A Sensitive Method for the Simultaneous Determination in Biological Fluids of Imipramine and Desipramine or Clomipramine and *N*-Desmethylclomipramine by Gas Chromatography Mass Spectrometry†

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A procedure is described which permits the simultaneous determination of imipramine and desipramine or clomipramine and *N*-desmethylclomipramine in serum or plasma for concentrations in the range of 1–200 ng ml^{-1}. Detection limits of 0.2 ng ml^{-1} for imipramine and 0.1 ng ml^{-1} for desipramine were demonstrated with a signal-to-noise ratio maintained at 2:1 or better. The method relies on the derivatization of the secondary amines with heptafluorobutyric anhydride and is based on the combined use of gas chromatography, electron impact mass spectrometry and computerized data handling. The assaying procedure is specific, accurate and precise. It is suitable for routine analyses and has sufficient sensitivity to permit monitoring the drug and metabolite levels in human plasma or serum resulting from a single therapeutic dose.

INTRODUCTION

Imipramine, 5-[3-(dimethylamino)propyl]-10,11-dihydro-5*H*-dibenz[*b*,*f*]azepine and clomipramine, 3-chloro-5-[3-(dimethylamino)propyl]-10,11-dihydro-5*H*-dibenz[*b*,*f*]azepine, as hydrochlorides, are the respective active ingredients in the widely prescribed tricyclic antidepressants Tofranil^{R} (Ciba-Geigy) and Anafranil^{R} (Geigy, Switzerland). These compounds, after oral administration to humans, demethylate to a certain degree to yield desipramine, 5-[3-(methylamino)propyl]-10,11-dihydro-5*H*-dibenz[*b*,*f*]azepine and *N*-desmethylclomipramine, 3-chloro-5-[3-(methylamino)propyl]-10,11-dihydro-5*H*-dibenz[*b*,*f*]azepine. The metabolites are also pharmacologically active and are found in plasma together with unchanged drug.[1]

The considerable interest in measuring plasma levels of these tricyclic drugs for pharmacologic studies and for therapeutic monitoring has prompted the development of several analytical methods. Included are procedures based on fluorescence,[2] direct densitometry of thin-layer chromatograms,[3] gas chromatography utilizing nitrogen detection,[4–7] selected ion monitoring by means of GCMS,[8–14] liquid chromatography utilizing UV detection[15] or combined with direct probe FIMS[16] and radioimmunoassay.[17]

The present work describes a selected ion monitoring GCMS method for the simultaneous determination of IP and DMI or CIP and DMCI. The desirability of monitoring the levels of imipramine and desipramine simultaneously is clinically important in the light of Gram's finding that a satisfactory antidepressant effect in patients is achieved only when steady-state plasma levels of both IP and DMI exceed a certain limit.[18] The method utilizes commercially available instrumentation and does not require the use of labeled standards. It is capable of monitoring both steady-state plasma levels and concentrations associated with single therapeutic doses, including levels (as low as 1 ng ml^{-1}) unassayable by most of the published procedures.

† Abbreviations: IP = imipramine; DMI = desipramine; CIP = clomipramine; DMCI = *N*-desmethylclomipramine; CDFA = chlorodifluoroacetyl; HFB = heptafluorobutyryl; PFP = pentafluoropropionyl.

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EXPERIMENTAL

Chemicals

The hydrochlorides of IP, DMI, CIP and DMCI were obtained from Ciba-Geigy (Summit, New Jersey or Ardsley, New York). Chlorodifluoroacetic anhydride (PCR, Gainesville, Florida), the perfluoro-anhydrides (Pierce Chem. Co., Rockford, Illinois), dichloromethane (nanograde; Mallinckrodt, St Louis, Missouri), *n*-heptane (chromatoquality; Matheson Coleman and Bell, Norwood, Ohio) and the remaining reagent grade commercial chemicals were used without any purification.

Standards and samples

Standard solutions of the tricyclic compounds were prepared by dilution in ethanol and/or distilled water. Samples were obtained by the addition of known amounts of standard solutions to serum or plasma. They were stored frozen in capped glass tubes. Samples for testing stability were prepared with fresh human plasma or serum.

CCC-0306-042X/79/0006-0200$02.50

Extraction

To the serum or plasma samples (0.2 to 2 ml) were added the internal standards (usually 72 ng of CIP and 72 ng of DMCI or 32 ng of IP and 22 ng of DMI). The samples were rendered alkaline with 2 ml of 2 M Na_2CO_3 and extracted with 9 ml of *n*-heptane containing 1% 3-methyl-1-butanol. The organic layer was mixed with 0.4 ml of 1 N H_2SO_4. After centrifugation and cooling in dry ice–ethanol, the supernatant organic phase was discarded. The aqueous extract was defrosted, rendered alkaline with 0.2 ml of 1 N NaOH and re-extracted with 1.4 ml of *n*-heptane. Following centrifugation and cooling, the organic phase was transferred into a 5 ml Reacti-vial (Pierce) and evaporated at 50 °C under a stream of N_2. The residue was subjected to derivatization.

Derivatization

The extraction residue was dissolved in 100 μl of dichloromethane containing 4% heptafluorobutyric anhydride. The container was capped, stirred briefly, and allowed to stand at room temperature for 1 h. This was followed by addition of dichloromethane (200 μl) and 2 N NaOH (20 μl), mixing, centrifugation, and cooling in dry ice–ethanol. The organic phase was transferred to a 1 ml Reacti-vial and allowed to evaporate at room temperature. The residue was dissolved in 15 μl of ethanol and an aliquot of this solution was injected for the analysis.

The acetyl, CDFA and PFP derivatives of the two secondary amines were prepared in a similar fashion with the appropriate anhydride.

Instrumentation

All analyses were performed on a Finnigan 3300 GCMS instrument, operated in the EI mode and equipped with a model 6100 data system.

Silanized glass columns, 1.5 m long (2 mm i.d.), were packed with 1.5% Poly S-179 on 80/100 mesh Chromosorb W AW DMCS or with 3% OV-17 on 80/100 mesh Chromosorb WHP. Helium flow was 20 ml min^{-1}. Temperatures were 255–260 °C for the injection port, 230–250 °C for the column oven, and 230–255 °C for the GCMS interface. They were adjusted to insure an imipramine retention time of 2.4–2.7 min. After each analysis, the column was purged for about 5 min at a temperature of 270–290 °C.

The MS conditions were: electron energy, 37 eV; emission current, 0.75–0.77 mA; continuous dynode electron multiplier voltage, 1.5 kV; preamplifier range, 10^{-9} AV^{-1}.

Revision H Finnigan software was used with the data system for all selected ion recordings.

Calibration curves and calculations

Daily calibration curves were prepared with 7–10 serum samples containing unchanged drug and metabolite of two known concentrations (usually 4 and 36 ng ml^{-1} each) as well as the appropriate internal standards. The average drug-to-standard ratios of peak heights (or peak areas) were plotted versus the two mean concentrations. Azea integrations were obtained by using the Finnigan software. Peak heights for each monitored ion were calculated from Eq (1), where P_i is the peak height of the ion; I_l, I_p and I_t are respectively the measured ion intensities of the leading baseline, the peak, and the trailing baseline; while S_l, S_p and S_t are the scan numbers corresponding to intensities I_l, I_p and I_t.

$$P_i = I_p - \left[I_l + (I_t - I_l) \times \frac{(S_p - S_l)}{(S_t - S_l)} \right] \quad (1)$$

RESULTS AND DISCUSSION

Gas chromatographic considerations

The GC elution of IP and CIP is quite satisfactory even for trace amounts of the compounds. This, however, is not the case with the more polar DMI and DMCI, which appear to be subject to column adsorption losses. Acylation improves their elution and has been used for the development of several assaying methods.[10–13]

A comparison was made for the GC retention times of the acetyl, CDFA, HFB and PFP derivatives of the two secondary amines. A summary of data is presented in Table 1. The HFB compounds are the fastest eluting derivatives of DMI and DMCI. They separate well from each other and from IP and CIP on Poly-S 179 and on OV-17 columns. On this basis, heptafluorobutyric anhydride was chosen as the preferred acylating agent and OV-17 or Poly-S 179 as a suitable column liquid phase.

Choice of ions for selected monitoring

Among fragments exceeding *m/z* 100, the EI mass spectrum of IP has been reported to exhibit most abundant ions at *m/z* 234[9,12] or 235.[10,11] In our experience, any one of the ions at *m/z* 194, 195, 234, or 235 may appear as the IP base peak. Similarly, any one of the ions at *m/z* 228, 229, 268 or 269 may appear as the most abundant ion in the clomipramine mass spectrum.

Mass spectral patterns are known to show variability because of factors such as the temperature of ionization[19] or the presence of active sites in the source.[20] It appears, however, that concentration can also be a contributing factor. Simultaneous selected monitoring of the ions at *m/z* 195, 220, and 234 for different amounts of injected IP shows a linear relationship between ion intensity and amount injected. There are slope differences, however, which are indicative of a fragmentation pattern changing as a function of concentration. For small IP amounts, the 220 and 195 mass peaks exceed significantly the one at *m/z* 234. Similar trends were observed with CIP, for low concentrations of which the ions at *m/z* 254 and 229 showed considerably higher intensities than mass peak 269. The effect of concentration on the fragmentation patterns of IP and CIP is illustrated by differences in the mass spectra for these compounds resulting from different sizes of injected samples (Figs. 1 and 2).

In Europe, Hirtz confirmed the dependence of IP fragmentation pattern on the size of sample introduced

Table 1. Gas chromatographic retention times of imipramine, clomipramine, desipramine, *N*-desmethylclomipramine and selected acyl derivatives

Compound	Retention times (relative to imipramine) for indicated liquid phases and temperatures		
	1.5% Poly-S 179		3% OV-17
	270 °C	230 °C	215 °C
Imipramine	1 (0.83 min)	1 (2.52 min)	1 (2.73 min)
Desipramine		1.59	1.22
N-Acetyldesipramine	10.60		
N-Chlorodifluoroacetyldesipramine	5.46		
N-Pentafluoropropionyldesipramine	1.71	2.01	
N-Heptafluorobutyryldesipramine	1.33	1.56	1.69
Clomipramine	1.78	1.89	1.86
N-Desmethylclomipramine			2.27
N-Acetyldesmethylclomipramine	19.30		
N-Chlorodifluoroacetyldesmethylclomipramine	10.48		
N-Pentafluoropropionyldesmethylclomipramine	3.23	4.28	
N-Heptafluorobutyryldesmethylclomipramine	2.52	3.37	3.16

into the Finnigan mass spectrometer.[21] He found, however, that this was not the case when IP mass spectra were generated with a Riber quadrupole instrument. In addition, such spectra gave a 220 mass peak of relatively small intensity. The analogous ion at *m/z* 254 was reported completely absent from the mass spectrum of CIP obtained with an LKB 2091 instrument.[14] Apparently, the mass spectral patterns can be affected by the type of instrumentation used for their generation.

The HFB derivatives of DMI and DMCI, whose mass spectra are presented in Fig. 3, exhibit base peaks at *m/z* 208 and 242, respectively. The abundance of these ions does not appear to be affected significantly by concentration changes.

In light of the aforementioned findings, the ions at *m/z* 220, 254, 208 and 242 were chosen for selected ion monitoring for IP, CIP, HFB-DMI and HFB-DMCI, respectively.

Extraction recovery and stability of samples

Liquid scintillation was used for measuring the amount of radioactivity extracted from plasma samples containing 4–100 ng ml^{-1} of [^{14}C]imipramine. HCl. Average recoveries equalled or exceeded 82% of added drug. This is consistent with recoveries of 80% for IP and 76% for DMI reported by Frigerio for plasma samples containing 5–200 ng ml^{-1} of each drug, alone or in combination.

The stability of IP, DMI, CIP and DMCI in frozen plasma or serum was tested for three months of frozen storage at −4 °C with samples containing 4–36 ng ml^{-1} of these compounds. All four showed good stability. Dubois has also reported that whole blood samples containing tricyclic antidepressants, including IP and DMI, can be stored effectively at −20 °C for at least 4 months.[13]

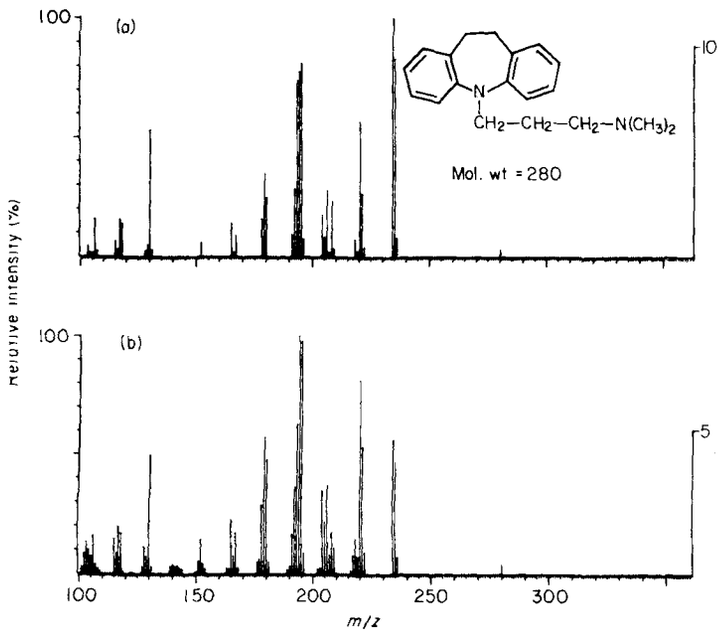

Figure 1. Electron impact mass spectra (37 eV) of imipramine obtained with samples of (a) 100 ng and (b) 10 ng.

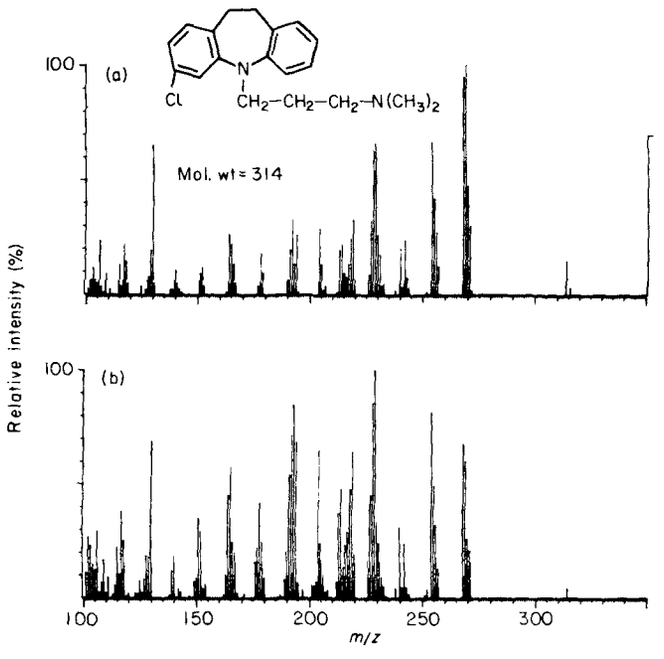

Figure 2. Electron impact mass spectra (37 eV) of clomipramine obtained with samples of (a) 100 ng and (b) 10 ng.

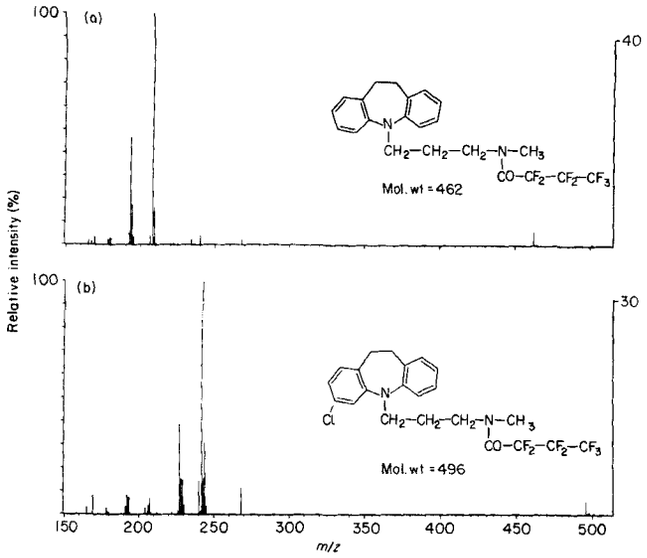

Figure 3. Electron impact mass spectra (37 eV) of the heptafluorobutyryl derivatives of (a) desipramine and (b) *N*-desmethylclomipramine.

Precision, accuracy and limits of detection

Precision refers to the reproducibility of determinations. It is affected by the experimental error, which represents indeterminate and unavoidable random errors associated with the nature of the assay. It can be measured conveniently by means of standard deviation.

A comparison of standard deviations was made for IP determinations based on peak height measurements and results obtained simultaneously by the use of peak area data. The results are summarized in Table 2. They indicate good precision for peak height results for the entire concentration range tested (1–80 ng ml^{-1}). Peak area results are comparable, except for concentrations lower than 4 ng ml^{-1} which are associated with a completely unacceptable 129% relative standard deviation. On the basis of these results, the peak height analytical approach was chosen for all calculations.

Accuracy refers to the correctness of determinations. The difference between true and found values results from the sum of random and determinate errors associated with the assay. When a sufficient number of measurements are taken, random errors tend to cancel out, while determinate errors introduce a degree of bias associated with the analytical procedure. If bias is absent or insignificant, precision can be used as an indicator for expected accuracy. If bias is present, its extent has to be assessed in order to determine the expected degree of accuracy.

Table 2. Precision of imipramine determinations obtained by selected ion monitoring and peak height or peak area measurements

Imipramine.HCl added to serum (ng ml^{-1})	Imipramine found, as % of added concentration: Peak height results Mean ± SD	Peak area results Mean ± SD	(N)
1–2	103.0 ± 19.9	141.4 ± 129.1	(5)
4–80	97.8 ± 11.8	96.1 ± 12.3	(26)

Table 3. Analytical results for imipramine and desipramine or for clomipramine and *N*-desmethylclomipramine, determined simultaneously in serum or plasma samples with drug and metabolite levels ranging from 1 to 200 ng ml^{-1}

Compound	Added ng ml^{-1}	Percent found Mean ± SD	(*N*)
Imipramine.HCl	1–3	101.1 ± 19.4	(17)
Imipramine.HCl	4–200	102.4 ± 10.4	(56)
Desipramine.HCl	1–2	124.5 ± 26.3	(10)
Desipramine.HCl	3–200	96.2 ± 10.7	(56)
Clomipramine.HCl	1	155.7	(2)
Clomipramine.HCl	2–200	100.4 ± 10.5	(35)
N-Desmethylclomipramine.HCl	1	120.6 ± 9.5	(4)
N-Desmethylclomipramine.HCl	2–200	101.3 ± 5.6	(36)

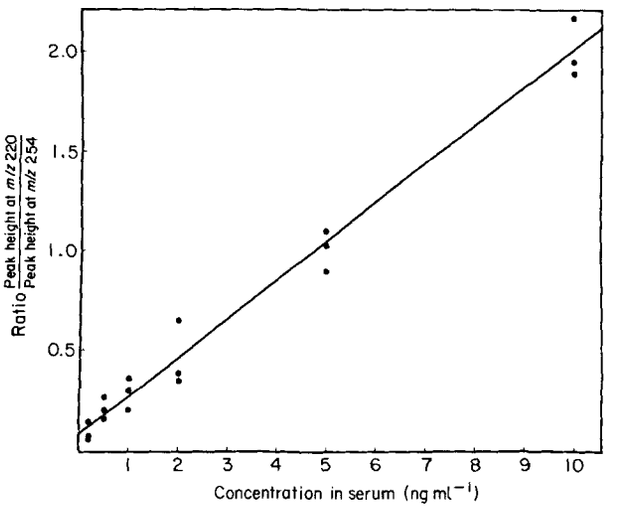

Figure 4. Standard curve for determining imipramine by selected ion monitoring, using 36 ng ml^{-1} of clomipramine as internal standard. Each point represents a single determination.

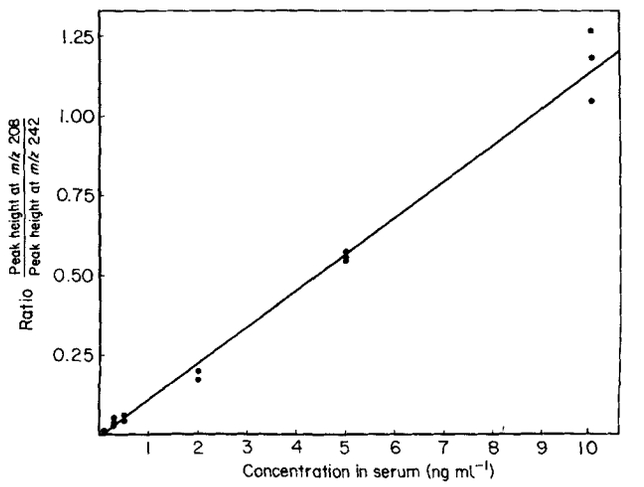

Figure 5. Standard curve for determining desipramine by selected ion monitoring, using 36 ng ml^{-1} of *N*-desmethylclomipramine as internal standard. Each point represents a single determination.

The precision and accuracy were tested for concentrations in the range of 1–200 ng ml^{-1}. The IP and DMI determinations included seven runs spaced within four months. Three of the runs involved samples with concentrations unknown to the analyst. The CIP and DMCI determinations were all part of a single run. All runs included at least one serum blank. In all instances, blank samples gave no signal for any of the drugs or standards, which is indicative of the lack of any interfering contaminants.

The results are summarized in Table 3. They show for all four compounds a precision associated with relative standard deviations of approximately 10%. Only at very low concentrations of IP (1–3 ng ml^{-1}) and DMI (1–2 ng ml^{-1}), the standard deviations became about twice as large. The results indicate also lack of any pronounced bias, except maybe for a tendency to overvalue levels of 1 ng ml^{-1}.

The limits of detection can be extended below the level of 1 ng ml^{-1} by using 4 ml samples and monitoring only two ions (drug and internal standard) at a time. Standard curves for IP and DMI, generated in this manner, are presented in Figs. 4 and 5. They extend to concentrations as low as 0.2 ng ml^{-1} for IP and 0.1 ng ml^{-1} for DMI. Blank serum samples, analyzed concurrently, gave no indication of interfering contaminants. The intercepts (−0.35 ng ml^{-1} for IP and 0.23 ng ml^{-1} for DMI) were calculated by regression analysis. They resulted from experimental error, which may be reduced by further development.

In conclusion, the judicious choice of ions and the optimization of experimental conditions have permitted the development of a reliable method for assessing drug and metabolite levels for an important group of tricyclic antidepressants. The assay offers the specificity, accuracy, precision and sensitivity required by pharmacokinetic and therapeutic level studies, and has been used routinely during the past two years for the IP and DMI determinations in our laboratory.

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Received 26 October 1978