ELSEVIER

Journal of Chromatography B, 772 (2002) 205–210

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

# Simultaneous determination of clomipramine and its desmethyl and hydroxy metabolites in plasma of patients by high-performance liquid chromatography after solid-phase extraction

R. Pirola[a], E. Mundo[b,1], L. Bellodi[b], S.R. Bareggi[a,*]

[a]*Department of Pharmacology, University of Milan, Via Vanvitelli 32, I-20129 Milan, Italy*
[b]*Department of Neuropsychiatric Sciences, San Raffaele Hospital, University of Milan School of Medicine, Via Prinetti 29, I-20127 Milan, Italy*

Received 4 July 2001; received in revised form 11 January 2002; accepted 6 February 2002

**Abstract**

Clomipramine (CMI) is a typical tricyclic antidepressant with a wide clinical spectrum, being used in major depressive, panic and obsessive-compulsive disorders. The relationship between clinical response and plasma levels of clomipramine and its N-desmethylated (*N*-desmethylclomipramine, DMCMI) and hydroxy-metabolites remains unclear. In particular, limited information is available on the correlation with clinical response in patients with obsessive-compulsive disorder (OCD). This study describes a new sensitive method to simultaneously determine CMI and its major N-desmethylated and hydroxy-metabolites present in human plasma by HPLC with a UV detector. After a solid-phase extraction from plasma (Isolute C2 columns) the separation of the compounds was performed on a Lichrospher CN column (250×4 mm, 5 μm with a 2-cm pre-column) by an eluent consisting of 10 m*M* $K_2HPO_4$–acetonitrile–methanol (35:25:40 v/v/v) at a flow of 1.5 ml/min. UV detector was set at 214 nm. The lower limit of quantification for all the analytes was at least 5 ng/ml. The coefficients of variation ranged between 2.0 and 4.9% with recovery rates between 97.0 and 100.3%. Linear regression analyses showed correlation coefficients between 0.98 and 0.99. This method is simple, fast and reliable with good specificity and sensitivity. Solid phase extraction is efficient and rapid, allowing the extraction of several plasma samples on the same day and may therefore be usefully and realistically applied in the clinical context. We thus investigated the relevance of plasma levels of CMI and its metabolites as a predictor of clinical outcome in a group of 15 patients with OCD. © 2002 Elsevier Science B.V. All rights reserved.

*Keywords:* Clomipramine; Desmethylclomipramine; Hydroxyclomipramine

## 1. Introduction

Clomipramine (CMI) is a typical tricyclic antidepressant with a wide clinical spectrum being used in major depressive, panic and obsessive-compulsive disorders (OCD). In spite of new atypical drugs such as those of the SSRI group (fluoxetine, fluvoxamine etc), clomipramine is still the reference compound in the treatment of these psychiatric disorders [1–3]. However, the relationship between clinical response and plasma levels of clomipramine and its N-des-

*Corresponding author.
*E-mail address:* sbareggi@mailserver.unimi.it (S.R. Bareggi).
[1]Present address: Centre for Addiction and Mental Health, R-30, University of Toronto, Toronto, ON M5T 1R8, Canada.

1570-0232/02/$ – see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S1570-0232(02)00089-2

methylated (*N*-desmethylclomipramine, DMCMI) and hydroxy-metabolites is still controversial: in particular, few studies have been carried out so far in OCD patients and the correlation with clinical response remains unclear [4–8]. This may be at least in part related to the fact that most studies measured only CMI and not DMCMI and/or hydroxy-metabolites. Several methods to determine CMI concentrations are currently available, only a few of them, however, can simultaneously determine its desmethyl and hydroxy-metabolites. Furthermore, this is accomplished by complex, expensive and time-consuming three-step liquid–liquid extraction [9,10] or by direct-injection methods which require more sophisticated equipment [11,12].

This study describes a new sensitive method capable of simultaneously determining CMI and its N-desmethylated and hydroxy-metabolites in human plasma by HPLC using a solid-phase extraction. The method was used to monitor the levels of CMI and its metabolites in a group of OCD patients.

## 2. Experimental

### 2.1. Chemicals and reagents

CMI, DMCMI, Maprotiline (internal standard, I.S.), were purchased from Sigma (St. Louis, MO, USA). 2-Hydroxyclomipramine, 2-HCMI, 2-hydroxydesmethylclomipramine and 8-hydroxydesmethylclomipramine, 2-HDMCMI and 8-HDMCMI, were kindly provided by Novartis (Basel, Switzerland). Acetonitrile and methanol were of HPLC-grade and the other reagents and solvents were of analytical grade (Merck, Darmstadt, Germany).

Isolute™ liquid–solid extraction columns packed with 100 mg of C2-bonded 40 μm silica with average pore size of 60 Å and with 1-ml reservoir (International Sorbent Technology, Mid Glamorgan, UK) were used to process plasma samples.

Stock solutions of drugs (1 mg/ml) in water were prepared weekly and stored at 4 °C protected from light. Standard working solutions at a concentration of 10 or 1 μg/ml (I.S. or compounds) were prepared daily immediately before use. Phosphate buffer was 50 m*M* potassium hydrogenphosphate pH 9.2.

### 2.2. Extraction of clomipramine and its metabolites from plasma

At the beginning of the analysis, three series of standard plasma samples spiked with low, medium and high concentrations of CMI and its metabolites (50, 100, 400 ng/ml of CMI and DMCMI; 25, 50, 100 ng/ml of the hydroxy-metabolites) and with the I.S. (quality control samples) were frozen at −25 °C and then one of each concentration level analyzed with every run of the unknown samples.

One ml of 50 m*M* phosphate buffer pH 9.2 and 0.1 ml of I.S. were added to 1 ml of plasma. After vortexing for 1 min and centrifuging for 10 min at 1500 *g*, the sample was applied to an Isolute C2 column placed on a Vac-Elut apparatus connected to vacuum. Before applying the sample, with the vacuum on, columns were washed with two column volumes of methanol, one volume of water and two volumes of 50 m*M* phosphate buffer pH 9.2. The vacuum was then turned off and, without allowing the column to dry, the sample was added to the column and the vacuum turned on. The columns were then washed with two volumes of 50 m*M* phosphate buffer and one volume of a mixture of water and acetonitrile (80:20 v/v). The columns were left to dry for 10 min and then the vacuum was turned off. A 6-ml conical centrifuge tube was placed in the VacElut rack under each column. The compounds were eluted with 1 ml of methanol with the vacuum turned on; 100 μl of the eluate were injected into the chromatograph.

Standard plasma samples were prepared by spiking 1 ml of drug-free plasma with known amounts of CMI, DMCMI, 2-HCMI, 2-HDMCMI and 8-HDMCMI and 1 μg of internal standard. Calibration curves were calculated by the integrator using linear regression analysis of the ratios of the area under the peak of CMI and its metabolites and those of I.S. versus CMI and metabolite concentrations in the standard samples and used to calculate the concentrations of CMI and its metabolites in unknown samples.

### 2.3. Liquid chromatography

HPLC analysis was carried out using a Shimadzu (Kyoto, Japan) system: LC-6A HPLC pump, SPD-

10A UV detector, SIL-6A autosampler and SCL-6A CR4-A controller integrator.

Wavelength was set at 214 nm. The column was a Lichrospher CN, 250×4 mm, 5 μm (Merck), with a 2-cm pre-column filled with the same material. The mobile phase was: 10 m*M* $K_2HPO_4$–acetonitrile–methanol (35:25:40 v/v/v). The flow was 1.5 ml/min.

In these conditions, peaks were eluted at the following times: CMI 4.5 min, DMCMI 8.0 min, 8-HCMI 4.0 min, 2-HDMCMI 6.6 min, 8-HDMCMI 7.2 min, MA (I.S.) 9.5 min.

### *2.4. Accuracy and precision*

Accuracy and precision were evaluated using values obtained from the analysis of five standard samples replicated 10 times on the same day (intra-day accuracy and precision) and from daily analyses of three quality control standard samples at three different concentrations for 5 days (inter-day accuracy and precision).

Accuracy was calculated as percentage of the measured versus the known concentrations. Precision was determined as the coefficient of variation (C.V.), i.e. the ratio between the mean of the observed concentrations and its standard deviation.

### *2.5. Recovery*

The absolute recovery of CMI and its metabolites from plasma samples was determined by comparing the areas of the peaks of standards not extracted with those of standards extracted according to the procedure described above.

### *2.6. Calculation of metabolic ratio for desmethylation and hydroxylation of CMI*

The main oxidative pathway of CMI is desmethylation to DMC, and plasma concentrations of CMI and HCMI are strongly dependent on N-desmethylation [13], which is regulated by different enzymes (CYP2C19, CYP3A4 and CYP1A2) from the one responsible for hydroxylation (CYP2D6) [14]. To minimize the effect of N-desmethylation in calculating the metabolic ratio, CMI and HCMI were not taken as independent parameters. Metabolic ratios (MR) were calculated as follows:

MR for N-desmethylation: MRD = CMI/DMCMI

MRs for hydroxylation: MR2H
= DMCMI/2HDMCMI and MR8H
= DMCMI/8HDMCMI

### *2.7. Patients*

A group of 15 DSM-III-R OCD patients responding to oral CMI who gave informed consent after full explanation of the experimental procedure were treated with oral CMI (150–300 mg/day) for 10 weeks [15]. Exclusion criteria were: the presence of major depressive, manic or hypomanic episodes, concomitant psychotherapy and psychotropic drug therapy with the exception of previously stabilized dosages of benzodiazepines. On week 10, blood samples were drawn in the morning 12 h after the last dose and before the morning administration to determine the levels of CMI and its metabolites. After centrifuging, plasma samples were stored frozen at −25 °C until analysis, which was carried out within 1 month. All patients obtained a reduction of the Yale–Brown Obsessive-Compulsive Scale (Y-BOCS) total score >35% and a CGI score at the "improvement" item <3 [15].

## 3. Results

No interfering peaks near the retention times of CMI, DMCMI, hydroxy-metabolites (8-HCMI, 2-HDMCMI, 8-HDMCMI) and I.S., were present in the chromatograms of blank or basal (time 0) plasma samples. Representative chromatograms are shown in Fig. 1. Psychotropic drugs most frequently used in OCD such as benzodiazepines, haloperidol and fluphenazine did not interfere with the analysis.

### *3.1. Linearity*

Six standard samples between 5 and 500 ng/ml (CMI), 5 and 500 ng/ml (DMCMI), 5 and 100 ng/ml (8-HCMI), 5 and 100 ng/ml (2-HDMCMI

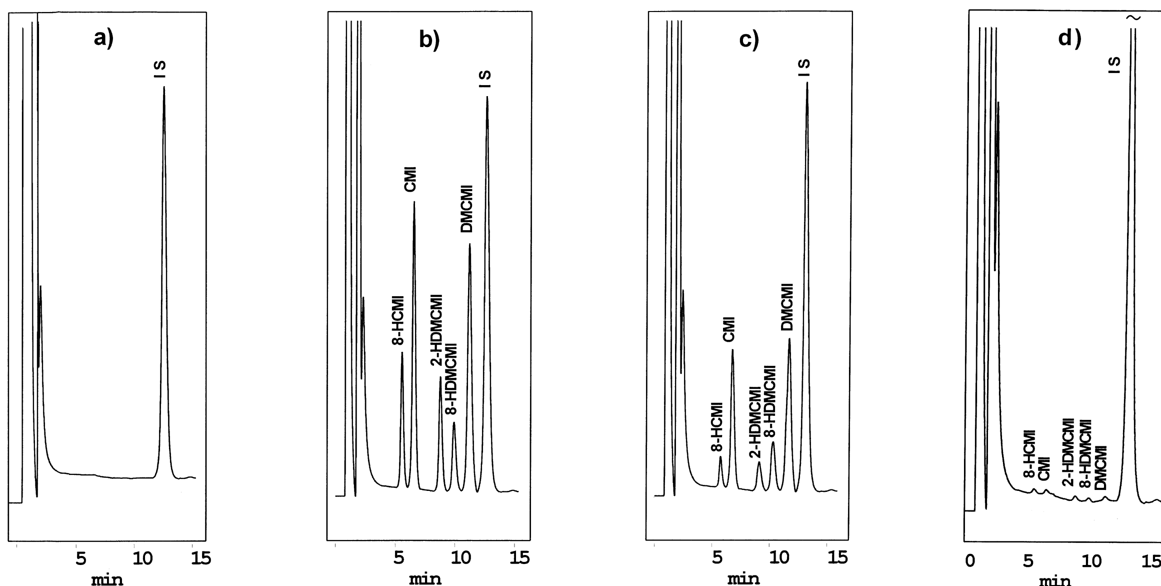


Fig. 1. (a) Plasma blank; (b) plasma standard: 2-HCMI 100 ng/ml, CMI 500 ng/ml, 2-HDMCMI 100 ng/ml, 8-HDMCMI 100 ng/ml, DMCMI 500 ng/ml, Maprotiline (1000 ng/ml, I.S.); (c) plasma sample: 2-HCMI 23.3 ng/ml, CMI 234.1 ng/ml, 2-HDMCMI 24.2 ng/ml, 8-HDMCMI 73.3 ng/ml, DMCMI 308.8 ng/ml; (d) CMI and metabolites 5 ng/ml, Maprotiline (I.S.) 1000 ng/ml.

and 8-HDMCMI) were analyzed in triplicate to determine the linearity of the assay.

The ratios of the areas of the peaks of each compound versus I.S. were linearly related to the concentrations in the range encountered in drug monitoring (CMI: $R = 0.03 + 1.02x$, $r^2 = 0.99$; DMCMI: $R = 0.01 + 0.89x$, $r^2 = 0.98$; 8-HCMI: $R = 0.02 + 0.49x$, $r^2 = 0.97$; 2-HDMCMI: $R = 0.03 + 0.49x$ $r^2 = 0.96$; 8-HDMCMI: $R = 0.01 + 0.70x$, $r^2 = 0.99$).

### 3.2. Accuracy and precision

The intra-assay coefficients of variation (C.V.s) of CMI and its metabolites were between 2.1 and 4.0% and the inter-assay C.V.s were between 2.2 and 4.9% for all compounds (Table 1). These results, therefore, validate the calibration curves used for each set of samples. Experimental results on the quality control samples kept at −25 °C (see inter-day accuracy above) guarantee the stability of the samples under our conditions.

### 3.3. Sensitivity and recovery

The lower limit of detection of CMI and its metabolites (i.e. with a signal three times that of the blank) were between 1 and 2 ng/ml. The lower limit of quantitation (C.V.<8%) of CMI and its metabolites was at least 5 ng/ml (Fig. 1).

The absolute recovery of CMI and its metabolites, determined by comparing the areas of the peaks of standards not extracted with those of standards extracted according to the procedure described above, was for all compounds higher than 97% (Table 1).

### 3.4. Steady-state concentrations of CMI and its metabolites

Concentrations of CMI and its metabolites in plasma of patients with OCD and the metabolic ratios for desmethylation and hydroxylation in human plasma at steady-state are shown in Table 2.

Table 1
Intra-day and inter-day accuracy for the assay of the CMI and its metabolites in plasma

| | Added concentrations (ng/ml) | Intra-assay precision $n=10$ | Inter-assay precision $n=6$ | Intra-assay accuracy (%) $n=10$ | Inter-assay accuracy (%) $n=6$ | Recovery (%)±S.D. $n=6$ |
|---|---|---|---|---|---|---|
| 8-HCMI | 25 | 4.2 | 2.8 | 100.3 | 99.8 | 99.7±6.2 |
| | 50 | 3.8 | 3.3 | 100.5 | 102.5 | 97.5±2.8 |
| | 100 | 4.0 | 4.1 | 97.9 | 103.1 | 99.1±3.5 |
| CMI | 50 | 4.0 | 2.7 | 98.9 | 101.1 | 99.7±1.9 |
| | 200 | 2.8 | 4.1 | 100.9 | 98.8 | 98.8±2.8 |
| | 400 | 3.5 | 3.2 | 100.6 | 100.5 | 99.6±2.5 |
| 2-HDMCMI | 25 | 2.9 | 4.0 | 97.8 | 97.7 | 98.9±5.1 |
| | 50 | 3.5 | 2.8 | 103.1 | 98.6 | 97.3±3.8 |
| | 100 | 2.3 | 4.4 | 100.6 | 99.3 | 97.5±4.2 |
| 8-HDMCMI | 25 | 3.7 | 3.8 | 98.7 | 97.8 | 97.0±0.5 |
| | 50 | 2.8 | 4.9 | 97.9 | 98.8 | 98.1±0.5 |
| | 100 | 3.5 | 3.5 | 99.6 | 102.3 | 97.8±0.5 |
| DMCMI | 50 | 2.0 | 2.5 | 102.5 | 99.3 | 100.3±3.0 |
| | 200 | 2.3 | 2.2 | 98.8 | 100.9 | 98.8±2.2 |
| | 400 | 2.1 | 2.6 | 101.1 | 99.8 | 99.5±1.7 |

## 4. Conclusions

The values of CMI and DMCMI concentrations are similar to those previously found with other methods [4–12]. Due to marked interindividual differences in hepatic metabolism, steady-state plasma concentrations of CMI, DMCMI and hydroxy-metabolites show a large variability among patients with standard therapeutic regimen; the few studies that calculated the MR values indicate that individual values have a large variation. This was the case also for our OCD patients, eight of them had higher levels of DMCMI relatively to CMI, whereas the others had equal or lower concentrations: our MR values are similar to those found by Szegedi et al. [16] and by Kramer Nielsen et al. [17] but lower than those of Shimoda et al. [9] in a Japanese population of psychiatric patients (MRD: 1.15±0.77; MRH: 2.26±2.19).

A larger number of samples is needed to observe clear correlations of drug or metabolite levels and clinical outcome. However, our female patients had higher concentrations of DMCMI, a lower metabolic rate for hydroxylation and a better response, particularly of the compulsion subscore of the Y-BOCS scale, as described elsewhere [18] and also previously reported [19]. This confirms the potential clinical usefulness of monitoring the levels of CMI and its desmethyl and hydroxy-metabolites in patients with OCD.

The HPLC method with solid-phase extraction employed in this study is simple, fast and reliable, with a good specificity and sensitivity. The methods presently available to simultaneously determine the desmethyl- and hydroxy-metabolites of CMI [9–12] are selective enough to avoid interference from other psychotropic drugs frequently co-administered in the treatment of major depression. In fact, the different drugs used in association with clomipramine to treat

Table 2
Steady-state plasma levels of CMI and its metabolites (ng/ml) and metabolic ratios (MRD, MR8H, see methods), in OCD patients (n=15; Mean (S.E.))

| Dose (mg/day) | 176.7 (8.3) |
|---|---|
| CMI | 133.9 (18.9) |
| DMCMI | 273.0 (45.0) |
| MRD | 0.63 (0.08) |
| 8-HCMI | 35.0 (4.2) |
| 8-HDMCMI | 64.6 (9.7) |
| MR8H | 5.4 (1.2) |

OCD patients did not interfere with the determination of CMI and its metabolites as performed in this study. We cannot exclude, however, that other compounds may interfere.

Compared to previous methods [9–12], our method has the advantage of efficient and rapid solid-phase extraction which allows the determination of CMI and its metabolites in several plasma samples in a few hours with higher sensitivity, better chromatographic resolution of the peaks, and without the need of more sophisticated equipment [11,12]. Solid-phase extraction is amenable to automated extraction as described for other psychotropic drugs [20] and, finally, can be used in association with other chromatographic methods beside HPLC.

This method can therefore be usefully and realistically applied to clinical studies investigating the relevance of plasma levels of CMI and its metabolites in the prediction of clinical outcome.

## References


[1] T.R. Insel, J. Zohar, in: H.Y. Meltzer (Ed.), Psychopharmacology: The Third Generation of Progress, Raven Press, New York, 1987, p. 1205.

[2] J. Zohar, T.R. Insel, J. Affect. Disord. 13 (1987) 193.

[3] P. Thoren, M. Asberg, L. Bertilsson, B. Mellstrom, F. Sjoqvist, L. Traskman, Arch. Gen. Psychiatry 37 (1980) 1289.

[4] R.S. Stern, I.M. Marks, D. Mawson, D.K. Luscombe, Br. J. Psychiatry 136 (1980) 161.

[5] T.R. Insel, D.L. Murphy, R.M. Cohen, I. Alterman, C. Kilts, M. Linnoila, Arch. Gen. Psychiatry 40 (1983) 605.

[6] T. Noguchi, K. Shimoda, S. Takahashi, J. Affect. Disord. 29 (1993) 267.

[7] Y. Kasvikis, I.M. Marks, Psychopharmacology 95 (1988) 113.

[8] M.R. Mavissakalian, B. Jones, S. Olson, J.M. Perel, J. Clin. Psychopharmacol. 10 (1990) 261.

[9] K. Shimoda, T. Minowada, T. Noguchi, S. Takahashi, J. Clin. Psychopharmacol. 13 (1993) 181.

[10] K. Kramer Nielsen, K. Brosen, Ther. Drug Monit. 15 (1993) 122.

[11] M. Banger, B. Hermes, S. Härtter, C. Hiemke, Pharmacopsychiatry 30 (1997) 128.

[12] H. Weigmann, S. Härtter, C. Hiemke, J. Chromatogr. B 710 (1998) 227.

[13] A.E. Balant-Giorgia, L.P. Balant, C. Genet, P. Dayer, J.M.O. Aeschlimann, G. Garrone, Eur. J. Clin. Pharmacol. 31 (1986) 449.

[14] K. Brosen, S.H. Sindrup, E. Skjelbo, K.K. Nielsen, L.F. Gram, Psychopharmacol. Ser. 10 (1993) 199.

[15] E. Mundo, S.R. Bareggi, R. Pirola, L. Bellodi, E. Smeraldi, J. Clin. Psychopharmacol. 17 (1997) 4.

[16] A. Szegedi, H. Wetzel, M. Leal, S. Härtter, C. Hiemke, J. Clin. Psychiatry 57 (1996) 257.

[17] the Danish University Antidepressant Group, K. Kramer Nielsen, K. Brosen, L. Gram, Eur. J. Clin. Pharmacol. 43 (1992) 405.

[18] E. Mundo, R. Pirola, L. Bellodi, E. Smeraldi, S.R. Bareggi, J. Clin. Psychopharmacol. (2002) in press.

[19] E. Mundo, S.R. Bareggi, R. Pirola, L. Bellodi, Biol. Psychiatry 45 (1999) 290.

[20] K.K. Åkerman, J. Jolkkonen, M. Parviainen, I. Penttilä, Clin. Chem. 42 (1996) 1412.