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INVESTIGATION OF THE METABOLITES OF TOFIZOPAM IN MAN AND ANIMALS BY GAS–LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

The metabolites of tofizopam [Grandaxin; 1-(3,4-dimethoxyphenyl)-4-methyl-5-ethyl-7,8-dimethoxy-5*H*-2,3-benzodiazepine] have been studied in patients and animals. The major pathway of the metabolic transformation of tofizopam was found to be demethylation. The position in which demethylation takes place and the rate of this process in various species were determined. Gas–liquid chromatography–mass spectrometry–mass chromatography was used for the identification of the metabolites.

INTRODUCTION

Benzodiazepines are widely used as psychotropic drugs[1] and include various chemically related derivatives such as diazepam, oxazepam, chlordiazepoxide, flurazepam and pivoxazepam. These compounds have sedative, tranquillizing, muscle relaxant and anticonvulsive effects.

A recent addition to this series is a drug with a completely different structure, 1-(3,4-dimethoxyphenyl)-4-methyl-5-ethyl-7,8-dimethoxy-5*H*-2,3-benzodiazepine (Grandaxin; tofizopam) (I) (Fig. 1), which was synthesized by Kőrösi and co-workers[2,3]. Pharmacology of tofizopam has been reported in detail[4–6]. As the clinically effective therapeutic doses of the compound are low, its metabolic transformation requires analytical methods suitable for the determination of the intact drug at nanogram levels and at the same time specific enough to differentiate the drug and its own metabolites in physiological fluids.

In this paper gas–liquid chromatographic (GLC) and GLC–mass spectrometric (GLC–MS) methods are described for the investigation of tofizopam and its metabolites in biological samples. It has been found that the only metabolic pathway for tofizopam[7,8] was the partial elimination of the methyl moiety of methoxyl groups. The position in which demethylation takes place and the rate of this process in various species were investigated.

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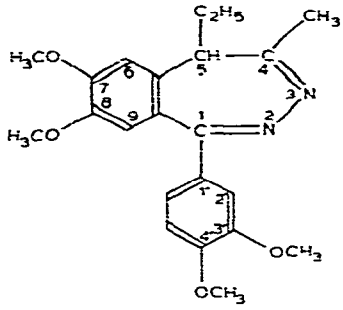
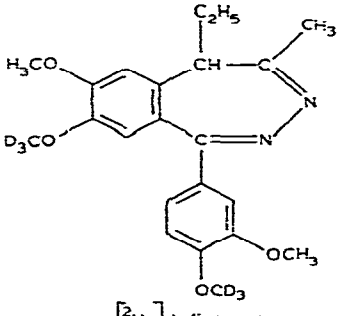
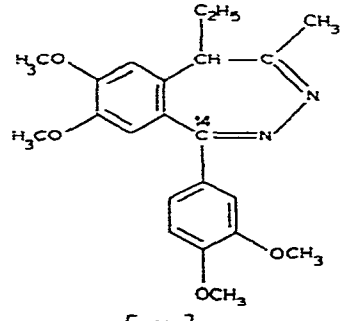


tofizopam (Grandaxin)
(I)



[$^{2}H_6$] tofizopam
(II)



[1-^{14}C]tofizopam
(III)

Fig. 1. Tofizopam and derivatives.

EXPERIMENTAL

Materials

N,O-Bistrimethylsilyltrifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (Pierce, Rockford, IL, U.S.A.) were used as silylating reagents. The stationary phases were OV-101, XE-60 and Gas-Chrom Q (80–100 mesh) (Applied Science Labs., State College, PA, U.S.A.). Tofizopam (I) (Fig. 1) was synthesized by EGYT Pharmacochemical Works (Budapest, Hungary). The 8,4′-ditrideuteromethoxy analogue of tofizopam (II) (Fig. 1) and [1-^{14}C]tofizopam (III) (Fig. 1) were prepared in the Institute for Drug Research (Budapest, Hungary)[9]. The solvents were of analytical-reagent grade.

Apparatus

An HP 5830 A gas chromatograph provided with a flame-ionization detector was used. Column A was a 6 ft. × 4.00 mm I.D. glass coil containing 2% XE-60 on Gas-Chrom Q (80–100 mesh); column B was a 2 ft. × 2.00 mm I.D. glass coil packed with 2% OV-101 on Gas-Chrom Q (80–100 mesh). The carrier gas was nitrogen at flow-rates of (A) 45 ml/min and (B) 25 ml/min. The column temperatures were (A) 240°C and (B) 200°C and the detector and injector temperatures were 250°C in each instance. The chart speed was 0.6 cm/min.

An HP 5990 A gas chromatograph–mass spectrometer equipped with column B was used with a column temperature of 200°C, an electron energy of 70 eV and a multiplier voltage of 3000 V.

Drug administration

Rats, dogs, rabbits and monkies were administered orally doses of 3, 15 and 30 mg/kg of a mixture of II and I (2:1). The composition of the mixture was controlled by GLC–MS. Human patients were administered orally a dose of 3 mg/kg of a mixture of II and I (2:1). Urines were collected after 24 h.

Extraction

A 10-ml volume of urine was extracted with 30 ml of chloroform. After centrifugation, the chloroform layer was separated, evaporated and examined by thin-layer chromatography (TLC). The extracts were spotted on silica gel PF-60 plates (20 × 20 cm) and developed in chloroform–*n*-hexane–ethanol–concentrated ammonia solution (40:40:20:3, v/v). The spots were detected by autoradiography or by exposure to UV light (254 nm) in the human experiments. In the animal experiments compound III (Fig. 1) was used as a tracer indicator.

The pH values of 10 ml of the aqueous phases containing conjugated metabolites were adjusted to 4.6 with borate buffer (1 *M*) and incubated for 72 h at 37°C with β-glucuronidase. The samples were extracted with chloroform, the chloroform layers were evaporated and aliquots of the concentrates were examined by TLC as described above. Radioactive spots were scraped off from the plates and eluted with chloroform–methanol (2:1) and concentrated to 0.5 ml. Aliquots of the samples were injected into the GLC–MS evaporator directly or 10 ml of the extracts were evaporated with a stream of nitrogen and 30 μl of BSTFA and 3 μl of TMCS were added to the samples, kept at 60°C in sand-bath for 30 min and injected into the equipment.

RESULTS AND DISCUSSION

Preliminary runs were carried out on stationary phases with different selectivities for the separation of tofizopam from its metabolites. As the major part of urinary metabolites were found to be conjugated with glucuronic acid, the samples were hydrolysed and purified using preparative TLC and analysed on polar and apolar stationary phases. The samples were analysed as TMS derivatives on XE-60 and OV-101 and without derivatization on OV-101. R_F values and relative retention data are given in Table I.

The metabolic pathway of tofizopam was assumed to be elimination of methyl group(s). Owing to interaction of these phenolic compounds with XE-60 stationary

TABLE I

R_F AND RELATIVE RETENTION TIMES (TOFIZOPAM = 1.00) OF METABOLITES OF TOFIZOPAM OBTAINED BY TLC AND GLC

*Compound**	R_F *values***	*Relative retention time****			
		XE-60		*OV-101*	
		t'	$t'_{1,2}$ ‡	t'	$t'_{1,2}$ ‡‡
IV	0.47	19.30	0.75	4.41	0.96
V	0.55	19.47	0.75	5.12	1.10
VI	0.47	20.59	0.79	4.41	0.96
VII	0.55	20.60	0.79	5.12	1.10
VIII	0.25	15.68	0.60	4.05	0.87
IX	0.21	14.87	0.57	5.12	1.10
I	0.89	26.20	1.00	4.61	1.00

* See Fig. 7 for structures.

** R_F values in solvent system chloroform–*n*-hexane–ethanol–concentrated ammonia solution (40:40:20:3).

*** t' = retention time (min); $t'_{1,2}$ = relative retention = (t'_2, metabolite)/(t'_1, tofizopam).

‡ Relative retention of TMS derivatives.

‡‡ Relative retention of samples without derivatization.

phase, they could be studied in the form of TMS derivatives. In the presence of the bulky TMS group the separation of the compounds was also insufficient when they were analysed without derivatization on OV-101. Comparing the relative retention data (Table I) on these two stationary phases, we established that compounds IV and V and VI and VII eluted with the same retention time on XE-60 but could be separated on OV-101.

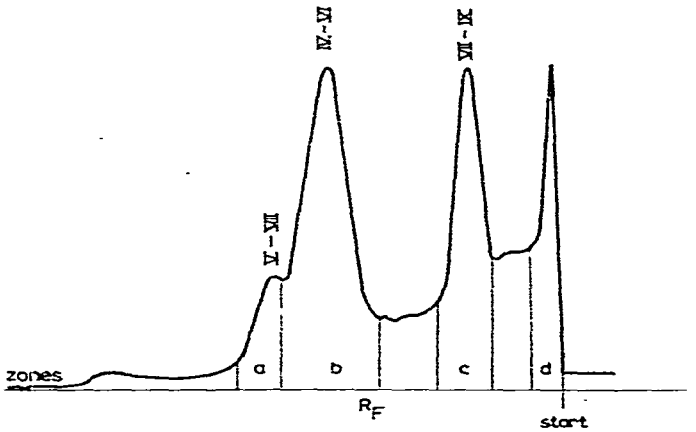


Fig. 2. Radio-scan of thin-layer chromatogram from urine extract of monkey. Zones a, b, c and d were scraped off the plate, eluted with chloroform–ethanol (2:1) and analysed by GLC–MS. The TLC solvent system was chloroform–*n*-hexane–ethanol–concentrated ammonia solution (40:40:20:3). Zone d was scraped off the plate eluted with chloroform–ethanol (2:1), treated with β-glucuronidase and investigated by TLC in a second run.

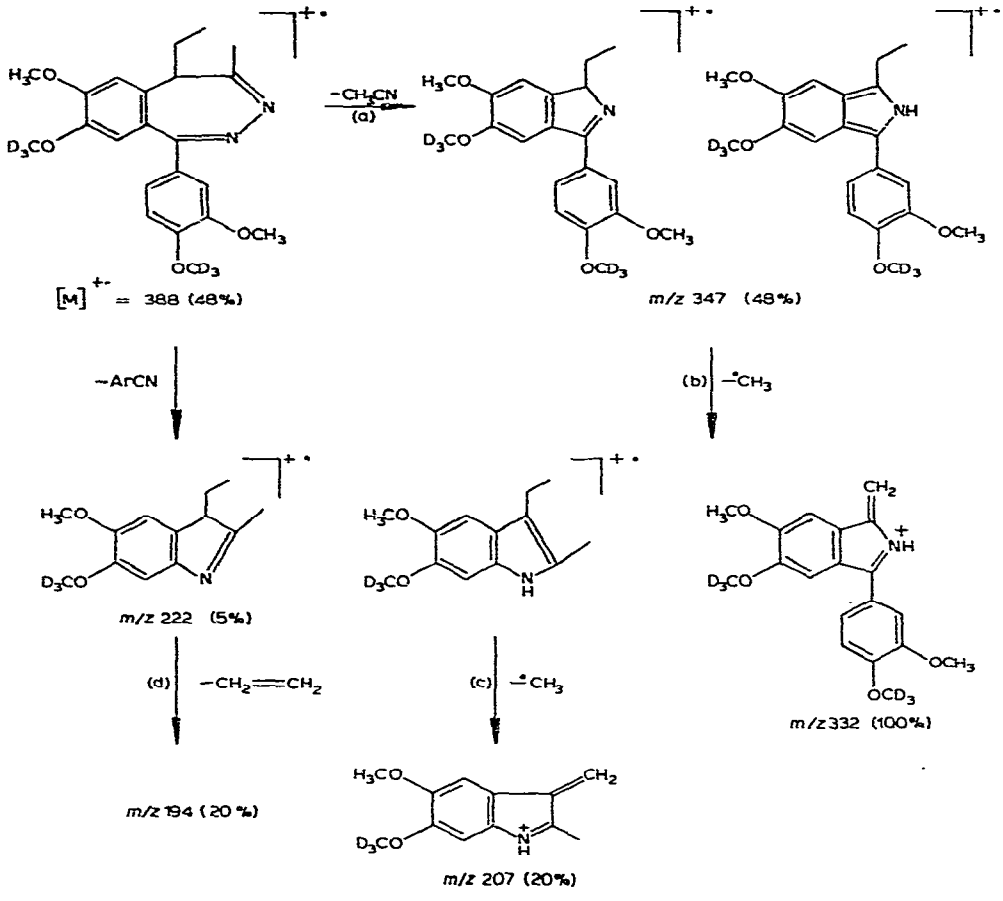


Fig. 3. The main fragmentation pathway of [$^2H_6$]tofizopam (II).

The extracts investigated by thin-layer chromatography (Fig. 2) or mass spectrometry only were found to be mixtures of various demethylated metabolites.

The mass spectral fragmentation of tofizopam has been reported earlier[3]. There is a possibility of differentiating between compounds demethylated at the C-1 aryl group and the condensed aromatic ring on the basis of the fragmentation pattern. In order to distinguish between the methoxyl groups on the same ring a labelled analogue of tofizopam, 8,4′-ditrideuteromethoxytofizopam (II) (Fig. 1) was synthesized[9]. In a quadrupole mass spectrometer we used an automatic peak selector to monitor more (up to twenty) ions and measured the peak heights of the ions of interest. The main fragmentation pathway of [$^2H_6$]tofizopam (II) is shown in Fig. 3.

The mass spectrum (Fig. 4*) of the peak eluted in 4.40 min on OV-101 (Table I) showed the deuterated and unlabelled tofizopam demethylated at position C-7 (IVa and IVb). These compounds were isolated as major metabolites from urine extracts of

* For Figs. 4, 5, 6, 8, 9 and 10 the notations of the principal fragments are given in Table II.

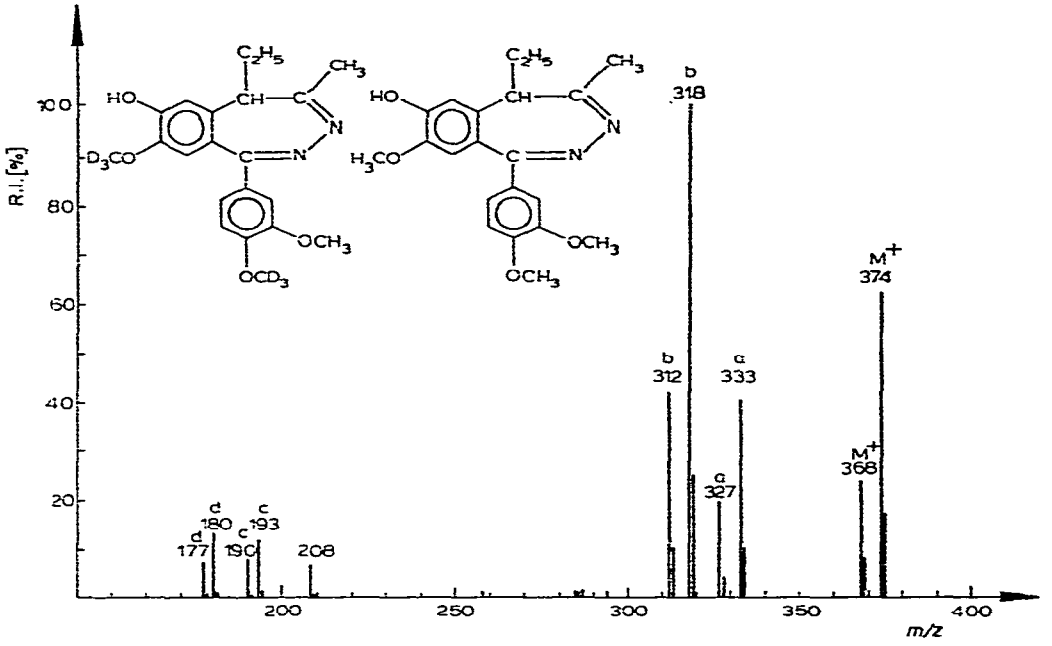


Fig. 4. Mass spectra of IVa and IVb. Conditions as specified in *Apparatus* (B).

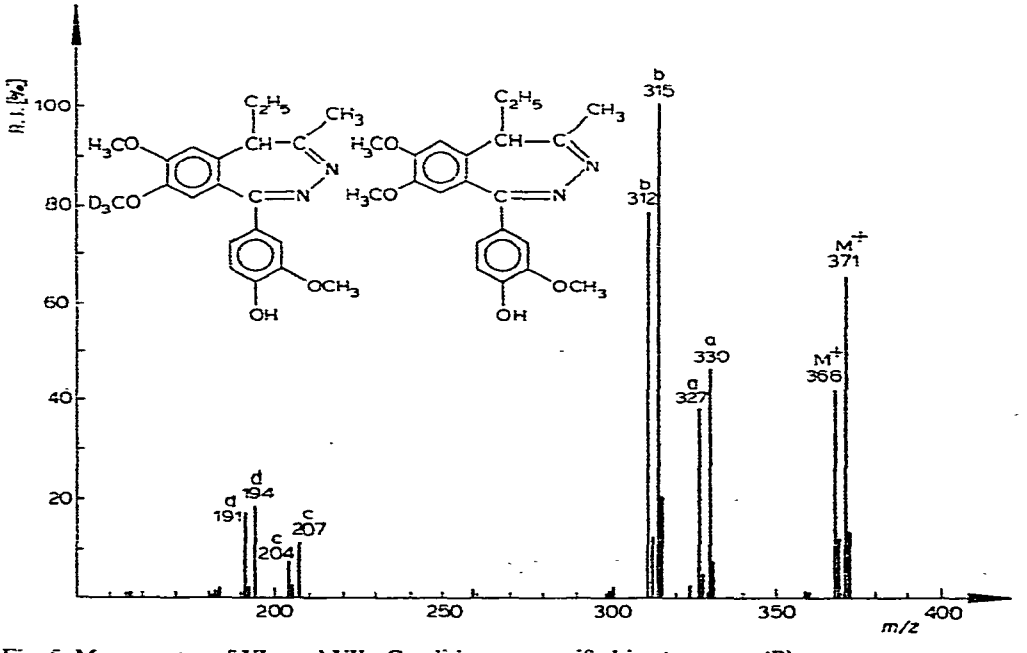


Fig. 5. Mass spectra of VIa and VIb. Conditions as specified in *Apparatus* (B).

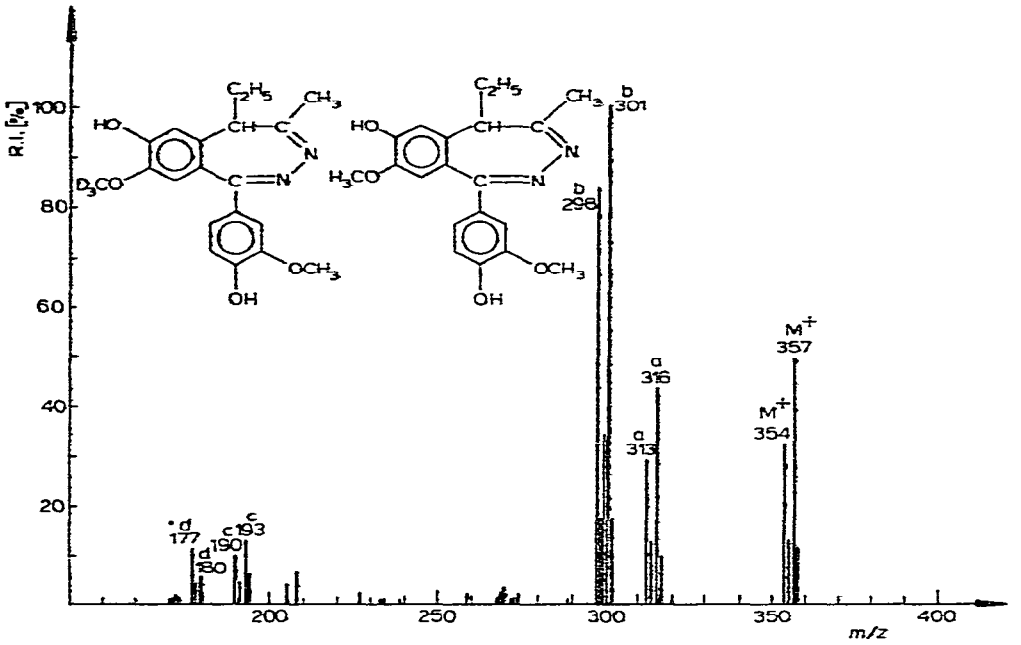


Fig. 6. Mass spectra of VIIIa and VIIIb. Conditions as specified in *Apparatus* (B).

monkies, rats and rabbits. The deuterated and unlabelled tofizopam demethylated at position C-4 (VIa and VIb) was eluted with the same retention time.

The compounds were identified with the aid of mass chromatography. The molecular ion of IVa is at *m/z* 374 and that of IVb at *m/z* 368. The base peak of the spectrum of IVa appears at *m/z* 318 and that of IVb at *m/z* 312. The characteristic ions $[M-ArCN-{}^{\bullet}CH_3]^+$ and $[M-ArCN-CH_2CH_2]^{+\bullet}$ appear at *m/z* 193 and 180 for compound IVa and at *m/z* 190 and 177, for compound IVb respectively. The molecular ion of VIa is at *m/z* 371 and that of VIb at *m/z* 368. The base peak of the spectrum of VIa (Fig. 5) appears at *m/z* 315 and that of VIb at *m/z* 312. The characteristic ions $[M-ArCN-{}^{\bullet}CH_3]^+$ and $[M-ArCN-CH_2CH_2]^{+\bullet}$ were at *m/z* 207 and 194 for compound VIa and at *m/z* 204 and 191 for compound VIb, respectively.

The peak eluted with 4.00 min on OV-101 (Table I) represents the deuterated and unlabelled tofizopam di-demethylated at positions C-7 and C-4 (VIIIa and VIIIb) (Fig. 6). The molecular ion of VIIIb is at *m/z* 354, the base peak of the spectrum of VIIIb appears at *m/z* 298 and characteristic ions $[M-ArCN-{}^{\bullet}CH_3]^+$ and $[M-ArCN-CH_2CH_2]^{+\bullet}$ are at *m/z* 190 and 177, respectively. The same ions are shifted to *m/z* 357, 301, 193 and 180, respectively, for compound VIIIa.

In the species investigated six metabolites were identified (Fig. 7). The relative abundances of principal fragments of all metabolites are given in Table II.

As can be seen from these data, the fragmentation pattern of the demethylated metabolites is the same as that of tofizopam. The identification of the metabolites that were found as minor components in the species investigated was based on mass chromatography. The high relative abundances of molecular ions and the base peaks in the spectra indicate the possibility of using the method in practice. Fragment ions

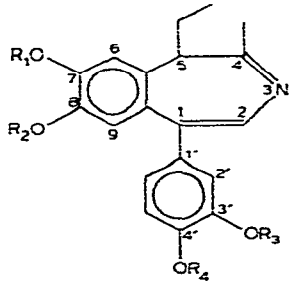
TABLE II

RELATIVE ABUNDANCES OF PRINCIPAL FRAGMENTS OF METABOLITES OF TOFIZOPAM (I) AND [$^2H_6$]TOFIZOPAM (II)

Compound	*Molecular ion*	*Relative intensity*	*m/z and relative intensities* of some fragment ions***
IVa	374	42	333[a](39), 318[b](100), 193[c](6), 180[d](11)
IVb	368	41	327[a](38), 312[b](100), 190[c](7), 177(6)
Va	374	46	333[a](31), 318[b](100), 207[c](11), 194[d](9)
Vb	368	44	327[a](32), 312[b](100), 204[c](9), 191[d](9)
VIa	371	45	330[a](31), 315[b](100), 207[c](11), 194[d](17)
VIb	368	44	327[a](30), 312[b](100), 204[c](10), 191[d](7)
VIIa	371	27	330[a](30), 315[b](100), 190[c](10), 177[d](8)
VIIb	368	28	327[a](29), 312[b](100), 204[c](10), 191[d](10)
VIIIa	357	58	316[a](46), 301[b](100), 193[c](13), 180[d](13)
VIIIb	354	63	313[a](27), 298[b](100), 190[c](9), 177[d](11)
IXa	360	56	319[a](45), 304[b](100), 193[c](13), 180[d](13)
IXb	354	62	313[a](26), 298[b](71), 190[c](19), 177[d](8)

* Relative intensities in parentheses.

** Notations: a = $[M-CH_3CN]^{+\cdot}$; b = $[M-CH_3CN-{}^{\cdot}CH_3]^+$; c = $[M-ArCN-{}^{\cdot}CH_3]^+$; d = $[M-ArCN-CH_2CH_2]^{+\cdot}$.



I $R_1 = R_2 = R_3 = R_4 = CH_3$
II $R_1 = R_3 = CH_3$; $R_2 = R_4 = CD_3$
IIIa $R_1 = H$; $R_2 = R_4 = CD_3$; $R_3 = CH_3$
IIIb $R_1 = H$; $R_2 = R_3 = R_4 = CH_3$
IVa $R_3 = H$; $R_2 = R_4 = CD_3$; $R_1 = CH_3$
IVb $R_3 = H$; $R_1 = R_2 = R_4 = CH_3$
Vb $R_4 = H$; $R_1 = R_2 = R_3 = CH_3$
VIa $R_4 = H$; $R_1 = R_3 = CH_3$; $R_2 = CD_3$
VIIa $R_2 = H$; $R_1 = R_3 = CH_3$; $R_4 = CD_3$
VIIb $R_2 = H$; $R_1 = R_3 = R_4 = CH_3$
VIIIa $R_1 = R_4 = H$; $R_2 = CD_3$; $R_3 = CH_3$
VIIIb $R_1 = R_4 = H$; $R_2 = R_3 = CH_3$
IXa $R_1 = R_3 = H$; $R_2 = R_4 = CD_3$
IXb $R_1 = R_3 = H$; $R_2 = R_4 = CH_3$

Fig. 7. Structures of metabolites of tofizopam (I and II).

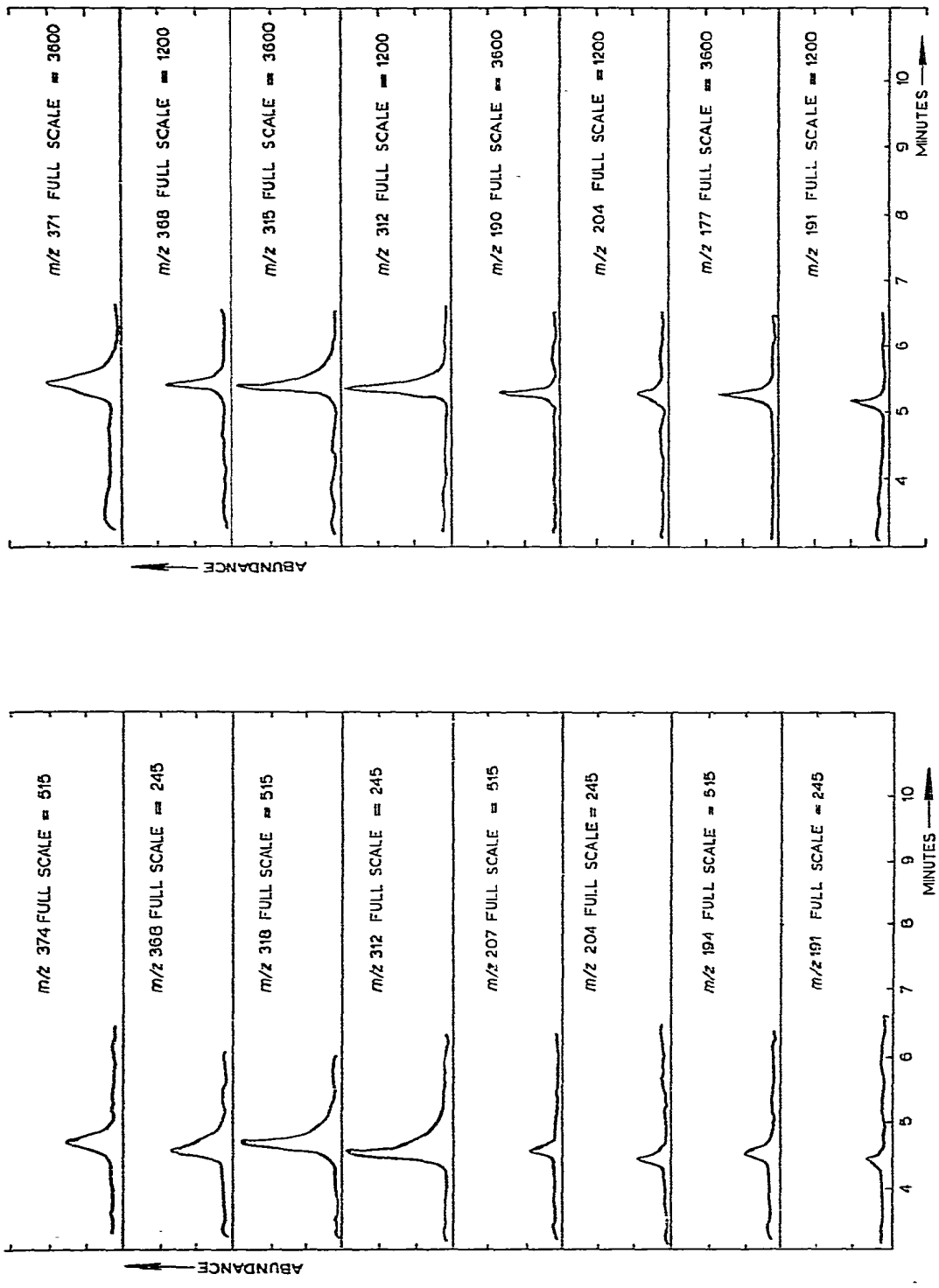


Fig. 8. Mass chromatograms of Va and Vb on an OV-101 column. Conditions as specified in *Apparatus* (B).

Fig. 9. Mass chromatograms of VIIa and VIIb on column B. Conditions as specified in *Apparatus* (B).

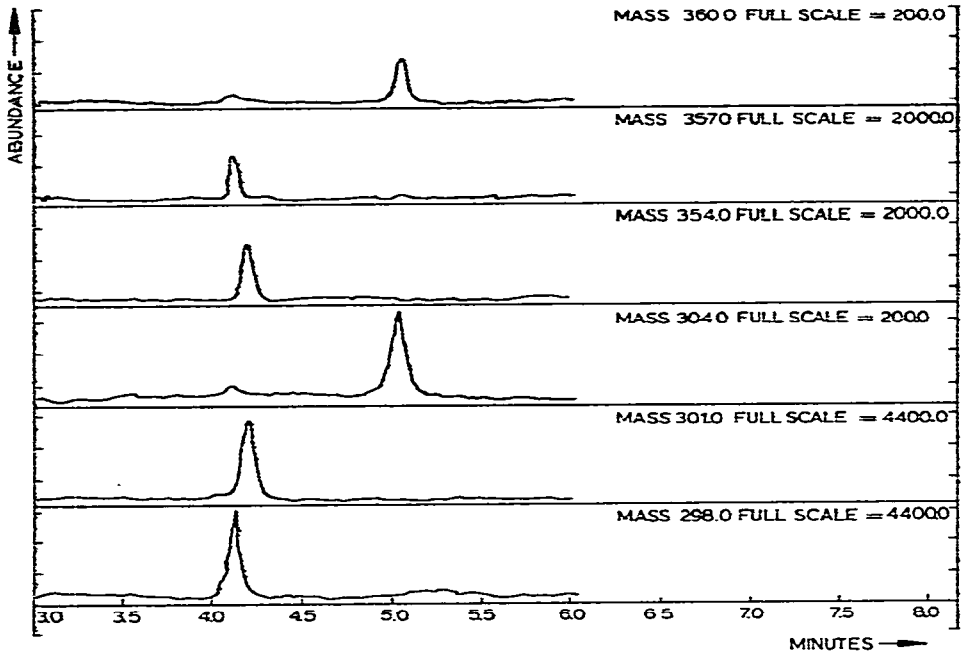


Fig. 10. Mass chromatograms of IXa, VIIIa and VIIIb. Conditions as specified in *Apparatus* (B).

of Va at *m/z* 374, 318, 207 and 194 that of Vb at *m/z* 368, 312, 204 and 191 were used to monitor the compounds. They eluted from column B in 5.1 min. Fig. 8 shows the mass chromatograms of compounds Va and Vb.

The mass chromatograms of compounds VIIa and VIIb are given in Fig. 9.

Fig. 10 shows the mass chromatograms of an extract from monkey urine. This sample contained VIIIa and VIIIb as major metabolites and IXa as minor metabolite.

TABLE III

PERCENTAGE OF METABOLITES OF TOFIZOPAM IN DIFFERENT SPECIES

Species	*Major component*	*%*	*Minor component*	*%*
Rat	IV	90	VI	5
			VIII	5
Dog	IV	65	VI	30
Monkey	IV	45	V	4
	VIII	30	VII	2
			VI	15
			IX	0.5
Rabbit	VI	55	V	4
	IV	30		
Man	VI	62	IV	15
			VIII	9

Four different monodemethylated metabolites were identified. According to the relative retention data given in Table II, the metabolites could be separated chromatographically from each other if they contained the phenolic group in a *p*- or *m*-position relative to C-1. The percentages of the metabolites that have been isolated from the urine of the various species investigated are given in Table III.

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