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# Simultaneous Determination of Imipramine, Desipramine, and Their 2-Hydroxy Metabolites in Plasma by Ion-Pair Reversed-Phase High-Performance Liquid Chromatography with Amperometric Detection

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**Abstract** □ An ion-pair reversed-phase high-performance liquid chromatographic (HPLC) method, using an electrochemical detector, is presented for the simultaneous and rapid quantitation of imipramine, desipramine, and their 2-hydroxylated metabolites in plasma. The drugs are extracted from 1 ml of plasma at pH 9.7 with ether, back-extracted into 0.1 *M* HCl, and reextracted into ether following alkalinization. An efficient electrochemical oxidation reaction at the detector electrode affords a low detection level of ~5 ng/ml in a mobile phase of acetonitrile–acetate buffer (40:60) containing 0.005 *M* heptanesulfonate. Patient data are presented as correlations between the plasma level of each hydroxy metabolite and its respective parent compound. The method is applicable to the laboratory experienced in HPLC.

**Keyphrases** □ Imipramine—simultaneous determination with desipramine and their 2-hydroxy metabolites, ion-pair reversed-phase high-performance liquid chromatography □ Desipramine—simultaneous determination with imipramine and their 2-hydroxy metabolites, ion-pair reversed-phase high-performance liquid chromatography □ High-performance liquid chromatography—simultaneous determination of imipramine, desipramine, and their 2-hydroxy metabolites

Considerable interest exists in the relationship between the plasma concentration of tricyclic antidepressant drugs and the therapeutic outcome or side effects, and this subject was reviewed recently (1, 2). There seems to be a consensus that there is a therapeutic range for nortriptyline and a minimum effective level for imipramine plus desipramine in patients with "endogenous-type depression"; a therapeutic range also was suggested for desipramine (3). Data for amitriptyline, however, are much more controversial (4), and other tricyclic antidepressants have not been studied adequately.

## BACKGROUND

Until recently, the role of the hydroxylated metabolites of the tricyclic antidepressant drugs has been largely ignored, because it was assumed (erroneously) that these metabolites were not psychoactive, did not cross the blood–brain barrier, and were rapidly excreted. Christianssen and Gram (5) demonstrated that these hydroxylated metabolites were present in the central nervous system in an acute overdose case. Several studies demonstrated considerable quantities of unconjugated and conjugated hydroxy metabolites of tricyclic antidepressants in the plasma of treated patients (6–11).

It is now known that the hydroxy metabolites of imipramine have strong cardiovascular activity (12, 13) and are essentially equipotent to the parent compound in the blockade of norepinephrine and 5-hydrox-

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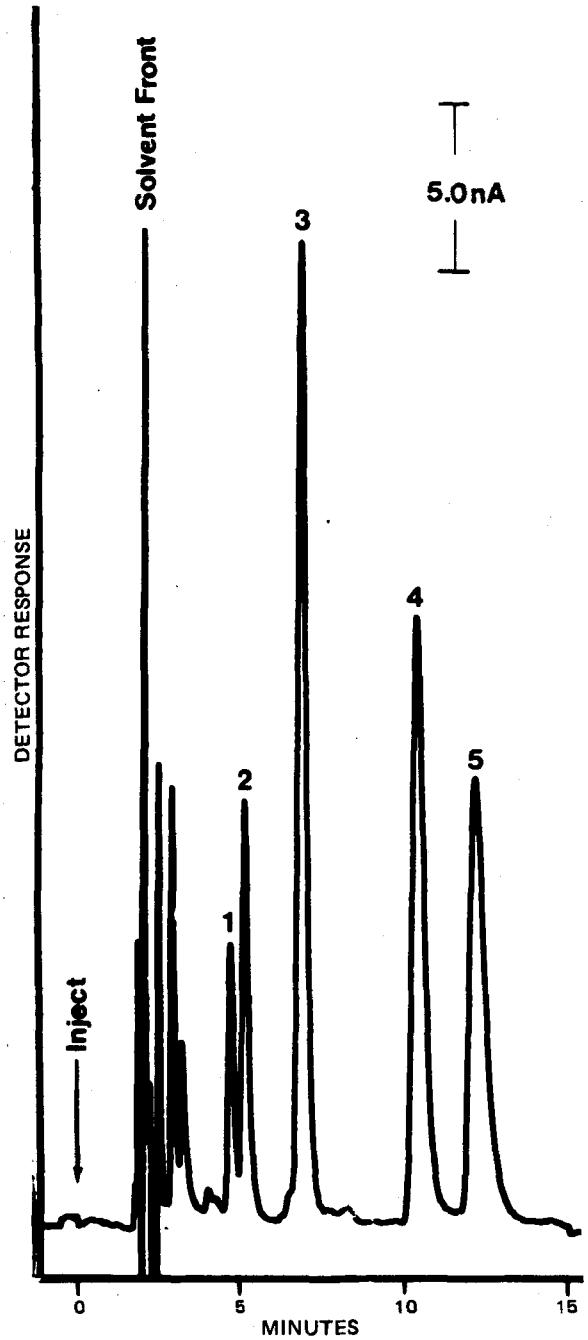


**Figure 1**—*Sample chromatogram of a 1-ml spiked plasma extract containing 25 ng of 2-hydroxydesipramine (1), 25 ng of 2-hydroxyimipramine (2), 100 ng of 8-hydroxyclomipramine (3), 50 ng of desipramine (4), and 50 ng of imipramine (5). One-half (50 μl) of the reconstituted extract was injected.*

ytryptophan receptors in rat brain slices, and isolated synaptosomal fractions (14–17). Also, the specificity of the respective hydroxylated metabolites is maintained, *e.g.*, 2-hydroxydesipramine was 100 times more potent in inhibiting norepinephrine than 5-hydroxytryptophan accumulation. These metabolites are present in the plasma in the same order of magnitude as the primary and secondary amines (10, 11). These data indicate that examination of the hydroxy metabolites may give insight into the clinical efficacy–side-effect relationship to the plasma drug levels.

The methods of analysis of the tricyclic antidepressants and their metabolites were reviewed recently (18). All methods involving high-performance liquid chromatography (HPLC) employed UV detection at various wavelengths. However, most of these methods suffer from poor resolution of the metabolites, interfering endogenous substances, or analysis time lasting >15 min. An assay for imipramine, desipramine,

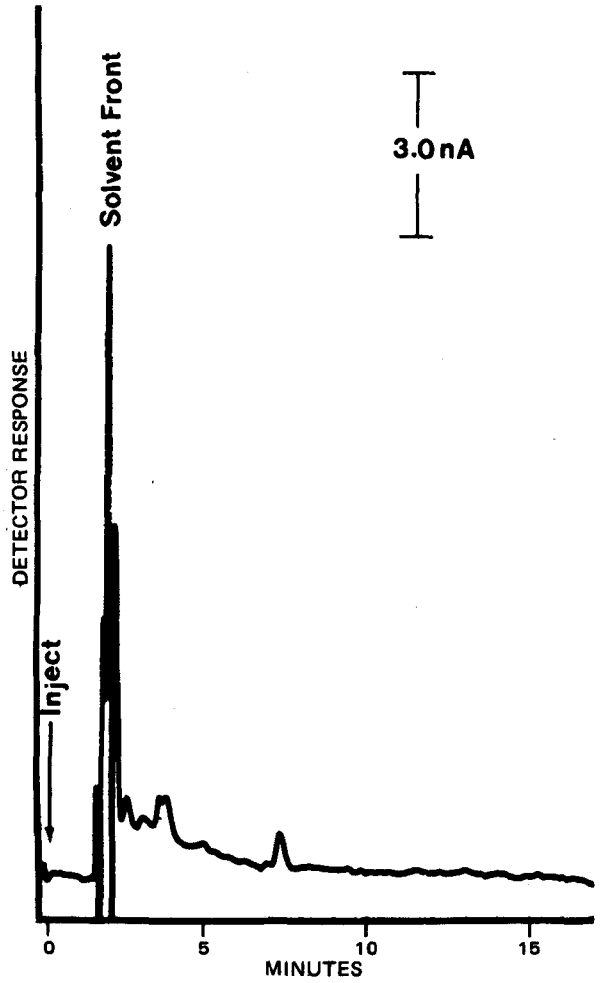


**Figure 2**—*Sample chromatogram of a 1-ml blank plasma extract. One-half (50 μl) of the reconstituted extract was injected.*

and their 2-hydroxylated metabolites in plasma was reported using a fluorescence detector and achieving a 1-ng/ml low level of detection (19). However, silica columns, as used in the preceding method, may deteriorate quickly when used at pH > 7.

Therefore, an assay was developed using ion-pair reversed-phase HPLC with amperometric detection for the rapid, sensitive, and simultaneous quantitations of imipramine, desipramine, and their 2-hydroxylated metabolites in plasma.

## EXPERIMENTAL

**Reagents**—Acetonitrile[1] was HPLC grade. Ether[1] was distilled prior to use. Acetic acid[1], sodium acetate[1], sodium carbonate[1], and sodium bicarbonate[1] were ACS reagent grade. Sodium heptanesulfonate[2] was used as received. Distilled water was passed through a water purification system[3] before use.

**Apparatus**—A high-performance liquid chromatograph[4] with a 2-ml syringe loading injector[5] was used. Chromatography was performed on a reversed-phase column[6]. The detector system consisted of a thin-layer flow-through electrochemical cell[7] with glassy carbon as the working and auxiliary electrodes and a silver–silver chloride reference electrode. The working electrode was directly opposite to the auxiliary electrode in the detector cell. This configuration was essential for increasing the linearity by several orders of magnitude to drug concentrations of >1 μg/ml. The potential and current response were monitored by an amperometric

[1] Fisher Scientific Co., Fair Lawn, N.J.
[2] Eastman Kodak Co., Rochester, N.Y.
[3] Milli-Q, Millipore Corp., Bedford, Mass.
[4] Model ALC/GPC 204, Waters Associates, Milford, Mass.
[5] UK6, Waters Associates, Milford, Mass.
[6] μBondapak $C_{18}$ (30 × 0.39 cm i.d.), Waters Associates, Milford, Mass.
[7] TL-5A, BioAnalytical Systems, West Lafayette, Ind.

**Table I—Recovery of Imipramine, Desipramine, 2-Hydroxyimipramine, and 2-Hydroxydesipramine (50 ng/ml Each) from 1 ml of Plasma**

| Compound | Recovery, % | SD | CV, % |
|---|---|---|---|
| 2-Hydroxyimipramine | 76 | 2.9 | 3.8 |
| 2-Hydroxydesipramine | 60 | 1.7 | 2.8 |
| Imipramine | 89 | 5.8 | 6.5 |
| Desipramine | 84 | 4.6 | 5.6 |

controller[8] and recorder[9], interfaced with a laboratory data acquisition system[10].

**Standards**—Stock solutions of imipramine hydrochloride[11] (1.13 mg/ml), desipramine hydrochloride[12] (1.13 mg/ml), 2-hydroxydesipramine free base[13] (1 mg/ml), and 2-hydroxyimipramine free base[13] (1 mg/ml) were prepared in methanol. The internal standard, 8-hydroxyclomipramine free base[13] (1 mg/ml), also was prepared in methanol.

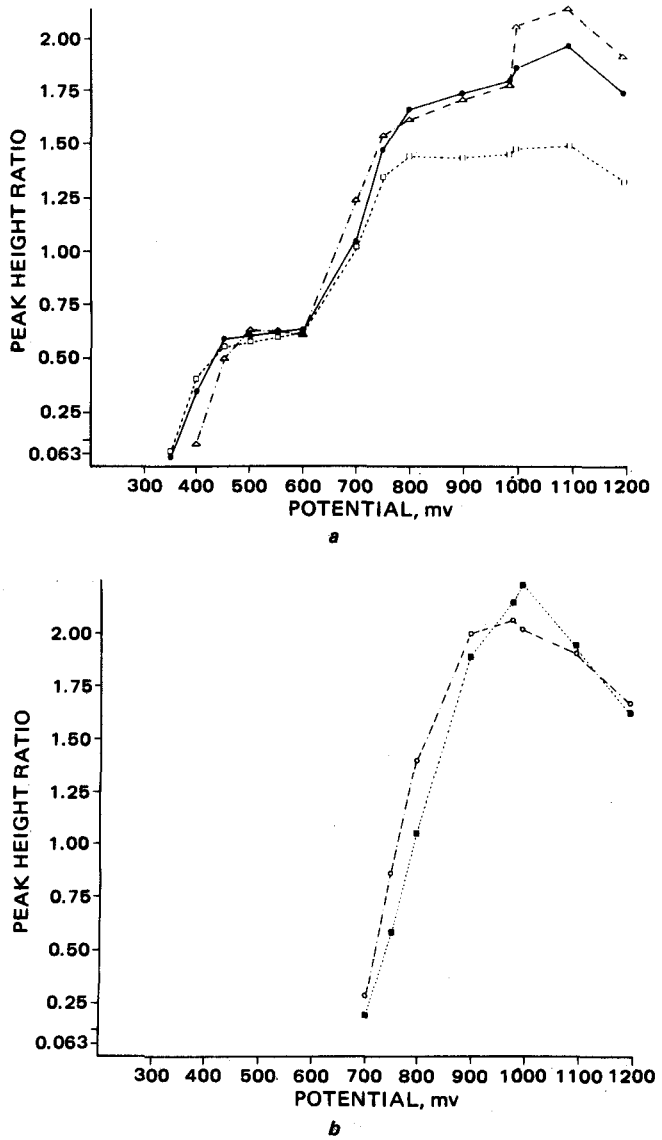


**Figure 3**—(a) *Plot of the peak height ratio against the potential* (versus *silver–silver chloride) for 2-hydroxyimipramine* (●), *2-hydroxydesipramine* (□), *and 8-hydroxyclomipramine* (△). *(b) Plot of the peak height ratio against the potential* (versus *silver–silver chloride) for desipramine* (○) *and imipramine* (■). *The chromatographic conditions were the same as described previously.*

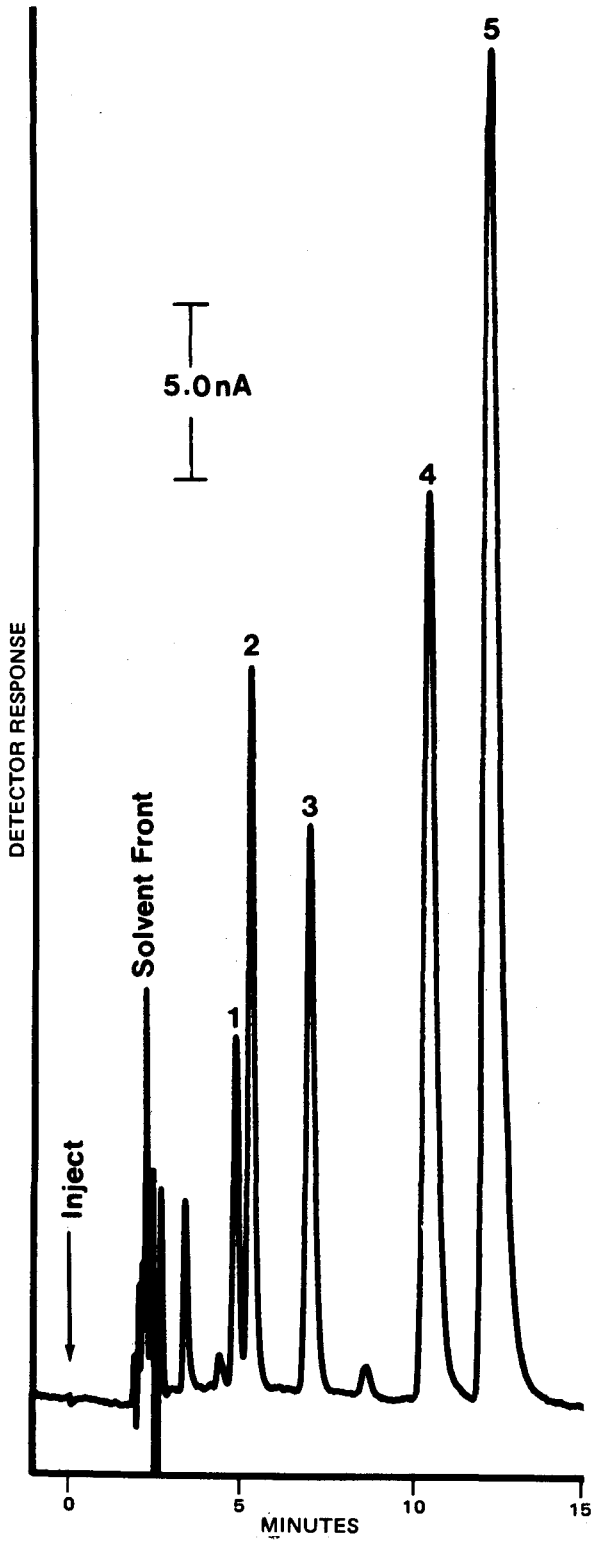


**Figure 4**—*Sample chromatogram of a 1-ml plasma sample from a patient receiving imipramine. One-quarter (25 μl) of the reconstituted extract was injected. The plasma levels were: 2-hydroxydesipramine (1), 54 ng; 2-hydroxyimipramine (2), 74 ng; internal standard (3); desipramine (4), 129 ng; and imipramine (5), 267 ng.*

[8] Metrohm V/A detector 611, Brinkmann Instruments, Westbury, N.Y.
[9] Houston Omniscribe model B5217B-2, Houston Instruments, Austin, Tex.
[10] Digital PDP/11/34 "Peak II" system, Digital Equipment Corp., Maynard, Mass.
[11] Ciba-Geigy Corp., Ardsley, N.Y.
[12] USV Pharmaceutical Corp., Tuckahoe, N.Y.
[13] A gift of Dr. Albert A. Manian, National Institute of Mental Health, National Institutes of Health, Rockville, Md.

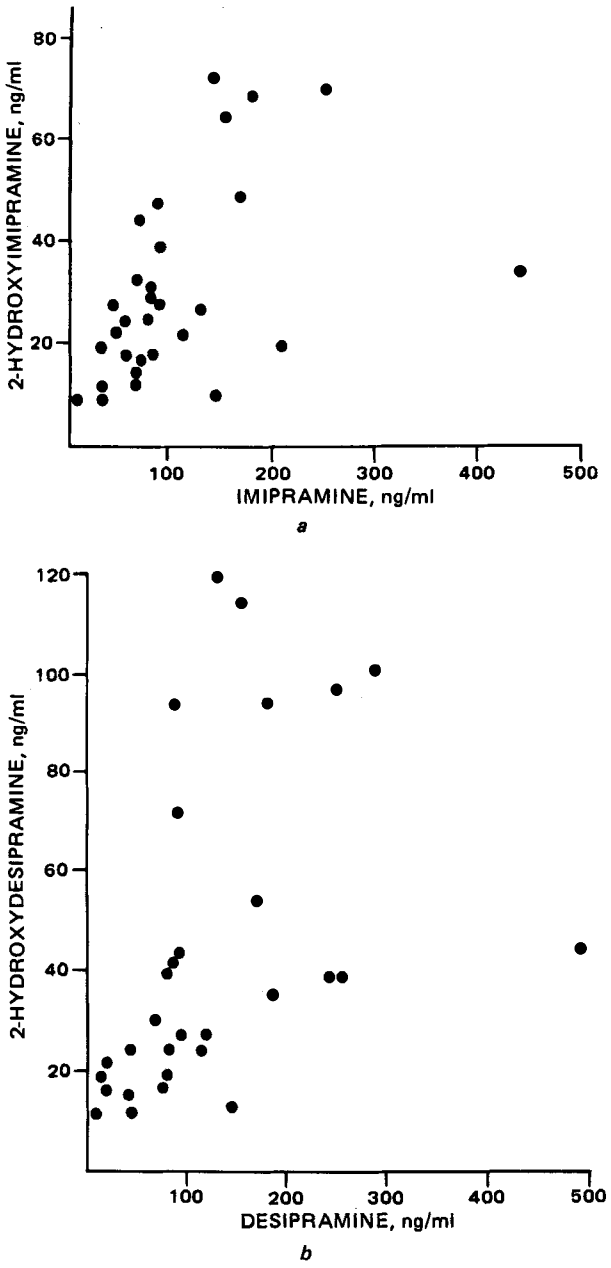


**Figure 5**—*(a) Relationship between plasma imipramine and 2-hydroxyimipramine levels in 30 randomly selected patients (r = 0.46). (b) Relationship between plasma desipramine and 2-hydroxydesipramine levels in the same patients (r = 0.40).*

Standard curves were prepared by the addition of the standards to drug-free plasma to give final concentrations of 25–400 ng of imipramine and desipramine/ml and of 10–200 ng of each of the 2-hydroxy metabolites.

**Extraction**—To 1.0 ml of plasma standard or unknown, 1.0 ml of water, 50 μl (100 ng) of the internal standard, and 1.0 ml of 0.6 *M* carbonate buffer (pH 9.7) were added in specially washed glassware[14]. Extraction was carried out by the addition of 8 ml of freshly distilled ether, and the solution was shaken and centrifuged for 15 min. The ether layer then was qualitatively transferred to 15-ml tapered centrifuge tubes containing 1.2 ml of 0.1 *M* HCl. After shaking and centrifuging for 10 min, the top layer was aspirated and the aqueous layer was transferred to 3-ml tapered, glass-stoppered, minicentrifuge tubes.

Neutralization resulted from the addition of 0.5 ml of 0.6 *M* carbonate buffer (pH 9.7). Ether (0.8 ml) was added, and the solution was shaken for 5 min and centrifuged for 5 min. The lower aqueous layer was discarded, and the ether layer was transferred to 1.0-ml reaction vials[15] and evaporated to dryness under a nitrogen stream at room temperature. The extract was reconstituted with 100 μl of the mobile phase, and the vial was capped.

**Chromatographic Conditions**—The mobile phase consisted of 0.1 *M* acetate buffer (pH 4.2) and acetonitrile (60:40). The paired-ion sodium heptanesulfonate (0.005 *M*) was added, and the mixture was filtered[16] and degassed prior to use. The flow rate was 1.3 ml/min, and the temperature was ambient. The effluent was monitored through the detector cell at a potential of +1.05 v *versus* the silver–silver chloride reference electrode.

**Quantitation**—All determinations were performed by calculating the peak height ratios of each compound to the internal standard. A standard curve was run with each set of samples.

## RESULTS AND DISCUSSION

Imipramine, desipramine, and their 2-hydroxylated metabolites were quantitated simultaneously in plasma. Each chromatogram took <15 min to elute all five compounds (Fig. 1).

The absolute sensitivity of this method was 1 ng injected for all four compounds (signal to noise ≃3). In practical terms, however, the lowest quantifiable levels were ~5 ng/ml of plasma. The blank plasma extracts showed no interfering endogenous substances (Fig. 2). This result was due to a careful sample cleanup, selectivity of the electrochemical detector, and the nature of the nonpolar bonded reversed phase, which eluted most hydrophilic biological material quickly.

The absolute recovery was checked by preparing a solution containing 50 ng of each 2-hydroxy metabolite, imipramine, and desipramine. The internal standard was added to five aliquots, and the sample was injected directly into the chromatograph. Then 1.0 ml of plasma was added to five additional aliquots, and the samples were processed routinely except for the internal standard, which was added to the vials after the samples were evaporated to dryness. The difference between the standard and the internal standard in the processed samples compared to the direct-injection sample gives a measure of the overall recovery (Table I).

The precision of the reported procedure was determined by spiking 10 1.0-ml aliquots of drug-free plasma with 25 ng of 2-hydroxyimipramine, 2-hydroxydesipramine, and 50 ng of imipramine and desipramine. After the addition of 100 ng of internal standard, each aliquot was processed as already described. The resulting coefficient of variation for each compound was 3.1% for 2-hydroxyimipramine, 8.7% for 2-hydroxydesipramine, 3.6% for imipramine, and 4.0% for desipramine.

8-Hydroxyclomipramine was chosen as the internal standard because of its structural similarity to all four compounds, its relative retention time, and its sensitive detector response. Its isomer, 2-hydroxyclomipramine, also may be used since the retention times differ by <1 min and it does not interfere with the assay.

Several drugs were tested using this assay to determine potential interference. When patient samples were tested, some high-dose major tranquilizers such as chlorpromazine, thioridazine, prochlorperazine, and loxapine did interfere with either the parent drug or, more frequently, the more polar metabolites. Low-dose major tranquilizers such as fluphenazine, perphenazine, and haloperidol and their metabolites are found therapeutically in very low concentrations in plasma and did not present significant interference problems.

Other tricyclic antidepressants such as nortriptyline, its 10-hydroxy metabolite, and protriptyline were not detected electrochemically but did appear on a simultaneous chromatogram with a UV detector. Amitriptyline and its 10-hydroxy metabolite were detected electrochemically but did not display a substantial response, as compared to the simultaneous UV run chromatogram. The benzodiazepines frequently are administered as adjuvants in these affective disorders and could interfere if UV detection were used. However, none of the benzodiazepines tested (chlordiazepoxide, diazepam, and flurazepam) is electrochemically active under the prevailing conditions. It would be prudent, however, to examine the medication profile of a patient prior to this assay (as with any other) to anticipate possible interferences.

The tricyclic antidepressant compounds containing the dibenzcycloheptane nucleus (*e.g.*, amitriptyline and nortriptyline) are devoid of, or have little, electrochemical reactivity under the described conditions. Therefore, the sensitivity of the electrochemically active dibenzazepine tricyclic antidepressants probably is due, at least in part, to the presence

[14] All glassware was treated as reported previously (20).

[15] Reactivials, Pierce Chemical Co., Rockford, Ill.

[16] Millipore type FH, 0.5 μm, Millipore Corp., Bedford, Mass.

of the ring nitrogen. A mechanism of electrochemical oxidation of these dibenzazepines seems best described by a two-step, three-electron ECE[17] process (21). The first step involves the transfer of one electron to form a radical, followed by dimerization. This compound then is oxidized further by an overall two-electron process at a more negative potential.

To determine the optimum positive potential for the five compounds used in this assay, current–potential curves were generated by injecting a standard solution of these compounds in methanol into the chromatograph at various potentials. A fixed-wavelength UV detector[18] (254 nm) was used as an "internal standard," connected in series preceding the electrochemical detector cell. The peak heights from the electrochemical response at various potentials were compared to the corresponding peak height from the UV detector response. These ratios were plotted against potentials. Figure 3*a* indicates that the 2-hydroxylated metabolites undergo oxidation at potentials more negative than the parent compound, probably due to the presence of the ring hydroxyl group. A second wave corresponds to the oxidation of the parent compounds (Fig. 3*b*).

Substantial levels of the 2-hydroxy metabolites can be found in patients receiving imipramine (Fig. 4). The relationships of the plasma concentrations of imipramine and desipramine to their respective 2-hydroxy metabolites are shown in Figs. 5*a* and 5*b*, respectively. These data show only a weak correlation, indicating the wide interindividual variations in the metabolic rates and pathways. A similar relationship between nortriptyline and 10-hydroxynortriptyline in plasma was reported with a somewhat stronger correlation ($r = 0.63$) (10).

These relationships may explain, in part, the variability in therapeutic response to the tricyclic antidepressants. Therefore, plasma level studies of these agents should include the active hydroxy metabolites when comparing the plasma levels and clinical efficacy and/or side effects.

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[17] An electron-transfer step followed by a chemical reaction and a second electron-transfer step.

[18] Model 440, Waters Associates, Milford, Mass.

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