High-Performance Liquid-Chromatographic Simultaneous Determination of Commonly Used Tricyclic Antidepressants

Henning F. Proelss,¹ Herman J. Lohmann,¹ and Daniel G. Miles²

We report a method for simultaneous measurement of five commonly used tricyclic antidepressant drugs (doxepin, desipramine, nortriptyline, imipramine, and amitriptyline) in serum by paired-ion high-performance liquid chromatography, with use of a reversed-phase column and ultraviolet detection at 254 nm. The drugs are extracted from 2 ml of serum at pH 14 into hexane/isoamyl alcohol (99/1 by vol) and re-extracted into 200 µl of 0.1 mol/liter HCl. An aliquot of the aqueous acid phase is chromatographed with use of a methanol/acetonitrile/water (41/15/44) solvent system, containing 5 mmol of pentanesulfonic acid per liter of phosphate buffer (0.1 mol/liter, pH 6.5), at a flow rate of 1.5 ml/min. Analytical recoveries of the drugs from serum increase with increasing concentration, from 62% at 25 µg/liter to 93% at 300 µg/liter. Linear response is observed for drug concentrations up to 1500 µg/liter and the detection limit is 2-3 µg/liter. Within-run precision ranges from 1.4 to 2.9% and day-to-day precision from 1.7 to 7%, depending on the specific drug. The entire procedure can be completed within 45 min and is well adapted to the routine clinical laboratory. Of 48 common basic and several neutral drugs tested for possible interferences, only three benzodiazepines, three phenothiazines, and three antihistamines interfere with the assay of doxepin, desipramine, and nortriptyline, respectively.

Increased use of tricyclic antidepressants for the treatment of endogenous depression in psychiatric hospitals and clinics and the growing evidence for the existence of a significant correlation between the total concentration of tricyclic amine in plasma and the clinical response of the patient (1–4) have greatly increased the demand for routine laboratory methods capable of assaying simultaneously the major tricyclic antidepressants and their therapeutically effective metabolites in serum (plasma) to monitor the course of tricyclic therapy in psychiatric patients.

Tricyclic drugs and their metabolites in serum are usually measured either by gas–liquid chromatography or high-performance liquid chromatography (HPLC). Gas–liquid chromatographic methods generally require a lengthy clean-up procedure to eliminate interferences from endogenous and exogenous sample constituents, resulting in variable extrac-

Materials And Methods

Apparatus

High-performance liquid chromatograph: Model 204, equipped with a Model 440 UV absorbance detector, a Model 6000A solvent delivery system, and a Model U6K injector (all from Waters Associates, Milford, Mass. 01757).

Column: C-18 reversed-phase column, 3.9 mm × 30 cm (µ-Bondapak, Waters Assoc.).

Additional equipment: 50-ml centrifuge tubes (polypropylene), and 15-ml, conical, Pyrex centrifuge tubes.

Reagents and Standards

All reagents were reagent grade (AR) unless otherwise specified.

Tricyclic stock standard, 1 g/liter of each of the following pure drugs in methanol: doxepin (Sinequan; Pfizer Laboratories), desipramine (Norpramin; Merrell-National Laboratories), nortriptyline (Pamelor; Sandoz Pharmaceuticals),...
imipramine (Tofranil; Geigy Pharmaceuticals), amitriptyline (Applied Science Laboratories, Inc.). Store at 4–8 °C.

Protriptyline stock standard, 1 g of protriptyline (Vivactil; Merck, Sharp and Dohme) per liter of methanol, stored at 4–8 °C.

Working standards (10 mg/liter), prepared by a 100-fold dilution of the stock standards with 0.1 mol/liter hydrochloric acid.

Hexane (UV-grade, distilled in glass; Burdick and Jackson Laboratories, Inc., Muskegon, Mich. 49442).


Acetonitrile (UV-grade, distilled in glass, Burdick and Jackson Laboratories).

Isoamyl alcohol (Fisher Scientific Co.).

n-Propylamine (98%; Mallinckrodt, Inc., Saint Louis, Mo. 63147).

Stock internal standard, β-naphthylamine (Sigma Chemical Co., Saint Louis, Mo. 63178), 5 g/liter of hydrochloric acid 0.1 mol/liter. Stored at 4 °C.

Working internal standard (1 mg/liter) is prepared by a 500-fold dilution of stock standard with 0.1 mol/liter hydrochloric acid.

Pentanesulfonic acid (PIC B-5, 5 mmol/liter; Waters Assoc.).

De-ionized water was further purified by chromatography over 2 × 50 cm columns packed with the neutral cross-linked polymer Amberlite XAD-2 (Applied Science Laboratories, State College, Pa. 16801).

Mobile phase, methanol/acetonitrile/phosphate buffer (0.1 mol/liter, pH 7.6), 41/15/44 by volume. Adjust the pH to 6.5 by dropwise addition of concentrated sodium hydroxide or glacial acetic acid. Filter the mixture through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass. 01730) under negative pressure and de-gas it by ultrasonic vibration.

Procedure

Glassware preparation: Just before use, we cleaned glassware ultrasonically in a hexane/n-propylamine mixture (99/1 by volume), followed by rinsing in a hexane/isoamyl alcohol mixture (99/1).

Method: Transfer 2 ml of serum into a 50-ml polypropylene centrifuge tube, add 100 μl of β-naphthylamine working internal standard, 200 μl of 1.5 mol/liter sodium hydroxide, and 10 ml of hexane/isoamyl alcohol mixture (99/1). Rotate-mix for 5 min (gentle agitation will prevent any emulsion formation during this step), centrifuge for 3 min at 500 × g, and transfer 9 ml of the organic (top) phase into a 15-ml conical centrifuge tube. Add 200 μl of 0.1 mol/liter hydrochloric acid, shake the mixture mechanically for 10 min, and centrifuge for 2 min at 500 × g. After discarding the organic layer, inject 85 μl of the aqueous phase into the chromatograph, using the following instrument settings:

- Flowrate: 1.5 ml/min
- Detector: UV, 254-nm filter
- Sensitivity: 0.005 A full scale

Quantitate by measuring the ratio of the absorbance of each tricyclic antidepressant drug to that of the internal standard β-naphthylamine in the unknown sample and comparing it to the same ratio obtained for a standard of known concentration.

Results

Extraction Procedure

We investigated solid-phase extractions with use of both Celite 545 (Fisher Scientific Co.) and XAD-2 (Applied Science Laboratories, Inc.) columns, and liquid-phase extractions with use of solvent systems of different solvent strengths, with respect to their efficiency, reproducibility, simplicity, and speed for the isolation of tricyclic antidepressants from serum in the clinical laboratory.

The solid-phase methods in general were too cumbersome; the eluates required extensive purification before chromatography, and analytical recoveries of the tricyclic amines were inadequate (40–70%).

The liquid–liquid extraction methods were much simpler and more suitable for routine applications; however, the recoveries of the tricyclic drugs were highly variable when a standard extraction/evaporation/reconstitution procedure was used. The more manipulations the procedure involved, the less reproducible it was. Apparently each manipulation (transfer of solvent layers by pipet, concentration by evaporation, etc.) increased the chances of variable losses due to adsorption. Neither treatment of all glassware used with ethanol/propanolamine to inactivate glass surfaces nor use of polypropylene labware for the entire extraction procedure improved day-to-day precision sufficiently.

Reproducibility was acceptable when the tricyclic bases were simply extracted from alkalized serum into hexane/isoamyl alcohol (99/1) and re-extracted into a small volume of dilute hydrochloric acid. An aliquot of the aqueous phase was injected directly into the chromatograph, circumventing the evaporation step, which apparently was responsible for much of the observed variability in recoveries.

Use of a relatively selective extraction solvent combined with re-extraction of the basic amines into dilute acid removes most acidic and neutral interfering substances and leads to a sufficiently “clean” extract that requires no further purification. The chromatogram of a serum blank extract (Figure 1) demonstrates this point. Of the solvent systems we studied,
hexane/isooamy alcohol (99/1) presented the best compromise with respect to extraction efficiency and selectivity. Higher proportions of isooamy alcohol increased the background interferences in the blank; lower proportions resulted in significant extraction losses of some of the tricyclic amines.

The concentration of sodium hydroxide used to alkalinize the serum sample is critical for optimum recovery; an excess decreases the recovery of the tricyclic amines.

**Chromatographic Conditions**

*Stationary phase*: To avoid the problems associated with normal phase silica columns in routine analysis of crude plasma extracts—i.e., excessively long retention of polar constituents, necessitating long column-recovery times in between injections—we used a C-18 bonded reversed-phase column (μ-Bondapak, Waters Assoc.).

*Mobile phase*: Water/methanol and water/methanol/acetoniitrile mixtures were tested as mobile phases in the pH range of 3–7. Although some of the drugs could be separated under these conditions, the selectivity factors were inadequate, especially for separating the tertiary amines from their homologous secondary bases. Also, the peak shapes of the longer-retained compounds were asymmetrical because of ionization of the amines in acidic and neutral solutions.

Addition of a base (ammonia, ethylamine, or propylamine) to the elution solvent did improve the peak shape by suppressing ionization, but the five drugs under study could not be completely resolved and column efficiency quickly deteriorated owing to channeling caused by gradual dissolution of the silica packing at the unfavorable pH.

![Chromatogram of a serum extract containing 50 µg of each of the tricyclic antidepressant drugs per liter of serum](image)

The elution sequence is (1) β-naphthylamine (internal standard), (2) doxepin, (3) desipramine, (4) nortriptyline, (5) imipramine, and (6) amitriptyline.

**Fig. 2.** Chromatogram of a serum extract containing 50 µg of each of the tricyclic antidepressant drugs per liter of serum

**Fig. 3.** Effect of pH on K' values of tricyclic antidepressant drugs

**Fig. 4.** Effect of mobile-phase velocity on column efficiency

**Paired-ion technique**: To eliminate the problems associated with ionization of the tricyclic amines and yet permit their elution at a pH below 7 to ensure a reasonable column life, we used the paired-ion technique, pentane sulfonate (5 mmol/liter, PIC B-5; Waters Assoc.) being used as counter-ion. Symmetrical peak shapes and suitable K' values were obtained for the tricyclic drugs by use of the solvent system methanol/acetonitrile/water (41/15/44). Figure 2 shows the chromatogram of an extracted serum sample, supplemented with the five tricyclic drugs at 50 µg of each per liter.

**Effect of pH**: The single most critical factor influencing the resolution of a tertiary and secondary amine of identical fused-ring structure is the pH of the solvent system, as shown in Figure 3. At pH values <5, the three tertiary amines—doxepin, imipramine, and amitriptyline—are completely re-
solved but the secondary amines, desipramine and nortriptyline, are not separated from their parent compounds, imipramine and amitriptyline. Above pH 5, however, the $K'$ values of the tertiary amines increase much faster with increasing pH than do those of their homologous secondary amines, so that all compounds can be completely resolved at a pH between 6.2 and 6.5.

**Column efficiency:** The theoretical plate heights obtained for the five tricyclic amines on the C-18 reversed-phase column at different mobile phase velocities are shown in Figure 4. The curves obtained for amitriptyline, desipramine, and nortriptyline are rather flat, and the theoretical plate heights are quite low (<0.3 mm) throughout the entire range of flow rates studied, indicating little loss of column efficiency with increasing flow rates, and good mass transfer. Doxepin and imipramine have significantly higher theoretical plate heights at all flow rates and steeper slopes in the low-velocity range (0.5-1 ml/min). The mobile-phase velocity chosen for the assay was 1.5 ml/min ($\approx$0.333 cm/s).

**Detection in the Ultraviolet**

The observed absorption maxima of the five tricyclic amines under study are about 235 nm for doxepin, 240 nm for nortriptyline and amitriptyline, and 250 nm for imipramine and desipramine. Although detection at 254 nm is less than optimal for doxepin, nortriptyline, and amitriptyline, sensitivity is adequate for all five drugs at this fixed wavelength. The molar absorptivities at 254 nm, measured under the conditions of this assay (pH, solvent mixture, counter ion), are 12 323 for doxepin, 11 038 for desipramine, 8994 for nortriptyline, 11 397 for imipramine, and 8791 for amitriptyline. The molar absorptivity of protriptyline at 254 nm ($c_{254} = 2853$) is too low to permit its detection at therapeutic concentrations, but it may be measured with good sensitivity at 280 nm ($c_{280} = 11 411$). In the absence of desipramine, which absorbs at 280 nm and elutes together with protriptyline, this tricyclic drug may be determined by using the same chromatographic conditions as outlined above, simply by replacing the 254-nm filter with a 280-nm filter.

**Precision and Accuracy**

**Reproducibility:** The within-run and day-to-day precision was established with fortified pools of serum at two different concentrations, corresponding roughly to the lower and upper limit of the therapeutic ranges reported for most tricyclic drugs. The results are listed in Table 1. Generally, the assay is more precise at higher concentrations because recovery is more complete. However, the CV's at lower concentrations are still within the generally accepted limits for drug assays.

**Recovery:** The analytical recovery of the five tricyclic drugs was measured at four different concentrations, ranging from 25 to 300 $\mu$g/liter. The results listed in Table 2 represent the average of five extractions of each of the four fortified serum pools.

The recoveries of all drugs increase with increasing concentrations, consistent with the findings of others (6).

**Extraction efficiencies** are quite comparable for the five bases at each concentration, although the tertiary amines show slightly higher yields than their corresponding $N$-dealkylated products (desipramine, nortriptyline).

**Linearity:** Figure 5 shows calibration curves, obtained by plotting the ratios of the peak-heights of each of the five tricyclic amines to that of the internal standard, $\beta$-naphthylamine, vs. their respective concentrations in seven different pools of serum, fortified to contain concentrations ranging from 25 to 1500 $\mu$g/liter. The relation is linear over this entire range. Table 3 lists the constants of the respective linear regression lines.

**Interferences:** We tested 48 commonly used basic and several neutral drugs that might be partly extracted into the acidic aqueous extract—and detectable if present in high concentration—for possible interference with the determination of the five tricyclic antidepressants (Table 4)
Table 3. Linear Regression Data for Calibration Curves

<table>
<thead>
<tr>
<th>Drug</th>
<th>Slope</th>
<th>y-intercept *</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxepin</td>
<td>0.0119</td>
<td>0.058</td>
<td>0.9998</td>
</tr>
<tr>
<td>Desipramine</td>
<td>0.0118</td>
<td>0.032</td>
<td>0.9997</td>
</tr>
<tr>
<td>Nortriptyline</td>
<td>0.0090</td>
<td>0.097</td>
<td>0.9992</td>
</tr>
<tr>
<td>Imipramine</td>
<td>0.0044</td>
<td>0.024</td>
<td>0.9994</td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>0.0042</td>
<td>0.054</td>
<td>0.9983</td>
</tr>
</tbody>
</table>

* Peak-height ratios are plotted on the y-axis, drug concentrations in µg/liter on the x-axis.

Table 4. K’ Values of Common Basic (and Neutral) Drugs Tested for Interference

<table>
<thead>
<tr>
<th>Drug</th>
<th>K’</th>
<th>Drug</th>
<th>K’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>0.13</td>
<td>Thioridazine</td>
<td>3.67</td>
</tr>
<tr>
<td>Acetylsalicylic acid</td>
<td>0.20</td>
<td>Chlorazepate,</td>
<td></td>
</tr>
<tr>
<td>Dihyline</td>
<td>0.20</td>
<td>Dipotassium</td>
<td>3.87</td>
</tr>
<tr>
<td>Theophylline</td>
<td>0.27</td>
<td>Doxepin</td>
<td>3.93</td>
</tr>
<tr>
<td>Caffeine</td>
<td>0.40</td>
<td>Promepitryline</td>
<td>4.27</td>
</tr>
<tr>
<td>Codeine</td>
<td>0.53</td>
<td>Propoxyphene</td>
<td>4.47</td>
</tr>
<tr>
<td>Ephedrine</td>
<td>0.60</td>
<td>Desipramine</td>
<td>4.87</td>
</tr>
<tr>
<td>Phenylephedrine</td>
<td>0.60</td>
<td>Promazine</td>
<td>5.13</td>
</tr>
<tr>
<td>Methoxyphenamine</td>
<td>0.73</td>
<td>Diethazine</td>
<td>5.53</td>
</tr>
<tr>
<td>Diphenhydramine</td>
<td>1.40</td>
<td>Promethazine</td>
<td>5.80</td>
</tr>
<tr>
<td>Methaprylone</td>
<td>1.80</td>
<td>Imipramine</td>
<td>6.47</td>
</tr>
<tr>
<td>β-Naphthylamine</td>
<td>2.07</td>
<td>Amitriptyline</td>
<td>7.67</td>
</tr>
<tr>
<td>Pyrilamine</td>
<td>2.07</td>
<td>Atropine</td>
<td></td>
</tr>
<tr>
<td>Quinine</td>
<td>2.20</td>
<td>Benzpropine</td>
<td></td>
</tr>
<tr>
<td>Carboxinamide</td>
<td>2.33</td>
<td>Chlorpromazine</td>
<td></td>
</tr>
<tr>
<td>Chlorphenoxamine</td>
<td>2.47</td>
<td>Chlorprothixine</td>
<td></td>
</tr>
<tr>
<td>Oxazepam</td>
<td>2.67</td>
<td>Fluphenazine</td>
<td></td>
</tr>
<tr>
<td>Dimenhydrinate</td>
<td>2.73</td>
<td>Perphenazine</td>
<td></td>
</tr>
<tr>
<td>Methaqualone</td>
<td>2.73</td>
<td>Procyclorperazine</td>
<td></td>
</tr>
<tr>
<td>Nitrazepam</td>
<td>2.73</td>
<td>Scopolamine</td>
<td></td>
</tr>
<tr>
<td>Chlorpheniramine</td>
<td>3.00</td>
<td>Thiorpantazol</td>
<td></td>
</tr>
<tr>
<td>Haloperidol</td>
<td>3.00</td>
<td>Thiopropazine</td>
<td></td>
</tr>
<tr>
<td>Flurazepam</td>
<td>3.40</td>
<td>Trifluoperazine</td>
<td></td>
</tr>
<tr>
<td>Chlordiazepoxide</td>
<td>3.53</td>
<td>Triflupromazine</td>
<td></td>
</tr>
<tr>
<td>Mesoridazine</td>
<td>3.67</td>
<td>Tripelennamine</td>
<td></td>
</tr>
</tbody>
</table>

* For this drug and all that follow, K’ is >9 or drug does not absorb at 254 nm.

Flurazepam (Dalmane), although ordinarily well resolved from doxepin, may interfere if present in high concentrations, as in overdose cases.

Although peak width increases with increasing K’ values, the number of interfering drugs decreases with increasing elution volumes and no interferences have been observed for amitriptyline and imipramine.

Sensitivity and Detection Limit

Because the described extraction procedure yields a relatively clean extract, the antidepressant drugs may be measured at the highest detector sensitivity (0.005 A full-scale). Under the conditions of this assay the detection limit (values exceeding three-fold the average background) is about 5 µg/liter for all five tricyclic amines; 2-3 µg/liter may be detected by increasing the injected sample volume from 55 to 150 µl.

Alternatively, the detection limit for amitriptyline, nortriptyline, and doxepin may be improved to about 2 µg/liter by using a variable-wavelength detector or filter with peak transmission at 240 nm, which is closer to the absorbance maxima for these compounds (235 nm for doxepin, 242 nm for amitriptyline and nortriptyline). However, in our studies of patients we have not encountered any steady-state concentration in serum of less than 10 µg/liter, and the lower limit of the therapeutic range for the five tricyclic drugs studied lies between 25 and 50 µg/liter, i.e., the method described with use of fixed-wavelength ultraviolet detection is adequately sensitive for all clinical applications.

Figure 6 shows a chromatogram of an extracted serum sample from a psychiatric patient who received 150 mg of amitriptyline daily for seven days, but was taken off this...
medication 24 h prior to collection of the specimen. For the last 3 days preceding the collection, the patient also received 200 mg of doxepin and 60 mg of oxazepam daily.

The serum drug concentrations, calculated from the chromatogram, are 16 µg/liter for amitriptyline, 45 µg/liter for nortriptyline, and 58 µg/liter for doxepin.

Discussion

With the described method, five of the most commonly used tricyclic antidepressants in serum can be simultaneously measured, including both the tertiary amines amitriptyline and imipramine and their major therapeutically active N-demethylated metabolites, nortriptyline and desipramine. Many psychiatrists treat their patients with combinations of several antidepressant drugs, and meaningful interpretation of patient response to therapy is thus possible only if concentrations of all drugs with antidepressant activity are known, including those produced endogenously by metabolism of a parent drug. Accordingly, there is a need for a fairly comprehensive routine profile of antidepressants in serum.

Our method is well suited for routine application in the clinical laboratory because the extraction procedure is simple and rapid and only standard HPLC equipment is used (standard high-performance liquid chromatograph with fixed-wavelength ultraviolet detection). The extraction takes 25 min and all five compounds are eluted within 15 min.

The assay provides adequate sensitivity and precision for monitoring therapeutic steady-state concentrations as well as the subtherapeutic concentrations encountered during the initial start-up phase of tricyclic therapy.

Since patients treated with tricyclic antidepressants frequently receive other psychoactive drugs, among them sedatives and tranquilizers that are structurally closely related to the antidepressants, the clinical usefulness of any method for monitoring antidepressants will be determined largely by the degree of freedom of interferences from these drugs. The chromatographic conditions used for this assay were designed to minimize interferences from such exogenous as well as from endogenous basic sample constituents. The range of K' values chosen for the tricyclic amines extends from 3.9 for doxepin to 7.7 for amitriptyline. Among the 48 drugs tested for interferences only three benzodiazepines, three phenothiazines, three anticholinergic agents, and propoxyphene have K' values in the range of 3.5–8.5, as compared to 25 drugs with K' values below 3.5. Most phenothiazines, especially those with halogen-substituents in their nucleus, have K' values above 9 and do not interfere.

The method described has been used successfully in our laboratory to monitor tricyclic antidepressants in the serum of psychiatric in-hospital patients for the past nine months, and a study is currently underway to correlate the clinical response of these patients with their total serum tricyclic drug concentration. The results of this clinical study will be reported separately.

We gratefully acknowledge the valuable suggestions and critical comments from Faye Doss, M.D., Chairman, Department of Psychiatry, Baptist Medical Center–Princeton, Birmingham, Alabama; and from Herbert Kohl, Ph.D., Director of Clinical Chemistry, Chemistry Department, Auburn University, Auburn, Alabama.

References