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AMITRIPTYLINE AND ITS BASIC METABOLITES DETERMINED IN PLASMA BY GAS CHROMATOGRAPHY

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SUMMARY

Gas chromatography was used to determine plasma levels of amitriptyline, nortriptyline and their 10-hydroxy derivatives after conversion to the dehydro compounds by heating with acid. The primary amine 10-hydroxydesmethylnortriptyline is also dehydrated and the dehydro compound coincides on the chromatogram with dehydronortriptyline. Treatment of the extract with salicylaldehyde selectively removed the primary amine, which was determined by difference. *Cis*- and *trans*-hydroxydesmethylnortriptyline were isolated from urine by thin-layer chromatography and used to standardize the estimation.

The stability of all the metabolites in plasma was investigated. Results are given for hydroxydesmethylnortriptyline levels in the plasma of 41 patients treated with amitriptyline.

INTRODUCTION

The hydroxy metabolites of amitriptyline (AT) and nortriptyline (NT) have recently been shown to be pharmacologically active [1—3], suggesting that where plasma levels of the parent drugs are monitored the 10-hydroxy derivatives should also be determined. We have modified our routine method for estimating AT and NT [4] to include the assay of these metabolites.

Gas chromatograpy (GC) has previously been used to determine 10-hydroxyamitriptyline (OHAT) and/or 10-hydroxynortriptyline (OHNT) in plasma, with electron-capture detection [5] or mass spectrometry [6—9]. In

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these methods *cis* and *trans* isomers were estimated together after dehydration to dehydroamitriptyline (DHAT) and dehydronortriptyline (DHNT), respectively. The methods for determining OHAT [8, 9] require mass spectrometry and are therefore unavailable to many laboratories.

Bertilsson and Alexanderson [10] separated and quantified the *cis* and *trans* isomers of OHNT in urine, using thin-layer chromatography (TLC). In plasma extracts, Breyer and Villumsen [11] demonstrated the partial separation of these isomers by TLC but without quantification. Breyer-Pfaff et al. [12] assayed *trans*-OHNT in plasma by TLC with reflectance spectrophotometry but the method was insufficiently sensitive to determine the other hydroxy metabolites.

High-performance liquid chromatography (HPLC) offers the possibility of determining AT and all its basic metabolites in plasma with separation of *cis* and *trans* isomers. Early work [13, 14] demonstrated the separation of the OHAT and OHNT isomers but provided no validated estimation procedures. In some later studies [15—18] sensitivity was inadequate and one or both pairs of isomers were unseparated. More recently, using methods with sufficient sensitivity, Edelbroek et al. [19] determined *cis*- and *trans*-OHAT together while Suckow and Cooper [20] and Bock et al. [21] separated both pairs of geometrical isomers.

Hydroxydesmethylnortriptyline (OHDMNT) has not previously been determined in plasma. It was identified in urine by Borgå and Garle [5] and shown by Alexanderson and Borgå [22] to constitute about 16% of the total metabolites recovered from the urine of patients treated with NT. These determinations were unreliable, however, as no reference OHDMNT isomers were available for standardization. Biggs et al. [23] used HPLC to determine AT, NT, desmethylnortriptyline (DMNT) and all their 10-hydroxy metabolites after dehydration, in urine. Standardizing with synthetic OHDMNT they found that conjugated and unconjugated OHDMNT together constituted about 13% of the metabolites recovered from the urine after single doses of AT. The sensitivity of the method was much too low for use with plasma.

We showed previously [24] that DMNT does not separate from NT on OV-17 but can be selectively removed by treatment of the plasma extract with salicylaldehyde (SA). Patients' plasma extracts, prepared by the present method and hence containing AT, NT, DHAT and DHNT, were treated with SA. Reductions occurred not only in the peak at the NT position but also in that in the position of DHNT, suggesting the presence of dehydrodesmethylnortriptyline (DHDMNT) formed from OHDMNT in the plasma. Pure samples of the OHDMNT isomers were not available to us. However, during the isolation of *cis*- and *trans*-OHNT from patients' urine by TLC, some of the fractions showed very large reductions in the peaks in the DHNT position on SA treatment. TLC with a different solvent produced two clear spots from such preparations. When material eluted from these spots was assayed by our usual procedure, SA treatment completely eliminated the dehydro compound peak from the front spots and had no effect on the corresponding peak from the rear spots. By successive TLC using three different solvents (Table II), two preparations were obtained from patients' urine, containing about 100 μg of *cis*- and *trans*-OHDMNT, respectively. These solutions were standardized on

the basis of their ultraviolet (UV) spectra and used to validate the determination of OHDMNT in plasma samples by the SA method.

MATERIALS AND METHODS

Drugs and metabolites

These were kindly donated as follows: amitriptyline hydrochloride by Merck Sharp & Dohme (Hoddesdon, U.K.). nortriptyline hydrochloride by E.R. Squibb and Sons (Twickenham, U.K.), desmethylnortriptyline and the *cis* and *trans* isomers of 10-hydroxyamitriptyline and 10-hydroxynortriptyline by Dr. A. Jørgensen of H. Lundbeck & Co. (Copenhagen, Denmark), maprotiline and desmethylclomipramine by Ciba-Geigy Pharmaceuticals (Horsham, U.K.).

Solvents

These were obtained from BDH (Poole, U.K.). *n*-Heptane, *n*-pentane, toluene (low in sulphur), all general purpose reagent grade, and diethylamine (analytical reagent grade) were distilled before being used for plasma extractions. Analytical reagent grade amyl alcohol was used without purification. For TLC, solvents were used without purification and consisted of general purpose reagent grade heptane and analytical reagent grade diethylamine, chloroform, *n*-butan-1-ol and glacial acetic acid.

Preparation of standard solutions

Stock solutions of drug compounds and metabolites in 0.01 *M* hydrochloric acid contained 250 μg/ml, expressed as base. Dilutions, containing 10 μg/ml or 1 μg/ml, were used when making additions to plasma. Standard solutions of the bases of AT, NT, maprotiline and desmethylclomipramine were prepared by extraction from 0.01 *M* hydrochloric acid into an equal volume of heptane containing 0.5% diethylamine [4]. A standard solution of DHAT and DHNT bases in the same solvent was prepared by mixing 5 ml stock OHAT, 5 ml stock OHNT and 10 ml of 1 *M* hydrochloric acid. After heating for 1 h in a boiling water bath, 3 ml of 4 *M* sodium hydroxide and 300 μl diethylamine were added and the solution was extracted with 20 ml heptane containing 0.5% diethylamine. The organic layer thus contained 625 ng/ml of DHAT and of DHNT, expressed in terms of OHAT and OHNT base, respectively. Mixtures of bases in the same solvent were prepared for calibrating the chromatograph. A 10-μl volume of each mixture contained 50 ng tetracosane, 150 ng maprotiline, 150 ng desmethylclomipramine and equal amounts (either 150 or 7.5 ng) of AT, NT, DHAT and DHNT.

Extraction from plasma

The three stoppered tubes required for each extraction were treated as previously described [4]. A 1.0-ml volume of a solution, prepared by diluting 125 ml of 4 *M* sodium hydroxide and 10 ml of diethylamine to 200 ml with water, was pipetted into a 35-ml tube, which was stoppered and rotated in the hand until the whole surface had been wetted with alkali. To the plasma sample (1—8 ml) in a polystyrene tube was added 1.0 ml of 0.01 *M* hydrochloric acid containing 1 μg of maprotiline as internal standard. After mixing, the plasma

was washed quantitatively into the 35-ml tube with water. Then 10 ml toluene and 0.1 ml amyl alcohol were added and water run in until the 35-ml tube was almost full. After mixing for 30 min on a rotary tumbler at 30 rpm, the tube was centrifuged for 15 min at 2000 *g*. The toluene layer was transferred to a 15-ml tube, using a Pasteur pipette that had been rinsed first with diethylamine and then with toluene. (Between successive samples the pipette was rinsed with toluene only.) After adding 2 ml of 0.05 *M* sulphuric acid the tube was tumbled at 30 rpm for 15 min and centrifuged briefly with stopper in place. Most of the toluene was aspirated, using a filter-pump. Pentane (10 ml) was added to the aqueous layer and the tube was stoppered, inverted several times and briefly left for the layers to separate. The pentane was removed quantitatively with the pump. Then 0.1 ml of 1 *M* hydrochloric acid was added and the tube, without stopper, placed in an ordinary domestic pressure cooker. The rack of tubes was covered with aluminium foil and heated at 1 bar excess pressure for 70 min. After cooling to room temperature, 1 μg of desmethylclomipramine in 0.1 ml of 0.01 *M* hydrochloric acid was added. Into a conical tube was pipetted 0.3 ml of a solution prepared by diluting 100 ml of 4 *M* sodium hydroxide and 45 ml diethylamine to 300 ml with water. The whole surface was wetted with the alkali as before. The acid solution from the 15-ml tube was poured into the conical tube and the final extraction of the compounds into 50 μl of heptane containing diethylamine and 250 ng tetracosane was carried out as previously described [4].

Treatment of the extract with salicylaldehyde was performed as described before [24].

Gas chromatography

Chromatography on OV-17 packed columns with flame ionization detectors was carried out as before [4]. The system was calibrated daily by injecting the standard mixtures of bases to obtain peak area ratios and corrections for losses in chromatography [4]. Results for plasma samples were then calculated assuming that the relative amounts of maprotiline and all the compounds to be estimated were the same in the plasma extract as in the original plasma sample. Desmethylclomipramine was not used in the calculation, having been introduced half-way through the extraction in order to minimize adsorptive losses of other compounds.

Table I shows that the retention times of NT and DHAT differ by a factor of only 1.08. Resolution of these peaks was incomplete (Figs. 1 and 2) and the integrator was used to measure only their combined area. This was allocated between the two individual peaks in proportion to their measured heights, using a weighting factor of 1.08 for DHAT.

With patients' plasma samples, the peak area of NT may be up to fifteen times greater than that of DHAT. On injecting a series of base mixtures, each containing 150 ng maprotiline and 15 ng DHAT per 10 μl, but with amounts of NT ranging from 15 to 300 ng, it was found that the measured DHAT peak area rose with increasing NT content. Presumably the NT peak tails slightly under the DHAT. When 2% of the measured NT area was subtracted from the measured area of DHAT, the resulting corrected value for DHAT was independent of the amount of NT present. DHAT peak areas corrected in this way were used when estimating plasma OHAT concentrations.

TABLE I

RELATIVE RETENTION TIMES

OV-17 packed columns. Compounds extracted from aqueous alkali into heptane.

Compound	RR_{AT}
Tetracosane	0.68
Amitriptyline (AT)	1.00
Nortriptyline (NT)	1.16(5)
Desmethylnortriptyline (DMNT)	1.16(5)*
Dehydroamitriptyline (DHAT)	1.26
Dehydrodesmethylnortriptyline (DHDMNT)	1.41(5)**
Dehydronortriptyline (DHNT)	1.43
Maprotiline	1.71(5)
trans-10-Hydroxyamitriptyline (OHAT)	1.88
cis-10-Hydroxyamitriptyline (OHAT)	1.97
trans-10-Hydroxynortriptyline (OHNT)	2.22
trans-10-Hydroxydesmethylnortriptyline (OHDMNT)	2.25
cis-10-Hydroxynortriptyline (OHNT)	2.36
Desmethylclomipramine	2.40
cis-10-Hydroxydesmethylnortriptyline (OHDMNT)	2.44
Cyclizine	0.59
Benzhexol	1.09
Zimelidine	1.16
Nomifensine	1.20
Didesmethylclomipramine (primary amine)	2.47
Flurazepam	7.32
Temazepam	no peak found
Lorazepam	no peak found
Atenolol	no peak found
Chlordiazepoxide	no peak found
Salbutamol	no peak found

*Also a minor peak at 1.30.
**Also a minor peak at 1.63.

Isolation of cis- and trans-hydroxydesmethylnortriptyline from urine

Glass plates (Merck), 20 × 20 cm with silica gel 60 containing fluorescent indicator were used in a tank lined with filter paper.

Morning samples of urine from patients being treated with AT were made alkaline with sodium hydroxide (0.1 *M* final concentration) and extracted with 0.33 vol toluene. The toluene was extracted with about 0.2 of its volume of 0.01 *M* hydrochloric acid and the acid layer was washed with pentane. After aspiration of the pentane and addition of sodium hydroxide to 0.1 *M* final concentration, the bases were reextracted from the aqueous layer into about 0.1 of its volume of toluene. This solution was evaporated to small volume under a stream of nitrogen and applied to the plate with a 5-μl capillary. After development the spots or bands seen under UV light were scraped off and eluted with 0.01 *M* hydrochloric acid. For further TLC this solution was made alkaline and the bases were extracted into toluene and concentrated as before.

Initial chromatography in solvent A (Table II) was used to isolate the OHAT isomers for other investigations. This solvent did not separate the primary from the secondary amines, and because the plates were grossly overloaded with

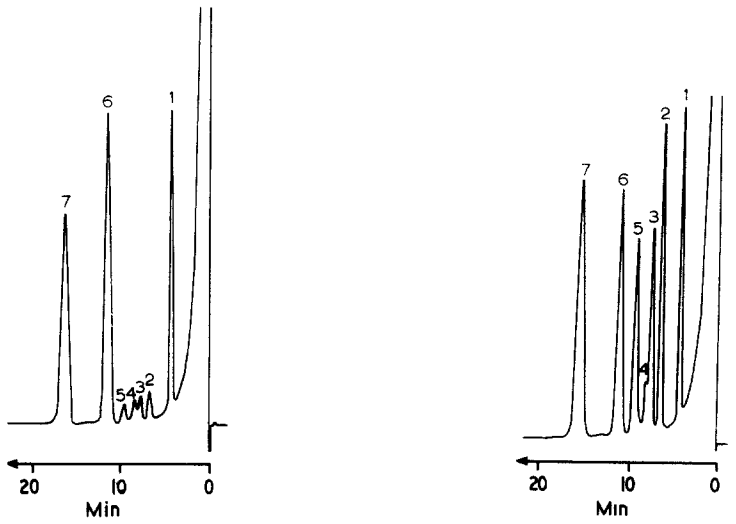

Fig. 1. Chromatogram from 10 μl of standard base mixture containing (1) 50 ng tetracosane; (2) 7.5 ng AT; (3) 7.5 ng NT; (4) DHAT from 7.5 ng OHAT; (5) DHNT from 7.5 ng OHNT; (6) 150 ng maprotiline, internal standard; and (7) 150 ng desmethyl clomipramine.

Fig. 2. Chromatogram from 10 μl of extract from 5 ml of a patient's plasma. Peaks numbered as in Fig. 1. Calculated concentrations: 130 ng/ml AT; 104 ng/ml NT; 26 ng/ml OHAT; 132 ng/ml OHNT.

TABLE II

THIN-LAYER CHROMATOGRAPHY

Compound	Distance above baseline (cm)		
	Solvent A*	Solvent B*	Solvent C*
cis-OHNT	1.6	7.2	3.0
cis-OHDMNT	**	8.5	**
trans-OHNT	2.1	7.2	3.8
trans-OHDMNT	**	8.5	**
cis-OHAT	6.0	5.5	9.0
DHNT	6.4	8.2	—
trans-OHAT	6.9	5.5	9.0
NT	8.0	8.3	9.0
DMNT	**	9.2	**
DHAT	13.6	6.4	—
AT	15.1	6.5	14

*Solvent A: heptane—diethylamine (7:1, v/v), run to top of 20-cm plate four times. Solvent B: butanol—glacial acetic acid—water (4:1:1, v/v/v), run on 20-cm plate once, for 4 h. Solvent C: heptane—chloroform—diethylamine (7:2:1, v/v/v), run to top of 20-cm plate three times.

**In solvents A and C the primary amines gave elongated spots in approximately the same positions as the corresponding secondary amines.

cis- and *trans*-OHNT these did not separate from each other. However, they were freed from the other bases listed in Table II as well as from various unidentified UV-absorbing materials.

Solvent B (Table II) was then used to separate OHDMNT from OHNT, followed by solvent C to separate the two isomers of OHDMNT. The final eluates were made alkaline and extracted with toluene. These bases were back-extracted into 0.01 *M* hydrochloric acid and traces of toluene removed by two successive extractions with pentane, mixing for 10 min each time. This procedure, applied to eluates of blank plate areas after TLC, gave solutions with zero UV absorbance.

The UV spectra of the preparations of OHDMNT from urine, together with those of AT, NT and the five metabolites of which pure samples were available, were observed in 0.01 *M* hydrochloric acid in 1-cm cuvettes in a Cecil spectrophotometer, Model CE 292.

RESULTS

Figs. 1 and 2 show chromatograms from a standard mixture of bases and from a patient's plasma extract.

Heparinized plasma obtained from drug-free human subjects gave no peaks in the region of interest. However, plasma prepared from out-dated blood-bank blood usually produced a small peak interfering with NT. Ox serum gave no peaks and was used for experiments except where heparinized plasma from volunteer human subjects is specified.

Compounds were added to ox serum, and extracts were prepared and injected before and after SA treatment. AT, NT, *cis*- and *trans*-OHAT and OHNT and the internal standards, tested separately and in various appropriate combinations, gave no interfering peaks. DMNT showed only the expected peak coinciding with NT, together with the minor peak of retention time 1.12 times that of the major peak [24]. *cis*- or *trans*-OHDMNT isolated from urine gave only a major peak of retention time 0.99 times that of DHNT, together with a minor peak of retention time 1.15 times that of the main peak. As with DMNT the ratio of minor to major peak areas was about 0.04 and both peaks disappeared completely on treatment with SA.

Determination of tertiary and secondary amines

Table III gives the results of assays of equal amounts of AT, NT and either *cis*- or *trans*-OHAT and OHNT, added to ox serum. The determinations were carried out on many different days, usually as single specimens in batches of patients' samples. Results for the *cis* and *trans* isomers were indistinguishable and have been combined.

Interactions between the different compounds were investigated by analysing samples containing 20 ng/ml of one substance, either alone or together with 200 ng/ml of the other three compounds. AT, NT and OHNT assays were unaffected by the presence of the other substances, while OHAT assays based on uncorrected peak areas were increased, as expected, by the presence of NT. When DHAT peak areas were corrected for NT tailing as already described, the OHAT assays were no longer influenced by the presence of NT. This was also tested using other concentrations of OHAT, with amounts of NT ranging from 1 to 20 times the OHAT present. A series of determinations of 20 ng/ml OHAT with 200 ng/ml NT gave a mean OHAT value of 19.2

TABLE III

SECONDARY AND TERTIARY AMINES DETERMINED IN SERUM SAMPLES CONTAINING EQUAL AMOUNTS OF ADDED COMPOUNDS

Amount added (ng/ml in 5 ml)	*n*	AT		NT		OHAT		OHNT	
		Mean	C.V. (%)	Mean	C.V. (%)	Mean	C.V. (%)	Mean	C.V. (%)
200	27	197.3	3.0	191.1	2.3	205.7	2.8	201.0	2.9
100	14	97.6	2.3	93.2	2.4	102.3	2.7	100.1	3.0
40	17	38.4	3.7	36.3	3.2	41.2	4.0	39.5	2.9
20	19	19.2	3.3	18.2	3.0	20.1	4.3	19.7	3.4
10	22	9 55	6.4	9.77	11.4	9.70	9.1	9.54	9.4

ng/ml with a coefficient of variation (C.V.) of 3.5% (n = 11), which is no greater than the value obtained for this concentration of OHAT with an equal amount of NT (Table III).

The quantity of serum or plasma present was shown not to affect the assay results for added compounds. Human plasma (1—8 ml) and ox serum (3—12 ml) all gave indistinguishable results for AT, NT and their *cis* and *trans* hydroxy metabolites.

These six compounds were found not to be adsorbed from human plasma (either fresh or previously frozen and thawed) on to glass pipettes or polystyrene blood tubes. Plasma was sucked up and down in five pipettes in series and poured from one tube to another, using five in series, without loss of compounds.

Determination of primary amines

Absorption spectra of the *cis*- and *trans*-OHDMNT preparations from urine are shown in Fig. 3, together with those of DMNT, NT, AT and *cis*-OHAT. The curves for *trans*-OHAT and both isomers of OHNT were extremely similar to those illustrated and have been omitted for clarity. All compounds showed maxima at 237 nm and minima at 228 nm. The close resemblance between the spectra of the preparations from urine and those of the pure compounds suggests that no other absorbing material contributed appreciably to the absorbance of the urine preparations. The molar extinction coefficients at 237 nm (Table IV) for all seven pure compounds were similar, the ratio of highest (*trans*-OHNT) to lowest (DMNT) being only 1.07. As neither the degree of methylation of the nitrogen atom, nor ring hydroxylation appreciably affected the maximum molar extinction coefficient, the mean value of 13,500 was taken to give a reasonably accurate measure of the concentration of OHDMNT in each of the preparations from urine.

To each of five serum samples 1.1 μg of *cis*-OHDMNT (based on this spectrophotometric standardization) were added and 1.1 μg of the *trans*-isomer to five more. After extraction with internal standards in the usual way, the peak areas of DHDMNT produced were found not to differ significantly between the two isomers. The mean value was 0.75 ± 0.03 (S.D.) times the peak area of DHNT produced by 1.1 μg of OHNT (*cis* or *trans*). The ratio is close to the value of 0.767 found previously [24] for the ratio of DMNT and NT peak areas

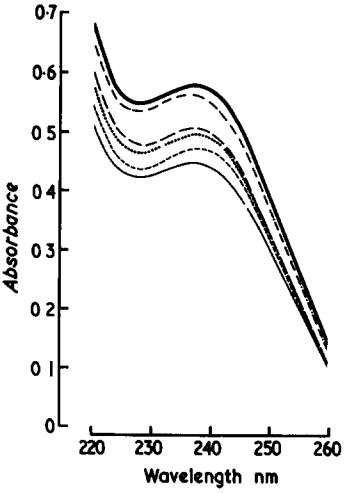

Fig. 3. Absorption spectra of compounds in 0.01 *M* hydrochloric acid. Traces: (—), *trans*-OHDMNT; (— • — • —), *cis*-OHDMNT; (— —), DMNT; (• • • • •), NT; (- - -), AT; (—), *cis*-OHAT.

TABLE IV

MOLAR EXTINCTION COEFFICIENTS AT PEAK WAVELENGTH (237 nm)

Measurements on 10 μg/ml solutions in 0.01 *M* hydrochloric acid.

Compound	$\varepsilon \cdot 10^{-3}$
AT	13.4
NT	13.4
DMNT	12.9
cis-OHAT	13.4
trans-OHAT	13.8
cis-OHNT	13.6
trans-OHNT	13.8
Mean	13.5

after extraction. This agreement supports the accuracy of the spectrophotometric standardization of the OHDMNT concentrations.

When serum extracts are treated with SA the peak areas of all the secondary amines decrease by a few percent relative to the tertiary amines and tetracosane [24]. Of the extracts prepared for the assays in Table III, six at each concentration from 20 to 200 ng/ml were treated with SA and rechromatographed. The peak area ratio of DHNT to maprotiline (which is a secondary amine) was found to be almost unaffected by SA treatment. It increased by 0.6% on average, with a standard deviation of 1.4% (n = 24).

Using symbols as before [24], F is defined as the ratio of apparent OHNT concentrations before and after SA treatment, using maprotiline as internal standard for the calculations. Let the ratio of the weight of OHDMNT to that of OHNT in the sample be r, and the ratio of the peak area of DHDMNT to that of DHNT (for equal weights of the two compounds in the sample) be a.

Then before SA treatment the apparent amount of OHNT in the sample is $(1 + ar)$ times the true amount. After SA treatment the apparent amount is 1.006 times the true amount. Hence $F = (1 + ar)/1.006$. Substituting the value of 0.75 found above for a, we have

$$F = 0.994 + 0.746r \quad (1)$$

This equation was verified using twenty determinations with *cis*-OHDMNT and twenty with the *trans* isomer. In each case the ratio r of OHDMNT to OHNT was held constant at 0.137, which is close to the mean value found in patients' plasma (see below). Five determinations were carried out at each of four OHNT concentrations, i.e. 20, 40, 100 and 200 ng/ml. F was found to be the same for *cis* and *trans* isomers and independent of concentration. Its mean value was 1.0943 ± 0.0166 (n = 40, S.E.M. = 0.0026), in good agreement with the value of 1.0962 calculated from eqn. (1) with r = 0.137. Thus, as in the determination of DMNT with NT [24], the relative amounts of primary and secondary amines extracted by the chosen procedure are independent of their absolute concentrations. The effect of SA treatment of extracts of mixtures of the two compounds agrees with predictions based on the relative peak areas of the compounds extracted separately. Hence, in patients' plasma samples, the ratio of OHDMNT to OHNT may be calculated as: $r = (F - 0.994)/0.746$.

Altogether 47 plasma samples were analysed, from 41 different patients being treated with AT at a wide range of daily dosage. OHNT concentrations ranged from 15 to 266 ng/ml. The mean value of F was 1.102 ± 0.0541 (S.D.) and no relationship between F and dose or OHNT concentration was apparent. The mean OHDMNT concentration was therefore 14.5 ± 7.25% of the OHNT level present, and before SA treatment the apparent OHNT concentration averaged 110.9 ± 5.4% of the true value.

Stability of the drugs and metabolites

Solutions in 0.01 *M* hydrochloric acid of AT, NT, DMNT and all six of their 10-hydroxy metabolites were stable in the refrigerator for many months and no detectable dehydration occurred. The standard base solutions in heptane containing 0.5% diethylamine, with AT, NT, DHAT, DHNT, maprotiline, desmethylclomipramine and tetracosane were stable at room temperature for at least one year. Both isomers of OHAT and of OHNT were stable as bases in toluene at room temperature for at least eight months. However, the OHDMNT isomers were unstable in tolune at room temperature. After two weeks only 80% of the *trans* and 60% of the *cis* compound remained.

In human plasma freshly separated from heparinized blood, AT, NT and their *cis* and *trans* hydroxy metabolites were reasonably stable at room temperature. When the compounds were added to drug-free plasma, NT declined slowly, losing 12% of its concentration in ten days at room temperature, while the other five compounds were unchanged. However, in plasma that had previously been frozen and thawed the compounds were much less stable, for unknown reasons. Perhaps the increase in pH caused by loss of carbon dioxide in freezing was responsible. Compounds were added to drug-free plasma that had been stored frozen. In eight days at room temperature, NT decreased on average by 22%, AT by 20% and the four hydroxy compounds by about 5%. Patients' plasma samples were also tested at room temperature and

gave similar results, both for freshly separated and for previously frozen and thawed samples. Thus there was no evidence of bases being liberated from conjugated forms in patients' plasma. In some samples that had been frozen for many months, much larger losses occurred after thawing, e.g. 55% of NT lost during ten days at room temperature.

The primary amines were less stable in plasma than the secondary and tertiary amines. When added to fresh plasma and kept at room temperature for seven days, DMNT declined by 38%, *cis*-OHDMNT by 25% and *trans*-OHDMNT by 16%. However, in 17 h these compounds showed negligible losses, so that estimations carried out promptly would be unaffected.

All the compounds were stable in plasma during freezing and thawing. AT, NT, DMNT and all six of their hydroxy metabolites, added to drug-free human plasma, showed no significant losses when frozen and thawed eleven times in succession and estimated directly after the final thawing. Patients' plasma samples treated similarly gave no evidence either of loss of bases or their liberation from conjugates.

The compounds were stable for long periods in frozen plasma. AT, NT, their four hydroxy metabolites and DMNT, when added to drug-free human plasma and frozen, were not detectably changed after six months storage, while *cis*- and *trans*-OHDMNT declined by about 10%. Patients' plasma samples stored frozen for periods up to one year showed no change in the tertiary and secondary amines. (Primary amines were not estimated in these samples, so that the stability of their conjugates was not tested.)

The compounds were not adsorbed appreciably by the precipitates that develop in frozen heparinized plasma on storage. A pool of stored plasma samples from patients treated with AT was thawed and centrifuged. Determinations of AT, NT, OHAT and OHNT before and after centrifugation showed no differences and assay of the precipitate showed negligible amounts of these compounds. Similarly, blank human plasma with added *cis*- and *trans*-OHDMNT, stored frozen for several months, showed negligible amounts of these compounds adhering to the precipitates.

All blood samples were equilibrated and centrifuged at 37°C as previously described [25], in order to prevent the redistribution of compounds between cells and plasma. Blood samples from six patients were divided into two. One aliquot was equilibrated and centrifuged at once, and the other after 24 h at room temperature. No significant differences in plasma levels of AT, NT, OHAT or OHNT were found.

Other drugs

Retention times relative to AT (RR_{AT}) were given previously [4] for 29 other drugs and some of their metabolites. In Table I, eleven further compounds are listed. The estimation of additional metabolites necessarily increases the likelihood of interference by other drugs. In the current method, peaks with RR_{AT} values between about 0.9 and 1.9 will interfere. The principal potential offenders are other tricyclics and related antidepressants, propranolol, benzhexol, possible metabolites of procyclidine, and the smaller, less polar benzodiazepines and phenothiazines. Most benzodiazepines and neuroleptics, however, have RR_{AT} values greater than 1.9.

The extraction, like the procedure [4] for AT and NT only, excludes small

polar bases as well as all non-basic compounds. Many large bases appear equally in extracts prepared by either technique, but in other cases the heating step causes complications. Some drugs (cyclizine, benztropine) are destroyed and give no detectable peak. Other drugs produce new peaks during heating. Chlordiazepoxide (RR_{AT} = 15.3), which does not itself extract into heptane, gives a peak at RR_{AT} = 0.81 during heating. Diazepam (RR_{AT} = 3.16) is largely excluded by the present extraction method with or without heating, and gives no other peak. However, in the plasma of patients taking diazepam there is apparently a metabolite which produces a peak at RR_{AT} = 0.81 during the heating step. Fortunately, peaks at this retention time do not interfere. Flurazepam (RR_{AT} = 7.32) gives an additional peak at RR_{AT} = 3.0 on heating.

DISCUSSION

Development of the method

Toluene was chosen for the extraction of compounds from plasma because heptane proved to be a poor solvent for the OHNT isomers. Partition coefficients between heptane and 0.1 *M* sodium hydroxide (with about 0.5% diethylamine in the heptane layer and 1.5% in the aqueous phase) were only 6.0 for *cis*-OHNT and 3.5 for the *trans* isomer.

The amount of diethylamine added to the plasma was adjusted so that the quantity passing from the toluene layer into the aqueous acid was about 0.065 mmol, neutralising about one-third of the available acid.

If hydrochloric acid was used instead of sulphuric acid for extracting the compounds from toluene, significant amounts of AT and NT were left in the organic phase. The higher the hydrochloric acid concentration, the more drug was left behind, suggesting ion pair formation with Cl^-. Sulphuric acid extracted all the compounds well, but in catalysing the dehydration of the hydroxy metabolites sulphuric acid was found to be no more effective than an equal molarity of hydrochloric acid. This is presumably because at pH $\leqslant$ 1 the HSO_4^- ion is largely undissociated and sulphuric acid acts as a monobasic acid. In order to minimise the sodium hydroxide required for final neutralisation, the compounds were extracted into sulphuric acid and hydrochloric acid was added before heating. In an earlier version of the method, 0.4 *M* hydrochloric acid was used and the mixture heated at 100°C for 1 h. However, results were less reproducible than expected and recoveries of DHNT were low; the high concentration of sodium chloride present in the final extraction was thought to be responsible. Use of dilute acid at a higher temperature greatly improved the results.

In the dehydration of hydroxy metabolites the *cis* isomers were found to react more slowly than the *trans*. However, the difference was neither large nor reproducible enough to be used for determining the ratio of *cis* to *trans* isomers in an extract. If either clomipramine or desmethylclomipramine was present during the heating step, about 30% of its peak area was lost and an interfering peak at RR_{AT} = 1.08 was produced from either compound.

Performance and results

The accuracy and precision of this method for the determination of the

tertiary and secondary amines are as good as those of other published methods [8, 17—21]. The measurement of low concentrations (e.g. 10—20 ng/ml) requires several millilitres of plasma, while volumes up to at least 8 ml can be used (if available) to make possible the determination of lower concentrations. Many other methods specify 1 ml plasma only, implying greater absolute sensitivity. However, in many cases their effective sensitivity is unclear as results with spiked samples at low concentrations have not been reported.

As in other GC methods, *cis* and *trans* isomers are determined together. Levels of total OHAT in plasma are so low [8, 9, 19—21] and the proportion of the *cis* form is so small [20, 21] that the determination of *cis*-OHAT by currently available methods [20, 21] is often impossible. Joint measurement of the isomers may therefore be preferable. Total OHNT levels in plasma are comparable with NT levels and the ratio of the isomers present differs considerably between individuals [19—21] so that separate determination would seem both desirable and feasible. However, the two available HPLC studies of plasma levels of *cis*- and *trans*-OHNT in patients treated with AT differ remarkably. Suckow and Cooper [20] found a mean *cis/trans* ratio of 0.30 (S.E.M. = 0.034, n = 11) while Bock et al. [21] obtained a mean of 0.14 (S.E.M. = 0.013, n = 27).

Relative retentions given in Table I show that resolution of all six hydroxy metabolites, as such, on OV-17 packed columns is impracticable. Whether or not the *cis* and *trans* isomers differ in antidepressive effect is unknown; pharmacological studies have given different results. While Bertilsson et al. [1] found *cis* and *trans* forms to be equally effective, Hyttel et al. [2] found differences in certain tests, while Potter et al. [3] obtained much greater activity from *cis*-OHNT (the minor form in plasma) than from *trans*-OHNT, the more abundant form. The test systems were different and their relevance to therapeutic action remains doubtful. Racemic mixtures of all metabolites were used although both the *cis*- and *trans*-OHNT formed in the body are known to be optically active [10]. It may perhaps be doubted whether the separate contributions to clinical effects of all the individual isomers of the various metabolites are likely to be evaluated in sufficient detail to justify the separate monitoring of all the compounds in patients' plasma.

The present study is the first to report plasma levels of OHDMNT. The average amount present was found to be 14.5 ± 7.25% of the OHNT. Thus, there is often at least as much OHDMNT as OHAT in the plasma and it seems surprising that the primary amines have not been identified on HPLC chromatograms. If they should happen to interfere with OHAT peaks, large errors could result. In the present method, the minor peak formed from DMNT during extraction will interfere with DHAT, but as its area is only about 4% of the DMNT area which is about 6% of the NT area [24] the errors will be unimportant.

Results with spiked serum samples showed that the standard deviation of OHDMNT determinations by the salicylaldehyde method was 0.0166 in F, corresponding to 0.022 in r, the ratio of OHDMNT to OHNT present. Expressed in relation to the mean level in patients' plasma, this gives a coefficient of variation of 15%. Most of the variation arises in the process of salicylaldehyde treatment and rechromatography, since when only NT was

present the S.D. observed for F was 0.014. Experimental error contributes only a small proportion of the total variation observed in the population of patients. Subtraction of variances leaves a standard deviation of 0.069 in r as the true inter-individual variation. This represents a coefficient of variation of 48% for inter-individual differences in the ratio of OHDMNT to OHNT.

Compared to DMNT estimations by a similar procedure [24], the OHDMNT method has a slightly smaller standard deviation. The coefficient of variation is considerably better because the mean amount of OHDMNT in plasma (in relation to OHNT) is 1.7 times as great as the mean amount of DMNT (in relation to NT). The present extraction method was not, however, found suitable for determining DMNT. Although DMNT peaks were of the expected area and disappeared completely on salicylaldehyde treatment, experiments with spiked serum samples containing a DMNT/NT ratio of 0.1 gave unsatisfactory results. For unknown reasons, the peak area reductions on SA treatment were too small. DMNT is therefore better determined by the original extraction procedure [24].

The stability of the secondary and tertiary amines in fresh plasma is such that samples can be sent by post without significant losses. The compounds are stable for several months in frozen plasma but, once thawed, samples should be extracted within a few hours or refrozen for further storage.

For the determination of primary amines, fresh plasma cannot be left at room temperature for more than a few hours before extraction. It may be stored frozen, if extracted immediately after thawing.

The methods described in this paper have been in routine clinical use for more than two years, with regular analysis of spiked plasma samples. Trouble from interfering peaks has been rare and has always been from drugs known to interfere on OV-17 [4], notably other tricyclics and procyclidine. Benztropine is a useful non-interfering drug that can be used in place of procyclidine or benzhexol. Benzodiazepines, most of which have long retention times and/or fail to extract, have not given trouble in practice; nor have interfering phenothiazines been encountered. The method is suitable for the many laboratories currently estimating AT and NT by GC, making possible the routine determination of the hydroxy metabolites that are believed to be pharmacologically active.

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