Gas-Chromatographic Analysis for Therapeutic Concentrations of Amitriptyline and Nortriptyline in Plasma, with Use of a Nitrogen Detector

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We describe a gas-chromatographic procedure for the simultaneous determination of amitriptyline and its active metabolite, nortriptyline, in therapeutic concentrations in human plasma, with use of a nitrogen detector. Both drugs are extracted at pH 10.5 into hexane/isoamyl alcohol, back-extracted into dilute HCl, and re-extracted into hexane/isoamyl alcohol after alkalization of the HCl. The solvent is evaporated and the residue gas-chromatographed. Protriptyline is used as the internal standard. As little as 5 μg of amitriptyline or nortriptyline can be detected per liter of plasma. The coefficients of variation, for a concentration of 200 μg/liter, are 4.6% and 4.3% within-day and 8.6% and 3.4% day-to-day for amitriptyline and nortriptyline, respectively. The procedure was applied to patients receiving therapeutic doses of both drugs and also to patients who had taken overdoses of amitriptyline.

Additional Keyphrases: values after overdose • toxicology

Amitriptyline (“Elavil,” Merck Sharp & Dohme) and its active N-desmethyl metabolite, nortriptyline, are a commercially available therapeutic agent (“Aventyl,” Lilly and Co.), among the most widely administered tricyclic antidepressants (Figure 1). There have been reports of large individual differences in steady-state concentrations in the plasma of patients receiving the same therapeutic doses of these tricyclics, and monitoring these compounds in plasma has become of interest in the treatment of depression (1–4). Concentrations of amitriptyline and nortriptyline in plasma after therapeutic doses range from 20 to 300 μg/liter (5), and, for nortriptyline, intermediate concentrations (50–140 μg/liter) may provide better clinical response than either lower or higher concentrations (6).

While mass fragmentography, with its superior sensitivity and specificity, is ideally suited for the determination of these drugs at such low concentrations (7), it is not widely available. Gas-liquid chromatography has been used to measure amitriptyline and nortriptyline (5, 8–11); however, in most of these procedures conventional flame-ionization gas chromatography is used, and the lower limit of detection has been at best 20 μg/liter, and involves a rather laborious extraction and derivatization (5). Borga and Garle (8) reported an electron-capture gas-chromatographic procedure that is sensitive to 10 μg/liter, but it was described only for nortriptyline and also requires an involved extraction and derivatization with heptafluorobutyric anhydride. Wallace et al. (11) recently reported another electron-capture gas-chromatographic assay sensitive to 5 μg/liter, but their method requires prior oxidation of both amitriptyline and nortriptyline to the same compound (anthraquinone), so that only a value for total tricyclics is obtained.

Here, we report a relatively simple gas-chromatographic procedure for the simultaneous, direct determination of underivatized amitriptyline and nortriptyline in human plasma at concentrations as low as 5 μg/liter, with use of a selective nitrogen (“alkali flame”) detector. Karmen and Guiffrida (12) originally described this type of detector.

Materials and Methods

Apparatus

We used a Model 3920 gas-liquid chromatograph equipped with a nitrogen/phosphorus detector (Perkin-Elmer Corp., Norwalk, Conn. 06852). The column was coiled glass (2 mm i.d., 1.8 m long) containing 3% OV-17 on Gas-Chrom Q, 100/120 mesh (Applied Science Laboratories, Inc., State College, Pa. 16801). Relative retention times and peak areas were determined with a PEP-1 data processor (Perkin-Elmer Corp.)

Reagents

All reagents were analytical (AR) grade.

Hexane. This was redistilled and then passed through a 4 cm by 26 cm column of alumina A-540, 80/200 mesh (Fisher Scientific Co., Pittsburgh, Pa. 15219).

Fig. 1. Structures of (A) protriptyline, (B) amitriptyline, and (C) nortriptyline

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Isoamyl alcohol. This was redistilled.
Methanol. This was redistilled.
Hexane/isomyl alcohol, 98/2 by vol.
Na₂CO₃, anhydrous.
Saturated Na₂CO₃. This reagent was washed with hexane/isomyl alcohol before use.
HCl, 0.1 mol/liter. The distilled water used to prepare this solution was first washed with hexane/isomyl alcohol.
Na₂SO₄, anhydrous.

Standards in Methanol

Store all standards in the freezer (−15 °C). The first three listed should be re-prepared at least once a month.
Protriptyline hydrochloride (internal standard), 1.00 g/liter. Dissolve 10 mg of protriptyline hydrochloride (Merck Sharp & Dohme, Rahway, N. J. 07066) in 10 ml of methanol.
Amritriline base, 1.00 g/liter. Dissolve 11.32 mg of amitriptyline hydrochloride (Merck Sharp & Dohme), equivalent to 10 mg of amitriptyline base, in 10 ml of methanol.
Nortriptyline base, 1.00 g/liter. Dissolve 11.38 mg of nortriptyline hydrochloride (Eli Lilly and Co., Indianapolis, Ind. 46206), equivalent to 10 mg of nortriptyline base, in 10 ml of methanol.
Mixed amitriptyline and nortriptyline standard, 100 mg/liter. Dilute 100 μl of each of the 1.00 g/liter solutions of amitriptyline and nortriptyline with 0.8 ml of methanol. Prepare this standard just before each run.
Dilute internal standard in hexane/isomyl alcohol, 100 μg/liter. Add 10 μl of the 1.00 g/liter protriptyline hydrochloride solution to each 100 ml of the hexane/isomyl alcohol prepared for the initial extraction. This provides 333 ng of internal standard per milliliter of plasma extracted. Prepare with each run.

Standards in Plasma

Concentrated standard in plasma, 500 μg/liter. Dissolve 100 μl of the mixed standard in 20 ml of drug-free plasma. We prepared the concentrated standard with each run, but it is stable for at least one month if frozen.
Working standards in plasma. For each run, appropriately dilute the concentrated standard with drug-free plasma to achieve the desired concentrations (usually 10–300 μg/liter).

Procedure

All glassware was washed with HCl (1 mol/liter) and rinsed with hexane/isomyl alcohol before use. Blood from patients receiving therapeutic doses of amitriptyline or nortriptyline was sampled into heparinized glass syringes. Blood from patients who had taken overdoses of amitriptyline was collected into evacuated glass tubes containing no anticoagulant (Vacutainers; Becton-Dickinson, Division of Becton, Dickinson and Co., Rutherford, N. J. 07070). If they were not to be analyzed promptly, the samples were centrifuged and the plasma (or serum) frozen (−15 °C).

Place 3 ml of plasma (samples, drug-free plasma, and plasma standards) into 15-ml glass tubes equipped with Teflon-lined screw caps. Adjust the pH to 10.5 by adding 0.5 ml of saturated Na₂CO₃. Add 10 ml of hexane/isomyl alcohol containing the internal standard and extract for 3 min. Centrifuge at 3000 rpm for 3 min and transfer the upper solvent layer to a clean 15-ml screw-capped tube. Add 1 ml of HCl (0.1 mol/liter), extract for 3 min, and, after centrifugation, aspirate and discard the upper solvent phase. Wash the acid for 1 min with 2 ml of hexane/isomyl alcohol (without internal standard), centrifuge, and discard the upper solvent layer. Add 1 scoopful (about 500 mg) of anhydrous Na₂CO₃ to saturate the aqueous phase, mix well, and extract for 3 min with 2 ml of hexane/isomyl alcohol (without internal standard). After centrifugation, dehydrate the upper solvent layer over anhydrous Na₂SO₄ in a 10-ml test tube. Transfer the solvent to a 5-ml glass centrifuge tube and evaporate it in a stream of nitrogen at room temperature.

Dissolve the residue in 20 μl of methanol and chromatograph 4 μl under the following conditions: column temperature, 235 °C (isothermal); injector temperature, 255 °C; nitrogen detector temperature, 300 °C (detector current setting of 5.9–6.9, corresponding to rubidium glass bead temperatures of about 350–400 °C); carrier gas (helium) flow rate, 20 ml/min; air flow, 400 ml/min; hydrogen, 1 ml/min; and amplifier setting, ×1. Calculate the peak-area ratio (amitriptyline or nortriptyline/protriptyline internal standard) for each sample and determine the plasma concentration by comparison to the peak-area ratios of the extracted standards.

Results

We analyzed 24 different drug-free plasmas, and 16 gave slight responses corresponding in retention time to amitriptyline and nortriptyline, equivalent to 3 μg/liter or less of both. The detection limit for each drug was thus considered to be about 5 μg/liter for the proposed method. The relationship between relative peak area and plasma concentration of amitriptyline and nortriptyline was linear over the range 5 to 300 μg/liter (r = 0.99). Nonlinearity was observed at concentrations exceeding 300 μg/liter, and samples were diluted and reanalyzed if above this concentration.

For precision studies, drug-free plasma was supplemented to a concentration of 200 μg each of amitriptyline and nortriptyline per liter. Twelve aliquots of this plasma were analyzed in one day to determine within-day precision and the remaining plasma was frozen (−15 °C). One aliquot of the frozen supplemented plasma was thawed and analyzed on 11 different days during 30 days, to determine day-to-day precision and sample stability. The coefficients of variation were 4.6% and 4.3% within-day and 8.6% and 3.4% day-to-day for amitriptyline and nortriptyline, respectively.

The analytical recovery was 45–50% for both amitriptyline and nortriptyline, determined by comparison of relative peak areas of extracted supplemented plasmas with those of nonextracted methanolic standards.
This incomplete recovery is corrected for by the use of extracted plasma standards in the proposed procedure.

The stability of the drugs in plasma was established to be at least one month when frozen at \(-15^\circ\)C. The extract residues were stable at least overnight when stored at \(4^\circ\)C in a desiccator.

When plasma was collected in heparinized glass syringes, essentially “clean” chromatograms were obtained, the background response being equivalent to 3 \(\mu\)g/liter or less for amitriptyline and nortriptyline. In addition, a small noninterfering peak at retention time 1.14 relative to the internal standard was usually seen (peak \(d\), Figure 2). This peak was much more pronounced in specimens that had been collected in Vacutainers.

Forty-seven commonly used basic drugs (Table 1) were screened for interference in the assay by comparing the relative retention time of their nonextracted methanolic solutions with those of amitriptyline (0.74) and nortriptyline (0.86) under the chromatographic conditions of the assay. (Acidic drugs, such as barbiturates, would not be recovered under the alkaline extraction conditions of the assay.) Doxepin (“Sinequan,” Pfizer) had a relative retention time identical to that of nortriptyline, and, when drug-free plasma was supplemented with doxepin, it extracted and chromatographed as nortriptyline. Desipramine, the active \(N\)-desmethyl metabolite of imipramine (“Tofranil,” Geigy), and itself a commercially available antidepressant (“Pertofrane,” USV Pharmaceutical; “Norpramin,” Lakeside), was extracted and chromatographed; its retention time corresponded to that of the internal standard, protriptyline. Thus, plasma from patients receiving doxepin would give falsly positive results for nortriptyline, and patients receiving either imipramine or desipramine in addition to amitriptyline or nortriptyline (an unlikely occurrence) would have falsely depressed values for the last two drugs because the response of the internal standard would be enhanced by desipramine. No other interfering drugs were found, although three of the drugs indicated in Table 1 had retention times near those of amitriptyline or nortriptyline, or both.

Plasmas from patients receiving therapeutic doses of the following medications (often administered concomitantly with the tricycles) were analyzed, to see whether their metabolites interfered: perphenazine, chlorpromazine, thioridazine, and benztpoline. None did.

We analyzed 12 plasmas from nine patients in whom therapeutic doses of amitriptyline and (or) nortriptyline had been stabilized (Table 2). In addition, we analyzed 25 samples from 17 patients, each of whom had ingested a nonfatal overdose of medications including amitriptyline. Plasma drug values in overdose ranged up to 1080 \(\mu\)g/liter for amitriptyline, up to 337 \(\mu\)g/liter for nortriptyline, and up to 1260 \(\mu\)g/liter for total tricyclics (sum of both). Data on three overdoses involving amitriptyline alone without other drugs are also shown in Table 2.

To validate the procedure further, we determined four consecutive values for amitriptyline and nortriptyline in one patient who had taken an overdose of amitriptyline, and drew decay curves for amitriptyline,
nortriptyline, and total tricyclics (the sum of both). All three semilog curves were linear (Figure 3).

Discussion

The interest in optimizing drug therapy by monitoring concentrations in blood is increasing, and this is true for the tricyclic antidepressants as well. Values for our series of patients, stabilized on therapeutic doses (Table 2), were similar to those obtained by others (1-3, 5, 6, 9, 13, 14), and they displayed moderate individual variation, as has also been previously reported (1-4). As expected, both amitriptyline and its desmethyl metabolite nortriptyline were found in the plasmas of patients receiving amitriptyline, but only nortriptyline in patients receiving nortriptyline. Some of our patients were on lower initial doses (50 mg) than in the other reported studies (1-3, 6, 9, 13, 14) so that initial values were occasionally lower in our series. As doses were increased, the concentrations in plasma also increased.

Values for amitriptyline after acute amitriptyline overdose were usually greater than those for nortriptyline, probably reflecting insufficient time for mono-N-demethylation of the parent drug. Except for three patients, all of our overdose cases had consumed other drugs in addition to amitriptyline, and so we do not now have sufficient data to make adequate clinical correlations. Fatalities have been frequent in other reports of amitriptyline overdose (10, 15-18); all our patients survived.

Because concentrations of amitriptyline and nortriptyline in plasma after therapeutic doses are low, conventional flame-ionization gas chromatography at best affords marginal sensitivity, and derivatization is required to enhance the response. These low values, however, are well within the range of measurement achieved by gas chromatography with a selective nitrogen detector, and derivatization is not required.

Because protriptyline is both an isomer of nortriptyline and a structural homolog of amitriptyline (Figure 1), has about the same solubility properties that they have, and is itself only a rarely administered tricyclic antidepressant, we selected it as the internal standard for the assay. It has been used as an internal

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Table 2. Plasma Concentrations of Amitriptyline and Nortriptyline in Patients

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Daily dose</th>
<th>Other medication</th>
<th>Amitriptyline</th>
<th>Nortriptyline</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Plasma concn, µg/liter</td>
<td></td>
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<tr>
<td>Patients on therapeutic doses of nortriptyline:</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>50 mg</td>
<td>none</td>
<td>0</td>
<td>31</td>
</tr>
<tr>
<td>2</td>
<td>50 mg</td>
<td>none</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>150 mg</td>
<td>none</td>
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<td>4</td>
<td>150 mg</td>
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<tr>
<td>5</td>
<td>200 mg</td>
<td>none</td>
<td>0</td>
<td>278</td>
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<tr>
<td>Patients on therapeutic doses of amitriptyline:</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>6</td>
<td>50 mg</td>
<td>a</td>
<td>18</td>
<td>52</td>
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<td>7</td>
<td>150 mg</td>
<td>b</td>
<td>64</td>
<td>73</td>
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<td>8</td>
<td>200 mg</td>
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<td>28</td>
<td>75</td>
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<td>9</td>
<td>250 mg</td>
<td>none</td>
<td>133</td>
<td>110</td>
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<td>Patients who had overdosed with amitriptyline alone:</td>
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<td>11</td>
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<td>12</td>
<td>1.25-250 g</td>
<td>none</td>
<td>740</td>
<td>210</td>
</tr>
</tbody>
</table>

* Chlorpromazine, 125-150 mg daily.

* Thoridazine, 200 mg daily; trihexyphenidyl, 4 mg daily; diphenylhydantoin, 300 mg daily.

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standard by others in the assay of amitriptyline and nortriptyline (9, 16).

Back-extraction of amitriptyline and nortriptyline into dilute HCl provides sufficiently “clean” extracts, free from neutral lipids, and has been used by others (9). Our day-to-day use of the nitrogen detector has been described previously (19).

We did not study the more polar 10-hydroxyamitriptyline and 10-hydroxynortriptyline metabolites. However, it is unlikely that they would be extracted under the conditions of the proposed method.

Of 47 common basic drugs, only doxepin and desipramine interfered. Doxepin and desipramine, however, themselves tricyclic compounds, would not be administered with amitriptyline or nortriptyline, although hypothetically they could be combined in an overdose situation.

We specifically analyzed plasma from patients receiving therapeutic doses of chlorpromazine, perphenazine, thioridazine, and benztpine (all included in Table 1), to exclude their metabolites as potential interferences, because these drugs are often given in combination with amitriptyline or nortriptyline and one (perphenazine) is combined with amitriptyline in two commercial preparations (“Triavil,” Merck Sharp & Dohme; “Etrafon,” Schering). No interferences were found. The noninterfering contaminant peak at relative retention time 1.14 (peak d, Figure 2) was considerably enhanced in specimens drawn into Vacutainers. It may represent tri(2-butoxyethyl)phosphate, which has been identified by others (20). When blood was collected into heparinized glass syringes, as was the case for all our patients on therapeutic doses of the tricyclics, this peak was small.

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References


