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Note

Determination of tofisopam in serum by high-performance liquid chromatography

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Tofisopam (Grandaxin^R, 1-(3,4-dimethoxyphenyl)-4-methyl-5-ethyl-7,8-dimethoxy-2,3-benzodiazepine) is a new type of tranquillizer valuable for the relief of anxiety and tension in a wide range of emotional disorders. There is no convenient method available for the determination of tofisopam in biological fluids.

On the other hand, several papers have already been published on the determination of diazepam, compounds chemically closely related to tofisopam, and its metabolites [1–3]. High-performance liquid chromatographic determination of various benzodiazepines and their derivatives [4–7] proved to be fast and reproducible.

In the present paper we describe a technique for the determination of tofisopam in human serum using high-performance liquid chromatography.

EXPERIMENTAL

Materials

Samples of tofisopam and internal standard [1-(3,4-dimethoxyphenyl)-4,5-

dimethyl-7,8-dimethoxy-2,3-benzodiazepine] (EGYT-2964)* were products of the Preparative Laboratory of EGYT Pharmacochemical Works (Budapest, Hungary). All other chemicals were of analytical grade.

Chloroform used for extraction was purified in the following way: 100 ml of 1 mol/l hydrochloric acid were added to 1 l of chloroform and shaken for 30 min. The phases were separated, then the organic phase was shaken with 100 ml of 1 mol/l sodium hydroxide for another 30 min, followed by extraction with 2 × 200 ml of distilled water. The washed chloroform was dehydrated by filtration through Whatman phase-separation paper.

Liquid chromatography

Chromatography was performed on a 25 × 0.21 cm LiChrosorb Si-60 (5 μm) column. The sample (20 μl) was applied by a Rheodyne 7120 injection valve.

The column was eluted isocratically, by *n*-heptane-isopropanol-methanol (70:10:1) at a flow-rate of 1 ml/min. The solvent was pumped by an Altex Type 110 pump, back pressure 35 bar. The effluent was monitored by a Spectro-Monitor II of Laboratory Data Control at 311 nm, where tofisopam shows maximum absorbance.

Standard solutions

Tofisopam was dissolved in methanol (1 mg/ml); this solution was diluted ten-fold with distilled water. The internal standard was dissolved in distilled water (100 μg/ml).

Serum standards

Serum standards for calibration were prepared by adding 0–40 μl of the 100 μg/ml tofisopam solution to 5.0 ml of human serum in extraction tubes (16 × 150 mm). To each mixture 20 μl of the internal standard solution were added.

In this way a series of serum standards was prepared containing 0–800 ng/ml tofisopam and 200 ng/ml internal standard each.

Extraction

The serum standards and samples (5 ml) were deproteinized by the addition of 10 ml of methanol containing 0.1 mol/l hydrochloric acid, and 5 ml of 1 mol/l hydrochloric acid. The precipitate was removed by centrifugation for 30 min at 1000 *g*.

The supernatants were transferred by syringe to test-tubes and evaporated to dryness over a water-bath at 60°C in a Rotavapor (Büchi) vacuum evaporator. The dry residue was dissolved in 2 ml of 0.1 mol/l hydrochloric acid and shaken with 10 ml of chloroform for 30 min in a shaking machine. The pH of the water phase was adjusted to 10 by the dropwise addition of 1 mol/l sodium hydroxide. The pH was checked using indicator paper. After adjustment of the pH, shaking was continued for another 10 min. The organic phase was withdrawn by syringe, and the alkaline water phase shaken with 5 ml of chloroform for 10 min. The organic phases were combined and washed with 5

*This compound is available on request from the Preparative Laboratory of EGYT Pharmacochemical Works.

ml of 5% sodium carbonate solution. The organic phase was filtered through Whatman phase-separation paper and evaporated to dryness in a stream of air at room temperature. The dry residue was washed into 0.3 × 3 cm conical test-tubes with 2 × 0.25 ml of methanol and dried again.

The dry residue was dissolved in 0.2 ml of mobile phase.

RESULTS AND DISCUSSION

By changing the polarity of the eluent we could adjust the retention time of tofisopam to 10 min. According to the elution pattern the tofisopam preparation contains a second component with a retention time of 12 min. This compound represents about 12% of the total and is thought to be a conformer of tofisopam [8].

An established way for checking extraction is the application of an internal standard. We used EGYT-2964 for this, since it has very similar structure and extraction properties to tofisopam yet is well separated from it. The retention

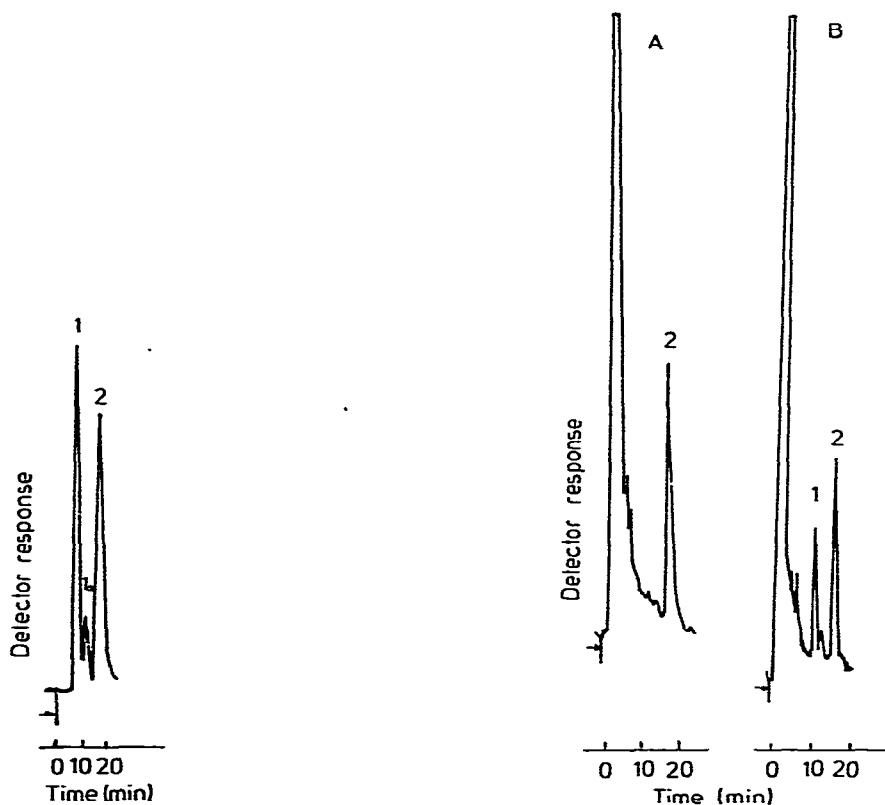


Fig. 1. Separation of tofisopam and internal standard. 1, 150 ng of tofisopam; 1a, tofisopam conformer; 2, 200 ng of internal standard. For separation conditions see Methods.

Fig. 2. Chromatograms of human serum extracts before (A) and after (B) tofisopam treatment. In each case 5 ml of serum were extracted. (A) Serum extract obtained from blood withdrawn before tofisopam treatment; (B) serum extract obtained from blood withdrawn 30 min after tofisopam treatment (100 mg, orally). 1 = Tofisopam; 2 = internal standard.

time of the internal standard was 17 min. A typical chromatogram of the mixture of tofisopam and internal standard is shown in Fig. 1.

A crucial aspect of quantitative techniques is the linearity of the calibration curve. We found that there was a linear relationship between the amount of tofisopam and the detector response expressed in peak height up to 800 ng of tofisopam injected. Likewise, a linear calibration curve was obtained after the extraction of different amounts of tofisopam and constant amounts (400 ng/ml) of EGYT-2964 from serum. The relationship between the peak heights of tofisopam and internal standard could be described by the equation $Y = 0.0054 + 0.0063X$ ($r^2 = 0.9906$) where Y is the ratio of the peak heights of tofisopam and internal standard, and X is the concentration of tofisopam (ng/ml) in serum. The drug concentration in the serum sample was calculated from this formula.

In human serum extracts tofisopam and internal standard separate well from other components extracted by the above technique (Fig. 2). The metabolic products of tofisopam do not interfere; they are eluted either before tofisopam or are strongly bound to the column.

Other contaminants are not retained; therefore, after elution of the internal standard the system is ready for the next sample. Thus the analysis of one sample takes about 20 min.

According to the extraction scheme presented, the efficiency in the concentration range 40–800 ng/ml was $34.6\% \pm 2.5$ (S.D.) with a coefficient of variation of 7.2%. The extraction efficiency of the internal standard was $38.2\% \pm 0.5$ (S.D.), the coefficient of variation 1.3%.

In preliminary pharmacokinetic experiments we could follow changes in the tofisopam concentration of human serum when Grandaxin^R was administered orally to healthy human volunteers (Table I). Since the aim of the method was

TABLE I

PLASMA TOFISOPAM CONCENTRATIONS FOLLOWING ORAL ADMINISTRATION OF 100 mg OF TOFISOPAM IN TABLET FORM

Concentrations are expressed in ng/ml tofisopam.

Subject No.	Time/h			
	0.5	1.0	1.5	2.0
1	<30	96	133	90
2	128	220	46	0
3	35	78	81	47
4	90	220	143	99
5	111	76	76	69
6	257	263	41	49
7	30	64	41	47
8	58	170	70	41
9	93	24	41	30
Mean	89	112	74	50
S.D.	±71	±80	±37	±27

the pharmacokinetic study of the drug, interference in the assay by other drugs was not tested. These pharmacokinetic studies are in progress and will be presented later.

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