COMBINED THIN-LAYER AND GAS–LIQUID CHROMATOGRAPHIC IDENTIFICATION OF TRICYCLIC ANTIDEPRESSANTS IN URINE

MICHAEL SHEEHAN and PATRICIA HAYTHORN

Department of Pathology, School of Medicine, University of California, Davis, Calif. 95616 (U.S.A.)

(First received May 11th, 1976; revised manuscript received July 19th, 1976)

SUMMARY

A combined thin-layer and gas–liquid chromatographic (TLC–GLC) procedure for determination of tricyclic antidepressant drugs in urine is described. GLC on 1.5% OV-17 was utilized to analyze basic urine extracts directly for the tricyclics and to confirm the identity of spots extracted from thin-layer chromatograms, and TLC was used to confirm the results of these GLC screens. Lower limits of sensitivity for the thin-layer spot extraction procedure were found to range from 2 μg/ml for amitriptyline and imipramine to 10 μg/ml for desipramine and nortriptyline. Turn-around-times for the GLC tricyclic screens were usually less than 1 h. The finding that nortriptyline and desipramine were chemically altered under various extraction conditions was used as a means of confirming the identity of these drugs, and the identity of these chemically altered derivatives was discussed with reference to their low resolution mass spectra.

INTRODUCTION

Tricyclic antidepressant drugs (amitriptyline, doxepin, imipramine, nortriptyline, and desipramine) are frequently used in the treatment of anxiety and depression, and it is not surprising that the incidence of self-poisonings with these drugs has increased dramatically in recent years[1,2]. Spiker *et al.*[1] reported recently that approximately 25% of all drug-overdosed patients seen in their emergency room were overdosed primarily with a tricyclic. The most dangerous complication in patients poisoned with these drugs is the cardiovascular toxicity[3–5]. Physostigmine has been reported[3,6] to rapidly reverse the cardiovascular action and antagonize the central nervous system effects of the tricyclic antidepressants, and as such, this drug has found frequent use in the treatment of patients overdosed with these drugs. However, because the administration of physostigmine itself is not without risk[7], it is important that identification of tricyclics be established prior to treatment with this drug. Unfortunately, most laboratory methods of analysis for tricyclics are either very time consuming or do not provide unequivocal identification of the tricyclics or both. Furthermore, no method has been reported which simultaneously analyzes for all five major tricyclics.

The rapid urine colorimetric test of Forrest et al.[8] is fairly specific, but it does not give positive reactions with doxepin, amitriptyline, or nortriptyline. UV spectrophotometric techniques[9-11] have found frequent use for the various individual tricyclics, but these techniques either employ tedious extraction procedures or require chemical transformation of the tricyclics prior to analysis. Thin-layer chromatography (TLC), although not very expedient, is undoubtedly the most widely used technique for identification of these drugs in urine, and several procedures have been reported[10,12,13]. Several gas chromatographic (GLC) procedures have also been reported[14-19], although again none of them have been used to analyze for all of the tricyclics simultaneously. The technique of combined GLC and mass fragmentography has been used to quantitate tricyclics in blood and urine[1,20-22], but this technique can hardly be considered practical for most routine laboratories.

This report describes a combined thin-layer and gas–liquid chromatographic (TLC–GLC) approach for the simultaneous identification of the tricyclics doxepin, amitriptyline, imipramine, nortriptyline, and desipramine in human urine. Herein is described not only a GLC method for the confirmation of the TLC results, but also a rapid GLC screen for tricyclics from urine directly.

MATERIALS AND METHODS

Apparatus

A Model 2100 (Varian Aerograph, Walnut Creek, Calif., U.S.A.) gas chromatograph equipped with dual flame ionization detectors was used in these studies. The instrument was fitted with U-shaped glass columns (1.83 m × 2 mm I.D.) packed with 1.5% OV-17 on 80–100 mesh Chromosorb G-HP (Applied Science Lab., State College, Pa. ,U.S.A.). The column was conditioned before use by heating to 300° overnight. Operating conditions were as follows: nitrogen as carrier gas was set at 30 ml/min, hydrogen at 30 ml/min, air at 300 ml/min, the injection port and detector temperatures at 280°, and the initial column oven temperature at 180°; the program rate was 10°/min up to 280° with 5 min isothermal at 280°.

Mass spectral analyses were performed on a Finnigan Model 3200 gas chromatograph–mass spectrometer with an electron energy of 60 eV and an emission current of 450 μA. The GLC conditions for this system were identical to those described above.

Reagents

All solvents were chromatoquality and were used without further purification unless otherwise indicated.

NH₄Cl–NH₄OH Buffer. To 100 ml of saturated NH_4Cl solution is added concentrated NH_4OH at 25° until pH 10 ± 0.1 is reached (approximately 80 ml).

Borate buffer. A solution of 100 ml of 25 mmoles/l Borax and 35.4 ml of 100 mmoles/l HCl is diluted to 200 ml with deionized water (pH 8.3 ± 0.1 at 25°).

Carbonate buffer. To 420 mg of $NaHCO_3$ is added 21 g Na_2CO_3 and 50 ml of deionized water. The pH is then adjusted to 11 ± 0.1 at 25° with 1 mole/l HCl or 1 mole/l NaOH.

Nalorphine standard. A 5 μg/μl aqueous standard was prepared by dissolving 5.0 mg nalorphine hydrochloride (Merck, Sharp and Dohme, West Point, Pa., U.S.A.) in 1 ml deionized water.

   Cyheptamide standard. A 0.5 μg/μl methanolic cyheptamide standard was prepared by dissolving 50 mg of cyheptamide (Ayerst Labs., New York, N.Y., U.S.A.) in 100 ml of absolute methanol.

Thin-layer chromatography

   All chromatography was performed on 10 × 20 cm glass chromatoplates coated with an absorbent layer of 250 μm silica gel 60 F_{254} (E. Merck, Darmstadt, G.F.R.). The developing solvent was prepared fresh daily and consisted of ethyl acetate, methanol, and concentrated ammonium hydroxide in the ratio of 170:20:10 ml, respectively.

Spray reagents

   The spray reagents and their composition were as follows: (a) 0.4% ninhydrin, prepared by dissolving 0.4 g ninhydrin in 100 ml of acetone, (b) 0.5% sulfuric acid, and (c) neutral iodoplatinate, prepared by adding 1 ml of 10% platinum chloride to 25 ml of a 4% solution of potassium iodide.

Extraction procedure (modification of the method of Davidow et al.[23])

   To 15 ml of urine in a 50-ml centrifuge tube were added 10 μl of the 5 μg/μl nalorphine standard and enough ammonium chloride–ammonium hydroxide buffer (one to five drops) to bring the pH of the urine to 8.5 $\pm$ 0.1. To this solution were added 15 ml of chloroform–isopropanol (9:1) solvent (AR grade), and the resultant mixture was shaken gently on a mechanical shaker for 5 min and centrifuged at 1500 rpm for 10 min. After aspirating the aqueous layer to waste, the organic layer was filtered through approximately 5 g of anhydrous sodium sulfate. Subsequently four drops of 0.5% methanolic sulfuric acid were added to this solution, and the extract was evaporated to dryness at 40° under a gentle stream of nitrogen.

Development and spraying procedure (modification of the method of Mulé[24])

   The residues from the extracts were reconstituted with 25 μl of methanol and spotted in entirety onto the silica gel plates. After allowing the spots to dry, the plates were developed in rectangular developing tanks by allowing the solvent front to migrate a distance of 10 cm from the point of application of the extracts. The plates were removed, air dried for 5 min, then oven dried at 100° for 5 min. The following sprays were then applied in succession: (1) 0.4% ninhydrin lightly, followed by UV for 10 min and 100° for 5 min, (2) 0.5% sulfuric acid lightly, and (3) iodoplatinate heavily. The plates were allowed to dry before recording R_F values.

TLC spot extraction procedure (modification of the method of Reynolds[25])

   After allowing the final spray to dry for at least 15 min, the appropriate spot was scraped into a small vial. To this solution were added 1 ml of the pH 8.3 borate buffer ,10 μl of the 0.5 μg/μl cyheptamide standard, and a "pinch" of Na_2SO_3. After swirling the mixture briefly and allowing it to stand for 5 min, 2 ml of chloroform–isopropanol (9:1) solvent (AT grade) were added and the contents of the vial shaken for 5 min on a mechanical shaker. The mixture was then centrifuged for 2 min at 2400 rpm, and the aqueous layer was aspirated to waste. After drying the organic layer over anhydrous Na_2SO_4 briefly, it was transferred to a clean vial and evaporated

to dryness at 50° under a gentle stream of nitrogen. The residue was reconstituted with 50 μl of methanol, and 5 μl of this solution were injected into the gas chromatograph.

GLC extraction procedure

To a 50-ml centrifuge tube were added 10 ml of urine and 20 μl of the 0.5 μg/μl cyheptamide standard. The pH of this mixture was adjusted to 9.0 ± 0.2 with carbonate buffer (around 1 ml of buffer is required for most urines), 10 ml of chloroform was added, and the resultant mixture shaken gently on a mechanical shaker for 5 min and centrifuged at 2000 rpm for 5 min. After removing the aqueous layer, 1 g of anhydrous Na_2SO_4 was added to the chloroform solution and it was swirled briefly. The chloroform solution was transferred to a dry tube and evaporated to dryness at 50° under nitrogen. The residue was reconstituted with 50 μl of methanol, and 5 μl of this solution was injected into the gas chromatograph.

RESULTS

R_F values for the tricyclic antidepressants and the more common drugs of abuse which were found to extract from urine at a basic pH (8.5) were determined, as shown in Table I. Assuming a maximum variation of ± 0.04 for these values, seven drugs were found to have chromatographic properties similar to those of the dimethylamine tricyclics (amitriptyline, imipramine, and doxepin), four to those of nortriptyline, and two to those of desipramine. Most of these drugs could be distin-

TABLE I

R_F VALUES OF VARIOUS DRUGS AND METABOLITES*

Drug	R_F value	Drug	R_F value
Amitriptyline metabolite**	0.10	Pentazocine metabolite**	0.58
Nortriptyline metabolite**	0.10	Nortriptyline	0.58
Doxepin metabolite**	0.12	Nicotine	0.62
Nicotine metabolite**	0.17	Doxepin metabolite	0.64
Morphine	0.17	Norpropoxyphene	0.66
Atropine	0.24	Meperidine	0.67
Desipramine metabolite**	0.25	Promethazine	0.68
Imipramine metabolite**	0.25	Promazine	0.70
Nalorphine	0.27	Diphenhydramine	0.70
Ephedrine	0.28	Imipramine	0.72
Phenylpropanolamine	0.28	Doxepin	0.72
Pentazocine metabolite**	0.30	Amitriptyline	0.74
Codeine	0.34	Pentazocine	0.74
Benztropine	0.40	Thioridazine	0.76
Quinine	0.46	Methaqualone	0.76
Desipramine	0.46	Chlorpromazine	0.78
Methamphetamine	0.48	Methadone	0.80
Amphetamine	0.52	Propoxyphene	0.80
Chlorpheniramine	0.54	Phencyclidine	0.83
Doxepin metabolite	0.54	Methadone metabolite	0.85
Hydroxyzine	0.56		

* R_F values relative to solvent front at 10 cm. See Materials and methods for details.
** Not confirmed with authentic sample.

guished from the tricyclics by their characteristic metabolite patterns and/or color reactions with the various spray reagents, but the possibility of multiple drug ingestion and the run-to-run variation in the TLC parameters prevents unambiguous identification of the tricyclics from these data alone. To confirm the identification of the tricyclics, the appropriate spots from the thin-layer plates after spraying were scraped off and extracted as described above. These extracts were concentrated and subjected to GLC analysis on an OV-17 column, and Table II lists the relative retention times (cyheptamide as standard) of authentic samples of drugs from Table I added to blank urines (also included in this list are drugs of abuse found in urine which might extract under the above conditions). With the exception of the drugs nortriptyline and desipramine (which are discussed in detail below), all drugs treated in this manner had the same relative retention times as corresponding authentic standards which had not been subjected to TLC prior to analysis by GLC; and it was concluded from these findings that the spot extraction procedure did not chemically alter these drugs. Allowing a variation of 0.04 in these relative retention times, only pentazocine and methaqualone had both TLC and GLC properties similar to doxepin, amitriptyline, and imipramine. Metabolite patterns and color reaction differences with the spray reagents make it possible to easily distinguish these drugs from the tricyclics, however.

In order to distinguish true negatives from those urines with low levels of drugs, the sensitivity of the spot extraction procedure was investigated. Known amounts of the appropriate drugs were added to blank urines and extracted at pH 9

TABLE II

RELATIVE RETENTION TIMES FOR VARIOUS DRUGS AND METABOLITES EX-
TRACTED FROM URINE

Drug	RRT*	Drug	RRT*
Nicotine	0.10	Imipramine	0.79
Barbital	0.19	Doxepin	0.81
Butalbital	0.30	Methaqualone	0.84
Butabarbital	0.31	Nortriptyline***	0.84, 1.19
Amobarbital	0.33	Nordiazepam	0.84
Meperidine	0.35	Desipramine***	0.85, 1.24
Pentobarbital	0.37	Pentazocine	0.85
Secobarbital	0.39	Promethazine	0.87
Diphenhydramine	0.44	Promazine	0.95
Phencyclidine	0.46	Norpropoxyphene	1.00
Meprobamate	0.51	Cyheptamide	1.00
Glutethimide	0.52	Oxazepam	1.05
Secondary methadone metabolite	0.54	Codeine	1.05
Caffeine	0.56	Chlorpromazine	1.10
Chlorpheniramine	0.61	Phenytoin	1.21
Primary methadone metabolite	0.62	Diazepam	1.22
Phenobarbital	0.66	Trifluoperazine	1.27
Methadone	0.70	Chlordiazepoxide	1.31
Propoxyphene**	0.46, 0.72	Flurazepam	1.37
Amitriptyline	0.76	Thioridazine	2.10

* Relative to cyheptamide as internal standard. See Materials and methods for GLC details.
** Thermally unstable, gives two peaks.
*** See Discussion for explanation of second peak.

as described above. These extracts were subjected to TLC, and the appropriate spots were removed, extracted, and subjected to GLC. Table III records the lowest concentrations of the drugs in urine at which the peak height of the drug was found to be equal to or greater than 10% of the peak height of the internal standard.

TABLE III

THIN-LAYER CHROMATOGRAPHY SPOT EXTRACTION EFFICIENCIES

Drug	Lower limit of sensitivity ($\mu g/ml$)*
Amitriptyline	2
Doxepin	5
Imipramine	2
Nortriptyline	10
Desipramine	10
Chlorpheniramine	2
Diphenhydramine	30
Meperidine	2
Methadone	2
Methadone metabolites	
Primary ·	20
Secondary	20
Nicotine	10
Norpropoxyphene	15
Pentazocine	5
Propoxyphene	5

* Based on initial concentration of pure drug in urine.

GLC analysis of extracted spots from thin-layer plates is a rather laborious process (4–8 h turn-around-time) and not one that is usually performed more than once a day. For a more rapid screen to rule out the presence of tricyclics, urine was extracted at pH 9 with chloroform and the extracts were analyzed directly by GLC without derivatization using cyheptamide as an internal standard. Since the GLC conditions were identical to those of the spot confirmation procedure, the data listed in Table II apply to both procedures. Screens were considered positive only when the peak height of the tricyclic drug was equal to or greater than 50% of the peak height of the internal standard. Under these conditions, normal endogenous metabolites frequently found in urine did not give chromatographic interference. Positive screens are routinely reported to the physicians and later confirmed by TLC.

DISCUSSION .

By using data from both TLC and GLC, the tricyclic antidepressant drugs could be easily distinguished from the other common drugs of abuse. Methaqualone was the only drug found to have chromatographic properties on both systems similar to the tricyclics doxepin, imipramine, and amitriptyline. In this case, however, the characteristic orange-brown color of methaqualone when over-sprayed with Dragendorff's spray provided a means of differentiating between these tricyclics and metha-

qualone. And although it is probably of little clinical importance, the combined TLC–GLC analysis of patient urine specimens makes it possible to distinguish between these tricyclics themselves by using the R_F values and relative retention times of both the parent drugs and their desmethyl metabolites (0.54 doxepin metabolite, nortriptyline, and desipramine, respectively). *In vivo* metabolism of these drugs is rapid[19], and in all the overdose cases involving these tricyclics that we have studied to date, both the parent drugs and their desmethyl metabolites have been present. Thus, imipramine and doxepin, even though they have very similar R_F values on TLC and relative retention times on GLC, are easily distinguished by virtue of the large differences in the corresponding R_F values and relative retention times for the desmethyl metabolites.

The rapid GLC screen for tricyclics in urine gave results in a more clinically useful time frame. Typically results could be returned to the physician in less than 1 h from receipt of specimen. *A priori* one might expect many false positives with such a screen. However, this proved not to be the case. Not only were normal urines relatively free of endogenous metabolites with relative retention times similar to those of the tricyclics, but by defining positive screens as described above, metabolites that did have similar retention times were excluded. To date we have found 23 patients as positive for tricyclics by this screen, and subsequently all 23 were later confirmed by TLC. The sensitivity of this screen was of some concern, however, since it has been reported[19] that only 0.4% of doxepin and 0.15% of amitriptyline appeared in the urine of normal volunteers on therapeutic doses of these drugs in the first 24 h following their administration. Table IV shows, however, that as far as our overdose cases are concerned, sensitivity has not been a problem. Patient 4, who purportedly only took four 25-mg tablets of amitriptyline, still had a peak height ratio of amitriptyline to internal standard greater than 0.5. Several urine specimens from patients on therapeutic doses of doxepin, amitriptyline, and imipramine were analyzed and found to consistently have peak height ratios of tricyclic to internal standard of less than 0.5. Nevertheless, insufficient data exist as yet to be certain that only overdosed patients and not those on therapeutic doses have ratios greater than 0.5, and it is probable,

TABLE IV

CLINICAL AND LABORATORY FINDINGS OF PATIENTS DURING THE FIRST 24 H AFTER OVERDOSE

Patient	Sex	Age	Drug taken	Amount taken by history	Other drugs	Patient status*	$\dfrac{h_{Tricyclic}}{h_{STD}}$ **
1	F	37	Doxepin	unknown	Codeine, salicylate	comatose	7.8
2	F	19	Amitriptyline	1500 mg		drowsy	6.0
3	F	34	Amitriptyline	600 mg	Alcohol, salicylate	semicomatose	2.2
4	M	14	Amitriptyline	100 mg	Diazepam, 60 mg	drowsy	0.6
5	M	56	Doxepin	>750 mg		awake	7.2
6	M	61	Imipramine	unknown	Phenytoin	confused	4.2
7	M	9	Doxepin	unknown		comatose	15.0
8	F	21	Amitriptyline	350 mg	Perphenazine	drowsy	2.0

* At time of initial examination in emergency room.
** Ratio of peak heights.

given the nature of drug metabolism and individual variations in excretion patterns, that some overlap exists. In an overdosed patients, however, it is the presence or absence rather than the amount of tricyclic that is currently used to determine whether or not to start physostigmine therapy. A large amount of urine was available on Patient 5, and ten consecutive extractions of his urine were chromatographed and found to give peak height ratios of doxepin to cyheptamide which ranged from 6.5 to 7.9. Thus it would appear that results from this screen have adequate reproducibility and are both sensitive and specific enough to be used as a guide to therapy in case of patients suspected of being poisoned with tricyclic antidepressant drugs.

As was mentioned earlier, nortriptyline and desipramine gave anomalous results upon GLC. That is ,when methanolic standards of these drugs were chromato-graphed directly, one peak was observed for each drug with a relative retention time of 0.85; whereas when these drugs were removed from thin-layer plates following chromatography and then analyzed by GLC, only peaks at 1.19 for nortriptyline and 1.24 for desipramine were observed (see Fig. 1). When the spots were scraped from the thin-layer plate without spraying and the material analyzed by GLC, however, both peaks were observed for each drug. Subsequently, it was found that when aqueous standards of these drugs were extracted between pH 8 and 9, only the peaks at 0.85 were observed; while for the same standards extracted at pH 10.5, small peaks at 0.85 and large peaks at 1.19 and 1.24 were observed when analyzed by GLC directly. Since these phenomena were reproducible for both aqueous standards and patient urines, it was concluded that a base catalyzed rearrangement and/or oxidation
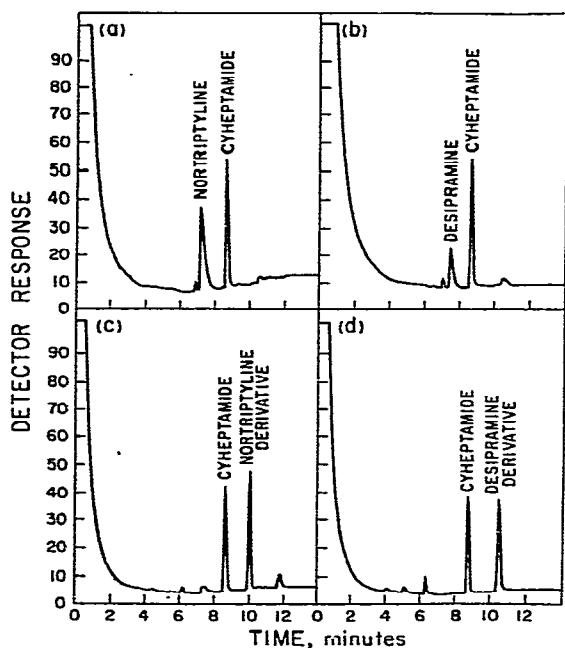

Fig. 1. Gas–liquid chromatograms of (a) equimolar methanolic solution of nortriptyline and cyheptamide; (b) equimolar methanolic solution of desipramine and cyheptamide; (c) spot extract from thin-layer chromatogram of (a); and (d) spot extract from thin-layer chromatogram of (b).

was occurring. Borga and Garle[16] reported the "*in vitro*" metabolism of nortriptyline and desipramine in acid via the 10-hydroxy intermediates to give 10,11-dehydro-desmethylnortriptyline (I) and 10,11-dehydrodesmethyldesipramine (II), respectively.

I   II
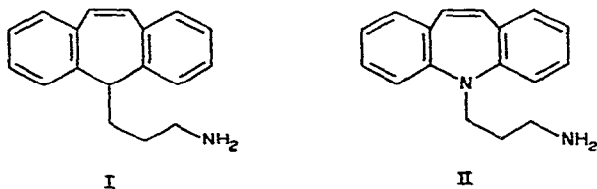
Under their chromatographic conditions (OV-1 column), these metabolites also had somewhat longer retention times than the parent drugs. Mass spectral analyses of the RRT 1.19 and 1.24 peaks, however, indicate that these derivatives are not the same as metabolites I and II seen by Borga and Garle. The published spectra for nortriptyline and desipramine[22] and the spectra for the derivatives shown in Fig. 2 all have intense
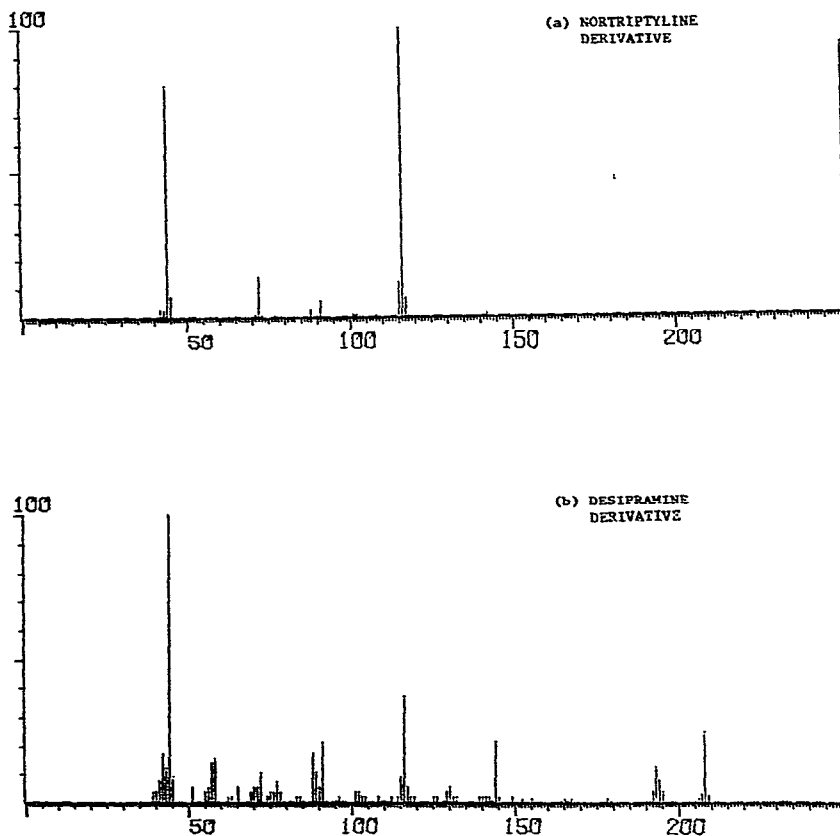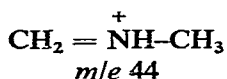

Fig. 2. Mass spectra of (a) compound with RRT of 1.19 in Fig. 1c (nortriptyline derivative) and (b) compound with RRT of 1.24 in Fig. 1d (desipramine derivative).

ions at m/e 44 characteristic of a-cleavage in secondary N-alkylmethylamines[26]. As is common in the mass spectra of alkylamines

$$\overset{+}{CH_2 = NH–CH_3}$$
$$m/e\ 44$$

no molecular ions were observed in either spectrum. The only ion of note besides the m/e 44 ion was that at m/e 116 (base peak for the nortriptyline derivative and second largest peak for the desipramine derivative). Although formation of this ion in each case is obviously energetically favorable, it is difficult to deduce their structures starting from the parent drugs and speculating probable metabolic pathways. High-resolution and chemical ionization mass spectral studies are now in progress to determine the structure(s) of this ion and to help establish the identities of these derivatives. The only conclusion possible at the present time is that the N-alkylmethylamine side chain of the parent drug in each case is still intact.

Even though the identities of these derivatives have not yet been established, the fact that their formation is reproducible has been found to be quite useful in identifying nortriptyline and desipramine. For instance, when a peak with a relative retention time of 0.84–0.85 is noted in the gas–liquid chromatogram of a urine extracted under normal conditions, the urine is re-extracted at pH 10.5 and analyzed again by GLC. If peaks at 1.18 or 1.24 appear, then positive identification of nortriptyline or desipramine is established.

ACKNOWLEDGEMENTS

REFERENCES

1 D. G. Spiker, A N. Weiss, S. S. Chang, J. F. Ruwitch and J. T. Briggs, *Clin. Pharmacol. Ther.*, 18 (1975) 539.
2 J. Noble and H. Matthew, *Clin. Toxicol.*, 2 (1969) 403.
3 T. L. Slovis, J. E. Ott, D. T. Teitelbaum and W. Lipscomb, *Clin. Toxicol.*, 4 (1971) 451.
4 R. G. Brackenridge, T. J. Peters and J. M. Watson, *Scot. Med. J.*, 13 (1968) 208.
5 C. M. Steel, J. O'Duffy and S. S. Brown, *Br. Med. J.*, 3 (1967) 663.
6 R. C. Duvoisin and R. Katz, *J. Amer. Med. Ass.*, 206 (1968) 1963.
7 R. W. Newton, *J. Amer. Med. Ass.*, 231 (1975) 941.
8 I. S. Forrest, F. M. Forrest and A. S. Mason, *Amer. J. Psychiat.*, 116 (1960) 1021.
9 J. E. Wallace and L. V. Dahl, *J. Forensic Sci.*, 12 (1967) 484.
10 G. Norheim, *Arch. Toxikol.*, 31 (1973) 7.
11 W. C. Randolph, S. S. Walkenstein, G. L. Joseph and H. P. Intoccia, *Clin. Chem.*, 20 (1974) 692.
12 K. K. Kaistha and J. H. Jaffe, *J. Pharm. Sci.*, 61 (1972) 679.
13 P. C. Reynolds, in I. R. Sunshine (Editor), *Methods for Analytical Toxicology*, CRC Press, Cleveland, Ohio, 1975, p. 142.
14 R. A. Braithwaite and J. A. Whatley, *J. Chromatogr.*, 49 (1970) 303.
15 H. B. Hucker and S. C. Stauffer, *J. Pharm. Sci.*, 63 (1974) 297.
16 O. Borgå and M. Garle, *J. Chromatogr.*, 68 (1972) 77.
17 G. Norheim, *J. Chromatogr.*, 88 (1974) 403.
18 L. A. Gifford, P. Turner and C. M. B. Pare, *J. Chromatogr.*, 105 (1975) 107.
19 L. J. Dusci and L. P. Hackett, *J. Chromatogr.*, 61 (1971) 231.

20 G. Belvedere, L. Burti, A. Frigerio and C. Pantarotto, *J. Chromatogr.*, 111 (1975) 313.
21 J. T. Biggs, W. H. Holland, S. S. Chang, P. P. Hipps and W. R. Sherman, *J. Pharm. Sci.*, 65 (1976) 261.
22 C. Hammar, B. Alexanderson, B. Holmstedt and F. Sjovist, *Clin. Pharmacol. Ther.*, 12 (1971) 496
23 B. Davidow, N. L. Petri, B. Quame, B. Searle, E. Fastlich and J. Savitzky, *Amer. J. Clin. Pathol.*, 46 (1966) 58.
24 S. J. Mulé, *J. Chromatogr.*, 55 (1971) 255.
25 P. C. Reynolds, in I. R. Sunshine (Editor), *Methods for Analytical Toxicology*, CRC Press, Cleveland, Ohio, 1975, p. 447.
26 H. Budzikiewicz, C. Djerassi and D. H. Williams, *Mass Spectrometry of Organic Compounds*, Holden-Day, San Francisco, 1967, p. 298.