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**Note**

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**Determination of desmethyl nortriptyline in plasma by gas chromatography before and after treatment with salicylaldehyde**

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During routine monitoring of patients' plasma levels of amitriptyline (AT) and nortriptyline (NT) by gas chromatography (GC) without derivatization [1], the corresponding primary amine desmethyl nortriptyline (DMNT) was found to have a retention time indistinguishable (within 1%) from that of NT, on OV 17 columns at 230°C. The extraction procedure (specific for lipophilic bases, see *Method* section below) extracted DMNT quantitatively from plasma and the primary amine was therefore being estimated as NT.

Fredricson Overø [2] used salicylaldehyde (SA) to eliminate interference by DMNT in the estimation of NT by radioactive acetylation. We found that SA, added to the final heptane extract of plasma, would react with DMNT and the SA, being phenolic, could then be removed by extraction with aqueous alkali. Chromatography before and after this treatment enabled DMNT to be determined by difference.

**EXPERIMENTAL***Materials*

DMNT hydrochloride and the *cis* and *trans* forms of 10-hydroxy-AT and 10-hydroxy-NT were kindly provided by Dr. A. Jørgensen of H. Lundbeck & Co (Copenhagen, Denmark). Salicylaldehyde (BDH, Poole, Great Britain), general purpose reagent grade, was used without further purification. A fresh solution was prepared daily, containing 0.75 ml salicylaldehyde in 4.25 ml *n*-heptane.

*Method*

To each plasma sample (1–10 ml, usually about 4 ml) the following in-

ternal standards were added: 2.5  $\mu\text{g}$  dextromethorphan, 1.0  $\mu\text{g}$  clomipramine, 1.0  $\mu\text{g}$  desmethyl clomipramine. The compounds were extracted [1] into 10 ml heptane, thence into 2 ml 0.1  $M$  hydrochloric acid and finally into 50  $\mu\text{l}$  heptane containing diethylamine (0.5%, v/v) and tetracosane (5  $\mu\text{g}/\text{ml}$ ). A 10- $\mu\text{l}$  aliquot of this extract was chromatographed as previously described [1] and the AT and apparent NT concentrations calculated, using dextromethorphan as internal standard. To the remaining extract, from which all of the original aqueous layer had been carefully removed by Pasteur pipette, 5  $\mu\text{l}$  of the SA solution were added. After standing for 20 min, 100  $\mu\text{l}$  of an aqueous solution containing 0.15 ml diethylamine in 10 ml 0.17  $M$  sodium hydroxide solution were added. The mixture was vibrated on a whirlmixer, centrifuged briefly with the stopper in place and the aqueous layer removed by Pasteur pipette. A 10- $\mu\text{l}$  aliquot of the organic phase was injected into the chromatograph.

## RESULTS

Extracts of drug-free plasma without added compounds gave no peaks on the chromatogram before or after SA treatment. No new peak appeared after SA treatment of extracts of plasma samples containing tetracosane, dextromethorphan, clomipramine, desmethyl clomipramine, AT, NT, or the *cis* or *trans* forms of the metabolites 10-hydroxy-AT and 10-hydroxy-NT. These compounds were tested separately and/or in various appropriate combinations.

DMNT hydrochloride, added to plasma and extracted by the usual technique, gave its peak at the same retention time as NT, together with a small extra peak at a retention time 1.12 times that of NT. This minor peak did not appear when DMNT hydrochloride was simply dissolved in chloroform and injected; it must therefore have been formed during the extraction procedure. Its peak area was 3–5% of that of the main peak. On SA treatment both peaks disappeared completely.

The peak area of NT, when present without DMNT, decreased on SA treatment by about 3% relative to internal standards. The only other secondary amine usually present, desmethyl clomipramine, decreased by about 5%. The relative peak areas of clomipramine, dextromethorphan, tetracosane and amitriptyline were unchanged by SA treatment. However, treatment greatly reduced both the *cis* and *trans* isomers of 10-hydroxy-NT. Plasma extracts from patients taking AT or NT usually contain small amounts of the hydroxy metabolites, which interfere with the clomipramine and desmethyl clomipramine peaks. Consequently dextromethorphan and tetracosane were chosen as internal standards for calculation of the effect of SA treatment on the apparent peak area of NT. Using the average of these two internal standards, the ratio  $F$  of apparent NT concentrations before and after treatment was calculated from

$$F = \frac{1}{2} \frac{N_1}{N_2} \left( \frac{dM_2}{dM_1} + \frac{T_2}{T_1} \right)$$

where  $dM_1$  and  $dM_2$  are the dextromethorphan peak areas before and after SA treatment, respectively, and  $T_1$  and  $T_2$  the corresponding peak areas of tetracosane.  $N_1$  and  $N_2$  are the NT peak areas, before and after treatment, corrected for chromatographic losses as previously described [1].

A total of 164 assays were carried out on drug-free plasma samples to which various amounts of NT and/or DMNT were added. Table I shows that, at a constant ratio of DMNT to NT,  $F$  was independent of the concentration of either component. In Fig. 1 the graph of  $F$  against the DMNT:NT ratio is shown to be a straight line. Thus both the relative extraction yield of DMNT and NT and the relative chromatographic response to these two compounds must be independent of concentration.

TABLE I

$F$  VALUES DETERMINED ON PLASMA SAMPLES CONTAINING KNOWN AMOUNTS OF NORTRIPTYLINE AND DESMETHYL NORTRIPTYLINE

$F$  is the ratio of apparent NT concentrations, before SA treatment/after SA treatment, using both dextromethorphan and tetracosane for internal standardization.

NT concentration (ng/ml in 5 ml plasma)		Ratio DMNT/NT			
		0	0.1	0.2	0.5
200	Mean $F$	1.029	1.109	1.189	—
	S.D.	0.022	0.015	0.020	
	$n$	10	10	10	
100	Mean $F$	1.032	1.105	1.185	1.434
	S.D.	0.021	0.020	0.018	0.027
	$n$	10	21	9	10
40	Mean $F$	1.029	1.101	1.192	—
	S.D.	0.011	0.020	0.025	
	$n$	10	10	9	
20	Mean $F$	1.028	1.113	1.175	1.424
	S.D.	0.034	0.043	0.026	0.048
	$n$	15	10	10	20
Total	Mean $F$	1.029	1.107	1.185	1.427
	S.D.	0.024	0.025	0.023	0.042
	$n$	45	51	38	30

Let the ratio of the weight of DMNT to that of NT in the plasma sample be  $r$  and let the ratio of the peak area of DMNT to that of NT, for equal weights of the two compounds in the sample, be  $a$ . Then the apparent amount of NT present before SA treatment is  $(1 + ar)$  times the true amount. Let the peak area of NT itself be reduced by the factor  $1/f$  by SA treatment. Then

$$F = \frac{1 + ar}{1/f} = f + afr$$

The graph of  $F$  against  $r$  is therefore linear, with intercept  $f$  and slope  $af$ ;  $r$  may be determined as  $(F - f)/af$  and the true amount of NT in the plasma

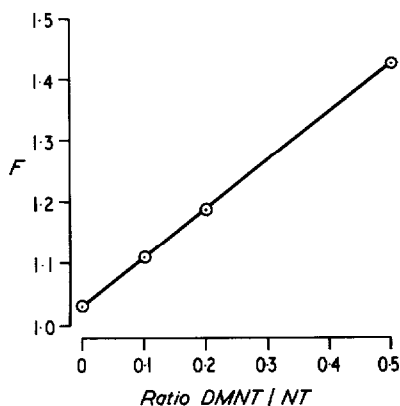


Fig. 1. Mean values of  $F$  (from Table I) plotted against  $r$ , the ratio of DMNT/NT in the plasma sample. The straight line represents the equation  $F = 1.03 + 0.79r$ .

is equal to the apparent amount as estimated before SA treatment, multiplied by the factor  $f/F$ . From Fig. 1,  $f = 1.03$  and  $af = 0.79$ .

The S.D. of determinations of  $F$  (Table I) was approximately constant at 0.02 for NT concentrations of 200, 100 and 40 ng/ml at DMNT:NT ratios of 0, 0.1 and 0.2. This represents a 2% coefficient of variation for the process of SA treatment and rechromatography. At 20 ng/ml NT this coefficient of variation rose to about 3.5%. At a DMNT:NT ratio of 0.5 the S.D. was slightly greater than when less DMNT was present, presumably because of an appreciable contribution from the variance of the proportion of DMNT extracted.

#### *Stability of DMNT*

In aqueous solution in 0.01 *M* hydrochloric acid in the refrigerator, DMNT was found to be stable over a period of six months. As a base, however, in solution in heptane containing 0.5% of diethylamine at room temperature, breakdown was appreciable in 24 h. The main DMNT peak diminished, the minor peak of later retention time increased and various new peaks formed.

Drug-free human plasma samples were freshly prepared from heparinized blood, spiked with NT and DMNT and frozen. Samples thawed and estimated at intervals for six months showed no change in drug content.

#### *Plasma levels of DMNT*

Sixty-four plasma samples were analysed from 54 different patients being treated with AT. AT concentrations ranged from 8 to 207 ng/ml and NT levels from 8 to 270 ng/ml. The mean value of  $F$  was  $1.097 \pm 0.041$  (S.D.) and no correlation could be discerned between  $F$  and either drug level. These values of  $F$  correspond to a DMNT:NT ratio of  $0.084 \pm 0.051$ , and an apparent NT concentration  $1.065 \pm 0.040$  times the true concentration. Plasma DMNT levels ranged from  $< 1$  to 20 ng/ml.

#### DISCUSSION

Plasma DMNT concentrations were determined by Borgå et al. [3] using

GC with mass spectrometry, a technique not available in many laboratories. In eight patients being treated with NT, NT plasma levels were 14 to 182 ng/ml and the mean DMNT:NT ratio may be calculated as  $0.085 \pm 0.046$ , a result closely similar to our own. No information was given on the precision of the method for DMNT, but for NT the coefficient of variation was 5.8%. Hence it seems that most if not all of the observed variation in the DMNT:NT ratio is accounted for by the assay variation and no estimate is possible of the true variation between plasma samples.

In the present study, an appreciable part of the overall variance is attributable to experimental error. Considering only those samples containing at least 200 ng of NT, the mean value of  $F$  was  $1.095 \pm 0.031$  (S.D.) ( $n = 35$ ) while in Table I the S.D. of such determinations was found to be approximately 0.020. Comparison of variances leaves a value of 0.024 for the S.D. of the true variation of  $F$  between plasma samples, equivalent to 0.030 in the ratio of DMNT to NT.

Both the average plasma content of DMNT and its variation between subjects are thus small compared with NT levels. GC without derivatization is in common use [4] for the determination of plasma NT and OV-17 (or the closely-related SP2250) are frequently-used liquid phases. However, since the amount of DMNT present averages only 8.4% of the NT, interference is unlikely to be of clinical significance in the estimation of NT levels unless the sensitivity of detection is heavily weighted in favour of DMNT. Our system, with flame-ionization detectors, gave a somewhat lower response to DMNT than to NT ( $a = 0.77$ ) reducing the average interference to 6.5%.

The salicylaldehyde method also allows DMNT to be determined separately. Whether this metabolite has significant pharmacological action is not known. However, if the hypothesis of the action of tricyclic antidepressants by the inhibition of amine uptake is accepted, then current evidence [5, 6] on the relative activity of AT, NT and their various metabolites suggests that DMNT is unlikely to contribute significantly when present in the low relative concentrations found in patients treated with AT or with NT.

#### ACKNOWLEDGEMENT

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