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Note

High-performance liquid chromatography for the determination of tacrine and its metabolites in plasma

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Tacrine (THA, 9-amino-1,2,3,4-tetrahydroacridine) (Fig. 1) is a potent cholinesterase inhibitor [1] which has been currently under investigation as a potential therapeutic agent in the treatment of Alzheimer's disease [2–4].

Several high-performance liquid chromatographic (HPLC) procedures for the determination of THA in biological samples have been reported. These methods employ ultraviolet (UV) [5–7], fluorescence [8] and electrochemical [9] detectors with a detection limit of 0.2–100 ng/ml of plasma. During the method development, unknown peaks were observed in monkey and human plasma [7,8] and microsomal preparations [9], but no information about the quantitation and identification of the unknowns was available.

Our laboratory recently reported that THA was extensively metabolized in rats after oral administration [10,11]. The major metabolic pathways involve the hydroxylation of the saturated ring at positions 1, 2 and 4 (Fig. 1). These hydroxylated metabolites are potent cholinesterase inhibitors and active in various animal models predictive of efficacy in Alzheimer's disease [11–13]. Recently, Ekman *et al.* [14] published an HPLC method utilizing UV detection to quantitate THA and its 1-hydroxy metabolite in plasma; however, that report did not describe the measurement of active 2- and 4-hydroxy metabolites. In this paper, we present a selective and sensitive analytical procedure for the quantitation of THA and its active metabolites in plasma.

EXPERIMENTAL

Materials

9-Amino-1,2,3,4-tetrahydroacridine hydrochloride (THA) was purchased from Aldrich (Milwaukee, WI, U.S.A.); 9-amino-1,2,3,4-tetrahydroacridin-1-ol (1-OH-THA), 9-amino-1,2,3,4-tetrahydroacridin-2-ol (2-OH-THA), 9-amino-1,2,3,4-tetrahydroacridin-4-ol (4-OH-THA) and N-methoxy-1,2,3,4-tetrahy-

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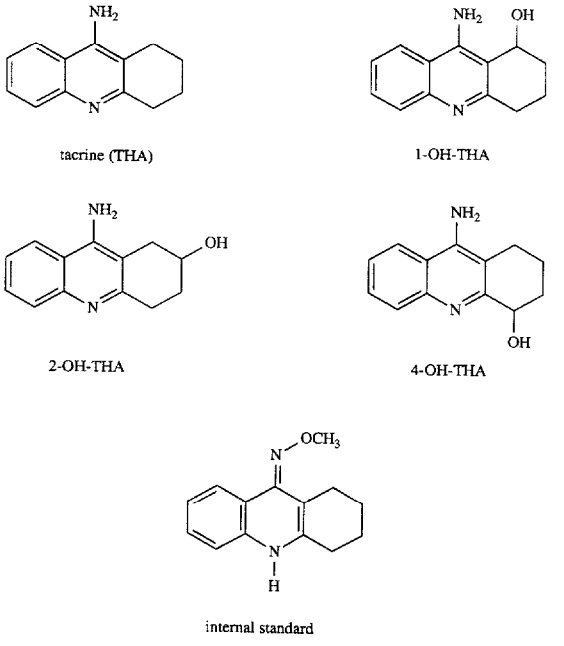

Fig. 1. Structures of 9-amino-1,2,3,4-tetrahydroacridine hydrochloride (THA), 9-amino-1,2,3,4-tetrahydroacridin-1-ol (1-OH-THA), 9-amino-1,2,3,4-tetrahydroacridin-2-ol (2-OH-THA), 9-amino-1,2,3,4-tetrahydroacridin-4-ol (4-OH-THA) and N-methoxy-1,2,3,4-tetrahydroacridin-9 (10H)-imine (internal standard).

droacridin-9 (10*H*)-imine (internal standard) (Fig. 1) were synthesized in the Chemical Research Department of Hoechst-Roussel Pharmaceuticals. Acetonitrile, cyclohexane, ethyl acetate, sodium hydroxide, formic acid and ammonium formate are all analytical grade and purchased from Fisher Scientific (Springfield, NJ, U.S.A.). The ammonium formate buffer was prepared by mixing formic acid and ammonium formate solution until the desired pH and concentration were obtained. Deionized water (Milli-Q® water purification system, Millipore, Bedford, MA, U.S.A.) was used throughout the study. Control rat plasma was obtained from male Wistar rats (Charles River Laboratory, Wilmington, MA, U.S.A.).

Plasma assay

Plasma samples (1 ml each) were added to test tubes containing 250 ng of internal standard and 0.5 ml of 0.5 *M* sodium hydroxide. The tubes were vortexed briefly and then 5 ml of cyclohexane–ethyl acetate (1:1, v/v) were added. The tubes were shaken for 10 min on an Eberbach shaker and centrifuged at 2000 *g* for 10 min in a Model J-6B centrifuge (Beckman Instruments, Fullerton, CA, U.S.A.). The organic phase was transferred to a tapered centrifuge tube and evaporated to dryness under a nitrogen stream in a water bath at 40°C. The

residue was then reconstituted in 0.2 ml of mobile phase and an aliquot of the sample analyzed by HPLC.

High-performance liquid chromatography

Plasma samples prepared according to the above procedures were analyzed by an HPLC system employing a Shandon Hypersil 3-μm phenyl column (100 mm $\times$ 4.6 mm I.D.). The samples were injected by a Waters WISP 710B autosample injector (Milford, MA, U.S.A.) and eluted with acetonitrile–0.02 *M* ammonium formate buffer, pH 2.75 (70:30, v/v) at a flow-rate of 1.5 ml/min. The elution was carried out at ambient temperature and column effluent was quantitated using a Kratos Model 773 UV spectrophotometer (Ramsey, NJ, U.S.A.) with detection at 240 nm. The chromatograms were integrated using ACCESS*CHROM Model 6000 system from Perkin Elmer Nelson (Cupertino, CA, U.S.A.).

Preparation of calibration curves

Calibration curves were constructed with six different plasma standards covering a concentration range of 1–500 ng/ml. Standard curves were established by analyzing five replicates at each concentration. Lincarity of thc calibration curves were determined by least-squares regression analysis.

Validation procedures

The recovery of THA, its metabolites 1-OH-THA, 2-OH-THA and 4-OH-THA, and internal standard from plasma was assessed by adding known amounts to blank rat plasma to give final concentrations of 5, 50 and 500 ng/ml. The samples (six replicates at each concentration) were extracted and analyzed by HPLC. Extraction efficiencies of THA, metabolites and internal standard were calculated by comparing the peak areas obtained from spiked plasma with the peak areas from injected standards in the mobile phase.

Assay precision was evaluated at concentrations of 5, 50 and 500 ng/ml. Plasma samples were prepared by spiking blank rat plasma with THA and metabolites and stored at -20°C until analyzed. Intra-assay variation was examined by analyzing six replicates at each concentration, and inter-assay precision was determined in triplicate on six occasions over a period of four weeks.

Animal experiments

Male Wistar rats (200–250 g) were orally administered with 10 mg/kg THA. Blood samples were collected at 0.25, 0.5, 1, 2, 4, 6, 8 and 16 h after dosing. Plasma was separated by centrifugation and stored at -20°C until assay.

RESULTS AND DISCUSSION

Selectivity

Plasma samples were analyzed by an isocratic reversed-phase HPLC system

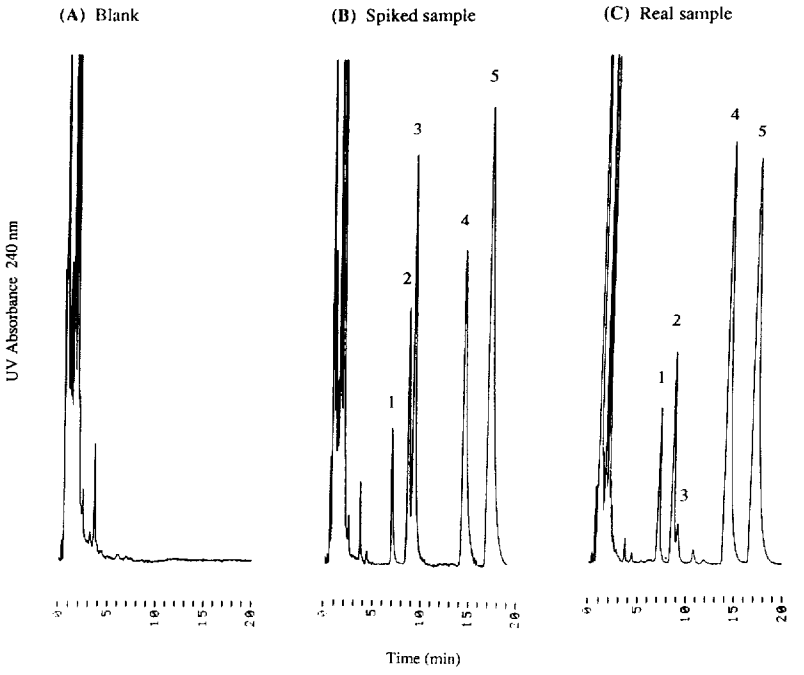

Fig. 2. Representative chromatograms of (A) blank rat plasma, (B) blank rat plasma containing 100 ng/ml THA, 1-OH-THA, 2-OH-THA, 4-OH-THA and 250 ng/ml internal standard and (C) rat plasma 0.25 h after oral administration of 10 mg/kg THA. Plasma samples were chromatographed on a Shandon Hypersil 3-μm phenyl column using acetonitrile–0.02 *M* ammonium formate buffer, pH 2.75 (70:30, v/v). Peaks: 1 = 2-OH-THA; 2 = 1-OH-THA; 3 = 4-OH-THA; 4 = THA; 5 = internal standard.

employing a Shandon analytical phenyl column and UV detection. Concentrations of THA and its metabolites were determined by internal standardization. Typical chromatograms for control blank, spiked rat plasma and dosed rat plasma are shown in Fig. 2. Using this chromatographic system, no endogenous components extracted from rat plasma interfere with THA, 1-OH-THA, 2-OH-THA, 4-OH-THA or the internal standard in the sample assay. Interfering sub-

TABLE I

ASSAY RECOVERY OF TACRINE AND METABOLITES FROM PLASMA

Concentration added (ng/ml)	Recovery (mean ± S.D., $n=6$) (%)			
	THA	1-OH-THA	2-OH-THA	4-OH-THA
5	92.0±4.5	71.5±6.3	33.1±2.0	84.9±2.6
50	95.9±4.3	69.9±3.3	34.7±3.9	83.4±4.5
500	93.2±4.1	67.3±3.1	32.1±2.7	86.3±5.2

stances were either eluted at the solvent front under the HPLC conditions or did not absorb UV at 240 nm under these conditions.

Recovery

Extraction efficiency was determined by comparing the peak areas from spiked samples with those obtained from injection in mobile phase (Table I). The recovery of THA, 1-OH-THA, 2-OH-THA and 4-OH-THA from plasma was approximately 92–96, 67–72, 32–35 and 83–86%, respectively, over a concentration range of 5–500 ng/ml. Recovery of internal standard (250 ng/ml) for the plasma assay was over 95%. The low recovery of 2-OH-THA could be due to the polar nature of the molecule; with two consecutive extractions, the recovery of 2-OH-THA could be doubled to *ca.* 60%.

Linearity and detection limit

Plasma standards were prepared from blank rat plasma spiked with THA and metabolites in the range 1–500 ng/ml. Calibration curves showed good linearity between peak-area ratios and concentrations from 1 to 500 ng/ml for THA, 1-OH-THA, 2-OH-THA and 4-OH-THA in plasma (THA, $y = 0.00549x + 0.00496$, $r = 0.99995$; 1-OH-THA, $y = 0.00275\ x + 0.00493$, $r = 0.99993$; 2-OH-THA, $y = 0.0001x + 0.00485$, $r = 0.99967$; 4-OH-THA, $y = 0.00499x + 0.0105$, $r = 0.99991$; y, x and r are peak-area ratio, concentration and correlation coefficient, respectively). The observed coefficient of variation (C.V.) for the stan-

TABLE II

INTRA- AND INTER-ASSAY PRECISION FOR TACRINE AND METABOLITES

Compound	Intra-assay ($n=6$)		Inter-assay ($n=6$)[a]	
	Concentration (mean ± S.D.) (ng/ml)	C.V. (%)	Concentration (mean ± S.D.) (ng/ml)	C.V. (%)
THA	5.1 ± 0.3	5.9	5.2 ± 0.4	7.7
	51 ± 2	3.9	52 ± 2	3.9
	520 ± 10	1.9	520 ± 22	4.2
1-OH-THA	4.9 ± 0.2	4.1	4.7 ± 0.3	6.4
	50 ± 2	4.0	51 ± 3	5.9
	505 ± 3	0.6	502 ± 12	2.4
2-OH-THA	5.5 ± 0.2	3.6	5.4 ± 0.3	5.6
	49 ± 2	4.1	51 ± 2	3.9
	501 ± 6	1.2	505 ± 18	3.6
4-OH-THA	4.8 ± 0.4	8.3	4.7 ± 0.2	4.3
	52 ± 1	1.9	52 ± 2	3.9
	502 ± 5	1.0	498 ± 20	4.0

[a] Individual values are the mean of three measurements.

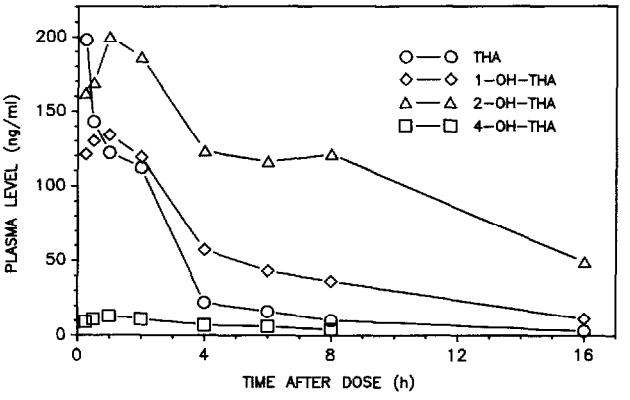

Fig. 3. Plasma concentration–time curves of THA, 1-OH-THA, 2-OH-THA and 4-OH-THA after the oral administration of 10 mg/kg THA.

dard curves, calculated from the least-squares regression equations, was less than 10%. The detection limit for the method, defined as the signal-to-noise ratio of 3, was 1 ng/ml for THA and its metabolites in plasma.

Method precision and plasma standard stability

The reproducibility of the plasma assay was determined by analyzing the plasma standards prepared at 5, 50 and 500 ng/ml. The results of intra-assay and inter-assay precision are expressed as C.V. The assay variations at 5, 50 and 500 ng/ml are less than 10% for THA and its metabolites (Table II).

Assay application

The plasma concentration–time curves of THA and its metabolites in rats following an oral administration of 10 mg/kg THA are shown in Fig. 3. THA was rapidly absorbed and extensively metabolized after oral dosing. THA, 1-OH-THA and 2-OH-THA reached maximal levels at 0.25, 0.5–1 and 1–2 h post-dose, respectively, and then disappeared biexponentially with half-lives of 0.5–2 and 4–8 h for the rapid and slower elimination processes. The plasma level of 4-OH-THA was relatively low compared to those of THA, 1-OH-THA and 2-OH-THA.

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