Istanbul J Pharm 52 (1): 37-46
DOI: 10.26650/IstanbulJPharm.2022.877867
Original Article

Determination of diazepam in human plasma by developed and validated a high-performance liquid chromatographic ultraviolet method

Emrah Dural[1], Beyza Nur Kaya[1]

[1]Sivas Cumhuriyet University, Faculty of Pharmacy,Department of Pharmaceutical Toxicology,Sivas, Turkiye

ORCID IDs of the authors: E.D. 0000-0002-9320-8008; B.N.K. 0000-0002-5576-3326

Cite this article as: Dural, E., & Kaya, B.N. (2022).Determination of diazepam in human plasma by developed and validated a high-performance liquid chromatographic ultraviolet method. *İstanbul Journal of Pharmacy, 52*(1), 37-46. DOI: 10.26650/IstanbulJPharm.2022.877867

ABSTRACT

Background and Aims: Diazepam is accepted as a safer drug to medicate in many serious cases, acting as an anticonvulsant, an anxiolytic and a treatment for many types of poisoning. Monitoring it is important in achieving successful treatment and reducing the risk of toxic effects. In this study, it is aimed to develop and validate a sensitive, repeatable, and reliable method based on high-performance liquid chromatographic analysis for the determination in human plasma.

Methods: Separation was carried out using a reverse-phase C18 column (4.0 mm x 150 mm, 3 µm) at 30 °C. The solution was prepared with a 10 mM phosphate buffer and acetonitrile (1:1, v/v) was employed as a mobile phase at the isocratic flow with 0.5 mL/min rate. Quantification was applied at 230 nm. A solid-phase extraction method was established and optimized, which was then used in the preparation of the plasma (0.5 mL) samples to the analysis.

Results: The method was found to be linear (r^2= 0.9805) between 100 and 1200 ng/mL. The analysis run was ≤12 min. Intra-day and inter-day accuracy were found between -5.78 and 5.93 and precision was ≤1.82%. The limit of detection and quantification were calculated as 20.42 and 61.86 ng/mL, respectively. Recovery was found between the range of 95.12% and 106.83%. The method was determined to be robust according to changes in UV, mobile phase organic solvent content, mobile phase pH, column temperature, and operator.

Conclusion: This simple, sensitive and reliable method is suggested for accredited-reference laboratories working on the therapeutic drug monitorization and/or overdose-toxicological quantified analysis of diazepam in human plasma.

Keywords: Diazepam, Solid-phase extraction, Method validation, High-performance liquid chromatography-ultraviolet detection

INTRODUCTION

Diazepam (*7-chlorine-1-methyl-5-phenyl-1,3-dihydrobenzo [e] [1,4] diazepin-2-one*), a benzodiazepine-derived drug (Figure 1-a), is primarily used in the treatment of mental anxiety (Zhang, Ouyang, Lipina, Wang, & Zhou, 2019), but it also used as a sedative-hypnotic (Cook, Flanagan, & James, 1984) and as an anticonvulsant (Chamberlain et al., 2014). It has skeletal muscle relaxant (Richards, Whittle, & Buchbinder, 2012) and anxiolytic (Faye et al., 2020) properties. It is accepted as a safer drug than others used in the treatment of anxiety (Zhang et al., 2019). It has a widespread use due to its high therapeutic index. Diazepam is used commonly in cholinestrase poisoning (Abou-Donia, Siracuse, Gupta, & Sobel Sokol, 2016), to alleviate some symptoms associated with alcohol (Weintraub, 2017) and barbiturate abstinence syndrome (Perry, Stambaugh, Tsuang, & Smith, 1981), in anti-histamine over-

Address for Correspondence:
Emrah DURAL, e-mail: emrahdural@cumhuriyet.edu.tr

Submitted: 10.02.2021
Revision Requested: 21.01.2022
Last Revision Received: 07.02.2022
Accepted: 17.02.2022
Published Online: 28.04.2022

dose (Montoro et al., 2013), black widow spider envenomation (Rogers, Stanford, & Dart, 2006) and chloroquine overdose (Hughes, 2020). In addition to that it could be used alone as an anaesthetic (McGrath et al., 2020) or in combination with other drugs for conscious sedation (Zanette et al., 2013), and also as an aid to anaesthesia (Wexler, Peyster, Hakkinen, & Pope, 2005).

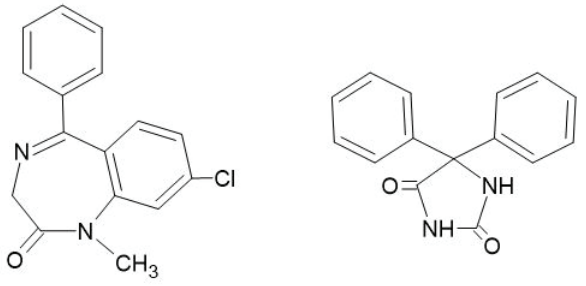

Figure 1. Chemical structures of diazepam (-a) and phenytoin (-b) which used as the internal standard.

Diazepam is commonly used via the oral, rectal, and parenteral routes (Bialer, 2007; Henney, Sperling, & Rabinowicz, 2014). It reaches maximum plasma concentration between 30 and 90 minutes (Gong, Liu, Xu, Fan, & Xue, 2015; Taysse et al., 2003). Diazepam is widely distributed throughout the body (Friedman et al., 1992). Drug interactions associated with protein binding are clinically insignificant, although they are 98% bound to plasma proteins.

Sedation is the most common side effect of the drug. In intravenous administration, the drug side effects emerge more quickly and the toxicity is usually iatrogenic. Chronic treatment with diazepam can lead to addiction. Also, withdrawal symptoms can be observed in cases where cessation and toxic effects occur too (Calcaterra & Barrow, 2014). Therefore, patients under treatment with diazepam need the medical monitoring. Overdose of diazepam in oral or parenteral administration causes a loss of consciousness, hypotension, bradycardia, coma and respiratory failure. Deaths have been reported when diazepam is used with other central nervous system depressants under combination treatment (Calcaterra & Barrow, 2014). Diazepam is reported as a genotoxic agent according to toxicity studies accomplished *in vitro*. Due to diazepam lipid solubility being significantly high, it is known that diazepam passes through the placenta during pregnancy, and it has the ability to cross the blood-brain barrier. Also, it is known that diazepam passes to the infant with maternal exposure by lactation (Ghosh, Reddy, Ramteke, & Rao, 2004; Wexler et al., 2005). For these reasons which could be evaluated as very serious for public health, development of a simple, sensitive and reliable monitoring of diazepam is very important.

Many methods have been developed for determining diazepam from biological samples and pharmaceutical preparations. These methods are based on thin layer chromatography (TLC) (Bakavoli & Kaykhaii, 2003), spectrophotometry (Morelli, 1997), gas chromatography mass spectrometry, high-performance liquid chromatography (HPLC) coupled with ultraviolet detector (UV) and tandem mass spectrometry (MS/MS) (Brieudes, Lardy-Fontan, Lalere, Vaslin-Reimann, & Budzinski, 2016; de Araujo, Bauerfeldt, Marques, & Martins, 2019; Gong et al., 2015; Kim et al., 2017; Miller, Wylie, & Oliver, 2008; Tran, Hu, & Ong, 2013), respectively. Also, various extraction methods such as protein precipitation (Pilli et al., 2020), liquid-liquid extraction (Kim et al., 2017), solid-phase extraction (SPE) (Borges, Freire, Martins, & de Siqueira, 2009; Mercolini et al., 2009), and solid-phase microextraction (Yuan & Pawliszyn, 2001) were employed in these investigations.

In this study, it was aimed to develop a high-performance liquid chromatographic method based on solid-phase extraction for monitoring diazepam from human plasma. Subsequently it was then validated in terms of linearity, repeatability, sensitivity, recovery and robustness according to the International Conference on Harmonization guideline Q2(R1) (ICH, 2005). On the other hand, it is aimed to develop a simple, fast, cheaper, repeatable, accurate and reliable high-performance liquid chromatography method for the quantitative determination of diazepam.

MATERIAL AND METHODS

Chemicals and reagents

Diazepam (Figure 1-a) and fenitoin (Figure 1-b) chemical standards (purity ≥ 99.0%) were donated from the Forensic Science Institute of Ankara University (Ankara, Turkey) and VEM pharmaceuticals (Istanbul, Turkey), respectively. Methanol and acetonitrile which were HPLC-grade and KCl, NaCl, NaOH and H_3PO_4 which were analytic grade were purchased from Sigma-Aldrich (Missouri, USA). Na_2HPO_4 and KH_2PO_4 were purchased from Merck (Darmstadt, Germany). The Sep-Pac® Vac 1 cc (100 mg) solid-phase C18 cartridge was obtained from Waters (Dublin, Ireland). Carboxymethyl cellulose was purchased from Biokim & Wenda Chemicals (Izmir, Turkey). Bovine serum albumine was purchased from Solarbio Life Science (Beijing, PRC). PTFE membrane filter (47 mm DIA, 0.45 µm pore size) was obtained from Millipore (MA, USA). Ultrapure water was supplied from Elga Purelab Water Purification System (Lane End, UK) performing purification as required by the reverse osmosis method. The conductivity and electrical resistance of the ultrapure water obtained were ≤0.055 µS/cm (25 °C) and ≤ 18.2 mΩ.cm, respectively. Membrane filters with a pore size of 0.45 um from Milipore (Massachusetts, USA) were used for the mobile phase filtration.

Instrumentation and chromatographic conditions

The analytical separation and quantification was achieved with an Agilent 1100 series HPLC system (CA, USA) coupled with a UV detector. A high-performance liquid chromatography (HPLC) was employed in this stud and consisted of a degasser (G1322A), a quadro gradient pump (G1311A), a manual injector (Rheodyne 7725i) with loop volume 20 µL, a column oven (G1316A, Colcom), and an ultraviolet detector (G1314A VWD). A 100 µL volume glass HPLC needle was utilized for applying samples to the system from the injector port.

A stainless steel end-cap ACE-5 reverse phase (RP) C18 analytical column (Aberdeen, Scotland) with 4.0 mm x 150 mm (i.d. x l) diameters 3 µm (p.s.) column filling material was employed for instrumental analytic separation. A 08.03 version ChemStation® software was used for data collection and system handling.

The column oven temperature was set at 30°C. According to the applied observations, the ultraviolet spectrum ultraviolet detector was set at 230 nm for both diazepam and phenytoin (Figure 2). The mobile phase composed of 10 mM KH_2PO_4 (pH 3.0) and acetonitrile (50:50, v/v) was applied to the analytical system with an isocratic, 0.5 mL/min constant flow. Before application to the system the mobile phase solution was filtered passing through a PTFE membrane disc filter at 20 kPa pressure using a vacuum filter system.

Analytic parameters were determined following the optimization study performed in terms of the mobile phase content and pH, analytical column and column oven temperature, respectively.

Preparation of chemical standards and simulated plasma

Both stock solution (1 mg/mL) and working solutions of diazepam 5, 10, 20, 40 and 60 µg/mL were prepared by dissolving them in methanol. Diazepam quality control samples (0.1, 0.2, 0.4, 0.8 and 1.2 µg/mL) were prepared by taking 10 µL of the respective working solution and dissolving it in 500 µL volume of simulated plasma sample. The working solution (10 µg/mL) of internal standard was prepared by diluting it with methanol from the main stock solution of phenytoin (1 mg/mL), weekly. Stock solutions were stored at -20°C until analysis and were observed to remain stable for at least 1 month.

During the development of solid-phase extraction and method validation steps, a simulated plasma, the preparation protocol of which is described by Mercolini et al. (2008) was used. According to this, 4 g bovine albumin, 20 mg KCl, 0.8 g NaCl, 20 mg KH_2PO_4, 144 mg Na_2HPO_4 was dissolved in 100 mL ultrapure water, then its pH was adjusted to 7.4 with 0.1 M KOH or H_3PO_4. Finally, the formed solution was split up into microtubes in 0.5 mL quantities and stored in -18 °C until use.

Determination of the internal standard

Clozapine, sodium valproate, chlorpromazine, flunarizine, cinnarizine and phenytoin chemicals were tested to determine the internal standard to be used in the analytical method. The obtained results from the selection of internal standard test study showed that the peak shape and structure of chlorpromazine and cinnarizine did not have enough sharpness to be accepted as an internal standard in these chromatographic conditions. Because the chromatographic peaks acquired from flunarizine analysis were so fragmental, and also the obtained clozapine and sodium valproate results were unsuitable in terms of the retention time according to diazepam, these 3 agents could not be utilized as an internal standard. It was decided to use phenytoin as an internal standard because it shows a precise peak structure even at low concentrations, its extraction recovery values are very successful, it gives a nice UV response at 230 nm, and has an acceptable retention time in the chromatogram (Figure 2).

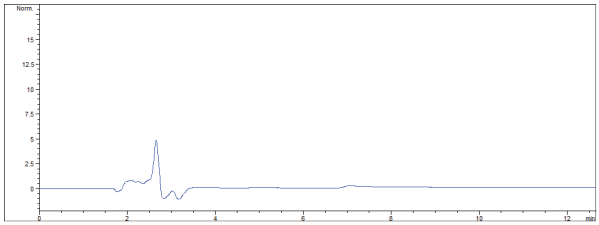

Figure 2. A chromatogram which belong to a blank simulated plasma sample extracted by the SPE method explained.

Optimization of the developed solid-phase extraction method

The solid-phase extraction procedure was optimized in terms of the cartridge conditioning, sample loading, cartridge washing and elution steps according to the results obtained from the comparative chromatographic analyses. As a rule, while performing the optimization study, changes were made in the intended step to monitor, while other variables related to extraction were kept constant.

In the optimization of the conditioning step, the methanol and ultrapure water application volume from 1 mL to 3 mL were tested for the determination of the best efficiency value for the activation of cartridge filling material. In the following step (called sample loading), an attempt was made to apply plasma samples both directly and diluted with water up to 1 mL into the cartridge. In addition, the volume of water to be used in the washing step tested in volumes varying (from 0.5 mL) up to 2 mL. In addition, the dilution values of the acetonitrile: methanol mixture (3: 1, v/v) used in the second stage of the washing step were evaluated at varying rates up to 12 times. The content ratios of the elution solution formed with acetonitrile and methanol were tested to determine the best efficiency value at ratios of 6:1, 3:1, 1:1, 1:3, 1:6 (v/v), respectively. Then, defining the best efficient solution volume value, it was tested from 1 up to 3 mL. The elution volume effect on the extraction efficiency was tested between 1 ml to 3 mL. The optimized extraction values were clearly given under *2.6.2. Solid-phase extraction* subtitle.

Extraction efficiency was evaluated by comparing the diazepam and phenytoin peak areas recovered from plasma to the results of standard solutions applied directly to HPLC without extraction by dissolving in the mobile phase. Since the internal standard was used in this study, the ratio value obtained by dividing the peak area of diazepam from the same chromatogram by the area of the internal standard was used in both recovery test and also other validation calculations.

Sample preparation

Preparation of spiked samples

10 µL diazepam and 10 µL internal standard (10 µg/mL) working solution were added to 500 µL of blank simulated plasma sample and then its volume was made up to 1 mL with ultrapure water. Then, it was stirred at 1200 rpm for 2 minutes with the vortex mixture before being used as a sample.

Solid-phase extraction

Initially, a C18 solid-phase cartridge was activated and conditioned by applying 3 mL of methanol and 3 mL of ultrapure

water, respectively. Then, 500 µL of the plasma sample were diluted with 1 mL of ultrapure water in a micro-tube containing 10 µL of diazepam and 10 µL of phenytoin internal standard, and this was applied to the solid-phase cartridge with 0.5 mL/min flow. After the samples were loaded into the cartridge, the remains were cleaned twice using 1 mL ultrapure water. Then, the cartridges were cleaned with 1 mL of the acetonitrile: methanol mixture (18:1, v/v) and again and they were fully dried using a constant vacuum. Under 0.5 mL/min constant flow, 1 mL acetonitrile: methanol (3:1, v/v) mixture was applied twice to the cartridges and they were then vacuumed (75 kPa) until completely dry. Finally, the collected extraction liquid (approximately 2 mL) was evaporated to complete dryness using the nitrogen evaporating system heated to 40 °C). After the remains were dissolved in 200 µL of the mobile phase by the vortex mixture at 3000 rpm, 1 minute, it was loaded into the HPLC system with a volume of 20 µL.

Method validation

The developed chromatographic method was validated in terms of linearity, sensitivity, accuracy, precision, recovery and robustness in accordance with the International Harmonization Conference (ICH) guidelines (ICH, 2005).

Linearity: Diazepam plasma samples in 5 different concentrations (100, 200, 400, 800 and 1200 ng/mL) that can be anticipated in plasma were prepared on the condition that 3 samples were found for each point. It was plotted according to the versus peak-area ratios to the internal standard. Each plasma sample prepared was delivered to the HPLC device for analysis. The calibration equation and the determination coefficient were calculated by drawing the calibration graph. The linearity test was designed to cover sub-therapeutic, therapeutic, overdose and toxic levels of the diazepam in plasma.

Sensitivity: The limit of detection (LOD) and limit of quantification (LOQ) were calculated according to the ICH recommendation based on standard deviation of the response and the slope of the calibration graph. 100 ng/mL was used in the test as the lowest calibration point of diazepam.

$$\mathrm{LOD} = 3.3\,\frac{\sigma}{S};\ \mathrm{LOQ} = 10\,\frac{\sigma}{S}$$

(σ: The standard deviation of the response; S: The slope of the calibration curve).

Accuracy and precision: The accuracy, defined as the relative error (RE%) was calculated as the percentage difference between the added and found diazepam quantity by 5 individual replicates both intraday and inter-day. The precision, defined as relative standard deviation (RSD%), was calculated by five separate replicates of diazepam both intraday and inter-day. Five replicate spiked samples were assayed intraday and inter-day at the three different concentrations (100, 400 and 1200 ng/mL).

Recovery: The efficiency of the extraction procedure from the plasma was determined by comparing pre-extraction spikes with the post-extraction spiked internal standard. Five individual replicates of spiked samples at low, middle and high concentrations (200, 400 and, 800 ng/mL, respectively) of diazepam were prepared with and without the internal standard. The extraction procedure was carried out as described previously in the sample preparation step.

Robustness: Robustness is a test for evaluating the response of the analytical method to some changes in the analysis parameters. Here, the method's response to changes in analytical parameters is evaluated in terms of reliability by examining the changes in the results obtained. It is the ability of a method to remain unaffected when small changes are applied. The robustness test was performed with 400 ng/mL of diazepam, which is the median concentration of the calibration interval. The response of the method of changes to ultraviolet wavelength (± 1 nm), mobile phase flow rate to column (± 0.1 mL/min), mobile phase organic solvent content (± 5%), column temperature (± 5 °C), mobile phase final pH (± 0.5) and also the operator changes were evaluated.

RESULTS

Selectivity and specificity

Initially the optimum conditions for mobile phase, column and UV-detector wavelength were determined and plasma samples containing diazepam and phenytoin were injected into HPLC under these conditions. Within the optimum analysis conditions, retention times of diazepam and phenytoin were determined as 4.9 and 10.8 minutes, respectively. Blank and sample chromatograms of 10 µg/mL phenytoin and 400 ng/mL diazepam are given in Figure 2 and 3, respectively.

By adding diazepam and phenytoin analytes into simulated plasma by the standard addition method, concentrations ranging from the lowest calibration point to the highest concentration of diazepam could be successfully detected and quantified from the relevant matrix. This can be clearly seen in the blank simulated plasma chromatogram. All observed analytes peaks were sharply, clearly and obviously detected.

As part of the selectivity study of the method validation, nitrazepam (100 ng/mL), diazepam (400 ng/mL), phenytoin (10 µg/mL) and lorazepam (100 ng/mL) were administered to HPLC. Quantitative determination of diazepam and phenytoin was successfully performed under the expressed chromatographic conditions. The chromatogram obtained is given in Figure 4.

Nitrazepam is used in disabling anxiety and insomnia. It has amnestic, sedative, anticonvulsant, and also skeletal muscle relaxant effects. It is defined as a hypnotic drug. It is classified in the benzodiazepine group of drugs (Yasui, et al., 2005). Lorazepam, is also a benzodiazepine medication, used in the treatment of severe agitation, anxiety disorders,

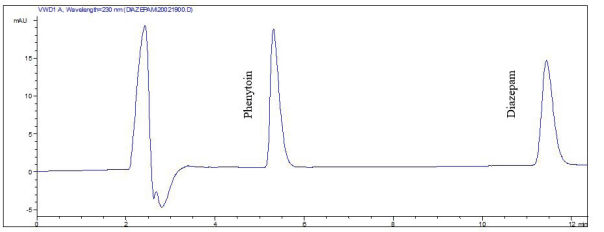

Figure 3. A typical chromatogram which exhibited phenytoin (10 µg/mL) and diazepam (400 ng/mL) peaks, respectively.

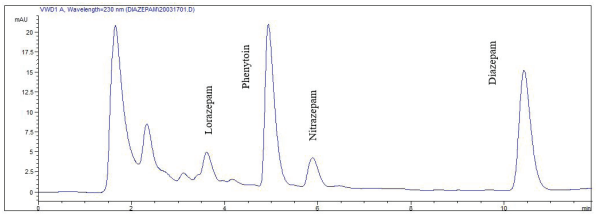

Figure 4. A typical chromatogram obtained in the selectivity study for method validation. Peaks of nitrazepam.

trouble sleeping, active seizures, alcohol withdrawal, and chemotherapy-induced nausea and vomiting (Herman, Van Pharm, & Szakacs, 1989). Nitrazepam and lorazepam were used in the selectivity study because they are structurally very close to diazepam.

Linearity

Calibration curves of diazepam drawn at five points (n=3) which are 100, 200, 400, 800 and 1200 ng/mL concentration versus the area of phenytoin as an internal standard by the standard addition method showed excellent correlation with $r^2 = 0.9805$, (Table 1). The linearity study was designed to cover sub-therapeutic, therapeutic and toxic drug levels of the drug. The wide linear range also had a positive effect on the use of the method since the obtained real blood results showed very high standard deviation.

System suitability parameters show that it has good resolution (Rs) and selectivity (α) values. Capacity factor (k') and theoretical plate number (*N*) show acceptable values for a successful determination of diazepam from plasma as it can be seen in Table 1.

Sensitivity

LOD and LOQ values were 20.42 ng/mL and 61.86 ng/mL, respectively. In addition to the LOD value being approximately 5 times lower than the lowest point on the calibration curve, LOQ value was found to be lower than the last point on the calibration curve. This result shows that the method can be used reliably for the analysis of diazepam in low concentrations in plasma Table 2.

Accuracy and precision

The repeatability study was performed at 100, 400 and 1200 ng/mL diazepam concentrations. The intraday repeatability study was performed with 5 replicate analyses for each concentration on the same day, and the accuracy was found between (RE%) (-5.78) and 3.30, and precision (RSD%) was found between 0.14 and 1.59. The repeatability study between days was carried out for 5 consecutive days and the accuracy was between (-5.78) and 5.93 (RE%), and precision (RSD%) was between 1.22 and 1.82. These data showed that when compared with the literature, this method has high repeatability and can obtain precise and accurate results in diazepam analysis from plasma in the intraday and inter-day reproducibility study (Table 3).

Recovery

The extraction yield values for the 3-replicates recovery study for diazepam concentrations of 200, 400 and 800 ng/mL were in the range of 95.12% to 106.83% (Table 4). These observed excellent values obtained from the extraction method developed and optimized suggest that the analytes, matrix (simulated plasma) and method (solid-phase) are perfectly compatibility. It is thought that it will make a significant contribution to the literature on diazepam analysis since the recovery value observed is 100.39% on average. The raw data used in the calculation of the recovery is given detailed in Table 4.

Robustness

No significant changes in the analytical signals were observed upon changing ultraviolet wavelength value (± 1 nm) (Table 5), mobile phase pH (± 0.5) (Table 6), mobile phase content (± 5%) (Table 7). Moreover, change of analysts, sources of chemicals and/or solvents did not lead to significant changes in chromatographic signals and results. The robustness of the experimental results demonstrated that the method is able to create data with acceptable precision and accuracy. Consistent data from selectivity studies and the robustness study demonstrated its suitability in the quantitative determination of diazepam from human blood.

DISCUSSION

Diazepam is widely used as an antiepileptic, muscle relaxant, hypnotic, and anesthetic inductor in pharmacotherapy. However, the

Table 1. Chromatographic properties and system suitability parameters of the suggested method. Because of phenytoin was used as an internal standard; calibration range, calibration equation, determination coefficient (r^2), selectivity factor (α), resolution (Rs) values belong to this agent did not be calculated.

Analyte	Retention time (min, t_R)	Capacity Factor (k')	Theoretical plate number (N)	Selectivity factor (α)	Resolution (Rs)	Calibration range (ng/mL)	Calibration equation	Determination coefficient (r^2)
Diazepam	10.8	5.35	3808.65	2.85	8.44	100 – 1200	y= 0.0019x + 0.1641	0.9805
Phenytoin	4.9	1.88	153.64	null	null	null	null	null

Capacity factor $(k') = \frac{tR-t0}{t0}$; Theoretical plate number $(N) = 16(\frac{tR}{Wt})^2$; Specificity factor $(\alpha) = \frac{k2}{k1}$; Resolution $(R_s) = \frac{\sqrt{N}}{4} \frac{(\alpha-1)}{\alpha} \frac{k}{(k+1)}$; Abbreviations: tR: retention time of the analyte peak; t0: retention time of first peak ; Wt: peak width

Table 2. Sensitivity test data applied at 100 ng/mL concentration of diazepam.

No	Concentration (ng/mL)	Area (mAu)		STD/ISTD				LOD (ng/mL)	LOQ (ng/mL)
		Diazepam	Phenytoin	Rationed Values	Average	SD	RSD%		
1	100	96.5	261.0	0.370					
2	100	98.6	266.8	0.370					
3	100	97.9	259.6	0.377					
4	100	94.6	259.9	0.364					
5	100	92.9	264.3	0.352	0.368	0.012	3.193	20.42	61.86
6	100	105.2	288.2	0.365					
7	100	109.7	303.2	0.362					
8	100	97.3	271.6	0.358					
9	100	105.5	274.8	0.384					
10	100	110.9	291.3	0.380					

Table 3. Confidence parameters of the method that including intraday and inter-day precision, accuracy and recovery values. These results were obtained from individual samples (n=3) prepared as quality control samples in real plasma.

Expected Concentration (ng/mL)	Intraday				Inter-day				Average Recovery (%)
	No. Obs.	Observed concentration ± SD (ng/mL)	Precision (RSD%)	Accuracy (RE%)	No. Obs.	Observed concentration ± SD (ng/mL)	Precision (RSD%)	Accuracy (RE%)	
100	5	99.82±3.30	1.59	-0.18	5	102.26±3.84	1.82	2.25	99.22
400	5	381.42±4.03	0.78	-4.65	5	384.97±6.43	1.22	-3.76	95.12
1200	5	1173.50±1.97	0.14	-2.21	5	1210.93±24.19	1.22	0.91	106.83

drug is connected with abuse due to its high potential for side effects such as addiction. Therefore, toxicological analysis of diazepam for therapeutic dose impression studies is important for forensic and clinical toxicologists. Some studies involving the monitoring of diazepam from various samples are summarized below.

In the study by Uddin et al. (2008), diazepam and 5 other benzodiazepines and two metabolites: in human plasma, urine and saliva by RP-HPLC-diode array detection method were determined. Analytes were quantitated at 240 nm. Methods were based on a solid phase extraction method. The separation was carried out on a C_8 (250 mm x 65 mm, 5 µm) analytical column with a mobile phase containing methanol, acetonitrile, and 50 mM ammonium acetate. Linearity was maintained in the range of 300-20000 ng/mL. r^2 was found ≥ 0.997. Intraday and inter-day precision test implementation were applied at concentrations of 2000, 4000 and 8000 ng/mL. Method precision values were found between 1.3% and 7.9% in plasma and between 2.1 and 7.8% in the urine. Linearity study of saliva was applied to 500, 1000 and 2000 ng/mL concentrations. The results were found between 2.2 and 8.1%, respectively. LOD and LOQ values were found as 20-470 and 70-1570 ng/mL, respectively (Uddin, Samanidou, & Papadoyannis, 2008).

Muchohi et al. (2001) developed an ultraviolet based reverse phase HPLC method to determine the amount of diazepam in plasma samples from children with severe malaria. After precipitation of plasma proteins, liquid-liquid extraction was performed with the mixture of acetonitrile, ethyl acetate and n-hexane. Diazepam was eluted from a reverse phase C_{18} column with an acidic (pH: 3.5) aqueous mobile phase (10 mM KH_2PO_4-acetonitrile, 69:31, v/v) at ambient temperature. The calibration curve between 10 and 200 ng/mL in the plasma after centrifugation was linear and the determination coefficient (r^2) was ≥0.99. Relative recovery values at 25 and 180 ng/mL were greater than 87%. The relative standard deviation during the day and between days was less than 15%.

Dragica Zendelovska et al. (2018) developed a high-performance liquid chromatographic (HPLC) method with UV-detection for direct determination of diazepam in whole blood and serum. The isolation of diazepam and internal standard bromazepam from serum and whole blood samples was performed using reverse phase cartridges with the solid-phase extraction method. The analytes were separated using a reverse phase C8 column with a mobile phase of 0.1% (v/v) triethylamine (pH 3.5) and acetonitrile (63:37, v/v) in water. The UV detector wavelength was

Table 4. Recovery data of the developed analysis method and obtained total recovery values.

Diazepam concentration (ng/mL)	Extraction	Area values (mAU)		STD/ISTD			Average Recovery (%)
		STD	ISTD	Rationed Values	Mean ($\bar{X}$)	SD	RSD%	
200	Non-extracted samples	150.7	327.7	0.461	0.455	0.007	1.440	99.22
		158.5	345.9	0.458				
		154.9	347.9	0.445				
		145.1	304.6	0.476				
200	Extracted samples	131.5	303.4	0.434	0.451	0.019	4.085	
		141.2	318.8	0.443				
400	Non-extracted samples	256.6	259.6	0.988	0.963	0.039	4.053	95.12
		259.0	261.0	0.992				
		246.8	271.9	0.908				
		264.4	288.9	0.915				
400	Extracted samples	275.4	300.7	0.916	0.915	0.001	0.078	
		278.9	305.1	0.914				
800	Non-extracted samples	561.9	327.2	1.717	1.720	0.002	0.008	106.83
		565.0	328.5	1.721				
		584.1	339.0	1.723				
		456.3	250.9	1.819				
800	Extracted samples	473.7	258.8	1.830	1.837	0.018	0.997	
		507.4	272.5	1.862				

Note: Diazepam as the analytic agent and the phenytoin used as the internal standard employed in the study were abbreviated as STD and ISTD, respectively. Standard deviation was abbreviated as SD and relative standard deviation was abbreviated as RSD% calculated with the formula

Table 5. Robustness test results by changing the detector wavelength ± 1 nm % within the standard optimization conditions.

UV (nm)	Area		STD/ISTD			
	Diazepam	Phenytoin	Rationed Values	Mean ($\bar{X}$)	SD	RSD%
229	278.0	330.8	0.840	0.842	0.002	0.170
	291.3	346.2	0.842			
	289.7	345.7	0.838			
230	304.7	309.5	0.985	0.937	0.040	4.289
	278.9	297.1	0.939			
	292.0	329.5	0.886			
231	262.8	232.2	1.132	1.132	0.003	0.248
	272.4	240.0	1.135			
	287.0	254.4	1.128			
Average ($\bar{X}$)				0.969		
Standard deviation (SD)				0.149		
RSD%				15.330		

240 nm. Linearity for serum and whole blood was achieved in the range of 10 -1000 ng/mL. In this method, after oral administration of 10 mg diazepam, plasma proteins were precipitated and applied to real biological samples.

Borges et al. (2008) developed a simultaneous HPLC method for determination of diazepam and individual 6 benzodiazepines from both human plasma, using the liquid-liquid and solid-phase extraction. 5 mM KH_2PO_4 buffer solution (pH 6.0):methanol:diethyl

Table 6. Robustness test results performed by changing mobile phase pH ± 0.5 % within the standard optimization conditions.

pH value	Area		STD/ISTD			
	Diazepam	Phenytoin	Rationed Values	Mean ($\bar{X}$)	SD	RSD%
	211.7	232	0.913			
pH 2.5	212.3	232.5	0.913	0.921	0.012	1.338
	224.6	239.2	0.939			
	304.7	309.5	0.985			
pH 3	278.9	297.1	0.939	0.937	0.040	4.289
	292	329.5	0.886			
	251.9	293.1	0.860			
pH 3.5	256.6	307.7	0.834	0.854	0.014	1.682
	247.7	285.5	0.868			
Average ($\bar{X}$)				0.969		
Standard deviation (SD)				0.149		
RSD%				15.330		

Table 7. Robustness test results performed by changing the mobile phase content ± 5% within the standard optimization conditions.

Mobile phase ingradients (Acetonitrile:KH2PO4, v/v)	Area		STD/ISTD			
	Diazepam	Phenytoin	Rationed Values	Mean ($\bar{X}$)	SD	RSD%
	276.7	332.8	0.832			
45:55	278.2	346.5	0.803	0.817	0.012	1.426
	272.2	333.0	0.818			
	304.7	309.5	0.985			
50:50	278.9	297.1	0.939	0.937	0.040	4.289
	292.0	329.5	0.886			
	278.0	318.1	0.874			
55:45	276.4	316.9	0.872	0.874	0.001	0.132
	287.0	328.0	0.875			
Average ($\bar{X}$)				0.876		
Standard deviation (SD)				0.060		
RSD%				6.812		

ether (55:40:5, v/v/v) mixture was used as a mobile phase at 0.8 mL/min flow rate. LC-18 DB column (250 mm x 4.6 mm, 5 µm) was used in isocratic conditions. The UV detector wavelength detector was set at 245 nm. When using liquid-liquid extraction, the best conditions were obtained by double extraction of 0.5 mL of plasma for pH 9.5 using ethyl acetate and Na_2HP0_4. Using SPE, the best conditions were achieved with 0.5 mL of plasma and 3 mL of 0.1 M borate buffer pH 9.5. In both methods, the solvent was evaporated under a stream of nitrogen at 40 °C. For LLE plasma linearity range 50–1200 ng mL and r^2 was found as 0.9927. Plasma linearity study was implemented in the range of 30–1200 ng/mL and r^2 was found as 0.9900 (Borges et al., 2009).

In this study, an optimization study was performed on wavelength, mobile phase, column and column furnace parameters in order to determine diazepam from plasma by HPLC coupled with UV. Optimized method: in accordance with the ICH Q2(R1) guidelines , it is validated in the parameters of linearity, precision, repeatability, recovery and robustness (ICH, 2005). All samples were prepared successfully with an optimized solid phase extraction method. Simulated plasma was used in all quantification steps, and the results obtained clearly showed that the artificial plasma provided appropriate results in the development and validation of the method.

Sample preparation in the study: this was made using simulated plasma and solid-phase extraction method. The determination coefficient for 5 points in the 100-1200 ng/mL diazepam concentration range was found to be greater than 0.98, which was found to be a good value for linearity. Sensitivity values

were 20.42 and 61.86 ng/mL for LOD and LOQ, respectively. The fact that the LOQ value was lower than the last point in the calibration curve suggests that the method can safely use for the analysis of diazepam at low concentrations. The intraday repeatability study was performed with 5 replicate analyses for each concentration on the same day, and the accuracy was between (RE%) (-5.78) and 3.30, and precision (RSD%) between 0.14% and 1.59%. The repeatability study between days was performed for 5 consecutive days, and the accuracy was between (RE%) (-5.78) and 5.93, and precision (RSD%) was between 1.22% and 1.82%.

The developed method has outstanding features when compared to the methods in the literature. This method was carried out between 100 and 1200 ng/mL concentrations including subtherapeutic, therapeutic, and toxic doses of diazepam in human blood. The fact that the related method has been developed using diazepam concentrations that can be encountered in human blood further increases the importance of the relevant validation tests. The values of sensitivity data determined in both LOD and LOQ (20.42 and 61.86 ng/mL, respectively) enable the developed method to work safely at sub-therapeutic doses. It was determined that the RE% (accuracy) values obtained both within and between days were maximum (-5.78) and 5.93. These values, obtained by the analysis of independent samples on 5 different days and on the same day, are a clear statement that the method can be used safely both during and between days. In addition, the value of 1.82, obtained as the maximum RSD% value within and between days, is one of the assertive values obtained in the literature. In addition, the low mobile phase flow (0.5 mL/min) applied to the HPLC system during the analysis, the high efficiency and ease of application obtained in the developed solid-phase extraction application, the low organic solvent volume used during the extraction (≤6 mL), are other important features that make the study stand out from other studies in the literature. It was seen that the yield values obtained from the recovery tests between 95.12% and 10.83% werre directly related to the successful values obtained in the basic validation parameters such as sensitivity, selectivity, robustness and reproducibility.

CONCLUSION

In order to perform diazepam analyses on plasma, an HPLC-UV analysis method was developed, which was simple, sensitive and reliable. The analysis method, which was developed and validated and applied to simulated plasma, has a simple sample preparation method with the use of 500 µL of plasma and 1 mL of solvent, and the total analysis time is less than 11 minutes. The method was found to be linear between 400 ng/mL and 1200 ng/mL. Recovery from plasma was performed at concentrations of 200, 400 and 800 ng/mL and a high recovery value was achieved with an average of 100.39%.

It was concluded that the developed and validated HPLC-UV method is a simple, fast, sensitive, and reliable analysis method that can be used in reference laboratories that make therapeutic and toxicological impressions of diazepam.

Acknowledgement: The authors kindly would like to thanks to Forensic Science Institute belong to Ankara University and VEM Pharmaceuticals (Tekirdağ, Turkey) for the donation of diazepam and phenytoin chemical standards, respectively. Besides that, the authors appreciate to "The School of Medicine Scientific Research Center" abbreviated as CÜTFAM which belongs to Sivas Cumhuriyet University for the open collaboration shown during this scientific research.

Peer-review: Externally peer-reviewed.

Author contributions: Conception/Design of Study- E.D., B.N.D.; Data Acquisition- E.D., B.N.D.; Data Analysis/Interpretation-E.D., B.N.D.; Drafting Manuscript- E.D., B.N.D.; Critical Revision of Manuscript- E.D., B.N.D.; Final Approval and Accountability- E.D., B.N.D.

Conflict of Interest: The authors have no conflict of interest to declare.

Financial Disclosure: Authors declared no financial support.

REFERENCES

- Abou-Donia, M. B., Siracuse, B., Gupta, N., & Sobel Sokol, A. (2016). Sarin (GB, O-isopropyl methylphosphonofluoridate) neurotoxicity: critical review. *Critical Reviews in Toxicology*, *46*(10), 845–875. https://doi.org/10.1080/10408444.2016.1220916
- Bakavoli, M., & Kaykhaii, M. (2003). Quantitative determination of diazepam, nitrazepam and flunitrazepam in tablets using thin-layer chromatography-densitometry technique. *Journal of Pharmaceutical and Biomedical Analysis*, *31*(6), 1185–1189. https://doi.org/10.1016/S0731-7085(03)00027-X
- Bialer, M. (2007). *Clinical pharmacology of parenteral use of antiepileptic drugs*. *48*, 46–48. https://doi.org/10.1111/j.1528-1167.2007.01348.x
- Borges, K. B., Freire, E. F., Martins, I., & de Siqueira, M. E. P. B. (2009). Simultaneous determination of multibenzodiazepines by HPLC/UV: Investigation of liquid-liquid and solid-phase extractions in human plasma. *Talanta*, *78*(1), 233–241. https://doi.org/10.1016/j.talanta.2008.11.003
- Brieudes, V., Lardy-Fontan, S., Lalere, B., Vaslin-Reimann, S., & Budzinski, H. (2016). Validation and uncertainties evaluation of an isotope dilution-SPE-LC-MS/MS for the quantification of drug residues in surface waters. *Talanta*, *146*, 138–147. https://doi.org/10.1016/j.talanta.2015.06.073
- Calcaterra, N. E., & Barrow, J. C. (2014). Classics in chemical neuroscience: diazepam (valium). *ACS Chemical Neuroscience*, *5*(4), 253–260. https://doi.org/10.1021/cn5000056
- Chamberlain, J. M., Okada, P., Holsti, M., Mahajan, P., Brown, K. M., Vance, C., … Baren, J. (2014). Lorazepam vs diazepam for pediatric status epilepticus: a randomized clinical trial. *JAMA*, *311*(16), 1652–1660. https://doi.org/10.1001/jama.2014.2625
- Cook, P. J., Flanagan, R., & James, I. M. (1984). Diazepam tolerance: effect of age, regular sedation, and alcohol. *British Medical Journal (Clinical Research Ed.)*, *289*(6441), 351–353. https://doi.org/10.1136/bmj.289.6441.351
- de Araujo, F. G., Bauerfeldt, G. F., Marques, M., & Martins, E. M. (2019). Development and validation of an analytical method for the detection and quantification of bromazepam, clonazepam and diazepam by UPLC-MS/MS in surface water. *Bulletin of Environmental Contamination and Toxicology*, *103*(2), 362–366. https://doi.org/10.1007/s00128-019-02631-z
- Faye, C., Hen, R., Guiard, B. P., Denny, C. A., Gardier, A. M., Mendez-David, I., & David, D. J. (2020). Rapid anxiolytic effects of RS67333, a serotonin type 4 receptor agonist, and diazepam, a benzodiazepine, are mediated by projections from the prefrontal cortex to the dorsal raphe nucleus. *Biological Psychiatry*, *87*(6), 514–525. https://doi.org/10.1016/j.biopsych.2019.08.009

- Friedman, H., Greenblatt, D. J., Peters, G. R., Metzler, C. M., Charlton, M. D., Harmatz, J. S., … Francom, S. F. (1992). Pharmacokinetics and pharmacodynamics of oral diazepam: effect of dose, plasma concentration, and time. *Clinical Pharmacology and Therapeutics*, *52*(2), 139–150. https://doi.org/10.1038/clpt.1992.123
- Ghosh, P., Reddy, M. M. K., Ramteke, V. B., & Rao, B. S. (2004). Analysis and quantitation of diazepam in cream biscuits by high-performance thin-layer chromatography and its confirmation by mass spectrometry. *Analytica Chimica Acta*, *508*(1), 31–35. https://doi.org/10.1016/j.aca.2003.11.058
- Gong, W., Liu, S., Xu, P., Fan, M., & Xue, M. (2015). *Simultaneous Quantification of Diazepam and Dexamethasone in Plasma by High-Performance Liquid Chromatography with Tandem Mass Spectrometry and Its Application to a Pharmacokinetic Comparison between Normoxic and Hypoxic Rats*. 6901–6912. https://doi.org/10.3390/molecules20046901
- Herman, R. J., Van Pham, J. D., & Szakacs, C. B. (1989). Disposition of lorazepam in human beings: enterohepatic recirculation and first-pass effect. *Clinical Pharmacology and Therapeutics*, 46(1), 18–25. https://doi.org/10.1038/clpt.1989.101
- Henney, H. R., 3rd, Sperling, M. R., Rabinowicz, A. L., Bream, G., & Carrazana, E. J. (2014). Assessment of pharmacokinetics and tolerability of intranasal diazepam relative to rectal gel in healthy adults. Epilepsy research, 108(7), 1204–1211. https://doi.org/10.1016/j.eplepsyres.2014.04.007
- Hughes, D. A. (2020). Acute chloroquine poisoning: A comprehensive experimental toxicology assessment of the role of diazepam. *British Journal of Pharmacology*, *177*(21), 4975–4989. https://doi.org/10.1111/bph.15101
- ICH, I. (2005). Q2 (R1): Validation of analytical procedures: text and methodology. *International Conference on Harmonization, Geneva*.
- Kim, D. H., Cho, J. Y., Chae, S. I., Kang, B. K., An, T. G., Shim, W. S., … Lee, K. T. (2017). Development of a simple and sensitive HPLC-MS/MS method for determination of diazepam in human plasma and its application to a bioequivalence study. *Translational and Clinical Pharmacology*, *25*(4), 173–178. https://doi.org/10.12793/tcp.2017.25.4.173
- McGrath, M., Hoyt, H., Pence, A., Jayakar, S. S., Zhou, X., Forman, S. A., … Raines, D. E. (2020). Competitive Antagonism of Etomidate Action by Diazepam. *Anesthesiology*, *133*(3), 583–594. https://doi.org/10.1097/ALN.0000000000003403
- Mercolini, L., Mandrioli, R., Amore, M., & Raggi, M. A. (2008). Separation and HPLC analysis of 15 benzodiazepines in human plasma. *Journal of Separation Science*, *31*(14), 2619–2626. https://doi.org/10.1002/jssc.200800212
- Mercolini, L., Mandrioli, R., Iannello, C., Matrisciano, F., Nicoletti, F., & Raggi, M. A. (2009). Simultaneous analysis of diazepam and its metabolites in rat plasma and brain tissue by HPLC-UV and SPE. *Talanta*, *80*(1), 279–285. https://doi.org/10.1016/j.talanta.2009.06.074
- Miller, E. I., Wylie, F. M., & Oliver, J. S. (2008). Simultaneous detection and quantification of amphetamines, diazepam and its metabolites, cocaine and its metabolites, and opiates in hair by LC-ESI-MS-MS using a single extraction method. *Journal of Analytical Toxicology*, *32*(7), 457–469. https://doi.org/10.1093/jat/32.7.457
- Montoro, J., Bartra, J., Sastre, J., Dávila, I., Ferrer, M., Mullol, J., … Valero, A. (2013). H-1 antihistamines and benzodiazepines. Pharmacological interaction and their impact on cerebral function. *Journal of Investigational Allergology & Clinical Immunology: Official Organ of the International Association of Asthmology (INTERASMA) and Sociedad Latinoamericana de Alergia e Inmunología*, *23*, 17–26.
- Morelli, B. (1997). Determination of diazepam and otilonium bromide in pharmaceuticals by ratio-spectra derivative spectrophotometry. *Fresenius' Journal of Analytical Chemistry*, *357*(8), 1179–1184. https://doi.org/10.1007/s002160050327
- Muchohi, S. N., Ogutu, B. R., Newton, C. R. J. C., & Kokwaro, G. O. (2001). High-performance liquid chromatographic determination of diazepam in plasma of children with severe malaria. *Journal of Chromatography B: Biomedical Sciences and Applications*, *761*(2), 255–259. https://doi.org/10.1016/S0378-4347(01)00284-5
- Perry, P. J., Stambaugh, R. L., Tsuang, M. T., & Smith, R. E. (1981). Sedative-hypnotic tolerance testing and withdrawal comparing diazepam to barbiturates. *Journal of Clinical Psychopharmacology*, *1*(5), 289–296. https://doi.org/10.1097/00004714-198109000-00004
- Pilli, N., Narayanasamy, S., Xu, L., Chockalingam, A., Shea, K., Stewart, S., … Matta, M. (2020). A high-throughput bioanalytical assay to support pharmacokinetic interaction study of oxycodone and diazepam in Sprague Dawley rats. *RSC Advances*, *10*, 886–896. https://doi.org/10.1039/C9RA05785D
- Qin, X., Xie, H., Wang, W., He, N., Huang, S., Xu, Z., … Zhou, H. (1999). Effect of the gene dosage of on diazepam metabolism in Chinese subjects. *Clinical Pharmacology & Therapeutics*, *66*(6), 642–646. https://doi.org/10.1053/cp.1999.v66.103379001
- Richards, B. L., Whittle, S. L., & Buchbinder, R. (2012). Muscle relaxants for pain management in rheumatoid arthritis. *The Cochrane Database of Systematic Reviews*, *1*, CD008922. https://doi.org/10.1002/14651858.CD008922.pub2
- Rogers, J. J., Stanford, C., & Dart, R. C. (2006, March). The use of visual analog pain scales in black widow spider envenomation. *Journal of Medical Toxicology, 2*(1), *46–47. https://doi.org/10.1007/BF03161013*
- Taysse, L., Cal, J., Bue, J., Christin, D., Delamanche, S., & Breton, P. (2003). Comparative efficacy of diazepam and avizafone against sarin-induced neuropathology and respiratory failure in guinea pigs : influence of atropine dose. *Toxicology, 188*(2-3), 197–209. https://doi.org/10.1016/S0300-483X(03)00086-6
- Tran, N. H., Hu, J., & Ong, S. L. (2013). Simultaneous determination of PPCPs, EDCs, and artificial sweeteners in environmental water samples using a single-step SPE coupled with HPLC-MS/MS and isotope dilution. *Talanta*, *113*, 82–92. https://doi.org/10.1016/j.talanta.2013.03.072
- Uddin, M. N., Samanidou, V. F., & Papadoyannis, I. N. (2008). Validation of SPE-HPLC determination of 1,4-benzodiazepines and metabolites in blood plasma, urine, and saliva. *Journal of Separation Science*, *31*(21), 3704–3717. https://doi.org/10.1002/jssc.200800342
- Weintraub, S. J. (2017). Diazepam in the treatment of moderate to severe alcohol withdrawal. *CNS Drugs*, *31*(2), 87–95. https://doi.org/10.1007/s40263-016-0403-y
- Wexler, P., Peyster, A. De, Hakkinen, P. J., & Pope, C. (2005). *Encyclopedia of toxicology*. 2944,(783-784).
- Yasui, M., Kato, A., Kanemasa, T., Murata, S., Nishitomi, K., Koike, … Abe, K. (2005). Pharmacological profiles of benzodiazepinergic hypnotics and correlations with receptor subtypes. *Japanese Journal of Psychopharmacology*, *25*(3), 143–151.
- Yuan, H., & Pawliszyn, J. (2001). Application of solid-phase microextraction in the determination of diazepam binding to human serum albumin. *Analytical Chemistry*, *73*(18), 4410–4416. https://doi.org/10.1021/ac010227s
- Zanette, G., Manani, G., Favero, L., Stellini, E., Mazzoleni, S., Cocilovo, F., … Facco, E. (2013). Conscious sedation with diazepam and midazolam for dental patient: priority to diazepam. *Minerva Stomatologica*, *62*(10), 355–374.
- Zendelovska, D., Pavlovska, K., Atanasovska, E., Gjorgjievska, K., & Petrusevska, M. (2018). High performance liquid chromatographic method for direct determination of diazepam in whole blood and serum - optimization of solid-phase extraction method. *Prilozi*, *38*(3), 89–96. https://doi.org/10.2478/prilozi-2018-0009.
- Zhang, Y., Ouyang, K., Lipina, T. V, Wang, H., & Zhou, Q. (2019). Conditioned stimulus presentations alter anxiety level in fear-conditioned mice. *Molecular Brain*, *12*(1), 28. https://doi.org/10.1186/s13041-019-0445-4.