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Short communication

Liquid chromatographic–tandem mass spectrometric method for the quantitation of huperzine A in dog plasma

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Abstract

A rapid and sensitive LC–MS–MS method for the determination of huperzine A in dog plasma using huperzine B as internal standard has been developed and validated. The analyte and internal standard were extracted from plasma using *n*-hexane–dichloromethane–2-propanol (300:150:15, v/v/v), chromatographed on a C_{18} column (5 μm, 50 mm × 4.6 mm i.d.) with a mobile phase consisting of acetonitrile–methanol–10 mM ammonium acetate (35:40:25, v/v/v), and detected using a tandem mass spectrometer with a TurboIonSpray ionization interface. The run time was only 2 min. The assay was linear over the concentration range 0.05–20 ng/ml and intra- and inter-day precision over this range were <5.3% with good accuracy. The limit of detection in plasma was 0.01 ng/ml. The method was successfully applied to define plasma concentration–time curves of huperzine A in dogs after the last dose of an intramuscular injection (10 μg/kg per day for 15 days) of a sustained-release formulation of huperzine A.

Keywords: Huperzine A

1. Introduction

Huperzine A [(5R,9R,11E)-5-amino-11-ethylidene-5,6,9,10-tetrahydro-7-methyl-5,9-methanocycloocta-[B]-pyridin-2(1H)-one] and its analogue huperzine B (Fig. 1) are Lycopodium alkaloids isolated from the Chinese herb *Huperzia serrata* (*Lycopodium serratum*). They act as reversible and selective cholinesterase inhibitors and are highly effective in improving memory in different animal models. Huperzine A is currently undergoing development as a drug for the treatment of Alzheimer's disease [1,2]. Compared to tacrine and donepezil, it shows favorable features such as high potency, long duration of action, high bioavailability and low toxicity [3–5].

Due to the low dosage required in human studies, an analytical technique with sufficient sensitivity and specificity to measure the low concentrations of huperzine A in biological fluids is required. The limited data on the pharmacokinetics of huperzine A in humans show the time course after a high oral dose (0.99 mg/kg) follows a one-compartment open model [6]. Assays based on high-performance liquid chromatography (HPLC) with diode-array or UV detection have been described with a limit of quantitation as low as 1.6 ng/ml [6–8]. This is adequate for determining the pharmacokinetic profile of huperzine A at high dosage but inadequate for studies involving therapeutic doses administered orally or as controlled release formulations.

Liquid chromatography with tandem mass spectrometry (LC–MS–MS) has been widely employed for the analysis of drugs in biological fluids because of its excellent specificity, speed, and sensitivity [9,10]. To our knowledge, there is no LC–MS–MS method for the determination of huperzine A. In the present paper, the development and validation of a sensitive and specific LC–MS–MS method for the determination of huperzine A in plasma is reported. The method has been successfully applied to a preclinical pharmacokinetic study in dog of a sustained-release formulation of huperzine A given by intramuscular injection.

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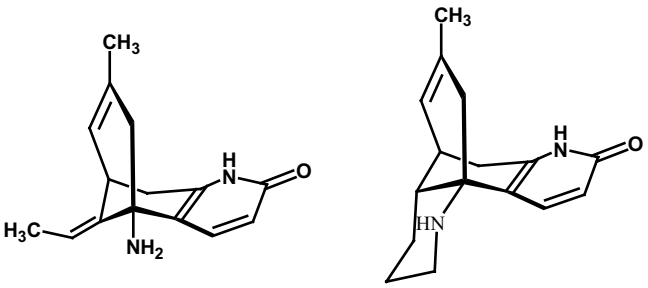

I II

Fig. 1. Structures of huperzine A (I) and huperzine B (II).

2. Experimental

2.1. Materials and chemicals

Huperzine A and B (Fig. 1) were kindly supplied by Zhulin Antun (Henan, China). The sustained-release formulation of huperzine A for intramuscular injection was a suspension of poly(DL-lactide-co-glycolide) (PLGA) microspheres (45 μm, drug loading 3.06%). The PLGA was a mixed polymer containing RESOMER grades RG 502 H and RG 503 H (1:1) purchased from Boehringer Ingelheim (Germany). Acetonitrile and methanol were HPLC grade, and all other chemicals were of analytical grade and used without further purification. Distilled water, prepared from demineralized water, was used throughout the study.

2.2. Sample preparation

Stock solutions of huperzine A and B (100 μg/ml) in methanol were used to prepare a series of six standard solutions of huperzine A (0.25–20 ng/ml) and an internal standard working solution of huperzine B (100 ng/ml) in methanol. QC solutions of huperzine A in methanol at three concentrations (0.5, 8 and 100 ng/ml) were also prepared and used to spike blank plasma in the preparation of QC samples for assay validation and analysis of samples. All solutions were stored at 4 °C and brought to room temperature before use.

To a 500 μl aliquot of dog plasma in a glass tube, 100 μl of the internal standard working solution, 100 μl methanol or a standard solution of huperzine A and 100 μl NaOH (1 M) were added. The mixture was extracted with 4 ml of *n*-hexane–dichloromethane–2-propanol (300:150:15, v/v/v) by shaking for 15 min. After centrifugation at 3000 × *g* for 5 min, the organic phase was transferred to another glass tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μl mobile phase, vortex mixed, transferred to plastic autosampler vials and injected into the LC–MS–MS system.

2.3. LC–MS

The HPLC system (Agilent 1100 series) consisted of a binary pump, an autosampler (20 μl injection) and a Nucleosil C_{18} column (5 μm, 50 mm × 4.6 mm i.d. from Dalian Johnsson Separation Science and Technology Corp., Dalien, China). Isocratic chromatography was performed at ambient temperature with a mobile phase consisting of acetonitrile–methanol–10 mM ammonium acetate (35:40:25, v/v/v) at a flow-rate of 0.40 ml/min. Detection was performed on an Applied Biosystems Sciex API 4000 mass spectrometer (Applied Biosystems Sciex, Ontario, Canada) using TurboIonSpray for ion production.

Electrospray ionization was performed in the positive ion mode (ion spray voltage 5000 V) with nitrogen as nebulizing (gas 1), heater (gas 2), curtain and collision gas (five units). High-flow gas flow parameters were optimized (nebulizer 40, heater 50 and curtain 10 units) by making successive flow injections while introducing mobile phase into the ionization source at 0.4 ml/min. The TurboIonSpray temperature was set at 500 °C, declustering potential at 40 V, collision energy at 35 V and collision cell exit potential at 10 V. The instrument response was optimized for huperzine A and B by syringe pump infusion of a constant flow (10 μl/min) of a solution of the two dissolved in mobile phase into the stream of mobile phase eluting from the column. The pause time was set at 10 ms and the dwell time at 200 ms.

2.4. Validation

Linearity was assessed by analyzing in triplicate six standards with concentrations in the range 0.05–20 ng/ml in plasma. The calibration curve was based on drug-internal standard product ion peak area ratios and was analyzed by weighted linear regression ($1/x^2$). The limit of detection (LOD) was determined as the concentration with a signal-to-noise ratio of 3:1. Intra- and inter-day precision and accuracy (given as relative standard deviation (R.S.D.)) were determined by analyzing QC samples ($n = 6$) at three different concentrations (0.1, 1.6, and 20 ng/ml in plasma) on three different days and subjecting the data to one-way analysis of variance (ANOVA). The extraction recovery of huperzine A at low, medium and high concentrations was determined by comparing the response of extracted samples of blank plasma spiked with analyte pre- and post-extraction. Stability of the analyte was assessed in spiked plasma standards stored at −20 °C for 3 months, stock solutions in methanol stored at 4 °C for 2 months and solutions in mobile phase in plastic autosampler vials at room temperature for 24 h. Huperzine A was shown to be stable under all storage conditions.

2.5. Application of the method

Six beagle dogs (10 ± 1.0 kg from the Laboratory Animal Center, Sichuan Academy of Medical Sciences) were

administered daily intramuscular injections (1 ml) of huperzine A (10 μg/kg per day for 15 days) after overnight fasts. Blood samples (3 ml) were collected into heparinized tubes before and at the following times after the last injection: 0.5, 1, 2, 4, 6, 8, 24 h and every 24 h for another 8 days. Plasma was separated immediately by centrifugation at 3000 × *g* for 10 min and stored at −20 °C until analysis. Plasma concentrations (mean ± standard deviation (S.D.)) were used to generate a concentration–time profile for huperzine A.

3. Results and discussion

3.1. Mass spectrometry

Analyte and internal standard responded best to positive ionization and protonated molecular ions $[M + H]^+$ were present as major peaks for both compounds. Small amounts of $[M + Na]^+$ were also detected. Product ion spectra of $[M + H]^+$ showed fragment ions at *m/z* 226, 210, 198 and 184 for huperzine A and at *m/z* 226, 198 and 170 for huperzine B (Fig. 2). The fragment ion at *m/z* 226 (formed by loss of NH_3) was present in the highest abundance and was suitable to quantitate huperzine B but was associated with considerable noise in blank plasma for huperzine A. However, the fragment ion at *m/z* 210 was suitable for quantitation of huperzine A and was chosen for multiple reaction monitoring acquisition. The most suitable collision energy was determined by observing the maximum response obtained for the two product ions.

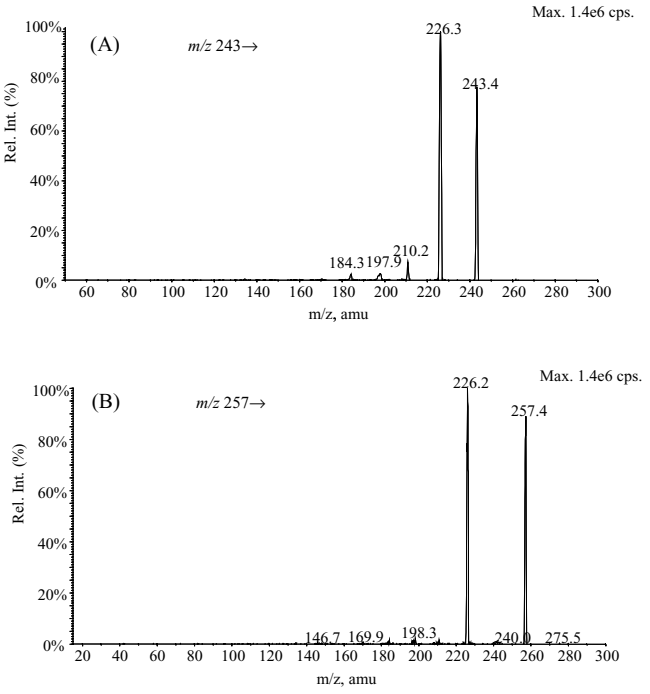

Fig. 2. Full-scan product ion spectra of $[M + H]^+$ for (A) huperzine A and (B) huperzine B.

3.2. Chromatography

Various combinations of acetonitrile, methanol, acetic acid and formic acid were investigated with a view to optimizing the mobile phase for sensitivity, speed and peak shape. Addition of acid reduced matrix effects but caused a decrease in response of the analyte. However, ammonium acetate reduced matrix effects without decreasing response and was included in the final mobile phase. A number of brands of C_{18} column (Nova-Pak, Hypersil and Zorbax) were evaluated but, compared to Nucleosil, gave poor chromatography or matrix effects. Under the optimum assay conditions, analyte and internal standard gave retention times of 1.64 and 1.69 min, respectively. The assay was linear ($r = 0.9991$) with an LOD of 0.01 ng/ml. The similarity of retention time of analyte and internal standard reduced the potential for different matrix effects and the run time of 2.0 min allowed a high sample throughput (150–200 samples per day).

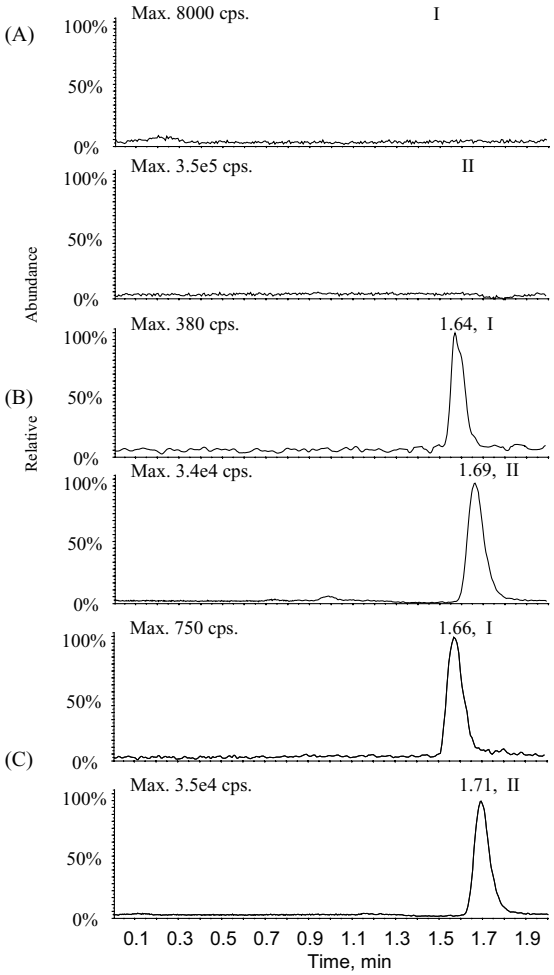

Fig. 3. Representative single reaction monitoring chromatograms of (A) blank plasma; (B) plasma spiked with huperzine A and huperzine B at the limit of quantitation (0.05 ng/ml); and (C) a plasma sample 192 h after an intramuscular injection (10 μg/kg) of a sustained-release formulation of huperzine A to a beagle dog. Peak I, huperzine A; peak II, huperzine B.

Table 1
Precision and accuracy for the determination of huperzine A in dog plasma (data are based on assay of six replicates on three different days)

Added concentration (ng/ml)	Found concentration (ng/ml)	Intra-day R.S.D. (%)	Inter-day R.S.D. (%)	Relative error (%)
0.10	0.101	3.72	4.96	0.93
1.6	1.61	3.39	3.16	0.53
20.0	20.5	2.44	5.33	2.25

3.3. Method validation

3.3.1. Specificity

Representative chromatograms of blank plasma, plasma spiked with huperzine A at the limit of quantitation (0.05 ng/ml) and a study sample containing a low concentration of huperzine A are shown in Fig. 3. The assay was free of interference from compounds in the biomatrix as shown by analysis of control plasma from each of the six dogs used in the study. Matrix effects were evaluated by comparing the peak areas of analyte in extracted samples of blank plasma spiked after extraction with the corresponding areas obtained by direct injection of standard solutions. No co-eluting "unseen" endogenous species interfered with the ionization of the analyte and internal standard.

3.3.2. Recovery, precision and accuracy

Various organic solvents were tested to develop an efficient liquid-liquid extraction procedure. Recovery was assessed by comparing the response of extracted samples of blank plasma spiked before and after extraction. Using *n*-hexane–dichloromethane–2-propanol (300:150:15, v/v/v) as extraction solvent resulted in clean extracts with good recovery. The extraction recoveries of huperzine A were 77.1 ± 4.0, 85.7 ± 2.8, and 86.3 ± 2.2% at plasma concentrations of 0.1, 1.6, and 20 ng/ml, respectively. Precision at these concentrations was <5.3% with good accuracy (Table 1).

3.4. Application of the method

The method was successfully applied to quantify huperzine A in dog plasma for 9 days following the final dose of daily intramuscular injections (10 μg/kg for 15 days) of a sustained-release formulation. The concentration versus time profile is shown in Fig. 4. The mean C_{max} was 0.36 ± 0.08 ng/ml occurring at 48 ± 24.5 h. The mean plasma elimination half life was 54.75 ± 5.61 h and the mean area under the plasma concentration versus time curve was 92.56 ± 4.54 ng h/ml.

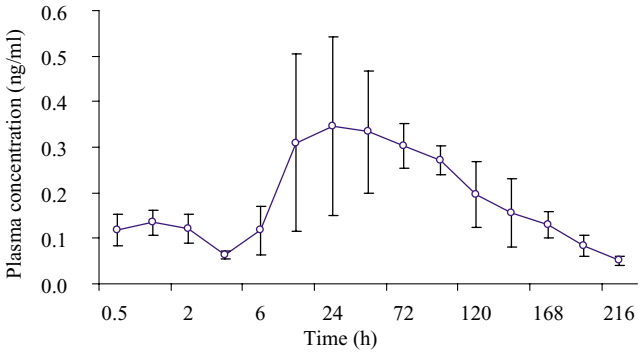

Fig. 4. Plasma concentration–time profile for huperzine A after the last dose of an intramuscular injection (10 μg/kg per day for 15 days) of a sustained-release formulation of huperzine A. Data are mean ± S.D. for six dogs.

4. Conclusion

A highly sensitive, selective and rapid method for the determination of huperzine A in dog plasma is reported using high-performance liquid chromatography with tandem mass spectrometric detection. The sensitivity was sufficient to determine the drug in dog plasma after injections of a sustained-release formulation of huperzine A making it suitable for use in pharmacokinetic studies. The method allows high sample throughput due to the short run time and relatively simple sample preparation procedure.

Acknowledgements

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