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Rapid and simple method for the determination of zolpidem in human plasma by high-performance liquid chromatography

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Abstract

A simple and reproducible method for the determination of zolpidem in human plasma is presented. This method involves protein precipitation with methanol (2 ml of methanol are added to 0.5 ml of plasma) and reversed-phase chromatography with fluorescence detection (excitation wavelength 244 nm, emission wavelength 388 nm). The mobile phase consists of methanol–30 m*M* dihydrogen potassium phosphate–triethylamine (30:69:1). pH of the aqueous part of the mobile phase is 6.8. No internal standard is required. Limit of quantitation is 1.5 ng/ml and the calibration curve is linear up to 400 ng/ml. Within-day and between-day precision expressed by relative standard deviation is less than 5% and inaccuracy also does not exceed 9%. The assay is useful for pharmacokinetic studies. © 1997 Elsevier Science B.V.

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1. Introduction

Zolpidem is an hypnotic agent with a chemical structure unrelated to benzodiazepines, barbiturates, or other drugs with known hypnotic properties. It is indicated for the short-term treatment of insomnia, it exhibits rapid onset of action and short elimination half-time [1].

A few chromatographic and capillary electrophoresis (CE) methods have been reported for determination of zolpidem in biological fluids. In high-performance liquid chromatography (HPLC) methods the sample pretreatment consists of liquid–liquid extraction [2–4] or is based on column-switching [5]. Gas chromatography (GC) methods [6,7] are suitable especially for toxicological examinations. An extractionless CE method was described for determination of zolpidem and its metabolites in urine [8].

The purpose of the present study was to develop a simpler procedure for sample preparation while maintaining the detection sensitivity.

2. Experimental

2.1. Chemicals

Methanol (Lichrosolv, for chromatography) and potassium dihydrogenphosphate (analytical grade) were manufactured by Merck (Darmstadt, Germany). *o*-Phosphoric acid (analytical grade) was obtained

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from Lachema (Brno, Czech Republic). Triethylamine (p.a.) was the product of Fluka Chemie (Buchs, Switzerland).

2.2. Apparatus

All HPLC instruments were obtained from Thermo Separation Products (Riviera Beach, FL, USA). The system consisted of a membrane degasser, ConstaMetric 4100 pump, AS 3000 automatic sample injector, FL2000 fluorescence detector and datastation with PC1000 software, version 2.5. The separation was performed on a Nucleosil 100-3 C_{18}, 150×4.6 mm I.D. column (Watrex, Prague, Czech Republic). A 10×4 mm I.D. pre-column packed with Nucleosil 120-5 C_{18} was used.

The mobile phase consisted of methanol–30 m*M* dihydrogen potassium phosphate–triethylamine (30:69:1) with 50 mg/l of sodium azide. pH of the aqueous solution was adjusted to 6.8 with concentrated *o*-phosphoric acid. The flow-rate of the mobile phase was 0.7 ml/min at 35°C. The fluorescence detector was operated at the following wavelengths: excitation 244 nm, emission 388 nm. The detector time constant was set to 2 s, the lamp flash rate was 100 Hz and the photomultiplier tube voltage was 600 V.

2.3. Preparation of standard solutions

Stock solutions of zolpidem hemitartrate were made by dissolving approximately 7 mg in 10 ml of methanol (factor 0.8036 for conversion to the free base). Separate solutions were prepared for calibration curve and quality control samples. Further solutions were obtained by serial dilutions of stock solutions with methanol. These solutions were added to drug-free plasma in volumes not exceeding 4% of the plasma volume.

All solutions were stored at −20°C and protected from light.

2.4. Preparation of the sample

The samples were stored in the freezer at −20°C and allowed to thaw at room temperature before processing.

A 2-ml volume of methanol was added to 0.5 ml of plasma, the tube was vortex-mixed at 1500 rpm for 10 s and centrifuged 5 min at 2600 *g*. 1 ml of the supernatant was transferred to the autosampler vial and 20 μl were injected into the chromatographic system.

2.5. Calibration curves

The calibration curve was constructed in the range 1.5–400 ng/ml to encompass the expected concentrations in measured samples. The calibration curves were obtained by weighted linear regression (weighing factor $1/y^2$): the zolpidem peak area was plotted versus zolpidem concentration in ng/ml.

3. Results and discussion

3.1. Chromatography

Under the chromatographic conditions described the retention time of zolpidem was 4.7 min ($k'=1.5$). The column efficiency expressed by the number of theoretical plates was 6500 and peak asymmetry measured at 5% of the peak height was 1.4.

Six blank plasma samples obtained from healthy volunteers were processed and chromatographed and were found to contain no interfering peaks. The typical chromatogram of blank plasma is shown in Fig. 1. The chromatogram of a plasma sample 8 h after administration of 10 mg zolpidem hemitartrate

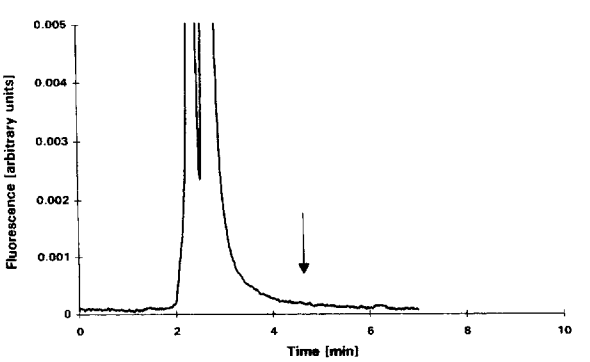

Fig. 1. Typical chromatogram of drug-free human plasma. The arrow indicates the retention time of zolpidem.

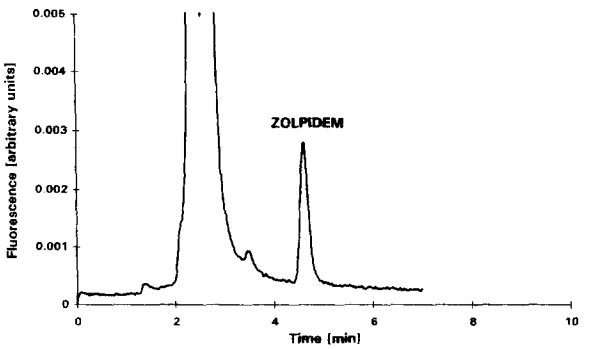

Fig. 2. Chromatogram of a plasma sample from a volunteer 8 h after administration of 10 mg of zolpidem hemitartrate. The measured concentration of zolpidem was 9.95 ng/ml.

to a volunteer is shown in Fig. 2. The concentration of zolpidem was 9.95 ng/ml.

3.2. Sample preparation

Zolpidem exhibits strong fluorescence and therefore no concentration of plasma samples is required. The simple precipitation of proteins was found to be sufficient to yield clean chromatograms. The following solvents were tested for protein precipitation: 10% perchloric acid (plasma–solvent ratio 1:0.3), acetonitrile (1:3) and methanol (1:4). With perchloric acid the recovery of zolpidem was only about 35%. The recovery with organic solvents was quantitative. However, the peak of zolpidem was deformed when the sample contained 75% of acetonitrile, because the elution strength of the sample solvent was substantially higher than that of the mobile phase. Therefore methanol was chosen as the best solvent for protein precipitation.

3.3. Linearity and limit of quantitation

The calibration curves were linear in the studied range. The calibration curve equation is $y=bx+c$, where y represents peak area of zolpidem and x represents the zolpidem concentration in ng/ml. The mean equation of the calibration curve ($n=7$) obtained from 6 points was $y=4810x+412$ (correlation coefficient $r=0.9999$).

The limit of detection was 1.5 pg with signal-to-noise ratio 3:1. The limit of quantitation was 1.5 ng/ml which corresponds to 6 pg injected onto the column. The precision, characterised by the relative standard deviation, was 6.0% and accuracy, defined as the deviation between the true and the measured value expressed in percent, was 2.4% at this concentration ($n=6$).

Table 1
Intra-day precision and accuracy

n	Concentration (ng/ml)		Bias (%)	R.S.D. (%)
	Added	Measured		
6	2.994	2.957	−1.2	2.8
6	20.87	21.53	3.2	2.6
6	324.9	351.1	8.1	2.7

n=number of samples.

3.3.1. Intra-day precision

Intra-day precision of the method is illustrated in Table 1. Six sets of quality control samples (low, medium and high concentration) were analysed with calibration samples on one day. Precision was better than 3%, whereas the accuracy was better than 9% at all levels.

3.3.2. Inter-day precision and accuracy

Inter-day precision and accuracy was evaluated by processing a set of calibration and quality control samples (three levels analysed twice, results averaged for statistical evaluation) on six separate days. The samples were prepared in advance and stored at −20°C. The respective data are given in Table 2.

Table 2
Inter-day precision and accuracy

n	Concentration (ng/ml)		Bias (%)	R.S.D. (%)
	Added	Measured		
6	2.994	2.993	0.0	3.1
6	20.87	21.56	3.3	4.4
6	324.9	339.9	4.6	1.9

n=number of days.

The precision was better than 5% and the inaccuracy did not exceed 5% at any level.

3.3.3. Stability study

Freeze and thaw stability. A 4-ml volume of a low and high concentration sample were stored in a flask at −20°C. The samples were subjected to three freeze–thaw cycles: during each cycle duplicate 0.5-ml aliquots were processed, analysed and the results averaged. The results are shown in Table 3. The concentration changes are less than 7%, indicating no significant substance loss due to repeated thawing and freezing.

Processed sample stability. Two sets of samples with low and high concentration of zolpidem were analysed on one day and left in the autosampler for 24 h at ambient temperature. The samples were re-analysed using freshly prepared calibration samples. The results are presented in Table 3. The difference between freshly prepared and old samples was less than 6%. The processed samples are stable at room temperature for 24 h.

Long term stability. Two sets of samples (low and high concentration of zolpidem) were stored in the freezer at −20°C for thirty days. The samples were then analysed using freshly prepared calibration samples. The results are presented in Table 3, the bias was lower than 8%. The samples are stable at −20°C for at least one month.

3.4. Application to biological samples

The proposed method was applied to the determination of zolpidem in plasma samples for the evaluation of the pharmacokinetics of zolpidem in humans. Nineteen healthy male volunteers participated in the study after having given their informed consent. Plasma samples were periodically collected up to 26 h after oral administration of one tablet containing 10 mg of zolpidem hemitartrate. Fig. 3 shows the mean plasma concentration of zolpidem. The plasma level of zolpidem reached a maximum 1

Table 3
Stability of the samples

Freeze and thaw stability

Sample concentration (ng/ml)	n	Cycle 1		Cycle 2		Cycle 3	
		Measured (ng/ml)	Bias (%)	Measured (ng/ml)	Bias (%)	Measured (ng/ml)	Bias (%)
5.013	2	5.025	0.2	5.015	0.0	4.909	−2.1
324.9	2	340.9	4.9	345.6	6.4	345.3	6.3

Processed sample stability (storage at room temperature)

Date	n	Concentration found (ng/ml)	R.S.D. (%)	Difference (%)
New	6	2.957	2.8	
24 h old	6	3.132	5.7	5.9
New	6	351.1	2.7	
24 h old	5	344.9	2.0	−1.8

Long-term stability (storage at −20°C for one month)

Concentration (ng/ml)	n	Concentration found (ng/ml)	R.S.D. (%)	Bias (%)
5.013	5	4.652	5.6	−7.2
324.9	6	338.0	1.8	4.0

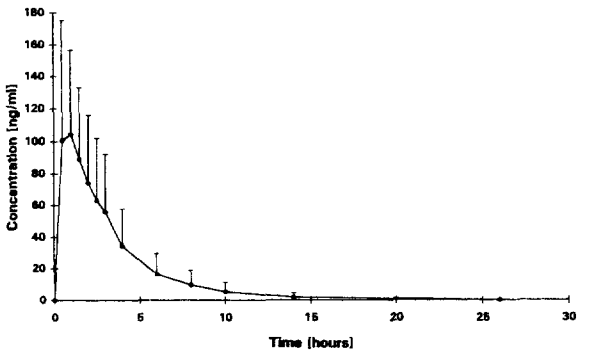

Fig. 3. Mean plasma concentrations (concentration+S.D.) of zolpidem after single oral dose administration of 10 mg of zolpidem hemitartrate (19 healthy volunteers).

h after the administration and thereafter the plasma level declined with an elimination half-time of ca. 1.5 h.

4. Conclusions

The results obtained indicate that this simple and rapid method for the assay of zolpidem in human plasma is sufficiently sensitive to follow the pharmacokinetics of this drug.

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