DETECTION OF ULTRA-LOW CONCENTRATIONS OF BROMODIHYDROCHLOROPHENYLBENZODIAZEPINE (PHENAZEPAM) AND ITS METABOLITES IN BIOLOGICAL OBJECTS

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In extreme situations, reliable detection of the minimum therapeutic concentrations of psychotropic substances is important, since this allows one to provide adequate resuscitation. The group of benzodiazepine derivatives, which includes bromodihydrochlorophenylbenzodiazepine (phenazepam), is widely used in clinical practice. Along with the positive clinical effect, phenazepam has numerous side effects, capable of causing poisoning, even death. The study was aimed to develop the method for detection of the phenazepam metabolites by high-resolution HPLC–TMS suitable for achieving the aims and objectives of forensic medical expertise in case of the ultra-low urine substance concentrations. Urine of six patients (males and females aged 28–40), who were prescribed phenazepam and took the drug at minimum therapeutic concentrations on an ad hoc basis, was used during the study. Optimum conditions for the analyte chromatography after the urine sample preparation were defined with the phenazepam retention time of 7.05 ± 0.06 min; specific ions (m/z) 179, 183, 206, 242, 271, 285, 320, 348 (main) were defined for identification of phenazepam.

Keywords: phenazepam, bromodihydrochlorophenylbenzodiazepine, 3-hydroxyphenazepam, high-performance liquid chromatography, forensic chemistry research, chemical toxicological analysis, urine

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Compliance with ethical standards: the study was planned and conducted in accordance with the requirements of the Declaration of Helsinki of the World Medical Association (2000) and subsequent revisions thereto; the informed consent was submitted by all study participants.

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ОБНАРУЖЕНИЕ БРОМДИГИДРОХЛОРФЕНИЛБЕНЗОДИАЗЕПИНА (ФЕНАЗЕПАМА) И ЕГО МЕТАБОЛИТА В БИОЛОГИЧЕСКОМ ОБЪЕКТЕ ПРИ СВЕРХНИЗКИХ КОНЦЕНТРАЦИЯХ

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В экстремальных ситуациях важна роль достоверного обнаружения психотропных веществ при минимальных терапевтических концентрациях, что позволит проводить адекватную реанимационную терапию. В медицинской практике широко используют группу производных бензодиазепинов, среди которых бромдигидрохлорфенилбензодиазепин (феназепам). Помимо положительного клинического эффекта феназепам обладает большим числом побочных эффектов, способных привести к отравлениям вплоть до летального исхода. Целью исследования было разработать методику обнаружения метаболитов феназепама методом ВЭЖХ-ТМС высокого разрешения для целей и задач судебно-медицинской экспертизы при наличии сверхнизких концентраций в моче. В исследовании использовали мочу шести пациентов (мужчин и женщин в возрасте 28–40 лет), принимавших феназепам в минимальных терапевтических концентрациях в разовых случаях, по назначению врача. По результатам исследования разработаны оптимальные условия хроматографирования аналитов после пробоподготовки мочи, с временем удерживания феназепама 7,05 ± 0,06 мин, выявлены характерные ионы (m/z) 179, 183, 206, 242, 271, 285, 320, 348 (основной) для идентификации феназепама.

Ключевые слова: феназепам, бромдигидрохлорфенилбензодиазепин, 3-гидроксифеназепам, высокоэффективная жидкостная хроматография, судебно-химическое исследование, химико-токсикологический анализ, моча

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Соблюдение этических стандартов: исследование спланировано и проведено с соблюдением требований Хельсинкской декларации Всемирной медицинской ассоциации (2000 г.) и последующих ее пересмотров; все участники подписали добровольное информированное согласие на участие в исследовании.

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In extreme situations, reliable detection of the minimum therapeutic concentrations of psychotropic substances is important, since this allows one to provide adequate resuscitation. Currently, benzodiazepine derivatives are among the most common psychotropic medications used in patients with anxiety disorders and other psychosomatic disorders. Bromodihydrochlorophenylbenzodiazepine (phenazepam) is the most potent benzodiazepine commonly used in medical practice [1–5]. Along with the positive clinical effect it has numerous side effects [6], which, in case of inappropriate medication use or non-medical use, may result in poisoning and even be fatal [7–9].

Bromodihydrochlorophenylbenzodiazepine is a benzodiazepine anxiolytic agent (tranquilizer) possessing anxiolytic, sedative-hypnotic, anticonvulsant and central muscle relaxant effects. The chemical name is 7-bromo-5-(orthochlorophenyl)-2,3-dihydro-1H-1,4-benzodiazepin-2-one; the molecular formula is $C_{15}H_{10}BrClN_2O$; in the Russian Federation, the compound is known mostly under the brand name of "Phenazepam". The dosage forms are 0.5, 1.0 and 2.5 mg tablets [10], and the 1 mg/mL solution, 0.5 and 1.0 mL [11]. Bromodihydrochlorop henylbenzodiazepine is a prescription drug (prescription form № 148–1/u-88). As a potent substance, in 2021 it became a controlled drug. To phenazepam, the risk of substance abuse and withdrawal syndrome remains rather high, which is the cause of the poisoning cases. Side effects, such as hallucinations, euphoria, etc., define the abuse potential of the drug [1, 9].

Phenazepam is used in small quantities because of its huge therapeutic effect. The possibility of the effect manifold potentiation by other substances entails low concentrations of the substance in the body's biological objects. Non-medical use, combinations with other psychotropic medications or alkohol, and side effects may result in acute or fatal phenazepam intoxication, that is why development of the method for detection and identification of the ultra-low substance concentrations in biological objects using the advanced techniques still remains an urgent challenge posed by forensic chemistry and chemical toxicology studies. Simultaneous detection of the phenazepam metabolites and native substance (bromodihydrochlorop henylbenzodiazepine) makes it possible to actually verify the substance use by patient/victim and confirm the lack of false positive result in case of falsification in the form of adding the substance to the biological object. The most common non-invasive biological object used in medical laboratories (including the chemical toxicology laboratories) is the biological fluid, urine [12, 13].

Today, high performance liquid chromatography–tandem mass spectrometry (HPLC–TMS) is successfully used as a reliable, selective and sensitive method for screening, identification and quantification of small molecules [14–16]. The study was aimed to develop the method for detection of phenazepam and its major metabolite by high-resolution (HR) HPLC–TMS based on the Orbitrap technology suitable for achieving the aims and objectives of forensic medical expertise in case of the ultra-low substance concentration in the biological object (urine).

METHODS

Urine samples were collected from patients (n = 6) in the morning on an empty stomach 8 ± 1 h after administration of the therapeutic concentrations of phenazepam (in tablets of 1 mg), prescribed due to psychosomatic disorder. Inclusion criteria: initial medication administration allowing to avoid deposition and concentration increase in the human body. Exclusion criteria: patients with comorbidities currently taking psychotropic medications; kidney disease; age over 45 or under 25. The average age of both male and female patients was 34 ± 6 years. The samples of biological fluid were collected anonymously using the non-invasive procedure on a voluntary basis, with the consent of the patient.

Bromodihydrochlorophenylbenzodiazepine, the active ingredient of phenazepam (LP-005121-191018), was assessed after purification of excipients. The working standards (WS) were prepared: alcohol solution containing 1 mg/mL of the test substance.

The following was used for assessment: high-resolution HPLC–TMS and Orbitrap technology [15, 16]; Orbitrap Exploris 120 mass spectrometer (ThermoFisher Scientific; USA), being the OrbitrapTM standalone unit with the atmospheric pressure chemical ionization (APCI) source for high-performance liquid chromatography–mass spectrometry.

Because of the phenazepam side effects being rather intense for benzodiazepine derivatives when referred to the forensic medical examination in order to perform chemical toxicological analysis, sample preparation was performed in accordance with the generally accepted procedure, developed for benzodiazepines derivatives. Phenazepam was isolated in two ways: 1) with no hydrolysis: 5 mL of native urine were alkalinized to pH 10 by adding sodium hydroxide (3–6 mL); 2) byhydrochloric acid hydrolysis and subsequent liquid–liquid extraction: 5 mL of concentrated hydrochloric acid were added to 5 mL of the first-void urine and heated in the closed tube in a boiling water bath for an hour (acid hydrolysis). Then the solution was neutralized by alkalinization with 60% sodium hydroxide to pH 10. Both options involved the subsequent chloroform extraction repeated two times: 10 mL in the separatory funnel, manually shaken moderately for 3 min. After settling chloroform was poured into the evaporating dish and evaporated to dryness on a water bath; solid residue was dissolved in 0.5 mL of acetonitrile and subjected to assessment.

Chromatographic conditions, HR HPLC–TMS

Thermo Scientific Xcalibur 4.4 software (Thermo Scientific; USA), TF Accucore PhenylHexyl column (100 × 2.1 mm, 2.6 mm) with the column temperature of 30 °C were used.

Table 1. Mobile phase gradient

Time, min	Liquid phase flow rate, mL/min	Mobile phase A, %	Mobile phase B, %
0	0.5	99.0	1.0
1.0	0.5	99.0	1.0
10.0	0.5	1.0	99.0
11.5	0.5	1.0	99.0
12.0	0.5	99.0	1.0
13.5	0.5	99.0	1.0

Table 2. Results of urine analysis by HR HPLC-TMS

Substance	Elemental composition	Theoretical mass of protonated ion, m/z	Experimental mass of protonated ion, m/z	Retention time, min (n = 6)	Statistical parameters of retention time*
Phenazepam	$C_{15}H_{10}BrClN_2O$	349.61	348.97	a = 7.05 (6.98; 7.05; 6.95; 7.15; 7.06; 7.11)	σ^2 = 0.00572; σ = 0.07563; V = 1.07 %; A/m_a = –0.07035; E/m_e = –2.14563; ā = 0.05667
3-hydroxyphenazepam	$C_{15}H_{10}BrClN_2O_2$	365.61	364.97	a = 6.77 (6.65; 6.88; 6.77; 6.85; 6.70; 6.77)	σ^2 = 0.00756; σ = 0.08695; V = 1.28 %; A/m_a = –0.08377; E/m_e = –2.12896; ā = 0.06333

Note: σ^2 — variance, σ — standard deviation; V — coefficient of variation; A/m_a — ratio of skewness to its error; E/m_e — ratio of kurtosis to its error; ā — mean deviation.

The combined mobile phase was previously prepared. To increase the speed of analysis and reduce the width of chromatographic peaks we used different gradients altering the composition of mobile phase and changed the flow rate of the delivered mobile phase. Mobile phase in the gradient mode: mobile phase A — 2 mM ammonium formate with 0.1% formic acid in water (pH 3.0), mobile phase B — 2 mM ammonium formate with 0.1% formic acid in the acetonitrile methanol mixture (1 : 1). The flow rate was 0.5 mL/min. The gradient mode details are provided in Table 1.

Detection was performed in the data-dependent acquisition mode. The full scan–ddMS2 mode was as follows: scan range 100–1000 m/z; RF Lens 50%, high resolution data acquisition (Orbitrap, 120,000 FWHM). Automatic scan range mode. Automatic detection of the ion injection time. Intensity threshold for fragmentation 2000. Peak detection 30%, isolation window 1 m/z, stepped collisional energy mode, absolute collision energy. Higher–energy collisional dissociation (HCD) of 15, 30, 45%. Fragment resolution of 30,000. Detection windows were adjusted by routine laboratory method in order to optimize the conditions of the qualitative and quantitative analysis. Ion exclusion mode was used after acquisition of one spectrum in 3 s.

The system was fitted with the ion source, the following settings were used: H-ESI ion source; positive-ion electrospray voltage of 3500 V; negative-ion electrospray voltage of 2500 V. Nebulizer gas — nitrogen 50 AU, auxiliary gas — nitrogen 13 AU. Capillary temperature — 280 °C; evaporation source temperature — 350 °C. Built-in mass calibration EASY-IC™ (fluoranthrene).

After cromatography, native compound, phenazepam, and its metabolite were identified using the mass spectral libraries: TRACEFINDER 5.1 SP1; TOXFINDER 1.0; EFS_HRAM_Compound_Database; Toxicology_HRAM_Compound_Database; Thermo Scientific™ mzVault HRAM MS/MS spectral library; COMPOUND DISCOVERER 3.1; MzCloud.

Student's t-test was used for statistical data processing. The differences were considered significant at $p < 0.05$.

RESULTS

The use of the selected scan mode made it possible to reach maximum sensitivity (due to the lack of collisional losses), which was essential for evaluation of the temporal detection windows, since the method, optimized for fast separation, could yield false-negative results due to low scan speed, whereas the significantly increased scan speed could result in the significantly reduced signal intensity, which was very important for detection of the low (therapeutic) concentrations of metabolites in biological fluids (urine). Chromatographic conditions, i.e. the mobile phase gradient combined with the temperature made it possible to reduce the quantity of compounds co-eluted from urine, and ensured reliable results (Fig. 1 and 3).

When performing isolation after hydrolisis, only the peak of phenazepam was observed (no 2-amino-5-bromo-2'-chlorobenzophenone was detected); peaks of phenazepam and 3-hydroxyphenazepam were observed when using no hydrolisis.

Phenazepam and its metabolite 3-hydroxyphenazepam were identified on the chromatograms using the selected ion approach. Detection was performed based on the retention time values with subsequent identification following the analysis of specific ions on the mass spectra of these compounds. Statistical analysis of the retention time values is provided in Table 2.

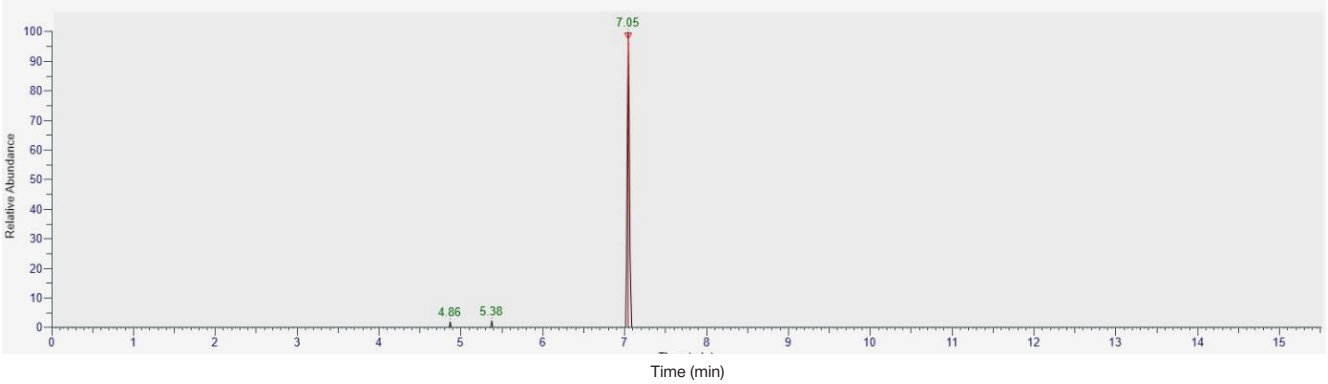

Fig. 1. Selected ion chromatogram (m/z 348) of the chloroform extract of urine containing phenazepam

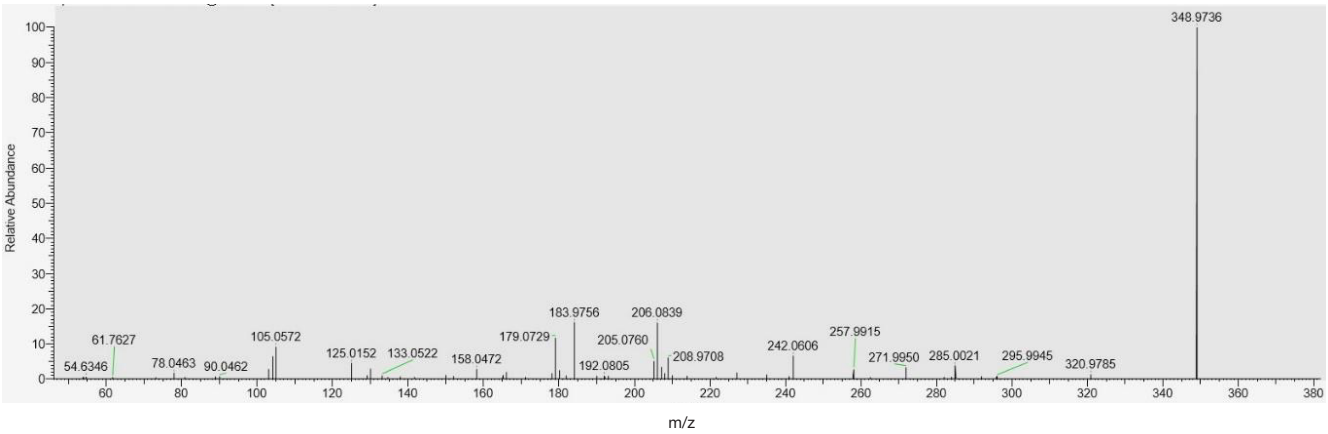

Fig. 2. Mass spectrum of phenazepam

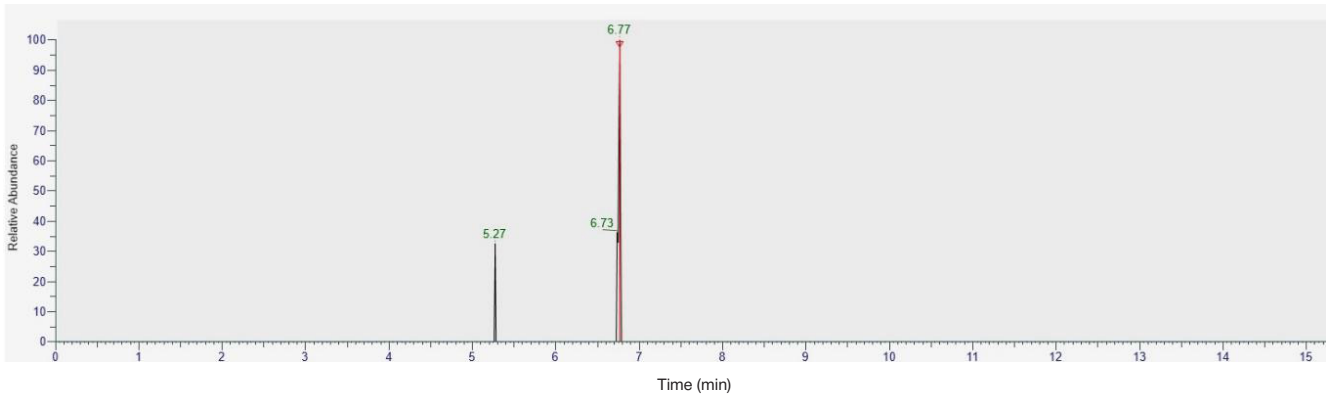

Fig. 3. Selected ion chromatogram (m/z 348) of the chloroform extract of urine containing 3-hydroxyphenazepam

In the body, phenazepam is metabolized to 3-hydroxyphenazepam. The percentage of native phenazepam excreted from the body is 47–89%, that is why identification is possible, and it is recommended to perform identification based on phenazepam.

Along with retention time, spectral characteristics of the test substances are considered a significant factor when performing identification (Fig. 2).

Statistical data processing and validation study (Table 3) were performed using the following parameters: analyte carryover assessment, determination of interference effects, ionization suppression/enhancement [17]. Parameters of validation characteristics met the acceptance criteria.

DISCUSSION

The acquired full range spectra of the test substances with the dissociated mass spectra of ions specific to the molecular fragments in various functional groups make it possible to distinguish ions specific to phenazepam: 179, 183, 206, 242, 271, 285, 320, 348 (main) m/z.

According to the data presented in Table 3, a specific ion could be distinguished for each test compound, however, identification requires the use of at least five. During the study it is possible to perform analysis in the selected ion monitoring (SIM) mode using the selected ions. Ion at m/z 348 is the most intense ion in the mass spectra of phenazepam. It should be noted that ions of the “non-informative” range (i.e. below 150 AMU) are present in both test compounds, that is why identification results are heavily influenced by the components of the studied sample matrix and the “background” of the chromatography column. Therefore, it is recommended to use optimum data exceeding 150 AMU.

CONCLUSIONS

The method for identification of phenazepam in urine extracts by HR HPLC-TMS using the Orbitrap technology, allowing one to detect the ultra-low concentrations of phenazepam in the human biological objects (such as urine), has been developed, to be used in forensic chemistry and chemical toxicology studies. The retention time of the metabolite 3-hydroxyphenazepam after oral administration have been discovered and determined, specific ions to be used for identification have been defined. The method has been validated using the following parameters: analyte carryover assessment, determination of interference effects, ionization suppression/enhancement; according to these parameters, the method meets the acceptance criteria.

Table 3. Assessment of the parameters of validation characteristics used for identification of phenazepam and its metabolite by HR HPLC-TMS

Parameter	Result
Analyte carryover assessment	No analyte carryover revealed for 10 ng/mL
Determination of interference effects	No interference effects in the group of medications — metabolites of other benzodiazepine receptor agonists (clobazam, zaleplon, phenazepam)
Ionization suppression/enhancement	from –4,1 to 1,1% RSD < 11%

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