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IDENTIFICATION AND DIFFERENTIATION OF BENZODIAZEPINES AND THEIR METABOLITES IN URINE BY COMPUTERIZED GAS CHROMATOGRAPHY–MASS SPECTROMETRY*

HANS MAURER* and KARL PFLEGER

Institut für Pharmakologie und Toxikologie der Universität des Saarlandes, D-6650 Homburg/Saar (F.R.G.)

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SUMMARY

A method for the identification and differentiation of the following benzodiazepines and their metabolites in urine after acid hydrolysis and acetylation is described: bromazepam, camazepam, chlordiazepoxide, clobazam, clonazepam, clorazepate, clotiazepam, cloxazolam, delorazepam, diazepam, ethylloflazepate, flunitrazepam, flurazepam, halazepam, ketazolam, loprazolam, lorazepam, lormetazepam, medazepam, metaclazepam, midazolam, nitrazepam, nordazepam, oxazepam, oxazolam, prazepam, quazepam, temazepam and tetrazepam. The acetylated extract was analysed by computerized gas chromatography–mass spectrometry. An on-line computer allowed rapid detection using ion chromatography with ions *m/z* 205, 211, 230, 241, 245, 249, 312 and 333. The identity of positive signals in the reconstructed ion chromatogram was confirmed by a comparison of the stored full mass spectra with reference spectra. The ion chromatograms, reference mass spectra and gas chromatographic retention indices (on OV-101) are documented.

INTRODUCTION

1,4- and 1,5-Benzodiazepines are used as tranquilizers, hypnotics, anticonvulsants or muscle relaxants and rank among the most frequently prescribed drugs [1]. The number of individual benzodiazepines has doubled in the last few years. They are often the cause of an intoxication, either alone or in combination with other drugs or ethanol. Many patients form a dependence on these drugs. Therefore, benzodiazepines are encountered frequently in clinical or forensic toxicological analysis. Concentrations of benzodiazepines and their metabolites in urine are higher than those in plasma, so that it is preferable to use urine for their identification. However, some of the benzodiazepines and their metabolites are

*Dedicated to Professor Dr. Walter Rummel, Homburg/Saar, on the occasion of his 65th birthday.

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excreted in urine in a completely conjugated form. The conjugates are usually cleaved by acid hydrolysis, which can be completed more quickly than enzymatic hydrolysis. In this process the benzodiazepine molecules are decomposed to benzophenones or analogues [2]. Detection of some of these hydrolysis products using thin-layer chromatography (TLC) [3,4], gas chromatography (GC) [3,5,6], high-performance liquid chromatography (HPLC) [7] and gas chromatography–mass spectrometry (GC–MS) has been described [8]. Other detection methods have been reviewed [4,9–11]. Some of the common benzophenones cannot be detected by the TLC procedures. The selectivity and specificity of TLC, GC and HPLC procedures are not sufficient for unequivocal identification. Therefore, these methods must be confirmed by a second independent method, specifically by GC–MS.

The GC–MS procedure of ref. 8 allowed the selective and specific detection of the hydrolysis products of sixteen benzodiazepines. However, the sensitivity for some of the hydrolysis products with primary amino or hydroxy groups was not sufficient for detection at low concentrations. We improved the sensitivity by acetylation.

A computerized GC–MS screening procedure for the identification and differentiation of at least 29 1,4- and 1,5-benzodiazepines and their metabolites in urine is described. This procedure has the additional advantage that several other categories of drugs may be detected simultaneously by searching for typical fragment ions in the stored spectra.

EXPERIMENTAL

Apparatus

A Hewlett-Packard (HP Waldbronn, F.R.G.) Series 5890 gas chromatograph was used in combination with an HP MSD Series 5970 mass spectrometer and an HP Series 59970 C workstation. 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°C to 310°C at 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.

The exact measurement of retention indices was performed on a Varian (Palo Alto, CA, U.S.A.) Series 3700 gas chromatograph. The column effluent was led into a flame-ionization detector and into a nitrogen-sensitive flame-ionization detector after a 1:1 split using 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 to those used for GC–MS, the temperature of the detectors was 270°C. Nitrogen was used as the carrier gas at a flow-rate of 30 ml/min.

Urine samples

The investigations were performed using urine samples from inpatients treated with therapeutic dosages of benzodiazepines. The data for clonazepam, flunitra-

zepam and nitrazepam were obtained from the analysis of urine samples from intoxicated patients. When suitable samples from man were not available, urine samples from rats were used (see Table I). Rats were administered between 20 and 80 mg/kg body weight of drugs in aqueous suspension by gastric intubation.

Hydrolysis and extraction procedure

A 10-ml volume of urine was refluxed with 3 ml of 37% hydrochloric acid for 15 min. Following hydrolysis, approximately 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–isopropanol–ethyl acetate (1:1:3). Phase separation was accomplished by centrifugation. The organic extract was decanted and evaporated to dryness under vacuum.

Acetylation

The extracted residue was acetylated for 30 min at 60°C with 100 μl of a mixture of three parts of acetic acid anhydride and two parts of pyridine. The acetylation mixture was evaporated to dryness, and the residue was dissolved in 100 μl of methanol. Between 0.5 and 2 μl of this sample were injected into the gas chromatograph.

GC–MS 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. The identity of positive signals in the reconstructed ion chromatogram was confirmed by a comparison of the full mass spectra with reference spectra (see Fig. 1).

RESULTS AND DISCUSSION

Table I summarizes the data collected during this study. Using the eight selected ions for constructing the ion chromatograms allowed the detection of at least 29 benzodiazepines and their metabolites. Most of the compounds are derivatized by acetylation following hydrolysis (see Table I). The mass spectra numbers referring to Fig. 1, the molecular masses, the species from which the urine was assayed and the retention indices are listed in the table. The latter indices were determined using temperature-programmed GC with flame-ionization detection (FID) and nitrogen-sensitive FID. In our experience, retention indices provide preliminary indications of drug class identity and may be useful to workers without a GC–MS facility. Additionally, they allow distinguishing between isomeric compounds, which give very similiar mass spectra.

Data are only given for metabolites which were frequently found. Not all the listed metabolites are detected in each sample due to inter-species (man/rat) or inter-individual variations in metabolism or due to variations in the time elapsed after administration. The mass spectra and retention indices of the less abundant metabolites will be included in a forthcoming handbook [12].

The data of alprazolam, brotizolam, triazolam and their human metabolites

TABLE I

MONITORING PROGRAMME FOR ACETYLATED HYDROLYSIS PRODUCTS OF BENZODIAZEPINES AND THEIR METABOLITES

MS No.	Mass	Drug/metabolite (M)	Derivative*		Species	*m/z* (relative intensities)**								Retention index (OV-101)
						205	211	230	241	245	249	312	333	
29	308	Alprazolam***			(PS)	+			+	+				3100
19	294	M (hydroxy-) $-CH_2O$			(PS)	++			+					3070
50	366	M (hydroxy-)		AC	(PS)				+					3180
33	318	Bromazepam + M (3-hydroxy-)	HY	AC	Man						++			2490
16	285	M (3-hydroxy-), artifact 1			Man	+					+			2255
22	299	M (3-hydroxy-), artifact 2			Man						+			2265
60	392	Brotizolam***			(PS)					+				3090
57	378	M (hydroxy-) $-CH_2O$			(PS)					+				3050
64	450	M (hydroxy-)		AC	(PS)					+				3140
4	245	Camazepam + M (temazepam)	HY		Man			+		+++				2100
10	273	M (oxazepam)	HY	AC	Man			+++						2245
10	273	Chlordiazepoxide	HY	AC	Man			+++						2245
12	274	Clobazam	HY		Man	+		+						2225
2	242	M (nor-)	HY		Man	+			+	+				2210
38	330	M (norhydroxymethoxy-)	HY	AC	Man					+			+	2615
23	300	M (norhydroxy-)	HY	AC	Man									3000
14	276	Clonazepam	HY		Man				+++					2470
39	330	M (amino-)	HY	AC	Man		++			+			+	2845
10	273	Clorazepate	HY	AC	Man			+++						2245
41	331	M (hydroxy-), isomer 1	HY	AC	Man			+			+		+	2560
42	331	M (hydroxy-), isomer 2	HY	AC	Man			+			+		+	2580
49	361	M (hydroxymethoxy-)	HY	AC	Man									2990
34	318	Clotiazepam§			Man									2540
55	376	M (hydroxy-)		AC	Man				+					2870
63	434	M (dihydroxy-)		AC	Man								+	2995
24	307	Cloxazolam + M (delorazepam)	HY	AC	Man			+++						2300
24	307	Delorazepam + M (hydroxy-)	HY	AC	Man			+++						2300
4	245	Diazepam	HY		Man			+		+++				2100
10	273	M (nor-) + M (oxazepam)	HY	AC	Man			+++						2245
18	291	Ethylloflazepate	HY	AC	Man			+			+++			2195

13	274	Flunitrazepam	HY				+							2370
31	314	M (noramino-)	HY	AC	Man		+	+++						2715
36	328	M (amino-)	HY	AC	Man	+++				+				2870
47	348	Flurazepam	HY											2555
18	291	M (desalkyl-)	HY	AC	Man			+			+++			2195
32	316	M (bisdesethyl-) $-H_2O$	HY	AC	Man		+			++				2460
44	335	M (hydroxyethyl-)	HY	AC	Man									2470
48	352	Halazepam			Rat				+					2335
30	313	Halazepam	HY		Rat					+		++		2380
1	240	M (oxazepam), artifact			Rat	+++			+					2060
10	273	M (desalkyl-)	HY	AC	Rat			+++						2245
53	371	M (hydroxy-), isomer 1	HY	AC	Rat			+		+		+	+	2350
54	371	M (hydroxy-), isomer 2	HY	AC	Rat			+		+		+	+	2375
62	401	M (hydroxymethoxy-)	HY	AC	Rat									2500
41	331	M (desalkylhydroxy-), isomer 1	HY	AC	Rat			+			+			2560
42	331	M (desalkylhydroxy), isomer 2	HY	AC	Rat			+			+			2580
49	361	M (desalkylhydroxymethoxy-)	HY	AC	Rat									2990
4	245	Ketazolam + M (diazepam)	HY		Man			+		+++				2100
1	240	M (oxazepam), artifact 1			Man	+++			+					2060
8	254	M (oxazepam), artifact 2			Man	+			+					2070
10	273	M (oxazepam)	HY	AC	Man			+++						2245
14	276	Loprazolam	HY		Rat				+++					2470
24	307	Lorazepam	HY	AC	Man			+++						2300
11	274	Artifact			Man	+			+					2170
15	279	Lormetazepam	HY		Man					+				2220
11	274	M (nor-), artifact			Man	+			+					2170
24	307	M (nor-)	HY	AC	Man			+++						2300
9	270	Medazepam				+		+	+	+				2235
10	273	M (nordazepam)	HY	AC	Man			+++						2245
21	298	M (nor-)		AC	Man			+						2470
59	392	Metaclazepam			Man								+	2640
56	378	M (nor-)			Man								++	2690
35	325	Midazolam										+		2580
58	383	M (hydroxy-)		AC	Man							+		2820
3	242	Nitrazepam	HY		Man				++					2365
20	296	M (amino- –)	HY	AC	Man		++							2985
10	273	Nordazepam + M (oxazepam)	HY	AC	Man		+++							2245

(Continued on p. 90)

TABLE I (continued)

MS No.	Mass	Drug/metabolite (M)	Derivative*		Species	*m/z* (relative intensities)**								Retention index (OV-101)
						205	211	230	241	245	249	312	333	
41	331	M (hydroxy-), isomer 1	HY	AC	Man			+			+		+	2560
42	331	M (hydroxy-), isomer 2	HY	AC	Man			+			+		+	2580
49	361	M (hydroxymethoxy-)	HY	AC	Man									2990
10	273	Oxazepam	HY	AC	Man			+++						2245
1	240	Artifact 1			Man	+++			+					2060
8	254	Artifact 2			Man	+		+						2070
10	273	Oxazolam + M (nordazepam)	HY	AC	Man			+++						2245
17	285	Prazepam	HY		Man			+	+	+				2410
10	273	M (desalkyl-)	HY	AC	Man			+++						2245
40	331	Quazepam + M (oxo-)	HY		Rat								+	1985
18	291	M (desalkyloxo-)	HY	AC	Rat			+			+++			2195
52	370	M (oxo-)			Rat					+				2255
4	245	Temazepam	HY		Man			+		+++				2100
10	273	M (nor-)	HY	AC	Man			+++						2245
6	249	Tetrazepam, isomer 1	HY		Man						+			2220
7	249	Tetrazepam, isomer 2	HY		Man						++			2280
4	245	M (dihydroxy-) $-2H_2O$	HY		Man			+		+++				2100
25	307	M (hydroxy-), isomer 1	HY	AC	Man			+			+			2380
26	307	M (hydroxy-), isomer 2	HY	AC	Man			+			+			2470
45	335	M (norhydroxy-)	HY	AC	Man			+			+			2500
27	307	M (hydroxy-), isomer 3	HY	AC	Man			+			+			2535
28	307	M (hydroxy-), isomer 4	HY	AC	Man			+			+			2560
46	342	Triazolam***			(PS)				+					3080
37	328	M (hydroxy-) $-CH_2O$		AC	(PS)				+					3000
61	400	M (hydroxy-)			(PS)				+					3200
43	332	(Androsterone)		AC	Man			+		+			+	2580 FID
51	368	(Cholesterol $-H_2O$)			Man	+				+				3030 FID
5	247	(Endogenous biomolecule)		AC	Man	+								1920

*HY = hydrolysed; AC = acetylated.

**+++ = >95% relative intensity; ++ = 50–95%; + = <50%.

***These data were recorded on pure substances (PS) only.

§Clotiazepam and its metabolites can only be detected after enzymatic hydrolysis.

were recorded only for the pure substances, because after therapeutic dosage the levels were too low and no human samples with toxic dosages were available. Rat studies were not suitable, because the detected metabolites were different from those described for man [13–15].

Clotiazepam and its metabolites are destroyed during acid hydrolysis. Clotiazepam is excreted in urine only in small quantities and its metabolites are extensively conjugated. The latter can be detected in urine only after enzymatic hydrolysis. The ions with *m/z* 217, 289, 318 and 434 are proposed for selective ion monitoring in an acetylated extract.

Flunitrazepam, flurazepam, medazepam and midazolam are excreted in urine almost exclusively as metabolites. In an overdose situation it is possible that the parent compounds are also detectable in urine. Therefore these data are included in the table.

Halazepam and oxoquazepam are not completely hydrolysed under the conditions used. Therefore, the data of the intact molecules have been included.

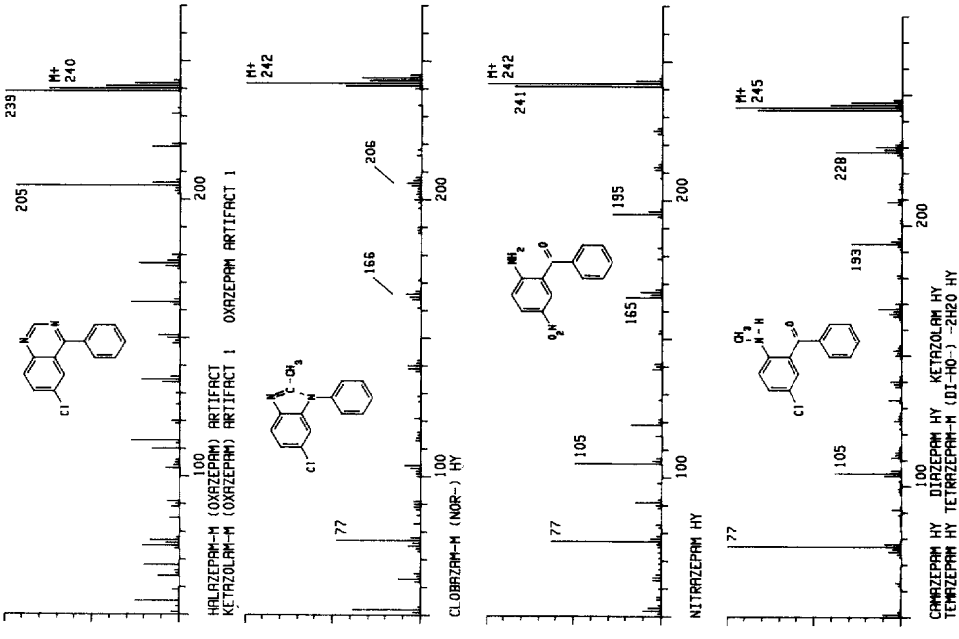
The data of androsterone, cholesterol and an unknown endogenous biomolecule are included, because the presence of these physiological compounds was indicated by the ion chromatograms.

Several artifacts were formed using the analytical procedure. The 1,4-benzodiazepinone molecules were decomposed to benzophenones and analogues. The α-hydroxy metabolites of alprazolam, brotizolam and triazolam were partly altered by elimination of formaldehyde (mass spectra 19, 37 and 57 in Fig. 1). Dehydration was observed for cholesterol and the hydrolysis products of bis(desethyl)flurazepam and dihydroxytetrazepam (mass spectra 4, 32 and 51). Hydroxybromazepam, lorazepam and oxazepam formed artifacts by rearrangement (mass spectra 1, 8, 11, 16 and 22). The normetabolites of clobazam were cleaved and rearranged to benzimidazole derivatives (mass spectra 2, 23 and 38) [8,16]. Tetrazepam and its two hydroxy metabolites (probably hydroxylated at C-3′ and C-4′) were transformed into two *cis/trans* isomeric hexahydroacridone derivatives each (mass spectra 6, 7, 25–28) [17,18]. Due to very low levels, the other isomers of the reacted norhydroxytetrazepam could not be detected.

The full mass spectra used for the identification of the compounds are shown in Fig. 1. They are listed in order of ascending mass of the highest ion. In case of identical nominal mass values, the spectra are arranged in order of ascending retention indices. Formulae are proposed for probable metabolite structures.

The identity of the peaks observed in the ion chromatograms can be positively confirmed by the comparison of the underlying mass spectra with spectra of standards (Fig. 1 and ref. 12). Therefore, interferences by other drugs are improbable. However, some benzodiazepines lead to the same metabolites and/or to the same hydrolysis products. In some cases the drug taken can be identified by the metabolite profile in urine. If necessary, the intact drug or its metabolites can be detected in an extract of urine after enzymatic hydrolysis. The mass spectra and indices of these compounds will be included in ref. 12.

The sensitivity of the method is sufficient to detect the majority of the benzodiazepines in urine following a single therapeutic administration. Clonazepam,


1
HALAZEPAM-M (OXAZEPAM) ARTIFACT
KETAZOLAM-M (OXAZEPAM) ARTIFACT 1
OXAZEPAM ARTIFACT 1
2
CLOBAZAM-M (NOR-) HY
3
NITRAZEPAM HY
4
CAMAZEPAM HY
TEMAZEPAM HY
DIAZEPAM HY
TETRAZEPAM-M (DI-HO-) -2H2O HY
KETAZOLAM HY
5
ENDOGENOUS BIOMOLECULE 2HC
6
TETRAZEPAM ISOMER 1 HY
7
TETRAZEPAM ISOMER 2 HY
8
KETAZOLAM-M (OXAZEPAM) ARTIFACT 2
OXAZEPAM ARTIFACT 2

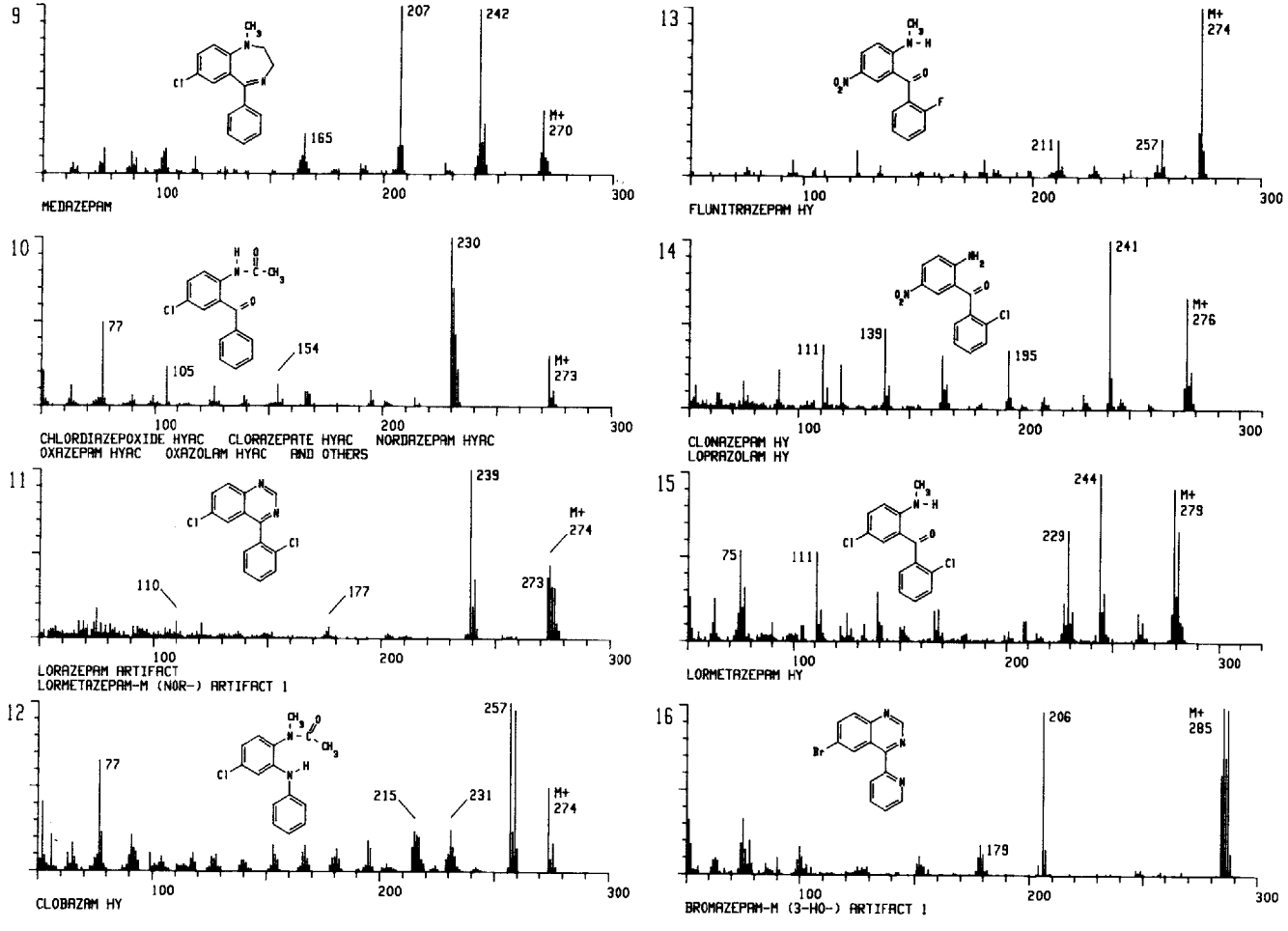


Fig. 1.

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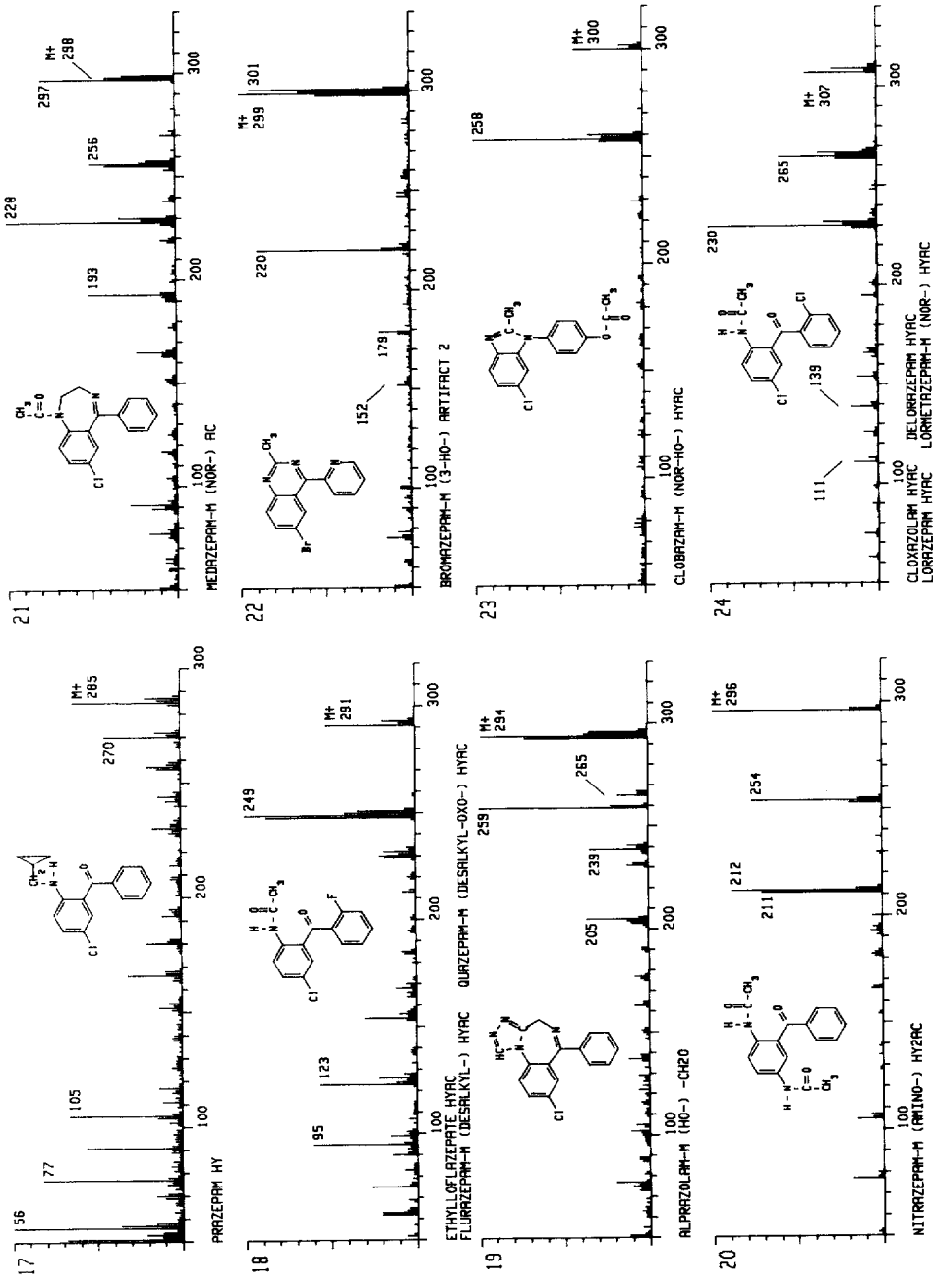
17
56
77
105
270
285
M+
100
200
300
DIAZEPAM HY
18
95
123
249
291
M+
100
200
300
ETHYLLOFLAZEPATE HYAC
FLURAZEPAM-M (DESALKYL-) HYAC
19
205
239
259
265
294
M+
100
200
300
QUAZEPAM-M (DESALKYL-OXO-) HYAC
ALPRAZOLAM-M (HO-) -CH2O
20
211
212
254
296
M+
100
200
300
NITRAZEPAM-M (AMINO-) HY2AC
21
193
228
256
297
298
M+
100
200
300
MEDAZEPAM-M (NOR-) AC
22
152
179
220
299
301
M+
100
200
300
BROMAZEPAM-M (3-HO-) ARTIFACT 2
23
258
300
M+
100
200
300
CLOBAZAM-M (NOR-HO-) HYAC
24
111
138
230
265
307
M+
100
200
300
CLOXAZOLAM HYAC
LORAZEPAM HYAC
DELORAZEPAM HYAC
LORMETAZEPAM-M (NOR-) HYAC

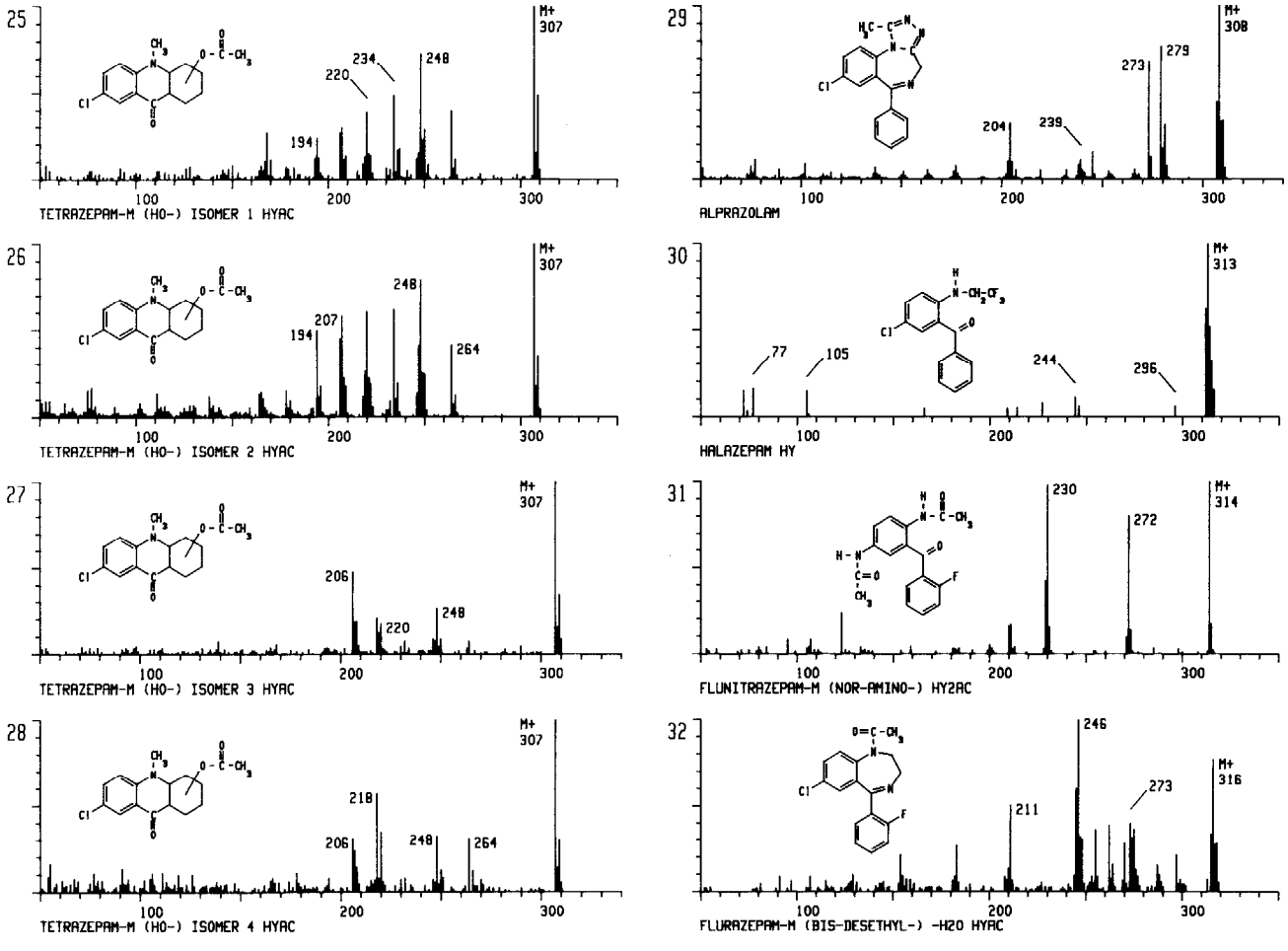

Fig. 1.

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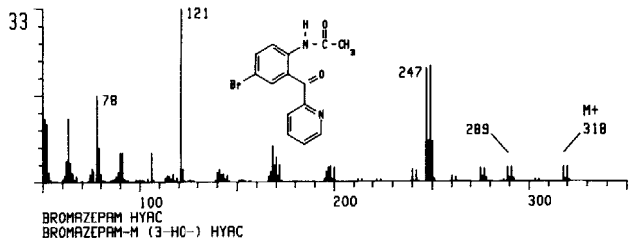
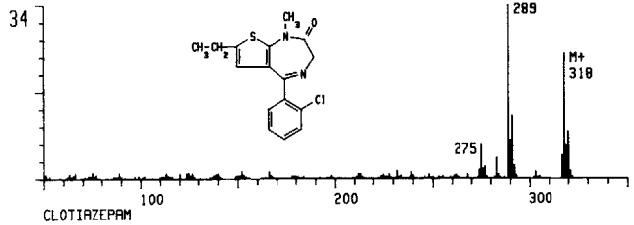
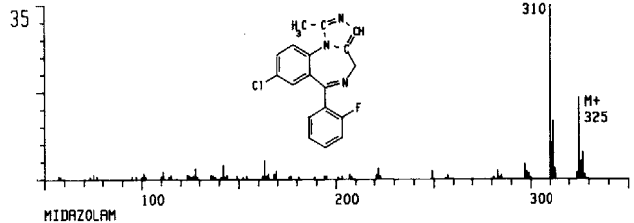


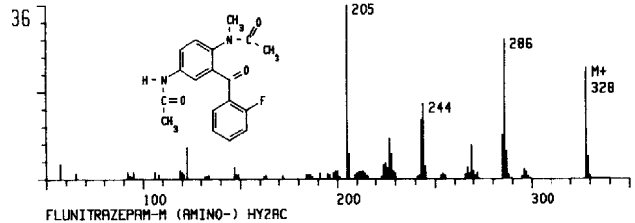
BROMAZEPAM HYAC
BROMAZEPAM-M (3-HC-) HYAC



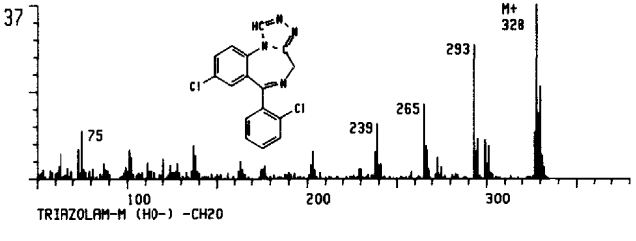
CLOTIAZEPAM



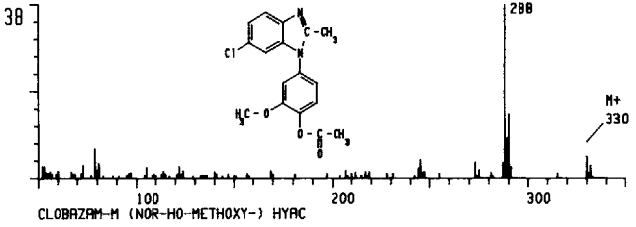
MIDAZOLAM



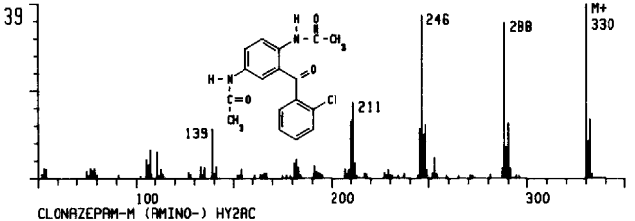
FLUNITRAZEPAM-M (AMINO-) HY2AC



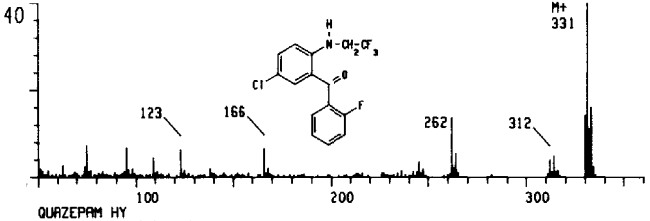
TRIAZOLAM-M (HO-) -CH2O



CLOBAZAM-M (NOR-HO-METHOXY-) HYAC



CLONAZEPAM-M (AMINO-) HY2AC



QUAZEPAM HY
QUAZEPAM-M (OXO-) HY

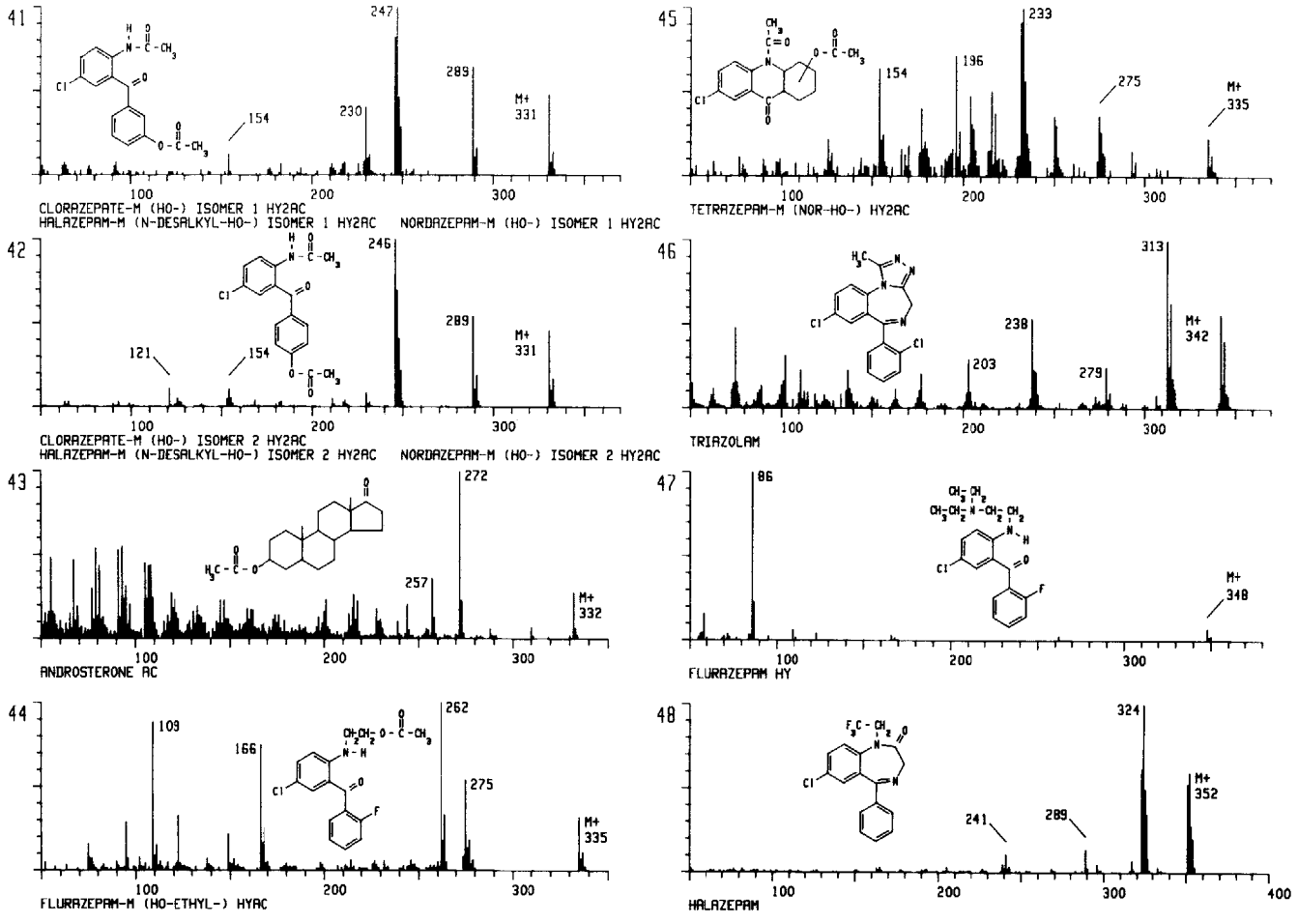


Fig. 1.

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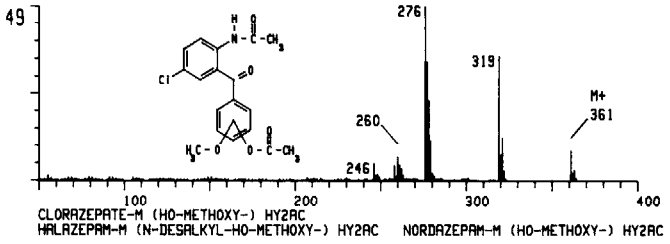
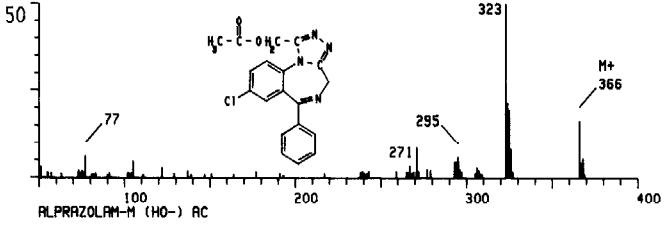
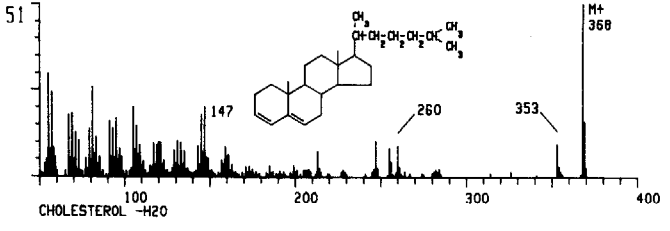
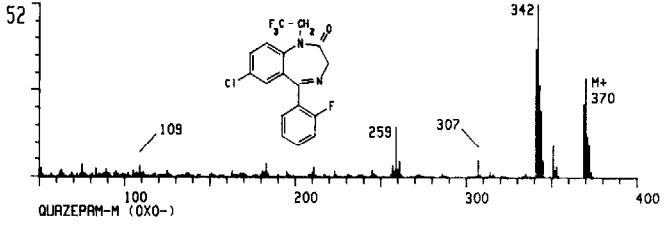
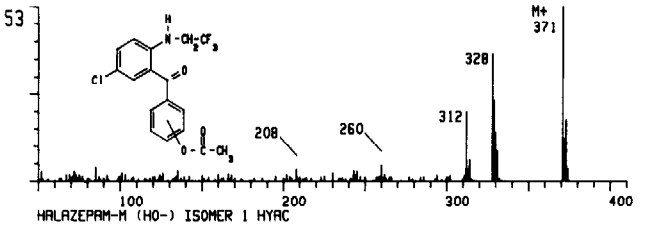
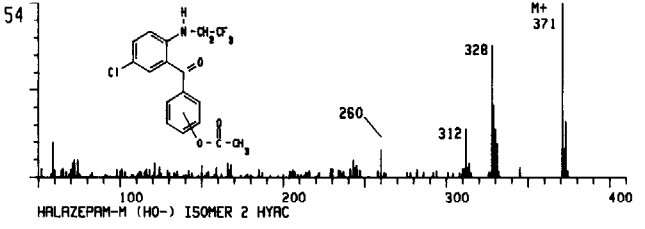

49
276
319
M+
361
260
246
100
200
300
400
CLORAZEPATE-M (HO-METHOXY-) HY2AC
HALAZEPAM-M (N-DESALKYL-HO-METHOXY-) HY2AC
NORDAZEPAM-M (HO-METHOXY-) HY2AC

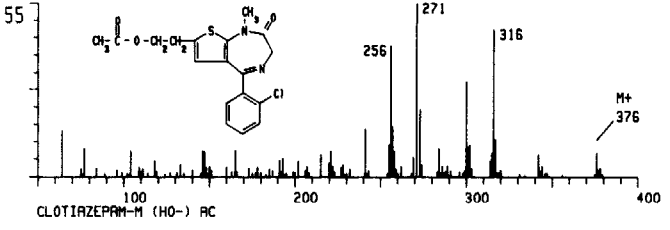

50
323
M+
366
77
295
271
100
200
300
400
ALPRAZOLAM-M (HO-) AC

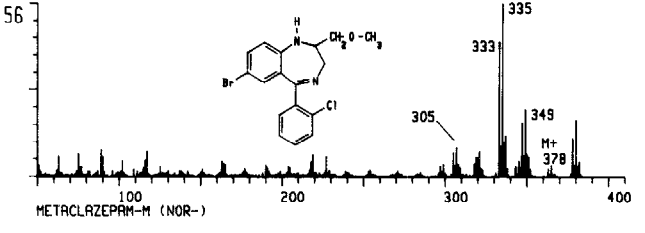

51
M+
368
147
260
353
100
200
300
400
CHOLESTEROL -H2O


52
342
M+
370
109
259
307
100
200
300
400
QUAZEPAM-M (OXO-)


53
M+
371
328
312
208
260
100
200
300
400
HALAZEPAM-M (HO-) ISOMER 1 HYAC


54
M+
371
328
260
312
100
200
300
400
HALAZEPAM-M (HO-) ISOMER 2 HYAC


55
271
256
316
M+
376
100
200
300
400
CLOTIAZEPAM-M (HO-) AC


56
335
333
349
305
M+
378
100
200
300
400

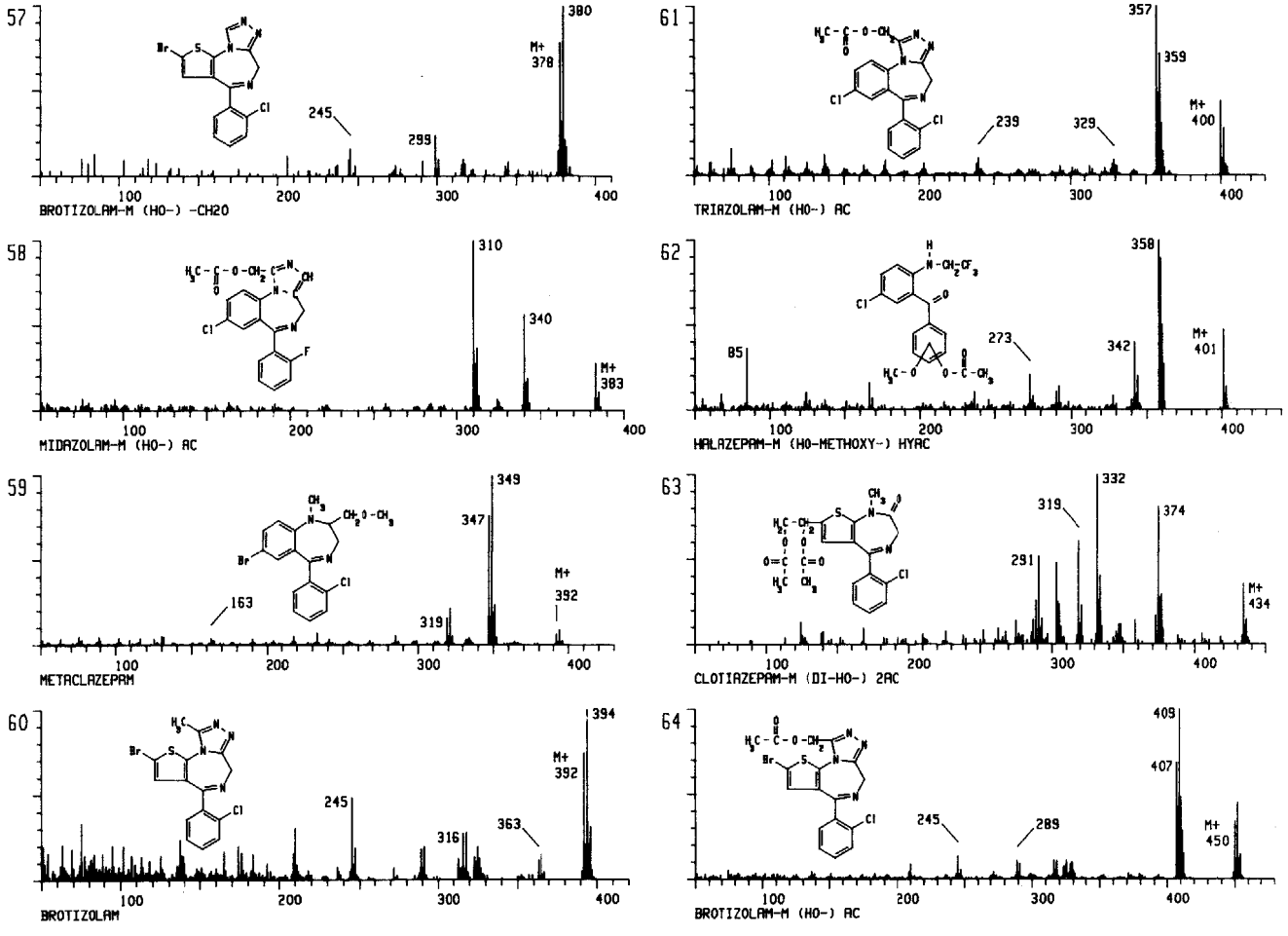


Fig. 1. Mass spectra of acetylated hydrolysis products of benzodiazepines and their metabolites identified in urine after acid hydrolysis, extraction and acetylation.

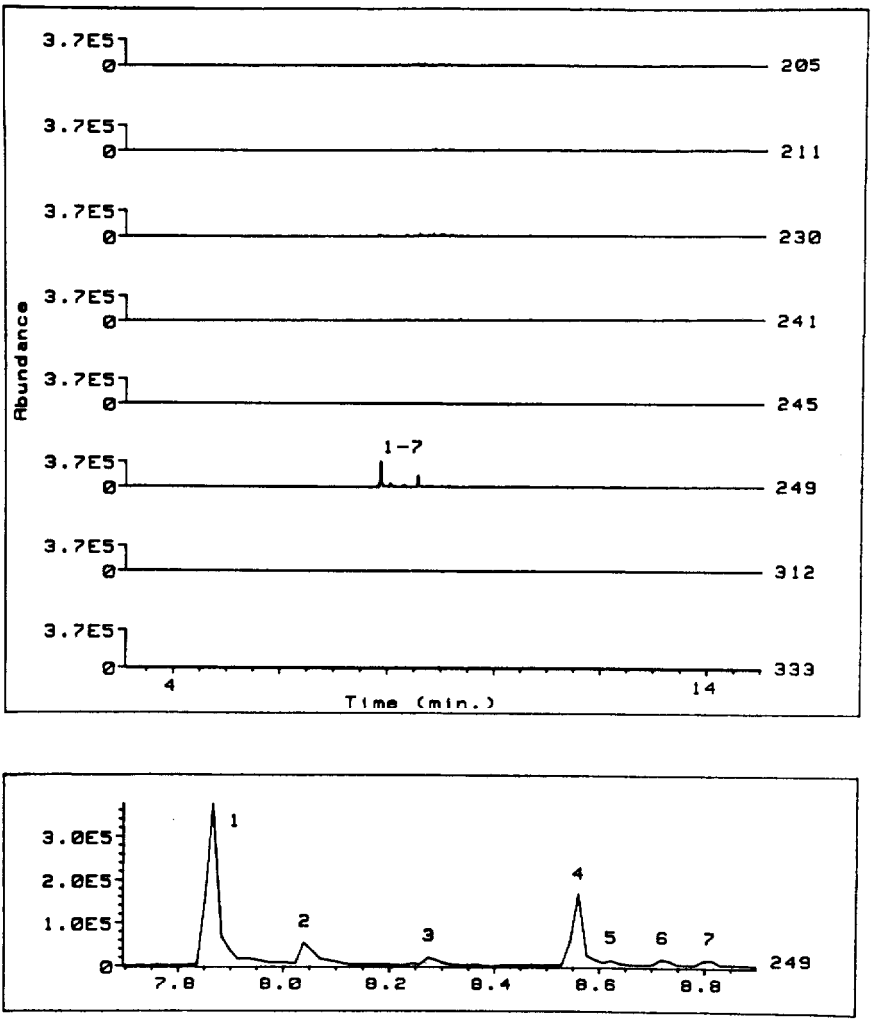


Fig. 2. Ion chromatograms indicating the presence of the hydrolysis products of tetrazepam and its metabolites (top). Amplified chromatogram in the region of the ion with m/z 249 (bottom).

flunitrazepam and nitrazepam were detected in human urine samples only after an overdosage.

To illustrate the method, the ion chromatograms from a urine sample of a patient suspected to be intoxicated with benzodiazepines is shown in Fig. 2 (top). The amplified chromatogram of the ion 249 is shown at the bottom of the figure. Peaks 1 and 2 indicate the two isomers of the hydrolysis product of tetrazepam and peaks 3–7 indicate the acetylated hydrolysis products of the metabolites of tetrazepam (mass spectra 6, 7, 25, 26, 45, 27 and 28).

CONCLUSIONS

The presented procedure allows the rapid and selective detection of at least 29 1,4- and 1,5-benzodiazepines and their metabolites in urine. The method has the

additional advantage that other classes of drugs can be detected simultaneously by searching for typical fragment ions in the stored spectra. Ion chromatograms typical for butyrophenones and bisfluorophenyl neuroleptics [19], anti-inflammatory analgesics [20], opioids and other potent analgesics [21], antidepressants [22], phenothiazine and analogous neuroleptics [23], antiparkinsonian drugs [24] and β-blockers [25] have been published previously. Detection and identification of antihistamines by GC–MS is in preparation [26]. Similar data for other compounds of toxicological interest will be collected. Through utilization of this technology nearly all relevant drugs will be detectable in urine or other biological materials within 1–2 h following administration.

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