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DETERMINATION OF CLOMIPRAMINE AND DESMETHYLCLOMIPRAMINE IN PLASMA BY MEANS OF LIQUID CHROMATOGRAPHY

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SUMMARY

A method is presented for the determination of clomipramine and its major metabolite desmethylclomipramine in plasma. After extraction with *n*-hexane, the components are separated by high performance liquid–solid chromatography on silica gel and detected with a UV detector. The detection limits are 2 ng/ml for clomipramine and 10 ng/ml for desmethylclomipramine. Recoveries from plasma exceed 95% for both drugs. In routine analysis, 30–40 samples can be handled in one day.

The practical use of the method is shown in plasma concentration–time curves after oral and intramuscular administration of clomipramine.

INTRODUCTION

Although the use of clomipramine (Anafranil®) in the treatment of certain depressions has been well established since its introduction in 1961, very little information is available on the pharmacokinetic properties of this drug because, despite various efforts, there does not seem to be a suitable analytical method for routinely monitoring the fate of the drug in the body.

The ideal assay procedure for clomipramine will be one that is reliable, rapid and economical, distinguishes between the parent component and its metabolites, and provides adequate quantitation of those components that are biologically active. The last aspect is particularly important with regard to the formation and the presence of N-desmethylclomipramine. So far, this compound has been reported to be the only metabolite with biological activity[1–3].

Very few assay procedures have been reported for the determination of clomipramine in biological fluids. Recently, gas chromatography–mass spectrometry with deuterated internal standards has been proposed for the determination of clomipramine and desmethylclomipramine[4,5]. Carnis *et al.*[6] and Jones and Luscombe[7]

have described a sensitive double radioisotope derivative technique. Although very elegant, this method is laborious, resulting in a rather limited throughput.

Liquid chromatography would seem to be a more suitable technique for these tricyclic antidepressants. It has the advantage that separations are carried out at room temperature so that the low volatility of the tricyclic components is not a limiting factor. Moreover, high-performance liquid chromatography (HPLC) combines high selectivity with sensitive detection modes such as the UV detector.

Lagerström *et al.*[8] have reported a liquid chromatographic determination of clomipramine and other tricyclic antidepressants by ion-pair partition chromatography. In this paper we describe a liquid chromatographic determination of clomipramine and its desmethyl metabolite, based on liquid–solid principles, which is also suitable for the analysis of other tricyclic antidepressants.

The technique is at present being applied in a combined pharmacokinetic and clinical study. Some preliminary results from this study are given in order to illustrate the practical use of the method.

MATERIALS AND METHODS

Chemicals and reagents

Clomipramine, desmethylclomipramine and imipramine were gifts from Ciba-Geigy (Basle, Switzerland). All solvents and other chemicals were of analytical-reagent grade and obtained from E. Merck (Darmstadt, G.F.R.). Dichloromethane was redistilled in glass before use. The glassware was cleaned by standing it overnight in chromic acid and then rinsing with distilled water.

Standard solutions

A standard solution of clomipramine and desmethylclomipramine as their hydrochlorides in distilled water was prepared at a concentration of 5 mg/ml. If necessary, this solution was diluted with water to produce solutions of the desired concentration. Spiked plasma samples, containing 5–400 ng/ml of both drugs, were prepared by adding 1 ml of the appropriate aqueous standard solution to 4 ml of citrated human plasma. The resulting mixture was mixed thoroughly.

An internal standard solution containing imipramine (380 ng/ml) was prepared by dissolving the required amount of imipramine hydrochloride in 0.1 *M* hydrochloric acid. An acidic solution was used to minimize losses due to adsorption on the glass surface.

Apparatus

A Spectra-Physics (Berkeley, Calif., U.S.A.) Model 3500 B liquid chromatograph was used in conjunction with a Model SF 770 variable-wavelength UV detector (Schoeffel), which was operated at 250 nm.

Injections were made with a Valco high-pressure injection valve fitted with a 100-μl sample loop. The separation was performed on a stainless-steel column, 10 cm × 4.6 mm I.D., packed with silica gel (LiChrosorb SI-60, particle diameter 5 μm; Merck) by a balanced-density slurry method[9].

A ternary mixture of *n*-hexane, dichloromethane and methanol (8:1:1) was used as the mobile phase at a flow-rate of 1.2 cm^3/min (pressure drop 28 atm) with

development being carried out at room temperature (22–25°). The mobile phase was de-gassed ultrasonically immediately before use. HETP values were calculated for clomipramine and desmethylclomipramine and appeared to be 20–30 μm for both components under the conditions described above.

Assay procedure

Blood samples (4–8 ml) were collected in heparinized glass tubes. Plasma was obtained by centrifugation. To 1–4 ml of plasma (depending on the drug concentration anticipated) were added 0.5 ml of the internal standard solution containing 190 ng of imipramine hydrochloride and 0.5 ml of 2 *M* sodium carbonate solution in a centrifuge tube of about 50 ml with a Quickfit stopper. This mixture (final pH 10) was extracted with 10 ml of *n*-hexane by shaking vigorously on a Vortex mixer for 1 min.

The phases were separated by centrifugation (10 min) at 6000 *g*. The tube was then dipped in liquid nitrogen for 45 sec, which resulted in freezing of the aqueous phase, and the organic layer was transferred by decantation into an evaporation tube, to which 2 ml of methanol were added in order to minimize adsorption losses during the evaporation step.

The *n*-hexane–methanol layer was subsequently evaporated at 60° under a gentle stream of nitrogen. The residue was re-dissolved in 300 μl of mobile phase solvent of which 100 μl were injected into the liquid chromatograph.

Calibration graph

Clomipramine and desmethylclomipramine concentrations were calculated with the aid of calibration graphs. Spiked plasma samples were processed as described in the assay procedure. Peak-height ratios were then calculated and plotted against the known plasma concentrations. The slopes and correlation coefficients were calculated by using a least-squares procedure.

Recovery

Recoveries of both drugs at different concentrations were determined by extracting spiked plasma samples, after which imipramine was added as an external standard.

The peak-height ratios of both drugs to the external standard (R_1) were compared with the ratios (R_2) obtained by direct injection of the same amount of the drugs and imipramine in mobile phase solvent.

$$\text{Recovery}\,(\%) = \frac{R_1}{R_2} \cdot 100$$

Determination of partition coefficients

Partition experiments were performed with equal volumes of aqueous and organic phase, saturated with each other, in centrifuge tubes with a Quickfit stopper. Phosphate buffers were used to control the pH of the aqueous phase. The tubes were shaken for 60 min at 25.0 $\pm$ 0.1° in a thermostated water-bath. The drug concentrations were determined spectrophotometrically in the aqueous phase before and after extraction, using a Beckman Model 25 spectrophotometer at 253 nm (the wavelength of maximum absorption). Prior to extraction, the pH of the aqueous phase was measured with a Radiometer PHM 62 digital pH meter.

RESULTS AND DISCUSSION

Choice of the phase system

Liquid–solid chromatography was chosen as the separation mode, because it offers excellent column stability, which is particularly important for routine analyses. Liquid–liquid systems are less suitable in this respect as several conditions have to be carefully standardized, which is difficult to maintain under routine conditions.

Various solid stationary phases were investigated. We choose silica gel as the adsorbent because of the high separation efficiency that could be obtained and which resulted in excellent separations between several tricyclic antidepressants. The retention times of some tricyclic antidepressants are summarized in Table I. As expected, this separation mode provides a high selectivity for components with small differences in functional groups. This resulted, for example, in a complete separation between imipramine and clomipramine. The order of elution of the components depends primarily on the polarity of the substances involved. Tertiary amines such as imipramine and clomipramine are eluted before their corresponding secondary analogues, owing to the more basic nature of the latter components.

TABLE I

LIQUID CHROMATOGRAPHIC RETENTION TIMES OF TRICYCLIC ANTIDEPRESSANT DRUGS

Free bases were injected into the liquid chromatograph and separated as described under Materials and Methods.

Compound	*Retention time (min)*
Trimipramine	2.0
Amytriptyline	3.1
Clomipramine	3.4
Doxepine	4.5
Imipramine	5.0
Nortriptyline	20
Desmethylclomipramine	23
Desmethylimipramine	30

Developments on chemically modified silica gel (LiChrosorb RP-8) also provided a suitable separation of clomipramine and desmethylclomipramine, using an appropriate solvent system. As the order of elution is reversed here, this system would provide a more sensitive method for desmethylclomipramine because of a smaller peak broadening of the latter. It should be emphasized, however, that reversed-phase systems are less selective with regard to small differences in the tricyclic ring system. Hence, these systems could not separate clomipramine and imipramine from one another, nor could they separate their desmethyl metabolites.

The use of a ternary solvent system as described here appeared to be very versatile, as has already been shown by Gonnet and Rocca[10]. For our purposes, *n*-hexane–dichloromethane–methanol mixtures proved to be the most suitable. The

TABLE II

INFLUENCE OF THE METHANOL CONCENTRATION IN THE MOBILE PHASE ON THE CAPACITY FACTORS (k') OF SOME TRICYCLIC ANTIDEPRESSANT DRUGS

Capacity factors were calculated form the retention times of the components and a non-retained compound (benzene). The dichloromethane concentration was kept at 10%.

Compound	*Volume percentage of methanol*				
	10	*7.5*	*5*	*2.5*	*1.5*
Clomipramine	2.4	2.8	3.2	6.4	44
Imipramine	3.5	3.9	4.7	7.9	—
Desmethylclomipramine	22	26	42	—	—

separation could be optimized by varying the mobile phase composition, in particular by changing the methanol concentration. Table II shows the influence of the methanol concentration on the capacity factor (k') of some tricyclic drugs.

Extraction

The isolation procedure is of great importance in bioanalysis, as drugs are usually present in very low concentrations in body fluids in which endogenous components are present in concentrations that are various orders of magnitude higher than that of the substances under investigation. Hence, high demands have to be made on this step in the total procedure. The isolation of tertiary and secondary amines such as clomipramine, imipramine and their demethylated analogues can be accomplished either by extraction of the uncharged form or by extraction of the ionized form as an association complex (ion pair)[11]. This is depicted schematically in Fig. 1. We investigated both approaches, after which extraction of the uncharged amines with *n*-hexane was chosen, because high extraction yields could be obtained with a single extraction step, while no interfering UV absorbing substances were co-extracted. The extraction yields (recoveries) of the amines at a given pH will depend on the pK_a and the partition coefficient (k_d) of the uncharged form between the two

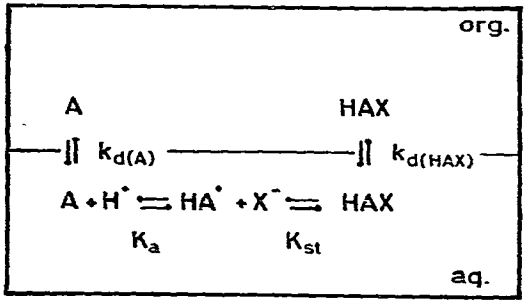

Fig. 1. Schematic representation of the main processes involved in the solvent extractions of amines (A) and the extraction of amines as ion pairs (HAX). Ion pairs can be formed in the aqueous phase between protonated amines (HA^+) if a suitable counter ion (X^-) is present. For reasons of simplicity, side-reactions have been omitted.

phases. Assuming partition of only the free bases (no ion-pair formation), the following equation can be derived:

$$D_a = \frac{k_{d(A)}\, K_a}{[H^+]}$$

where D_a is the distribution coefficient of a component A.

Quantitative extractions (99%), using equal phase volumes, can be obtained from water with distribution coefficients of more than 100. The partition coefficients for various organic solvent–water systems and the pK_a values of clomipramine and desmethylclomipramine are given in Table III. With the aid of these values, it can be calculated that above pH 9 both clomipramine and desmethylclomipramine can be extracted quantitatively from water with *n*-hexane, using equal phase volumes.

TABLE III

PARTITION COEFFICIENTS AND DISSOCIATION CONSTANTS OF CLOMIPRAMINE AND DESMETHYLCLOMIPRAMINE

Compound	*Log k_d**				*pK_a***
	$CHCl_3$	*CH_2Cl_2*	*Diethyl ether*	*n-Hexane*	
Clomipramine	7.1	6.7	5.2	4.8	9.4
Desmethylclomipramine	6.3	5.8	4.2	3.9	10.2

* Calculated from log $k_d \cdot K_a$.

** Taken from ref. 12.

In order to be able to determine drug concentrations at the lower nanogram per millilitre level, the organic phase must be evaporated and the residue re-dissolved in a minimum amount of mobile phase solvent. The residue could be readily re-dissolved in 300 μl of mobile phase solvent mixture by dipping the evaporation tube into an ultrasonic bath for a few seconds. Imipramine was used as the internal standard throughout this study because of its general availability and its structural resemblance. However, if imipramine is co-prescribed as medication or if its presence in the sample is suspected for other reasons, another tertiary tricyclic antidepressant can be used equally well.

Recoveries from plasma were determined for clomipramine in the range 10–400 ng/ml and for desmethylclomipramine in the range 20–400 ng/ml. Both recoveries appeared to be constant over these ranges, with mean values of 97 $\pm$ 2.7% for clomipramine and 95 $\pm$ 5.2% for desmethylclomipramine (mean $\pm$ S.D., $n = 24$).

Sensitivity, linearity and precision

The calibration graph for clomipramine and desmethylclomipramine using imipramine as internal standard is shown in Fig. 2. Each point represents the average of four replicate determinations. The curves were linear over a large concentration range for both components. The correlation coefficients of the calibration line were found to be 0.995 for clomipramine and 0.990 for desmethylclomipramine ($n = 20$).

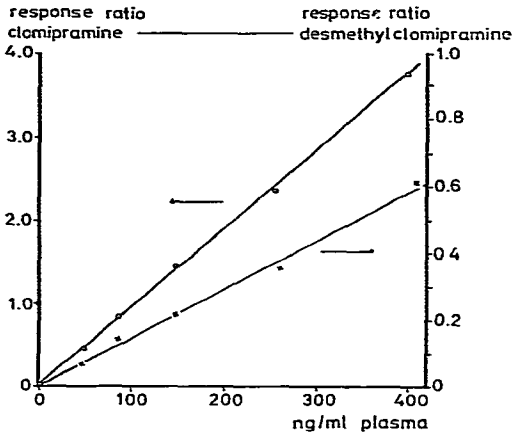

Fig. 2. Calibration graph for the determination of clomipramine and desmethylclomipramine in plasma using imipramine as internal standard. Each point represents the mean value of four replicate determinations. Sample size, 2 ml of plasma.

The lower limits of detection in plasma are 2 ng/ml for clomipramine and 10 ng/ml for desmethylclomipramine using 2 ml of plasma. Although both components have the same sensitivity in the detector, the limit for desmethylclomipramine is higher as a result of peak broadening. Although clomipramine and desmethylclomipramine show higher absorbances at 220 nm, the wavelength of 250 nm (secondary maximum) was chosen in order to prevent interferences from endogenous plasma compounds and solvent impurities.

These interferences have a strong influence on the signal-to-noise ratio, which ultimately determines the detection limit, as can be seen in Table IV. Fig. 3 illustrates a chromatogram obtained from the analysis of a plasma sample containing 25 ng/ml of clomipramine and 50 ng/ml of desmethylclomipramine using the procedure described above.

TABLE IV

INFLUENCE OF THE DETECTION WAVELENGTH ON THE SIGNAL-TO-NOISE RATIO AS DETERMINED FOR CLOMIPRAMINE

Parameter	*Wavelength (nm)*		
	220	*250*	*280*
Relative noise (N)	3.2	1.4	1.1
Relative peak height (S)	21.2	10.8	7.9
S/N (relative)	6.6	7.7	7.2

The proposed method is selective, rapid and simple and especially suitable for routine analysis, as 30–40 samples can be handled in one day. With regard to simplicity, speed and costs it compares favourably with other recently introduced methods, namely the double radioisotope derivative technique[6,7] and gas chromatography–mass spectrometry using deuterated internal standards[4,5].

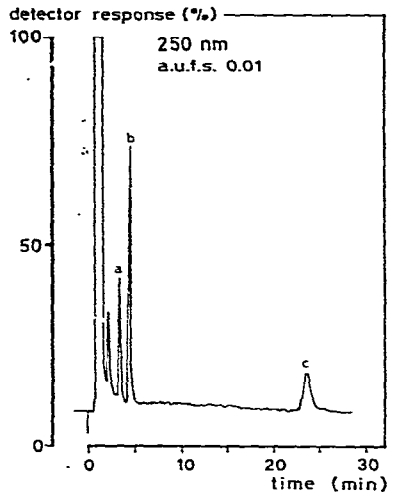

Fig. 3. Liquid chromatogram of a spiked plasma extract. (a) Clomipramine, 25 ng/ml; (b) internal standard (imipramine); (c) desmethylclomipramine, 50 ng/ml.

Application

The method is at present being used to follow clomipramine and desmethylclomipramine levels in plasma after intravenous, intramuscular and oral administration of clomipramine to volunteers as well as to depressive patients. In single-dose experiments in volunteers, clomipramine appeared rapidly in the general circulation after oral administration of 100 mg of the hydrochloride. Peak plasma levels were generally reached 1–2½ h after intake and were in the order of 70–140 ng/ml. Assuming a two-compartment model, the plasma half-lives of clomipramine were found to be

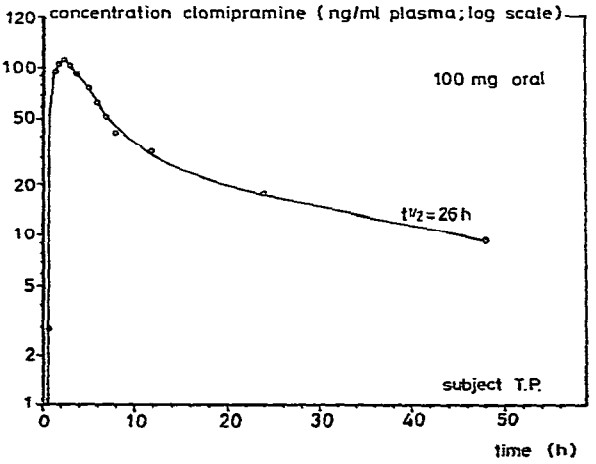

Fig. 4. Plasma levels (semi-logarithmic scale) of clomipramine in subject T.P. after a single oral dose of 100 mg of clomipramine hydrochloride. The data were fitted with the NAFFIT-I calculation programme.

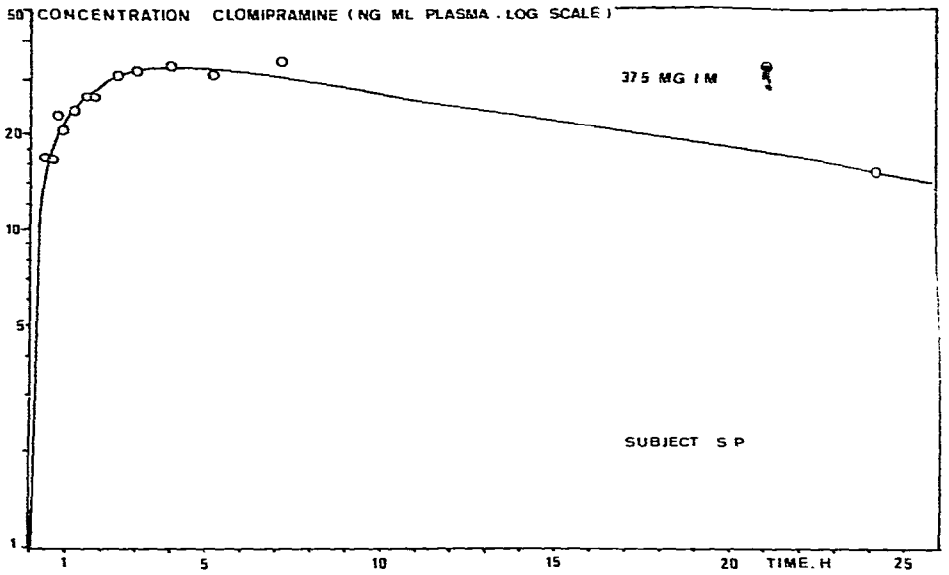

Fig. 5. Plasma levels (semi-logarithmic scale) of clomipramine in subject S.P. after a single intramuscular dose of 37.5 mg of clomipramine hydrochloride. The data were fitted with the NAFFIT-1 calculation programme.

in the order of 20 h, and metabolism to desmethylclomipramine was found to be of little importance. In all samples, desmethylclomipramine concentrations did not exceed 10 ng/ml. Single-dose intramuscular administration of 37.5 mg of the hydrochloride resulted in long-lasting plateau levels in the plasma ranging from 25–50 ng/ml, up to at least 8 h after administration. This was followed by slow elimination, which confirmed the relatively long half-lives found in the oral experiments. Figs. 4 and 5 show examples of plasma concentration–time curves on a semi-logarithmic scale after oral and after intramuscular administration. The data were fitted with the NAFFIT-1 calculation programme developed in our Institute[13].

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