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GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC DETERMINATION OF AMITRIPTYLINE AND ITS MAJOR METABOLITES IN HUMAN SERUM

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SUMMARY

A gas chromatographic—electron-impact ionization mass spectrometric method has been developed for the determination of amitriptyline (AMT) and its metabolites, nortriptyline (NT), 10-hydroxyamitriptyline (10-OH-AMT) and 10-hydroxynortriptyline (10-OH-NT) in human serum. The lower limit of detection was 2 ng/ml for all compounds except 5 ng/ml for 10-OH-NT. The calibration curves for AMT and 10-OH-AMT were linear up to 100 ng/ml, and up to 200 ng/ml for NT and 10-OH-NT. The accuracy of the assay in terms of coefficient of variation was less than 7%. The extraction efficiency was almost quantitative for all compounds except 60% for 10-OH-NT.

Using this method, human serum samples which had been collected after oral administration of a single 50-mg dose of AMT were analyzed. Ratios of the conjugation of each metabolite were estimated, including AMT.

INTRODUCTION

The tricyclic antidepressant amitriptyline (AMT) is mainly metabolized to nortriptyline (NT) via N-demethylation, and their hydroxy metabolites by oxidation at position 10 [1—3]; the structures of these compounds are shown in Fig. 1. As minor metabolites, desmethylnortriptyline and amitriptyline N-oxide have been identified in human urine. The *trans* and *cis* enantiomers of the 10-hydroxylated metabolite, which are essentially equipotent to the parent compound in terms of blockade of norepinephrine uptake [4], can be analyzed stereoselectively using high-performance liquid chromatography [5—8]. The hydroxy metabolites have been demonstrated to be conjugated in considerable quantities in human urine or plasma [1, 2, 9]. The presence of the N-glucuronide of AMT in human urine was suggested by Breyer-Pfaff et al. [2],

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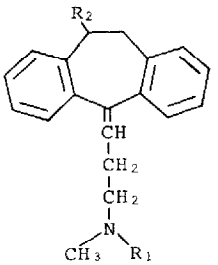


	R_1	R_2
I	CH_3	H
II	H	H
III	CH_3	OH
IV	H	OH

Fig. 1. Structures of amitriptyline (I), nortriptyline (II), 10-hydroxyamitriptyline (III) and 10-hydroxynortriptyline (IV).

but no information has been reported about conjugation of AMT in human plasma.

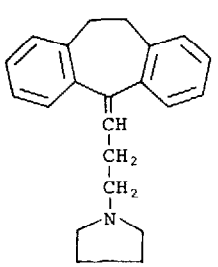
Although various methods have been available for the determination of AMT and its metabolites in biological fluids, including thin-layer chromatography [3, 10], gas chromatography [11, 12], high-performance liquid chromatography [5—8, 13—15] and gas chromatography—mass spectrometry (GC—MS) [9, 16—18], the GC—MS method is the most suitable for the separative determination because of its high sensitivity and specificity. A GC—electron-impact (EI) MS method has been described by Alván et al. [17] for the measurement of NT and 10-OH-NT and by Biggs et al. [18] for tricyclic antidepressants including AMT and NT. Garland et al. [9] have published a GC—chemical ionization (CI) MS method for the quantitation of AMT and its metabolites in plasma. The method was not readily applied because the standard curves of hydroxy metabolites were not linear due to use of monodeuterated analogues as internal standards.

Therefore, we have developed a more convenient method for the analysis of AMT and its major metabolites in serum using GC—EI-MS, and then applied the procedure to evaluate the conjugation ratios of these compounds.

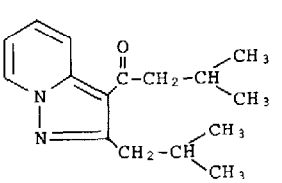
EXPERIMENTAL

Materials

Hydrochlorides of AMT and NT, *cis* and *trans* isomers of 10-OH-AMT and 10-OH-NT, and IS-1 (Fig. 2) were all gifts from H. Lundbeck, Denmark. IS-2 was synthesized according to a method described elsewhere [19]. Analytical reagent grade *n*-hexane and benzene were distilled in a glass still before use.



IS—1



IS—2

Fig. 2. Structures of internal standards.

Distilled water was passed through Amberlite MB-3. Other reagents and solvents were used as received. β-Glucuronidase—arylsulphatase was purchased from Boehringer Mannheim (Mannheim, F.R.G.).

Apparatus

A JEOL Model JMS-D300 mass spectrometer was employed in conjunction with the manufacturer's Model JGC-20K gas chromatograph and JMA-3100 mass data analysis system. Aliquots of each standard compound were injected into the GC—MS system after trifluoroacetylation, and then EI ionization mass spectra were recorded under the following conditions. A glass column, 1 m × 2 mm I.D., was packed with 2% OV-17 on 80—100 mesh Chromosorb W HP (Gasukuro Kogyo, Tokyo, Japan). Oven and injection port temperatures were 235°C and 250°C, respectively. Helium was used as a carrier gas, maintaining a column head pressure to 0.9 kg/cm^2. The MS conditions were: ionization current, 300 μA; ion source temperature, 200°C; separator temperature, 280°C; EI mode. For selected-ion monitoring, the mass spectrometer was set to monitor *m/z* 58 for AMT and 10-OH-AMT and *m/z* 84 for IS-1 at an ionization voltage of 24 eV, and *m/z* 232 for NT, 230 for 10-OH-NT and 201 for IS-2 at 70 eV. Other GC—MS conditions were the same as those described above, except that mass fragmentograms were recorded on a Rikadenki Model R-14 four-pen recorder.

Extraction

To a 1-ml aliquot of standard or unknown serum sample were added 200 μl of aqueous solution containing IS-1 (1.5 μg free base per ml), 200 μl of IS-2 in *n*-hexane (0.4 μg/ml) and 0.1 ml of 5 *M* NaOH. The serum was then extracted with 6 ml of *n*-hexane by shaking for 10 min in a mechanical shaker. After centrifuging at 1300 *g* for 10 min, 5 ml of the *n*-hexane layer were evaporated to dryness. To the residue were added 100 μl of 0.1% triethylamine in *n*-hexane and of trifluoroacetic anhydride. The mixture was reacted at 50°C for 10 min. After the removal of solvent by evaporation, the residue was vortexed with 0.1 ml of benzene and 0.1 ml of distilled water followed by centrifugation. An aliquot of the resultant benzene layer was injected into the GC—MS system.

For the determination of total quantity including conjugate, serum samples were treated with 1 *M* acetate buffer solution (pH 5.2) containing 0.1 U of β-glucuronidase and 0.05 U of arylsulphatase at 37°C for 16 h prior to the above extraction.

Quantitation

Quantitation of AMT and 10-OH-AMT was separately performed from that of NT and 10-OH-NT. All determinations were carried out by calculating a peak height ratio to internal standard.

Serum specimens

Blood samples were withdrawn from one male healthy volunteer, who had received a tablet of AMT (50 mg, Tryptanol®, Merck-Banyu, Japan) with a glass of water. Serum samples were prepared as usual and then kept at −20°C until assayed.

RESULTS AND DISCUSSION

Mass spectra of AMT, trifluoroacetyl (TFA)-NT, TFA-10-OH-AMT, TFA-10-OH-NT, IS-1 and IS-2 are presented in Fig. 3. Both AMT and TFA-10-OH-AMT gave one major fragment ion *m/z* 58 $[CH_2N(CH_3)_2]^+$, while TFA-NT and TFA-10-OH-NT showed several fragment-ion peaks with *m/z* 232 and 230 [M—(NCH_3COCF_3, H)] as base peak, respectively. IS-1 gave one major fragment ion, *m/z* 84 $[CH_2N\square]^+$, whereas IS-2 gave several fragment ions in addition to the base peak ion *m/z* 201 $[M—CH_2CH(CH_3)_2]^+$. In selected-ion monitoring, these base peak ions were monitored.

Under the GC—MS conditions employed here, a slight peak separation was observed between enantiomers of each hydroxy metabolite, but the retention

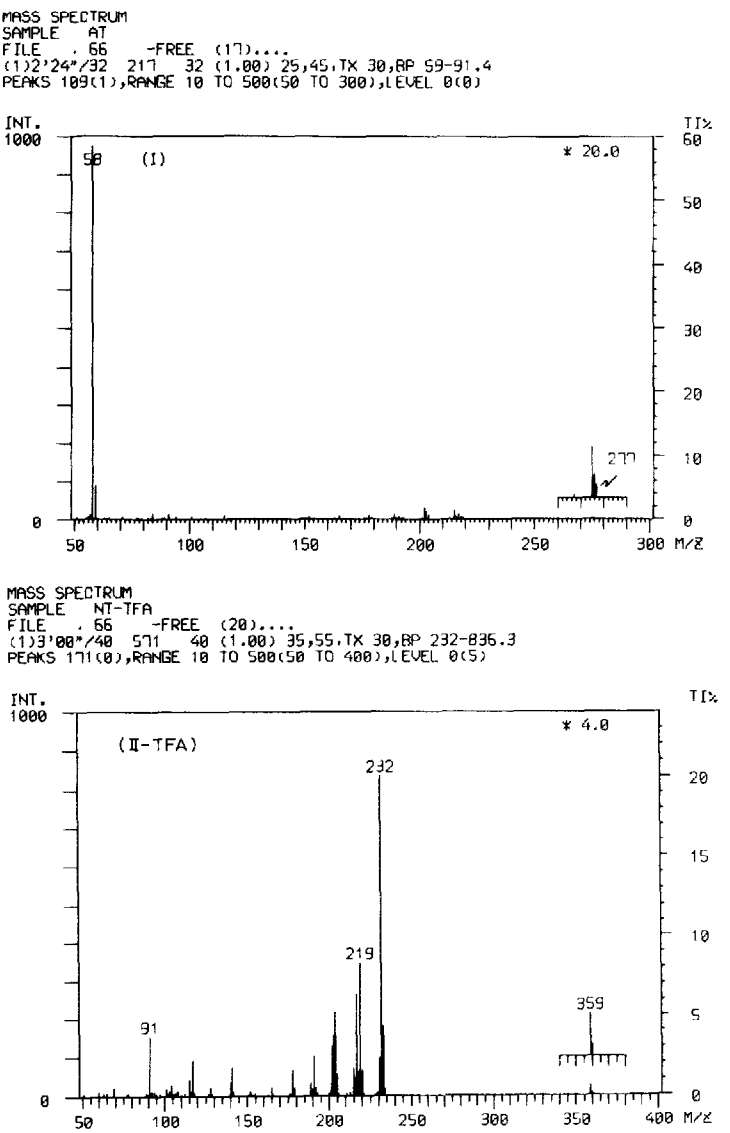
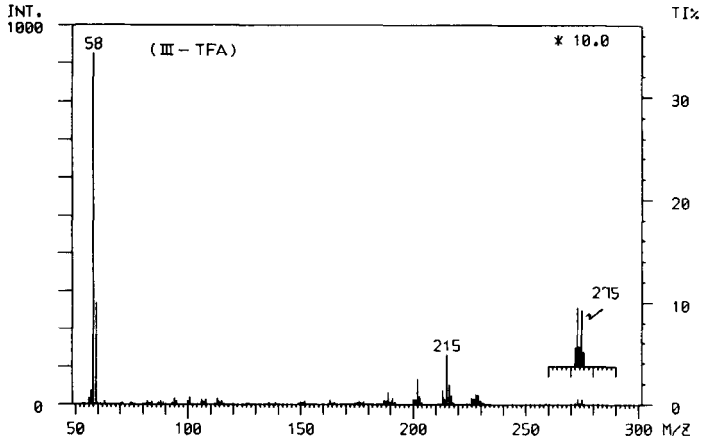
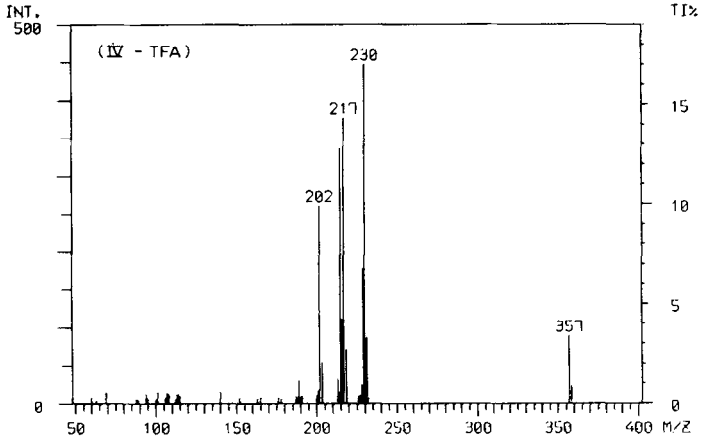


Fig. 3

MASS SPECTRUM
SAMPLE : 10-OH-AT-TFA
FILE : 66 -FREE (18)....
(1)3'09"/42 367 42 (1.00) 35,50;TX 30,BP 59-268.9
PEAKS 139(1),RANGE 10 TO 500(50 TO 300),LEVEL 0(3)



MASS SPECTRUM
SAMPLE : 10-OH-NT-TFA
FILE : 66 -FREE (21)....
(2)3'27"/46 392 46 (1.00) 40,60;TX 30,BP 230-447.7
PEAKS 118(0),RANGE 10 TO 500(50 TO 400),LEVEL 0(5)



MASS SPECTRUM
SAMPLE : IS-1
FILE : 66 -FREE (23)....
(2)2'51"/38 247 38 (1.00) 30,45;TX 30,BP 85-99.0
PEAKS 141(1),RANGE 10 TO 500(50 TO 350),LEVEL 0(1)

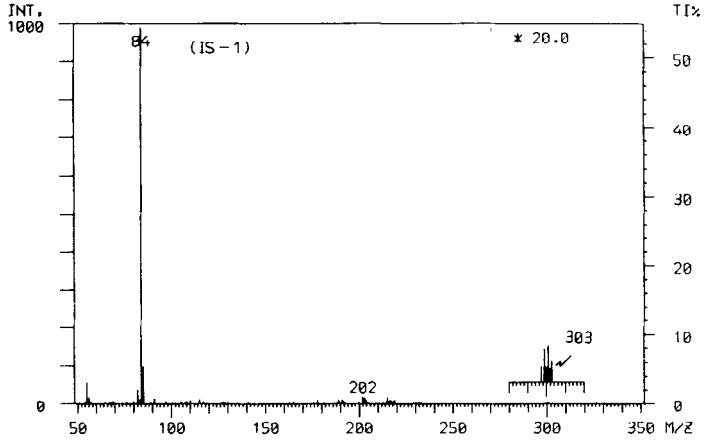


Fig. 3.

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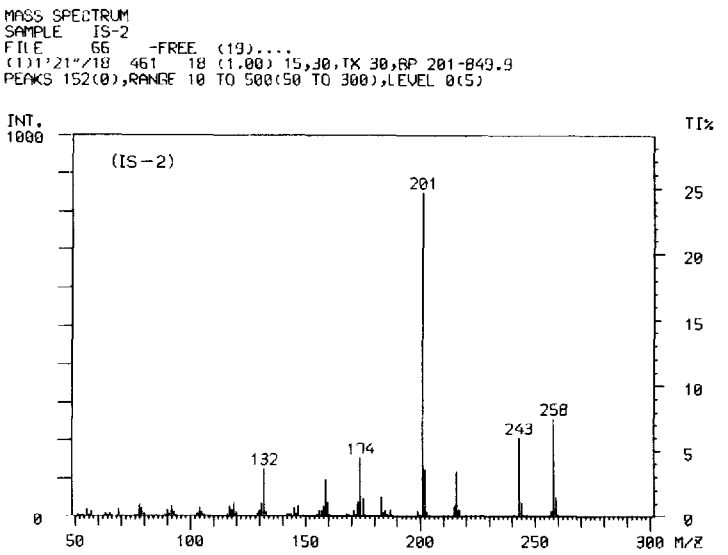

Fig. 3. Mass spectra of amitriptyline (I), nortriptyline (II), 10-hydroxyamitriptyline (III), 10-hydroxynortriptyline (IV), IS-1 and IS-2. II, III, IV are trifluoroacetyl derivatives.

times and mass spectra were identical after trifluoroacetylation. This was due to the conversion from the stereospecific hydroxy compound to a non-chiral compound by this reaction, as illustrated in Fig. 4; i.e. the stereospecific hydroxy compounds were dehydrated to the corresponding 10,11-dienes, which were confirmed by the mass spectra. Kraak and Bijster [6] demonstrated the presence of the *trans* form of each hydroxy metabolite in human serum, and Suckow and Cooper [7] suggested the presence of both configurations in human serum using high-performance liquid chromatography. As apparent from the above evidence, our method was not so stereospecific that it gave the total quantity of the two isomers.

Typical mass fragmentograms for the measurement of AMT and 10-OH-AMT, and of NT and 10-OH-NT are presented in Fig. 5A and Fig. 5B, respectively. Mass fragmentograms obtained from blank serum (Fig. 5a) indicated that the determination was not disturbed by human serum. Similar mass fragmentograms were obtained even after the incubation of blank serum with

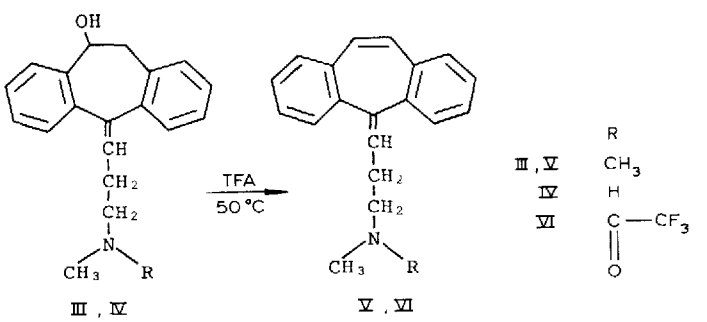

Fig. 4. Trifluoroacetyl derivatives of 10-hydroxyamitriptyline (III) and 10-hydroxynortriptyline (IV). V = III-TFA, VI = IV-TFA.

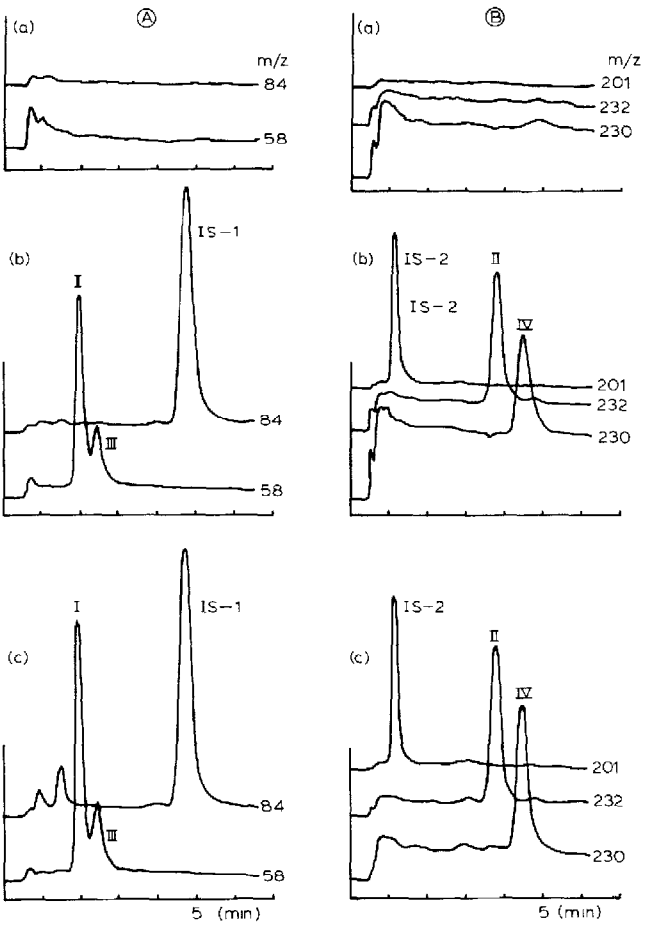

Fig. 5. Mass fragmentograms for measurement of (A) amitriptyline (I) and 10-hydroxyamitriptyline (III), and (B) nortriptyline (II) and 10-hydroxynortriptyline (IV). (a) Blank serum, (b) blank serum spiked with standard compounds, 50 ng (c) serum treated with amitriptyline.

β-glucuronidase—arylsulphatase. Fig. 5b and c are mass fragmentograms obtained from serum spiked with authentic compound and internal standard, and serum sample obtained after drug administration, respectively.

Dynamic range, accuracy and recovery in the determination of each compound are summarized in Table I. Linear calibration curves were obtained over the dynamic range. To check the accuracy and precision of the method, four serum samples spiked with authentic samples were analyzed at two concentration levels, 10 and 100 ng/ml. Coefficients of variation at these levels were less than 7%. This result indicates that the method is accurate and precise. To determine recovery, *n*-hexane extracts of blank serum containing internal standard alone were spiked with authentic sample and then treated according to the above method. The slope was compared with that found with serum samples containing authentic sample and internal standard. As shown in Table I, the overall percentage recovery of AMT, 10-OH-AMT and NT was greater than 90% and that of 10-OH-NT was about 60%. These recoveries are better

TABLE I

DYNAMIC RANGE, COEFFICIENT OF VARIATION (C.V.) AND RECOVERY OF AMITRIPTYLINE (I), NORTRIPTYLINE (II), 10-HYDROXYAMITRIPTYLINE (III) AND 10-HYDROXYNORTRIPTYLINE (IV)

Compound	Dynamic range (ng/ml)	C.V. (%)		Recovery (%)
		10 ng/ml	100 ng/ml	
I	2—100	2.8	2.3	100
II	2—200	2.2	3.4	96
III	2—100	4.6	5.2	94
IV	5—200	6.9	3.1	58

TABLE II

SERUM CONCENTRATIONS OF AMITRIPTYLINE (I), NORTRIPTYLINE (II), 10-HYDROXYAMITRIPTYLINE (III) AND 10-HYDROXYNORTRIPTYLINE (IV) AFTER ORAL ADMINISTRATION OF AN AMITRIPTYLINE PREPARATION (TRYPTANOL, 50 mg) TO A HUMAN SUBJECT

Results are expressed in ng/ml.

Compound		Time (h)						
		2	4	6	12	15	24	32
I	Free	23	23	14	8	13	7	3
	Conjugated	18	9	18	11	3	23	3
II	Free	7	10	8	11	7	9	4
	Conjugated	nd*	nd	nd	nd	nd	nd	nd
III	Free	15	15	4	nd	5	nd	nd
	Conjugated	91	169	108	93	31	21	18
IV	Free	15	18	14	26	18	16	11
	Conjugated	8	26	32	28	23	16	5

*nd = not determined.

than those of methods published previously. For example, Garland et al. [9] had to add a substantial amount of chlorpromazine to the plasma sample prior to extraction in order to increase extraction efficiency. Even so, their recovery ranged from 36 to 69%. On the other hand, our method did not require any addition of such a carrier substance. The assay limits were 2 ng/ml for AMT, NT and 10-OH-AMT, and 5 ng/ml for 10-OH-NT when a 1-ml aliquot of serum was extracted. These sensitivities are inferior to those of the GC—CI-MS method [9], but superior to those of high-performance liquid chromatography [7]. It has also been recognized that GC—CI-MS [9] gives a lower sensitivity for 10-OH-NT than for other metabolites.

The serum concentrations of unconjugated and conjugated AMT, NT, 10-OH-AMT and 10-OH-NT after a single oral dose of 50 mg of AMT, are presented in Table II. Quantities of conjugated form were calculated as the difference between total and free quantities. The total quantities were obtained

from samples which had been treated with β-glucuronidase—arylsulphatase before extraction. AMT was conjugated to a level of half the total amount during the period of measurement. The conditions of enzymatic hydrolysis were checked with three levels of enzymes (0.104 and 0.052 U, 0.208 and 0.104 U, and 0.520 and 0.260 U of β-glucuronidase and of arylsulphatase, respectively) using urine samples of a patient treated with AMT. As shown in Fig. 6, the lowest level of enzyme employed in this study was sufficient for hydrolysis of conjugates of two hydroxy metabolites, but the enzyme level was not good enough for conjugates of AMT and NT. Therefore, the values for the conjugation of AMT and NT presented in Table II were underestimated. The type of conjugation of AMT in serum has not been identified. It is probable that the type is N-glucuronide because conjugated AMT in serum was hydrolyzed to intact AMT by treatment with β-glucuronidase—arylsulphatase as shown by this study, and because the N-glucuronide of AMT was suggested to be present in human urine by Breyer-Pfaff et al. [2]. Such a quaternary ammonium N-glucuronide has been identified as a unique metabolite of cyproheptadine [20] and tripelennamine [21] in human urine. Axelrod et al. [22] reported that N-glucuronide resisted hydrolysis with β-glucuronidase, which was rather non-enzymatic. The resistance to β-glucuronidase was also confirmed in hydrolysis of conjugate of AMT, but its hydrolysis was enzymatic as shown by the dependence of the hydrolysis rate on enzyme concentration. A first-order kinetic analysis of Fig. 6 indicated that over 80% of AMT was conjugated in human urine. The conjugation of NT in serum was not detected, although the conjugation of NT was suggested in urine as shown in Fig. 6. It is

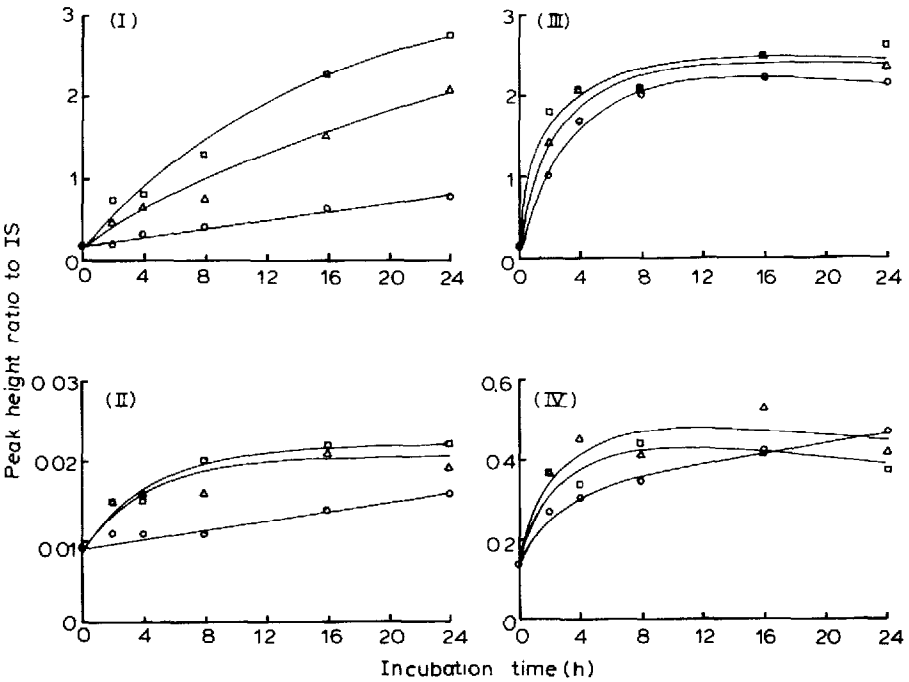

Fig. 6. Releasing profiles of amitriptyline (I), nortriptyline (II), 10-hydroxyamitriptyline (III) and 10-hydroxynortriptyline (IV) after incubation of urine of a patient (dosed with amitriptyline) with 0.104 and 0.052 U (○), 0.208 and 0.104 U (△), and 0.520 and 0.260 U (□) of β-glucuronidase and arylsulphatase, respectively.

considered that secondary amine is more easily conjugated than tertiary amine, but to our knowledge it has not been described that NT is conjugated in serum. In contrast to NT, 10-OH-AMT was almost completely conjugated, though the free component could be detected within a few hours after dosing. This extensive conjugation is in agreement with results obtained in similar studies by other workers [9], who found that more than 85% of 10-OH-AMT was conjugated. About 60% of total 10-OH-NT was conjugated, which was consistent with data obtained by other workers. Kragh-Sørensen et al. [23] showed that 51—85% of 10-OH-NT was conjugated in human plasma after oral administration of NT. From the data of Garland et al. [9] it was estimated that the conjugation ratio of 10-OH-NT ranged from 56 to 84% in human plasma after oral administration of AMT.

It is well known that there are wide inter-patient variations in the serum concentration of AMT. Many studies have been devoted to establishing the relationship between serum concentrations of AMT and/or NT and clinical response, but the data are still conflicting. In order to clarify these questions it would be important to measure not only intact drug but also its metabolites, because the hydroxy metabolites have pharmacological activities comparable with AMT, and the biological fate of the drug depends on individual metabolic activity.

REFERENCES

1 E. von Eschenhof and J. Rieder, Arzneim.-Forsch., 19 (1969) 957.
2 U. Breyer-Pfaff, M. Evert and R. Wiatr, Arzneim.-Forsch., 28 (1978) 1916.
3 G. Santagostino, R.M. Facino and D. Pirillo, J. Pharm. Sci., 63 (1974) 1690.
4 L. Bertilsson, B. Mellström and F. Sjöqvist, Life Sci., 25 (1979) 1285.
5 G.A. Smith, P. Schulz, K.M. Giacomini and T.F. Blaschke, J. Pharm. Sci., 71 (1982) 581.
6 J.C. Kraak and P. Bijster, J. Chromatogr., 143 (1977) 499.
7 R.F. Suckow and T.B. Cooper, J. Chromatogr., 230 (1982) 391.
8 B. Mellström and R. Braithwaite, J. Chromatogr , 157 (1978) 379.
9 W.A. Garland, R.R. Muccino, B.H. Min, J Cupano and W.E. Fann, Clin. Pharmacol. Ther., 25 (1979) 844.
10 U. Breyer and K. Villumsen, Eur. J. Clin. Pharmacol., 9 (1976) 457.
11 L.A. Gifford, P. Turner and C.M.B. Pare, J. Chromatogr., 105 (1975) 107.
12 A. Jørgensen, Acta Pharmacol. Toxicol., 36 (1975) 79.
13 F.L. Vandemark, R.F. Adams and G.J. Schmidt, Clin. Chem., 24 (1978) 87.
14 H.F. Proelss, H.J. Lohmann and D.G. Miles, Clin. Chem., 24 (1978) 1948.
15 S.R. Biggs, L.F. Chasseaud, D.R. Hawkins and I. Midgley, Drug Metab. Dispos., 7 (1979) 233.
16 R.G. Jenkins and R.O. Friedel, J. Pharm. Sci., 67 (1978) 17.
17 G. Alván, O. Borgå, M. Lind, L. Palmér and B. Siwers, Eur. J. Clin. Pharmacol., 11 (1977) 219.
18 J.T. Biggs, W.H. Holland, S. Chang, P.H. Hipps and W.R. Sherman, J. Pharm. Sci., 65 (1976) 261.
19 T. Irikura, M. Hayashi, K. Koshirae, J. Hatayama, E. Hetsugi, Ger. Offen., 2,315,801; Chem. Abstr., 80 (1974) 432.
20 C.C. Porter, B.H. Arison, V.F. Gruber, D.C. Titus and W.J.A. Vandenheuvel, Drug Metab. Dispos., 3 (1975) 189.
21 N.K. Chudhuri, O.A. Servando, M J. Manniello, R.C. Luders, D.K. Chao and M.F. Bartlett, Drug Metab. Dispos., 4 (1976) 372.
22 J. Axelrod, J.K. Inscoe and G.M. Tomkins, J. Biol. Chem., 232 (1958) 835.
23 P. Kragh-Sørensen, O. Borgå, M. Garle, L.B. Hansen, C.E. Hansen, E.F. Hvidberg, N.E. Larsen and F. Sjöqvist, Eur. J. Clin. Pharmacol., 11 (1977) 479.