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Identification and differentiation of barbiturates, other sedative-hypnotics and their metabolites in urine integrated in a general screening procedure using computerized gas chromatography–mass spectrometry[a]

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ABSTRACT

A gas chromatographic–mass spectrometric procedure is described for the identification and differentiation of sedative-hypnotics and their metabolites in urine. The following 24 barbiturates and thirteen other hypnotics could be detected: acecarbromal, allobarbital, amobarbital, aprobarbital, barbital, brallobarbital, bromisoval, (sec)butabarbital, butalbital, butobarbital, carbromal, clomethiazole, crotylbarbital, cyclobarbital, cyclopentobarbital, diethylallylacetamide, dipropylbarbital, glutethimide, guaifenesin, ethinamate, heptabarbital, hexobarbital, meprobamate, methaqualone, metharbital, methohexital, methylphenobarbital, methyprylone, pentobarbital, phenobarbital, propallylonal, pyrithyldione, secobarbital, thiobutabarbital, thiopental, vinbarbital and vinylbital. The procedure presented is integrated in a general screening procedure (general unknown analysis) for several groups of drugs detecting over 300 drugs and over 1000 of their metabolites. It includes cleavage of conjugates by acid hydrolysis, isolation by liquid–liquid extraction, derivatization by acetylation, separation by capillary gas chromatography, and identification by computerized mass spectrometry. Using mass chromatography with the selected ions m/z 83, 117, 141, 167, 169, 207, 221 and 235, the presence of barbiturates, other hypnotics and/or their metabolites was indicated. The identity of positive signals in the reconstructed mass chromatograms was confirmed by a visual or computerized comparison of the stored full mass spectra with the reference spectra. The sample preparation, mass chromatograms, reference mass spectra and gas chromatographic retention indices are documented.

INTRODUCTION

The sedative-hypnotic drugs are one of the largest groups of drugs, usually classified into barbiturates, bromureids, benzodiazepines, and antihistamines. They are widely used for the treatment of insomnia, anxiety states and convulsive disorders as well as for anaesthetic and preanaesthetic medication. Because of their central nervous and respiratory depressant effect, they may cause, alone or in combination with other drugs and/or ethanol, severe accidental or suicidal

[a] Some of these results were reported at the *1st German–German Symposium of the Gesellschaft für Toxikologische und Forensische Chemie (GTFCh) and the Arbeitsgemeinschaft Toxikologische Chemie der DDR, Leipzig, July 3–5, 1990* [1].

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intoxication for which treatment is necessary. Furthermore, they may impair the fitness to drive a car and to work at machines even after therapeutic doses. In particular, the barbiturates and benzodiazepines may lead to a drug dependence. The medical use of barbiturates has been declining in recent years, because their margin of therapeutic safety is relatively narrow [2]. However, they are still misused by heroin fixers to ease the withdrawal symptoms from heroin or to augment to effects of "weak heroin".

For all these reasons, sedative-hypnotics may be encountered in clinical or forensic toxicological analysis. Before quantification in plasma, the drugs, which are usually unknown, must be first identified. Identification methods for benzodiazepines [3] and antihistamines [4–7] have already been published. Detection of some of the barbiturates and other hypnotics using thin-layer chromatography (TLC) [8–11], high-performance liquid chromatography (HPLC) [8, 12–15], gas chromatography (GC) [8,16–20] and gas chromatography–mass spectrometry (GC–MS) [16,21] has been described. Furthermore, fluorescence polarization immunoassays [22], enzyme immunoassays [23] or radioimmunoassays [24] are commercially available for the detection and quantification of barbiturates. However, in clinical and forensic toxicology, immunological results must be confirmed by a non-immunological method, especially by GC–MS. However, none of these procedures allows the rapid and specific identification and differentiation of all barbiturates and other sedative-hypnotics.

The aim of this study was to investigate whether and how barbiturates and other sedative-hypnotics can be detected within the "general-unknown" analysis procedure that has proved to be successful for several hundred psychotropic, antihistaminic, analgesic and cardiovascular drugs and their metabolites [3–7,25–36]. Urine was used for this general screening procedure, because the concentrations of drugs and their metabolites are higher in urine than in plasma. Because most of the toxicologically relevant drugs are excreted in urine in a completely metabolized and conjugated form, at least in the later phase of excretion, conjugates were cleaved by acid hydrolysis, which can be completed more quickly than enzymic hydrolysis. Polar metabolites were derivatized by acetylation.

This paper describes a computerized GC–MS method for the identification and differentiation of barbiturates, other sedative-hypnotics and their metabolites in urine, integrated in a general unknown analysis procedure.

EXPERIMENTAL

Apparatus

A Hewlett-Packard (HP) Series 5890 gas chromatograph, combined with an HP MSD Series 5970 mass spectrometer and an HP Series 59970 C workstation, was used. The GC conditions were as follows: splitless injection mode; column, HP capillary (12 m × 0.2 mm I.D.), cross-linked methylsilicone, 0.33 μm film thickness; column temperature, programmed from 100 to 310°C in 30°C/min,

initial time 3 min, final time 5 min; injection port temperature, 270°C; carrier gas, helium; flow-rate 1 ml/min.

The MS conditions were as follows: scan mode; ionization energy, 70 eV; ion-source temperature, 220°C; capillary direct interface heated at 260°C.

Exact measurement of retention indices was performed on a Varian Series 3700 gas chromatograph. The column effluent went to a flame-ionization detector and a nitrogen-sensitive flame-ionization detector after a 1:1 split by a splitter made from nickel tubing. The column was a steel tube (60 cm × 2 mm I.D.) packed with Chromosorb G HP (100–120 mesh) coated with 5% OV-101. The column and injector temperatures were identical with those used for GC–MS, and the temperature for the detectors was 270°C. Nitrogen was used as carrier gas at a flow-rate of 30 ml/min.

Urine samples

The investigations were performed on urine from in-patients treated with therapeutic doses of hypnotics.

Hydrolysis and extraction procedure

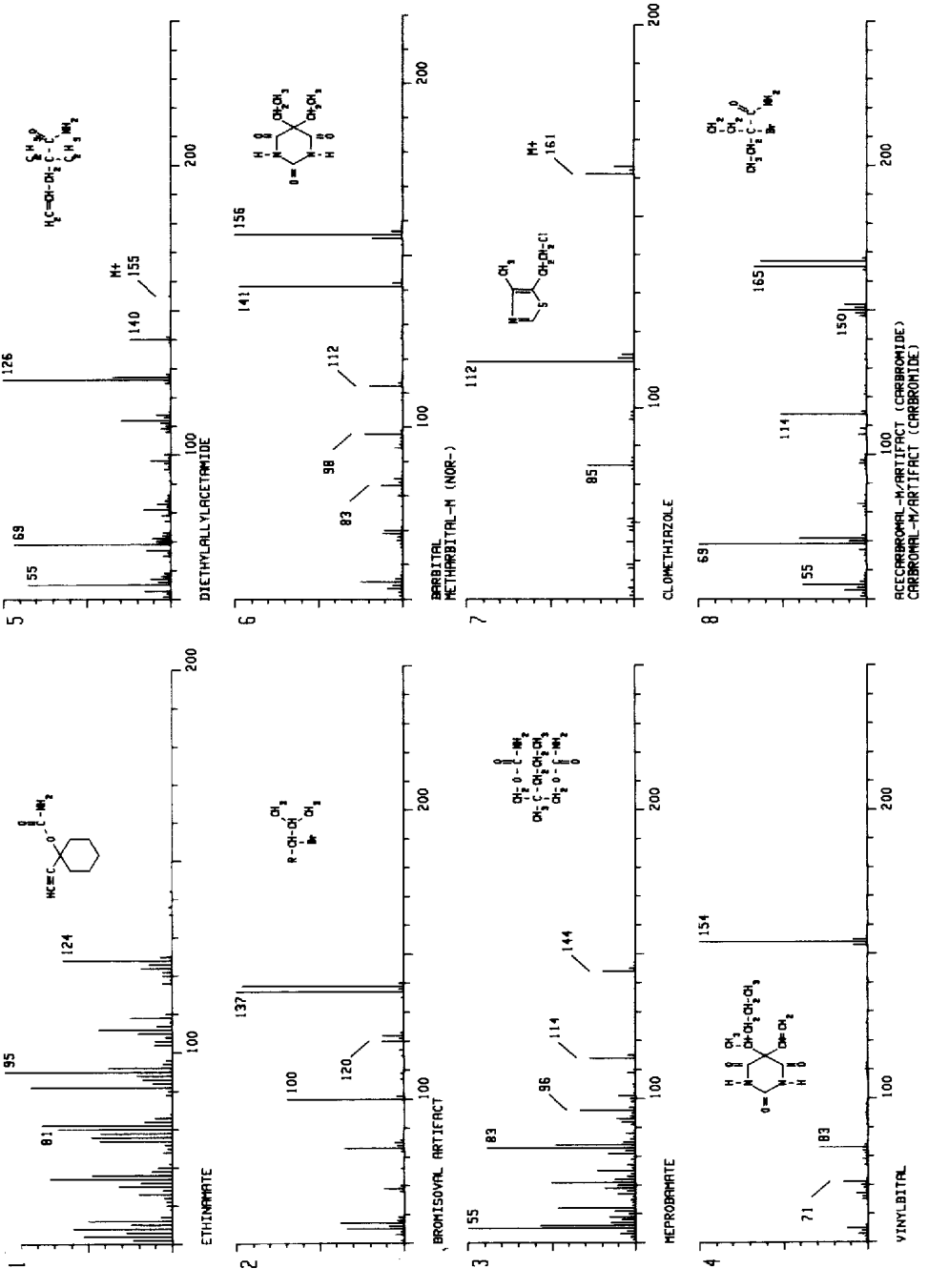
A 10-ml volume of urine was refluxed with 3 ml of 37% hydrochloric acid for 15 min. Following hydrolysis, *ca.* 3 g of potassium hydroxide pellets were added and the resulting solution was mixed with 10 ml of 30% aqueous ammonium sulphate to obtain a pH between 8 and 9. This solution was extracted with a 10-ml portion of dichloromethane–2-propanol–ethyl acetate (1:1:3). Phase separation was accomplished by centrifugation. The organic extract was transferred to a pear-shaped flask and carefully evaporated to dryness. The temperature should not be too high and the vacuum not too strong, because hypnotics with relatively low molecular masses can be partly evaporated.

Acetylation

The extracted residue was acetylated for 30 min at 60°C with 100 μl of acetic acid anhydride pyridine (3:2). The acetylation mixture was then carefully evaporated to dryness and the resultant residue dissolved in 100 μl of methanol. A 0.5–2 μl volume of this sample was injected into the gas chromatograph.

Gas chromatographic–mass spectrometric analysis

Full mass spectra were recorded at a speed of 1 scan/s and stored on a hard disk during the temperature-programmed GC analysis. Using mass chromatography with the ions *m/z* 83, 117, 141, 167, 169, 207, 221 and 235, the stored spectra were evaluated. The identity of positive signals in the reconstructed mass chromatograms was confirmed by a visual comparison of the full mass spectra with reference spectra (Fig. 1) or by a computer library search [37]. The reconstruction of the mass chromatograms, the recognition of the top of the peaks and the library search of the peak underlaying mass spectra can be automated by connection of the corresponding computer programs using so-called macros [25].


1
ETHINAMATE
2
BROMISOVAL ARTIFACT
3
MEPROBAMATE
4
VINYLBITAL
5
DIETHYLALLYLACETAMIDE
6
BARBITAL
METHARBITAL-M (NOR-)
7
CLOMETHIAZOLE
8
ACECARBROMAL-M/ARTIFACT (CARBROMIDE)
CARBROMAL-M/ARTIFACT (CARBROMIDE)

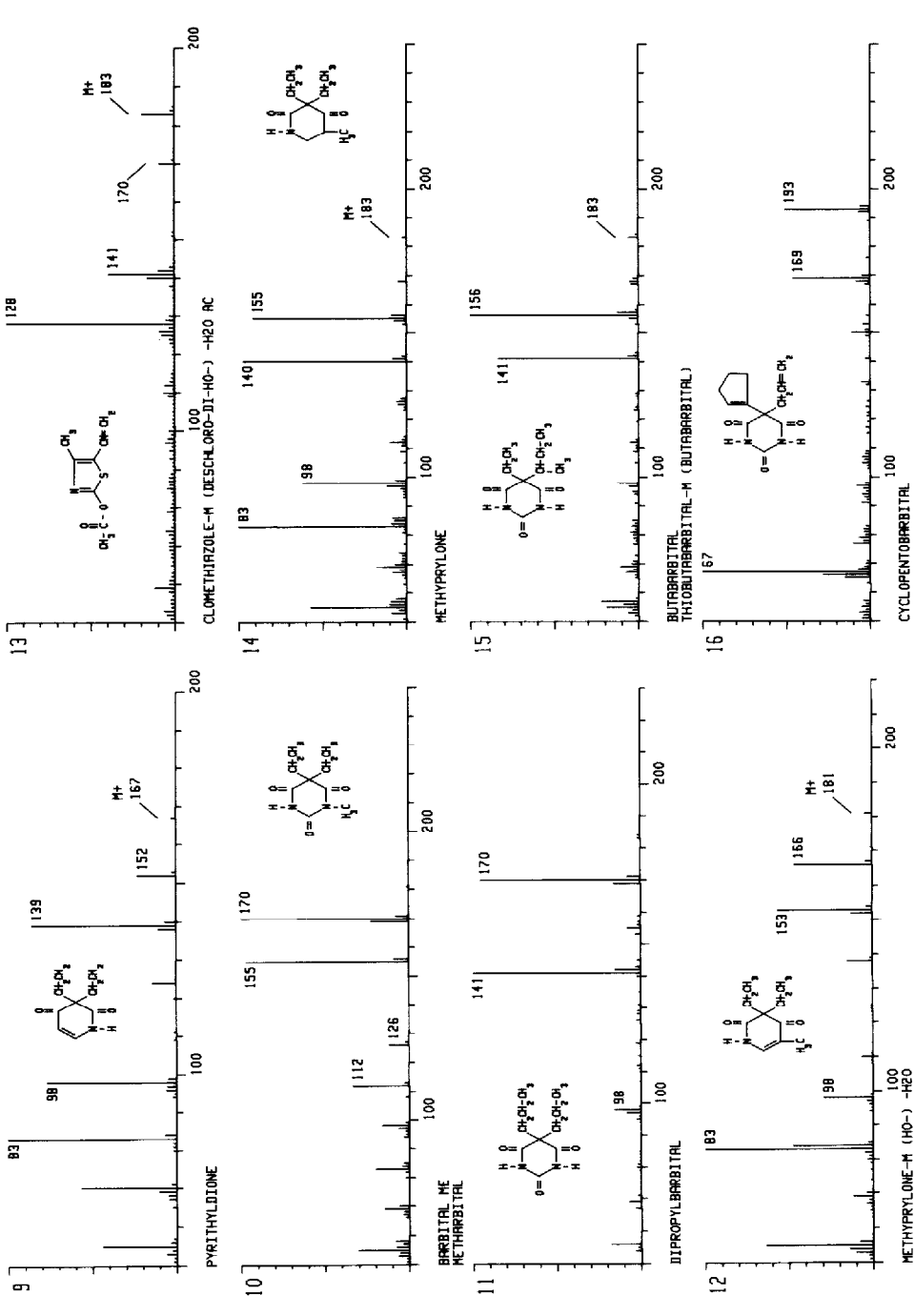
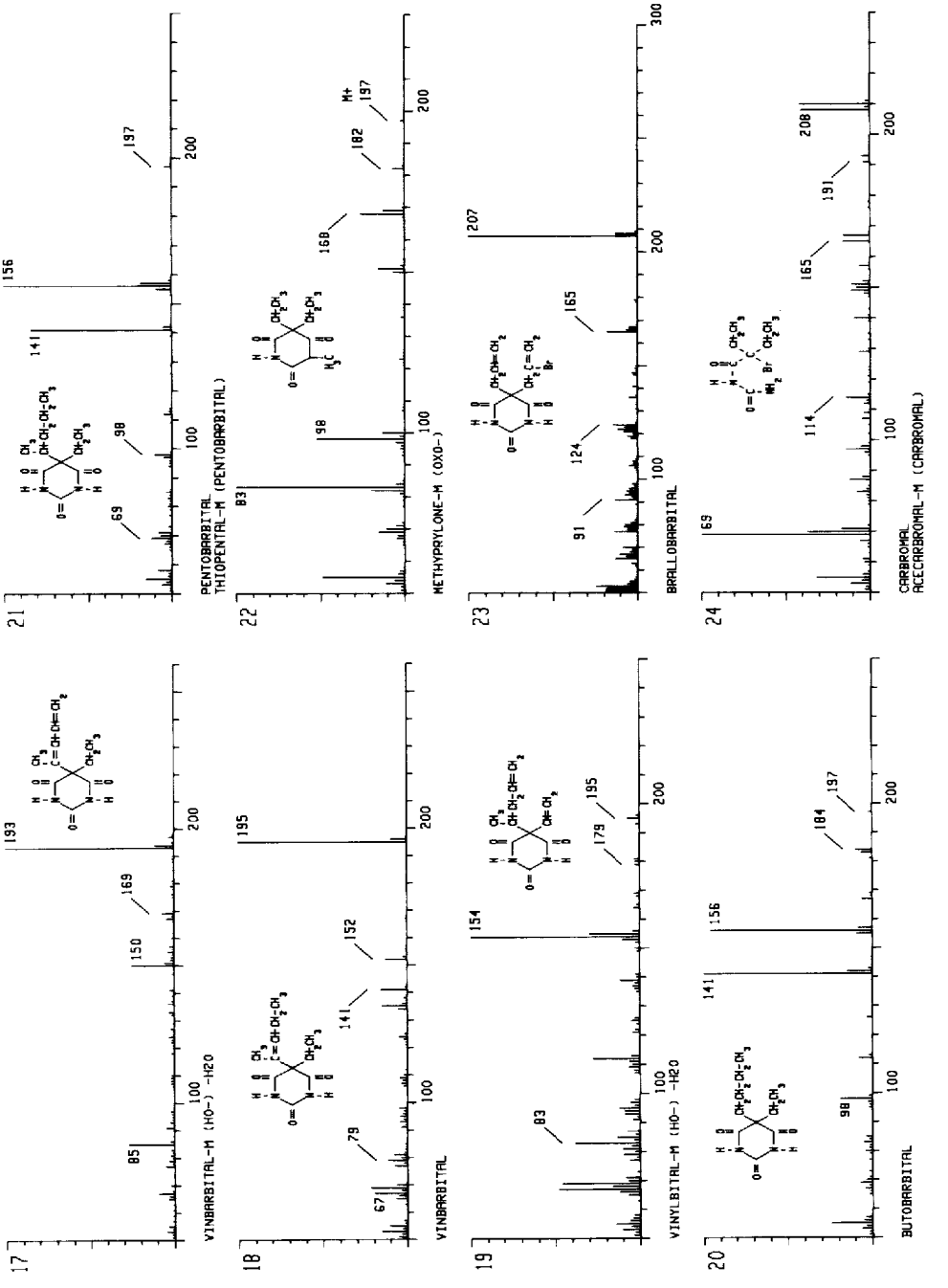


Fig. 1.

(Continued on p. 312)


17
193
169
150
85
100
200
VINBARBITAL-M (HO-) -H2O
18
195
152
141
79
67
100
200
VINBARBITAL
19
154
83
179
195
100
200
VINYLBITAL-M (HO-) -H2O
20
141
156
197
184
98
100
200
BUTOBARBITAL
21
156
141
197
98
69
100
200
PENTOBARBITAL
THIOPENTAL-M (PENTOBARBITAL)
22
83
98
168
182
M+
197
100
200
METHYPRYLONE-M (OXO-)
23
207
165
124
91
100
200
BRALLOBARBITAL
24
69
165
114
191
208
100
200
CARBROMAL
ACECARBROMAL-M (CARBROMAL)

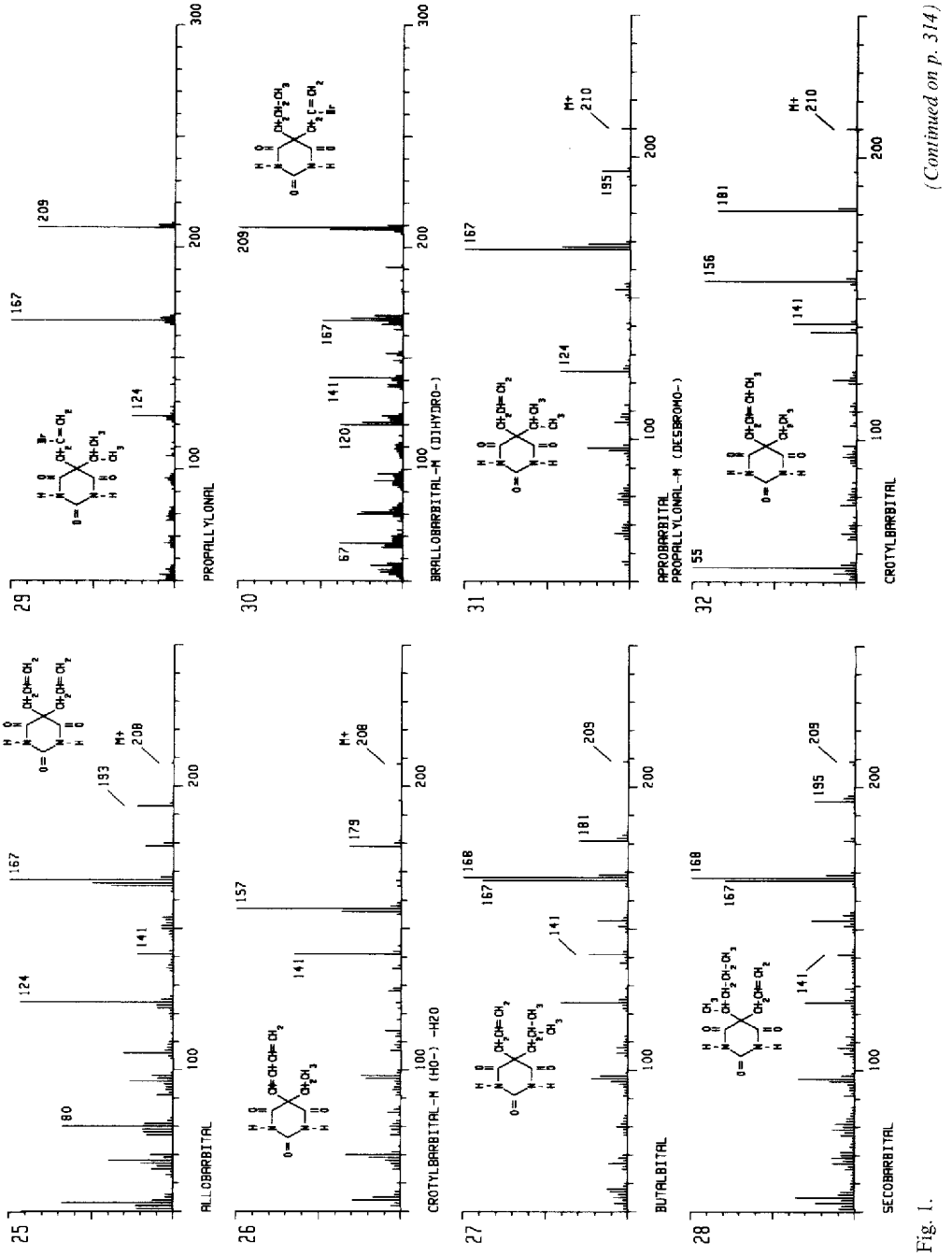
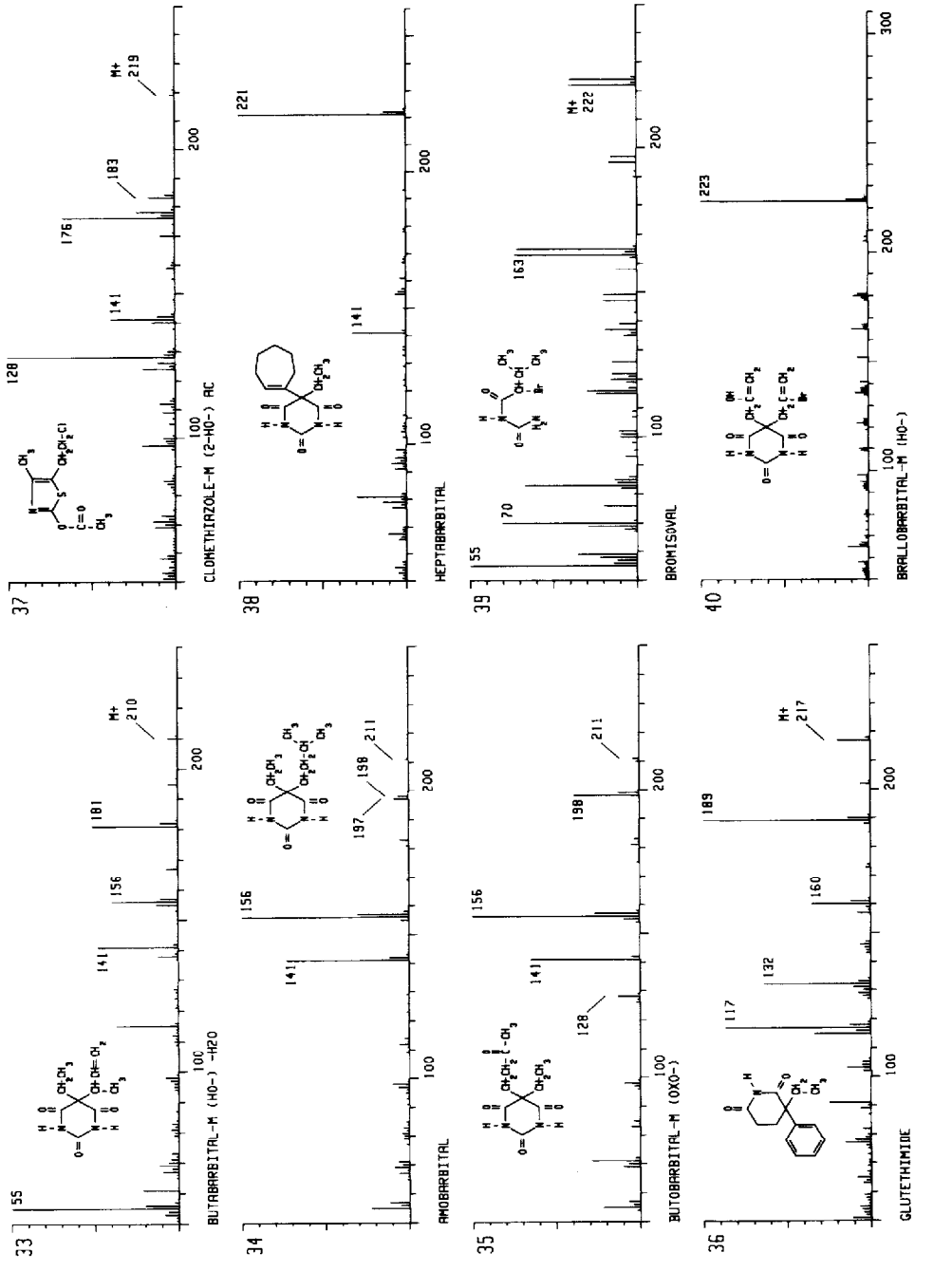


Fig. 1.

(Continued on p. 314)


33
BUTABARBITAL-M (HO-) -H2O
34
AMOBARBITAL
35
BUTOBARBITAL-M (OXO-)
36
GLUTETHIMIDE
37
CLOMETHIAZOLE-M (2-HO-) AC
38
HEPTABARBITAL
39
BROMISOVAL
40
BRALLOBARBITAL-M (HO-)

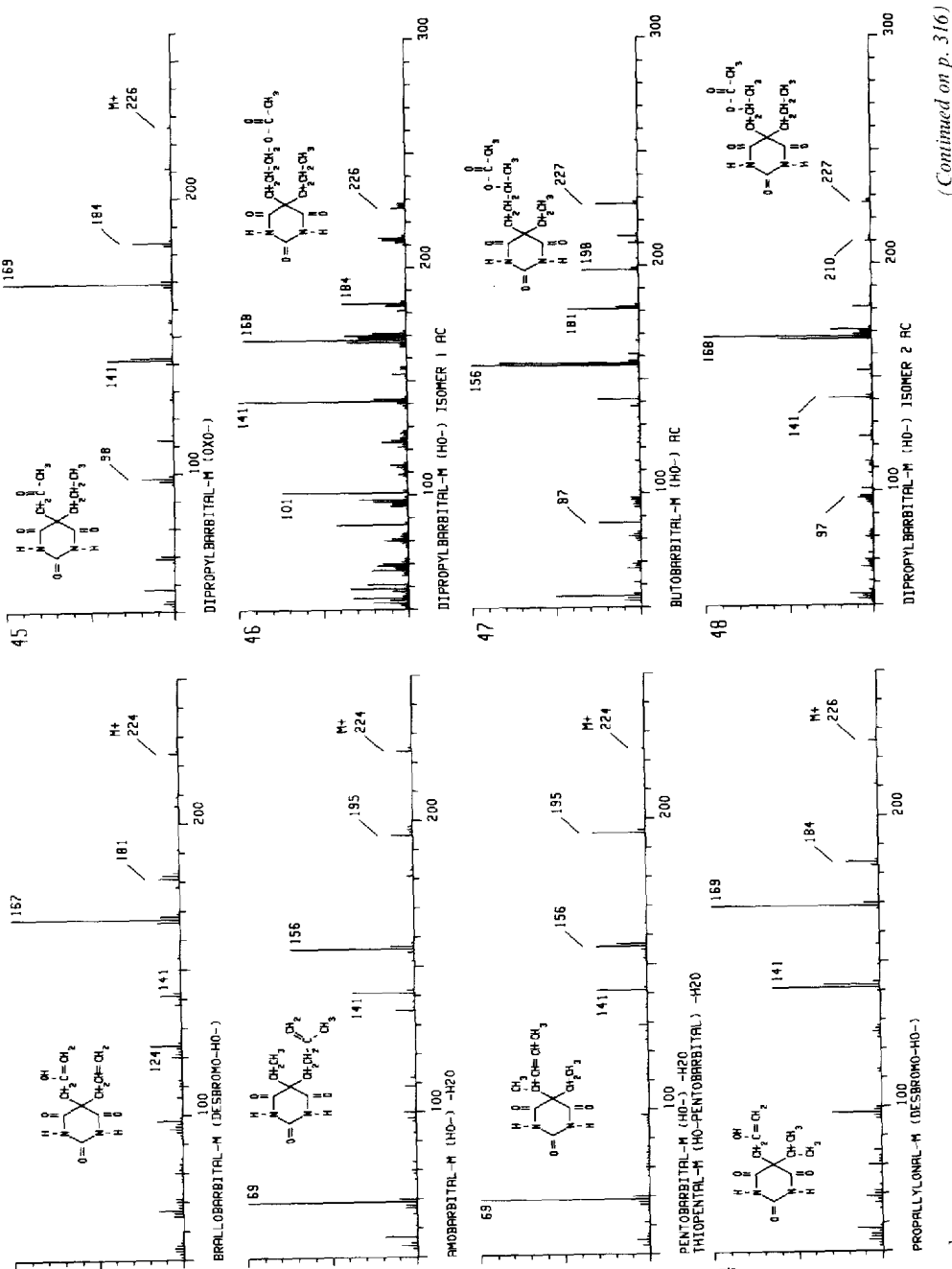
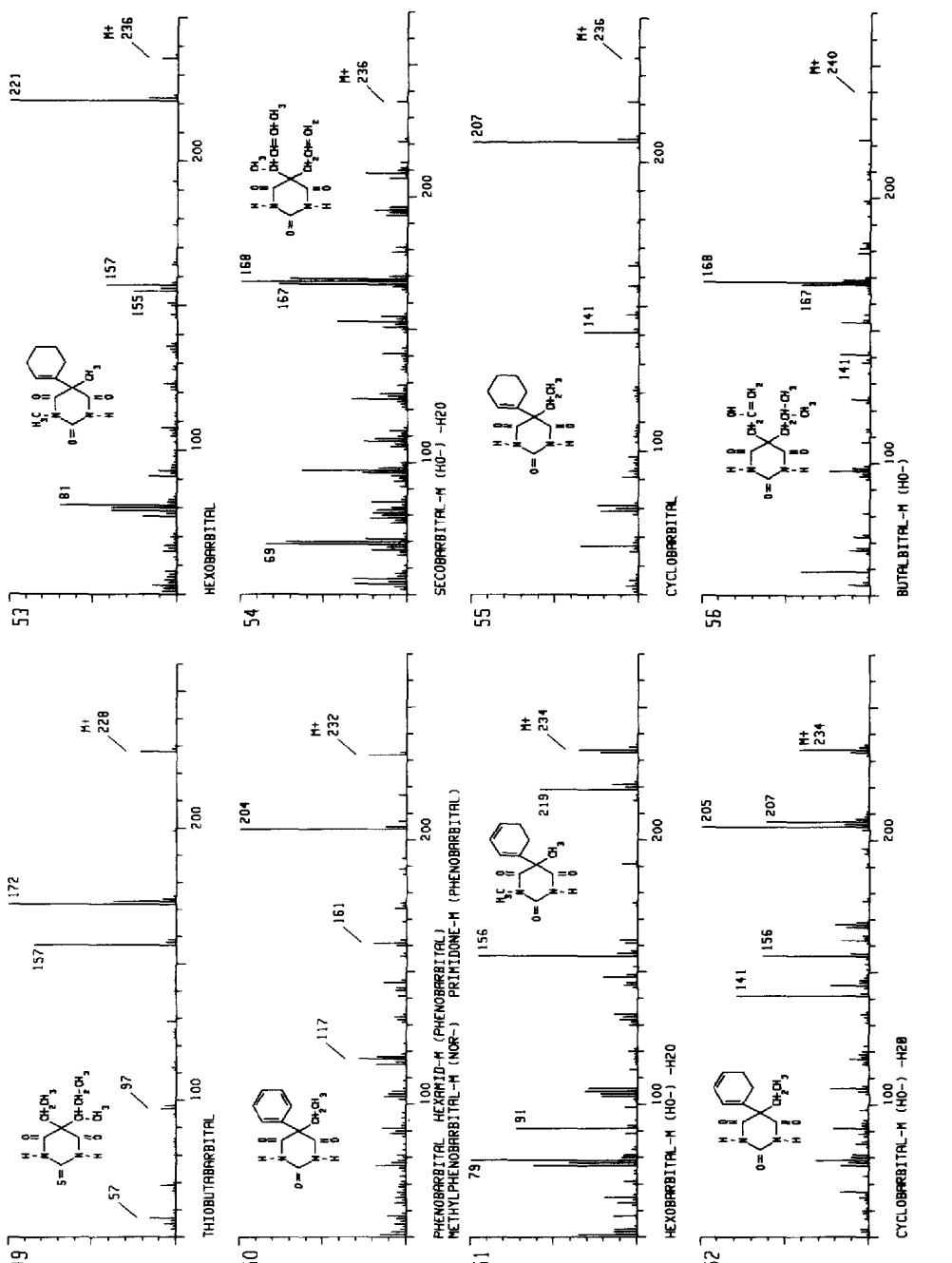


Fig. 1.

(Continued on p. 316)


49
THIOBUTABARBITAL
50
PHENOBARBITAL HEXAMID-M (PHENOBARBITAL)
METHYLPHENOBARBITAL-M (NOR-) PRIMIDONE-M (PHENOBARBITAL)
51
HEXOBARBITAL-M (HO-) -H2O
52
CYCLOBARBITAL-M (HO-) -H2O
53
HEXOBARBITAL
54
SECOBARBITAL-M (HO-) -H2O
55
CYCLOBARBITAL
56
BUTALBITAL-M (HO-)

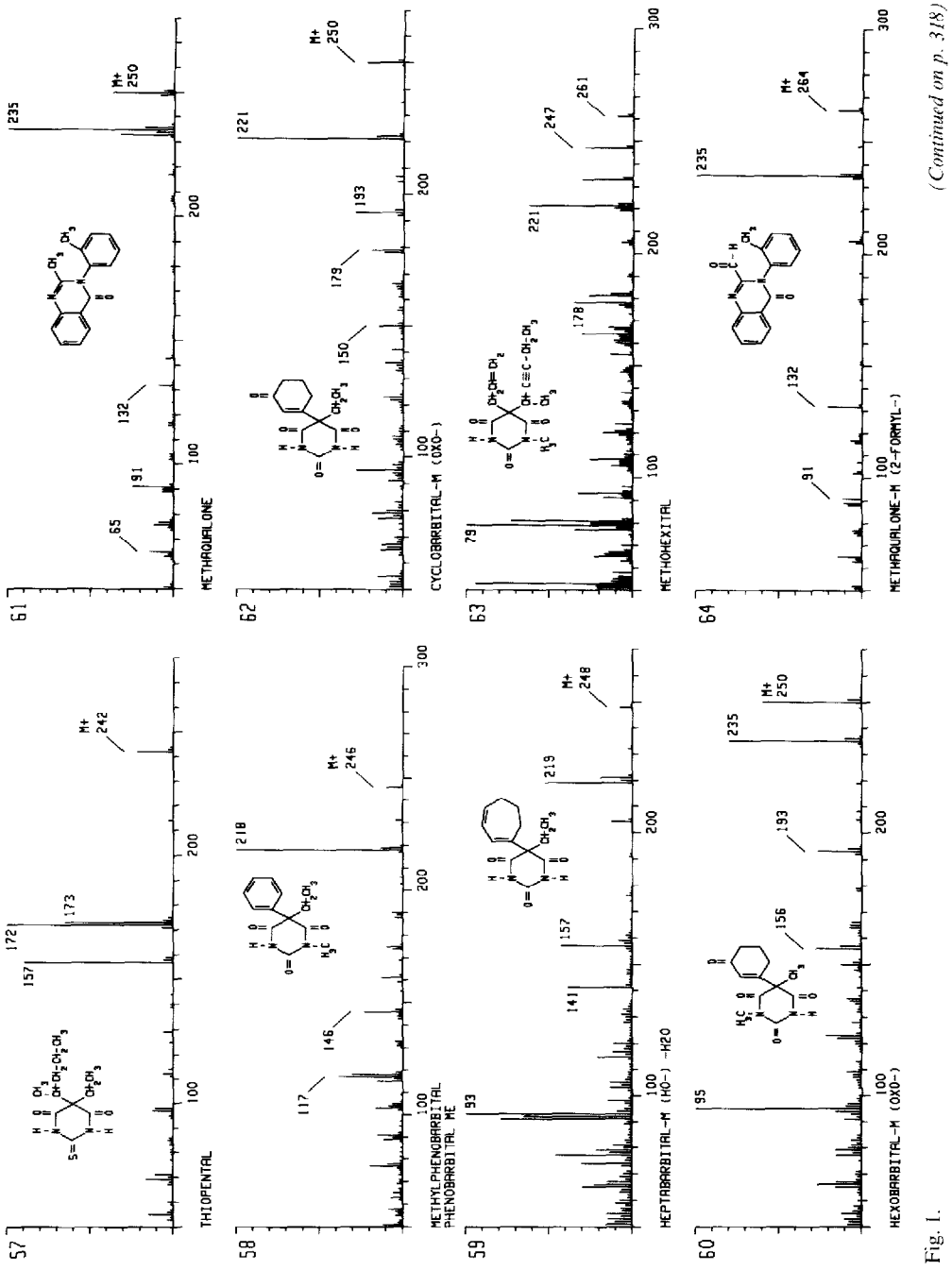
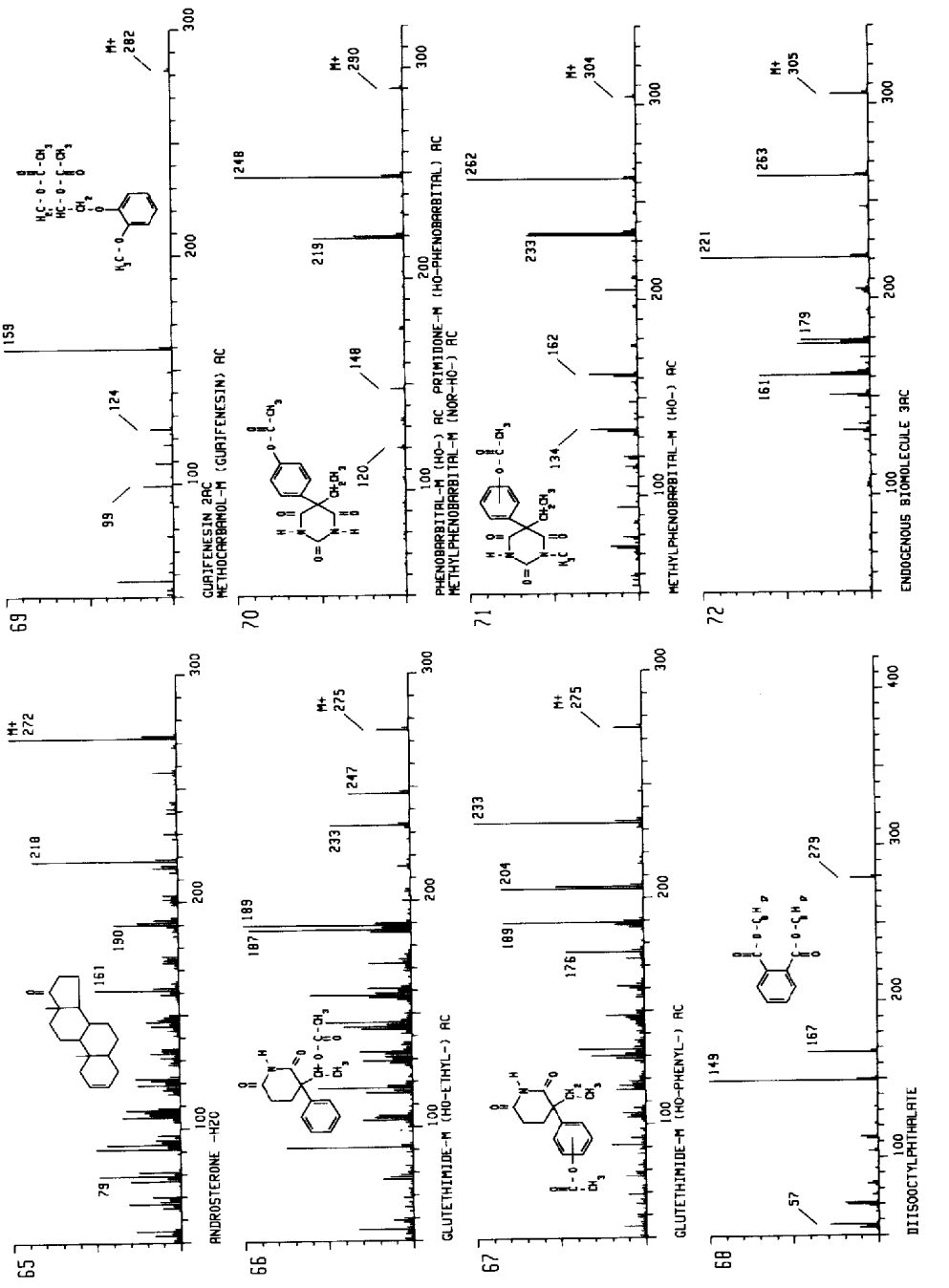


Fig. 1.

(Continued on p. 318)


65
ANDROSTERONE -H2O
66
GLUTETHIMIDE-M (HO-ETHYL-) AC
67
GLUTETHIMIDE-M (HO-PHENYL-) AC
68
DIISOOCTYLPHTHALATE
69
GUAIFENESIN 2AC
METHOCARBAMOL-M (GUAIFENESIN) AC
70
PHENOBARBITAL-M (HO-) AC PRIMIDONE-M (HO-PHENOBARBITAL) AC
71
METHYLPHENOBARBITAL-M (NOR-HO-) AC
METHYLPHENOBARBITAL-M (HO-) AC
72
ENDOGENOUS BIOMOLECULE 3AC

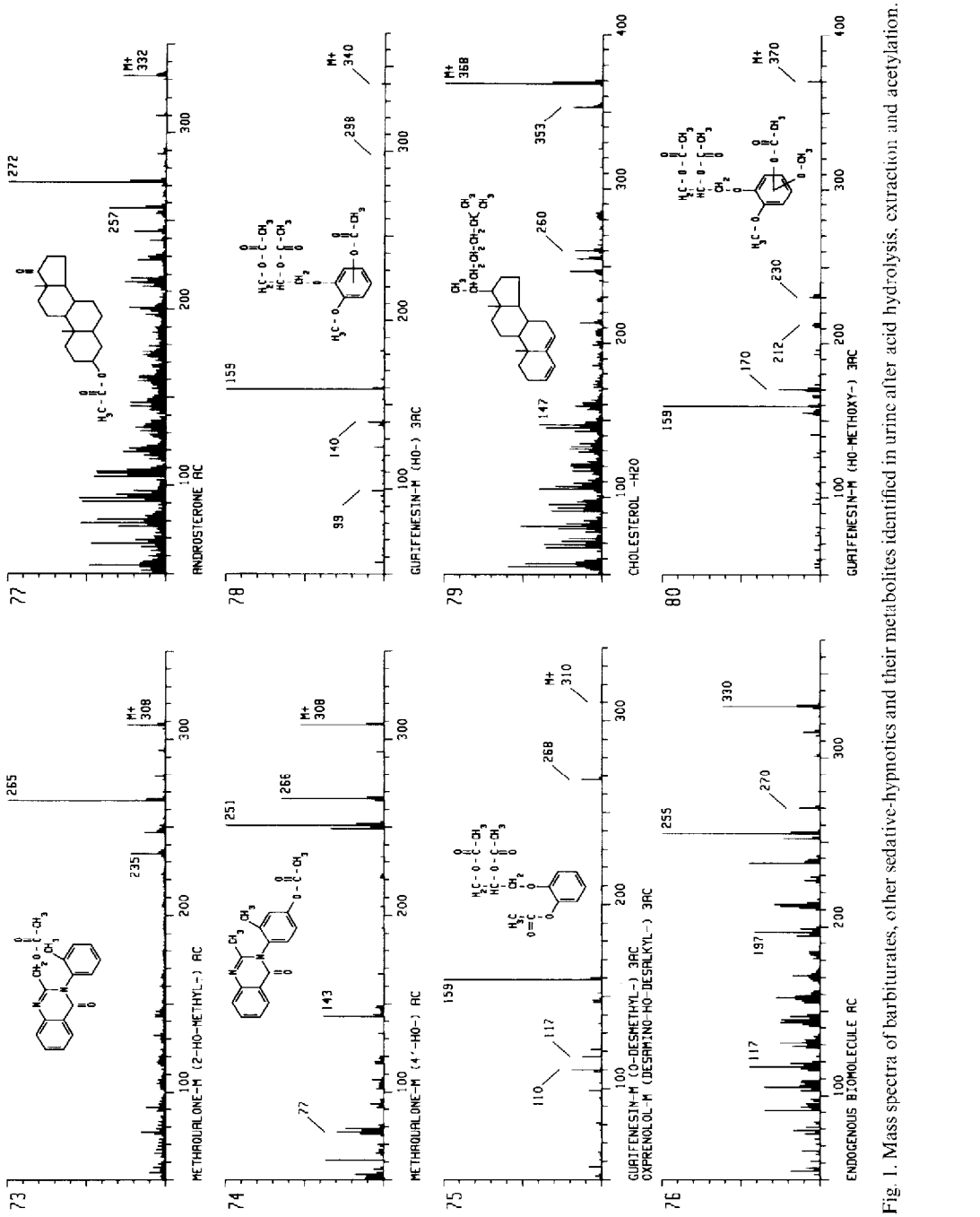


Fig. 1. Mass spectra of barbiturates, other sedative-hypnotics and their metabolites identified in urine after acid hydrolysis, extraction and acetylation.

RESULTS AND DISCUSSION

The results of the studies are summarized in Table I. Using mass chromatography with the eight proposed ions, the possible presence of barbiturates, other sedative-hypnotics and/or their metabolites in urine could be indicated selectively. In contrast to mass fragmentography, mass chromatography is based on the full scan mode and, therefore, the specific identification is carried out by comparison of the peak underlying full mass spectrum with reference spectra.

Some of the compounds are derivatized by acetylation (see Derivative column in Table I). The mass spectra numbers in Fig. 1, the molecular masses and the GC retention indices are given. These indices were determined using temperature-programmed GC combined with flame ionization detection (FID) and nitrogen-sensitive FID. In our experience, retention indices provide preliminary indications of the possible presence of the compounds and may be useful to workers without a GC–MS facility.

Data are given for only those metabolites that were frequently found. All of the listed metabolites are not detected in every sample owing to inter-individual differences in metabolism, or variable times which elapsed after administration. The mass spectra and retention indices of the less abundant metabolites will be included in a forthcoming handbook and computer library [37,38].

The data for androsterone, the dehydration products of androsterone and cholesterol, two unidentified endogenous biomolecules and diisooctylphthalate – a widespread softener of plastics – are included, because these compounds can be indicated by the mass chromatograms.

Some artifacts were formed during sample preparation and/or GC. Acecarbromal and carbromal were partly altered to the corresponding amide carbromide. Bromisoval was also altered in part to an artifact, probably also to the amide. Alcoholic hydroxy groups were dehydrated to the corresponding alkenes ($-H_2O$ in Table I and Fig. 1). Because the dehydration artifacts were formed during acid hydrolysis, the corresponding intact molecules were found after enzymic hydrolysis. The data for the intact molecules will be included in a forthcoming handbook and computer library [37,38].

The full mass spectra for the specific identification of the selectively indicated compounds are shown in Fig. 1. They are listed primarily in order of ascending mass of the ion with the largest mass detected in the spectrum. For the same nominal mass value, the spectra are arranged in order of ascending retention indices. Formulae are proposed for probable metabolite structures.

Interferences by other drugs are improbable because the identity of the peaks observed in the mass chromatograms can be positively confirmed by a visual comparison of the underlying mass spectrum with reference spectra (Fig. 1 and ref. 38) or by a computer library search [37]. The following drugs lead to common metabolites and/or artifacts: acecarbromal and carbromal, aprobarbital and propallylonal, barbital and metharbital, butabarbital and thiobutabarbital, pento-

TABLE I

MONITORING PROGRAMME FOR THE DETECTION OF BARBITURATES, OTHER SEDATIVE-HYPNOTICS AND THEIR METABOLITES IN URINE AFTER ACID HYDROLYSIS AND ACETYLATION

Mol. Mass	Drug/metabolite (M)	Derivative[a]	Relative intensity (%)								Retention index	MS No.
			m/z 83	*m/z* 117	*m/z* 141	*m/z* 167	*m/z* 169	*m/z* 207	*m/z* 221	*m/z* 235		
193	Accarbromal-M/artifact (carbromide)		5			63					1215	8
236	M (Carbromal)		7	0.1	0.9	15		0.2			1515	24
208	Allobarbital		1		22	100	1				1595	25
226	Amobarbital		3		73		1				1710	34
224	M (HO–) −H20		2		37						1830	42
210	Aprobarbital		4	0.5	2	100	25				1610	31
184	Barbital		12		97		0.2				1500	6
286	Brallobarbital		0.7		2	5	0.1	100			1850	23
224	M (Desbromo–HO–)		2		13	100	1				1795	41
288	M (Dihydro-)		6		45	49	17	5			1970	30
302	M (HO–)				4	3		1			2040	40
222	Bromisoval		67	5							1540	39
	Bromisoval artifact		35	2							1510	2
212	Butabarbital		3		83	4	2				1655	15
210	M (HO–) −H20		4		48	6	0.2	0.2			1905	33
224	Butalbital		2		24	88	18				1690	27
240	M (HO–)				18	41	16	2			1940	56
212	Butobarbital		5		100	6	1				1665	20
226	M (Oxo-)		5		65						1880	35
270	M (HO–)	AC	2		25	2	0.3	0.2	0.3		1940	47
236	Carbromal		7	0.1	0.9	15		0.2			1515	24
193	M/Artifact (carbromide)		5			63					1215	8
161	Clomethiazole										1230	7
183	M (Deschloro-di-HO–) −H20	AC	1	0.6	39		0.3				1420	13
219	M (2-HO–)	AC	2	0.1	38				1		1590	37

(Continued on p. 322)

TABLE I *(continued)*

Mol. Mass	Drug/metabolite (M)	Derivative[a]	Relative intensity (%)								Retention index	MS No.
			m/z 83	*m/z* 117	*m/z* 141	*m/z* 167	*m/z* 169	*m/z* 207	*m/z* 221	*m/z* 235		
210	Crotylbarbital		3		39	2					1620	32
208	M (HO–) −H20				65	3					1600	26
236	Cyclobarbital			1	33			100	7		1970	55
234	M (HO–) −H20			11	79	13	7	62		6	2170	52
250	M (Oxo-)		5	1	7	1		5	100	2	2190	62
234	Cyclopentobarbital		2	2	3	3	46				1865	16
155	Diethylallylacetamide[c]		8		2						1285	5
212	Dipropylbarbital		0.5		99		15				1650	11
226	M (Oxo-)				39	1	100	0.3			1870	45
270	M (HO–) ISOMER 1	AC	3		100	35	28		0.1		1950	46
270	M (HO–) ISOMER 2	AC	2		26	56	11				2000	48
217	Glutethimide			87							1830	36
275	M (HO-ethyl-)	AC	5	56	6		4				2060	66
275	M (HO-phenyl-)	AC	2	15	4		1	1		3	2250	67
282	Guaifenesin	AC		4							1865[b]	69
310	M (O-desmethyl-)	AC		12			0.5				1920[b]	75
340	M (HO–)	AC		1	1						2235[b]	78
370	M (HO-methoxy-)	AC		1	6		9				2265[b]	80
167	Ethinamate[c]		9								1395[b]	1
250	Heptabarbital				32	2	1		99		2070	38
248	M (HO–) − H20		4	12	38	1			19		2300	59
236	Hexobarbital		4	1					100		1855	53
234	M (HO–) −H20		3	2					15	5	1970	51
250	M (OXO-)		6	1	3					80	2055	60
218	Meprobamate		88								1785[b]	3
250	Methaqualone			3		0.4		2	1	100	2155	61
264	M (2-Formyl-)			7				2		100	2240	64
308	M (2-HO-methyl-)	AC	4	5	2	2	1	1	1	22	2440	73
308	M (4'-HO–)	AC		6		4			2		2530	74

198	Metharbital		20				22				1455	10
184	M (Nor-)		12		97		0.2				1500	6
262	Methohexital			4		14	1	1	62		1780	63
246	Methylphenobarbital		2	38			2				1895	58
232	M (Nor-)		2	29	5		2	5			1965	50
304	M (HO–)	AC	1			0.2	0.3	1		7	2330	71
290	M (Nor-HO–)	AC						0.3	4		2360	70
183	Methyprylone		100		8						1525	14
181	M (HO–) −H20		100	0.1		4					1540	12
197	M (Oxo-)		100		0.6	0.9	12				1870	22
226	Pentobarbital		2		84	0.4					1740	21
224	M (HO–) −H20		1		29						1890	43
232	Phenobarbital		2	29	5		2	5			1965	50
290	M (HO–)	AC						0.3	4		2360	70
288	Propallylonal		2		1	99	2				1875	29
210	M (Desbromo-)		4	0.5	2	100	25				1610	31
226	M (Desbromo-HO-)		1		63		98				1770	44
167	Pyrithyldione [c]		100			3					1520	9
238	Secobarbital		5	1	10	78	17				1795	28
236	M (HO–) −H20		9		15	77	70	11	6		1970	54
228	Thiobutabarbital		1	0.9	0.6	0.2	0.4				1790	49
212	M (Butabarbital)		3		83	4	2				1655	15
242	Thiopental		2								1855	57
226	M (Pentobarbital)		2		84	0.4					1740	21
224	M (HO-pentobarbital) −H20		1		29						1890	43
224	Vinbarbital		1	0.5	16	1					1765	18
222	M (HO–) −H20		1	0.3	2	4	6				2020	17
224	Vinylbital		28		0.5	0.2					1745	4
222	M (HO–) −H20		38		2		2				1995	19
332	Androsterone	AC	13	14	6	8	4	4	1	2	2580[b]	77
272	Androsterone −H20			10	3						2240[b]	65
368	Cholesterol −H20		23	19	3	1	2	4			3030[b]	79
390	Diisooctylphthalate		6		0.5	41	0.4		0.5		2540[b]	68
	Endogenous biomolecule	AC	0.2	0.9		2			100	0.5	1950	72
	Endogenous biomolecule	AC	2	44	6	1	6				2575[b]	76

[a] AC = acetylated.

[b] These compounds cannot be detected by nitrogen FID.

[c] These drugs can be detected only in a direct extract of urine.

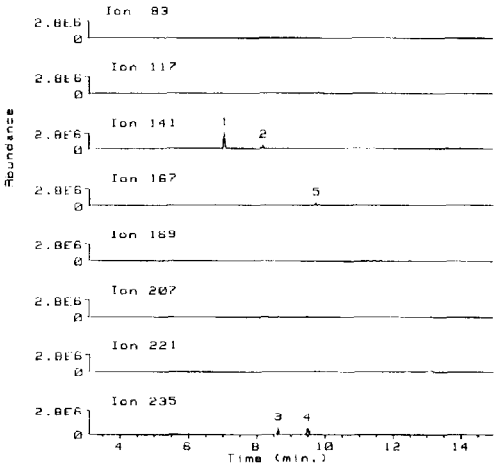


Fig. 2. Mass chromatograms from the urine of a patient suspected of misusing hypnotics, indicating pentobarbital (1) and its dehydrated hydroxy metabolite (2), methaqualone (3) and its 2-hydroxy metabolite (4), and diisooctylphthalate (5), a widespread softener of plastics (mass spectra Nos. 21, 43, 61, 73 and 68 in Fig. 1).

barbital and thiopental, and phenobarbital, methylphenobarbital and the anticonvulsant primidone [36]. Guaifenesin has a common metabolite with the muscle relaxant methocarbamol and a common metabolite with the β-blocker oxprenolol [33]. However, the detection of the parent drugs and/or further unique metabolites allowed a differentiation. Acecarbromal and carbromal can only be differentiated in a direct extract of native urine [37–39]

The detection limits of the parent compounds were between 10 and 20 ng/ml of urine. The sensitivity of the method was sufficient to detect therapeutic concentrations of the studied drugs. Because urine concentrations of sedative-hypnotics are relatively high, the relatively poor analytical recovery of only 40–80% caused by hydrolysis and weakly alkaline pH is of minor relevance. However, diethylallylacetamide, ethinamate and pyrithyldione could only be detected in a direct extract of native urine [39].

To illustrate the method, mass chromatograms from the urine of a patient suspected of misusing hypnotics is shown in Fig. 2. Peaks 1 and 2 indicate pentobarbital and its dehydrated hydroxy metabolite, peaks 3 and 4 methaqualone and its 2-hydroxy metabolite, and peak 5 indicates diisooctylphthalate (mass spectra Nos. 21, 43, 61, 73 and 68 in Fig. 1).

CONCLUSIONS

The procedure presented allows the identification and differentiation of therapeutic concentrations of barbiturates, other sedative-hypnotics and their metabolites in urine, integrated in a general unknown analysis procedure for the detec-

tion of several categories of drugs like benzodiazepines [3,26], antihistamines [4–7], butyrophenone neuroleptics [27], analgesics [28], opioids [29], antidepressants [30], neuroleptics [31], antiparkinsonians [32], β-blockers [33], antiarrhythmics [34], laxatives [35] and anticonvulsants [36]. The identified sedative-hypnotics can then be quantified in plasma using methods described by Mangin *et al.* [14], Rop *et al.* [15], Svinarov and Dotchev [40] or reviewed by Gupta [41].

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