Measurement of Clomipramine, N-Desmethylclomipramine, Imipramine, and Dehydroimipramine in Biological Fluids by Selective Ion Monitoring, and Pharmacokinetics of Clomipramine

Jean-Pierre Dubois, Werner Küng, Walter Theobald, and Bernard Wirz

To quantitatively determine tricyclic antidepressant agents, we used a combined gas chromatograph/mass spectrometer system, and deuterium-labeled internal standards. Recovery exceeds 95% and the coefficient of variation is less than 4% for human whole-blood samples supplemented with 5 to 15 ng of clomipramine hydrochloride or 20 to 60 ng of dehydroimipramine hydrogen fumarate per milliliter. For both amines, the detection limit is 0.3 µg/liter. Six healthy volunteers who received a single oral dose of 50 mg of clomipramine hydrochloride showed peak drug concentrations in the blood 3 to 5 h after administration, ranging between 14.4 and 30.1 µg/liter. Plasma/whole blood concentration ratios varied from 0.70 to 1.20, and the cumulative renal elimination from 0 to 72 h is less than 0.2% of the dose. This method is suitable for in vivo bioavailability studies of unchanged clomipramine, dehydroimipramine, and imipramine after a single oral dose of as little as 25 mg.

Additional Keyphrases: whole blood • urine • gas chromatography/mass spectrometry • monitoring tricyclic antidepressants

Clomipramine, 3-chloro-5-[3-(dimethylamino)-propyl]-10,11-dihydro-5H-dibenz[b,f]azepine-hydrochloride, the active ingredient in “Anafranil” (Geigy, Switzerland), and imipramine, 5-[3-(dimethylamino)-propyl]-10,11-dihydro-5H-dibenz[b,f]azepine-hydrochloride, the active ingredient in “Tofranil” (Geigy, Switzerland), are well-known antidepressant agents.

Different methods for measuring clomipramine and imipramine in biological fluids have been reported: fluorescence (1, 2), thin-layer chromatography/densitometry (3–5), radioimmunoassay (6), gas chromatography (7, 8), and gas chromatography/mass spectrometry (9–11), but only the last one of these methods is sensitive and specific enough for monitoring concentrations of the unchanged active compound in whole blood after a single oral dose of 50 or even 25 mg of a tricyclic antidepressant agent.

We describe here a gas chromatographic/mass spectrometric method for quantitatively determining clomipramine, desmethylicomipramine, imipramine, or dehydroimipramine in 1 ml of human whole blood, down to a concentration of 0.3 µg/liter. Deuterium-labeled internal standards are added to the biological sample before the extraction process. Urine samples have been analyzed in the same way.

Materials and Methods

Instrument

Analyses were done with a Model 9000 combined gas chromatograph/mass spectrometer system (LKB Producter AB, S-161 25 Stockholm-Bromma 1, Sweden) equipped with a mass marker and an accelerating voltage alternator from LKB, and a Speedomax G Model S millivolt high-speed recorder (Leeds & Northrup, North Wales, Pa. 19454). The chromatograph was fitted with 2.1-m glass columns, 3 mm i.d., packed with 3% OV-101 on 80–100 mesh Gas Chrom Q for clomipramine and desmethylicomipramine determinations, and 3% OV-17 on 80–100 mesh Supelcoport from Supelco, Bellefonte, Pa., for imipramine and dehydroimipramine determinations. Helium flow was 30 ml/min. Operating temperatures were: flash heater, 270 °C; column, 270 °C; molecular separator, 285 °C; ion source, 310 °C. The trap current was 60 µA, and the electron energy 30 eV. Entrance slit width was 0.4 mm, collector slit width, 0.6 mm. The accelerating voltage alternator was adjusted to monitor the intensities of two molecular ions: clomipramine (m/e = 314) and D₄-clomipramine containing one 37Cl atom (m/e = 320); the pentafluoropropyl derivatives of desmethylicomipramine (m/e = 446) and D₅-desmethylicomipramine containing one 37Cl atom (m/e = 453); imipramine (m/e
Fig. 1. Synthesis of D₂-imipramine (II) and D₂-dehydroimipramine (mixture of isomers VI and VII)

= 280) and D₂-imipramine (m/e = 282); dehydroimipramine (m/e = 278) and D₂-dehydroimipramine (m/e = 280). The third channel of the accelerating voltage alternator was used to control the stability of the magnetic field strength.

Reagents

All chemicals were of analytical grade and were specially tested for purity with blank runs.

Synthesis of the Deuterium-Labeled Internal Standards

The reaction pathway for the preparation of the deuterium-labeled internal standards is outlined in Figures 1 and 2.

D₂-imipramine (II) carrying the deuterium atoms at one benzyl position of the azepine ring, was obtained by reduction of 5-[3-(dimethylamino)-propyl]-10,11-dihydro-5H-diben[b,f]azepin-10-one (I) with lithium aluminum deuteride.

D₂-Dehydroimipramine, D₄-clomipramine, and D₅-desmethylocimipramine were labeled with the deuterium atoms attached to the amino-propyl side chain.

D₂-Dehydroimipramine (mixture of isomers VI and VII) was obtained by reduction of 3-dimethylamino-propanoic acid methyl ester III with lithium aluminum deuteride, conversion of the resulting 3-dimethylamino-propanol IV to the chloride V, which was used as alkylating reagent in the N-alkylation of 5H-diben[b,f]azepin, the deuterium label of the resulting product is distributed in equal amounts to the 1- and 3-position of the propyl side chain, as shown by nuclear magnetic resonance and mass spectrometry. Confirmation of the specific deuterium labeling in the intermediate chloride V has also been established by nuclear magnetic resonance and mass spectrometry. The rearrangement of the deuterium atoms can be rationalized assuming the formation of an azetidinium intermediate VIII, followed by displacement on either bond "a" or bond "b" of VIII during the alkylation reaction (12), yielding the two isomers VI and VII. Solomon et al. (13) have reported the preparation of deuterated promazine, specifically labeled in either the 1-, 2-, or 3-position of the propyl side chain, by condensation of the corresponding 3-dimethylamino-propyl-4-toluenesulfonates with phenothiazine. No evidence is given, however, for the specific labeling of the resulting products.

D₄- and D₅-Clomipramine (XXIII and XVIII). Di-deutero- and tetradeutero-3-iodopropanoic acid XVIII and XII were prepared by reaction of D₄-ethyleneglycol IX to ethylene bromohydrin X and hydracrylonitrile XI, and hydrolysis of the latter with hydroiodic acid or deuteroiodic acid, respectively. After methylation of the two 3-iodopropanoic acids and reaction of the esters XIX and XIII with dimethylamine, the same reaction pathway was followed as described above for dehydroimipramine. During substitution of tetradeutero-3-iodopropanoic acid methyl ester XIII with nondeuterated dimethylamine, one deuterium atom was lost, because of the elimination-addition mechanism of this substitution (14).

D₅-Desmethylocimipramine (XXV) was obtained after reaction of D₅-clomipramine XVII with ethyl chlorofomate and hydrolysis of the carbamic acid ester XXIV.

Extraction Procedure

At pH values exceeding 10, the amines are extracted in high yield with n-heptane/isooamy alcohol (99/1 by vol); at pH values below 2, the amines can be re-extracted from the solvent.
Extraction scheme: Mix 0.5 ml of a 200 μg/liter solution of the internal standard in 10 mmol/liter HCl with 1 ml of the biological fluid. Add 1 ml of 2 mol/liter Na2CO3 and 4.5 ml of n-heptane/isoamyl alcohol (99/1 by vol). Agitate for 15 min and centrifuge for 10 min at 1100 × g. Remove the n-heptane phase and extract into 0.5 ml of 50 mmol/liter H2SO4. Discard the organic phase and add 0.3 ml of 1 mol/liter NaOH and 1.6 ml of n-heptane. Remove the n-heptane phase and evaporate under nitrogen. Redissolve the residue in 6 to 10 μl of pyridine. The sample is now ready for analysis.

If the concentration of the sample lies outside of the calibration range, as for urine samples, better results are obtained by adding more internal standard to 1 ml of the liquid and diluting this mixture to a concentration that is within the calibration range than by adding the usual amount of internal standard solution to a diluted biological sample.

Derivatization of Desmethylclomipramine

Desmethylclomipramine reacts with pentafluoropropionic anhydride at 30 °C to give the monopentafluoropropyl derivative. At 40 °C, the principal reaction product is a β,γ-unsaturated bis(pentafluoropropyl) derivative, which corresponds to the bis(tri-fluoroacety1) derivative of desipramine described by Walle et al. (15). At 60 °C even clomipramine reacts with pentafluoropropionic anhydride to form a β,γ-unsaturated monopentafluoropropyl derivative (Figure 3).

Desmethylclomipramine can also be derivatized in the n-heptane phase (concentration about 100 ng in 1.6 ml of n-heptane) by adding 20 μl of dimethylformamide, 20 μl of pyridine, and 100 μl of pentafluoropropionic anhydride to the n-heptane phase and heating at 60 °C for 1 h. The n-heptane phase is then washed with 1.5 ml of a water/methanol mixture (2/1 by vol) and evaporated. This method has been reported for the formation of the heptafluorobutyl derivative of desmethylnortriptyline by Borgå et al. (16).

![Fig. 3. Principle reaction products of desmethyl-clomipramine and clomipramine with pentafluoropropionic anhydride at different temperatures](image)

![Fig. 4. Calibration curves for clomipramine hydrochloride with D₄-clomipramine hydrochloride as internal standard](image)

Table 1. Calibration Curves for Dehydroimipramine-C₇H₁₁O₄, Imipramine-HCl, and Desmethylclomipramine-HCl (Pentafluoropropyl Derivative)³

<table>
<thead>
<tr>
<th>Substance and internal standard</th>
<th>Calibration points</th>
<th>N</th>
<th>Least-squares line Y = mX + b</th>
<th>Sᵧ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dehydroimipramine-C₇H₁₁O₄ and D₄-Dehydroimipramine-C₇H₁₁O₄</td>
<td>A) 0/20; 5/20; 10/20; 15/20</td>
<td>8</td>
<td>Y = 0.0454X + 0.023</td>
<td>0.0070</td>
</tr>
<tr>
<td></td>
<td>B) 0/100; 20/100; 40/100; 60/100</td>
<td>14</td>
<td>Y = 0.00906X + 0.028</td>
<td>0.0060</td>
</tr>
<tr>
<td></td>
<td>Scaling factor 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imipramine-HCl and D₄-Imipramine-HCl</td>
<td>A) 0/12; 1/12; 2/12; 4/12; 6/12</td>
<td>10</td>
<td>Y = 0.0824X + 0.028</td>
<td>0.0035</td>
</tr>
<tr>
<td></td>
<td>B) 0/120; 20/120; 40/120; 60/120</td>
<td>14</td>
<td>Y = 0.00841X + 0.024</td>
<td>0.0035</td>
</tr>
<tr>
<td></td>
<td>Scaling factor 10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desmethylclomipramine·HCl and D₄-Desmethylclomipramine·HCl (pentafluoropropyl derivative)</td>
<td>A) 0/20; 0.5/20; 1/20; 1.5/20; 2/20</td>
<td>10</td>
<td>Y = 0.138X + 0.053</td>
<td>0.0051</td>
</tr>
<tr>
<td></td>
<td>B) 0/100; 5/100; 10/100</td>
<td>6</td>
<td>Y = 0.0270X + 0.046</td>
<td>0.0051</td>
</tr>
</tbody>
</table>

³ A) Direct injection of salt-mixtures in pyridine (a/b means: a ng substance + b ng internal standard injected). B) Injection of about 1/5 of the extract of 1 ml of drug-supplemented human whole blood (a/b means: a ng substance + b ng internal standard in 1 ml of human whole blood).
Quantitative Evaluation

For quantitation, the peak-height ratio of the mass spectrometer response at the specific masses of the molecular ions of the substance to be analyzed and of the internal standard is compared with the calibration curve, which is prepared with each assay. With use of deuterium-labeled internal standards, equivalent calibration curves can be obtained by directly injecting mixtures of the salts of the substance to be analyzed and of the internal standard, or by injecting a portion of the extract of 1 ml of drug-supplemented samples of human whole blood.

The solutions containing 1 mg of labeled or nonlabeled clomipramine hydrochloride, imipramine hydrochloride, or dehydroimipramine hydrogen fumarate in 10 ml of pyridine are stable for a year at 4 °C. Therefore, we directly injected mixtures of these solutions for calibration and used the supplemented human whole blood samples, which were also run with each assay, for precision and accuracy documentation.

Results

Method

Figure 4 shows the calibration curves for clomipramine hydrochloride by direct injection of salt mixtures in pyridine (A) and by injection of a portion of the extract from 1 ml of drug-supplemented samples of human whole blood (B). Each least-squares line is characterized by three parameters: the slope m, which is influenced by a proportional error, the y-intercept b, which is influenced by a constant error, and the standard error of estimate S_y, which quantitates the random error (17, 18).

The parameters m, b, and S_y of the calibration curves of dehydroimipramine hydrogen fumarate, imipramine hydrochloride, and the pentafluoropropyl derivatives of desmethylclomipramine are listed in Table 1. Day-to-day precision and recovery for clomipramine hydrochloride and dehydroimipramine hydrogen fumarate obtained from repeated analyses of 1 ml of drug-supplemented human whole blood are given in Tables 2 and 3. For both amines, the detection limit, equal to twice the standard deviation of the day-to-day precision of the samples supplemented with the lowest amount of drug, is 0.3 μg/liter.

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Table 2. Precision and Recovery for Clomipramine-HCl Determinations in 1-ml Samples of Supplemented Human Whole Blood Samples

<table>
<thead>
<tr>
<th>Added, ng</th>
<th>4.8</th>
<th>9.6</th>
<th>14.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Found mean, ng</td>
<td>4.6</td>
<td>9.3</td>
<td>14.2</td>
</tr>
<tr>
<td>SD, ng</td>
<td>0.16</td>
<td>0.31</td>
<td>0.48</td>
</tr>
<tr>
<td>CV, %</td>
<td>3.5</td>
<td>3.3</td>
<td>3.4</td>
</tr>
<tr>
<td>% recovery</td>
<td>95.8</td>
<td>96.9</td>
<td>98.6</td>
</tr>
</tbody>
</table>

*Single determinations of each of the three concentrations done on twelve different days (n = 12).

Table 3. Precision and Recovery for Dehydroimipramine-C₆H₁₂O₂ Determinations in 1-ml Sample of Supplemented Human Whole Blood

<table>
<thead>
<tr>
<th>Added, ng</th>
<th>2.0</th>
<th>20.0</th>
<th>40.0</th>
<th>60.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Found mean, ng</td>
<td>2.0</td>
<td>19.9</td>
<td>39.8</td>
<td>60.2</td>
</tr>
<tr>
<td>SD, ng</td>
<td>0.16</td>
<td>0.47</td>
<td>1.56</td>
<td>0.93</td>
</tr>
<tr>
<td>CV, %</td>
<td>8.0</td>
<td>2.4</td>
<td>3.9</td>
<td>1.5</td>
</tr>
<tr>
<td>% recovery</td>
<td>100.0</td>
<td>99.5</td>
<td>99.5</td>
<td>100.3</td>
</tr>
</tbody>
</table>

*Single determinations of each of the three concentrations, 20.0, 40.0, and 60.0 ng, done on 12 different days (n = 12). For 2.0 ng, n = 10.

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Fig. 5. A and B: Concentrations of unchanged clomipramine hydrochloride (ng/ml) in whole blood after a single oral dose of 50 mg of clomipramine hydrochloride (two 25-mg "Anafranil" sugar-coated tablets) to six healthy volunteers.
Pharmacokinetic Study with Clomipramine in Man

In a clinical study, six healthy volunteers received a single oral dose of 50 mg of clomipramine hydrochloride in the form of two 25-mg "Anafranil" commercial sugar-coated tablets. Whole-blood samples were drawn just before and 0.5, 1, 2, 3, 5, 7, 10, 24, 34, 48, and 72 h after administration, and urine was collected before and during the time intervals 0 to 24, 24 to 48, and 48 to 72 h. All these samples were kept frozen at -20 °C and analyzed within four weeks.

Figure 5 shows the whole blood concentrations of unchanged clomipramine hydrochloride, Table 4 contains the areas under the whole blood concentration curves from 0 to 72 h and the plasma/whole blood concentration ratios, and Figure 6 shows the cumulative renal elimination from 0 to 72 h for each of the six healthy volunteers.

Discussion

Gas Chromatograph/Mass Spectrometer System

Excellent day-to-day precision of the gas chromatograph/mass spectrometer system has been found by directly injecting salt-mixtures of the substance and the internal standard in pyridine. The coefficient of variation of the slope m of these calibration curves was less than 1%

For imipramine and dehydroimipramine, where internal standards labeled with two deuterium atoms were used, the calibration range for a given quantity of internal standard is limited because of the natural 13C content of the nonlabeled material (19). To avoid a significant contribution of the substance to the signal on the specific mass of the internal standard, an excess of internal standard must be added to the biological sample. The excess of deuterium-labeled internal standard serves also as a carrier to minimize losses during the extraction procedure.

Drug-Supplemented Samples of Human Whole Blood

Comparison of the calibration curves obtained by injecting the extracts of drug-supplemented samples or by directly injecting salt-mixtures in pyridine permits adequate differentiation between random, constant, and proportional errors.

Random error. Equal values for the standard error of estimate $S_e$ have been found for both types of calibration curves.

Constant error. Impurities contained in the solvent used before the evaporation may cause a constant error, indicated by a parallel shift of the least-squares line. Such constant errors can be eliminated by running blanks and zero values with each assay.

Proportional errors, indicated by a different slope of the least-squares line, have frequently been observed during the elaboration of this method; they are due to problems in preparation of solutions of the tricyclic antidepressant agents. Up to 20% of the substance have been lost when we prepared solutions containing 200 μg of substance per liter of water, ethanol, or n-heptane; for solutions in 10 mmol/liter HCl, these losses were less than 5%. The best results, however, were obtained by directly dissolving the substance in human whole blood. One-milliliter portions stored at -20 °C showed an analytical recovery of about 96% after two months and of about 90% after four months.

Pharmacokinetic Study with Clomipramine in Man

The rapid passage of the clomipramine to the body tissues and metabolism of the major part of the drug are the reasons for the low concentrations of unchanged clomipramine hydrochloride found in biological fluids (20). Nevertheless, the whole blood concentrations observed from 0 to 48 h after administration of a single oral dose of 50 mg of clomipramine hydrochloride to six healthy volunteers are two- to 100-fold the detection limit of the method, and the six areas under the whole

| Table 4. Area under the Concentration Curve for Whole Blood after a Single Oral Dose of 50 mg of Clomipramine-HCl Given to Six Healthy Volunteers, and Plasma/Whole Blood Concentration Ratio |
|---|---|---|
| Volunteer | Body weight [kg] | Area under concn curve from 0 to 72 h [ng ml⁻¹ * hours] | Concentration ratio (Plasma/Whole blood) | Mean value (N = 4) | SD |
| 1 | 70 | 330.7 | 1.00 | 0.14 |
| 2 | 77 | 330.7 | 1.14 | 0.06 |
| 3 | 76 | 295.9 | 0.70 | 0.08 |
| 4 | 70 | 276.9 | 0.83 | 0.10 |
| 5 | 79 | 521.3 | 1.20 | 0.13 |
| 6 | 64 | 316.8 | 1.04 | 0.12 |

* Measured 2, 5, 7, and 24 h after administration.

Fig. 6. Cumulative renal elimination of unchanged clomipramine hydrochloride (μg) after a single oral dose of 50 mg clomipramine hydrochloride given to six healthy volunteers.
blood concentration curves differ by a factor of less than two.

The peak blood concentrations of unchanged clomipramine hydrochloride range between 14.4 and 30.1 μg/liter and the cumulative renal elimination from 0 to 72 h varies between 33.0 and 77.8 μg, corresponding to 0.07% and 0.16% of the dose. These results accord with previously reported studies on the pharmacokinetics of clomipramine (20, 21) and demonstrate the possibility to study in vivo the bioavailability of unchanged clomipramine hydrochloride in human whole blood after a single oral dose of 50 or even 25 mg with the gas chromatography/mass spectrometry method when a deuterium-labeled internal standard is used.

We thank Mr. R. Ackermann for skillful technical assistance.

References