Liquid-Chromatographic Determination of Amitriptyline and Its Metabolites in Serum, with Adsorption onto Glass Minimized

Peter M. Edelbroek, Ed J.M. de Haas, and Frederik A. de Wolff

To study correlations between the concentrations, in serum, of amitriptyline and its most important metabolites with clinical response in patients, we developed a "high-performance" liquid-chromatographic method for routine determination of amitriptyline, nortriptyline, total 10-hydroxyamitriptyline, desmethylnortriptyline, and E(trans)- and Z(cis)-10-hydroxyamitriptyline. These compounds are extracted from 1 mL of alkalized serum into hexane/isooamyl alcohol (99/1 by vol). Perazine is the internal standard. To minimize irreversible adsorption of the drugs onto the glassware, 5 µg of maprotiline is added to the organic phase just before evaporation. After a 10-min resolution on a silica column eluted with acetonitrile/methanol/NH₄OH (1 mol/L), absorbance is measured at 240 nm. Only chlorazipramine, doxepin, procainamide, and N-acetylpromoxinamide may interfere with assay of the compounds that probably are pharmacologically relevant: amitriptyline, nortriptyline, and E-10-hydroxyamitriptyline. Uremia, lipemia, and icterus also do not affect the analysis.

Additional Keyphrases: drug assay • analytical error • sample handling • monitoring therapy • tricyclic antidepressants • dosage-compliance test

The tricyclic compound amitriptyline (AT)⁴ is widely used to treat mental depression. It is extensively metabolized (f), mainly by N-demethylation, hydroxylation, and N-oxidation (Figure 1).

Results from animal studies (2, 3) indicate that not only are AT and NT active, but also their 10-hydroxy metabolites may have antidepressive activity in man.

Methods for assay of AT and other tricyclic antidepressants were recently reviewed (4, 5). In only one gas-chromatographic-mass spectrometric method (6) and a few HPLC methods (7–9) could AT and NT and its hydroxylated metabolites be determined. Moreover, the procedures reported (6–9) are laborious and incomplete; e.g., drug interferences were not tested. For several tricyclics, irreversible adsorption onto glass has been described. Efforts (4, 5, 10–13) to prevent adsorption—such as silanization and siliconizing of glassware, replacement by plastic, pretreatment of glassware with alkylamides or alkyl alcohols, or addition of these reagents to the solution for evaporation—gave variable results. To assess correlation between concentrations of AT and its most important metabolites in serum with clinical response in patients, we developed an HPLC method for determination of the drug and its metabolites, including a simple and effective procedure to minimize irreversible adsorption of the drugs onto the glassware.

Materials and Methods

Drugs and reagents. All chemicals were of analytical grade. Hexane, isooamyl alcohol, methanol, sodium hydroxide, and ammonium hydroxide were purchased from E. Merck AG, Darmstadt, F.R.G.; acetonitrile (ChromAR grade) from Mallinckrodt Inc., Paris, KY 40361. AT, HC1, NT, AT-NO, Z-10-OH-AT, Z-10-OH-NT, Z-10-OH-NT, and DNT were kindly supplied by Lundbeck & Co., Amsterdam, The Netherlands. Perazine dimaleate was a gift from Bijk, Zwartenburg, and maprotiline from Ciba-Geigy, Arnhem, The Netherlands.

We prepared stock solutions of AT, its metabolites, and perazine dimaleate in ethanol to give a concentration of 1 g/L in terms of the free base. Maprotiline was as reported, 2.5 g/L solution in methanol. Serum standards of 25, 50, 100, 200, and 300 µg/L were made up by adding AT, NT, Z-10-OH-AT, DNT, E-10-OH-NT, and Z-10-OH-NT to drug-free pooled serum, then stored at –20 °C in 1-mL aliquots in conical glass tubes with Teflon-lined screw-caps.

Glassware. The tubes and their caps were washed with laboratory detergent (Liquinox; Alconox Inc., New York, NY 10003), cleaned with dichromate-sulfuric acid, rinsed with tap water and distilled water, and dried.

For collecting blood samples and concentrating the extract, we used disposable conical glass tubes without further cleaning.

Apparatus. The chromatograph used was a laboratory-assembled instrument consisting of: a pump of reciprocating piston-type with flow feedback control (Model 740; Spectra Physics, Mountain View, CA 94042), an ultraviolet detector with wavelength variable (GM 770; Schoeffel, Westwood, NJ 07675), and a syringe-loading sample injector with a 175-µL loop and variable injection volume (Model 7105; Rhodyne Cotati, CA 94928). For all separations we used a 10 × 0.3 cm column, packed in our laboratory with 5-µm (av. particle di-

---


Fig. 1. Pathways of amitriptyline metabolism

ameter) silica (Lichrosorb SI60, E. Merck AG) by a slurry technique (14). The column was eluted at ambient temperature with a solvent system consisting of acetonitrile/methanol/NH₄OH (1 mol/L), 250/55/13 by vol, at 1.2 mL/min. Absorbance of the eluate was measured at 240 nm, at a detector sensitivity of 0.01 A full-scale, by a flat-bed recorder (Kipp BD 8), with a measuring range of 20 mV and a chart speed of 0.5 cm/min.

Patients. Depressed outpatients received 150 mg of AT (Sarotex; Lundbeck & Co., Denmark) daily for three months. Patients being co-medicated with enzyme-inducing agents or with other antidepressants or phenothiazines were excluded from the trial. At days 0, 2, and 7 and weeks 3, 6, 9, and 13 after this treatment was begun, blood was sampled from patients into disposable glass tubes 3 h after the morning dose. We avoided using Vacutainer tubes, as recommended by Scoggins et al. (4). Serum was promptly separated, frozen, and stored at −20 °C until analyzed.

A urine specimen collected by the patient in the morning was analyzed for riboflavin as a compliance check, riboflavin having been incorporated into the medication formulation.

We developed a simple, quick “high-performance” thin-layer chromatographic method to differentiate riboflavin from interfering background fluorescence: 25 μL of urine is spotted directly on an HPTLC-plate with concentrating zone (Kieselgel 60 F 254, 10 × 20 cm, cat. no. 13728; E. Merck AG). The plate is developed in butanol/ethanol/H₂O (70/20/10 by vol) until the front is 5 cm from the concentrating zone, dried at 60 °C, and viewed under ultraviolet light at 366 nm. The RF of riboflavin is 0.36; the detection limit is approximately 0.05 μg per spot.

Procedure. To 1 mL of serum (standard or patient) in tubes with Teflon-lined screw-caps, add with an automatic pipettor (Clinipette; Labora Mannheim, F.R.G.) 200 μL of 1.5 mol/L NaOH and vortex-mix for 5 s. Add 6 mL of hexane/isoamyl-alcohol (99/1 by vol) and 200 μL of the internal standard (1.0 mg of perazine dimalonate per liter of ethanol) with an automatic pipettor (Capilettor, Labora Mannheim), mix the contents of the tubes on a roller mixer at 60 rpm for 30 min, and centrifuge for 4 min at 3000 × g. Transfer the organic (upper) phase with a Pasteur pipette into a clean conical tube and add 20 μL of maprotiline (2.5 g/L, in methanol). After vortex-mixing for 5 s, evaporate quickly at 40 °C in a water bath, under a stream of nitrogen. Dissolve the residue in 50 μL of eluent and immediately inject 20 μL of the solution.

Construct calibration curves of the peak-height ratios of amitriptyline and its metabolites to perazine (the internal standard) vs the concentration of amitriptyline and its metabolites. For every series of assays, a new calibration curve must be prepared.

Results

Assay Characteristics

Chromatographic conditions. The solvent system described suitably resolved AT and its metabolites (Table 1).

Detection at 240 nm is optimal for AT, NT, DNT, and the

<table>
<thead>
<tr>
<th>Table 1. Retention Times of Amitriptyline and Its Metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>AT</td>
</tr>
<tr>
<td>E/Z-10-OH-AT</td>
</tr>
<tr>
<td>DNT</td>
</tr>
<tr>
<td>Perazine (inter. std.)</td>
</tr>
<tr>
<td>NT</td>
</tr>
<tr>
<td>E-10-OH-NT</td>
</tr>
<tr>
<td>AT-NO</td>
</tr>
<tr>
<td>Z-10-OH-NT</td>
</tr>
</tbody>
</table>

* See text footnote 1.
10-hydroxy components. Changing the acetonitrile/methanol ratio strongly influenced the selectivity of the system, because methanol has better proton-acceptor properties than acetonitrile. The efficiency of our columns was insufficient to separate Z- and E-10-OH-AT; therefore, we chose to use an acetonitrile/methanol ratio that would not separate E- and Z-10-OH-AT, both to facilitate measurement and because, for our purposes, their resolution was not necessary. These isomers are extracted with equivalent efficiency and their molar absorptivities at 240 nm differ by less than 5%, so the combined peak is a valid reflection of total 10-OH-AT.

Under our conditions we do not expect any serious interference of AT-NO with E-10-OH-NT in the therapeutic range. The polar metabolite AT-NO is poorly extracted (Table 2) and has a 20-fold lower molar absorptivity than E-10-OH-NT in the mobile phase at 240 nm. Figure 2 shows the chromatograms of a standard mixture, an extract of drug-free serum, and an extract of the serum of a patient receiving AT. Under our conditions we saw no rapid deterioration of the column, and hundreds of serum extracts could be analyzed without problems. When starting with a new column, stabilize it with the mobile phase for at least one day; the resolution of 10-OH-AT and DNT is incomplete with newly made columns.

**Extraction procedure.** Irreversible adsorption onto the glass surface after extraction from alkalinized serum with hexane/isooamyl alcohol (99/1) gave artifactually low results for control sera, and nonlinear calibration curves for AT and its metabolites. Especially after evaporation, this variable loss was evident, DNT being the most extensively adsorbed compound.

Testing with NT, we tried several proposed methods (4, 5, 10, 13) for minimizing adsorption of tricyclic antidepressants. Silanization of the glassware with dichlorodimethylsilane, silanization with Silicaid (Clay Adams, Parsippany, NJ), treatment of the glassware with triethylamine in methanol (200 mL/L), removal of traces of heavy metals and then silanizing (15), and coating the glassware with glycidoxypropyltrimethoxysilane were all ineffective.

To solve this problem, we tried adding an excess of a structurally related compound to compete with AT and its metabolites for the adsorptive sites on the glass surface. Use of β-naphthylamine decreased adsorption but interfered with AT and its metabolites in the chromatogram. The tricyclic protriptyline and the tetracyclic maprotiline were also effective in reducing adsorption. We chose the latter because its peak appears immediately after the last peak (E-10-OH-NT) in the chromatogram.

Addition of a 100-fold excess of maprotiline (with respect to AT) just before the evaporation after the extraction with hexane/isooamyl alcohol resulted in reproducible calibration curves and maximum recoveries. For optimum results, however, it is desirable to minimize evaporation time and to dissolve the residue in the eluent just before injection, even though maprotiline is also present in it.

To quantify the effectiveness of maprotiline in minimizing adsorption of AT and its metabolites onto the glass surface, we compared the results of analysis, with and without added maprotiline, of 30 serum samples from patients who were receiving AT. Extraction with maprotiline present gave significantly higher concentrations for AT, NT, total 10-OH-AT (p <0.00001), and E-10-OH-NT (p <0.0004) than

---

**Table 2. Analytical Recovery and Precision of the Method for Amitriptyline and Its Metabolites**

<table>
<thead>
<tr>
<th>Recovery, % a</th>
<th>Within-day precision b</th>
<th>Day-to-day precision b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean, µg/L</td>
<td>CV, %</td>
<td>n</td>
</tr>
<tr>
<td>AT c</td>
<td>98.8</td>
<td>33.9</td>
</tr>
<tr>
<td>E-10-OH-AT</td>
<td>94.7</td>
<td>—</td>
</tr>
<tr>
<td>Z-10-OH-AT</td>
<td>94.5</td>
<td>28.6</td>
</tr>
<tr>
<td>DNT</td>
<td>53.2</td>
<td>33.5</td>
</tr>
<tr>
<td>Perazine</td>
<td>90.7</td>
<td>—</td>
</tr>
<tr>
<td>NT</td>
<td>89.1</td>
<td>30.3</td>
</tr>
<tr>
<td>E-10-OH-NT</td>
<td>74.3</td>
<td>31.2</td>
</tr>
<tr>
<td>AT-NO</td>
<td>5.5</td>
<td>—</td>
</tr>
<tr>
<td>Z-10-OH-NT</td>
<td>77.3</td>
<td>28.5</td>
</tr>
</tbody>
</table>

* a Mean of three extractions each of sera supplemented with 25, 50, 100, 200, or 300 µg of drug per liter. b As determined with two standards, 30 and 150 µg/L, for each drug. c See text footnote 1.
without it (two-sided t-test for the mean difference of paired data).

**Analytical Variables**

*Linearity.* The linear regression data listed in Table 3 are representative of calibration curves for AT and its metabolites in the range from 25 to 300 \(\mu\)g/L. These curves were obtained by plotting the ratios of the peak-height of each compound to that of the internal standard, perazine, for different concentrations. This linear range can be extended to 2500 \(\mu\)g/L.

*Recovery and precision.* Table 2 shows the analytical recoveries and precision for AT, NT, DNT, and the hydroxylated compounds. The recovery of each drug was constant and independent of the concentration of the drug over the range 25 to 300 \(\mu\)g/L.

Within-day and day-to-day precision were determined by assaying two serum standards, one supplemented with 50 \(\mu\)g and the other with 150 \(\mu\)g of AT, Z-10-OH-AT, DNT, NT, E-10-OH-NT, and Z-10-OH-NT per liter.

*Interferences.* We tested 83 drugs and metabolites for possible interference with the measured substances (Table 4). Such interference can be expected when the relative retention times of two peaks with comparable peak heights differ by less than about 5%. As is shown in this Table, 12 drugs and metabolites appear in the chromatogram, but only four of them actually interfere with AT, NT, or E-10-OH-NT.

We observed no interfering peaks after extracting uremic, lipemic, and icteric sera.

*Sensitivity.* The detection limit, determined as the concentration of the compound necessary to produce a signal three times stronger than the background noise under the condition of the assay, is 5 \(\mu\)g/L for AT and 10-0H-AT; 10 \(\mu\)g/L for DNT, NT, and 10-OH-NT; and 2.5 mg/L for AT-NO in serum.

**Observations in Patients**

Results of our study of outpatients treated with AT will be published elsewhere in detail. Figure 3 shows the concentration/time curves of AT and its metabolites in two representative patients.

An intermediate evaluation of the results of the clinical study with AT in our hospital showed that steady-state concentrations of AT, NT, and E-10-OH-NT differed widely among 14 patients. At 13 weeks after starting therapy with AT, the mean concentrations (and range) were (\(\mu\)g/L): AT, 108.1 (29.7–188.2); NT, 97.5 (40.4–205.9); and E-10-OH-NT, 108.9 (20.4–191.6). The correlation between steady-state concentrations of AT or NT and E-10-OH-NT in serum was not significant (\(p > 0.10\)), but it was significant between AT and NT (\(r = 0.79\); \(p < 0.01\)).

Mean concentrations (and range) of Z-10-OH-NT and total 10-OH-AT were 19.1 (9.6–28.5) and 18.5 (<5–40.2) \(\mu\)g/L, respectively.

There was no correlation between the E- and Z-isomer concentration of 10-OH-NT (\(r = –0.0053\)).

Steady-state DNT concentrations in patients, not shown in Figure 3, were usually near background values (<15 \(\mu\)g/L).

**Discussion**

The liquid–liquid extraction procedures for tricyclic drugs generally involve either one or three steps.

With the three-step method it is possible to omit the evaporation step after extracting alkalized serum by back-extracting with an aqueous acid phase (11, 13, 16). This procedure not only removes most acidic and neutral interfering substances, it also improves the reproducibility of the assay.
However, this method is laborious and extraction recovery of the drug is poorer.

The one-step method necessarily involves concentration by evaporation, and special care is necessary to prevent adsorption.

On comparing the described methods (4, 5, 10-13) for preventing adsorption with our method of maprotiline addition, we found all of them to be ineffective or inferior to our method. We expect that the principle of adding an excess of a structurally related tri- or tetracyclic in the prevention of adsorption is not only applicable for AT but also for other tricyclics. However, this procedure does not preclude adsorption of the drugs during collection and storage of patients' samples and during the preparation of serum standards. We did not add the excess of maprotiline to the serum instead of the extraction solvent, because we would then expect a low extraction recovery of AT and its metabolites.

The procedure presented here is sufficiently accurate, precise, and sensitive for reliable measurements of concentrations of AT, total 10-OH-AT, NT, Z-10-OH-NT, and E-10-OH-NT in human serum, and it permits therapeutic drug monitoring of AT and its active metabolites.

Analysis for DNT is unreliable as a result of incomplete extraction and high variability. However, the concentrations of DNT in the serum of patients seem to be very low. Analysis for AT-NO is not possible because of poor extraction recovery and a high detection limit.

Møller-Jensen (17) successfully extracted AT-NO with dichloromethane from alkalized aqueous serum after hexane extraction.

For our purpose, measurement of DNT and AT-NO is probably not required, or even desirable, because animal studies (2, 3) lead us to expect that these two metabolites have no considerable antidepressant activity.

Our observations in patients show that, of the hydroxylated metabolites, only E-10-OH-NT reaches high concentrations in serum and must certainly be taken into account in studies correlating concentrations in serum and clinical response, especially because we found no linear correlation between steady-state concentrations in serum of AT or NT and E-10-OH-NT and because the latter may be assumed to have antidepressant activity (2, 3).

The lack of a correlation between NT and E-10-OH-NT in serum is not in agreement with the findings of Bertilsson et al. (2) in an uncontrolled study in which AT or NT was given to patients.

We did not find any correlation between Z-10-OH-NT and E-10-OH-NT concentrations. Recently Mellström et al. (18) suggested that different enzymatic mechanisms are possibly involved in the hydroxylation of NT in E- and Z-10-OH-NT. They selected eight healthy subjects (who were phenotyped with a debrisoquine hydroxylation test) to cover a wide range in the ratio between debrisoquine and 4-hydroxydebrisoquine in the urine. After a single oral dose of NT the metabolic clearance by 10-hydroxylation in the E-position, but not in the Z-position, correlated closely with the metabolic ratio.
We thank Professor Dr. H. G. M. Rooymans and his staff, Dept. of Psychiatry, University Hospital of Leiden, for their cooperation in the clinical part of this study, and gratefully acknowledge the valuable suggestions by Dr. Ubbo R. Tjaden, Dept. of Analytical Chemistry and Pharmaceutical Analysis, University of Leiden. Professor H. de Jonge, Dept. of Medical Statistics, University Hospital of Leiden, performed the statistical analyses. We also thank Mr. B. Muller, Lundbeck & Co., Amsterdam, for the supply of Sarotex coated tablets with riboflavin.

References