RAPID SPECTROPHOTOMETRIC METHOD FOR DIAZEPAM QUANTIFICATION IN PHARMACEUTICAL FORMULATIONS USING ION PAIR FORMATION WITH METHYL ORANGE AND BROMOPHENOL BLUE**

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An extractive visible spectrophotometric procedure has been developed for diazepam (DZ) determination in pure and pharmaceutical forms using methyl orange (MO) and bromophenol blue (BPB) dyes. The proposed method was based on the formation of an ion-pair colored complex between diazepam and indicators via extracting them into chloroform whereby absorbance measured at 411 and 415 nm, respectively. The extracts are intensely colored and very stable at room temperature. The molar absorptivity for DZ–MO and DZ–BPB ion pairs were determined to be 5.3×10^3 and 6.66×10^3 L/mol ·cm, respectively. The stoichiometry of the reaction was found to be 1:1 in all cases and the conditional stability constant (K_f) of the complexes was calculated. The effective range of concentration for an accurate determination as ascertained from Ringbom's plot was obtained at 16.67–50 μg/mL. The proposed method has been applied successfully to the analysis of drug dosage forms and no interference was observed from common excipients present in pharmaceutical formulations. The results obtained by the proposed method were statistically compared by means of the student t-test and by the variance ratio, and F-test with the HPLC method. Here it is shown to be in excellent agreement with the official method.

Keywords: *diazepam, spectrophotometry, methyl orange, bromophenol blue, ion-pair complexation.*

СПЕКТРОФОТОМЕТРИЧЕСКИЙ ЭКСПРЕСС-МЕТОД КОЛИЧЕСТВЕННОГО ОПРЕДЕЛЕНИЯ ДИАЗЕПАМА В ФАРМАЦЕВТИЧЕСКИХ ПРЕПАРАТАХ С ИСПОЛЬЗОВАНИЕМ ИОННЫХ ПАР С КРАСИТЕЛЯМИ МЕТИЛОВЫМ ОРАНЖЕВЫМ И БРОМФЕНОЛОВЫМ СИНИМ

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Разработан спектрофотометрический экспресс-метод определения диазепама (ДЗ) в чистом виде и фармацевтических препаратах с использованием красителей метилового оранжевого (МО) и бромфенолового синего (БФС). Предлагаемый метод основан на образовании окрашенного комплекса ионных пар ДЗ и указанных индикаторов путем экстрагирования их в хлороформ, при этом оптическую плотность измеряют при 411 и 415 нм соответственно. Экстракты интенсивно окрашены и очень стабильны при комнатной температуре. Молярная абсорбционная способность для ионных пар ДЗ–МО и ДЗ–БФС составляет 5.3×10^3 и 6.66×10^3 л/моль·см. Во всех случаях установлена стехиометрия реакции 1:1 и рассчитана условная константа устойчивости (K_f) комплексов. Оптимальный диапазон концентраций, определенный по графику Рингбома, составляет 16.67–50 мкг/мл. Предлагаемый метод успешно применен для анализа лекарственных форм. Помех со стороны обыч-

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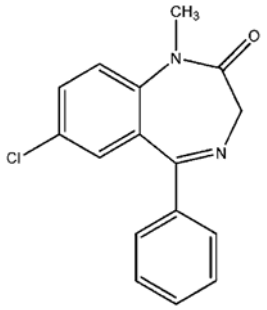
ных вспомогательных веществ, присутствующих в фармацевтических препаратах, не наблюдалось. Статистическое сравнение с использованием критерия Стьюдента, коэффициента дисперсии и F-критерия предлагаемого метода с методом ВЭЖХ показало их хорошее согласие.

Ключевые слова: *диазепам, спектрофотометрия, метиловый оранжевый, бромфеноловый синий, комплексообразование ионных пар.*

Introduction. Benzodiazepines are a large group of drugs with important clinical applications. They are prescribed worldwide as anxiolytic-sedative, hypnotics, anticonvulsive, and sleep regulator agents. Benzodiazepines are depressants used therapeutically to produce sedation, induce sleep, relieve anxiety and muscle spasms, and prevent seizures. In general, benzodiazepines act as hypnotics in high doses, anxiolytics in moderate doses, and sedatives in low doses, being among the most widely prescribed medications [1]. One of the benzodiazepines is diazepam with the chemical name of diazepam being 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one (I)



which enhances the activity of gamma-aminobutyric acid, the most common inhibitory neurotransmitter in the central nervous system. It is used in the treatment of severe anxiety disorders, as a hypnotic in the short-term management of insomnia, as a sedative and premade-cant, as an anticonvulsant, and it is used in the management of alcohol withdrawal syndrome [2].

Based on the wide application of diazepam in the world, it could be concluded that it is necessary to develop a method for analyzing diazepam in pure, biological, and pharmaceutical samples. Varieties of primitive and novel methods have been used for the determination of diazepam such as room temperature phosphorescence [3], second-order derivative spectrophotometry [4], ion chromatography [5–7], high-performance liquid chromatography [8–10], potentiometry [11], polarography [9, 12], fluorimetry [13], RP-HPLC [14–16], solid phase extraction [17], anodic stripping voltammetry [18], chemiluminescence [19], modified electrodes [20], and capillary electrophoresis [21]. However, all these methods have different disadvantages such as low sample frequency, the need to use large volumes of toxic reagents and solvents, dependency on complicated systems, and that it is expensive and time-consuming. However, spectrophotometric methods can be implemented on very simple, rapid, and inexpensive equipment providing a low-level determination. These advantages cause them to remain an attractive technique. On the other hand, some visible spectrophotometric methods have been reported for diazepam quantification. Still, these methods suffer from disadvantages of which some of them are as follows: Beer's law obeys low concentration range [22–24], some need acid hydrolysis of diazepam before analysis [24, 25] and some use reagents that are expensive and inaccessible [26]. In most previously reported spectrophotometric methods, a relatively narrow linear range or high limit of quantification has been obtained. The present study deals with the development and validation of a sensitive extractive spectrophotometric method using methyl orange (MO) and bromophenol blue (BPB) dyes as an ion-pair complexion reagent. The reagents are relatively cheap and have also been used before for the determination of some other drugs [27–29]. Spectrophotometric methods are rapid, cost-effective, simple, and acceptable and use very routine instruments available in any quality control laboratory. The validated method could be successfully used for routine quality control analysis without any special sample preparation. The goal of this research is to develop an accurate, reproducible, and adequate spectrophotometric method based on the formation of two chloroform soluble ion–association complexes DZ–MO and DZ–BPB in an acidic medium, and finally, to introduce the dyes as in-hand reagents to develop new, simple, sensitive and selective spectrophotometric methods for determining trace amounts of diazepam in pure form, pharmaceutical samples, and biological fluids. The obtained results of the proposed procedure in tablets were compared with those of the reference method HPLC to assess diazepam content.

Materials and methods. All of the reagents were obtained from Merck (Germany). Pure powder of diazepam was obtained from Sobhan (pharmaceutical company, Rasht, Iran). The diazepam pharmaceutical tablets (10 mg) were obtained from Abidi and Loghman pharmaceutical company. Double distilled water was utilized to prepare all solutions. All of the chemicals used were of analytical or pharmaceutical grade and used without further purification. Jenway 6715 UV/Vis Spectrophotometer with 1 cm matched quartz was used for all measurements. A Jenway 4330 digital pH-meter was used for pH measurements. C1 biotech 100 and 50 μL syringes were used for diluting and picking the solutions up. All of the used filter papers were Whatman 41 ashless (125 mm diameter).

Experimental. A stock solution of diazepam was prepared by dissolving a certain amount of pure drug in a few drops of dilute hydrochloric acid (0.1 M into a 100-mL calibrated flask which was then diluted to the mark with distilled water). The freshly prepared solution was kept in a dark bottle in the refrigerator. The different concentrations of diazepam were prepared daily from the stock solutions. Buffer solutions with different pH values were prepared by standard procedures (Britton-Robinson's instruction [30]). Methyl orange and bromophenol blue solutions (0.1% w/v) were prepared in double distilled water.

Preparation of pharmaceutical tablet solutions for proposed method. 10 numbers diazepam tablets (10 mg) were carefully weighed and ground to a fine powder. Accurate weights equivalent to 10 mg of diazepam were transferred into a 100-mL flask and a few drops of diluted HCl (0.1 M) were added in. Then, the obtained mixture was stirred with a mechanical stirrer for 5 min and left at room temperature for 10 min, after which it was filtered to collect the undissolved part of the tablets. The obtained filtrate was diluted to the mark (50 mL) with distilled water. Different concentrations of the samples were prepared by diluting the stock solution with double distilled water.

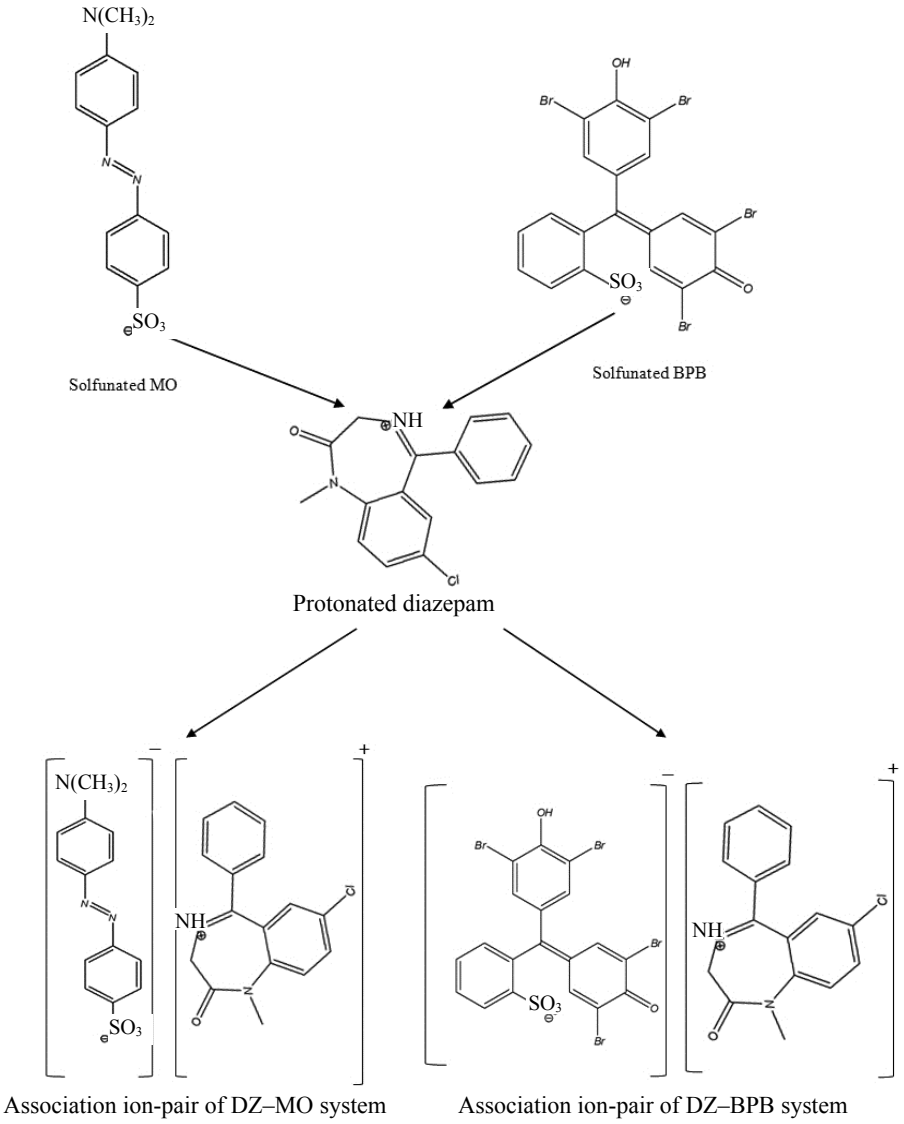
Preparation of pharmaceutical tablet solutions for HPLC method. 10 numbers diazepam tablets (10 mg) were accurately weighed and finally powdered. A distinctive amount of achieved powder, equivalent to 10 mg of diazepam, was weighed and transferred into 100 volumetric vessels. A sufficient amount of methanol was added in and the mixture was stirred with a mechanical stirrer for 10 min. The obtained mixture was sonicated for 15 min in an ultrasonic bath to complete the dissolution of the analyte and then filtered. Finally, the filtered mixture was diluted to the mark with methanol and used for injection into the HPLC instrument [31].

An aliquot of a standard solution of diazepam was transferred to a 50-mL separating funnel, after which 2 mL of potassium hydrogen phthalate – HCl buffer (pH 4.2, was added for MO indicator or 2 mL of mentioned buffer (pH 2.8 [30]) was added for the BPB indicator, followed by 2 mL of indicator solution (0.1% w/v), and finally 5 mL of chloroform was added. The contents were shaken vigorously for 3 min and then allowed to be separated. The colored chloroform phase was extracted and the absorbance of the organic phase was measured at 411 nm for MO and 415 nm for BPB against a reagent blank, finally. All measurements were made at room temperature.

Results and discussion. In the acidic pH values, the molecules of MO and BPB were changed to anionic form and coupled with cationic nitrogen groups of molecules of diazepam, forming an association ion-pair colored complex. Negatively charged sulfonated groups of MO or BPB formed a light-yellow ion-pair complex with positively charged nitrogen of diazepam. Neutralized ion-pair association complex was extracted into the organic phase (chloroform) and absorbance of the colored organic phase was measured in λ_{max}. The proposed reaction mechanism is given in Scheme 1.

The maximum absorbance of DZ–MO and DZ–BPB were obtained at 411 and 415 nm, respectively, and at these wavelengths, the individual molecules of methyl orange, BPB and DZ showed no absorbance. Therefore, all measurements were done at λ_{max} 411 nm for DZ–MO and λ_{max} 415 nm for DZ–BPB (Fig. 1).

Different conditions, that affected the proposed method, were carefully studied. The effect of each parameter was investigated at λ_{max}, while other parameters were kept constant. The effect of pH on the formation of the ion–pair complex of the DZ–MO system was investigated by varying the pH values in the range of 2.2–5.5. The potassium hydrogen petal at HCl was used as an acidic buffer solution. The maximum absorbance was observed at pH 4.2; therefore, this pH value was selected and used for DZ–MO system. For the DZ–BPB system, the pH values in the range of 1.6–3.5, with the same buffer, were tested and pH 2.8 was selected as the optimum pH value.



Scheme 1. Probable mechanism for the formation of 1:1 association ion-pair of DZ–MO and DZ–BPB.

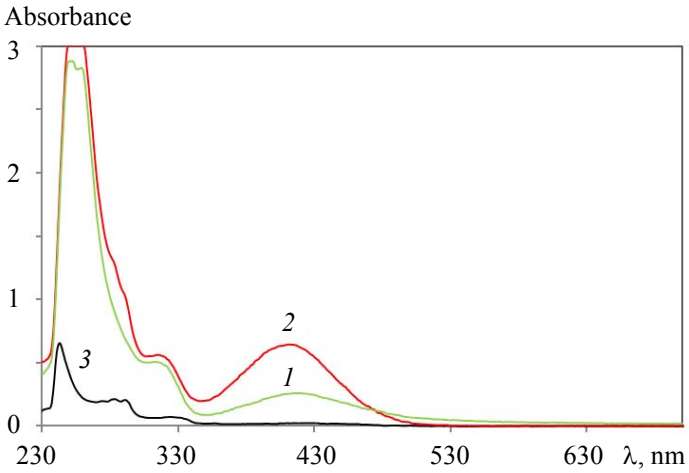


Fig. 1. Absorption spectra of association ion pair of DZ–MO (1), DZ–BPB (2) systems and pure DZ (3) extracted to chloroform (DZ: 1.43×10^{-4} M, MO: 2 mL of 0.1% w/v and BPB: 2 mL of 0.1% w/v).

The calibration graph of diazepam was prepared under optimum experimental conditions, with the calibration equation of $Y = bC + a$, in which C is the concentration of diazepam, and b, a is the slope and intercept of calibration equation, respectively (Fig. 2). The value of correlation coefficient indicates good linearity of the calibration graph. Other parameters due to calibration are given in Table 1.

TABLE 1. Sensitivity and Regression Parameters

Parameter	MO	BPB
Wavelength, nm	411	415
Beer's law limits, μg/mL	4–50	6.1–55.5
Molar absorptivity, $L \cdot mol^{-1} \cdot cm^{-1}$	5.37×10^{3}	6.66×10^{3}
Sensitivity of Sandell, $\mu g \cdot cm^{-2}$ [a]	67.61×10^{-3}	44.95×10^{-3}
Intercept (a)	0.12	–0.027
Slope (b)	0.007	0.023
LOD, μg/mL	0.54	0.29
LOQ, μg/mL	1.64	0.90
Confidence limit of intercept, CL_a	±0.032	±0.064
Confidence limit of slop, CL_b	$\pm1.17\times10^{-3}$	$\pm1.84\times10^{-3}$
SD of regression, $S_{y/x}$	0.016	0.037
SD of intercept, S_a	0.010	0.027
SD of slop, S_b	3.69×10^{-4}	7.82×10^{-4}
Variance, S_a^2	1.10×10^{-4}	7.40×10^{-4}
Regression coefficient, R^2	0.9950	0.9930

N o t e. Limit of determination as the weight in μg per mL of solution, which corresponds to an absorbance of $A = 0.001$ measured in a cuvette of cross-sectional area 1 cm^2 and $l = 1$ cm.

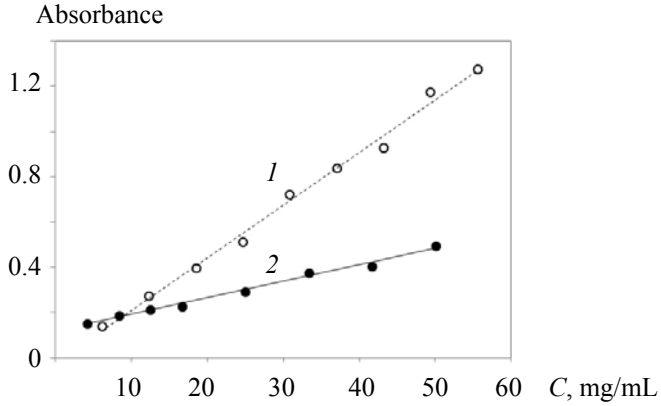


Fig. 2. Calibration graph for the determination of DZ under the optimum conditions. The maximum absorbance of DZ–MO (1) and DZ–BPB (2) determined at λ_{max} = 411 and 415 nm.

Different analytical performance characteristics for validation of the procedure, such as linearity, LOD, LOQ, precision, and accuracy, were studied and tabulated. The LOD and LOQ were determined from calibration data. LOD was calculated as 3 σ/s and LOQ came from 10 σ/s, where σ is the standard deviation of blank absorbance values and s is the slope of the calibration curve [32]. The molar absorptivity and sensitivity of Sandell were calculated and reported (see Table 1 for the calculated results).

Job's method of continuous variation At first, equimolar solutions of the drug and indicator were prepared, and then certain proportions of the drug and indicator were applied (with a fixed total volume). For two association ion-pair colored complexes, the plots of absorbance versus molar fraction ($V_{reagent}/V_{drug}+V_{reagent}$) reached its maximum value at a mole fraction of 0.5, which indicated that the association ion–pair complex formed between drug and indicator is 1:1 (Fig. 3).

In the mole ratio method, different solutions were prepared with a constant volume of diazepam and a variable volume of two selected indicators. The association ion-pair complexes between diazepam and indi-

cators were extracted to chloroform and their absorbance was measured at the selected λ_{max}. From the graphs, it is observed that one mole of indicator and one mole of the drug participate in the complex formation, which is in good agreement with the results of Job's method of continuous variation (Fig. 4).

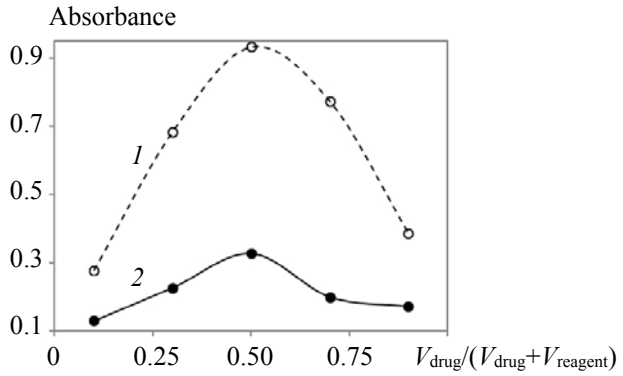


Fig. 3. Job's method of continuous variations. Concentrations of DZ–MO (1) and DZ–BPB (2) were 0.75×10^{-4} and 1.5×10^{-4} M, respectively.

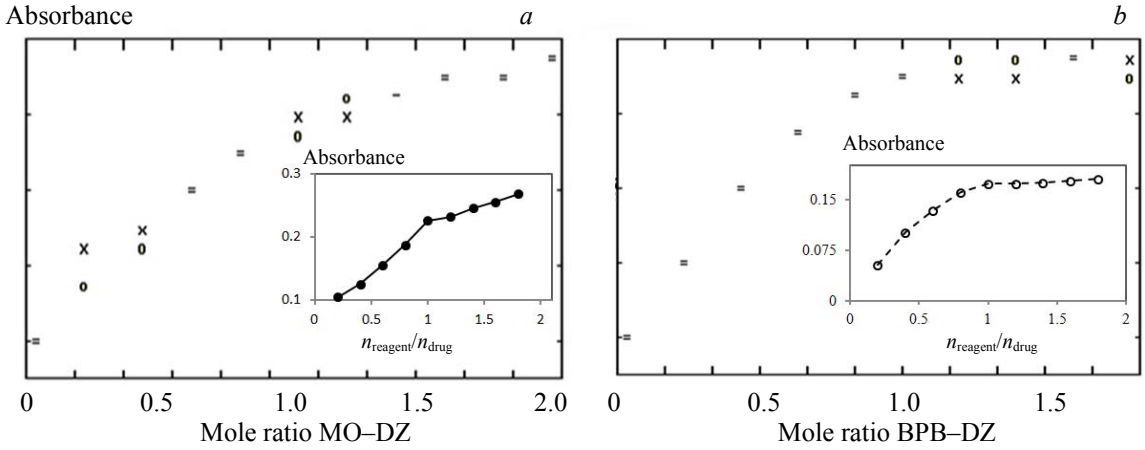


Fig. 4. Computer fit of absorbance vs. MO–DZ mole ratio (a) and BPB–DZ mole ratio (b) in chloroform; calculated point (o), experimental point (×), (▪) experimental and calculated points are the same within the resolution of the plots. Inception plot: molar ratio method of DZ–reagent complex. DZ: 2 mL of 10^{-4} mol/L; MO: 0.04–0.4 mL of 10^{-3} M; BPB: MO: 0.2–1.8 mL of 10^{-4} M.

Conditional stability constant of the ion pair complex. The corresponding complexes' stability constant was obtained by the nonlinear least square program KINFIT as described previously [32]. The formation constant for ion-pair association complex was extracted via the mole ratio technique and standard free energy changes were calculated from the following equation:

$$\Delta G^\circ = -2.303RT\log K_f, \quad (1)$$

where ΔG° is the free energy change of the complex, R is the gas constant, T is temperature in Kelvin and K_f is the association constant of drug-reagent ion-pair complex [32]. The obtained results are summarized in Table 2.

TABLE 2. Parameters Determined by Job, Mole Ratio, and Ringbom Plot

Parameter	MO	BPB	Parameter	MO	BPB
Complex ratio	1:1	1:1	Slope	0.696	0.558
LogK_f± SD, L/mol	3.274±0.071	3.993±0.099	Regression coefficient R^2	0.9981	0.9850
$-\Delta G^\circ$, kJ/mol	4.458	5.445	Effective range of concentration, μg/mL	16.67–50	6.17–43.21
Intercept	–0.285	–0.279	RE in concentration	0.032	0.023

A Ringbom plot is an established standard method to give the optimum range of concentration for a system that obeys Beer's law. The Ringbom plot was drawn between log *C* and 1–*T*, where *T* was the transmittance and *C* displayed drug concentrations. The Ringbom plot of the DZ–MO system has a camber shape with the liner segment at an intermediate absorbance value of 0.228–0.496. The effective range of concentration for accurate determination was ascertained from the Ringbom plot reported in Table 2. The slope of the Ringbom plot for the DZ–MO system was 0.693. Based on this value, the ratio between the relative error in concentration and photometric error was 3.32. For a photometric error, $\Delta P = 0.01$, the relative error in concentration is 0.032. The effective range concentration and photometric error for DZ–BPB system were obtained from the Ringbom plot as described previously and reported in Table 2.

The accuracy of the proposed method was determined by the recovery of drugs at different concentrations in the linearity concentration range. Three levels of drug concentrations (low concentration, medium concentration, and high concentration) were selected in a linear calibration range and each of them was replicated 4 times for DZ–MO and 5 times for DZ–BPB systems within-day to determine the repeatability (intra-day precision) and between-day to determine the intermediate precision (inter-day precision). The accuracy of the proposed method was determined by the percent relative error (%RE). The RE comes from bias calculation. The calculated parameters for accuracy and precision are summarized in Table 3.

TABLE 3. Evaluations of Inter-day and Intra-day Accuracy and Precision for DZ in Pure Form

Indicator	Parameter	Intra-day accuracy and precision (within-day)			Inter-day accuracy and precision (between-day)		
MO (n = 4)	Taken, μg/mL	8.33	25	41.66	8.33	25	41.66
	Found, μg/mL	8.43	25.14	40.00	8.14	24.28	41.28
		8.00	24.57	42.43	8.57	25	41.43
		8.86	25.28	39.57	7.85	24.71	41.43
		7.86	24.43	43.28	8.00	25.28	42
	Average	8.28	24.85	41.32	8.14	24.81	41.53
	Recovery, %	99.39	99.44	99.18	97.72	99.28	99.71
	SD	0.454	0.417	1.81	0.307	0.423	0.318
	RSD	0.054	0.016	0.043	0.038	0.017	0.007
	RE	0.005	0.038	0.008	0.022	0.018	0.003
BPB (n = 5)	Taken, μg/mL	8.33	25	41.66	8.33	25	41.66
	Found, μg/mL	8.47	25.73	40.39	8.69	26.26	41.95
		9.13	26.01	41.34	9.21	25.17	41.09
		8.78	26.73	40.87	8.56	25.82	39.95
		8.22	26.86	40.65	8.22	25.39	41.34
		9.21	24.43	40.04	9.3	24.65	38.32
	Average	8.76	25.95	40.65	8.79	25.45	40.63
	Recovery, %	105.1	103.8	99.97	105.5	101.8	97.53
	SD	0.482	0.974	0.490	0.453	0.615	1.244
	RSD	0.053	0.037	0.012	0.052	0.024	0.030
	RE	0.075	0.038	0.024	0.056	0.018	0.024

The developed method was applied for the determination of the claimed diazepam in two commercial pharmaceutical formulations and the obtained results were compared with the HPLC method (Fig. 5). Diazepam tablets (10 mg) were used as dosage forms.

The recovery experiments are shown in Table 4. The results indicate that there is a good agreement between the diazepam contents which was determined via the proposed and HPLC methods. In addition, the obtained results offer comparable accuracy (*t*-test) and precision (*F*-test), since the calculated values of *t* and *F* are less than the theoretical values (probability %95).

The specificity of the method was evaluated by investigating the interference liabilities from the common excipients that might be added during pharmaceutical formulation. The extent of interference by commonly associated excipients such as starch, sucrose, glucose, and fructose was determined by measuring the absorbance of a solution containing 25 μg/mL of DZ with MO and 35 μg/mL of the drug with BPB.

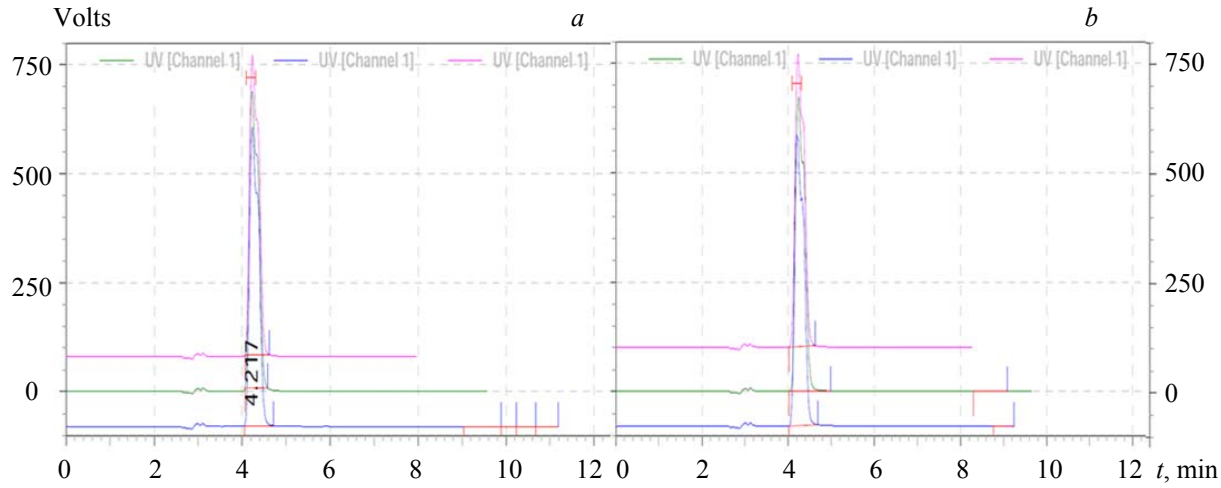


Fig. 5. HPLC chromatograms peak. Three replicates of 10 mg dosage DZ tablets from Loghman (a) and Abidi (b). Column: ODS (MZ, 5mm, 250×4.6 mm). Mobile phase: methanol. Volume of injection: 20 μL. Detector: UV 2690. Detection: spectrophotometer at 242 nm.

TABLE 4. Determination of DZ in 10 mg Tablets Using the Proposed Method and Comparison to Official Method (HPLC) (n = 3)

Statistical	MO		BPB	
parameters	HPLC method	Proposed method	HPLC method	Proposed method
Abidi				
Found, mg	9.91	10.08	9.91	9.99
Recovery, %	99.1	100.96	99.1	99.9
SD	0.035	0.062	0.035	0.026
RSD, %	0.353	0.628	0.353	0.270
t-test	4.29		3.23	
F-test	3.14		1.81	
Loghman				
Found, mg	9.99	10.14	9.66	9.85
Recovery, %	99.9	101.4	96.6	98.5
SD	0.026	0.087	0.167	0.264
RSD, %	0.270	0.860	1.73	2.47
t-test	3.25		1.26	
F-test	11.19		2.50	

N o t e. The theoretical values of t and F at p = 0.05 are 4.30 and 19.0, respectively [32].

Deviation swings lower than ±5% in the absorbance readings were accepted as tolerable determinations. The proposed method was found to be free from common usage pharmaceutical fillers. In order to test the statistical parameters of the method in the presence of interference, the analysis of laboratory-prepared samples was carried out four times using the general recommended procedure and the RE and RSD values were calculated. The obtained results are given in Table 5. These data confirmed the absence of interference of the common excipients with the determination of diazepam by the proposed spectrophotometric method.

The recommended procedure for the determination of diazepam in human serum was applied to evaluate its effectiveness. Diazepam was determined in human serum samples using the standard addition method. The recovery results for 21.73 μg/mL were achieved 103.86±1.6% for MO–DZ system and 90.36±2.6% for BPB–DZ system, with RSD < 2.9% (n = 3), confirming the proposed method is reliable for the determination of diazepam in the human serum sample.

TABLE 5. Analysis of DZ in the Presence of Common Excipients by the Proposed Method (n = 4)

Excipients	Maximum tolerance limit of excipients, μg/mL		RE, %		RSD, %	
	MO	BPB	MO	BPB	MO	BPB
Glucose	3000	3500	3.41	0.57	±4.71	±2.5
Sucrose	3500	3500	4.68	2.28	±3.26	±1.12
Fructose	3000	3000	2.72	1.28	±4.76	±1.66
Starch	2500	3000	1.68	0.21	±5.94	±1.81

N o t e. Tolerance limit refers to the concentration interfering with the determination of DZ (25 μg/mL of DZ with MO and 35 μg/mL of DZ with BPB indicators).

Conclusions. A sensitive spectrophotometric determination of diazepam is described based on ion-pair formation between diazepam and two dyes. The important advantage of the spectrophotometric technique versus the official method, like HPLC and GC, is its simplicity. This technique is not expensive and does not need complex instrumentations and is easy to use and does not need an advanced training course. This aspect of spectrophotometric analysis is of major interest in analytical pharmacy since it offers distinct possibilities in the assay of a particular component in complex dosage formulations. The suggested reagent in the proposed method is cheap and can be readily available. The offered method does not evolve any critical reaction conditions or difficult sample preparation. The proposed method is accurate, reproducible, adequately sensitive, and free from interference effects by common additives and excipients. Therefore, the validated method could be useful for routine quality control assays of diazepam in pharmaceutical raw materials and dosage forms.

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