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Simultaneous determination of trimipramine and desmethyl- and hydroxytrimipramine in plasma and red blood cells by capillary gas chromatography with nitrogen-selective detection

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ABSTRACT

A simple procedure is described that permits the simultaneous determination of trimipramine and its two major metabolites, desmethyl- and hydroxytrimipramine, in human plasma or red blood cells (RBCs) at therapeutic concentrations. The extracted biological fluids are injected into a capillary gas chromatograph with an OV-1 fused-silica column coupled to a nitrogen–phosphorus-selective detector. The limit of determination for trimipramine is 3 ng/ml and for that desmethyl- and hydroxytrimipramine is 4 ng/ml. The method permits the RBC/plasma ratios to be determined and to be correlated with the clinical response.

INTRODUCTION

Important advances have been made in using plasma antidepressant (ADP) levels to guide individual therapy, in exploring higher doses when ordinary doses prove ineffective and in recognizing a broadening range of possible indications. Trimipramine is a tricyclic ADP with an efficacy in the treatment of major depressive illness equal to that of other currently available tricyclics [1]. The identification and determination of ADP and some major metabolites should provide useful information in relation to metabolism and maintenance of activity [2]. Non-linear kinetics of trimipramine in depressed patients highlight the value of monitoring blood levels of ADP and metabolite drugs [3]. An increase in the dose of trimipramine results in a markedly disproportionate increase in the steady-state plasma concentrations of the major active desmethyltrimipramine metabolite. This is most likely due to the saturation within the therapeutic dosage range of the subspecies of cytochrome P-450 responsible for hydroxylation of desmethyltrimipramine [3]. Moreover, the hydroxy-tricyclic ADP metabolites have pharmacological properties similar to those of the parent compounds, may be particularly cardiotoxic [4,5], have relatively long elimination half-lives [6] and are rarely measured in clinical assays of plasma ADP concentrations [7–9]. The red blood cell (RBC)/plasma ratio of drug concentration is a function of the protein binding of the drug in the plasma and of the affinity of the erythrocytes for the drug [10,11]. The apparent variability in relative affinities for tricyclic ADP and their desmethyl metabolites may reflect a similar variability in the central nervous system [12]. A number of studies have demonstrated a significant correlation between respon-

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ders and non-responders in the various blood-level parameters. Plasma and RBC ratios of the desmethyl metabolite to the parent drug were reported to be greater in responders than non-responders [11]. These blood-level parameters may permit the correlation between RBC/plasma ratios and clinical response to be determined.

Trimipramine as other tricyclic ADP, is often assayed by GC or HPLC techniques [13,14]. More recently, mass spectrometry has greatly improved the sensitivity and selectivity but this technique is not available in many laboratories [15–17]. Although immunological assays are now largely developed in routine applications, the lack of specificity and problems of antibody cross-reactivity with other tricyclic ADP drugs make them suitable mostly for research purposes. We describe here a sensitive, rapid and accurate method for the simultaneous determination of trimipramine and desmethyl- and hydroxytrimipramine in human plasma and RBCs by gas–liquid chromatography using thermionic nitrogen-selective detector.

EXPERIMENTAL

Chemicals

Trimipramine and its major metabolites, monodesmethyltrimipramine and 2-hydroxytrimipramine, were kindly supplied by Rhône Poulenc Rorer (Vitry sur Seine, France). The internal standards (I.S.), clomipramine and maprotiline, were provided by Ciba Geigy (Basle, Switzerland).

Ammonia solution (minimum 20%), absolute ethanol, dichloromethane and isoamyl alcohol were RP Normapur products from Prolabo (Paris, France), and hexane was 99% pestipur grade from SDS (Valdonne-Peypin, France).

Glassware

Polycyclic amines have a tendency to adhere to acidic glass surfaces, adsorption being particularly significant with the more polar metabolites. If the tubes are wetted with aqueous alkali prior to the transfer of the amine-containing aqueous solution, these losses can be prevented [13,19]. We added 2 ml of 20% ammonia solution before addition of plasma and initial extraction. Further, it is advisable to use tubes with a capacity of at least twice the volume of liquid to be shaken.

Instrumentation

A Hewlett-Packard Model 5890 A gas chromatograph equipped with a nitrogen–phosphorus-selective detector and a split–splitless injector system was used. An OV-1 fused-silica capillary column (25 m × 0,32 mm I.D.) with a film thickness of 0.2 μm (Spiral, Dijon, France) was employed. The GC conditions were as follows: injector and detector temperature, 280°C, column temperature, 195°C; injection mode, split with a splitting ratio of 3; and purge, on. The carrier gas was helium at a flow-rate of 2.5 ml/min; other flow-rates were make-up gas 33 ml/min, hydrogen 2.3 ml/min and air 55 ml/min. The reporting integrator used peak-height ratios to calculate drug concentrations.

Standard solutions

Trimipramine maleate (13.94 mg; equivalent to 10 mg of trimipramine), desmethyltrimipramine maleate (14.13 mg; equivalent to 10 mg), 2-hydroxytrimipramine fumarate (11.87 mg; equivalent to 10 mg of 2-hydroxytrimipramine) and internal standard solutions of clomipramine hydrochloride (11.15 mg; equivalent to 10 mg of clomipramine) and maprotiline hydrochloride (11.31 mg; equivalent to 10 mg of maprotiline) were dissolved in 100 ml of absolute ethanol to yield stock standard solutions (100 mg/l). The solutions were stored in the dark in glass-stoppered bottles at −25°C and were stable for at least three months. These solutions were further freshly diluted before analysis at a ratio of 1:100 to yield working standard solutions of trimipramine, desmethyltrimipramine and hydroxytrimipramine (1 mg/l) and at a ratio of 1:10 to yield the working standard solutions of clomipramine and maprotiline (10 mg/l). Drug-free plasma was obtained from healthy volunteers and kept at −25°C.

Extraction procedure

A 2-ml volume of 20% ammonia solution was placed in a 20-ml glass stoppered tube and the tube was agitated gently in an upright position on a vortex mixer for 15 s. To this were added 2 ml of plasma, 50 μl of 10 mg/l clomipramine solution (I.S. for trimipramine and 2-hydroxytrimipramine), 80 μl of 10 mg/l maprotiline solution (I.S. for desmethyltrimipramine) and 9 ml of hexane–dichloromethane–isoamyl alcohol (57:42:1). The tube was shaken mechanically for 15 min and centrifuged for 10 min at 1600 *g*. The upper organic layer was collected with a Pasteur pipette and evaporated to dryness in a stream of air at ambient temperature. The residue was dissolved in 50 μl of absolute ethanol and 1–4 μl of this solution were injected into the gas chromatograph.

To determine the same compounds in erythrocytes 5 ml of 0.9% NaCl solution were added to 2 ml of erythrocytes. After gentle agitation, the tube was centrifuged and the upper phase discarded. The erythrocytes were then transferred into a glass tube containing 2 ml of 20% ammonia solution and 4 ml of deionized water.

Calibration

Concentrations of specific compounds (trimipramine, and the desmethyl and 2-hydroxy metabolites) were determined from calibration graphs for each compound. These graphs were derived from determinations of standard concentrations (50, 100, 150, 200 and 250 ng/ml) added to drug-free control plasma. The ratios of the peak height of each compound to that of internal standard (y) were plotted against the standard concentrations of each compound (x). These calibration graphs were linear across the entire concentration range for all compounds.

RESULTS

The procedure provided good resolution of trimipramine from its major metabolites. Typical chromatograms obtained from the analysis of standards and samples from patients receiving medication with trimipramine are shown in Figs. 1 and 2. Trimipramine and its two major metabolites were separated in a single chromatogram in less than 20 min.

Two internal standards were incorporated in the specimens to aid in quantification. We used a tertiary ADP (clomipramine) for the measurement of tertiary amines (trimipramine, hydroxytrimipramine) and a secondary ADP (maprotiline) for the measurement of the secondary amine (desmethyltrimipramine). This choice was based on methodological considerations relating to extraction and chromatographic performance: the variability of the assay, as evidenced by the coefficient of variation (C.V.) for peak-height ratios, was always better when the tertiary amine was measured with a tertiary tricyclic ADP as I.S. and the secondary amine with a secondary tricyclic ADP as I.S. The inclusion of a third I.S. for the hydroxy metabolite was not necessary. Although trimipramine and hydroxytrimipramine behaved differently in their extraction properties, the hydroxy metabolite was recovered with a relatively high precision with clomipramine as I.S.

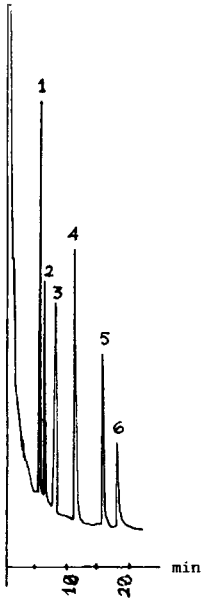

Fig. 1. Chromatogram of a standard mixture containing 150 ng/ml **1** and 100 ng/ml **2**, **3**, **4**, **5** and **6** in ethanol. **1** = Trimipramine; **2** = desmethyltrimipramine; **3** = maprotiline; **4** = clomipramine; **5** = 2-hydroxytrimipramine; **6** = 2-hydroxydesmethyltrimipramine (not determined).

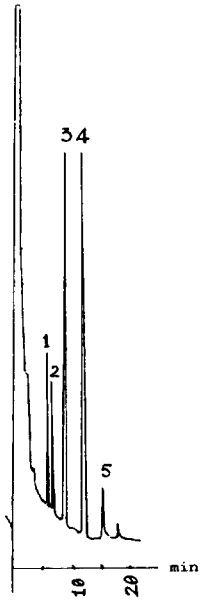

Fig. 2. Chromatogram of a 2-ml plasma extract containing 57 ng/ml **1**, 61 ng/ml **2** and 8 ng/ml **5**; **3** and **4** are the I.S. (compound numbers as in Fig. 1). A patient was treated with chronic administration of 50 mg of trimipramine per day. Time after administration: 3 h.

Precision and accuracy

The data presented in Table I show the precision and accuracy of this assay. The intra-assay precision was determined at three concentration levels, 50, 150 and 250 ng/ml (n = 10), for trimipramine, desmethyltrimipramine and hydroxytrimipramine in plasma. In Table II the inter-assay precision (n = 5) is given for the same concentrations. The precision of the method (mean C.V.) was 1.8–3.2 and 2.9–4.1% for intra- and inter-assay, respectively.

Recovery

The overall recoveries in Table III were calculated by comparing the peak heights of a series of trimipramine- and trimipramine metabolite-spiked samples after their extraction from plasma without an I.S. with the peak heights of a series of the same unextracted products.

TABLE I

INTRA-ASSAY PRECISION AND ACCURACY

Concentration added (ng/ml)	Concentration found (mean ± S.D., n = 10) (ng/ml)	C.V. (%)	Difference between added and found concentrations (%)
Trimipramine[a]			
50	47.8 ± 1.1	2.3	−4.4
150	154.9 ± 1.5	1.0	3.2
250	243.4 ± 3.3	2.3	−2.7
		Mean: 1.8	
Desmethyltrimipramine[b]			
50	52.0 ± 1.1	2.1	4.0
150	143.6 ± 3.3	2.2	−4.3
250	245.6 ± 2.8	1.1	−1.8
		Mean: 1.6	
Hydroxytrimipramine[c]			
50	50.2 ± 1.8	3.5	0.4
150	138.8 ± 6.1	4.4	−7.5
250	258.7 ± 4.8	1.8	3.4
		Mean: 3.2	

[a] Linear regression line: $y = 3.97 \cdot 10^{-3}x + 0.0001$ (r = 0.999).
[b] Linear regression line: $y = 4.01 \cdot 10^{-3}x - 0.010$ (r = 0.999).
[c] Linear regression line: $y = 2.72 \cdot 10^{-3}x - 0.051$ (r = 0.973).

TABLE II

INTER-ASSAY PRECISION AND ACCURACY

Concentration added (ng/ml)	Concentration found (mean ± S.D., n = 5) (ng/ml)	C.V. (%)	Difference between added and found concentrations (%)
Trimipramine[a]			
50	51.0 ± 2.9	5.6	2.0
150	151.7 ± 7.6	5.0	1.1
250	248.2 ± 4.5	1.8	−0.7
		Mean: 4.1	
Desmethyltrimipramine[b]			
50	43.6 ± 1.1	2.5	−12.8
150	152.5 ± 6.1	4.0	1.6
250	246.2 ± 5.8	2.3	−1.5
		Mean: 2.9	
Hydroxytrimipramine[c]			
50	49.7 ± 3.3	6.6	−0.6
150	143.0 ± 4.5	3.1	−4.7
250	249.2 ± 4.3	1.7	−0.3
		Mean: 3.8	

[a] Linear regression line: $y = 4.03 \cdot 10^{-3}x + 0.018$ ($r = 0.997$).
[b] Linear regression line: $y = 3.93 \cdot 10^{-3}x + 2.3 \cdot 10^{-3}$ ($r = 0.998$).
[c] Linear regression line: $y = 2.15 \cdot 10^{-3}x - 0.020$ ($r = 0.993$).

Limit of detection

Under the adopted conditions the limits of detection using a 2-ml plasma sample and a 1-μl injection were 3 ng/ml for trimipramine, 4 ng/ml for desmethyltrimipramine and 4 ng/ml for hydroxytrimipramine, with a signal-to-noise ratio greater than 3:1.

Linearity

The linearity of the method was tested on the basis of extracted samples in the concentration range 0–250 ng/ml for each drug. A linear relationship ($r = 0.99$) was observed for each drug over the range studied. To test the limit of linearity of the method, known high amounts of the drugs were added to blank serum samples and linearity limits were evaluated graphically: 1000 ng/ml for trimipramine, 930 ng/ml for desmethyltrimipramine and 810 ng/ml for hydroxytrimipramine.

Plasma interference and selectivity

A typical chromatogram obtained from the analysis of samples from patients receiving medication with trimipramine is shown in Fig. 2. There is no interference from the normal constituents of blood, as Fig. 3 shows. Interference was tested with numerous compounds whose chemical structure or concurrent usage could cause concern over potential interference with the assay. The interference of a compound was determined by analysing drug-free pooled human serum spiked with therapeutic concentrations of the compound to be tested. Only imipramine interferes with trimipramine and diazepam with clomipramine.

The following compounds do not interfere with trimipramine, desmethyltrimipramine, hydroxytrimipramine and their I.S. (some of them give a response under the same analytical conditions, but have different retention times): desipra-

TABLE III

RECOVERIES

Concentration (ng/ml)	Peak height		Recovery (%)
	Set A = authentic standards	Set B = standards extracted from plasma	
Trimipramine			
Blank	N.D.[a]	N.D.	
50	0.241	0.231	95.8
150	0.743	0.650	87.4
250	1.173	1.040	88.6
			Mean[b]: 90.6 ± 4.5
Desmethyltrimipramine			
Blank	N.D.	N.D.	
50	0.227	0.208	91.6
150	0.597	0.503	84.2
250	1.041	1.020	97.9
			Mean[b]: 91.2 ± 6.8
Hydroxytrimipramine			
Blank	N.D.	N.D.	
50	0.151	0.127	84.1
150	0.289	0.247	85.4
250	1.070	0.920	85.9
			Mean[b]: 85.1 ± 0.9

[a] N.D. = not detectable.

[b] Mean overall recovery ± S.D.

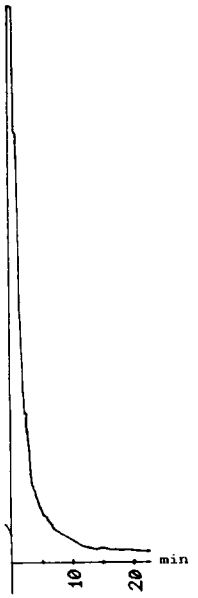

Fig. 3. Chromatogram of a drug-free blood sample.

mine, amitriptyline, nortriptyline, medifoxamine, fluoxetine, alimemazine, cyamemazine, promethazine, levopromazine, carbamazepine, clobazam, clonazepam, desmethyldiazepam, prazepam, lorazepam, bromazepam and nitrazepam. Other drugs in co-medications give no response: phenobarbital, phenytoin, sodium valproate, lithium, theophylline, digoxin, digitoxin, amikacin, gentamicin and hydroquinidine.

DISCUSSION

The tricyclic antidepressant drugs are strong lipophilic bases that are readily extracted from an aqueous phase into an organic phase at pH > 10. The simple addition of ammonia solution before the initial introduction of plasma and other reagents for extraction serve as a "scavenger" function by preventing adsorption of tertiary and especially secondary amines on glass surfaces, and also made the biological material just alkaline (pH > 10) for the initial extraction. This method is easier than and as efficient as those with addition of volatile low-molecular-mass amines such as triethylamine, trimethylamine, diethylamine or triethanolamine, or by silanizing glassware or soaking it in ethanol [13]. Hexane and heptane are the best solvents for extraction, compared with diethyl ether and light petroleum, owing to their decreased tendency to form emulsions and their increased capacity for extracting neutral lipids. Although less likely to form emulsions than diethyl ether, hexane and heptane can still do so, especially when the ratio of organic to aqueous phase is low. We circumvented this problem with the addition of 1% of isoamyl alcohol (IAA); a concentration >1% resulted in a yellow final residue that led to differences in chromatographic performance. However, hexane–IAA (99:1) did not extract the hydroxylated metabolite.

Previously reported methods to assay hydroxy metabolites of ADP drugs include liquid chromatography [7–9], gas chromatography–mass spectrometry [17] and rarely gas chromatography [18]. Jones *et al.* [18] added to hexane extractant a more polar solvent, 2-butanol, for the determination of hydroxyamitriptyline, and we similarly

accomplished the simultaneous determination of trimipramine, desmethyltrimipramine and hydroxytrimipramine by addition of dichloromethane, and finally adopted as the extraction solvent hexane–dichloromethane–IAA (57:42:1). This mixture allows essentially complete recovery and is volatile enough that the solvent can be readily evaporated prior to the last reconstitution step. Whereas three-step extraction procedures are often required [14], only one analytical run is needed here for all the compounds of interest.

The benefit of capillary gas chromatography resides in the versatility of this high-resolution technique and diminishes the risk of interference from other psychoactive drugs used in conjunctive therapy. Selection of another tricyclic antidepressant as the I.S. has several distinct advantages: these molecules are readily obtainable, they behave chemically in a manner similar than the compounds of interest and they are virtually never co-prescribed in the treatment of depression. In acute overdose situations in which multiple ingestion of antidepressants is suspected, the selection of a tricyclic compound not currently prescribed would be appropriate.

CONCLUSION

Advantages of the proposed method include rapid analysis, very simple procedure for deactivation of the glassware, simultaneous determination of trimipramine and its two major metabolites without derivatization, extension to RBC analysis and acceptable specificity. As can be seen from the results, the technique allows precise and reliable measurement of the drug at concentrations as low as 3 ng/ml in plasma or RBCs. The method has adequate sensitivity for measuring drug levels in the blood of patients receiving therapeutic doses.

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