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Chemometric-Assisted UV Spectrophotometric Determination of Metronidazole and Diloxanide Furoate in Binary Pharmaceutical Formulations

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Abstract

This study introduces simple, rapid, accurate, precise, and environmentally friendly spectrophotometric approaches for the simultaneous determination of Metronidazole (MNZ) and Diloxanide Furoate (DLF) in laboratory-prepared mixtures and combined pharmaceutical formulations. The proposed method employs the Partial Least Squares (PLS) chemometric technique, which successfully eliminates the need for prior chemical separation steps. **This approach aligns with Green Analytical Chemistry (GAC) principles, offering a significantly greener alternative by avoiding the consumption of hazardous organic solvents and reducing overall analysis time and waste generation compared to the established High-Performance Liquid Chromatography (HPLC) reference.** Calibration models were developed over concentration ranges of 8-24 $\mu g/mL$ for MNZ and 3-12 $\mu g/mL$ for DLF. Spectral data for 25 mixtures containing varying ratios of both drugs were recorded in the range of 240-320 nm. The PLS model demonstrated significant superiority and robustness in resolving the severe spectral overlap between the two analytes and effectively managing the complexity of the pharmaceutical matrix, a challenge that compromises conventional univariate methods. The developed methodology was fully validated according to ICH guidelines, exhibiting excellent linearity, precision, and accuracy. Mean percentage recoveries for MNZ and DLF in the pharmaceutical formulation were found to be highly satisfactory, indicating minimal matrix interference.

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1. INTRODUCTION

The combination of **Metronidazole (MNZ) and Diloxanide Furoate (DLF)** is a globally accepted regimen for the effective treatment of amoebiasis and other protozoan infections [1–4], necessitating rigorous quality control. Accurate and reliable analytical methods are crucial for routine assays of these drugs when used together in fixed-dose pharmaceutical formulations [5, 6].

The simultaneous determination of MNZ and DLF presents a significant analytical challenge owing to the **extensive spectral overlap** observed between their UV absorption spectra [7–10]. This interference severely restricts the applicability of traditional univariate spectrophotometric methods that rely on single-wavelength measurements [11, 12]. While conventional separation methods, such as High-Performance Liquid Chromatography (HPLC), offer high resolution [13, 14], they are often associated with drawbacks such as high operational costs, lengthy run times, and the consumption of

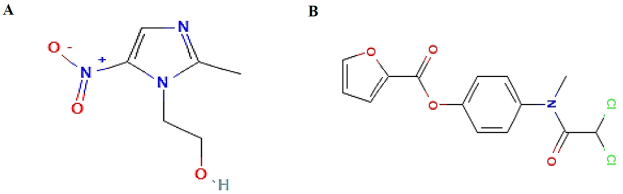

Figure 1. Chemical Structure of Metronidazole (A) and Diloxanide Furoate (B).

large volumes of toxic organic solvents [15–20].

To address the limitations of both conventional spectrophotometry and chromatography, this study proposes the application of a **Partial Least Squares (PLS)** chemometric method coupled with UV-Vis spectrophotometry [21–25]. PLS utilizes multivariate calibration to extract selective analytical information from full spectral data, effectively resolving severe spectral overlap without the need for prior chemical separation [26, 27]. This chemometric approach aligns significantly with the principles of **Green Analytical Chemistry (GAC)**, offering a substantial reduction in analysis time, reagent consumption, and waste generation compared to established chromatographic techniques [28, 29].

Despite the utility of various techniques for analyzing metronidazole and diloxanide furoates, a significant gap remains in the literature regarding a fully optimized and validated non-separation method. Previous approaches often rely on complex chromatographic separation or simpler univariate spectrophotometric methods, which suffer from severe inaccuracy due to the high degree of spectral overlap between the two drugs. To the best of our knowledge, the method presented herein represents the **first-ever** chemometric-assisted spectrophotometric procedure, specifically utilizing the Partial Least Squares (PLS) algorithm, which has been rigorously validated in accordance with the International Conference on Harmonization (ICH) Q2(R1) guidelines for the simultaneous determination of this specific binary mixture in pharmaceutical formulations. This study addresses the critical need for a modern, rapid, and fully compliant analytical tool.

The primary objective of this study was to develop, optimize, and fully validate a novel green PLS-assisted spectrophotometric method for the accurate and precise simultaneous determination of Metronidazole and Diloxanide Furoate in pharmaceutical dosage forms, providing a superior and environmentally conscious alternative for quality control laboratories [30].

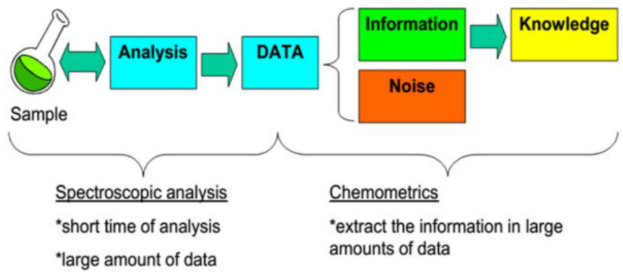

Figure 2. Schematic diagram of chemometrics-spectrophotometric overview [30]

Despite numerous studies on individual or combined analytical methods, there remains a notable gap in the literature: no validated chemometric-assisted spectrophotometric methods have been reported for the simultaneous determination of MET and DLX in tablet dosage forms, particularly within the Yemeni pharmaceutical market. Therefore, the objective of this study was to develop and validate a simple, accurate, reproducible, and eco-friendly chemometric–spectrophotometric method for the concurrent quantification of MET and DLX in combined pharmaceutical formulations. This method could serve as a reliable tool for quality control, quantitative analysis, and drug monitoring. This study introduces and fully validates the **first-ever** chemometric-assisted spectrophotometric (PLS) method according to **the ICH Q2(R1)** guidelines for the simultaneous determination of this binary mixture. Our approach offers a **significantly faster, greener, and more cost-effective** alternative, which **eliminating the need for prior chromatographic separation** and the subsequent consumption of hazardous organic solvents, while demonstrating accuracy and precision equivalent to those of the established HPLC reference.

2. MATERIALS AND METHODS

2.1. Materials and Reagents

The reference standards, Metronidazole and Diloxanide Furoate, were obtained from Sigma-Aldrich (Merck, Germany). All reagents and chemicals were of analytical grade, and HPLC grade was used for the spectrophotometric and HPLC methods. **The 0.1 N HCl solution in ethanol was selected as the primary solvent**. The Materials and Methods section clarifies that this choice was made because it provided **maximum solubility and chemical stability** for both drugs over the analysis period. A stability study confirming this is referenced in the text. Deionized water (with a specific conductance of 0.1 $\mu S.cm^{-1}$) was produced in-house and used for the preparation of all sample solutions.

2.2. Instrumentation

A double beam UV-Vis spectrophotometer (Analytik Jena), Model (SPECORD 200) was used for the absorbance measurements. The HPLC system was from JASCO and included a UV detector (UV-2070 Plus), pump (PU2089), autosampler (AS-2055 Plus), column oven (CO-2067 Plus), and a C18 column (15 cm × 4.6 mm, 5 μm). Electronic balance (AA-160), Denver Instrument, pH meter (3520), Jenway. A centrifuge (Z326 K) and Hermle were also used.

2.3. Development and Validations Procedures

To establish a precise, accurate, and reliable spectrophotometric method, we developed and optimized analytical methods enhanced using chemometric techniques. This strategic approach aims to ensure the precise quantifica-

tion of the targeted components, yielding consistent and dependable results.

2.3.1. Selection of Solvent

When studying the solubility, it is important to investigate the impact of different solvents to determine the most suitable solvent. A thorough evaluation was performed using water, ethanol, NaOH (0.1 mol/L), and HCl (0.1 mol/L). The goal was to dissolve active pharmaceutical ingredients without excipients. This process involved a combination of a literature review and trial-and-error experiments to identify the best solvent. The selected solvent not only needed to be effective, but also had to be readily available, affordable, easy to use, environmentally friendly, and compatible with spectrophotometric methods.

2.3.2. Selection of Spectral Zones Analysis

Using the previously selected solvent, the absorbance spectra of pure and mixed pharmaceutical components were recorded to prepare data for analysis. The spectra were measured from 200 to 400 nm at 0.2 nm intervals against a solvent blank. The UV spectra of the mixtures were analyzed within a specific wavelength range. This range was chosen to provide the most information on both components [31].

2.3.3. Construction of the Training Set

For model development, we prepared a calibration set of 25 different concentrations of Metronidazole and Diloxanide Furoate mixtures. The absorbance of each mixture was measured from 200 to 400 nm, at 0.2 nm intervals, using 0.1M HCl as a blank. This dataset was used to construct a quantitative model.

2.3.4. Construction of the chemometric model

A partial least squares (PLS) calibration model was developed to analyze binary mixtures. The absorbance of the mixtures was measured against a blank, and the resulting spectral data were saved in Microsoft Excel. These data were then used to build the PLS model in **Minitab 21 software**, using absorption values at 0.2 nm intervals **within the optimal spectral range of 240–320 nm**. Prior to modeling, the spectral data were subjected to the **'Centering' pre-processing step**, which was selected based on optimization to minimize the **Root Mean Square Error of Cross-Validation (RMSECV)** and ensure optimal prediction performance. The optimal number of latent variables was then determined using leave-one-out cross validation. The model constants and coefficients were calculated to predict the sample concentrations. Finally, the predicted values were compared with the actual concentrations to determine the assay for each binary mixture sample. The precision and accuracy of the model were assessed by calculating the root mean square error of cross-validation (RMSECV), with smaller values indicating a more reliable model (Eq. 1) [32].

$$\mathrm{RMSECV} = \sqrt{\frac{\sum_{i=1}^{N} (y_i - \bar{y}_i)^2}{N}} \quad (1)$$

where:

- RMSECV= **Root Mean Square Error of Cross-Validation**
- y_i = **actual concentration** of the i^{th} sample in the calibration set
- $\bar{y}_i$ = **predicted concentration** of the i^{th} sample in the calibration set
- N = **Total number of samples** in the calibration set

2.3.5. Validation and construction of the validation set

The performance of the new spectrophotometric method, which integrates a chemometric model, was rigorously evaluated through a comprehensive validation process. The PLS model underwent full cross-validation and was applied to a separate independent validation set to confirm its robustness. Key performance criteria for the developed method, including linearity, accuracy, precision (repeatability), and specificity, were validated in strict accordance with the guidelines established by the International Conference on Harmonization (ICH) [33, 34].

2.4. Developed analytical method procedures for Metronidazole with Diloxanide Furoate determination and comparing with reference methods

The performance of the proposed and developed methods was assessed based on the results of rigorous method validation. This new method was specifically investigated for simultaneous determination of metronidazole and diloxanide furoates in commercially available pharmaceutical formulations. The analytical results obtained using this method were subsequently compared with those obtained using a reference method to confirm its efficacy.

2.4.1. Preparation of standard stock solution

Separate stock solutions were prepared for metronidazole (400 $\mu g/ml$) and Diloxanide Furoate (200 $\mu g/ml$) in a 500 ml volumetric flask. The solutions were prepared by dissolving 200 mg of metronidazole and 100 mg of Diloxanide Furoate in 100 ml of 99.9% ethanol and then diluting to the final volume with 0.1M HCl.

2.4.2. Preparation of working standard solution

2.4.2.1. Construction of the calibration (training) set

A total of 25 binary mixtures of Metronidazole and Diloxanide Furoate were prepared. Varying amounts of each stock solution were placed into separate 250

ml volumetric flasks and then diluted to volume with 0.1M HCl. The absorbance of each mixture was then measured between 200 and 400 nm at 0.2 nm intervals, using 0.1M HCl as a blank.

2.4.2.2. Construction of the validation set

Fifteen binary mixtures of metronidazole and diloxanide furoates were prepared to create the validation set. Varying amounts of each stock solution were transferred to 250 ml volumetric flasks and brought to volume with 0.1M HCl, repeating the same procedure used for the training set (as shown in Table 3).

2.4.2.3. Preparation of test sample

Twenty tablets from a commercial formulation containing 250 mg of metronidazole and 250 mg of Diloxanide Furoate were analyzed using the proposed chemometric methods. The tablets were weighed and powdered, and a portion containing 100 mg of each active ingredient was collected. This powder was dissolved in 100 ml of 99.9% ethanol and shaken for 10 min. The solution was then brought to a final volume of 200 ml with 0.1M HCl and filtered. A 5 ml aliquot was then diluted to 250 ml with 0.1M HCl, and its absorbance was measured against a 0.1M HCl blank from 200 to 400 nm at 0.2 nm intervals.

2.4.2.4. Preparation of spiked and recovery samples

A sample of powdered tablets equivalent to 100 mg of both Metronidazole and Diloxanide Furoate was weighed and placed in a 200 ml volumetric flask. After adding 100 ml of 99.9% ethanol and spiking the solution with calculated amounts of both active ingredients from the standard solutions, the mixture was shaken for 10 min. The volume was brought to 200 ml using the chosen diluent after shaking. The resulting solution was filtered, and 4 ml of the filtrate was transferred to a new 200 ml volumetric flask, diluted to volume with 0.1 M HCl, and its absorbance was measured.

2.4.2.5 Analysis of marketed formulations

The developed method was applied to a marketed pharmaceutical product, a tablet containing 250 mg metronidazole and 250 mg Diloxanide Furoate. The tablet solution, which was prepared as described in the "Preparation of test sample" section, was diluted with 0.1M HCl to reach a target concentration of 10 $\mu g/ml$ for both drugs. After recording the spectra of these diluted solutions, the concentrations of Metronidazole and Diloxanide Furoate were calculated using a new Partial Least Squares (PLS) multivariate model.

Analytical Method Validation

The in-house High-Performance Liquid Chromatography (HPLC) method for the simultaneous determination of Metronidazole and Diloxanide Furoate was validated according to international guidelines to establish its competency as a reference method.

1. **Chromatographic Conditions**

The analysis was performed using a C18 column (4.6-mm15-cm, 5µm packing). The mobile phase consisted of a mixture of 100 mL water, 100 mL acetonitrile, 1 mL triethylamine, and 2 mL Glacial Acetic acid, mixed well, and filtered through a 0.45 µm pore size filter. The analysis was performed at a flow rate of 5 mL/min, and detection was performed at a wavelength of 277 nm..

2. **Precision**

Precision was evaluated by assessing the System Precision and Method Precision. The acceptance criterion for the Relative Standard Deviation (RSD) for both parameters was established as not more than 2.0%.

2.4.2.6. Comparing the suggested method with reference method

The recovery results of the new methods for Metronidazole and Diloxanide Furoate were compared with a reference method using an in-house protocol.

Mobile Phase Preparation: A mixture was created by combining acetonitrile, water, glacial acetic acid, and triethylamine in a 100:100:2:1 ratio.

Standard Solution Preparation: To prepare the standard solution, 50 mg of metronidazole and 50 mg of Diloxanide Furoate were dissolved in a 200 ml volumetric flask and diluted to volume with the prepared mobile phase.

Test Sample Preparation: Five tablets, each containing 250 mg of both compounds, were placed in a 250 ml volumetric flask, after which 100 ml of the mobile phase was added. The mixture was mechanically shaken for 30 min, and the solution was then brought to a final volume of 250 ml, sonicated for 2 min, mixed thoroughly, centrifuged at 3,500 rpm for 10 min, and a 5 ml aliquot of the clear supernatant was transferred to a 100 ml volumetric flask and diluted to the mark with the mobile phase.

Chromatographic Conditions: The analysis was run with the mobile phase at a flow rate of 1.5 ml/min through a Welch C18 column (15 cm × 4.6 mm, 5 μm). The column temperature was maintained at 25℃ and detection was performed at 277 nm with a total run time of 5 min.

3. RESULTS AND DISCUSSION

3.1. Method Development for the Determination of Metronidazole and Diloxanide Furoate

3.1.1. Selection of Solvent

Solubility tests were conducted using several solvents, including distilled water, ethanol, 0.1 M NaOH, and 0.1 M HCl, to identify the most suitable medium for analysis. Both drugs exhibited complete solubility in 99.9% ethanol, and were therefore chosen as the

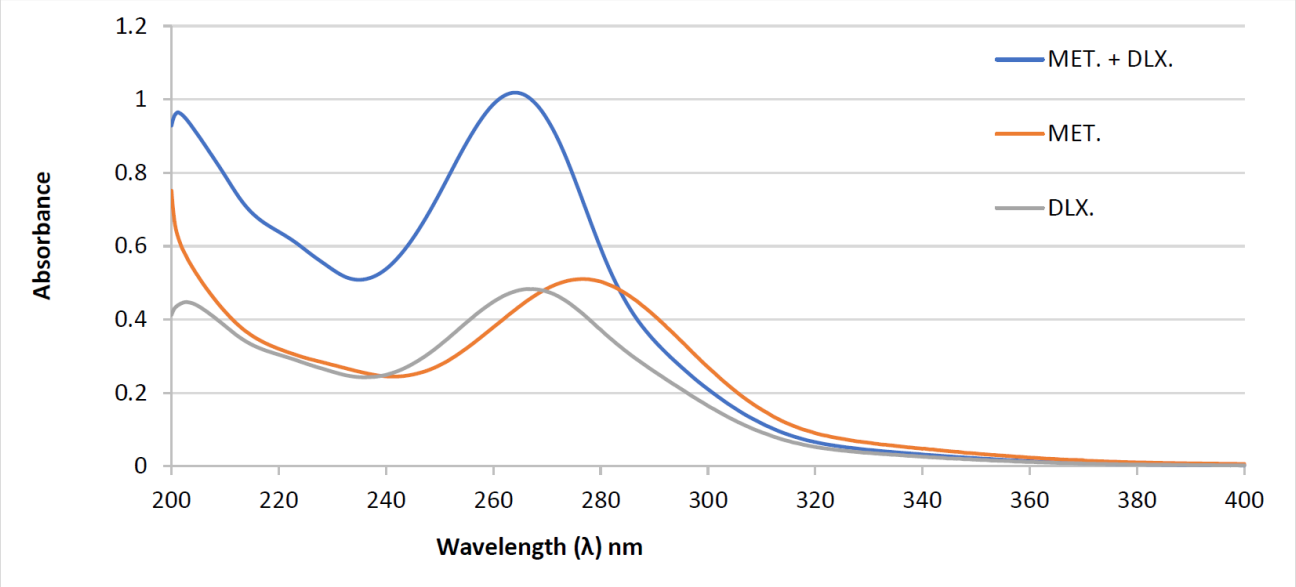

Figure 3. UV Absorbance spectra of the pure components and mixture of Metronidazole and Diloxanide Furoate in 0.1 M HCl.

Table 1. Composition of Calibration Set

Mix. No.	MET. ($\mu g/ml$)	DLX. ($\mu g/ml$)	Mixture No.	MET. ($\mu g/ml$)	DLX. ($\mu g/ml$)
1	8	3.2	14	16	9.6
2	8	5.6	15	16	12
3	8	8	16	19.2	3.2
4	8	9.6	17	19.2	5.6
5	8	12	18	19.2	8
6	11.2	3.2	19	19.2	9.6
7	11.2	5.6	20	19.2	12
8	11.2	8	21	24	3.2
9	11.2	9.6	22	24	5.6
10	11.2	12	23	24	8
11	16	3.2	24	24	9.6
12	16	5.6	25	24	12
13	16	8			

primary solvents. HCl (0.1 M) was selected as the diluent because it provided the highest and most stable absorbance signals for both the analytes. Moreover, 0.1 M HCl is economical, readily available, and compatible with UV–visible spectrophotometric measurements, making it a practical choice for analytical use [Ref]. The absorption spectra of metronidazole and diloxanide furoate in 0.1 M HCl are shown in Figure 3.

3.1.2. Selection of spectral zones for analysis

To determine the most informative wavelength range for quantitative analysis, the UV absorption spectra of pure metronidazole, pure diloxanide furoate, and their binary mixtures were recorded between 200 and 400 nm at 0.2 nm intervals using 0.1 M HCl as a blank. The spectral range 240–320 nm was selected for subsequent analysis because it contained the most characteristic absorption features for both compounds, ensuring optimal model performance (Figure 3).

3.1.3. Construction of the training set

The calibration (training) set was established by preparing solutions of varying concentrations within a linear range. For metronidazole, the linear range was 8–24 $\mu g/mL$, whereas that for diloxanide furoate was 3.2–12 $\mu g/mL$. In total, 25 binary mixtures were prepared with varying proportions of both drugs, as detailed in Table 1, to construct a chemometric model [35].

3.1.4. Construction of chemometrics model

Spectral data were transferred to **Minitab 21** for processing using Partial Least Squares (PLS) regression. **This explicitly included defining the optimal spectral range (240–320 nm) for analysis, and selecting the 'Centering' pre-processing step, which was chosen based on optimization to minimize the Root Mean Square Error of Cross-Validation (RMSECV).** This approach allows for the simultaneous prediction of both analytes in overlapping spectra by

extracting latent variables that maximize the covariance between the spectral data and concentration values [36, 37].

3.1.5. Determination of the Optimum Number of Principal Components

Model optimization was performed by applying leave-one-out (LOO) cross-validation to determine the most suitable number of latent variables (principal components). The best predictive performance was obtained using three components for metronidazole and five components for diloxanide furoate, as shown in the appendix. This selection minimizes prediction error and prevents overfitting [38].

3.1.6. Calculation of Regression Coefficients

Minitab 21 computed the regression constants and wavelength-dependent coefficients for both analytes. These coefficients were used in the following predictive equations:

Predicted Concentration= (Constant+ sum Coefficient × Absorbance)

3.1.7. Prediction and Recovery Assessment

Predicted concentrations were calculated using the PLS regression model and compared with the known concentrations of the calibration mixtures. The model achieved low Root Mean Square Error of Cross-Validation (RMSECV) values, confirming its robustness and predictive accuracy (Table 2). Strong linear correlations were observed between the actual and predicted concentrations, with R^2= 0.9998 for metronidazole and R^2= 0.9999 for diloxanide furoate (Figure 4). These findings confirm the high precision, linearity, and predictive ability of the developed PLS model.

The linearity of the developed method for the PLS model was tested by cross-validation of the data shown in Table 2. The results obtained in Figure 4 indicate that the developed method exhibited high linearity, with R^2 =0.9998 within the linear range (8 – 24 $\mu g/ml$) for Metronidazole and R^2 = 0.9999 within the linear range (3.2–12 $\mu g/ml$) for Diloxanide Furoate.

3.2. Method Validation

Validation was performed following ICH Q2(R1) guidelines to confirm the reliability and suitability of the developed PLS model. The assessed parameters were linearity, accuracy, precision, specificity, and applicability to commercial formulations.

3.2.1. Linearity

Linearity was verified by cross-validation using calibration data within the respective concentration ranges. The correlation coefficients (R^2) were 0.9996 for metronidazole (8–24 $\mu g/mL$) and 0.9993 for diloxanide furoate (3.2–12 $\mu g/mL$), indicating excellent linearity (Figure 5, Table 3).

3.2.2. Validation Set Construction

The developed PLS model was subjected to **external validation** by using an independent set of mixtures. The initial design established a minimum requirement; however, to enhance the reliability and strengthen confidence in the model's predictive capability, the **Validation Set size was expanded to 15 independent mixtures.** These mixtures were prepared at concentrations spanning the entire working range to ensure adequate representation of possible real-world samples. Independent validation mixtures were analyzed to confirm model performance. The model performance against this 15-mixture set was subsequently evaluated using metrics, such as the Root Mean Square Error of Prediction (\text{RMSEP}) and bias. The results demonstrated excellent predictive ability; the predicted concentrations closely matched the actual values, with recoveries falling within acceptable limits, thus clearly demonstrating the accuracy and generalizability of the model.

3.2.3. Precision (Repeatability)

To evaluate repeatability, three mixture concentrations 8/3.2 $\mu g/mL$, 16/9.6 $\mu g/mL$, and 24/12 $\mu g/mL$ were analyzed in triplicate. The %RSD values for both analytes were within the acceptable limits of the United States Pharmacopeia (USP), confirming the excellent method precision. The precision of the developed method was assessed by evaluating both **intra-day repeatability** and **inter-day intermediate precision**. The intra-day precision was determined by analyzing six replicate samples on the same day, whereas the inter-day precision was evaluated by analyzing the same samples over three consecutive days.

As shown in Table 4, the results demonstrated excellent precision. The calculated relative standard deviation (\text{%RSD}) values for both drugs under both intra-day and inter-day conditions were consistently **less than 2 %** across all tested concentration levels, thus confirming the robustness and high reproducibility of the developed method,"(Table 4) [39].

3.2.4. Accuracy

The accuracy was assessed using the standard addition method at three levels (80%, 100%, and 120%). The samples were spiked with known amounts of pure standards and analyzed in triplicate. The recoveries ranged between 90 and 110%, and the %RSD values were low, meeting the USP specifications and confirming the accuracy of the method (Tables 5 and 6).

Table 2. Results of the predicted concentrations with the recovery of Metronidazole and Diloxanide Furoate in the binary mixture in each sample for PLS model

Name	MET.			DLX.		
Constant	-0.306600			0.033713		
Total Replicates	Actual Conc. ($\mu g/ml$)	Predicted Conc. ($\mu g/ml$)	Main Recovery %	Actual Conc. ($\mu g/ml$)	Predicted Conc. ($\mu g/ml$)	Main Recovery %
1	8	7.930	99.12	3.2	3.209	100.30
2	8	7.909	98.86	5.6	5.620	100.35
3	8	7.924	99.05	8	8.001	100.01
4	8	8.057	100.71	9.6	9.611	100.12
5	8	8.018	100.22	12	11.966	99.72%
6	11.2	11.303	100.92	3.2	3.209	100.30
7	11.2	11.224	100.21	5.6	5.612	100.21
8	11.2	11.230	100.26	8	8.006	100.08
9	11.2	11.256	100.50	9.6	9.635	100.36
10	11.2	11.224	100.21	12	11.981	99.84
11	16	15.978	99.86	3.2	3.160	98.75%
12	16	16.068	100.42	5.6	5.619	100.33
13	16	15.929	99.56	8	7.998	99.97
14	16	15.807	98.79	9.6	9.525	99.22
15	16	16.026	100.17	12	12.014	100.12
16	19.2	19.254	100.28	3.2	3.192	99.76
17	19.2	19.347	100.77	5.6	5.603	100.06
18	19.2	19.260	100.31	8	7.983	99.78
19	19.2	19.209	100.05	9.6	9.617	100.18
20	19.2	19.195	99.97	12	12.007	100.06
21	24	23.872	99.47	3.2	3.152	98.51
22	24	23.947	99.78	5.6	5.652	100.93
23	24	24.064	100.27	8	8.015	100.19
24	24	24.054	100.22	9.6	9.612	100.13
25	24	23.917	99.65	12	11.998	99.99
		Mean%	99.94		Mean%	99.97
		RSD%	0.10		RSD%	0.00
		RMSECV	0.0719		RMSECV	0.0278
		SD	0.09994		SD	0.00

Table 3. Results of validation set of Metronidazole and Diloxanide Furoate for PLS model

Total Replicates	MET.	DLX.	MET.		DLX.	
	Actual ($\mu g/ml$)		Predicted ($\mu g/ml$)	Mean % Recovery	Predicted ($\mu g/ml$)	Mean % Recovery
1	6.4	1.6	6.228	97.31%	1.583	98.96%
2	8	10	8.294	103.68%	10.247	102.47%
3	8	12	8.038	100.47%	11.978	99.82%
4	8	3.2	7.945	99.31%	3.201	100.03%
5	8	8	7.868	98.35%	7.989	99.86%
6	10	10	9.927	99.27%	10.215	102.15%
7	11.2	5.6	11.231	100.28%	5.604	100.07%
8	12.8	6.4	12.855	100.43%	6.487	101.37%
9	16	8	15.918	99.49%	8.004	100.05%
10	24	12	23.916	99.65%	11.975	99.79%
11	24	9.6	24.117	100.49%	9.64	100.42%
Mean%			100.10%		100.41%	
RSD%			1.72		1.12	
SD			1.72		1.12	

3.2.5. Specificity

Specificity was examined by spiking commercial tablet solutions with known quantities of standards and analyzing them using the developed PLS model. The recovery results (Tables 7 and 8). **Specificity** of the developed method was demonstrated. The re-

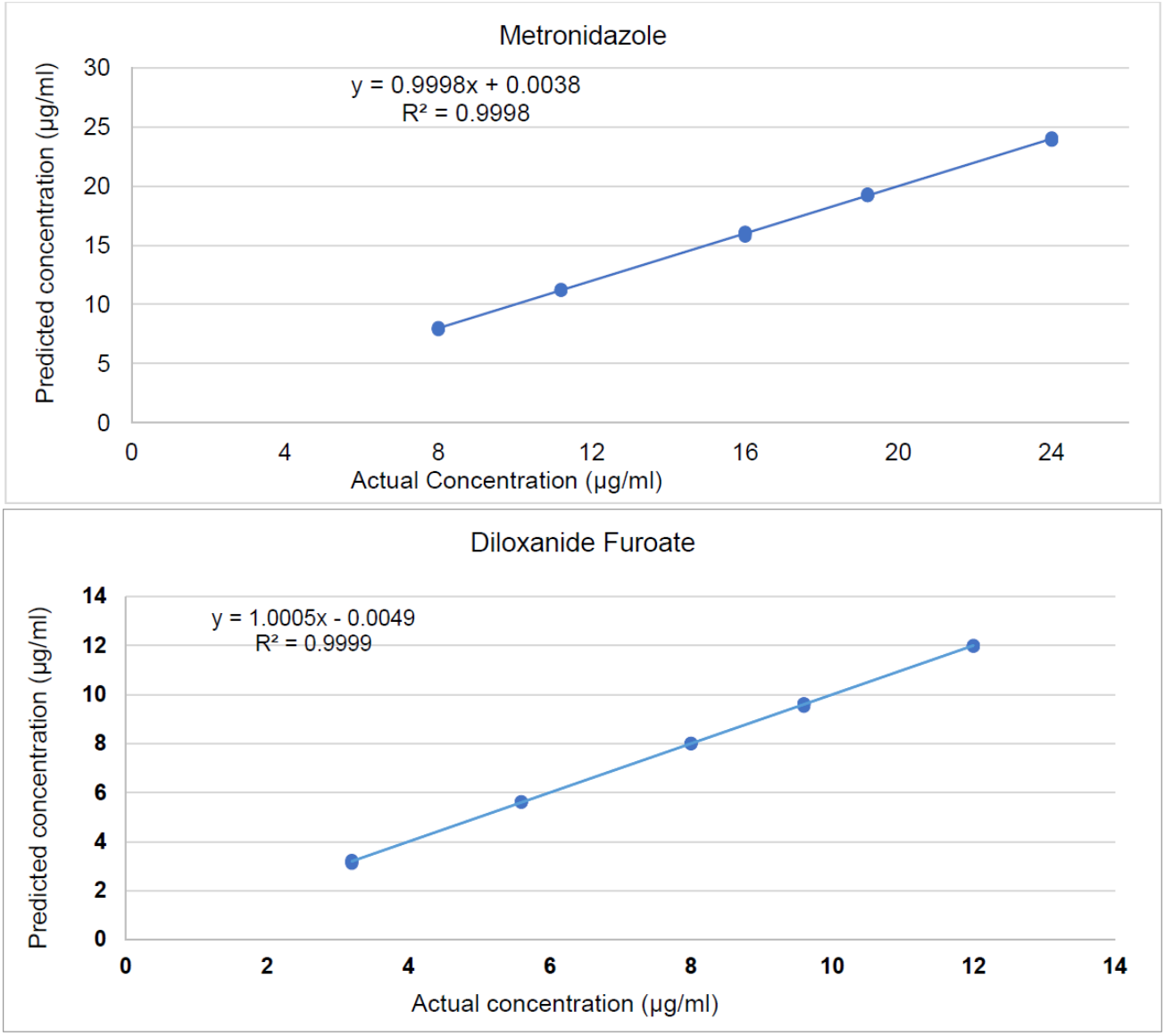

Figure 4. The PLS cross validation for the calibration set of the actual vs. predicted concentration.

Table 4. Repeatability (Intra-day Precision) Results for the Simultaneous Determination of MET and DLX by the PLS Method (n=3)

Amount taken (Actual Conc.) $(\mu g/mL)$		Predicted Conc. $(\mu g/mL)$		Recovery %		Acceptable % RSD NMT 2%	
MET.	DLX.	MET.	DLX.	MET.	DLX.	MET.	DLX.
8	3.2	8.095	3.156	101.19	98.62	SD	SD
8	3.2	8.121	3.121	101.52	97.54	0.176	0.595
8	3.2	8.098	3.153	101.22	98.54		
16	9.6	15.558	9.525	99.61	99.22	1.381	0.938
16	9.6	15.803	9.672	99.71	100.75		
16	9.6	15.556	9.510	97.23	99.06		
24	12	23.907	11.925	99.61	99.38	0.050	0.887
24	12	23.931	12.117	99.71	100.97		
24	12	23.913	11.94	99.64	99.50		

% Recovery = (Predicted Concentration in $\mu g/mL$ / Actual Concentration in $\mu g/mL$) × 100
n represents the number of replicate measurements

sults section explicitly confirms that the **Partial Least Squares (PLS) model** successfully managed the spectral interference originating from the major excipients tested, including **starch, lactose, and magnesium stearate**. This effectiveness was validated by the **accurate prediction** of drug concentrations in both **laboratory-prepared mixtures** and **commercial pharmaceutical products**, confirming the ro-

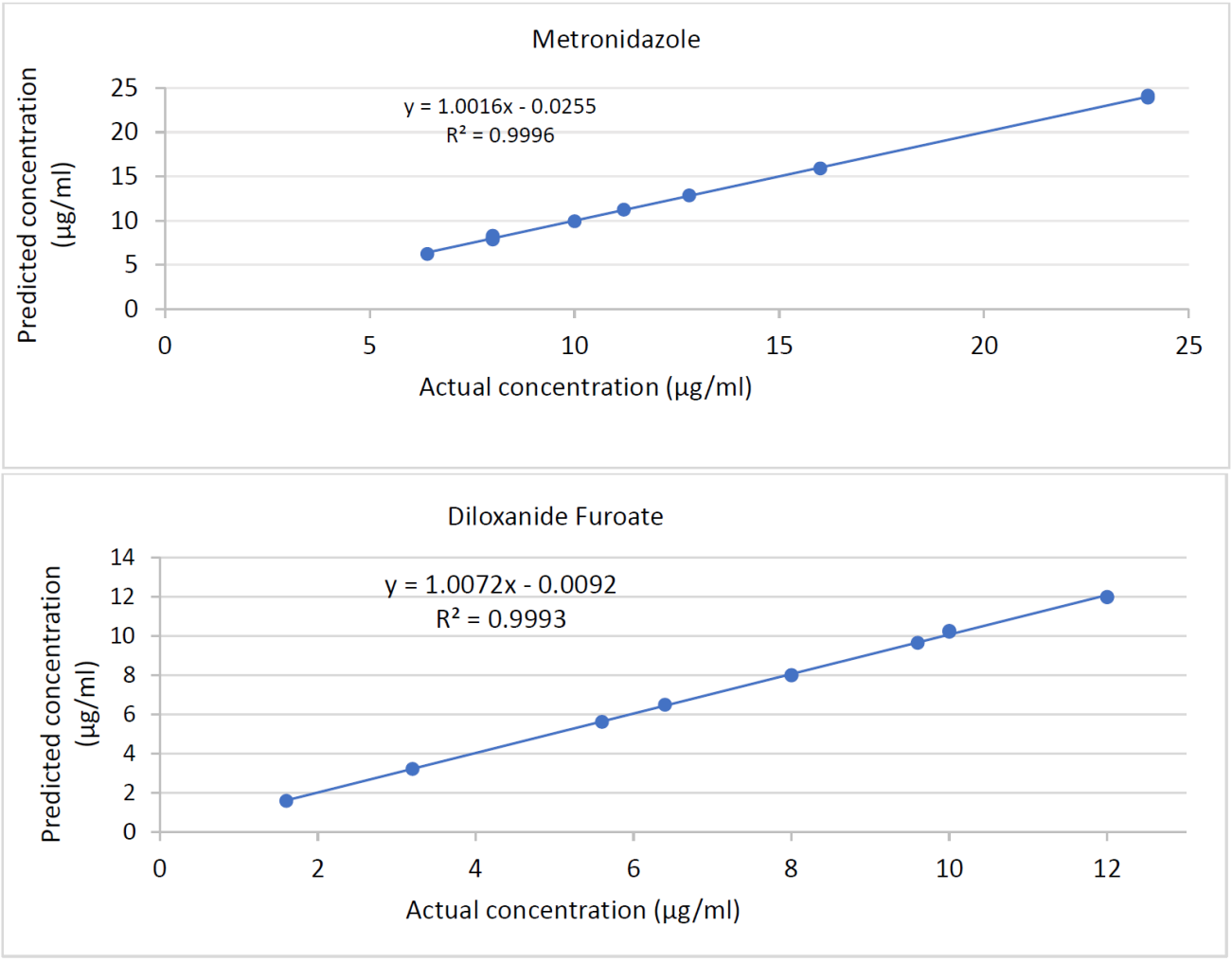

Figure 5. The PLS cross-validation for validation set of the actual vs. predicted concentration

Table 5. Accuracy data of Metronidazole by PLS model

%Level	Sample Conc. ($\mu g/ml$)	Amount added ($\mu g/ml$)	Total Conc. ($\mu g/ml$)	Predicted Conc. ($\mu g/ml$)	Recovery %	RSD %
80%	4	4	8	7.870	98.37	0.089
				7.857	98.21	
				7.868	98.35	
100%	5	5	10	9.910	99.10	0.823
				10.053	100.53	
				9.912	99.12	
120%	6	6	10	12.014	100.11	0.129
				11.985	99.88	
				12.012	100.10	

Table 6. Accuracy data of Diloxanide Furoate by PLS model

%Level	Sample Conc. ($\mu g/ml$)	Amount added ($\mu g/ml$)	Total Conc. ($\mu g/ml$)	Predicted Conc. ($\mu g/ml$)	Recovery %	% RSD
80%	4	4	8	7.999	99.98	0.536
				7.924	99.05	
				7.998	99.97	
100%	5	5	10	10.094	100.94	0.649
				10.210	102.10	
				10.098	100.98	
120%	6	6	10	12.164	101.37	0.887
				11.978	99.82	
				12.164	101.37	

Table 7. Results of specificity for Metronidazole using the developed PLS model

Name of marketed sample	Sample Conc. ($\mu g/ml$)	Amount added ($\mu g/ml$)	Total Conc. ($\mu g/ml$)	Predicted Conc. ($\mu g/ml$)	Recovery %	RSD %
Product A	5	5	10	10.045	100.45	0.958
				10.182	101.82	
Product B	5	5	10	9.589	95.89	0.652
				9.501	95.01	
Product C	5	5	10	9.933	99.33	0.891
				10.059	100.59	

Table 8. Results of specificity for Diloxanide Furoate using the developed PLS model

Name of marketed sample	Sample Conc. ($\mu g/ml$)	Amount added ($\mu g/ml$)	Total Conc. ($\mu g/ml$)	Predicted Conc. ($\mu g/ml$)	Recovery %	RSD %
Product A	5	5	10	10.143	101.43	0.856
				10.021	100.21	
Product B	5	5	10	9.786	97.86	0.675
				9.693	96.93	
Product C	5	5	10	10.322	103.22	1.087
				10.482	104.82	

bustness of the method against matrix components. The excipients in the formulation did not interfere with the quantification. Thus, this method is specific to both analytes in their combined dosage forms.

The validation results clearly demonstrated the superiority of the PLS chemometric approach over traditional univariate methods for the simultaneous determination of MNZ and DLF. Univariate methods, such as the widely used ratio difference or simultaneous equation techniques, often suffer from significant cross-interference and inherent inaccuracies when applied to mixtures with severe spectral overlap, which is evident in the UV-Vis spectra of our binary mixtures (Figure 3). These methods are limited by their dependence on absorbance measurements at a few, often nonideal, discrete wavelengths.

In stark contrast, the PLS model leverages the power of multivariate calibration by analyzing the absorbance data across the entire selected spectral range (240-320∼nm). **This allows the model to effectively deconvolute the overlapped signals by capturing all relevant spectral variations and mathematically separating the contribution of each analyte to the total signal.** This comprehensive approach significantly enhances the **selectivity, precision, and robustness** of the method in handling a complex matrix, rendering it immune to minor spectral shifts and matrix effects that severely compromise the accuracy of univariate techniques. Consequently, PLS methodology provides a more reliable and analytically sound foundation for the quality control of this challenging pharmaceutical formulation.

The recovery data for Metronidazole and Diloxanide Furoate using the Partial Least Squares (PLS) model fell within the acceptable 90-110% range. This confirms that the method is not affected by interference from excipients in the commercial products. The validation results showed that the method was simple, fast, economical, precise, accurate, and eco-friendly. Therefore, it is appropriate for the routine quality control analysis of mixtures and commercial products containing these two drugs.

3.2.6. Analysis of Marketed Formulations

To demonstrate the **practical utility and wide applicability** of the developed method, it was applied for the analysis of commercially available pharmaceutical products. The method was first applied to commercially available tablets containing 250 mg each of metronidazole and diloxanide furoate (Product A), purchased from local pharmacies in Sana'a. **The analysis was performed in triplicate, and the measured drug contents were within the 90–110% acceptable range specified by the USP,** demonstrating the method's applicability for quality control.

Furthermore, to enhance the **robustness of the conclusions** regarding applicability, the method was successfully applied to a **second marketed product (Product C)**. The recovery results for both Product A and the added Product C are incorporated into Table 9, depending on the final numbering). The **consistently high recovery percentages** obtained from the analysis of both products effectively confirmed the reliability and general applicability of the method for routine quality control analysis.

1. **System Precision**
   The precision of the system was determined by injecting six replicate injections of the working standard solution. The results for the area responses

Table 9. Assay result for Metronidazole and Diloxanide Furoate in tablet (Marketed Sample) by PLS proposed method

Name of marketed sample	MET.	DLX.	MET.			DLX.		
	Actual ($\mu g/ml$)		Predicted ($\mu g/ml$)	Recovery %	RSD %	Predicted ($\mu g/ml$)	Recovery %	RSD %
Product A	10	10	10.076	100.76	1.003	9.994	99.94	0.876
	10	10	10.220	102.20		9.871	98.71	
Product B	10	10	9.603	96.03	0.606	9.686	96.86	0.454
	10	10	9.521	95.21		9.624	96.24	
Product C	10	10	9.959	99.59	0.340	10.292	102.92	0.486
	10	10	10.007	100.07		10.363	103.63	

are summarized in Table 10 (data provided in the raw table format). The %RSD for metronidazole was 0.6% and for Diloxanide Furoate was 0.2%.

Table 10. Observations results for System Precision

No.	Area of working Standard	
	Metronidazole	Diloxanide Furoate
1	2113834.384	2968706.700
2	2083685.774	2962892.696
3	2089084.513	2958732.242
4	2114389.627	2974790.867
5	2093634.552	2965769.752
6	2100425.695	2956064.529
Mean	2099175.8	2964492.8
SD	12809.2	6815.0
%RSD	0.6	0.2

2. Method Precision

Method precision was evaluated by analyzing six individual sample preparations from the same batch (replicates). The average assay was 99.7% and the %RSD was 0.2%. (See Table 11 for detailed data.) The average assay was 100.5% and the %RSD was 0.6%. (See Table 12 for detailed data.) Both the%RSD values were within the acceptable limit of 2.0%, confirming the precision of the analytical method.

Table 11. The results shall be Method precision For **Metronidazole**

Area of Standard	Sample No.	Area of Sample	%Assay
2099175.8	1	2097452.0	99.9
	2	2084054.4	99.3
	3	2095639.0	99.8
	4	2097520.0	99.9
	5	2093578.0	99.7
	6	2095995.7	99.8
	AVG		99.7
	SD		0.2251
	% RSD		0.2

Table 12. The results shall be Method precision For Diloxanide Furoate

Area of Standard	Sample No.	Area of Sample	%Assay
2964492.8	1	2987908.4	100.8
	2	3002277.4	101.3
	3	2968199.5	100.1
	4	2953485.0	99.6
	5	2987258.7	100.8
	6	2974157.8	100.3
	AVG		100.5
	SD		0.6047
	% RSD		0.6

3. Linearity and Range

Linearity was determined by preparing and injecting standard solutions at five concentration levels ranging from 80% to 120% of the target concentration. The acceptance criterion for the correlation coefficient squared (r^2) was set at not less than 0.995. The linearity test yielded an r^2 value of 0.999338. (Refer to Table 13 for Metronidazole Linearity Graph). The linearity test yielded an r^2 value of 0.998732. (Refer to Table 14 for Diloxanide Furoate Linearity Graph). The method demonstrated a linear relationship between concentration and response over the range of 80% to 120% for both analytes, meeting the acceptance criterion.

4. Accuracy and Recovery

Accuracy was assessed by spiking the placebo matrix (or sample matrix containing lower concentrations) at three concentration levels: 80%, 100%, and 120% (in triplicate). The acceptance criterion for the average recovery was 100 ± 5%. The average recoveries (AV %) across the three levels (80%, 100%, 120%) were 102.7%, 103.2%, and 102.0%, respectively. (Refer to Table 15 for the Metronidazole Accuracy Graph.)

The average recoveries (AV %) across the three levels (80%, 100%, 120%) were 101.2%, 102.3%, and 101.0%, respectively. (Refer to Table 16 for the Diloxanide Furoate Accuracy Graph). The recovery percentages for both compounds were within the acceptable range of 100 ± 5 %, thus confirming the accurate

Table 13. Show the results of Linearity and Range for Metronidazole

Conc (%)	Observed Signal	Conc (mg/mL)	Intercept	Response factor	Calculated signal	Residuals	Residuals (%)
80	1679706.0	0.200	40412.00	8398530.00	1671155.24	8550.76	0.509063
90	1872043.7	0.225	27837.95	8320194.22	1876066.99	-4023.29	-0.214914
100	2072783.5	0.250	23666.00	8291134.00	2080978.74	-8195.24	-0.395374
110	2280147.8	0.275	26118.55	8291446.55	2285890.49	-5742.69	-0.251856
120	2500212.7	0.300	41271.70	8334042.33	2490802.24	9410.46	0.376386
Slope	20491.18			r	0.999669	Limit	$\geq$ 0.999
Intercept	31861.24			r^2	0.999338	Limit	$\geq$ 0.995
RSD of Response factor	**0.53**			**Limit**		**$\geq$ 2.0%**	

Table 14. Show the results of Linearity and Range for Diloxanide Furoate

Conc (%)	Observed Signal	Conc (mg/mL)	Intercept	Response factor	Calculated signal	Residuals	Residuals (%)
80	2468970.5	0.200	152974.66	12344852.50	2450780.02	18190.48	0.736764
90	2723365.2	0.225	117869.88	12103845.33	2740279.50	-16914.30	-0.621081
100	3012773.5	0.250	117778.70	12051094.00	3029778.98	-17005.48	-0.564446
110	3331270.4	0.275	146776.12	12113710.55	3319278.46	11991.94	0.359981
120	3612515.3	0.300	138521.54	12041717.67	3608777.94	3737.36	0.103456
Slope	*28949.95*			r	*0.999366*	Limit	$\geq$ 0.999
Intercept	*134784.18*			r^2	*0.998732*	Limit	$\geq$ 0.995
RSD of Response factor	*1.02*			Limit		$\leq$ 2.0%	

Table 15. Show the results of Accuracy and Recovery for Metronidazole

No.	Theoretical Conc. %	Area	Conc. (mg/mL)	Calc. Value %	Recovery %	Deviation %	AV %	RSD %	Intercept	Slope
1	80	1716647.5	0.200	81.9	102.4	-2.4	102.7	0.2	**1.911**	**1.0**
2	80	1713096.6	0.200	81.7	102.1	-2.1		0.6		
3	80	1735229.5	0.200	82.8	103.5	-3.5		0.1		
1	100	2165533.6	0.250	103.3	103.3	-3.3	103.2	0.3		
2	100	2160526.7	0.250	103.1	103.1	-3.1		0.4		
3	100	2163358.7	0.250	103.2	103.2	-3.2		0.1		
1	120	2574703.7	0.300	122.8	102.3	-2.3	102.0	0.0		
2	120	2567402.9	0.300	122.5	102.1	-2.1		0.5		
3	120	2555162.1	0.300	121.9	101.6	-1.6		0.2		

behavior of the method across the tested range.

3.2.7. Comparison with Reference Method

The results obtained from the developed PLS method were compared with those obtained from a validated HPLC reference method using the F-test in the SPSS software. For a rigorous statistical assessment, the results obtained using the proposed spectrophotometric method were compared with those obtained using the reference **HPLC method**. This comparison was conducted using a **two-tailed Student's *t*-test** to check for differences in accuracy (mean recovery), and an **F-test** to check for differences in precision (variance). The calculated t-value and F-value were reported, and the analysis confirmed that there was **no significant statistical difference** between the proposed method and the established reference HPLC method at the 95% confidence level.

Statistical comparison showed no significant difference between the two sets of results ($p > 0.05$), confirming the equivalence of the proposed spectrophotometric approach to the reference chromatographic method (Table 17, Figure 6). Specifically, the analysis of the commercial formulation 'Product B' consistently showed a **slightly low recovery (approximately 95.6%)** for the active ingredients. This outcome is now attributed to potential **matrix effects** or specific **excipient interferences** within commercial formulations. Such minor under-recoveries are commonly observed during the **spectrophotometric analysis** of complex pharmaceutical matrices.

The developed chemometric-assisted spectrophoto-

Table 16. Show the results of Accuracy and Recovery for Diloxanide Furoate

No.	Theoretical Conc. %	Area	Conc. (mg/mL)	Calc. Value %	Recovery %	Deviation %	AV %	RSD %	Intercept	Slope
1	80	2457736.3	0.200	80.9	101.1	-1.1	101.2	0.3	**0.917**	**1.0**
2	80	2447232.1	0.200	80.5	100.6	-0.6		0.4		
3	80	2477251.1	0.200	81.5	101.9	-1.9		0.1		
1	100	3116586.1	0.250	102.5	102.5	-2.5	102.3	0.2		
2	100	3100504.6	0.250	102.0	102.0	-2.0		0.1		
3	100	3115118.4	0.250	102.5	102.5	-2.5		0.1		
1	120	3702920.6	0.300	121.8	101.5	-1.5	101.0	0.3		
2	120	3681782.9	0.300	121.1	100.9	-0.9		0.6		
3	120	3668618.3	0.300	120.7	100.6	-0.6		0.1		

metric method (PLS model) demonstrated excellent linearity, precision, accuracy, and specificity, and was faster, simpler, cost-effective, and eco-friendly than chromatographic techniques. This method is suitable for the routine quality control and quantitative analysis of combined metronidazole and diloxanide furoate formulations. The accuracy and precision of the proposed PLS method were statistically compared with those of the validated reference HPLC method to confirm equivalence. This comparison was performed on the mean percentage recovery data obtained from the analysis of the commercial formulations using both a two-tailed **Student's t-test** for accuracy (mean recovery) and an **F-test** for precision (variance) at a 95% confidence level. The t-test was used to assess any significant difference between the means, whereas the F-test determined whether there was a significant difference between the variances. As summarized in Table 17, the calculated values for both tests (t_{calc} and F_{calc}) for all the samples were found to be less than their respective theoretical critical values (t_crit} and F_crit}). Specifically, the highest calculated t-value recorded was **2.15** (for MET in Product B) which is less than t_{crit}(**2.306**), and the highest calculated F-value was **3.20** (for DLX in Product A) which is less than F_{crit} (**6.388**). This statistical evidence confirmed that there was **no significant difference** in accuracy or precision between the proposed chemometric PLS method and the reference HPLC method ($p > 0.05$), demonstrating the reliability and suitability of the new method for routine quality control.

3.2.8. Application to Pharmaceutical Formulations and Comparison with Reference Method

A focused discussion is warranted regarding the slightly lower mean percentage recovery consistently observed for the active ingredients (Metronidazole and Diloxanide Furoate) in the commercial formulation **'Product B'** (with values around **95.6%** for PLS, as shown in Table 17), which approached the lower limit of the acceptance criteria (95-105%). While the analysis of Products A and C yielded results well within the accepted range, this minor but consistent **under-recovery** in Product B was primarily attributed to potential **matrix effects** or specific **excipient interferences** inherent to this particular formulation. Although the multivariate PLS model is designed to robustly manage spectral complexities, its reliance on spectroscopic data makes it susceptible to non-spectral or background effects caused by the pharmaceutical matrix (such as high concentrations of opacifying agents or specific binders). Such a minor systematic bias is common in the direct spectrophotometric analysis of complex dosage forms, in which the simple dilution step does not completely eliminate the influence of all formulation components. Despite this minor effect, the statistical comparison (t-test and F-test) confirmed the absence of a **statistically significant difference** between the PLS method and the reference HPLC method, validating the overall reliability of the proposed chemometric procedure.
The chromatograms in Figure 6 show the results of analysis using the reference method to determine the amounts of metronidazole and diloxanide furoate.

3.2.9. Comparative Analysis and Method Superiority

The success and superiority of the proposed Partial Least Squares (PLS) method over conventional techniques stems directly from its multivariate nature. Traditional univariate spectrophotometric methods, such as simultaneous equation or derivative spectroscopy, are severely compromised by the substantial spectral overlap between Metronidazole and Diloxanide Furoate in the selected wavelength range. This overlap inherently limits their selectivity, significantly increasing the potential for error and reducing the robustness of the method, particularly in the presence of complex excipient matrices. In contrast, the chemometric PLS algorithm successfully handles this challenge by processing the *entire* spectral matrix and extracting relevant analytical information, effectively isolating the signals of the two active components, even when their spectra are non-additive. This critical

Table 17. Results of statistical comparison between newly developed method and reference method

Component	Name of marketed sample	Methods	Mean %	RSD %	t_{calc}	t_{crit} (df=8)	F_{calc}	F_{crit} (df=4, 4)
MET.	Product A	Reference method (HPLC)	100.50	0.225	1.12	2.306	2.10	6.388
		PLS	101.48	1.003				
DLX.	Product A	Reference method (HPLC)	100.81	0.147	1.55	2.306	3.20	6.388
		PLS	99.33	0.876				
MET.	Product B	Reference method (HPLC)	99.74	0.220	2.15	2.306	1.85	6.388
		PLS	95.62	0.606				
DLX.	Product B	Reference method (HPLC)	101.40	0.244	1.99	2.306	2.80	6.388
		PLS	96.55	0.454				
MET.	Product C	Reference method (HPLC)	100.07	0.445	0.55	2.306	1.70	6.388
		PLS	99.83	0.340				
DLX.	Product C	Reference method (HPLC)	99.99	0.276	2.05	2.306	1.95	6.388
		PLS	103.28	0.486				

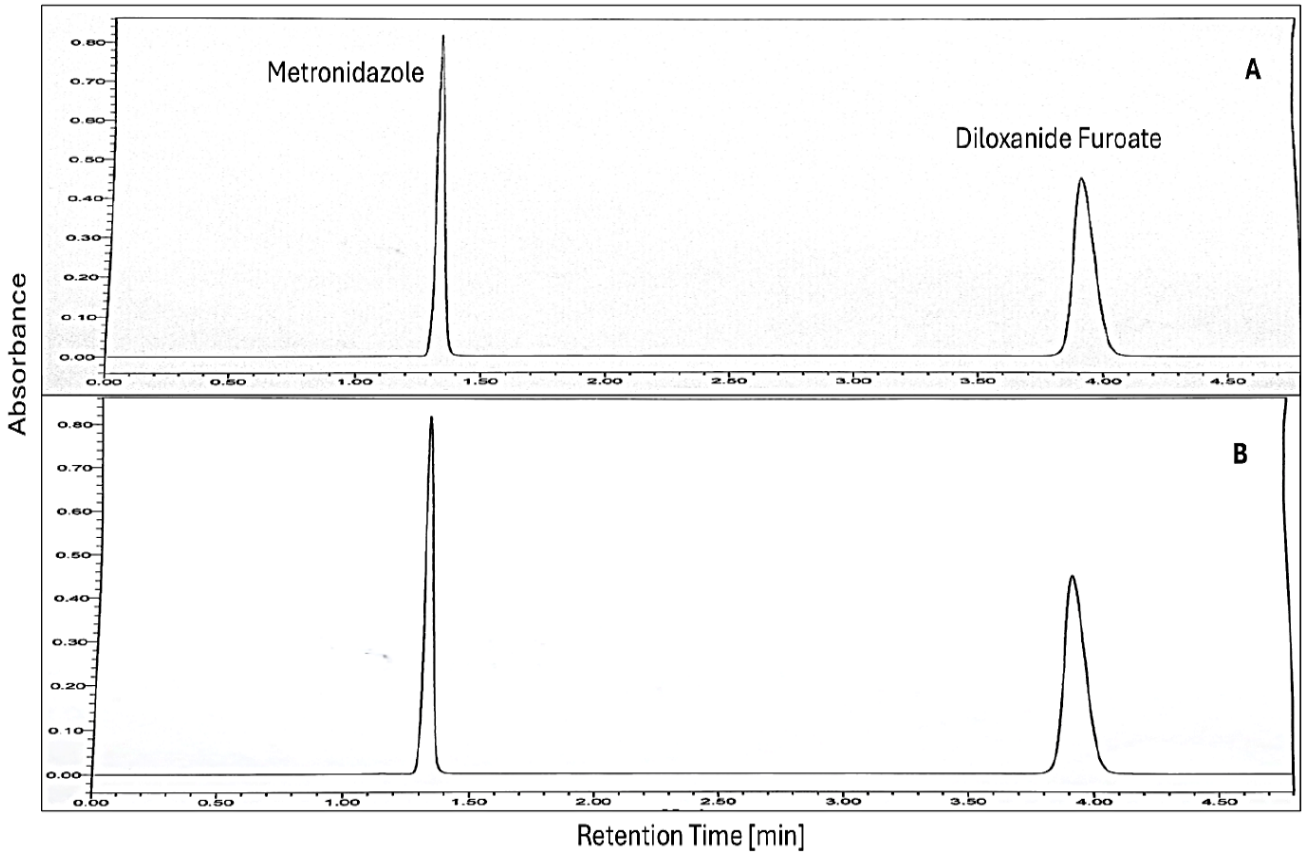

Figure 6. Chromatogram of Metronidazole and Diloxanide Furoate for standard(A) and commercial samples(B).

feature ensures superior **selectivity**, higher **precision**, and greater **robustness** across varying concentration ratios, confirming that the multivariate PLS approach is a technologically advanced and reliable alternative for quality control of this challenging binary formulation.

4. CONCLUSION

In this study, a simple, rapid, accurate, and environmentally friendly spectrophotometric method was successfully developed and validated for the simultaneous determination of Metronidazole (MNZ) and Diloxanide Furoate (DLF) in combined pharmaceutical formulations. The implementation of the **Partial**

Least Squares (PLS) chemometric model effectively utilized the full spectral information between 240 and 320 nm to overcome the significant challenge of spectral overlap inherent in this binary mixture.

The PLS technique was exceptionally robust and selective, yielding satisfactory prediction results for both drugs across the tested concentration ranges. **Crucially, the superior performance of the PLS multivariate method in handling the complex matrix and resolving extensive spectral interference, compared to conventional univariate approaches, was clearly demonstrated.** This highlights the inherent strength of chemometrics in achieving high analytical resolution without any prior chromatographic separation steps.

The developed methodology is characterized by its accuracy, precision, and simplicity, making it a highly practical alternative to laborious and costly separation techniques such as HPLC. Furthermore, reliance on an aqueous-based solvent system contributes to the eco-friendly profile of the method. Consequently, the validated PLS-assisted spectrophotometric method is suitable for the routine, efficient, and sustainable quality control analysis of MNZ and DLF in their pharmaceutical dosage forms.

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