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Determination of tacrine and its metabolites in human plasma and urine by high-performance liquid chromatography and fluorescence detection

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Abstract

A new method for the simultaneous quantitation of tacrine and the three metabolites, 1-hydroxytacrine (velnacrine, maleate), 2-hydroxytacrine and 4-hydroxytacrine, in human plasma and urine has been developed. The method was based on simple one-step liquid–liquid extraction with ethyl acetate followed by isocratic, reversed-phase high-performance liquid chromatography and fluorescence detection (excitation: 330 nm and emission: 365 nm). The limit of detection in plasma was 0.5 n*M* for 2-hydroxytacrine and 4-hydroxytacrine, 2 n*M* for 1-hydroxytacrine and tacrine. In urine it was 60 n*M* for 2-hydroxytacrine and 4-hydroxytacrine, 30 n*M* for 1-hydroxytacrine and 80 n*M* for tacrine. The limit of quantification in plasma was 2.5 n*M* for 2-hydroxytacrine and 4-hydroxytacrine, 10 n*M* for 1-hydroxytacrine and 2 n*M* for tacrine. In urine it was 120 n*M* for all components. The overall mean recoveries ranged from 84 to 105% in plasma and from 64 to 100% in urine for all four compounds. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Tacrine; Hydroxytacrine

1. Introduction

Tacrine (1,2,3,4-tetrahydro-9-aminoacridine) is a potent, centrally active, reversible acetylcholinesterase inhibitor, and it was the first drug licensed for the treatment of mild to moderate Alzheimer's disease [1]. In humans tacrine undergoes extensive oxidative metabolism, and the primary metabolites are 1-, 2- and 4-hydroxytacrine [2]. Other metabolites include 3- and 7-hydroxytacrine, phenoltacrine and dihydroxytacrine [3]. In vitro studies have shown, tacrine cytochrome P4501A2 (CYP1A2) is the major enzyme catalysing the oxidation of tacrine [2]. Thus the wide interindividual variations in tacrine plasma level following oral administration most likely mirror differences in CYP1A2 activity. Both the desired effects and the cholinergic side effects [4] seem to be dose dependent. The clinical use of tacrine is hampered by the development of asymptomatic serum transaminase elevations (transaminitis) in about 40–50% of the patients [5] but whether this is related to the parent compound or to one or more of its metabolites has not been settled. Thus CYP1A2 may play a key role in both the desired and undesired effects.

There are several published methods on the HPLC

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assay in plasma of tacrine alone [6–8], tacrine and its major metabolite, 1-hydroxytacrine [9–11] and tacrine and its 1-, 2- and 4-hydroxylated metabolites [12–14]. In the present paper we describe an isocratic and very sensitive HPLC method for the assay of tacrine and 1-, 2- and 4-hydroxytacrine in plasma and urine. In similar methods previously published, hazardous agents such as chloroform [13] were used for the extraction. In our method we use a simple one-step extraction procedure with the much less dangerous solvent, ethyl acetate. Furthermore we achieved a total baseline separation of tacrine and three metabolites and improved the sensitivity, compared to other methods [12,14].

2. Experimental

2.1. Chemicals and reagents

Tacrine, HCl (M_w=252.74 g mol^{-1}), velnacrine, maleate (M_w=330.34 g mol^{-1}), 2-hydroxytacrine (M_w=214.63 g mol^{-1}) and 4-hydroxytacrine (M_w=214.27 g mol^{-1}) were kindly donated by Parke Davis, Pharmaceutical Research, 2800 Plymouth Road, Ann Arbor Michigan, USA. 1,2,3,4-tetrahydro-9-acridanone (internal standard) were purchased from Aldrich Chemical Co. Inc., P.O. Box 355, Milwaukee, Wisconsin 53233, USA. Acetonitrile, HiPerSolv™ for HPLC, methanol, HiPerSolv™ for HPLC and ethylacetate, HiPerSolv™ for HPLC were purchased from BDH (Poole, England). Sodium acetate anhydrous (CH_3COONa), perchloric acid p.a. ($HClO_4$, 60%), sodium hydroxide (NaOH) and hydrochloric acid (HCl), Titrisol (1 *M*) were purchased from Merck (Darmstadt, Germany). Water was purified by osmosis and distillation.

Sodium acetate buffer solution: CH_3COONa (M_w=82.03 g mol^{-1}), 0.2 *M* solution contains 16.406 g l^{-1}, adjusted to pH 4.0 with $HClO_4$, 60%. The aqueous solution was filtered through a Millipore filter (Type HA, pore size: 0.45 μm; filter system from Waters Millipore).

2.2. Preparation of standard solutions

Stock solutions of tacrine, and the three metabolites; 1-hydroxytacrine, 2-hydroxytacrine and 4-hydroxytacrine (1 m*M* of each) and internal standard (1 m*M*) were prepared. Tacrine and 1-hydroxytacrine were prepared in distilled water, 2-hydroxytacrine, 4-hydroxytacrine and internal standard was prepared in 1 *M* HCl and water (1:7% v/v). The stock solutions were used to prepare the appropriate standards in plasma and urine.

All solutions were stored at −20°C in dark glasses. Pure standards prepared for the recovery calculations had to be diluted in acid (0.02 *M* HCl) otherwise they were unstable.

2.3. Extraction procedure

2.3.1. Plasma

To 500 μl of plasma samples (unknown as well as blank) were added 25 μl of a 10 μ*M* internal standard and 250 μl 0.5 *M* NaOH in glass tubes (10 ml) with screwcaps, and finally 5 ml ethylacetate was added. The mixture was shaken (IKA® Shaker, IKA Labortechnik, Staufen, Germany) for 10 min, and after separation of the two phases by centrifugation at 1.100×*g* for 10 min, the tubes were kept at −30°C until the aqueous phase was frozen (1 min).

The organic phase was transferred to conical glass tubes and evaporated to dryness under a nitrogen stream at 40°C (45 min).

The residue was reconstituted in 300 μl mobile phase, vortexes for 30 s and centrifuged (MSE Mistral 6 L, Micro Technic, Odense, Denmark) for 1 min at 1.100 *g*. The sample was transferred to 1.5 ml vials and centrifuged (Sigma 2–15, Struers Kebolab, Albertslund, Denmark) for 15 min at 13 000 *g*.

A 100 μl volume of the sample was taken from the middle and transferred to HPLC-vials (Mikrolab Aarhus, Aarhus, Denmark).

2.3.2. Urine

To 500 μl of urine samples (unknown as well as blank) were added 25 μl of a 100 μ*M* internal standard and 250 μl 0.5 *M* NaOH in glass tubes (10 ml) with screwcaps, and finally 5 ml ethyl acetate was added. The mixture was shaken (Marius) for 10 min, and after separation of the two phases by centrifugation (MSE Mistral 6 L, Micro Technic, Odense, Denmark) at 1.100 *g* for 10 min, the tubes were placed in −30°C until the aqueous phase was frozen (1 min).

The organic phase was transferred to conical glass tubes and evaporated to dryness under a nitrogen stream at 40°C (45 min). The residue was reconstituted in 900 μl mobile phase and vortexes for 30 s.

A 100 μl volume was transferred to HPLC vials (Mikrolab Aarhus, Aarhus, Denmark). Some of the samples had to be diluted because of the concentration. This means in practice that each sample has to be analysed twice.

2.3.3. Apparatus and chromatographic conditions

Chromatography was performed using LaChrom® instruments (Merck Hitachi, Tokyo, Japan): an L-7200 autosampler with a 100 μl injector loop, a L-7350 column thermostat, a L-7100 intelligent Pump and a L-7480 fluorescense Detector. The system was controlled through an interface module HPLC System Manager (HSM) and a personal computer (IBM).

Separations were achieved using a LiChrospher® 60 RP-select B, 5 μm (250×4 mm I.D.) column (Merck, Darmstadt, Germany) with a LiChrospher® 60 RP-select B guard column (Merck, Darmstadt, Germany).

The mobile phase consisted of acetate buffer (0.2 M, pH 4.0) and acetonitrile (87:13, v/v). The aqueous phase was filtered through a Millipore filter (0.45 μm) and the mobile phase was degassed prior to use.

The analytical time per run was 40 min; the flow-rate was kept at 1.25 ml min^{-1} from 0–16 min, and at 2.5 ml min^{-1} from 16–40 min. The normal operating pressure was 142–280 bar and the column temperature was 30°C. The column effluent was quantified at a wavelength of excitation of 330 nm and emission at 365 nm.

3. Results

3.1. Selectivity

Baseline separation was obtained between the four compounds and the internal standard under the applied conditions (Fig. 1B and Fig. 2B). About 40 min was required for the analysis. The retention times in plasma were 11.01, 12.40, 14.83, 30.39, 32.93 for 2-hydroxytacrine, 1-hydroxytacrine, 4-hydroxytacrine, internal standard and tacrine respectively and in urine the retention times in plasma were 11.21, 12.39, 14.95, 29.30, 31.71 for 2-hydroxytacrine, 1-hydroxytacrine, 4-hydroxytacrine, internal standard and tacrine respectively. A blank chromatogram is shown in Fig. 1A and Fig. 2A. A typical chromatogram after extraction from a sample from a volunteer is shown in Fig. 1C and Fig. 2C.

3.2. Recovery

The absolute recoveries of the compounds were assessed ($n=10$) at five concentration levels by comparing the peak area after extraction with the peak area obtained from direct injection of equivalent quantities of pure standard. In plasma the five concentration levels were 6, 15, 30, 45 and 60 n*M* for 2- and 4-hydroxytacrine, and 24, 60, 120, 180 and 240 n*M* for 1-hydroxytacrine and tacrine. Table 1 shows the mean recoveries of the four compounds at the five concentrations tested. The mean recovery for the internal standard in plasma was 95.2% (C.V.: 2.1%).

In urine the five concentration levels were 0.12, 0.3, 0.9, 1.5, and 2.1 μ*M* for 2-hydroxytacrine, 4-hydroxytacrine and to 0.48, 1.2, 3.6, 6.0, and 8.4 μ*M* for 1-hydroxytacrine. Table 1 shows the mean recoveries of the four compounds at the five concentrations tested. The mean recovery for the internal standard in urine was 88.4% (C.V.: 6.0%)

3.3. Linearity

The linearity of detector response to variations in concentration of each compound was determined at plasma concentrations of 6, 15, 30, 45 and 60 n*M* for 2-hydroxytacrine and 4-hydroxytacrine, and 24, 60, 120, 180 and 240 n*M* and for 1-hydroxytacrine and tacrine. The urine was determined at urine concentrations of 0.12, 0.3, 0.9, 1.5 and 2.1 μ*M* for 2-hydroxytacrine and 4-hydroxytacrine, and of 0.48, 1.2, 3.6, 6.0 and 8.4 μ*M* for 1-hydroxytacrine and tacrine. The standard curves [plots of peak area ratio (compound/internal standard) versus concentration] for all components were linear over the investigated concentration range (Fig. 2).

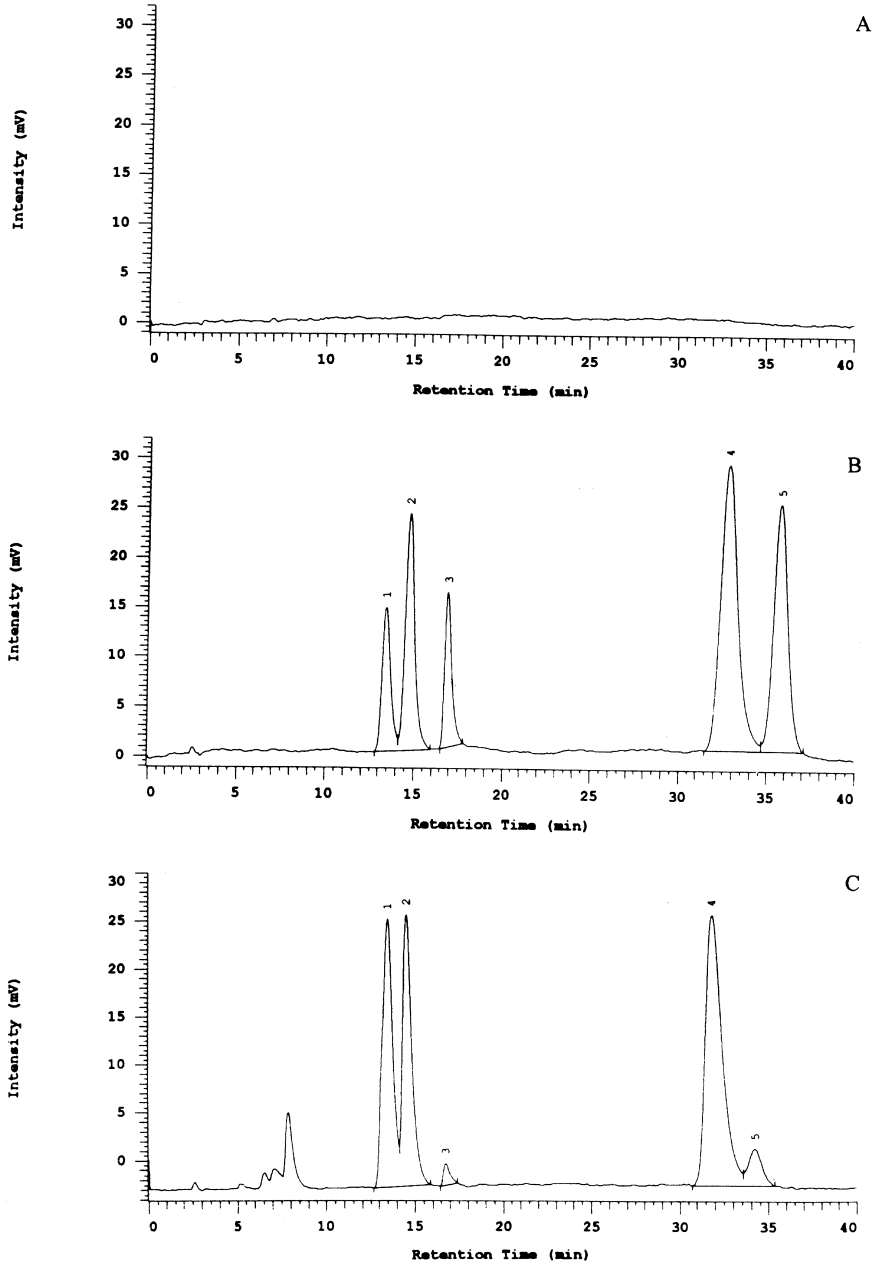

Fig. 1. (A) Blank plasma from a volunteer. (B) Extraction of standard mixture spiked in plasma to 25 n*M* for (1) and (3) and to 100 n*M* for (2) and (5); 25 μl of 10 μ*M* (4) was added before extraction. The retention times were 13.69 (1), 14.68 (2), 16.87 (3), 32.65 (4) and 35.65 (5). (C) Chromatogram of a 500-μl plasma extract from a healthy subject 1 h after ingestion of 20 mg tacrine: (1) 62 n*M*, (2) 130 n*M*, (3) 4 n*M* and (5) 16 n*M*; 25 μl of 10 μ*M* (4) was added before extraction. The retention times were 13.50 (1), 14.55 (2), 16.74 (3) and 34.19 (5). (D) Blank urine from a healthy volunteer. (E) Extraction of standard mixture spiked in urine to 0.4 μ*M* for (1) and (3) and to 1.6 μ*M* for (2) and (5); 25 μl of 100 μ*M* (4) was added before extraction. The retention times were 11.19 (1), 12.41 (2), 14.89 (3), 29.35 (4) and 31.70 (5). (F) Chromatogram of a 500 μl urine extract from a healthy patient 6 h after ingestion of 20 mg tacrine: (1) 3.05 μ*M*, (2) 3.29 μ*M*, (3) 0.18 μ*M* and (5) 0.164 μ*M*; 25 μl of 100 μ*M* (4) was added before extraction. The retention times were 11.78 (1), 12.71 (2), 15.30 (3) and 32.53 (5). Peak identification: (1) 2-hydroxytacrine, (2) 1-hydroxytacrine (velnacrine, maleate), (3) 4-hydroxytacrine, (4) 1,2,3,4-tetrahydro-9-acridanon and (5) tacrine.

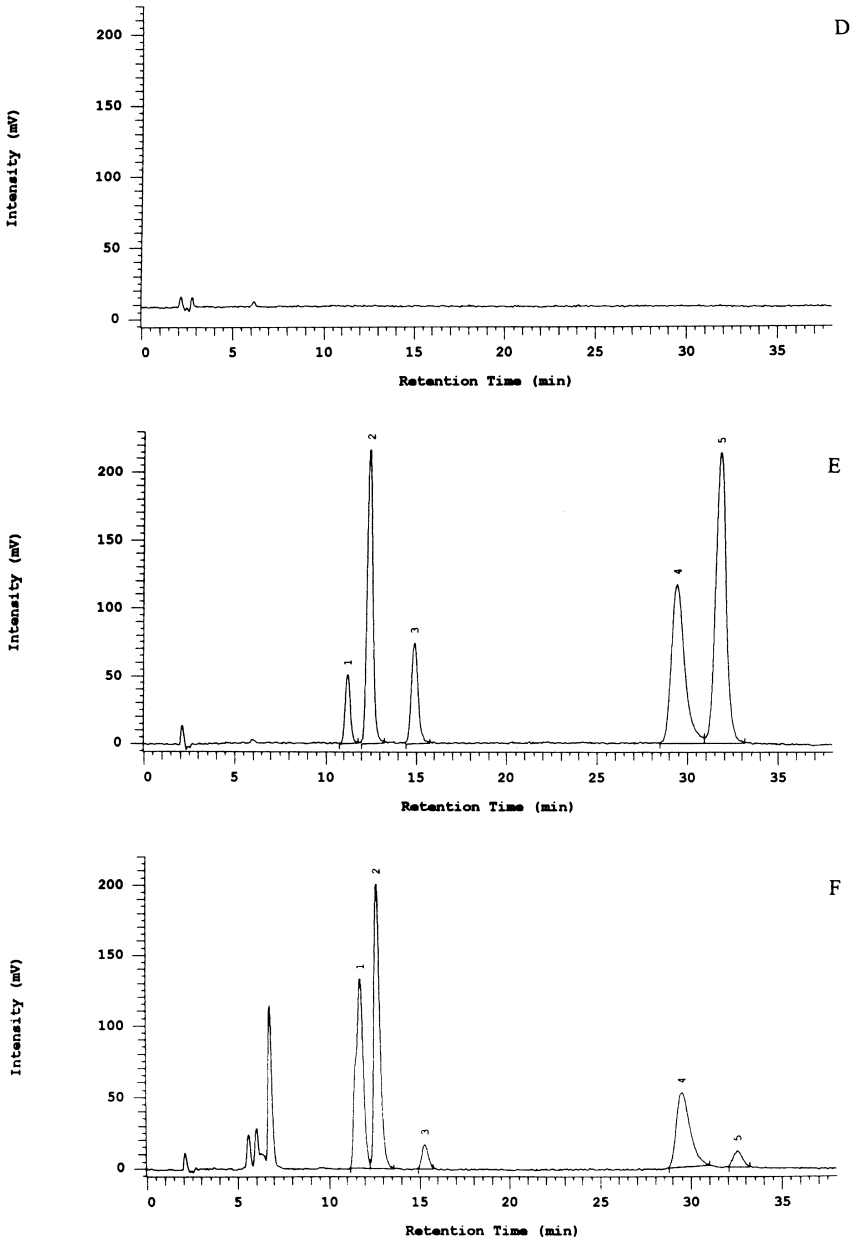

Fig. 1. (*continued*)

Prior to analysis of unknown samples, calibration curves of five standard levels were prepared. The linear calibration curves were fitted through the data point by linear regression. The quantitative analysis of an unknown sample was derived with reference to the internal standard.

3.4. Reproducibility

Inter-day reproducibility was assessed for five following days at three concentration levels. The levels were 15, 25 and 45 n*M* for 2-hydroxytacrine and 4-hydroxytacrine, and 60, 100 and 180 n*M* for

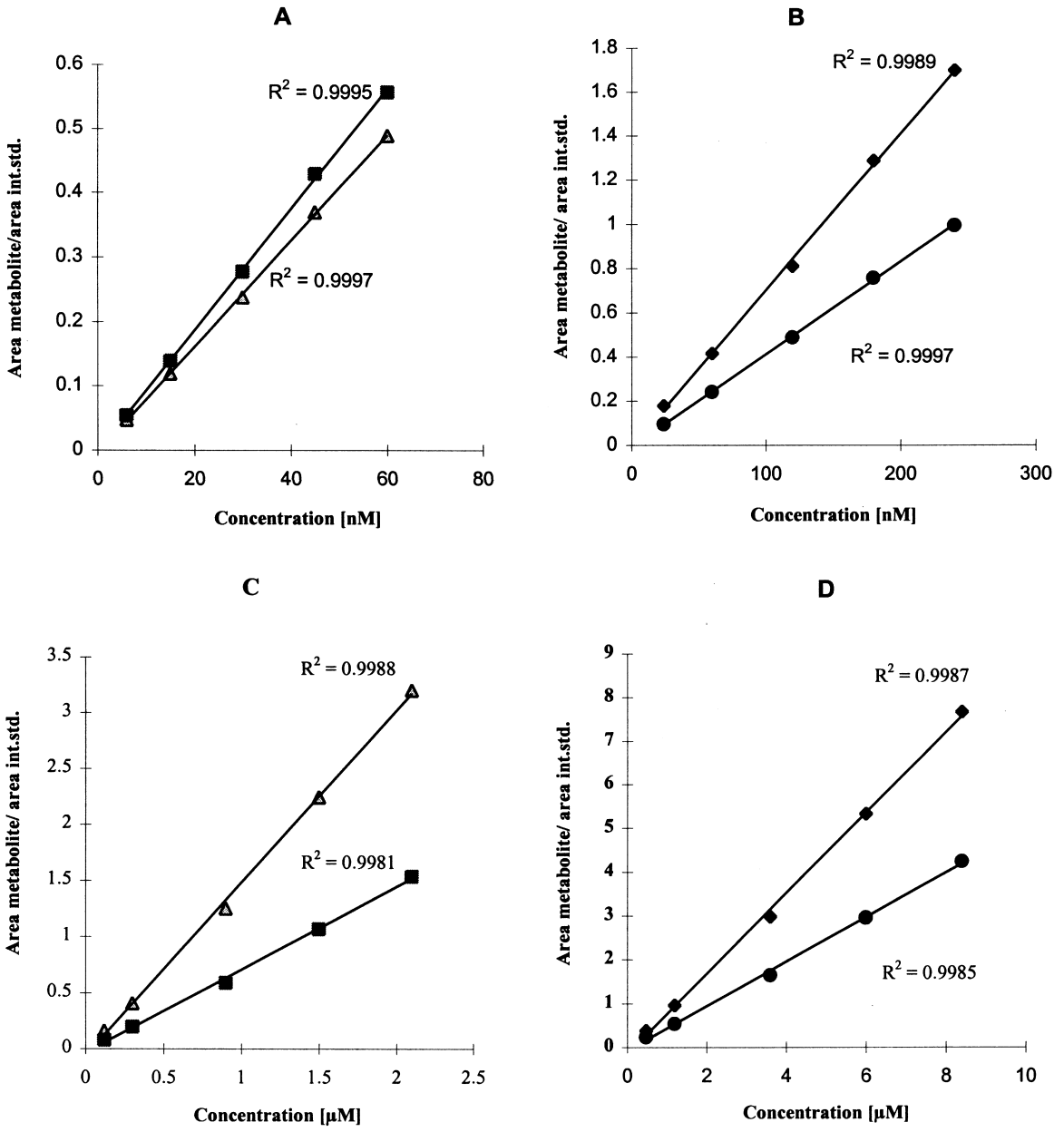

Fig. 2. Linearity of detector response to variation in concentration. A and B spiked plasma. C and D spiked urine. △ 4-hydroxytacrine, ■ 2-hydroxytacrine, ● 1-hydroxytacrine, and ♦ Tacrine.

1-hydroxytacrine and tacrine in plasma, and 0.12, 0.9 and 2.1 μ*M* for 2-hydroxytacrine and 4-hydroxytacrine, and 0.48, 3.6 and 8.4 μ*M* for 1-hydroxytacrine and tacrine in urine. The coefficients of variation are shown in Table 2.

3.5. Accuracy

Samples spiked to three concentration levels were analysed once a day for five days. The concentrations were 15, 25 and 45 n*M* for 2-hydroxytacrine

Table 1
Mean recoveries ($n=10$) of 2-hydroxytacrine, 4-hydroxytacrine, 1-hydroxytacrine and tacrine from human plasma and urine

Plasma			Urine		
Concentration (n*M*)	Recovery (%) Mean±S.D.	C.V. (%)	Concentration (μ*M*)	Recovery (%) Mean±S.D.	C.V. (%)
2-hydroxytacrine			2-hydroxytacrine		
6	84±4	5	0.12	64±6	9
15	88±3	3	0.3	76±4	5
30	88±2	2	0.9	76±3	4
45	89±3	3	1.5	80±4	4
60	85±3	4	2.1	78±2	2
4-hydroxytacrine			4-hydroxytacrine		
6	99±6	6	0.12	90±8	9
15	99±3	3	0.3	98±5	5
30	99±2	2	0.9	95±2	2
45	100±3	3	1.5	100±4	4
60	98±3	3	2.1	97±2	2
1-hydroxytacrine			1-hydroxytacrine		
24	99±4	5	0.48	71±4	5
60	96±3	3	1.2	90±3	3
120	96±2	2	3.6	89±3	3
180	96±3	3	6.0	93±4	4
240	94±3	3	8.4	91±1	2
Tacrine			Tacrine		
24	102±4	4	0.48	95±7	7
60	104±3	3	1.2	95±4	4
120	103±6	6	3.6	94±3	3
180	105±3	3	6.0	99±5	5
240	101±2	2	8.4	96±1	1

The table shows recoveries (%), mean and standard deviations (mean±S.D.) and coefficient of variation C.V. (%).

and 4-hydroxytacrine, and 60, 100 and 180 n*M* for 1-hydroxytacrine and tacrine in plasma, and 0.12, 0.9 and 2.1 μ*M* for 2-hydroxytacrine and 4-hydroxytacrine, and 0.48, 3.6 and 8.4 μ*M* for 1-hydroxytacrine and tacrine in urine. The mean estimates and deviations from the spiked values are shown in Table 2.

3.6. Limit of detection and quantification

The limit of detection, based on a signal-to-noise ratio of 3:1 was 0.5 n*M* for 2-hydroxytacrine and 4-hydroxytacrine and 2 nm for 1-hydroxytacrine and tacrine in plasma and 60 n*M* for 2-hydroxytacrine and 4-hydroxytacrine, 30 n*M* for 1-hydroxytacrine and 80 n*M* for tacrine in urine.

The limit of quantification based on a coefficient of variation of less than (20%) was 5 n*M* for 2-hydroxytacrine and 4-hydroxytacrine, 10 n*M* for 1-hydroxytacrine and 2 n*M* for tacrine in plasma and 120 n*M* for all components in urine.

4. Discussion

This study describes an isocratic reversed-phase HPLC method developed for simultaneous quantitation of tacrine and its three metabolites in plasma and urine. In an attempt to improve safety in the laboratory we have developed a method where the less hazardous compound ethyl acetate is used instead of chloroform as an extraction solvent. The method shows good overall recovery, accuracy and

Table 2
Between-day variation ($n=5$) of 2-hydroxytacrine, 4-hydroxytacrine, 1-hydroxytacrine and tacrine in plasma and urine

Compound	15 n*M*			25 n*M*			45 n*M*		
	Mean	C.V. (%)	Accuracy (%)	Mean	C.V. (%)	Accuracy (%)	Mean	C.V. (%)	Accuracy (%)
Plasma									
2-hydroxytacrine	15	4.3	0	24	4.1	4	44	2.4	2.2
4-hydroxytacrine	14	3.7	6.7	24	3.5	4	42	6.8	6.7
	60 n*M*			100 n*M*			180 n*M*		
	Mean	C.V. (%)	Accuracy (%)	Mean	C.V. (%)	Accuracy (%)	Mean	C.V. (%)	Accuracy (%)
1-hydroxytacrine	58	1.3	3.3	98	3.1	2	181	0.9	0.6
Tacrine	61	1.9	1.7	100	1.5	0	181	0.5	0.6
Urine	0.12 μ*M*			0.9 μ*M*			2.1 μ*M*		
	Mean	C.V. (%)	Accuracy (%)	Mean	C.V. (%)	Accuracy (%)	Mean	C.V. (%)	Accuracy (%)
2-hydroxytacrine	0.12	6.8	0.8	0.9	3.7	3.3	2.2	1.8	3.8
4-hydroxytacrine	0.11	3.7	5.8	0.9	3.6	7.4	2.2	4.1	2.4
	0.48 μ*M*			3.6 μ*M*			8.4 μ*M*		
	Mean	C.V. (%)	Accuracy (%)	Mean	C.V. (%)	Accuracy (%)	Mean	C.V. (%)	Accuracy (%)
1-hydroxytacrine	0.46	4.4	4.4	3.6	3.7	0.9	8.7	4.1	3.0
Tacrine	0.44	6.1	8.8	3.5	3.6	1.8	8.5	4.4	1.4

The table shows means, coefficients of variation (C.V. %) and accuracy (%).

precision, and low detection limits of all components.

After the validation we applied the method on plasma and urine samples of 18 healthy subjects who had taken single oral doses of tacrine on three separate occasions. Among the many samples we discovered an unknown peak with nearly the same retention time as 2-hydroxytacrine in some of the chromatograms. The unknown peak and 2-hydroxytacrine could be separated by using a gradient method (100% A 10:90% v/v CH_3CN: 0.2 *M* sodium acetate buffer pH 4.0 from 0–15 min at 0.8 ml min^{-1}, then 100% A, but from 15–23 min the flow-rate was 1.5 ml min^{-1}). During the last minutes (23–45 min) the composition was changed to 100% B (13:87% v/v CH_3CN: 0.2 *M* sodium acetate buffer pH 4.0) at a flow-rate of 2.5 ml min^{-1}.

When using this more complicated and farmore costly method we found a very constant quotient between 2-hydroxytacrine and the unknown peak. The quotient was 1: 2.41 (C.V. 12.9%) in 66 samples from three subjects. Thus dividing the apparent concentration assessed the concentration of 2-hydroxytacrine by (1 plus 2.41), equal to 3.41. Therefore tacrine and metabolites were assayed by the fully validated isocratic method and the concentration of 2-hydroxytacrine was calculated through correction by the quotient as mentioned above. The unknown peak might be one of the other metabolites described by Ref. [3].

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