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Simultaneous determination of ZT-1 and its metabolite Huperzine A in plasma by high-performance liquid chromatography with ultraviolet detection

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Abstract

ZT-1 is a novel acetylcholinesterase (AChE) inhibitor. It is rapidly transformed to Huperzine A (Hup A) in vitro. A simple and rapid HPLC-UV method for the simultaneous determination of ZT-1 and its metabolite Hup A in plasma is described. The chromatographic separations were achieved on a C_{18} ODS column (250 mm × 4.6 mm ID) using methanol-1 mmol/L ammonium acetate (70:30,v/v) as mobile phase. The flow rate was 0.7 mL/min, the detection wavelength was 313 nm and the column temperature was kept at 35 °C. Plasma samples were prepared as rapidly as possible and extracted immediately with 5 mL of chloroform:iso-propyl alcohol mixture (v/v, 9:1).The retention times of ZT-1 and Huperzine A (Hup A) were 18.7 and 14.4 min, respectively. The mean absolute recoveries of two analytes were >90%. Quantification limits were all 0.02 nmol/mL for ZT-1 and Hup A. This analytical method was reliable and convenient procedure that meets the criteria for the pharmacokinetic evaluation of ZT-1 on experimental animals.

Keywords: ZT-1; Huperzine A

1. Introduction

ZT-1, *N*-[2-hydroxy-3-methoxy-5-chlorobenzilidene] Huperzine A is a novel potent cholinesterase (ChE) inhibitor, which is rapidly transformed into the active metabolite Huperzine A (Hup A). Originally isolated from Chinese club moss by Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Hup A was demonstrated to have the neuroprotective and potent anticholinesterase properties [1,2]. Because of its optimal profile and potency, ZT-1 was selected out of over 100 Hup A derivatives initially identified at this institute. In vitro pharmacological studies have showed that ZT-1 produces a marked concentration dependent inhibition of acetylcholinesterase (AchE). In vivo investigations conducted in mice, rats and monkeys have showed that ZT-1 is equipotent to Hup A and far potent than donepezil and tacrine [3,4]. Fig. 1 shows the chemical structures of ZT-1 and Hup A. The preclinical studies have been finished in China and phase I clinical studies have also been completed in Switzerland and China. A phase II for the treatment of Alzheimer's disease (AD) is being conducted in patients with mild moderate AD [4]. The determination of Hup A in plasma has previously been described [5–7], but the HPLC-UV method for the determination of ZT-1 has not been reported. In this paper, we described a simple, accurate and precise method based on HPLC-UV technique for the simultaneous determination of ZT-1 and its active metabolite Hup A in plasma for the first time, which could successfully be applied to the pharmacokinetic evaluation of ZT-1 on experimental animals.

2. Experimental

2.1. Materials

ZT-1 and Hup A were supplied by Shanghai Institute of Materia Medica, Chinese Academy of Sciences. ZT-1 (Batch No. 20000307, Purity: >99.6%). Hup A (Batch No: 20010116, Purity: >99.5%). Polyvinylpyrrolidone (PVP) solubility-promoted ZT-1 (PVP/ZT-1, Batch No: 20000515, Content of ZT-1: 2.07%) was also supplied by the institute.

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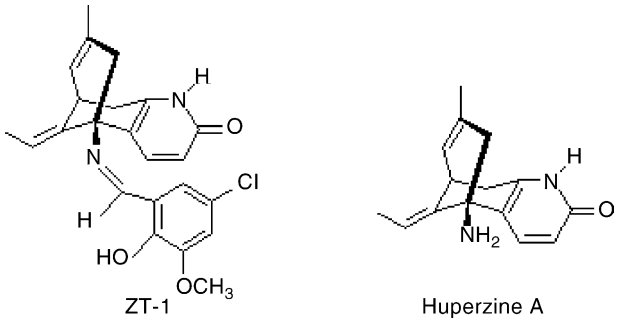

Fig. 1. Chemical structures of ZT-1 and Huperzine A.

HPLC-grade methanol was obtained from Concord Tech, Tianjin, China. Analytical grade ammonium acetate, analytical grade chloroform and analytical grade isopropanol were obtained from Tianjin Chemical Reagent Co., Tianjin, China.

The water was deionized (water resistivity: 6–7 MΩ) with Model 2 Millipore (Millipore II, Japan).

2.2. Animals

Sprague-Dawley rats (male and female, body weight 180–200 g) were supplied by The Experimental Animal Department of Tianjin Institute of Pharmaceutical Research (Accreditation No.: Jinkedonggan 001; Tianjin).

Beagles dogs (male and female, body weight: 10–12 kg) were supplied by Laboratory Animals Center, Military Medical Academy of Sciences (Accreditation No.: Yidongguan 01-3042; Beijing).

2.3. Instrument and chromatographic conditions

The liquid chromatographic system consisted of a Shimadzu LC-10A series liquid chromatograph (including SCL-10A system controller, SIL-10AXL auto injector, LC-10AT pump, thermostated column compartment) with a Gilson 118 UV–vis detector. The system runs by ChemStation software.

A stainless-steel column (250 mm × 4.6 mm ID) was packed with Nucleosil C18, particle size 5 μm. The column was maintained at 35 °C and UV detection was set at 313 nm. The mobile phase, consisting of 1 mmol/L ammonium acetate–methanol (70:30,v/v), was delivered at a flow rate of 0.7 mL/min.

2.4. Preparation of standards

Stock solutions of ZT-1 and Hup A of both 20.0 nmol/mL were prepared in methanol, respectively. They were placed in closed volumetric flask and kept at 4 °C. No change in stability over a period of 7 days was observed. The working solutions were prepared by diluting appropriate portions of these solutions with methanol at the concentrations of 2.0, 0.5, 0.2, 0.05 and 0.02 nmol/mL for ZT-1 and 5.0 2.0, 0.5, 0.2, 0.05 and 0.02 nmol/mL for Hup A.

2.5. Sample preparation

Since ZT-1 is not stable and can be rapidly transformed to Huperzine A (Hup A) in water or aqueous organic solvents, we constructed the calibration curves of ZT-1 and Hup A independently in order to prevent interferes from the transformation of ZT-1 to Hup A.

The solutions of ZT-1 was prepared with 2 mL methanol at followed concentrations: 2.0, 0.50, 0.20, 0.05 and 0.02 nmol/mL and dried with nitrogen before adding 2.0 mL rat plasma. Plasma samples were prepared as rapidly as possible. After mixed, the rat plasma was extracted immediately with 5 mL chloroform:isopropyl alcohol mixture(v/v, 9:1). The mixture was vortex mixed for 2 min and centrifuged at 15000 rpm for 5 min, then the 4 mL organic layer was transferred to a second tube and dried with nitrogen. The residue was reconstituted with 0.16 mL methanol and an aliquot (50 μL) was used for HPLC assay.

The solution of Huperzine A was prepared with 2.0 mL of methanol at followed concentration: 5.0, 2.0, 1.0, 0.50, 0.20, 0.10, 0.05 and 0.02 nmol/mL and dried with nitrogen before adding 2.0 mL rat plasma. After the same process of extraction as described for ZT-1, a volume of 50 μL was injected for HPLC analysis.

2.6. Calibration curves

The calibration ranges were 0.02–2.0 nmol/mL for ZT-1 and 0.02–5.0 nmol/mL for HupA, respectively. Calibration curves of ZT-1 and Hup A were obtained by plotting the peak area versus concentration. The regression equations were calculated by the least-squares method.

3. Results

3.1. Method development

Typical chromatograms obtained from standard and extracted rat plasma are illustrated in Fig. 2A–D. Under the chromatographic conditions described, the peaks of ZT-1 and Hup A were well resolved, their retention times were 18.7 and 14.4 min, respectively. Endogenous rat plasma components did mot give any interfering peaks.

3.2. Method validation

3.2.1. Linearity

Linearity was tested at five concentration points ranging from 0.02 to 2.0 nmol/mL for ZT-1 and at six concentration points ranging from 0.02 to 5.0 nmol/mL for Hup A in rat plasma. Respective regression equations were: $y = 1462 + 3363936x$ for ZT-1 and $y = 29503 + 3261455x$ for Hup A. The correlation coefficients were 0.997 and 0.999, respectively.

3.2.2. Limit of quantification

The limit of quantification was defined as the lowest amount detectable with a precision of less than 15% ($n = 5$) and an accuracy of ±15% ($n = 5$). The limits of quantification and LOQ

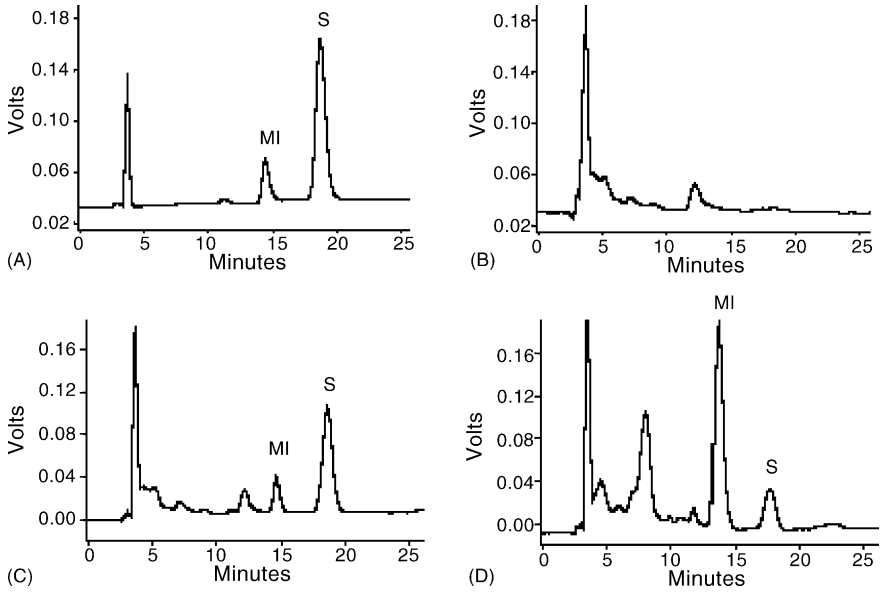

Fig. 2. Typical chromatograms of ZT-1 and Hup A. (A) standard spiked with ZT-1 2 nmol/mL and Hup A 0.5 nmol/mL; (B) blank rat plasma; (C) blank rat plasma spiked with ZT-1 2 nmol/mL and Hup A 0.5 nmol/mL; (D) a rat plasma 10 min after ig administration of 5.0 mg/kg of PVP/ZT-1. Peaks: S = ZT-1; M_1 = Hup A.

were found to be 0.02 nmol/mL for both ZT-1 and Hup A in rat plasma.

3.2.3. Recovery

The absolute recoveries of ZT-1 and Hup A were evaluated by comparing the areas found in rat plasma spiked with known amounts of the analyte, which had been processed through the entire extraction procedure, to the areas from the unextracted standard solutions prepared in methanol. The absolute recoveries were 107.8, 91.9 and 90.0% at the concentrations of 0.02, 0.5 and 2.0 nmol/mL for ZT-1 and 98.9, 101.7 and 96.6% at the concentrations of 0.02, 0.5 and 5.0 nmol/mL for Hup A, respectively. The results were provided in Table 1.

3.2.4. Precision and accuracy

The precision and accuracy were assessed by the analysis of five samples at three concentrations (low, medium and high). The precision was based on the calculation of the relative standard deviation (R.S.D.). An indication of accuracy was based on the calculation of the relative error (R.E.) of the mean found concentration as compared to the nominal concentration.

The intra-day precision and accuracy of ZT-1 are shown in Table 2. The R.S.D. for all samples analyzed were within 9.5% and the R.E. ranged from –3.5 to 5.0% of the nominal concentrations.

The intra- and inter-day precision and accuracy of Hup A are shown in Table 3. The R.S.D. for all samples analyzed were within 15.0% and the R.E. ranged from 0 to 5.0% of the nominal concentrations.

3.2.5. Transformation of ZT-1 in water, rat plasma and rat homogenate

ZT-1 was found to be stable only in methanol. The compound, however, is rapidly transformed into Huperzine A (Hup A) in water or aqueous organic solvents. So the transformation tests of ZT-1 in vitro were carried out in water, rat plasma

Table 1
Recoveries of ZT-1 and Hup A from rat plasma ($n = 5$)

Drug	Concentration (nmol/mL)	Recovery (%)	R.S.D. (%)
ZT-1	0.02	107.8	9.0
	0.5	91.9	2.4
	2.0	90.0	2.1
Hup A	0.02	98.9	1.8
	0.5	101.7	1.8
	5.0	96.6	1.8

Table 2
Intra-day precision and accuracy of ZT-1 in rat plasma ($n = 5$)

Nominal concentration (nmol/mL)	Mean found concentration (nmol/mL)	R.S.D. (%)	R.E. (%)
0.02	0.021	9.5	5.0
0.5	0.51	2.0	2.0
2.0	1.93	2.6	–3.5

Table 3
Intra- and inter-day precision and accuracy of HupA in rat plasma ($n = 5$)

Nominal concentration (nmol/mL)	Mean found concentration (nmol/mL)	R.S.D. (%)	R.E. (%)
Intra-day			
0.02	0.021	14.3	5.0
0.5	0.51	5.9	2.0
5.0	5.16	4.5	3.2
Inter-day			
0.02	0.020	15.0	0
0.5	0.50	2.0	0
5.0	5.12	5.5	2.4

Table 4
Transformation of ZT-1 into Hup A in different matrixes ($n = 3$, Mean ± S.D.)

Time (min)	10	20	40	60	120	180
H_2O						
$Fn_{(ZT)}$[a]	0.67 ± 0.01	0.47 ± 0.07	0.27 ± 0.03	0.17 ± 0.06	0.15 ± 0.09	0.02 ± 0.001
$Fn_{(A)}$[b]	0.20 ± 0.02	0.35 ± 0.03	0.51 ± 0.01	0.60 ± 0.07	0.67 ± 0.07	0.68 ± 0.001
Rat plasma						
$Fn_{(ZT)}$[a]	0.77 ± 0.05	0.70 ± 0.06	0.61 ± 0.05	0.55 ± 0.05	0.27 ± 0.03	0.21 ± 0.04
$Fn_{(A)}$[b]	0.16 ± 0.01	0.24 ± 0.03	0.36 ± 0.02	0.45 ± 0.06	0.66 ± 0.07	0.79 ± 0.04
Rat liver homogenate						
$Fn_{(ZT)}$[a]	0	0	0	0	0	0
$Fn_{(A)}$[b]	0.29 ± 0.01	0.48 ± 0.03	0.58 ± 0.01	0.61 ± 0.02	0.62 ± 0.02	0.63 ± 0.03

[a] $Fn_{(ZT)}$: fraction of ZT-1 concentration at different time divided by ZT-1 concentration at 0 min.
[b] $Fn_{(A)}$: fraction of Hup A concentration at different time divided by ZT-1 concentration at 0 min.

and rat liver homogenate. The samples mixed with 2.0 nmol/mL ZT-1 were incubated at 37 °C and taken out for assay at 0, 10, 20, 40, 60, 120 and 180 min, respectively. The concentration of ZT-1 measured at 0 min was considered as 1. Then fractions of ZT-1 and Huperzine A concentrations at different time points based on ZT-1 concentration at 0 min were calculated, i.e. $Fn_{(ZT)} = Cn_{(ZT)}/C_{0(ZT)}$, where $Fn_{(ZT)}$ was fraction of ZT-1 concentration at different time divided by ZT-1 concentration at 0 min, $C_{0(ZT)}$ was the beginning concentration of ZT-1, $Cn_{(ZT)}$ was the concentration of ZT-1 measured at different time; $Fn_{(A)} = Cn_{(A)}/C_{0(ZT)}$, where $Fn_{(A)}$ was fraction of Hup A concentration at different time divided by ZT-1 concentration at 0 min, $C_{0(ZT)}$ was the beginning concentration of ZT-1, $Cn_{(A)}$ was the concentration of Hup A measured at different time. The results (Table 4) indicated that ZT-1 was rapidly transformed into Hup A. After individual incubation of ZT-1 with water, rat plasma or rat homogenate for 3 h, $Fn_{(ZT)}$ were 0.02, 0.21 and 0 and $Fn_{(A)}$ were 0.68, 0.79 and 0.63, respectively. Since ZT-1 was rapidly transformed into Hup A in rat liver homogenate, it could not be detected from rat liver homogenate extracts. Typical chromatograms of Hup A extracted from rat liver homogenates were shown in Fig. 3.

3.2.6. Application

3.2.6.1. Determination of concentrations of ZT-1 and Hup A in rat plasma. To prevent the transformation of ZT-1 to Hup A, the PVP/ZT-1 solutions were prepared fresh in ice-cold saline (1 mL for 5 mg/kg dose) and used within 2 min. The rats were fasted for 15 h and the blood samples were collected from eye vein before and subsequently at 0.17, 0.25, 0.5, 1, 2, 4, 6, 8, 12 and 20 h after ig administration of a single dose of PVP/ZT-1. The blood samples were immediately extracted and analyzed by the proposed method. The time courses of plasma ZT-1 and Hup A concentrations were shown in Table 5.

3.2.6.2. Pharmacokinetic study following ig administration of ZT-1 to dogs. Four Beagle dogs were used for the pharmacokinetic study following ig administration of 2.5 mg/kg of PVP/ZT-

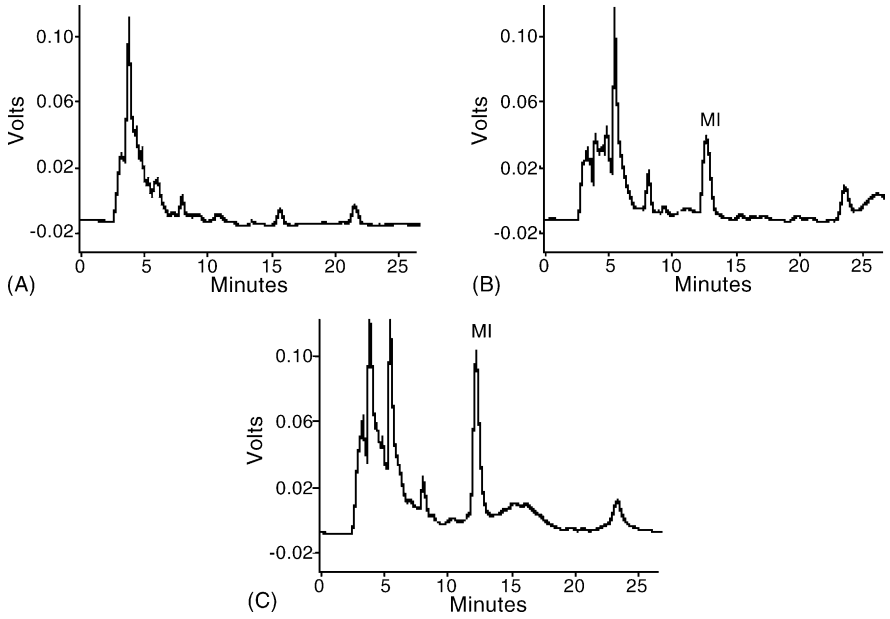

Fig. 3. Typical chromatograms of Hup A. (A) Blank rat liver homogenate; (B) blank rat liver homogenate spiked with Hup A 0.5 nmol/mL; (C) a rat liver homogenate spiked with ZT-1 2.0 nmol/mL at 20 min after incubation. Peaks: M_1 = Hup A.

Table 5
Plasma concentrations of Hup A and ZT-1 in rats after a single oral dose of 5 mg/kg PVP/ZT-1 ($n = 5$, Mean ± S.D.)

Time (h)	Hup A (nmol/mL)	ZT-1 (nmol/mL)
0.17	1.40 ± 0.74	0.145 ± 0.144
0.25	2.29 ± 0.23	0.036 ± 0.012
0.5	1.82 ± 0.14	0.029 ± 0.008
1.0	1.63 ± 0.68	ND
2.0	1.57 ± 0.33	ND
4.0	0.88 ± 0.40	ND
6.0	0.68 ± 0.42	ND
8.0	0.61 ± 0.42	ND
12.0	0.42 ± 0.20	ND
20.0	0.15 ± 0.06	ND

ND: not determined.

Table 6
Plasma concentrations of Hup A after ig administration of PVP/ZT-1 (2.5 mg/kg) in Beagle dogs ($n = 4$, Mean ± S.D.)

Time (h)	Hup A (nmol/mL)
0.25	0.68 ± 0.60
0.5	1.73 ± 1.65
1.0	2.21 ± 1.27
2.0	2.91 ± 0.59
3.0	2.65 ± 0.43
4.0	2.00 ± 0.41
6.0	1.52 ± 0.45
8.0	0.99 ± 0.37
12.0	0.54 ± 0.23
24.0	0.13 ± 0.06

1. To prevent the transformation of ZT-1 to Hup A, the PVP/ZT-1 solutions were prepared fresh in ice-cold saline (1 mL for 1 kg body weight) and used within 2 min. The dogs were fasted for 15 h and the blood samples were collected from forefoot hypodermic cephalic vein with sterile syringes at 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h after ig administration of a single dose of PVP/ZT-1. The blood sample was immediately extracted and analyzed by the determined method.

The assay of Hup A for dog plasma was validated with the same method as for rat plasma. The typical chromatograms of Hup A in dog plasma were shown in Fig. 4. The typical calibration equation was $y = 50008 + 3124213x$ ($r = 0.9995$).

The plasma concentrations of ZT-1 were not determined and that of Hup A were shown in Table 6. The results showed that ZT-1 was rapidly transformed to Hup A following ig administration of PVP/ZT-1. The peak concentrations (C_{max}) of Hup A were 2.91 ± 0.59 nmol/mL and achieved at 2 h after dosing, then the plasma levels declined to 0.13 ± 0.06 nmol/mL at 24 h after dosing.

4. Discussion and conclusion

Hup A, a biologically potent, reversible AchE inhibitor for the treatment of Alzheimer disease is being used in clinical application. In vitro and in vivo tests showed that ZT-1 was rapidly degraded to Hup A and possessed prodrug characteristics. A number of HPLC methods have been used for the pharmcokinetic evaluation of Hup A in animals and human subjects [5–7]. In this paper, we described a simple, accurate and precise method based on HPLC-UV technique for the simultaneous determination of ZT-1 and its active metabolite Hup A in plasma for the first time. The intra-day precision and accuracy of ZT-1 showed that the R.S.D. for all samples analyzed were within 9.5% and the R.E. ranged from −3.5 to 5.0% over the concentration range of 0.02–2.0 nmol/mL. The intra- and inter-day precision and accuracy of Hup A showed that the R.S.D. for all samples analyzed were within 15.0% and the R.E. ranged from 0 to 5.0% over the concentration range of 0.02–5.0 nmol/mL. According to Li's paper [8], the intra-day and inter-day variation for ZT-1 (0.1–10 ng/mL) and Hup A (0.5–50 ng/mL) were all within 10.4% with LC/MS/MS approach. This

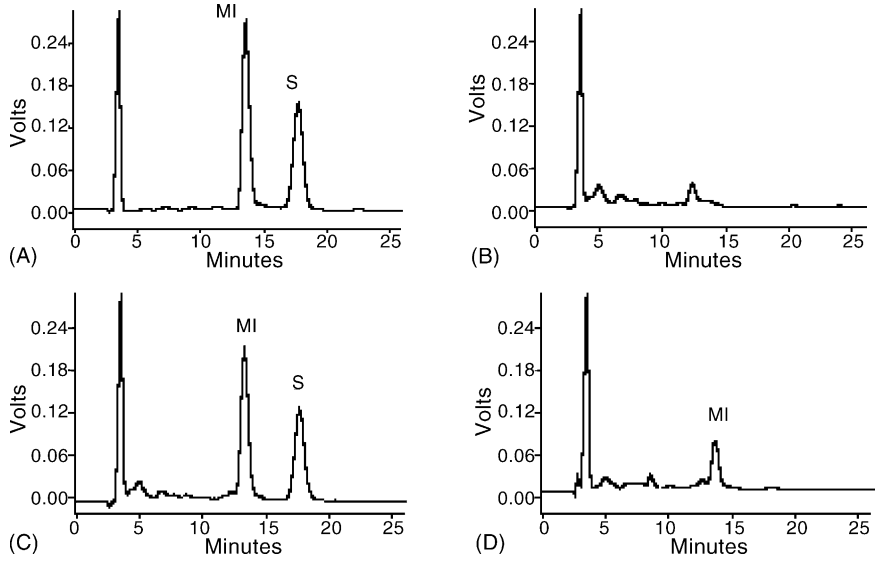

Fig. 4. Typical chromatograms of ZT-1 and Hup A. (A) Standard spiked with ZT-1 2.0 nmol/mL and Hup A 2.0 nmol/mL; (B) blank dog plasma; (C) blank dog plasma spiked with ZT-1 2.0 nmol/mL and Hup A 2.0 nmol/mL; (D) a dog plasma 2 h after ig administration of 2.5 mg/kg of PVP/ZT-1. Peaks: S = ZT-1; M_1 = Hup A.

comparison showed that this method was simple and accurate enough and could be adopted in routine pharmacokinetics analysis.

In conclusion, the described method for the analysis of ZT-1 and Hup A in plasma was specific, sensitive and accurate. This method was successfully applied to the pharmacokinetic evaluation of ZT-1 on experimental animals.

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References

[1] A. Zangara, Pharmacol. Biochem. Behav. 75 (2003) 675.
[2] R. Wang, H.Y. Zhang, X.C. Tang, Eur. J. Pharmacol. 421 (2001) 149.
[3] D.Y. Zhu, X.C. Tang, J.L. Lin, C. Zhu, J.K. Shen, G.S. Wu, S.H. Jiang, T. Yamaguch, K. Tananka, T. Wakamastu, H. Nishimura. Huperzine A Derivatives, their Preparation and their Use. US Patent 5929084 07/27/1999. Chinese Patent 95196884.X, 05/ 10/2000.
[4] ZT-1, a new treatment for Alzheimer's disease. http://www.debiopharm.com.
[5] J. Ye, S. Zeng, W. Zhang, G. Chen, J. Chromatogr. B 817 (2005) 187.
[6] Y.E. Wang, J. Feng, W.H. Lu, et al., Acta Pharmacol. Sin. 9 (1988) 193.
[7] B.C. Qian, M. Wang, Z.F. Zhou, K. Chen, R.R. Zhou, G.S. Chen, Acta Pharmacol. Sin. 16 (1995) 396.
[8] C. Li, F.F. Du, C. Yu, et al., Rapid Commun. Mass Spectrom. 18 (2004) 651.