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Simultaneous determination of *N*-butyramide-tacrine and tacrine in mouse plasma and brain homogenate by high-performance liquid chromatography with a simple gradient solvent system

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Abstract

A novel reversed-phase HPLC method was developed for the simultaneous determination of tacrine (THA) and the newly synthesized prodrug (*N*-butyramide-THA, BTHA) in mouse plasma and brain homogenate. The assay involves deproteinisation and subsequent detection at 240 nm with a gradient solvent system. Retention times were 18.5 and 9.3 min for BTHA and THA, respectively. Average recoveries for the analytes were 80.7% (BTHA) and 76.6% (THA) from plasma, and 75.0% (BTHA) and 68.4% (THA) from brain homogenate. Linear responses were observed over a wide range (0.25–20 μg/ml for BTHA in plasma and in brain homogenate, 0.025–20 μg/ml for THA in both matrices). Both BTHA and THA degraded from the prodrug can be detected even 12 h after intravenous administration of BTHA, indicating that BTHA is a promising prodrug for brain targeting.

Keywords: *N*-Butyramide; Tacrine

1. Introduction

Tacrine (THA, Fig. 1, I) is a potent acetylchlorinesterase inhibitor, which had been employed in the treatment of Alzheimer's disease for a long time. However, the clinical use of THA has been hampered by its low therapeutic index, short half-life and liver toxicity [1]. Therefore, *N*-butyramide-THA (BTHA, Fig. 1, II) was synthesized as the prodrug of THA to achieve brain-targeted delivery [2]. Since the octanol–water partition coefficient of BTHA was 80-fold higher than that of THA, transport of BTHA across the blood–brain barrier (BBB) was increased significantly. After entering the brain, BTHA is degraded into THA under the catalyzation of esterase to exert pharmaco-active effects in the brain, with systemic side-effects consequently being reduced.

There have been several published methods on the HPLC assay of THA alone [3–5] and the simultaneous determination of THA and its metabolites [6–9]. These were similar methods where acetonitrile was used as the mobile phase and the pH of the mobile phase was adjusted to acidity to keep THA in the ionized form to obtain an appropriate retention time. But these methods were not suitable for simultaneous determination of THA and BTHA due

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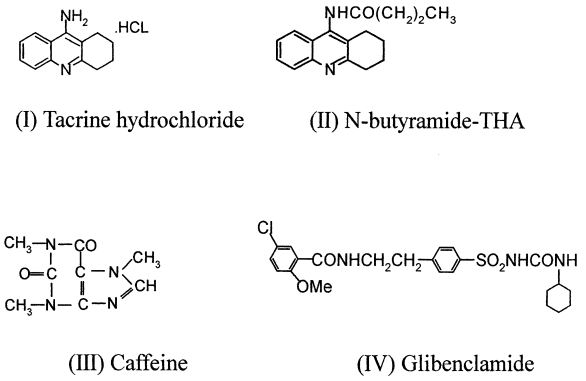

Fig. 1. Chemical structure of analysts. (I) Tacrine hydrochloride; (II) BTHA; (III) caffeine (internal standard); (IV) glibenclamide (internal standard).

to the high lipophilicity of the prodrug: the retention time of BTHA would be too long for convenient analysis under acid conditions.

Since BTHA is a newly synthesized compound, a suitable assay method has not previously been developed. Therefore, the objective of this study is to establish a simple and novel HPLC method for the simultaneous assay of BTHA and the metabolite, THA, and to evaluate the BBB penetration ability of BTHA. A one-step extraction procedure with a gradient solvent system was employed in the present study. Furthermore, according to the basic characteristic of THA, the pH of the mobile phase was adjusted to neutrality to keep THA in the undissociated form and prolong the retention time of THA. Under these conditions, THA can be assayed simultaneously with its prodrug BTHA by the reversed-phase HPLC method. Caffeine and glibenclamide were chosen as internal standards of THA and BTHA, respectively.

2. Experimental

2.1. Chemicals and reagents

Tacrine hydrochloride (9-amino-1,2,3,4-tetrahydroacridine) was purchased from Cayman Chemical (USA). Tacrine hydrochloride was added into ammonia and extracted by chloroform to obtain the undissociated form. BTHA was synthesized in our laboratory (99.37% purity). Internal standard caffeine (Fig. 1, III) was obtained from the First Pharmaceutical Company of West China (Ref. No. AJR0006-20-11-8), while another internal standard, glibenclamide (Fig. 1, IV) was obtained from Suchi Pharmaceutical (Ref. No. 0101100). Methanol, phosphoric acid and triethylamine were purchased from Bioway (USA). Water was prepared in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Preparation of standard solutions

Concentrated stock solutions of THA and BTHA were prepared separately at concentrations of 4025 and 1011 μg/ml in methanol for the preparation of plasma and brain homogenate calibration standards. All solutions were stored at −20 °C in dark glass. Pure standards prepared for the recovery calculations were diluted with methanol to appropriate concentrations.

2.3. Preparation of calibration standards

Plasma calibration curves were constructed with ten different plasma standards covering the expected concentration ranges (0.025–20.00 μg/ml for THA and 0.25–20.00 μg/ml for BTHA).

Plasma samples (500 μl each) were transferred to test tubes, which contained 100 μl of 250 μg/ml glibenclamide, 100 μl of 250 μg/ml caffeine, and 4 ml of methanol. These tubes were shaken vigorously for 3 min and centrifuged at 1500 *g* for 10 min. The organic phase was removed under a nitrogen stream in a water bath at 40 °C. Then the residue was reconstituted in 0.2 ml of methanol, vortexed for 2 min and centrifuged for 5 min at 1500 *g*. A 20-μl volume of supernatant fluid was subjected to HPLC analysis. Calibration curves were constructed using peak area ratios of analyte to internal standard.

Brain homogenate calibration curves were constructed with ten different brain homogenate standards covering the expected concentration ranges (0.025–20.00 μg/ml for THA and 0.25–20.00 μg/ml for BTHA). The preparation method was exactly

the same as for plasma standards method. Similarly, 20 μl of supernatant fluid was subjected to HPLC analysis.

2.4. Apparatus and chromatographic conditions

Analysis was performed using Shimadzu® instruments (Chiyoda-Ku, Kyoto, Japan) consisting of a 20-μl injector loop, a CTO-10A column thermostat, two LC-10AT pumps and an SPD-10A UV detector. The system was controlled through an SCL-10A system controller and a personal computer.

Separations were carried out using a Shimadzu Shim-Pack C_{18} reverse phase column (150×6 mm I.D.) with 5-μm pore size column (Chiyoda-Ku, Kyoto, Japan) protected by a Shimadzu Shim-Pack G guard column (4×1 mm I.D.) (Chiyoda-Ku, Kyoto, Japan).

A gradient solvent system of methanol in 0.1 *M* phosphate buffer solution (pH 6.1) was used as the mobile phase. The content of methanol in the mobile phase was 35% (v/v) for 5 min followed by a linear increase to 70% in 15 min which was maintained for another 15 min.

The mobile phase was filtered, degassed by sonication and pumped through the system at a flow rate at 1.0 ml/min at 40 °C. The normal operating pressure was 97–105 MPa and the analytical time was ~30 min. The column effluent was detected at 240 nm.

2.5. Animal experiments

Male mice (19–22 g) of Kunming strain were divided into test group and control group at random. The test group was intravenously administered 13.5 mg/kg BTHA, and the control group was intravenously administered 10 mg/kg THA. The mice were sacrificed at 5, 15, 30, 60, 120, 360, 480 and 720 min after injection. The blood samples were rapidly collected and plasma was separated by centrifugation and stored at −40 °C until assay. The whole brain was immediately removed and weighed. Brain tissue samples were homogenized and diluted with saline solution to 1:2 (g/ml). The homogenates were also stored at −40 °C until assay.

3. Results and discussion

3.1. Chromatography

Good chromatographic results were obtained with the extracts of both matrices. Typical chromatograms obtained from blank plasma, blank brain homogenate, plasma sample at 15 min after injection, and the brain homogenate sample at 12 h post injection are shown in Fig. 2. Under the chromatographic conditions described previously, BTHA and THA have retention times of 18.5 and 9.3 min, respectively, with no interfering co-elution of endogenous substances.

3.2. Recovery

Extraction recoveries of the components were assessed at three concentration levels by calculating the ratio (analyte:internal standard) of the peak area in extracts and comparing them with the same ratio in standard solutions. Concentration levels of 0.25, 5.00 and 20.00 μg/ml for BTHA and 0.025, 1.00 and 20.00 μg/ml for THA in both matrices were applied to analysis. All analyses were performed in triplicate. Table 1 shows the mean recoveries of THA and BTHA at the concentrations tested. The mean recoveries were 71.3 and 73.8% for caffeine and 76.1 and 70.7% for glibenclamide in plasma and brain homogenate, respectively.

3.3. Linearity and detection limit

Calibration curves for THA and BTHA in both matrices were generated by linear regression of peak area ratios against their respective concentrations. The calibration curves showed good linearity between peak–area ratios against concentrations over the calibration ranges in plasma (BTHA, $y = 0.00328 + 0.10174x$, $r^2 = 0.999$; THA, $y = 0.00024 + 0.02956x$, $r^2 = 0.998$) and in brain homogenate (BTHA, $y = 0.01308 + 0.09504x$, $r^2 = 0.999$; THA, $y = 0.00079 + 0.01164x$, $r^2 = 0.998$). The detection limit for the methods, defined as a signal-to-noise ratio of 3:1, was 0.20 μg/ml for BTHA and 0.02 μg/ml for THA in both matrices.

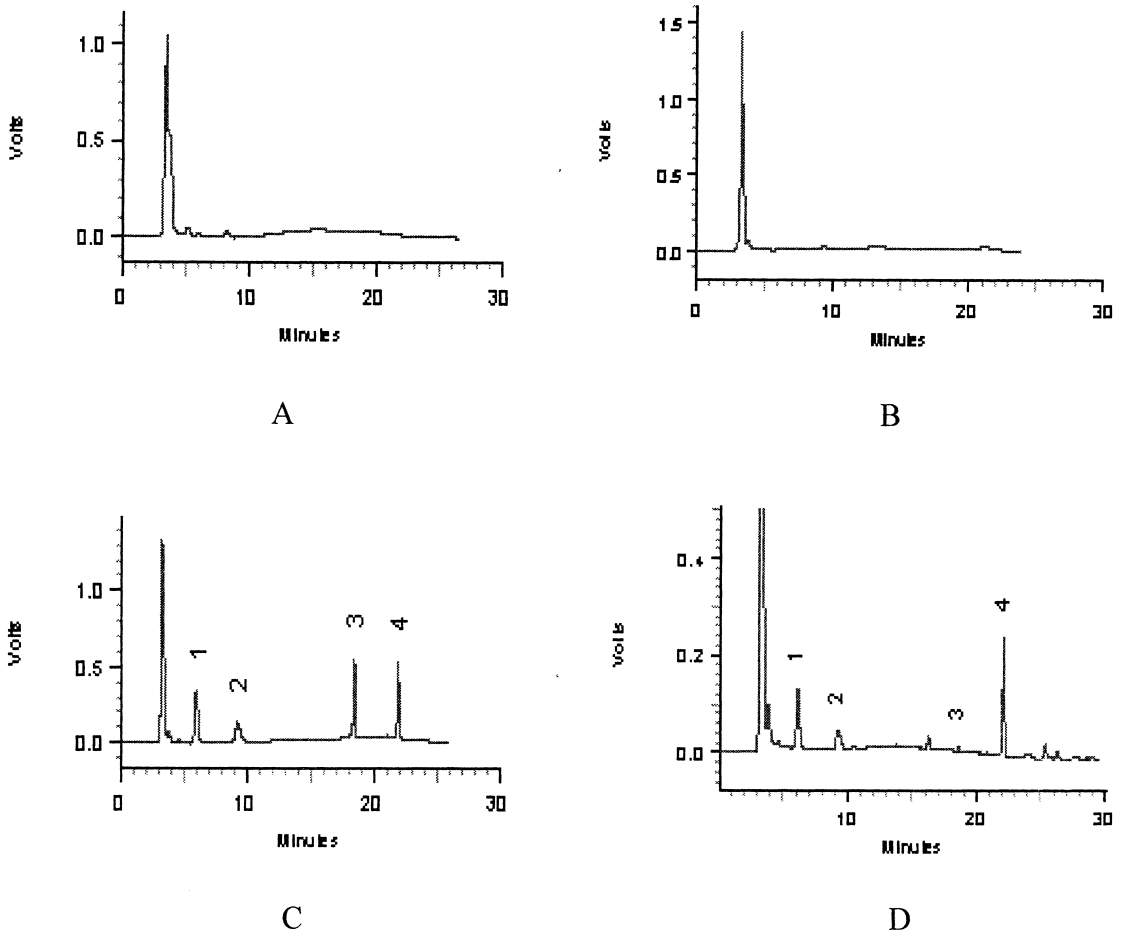

Fig. 2. Chromatograms of (A) extracted blank mouse brain homogenate, (B) extracted blank mouse plasma, (C) extracted mouse plasma sample at 15 min after injection of BTHA, (D) extracted mouse brain homogenate sample at 12 h after injection of BTHA. Chromatographic peaks: (1) caffeine (internal standard), (2) THA, (3) BTHA and (4) glibenclamide (internal standard).

Table 1
Assay recoveries of *N*-butyramide-THA and THA from plasma and brain homogenate

Compound		Concentration (μg/ml)	% Recovery (mean±SD, $n=3$)	Compound		Concentration (μg/ml)	% Recovery (mean±SD, $n=3$)
BTHA	Plasma	0.25	90.5±3.5	THA	Plasma	0.025	70.8±5.5
		5.00	80.4±5.1			1.00	83.7±4.6
		20.00	74.5±3.9			20.00	72.7±3.6
	Brain	0.25	68.5±6.4		Brain	0.025	73.1±4.9
		5.00	84.3±4.3			1.00	70.7±4.0
		20.00	76.6±4.8			20.00	72.9±3.6

3.4. Method precision and standard stability

The reproducibility of the plasma and brain homogenate assay was determined by analyzing the plasma and brain homogenate standards prepared at 0.25, 5.00 and 20.00 μg/ml for BTHA and 0.025, 1.00 and 20.00 μg/ml for THA. The results of intra-assay and inter-assay precision are expressed as the coefficient of variation (C.V.). The assay was reproducible with an average intra-day coefficient of variation less than 5% and an average inter-day coefficient of variation less than 10% (Table 2).

3.5. Assay application

Mice brain homogenate concentration–time curves of BTHA and the metabolite THA following intravenous administration of 13.5 mg/kg BTHA and curves of THA following injection of 10.00 mg/kg THA are shown in Fig. 3A. The mice plasma concentration–time curves of BTHA and THA following intravenous administration of 13.5 mg/kg BTHA and curves of THA following injection of 10.0 mg/kg THA are shown in Fig. 3B.

The concentrations of the parent drug THA and prodrug BTHA in plasma and in brain homogenate after injection were compared. In the test group, it was found that BTHA could be detected in brain homogenate and in plasma even 12 h after administration and the THA degraded from BTHA could also be detected after 12 h. But in the control group, no THA could be detected in brain 6 h after administration. Therefore, BTHA provides the possibility of sustained release of THA in brain. The THA concentration in brain 4 h after administration of BTHA was higher than the concentration after administration of THA at equivalent dose after 4 h, indicating that BTHA has enhanced permeability across BBB compared with THA. It also was observed that 100% of mice died within 5 min of intravenous injection of 20 mg/kg (1×10^{-4} mol/kg) THA, but all mice survived after intravenous injection of 27 mg/kg (1×10^{-4}mol/kg) BTHA.

4. Conclusion

The assay described is a useful, simple HPLC assay for simultaneous quantification of BTHA and the metabolite THA in plasma and brain homogenate. The method shows good overall recovery, accuracy, precision, and low detection limits of two compounds. BTHA and THA were assayed with appropriate retention times under the same chromatographic conditions, although there is a great difference in lipophilicity between the two compounds,

Table 2
Intra- and inter-assay precision during assay for BTHA and THA

Compound		Intra-assay ($n=3$)		Inter-assay ($n=3$)	
		Concentration (mean±SD) (μg/ml)	C.V. (%)	Concentration (mean±SD) (μg/ml)	C.V. (%)
BTHA	Plasma	0.28±0.01	3.6	0.24±0.02	8.3
		4.93±0.15	3.0	4.95±0.29	5.9
		19.5±0.57	2.9	19.2±1.37	7.1
	Brain	0.26±0.01	3.8	0.27±0.01	3.7
		4.94±0.09	1.8	4.79±0.22	4.6
		19.2±0.81	4.2	19.6±1.45	7.4
THA	Plasma	0.027±0.0005	1.8	0.026±0.0007	2.7
		1.02±0.04	3.9	1.06±0.07	6.6
		19.6±0.58	3.0	20.9±1.96	9.4
	Brain	0.025±0.0006	2.4	0.027±0.0008	3.0
		1.07±0.03	2.8	0.99±0.06	3.1
		20.8±0.92	4.4	21.3±1.77	8.3

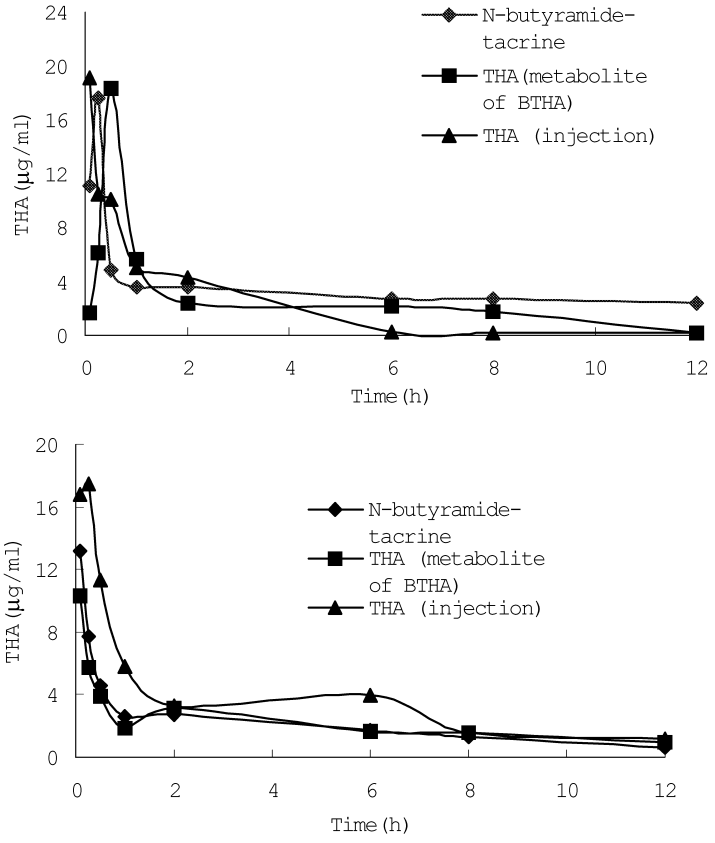

Fig. 3. (A) Brain concentration-time profile of BTHA (♦-♦), THA (the metabolite of BTHA, ■-■) after intravenous injection of 13.5 mg/kg BTHA and the concentration-time profile of THA (▲-▲) after intravenous injection of 10.00 mg/kg of THA. (B) Plasma concentration of BTHA (♦-♦), THA (the metabolite of BHTA, ■-■) after intravenous injection of 13.5 mg/kg BTHA and the concentration of THA (▲-▲) after intravenous injection of 10.00 mg/kg of THA.

indicating that it is valid to adjust the mobile phase to neutrality and apply the gradient solvent system described above.

The results of tissue distribution study show that the prodrug at the N-position of THA may provide a promising approach for brain-targeted delivery, with sustained brain levels of THA and significantly reduced toxicity of the parent drug.

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