Liquid-Chromatographic Procedure for Tricyclic Drugs and Their Metabolites in Plasma

F. L. Vandemark, Reginald F. Adams,¹ and G. J. Schmidt

We describe a procedure for determining amitriptyline and imipramine and their active metabolites nortriptyline and desipramine, respectively, at therapeutic concentrations in human plasma by use of liquid chromatography. The drugs are extracted at pH 10.5 into hexane/isoamyl alcohol, which is evaporated and the residue chromatographed. Protriptyline is used as the internal standard. As little as 10 µg of each drug per liter could be detected in plasma, the limit being established by variability in drug-free plasmas. The day-to-day coefficient of variation for each drug at a concentration of about 100 µg/liter was about 7%. Doxepin and diphenhydramine interfere with the analysis of amitriptyline. Total analysis time for a single sample is 20 min.

The tricyclic antidepressants are probably the type most widely prescribed. Several reports have shown large individual variations in plasma concentrations among patients receiving the same dosage of these tricyclic drugs (1–6); although most patients show concentrations of about 50–300 µg/liter, reported therapeutic concentrations of these drugs in plasma range from 15 to 500 µg/liter (7, 8). There is much interest in their measurement in plasma so that therapy can be made optimal. Various techniques for doing so have been used: isotope dilution (9), thin-layer chromatography (10), colorimetry (11), and spectrofluorometry (12)—methods that are either time-consuming, lack adequate sensitivity, or are prone to interference. In earlier gas–liquid chromatographic procedures for these drugs (13–15) flame ionization detectors were used that limit sensitivity to about 20 µg/liter and require considerable sample pretreatment. Electron capture detectors have been used with gas chromatography for analysis for amitriptyline (16, 17). These procedures are sensitive but give little or no information on metabolite concentrations. Mass fragmentography (18, 19) is sensitive and specific but not easily adaptable to the clinical laboratory. Recently reported gas-chromatographic procedures involving a nitrogen selective detector (20–22) for the determination of these drugs offer good sensitivity and specificity, but the four compounds cannot be determined in a single analytical run, and in one procedure derivatization is required for desipramine before chromatography. Liquid-chromatographic procedures have been used for the analysis for tricyclics (23–25), but have only been applied to aqueous samples or are too time-consuming to be performed routinely.

We report a procedure for the simultaneous determination of amitriptyline, imipramine, desipramine, and nortriptyline in plasma samples by liquid chromatography. Sample pretreatment is fairly rapid and 10 µg of each drug per liter can be detected.

Materials and Methods

Apparatus

We used a Model 601 liquid chromatograph equipped with a Model LC-55 UV detector and a Rheodyne 7105 injection valve (Perkin-Elmer Corp., Norwalk, Conn. 06856). All analyses were performed on a 25 × 0.46 cm column packed with 5-µm (particle diameter) Silica B/5, Part No. 089-0721 (Perkin-Elmer Corp.). Chromatograms were recorded on a Model 56 recorder, set at 10 mV.

Other equipment included 16 × 100 mm Teflon-lined screw-capped culture tubes, 5-ml conical centrifuge tubes, a bench-top centrifuge, a Tempblock heating block, an evaporation manifold (dry air or nitrogen), and a rotator rack.

Reagents and Standards

All reagents were analytical (AR) grade, unless otherwise stated.

Hexane and acetonitrile, distilled in glass, ultraviolet grade, were from Burdick and Jackson, Muskegon, Mich. 49442.

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Isoamyl alcohol, redistilled before use.
Hexane/isooamyl alcohol, 98/2 by vol.
Ammonium hydroxide, concentrated.

Mobile phase, prepare the mobile phase solution by mixing acetonitrile and concentrated ammonium hydroxide in the ratio of 99.3 to 0.7, by volume.
Sodium carbonate, saturated aqueous solution.
Amitriptyline, imipramine, nortriptyline, and desipramine2 (United States Vitamin Pharmacopeial Convention, Inc., 12601 Twin Brook Parkway, Rockville, Md.). Make individual working standards by dissolving 5 mg of the drug in 100 ml of methanol. Store at 4 °C.

Protriptyline, internal standard (The United States Vitamin Pharmacopeial Convention). Prepare the stock solution by dissolving 1.0 mg in 100 ml of methanol. Prepare the working internal standard solution by evaporating 1 ml of the stock solution and adding 100 ml of the hexane/isooamyl alcohol solution to the residue. Each milliliter of the working solution contains 100 ng of protriptyline.

Column test solution, combine 0.5 ml of each of the four 50 mg/liter standards, evaporate, and reconstitute the residue with 1.0 ml of the mobile phase. The solution contains 25 mg of each drug per liter. Store at 4 °C.

Procedure

Sample extraction. Transfer a 2-ml plasma sample into a culture tube and add 0.5 ml of the saturated Na₂CO₃ solution. After gentle mixing, add 5 ml of the hexane/isooamyl alcohol and the internal standard solution, place the mixture on a rotator rack, and extract for 3 min. After centrifugation (2000 rpm, 2 min) transfer the hexane/isooamyl alcohol (upper) layer to a 5-ml conical centrifuge tube. Evaporate the solvent carefully with dry air at 60 °C in a Tempblock heater. Dissolve the residue in 10 µl of the acetonitrile/ammonium hydroxide mobile phase, mix, and inject 5 to 7 µl of the extract into the chromatograph.

Chromatography. The mobile phase flow rate is 1.5 ml/min. The column temperature is set at 65 °C and the column effluent is monitored at 211 nm at a recorder scale of 0.05 A full scale. As a quality-control measure the performance was tested each day for resolution and sensitivity by injecting 2 µl of the column test solution containing a mixture of 50 ng of each tricyclic drug. Figure 1 shows a typical chromatogram.

Quantitation. Standard curves were prepared by adding amitriptyline, imipramine, nortriptyline, and desipramine to drug-free plasma, and processing these standards according to the procedure. After peak areas are measured for the standards, the peak-area ratio is calculated (analyte/protriptyline internal standard) for each standard. A working curve (Figure 2) for each drug relates peak-area ratio to drug concentration in the standard. Plasma concentrations in the unknown sample chromatograms are determined from the peak-area ratios by using the working curve.

Results

Peak-area ratios were linearly related to concentration for each drug over the range 50–800 µg/liter (Figure 2). However, in practice in our laboratory, most patients’ samples were found to contain drugs in the concentration range of 50–300 µg/liter. A calibration curve over the range of 25–300 µg/liter was found to be more useful.

Reproducibility of the procedure was determined by performing 10 replicate analyses on aliquots of a drug-free plasma to which the tricyclic drugs were added to give a concentration of 100 µg/liter. Day-to-day precision was estimated for the same test material over a two-week period. The results are shown in Table 1.
Extraction recoveries. Aliquots of drug-free plasma were supplemented with the four drugs to give concentrations of 100 and 500 μg/liter. Three aliquots at each concentration were taken through the complete procedure, the residues were reconstituted with 20 μl of methanol, and a 10-μl aliquot was injected into the liquid chromatograph. The peak areas for each of the extracted drugs were compared with the peak areas obtained by injecting 200 ng of each of the pure drug standards into the chromatograph. The recoveries ranged around 65% (Table 2). Incomplete recovery is corrected for by use of the internal standard and extracted plasma standards.

Background. We analyzed 10 different drug-free plasma samples by our procedure. They showed background values at the retention times of the drugs that ranged from 0 to 2 μg/liter. Because this background sets the lower limit of sensitivity, we have conservatively taken 10 μg/liter of each drug to be the minimum concentration reliably detected by this procedure.

Twenty drugs (Table 3) were tested for potential interference with our procedure by comparison of the retention times of methanolic standards of these drugs relative to those of amitriptyline, imipramine, nortriptyline, and desipramine. We did not determine the extraction of these materials from plasma. Table 3 shows retention times relative to that of the protriptyline internal standard. Caffeine, also tested for interference, had a relative retention of 0.14. There is a risk of interference for compounds that differ in relative retention time by less than 6% for relative retentions greater than 0.2. For example, doxepin and diphenhydramine interfered with the analysis of amitriptyline.

Plasma samples from patients on tricyclic therapy were analyzed by our procedures. The chromatograms from several of the patients are shown in Figure 3.

### Table 1. Precision of the Method

<table>
<thead>
<tr>
<th>Method</th>
<th>Amitriptyline</th>
<th>Imipramine</th>
<th>Nortriptyline</th>
<th>Desipramine</th>
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<tr>
<td>Within-day</td>
<td>µg/liter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>101</td>
<td>100</td>
<td>101</td>
<td>99</td>
</tr>
<tr>
<td>SD</td>
<td>5.0</td>
<td>4.9</td>
<td>5.9</td>
<td>6.4</td>
</tr>
<tr>
<td>CV%</td>
<td>5.0</td>
<td>4.9</td>
<td>5.9</td>
<td>6.4</td>
</tr>
<tr>
<td>Day-to-day</td>
<td>µg/liter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>101</td>
<td>100</td>
<td>101</td>
<td>99</td>
</tr>
<tr>
<td>SD</td>
<td>6.1</td>
<td>5.8</td>
<td>6.8</td>
<td>7.8</td>
</tr>
<tr>
<td>CV%</td>
<td>6.0</td>
<td>5.8</td>
<td>6.7</td>
<td>7.9</td>
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</tbody>
</table>

* n = 10 measurements.
* b n = 7 different days.

### Table 2. Analytical Recovery

<table>
<thead>
<tr>
<th>Method</th>
<th>100 µg/liter</th>
<th>500 µg/liter</th>
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<tr>
<td>Recovery, %</td>
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<td></td>
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<tr>
<td>Amitriptyline</td>
<td>68</td>
<td>69</td>
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<td>Imipramine</td>
<td>60</td>
<td>62</td>
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<tr>
<td>Nortriptyline</td>
<td>64</td>
<td>63</td>
</tr>
<tr>
<td>Desipramine</td>
<td>63</td>
<td>61</td>
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</table>

### Table 3. Relative Retention (r_{16}) for some Common Drugs Tested with This Procedure

<table>
<thead>
<tr>
<th>Drug</th>
<th>r_{16}</th>
<th>Drug</th>
<th>r_{16}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>0.02</td>
<td>Amitriptyline</td>
<td>0.22</td>
</tr>
<tr>
<td>Phenacetin</td>
<td>0.03</td>
<td>Diphenhydramine</td>
<td>0.22</td>
</tr>
<tr>
<td>Scopolamine</td>
<td>0.03</td>
<td>Doxepin</td>
<td>0.22</td>
</tr>
<tr>
<td>Cocaine</td>
<td>0.07</td>
<td>Imipramine</td>
<td>0.28</td>
</tr>
<tr>
<td>Diazepam</td>
<td>0.07</td>
<td>Meperidine</td>
<td>0.34</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>0.07</td>
<td>Trifluoperazine</td>
<td>0.39</td>
</tr>
<tr>
<td>Propoxyphene</td>
<td>0.11</td>
<td>Perphenazine</td>
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<tr>
<td>Oxazepam</td>
<td>0.12</td>
<td>Prochlorperazine</td>
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<td>Flurazepam</td>
<td>0.14</td>
<td>Nortriptyline</td>
<td>0.49</td>
</tr>
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<td>Chlordiazepoxide</td>
<td>0.16</td>
<td>Codeine</td>
<td>0.61</td>
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<tr>
<td>Chlorpromazine</td>
<td>0.17</td>
<td>Desipramine</td>
<td>0.71</td>
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<td>Methadone</td>
<td>0.17</td>
<td>Chlorpheniramine</td>
<td>0.76</td>
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</table>

* a Relative to protriptyline, the internal standard, after adjusting the retention time by subtracting 0.70 min mobile phase holdup time.
* b Extracts and chromatograms like amitriptyline.

**Discussion**

Interest is increasing in determining optimum drug dosage, especially for the tricyclic drugs, where relationships between concentration in plasma and clinical response are still being studied. This has been studied infrequently in the past, partly because routine methods that possess the required sensitivity and specificity for those analyses were not available.

Application of liquid chromatography for the determination of tricyclic drugs has had several limitations for its general use. The first problem has been one of sensitivity for these drugs. Use of an ultraviolet detector at 240 nm (24) is not sensitive enough for lower concentrations in plasma. Fluorescence detectors show excellent sensitivity but are only applicable to two of the tricyclic drugs, imipramine and desipramine. That the sensitivity for many analyses can be improved by using the far ultraviolet has been well documented (26–28). The tricyclic drugs exhibit maxima at about 245 nm, but the absorbance increases again in the far ultraviolet and the analysis at 211 nm provides an enhancement in sensitivity of about fourfold, which allows a detection limit of about 10 μg/liter in plasma.

Besides the problem of sensitivity, chromatography of these compounds has presented various other difficulties. They are partly ionized in aqueous solutions as weak bases. Therefore, reversed-phase chromatography gives peaks that are not adequately resolved because of peak tailing. Increasing the pH of the mobile phase to suppress ionization resulted in good separations but, in our own hands, if the pH of the mobile phase exceeded 7.2, column performance degraded after about 50 runs. Paired-ion chromatography techniques (29) permitted separations of most of these compounds without column degradation, but the reagents used for ion-pairing alkaline compounds have ultraviolet cut-offs at about 240 nm, which prevents use of the more sensitive short-wavelength absorption.

Columns of activated silica, for adsorption chromatography, are often used to separate compounds of
closely related structure, as are the four tricyclic drugs and the internal standard. Hexane/isopropanol, with small amounts of ammonium hydroxide, separated the compounds well, but matrix components co-extract from the plasma samples and elute close to the amitriptyline.

Experiments with the mobile phase indicated that increased amounts of ammonium hydroxide and decreased solvent strength eliminated matrix interferences. Thus, use of acetonitrile with ammonium hydroxide (7 ml/liter) results in good separations and lengthens the retention time of the drugs, removing the interference of plasma matrix components. Despite the harsh nature of this mobile phase, the column yielded more than 200 analyses with only slight decrease in column efficiency. We subsequently evaluated a modification of the mobile phase reported. The addition of 10% isopropanol to the acetonitrile changes the solvent strength only slightly but apparently modifies the silica surface, allowing reduction of ammonium hydroxide to 2 ml/liter. This mobile phase gives similar separation and analysis time as the reported mobile phase; however, studies on patient samples have not been completed.

As indicated previously, doxepin and diphenhydramine interfere with the analysis of amitriptyline. Combined use of the two tricyclic drugs is rare except in cases of drug abuse.

We did not study the hydroxy metabolites of the tricyclic drugs, but we believe that these metabolites would not be extracted in the procedure described.

References