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Note

Assay of tricyclic structured drugs and their metabolites in urine by high-performance liquid chromatography

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Previous reports of the assay of amitriptyline (AMI) and nortriptyline (NOR) in biological fluids have included thin-layer chromatography[1,2], gas–liquid chromatography (GLC)[3–5], and GLC–mass spectrometry[6,7] for fractionation and quantitation. More recently, high-performance liquid chromatography (HPLC) was applied to assay of these compounds in plasma[8], and this report constitutes a modification of this procedure, to permit analysis of tricyclic structured drugs and their metabolites in urine. Two other tricyclic drugs, imipramine and chlorpromazine, and their metabolites have also been chromatographed.

EXPERIMENTAL

Materials

Reagents and solvents were all BDH (Poole, Great Britain) AnalaR grade, except dichloromethane, which was obtained from Rathburn (Walkerburn, Peebleshire, Great Britain). Drugs and metabolites were given by the following: Drs. I. Stevenson, M. Turnbull, J. Moody, and Mr. R. Sparks (Ninewells Hospital and Medical School, Dundee, Great Britain); Dr. A. A. Manian (U.S. National Institute of Mental Health); H. Lundbeck (Copenhagen-Valby, Denmark); Smith, Kline and French Labs. (Philadelphia, Pa., U.S.A.); May and Baker (Dagenham, Great Britain).

Methods

Chromatographic conditions. A Varian Model 8500 chromatograph with a Varian Techtron 635 series variable-wavelength detector was used. The column (25 cm × 2 cm I.D.) was stainless steel (316 type) and packed with Micropak SI5 (Varian, Palo Alto, Calif., U.S.A.), particle size 5 μm.

HPLC operating conditions. The pressure was 2750 p.s.i., the flow-rate 60 ml/h, the chart speed 25 cm/h, the band pass 2 nm, the wavelength 240 nm, and the absorbance range 0–1.0. The solvent used was dichloromethane–propan-2-ol–ammonia (100:10:0.2).

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Extraction procedure

Unconjugated metabolites. A 10-ml volume of urine, containing 5 μg of protriptyline (PRO) internal standard (1 mg/ml), was brought to pH 12.0 with 5 *M* NaOH and extracted with 25 ml of dichloromethane. The organic phase was filtered through Whatman No. 2 paper and the filtrate evaporated to dryness under nitrogen at 50°. Residues were taken up in 25 μl methanol and applied to the column in quantities of 5 μl.

Conjugated metabolites. After the extraction of unconjugated metabolites, the aqueous phase was acidified to pH 4.5, mixed with 5 ml 2.5 *M* acetate buffer, pH 4.8, and a pinch of limpet acetone powder was added. After incubation at 37° for 48 h, the particulate limpet powder was removed by filtration and the free bases extracted as previously described.

Calculation of results

Calibration curves were constructed for amitriptyline and nortriptyline by calculating the ratios of peak areas to the peak area of the internal standard. Pure standards of the other urinary metabolites were not available for the determination of linearity and therefore these compounds were expressed as "equivalents to AMI".

RESULTS

Linearity and recovery

Analysis of AMI and NOR demonstrated a linear response over the range 10 ng–5.5 μg on column weight. Relative to the internal standard, recovery was 90 ± 8%.

Precision

Precision studies on urine from a patient treated with AMI produced the following coefficients of variation ($n = 6$): (I) chromatographic precision, ±5%; (II) estimation of unconjugated metabolites, 3.8–14.5%; (III) estimation of conjugated metabolites, 4.5–34.6%. The lower coefficients of variation are associated with the larger peaks.

Resolutions achieved

Table I lists retention times relative to PRO of various tricyclic structured drugs and their metabolites chromatographed in this solvent system.

Fig. 1 shows a typical trace from a patient chronically treated with amitriptyline. The identities of peaks 1, 3, and 4 are based on comparison of relative retention data with relative data of standards. Analysis of the urine of a subject dosed with NOR allowed identities to be assigned to peaks 2 and 9.

DISCUSSION

This method is a modification of a procedure applied by us to the assay of AMI and NOR in plasma[8]. Modification was required to reduce retention times of the more polar metabolites of these drugs whilst retaining the accuracy and precision of the plasma method.

TABLE I

RELATIVE RETENTION TIMES FOR DIFFERENT TRICYCLIC STRUCTURED DRUGS AND THEIR METABOLITES

Group	*Identity*	*Retention relative to PRO**
	Solvent front	0.06
Urinary	1. Amitriptyline	0.17
Amitriptyline	2. ? Desmethylnortriptyline	0.30
Metabolites	3. 10-Hydroxyamitriptyline	0.36
	4. Nortriptyline	0.46
	5. Unknown metabolite	0.59
	6. Endogenous	0.66
	7. Unknown metabolite	0.79
	8. Endogenous	0.90
	Protriptyline	1.00
	9. 10-Hydroxynortriptyline	1.23
	10. Unidentified desmethylated metabolite	1.61
Amitriptyline and metabolites	Amitriptyline	0.18
	10-Hydroxyamitriptyline	0.36
	Amitriptyline-N-oxide	k' large
	Nortriptyline	0.47
	Protriptyline	1.0
Imipramine and metabolites	Iminodibenzyl	0.10
	2-Hydroxyiminodibenzyl	0.10
	Imipramine	0.24
	Didesmethylimipramine	0.36
	Desmethylimipramine	0.71
	10-Hydroxyimipramine	0.76
	2-Hydroxyimipramine	0.88
	Imipramine-N-oxide	0.93
	10-Hydroxydesmethylimipramine	3.13
	2-Hydroxyimipramine	3.32
Chlorpromazine and metabolites	Chlorpromazine	0.13
	Didesmethylchlorpromazine	0.20
	6-Hydroxychlorpromazine	0.46
	Desmethylchlorpromazine	0.56
	Chlorpromazine-N-oxide	0.65
	7-Hydroxychlorpromazine	0.73
	Chlorpromazine sulphoxide	0.90
	8-Hydroxychlorpromazine	k' large

* The retention volume of PRO was 7.2 ml.

10-Hydroxyamitriptyline is eluted prior to NOR in this solvent system, which is reversal of the order observed using the plasma method[8]. This effect is related to the change in propan-2-ol concentration[9].

Peak 9 has been identified as 10-hydroxynortriptyline, on the basis of retention relative to NOR, compared with the retention of 10-hydroxy-AMI to AMI. The percentage of total metabolite excretion represented by peak 9 in a subject taking NOR was 89%. This compares with the 80% reported by Alexanderson and Borga[10] of total dose excreted as 10-hydroxy-NOR in such subjects.

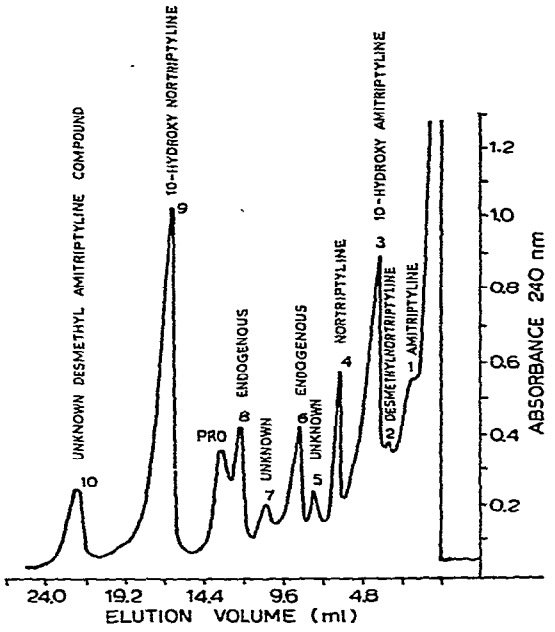

Fig. 1. Typical separation of unconjugated metabolites from the urine of a patient on amitriptyline.

The provisional identity of desmethylnortriptyline has been assigned to peak 2. Reference to Table I will show that the order of elution for chlorpromazine and imipramine metabolites is tertiary amine — primary amine — secondary amine. Peak 2 has an elution volume consistent with the volume to be expected of desmethyl-NOR, the primary amine.

The well known ability of silica gel to separate isomers is demonstrated in the wide range of relative retention times found for the respective isomeric hydroxyl metabolites.

Peaks 5, 7 and 10 have not yet been identified. From examination of urinary nortriptyline metabolites, a peak with an elution volume equal to peak 10 was found. This is therefore a desmethylated amitriptyline metabolite. The identities of the unknown peaks, and confirmation of the identities of the other peaks, can be made by mass spectrometry. We are currently investigating this approach.

This profiling of urinary tricyclic metabolites may be used to study the metabolic fate of the drug. Correlation of therapeutic response to plasma levels has been attempted[3,11], but is possibly premature until better understanding of the drug metabolism of the individual is studied by estimation of the urinary metabolites.

This method is sensitive and precise with good recovery and linearity and is applicable to a range of psychotropic drugs. It is hoped that this will lead to a better understanding of the variation of clinical response to treatment with tricyclic structured drugs.

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