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# QUANTIFICATION OF AMITRIPTYLINE, NORTRIPTYLINE, AND 10-HYDROXY METABOLITE ISOMERS IN PLASMA BY CAPILLARY GAS CHROMATOGRAPHY WITH NITROGEN-SENSITIVE DETECTION

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## SUMMARY

A selective, sensitive method for the determination of amitriptyline and its metabolites is described. This method involves liquid—liquid extraction and capillary gas chromatography with nitrogen-sensitive detection. The detection limits of amitriptyline, nortriptyline, 10-hydroxy(*E*)amitriptyline, 10-hydroxy(*E*)nortriptyline, and 10-hydroxy(*Z*)nortriptyline were slightly less than 0.5 ng/ml in 1.0-ml plasma samples. The coefficients of variation for within-run and between-run analyses of samples containing 100 ng/ml were less than 12% and 9%, respectively. The method offers rapid analysis of individual isomers, increased sensitivity over high-performance liquid chromatographic methodology and the conveniences of the gas chromatographic technique.

## INTRODUCTION

In recent years the tricyclic antidepressants (TCAD) have been widely used for the treatment of endogenous depression. Two of the most prescribed TCADs are amitriptyline (Ami) and its active desmethyl metabolite, nortriptyline (Nor). Amitriptyline metabolism in humans yields nortriptyline via N-demethylation, or 10-hydroxyamitriptyline (*E* or *Z* isomers) via hydroxylation. Nortriptyline, in turn, can be metabolized to 10-hydroxynortriptyline (*E* or *Z* isomers). These structures and metabolic pathways are illustrated in Fig. 1.

Plasma levels of amitriptyline and nortriptyline have been measured clinically in attempts to establish a therapeutic range of concentration in plasma [1]. However, correlations between steady-state plasma level and clinical response have been questioned [2, 3]. Low correlation can be attributed

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to many factors. Among these are activities of unmeasured metabolites, inaccuracies in measurements, differences in specimen collection and handling, and heterogeneity of response to drug.

Recently, studies have shown that 10-hydroxy metabolites of amitriptyline and nortriptyline possess pharmacological activities similar to their parent compounds in inhibiting norepinephrine and serotonin uptake in vitro. However, these metabolites have been shown to possess different degrees of pharmacological activity [4, 5]. If these 10-hydroxy metabolites exhibit pharmacological activity in vivo, the quantitation of these metabolites should be included in studies involving efficacy versus plasma levels.

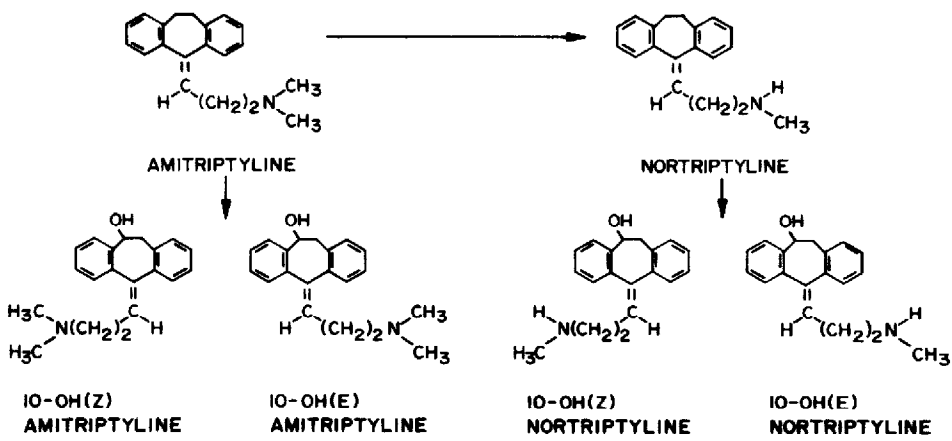



Fig. 1. Metabolic pathways of amitriptyline.

Methods have been developed to quantitate 10-hydroxy metabolites of amitriptyline and nortriptyline. Initial gas chromatographic (GC) methods [6—10] involved dehydration of these metabolites to unsaturated products having suitable GC properties since the isomers tail and are poorly resolved on conventional packed columns. In the case of 10-hydroxyamitriptyline, this dehydration of both *E* and *Z* isomers yielded the drug cyclobenzaprine (CAS-6202-23-9). These initial methods lacked the ability to distinguish and quantitate the individual isomers of 10-hydroxyamitriptyline or 10-hydroxynortriptyline. On the other hand, these individual isomers have been quantitated by recently developed high-performance liquid chromatography (HPLC) methodology [11—15]. Limits of sensitivity of these methods are approximately 5—10 ng/ml using 1—3 ml of serum.

In order to utilize the sensitivity of nitrogen—phosphorus detection and alternatively, the convenient interfacing for mass spectrometry afforded by GC methodology, we developed a GC method using fused-silica capillary columns. In this method we report an assay that offers greater sensitivity, superior resolution, and rapid analysis of amitriptyline, nortriptyline, and the 10-hydroxy isomers in physiological samples.

## EXPERIMENTAL

### *Materials*

Amitriptyline {10,11-dihydro-5-[3-(dimethylamino)propylidene]-5H-dibenzo-[*a*,*d*]cycloheptene hydrogen chloride} (CAS-549-18-8) and nortriptyline {10,11-dihydro-5-[3-(methylamino)propylidene]-5H-dibenzo[*a*,*d*]cycloheptene hydrogen chloride} (CAS-894-71-3) were obtained from the United States Pharmacopeial Convention (USPC). The hydroxy metabolites, 10-hydroxy-(*E*)amitriptyline {(*dl*)-(*E*)-10,11-dihydro-5-[3-(dimethylamino)propylidene]-5H-dibenzo[*a*,*d*]cyclohepten-10-ol} (CAS-64520-05-4), 10-hydroxy(*E*)nortriptyline {(*dl*)-(*E*)-10,11-dihydro-5-[3-(methylamino)propylidene]-5H-dibenzo[*a*,*d*]-cyclohepten-10-ol hydrogen maleate} (CAS-47132-16-1), and 10-hydroxy-(*Z*)nortriptyline {(*dl*)-(*Z*)-10,11-dihydro-5-[3-(methylamino)propylidene]-5H-dibenzo[*a*,*d*]cyclohepten-10-ol oxalate ethanolate} (CAS-47132-19-4) were synthesized by a previous method [16] and were gifts from Merck Sharp and Dohme (Rahway, NJ, U.S.A.). The 10-hydroxy(*Z*)amitriptyline isomer was unavailable. The two internal standards, protriptyline [5-(3-methylamino-propyl)-5H-dibenzo[*a*,*d*]cycloheptene hydrogen chloride] (CAS-1225-55-4) and chlorprothixene [2-chloro-9-(3-dimethylaminopropylidene)thioxanthene] (CAS-113-59-7) were also purchased from the USPC.

### *Reagents*

Hydrochloric acid and sodium hydroxide were analytical grade. Hexane, 2-butanol, methanol, water and *n*-butyl acetate were HPLC solvent grade.

### *Standards*

Stock standards of each compound were prepared as 1 mg free base per ml of methanol. All other working standards (1, 10, 100 ng/μl methanol) were prepared from these stock standards. A working solution containing both internal standards was diluted with water to obtain a concentration of 0.5 ng/μl.

### *Extraction procedure*

For each analysis, 1 ml of plasma or serum was placed into a silanized 15-ml culture tube and a 100-μl aliquot of the internal standard solution containing protriptyline and chlorprothixene was added to produce a concentration of 50 ng/ml of plasma. Next, the solution was adjusted to pH 14 by addition of 1 ml of 4 *M* sodium hydroxide and was vortex-mixed for 10 sec. This mixture was extracted with 8 ml of hexane—2-butanol (98:2, v/v), mixed for 2 min, and centrifuged for 2 min. The hexane—2-butanol (upper phase) was transferred to a second silanized 15-ml culture tube. One ml of 0.001 *M* hydrochloric acid was added to the hexane—2-butanol that contained the extracted drug. This solution was mixed for 2 min and centrifuged for 2 min. Then the organic layer was aspirated and discarded. Using a borosilicate Pasteur pipette, the acid phase was transferred to a 15-ml conical-tipped tube containing 0.5 ml of 4 *M* sodium hydroxide and the solution was mixed for 10 sec. Next, a 100-μl aliquot of *n*-butyl acetate was added

to this mixture. This solution was mixed for 2 min and centrifuged for 2 min. Then most of the aqueous phase was withdrawn and discarded using a borosilicate Pasteur pipette. A fraction of the *n*-butyl acetate phase (0.5—8.0 μl) was removed and injected into the gas chromatograph.

*Equipment*

The gas chromatograph was a Hewlett-Packard 5710 equipped with a 18740B capillary injector and a 18789A nitrogen—phosphorus detector (Hewlett-Packard, Palo Alto, CA, U.S.A.). A Hewlett-Packard 3390A integrator was used that printed peak height values after each run.

The column was a 15 m × 0.32 mm I.D. Durabond® capillary column with DB-5 stationary phase (J & W Scientific, Rancho Cordova, CA, U.S.A.). The DB-5 stationary phase is a non-extractable bonded phase equivalent to SE-54. Injector and detector temperatures were 200°C and 300°C, respectively. The fused-silica injection port insert was silanized before use. After injection in splitless mode, the split was opened 40 sec later. The column temperature raised from 120°C to 250°C at 32°C/min upon injection. Helium (carrier) pressure to the column was 69 kPa resulting in a column linear velocity of 48 cm/sec. Detector gas flow-rates were hydrogen, 3 ml/min; air, 50 ml/min; and helium (make-up), 30 ml/min. Detector voltage offset was adjusted to 10% at attenuation 32 when the oven temperature was 250°C.

*Quantitation*

The internal standards, protriptyline and chlorprothixene, were incorporated into the specimens to aid in quantitation. Concentrations for specific compounds (amitriptyline, nortriptyline, or the 10-hydroxy metabolites) were determined from standard curves for each compound. These curves were derived from analyses of standard concentrations (10, 25, 50, 100, 250, 500, and 1000 ng/ml) added to drug-free plasma. The ratios of the peak height of each compound to that of protriptyline were plotted versus the standard concentrations of each compound. These standard curves were linear across the entire concentration range for all compounds.

*Precision*

To evaluate the precision of this method, within-run and between-run coefficients of variation (C.V.) were calculated. For both calculations, data were used from two concentrations (10 ng/ml and 100 ng/ml) of amitriptyline, nortriptyline and each 10-hydroxy metabolite in plasma. To estimate the within-run C.V., concentrations were calculated from six or seven assays of each of these two plasma samples using a single standard curve for each compound.

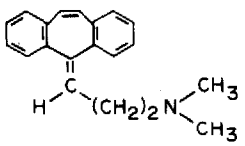
To calculate the between-run C.V., seven tubes of each of the two concentrations were frozen for analysis in seven subsequent runs. A standard curve was constructed for each run. Concentrations were determined in duplicate, and mean values from each of the seven runs were used to calculate the between-run C.V.

*Efficiency*

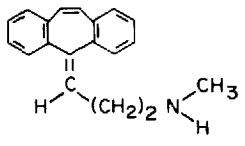
The analytical efficiency of the method was calculated using the internal standard, chlorprothixene. Reference standards were prepared containing known amounts of amitriptyline, nortriptyline, each of the 10-hydroxy metabolites and protriptyline in conical-tipped tubes. A known amount of chlorprothixene (50 ng) was added to each. Then *n*-butyl acetate was added to these reference standards to obtain a final volume of 100 μl. Plasma samples were prepared for extraction containing amounts of each drug identical to the reference standards. Samples of each concentration were extracted and reconstituted with 100 μl of *n*-butyl acetate containing 50 ng of chlorprothixene. Sample peak height ratios of each compound to chlorprothixene were compared to the peak height ratios from the reference standards to determine the percentage analytical efficiency.

*Dehydration*

To obtain dehydration of the 10-hydroxy metabolites, each metabolite was treated at room temperature for 2 h with 12 *M* hydrochloric acid to obtain the products illustrated in Fig. 2. The solutions were adjusted to pH 14 with 4 *M* sodium hydroxide, extracted and analyzed as above.




DEHYDRATED FORM OF
10-OH AMITRIPTYLINE




DEHYDRATED FORM OF
10-OH NORTRIPTYLINE

Fig. 2. Structures resulting from dehydration of 10-hydroxy metabolites of amitriptyline and nortriptyline.

## RESULTS AND DISCUSSION

Since the TCAD and metabolites had terminal amines (secondary or tertiary), the extraction under basic conditions eliminated acidic materials while the subsequent acidic extraction eliminated neutral organic impurities. The final partitioning of drug into *n*-butyl acetate following alkalinization allowed injections to be made directly from this solvent. This technique obviated evaporation of samples to dryness, which requires a significant amount of time, introduces undesirable variation, and results in sample loss [17]. Chromatograms with the retention times of each of the compounds are displayed in Fig. 3 along with a chromatogram from drug-free plasma.

*Standardization*

Two internal standards, protriptyline and chlorprothixene, were added to all samples to normalize extraction efficiency in the quantitation of each compound. The former, a secondary amine, and the latter, a tertiary amine, were used due to their structural similarities to nortriptyline and amitrip-

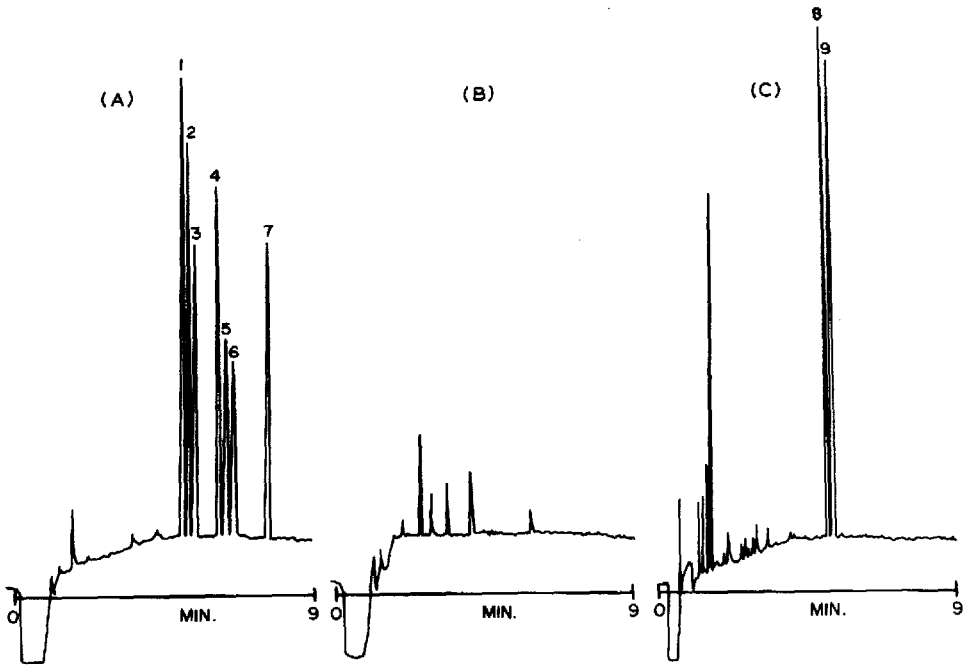



Fig. 3. Chromatograms of (A) amitriptyline, metabolites, and internal standards protriptyline and chlorprothixene; (B) drug-free plasma extracts; (C) dehydrated products from 10-hydroxyamitriptyline and -nortriptyline. Peaks: 1 = amitriptyline (5.05 min); 2 = nortriptyline (5.16 min); 3 = protriptyline (5.40 min); 4 = 10-hydroxy(*E*)amitriptyline (6.17 min); 5 = 10-hydroxy(*E*)nortriptyline (6.37 min); 6 = 10-hydroxy(*Z*)nortriptyline (6.50 min); 7 = chlorprothixene (7.66 min); 8 = dehydrated form of 10-hydroxyamitriptyline (5.35 min); 9 = dehydrated form of 10-hydroxynortriptyline (5.41 min).

tyline, respectively. We have found that protriptyline was the better internal standard for quantitation of all compounds as assessed by the standard curve linearity. Each line was fitted using least-squares regression analysis. Typical standard curves, using protriptyline as the internal standard are shown in Fig. 4.

*Dehydration*

Each of the 10-hydroxy metabolites has been shown to dehydrate readily in a strong acidic environment. A concentration of 0.01 *M* hydrochloric acid or greater produced detectable quantities of the dehydrated compounds in the chromatogram, as shown in Fig. 3. Note that the dehydrated products of 10-hydroxynortriptyline elute with protriptyline and interfere with the quantitation of every compound. On the other hand, we have found no detectable formation of dehydrated products with 0.001 *M* hydrochloric acid as used in our method. This allows individual quantitation of the 10-hydroxy isomers.

*Precision*

The precision of this method was determined using within-run and between-run C.V. of plasma samples containing 10 and 100 ng/ml of each com-

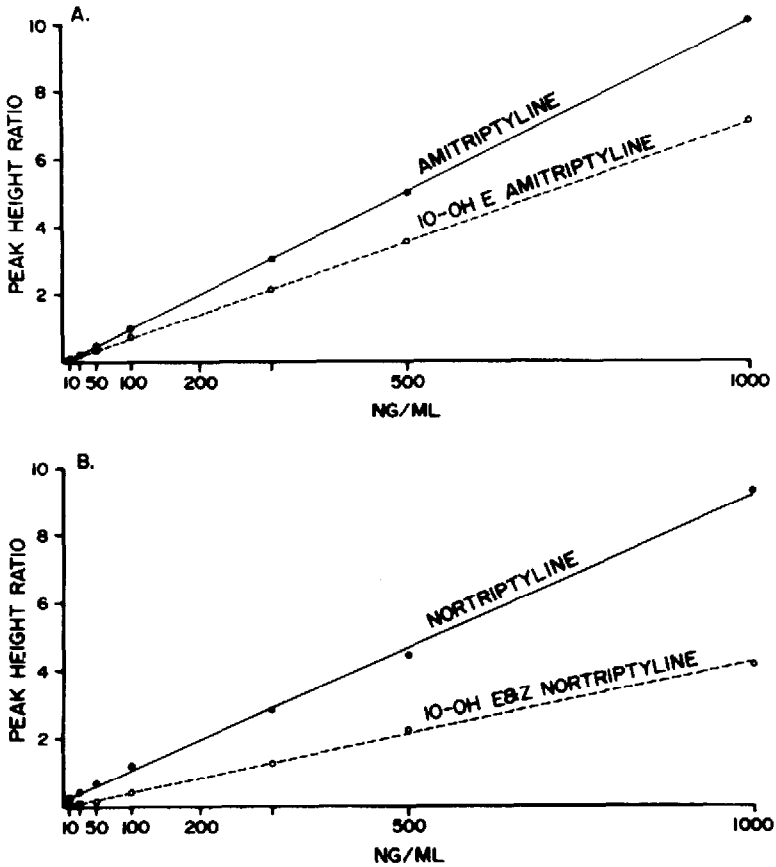



Fig. 4. Standard response curves for (A) amitriptyline and 10-hydroxy metabolite; (B) nortriptyline and 10-hydroxy metabolites.

TABLE I

WITHIN-RUN AND BETWEEN-RUN COEFFICIENTS OF VARIATION (%)

| Concentration (ng/ml) | Ami | Nor | 10-Hydroxy-(*E*)ami | 10-Hydroxy-(*E*)nor | 10-Hydroxy-(*Z*)nor |
|---|---|---|---|---|---|
| 10* | 10.0 | 17.0 | 13.8 | 14.0 | 10.0 |
| 100* | 11.3 | 5.2 | 4.7 | 4.9 | 4.2 |
| 10** | 21.8 | 17.0 | 17.8 | 14.0 | 20.0 |
| 100** | 8.9 | 4.9 | 5.7 | 6.0 | 7.1 |

*Relative standard deviations of seven spiked samples at each concentration which were analyzed using a single standard curve for each compound.

**Relative standard deviations of means of duplicate spiked samples analyzed and standardized on seven different days.

pound, shown in Table I. The within-run C.V. values represent the variability of several samples using a single standard curve. Note that at the lower concentration, which approaches the limit of detection of HPLC methods, the maximum C.V. value was only 17.0%. At a typical therapeutic concentration, 100 ng/ml, the C.V. values were approximately 5% with the exception of amitriptyline.

The between-run C.V. values represent the variability of the means of duplicate samples determined using a different standard curve for each pair of samples. The C.V. values were 21.8% or less for a concentration of 10 ng/ml plasma and 8.9% or less for a concentration of 100 ng/ml plasma. This increase in relative variation includes variability contributed by the multiple standard curves and by sample-to-sample variation.

*Efficiency*

In calculating the analytical efficiency of our method, the peak height ratios of reference standards represented 100% extraction efficiency. Peak height ratios of extracted plasma samples, expressed as percentages of ratios from reference standards, are listed as analytical efficiencies in Table II. The average extraction efficiency from 10—1000 ng/ml plasma was greater than 80% for all compounds except for amitriptyline, which was 73%. These values indicate a reasonably efficient and consistent extraction throughout the entire concentration range. It is of interest to note that the extraction of amitriptyline was the least efficient as well as the most variable. No correction for analytical efficiency was applied in routine quantitation other than that implicit in standardization.

TABLE II

EXTRACTION EFFICIENCIES

Extracted spiked samples were compared to non-extracted reference standards.

| Concentration (ng/ml) | Recovery (%) | | | | | |
|---|---|---|---|---|---|---|
| | Ami | Nor | 10-Hydroxy-(*E*)ami | 10-Hydroxy-(*E*)nor | 10-Hydroxy-(*Z*)nor | Pro |
| 10 | 81 | 92 | 90 | 88 | 112 | 122 |
| 25 | 60 | 73 | 80 | 90 | 90 | 84 |
| 50 | 76 | 82 | 81 | 83 | 80 | 89 |
| 100 | 64 | 80 | 71 | 103 | 106 | 96 |
| 250 | 79 | 83 | 82 | 79 | 80 | 87 |
| 500 | 72 | 87 | 89 | 88 | 92 | 85 |
| 1000 | 78 | 97 | 82 | 92 | 91 | 98 |
| Mean | 73 | 85 | 82 | 89 | 94 | 94 |
| S.D. | 8.0 | 8.0 | 6.3 | 7.6 | 10.9 | 13.3 |

*Sensitivity*

The detection limit of drug in plasma was somewhat less than 0.5 ng/ml. The peak heights at this concentration were greater than five times the baseline peak–peak noise for each compound.

In conclusion, we have developed a method to resolve and quantitate amitriptyline and metabolites by capillary GC. Advantages of our method include rapid analysis of individual isomers, increased sensitivity over HPLC, acceptable precision, and the conveniences of the GC technique including the potential for direct interfacing with mass spectrometers.

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