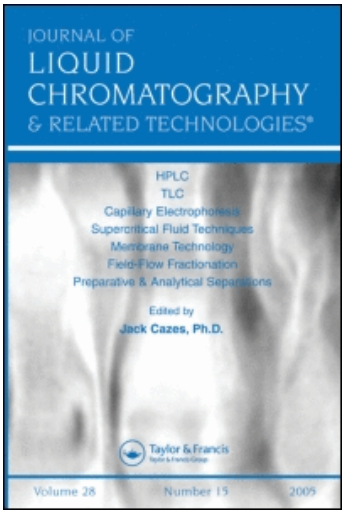

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HPLC Method for Simultaneous Determination of 1,4-Benzodiazepines and Tricyclic Antidepressants in Pharmaceutical Formulations and Saliva—A Useful Tool in Medicinal Chemistry

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Abstract: Herein, a sensitive and rapid reversed phase HPLC assay was developed for the simultaneous determination of 1,4-benzodiazepines and tricyclic antidepressants in human saliva and pharmaceutical formulations using colchicine as internal standard. Diode array detection (DAD) was carried out at a monitoring wavelength of 240 nm. The analytes and the internal standard were extracted by Nexus (Varian) SPE cartridge and separated by 15 min, utilizing a Kromasil C_8 (150 × 4.6 mm; 5 μm) column with a gradient mobile phase containing methanol, acetonitrile, and 0.05 M ammonium acetate, delivered at a flow rate of 1.0 mL/min. Calibration curves were linear up to the examined range of 20 ng/μL with coefficients of determination (r^2) 0.999. The limits of detection (LOD) and quantification (LOQ) were 0.08–0.34 and 0.28–1.13 ng/mL, respectively, using 20 μL injected volumes. The mean relative recoveries of all analytes in saliva were 85–105% ($n = 6$) and 86–84 % ($n = 6$) for intra- and inter day, respectively. Analysis for benzodiazepines and tricyclic antidepressants gave good precision (RSD <10%). The sample preparation was very simple and the method was sensitive enough and reproducible. The method described here is a useful tool in medicinal chemistry, suitable for use both in clinical analysis, as well as for quality control of pharmaceutical formulations.

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Keywords: Benzodiazepines, HPLC, Pharmaceuticals, Saliva, SPE, Tricyclic antidepressants

INTRODUCTION

Both benzodiazepines and tricyclic antidepressants have been introduced for the treatment of a wide spectrum of clinical disorders and constitute large and important classes of pharmaceutical active drugs. Since discovery of diazepam (Valium) and chlordiazepoxide (Librium), more than thirty benzodiazepines have become the drugs of choice for the treatment of anxiety, sleep disorders, status epilepsy, insomnia, frequent nocturnal awakenings, early morning awakenings, and other convulsive disorders as they possess antiepileptic hypnotic, tranquillizing, anticonvulsant, sedative, muscle relaxant, and amnesic properties.[1–4] Alprazolam, the prominent member of benzodiazepines belonging to the new class of triazolobenzodiazepines, has gained widespread use due to its potent action at low doses. It also shows antidepressant activity in addition to the pharmacological action common to other benzodiazepines.[5] Extensive use of benzodiazepines has lead to overuse and abuse, resulting in toxicity and causing profound behavioural effects.[6] They may also cause or contribute to sudden death if misused.[7] Furthermore, tricyclic antidepressants (TCAs), prominently amitriptyline, clomipramine, doxepine, are the most popular class of drugs in the treatment of depression. For the medical treatment of depression and neurosis, the concomitant administration of tricyclic antidepressants and benzodiazepines is widely used. The concomitant use of these drugs had no effect on the drug metabolizing system; also interaction of these drugs presented no serious problem. The efficacy of monitoring TCAs to assess compliance, toxicity, clinical response, and drug interactions is well documented.[8] Unrestricted use of these drugs concomitantly with other drugs warrants careful monitoring to avoid the toxic effects of drug interactions. In view of the above considerations, a versatile analytical procedure is needed to assay both classes of drugs, particularly in a setting where a patient has taken them concurrently. Therefore, in clinical and forensic cases their measurement is widely practiced.[9,10] Though numerous HPLC methods for the determination of benzodiazepines or tricyclic antidepressants have been reported, none of the methods is applicable to their simultaneous determination.

Blood and urine are the conventional specimens to document Drug Facilitated Crimes,[11] but there have been few reports in practice for oral fluid.[12–15] The advantages of oral fluid over traditional specimens like blood or urine are the non-invasive, easy collection protocol, and the fact that sampling can be achieved under close supervision and can

be considered as an additional source of information.[16] Oral fluid is rather recommended for workplace drug testing or for roadside testing of intoxicated drivers.[17,18] The most common way for drug molecules to pass from blood to oral fluid is passive diffusion through lipid membranes, which is restricted to molecules that are uncharged and lipophilic, and not highly bound to plasma proteins. Since the salivary drug concentration correlates with the free and pharmacologically active concentration of drugs in plasma, for certain drugs the concentration in oral fluid may reflect drug effects better than the total plasma concentration, suggesting the qualitative measurements in saliva may be a valuable technique to determine the current degree of exposure to a definite drug at the time of sampling.[14,15]

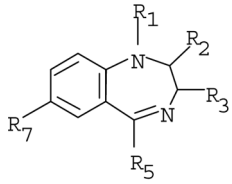
Benzodiazepines undergo extensive biotransformation in the liver, mainly through N-demethylation, 1 or 3-hydroxylation and glucuronidation, and in the case of nitro benzodiazepines by the reduction of the nitro group to an amine with subsequent acetylation.[17–20] Due its high sedative potency, therapeutic doses of benzodiazepines are low and the effective concentration in plasma or serum is correspondingly low (ng/mL to ng/μL)[21] consequently to saliva (μg/L range, even in the ng/L range),[22] which is related to the therapeutic dose.[23,24] Since the plasma concentration of the unchanged drug is usually at low (ng/mL) levels, a sensitive analytical technique is required in both pharmacokinetic study and therapeutic or forensic assay.[25]

Routine analysis of benzodiazepines and tricyclic antidepressants comprises various techniques such as spectrophotometry and spectrofluorimetry,[26,27] potentiometry,[28] indirect kinetic catalytic method by stopped flow technique,[29] non-aqueous capillary electrophoresis,[30] modified carbon paste electrodes as sensors,[31] immunoassay,[32,33] radioreceptor assay,[34] separation techniques TLC densitometry, [35,36] and RPLC electrospray ionization mass spectrometry,[37,38] UPLC-MS/TOF,[39] micellar electrokinetic chromatography (MEKC),[40,41] GC-nitrogen phosphorus detection (GC/NPD),[42] GC electron capture detection (GC-ECD),[43] GC mass spectrometry detection (GC/MS),[44,45] GC-FID[46] and HPLC coupled with fluorescence detector,[47] chemiluminescence detector,[48] UV-VIS detector or HPLC-DAD,[49,50] HPLC-ECD,[51] more recently the coupling of HPLC-MS[52,53] or HPLC-MS/ESI,[54] HPLC-MS/MS[55] offering interesting selectivity, specificity, reliability, and precision for the determination of both benzodiazepines, tricyclic antidepressants and their metabolites in tablets, hair and biological fluids.

Either liquid-liquid extraction,[12–15,56] solid phase extraction,[24,57,58] or solid phase micro extraction[59] was usually employed to perform matrices removal and analyte preconcentration prior to HPLC separation, which could enrich the analyte by several folds even 1–2 orders of

magnitude. Among them, SPE still has been used widely, due to its advantages such as less solvent use and time saving. Furthermore, selective elution of SPE provides the additional advantage in fractionation of two different class drugs. Authors improved a method for the separation of both drugs, either by single step or in step wise (sequential) elution from plasma and urine samples.[60] As per our knowledge, only one method was reported using SPE for oral fluid. Therefore, the aim of this study was to develop a relatively simple, sensitive, rapid method for the simultaneous determination of six 1,4-benzodiazepines and three tricyclic antidepressants (Table 1) in a single chromatogram from human saliva samples after SPE. The determination of these drugs in pharmaceutical formulations has been described as well. The analysis can be applied for drug determination in tablet formulations in a reasonable time, eliminating interferences due to possible impurities.

Table 1. Chemical structure of analytes



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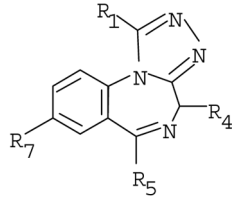
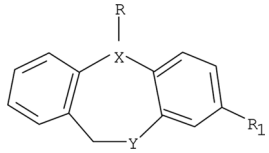


II

Benzodiazepines	Group	R_1	R_2	R_3/R_4	R_5	R_7
Bromazepam (BRZ)		H	=O	H	2′-pyridyl	Br
Clonazepam (CLZ)	I	H	=O	H	2-Cl-phenyl	NO_2
Diazepam (DZP)		CH_3	=O	H	phenyl	Cl
Flunitrazepam (FNZ)		CH_3	=O	H	2-F-phenyl	NO_2
Lorazepam (LRZ)		H	=O	OH	2-Cl-phenyl	Cl
Alprazolam (APZ)	II	CH_3	–	H	phenyl	Cl



Tricyclic antidepressants	X	Y	R	R_1
Amitriptyline	C	C	$=CHCH_2CH_2N(CH_3)_2$	H
Clomipramine	N	C	$-CH_2CH_2CH_2N(CH_3)_2$	Cl
Doxepine	C	O	$=CHCH_2CH_2N(CH_3)_2$	H

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 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). Doxepine (DXP), Amitriptyline (ATP), and Clomipramine (CLP), were purchased from Sigma (St. Louis, MO, USA). Pharmaceutical formulations commercially available in Greece were analysed to check the applicability of the method: Sinequan discs (25 mg) for DXP by Pfizer (Athens, Greece), Saroten capsules (25 mg) for ATP by H. Lundbeck A/S (Copenhagen, Denmark), Anafranil discs (10 mg) and Anafranil injection (25 mg/2 mL) for CLP by Novartis Pharma AG(Basle, Switzerland). Hipnosedon (1 mg) by Roche (Basel, Switzerland) for flunitrazepam (FNZ), Stedon (5 mg) by Adelco S. A. (Moschato, Greece) for Diazepam (DZP), Lexotanil (3 mg) by Roche for Bromazepam (BRZ), and Xanax (0.05 mg) by Pfizer Hellas (Athens, Greece) for Alprazolam) ALP, Rivotril (0.5 mg) by Roche Hellas (Maroussi, Greece) for Clonazepam (CLZ), and Tavor (1.0 mg) by Wyeth Hellas (Athens, Greece) for Lorazepam (LRZ). 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. LC-18 cartridges were supplied from Varian and Oasis from Waters (Milford, MA, USA). Saliva samples were provided from healthy volunteers. Pooled samples were prepared.

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, complied with Data acquisition software Lab Solutions-LC solutions by Shimadzu. Degassing of the

mobile phase was achieved by helium sparging in the solvent reservoirs by a DGU-10B degassing unit. The system was controlled and data were analyzed on a computer equipped with LC solution software.

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). SPE was carried out on a 12 port-vacuum manifold from Supelco. All evaporations were performed with a Supelco 6-port Mini-Vap concentrator/evaporator.

Saliva Collection

An oral fluid sample was collected from one male volunteer in a polypropylene tube over 240 min by spitting at fixed time intervals, without any stimulation of the saliva production, to obtain approximately 3 mL of sample. For a period of 30 min before sampling, no food or drink was taken by the donor and the mouth was rinsed with fresh water before spitting.[15,22] As soon as possible after collection without any preservative, the supernatant of saliva, obtained after centrifugation at 2500 g for 10 min to remove potential food debris, was separated into 2 mL aliquots in Eppendorf tubes and stored at −20°C until analysis (performed within 15 days).

Chromatographic Conditions

Prior to analysis, the reversed phase HPLC columns were equilibrated with mobile phase CH_3OH: 0.05 M CH_3COONH_4:CH_3CN by the initial ratio of 30:50:20 (v/v/v). A gradient program, as presented in Table 2, at a flow rate of 1.0 mL/min at ambient temperature was applied. The ammonium acetate solution was filtered in vacuo, and the mobile phase was degassed prior to use by a stream of helium. The detector setting on the DAD was wavelength of 240 nm to monitor the column effluent. The injection volume was 20 μL. 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.

Standard Solutions

Authentic standards of BDZs, TCAs, and internal standard were accurately weighed, transferred to volumetric flasks, and dissolved in methanol to

Table 2. Gradient programs examined in this study

Program	Time (min)	Solvent (0.05 M) CH_3OH	CH_3CN	CH_3COONH_4	Flow rate (mL/min)	R.time (min)
A*	0.01	30.00	15.00	55.00	1.0	14.61 ± 0.13
	2.00	50.00	16.60	33.40		
	6.00	30.00	20.00	50.00		
	9.00	55.00	22.50	22.50	1.1	13.68 ± 0.05
	12.00	55.00	25.00	20		
B	0.01	30.00	15.00	55.00	1.00	13.08 ± 0.012
	2.00	50.00	17.30	32.70		
	6.00	30.00	22.00	48.00		
	9.00	60.00	26.00	14.00		
	12.00	60.00	30.00	10		

*Program with flow rate 1.00 mL/min has been followed throughout the experiment.

make individual stock solutions of 100 ng/μL. These solutions were thoroughly mixed and stored tightly closed at 4°C until use. These were stable for at least two months. Interim mixture solutions were prepared daily at five working concentrations levels in range 0.2–20.0 ng/μL of each analyte and diluted with methanol, along with the addition of internal standard, so as to gain a constant concentration of 4 ng/μL.

Pharmaceutical Formulations

Five tablets or the content of six capsules were finely ground and powdered. An accurately weighed portion equivalent to 100 ng/μL solution for each compound, was transferred to volumetric flasks, dissolved, 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. All stock solutions were stored at 4°C in the refrigerator. Dilution was done to accurately measured aliquots of the stock solution with methanol to give final concentrations of 5.0, 7.5, 10.0 ng/μL of individual analyte, each containing 4.0 ng/μL internal standard.

SPE Protocol

Selection of Sorbent and Eluting Solvents

Solid phase extraction protocols were optimized in terms of recovery studies of analytes prior to the application to oral 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. After investigating various SPE sorbents and elution systems, the optimum conditions were found using Nexus (Varian) cartridge and methanol as the elution solvent. Optimum protocol yielded high recovery rates of 103.7% for BRZ, 105.6 for CLZ, 101.9% for FNZ, 99.1% for LRZ, 106.3% for ALP, 99.1% for DXP, 98.1% for DZP, 98.7% for ATP, and 98.5% for CLP.

Sample Preparation

Preparation of Spiked Samples

Of experimental standard stock solution 200 μL at seven concentration levels of 0.2–20 ng/μL for spiked samples or 200 μL of methanol for blank samples was added to aliquots of 500 μL saliva.

Solid Phase Extraction

A spiked saliva sample was applied to the SPE cartridge, preconditioned previously with 2 mL of methanol and 2 mL of water, for extraction without protein removal, as oral fluids contain a low amount (0.3%) of protein. After washing with 1 mL of water, retained drugs were eluted with (2 × 1) mL MeOH, followed by the evaporation to dryness under a gentle flow of N_2 at 30°C. The residues were reconstituted with 200 μL of methanol prior to their injection into the chromatographic system or the tubes were closed before being stored at −20°C. Aliquots of 20 μL were injected onto column.

Validation of the Method

The method was validated in terms of ICH analytical performance parameters;[61] precision, accuracy, specificity, limit of detection, limit of quantitation, linearity and range, suitability and robustness.

RESULTS AND DISCUSSION

The validated method developed here was applied to saliva samples spiked at various concentrations for determining the content of both

BDZs and TCAs. The linearity and sensitivity data are presented in Table 3, the values of the overall drug percentage recoveries and RSD values in Tables 4 and 5, system suitability and robustness analytical data

Table 3. Linearity and sensitivity data for proposed method

Analyte	Linearity equation	r^2	LOQ (ng/μL)	LOD (ng/μL)	Upper limit (ng/μL)
	Standard				
Bromazepam	$y = (0.313 \pm 0.001)x - (0.059 \pm 0.01)$	0.999	0.32	0.097	20
Clonazepam	$y = (0.201 \pm 0.001)x + (0.009 \pm 0.008)$	0.999	0.40	0.12	
Flunitrazepam	$y = (0.159 \pm 0.001)x + (0.013 \pm 0.008)$	0.999	0.50	0.15	
Lorazepam	$y = (0.232 \pm 0.001)x + (0.037 \pm 0.01)$	0.999	0.43	0.13	
Alprazolam	$y = (0.206 \pm 0.001)x - (0.011 \pm 0.006)$	0.999	0.28	0.08	
Doxepine	$y = (0.103 \pm 0.001)x + (0.014 \pm 0.005)$	0.999	0.55	0.16	
Diazepam	$y = (0.273 \pm 0.001)x + (0.016 \pm 0.01)$	0.999	0.47	0.14	
Amitriplyline	$y = (0.135 \pm 0.001)x + (0.078 \pm 0.01)$	0.999	0.80	0.24	
Clomipramine	$y = (0.058 \pm 0.001)x + (0.040 \pm 0.006)$	0.999	1.11	0.31	
	Saliva				
Bromazepam	$y = (0.291 \pm 0.002)x + (0.020 \pm 0.02)$	0.999	0.88	0.26	20
Clonazepam	$y = (0.172 \pm 0.001)x + (0.049 \pm 0.01)$	0.999	0.53	0.16	
Flunitrazepam	$y = (0.121 \pm 0.001)x + (0.158 \pm 0.01)$	0.999	0.90	0.27	
Lorazepam	$y = (0.220 \pm 0.001)x - (0.004 \pm 0.01)$	0.999	0.55	0.17	
Alprazolam	$y = (0.178 \pm 0.001)x - (0.054 \pm 0.009)$	0.999	0.54	0.16	
Doxepine	$y = (0.127 \pm 0.001)x + (0.110 \pm 0.006)$	0.999	0.52	0.16	
Diazepam	$y = (0.274 \pm 0.002)x - (0.271 \pm 0.03)$	0.999	1.13	0.34	
Amitriplyline	$y = (0.140 \pm 0.001)x - (0.040 \pm 0.006)$	1.00	0.43	0.13	
Clomipramine	$y = (0.046 \pm 0.001)x + (0.082 \pm 0.003)$	0.999	0.78	0.23	

Table 4. Intra-day analytical data for the proposed method

	Standard solution				Spiked saliva			
Analytes	Added	Found ± SD	Recovery (%)	RSD	Added	Found ± SD	Recovery (%)	RSD
Bromazepam	2.00	1.99 ± 0.01	99.6	1.9	1.00	1.01 ± 0.02	100.5	3.7
	5.00	4.98 ± 0.07	99.6	4.4	2.00	1.97 ± 0.05	98.6	4.1
	10.00	10.04 ± 0.04	100.4	1.2	4.00	4.01 ± 0.11	100.3	4.6
Clonazepam	2.00	1.94 ± 0.01	97.0	2.8	1.00	0.99 ± 0.02	99.3	4.3
	5.00	5.22 ± 0.007	104.4	0.1	2.00	1.99 ± 0.03	99.9	3.5
	10.00	9.99 ± 0.03	99.9	1.4	4.00	3.93 ± 0.05	98.3	3.6
Flunitrazepam	2.00	1.90 ± 0.02	95.1	7.2	1.00	0.99 ± 0.01	99.0	3.7
	5.00	5.25 ± 0.01	105.1	1.5	2.00	2.04 ± 0.04	101.8	6.0
	10.00	9.97 ± 0.02	99.8	1.3	4.00	3.99 ± 0.05	99.8	4.4
Lorazepam	2.00	1.94 ± 0.04	96.9	0.9	1.00	1.01 ± 0.02	100.5	5.7
	5.00	5.28 ± 0.02	105.5	1.3	2.00	2.01 ± 0.04	100.3	4.6
	10.00	10.02 ± 0.02	100.2	0.7	4.00	4.02 ± 0.07	100.5	4.2

Alprazolam	2.00	1.92 ± 0.005	96.1	1.3	1.00	1.01 ± 0.01	100.9	4.4
	5.00	4.98 ± 0.006	99.7	0.6	2.00	2.10 ± 0.03	105.1	4.5
	10.00	10.00 ± 0.04	100.1	1.9	4.00	4.14 ± 0.04	103.5	2.7
Doxepine	2.00	1.95 ± 0.008	97.4	3.8	1.00	0.96 ± 0.01	95.6	1.5
	5.00	4.85 ± 0.004	97.0	0.8	2.00	1.79 ± 0.03	89.3	4.6
	10.00	10.14 ± 0.02	101.4	1.9	4.00	3.42 ± 0.03	85.6	2.8
Diazepam	2.00	1.90 ± 0.006	95.1	1.2	1.00	1.05 ± 0.01	105.5	2.3
	5.00	5.24 ± 0.03	104.7	1.9	2.00	2.01 ± 0.04	100.4	4.5
	10.00	9.98 ± 0.06	99.8	2.1	4.00	3.94 ± 0.04	98.5	2.3
Amitriplyline	2.00	2.08 ± 0.02	104.1	6.4	1.00	0.93 ± 0.01	92.8	4.9
	5.00	5.16 ± 0.07	103.1	9.0	2.00	1.91 ± 0.01	95.3	2.7
	10.00	9.99 ± 0.05	99.9	3.6	4.00	3.76 ± 0.03	94.1	3.2
Clomipramine	2.00	2.10 ± 0.01	104.9	7.9	1.00	0.87 ± 0.01	86.9	3.9
	5.00	4.71 ± 0.005	94.2	1.7	2.00	1.86 ± 0.01	92.9	4.0
	10.00	10.10 ± 0.01	101.1	2.3	4.00	3.72 ± 0.01	93.0	1.9

Table 5. Inter-day analytical data for the proposed method

	Standard solution				Spiked saliva			
Analytes	Added	Found ± SD	Recovery (%)	RSD	Added	Found ± SD	Recovery (%)	RSD
Bromazepam	2.00	1.97 ± 0.03	98.7	5.5	1.00	1.01 ± 0.01	101.1	0.7
	5.00	5.02 ± 0.06	100.3	3.9	2.00	1.98 ± 0.05	99.0	4.3
	10.00	9.31 ± 0.03	93.1	0.9	4.00	4.01 ± 0.14	100.2	5.9
Clonazepam	2.00	1.92 ± 0.03	96.0	8.7	1.00	0.98 ± 0.02	98.4	6.1
	5.00	4.70 ± 0.03	94.1	3.3	2.00	2.00 ± 0.02	100.2	2.7
	10.00	9.43 ± 0.11	94.3	5.7	4.00	3.91 ± 0.08	97.8	5.6
Fluni-	2.00	1.95 ± 0.03	97.6	9.4	1.00	1.00 ± 0.02	100.0	4.7
trazepam	5.00	4.75 ± 0.02	94.9	2.7	2.00	2.04 ± 0.03	101.8	4.0
	10.00	9.40 ± 0.10	94.0	6.8	4.00	3.95 ± 0.06	98.8	5.5
Lorazepam	2.00	1.89 ± 0.02	94.4	4.5	1.00	1.01 ± 0.01	100.5	3.1
	5.00	4.72 ± 0.10	94.4	9.5	2.00	1.98 ± 0.06	99.2	7.0
	10.00	9.68 ± 0.03	96.8	1.1	4.00	4.01 ± 0.10	100.3	5.8
Alprazolam	2.00	1.91 ± 0.02	95.3	4.7	1.00	1.03 ± 0.01	102.8	2.2
	5.00	4.66 ± 0.04	93.3	4.2	2.00	2.08 ± 0.03	103.9	5.1
	10.00	9.30 ± 0.11	93.0	5.7	4.00	3.96 ± 0.08	99.0	6.2
Doxepine	2.00	1.89 ± 0.02	94.7	8.9	1.00	0.86 ± 0.02	86.2	5.9
	5.00	4.06 ± 0.02	92.1	4.6	2.00	1.84 ± 0.02	91.9	4.2
	10.00	9.71 ± 0.05	97.1	4.6	4.00	3.53 ± 0.06	88.3	6.3
Diazepam	2.00	1.92 ± 0.006	96.0	1.2	1.00	1.04 ± 0.02	103.8	6.8
	5.00	4.72 ± 0.04	94.5	3.3	2.00	2.04 ± 0.04	102.1	5.1
	10.00	9.43 ± 0.03	94.3	1.1	4.00	4.13 ± 0.08	103.4	4.0
Amitriplyline	2.00	2.18 ± 0.03	108.9	7.2	1.00	0.94 ± 0.01	93.8	5.6
	5.00	5.12 ± 0.05	102.5	6.2	2.00	1.91 ± 0.02	95.4	4.8
	10.00	10.40 ± 0.12	104.0	7.8	4.00	3.62 ± 0.06	90.6	6.6
Clomipramine	2.00	2.13 ± 0.05	106.4	3.3	1.00	0.86 ± 0.01	86.2	6.2
	5.00	5.02 ± 0.02	100.3	7.4	2.00	1.84 ± 0.01	91.8	3.2
	10.00	10.53 ± 0.06	105.3	9.2	4.00	3.66 ± 0.02	91.4	4.2

in Table 6. Column efficiency data shown in Table 7 indicate that these values are acceptable and the method is accurate and precise.

Chromatography

Separation of benzodiazepines and their metabolites has been achieved by a number of workers on a reversed phase column utilizing a variety of solvent combinations. Similarly, there is an abundance of literature on the separation of TCAs by reversed phase chromatography. Recently, Minder et al.[62] reported a common isocratic mobile phase for the

Table 6. Analytical data of suitability and robustness as validation parameters

		Suitability (RSD)			Resolution factor		
				R_s at different buffer conc.	Program A		Program B
Analytes	Retention time	R_s	A. Ratio		F.R 1.0 (mL/min)	F.R 1.1 (mL/min)	F.R 1.0 (mL/min)
BRZ	6.72 ± 0.05	0.9	0.6	0.2	2.9	2.4	2.2
CLZ	8.07 ± 0.04	0.8	1.7	0.7	2.0	1.6	1.7
FTZ	8.85 ± 0.04	1.0	1.8	0.9	1.0	0.9	0.9
LRZ	10.57 ± 0.05	0.5	1.6	0.7	2.7	1.9	2.1
ALZ	11.11 ± 0.05	0.9	1.5	0.5	1.1	1.7	1.2
DXP	11.65 ± 0.51	1.1	1.2	0.9	1.2	2.0	1.1
DZP	12.83 ± 0.01	0.6	1.6	0.3	2.8	3.2	2.2
ATP	13.63 ± 0.03	0.1	1.3	0.7	1.7	1.4	1.2
CLP	14.73 ± 0.04	0.9	1.9	0.7	2.1	2.0	1.4

	(% Recovery)					
	Program A		Program B	Buffer effect (CH_3COONH_4)		
Analytes	F.R 1.0 (mL/min)	F.R 1.1 (mL/min)	F.R 1.0 (mL/min)	0.04 M	0.05 M	0.06 M
BRZ	102.3	101.9	107.9	108.4	107.9	103.7
CLZ	104.5	103.4	100.9	104.2	104.8	107.4
FTZ	103.4	103.9	101.4	101.8	103.3	104.3
LRZ	99.3	98.8	99.7	103.4	106.3	107.6
ALZ	99.2	94.3	94.9	99.4	100.3	102.3
DXP	100.1	96.7	94.4	97.6	99.1	98.8
DZP	101.4	99.7	101.2	109.3	107.9	105.9
ATP	93.3	96.7	94.5	104.1	98.3	94.2
CLP	95.6	100.5	96.9	97.7	103.2	96.6

separation of both benzodiazepines and antidepressants with photodiode array detection.

Herein, a reversed phase liquid chromatography method has been developed using a simple mobile phase to provide a rapid, quality control simultaneous determination of benzodiazepines and tricyclic antidepressants in tablets and saliva samples. All samples were analyzed using the assay chromatographic conditions described in the experimental section. By injecting two test mixtures the effect of various chromatographic conditions on separation were evaluated: one containing six major benzodiazepines with internal standard, another containing four TCAs with internal standard. Using the proposed chromatographic

Table 7. Column efficiency parameter values

Analytes	Efficiency parameters				
	Retention factor (k)	Separation factor (α)	Resolution factor (R_s)	Theoretical plate (N)	Tailing factor (T_f)
Bromazepam	1.4	1.5	2.9	2.832×10^3	1.3
Clonazepam	1.8	1.3	2.0	2.662×10^3	1.0
Flunitrazepam	2.1	1.1	1.0	2.349×10^3	1.0
Lorazepam	2.7	1.3	2.7	3.407×10^3	1.2
Alprazolam	2.9	1.1	1.1	5.361×10^3	1.3
Doxepine	3.2	1.1	1.2	8.721×10^3	1.3
Diazepam	3.7	1.1	2.8	16.940×10^3	1.1
Amitriplyline	3.9	1.1	1.9	12.019×10^3	1.3
Clomipramine	4.3	1.1	2.1	13.914×10^3	1.3

conditions IMP and DZP peaks co-eluted at the same retention time, therefore imipramine was omitted. The separation of six benzodiazepines and three antidepressants, along with the internal standard, was achieved in less than 15 min. Under the assay conditions described above, the examined drugs were well resolved, providing better separation and resolution. Variations in the composition of the mobile phase, flow rate, as well as the concentration of buffer, were examined. Higher concentrations of acetonitrile have a predominant effect on the separation of benzodiazepines with very little effect with pH variations of mobile phase. Representative chromatograms of standard solutions are presented in Figure 1, as indication that the method provides better resolution for examined analytes. Figure 2 shows the chromatogram of spiked saliva (4 ng/μL) against saliva blank in the presence of colchicine (4 ng/μL) as the internal standard. Retention times of the examined drugs are presented in Table 6. Precision of retention times and peak area were 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 analytes, which revealed RSD values of 0.1–1.1% and 0.6–1.9%, respectively.

Column Efficiency

The following parameters have been calculated for one of the representative chromatograms to check the column efficiency: retention factor ($k = t_R - t_0/t_0$), separation factor ($\alpha = t_{R2} - t_0/t_{R1} - t_0$), resolution factor, $R_s = 2(t_{R2} - t_{R1})/(w_2 + w_2)$ theoretical plate number, $N = 16(t_R/w)^2$,

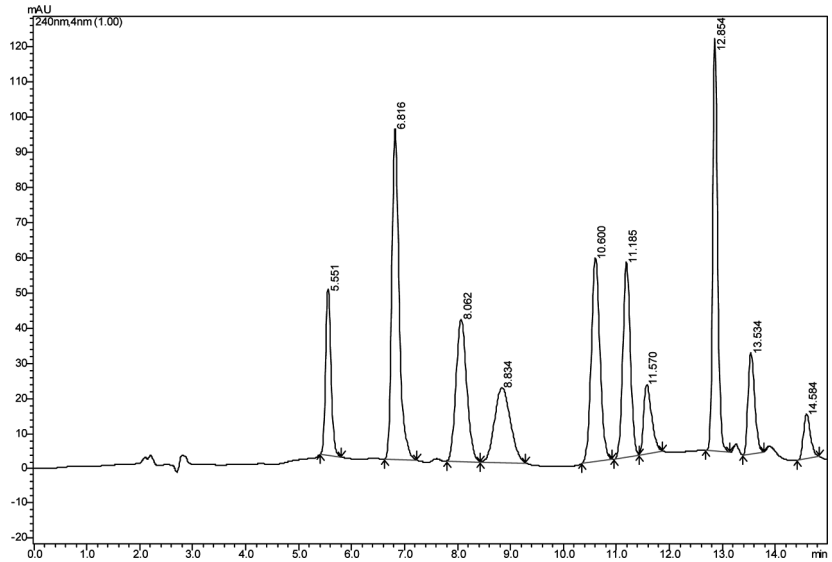


Figure 1. High performance liquid chromatogram of standard (8 ng/μL) in the presence of colchicine (4 ng/μL) as the internal standard according to the conditions described in the text. Peak designation: IS 5.56, BRZ 6.82, CLZ 8.06, FNZ 8.83, LRZ 10.60, ALP 11.18, DXP 11.57, DZP 12.85, ATP 13.53, and CLP 14.58 min.

and tailing or asymmetry factor ($T_f = b_{0.1}/a_{0.1}$). Where, t_0, t_{R1}, and t_{R2} are the retention times and w_1 and w_2 the baseline peak width of successive peaks. Results given in Table 7 show the excellent performance of the column.

Solid Phase Extraction

For precise and critical application, SPE protocol was optimized. The method described here for the analysis of benzodiazepines and TCAs involves the combination of two steps. The first step was the selective adsorption of these drugs on a bonded phase column, removal of hydrophilic and hydrophobic impurities from the saliva, and a very selective elution of the benzodiazepines and TCAs. Several combinations of wash mixtures containing different concentrations of methanol-water or acetonitrile-water were evaluated for removing endogenous impurities and salts to obtain a clean solvent front. Pure water proved specific for washing the impurities, which would appear in the HPLC solvent front. The total volume of wash was optimized to 1 mL. The BDZs and TCAs retained on the sorbents due to non-polar interaction

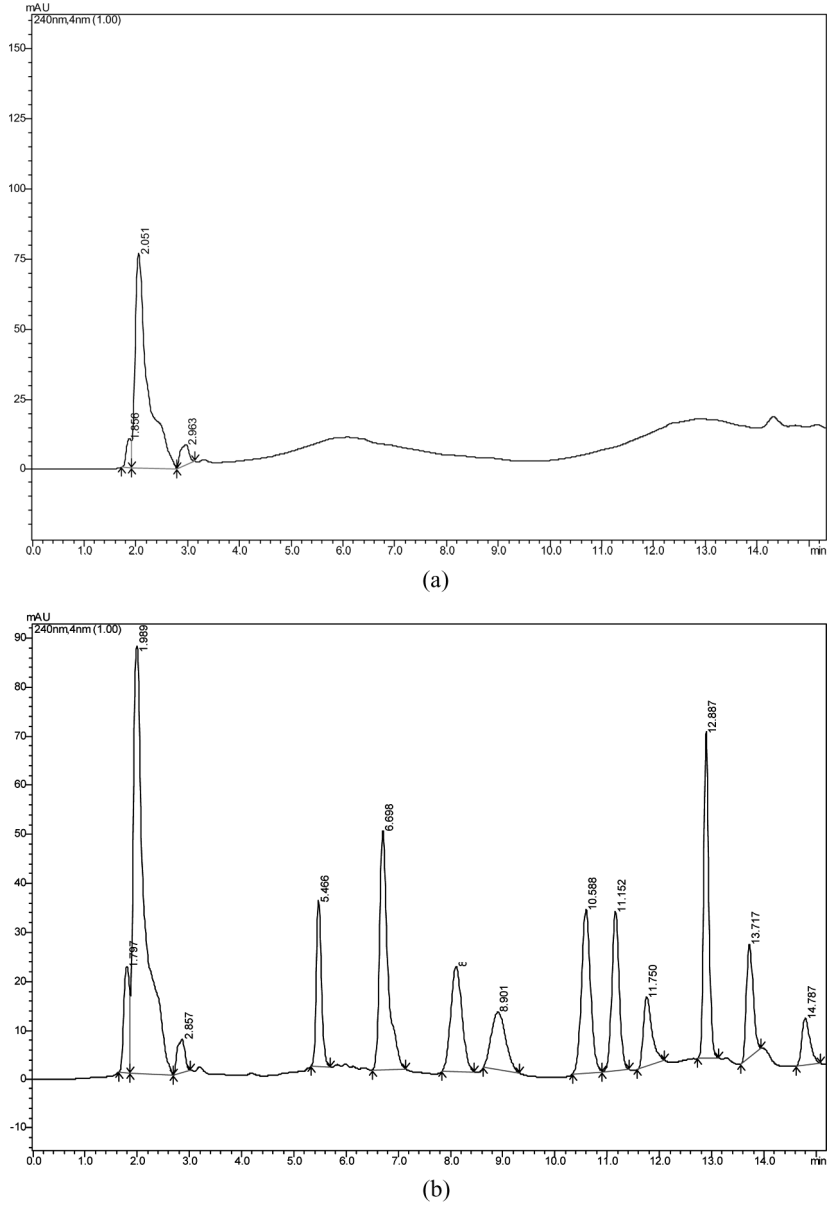


Figure 2. High performance liquid chromatogram of (a) blank saliva, (b) spiked saliva (4 ng/μL), in presence of colchicine (4 ng/μL) as the internal standard according to the conditions described in the text. Peak designation: IS 5.46, BRZ 6.69, CLZ 8.10, FNZ 8.90, LRZ 10.58, ALP 11.15, DXP 11.75, DZP 12.88, ATP 13.72, and CLP 14.78 min.

(benzodiazepines) and a combination of non-polar and polar interaction (strong hydrogen bonding interaction between the amino side chain of the TCAs with the free silanol group of the sorbents) with the solid phase presented a challenge for selective elution. This approach was evaluated using different solvents, methanol and acetonitrile and their combinations. Addition of acetonitrile drastically decreases the elution of TCAs. Recovery results of the investigation with various sorbents and eluents are given in Table 8, showing both BDZs and TCAs are completely eluted by methanol with either Varian (applied in this study) or Lichrolut RP-18 cartridges. Hence, a single step elution with methanol gave total determination of nine drugs from the mixture under described chromatographic conditions. Investigation of various elution systems, the optimum conditions were found using Varian or Lichrolut RP cartridges, and mixture of acetonitrile and methanol by 50:50 as

Table 8. SPE-recovery of different sorbents and different eluents with Nexus sorbent

	Sorbents			
Analyte	Varian	LC-18	DSE-18	Lichrolut
Bromazepam	103.7	106.3	95.2	96.8
Clonazepam	105.6	94.4	98.8	100.1
Flunitrazepam	101.9	92.6	92.4	107.4
Lorazepam	99.1	90.1	92.1	107.2
Alprazolam	106.3	94.1	105.3	106.3
Doxepine	99.1	17.9	57.1	98.0
Diazepam	99.1	96.8	103.1	104.6
Amitriplyline	98.7	19.5	47.4	95.9
Clomipramine	98.5	18.1	50.6	108.2

	Eluents						
Analyte	MeOH: ACN 100:00	MeOH: ACN 70:30	MeOH: ACN 60:40	MeOH: ACN 50:50	MeOH: ACN 40:60	MeOH: ACN 30:70	MeOH: ACN 00:100
Bromazepam	103.7	100.7	103.5	105.6	93.8	88.5	66.2
Clonazepam	105.6	103.9	105.5	106.9	92.2	90.5	85.0
Flunitrazepam	101.9	101.8	101.3	103.7	92.7	90.5	84.7
Lorazepam	99.1	99.8	100.3	99.8	88.6	87.0	72.6
Alprazolam	106.3	106.5	104.3	105.5	86.6	72.6	46.5
Doxepine	99.1	13.4	3.9	5.5	1.4	2.9	0.0
Diazepam	99.1	104.6	102.3	100.4	86.8	84.3	52.3
Amitriplyline	98.7	10.3	5.4	3.4	1.8	0.0	0.0
Clomipramine	98.5	11.9	2.6	3.5	1.9	2.6	0.0

eluent, gave complete elution of all the benzodiazepines with the total retention of TCAs on the column, which was subsequently eluted by methanol. Based on this sequential elution, both drug classes have been determined in plasma and urine.[60]

Validation of the Method

Linearity

Linearity was determined by constructing the calibration curves for both of standard solutions and oral fluid. Aliquot volumes of stock solutions in methanol were diluted to produce mixture solutions at six concentration levels in the range 0.5–20.0 ng/μL for each drug, along with a volume to be equivalent to 4 ng/μL of IS. Calibration standards of each concentration were analyzed in triplicate and curves of analytes were constructed using peak area ratio of drug to the internal standard versus nominal concentrations of the analytes. Least square linear regression analysis of the date gave slopes, intercept, and coefficient of determination.

The calibration curves were linear up to the examined range 20 ng/μL with the coefficient of determination (r^2) of 0.999 for all analytes. The representative linear equation, a coefficient of determination, intercept are given in Table 2.

Recovery/Accuracy

The accuracy of the method was tested by analyzing different concentrations of standard BDZs and TCAs. A 20 μL of the selected assay standard at the concentration levels 2.0, 5.0, 10.0 ng/μL or spiked saliva at the concentration levels 1.0, 2.0, 4.0 ng/μL were injected into the HPLC system, and triplicate measurements were recorded for both intra-day and inter-day assays. The contents of the drug in each solution were calculated from the respective 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 study mentioned before shows the results; 93–109% for standard, 86.0–105.0% for spiked samples, indicating good accuracy agreement.

Precision

The precision of the method was determined by calculating the relative standard deviation (RSD) for the repeated measurements for both

intra-day and inter-day assays. Intra-day repeatability was determined at three concentrations levels of 2.0, 5.0, 10.0 ng/μL in standard solutions and 1.0, 2.0, 4.0 ng/μL in spiked saliva in the same day, taking six measurements for each. The procedure was repeated in triplicate, at the same concentration levels on six different days, to determine between-day repeatability. The RSD results obtained in the intra-day assay were 0.1–7.9% for standard solutions and 1.5–6.0% for spiked saliva, and in inter-day corresponding values were 1.0–9.5% and 0.7–6.8%, indicating the proposed method to be significantly precise.

Specificity

Specificity is the ability of the method to measure the analytes in the presence of potential impurities. The specificity was demonstrated showing that drugs were free of interference from degradation products, and in saliva samples by the analysis of five blank matrices, and it was assessed by the absence of interference in the same chromatographic windows as examined for analytes. The absence of any peak in blank oral 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.

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.[63] The limit of detection (LOD) and the limit of quantitation (LOQ) were 0.1–0.3 and 0.3–1.1 ng/μL for standard, and 0.1–0.3 and 0.4–1.1 ng/μL for spiked saliva, respectively, as shown in Table 3.

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 and oral fluid samples. Precision of retention time and peak area ratios was examined to evaluate the system suitability. RSD of the peak area ratios 0.6–1.9 and retention time 0.1–1.1%, and that of retention time at three buffer concentrations levels at triplicate measurements,

each shows the values 0.23–0.93% indicate excellent suitability of the system.

Stability of Standard Solution

The stability of standard solutions was tested by the proposed HPLC method over a period of 90 days. The freshly prepared solutions at room temperature, and the 90 days stored samples in a refrigerator at 4°C, were analyzed. Respective chromatograms and recoveries were compared. No degradation products were observed and the drugs were stable at 4°C for at least 90 days, indicating the possibility of using BDZs and TCAs samples over a period of 90 days stored at refrigerator without degradation.

Freeze-Thaw Cycle of Spiked Samples

Saliva samples spiked with 2 ng/μL of examined drugs were stored at −20°C. Each sample was analyzed for intact compounds, once daily for seven successive days after the freeze-thaw cycle, for investigation of stability when spiked samples were allowed to thaw at room temperature. Recovery of analytes for the stored samples were calculated and compared to that of freshly prepared samples. The results showed that BDZs, up to five cycles and TCAs, up to three cycles were almost stable. Recovery of BDZs, after fifth cycle and that of TCAs, after third cycle, was decreased by less than 10% and 20%, respectively. Results have been presented graphically in Figure 3.

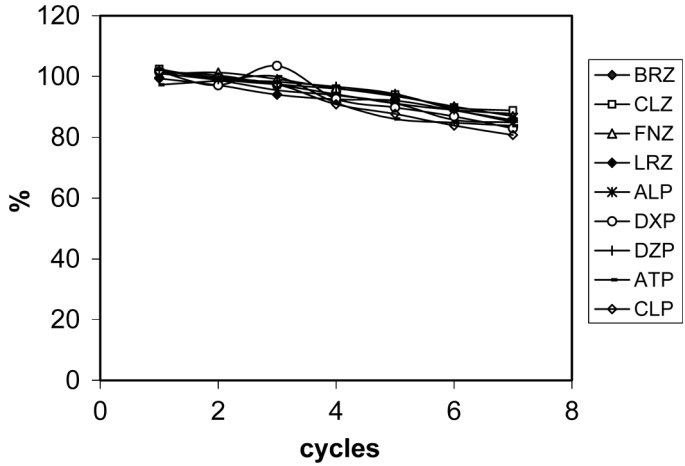


Figure 3. Stability study through freeze-thaw cycle.

Robustness

Robustness is the capacity of the method to remain unaffected by small deliberate variations in method parameters. The method robustness was tested by varying several chromatographic parameters. The optimum HPLC conditions for this method have been slightly modified by the small changes in flow rate buffer concentration to justify the effect (if any) on the results of the method as a means to evaluate the method's robustness. It was found that the percent recoveries of compounds were excellent under most conditions, and remained unaffected by small deliberate changes of flow rate (Table 6).

Buffer Concentration

The effect of varying buffer concentrations from 0.04–0.06 M was examined. Despite varying the buffer concentrations, the peak area, retention time, resolution factor didn't significantly alter. Furthermore, working within range didn't lead to any decrease in column performance.

Flow-Rate

The study of variation of the flow rate from 1.0 to 1.1 mL/min showed that the peak area decreased with increasing flow rate for all the compounds. It might be due to the fact that at high flow rates, the compounds did not have enough time to penetrate the pores of particles and were retained by the hydrophobic inner surface of the particles before being eluted by the analytical mobile phase. Also, at high flow rate, retention time of each peak was reduced with subsequent change in the separation and resolution factor, without affecting their resolution. However, using a flow rate of 1.0 mL/min seems to be a good compromise when considering the chromatographic system and total retention time. At this flow rate, reproducibility of retention time ($n = 6$) was always better than 1%.

Results of 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.

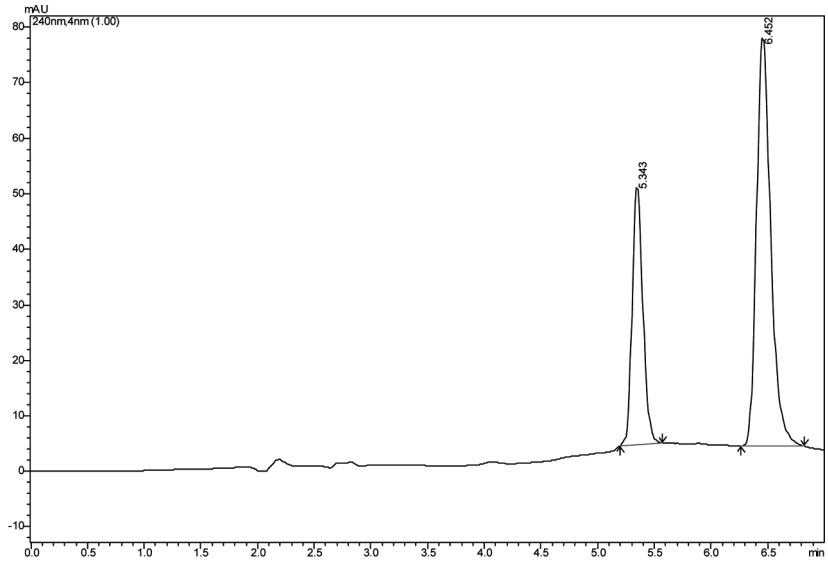
Application to Pharmaceutical Products

The method developed herein was applied to pharmaceutical products at various concentrations (5.0, 7.5, 10.0 ng/μL) levels for determining

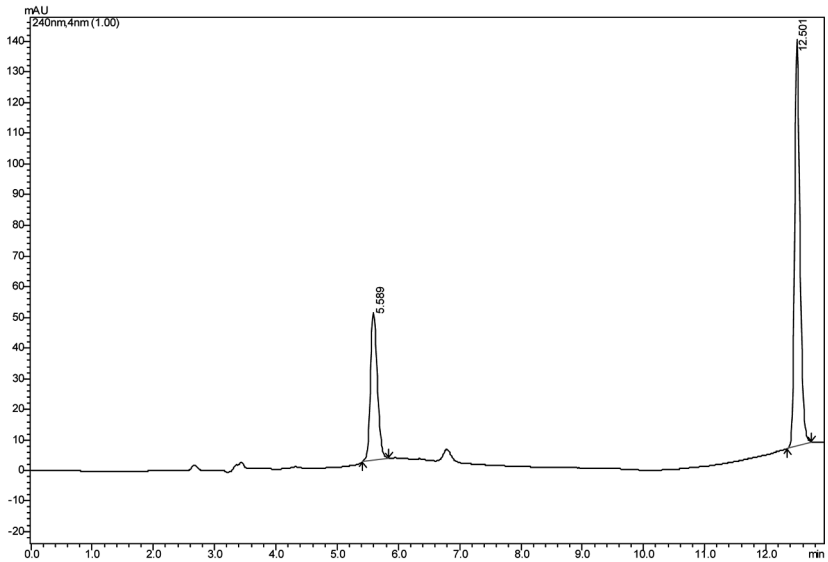
the content of BDZs and TCAs. The values of the overall drug percentage recoveries are 95–107% for BDZs, 81–106% for TCAs, and the RSD value from 0.2–7.2% for both drugs, as presented in Table 9, indicating that these values are acceptable and the method is accurate and precise. Moreover determination 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

Table 9. Analytical data of pharmaceutical formulations

Analytes	Tablets	Added (ng/mL)	Found ± SD (ng/mL)	RSD	Recovery (%)	Mean recovery (%)	Per tablet (mg)
Bromazepam	Laxotanil	5.00	5.30 ± 0.08	5.3	106.0		
	(3.0 mg)	7.50	7.67 ± 0.01	0.4	102.2	103.0	3.1
		10.00	10.09 ± 0.01	0.4	100.9		
Clonazepam	Rivotril	5.00	4.84 ± 0.03	3.1	96.9		
	(0.50 mg)	7.50	7.44 ± 0.01	0.5	99.2	97.7	0.49
		10.00	9.70 ± 0.02	1.0	97.0		
Fluni-	Hipnosedon	5.00	5.38 ± 002	2.6	107.6		
trazepam	(1.0 mg)	7.50	7.42 ± 0.03	2.5	990	102.2	1.02
		10.00	10.00 ± 0.06	3.8	100.0		
Lorazepam	Tavor	5.00	4.77 ± 0.04	3.9	95.5		
	(1.0 mg)	7.50	7.46 ± 0.06	3.5	99.4	98.5	0.98
		10.00	10.05 ± 0.10	4.3	100.5		
Alprazolam	Xanax	5.00	5.06 ± 0.01	1.2	101.3		
	(0.50 mg)	7.50	7.90 ± 0.01	0.9	105.3	104.1	0.52
		10.00	10.59 ± 0.09	4.0	105.9		
Doxepine	Sinequan	5.00	4.27 ± 0.02	4.3	85.5		
	(discs,	7.50	7.15 ± 0.02	2.7	95.3	90.7	22.7
	25 mg)	10.00	9.13 ± 0.08	7.9	91.3		
Diazepam	Stedon	5.00	4.87 ± 0.04	3.0	97.4		
	(5.0 mg)	7.50	7.24 ± 0.07	3.7	96.6	99.3	5.0
		10.00	10.39 ± 0.09	3.0	103.9		
	Valium	5.00	5.06 ± 0.30	3.3	101.2		
	(5.0 mg)	7.50	7.25 ± 0.02	4.1	96.7	97.7	4.9
		10.00	9.51 ± 0.03	3.1	95.1		
Amitriplyline	Saroten	5.00	4.07 ± 0.02	2.4	81.4		
	(capsules,	7.50	7.18 ± 0.02	2.2	95.7	87.9	22.0
	25 mg)	10.00	8.66 ± 0.02	1.7	86.6		
Clomipramine	Anafranil	5.00	4.05 ± 0.007	2.7	80.9		
	(discs,	7.50	6.90 ± 0.01	3.0	91.9	87.9	8.8
	10 mg	10.00	9.07 ± 0.01	1.8	90.7		
	Anafranil	5.00	4.68 ± 0.02	7.2	93.5		
	(ampoules,	7.50	7.97 ± 0.01	0.2	106.3	98.2	24.5
	25 mg/	10.00	9.47 ± 0.03	5.6	94.7		
	2 mL)						

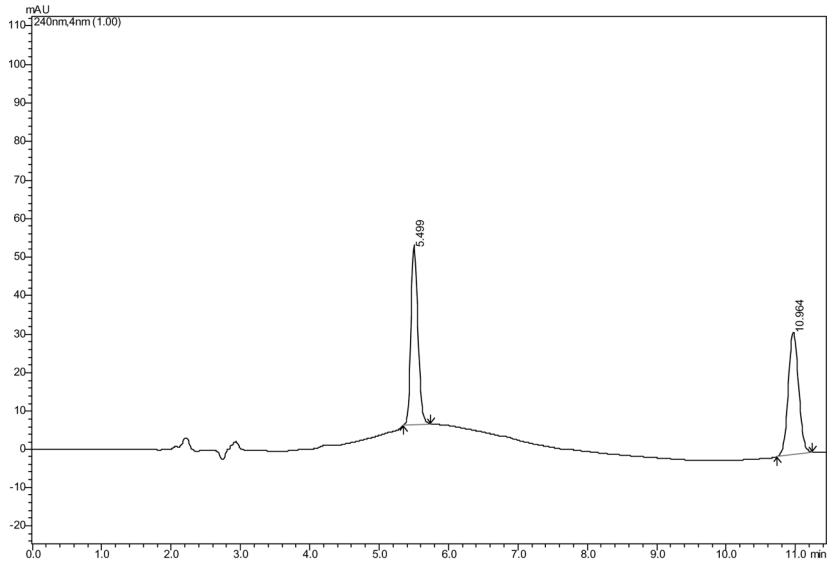


(a)

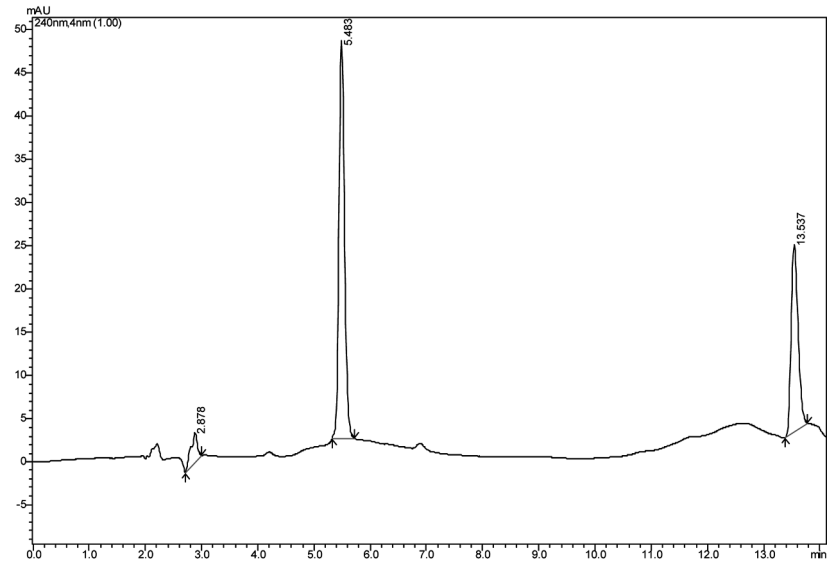


(b)

Figure 4. High performance liquid chromatogram of pharmaceutical formulations: (a) BRZ (8 ng/μL) 6.45 min, (b) DZP (10 ng/μL) 12.50 min, (c) ALP (5 ng/μL) 10.96 min, (d) ATP (8 ng/μL) 13.54 min., (e) CLP (10 ng/μL) 14.79 min. in the presence of colchicine (4 ng/μL) as the internal standard according to the conditions described in the text.



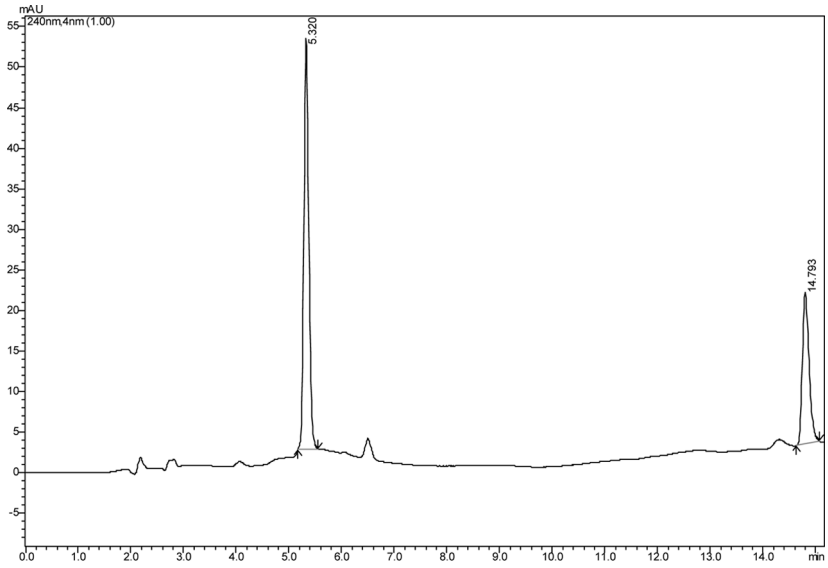
(c)



(d)

Figure 4. Continued.

the method. In the case of TCAs, results are found a bit lower than labelled values. Figure 4, shows some chromatograms of pharmaceuticals in the presence of colchicine as internal standard.



(e)

Figure 4. Continued.

CONCLUSION

The method presented herein is simple, rapid, and permits the analysis of common benzodiazepines along with the commonly used tricyclic antidepressants in saliva. Isolation of examined drugs was performed by means of SPE and separation was achieved in a single chromatogram. The described procedure, subsequently, was used for experimental tablets and capsule formulations. The simplicity, specificity, and sensitivity of this method may have wide applications in clinical settings for routine or immediate analysis. The present method has a comparable sensitivity with respect to existing methods for the determination of both drugs, with the great advantage of a routine and less expensive instrumentation. The method is suitable for the pharmacokinetics and bioavailability studies and drug quality control. The analysis can be applied for drug determination in composite tablet formulations in a reasonable time, eliminating interferences due to possible impurities. In a clinical situation of concomitant use of these drugs, patient samples can be monitored by a single procedure. The applicability of this method is of significant value in view of their increasing importance in forensic toxicology.

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