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New method for high-performance liquid chromatographic determination of amantadine and its analogues in rat plasma

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Abstract

A novel precolumn derivatization method is described for the quantitative determination of amantadine, rimantadine and memantine in biological samples by HPLC with UV detection. The derivatization was performed at room temperature using anthraquinone-2-sulfonyl chloride (ASC) as reagent for only 10 min and without postderivatization treatment to inactivate excess reagent. The derivatives were analyzed by isocratic HPLC with a UV detector at 256 nm on a Lichrosper C18 column. The linear range for the determination of three drugs spiked in plasma (0.2 ml) was 0.05–5.0 μg/ml for amantadine and rimantadine, 0.05–2.0 μg/ml for memantine, respectively. The limits of detection and quantification were 20 and 50 ng/ml for the analytes, respectively. Application of the method to the analysis of amantadine, rimantadine and memantine in rat plasma and pharmacokinetic studies are demonstrated and proved feasible.

Keywords: Derivatization; Anthraquinone-2-sulfonyl chloride; Amantadine; Rimantadine; Memantine; Rat plasma

1. Introduction

The tricyclic antiviral drugs constitute a small, but unique part of a diverse class of nonnucleoside therapeutic agents that are effective in the prophylaxis and treatment of influenza A virus infections. Amantadine (AT) is clinically used for the treatment of influenza A virus infections and Parkinsonism [1]. Memantine (MT) and rimantadine (RT) are derivatives of AT. MT is used for the treatment of Alzheimer's disease [2] and RT for the treatment of the infections resulting from influenza A virus [3].

The three drugs are primary amines possessing neither prominent UV absorption, nor fluorescence properties nor even electroactive groups. Sample pretreatment with organic solvent extraction and derivatization techniques coupled with GC [4–6], HPLC [7–12], CE [13] are frequently used for enhancing sensitivity, especially for their determination in biological samples. Although GC–MS and LC–MS methods were suitable for microdetermination [14–16], the instruments used are not always available in a common laboratory. Analytical derivatization of AT, RT and MT has been performed [4–13]. In these methods high temperature [4–6,8–10,13], long reaction time [4–6,11,13], or complicated postderivatization treatment to inactivate excess reagents [6,12] or special facilities for the use of radioactive compounds [4–6] were usually required. Higashi et al. [7] reported a derivatization method for simultaneous determination of AT and RT in rat plasma at room temperature, but re-extraction cycles made it laborious. The aim of this study was to develop and optimise a simple, selective and sensitive HPLC-UV method for the quantitative determination of the three drugs in biological fluids using the recently described anthraquinone-2-sulfonyl chloride (ASC) [17] as a very stable precolumn derivatization reagent.

2. Experimental

2.1. Chemicals and solutions

Amantadine hydrochloride (≥99.0% purity), rimantadine hydrochloride (>99.0% purity) and memantine hydrochloride (>99.0% purity) were supplied by ZHE JIANG KANG YU pharmaceutical Co. (Hengdian, China). RT is used as the internal standard (IS) for AT analysis and AT as the internal standard

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for RT and MT analysis. ASC was prepared according to the authors' reported method [17]. Doubly distilled water was used. Methanol, acetonitrile, chloroform, ethyl acetate, ethyl ether, toluene, dichloromethane, sodium hydroxide and other chemicals employed were all of analytical grade and from Nanjing Chemical Co. (Nanjing, China).

Solutions of AT, RT and MT were prepared by dissolving the appropriate amounts of the respective compounds in water. Solution of ASC in various concentrations was prepared in dichloromethane. Solution of sodium hydroxide was prepared in water.

2.2. Instrumentation and conditions

The HPLC analysis was performed with a Shimadzu HPLC system equipped with a LC-10ATvp pump, a Shimadzu SPD-10A UV detector ($\lambda = 256$ nm). Chromatography was performed on a Lichrospher C18 column (150 mm × 6 mm i.d., 5 μm, Hanbon Ltd, Jiangsu, China) with methanol–water (85:15, v/v) as mobile phase at a flow rate 1.0 ml/min. The column was maintained at 25 °C. A Shimadzu UV-260 spectrometer (Shimadzu, Kyoto, Japan) was used to record the UV spectra of standard derivatives.

The mass spectral analysis of the standard derivatives was performed on Thermo Electron TSQ Quantum ultra tandem triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) source (San Jose, CA, USA). Mass spectrometer was operated in the negative ion mode. The spray voltage was set at 5.0 kV. Nitrogen was used as the sheath gas (35 psi). The heated capillary temperature was set at 300 °C.

2.3. Preparation and characteristics of the standard derivatives

In order to optimize the derivatization conditions and identify the peak position of the derivatives, the standard derivatives were synthesized as follows.

In a 100-ml flask equipped with a magnetic stirrer, 2.0 mmol of each drug was dissolved in 10 ml water containing 0.2 g of NaOH. 15 ml dichloromethane was added and the ASC solution (1.0 mmol in 25 ml dichloromethane) was dropped into the flask within 30 min under stirring. The mixture was stirred for further 1 h. Then, the aqueous phase was discarded. The organic phase was washed three times with 5–5 ml of 1 M hydrochloric acid aqueous solution, and dried over anhydrous Na_2SO_4. Dichloromethane was removed by rotary evaporation to obtain the standard derivatives.

The mass spectra of the three standard derivatives were obtained by ESI-MS in negative ion mode and exhibited diagnostic peaks: for the AT derivative, a base peak *m/z* 420.08 $[M–H]^-$; for the RT derivative, a base peak *m/z* 448.08 $[M–H]^-$; for the MT derivative, a base peak *m/z* 448.08 $[M–H]^-$. The standard derivatives of AT, RT and MT all have UV absorption maxima at 256 and 325 nm. The UV spectra of the derivatives were similar to those of ASC itself and ASC related derivatives previously reported [17]. The UV absorption at 256 nm is much stronger than at 325 nm, and 256 nm was thus selected as the detection wavelength in HPLC. The UV spectrum of AT standard derivative is shown in Fig. 1. The UV spectra of RT and MT derivatives (not shown) are similar to that of AT derivatives.

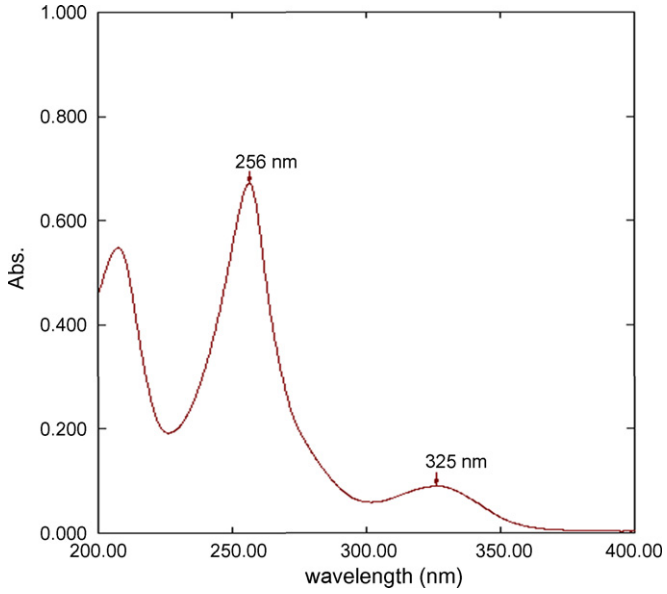

Fig. 1. The UV spectrum of derivatized AT standard. Solvent: methanol; concentration: 10 μg/ml).

2.4. Extraction and derivatization

A 200 μl aliquot of plasma was rendered alkaline by addition of 200 μl 2 M NaOH solution. Twenty microliters of IS solution (50 μg/ml) and 1 ml of dichloromethane were added. Then, the mixture was vortex-extracted for 3 min and centrifuged at 4000 × *g* for 10 min. An aliquot (0.8 ml) of dichloromethane layer was removed and evaporated to dryness under nitrogen. The residue was reacted with 0.6 ml of ASC solution (0.05 mg/ml in dichloromethane) at room temperature for 10 min, after the addition of 0.3 ml 2 M NaOH aqueous solution. After derivatization, 0.4 ml of the organic layer was removed and evaporated to dryness under nitrogen. The residue was reconstituted with 100 μl of mobile phase vortexed for 3 min, and a 20 μl aliquot of the solution was subjected to HPLC analysis. The proposed derivatization scheme is shown in Fig. 2.

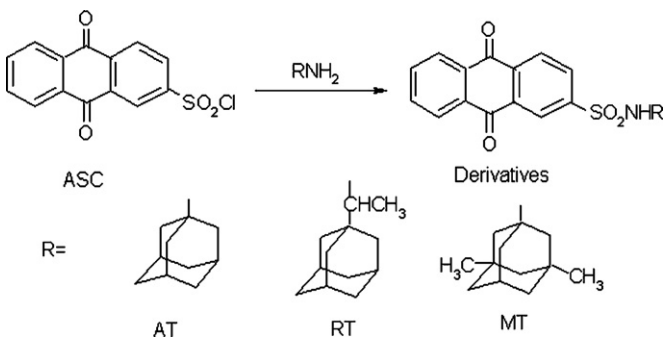

Fig. 2. Reaction scheme for the derivatization of amantadine (AT), rimantadine (RT) and memantine (MT) with anthraquinone-2-sulfonyl chloride (ASC). RNH_2 stands for AT, RT or MT.

2.5. Calibration standards and quality control samples

The quantitative applicability of the method was validated by six-point calibration standard curves over the range of 0.05–5.0 μg/ml for AT and RT, 0.05–2.0 μg/ml for MT by adding solutions of AT, RT and MT to 200 μl blank plasma. The quality control (QC) samples were prepared using different solutions of the analytes to obtain plasma concentrations of 0.1, 1.5, 4.5 μg/ml for AT and RT, 0.1, 0.5, 1.5 μg/ml for MT representing low, medium and high concentration of QC samples, respectively.

The spiked plasma samples (standards and quality controls) were extracted and analyzed using the procedure described in Section 2.3. Calibration curves based on the peak area ratios of AT, RT and MT to IS were prepared in duplicate for each sample.

2.6. Application of the assay

The developed method was used to determine the analytes in plasma samples at 0–12 h after oral (p.o.) administration of 100 mg/kg AT·HCl and RT·HCl, respectively, and at 0–12 h after intraperitoneal (i.p.) injection of 50 mg/kg MT·HCl to Sprague-Dawley male rats (8–10 weeks, 200–240 g). AT, RT and MT were dissolved in water for administration. Under light anesthesia by diethyl ether, blood samples were drawn to heparinized Eppendoff tubes at 0.083, 0.25, 0.5, 1, 2, 3, 4, 6, 8 and 12 h for AT and RT after p.o administration, and at 0.083, 0.176, 0.25, 0.5, 1, 2, 3, 4, 6, 8 and 12 h for MT after i.p administration. Blood samples were centrifuged at 3000 × *g* for 10 min to obtain the plasma. In the same manner, drug-free pooled plasma samples were obtained from rats. The plasma samples were immediately frozen and stored at −20 °C until analysis.

3. Results and discussion

3.1. Optimization of the derivatization reaction

The effect of solvent, ASC concentration and derivatization time, etc. was investigated. The reaction was carried out at room temperature and evaluation was based on the absolute peak areas of the resulting derivatives. Concentrations of AT, RT and MT spiked in plasma were 5.0 μg/ml.

The solution of each drug was mixed with NaOH aqueous solution and ASC solution prepared by different organic solvents. The mixture was vortex-mixed and centrifuged. The organic phase was separated and evaporated to dryness under nitrogen. The residue was dissolved in mobile phase and analyzed by the HPLC-UV system.

Chloroform, ethyl acetate, ethyl ether, toluene, and dichloromethane were tested as the organic solvents and dichloromethane was found to be the most suitable solvent. The influence of ASC concentrations (0.02–0.5 mg/ml) on the derivatization indicated that 0.05 mg/ml afforded maximum yield; increasing the excess of the reagent beyond this level had no significant effect on yield. The effect of derivatization time (0.5–20 min) show that the plateau formation of the derivatives is attainable in 5 min. Ten minutes were used to guarantee complete reaction in various matrixes. The volume of ASC solution to 0.3 ml of aqueous solution was examined. The best result was obtained when the volume ratio between the organic layer and water layer was 2:1. The NaOH solution was used to maintain the analytes in the free base form in order to keep their nucleophilic character. The effect of NaOH concentration was investigated from 0.5 to 5 M and 2 M was found optimum. The results of optimized derivatization conditions are listed in Table 1.

Table 1
Optimized derivatization conditions of AT, RT and MT

	AT/RT/MT
ASC concentration (mg/ml)	0.05
Organic solvent	Dichloromethane
Derivatization time (min)	10
Volume ratio of organic and aqueous solutions	2/1
NaOH concentration (M)	2

The volume of dichloromethane (0.5–2.0 ml) for extraction the analytes from alkalinized plasma samples (2 M NaOH solution, 100 μl) was also studied. The extraction efficiency reached maximum when the volume of dichloromethane was 1.0 ml.

3.2. Inactivation of excess ASC

When the reaction mixture is evaporated to dryness and reconstituted in the mobile phase (methanol–water, 85:15) the excess of ASC reacts with the methanol component of the eluent to form methyl anthraquinone-2-sulfonylate (MAS). Its chromatographic peak (R.T. 4.3 min) does not interfere with those of the analytes.

3.3. Stability of the derivatives

The stability of AT, RT and MT derivatives in methanol–water (85:15) at room temperature was studied over a period of 48 h. No significant change in the absolute peak area of AT, RT or MT was found, indicating that the derivatives are sufficiently stable during the time required for analysis.

3.4. Analytical calibration with spiked plasma samples

The quantitative applicability of the method for the analytes spiked in plasma was validated by six-point calibration standard curves over the range of 0.05–5.0 μg/ml for AT and RT, 0.05–2.0 μg/ml for MT. Linear regression equations were obtained as follows: $y = (0.0086 \pm 0.001) + (0.8677 \pm 0.006)\ x$ $(n = 5)$ for AT, $y = (0.0356 \pm 0.002) + (1.121 \pm 0.005)\ x$ $(n = 5)$ for RT and $y = (0.0652 \pm 0.003) + (1.302 \pm 0.017)\ x$ $(n = 5)$ for MT, $r > 0.999$, where y represents the peak-area ratios of AT, RT or MT derivative to IS and x represents the concentrations of the analytes spiked.

The limit of detection (LOD) was determined as the lowest concentration with a signal-to-noise ratio S/N = 3. The LOD of AT, RT and MT were 20 ng/ml. The limit of quantitation (LOQ), defined as S/N = 10 was 50 ng/ml with accuracy ranged

Table 2
Analytical results AT, RT and MT spiked in rat plasma

	Added C^a. (μg/ml)	Within batch ($n=5$)			Between batches ($n=3$)		
		Found C. (mean ± S.D.,μg/ml)	Mean accuracy (%)	RSD[b] (%)	Found C. (mean ± S.D., μg/ml)	Mean accuracy (%)	RSD (%)
AT	0.1	0.11 ± 0.0071	114	6.26	0.095 ± 0.0057	95.4	5.97
	1.5	1.57 ± 0.035	105	2.23	1.53 ± 0.024	102	1.56
	4.5	4.42 ± 0.022	98.2	0.50	4.48 ± 0.052	99.6	1.16
RT	0.1	0.11 ± 0.0081	109	7.44	0.12 ± 0.063	119	5.30
	1.5	1.61 ± 0.055	107	3.42	1.47 ± 0.043	98.1	2.92
	4.5	4.45 ± 0.012	98.9	0.27	4.54 ± 0.026	101	0.57
MT	0.1	0.09 ± 0.0062	89.7	6.92	0.091 ± 0.059	91.3	6.47
	0.5	0.51 ± 0.011	103	2.15	0.54 ± 0.016	109	2.95
	1.5	1.44 ± 0.047	97.3	3.22	1.49 ± 0.036	99.1	2.42

[a] within- and between-batch analyses, respectively for five replicate analyses and three consecutive days.
[b] RSD, for relative standard derivation.

from −4.0 to 7.2% and precision within 5.5% for AT, accuracy ranged from –6.3 to 3.7% and precision within 5.1% for RT and accuracy ranged from −8.9 to 4.3% and precision within 6.8% for MT.

The within- and between-batch precision and accuracy are shown in Table 2 based on the analysis of AT and RT each three levels of 0.1, 1.5, 4.5 μg/ml, of MT each three levels of 0.1, 0.5, and 1.5 μg/ml.

Fig. 3A–C show the chromatograms of blank plasma and plasma spiked with 50 ng/ml for AT, RT and MT, respectively, and indicated no significant interferences at the retention positions of AT, RT and MT derivatives.

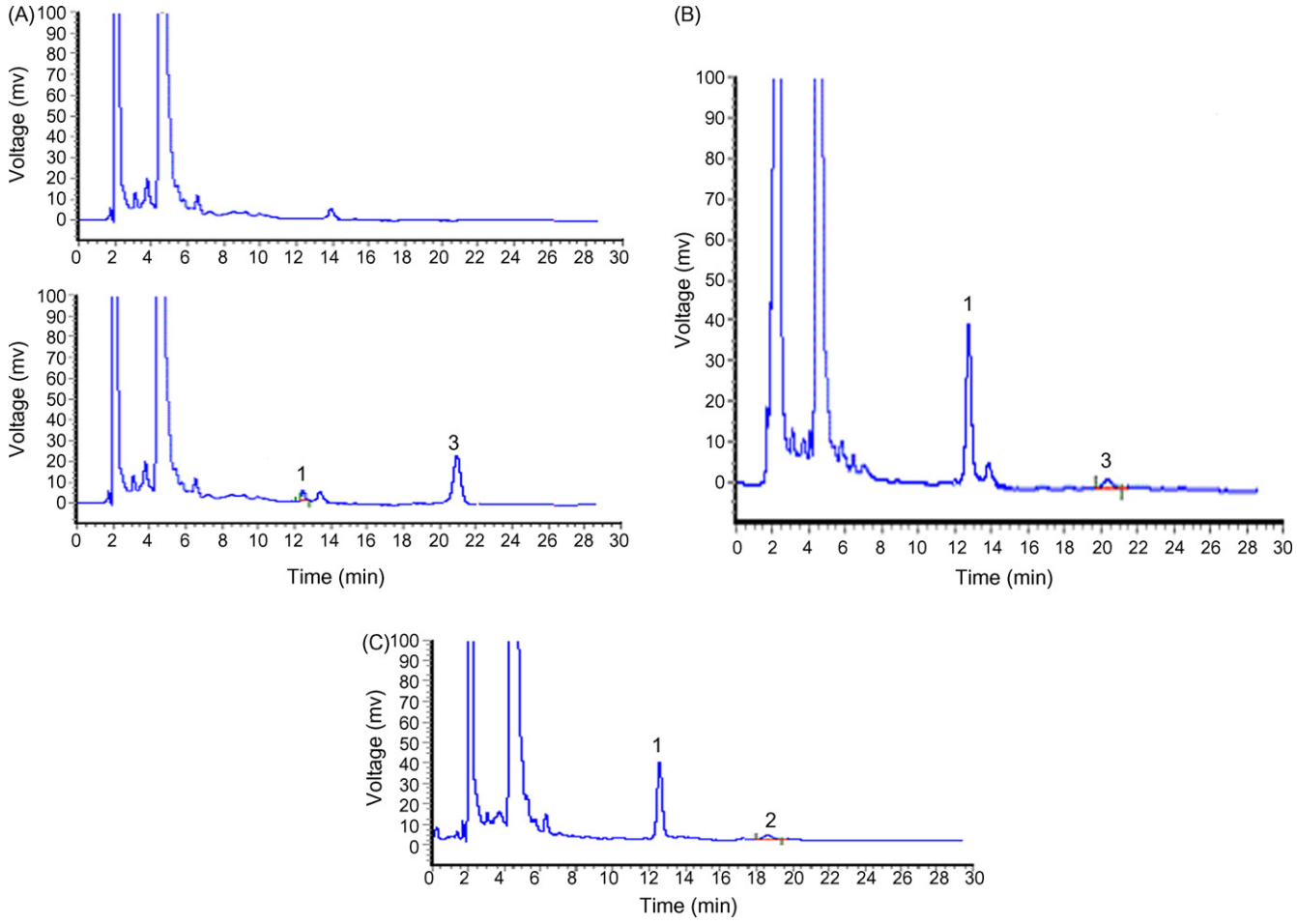

Fig. 3. Chromatograms of plasma samples spiked with analyte (50 ng/ml) and IS (5.0 μg/ml), (A) amantadine (top blank plasma), (B) rimantadine and (C) memantine. Peaks: 1 = AT derivative, 2 = MT derivative and 3 = RT derivative.

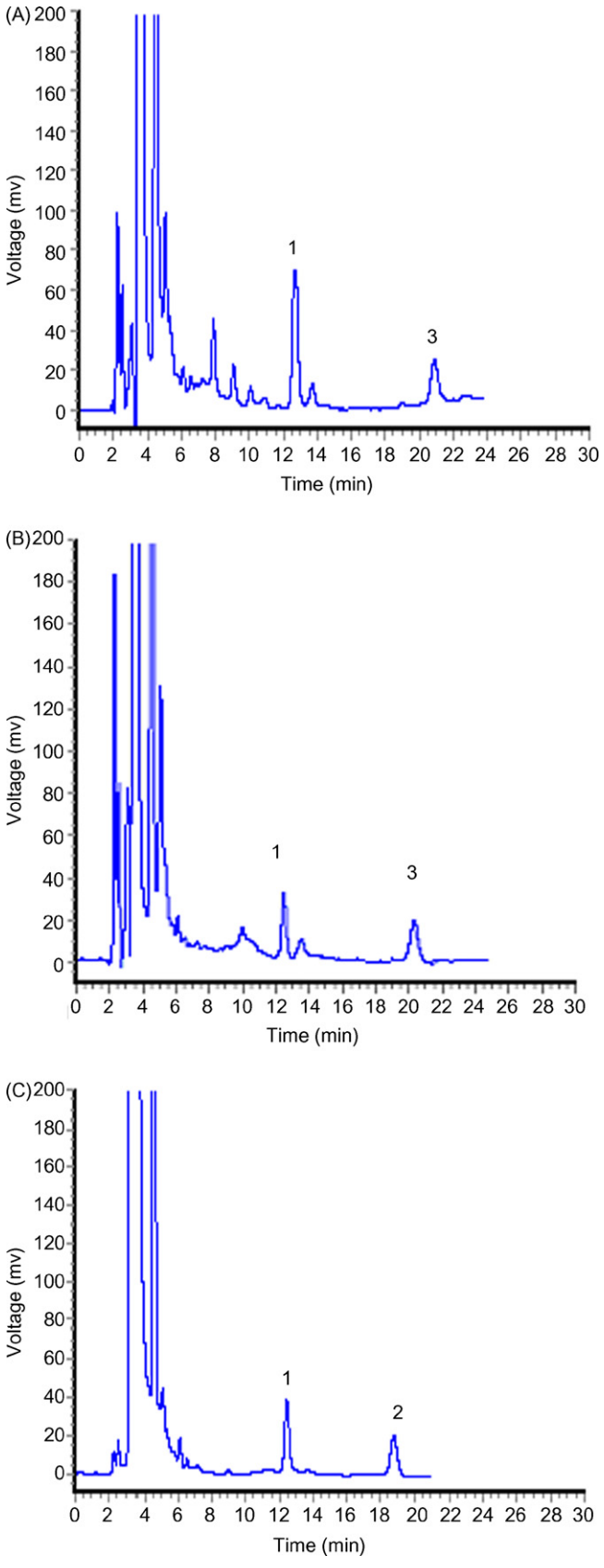

Fig. 4. Chromatograms of amantadine (A), rimantadine (B) and memantine (C) extracted from plasma sample 2 h for AT and RT, 1 h for MT after administration and derivatized with ASC. Peaks as in Fig. 3.

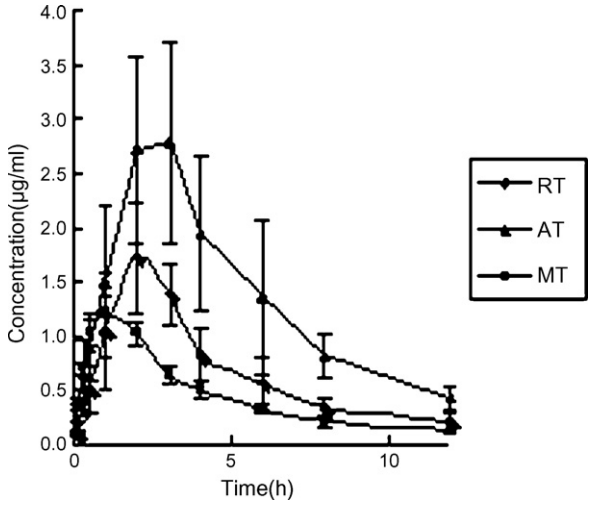

Fig. 5. Mean plasma concentration-time curves of AT, RT and MT in Sprague-Dawley rats ($n = 6$).

3.5. Application

The method described above was successfully applied to the pharmacokinetic studies of AT, RT and MT in rat plasma. Because the half-life of MT was more than 60 h with p.o. administration [18], and according to China State Food and Drug Administration (SFDA) guidance for Nonclinical Pharmacokinetic Studies of Chemical drugs [19], it needs 3–5 elimination half-lives for study. The experimental period is obviously too long to guarantee the parallelism and credibility of experiment results. MT is therefore administrated i.p. in this study.

Fig. 4 shows typical chromatograms of the analysis of AT, RT and MT in rat plasma. It is seen that the method is suitable for analysis of each drug individually using the other drug as an internal standard. The figures indicate that the three derivatives can be separated with a good resolution, which implies that the three analytes could be determined simultaneously. The pharmacokinetic profiles of AT, RT and MT in rats are shown in Fig. 5. The pharmacokinetic parameters were calculated according to the concentration-time curves and are shown in Table 3. No significant difference in the T_{max} values of AT and RT was observed compared with a previous report [7]. The MRT values of AT and MT were higher than those in the literature [7]. To our knowledge, there was no information about the kinetic parameters of MT

Table 3
Pharmacokinetic parameters of AT, RT and MT after administration in rats

	C_{max} [a] (μg/ml)	T_{max} [b] (h)	MRT[c] (h)	AUC[d] $_{0-12}$ (μg h/ml)
AT	3.27 ± 0.33	2.7 ± 0.8	6.12 ± 1.20	16.1 ± 1.5
RT	1.86 ± 0.32	2.5 ± 0.6	5.78 ± 1.46	7.97 ± 1.13
MT	1.20 ± 0.17	0.9 ± 0.2	5.46 ± 0.69	5.36 ± 0.43

Each value represents the mean ± S.D. of six rats.
[a] Maximum plasma concentration.
[b] Time to reach C_{max}.
[c] Mean residence time.
[d] Area under curve (AUC) calculated as 12 h.

in rat plasma. In humans, the T_{max} values of MT (20 mg, p.o. dose) were within 4–11 h according to previous data [18].

4. Conclusion

A new derivatization method was established for the analysis of AT, RT and MT. The derivatization conditions are mild and the derivatives are stable. Application of the method to the analysis of AT, RT and MT in plasma proved feasible. From the results obtained, it is hopeful that the method can be applied to determine AT, RT and MT in other samples such as pharmaceutical formulations, urine or tissues. Application of the reagent to the trace analysis of other biological/pharmaceutical analytes with amino function is being studied.

Compared with the derivatization methods of the three drugs reported previously [4–13], the proposed method is completed rapidly at room temperature and without complicated postderivatization treatment to inactivate excess reagents. The LOQ of the method was 50 ng/ml and the sensitivity was relatively high compared with another method for the determination of AT in plasma [10].

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