



Original Article

ISSN : 2277-3657
CODEN(USA) : IJPRPM

UV-Spectrophotometry for Determination of Diazepam by Comparative Estimation of Methods of Calibration Curve and Reference Standard

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ABSTRACT

The objective of the current work was to compare the methods of the calibration curve (MCC) and the external reference standard (MRS) for estimating the analytical parameters accuracy and precision in order to validate the UV-spectrophotometric method for the determination of diazepam at $\lambda_{max} = 328 \text{ nm}$ in 95% ethanol. The fact that the detected absorbance at $\lambda_{max} = 328 \text{ nm}$, a particular wavelength for diazepam, could not be found in the UV spectra of the blank solution was provided as evidence of selectivity. Analysis using linear regression was performed on the experimental findings: $y = 14387.x + 0.05$. An analysis of linearity was conducted by measuring the linear regression coefficient: $R^2 > 0.998$. $LOD = 1.84 \cdot 10^{-5} \text{ g/ml}$; $LOQ = 1.04 \cdot 10^{-5} \text{ g/ml}$. Accuracy was reflected and determined by recovery degree $R [\%] \pm RSD [\%]$ as per ICH guidelines: MCC: $100.49 \% \pm 1.35 \%$; MRS: $105.56 \% \pm 1.37 \%$. The fact that SD and RSD were less than 1.5 confirmed that the findings and actual values were in agreement. From the precision assessment, all values for the content of Diazepam, obtained both by MRS and MCC at the confidence possibility $P = 98\%$, suit the relevant confidence interval: MCC: $4.88 \text{ mg} \div 4.96 \text{ mg}$; MRS: $5.12 \text{ mg} \div 5.20 \text{ mg}$. Through the use of all of these data, it was demonstrated that the validated approach was reliable and accurate for determining the dose of Diazepam in medication formulations.

Key words: Diazepam, Spectrophotometry, Validation, Accuracy, Precision

INTRODUCTION

Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one (**Figure 1**) is applied commonly for therapy of features associated with alcohol, opiate, and benzodiazepine withdrawal, anxiety and trouble sleeping [1]. The drug is used as miorelaxant [2] for muscle spasms and induced by stroke paresis [3]. Diazepam possesses effect on the growth of *Chrysomya albiceps* in rabbit carcass [4]. Diazepam is applied as an anesthetic for preoperative premedication before endoscopic or surgical procedures like open-heart surgery [5] or aortic valve implantation [6].

In European Pharmacopoeia [7] and British Pharmacopoeia [8] for the determination of Diazepam substance is applied acid-base neutralization non-water method in the medium of acetic anhydride and titration with 0.1 M perchloric acid by using indicator Nile blue [7] or potentiometrically [8] with Diazepam ion-selective electrode [9]. For simultaneous potentiometric quantification of Diazepam, Bromazepam, and Clonazepam has been described as the method with solid contact ion-selective electrodes [10]. Electrochemical assay of Diazepam in pharmaceutical products by polarography has been reported [11].

The advantages of the titrimetric method are the short time of analysis, quick and easy performance, cost-effectivity, and simple instrumentation [12]. The disadvantage of titrimetric methods is the lack of selectivity for the analysis of combinations in comparison with the separation techniques: gas chromatography, HPLC, and capillary electrophoresis, which offer significant advantages, such as high separation efficiency, fast analysis, and small sample volumes [13].

Diazepam dose estimation in tablet form using the RP-HPLC technique has been introduced [3]. For the simultaneous measurement of Diazepam and Otilonium bromide in tablets, reversed-phase liquid chromatography and capillary electrophoresis have been devised [14, 15].

Fluorimetry has been applied for the quantification of Diazepam in tablets and injections [16] and of other drugs [17]. Spectrophotometric and fluorimetric methods for the assay of Diazepam, Bromazepam, and Clonazepam in pharmaceutical and urine samples have been used [18].

The drawback of HPLC is that it requires a trained technician for monitoring. In comparison with HPLC, the advantages of UV-spectrophotometric methods are simplicity of procedures and economy [19].

The following spectrophotometric techniques for analyzing Diazepam have been published:

1. first-order derivative UV-spectrophotometry for determination of Diazepam alone [20] and for simultaneous quantification in combination with Diazepam and Otilonium bromide [21],
2. second-order-derivative spectrophotometry for analysis of mixtures of 1,4-benzodiazepines [22],
3. ratio-spectra derivative spectrophotometry for simultaneous assay of Diazepam and Otilonium bromide [23],
4. visible spectrophotometry for Diazepam in pure form, tablets, and ampoules after the interaction with picric acid at $\lambda = 475$ nm and with 3,5-dinitrobenzoic acid and 2,4-dinitrobenzoic acid at $\lambda = 500$ nm [24].

Mass spectrometry is also used for drug analysis [25]. Derivative spectrophotometry has the drawback of being sensitive to adjustments in the settings of the device. Particularly in the zero-crossing approach, which lacks repeatability due to inaccuracies in the registration of the spectrum, small variations in the wavelength setting have a significant impact on the outcome [26]. Chemometric assisted spectrophotometric method is applied for the simultaneous evaluation of drugs, such as of Amlodipine Besylate and Candesartan Cilexeti [27]. Contrary to the UV-derivative approach, the standard UV method has lower sensitivity to changes in the apparatus settings and is one of the most widely used methods for pharmaceutical analysis because it is quick, easy, specific, accurate, and speedy [19].

Due to the content chemical structure of chromophore groups, Diazepam is developed UV-spectrophotometric methods for direct determination in the ultraviolet region without the need for a derivatization reaction. UV-spectrophotometric method for analysis of Diazepam substance in solvent mixture methanol: distilled water = 1:1 at $\lambda = 231$ nm has been described [26]. UV-spectrophotometric methods for quantification of Diazepam and Sodium benzoate in dosage drug forms at $\lambda = 306$ nm [28] and for simultaneous assay of Diazepam, Caffeine, and Phenylpropanolamine hydrochloride in tablets have been developed [29].

Following British Pharmacopoeia the determination of Diazepam in tablets is spectrophotometrically in UV-area in 0.5 % methanolic sulphuric acid a by method of specific absorbance: $A(1\%, 1 \text{ cm}) = 450$ at $\lambda = 284$ nm [7].

By estimating analytical characteristics such as selectivity, linearity, LOD, LOQ, accuracy, and precision, the current study aimed to validate and compare traditional UV-spectrophotometric techniques of calibration curves and methods of external standards for the measurement of Diazepam in ethanol.

MATERIALS AND METHODS

Materials

1. Reference standard (RS): Diazepam.
2. Purity-graded chemicals for analytical use: SZBD 0500 V UN 1170, Sigma Aldrich, 95% ethanol.

Method. UV-Spectrophotometry

1. Equipment.

UV-VIS diode array spectrophotometer (Hullett Packard N: 8452 A).

2. Preparation of blank solution for estimation of an analytical parameter selectivity.

To estimate the analytical parameter selectivity, a blank solution in 95% ethanol was made. Diazepam-free supplement starch, which is utilized in tablet manufacturing, was added to blank solutions. A volumetric flask was used to dissolve 0.05 g of supplement starch that had been precisely weighed in 95% ethanol to a volume of 25.0 ml, and a portion of the solution equal to 1.0 ml was divided into 10.0 ml and then diluted again in 95% ethanol. 95% ethanol was applied as a blank solution. The data for the absorbance were measured at $\lambda = 328$ nm.

3. *Preparation of solutions of reference standard Diazepam in 95% ethanol for estimation of analytical parameter linearity.*

95% ethanol was used to dissolve a precisely measured amount of the reference standard Diazepam (25 mg, 37.5 mg, 50 mg, 75 mg, 100 mg, 125 mg, and 175 mg), and the same solvent was then used to dilute the solution to 250.0 ml in volumetric flasks. Aliquot portions of 10.0 ml of the obtained solutions were diluted in 95% ethanol to 100.0 ml in volumetric flasks, resulting in solutions with equivalent concentrations of diazepam: 1.10^{-5} g/ml, $1.5.10^{-5}$ g/ml, 2.10^{-5} g/ml, 3.10^{-5} g/ml, 4.10^{-5} g/ml, 5.10^{-5} g/ml and 7.10^{-5} g/ml. All solutions were analyzed at $\lambda = 328$ nm against 95% ethanol and the absorbances were measured.

4. *Preparation of model mixture with Diazepam for validation of the UV-method for analytical parameters accuracy and precision (repeatability).*

By adding an active ingredient, Diazepam, equal to 100% (5 mg) of the theoretical concentration of Diazepam in tablets (5 mg), a model combination was created from the supplement in tablet form (starch). The model combination weighed 0.05 g on average. The model mixture amount, which is equal to 5 mg of benzodiazepine, was precisely measured to create six identical, homogeneous samples. All samples were dissolved in 95% ethanol and were diluted with 95% ethanol to 25.0 ml in volumetric flasks. The produced solutions were diluted in volumetric flasks with aliquots of 1.0 ml each in 95% ethanol to 10.0 ml. As the blank solution, 95% ethanol was used to measure the absorbances of the produced solutions at $\lambda = 328$ nm.

5. *Preparation of reference solution of Diazepam for the method of an external standard.*

In volumetric flasks, 95% ethanol was used to dilute a precisely measured quantity of 5 mg of the reference standard benzodiazepine to 25.0 ml. Diazepam solutions with various concentrations were created by diluting aliquot portions of the resultant solution, which included 1.0 ml, in 95% ethanol to 10.0 ml in a volumetric flask: 2.10^{-5} g/ml. The absorbance of the final solution was detected at $\lambda = 328$ nm applying 95% ethanol as compensation.

6. *Root limit mean square error method (RMSE) for the determination of limit of detection (LOD) and limit of quantitation (LOQ).*

The examination of solutions with low concentrations was used to create calibration curves. After the data underwent linear regression analysis, the linear correlation coefficients (R^2) were discovered. The regression equation: $y = a.x + b$ was applied for obtaining the data for the predictable absorbance (A_p); the error $E = |A_p - A|$; $E^2 = [|A_p - A|]^2$, $E_1 = \frac{\sum E^2}{n - 2}$; $RMSE = \sqrt{E_1}$, $LOD = 3.RMSE/a$, $LOQ = 10.RMSE/a$ [30].

RESULTS AND DISCUSSION

Validation of UV-spectrophotometric method [31-34]

1. Estimation of analytical parameter selectivity

When a component that could be present in the sample matrix is present, the analyte is quantitatively detected by selectivity [35, 36]. Selectivity was demonstrated by the absence of measurable absorption at the targeted wavelengths in the UV spectra of blank solutions.

2. Investigation of analytical parameter linearity, accuracy and precision.

Application of the method of linear regression analysis

Linearity is the portion of a detector-derived signal that continues to exhibit a linear relationship with the analyte's concentration [31-35].

A series of solutions with increasing concentrations (1.10^{-5} g/ml \div 7.10^{-5} g/ml) were created in order to investigate the linearity of analytical parameters from the reference standard Diazepam. For the investigation of analytical

parameter accuracy and repeatability 6 mixtures containing Diazepam were prepared. The absorbances (A) at wavelength $\lambda = 328$ nm were measured and UV-spectra were detected and were demonstrated on **Figure 1**.

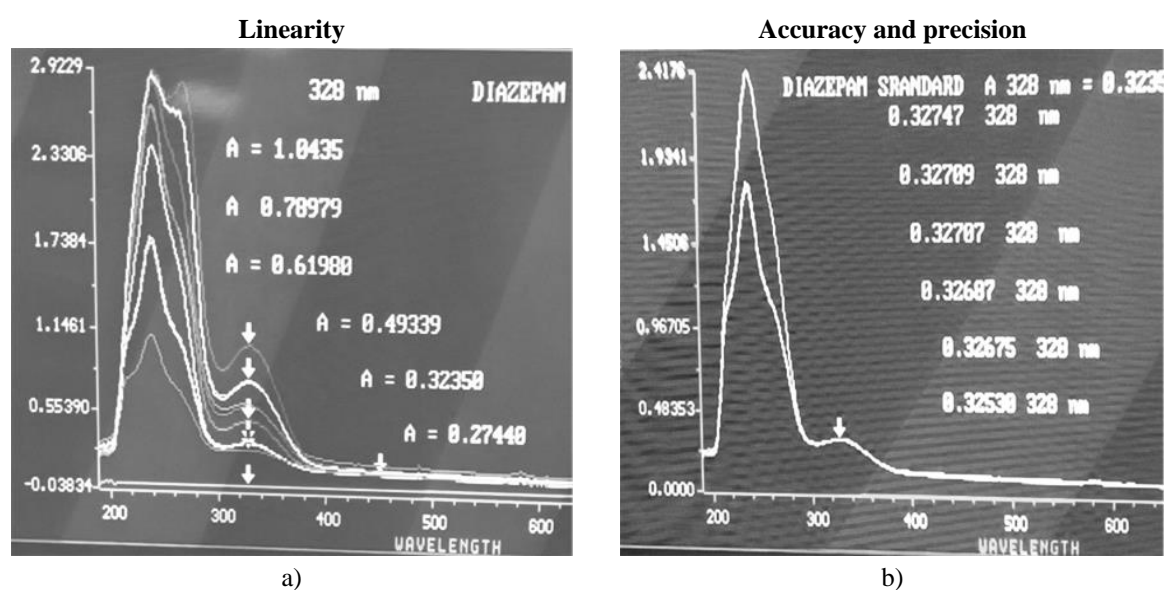


Figure 1. UV-spectra for standard solutions of Diazepam.

Table 1 provides an overview of the experimental findings for the observed absorbances of a number of solutions with escalating Diazepam doses.

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 obtained experimental results for absorbances of reference standards of Diazepam were used to linear regression analysis. The regression equation's parameters, which showed a linear connection between absorbances and concentrations at the relevant concentration intervals, were given 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	$y = 14387.49 \cdot x + 0.05$
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 depicts the calibration curve, which demonstrates the linear connection between A and concentration C [g/ml]. Linear regression coefficients serve as a measure of linearity: $R^2 > 0.9980$.

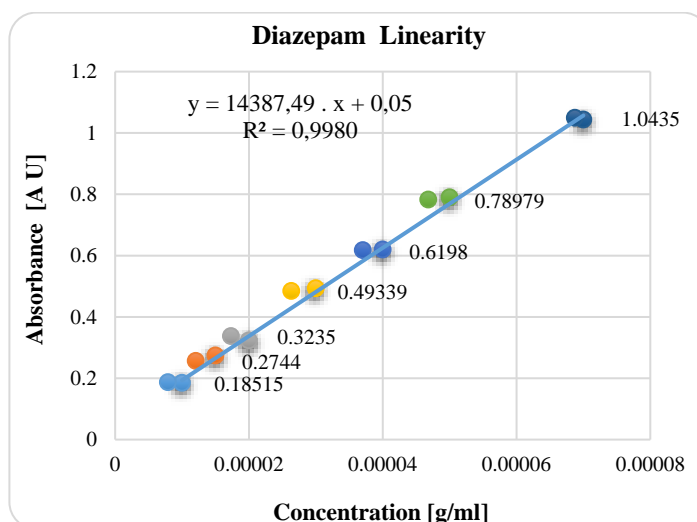


Figure 2. Calibration curve for linearity for Diazepam.

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

Table 3 includes the findings from the examination of standard solutions containing progressively higher concentrations of Diazepam to investigate LOD and LOQ: C [g/ml] – concentration, A – measured absorbance, A_p – calculated by calibration curve absorbance, $E = |A_p - A|$, $E^2 = [(A_p - A)]^2$, $RMSE = \sqrt{E1}$, $LOD = 3 \cdot RMSE/a$, $LOQ = 10 \cdot RMSE/a$. LOD and LOQ are based on the regression equation: $y = 14387.49 \cdot x + 0.05$ by application of RMSE-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.	$1 \cdot 10^{-5}$	0.18515	0.19387	0,00872	0.00008
2.	$1.5 \cdot 10^{-5}$	0,27440	0.26581	0,00859	0.00007
3.	$2 \cdot 10^{-5}$	0.32350	0.33775	0,01425	0.00020
4.	$3 \cdot 10^{-5}$	0.49339	0.48162	0,01177	0.00014
5.	$4 \cdot 10^{-5}$	0.61980	0.62550	0,00570	0.00003
6.	$5 \cdot 10^{-5}$	0.78979	0.76937	0,02042	0.00042
7.	$7 \cdot 10^{-5}$	1.04350	1.05712	0,01362	0.00019
$\sum E^2 = 0.00113$		$E1 = \frac{\sum E^2}{n-2} = 0.000226$		$RMSE = \sqrt{0.000226} = 0.015$	
LOD = $(3 \cdot 0.015)/14387.49 = 3.13 \cdot 10^{-6}$ g/ml			LOQ = $(10 \cdot 0.015)/14387.49 = 1.04 \cdot 10^{-5}$ g/ml		

4. Estimation of analytical parameters accuracy and precision for Diazepam.

Table 4 are presented values for: 1) included content of Diazepam; 2) weighed quantity of Diazepam for analysis; 3) absorbances: A, $A_{St} = 0.32350$.

Table 4. Added and content and 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
\bar{X}			0.32676
SD			0.0008
RSD [%]			0.24

By using the calibration curve technique (MCC) and the external reference standard method (MRS) in **Table 5**, the amount of Diazepam was determined. It is indicated as N – number of the individual measurements ($n = 6$); C – received content of Diazepam; UC – Schöveneou's value for obtained quantity (UC); R (%) – level of recovery (RC); \bar{X} – arithmetical mean; SD – standard deviation; RSD [%] – relative standard deviation; $S\bar{X}$ – mean quadratic error; $\bar{X} - t.S\bar{X} \div \bar{X} + t.S\bar{X}$ – confidence interval (CI); E (%) – relative error. For the calculation of the confidence interval are used: the confidence probability was 98% and the coefficient of Student was 3.37.

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.

N:	Method of the calibration curve			Method of external standard		
	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
$\bar{X} \pm SD$	4.92 \pm 0.03			5.16 \pm 0.03		
R [%] \pm RSD [%]	100.49 \pm 1.35			105.56 \pm 1.37		
SD	0.03	1.36		0.03	1.45	
RSD	0.61	1.35		0.58	1.37	
$S\bar{X}$	0.012	0.56		0.012	0.59	
$t.S\bar{X}$	0.04	1.89		0.04	1.99	
$\bar{X} - t.S\bar{X} \div \bar{X} + t.S\bar{X}$	4.88 \div 4.96	98.60 \div 102.38		5.12 \div 5.20	103.57 \div 107.55	
E [%]	0.24	0.56		0.23	0.56	

The data for Chauvenet's criteria are lower for all of the observed experimental findings than the highest permitted standard requirement: $U < 1.73$ ($n = 6$) is used to determine if findings with sharp discrepancies should be eliminated.

Accuracy

A sample standard deviation (SD) is determined for the estimation of accuracy and precision by using Bessel's adjustment.

Accuracy is the level of agreement between the mean result of the repeated measurements and the real values. By carrying out the recovery for 6 samples at a level equal to 100% of the nominal concentration, the accuracy of the procedure was assessed. R [%] \pm RSD [%] was used as a measure of accuracy in accordance with ICH recommendations [31-34]: MCC: 100.49 % \pm 1.35 %; MRS: 105.56 % \pm 1.37 %. Results indicate that all data for degree of recovery fit respective confidence intervals at the specified confidence level $P = 98\%$: MCC: 98.60 % \div 102.38 %; MRS: 103.57 % \div 107.55 %. SD and RSD were less than 1.5, demonstrating agreement between the findings and real values.

Precision (repeatability)

The uncertainty of the outcome, which is calculated by the SD, RSD, and CI [30-36], is employed for the estimate of an analytical parameter with accuracy (repeatability). By scanning samples of Diazepam ($n = 6$) repeatedly, repeatability was achieved. It is clear from the assessment of precision that all values for the content of Diazepam, obtained by MES and MCC at the confidence level $P = 98\%$, are consistent with the applicable confidence interval: MCC: 4.88 mg \div 4.96 mg; MRS: 5.12 mg \div 5.20 mg. Due to the close proximity of the findings to the average value and the corresponding small confidence interval, all values for SD are less than $SD = 1.5$, indicating good accuracy.

CONCLUSION

The current UV-spectrophotometric technique was validated in accordance with the ICH criteria for the analytical parameters of selectivity, linearity, LOD, LOQ, accuracy, and precision for the measurement of diazepam in 95% ethanol using the method of the calibration curve and the methodology of the external standard. The consistency of the results for repeatability and accuracy within the appropriate confidence intervals demonstrated that the validated technique was determined to be precise and accurate for use in determining the amount of diazepam in the dose medication formulation.

ACKNOWLEDGMENTS : None

CONFLICT OF INTEREST : None

FINANCIAL SUPPORT : None

ETHICS STATEMENT : None

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