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ORIGINAL RESEARCH

Analysis of tofisopam in human serum by column-switching semi-micro high-performance liquid chromatography and evaluation of tofisopam bioequivalency

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ABSTRACT: A rapid and sensitive column-switching semi-micro HPLC method is described for the direct analysis of tofisopam in human serum. The sample (100 μL) was directly injected onto the precolumn (Capcell Pak MF Ph-1), where unretained proteins were eluted to waste. Tofisopam was then eluted into an enrichment column using 13% acetonitrile in 50 mM phosphate buffer (pH 7.0) containing 5 mM sodium octanesulfonate and subsequently into the analytical column using 43% acetonitrile in 0.1% phosphoric acid containing 5 mM sodium octanesulfonate. The detection limit (2 ng/mL), good precision (CV $\leq$ 4.2%) and speed (total analysis time 24 min) of the present method were sufficient for drug monitoring. This method was successfully applied to a bioequivalence test of two commercial tofisopam tablets. Copyright © 2002 John Wiley & Sons, Ltd.

INTRODUCTION

Tofisopam [1-(3,4-dimethoxyphenyl)-4-methyl-5-ethyl-7,8-dimethoxy-2,3-benzodiazepine] (Fig. 1) is a minor tranquilizer used in the treatment of emotional disorders. There are few methods described for the determination of tofisopam in biological fluids, include normal-phase high performance liquid chromatography (HPLC; Sajgo *et al.*, 1981), gas–liquid chromatography (GLC) with nitrogen-phosphorous detection (Gaillard *et al.*, 1993) or mass spectrometry (GLC-MS; Tomori *et al.*, 1982). These methods required large sample volumes (1–5 mL) and time-consuming sample clean-up procedures such as protein precipitation, liquid–liquid extraction or solid-phase extraction prior to instrumental analysis. Therefore, the aim of the present study was to develop a rapid, accurate and convenient method to determine tofisopam in human serum using column-switching HPLC. The applicability of the assay method was demonstrated in the study of tofisopam bioequivalency to identify pharmaceutical equivalents of the two tofisopam formulations.

EXPERIMENTAL

Materials and reagents. Tofisopam was obtained from Charm Pharmaceutical Co. (Seoul, Korea). HPLC grade methanol and acetonitrile were purchased from Burdick & Jackson Inc. (Muskegon, MI, USA). Stock solution of tofisopam was prepared by dissolving tofisopam in acetonitrile (100 μg/mL) and aliquots were spiked to drug-free human blank serum to obtain the calibration serum standards at five concentrations of 5, 10, 50, 100 and 200 ng/mL. Serum samples were filtered with disposable low protein binding membrane syringe filter (0.22 μm, PVDF, Millipore, Bedford, MA, USA) before HPLC analysis.

Chromatographic system. The HPLC system consisted of the Nanospace SI-1 (Shiseido, Tokyo, Japan), ie two 2001 pumps, a 2002 UV–vis detector, a 2003 autosampler, a 2004 column oven, a 2012 high pressure switching valve and a 2009 degassing unit. The data was processed by S-MC 4.1 (Shiseido) using a personal computer. A block diagram of column switching HPLC system is shown in Fig. 2.

The columns used in this on-line extraction system were a

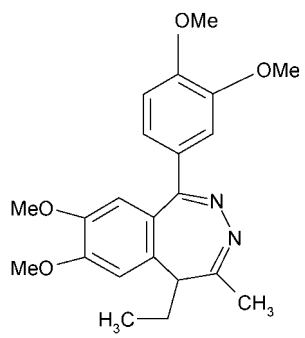

Figure 1. Chemical structure of tofisopam.

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Abbreviations used: LOD, limit of detection.

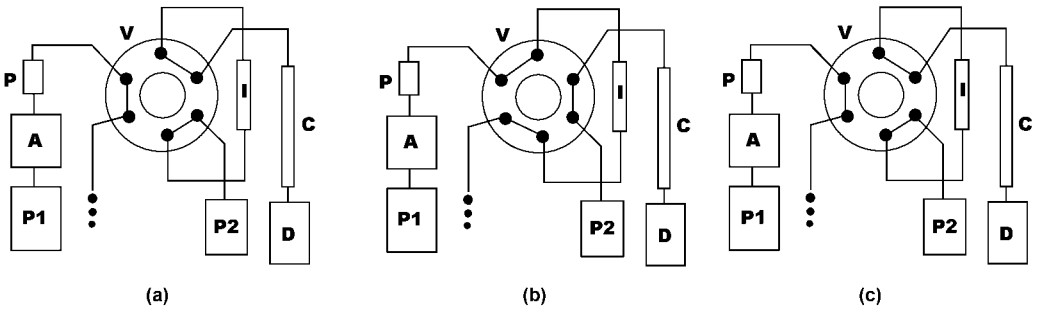

Figure 2. Flow diagrams of the three steps in the column-switching technique. (a) Sample injection and primary separation. (b) Enrichment of tofisopam into the enrichment column. (c) Chromatographic separation. A, autosampler; C, analytical column; D, detector; I, intermediate column; P, precolumn; P1, pump 1; P2, pump 2; V, switching valve.

precolumn (Capcell Pak MF Ph-1, 20 × 4 mm i.d., 5 μm, Shiseido, polymer-coated mixed function pre-column), an enrichment column (Capcell Pak C_{18} UG 120, 35 × 2 mm i.d., 5 μm) and an analytical column (Capcell Pak C_{18} UG 120, 250 × 1.5 mm i.d., 5 μm).

The mobile phase for the primary separation of tofisopam in the precolumn and concentration in the enrichment column (pump 1) was 13% acetonitrile in 50 mM phosphate buffer (pH 7.0) containing 5 mM sodium octanesulfonate with a flow rate of 0.5 mL/min. The mobile phase for the analytical column (pump 2) was 43% acetonitrile in 0.1% phosphoric acid containing 5 mM sodium octanesulfonate at a flow rate of 0.1 mL/min. The precolumn and the analytical column were maintained at 35°C and the enrichment column was run at room temperature. The detection wavelength was 310 nm.

Analytical procedure. The operation of this column-switching semi-micro HPLC consists of three main steps: sample loading and primary separation, enrichment of the analyte fraction and chromatographic separation (Lee *et al.*, 1998; Tagawa *et al.*, 1999; Jeong *et al.*, 2000).

When the column-switching valve was at the A position, an aliquot of filtered serum sample (100 μL) was loaded to precolumn and primary separation of tofisopam from serum proteins were performed using the mobile phase for the precolumn [Fig. 2(a)]. Subsequently, the valve was switched to the B position and tofisopam fraction was eluted from the precolumn and concentrated in the enrichment column by the mobile phase for the precolumn [Fig. 2(b)]. Position B was maintained for 6.0–12.0 min after injection of serum sample. Then, the valve position was returned to A and tofisopam concentrated in the enrichment column were transferred into the analytical column in the back-flush mode using the mobile phase for the analytical column [Fig. 2(c)].

Method validation. Limit of detection (LOD) for tofisopam was determined as the concentration of drug giving a signal-to-noise ratio greater than 3:1. The mean recovery of tofisopam from serum was determined by the analysis of tofisopam-spiked serum, followed by replicate injection of the same amount of a standard in mobile phase directly onto the microbore column providing the 100% value. Five tofisopam-spiked serum standard samples over the concentration range of 5–200 ng/mL were quantified to evaluate the linearity, precision [the coefficient of variation (CV) of replicate analysis] and accuracy (the bias between theoretical and actual concentration). Within-day and day-to-day reproducibility were evaluated from six experiments in a day and six consecutive days, respectively.

Bioequivalence test of tofisopam. Fourteen healthy male volunteers (age 22.4 ± 2.2 years) were selected after passing a physical examination, blood pathological test and serum biochemical examination. Volunteers were assigned in a random manner into group 1 ($n = 7$) and group 2 ($n = 7$). The study design was Latin-square two-period crossover evaluation of the bioavailability of two tablet formulations of tofisopam, Grandaxin® (Hwanin Pharm. Co., Seoul, Korea, reference drug) and Puri® (Charm Pharm. Co., test drug). Volunteers, fasted for 12 h, received a single oral dose of test or reference tofisopam tablet (50 mg) with 20 mL of water. There was a 7 day washout period between medications. Serial blood samples (2 mL) were withdrawn by venipuncture at 0, 0.33, 0.67, 1, 1.5, 2, 3, 5, 7 and 11 h post dosing. Light meals were served 4 h after drug administration. The serum samples were separated from the blood by centrifugation (3000***g***, 15 min, 4°C) and kept at −70°C until HPLC analysis. Drug concentrations were determined as the mean of duplicate samples by this method.

The bioequivalence evaluation of the two tablet formulations of tofisopam was based on the pharmacokinetic parameters such as area under the serum concentration–time curve (AUC), the peak serum concentration (C_{max}) and the time of peak serum concentration (T_{max}). C_{max} and T_{max} were determined by visual inspection from each volunteer's serum concentration–time curve for tofisopam, and AUC was calculated by the linear trapezoidal method from 0 to 11 h. Analysis of variance was performed to evaluate the statistical significance of the mean values of pharmacokinetic parameters.

RESULTS AND DISCUSSION

Chromatography and column-switching procedure

Reversed-phase as well as normal-phase HPLC techniques were used for the identification of potential impurities of tofisopam drug substance (Patthy and Salát,

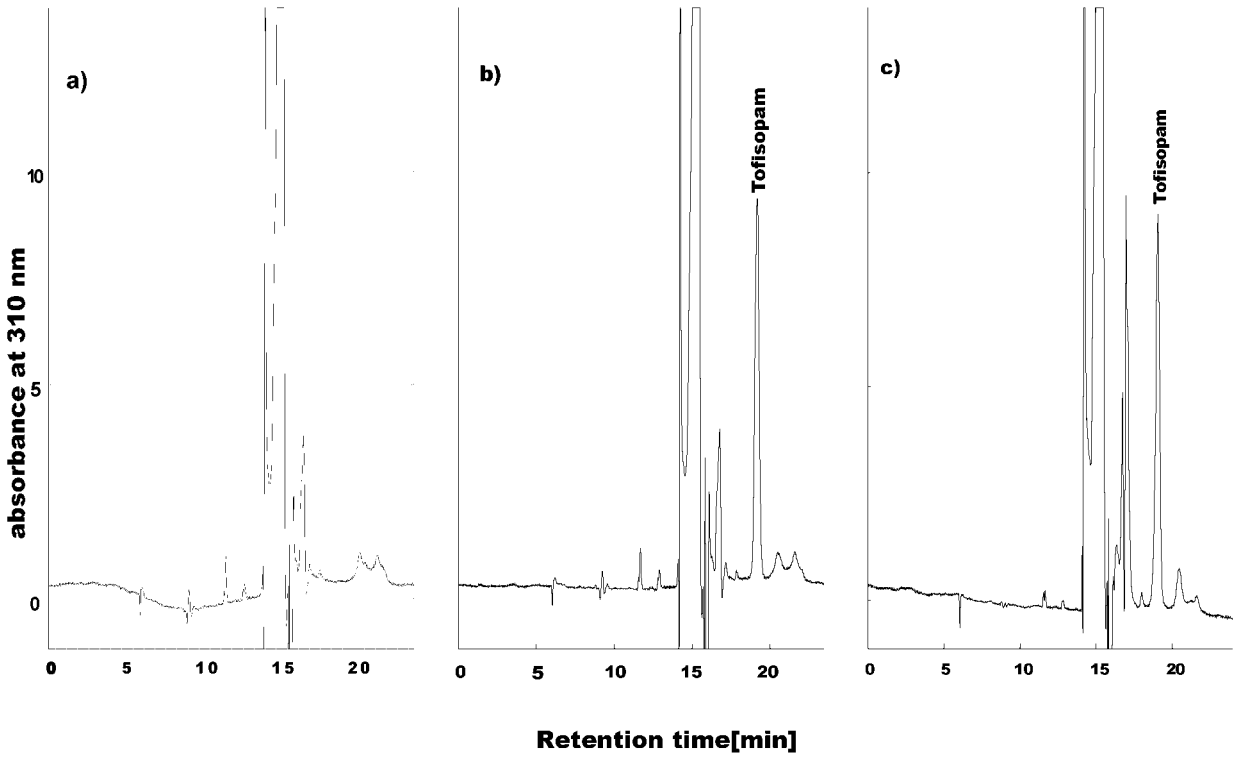

Figure 3. Representative chromatograms of (a) blank serum, (b) blank serum containing tofisopam (100 ng/mL), and (c) serum sample (tofisopam = 100.2 ng/mL) obtained 1 h after an oral dose of 50 mg tofisopam to a healthy male volunteer.

1981; Maianna *et al.*, 1993). However, normal-phase HPLC was reported for the analysis of serum tofisopam concentrations (Sajgó *et al.*, 1981), This reversed-phase semi-micro HPLC method using column-switching produced some advantages over the previous normal-phase HPLC method, ie increased sensitivity (0.2 *vs* 150 ng), small serum volume (100 μL *vs* 5 mL), no sample pre-purification step, reliability and lower organic solvent consumption.

Tofisopam was eluted as double peaks in some reverse-phase systems (Patthy and Salát, 1981; Maianna *et al.*, 1993), because tofisopam molecules in solution exist as a mixture of two boat conformers. The ratio of conformers is a function of time, temperature and solvent (Symonyi and Fitos, 1983; Maianna *et al.*, 1993). To obtain good resolution of tofisopam as a conformer in serum samples, 43% acetonitrile in 0.1% phosphoric acid containing 5 mM sodium octanesulfonate was chosen as mobile phase for analytical column (Fig. 3).

The separation profile of tofisopam in serum on the precolumn was evaluated using 15% acetonitrile in 50 mM phosphate buffer (pH 7.0) containing 5 mM sodium octanesulfonate to obtain good recovery and to determine the appropriate time for column-switching. Tofisopam was retained in the precolumn during the exclusion of the serum proteins, which were seen at the position of void volume, and then the peak of tofisopam appeared from 6 to 12 min. Therefore, the eluate of precolumn from 6.0 to 12.0 min after injection of samples was transferred to the enrichment column by switching the valve to the B position. The enrichment column was used in order to protect the precolumn from high pressure and save analysis time. Without an enrichment column, it might take 30 min to transfer the tofisopam fraction from the precolumn to the analytical column at a flow rate of 0.1 mL/min.

Typical chromatograms of blank serum, serum spiked with tofisopam and serum sample obtained after a single oral administration of tofisopm tablet are shown in Fig. 3. There was no interference peak at the retention time (19.2 min) of tofisopam.

MF Ph-1 precolumn was exchanged after injection of 40 serum samples (equivalent to 4.0 mL serum). The efficiency of intermediate and main columns began to be decreased after the analysis of 400 serum samples.

Method validation

Mean absolute recovery of tofisopam from serum samples was 93.7 ± 2.1%. LOD of tofisopam was 2 ng/mL using 100 μL serum, and therefore this method can be used to characterize the pharmacokinetics of tofisopam in cases where biological sample volumes are limited. The calibration curve of peak area *vs* the concentrations of tofisopam in serum was linear over the range 5–200 ng/mL, giving a correlation coefficient of 0.999. The within-

Table 1. Reproducibility of tofisopam in human serum samples ($n = 6$)

Theoretical concentration (ng/mL)	Concentration found (ng/mL)		CV(%)	
	Within-day	Day-to-day	Within-day	Day-to-day
5	5.1	5.0	3.8	4.2
10	9.9	10.1	3.6	3.9
50	49.9	50.2	2.5	2.3
100	100.5	101.0	2.2	2.0
200	201.1	200.9	1.5	1.6

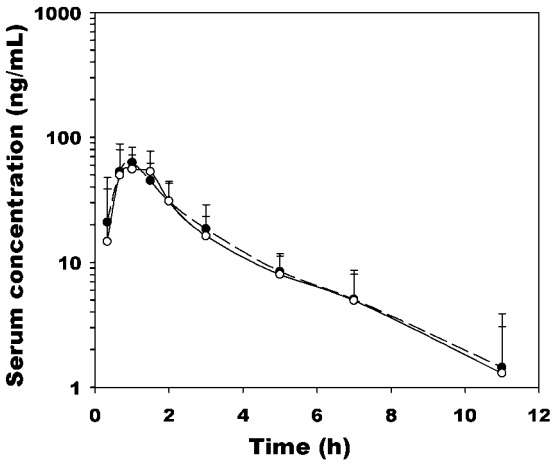

Figure 4. Mean serum concentration–time plots of tofisopam following an oral administration of the two tofisopam products (50 mg as tofisopam) in 14 male healthy volunteers. The each point represents the mean ± SD. (●) Test drug: (○) reference drug.

day and day-to-day precision (CV) and accuracy of the assay are shown in Table 1. The amount found deviated from −1.0 to 2.0% of the theoretical amount in the spiked serum samples and the assay was precise because CV was less than 4.2%.

Bioequivalence test of tofisopam products

The present method was successfully applied to the determination of tofisopam in human serum samples for the bioeuivalence evaluation of two commercial tofisopam tablets. Figure 4 shows the mean serum concentration–time curves of tofisopam after a single oral dose of Grandaxin® (reference drug) and Puri® (test drug), 50 mg as tofisopam, by 2×2 Latin square crossover method. The pharmacokinetic parameters such as AUC, C_{max} and T_{max} of the test drug were similar to those of the reference drug, as shown in Table 2, which proved that there was no significant difference between the bioavailability of Puri® (test drug) and Grandaxin® (reference drug), and therefore the two tofisopam products are bioequivalent.

Table 2. The pharmacokinetic parameters of tofisopam for the test and reference drug (each value presents the mean ± SD of 14 volunteers)

Products	Test drug	Reference drug
AUC (ng h/mL)	158.7 ± 29.4	152.0 ± 18.9
C_{max} (ng/mL)	77.9 ± 21.0	74.7 ± 21.7
T_{max} (h)	1.06 ± 0.48	1.08 ± 0.40

CONCLUSION

For the direct analysis of tofisopam from human serum samples, a reversed-phase semi-micro HPLC method using column-switching has been developed without pre-purification step. This method has shown many advantages such as excellent sensitivity (2 ng/mL) using small sample volume (100 μL), reproducibility, specificity and speed (24 min per sample). Using this method, the study of bioequivalence test for the two commercial tofisopam formulations was successfully performed.

Acknowledgements

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REFERENCES

Gaillard Y, Gay-Montchamp J-P and Ollagnier M. Simultaneous screening and quantitation of alpidem, zolpidem, buspirone and benzodiazepines by dual-channel gas chromatography using electron-capture and nitrogen-phosphorous detection after solid-phase extraction. *Journal of Chromatography* 1993; **622**: 197.

Jeong CK, Kim SB, Choi SJ, Sohn DH, Ko GI and Lee HS. Rapid microbore liquid chromatographic analysis of biphenyldimethyl dicarboxylate in human plasma with on-line column switching. *Journal of Chromatography B* 2000; **738**: 175.

Lee HS, Kim JH, Kim K and Do KS. Determination of myristicin in rat serum samples using microbore high performance liquid chromatography with column-switching. *Chromatographia* 1998; **48**: 365.

Maianna V, Erika K and László L. Investigation of potential impurities of tofisopam drug substance by high performance liquid chromatography. *Acta Pharmacia Hungarica* 1993; **61**: 57.

Patthy M and Salát J. High-performance liquid chromatographic determination of grandaxin and its trace impurities. *Journal of Chromatography* 1981; **210**: 159.

Sajgó M, Gesztesi A and Sido T. Determination of tofisopam in serum by high-performance liquid chromatography. *Journal of Chromatography* 1981; **222**: 303.

Symonyi M and Fitos I. Stereoselective binding of a 2,3-benzodiazepine to human serum albumin. *Biochemical Pharmacology* 1983; **32**: 1917.

Tagawa N, Tsuruta H, Fujinami A and Kobayashi Y. Simultaneous determination of estriol and estriol 3-sulfate in serum by column-switching semi-micro high-performance liquid chromatography with ultraviolet and electrochemical detection. *Journal of Chromatography B* 1999; **723**: 39.

Tomori E, Horváth Gy, Elekes I, Láng T and Körösi J. Investigation of the metabolites of tofizopam in man and animals by gas-liquid chromatography-mass spectrometry. *Journal of Chromatography* 1982; **241**: 89.

