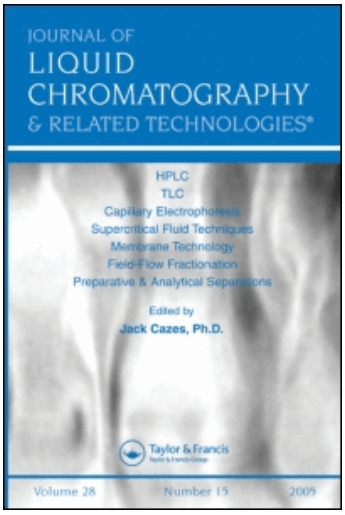

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DETERMINATION OF GIRISOPAM (2,3-BENZODIAZEPINE COMPOUND) AND ITS FOUR METABOLITES IN HUMAN AND RAT PLASMA BY GRADIENT RP-HPLC METHOD

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ABSTRACT

A gradient RP-HPLC bioanalytical method has been developed for the human pharmacokinetic studies of girisopam, the new 2,3-benzodiazepine compound with anxiolytic effect that has no myorelaxant and anticonvulsive side effects. The compound is an analogue of tofizopam (Grandaxin®, EGIS Pharmaceuticals Ltd., Budapest, Hungary). The method was found to be appropriate for the purposes of human pharmacokinetic studies performed at 25, 50, 100, 200, 325, and 525 mg dose levels. The method allowed the simultaneous determination of girisopam (G) and its four metabolites (4'-hydroxy-G, 7-demethyl-G, 4-hydroxymethyl-G and 4-demethyl-4-oxo-G) identified in previous studies in human plasma. The solutes were separated on Hypersil BDS C18 column and quantified by UV detection at 238 nm. A solid phase extraction (SPE) method using reversed-phase cartridges was developed for sample processing, whereby girisopam and the much more polar metabolites, as well as the internal standard could be extracted in a single step.

The limit of quantitation (LLOQ) was: 1 ng/mL in the case of Girisopam (G), 4-hydroxymethyl-G, 4-demethyl-4-oxo-G and 4'-hydroxy-G. In the case of 7-demethyl-G, LLOQ amounted to 2 ng/mL. The calibration curves showed good linearity; r = 0.9959, 0.9928, 0.9954, and 0.9974 in the concentration range of 1-500 ng/mL and r = 0.9959 in the range of 2-500 ng/mL respectively. The validation results obtained for all the five solutes indicated that the present method complied with internationally accepted criteria and ensured quantitative determinations of appropriate accuracy and reproducibility. After small modification and validation, the developed method was applied for determination of girisopam and its metabolites in rat plasma in a toxicological study (in vivo rat liver micronucleus test) at 600 and 1200 mg/kg dose levels. The LLOQ was 10 ng/mL for girisopam and 50 ng/mL for metabolites in rat plasma. The validation parameters for determination of solutes in rat plasma were internationally acceptable. The linearity was good for all components ($r \geq 0.992$) in the wide calibration range of 10-18000 ng/mL and 50-6000 ng/mL in the case of girisopam and its metabolites respectively. The absorption of girisopam was verified by the measuring of girisopam and its metabolite (7-demethyl-G) in the plasma samples of toxicological study (micronucleus test).

INTRODUCTION

Girisopam is a new 2,3-benzodiazepine derivative having selective anxiolytic effect. Unlike most anxiolytic agents, the compound has no anticonvulsive and myorelaxant side-effects.[1,2] Girisopam is an analogue of another 2,3-benzodiazepine compound the tofizopam (Grandaxin®, Egis Pharmaceuticals Ltd., Budapest, Hungary) which also has selective anxiolytic pharmacological effect. These compounds have no affinity to the 1,4-benzodiazepine receptors.[1,3,4] Girisopam has been absorbed rapidly in humans after about 20 minutes latency period. The C_{max} value was 178 ± 97 ng/mL at t_{max}=2.0 ± 0.7 hours and the $AUC_{0-\infty}$ amounted to 1733 ± 836 ng*h/mL after 100 mg oral dose of girisopam in healthy volunteers. The $t^{\beta}_{1/2}$ value was 22.2 ± 9.7 hours. A solid-phase extraction and gradient RP-HPLC method was elaborated for simultaneous determination of girisopam (G) and its three main [4'-hydroxy-G (EGIS-7875), 7-demethyl-G (EGIS-7872), 4-hydroxymethyl-G (EGIS-7874)] and one minor [4-demethyl-4-oxo-G (EGIS-7873)] metabolites in human plasma for pharmacokinetic studies at 25, 50, 100, 200, 325, and 525 mg dose levels. The calibration range was 1-500 ng/mL for G and for

metabolites, except for 7-demethyl-G the range was 2-500 ng/mL. The metabolites examined were identified during previous studies[1] by HPLC-MS, MS, GC-MS, and GC methods in human plasma and urine. The previous bioanalytical methods were less sensitive and/or did not allow the simultaneous determination of metabolites and G.[1]

The new method was also applied in a toxicological study (in vivo rat liver micronucleus test) of girisopam in rats at high toxicological dose levels of 600 and 1200 mg/kg. The calibration range (10-18000 ng/mL for girisopam and 50-6000 ng/mL for metabolites) and the gradient profile were modified, compared to the original method, for analysis of rat plasma samples. The method was also validated for human and rat plasma.

MATERIALS

Girisopam (G), 4'-hydroxy-G, 7-demethyl-G, 4-hydroxymethyl-G, 4-demethyl-4-oxo-G and nerisopam (as internal standard) were prepared at EGIS Pharmaceuticals Ltd. (Budapest, Hungary). Structures are shown in Figure 1. Acetonitrile (ACN), methanol, HPLC-grade water were produced by J. T. Baker, Deventer, The Netherlands). Ammonium-carbonate ammonium-chloride were purchased from Fluka Chemie AG. (Buchs, Switzerland). The solvents used were of chromatography grade, the other chemicals were of analytical grade. Solid-Phase extraction was carried out by using Chromabond C18 (100 mg) SPE columns (Macherey-Nagel, Düren, Germany).

METHODS

Instruments and Chromatographic Parameters

A HP 1090 M Series II liquid chromatograph (Hewlett-Packard, Palo Alto, CA. USA) with autosampler, terner gradient pump, diode array detector (DAD), was used under control of HP ChemStation Pascal Series software. The separation of the solutes was achieved on a Shandon Hypersil BDS C18 analytical column 250 x 2.0 mm which was equipped with Hypersil BDS C18 guard cartridge 4 x 2 mm (Shandon HPLC, Life Sciences International Ltd., Runcorn, U.K.). The separation was performed at 40°C. The flow rate was 0.55 mL/minute. The UV (DAD) monitoring was done at 238 nm (4 nm bandwidth) and the reference wavelength was 450 nm (100 nm bandwidth). The mobile phase A consisted of 200 mL of ACN, 120 mL of methanol, 800

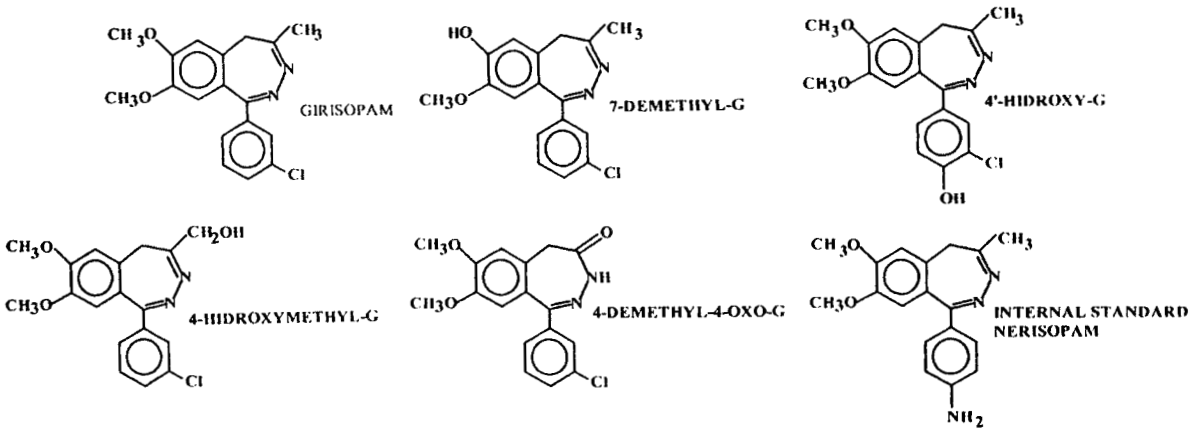

Figure 1. Chemical structures of solutes.

mL of water, 0.98 g of ammonium-carbonate and 0.90 g of ammonium-chloride. Solvent B consisted of 185 mL of ACN, 110 mL of methanol, 200 mL of water, 0.33g of ammonium-carbonate, and 0.37g of ammonium-chloride. The solvents were filtered through Durapore membrane of 0.45 μm pore size and bubbled with high purity Helium.

Gradient program for the human plasma analysis was 98 % A and 2% B 0-4.2 minutes; B increased to 40% at 7 minutes, 45% at 10 minutes, and 50% at 14 minutes. The post time was 5 minutes after a run. The gradient profile was modified for analysis of rat plasma as follows: 95% A and 5% of B solvent 0-3.5 minutes; B solvent changed to 40% at 10 min., 60% at 12 min., A 10%, B 60%, and C solvent (methanol) 30% between 18-20 minutes. The post time was 3 minutes in this case.

Solutions

500 μg/mL concentration of standard solutions were prepared by dissolving the girisopam in methanol and in the case of other substances, in methanol-acetonitrile (1:1) mixture. Further dilutions of stock solutions for calibration were prepared with acetonitrile-methanol (4:1) mixture.

Preparation of Spiked Human Plasma Samples

Nine mL of freshly collected drug free human blood was supplemented with 1 mL of 2% (g/v) disodium EDTA solution as anticoagulant and the sample was centrifuged at 2000 r.p.m. for 10 minutes. The resulting plasma was aspirated off with a pipette and transferred into 10 mL tubes which have ground glass stoppers and stored at -20°C until processing. Sample analysis requires 1 mL of human plasma.

Calibration levels were 1, 5, 20, 50, 100, 200, and 500 ng/mL for all components except 7-demethyl-girisopam, which was measured in the calibration range of 2-500 ng/mL. The concentration of the internal standard was 100 ng/mL in each sample.

Preparation of Spiked Rat Plasma Samples

Blank plasma obtained from Wistar SPF rats (150-160 g) was used. The frozen plasma which was previously stored at -20°C, was allowed to thaw in a water bath at a temperature approximately 30°C. Calibration range was 10-

18000 ng/mL for girisopam and 50-6000 ng/mL for metabolites. The concentration of the internal standard (ISTD) was 500 ng/mL. The rat plasma samples were spiked with the appropriate volumes of diluted stock solutions prepared in solvent A.

Sample Processing for Human Plasma

Solid-phase extraction was carried out using C_{18} phase cartridges. The sample applied onto the column was sucked through by means of a vacuum extraction manifold with an approximately rate of 1 mL/min. The SPE phase was activated by sucking 2 mL of methanol and 2 mL of HPLC grade water through the column. To 1 mL of plasma 200 μL of 0.12% g/v ammonium-carbonate solution was added and after the sample was applied onto it, the column was washed with 2 mL of water, 200 μL of 0.12% g/v ammonium-carbonate solution, and 30 μL of methanol. The elution was carried out with 2 x 200 μL of methanol. The eluate was evaporated to dryness under stream of nitrogen at ca. 40°C. The dry residue was dissolved in 400 μL of solvent A and 60 μL was injected into HPLC system.

Sample Processing for Rat Plasma

Solid-phase extraction was carried out using a C_{18} cartridge and a vacuum extractor. The SPE column was activated by sucking 2 mL of methanol and 2 mL of water through the column. To 1 mL of plasma 200 μL of water and 200 μL of 0.12% (g/v) ammonium-carbonate solution was added and after the sample was applied onto it, the column was washed with 2 mL of water, 200 μL of ammonium-carbonate, and 30 μLof methanol. The elution was carried out with 1 mL of methanol-acetonitrile (1:1). The eluate was evaporated to dryness under nitrogen stream at ca. 40°C. The dry residue was dissolved in 400 μL of solvent A and 60 μL of the resulting sample was injected into the chromatograph.

Linearity Test

Quantitative determination of girisopam and its metabolites was performed in the concentration range of 1-500 ng/mL, whereas the calibration for 7-demethyl-girisopam covered the range of 2-500 ng/mL in human plasma. In the case of rat plasma samples the linearity was investigated in the calibration range of 10-18000 ng/mL for G and 50-6000 ng/mL for its

metabolites. For constructing the calibration curves, peak area ratios of the solute and the internal standard were plotted against nominal concentration values. The calibration curves were fitted by least square method using $1/y^2$ weighting. The acceptance criteria of linearity was $r>0.990$ and the calculated concentration value, using the equation of calibration curve, should not differ from the nominal concentration value by more than 20% (accuracy).

Method Validation

The HPLC method was validated according to internationally accepted criteria.[5,6,7,8]

Intraday precision and accuracy were determined at three concentration levels (20, 50, and 100 ng/mL at human plasma and 50, 500, and 1000 ng/mL at rat plasma, n=5). For establishing interday precision and accuracy, five independent calibrations were measured in each case. Precision and accuracy were accepted at values lower than 20% (R.S.D.% and Diff.%, respectively).

The suitability of the chromatographic system was determined by 5 parallel injections of two different concentration spiked plasma samples (50 and 200 ng/mL for human plasma and 50 and 100 ng/mL for rat plasma) of girisopam.

The percentile value of absolute recovery was determined and calculated as the area ratio of the chromatographic peaks obtained from the extracted and the standard samples.

Stock solutions and their dilutions used for preparing spiked plasma samples, were stored in a refrigerator at 4°C. The stability of the solutes during storage was checked by comparing the peak area values obtained with freshly made solutions to those recorded after storage. The solutions were regarded as stable until the difference between the peak area values did not exceed 5%.

The stability of the solutes in human plasma stored at -20°C was tested at 10, 50, and 200 ng/mL concentration levels with triplicate determination every month during a three month storage period. The stability of solutes in rat plasma at 100 and 1000 ng/mL concentrations were investigated after seven week storage at -20°C. The samples were supplemented with the internal standard right prior to sample processing. The solutes were regarded as stable under these storage conditions if the R.S.D. values of the determinations fell below 20% and the samples could be determined with an accuracy value lower than 20%.

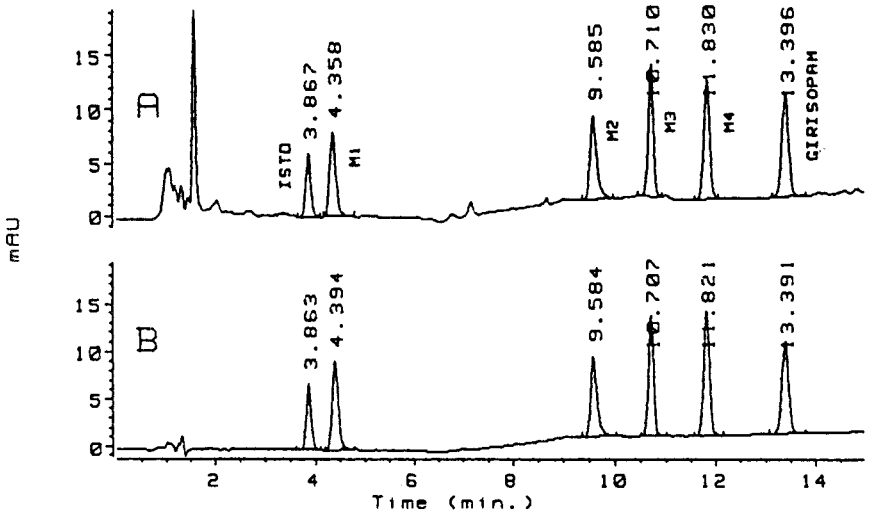

Figure 2. HPLC chromatograms: A/ Extracted human plasma sample containing 50 ng/mL of each of investigated component and 100 ng/mL ISTD. B/ Standard sample containing 50 ng/mL of each of investigated component and 100 ng/mL ISTD. M1: 4'-hydroxy-G, M2: 7-demethyl-G, M3: 4-hydroxymethyl-G, M4: 4-demethyl-4-oxo-G.

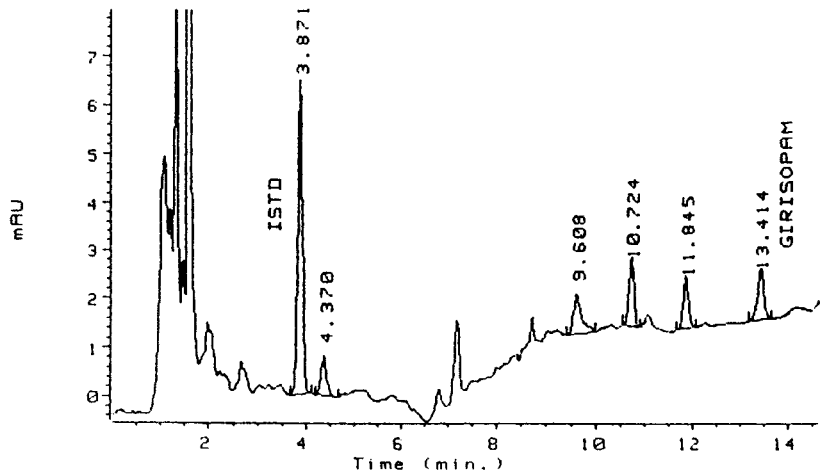

Figure 3. HPLC chromatogram: Extracted human plasma sample containing 5 ng/mL of each of investigated component and 100 ng/mL ISTD. Peak identification in Figure 2.

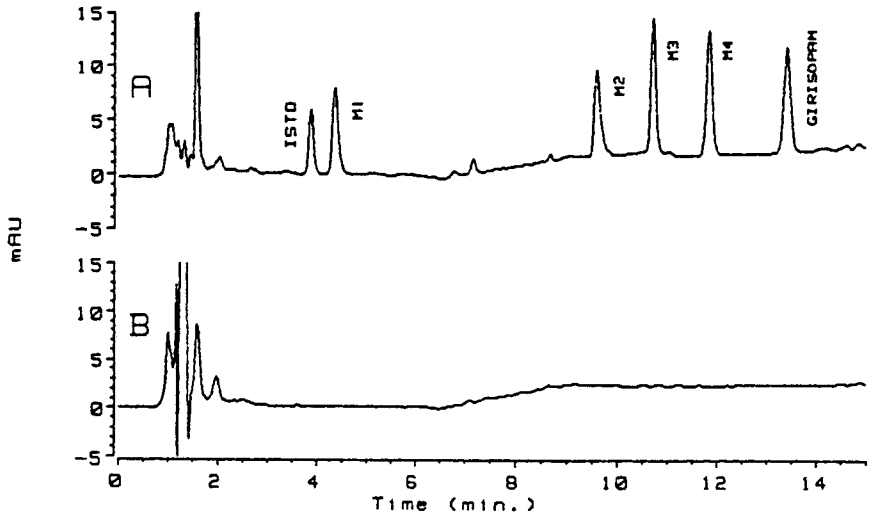

Figure 4. HPLC Chromatograms: A) Extracted human plasma sample containing 50 ng/mL each of investigated component and 100 ng/mL ISTD. B) Blank human plasma without internal standard. Peak identification in Figure 2.

RESULTS

Girisopam and its metabolites were separated in a gradient reversed-phase (C_{18}) chromatographic system using UV (diode array) detection at 238 nm. Girisopam was eluted with a retention time of 13.4, while the metabolites (4'-hydroxy-G, 7-demethyl-G, 4-hydroxymethyl-G, and 4-demethyl-4-oxo-G) and internal standard were detected at 4.3, 9.6, 10.7, 11.8, and 3.8 minutes respectively (Figure 2 and 3). The chromatogram (Figure 4) of blank human plasma extract did not show any peak that would interfere with the investigated compounds.

The calibration curves showed good linearity; $r \geq 0.993$ (Figure 5). The limit of quantitation amounted to LLOQ = 1 ng/mL for girisopam and metabolites, except in the case of 7-demethyl-G, the LLOQ amounted to 2 ng/mL. The precision and accuracy fulfilled the internationally accepted criteria for girisopam determination.

The intraday precision varied between 4.4-14.6 RSD% and accuracy values were -2.9 - 0.2%. The interday precision and accuracy were between 3.2-17.5 RSD% and -6.6-13.4% respectively (Table 1).

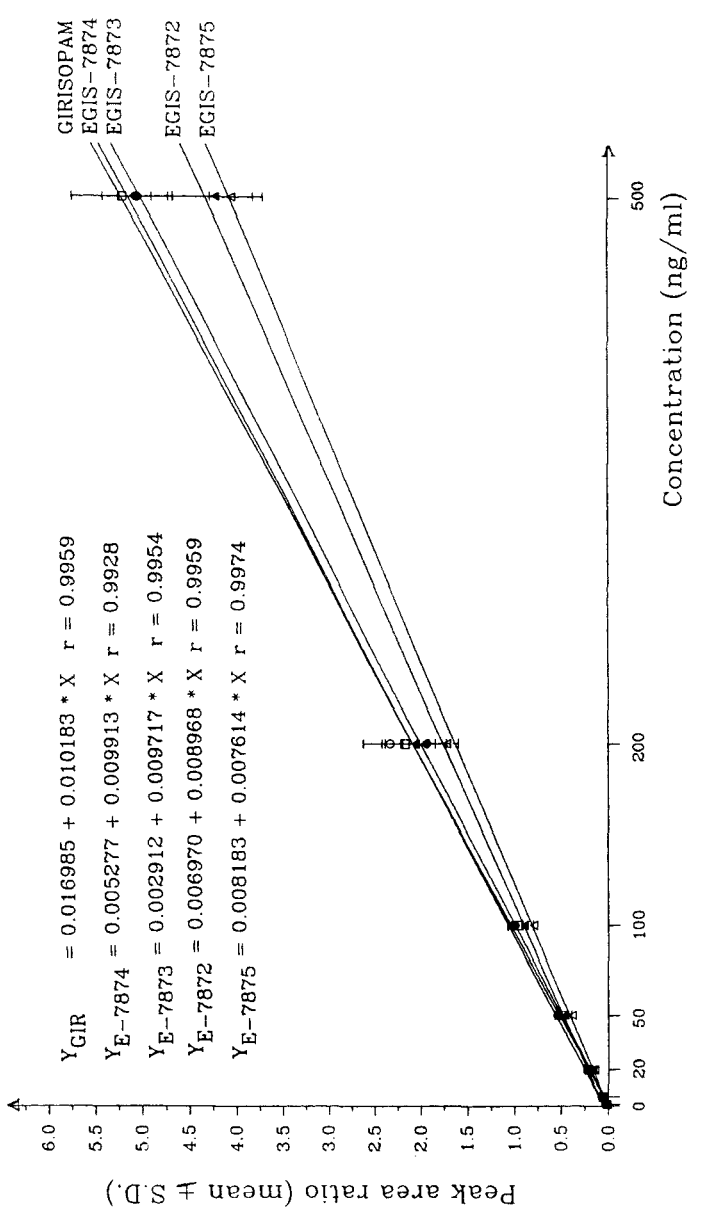

Figure 5. Calibration curves of girisopam and its metabolites in human plasma.

Table 1

Precision and Accuracy for the Analysis of Girisopam in Human Plasma

Nominal Conc. (ng/mL)	Measured Conc. ±S.D. (n=5) (ng/mL)	Intraday Accuracy (%)	Intraday CV (%)	Measured Conc. ±S.D. (n=5) (ng/mL)	Interday Accuracy (%)	Interday CV (%)
1	1.09 ± 0.12	9.0	11.0	1.04 ± 0.15	4.0	14.5
5	4.87 ± 0.68	2.6	14.0	4.89 ± 0.85	2.2	17.5
20	19.52 ± 2.84	-2.4	14.6	19.57 ± 1.65	2.2	8.4
50	48.55 ± 2.11	-2.9	4.4	52.02 ± 5.77	4.0	11.1
100	100.19 ± 4.40	0.2	4.4	93.36 ± 3.26	-6.6	3.5
200				226.83 ± 14.29	13.4	6.3
500				488.02 ± 15.42	-2.4	3.2

Table 2

Precision and Accuracy for the Analysis of Girisopam in Rat Plasma

Nominal Conc. (ng/mL)	Measured Conc. ±S.D. (n=5) (ng/mL)	Intraday Accuracy (%)	Intraday CV (%)	Measured Conc. ±S.D. (n=5) (ng/mL)	Interday Accuracy (%)	Interday CV (%)
10	10.16 ± 1.13	1.6	11.1	10.13 ± 1.16	1.3	11.5
50	49.87 ± 2.15	0.3	4.3	51.36 ± 3.52	2.2	6.9
100				105.75 ± 13.01	5.8	12.3
500	509.80 ± 18.57	2.0	3.6	522.95 ± 12.64	4.6	2.4
1000	1103.24 ± 92.16	10.3	8.4	1121.12 ± 141.21	12.1	12.6
4000				4041.33 ± 292.52	13.4	7.2
6000				5502.99 ± 429.94	-8.3	7.8
18000				16831.91 ± 10.9121	-6.5	6.5

The intraday precision for main metabolite (M3) of girisopam determination varied between 6.3-17.2 RSD% and the accuracy values were between -6.3 - 15.5%. The interday precision and accuracy were between 6.0-15.6 RSD% and -5.9-14.2% respectively.

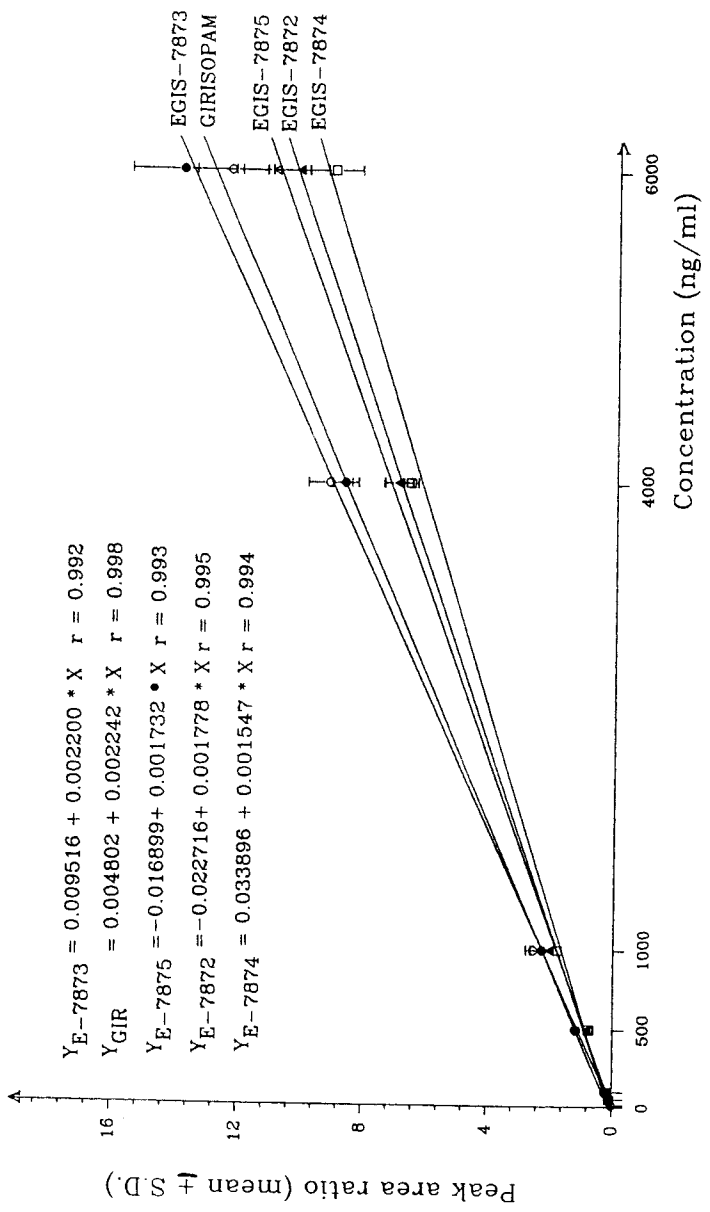

Figure 6. Calibration curves of girisopam and its metabolites in rat plasma.

The reproducible operation of the chromatographic system was verified by system suitability test, the results were 1.28 and 1.78 RSD% at 50 and 200 ng/mL human plasma concentration respectively. The extraction efficiencies were 98.5 ± 3.3% for girisopam, 85 ± 2% for internal standard, and between 82.5 ± 4% - 93.3 ± 2.9% for metabolites. The stock solutions of standards were stable for two weeks. According to the results of stability test, girisopam and its metabolites proved to be stable in human plasma stored at -20°C for 3 months.

After small modification and validation the method was used for a toxicological study of girisopam in rats. The LLOQ was 10 ng/mL for girisopam and 50 ng/mL for metabolites. Linearity of calibrations was acceptable in every case ($r \geq 0.992$) (Figure 6). The precision and accuracy of the method for rat plasma analysis of girisopam can be seen in Table 2.

The intraday precision varied between 5.4-10.6 RSD% and accuracy was -5.6 - 15.7% for the determination of girisopam metabolites. The interday precision and accuracy were between 7.1-18.6 RSD% and 8.2-17.3% respectively.

The extraction efficiencies were the following: internal standard 92.4 ± 2.2%, girisopam 90.1 ± 4.0%, and metabolites between 66.0 ± 3.5% - 93.2- ± 3.0%. According to the results of stability test, girisopam and the metabolites proved to be stable in rat plasma stored at -20°C for 7 weeks.

Apart from girisopam and 4-hydroxymethyl-G metabolite, none of the other solutes appeared in the chromatograms, which was in agreement with previous metabolism studies in rat performed after the administration of a lower dose (30mg/kg).[1] Since no metabolism study had been performed with 600 and 1200 mg/kg oral doses earlier, data about the expected plasma levels of metabolites were not available, therefore, all the possible metabolites were taken into consideration during method development. Chromatograms of standard mixture, blank rat plasma, and extracted rat plasma samples are shown on Figure 7, 8 and 9.

DISCUSSION

On the basis of parameter values obtained during method validation, the developed method proved to be suitable for the sensitive simultaneous determination of girisopam and its metabolites in human and rat plasma. The method was found to be appropriate for the purposes of pharmacokinetic and/or

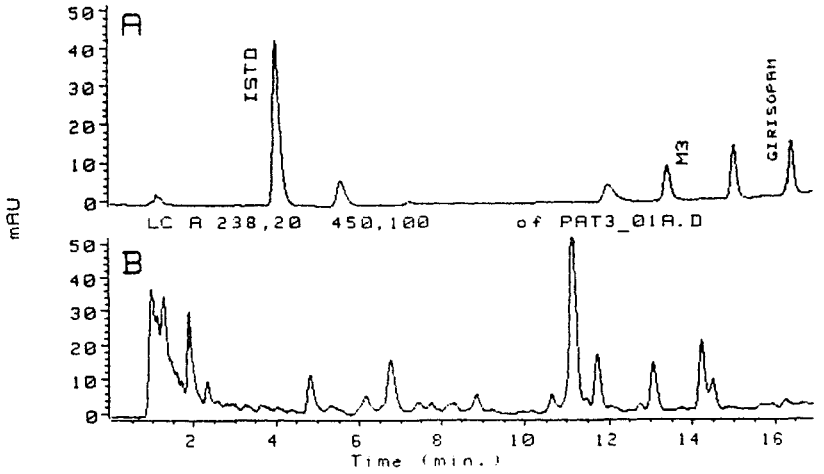

Figure 7. HPLC Chromatograms: A/ Standard sample sample containing 50 ng/mL of girisopam and metabolite M3 and 500 ng/mL of ISTD. B/ Blank rat plasma sample.

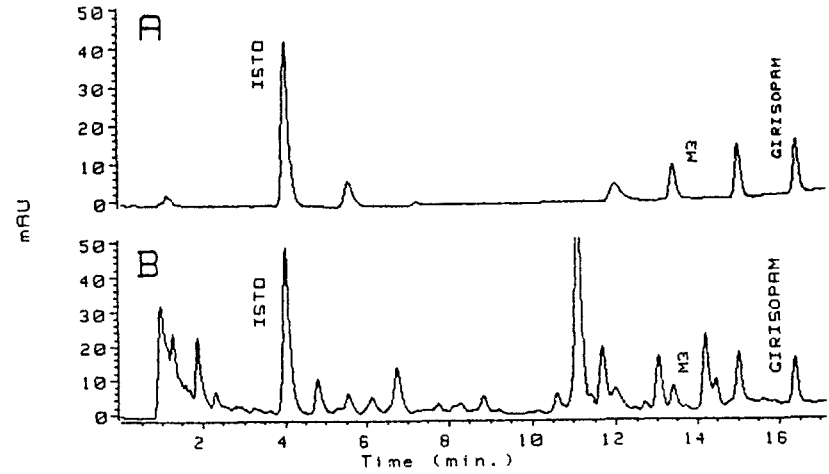

Figure 8. HPLC Chromatograms: A/ Standard sample containing 50 ng/mL of girisopam and metabolite M3 and 500 ng/mL of ISTD. B/ Spiked rat plasma sample containing 50 ng/mL of girisopam and metabolite M3 and 500 ng/mL of ISTD. Peak identification in Figure 2.

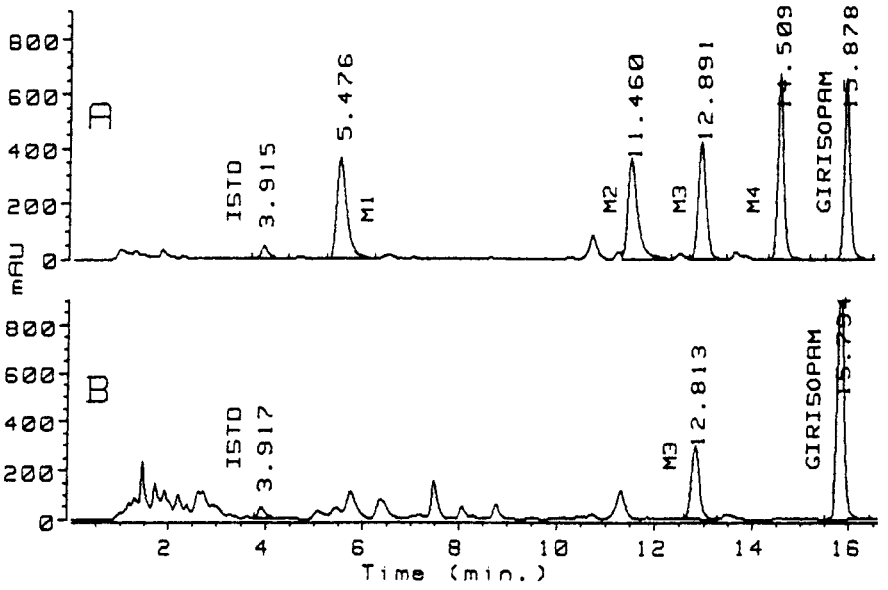

Figure 9. HPLC Chromatograms: A/ Extracted rat plasma sample containing 4000 ng/mL of girisopam and metabolites and 500 ng/mL of ISTD. B/ Extracted rat plasma sample collected at 72 hours after treatment with 600 mg/kg of girisopam in toxicokinetic study. Peak identification in Figure 2.

metabolitekinetic studies of girisopam. The method was suitable for toxicologic (toxicokinetic) study (in vivo rat liver micronucleus test) of girisopam in rats with small modification. The parent drug and one metabolite (4-hydroxymethyl-G) have been determined in the rat plasma to justify girisopam exposition in the toxicological study.

Compounds of wide polarity range were analyzed together by this method, so the gradient chromatographic system and the solid phase extraction can be a basis to analyse other 2,3-benzodiazepine structure compounds.

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