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Chemical and Toxicological Diagnosis of Acute Poisonings with Phenazepam

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BACKGROUND The relative availability of Phenazepam makes it a frequent cause of overdose, suicide and non-medical use. At the same time, it remains insufficiently studied in chemical and toxicological terms.

THE AIM OF STUDY to create an accessible, rapid method for detecting Phenazepam in biological matrices of patients with acute poisoning.

MATERIALS AND METHODS We used thin-layer chromatography (TLC), gas chromatography with a mass selective detector (GC-MS), high performance liquid chromatography with a tandem mass-selective detector (LC-MS/MS) and immunochromatographic analysis (ICA). The preparation of samples of intact urine with the addition of standard solutions of Phenazepam and real urine samples of patients with acute poisoning with Phenazepam was carried out using liquid-liquid extraction or precipitation of related components of the sample with acetonitrile. Hydrolysis and derivatization were also added in GC-MS analysis.

RESULTS The analysis of statistics of the Department of Acute Poisonings of the N.V. Sklifosovsky Research Institute for Emergency Medicine in 2014–2016 showed that Phenazepam poisonings averaged 9.2% of the total number of admissions and mainly occurred as suicidal attempts. A technique has been developed for the detection of Phenazepam by TLC, which gives more objective results than ICA. For confirmatory analysis, it is advisable to use LC-MS/MS method for the native substance and GC-MS for the products of hydrolysis after derivatization. Compared to confirmatory methods, the developed TLC-screening technique is expressive, does not require the use of expensive high-tech equipment, difficult sample preparation, and makes it possible to reliably detect toxic and lethal concentrations of Phenazepam.

Key words: phenazepam, acute poisoning, thin-layer chromatography, immune analysis, HPLC-MS/MS, GC-MS

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GC-MS – gas chromatography mass-spectrometry
ICA – immune chromatographic analysis
HPLC-MS/MS – high performance liquid chromatography with tandem mass spectrometry
TLC – thin-layer chromatography

INTRODUCTION

The popularity of benzodiazepine derivatives is associated with their relatively low toxicity [1]. However, it has now been found that their long-term use can lead to the development of tolerance, as well as cognitive disorders and drug addiction [1]. However, there are cases of taking drugs derivatives of benzodiazepine in order to obtain euphoria or relieve withdrawal symptoms in the absence of ability to take an addictive substance in time [2]. The most common in the Russian Federation is the use of the domestic drug of this group Phenazepam. The relative availability makes it a frequent cause of overdose, suicide, and non-medical use, which is a concern for the Ministry of Health [3].

Phenazepam remains poorly studied in chemical-toxicological terms. It can be detected by screening with the thin-layer chromatography (TLC) [4, 5]. In these works, the proposed mobile phases and developers are not selective for the detection of Phenazepam. Immune and chemical methods, in particular, immune chromatographic analysis (ICA), are actively used for screening 1,4-benzodiazepine derivatives in biological samples for acute poisoning [6–8]. The advantages of this group of methods include high sensitivity, however, the number of false-positive results caused by cross-reactivity with other toxicants is high, and false-negative results are also encountered. A significant disadvantage of ICA is the lack of reliable information about the sensitivity and selectivity of the method when detecting Phenazepam, as it is a domestic drug that does not appear in foreign studies on the analysis of poisoning with 1,4-benzodiazepine derivatives, and the relevant data in Russian sources is not enough. Gas chromatography with mass spectrometry (GC-MS) [9–11] and high-performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS) have been proposed for confirmatory chemical toxicological analysis of poisonings with derivatives of 1,4-benzodiazepine [7, 8, 11–13].

The aim of study is to create an accessible rapid procedure for detecting Phenazepam in biological fluids of patients with acute poisoning.

MATERIAL AND METHODS

For the preparation of the original standard solution of Phenazepam in ethanol (10 mg/ml), the substance-powder of Phenazepam (Usolye-Siberian CPP, purity 99.0%) was used. The resulting solution was used then for preparation of working solutions and samples with concentrations of 100 μg/ml, 10 μg/ml, 1 μg/ml, 0.5 μg/ml, 50 ng/ml and 20 ng/ml.

For preliminary testing of urine samples of patients, immune chromatographic strips "ImmunoChrom BENZODIAZEPINE Express" were used. Series 136071. Sensitivity 300 ng/ml.

Thin layer chromatography was performed on *Macherey–Nagel POLYGRAM®SIL G/UV*$_{254}$ plates. The following mobile phases were compared: benzene, benzene – ethyl acetate (3:1), toluene – acetone – ethanol – 25% ammonia (45:45:7.5:2.5) and ethyl acetate – toluene – acetone (20:40:10). The hydrolysis of Phenazepam was carried out on a plate under the action of 2H hydrochloric acid or a solution of concentrated sulfuric acid in ethanol (1:1) at a temperature of 130° C. The hydrolysis time varied from 10 to 30 minutes. Ultraviolet radiation and the azo dye formation reaction were used for detection, including sequential application to the plate of a 0.1% sodium nitrite solution and either a 2% alkaline ß-naphthol solution or a 0.1% (N-1-naphthyl)-ethylenediamine dihydrochloride solution [14, 15].

GC-MS and/or HPLC-MS/MS were used as confirmatory research methods.

Analysis conditions by GC-MS method: *ThermoTraceGCUltra* gas chromatograph with a *DSQ* II mass spectrometric detector. Column *TR*-5 *MS*, length 30 m, internal diameter 0.25 mm, the film thickness of the stationary liquid phase 0.25 microns. The carrier gas is helium. Column temperature program: 50° C — 3 min, heating 100° C/min to 100° C, 100° C — one min, heating 15° C/min to 280° C, 280° C — 20 min. Injector temperature — 220 ° C. Detection is based on the total ion current in the *m/z* range of 45–650, and ionization is produced by an electron impact with an energy of 70 *eV* .

The method for detecting Phenazepam in the urine using the HPLC-MS/MS method was developed using the algorythm described in studies on detecting 1,4-benzodiazepine derivatives in biological objects by this method [8, 12, 13, 16]. Analysis conditions by HPLC-MS/MS: *Nexera* high performance liquid chromatograph with a tandem mass spectrometer *LCMS*-8040 (*QQQ*), *Shimadzu* (Japan). Column: *Waters Xbridge C* 18 50x4.6; 3.5 microns. Mobile phase: eluent *A* — deionized water (electrical resistance 18.2 MOhm · cm) with the addition of 0.1% formic acid (*Fluka*, *for mass-spectrometry*), eluent *B* — acetonitrile (*Panreac, supergradient for UPLC*) with the addition of 0.1% formic acid. Elution was performed in a gradient mode. Ionization by the electrospray method in a positive mode, spraying gas (nitrogen) — 3 l/min, drying gas (nitrogen) — 20 l/min, heating unit temperature — 400° C, desolvation line temperature — 200° C, capillary voltage — 5 kW. The detection of Phenazepam was conducted in multiple reaction monitoring mode (*MRM*$^+$): *m/z* 351.10→*m/z* 185.90 и *m/z* 351.10→ *m/z* 206.10. Conditions for the detection of Phenazepam were selected experimentally.

During the sample preparation, chloroform, ethyl acetate, diethyl ether and acetonitrile were used. All solvents and reagents used were "c.p." (chemically pure).

Sample preparation in the analysis by TLC. Ten ml of the urine sample or model sample was made alkaline with 10% ammonia solution to pH 8–9 and extracted with two portions of chloroform 10 ml each. The combined chloroform extracts were evaporated. The dry residue was redissolved in 0.3 ml of chloroform and 5 μl of the resulting solution were applied onto the starting line of plate.

To assess the sensitivity and objectification of semi-quantitative determination of Phenazepam using the presented TLC method, extracts from model samples were used, obtained by adding to the intact urine of working standard solutions of Phenazepam to get concentrations of 100 μg/ml, 10 μg/ml, 1 μg/ml, 0.5 μg/ml. Sample preparation and analysis of model samples were performed by the same method as in the case of the study of urine samples of patients.

Sample preparation for GC-MS when determining the native Phenazepam. We added 1 g of sodium chloride, 50 μl of 25% ammonia solution, 50 μl of diphenylamine solution (internal standard at a concentration of 100 μg/ml) and 2.5 ml of a mixture of ethyl acetate - diethyl ether (1:1) to 3 ml of urine. Then, it was extracted for 10 min with stirring in a shaker, then the layers were separated by centrifugation for 1 min at a speed of 3,500 rpm. The organic layer was transferred to glass vials, evaporated to dryness under vacuum, and the residue was redissolved in 100 μl of ethyl acetate. Two μl of the resulting solution were put into the chromatograph .

Sample preparation for GC-MS when determining Phenazepam by the product of hydrolysis — 2-amino-2-bromo-5-chlorobenzophenone. We added 0.5 ml of concentrated hydrochloric acid into 3 ml of urine and placed it into a thermostat for 30 minutes at 120° C to carry out the hydrolysis of Phenazepam. One gramm of sodium chloride was added to 3 ml of hydrolyzate placed in a glass vial, the pH was adjusted to 8–10 with 25% ammonium hydroxide solution and extracted with 2.5 ml of ethyl acetate – methylene chloride – isopropanol (6:3:1). The layers were separated by centrifugation (10 min, 3,000 rpm/min). The upper layer (2 ml) was removed and evaporated to dryness under a stream of nitrogen. Then, 50 μl were added to the dry residue *MBTFA* (N-methyl-bis(trifluoroacetamide), incubated at 120° C for 30 min and cooled to room temperature. The residue was redissolved in 50 μl of ethyl acetate. The volume of the sample was 2 μl.

Sample preparation for HPLC-MS/MS. We added 900 μl of acetonitrile to 300 μl of urine or model sample of Phenazepam in intact urine with a concentration of 50 ng/ml and 20 ng/ml, mixed by a vortex- shaker, and

centrifuged at 15,000 rpm for 15 minutes. Then, 2 µl of a supernatant fluid were put into the chromatograph depending on a preliminary assessment of the concentration of Phenazepam.

RESULTS AND DISCUSSION

The analysis of case histories of patients of the Department of Acute Poisoning Treatment of the N.V. Sklifosovsky Research Institute for Emergency Medicine in 2014–2016 showed that Phenazepam poisoning averaged 9.2% of the total intake. In most cases, the cause of the poisoning was a suicidal attempt (94% in 2014, 98% in 2015, 92.5% in 2016). Approximately with the same frequency, Phenazepam was taken for the purpose of intoxication (~ 2.7%) and self-treatment (~ 2.3%). About half of patients with Phenazepam poisoning were intoxicated. In 8.5% of cases, Phenazepam was taken together with other drugs. The most common were drugs such as Amitriptyline (12.5%), Corvalol (12.5%), Donormil (10.2%) and Carbamazepine (7%).

The findings confirm the relevance of a more detailed study of acute poisoning with Phenazepam and indicate the need to improve methods of detecting it in the biological fluids of patients.

The analysis of Phenazepam by TLC was performed in the systems listed in Table 1, which also presents the corresponding values of the mobility coefficients (R_f) and the chromatography times established during the experiment.

Table 1

R_f and time of Phenazepam chromatographic procedure in various solvent systems

	A	B	C	D
Mobile phase composition	Toluene-acetone-ethanol-25% ammonia solution (45:45:7.5:2.5)	Ethyl acetate-toluene-acetone (20:40:10)	Benzene	Benzene-ethyl acetate (3:1)
R_f	0.76	0.48	0.43	0.25
Time of chromatographic procedure, min	6	8	6	10

As can be seen from the Table 1, the use of the toluene – acetone – ethanol – 25% ammonia solution (45:45:7.5:2.5) is optimal for the detection of Phenazepam. This mobile phase is a common solvent system for basic substances [14]. Its use in a non-directional chemical-toxicological study allows to identify Phenazepam along with other substances, without changing the general course of the study. Chromatography time of 6 minutes satisfies the express requirement of the analysis. Benzene with the same chromatography time is a particular system for the directional detection of 1,4-benzodiazepine derivatives [14], which limits its scope.

The chromatographic zone of Phenazepam was manifested by yellow-green fluorescence in UV light at a wavelength of 254 nm, which was enhanced after the plate was treated with a mixture of concentrated sulfuric acid and alcohol (1:1) due to the partial formation of 2-amino-2-bromo-5-chlorobenzophenone, which increased the detection sensitivity [15].

The intensity of the staining area of Phenazepam after hydrolysis, carried out for 10, 15 and 30 min, did not differ. Thus, the time of hydrolysis may be reduced to 10 minutes without loss of sensitivity, which is essential in the conditions of the rapid diagnosis of acute poisoning.

After the hydrolysis, the azo dye was formed by sequentially dripping a 0.1% solution of sodium nitrite and an alkaline solution of ß naphthol or a 0.1% solution of (N-1-naphthyl)-ethylenediamine dihydrochloride. Depending on the reagent used, Phenazepam formed an orange or crimson azo dye, respectively. The use of sulfuric acid in alcohol for hydrolysis and (N-1-naphthyl)-ethylenediamine dihydrochloride for the formation of azo dye allowed Phenazepam to be detected in a model mixture with a concentration of 1 µg/ml, while the result of detection using α-naphthol could be questioned. When analyzing the intact urine samples according to the indicated method, no chromatographic spots were observed on the chromatogram corresponding to Phenazepam for the *Rf* value and color, which confirms the selectivity of the technique.

It should be noted that when analyzing samples with a concentration of 0.5 µg/ml, the zone of Phenazepam did not appear in both variants of the reaction. Therefore, the concentration of Phenazepam 1 µg/ml can be considered as the detection limit for this technique.

The study of samples by the described method of detection of Phenazepam was not complicated by the presence of other toxicants in the sample. Native Phenazepam detection only by fluorescence in UV light is difficult if such substances as chlorprothixen and carbamazepine are present in the sample, as they have their own fluorescence and close R_f values (0.60–0.70), so they can mask the Phenazepam stain. Conducting on-plate hydrolysis and the formation of azo dye with a solution (N-1-naphthyl)-ethylenediamine dihydrochloride allowed Phenazepam to be reliably differentiated.

This technique was used in the study of urine samples of 13 patients with suspected poisoning with Phenazepam. The results are shown in Table 2. In addition to TLC, in these cases, screening was performed by ICA, GC-MS and HPLC-MS/MS were used to confirm the detection of Phenazepam.

Table 2

The results of the biological samples study in patients with suspected poisoning with Phenazepam

	Detection of Phenazepam in the urine	Detection of other substances

	ICA	TLC	GC-LS of intact material	GC-LS of hydrolysis products	LC-MS/MS	Ethanol in blood	Ethanol in urine	Other substances
Patient 1	not found	found	-	-	found	not found	not found	barbiturates
Patient 2	not found	found	not found	found	-	1.77	3.08	
Patient 3	found	found	found	-	found	not found	not found	barbiturates
Patient 4	found	found	-	-	found	not found	not found	
Patient 5	not found	found	not found	found	-	not found	not found	
Patient 6	found	found	not found	found	-	not found	not found	barbiturates, amphetamine
Patient 7	found	found	-	-	found	not found	not found	phenothiazines
Patient 8	not found	found	-	-	found	not found	not found	
Patient 9	not found	not found	-	-	found	2.15	4.55	
Patient 10	not found	not found	not found	found	found	2.03	2.83	
Patient 11	not found	not found	not found	not found	-	1.65	3.61	
Patient 12	not found	not found	-	not found	-	not found	not found	barbiturates
Patient 13	not found	not found	-	not found	-	not found	not found	barbiturates

Notes: "-" — not performed; GS-MS — gas chromatography with a mass selective detector; HPLC-MS/MS — high performance chromatography with a mass-selective detector; ICA — immune-chromatographic analysis; TLS — thin-layer chromatography

Immune and chemical tests, despite their high sensitivity and group selectivity for 1,4-benzodiazepine derivatives [6–8], are not always able to give a positive result when Phenazepam is detected. Of the 13 samples analyzed, the ICA result was false-negative in 6 cases, while the TLC result was false-negative only in 2 cases. In the two samples indicated, Phenazepam was detected only by the HPLC-MS/MS method. The "trace" amounts of Phenazepam found at the same time had no clinical value. It should be noted that neither ICA nor TLC gave false-positive results.

From the above it can be seen that the developed TLC method gives more objective results of Phenazepam detection than ICA, which makes it possible to more reliably detect cases of Phenazepam poisoning at the stage of preliminary research.

When conducting a confirmatory analysis, it is worthwhile to give preference to HPLC-MS/MS, since this method provides high sensitivity. The analysis of the model samples showed that the detection limit of Phenazepam was 20 ng/ml, which made it possible to determine Phenazepam not only in toxic concentrations, but also at the therapeutic level, and the analysis was carried out using the native compound. A simple and fast sample preparation procedure was proposed, and an analysis time of 7 minutes gave the right to consider HPLC-MS/MS as an express method for the detection of Phenazepam.

GC-MS in most cases gives false-negative results when determining Phenazepam in an unchanged form. This deficiency is not observed when analyzing Phenazepam using the GC-MS method for hydrolysis products after derivatization, but this approach significantly increases the complexity and analysis time.

Compared with confirmatory methods, the developed TLC screening technique is expressive, does not require the use of expensive high-tech equipment, complex sample preparation, in particular derivatization, and allows you to confidently detect Phenazepam in the urine at a concentration above 1 μg/ml, which is observed in acute poisonings.

FINDINGS

1. The proposed TLC-method for the detection of Phenazepam, which includes chromatography in a toluene – acetone – ethanol system – 25% ammonia solution (45:45:7.5:2.5), hydrolysis on a plate with a mixture of concentrated sulfuric acid and alcohol (1:1) at a temperature of 120° C for 10 minutes and the formation of an azo dye by successively applying a 0.1% solution of sodium nitrite, a 0.1% solution (N-1-naphthyl)-ethylenediamine dihydrochloride as an azo-component is available, express, convenient and well suited for chemical toxicological screening. The analysis of this substance in the urine is for patients with acute poisoning with levels of Phenazepam of more than 1 μg/ml.

2. The developed TLC-method gives more objective results of Phenazepam detection at the stage of preliminary analysis, than immune-chromatographic analysis.

3. The proposed high-performance liquid chromatography technique for detecting Phenazepam in the urine combines high sensitivity (detection limit of 20 ng/ml), proof and rapidity, and also does not require time-consuming sample preparation .

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