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UV-Spectrophotometric Analysis of Diazepam Using Calibration Curve and Reference Standard Methods

Dobrina Tsvetkova[1]*, Stefka Ivanova[2], Maria Chaneva[3]

[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Medical University-Pleven, Sofia 1000, Bulgaria.
[2]Bulgarian Scientific Society of Pharmacy, Bulgaria.
[3]Clinic of Inrermnal Medicine, UMBALSM "N. I. Pirogov" – Sofia, Bulgaria.

***E-mail** ✉ dtsvetkova@pharmfac.mu-sofia.bg

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ABSTRACT

This study aimed to evaluate the method of the calibration curve (MCC) and the external reference standard method (MRS) to assess the accuracy and precision to validate the UV-spectrophotometric technique for quantifying diazepam at λ_{max} = 328 nm in 95% ethanol. Selectivity was confirmed by the absence of absorbance at λ_{max} = 328 nm in the UV spectrum of the blank solution, whereas this wavelength was distinctly observed for diazepam. The experimental data were analyzed using linear regression, yielding the equation y = 14387.x + 0.05. The linearity assessment included the calculation of the linear regression coefficient, which resulted in $R^2 > 0.998$. The limit of detection (LOD) and limit of quantification (LOQ) were determined as 1.84×10^{-5} g/ml and 1.04×10^{-5} g/ml, respectively. The accuracy was established by the recovery rate R (%) ± RSD (%) according to ICH guidelines: MCC yielded 100.49 ± 1.35%, while MRS resulted in 105.56 ± 1.37%. The standard deviation (SD) and relative standard deviation (RSD) values remained below 1.5, indicating a strong agreement between the measured results and the actual values. The precision evaluation showed that all values of diazepam content obtained using MCC and MRS at a confidence probability of P = 98% fell within the respective confidence intervals: MCC ranged from 4.88 mg to 4.96 mg, while MRS varied between 5.12 mg and 5.20 mg. Overall, these findings confirmed that the validated method is both accurate and reliable for the determination of diazepam concentrations in pharmaceutical formulations.

Keywords: Precision, Diazepam, Validation, Spectrophotometry, Accuracy

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Introduction

Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one) **(Figure 1)** is commonly utilized in clinical practice to manage symptoms associated with alcohol, opioid, and benzodiazepine withdrawal, as well as to treat anxiety and sleep disorders [1]. It also functions as a miorelaxant [2], prescribed for muscle spasms and conditions such as stroke-induced paresis [3]. Studies have also investigated its effect on the development of Chrysomya albiceps in decomposing rabbit tissue [4]. Moreover, diazepam is frequently administered as a pre-anesthetic sedative before procedures such as open-heart surgery [5] and aortic valve implantation [6].

Pharmacopoeial methods for analyzing diazepam are described in the European Pharmacopoeia [7] and British Pharmacopoeia [8], where a non-aqueous acid-base titration approach is used. This involves acetic anhydride as the reaction medium and 0.1 M perchloric acid as the titrant, with detection achieved either through the Nile blue indicator [7] or potentiometric techniques utilizing a diazepam ion-selective electrode [8, 9]. A potentiometric system with solid-contact ion-selective electrodes has been introduced for the concurrent determination of

diazepam, clonazepam, and bromazepam [10]. Additionally, electrochemical methods such as polarography have been employed for measuring diazepam in pharmaceutical preparations [11].

Titrimetric techniques offer advantages including rapid analysis, straightforward operation, and cost-effectiveness [12], but they lack specificity for complex mixtures. In contrast, separation-based approaches such as gas chromatography, HPLC, and capillary electrophoresis provide enhanced resolution, shorter analysis time, and minimal sample requirements [13]. Quantification of diazepam in tablet formulations has been successfully conducted using RP-HPLC [3], while reversed-phase liquid chromatography and capillary electrophoresis have been applied for simultaneous analysis of diazepam and otilonium bromide [14, 15].

Fluorimetry has been employed for measuring diazepam in both tablet and injectable forms [16] and for detecting other pharmaceutical compounds [17]. Spectrophotometric and fluorimetric techniques have also been utilized for analyzing diazepam, clonazepam, and bromazepam in pharmaceutical products and biological specimens, including urine [18]. While HPLC provides high precision, it requires specialized training for operation, whereas UV-spectrophotometry offers a more economical and user-friendly alternative [19].

A range of spectrophotometric methods has been utilized for diazepam analysis, including:

1. First-order derivative UV-spectrophotometry for individual assessment [20] and simultaneous detection with Otilonium bromide [21].
2. Second-order derivative spectrophotometry for evaluating 1,4-benzodiazepine mixtures [22].
3. Ratio-spectra derivative spectrophotometry for concurrent analysis of diazepam and Otilonium bromide [23].
4. Visible spectrophotometry for diazepam in different forms, including tablets and ampoules, after reacting with picric acid (λ = 475 nanometers) or with 3,5-dinitrobenzoic acid and 2,4-dinitrobenzoic acid (λ = 500 nanometers) [24].

Mass spectrometry is also a widely used technique for drug quantification [25]. However, derivative spectrophotometry has certain drawbacks, as it is highly sensitive to variations in instrument parameters. The zero-crossing technique, in particular, lacks reliability due to its susceptibility to small wavelength fluctuations, which can significantly alter outcomes [26]. Chemometric-assisted spectrophotometric methods have been applied for multi-drug analysis, such as for amlodipine besylate and candesartan cilexetil [27]. Unlike derivative methods, conventional UV-spectrophotometry remains a preferred approach due to its simplicity, precision, and reduced dependence on instrument conditions [19].

Because diazepam contains chromophoric groups, direct UV-spectrophotometric techniques allow its quantification in the ultraviolet region without requiring derivatization. One such method involves analysis in a methanol-distilled water mixture (1:1) at λ = 231 nanometers [26]. Other approaches have been developed to determine diazepam alongside Sodium benzoate in pharmaceutical formulations at λ = 306 nanometers [28] and to measure Diazepam with Caffeine and Phenylpropanolamine hydrochloride in tablet form [29].

According to the British Pharmacopoeia, diazepam quantification in tablets is performed through UV spectrophotometry in 0.5% methanolic sulfuric acid, with a specific absorbance of A (1%, 1 cm) = 450 at λ = 284 nanometers [7]. This research aimed to validate and compare UV-spectrophotometric techniques by assessing the calibration curve approach and the external standard method for diazepam determination in ethanol. The evaluation included parameters such as linearity, selectivity, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy.

Materials and Methods

Materials

1. Reference standard (RS): Diazepam.
2. Analytical-grade chemicals: SZBD 0500 V UN 1170, Sigma Aldrich, and 95% ethanol.

Methodology: UV-spectrophotometry

1. Apparatus: A UV-VIS diode array spectrophotometer (Hullett Packard N: 8452 A) was used.
2. Blank solution for analytical parameter selectivity: A blank solution was prepared with 95% ethanol, supplemented with starch that does not contain diazepam, typically used in tablet formulations. 0.05 g of starch was precisely weighed and dissolved in 95% ethanol to a final volume of 25.0 ml in a volumetric flask. A 1.0

ml aliquot of this solution was diluted to 10.0 ml with 95% ethanol. The blank solution was considered to be 95% ethanol, and absorbance was recorded at λ = 328 nm.

3. Preparation of diazepam Solutions for Linearity Testing: To prepare solutions for analyzing the linearity of the method, different quantities of diazepam (25, 37.5, 50, 75, 100, 125, and 175 milligrams) were dissolved in 95% ethanol and the final volume was made up to 250.0 ml. Aliquots of 10.0 ml were taken from each solution and further diluted to 100.0 ml with 95% ethanol. The resulting diazepam concentrations were: 1.10–5, 1.5.10–5, 2.10–5, 3.10–5, 4.10–5, 5.10–5, and 7.10–5 grams per milliliter. Absorbance was measured at λ = 328 nm against 95% ethanol.
4. Model mixture with diazepam for accuracy and precision validation: To validate the method's precision and accuracy, a model mixture was prepared by adding 5 mg of diazepam to a starch supplement, representing the theoretical concentration found in tablets. The model mixture, weighing around 0.05 g, was dissolved in 95% ethanol, and aliquots of 1.0 ml were diluted to 10.0 ml. Absorbance was measured at λ = 328 nm, with 95% ethanol used as the blank solution.
5. Reference diazepam solution for external standard method: For the external standard method, 5 mg of diazepam was dissolved in 95% ethanol, and the final volume was made up to 25.0 ml. Different concentrations were prepared by diluting 1.0 ml aliquots of this solution in 95% ethanol to 10.0 ml, resulting in a concentration of 2.10–5 g/ml. Absorbance was then measured at λ = 328 nm, with 95% ethanol used as a blank for compensation.
6. RMSE method for determining the limit of detection (LOD) and limit of quantitation (LOQ)

Calibration curves were generated by analyzing solutions at low concentrations. Upon conducting linear regression analysis on the resulting data, the linear correlation coefficients (R^2) were determined. The regression equation: y = a.x + b was applied to obtain the data for the predictable absorbance (Ap); the error E = |Ap - A|; E^2 = [|Ap - A|]^2, $E_1=\frac{\sum E^2}{n-2}$; RMSE =$\sqrt{E1}$, LOD = 3.RMSE/a, LOQ = 10.RMSE/a [30].

Results and Discussion

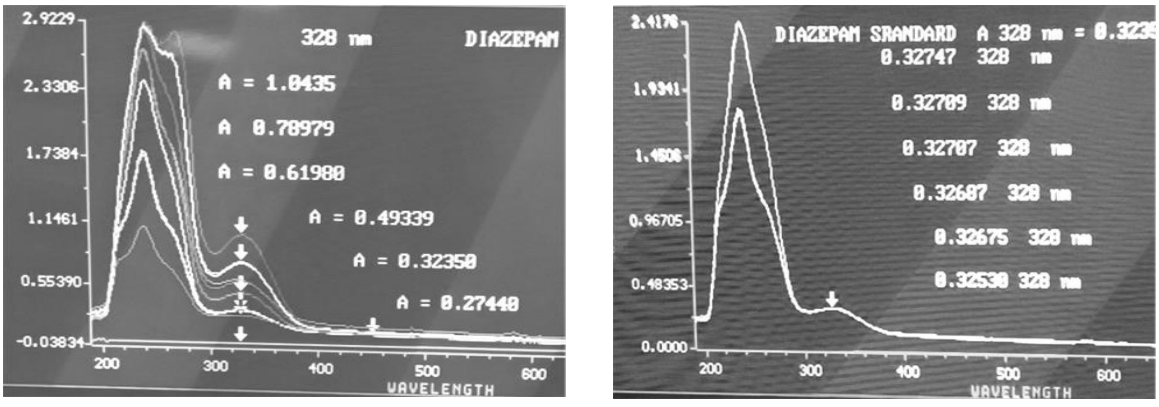
Validation of UV-spectrophotometric method [31-34]

Estimation of analytical parameter selectivity

Selectivity was assessed by ensuring no detectable absorption at the designated wavelengths in the UV spectra of blank solutions, confirming that the method can reliably distinguish the analyte from other potential components in the sample matrix [35, 36].

Investigation of analytical parameter linearity, precision, and accuracy

To test for linearity, solutions with concentrations between 1.10-5 g/ml and 7.10-5 g/ml were prepared using diazepam as the reference standard. A linear relationship between concentration and signal output was confirmed using regression analysis [31-35]. Accuracy and repeatability were evaluated by preparing six diazepam mixtures, with absorbances measured at λ = 328 nm. The UV spectra for these samples are illustrated in **Figure 1**.



a) b)

Figure 1. UV-spectra for standard solutions of diazepam. a) Linearity, b) Accuracy and precision

Table 1 presents a summary of the experimental results, showing the observed absorbance values for various solutions with increasing concentrations of diazepam.

Table 1. Concentrations and absorbances for reference standards of diazepam in 95% ethanol for estimation of analytical parameter linearity.

N:	C [g/ml]	A
1.	1.10^{-5}	0.18515
2.	$1.5.10^{-5}$	0.27440
3.	2.10^{-5}	0.32350
4.	3.10^{-5}	0.49339
5.	4.10^{-5}	0.61980
6.	5.10^{-5}	0.78979
7.	7.10^{-5}	1.0435

The experimental absorbance values for the diazepam reference standards were subjected to linear regression analysis. The parameters of the regression equation, which demonstrated a linear relationship between absorbance and concentration within the specified concentration ranges, are provided in **Table 2**.

Table 2. Parameters of the regression equation for diazepam.

N:	Parameter	Result
1.	The linear interval [g/ml]	$1.10^{-5} \div 7.10^{-5}$
2.	Regression equation	y = 14387.49. x + 0.05
3.	Slope (a)	14387.49
4.	Standard slope error	286.9014
5.	Inrersept (b)	0.049782
6.	Standard intercept error	0.011178
7.	Correlation coefficient (R^2)	0.9980

Figure 2 illustrates the calibration curve, showing the linear relationship between absorbance (A) and concentration (C) in [g/ml]. The linearity is quantified by the regression coefficient, with $R^2 > 0.9980$.

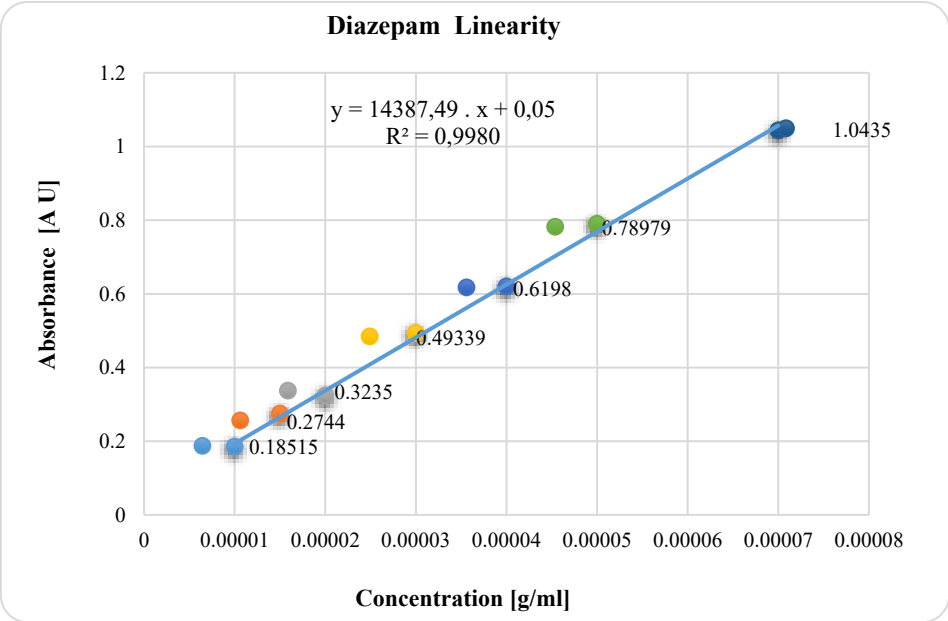


Figure 2. Calibration curve for linearity for diazepam.

Estimation of analytical parameters limit of detection (LOD) and limit of quantitation (LOQ)

The analysis of standard solutions with progressively increasing diazepam concentrations, aimed at determining the LOD and LOQ, is summarized in **Table 3**. The concentration (C) [g/ml] and the measured absorbance (A) are provided, along with the absorbance values calculated from the calibration curve (Ap). The deviation between the calculated and measured absorbance is represented by $E = |Ap - A|$, and the squared deviation is $E^2 = [|Ap - A|]^2$. RMSE is computed as the square root of E^2. The LOD and LOQ are then calculated using the formulas: $LOD = 3 \times RMSE / a$ and $LOQ = 10 \times RMSE / a$. These values were derived from the regression equation $y = 14387.49x + 0.05$, utilizing the RMSE-based method [30].

Table 3. RMSE method for LOD and LOQ for diazepam in 95% ethanol.

N:	C [g/ml]	A	A_p	$E = \|A_p - A\|$	$E^2 = [\|A_p - A\|]^2$
1.	1.10^{-5}	0.18515	0.19387	0,00872	0.00008
2.	$1.5.10^{-5}$	0,27440	0.26581	0,00859	0.00007
3.	2.10^{-5}	0.32350	0.33775	0,01425	0.00020
4.	3.10^{-5}	0.49339	0.48162	0,01177	0.00014
5.	4.10^{-5}	0.61980	0.62550	0,00570	0.00003
6.	5.10^{-5}	0.78979	0.76937	0,02042	0.00042
7.	7.10^{-5}	1.04350	1.05712	0,01362	0.00019
$\sum E2 = 0.00113$		$E1 = \frac{\sum E2}{n-2} = 0.000226$		RMSE= $\sqrt{0.000226} = 0.015$	
LOD = (3.0.015)/14387.49 = $3.13.10^{-6}$ g/ml				LOQ = (10.0.015)/14387.49 = $1.04.10^{-5}$ g/ml	

Estimation of analytical parameters precision and accuracy for diazepam

Table 4 provides the values for the following parameters: 1) the content of diazepam included in the sample, 2) the weighed amount of diazepam used for analysis, and 3) the absorbance values, including A and ASt, which are 0.32350.

Table 4. Added and content aud absorbances for mixtures of diazepam.

N	Added content [mg]	Weight content [g]	Absorbance A
1.	4.84	0.0484	0.32530
2.	4.86	0.0486	0.32675
3.	4.88	0.0488	0.32687
4	4.91	0.0491	0.32707
5.	4.92	0.0492	0.32709
6.	4.94	0.0494	0.32747
$\overline{X}$			0.32676
SD			0.0008
RSD (%)			0.24

Using the calibration curve (MCC) and external reference standard (MRS) methods, the amount of diazepam was calculated and is summarized in **Table 5**. The table includes: N – the number of individual measurements (n = 6); C – the determined diazepam content; UC – Schöveneou¢s value for the obtained quantity (UC); R (%) – recovery percentage (RC); $\overline{X}$ – the average value; SD – standard deviation; RSD (%) – relative standard deviation; $S\overline{X}$ – the standard error of the mean; and the confidence interval (CI), calculated as $\overline{X} \pm t.S\overline{X}$, with a confidence level of 98% and a Student's t value of 3.37. E (%) represents the relative error in the results.

Table 5. Obtained quantity (C), recovery (R), and Schöveneou's criterion (U) for C –estimation by methods of the calibration curve and of external standards.

	Method of the calibration curve			Method of external standard		
N:	Obtained quantity C (mg)	R C (%)	U C	Obtained quantity C (mg)	R (%)	U C

1.	4.94	102.07	0.67	5.19	107.23	1.00
2.	4.95	101.85	1.00	5.20	107.00	1.33
3.	4.93	101.02	0.33	5.18	106.15	0.67
4.	4.90	99.80	0.67	5.15	104.89	0.33
5.	4.89	99.39	1.00	5.14	104.47	0.67
6.	4.88	98.79	1.33	5.12	103.64	1.33
$\overline{X}$± SD	4.92 ± 0.03			5.16 ± 0.03		
R (%) ± RSD (%)		100.49 ±1.35			105.56 ±1.37	
SD	0.03	1.36		0.03	1.45	
RSD	0.61	1.35		0.58	1.37	
S$\overline{X}$	0.012	0.56		0.012	0.59	
t.S$\overline{X}$	0.04	1.89		0.04	1.99	
$\overline{X}$– t.S$\overline{X}$÷$\overline{X}$+ t.S$\overline{X}$	4.88 ÷ 4.96	98.60 ÷102.38		5.12 ÷ 5.20	103.57 ÷107.55	
E (%)	0.24	0.56		0.23	0.56	

The Chauvenet's criterion values for all the experimental data are below the highest allowable threshold, with U < 1.73 (n = 6), ensuring that any outliers with significant discrepancies are excluded from the analysis.

Accuracy

To assess accuracy, the standard deviation (SD) is calculated using Bessel's correction. Accuracy is defined as the degree of agreement between the mean of repeated measurements and the true values. Recovery tests conducted on six samples at the nominal concentration of 100% helped evaluate the procedure's accuracy. The accuracy was expressed as R (%) ± RSD (%), in line with ICH guidelines [31-34]: MCC: 100.49% ± 1.35%, MRS: 105.56% ± 1.37%. These results confirm that all recovery data fall within their respective confidence intervals at a 98% confidence level: MCC: 98.60% – 102.38%; MRS: 103.57% – 107.55%. SD and RSD values were both below 1.5, indicating strong agreement between the experimental results and the true values.

Precision (repeatability)

The precision of the analytical method, assessed through SD, RSD, and confidence intervals [30-36], was evaluated by repeated scanning of diazepam samples (n = 6). The results show that the content values for diazepam, obtained through both MES and MCC methods at a 98% confidence level, are within the respective confidence intervals: MCC: 4.88 mg – 4.96 mg; MRS: 5.12 mg – 5.20 mg. The proximity of the results to the mean and the narrow confidence intervals indicate low uncertainty, with all SD values under 1.5, demonstrating excellent repeatability and accuracy.

Conclusion

The UV-spectrophotometric method was validated following ICH guidelines for key analytical parameters, including linearity, selectivity, LOQ, LOD, accuracy, and precision, in the determination of diazepam in 95% ethanol. Both the calibration curve and external standard methods were used for the validation process. The results for accuracy and repeatability, within the specified confidence intervals, confirmed that the method is precise and accurate, making it suitable for quantifying diazepam in dosage forms.

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Conflict of Interest: None

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Ethics Statement: None

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