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Short communication

Determination of Bis(9)-(−)-Meptazinol, a bis-ligand for Alzheimer's disease, in rat plasma by liquid chromatography–tandem mass spectrometry: Application to pharmacokinetics study

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ABSTRACT

A rapid, simple and sensitive LC–MS/MS method was developed and validated for the determination of Bis(9)-(−)-Meptazinol (B9M) in rat plasma. Protein precipitation method was used for sample preparation, using five volumes of methanol as the precipitation agent. The analytes were separated by a Zorbax Extend-C18 column with the mobile phase of methanol-water (containing 5 mM ammonium formate, pH 9.8) (95:5, v/v), and monitored by positive electrospray ionization in multiple reaction monitoring (MRM) mode. Retention time of IS (Bis(5)-(−)-Meptazinol) and B9M were 1.9 min and 3.3 min, respectively. The limit of detection was 0.1 ng/ml and the linear range was 1–500 ng/ml. The relative standard deviation (RSD) of intra-day and inter-day variation was 4.4–6.2% and 6.2–8.9%, respectively. The extraction recoveries of B9M in plasma were over 95%. The method proved to be applicable to the pharmacokinetic study of B9M in rat after intravenous and subcutaneous administration.

1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease in aging populations, characterized by progressive abnormalities of memory, behavior, and cognition [1]. Although the precise etiology remains unknown, the cholinergic [2] and β-Amyloid peptide (Aβ) hypotheses [3] are widely recognized. Acetylcholinesterase inhibitors (AChEIs), including tacrine [4], rivastigmine [5], donepezil [6] and galantamine [7] have so far been approved by FDA for the treatment of AD. However, these AChEIs can only alleviate the symptoms of AD in general rather than reverse its pathological processes. Therefore, intensive researches are now focusing on the development of new drugs that can not only alleviate the clinical symptoms of AD but also selectively modify pathological process [8].

Existing evidence has demonstrated that AChE has a narrow deep active-site gorge, the bottom and opening regions of which are known as catalytic anionic site (CAS) and peripheral anionic site (PAS). CAS is a catalytic center of ACh, while PAS is involved in the allosteric modulation of CAS and a number of non-classical functions, in particular, amyloid deposition, cell adhesion and neurite outgrowth [9]. The AChEIs simultaneously blocking both the CAS and PAS might not only alleviate the cognitive deficit of AD patients by elevating ACh levels but also act as disease-modifying agents by inhibiting the formation of amyloid plaque [4,10]. Therefore, bivalent ligand strategies targeting both of the sites have been utilized for the design of AChEIs with dual binding site [11–16].

Our research team has designed and synthesized a novel bis-ligand AChEI, Bis(9)-(−)-Meptazinol (B9M), by connecting two lead compound (−)-Meptanizols with 9 alkylenes. B9M was capable of inhibiting mouse brain AChE activity at nanomolar levels ($IC_{50} = 3.9 \pm 1.3$ nM). Enzyme kinetic and molecular docking studies revealed that B9M could simultaneously bind to both the CAS and PAS of AChE [17]. The inhibitory activity of B9M against AChE-induced Aβ aggregation further confirmed its affinity to PAS [18,19]. The in vivo experiments showed that subcutaneous injection of B9M significantly reversed memory deficits in scopolamine-treated mice and senescence accelerated mice (SAMP8). The decrease in Aβ deposition and tau protein hyperphosphorylation, and the protection against neuronal degeneration were also observed in the hippocampus of B9M-treated SAMP8 mice (unpublished), indicating B9M as a potential disease-modifying agent for AD.

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Although the pharmacodynamics results were encouraging, the pharmacokinetic profile of B9M had not been studied yet. High performance liquid chromatography–UV (HPLC–UV) and Liquid chromatography–mass spectrometry (LC–MS/MS) methods have been described for the quantification of meptazinol in rat plasma [20,21]. However, the physicochemical property of B9M is quite different from that of meptazinol due to the existence of a nine-carbon spacer. Therefore, in this study, a high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method was developed, validated and successfully applied to the evaluation of the absolute bioavailability of B9M in rat after intravenous and subcutaneous administration (0.65 mg/kg).

2. Experimental

2.1. Reagents and chemicals

B9M and IS (Bis(5)-(−)-Meptazinol) were synthesized by School of Pharmacy, Fudan University (Shanghai, China). The purity of the compounds was higher than 99.8% by HPLC analysis. Methanol of HPLC grade was supplied by Burdick & Jakson (NJ, USA). Aqueous ammonia and ammonium formate were HPLC grade and purchased from Tedia (OH, USA). Water of HPLC grade was purchased from ROE (New castle, USA). All other chemicals and reagents were of analytical grade.

2.2. Animals

Sprague–Dawley rats (200–220 g) were supplied by the SLAC Laboratory Animal Co. Ltd. (Shanghai, China), allowed to acclimate to their environmentally controlled quarters (20–22 °C, 12:12 h light–dark cycle) for 3 days before experiment, and fasted overnight with water ad libitum before dosing.

Animal study was approved by the Animal Ethics Committee of Shanghai Jiaotong University School of Medicine (Shanghai, China).

2.3. LC–MS/MS instrument and conditions

The LC–MS/MS system was comprised of an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies, Inc., USA) equipped with an electrospray ionization (ESI) system, and an Agilent 1200 HPLC system (Agilent Technologies, Inc., USA). The separation was carried out using a Zorbax Extend-C18 column (2.1 mm × 100 mm, 3.5 μm; Agilent Technologies, Inc., USA) maintained at 30 °C. The elution was performed isocratically with the mobile phase consisting of methanol and water (95:5, v/v, the aqueous phase contained 5 mM ammonium formate, and was adjusted to pH 9.8 using aqueous ammonia). The flow rate was set at 0.3 ml/min. Data were acquired in the positive ionization mode using multiple reaction monitoring (MRM). Two MRM transitions, B9M (*m*/*z* 563.5 to 107.1, fragmentor 210 eV, collision energy 65 eV) and IS (*m*/*z* 507.5 to 107.1, fragmentor 210 eV, collision energy 65 eV) were monitored. The ESI–MS/MS working parameters were as follows: temperature 350 °C, drying-gas flow rate 8 L/min, capillary voltage 4000 V, nebulizer pressure 25 psi. Mass data processing was performed using the MassHunter software package (Version B.04.00, Agilent Technologies, Inc., USA).

2.4. Preparation of calibration standards and quality control (QC) samples

Stock solutions of B9M and IS were prepared in methanol at the concentrations of 1 mg/ml, and stored at −20 °C. Working solutions for spiking plasma ranging from 5 ng/ml to 2500 ng/ml were all freshly prepared by stepwise dilution of the stock solution with methanol. A series of calibration standards were then prepared by spiking blank plasma samples (50 μl) with 10 μl aliquots of standard solutions to yield the concentrations of 1, 5, 10, 50,100, 200 and 500 ng/ml. QC samples were prepared at three levels (low 2.5 ng/ml, medium 75 ng/ml, high 450 ng/ml) independently in the same way. All QC samples were stored at −70 °C. The IS working solution (20 ng/ml) was made up by diluting the stock solution with methanol.

2.5. Sample pretreatment

Frozen plasma samples were thawed at room temperature. Ten microliter of methanol and 250 μl methanol containing IS at the concentration of 20 ng/ml were added to 50 μl plasma. The mixture was vortexed for 1 min and centrifuged at 15,000 × *g* for 5 min with the supernatant (5 μl) subjected to the LC–MS/MS analysis.

2.6. Method validation

Selectivity was assessed by using blank plasma from six different rats with and without B9M and IS. Calibration curves were constructed at the range of 1–500 ng/ml by plotting peak area ratio (*y*) of B9M to IS vs nominal concentration (*x*), and the linearity was assessed at the weighting factor of 1/*x*. Five replicates of QC samples (low, middle and high concentrations) on three separate days were assayed to evaluate the intra-day and inter-day precision (expressed as relative standard deviation, RSD) and accuracy (expressed as relative error, RE). The lower limit of quantification (LLOQ) was defined as the lowest concentration in the calibration curve. Absolute recoveries were evaluated by comparing the peak area of B9M in spiking post-deproteinized blank plasma samples with the corresponding spiking samples. Matrix effect was evaluated by comparing spiking post-deproteinized blank plasma samples with corresponding standard neat solutions. Room-temperature stability, auto-sampler stability, long-term stability and freeze/thaw stability were all studied at three levels of QC samples in five replicates under our experimental conditions.

2.7. Pharmacokinetic study

Twelve Sprague–Dawley rats (200–220 g) were randomly divided into the intravenous and subcutaneous administration groups (evenly divided between male and female). Blood samples (most were 0.2 ml or less) were collected via retro-orbital puncture at pre-dose (0 h) and 0.083, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8,12, 24, 32, 48 and 72 h after administration of 1 mg/kg B9M tartrate in saline (identical with 0.65 mg/kg B9M in free form). Heparinized plasma was separated immediately by centrifugation at 3000 g at 4 °C for 10 min and stored at −70 °C until analysis. The pharmacokinetic parameters were calculated using WinNonLin (Version 6.1, Pharsight, Mountain View, CA, USA) according to non-compartmental model. The absolute bioavailability was calculated as follows:

$$F = \frac{AUC_{(0-\infty)(sc)}}{AUC_{(0-\infty)(iv)}} \times 100\%$$

3. Results and discussion

3.1. Method development

In our experiment, we selected Bis(5)-(−)-Meptazinol, an analog of B9M as IS due to its similar physicochemical property and mass spectrometric behavior with B9M. The response in positive mode was much higher than that in negative mode in both acidic and basic elution systems. Both mono- and double-protonated ions were found in full scan mode, but the intensity of $[M+H]^+$ (563.5)

Table 1
Intra-day and inter-day precision, accuracy, absolute recovery and matrix effect of the method for determination of B9M in rat plasma ($n = 5$).

Conc. (ng/ml)	Intra-day (%)		Inter-day (%)		Absolute recovery (%)		Matrix effect (%)	
	RSD	RE	RSD	RE	Mean	RSD	Mean	RSD
1	6.7	12.9	5.3	11.8	NA[a]	NA[a]	NA[a]	NA[a]
2.5	4.4	4.8	8.9	−0.6	96.4	2.8	100.2	6.4
75	4.7	−2.4	7.3	5.5	99.2	1.2	97.3	2.0
450	6.2	−2.0	6.2	−0.6	95.6	2.1	96.4	3.1

[a] NA, not available.

was stronger than that of $[M+2H]^+$ (282.3), so $[M+H]^+$ 563.5 was selected for further fragmentation. The product ions and other mass parameters were obtained automatically using MassHunter optimizer software (Version B.03.01, Agilent Technologies, Inc., USA). The product ion spectrum and its allocation were shown in Fig. 1.

B9M is an amphoteric compound with acidic (phenolic hydroxy) and basic group (tertiary amine), so pH is very important for a good peak shape. Under acidic condition, no retention (RT < 1 min) was achieved for B9M on reversed-phase columns even adjusting the aqueous phase to 95% (v/v) where tailed peak obtained. When the pH of the aqueous phase adjusted to 9.8, the peaks of B9M and IS became very sharp and symmetrical. Thus, Zorbax Extend-C18 column, which is able to tolerate mobile phases with pH up to 11.5, was used in our study. In addition, an eluent consisting of very high ratio of methanol (95%, v/v) was chosen due to the strong retention of B9M and IS (RT ≥ 26 min) with 80% organic phase (v/v). This high percentage of methanol not only minimized the retention times of B9M and IS to 1.9 min and 3.3 min, respectively, but also significantly enhanced the intensity of peak due to better ionization.

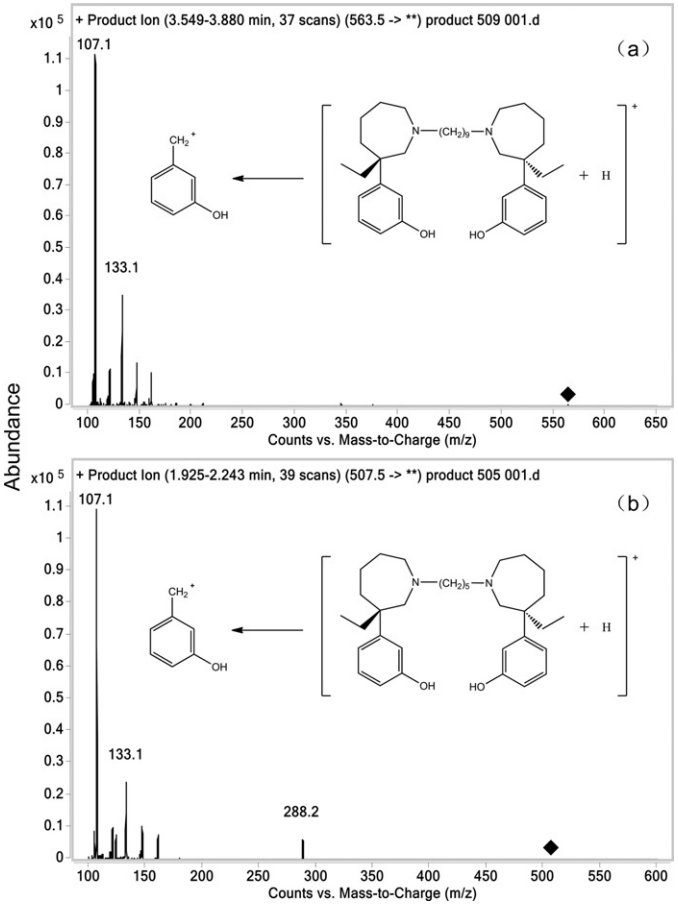

Fig. 1. Collision-induced dissociation mass spectra of B9M (a) and IS (b).

In order to save time and labor, protein precipitation with five volumes of methanol was adopted for sample preparation. The supernatant after centrifugation was directly injected to the LC–MS/MS system because of the similar high percentage of organic solvent in the mobile phase.

3.2. Method validation

The method was validated with respect to selectivity, linearity, LLOQ, precision, accuracy, recovery, matrix effect and stability according to the US Food and Drug Administration (FDA) bioanalytical method validation guidance [22].

The chromatograms of blank matrix from six individuals showed no endogenous interference at the retention times of B9M and IS. The representative MRM chromatograms of blank rat plasma, blank rat plasma spiked with B9M and IS, and the plasma from a rat 5 min after an intravenous injection of 0.65 mg/kg B9M were illustrated in Fig. 2.

The calibration curve exhibited a good linear correlation within the concentration range of 1–500 ng/ml ($y = 0.0122x + 0.0123$). The coefficient of determination (R^2) was higher than 0.998. The limit of detection (LOD) for B9M, with a signal-to-noise ratio at 3, was 0.1 ng/ml. LLOQ was established at 1 ng/ml with an intra-day precision (% RSD) and accuracy (% RE) of 6.7% and 12.9%, respectively. Accuracy and precision of intra- and inter-assay were summarized in Table 1. The absolute recoveries were 96.4% (2.5 ng/ml), 99.2% (75 ng/ml) and 95.6% (450 ng/ml) for B9M, and the matrix effect were 100.2% (2.5 ng/ml), 97.3% (75 ng/ml) and 96.4% (450 ng/ml), respectively.

Plasma B9M was stable at −70 °C for 2 months and after three freeze/thaw cycles. B9M was also found to be stable in the processed samples at room temperature for 4 h and in the autosampler for at least 24 h. The RSD and RE were in the range of 3.8% to 9.1% and −8.1% to 7.0%, respectively. The method was claimed to be credible and robust under our experiment conditions.

3.3. Application to a pharmacokinetic study

This method was successfully applied to determine the plasma concentration of B9M in rats following intravenous and subcutaneous administrations (0.65 mg/kg). The mean plasma concentration-time curves were illustrated in Fig. 3. The main pharmacokinetic parameters for B9M in rats were presented in Table 2. The volume of distribution at terminal phase (V_Z) was 14.8 ± 2.0 l/kg, much larger than the total body water volume (0.67 l/kg), suggesting that B9M distributed extensively into extravascular tissues such as fat and brain due to its high lipophilicity (ClogP 8.73). The $T_{1/2}$ of 18.6 h was long, which might also be attributed to the extensive distribution of B9M in some tissues and its slow clearance from the reservoir. Such suggestion was justified by our later tissue distribution study (data not shown). B9M reached peak plasma concentration rapidly after subcutaneous administration (T_{max} = 6.6 min). The absolute bioavailability was up to 85.6%. These data indicated that the subcutaneous administration was an optimal route for the administration for B9M, and it was

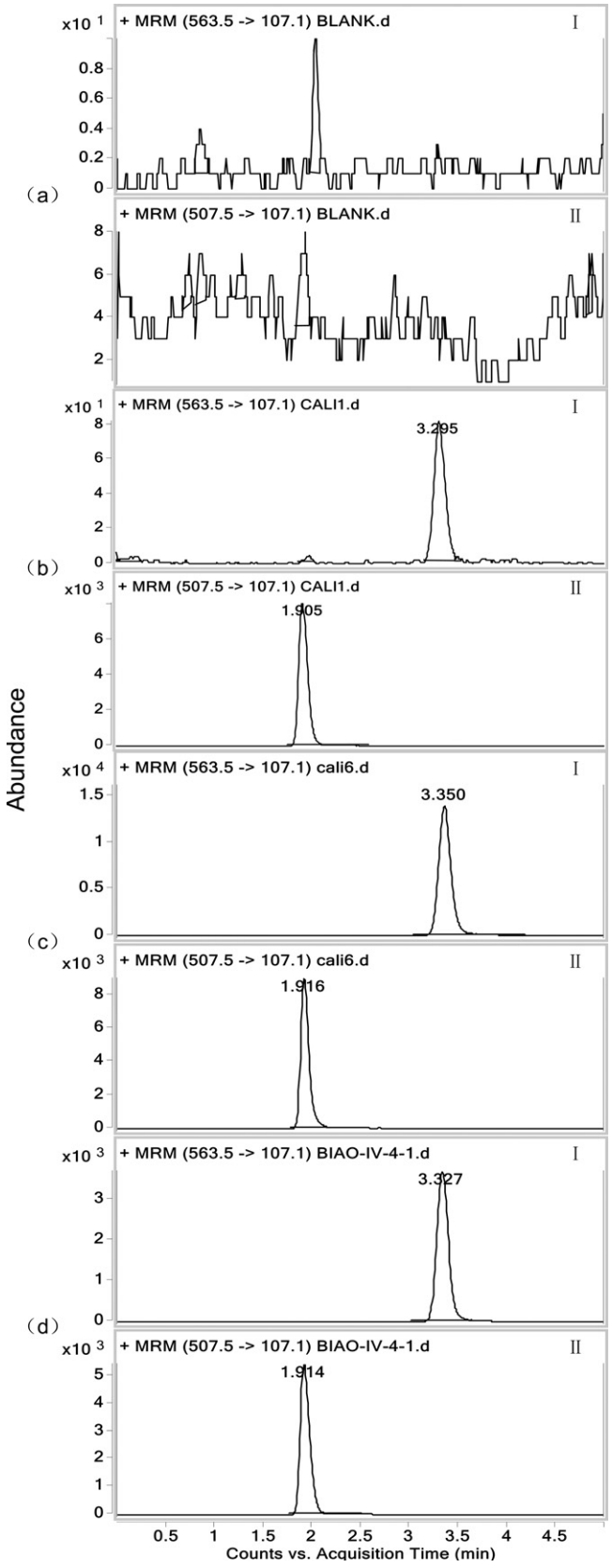

Fig. 2. Representative MRM chromatograms of (a) a blank rat plasma sample (non-spiked), (b) a blank rat plasma sample spiked with B9M at LLOQ level (1 ng/ml), (c) a blank rat plasma sample spiked with B9M (200 ng/ml) and (d) a rat plasma sample obtained 5 min after intravenous injection of B9M (0.65 mg/kg). Peak I, B9M; Peak II, IS.

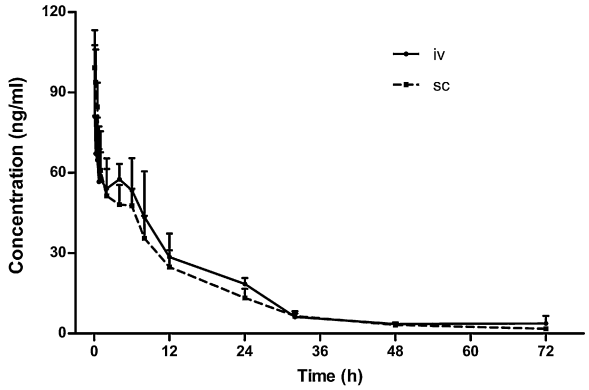

Fig. 3. Concentration (Mean ± S.D., $n = 6$) – time profiles of B9M in rats with two routes of administration (intravenous, iv; subcutaneous, sc) at 0.65 mg/kg.

Table 2
The pharmacokinetic parameters of B9M in rats with two routes of administration at 0.65 mg/kg ($n = 6$, Mean ± S.D.).

Parameters	Intravenous	Subcutaneous
$AUC_{(0-72)}$ (ng h/ml)	1072 ± 185	977.0 ± 174.5
$AUC_{(0-\infty)}$ (ng h/ml)	1194 ± 144	1022 ± 170
$MRT_{(0-72)}$ (h)	14.41 ± 2.59	14.97 ± 1.04
$T_{1/2}$ (h)	18.6 ± 0.9	17.5 ± 2.6
T_{max} (h)	0.08 ± 0.00	0.11 ± 0.07
C_{max} (ng/ml)	82.58 ± 20.90	100.62 ± 13.28
$V_{Z/F}$ (l/kg)	14.8 ± 2.0	16.7 ± 4.9
CL/F (ml/h/kg)	550.8 ± 65.1	651.6 ± 111.0

possible and significant to develop a subcutaneously-implantable formulation of B9M for Alzheimer's patients in our further study.

4. Conclusion

A sensitive, rapid and specific method was firstly reported for the quantification of B9M, a new anti-Alzheimer's dimer, in rat plasma. The method showed excellent performance as follows: low LLOQ (1 ng/ml), wild range (1–500 ng/ml), small sample volume (50 μl), short running time (5 min) and simple preparation process. It was successfully applied to the pharmacokinetic study of B9M in rat plasma, and could be easily extended to the pharmacokinetic study in other species of animal or other biological matrix.

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