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DETERMINATION OF CLOMIPRAMINE OR IMIPRAMINE AND THEIR MONO-DEMETHYLATED METABOLITES IN HUMAN BLOOD OR PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method for the quantitative assay of clomipramine or imipramine and their mono-demethylated metabolites in human blood or plasma is described. After addition of the internal standards, imipramine for clomipramine, desipramine for desmethylclomipramine and conversely, the compounds are extracted from blood or plasma at pH 10 into heptane containing 1% isoamyl alcohol; they are then back-extracted into an acidic aqueous phase and re-extracted at a basic pH into heptane. After evaporation, the residue is dissolved in 300 μl of mobile phase and 150 μl is injected.

Both drugs and their mono-demethylated metabolites are well separated from the blood or plasma components and the other metabolites on a silica gel column using ethanol–hexane–dichloromethane–diethylamine ($30:62:8:5 \cdot 10^{-3}$) as the mobile phase at a flow-rate of 1.5 ml/min. The limit of sensitivity is 5 ng/ml for clomipramine and imipramine and 10 ng/ml for the corresponding mono-demethylated metabolites.

This method has been used to analyse plasma from subjects given therapeutic doses of clomipramine or imipramine.

INTRODUCTION

Several liquid chromatographic procedures have been described for the analysis of the tricyclic antidepressant drugs clomipramine (CMI) or imipramine (IMI) and their respective demethylated metabolites (Fig. 1), desmethylclomipramine (DCMI) and desipramine (DMI)[1–27].

A few of these methods have been applied only to pure solutions[1–5]. Those described for the assay in biological materials involved either ion-pair partition chromatography[6–11], adsorption[12–22] or reversed-phase chromatography[23–27]. The sensitivity ranged from 1 ng/ml with fluorimetric detection[16,25] to 5–20 ng/ml with UV detection[6–10,12,13,17,18,23,24].

We tried to reproduce the method described by Westenberg and co-workers[12,13] for CMI and DCMI in plasma because it was reported as being "selective, rapid and simple, and especially suitable for routine analysis"[12]. It requires a single-step extrac-

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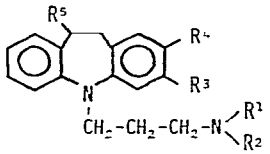

Compound	R^1	R^2	R^3	R^4	R^5
Clomipramine[1] (CMI)	CH_3	CH_3	Cl	H	H
Desmethyl-clomipramine (DCMI)	CH_3	H	Cl	H	H
Bis-desmethyl-clomipramine	H	H	Cl	H	H
Imipramine[2] (IMI)	CH_3	CH_3	H	H	H
Desipramine[3] (DMI)	CH_3	H	H	H	H
2-hydroxy-imipramine (2-OH-IMI)	CH_3	CH_3	H	OH	H
2-hydroxy-desipramine (2-OH-DMI)	CH_3	H	H	OH	H
10-hydroxy-imipramine (10-OH-IMI)	CH_3	CH_3	H	H	OH
10-hydroxy-desipramine (10-OH-DMI)	CH_3	H	H	H	OH
Bis-desmethyl-imipramine (DDMI)	H	H	H	H	H

1 Anafranil® Ciba-Geigy.
2 Tofranil® Ciba-Geigy.
3 Pertofran® Ciba-Geigy.

Fig. 1. Clomipramine, imipramine and their metabolites.

tion before injection, and IMI is used as internal standard for *both* CMI and DCMI. We applied this method using chromatographic conditions very similar to those described and the reported extraction procedure, but were not able to obtain acceptable and reproducible blank plasma extracts. An interfering peak having the same retention time as CMI was observed with eight different samples of blank human plasma; its height was subject-dependent. This interfering compound could be removed only by using a three-step extraction procedure.

This paper describes an high-performance liquid chromatographic (HPLC) method for the simultaneous determination of CMI and DCMI or IMI and DMI in blood and plasma with new extraction conditions. As already suggested by Gupta and Molnar[28], two internal standards were used to improve the reproducibility: IMI for CMI, DMI for DCMI and conversely. This method was applied to plasma samples from patients given repeated doses of clomipramine and from healthy subjects given a single oral dose of imipramine.

EXPERIMENTAL

Chemicals

CMI, IMI and their metabolites (Fig. 1) were supplied by Ciba-Geigy (Basle, Switzerland). Dichloromethane, *n*-heptane, isoamyl alcohol, pH 10 buffer (Titrisol) and 5-μm LiChrosorb Si 60 were purchased from E. Merck (Darmstadt, G.F.R.), ethanol from Prolabo (Paris, France), diethylamine from Fluka (Buchs, Switzerland)

and hexane (Spectrosol quality) from S.D.S. (Valdonne, France). Internal standards and calibration solutions were prepared in 0.01 *N* hydrochloric acid from the hydrochlorides.

Chromatography

The chromatography was performed on a Hewlett-Packard Model 1084 B instrument, equipped with a fixed-wavelength detector (254 nm) and a variable-volume injector. The column was a stainless-steel tube (12.5 cm × 4.6 mm) filled with 5-μm LiChrosorb Si 60 (Merck 9388) using the balanced-density slurry packing technique. The slurry, made of 1.8 g 5-μm LiChrosorb Si 60 dispersed in 7.8 ml carbon tetrachloride, was forced into the column with methanol. The degassed mobile phase, ethanol–hexane–dichloromethane–diethylamine ($30:62:8:5\cdot10^{-3}$), was used at a flow-rate of 1.5 ml/min.

With a new column, the retention times were 3.3, 4.1, 11.3 and 15.3 min for CMI, IMI, DCMI and DMI, respectively. The maximum pressure was about 25 bar. After about 800 injections, the pressure increased to 40 bar and the retention times decreased to 2.3, 2.8, 7.5 and 10.1 min, respectively. The mobile phase and the column were at room temperature.

Sample preparation

To 1 ml plasma or blood in a 10-ml glass tube were added 100 μl internal standard solution (added amount: CMI, 60 ng; DCMI, 150 ng; IMI, 35 ng; DMI, 100 ng), 1 ml buffer (0.2 *M* boric acid, 0.2 *M* potassium chloride, 0.176 *M* sodium hydroxide), pH 10 and 5 ml *n*-heptane–isoamyl alcohol (99:1). The stoppered tube was shaken mechanically for 5 min at 3000 rpm (Infors shaker) and centrifuged at 1400 *g* for 10 min. The *n*-heptane–isoamyl alcohol phase was transferred with a Pasteur pipette into another tube and 1 ml 0.1 *N* H_2SO_4 was added. The tube was shaken for 5 min and centrifuged for 3 min. It was then dipped into a mixture of methanol and solid CO_2 to freeze the aqueous phase, and the organic phase was discarded.

The acidic aqueous phase was left to thaw at room temperature. Then 0.5 ml 1 *N* NaOH and 5 ml *n*-heptane–isoamyl alcohol were added. The tube was shaken for 5 min and centrifuged for 3 min. The *n*-heptane–isoamyl alcohol phase was transferred into a conical glass tube and evaporated to dryness under nitrogen at 60°C. The residue was dissolved in 300 μl of mobile phase.

This solution must be prepared just before injection. After mixing for 20 sec on a Vortex mixer, 150 μl were injected.

Calibration curves

Samples were prepared by adding 100 μl of reference solutions of both compounds, CMI and DCMI or IMI and DMI, to 1 ml of blood or plasma. These samples were then worked up according to the procedure described above. The calibration curves were obtained by plotting either the peak area ratio for CMI and IMI or the peak height ratio for DCMI and DMI against concentration.

Acidic solutions of both the internal standards and the reference compounds have to be used to minimize adsorption on glass or plastic surfaces, which occurs with solutions in distilled water[29].

RESULTS AND DISCUSSION

Plasma interference

When a single-step extraction (heptane + buffer pH 10) as indicated by Westenberg *et al.*[12] was used, an interfering peak having the same retention time as CMI was observed with eight different samples of blank human plasma; its height was subject-dependent. This interfering compound could be removed only by a three-step extraction procedure: extraction into heptane at a basic pH, back-extraction into an acidic aqueous phase and re-extraction into heptane at a basic pH.

The chromatograms corresponding to the extract of a 1-ml sample of blank human plasma or of plasma spiked with CMI, IMI, DCMI and DMI are shown in Fig. 2. Similar chromatograms are obtained with blank human blood.

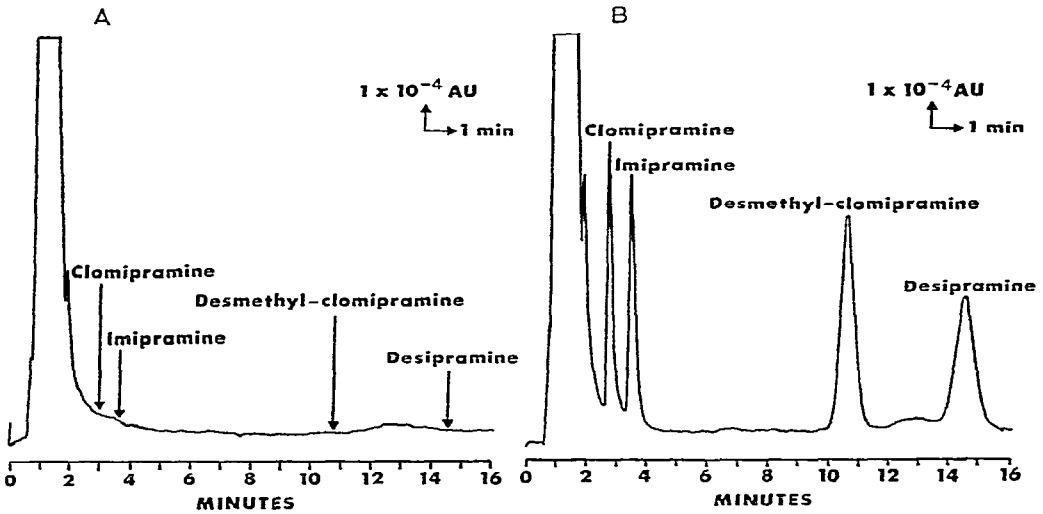

Fig. 2. Chromatograms corresponding to the extract of 1 ml of blank plasma (A), and 1 ml of plasma spiked with 30 ng of IMI, 60 ng of DMI, 35 ng of CMI and 100 ng of DCMI (B).

Choice of internal standard

In most of the published methods, a single internal standard, either a tertiary or a secondary amine, was used for the estimation of both tertiary (CMI and IMI) and secondary (DCMI and DMI) bases: protriptyline[14,18,21], DCMI[6,11,16], DMI[6], CMI[6,15], IMI[12,13], trimipramine[7,8,25], maprotiline[24], promazine[15,22], β-naphthylamine[10] and mesopridazine[26].

Using IMI as internal standard for DCMI, we were unable to obtain a good linear relationship between the peak height ratio, DCMI/IMI, and the concentration of DCMI, and the results were not reproducible. The use of a secondary amine (DMI) as internal standard for DCMI gave more reliable results. The losses of CMI and DCMI during the analytical procedure were well compensated by IMI and DMI, respectively.

Linearity

The calibration curves were established as described above. Their equations were calculated by least-squares linear regression. A good linear relationship was obtained in the range of 10–400 ng/ml for DCMI and DMI, and in the ranges of 5–50 and 50–400 ng/ml for CMI and IMI.

For routine analysis, a calibration curve is established every week, and test samples are analysed during the week to validate the method.

Accuracy, precision and sensitivity

The results summarized in Tables I and II show that concentrations down to 5 ng of CMI or IMI can be accurately and precisely determined in 1 ml of plasma by the present method. This corresponds to a peak height ranging from 0.4 to 1 cm. The sensitivity limit in blood is 10 ng/ml for CMI and 5 ng/ml for IMI. For DCMI and DMI, the sensitivity limit in blood or plasma is 10 ng/ml (Tables I and II): this corresponds to a peak height ranging from 0.4 to 1.5 cm.

Lower concentrations can be estimated, but the accuracy is poorer (Table I).

TABLE I

ACCURACY, PRECISION AND WITHIN-DAY REPRODUCIBILITY FOR CMI AND DCMI (SPIKED HUMAN PLASMA SAMPLES)

Compound	*CMI*				*DCMI*			
Amount added (ng/ml)	2.5	5	50	100	10	15	100	300
Amount found (ng/ml)	3.9	5.4	50.5	98.5	9.9	14.0	106	299
(*n* replicates)	(6)	(6)	(10)	(6)	(6)	(6)	(6)	(6)
Coefficient of variation (%)	9.5	9.5	6.1	3.0	16.0	9.5	4.2	5.4
Mean recovery (%)	158	108	101	98.5	99	93	106	99.8

TABLE II

ACCURACY, PRECISION AND WITHIN-DAY REPRODUCIBILITY FOR IMI AND DMI (SPIKED HUMAN PLASMA SAMPLES)

Compound	*IMI*				*DMI*			
Amount added (ng/ml)	5	10	30	200	5	10	150	400
Amount found (ng/ml)	5.1	9.7	29.4	203	4.2	9.6	157	392
(*n* replicates)	(10)	(9)	(7)	(7)	(12)	(12)	(12)	(12)
Coefficient of variation (%)	6.6	2.7	2.1	3.3	11.0	7.3	2.8	2.9
Mean recovery (%)	102	97	98	102	84	96	104	98

Specificity

Clomipramine. N-Demethylation is a main pathway of biotransformation of clomipramine in man[30,31]. Hydroxylation occurs at various positions of the molecule, but the hydroxylated metabolites, eliminated as glucuronides or sulphates in urine, are only present in small amounts in the plasma.

As bis-desmethylclomipramine does not interfere in the assay of CMI and DCMI, the described method can be considered specific.

Imipramine. N-Demethylation and hydroxylation at two positions of the molecule are the main pathways of biotransformation of imipramine[32]. Desipramine is a therapeutically active metabolite, but recently the 2-hydroxy metabolites of both IMI and DMI have been shown to be biologically active[16,33–35].

As all the hydroxylated and demethylated metabolites are well separated from the parent drug and the internal standards under the chromatographic conditions described in the present method (see Fig. 3), their determination together with the parent drug was investigated with CMI as internal standard for IMI, 2-OH-IMI and 10-OH-IMI, and DCMI as internal standard for DMI and DDMI; the hydroxylated metabolites of desipramine, 2-OH-DMI and 10-OH-DMI, which were eluted as broadened peaks, were not considered.

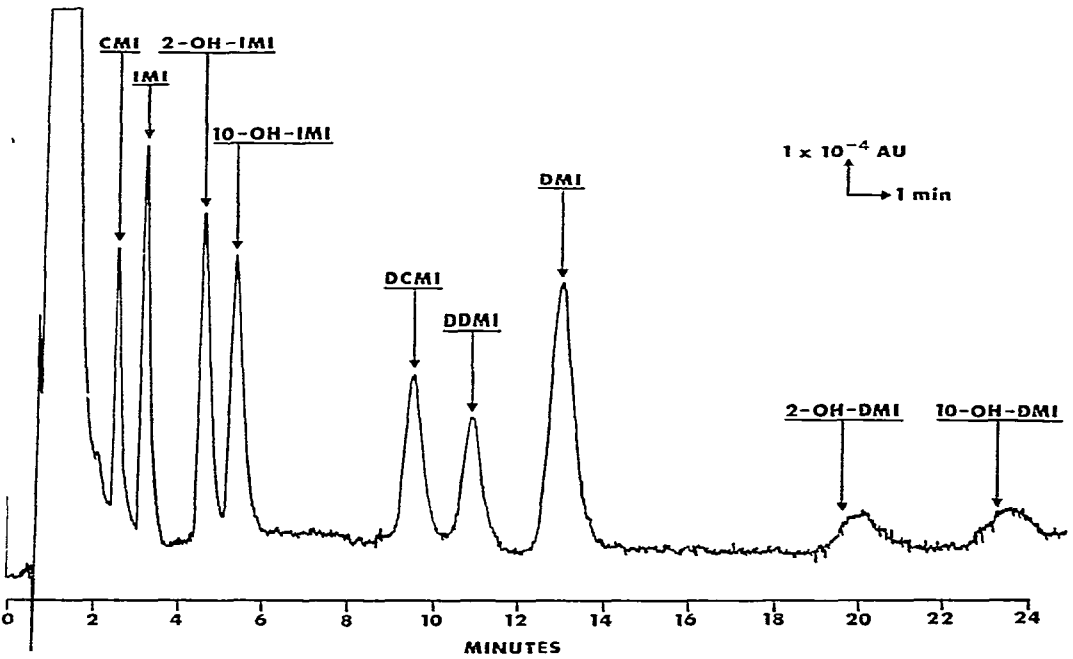

Fig. 3. Separation of CMI, IMI and their metabolites. Spiked calf plasma sample extracted with dichloromethane–diethyl ether (1:2).

When heptane with isoamyl alcohol was used as extraction solvent, 2-OH-IMI was not extractable from plasma. The solvent mixtures *n*-butanol–heptane (20:80) or dichloromethane–diethyl ether (40:60) increased the extraction yield, but resulted in an impure extract of blank human plasma: an interfering peak having the same retention time as that of CMI was observed. All attempts to eliminate this interfering compound were unsuccessful.

It is likely that 10-OH-IMI could be assayed with the present extraction and chromatographic conditions, but this remains to be verified. DDMI was extracted into heptane with good yield, but could not be accurately assayed because the reproducibility of the DDMI/DCMI ratio was poor. Only imipramine and desipramine can be precisely and specifically assayed by the present HPLC method.

Stability

As mentioned previously, the dry residue must be dissolved in 300 μl of the mobile phase just before injection to ensure good reproducibility. However, the dry evaporation residue remains stable for 5 days when stored in the refrigerator.

Application

Steady-state plasma levels of CMI and DCMI. The present method was used to determine the steady-state plasma levels of CMI and DCMI reached in patients given daily 75–150-mg doses of clomipramine. Blood samples were taken in the morning on days 26 and 27 of treatment, just before the first daily administration. Plasma was separated and stored at −20°C until analysis. The results summarized in Fig. 4 show that on days 26 and 27 the pre-dose mean plasma levels of CMI and its metabolite DCMI were not linearly related to the daily dose, and the concentrations of the metabolite DCMI were larger than those of the unchanged drug during chronic treatment with clomipramine.

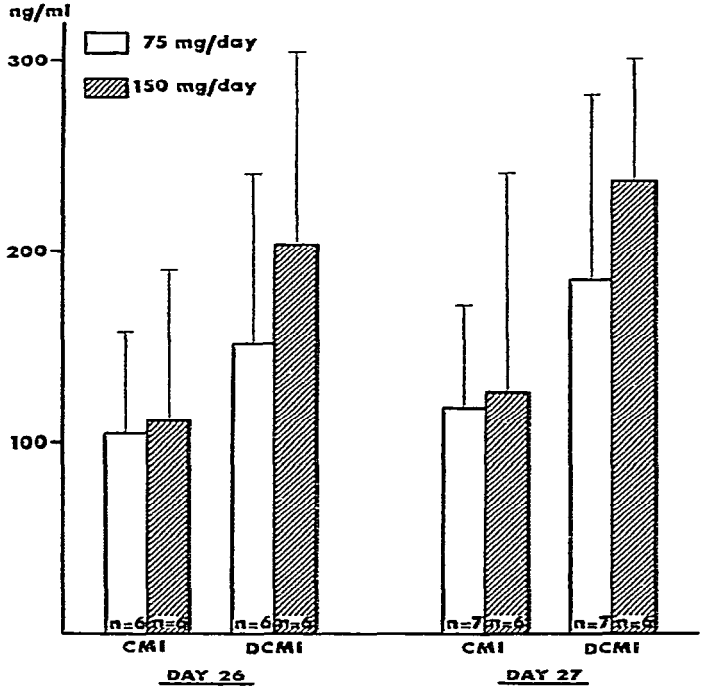

Fig. 4. Mean (± S.D.) steady-state plasma levels of CMI and DCMI achieved in patients given daily 75- or 150-mg doses of clomipramine (25-mg Anafranil tablets).

Imipramine plasma levels after oral administration of a single 50-mg dose. The time course of the plasma levels of imipramine following the administration of a single 50-mg oral dose of imipramine hydrochloride was determined in six healthy volunteers. The average curve is shown in Fig. 5. IMI levels peaked at 18 ng/ml 4 h after dosing and declined to 5 ng/ml at 24 h. DMI levels were lower than 10 ng/ml.

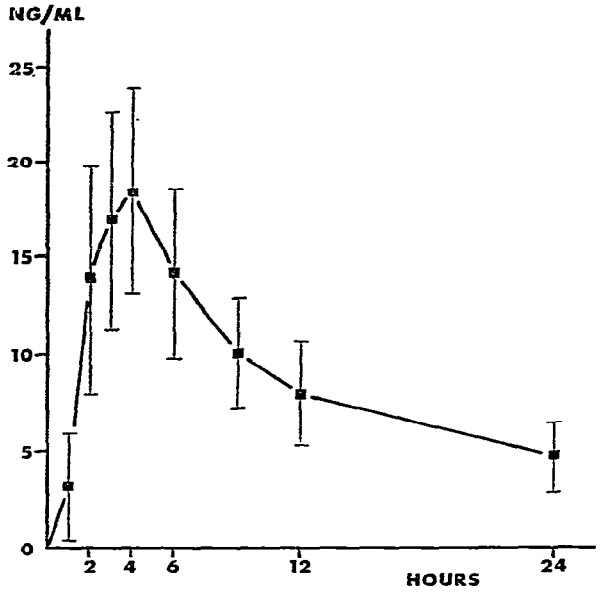

Fig. 5. Time course of plasma concentrations of imipramine following administration of a single 50-mg oral dose of imipramine hydrochloride (25-mg Tofranil tablets). Average curve ± S.D. from six healthy subjects.

CONCLUSION

The described method is sensitive enough for clinical pharmacology studies and for drug monitoring: concentrations down to 5–10 ng/ml can be determined using 1-ml blood or plasma samples.

REFERENCES

1 C. Gonnet and J. L. Rocca, *J. Chromatogr.*, 120 (1976) 419.
2 M. R. Detaevernier, L. Dryon and D. L. Massart, *J. Chromatogr.*, 128 (1976) 204.
3 K.-G. Wahlund and A. Sokolowski, *J. Chromatogr.*, 151 (1978) 299.
4 J. E. Greving, H. Bouman, J. H. G. Jonkman, H. G. M. Westenberg and R. A. de Zeeuw, *J. Chromatogr.*, 186 (1979) 683.
5 J. H. Knox and J. Jurand, *J. Chromatogr.*, 103 (1975) 311.
6 P. O. Lagerstrom, I. Carlsson and B. A. Persson, *Acta Pharm. Suecica*, 13 (1976) 157.
7 B. Mellström and S. Eksborg, *J. Chromatogr.*, 116 (1976) 475.
8 B. Mellström and G. Tybring, *J. Chromatogr.*, 143 (1977) 597.
9 H. J. Lohmann, H. F. Proelss and D. G. Miles, *Clin. Chem.*, 24 (1978) 1006 (abstract No. 092).
10 H. F. Proelss, H. J. Lohmann and D. G. Miles, *Clin. Chem.*, 24 (1978) 1948.

11 B.-A. Persson and P.-O. Lagerström, *J. Chromatogr.*, 122 (1976) 305.
12 H. G. M. Westenberg, B. F. H. Drenth, R. A. de Zeeuw, H. de Cuyper, H. M. van Praag and J. Korf, *J. Chromatogr.*, 142 (1977) 725.
13 H. G. M. Westenberg, R. A. de Zeeuw, H. de Cuyper, H. M. van Praag and J. Korf, *Postgrad. Med. J.*, 53 (1977) 124.
14. R. B. Moyes and I. C. A. Moyes, *Postgrad. Med. J.*, 53 (1977) 117.
15 J. H. M. van den Berg, H. J. J. M. de Ruwe, R. S. Deelder and Th. A. Plomp, *J. Chromatogr.*, 138 (1977) 431.
16 T. A. Sutfin and W. J. Jusko, *J. Pharm. Sci.*, 68 (1979) 703.
17 A. Bonora and P. A. Borea, *Experientia*, 34 (1978) 1486.
18 F. L. Vandemark, R. F. Adams and G. J. Schmidt, *Clin. Chem.*, 24 (1978) 87.
19 I. D. Watson and M. J. Stewart, *J. Chromatogr.*, 134 (1977) 182.
20 R. A. de Zeeuw and H. G. M. Westenberg, *J. Anal. Toxicol.*, 2 (1978) 229.
21 H. J. Kuss and M. Nathmann, *Arzneim.-Forsch.*, 28 (1978) 1301.
22 D. R. A. Uges and P. Bouma, *Pharm. Weekbl. Sci. Ed.*, 1 (1979) 417.
23 F. L. Vandemark, R. F. Adams, G. J. Schmidt and W. Salvin, *Clin. Chem.*, 23 (1977) 1139 (abstract No. 112).
24 P. B. Bondo, J. J. Thoma and G. A. Beltz, *Clin. Chem.*, 25 (1979) 1118 (abstract No. 279).
25 P. A. Reece and R. Zacest, *J. Chromatogr.*, 163 (1979) 310.
26 G. Sivorinovsky, *Clin. Chem.*, 25 (1979) 1144.
27 L. P. Hackett and L. J. Dusci, *Clin. Toxicol.*, 15 (1979) 55.
28 R. Gupta and G. Molnar, *Drug Metab. Rev.*, 9 (1979) 79.
29 G. Carnis, J. Godbillon and J. P. Metayer, *Clin. Chem.*, 22 (1976) 817.
30 J. W. Faigle and W. Dieterle, *J. Int. Med. Res.*, 1 (1973) 281.
31 J. W. Faigle and W. Dieterle, personal communication.
32 B. Herrmann, *Neuropsycho-Pharmacology, Proc. 5th Meeting, C.I.N.P., Washington, 1966, Int. Congr. Ser. No. 129*, Excerpta Medica, Amsterdam, 1967, pp. 347–351.
33 L. F. Gram, *Commun. Psychopharmacol.*, 2 (1978) 373.
34 J. M. Perel, R. L. Stiller and A. H. Glassmann, *Commun. Psychopharmacol.*, 2 (1978) 429.
35 W. Z. Potter, H. Calil, A. Zavadil, W. Jusko and T. Sutfin, *Clin. Pharmacol. Ther.*, 25 (1979) 242.