A GAS CHROMATOGRAPHIC METHOD FOR THE QUANTITATIVE DETERMINATION OF NORTRIPTYLINE AND SOME OF ITS METABOLITES IN HUMAN PLASMA AND URINE

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SUMMARY

A gas chromatographic method has been developed which enables accurate and specific quantitative determinations in plasma and urine of the tricyclic antidepressant drug nortriptyline and some of its desmethylated or hydroxylated metabolites and their conjugates formed *in vivo*. The compounds are extracted as bases into hexane (the conjugated metabolites after hydrolysis) and, after a clean-up procedure, derivatized with heptafluorobutyric anhydride. The heptafluorobutyryl derivatives are separated on a gas chromatograph equipped with an electron capture detector. Accurate determinations are possible in concentrations down to 10 ng/ml.

INTRODUCTION

A quantitative method for the determination of the tricyclic antidepressant drug desmethylimipramine in plasma samples from patients, was described by HAMMER AND BRODIE[1]. This secondary amine was extracted and derivatized by acetylation with tritium-labelled acetic anhydride and the yield of radioactivity of the amide formed was determined. The procedure has been applied also to the determination of nortriptyline[2].

The latter drug and its metabolite desmethylnortriptyline has also been determined by the recently developed technique mass fragmentography, which is based on the combined use of gas chromatography and mass spectrometry[3].

Gas chromatography using electron-capture (EC) detection has been used for the determination of the tricyclic antidepressant drugs protriptyline[4], desmethylimipramine[5] and nortriptyline[6]. These methods were restricted to the determination of the drugs themselves and did not cover their metabolites.

The purpose of our work was to develop a method by which nortriptyline as well as its metabolites (Fig. 1) could be determined in plasma and urine. There is a need for such methods in work aiming to explain the great individual differences in the rate by which drugs of this type undergo biotransformation in the body[2,7].
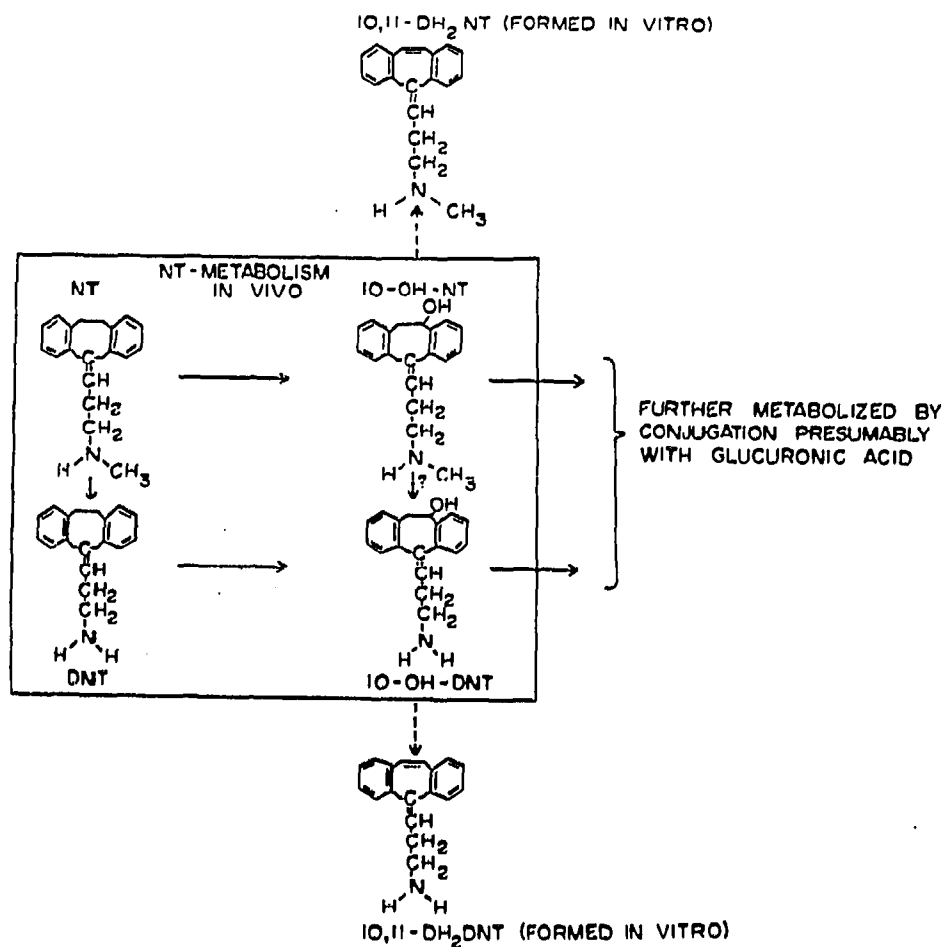
Fig. 1. Metabolites formed from nortriptyline in man according to various authors (for references see HAMMAR et al.[8]).
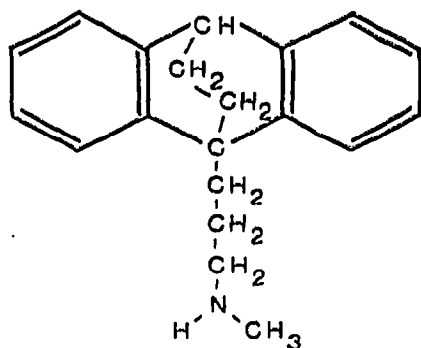

Fig. 2. Structural formula of Ciba 34 276.

EXPERIMENTAL

Reagents, solvents and glassware

n-Hexane of a low grade of purity was distilled once using an efficient column to give a gas chromatographically pure product. Pyridine (Mallinckrodt), N,N-dimethylformamide (Koch-Light), methanol (Merck), diethyl ether (Mallinckrodt) and heptafluorobutyric anhydride (Merck) were all of analytical grade.

Glassware was cleaned by ordinary dish-washing. An additional rinse with hexane was found necessary to minimize interference from an unidentified compound with high EC-response adsorbed in minute amounts on the glass. The compound seemed to originate from the tap water.

Reference drugs and metabolites

For structural formulae see Figs. 1 and 2. The stereoisomers of 10-hydroxynortriptyline (10-OH-NT) 1,5-naphthalene disulphonate (provisionally designated as isomer I and isomer II since the absolute configurations are not known), 10,11-dehydrodesmethylnortriptyline (10,11-DH$_2$DNT) hydrochloride and 10-oxonortriptyline hydrochloride* were synthesized and donated by Merck & Co., Inc., Pennsylvania. Desmethylnortriptyline (DNT) hydrochloride, nortriptyline (NT) hydrochloride and 10,11-dehydronortriptyline (10,11-DH$_2$NT) were donated by Pharmacia, Uppsala, Sweden. Ciba 34276 hydrochloride (Fig. 2) was donated by Ciba, Ltd., Basle, Switzerland.

Stock solutions

Stock solutions of drugs and metabolites were prepared in water (10-OH-NT) or 0.01 *M* HCl (NT, DNT, 10,11-DH$_2$NT, 10,11-DH$_2$DNT, 10-oxonortriptyline and Ciba 34276).

Samples for standard curves

Known amounts of the above mentioned drugs and metabolites were added in increasing concentrations to plasma or urine blanks. For plasma, standard curves from 0.01 to 0.4 μg/ml of the compound were used; for urine two standard curves were prepared, one from 0.01 to 0.2 μg/ml and one from 0.2 to 4 μg/ml. The concentrations of internal standard (Ciba 34276) in the samples were 0.36 μg/ml and 3.6 μg/ml, respectively. (Concentration values refer to the free base.)

Preparation of heptafluorobutyryl (HFB) derivatives

The HFB derivatives were prepared in crystalline form by reacting the free amine (NT, DNT and Ciba 34276) with heptafluorobutyric anhydride according to the analytical procedure described below, or with heptafluorobutyrylimidazole (10,11-DH$_2$NT) using hexane as a solvent. Recrystallizations were performed in *n*-hexane or ethanol. The melting points of the derivatives were: NT, 92–94°; DNT, 115–116°; 10,11-DH$_2$NT, 52–55°; and Ciba 34276, 72–74°. Too little of the 10,11-DH$_2$DNT reference compound was available to make the synthesis of the pure HFB derivative possible.

Gas chromatography

A modified (all-glass system) Varian Aerograph 204 with on-column injection, equipped with a ^{3}H-electron capture detector, was used. Silanized glass columns 320 cm × 1.8 mm (I.D.) were packed with: (a) 0.75 % OV-17 or (b) 0.5 % XE-60 and 0.25 % DC LSX-3-0295 on 80–100 mesh silanized Chromosorb G (support and stationary phases were from Applied Science Lab., Inc., State College, U.S.A.).

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* This compound had the same configuration as isomer I of 10-OH-NT.

The nitrogen carrier gas flow rate was 10–15 ml/min. Operational temperatures were: injection port 255°, column oven 245° and detector oven (measured close to the foil) 210°.

Analysis of unconjugated metabolites

An idea of the expected concentration of the compound to be determined was necessary, since samples with concentrations above 4 μg/ml had to be diluted prior to analysis. Usually two samples were analyzed, one with the high and one with the low concentration of internal standard added, to permit quantitation even of the minor components.

To 4.00 ml urine or plasma in a 50-ml glass-stoppered centrifuge tube was added 0.100 ml of a solution of the internal standard and 1 ml of 2 M bicarbonate buffer (pH 10.5). The sample was shaken for 10 min with 20 ml of *n*-hexane. After centrifugation, 18 ml of the organic phase were transferred to another 50-ml centrifuge tube. The water phase was extracted once more in the same manner after a further addition of 18 ml of hexane. The water phase was saved for the analysis of conjugated metabolites (see below).

The combined hexane extracts (36 ml) were shaken with 1.5 ml of 0.050 M sulphuric acid for 15 min (10-OH-NT is stable under these conditions). After centrifugation, 1.00 ml of the acid phase was taken out and to this 1.00 ml of 0.10 M NaOH and 0.5 ml of 2 M bicarbonate buffer (pH 10.5) were added. The alkaline water phase was extracted three times with 1.5 ml of hexane. To the combined hexane phases in a 15-ml glass-stoppered tube 20 μl of N,N-dimethylformamide, 20 μl of pyridine and 100 μl of heptofluorobutyric anhydride were added. The stoppered tube was kept for 60 min at 63° in a water bath. Then 1.5 ml of a methanol–water mixture (1:2) was added and the tube shaken for 10 min. After centrifugation the water phase was aspirated off and discarded. The hexane solution was refrigerated until analyzed.

Usually 0.5–1 μl of this solution was injected into the chromatograph. For plasma analysis and for determinations of low urine concentrations ($<$0.2 μg/ml), the hexane solution was concentrated by evaporation in a stream of nitrogen to give a volume of 0.1–1 ml. The hexane solution was then shaken once more with an equal volume of methanol–water before analysis by gas chromatography.

Analysis of conjugated metabolites

The extracted urine (or plasma) sample from above was frozen and the residue of hexane aspirated off by means of a Pasteur pipette. After thawing, the sample was washed with 5 ml of diethyl ether and 0.100 ml of an adequate concentration of the internal standard solution was added. The conjugates were hydrolyzed by one of the following procedures: (a) Enzymatic hydrolysis was achieved by incubation for 24 h (in a "shaking" water bath at 37°) with 100 μl of Glusulase Boehringer, containing β-glucuronidase and arylsulphatase activity; pH was kept at 5.5 by use of a 0.5 M phosphate–citric acid buffer; (b) Acid hydrolysis was obtained by adding 1.0 ml of 5.0 M HCl and allowing the reaction to proceed at 95° for 60 min. After cooling, the sample was neutralized with 1.0 ml of 5.0 M NaOH.

After hydrolysis according to (a) or (b) the analysis proceeded according to the method described for the unconjugated metabolites.

Studies of the reaction with HFBA

Known concentrations in hexane of the investigated compounds were reacted with HFBA for various lengths of time. To stop the reaction instantaneously, 1.5 ml of a methanol–water mixture (1:2) were added to the warm reaction solution, which was then shaken for 15 min. An equal volume of a solution of an appropriate internal standard in hexane was then added and the sample analyzed by GLC. The HFB derivative of Ciba 34276 was used as internal standard for NT, DNT and 10,11-DH$_2$NT, while the HFB derivative of DNT was used when studying the reaction of Ciba 34276 and the isomers of 10-OH-NT.

For calculation of the reaction yield of a compound, standard curves prepared from solutions of known concentrations of the pure HFB derivatives were used.

RESULTS AND DISCUSSION

Derivatization products

It has been shown earlier by HAMMAR et al.[8] that 10-OH-NT and (tentatively) 10-hydroxydesmethylnortriptyline (10-OH-DNT) are formed in man by the metabolism of NT. They are excreted in the urine partly as conjugates (Fig. 1).

These 10-hydroxylated compounds readily lose water when reacted with trifluoroacetic anhydride, to give the trifluoroacetyl (TFA) derivatives 10,11-DH$_2$NT and 10,11-DH$_2$DNT, respectively[8].

That the same dehydration reaction occurred in the derivatization procedure used by us was shown by reacting 10-OH-NT and 10,11-DH$_2$NT with HFBA and analyzing the products by combined gas chromatography–mass spectrometry. The mass spectra of the derivatives of the two compounds as well as their retention times on two different stationary phases (Table I) were identical.

TABLE I

RETENTION TIMES

Compound	OV-17		XE-60/DC LSX-3-0295[a]	
	Retention time (min)	Relative retention time	Retention time (min)	Relative retention time
10-Oxo-NT I	2.46	0.66	3.65	0.90
DNT	2.97	0.80	5.61	1.38
10-OH-DNT[b]	3.47	0.93	6.08	1.50
10,11-DH$_2$DNT	3.41	0.92	—	—
NT	3.73	1.00	4.05	1.00
10-OH-NT I	4.53	1.22	4.83	1.19
10-OH-NT II	4.53	1.22	4.83	1.19
10,11-DH$_2$NT	4.53	1.22	4.83	1.19
Ciba 34276	5.89	1.58	8.31	2.05

[a] Injector 240°, column oven 217°.
[b] From chromatograms of urine from a patient treated with nortriptyline.

10-Oxonortriptyline forms a derivative containing two HFB groups, one on the nitrogen and the other probably on the enolic oxygen in the 10-position. This interpretation is in accordance with the mass spectrum which shows a molecular

ion at m/e 669 and a prominent peak at m/e 442 corresponding to the cleavage of the bond α to the nitrogen (M—227).

Extraction recoveries

The distribution ratio of the most polar of the compounds studied (with the exception of 10-OH-DNT), namely the isomers of 10-OH-NT, between hexane and 0.4 M bicarbonate buffer of pH 10.5, was found to be 1.6 for isomer I and 3.4 for isomer II. It is possible to calculate that the recovery in the initial hexane extraction of urine of 10-OH-NT should be 98 % for isomer I and 99 % for isomer II. In the hexane extraction procedure following the re-extraction with sulphuric acid the theoretical recoveries should be 94 % and 99 %, respectively. A similar recovery is expected when analyzing plasma, since 10-OH-NT is negligibly bound to plasma proteins[9].

Time course and yield of the reaction with HFBA

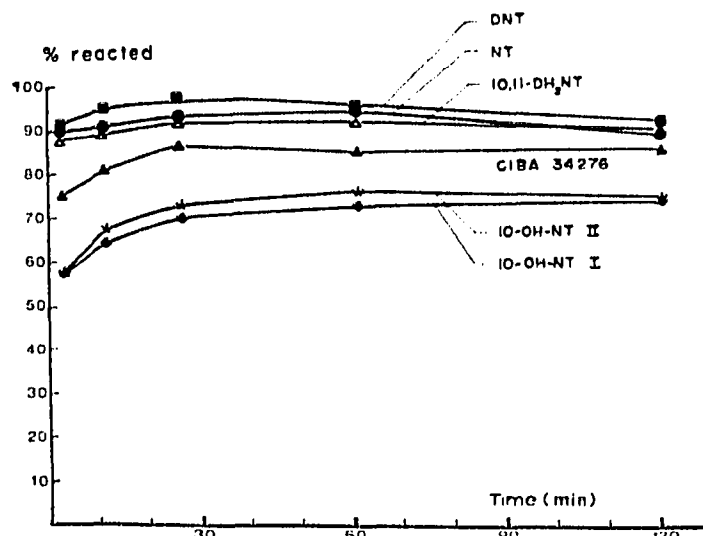The reaction was rapid with NT, DNT and 10,11-DH$_2$NT while Ciba 34276 and

Fig. 3. Product formation (% of theoretical yield) time curve for the derivatization of various amines with HFBA. Each point represents one determination.

TABLE II

YIELD IN THE DERIVATIZATION PROCEDURE

After 60 min of reaction.

Compound	Yield (percent $\pm$ S.E.[a])
DNT	98 $\pm$ 2
NT	97 $\pm$ 1
10-OH-NT I	76 $\pm$ 3
10-OH-NT II	75 $\pm$ 2
10,11-DH$_2$NT	96 $\pm$ 2
Ciba 34276	82 $\pm$ 2

[a] Five determinations were made on a hexane solution of known concentration of each amine.

the two isomers of 10-OH-NT reacted more slowly (Fig. 3). From these data it was concluded that a reaction time of 60 min would be suitable for all the compounds studied (10-oxonortriptyline was not studied). The reproducibility and yield of the reaction step at a reaction time of 60 min are presented in Table II. The yields for Ciba 34276 and the isomers of 10-OH-NT were considerably less than 100%, although it is evident that the product formation did not proceed further after 30–60 min of reaction (Fig. 3).

EC response of the HFB derivatives

The smallest detectable amount of NT-HFB was 10 pg. There was a more than two-fold difference in the relative molar response (calculated on peak areas) between the pure HFB-derivatives of the found compounds (Table III). It is evident from these data that the response of the HFB derivative of 10-OH-DNT ($10,11\text{-DH}_2\text{DNT-HFB}$) cannot be predicted and that the exact determination of this metabolite in biological material requires that the reference compound is available.

TABLE III

MOLAR RESPONSE RELATIVE TO NT-HFB OF THE HFB DERIVATIVES

HFB derivative	Relative molar response (mean ± S.E., n = 4)	
	Peak height	Peak area
DNT	0.72 ± 0.02	0.62 ± 0.01
NT	1.00	1.00
$10,11\text{-DH}_2\text{NT}$	1.17 ± 0.01	1.38 ± 0.01
Ciba 34 276	0.46 ± 0.002	0.69 ± 0.01

Stability of the HFB derivatives

Analyzed samples containing HFB derivatives of DNT, NT, $10,11\text{-DH}_2\text{NT}$ and the internal standard in hexane were stored for four weeks in the refrigerator and the analyses repeated. The difference in peak ratio for each of the compounds (compared to the internal standard) between the two occasions was less than 3%. HFB derivatives of NT and DNT stored at 4° in hexane solutions for one year showed no significant decomposition. The stability of the compounds makes it possible to derivatize a large number of samples and store them until time is available for their analysis.

Retention times

Retention times of reference compounds are presented in Table I. The HFB derivatives of primary amines were eluted after the corresponding secondary amines on the XE-60 stationary phase, while the opposite was true for the OV-17 phase. On both phases the 10,11-unsaturated compounds appeared later than the corresponding saturated compounds. The short retention times obtained with the di-HFB derivative of 10-oxonortriptyline is in agreement with the finding that the introduction of a second HFB group in desmethylimipramine gives a reduction of the retention time by almost 50% on an OV-17 stationary phase[6].

Urine and plasma analysis

Typical chromatograms from analysis of non-conjugated metabolites in urine and plasma from subjects given nortriptyline are shown in Figs. 4 and 5.
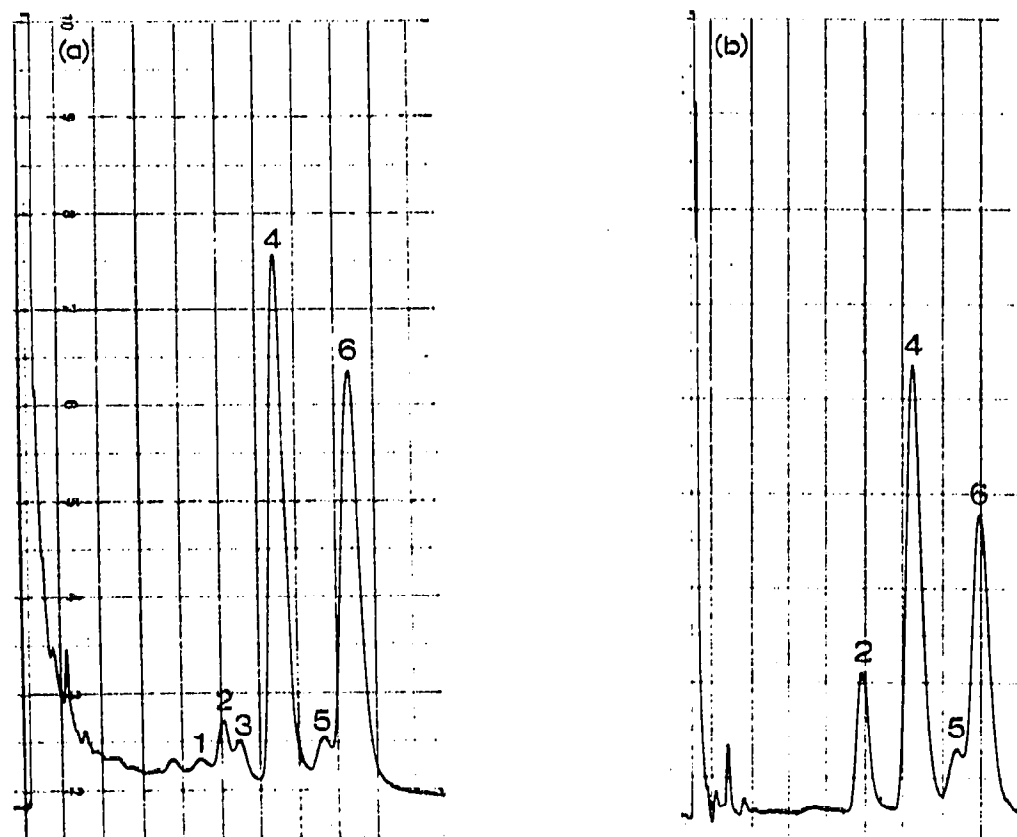

Fig. 4. Gas chromatographic analysis on OV-17 stationary phase of HFBA derivatized extract of urine from a subject given a single oral dose (1 mg/kg) of NT. (a) Unconjugated metabolites. The concentration of 10-OH-NT was 18.5 μg/ml and that of NT 0.76 μg/ml (determined by adding 1/10 as much of the internal standard); (b) Conjugated metabolites. The 10-OH-NT concentration was 11.2 μg/ml. 1 = DNT, 2 = 10-OH-DNT, 3 = NT, 4 = 10-OH-NT, 5 = unidentified impurity, 6 = Ciba 34 276 (internal standard).

The identities of the chromatographic peaks have been ascertained by comparing the retention times with those of reference compounds, and by the combined gas chromatography–mass spectrometry of derivatized samples of urine from subjects taking NT. The metabolites DNT and unconjugated and conjugated 10-OH-NT, as well as unchanged NT, could be identified, thus confirming the results of HAMMAR *et al.*[8]. These workers also demonstrated the presence of the metabolite 10-OH-DNT in urine although it was stated that the identification was only tentative due to the lack of a reference compound. That 10-OH-DNT is a metabolite of NT was shown as follows. A urine sample from a human subject given a single oral dose of NT hydrochloride (1 mg/kg body weight) was treated according to the analytical procedure. The HFB derivatives formed from the hydrolyzed conjugated fraction were subjected to combined gas chromatography–mass spectrometry. The mass spectrum of the tentatively identified 10-OH-DNT derivative showed a close agreement with that of reference 10,11-DH$_2$DNT-HFB (Table IV). It was also shown that the main metabolite

in urine from rats given DNT intravenously (10 mg/kg) gave a HFB derivate with the same mass spectrum.

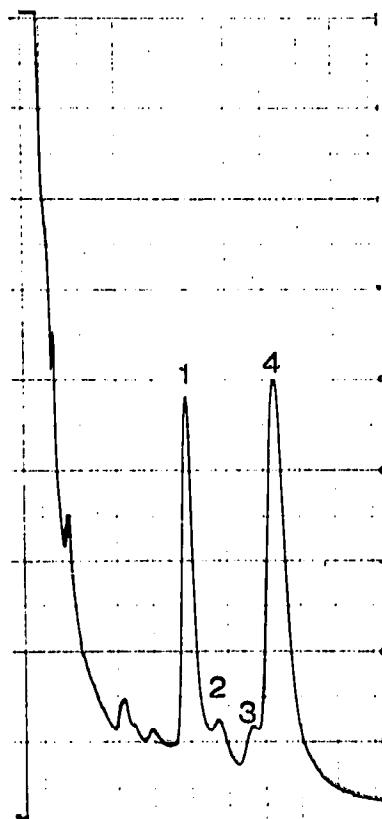The possibility that the 10,11-unsaturated compounds are metabolites occurring

Fig. 5. Gas chromatographic analysis of unconjugated metabolites in plasma from patient treated with NT. The plasma concentration of NT was 131 ng/ml. 1 = NT, 2 = 10-OH-NT, 3 = unidentified impurity, 4 = Ciba 34276 (internal standard).

TABLE IV

MASS SPECTRA FROM COMBINED GAS CHROMATOGRAPHY–MASS SPECTROMETRY

(a) HFB derivative of 10,11-DH$_2$DNT; (b) and (c) HFBA-treated extracts from the hydrolyzed conjugated fraction of urine collected from rat treated with DNT (b) and human treated with NT (c). The LKB 9000 combined instrument was used. Gas chromatographic conditions were similar to those described in the text. The ionization energy was 70 eV. Only the most prominent peaks with m/e values > 100 are listed.

m/e	Relative peak height (%)		
	(a)	(b)	(c)
202	58	61	64
215	88	93	94
216	16	36	36
217	100	100	100
218	11	20	21
229	33	43	46
230	75	64	73
443	33	43	31

simultaneously as 10-OH-NT and 10-OH-DNT has been considered. This was investigated by HAMMAR et al.[8] who found no gas chromatographic evidence for $10,11$-DH_2NT in an underivatized extract of urine from a patient treated with NT, while the same extract contained the trifluoroacetyl derivative of $10,11$-DH_2NT after treatment with trifluoroacetic anhydride.

BERTILSSON AND ALEXANDERSON[10] have used a combination of TLC and GLC to investigate the possibility of formation of $10,11$-DH_2NT in vivo in humans. They concluded that $10,11$-DH_2NT, if formed at all in vivo, accounted for less than 1 % of the total amount of 10-OH-NT and $10,11$-DH_2NT excreted in the urine.

A compound with retention time identical to that of 10-oxo nortriptyline has been observed when analyzing urine from two volunteers taking a single oral dose of NT. However, the amounts were small, and so far no positive identification has been made of this metabolite. The metabolite has, however, recently been found after incubation of 10-OH-NT with rat liver microsomes[11].

Interference by other compounds

Plasma and urine from persons not taking any drug, as well as homogenates of tissues from rat (brain, lung, muscles and liver) gave no peaks which could interfere with the determination of NT, 10-OH-NT or 10-OH-DNT when OV-17 was used as stationary phase. However, occasionally a peak with the same retention time as DNT has been found when analyzing blank plasma. The XE-60 stationary phase was used mostly for identification purposes as the tailing from the solvent peak made the quantitation of NT and 10-OH-NT peaks less reliable in samples with low concentrations.

On most chromatograms (on OV-17) a peak with a retention time between that of 10-OH-NT and Ciba 34276 was observed (Figs. 4 and 5). It could be greatly reduced by hexane washing of all glassware. The compound, which by mass spectrometry forms prominent peaks at m/e 149 (base peak) and 279 (molecular ion?) is as yet unidentified.

Hydrolysis of conjugated metabolites

The metabolites remaining in the water phase after the urine had been subjected to repeated hexane and ether extractions can be considered as rather polar. Hydrolysis of the water phase by β-glucuronidase containing arylsulphatase gave high amounts of 10-OH-NT and 10-OH-DNT.

Acid hydrolysis, on the other hand, gave $10,11$-DH_2NT and $10,11$-DH_2DNT. It is obvious that these compounds are formed via the released 10-hydroxylated compounds or directly from their conjugates.

Urine samples obtained from a dosed subject were hydrolyzed by the two different procedures and the released 10-OH-NT was determined (Table V). Higher yields were obtained by the acid hydrolysis.

It is also our experience that the enzymatic hydrolysis is less reproducible. The nature of the conjugates is still unknown. It has been claimed by McMAHON et al.[12], who were the first to describe the formation of 10-OH-NT as a metabolite of NT in rats, that 10-OH-NT is conjugated with glucuronic acid. The same has been claimed for 10-OH-NT formed in the metabolism of amitriptyline in man[13]. However, conclusive evidence for the formation of 10-OH-NT-glucuronide is still lacking.

TABLE V

COMPARISON OF ACID AND ENZYMATIC HYDROLYSIS OF 10-OH-NT CONJUGATES IN URINE

Urine was fractionally collected from a subject who took 75 mg of nortriptyline hydrochloride. The time refers to the mid-point of each sampling interval, taking the time for ingestion of the drug as zero.

Time (h)	10-OH-NT (μg/ml)	
	Enzymatic hydrolysis	Acid hydrolysis
0.5	0.5	0.5
1.5	6.4	6.1
3.0	10.8	14.5
6.0	10.2	18.5
10.0	9.1	23.9
13.8	10.2	22.2
20.0	15.1	17.9
36.0	7.0	14.0
54.0	1.5	7.0
79.5	2.6	4.3

Quantitative determinations

From the chromatograms of samples of known amounts of the compounds (NT, DNT or 10-OH-NT), standard curves were prepared by plotting the ratio of the peak height of the compound divided by the peak height of the internal standard against the concentration of the compound (calculated as free base), (Fig. 6). Linear relationships were obtained, provided that the linear range of the EC detector was not exceeded. As only the peak ratio was measured, this was readily accomplished by adjusting the injected amount and/or adjusting the concentration of the sample by diluting or evaporating procedures.
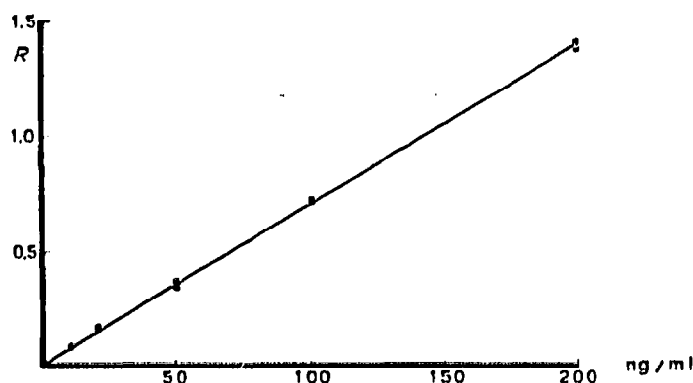
Fig. 6. Standard curve for NT in plasma. R = ratio between the peak heights of NT and the internal standard.

The lower limit for accurate determinations of NT, DNT and 10-OH-NT in plasma or urine has been found to be approximately 10 ng/ml.

Concentrations of NT and 10-OH-NT in plasma from patients are usually above this limit. Plasma concentrations of DNT, measured by combined gas chromatography–mass spectrometry, in 9 patients were all in the range 1–12 ng/ml[3].

Precision of the method

The relative standard deviation for determination of 10-OH-NT in urine, calculated from duplicate analyses of 81 urine samples obtained from experiments in four healthy volunteers given a single oral dose of NT-hydrochloride (1 mg/kg body weight) varied from 2.8 to 6.2 % in the 0.4–30 μg/ml range.

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