Journal of Chromatography, 230 (1982) 391—400
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1264

SIMULTANEOUS DETERMINATION OF AMITRIPTYLINE, NORTRIPTYLINE AND THEIR RESPECTIVE ISOMERIC 10-HYDROXY METABOLITES IN PLASMA BY LIQUID CHROMATOGRAPHY

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(First received September 17th, 1981; revised manuscript received February 18th, 1982)

SUMMARY

An ion-pair reversed-phase liquid chromatographic method for the determination of the tricyclic antidepressant amitriptyline, its demethylated metabolite nortriptyline, and their respective *cis*- and *trans*-hydroxylated metabolites in plasma is presented. After extraction from 1 ml of plasma, the reconstituted residue was chromatographed on a trimethylsilyl packed column using a mobile phase of acetonitrile and acetate buffer with sodium heptanesulfonate and triethylamine. Recovery of the drugs and its metabolites from plasma ranged from 56 to 99%. The method is suitable for determining plasma concentrations as low as 5 ng/ml (C.V. < 9%) for all six compounds.

Plasma concentrations of amitriptyline and its metabolites from eleven different patients are presented.

INTRODUCTION

It has been repeatedly demonstrated that individuals treated with a fixed dose of antidepressant medication have a 10—20 fold variation in their steady state plasma concentration and that this variation is genetically determined [1]. The investigation of plasma drug concentrations in a variety of studies has produced a wealth of important information on the pharmacokinetics and pharmacodynamics of these drugs but there remains a great deal of controversy in the relevance of routine plasma level monitoring of the tricyclic antidepressant drugs in clinical practice [2]. Brodie [3] in 1964 suggested that if one was unable to demonstrate clear relationships between the plasma concentration of the parent drug and its pharmacological activity then an investigation of the metabolites of that drug was indicated. Christiansen and Gram [4] demonstrated that there were hydroxylated metabolites present in the central nervous system in an acute overdose case and several studies have demonstrated

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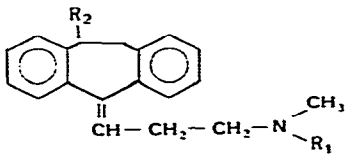
considerable quantities of unconjugated and conjugated hydroxy metabolites of tricyclic antidepressants in the plasma of treated patients [5—12]. It is now known that the hydroxy metabolites have strong cardiovascular activity [13], and are essentially equipotent to the parent compound in terms of blockade of norepinephrine and serotonin receptors in rat brain slices and isolated synaptosomal fractions [14—17]. The specificity of the respective hydroxylated metabolite is maintained, e.g. 2-hydroxydesmethylimipramine is 100 times more potent in inhibiting norepinephrine than serotonin accumulation. Finally, these metabolites are present in the plasma in the same order of magnitude as the primary and secondary amines [9, 11].



	R_1	R_2
AMI	CH_3	H
trans-10-OH-AMI	CH_3	OH
cis-10-OH-AMI	CH_3	OH
NOR	H	H
trans-10-OH-NOR	H	OH
cis-10-OH-NOR	H	OH

Fig. 1. Chemical structures of amitriptyline (AMI) and metabolites.

Clearly, therefore, there is a need for adequate analytical methodology for the quantitation of the hydroxy metabolites of these psychotropic drugs. A method for the analysis of amitriptyline (AMI) and its hydroxylated metabolites (for structures see Fig. 1) using gas chromatography—mass spectrometry (GC—MS) was recently published by Garland et al. [18] but this procedure carefully dehydrated the hydroxylated compounds to form the 10,11-diene before GC—MS; thus the differentiation between the *E* and *Z* (*trans* and *cis*) forms of the 10-hydroxylated metabolites were obscured. A similar procedure has been recommended by Alvan et al. [7] for the drug nortriptyline (NOR) on the basis that the *E* and *Z* enantiomers were equipotent in terms of their blockade of norepinephrine, a point recently emphasized by Bertilsson et al. [9]. Despite these rather convincing data it appears to us that as the mode of action of these tricyclic antidepressant drugs is again being questioned that we should, if possible, develop a technique capable of separating these enantiomers.

Various methods have been reported for the analysis of AMI and metabolites and have been reviewed recently [19]. Liquid chromatography (LC) has, thus far, been the only method available for the separation of the *cis—trans* isomers of the 10-hydroxylated metabolites of AMI and NOR. However, the LC methods available lack either sensitivity, complete resolution of all metabolites, or many interfering and/or unidentified peaks [20, 21].

Therefore, we have developed a convenient method for the determination of AMI, NOR and their respective isomeric 10-hydroxylated metabolites in plasma using reversed-phase ion-pair LC.

EXPERIMENTAL

Reagents

Acetic acid, sodium acetate, sodium hydroxide and hydrochloric acid were all analytical reagent grade. Sodium heptanesulfonate (Eastman-Kodak, Rochester, NY, U.S.A.), triethylamine (Aldrich, Milwaukee, WI, U.S.A.) and isoamyl alcohol (Sigma, St. Louis, MO, U.S.A.) were all used as received. Acetonitrile was HPLC—UV grade from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.) and *n*-heptane was obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). Distilled water was passed through a Milli-Q Water Purification System (Millipore, Bedford, MA, U.S.A.) before use.

Apparatus

Chromatography was performed using a Model 6000A solvent delivery pump, a U6K injector or WISP 710A automatic sampler and a Model 440 UV absorbance detector at 254 nm (Waters Assoc., Milford, MA, U.S.A.). The separations were achieved with a 25 cm × 4.6 mm I.D. LC-1 (5 μm) particle size column (Supelco, Bellefonte, PA, U.S.A.). Chromatograms were recorded on a Model B5217-5 Ombiscribe recorder (Houston Instruments, Austin, TX, U.S.A.) and the data processed by a PDP 11/34 data acquisition system (Digital Equipment, Maynard, MA, U.S.A.). The detector signal output to the PDP 11/34 was amplified 50 × by an operational amplifier circuit.

Standards

Stock solutions of 1 mg/ml of each of the *cis* and *trans* isomers of the 10-hydroxyamitriptyline and 10-hydroxynortriptyline (H. Lundbeck and Co., Copenhagen, Denmark) were prepared as the free base in 0.01 *N* hydrochloric acid. Working solutions of 1 ng/μl were subsequently prepared. Amitriptyline · HCl (Merck, Sharp and Dohme, West Point, PA, U.S.A.) and nortriptyline · HCl (Eli Lilly, Indianapolis, IN, U.S.A.), stock solutions of 1 mg/ml were prepared in 0.1 *N* hydrochloric acid. The stock solutions were diluted with 0.01 *N* hydrochloric acid to give working solutions of 2 ng/ml. Loxapine succinate (Lederle Labs., Pearl River, NY, U.S.A.) stock solutions of 1 mg/ml in 0.1 *N* hydrochloric acid were prepared, then further diluted to 1 ng/ml in 0.01 *N* hydrochloric acid for use as the internal standard.

Standard curves in plasma were prepared containing five concentrations of spiked samples: 25, 50, 100, 200 and 400 ng/ml of amitriptyline and nortriptyline and 12.5, 25, 50, 100 and 200 ng/ml of each of the 10-hydroxy isomers of amitriptyline and nortriptyline. Each set of standards included a blank.

Extraction

To 1 ml of plasma standard or unknown sample, 100 μl (100 ng) of internal standard loxapine and 0.5 ml of 0.5 *N* sodium hydroxide were added to specially washed glassware. Eight millilitres of 1.5% (v/v) isoamyl alcohol in *n*-heptane were added and the mixture was shaken for 15 min and centrifuged at 1500 *g* for 10 min. The organic layer was then transferred to a 15-ml tapered centrifuge tube containing 1.2 ml 0.1 *N* hydrochloric acid. After mixing for 10

TABLE I

RECOVERY OF AMITRIPTYLINE AND METABOLITES FROM 1 ml PLASMA (n = 7)

	5 ng		25 ng		50 ng		200 ng		400 ng	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
trans-10-OH-NOR	56	10.4	66	4.7			58	5.4		
trans-10-OH-AMI	84	7.0	89	4.8			91	5.8		
cis-10-OH-NOR	65	7.6	79	9.0			66	5.8		
cis-10-OH-AMI	94	4.9	91	5.4			92	4.3		
NOR	63	9.0			79	5.1			78	5.0
AMI	94	4.2			99	5.2			91	3.5

TABLE II

WITHIN-RUN PRECISION OF ASSAY BASED UPON PEAK-HEIGHT RATIOS AT VARIOUS CONCENTRATIONS (n = 5)

	5 ng/ml		25 ng/ml		50 ng/ml		100 ng/ml		200 ng/ml		400 ng/ml	
	Pk.ht. ratio	C.V. (%)	Pk.ht. ratio	C.V. (%)	Pk.ht. ratio	C.V. (%)	Pk.ht. ratio	C.V. (%)	Pk.ht. ratio	C.V. (%)	Pk.ht. ratio	C.V. (%)
trans-10-OH-NOR	0.10	8.9	0.35	6.7	0.95	0.8	2.01	6.4	4.08	7.3		
trans-10-OH-AMI	0.06	8.8	0.27	6.7	0.61	1.5	1.30	6.2	2.65	6.9		
cis-10-OH-NOR	0.08	6.9	0.30	9.4	0.65	3.0	1.37	7.0	2.77	6.7		
cis-10-OH-AMI	0.08	6.3	0.38	7.5	0.80	1.8	1.67	6.3	3.37	6.3		
NOR	0.05	6.4	0.24	3.3	0.38	6.4	0.89	6.4			3.40	6.2
AMI	0.04	7.3	0.20	5.0	0.35	1.9	0.78	6.9			3.05	5.6

min and centrifuging at 1500 *g* for 10 min, the organic layer was aspirated, the aqueous portion transferred to a 3-ml tapered glass-stoppered minicentrifuge tube and neutralized with 0.5 ml 0.5 *N* sodium hydroxide. One half (0.5) millilitre 1.5% isoamyl alcohol in *n*-heptane was added and the tube stoppered, shaken for 10 min and centrifuged for 5 min at 1500 *g*. The lower aqueous layer was discarded and the organic layer transferred to a 1.0-ml Reactivial (Pierce, Rockford, IL, U.S.A.). The contents were evaporated to dryness in a Model SVC-100 M Speed Vac Concentrator (Savant Instruments, Hicksville, NY, U.S.A.) at 45°C. The extract was then reconstituted with 100 µl of mobile phase, capped and mixed.

Chromatographic conditions

The mobile phase consisted of 0.1 *M* acetate buffer (pH 4.2)—acetonitrile (70:30) with 0.005 *M* heptanesulfonate and 0.01 *M* triethylamine added. The mixture was filtered and degassed prior to use. The flow-rate was 1.8—2.0 ml/min and the temperature ambient. The effluent was monitored through a UV detector at 254 nm.

Quantitation

All determinations were performed by calculating the peak height and/or area ratios of each compound to the internal standard. A linear regression analysis for each of the standard curves was performed by a computer program resulting in the calculation of slope, *x*-intercept, correlation coefficient, and standard errror of this estimate.

RESULTS AND DISCUSSION

The report describes the simultaneous determination of AMI and five metabolites by liquid chromatography with UV detection. This method is able to separate and quantitate the *cis* and *trans* isomers of 10-OH-AMI and 10-OH-NOR. A sample chromatogram appears in Fig. 2. Peak symmetry was enhanced by the addition of triethylamine. Otherwise, peak tailing and broadening occurs as well as loss of resolution among the *cis*—*trans*-hydroxy metabolites of AMI and NOR. Fig. 3 illustrates a chromatogram of a drug-free patient plasma. No interfering endogenous peaks were detected. This is a result of careful sample clean-up and the selectivity of this column.

The absolute recovery for all the compounds was checked at the sensitivity limit (5 ng/ml), at midpoint, and at the upper limit of linearity. The results are shown in Table I.

The precision of the reported procedures was determined by spiking 1-ml plasma aliquots of drug-free plasma with various concentrations of the 10-hydroxy metabolites of AMI and NOR and of AMI and NOR with the internal standard. The samples were processed as described. The resulting coefficients of variation for each compound appear in Table II. Day-to-day reproducibility for the method was assessed by analysis of the peak height ratios of each compound generated at each concentration point on the calibration curve. These results appear in Table III.

TABLE III

DAY-TO-DAY PRECISION OF THE ASSAY FOR AMI AND METABOLITES AT ALL CONCENTRATION POINTS ON THE LINEAR REGRESSION CURVE (n = 5)

	12.5 ng/ml		25 ng/ml		50 ng/ml		100 ng/ml		200 ng/ml		400 ng/ml	
	Pk.ht. ratio	C.V. (%)	Pk.ht. ratio	C.V. (%)	Pk.ht. ratio	C.V. (%)	Pk.ht. ratio	C.V. (%)	Pk.ht. ratio	C.V. (%)	Pk.ht. ratio	C.V. (%)
trans-10-OH-NOR	0.21	7.4	0.38	6.0	0.77	3.3	1.57	0.8	3.39	4.6		
trans-10-OH-AMI	0.13	12.9	0.28	8.7	0.55	5.2	1.15	5.7	2.43	5.8		
cis-10-OH-NOR	0.17	5.9	0.31	13.0	0.61	6.0	1.19	5.0	2.51	4.0		
cis-10-OH-AMI	0.18	7.3	0.36	7.7	0.72	3.5	1.49	3.1	3.12	5.2		
NOR			0.20	12.8	0.38	13.1	0.79	8.2	1.56	9.0	3.28	6.5
AMI			0.18	7.3	0.36	5.8	0.70	5.0	1.39	5.2	2.78	4.5

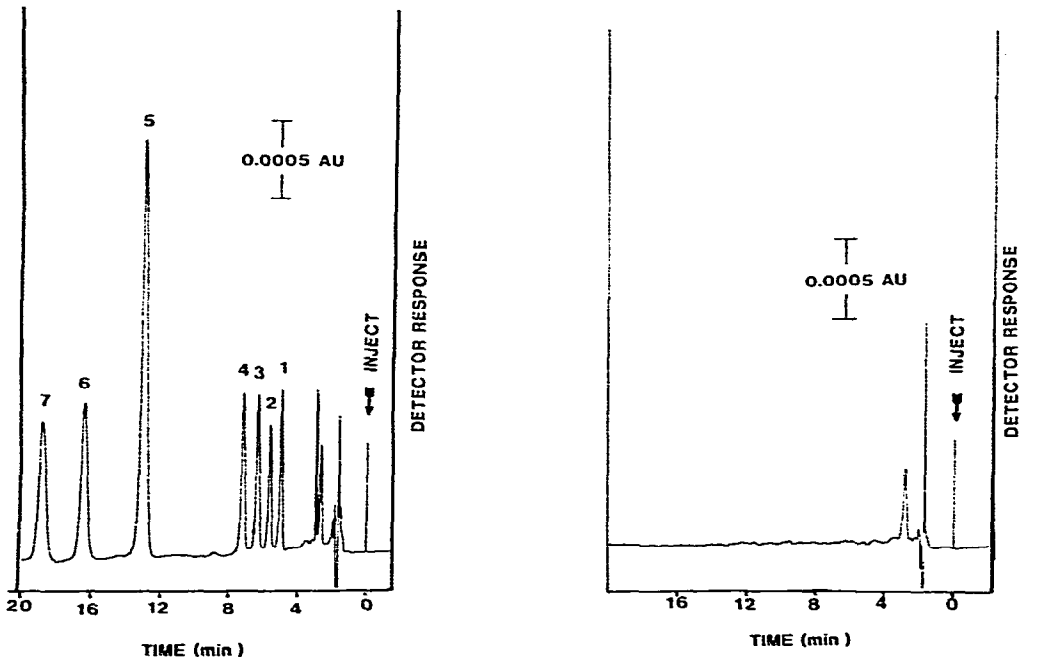


Fig. 2. Sample chromatogram of 1 ml plasma containing (1) *trans*-10-OH-NOR 25 ng, (2) *trans*-10-OH-AMI 25 ng, (3) *cis*-10-OH-NOR 25 ng, (4) *cis*-10-OH-AMI 25 ng, (5) loxapine 100 ng, (6) NOR 50 ng, and (7) AMI 50 ng. Chromatographic conditions as described in text. Entire reconstituted extract was injected.

Fig. 3. Sample chromatogram of 1 ml plasma blank. Entire reconstituted extract was injected.

Although loxapine was chosen as the internal standard for this method, several other readily available compounds such as doxepin, desmethyldoxepin or desmethylimipramine are among a few alternatives that were found to be suitable.

Because there are seven peaks of importance in this determination, the possibility of an interfering peak from another compound co-eluting is obviously greater than for a determination of one or two compounds. Probably the most frequently found interfering peak results from the presence of the benzodiazepines and their metabolites. For example, plasma from a patient receiving Limbitrol® (a combination drug containing chlordiazepoxide and amitriptyline) could not be analyzed for the 10-hydroxy metabolites of AMI because chlordiazepoxide and its three major metabolites (N-desmethylchlordiazepoxide, demoxapam, and desmethyldiazepam) co-extract, have similar retention times to the 10-hydroxy metabolites and are readily detected at 254 nm. A prior knowledge of the patient medication profile may help to eliminate these interferences by minor adjustments in the chromatographic conditions.

The detection limit for AMI and all of its metabolites in this assay is ca. 5 ng/ml. The detector response remained linear from 5 ng to about 500 ng, far exceeding the normal therapeutic range for these compounds.

The question of the stability of the 10-hydroxymetabolites of AMI and NOR

during the sample processing was resolved by subjecting 100 ng of each isomer of both hydroxy metabolites to separate extractions. The purpose was to determine if there was any conversion of the geometric isomers from one to another. Only one peak occurred from each hydroxy compound after routine processing thus indicating no conversion.

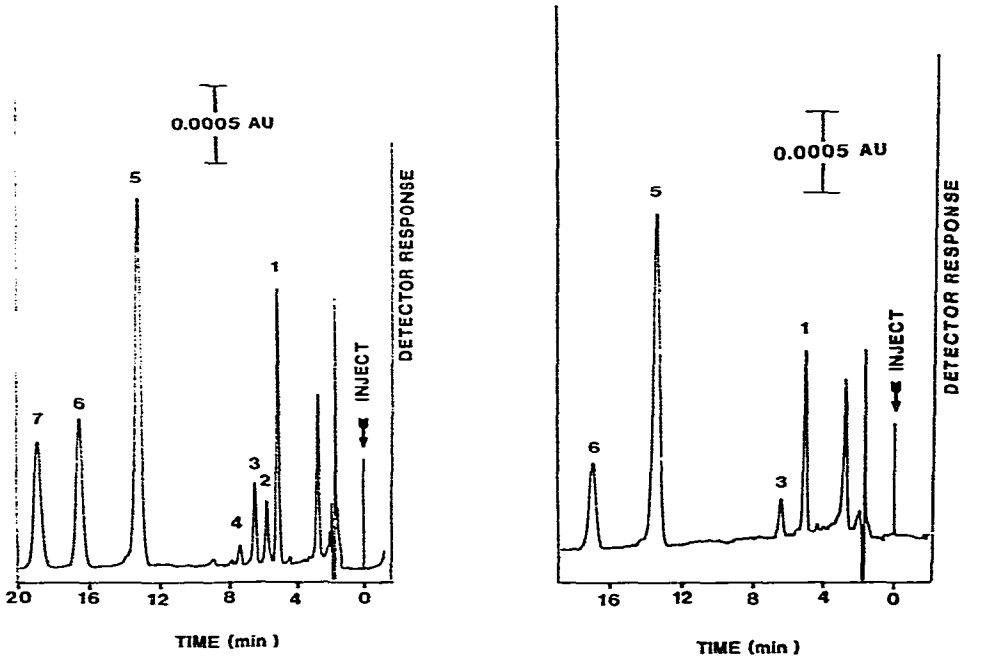


Fig. 4. Sample chromatogram of a 1-ml plasma sample from a patient receiving AMI 150 mg daily for 6 weeks. Concentrations were found to be (1) *trans*-10-OH-NOR 46 ng, (2) *trans*-10-OH-AMI 13 ng, (3) *cis*-10-OH-NOR 17 ng, (4) *cis*-10-OH-AMI trace (<5 ng/ml), (5) loxapine 100 ng, (6) NOR 51 ng, and (7) AMI, 51 ng. Chromatographic conditions as in Fig. 2.

Fig. 5. Sample chromatogram of a 1-ml plasma sample from a patient receiving NOR 75 mg daily for 2 days. Levels were found to be (1) *trans*-10-OH NOR 37 ng, (3) *cis*-10-OH NOR 11 ng, (5) loxapine 100 ng, and (6) NOR 40 ng. Chromatographic conditions as in Fig. 2.

A chromatogram of a plasma sample of an actual steady state patient receiving AMI is shown in Fig. 4. The concentration of *trans*-10-OH-NOR exceeds the concentration of both isomers of OH—AMI metabolites as well as the *cis* isomers of OH-NOR. This is generally the case as shown in Table IV. The concentrations of *trans*-10-OH-AMI are usually low in comparison with the *trans*-10-OH-NOR. The *cis*-10-OH-AMI isomer is found in even lower concentrations than the *trans* isomer or is not detected. This is in agreement with previous published data [9, 18, 20, 22]. Fig. 5 illustrates that patients receiving NOR only, can also be monitored for the two hydroxy isomers of NOR as well as the parent compound.

Since it is known that the unconjugated hydroxy metabolites of AMI and NOR are pharmacologically active, their measurement in plasma should be included in

TABLE IV

PLASMA LEVELS (ng/ml) OF AMI AND METABOLITES IN ELEVEN DIFFERENT PATIENTS RECEIVING AMI OR NOR

Subject	AMI	NOR	*trans*-10-OH-AMI	*cis*-10-OH-AMI	*trans*-10-OH-NOR	*cis*-10-OH-NOR
1	85	98	T**	ND***	25	14
2	80	48	20	ND	42	9
3	40	19	17	5	25	7
4	51	51	13	T	46	17
5*		159			93	31
6	163	143	37	10	231	62
7	65	114	13	ND	161	35
8	231	251	53	13	293	83
9	48	36	22	ND	64	7
10*		50			56	17
11*		51			43	15

*Patient receiving NOR only.
**T = Level < 5 ng/ml.
***ND = No detectable levels.

the evaluation of clinical response vs. plasma concentrations. This method permits the clinician to determine the individual patient's metabolic profile with respect to AMI and/or NOR hydroxylation.

ACKNOWLEDGEMENTS

The work was supported in part by Public Health Service Grant RR 05651-10 from the Department of Health and Human Services.

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