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SPECTROPHOTOMETRIC METHODS FOR DETERMINATION OF NARINGIN, AMLODIPINE, AND NIFEDIPINE USING CHEMOMETRIC TECHNIQUES

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ABSTRACT

Background: Chemometrics articulates statistical and mathematical aspects to analyse the effectiveness of chemical data, playing a pivotal role in spectroscopy. Among all the chemometrics techniques, this study utilizes the Orthogonal partial least squares (OPLS) model for the simultaneous analysis of naringin, amlodipine, and nifedipine, a well-established calcium channel blocker. Naringin, a citrus flavonoid exhibiting notable pharmacological activities. **Methodology:** This research employs UV-visible spectrophotometry in conjunction with the OPLS method for both calibration and prediction sets in simultaneous studies of Amlodipine–Naringin and Nifedipine–Naringin, aiming to develop a precise model for measuring drug concentrations. A linear dynamic range of 5-20 µg/mL was achieved for standard solutions, while calibration sets were developed using factorial designs. **Result and Discussion:** The OPLS model had significant predictive performance with R^2 values within the range of 0.9947-0.9976 for calibration and 0.9947-0.9985 for prediction, and low root mean square error of cross validation (RMSECV) values of 0.6191- 0.4353 for NIF-NAR, and 0.3978- 0.4418 for AML-NAR, indicating robust model performance. The model validation process, using Hotelling's T2 test, DModx, established no significant outliers, and permutation analysis validated the model's reliable fit. The recovery studies showed values close to 100%, thus verifying the effectiveness of the methodology. **Conclusion:** The research demonstrated OPLS (Orthogonal Partial Least Squares) as a powerful solution for resolving overlapping spectral data, providing high-precision drug analysis with minimal interference. The development of chemometrics methods demonstrated efficiency and precision in pharmaceutical analysis while also offering cost-effectiveness for quality control and formulation development.

INTRODUCTION

Chemometrics combines statistical and mathematical techniques to analyze chemical data quantitatively across multiple disciplines [1]. The statistical methods provide informative analysis and optimize the information derived from chemical data, which entails the application of multivariate mathematical and statistical approaches for data assessment [2]. This further determines the inter-object links, facilitates pattern recognition

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for classification, and enables the prediction of new and unclassified objects [3]. From the mid-1970s [4], chemometrics has become a key element in spectroscopy and related analytical fields through its capability to process multivariate data effectively. The field has developed further due to advances in computational technology and the creation of sophisticated instruments that produce multivariate responses [5] [6] [7]. Orthogonal Partial Least Squares (OPLS) stand out among chemometric methods due to their widespread use in multivariate calibration [8] [9] [10] [11], which boosts classification precision by reducing systematic variations that do not correlate with sample composition [12] [13]. The strength of the OPLS method has been demonstrated through its applications in modeling complex relationships involving overlapping orthogonal variations of analytical difficulties, such as calibration transfers and detection limits [14] [15]. OPLS facilitates interpretation by separating the variation, such as the descriptor variables, into two components: "predictive" variation associated with the response and "orthogonal" variation not associated with the response. OPLS is applicable even when the number of observations is less than the number of variables; thus, it can tolerate correlated variables, noisy variables, and missing values. This string-adaptable technique finds application in a broad variety of analytical tasks, such as regression [16] [17] [18]. Thus, this study explored and validated chemometric techniques that enabled scientists to simultaneously analyse Naringin with Amlodipine and Naringin with Nifedipine conveniently.

Naringin (NAR), a citrus flavonoid [19], as shown in Figure 1 (a), is chemically 4',5',7-trihydroxy flavanone-7-β-L-rhamnoglucoside, a flavanone glycoside [20] [21]. Naringin is quoted as the "Bitter principle" for the grape family. Naringin exhibits notable biological and pharmacological properties, including antioxidant, anti-carcinogenic, and anti-diabetic properties, as well as the ability to inhibit several cytochrome P450 enzymes [22], such as CYP3A4 and CYP1A2 [23], which may potentially result in various in vitro drug interactions.

Nifedipine (NIF), a dihydropyridine subclass calcium channel blocker, as shown in Figure 1(b), is primarily used as an antihypertensive agent [24], a calcium channel blocker that is responsive to various cardiovascular disorders, including chronic hypertension and angina pectoris. NIF and other dihydropyridines [25] are primarily considered selective for the L-type calcium channel and may have a nonspecific action towards additional voltage-dependent calcium channels [26]. It undergoes hepatic metabolism via the CYP3A4 pathway.

Amlodipine (AML), an oral dihydropyridine calcium channel blocker depicted in Fig. 1(c), maintains efficacy by inhibiting voltage-dependent L-type calcium channels [27]. This impact impedes the first calcium influx, distinguishing it within its pharmacological classification. AML undergoes significant hepatic metabolism to form inactive metabolites [28]. Cytochrome P-450 (CYP) enzymes CYP3A4 [29] and CYP3A5 [30] are substantial in the metabolism of amlodipine [31]. The history of calcium channel blockers such as Diltiazem and Verapamil, along with their relationship to naringin, has been thoroughly researched [32] [33]. The study highlighted their interactions, which affected the bioavailability of the drugs when used concomitantly with Naringin by inhibiting the Cytochrome P450 enzyme, specifically CYP3A4. Thus, this established interaction helped expand the study to include Dihydropyridine calcium channel blockers, such as Amlodipine (AML) and Nifedipine (NIF). As both drugs overlap the metabolic pathways of VER and DIL, an exploration of possible pharmacokinetic interactions and their effects on drug bioavailability, which is clinically essential for efficacy and safety, has been pursued. With this background, the current study aims to create a novel chemometric strategy using OPLS in UV-Visible spectroscopy to evaluate the simultaneous use of NAR with AML and NAR with NIF. It demonstrates the first-time application of OPLS for resolving overlapping spectral data in such a dual system, while also establishing a robust statistical framework for model validation. This study presents a cost–effective, precise, and interference-minimized method with high potential for pharmaceutical quality control and formulation development.

MATERIALS AND METHODS

All the chemicals, NAR, AML, and NIF, were of high grade and were purchased from Yarrow Chem Products in Mumbai, with purity levels exceeding > 99%. Milli Q was the source of water for its optimum quality, while Methanol (AR Grade) was acquired from S.D. Fine Chemicals Ltd. in Mumbai. The other ingredients and reagents were all commercially available and of AR quality. A UV-visible spectrophotometer (Shimadzu-Kyoto, Japan, Model 1800, double-beam with corresponding 1 cm pathlength quartz cells) was employed to quantify AML, NIF, and NAR at wavelengths ranging from 200 to 400 nm, using

methanol as the solvent. The moieties' unique properties were their molar absorptivity, melting temperature, and solubility, which were their distinguishing characteristics. To establish the orthogonal partial least squares (OPLS) model, the process was carried out using the UV-Visible technique with software UV Probe 2.34 and SIMCA (Sartorius, version 17, running on Windows 10 Pro). This methodology fulfilled the criteria established by the International Conference on Harmonisation (ICH) for analytical standards [34], including linearity, precision, and accuracy.

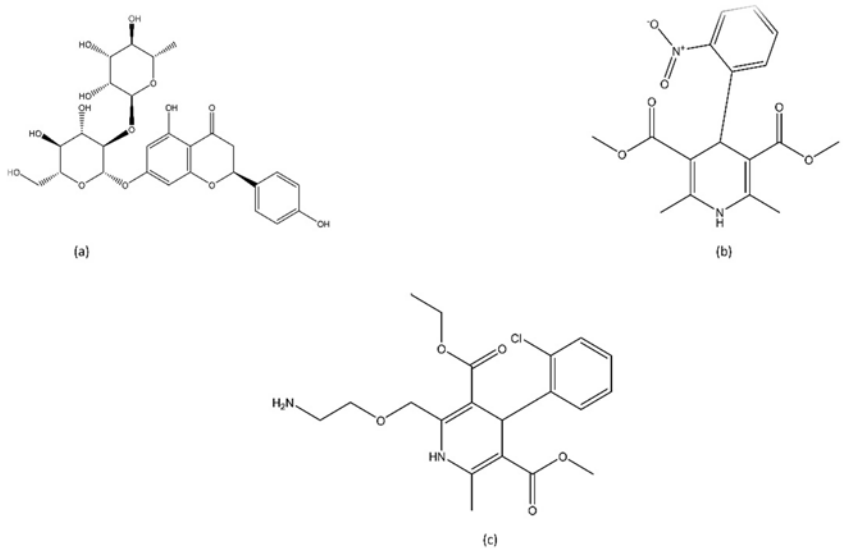

Figure 1: Structures of the different compounds, a) NAR, b) NIF, c) AML

PREPARATION OF STANDARD SOLUTION

After precisely weighing 10.0 mg of NAR and AML each, the stock solution was prepared by dissolving AML in 10.0 mL of methanol and adjusting the volume using the same solvent system. The stock solutions of AML and NAR were prepared at varying concentrations ranging from 5 to 20 µg/mL. The zero-order spectra were obtained at wavelengths ranging from 200 to 400 nm, in comparison to a blank solution, and accurately weighed 10.0 mg of NAR and NIF, respectively. The stock solution was prepared by dissolving the NIF in 10 mL of methanol, followed by the addition of the necessary volume using the same solvent. NAR and NIF stock solutions were formulated with concentrations varying from 5 to 20 µg/mL. The zero-order spectra were acquired at wavelengths between 200 and 400 nm relative to a blank solution

ONE COMPONENT CALIBRATION

The spectral absorption for the linear dynamic range (LDR) was measured at 200-400 nm by adding different concentrations of stock solutions to a 10.0 mL volumetric flask, using methanol to make up the volume. The absorbance vs. concentration graph analyzed the LDR, which was determined to be between 1 and 50 µg/mL.

STANDARD CALIBRATION AND PREDICTION SAMPLES SET OF AML- NAR AND NIF- NAR

The method by Sonawane et al. [35] was further modified, where the NAR was combined with AML in several different proportions, corresponding to their linear concentration ranges, to create calibration and prediction mixes. A traditional factorial design with two factors and four levels each was used to determine the concentration of the calibration set. In contrast, a design with two factors and three levels each was used for the prediction set. Nine prediction mixes and sixteen calibration mixes were produced separately. The different sets, i.e., calibration (5-20 µg/mL) and a prediction set of three-component combinations (2.5-15 µg/mL), were created as shown in Table 1. A weighed amount of powder equivalent to 10.0 mg was added to a 10.0 mL volumetric flask for analysis. Methanol was used to dissolve the powder and bring the sample volume to 10.0 mL. 2.5 mL of the sample was pipetted out from the stock solution (10.0 mg/10.0 mL), which was then poured into a 25 mL volumetric flask. The sample was further analyzed at 200-400 nm at 1-nm intervals, compared to the blank methanol. The amount of methanol was subsequently modified to meet the AML-NAR and NIF-NAR standards of the calibration and prediction samples set. These sets were created

using the multilevel, multi-factor design. All these data were analysed in the UV Probe and imported to SIMCA 17 for data analysis. Further, a calibration model was constructed. Recovery and mean recovery calculations were also performed.

Table 1: Different concentrations of AML-NAR and NIF-NAR used as calibration and prediction sets in the OPLS method of analysis.

Sl no.	Calibration		Prediction		Calibration		Prediction	
	AML (µg/mL)	NAR	AML (µg/mL)	NAR (µg/mL)	NIF (µg/mL)	NAR (µg/mL)	NIF (µg/mL)	NAR (µg/mL)
1.	20	5	9	12.5	20	5	9	12.5
2.	1	10	15	12.5	1	10	15	12.5
3.	20	10	9	2.5	20	10	9	2.5
4.	20	20	15	2.5	20	20	15	2.5
5.	10	1	3	12.5	10	1	3	12.5
6.	10	5	9	7.5	10	5	9	7.5
7.	10	10	3	7.5	10	10	3	7.5
8.	5	10	15	7.5	5	10	15	7.5
9.	1	20	3	2.5	1	20	3	2.5
10.	20	1			20	1		
11.	5	20			5	20		
12.	5	1			5	1		
13.	1	1			1	1		
14.	10	20			10	20		
15.	1	5			1	5		
16.	5	5			5	5		

PRE-PROCESSING

A methodical preprocessing approach has been employed in this research to enhance the accuracy and reliability of the chemometric analysis. The analysis was recorded using UV-Vis spectroscopy over the range of 200-400 nm at an interval of 1 nm. A Shimadzu 1800 double-beam spectrophotometer was used, with methanol as the solvent system. The raw data set was then transferred into SIMCA (version 17). Firstly, the dataset was standardized using an automated scaling. Different filters, including Moving Average (MA), Norris Derivative (NA), Exponentially Weighted Moving Average (EWMA), and Savitsky-Golay (SG), were used. Still, they didn't result in removing the noise, as a result of which out of all the filters, the SG-EWMA (Savitsky-Golay Exponentially Weighted Moving Average) filter was applied, which excluded the spectrum overlap, particularly at the range of 207-332nm, which enhanced the model performance and signal quality. Furthermore, the dataset was evaluated for overfitting and processed for cross-validation to determine the model's robustness. Thus, the calibration and the prediction sets were developed. After building the OPLS model (orthogonal projection to latent structure), statistical tests, including Hotelling's T2, DModx, Score, and permutation analysis, were performed to validate the results further. The high spectral resolution facilitated the obtaining of easier minute spectral variations, providing precise and reproducible analysis.

BUILDING AN OPLS MODEL

A multivariate calibration technique, formulated by Abdallah FF et al. [36] and Ríos-Reina et al. [37] using the OPLS method of analysis, was further modified by employing different filter derivatives to mitigate overlap among the pharmaceuticals under study. During preprocessing, the calibration set data were automatically scaled for use in the OPLS filter, primarily the Savitzky-Golay Exponentially Weighted Moving Average (SG-EWMA), where the concentration range was taken from 207 to 332 nm. The cross-validation procedure involved omitting samples and subsequently analysing the remaining data. The optimal quantity of the module is crucial for the OPLS methodology; excessive components introduce noise, while

insufficient components diminish it. The established model, developed through the OPLS method, helped characterize the latent variables. A calibration plot is generated by juxtaposing the anticipated concentrations with the actual concentrations.

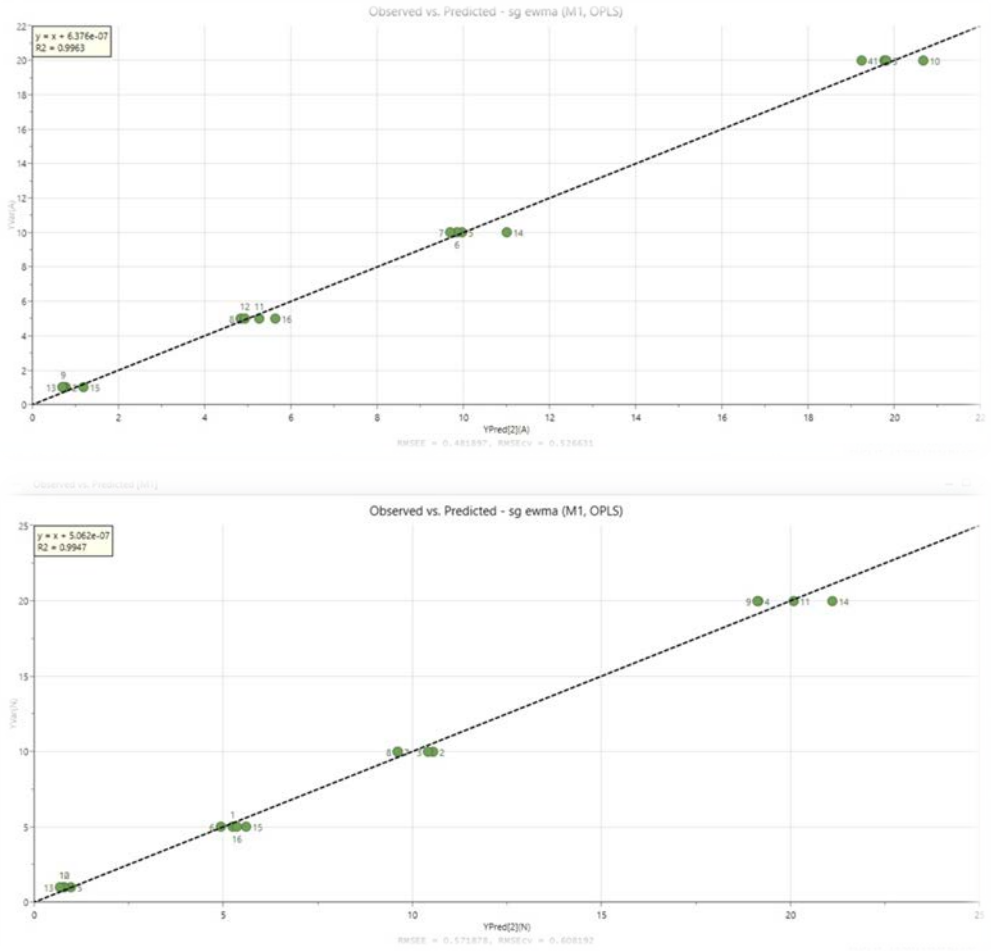

Figure 2: Observation Vs Prediction of AML and NAR using SG EWMA filters.

Note: OPLS models with SG-EWMA preprocessing are illustrated in the calibration figure, which helps clarify the correlation between the actual and predicted concentrations of AML and NAR. The predictive accuracy of the model is determined by its high degree of linearity ($R^2 > 0.99$) [38].

ASSAY OF SYNTHETIC MIXTURE OF AML-NAR AND NIF-NAR:

To prepare the synthetic mixture, a calibrated 10.0 mL volumetric flask was filled with AML-NAR in a 1:1 powder ratio, equivalent to 10.0 mg, for the analysis, which was then further dissolved in methanol. 2.5 mL was pipetted out of the prepared stock solution (10.0 mg/10.0 mL) and put into a 25 mL volumetric flask (100 µg/mL), which was then further diluted with methanol. From the prepared solution, further concentrations were formed and analysed. Similarly, the synthetic combination of NIF and NAR was also established.

MODEL VALIDATION

The weak, moderate outliers were all identified with the help of parameters like Hotelling's T^2 [39] test and DModX [40], which would also confirm the lack of outliers. Hotelling's T^2, a multivariate extension of the classical Student t-test, measured the distance of each observation from the model centre. In this study, all data for both the AML-NAR and NIF-NAR models fell within the 95% confidence ellipse, indicating that no significant outliers were present. DModX values were below the Critical limit, confirming that the samples fit well within the established model. Permutation analysis assesses the robustness and statistical significance of the model. The study revealed that all permuted Q^2 values were negative, whereas R^2 values were about 1 or lower, validating that the model was not overfitted and has real predictive power [38]. The score plot enables the observation of all samples located within Hotelling's range. The result of the two models, i.e., AML-NAR and NIF-NAR, was effective as each optimization approach satisfied the criteria.

The Score representation, DModx, permutation plot, and Hotelling's range are illustrated in the aforementioned figures for both the calibration and prediction sets. All experimental procedures, including calibration and prediction set preparations, as well as all analytical measurements, were carried out in triplicate to ensure the reproducibility, reliability, and statistical robustness of the results determined through the chemometric validation procedure.

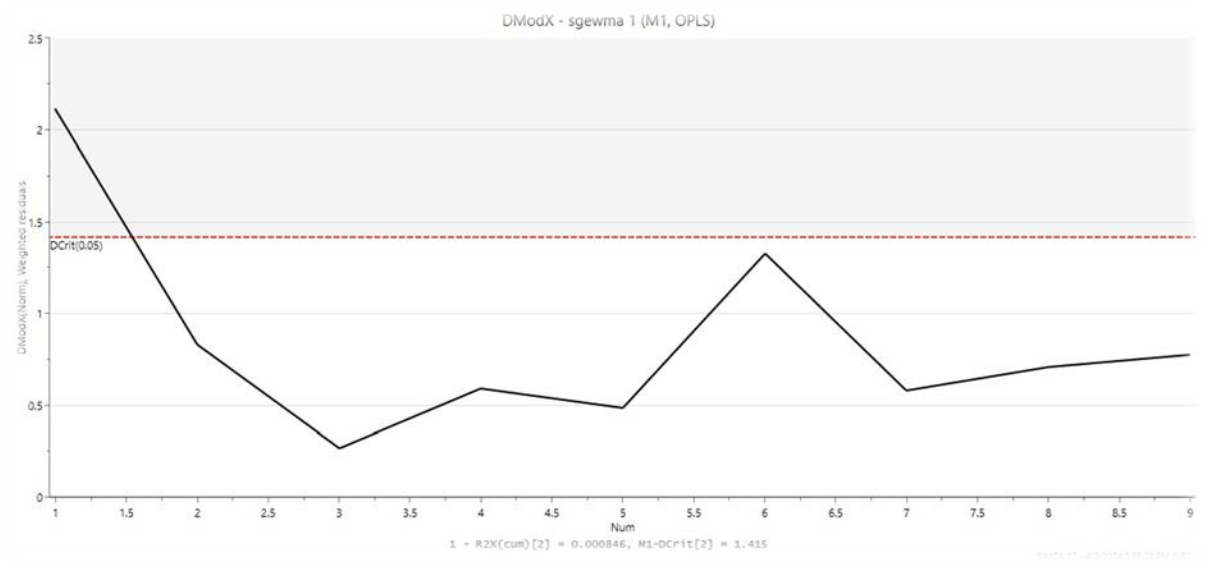

Figure 3: DModX plot for each observation in the OPLS model.

Note: DModX plot of the OPLS model determines how well the samples fit the model. There are no noticeably outlying values as the sample falls within the critical level of 0.05 [41].

STATISTICAL PARAMETERS

The model of regression can be characterized in various ways. To determine the RMSECV, RMSEP, and R^2 values, the OPLS technique was applied to the validation dataset corresponding to the specified combination. An acceptable range was identified for the mean percent recovery of the various medication combinations, as well as the validation of the calibration parameters. Standardized medications were incorporated into the exact amounts of a synthetic combination with a specified concentration for recovery trials. Standard working solutions of predetermined volumes were added. Once the sample volume or concentration for addition was specified. Methanol was subsequently introduced into the flask.

The recovery of the medications in the mixtures was evaluated using chemometric techniques. The root mean square deviation was also assessed using the OPLS technique. The results of the recovery research showed that the procedures were validated, as the recovery study achieved nearly 100% recovery, and the percentage root mean square deviation (RSD) was less than 2%. For both drugs in the prediction set, it can be concluded that OPLS, the optimized approach, is successful, proving its effectiveness and potent predictive power.

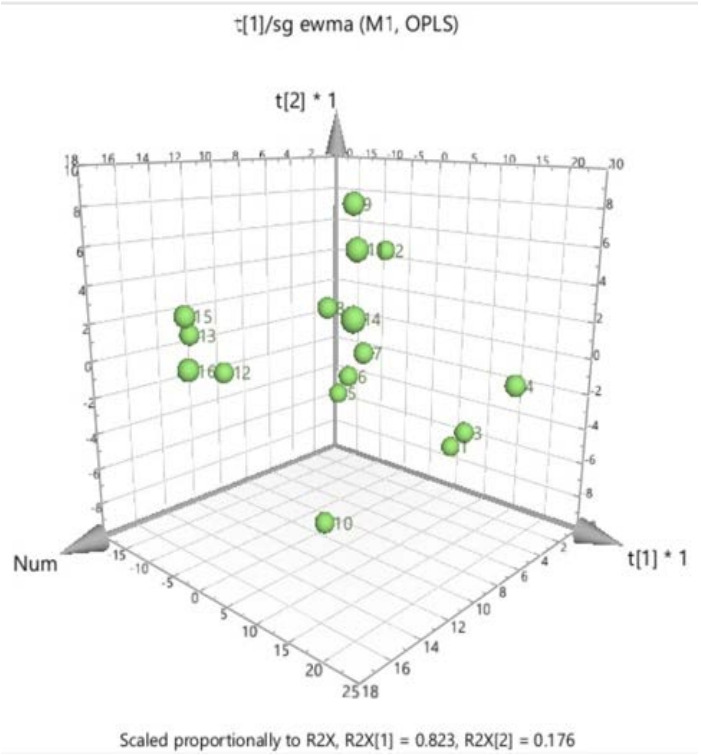

Figure 4: 3D score plot from OPLS analysis showing the distribution of 16 sample sets.

Note: A score plot evaluates the degree of separation between various sample classes and helps decipher the data's underlying structure [38].

RESULTS AND DISCUSSION

Table 2: Statistical parameters for the OPLS method for AML-NAR and NIF-NAR (Calibration and Prediction set).

SETS	DRUGS	R^2	RMSECV	RMSEE
	AML	0.9963	0.5266	0.4818
	NAR	0.9947	0.6081	0.5718
Calibration	NIF	0.9935	0.7034	0.6613
	NAR	0.9976	0.4051	0.5718
	AML	0.9978	0.3978	0.4083
	NAR	0.9982	0.4418	0.3929
Prediction	NIF	0.9974	0.6191	0.4872
	NAR	0.9985	0.4353	0.4199

Table 3: Representation of prediction outcomes from the OPLS model for NAR and AML in the Prediction datasets, along with the % recovery of the model.

AML			NAR		
Known concentration (µg/mL)	**Predicted value**	**% recovery**	**Known concentration (µg/mL)**	**Predicted value**	**% recovery**
20	19.7	98	5	5.2	104
20	19.2	96	20	19.1	95
10	9.9	99	1	0.9	95
10	9.6	96	10	9.6	96
1	0.9	97	20	19.1	95
20	19.9	99	1	0.9	91
5	4.9	98	1	0.9	95
1	0.9	99	5	5.0	100
5	4.5	91	5	5.2	104

The predicted values closely match the actual concentration, demonstrating the model's precision and accuracy.

Table 4: **Analytical parameters (LOD, LOQ, SD, SE) for NAR, NIF and AML**

	NAR	NIF	AML
Standard Error	0.003	0.002	0.004
Standard Deviation	0.007	0.006	0.011
LOD (µg/mL)	2.092	1.753	1.861
LOQ (µg/mL)	6.338	5.312	5.639

The current study demonstrates the effectiveness of OPLS chemometric-assisted UV-visible spectrophotometry in simultaneously determining Naringin-Amlodipine and Naringin-Nifedipine in synthetic mixtures. This chemometric method effectively addresses the challenges posed by traditional univariate spectrophotometric techniques [42], such as spectral overlap, which significantly enhances the accuracy of quantifying multiple components in a single analysis. It also improves sensitivity to experimental conditions, selectivity, and detection limits [43] [44] [45]. Calibration and prediction set design were a critical aspect of this analysis. As shown in Table 1, a comprehensive range of concentrations for both AML-NAR and NIF-NAR combinations was systematically selected, ensuring coverage across the linear dynamic range (5-20 µg/mL for calibration and 2.5-15 µg/mL for the prediction set). The present work was optimized using Savitzky-Golay and EWMA filters, which helped to resolve the issue of overlapping and enabled an accurate unfolding of each analyte's contributions. We validated the OPLS models using statistical measures, including R^2 (the coefficient of determination), RMSECV (Root Mean Square Error of Cross-Validation), and RMSEE (Root Mean Square Error of Estimation), for both calibration and prediction sets. The impressive R^2 values of over 0.99 for both the calibration and prediction sets demonstrate the model's strength. In contrast, the low RMSECV and RMSEE values, both below 0.99, indicate that errors are minimal and the predictive power is substantial. The lower values of RMSECV suggested that the model was not overfitted and was capable of accurately predicting new, unseen samples. Additionally, validation parameters like Hotelling's T^2 test and DModX analysis were used to confirm that no significant outliers were skewing the model's performance, as all data points fell within the 95% confidence ellipse and below the critical DModX limits [46]. Permutation analysis further supported the model's reliability, revealing that the fitting wasn't just a fluke, as all permuted Q^2 values were negative and R^2 values stayed close to 1. The recovery studies, which produced results of 100%, further validated the model's accuracy and precision. The average recovery percentages and percentage root mean square deviation (RSD < 2%) [47] fell well within the acceptable analytical range, making this method a novel fit for pharmaceutical applications. This method showed a consistent linear response across the AML-NAR and NIF-NAR concentration ranges of 5-20 (µg/mL) with correlation values ranging from 0.9947-0.9963 and 0.9935-0.9976, respectively. Higher linearity indicated that the model could generalize across various sample compositions and concentration ranges, making it a robust one. With mean recoveries of 98.7% for AML and 99.2% for NIF in synthetic

combination, recovery investigations confirmed the approach. All the parameter results were within the range of ICH guidelines, which benefits the present OPLS approach for reducing noise, managing interferents, and enhancing exploratory data analysis, particularly for complex pharmaceutical mixtures. When compared to traditional spectrophotometric methods [48], this approach significantly lessens interference effects and boosts the reliability of simultaneous drug quantification. However, the limit of detection (LOD) and Limit of quantitation (LOQ) values were relatively high; as such, the application of the particular method was limited to pharmaceutical formulation and its utility on biological matrices.

CONCLUSION

In searching for weak and moderate outliers, Hotelling's T^2 test and DModX encountered no deviance, suggesting that no outliers were detected (24). All of the permuted Q^2 values were less than 0, and R^2 values were less than or equal to 1, as shown by the permutation analysis. The model fitting was probably correct, as Q^2 and R^2 [49] were less than the initial values. Looking at the score plot, we can observe all the samples that are inside Hotelling's range.

This leads us to believe that the model is effective, as all optimization strategies meet the specified criteria. At long last, we may make educated guesses on the repeatability of the approaches based on the outcomes of several evaluations. There was no deviation from the ICH standards in any of the above analyses. The cost-effectiveness, multicomponent analysis, and the removal of interface overlap in the overlapping UV spectra for NAR, AML, and NIF were all considered significant advantages of the OPLS method, which allows for the precise determination of all three compounds. This method provided adequate sensitivity in the 5-20 (µg/mL) range, which is ideal for pharmaceutical manufacturing quality control.

Lastly, it should be noted that although the techniques achieved excellent results in pharmaceutical formulations, they have a restricted application in biological matrices, such as plasma, due to relatively high LOD and LOQ values, which could likely arise from increased matrix complexity or spectral interferences in the biological samples. Subsequent work on lowering these limits may improve the capability of accurately measuring the compounds of interest in biological specimens.

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FINANCIAL ASSISTANCE

NIL

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Vishala Rani Baraily contributed to the development of the technique, execution of the experimental data, writing of the original draft, and data analysis. Jithendar Reddy Mandhadi supervised the research, provided critical insights, and contributed to manuscript writing and revisions. Bhupendra Shrestha contributed to experimental work, data interpretation, manuscript preparation, and revisions. All the authors have read and approved the final manuscript.

LIST OF ABBREVIATIONS

AML: Amlodipine, NIF: Nifedipine, NAR: Naringin, OPLS: Orthogonal Partial Least Squares, RMSEP: Root mean square error of prediction, RMSECV: Root mean square error of Cross-validation, RMSEE: Root mean square error, ICH: International Council for Harmonization, RSD: Root mean square deviation, UV: Ultra-Violet, LDR: Linear dynamic range, LOD: Limit of detection, LOQ: Limit of quantification

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