Journal of Chromatography, 381 (1986) 315—322
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3211

ISOTHERMAL GAS CHROMATOGRAPHIC DETERMINATION OF NANOGRAM AMOUNTS OF CHLORIMIPRAMINE, CHLORPROMAZINE AND THEIR N-DESMETHYL METABOLITES IN PLASMA USING NITROGEN-SELECTIVE DETECTION

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(First received December 11th, 1985; revised manuscript received April 18th, 1986)

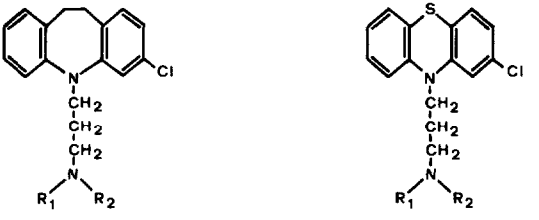
SUMMARY

A gas chromatographic method using nitrogen-selective detection for the quantitative determination of nanogram amounts of chlorimipramine, chlorpromazine and their nor_1 and nor_2 derivatives in plasma is described. Derivatization with trifluoroacetic anhydride of nor_1 and nor_2 metabolites allowed the chromatographic separation of these compounds. A three-step solvent extraction procedure was performed using *n*-heptane containing 1% isoamyl alcohol and *n*-hexane and compared with a plasma clean-up procedure using C_{18} Sep-Pak cartridges. The two procedures were characterized by similar degrees of precision. The use of C_{18} Sep-Pak cartridges, however, produced a significant time and material saving over the conventional extraction method.

0378-4347/86/$03.50

INTRODUCTION

N-Dealkylation of tricyclic psychotropic compounds represents an important metabolic pathway in mammals. The formation of N-desmethyl metabolites has been shown to play an important role in overall therapeutic efficacy of antidepressant drugs. In depressed patients chronically medicated with chlorimipramine (CI), not only were plasma levels of desmethyl-CI much higher than those of the parent drug but they were also found to be related to the clinical response [1]. In psychotic patients under chlorpromazine (CPZ) medication, appreciable amounts of N-desmethyl-CPZ have been evidenced in plasma [2, 3]; however, their role in the therapeutic efficacy of CPZ remains to be established. The analytical methods employed in the above-mentioned studies, however, could only measure the sum of nor_1 and nor_2 metabolites, thus giving no information on the relative role played independently by each one of the nor metabolites of both CI and CPZ. Recently, in order to perform a study in man to investigate N-dealkylation of both drugs [4], the following gas chromatographic (GC) assay method was developed, which enabled plasma levels of parent drugs and their nor_1 and nor_2 derivatives (Fig. 1) to be estimated in nanogram amounts.



	R_1	R_2		R_1	R_2
CI	CH_3	CH_3	CPZ	CH_3	CH_3
CI-nor_1	H	CH_3	CPZ-nor_1	H	CH_3
CI-nor_2	H	H	CPZ-nor_2	H	H

Fig. 1. Chemical structures of chlorimipramine (CI), chlorpromazine (CPZ) and their nor_1 and nor_2 metabolites.

EXPERIMENTAL

Reagents and materials

All reagents were of analytical grade and used without further purification. Chemicals were from Merck (Darmstadt, F.R.G.) except for: methanol (Aristar grade) from BDH (Poole, U.K.); trifluoroacetic anhydride (TFAA) from Pierce Eurochemie (Rotterdam, The Netherlands); dichlorodimethylsilane (Silyl 8) from Pierce (Rockford, IL, U.S.A.); CI · HCl, CI-nor_1 · HCl and CI-nor_2 · HCl from Ciba Geigy (Milan, Italy); protriptyline · HCl (PRT) from Lepetit (Milan, Italy); CPZ · HCl and promazine · HCl (PZ) from Arcispedale S.M.N. (Florence, Italy); CPZ-nor_1 · HCl, CPZ-nor_2 · HCl and CPZ-sulphoxide · HCl (CPZ-SO) from NIMH (Rockville, MD, U.S.A.). C_{18} Sep-Pak cartridges were purchased from

Waters Assoc. (Milford, MA, U.S.A.). Stock solutions of CI · HCl, CPZ · HCl and their respective nor_1 and nor_2 derivatives were prepared in methanol at a concentration of 100 ng μl^{-1}. The concentration of PRT, used as internal standard for the assay of either CI and nor metabolites or CPZ and nor metabolites, was 50 ng μl^{-1}. Stock solutions were stored in the dark at −20°C in silylated screw-capped glass tubes. Appropriate dilutions of the standard solutions were added to human blood plasma.

Glassware

The glassware was cleaned with dichromate—sulphuric acid, rinsed with distilled water and dried. It was then silylated with 5% Silyl 8 in toluene and rinsed with toluene.

Apparatus

A Sigma 3B gas chromatograph (Perkin Elmer, New Haven, CO, U.S.A.) equipped with a nitrogen—phosphorus-sensitive detector and a Sigma 15 data station was used. The glass columns (2000 × 2 mm I.D.) were packed with 3% OV-17 on 100-120 mesh Chromosorb W HP or with 3% SP-2250 on 80—100 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.) and conditioned overnight at 315°C. The columns were then silylated by injecting 20 μl of Silyl 8 reagent. Purified nitrogen was used as carrier gas at a flow-rate of 28 and 22 ml min^{-1} for the assay of CI/metabolites and CPZ/metabolites, respectively. Air and hydrogen flow-rates were 100 and 2.5 ml min^{-1}, respectively. The injector and detector temperatures were set at 300°C, and the column temperature at 265 and 275°C for the assay of CI/metabolites and CPZ/metabolites, respectively.

Sample preparation

Solvent extraction procedure. This consisted in a modification of the method described by Hucker and Stauffer [5]. A 2-ml volume of plasma and 50 μl of methanol containing the internal standard PRT were combined in a 45-ml glass-stoppered centrifuge tube and made alkaline with 1 ml of 0.5 *M* sodium hydroxide. After adding 15 ml of *n*-heptane containing 1% isoamyl alcohol, the mixture was extracted for 15 min on a mechanical shaker and centrifuged at 1000 g for 5 min. The organic layer was then transferred to a second 45-ml glass-stoppered tube and mixed with 5 ml of 0.2 *M* hydrochloric acid. The mixture was shaken for 15 min and centrifuged as above. The organic layer was discarded by aspiration and most of the aqueous phase was transferred to a 25-ml glass-stoppered centrifuge tube and made alkaline with 3 ml of 0.5 *M* sodium hydroxide. A 6-ml volume of redistilled *n*-hexane was added and the tube shaken for 15 min. After centrifugation, as much as possible of the hexane layer was transferred into a fine-pointed 10-ml screw-capped centrifuge tube. The hexane was evaporated at 60°C under a gentle stream of nitrogen. The extract residue was subsequently derivatized with TFAA.

C_{18} Sep-Pak clean-up procedure. C_{18} Sep-Pak cartridges were activated with 2 ml of methanol followed by 2 ml of distilled water at a flow-rate of 5 ml min^{-1}, as already described by Narasimhachari [6]. A 50-μl volume of methanol containing the internal standard PRT and a 0.5-ml volume of 0.1 *M*

sodium carbonate—bicarbonate buffer (pH 9.8) were added to 2 ml of plasma. The mixture was vortexed and passed through the cartridges. The cartridges were then washed with 0.5 ml of distilled water and the compounds were subsequently eluted with 10 ml of a mixture of organic solvents. A mixture of methanol—*n*-hexane (5:95) was used for the assay of CPZ/metabolites and one of acetone—*n*-hexane (30:70) for the assay of CI/metabolites. The organic layer was transferred to a centrifuge tube and evaporated to dryness at 60°C under nitrogen. The extract residue was then derivatized with TFAA.

Derivatization procedure

The residues obtained through either of the sample preparation procedures were dissolved in 0.5 ml of toluene, 10 μl of pyridine, 25 μl of TFAA and vortexed for 30 s. The reaction mixture was added to 1 ml of 0.1 *M* sodium phosphate buffer (pH 6.2), vortexed again and centrifuged for 5 min at 1000 *g*. The organic layer was then transferred to a fine-pointed 10-ml screw-capped centrifuge tube and evaporated to dryness under a stream of nitrogen. The extract residue was dissolved in 25 μl of methanol. Aliquots of 1—4 μl of this solution were gas chromatographed under the conditions described above.

Quantitative analysis

Standard calibration curves were constructed by adding to 2-ml plasma samples 50 μl of methanol containing different amounts of standard drug/metabolite in the range 2—500 ng ml^{-1} and a fixed amount of the internal standard PRT. The samples were processed as described above. The peak-area ratio of each standard drug/metabolite to the internal standard were plotted against quantities of standard amounts of drug/metabolite. A regression analysis of the peak-area ratios obtained was performed to determine the linearity of the response with respect to concentration [7]. The resulting linear regressions were used to evaluate the precision of the estimation of various drug/metabolite concentrations. The recoveries of the two procedures for CI, CPZ and their metabolites were determined at different plasma concentrations by comparing the peak areas of the compounds obtained from extracted plasma samples with those obtained from the injection of organic standard solutions. A similar procedure was used to evaluate the recovery of the internal standard.

RESULTS AND DISCUSSION

A number of methods using GC [8], thin-layer chromatography [9], high-performance liquid chromatography [10—12] or GC—mass spectrometry [13, 14] are available from the literature for the assay of CI or CPZ and of the sum of their respective nor_1 and nor_2 metabolites. To our knowledge, only a few methods have been developed for the separation and quantitation of CPZ-nor_1 and CPZ-nor_2 [3, 15], but none is available for both series of compounds. Among some derivatizing agents tested (heptafluorobutyric anhydride, pentafluoropropionic anhydride and TFAA), TFAA was selected because derivatization was quantitative, fast and applicable to the nor metabolites of both CI and CPZ. Using the GC conditions as outlined in the

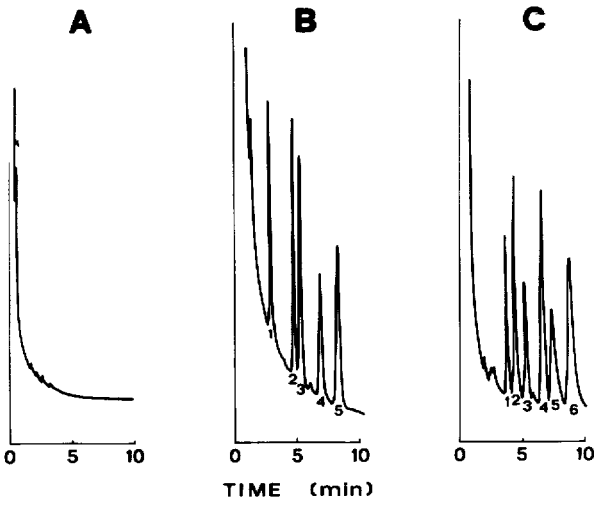


Fig. 2. Chromatograms of blank plasma (A) and of samples containing a mixture of drug/metabolites and internal standards (B and C). (B) Samples containing 10 ng of each of the following compounds: amitriptyline (peak 1); chlorimipramine (peak 2); protriptyline (peak 3); chlorimipramine-nor$_2$ (peak 4); chlorimipramine-nor$_1$ (peak 5). (C) Samples containing 10 ng of each of the following compounds: promazine (peak 1); protriptyline (peak 2); chlorpromazine (peak 3); chlorimipramine-nor$_1$ (peak 4); chlorpromazine-nor$_2$ (peak 5); chlorpromazine-nor$_1$ (peak 6).

Experimental section, the mono- and di-desmethyl derivatives of both CI and CPZ could be separated from their respective parent drugs and the internal standard. Plasma samples processed by either of the two methods, the solvent extraction or the C_{18} Sep-Pak cartridge clean-up procedure, showed clean chromatograms free of interfering peaks. Typical chromatograms of CI/metabolites, CPZ/metabolites, PRT, amitriptyline (AMI) and PZ are shown in Fig. 2.

TABLE I

RANGE OF SLOPES AND AVERAGE STANDARD DEVIATION OF EACH SLOPE FOR STANDARD CURVES OBTAINED ON DIFFERENT DAYS FROM SIX TO EIGHT KNOWN SAMPLES EACH DAY USING SOLVENT EXTRACTION PROCEDURE

Compound	Number of standard curves	Concentration range (ng ml^{-1})	Slope of regression lines		C.V.** (%)
			Range (× 10^2)	Average S.D.* (× 10^2)	
Chlorimipramine	4	6.5—500	3.31—5.15	0.19	3— 6
Chlorimipramine-nor$_1$	3	6.5—500	3.46—5.11	0.30	6— 9
Chlorimipramine-nor$_2$	3	6.5—500	1.62—2.80	0.20	7—12
Chlorpromazine	4	12.5—200	1.20—2.04	0.10	5—8
Chlorpromazine-nor$_1$	3	12.5—200	2.88—3.30	0.05	1—2
Chlorpromazine-nor$_2$	3	12.5—200	1.56—2.06	0.07	3—5

*Calculated by the formula $\sqrt{\Sigma_{i=1}^{n} s_i^2/n}$, were s_i is the standard deviation of the slope of the regression line for the ith standard curve, and n is the number of standard curves.
**Coefficient of variation of slopes.

Standard curves used for quantitation exhibited a good linearity over the concentration ranges employed and the intersections with the y-axis were not significantly different from the origin. Ranges of slopes and average standard deviations for standard curves obtained on different days from four to eight known samples each day, using the solvent extraction and the clean-up

TABLE II

RANGE OF SLOPES AND AVERAGE STANDARD DEVIATION OF EACH SLOPE FOR STANDARD CURVES OBTAINED ON DIFFERENT DAYS FROM FOUR TO FIVE KNOWN SAMPLES EACH DAY USING C_{18} SEP-PAK CLEAN-UP METHOD

Compound	Number of standard curves	Concentration range (ng ml^{-1})	Slope of regression lines		C.V.** (%)
			Range ($\times 10^2$)	Average S.D.* ($\times 10^2$)	
Chlorimipramine	7	5—300	4.0—6.7	0.47	7 —12
Chlorimipramine-nor$_1$	7	5—300	1.5—6.4	0.30	5 —20
Chlorimipramine-nor$_2$	5	2—300	0.8—4.0	0.12	3 —15
Chlorpromazine	5	5—200	0.8—1.6	0.05	3 — 6
Chlorpromazine-nor$_1$	4	10—200	0.4—2.5	0 01	0 4— 2
Chlorpromazine-nor$_2$	3	5—200	2.6—9.3	0.46	5 —18

*Calculated as reported in Table II. Elution mixtures were: acetone—n-hexane (30:70) for chlorimipramine and metabolites; methanol—n-hexane (5:95) for chlorpromazine and metabolites.
**Coefficient of variation of slopes.

TABLE III

RECOVERY AND PRECISION OF SOLVENT EXTRACTION PROCEDURE FOR CHLORIMIPRAMINE, CHLORPROMAZINE AND THEIR METABOLITES

Figures in parentheses represent 95% confidence interval of the recovery. n = 15

Compound	Recovery (%)		
	25 ng ml^{-1}	65 ng ml^{-1}	400 ng ml^{-1}
Chlorimipramine	78.1 (58.6—98.3)		63.3 (58.3—68.7)
Protriptyline	61.5 (46.3—80.0)		66.9 (60.4—74.2
Chlorimipramine-nor$_2$	57.8 (42.0—75.8)		49.3 (43.7—55.2)
Chlorimipramine-nor$_1$	70.1 (52.9—87.9)		58.1 (52.8—63.8)
Promazine	82.0 (74.2—90.7)	69.4 (55.2—84.9)	56.6 (46.2—70.3)
Chlorpromazine	65.1 (53.1—77.6)	70.7 (61.3—80.6)	68.5 (56.7—84.3)
Chlorpromazine-nor$_2$	39.3 (29.7—49.7)	64.6 (50.5—80.4)	45.9 (33.8—60.7)
Chlorpromazine-nor$_1$	68.8 (54.5—84.3)	81.2 (70.5—92.9)	64.9 (54.9—67.1)

procedure, are shown in Tables I and II, respectively. The extraction efficiency of the solvent method is reported in Table III. Mean recoveries of CI, CI-nor$_1$, CI-nor$_2$ and the internal standard PRT ranged between 49.3 and 78.1%. Mean recoveries of CPZ and metabolites ranged between 39.3 and 81.2%. The extraction efficiency of the C_{18} Sep-Pak cartridges was studied using different eluting systems. The presence of a polar solvent in the system was crucial for the recovery of the nor derivatives. However, an excess of it was shown to produce interfering peaks, which compromised the GC analysis. Among several polar solvents tested (acetone, methanol, 2-propanol and dichloromethane) in combination with *n*-hexane, methanol and acetone (see Experimental) proved to be the most efficient ones. The recoveries of the analysed compounds processed through the C_{18} Sep-Pak clean-up procedure are reported in Table IV. The extraction efficiency of this method was better or at least equal to that of the solvent extraction procedure for all compounds except for the nor$_2$ derivatives of CI and CPZ. The precision of both procedures was studied over a concentration range of 2—300 ng ml^{-1}. The results reported in Table V indicate that both procedures were characterized by an overall similarity in precision. However, the use of C_{18} Sep-Pak cartridges resulted in significant time and material saving over the conventional solvent extraction method.

In the present study, the analysis of CPZ-SO, a metabolite of CPZ formed either in experimental animals or in man, has not been taken into account. Recently, however, its decomposition during GC analysis, which gives rise to

TABLE IV

RECOVERY AND PRECISION OF SEP-PAK CLEAN-UP PROCEDURE FOR CHLORIMIPRAMINE, CHLORPROMAZINE AND THEIR METABOLITES WITH USE OF DIFFERENT ELUTION MIXTURES

Figures in parentheses represent 95% confidence interval of the recovery. Elution mixtures: I = methanol—*n*-hexane (5:95), n = 8; II = acetone—*n*-hexane (10:90), n = 11; III = acetone—*n*-hexane (30:70), n = 4—8. N.S. = not significant

Compound	Recovery of 400 ng ml^{-1} (%)		
	I	II	III
Chlorimipramine	67.0 (59.8—74.4)		73.2 (65.0—81.7)
Protriptyline	51.7 (46.0—57.4)	56.0 (N.S.)	56.0 (48.3—63.7)
Chlorimipramine-nor$_2$	35.9 (27.0—44.5)		39.5 (34.5—44.3)
Chlorimipramine-nor$_1$	61.8 (57.1—66.4)		69.2 (61.1—77.5)
Promazine	83.5 (78.5—88.9)	70.3 (58.9—79.4)	29.3 (N.S.)
Chlorpromazine	80.6 (72.0—90.5)	83.0 (71.9—94.2)	58.7 (30.1—92.6)
Chlorpromazine-nor$_2$	27.2 (23.4—31.1)	33.7 (27.6—40.5)	31.0 (15.8—48.4)
Chlorpromazine-nor$_1$	54.5 (49.8—59.2)	35.3 (30.0—40.6)	53.1 (29.2—79.6)

TABLE V

PRECISION OF THE SOLVENT EXTRACTION PROCEDURE (A) AND CLEAN-UP PROCEDURE (B) FROM REPLICATE ANALYSES OF BLOOD BANK PLASMA SAMPLES CONTAINING KNOWN AMOUNTS OF CHLORIMIPRAMINE, CHLORPROMAZINE AND THEIR NOR METABOLITES

Compound	Nominal concentration (ng ml^{-1})		Mean concentration found (ng ml^{-1})		C V (%)		n	
	A	B	A	B	A	B	A	B
Chlorimipramine	2—100	5—300	2 4— 96 8	5 3—300 8	6 9—25 0	5 0—36 0	4— 6	3— 9
Chlorimipramine-nor$_1$	2—100	5—300	2 4—102 7	5 4—297 8	6 4—15 8	12 6—29 1	3— 4	3—10
Chlorimipramine-nor$_2$	2—100	5—300	2 5—101 5	6 3—303	10 0—44 2	7 5—24 9	3— 4	3— 5
Chlorpromazine	12—100	10—150	12 7—109 1	12 9—151 9	12 2—16 7	3 6— 9 5	8—12	3— 6
Chlorpromazine-nor$_1$	12—100	10—150	12 1—102 1	13 1—150 2	6 3—22 5	1 6—28 6	7—11	3— 4
Chlorpromazine-nor$_2$	12—100	25—150	11 8—100 7	26 5—153 1	11 3—19 0	5 0—12	7—10	3

the formation of CPZ, has been demonstrated [16]. Experiments performed in this laboratory have shown that the conversion of CPZ-SO into CPZ averaged 4.8 ± 0.5% (mean ± S.D.; n = 8) and 6.4 ± 0.5% (n = 12) when the injection port temperature was maintained at 270 and 300°C, respectively. This conversion did not seem to occur during either of the plasma preparation procedures.

In conclusion, this method has been proven to be sufficiently sensitive, accurate and reliable for the determination in plasma of nanogram amounts of CI, CPZ and their respective nor$_1$ and nor$_2$ metabolites. Furthermore, this method could be extended to the determination of some of the many other metabolites formed by both CI and CPZ in mammals. By using this method, in fact, we could ascertain very recently that PZ was a major plasma metabolite of CPZ in a population of chronic schizophrenics [17].

ACKNOWLEDGEMENTS

We acknowledge the financial support for this work given by M.P.I. (40% + 60%) and C.N.R. (Contr. 84.00774.04 and 85.00545.04), Rome, Italy.

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