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Short communication

# Automated determination of clomipramine and its major metabolites in human and rat serum by high-performance liquid chromatography with on-line column-switching

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**Abstract**

A fully automated method including column-switching and isocratic high-performance liquid chromatography (HPLC) was developed for simultaneous determination of the tricyclic antidepressant clomipramine and its metabolites demethylclomipramine, 2-, 8-, and 10-hydroxyclomipramine, 2-, and 8-hydroxydemethylclomipramine and didemethylclomipramine in serum. After serum injection into the HPLC system and on-line sample clean-up on a clean-up column (Hypersil CN; 10×4.6 mm) by an eluent consisting of 35% acetonitrile and 65% deionized water, the chromatographic separation was performed on an analytical column (LiChrospher CN; 250×4.6 mm I.D.) by an eluent consisting of 38% acetonitrile and 62% aqueous sodium perchlorate (0.02 *M*, pH 2.5). The UV detector was set at 260 nm. The limit of quantification was about 15 ng/ml for all analytes. The coefficients of variation ranged between 3 and 12% with recovery rates between 64 and 110%. Linear regression analyses revealed coefficients of correlation between 0.98 and 0.99. The method could be applied to therapeutic drug monitoring as well as metabolism studies in man and rat. 

*Keywords:* Clomipramine

## 1. Introduction

Clomipramine, a tricyclic tertiary amine has been applied for the therapy of depression and obsessive compulsive disorders. Monitoring serum or plasma concentrations is helpful to improve the response and minimize the occurrence of side effects.

Most methods for the determination of antidepressants in human serum focus on the parent compound. Demethylated and hydroxylated metabolites of clomipramine, however, are pharmacologically active too, and their activities are different from that of clomipramine itself [1,2]. It has been reported that clinical outcome, as well as side effects, can be correlated to the hydroxylated metabolites of clomipramine [3,4].

Gas chromatography (GC) or high-performance liquid chromatography (HPLC) methods have been developed to measure clomipramine and several metabolites in biological fluids (for a review see Ref. [5]). So far, there is only one published method that determines clomipramine, demethylclomipramine, 2-, 8-, and 10-hydroxyclomipramine, 2-, 8-, and 10-hydroxydemethylclomipramine, didemethylclomi-

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pramine and clomipramine N-oxide simultaneously [6]. It uses laborious, expensive and time consuming three-step liquid–liquid extraction as sample pretreatment.

The aim of this study was to establish a fully automated HPLC method, that enables the quantitative analysis of clomipramine and its major metabolites for drug disposition studies.

## 2. Experimental

### 2.1. Chemicals

Clomipramine, demethylclomipramine, 2-, 8-, and 10-hydroxyclomipramine, 2-, and 8-hydroxydemethylclomipramine, and didemethylclomipramine were kindly supplied by Novartis (Basel, Switzerland). Acetonitrile (HPLC grade) and sodium perchlorate monohydrate (p.a.) were obtained from Merck (Darmstadt, Germany). Water was deionized and filtered through a Milli-Q water processing system (Millipore, Eschborn, Germany).

### 2.2. Standards

Stock solutions were prepared by dissolving 10 mg of substance in 10 ml methanol each. They were diluted with deionized water and mixed with drug free plasma from healthy volunteers to obtain calibration standards of free bases at six different concentrations each. The hydroxylated metabolites and didemethylclomipramine were used at final concentrations of 25, 50, 100, 200, 400 and 800 ng/ml, clomipramine at final concentrations of 50, 100, 200, 400, 800 and 1600 ng/ml and demethylclomipramine at final concentrations of 100, 200, 400, 800, 1600 and 3200 ng/ml. All standards could be stored in the dark at −20°C for several months without measurable decomposition.

### 2.3. Plasma or serum samples

Human plasma or serum samples were obtained from healthy nontreated volunteers or from patients treated with clomipramine. Patients' blood for preparation of serum was collected in the morning immediately before the first daily dose.

Male Sprague–Dawley rats received five oral doses of clomipramine 20 mg/kg every 4 h. After decapitation, truncal blood was collected for preparation of serum.

Serum samples could be stored frozen at −20°C for several months without measurable decomposition. When stored for 24 h at room temperature no change in concentrations of analytes could be observed.

### 2.4. Instrumentation

The HPLC system consisted of a CMA/200 autosampler (CMA/Microdialysis, Stockholm, Sweden), an electric six-port switching valve (Besta, Wilhelmsfeld, Germany) coupled to the autosampler and two HPLC pumps. One HPLC pump (Constametric III, LDC Analytical, Gelnhausen, Germany) was used for loading plasma or serum onto the clean-up column and subsequent washing. A second pump (Type 2200, Bischoff, Leonberg, Germany) was used to pump the analytical mobile phase through the analytical column. A variable wavelength ultraviolet detector, type SPD-10A (Shimadzu, Kyoto, Japan) was used to monitor absorption at two different wavelengths (214 and 260 nm) simultaneously. Quantitative evaluation of chromatograms was performed at 260 nm using a Kontron chromatogram integration software PCIP (Kontron, Milan, Italy).

The analytical column (250×4.6 mm I.D.) was packed with LiChrospher CN (5 μm particle size) by MZ-Analysentechnik (Mainz, Germany). The clean-up column (10×4.6 mm) was filled with 10 μm particles of cyanopropyl (CN) bonded material, Hypersil CN (ICT, Frankfurt, Germany)

### 2.5. Chromatographic procedure

Sample clean-up and chromatographic separation were performed at room temperature.

#### 2.5.1. 0–5 min

After recentrifugation of serum (10 000 *g* for 5 min), 100 μl of the supernatant was injected onto the clean-up column. Proteins and other interfering compounds were washed to waste by using deionized

water containing 35% (v/v) acetonitrile at a flow-rate of 1.5 ml/min.

*2.5.2. 5–8 min*

After the electric six-port valve had been switched at 5 min, the analytical run was started. The analytes to be determined were eluted onto the analytical column (back flush) and separated by the analytical mobile phase (second HPLC pump) consisting of 38% acetonitrile and 62% sodium perchlorate solution (0.02 *M*), adjusted to pH 2.5 by $HClO_4$ at a flow-rate of 1.5 ml/min.

*2.5.3. 8–30 min*

Three min after the start of the analytical run (at 8 min) the switching valve was reset.

*2.5.4. 30–60 min*

After each analytical run 100 μl of a 50% (v/v) aqueous sodium perchlorate (0.1 *M*), containing 50% acetonitrile was injected instead of a sample to rinse the clean-up column. The clean-up column was replaced after injection of 20–30 serum or plasma samples.

*2.6. Interferences*

To control for possible interferences with drugs that are frequently used in combination with clomipramine, the suggested interfering compounds were dissolved in water to obtain two different concentrations (100, 1000 ng/ml).

*2.7. Calculations*

The peak heights obtained from spiked serum or plasma, containing known amounts of drugs, were subjected to weighted ($1/y^2$) linear regression analysis for the calculation of correlation coefficients, slopes and intercepts. Drug concentrations in samples containing unknown amounts of drug were calculated on the basis of the computed regression lines.

*2.8. Precision, accuracy and recovery*

Precision and accuracy were evaluated by six replicate analyses of quality-control samples within one series to asses intraassay variability and on six different days to evaluate interassay variability. Precision was calculated as coefficients of variation, while accuracy was determined from the difference between nominal and determined concentrations.

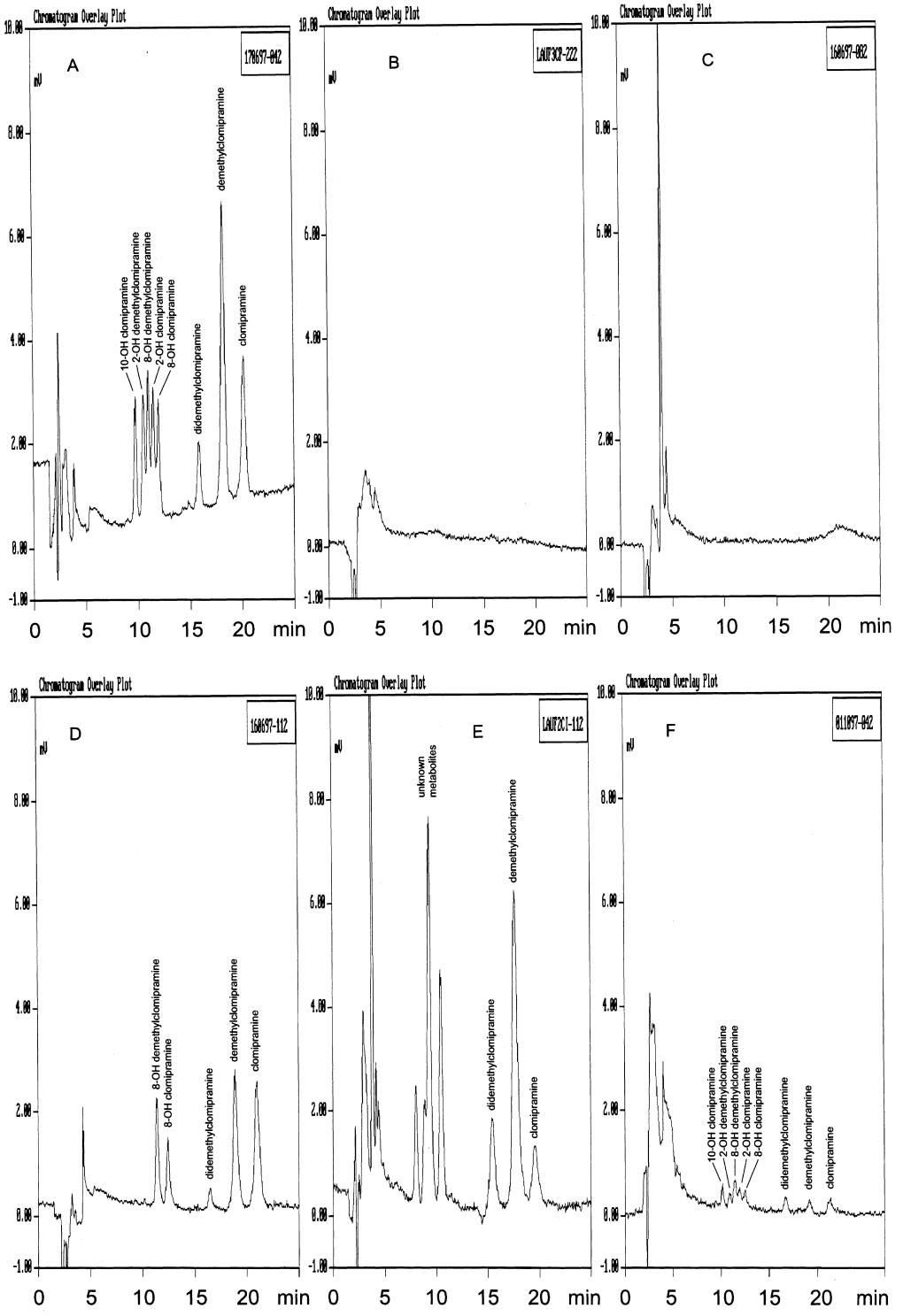
Recovery was determined for each analyte at two different concentrations by comparing peak heights of the analytes from calibrated plasma samples after clean-up, using column-switching with peak heights of analytes from standard analytical eluent solutions of the same concentration injected directly onto the analytical column.

## 3. Results and discussion

Column-switching techniques are most useful for automated and rapid analysis of drugs in complex matrices [7–15]. In addition to a conventional isocratic HPLC system, only a precolumn for sample clean-up, a second HPLC pump and a six-port switching valve are needed. The procedure can be easily automated and urgent samples can be analysed within 1 h.

There are many methods for the determination of clomipramine and demethylclomipramine by GC or HPLC (for review see Ref. [5]) but to our knowledge only three methods [1,16,17] allow the simultaneous determination of hydroxylated metabolites. Only one method is able to measure 2-, 8-, and 10-hydroxy-metabolites [6]. The latter uses a time consuming three-step liquid–liquid extraction as sample pretreatment which is difficult to automate.

We injected human or rat serum directly into the system. Separation could be obtained by HPLC for all compounds to be determined within less than 25 min on a CN column (Fig. 1A). Baseline separation could not be reached for 8-hydroxy and 2-hydroxy metabolites when determined simultaneously, but in patients treated with clomipramine only 8-hydroxy metabolites were observed besides demethylclomipramine and didemethylclomipramine. These compounds could be well separated (Fig. 1D). To reach a similar quality for the separation of hydroxylated metabolites of clomipramine on a reversed-phase $C_{18}$ column, about 40 min were required by others [18]. For clomipramine the retention time is twice as long as in our described method (Fig. 1A, B, D).


Chromatogram Overlay Plot
A
178697-042
10-OH clomipramine
2-OH demethylclomipramine
8-OH demethylclomipramine
2-OH clomipramine
8-OH clomipramine
didemethylclomipramine
demethylclomipramine
clomipramine
B
LAUF302-222
C
168697-082
D
168697-112
8-OH demethylclomipramine
8-OH clomipramine
didemethylclomipramine
demethylclomipramine
clomipramine
E
LAUF201-112
unknown metabolites
didemethylclomipramine
demethylclomipramine
clomipramine
F
811897-042
10-OH clomipramine
2-OH demethylclomipramine
8-OH demethylclomipramine
2-OH clomipramine
8-OH clomipramine
didemethylclomipramine
demethylclomipramine
clomipramine
mV
min

In serum of rats treated with clomipramine hydroxylated metabolites could not be detected. Peaks appearing between 7 and 11 min were unlikely to be hydroxylated metabolites of clomipramine since their absorption spectra at 214 and 260 nm differed from those of the reference compounds. The failure to detect 2-, 8- or 10-hydroxylated metabolites was inconsistent with the results from in vitro incubations of clomipramine and rat microsomes [18]. In vitro, hydroxylated metabolites are found [18]. These hydroxylated metabolites might have been eliminated by fast phase II reactions, and did thus not appear in rat serum at concentrations above the detection limit.

In sample pretreatment of serum or plasma samples with column-switching techniques, the portion of organic solutions in the clean-up eluent usually did not exceed 20% to prevent protein precipitation. In the described method, however, we used 35% acetonitrile solution as clean-up eluent, since, at lower concentrations of acetonitrile, interfering peaks occurred. Nevertheless, the clean-up column had not to be changed more frequently than with a clean-up eluent containing lower acetonitrile concentrations.

Analysing blank serum samples at 214 nm, a peak was detected, coeluting with 2-hydroxyclomipramine. Since the interfering peak showed no absorption at 260 nm, we used 260 nm for routine determination.

Intra-assay and inter-assay variabilities ranged between 3 and 12%. The inaccuracies were between 1 and 15%, coefficients of variation between 4 and 12%. Recoveries ranged between 64 and 110% (Table 1). This was similar to those reported by others [6,16,17]. A precision below 15% was considered acceptable for therapeutic drug monitoring, since they were in the range recommended for determination of antiepileptics according to legal requirements [19,20].

At concentrations of 15 ng/ml (Fig. 1F) the analytes showed intraassay variabilities between 3 and 32% (Table 1). They were high for the hydroxylated metabolites in mixtures containing analytes that were not separated to baseline (Fig. 1A). In serum of patients treated with clomipramine, however, only the 8-hydroxylated metabolites were found (Fig. 1A). Therefore the intra-assay variabilities were always below 10% when analysing patients' samples. Under these routine conditions quantification of all analytes was possible down to 15 ng/ml.

When analysing blank plasma samples spiked with 50 to 1600 ng/ml of clomipramine, 100 to 3200 ng/ml of demethylclomipramine 25 to 800 ng/ml of didemethylclomipramine and of each of the hydroxylated metabolites of clomipramine, the detector responses were linear for all substances. Linear regression analyses revealed acceptable coefficients of correlation between 0.98 and 0.99 for all analytes except didemethylclomipramine with a coefficient of correlation of 0.95.

When testing standard solutions containing other psychotropic drugs that may be applied in combination with clomipramine, no interferences could be observed with clomipramine or demethylclomipramine. The latter are usually measured for therapeutic drug monitoring (for review see Ref. [5]). Clozapine and its metabolite clozapine N-oxide showed similar retention times as 8-hydroxyclomipramine and 8-hydroxydemethylclomipramine, respectively. The combination of clomipramine and clozapine however, is most rare in clinical practice because of cardiotoxic side effects. *levo*-Mepromazine, perazine and haloperidol interfered with

Fig. 1. Representative chromatograms of serum or plasma samples analysed by the described method for clomipramine and metabolites (detection at 260 nm): (A) Blank human plasma sample spiked with 100 ng/ml of hydroxylated clomipramine and demethylclomipramine metabolites, 100 ng/ml of didemethylclomipramine, 400 ng/ml of demethylclomipramine and 200 ng/ml of clomipramine; (B) Plasma of a nontreated healthy subject; (C) Serum of a nontreated rat; (D) Serum obtained from a patient receiving 225 mg/day clomipramine orally. In this sample the serum concentrations were 139 ng/ml for 8-OH demethylclomipramine, 120 ng/ml for 8-OH clomipramine, 35 ng/ml for didemethylclomipramine, 212 ng/ml for demethylclomipramine and 204 ng/ml for clomipramine; (E) Serum obtained from a rat receiving clomipramine 20 mg/kg orally, five times, every 4 h, 2 h after the last dose. In this sample the serum concentrations were 82 ng/ml for didemethylclomipramine, 409 ng/ml for demethylclomipramine and 101 ng/ml for clomipramine. The other peaks with retention times between about 7 and 11 min are not likely to represent hydroxylated metabolites of clomipramine, as suggested from a comparison between different absorptions at 214 and 260 nm suggests; (F) Blank human plasma sample spiked with 15 ng/ml of all analytes.

Table 1
Precision, accuracy and recovery for the determination of clomipramine and metabolites by the described procedure

| Analyte | Nominal concentration (ng/ml) | Intra-assay variability (%) | Inter-assay variability (%) | Intra-assay inaccuracy (%) | Inter-assay inaccuracy (%) | Recovery±S.D. (%) |
|---|---|---|---|---|---|---|
| Clomipramine | 15 | | 8.5 | | −4.4 | |
| | 200 | 5.0 | 5.7 | −1.6 | +2.3 | 88±2.7 |
| | 800 | 5.8 | 5.1 | −4.3 | −8.0 | 82±4.1 |
| Demethylclomipramine | 15 | | 9.3 | | −3.3 | |
| | 400 | 9.0 | 11.9 | +9.3 | +6.0 | 86±8.7 |
| | 1600 | 6.7 | 5.6 | −15.1 | −10.5 | 69±3.8 |
| Didemethylclomipramine | 15 | | 13.7 | | −11.0 | |
| | 100 | 9.2 | 6.4 | +1.2 | +3.9 | 73±4.6 |
| | 400 | 4.5 | 5.2 | −7.4 | −8.5 | 68±3.6 |
| 8-OH Clomipramine | 15 | | 13.4 | | −23.3 | |
| | 100 | 6.8 | 3.0 | +3.3 | +8.3 | 86±2.6 |
| | 400 | 8.7 | 4.8 | −3.4 | −11.0 | 80±3.8 |
| 2-OH Clomipramine | 15 | | 32.0 | | −6.0 | |
| | 100 | 5.7 | 5.6 | −1.5 | +7.3 | 83±4.7 |
| | 400 | 12.1 | 5.2 | −1.2 | −10.7 | 83±4.3 |
| 8-OH Demethylclomipramine | 15 | | 30.5 | | −2.6 | |
| | 100 | 4.2 | 4.3 | 2.9 | +6.7 | 85±3.7 |
| | 400 | 7.7 | 4.0 | −6.5 | −8.4 | 76±3.1 |
| 2-OH Demethylclomipramine | 15 | | 24.2 | | +2.6 | |
| | 100 | 7.2 | 9.0 | −2.6 | +2.7 | 64±5.8 |
| | 400 | 8.7 | 6.6 | +0.9 | −8.5 | 64±4.2 |
| 10-OH Clomipramine | 15 | | 3.2 | | +9.5 | |
| | 100 | 7.4 | 10.7 | −2.9 | +3.8 | 102±10.9 |
| | 400 | 9.0 | 6.4 | +1.9 | −7.7 | 110±7.1 |

S.D.=Standard deviation.
Clomipramine and metabolites were evaluated by six replicate analyses of quality-control samples within one series to assess intra-assay variability and on six different days to determine inter-assay variability.
Precision was calculated as the percentage of coefficients of variation, while accuracy was determined from the difference between nominal concentrations and concentrations determined.
Recovery was determined for each analyte by comparing peak heights of the analytes from quality-control samples after clean-up, by means of column-switching, with peak heights of analytes from standard analytical eluent solutions of the same concentration injected directly onto the analytical column.

the detection of didemethylclomipramine. To separate *levo*-mepromazine or perazine and didemethylclomipramine, the acetonitrile content of the analytical eluent should be reduced from 38 to at least 35%.

The method described here offers the possibility of analysing samples automatically. Batchwise analysis, which is usual for routine procedures that include off-line preextractions, is therefore not necessary for our procedure. Using the described method, clomipramine and seven metabolites can be analysed in human, as well as rat serum, simultaneously. Results can be obtained within 1 h.

In conclusion, the fully automated column-switching HPLC method described here seems advantageous over other methods reported so far in literature. The described method has sufficient accuracy and precision. It may be applied not only for therapeutic drug monitoring of clomipramine plus demethylclomipramine, but also to hydroxylated clomipramine metabolites suggested to play a role for clinical outcome and/or side effects [2–4].

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