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Development of a liquid chromatographic method to monitorization of medazepam and lorazepam in plasma and its validation

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ABSTRACT

This study, it was aimed to develop a simple, sensitive and reliable high-performance liquid chromatographic method for simultaneous analysis of medazepam and lorazepam based on the solid-phase extraction from human blood. For the pretreatment of (500 µL) plasma sample, an efficient extraction method was developed and optimized. Separation was carried out with an ODS reverse phase C18 analytical column (150x4.0mm, 3µm). The composition of 20 mM KH_2PO_4 buffer and methyl cyanide (6:4, v/v) was employed as the mobile phase in the chromatographic system. The ultraviolet detector was set at 220nm. Determination of coefficients values was found as 0.9928 (r2) between 500-2500 ng/mL concentrations for medazepam and 0.9983 between 20-300 ng/mL for lorazepam. It was observed that the method has successful validation test results from accuracy, sensitivity, recovery, precision, and robustness in accordance with ICH Q2R1 guidelines. The method is recommended for monitoring blood levels of lorazepam and medazepam in toxicology laboratories.

Keywords: Medazepam, lorazepam, solid-phase extraction, HPLC-UV, method validation

INTRODUCTION

Medazepam, (7-chloro-2,3-dihydro-1-methyl-5-phenyl-1,4-benzodiazepine), and lorazepam (7-chloro-5-(2-chlorophenyl)-1,3-dihydro-3-hydroxy-2H-1,4-benzodiazepine-2-one) are benzodiazepine group drugs, they are used as a sedative, tranquillizer, anxiolytic, anticonvulsant, hypnotic or muscle-relaxant[1]. Medazepam (Figure 1-a) is metabolised to its active metabolites named

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diazepam, desmethyldiazepam, and oxazepam by oxidation. The elimination half-life is 2-5 hours. Lorazepam (Figure 1-b) is eliminated by the glucuronide conjugation pathway and its elimination half-life is 10-40 hours[2].

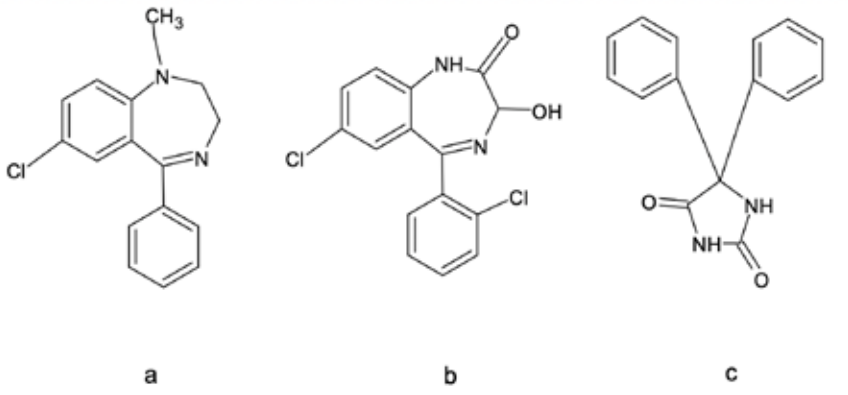

Figure 1. Representation of the chemical construction of medazepam (-a), lorazepam (-b) and phenytoin (-c) used as the internal standard.

Medazepam treatment leads to a wide range of toxicologically important effects ranged from enzyme induction-inhibition to genotoxic effects. Its chronic treatment is responsible for reducing human liver CYP2E1 enzyme activity. CYP2E1 inhibition can modulated the toxicity since some clinically used drugs or non-drug xenobiotics are mretabolised by CYP2E1[3]. In addition, the human 20α-hydroxysteroid dehydrogenase (AKR1C1) inhibition by medazepam treatment was reported[4]. Medazepam treatment is caused to a CYP3A4 induction in the study accomplished in the primary cultures of human hepatocytes in both therapeutics and intoxicated concentrations. Medazepam was found to activate the pregnane X receptor (PXR) in hepatocarcinoma cells[5]. In Chinese hamster cell culture, medazepam treatment produces chromosomal abnormalities and hyperdiploidy, including a dose-dependent reduction in diploid cell count[6]. Chronic exposure to medazepam (5 mg/kg/day, i.p.) resulted in significant reductions in the binding affinity and receptor binding capacity of rats to muscarinic receptors. In contrast, the number of muscarinic receptor binding sites in the hippocampus was decreased[7]. High medazepam concentrations were detected in the plasma of newborn babies whose mothers had been used high doses of medazepam during pregnancy. Thus, placenta seems not to be a strong barrier in the transmission of medazepam. Congenital anomallies behaviorial deviations and teratogenic effects have also been found to be associated with the high-dose medazepam use during pregnancy[8].

In a study, Iakovidou-kritsi et al. (2009), the cytostatic and cytotoxic properties of lorazepam were investigated in human lymphocyte cultures at concentrations equivalent to a daily oral dose of 1-6 mg/day[9]. It was observed that lorazepam caused genotoxic effects at these concentrations[9]. Lorazepam, like other benzodiazepines, has been reported to cause brain dysfunction, prolongation of hospital stay, coma, and even death due to overdose or suicide, and the importance of monitoring plasma drug levels in the treatment of intoxications is important[10]. It has been stated that when lorazepam is used together with sedative herbs such as valerian and passionflower, it may cause intoxications with synergistic effects by increasing the GABA-A receptor activity on the central nervous system[11]. When mouse embryonic stem cell-derived cardiomyocyte cultures and embryonic chick heart micromass (MM) were administered with a range of lorazepam concentrations, the highest lorazepam concentration was observed to cause cytotoxic effects in the embryonic chick heart micromass. It was also observed that lorazepam causes teratogenic effects in mouse embryonic stem cells[12]. Therefore, the development of a reliable, sensitive and simple method for analysis of medazepam and lorazepam is critical in terms of monitoring and treatment of possible intoxication could be considered very serious in terms of public health.

There are some methods for the individual determination of medazepam and lorazepam in the literature. These methods are based on the gas chromatography mass spectrometry (GC-MS)[13], micellar elektrokinetic capillary chromatography (MECC)[14], electrospray ionization-mass spectrometry (ESI-MS)[15], and thermal desorption direct analysis real time mass spectrometry (TD-DART-MS)[16]. Additionally, immunoassay[17], chiral column with UV and circular dichroism (CD) detection[18], voltametry[19], fourier transform infrared spectrophotometry (FTIR)[20], gas chromatography (GC)[21], high-performance liquid chromatography-electrospray tandem mass spectrometry (HPLC/MS/MS)[22], liquid chromatography-tandem mass spectrometry (LC-MS/MS)[23,24], capillary electrophoresis[25] methods were suggested for lorazepam determination. In addition, in the literature, some liquid chromatography-tandem mass spectrometry methods (LC–MS/MS) are recommended for the simultaneous determination of medazepam and lorazepam[26-28]. Described extraction methods are based on liquid-liquid microextraction[29], solid-phase extraction[30], and fiber liquid-phase microextraction (LPME)[31]. However, the fact that these are sophisticated instruments make difficult to carry out the relevant analyzes.

Most of these methods may be complicated, time-consuming, non-green and also expensive, that may require special sample preparation techniques and so-

phisticate instruments. HPLC is a relatively simple, repeatable and cost-effective method as compared to the other analysis techniques. Therefore, it not only provides excellent recovery with high sensitivity for a wide range of pharmaceutical compounds, but also provides a good separation opportunity for similar endogenous chemical structures and metabolites. It allows simultaneous determination of both main chemicals and metabolites and endogen metabolic products in biological samples. HPLC allows the separation, identification, quantitative measurement and purification of the components-analytes that make up a mixture that could be a biological, chemical or pharmaceutical sample.

Medazepam and lorazepam are used as benzodiazepine derivatives for different conditions and treatment purposes such as depression, alcohol withdrawal, sleep disorder, anxiety treatment, sedation, skeletal muscle relaxant, antiepileptic, chemotherapy-induced nausea and vomiting. Considering the toxicological risks that may be caused by the intoxications of these active substances, it is thought that it would be important to analyze medazepam and lorazepam together.

In this study, a repeatable, sensitive and reliable high-performance liquid chromatographic method based on solid-phase extraction for the monitoring of medazepam and lorazepam from human blood was developed. The developed method was validated in terms of of sensitivity, recovery, linearity, intraday and inter-day repeatability (accuracy and precision were subtitles) and robustness testsaccording to the International Conference on Harmonization guideline Q2(R1) and subsequent revisons[32].

METHODOLOGY

Chemicals and reagents

Chemical standards of medazepam and lorazepam were obtained from the Toxicology Department of Ankara University (Ankara, Türkiye) and phenytoin was donated from VEM pharmaceuticals (Istanbul, Türkiye). Analytical grade potassium chloride, sodium chloride, and sodium hydroxide were from obtained from Sigma Aldrich (Missouri, USA). Methyl cyanide (≥99.9) and methyl alcohol (≥99.9) were purchased from Riedel de Haen (Seelze, Germany). Di-sodium hydrogen phosphate and potassium dihydrogen phosphate were took from Merck (Darmstadt, Germany). Bovine albumin was provided by Solarbio Life Science (Beijing, China). Carmellose (carboxymethyl cellulose) was taken from Wenda (Izmir, Türkiye). Nylon membrane filter (0.45 µm p.s., 47 mm DIA) was supplied from Millipore (MA, USA). Dionized water was gained from the Water Purification System (Buckinghamshire, UK). The solid-phase C_{18} cartridge, Sep-Pac® Vac 1 cc (0.1 g), was purchased from Waters (Dublin, Ireland).

Instruments and equipments

Pretreatment of the specimens was achieved by a solid-phase extraction vacuum manifold that has 12- cartridge position coupled with an air pump. High-performance liquid chromatography (HPLC) system, Agilent 1100 series (CA, USA) coupled with an ultraviolet detector (G1314A, VWD), a gradient pump (G1311A, QuadPomp), a degasser (G1322A), a port of injection (Rheodyne 7725i) 20 µL sample loop and a separation oven (G1316A, Colcom) was used performing analytical separation. Separation was achieved by a C18 (150 x 4.0 mm, 3.0 µm particle size) column which was commercially named the ACE 3 (Tokyo, Japan) analytical column. The column oven temperature was set at 40 °C during the chromatographic run. The detection and quantitation was carried out with an ultraviolet detector at 220 nm. The mobile phase was prepared with potassium dihydrogen phosphate buffer (20 mM) pH 2.0 and acetonitrile (6:4, v/v). The phosphoric acid (0,1 and 1 M) solutions were used for pH adjustment of the mobile phase buffer. Mobile phase composition was applied isocratically to the column with 0.5 mL/min flow rate. The result composition of mobile phase solution was filtered with a nylon membrane filtered with a special vacuum system. Following, it was degassed for 20 minutes in an ultrasonic bath MRC ACP (London, UK). The elapsed time between the two analyzes was 5 minutes. The ChemStation® 08.3v software was used in the system control and data integration.

Stock solutions preparation

Medazepam and lorazepam were prepared as 5 mg/mL, and also phenytoin (Figure 1-c) main stock solutions were prepared as 1 mg/mL by dissolving in methyl alcohol. For this purpose, 50 mg medazepam and lorazepam, and 10 mg phenytoin were dissolved individually in 10 mL flasks. Chemical standards solutions were held at -18 °C during the study.

Artificial plasma preparation

In order to the preparation of the artificial plasma, 20 mg KCl, 20 mg KH_2PO_4, 180.6 mg $Na_2HPO_4.2H_2O$, and 0.8 g NaCl and 4 g bovine albumin were dissolved in 95 mL of deionized water. Then, the solution pH was adjusted with 0.1 M KOH to 7.4 and finally, its volume made up of 100 mL[33]. Finally, the resulting solution was divided into 500 µL microtubes and held at -18°C till to the analysis.

Determination of the internal standard

In order to using as an internal standard in this investigation, clozapine, cinnarizine, chlorpromazine, flunarizine, phenytoin and sodium valproate chemical standards were individually tested. The obtained results at the indicated

chromatographic conditions were explained in the following: It was observed that cinnarizine and clozapine have unfavourable retention times. The peak widths of cinnarizine and clozapine obtained were 3 minutes, with an unsuitable width to cause disruption of the chromatogram. When they were applied to column with medazepam individually, they caused overlapping and chromatographic problems. The sodium valproate peak was not seen in this chromatographic condition, which was thought to be related to the use of the UV wavelength. In addition to this, the peak shape and structure of chlorpromazine and flunarizine were fragmented, so they did not have enough peak sharpness for use as an internal standard.

The retention time of phenytoin in the chromatogram was between the peaks of medazepam and lorazepam. It was not showed any interaction with these peaks. It has a sharp peak structure, and the efficiency obtained in extraction. The best and reproducible recovery values were obtained with phenytoin in extraction applications. Depending of these factors, phenytoin was decided to use as an internal standard in the analysis method.

Development of the extraction method

At the begining of the sample preparation studies, liquid-liquid extraction and solid-phase extraction methods were applied both STDs and ISTD. In the liquid-liquid extraction method, the human plasma sample (0,5 mL) was treated with 1000 μL ethyl acetate. After centrifugation, the organic layer was separated to a clean micro test tube. It was evaporated at room temperature under a stream of nitrogen and redissolved in 100 μL mobile phase. Lorazepam did not have the appropriate peak area and peak height compared to the solid-phase extraction method in all liquid-liquid extraction methods that were experimented. In addition, the observed peaks had a shape that was not suitable for quantitative analysis, and they were segmented and not sharp.

The suggested SPE method were optimized in terms of the sampling, cartridge washing, elution and conditioning of the cartridge. In the conditioning step, methanol and ultrapure water application volumes were individually tested from 1 to 5 mL for the determination of the best efficiency value. The borate buffers (pH: 8.0, 8.30 and 9.0) and water were tested for the dilution of plasma samples before application to the solid phase cardridge. Also, water and borate buffers (pH: 8.0, 8.30 and 9.0) were tested for use in the elution step of SPE in volumes ranging from 1 mL to 3 mL.

Preparation of the working solutions

Working solutions of medazepam and lorazepam were prepared weekly from

main stock solutions (5 mg/mL). Medazepam were prepared as 25, 50, 75, 100, 125 μg/mL and lorazepam were prepared as 1, 2.5, 5, 10, 15 μg/mL concentrations. The main stock solution of phenytoin (1 mg/mL) was diluted by methyl alcohol to yield a working solution (50 μg/mL). Validation samples were prepared by taking 10 μL of the working solutions and dissolving them in a 500 μL virtual plasma sample. Following, it was mixed at 1800 rpm for 1 minute. Ultraviolet spectrums of medazepam (a), lorazepam (b), and phenytoin (c) were plotted at concentrations of 1 μg/mL (Figure 2).

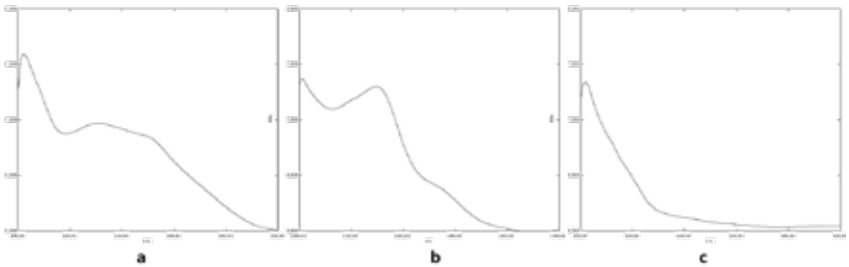

Figure 2. Ultraviolet spectrums of medazepam (a), lorazepam (b) and phenytoin (c).

Solid-phase extraction

Initially, samples used in the validation steps were prepared. For this purpose, 10 μL of the aliquot of the working solutions of medazepam, lorazepam and phenytoin were taken separately and transferred to 500 μL of virtual plasma. Then, 0.5 mL of ultrapure water was added into the plasma solutions and this composition were mixed at 3500 rpm for 3 minutes.

Following, these prepared quality control samples were used in the validation steps 1- 4.

Step-1. Conditioning: Initially, methanol (3 mL) and then 3 mL ultrapure water were applied appropriately to the cartridge.

Step-2. Sample loading: The plasma solution, the preparation of which was described above, was carefully applied to the cartridge.

Step-3. Washing: Ultrapure water (2 times x 1 mL) was used for the washing of the cartridge and then it was dried with airflow in the air pump application for 3 minutes.

Step-4. Elution: The sample collection tube was settled in the extraction manifold and 1 mL methanol (2 times) was gently applied to the cartridge then it was vacuumed (75 kPa) until reached completely for 3 minutes to recover all liquid.

The elution (2 mL) was collected in test tube and it was placed in the heated block at 40 °C then evaporated to complete dryness with the constant flow of nitrogen (1 kpascal). 200 μL mobile phase was added on the residue. Then it was shaked with the rotative shaker at 3500 rpm for 3 minutes. Finally, fhe solutions were injected into the liquid chromatography by a manual injection system of 20 μL.

RESULTS AND DISCUSSION

Method validation

The optimum conditions of UV wavelength, mobile phase content and column were determined and plasma samples which containing analytes and internal standard (phenytoin, medazepam and lorazepam) were loaded into the HPLC system under these conditions. The method was validated in accordance with the International Harmonization Conference (ICH) guidelines in terms of the recovery, linearity, accuracy, sensitivity, precision and robustness[32]. A validation protocol was applied, taking into account the reproducibility of the method, to obtain accurate and precise measurements.

Under these chromatographic conditions, analyte-free blank plasma samples were applied to the HPLC system for the determination of endogenous transitions from plasma. A chromatogram sample was given in Figure 3. Retention times of medazepam, phenytoin and lorazepam were 5.2, 6.9 and 8.1 minutes, respectively, under the determined analysis conditions. A sample chromatogram of medazepam (1500 ng/mL), lorazepam (100 ng/mL) and phenytoin (1000 ng/mL) was given in Figure 4. During the analyses, it was observed that the pressure in the system varied between 85-115 bar.

Linearity

After the chromatographic conditions were established, calibration curves for medazepam and lorazepam were formed in concentrations over the range 500 to 2500 ng/mL and 20 to 300 ng/mL versus peak-area ratios to the internal standard (phenytoin). The calibration points (n=5) were 500, 1000, 1500, 2000 to 2500 ng/mL for medazepam and 20, 50, 100, 200 to 300 for lorazepam, and they were prepared in drug-free plasma according to the standard addition method. After extraction procedures were performed, samples were applied to the HPLC system described conditions, and obtained data/values were evaluated and processed.

Calibration curves were constituted at 5 points (n=3) for both medazepam and lorazepam. They were drawn versus the area of phenytoin as an internal stand-

ard by the standard addition method and showed good correlations with r^2 = 0.9928 and 0.9983, respectively.

System suitability parameters were shown that it has a nice selectivity (α) and resolution (Rs). Theoretical plate number (N) and capacity factor (k') showed the good values for a successful determination of medazepam and lorazepam from plasma (Table 1).

Table 1. System suitability parameters and chromatographic characteristics of the developed method. Phenytoin was used as the internal standard in the method. For this reason determination coefficient (r2), selectivity factor (α), calibration range, calibration equation, and resolution (Rs) values belonging to this agent did not be calculated.

Analyte	Retention time (tR)	Capacity Factor (k')	Theoretical plate number (N)	Selectivity factor (α)	Resolution (Rs)	Calibration range (ng/mL)	Calibration equation	Determination coefficient (r^2)
Medazepam	5.0	1.2	7145	1.6	4.3	500-2500	y=0.9323x+0.1094	0.9928
Lorazepam	9.0	2.5	10462	0.3	4.2	20-300	y=2.6179x+0.0135	0.9983
Phenytoin	7.1	1.9	8568	null	null	null	null	null

Abbreviations: Theoretical plate number (N) = $16(\frac{tR}{Wt})^2$; t_R: Retention time of the analyte peak; t_o: retention time of first peak ; Wt: peak width;

Capacity factor (k') = $\frac{tR-t0}{t0}$; Resolution (R_s) = $\frac{\sqrt{N}}{4}\frac{(\alpha-1)}{\alpha}\frac{k}{(k+1)}$;

Selectivity factor (α) = $\frac{k2}{k1}$.

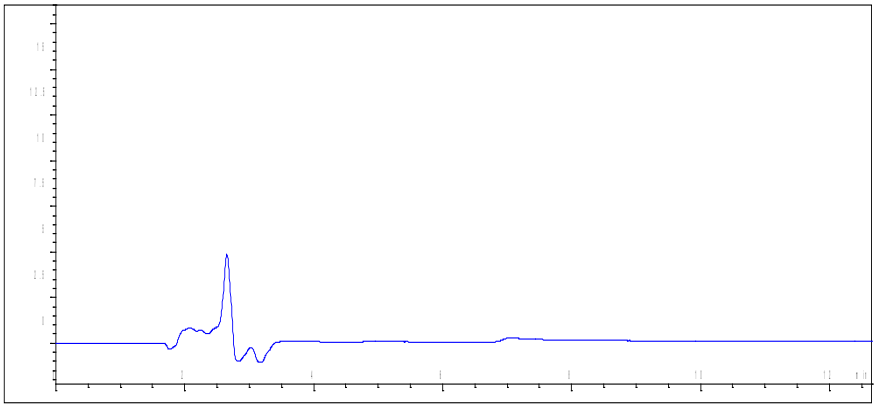

Figure 3. Chromatogram of an empty artificial plasma sample extracted by SPE method and prepared for analysis

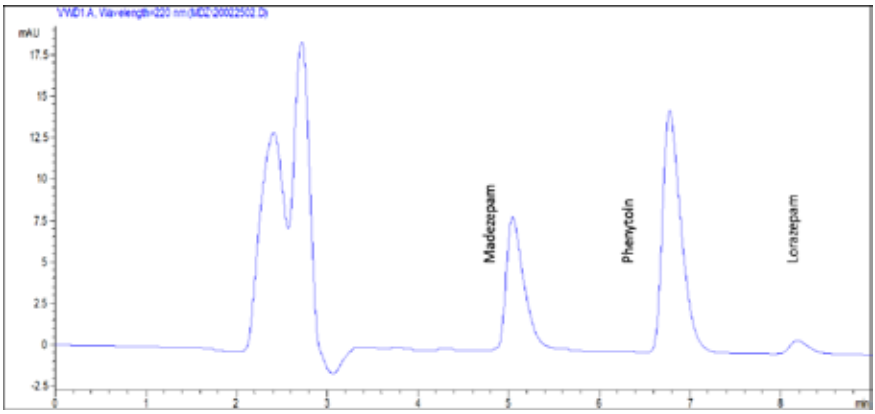

Figure 4. A typical chromatogram was obtained from the method exhibited medazepam (1500 ng/mL) phenytoin (1000 ng/mL) and lorazepam (100 ng/mL) peaks, respectivel

Sensitivity

The quantification limit (LOQ) and detection limit (LOD) were computed based on the standard deviation of the response and the slope of the calibration graph which according to the ICH recommendation. Calculations were made according to the formulas given below.

LOQ = 10 σ/S ; LOD = 3.3 **σ/S**

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

The concentration of 20 ng/mL lorazepam and 500 ng/mL medazepam were used as lowest calibration points in the determination of LOD and LOQ. A total of 10 samples prepared as described above were analyzed at the same day.

Results demonstrated that the suggested method has very low sensitivity values. LOQ and LOD values of MDZ and LRZ were calculated to range between 2.86–39.69 ng/mL and 8.67–120.29 ng/mL, respectively (Table 2).

Table 2. Sensitivity test data applied of medazepam (500 ng/mL) and lorazepam (20 ng/mL). These results were obtained from individual samples (n=10) prepared as quality control samples in plasma.

Analyte	Concentration (ng/mL)	STD/ISTD				LOD (ng/mL)	LOQ (ng/mL)
		Average	SD	Ratio	RSD%		
Medazepam	500	0.442	0.010	0.500	2.448	39.69	120.29
Lorazepam	20	0.069	0.002	0.537	3.267	2.86	8.67
Phenytoin	1000	null	null	null	null	null	null

Accuracy and precision

The accuracy defined as the relative error (bias%) was carried out with individual replicates (n=5) both in interday and intraday. The precision, defined as the relative standard deviation (RSD%), was calculated by five separate replicate analyses of medazepam and lorazepam both intraday and inter-day. Five replicate spiked samples were assayed intraday and inter-day at the three different concentrations (500, 1500 and 2500 ng/mL for medazepam and 20, 100 and 300 ng/mL for lorazepam) for all analytes.

In order to the observing of the matrix effect, blank-plasma samples were used during the validation tests and validation test samples were prepared with the standard addition method. Accuracy was calculated and presented by the recovery percentage (RE%) and it was found between -1.16 and 4.81%. Relative standard deviations (RSD%) for precision were less than 4.11. These data showed that the method can gain precise and accurate results in plasma analysis of medazepam and lorazepam. Observed results were given in Table 3.

Table 3. Confidence parameters of the method include recovery and intraday - inter-day accuracy and precision values. Results were get from individual samples (n=3) prepared as quality control samples in artificial plasma.

Analyte	Expected concentration (ng/mL)	Intra-day				Inter-day			
		No. Obs.	Observed concentration $\bar{x}$ ± SD (ng/mL)	Precision (RSD%)	Accuracy (RE%)	No. Obs.	Observed concentration $\bar{x}$ ± SD (ng/mL)	Precision (RSD%)	Accuracy (RE%)
Medazepam	500	5	524.4 ± 17.2	2.41	4.81	5	517.2±5.5	1.31	4.03
	1500	5	1569.1 ± 22.6	1.23	4.62	5	1553.6±6.9	4.11	4.56
	2500	5	2557.1 ± 23.6	0.84	4.25	5	2553.4±6.9	0.37	4.13
Lorazepam	20	5	20.4 ± 0.8	2.91	2.42	5	20.7±0.5	2.02	3.57
	100	5	103.9±0.6	0.52	3.94	5	101.9±2.6	2.46	1.96
	300	5	296.6±9.1	2.78	-1.16	5	302.9±5.5	2.43	1.88

Recovery

The recovery of extraction procedures from simulated plasma was determined by comparing pre-extraction spikes with post-extraction spikes. Individual replicates of spiked samples (n=5) at high, middle and low concentrations of medazepam (2500, 1500 and 500 ng/mL, respectively) and also lorazepam (200, 100 and 20 ng/mL, respectively) were prepared with and without internal standard. The obtained data were evaluated and processed.

Recovery values for medazepam and lorazepam were calculated with 3 replications of samples prepared separately from each other. Recovery values were calculated as between 96.20% and 98.25% for each analyte tabulated in Table 4. Absolute recoveries of medazepam and lorazepam were found as 97.23% and 96.87%, respectively.

Table 4. Observed raw recovery data and calculated recovery values of the developed analysis method

Analyte	Concentration (ng/mL)	STD/ISTD						Recovery (%)
		Non-extracted samples			Extracted samples			
		Mean (x^-)	SD	RSD%	Mean (x^-)	SD	RSD%	
Medazepam	500	510.22	22.59	4.84	490.85	0.016	3.13	96.20
	1500	1472.24	39.91	2.83	1446.51	0.122	9.39	98.25
	2500	2544.46	46.98	4.89	2474.64	0.081	4.44	97.25
Lorazepam	20	21.32	0.003	5.45	20.57	0.001	1.85	96.48
	100	101.63	0.011	4.18	99.21	0.005	1.87	97.61
	300	297.77	0.016	2.07	287.41	0.042	5.36	96.52

Robustness

Ultraviolet wavelength value (±1 nm), mobile phase organic solvent component (± 5%), buffer pH (± 0.5) and interpersonal variations did not cause any significant changes in the analysis results (Table 5). In addition, changes in analysts did not lead to significant changes in chromatographic signals, too. Separation robustness experiments demonstrated that the method created data of acceptable precision and accuracy. Robustness results were given in Table 5.

A ±1 change in UV wavelength caused a 2.68% (RSD%) change in the quantitative medazepam measurement, and a 0.21% (RSD%) change in the lorazepam measurement. A 5% solvent change in the mobile phase content caused a 1.08% RSD% change in the quantitative medazepam measurement and a 2.30% RSD% change in the lorazepam measurement. A change of 0.5 unit in mobile phase buffer pH caused a 0.72% RSD% change in the quantitative medazepam measurement and a 2.76% RSD% change in the lorazepam measurement. In the robustness test application, where the operator's effect on the method was investigated in the process from sample preparation to sample ejection and calculation, the highest RSD% value for medazepam measurement was ≤3.03% and the highest RSD% value for lorazepam measurement was observed to be ≤4.56%.

Table 5. Robustness test results were performed by changing the detector wavelength ± 1 nm, the mobile phase content ± 5%, the buffer solution pH ± 0.5 % and the interpersonal exchange within the standard optimization conditions.

UV (nm)	MDZ (Area-AU)	Lorazepam (Area-AU)	MP Comp. (ACN:KH_2PO_4, v/v)	Medazepam (Area-AU)	Lorazepam (Area-AU)	Buffer pH value	Medazepam (Area-AU)	Lorazepam (Area-AU)	Interpersonal	Medazepam		Lorazepam	
										P1	P2	P1	P2
219	1456.54	97.69	55:45	1487.80	102.41	pH 1.5	1489.19	99.12	null	1511.57	1512.87	105.45	107.98
220	1504.29	101.78	60:40	1507.71	98.67	pH 2.0	1513.58.	98.45	null	1512.21	1503.69	99.42	98.54
221	1536.97	103.97	65:35	1520.11	98.31	pH 2.5	1504.56	103.56	null	1482.52	1495.37	101.25	102.96
Mean (x^-)	1499.26	101.15	Mean (x^-)	1505.21	98.48	Mean (x^-)	1502.44	100.38	Mean (x^-)	1506.76	1501.97	101.70	101.46
SD	40.44	3.18	SD	16.29	2.27	SD	10.86	2.77	SD	16.95	8.75	3.09	4.72
RSD%	2.68	0.21	RSD%	1.08	2.30	RSD%	0.72	2.76	RSD%	1.12	0.58	3.03	4.65

Abbreviation: UV: Ultraviolet; MDZ: Medazepam; LRZ: Lorazepam; P1: Person-1; P2: Person-2; MP-Comp.: Mobile Phase Composition; ACN: Acetonitrile; KH_2PO_4: Potassium Dihydrogen Phosphate Buffer

An accurate, sensitive, and rapid analytical method was developed to be used for the analysis and separation of two benzodiazepine compounds called medazepam and lorazepam. The chromatographic conditions were as follows: a reverse phase C_{18} column was used and the column temperature was set to 40°C; the mobile phase consisted of 40% acetonitrile, and 60% 20 mM potassium dihydrogen phosphate buffer (pH: 2) which was adjusted by adding an phosphoric acid solution (100 mM). The mobile phase flow rate was 0.5 mL/min and the UV detector wavelength was set to 220 nm. Some prominent studies on the subject and their results are given below.

Al-Hawasli et al. (2012) developed a method for the quantitative determination of bromazepam, medazepam and midazolam from the mixture content and in different pharmaceutical preparations. It was used an analytical C_{18} column (250 x 4.6 mm, 3 µm) for separation and the oven was held at 50°C in the analysis. The mobile phase component was prepared with methyl alcohol: acetonitrile: 50 mM ammonium acetate-pH 9.0 (9:5:6, v/v/v). The UV detector was set to 240 nm. They used a solid phase extraction method in their work. The fact that the working range of medazepam is quite narrow, such as 80-120 µg/mL, draws attention in a negative way. On the other hand, the

developed correlation coefficient was calculated as 0.994 (r^2). Medazepam's retention time in the chromatogram was as high as ≥8.2 minutes. The LOD and LOQ were computed as 3.03 μg/mL and 10.12 μg/mL, respectively, which were lower than the sensitivity of our proposed method[34].

Jinno et al. (1998) was developed an HPLC (SPME/LC) and solid-phase microextraction for simultaneous detection of benzodiazepine, including medazepam, in human urine samples. The benzodiazepine compounds were separated Superiorex ODS column (250 × 1.5 mm i.d) at 35 °C. Acetonitrile and water (35:65, v/v) were used to form the mobile phase, and the UV wavelength was set to 220 nm for the quantitative determination. The mobile phase flow rate was 1 mL/min. The method calibration was established between concentrations of 20 and 2400 ng/mL and the determination coefficient (r^2) was calculated as 0.996. This calibration range represents a very difficult range to be linear. The limit of detection (LOD) was given as 6 ng/mL and the relative standard deviation was calculated as <15.0 (RSD%). In this method, the retention time of medazepam was 125 minutes. This value makes the efficiency of the method controversial in terms of lost mobile phase amount and time[35].

Muchohi et al. (2005) developed a method based on UV detection-high-performance liquid chromatography for the determination of lorazepam in child plasma. Oxazepam was used as an internal standard. Analytic separation was achieved by a reversed-phase column (150x 4.6 mm, 4 μm i.d.) and acetonitrile and 10 mM (pH 2.4) phosphate buffer (13:7 v/v) was used for the mobile phase. The flow rate was 2.5 mL/min. In the single-step liquid-liquid extraction protocol, n-hexane and dichloromethane (7:3 v/v) were used an organic solvent. Lorazepam-retention time was 11.9 min. A calibration curve was linear between 10 to 300 ng/mL with correlation coefficients higher than 0.99. The limits of detection and quantification were 2.5 and 10 ng/mL, respectively. The relative recoveries of lorazepam were 84.1±5.5% (n=6) and 72.4±5.9% (n=7)[36].

In Sreeram et al. (2012) was developed a high-performance liquid chromatographic (HPLC) method for the determination of lorazepam in some pharmaceutical formulations. Chromatographic analysis was performed using reversed phase ODS C_{18} column in isocratic mode with mobile phase containing methanol: water (13: 7, v/v) was used. The column was kept at ambient temperature. The mobile phase flow was 1.0 mL/min. In the determination, ultraviolet wavelength was held at 230 nm. LOD was found as 35 μg/mL and the LOQ was found as 55 μg/mL. The recovery was higher than 91%. The relative standard deviations (RSD%) was less than 5.0[37].

Uddin et al (2008) was developed a method based on the ultraviolet detection, reversed-phase high-performance liquid chromatographic method. The UV detector wavelength was set at 240 nm. In the method, they were developed an analytical method for the separation and quantification of alprazolam, bromazepam, clonazepam, diazepam, flunitrazepam, lorazepam from both pharmaceutical and biological matrices. Colchicine was used as internal standard. A Kromasil C8 column (250 × 4 mm, 5 µm i.d), equilibrated with the mobile phase methanol, ammonium acetate and 0.05 M acetonitrile (6:11:3 v/v/v) was used in the study. Mobile phase was applied to the column at the ambient temperature with a gradient flow program. A LC-18 cartridges 500 mg/3 mL and DSC-18 500 mg/3 mL were used in the determination method for the lorazepam. The calibration curve was linear in the range of 0.2 to 15 ng/mL. The correlation coefficient of calibration (r^2) was 0.990. Within-day recovery from pharmaceutical/biological samples was between 88-111% and with RSD% in the range of 0.5–11. Between-day recovery from pharmaceutical/biological samples was between 93–110% and RSD values were in the range of 1.0–13%. The LOD and LOQ values for lorazepam were found as 0.2 – 0.5 ng/mL, respectively[38].

First of all, it should be emphasized that the developed method for the determination of lorazepam (20 – 300 ng/mL) and medazepam (500 - 2500 ng/mL) in human blood is suitable not just only for the determination in the therapeutic range but also for toxic and subtherapeutic ranges of these drugs. The ability to analyze low and high concentrations of lorazepam and medazepam with the method further increased the importance of the data from the validation test in terms of the reliability of the method. The LOD and LOQ data obtained for lorazepam were 2.86 and 8.67 ng/mL, respectively, and 39.69 and 120.29 ng/mL for medazepam, indicating that the developed analysis method could be used safely at subtherapeutic doses. It was observed that the RE% (accuracy) values obtained both intraday and interdays were (-1.16) and 4.56 considering both analytes.

These data, obtained by analyzing the individual prepared samples on five different days (between days) and on the same day (within days), are a clear indication that the proposed method is repeatable and reliable both within and between days. In addition, fast run time (< 9 min), low mobile phase flow (0.5 mL/min) applied to the HPLC system during analysis, high efficiency and ease of application obtained in the extraction application, low volume of organic solvent used during extraction (≤4 mL) , are other important features that distinguish the study from other chromatographic studies in the literature. It was seen that the yield values between 96.20% and 98.25% obtained from the recovery tests

were directly related to the successful values obtained in the validation parameters such as sensitivity, selectivity, robustness and reproducibility.

The method of applying the mobile phase to the analytical column with isocratic flow has shown very significant positive effects on increasing the intraday and interday repeatability of the method (accuracy and precision). The fact that the intraday and interday precision value was ≤ 4.11 (RSD%) and the accuracy value was between -1.16 and 4.81 showed the consistency of the mobile phase content with the analysis performed. The reflections of these positive effects were also observed in the sensitivity (LOD and LOQ) values of the method. At the same time, the isocratic flow prevented the loss of mobile phase between analyzes according to the gradient flow. As it is known, at the end of the gradient flow, the change of the initial mobile phase composition should be balanced and the column should reach the initial mobile phase values again. This situation causes both cost and time consumption in analysis. Isocratic flow showed positive effects in preventing this situation and stabilizing the validation data. The suitability of the mobile phase composition and pH value of the mobile phase for analysis was observed both with the preliminary analyzes performed and with the validation test results obtained.

The analysis method developed and validated and applied to simulated plasma has a practical, economical and environmentally friendly sample preparation method with the use of 0.5 mL plasma and a total of 4 mL organic solvent for extraction, and the total analysis time is less than 8.5 minutes. The method was found to be linear between 0.5 and 2.5 µg/mL for medazepam and between 20 and 300 ng/mL for lorazepam. Recovery tests (n=3) have an average recovery value of 103.7% for medazepam and 100.1% for lorazepam.

The purpose of this study develops a sensitive, sensitive and rapid analytical method. The developed method provided successful separation and detection of medazepam and lorazepam. This RP-HPLC-UV analysis method can be used in toxicological laboratories that make therapeutic and toxicological impressions of medazepam and lorazepam.

STATEMENTS OF ETHICS

Ethical approval was not required to perform this study.

CONFLICT OF INTEREST STATEMENT

None of the authors of this article has a personal or financial relationship with any organizations that may inappropriately affect or bias the content of the paper. All authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Conception/Design of Study- E.D.; Data Acquisition- E.D.; Data Analysis/Interpretation-E.D., M.A.; Drafting Manuscript- E.D., M.A.; Critical Revision of Manuscript- E.D., M.A.; Final Approval and Accountability- E.D.

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REFERENCES

1. Papoutsis II, Athanaselis SA, Nikolaou PD, Pistos CM, Spiliopoulou CA, Maravelias CP. Development and validation of an EI-GC-MS method for the determination of benzodiazepine drugs and their metabolites in blood: Applications in clinical and forensic toxicology. J Pharm Biomed Anal. 2010;52(4):609–14. https://doi.org/10.1016/j.jpba.2010.01.027

2. Dailly E, Bourin M. The use of benzodiazepines in the aged patient: Clinical and pharmacological considerations. Pak J Pharm Sci. 2008;21(2):144–50.

3. Tassaneeyakul W, Birkett DJ, Miners JO. Inhibition of human hepatic cytochrome P4502E1 by azole antifungals, CNS-active drugs and nonsteroidal anti-inflammatory agents. Xenobiotica. 1998;28(3):293–301. https://doi.org/10.1080/004982598239579

4. El-Kabbani O, Dhagat U, Hara A. Inhibitors of human 20α-hydroxysteroid dehydrogenase (AKR1C1). J Steroid Biochem Mol Biol. 2011;125(1–2):105–11. https://doi.org/10.1016/j.jsbmb.2010.10.006

5. Vrzal R, Kubesova K, Pavek P, Dvorak Z. Benzodiazepines medazepam and midazolam are activators of pregnane X receptor and weak inducers of CYP3A4: Investigation in primary cultures of human hepatocytes and hepatocarcinoma cell lines. Toxicol Lett. 2010;193(2):183–8. https://doi.org/10.1016/j.toxlet.2010.01.004

6. Lafi A, Parry JM. A study of the induction of aneuploidy and chromosome aberrations after diazepam , medazepam , midazolam and bromazepam treatment. Mutagenesis. 1988;3(l):23–7. https://doi.org/10.1093/mutage/3.1.23

7. Popova J, Petkov V V., Tokuschieva L. The effect of chronic diazepam and medazepam treatment on the number and affinity of muscarinic receptors in different rat brain structures. Gen Pharmacol. 1988;19(2):227–31. https://doi.org/10.1016/0306-3623(88)90066-3

8. Gidai J, Ács N, Bánhidy F, Czeizel AE. A study of the effects of large doses of medazepam used for self-poisoning in 10 pregnant women on fetal development. Toxicol Ind Health. 2008;61–8. https://doi.org/10.1177/0748233708089016

9. Iakovidou-kritsi Z, Akritopoulou K, Ekonomopoulou MT, Mourelatos D. *In vitro* genotoxicity of two widely used benzodiazepines : alprazolam and lorazepam. Aristotle Univ Med J. 2009;36:39–44.

10. Pandharipande PP, Pun BT, Herr DL, Maze M, Girard TD, Miller RR, et al. Effect of sedation with dexmedetomidine vs lorazepam on acute brain dysfunction in mechanically ventilated patients: The MENDS randomized controlled trial. J Am Med Assoc. 2007;298(22):2644–53. https://doi.org/10.1001/jama.298.22.2644

11. Jang MH, Piao XL, Kim JM, Kwon SW, Park JH. Inhibition of cholinesterase and amyloid-&bgr; aggregation by resveratrol oligomers from Vitis amurensis. Phyther Res. 2008;22(4):544–549. https://doi.org/10.1002/ptr.2406

12. Ahir BK, Pratten MK. Association of anxiolytic drugs diazepam and lorazepam, and the antiepileptic valproate, with heart defects-Effects on cardiomyocytes in micromass (MM) and embryonic stem cell culture. Reprod Toxicol. 2011;31(1):66–74. https://doi.org/10.1016/j.reprotox.2010.09.008

13. Scott KS, Nakahara Y. A study into the rate of incorporation of eight benzodiazepines into rat hair. Forensic Sci Int. 2003;133(1–2):47–56. https://doi.org/10.1016/s0379-0738(03)00049-5

14. Hancu G, Gáspár A, Gyéresi Á. Separation of 1,4-benzodiazepines by micellar elektrokinetic capillary chromatography. J Biochem Biophys Methods. 2007;69(3):251–9. https://

doi.org/10.1016/j.jbbm.2006.02.003

15. Figueiredo EC, Sparrapan R, Sanvido GB, Santos MG, Zezzi Arruda MA, Eberlin MN. Quantitation of drugs via molecularly imprinted polymer solid phase extraction and electrospray ionization mass spectrometry: Benzodiazepines in human plasma. Analyst. 2011;136(18):3753–7. https://doi.org/10.1039/c1an15198c

16. Jones S, Sisco E, Marginean I. Analysis of benzodiazepines by thermal desorption direct analysis in real time mass spectrometry (TD-DART-MS). Anal Methods. 2020;12(45):5433–41. https://doi.org/10.1039/D0AY01650K

17. Smink BE, Mathijssen MPM, Lusthof KJ, De Gier JJ, Egberts ACG, Uges DRA. Comparison of urine and oral fluid as matrices for screening of thirty-three benzodiazepines and benzodiazepine-like substances using immunoassay and LC-MS(-MS). J Anal Toxicol. 2006;30(7):478–85. https://doi.org/10.1093/jat/30.7.478

18. Kanazawa H, Kunito Y, Matsushima Y, Okubo S, Mashige F. Stereospecific analysis of lorazepam in plasma by chiral column chromatography with a circular dichroism-based detector. J Chromatogr A. 2000;871(1–2):181–8. https://doi.org/10.1016/S0021-9673(99)01244-3

19. Norouzi P, Ganjali MR, Meibodi ASE. A novel adsorptive square wave voltammetric method for pico molar monitoring of lorazepam at gold ultra microelectrode in a flow injection system by application of fast Fourier transform analysis. Anal Lett. 2008;41(7):1208–24. https://doi.org/10.1080/00032710802052601

20. Konoz E, Sarrafi AHM, Samadizadeh M, Boreiri S. Quantitative analysis of lorazepam in pharmaceutical formulation through FTIR spectroscopy. E-Journal Chem. 2012;9(4):2232–8. https://doi.org/10.1155/2012/914974

21. Perez ER, Knapp JA, Horn CK, Stillman SL, Evans JE, Arfsten DP. Comparison of LC-MS-MS and GC-MS analysis of benzodiazepine compounds included in the drug demand reduction urinalysis program. J Anal Toxicol. 2016;40(3):201–7. https://doi.org/10.1093/jat/bkv140

22. Zhu H, Luo J. A fast and sensitive liquid chromatographic-tandem mass spectrometric method for assay of lorazepam and application to pharmacokinetic analysis. J Pharm Biomed Anal. 2005;39(1–2):268–74. https://doi.org/10.1016/j.jpba.2005.02.024

23. Goucher E, Kicman A, Smith N, Jickells S. The detection and quantification of lorazepam and its 3-O-glucuronide in fingerprint deposits by LC-MS/MS. J Sep Sci. 2009;32(13):2266–72. https://doi.org/10.1002/jssc.200900097

24. Turfus SC, Braithwaite RA, Cowan DA, Parkin MC, Smith NW, Kicman AT. Metabolites of lorazepam: Relevance of past findings to present day use of LC-MS/MS in analytical toxicology. Drug Test Anal. 2011;3(10):695–704. https://doi.org/10.1002/dta.305

25. Baldacci A, Thormann W. Analysis of lorazepam and its 3O-glucuronide in human urine by capillary electrophoresis: Evidence for the formation of two distinct diastereoisomeric glucuronides. J Sep Sci. 2006;29(1):153–63. https://doi.org/10.1002/jssc.200500268

26. Moretti M, Freni F, Tomaciello I, Vignali C, Groppi A, Visonà SD, et al. Determination of benzodiazepines in blood and in dried blood spots collected from post-mortem samples and evaluation of the stability over a three-month period. Drug Test Anal. 2019;11(9):1403–11. https://doi.org/10.1002/dta.2653

27. Morini L, Vignali C, Polla M, Sponta A, Groppi A. Comparison of extraction procedures for benzodiazepines determination in hair by LC-MS/MS. Forensic Sci Int. 2012;218(1–3):53–6. https://doi.org/10.1016/j.forsciint.2011.10.013

28. Kim J, Lee S, In S, Choi H, Chung H. Validation of a simultaneous analytical method for the detection of 27 benzodiazepines and metabolites and zolpidem in hair using LC-MS/MS and its application to human and rat hair. J Chromatogr B Anal Technol Biomed Life Sci. 2011;879(13–14):878–86. https://doi.org/10.1016/j.jchromb.2011.02.038

29. Molaei K, Asgharinezhad AA, Ebrahimzadeh H, Shekari N, Jalilian N, Dehghani Z. Surfactant-assisted dispersive liquid-liquid microextraction of nitrazepam and lorazepam from plasma and urine samples followed by high-performance liquid chromatography with UV analysis. J Sep Sci. 2015;38(22):3905–13. https://doi.org/10.1002/jssc.201500586

30. Lemmer P, Schneider S, Mühe A, Wennig R. Quantification of lorazepam and lormetazepam in human breast milk using GC-MS in the negative chemical ionization mode. J Anal Toxicol. 2007;31(4):224–6. https://doi.org/10.1093/jat/31.4.224

31. de Bairros AV, de Almeida RM, Pantaleão L, Barcellos T, Silva SM e., Yonamine M. Determination of low levels of benzodiazepines and their metabolites in urine by hollow-fiber liquid-phase microextraction (LPME) and gas chromatography-mass spectrometry (GC-MS). J Chromatogr B Anal Technol Biomed Life Sci. 2015;975:24–33. https://doi.org/10.1016/j.jchromb.2014.10.040

32. ICH I. Q2 (R1): Validation of analytical procedures: text and methodology. In: International Conference on Harmonization, Geneva. 2005.

33. Mercolini L, Mandrioli R, Amore M, Raggi MA. Separation and HPLC analysis of 15 benzodiazepines in human plasma. J Sep Sci. 2008;31(14):2619–26. https://doi.org/10.1002/jssc.200800212

34. Al-Hawasli H, Al-Khayat MA, Al-Mardini MA. Development of a validated HPLC method for the separation and analysis of a Bromazepam, Medazepam and Midazolam mixture. J Pharm Anal. 2012;2(6):484–91. https://doi.org/10.1016/j.jpha.2012.05.001

35. Jinno K, Taniguchi M, Hayashida M. Solid phase micro extraction coupled with semimicrocolumn high performance liquid chromatography for the analysis of benzodiazepines in human urine. J Pharm Biomed Anal. 1998;17(6–7):1081–91. https://doi.org/10.1016/S0731-7085(98)00074-0

36. Muchohi SN, Obiero K, Kokwaro GO, Ogutu BR, Githiga IM, Edwards G, et al. Determination of lorazepam in plasma from children by high-performance liquid chromatography with UV detection. J Chromatogr B Anal Technol Biomed Life Sci. 2005;824(1–2):333–40. https://doi.org/10.1016/j.jchromb.2005.07.040

37. Sreeram V, Basaveswara Rao M, Nagendrakumar A, Sivanath M, K S. Validated RP-HPLC Method for the estimation of lorazepam in pharmaceutical formulation. J Pharm Sci Innov. 2012;1(6):5–8.

38. Uddin MN, Samanidou VF, Papadoyannis IN. Development and validation of an HPLC method for the determination of six 1,4-benzodiazepines in pharmaceuticals and human biological fluids. J Liq Chromatogr Relat Technol. 2008;31(9):1258–82. https://doi.org/10.1080/10826070802019574