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# Quantitative Determination of Amitriptyline in Blood

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**Abstract** □ An assay was developed and standardized for amitriptyline and its hydroxylated tertiary amine metabolites in blood and other biological tissues. This method is capable of determining 5–15 ng of these compounds/ml and is based on reacting the drug as base with 9-bromomethylacridine to form a quaternary product which, on photolysis, yields fluorescence in a stoichiometric fashion. The precision of the method is usually around ±5%.

**Keyphrases** □ Amitriptyline—fluorometric analysis, blood and tissues □ Fluorometry—analysis, amitriptyline, blood and tissues □ Antidepressants—amitriptyline, fluorometric analysis, blood and tissues

Blood amitriptyline levels in humans following a single oral dose are generally 20 ng/ml or less (1). These low levels were difficult to determine until recently, primarily because of a lack of appropriate methods with adequate sensitivity and precision.

Several methods were reported (1–6), but only three were capable of assaying blood levels of less than 20 ng/ml. A GLC method (3) has not yet been used to study amitriptyline pharmacokinetics. A method based on UV reflectance photometry was used for various psychoactive drugs including amitriptyline (5), but its ability to determine blood levels of this tricyclic compound following a single oral dose has not been demonstrated. One GLC method (6) was reported to exhibit reasonably good sensitivity and precision.

A method based on a fluorometric approach (7–9) could determine the absorption and elimination profiles of amitriptyline in humans following a single oral dose of 1 mg/kg (4). In this method, amitriptyline extracted from blood is reacted with 9-bromomethylacridine to form a quaternary ammonium product which, on photolysis, yields fluorescence. Both quaternization and photolysis are stoichiometric with a high degree of precision.

This paper describes the development of a fluorometric assay capable of determining levels as low as 5 ng of amitriptyline/ml in biological fluids. The method has been applied to assaying patient blood samples and is being used to generate basic pharmacokinetic data on amitriptyline in animals and humans.

## EXPERIMENTAL

**Materials and Equipment**—The acridine reagent was synthesized as described earlier (8). Various chemicals and solvents, the laboratory equipment, and other instrumentation were essentially the same as described previously (9). Amitriptyline[1] was used as a hydrochloride salt.

**Table I—Percent Maximum Extraction of Amitriptyline from Blood ($n$ = 4)**

| Solvent | Relative Fluorescence | Blood Blank | Maximum Extraction, % |
|---|---|---|---|
| Hexane | 2762 ± 345[a,b] | 100 ± 21[a,c] | 100 |
| Hexane–1.5% isoamyl alcohol | 2707 ± 242 | 86 ± 5 | 98 |
| Toluene–1.5% isoamyl alcohol | 2020 ± 106 | 180 ± 0 | 73 |
| Benzene–1.5% isoamyl alcohol | 2435 ± 78 | 202 ± 53 | 88 |
| Toluene | 2030 ± 259 | 138 ± 30 | 73 |
| Benzene | 2148 ± 43 | 108 ± 11 | 78 |

[a] Mean ± *SD*. [b] Formed emulsions. [c] $n$ = 2.

***General Procedure***—*Amitriptyline, as the base, was reacted with* excess 9-bromomethylacridine in 200 μl of acetonitrile in the presence of glass beads as the catalyst. The reaction mixture was incubated at 50° for 18 hr, evaporated to dryness, and finally reconstituted in 100 μl of acetonitrile. An aliquot was subjected to TLC to separate the quaternary salt. The separated quaternary salt was photolyzed under UV light, and the fluorescent material was eluted with methanolic sulfuric acid for fluorometric determination[2].

**Standardization of Assay**—Various steps, starting from the isolation of amitriptyline from blood, its reaction with the acridine reagent, and finally fluorometric determination, were standardized to derive the optimum conditions. Since the assay primarily would be applied to blood, all standardization steps were carried out in the presence of a blank blood extract, *i.e.*, amitriptyline was added either to the blank whole blood and extracted out or to the evaporated extract of 3-ml blank blood, prior to reaction with the acridine reagent.

**Extraction from Blood**—Of the organic solvents examined, $n$-hexane containing 1.5% isoamyl alcohol was optimum for single extraction (Table I). Other optimum conditions included a blood-to-solvent ratio of 3:20, an extraction pH of 13 ± 0.5, and a shake time of 30 min at 150 cpm (Table II).

**Quaternization Reaction**—A range of 9-bromomethylacridine concentrations was reacted with amitriptyline and assayed. The optimum acridine concentration for the quaternization of 0.1–1 μg of amitriptyline was $0.47 \times 10^{-3}$ $M$ and higher (Table III).

**TLC and Photolysis**—A 20-μl aliquot of the reconstituted mixture was spotted on the synthetic-backed 100-μm silica gel plate. The plate was dried and developed in many trial solvents, including ethyl acetate–methanol–water–acetic acid–cyclohexane (100:20:12:5:5). This solvent system caused a good separation of the amitriptyline quaternary product ($R_f$ 0.42) from the quaternary products of 10-hydroxy and

[1] Supplied by Merck Sharp & Dohme Research Laboratories, West Point, Pa.
[2] Aminco-Bowman spectrophotofluorometer.

**Table II—Effect of Varying the Blood-to-Solvent Volume Ratio and Shake Time on the Extraction Efficiency Based on 3-ml Blood Samples Containing 100 ng of Amitriptyline/ml**

| Solvent Volume[a], ml | Maximum Extraction at Shake Time ($n$ = 3), % | | |
|---|---|---|---|
| | 15 min | 30 min | 60 min |
| 10 | 83 ± 7.4 | 83 ± 7.3 | 90 ± 8.0 |
| 15 | 86[b] | 88 ± 5.0 | 97 ± 9.1 |
| 20 | 92 ± 10.8 | 97 ± 4.7 | 100 ± 1.6 |

[a] Extraction solvent was hexane containing 1.5% isoamyl alcohol. [b] $n$ = 1.

10,11-dihydroxy metabolites of amitriptyline. Both hydroxy compounds moved to the same spot ($R_f$ 0.26).

The optimum exposure time for photolysis appeared to be approximately 3 min (Table IV). The photolysis products were reconfirmed to be the same as previously reported (4). Both quaternization and photolysis were stoichiometric over a wide concentration range with a high degree of precision (coefficient of variation ≤5).

**Stability of Amitriptyline**—Since, in actual practice, the blood samples are generally stored until assayed and since the described assay involves rendering the blood to pH 13, amitriptyline stability was studied in aqueous solution as well as in whole blood at pH 7.4 and 13. These solutions were maintained at 5 and 22° for up to 72 hr. Additional quantitative chromatographic procedures were developed for this work to separate and detect possible decomposition products.

**Optimum Assay Procedure**—A 3-ml sample of blood was sonicated, adjusted to pH 13 ± 0.05 in a 35-ml screw-capped centrifuge tube by adding 2 ml of 2 $N$ NaOH, and treated with 20 ml of $n$-hexane containing 1.5% isoamyl alcohol. Then the tube was capped and shaken horizontally at 150 cpm for 30 min. Following centrifugation at 2250 rpm for 10 min, an aliquot (19 ml) was transferred to an evaporation tube. The extract was evaporated at room temperature under nitrogen to 0.5 ml and quantitatively transferred into a 1.5-ml tapered polypropylene tube. The contents were then evaporated to dryness under nitrogen.

Fifty milligrams of 200-μm glass beads and 0.2 ml of the acridine reagent ($0.94 \times 10^{-3}$ $M$ 9-bromomethylacridine in acetonitrile) were added to the reaction tube. The tube was capped, vortexed for 20 sec, and placed in an oven at 50° for 18 hr. Following centrifugation, the contents were evaporated to dryness under nitrogen at room temperature and reconstituted in 100 μl of acetonitrile. A 20-μl aliquot was spotted on a scribed, plastic-backed, 100-μm silica gel TLC plate without fluorescent indicator and developed in ethyl acetate–methanol–water–acetic acid–cyclohexane (100:20:12:5:5) in a saturation chamber.

The plate was dried at 85° for 15 min and placed under a UV lamp ($\lambda_{max}$ 254 nm) at 12–14 cm for 3 min to generate fluorescence. Then the fluorescent spot was cut out and eluted by vortexing for 20 sec into 10 ml of 0.01 $N$ $H_2SO_4$ containing 20% methanol in a test tube. The fluorescence was then quantitated on a spectrofluorometer set at an excitation wavelength of 350 nm and an emission wavelength of 474 nm.

Standard curves were obtained by adding known amounts of amitriptyline and/or 10-hydroxyamitriptyline to whole blood and assaying the samples in replicate sets as described. The blanks were run similarly, except that no amitriptyline was added to the blood sample.

## RESULTS AND DISCUSSION

The data scatter in the stability experiments was well within the range of experimental error. Thus, no significant loss of amitriptyline in aqueous and blood solutions at basic pH values was observed for up to 72 hr.

**Table III—Optimum Concentration of 9-Bromomethylacridine (I) Required for Quaternization of Amitriptyline**

| I Concentration $\times 10^{-3}$ $M$ | Relative Fluorescence ± $SD$ ($n$ = 3) | | Maximum Yield, % |
|---|---|---|---|
| | Blood Blank | Drug[a] | |
| 0.16 | 90 ± 14 | 4670 ± 177 | 64 ± 3.8 |
| 0.31 | 103 ± 11 | 6731 ± 116 | 92 ± 1.7 |
| 0.47 | 160 ± 64 | 7440 ± 173 | 100 ± 2.3 |
| 0.63 | 130 ± 0 | 7187 ± 301 | 98 ± 4.2 |
| 0.78 | 230 ± 85 | 7103 ± 450 | 97 ± 6.3 |
| 0.94 | 205 ± 14 | 7311 ± 368 | 100 ± 5.0 |
| 1.25 | 203 ± 4 | 7313 ± 202 | 100 ± 2.8 |

[a] Amitriptyline, 1 μg, reacted in the presence of blood extract.

**Table IV—Effect of Varying the Duration of Photolysis of the Amitriptyline Quaternary Product in the Presence and Absence of Blank Blood Extract**

| Minutes | Photolytic Yield (Direct Reaction) | | Photolytic Yield (Extracted from Blood) | |
|---|---|---|---|---|
| | Relative Fluorescence | % Maximum | Relative Fluorescence | % Maximum |
| 0.5 | 1373 ± 32[a] | 78 | 1023 ± 15[a] | 76 |
| 1.5 | 1733 ± 6 | 98 | 1320 ± 36 | 98 |
| 2.5 | 1750 ± 10 | 99 | 1337 ± 6 | 99 |
| 3.5 | 1763 ± 21 | 100 | 1353 ± 12 | 100 |
| 4.0 | 1763 ± 6 | 100 | 1340 ± 26 | 99 |
| 5.5 | 1710 ± 10 | 97 | 1327 ± 35 | 98 |
| 6.0 | 1730 ± 0 | 98 | 1297 ± 29 | 96 |
| 8.0 | 1677 ± 6 | 95 | 1260 ± 20 | 93 |
| 10.0 | 1633 ± 21 | 93 | 1143 ± 55 | 84 |

[a] $n$ = 3–5.

The previously used solvent system, acetonitrile–water (9:1), separated the quaternary products of chlorpromazine (9) and amitriptyline from the reactants rather well. However, it did not separate the amitriptyline product from the quaternary products of the 10-hydroxy and 10,11-dihydroxy metabolites of amitriptyline, which may be present *in vivo*. Of the various solvent systems tried, ethyl acetate–methanol–water–acetic acid–cyclohexane (100:20:12:5:5) proved effective in solving the separation problem. Thus, in the developed method, the hydroxy tertiary amine metabolites do not interfere. As with chlorpromazine (9), the desmethyl metabolites of amitriptyline also do not interfere.

**Blanks, Sensitivity, and Precision**—Based on a 3-ml sample, the relative fluorescence values from blank blood and plasma were approximately numerically equivalent to those of the reagent blank. The fluorescence values obtained from analyses of blank organ homogenates were generally greater than those for plasma or blood. However, due to the good precision of the method, it is possible to quantitate accurately low nanogram amitriptyline concentrations in organ homogenates. Since a larger blood or plasma sample did not yield higher blank values, quantities of amitriptyline in the range of 5–15 ng/ml could be assayed by utilizing 6–9-ml samples. The precision within an experiment on the blood and plasma samples was usually within ±5% but occasionally was as high as 10%. Abnormal values (> ±10%) generally warrant repeat analyses.

**Addition and Recovery**—When known concentrations of amitriptyline, ranging from 20 to 200 ng/ml, were assayed, recoveries of 75–89% were obtained based on the described single-extraction procedure. However, nearly 100% recoveries were attained on triple extraction.

The plasma yielded 76–86% recoveries for a 25–100-ng/ml concentration range. For concentrations of 10 ng/ml or less, better precision and sensitivity could be achieved when larger plasma samples were used for assay, *i.e.*, 6–9 ml instead of 3 ml.

The addition and recovery data from various organs are summarized in Table V.

The addition–recovery experiments were also carried out in whole mouse homogenates (Table VI) to derive the amitriptyline metabolism rate profile in the whole organism.

**Hydroxylated Metabolites**—Since a solvent system capable of separating from each other the quaternary products of amitriptyline and its two hydroxylated metabolites has not yet been found, it is not possible to quantitate individually 10-hydroxyamitriptyline and 10,11-dihydroxyamitriptyline simultaneously. However, one solvent system did separate the free base of each of the three substances. The free bases can be eluted, evaporated, and then reacted with the acridine reagent to accomplish quantitation.

This approach was used to quantitate the tertiary amine hydroxylated

**Table V—Percent Recovery of Amitriptyline from Various Organ Homogenates of Dog [a]**

| Concentration, ng/ml | Percent Recovery from (Mean ± $SD$, $n$ = 3) | | | | |
|---|---|---|---|---|---|
| | Heart | Liver | Lung | Muscle | Brain |
| 25 | 77 ± 12 | 115 ± 17 | 90 ± 6 | 80 ± 8 | 82 ± 11 |
| 50 | 70 ± 11 | 91 ± 13 | 81 ± 2 | 88 ± 4 | 86 ± 1 |
| 100 | 65 ± 3 | 88 ± 2 | 80 ± 4 | 86 ± 5 | 76 ± 6 |

[a] The tissues were homogenized in 20 ml of 0.1 $N$ NaOH/g of tissue.

**Table VI—Percent Recovery of Amitriptyline from Whole Mouse Homogenate** [a]

| Concentration, ng/ml | 25 | 50 | 100 | 200 | 400 | 600 | 1000 |
|---|---|---|---|---|---|---|---|
| Percent recovery ± *SD* | 74 ± 14[b] | 89 ± 7 | 92 ± 2 | 89 ± 1 | 89 ± 1 | 91 ± 1 | 92 ± 5 |

[a] The mice were homogenized in 20 ml of 0.1 *N* NaOH/g of body weight. [b] $n$ = 3.

**Table VII—Blood Amitriptyline and 10-Hydroxyamitriptyline Levels in Subjects Receiving 100 mg of Amitriptyline at Bedtime**

| Subject | Time since Last Dose, hr | Amitriptyline, ng/ml | 10-Hydroxyamitriptyline, ng/ml |
|---|---|---|---|
| G.M. | 19 | 42 ± 1 | 6 ± 1 |
| K.F. | 11 | 34 ± 3 | 10 ± 1 |
| S.B. | 17 | 165 ± 6 | 6[a] |
| M.F. | 10 | 33 ± 2 | 6 ± 0 |

[a] Single spot only. All other values are mean ± *SD* of values from duplicate spots derived from a single blood sample per subject.

metabolites added to blood[3]. In the blood samples from both humans and dogs, 10,11-dihydroxyamitriptyline could not be detected. Therefore, the hydroxylated metabolite data may be assumed to represent 10-hydroxyamitriptyline only.

**Application to Biological Specimens**—*Dog Studies*—Blood levels of amitriptyline were determined in a 9.1-kg dog following a rapid dose of 1 mg/kg iv. Blood specimens were drawn at suitable intervals up to 6 hr. Figure 1 shows the blood level–time profile of amitriptyline and one metabolite, 10-hydroxyamitriptyline. The blood amitriptyline levels declined very rapidly and had an apparent elimination half-life of about 3 hr.

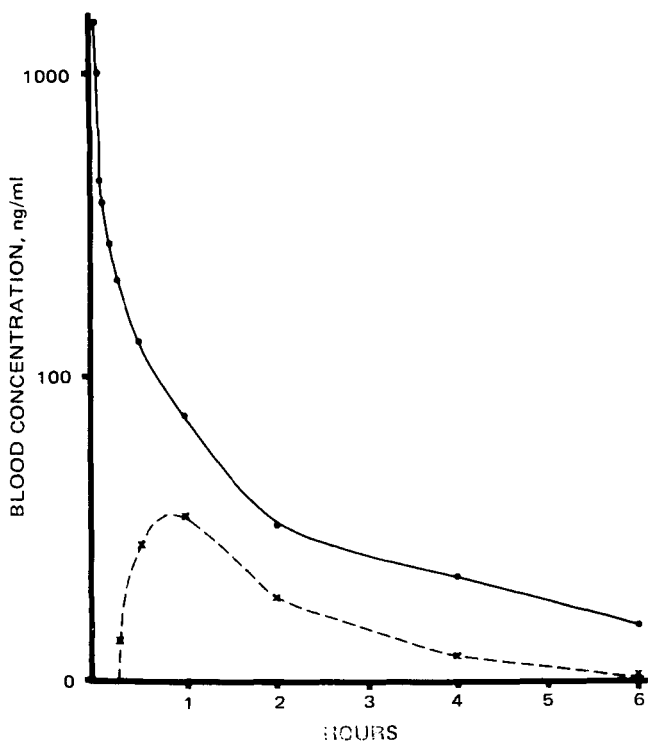


**Figure 1**—*Blood levels of amitriptyline (●) and 10-hydroxyamitriptyline (×) after 1 mg/kg iv.*

[3] Unpublished results.

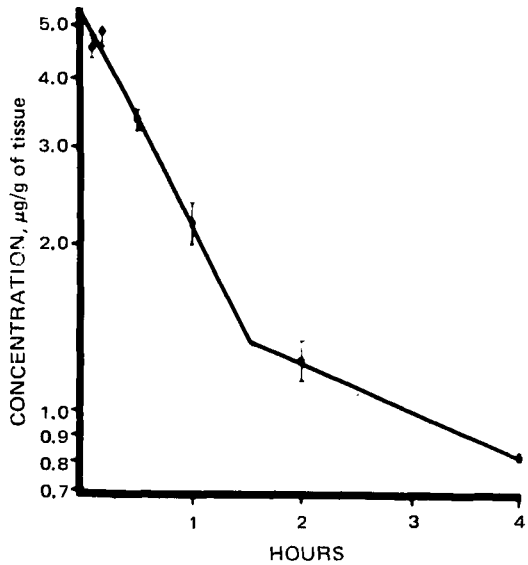


**Figure 2**—*Disappearance with time of amitriptyline in whole mouse given 5 mg/kg iv. Each point is the mean of two mice with a triplicate analysis on each mouse.*

The 10-hydroxyamitriptyline data indicate a rapid appearance in blood, with a measurable level seen within 3 min. The observed peak blood level was 36 ng/ml and occurred at 1 hr. The metabolite had an apparent elimination half-life of approximately 8 hr. A detailed pharmacokinetic analysis of these data will be published.

*Mouse Studies*—The method is also amenable to the analysis of biological materials other than blood and plasma. Figure 2 shows the results of applying the method to the determination of amitriptyline from whole mouse homogenates. The mice received a 5-mg/kg iv dose and were subsequently sacrificed at intervals up to 4 hr. Duplicate mice were assayed for each time period. The data show a biphasic decline of the amitriptyline levels. The first phase had a half-life of approximately 0.5 hr, and the second phase had a half-life of approximately 3.5 hr.

*Human Studies*—Table VII shows the blood levels of amitriptyline and 10-hydroxyamitriptyline in subjects described as suffering from depressive neurosis. Each subject received 100 mg po of amitriptyline at bedtime. Each subject had been receiving treatment for at least 1 week.

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