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Journal of Chromatography B, 678 (1996) 63–72

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Simultaneous determination of nerisopam, a novel anxiolytic agent showing polymorphic metabolism, and its N-acetyl metabolite from human plasma by a validated high-performance liquid chromatographic method

K. Róna[a,*], K. Ary[a], B. Gachályi[a], I. Klebovich[b], É. Tomori[c]

[a]*Haynal Imre University of Medical Sciences, Department of I. Medicine, P.O. Box 112, 1389 Budapest, Hungary*
[b]*EGIS Pharmaceuticals Ltd., Department of Pharmacokinetics, P.O. Box 100, 1475 Budapest, Hungary*
[c]*Institute for Drug Research, P.O. Box 82, 1325 Budapest, Hungary*

Abstract

A sensitive reversed-phase high-performance liquid chromatographic method with ultraviolet absorbance detection has been developed to simultaneously determine the concentrations of nerisopam (EGIS-6775) and its N-acetyl metabolite (EGIS-7649) from human plasma. The separation of the investigated compounds and internal standard was achieved on a Nucleosil 7 C_{18} column with 2 m*M* heptanesulphonic acid containing 0.04 *M* phosphoric acid–acetonitrile–methanol (70:25:5, v/v), pH 2.7 mobile phase. The detection was performed at 385 nm. The compounds were isolated from plasma by Bakerbond C_{18} solid-phase extraction. The limit of quantitation was 10 ng/ml plasma for each compound investigated. The assay has been validated with respect to accuracy, precision and system suitability. All validated parameters were found to be within the necessary limits. On the basis of the sensitivity, linearity and validation parameters, the developed analytical method was found to be suitable for the determination of nerisopam and its N-acetyl metabolite from human plasma and for application in pharmacokinetic studies and human drug monitoring. The pharmacokinetic parameters obtained from twelve human volunteers are reported. It was found that nerisopam acetylation is polymorphic: the volunteers with fast or slow acetylator phenotypes produced significantly different plasma concentrations. In slow acetylator phenotypes the concentration of nerisopam was considerably higher in plasma, while the level of its acetyl metabolite was higher in plasma of fast acetylators.

Keywords: Nerisopam; N-Acetylnerisopam

1. Introduction

It has long been known that a part of the population reacts to certain drugs in an unusual way. The observed individual reactions towards drugs are principally caused by different metabolising abilities. In the clinico-pharmacological centres dealing with the development of new drugs, the metabolism of a new compound is studied in phase I testing. Given the existence of these varying metabolic rates, it seems reasonable to screen volunteers participating in the

* Corresponding author.

0378-4347/96/$15.00
SSDI 0378-4347(95)00289-9

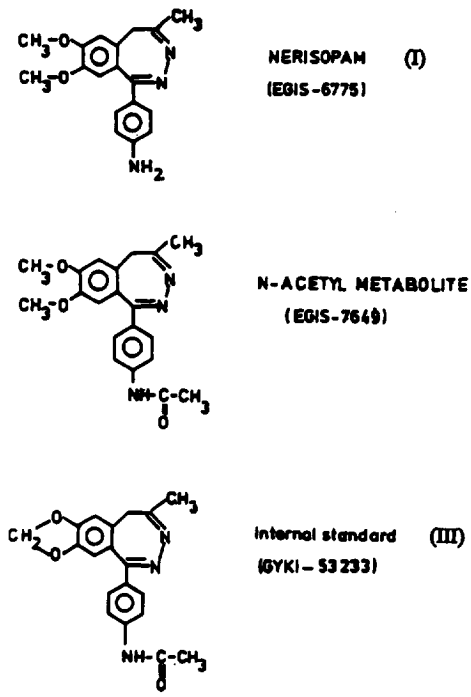

Fig. 1. Chemical structures of EGIS-6775 (I), EGIS-7649 (II) and GYKI-53233 (internal standard) (III).

studies on the basis of the pharmacogenetic polymorphism.

The monogeneous determination of drug acetylation has been known since the 1950s [1,2]. The rate of acetylation shows a bimodal distribution: a sub-population is made up of either fast or slow acetylator phenotypes. Acetylator phenotypes of subjects living under different geographical conditions differ significantly: 95–100% of the Eskimos living in Canada, 93% of Polynesians, about 90% of Koreans and Japanese, and 40–55% of white Europeans belong to the fast acetylator type [3]. In Hungary, according to our own studies, the frequency of fast acetylators is 43% [4]. In the study of the human tolerance of nerisopam with its 4-aminophenyl structure, the original compound was expected to undergo acetylation (Fig. 1). Therefore, half of the volunteers participating in the study were chosen from the fast group and the other half from the slow group.

5H-2,3-Benzodiazepine derivatives synthesized by Láng and Körösi at the Institute for Drug Research (Budapest, Hungary) [5] represent a new subclass among benzodiazepines. Nerisopam is a member of these new 2,3-benzodiazepines which differs in anxiolytic activity from that of the classical 1,4-benzodiazepines [6].

During the Phase I clinical trials the plasma concentrations were measured after the administration of different doses of nerisopam. For this study a new analytical method suitable for measuring the plasma concentrations of both the original compound and its N-acetyl metabolite was developed.

2. Experimental

2.1. Materials

Nerisopam (EGIS-6775, I) [1-(4-aminophenyl)-4-methyl-7,8-dimethoxy-5H-2,3-benzodiazephyne], N-acetyl metabolite (EGIS-7649, II) [1-(4-acetylaminophenyl)-4-methyl-7,8-dimethoxy-5H-2,3-benzodiazephyne] and internal standard [1-(4-acetaminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazephyne] (code: GYKI-53233, III) substances were obtained from EGIS Pharmaceuticals (Budapest, Hungary). The structural formulae of the substances are shown in Fig. 1. Methanol, acetonitrile (LiChrosolv) and ethylamine were from Merck (Darmstadt, Germany), Bakerbond SPE C_{18} 1-ml extraction cartridges were manufactured by Baker (Phillipsburg, NJ, USA). Heptanesulphonic acid sodium salt was purchased from Sigma (St. Louis, MO, USA). Phosphoric acid (85%), disodium hydrogen phosphate, potassium dihydrogen phosphate, EDTA-Na were purchased from Reanal (Budapest, Hungary). All chemicals and solvents were of analytical and HPLC-solvent grade, respectively.

2.2. Chromatographic conditions

LC-6A pump, SPD-10 UV detector and C-R6A integrator were used for liquid chromatography. The injection was carried out with a SIL-6B automatic sample injector (equipped with SCL-6B system controller) through a 20-μl loop.

All instruments were from Shimadzu (Kyoto, Japan).

For checking the homogeneity and identity of chromatographic peaks, an HP 1090 liquid chromatograph equipped with a diode-array detector (DAD) and a HP 85B system master (with HP 9121 dual disc drive) were used. The loop volume was 20 μl. Area integrations, calculations and plotting of the chromatogram and spectrum were carried out by an HP 3390A integrator and an HP 7470 plotter. All of them were from Hewlett-Packard (Palo Alto, CA, USA).

The separation was performed at room temperature (air-conditioned room, 20 ± 2°C) on a Nucleosil 7 C_{18} (250×4 mm I.D.) column supplied with a Nucleosil 7 C_{18} (30×4 mm I.D.) guard column (BST Budapest, Hungary). The flow-rate was 1.0 ml/min. The UV monitoring was done at 385 nm. The mobile phase was 0.04 *M* phosphoric acid–acetonitrile–methanol (70:25:5, v/v) containing 2 m*M* heptanesulphonic acid sodium salt; the pH of the eluent was adjusted to 2.7 with ethylamine. The eluent was filtered through a 0.45-μm Nylon 66 membrane (Supelco, Bellefonte, PA, USA) and degassed by sparging with nitrogen.

2.3. Solutions

The 1 mg/ml stock solution of I was prepared in 0.04 *M* phosphoric acid. The dilutions for the working solutions were made with 0.04 *M* phosphoric acid (1 μg/ml). The 1 mg/ml stock solution of II was prepared in methanol. Further dilutions of the working solutions were made with 0.04 *M* phosphoric acid (2 μg/ml). The internal standard stock solution (1 mg/ml final concentration) was prepared in methanol. Further dilutions were made with 0.04 *M* phosphoric acid (10 μg/ml).

All three stock solutions were stable for 1 month when stored at −20°C. The working solutions were freshly made each week and stored at 4°C in a refrigerator.

Phosphate buffer (0.1 *M*, pH 7.0) was prepared according to Sörensen: KH_2PO_4–Na_2HPO_4 (4:6, v/v).

Human plasma was obtained from blood samples collected from the cubital vein. Coagulation was prevented by adding 15 mg of EDTA-sodium salt to 15 ml of blood. The anticoagulant was previously dissolved in 0.15 ml of distilled water. The blood treated with anticoagulant was centrifuged at 1500 *g* for 10 min, and the drug-free plasma was stored at −20°C before use.

2.4. Plasma extraction

To 1 ml of plasma 500 ng of internal standard (from 10 μg/ml 0.04 *M* phosphoric acid solution in 50 μl volume) and 1 ml of 0.1 *M* pH 7.0 phosphate buffer were added. The sample was homogenized by vortex-mixing for 5 s, then poured onto a Bakerbond C_{18} (1 ml) extraction column (SPE) previously activated with 1 ml methanol and 1 ml 0.05 *M* phosphate buffer (pH 7.0). The column was washed with 2.0 ml of 0.1 *M* phosphate buffer (pH 7.0), and the compounds under investigation were eluted with $2 \times$ 0.1 ml methanol. From the methanol eluate (without evaporation) 20-μl aliquots were injected onto the column.

2.5. Method validation

2.5.1. System suitability test

All three compounds were injected five times from their own working solutions. The concentration of the working solutions was: 20-μl loop = 20 ng of I, 40 ng of II, 200 ng of internal standard.

2.5.2. Calibration curves

For calibration, "spiked" samples were made from collected drug-free plasma by adding a known amount of the diluted stock solution. To 1 ml of drug-free plasma increasing amounts of I (0, 10, 20, 30, 50, 100, 500, 1000, 2000 ng) and of II (0, 10, 30, 100, 250, 500, 1000, 1500 ng), and on both occasions 500 ng of internal standard, were added.

The extraction and liquid chromatography were carried out as previously described. The ratio of the peak areas of investigated compounds I and II and the internal standard III was plotted against plasma concentration. The cali-

bration curves were fitted to the measured points by the least squares method using an HP 85 B (Hewlett-Packard) computer. The F test for linearity and linear regression analysis were chosen for testing linearity.

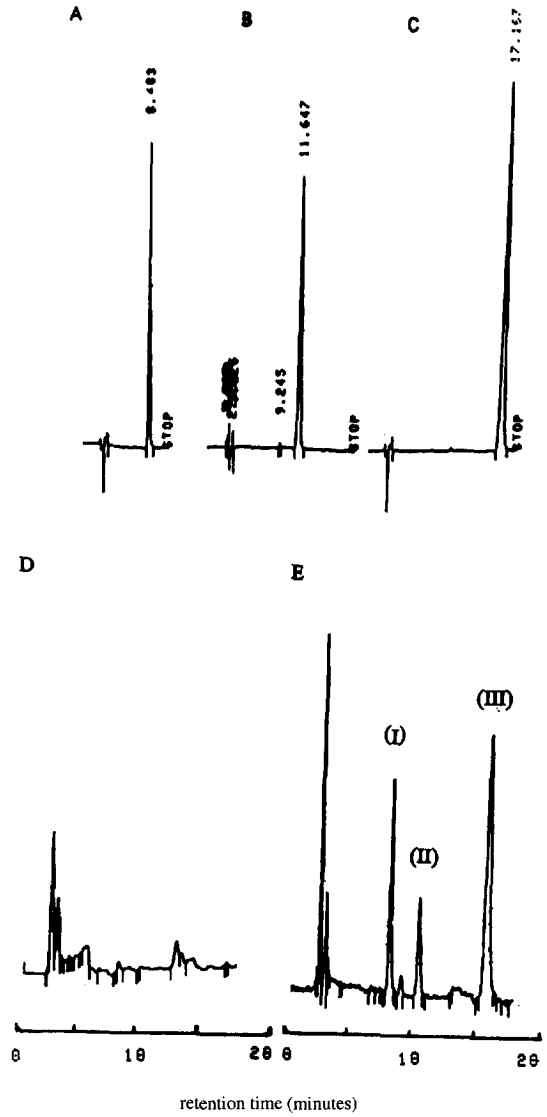

Fig. 2. Chromatograms of standard solution of (A) 1 μg/ml of EGIS-6775 (I), (B) 2 μg/ml of EGIS-7649 (II) and (C) 10 μg/ml of internal standard (III) plus chromatograms of (D) a typical blank plasma sample and (E) "spiked" sample containing 100 ng/ml of I, 100 ng/ml of II and 500 ng/ml of III. See text for instrumentation and chromatographic conditions.

2.5.3. Within-day precision

Amounts of 10, 50, 100 ng of I and 500 ng of internal standard, as well as 30, 100, 1500 ng of II and 500 ng of internal standard, were added to 1 ml of plasma. The analysis was performed with six replicates at each concentration.

2.5.4. Between-day precision

Amounts of 10, 50, 100 ng of I and 500 ng of internal standard, as well as 30, 100, 250, 500, 1000, 1500 ng of II and 500 ng of internal standard, were added to 1 ml of plasma. The

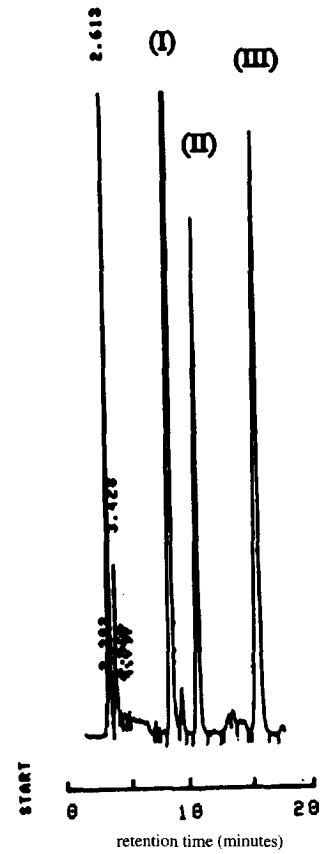

Fig. 3. Representative chromatogram of a plasma extract of volunteer no. 10 obtained 0.5 h after administering a 50-mg dose of nerisopam (966.5 ng/ml of I, 341.1 ng/ml of II and 500 ng/ml of III). For chromatographic conditions, see text.

determinations were made over 2 × 3 consecutive days.

2.5.5. Determination of the extraction efficiency

Amounts of 10, 20, 50, 100 ng of I and 30, 100, 250, 500, 1000, 1500 ng of II were added to 1 ml of plasma. The solid-phase extraction was done without internal standard, and 500 ng of internal standard were added to the methanol eluate after extraction. The peak-area ratios were compared

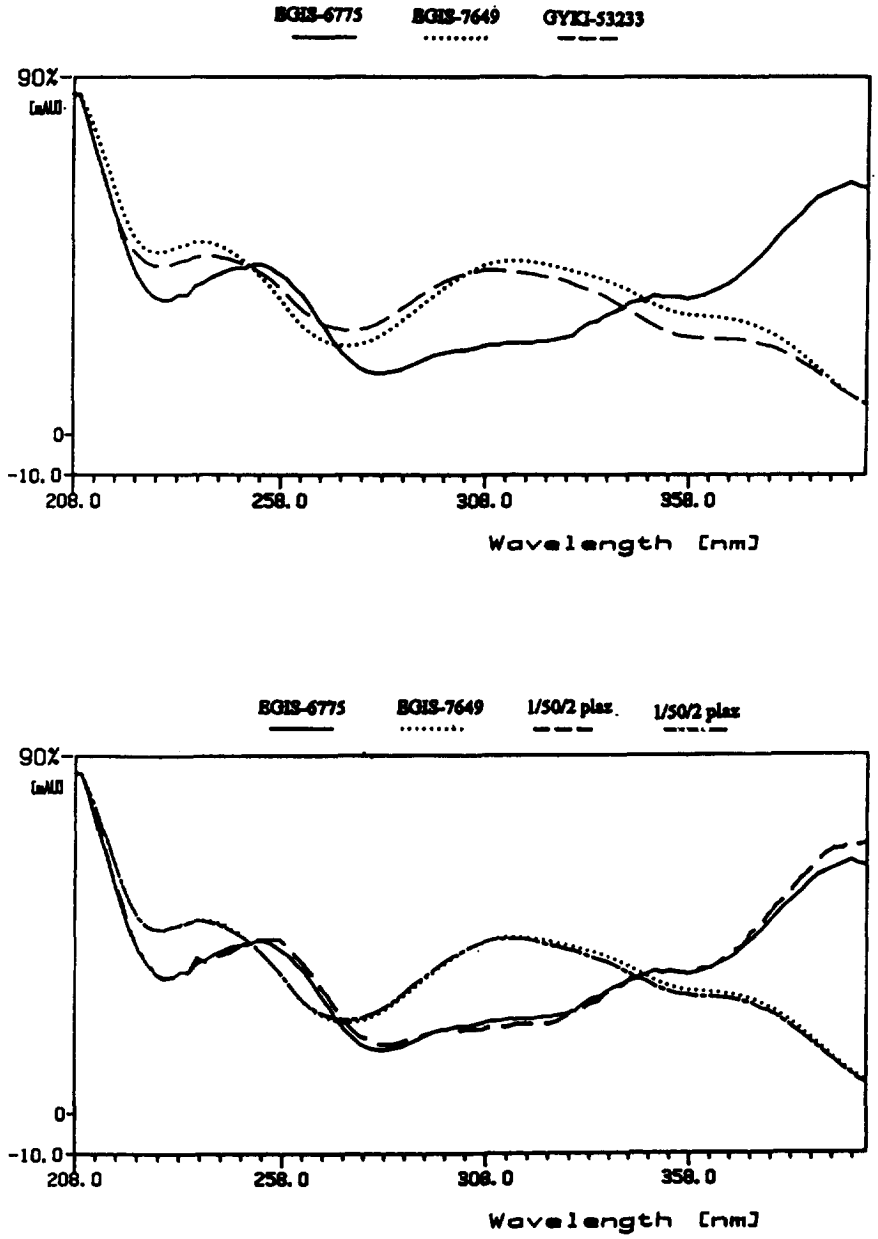

Fig. 4. Top: UV spectra of the investigated compounds. Bottom: UV spectra at the apex of the chromatographic peaks of EGIS-6775 (I) and EGIS-7649 (II) from volunteer no. 1 and of the authentic reference materials.

to the ratio of the standard aqueous samples without extraction. Five replicate samples were determined at each point.

2.5.6. Stability

The stability of the 50 and 1000 ng of I/ml plasma samples and that of 50 and 1000 ng of II/ml plasma samples was studied after two and four weeks of storage at −20°C. The internal standard was added immediately before the extraction of the samples.

Three replicate determinations were made in each case and at each time.

2.6. In vivo experiments

The investigated persons were fasted overnight and received 2.5 to 100 mg of nerisopam orally with 100 ml of tap water. Compounds I and II were determined in the plasma samples between 0 and 48 h.

3. Results and discussion

An HPLC method was carried out for the quantitative determination of nerisopam I and its N-acetyl metabolite II from human plasma by UV absorbance detection (385 nm).

By using a Nucleosil 7 C_{18} column and a ternary mobile phase [2 m*M* heptanesulphonic acid, 0.04 *M* phosphoric acid–acetonitrile–methanol (70:25:5, v/v), pH 2.7] symmetrical peaks were obtained both for the compounds to be determined and the internal standard (Fig. 2A–C). The reproducibility of the chromatographic parameters (retention time, peak area) was determined from replicate injections ($n = 5$) of 20 μl of standard samples containing 20 ng of I, 40 ng of II and 200 ng of III. Based on the system suitability test, the relative standard deviations for peak areas of I, II and III were 1.708%, 1.751% and 2.069%, respectively. The mean retention times for I, II and the internal standard III were 8.48 (0.017), 11.65 (0.010) and 17.09 (0.045) min, respectively.

Fig. 2D and E show the chromatograms of solid-phase extraction with a Baker C_{18} (1 ml) cartridge resulting in typical blank plasma extract (panel D) as well as the spiked sample chromatograms of 100 ng of I, 100 ng of II and 500 ng of internal standard III in 1 ml of plasma (panel E). Fig. 3 shows a typical chromatogram of a volunteer's plasma sample. Endogeneous compounds interfered with the determination of I and II, and internal standard was not detected in the chromatogram.

The identity of the chromatographic peaks was checked using a diode-array detector: UV absorbance spectra at the apex of each investigated peak were taken during the chromatographic elution, and UV absorbance spectra were drawn after normalization in one coordinate system (Fig. 4, upper panel). Fig. 4, bottom panel shows the identity of the UV absorbance spectra at the apex of the chromatographic peaks of two compounds examined, i.e. nerisopam and its N-acetyl metabolite II from the human plasma (label 1/50/2, 1st volunteer, 50 mg dose, 2-h sample) of one of the volunteers, compared to the authentic reference materials.

The detailed validation data are given in Tables 1–4. The reproducibility of the method

Table 1
Summary of the calibration curve of compounds I and II

Nominal concentration (ng/ml)	I or II/I.S. peak-area ratios (mean ± S.D.)	R.S.D. (%)	*n*
Compound I			
10	0.068 ± 0.013	19.11	6
20	0.140 ± 0.025	17.86	6
30	0.208 ± 0.019	9.13	6
50	0.347 ± 0.018	5.19	6
100	0.703 ± 0.051	7.25	6
500	3.693 ± 0.175	4.74	6
1000	7.505 ± 0.166	2.21	6
2000	15.191 ± 0.268	1.76	6
Compound II			
10	0.022 ± 0.004	17.93	6
30	0.072 ± 0.008	11.11	6
100	0.221 ± 0.019	8.59	6
250	0.607 ± 0.053	8.73	6
500	1.093 ± 0.070	6.40	6
1000	2.184 ± 0.035	1.60	6
1500	3.369 ± 0.171	5.07	6

Table 2
Within-day precision of the method for compounds I and II

Nominal concentration (ng/ml)	Measured concentrations (mean ± S.D.) (ng/ml)	Accuracy (%)	R.S.D. (%)	n
Compound I				
10	9.43 ± 0.52	94.3	5.55	6
50	46.44 ± 1.30	92.9	2.81	6
100	100.03 ± 2.91	100.03	2.90	6
Average			3.75	
Compound II				
30	32.57 ± 3.64	108.56	11.17	6
100	102.66 ± 2.84	102.66	2.76	6
1500	1441.10 ± 28.76	96.07	1.99	6
Average			5.30	

Table 3
Between-day precision of the method for compounds I and II

Nominal concentration (ng/ml)	Measured concentrations (mean ± S.D.) (ng/ml)	Accuracy (%)	R.S.D. (%)	n
Compound I				
10	10.14 ± 1.41	101.40	13.90	6
50	47.91 ± 3.10	95.82	6.48	6
100	99.01 ± 2.23	99.01	2.25	6
Average			7.54	
Compound II				
30	30.63 ± 3.11	102.10	10.15	6
100	96.99 ± 4.88	96.99	5.03	6
250	275.17 ± 26.31	110.07	9.56	6
500	494.03 ± 14.25	98.80	2.88	6
1000	959.44 ± 24.63	95.94	2.57	6
1500	1441.10 ± 28.76	99.89	6.23	6
Average			6.07	

was within the accepted limit of 20% R.S.D. According to the validation the limit of quantitation of I and II was 10 ng/ml plasma, and the limit of detection was 5 ng/ml plasma, respectively. The calibration curves of area ratios versus the concentration were linear in the 10–2000 ng/ml ($y = 0.040 + 0.008x$; $r^2 = 0.999$) and 10–1500 ng/ml ($y = 0.005 + 0.002x$; $r^2 = 0.999$) plasma concentration range for nerisopam and its N-acetyl metabolite, respectively [y = the ratio of the peak areas of investigated compounds/I.S., x = the concentration of the investigated compounds (ng/ml plasma)]. The data of the calibration curves are summarized in Table 1.

The F test for linearity [$F(6,40) = 0.6482$, p =

Table 4
Determination of extraction recovery of I and II ($n = 5$ at each concentration)

Nominal concentration (ng/ml)	Peak-area ratio of I or II/I.S. after extraction	Peak-area ratio from the same concentration standard aqueous solution of I or II/I.S.	Calculated extraction efficiency (%)
Compound I			
10	0.051	0.059	86.4
20	0.101	0.113	89.3
50	0.255	0.274	93.0
100	0.481	0.578	83.2
Average			87.97
Compound II			
30	0.068	0.0759	89.5
100	0.209	0.2280	91.7
250	0.565	0.5824	97.0
500	1.008	1.2047	83.6
1000	1.937	2.1840	88.7
1500	3.113	3.3690	92.4
Average			90.48

0.691 for I and $F(5,35) = 1.211$, $p = 0.324$ for II] indicated that the calibration curves determined by the least squares regression model were linear over the calibration range investigated.

The R.S.D. values of the slope of the calibration curve were 2.14% for I and 1.79% for II.

Tables 2 and 3 demonstrate the within-day and between-day precision and accuracy of the method. The average R.S.D. values of within-assay analysis were 3.75% and 5.30% for I and II, respectively. Acceptable accuracy was achieved for all concentrations investigated. The between-

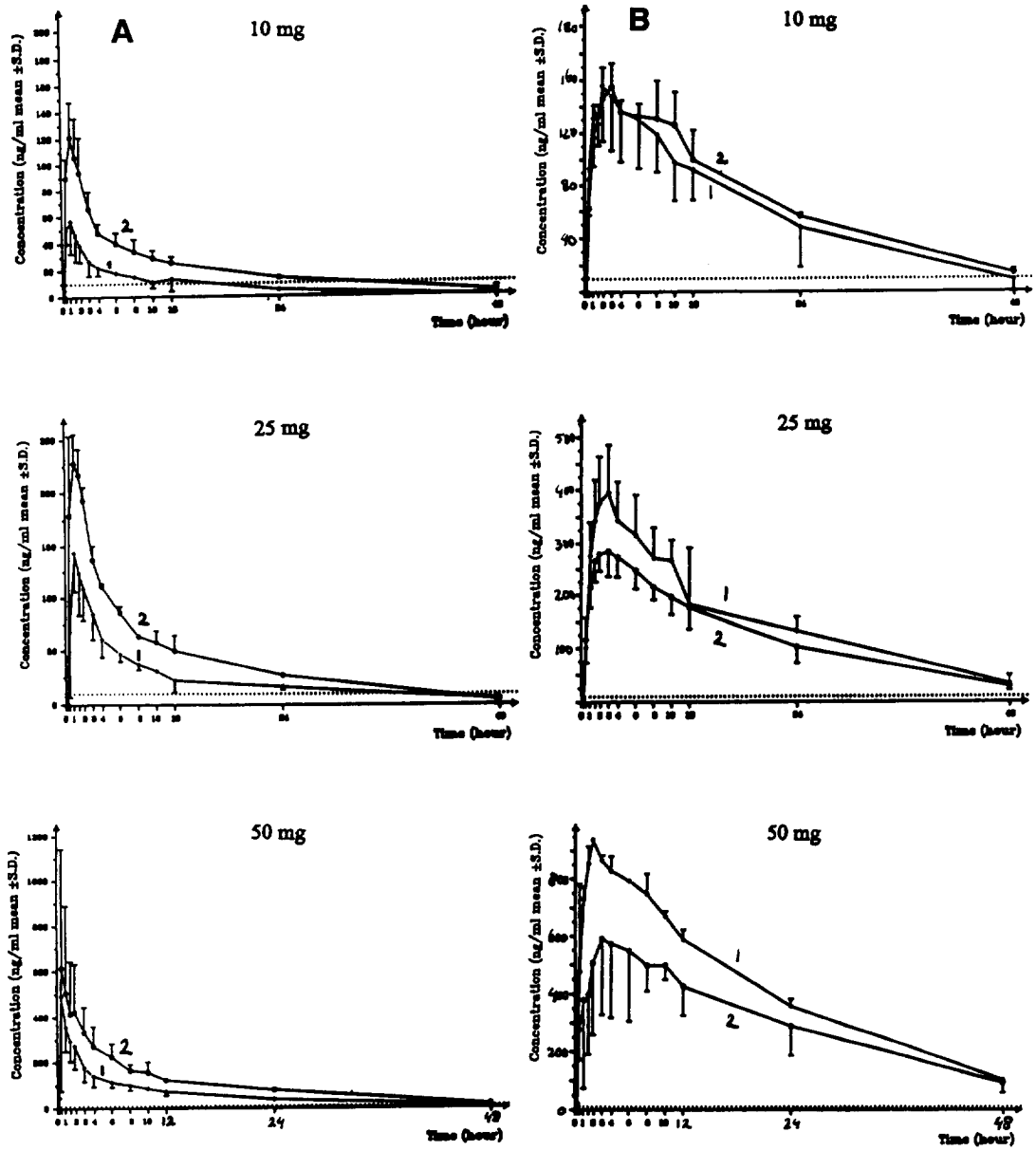

Fig. 5. Pharmacokinetic curves of (A) nerisopam (I) and (B) its N-acetyl metabolite (II) in fast (1) and slow (2) acetylator phenotypes after some different EGIS-6775 (I) doses; $n = 3$. Dotted line represents the lower quantification limit by validation process.

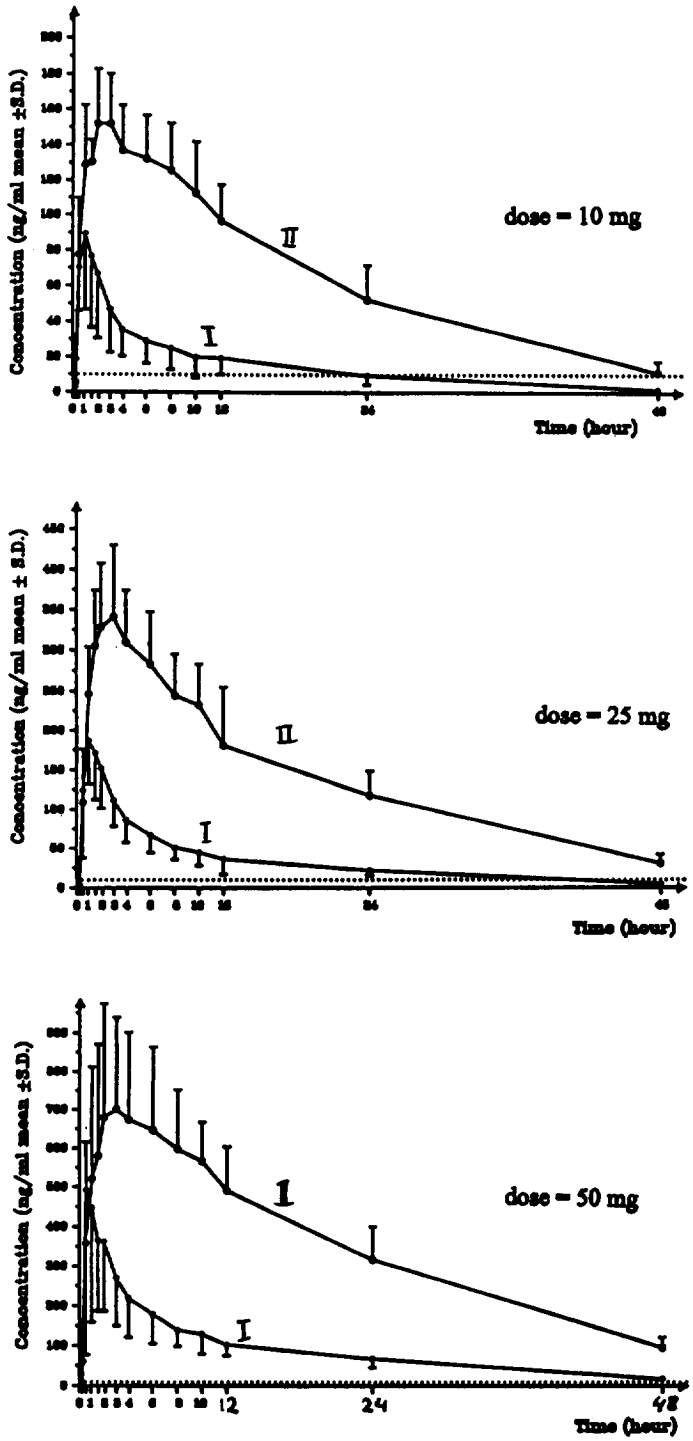

Fig. 6. Pharmacokinetic curves of nerisopam (I) and its N-acetyl metabolite (II) after some different EGIS-6775 (I) doses ($n = 6$ volunteers at each dose). Dotted line represent the lower quantification limit by validation process.

assay precision was calculated from the data obtained on six different days. The average R.S.D. values were 7.54% and 6.07% for I and II, respectively. The accuracy of the assays ranged from 95.82% to 110.07%, which is acceptable for biological samples.

Data concerning the extraction recoveries of the compounds are presented in Table 4. The average recovery was 87.97% for I and 90.48% for II ($n = 5$ at each concentration).

Nerisopam and its N-acetyl metabolite in human plasma showed no sign of significant degradation during four weeks of storage at −20°C. The accuracy of I and II in the plasma after storage was between 93.08% and 102.20%.

Three different doses (10, 25 and 50 mg) were studied. A significantly lower original compound (nerisopam) and higher N-acetyl metabolite levels (Fig. 5) were measured after administration to fast acetylators than to slow acetylators. The limit of quantitation is shown by a dotted line in the figures.

The results of the analytical measurements prove that nerisopam is N-acetylated through polymorphic metabolisation. It was found that the kinetics of nerisopam could be described by a two-compartment open model, and that of the N-acetyl metabolite by a one-compartment model. Since nerisopam acetylation is polymorphic, the fast or slow acetylator phenotypes produced significantly different plasma concentrations. In slow acetylators the concentration of I was higher in plasma, while the level of II (the N-acetyl metabolite) was higher in the fast acetylators' plasma. The elimination phases of both test compounds were found to be practically parallel at the same time, although the C_{max} and AUC values differed considerably. Both compounds were quickly absorbed, but II appeared in blood very rapidly (Fig. 6). On the basis of these results, it can be claimed that nerisopam shows significant "first-pass" metabolism, with varying intensity in various pharmaco-genetical phenotypes. Therefore, during further studies of the compound, the acetylator phenotypes of the examined populations must be taken into account.

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