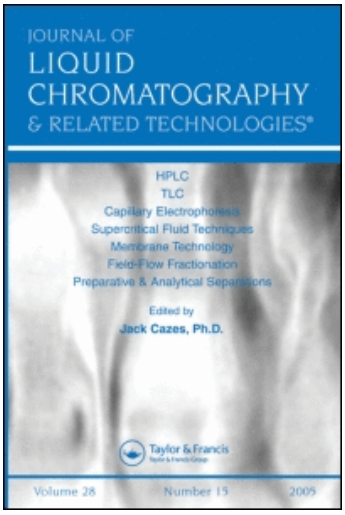

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Development and Validation of an HPLC Method for the Determination of Six 1,4-Benzodiazepines in Pharmaceuticals and Human Biological Fluids

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Abstract: A sensitive, fast, and accurate reversed phased High Performance Liquid Chromatographic method, coupled with UV/DAD set at 240 nm has been developed for the separation and quantification of six benzodiazepines (alprazolam, bromazepam, clonazepam, diazepam, flunitrazepam, lorazepam) in pharmaceutical and biological matrices after solid phase extraction. The separation was achieved within 10 min. on a Kromasill (C_8-5 μm, 250 × 4 mm, 100) analytical column using a mobile phase consisting of CH_3OH:0.05M, CH_3COONH_4:CH_3CN delivered under a gradient program at a constant flow rate of 1.0 mL/min. Colchicine (4 ng/μL) was used as internal standard. The drug/internal standard peak area ratios were linked in quadratic relationships to its concentrations giving a linearity range of 0.20–15.00 ng/μL for each BDZ, except BRZ that of which was 0.1–18.0 ng/μL with correlation coefficients greater than 0.993. The precision and accuracy were evaluated at three concentrations. Validation was also performed in terms of selectivity, extraction recovery, stability, and robustness. Recoveries from pharmaceutical/biological samples were between 88–113% and intra-inter day RSD were 0.5–12%. The method was also applied to a real urine sample from a patient under treatment with alprazolam.

Keywords: Benzodiazepines, Alprazolam, Diazepam, Flunitrazepam, Bromazepam, Clonazepam, Lorazepam, Pharmaceuticals, Biological fluids, Plasma, Urine, HPLC, SPE

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INTRODUCTION

Benzodiazepines (BDZs) belong to a group of substances known for their sedative, anti-depressive, muscle relaxant, tranquilizer, hypnotic, and anticonvulsant properties. They have become the most worldwide commonly prescribed medicines in the therapy of anxiety, sleep disorders, and convulsive attacks[1,2] as being relatively safe with mild side effects. Their sedative-hypnotic, anxiolytic, tranquilizing, and anticonvulsant effects are mediated by binding to a specific subtype of the GABAs receptor, which is mainly expressed in cortical areas and in the thalamus of all vertebrates.[3] The existence of endogenous ligands for the binding sites could be responsible for the physiological regulation of sleep, muscle tensions, and anxiety.[4]

However, on account of their excessive utilization and their implication in many cases of multi-drug abuse, BDZs are often found in fatal cases of drug intoxication. Indeed, apart from their therapeutic applications, BDZs are often used abusively with other classes of compounds such as opiates, antidepressants, and alcohol by drug addicts.[2] Because of their drug abuse potential, BDZs are frequently present in the blood of drivers involved in traffic accidents.[5] Finally, some BDZs such as flunitrazepam are deliberately misused in cases of chemical submission during sexual assault. They are often been abused by the young illicit drug user sometimes in large doses causing profound behavioral effects and their continued abuse leads to dependence.[6] The older population is not immune to using BDZs and is also often dependent on their effects.[7] BDZs may also cause or contribute to sudden death if misused.[7] Moreover, there is accumulating evidence that benzodiazepines could be regarded as natural drugs, since they have been found in trace amount also in plants and various tissues of different animal species.[8]

Since BDZs are widely seen in clinical and forensic cases,[9,10] for the bio-pharmacological, clinical, and toxicological studies of these drugs the availability of rapid, sensitive, and selective analytical methods for their determination in biological fluids and pharmaceutical formulations is essential. Researchers focused on the development of rapid, accurate, and time and cost economic methods, for the determination of BDZs. In recent years, a large number of analytical and pharmacological studies on determination of some benzodiazepines and their metabolites were reported in the literature using different techniques, such as spectrophotometry and spectrofluorimetry,[11–13] potentiometry,[14,15] indirect kinetic catalytic method by stopped flow technique,[16] modified carbon-paste electrodes as sensors,[17] immunoassay,[18] radioreceptor assay,[19,20] and chromatography (Thin Layer Chromatography (LC), High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), and Micellar Electrokinetic Chromatography (MEKC). Chromatographic methods are generally preferred for their greater selectivity and simplicity.

Chromatographic techniques, particularly HPLC, and GC have been reported for the determination of alprazolam (APZ),[21] bromazepam

(BRZ),[22] clonazepam (CLZ),[23] diazepam (DZP),[24] flunitrazepam (FNZ),[25] and lorazepam(LRZ).[26] TLC-densitometry[24] and RPLC-electrospray ionization Mass Spectrometry[27] techniques were also used for the determination of some benzodiazepines and their metabolites in tablets, rat hair, and plasma. Separation and determination of some benzodiazepines have also been performed by Micellar electrokinetic chromatography (MEKC).[28,29] Several methods using GC-nitrogen phosphorus detection (GC/NPD),[30] GC-electron capture detection (GC-ECD),[31] GC-mass spectrometry detection (GC/MS)[32–34] are available in literature. HPLC methods are effective in monitoring drugs with respect to reliability and precision and have been reported for the determination of BDZs concentration in various pharmaceutical and biological matrices coupled with UV-VIS detector or HPLC-DAD,[35–39] more recently the coupling of HPLC-MS[40] offers interesting selectivity and specificity.

Since HPLC-UV methods are inexpensive and widely utilized, has carved out a place of choice in the analysis of BDZs for simultaneous separation and quantitation. This method also serves as an advantageous alternative to those proposed earlier for the same purpose and requiring a larger sample volume, more complicated and lengthier sample preparation steps, and/or detection with a fluorescence, chemiluminescence, or electrochemical detector. Nevertheless, GC needs a derivatization step to increase the volatility of BDZs on account of their thermolability.

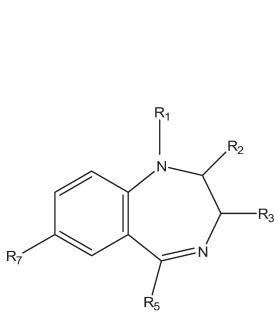
Common HPLC procedures need sample pretreatment based on protein precipitation liquid-liquid extraction,[41,42] solid-phase extraction.[43] Some 1,4-benzodiazepines have been determined in serum by direct on-column injection,[44,45] automated on-line SPE,[46] SPME,[47,48] on-line dialysis,[49] direct LC analysis using restricted access extraction materials,[37,38] column switching techniques,[50,51] as well as liquid phase microextraction.[52]

The use of SPE columns to isolate BDZs from biological samples appears to be efficient and time saving. A method for the simultaneous determination of four BDZs was previously developed by the authors.[53] The objective of this study is to considerably reduce the duration of analysis while maintaining the sensitivity required for the detection and the rapid determination of six benzodiazepines: alprazolam, bromazepam, clonazepam, diazepam, flunitrazepam, and lorazepam in their therapeutic range. The chemical structures of examined BDZs are shown in Figure 1.

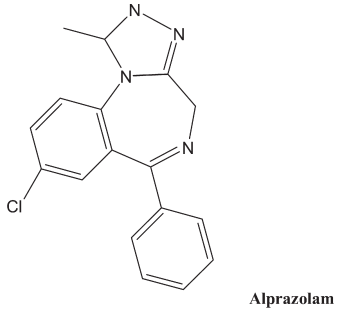
EXPERIMENTAL

Materials

Colchicine, used as the internal standard, was supplied by Merck (Darmstadt, Germany). HPLC grade methanol and ACN were supplied by Carlo Erba (Milano, Italy). Water used throughout the study was purified by the reverse



Benzodiazepine



Alprazolam

Benzodiazepine	R_1	R_2	R_3	R_5	R_7
a. Bromazepam	H	= O	H	2`-pyridyl	Br
b. Clonazepam	H	= O	H	2-Cl-phenyl	NO_2
c. Flunitrazepam	CH_3	= O	H	2-F-phenyl	NO_2
d. Lorazepam	H	= O	OH	2-Cl-phenyl	Cl
e. Diazepam	CH_3	= O	H	phenyl	Cl

Figure 1. Chemical structures of examined benzodiazepines.

osmosis method to gain high purity water with a Milli-Q water purification system from Millipore (Millipore, Bedford, MA, USA). Ammonium acetate was supplied from Riedel-de Haen (Buchs, SG, Switzerland). Six pharmaceutical formulations commercially available in Greece were used to check the applicability of the method: Hipnosedon (1 mg) by Roche (Basel, Switzerland) for FNZ, Stedon (5 mg) by Adelco S. A. (Moschato, Greece) for DZP, Lexotanil (3 mg) by Roche for BRZ, and Xanax (0.05 mg) by Pfizer Hellas (Athens, Greece) for ALP, Rivotril (0.5 mg) by Roche Hellas (Maroussi, Greece) for CLZ, and Tavor (1.0 mg) by Wyeth Hellas (Athens, Greece) for LRZ. SPE cartridges LC-18 500 mg/3 mL were supplied from Supelco (Bellefonte, PA, USA). Blood plasma samples were kindly provided from the Blood Donation Unity of a State Hospital, while urine samples were provided by healthy volunteers. Pooled samples were prepared. Urine samples containing ALP were obtained from one volunteer after oral administration.

Instrumentation

A Shimadzu (Kyoto, Japan) quaternary low pressure gradient system was used for the chromatographic determination of the examined analytes. The solvent lines were mixed in an FCV-10AL_{VP} mixer. An LC-10AD_{VP} pump equipped with a Shimadzu SCL-10AL_{VP} System Controller, permitting fully automated operation, was used to deliver the mobile phase to the analytical column.

Sample injection was performed via a Rheodyne 7725i injection valve (Rheodyne, Cotati California, U.S.A) equipped with a 20 μL loop. Detection was achieved by an SPD-M10A_{VP} Photodiode Array Detector, supplied with data acquisition software LabSolutions-LCsolutions by Shimadzu. Degassing of the mobile phase was achieved by helium sparging in the solvent reservoirs by a DGU-10B degassing unit.

The analytical column, a Kromasil, C-8 5 μm, 250 × 4 mm, was purchased from MZ-Analysentechnik (Mainz, Germany). A glass vacuum filtration apparatus obtained from Alltech Associates was employed for the filtration of the buffer solution, using 0.2 μm membrane filters obtained from Schleicher and Schuell (Dassel, Germany). The SPE study was performed on a Vac-Elut vacuum manifold column processor, purchased from Analytichem International, Varian (Harbor City, CA, USA). LC-18 cartridges 500 mg/3 mL and DSC-18 500 mg/3 mL were supplied from Supelco (Bellefonte, PA, USA), Abselut Nexus (30 mg/1 mL) from Varian, and Lichrolut RP-select B (200 mg/3 mL) from Merck. All evaporations were performed with a Supelco 6-port Mini-Vap concentrator/evaporator.

Chromatographic Conditions

Prior to analysis the reversed phase HPLC columns were equilibrated with mobile phase CH_3OH:0.05M, CH_3COONH_4:CH_3CN by the initial ratio of 30:55:15(v/v). CH_3COONH_4 solution of 0.05 M was filtered in vacuum, and the mobile phase was degassed ultrasonically prior to use, then by a stream of helium during use. The detector setting was on a UV detector at wavelength of 240 nm to monitor the column effluent. The injection volume was 20 μL. The mobile phase was pumped at a flow rate of 1.0 mL/min under ternary gradient elution at ambient temperature. Experiments were performed to determine the optimum solvent gradient that would result in the maximum number of detectable peaks with high resolution in the chromatogram. The optimum gradient elution program was as presented in Table 1.

Standard Solutions

Standards of BDZs were accurately weighed, transferred to volumetric flasks, and dissolved in methanol to make individual stock solutions of 1.0 mg/10 mL. These solutions were thoroughly mixed and stored at 4°C, tightly closed, until use, which were stable for at least a month. Interim mixture solutions were prepared daily at the working concentrations of 0.2, 0.5, 1.0, 3.0, 5.0, 10.0, 15.0, and 20.0 ng/μL of each BDZ and diluted with methanol along with the addition of internal standard, so as to gain a

Table 1. Gradient programs for the separation of the examined benzodiazepines

		Solvent				
Program	Time	CH_3OH	CH_3CN	0.05M CH_3COONH_4	Flow rate (mL/min)	R. time (min)
A[a]	0.01	30.00	15.00	45.00	1.0	9.50
	2.00	50.00	18.30	31.70		
	3.00	50.00	20.00	30.00		
B[b]	0.01	30.00	15.00	45.00	1.00	10.60
	3.00	50.00	16.90	33.10	1.10	10.00
	8.00	50.00	20.00	30.00		

[a]Program A has been followed throughout the experiment.
[b]Program B was followed for robustness study.

constant concentration of 4 ng/μL for each working solution. The internal standard solution was also prepared in methanol yielding a concentration of 1.0 mg/10 mL.

Optimization of SPE Protocol

Solid phase extraction protocols were optimized in terms of recovery studies of BDZs prior to the application to biological fluids. Various extraction protocols to isolate the analytes from samples and different solvents or mixture of solvents at different compositions for elution of the adsorbed analytes were tested. Recovery after SPE was measured by comparison of peak area ratios of extracted standard solutions versus non-extracted solutions.

Sample Preparation and Analysis

Pharmaceutical Samples

Ten tablets were finely ground and powdered. An accurately weighed portion equivalent to 1 mg/10 mL solution for each compound, was transferred to a volumetric flask, and diluted up to the mark with methanol. The solution was sonicated for 15 min and centrifuged at 3000 rpm for 10 min, and filtered through a 0.2 μm filter. The solution was stored at 4°C in the refrigerator. Accurately measured aliquots of the supernatant were transferred to 25 mL flasks and diluted with methanol to give final concentrations of 0.5, 3.0, 5.0 ng/μL of analytes, each containing 4.0 ng/μL internal standard.

Biological Samples

Sample pretreatment: Prior to extraction, biological fluids were pretreated for the removal of proteins by precipitation with the addition of 200 μL of acetonitrile to aliquots of 50 μL of pooled blood plasma, or 100 μL of urine samples containing 200 μL of methanol for blanks, or 200 μL standard solutions at six concentration levels of 0.5, 1, 2, 3, 5 and 10 ng/μL of experimental standard solutions for spiked samples. After centrifugation for 15 min at 3000 rpm, the clear supernatant was transferred to glass tubes and evaporated to 50 μL under a nitrogen flow at 30°C. With addition of 2 mL distilled water it was applied to a cartridge preconditioned previously with 2 mL of methanol and 2 mL of water for extraction. Retained analytes were eluted with MeOH:CH_3CN (50:50) followed by the evaporation of elution to dryness under a gentle flow of N_2 at 30°C. Analytes were reconstituted with 200 μL of MeOH and aliquots of 20 μL were injected onto the column.

Validation of Method

The parameters linearity, selectivity, extraction recovery, precision, accuracy, stability, and robustness were studied for the validation of the method.

Linearity

Linearity was determined by constructing the calibration curves. Aliquot volumes of stock solutions of benzodiazepines in methanol were transferred to a series of 25 mL volumetric flasks to produce mixture solutions at concentration levels 0.2, 0.5, 1.0, 3.0, 5.0, 8.0, 10.0, 15.0, 20.0 ng/μL for each drug. A volume equivalent to 4 ng/μL of IS was added to each flask and the solution was diluted to the mark with methanol. Calibration standards of each concentration were analyzed in triplicate and curves of BDZs were constructed using peak area ratio of drug to the internal standard versus nominal concentrations of the analytes. For biological fluids, the calibration curve was constructed using the same working solutions at conc. 0.5, 1.0, 3.0, 5.0, and 10.0 ng/μL for human plasma and 1.0, 2.0, 3.0, 5.0, 8.0, 10.0 ng/μL for urine, followed by the pretreatment step. Least square linear regression analysis of the date gave slops, intercept, and correlation coefficient.

Recovery

Aliquots of 20 μL of the selected assay solutions at the concentration levels 0.05, 1.0, 5.0 ng/μL were injected into the HPLC system and triplicate measurements were recorded for each concentration. The nominal contents of the drug in each solution were calculated from the linear regression equations. The results were expressed as percent recoveries of the particular

components in the samples as [mean found concentration/theoretical concentration] × 100.

The recovery of the solid phase extraction procedure was assessed by analyzing extracts of spiked (plasma and urine) samples containing benzodiazepines at conc. 1.0, 3.0, 5.0 ng/μL and the extract efficiency was determined based on the peak area ratios obtained for the linearity study. The peak area ratios for solutions of BZDs to spiked samples were compared with those derived from non-extracted standard solutions in methanol at same concentration levels. Assuming the detector response ratios for solutions in methanol to represent 100% recovery, the recovery of each compound from spiked samples was calculated using the equation (ratio of spiked samples × 100/ratio of standard solution in methanol).

Precision/Accuracy

The precision of the method was determined by calculating the relative standard deviation (RSD) for the repeated measurements, and the accuracy as the standard deviation (SD) between nominal and measured concentrations. The accuracy of the method was tested by analyzing different concentrations of standard BDZs.

Within-day and between-day precision and accuracy of the assay were assessed by performing replicate analyses of BDZs samples in standard solutions and biological fluids (plasma and urine) against a calibration curve. Within-day repeatability was determined in six replicates at three concentrations levels of 0.5, 1.0, 5.0 ng/μL of BDZs in both standards and in spiked samples at 1.0, 3.0, 5.0 ng/μL levels in the same day. The procedure was repeated on six different days, in standard solution and spiked samples at the respective concentration levels, to determine between-day repeatability.

Specificity

The specificity was demonstrated showing that BDZs were free of interference from degradation products and in biological samples by the analysis of five blank matrices, and it was assessed by the absence of interference in the same retention times as examined for benzodiazepines.

Sensitivity

Sensitivity of the method has been tested by examining the Limit of Detection (LOD) and Limit of Quantification (LOQ) values. The LOD, the lowest absolute concentration of analyte in a sample that can be detected but not necessarily quantified and the LOQ, the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy, were calculated. The limit of detection was calculated from the calibration

graph by the formula; $LOD = 3\ S_{xy}/a$, and the limit of quantification; $LOQ = 10 \cdot S_{xy}/a$, where S_{xy} is the standard deviation and a is the slope.

Stability of Standard Solution

The stability of sample solutions was tested by the proposed HPLC method over a period of 30 days. The freshly prepared solutions at room temperature, and the 30 days stored samples in a refrigerator, were analyzed. Increased biological plasma and urine samples spiked with 3 ng/μL of benzodiazepine were subjected to deproteinization and stored at ambient temperature and in a freezer at 4°C for 30 days. Short time stability was assessed after 12 h at room temperature, 24 h of storage in a refrigerator, and for long term assay after 5, 7, and 30 days refrigerated. Each sample was analyzed for intact BDZs compounds once daily after a freeze thaw cycle for investigation of stability, when spiked samples were allowed to thaw at room temperature and analyzed. Recovery and RSD of the stored samples were calculated and compared to that of freshly prepared samples.

Robustness

The optimum HPLC conditions set for this method have been slightly modified by the small changes of the flow rate and gradient program, as a means to evaluate the method's robustness.

RESULTS AND DISCUSSIONS

The validated method developed here was applied to various concentrations taken from the pharmaceutical products and spiked biological samples for determining the content of BDZs. The linearity and sensitivity data are presented in Table 2. System suitability, robustness, and stability analytical data are shown in Tables 3, and 4, respectively. All analytes were found to be stable for seven freeze thaw cycles except for lorazepam. All analytes were found to be stable for one month except for bromazepam, clonazepam, and flunitrazepam which were stable for one week. Table 5 summarizes precision and accuracy data, indicating that these values are acceptable and the method is accurate and precise.

Chromatographic Conditions

The mobile phase was a mixture of CH_3OH, 0.05M CH_3COONH_4, and CH_3CN equilibrated initially by 30:45:15 and gradient pressure was used. The separation of six benzodiazepines and the internal standard was achieved in less than 10 min. Under the assay conditions described above the examined drugs were

Table 2. Linearity and sensitivity data for proposed method (x = ng/μL)

Analyte	Linearity equation	r^2	LOQ (ng/μL)	LOD (ng/μL)	Upper limit (ng/μL)
Standard					
Bromazepam	$y = 0.2400x - 0.0476$	0.998	0.2	0.067	18
Clonazepam	$y = 0.3475x - 0.067$	0.996	0.1	0.033	15
Flurinitrazepam	$y = 0.2075x - 0.0066$	0.998	0.2	0.067	15
Lorazepam	$y = 0.3332x - 0.0325$	0.990	0.1	0.033	15
Alprazolam	$y = 0.1944x + 0.0239$	0.996	0.2	0.067	15
Diazepam	$y = 0.3589x + 0.0102$	0.993	0.1	0.033	15
Plasma					
Bromazepam	$y = 0.2342x - 0.0269$	0.9997	0.5	0.167	18
Clonazepam	$y = 0.1805x + 0.0392$	0.998	0.2	0.067	15
Flunitrazepam	$y = 0.1683x + 0.0318$	0.9990	0.5	0.167	15
Lorazepam	$y = 0.2343x + 0.0915$	0.9990	0.2	0.067	15
Alprazolam	$y = 0.1746x + 0.0123$	0.998	0.5	0.167	15
Diazepam	$y = 0.2671x + 0.1303$	0.9990	0.2	0.067	15
Urine					
Bromazepam	$y = 0.1703x + 0.0355$	0.997	0.5	0.167	18
Clonazepam	$y = 0.144x + 0.0373$	0.9996	0.2	0.067	15
Flunitrazepam	$y = 0.1581x - 0.0006$	0.9990	0.5	0.167	15
Lorazepam	$y = 0.2102x + 0.0049$	0.998	0.2	0.067	15
Alprazolam	$y = 0.1526x - 0.0316$	0.9990	0.5	0.167	15
Diazepam	$y = 0.2339x + 0.031$	0.9996	0.2	0.067	15

Table 3. System suitability and robustness data

	System suitability (RSD)		Robustness (% recovery)		
			Program A	Program B	
Analyte	R_t	Area ratio	F. R 1.0 mL/min	F. R 1.0 mL/min	F. R 1.1 mL/min
BRZ	0.4	3.4	107.0	107.7	108.4
CLZ	0.6	5.0	109.3	110.3	111.1
FTZ	0.6	9.2	98.0	107.2	110.7
LRZ	0.6	1.0	92.7	100.4	99.6
ALZ	0.6	7.7	103.5	101.6	101.9
DZP	0.7	6.3	106.0	102.2	107.8

Table 4. Long-term stability and freeze-thaw stability data

	Analyte					
	Bromazepam	Clonazepam	Flunitrazepam	Lorazepam	Alprazolam	Diazepam
Freeze-thaw stability % recovery of analytes at different freeze-thaw cycles						
1	103.3	109.5	99.4	102.6	99.2	98.0
2	95.2	93.4	99.8	106.0	98.5	106.8
3	99.9	105.4	105.0	107.6	107.2	114.0
4	102.0	95.7	97.7	106.1	99.5	108.1
5	99.78	99.3	103.5	105.7	96.9	100.4
6	99.8	103.2	98.0	102.7	104.8	95.1
7	95.7	96.8	100.4	92.4	98.5	95.1
Long-term stability % recovery of analytes at different days						
1d	109.4	101.1	100.9	108.0	93.6	103.3
2d	104.6	103.5	108.5	107.6	115.1	113.1
5d	97.8	98.7	100.3	108.2	112.9	112.0
7d	95.1	93.0	91.2	101.5	96.9	104.8
1m	94.5	91.9	91.2	105.8	102.3	105.3

Table 5. Within-day (n = 6) and between-day (n = 6) precision and accuracy results

		Within-day			Between-day		
Analyte	Added (ng/μL)	Found ± SD (ng/μL)	Recovery (%)	RSD	Found ± SD (ng/μL)	Recovery (%)	RSD
Standard solution							
BRZ	0.50	0.54± 0.04	107.1	6.9	0.53 ± 0.03	106.6	5.6
	1.00	0.93 ± 0.06	93.0	6.9	1.04 ± 0.09	103.9	8.2
	5.00	0.93 ± 0.06	110.8	2.4	5.28 ± 0.34	105.5	6.5
CLZ	0.50	0.54 ± 0.02	107.3	3.5	0.55 ± 0.06	110.1	10.7
	1.00	0.93 ± 0.04	93.2	4.7	0.94 ± 0.06	94.5	6.6
	7.50	7.72 ± 0.18	103.0	2.3	7.83 ± 0.56	104.4	7.3
FTZ	0.50	0.46 ± 0.02	92.0	5.6	0.52 ± 0.04	103.3	10.0
	1.00	0.93 ± 0.06	93.3	6.6	0.93 ± 0.06	93.0	7.7
	5.00	4.95 ± 0.17	99.0	3.4	5.07 ± 0.38	101.4	7.5
LRZ	0.50	0.47 ± 0.02	94.8	4.9	0.49 ± 0.04	98.4	8.4
	1.00	1.07 ± 0.02	107.0	2.2	0.93 ± 0.07	95.1	8.0
	5.00	5.39 ± 0.08	107.8	1.5	5.44 ± 0.41	108.9	7.5
ALZ	0.50	0.47 ± 0.04	93.9	10.9	0.49 ± 0.04	97.8	9.6
	1.00	0.97 ± 0.05	97.0	6.0	1.08 ± 0.05	107.6	4.3
	5.00	5.46 ± 0.11	109.2	2.0	5.11 ± 0.44	102.2	7.8
DZP	0.50	0.52 ± 0.05	104.6	9.6	0.47 ± 0.04	93.6	10.0
	1.00	1.03 ± 0.08	103.5	8.6	0.98 ± 0.05	98.3	5.0
	5.00	5.44 ± 0.10	108.8	1.9	5.52 ± 0.42	110.4	7.6

(*continued*)

Table 5. Continued

Analyte	Added (ng/μL)	Within-day			Between-day		
		Found ± SD (ng/μL)	Recovery (%)	RSD	Found ± SD (ng/μL)	Recovery (%)	RSD
Spiked plasma							
BRZ	1.00	0.97 ± 0.03	97.0	6.3	0.96 ± 0.02	96.5	10.6
	3.00	3.22 ± 0.03	107.3	4.1	2.97 ± 0.09	99.1	12.0
	5.00	4.84 ± 0.12	96.8	10.1	5.24 ± 0.16	104.9	13.7
CLZ	1.00	1.11 ± 0.02	111.3	6.6	1.70 ± 0.01	107.0	5.3
	3.00	3.24 ± 0.05	108.0	7.4	2.26 ± 0.06	108.8	9.3
	5.00	4.89 ± 0.02	97.7	2.3	4.74 ± 0.03	94.7	3.4
FTZ	1.00	1.90 ± 0.03	109.0	11.6	1.03 ± 0.02	102.6	10.6
	3.00	3.13 ± 0.02	104.4	4.1	3.18 ± 0.02	106.0	3.9
	5.00	4.74 ± 0.03	94.8	3.6	4.73 ± 0.03	94.6	3.2
LRZ	1.00	1.09 ± 0.03	108.9	9.0	0.99 ± 0.01	99	2.0
	3.00	3.17 ± 0.04	105.6	4.4	3.22 ± 0.01	107.4	0.8
	5.00	5.00 ± 0.01	100.0	0.5	4.90 ± 0.02	98.0	2.0
ALZ	1.00	1.00 ± 0.01	100.0	4.8	1.13 ± 0.01	113	1.8
	3.00	3.11 ± 0.02	103.8	3.8	3.24 ± 0.01	108.1	1.5
	5.00	5.08 ± 0.01	101.7	0.5	4.77 ± 0.02	95.4	2.1

DZP	1.00	0.91 ± 0.03	91.0	9.4	0.99 ± 0.02	99.0	7.2
	3.00	3.29 ± 0.02	109.7	1.8	3.21 ± 0.07	107.0	6.6
	5.00	5.22 ± 0.08	104.4	5.3	5.18 ± 0.05	103.8	3.1
Spiked urine							
BRZ	1.00	0.92 ± 0.02	92.0	11.3	0.90 ± 0.01	90.0	7.7
	3.00	2.94 ± 0.04	98.2	8.4	2.82 ± 0.05	94.0	9.1
	5.00	5.15 ± 0.05	103.2	5.5	4.87 ± 0.05	97.3	5.9
CLZ	1.00	0.88 ± 0.01	88.0	6.2	0.90 ± 0.01	90.0	7.5
	3.00	2.97 ± 0.03	99.1	5.8	2.94 ± 0.01	97.9	3.0
	5.00	5.08 ± 0.03	101.7	3.3	4.85 ± 0.05	97.0	6.7
FTZ	1.00	1.05 ± 0.01	105.0	6.2	1.07 ± 0.01	107.0	5.6
	3.00	2.95 ± 0.04	98.4	7.7	2.86 ± 0.03	95.4	5.2
	5.00	4.87 ± 0.05	97.4	6.4	4.46 ± 0.04	89.2	5.4
LRZ	1.00	0.91 ± 0.01	91.0	7.4	1.10 ± 0.01	110.0	1.3
	3.00	3.21 ± 0.02	107.1	3.4	3.07 ± 0.03	102.6	4.0
	5.00	5.33 ± 0.04	106.6	3.0	4.85 ± 0.08	96.9	7.8
ALZ	1.00	1.01 ± 0.01	101.0	5.6	1.12 ± 0.01	112.0	3.7
	3.00	3.13 ± 0.01	104.2	6.3	2.89 ± 0.01	96.4	3.4
	5.00	5.26 ± 0.08	105.3	10.2	4.49 ± 0.01	97.7	1.2
DZP	1.00	1.00 ± 0.01	100.0	3.3	0.99 ± 0.03	99.0	11.9
	3.00	3.03 ± 0.06	101.2	9.4	3.03 ± 0.02	101.1	2.2
	5.00	5.52 ± 0.08	110.5	5.8	4.97 ± 0.06	99.5	5.1

well resolved. Resolution factors were in the range 0.6–4.3, indicating a sufficient separation. R_s values were calculated by the formula:

$$R_s = 2(t_2 - t_1)/(w_2 + w_2)$$

where, t_1 and t_2 are the retention times and w_1 and w_2 the baseline peak width of successive peaks. Retention times of the examined benzodiazepines are 5.28 ± 0.06 for the internal standard, 6.18 ± 0.02 for BRZ, 6.63 ± 0.04 for CLZ, 6.85 ± 0.04 for FNZ, 7.26 ± 0.05 for LRZ, 7.60 ± 0.05 for ALP, and 9.44 ± 0.07 min for DZP. Precision of retention times was examined to evaluate system suitability from within-day repeatability (mean value of six measurements, n = 18) and between-day precision (mean value of three measurements during six days, n = 54) at 3.0 ng/μL level of benzodiazepines, which revealed RSD values of 0.4–0.7%. A typical chromatogram of standard solution is presented in Figure 2.

Optimization of SPE Protocol

After investigating various SPE sorbents and elution systems, the optimum conditions were found using LC-18 cartridges and mixture of acetonitrile

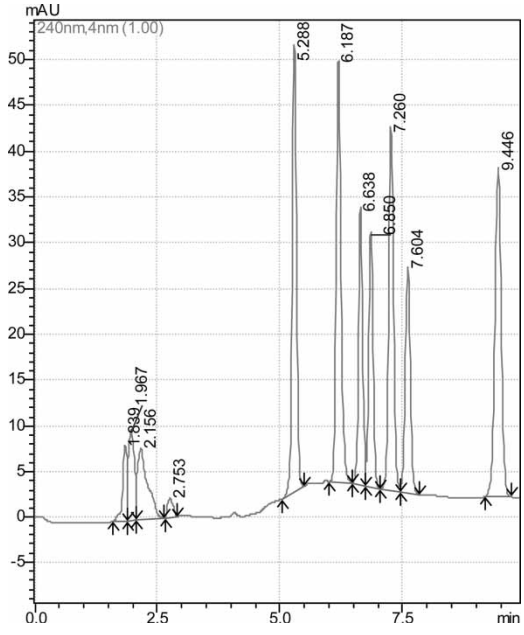


Figure 2. Typical HPLC chromatogram of the six examined benzodiazepins (3 ng/μL) in the presence of colchicine (4 ng/μL) as internal standard. Chromatographic conditions are described in text. Peaks: 5.288 min (IS), 6.187 min (BRZ), 6.638 min (CLZ), 6.850 min (FNZ), 7.260 min (LRZ), 7.604 min (APZ), and 9.446 min (DZP).

and methanol by 50:50 as eluent. Optimum protocol yielded high absolute recovery rates of 100.2% for BRZ, 86.5 for CLZ, 91.3% for FNZ, 95.8 for LRZ, 90.0% for ALP, and 93.1% for DZP. Recovery results using different sorbents and eluents are given in Tables 6 and 7.

Validation of the Method

The described reversed phase liquid chromatography method was developed using a simple mobile phase to provide a rapid quality control determination of benzodiazepines in tablets and biological samples. All samples were analyzed using the assay chromatographic conditions described.

Linearity

The calibration curves were linear in the range of 0.2–15 ng/μL for all but BRZ, the range of which was 0.2–18 ng/μL. The coefficients of determination (r^2) were greater than 0.993 for all analytes. Results are summarized in Table 2.

Recovery, Precision, and Accuracy

The results obtained from the within-day accuracy study at three concentrations ($n = 6$) indicated high recoveries of BDZs by the proposed method: 92–109% for standards, 91–111% for spiked plasma and 88–110% for spiked urine, with RSD in the range 0.5–11%. Between-day recovery was 93–110% for standards, 94–113% for spiked plasma and 89–112 % for urine samples. RSD values were in the range 1.0–13% indicating high precision.

Specificity

Specificity is the ability of the method to measure the analyte in the presence of its potential impurities. The specificity was demonstrated showing that BDZs was free of interference from degradation products and absence of

Table 6. Recovery of BDZs using different SPE cartridges

	Recovery (%)					
Adsorbents	BRZ	CLZ	FTZ	LRZ	ALP	DZP
Nexus	99.3	88.7	90.5	91.0	90.1	94.1
LC-18	100.2	86.5	91.3	95.8	90.0	93.1
DSC-18	61.1	50.1	71.9	73.0	55.2	79.2
RP select B	62.7	43.0	45.0	60.4	64.0	64.4

Table 7. Recovery of BDZs using different eluents LC-18 SPE cartridge

	Recovery (%)					
Eluents	BRZ	CLZ	FTZ	LRZ	ALP	DZP
MeOH	67.2	61.2	93.6	92.3	96.1	100.9
ACN	111.8	84.5	133.8	111.3	133.1	143.6
MeOH:ACN = 50:50	99.3	88.7	90.5	91.0	90.1	94.1
MeOH:ACN = 40:60	105.4	81.9	84.8	85.9	86.2	90.3
MeOH:ACN = 30:70	83.6	77.8	85.6	81.5	84.9	87.4

any peak in blank biological fluids coincides to that of any analyte, as well as IS indicates the high specificity of the method and can be used in a stability assay. Typical chromatograms of blank and spiked plasma samples are illustrated in Figures 3a and 3b, respectively, while those for blank and spiked urine samples are presented in Figures 4a and 4b.

Sensitivity

The limit of detection (LOD) and the limit of quantitation (LOQ) for BDZs were 0.033–0.167 ng/μL and 0.2–0.5 ng/μL, respectively, for standard and spiked samples. The results of the statistical analysis of the

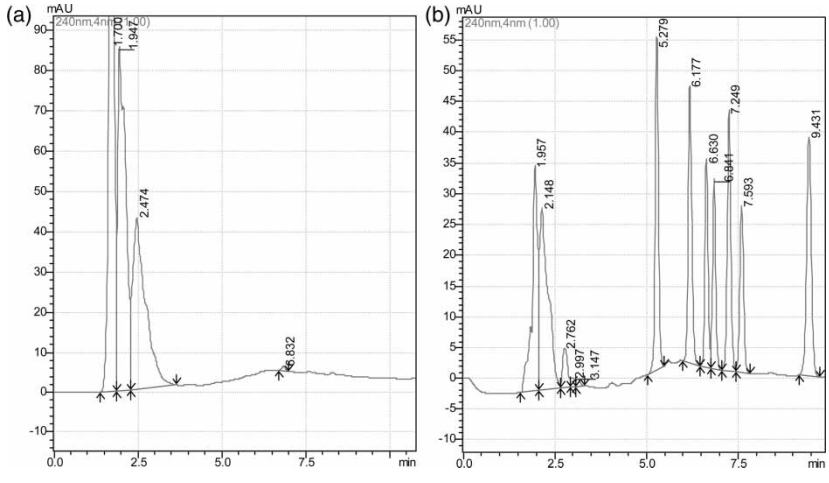


Figure 3. (a) High performance liquid chromatogram of benzodiazepines in blank human plasma and (b) spiked human plasma at 3 ng/μL, after SPE using the conditions described in text. Peaks: 5.279 min (IS), 6.177 min (BRZ), 6.630 min (CLZ), 6.841 min (FNZ), 7.249 min (LRZ), 7.593 min (APZ), and 9.431 min (DZP).

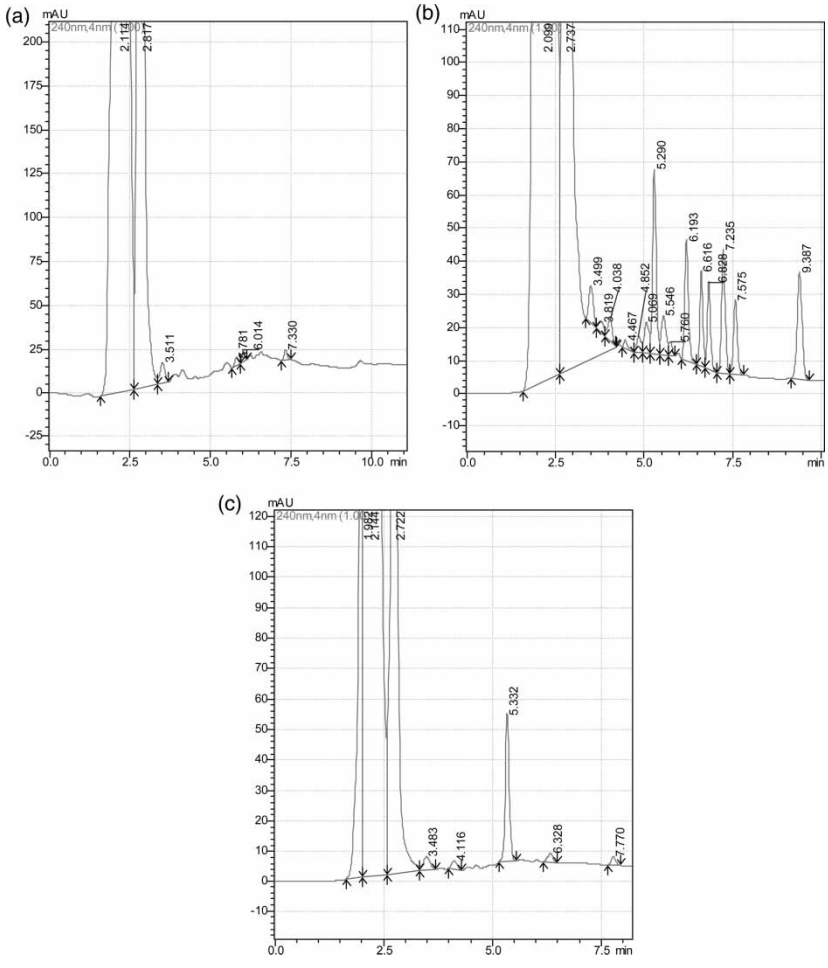


Figure 4. High performance liquid chromatogram of (a) blank human urine, (b) benzodiazepines in spiked human urine at 3 ng/μL, after SPE using the conditions described in text. Peaks: 5.290 min (IS), 6.193 min (BRZ), 6.616 min (CLZ), 6.828 min (FNZ), 7.235 min (LRZ), 7.575 min (APZ), and 9.387 min (DZP). (c) urine from a human volunteer after oral administration: 7.770 min (ALP).

experimental data, such as the slopes, the intercepts, and sensitivity data are shown in Table 2.

Stability of Solution

The stability of sample solutions and solutions in spiked samples were tested by the proposed HPLC method over a period of 30 days for freshly prepared solutions at room temperature, and up to 30 days of stored samples in a

refrigerator. From the comparison of results, we can conclude that there were no degradation products and the drug was stable at 4°C for at least 30 days, indicating the possibility of using BDZ samples over a period of 30 days at refrigeration without degradation.

Robustness

It was found that the percent recoveries of BDZs were excellent under most conditions, and remained unaffected by small deliberate changes of experimental parameters including the flow rate and gradient program (Table 1). Variation in the experimental parameters, as well as carrying out the experiment at room temperature, provided an indication of its reliability during normal use and concluded that the method was robust.

System Suitability

The system suitability was assessed by replicate injections of the sample at a concentration of 3 ng/μL including intra-inter day assessments for standard, spiked plasma and urine samples. Precision of retention time and peak area ratios was examined to evaluate the system suitability. RSD of the peak area ratios 1.0–9.2, and that of retention time 0.6–0.7, indicates excellent suitability of the system.

Application to Real Samples

Application to Pharmaceutical Products

The method developed here was applied to various concentrations (1.0, 3.0, 5.0 ng/μL) of solutions made from pharmaceutical products for determining the content of BDZs. The values of the overall drug percentage recoveries and the RSD value of BDZs are 89–109% and 0.3–12%, respectively, as presented in Table 8, indicating that these values are acceptable and the method is accurate and precise. Moreover determination was free of interference from degradation products and no interference from the sample excipients could be observed at this detection wavelength, indicating the high specificity of the method.

Application to Urine Samples after Oral Administration

The method was applied to the determination of ALP in urine samples taken from one volunteer under regular treatment (0.25 mg). Standard addition method was applied to the analysis of urine sample. Regression equation after standard addition to the urine sample yielded ALP a concentration of

Table 8. Results of pharmaceuticals' analysis

Real sample	Analytes	Tablets	Added (ng/μL)	Found ± SD (ng/μL)	RSD	Recovery (%)	Per tablet (mg)	Mean
Pharmaceutical formulations	Bromazepam	Lexotanil (3.0 mg)	1.00	1.07 ± 0.07	6.6	107.5	3.22	3.18 ± 0.11
			3.00	3.06 ± 0.35	11.6	101.9	3.06	
			5.00	5.46 ± 0.11	2.0	109.2	3.27	
	Clonazepam	Rivotril (0.50 mg)	1.00	1.10 ± 0.03	0.1	90.2	0.45	0.48 ± 0.03
			3.00	2.95 ± 0.07	0.1	98.4	0.49	
			5.00	5.03 ± 0.05	0.03	100.6	0.50	
	Flurinitrazepam	Hipnosedon (1.0 mg)	1.00	1.09 ± 0.05	4.2	109.2	1.09	1.03 ± 0.12
			3.00	3.54 ± 0.18	5.2	10.7	1.11	
			5.00	4.51 ± 0.26	5.7	90.2	0.90	
	Lorazepam	Tavor (1.0 mg)	1.00	0.93 ± 0.01	0.03	93.0	0.93	0.91 ± 0.02
			3.00	2.72 ± 0.03	0.04	90.5	0.91	
			5.00	4.42 ± 0.03	0.02	89.4	0.88	
	Alprazolam	Xanax (0.50 mg)	1.00	1.06 ± 0.07	6.5	105.6	0.53	0.50 ± 0.04
			2.50	2.23 ± 0.04	1.6	90.1	0.45	
			5.00	5.25 ± 0.54	10.4	105.1	0.52	
	Diazepam	Stedon (5.0 mg)	1.00	0.94 ± 0.02	2.5	94.2	4.71	5.11 ± 0.40
			3.00	3.30 ± 0.09	2.6	110.0	5.50	
			5.00	5.11 ± 0.63	12.3	102.2	5.11	
		Valium (5.0 mg)	1.00	0.95 ± 0.03	3.1	94.5	4.73	5.15 ± 0.38
			3.00	3.15 ± 0.19	6.1	105.0	5.25	
			5.00	5.46 ± 0.34	6.3	109.2	5.46	

Table 9. Results of urine analysis after oral administration of Xanax (ALP)

Added (ng/μL)	Found ± SD (ng/μL)	RSD	ALP concentration (ng/μL)	Mean concentration (ng/μL)
0.00	0.64 ± 0.01	5.51	0.64	0.66 ± 0.03
1.00	1.62 ± .01	3.64	0.62	
3.00	3.68 ± .02	4.53	0.68	
5.00	5.69 ± .02	2.90	0.69	

0.66 ng/μL as shown in Table 9. A chromatogram of urine analysis after oral drug administration is shown in Figure 4c.

CONCLUSION

The method described herein is a simple and rapid assay for the quantitative determination of six benzodiazepines: ALP, BRZ, CLZ, DZP, FNZ, and LRZ in pharmaceuticals and biological matrices. Selective extraction of analytes from the matrix containing the drugs and specific separation among benzodiazepine drugs were achieved by means of SPE. Repeatability ($n = 6$) and between-day precision over a period of 6 days revealed RSD values lower than 12%. Recoveries ranged from 88 to 113%. The detection limit of the method was calculated as 0.033–0.167 ng/μL in standard, blood plasma and urine for 20 μL of injection volume. The present method has a comparable sensitivity with respect to existing methods for the determination of benzodiazepines, with the great advantage of a routine and less expensive instrumentation. The method was successfully applied to real urine samples after alprazolam oral administration. The method is suitable for the pharmacokinetics and bioavailability studies of benzodiazepines and drug quality control. It is also a useful tool in human medicine for estimating and personalizing the effective drug dose in patients. Moreover, it can be used in forensic toxicology.

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