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INVITED ARTICLE

CHEMOMETRIC ASSISTED SPECTROPHOTOMETRIC AND HPLC METHODS FOR THE ESTIMATION OF AMLODIPINE BESYLATE AND TELMISARTAN MARKETED FORMULATION

Khushboo Khatri, Prachi R. Bhamre and S. J. Rajput*

Quality Assurance Laboratory, Centre of Relevance and Excellence in Novel Drug Delivery System, Pharmacy Department, G. H. Patel Building, Donor's Plaza, The Maharaja Sayajirao University of Baroda, Fatehgunj, Vadodara – 390 002, Gujarat, India.

Abstract

Chemometric assisted spectrophotometry and HPLC methods have been developed for the simultaneous determination of Amlodipine besylate and Telmisartan. The two chemometric methods i.e. Inverse least square (ILS) and Classical least square (CLS) methods were successfully applied to quantify each drug in the mixture using the information included in the UV absorption spectra of appropriate solutions in the range of 230-302 nm with the intervals of 4 nm at 19 wavelengths. The HPLC method was developed and the three methods (ILS, CLS and HPLC) were successfully applied to marketed formulation and the results were compared statistically which showed no significant difference among the results.

Keywords: *HPLC, chemometrics, spectrophotometric, amlodipine besylate, telmisartan, CLS, ILS*

***Correspondent Author:** Email: sjrajput@gmail.com, Phone: +91-9998309662*

Introduction

Amlodipine besylate (AML) is chemically described as (3-Ethyl 5-methyl (4RS)-2-[(2-aminoethoxy) methyl] - 4 - (2-chlorophenyl) - 6 - methyl - 1, 4 - dihydropyridine - 3, 5 - dicarboxylate benzene sulfonate). AML is a calcium-channel blocking agent; a dihydropyridine derivative with an intrinsically long duration of action and can be given once daily.AML is an antihypertensive and used as a prophylaxis for angina.

Telmisartan (TEL) is an angiotensin II receptor (type AT1) antagonist used in the management of hypertension. TML prevents the constriction (narrowing) of blood vessels (veins and arteries). It is a non-peptide molecule and chemically described as 4'-[(1,7'-Dimethyl-2'-propyl-1H,3'H -2,5'-bibenzimidazol-3'-yl) methyl]-2-biphenyl carboxylic acid. The combination of AML and TEL has been reported to show substantial and sustained 24 hour blood pressure reduction and is well-tolerated in a range of patients with hypertension and at risk of cardiovascular events. AML and TEL are available in combined tablet dosage form for the treatment of hypertension.

Literature review reveals several analytical methods for the estimation of AML and TEL alone or in combination with other drugs. Several spectrophotometric methods [1-3], HPTLC method [4] chromatographic methods [5-15], stability indicating analytical methods [16, 17] and methods for determination of AML and TEL in human plasma [18-20] have been reported.

The UV spectra of AML and TEL are highly overlapping and hence very few spectrophotometric methods are available in the literature for their simultaneous estimation. In the present study the chemometric approach has been applied to resolve the spectra and enable the simultaneous determination of AML and TEL in the marketed formulation alongwith the HPLC method. The results obtained by chemometric methods have been statistically compared with the HPLC method to confirm the results.

Materials and Methods

Instrumentation

Chemometric spectrophotometry

A double-beam Shimadzu UV-1700 spectrophotometer (Kyoto, Japan) connected to a computer loaded with Shimadzu UV Probe 2.10 software was used for all the spectrophotometric measurements. The absorbance spectra of the reference and test solutions were carried out in 1cm quartz cells over the range of 200-400 nm. The numerical calculations for ILS and CLS methods were performed by using MATLAB R2007a Software and Excel.

HPLC

Chromatography was performed on Shimadzu (Shimadzu Corporation, Kyoto, Japan) chromatographic system equipped with Shimadzu LC-20AT pump and Shimadzu SPD-20AV absorbance detector. Samples were injected through a Rheodyne 7725 injector valve with fixed loop at 20µL. Separation and quantitation were made on a reversed-phase, Hypersil BDS

C_{18} column (250×4.6 mm i.d, 5 µm particle size). Detector was set at 238nm. Data acquisition and integration was performed using Spinchrome software (Spincho Biotech, Vadodara).

Materials and Reagents

AML and TEL (Bulk drugs) were kindly gifted by Cadila pharmaceutical limited, Ahmedabad. HPLC grade methanol (Qualigens fine chemicals, Mumbai) was used for HPLC and AR grade methanol (Qualigens fine chemicals, Mumbai) was used for spectrophotometry. Potassium dihydrogen orthophosphate KH_2PO_4 (AR grade: Qualigens fine chemicals, Mumbai), ortho phosphoric acid (AR grade: Qualigens fine chemicals, Mumbai), acetonitrile (HPLC grade, Qualigens fine chemicals, Mumbai) were used for HPLC. Double distilled water filtered through nylon filter paper 0.2 µm pore size and 47 mm diameter (Pall Lifesciences, Mumbai, India), was used throughout the analysis. The marketed formulation of Telma-AM tablet (Glenmark Pharmaceuticals Ltd.) containing 5mg AML and 40mg TEL per tablet was taken for the study.

Experimental conditions
Chemometric spectrophotometry

The UV absorption spectra of appropriate solutions were recorded in the wavelength range 200-400nm with the intervals of 4nm ($\Delta\lambda$ = 4nm) at 19 wavelength points. The scanning range selected was 230-302nm.

HPLC

The mobile phase was prepared by mixing acetonitrile and 0.01M phosphate buffer. The phosphate buffer was prepared by dissolving 0.27 g of potassium dihydrogen phosphate in sufficient double distilled water to produce 100 mL, 0.4 ml of triethylamine (TEA) was added and the pH was adjusted to 2.5 with orthophosphoric acid and filtered through 0.2 µm nylon filter paper. Acetonitrile and phosphate buffer were mixed in a ratio of 45:55% v/v and degassed by sonicating for 5 min in ultrasonic bath. The flow rate was maintained at 1.0 mL/min. All determinations were performed at ambient temperature. Quantitation based on peak area was achieved with UV detection at 238nm. The injection volume was 20 µL.

Standard solutions and calibrations

AML stock solution (1000 ppm) was prepared by dissolving 13.9mg of Amlodipine Besylate (equivalent to 10 mg of AML) in 10 mL methanol. TEL stock solution (1000 ppm) was prepared by dissolving 10 mg of TEL in 10 mL methanol. The stock solutions were ultrasonicated for 1 min. The working solutions of AML and TEL (100 ppm) were prepared by transferring 1 mL aliquot from stock solution to a 10 mL volumetric flask and making up the volume with mobile phase in case of HPLC and making up the volume with methanol in case of chemometric spectrophotometry.

Preparation of solution mixture sets for chemometric estimation

A calibration set of 24 mixtures was prepared in methanol, applying a multilevel multifactor design in which two levels of concentrations of AML and TEL

Table 1: Calibration Set and Validation Set

Calibration Set			Validation Set		
Sr. No.	AML	TEL	Sr. No.	AML	TEL
1	2	16	1	5	2
2	4	16	2	5	4
3	6	16	3	5	6
4	8	16	4	5	8
5	7	2	5	2	14
6	7	4	6	4	14
7	7	6	7	6	14
8	7	8	8	8	14
9	2	0	9	30	4
10	4	8	10	30	6
11	0	16	11	30	8
12	0	2	12	30	10
13	25	4	13	15	5
14	25	6	14	25	5
15	25	8	15	35	5
16	25	10	16	45	5
17	25	12	17	1	8
18	0	7			
19	10	7			
20	20	7			
21	30	7			
22	40	7			
23	50	7			
24	60	0			

Concentrations in µg/mL

within the linearity range were introduced as shown in Table 1. A validation set containing 17 synthetic mixture solutions was prepared as shown in Table 1. The linearity range of AML was found to be 1-60 µg/mL and of TEL was found to be 1-18 µg/mL. UV spectra were recorded in the wavelength range 200-400 nm versus solvent blank and digitalized absorbance was recorded at 4 nm intervals.

Preparation of calibration curve for HPLC

Aliquots ranging from 0.1 mL to 1 mL of AML and 0.8 mL to 8 mL of TEL were

taken, from working stock solution, in 10 mL volumetric flask and diluted upto the mark with the mobile phase to obtain the final concentration of binary mixtures as marketed available formulation. Triplicate 20 µL injections were made for each concentration and chromatogram was obtained under the specified chromatographic conditions described previously. The calibration graph was constructed by plotting peak area versus concentration of each drug and the regression equation was calculated.

Sample preparation

Twenty tablets were weighed and finely powdered. The powder equivalent to one tablet (5 mg AML and 40 mg TEL) was accurately weighed and transferred to volumetric flask of 100 mL containing 50 mL of the methanol and sonicated for 15 min at cool temperature. The above solution was carefully filtered through whatman filter paper (No. 41) to 100mL volumetric flask and residue were washed thrice with methanol , the combined filtrate was made up to the mark with methanol.

For chemometric spectrophotometry, an aliquot of 0.2 mL was pipette out from above prepared solution and transferred to volumetric flask of 10 mL. Volume was made up to the mark with methanol to give a solution containing 1: 8 µg/mL of AML:TEL. This solution was used for the estimation of AML and TEL.

For HPLC, an aliquot of 1 mL was pipette out from above prepared solution and transferred to volumetric flask of 10 mL. Volume was made up to the mark with mobile phase to give a solution containing 5: 40 µg/mL of AML:TEL. This solution was used for the estimation of AML and TEL.

Results and Discussion

Chemometric spectrophotometry

Figure 1 shows the overlain zero-order absorption spectra of AML, TEL and their binary mixture solution in methanol in the 200-400nm absorption region.

The ratio of AML and TEL in commercial tablet is 1:8. The zero-order absorption spectra (Figure 1) of AML and TEL completely overlap with each other making it difficult to use conventional spectrophotometric techniques for simultaneous analysis. Thus the ILS and CLS chemometric spectrophotometric methods were found to be appropriate for determination of AML and TEL in binary mixture. A training set (calibration set) of 24 synthetic binary mixture solutions (Table 1) and a validation set containing 17 synthetic binary mixture solutions (Table 1) in the possible combinations were prepared with methanol (AR grade). The UV absorption spectra of appropriate solutions were recorded in scanning range of 200-400nm and the wavelength range selected was 230-302nm with the intervals of 4nm ($\Delta\lambda$ = 4nm) at 19 wavelength points.

CLS method

CLS is one of the simplest methods, based on a linear relationship between the absorbance and the component

concentrations at each wavelength. In matrix notation, the Beer's law models for m calibration standards containing l chemical components with spectra of n digitised absorbance is given by

$A = C \times K + EA$ ..... *(Eq. 1)*

where A is the m x n matrix of calibration spectra, C is the m x l matrix of component concentrations, K is the l x n matrix of absorbance-concentration proportionality constants (absorptivity-pathlength) and EA is the in m x n matrix of spectral errors or residuals not fit by the model.

In this method, the coefficient matrix (K) was calculated by using the linear equation system between the absorbance data and training set. The observed absorbance values of the compounds in the binary mixture solutions, at the 19 wavelength points with the interval of 4nm ($\Delta\lambda$ = 4nm) in the spectral region from 230nm to 302nm, were replaced in the equation 1 and the amount of AML and TEL in the synthetic mixtures (validation set) were calculated.

ILS method

In this method, the coefficient matrix (P) was obtained from the linear equation system using the absorbance data and the calibration set. Introducing (P) into the linear equation system we obtain the calibration for ILS, as this method treats concentration as a function of absorbance. The inverse of Beer's law model for m calibration standards with spectra of n digitised absorbance is given by

$C = A \times P + Ec$ ..... *(Eq. 2)*

Where C and A are as before, P is the n x l matrix of unknown calibration co-efficient relating the l component concentrations of the spectral intensities and Ec is the m x l vector of errors. Since in ILS the number of wavelengths cannot exceed the total number of calibration mixtures, stepwise multiple linear regressions have been used for the selection of wavelengths. The observed absorbance values of the compounds in the binary mixture solutions, at the 19 wavelength points in the spectral region from 230nm to 302nm, were replaced in the equation 2 and the amount of AML and TEL in the synthetic mixtures (validation set) were calculated.

Validation of chemometric methods

The tested mixtures for ILS and CLS were subjected to recovery studies. The results obtained were in good agreement with the true values. These results of percentage recoveries and standard deviation are shown in (Table 2) which determines the accuracy of the chemometric methods. The numerical values of the statistical parameters (Table 3), such as plots of predicted versus true value (their regression coefficient), RMSEP (Root mean square error of prediction) value indicate that the proposed techniques are suitable for the determination of these drugs in the tablet formulation as excipients do not interfere. Moreover the plots of residual value versus predicted concentration show that the residual values for the model are close to zero and more randomly distributed (Figure 2). The ILS and CLS models were then applied to estimate AML and TEL in commercial

tablet preparations. The results are shown in (Table 4).

Selection and optimization of chromatographic condition

Optimization of chromatographic procedure

Different solvents in mixtures of varied proportions, at different flow rate and wavelength were tried to select the mobile phase. The mobile phase with water:acetonitrile 50:50 gave broad peaks. Hence trials were carried out with different pH of phosphate buffer and acetonitrile at pH 2.5, 4.5 and 6. Among these trials the most appropriate peak shape and resolution alongwith appropriate system suitability parameters (according to ICH guidelines) were obtained with pH 2.5 with a ratio of phosphate buffer:acetonitrile 55:45 with 0.4% TEA (Table 5). TEA was added to minimise the tailing of the peaks. The optimized chromatogram of the laboratory sample solution has been shown in Figure 3.

Selection of common wavelength

The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. An ideal wavelength is the one that gives good response for the drugs that are to be detected. In the present study individual drug solutions of 1µg/mL of AML and 8µg/mL TEL were prepared in methanol. As the absorbance and concentration of AML is less than TEL its absorbance maxima 238nm (Figure 1) was selected as the common wavelength for simultaneous estimation of the two drugs.

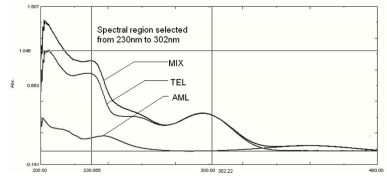

Figure 1: The overlain zero-order absorption spectra of AML(1 ppm), TEL(8 ppm) and their binary mixture(AML:TEL 1:8 ppm) in methanol

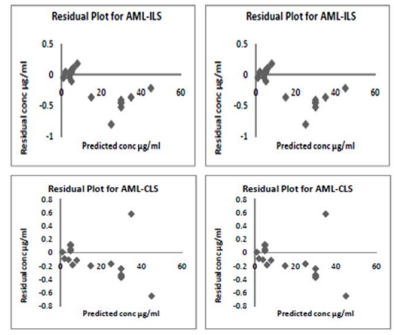

Figure 2: Concentration residuals vs actual concentration plot for AML and TEL for CLS and ILS

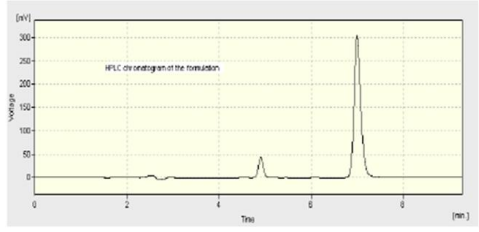

Figure 3: HPLC chromatogram of the formulation

Validation of HPLC method

Validation of the HPLC method was carried out according to ICH guideline [21].

Linearity and Range

The linearity study was carried out for both drugs at ten different concentration levels. The linearity of AML and TEL were in the

range of 1-10 µg/mL and 8-80 µg/mL respectively.

Precision

Precision was estimated by the determination of the repeatability of the method. Binary mixtures containing three different concentrations were prepared with mobile phase. 20 µL of the standard solutions were injected and chromatograms were recorded. The peak area of AML and TEL were calculated for each trial. The experiment was repeated three times in a day (intra-day precision) and repeated on three different days (inter-day precision). The average % RSD (relative standard deviation) values of the results were calculated which were found to be less than 2%, which confirms that the method is precise.

Accuracy

Accuracy of the method was confirmed by recovery study from marketed formulation at three level of standard addition from 80 % to 120 % of label claim. The results are shown in Table 4. Recovery greater than 98 % with low SD (standard deviation) justifies the accuracy of the method.

Table 2: Percentage recovery results of AML and TEL in synthetic mixtures (validation set) by the proposed ILS and CLS chemometric methods

Sr. No.	Mixture Composition (µg/mL)		%Recovery			
			ILS		CLS	
	AML	TEL	AML	TEL	AML	TEL
1	5	2	102.08	98.11	97.54	97..62
2	5	4	98.46	99.06	97.8	99.77
3	5	6	98.96	100.55	98.97	99.71
4	5	8	99.15	98.78	99.388	98.28
5	2	14	97.68	96.88	104.37	99.4
6	4	14	101	98.23	102.64	98.59
7	6	14	98.16	99.04	103.025	98..08
8	8	14	97.75	98.8	101.4	98.56
9	30	4	101.75	101.38	101.22	101.77
10	30	6	101.36	101.73	100.79	100.56
11	30	8	101.52	102.19	101.1	102.44
12	30	10	101.5	101.69	101.17	102.62
13	15	5	102.43	101.65	101.29	102.31
14	25	5	101.22	101.99	100.66	102.1
15	35	5	101.04	102.99	98.33	102.38
16	45	5	100.48	97.12	101.43	101.76
17	1	8	104.79	98.27	99	97.31
	AVG		100.5488	99.90941	100.5955	100.504
	SD		1.925714	1.949287	1.872517	1.807041
	%RSD		1.915203	1.951054	1.861433	1.797979

Table 3: Statistical parameters of AML and TEL in synthetic mixtures (validation set) using the proposed ILS and CLS chemometric methods

Statistical Parameter	AML		TEL	
	CLS	ILS	CLS	ILS
Range (µg/mL)	2-60		1-18	
Regression equation*	y =1.007x - 0.013	y=1.014x - 0.043	y=0.979x + 0.147	y=0.975x + 0.164
Regression Coefficient	0.999	0.999	0.998	0.998
RMSEP	0.176	0.344	0.155	0.176

*Regression equation of predicted Vs true concentration

Table 4: Summary of the validation parameters.

Parameters	AML	TEL
Wavelength (nm)	238nm	
Linearity range (µg/mL)	1-10	8-80
Accuracy*	100.45 ± 0.69	100.72 ± 1.33
Precision(%RSD)		
Intraday	0.60	0.40
Interday	0.71	0.40
Regression Equation: y=mx+c	y = 23.8663x + 64.5460	y = 57.6180x - 3.5013
Correction coefficient	0.9990	0.9998
LOD (µg/mL)	0.210	0.041
LOQ(µg/mL)	0.639	0.125

*Average of 80, 100 and 120% standard addition

Table 5: Results of system suitability parameters

Parameters	AML	TEL
Theoretical Plates ± RSD	9080.9±1.81	9666.3 ± 1.76
Asymmetry Factor± RSD	1.155± 0.162	1.189± 0.345
Resolution ± RSD	--	9.05± 0.458

Table 6: Assay results of the commercial tablet preparation (Telma-AM) by the proposed ILS, CLS chemometric methods and HPLC method

Formulation	TELMA-AM (Glenmark)					
Drug	**Amlodipine**			**Telmisartan**		
Labeled claim	5(mg/tab)			40(mg/tab)		
Methods	HPLC	CLS	ILS	HPLC	CLS	ILS
Amount Found (mg)	5.007	5.09	5.06	40.032	39.65	39.8
% Amount Found	100.14 ± 1.05	101.8 ± 1.74	101.13 ± 2.04	100.08 ± 1.37	99.13 ± 1.96	99.50 ± 1.80
P-value		0.615			0.528	
F-value		0.527			0.712	

Average of 5 determinations for HPLC
Average of 3 determinations for CLS and ILS
F-crit value 5.14

Limit of detection (LOD) and Limit of quantitation (LOQ)

Calibration curve was repeated 5 times and the SD of the intercepts was calculated. Then LOD and LOQ were measured as follows

$$LOD = \frac{3.3 \times S.D.}{Slope\ of\ calibration\ curve} \quad (Eq.\ 3)$$

$$LOQ = \frac{10 \times S.D.}{Slope\ of\ calibration\ curve} \quad (Eq.\ 4)$$

SD = Standard deviation of intercepts

The theoretical values were assessed practically. The detection limits were found to be 0.21 and 0.041 µg/mL for AML and TEL, respectively. The quantitation limits were found to be 0.639 and 0.125 µg/mL for AML and TEL, respectively. (Table 4)

Robustness

Robustness of the method was examined by changing factors at three levels (-1, 0, 1) such as: flow rate (0.9, 1.0, 1.1 mL/min), mobile phase ratio (acetonitrile composition: 44, 45, 46%) and pH of the buffer (2.4, 2.5, 2.6). One factor at the time was changed to estimate the effect. Thus replicate injections (n=3) of standard solution at three concentration levels were performed under small changes of three chromatographic parameters (factors). Results indicate that the selected factors remained unaffected by small variation of these parameters. It was also found that acetonitrile of different lots from the same manufacture had no significant influence on the method. Insignificant difference in asymmetric factor and less variability in retention time were observed.

Stability in sample solutions

Binary mixtures containing two different concentrations of AML and TEL were prepared from standard stock solution and stored at room temperature for 24hrs. They were then injected in to LC system. There was no change in signal intensity and no additional peak found in chromatogram indicating the stability of AML and TEL in the sample solution.

System suitability

The system suitability parameters including resolution (Rs), asymmetric factor (As) and theoretical plates were established by 10 replicates of binary mixture of AML and TEL (1:8 μg/mL, respectively).

Analysis of commercial tablet preparation

The proposed multivariate ILS and CLS spectrophotometric methods and HPLC method were applied to the simultaneous determination of AML and TEL in commercial Telma-AM tablet preparation. Satisfactory results were obtained for each compound which were in good agreement with the label claim. The assay results of the proposed ILS and CLS spectrophotometric methods were compared to those of the proposed HPLC method. The results obtained were statistically analyzed by one way ANOVA at 95% confidence level. The results are shown in Table 6. The calculated values did not exceed the theoretical ones; indicating that there was no significant difference between the methods compared.

Conclusion

RP-HPLC techniques are generally used for separation and determination of components in final pharmaceutical preparations and are superior with regard to identification and specificity. However, the chemometric methods are less expensive by comparison and do not require sophisticated instrumentation nor any prior separation step. The proposed chemometric-assisted spectrophotometric methods are applicable, prompt and specific for the simultaneous determination of AML and TEL in their synthetic mixtures and commercial pharmaceutical tablets. The results obtained were compared with the proposed RP-HPLC method and good coincidence in the means of recovery was observed as there was no significant difference between the methods compared. The three proposed methods were accurate, precise with good reproducibility and sensitivity; hence can be used for the routine analysis of AML and TEL in their combined pharmaceutical formulations.

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