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VALIDATION OF A GAS CHROMATOGRAPHY/MASS SPECTROMETRY METHOD FOR QUANTITATIVE DETERMINATION OF ZOLPIDEM IN WHOLE BLOOD

E. A. Krylova,[1] S. S. Kataev,[1] Yu. A. Khomov,[2] and O. N. Dvorskaya[2]

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Zolpidem was determined quantitatively in blood using a GC/MS method with an internal standard of imipramine hydrochloride. The multiple regression coefficient for the obtained calibration curve was $r = 0.999$. Metrological assessment of the method used eight statistical parameters. The extraction efficiencies of zolpidem at three concentrations were determined in model blood. Yields of 96 – 99% of the test compound were observed. The method was validated for specificity, detection limit (DL), limit of quantitation (LOQ), linearity, analytical range, accuracy, and precision (repeatability and intralaboratory precision). Statistically processed results were within acceptance limits. The DL for zolpidem in blood was 24 ng/mL; LOQ, 72.7 ng/mL.

Keywords: zolpidem, quantitative determination, validation.

Zolpidem is a soporific drug that has recently been widely used in the Russian Federation [1]. According to pharmacoepidemiological data supplied by researchers from various countries, the frequency of use of zolpidem to treat insomnia is constantly rising. Thus, Australian researchers noted that use of the drug practically doubled in 2003 – 2007 (from 11.3 to 20.3% relative to other soporifics) [2]. German researchers indicated that the number of introductory drug packages prescribed by physicians in 1993 – 2007 increased by 2.6 times [3]. Other researchers noted that zolpidem was second only to lorazepam for treating insomnia in Taiwan [4]. According to the USA National Outpatient Medical Service, zolpidem is the most frequently prescribed drug for insomnia among persons over 65 years old [5]. Because of the pharmacodynamics [interaction with benzodiazepine receptors subtype 1 (BZ_1)] [6] and pharmacokinetics (rapid attainment of the peak blood concentration, short elimination half-life) [7] of zolpidem in addition to potentiation of the sedative effect when combined with other psychotropic drugs, alcohol, and caffeine, zolpidem has been used more than once to commit illegal acts [8]. Instances of abuse of this drug with development of physical and psychological dependency were reported [9]. Therefore, identification of this drug in biological materials and quantitative analysis of its blood content are exceedingly important for chemical, toxicological, and forensic analysis. Methods for this purpose exist and use gas [10, 11] and liquid [12, 13] chromatography with various detection modes and immunoassay [14]. The use of one method or another depends on the technical and materials capabilities of the laboratory as influenced by traditional preferences. We previously developed and described a method for quantitative determination of zolpidem in whole blood that required solid-phase extraction (SPE) followed by gas chromatography and mass spectrometry (GC/MS) [15]. The present work addresses the validation of that method.

EXPERIMENTAL PART

GC/MS used an Agilent 7820 GC, an Agilent 5975 mass-selective detector (USA), an HP-5ms capillary column (30 m × 0.25 mm × 0.25 μm). SPE used a vacuum manifold with 12 ports (Supelco), an Air Cadet Vacuum/Pressure Pump (USA), and SampliQ Evidex cartridges (200 mg, 3 mL, Agilent, USA).

[1] Perm Regional Bureau of Forensic Medicine Examination, Perm, 614977 Russia.
[2] Perm State Pharmaceutical Academy, Ministry of Public Health of the RF, Perm, 614990 Russia.

Reagents used in the work included zolpidem tartrate (powdered substance, Spain, ND 42-13447-05), Melipramin (ampuls with imipramine hydrochloride, 12.5 mg/mL, Egis Pharmaceuticals), Relanium (2-mL ampuls with diazepam, 5 mg/mL, Polfa, Poland), and phosphate buffer, 1/15 M, pH 6.0. The organic solvents were CH_2Cl_2 (chemically pure, cp), *i*-PrOH (cp), 95% EtOH (cp), hexane (high purity, hp), EtOAc (cp), and 99.6% MeOH (cp). Other reagents included 25% NH_4OH solution (SP, XIIth Ed.), N_2 (hp), and He (hp).

Sample preparation. Blood samples (500 μL) were treated with imipramine hydrochloride internal standard (25 μL, 0.02 mg/mL) and phosphate buffer (3 mL, 1/15 M, pH 6.0) and centrifuged at 3,000 rpm for 10 min. Then, SPE was performed as follows. The sorbent was conditioned by rinsing successively with EtOH (95%, 2 mL) and phosphate buffer (2 mL, 1/15 M, pH 6.0). The blood analyte was loaded onto the sorbent at 1 mL/min followed by rinsing successively with phosphate buffer (1 mL, 1/15 M, pH 6.0), HOAc solution (1 mL, 0.1 M), and EtOH (1 mL, 10%). The cartridge was eluted initially by EtOAc:*n*-hexane (1:3) (2 × 2 mL) (eluate I) and then by MeOH (2 mL). The next elution into a separate tube used CH_2Cl_2:*i*-PrOH:NH_4OH (25%) (4:1:0.1) (2 × 2 mL) at 1 mL/min (eluate II). Eluate II was evaporated to dryness in a stream of N_2 at 60°C. The dry residue was treated with EtOAc (200 μL) containing internal standard diazepam (0.001 mg/mL). The resulting solution (1 μL) was injected into the GC/MS.

The GC/MS operating regime used He carrier gas (1.5 mL/min), split/splitless mode (flow division 15:1 with a starting delay of 1 min after sample injection), chromatograph vaporizer and detector interface temperatures of 300 and 280°C, respectively. The column temperature started at 70°C for 1 min with heating to 230°C at 40°C/min, heating to 300°C at 20°C/min, and holding constant at the final temperature for 2.5 min. Mass spectra were recorded with selected ion monitoring (SIM) for zolpidem ions with *m*/*z* 235, 307, and 219; imipramine, 234, 235, and 280 (internal standard); and diazepam, 256 and 283 (external standard). Chromatograms were processed in order to identify sample components using the ChemStation G1701DA program.

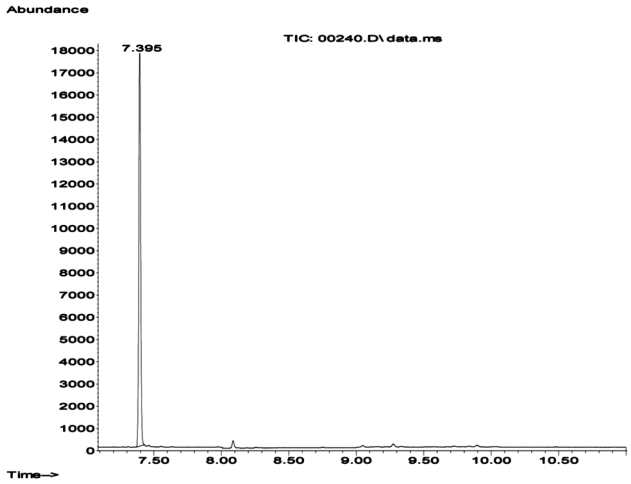

Fig. 1. General chromatogram obtained for blood without zolpidem (peak with retention time 7.395 min is for imipramine).

Calibration curve construction. A calibration curve for quantitative determination of zolpidem in blood was constructed by preparing and analyzing calibration standards. The standards were prepared by treating blank whole blood (0.5 mL) with standard solutions containing 20, 75, 125, 250, and 750 ng of zolpidem with internal standard imipramine

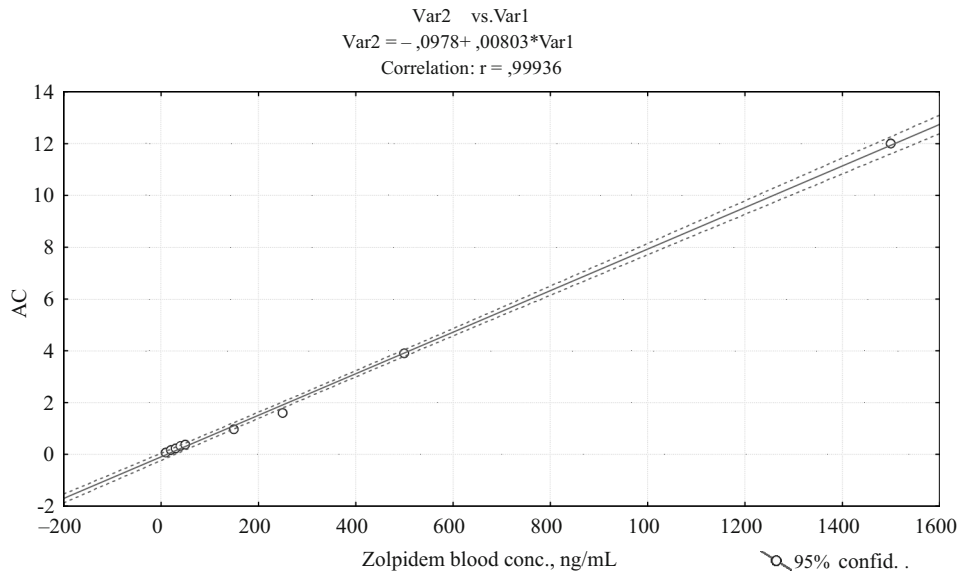

Fig. 2. Calibration curve with additional points corresponding to zolpidem blood concentrations near the detection limit.

TABLE 1. Metrological Assessment of Quantitative Determination Method for Zolpidem in Blood (n = 15, p = 95%)

μ, ng/mL	f	$\bar{x}$	s^2	s	$s_{\bar{x}}$, %	$t(P,f)$ table	$\Delta\bar{x}$	$\bar{\varepsilon}$	t_{calc}	δ, %
150	14	157.95	513.29	22.656	14.3	2.15	12.58	7.96	9.91	38.6
500		543.70	5967.56	77.250	14.2		42.88	7.89	2.19	8.74
1500		1577.52	34137.4	184.763	11.7		102.57	6.50	1.62	-

hydrochloride (500 ng). Two samples were analyzed for each concentration.

The method was validated according to State Pharmacopoeia XIIth Ed. [16]. Model blood samples with three zolpidem concentrations and three samples for each concentration were prepared for the validation.

RESULTS AND DISCUSSION

Metrological assessment of the method. Table 1 presents the quantitative determination results for zolpidem in the model blood samples using GC/MS where μ is the nominal zolpidem concentration; f, the number of degrees of freedom; s^2, the scatter; s, the standard deviation; $s_{\bar{x}}$, the relative standard deviation of the mean (variation coefficient) (%); $t(P, f)$ (table), the tabular Student criterion for a given probability level p and number of degrees of freedom f; $\Delta\bar{x}$, the half-width of the confidence interval of the mean; $\bar{\varepsilon}$, the relative error (uncertainty) of the mean (%); t_{calc}, the calculated Student criterion; and δ, the systematic error.

Due to the fact that $|\mu - \bar{x}| > 0$ for the tests at each zolpidem concentration, it was necessary to decide if systematic error was or was not present. For this, Student criteria were calculated using the formula:

$$t_{calc} = \frac{|\mu - \bar{x}|\sqrt{n}}{s}. \quad (1)$$

The resulting t_{calc} values were assessed for p = 95% and $f = n - 1 = 14$. The inequality $t_{calc} > t_{table}$ was true for model blood samples with concentrations 150 and 500 ng/mL. Therefore, the obtained results at these concentrations contained systematic errors, the magnitude δ of which was calculated using Eq. (2). Table 1 presents the results.

$$\delta = \frac{|\bar{x} - \mu|}{\mu} 100\ \%. \quad (2)$$

Extraction efficiency (yield) of zolpidem (*R*). Table 2 presents the results for SPE isolation of zolpidem from the studied blood samples.

Thus, zolpidem was nearly 100% extracted from the blood by using SPE.

The specificity of the method for zolpidem detection was determined using GC/MS as follows. Five blood samples from persons not receiving zolpidem beforehand were investigated. The samples were prepared by the aforementioned methods. Then, they were analyzed in random order by GC/MS both by scanning the total mass range 45 – 450 amu and in SIM mode. Characteristic ions with m/z 235, 307, and 219 in each obtained chromatogram were analyzed in portions corresponding to the zolpidem retention time ±5% [(15.40 ± 0.77) min for Scan mode and (9.73 ± 0.49) min for SIM mode]. Extraneous peaks with signal/noise ratio >3:1 were not observed in all chromatograms in this range of retention times. Thus, it was demonstrated that neither matrix components nor possible impurities distorted the quantitative determination results (Fig. 1).

TABLE 2. Extraction Efficiency of Zolpidem from Model Blood Samples

Sample No.	R, %		
	150 ng/mL	500 ng/mL	1500 ng/mL
1	100.212	94.81662	114.0456
2	91.2548	95.28679	82.71029
3	90.5392	102.3197	93.69478
4	104.6668	97.15002	102.6618
5	93.56654	91.95916	93.72303
6	98.97796	94.33791	106.0756
$\bar{R}$, %	96.54	95.98	98.82
s	5.63	3.53	11.05
$s_{\bar{x}}$, %	5.83	3.68	11.18

TABLE 3. Characteristics for Maintaining Linearity During Quantitative Determination of Zolpidem in Blood

Sample size, n = 5	Concentration of model blood sample, ng/mL				
	40	150	250	500	1500
AS magnitude	0.3126	0.9649	1.5986	3.8950	12.0080
Correlation coefficient, r			0.999		
Slope, b			0.00813		
Intersection with the ordinate, a			– 0.2129		
Residual sum of squared deviations			0.03106		

TABLE 4. Quantitative Determination of Zolpidem in Model Blood Samples for Confirmation of Accuracy

Parameter	Zolpidem sample concentration								
	150 ng/mL			500 ng/mL			1500 ng/mL		
Determination No.	1	2	3	1	2	3	1	2	3
Found zolpidem conc., ng/mL	148.42	161.18	150.34	520.94	501.80	487.88	1485.44	1511.86	1781.68
Average, $\bar{x}$, ng/mL		153.31			503.54			1592.99	
Systematic error, ng/mL	– 4.89	7.87	– 2.97	17.4	– 1.74	– 15.66	– 107.55	– 81.13	188.69
Standard deviation, s		6.880			16.599			163.941	
Variation coefficient, $s_{\bar{x}}$, %		4.5			3.3			10.3	
Student criterion (0.05, n^{-1})					4.30				
Half-width of confidence interval, ng/mL		17.08			41.21			407.00	
Confidence interval, ng/mL		54.71 ± 3.49			152.19 ± 29.82			391.20 ± 84.43	
Acceptance criterion	1.23	1.98	0.75	1.82	0.18	1.63	1.14	0.86	1.99

Detection limit (DL) and limit of quantitation (LOQ) for zolpidem in blood. The DL and LOQ of the quantitative determination method for zolpidem in blood were calculated using a calibration curve and the standard deviation of the analytical signal (AS).

The DL and LOQ for blood were validated by analyzing five model blood samples with concentrations presumably close to the DL (10, 20, 30, 40, and 50 ng/mL). Figure 2 graphs the resulting function of AS intensity vs. amount of zolpidem in blood, which obeyed the multiple regression equation:

$$y = 0.00803x - 0.0978. \quad (3)$$

Then, the DL and LOQ [18] of zolpidem in blood were calculated using the formulas:

$$\mathrm{DL} = 3.3\frac{S}{b};\ \mathrm{LOQ} = 10\frac{S}{b},$$

where S is the standard deviation of the AS; b, the sensitivity coefficient given as the ratio of the AS to the determined value (slope of the calibration curve). The DL and LOQ of zolpidem in blood were

TABLE 5. Quantitative Determination of Zolpidem in Model Blood Samples for Confirmation of Repeatability

Parameter	Zolpidem sample concentration, ng/mL								
	150			500			1500		
Determination No.	1	2	3	1	2	3	1	2	3
Found zolpidem conc., ng/mL	144.02	130.92	162.5	443.48	414.3	479.72	1573.28	1395.46	1452.32
Average, $\bar{x}$, ng/mL		145.81			445.83			1473.69	
Standard deviation, s		15.866			32.773			90.815	
Variation coefficient, $s_{\bar{x}}$, %		10.9			7.4			6.2	
Student criterion (0.05, n^{-1})					4.30				
Half-width of confidence interval, ng/mL		39.39			81.36			225.46	
Confidence interval, ng/mL		145.81 ± 39.39			445.83 ± 81.36			1473.69 ± 225.46	

TABLE 6. Quantitative Determination of Zolpidem in Model Blood Samples for Determination of Intralaboratory Precision

Zolpidem sample conc., ng/mL	Determination No.	Found zolpidem conc., ng/mL					Average, $\bar{x}$, ng/mL	Standard deviation, s	Variation coefficient, $s_{\bar{x}}$, %
		1 d	2 d	3 d	4 d	5 d			
150	1	150.22	144.02	148.42	182.2	136.98	157.95	22.656	14.3
	2	214.24	130.92	161.18	157.94	126.56			
	3	189.10	162.5	150.34	147.77	154.98			
500	1	561.32	443.48	520.94	647.54	581.02	543.70	77.250	14.2
	2	550.02	414.3	501.8	590.70	631.92			
	3	489.88	479.72	487.88	690.02	564.92			
1500	1	1178.32	1573.28	1485.44	1629.14	1517.02	1577.52	184.763	11.7
	2	1818.82	1395.46	1511.86	1636.44	1733.34			
	3	1912.56	1452.32	1781.68	1475.28	1561.84			

$$\mathrm{LOQ} = 10 \times \frac{0.0583905}{0.00803} = 72.7 \text{ ng/mL};$$

$$\mathrm{DL} = 3.3 \times \frac{0.0583905}{0.00803} = 24.0 \text{ ng/mL}.$$

Analytical range of the method and linearity. The linearity was studied using five zolpidem concentrations (in the range 40 – 1500 ng/mL) and GC/MS to determine zolpidem in model blood samples after SPE. The acceptance criterion was a correlation coefficient satisfying the condition $r \geq 0.99$. The linear regression correlation coefficient was 0.999 for statistical processing of the linear dependence of the obtained AS values[1] vs. zolpidem concentration according to Eq. (3). Based on the results (Table 3), it was confirmed that linearity was maintained in the range used for the method.The analytical range of the method could be established from the range of experimental data satisfying the linear model taking into account the LOQ. The analytical range of the zolpidem quantitative determination method to be validated corresponded to the concentration range 70 – 1500 ng/mL. The lowest zolpidem concentration was limited by the LOQ found for it.

Determination of the method accuracy. The accuracy of the analytical method for quantitative determination of zolpidem in blood was demonstrated over the whole useful range. Determinations were made by analyzing model blood samples with concentrations of 150, 500 and 1500 ng/mL. The calculated differences between the average and nominal values were assessed taking into account the corresponding confidence intervals of the mean. Table 4 presents the results from nine tests (three concentrations covering the working range with three determinations at each concentration).

[1] The AS was equal to the ratio of the peak area of the zolpidem fragment ion with *m*/*z* 235 to that of the internal standard (imipramine) fragment ion with *m*/*z* 234.

Thus, the results using the quantitative method for determining zolpidem in blood could be considered accurate because the found concentrations taken as the true ones fell within the confidence interval of the corresponding analytical mean obtained experimentally by the corresponding method. Furthermore, a method is considered accurate if its systematic error is insignificant, which occurs if the inequality $(d_i\sqrt{n})/s < t(P, f)$ is fulfilled [17]. In this instance, this value was significantly less (in the range 0.37 – 2.00) than the tabular value of the Student criterion (4.30 for confidence probability $p = 95\%$ and $f = n - 1$).

Determination of method precision (repeatability and intralaboratory precision). The repeatability (convergence) was confirmed by analyzing nine model blood samples with three replicates at each concentration. Samples were prepared and analyzed on the same day by the same operator on the same equipment. The acceptance criterion was a variation coefficient <15% [18].

Table 5 presents the determination results and the metrological characteristics and confirms the precision of the method at the repeatability level because the variation coefficient for all tests was 6.2 – 10.9%.

The intralaboratory precision over one factor (time) was determined by preparing and analyzing a series of samples over five days. Each day, nine model blood samples with zolpidem added at three concentrations, each of which was repeated three times, were tested.

Table 6 presents the results confirming the intralaboratory precision of the method for quantitative determination of zolpidem in blood. It can be seen that the variation coefficient at all concentrations was <15%.

The validation tests confirmed that the method for quantitative determination of zolpidem corresponded to established criteria and could reliably determine the content of this soporific in blood samples.

Thus, the proposed method for quantitative determination of zolpidem in blood corresponded to required accep-

tance criteria, was highly specific and sensitive, and could be used successfully to assess the intoxication level in toxicological and forensic medical practice.

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