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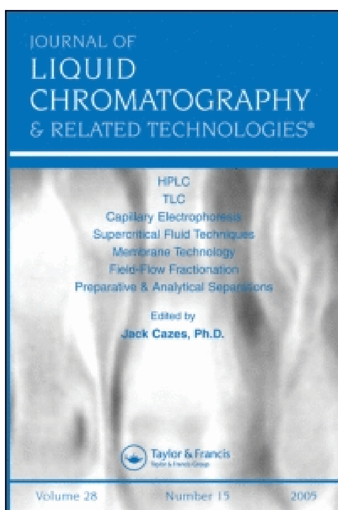

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RAPID AND SIMPLE METHOD FOR THE DETERMINATION OF ZOPICLONE IN PLASMA BY HPLC

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ABSTRACT

A simple, accurate, and reproducible high performance liquid chromatography method for the determination of zopiclone in plasma is presented. This method involves protein precipitation with acetonitrile and reversed-phase chromatography with ultraviolet detection at 304 nm. The mobile phase was 0.067 M phosphate buffer, pH 7.95-acetonitrile (55:45, v/v). Diazepam was applied as an internal standard.

The method was tested for linearity (over the range 2.5-200.0 ng/mL). The recovery (mean ± SD, n=7) was 96.76 ± 1.89%. Inter- and intra-day precision was less than 3% (n=6).

The described method has been applied to the quantitative determination of zopiclone in plasma and should be useful for bioavailability investigations.

INTRODUCTION

Zopiclone, 6-(5-chloro-2-piridyl)-6,7-dihydro-7-oxo-5H-pyrrolo[3,4-b]-pyrazin-5-yl-4-methylpiperazine-1-carboxylate (Figure 1a), is a non-benzodiazepine hypnotic drug of the cyclopyrrolone class possesing a short duration of

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action and few associated side effects. Zopiclone binds to the GABA-A-benzodiazepine receptor complex. In adults, the therapeutic dose is 7.5 mg of zopiclone per os and plasma levels usually lie in the range 20-80 ng/mL.[1,2]

Several methods have been described to detect zopiclone in biological fluids (plasma, serum, urine): gas chromatography (GC),[1-4] gas chromatography-mass spectrometry (GC-MS),[5] capillary electrophoresis (CE),[6] and high performance liquid chromatography (HPLC) with fluorescence detection[7-13] or with ultraviolet detection.[14,15]

Liquid-liquid extraction is the most frequently used method to extract zopiclone from biological samples, using mainly dichloromethane,[2,7,14] dichloromethane-propan-2-ol (9:1),[9,10] hexane-dichloro-methane (4:3),[3] n-butyl chloride,[15] or chloroform-propan-2-ol (9:1)[6] in basic media. Solid-phase extraction was also applied to the isolation of zopiclone from plasma. C18 extraction columns were used.[1,13]

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This paper presents a simple, rapid, sensitive, and easy to perform HPLC method for the determination of zopiclone in plasma. The method uses ultraviolet detection with a limit of quantitation of 2.5 ng/mL.

EXPERIMENTAL

Reagents and Materials

Zopiclone was purchased from Sigma (St.Louis MO, USA), the internal standard (I.S.) diazepam (Figure 1b) was obtained from Pharmaceutical Works “Polfa” (Tarchomin, Poland).

Acetonitrile LiChrosolv® for chromatography (E.Merck, Darmstadt, Germany) was applied. All the other reagents were of analytical grade. Water was purified by double distillation.

Heparinized human whole blood was provided by the District Blood Centre (Lublin, Poland). Blood samples were centrifuged and the plasma, thus obtained, was stored at -18°C.

Apparatus

A Waters HPLC system (Milford, MA, USA), consisting of a Model 515 high-pressure pump and a Model 2487 variable wavelength detector (UV-VIS), was used. Manual injections were made using a Rheodyne injectable valve (20 μL loop).

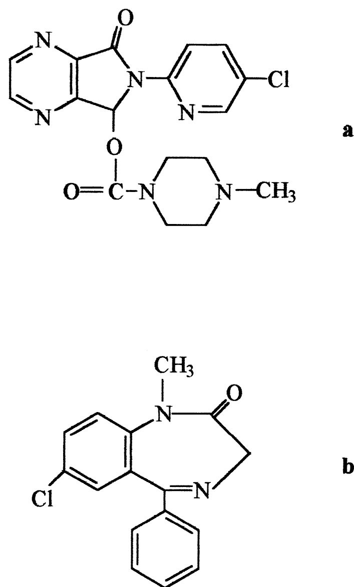

Figure 1. Structures of (a) zopiclone and (b) diazepam (I.S.).

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The data were collected and analyzed with a Millenium 32 system software on Pentium MMX 166 MHz computer.

A high-speed refrigerated centrifuge, type K24D (MLW, Engelsdorf, Germany), with 10 mL propylene centrifuge tubes was used. PolyPure polypropylene membranes (Alltech Associates Inc. USA) were applied.

Chromatographic Conditions

Sample volumes of 20 µL were injected into the liquid chromatograph. The chromatographic separations were performed at room temperature on a Nova-Pak C18 column (150 x 3.9 mm, dp = 4 µm) (Waters, Milford, MA, USA).

The mobile phase was a mixture of acetonitrile and 0.067 M phosphate buffer pH 7.95 (45:55, v/v), filtered and degassed prior to use, and flowing at the rate of 1 mL/min. Detection was by UV absorption at 304 nm.

Solutions

Stock solutions (1.0 mg/mL) of zopiclone and diazepam (I.S.) were prepared by dissolving appropriate amounts of these substances in acetonitrile. These solutions were stable for at least 2 months at 4°C. Working solutions were prepared by dilution with acetonitrile.

Calibration Procedure

Using the working solutions of zopiclone and diazepam (I.S.), samples were spiked with both compounds at concentrations ranging from 2.5 to 200 ng/mL for zopiclone, and with a fixed concentration of I.S. (500 ng/mL). A 20 μL volume of each sample was injected into the analytical column. All measurements were repeated five times at each concentration.

A calibration curve, based on the peak area ratios of zopiclone to I.S., was constructed using seven different concentrations of zopiclone. The data was subjected to linear-regression analysis in order obtain the appropriate calibration factors.

Sample Preparation

To seven centrifuges tubes containing 1.0 mL of plasma, zopiclone (from 10 ng to 800 ng) was added. Then, 2 μg of I.S. was added to each sample and the volume was completed up to 4 mL using acetonitrile. The mixtures were centrifuged for 15 min at approximately 1100 g. Then, 20 μL volumes of the supernatants were injected into the analytical column using PolyPure polypropylene membranes. Drug-free plasma was analysed in the same way as a control. All measurements were repeated five times at each concentration.

Absolute recovery was determined by comparing the average peak area for extracted plasma samples at each standard concentration of zopiclone and the I.S., with those for unextracted samples with an identical content of both substances.

Precision

Samples were prepared for inter- and intra-day validation. Five samples at each of the following concentrations (2.5, 50.0, and 200.0 ng/mL) were prepared for calculation of the coefficient of variation.

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Accuracy

The accuracy of the method was determined by injecting samples containing theoretical amounts of zopiclone at the same concentrations as those used for the calibration curve. Calculated values were compared with theoretical values and the percentage error was determined.

RESULTS AND DISCUSSION

A reversed-phase isocratic procedure was proposed as a suitable method for the analysis of zopiclone in plasma. Zopiclone is partly metabolised in the liver to form an inactive N-demethylated derivative and an active N-oxide metabolite. These metabolites are there in urine. In human plasma they are not detectable. This is presented in the papers.[16,17]

This study uses a mobile phase, an internal standard, and a technique of isolation of zopiclone from plasma which is different from previous HPLC methods. Besides, precision of our method is higher (CV < 3%) than the precision given in the literature of HPLC methods with ultraviolet detection (CV < 7%).[14,15]

The method described in this work is rapid, simple, and sensitive. The zopiclone is measured by ultraviolet detection with a limit of quantitation of 2.5 ng/mL. This value is adequate for clinical analysis. Boniface et al.[15] and Royer-Morrot et al.[14] determined zopiclone with a limit of quantitation of 4 ng/mL and 5 ng/mL, respectively.

A mixture of 0.067 M phosphate buffer, pH 7.95-acetonitrile (55:45, v/v) at a flow rate of 1 mL/min was found to be an appropriate mobile phase, allowing adequate and rapid separation of analyte and the I.S.. The retention times for zopiclone and diazepam were 2.60 and 4.65 min, respectively.

The selectivity of the chosen chromatographic system was also ascertained. Five blank plasma samples were processed and chromatographed and were found to contain no interfering peaks. The typical chromatogram of blank plasma is shown in Figure 2A.

The chromatogram of a plasma sample containing zopiclone, and the I.S. added from standard solutions, is shown in Figure 2B. The substances were eluted forming well shaped, symmetrical single peaks, well removed from the solvent front. Diazepam, chosen as an internal standard, was clearly separated from zopiclone, and the total analysis time was 6.0 min.

Over the concentration range 2.5-200.0 ng/mL, the relationship between the peak area ratios of zopiclone to the I.S. and the concentration of the drug

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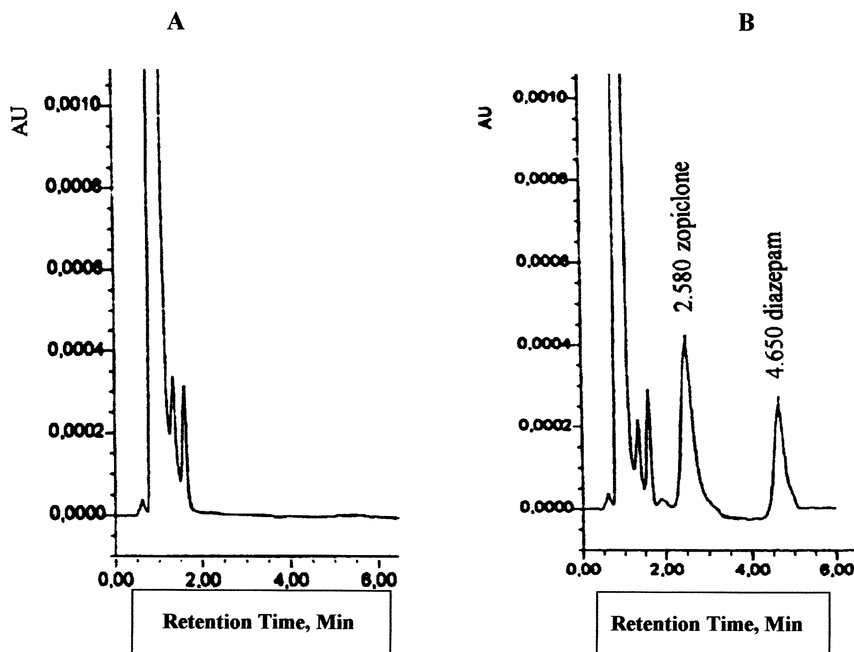

Figure 2. Typical chromatograms of extracted plasma samples. (A), drug-free plasma; (B), plasma sample containing 50 ng/mL of zopiclone and 500 ng/mL of diazepam (the internal standard).

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was linear. The regression equation for standard solutions was y = 0.0101x + 0.0047 (correlation coefficient, r = 0.9999), the regression equation for plasma samples was y = 0.0099x - 0.0002 (r = 0.9999), where y = peak area ratio of zopiclone to that of the I.S. and x = concentration of zopiclone, in ng/mL. The results indicate a good linear proportionality between the detector response and the concentration of zopiclone in plasma.

The following solvents were tested for protein precipitation: 6% perchloric acid (plasma solvent ratio 1:1), acetonitrile (1:3) and methanol (1:3). With acetonitrile, the recovery of zopiclone was quantitative and, therefore, it was chosen as the best solvent for protein precipitation.

PolyPure polypropylene membranes were successfully applied to the isolation of zopiclone and I.S. from plasma. They gave the best reproducibility and good recovery for both substances, i.e., zopiclone and I.S. The mean absolute recovery (± SD) over the tested range was 96.76 ± 1.89% (C.V.

1.95%),(n=7) and 98.26 ±0.75% (C.V. 0.76%),(n=6) for zopiclone and the I.S., respectively.

The intra- and inter-day precision are presented in Table 1. For intra-day precision five sets of samples (low, medium, and high concentration) were analysed on one day. Precision was better than 2%. For inter-day precision five sets of samples (three levels) were analysed on five separate days. The accuracy of the method, calculated by determining seven concentrations of zopiclone, is given in Table 2. The limit of quantitation was 2.5 ng/mL (S/N=10).

Some other drugs were tested for possible interference with the described zopiclone assay. Their retention times, under the same chromatographic conditions and UV detection wavelength as used for the zopiclone determination, are given in Table 3. Figure 3 shows a chromatogram obtained on a Nova-Pak C18 column with standard solution containing a mixture of some tested drugs.

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Table 1

Intra- and Inter-Day Validation of HPLC Method (Precision of the Method)

Number of Runs per Day	Amount Added (ng/mL)	Amount Found (Mean ± SD)[a] (ng/mL)	Coefficient of Variation (%)
Intra-Day Validation			
5	2.5	2.35 ± 0.041	1.74
5	50.0	48.45 ± 0.049	0.10
5	200.0	197.95 ± 0.038	0.02
Inter-Day Validation			
Number of Days			
5	2.5	2.40 ± 0.075	3.12
5	50.0	48.35 ± 0.105	0.22
5	200.0	198.15 ± 0.070	0.04

[a] n = 5.

Table 2

Accuracy of the Determination of Zopiclone in Plasma

Theoretical Concentration (ng/mL)	Concentration Found (Mean±SD, n=5)	Error (%)
2.5	2.35 ± 0.041	6.0
5.0	4.75 ± 0.052	5.0
25.0	23.95 ± 0.117	4.2
50.0	48.45± 0.049	3.1
100.0	98.65 ±0.069	1.3
150.0	147.00 ± 0.210	2.0
200.0	197.95 ± 0.038	1.0

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The advantages of the proposed method for the determination of zopiclone are its short analysis time and the simple procedure used for sample preparation.

In conclusion, the described method of determination of zopiclone in plasma is rapid, linear over a wide range, sensitive, and reproducible. With

Table 3

Drug Tested for Potential Assay Interference

Compound	Retention Time (Min)
Diazepam	4.65
Clonazepam	2.21
Lorazepam	2.11
Lormetazepam	3.06
Moclobemide	1.78
Nitrazepam	2.08
Oxazepam	2.03
Paroxetine	6.45
Temazepam	2.88
Zopiclone	2.60

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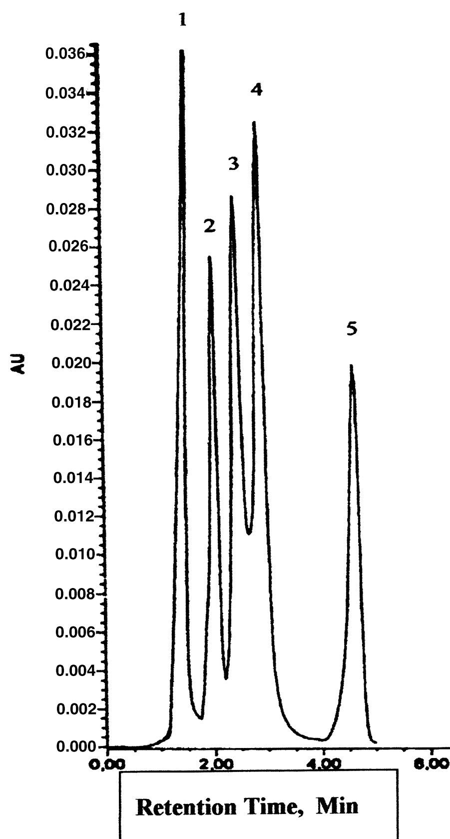

Figure 3. Chromatogram obtained on Nova-Pak C18 column with standard solution containing a mixture of drugs. Peaks: 1 = moclobemide; 2 = oxazepam; 3 = zopiclone; 4 = temazepam; 5 = diazepam.

regard to its analytical criteria, it is valuable for pharmacokinetic studies and for monitoring zopiclone.

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