Quantitative Liquid-Chromatographic Technique for the Simultaneous Assay of Tricyclic Antidepressant Drugs in Plasma or Serum

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A quantitative "high-pressure" liquid-chromatographic assay for tricyclic antidepressant drugs in plasma or serum is described in which amitriptyline, nortriptyline, imipramine, desipramine, doxepin, and desmethyldeoxepin are separated with a 10-μm particle size silica column and a methanol/NH₄OH/NH₄NO₃ solvent system. The drugs and two internal standards are extracted with hexane/isooamyl alcohol, the solvent layer is evaporated at 40 °C, and the drugs are detected at 254 nm. Drug concentrations are linear with absorbance from 25 to 1000 μg/L; within-assay and between-assay CVs are ≤ 10%.

Additional Keyphrases: use of two internal standards - drug assay

The tricyclic antidepressant drugs amitriptyline (AT), nortriptyline (NT), imipramine (IMI), desipramine (DES), and doxepin (DOX) are widely used in the treatment of depression. Although precise therapeutic ranges are ill-defined for these drugs, clinically useful concentration ranges in plasma have been reported (1-9). Plasma concentrations for these drugs are unpredictable; marked differences in individual hepatic metabolism of the drugs, age, and concurrent administration of other drugs can cause considerable variation in steady-state plasma concentrations (1, 8-12). Thus, for many individuals a standard dose is inadequate for achieving effective plasma concentrations, or may result in undesirably high concentrations of the parent drug if metabolism is slow or inhibited. Because the tricyclic antidepressant drugs may cause undesirable anticholinergic side effects and cardiac changes, the plasma concentrations for many patients should be kept in the lowest effective range.

Most techniques for tricyclic antidepressant assays involve gas-liquid chromatography (GLC) or "high-pressure" liquid chromatography (HPLC). The most sensitive and specific methods use chemical ionization/mass spectrometry (GC-MS-CI), but unfortunately the complexity and expense of this instrument limits its routine use in clinical laboratories (1, 13). Other gas-chromatographic techniques require lengthy and multiple extractions to eliminate interferences, or derivatization to improve separation and identification of the drugs (14, 15). HPLC methods reported include adsorption, ion-pairing, and reversed-phase chromatography (14, 16-19). Critical reviews of tricyclic antidepressant drug assays have been published (14, 20).

Complete resolution of the common tricyclic antidepressant drugs and their major metabolites, AT, IMI, DOX, NT, DES, and desmethyldeoxepin (DODOX), is necessary so that they may be assayed in a single procedure that is free from interferences caused by the coelution of two or more of the drugs or their metabolites. Most methods reported for tricyclic antidepressant assays in plasma do not resolve all six drugs and their metabolites. Thoma et al. (19) published an HPLC technique involving a CN-bonded-phase column and a buffered acetoniitrile mobile phase; however, AT and NT interfere with DOX and DODOX, respectively. Proless et al. (18) report an ion-pairing technique with pentanesulfonic acid and reversed-phase chromatography; long chromatography time, however, allows only four injections per hour, and the resolution of DES and PT is incomplete (DODOX data was not reported). A technique described by Vandemark et al. (16), involving a silica column and an acetoniitrile/NH₄OH mobile phase, does not separate DOX and AT, and chromatography time is 20 min per injection. Wallace et al. (17) described a reversed-phase HPLC technique; however, chromatography time is long, and PT, DOX, and DES have similar retention times. Recently, Kabra et al. (21) reported an HPLC method that resolves the tricyclic antidepressants; however, the chromatography time is 13 min per injection.

With the HPLC method we report, involving a silica column and a methanol/NH₄OH/NH₄NO₃ mobile phase, all of the commonly prescribed tricyclic antidepressant drugs, AT, NT, IMI, DES, DOX, and DODOX, are simultaneously assayed within 7 min. None of these drugs coelute with one another. The procedure requires a single organic solvent extraction of the drugs from plasma and includes two internal standards, a tertiary amine and a secondary amine, to provide accurate precision and accuracy for the respective tertiary and secondary antidepressant drugs.

Materials and Methods

Apparatus

A high-pressure liquid chromatograph with a variable-wavelength detector (Model 1084-B: Hewlett-Packard Co., Palo Alto, CA 94304) and a liquid chromatograph (Varian 500) with a Varichrom detector (Varian Associates, Walnut Creek, CA 94598) were used with a Varian SI-10 column (10-μm, 30 cm X 4 mm). Column temperature was set at 25 °C, and the flow rate was 1.70 mL/min. Sensitivity was attenuated at 2 or 3 for the Hewlett-Packard and the absorbance set at 0.02 A (full-scale) for the Varian. Eluting compounds were detected at 254 nm.

Reagents and Glassware

Hexane and toluene (Nanograde quality, Mallinkrodt, Inc., St. Louis, MO 63147).

Isoamyl alcohol ("Baker analyzed") and HPLC-grade water (J.T. Baker Chemical Co., Phillipsburg, NJ 08865).

Me thanol (LiChrosolv, E. Merck, Cincinnati, OH 45212). NH₄OH, 2 mol/L. Place 35.5 mL of 58% NH₄OH in a 250-mL volumetric flask; dilute to volume with HPLC-grade water.

NH₄NO₃, 1 mol/L. Dissolve 20 g of NH₄NO₃ in 250 mL of HPLC-grade water.

Borate buffer, saturated, pH 9. Dissolve approximately 300
g of sodium borate in 1 L of distilled water. Stir and heat until dissolved. Cool before use.

Extraction solvent: hexane/isoamyl alcohol (98/2 by vol).

Mobile phase: methanol, 2 mol/L NH₂OH, and 1 mol/L NH₄NO₃ (95/3/2 by vol). Filter and briefly de-gas before use.

Extraction tubes, glass, with Teflon-lined caps, 16 × 100 mm (no. 9826; Corning Glass Works, Corning, NY 14830).

Evaporation tubes, glass, 16-mL conical (no. 380; Scientific Products).

Venajet blood-collection tubes, 7 mL, containing sodium fluoride and potassium oxalate (no. B3046-3, Scientific Products).

Glassware preparation. Prepare a 100 mL/L solution of dichlorodimethylsilane (Applied Science Laboratories, Inc., State College, PA 16801) in toluene. Fill extraction and evaporation glassware with this solution, let stand for 30 min before emptying, rinse with methanol, and dry. Rinse glassware with hexane/isoamyl alcohol and dry before use.

Drugs and metabolites. The drugs, as their hydrochloride salts, were obtained from the following sources: AT, 10-hydroxy-AT, and protriptyline (PT) (Merck, Sharp and Dohme Research Laboratory, Rahway, NJ 07065); NT (Eli Lilly and Co., Indianapolis, IN 46206); IMI (CIBA Pharmaceutical Co., Summit, NJ 07901); DES (USV Pharmaceutical Corp., Tuckahoe, NY 10707); and DOX and DDOX (Pfizer Laboratories, New York, NY 10017). Trimipramine (TR) maleate was obtained from IVES Laboratories, Inc., New York, NY 10017.

Standards

Stock standards. The drugs and metabolites are dissolved in methanol to produce stock standards containing 1 g of the free base drug per liter. Two stock combination standards, A and B, are prepared by appropriate combination and dilution of the drug standards. Combination standard A contains 200 mg of AT, NT, IMI, and DES per liter, and is prepared by combining equal volumes (1 mL) of 1 g/L stock standards of AT, NT, IMI, and DES with 1.0 mL of methanol. Combination standard B contains 200 mg of DOX and DDOX per liter, and is prepared by combining 1.0 mL each of the 1 g/L stock standards of DOX and DDOX with 3.0 mL of methanol.

Working standards. Standards A and B are diluted 10-, 20-, 40-, 80-, 160-, 320-, and 640-fold. These working standards contain drug concentrations of 20, 10, 5, 2.5, 1.25, 0.625, and 0.3125 mg/L, producing drug concentrations in plasma equivalent to 1000, 500, 250, 125, 62.5, 31.25, and 15.6 µg/L, when working standards from Set A and Set B are used as described in Procedure.

Internal standards. Prepare stock standards of trimipramine (TR) and protriptyline (PT) by dissolving the drugs in methanol to produce stock standard containing 1 g of free-base drug per liter. To a 10-mL volumetric flask containing approximately 5 mL of methanol add 30 µL of TR stock standard and 200 µL of PT stock standard; add methanol to a final volume of 10 mL. This standard contains 3 mg of TR and 20 mg of PT per liter. Store all standards at −4°C.

Procedure

To 2.0 mL of plasma or serum in silanized 16 × 100 mm extraction tubes with Teflon-lined screw caps, add 100 µL of the working internal standard containing TR and PT. Vortex-mix. Add 1.0 mL of borate buffer and vortex-mix again. Add 6.0 mL of hexane/isoamyl alcohol (98/2 by vol) and invert or shake for 10 min. Centrifuge at 2000 rpm for 10 min to separate the phases. Transfer the solvent layer to silanized 16.0-mL conical evaporation tubes and evaporate to 40°C under a stream of air. Remove the tubes promptly when dry. Reconstitute the residue in 100 µL of mobile phase solvent and inject 25 µL onto the column. Calculate appropriate peak height ratios for the drug(s) present: AT/TR, IMI/TR, DOX/TR, and NT/PT, DES/PT, and DDOX/PT. Drug concentrations are determined from standard curves in which peak height ratios of the standards are plotted against concentrations. The standard curves are prepared by adding 100 µL of the working standards from either Set A or B to 2.0 mL of drug-free plasma and assaying by the described procedure.

Patients' Samples

Patients' blood samples used for correlation studies were collected in Venajet tubes containing sodium fluoride and potassium oxalate. After centrifugation the plasma was transferred to glass tubes sealed with Teflon-lined caps. The plasma was stored at −10°C until assayed by GC-MS-CI and refrozen until assayed by the HPLC procedure.

Results

Figure 1 represents typical chromatograms of a standard containing 40 ng of TR, AT, DOX, IMI, DDOX, and DES and 200 ng of PT injected on column; a blank plasma with internal standard added; and a patient's sample containing IMI and DES.

The standard curves for the six drugs and metabolites are linear over the concentration range 25 to 1000 µg/L (r > 0.99), when peak height ratios are plotted against concentration and applied to a least-squares regression equation. Regression

Table 1. Linearity of Peak Height Ratio (y) with Concentration (x) in Plasma Standards (25–1000 µg/L) *

<table>
<thead>
<tr>
<th>Drug</th>
<th>y Intercept</th>
<th>Slope</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amitriptyline</td>
<td>−0.280</td>
<td>0.006</td>
<td>0.9998</td>
</tr>
<tr>
<td>Nortriptyline</td>
<td>−0.031</td>
<td>0.006</td>
<td>0.9999</td>
</tr>
<tr>
<td>Imipramine</td>
<td>0.024</td>
<td>0.005</td>
<td>0.9998</td>
</tr>
<tr>
<td>Desipramine</td>
<td>−0.017</td>
<td>0.005</td>
<td>0.9999</td>
</tr>
<tr>
<td>Doxepin</td>
<td>−0.0202</td>
<td>0.004</td>
<td>0.9998</td>
</tr>
<tr>
<td>Desmethyldoxepin</td>
<td>0.0416</td>
<td>0.004</td>
<td>0.9999</td>
</tr>
</tbody>
</table>

* Calculations based on six standards per drug.
correlation data for the individual drugs are given in Table 1. All of the drugs are detectable at concentrations of ≤15 μg/L in plasma. Quantitative sensitivity is established at 25 μg/L (signal-to-noise ratio of 6:1).

Within-assay and between-assay precision data are listed in Table 2 for the individual drugs; within-assay and between-assay CVs are <10% for all of the drugs.

Various controls were analyzed by GC-MS-Cl (13) to determine if there was any discrepancy between the added concentration and the analyzed concentration. Table 3 shows the results of these comparisons.

Patients' samples analyzed by GC-MS-Cl were also analyzed by the described HPLC method. Correlation data for AT, NT, IMI, and DES are presented in Figure 2. An insufficient number of samples have been analyzed for accurate correlation of DOX and DDOX.

Discussion

Resolution of AT, NT, IMI, DES, DOX, and DDOX is essential for detection and accurate quantitation of these drugs when multiple tricyclic antidepressant drugs and metabolites are present in a single plasma sample. The described tricyclic antidepressant drugs have been analyzed in our laboratory; generally more than one drug is detected in plasma samples when drug therapy is being changed to a different tricyclic drug. Less frequently, but equally important, multiple tricyclic antidepressant drugs have been detected when two different tricyclic antidepressant drugs were prescribed simultaneously. Although baseline resolution is not achieved between AT and DOX, they are sufficiently separated for specific detection.

More than 40 drugs were analyzed for their possible interferences in the procedure. Only a few cause potential problems. Table 4 lists the drugs that do not interfere at typical plasma concentrations (22). Table 5 lists the relative retention times of the tricyclic antidepressant drugs and those drugs with similar retention times that could interfere at typical plasma concentrations. Amoxapine, meperidine, chlorimipramine, and chlorpromazine will coelute with AT or DOX quantitation; quinidine and quinine will coelute with DOX, and thioridazine coelutes with IMI. Except for chlorpromazine, it is unlikely that these drugs would be administered to patients on tricyclic antidepressant drug therapy.

The 10-hydroxylated metabolites of AT and NT were extracted and chromatographed with this procedure. Although AT and 10-hydroxy-AT have similar retention times, interference from the latter is unlikely because its concentration in the unconjugated form in plasma is extremely low (23). 10-Hydroxy-NT is well resolved from NT. Neither of these compounds have been detected in any of the patients' samples analyzed.

Table 3. Comparison of GC-MS-Cl and HPLC Values of Various Known Control Sera

<table>
<thead>
<tr>
<th>Drug</th>
<th>Added</th>
<th>GC-MS-Cl a</th>
<th>HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amitriptyline</td>
<td>50</td>
<td>54</td>
<td>49 b</td>
</tr>
<tr>
<td>Nortriptyline</td>
<td>50</td>
<td>45</td>
<td>49 b</td>
</tr>
<tr>
<td>Imipramine</td>
<td>50</td>
<td>55</td>
<td>48 b</td>
</tr>
<tr>
<td>Desipramine</td>
<td>50</td>
<td>40</td>
<td>48 b</td>
</tr>
<tr>
<td>Doxepin</td>
<td>100</td>
<td>106</td>
<td>97 c</td>
</tr>
<tr>
<td>Desmethyldoxepin</td>
<td>100</td>
<td>116</td>
<td>96 c</td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>150</td>
<td>166</td>
<td>164 d</td>
</tr>
<tr>
<td>Nortriptyline</td>
<td>100</td>
<td>92</td>
<td>94 d</td>
</tr>
<tr>
<td>Imipramine</td>
<td>225</td>
<td>242</td>
<td>232 d</td>
</tr>
<tr>
<td>Desipramine</td>
<td>160</td>
<td>148</td>
<td>148 d</td>
</tr>
</tbody>
</table>

a Single determination. b Mean of nine determinations. c Mean of four determinations. d Mean of 10 determinations.

Fig. 2. GC-MS-Cl and HPLC correlation of drug concentrations in patients' samples.
The single extraction and evaporation procedure is a rapid and reliable method of sample preparation. Drug recovery from plasma provides sensitivity to 15 μg/L and is adequate for therapeutic drug monitoring. Occasionally, extraneous peaks are detected in drug-free plasma and patients' samples, but are generally well-resolved from the peaks for the tricyclic antidepressant drugs. A more frequently encountered peak, as yet unidentified, elutes near IMI. When this peak elutes in IMI-containing samples, it appears as a shoulder on the downward slope of the IMI peak; therefore, its influence on peak height measurement is negligible. Possibly in the absence of IMI, this peak could be misconstrued to be IMI. Comparison of relative retention times (relative to PT) of this peak and IMI peaks, however, can differentiate the unidentified plasma peak from IMI.

Developing this procedure, we noted variability in drug recoveries; variations in drug recovery from extraction-evaporation procedures have also been reported by others (1, 16, 21). We subsequently minimized this variability by using silanized glassware, carefully controlling the evaporation temperature, promptly removing the evaporation tubes from the water bath after the solvent had evaporated, and by using two internal standards (a tertiary amine and a secondary amine) for quantitation. Although the internal standards are available as antidepressant drugs, and their presence in patients' plasma or serum would interfere with the assay, neither drug is widely used as a therapeutic agent. If, however, there is concern that TR or PT may be present in a sample, the TR/PT ratio can be calculated and compared with those ratios calculated for the standards. Although some variations of the TR/PT ratios will be seen, owing to the differential evaporation losses of the drugs, an extreme change in the ratio would indicate the presence of one of these drugs.

Detection at 254 nm provides a useful signal with minimum noise. The NH₄NO₃ in the solvent system limits the usable wavelength to 240 nm or greater. Although 245 nm provided better sensitivity for AT, NT, DOX, and DDOX, fewer interferences from extraneous peaks were detected at 254 nm; thus we selected the latter as the optimum wavelength.

The described system requires only 7 min for complete resolution, and there is no noticeable deterioration of the column for up to 10 months (approximately 500 h of operation). A similar column and solvent system were used for quinidine determinations, and column performance was unaltered over 12 months (24).

In summary, with the described HPLC technique, resolution of the commonly prescribed tricyclic antidepressant drugs is achieved within a short chromatographic time. The assay provides the accuracy and sensitivity needed for clinical assays of the tricyclic antidepressant drugs and their active metabolites, and requires minimal sample preparation.

We thank the Hewlett-Packard Foundation for their generous gift of the liquid chromatograph used in the development of this assay.

References
9. Jatlow, P., Therapeutic monitoring of plasma concentrations of...


