GLC Identification and Determination of 3,4-Methylenedioxyamphetamine (MDA) from Plasma and Urine

K. K. MIDHA ˣ, I. J. McGILVERAY, S. P. BHATNAGAR, and J. K. COOPER

Abstract □ A GLC method for qualitative and quantitative determination of 3,4-methylenedioxyamphetamine in biological fluids is described. After extraction of the drug and the internal standard, 4-methoxyamphetamine, from plasma and urine, the solvent is evaporated and the residue is mixed with 20 μl of freshly distilled ether. Aliquots (1–2 μl) then are injected into the gas chromatograph. Both the drug and the internal standard give well-separated symmetrical peaks. Flame-ionization detection allows concentrations of 0.125 μg of 3,4-methylenedioxyamphetamine from plasma to be determined with a precision of 3.16%. The method is applicable to the estimation of 4-methoxyamphetamine, employing 3,4-methylenedioxyamphetamine as the internal standard. For identification purposes, various derivatives such as Schiff bases, isothiocyanates, trifluoroacetates, and heptafluorobutyrates of both compounds were formed following extraction from urine and their GLC behavior was investigated. Derivative formation was confirmed by GLC–mass spectrometry. Mass spectral data for these derivatives are presented.

Keyphrases □ 3,4-Methylenedioxyamphetamine—GLC analysis, mass spectrum, plasma and urine, human, dog □ 4-Methoxyamphetamine—GLC analysis, mass spectrum, plasma and urine, human, dog □ GLC—analysis, 3,4-methylenedioxyamphetamine and 4-methoxyamphetamine, plasma and urine, human, dog

3,4-Methylenedioxyamphetamine (MDA) (I) is chemically related to both mescaline and amphetamine. Some investigators found that the drug possesses stimulant and minor sympathomimetic activity; I inhibited monoamine oxidase (1) and increased muscular rigidity in a patient with Parkinson's disease (2). It was evaluated as an adjunct to psychotherapy (3), and another study (4) compared the subjective effects of I with those of amphetamine.

Because of the widespread abuse of I on the street (under such names as Mellow Drug of America, Love Drug, Love Pill, or MDA), it was felt that there was a need for its toxicological identification and quantitative estimation from biological fluids such as urine and plasma. A literature review on the analysis of this drug showed only one published method (5), which lacks details for quantitation from biological fluids. The present paper describes a GLC procedure for the positive identification and quantitation of I from plasma and urine.

Table I—Recovery of I and II from Plasma Determined by GLC Assay[a]

Micrograms Added to 1 ml of Plasma	Mean Micrograms Recovered	Mean Percent Recovery	*SD* of Percent Recovery
I			
1.04	1.00	99.72	2.19
4.00	4.04	100.86	0.96
Mean 100.29 ± 1.15%			
II			
1.0	1.01	100.24	1.23
4.0	4.02	100.42	1.36
Mean 100.30 ± 0.62%			

[a] $n = 4$.

Table II—Estimation of I Added to Plasma by GLC

I Added, μg	*n*	Mean Peak Height Ratio	*SD*	*CV*, %[a]
0.125	6	0.053	0.002	3.16
0.250	6	0.107	0.003	2.54
0.50	4	0.206	0.006	2.83
1.0	4	0.406	0.002	0.53
2.0	4	0.820	0.012	1.47
4.0	4	1.663	0.046	2.76

[a] Mean $CV = 2.22\%$, $y = mx$, where $m = 0.415 \pm 0.004$, $r^2 = 1$.

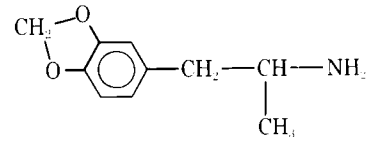
EXPERIMENTAL

Reagents—Acetone[1], ethyl acetate[1], carbon disulfide[2], and ether[3] were distilled in glass prior to use. Heptafluorobutyric anhydride[4] and trifluoroacetic anhydride[4] were refluxed on phosphorus pentoxide for 3 hr before glass distillation. The I[5], II[5], *dl*-amphetamine[5], methamphetamine[5], and *n*-propylamphetamine[5] were donated. Pyridine[4] was silylation grade, and all other chemicals employed were analytical grade.

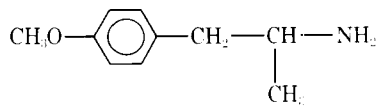
Stock solutions of I (100 μg/ml) and the internal standard, 4-methoxyamphetamine (II) (100 μg/ml), were prepared fresh weekly by dissolving their hydrochloride salts in water. Appropriate dilutions of each were made fresh daily as required.

Plasma Level Study—3,4-Methylenedioxyamphetamine hydrochloride, 10 mg/kg, was administered to a male dog weighing 8.38 kg. Samples of blood (10 ml) were withdrawn from the femoral vein by means of heparinized tubes[6] at 12 appropriate time intervals. The blood samples were centrifuged, and the plasma was transferred to another tube before storing at −10°.

General Procedure for Extraction of I—To the sample [human or canine plasma (2 ml) or urine (5 ml) spiked with I or biological fluids from dosed animals], placed in screw-capped[7] cen-



I



II

[1] Caledon Laboratories Ltd., Georgetown, Ontario, Canada.
[2] Fisher Scientific Co., Montréal, Québec, Canada.
[3] Mallinckrodt Chemical Works Ltd., Montréal, Québec, Canada.
[4] Pierce Chemical Co., Rockford, Ill.
[5] Dr. Keith Bailey, Pharmaceutical Chemistry Division, Drug Research Laboratories, Health Protection Branch, Ottawa, Canada.
[6] Vacutainers, Becton Dickinson and Co., Mississauga, Ontario, Canada.
[7] Lined with Teflon (du Pont).

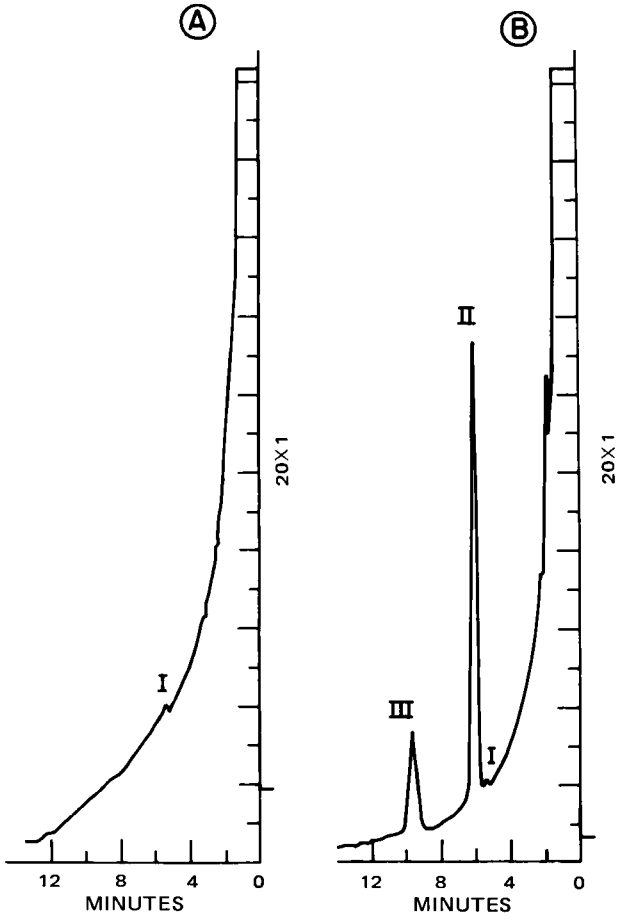


Figure 1—*Gas-liquid chromatogram of human plasma. Key: A, control plasma; B, plasma spiked with I (0.5 μg/ml) and II (3.0 μg/ml); peak I, endogenous material; peak II, II; and peak III, I.*

trifuge tubes (20 ml), are added 1 ml of II (3.0 μg/ml), 5 ml of 0.2 *M* phosphate buffer (pH 7.2), 1 ml of 1 *N* HCl, and 5 ml of ether. The sample is extracted by mixing[8] for 5 min at 40 rpm, followed by centrifugation at 3000 rpm for 5 min. The ethereal layer is removed by aspiration, and the extraction is repeated with another 5 ml of ether.

Following centrifugation, the ethereal layer is discarded and an aliquot of the aqueous layer is transferred (8 ml for plasma and 11 ml for urine) into another similar centrifuge tube containing 2 ml of 1 *N* NaOH. The sample is extracted with 6 ml of ether by mixing as before for 10 min at 40 rpm, followed by centrifugation at

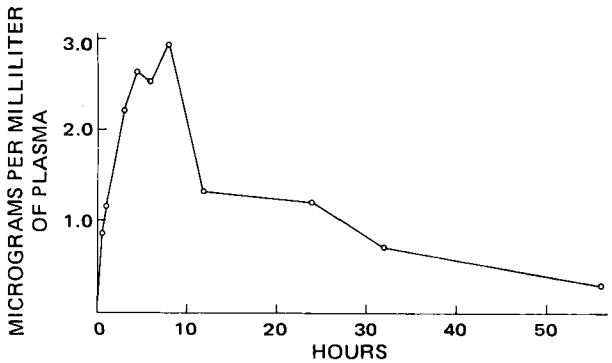
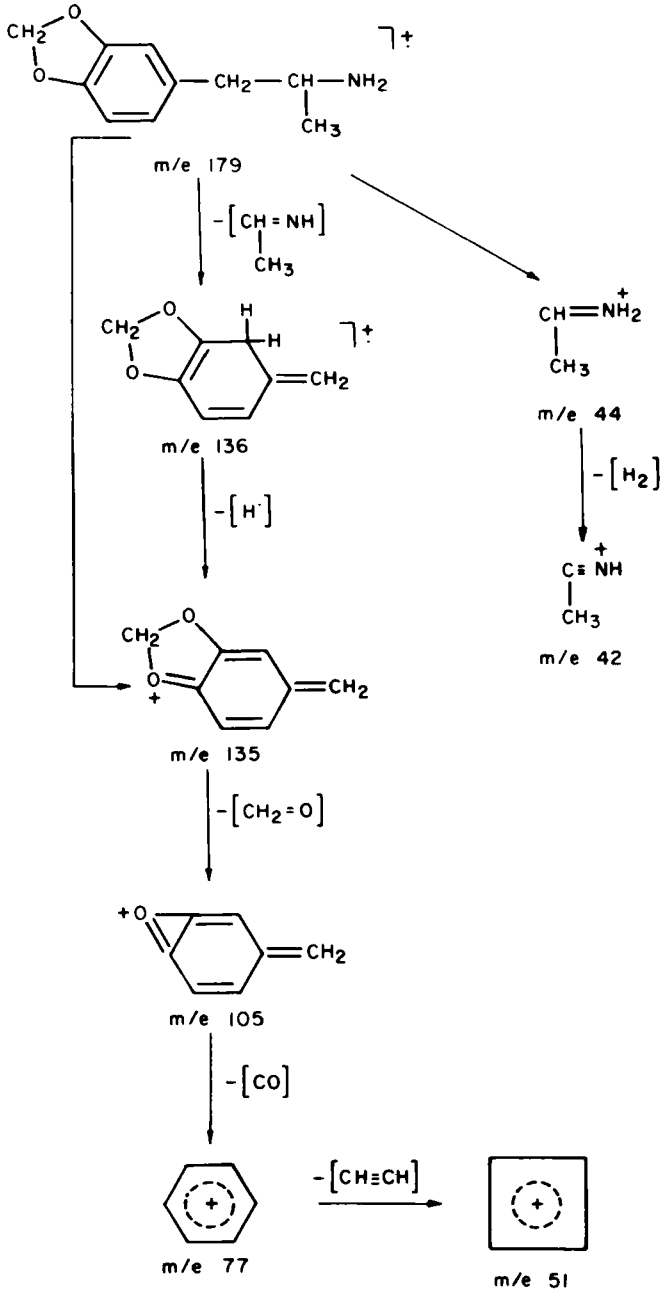


Figure 2—*Plasma concentration of I in a dog weighing 8.38 kg following a single oral dose of I-HCl (10 mg/kg).*

[8] Roto-Rack, Fisher Scientific Co., Montréal, Québec, Canada.



Scheme I—Postulated fragmentation of I

3000 rpm for 5 min. Five milliliters of the ethereal layer is transferred into a custom-made evaporating tube (6) containing a small antibumping granule[9].

The extraction is repeated as before with another 6 ml of ether. Six milliliters of the ethereal layer is removed and combined with the first extract. The combined extracts are concentrated[10] under a stream of dry nitrogen at 40° to a volume of approximately 20–30 μl. Aliquots (1–2 μl) then are injected into the gas chromatograph[11] equipped with a flame-ionization detector.

Derivatization—*Acetone Schiff Bases and Isothiocyanate Derivatives*—To the concentrated ethereal extracts from plasma or urine is added 0.5 ml of acetone (for Schiff base) or carbon disulfide (for isothiocyanate). The tubes are swirled on a mixer[12] for 30 sec and then concentrated to 50 μl at 60° under a stream of dry ni-

[9] British Drug House, Toronto, Ontario, Canada.
[10] Thermolyne Dri-Bath, Fisher Scientific Co., Montréal, Québec, Canada.
[11] Model F/11, Perkin-Elmer, Montréal, Québec, Canada.
[12] Vortex Genie, Fisher Scientific Co., Montréal, Québec, Canada.

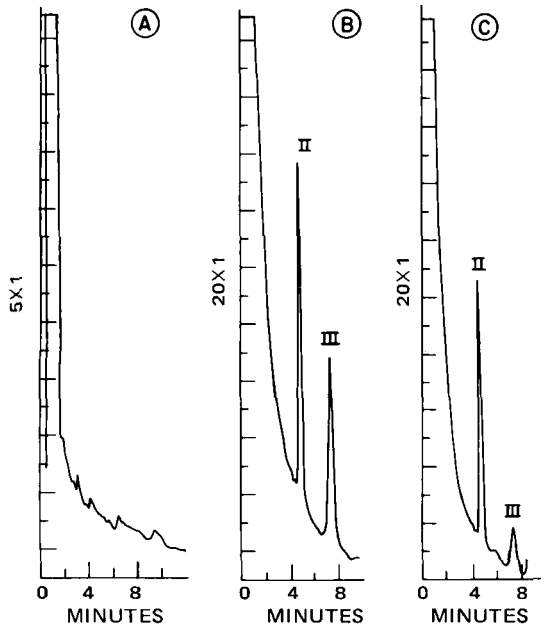
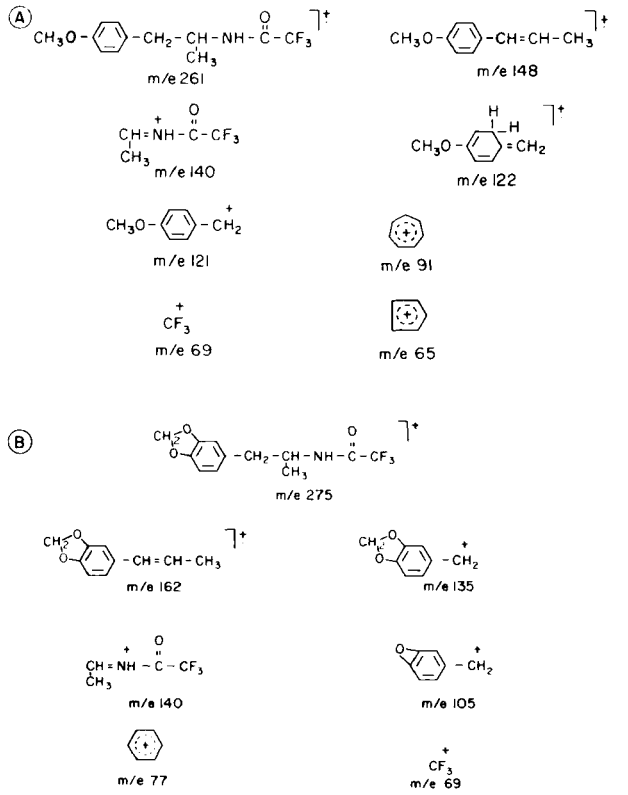


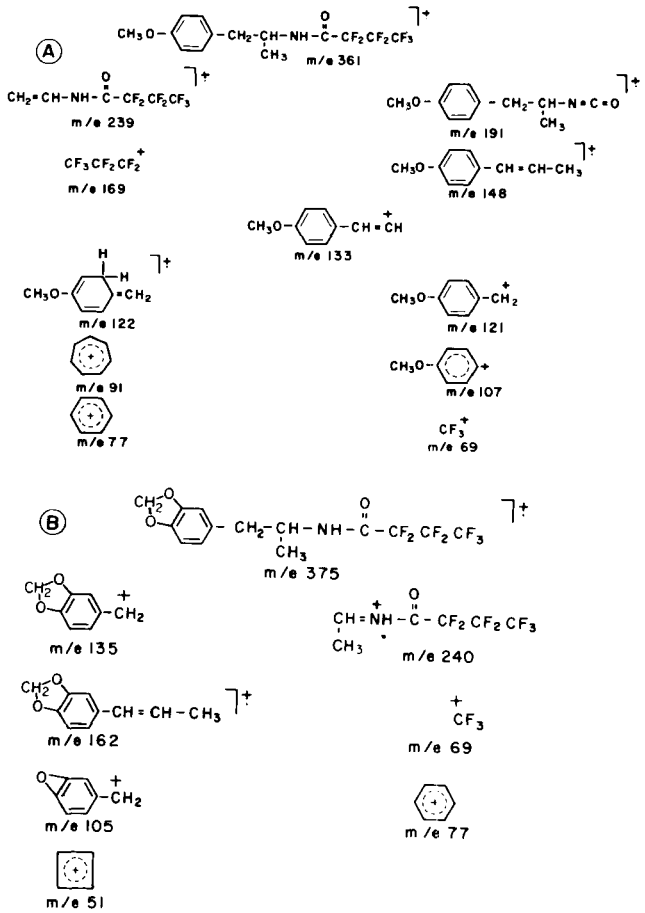
Figure 3—*Gas–liquid chromatogram of dog plasma. Key: A, control plasma; B, control plasma spiked with I (2.0 μg/ml) and II (5.0 μg/ml); C, plasma (12 hr) from a dog given 10 mg/kg po of I-HCl containing I (1.32 μg/ml) and II (5.0 μg/ml); peak II, II; and peak III, I.*

trogen. Aliquots (1–2 μl) are injected into the gas chromatograph[13].

Heptafluorobutyrate Derivatives—To dried ethereal extracts from plasma or urine are added 100 μl of freshly distilled ethyl acetate and 50 μl of heptafluorobutyric anhydride. The tubes are stoppered tightly, swirled on a mixer[12] for 30 sec, and then incubated at 60° for 1 hr. Concentrated ammonium hydroxide (0.5 ml) is added, and the mixture is extracted with 2.5 ml of glass-distilled benzene. The benzene extract is concentrated to 50 μl under a stream of dry nitrogen at 60°, and aliquots (1–2 μl) then are injected into the gas chromatograph[13].



Scheme II—Postulated fragmentations of trifluoroacetyl derivatives of II (A) and I (B)



Scheme III—Postulated fragmentations of heptafluorobutyryl derivatives of II (A) and I (B)

Trifluoroacetyl Derivatives—Dried ethereal extracts were treated in the same manner as described for heptafluorobutyrate derivatives, except that trifluoroacetic anhydride was used instead of heptafluorobutyric anhydride.

GLC—GLC was effected on two gas chromatographs[11,13], both equipped with flame-ionization detectors. System I[11] consisted of a glass column, 1.8 m × 0.3 cm (6 ft × 0.125 in.) i.d. It was packed with saturated hydrocarbon lubricant[14] (20%) along with potassium hydroxide (10%) on acid-washed, dimethyldichlorosilane-treated, high performance flux-calcined diatomite support[15] (80–100 mesh).

The column was conditioned for 20 hr at 290° (oven) with a low nitrogen flow. The conditions of analysis for the assay were: column oven temperature, 190°; injection port temperature, 250°; and detector block temperature, 240°. The carrier gas was nitrogen at a flow rate of 60 ml/min. The hydrogen and compressed air were adjusted to give maximum response.

System II[13] consisted of a glass column, 1.8 m × 0.25 cm (6 ft × 0.10 in.) i.d., silanized with a 5.0% solution of trimethylchlorosilane[16] and hexamethyldisilazane[16] (1:1) in ether. It was packed with 5.0% phenylmethyldimethyl silicone[17] coated on acid-washed, dimethyldichlorosilane-treated, high performance flux-calcined diatomite support[15]. The column was conditioned by injecting a silylating mixture[18] (25 μl) and maintaining the column at 300° for 18 hr under low nitrogen flow. The injection port temperature and

[13] Model 3920, Perkin-Elmer, Montréal, Québec, Canada.

[14] Apiezon-L, Apiezon Products Ltd., 8 York Road, London, England.

[15] Chromosorb W, Chromatographic Specialties, Brockville, Ontario, Canada.

[16] Chromatographic Specialties, Brockville, Ontario, Canada.

[17] OV-7, Chromatographic Specialties, Brockville, Ontario, Canada.

[18] Silyl-8, Pierce Chemical Co., Rockford, Ill.

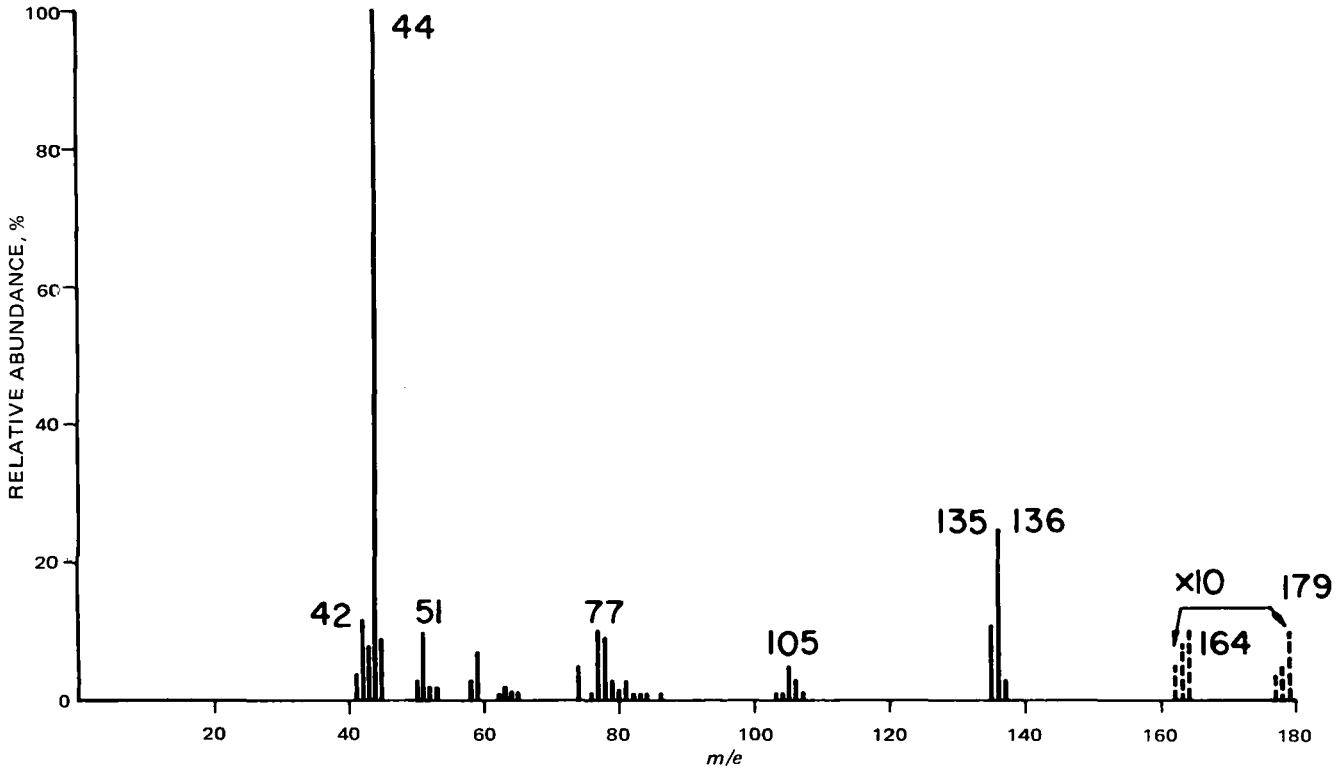


Figure 4—*GLC–mass spectra of I (peak III, Fig. 3C).*

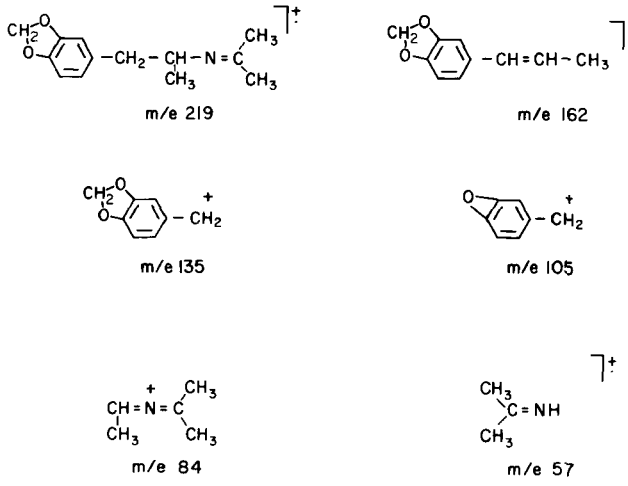
detector block temperature were 240 and 230°, respectively. The oven temperatures employed ranged from 170 to 200°. Nitrogen was the carrier gas at a flow rate of 63 ml/min. Hydrogen and compressed air were adjusted to give maximum response.

Calculations—Peak height ratios were calculated by dividing the height of the peak due to I by the height of the peak due to II. Calibration curves were constructed from the results of spiked control plasma samples by plotting the concentration of I in micrograms per milliliter against the respective peak height ratios.

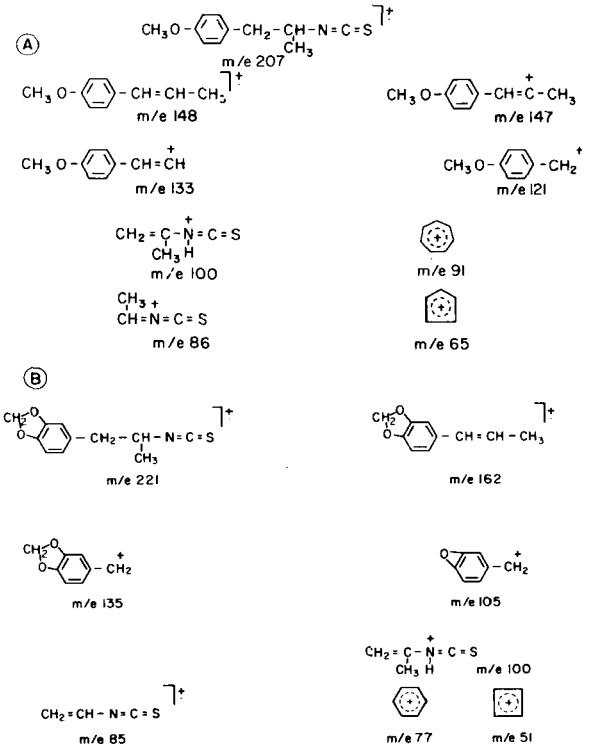
RESULTS AND DISCUSSION

Figure 1A shows a typical chromatogram obtained by processing control blank human plasma, as described in the general procedure, in which the internal standard II was omitted. The extraction procedure provided a relatively clean chromatogram, with only one extraneous peak from endogenous material in plasma with a retention time of 5.6 min (peak I, Fig. 1A). A chromatogram obtained when the method was applied to spiked human plasma containing 0.5 μg of I and 3.0 μg of II is shown in Fig. 1B; it is clear that the extraneous peak I did not interfere with the peak of II (6.04 min) or I (peak III, 9.6 min). An analysis time of 10 min was achieved.

The response of the flame-ionization detector was linear with concentrations in the 0.125–4.0-μg/ml range. The peak height ratio



Scheme IV—Postulated fragmentations of Schiff base of I



Scheme V—Postulated fragmentations of the isothiocyanate derivatives of II (A) and I (B)

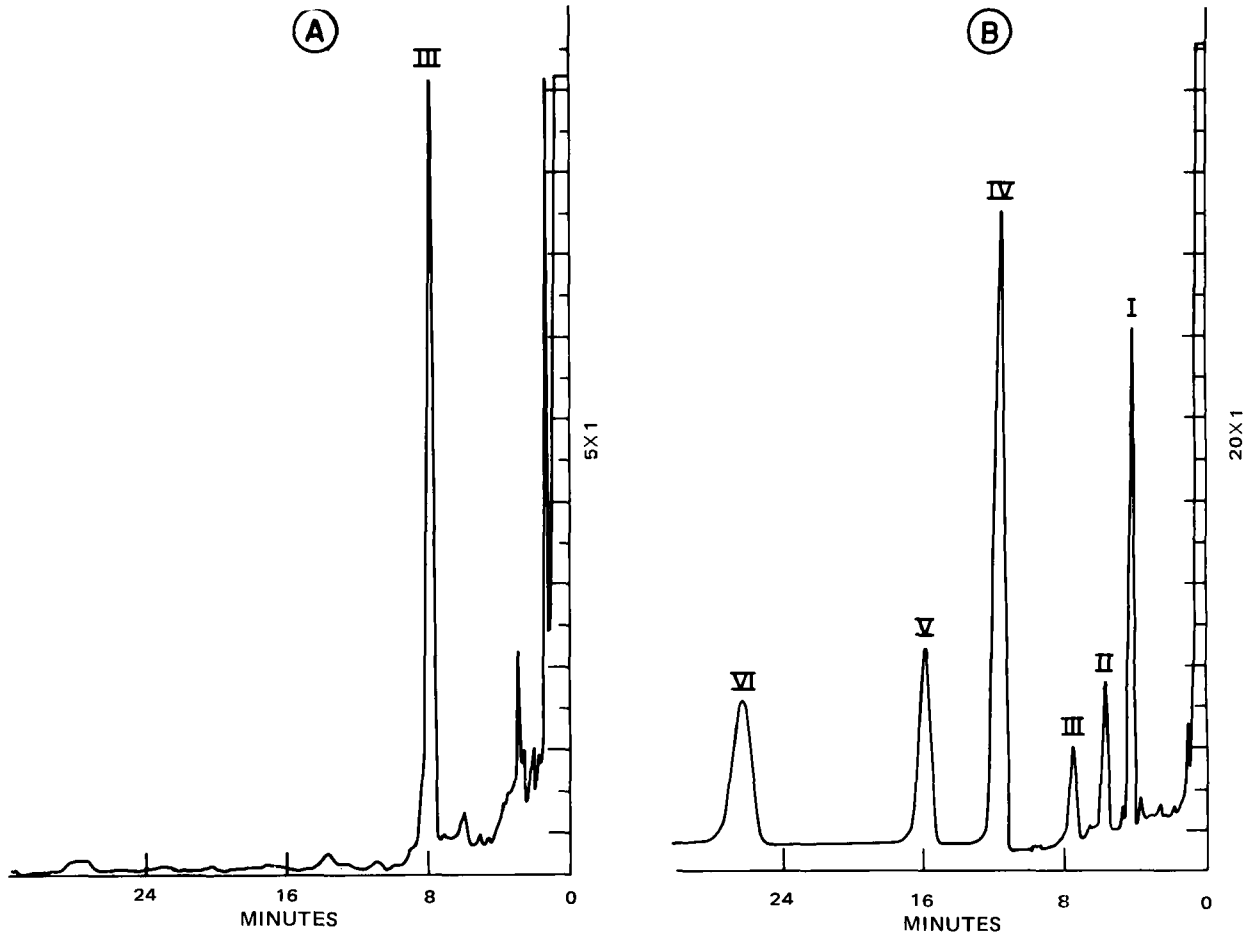


Figure 5—*Gas-liquid chromatograms of human urine extracts. Key: A, control urine; B, control urine spiked with* dl-*amphetamine, methamphetamine,* n-*propylamphetamine, II, and I; peak I,* dl-*amphetamine; peak II, methamphetamine; peak III, endogenous material; peak IV,* n-*propylamphetamine; peak V, II; and peak VI, I.*

of I and II was used as the index of the detector performance and overall efficiency of the analytical procedure. The overall recoveries of I and II from plasma were about 100.29 ± 1.15 and 100.30 ± 0.64%, respectively (Table I).

The accuracy and precision of the GLC assay for I are demonstrated in Table II. Results are based on at least four determinations of each I concentration, ranging from 0.125 to 4.0 μg/ml, which were treated as described in the general procedure. The overall coefficient of variation was 2.22%. The calibration curve obtained by plotting the peak height ratio of I–II *versus* the concentration of I was linear ($y = mx$) in the concentration range of 0.125–4 μg of I/ml of plasma and passed through the origin. A mean slope value of 0.415 ± 0.004 ($r^2 = 1$) was obtained.

Application of this method is demonstrated in Fig. 2. A 10-mg/kg dose of I-HCl was given orally to a dog (8.4 kg), and plasma was withdrawn at intervals over 56 hr and analyzed for I by the described GLC procedure. No interference was observed from the endogenous materials in dog plasma (Fig. 3). Retention times for I and II were slightly shorter due to a change in the carrier gas pressure.

To confirm that I and II were being measured in the dog study and not any endogenous materials from plasma, plasma extracts from the dog were pooled together and injected into the gas chromatograph. Mass spectra[19] were obtained for peaks III and II (Fig. 3C). The normalized mass spectrum of the compound giving rise to peak III is shown in Fig. 4. Structures for the molecular ion at *m/e* 179, the base peak at *m/e* 44, and other abundant ions at *m/e* 136, 135, 105, 77, 51, and 42 are postulated in Scheme I. The mass spectrum of the compound giving rise to peak II (Fig. 3C) was identical to that of synthetic II (7). These observations confirm that intact I

[19] Perkin-Elmer model 900 gas chromatograph attached to a Hitachi Perkin-Elmer model RMSU mass spectrometer through a jet-separator.

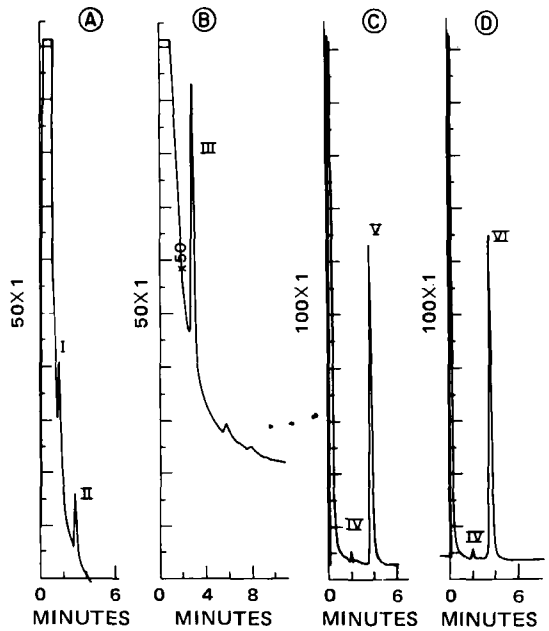


Figure 6—*Gas-liquid chromatograms of trifluoroacetyl and heptafluorobutyryl derivatives of I and II. Key: A, trifluoroacetyl derivative of I; B, heptafluorobutyryl derivative of I; C, trifluoroacetyl derivative of II; D, heptafluorobutyryl derivative of II; peak I, I; peak II, trifluoroacetyl derivative of I; peak III, heptafluorobutyryl derivative of I; peak IV, II; peak V, trifluoroacetyl derivative of II; and peak VI, heptafluorobutyryl derivative of II.*

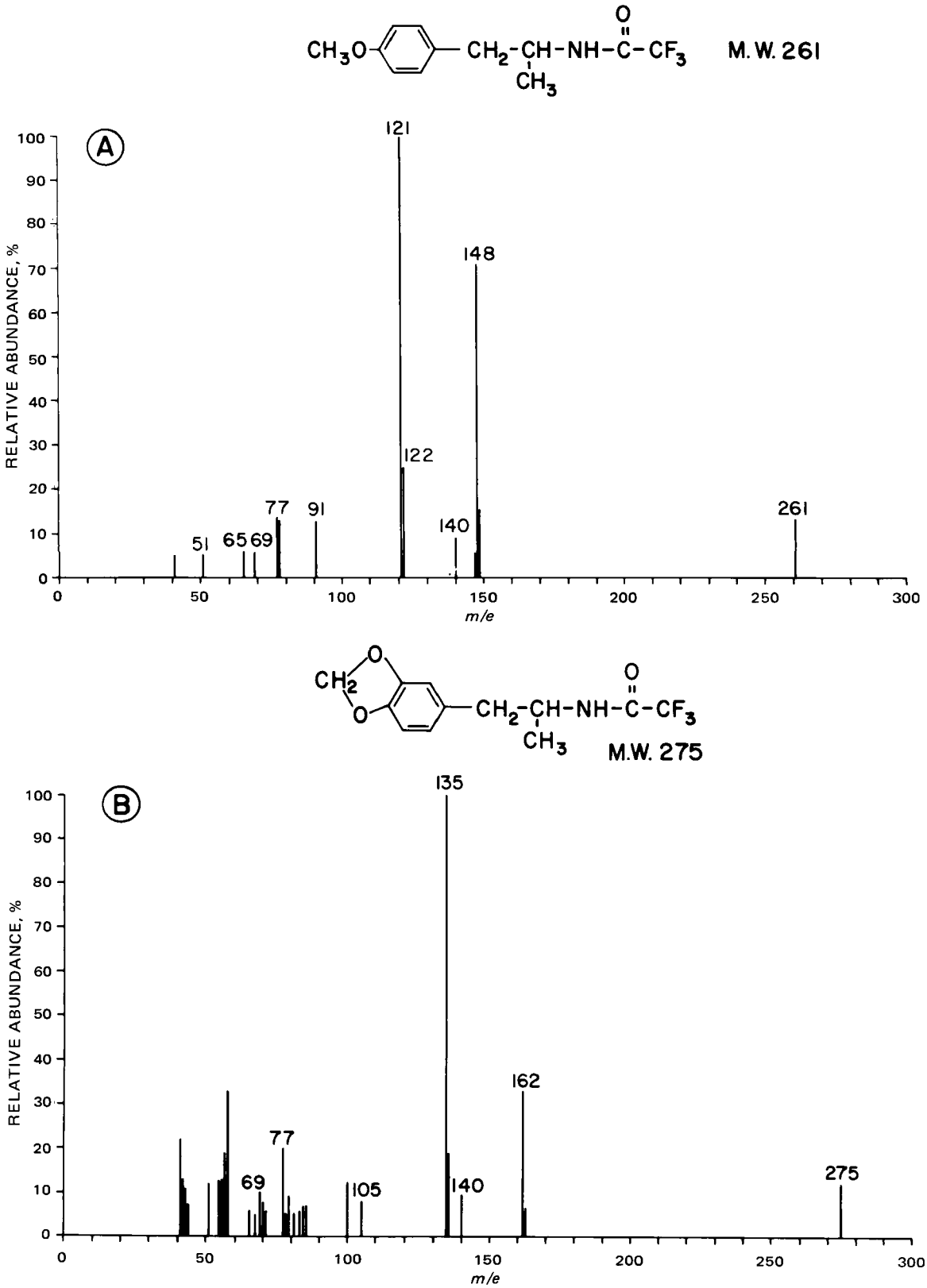


Figure 7—*GLC–mass spectra of trifluoroacetyl derivatives of II (A) and I (B).*

and II, devoid of any plasma interference, are measured in this assay.

The described GLC procedure is also applicable to the estimation and identification of *dl*-amphetamine[5], *N*-methylamphetamine[5], *n*-propylamphetamine[5], I, and II from human urine (Fig. 5). Blank control urine spiked with these compounds was processed according to the procedure given under *Experimental* and analyzed on chromatographic System I with a column oven temperature of 160°. The extraneous peak III (retention time 7.60 min, Fig. 5A) from urine did not interfere with the peaks due to *dl*-amphetamine, *N*-methylamphetamine, *n*-propylamphetamine, II, and I, *i.e.*, peaks I, II, IV, V, and VI, respectively (Fig. 5B). Any

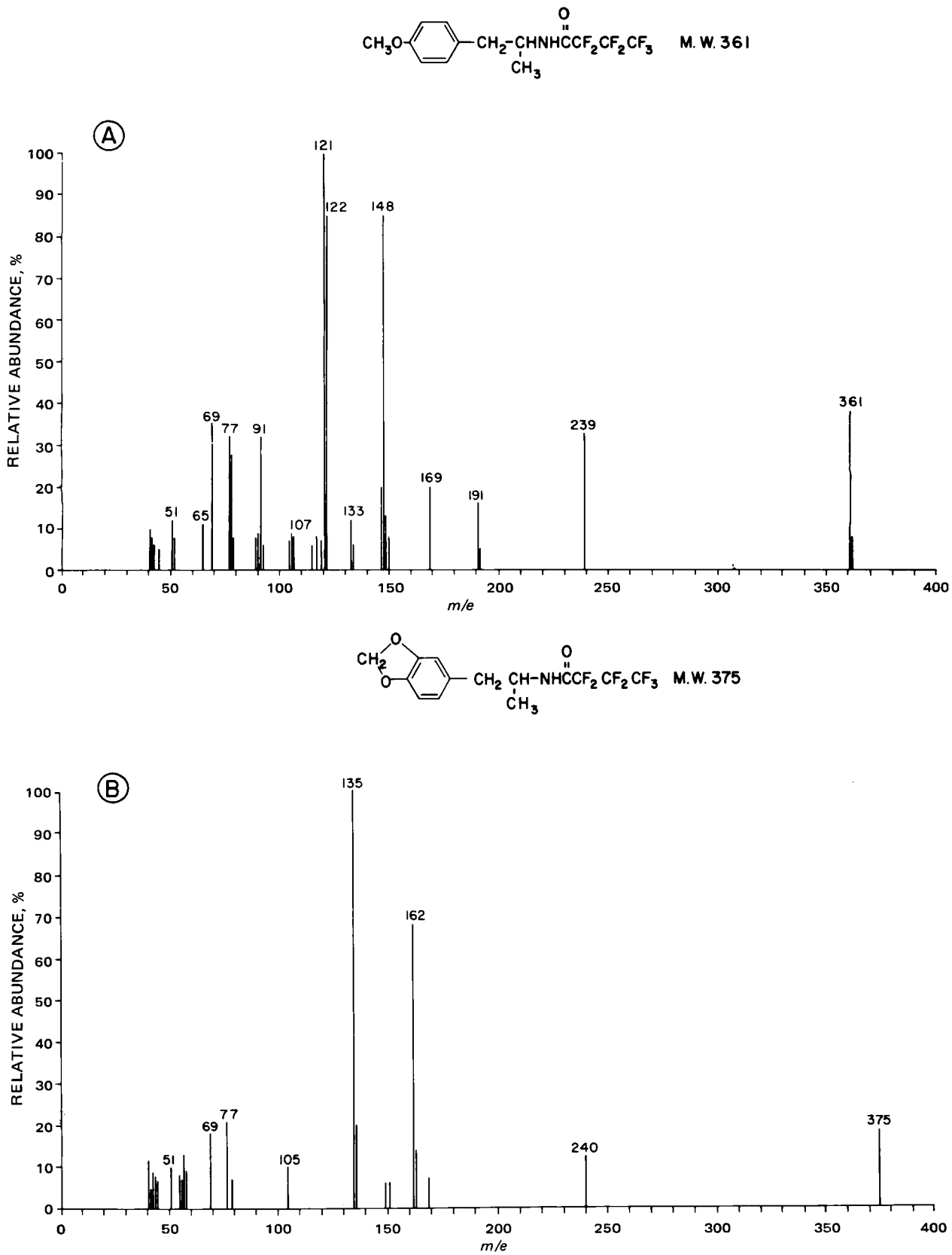


Figure 8—*GLC–mass spectra of heptafluorobutyryl derivatives of II (A) and I (B).*

of these compounds can be estimated in urine by choosing an internal standard. For quantitative analysis of I, II was chosen as the internal standard. The column oven temperature (System I) was raised to 190° to shorten analysis time to 10 min.

Fluoroacyl derivatives formed from trifluoroacetic and heptafluorobutyric anhydrides give good electron-capture response and have been used in the quantitative analysis of amphetamines and their metabolites in rat brain (8) and in biological fluids (9–11) at the nanogram level. However, use of these derivatives has not been explored in the qualitative or quantitative analysis of II and I. In

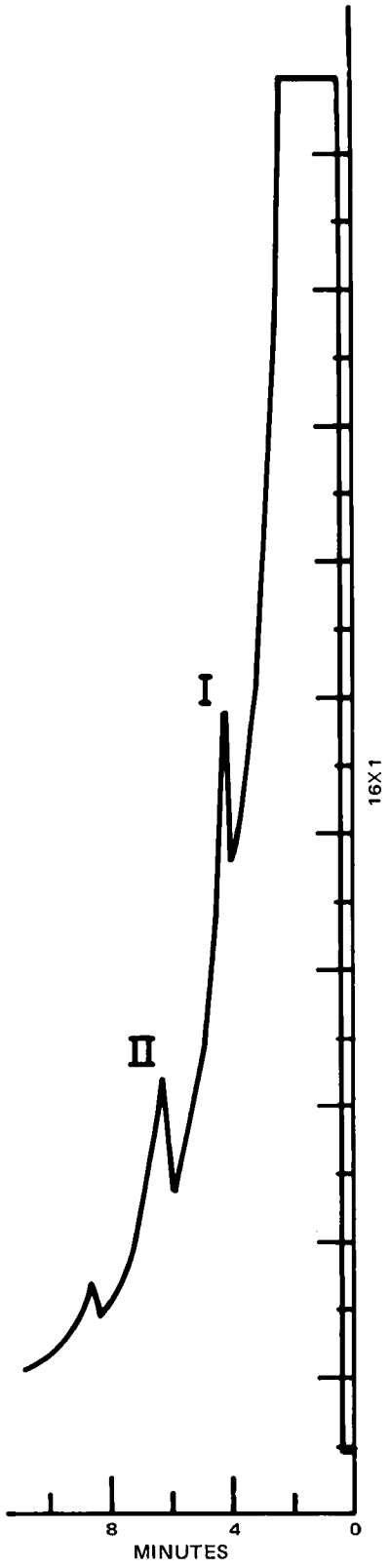


Figure 9—*Gas–liquid chromatogram of heptafluorobutyrates of II (2.5 ng/ml) and I (2.5 ng/ml) with electron-capture detector. Key: peak I, heptafluorobutyrate derivative of II; and peak II, heptafluorobutyrate derivative of I. The conditions were: glass column, 1.8 m × 2 mm i.d. packed with OV-7 on Chromosorb W (80–100 mesh); pulse, 3.5; nitrogen purge gas, 20 ml/min; electron-capture detector temperature, 310°; oven temperature, 165°; and injection port temperature, 220°.*

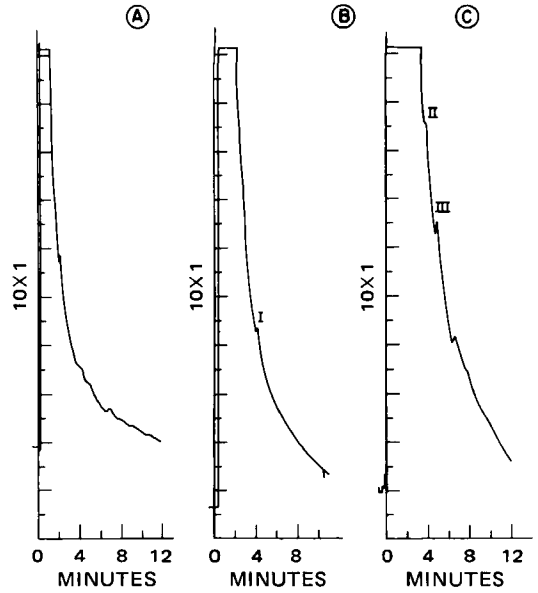


Figure 10—*Gas–liquid chromatograms of Schiff bases of II and I from human urine extract. Key: A, control urine; B, control urine spiked with II (0.25 μg/ml); C, control urine spiked with I (0.25 μg/ml); peak I, Schiff base of II; peak II, I; and peak III, Schiff base of I.*

the present study, these derivatives of II and I were formed and their GLC behavior was examined.

Figure 6 shows the trifluoroacetyl and heptafluorobutyryl derivatives of II and I on System II. The column oven temperature was 190°. The trifluoroacetate derivative of I (peak II, Fig. 6A) and the heptafluorobutyrate derivative of II (peak III, Fig. 6B) had retention times of 3.20 and 2.90 min, respectively. The trifluoroacetyl (peak V, Fig. 6C) and heptafluorobutyryl (peak VI, Fig. 6D) derivatives of II had retention times of 3.58 and 3.43 min, respectively, with a column oven temperature of 170° on System II. GLC–mass spectral[19] evidence of the formation of the trifluoroacetyl derivatives of II and I was obtained. Normalized mass spectra of trifluoroacetyl derivatives of II and I are shown in Figs. 7A and 7B. The molecular ion in each case is present, and other major ions are postulated for these derivatives in Scheme II. These ions indicate the formation of trifluoroacetyl derivatives of II and I.

Similarly, GLC–mass spectral[19] evidence for the formation of heptafluorobutyryl derivatives of II and I was obtained. Normalized mass spectra of these derivatives are shown in Fig. 8. The mass spectrum of the heptafluorobutyryl derivative of II (Fig. 8A) showed the molecular ion at *m/e* 361 and other major ions at *m/e* 239, 191, 169, 148, 122, 121, 107, 91, 77, 69, 65, and 51, indicative of

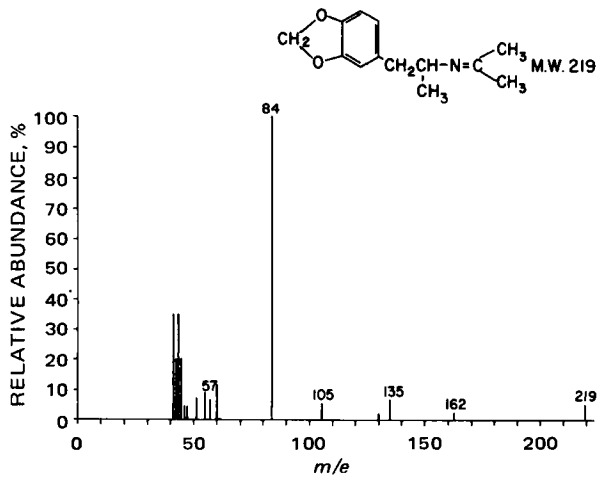


Figure 11—*GLC–mass spectra of Schiff base of I (peak III, Fig. 10C).*

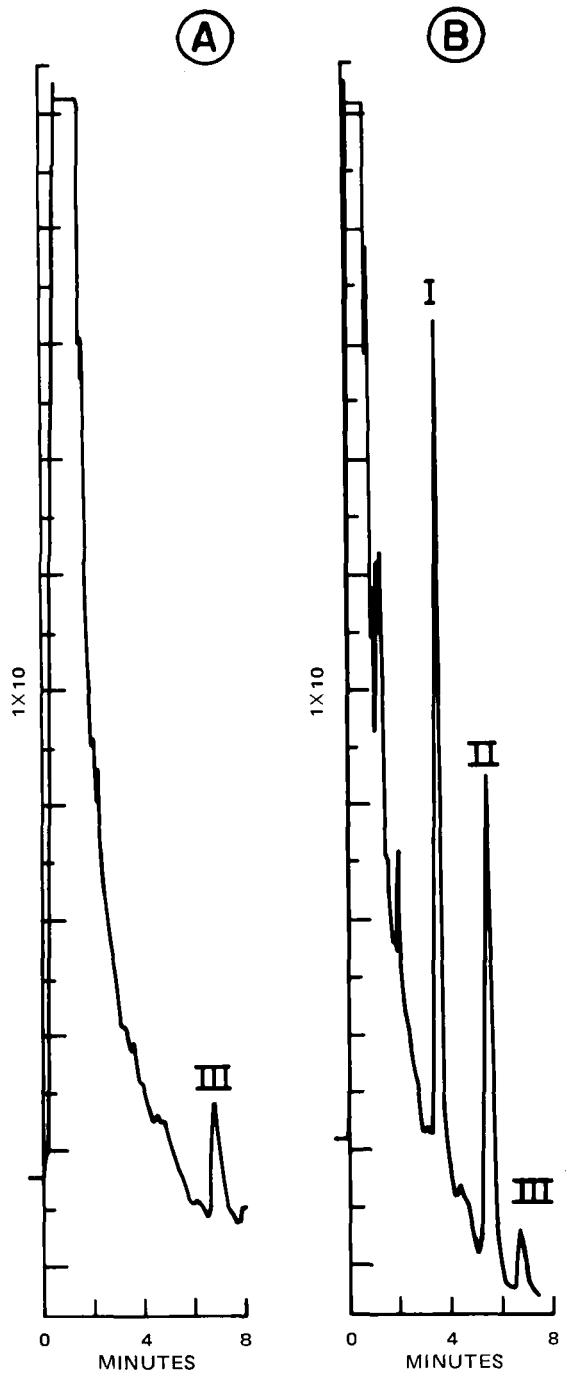


Figure 12—*Gas-liquid chromatograms of isothiocyanate derivatives of II and I from human urine extract. Key: A, control urine; B, control urine spiked with I and II (0.25 μg/ml of each); peak I, isothiocyanate derivative of II; peak II, isothiocyanate derivative of I; and peak III, endogenous material.*

the formation of the II derivative. Structures for these ions are postulated in Scheme IIIA. The heptafluorobutyryl derivative of I (Fig. 8B) also showed the molecular ion at *m/e* 375 and other diagnostic ions at *m/e* 240, 162, 135, 105, 77, 69, and 51. Postulated structures for these diagnostic ions are shown in Scheme IIIB, which confirm the formation of the heptafluorobutyryl derivative of I.

The heptafluorobutyryl derivatives showed tremendous electron-capture response. Levels as low as 1 ng can be detected and estimated. Figure 9 shows the chromatogram of 2.5 ng of I and II added to plasma, extracted according to the method of Walle and Ehrsson (11), and derivatized according to the details given under *Experimental*. By employing a ^{63}Ni-electron-capture detector in System II with a column oven temperature of 165°, 1–5 ng of II and I may be quantitated.

Qualitative identification of a substance in biological fluids by GLC is generally accompanied by comparison of its retention times after extraction from the fluids with those of the reference material on different columns. An alternative approach is to form derivatives of the unknown compound after extraction and to compare the retention times of similar derivatives of the reference material on the same column. For qualitative identification of II and I from urine, their Schiff bases and isothiocyanate derivatives were prepared after extraction from urine according to the method of Beckett *et al.* (12). Figure 10B shows urine spiked with II, and Fig. 10C shows urine spiked with I. No interference was observed from either I or II. System II was employed for chromatographic analysis with a column oven temperature of 180°. The Schiff bases for I and II had retention times of 6.65 (peak III, Fig. 10C) and 3.79 (peak I, Fig. 10B) min, again with System II with the oven at 180°. To confirm that the Schiff bases of I and II were formed (which would also afford conclusive proof of their presence in biological fluids), the mass spectrum of peak III (Fig. 10) was obtained[19]. The normalized mass spectrum of the compound giving rise to peak III is shown in Fig. 11. The molecular ion at *m/e* 221 and other major ions at *m/e* 185, 162, 105, 84, and 57 were present, consistent with formation of the Schiff base of I. Structures for these ions are postulated in Scheme IV. Similar confirmation of the formation of the Schiff base of 4-methoxyamphetamine was obtained.

Figure 12 shows the chromatograms of isothiocyanate derivatives of II (peak I, 3.38 min) and I (peak II, 5.43 min) along with that of blank control urine treated in the same manner as described under *Experimental*. Peak III (Fig. 12A) from endogenous material in blank control urine did not interfere with peaks due to isothiocyanate derivatives of II and I (peaks I and II) in System II with a column oven temperature of 200°. To confirm the formation of these isothiocyanate derivatives, eluates from the GLC column System II were fed directly into the mass spectrometer[19], and the mass spectra of compounds giving rise to peaks I and II were recorded (Fig. 13).

The mass spectrum of the isothiocyanate derivative of II (Fig. 13A) showed the molecular ion at *m/e* 207 and the base peak at *m/e* 121. Structures for other major ions at *m/e* 148, 147, 133, 100, 91, 86, and 65 are postulated in Scheme VA. The normalized mass spectrum of the isothiocyanate derivative of II is shown in Fig. 13B. Structures for the molecular ion at *m/e* 221, the base peak at *m/e* 135, and other major ions at *m/e* 162, 105, 100, 85, 77, and 51 are postulated in Scheme VB. These mass spectra indicate that isothiocyanate derivatives are formed and can aid in the identification of II and I from biological fluids.

The described GLC procedure is adequate for the qualitative as well as quantitative analysis of I from biological fluids. Formation of various derivatives of hallucinogenic amines, as well as the mass spectral behavior of these derivatives, aids in conclusive identification of these drugs of abuse. The procedure described gives high recoveries of these amines from biological fluids and is of sufficient sensitivity, using the conventional flame-ionization detector, to determine 0.125 μg of I/ml of plasma. The method is also applicable to quantitative estimation of II from biological fluids by using I as an internal standard. It can also be employed for estimating both I and II from biological fluids by using *n*-propylamphetamine as an internal standard. Analysis time with GLC is short (10 min), and multiple determinations can be performed on the same day.

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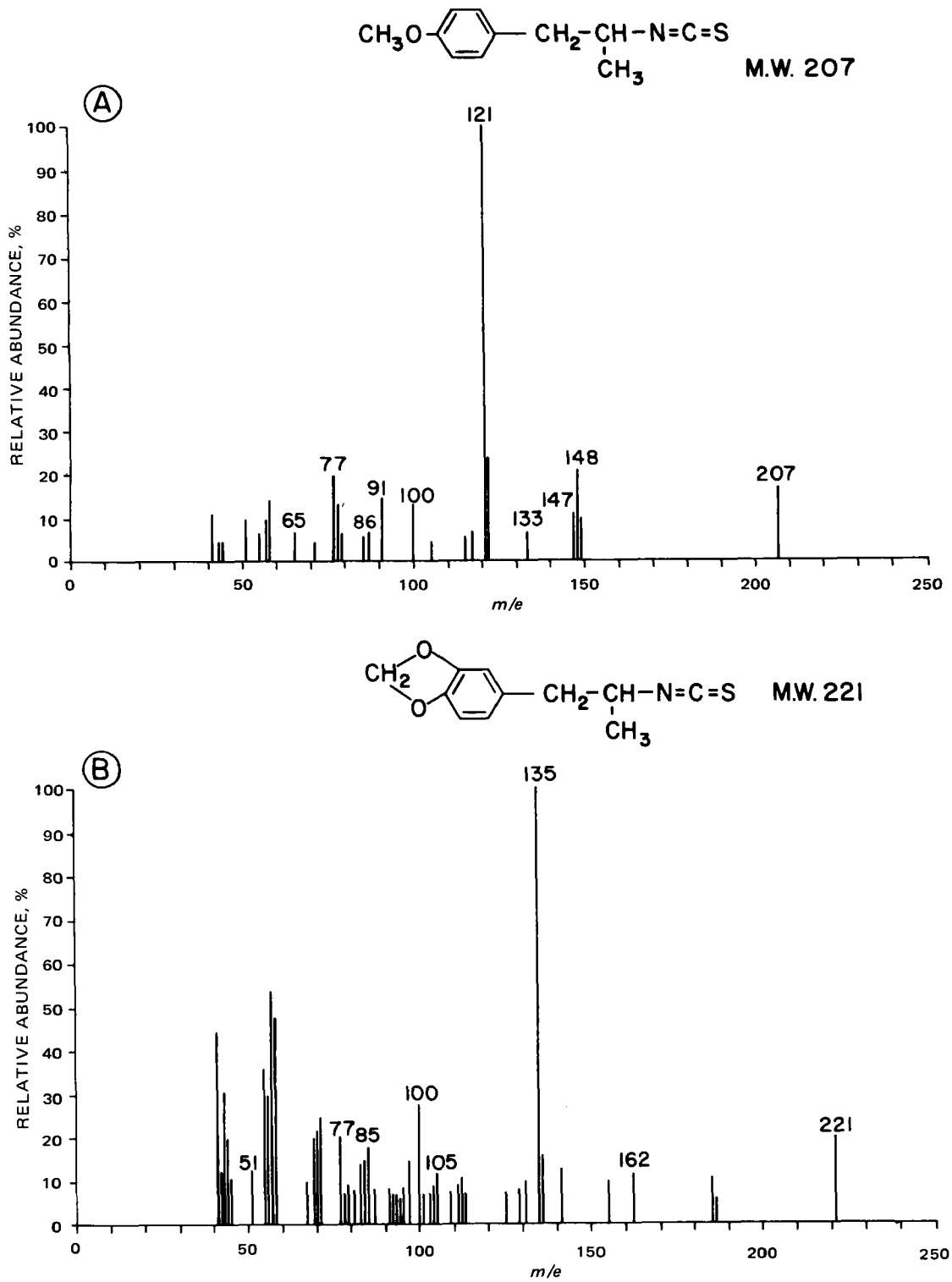

Figure 13—*GLC–mass spectra of isothiocyanate derivatives of II (A) and I (B).*

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