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High-performance liquid chromatographic determination of amitriptyline and its main metabolites using a silica column with reversed-phase eluent

Application in mice

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ABSTRACT

A method was developed for the assay of amitriptyline, amitriptyline-N-oxide, nortriptyline, desmethylnortriptyline and *E* (*trans*) and *Z* (*cis*) isomers of 10-hydroxyamitriptyline and of 10-hydroxynortriptyline in plasma and brain of animals, using high-performance liquid chromatography with ultraviolet detection (254 nm). Single extraction was performed at pH 10.5 from 0.25 ml of plasma or 1 ml of brain mixture. Chromatographic separations were achieved with a silica column and an aqueous methanol mobile phase containing ammonia. This procedure offers high sensitivity (8–10 ng/ml), high linearity ($r > 0.99$) and acceptable precision (coefficient of variation $\leq 13.3\%$). The method was used to determine levels of amitriptyline and its major metabolites in mice 30 min after a single intraperitoneal administration of amitriptyline (20 mg/kg).

INTRODUCTION

Demethylation and hydroxylation are the main metabolic reactions undergone by tricyclic antidepressants (TCAs) [1–4]. In animals, hydroxylated metabolites possess neuropharmacological activities which for the *E* (*trans*) isomers are different from the *Z* (*cis*) isomers [5–7]. We have developed an appropriate analytical procedure to determine the plasma and brain levels of all these compounds.

High-performance liquid chromatography (HPLC) [8,9] and gas chromatography (GC) [10] are the most widely used methods for determining TCA levels [11]. Several studies using these methods have included measurements of hydroxylated metabolites [12–15] but few have involved separation of *E* and *Z* isomers [16–21]. For this purpose, capillary GC with a nitrogen–phosphorus detector [16] or reversed-phase liquid chromatography [17–20] have been proposed. How-

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ever, use of silica columns and reversed-phase aqueous eluents have also found favour because of their numerous advantages including high efficiency, suitability for evaluation of metabolism, and convenience for the analysis of basic drugs [21–24]. We used this method to separate amitriptyline (AMI), amitriptyline-N-oxide (AMI-N-O), *E*- and *Z*-10-hydroxyamitriptyline (*E*- and *Z*-10-OH-AMI), nortriptyline (NOR), *E*- and *Z*-10-hydroxynortriptyline (*E*- and *Z*-10-OH-NOR) and desmethylnortriptyline (DM-NOR). Also, a single-step extraction was included, providing an easy and inexpensive procedure for the determination of AMI and all its metabolites from small amounts of plasma from rodents.

EXPERIMENTAL

Reagents

AMI, NOR, AMI-N-O, *E*- and *Z*-10-OH-AMI, *E*- and *Z*-10-OH-NOR, DM-NOR and the internal standard (imipramine) were gifts from H. Lundbeck (Copenhagen, Denmark). The chemical structures are shown in Table I. Hexane, methanol and isopropanol (all HPLC solvent grade) were purchased from Rathburn (Walkerburn, UK), isoamyl alcohol from Merck (Darmstadt, Germany) and butylamine from Fluka (Buchs, Switzerland). Sodium bicarbonate, sodium hydroxide, ammonium hydroxide and acetic acid, all analytical-reagent grade, were obtained from Merck. Distilled water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Bicarbonate buffer (pH 10.5) was prepared by dissolving 8 g of sodium bicarbonate in water in a 100-ml volumetric flask and adjusting the pH to 10.5 with 0.1 *M* sodium hydroxide.

Standards

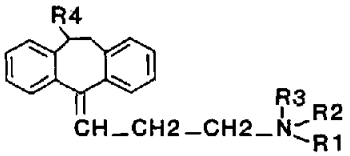
Stock solutions (1 mg/ml) of AMI, demethylated metabolites and imipramine were obtained by weighing out AMI hydrochloride (22.64 mg), NOR hydrochloride (22.76 mg), DM-NOR hydrochloride (22.80 mg) and imipramine hydrochloride (22.60 mg) and dissolving these in 20 ml of methanol. Stock solutions of AMI-N-O, *E*- and *Z*-isomers of 10-hydroxylated metabolites of AMI and of NOR were prepared as 1 mg free base per ml of methanol. All the stock solutions were diluted in methanol to give working solutions containing 2 ng/ml.

Sample preparation

Blood samples were collected in polypropylene tubes and immediately centrifuged at 3000 *g* for 3

TABLE I

CHEMICAL STRUCTURES OF AMITRIPTYLINE AND ITS METABOLITES



Compound	Abbreviation	R1	R2	R3	R4
Amitriptyline	AMI	CH_3	CH_3	–	–
E-10-Hydroxyamitriptyline	*E*-10-OH-AMI	CH_3	CH_3	–	OH
Z-10-Hydroxyamitriptyline	*Z*-10-OH-AMI	CH_3	CH_3	–	OH
Amitriptyline-N-oxide	AMI-N-O	CH_3	CH_3	O	–
Nortriptyline	NOR	CH_3	H	–	–
E-10-Hydroxynortriptyline	*E*-10-OH-NOR	CH_3	H	–	OH
Z-10-Hydroxynortriptyline	*Z*-10-OH-NOR	CH_3	H	–	OH
Desmethylnortriptyline	DM-NOR	H	H	–	–

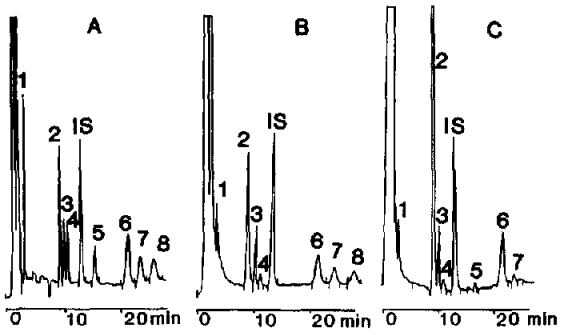


Fig. 1. Representative chromatograms of (A) spiked mouse plasma extract containing AMI (400 ng/ml), *E*-10-OH-AMI (200 ng/ml), *Z*-10-OH-AMI (200 ng/ml), AMI-N-O (200 ng/ml), NOR (400 ng/ml), *E*-10-OH-NOR (200 ng/ml), *Z*-10-OH-NOR (200 ng/ml), DM-NOR (200 ng/ml) and imipramine (IS), (B) plasma and (C) brain extracts from a mouse, 30 min after a single intraperitoneal injection of amitriptyline (20 mg/kg). Peaks: 1 = AMI-N-O; 2 = AMI; 3 = *E*-10-OH-AMI; 4 = *Z*-10-OH-AMI; 5 = DM-NOR; 6 = NOR; 7 = *E*-10-OH-NOR; 8 = *Z*-10-OH-NOR; IS = internal standard, imipramine. Experimental conditions: column, silica Si 5-μm Ultrasphere (250 mm × 4.6 mm I.D.); precolumn, silica Si 5-μm (45 mm × 4.6 mm I.D.); eluent, methanol–water (80:20, v/v) and ammonia (27 μM); flow-rate, 2 ml/min. Detection, 254 nm.

min; brains were removed, weighed and kept frozen with plasma samples at −20°C until analysis.

Glassware

Glass tubes and caps were thoroughly washed with laboratory detergent (Dubernard Hospital, Bordeaux, France), placed in dichromate–sulphuric acid for 12 h and then for 15 min in an ultrasonic bath. They were rinsed with distilled water and dried at 150°C.

Extraction procedure

For plasma analysis, 0.25 ml of plasma sample, 0.02 ml (40 ng) of working solution of internal standard (imipramine), 0.75 ml of twice-distilled water and 0.25 ml of bicarbonate buffer (pH 10.5) were successively added.

For brain analysis, samples were homogenised in 3 ml of 0.1 *M* acetic acid with an Ultra Turrax Ika-Werk (Heitersheim, Germany) grinder. A 0.10-ml volume (200 ng) of working solution of internal standard (imipramine) and 1 ml of bicarbonate buffer (pH 10.5) were added to 1 ml of the mixture.

Extraction was carried out by shaking the mixture mechanically for 10 min with 5 ml of hexane–isoamyl alcohol (99.5:0.5, v/v). After centrifugation for 10 min at 10 000 *g*, the organic phase was removed and quickly evaporated to dryness under a stream of nitrogen. For both the plasma and the brain extract, the residue of the evaporation was redissolved in 0.06 ml of methanol–water (80:20, v/v) and thoroughly vortex-mixed. A 0.02-ml aliquot of this solution was then immediately injected into the analysis system.

Apparatus

Chromatography was performed using a Shimadzu (Tokyo, Japan) LC-5A solvent delivery pump, equipped with a Waters (Milford, MA, USA) U6K injector and fitted to a Shimadzu SPD-2A variable-wavelength UV detector. Chromatograms were recorded on a Shimadzu C-R3A integrator.

Separations were achieved on an Ultrasphere Si 5-μm (Beckman, Frankfurt/Main, Germany) column (250 mm × 4.6 mm I.D.) fitted with a guard column (45 mm × 4.6 mm I.D.) containing the same stationary phase. The mobile phase, methanol–water (80:20, v/v) and ammonia (27 μM), was filtered through a 0.22-μm PTFE membrane filter (Millipore) and degassed for 15 min in an ultrasonic bath immediately before use. All separations were performed isocratically at room temperature and the eluent was monitored at 254 nm. The flow-rate was 2 ml/min. After each set of experiments, all the apparatus was washed with methanol for 30 min.

Quantification

Plasma and brain calibrations were constructed using four concentrations of spiked samples. Plasma concentrations ranges were 50–800 ng/ml of AMI and NOR and 25–400 ng/ml of AMI-N-O, *E*- and *Z*-10-OH-AMI, *E*- and *Z*-10-OH-NOR and DM-NOR. Each was done in 0.25 ml of drug-free plasma. Brain calibration standards were constructed to obtain concentration ranges of 25–1600 ng of AMI and NOR and 12.5–800 ng

of AMI-N-O, *E*- and *Z*-10-OH-AMI, *E*- and *Z*-10-OH-NOR and DM-NOR per ml of brain mixture.

These calibration samples were extracted before each series of assays by the method described above. Quantitation was performed by calculating the analyte/internal standard peak-area ratio, and a straight-line regression model was fitted to the peak-area ratio of each compound to internal standard *versus* concentration.

RESULTS AND DISCUSSION

Evaluation of the method

Analysis of AMI and all its metabolites, particularly the isomeric 10-hydroxylated metabolites, was performed using a silica column and an aqueous eluent. Also, for more effective separation and satisfactory peak shape, an amine modifier, ammonia, was added to the methanol–water eluent. Stabilisation of new columns was necessary with isopropanol and with mobile phase for at least 30 min and half a day, respectively. Retention times of both compounds are shown in Fig. 1. No interfering endogenous peaks were detected. Marked differences in retention times, and consequently in the ability to separate and quantitate metabolites of AMI, are in agreement with previous reports by Law [24]. This author showed that N-oxidation of AMI reduced the retention time relative to the original amine, unlike ring-hydroxylation and monodemethylation which increased the retention time. This could mainly be explained by an ion-exchange mechanism, but other mechanisms are probably also implicated.

The extraction procedure allowed quantitation of AMI and metabolites from small amounts of plasma from rodents (0.25 ml). The combination of a single-step extraction, isoamyl alcohol in hexane and a short evaporation time was used to avoid irreversible glass adsorption and to opti-

TABLE II

STANDARD CURVE PARAMETERS FOR THE ASSAY OF AMITRIPTYLINE AND ITS METABOLITES IN PLASMA AND BRAIN OF MICE (n = 8)

Compound	Concentration range (ng/ml of plasma or brain mixture)	Slope (mean ± S.E.M.) ($\times 10^{-3}$)	y-intercept (mean ± S.E.M.)	Correlation coefficient (mean ± S.E.M.)
Plasma				
AMI	50–800	8.4 ± 0.3	0.067 ± 0.057	0.9979 ± 0.0010
E-10-OH-AMI	25–400	7.8 ± 2.5	−0.029 ± 0.028	0.9984 ± 0.0009
Z-10-OH-AMI	25–400	9.6 ± 7.2	−0.026 ± 0.030	0.9986 ± 0.0008
AMI-N-O	25–400	1.4 ± 0.4	0.027 ± 0.018	0.9917 ± 0.0044
NOR	50–800	2.8 ± 0.8	−0.060 ± 0.084	0.9967 ± 0.0021
E-10-OH-NOR	25–400	2.3 ± 0.5	−0.029 ± 0.058	0.9954 ± 0.0020
Z-10-OH-NOR	25–400	1.9 ± 0.7	−0.093 ± 0.132	0.9976 ± 0.0009
DM-NOR	25–400	5.7 ± 2.0	−0.003 ± 0.016	0.9947 ± 0.0027
Brain				
AMI	25–1600	6.6 ± 0.5	−0.042 ± 0.023	0.9980 ± 0.004
E-10-OH-AMI	12.5–800	6.7 ± 3.5	−0.052 ± 0.130	0.9940 ± 0.009
Z-10-OH-AMI	12.5–800	7.2 ± 3.0	0.058 ± 0.090	0.9954 ± 0.010
AMI-N-O	12.5–800	1.9 ± 1.1	0.044 ± 0.038	0.9910 ± 0.032
NOR	25–1600	2.2 ± 0.2	0.015 ± 0.008	0.9994 ± 0.018
E-10-OH-NOR	12.5–800	2.3 ± 0.8	0.009 ± 0.007	0.9984 ± 0.005
Z-10-OH-NOR	12.5–800	1.8 ± 0.2	0.009 ± 0.036	0.9978 ± 0.012
DM-NOR	12.5–800	3.3 ± 0.8	−0.002 ± 0.030	0.9935 ± 0.020

mise the procedure. Extraction recoveries, analysed by comparing the peak heights of extracted with those of non-extracted spiked standards, were in the range 65–95% for AMI, *E*- and *Z*-10-OH-AMI and in the range 61–92% for NOR, *E*- and *Z*-10-OH-NOR and DM-NOR. These results are similar to those of Jones *et al.* [16], Kraak and Bijster [17] and Suckow and Cooper [18], who used a second extraction step. As already described by Edelbroek *et al.* [21], AMI-N-O was more weakly extracted (54%).

Determined as the concentration producing a signal-to-noise ratio of 2:1, the detection limits were 8 ng/ml for AMI and NOR, 10 ng/ml for hydroxylated metabolites, AMI-N-O and DM-NOR. These values were comparable to those reported by Dapgar and Power [13] with a direct injection technique (5–10 ng/ml using 0.250 ml of plasma). Hence these detection limits were very satisfactory, in view of the small sample requirement and the UV detector used. A lower wavelength provides better sensitivity but the interfer-

TABLE III

WITHIN-DAY AND DAY-TO-DAY VARIABILITY (n = 8)

Compound	Amount added (ng/ml of plasma or brain mixture)	Plasma		Brain	
		Within-day C.V. (%)	Day-to-day C.V. (%)	Within-day C.V. (%)	Day-to-day C.V. (%)
AMI	50	0.8		7.4	
	100	2.2		3.5	
	200	1.9	6.1	2.6	6.0
	400	0.3	4.0	0.4	2.9
E-10-OH-AMI	25	4.5		6.4	
	50	7.9		4.2	
	100	0.8	4.7	3.8	5.6
	200	3.9	2.5	0.6	3.6
Z-10-OH-AMI	25	1.1		4.8	
	50	3.8		1.8	
	100	2.2	3.9	0.6	3.0
	200	4.3	1.8	0.4	1.8
AMI-N-O	25	13.3		11.9	
	50	5.7		7.5	
	100	8.0	5.6	9.8	8.2
	200	3.8	4.7	8.4	7.5
NOR	50	6.7		4.2	
	100	7.4		3.0	
	200	8.5	2.7	6.1	8.8
	400	1.6	1.9	1.2	5.2
E-10-OH-NOR	50	5.2		5.6	
	100	3.2		7.8	
	200	2.5	1.9	1.5	3.0
	400	0.4	1.2	0.8	5.2
Z-10-OH-NOR	50	9.3		7.9	
	100	0.6		1.7	
	200	3.6	1.3	0.4	2.9
	400	0.8	1.0	0.4	4.3
DM-NOR	25	8.5		5.8	
	50	9.5		3.0	
	100	5.1	2.5	0.6	5.9
	200	2.9	1.1	10.4	12.3

ing endogenous peaks mentioned earlier are present. Also, some authors report similar detection limits but using samples of 1 ml or more; Fazio *et al.* [8], Dixon and Martin [15], Suckow and Cooper [18], Lundgreen *et al.* [20] and Edelbroek *et al.* [21] obtained 5, 25, 5–10, 15 and 5–20 ng/ml, respectively. Considering mice brain weights (0.45 ± 0.05 g) and the ratio of volume of brain mixture used for extraction to total volume, brain detection limits of AMI and both metabolites were approximately 50 ng/g.

A linearity study, performed by spiking different amounts of each compound, showed correlation coefficients for straight-line regression models in the range 0.9935–0.9994, except for AMI-N-O (r = 0.9917 ± 0.0044 and r = 0.9910 ± 0.032 in plasma and brain, respectively) (Table II).

Within-day and day-to-day precision were determined by analysing either several extracts of a spiked sample with the same calibration curve, or an aliquot of a spiked pool at different times over a two-week period. Whatever the amount added, the coefficient of variation (C.V.) never exceeded 13.3 and 6.1% in plasma or 11.9 and 12.3% in brain mixture, for within-day and day-to-day analysis, respectively (Table III).

TABLE IV

PLASMA AND BRAIN CONCENTRATIONS OF AMITRIPTYLINE AND ITS MAIN METABOLITES IN MICE, 30 min AFTER RECEIVING 20 mg/kg AMITRIPTYLINE INTRAPERITONEALLY (n = 8)

Compound	Plasma concentration (mean ± S.E.M.) (ng/ml)	Brain concentration (mean ± S.E.M.) (ng/g)
AMI	425 ± 34	9630 ± 1060
E-10-OH-AMI	200 ± 17	1471 ± 460
Z-10-OH-AMI	38 ± 5	314 ± 106
NOR	195 ± 24	1565 ± 190
E-10-OH-NOR	190 ± 23	58 ± 20
Z-10-OH-NOR	140 ± 25	T[b]
DM-NOR	T[a]	61 ± 20

[a] T = mean plasma level < 10 ng/ml.
[b] T = mean brain level < 50 ng/g.

Application of the method

Plasma and brain levels of AMI and its metabolites were studied in Swiss CD-1 male mice, 30 min after an acute intraperitoneal administration of AMI (20 mg/kg). Analysis was performed by the method described. Determination of AMI-N-O was of no interest because this metabolite does not possess any appreciable neuropharmacological activity [5], so it was not taken into account here.

The results (Table IV) show that AMI remains the main compound; its value at 30 min was in good agreement with previously reported pharmacokinetics [25,26]. In addition, plasma profiles revealed the closely similar concentrations of *E*-10-OH-AMI (200 ± 17 ng/ml), NOR (195 ± 24 ng/ml), *E*-10-OH-NOR (190 ± 23 ng/ml) and *Z*-10-OH-NOR (140 ± 25 ng/ml). In brain, the main compounds were AMI (9630 ± 1060 ng/g) and NOR (1565 ± 190 ng/g). Except for *E*-10-OH-AMI (1471 ± 460 ng/g), hydroxylated metabolites and DM-NOR were less abundant.

This easy, sufficiently sensitive and selective method allows quantitation of AMI and its more important metabolites, especially the *E* and *Z* isomers of hydroxylated metabolites, and enables individual investigation of *in vivo* pharmacokinetics in small animals, especially rodents, the main species used in screening tests of psychoactive drugs.

REFERENCES

1 M. H. Bickel, in F. Hoff and G. Stille (Editors), *Psychotropic Agents, Handbook of Experimental Pharmacology*, Vol. 55/I, Springer-Verlag, New-York, 1980, p. 550.
2 R. Krüger, G. Hölzl, H. J. Kuss and L. Schefold, *Psychopharmacology*, 88 (1986) 505.
3 S. Caccia and S. Garattini, *Clin. Pharmacokin.*, 18 (1990) 434.
4 C. S. Smith, R. K. Abramson and S. L. Morgan, *J. Liq. Chromatogr.*, 9 (1986) 1727.
5 J. Hyttel, A. V. Christensen and B. Fjalland, *Acta Pharmacol. Toxicol.*, 47 (1980) 53.
6 W. Z. Potter, M. V. Rudorfer and E. A. Lane, in E. Usdin (Editor), *Frontiers in Biochemical and Pharmacological Research in Depression*, Raven Press, New York, 1984, p. 373.
7 R. C. Young, *Psychopharmacologia*, 27 (1991) 521.
8 A. Fazio, E. Spina and F. Pisani, *J. Liq. Chromatogr.*, 10 (1987) 223.

9 S. H. Y. Wong, *Clin. Chem.*, 34 (1988) 848.
10 N. Rifai, C. M. Howlett, C. B. Levtzow, J. C. Phillips, N. C. Parker and R. E. Cross, *Ther. Drug Monit.*, 10 (1988) 194.
11 R. N. Gupta, *J. Chromatogr.*, 576 (1992) 183.
12 R. Ishida, T. Osaki, H. Uchida and T. Irikura, *J. Chromatogr.*, 305 (1984) 73.
13 D. Dapgar and A. Power, *J. Chromatogr.*, 416 (1987) 99.
14 S. R. Biggs, L. F. Chasseaud, D. R. Hawkins and I. Midgley, *Drug Metab. Dispos.*, 7 (1979) 233.
15 R. Dixon and D. Martin, *Res. Commun. Chem. Pathol. Pharmacol.*, 33 (1981) 537.
16 D. R. Jones, B. J. Lukey and H. E. Hurst, *J. Chromatogr.*, 278 (1983) 291.
17 J. C. Kraak and P. Bijster, *J. Chromatogr.*, 143 (1977) 499.
18 R. F. Suckow and T. B. Cooper, *J. Chromatogr.*, 230 (1982) 391.
19 J. S. Kiel, R. K. Abramson, R. C. Smith and S. L. Morgan, *J. Chromatogr.*, 383 (1986) 119.
20 T. I. Lundgreen, L. Slordal, R. Jæger, J. E. Whist and J. Aarbakke, *Pharmacol. Toxicol.*, 67 (1990) 132.
21 P. M. Edelbroek, E. J. M. de Haas and F. A. de Wolff, *Clin. Chem.*, 28 (1982) 2143.
22 B. A. Bidlingmeyer, J. K. Del Rios and J. Korpi, *Anal. Chem.*, 54 (1982) 442.
23 B. A. Bidlingmeyer, J. Korpi and J. N. Little, *Chromatographia*, 15 (1982) 83.
24 B. Law, *J. Chromatogr.*, 407 (1988) 1.
25 G. B. Cassano, S. E. Sjöstrand and E. Hansson, *Psychopharmacologia*, 8 (1965) 1.
26 R. K. Glotzbach and S. H. Preskorn, *Psychopharmacology*, 78 (1982) 25.