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ION-PAIR LIQUID CHROMATOGRAPHY OF STEADY-STATE PLASMA LEVELS OF CHLORIMIPRAMINE AND DEMETHYLCHLORIMIPRAMINE

BRITT MELLSTRÖM and GUNNEL TYBRING

Department of Clinical Pharmacology, Karolinska Institutet, Huddinge Hospital, S-141 86 Huddinge (Sweden)

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SUMMARY

A method for the determination of chlorimipramine and its metabolite demethylchlorimipramine in the plasma of depressed patients during treatment is described. The method involves extraction of the parent drug, its metabolite and the internal standard from plasma, back-extraction into an acidic aqueous phase and re-extraction into a small volume of organic phase. Separation and quantitation are carried out by ion-pair partition chromatography with UV detection. Accurate determination is possible down to levels of 30 and 60 nmole per liter of plasma for chlorimipramine and the metabolite, respectively, when 1 ml of plasma is used.

The coefficient of variation is 7.3% or less at different levels for chlorimipramine and demethylchlorimipramine. Plasma levels of the parent drug and the metabolite measured by this liquid chromatographic method and by a gas chromatographic procedure with electron-capture detection were in good agreement ($r = 0.98$).

The steady-state plasma level of the metabolite was always higher than that of the parent drug in the 34 depressed patients investigated. The mean ratio between the metabolite and the parent drug was 2.7 ± 1.1 (S.D.) Large inter-individual differences in the levels of the two compounds in patients receiving similar doses were found.

INTRODUCTION

The measurement of tricyclic antidepressant plasma levels is of significant clinical importance during therapy [1, 2]. When tertiary amines are given, the therapeutic effect will depend not only on the level of the parent drug but also on the level of the pharmacologically active demethylated metabolite formed. Tertiary tricyclics are more potent inhibitors of serotonin uptake than are their secondary amine metabolites, whereas the opposite is true for the uptake into noradrenergic neurons [3, 4]. Chlorimipramine is of special interest because it is the most potent serotonergic re-uptake blocker of the tricyclic antidepressants both in vitro [3, 4] and in vivo in man [5, 6].

Several methods have appeared for the simultaneous quantitation of amitriptyline or imipramine and their secondary amine metabolites. These methods include gas chromatography with flame-ionization [7, 8], alkali flame-ionization [9—11] or electron-capture [12] detection, mass fragmentography [13—15], thin-layer chromatography [16, 17] and liquid chromatography [18, 19].

Chlorimipramine and its demethylated metabolites have been determined by thin-layer chromatography [20] and mass fragmentography [21]. Recently, methods have been developed using ion-pair partition chromatography [22, 23]. This paper deals with the development and evaluation of a modified ion-pair liquid chromatographic method and its application to plasma samples from patients treated with Anafranil.

EXPERIMENTAL

Apparatus and materials

The chromatograph consisted of a pump (LDC 711-26 Solvent Delivering System), an injector (Model 905-19 Syringe Loading Sample Injector) and a UV detector (Model 153 Analytical UV Detector) operated at 254 nm (cell volume 8 μl, path length 10 mm). The injector and the detector were obtained from Altex Scientific, Berkeley, Calif., U.S.A. The separation column was a 75 × 3 mm I.D. stainless-steel column, packed with Partisil 10 (10 μm diameter, 400 m^2/g; Reeve Angel, Clifton, N.J., U.S.A.). For equilibration of the mobile phase, a 300 × 9 mm I.D. pre-column made of stainless steel was used. The pre-column was packed with Porasil C (37—75 μm diameter; Waters Assoc., Milford, Mass., U.S.A.). The whole system, except the pump, was thermostated at 23°.

The stationary phase consisted of 0.1 *M* hydrochloric acid containing 0.01 *M* tetrapropylammonium hydrogen sulphate, (Hässle, Mölndal, Sweden) and the mobile phase consisted of 13% 1-butanol in *n*-hexane. The phases were carefully equilibrated with each other at 23° by stirring overnight.

Preparation of columns

The separation column was packed by the balanced density slurry technique described by Majors [24]. The column was loaded with the stationary phase by precipitation from an acetone solution (75%, v/v) pumped through the column with saturated hexane [25]. The pre-column was dry-packed with the support previously equilibrated with 50% (w/v) of the stationary phase. The volume of the stationary phase (0.15 ml) in the separation column was determined by elution with methanol, followed by determination of the water content by Karl Fisher titration. The interstitial volume of the column (0.35 ml) was determined by injection of the non-retained solute benzene. With a flow-rate of 0.4—0.6 ml/min, the pressure never exceeded 100 p.s.i. for the entire system.

Chemicals

n-Hexane was of spectroscopic grade (Uvasol; E. Merck, Darmstadt, G.F.R.). All other solvents and chemicals were of analytical grade.

Stock solutions of chlorimipramine (Cl) hydrochloride, demethylchlorimipramine (DMCl) hydrochloride (both from Ciba-Geigy, Basle, Switzerland) and trimipramine maleate (Leo, Helsingborg, Sweden) were prepared in 0.01 *M* hydrochloric acid.

Determination of partition coefficients and extraction constants

The partition experiments with the amines were performed with equal volumes of aqueous and organic phases (pre-equilibrated with each other [26]) in centrifuge tubes and with an equilibration time of 30 min at 23°. After centrifugation, the concentration of the amines was measured by photometry at the absorbance maximum in both the aqueous and the organic phase, either directly or after re-extraction into 0.1 *N* sulphuric acid.

Analytical method

To a plasma sample of 1 ml, 100 µl of the internal standard solution (containing 0.49 nmole of trimipramine maleate) and 0.2 ml of 2.5 *M* sodium hydroxide solution were added. The sample was extracted with 5 ml of diethyl ether for 40 min at 23° and centrifuged at 1000 *g* for 10 min.

About 4.5 ml of the organic phase were transferred into another tube containing 1 ml of 0.25 *N* sulphuric acid and the tube was shaken for 10 min and centrifuged. The ether layer was aspirated and 0.1 ml of hexane was added. The phases were mixed and the hexane phase was removed after centrifugation. (Instead of the extraction step with hexane the tube can be left uncapped overnight to remove trace amounts of ether.) The aqueous phase was transferred into a narrow 2-ml tube and was made alkaline with 0.2 ml of 2.5 *M* sodium hydroxide solution, then 75 µl of the mobile phase were added. The tube was slowly rotated for 10 min and centrifuged and an aliquot of the upper organic phase (40 µl) was injected into the column.

A standard graph was prepared by analysis according to the above procedure of 1-ml serum samples spiked with chlorimipramine and demethylchlorimipramine. Peak-height ratios with the internal standard were calculated.

Plasma samples

Blood samples were drawn from patients into heparinized plastic tubes immediately before the morning dose and centrifuged. The plasma was transferred into glass vials and stored at −20° until analysed.

The influence of storage time on the two compounds was checked when 295 plasma samples stored for 1—40 months had been analysed. No correlation was found between storage time and plasma level of either Cl (r = 0.08) or DMCl (r = 0.01).

RESULTS AND DISCUSSION

The analytical procedure

The determination of chlorimipramine and demethylchlorimipramine in biological material is preferably effected by ion-pair partition chromatography as described in a preliminary report [12]. The ion pair consists of the protonated compound HA^+ and a chloride ion Cl^- which is partitioned between

the stationary and the mobile phase. The extraction of the cation HA^+ as an ion pair with the counter ion X^- can be quantitatively expressed by the distribution ratio, $D_{HAX} = E_{HAX} \cdot C_X$. The conditional extraction constant E^x is defined as $E^x_{HAX} = C_{HAX org} \cdot (C_{HA} \cdot C_X)^{-1}$.

An increase in the detection sensitivity compared with the preliminary chromatographic system has been obtained by minor modifications. Changing the particle diameter of the support from 37—44 to 10 μm decreased the factor $N^{-\frac{1}{2}}$ and shortening of the column length decreased the ratio $V_m \cdot N^{-\frac{1}{2}}$, which resulted in an increase in the detectability according to the equation [27]

$$A = V_m \cdot (1 + k') \cdot N^{-\frac{1}{2}}$$

where

A = amount of sample giving a certain detector response;

V_m = volume of the mobile phase in the column;

k' = capacity factor;

N = number of theoretical plates.

Owing to tailing of the peaks and higher capacity factors obtained when using Partisil 10, an increase in the solvation of the ion pair in the mobile phase was necessary. The addition of methylene chloride to a mixture of hexane and alcohol results in a smaller separation factor between the amines than does an increase in the alcohol content. On the addition of butanol, the separation of a substance in front of DMCI present in the plasma of patients was achieved (Fig. 1). The identification of this peak as the didemethylated metabolite of chlorimipramine was supported by the following two experiments. Upon analysis of plasma samples by mass fragmentography after trifluoroacetylation, a small peak appears in front of DMCI when focusing the mass spectrometer on the ion of *m/e* 269 (cleavage in the α-position to the nitrogen atom). The relative retention in the liquid chromatographic system is about the same for the proposed didemethylated metabolite and demethylchlorimipramine as for demethylnortriptyline and nortriptyline.

The ratio between k'_f and k'_c was found to be slightly more than unity (Table I), indicating some influence of the support on the partition process in the column. Low values of the capacity factors are necessary in order to achieve high detection sensitivity [27] and a rapid separation (8 min) with a low flow-rate (0.45 ml/min), which improves the column efficiency [28].

The partition coefficients for chlorimipramine and demethylchlorimipramine as the free bases are very high with diethyl ether or hexane—butanol as the organic phase (Table II). The extraction of the amines from plasma still requires a long period, as mentioned previously [22] and later confirmed by Lagerström et al. [23].

Depressed patients are often treated with other drugs, e.g., benzodiazepines and barbiturates. Benzodiazepines were shown not to give any peaks in this system. Barbiturates and other acidic drugs cannot be extracted from an alkaline aqueous phase and therefore will not interfere.

Drugs that have chemical properties similar to those of chlorimipramine, e.g., other tricyclic antidepressants or phenothiazines, can be determined with this method. The separation of some common tricyclic antidepressants

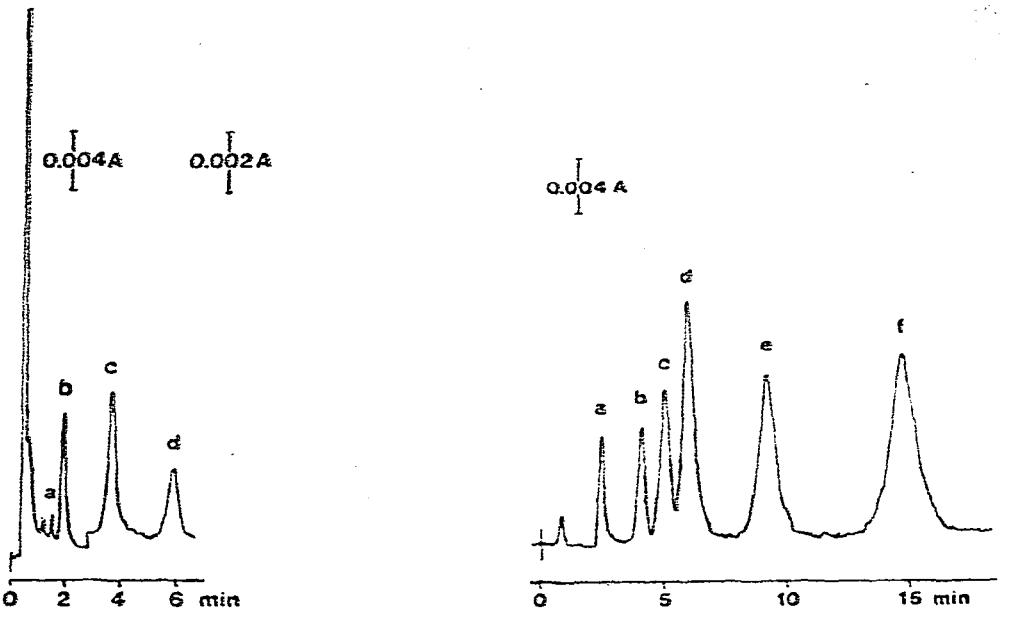

Fig.1. Chromatogram of a plasma sample from a patient receiving chlorimipramine hydrochloride. Peaks: (a) the didemethylated metabolite of chlorimipramine; (b) 456 nmole/l of demethylchlorimipramine; (c) 285 nmole/l of chlorimipramine; and (d) trimipramine (internal standard). The amplification was increased 2-fold after 2.5 min.

Fig.2. Separation of tricyclic antidepressant drugs and their demethylated metabolites. Reference compounds: (a) demethylchlorimipramine; (b) nortriptyline; (c) chlorimipramine; (d) desipramine; (e) amitriptyline; (f) imipramine. Chromatographic system as described under Experimental.

TABLE I

CAPACITY FACTORS AND EFFICIENCY OF THE CHROMATOGRAPHIC SYSTEM

Mobile phase: 1-butanol—*n*-hexane (13:87). Stationary phase: 0.1 *M* hydrochloric acid, containing 0.01 *M* tetrapropylammonium hydrogen sulphate. Support: Partisil 10. Column length: 75 mm. Flow-rate: 0.45 ml/min.

Amine	k'_c*	k'_f	k'_f/k'_c	H (mm)**
Demethylchlorimipramine	1.4	2.0	1.4	0.17
Chlorimipramine	3.0	5.0	1.7	0.09
Trimipramine	7.0	9.3	1.3	0.07

*$k'_c = V_s.(Vm.\Sigma^{x}HAX \cdot C_X)^{-1}$.
**H = theoretical plate height.

TABLE II

PARTITION AND EXTRACTION CONSTANTS

Amine	pK'_{HA}	log $k_{d(A)}$*		log $E^{\pm}$
		diethyl ether	1-butanol—*n*-hexane (13:87)	
Demethylchlorimipramine	10.2**	4.2**	4.3	0.55
Chlorimipramine	9.4**	4.9**	5.1	0.22
Trimipramine	9.5***	5.1	5.2	−0.15

*Calculated from log $k_{d(A)} \cdot K'_{HA}$ determined according to ref. 36.

$K_{d(A)} = \frac{[A]_{org}}{[A]_{aq}}$

K'_{HA} = dissociation constant of the acid.

$E^{\pm}_{HACl} = C_{HACl} \cdot (C_{HA} \cdot C_{Cl})^{-1}$ (conditional extraction constant of the amine as chloride ion pair).

**From ref. 23.

***From ref. 35.

is shown in Fig. 2. The reproducibility of the method was determined from measurements on 16 duplicate plasma samples. The coefficient of variation was 6.5% for CI at the lowest level (70—140 nmole/l) and 7.3% for DMCI (295—445 nmole/l). All other concentration levels gave better reproducibility.

The specificity of this liquid chromatographic method was checked by comparison with an electron-capture gas chromatographic method, based on the conversion of the two amines into trichloroethyl carbamates according to a method for amitriptyline and nortriptyline [12]. The two independent methods gave very similar plasma levels, as shown in Fig. 3.

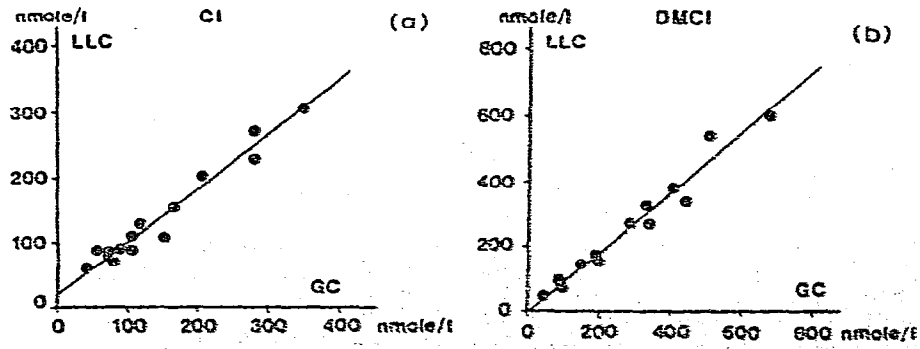

Fig.3. Comparison of results obtained by gas chromatography (abscissa) and liquid chromatography (ordinate) for chlorimipramine (CI) and demethylchlorimipramine (DMCI) in plasma. (a) CI (n= 15, r = 0.98). Line of best fit: $y = 0.83\,x + 16.4$. (b) DMCI (n = 13, r = 0.98). Line of best fit: $y = 0.94\,x - 12.0$.

Determination of plasma levels

When blood was collected in Vacutainer tubes, Cotham and Shand [29] found lower levels of propranolol in plasma compared with blood collected in an all-glass system. This result was explained by the presence of a substance in the rubber stopper that reduced plasma protein binding of the drug. They suggested that this effect might be seen with other highly protein-bound basic drugs that distribute significantly into red blood cells in proportion to the free fraction of the drug. Significantly lower plasma levels of chlorimipramine and demethylchlorimipramine (p = 0.032 and 0.021, respectively) were found when blood from five patients was drawn into Vacutainer tubes compared with blood collected in the usual plastic tubes from the other arm at the same time. The decrease in the mean ± S.D. was 28 ± 13% and 17 ± 10% for the two compounds, respectively. This drug-displacing substance has recently been proposed to be trisbutoxyethyl phosphate [30].

The plasma concentration graph for a patient treated with chlorimipramine (Anafranil, three 50-mg doses per day) for a period of 4 weeks is shown in Fig. 4. The steady-state level of CI was reached within 1 week, but the level of the metabolite had a tendency to increase during the 4-week period. This effect was also seen in some other patients. The plasma concentration of the metabolite was higher than that of the parent drug in all of the patients studied. The concentrations of CI and DMCI during the fourth week of treatment in 34 patients given a dose of 150 mg per day are shown in Fig. 5. The concentration of CI was 248 ± 91 (S.D.) nmole/l with a range of 63—900 nmole/l, which is a 14-fold variation, and the level of DMCI was 561 ± 288 (S.D.) nmole/l in the range 116—1178 nmole/l, a 10-fold variation. A large inter-

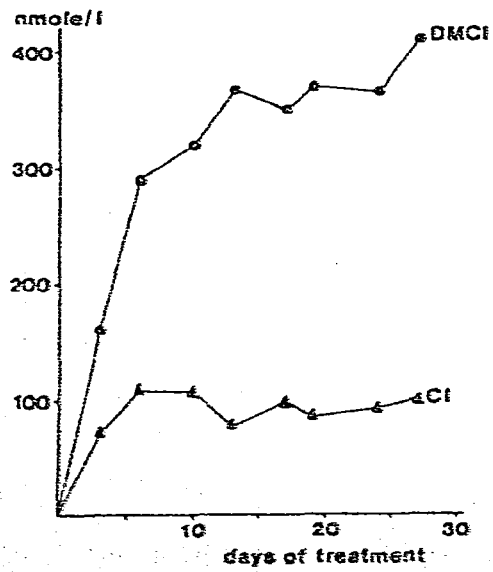

Fig.4. Plasma levels of chlorimipramine (CI) and demethylchlorimipramine (DMCI) during 4 weeks in a patient receiving 50 mg of CI·HCl three times a day.

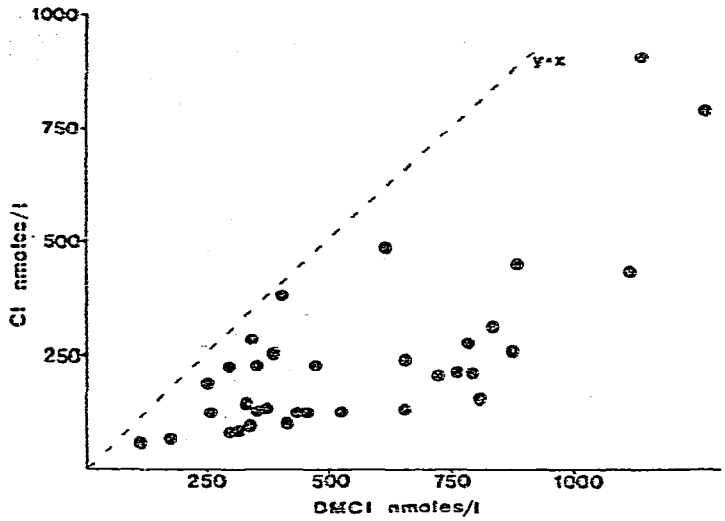

Fig.5. Fourth-week plasma levels of chlorimipramine (Cl) and demethylchlorimipramine (DMCl) in 34 patients receiving 50 mg Cl-HCl three times a day.

individual variation in plasma levels has been shown for other tricyclic antidepressant drugs: nortriptyline [31], amitriptyline [32], desipramine [33] and imipramine [34]. However, the ratio between metabolite and parent drug is always greater than unity (2.7 ± 1.1 (S.D.), range 1.05—5.24) for chlorimipramine, which differs from amitriptyline (1.0 ± 0.3 (S.D.), range 0.58—1.65) [32] and imipramine (3.4 ± 4.3 (S.D.), range 0.25—18.91) [34].

The relationship between the levels of Cl and DMCl presented in this paper and the clinical effect of chlorimipramine in depressed patients is now under evaluation.

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