High-Pressure Liquid Chromatographic Determination of Amitriptyline and Its Major Metabolites in Human Whole Blood

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Abstract □ A sensitive, specific, high-pressure liquid chromatographic method using an internal standard was developed for the determination of amitriptyline and its major metabolites in whole blood. Analysis was carried out on a microparticulate silica column with a mobile phase consisting of acetonitrile–methanol–aqueous ammonium hydroxide (93:7:0.4). Linear calibration curves ranging to 250 ng/ml were obtained for all compounds using UV absorbance detection at 220 nm. The lower limit of detection was 2 ng/ml for amitriptyline and 10-hydroxyamitriptyline, and 6 and 16 ng/ml for nortriptyline and its 10-hydroxylated metabolite, respectively. Human whole blood samples collected after single intravenous and single oral doses can be analyzed using this procedure.

Keyphrases □ High-pressure liquid chromatography—determination of amitriptyline and its major metabolites in whole human blood □ Amitriptyline—high-pressure liquid chromatographic determination in whole human blood, major metabolites determined □ Metabolites—of amitriptyline, determination by high-pressure liquid chromatography

Amitriptyline, 10,11-dihydro-*N*,*N*-dimethyl-5*H*-dibenzo[*a*,*d*]cycloheptene-Δ^5,γ-propylamine (I) is a commonly used tricyclic antidepressant. It undergoes extensive metabolism in humans, the major pathways being *N*-demethylation and hydroxylation at the 10-position. The major metabolites of I, 10-hydroxyamitriptyline (II), nortriptyline (IV), and the *trans* (V) and *cis* (VI) isomers of 10-hydroxynortriptyline are shown in Fig. 1.

Various methods have been described for measuring I and its major metabolites in biological fluids, including radioimmunoassay (1), UV spectrometry (2), GC with flame ionization (2, 3) or nitrogen detection (3, 4), GC–mass spectrometry (5), and high-pressure liquid chromatography (HPLC) using UV absorbance detection (6, 7). Except for the procedure using mass spectrometry, no method has sufficient sensitivity to separate and quantitate I and its major metabolites in single-dose pharmacokinetic studies.

This paper describes a specific HPLC method for the measurement of I, II, IV, V, and VI in human whole blood. Detection is by UV absorbance and no derivitization is required prior to analysis. The sensitivity is sufficient to measure accurately the low drug and metabolite concentrations observed during single-dose pharmacokinetic studies in humans.

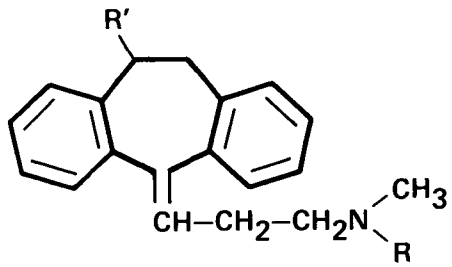
EXPERIMENTAL

Reagents and Materials—Propranolol hydrochloride[1] (internal standard) was obtained commercially and was used as the salt. Amitriptyline hydrochloride[2], [^{14}C]amitriptyline hydrochloride (specific activity 7.1×10^{-3} mCi/mg), nortriptyline hydrochloride[3], 10-hydroxyamitriptyline[2], and *cis*- and *trans*-10-hydroxynortriptyline[2] were used as provided. Acetonitrile and methanol were purchased as glass-distilled solvents[4]. The water used in extraction was glass-distilled. Aqueous ammonium hydroxide (58%)[5] was analytical reagent grade.

Standard Solutions—Methanolic solutions of amitriptyline hydrochloride, nortriptyline hydrochloride, and the hydroxylated metabolites were prepared at a concentration of 5 ng/μl. The concentration of the internal standard was 10 ng/μl, also in methanol. New solutions of amitriptyline hydrochloride and nortriptyline hydrochloride were made every 3 weeks; the remainder were freshly prepared every 6–8 weeks. All were stored at 4°, with no detectable decomposition during the storage period.

Sample Preparation—A 10-μl aliquot containing 100 ng of the internal standard was dispensed with a syringe[6] into a 15-ml culture tube fitted with a polytef-lined screw cap. One milliliter of hemolyzed whole blood, 1.0 ml of water, 100 μl of 5.0 *M* NaOH, and 6.0 ml of hexane–*n*-butanol (98:2) were added. The mixture was shaken[7] for 10 min followed by centrifugation at 2800 rpm for 5 min. The tube was then immersed in an acetone–dry ice bath until the aqueous phase was frozen. The organic phase was poured into a second tube with an elongated cone (capacity ~50 μl) at its base, and the still-frozen aqueous phase was quickly washed with another 1.0 ml of hexane–*n*-butanol which was then added to the extract. This combined extract was evaporated to dryness at 40° under vacuum on a vortex evaporator[8]. One-hundred microliters of the mobile phase used in the chromatography was added to the resulting residue. The tube was capped, vortexed for 30 sec, and centrifuged for 5 min. All of the mixture was drawn into a 100-μl syringe[6] and injected into the chromatograph.

Apparatus—The modular high-pressure liquid chromatograph consisted of a constant-flow pump[9], a valve-type injector[10], a variable



	R	R'
I	CH_3	H
II	CH_3	OH
IV	H	H
V,VI	H	OH

Figure 1—*Amitriptyline (I), 10-hydroxyamitriptyline (II), nortriptyline (IV) and* cis- *and* trans-*10-hydroxynortriptyline (V, VI, respectively).*

[1] Sigma Chemical Co., St. Louis, Mo..
[2] Merck, Sharp & Dohme Research Laboratories, Rahway, N.J.
[3] Eli Lilly and Co., Indianapolis, Ind.
[4] Burdick and Jackson Laboratories, Muskegon, Mich.
[5] Mallinckrodt Chemical Works, St. Louis, Mo.
[6] Hamilton Co., Reno, Nev.
[7] Labquake reciprocating shaker, Labindustries, Berkeley, Calif.
[8] Buchler Instruments (Div. of Searle Analytic Inc.), Fort Lee, N.J.
[9] Chromatography pump, model 6000A, Waters Associates, Milford, Mass.
[10] Sample injection valve, model U6K, Waters Associates, Milford, Mass.

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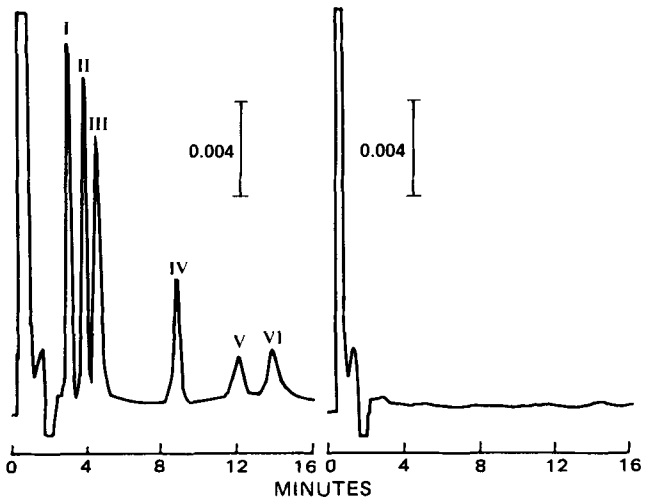

Figure 2—*Chromatograms of extracts from blank human whole blood (right) and blank blood spiked with 100 ng of I, II, III (internal standard), IV, V, and VI per milliter (left).*

wavelength absorbance detector[11] (set at 220 nm), and a strip-chart recorder[12] (0.5 cm/min). A stainless-steel column (250 mm × 2.0-mm i.d.) packed with 5-μm silica[13] was obtained commercially and fitted with a water-jacket[14].

Chromatographic Conditions—The mobile phase was acetonitrile–methanol–aqueous ammonium hydroxide (93:7:0.4). The flow rate was 1.5 ml/min with a column inlet pressure of 3100 psi. Column temperature was maintained at 22° by using a water-jacket connected to a constant temperature water bath. Overnight the flow rate was reduced to 0.1 ml/min.

Calibration—The assay was calibrated by analyzing 1.0-ml aliquots of blank hemolyzed whole blood to which 5–250 ng of I and II, 10–250 ng of IV, V, and VI, and 100 ng of the internal standard had been added. In each sample, the peak height ratio of each tricyclic component relative to the internal standard was calculated. These ratios were plotted against concentration to determine the linearity of the extraction and detector response for each species. In addition, each ratio was divided by the amount of the corresponding component in that sample to give a normalized peak height ratio. These normalized ratios were averaged, and the mean value for each tricyclic component was used to determine the amount present in unknown samples. The precision of the assay was estimated by calculating the coefficient of variation (*CV*) for each set of normalized peak height ratios.

To determine whether the method was valid for plasma samples as well as for whole blood, a calibration curve was prepared using duplicate 1.0-ml plasma aliquots spiked to 25, 100, and 250 ng/ml with each species. The linearity and precision of this assay were calculated as described.

Reproducibility—Aliquots (1 ml) of hemolyzed normal human whole blood were spiked with 25, 100, or 200 ng of each species and assayed in quintuplicate using the described method. Absolute peak heights were measured and the *CV* was calculated for each concentration.

To ascertain whether the assay would be useful if it were carried out from various blood volumes, the same procedure was used to determine the reproducibility of extraction from 0.5, 1.5, and 2.0 ml of whole blood spiked with 100 ng/ml of I and its metabolites. Each volume was assayed in quadruplicate. The rationale for this experiment was that clinically and in pharmacokinetic studies, concentrations of I and its metabolites in a particular blood sample may be lower or higher than those of the standards. To keep the concentrations within the range of the standards, it is necessary to use variable blood volumes.

Efficiency—The efficiency of extraction of I from hemolyzed whole blood into the organic phase was determined by spiking 1.0-ml aliquots of blood with ^{14}C-I, and determining the amounts of ^{14}C-I in the evaporated hexane–*n*-butanol extract by liquid scintillation counting. The extraction procedure was identical to that in the other parts of the study. Concentrations of 20, 50, 100, and 200 ng of ^{14}C-I/ml were extracted in duplicate. Results obtained as counts per minute were converted to disintegrations per minute using the external standard method. These values were compared with those obtained by adding the same amounts of labeled I directly to scintillation fluid, and the resulting ratios were expressed as the percent extraction.

Recovery—Aliquots (1 ml) of hemolyzed whole blood were spiked with known tricyclic quantities (50 and 200 ng/ml, five replicates for each concentration). After the samples were extracted and chromatographed as described, the peak heights for I and its metabolites were compared with the peak heights obtained when the same amount of each component in a standard solution was injected directly onto the column.

RESULTS

Chromatography—The retention times for I, its metabolites, and the internal standard are shown in Fig. 2. Occasional day-to-day adjustment of the methanol concentration (±0.1%) was necessary to obtain optimal separation between II and III. Column temperature was also a critical factor in maintaining this separation. Since room temperature fluctuated daily between 22 and 32°, the use of a column water-jacket maintained at 22° greatly improved baseline stability and peak resolution. In some samples from human subjects, a broad contaminant peak appeared between III and IV; careful manipulation of the methanol concentration was required to prevent it from eluting with one of the peaks of interest.

Calibration—A typical calibration curve relating peak height ratio to concentration was linear for all species over the concentration range studied. The *CV* for the estimation of I averaged 5.2%; corresponding values for the metabolites ranged from 3.6 to 10.2%. A standard curve was assayed with each set of unknowns. The limits of detection of an extracted standard, defined as five times the baseline noise, were 2 ng/ml for I and II, 6 ng/ml for IV, and 16 ng/ml for V and VI.

For the standards prepared in plasma, plots of concentration *versus* peak height ratio were linear with *CV* values of 1.8% for I, and 2.2–10.8% for the metabolites.

Reproducibility—Data showing the reproducibility of the assay are summarized in Table I. All species extracted consistently at three different concentrations from 1.0 ml of hemolyzed whole blood. The averaged normalized peak height values showed no trend with increasing concentration.

For any given sample size, extraction of all components was highly reproducible. There was a trend towards a lower percentage of extraction for all species with increasing sample size. This was probably due to a change in the volume ratio of aqueous to organic phase.

Efficiency and Recovery—Extraction efficiency was compared with overall recovery as described. For 20–200 ng/ml concentrations of ^{14}C-I, the amount of labeled drug partitioning into the organic phase varied from 96 to 100%. The total amount of I recovered from the assay procedure was 61% of an original 50 ng/ml concentration, and 53.5% for 200 ng/ml. Recovery of the metabolites was also consistent (46–69%).

DISCUSSION

Previously reported high-pressure liquid chromatographic assays for I have been unable to separate all of the metabolites of interest using a single procedure. As such, they were inadequate for studying the disposition of I in humans, since the metabolites are pharmacologically active, and are often present at concentrations comparable to those of the unchanged drug. To analyze blood samples from subjects participating in a single-dose pharmacokinetic study, it was necessary to develop a simple and rapid procedure which could resolve the parent drug from its demethylated and 10-hydroxylated metabolites. Since unchanged I was the major component of interest in the separation, a normal-phase chromatographic procedure was developed which would elute I first (as the most nonpolar species), giving maximum sensitivity for quantitation. It was possible, to separate I, II, IV, V, and VI in a total run time of 15 min by careful adjustment of the methanol concentration.

Propranolol was chosen as the internal standard, since it did not interfere chromatographically with any of these compounds. However, zymelidine, which eluted at 14 min under the described conditions, could have been used for samples in which VI was not present. Aqueous ammonium hydroxide was used at a low concentration in the mobile phase to decrease chemisorption on the silica column, which leads to peak tailing.

Although this paper describes chromatography on 5-μm packing material, acceptable peak resolution can be obtained using 10-μm silica with slight modifications of the chromatographic conditions. In this laboratory, a 250 × 2.0-mm i.d. column packed with 10-μm silica[15] was used for

[11] Model 450, Waters Associates, Milford, Mass.
[12] Model 9176, Varian Instruments, Palo Alto, Calif.
[13] MicroPak Si-5, Varian Instruments, Walnut Creek, Calif.
[14] Varian Instruments, Walnut Creek, Calif.
[15] MicroPak Si-10, Varian Instruments, Walnut Creek, Calif.

Table I—Reproducibility of Recovery of Amitriptyline and Metabolites from Spiked Hemolyzed Whole Blood

Compounds Added to Whole Blood	Normalized Peak Heights × 10^2[a]					
	25 ng[b]	100 ng[b]	200 ng[b]	0.5 ml[c]	1.5 ml[c]	2.0 ml[c]
I	1.69 ± 0.05	1.83 ± 0.06	1.73 ± 0.04	1.78 ± 0.06	1.57 ± 0.06	1.37 ± 0.08
II	1.53 ± 0.06	1.57 ± 0.06	1.53 ± 0.03	1.56 ± 0.05	1.47 ± 0.03	1.38 ± 0.06
IV	0.56 ± 0.06	0.61 ± 0.02	0.64 ± 0.01	0.65 ± 0.01	0.55 ± 0.03	0.48 ± 0.02
V	0.18 ± 0.02	0.15 ± 0.01	0.16 ± 0.004	0.18 ± 0.005	0.14 ± 0.004	0.13 ± 0.007
VI	0.21 ± 0.02	0.20 ± 0.01	0.23 ± 0.03	0.23 ± 0.005	0.19 ± 0.01	0.18 ± 0.01

[a] Mean ± *SD*, $n = 5$ for columns 1–3, $n = 4$ for columns 4–6. [b] Amount of I and metabolites added to 1.0 ml of blank, hemolyzed whole blood. [c] Volumes of blank, hemolyzed whole blood to which 100 ng of each component was added.

several months for quantitation of I and all of its metabolites. The use of a water-jacket to maintain column eqilibrium should not be necessary if laboratory ambient temperature fluctuates 3° or less daily.

Plasma samples can be analyzed satisfactorily using the same procedure as that followed for blood samples. As evidenced by the reproducibility of the normalized peak height values of I and the fact that there was no trend in these values with concentration, adsorption to glassware, if it occurred, did not present a problem with the calibration of the assay. This is in contrast to other extraction procedures where adsorption to glassware has resulted in erratic recoveries (7). Butanol was added to the extracting solvent to prevent emulsification and to improve extraction of the more polar metabolites and the internal standard. It is likely that this short-chain alcohol acts like isoamyl alcohol in decreasing adsorption of basic drugs to glass. When standard solutions were assayed, all methanol was evaporated off before the addition of whole blood, as it otherwise affected the cleanliness of the extract. Maximum recovery depended on careful attention to every step of the extraction, particularly the pooling of the organic phase, evaporation, and final vortexing.

Although the addition of bases to mobile phases used with silica columns is known to decrease column life (8), the low concentration of aqueous ammonium hydroxide used in this procedure caused little deterioration of the packing material. A greater problem arose from column contamination by sample extracts, resulting in broadening or even splitting of the peaks. This condition was rectified periodically by removing the top few millimeters of packing material and replacing it with glass microbeads. The use of a guard column packed with pellicular silica should circumvent this problem.

The present method offers several advantages over some previously published procedures. The extraction is simple and rapid, the chromatography is short (15 min), yet allows good separation of I and its known active metabolites. The sensitivity is good, which should allow pharmacokinetic studies to be carried out; the procedure can be applied to either whole blood or plasma. For high-clearance compounds such as I, whole blood measurements may be preferable, obviating the need for determining the red cell to plasma partition coefficient, which may be concentration-dependent.

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NOTES

Analysis of Conjugated Estrogens in a Vaginal Cream Formulation by Capillary Gas Chromatography

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Abstract □ A capillary gas-chromatographic method is described for the quantitative analysis of nine equine estrogens in a vaginal cream formulation. The sodium sulfate ethers of the estrogens were selectively extracted from the formulation, subjected to enzyme hydrolysis, and derivatized to their oxime-trimethylsilyl esters. Resolution of the resulting derivatives was achieved on a short (15 m) capillary column, wall-coated with cyanopropylmethyl silicon stationary phase.

Keyphrases □ Estrogens—conjugated, analysis of a vaginal cream formulation by capillary gas chromatography □ GC—capillary, analysis of conjugated estrogens in a vaginal cream formulation □ Vaginal cream formulation—conjugated estrogens, analysis by capillary gas chromatography

The pharmaceutical product known as conjugated estrogens, is a mixture of nine or more equine estrogens isolated from the urine of pregnant mares (1). As their sodium sulfate salts, they have been used in tablet, injectable, and cream formulations for relieving symptoms associated with menopause. Application of the vaginal cream