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Research Article

Development and Validation of Analytical Methods for Simultaneous Estimation of Lercanidipine Hydrochloride and Enalapril Maleate

Archita J. Patel[1*], Jamila J. Patel[1] and Samir G. Patel[1]

[1]*Department of Pharmaceutical Chemistry and Analysis, Ramanbhai Patel College of Pharmacy, Charotar University of Science and Technology, CHARUSAT Campus at & Post: Changa, Tal: Petlad, Dist: Anand, Gujarat-388 421.*

[1*]*Assistant Professor, Department of Pharmaceutical Chemistry and Analysis, Ramanbhai Patel College of Pharmacy, Charotar University of Science and Technology, CHARUSAT Campus, At & Post: Changa, Tal: Petlad, Dist: Anand, GUJARAT-388 421, INDIA*

Abstract: The fixed dose of lercanidipine hydrochloride(LH) and enalapril maleate (EM) is used to provide more effective control of hypertension. The research work was aimed at the development and validation of simple and sensitive analytical methods for their quantification from tablets. The simultaneous equation, first-order derivative, and multivariate spectrophotometric methods were developed for the simultaneous estimation of LH and EM in tablets. The simultaneous equation method involves a determination of LH and EM at 236 nm and 207 nm, respectively. First order derivative UV spectrophotometry method involves using a zero crossing point of LH at 330 nm and EM at 219 nm. Both methods were validated as per ICH Q2 (R2) guidelines and found to be accurate and precise as they exhibited <2% relative standard deviation. The limit of detection (LOD) and limit of quantitation (LOQ) for LH were found to be 0.26 µg/ml and 0.78 µg/ml respectively whereas LOD and LOQ for EM were found to be 0.41 µg/ml and 1.24 µg/ml respectively for simultaneous equation method. The LOD and LOQ for LH were found to be 0.39 µg/ml and 1.19 µg/ml respectively whereas LOD and LOQ for EM were found to be 0.31 µg/ml and 0.97µg/ml respectively for the first-order derivative method. Three Chemometric methods namely classical least square (CLS), principal component regression (PCR), and partial least square (PLS) were studied for the simultaneous determination of LH and EM in tablets using spectrophotometry. A set of 25 standard mixtures containing both drugs were prepared in the range of 5–25 µg/ml for both drugs. The analytical figure of merit (FOM) was determined for all three chemometric methods. The LOD values for LH were found to be 0.97, 0.93, and 0.94 µg/ml and 0.90,0.88, and 0.89 µg/ml for EM using CLS, PCR, and PLS modeling techniques. The proposed methods were user-friendly, rapid, and sensitive enough for the determination of LH and EM.

Keywords: Lercanidipine Hydrochloride, Enalapril maleate, Simultaneous Equation Method, First Order Derivative Method, Chemometrics

***Corresponding Author**

Archita J. Patel , Assistant Professor, Department of Pharmaceutical Chemistry and Analysis, Ramanbhai Patel College of Pharmacy, Charotar University of Science and Technology, CHARUSAT Campus, At & Post: Changa, Tal: Petlad, Dist: Anand, GUJARAT-388 421, INDIA

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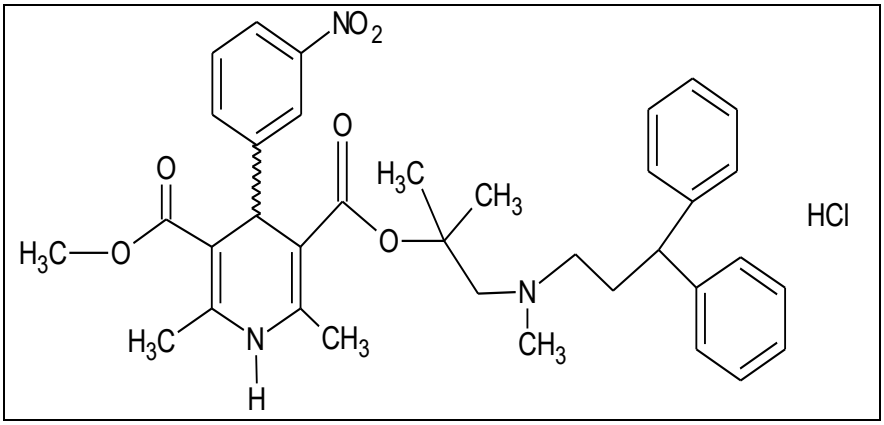
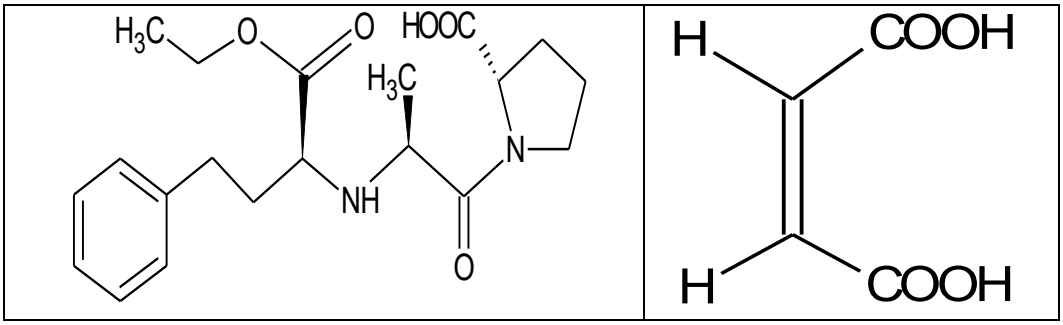
1. INTRODUCTION

Lercanidipine Hydrochloride (LH) is a dihydropyridine calcium-channel blocker used in the treatment of hypertension. LH is chemically 3, 5-pyridine dicarboxylic acid, 1,4- dihydro-2,6-dimethyl- 4-(3- nitro phenyl) 2 [(3,3diphenyl propyl) methyl amino]1,1-dimethylethyl methyl ester hydrochloride (Figure 1A). LH is not official in any of the pharmacopeias.[1-4] Enalapril Maleate (EM) is an angiotensin-converting enzyme (ACE) inhibitor. EM is used to treat high blood pressure (hypertension) and congestive heart failure. EM is chemically (2S)-1-[(2S)-2-[[(1S)-1-(ethoxycarbonyl)-3phenylpropyl] amino] propanoyl] pyrrolidine-2-carboxylic acid (Z) butenedioate (Figure 1B). EM is official in Indian Pharmacopoeia, United State Pharmacopoeia, and British Pharmacopoeia.[5-7]



(A)



(B)

Fig 1: Structure of (A) Lercanidipine hydrochloride, (B) Enalapril Maleate

The marketed formulations of this drug combination are ZANIPRESS (Merckle Recordati) 10 mg/10 mg tablet (White film coated) containing 10mg of LH and 10 mg of EM per tablet, and ZANIPRESS (Merckle Recordati) 10 mg/20 mg tablet (Yellow film coated) containing 10 mg of LH and 20 mg EM. The formulations are used in the treatment of Hypertension. Based on the literature review, it was found that several methods are available for the estimation of LH and EM individually as well as in combination with other drugs. Spectrophotometric [8] and HPLC [9-13] methods have been used for the estimation of LH as well as [14-19] EM. So far, HPLC [20-22] HPTLC [23] methods have been reported for the estimation of LH and EM in their synthetic mixture. However, none of the reported methods showed the estimation of these drugs from the tablet dosage form, and hence, the available methods were not applied for the estimation of the aforementioned drugs from their approved dosage forms. The available methods for simultaneous estimation of these drugs include mostly the use of chromatographic methods, hence it was necessary to develop and validate simple yet sensitive analytical methods for estimating LH and EM from their combined dosage form. The current research work was aimed at the development and validation of simple spectrophotometric methods using derivative spectrophotometry and simultaneous equation method (SEM) of UV/Vis spectroscopy. It is an instrumental technique of choice for the mentioned purpose in industrial laboratories due to its simplicity and ease of operation. But, the limitation of UV spectrophotometric methods is their sensitivity. This limitation could be overcome by using chemometric techniques that have been reported to be more sensitive than chromatographic techniques. In recent years, multivariate calibrations such as classical least squares (CLS), Principal component regression (PCR), and Partial least squares (PLS) have been employed extensively in quantitative spectral analysis to get selective information for unselective data. These methods are widely accepted, as they give the best results in cases of complex mixture solutions. These methods can be applied for the simultaneous spectrophotometric estimation of drugs in pharmaceutical formulations containing two or more drug compounds. CLS, the simplest method, has a multivariate least square procedure based directly on Beer's law. PCR and PLS are factor analysis methods that are used to establish a relationship between matrices of the chemical data. PLS is related to PCR in that spectral decomposition is performed. PCR decomposition is significantly influenced by variations, which are irrelevant to the analyte concentration, whereas PLS spectral decomposition is weighted to concentration.[24-26] As the literature review did not reveal any analytical method for the simultaneous estimation of LH and EM from its pharmaceutical formulation, it was thought of

interest to combine the advantage of statistics and UV spectroscopy to develop simple yet sensitive methods for the simultaneous analysis of these drugs.

2. EXPERIMENTAL

2.1. Instruments and Software

A double beam UV-spectrophotometer, UV-1800 (Shimadzu, Japan) equipped with 1 cm quartz cells and 2 nm fixed slit width connected to a computer loaded with Shimadzu UPVC software was used. An analytical balance New Classic MF (Model No. ML204/A01 METTLER TOLEDO, made in Switzerland) was used to weigh the standard and test samples accurately. The additional PLS-tool box software (EIGENVECTOR) was used for the statistical treatment of data. The zero-order spectra were recorded over 200–400 nm wavelength with one data point/nm for simultaneous first-order derivative and chemometric methods.

2.2. Chemicals

Standards for LH and EM were obtained as gift samples from Torrent Pharmaceuticals, Ahmedabad (India). Methanol of analytical grade purity was purchased from AR grade, Loba Chemie, India. Double distilled water (In house) was used throughout the study for UV-visible spectrophotometry.

2.3. Standard stock solution of drugs

Standard stock solutions of LH and EM (1000 µg/ml) were prepared individually by dissolving 100 mg in 100 mL of methanol. The working standard solutions of LH and EM (100 µg/ml) were prepared individually by further diluting the standard stock solution ten times with methanol.

2.4. Spectrophotometric methods

Preparation of solutions to study linearity and range: The solutions were prepared by diluting the suitable aliquots (0.4,0.8,1.0,1.2,1.4 mL for LH) and (0.4,0.8,1.0,1.2,1.4 and 1.6 mL for EM) of working standard solution (100 µg/ml) of both drugs with methanol in 10 mL volumetric flasks separately to get a concentration range 4-14 µg/ml for LH and 4-16 µg/ml for EM.

2.4.1. Method A: Simultaneous Equation Method

The simultaneous equation method involves the measurement of absorbance at the respective wavelength maxima of LH and EM respectively. The wavelength maxima were determined by scanning the working standard solution of each drug in the 200-400 nm range. The wavelength maxima were found to be 207 nm and 236 nm for EM and LH respectively. The absorbance of prepared solutions in the concentration range of 4 - 14 µg/ml for LH and 4-16 µg/ml for EM were recorded at these wavelengths. Then, the absorptivity values were determined using Beer-Lambert's law. The experiment was repeated six times. Then, the average value of absorptivity was put in the following equations. The concentration of the unknown sample containing EM and LH was determined using the following simultaneous equations.

C1 = A2 (719.35) - A1 (446.76) / (719.35) (68.99) - (714.2) (446.76) _____ (1)
C2 = A1 (68.99) - A2 (714.2) / (719.35) (68.99) - (714.2) (446.76) _____ (2)

Where, C1 and C2 are concentrations of EM and LH respectively, in µg/ mL, in the sample solution. A1 & A2 are the absorbances of the mixture at 207 nm and 236 nm respectively.

2.4.2. Method B: First-Order Derivative Spectroscopy Method

LH and EM exhibited overlapping UV spectra as evident from their wavelength maxima values. Hence, to estimate them more precisely and to overcome the spectral interference from the other drug; first-order derivative spectroscopy can be used. Zero-order spectra of both drugs are converted to first-order derivative spectra with the help of the spectra manager software of the instrument. It was observed that LH showed dA/dλ= 0 at 219 nm in contrast to EM which has considerable dA/dλ at this wavelength. Further, EM has dA/dλ=0 at 330 nm while at this wavelength LH has significant dA/dλ. Therefore, wavelengths 219 nm and 330 nm were employed for the determination of LH and EM respectively without the interference of the other drug. The calibration curves were plotted at these wavelengths of concentrations against dA/dλ separately (Figure 2).

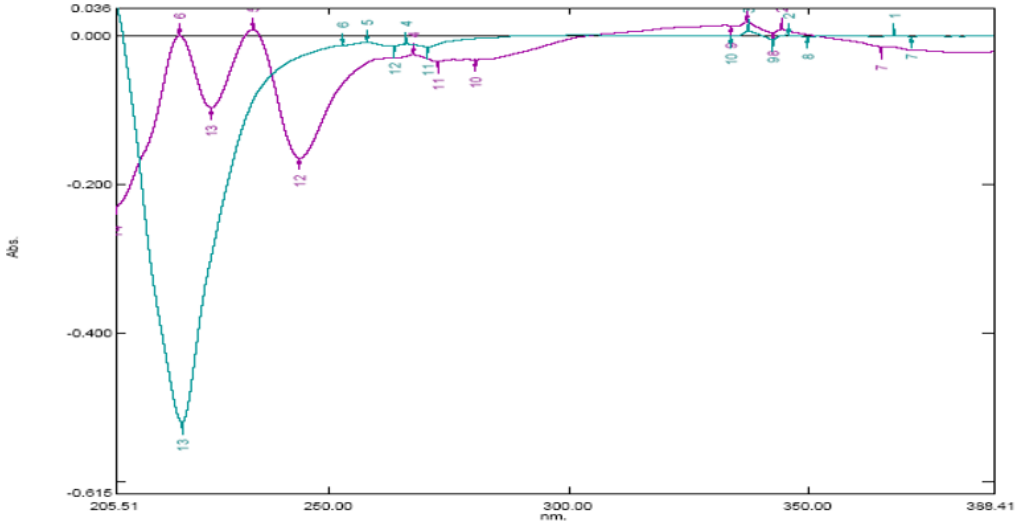


Fig 2: Overlay Spectra of LH and EM for first Order derivative method

2.5. Validation of UV-VIS Spectrophotometric methods

2.5.1. Linearity and range

Aliquots of working stock solutions of LH and EM were diluted with methanol to get final concentrations in the range of 4-14 µg/ml for LH and 4-16 µg/ml for EM for both methods. The study was performed five times, and average absorbance was calculated for respective wavelengths. The calibration curves were prepared for LH and EM at wavelengths by plotting absorbance vs. concentration. The correlation coefficient value (r^2) and the calibration equations were generated.

2.5.2. Precision

The precision of the method was obtained by performing method repeatability studies, intraday variation, and interday variation. In the repeatability study, single concentrations (8 µg/ml LH and 10 µg/ml EM) of both drugs were analyzed six times for the simultaneous equation method whereas (6 µg/ml LH and 12 µg/ml EM) of both the drugs were analyzed six times for first derivative method. Intraday and interday variation, the absorbances of three different concentration mixtures of LH and EM were measured three times a day for three consecutive days. The results obtained were used to calculate % RSD.

2.5.3. Limits of detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ of the developed method were calculated using the following equations:

Limit of detection

$$LOD = 3.3\ \sigma/S \quad (3)$$

Limit of quantification

$$LOQ = 10\ \sigma/S \quad (4)$$

Where σ is the standard deviation of the response and S is the slope of the calibration curve.

2.5.4. Accuracy

The accuracy of the proposed method was determined using the standard addition method at 80%, 100%, and 120% levels of the test solution. The test samples were spiked with known amounts of standards repetitively (n = 3) and mean percentage recoveries and % RSD was calculated.[27]

2.6. Chemometric-Assisted UV-Visible Spectrophotometric Methods

2.6.1. Preparation of working standard stock solutions:

The working standard solutions were prepared by diluting 0.5, 1.0,1.5, 2, and 2.5 mL aliquots of working standard solution (100 µg/ml) of both the drugs with methanol in 10 mL volumetric flasks separately to obtain a concentration range 5–25 µg/ml for LH and EM.

2.6.2. Experimental design for CLS, PLS, and PCR calibration

Preparation of a mixture of standard solutions

A set design of concentration data corresponding to the LH and EM mixture was organized statistically to maximize the information content in the spectra and to minimize the error of multivariate calibrations as shown in Table 1. The set of 25 standard mixture solutions, with different concentration ratios of LH and EM, was systemically prepared in the range of 5–25 µg/ml for LH and EM. From the data set, 9 mixtures have been randomly selected for validation purposes. The UV absorption spectra were recorded over selected wavelength points. The computations were made using EIGENVECTOR SOLO software for PLS, PCR, and CLS methods.

Table 1: Concentration matrix of the mixtures containing two drugs (calibration data set)

Calibration Dataset					
Sr. No.	LH (µg/ml)	EM (µg/ml)	Sr. No.	LH (µg/ml)	EM (µg/ml)
1[a]	5	5	**14 [a]**	15	20
2	5	10	15	15	25
3	5	15	16	20	5
4	5	20	17	20	10
5	5	25	**18 [a]**	20	15
6	10	5	**19 [a]**	20	20
7	10	10	20	20	25
8	10	15	21	25	5
9	10	20	**22 [a]**	25	10
10[a]	10	25	**23 [a]**	25	15
11	15	5	**24 [a]**	25	20
12	15	10	**25 [a]**	25	25
13	15	15	**Mixtures marked with [a] were selected for the validation data set**		

CLS

The CLS is the easiest method was developed using Beer-Lambert's law and the converse expressions of UV-Visible spectroscopy using multiple linear regression functions. Briefly, the absorbance values obtained against different concentrations of drugs were converted into a matrix that can be represented as A= K X C for CLS, where A is the zero-order absorbance, C is the concentration matrix, and K & P are the calibration coefficient for the selected concentration range. The method has a limitation in that it is an inflexible

model and it is required that there is no interaction between the matrix components in comparison with PLS and PCR. [28]

PLS

The PLS calibration can be performed using the PLS algorithm initially developed by Ward and later on elaborated by Martens and Naes. It involves simultaneous treatment of the independent and dependent variables for data compression and decomposition of recorded spectra of drugs. In spectrophotometry, it is done by decomposing both the concentration and absorbance matrix into latent variables. The method is based on factors analysis and results in loading and score plots to derive meaningful information from the spectra of drugs. [29]

PCR

The PCR calibration method is also based on factor analysis, and here the original data is obtained in terms of absorbances (A) that is smoothed using the Savitzky–Golay smoothing method and concentration (C) of the analyte is treated by the mean-cantering method. Then, the co-variances will be calculated using the dispersion matrix of the central matrix of absorbance. The eigenvectors with the highest scores of eigenvalues were considered, and the other eigenvalues and their corresponding eigenvectors were eliminated from this study. [30] PCR and PLS are comparable, and there are several reports wherein authors have compared both methods for the analysis of drugs. Scientists Wentzell and Montano, have reported a few instances which indicated that PLS gave better results than PCR, and in most of the studies, there is no substantial difference in the performance of the developed models. [31]

2.7. Validation of the Chemometric-Assisted Spectrophotometric methods

For the validation of mathematical models of CLS, PCR, and PLS, statistical analysis was applied. Various Parameters like Root mean standard error of calibration (RMSEC), Root mean standard error of cross-validation (RMSECV), Root mean standard error of prediction (RMSEP), Predicted Residual Error Sum of Square (PRESS), and correlation coefficient (r^2) were determined. The figure of merits (FOM) is necessary for validating chemometric methods. FOM such as sensitivity (SEN), analytical sensitivity (c), the limit of detection (LOD), and the limit of quantitation (LOQ) can be estimated and used to compare analytical methods.

2.8. Analysis of tablets

A total of 10 tablets were accurately weighed and powdered by using a mortar & pestle. A quantity of powder equivalent to one tablet (containing 10 mg of LH and EM) was weighed accurately and dissolved in 10 ml methanol by sonication for 20 minutes. Then it was transferred to a 10 ml volumetric flask through a Whatman No. 40 filter paper and the residue on the filter paper was thoroughly washed with methanol and the filtrate was made up to the mark with methanol to make standard stock solutions of 1000 µg/ml of LH and EM. The working solution of the tablet was prepared by transferring 1 ml from the standard stock solution to a 10 ml volumetric flask, and then the volume was made up of methanol. The final sample solution was prepared by diluting 0.6 ml of the working solution up to 10 ml with methanol in a 10 ml volumetric flask to get 6 µg/ml of LH and 6 µg/ml of EM respectively. The absorbance of the test solution was measured at the selected wavelengths and concentrations were determined by UV Methods and chemometric methods.

2.9. Selectivity

The selectivity of the developed simultaneous equation method and first-order derivative spectroscopy was determined as mentioned below. The selectivity was determined by preparing a synthetic mixture of 10 mg of pure EM and LH with commonly used excipients for tablet formulation namely starch, lactose, talc, and magnesium stearate. The mixture was homogenized using mortar and pestle, and prepared samples were subjected to UV-spectrophotometry using the same procedure as mentioned in "analysis of tablets" for simultaneous equation and first-order derivative methods as well as chemometric methods. [32]

3. RESULTS AND DISCUSSION

3.1. Selection of solvent for all spectroscopic methods

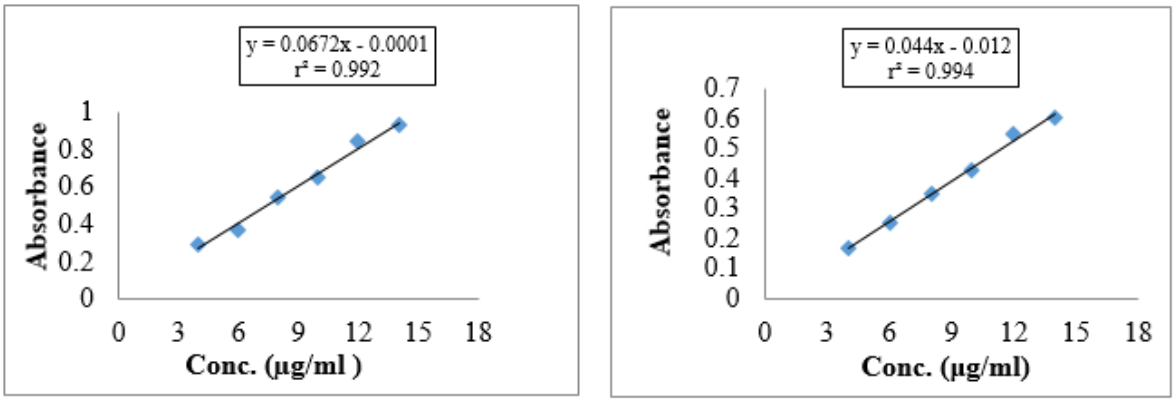
LH is soluble in chloroform and dichloromethane but practically insoluble in water whereas, EM is sparingly soluble in water and practically insoluble in dichloromethane. But both drugs were soluble in methanol. So, methanol was taken as a common solvent for the study as it doesn't shift the absorption maxima of both drugs. The solubility study revealed that both drugs are freely soluble in methanol. So, methanol was selected for further analytical method development. Both methods were validated as per ICH guidelines.

3.2. Validation of UV Spectrophotometric Methods

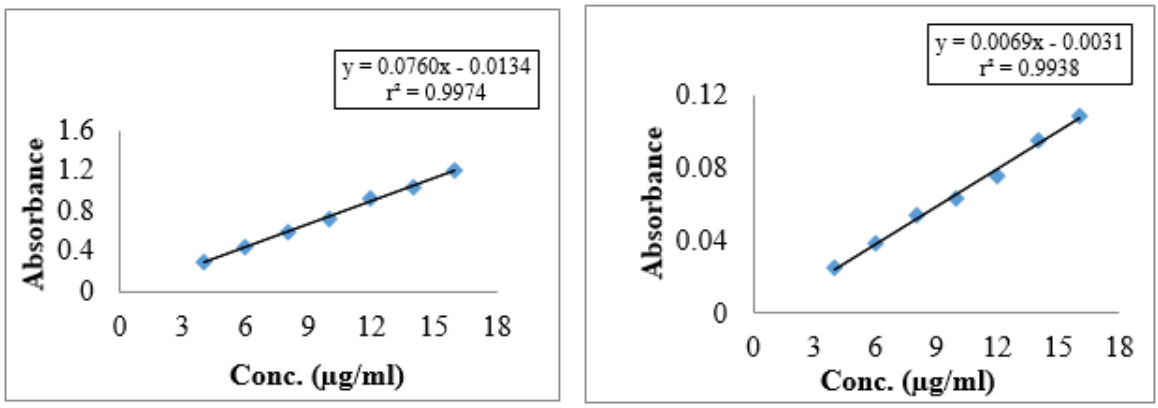
The UV absorption spectra of working solutions were recorded in the wavelength range of 200–400 nm, keeping the highest dilution as blank. LH shows maximum absorbance at 236 nm whereas EM showed absorbance maxima at 207 nm.

3.2.1. Linearity

The calibration curves of LH and EM were linear in the range of 4-14 µg/ml and 4-16 µg/ml respectively (Fig. 3 and 4) for both methods. The calibration curve of LH at 207 nm and 236 nm and EM at 207 nm and 236 nm for the simultaneous equation method were determined, and correlation coefficients were found to be 0.992, 0.994, 0.9974, and 0.9938 respectively (Fig. 3). The calibration curve of LH at 330 nm and EM at 219 nm for First derivative method were determined, correlation coefficients were found to be 0.9997, 0.9982 respectively (Fig. 4).



(A)



(B)

Fig 3: Calibration curve of LH and EM for Simultaneous Equation Method (A) Calibration curve of LH at 207 nm and 236 nm. (B) Calibration curve of EM at 207 nm and EM at 236 nm

3.2.2. Precision

The precision study is a necessary part of analytical method validation as per ICH guidelines wherein the consistency in the performance of the method is checked by performing it repeatedly. The % relative standard deviation (% RSD) was calculated for repeatability studies, intraday, and interday precision. Results of repeatability studies were 1.27% and 0.91% for the simultaneous equation method and 1.16% and 1.40% for the first derivative method, intraday precision was 1.15% and 1.13% for the simultaneous equation method 1.16%, 1.14 for First derivative method, interday precision 1.4% and 0.78%, for the simultaneous equation method and 1.12% and 1.38% for First derivative method, for LH and EM respectively. The values of % RSD were found to be less than 2% which made this method more acceptable.

3.2.3. Accuracy

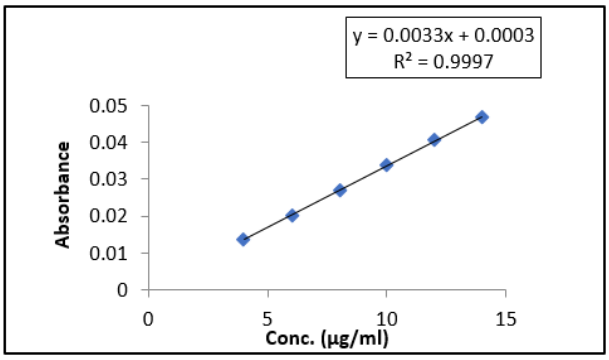
The percentage recoveries of drugs from marketed formulations were determined by the standard addition of pure drugs at 80%, 100%, and 120% of known concentration, and good recoveries were obtained at each level. The percent recovery for LH and EM were found to be 99.19 ± 1.08 and 100.89 ± 0.78 for the simultaneous equation method and 99.42 ± 0.59 and 98.62 ± 0.62 respectively for the first derivative method.

3.2.4. Selectivity

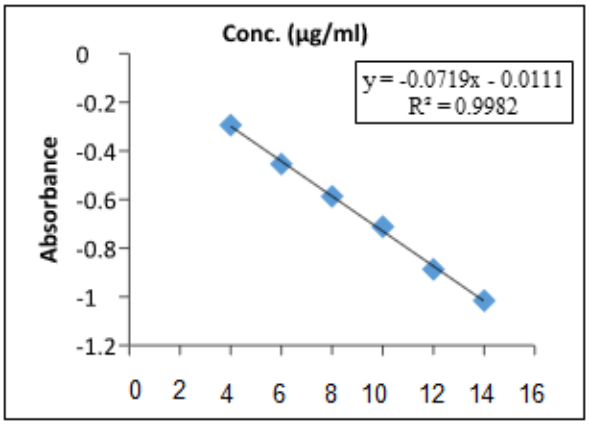
The selectivity/specificity of the methods was evaluated by analysis of LH and EM in the placebo solution as shown under "Procedure for tablets" and the resulting absorbance values in simultaneous equation method, first-order derivative, and chemometric methods. There was no interference from the placebo when the spectra were recorded. Hence, the developed methods can selectively analyze both drugs without interference from excipients. [31]

3.2.5. Limits of detection (LOD) and limits of quantification (LOQ)

LOD and LOQ have been obtained from the equations where σ is the standard deviation of the intercept and S is the mean of the slope of the calibration curve. The LOD and LOQ for LH were found to be 0.26µg/ml and 0.78µg/ml respectively and LOD and LOQ for EM were found to be 0.41µg/ml and 1.24µg/ml respectively for the Simultaneous equation method. The LOD and LOQ for LH were found to be 0.39µg/ml and 1.19µg/ml respectively and LOD and LOQ for EM were found to be 0.31 µg/ml and 0.97µg/ml respectively for the First derivative method.[27,33-37]



(A)



(B)

Fig 4: Calibration curve of LH and EM for First Derivative Method (A) Calibration curve of LH at 330 nm (B) Calibration curve of EM at 219 nm

3.3. Validation of Chemometric-Assisted UV Spectroscopic Methods

Calibration and validation sets were prepared as mentioned and zero-order spectra were scanned over a range of 200–400 nm. To obtain minimum RMSEC, RMSECV values, and wavelength ranging from 200 nm to 288 nm were selected[37-40].

3.3.1. CLS method

Absorbance matrix A was constructed using recording zero-order spectra between 200 nm and 288 nm at 10 nm intervals. Thus, 9 wavelength points were selected. The CLS model was created by introducing absorbance (A) and concentration matrix (C) data in the PLS toolbox, version 2 software. Absorbance values of samples at 9 different wavelength points have been incorporated in the constructed model and quantities of LH and EM in the validation data set as well as tablets were predicted.

3.3.2. PCR and PLS methods

PCR and PLS calibrations were constructed by using the respective algorithms. Absorbance (A) and Concentration (C) data matrices were introduced in PCR and PLS models in the PLS toolbox, version: 2 software, and the quantities of LH and EM in the systemic mixture prediction (validation) set and tablets were predicted.

Table 2: Composition and results of the prediction set by CLS, PLS, PCR

Prediction set Concentration			Amount of drugs (µg mL^{-1})					
			CLS		PLS		PCR	
Sr. No	LH	EM	LH	EM	LH	EM	LH	EM
1	5	5	5.23	5.44	5.28	5.43	5.28	5.50
2	10	25	9.84	24.99	9.83	25.07	9.83	25.16
3	15	20	14.89	19.81	14.86	19.95	14.91	19.81
4	20	15	20.40	15.02	20.33	15.02	20.35	14.98
5	20	20	20.55	19.75	20.48	20.04	20.58	19.75
6	25	10	25.77	10.69	25.65	10.81	25.71	10.69
7	25	15	25.50	14.23	25.39	14.24	25.42	14.18
8	25	20	26.12	19.81	26.02	19.85	25.96	20.02
9	25	25	24.65	24.60	24.54	24.93	24.62	24.70

3.3.3. Statistical analysis in selecting the number of principal components or factors

An appropriate choice of the number of principal components or factors is necessary for PCR and PLS calibrations. The number of factors should account as much as possible for the experimental data without resulting in overfitting. Various criteria like RMSEC, RMSECV, and PRESS have been used to select the optimum number. Cross-validation methods leaving out one sample at a time were employed, with a calibration set of 25 mixtures. CLS, PCR, and PLS calibration on 25 mixtures were performed using this calibration, and the concentration of sample left out during the calibration process was determined. This process was repeated 25 times until each calibration sample had been left once. The predicted concentrations were compared with the known concentration of compounds in each calibration sample. To validate the model, both RMSECV and RMSEP were considered. Both values must be as low as possible for a particular model. Both the values were calculated as follows:

$$RMSECV = \sqrt{PRESS/n} \quad \text{_____(5)}$$

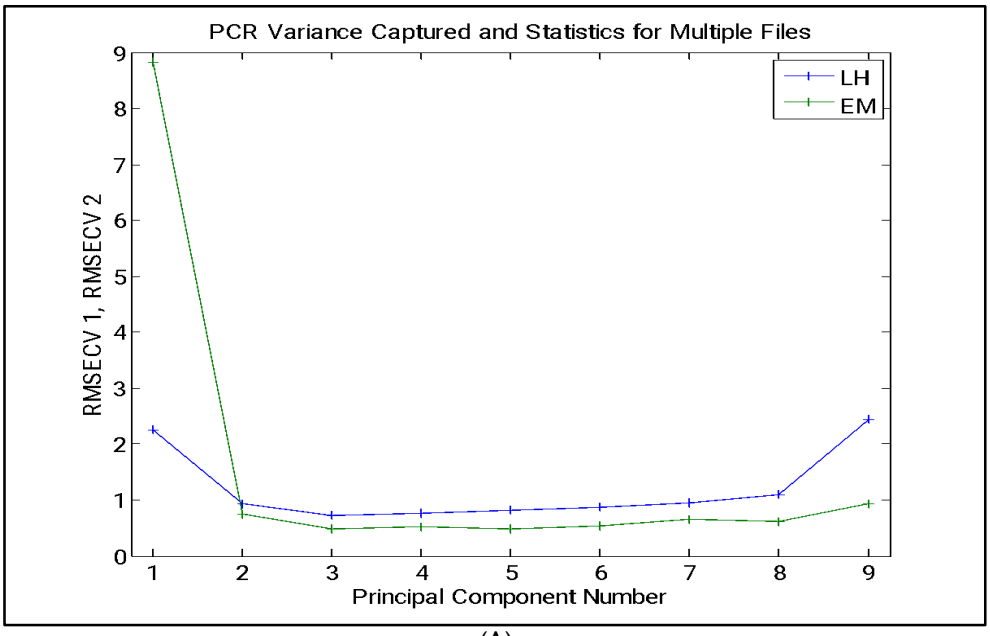
Where n is the number of training samples

$$PRESS = \sum (Ypre - Y_{true})^2 \quad \text{_____(6)}$$

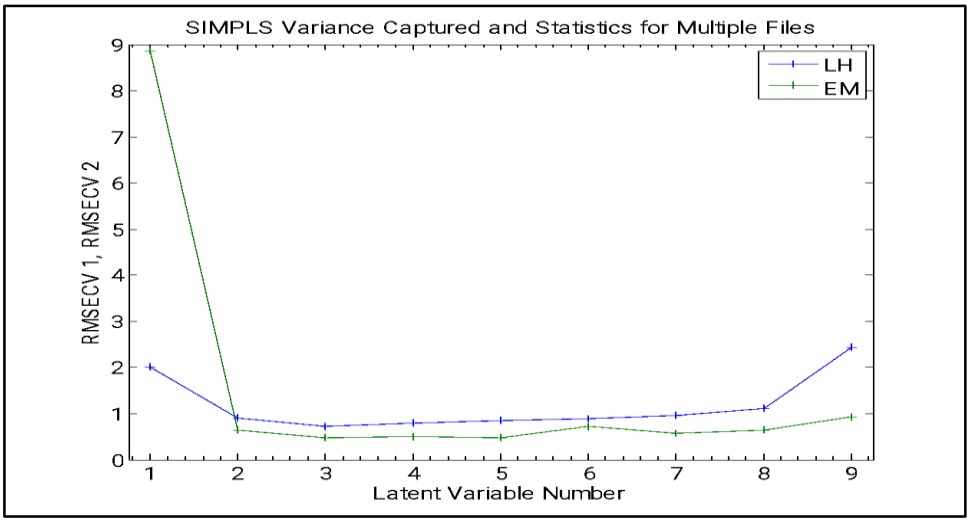
Ypre and Ytrue are predicted and true concentrations in µg/ml respectively. The RMSECV value was used as a diagnostic test for examining the errors in the predicted concentrations. It indicates both the precision and accuracy of predictions. Appropriate selection of the number of factors to construct the model is the key to achieving correct quantitation in PCR and PLS calibrations. The usual procedure for this purpose involves choosing the number of factors that result in the minimum RMSECV. The method developed by Haaland and Thomas was used to select the optimum number of factors that results in a negligible difference between the corresponding RMSECV and the minimum RMSECV. Fig. 5 shows the variation of RMSECV as a function of several components and RMSECV as a function of latent variables for PCR and PLS respectively. Two factors were found to be optimum for each component in the mixture by PCR and PLS methods. Predicted values and estimated values can be correlated by Score plot with the help of the PLS Toolbox version 5 demo (Fig. 6). As seen in figure 6, there is a good correlation between predicted and measured values indicated by r2 values close to 1. Two methods were employed for evaluation where the first method was carried out by plotting the known concentration against the predicted concentration. A satisfactory correlation coefficient (r**2**) value was obtained for each drug by the mentioned chemometric approaches (Table 2), and the second method was carried out by determining the Root mean standard error of calibration (RMSEC) and Root mean standard error of prediction (RMSEP) by the following expression:

$$SEC(SEP) = \sqrt{\frac{\sum_{i=1}^{n}\left(C_i^{Added} - C_i^{Found}\right)^2}{n-1}} \quad (7)$$

Here, I added represents the added concentration, found denotes the determined concentration and n is the total number of samples. The RMSECV, RMSEC, and RMSEP values obtained by optimizing the calibration matrix of absorption spectra for CLS, PCR, and PLS methods are shown in Table 3, indicating good accuracy and precision. The values of RMSEC, RMSECV, RMSEP, and PRESS were found to be minimum for the PLS method. Hence, it could be concluded that PLS is the most suitable method among developed chemometric methods. [41-45]



(A)



(B)

Fig 5: Plot of RMSECV vs. Number of components for calibration set prediction using cross-validation of (A) PLS model and (B) PCR model

Plots of actual Vs predicted values for LH and EM by (A) CLS (B) PLS and (C)PCR methods called Scored plots and plotting the concentration residuals against the predicted concentrations which were again used to carry out the residual plot (Fig. 7). All the residuals were distributed in between +2 to -2. Chemometric is the technique of separation of the necessary information from whole spectra at multiple wavelengths and removal or reduction of the noise.

3.3.4. Validation of Chemometric Methods

The analytical figure of merits (FOM) is very important to quantify the quality of a given methodology or for method comparison. In multivariate calibration, several FOM has been reported e.g. sensitivity (SEN), analytical sensitivity (γ**-1**), the limit of detection (LOD), and limit of quantitation (LOQ). Results of FOM are shown in Table 3. [41-45]

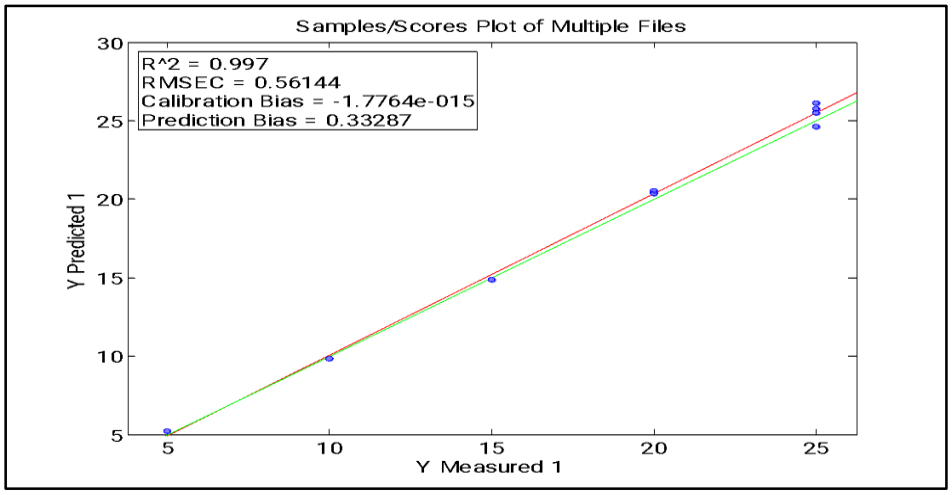
Table 3: Statistical parameters and figure of merits

Parameter	LH			EM		
	CLS	PCR	PLS*	CLS	PCR	PLS*
RMSEC	0.5614	0.5742	**0.5570**	0.4883	0.5424	**0.4761**
RMSECV	0.9174	0.8965	**0.8796**	0.7310	0.6455	**0.6445**
RMSEP	0.5581	0.5097	**0.5065**	0.4209	0.4152	**0.4032**
PRESS	0.3083	0.2575	**0.2547**	0.1730	0.1720	**0.1617**
r^2	0.9917	0.9916	**0.9911**	0.9956	0.9953	**0.9942**
Slope	1.0238	1.0182	1.0162	0.956	0.9634	0.9646
Noise ‖ ε ‖	0.287334	0.27805	0.27981	0.287334	0.27805	0.27981
Sensitivity (ml/µg)	0.9767	0.9821	0.9840	1.0460	1.0379	1.0366
Analytical sensitivity γ (µg/ml)	3.3993	3.5320	3.5168	3.6404	3.7329	3.7049
LOD (µg/ml)	0.9707	0.9342	0.9383	0.9064	0.8840	0.8906
LOQ (µg/ml)	2.9417	2.8312	2.8434	2.7469	2.6788	2.6990

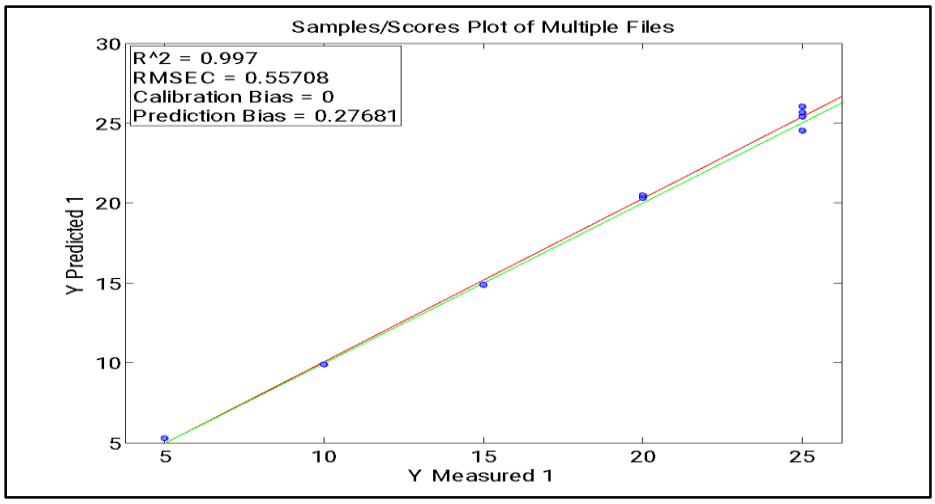
The figures in bold indicate that the PLS model was found to be the best model among all models.

3.4. Analysis of marketed formulation

The developed methods were applied for analyzing the LH and EM in marketed formulations and the study was repeated three times. The results obtained are shown in Table 4. The content of LH was found in the range of 98.7-100.5% and 99-99.4%. for EM. The results obtained were complying with the label claim.



(A)



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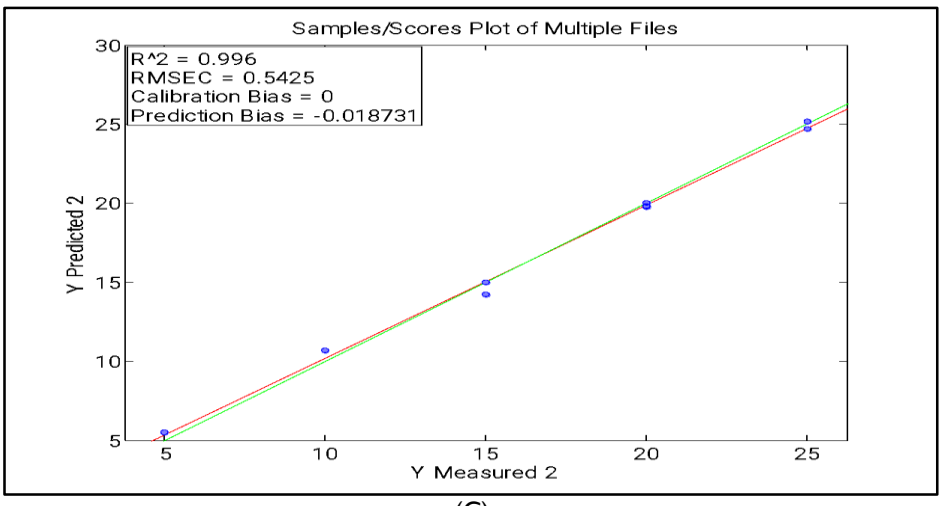
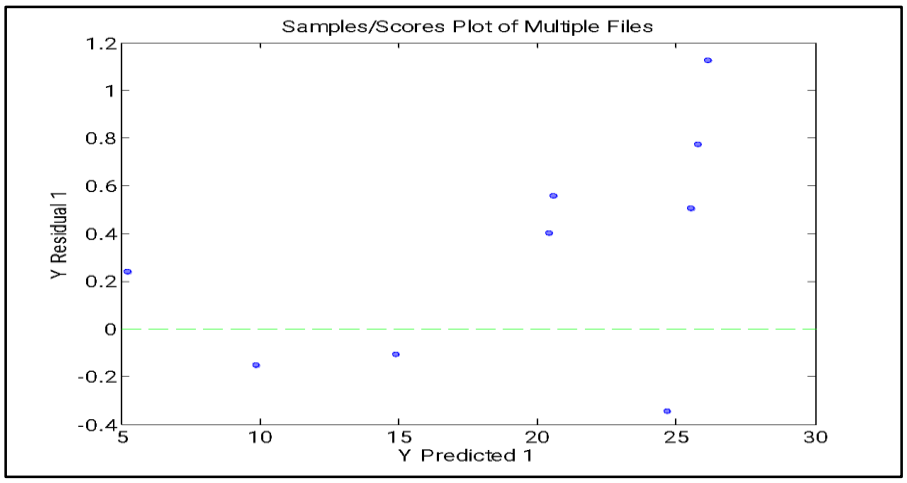


(C)

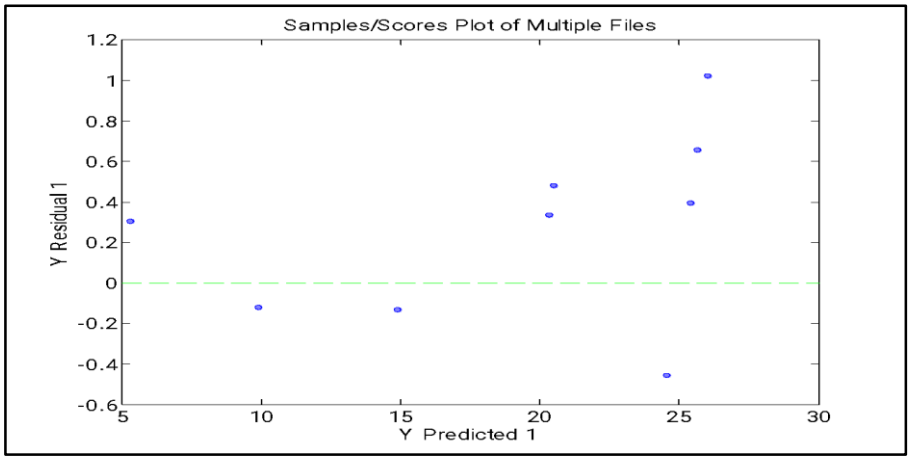
Fig 6: Plots of actual vs predicted values for LH and EM by (A) CLS (B) PLS (C)PCR

Table 4: Assay of marketed formulation by proposed methods

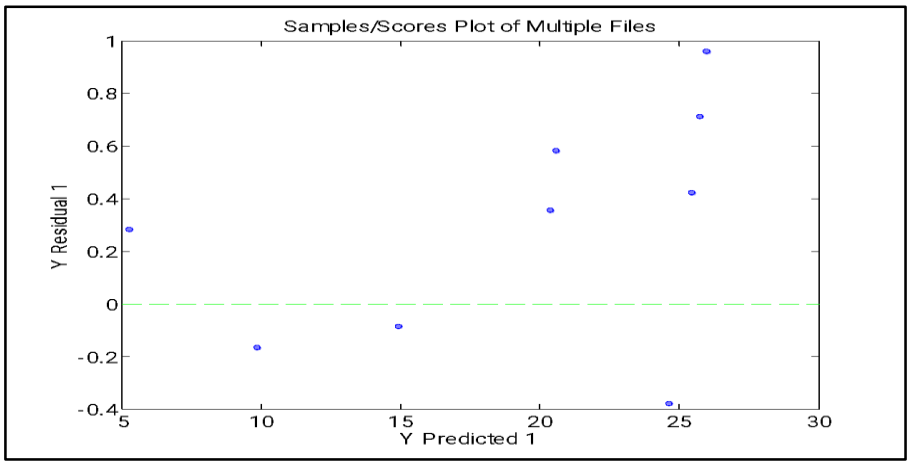
Formulation Name	Methods	LH			EM		
		Label Claim (mg)	Amount Found (mg)	Mean ± RSD (%)	Label Claim (mg)	Amount Found (mg)	Mean ± RSD (%)
ZANIPRESS (10/10 mg)	SEM	10	10.05	10.05 ± 1.34	10	9.90	9.90±0.47
	FDM	10	10.19	10.19±0.396	10	9.49	9.49±0.81
	CLS	10	9.89	9.89 ±1.16	10	9.94	9.94±0.54
	PCR	10	9.84	9.84±1.18	10	9.93	9.93±0.56
	PLS	10	9.87	9.87±1.16	10	9.92	9.92±0.50



(A)



(B)



(C)

Fig 7: Plot of Concentration Residual vs. Actual Concentration for LH and EM by (A)CLS, (B) PLS, (C) PCR methods

4. Statistical comparison of developed methods

Statistical comparison between developed chemometric methods by ANOVA to compare the differences between methods, the one-way ANOVA test was applied to five sets (f I've r replicates) obtained from the assay results for each tablet. In this procedure, Snedecor's F-values were computed and compared with the tabulated F value (p = 0.05). The same computation process was repeated for both drugs. In the standard table, the F-value was given as 2.86. The results of the ANOVA test were found to be 2.43 for LH and 0.880 for EM (Table 5). The calculated F-values did not exceed the tabulated F-value in the variance analysis. Hence, it was concluded that there is no significant difference between the newly developed methods. The numerical values of all the statistical parameters indicated that developed methods are suitable for the simultaneous determination of drugs.[41-45]

Table 5: ANOVA Results of LH and EM by Chemometric and UV Methods

Source of Variation		LH	EM
Sum of Squares	Between group	0.154	0.017
	Within group	0.318	0.095
	Total	0.473	0.112
Degrees of freedom	Between group	4	4
	Within group	20	20
	Total	24	24
Mean squares	Between group	0.038666	0.004204
	Within group	0.015906	0.004772
F-Test	F-calculated	2.430	0.880
	F-tabulated	2.866	2.866

5. CONCLUSION

Two UV spectrophotometric methods (SEM and FDM) and three novel chemometric methods were developed and validated successfully for the simultaneous estimation of LH and EM. The statistical comparison showed that there was no significant difference between developed methods. The developed methods can serve as good alternatives to the available chromatographic methods as evidenced by their LOD and LOQ values for the detection of LH and EM. Most of the available chromatographic methods were involving the use of a synthetic mixture of LH and EM whereas this method involved the determination of LH and EM from its tablet dosage form. The methods are beneficial in terms of ease of performing and reduced cost and time of analysis for routine quality control of synthetic mixture and commercial preparation containing these two drugs.

6. ACKNOWLEDGEMENT

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7. AUTHORS CONTRIBUTION STATEMENT

Dr. Samir Patel designed the whole study, Ms. Jamila Patel performed the necessary experiments in the laboratory and Dr. Archita Patel prepared and communicated the manuscript. All the authors have read and finalized the manuscript.

8. CONFLICT OF INTEREST

Conflict of interest declared none.

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