BIOMEDICAL CHROMATOGRAPHY, VOL. 7, 59–63 (1993)

Drug Monitoring of Clomipramine and Desmethylclomipramine in Depressed Patients Using a New Liquid Chromatographic Assay

Bertrand Diquet,* Patrick Thomaré, Muriel Bocquentin and Catherine Diviné

Labortoire de Pharmacocinétique, Département de Pharmacologie Clinique, Groupe Hospitalier Pitié-Salpêtrière, 47 Boulevard de l'Hôpital, 75013 Paris, France

A method has been developed for the quantitative analysis of clomipramine and its major metabolite desmethylclomipramine in plasma, using normal phase chromatography with UV detection at 254 nm. This rapid (6 min) and highly sensitive methodology (detection limits 0.5 ng/mL and 2 ng/mL for clomipramine and desmethylclomipramine, respectively; $S/N = 3$, 0.001 aufs) allows pharmacokinetic studies and drug monitoring of the two compounds. Using the described methodology we report an application which involved 10 depressed inpatients.

INTRODUCTION

Clomipramine (CMI) is one of the most widely prescribed tricyclic antidepressants (TCA) for the treatment of depression in Western Europe since the sixties. In some other countries, e.g. the United States, it has only recently been approved for the treatment of obsessive compulsive disorders (McTavish and Benfield, 1990) according to DSM III classification. CMI is considered to be the most potent inhibitor of serotonin uptake among all the TCA, while its main hepatic metabolite desmethylclomipramine (DCMI) has been shown to be a stronger inhibitor of norepinephrine and a weaker inhibitor of 5-HT uptake than the parent drug.

Numerous studies have been conducted with the objective of finding a relationship between blood concentrations of CMI and/or DCMI and clinical outcome, but despite all efforts this remains highly controversial (Montgomery, 1980; Balant-Gorgia *et al.*, 1991). The problems encountered were the choice of a daily dose, the existence of at least two active principles with different pharmacological profiles whose ratio is under strong genetic control (Balant-Gorgia *et al.*, 1989) and the influence of the analytical method on the outcome of the studies (Piollet *et al.*, 1988). It appears that studies using radioimmunoassay have consistently failed to demonstrate any relationship between concentration and effect (Jones and Luscombe, 1977; Montgomery *et al.*, 1980; Moyes *et al.*, 1980), while specific methods, gas–liquid chromatography (GLC) or high performance liquid chromatography (HPLC), were more successful in detecting such a relationship (Träskman *et al.*, 1979; Vandel *et al.*, 1982; Favarelli *et al.*, 1984; Diquet *et al.*, 1986; De Oliveira *et al.*, 1989). Nevertheless, most authors believe that the great inter-individual variability of the pharmacokinetics of CMI, associated with its rather narrow therapeutic margin, makes CMI an ideal candidate for blood concentration monitoring. CMI and DCMI plasma concentrations should be determined at steady state. Whole-blood, "unbound" concentrations or the measurement of hydroxylated metabolites are inadequate as a guide to treatment adequacy (Balant-Gorgia *et al.*, 1991).

Accordingly, drug monitoring, pharmacological studies and determination of pharmacokinetic parameters in animals and human subjects require a highly selective and sensitive assay. The aim of the present paper is to describe an HPLC technique with UV detection in order to monitor very low concentrations of CMI and DCMI in plasma.

EXPERIMENTAL

Chemicals. Clomipramine HCl, desmethylclomipramine HCl and imipramine HCl, used as internal standard (IS), were kindly supplied by Ciba-Geigy (Rueil Malmaison, France). Stock solutions of CMI and DCMI were prepared in 0.01 M HCl at two concentrations expressed as base: 10 μg/mL and 1 μg/mL. Imipramine stock solution was also prepared in 0.01 M HCl at a concentration of 1 μg/mL. Solutions were stored at +4 °C and were stable for several weeks. Analytical reagent grade carbonate disodium was obtained from UCB Laboratory (Leuven, Belgium). Acetonitrile, methanol (Merck, Darmstadt, Germany) and *n*-hexane (Carlo Erba, Milano, Italy) were UV grade. Diethylamine Rectapur was obtained from Prolabo (Paris, France). Glassware and centrifuge tubes were cleaned by soaking overnight in sulphochromic acid, then ultrasonically cleaned and rinsed twice with bidistilled water (PCH, Paris, France) and methanol.

Instruments and chromatographic conditions. The liquid chromatographic system consisted of a Beckman Model 114A pump (Beckman Instruments, San Ramon, USA) connected to a Beckman Model 507 autosampler and a Beckman Model 160 absorbance detector provided with a 254 nm filter and a mercury lamp. The HPLC system was controlled by a Gold Beckman system.

The eluent was methanol:acetonitrile:distilled water:diethylamine ($98.74:1:0.2:6\times10^{-2}$ v/v). Before analysis, the mobile phase was filtered through a 0.22 μm filter (Durapore

* Author to whom correspondence should be addressed.

0269–3879/93/020059–05 $07.50

Received 20 March 1992
Accepted (revised) 31 July 1992

GVWP 047, Bedford, MA, USA) with a Pyrex filter holder (Millipore, Bedford, MA, USA) and then degassed ultrasonically.

Separation was achieved at room temperature using a normal phase Resolve spherical silica column (15.0 cm × 3.9 mm i.d., particle size 5 μm) purchased from Waters Millipore (Bedford, MA, USA). The flow rate was set at 1.0 mL/min with an average operating pressure of 49 bars (700 psi).

Plasma extraction procedure. In a 15 mL centrifuge tube, 1 mL of plasma (from human heparinized blood) was alkalinized with 0.5 mL of 2 M Na_2CO_3 (pH = 11.3). Each plasma sample was then extracted with 10 mL of *n*-hexane after addition of 0.1 mL of the IS working solution (100 ng). The tube was capped, shaken horizontally for 15 min and then centrifuged for 10 min at 1000 g. The hexane layer was transferred into another tube and evaporated to dryness under a stream of nitrogen (30 °C). The drug residue was dissolved in 1 mL of *n*-hexane, vortexed and re-evaporated. Finally, the residue was reconstituted in 100 μL of freshly prepared mobile phase, 50 μL of which were injected into the liquid chromatographic (LC) system.

Calibration curve. A calibration curve based on peak height ratio (CMI or DCMI) was constructed for each assay by adding known amounts of CMI or DCMI to 1 mL of drug-free human plasma. Concentrations of the two drugs equivalent to 0, 5, 10, 20, 50, 100, 200 and 300 ng/mL of CMI and DCMI as base were assayed.

Pharmacokinetic study. All patients had a four day wash-out period and one blood sample was assayed prior to the drug administration in order to check the absence of CMI and DCMI. Ten patients were treated with a 75 mg intravenous dose of clomipramine as a 2.50 h infusion. Venous blood samples were collected in "vacutainer" tubes containing sodium heparinate at time 3, 6, 18 and 24 h. They were immediately centrifuged and plasma samples were stored at +4 °C until assay. The concentration vs. time plot was used for the estimation of the pharmacokinetic parameters.

RESULTS

Quantification, separation and interferences

Chromatograms obtained from drug-free plasma, from drug-free plasma spiked with CMI, DCMI and IS and from a patient sample following administration of CMI are shown in Fig. 1. The retention times were ca. 2.1 min, 2.5 min and 5.2 min for CMI, IS and DCMI respectively. The three peaks were adequately resolved without any interference from endogenous compounds. Capacity factors were found to be $k'_1 = 2.00$, $k'_2 = 2.57$, $k'_3 = 6.43$ for CMI, IS and DCMI, respectively. No interfering peak was detected after extraction and injection into the LC system related to TCA or other drugs which could be coadministered with CMI (Table 1). As far as the metabolites of CMI are concerned, the resolution for DCMI is quite satisfactory, and both hydroxydesmethylclomipramine are not eluted within 30 min under the described conditions.

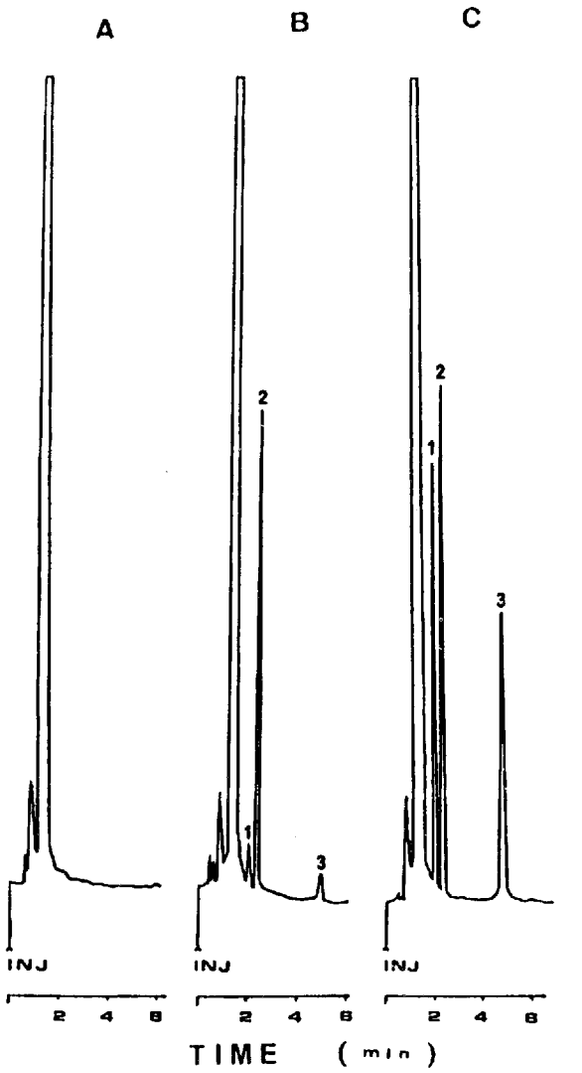

Figure 1. Chromatograms of (A) an extract of drug-free human plasma, (B) drug-free plasma spiked with 5 ng/mL each of CMI, DCMI and IS and (C) a plasma patient sample and the IS. Peak 1 is CMI (retention time: 2.1 min), peak 2 is imipramine (IS) (2.5 min) and peak 3 is DCMI (5.2 min).

Linearity, accuracy, recovery and sensitivity

The equations for the linear regression were for CMI and DCMI respectively: y_1 (peak height ratio) = 0.006877x (amount of CMI injected, ng) + 0.009188 (CV = 4.1%, $n = 10$, $r^2 = 0.9992$); y_2 (peak height ratio) = 0.003797x (amount of DCMI injected, ng) + 0.02948 (CV = 4.5%, $n = 10$, $r^2 = 0.9974$).

Precision has been estimated for both within-day and day-to-day variability (Table 2) as the coefficient of variation (CV) of the peak height ratio for three concentrations of CMI and DCMI. The extraction recovery determined by comparing the peak height ratios of the extracts with those obtained by direct injection of the same amount of drug was estimated at 90% and 85% for CMI and DCMI, respectively (three independent determinations).

The limits of detection under the previously described analytical procedures are 0.5 ng/mL for CMI and 2.0 ng/mL for DCMI (signal-to-noise ratio = 3, 0.001 aufs). The limit of quantification of the method under routine conditions with a CV < 10% is 2 ng/mL and 5 ng/mL for CMI and DCMI, respectively.

Table 1. Capacity factors (k') of some drugs tested for interference

Drug	k'	Drug	k'
Propericiazine	0.68	Alpidem	NE[a]
Haloperidol	1.06	Amineptine	NE
Viloxazine	1.25	Buspirone	NE
Trimipramine	1.34	Clobazam	NE
Levomepromazine	1.43	Clonazepam	NE
Alimemazine	1.48	Clorazepate, dipotassium salt	NE
Amitriptyline	1.81	Desmethyldiazepam	NE
Chlorpromazine	1.84	Diazepam	NE
Clomipramine	2.00	Flunitrazepam	NE
Imipramine	2.57	Hydroxyclomipramine	NE
Fluvoxamine	3.31	Hydroxydesmethylclomipramine	NE
Fluoxetine	4.22	Lorazepam	NE
Norfluoxetine	4.59	Nitrazepam	NE
Nortriptyline	5.44	Oxazepam	NE
Desmethylclomipramine	6.43	Zolpidem	NE
Desipramine	8.78	Zopiclone	NE

[a] NE = not eluted within 30 min.

Clinical application

A pharmacokinetic elimination curve was drawn for each patient; the individual pharmacokinetic data of 10 patients are shown in Table 3. The mean CMI elimination half-life calculated for the 10 patients is 13.7 h (SD = 2.9 h).

DISCUSSION

Numerous analytical methods have been proposed to measure CMI and DCMI concentrations in biological fluids (Scoggins *et al.*, 1980; Norman and Maguire, 1985; Balant-Gorgia *et al.*, 1991). Radioimmunoassays and immunoenzymatic assays take advantage of the relatively good sensitivity provided by immunological techniques (Flouvat, 1985). However, they lack specificity since the major metabolite DCMI is not quantified alone due to the cross-reactivity of antisera. Gas–liquid chromatographic methods with flame ionization detection have long been used for routine determination of steady-state concentrations of CMI and DCMI (Broadhurst *et al.*, 1977; Nyberg and Martensson, 1977). Their sensitivity has been improved by using derivatization procedures and/or nitrogen–phosphorus (Gifford *et al.*, 1975; Dawling and Braithwaite, 1978; Rovei *et al.*, 1980; Bredesen *et al.*, 1981; Ninci and Sgaragli, 1986; Balant-Gorgia *et al.*, 1989; Gupta *et al.*, 1983) or electron capture detectors (Biggs *et al.*, 1976; Jenkins and Fridel, 1978), but they become then time consuming. Benchtop gas chromatography–mass spectrometry (GC–MS) (Dubois *et al.*, 1976; Alfredsson *et al.*, 1977; Sioufi *et al.*, 1988) is probably suitable for routine analysis. However, GC–MS remains rather expensive as compared to a basic HPLC device. LC methods with UV detection have expanded because of various advantages: low limit of quantification (5 ng/mL or less), no derivatization step and good resolution especially for metabolites (Lagerström *et al.*, 1976; Mellström and Eksborg, 1976; Persson and Lagerström, 1976; Westenberg *et al.*, 1977; Proless *et al.*, 1978; Diquet *et al.*, 1982) and/or other TCA (Moyes and Moyes, 1977, 1980; Mellström and Tybring, 1977; Uges and Bouma, 1979; Godbillon and Gauron, 1981; Lagerström *et al.*, 1983; Sutfin *et al.*, 1984; Visser *et al.*, 1984). Two very sensitive LC assays with electrochemical detection which allow quantification down to 0.2 ng/mL have been published by Balant-Gorgia *et al.* (1986) and Spreux-Varoquaux *et al.* (1987).

The advantages of our technique are very good practicability and standard apparatus. Under the conditions we use (normal phase) the adsorbant phase activity is standardized, applying the solvent isohydricity theory developed by Thomas *et al.* (1977). Actually, in liquid–solid phase chromatography, the adsorbant phase activity is mainly dependent on the

Table 2. Precision of the analytical method

		Intraday ($n = 10$)		Day-to-day ($n = 10$)[a]	
	Antidepressant added (ng/mL)	Antidepressant found (ng/mL)	CV (%)	Antidepressant found (ng/mL)	CV (%)
CMI	10	10.4	3.2	10.6	3.5
	100	100.0	3.5	102.0	3.8
	300	304.0	2.0	299.0	2.9
DCMI	10	9.8	4.0	10.1	4.7
	100	101.0	4.9	97.0	3.5
	300	298.0	3.7	297.0	3.0

[a] Day-to-day assays were performed over a ten-week period using spiked human plasma samples.

Table 3. Patient plasma concentrations and individual pharmacokinetic data

Patient number	Sex	Age (year)	Weight (kg)	CMI C (max) (ng/mL)	CMI C (residual 24 h) (ng/mL)	$t_{1/2}$ (h)
1	F	36	61	134	14	12.2
2	F	27	61	146	23	15.2
3	F	33	55	132	34	14.9
4	F	28	52	195	36	18.5
5	F	37	77.5	135	9.5	9.7
6	M	30	86	108	32	18.3
7	F	56	67	240	47	11.7
8	M	28	67.5	102	19	12.9
9	F	58	57	125	17	12.2
10	M	27	68	99	15	11.7

percentage of water. Accordingly, Schlitt and Geiss (1972) recommended the addition of a small amount of water to the mobile phase in order to improve the analytical reproducibility (Diquet *et al.*, 1982). We were able to perform more than 1200 assays on the same column without any significant reduction of the assay quality.

Although 254 nm is not the maximum absorption wavelength for CMI, the lower background noise (due to reagents and plasma components) obtained under such conditions highly improves the signal-to-noise ratio and explains the high sensitivity of the method (0.5 ng/mL for CMI and 2 ng/mL for DCMI). It avoids most of the pitfalls of previously described techniques such as double extraction, thermostated column requirement and time consumption (Balant-Gorgia *et al.*, 1991). The limit of quantification is further improved, strongly reducing the sample volume required. The same method with few modifications (flow rate of the mobile phase) allows drug monitoring of numerous other TCA and their active metabolites. This technique can successfully be applied at 222 nm, the maximum wavelength of various TCA. With a slight modification of the calibration curve, i.e. by adding known amounts of CMI and DCMI to whole heparinized blood, the technique can be applied to blood determination. This point might be useful when the volume of the sample is smaller than 1 mL in clinical or in animal studies.

As an application, we determined the CMI pharmacokinetic parameters in a group of 10 depressed patients. These patients did not receive any other psychotropic drug except benzodiazepines during the study. After administration of CMI (75 mg intravenously), the residual concentrations at time 24 h were low (Table 3) which points out the necessity of a highly sensitive assay for the determination of pharmacokinetic parameters. The apparent elimination half-life was determined: 13.7 h (range: 9.7–18.5). These large interindividual variations of plasma levels and elimination half-lives have been reported by various authors but essentially after oral single dose administration: Dawlings *et al.* (1980) and Viala (1982) reported half-lives of 22–84 h and 15–40 h, respectively.

In this study, half-life determination is realized on a 24 h period because the last blood sample must be collected just before the next CMI infusion in order to adapt the dose. This short time collection period therefore artificially reduces the CMI half-life value, which probably takes into account the distribution phase. Moreover, owing to its slower elimination phase as compared to parent drug (elimination rate limited) the determination of DCMI kinetic parameters was not possible with sufficient precision.

Antidepressant clinical efficacy is tightly linked to obtaining CMI and DCMI plasma levels within a therapeutic range. Poor clinical improvement is reported when CMI and DCMI levels were found to be too low (i.e. <150 ng/mL) or too high (i.e. >450 ng/mL) (Diquet *et al.*, 1986; Vandel *et al.*, 1982; Balant-Gorgia *et al.*, 1989). Taken together with the important interindividual variability (Luscombe and John, 1980; Balant-Gorgia *et al.*, 1986), this suggests the advantage of being able to adapt the dose rapidly and individually. The availability of a rapid, selective and highly sensitive CMI assay should allow the prediction of such posology, by performing a single kinetic analysis after the first dose. Other applications, such as a paediatric monitoring (using microsamples), brain level determinations in rat or mouse (Varoquaux *et al.*, 1985) and chronopharmacological studies (Nakano and Hollister, 1983) are also interesting.

Acknowledgements

The authors thank Dr. M. Betes de Toro and Mr. G. Gaudel for suggestions and technical assistance.

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