Research Article

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Chemical and Toxicological Diagnosis of Acute Poisoning with Doxylamine, Zaleplon, and Phenazepam

***M.V. Belova*[1, 2, 3] ✉, *D.V. Drozdovskaya*[2], *E.A. Kluev*[1], *E.S. Melnikov*[2]**

Department of Acute Poisoning and Somatopsychiatric Disorders
[1] N.V. Sklifosovsky Research Institute for Emergency Medicine
3, Bolshaya Sukharevskaya Sq., Moscow, 129090, Russian Federation
[2] I.M. Sechenov' First Moscow State Medical University (Sechenov University)
2, bldg. 4, Bolshaya Pirogovskaya Str., Moscow, 119991, Russian Federation
[3] Russian Medical Academy of Continuous Professional Education
2/1, bldg. 1, Barrikadnaya Str., Moscow, 125993, Russian Federation

✉ **Contacts:** Maria V. Belova, Doctor of Biological Sciences, Associate Professor, Leading Researcher, Department of Acute Poisoning and Somatopsychiatric Disorders, N.V. Sklifosovsky Research Institute for Emergency Medicine.
Email: maniabel@gmail.com

BACKGROUND The market of hypnotic and sedative drugs is being updated due to the high toxicity of barbiturates and the limitations of their use. Currently, safer drugs such as Z-drugs, Doxylamine, and some benzodiazepine derivatives are often prescribed for the treatment of anxiety and insomnia, but they can cause acute poisoning if overdosed or in case of nonmedical use.

AIM To establish an affordable express thin-layer chromatography (TLC) technique for preliminary screening detection of Doxylamine, Phenazepam and Zaleplon in order to diagnose acute poisoning.

MATERIAL AND METHODS Thin-layer chromatography (TLC) and gas chromatography with mass selective detection (GC-MS) methods were used. Urine samples from patients with symptoms of acute Doxylamine, Zaleplon, Phenazepam poisoning, and model urine samples were prepared by liquid-liquid extraction at pH 9.0 with chloroform for TLC analysis, with ethyl acetate-diethyl ether mixture (1:1) for GC-MS.

RESULTS We developed the TLC method of Doxylamine, Zaleplon and Phenazepam detection which helps reveal their presence in the patient's urine, as well as distinguish one from another in case of similar toxic symptoms. The GC-MS method was used for confirmatory analysis. Compared to confirmatory methods, the developed technique of TLC screening is expressive, does not require expensive high-tech equipment, while allowing to differentiate Doxylamine, Zaleplon and Phenazepam from each other and from other toxicologically significant psychoactive substances detected in general screening.

Keywords: doxylamine, zaleplon, phenazepam, acute poisoning, TLC, GC-MS

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Affiliations

Maria V. Belova	Doctor of Biological Sciences, Leading Researcher, Department of Acute Poisoning and Somatopsychiatric Disorders, N.V. Sklifosovsky Research Institute for Emergency Medicine; Professor, A.P. Arzamastsev Department of Pharmaceutical and Toxicological Chemistry, I.M. Sechenov First Moscow State Medical University; Associate Professor, Department of Clinical Toxicology, Russian Medical Academy of Continuous Professional Education; https://orcid.org/0000-0002-0861-5945, maniabel@gmail.com; 30%, scientific guidance, idea and design of the study, discussion of the results, text writing, approval of the final version of the article
Daria V. Drozdovskaya	Student, I.M. Sechenov First Moscow State Medical University; https://orcid.org/0009-0000-0333-3546, kvortehvost1988@bk.ru; 30%, development of methodology and analysis by thin layer chromatography, drafting the primary text of the article
Evgeny A. Kluev	Medical Laboratory Scientist, Chemical and Toxicological Laboratory, N.V. Sklifosovsky Research Institute for Emergency Medicine; https://orcid.org/0000-0002-9684-8764, 89035601642@mail.ru; 20%, analysis by gas chromatography-mass spectrometry
Evgeny S. Melnikov	Candidate of Pharmaceutical Sciences, Assistant, A.P. Arzamastsev Department of Pharmaceutical and Toxicological Chemistry, I.M. Sechenov First Moscow State Medical University; https://orcid.org/0000-0002-8993-4808, evgeniy.melnikov@gmail.com; 20%, discussion of the results, text editing

GC-MS – gas chromatography-mass spectrometry
TLC – thin layer chromatography
UV – ultraviolet light
Rf – retention factor
Z-drugs – a generic name for 3rd generation sleeping pills (zaleplon, zopiclone, zolpidem)

INTRODUCTION

Poisoning with hypnotic and sedative drugs is a rather urgent problem today. This is due to both the treatment for insomnia and the non-medical use of such drugs. Many of them, even administered for therapeutic purposes, can be addictive and develop tolerance [1–3] which leads to their constant intake, sometimes in ever-increasing doses. This results in overdose and acute poisoning.

After significant limitation of the use of barbiturates and first-generation benzodiazepines for the treatment of insomnia, non-bezodiazepine hypnotics began to be widely used - the so-called Z-drugs, such as zaleplon, an antihistamine drug with a sedative effect - doxylamine, relatively new members of the benzodiazepine class, in particular, phenazepam [2-6]. However, these drugs, when administered for suicidal [7–9] and other non-medical purposes, cause acute poisoning. There have been cases of their abuse in high doses among people suffering from drug addiction, which lead to acute poisoning [7]. It should also be noted that the most well-known complications of taking benzodiazepine tranquilizers even at therapeutic dosing are the development of drug dependence, withdrawal syndrome, daytime sleepiness and mental confusion in some groups of patients. For example, in elderly and senile patients, a high risk of falls due to the therapy is possible [10-12]. Zaleplon produces a rapid but short-lived hypnotic effect and does not result in daytime sleepiness or decreased performance in most patients [13]. However, the literature mentions cases of both fatal and non-fatal poisoning with zaleplon and other Z-drugs, despite their low lethal toxic index compared to benzodiazepines [7, 14, 15]. It should be noted that during chemical and toxicological analysis, difficulties arise in the identification of these drugs, especially when they are present together.

Previously, approaches were proposed for the separate detection of doxylamine, zaleplon, and phenazepam in screening procedures using thin layer chromatography (TLC) [7, 16–21]. However, the proposed mobile phases and detection methods are non-selective for detection of the present substances. Immunochemical methods for doxylamine and Z-drugs have not been developed and are not used. Existing test systems for 1,4-benzodiazepine derivatives have group specificity and do not always detect phenazepam [22–25]. Thus, the development of comprehensible informative screening methods for the detection of these drugs in biological media in cases of acute poisoning is relevant.

The **aim** of the research is to develop a TLC technique for the preliminary screening detection of doxylamine, phenazepam and zaleplon in order to diagnose acute poisoning by these drugs.

MATERIAL AND METHODS

The following substances were used in the study: phenazepam powder substance (Usolye-Siberian Chemical and Pharmaceutical Factory, purity 99.0%), Donormyl 15 mg effervescent tablets (Bristol-Myers Squibb, France, valid until 11.2022) and Andante 10 mg capsules (Gedeon Richter PLC, Hungary, valid until 05.2022).

The initial standard solution of phenazepam with a concentration of 0.5 mg/ml was prepared by dissolving the precisely weighed quantity of 5 mg of the substance in 10 ml of ethanol.

The initial solution of doxylamine with a concentration of about 1.5 mg/ml was prepared by dissolving an Donormyl 15 mg effervescent tablet in distilled water with extraction of the substance by double chloroform extraction at pH equal to 9 (pH regulator — 10% ammonia solution) [18]; the resulting chloroform layer was separated by centrifugation, the combined chloroform extracts were evaporated to dryness in a stream of nitrogen; and the dry residue was redissolved in 10 ml of ethanol. To determine the limit of detection, solutions of doxylamine with concentrations of 0.5 mg/ml and 1.0 mg/ml were obtained by appropriate dilution of the stock solution with ethanol.

An initial solution of zaleplon with a concentration of 0.5 mg/ml was prepared from the contents of an Andante 10 mg capsule; zaleplon was extracted from the powder by double chloroform extraction at pH equal to 10 [21], the chloroform layer was separated by centrifugation, the combined extracts were evaporated to dryness in a stream of nitrogen; and the dry residue was redissolved in 20 ml of ethanol.

The initial mixed solution was prepared by mixing 2 ml of each solution with concentrations of doxylamine 1.5 mg/ml, zaleplon 0.5 mg/ml and phenazepam 0.5 mg/ml. The resulting concentration of the substances is presented in Table 1.

To prepare a model urine sample, 9 ml of intact biological fluid was added to 1 ml of the initial mixed solution. The resulting concentration of the substances is shown in Table 1.

Table 1

The concentration of Doxylamine, Zaleplon and Phenazepam in standard samples

Substance name	Concentration, mg/ml		
	Initial solutions	Initial mixed solution	Model urine sample
Doxylamine	0.5; 1.0; 1.5	0.5	0.05
Zaleplon	0.5	0.17	0.017
Phenazepam	0.5	0.17	0.017

TLC SilicaGel 60 F254 plates on a flexible aluminum substrate (Merck) were used as the stationary phase in TLC. The following mobile phases were compared: ethyl acetate-ethanol-25% ammonia solution (10:30:1), toluene-acetone-methanol-25% ammonia solution (45:45:7.5:2.5), ethyl acetate-methanol-25 % ammonia solution (17:2:1), methanol-diethylamine (9.5:0.5) and chloroform-methanol-diethylamine (9.5:0.5:0.25). Ultraviolet (UV) light with a wavelength of 254 nm, Dragendorff's reagent, and azo dye formation reaction were used for detection. To obtain an azo dye, the chromatographic zones were treated with a solution of sulfuric acid in ethanol (1:1) and subjected to hydrolysis at a temperature of 130ºC for 20 minutes; then a 0.1% sodium nitrite solution and a 0.1% solution of (N-1-naphthyl)-ethylenediamine dihydrochloride were sequentially applied to the plate; and the coloring of the chromatographic zones was observed. To determine the retention factor (Rf) values, the samples were evaluated in the selected system in triplicate.

GC–MS was used as confirmatory methods in the analysis of urine samples from patients with acute poisoning.

GC–MS analysis conditions: Thermo Trace GC Ultra gas chromatograph with DSQ II mass spectrometer. TR-5MS column, length 30 meters, inner diameter 0.25 mm, film thickness of the stationary liquid phase - 0.25 µm. The carrier: helium. Column temperature program: 50°C - 3 minutes, heating 100°C/min to 100°C, 100°C - 1 minute, heating 15°C/min to 280°C, 280°C - 20 minutes. The injector temperature: 220°C. Total ion current detection at m/z 45–650, electron impact ionization with an energy of 70 eV. The duration of the analysis was 18 minutes.

Sample preparation for TLC analysis. 5 ml of the test urine sample or model sample was alkalinised with 10% ammonia solution to pH 9; 5 ml of chloroform was added; and then extracted for 3 minutes; the layers were separated by centrifugation for 5 minutes at 3500 rpm. After centrifugation, the organic phase layer was evaporated to dryness under nitrogen. The dry residue was redissolved in 0.5 ml of ethanol. 50 µl of the sample and 30 µl of each of the initial solutions of individual substances were applied to the start line of the plate.

Sample preparation for GC-MS analysis. 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 ethyl acetate-diethyl ether mixture (1:1) were added to 3 ml of urine; extracted for 10 minutes with stirring in a shaker, then the layers were separated by centrifugation for 10 minutes at 3500 rpm. The organic layer was transferred to glass vials, evaporated to dryness under vacuum and the residue was dissolved in 100 µl of ethyl acetate. 2 µl of the resulting solution was injected into a chromatography detector.

RESULTS AND DISCUSSION

DEVELOPMENT OF THE TLC METHOD FOR THE DETECTION OF DOXYLAMINE, ZALEPLON AND PHENAZEPAM

The choice of the mobile phase was made taking into account the influence of the composition and polarity on the mobility of the substances and the resolution of the chromatographic system. Rf values of doxylamine, zaleplon and phenazepam in the compared systems, established during the experiment, are presented in Table 2.

Table 2

Rf values of Doxylamine, Zaleplon and Phenazepam in compared mobile phases for thin layer chromatography

Substance name	Rf value in the TLC developing solvent				
	Ethyl acetate-ethanol – 25% ammonia solution (10:30:1)	Toluene-acetone-methanol – 25% ammonia solution (45:45:7.5:2.5)	Ethyl acetate - methanol – 25% ammonia solution (17:2:1)	Methanol-diethylamine (9.5:0.5)	Chloroform-methanol-diethylamine (9.5:0.5:0.25)
Doxylamine	0.29±0.02	0.46±0.02	0.47±0.03	1.0	0.96±0.04
Zaleplon	0.85±0.04	0.52±0.02	0.78±0.05	0.88	0.88±0.04
Phenazepam	0.87±0.04	0.63±0.03	0.88±0.04	0.91	0.85±0.05
Duration of chromatography, min	27	15	20	25	26

Based on the data in Table 2, among the systems studied, we chose the toluene-acetone-methanol-25% ammonia solution (45:45:7.5:2.5) system, since it provides complete separation of all three substances under analysis, because the chromatographic zones have clear contours. The duration of chromatography was 15 minutes, which satisfies the requirement of rapidity. This system is recommended for TLC studies of some other toxicologically significant substances basic in nature [26], which makes it possible to detect those substances without disturbing the general course of the research. In other systems, Rf values of all the substances or the pair of zaleplon and phenazepam had similar values, which, in case of their simultaneous presence, did not allow researchers to clearly separate them.

The applied methods for detecting the chromatographic zones of the studied substances and the minimum detectable concentrations are presented in Table 3.

Table 3

Evaluation of different detection methods for Phenazepam, Zaleplon and Doxylamine

Substance name, detection limit	Detectors				
	UV, 254 nm	A mixture of concentrated sulfuric acid and ethanol (1:1)	UV, 254 nm after hydrolysis	Azo dye formation reaction	Dragendorff's reagent
Doxylamine 10 mcg	Fluorescence quenching	–	Bright orange fluorescence	–	Orange coloring
Zaleplon 10 mcg	Bright blue fluorescence	Light green coloring	Green fluorescence	Pink coloring	Orange coloring
Phenazepam 10 mcg	–	Yellow coloring	Green-blue fluorescence	Purple coloring	Orange coloring

Note: УФ – ultraviolet light

According to the results presented in Table 3, it can be concluded that zaleplon has its own fluorescence, and doxylamine quenches the fluorescence, thus, they can be detected. After hydrolysis, doxylamine and phenazepam form products that can be detected by the characteristic fluorescence in UV light. Subsequent treatment of the chromatographic zones of zaleplon and phenazepam with 0.1% sodium nitrite solution and 0.1% solution of (N-1-naphthyl)-ethylenediamine dihydrochloride led to the formation of colored products. For phenazepam, this is a known azo-coupling product [16, 26], and for zaleplon, the structure of the product has not been determined, which requires further study. The Dragendorff's reagent was a universal detector for all three substances, since it was used without preliminary hydrolysis. Rf had stable values in the analysis of both solutions of individual substances and their mixed solution.

During chromatography of the sample containing all three compounds under study, the Rf values practically did not change compared to those during chromatography of the initial solutions of the individual substances, falling within the calculated and given in Table 2 deviations.

To assess the applicability of the technique for detecting substances after extraction from the biological fluid (urine), model urine samples containing a mixture of doxylamine, zaleplon, and phenazepam at the indicated

concentrations were subjected to extraction (see Table 1). The experiment was performed in triplicate. Extraction and development of samples was carried out according to the method described above. The deviation of Rf did not exceed 10% of the values established for solutions of the individual substances.

The technique developed by us was used in the study of urine samples from patients with suspected doxylamine, phenazepam, or zaleplon poisoning. In all the experiments, the spots corresponding to the studied substances on the chromatograms of extracts from the urine of patients with acute poisoning had coloring and Rf values similar to those established in the model experiment. The results are shown in Table 4.

Table 4

The results of analyzing biological samples of patients with suspected Doxylamine and Phenazepam poisoning

Sample #	Detection of other substances					
	TLC	GC-MS	Ethanol in blood, g/l	Ethanol in urine, g/l	Other substances	Notes
1	doxylamine, phenazepam	doxylamine, phenazepam	n/d	n/d	bisoprolol, verapamil	
2	doxylamine, phenazepam	doxylamine, phenazepam	2.37	4.65		
3	n/d	doxylamine	1.85	2.48		low concentration in the sample

Notes: ГХ–МС — gas chromatography with mass selective detection; н/о — not detected; ТСХ — thin layer chromatography

The results obtained were confirmed by GC–MS analysis of biological samples.

As can be seen from Table 4, using the TLC technique developed by the authors, doxylamine and phenazepam were detected in two analyzed samples. Moreover, their detection was not complicated by the presence of other toxicants in the sample. In one of the samples examined, the proposed TLC technique gave negative results for doxylamine, while it was found during the confirmatory testing. The reason for the false-negative result, most likely, was the low concentration of the drug in the sample. Since ethanol (1.85 g/l in blood and 2.48 g/l in urine) was also found in the biological media of this patient, the severity of the patient's condition was probably due to the combined synergistic effect of the therapeutic dose of doxylamine and ethanol.

Compared to confirmatory methods, the developed TLC technique is simple, fast, and does not require the use of expensive high-tech equipment, while it allows researchers to confidently detect doxylamine, zaleplon, and phenazepam in one mixture, which can be observed in acute poisoning.

CONCLUSION

The proposed method for the detection of doxylamine, zaleplon and phenazepam by thin-layer chromatography, including chromatography in the system toluene-acetone-methanol-25% ammonia solution (45:45:7.5:2.5), use as a detector of ultraviolet light with a wavelength of 254 nm, followed by treatment of the plate with Dragendorff's reagent, as well as the formation of colored products by azo coupling reaction after the hydrolysis of zaleplon and phenazepam on the plate, can be used for screening in acute poisoning with doxylamine, phenazepam and zaleplon due to its rapidity, informative value, sufficient sensitivity, relative simplicity and reliability.

FINDINGS

1. We developed a thin-layer chromatography technique for the detection of doxylamine, zaleplon and phenazepam in a biological fluid (urine), suitable for rapid diagnosis of acute poisoning with these drugs.
2. The proposed method is easy to use, affordable, not requiring complex equipment and reagents, allowing the detection and differentiation of doxylamine, zaleplon and phenazepam in their joint presence.
3. Sufficient sensitivity and informative value of the developed technique as a stage of preliminary research is confirmed by the results of parallel analysis of urine samples of patients by gas chromatography-mass spectrometry.

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