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Pharmacokinetic and metabolism studies on girisopam by chromatographic and spectrometric methods in humans

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ABSTRACT

Girisopam possesses selective anxiolytic action without muscle relaxant and anticonvulsive activity. After a 100-mg oral dose of ^{14}C-labelled girisopam to seven male subjects, the mean recovery of ^{14}C radioactivity was 51% in urine and 33% in faeces. A high-performance liquid chromatographic method has been developed for studying girisopam in single-dose pharmacokinetic studies. The serum extract was chromatographed on a normal-phase column using a mobile phase of hexane–ethanol–diethyl ether (66:9:25, v/v) and ultraviolet detection at 235 nm. The recovery was 60% and the detection limit was 3 ng/ml, using 1 ml of serum. After a 20-min delay, girisopam is rapidly absorbed. After reaching a mean serum level of 178 ng/ml at a mean time of 2.0 h, the serum concentration of girisopam decreased with a mean elimination half-time of 22.2 h. The metabolites were separated by high-performance liquid chromatography, radio thin-layer chromatography and gas chromatography. Their structures were determined by liquid chromatography–mass spectrometry, mass spectrometry and gas chromatography–mass spectrometry. Their chemical structures were confirmed by comparison with synthesized reference compounds. The major urinary metabolites were 7-demethylgirisopam (I), 4′-hydroxygirisopam (II) and 4-hydroxymethyl-4-demethylgirisopam (III), which were in conjugated form, and 4-carboxy-4-demethylgirisopam (V), a compound with an open-chain structure (VII) and traces of 4-demethyl-4-oxogirisopam (VIII) and 4-hydroxymethyl-4-demethylgirisopam (III), which were in non-conjugated form. The metabolic profile in the serum consisted predominantly of the glucuronides of I, II and III. The non-conjugated metabolites were the metabolite with the open-chain structure (VII), III and V. Besides the parent compound, the faeces sample contained conjugates of I and II.

INTRODUCTION

Girisopam (G) (GYKI-51 189, 1-(3-chlorophenyl)-4-methyl-7,8-dimethoxy-5*H*-2,3-benzodiazepine, is a new analogue of tofizopam. The pharmacology of tofizopam has been reported in detail [1–5]. The main metabolic pathway for tofizopam has been found to be demethylation of the methoxyl groups [6,7]. G displays a peculiar spectrum of pharmacological activity [8]. Tofizopam and G both differ from the traditional 1,4-benzodiazepines in many respects, *e.g.* they possess selective anxiolytic action without muscle relaxant and anticonvulsive activity, and they show no affinity for the 1,4-benzodiazepine receptors.

For the determination of tofizopam in human serum a high-performance liquid chromatographic (HPLC) technique has been described by Sajgó *et al.* [9], and its trace impurities were analysed by HPLC by Patthy and Salát [10]. This paper reports normal- and reversed-phase HPLC methods for the pharmacokinetic investigation of G. For the metabolism study, HPLC, liquid chromatography–mass spectrometry (LC–MS), mass spectrometry (MS), gas chromatography (GC) and GC–MS were used.

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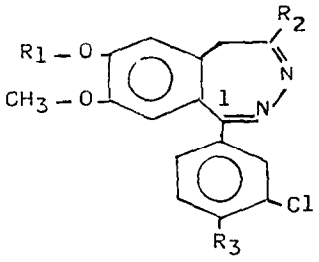
TABLE I

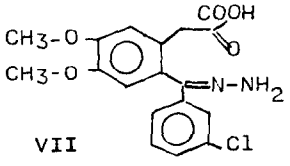
STRUCTURES AND RETENTION TIMES OF GIRISOPAM AND ITS METABOLITES



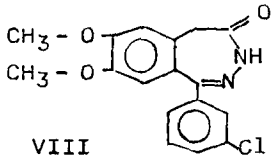
Compound	R_1	R_2	R_3	Retention time HPLC[a] (min)
Girisopam	CH_3	CH_3	H	38.5
I	H	CH_3	–	34.1
II	CH_3	CH_3	OH	32.0
III	CH_3	CH_2OH	H	33.8
IV	CH_3	CHO	H	35.8
V	CH_3	COOH	H	31.5
VI	CH_3	H	H	36.6
VII[b]				35.1
VIII[c]				
IX	H	CH_3	OH	28.1
VII				18.0

[a] Conditions as Fig. 2.

[b] Compound VII could not be synthesized.



VII



VIII

EXPERIMENTAL

Materials

Girisopam and its possible metabolites (I, II, III, IV, V, VI, VIII and IX) (Table I) were synthesized in the Institute for Drug Research (Budapest, Hungary). Radiolabelled girisopam contained a ^{14}C atom at position 1 (Table I). The internal standard, 1-(3'-chloromethyl)-4-methyl-6,7-dimethoxyphthalazine (GYKI-51 330), was synthesized in the Institute for Drug Research. A LiChroprep RP-8, 15–25 μm, 40 mm × 4 mm I.D. precolumn, a Polygosil-60 C_{18}, 10 μm, 250 mm × 4 mm I.D. analytical column, a Polygosil-60 Si, 10 μm, 250 mm × 4 mm I.D. analytical column and Samplex C_{18} cartridges were supplied by Bioseparation Technologies (Budapest, Hungary). Stationary phases, 3% OV-1 on

Gas Chrom Q (80–100 mesh) and 2% OV-225 on Gas Chrom Q (80–100 mesh), were supplied by Applied Science Labs. (State College, PA, USA). The thin-layer chromatographic (TLC) plates (20 cm × 20 cm, thickness 0.25 mm, Kieselgel 60-F-254, silanized) and the Extrelut 20 columns were supplied by Merck (Darmstadt, Germany).

Drug administration

Seven healthy volunteers, aged 35 to 50 years and within 9% of desirable weight for their height and body build, were administered orally 100 mg of G–[^{14}C]-G (98:2). Blood samples were taken at 0.16, 0.25, 0.33, 0.66, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0, 16.0, 20.0, 24.0, 36.0, 48.0 and 72.0 h after administration. Urine samples were collected in 4-h intervals up to 24 h, and thereafter daily up to five days. Faeces was collected daily up to three days. Serum, urine and faeces samples were stored at −20°C.

High-performance liquid chromatography

The apparatus consisted of an LKB (Bromma, Sweden) high-performance liquid chromatograph, three Model 2150 LKB pumps, a Model 2152 controller, a Model 7125 Rheodyne injector for sampling, a Model 7010 Rheodyne injector for column switching, an LKB 2151 multiwavelength UV–visible detector (at 235 nm), a Model 2210 LKB recorder (10 mV), a LiChroprep RP-8, 15–25 μm, 40 mm × 4 mm I.D. pre-column, a Polygosil-60 C_{18}, 10 μm, 250 mm × 4 mm I.D. analytical column and a Model 2140 LKB diode array detector.

The eluents used for the metabolism study were: eluent A, methanol–0.1 *M* ammonium acetate buffer (pH 5.8) (3:7, v/v); eluent B, methanol–0.1 *M* ammonium acetate buffer (pH 5.8) (4:1, v/v). At each injection, eluent A was pumped through the HPLC system at a flow-rate of 1 ml/min. After 12 min the solvent programmer produced a gradient from 100% eluent A to 100% B during 30 min. Then 100% eluent B was applied for 15 min. After the termination of a chromatographic run there was an equilibrium time of 5 min before the next injection, during which 100% eluent A was pumped through the system. For the pharmacokinetic study the separation was performed on a 250 mm × 4 mm I.D. Polygosil-60 Si column (10 μm). The column was eluted isocratically with *n*-hexane–ethanol–diethyl ether (66:9:25, v/v) at a flow-rate of 1 ml/min. The effluent was monitored with an LKB UV–visible detector at 235 nm.

Liquid chromatography–mass spectrometry

A Hewlett-Packard (HP) 5988A mass spectrometer (Palo Alto, CA, USA) fitted with a thermospray (TSP) interface (Vestec, Houston, TX, USA) was used. The vapourizer temperature was 95°C, the tip temperature 200°C, and the source temperature 260°C. Data were acquired and processed by using the standard software supplied by Hewlett-Packard. Scanning data were acquired in the mass range 150–530 to avoid high background from solvent cluster ions of lower mass. An HP 1090 liquid chromatograph was equipped with a binary pumping system. The pre-column, the analytical column, the gradient run and all other conditions were the same as for HPLC analysis.

Gas chromatography

For GC investigation, an HP 5710 gas chromatograph with a frequency-modulated ^{63}Ni electron-capture detector was used. The column, a 1.6 m × 4 mm I.D. glass coil, was packed with 2% OV-225 on Gas Chrom Q (80–100 mesh). The carrier gas was argon–methane (95:5) at a flow-rate of 50 ml/min. Temperatures were: injection port, 250°C; detector, 300°C; column, 235°C.

Gas chromatography–mass spectrometry

An HP 5990A gas chromatograph–mass spectrometer equipped with a column of 1.6 m × 4 mm I.D. glass coil containing 3% OV-1 on Gas Chrom Q (80–100 mesh) was used. The instrument parameters were: injector temperature, 240°C; column temperature, 240°C; electron energy, 70 eV; multiplier voltage, 3000 V. The column was fitted to the ion source by a jet separator.

Mass spectrometry

A Finnigan MAT 8430 mass spectrometer (Bremen, Germany) with direct sample introduction was used. Evaporation was controlled by the total ion current. The instrument parameters were: electron energy, 70 eV; ion source temperature, 250°C; resolution, 1250 *vs.* 10 000. For the fast atom bombardment (FAB) measurements, an 11NF type FAB source (Ion Tech, Middlesex, UK) was used. The matrix was glycerol–*m*-nitrobenzyl alcohol, with some drops of phosphoric acid. For high-resolution mass measurement, PFK was used as the reference standard.

Extraction procedures

Isolation of metabolites from urine. Aliquots of 100 ml of pooled urine samples were freeze-dried, and the residues were dissolved in 40 ml of methanol and filtered. The methanolic solutions were evaporated to dryness, and the residues were taken up in 10 ml of water and poured onto Samplex C_{18} cartridges activated with 5 ml of methanol. Each methanolic phase was subjected to TLC and the plates were developed with methanol–water (3:2, v/v) and visualized by autoradiography. Spots showing radioactivity were eluted with methanol (recovery 80 ± 5%). The remaining residue was digested with β-glucuronidase–arylsulphatase and chromatographed by HPLC and TLC.

On-line urine or serum analysis. Aliquots of 1 ml of pooled urine or 1 ml of serum were injected into a LiChroprep RP-8 pre-column and rinsed with 3 ml of water. The sample was then back-eluted with methanol–0.1 *M* ammonium acetate buffer, pH 5.8 (3:7, v/v) to a Polygosil-60 C_{18} analytical column. The column switching was actuated manually. The column effluent was continuously monitored by a UV detector, and 1-ml fractions were collected for measurement of radioactivity. (The recoveries for urine and serum samples were 80 and 45%, respectively.) For further examination, appropriate HPLC fractions were pooled and the solvent was removed with a stream of nitrogen in a sand-bath at 40°C. The residue was redissolved and analysed by HPLC or MS. The remaining residue was digested with β-glucuronidase–arylsulphatase and chromatographed by HPLC and TLC.

Isolation of metabolites from faeces. Pooled faeces samples (200 g) were suspended in 300 ml of methanol and homogenized. The slurry was filtered and the solvent was evaporated *in vacuo*. The recovery of ^{14}C activity was 60%. The solid residue was dissolved in methanol, and an aliquot was analysed by TLC. The remaining methanolic solution was evaporated to dryness and the parent compound was extracted with chloroform. The residue was dissolved in acetate buffer (pH 4.7) and digested with β-glucuronidase–arylsulphatase. The buffer solution containing the metabolites was neutralized with NaOH solution then loaded on an Extrelut 20 column. After 30 min the metabolites were eluted with 40 ml of chloroform–methanol (9:1, v/v). The effluent was evaporated to dryness, and the solid residue was dissolved in the same solution. The metabolites could then be isolated by TLC as described for urine samples.

Extraction of serum for metabolism and pharmacokinetic studies. To 1-ml serum samples, 1 ml of saturated sodium sulphate solution and 1 mg of sodium sulphate were added. The samples were extracted with 2 ml of ethyl acetate. After centrifugation at 2000 *g* for 15 min the aqueous phase was frozen in an acetone–dry ice bath. The organic phase was evaporated under a stream of nitrogen. The residue was taken up in 25–50 μl of hexane or HPLC eluent and several microlitres were subjected to GC–MS or HPLC. For the pharmacokinetic study, 150 ng of internal standard were added to the 1-ml serum samples before the extraction.

RESULTS AND DISCUSSION

Pharmacokinetic studies in humans

Preparation of calibration curve. The ratio of the peak area of G to that of the internal standard (150 ng) was plotted against the amount of G added to 1 ml of serum samples. The curve was linear in the range 5–400 ng. Triplicate measurements of seven different concentrations were carried out. The mean regression equation obtained

from three replicate curves was $y = 8.45 \cdot 10^3 x + 2.56 \cdot 10^{-3}$ ($r = 0.9984$). The values of the mean intercept of the regression line (b value) were in the range ±2%.

Analytical recovery, precision and accuracy. The absolute recovery was assessed from three replicate analyses using samples prepared by adding known amounts of G to human blank serum to give final concentrations of 10, 50 and 100 ng/ml (Table II).

The within-day and between-day variations were examined by analysing the samples prepared by adding G to the plasma at concentrations of 10, 20 and 100 ng/ml (Table III). The detection limit of G in serum was found to be 3 ng/ml, with a precision of 22.40% and an accuracy of −1.00%.

The stability of G in serum was investigated. Aliquots of serum were spiked with G to concentrations of 50, 100 and 300 ng/ml and then stored frozen at −20°C. At various time intervals (from 1 to 48 days), three samples from each concentration group were assayed for G as described. The data generated on days 1 and 48 revealed no significant change in G concentrations.

Our HPLC assay method has been used to study the pharmacokinetics of G after a single oral dose of 100 mg of G plus [^{14}C]-G to humans. The elimination pattern of G from human serum displayed a typical two-compartment pharmacokinetic profile (Fig. 1). After a delay of 0.27 ± 0.08 h (S.D.) G is rapidly absorbed with a mean absorption half-life (t_A) of 0.6 ± 0.31 h (S.D.) and eliminated with a half-life ($t_{1/2\beta}$) of 22.2 ± 9.70 h (S.D.) after reaching a mean serum level (C_{max}) of 178 ± 97 ng/ml (S.D.) at a mean time

TABLE II

ABSOLUTE RECOVERY OF GIRISOPAM

Concentration (ng/ml)	Recovery (mean ± S.D., $n = 3$) (%)
10.0	60.0 ± 15.0
50.0	62.0 ± 13.1
100.0	66.3 ± 10.2

TABLE III

WITHIN-DAY AND BETWEEN-DAY PRECISION AND ACCURACY ($n = 3$)

Concentration (ng/ml)	Precision (%)		Accuracy (%)	
	Within	Between	Within	Between
10.0	17.87	21.90	−4.40	−6.40
20.0	15.46	21.79	−1.75	+0.50
100.0	2.38	5.67	+2.46	+3.10

(t_{max}) of 2.0 ± 0.7 h (S.D.). These serum parameters agreed well with those determined by radioactivity analysis. In that study, after the same treatment (100 mg of G containing 3.7 MBq of [^{14}C]-G), the mean serum C_{max} of 665 ± 280 ng equiv./ml (S.D.) occurred at 2.1 ± 0.7 h (S.D.) after dosing: the mean $t_{1/2\beta}$ was 23.3 ± 4.3 h (S.D.). Of the orally administered dose, 51 ± 9.0% (S.D.) was eliminated in the urine and 33 ± 8.0% (S.D.) in the faeces in healthy male volunteers. The mean plasma levels of G and [^{14}C]-G in humans are shown in Fig. 1.

Metabolism study

Investigation of metabolites in urine. Table I shows the retention times of the synthesized metabolites I, II, III, IV, V, VI, VIII and IX. The electron-impact (EI) MS data of G and its possible metabolites synthesized are given in Table IV. Fig. 2 shows the HPLC chromatogram obtained by on-line analysis of pooled urine sam-

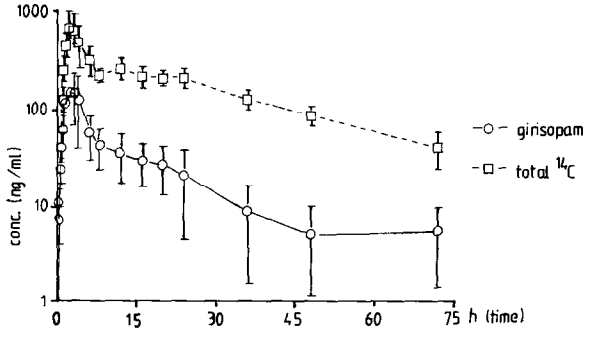


Fig. 1. Serum girisopam levels in humans. Each subject received a single oral dose of 100 mg of G-[^{14}C]-G. Values are mean ± S.D. ($n = 7$).

TABLE IV

EI MASS SPECTRA OF GIRISOPAM AND ITS METABOLITES

Values are *m/z* (relative intensity, %). a = $M^{+\cdot}$; b = $[M - R_2CN]^{+\cdot}$; c = $[M - R_2CN - {}^{\cdot}CH_3]^+$; d = $[M - R_2CN - {}^{\cdot}CH_3 - CO]^+$; e = $[b - {}^{\cdot}Ar]^+$.

Compound	MW	a	b	c	d	e	Others
Girisopam	328	328 (100)	287 (30)	272 (28)	244 (18)	176 (14)	
I	314	314 (100)	273 (82)	258 (90)	230 (80)	162 (36)	
II	344	344 (100)	303 (62)	288 (64)	260 (24)	176 (28)	
III	344	344 (100)	287 (37)	272 (24)	244 (12)	176 (11)	315 (21) $[M - {}^{\cdot}CHO]^+$
IV	342	342 (100)	287 (10)	272 (8)	–	176 (12)	313 (18) $[M - {}^{\cdot}CHO]^+$
							231 (17) $[M - {}^{\cdot}Ar]^+$
V	358	358 (14)	287 (32)	272 (22)	–	176 (9)	314 (100) $[M - {}^{\cdot}CO_2]^+$
							299 (14) $[M - {}^{\cdot}CO_2 - {}^{\cdot}CH_3]^+$
VI	314	314 (100)	287 (24)	272 (18)	244 (11)	176 (6)	313 (22) $[M - {}^{\cdot}H]^+$
VIII	330	330 (100)	–	–	–	–	301 (73) $[M - {}^{\cdot}N_2H]^+$
IX	330	330 (100)	289 (64)	274 (90)	246 (71)	162 (27)	

ples of 4–8 h. The radioactive metabolites were mapped by an HPLC (off-line) radioactivity method. The compounds detected by radioactivity are indicated as A, B, C and D in the chromatogram of Fig. 2. The sample preparation method ensured that 89% of the radioactivity applied to the column was recovered. The cumulative percentage of the [^{14}C]-G dose recovered by radioactivity measurements of 1-ml pooled

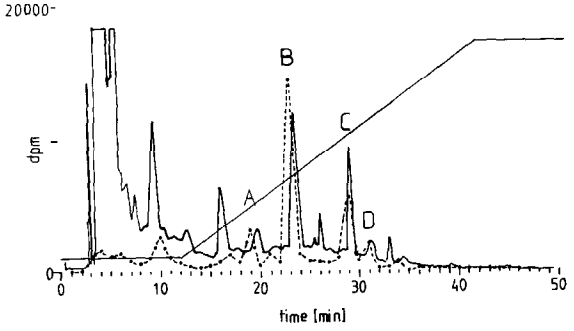


Fig. 2. HPLC chromatogram of a 1-ml urine sample (after a 100-mg single oral dose of G-[^{14}C]-G) monitored by (——) UV and (– – –) radioactive detection. Conditions: mobile phase A, methanol–0.1 *M* ammonium acetate buffer, pH 5.8 (3:7, v/v); mobile phase B, methanol–0.1 *M* ammonium acetate buffer, pH 5.8 (8:2, v/v). Gradient elution was used: 0 to 12 min, 100% A, 12 to 42 min, from 0 to 100% B; 42 to 47 min, 100% B; and 47 to 52 min, from 100% B to 100% A at a flow-rate of 1 ml/min. The analytical column was a Polygosil-60 C_{18}, 10 μm, 250 mm × 4 mm I.D. The UV detector was set at 235 nm.

HPLC fractions for assumed metabolites were as follows: A, 3.8 ± 1.03%; B, 11.9 ± 2.14%; C, 6.7 ± 1.14%; D, 1.7 ± 0.6%; non-identified metabolites, 3.5 ± 1.52%.

As can be seen in Fig. 2, the radioactive compounds A, B and C do not correspond to the retention time of any synthesized reference compounds (Table I) but peak D appeared with the same retention time as the reference standard V. The parent compound G could not be detected in urine by HPLC. In the TSP mass spectrum of peak A eluting at 18 min, the ions *m/z* 377/379 ($[M + H]^+$) and *m/z* 359/361 ($[M + H - H_2O]^+$) were found, suggesting structure VII (Table I) for this metabolite. In the TSP mass spectrum of peak B eluting at 23 min, the fragment ions *m/z* 345/347 and 315/317 were present, indicating the presence of two compounds in this peak. The *m/z* 315 and 345 values correspond to the $[M + H]^+$ ion of compounds I and II, but do not correspond to the retention times of these compounds, so we suggest that these compounds are conjugates. As direct TSP ionization failed to provide molecular mass information, the FAB-MS method was used to confirm the structures of these metabolites.

The compounds from the U/1 and U/2 spots on TLC (Fig. 3A) were isolated and analysed by HPLC and LC–MS. One of the two radioactive

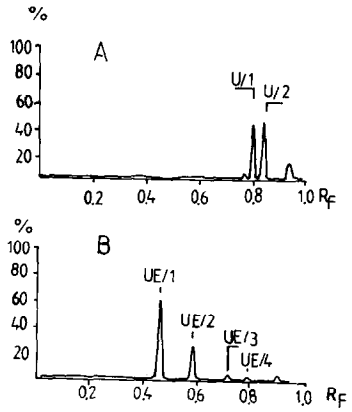


Fig. 3. (A) Radio scan of TLC chromatogram from urine extract following oral administration of 100 mg of G-[^{14}C]-G. (B) Radio scan of TLC chromatogram from digested urine following oral administration of 100 mg of G-[^{14}C]-G. The TLC solvent system was methanol–water (2:1, v/v). The spots U/1, U/2, UE/1, UE/2, UE/3 and UE/4 were scraped off the plate eluted with methanol.

peaks obtained from the zone U/1 was eluted at the same retention time (23 min) as peak B in Fig. 2, and in its TSP mass spectrum the same fragments ions *m/z* 345/347 were found as in peak B. The radioactive zone U/1 contained a second compound, which was eluted with the same retention time as peak C (28 min) indicating that the TLC method failed to separate these two possible conjugated metabolites. In the FAB spectrum of the isolated zone U/1, the quasi-molecular ion *m/z* 521/523 confirmed the presence of hydroxylated G-glucuronide conjugates. The compound isolated from the radioactive zone U/2 was eluted at the same retention time (23 min) and gave the same ions *m/z* 315/317 in the TSP mass spectrum as the HPLC peak B. In its FAB mass spectrum the quasi-molecular ion *m/z* 491/493 appeared, indicating the presence of a demethyl G-glucuronide derivative (I-glucuronide). It should be noted that in the FAB method, when using a glycerol–*m*-nitrobenzylalcohol matrix, the isolated glucuronides gave $[M + 2Na]^+$ quasi-molecular ions: *m/z* 565/567 for sample U/1 and *m/z* 535/537 for sample U/2. To obtain the $[M + H]^+$ ions H_3PO_4 was added to the matrix, resulting in partial hydrolysis of the conjugates giving the unconjugated quasi-molecular ions *m/z* 345/347 for U/1 and *m/z* 315/317 for U/2.

To obtain the free metabolites the HPLC fraction B was hydrolysed with β-glucuronidase–arylsulphatase and chromatographed. On the HPLC chromatogram two peaks appeared with retention times of 34 and 31 min, respectively, identical with those of compounds I and II (Table I). The digested urine sample was subjected to TLC (Fig. 3B). The compound isolated from spot UE/1 eluted at the same retention time as metabolite I in the HPLC system and one of the compounds observed by on-line LC–MS analysis of the digested peak B. In its EI mass spectrum the molecular ion at *m/z* 314/316 and fragment ions at *m/z* at 273/275, 258/260, 230 and 162 suggest structure I (Table IV).

The main EI-MS fragmentation pattern of G and compounds I–IV and IX is depicted in Fig. 4, and the LC–MS data for G and its metabolites are given in Table V. The compound isolated from spot UE/2 (Fig. 3B) eluted at the same retention time (32 min) as one of the peaks obtained by on-line TSP LC–MS and HPLC analysis of digested peak B. In its TSP mass spectrum the quasi-molecular ion $[M + H]^+$ (*m/z* 345/347) appeared in accordance with structure II, which was confirmed by its EI mass and ^{1}H NMR spectra. The presence of ions *m/z* 344/346, 303/305

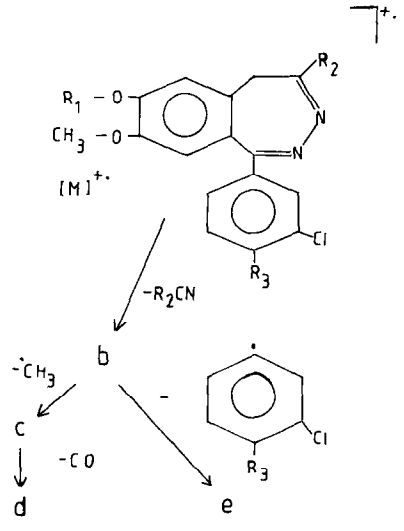


Fig. 4. EI-MS fragmentation of metabolites I–IV and IX.

TABLE V

TSP MASS SPECTRAL DATA FOR GIRISOPAM AND ITS METABOLITES

Compound	MW	*m/z* (relative intensity, %)	
		$M + H^+$	Other ions
Girisopam	328	329 (100)	
I	314	315 (100)	
II	344	345 (100)	
III	344	345 (100)	343 (70)
IV	342	343 (100)	
V	358	359 (60)	315 (100) $[M + H - CO_2]^+$
VII	376	377 (80)	359 (100) $[M + H - H_2O]^+$
VIII	330	331 (100)	
IX	330	331 (100)	
III-Glucuronide	520	521 (20)	345 (100) [M + H-glucuronide]$^+$ 343 (20)[a]

[a] Metabolite III is converted into metabolite IV at the interface temperature.

and 176 (Table IV) indicated hydroxylation on the chlorophenyl ring. The pattern of aromatic protons in the ^1H spectrum of the isolated metabolite was identical with that of compound II, which indicated 4'-hydroxyl substitution.

The peak C obtained by on-line analysis of urine eluted at 28 min (Fig. 2) was examined by LC–MS. In its TSP mass spectrum the fragment ions *m/z* 345/347, 343/345 and the assumed quasi-molecular ion *m/z* 521/523 were found, which indicates that this compound is a hydroxylated G derivative in glucuronide form, probably the glucuronide of III. However, when fraction C was digested and analysed by HPLC and TLC, more peaks and spots appeared on the chromatograms, revealing the degradation of compound III during the digestion procedure. The compound isolated from the radioactive zone U/1 eluted at the same retention time (28 min) as compound C (Fig. 2), and the ions *m/z* 345/347, 343/345 and 521/523 appeared in its TSP mass spectrum, indicating the presence of the glucuronide of III.

In the TSP mass spectrum of peak D, eluting at 31 min (Fig. 2), the quasi-molecular ion *m/z* 359/361 and a fragment ion $[M + H - CO_2]^+$ (*m/z* 315/317) were detected. On the basis of TSP mass spectrum and retention time, peak D is identical with the synthesized compound V (Table I).

The compound of the radioactive zone UE/4 eluted with an identical retention time in the HPLC system as metabolite V. The presence of the quasi-molecular ion *m/z* 359/361 and the fragment ion *m/z* 315/317 in its TSP mass spectrum also proved the structure of metabolite V.

The compound isolated from the radioactive zone UE/3 showed a retention time of 28 min in HPLC, and a quasi-molecular ion *m/z* 331/333 in its TSP mass spectrum suggests the structure IX. The synthesized compound IX gave the same chromatographic and MS data as metabolite IX.

GC was used to find minor metabolites in urine. In Fig. 5, a gas chromatogram obtained from a blank urine extract and from an extract of a urine sample containing G metabolites are shown. The latter shows peaks X_1 and X_2 at 9 and 18 min, respectively. In GC–MS (Fig. 6) the ions *m/z* 330/332 and 301/303 were detected for the peak at 18 min. According to high-resolution mass measurements this loss of 29 mass units corresponds to $^{\bullet}N_2H$, suggesting structure VIII for this metabolite. Compound VIII was synthetized and analysed by GC and GC–MS. The synthetic compound VIII gave identical chromatographic and MS data (Table IV) as metabolite X_2. The GC retention time of metabolite X_1 was found to

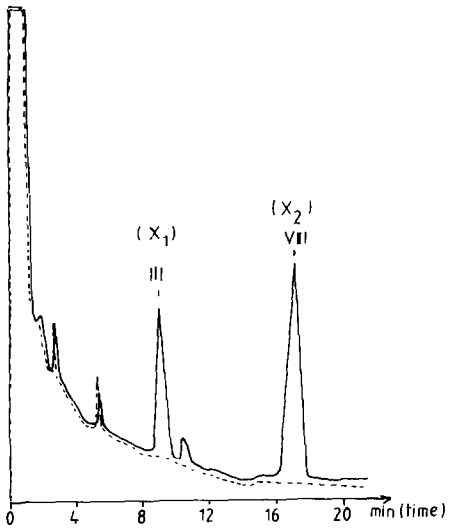


Fig. 5. (——) GC of urine extract following oral administration of G-[^{14}C]-G (4–8 h); (– – –) GC of the extract of a blank urine on a 2% OV-225 column at a flow-rate of 50 ml/min at 225°C. Injection and detector temperatures were 250 and 300°C, respectively.

be identical with that of compound III. The relative amounts of the urinary metabolites are listed in Table VI.

Investigation of metabolites in serum. Fig. 7 shows the HPLC chromatogram of the extract of a serum sample obtained at 1.5 h from a subject. The serum extract contains the same radioactive peaks as the undigested urine samples (Fig. 2). In LC–MS the same ions were monitored as for the metabolites found in urine. The compounds appearing at 18, 23 and 28 min were assigned the structures VII, I + II conjugated and III conjugated, respectively.

A less polar radioactive component E was also found in serum samples. In the TSP spectrum of the HPLC peak at 34 min the ions *m/z* 345/347 and 343/345 occur, but the retention time of this compound was different from that of metabolite II. The compound was thought to be a G metabolite hydroxylated at the C-4 methyl group (metabolite III). The HPLC fraction containing peak E was analysed by GC and gave a major peak with the same retention time (9 min) as compound X_1 in urine (Fig. 5).

Compound III was also synthesized and showed the same retention time as X_1 in both GC

TABLE VI

RELATIVE AMOUNTS OF THE METABOLITES IN URINE

Compound	Structure	Percentage of metabolite
A	VII	13.6
B	I conjugate	22.7
	II conjugate	20.2
C	III conjugate	24.2
D	V	6.1
	VIII	1[a]
	III	1[a]
Unidentified conjugated compounds and the IX conjugate		*ca.* 11.6

[a] The radioactivity chromatography method was not sensitive enough for detection of this metabolite.

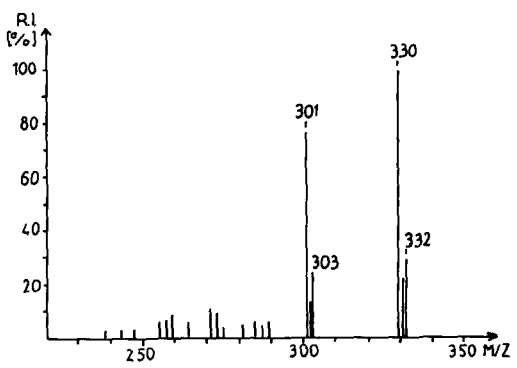


Fig. 6. GC–MS spectrum of metabolite VIII from urine extract on 3% OV-1 at 240°C. Injector temperature, 240°C; electron energy, 70 eV; multiplier voltage, 3000 V.

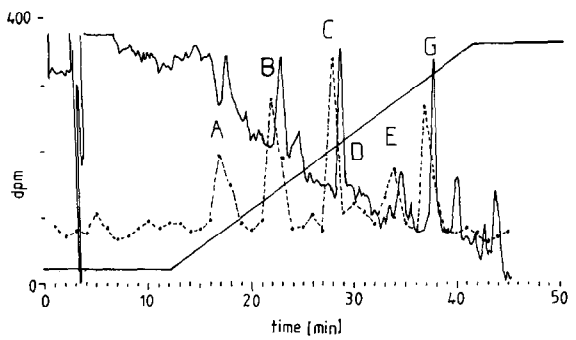


Fig. 7. HPLC of 1 ml of serum monitored by (——) UV and (– – –) radioactive detection. For HPLC conditions see Fig. 2.

and HPLC. However, in the GC–MS spectrum of X_1 (Fig. 8) and the synthetic compound III a molecular ion of m/z 342/344 was found instead of the expected m/z 344/346. Compound IV (Table I) gave identical GC and GC–MS data to metabolite X_1 (*cf.* III, peak E). This can be explained by thermal degradation of III to IV in the injector block of the gas chromatograph. The behaviour of metabolite X_1 and compound III is also similar in solution. After standing in methanol for a short period, both gave a new GC peak at 18 min corresponding to a compound that was also found in urine (X_2, compound VIII). On the basis of the above data, peak E in the HPLC chromatogram (Fig. 7) of human serum and peak X_1 on the chromatogram of human urine (Fig. 5) is metabolite III (also denoted by X_1). The compound eluted at 38 min (Fig. 7) proved to be the parent compound, G, by its retention time and the ions of m/z 329/331 in its TSP spectrum.

Investigation of metabolites in faeces. Radio-TLC of a digested faeces sample showed three main spots: F/0 (R_F = 0.35), F/1 (R_F = 0.45), and F/2 (R_F = 0.55). The mass spectrum of the compound isolated from F/0 proves the presence of the parent compound, G, in faeces. The compound isolated from zone F/1 eluted in the HPLC system with the same retention time as metabolite I, and the ion m/z 315/317 in its TSP mass spectrum proved the presence of metabolite I. The isolated compound from zone F/2 was analysed by LC–MS, ions m/z 315/317 and 345/347 showing that two compounds were present: metabolite II and a small amount of metabolite I. The presence of metabolite II was confirmed by EI-MS.

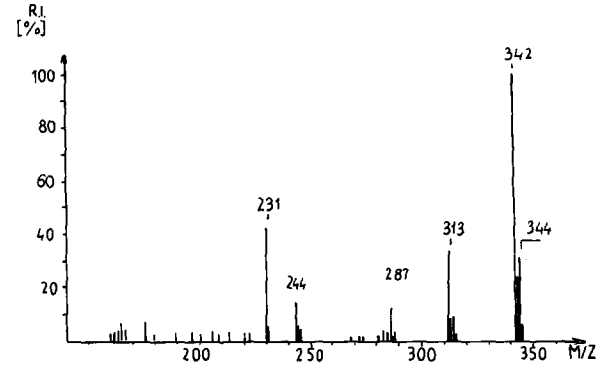


Fig. 8. GC–MS spectrum of metabolite III (X_1). For GC–MS conditions see Fig. 6.

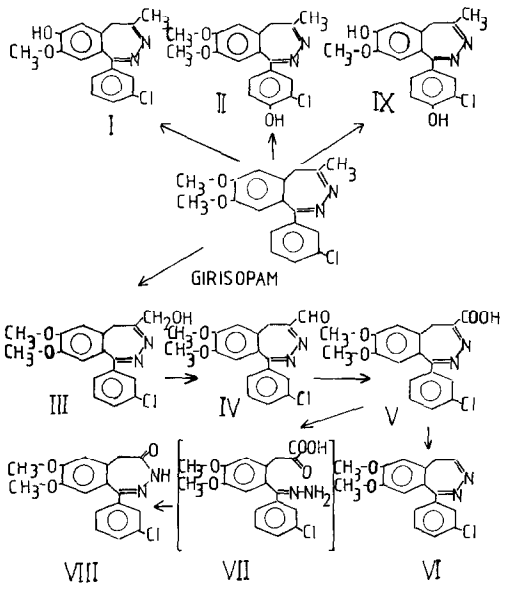


Fig. 9. Pathways of girisopam metabolism in humans.

In these experiments the following four metabolic routes have been observed (Fig. 9): (A) demethylation of the ether group at position C-7; (B) hydroxylation of the aromatic ring at position C-4′; (C) combination of A and B; and (D) enzymic oxidation of the methyl group at C-4 position.

Major metabolites are formed in routes A and B (I + II). Metabolite IX, formed in route C, is a minor one. Most of the metabolites formed in route D (III, V, VII and VIII) are relatively unstable.

REFERENCES

1 I. Kosóczky, L. Petőcz, E. Komlós, J. Kőrösi and T. Láng, in G. P. Leszkovszky (Editor), *Fifth Hungarian Conference on Therapy and Pharmacological Research, Budapest, Oct. 1–5, 1968*, Publishing House of the Hungarian Academy of Sciences, Budapest, 1971, pp. 183–187.
2 L. Petőcz and I. Kosóczky, *Ther. Hung.*, 23 (1975) 134.
3 I. Elekes, Gy. Horváth, T. Láng, E. Csányi, É. Tomori and J. Kőrösi, *Seventh European Workshop on Drug Metabolism, Zürich, Oct. 5–10, 1980*, Abstracts, p. 427.

4 I. Elekes, Gy. Horváth, T. Láng, E. Csányi and J. Kőrösi, *First European Symposium on Organic Chemistry, Köln, Aug. 20–23, 1979*, Abstracts, p. 512.
5 G. Zólyomi, J. Kőrösi and T. Láng, *First European Symposium on Organic Chemistry, Köln, Aug. 20–23, 1979*, Abstracts, p. 544.
6 É. Tomori, Gy. Horváth, I. Elekes, T. Láng and J. Kőrösi, *J. Chromatogr.*, 241 (1982) 89.
7 É. Tomori, I. Elekes, T. Láng and Gy. Horváth, *Pol. J. Pharmacol. Pharm.*, 36 (1984) 423.
8 F. Andrási, K. Horváth, E. Sineger, P. Berzsenyi, J. Borsy, Á. Kenessey, M. Tarr, T. Láng, J. Kőrösi and T. Hámori, *Arzneim.-Forsch.*, 37 (1987) 1119.
9 M. Sajgó, A. Gesztesi and T. Sidó, *J. Chromatogr.*, 222 (1981) 303.
10 M. Patty and J. Salát, *J. Chromatogr.*, 210 (1981) 159.