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MASS FRAGMENTOGRAPHIC DETERMINATION OF LOFEPRAMINE AND ITS METABOLITES IN HUMAN PLASMA AND URINE USING DEUTERATED INTERNAL STANDARDS

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SUMMARY

A sensitive and specific method for the determination of lofepramine and its metabolites, desipramine and 2-hydroxydesipramine, in human plasma and urine is described. Lofepramine, desipramine and 2-hydroxydesipramine were derivatized to ethyl *p*-chlorobenzoate, the bis(heptafluorobutyryl) derivative and the N,O-bis(trifluoroacetyl) derivative, respectively, and then analysed by gas chromatography—mass fragmentography. Corresponding deuterated compounds were used as internal standards. Determination was possible at levels as low as 2 ng/ml for lofepramine and desipramine and 20 ng/ml for 2-hydroxydesipramine.

INTRODUCTION

Lofepramine [N-methyl-N-(4'-chlorophenacyl)-3-(10,11-dihydro-5H-dibenz-[*b*, *f*]azepin-5-yl)propylamine hydrochloride] is a tricyclic antidepressant drug, an imipramine analogue in which the amino group of the side-chain is substituted by a *p*-chlorophenacyl group [1]. This compound is apparently more lipophilic than imipramine and is expected to be absorbed and distributed faster into the body [2, 3]. Clinical reports have shown that lofepramine has similar antidepressive activities to imipramine, but a lower toxicity [4]. These properties indicate that lofepramine would be a more desirable antidepressant drug. In recent years, the determination of plasma levels of the drug in man after a single oral administration or during chronic treatment has become more important, because it affords valuable information concerning the bioavailability of the drug and its therapeutic and toxic thresholds. The method for the determination of lofepramine and its metabolite, desipramine, in plasma by gas chromatography has been already reported [5]. However, the method requires skilful technique to control the conditions of the procedure. In the work described here, a mass fragmentographic method with deuterated internal standards was devised for the sensitive and specific determination of lofe-

pramine, desipramine and 2-hydroxydesipramine in plasma and urine. 2-Hydroxydesipramine was shown, in a preliminary metabolic experiment, to be the main unconjugated metabolite of lofepramine in human urine.

EXPERIMENTAL

Reference drugs

The hydrochlorides of lofepramine, desipramine and 2-hydroxydesipramine were gifts from AB Leo (Hersinborg, Sweden). *p*-Chlorobenzoic acid was purchased from Tokyo Kasei (Tokyo, Japan).

Chemicals

Pentadeuteriochlorobenzene and deuterium oxide were purchased from Merck Sharp & Dohme (Quebec, Canada); 10, 11-dihydro-5H-dibenz[*b,f*]-azepine from Aldrich (Milwaukee, Wisc., U.S.A.); heptafluorobutyric anhydride and trifluoroacetylimidazole from Tokyo Kasei; and deuterium gas from Showa Denko (Tokyo, Japan).

Deuterated internal standards

4-Chloro-2,3,5,6-tetradeuteriobenzoic acid (p-chlorobenzoic acid-d_4). This compound was prepared according to the method of Gross et al. [6]. Pentadeuteriochlorobenzene (isotopic purity: >99 atom-% ^{2}H) was allowed to react with dichloromethylenedioxybenzene in methylene chloride in the presence of anhydrous aluminium trichloride. The reaction product was hydrolysed with 25% potassium hydroxide solution to give *p*-chlorobenzoic acid-d_4. 2-Chloro-3,4,5,6-tetradeuteriobenzoic acid appeared not to be formed as a by-product. The product was characterized by thin-layer chromatography (TLC) and, after derivatization to its methyl ester, by gas chromatography—mass spectrometry (GC—MS). The isotopic distribution was as follows: d_4, 72.2%; d_3, 23.1%; d_2, 4.3%; d_1, 0.4%; d_0, 0.03%.

10,11-Dideuteriodesipramine (DMI-d_2) hydrochloride. 10,11-Dihydro-5H-dibenz[*b,f*]azepine was converted into 5H-dibenz[*b,f*]azepine by the method of Schindler and Blattner [7]. 5-(N-Methylaminopropyl)-5H-dibenz[*b,f*]azepine (dehydro-DMI) hydrochloride was synthesized from 5H-dibenz[*b,f*]azepine as described by Geigy [8]. DMI-d_2 hydrochloride was obtained as follows. Dehydro-DMI hydrochloride in deuterium oxide was stirred under deuterium gas (isotopic purity: >99.5%) in the presence of 5% palladium on carbon. The reaction mixture was frequently monitored by TLC during hydrogenation. After 6 h, the reaction was stopped, the catalyst was removed by filtration and the filtrate was evaporated to dryness. The residue was recrystallized from ethanol—light petroleum (b.p. 30—70°). The product was characterized as DMI-d_2 hydrochloride by TLC and GC—MS. The isotopic distribution was as follows: d_2, 81.6%; d_1, 9.0%; d_0, 0.6%.

2-Hydroxy-10,11-dideuteriodesipramine (2-OH-DMI-d_2). This compound was obtained as a metabolite of 10,11-dideuteriolofepramine from rat urine as follows. A suspension of 10,11-dideuteriolofepramine hydrochloride in 0.5% CMC(sodium carboxy methyl cellulose) solution was administered orally to male Wistar rats. The urine was collected during 24 h after administration and

extracted with chloroform under alkaline medium. From the extract, 2-OH-DMI-d_2 was separated and purified by preparative TLC. The product was characterized by TLC and, after preparation of its N,O-bis(trifluoroacetyl) derivative, by GC—MS. The isotopic distribution was as follows: d_2, 77.2%; d_1, 13.0%; d_0, 2.5%. 10, 11-Dideuteriolofepramine hydrochloride was prepared from DMI-d_2 by the method of Ericksoo and Rothe [1].

Gas chromatography—mass fragmentography

A Hitachi Model RMU-6MG mass spectrometer equipped with a gas chromatograph was used. The GC conditions for ethyl *p*-chlorobenzoate (derived from lofepramine) were as follows: glass column (2 m × 3 mm I.D.) containing 2% PEG-20M on Gas-Chrom Q (80—100 mesh); temperatures of the oven, injection port and separator, 110°, 210° and 250°, respectively. The GC conditions for the derivatives of DMI and 2-OH-DMI were as follows: glass column (1 m × 3 mm I.D.) containing 1% OV-101 on Gas-Chrom Q (80—100 mesh); temperatures of the oven, injection port and separator, 220°, 260° and 265°, respectively. The carrier gas (helium) flow-rate was 30 ml/min in all instances. The MS conditions in all instances were as follows: ionization voltage, 30 eV; target current, 80 μA; ion source temperature, 230°; multiplier potential, 1.5—1.7 kV. For mass fragmentography, multiple ion detection was employed. The following ion *m/e* focusing was used: *m/e* 139 for ethyl *p*-chlorobenzoate; *m/e* 143 for ethyl *p*-chlorobenzoate-d_4; *m/e* 459 for the bis(heptafluorobutyryl) derivative of DMI; *m/e* 461 for the bis(heptafluorobutyryl) derivative of DMI-d_2; *m/e* 320 for the N,O-bis(trifluoroacetyl) derivative of 2-OH-DMI; and *m/e* 322 for the N,O-bis(trifluoroacetyl) derivative of 2-OH-DMI-d_2 (see Figs. 1—3).

Analytical procedure

Lofepramine. To 1 ml of heparin-treated plasma, which had previously been acidified with 0.06 ml of 2 *M* hydrochloric acid and stored at −20°, 0.2 ml of 0.5 *M* sodium hydroxide solution and 1 ml of 0.2 *M* sodium hydrogen carbonate–sodium carbonate buffer (4:6) pH 10, were added. The solution was extracted with 5 ml of *n*-hexane by shaking for 10 min on an automatic shaker. After centrifugation, 4 ml of the organic phase were transferred into a glass tube and the solvent was evaporated under reduced pressure. To the residue, 1 ml of ethanol containing 0.1 ml of 30% hydrogen peroxide solution was added, and the mixture was allowed to stand for 1 h at room temperature. To the reaction mixture, 0.5 ml of distilled water and 50 μl of the internal standard solution (4 μg/ml of *p*-chlorobenzoic acid-d_4 in ethanol) were added. The mixture was concentrated under reduced pressure to a volume of about 0.5 ml, then 2 ml of 1 *M* hydrochloric acid and 5 ml of benzene were added. The tube was shaken mechanically for 10 min. After centrifugation, the organic phase was transferred into a glass-stoppered tube and the solvent was evaporated. The residue was dissolved in 0.2 ml of ethanol and 1 ml of ethanol saturated with hydrogen chloride gas. The mixed solution was heated at 60° for 1 h, then the reaction mixture was cooled in ice-cold water and 0.2 ml of isoamyl alcohol was added. The mixture was concentrated under reduced pressure to a volume of about 0.3 ml at room temperature. To the concentrate

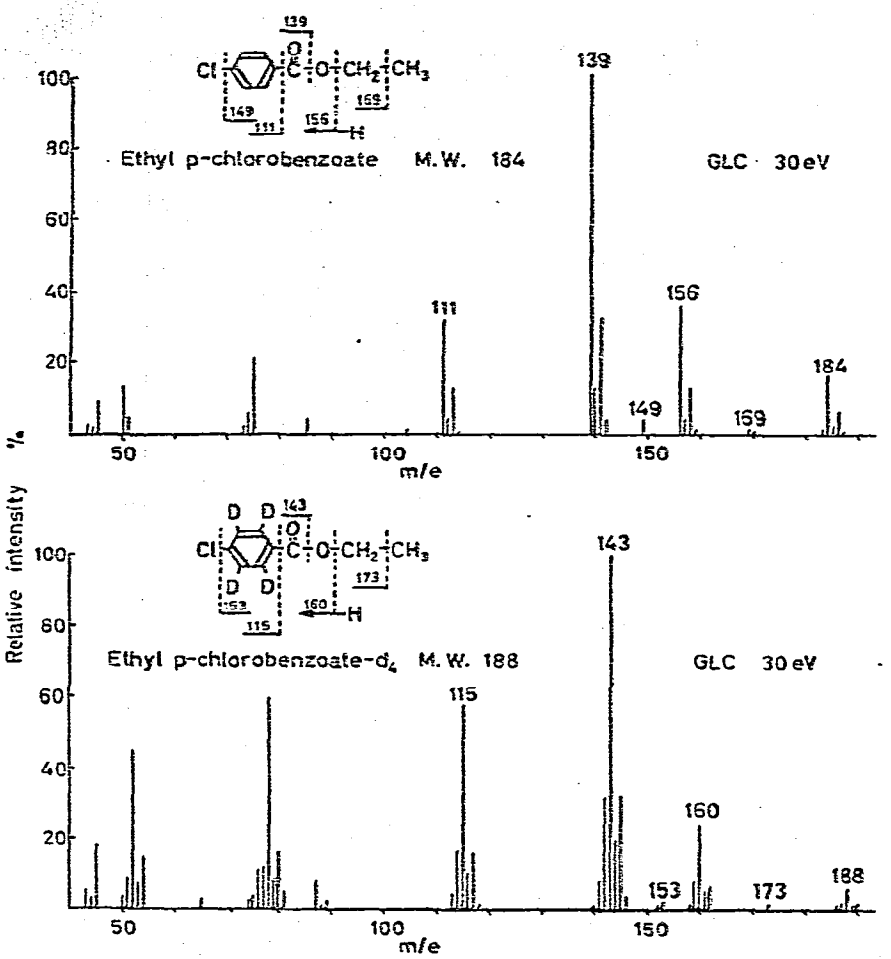

Fig. 1. Mass spectra of ethyl *p*-chlorobenzoate (derived from lofepramine) (above) and ethyl *p*-chlorobenzoate-d_4 (below).

5 ml of *n*-hexane and 2 ml of 5% sodium hydrogen carbonate solution were added. The tube was carefully shaken for 2 min and the organic phase was transferred into a glass tube and concentrated to a volume of about 50 µl under reduced pressure. A volume of 3 or 4 µl of this solution was injected into the GC column.

To 1 ml of urine sample, stored at −20°, 1 ml of the carbonate buffer was added, the mixture was extracted with 5 ml of *n*-hexane, and then processed as described for the plasma sample.

Desipramine. To 1 ml of plasma sample, 20 µl of the internal standard solution (10 µg/ml of DMI-d_2 hydrochloride in 0.1 *M* hydrochloric acid) and 1 ml of 0.2 *M* hydrochloric acid were added. The solution was shaken with 5 ml of dichloroethane for 10 min and, after centrifugation, the organic phase

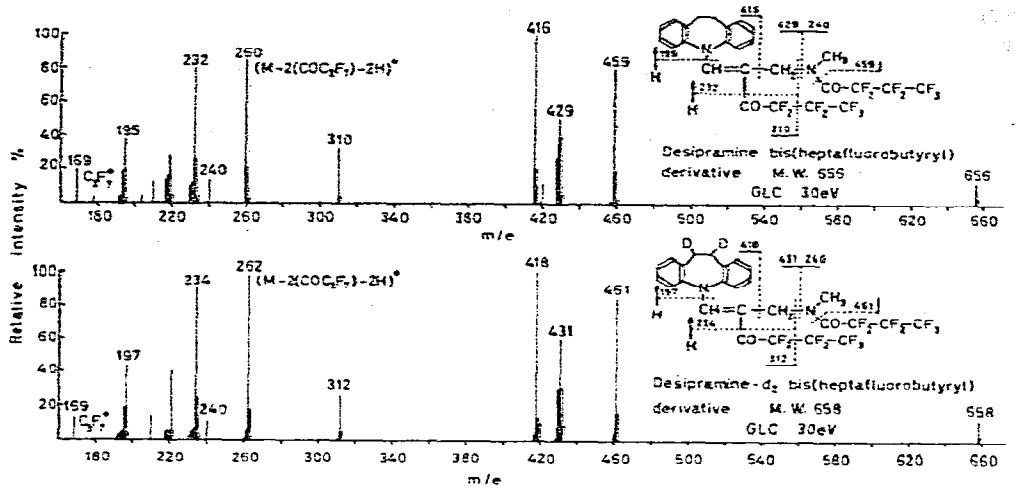

Fig. 2. Mass spectra of desipramine bis(heptafluorobutyryl) derivative (above) and desipramine-d_2 bis(heptafluorobutyryl) derivative (below).

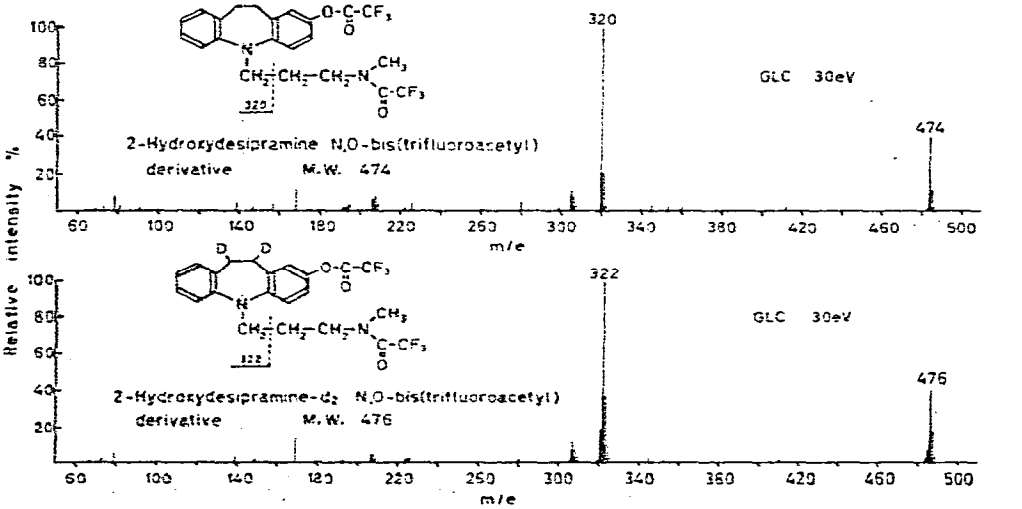

Fig. 3. Mass spectra of 2-hydroxydesipramine N,O-bis(trifluoroacetyl) derivative (above) and 2-hydroxydesipramine-d_2 N,O-bis(trifluoroacetyl) derivative (below).

was removed and discarded. The aqueous phase was made alkaline by adding 0.5 ml of 5 *M* sodium hydroxide solution and extracted with 5 ml of *n*-hexane, and then the mixture was centrifuged. All glassware in the following procedure was rinsed with isoamyl alcohol prior to use. The organic phase was dried over 1 g of anhydrous sodium sulphate and then evaporated to dryness under reduced pressure. To the residue, 200 μl of ethyl acetate and 40 μl of heptafluorobutyric anhydride were added and the reaction mixture was allowed to stand overnight in a tube with a stopper at room temperature. The mixture was evaporated to dryness under reduced pressure. The residue was re-dissolved

in 50 µl of ethyl acetate and an aliquot of 2—4 µl was injected into the GC column.

To a 3-ml urine sample, 50 µl of the internal standard solution as mentioned above were added. The solution was made alkaline by addition of 0.5 ml of 5 *M* sodium hydroxide solution and extracted twice with 3-ml portions of *n*-hexane. After centrifugation, the extracts were combined and then processed as described for the plasma sample.

2-Hydroxydesipramine. To 1 ml of urine sample, 10 µl of the internal standard solution (15 µg/ml of 2-OH-DMI-d_2 in methanol) were added and the pH was adjusted to 10 with 1 ml of 0.2 *M* carbonate buffer. The solution was shaken with 5 ml of dichloroethane for 10 min. After centrifugation, the organic phase was transferred into a glass tube and evaporated to dryness under reduced pressure. The residue was dissolved in 200 µl of acetonitrile, mixed with 20 µl of N-trifluoroacetylimidazole and stood at 80° for 3 h. An aliquot of 1—4 µl of the reaction mixture was used for GC.

Synthesis and spectral data of authentic 10,11-dihydro-5-[3-(N-heptafluorobutyryl-N-methylamino)-2-heptafluorobutyryl-1-propenyl]-5H-dibenz[b,f]azepine

To a suspension of 100 mg of desipramine hydrochloride in 2 ml of ethyl acetate, 1 ml of heptafluorobutyric anhydride was added. The reaction mixture was allowed to stand for 15 h at room temperature, and then evaporated to dryness under reduced pressure. The residue was chromatographed on 4 g of silica gel and eluted with benzene. From the main fraction, 65 mg of the bis-(heptafluorobutyryl) derivative were obtained as pale yellow crystals, m.p. 68—70°; infrared (film), 1680, 1640 (C=O), 1560 cm^{-1} (C=C and C=O); nuclear magnetic resonance ($CDCl_3$), 8.13 (s. 1H, vinylic), 3.85 (s. 2H, $-CH_2-N$), 2.7—3.8 (m. 4H, $-CH_2-CH_2-$), 3.0 (broad s. 3H, $-N-CH_3$), 7.28 ppm (s. 8H, aromatic); ultraviolet $\lambda_{max.}$ (ethanol) 319 nm ($\epsilon = 4.7 \cdot 10^4$). The mass spectrum is shown in Fig. 2. The elemental composition was confirmed by high-resolution MS.

RESULTS AND DISCUSSION

The GC analysis of lofepramine itself has not been successful because it is decomposed at the high temperatures usually employed. Lundgren et al. [5] reported a method for the determination of lofepramine, in which *p*-chlorobenzaldehyde derived from lofepramine was detected by GC with an electron-capture detector. In this method, the steam-distillation procedure for cleaning up samples had to be operated with much caution because of the high volatility of the aldehyde.

In our study, it was found that lofepramine was very unstable under oxidation conditions and readily formed *p*-chlorobenzoic acid in hydrogen peroxide solution in 80% yield (standard deviation 5%). By determining the *p*-chlorobenzoic acid thus generated, the amount of lofepramine could be calculated. The oxidation reaction was completed in 1 h. The resulting *p*-chlorobenzoic acid was converted into its ethyl ester and determined by mass fragmentography, monitoring the *p*-chlorobenzoyl ion, *m/e* 139 (the base peak ion).

The mass spectrum of the methyl ester gave an analogous pattern to that of the ethyl ester but, as long as *m/e* 139 ion was monitored, the methyl ester was found to be an unsuitable derivative for this analysis because methyl *p*-chlorobenzoate-d_4, used as an internal standard, also gave an ion at *m/e* 139 due to the fragment $[M-Cl]^+$. As shown in Fig. 1, such a disturbance was not encountered with the ethyl ester (Fig. 4A).

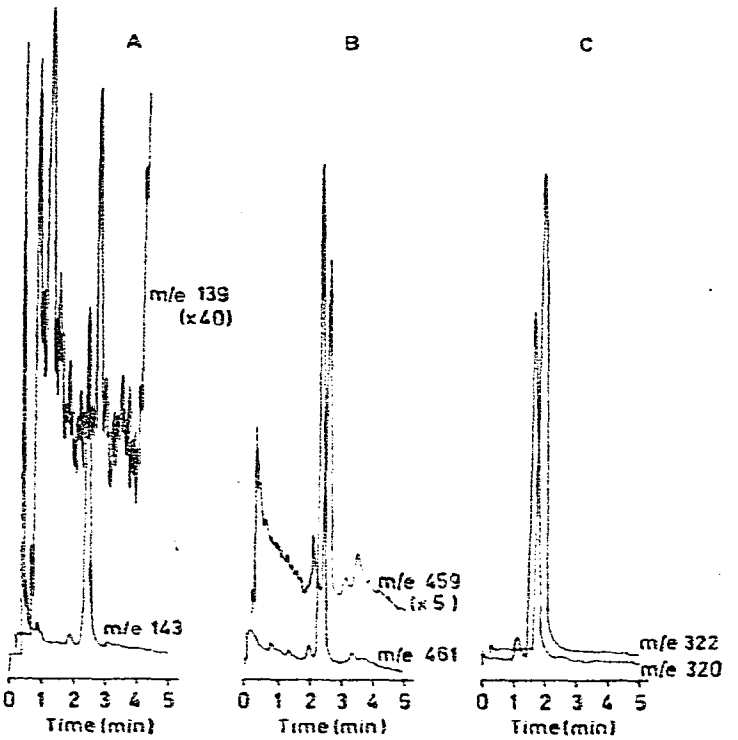

Fig. 4. Mass fragmentograms obtained from plasma or urine of a healthy volunteer dosed with lofepramine. (A) Lofepramine in plasma; (B) desipramine in plasma; (C) 2-hydroxydesipramine in urine. Samples were processed as described under *Analytical procedure*.

The kinetics of the esterification reaction in ethanol saturated with hydrogen chloride are shown in Fig. 5. The peak-height ratio was linear over the range 2—80 ng/ml in plasma or urine (Fig. 7A). A typical mass fragmentogram obtained from human plasma containing lofepramine is shown in Fig. 4A. The overall recovery of lofepramine in this procedure was about 60%.

Although the internal standard was added after oxidation, the determination of lofepramine was performed accurately because the recovery of lofepramine during the extraction procedure was shown to be more than 95% in preliminary experiments in which known amounts of lofepramine hydrochloride were added in 0.1 *M* borate buffer (pH 10) and extracted with *n*-hexane, the recovery being determined by ultraviolet spectrophotometry [9]. The concomitant metabolite, *p*-chlorobenzoic acid, in biological material remained in the aqueous phase under these extraction conditions.

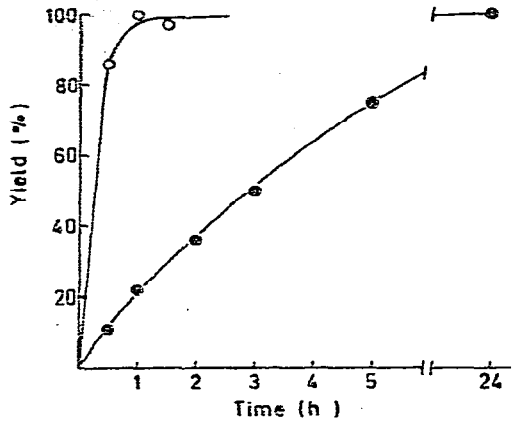

Fig. 5. Yields from the esterification reaction of *p*-chlorobenzoic acid at (•) 25° and (○) 60°.

Desipramine is the active metabolite of lofepramine that retains antidepressant activity. Therefore, the determination of plasma levels of desipramine would also afford valuable biological information. Several methods for the GC determination of desipramine in biological fluids have been described [10–15]. In those methods, desipramine was converted into its acyl derivative in order to avoid adsorption on the stationary phase. It has been shown that the N-heptafluorobutyryl derivative (monoacyl derivative) of DMI is more effective for avoiding adsorption than the acetyl or the trifluoroacetyl derivative. However, under the usual reaction conditions for heptafluorobutyrylation, the bis(heptafluorobutyryl) derivative of desipramine was also formed in considerable amounts and it was difficult to control the reaction to form the N-heptafluorobutyryl derivative selectively. By using ethyl acetate as the reaction solvent, the bis(heptafluorobutyryl) derivative could be obtained more easily and quantitatively. This bis(heptafluorobutyryl) derivative was found to be far less adsorbed than the N-heptafluorobutyryl derivative on the GC column and gave a symmetrical peak in the gas chromatogram. It was also stable to moisture.

These properties of the bis(heptafluorobutyryl) derivative suggested that it was the most suitable derivative for the GC analysis of desipramine. The infrared, nuclear magnetic resonance and ultraviolet spectra of this compound were similar to those of the bis(trifluoroacetyl)derivative of desipramine reported by Walle et al. [16]. The mass spectrum is shown in Fig. 2. These spectral data suggest that the chemical structure of the bis(heptafluorobutyryl) derivative is 10,11-dihydro-5-[3-(*N*-heptafluorobutyryl-*N*-methylamino)-2-heptafluorobutyryl-1-propenyl]-5H-dibenz[*b*,*f*]azepine, the compound in which the trifluoroacetyl groups in Walle et al. 's compound are replaced with heptafluorobutyryl groups. The kinetics of the derivatization reaction are shown in Fig. 6. The reaction was completed in 6 h. The ion corresponding to $[M-C_3F_7CO]^+$, *m/e* 459 for the sample and *m/e* 461 for the internal standard, was monitored (Fig. 4B). The ratio peak height of DMI to that of DMI-d_3 was linear over the range 2–60 ng/ml in plasma or urine (Fig. 7B).

For the selective detection of desipramine in biological fluids, it was nec-

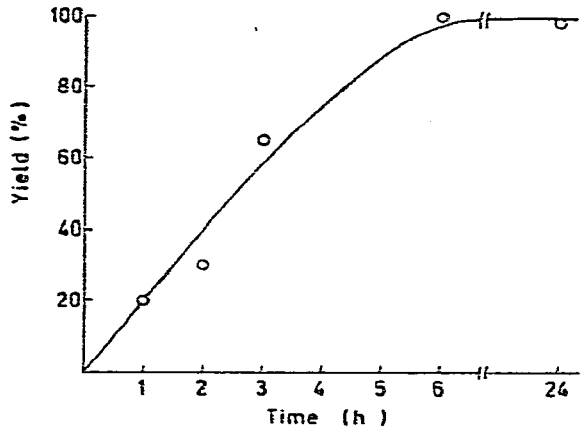

Fig. 6. Yields from the bis(heptafluorobutyryl) derivative formation reaction of desipramine.

essary to remove lofepramine, which readily liberated desipramine during the cleaning up process. Lofepramine was removed by extracting the sample with dichloroethane just after DMI-d_2 was added as an internal standard. Addition of the deuterio-labeled internal standard at the beginning of the procedure contributed to a more precise and accurate determination of plasma or urine levels of DMI.

When 2-OH-DMI was subjected to reaction with N-trifluoroacetylimidazole at 80° for 3 h, the N,O-bis(trifluoroacetyl) derivative was formed quantitatively. When examined by GC, the product gave a single symmetrical peak. With 2-OH-DMI, the tris(heptafluorobutyryl) derivative corresponding to the bis(heptafluorobutyryl) derivative of DMI was found to be unstable under the GC conditions. The mass spectra of the N,O-bis(trifluoroacetyl) derivatives of 2-OH-DMI and of 2-OH-DMI-d_2 are shown in Fig. 3. In the mass fragmentographic determination, the ions monitored were *m/e* 320 for the sample and *m/e* 322 for the internal standard (Fig. 4C). The standard graph was linear over the range 20 ng/ml to 1 μg/ml in urine (Fig. 7C).

The utility of these methods was demonstrated by applying them to clinical

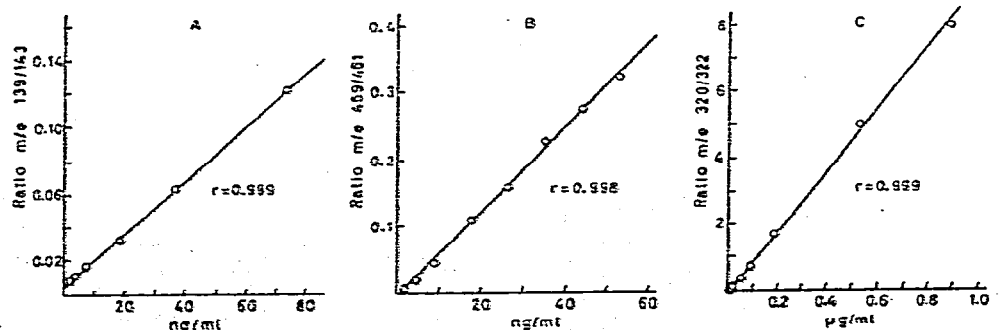

Fig. 7. Standard graphs: (A) lofepramine in human plasma; (B) desipramine in human plasma; (C) 2-hydroxydesipramine in human urine. The standard graphs were obtained by adding known amounts of lofepramine, desipramine or 2-hydroxydesipramine to 1 ml of human plasma or urine and processing them as described under *Analytical procedure*.

experiments with human volunteers receiving single or multiple doses of lofepramine. The clinical significance of these results has been discussed previously [17]. Representative results are shown in Fig. 8.

The results demonstrate that the technique described here seems to be suitable for determining the plasma levels and urinary excretion of lofepramine in depressed patients undergoing chronic treatment.

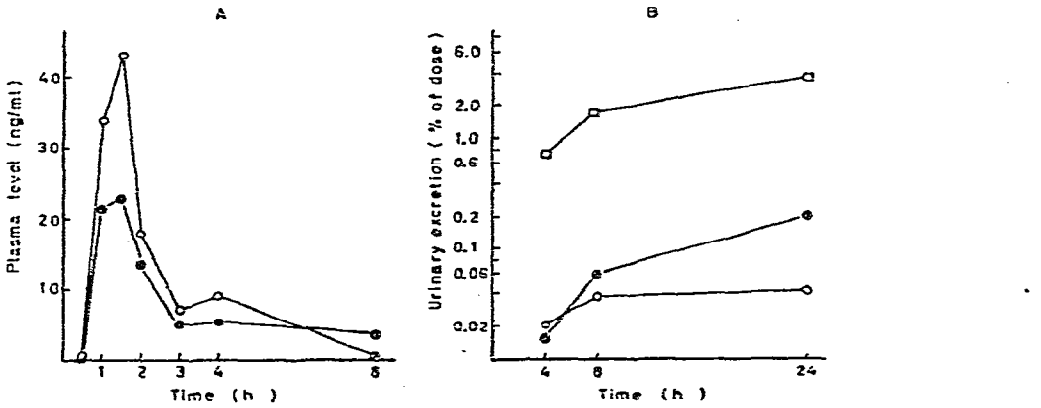

Fig. 8. (A) Plasma levels of lofepramine and desipramine in a healthy volunteer (subject K.O., male, age 27, weight 55 kg) after a single oral dose of 50 mg of lofepramine. (B) Urinary excretion of lofepramine and its metabolites in the same experiment. ○, Lofepramine ●, desipramine; □, 2-hydroxydesipramine.

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