Identification of a Major Metabolite of Nortriptyline in Human Urine

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Abstract □ Urine samples taken from a person who ingested nortriptyline·HCl (4 × 25 mg. daily) were extracted by a two-phase procedure with ether. A first screening by UV spectrophotometry showed the presence of compound(s) with basic character but different from free nortriptyline. The TLC separation of aliquots of extracts revealed traces of free nortriptyline, three minor metabolites, and a more polar major metabolite. These results were confirmed by GLC, which was performed on the acetylated extracts using two different columns. Elucidation of the structure of the major metabolite was attempted in two ways. A 1-hr. treatment of the extract with 1 *N* HCl at 100° caused a β-elimination and transformed the compound to a polyene structure demonstrated by the typical UV spectral shift. A microcollection technique for trapping GLC effluents was developed to obtain the acetylated derivative of the metabolite in a highly purified form. From the trapped compound, a micro-IR and low- and high-resolution mass spectra were recorded; these permitted a thorough interpretation. These experiments indicated that 10-hydroxynortriptyline is excreted in human urine as a major metabolite from nortriptyline. Desmethylnortriptyline was not detected in the urine.

Keyphrases □ Nortriptyline—identification of 10-hydroxynortriptyline as major metabolite in human urine □ 10-Hydroxynortriptyline—identification as major metabolite of nortriptyline in human urine, GLC

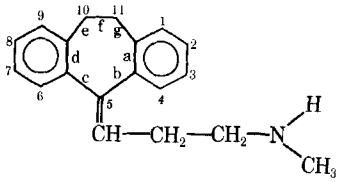
Nortriptyline (I) is a dibenzocycloheptadiene antidepressant (1). It is the monodesmethyl derivative of amitriptyline and is a metabolite of amitriptyline in animals as well as in man (2). From the structural formula, it may be deduced that the free base should be extractable from alkalized biological fluids with a nonpolar organic solvent such as ether. The less polar metabolites may also be expected in the same extract.

McMahon *et al.* (3) reported that nortriptyline is metabolized in the rat to 10-hydroxynortriptyline. Amundson and Manthey (4) published an excellent study on nortriptyline excretion by man. With a different experimental approach, the objective of the present study was to obtain corresponding information about the metabolism of nortriptyline in man.

EXPERIMENTAL

The total urine output from a 50-year-old man, who ingested 4 × 25 mg. of nortriptyline·HCl daily for 7 months, was collected on 4 successive days. Each urine sample was immediately placed in the refrigerator, and a 200-ml. aliquot of the 24-hr. collection was extracted prior to analysis without any delay.

Extraction Procedure—A 200-ml. aliquot of each urine sample was acidified with 2 *N* HCl and extracted twice under nitrogen atmosphere with 400 and 200 ml. of peroxide-free ether (freshly distilled over hydroquinone); the ether phases were rejected. The aqueous phase was alkalized with 10 *N* NaOH and again extracted twice under nitrogen atmosphere with 200 ml. of ether. The ether layers, which contained nortriptyline and less polar metabolites,



5-(3-monomethylaminopropylidene)-5*H*-dibenzo[*a*,*d*][1,4]-cycloheptadiene

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were washed with 5-ml. portions of 0.001 *N* NaOH until the aqueous layer remained colorless. The washed ether was evaporated in a rotatory evaporator to a final volume of about 100 ml., saturated with nitrogen, and extracted twice with 10 ml. of 0.1 *N* HCl. These acid extracts were first used for UV analysis. The total acid extracts were made basic with 1 ml. of 10 *N* NaOH, saturated with nitrogen, and extracted twice with 40 ml. of peroxide-free ether. The combined ether phases were dried over Na_2SO_4, filtered, and evaporated on a water bath at 40° under a slow stream of nitrogen. The residue was dissolved in 10 ml. of ethanol and kept in a well-closed tube at −15° for further analysis ("alcoholic extraction liquid").

UV Spectrophotometry—The UV spectra of the acidic extracts were recorded with a double-beam Unicam SP 800 spectrophotometer in the wavelength range of 450–190 nm.

TLC—A 1-ml. aliquot of each alcoholic extract was evaporated on a water bath at 40° to a small volume with the aid of a stream of nitrogen. The extract, together with 10 mcg. of the reference compounds nortriptyline·HCl and desmethylnortriptyline·HCl (0.1% ethanolic solutions), was spotted separately on a 250-μ thin-layer plate prepared with equal parts of silica gel H 254 and cellulose MN 300. Chloroform, acetone, and 25% ammonia (50:50:1 in milliliters) was used for development (5). Then the plate was viewed under 254-nm. light to determine R_f values. A more selective visualization was performed by spraying the plate with iodoplatinate reagent, prepared by mixing chloroplatinic acid 5% (w/v), potassium iodide 10% (w/v), and distilled water (5:45:100 in milliliters) (6).

GLC—GLC analysis was performed with Hewlett-Packard research gas chromatograph 5750, equipped with dual flame-ionization detectors (FID). Two packed columns of different polarity were installed for simultaneous single-column operation. One was prepared with 2% FFAP (a reaction product of carbowax 20M and *m*-dinitroterephthalic acid) and the other with 5% OV-1 (a methylsilicone polymer) by coating the liquid phases on Diatoport S (acid and silane treated), 80–100 mesh, as a support.

The working conditions were: glass spiral columns, 1.80-m. length, 4-mm. i.d.; carrier gas, nitrogen, 70 ml. $min.^{-1}$ (bubble flowmeter); oven temperature, 240° for analysis on 2% FFAP (polar column), 215° for analysis on 5% OV-1 (apolar column); injector block temperature, 245° (FFAP), 220° (OV-1); detector block temperature (FID), 245° (FFAP), 220° (OV-1); and air and hydrogen, calibrated for optimum sensitivity.

The compounds were transformed to their respective acetylated derivatives before injection, because the presence of a secondary amine function in the side chain causes irreversible retention on the polar 2% FFAP column. For that reason, 1-ml. aliquots of the individual extracts were transferred to conical siliconized tubes of 15-ml. capacity and evaporated on a water bath at 40° under a slow stream of nitrogen. To the final residues, 0.2 ml. of pyridine (refluxed and distilled over KOH) and 0.2 ml. acetic acid anhydride

Table I—Volume and pH of Collected Urine Samples

Sample	Volume of Urine, ml./24 hr.	pH
1 (1st day)	1650	6.0
2 (2nd day)	1950	5.5
3 (3rd day)	1800	6.5
4 (4th day)	1950	6.5

(refluxed and distilled over CaC_2) were added. After a 1-hr. reaction time in a P_2O_5 desiccator, the reaction mixtures were evaporated again under the mentioned conditions. The residues obtained were dissolved in a small volume of ethyl acetate (50 μl.) with the aid of a Whirlmixer. The standards, nortriptyline·HCl and desmethylnortriptyline·HCl (10-mg. amounts), were treated in the same way, but the residues obtained were taken up in 10 ml. of ethyl acetate (0.1% solutions).

Trapping Technique of GLC Effluents on Microscale—A simple system for trapping GLC effluents arising from 20–200 mcg. of injected samples was developed. A splitting system[1] was installed at the exit of each column, splitting the effluent gas so that 10 parts went to the FID and 100 parts entered the catharometer block. This split ratio was obtained by passing the effluent through two parallelly mounted capillaries of 101.6- (FID) and 10.16- (catharometer) cm. (40- and 4-in.) lengths, respectively.

The catharometer detector was heated at the same temperature as the oven and only used as a transfer zone (bridge current switched off). As a collection device, a Pyrex capillary of 12.5-cm. length and 1.5-mm. i.d., fixed with two Teflon O-rings in a brass tube of 10.5-cm. length, 7-mm. o.d., and 4-mm. i.d., provided with a 0.42-cm. (0.125-in.) swagelok nut, was used. For each collection, a brass tube was first loaded with a carefully cleaned capillary (stored overnight in chromic acid, rinsed six times with distilled water, and dried at 120° in a drying stove), and the assembly was attached to the exit of the catharometer block approximately 1 min. before the desired compound was eluted. The simultaneous recording of the chromatogram allowed us to follow the chromatographic process and indicated the moment at which the collection device could be unscrewed from the chromatograph. The brass tube served as a temperature gradient which avoided high losses of trapped compounds through aerosol formation.

IR Spectrophotometry—A 1-ml. aliquot of the alcoholic extract was acetylated, and the residue was dissolved in 50 μl. of ethyl acetate. This solution was at once preparatively chromatographed on the 5% OV-1 column at 215°. After collection, the condensed parent metabolite was rinsed from the capillary with 2 × 10 μl. of ethanol. The ethanol solutions were poured on 27 mg. of KBr in an agate mortar, and the solvent was allowed to evaporate. A micropellet was prepared by means of a punched disk (2 × 10-mm. hole); the IR spectrum was recorded from 625 to 4000 $cm.^{-1}$ with a Perkin-Elmer 257 spectrophotometer, equipped with microbeam condenser RIIC C-41.

Degradation Reaction—The product, 10-hydroxyamitriptyline, is known to give the reaction product 10,11-dehydroamitriptyline when heated in 1 *N* HCl (3). Two solutions were prepared containing, respectively, 100 mcg. of 10α-hydroxyamitriptyline and 100 mcg. of 10β-hydroxyamitriptyline in 10 ml. of 1 *N* HCl. After heating the solutions for 1 hr. on a boiling water bath, the UV spectra of the reaction mixtures were recorded. The same reaction was carried out on 1 ml. of the alcoholic extracts.

Mass Spectrometry—A low-resolution mass spectrum was obtained using a fragment of the KBr micropellet (containing the acetylated compound) with an AEI MS 902 mass spectrometer. The remaining part of the pellet was eluted with ethyl acetate. The solution was employed for recording high-resolution mass spectral data by means of the same spectrometer, provided with the AEI DS 10 data acquisition system.

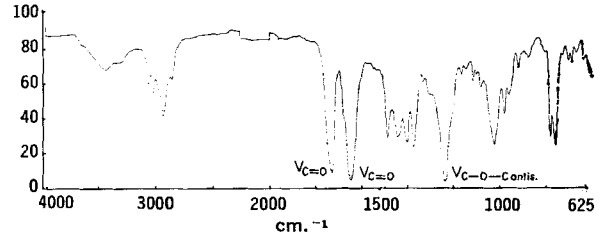


Figure 1—*IR spectrum of trapped major metabolite (acetylated compound).*

[1] Hewlett-Packard.

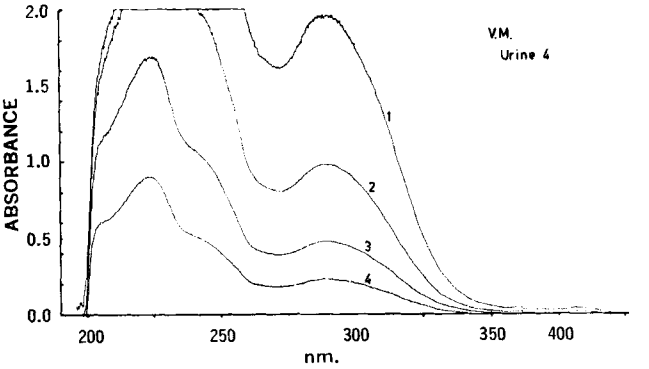


Figure 2—*UV spectrum of the extract of Sample 4 after treatment with 1 N HCl at 100° for 1 hr.*

RESULTS AND DISCUSSION

The volume and pH of the urine samples were determined just after each 24-hr. collection. These data are summarized in Table I.

The UV spectra of all samples showed a striking correspondence with the reference spectra of 10α-hydroxyamitriptyline ($\lambda_{max.}$ 209 and 239 nm. with ε 37,378, and 14,885; $\lambda_{min.}$ 230 nm.) and β-hydroxyamitriptyline ($\lambda_{max.}$ 209 and 239 nm. with ε 40,355 and 15,878; $\lambda_{min.}$ 229 nm.).

More details concerning the value and interpretation of UV spectra obtained on biological extracts were published earlier (7).

A small amount of free nortriptyline was detected by TLC only in the extract of Sample 4 (R_f 0.25 brown). A constant metabolic pattern was found in all extracts; three minor metabolites (R_f 0.04 brown, 0.50 blue-gray, and 0.53 brown) besides one major metabolite (R_f 0.16 brown) were present. No spots occurring in the extracts could be assigned to desmethylnortriptyline (R_f 0.62 brown).

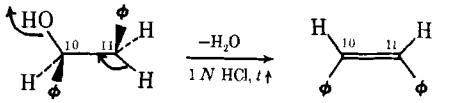
GLC analysis on the 2% FFAP column (240°) showed the presence of free nortriptyline (R_t 23 min. 25 sec., theoretical plates 2500) in the fourth urine sample. On the 5% OV-1 column (215°), free nortriptyline (R_t 12 min. 18 sec., theoretical plates 2100) was also revealed in Sample 4; one minor metabolite, with a relative retention time of 1.65, and one major metabolite with a relative retention time of 1.94, were noted in all extracts examined. These results correlate with the TLC analysis, except that the later technique detected two more metabolites which probably are adsorbed irreversibly on the GLC columns.

The IR spectrum (Fig. 1) of the acetylated metabolite isolated by GLC gave the following structural information:

1. A strong absorption band at 1650 $cm.^{-1}$ corresponds to the ketone-stretching vibration ($\nu_{C=O}$) of a tertiary amide structure (6); the last was caused by the acetylation treatment which converted the secondary amine group of the side chain to the respective acetamide function [—N(—CH_3)—C(=O)—CH_3].

2. Two other strong absorption bands occur at 1735 and 1240 $cm.^{-1}$. The former is explained as a carbonyl-stretching vibration ($\nu_{C=O}$) of an alcoholic acetylester, which is further confirmed by the second band owing to an ester antisymmetrical stretching vibration (ν_{C-O-C} stronger and broader than $\nu_{C=O}$ of the same group) (8). Here the acetylation reaction transformed the alcoholic function to the acetylester group [≡C—O—C(=O)—CH_3].

The degradation reaction can be understood as a *trans*-diaxial β-elimination, which results in the formation of a supplementary double bond between C_{10} and C_{11} (Scheme I). The 10,11-dehydro derivative possesses an extensive conjugation system, which may be



10-hydroxy compound 10,11-dehydro compound

Scheme I

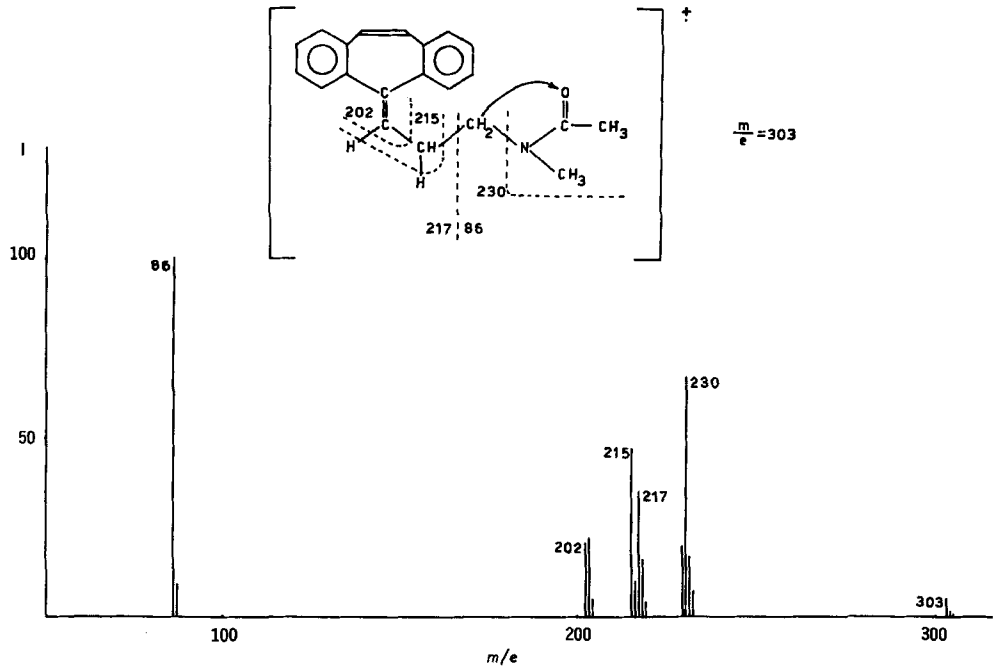
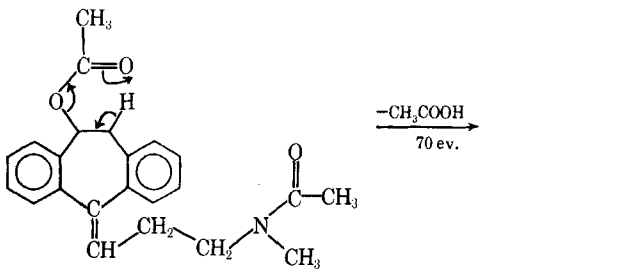


Figure 3—*Mass spectrum of trapped major metabolite (acetylated compound).*

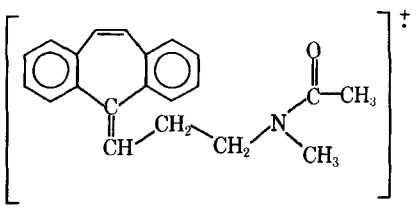
observed as a typical UV spectral shift. In this way, the UV spectra from 10α- and 10β-hydroxyamitriptyline reference solutions were highly different from those obtained before reaction, and both showed two absorption maxima at 225 and 290 nm. and an absorption minimum at 266 nm. A completely similar effect was found with the extracts of Samples 1 and 4 (Fig. 2).

Final evidence was derived from following mass spectral data (70 ev., 150°): *m/e* (relative intensity) 303(5) $C_{21}H_{21}NO$, 230(67) $C_{18}H_{14}$, 217(37) $C_{17}H_{13}$, 215(47) $C_{17}H_{11}$, 202(21) $C_{16}H_{10}$, 86(100) C_4H_8NO. The peak at 303 corresponded to nortriptyline *O,N*-diacetate minus a molecule of CH_3COOH. Loss of the molecular ion peak is known for acetates with long alkyl chains (9, 10). The process occurring for the unknown apparently may be envisioned as shown in Scheme II.

The mass spectral fragmentation pattern (Fig. 3) is conclusive for accepting an unchanged side chain of nortriptyline metabolite. All these experiments indicate the presence of a parent metabolite with a similar basic structure but of higher polarity than the free compound nortriptyline. The IR spectrophotometric analysis of the acetylated and trapped metabolite led to the conclusion of an *O,N*-diacetate structure. Therefore, the metabolite had to possess an alcoholic OH function besides its original secondary amine group.



expected *m/e* = 363



m/e = 303

Scheme II

The location of this OH function could not be on one of the two phenyl groups as seen from the IR spectrum [that is, absorption at 1735 cm.$^{-1}$ is typical for saturated acetylesters as compared to 1760 cm.$^{-1}$ reported for phenol acetate esters (8)]. The remaining positions were then C_{10}, C_{11} or the alkyl side chain. The former possibility was strongly supported by a β-elimination reaction followed by UV spectrophotometric analysis, which gave exactly the same result for the major metabolite as for the model compounds 10α- and 10β-hydroxyamitriptyline. Complete confirmation of former interpretations was given by mass spectral analysis. Therefore, it was concluded that 10-hydroxynortriptyline is the structure of the major metabolite.

REFERENCES

(1) L. S. Goodman and A. Gilman, "The Pharmacological Basis of Therapeutics," 3rd ed., Macmillan, New York, N. Y., 1965.

(2) R. A. Braithwaite and J. A. Whatley, *J. Chromatogr.*, **49,** 303(1970).

(3) R. E. McMahon, J. F. Marshall, H. W. Culp, and W. M. Miller, *Biochem. Pharmacol.*, **12,** 1207(1963).

(4) M. E. Amundson and J. A. Manthey, *J. Pharm. Sci.*, **55,** 277(1966).

(5) A. Heyndrickx and A. De Leenheer, *J. Pharm. Belg.*, **3, 4,** 109(1967).

(6) A. Stolman, "Progress in Chemical Toxicology," vol. 2, Academic, New York, N. Y., and London, England, 1965.

(7) A. Heyndrickx and A. De Leenheer, *Proc. Eur. Soc. Study Drug Toxicity, Xth*, **1969,** 87–93.

(8) K. Nakanishi, "Infrared Absorption Spectroscopy–Practical," Holden Day, San Francisco, Calif., and Nankodo Co. Ltd., Tokyo, Japan, 1964.

(9) A. G. Sharkey, J. L. Schultz, and R. A. Friedel, *Anal. Chem.*, **31,** 1, 87(1959).

(10) J. H. Beynon, R. A. Saunders, and A. E. Williams, *ibid.*, **33,** 2, 221(1965).

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