


Liquid-Chromatographic Determination of Eight Tri- and Tetracyclic Antidepressants and Their Major Active Metabolites

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A "high-performance" liquid-chromatographic method is presented for monitoring the therapeutic concentrations, in plasma, of eight tri- and tetracyclic antidepressants (amitriptyline, nortriptyline, imipramine, desipramine, doxepin, clomipramine, maprotiline, and protriptyline) and their major active metabolites. One of two internal standards is added to 0.5 mL of drug-containing plasma, the pH is adjusted to about 9.5 with borate buffer, and the sample is extracted with isoamyl alcohol in hexane. The extracts are chromatographed on a column of silica and absorbance of the effluent is measured at either 214 or 254 nm. Chromatographic response is linearly related to concentration for all components over a 5–500 ng range. Analytical recovery of the drugs and metabolites from plasma is approximately 75 to 85% at low and high concentrations. Within-run and day-to-day precision (CV) is <5% for both high and low concentrations of most of these drugs and metabolites. Parallel analysis of clinical samples by gas chromatography indicates that results by the two techniques are comparable. We report some results of therapeutic monitoring of clinical samples.

Additional Keyphrases: monitoring therapy · drug assay · gas chromatography

The increased interest in monitoring the concentrations of tri- and tetracyclic antidepressants in plasma, and in the potential clinical significance of active drug metabolites, indicates the need for sensitive and specific assays capable of measuring, concurrently, several antidepressants and their major active metabolites. Scoogins et al. (1) reviewed 59 gas-chromatographic, "high-performance" liquid-chromatographic, and radioimmunoassay methods of analysis for tricyclic antidepressants. Only eight of the procedures can measure more than one antidepressant, and three-fourths of the 48 chromatographic methods require 2 to 5 mL of plasma. The well-known potential problem of cross reactivity between parent drug and metabolites in RIA can be particularly significant when the metabolite exhibits a different pharmacological activity and (or) potency relative to the parent drug. More recently, at least two methods, one liquid-chromatographic and one gas-chromatographic, have been reported for simultaneous analysis for several antidepressants and metabolites (2, 3). The gas-chromatographic method requires a nitrogen-sensitive detector and derivatization; the liquid-chromatographic method involves reversed-phase and requires that column temperature be kept high. Neither method includes the separation and identification of the active hydroxy metabolites of both amitriptyline and imipramine.

We previously reported a liquid-chromatographic method for assay of imipramine, desipramine, and their 2-hydroxy metabolites (4). This method has now been modified and extended to include the analysis of eight additional drugs and metabolites. It is applicable for therapeutic drug monitoring and for screening for toxicity in overdose cases.

Materials and Methods

Apparatus

The chromatographic system consists of a Model 6000A solvent delivery system, a Model U6K injector, a Model 441
ultraviolet absorbance detector with measurement capability at both 214 and 254 nm, and an M730 Data Module (all from Waters Associates, Milford, MA 01757). The 4.6 mm × 25 cm chromatographic column contains Zorbax Sil (DuPont Instruments, Wilmington, DE 19898). The mobile phase is ammonium hydroxide in methanol (2/998 by vol) and the flow rate is 1.5 mL/min.

Reagents

**Stock borate buffer**, pH 9.0, is prepared according to deSilva and Puglisi (5). The buffer, stored in a polyethylene bottle at 35–37 °C to prevent crystallization of the salts, is prepared freshly every day. Aliquots of the stock buffer for use in the plasma extraction procedure are adjusted to pH 10.0 with 6 mol/L sodium hydroxide.

The extraction solvent consists of isooamyl alcohol/hexane (5/95 by vol).

Two separate internal standards are used: N-desmethylclomipramine HCl and desipramine HCl, each at 2 mg/L of methanol.

Hexane and methanol are both glass-distilled (Burdick and Jackson, Muskegon, MI 49442). The methanol is filtered through a Millipore HF filter and apparatus (Millipore, Bedford, MA 01730) before the mobile phase is prepared. All other chemicals and solvents are either certified ACS grade (Fisher Scientific Co., Fair Lawn, NJ 07410) or "Baker Analyzed" grade (J. T. Baker Chemical Co., Phillipsburg, NJ 08865).

Standards

Prepare separate stock solutions of the following drugs or metabolites in methanol to give free-base concentrations of 1 g/L: amitriptyline HCl, protriptyline HCl (Mercier, Sharp & Dohme, West Point, PA 19486); nortriptyline HCl (Eli Lilly & Co., Indianapolis, IN 46302); imipramine HCl, 2-hydroxy-desipramine oxalate, clomipramine HCl, N-desmethylclo-

Clomipramine hydrochloride, desmethyldoxepine HCl, maprotiline HCl (Ciba-Geigy Corp., Summit, NJ 07901); desipramine HCl (USV-Pharmaceutical Corp., Tarrytown, NY 10591); 2-hydroxyimipramine (Dr. Albert A. Manian, National Institute of Mental Health, Rockville, MD 20857); and doxepin HCl, desmethyldoxepin HCl (trans isomer) (Pfizer Inc., Brooklyn, NY 11206).

Using these, prepare three separate standards, in plasma:

**Standard I**: amitriptyline, nortriptyline, imipramine, desipramine, 2-hydroxyimipramine, and 2-hydroxydesipramine.

**Standard II**: doxepin and desmethyldoxepin.

**Standard III**: clomipramine, desmethyldoxipramine, maprotiline, and protriptyline.

Add diluted methanol solutions to drug-free, citrated human plasma to give a final concentration of each component of the calibration standards of 150 ng/mL, and of the (aqueous) controls at 30 and 300 ng/mL. Prepare the standards and controls every three months and store in polypropylene tubes at −4 °C.

Extraction and Chromatographic Procedures

To 0.5-mL of each plasma-based standard, control, and unknown, in polypropylene tubes, add the appropriate internal standard, 0.5 mL of borate buffer (pH 10), and 1.5 mL of the extraction solvent. N-Desmethylclomipramine is the standard for the assay of compounds in standards I and II; desipramine is the internal standard for the compounds in standard III. Shake the mixtures for 10 min. Using disposable glass pipets, transfer the organic layers to clean polypropylene tubes. Evaporate the extracts at ambient temperature, under nitrogen. Reconstitute each residue with 100 µL of methanol and inject 50 µL onto the chromatographic column. Measure the absorbance of compounds in standards I and II at 254 nm and standard III at 214 nm.

Flush the entire system with filtered methanol at the end of the analytical day. Equilibration of the system with fresh mobile phase requires less than 1 h.

Assay Comparisons

Serum samples containing the drugs and metabolites of interest, which had been previously assayed by the gas-chromatographic method of Gupta et al. (6), were kindly provided by Dr. R. N. Gupta (St. Josephs Hospital, Hamilton, Ontario). Since their initial analysis, the samples had been stored frozen in various types of tubes (glass, polyethylene, or polypropylene) and showed various degrees of hemolysis and lipemia. We re-analyzed these samples by the present procedure and used the weighted perpendicular least squares method (7) to correlate these results, a method that is appropriate when both variables contain experimental error.

Results and Discussion

New highly sensitive and specific liquid-chromatographic assays have enhanced the capability for routine therapeutic monitoring of several antidepressant drugs and their metabolites. Our normal-phase system provides the most comprehensive assay for tri- and tetracyclic antidepressants and their major demethylated and hydroxylated metabolites to date.

Figures 1 and 2 show chromatograms of extracts of the plasma-based standards and of serum from patients who had received the various antidepressants. Use of normal-phase chromatography allows these antidepressants and their metabolites to be simultaneously measured. The specific metabolite patterns, characteristic of each drug, aid identification of specimens. In vitro and in vivo experimental evidence (9–11) indicates that the 2-hydroxy metabolites of imipramine and desipramine, and the 10-hydroxy metabolites of amitriptyline and nortriptyline have pharmacological activity. Therefore, the metabolites may contribute to the therapeutic and (or) toxic effects of the parent drugs. Results of a single-dose pharmacokinetic study of imipramine in normal volunteers predicted little accumulation of the 2-hydroxy metabolites (12). However, Potter et al. (13) reported significant concentrations in plasma of patients at steady state. Although currently there is no need to report concentrations of the hydroxy compounds, they should be resolved and distinguished from the parent drugs.

Recent reports also indicate different pharmacological potencies for the cis and trans isomers of doxepin and desmethyldoxepine (14). The demethylated metabolite of maprotiline may also be pharmacologically active, but confirmatory studies are needed.

The Zorbax Sil columns perform efficiently for at least five to six months with almost daily exposure to 1 L of mobile phase. One typical column exhibited a decrease of only 2.8% in relative retention after nine months of use.

Linearity of detector response to the amount of compound being detected, as determined by direct injection of constant volumes of methanolic solutions of each drug and metabolite, was verified over the range of 5 to 500 ng for each.

Analytical recoveries of high and low (as compared with the usual therapeutic) concentrations of drugs and metabolites from plasma ranged from 75 to 85%.

Within-run precision (CV), based on analysis of 10 separate aliquots of plasma for each drug and metabolite, was 5% or less for most compounds. Day-to-day precision (CV), based on repeat analyses during two to three months, was <10% for all drugs and metabolites. As little as 10 ng of
each compound per milliliter can be accurately quantified in 0.5 mL of plasma.

We examined potential interference from benzodiazepines—frequently administered with tricyclic antidepressants—by directly injecting methanolic solutions of chlordiazepoxide, diazepam, flurazepam, and oxazepam. All the peaks associated with these compounds were eluted with the solvent front. Moreover, plasma samples from patients who were being treated with chlordiazepoxide or diazepam also showed no apparent interfering peaks. Phenothiazines are occasionally administered with tricyclic antidepressants, and they pose the greatest problem of potential interference because of their numerous and highly ultraviolet-absorbing metabolites. We analyzed several plasma samples from patients known to be taking a phenothiazine and an antidepressant. Figure 1 depicts a chromatogram from the plasma of a patient taking doxepin and thioridazine and exemplifies the type of pattern usually observed in these patients. Despite the cluster of unidentified peaks eluting in the middle of the chromatogram, the antidepressant concentrations can still be accurately estimated. However, the analyst should be informed about all medications that a patient is taking, so that interference from extraneous peaks can be anticipated.

We used this method on more than 100 clinical plasma samples previously analyzed elsewhere by a gas-chromato-

graphic method (6). The narrow ranges of concentrations and the small numbers of samples in some cases preclude precise interpretation of the results of regression analysis. Many factors, including different methods of standard calibration, the purity of the standards, differences in assay specificity and precision, and sample handling and storage between analyses, could have affected the results. However, the correlations shown in Figure 3 indicate generally good agreement between the two methods.

Our results for 115 other samples from patients receiving these drugs, and their estimated therapeutic ranges, are shown in Figure 4. More than half of the results fell below the therapeutic ranges; less than a quarter were within the ranges. However, the concentrations typically exceeded the minimum concentration that can be measured (10 ng/mL) and fell within the range of standards and controls (30–300 ng/mL).

Therapeutic monitoring of antidepressants has been recommended to check for compliance, to detect cases of unusu-
Fig. 3. Correlation between the present method and a gas-chromatographic method in analysis for six antidepressants in plasma
Abbreviations as in Figs. 1, 2. Slopes and intercepts of regression lines for each compound are: AMT, 1.360; -12.7; NRT, 0.962; 0.0; DXP, 0.828; 3.8; DMX; 0.880; 4.8; IMI, 0.934; 1.7; DMI, 0.920; -11.5; and MPT, 1.020; 7.6

Fig. 4. Results by this method for serum from patients receiving chronic therapy with antidepressants
Results shown are the sums of the parent drugs and demethylated metabolites, or parent drug alone (MPT). The brackets represent therapeutic ranges. Abbreviations as in Figs. 1, 2

al drug disposition, to ascertain the causes of unusual side effects, to avoid excessive concentrations in the very young or elderly, to prevent or detect complications due to drug interactions or disease, and ultimately to optimize dosage regimens. Our normal-phase chromatographic system and single-step extraction provides a method for simultaneously measuring the most widely used tri- and tetracyclic antidepressants and their major active metabolites. The method is applicable to both therapeutic monitoring and to screening for toxicity.

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References