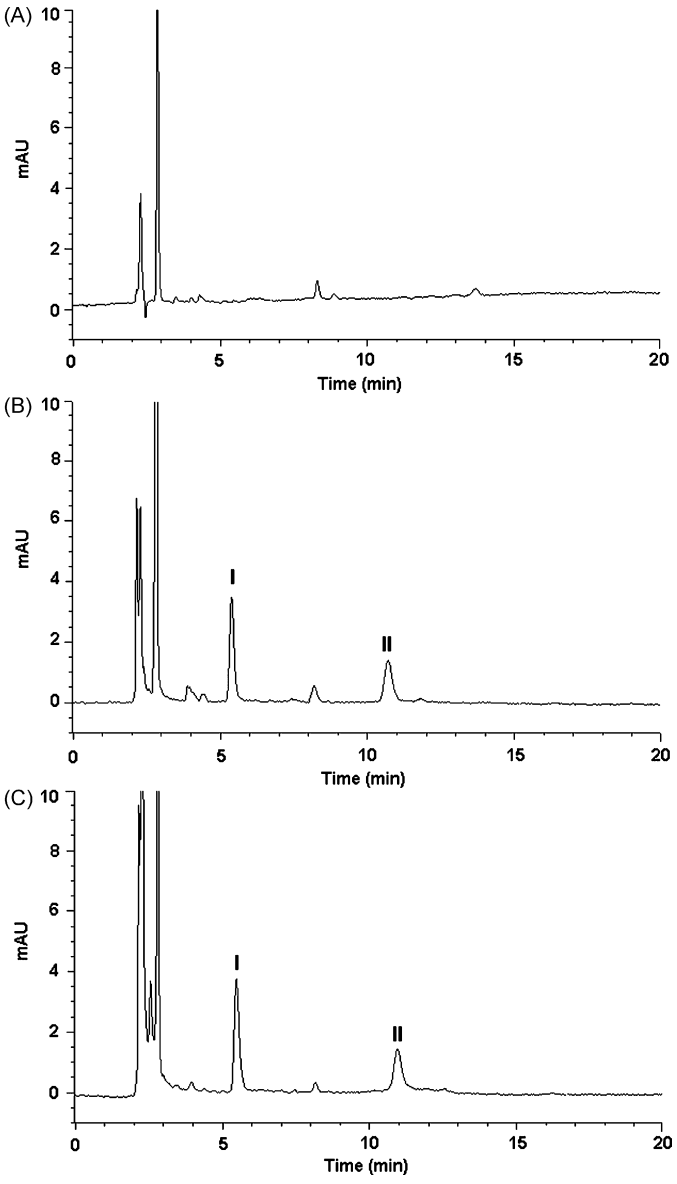


Fig. 2. HPLC chromatograms of (A): blank blood, (B): blood spiked with B12H (1.0 μg/mL) and IS (1.0 μg/mL) and (C): blood sample at 5 min after intravenous administration of B12H (5 mg/kg). Peak I: B11H (IS); Peak II: B12H.

2.6. Pharmacokinetic study

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 the controlled temperature at 20–22 °C and relative humidity of 50% with 12-h light/dark cycle. One day prior to drug administration, rats were surgically cannulated with polyethylene catheters (polyethylene tubing, 0.4 mm I.D., 0.8 mm O.D., Portex Ltd., Hythe, Kent, England) on the right jugular veins under anesthesia with 10% chloral hydrate. The animals recovered in individual metabolic cages and fasted but were allowed to have free access to water overnight.

Three groups of conscious cannulated rats with at least six in each group were dosed with B12H intravenously (5 mg/kg) and intraperitoneally (10 and 20 mg/kg), respectively. Serial venous blood samples (0.25 mL) were collected from the right jugular vein via the cannulated catheter into heparinized tubes at: (i) 1, 3, 5, 8, 15, 30, 60, 120, 180 and 240 min after i.v. administration and (ii) 1, 3, 5, 8, 15, 30, 60, 120, 180, 240 and 360 min after i.p. administration. After each blood sampling, an equivalent volume of heparinized saline (25% v/v, 0.25 mL) was injected into the animals to maintain a constant blood volume. The blood samples were stored at −20 °C until analysis.

Pharmacokinetic parameters, including area under blood concentration–time curve (*AUC*), mean residence time (MRT), apparent blood clearance (*CL*), apparent volume of distribution (V_d), elimination half-life ($t_{1/2}$), and maximum blood concentration and time (C_{max} and t_{max}) were calculated by the commercially available software program of WinNonlin™ Professional Version 5.0.1 (Pharsight, Mountain View, CA, USA) with two-compartmental and/or non-compartmental approach. Absolute bioavailability (*F*) was calculated as the ratio of the dose-normalized $AUC_{0\rightarrow\infty}$ after i.p. injection (10 or 20 mg/kg) to that after bolus i.v. injection (5 mg/kg) of B12H.

3. Results and discussion

3.1. Selection of the internal standard

Due to the high accuracy requirement for biological samples, assay method with a suitable IS is often adopted. Based on its similar structure to B12H, B11H was synthesized to serve as the internal standard for the current study. As shown in Fig. 2, B12H could be chromatographically separated from the IS with no observed interference.

3.2. Analytical method

In the current study, an HPLC-DAD method was optimized and validated for the quantitative determination of B12H in rat blood. The representative chromatograms of blank blood, blank blood sample spiked with B12H, and a blood sample obtained from a B12H-dosed rat (i.v., 5 mg/kg, 5 min) were shown in Fig. 2. Under the present assay conditions, B12H could be well separated from the IS with no endogenous interference from blood observed at the retention times of both the analyte and the IS.

Table 1
Intra- and inter-day precision and accuracy (mean ± S.D., $n = 6$) for the quantification of bis(12)-hupyridone in rat blood.

Nominal concentration (μg/mL)	Determined concentration (mean ± S.D.; μg/mL)	R.S.D.[a] (%)	R.E.[b] (%)
Intra-day ($n = 6$)			
0.1	0.10 ± 0.01	4.22	0.7
1.0	0.97 ± 0.04	4.02	−3.4
7.5	7.38 ± 0.12	1.60	−1.6
Inter-day ($n = 5$)			
0.1	0.10 ± 0.01	13.30	3.6
1.0	0.96 ± 0.04	4.18	−4.3
7.5	7.43 ± 0.18	2.39	−0.94

[a] R.S.D. (%) (relative standard deviation) = (S.D./mean concentration) × 100.
[b] R.E. (%) = [1 − (mean concentration determined/concentration spiked)] × 100.

Table 2
Comparison on the pharmacokinetic parameters of B12H and ^{3}H-Hup A after intravenous administration to rats.

Parameters	B12H (5 mg/kg)[a]	^{3}H-Hup A (13.9 MBq/kg)[b]
α (min^{-1})	0.43 ± 0.11	0.107 ± 0.016
β (min^{-1})	0.0075 ± 0.0065	0.006 ± 0.003
$t_{1/2\alpha}$ (min)	1.68 ± 0.44	6.6 ± 1.1
$t_{1/2\beta}$ (min)	92.86 ± 7.89	149 ± 96
V_d (L/kg)	7.54 ± 0.88	3.6 ± 1.0
CL (L/(min kg))	0.067 ± 0.006	0.020 ± 0.006

α: Distribution rate constant; β: elimination rate constant; $t_{1/2\alpha}$: distribution half-life; $t_{1/2\beta}$: elimination half-life; V_d: the volume of distribution; CL: total blood clearance.
[a] Data were calculated by using two-compartmental analysis and expressed as mean ± S.D. ($n = 6$).
[b] Data were obtained from Ref. [21].

The intra-day and inter-day precision and accuracy of the current developed assay are listed in Table 1. The intra-day R.S.D. for B12H was below 4.22% and inter-day R.S.D. was below 13.3% in the tested concentration range. In addition, the calibration curve for B12H exhibited a good linear response within the range of concentrations from 0.1 to 7.5 μg/mL ($y = 0.9784x + 0.0191$; $r^2 = 0.9991$; y: concentrations, x: peak area ratio).

In order to optimize the sample preparation method, a single-step liquid–liquid extraction with different solvents or mixture (include ACN, chloroform, petroleum ether, ethyl ether, ethyl acetate, water:ACN/1:1) had been employed to compare the extraction efficiency of B12H from rat blood sample. Among the tested solvents, ethyl acetate exhibited the best extraction recovery (~80%). In addition, a significant difference was found in the absolute recoveries between the extraction with and without alkalization of the samples. B12H could be extracted much more efficiently after addition of 20 μL sodium hydroxide (1.0 M) than extracted without sample alkalization (extraction recovery <10%). With the optimized sample preparation conditions, the absolute recoveries ranged from 79.3% to 84.2% for the extraction of B12H from blood samples at low, medium and high concentrations, and 82.3% for B11H (IS) (1.0 μg/mL), respectively.

Under the current assay conditions, the LOQ was 0.1 μg/mL and the LOD was 0.05 μg/mL for B12H.

The related stability experiment indicated that B12H was very stable for at least three freeze–thaw cycles (with 102.0–99.1% remained for concentrations ranged from 0.1 to 7.5 μg/mL). In addition, B12H was found to be very stable (97.6–102.2% remained for the concentrations from 0.1 to 7.5 μg/mL) in the prepared samples after being placed in the auto-sampler at room temperature for 24 h.

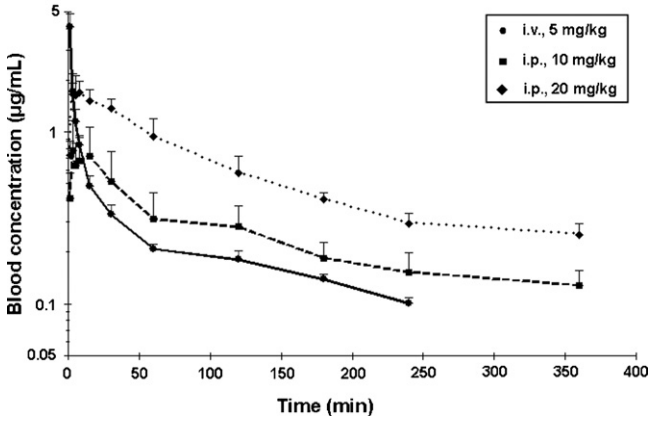


Fig. 3. Pharmacokinetic profiles of bis(12)-hupyridone in rats after intravenous (●: 5 mg/kg) and intraperitoneal (■: 10 mg/kg and ◆ : 20 mg/kg) administration ($n = 6$).

3.3. Pharmacokinetics of B12H after intravenous and intraperitoneal administrations

The blood concentrations of B12H from 0 to 240 min and/or 360 min after i.v. or i.p administration in rats were quantitatively determined by the developed HPLC-DAD method. Comparison of the pharmacokinetic parameters of B12H to that of Hup A, the monomer, was summarized in Table 2.

As shown in Fig. 3, the pharmacokinetic profile of B12H after the i.v. bolus injection (5 mg/kg) suggested a first-order kinetic process in rats with a two-compartment model. The blood level of B12H

Table 3
Pharmacokinetic parameters (mean ± S.D., $n = 6$) of B12H in rats after different administration routes.

Pharmacokinetic parameters	Route of administration		
	Intravenous	Intraperitoneal	
	5 mg/kg	10 mg/kg	20 mg/kg
Lambda_z (L/min)	0.0050 ± 0.0005	0.0045 ± 0.0009	0.0059 ± 0.0008
$AUC_{0\to t}$ (min μg/mL)	62.62 ± 5.47	96.15 ± 20.67	219.92 ± 8.78
$AUC_{0\to\infty}$ (min μg/mL)	82.50 ± 8.55	125.73 ± 28.92	266.12 ± 13.14
MRT (h)	2.67 ± 0.18	3.84 ± 0.37	3.14 ± 0.37
C_{max} (μg/mL)	–	0.93 ± 0.23	1.91 ± 0.29
t_{max} (min)	–	9.33 ± 6.25	4.75 ± 2.36
$t_{1/2}$ (h)	2.35 ± 0.29	2.63 ± 0.49	1.99 ± 0.29
CL (L/(min kg))	0.061 ± 0.006	0.055 ± 0.016	0.093 ± 0.005
V_d (L/kg)	12.35 ± 1.24	12.09 ± 1.72	16.06 ± 1.86
F (%)	–	76.2	80.7

Data were calculated by using non-compartmental analysis. $AUC_{0\to t}$: area under the concentration–time curve from time 0 to 4 h (i.v.) or 6 h (i.p.); $AUC_{0\to\infty}$: area under the concentration–time curve from time 0 to infinity; MRT: mean residence time; C_{max}: maximum blood concentration; t_{max}: time to peak concentration; $t_{1/2}$: elimination half-life; V_d: the volume of distribution; CL: total blood clearance; F: absolute bioavailability.

exhibited a biexponentially decline from rat blood with a very rapid distribution to the extravascular tissues ($t_{1/2\alpha}$: 1.7 ± 0.4 min) which was faster than that of Hup A ($t_{1/2\alpha}$: 6.6 ± 1.1 min) [21]. As the lipophilicity of B12H ($\log P_{pH7.7}$: 1.61) [18] was found to much higher than that of Hup A ($\log P_{pH7.7}$: 1.15) [22], it is expected that B12H exhibited greater volume of distribution in rat (V_d: 7.54 ± 0.88 L/(min kg)) than Hup A (V_d: 3.6 ± 1.0 L/(min kg)) [21]. The mean blood clearance of 0.067 ± 0.006 L/(min kg) and the elimination half-life ($t_{1/2\beta}$) of 92.9 ± 7.9 min for B12H suggested its moderate elimination in rat after i.v. administration.

The pharmacokinetic parameters of B12H after i.v. (5 mg/kg) and i.p. (10 and 20 mg/kg) were calculated by WinNonlin on basis of the non-compartmental analysis and summarized in Table 3. B12H was absorbed quickly after i.p. injection (Fig. 3) with the maximum blood concentrations (0.93 ± 0.23 μg/mL and 1.91 ± 0.29 μg/mL) reached within 9.33 ± 6.25 min and 4.75 ± 2.36 min after 10 and 20 mg/kg dosing. Comparing the pharmacokinetic parameters obtained from the two i.p. dosing, similar pharmacokinetic processes were observed for $t_{1/2}$ (2.63 ± 0.49 h and 1.99 ± 0.29 h), CL (0.055 ± 0.016 L/min/kg and 0.093 ± 0.005 L/(min kg)), V_d (12.09 ± 1.72 L/kg and 16.06 ± 1.86 L/kg) and MRT (3.84 ± 0.37 h and 3.14 ± 0.37 h). The values of $AUC_{0\to\infty}$ (125.73 ± 28.92 min μg/mL and 266.12 ± 13.14 min μg/mL) from the two i.p. dosing indicated the dose-dependent manner of B12H after i.p administration. Comparing the $AUC_{0\to\infty}$ values from i.p. to that of i.v. administration, the absolute bioavailabilities were calculated to be 76.2% and 80.7% for 10 and 20 mg/kg, respectively. In summary, B12H could be well absorbed and most of the administrated drug could enter into the systemic circulation after extravascular injection.

4. Conclusion

In summary, a novel HPLC-DAD analytical method for quantitative determination of B12H in the rat blood is established and successfully applied to measure the blood samples obtained from the B12H-dosed rats with no interference from endogenous substances. The developed assay is simple, accurate and specific with good reproducibility. With this method, the pharmacokinetic profiles of B12H after i.v. (5 mg/kg) and i.p. (10 and 20 mg/kg) administration in rats were first studied and reported. The current investigation contributes not only to the determination of B12H in rat blood by HPLC but also to our understanding of the linear pharmacokinetic characteristics of B12H over dose range studied in rats after intravascular and extravascular administration.

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