Journal of Chromatography, 416 (1987) 311–319
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3568

DETERMINATION OF CLOMIPRAMINE AND ITS HYDROXYLATED AND DEMETHYLATED METABOLITES IN PLASMA AND URINE BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

ODILE SPREUX-VAROQUAUX*, DOMINIQUE MORIN, CHARLES ADVENIER and MICHEL PAYS

Département de Biochimie et de Pharmacologie, Hôpital A. Mignot, 177, Rue de Versailles, F-78157 Le Chesnay Cedex (France)

(First received July 28th, 1986; revised manuscript received December 27th, 1986)

SUMMARY

A procedure for the determination of clomipramine and its 8-hydroxy, demethyl, 8-hydroxydemethyl and didemethyl metabolites in plasma and urine by high-performance liquid chromatography with electrochemical detection is described. A 1-ml plasma or urine sample is made alkaline with a carbonate buffer (pH 9.8) and extracted with 20% ethyl acetate in *n*-heptane. After back-extraction into an acid phosphate buffer (pH 2.4), an aliquot is injected into a 5-μm ion-paired reversed-phase column and eluted with a mobile phase containing a phosphate buffer with tetramethylammonium chloride–acetonitrile (57:43). The detection is coulometric with a first cell at +0.40 V, a second at +0.73 V and a guard cell set at 0.75 V for oxidation of the mobile phase. The method provides recoveries in the general range of 80–110% and a day-to-day precision of 3.7–8.8%, depending on the compound. The minimum quantifiable level for all compounds was 0.2 ng/ml with a 20-μl injection. Steady-state plasma concentration data and urinary levels are reported for 24 depressed patients receiving daily either 75–150 mg orally or 50–75 mg by infusion.

INTRODUCTION

Antidepressant drug plasma level determination is widespread [1,2]. Most methods focus on the parent compound so as to monitor the dosage regimen, whereas others include identification and quantification of metabolites, some of which are potentially active [3–5]. For instance, the demethylated and hydroxylated metabolites of clomipramine are pharmacologically active, although their spectrum of activity may be different from that of clomipramine itself [6–8].

Antidepressant drug metabolite determination has sometimes required combined gas chromatography–mass spectrometry (GC–MS) to achieve sufficient

0378-4347/87/$03.50

sensitivity after single doses. These techniques are laborious, requiring many extraction steps and the synthesis of many derivatives. A limit of detection below 2 ng/ml has been achieved for nortriptyline [4–9] and amitriptyline [10,11], but not for clomipramine metabolites. More recently, methods using reversed phase high-performance liquid chromatography (RP-HPLC) with UV detection for separating metabolites have been reported, with a limit of detection of 5–10 ng/ml for imipramine derivatives [12,13] and 2 ng/ml for maprotiline derivatives [14]. One group has separated imipramine, mianserin and trimipramine hydroxylated derivatives by RP-HPLC and used amperometric detection to achieve a limit of detection of 5 ng/ml for the first two compounds and 2 ng/ml for the third [15–17]. Linnoila et al. [8] reported the detection in plasma of 8-hydroxyclomipramine and demethylclomipramine using HPLC separation and electrochemical detection (ED) with a limit of detection of 2 ng/ml, although the specific methodology was not described.

This paper describes an RP-HPLC method with coulometric detection for the separation and quantification of clomipramine and its metabolites: demethyl, didemethyl, 8-hydroxyclomipramine and 8-hydroxydemethylclomipramine. The detection system consists of a dual-electrode cell placed in line with three electrodes and a guard cell before the injector, in order to electrolyse mobile phase components that could contribute to background currents. The principle of coulometric analysis (100% electrolysis) and the apparatus (dual cell, reading electrode with a large active surface) allow much greater sensitivity, i.e. down to 0.2 ng/ml in a urine or blood sample, than that achieved by other previously described HPLC methods.

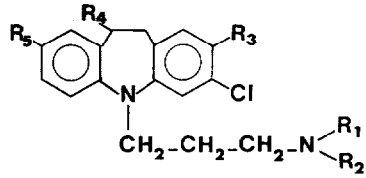
Our new method is comparable with combined GC–MS, but is much easier and is more practical for clinical and animal pharmacological studies requiring small sample volumes (125 μl).

EXPERIMENTAL

Chemicals

Clomipramine, its metabolites (Fig. 1) and demethylimipramine, which was used as an internal standard, were supplied by Ciba-Geigy (Basle, Switzerland), *n*-heptane, ethyl acetate, acetonitrile, methanol and tetramethylammonium chloride were purchased from E. Merck (Darmstadt, F.R.G.), sodium carbonate, sodium bicarbonate and potassium dihydrogen phosphate from Prolabo (Paris, France) and phosphoric acid from Riedel de Hahn (Hannover, F.R.G.). Distilled water was passed through an autostill Jencons-Bioblock scientific water-purification system before use.

Stock solutions (1 mg/ml) of clomipramine chlorhydrate (CMI), demethylclomipramine chlorhydrate (DCMI), 8-hydroxyclomipramine (8-OHCMI), 8-hydroxydemethylclomipramine (8-OHDCMI) and di-demethylclomipramine (DDCMI) were prepared in methanol. All stock solutions were further diluted with methanol to give working solutions of 1.5 ng/μl for the metabolites and 3 ng/μl for CMI and DCMI.



Compound	R_1	R_2	R_3	R_4	R_5
Clomipramine (CMI)	CH_3	CH_3	H	H	H
Demethylclomipramine (DCMI)	CH_3	H	H	H	H
2-Hydroxyclomipramine (2-OHCMI)	CH_3	CH_3	OH	H	H
10-Hydroxyclomipramine (10-OHCMI)	CH_3	CH_3	H	OH	H
8-Hydroxyclomipramine (8-OHCMI)	CH_3	CH_3	H	H	OH
8-Hydroxydemethylclomipramine (8-OHDCMI)	CH_3	H	H	H	OH
Di-demethylclomipramine (DDCMI)	H	H	H	H	H

Fig. 1. Structures of clomipramine and its metabolites.

Chromatography

Chromatography was performed using a dual-piston solvent delivery Knauer pump with a 20-μl fixed-volume injector. The column (15 cm$\times$4.6 mm I.D.) was packed with 5-μm ion-paired reversed-phase Ultrasphere-I.P. (Beckman). The ESA detector system consisted of a thin-layer flow-through electrochemical ESA coulochem detector (Model 5100 A) using dual cells (Analytical Cell ESA Model 5011) containing two working electrodes in porous graphite, together with associated silver–silver chloride reference and counter electrodes. Carbon filters were placed in front of each analytical cell. A guard cell (Model 5020 ESA) was put between the solvent-delivery system and the injector. The current response was shown by a recorder for each detector.

The mobile phase was a mixture of an acidic aqueous solution (0.01 *M* potassium dihydrogen phosphate, 5 m*M* tetramethylammonium chloride adjusted to pH 2.4 with phosphoric acid 85%) and acetonitrile (57:43). The filtered and degassed mobile phase was used at a flow-rate of 1.3 ml/min. The guard cell was set at +0.75 V. The analytical cells were set at +0.40 V for the first detector and at +0.73 V for the second detector. The mobile phase and the column were at room temperature.

Sample preparation

To 1.0 ml (or 0.5 ml) of plasma or urine, the internal standard, demethylimipramine (25 μl=25 ng) and 0.5 ml of 0.6 *M* sodium carbonate–sodium bicarbonate buffer (pH 9.8) were added. After addition of 5 ml of 20% (v/v) ethyl acetate in *n*-heptane, the vials were capped and mixed vigorously for 1.5 min, then centrifuged at 3000 *g* for 10 min. The organic layer was transferred to another tube containing 0.50 ml of acidic phosphate buffer (0.025 *M* potassium dihydrogen phosphate adjusted to pH 2.4 with 85% phosphoric acid, then mixed for 1 min and centrifuged at 3000 *g* for 10 min. The organic layer was discarded, and a 20-μl aliquot of the aqueous phase was injected for chromatographic separation.

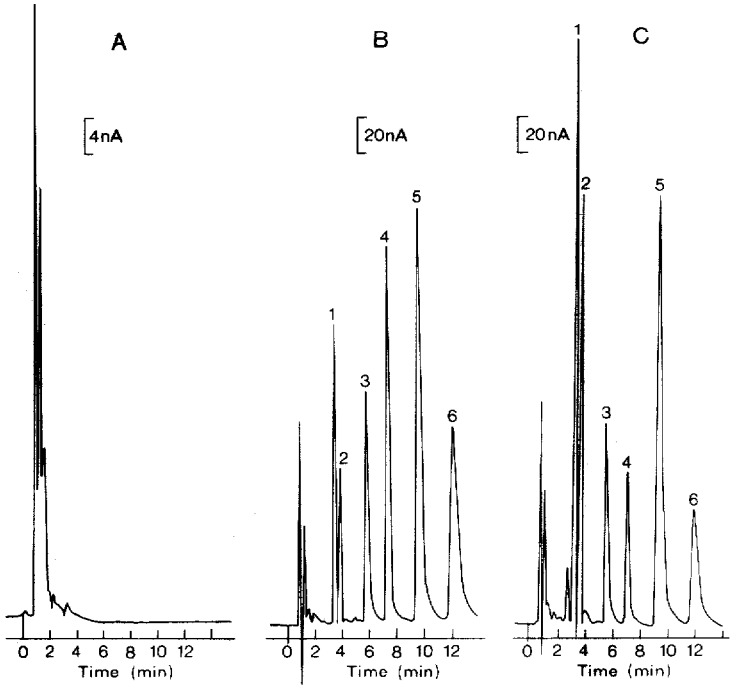


Fig. 2. (A) Chromatogram of blank sample: the gain is five-fold greater than in B and C. (B) Chromatogram of a spiked 1-ml plasma extract containing 75 ng of 8-OHDCMI, 8-OHCMI and DDCMI, 150 ng of DCMI and CMI and 50 ng of internal standard (I.S. = DMI), with retention times of 3.6, 4.0, 6.2, 7.7, 10.0 and 12.7 min, respectively. (C) Chromatogram of a 1-ml plasma extract from a patient receiving orally 150 mg of clomipramine per day for two years, and containing 10-OHCMI, 8-OHDCMI (170 ng/ml), 8-OHCMI (85 ng/ml), DMI (I.S.), DDCMI (35 ng/ml), DCMI (180 ng/ml) and CMI (100 ng/ml). Peaks: 1 = 8-OHDCMI; 2 = 8-OHCMI; 3 = DMI; 4 = DDCMI; 5 = DCMI; 6 = CMI.

Calibration curves

Standard curves were prepared using four concentrations of spiked samples: 30, 75, 150 and 300 ng/ml for 8-OHCMI, 8-OHDCMI and DDCMI, and 60, 150, 300 and 600 ng/ml for CMI and DCMI. These samples were then prepared according to the procedure described above. Quantification was performed by calculating the peak-height ratios of each compound to the internal standard.

RESULTS AND DISCUSSION

Clomipramine and its major metabolites were separated on the same chromatogram within 16 min. Fig. 2B shows a chromatogram of a spiked plasma sample. The chromatogram corresponding to the extract of a 1-ml patient plasma with high level of metabolites is shown in Fig. 2C.

Another peak at 3.0 min retention time was found at low concentrations in the plasma of patients studied, and was identified as the 10-OHCMI metabolite. The 2-OHCMI metabolite could be detected at the same retention time as 8-OHDCMI.

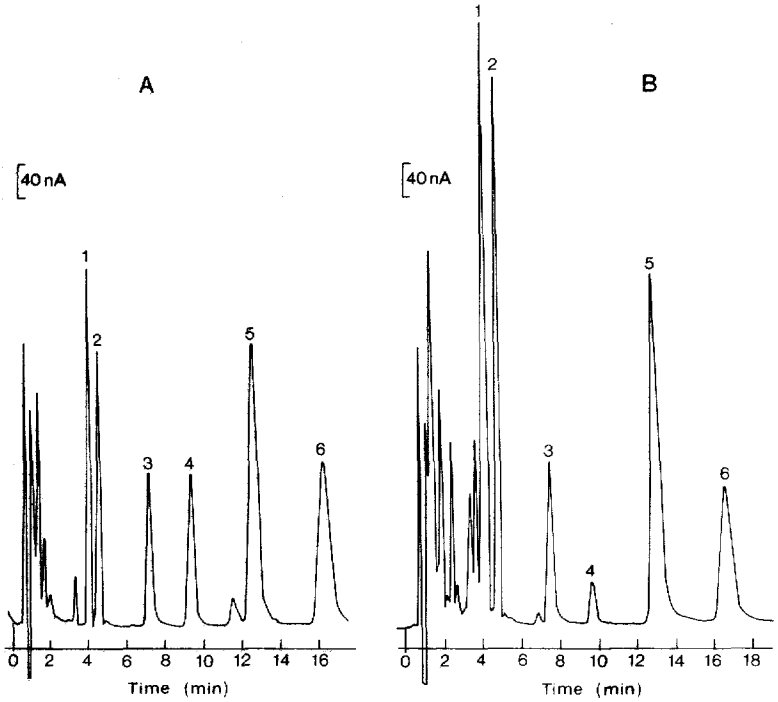


Fig. 3. Chromatograms of (A) a spiked 1-ml urine extract containing 150 ng each of 8-OHDCMI, 8-OHCMI, DDCMI, DCMI, CMI and DMI (I.S.) and (B) a 1-ml urine sample from a patient containing 8-OHDCMI (231 ng/ml), 8-OHCMI (213 ng/ml), DDCMI (13 ng/ml), DCMI (180 ng/ml), CMI (120 ng/ml), as unconjugated compounds. For peak identification see Fig. 2.

The 2-OHCMI is present only in negligible amounts as the unconjugated form in plasma and urine (less than 3% of 8-OHDCMI in our patients). A blank plasma extract showed no endogenous interfering peaks (Fig. 2A). Fig. 3 shows chromatograms obtained from urine samples.

The optimum range of oxidation potential was determined to be 0.70–0.80 V. The presence of hydroxy groups lowers the oxidation potential.

Accuracy, precision and sensitivity

The precision of this method was determined by spiking six 1.0-ml aliquots of drug-free plasma with various levels of drugs and metabolites. After the addition of 25 ng of the internal standard, samples were processed according to the procedure above.

The study of within-day reproducibility is shown in Table I. The coefficients of variation (C.V.) and the absolute recovery of 77–125% are satisfactory. Day-to-day accuracy was checked after runs over 21 consecutive days ($n=10$; Table II).

The absolute sensitivity of this method is 15 pg injected for all compounds. In practical terms, however, the lowest quantifiable levels in plasma are 200 pg/ml for 8-OHCMI and 8-OHDCMI and 300 pg/ml for CMI, DCMI and DDCMI, with the detector set at a gain of 12 nA full scale and a sample size of 2 ml of plasma.

TABLE I

ACCURACY, PRECISION AND WITHIN-DAY REPRODUCIBILITY FOR CMI AND ITS METABOLITES

From 1 ml of plasma; $n=6$; I.S. = internal standard; C.V. = coefficient of variation.

Compound	Amount added (ng/ml)	Peak-height ratio compound/I.S.	C.V. (%)	Mean recovery (%)	Regression equation	r
CMI	0.2	0.00629	18.7	97	$y=0.0128x-0.006$	0.9999
	1	0.0169	9.8	99		
	10	0.119	5.8	80		
	50	0.60	4.6	80		
	100	1.27	2.5	80		
	300	3.85	2.0	78		
	500	6.36	4.8	77		
DCMI	0.2	0.00773	6.5	95	$y=0.0233x-0.065$	0.9996
	1	0.0268	6.3	95		
	10	0.186	5.5	83		
	50	1.068	4.7	91		
	100	2.23	2.4	89		
	300	6.68	1.9	84		
	500	11.74	1.6	88		
8-OHCMI	0.2	0.00706	10.6	103	$y=0.0253x+0.019$	0.9995
	1	0.0295	2.7	106		
	10	0.207	6.7	94		
	50	1.19	3.9	105		
	100	2.59	2.4	109		
	300	7.36	2.4	98		
	500	12.83	2.2	103		
8-OHDCMI	0.2	0.00706	16.2	102	$y=0.0329x-0.138$	0.9999
	1	0.034	17.0	99		
	10	0.274	11.1	96		
	50	1.46	3.7	121		
	100	3.09	3.6	125		
	300	9.08	2.6	111		
	500	16.73	2.4	117		
DDCMI	0.067	0.00394	16.7	91	$y=0.0366x-0.05$	0.9999
	0.333	0.0132	5.5	94		
	3.33	0.096	5.2	78		
	16.67	0.5425	8.8	87		
	33.33	1.15	2.4	87		
	100	3.37	2.1	79		
	167	6.21	1.6	88		

This corresponds to a peak height ranging from 1.5 to 2 cm. Lower concentrations could be estimated using an injection volume of 100 μl instead of 20 μl.

Linearity

The calibration curves were established as described above. A good linear relationship was obtained in the range 0.2–500 ng/ml for all compounds ($r=0.999$). For routine analysis, a calibration curve is established each day. The linear regression equations are given in Table I.

TABLE II

ACCURACY AND DAY-TO-DAY REPRODUCIBILITY FOR CMI AND ITS METABOLITES

From 1 ml of plasma; $n = 10$, in 21 consecutive days.

Compound	Amount added (ng/ml)	Amount found (ng/ml)	Coefficient of variation (%)
CMI	375	375	3.71
DCMI	375	379	5.38
DDCMI	37.5	37.4	7.35
8-OHCMI	37.5	40.1	5.11
8-OHDCMI	37.5	39.9	8.79

Selectivity

Interfering peaks occurred in samples from patients receiving imipramine and desipramine. No interference was found in samples from patients treated with propericiazine, chlorpromazine, levomepromazine, cyamepromazine, haloperidol, amitriptyline, nortriptyline, amineptine, mepronizine, chlorazepate, diazepam, lorazepam, flunitrazepam, alimemazine, heptaminol and dihydroergotamine.

Stability

The plasma aqueous acidic phase extract remains stable for five days when stored at +4°C.

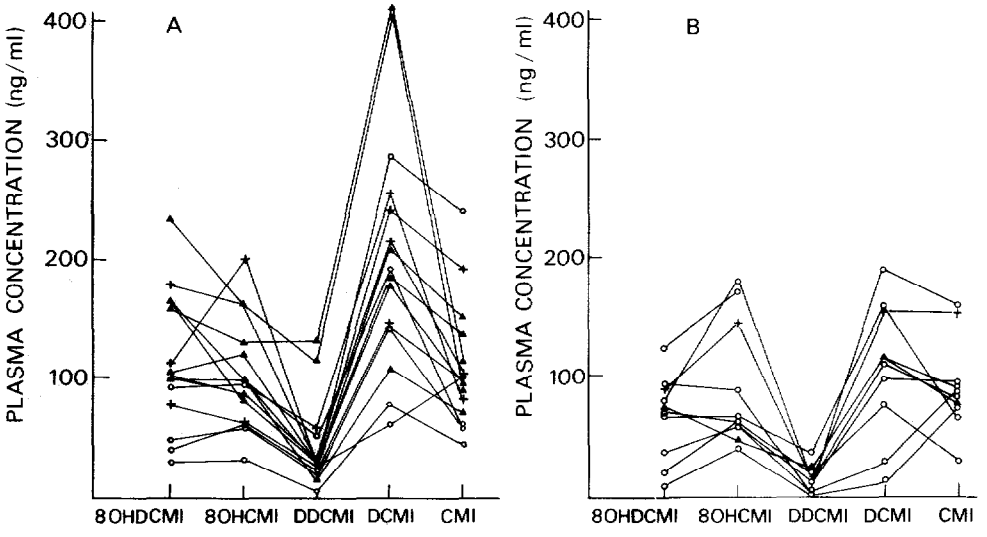


Fig. 4. Plasma levels of clomipramine and of its metabolites in plasma of patients receiving clomipramine daily, either (A) orally ($n = 12$) in doses of 75 (○), 100 (+) or 150 (▲) mg or (B) intravenously ($n = 10$) in doses of 50 (▲), 75 (○) or 100 (+) mg.

TABLE III

CONCENTRATIONS OF CMI AND ITS UNCONJUGATED METABOLITES FROM URINE OF PATIENTS SUFFERING FROM INTOXICATION BY CLOMIPRAMINE

Subject	Volume of urine (0–4 h) (ml)	Concentration (μg/l)				
		CMI	DCMI	DDCMI	8-OHCMI	8-OHDCMI
1	400	6	12	4	11	228
2	400	375	321	11	552	270
3	400	96	195	25	210	480
4	1130	480	261	18	520	180

Application

Steady-state unconjugated plasma levels of CMI and metabolites. The present method was used to determine the steady-state unconjugated plasma levels of CMI and its hydroxylated and demethylated metabolites reached in patients given clomipramine daily, either 75–150 mg orally or 50–75 mg by infusion. Blood samples were collected into heparinized plastic tubes in the morning just before the first daily administration, i.e. 12 h after the last oral dose or 20 h after the end of the last infusion. Plasma was separated and stored in plastic tubes at −20°C until analysis.

The results are summarized in Fig. 4. Following oral administration, DCMI is the predominant form as shown by the high steady-state concentrations. In contrast, following repeated intravenous administration, plasma DCMI concentrations are only slightly higher than those of CMI; 8-OHCMI and 8-OHDCMI plasma levels are similar to those of CMI. At any particular dose, inter-individual variations of up to five-fold were observed even in this small sample.

Urinary levels of clomipramine and its metabolites. Table III lists the urinary levels of the unconjugated derivatives of clomipramine and its metabolites from patients under intensive care after clomipramine intoxication.

ACKNOWLEDGEMENTS

The authors are indebted to Ciba-Geigy (Basle, Switzerland) for the generous gift of clomipramine and its metabolites, to J. Stephen Kennedy, Ph.D. (Neurosciences Research Branch, National Institute of Mental Health, Rockville, MD U.S.A.) for his kind supply of 8-hydroxydemethylclomipramine, and to William Z. Potter (Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, MD, U.S.A.) for his helpful discussion.

This work was supported by a grant from the Direction de la Recherche du Ministère de l'Education Nationale (France).

REFERENCES

1 T.R. Norman and K.P. Maguire, J. Chromatogr., 340 (1985) 173.
2 E. Spina, O. Ericsson and C. Nordin, Ther. Drug Monit., 7 (1985) 242.
3 L. Bertilsson, B. Mellstrom and F. Sjöqvist, Life Sci., 25 (1979) 1285.

4 W.Z. Potter, H.M. Calil, A.A. Manian, A.P. Zavadil and F.K. Goodwin, Biol. Psychiatry, 14 (1979) 601.
5 J.C. Nelson, J.L. Bock and P.I. Jatlow, Clin. Pharmacol. Ther., 33 (1983) 183.
6 L. Bertilsson, M. Asberg and P. Thoren, Eur. J. Clin. Pharmacol., 7 (1974) 365.
7 L. Traskman, M. Aberg, L. Bertilsson, B. Cronholm, B. Mellstrom, L.M. Neckers, F. Sjöqvist, P. Thoren and G. Tybring, Clin. Pharmacol. Ther., 26 (1979) 600.
8 M. Linnoila, T. Insel, C. Kilts, W.Z. Potter and D.L. Murphy, Clin. Pharmacol. Ther., 32 (1982) 208.
9 O. Borga and M. Garle, J. Chromatogr., 68 (1972) 77.
10 W.A. Garland, R.R. Muccimo, B.I.H. Mim, J. Cupano and W.E. Fann, Clin. Pharmacol. Ther., 25 (1979) 844.
11 P. Bauman, L. Koeb, D. Tinguely and L. Rivier, Eur. J. Mass Spectrom. Biochem. Med. Environ. Res., 2 (1982) 19.
12 S.C. Bogema and N. Narasimhachari, Clin. Chem., 29 (1983) 1249.
13 P.P. Rop, T. Conquy, G. Gouézo, A. Viala and F. Grimaldi, J. Chromatogr., 375 (1986) 339.
14 H.J. Kuss and E. Feistenauer, J. Chromatogr., 204 (1981) 349.
15 R.F. Suckow and T.B. Cooper, J. Pharm. Sci., 70 (1981) 257.
16 R.F. Suckow, T.B. Cooper, F.E. Quitkin and J.W. Stewart, J. Pharm. Sci., 71 (1982) 889.
17 R.F. Suckow and T.B. Cooper, J. Pharm. Sci., 73 (1984) 1745.