Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 44 (2007) 309–312
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Determination of Huperzine A in rat plasma by high-performance liquid chromatography with a fluorescence detector

Peng Yue, Tao Tao *, Yan Zhao, Jinfeng Ren, Xuyu Chai

Division of Pharmaceutics, Shanghai Institute of Pharmaceutical Industry, ZhongShanBeiYi Road 1111, Shanghai 200437, China

Received 18 October 2006; received in revised form 14 February 2007; accepted 16 February 2007
Available online 23 February 2007

Abstract

A simple reversed-phase high-performance liquid chromatography (HPLC)-fluorescence method for the determination of Huperzine A in rat plasma was developed and validated. Separation was achieved on Kromasil C(8) column (5 μm, 150 mm × 4.6 mm i.d.). The mobile phase, methanol–water–triethanol amine (45:55:0.05, v/v/v), was delivered at a flow rate of 1.0 ml/min. The eluent was monitored by a fluorescence detector with excitation wavelength at 310 nm and emission wavelength at 370 nm. No interfering peaks were observed in rat blank plasma. The relationship between Huperzine A concentration and peak-area ratio of Huperzine A to the IS was linear over the range of 2.5–250 ng/ml. The intra- and inter-day coefficients of variation were ≤10.1% and ≤14.1%, respectively. The method and extraction recovery of Huperzine A were 101.9–108.9% and 73.5–84.6%, respectively. Huperzine A was found to be stable for at least 5 h at RT and 1 week at −20 °C. The method was applied to a pharmacokinetic study of Huperzine A in rats following intranasal administration.

Keywords: Huperzine A; HPLC fluorescence; Rat plasma; Determination

1. Introduction

Huperzine A (Fig. 1) is a natural compound originally isolated from Chinese medicine Huperzia serrata in 1986, which is a potent, reversible and selective inhibitor of AChE with a rapid absorption and penetration into the brain in animal tests [1]. It exhibits memory-enhanced activities in animal and clinical trials. It has been approved as a drug for the treatment of Alzheimer disease (AD) in China, and marketed in USA as a dietary supplement [2]. Researches had revealed that 73% of the administered drugs were excreted from kidney swiftly [3]. Results of clinical trials demonstrated that Huperzine A was not only efficient and reliable in treatment of patients with mild to moderate AD if used properly, but also enhanced the memory and learning performance of adolescent students. Currently, the marketed medicines of Huperzine A are orally administered products, including tablets and capsules.

Recently, much interest had been arisen to the exploitation of the nasal route for delivery of drugs to the brain, and the intranasal administration was believed to be a promising method which can circumvent the blood–brain barrier [4,5]. Our institute had developed a Huperzine A in situ gel intranasal spray, which was supposed to deliver more drugs to the CNS, while decreasing the peripheral cholinergic side effects compared with the orally administered products. Huperzine A is a potent molecule (50 μg/tablet; maximum recommended human daily dose: 450 μg). The blood concentration was very low and difficult to detect by routine HPLC-UV method. For this reason, there was little information available for the pharmacokinetic study of Huperzine A [6–9].

In order to characterize the Huperzine A concentration–time profiles and study pharmacokinetics of Huperzine A intranasal spray in rats, a simple and sensitive method for determination of Huperzine A in plasma was established and validated. Our laboratory had already used it in Huperzine A determination in cerebrospinal fluid of rats [10]. Previous work confirmed that this method was more sensitive than the HPLC-UV method. In addition the low cost could make this method a routine usage for Huperzine A pharmacokinetics analysis.

* Corresponding author. Tel.: +86 21 55514600x124; fax: +86 21 65420806.
E-mail address: taotaosipi@hotmail.com (T. Tao).

0731-7085/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2007.02.019

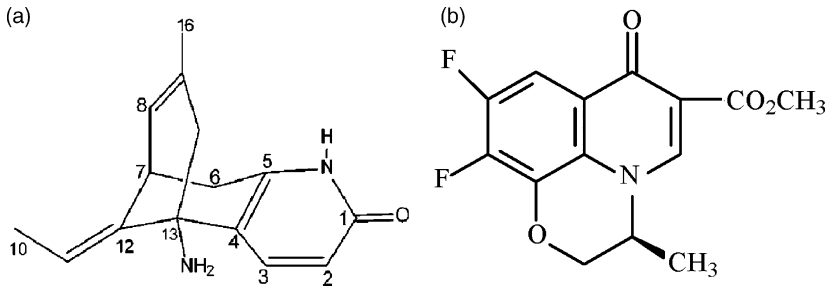

Fig. 1. Structure of Huperzine A (a) and internal standard (Shix, b).

2. Experimental

2.1. Materials and chemicals

Huperzine A was provided by Wan Bang Pharmaceutical Co. (Zhejiang, China). Its purity was 99.6%, which was verified by Zhejiang Provincial Institute for Drug Control. Huperzine A intranasal spray (2 mg/ml) was developed by Pharmaceutical Department of Shanghai Institute of Pharmaceutical Industry. The internal standard: (*S*)-9,10-difluoro-3-methyl-7-oxo-2,3-dihydro-7*H*-pyrido[1,2,3-de][1,4]benzoxazine-6-carboxylic acid methyl ester (Shix), was acquired from Chemical Department of Shanghai Institute of Pharmaceutical and Industry which has similar distribution coefficient between chloroform and water with Huperzine A (Fig. 1).

Borax, sodium carbonate, and triethanol amine were of analytical grade. Chloroform and methanol were chromatographic grade. Demineralized water was used throughout the study. Sprague–Dawley rats were obtained from experimental animal center of Fudan University.

2.2. Equipment and chromatographic condition

The Shimadzu HPLC equipment consisted of two LC-10ADVP pumps, one RF-10AXL fluorescence detector, a manual 7725i injection with a 20 μl fixed loop, a CTO-10AVP column oven, a SCL-10VP system controller and a CLASS-VP HPLC work station. The analytical column was a Kromasil ODS C (8) (150 mm × 4.6 mm, 5 μm). The mobile phase was a mixture of methanol–water–triethanol amine (45:55:0.05, v/v/v), with a flow rate of 1.0 ml/min. Fluorescence detector has excitation wavelength at 310 nm and emission wavelength at 370 nm and the temperature of column oven was set at 40 °C.

2.3. Sample preparation

Blood samples were collected from femoral artery of rats, and then processed for plasma by centrifugation at 3000 × *g* for 10 min. Plasma samples were frozen and maintained at −20 °C until analysis.

Stock solutions of Huperzine A (1 mg/ml) and Shix (100 μg/ml) in methanol were used to prepare a series of seven standard solutions of Huperzine A (20–2000 ng/ml) and an internal standard working solution (2 μg/ml) in methanol. All solutions were stored at −4 °C and brought to room temperature before use. Standard plasma samples in concentrations (2.5, 6.25, 12.5, 25, 62.5, 125 and 250 ng/ml) were made from 175 μl of blank rat plasma with 25 μl of Huperzine A standard solution. Three concentrations (6.25, 62.5 and 125 ng/ml) were selected as QC samples for assay validation.

Standard plasma samples with 10 μl of the internal standard working solution and 100 μl of borax–sodium carbonate buffer solution (pH 11.8) were added to a glass tube. After briefly vortex, the mixture was extracted with 2 ml of chloroform by vortex-mixing for 5 min. After centrifugation at 3000 × *g* for 10 min, the organic phase was transferred to another clean spiky bottom glass tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 50 μl mobile phase, and an aliquot of 20 μl of the resulting solution was injected into the HPLC system.

2.4. Application of the assay

The present assay has been used to quantify the rat plasma concentrations of Huperzine A in pharmacokinetic studies of newly developed Huperzine A intranasal spray. A 25 μl of Huperzine A intranasal spray (2 mg/ml) was given to one nostril of the anesthetized rat with a microinjector which connected with an about 2 cm PE-20 plastic tube. In the study, 0.4 ml blood samples were collected from the femoral artery of each rat at 5, 15, 30, 45, 60, 90, 120, 180, 240 and 360 min, respectively. After sampling, 200 μl plasma samples were analyzed as described above.

3. Results

3.1. Assay specificity

The specificity of the assay was evaluated with six independent sources of plasma. The results were shown in Fig. 2 indicated that the method was specific for determining Huperzine A under the chromatographic conditions employed. The peak locations of Huperzine A and IS were not interfered by the endogenous compounds in plasma. Huperzine A and IS were absolutely separated from each other with retention time of approximately 6.8 and 8.8 min, respectively.

3.2. Calibration curves and LOQ

Evaluation of the assay was performed with a seven-point calibration curve over the concentration range 2.5–250 ng/ml. The

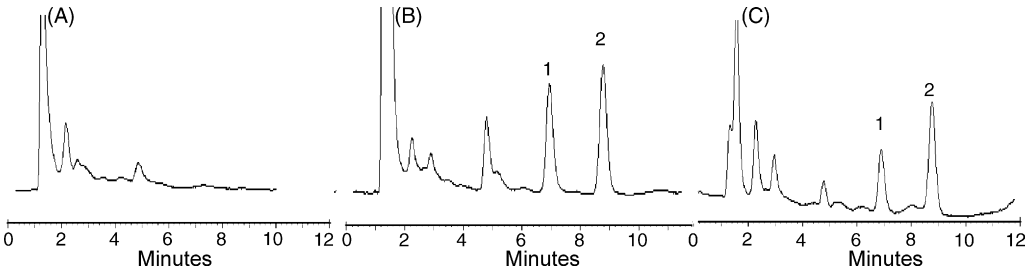

Fig. 2. HPLC chromatograms of: (A) blank plasma sample; (B) blank plasma spiked with Huperzine A (62.5 ng/ml) and IS; (C) rat plasma sample 15 min after intranasal administration. Peak 1, Huperzine A; peak 2, IS.

slope and intercept of the calibration graphs were calculated by weighted least squares linear regression. The regression equation was $Y = 90.024X - 2.1397$ and coefficient of correlation (r) was 0.9998. Y is the Huperzine A plasma concentration, and X is the peak-area ratios of Huperzine A to IS. In addition, three calibration curves of independent sources of blank plasma were analyzed for three consecutive days, respectively. The results showed that the calibration curve had good reproducibility. The limit of detection (LOD) measured based on signal/noise (S/N) ≥ 3 was 2.5 ng/ml in plasma for this assay which was the lowest concentration of the calibration curve. The limit of quantification (LOQ) of this method was set at 6.25 ng/ml measured based on signal/noise (S/N) ≥ 10 with acceptable precision (R.S.D. was 14.06%).

3.3. Accuracy and precision

The method recovery was obtained by comparing the peak-area ratios of Huperzine A to IS of the QC samples with which calculated from the calibration curve. While extraction recovery was determined by comparing the peak-area ratio of Huperzine A to IS of QC samples with the ratios obtained from direct injections of a standard solution containing the same Huperzine A and IS concentrations. The average method and extract recovery of assay were 101.9–108.9% and 73.5–84.6%, respectively.

Five QC samples at each concentration were prepared for intra- and inter-day precision study. They were detected on the same day for intra-day or on five consecutive days for inter-day assay. The precision was evaluated by the R.S.D. of intra- and inter-day assay. The R.S.D. was $\leq$10.1% for intra-day and $\leq$14.1% for inter-day (Table 1).

3.4. Stability

The stability of the assay was evaluated by determining the blank plasma spiked with Huperzine A after stored for 1 day or 1 week at −20 °C conditions (Table 2). No obvious degradations were observed and all the pharmacokinetic samples were assayed in at most 1 week.

Table 2
Stability of Huperzine A in rat plasma in two different storing conditions ($n = 3$)

Concentration added (ng/ml)	Concentration of plasma sample	
	−20 °C 1 day	−20 °C 30 days
60	61.2 ± 2.1	59.7 ± 3.2
30	31.1 ± 1.9	27.9 ± 2.2
20	21.3 ± 2.1	19.8 ± 1.6

3.5. Pharmacokinetic applicability

This validated method was applied to monitoring the plasma concentrations of Huperzine A in rats after a single intranasal administration of a 50 μg dose. Fig. 2(C) showed the typical chromatogram of plasma collected after Huperzine A intranasal administration. The pharmacokinetic study results showed swift absorption happened right after administration with C_{max} of approximate 60 ng/ml and $T_{1/2}$ of about 1.4 h calculated from Kinetic 2000 computer program. The mean plasma concentration–time profile is illustrated in Fig. 3.

4. Discussion

Many compounds have UV absorption, but few of them have fluorescence activities. Huperzine A is such a molecular with necessary conjugated π–π* transitional structure for triggering fluorescence absorption. The HPLC-fluorescence method is much more sensitive than the UV method because of its two terminal restrictions to the compound at both excitation and emission. And there were some endogenous compounds with extreme fluorescence activity observed not in rat plasma but in rat cerebrospinal fluid [10], so it was futile for worrying

Table 1
Accuracy and precision for assay of Huperzine A ($X \pm s$, %, $n = 5$)

Concentration (ng/ml)	Recovery (%)		Precision (%)	
	Method recovery ($X \pm s$, %)	Extract recovery ($X \pm s$, %)	Intra-day	Inter-day
6.25	105.9 ± 9.9	84.6 ± 8.8	7.1	14.1
25	108.9 ± 6.7	79.1 ± 11.2	4.9	9.2
125	101.9 ± 11.4	73.5 ± 1.3	10.1	11.9

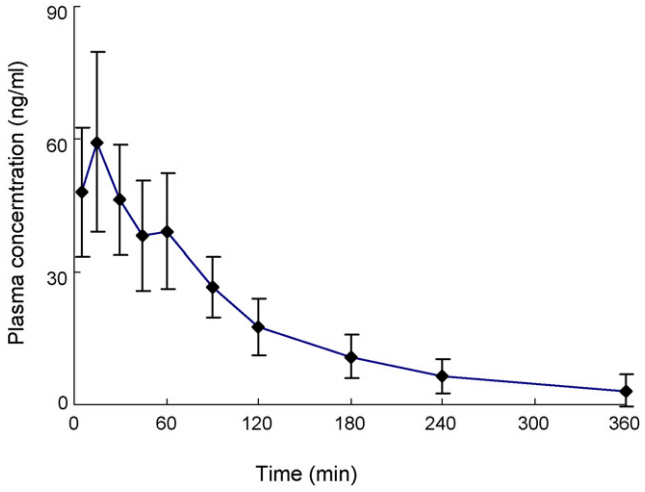

Fig. 3. Plasma concentration–time profile of Huprezine A after intranasal administration. Data are mean ± S.D. ($n = 6$).

about the possibility that the Huperzine A determination could be interfered.

Because the Huperzine A is a natural weak alkaloid, the plasma was adjusted to alkalescence for increasing extraction of Huperzine A. And small amount of triethanol amine added into the mobile phase helped separating the Huperzine A from IS and diminishing the tailing of Huperzine A. But chromatography column should be carefully selected to maintain good performance under long term of alkalescence condition.

In comparison of the recently published articles concerning the determination of Huperzine A [6–9], considering the factors such as plasma volume sampled and the volume injected, the absolute amount of the drugs could be detected in this method was more than the UV detector and less than the MS with the similar preparation and extraction procedures.

5. Conclusion

This paper describes a specific, simple, and sensitive HPLC method with fluorescence detector for the determination of Huperzine A in rat plasma after intranasal administration. Both sensitive and cheap characteristics will make this method a preferred choice for pharmacokinetic study of Huperzine A.

Acknowledgement

This work was supported by the 863 National Natural Science Foundation of China.

References

[1] D. Bei, X. Tang, X. He, Curr. Med. Chem. 7 (2000) 355–374.
[2] H. Jiang, X. Luo, D. Bai, Curr. Med. Chem. 10 (2003) 2231–2252.
[3] Y.E. Wang, J. Feng, W.H. Lu, X.C. Tang, Acta Pharmacol. Sinica 9 (1988) 193–196.
[4] L. Illum, Eur. J. Pharm. Sci. 11 (2000) 1–18.
[5] M.I. Ugwoke, N. Verbeke, R. Kinget, J. Pharm. Pharmacol. 53 (2001) 3–22.
[6] Y. Wang, D. Chu, J. Gu, J.P. Fawcett, Y. Wu, W. Liu, J. Chromatogr. B 803 (2004) 375–378.
[7] J.C. Ye, S. Zeng, W.G. Zhang, J. Chromatogr. B 817 (2005) 187–191.
[8] G.L. Wei, S.H. Xiao, R.C.X. Lu, J. Chromatogr. B 830 (2006) 120–125.
[9] A.B. Hameda, S. Elosta, J. Havel, J. Chromatogr. A 1084 (2005) 7–12.
[10] P. Yue, Y. Zhao, T. Tao, Chin. Pharm. J. 40 (2005) 1503–1505.