Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 1, 2014

Research Article

SIMULTANEOUS DETERMINATION OF AMLODIPINE BESYLATE AND ATORVASTATIN CALCIUM BY USING SPECTROPHOTOMETRIC METHOD WITH MULTIVARIATE CALIBRATION AND HPLC METHOD IMPLEMENTING "DESIGN OF EXPERIMENT"

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Received: 13 Oct 2013, Revised and Accepted: 05 Nov 2013

ABSTRACT

Objectives: The aim of the present work is to develop a rapid and simple method for the simultaneous determination of amlodipine besylate and atorvastatin calcium mixture by two different methods. First, Multivariate calibration methods using preprocessing to enhance results in case of presence of any interference in samples. Second, RP-HPLC method for achieving a good separation of the mixture with accepted system suitability parameters with using design of experiment in the robustness study according to Plackett-Burman design.

Methods: The method is based on the spectrophotometric measurements of the drugs in the range of 200-400 nm together with multivariate calibration methods. Resolution of the binary mixture under investigation has been accomplished mainly by using partial least squares (PLS) and principal component regression (PCR). The proposed RP-HPLC method utilizes a YMC-pack pro C18 ODS-A (25 cm x 4.6 mm, 5 µm) column, at room temperature, optimum mobile phase consisted of methanol and 0.01 M sodium dihydrogen phosphate buffer (75:25, v/v), pH adjusted to 3.5 with orthophosphoric acid solution. The flow rate was monitored at 1.2 ml/min, and UV detection at 239 nm.

Results: The recovery percentage for amlodipine besylate and atorvastatin calcium in tablets dosage form were found to be in PLS method (98.98 ± 0.85, 99.68 ± 1.35), PCR method (99.16 ± 0.75, 99.60 ± 1.40) and RP-HPLC method (98.82 ± 0.62, 101.19 ± 0.69), respectively.

Conclusion: The methods were validated as per ICH guidelines. All the results obtained were found to be within the acceptable limits. The methods were successful to estimate amlodipine besylate and atorvastatin calcium in bulk powder and pharmaceutical preparation Caduet®.

Keywords: Amlodipine besylate, Atorvastatin calcium, PCR, PLS, RP-HPLC, Design of experiment.

INTRODUCTION

Amlodipine besylate (AM) [3-Ethyl 5-methyl (4RS)-2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)- 6-methyl-1,4-dihydropyridine-3,5-dicarboxylate benzenesulphonate] [1]. Amlodipine besylate is a calcium channel blocker that inhibits the trans membrane influx of calcium ions into vascular smooth muscle and cardiac muscle [2]. The chemical structure of amlodipine besylate is shown in (Figure 1).

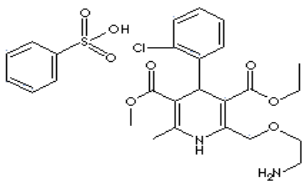


Fig. 1: Chemical structure of amlodipine besylate (AM)

Atorvastatin calcium (AT) [[R-(R*,R*)]-2-(4-fluorophenyl)-β,δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino)carbonyl]-1Hpyrrole-1-heptanoic acid, calci- um salt (2:1) trihydrate] [3]. Atorvastatin calcium is an inhibitor of 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-Co A) reductase. This enzyme catalyses the conversion of HMG-Co A to mevalonate, an early and rate limiting step in cholesterol biosynthesis [4,5]. The combination dosage form of amlodipine besylate and atorvastatin calcium are available in the market for the treatment of hypertension, Coronary artery disease (CAD), hyperlipidemia and the prevention of cardiovascular disease. The chemical structure of atorvastatin calcium is shown in (Figure 2).

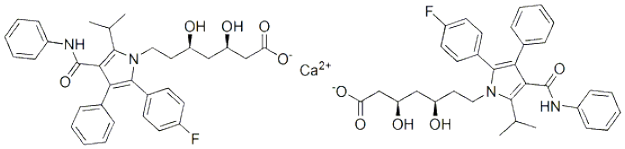


Fig. 2: Chemical structure of atorvastatin calcium (AT)

According to the international chemometrics society (ICS), chemometrics is defined as the science of relating measurements made on a chemical system or process to the state of the system via application of mathematical or statistical methods [6]. In this study two techniques of chemometrics were used, the first is the multivariate calibration in spectrophotometry where the absorption spectra of AM and AT have a severe overlap that could not be resolved by the simple univariate methods as shown in (Figure 3). And the second is the design of experiment in RP-HPLC method.

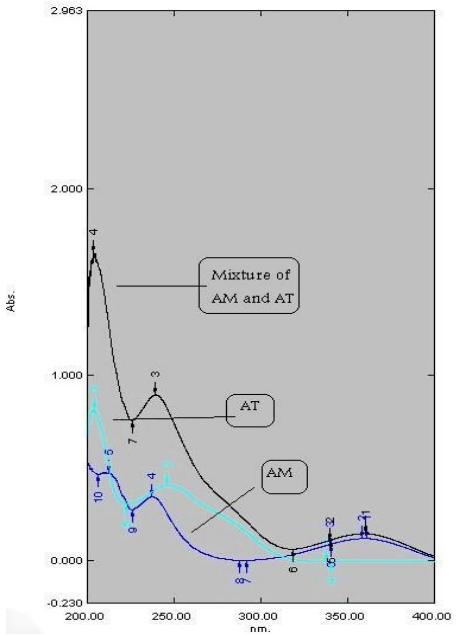


Fig. 3: Absorption spectrum of AM and AT showing the overlapping

Quantitative determination using spectrophotometric methods has been greatly improved by the use of multivariate methods. The methods used were principal component regression (PCR) and partial least squares regression (PLS), both are the most popular chemometric tools used for quantitative modeling of the multidimensional spectroscopic data [7]. The method involves calibration; which is the relation between spectra and component concentrations. The validation set and the unknown set can be predicted from a set of reference samples.

Design of experiments (DOE) remains a core area of study in chemometrics [8,9] and it is a well established statistical method. There are levels of design which can be applied: these range from the simplest fractional factorial (which includes experiments to identify which factors are most critical), followed by full factorial (which enables identification of significant interactions between factors), and the more complex surface area design (which facilitates optimization of factors). Accordingly, the used type is the simplest which is two-level Plackett-Burman design [10] which is a class from a fractional factorial design to identify the most critical factors to examine the method robustness. The design allows examining N-1 factors in N experiments, which will lead to save time and costs. And also factorial principles were used in designing the calibration and validation sets in multivariate calibration. Several analytical methods of AM and AT quantification in pharmaceuticals have been developed. The most recent methods include UV-VIS spectrophotometry [11-17] and HPLC [18-25].The proposed methods differ from the already published methods by using the multivariate calibration in spectrophotometric method and DoE in the chromatographic method and compare between them.

MATERIALS AND METHODS

Chemicals and reagents

AM and AT were supplied by E.I.P.I.Co. (Egyptian International Pharmaceutical Industries Company). Methanol was supplied from (Sigma-Aldrich, Germany) and was of HPLC grade, while sodium dihydrogen phosphate and orthophosphoric acid were supplied from (Adwic, Egypt). Caduet® tablets (B.N. 1172110) was manufactured by Pfizer. It is labeled to contain 10 mg of both AM and AT and was purchased from the local market.

Instruments

A double beam UV-Visible spectrophotometer (SHIMADZU, Kyoto, Japan) model UV-1601 PC. The bundled software was UV-PC personal spectroscopy software version 2.10 (shimadzu). The spectral bandwidth was 0.1 nm and wavelength-scanning speed 1800 nm/min. The absorption spectra of the standard are recorded in 1 cm quartz cells over the range of 200-400 nm at room temperature. Data handling was done using PLS toolbox, Solo version 7.0.3.

An HPLC system equipped with an isocratic pump UV detector Agilent 1100. Chromatographic signals were acquired processed by Agilent LC chemstation software 1100. YMC- pack pro C18 ODSA (25 cm x 4.6 mm, 5 µm) column was used for separation.

Preparation of standard solutions

Stock standard AM and AT solution were prepared by accurately weighed 10 mg of either AM and AT in 100 mL volumetric flask volume, volume was completed to 100 mL using methanol to have a final concentration of 100 µg/mL.

Standard solution for multivariate calibration

Calibration and validation sets for two component systems were designed according to factorial principles. Solutions containing drug concentrations in the range 5-15 µg/mL for AM and AT were produced by dilution of the working standard solutions (100 µg/mL). A five level factorial design was used to produce a full set of 25 samples as shown in (Table 1). One third of the samples (Eight samples) were randomly chosen and used for external validation (validation set) and the rest of the samples were used for construction of the regression model (calibration set).

Chromatographic conditions for RP-HPLC

Different mobile phase proportions were tried for obtaining optimum resolution of the analytes. The mobile phase that was found to obtain the best results was consisted of methanol: 0.01M phosphate buffer at pH=3.5; (75:25, v/v).The phosphate buffer was prepared by adding 10 mL of triethylamine to the prepared buffer (0.01M). The pH adjusted using orthophosphoric acid. The mobile phase was prepared, filtered through 0.45µm membrane filter and degassed before use then delivered at flow rate 1.2 mL/min. The detection wavelength was done at 239nm.

Application to pharmaceutical preparation Caduet®

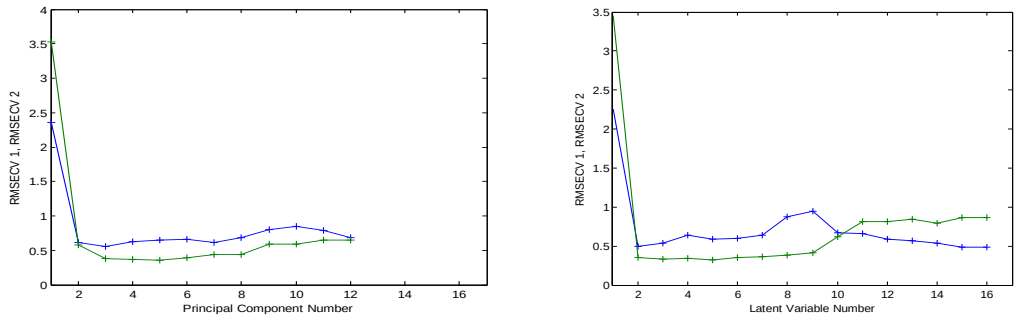
The method was applied on Caduet® tablets which are present in the market labeled to contain 10 mg for both AM and AT per tablet.

Twenty tablets were weighed and finely grinded, an amount of the powder equivalent to 10 mg of AM and AT was placed in a 100 mL volumetric flask and dissolved in 50 mL of 50% v/v methanol: water and the solution was sonicated for 10 minutes. The volume was completed with the same solvent and the solution was then filtered through a filter paper to a concentration of 100µg/mL then centrifuged for 10 minutes. An aliquot of (1mL) was taken and diluted to a 10 mL volumetric flask with methanol to have a final concentration of 10µg/mL.

RESULTS AND DISCUSSION

Multivariate calibration

In PCR and PLS methods, a data were mean centered as a preprocessing step and leave one out was applied as a cross validation [26] method. To select the optimum number of PCs and LVs, F statistics [27] was used in which the root mean square error of cross validation (RMSECV) values were compared and the selected model was that with the smallest number of factors such that RMSECV for that model was not significantly greater than RMSECV from the model with an additional factor (LV). If we decided to retain more factors than we should, we would be retaining some factors that can only add more noise to our data. On the other hand, if we don't keep enough factors, we will be discarding potentially meaningful information that could be necessary for a successful calibration. It was found that the optimum number of PCs and LVs were two as shown in (Figure 3).



(a) **(b)**

Fig. 3: Plot of RMSECV versus (a) the number of principal components using PCR model and (b) the number of Latent variable using PLS model.

To validate the suggested models, several diagnostic tools were used for predicting a mixture containing different ratios of both drugs. The predicted validation set where shown with their recoveries, standard deviation and relative standard deviations values using PLS and PCR models and summarized in (Table 2).

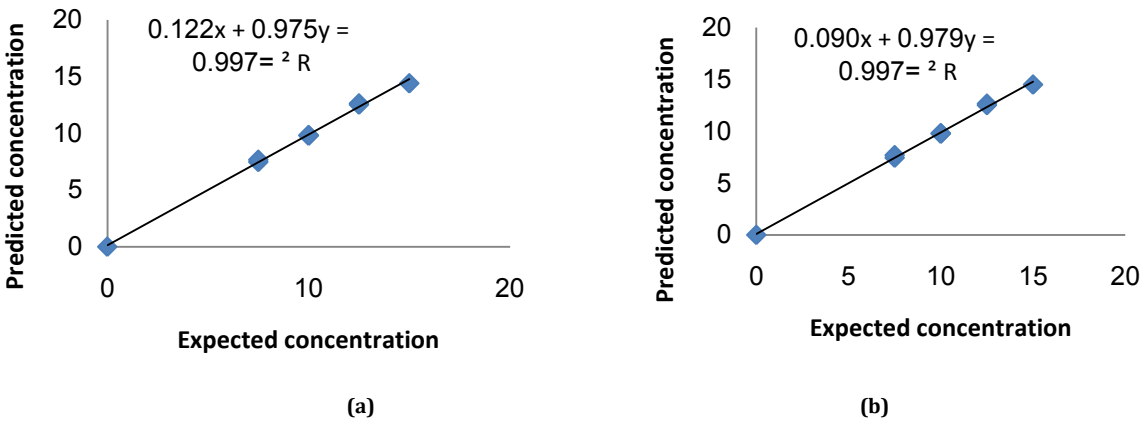
In order to assess the predictive ability of the developed models, each model was applied for determination of AM and AT in the validation set. (Figures 4 and 5) shows the expected concentration versus the predicted concentrations for each compound. Root mean squared error of cross-validation (RMSECV), of calibration (RMSEC) and prediction (RMSEP), together with the statistical parameters of the linear relationship between the predicted and the expected concentration of AM and AT in the validation set are represented in (Table 3).

Table 1: The five level two factor experimental design of the calibration and validation set mixtures.

Sample No.	AM (µg/ml)	AT (µg/ml)
1	10	10
2	5	10
3	5	5
4	15	5
5	7.5	15
6	15	7.5
7	10	15
8	7.5	10
9	7.5	7.5
10	12.5	7.5
11	15	12.5
12	12.5	15
13	10	12.5
14	15	10
15	15	15
16	5	15
17	12.5	5
18	5	12.5
19	10	15
20	12.5	10
21	12.5	12.5
22	7.5	12.5
23	5	7.5
24	7.5	5
25	10	7.5

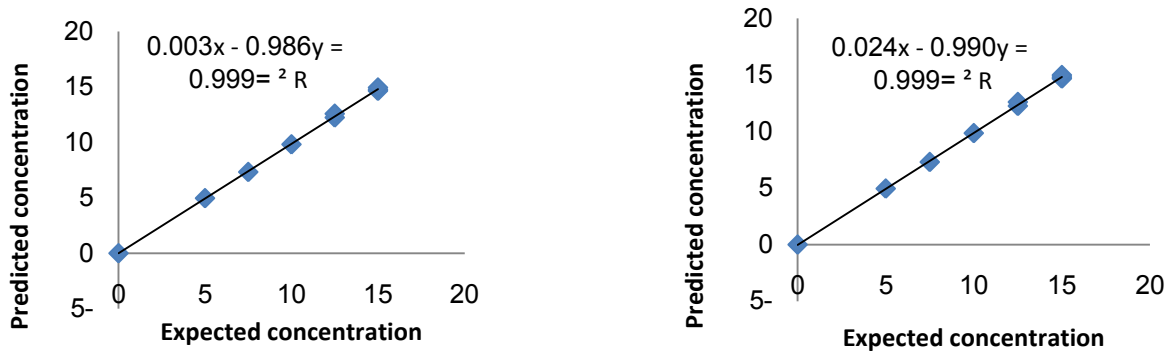
Table 2: Percent recoveries of AM and AT in validation set using PCR and PLS regression models

No.	Added µg/mL		Found µg/mL				Recovery %			
			PCR		PLS		PCR		PLS	
	AM	AT	AM	AT	AM	AT	AM	AT	AM	AT
1	10	10	9.78	9.83	9.76	9.85	97.80	98.30	97.60	98.50
2	10	15	9.83	15.02	9.80	15.10	98.30	100.13	98.00	100.66
3	12.5	7.5	12.62	7.32	12.64	7.30	100.96	97.60	101.12	97.33
4	15	15	14.51	14.73	14.48	14.80	96.73	98.20	96.53	98.66
5	10	15	9.80	14.93	9.76	14.98	98.00	99.53	97.60	99.86
6	12.5	12.5	12.51	12.23	12.5	12.25	100.08	97.84	100.00	98.00
7	7.5	12.5	7.46	12.57	7.43	12.60	99.46	100.56	99.06	100.80
8	7.5	5	7.63	4.96	7.68	4.95	102.13	99.20	101.73	99.00
						Mean ± SD	99.18±1.80	98.92±1.09	98.95±1.84	99.10±1.24
						RSD	1.81	1.10	1.85	1.25



(a) **(b)**

Fig.4: Plot of expected concentrations (µg/ml) versus predicted concentrations (µg/ml) for AM using (a) PCR, (b) PLS models.



(a) **(b)**

Fig. 5: Plot of expected concentrations (µg/ml) versus predicted concentration (µg/ml) for AT using PCR and PLS models.

Table 3: Root mean squared error of cross-validation (RMSECV), of calibration (RMSEC) and prediction (RMSEP), together with linear regression parameters of the linear relationship between the expected and the predicted values of AM and AT in the validation set by the proposed chemometric methods.

	PCR (2)[a] model		PLS (2)[b] model	
	AM	**AT**	**AM**	**AT**
Root mean square error				
RMSEC	0.43	0.32	0.41	0.28
RMSECV	0.61	0.58	0.49	0.35
RMSEP	0.25	0.21	0.28	0.18
Regression parameters				
Slope	0.957	0.986	0.979	0.990
Intercept	0.122	-0.003	0.090	-0.024
Correlation coefficient (r)	0.9987	0.9997	0.9988	0.9996

[a] The number of PCR factors (PCs) are represented in parentheses

[b] The number of PLS factors (LVs) are represented in parentheses.

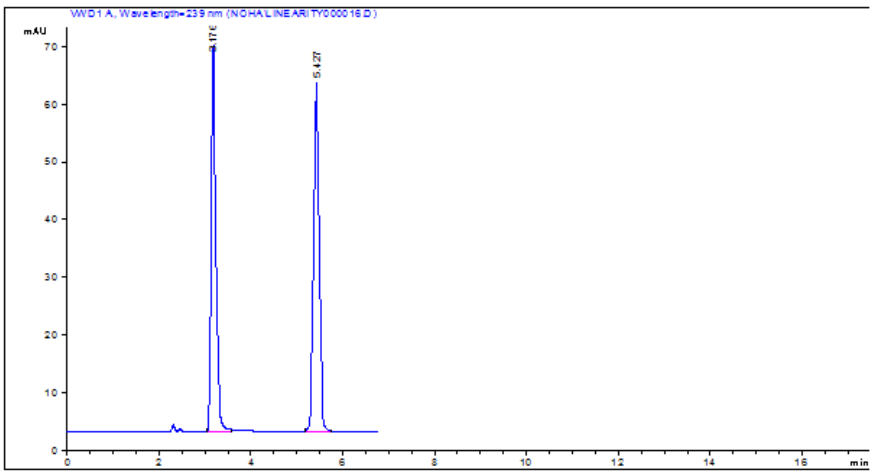


Fig. 6: HPLC chromatogram of a resolved mixture of AM (t_R= 3.17) and AT (t_R=5.42), mobile phase (Methanol: phosphate buffer pH=3.5, 75:25 v/v)

RP-HPLC method

A validated HPLC method was developed for quantitative determination of AM and AT in bulk powder and in pharmaceutical preparation.

The chromatograms for both amlodipine besylate and atorvastatin calcium are shown in (Figure 6).

The method was validated for linearity, precision (Repeatability and intermediate precision), LOD (Limit of detection), LOQ (Limit of quantitation) and accuracy as per the ICH guideline [27]. The robustness study was carried out statistically by the design of experiments using MODDE 9.0 (trial version).

Linearity

Different aliquots (0.25-1.75 mL) of AM and AT standard solution (100µg/mL) were transferred into two series of 10 mL volumetric flasks; each was diluted with the mobile phase to reach a final concentration range of (2.5-17.5 µg/mL). Solutions were injected in triplicate with 20 µL injection volume. Linearity regression equations were

y= 46.984x+ 1.4429 (r= 0.9999) for AM and y= 42.043x+ 8.8571 (r= 0.9999) for AT, respectively.

Accuracy

The accuracy of the method was demonstrated by analyzing different concentrations covering the points in the calibration range

(5, 7.5, 10, 12.5,15 µg/mL) for AM and AT. Each concentration was injected in triplicate. The average percentage recovery at each concentration level was determined, the mean percentage recovery and RSD values were calculated as shown in (Table 4).

Precision

Repeatability

Repeatability was demonstrated by assaying three freshly prepared solutions in triplicates on the same day at concentrations (7.5, 10, 12.5 µg/mL) for both concentrations. RSD percentage values were 0.753 and 0.780 for AM and AT, respectively.

Intermediate precision

Intermediate precision was studied by assaying the previously mentioned concentration under repeatability within different days. RSD percentage values were 1.253 and 0.712 for AM and AT, respectively.

Lower detection limit (LoD)

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. Values were found to be 0.207 and 0.364 for AM and AT, respectively. And were calculated as shown:

LoD =3.3 *σ/S

where σ = the standard deviation of the response

S = the slope of the calibration curve

Lower quantitation limit (LoQ)

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products. Values were found to be 0.628 and 1.106 for AM and AT, respectively. And were calculated as shown.

LoQ =10* σ/S

where σ = the standard deviation of the response

S = the slope of the calibration curve

The summary of Regression and validation parameters for determination of AM and AT are shown in (Table 5)

Suitability parameters

System suitability parameters were calculated for each chromatographic run for both drugs as shown in (Table 6).

Statistically Designed Robustness Experiments

The concept of DOE was used for determining the effect of 4 factors in only 11 experiments. The design of experiment (DOE) used was two-level plackett-Burman design, the effect of each factor was computed by MODDE 9.0 trial version with respect to resolution, symmetry and selectivity. Factors applied to robustness with their two levels as shown in (Table 7). All factors were found to be non-significant ($P<0.05$) as shown in the coefficient plot as presented in (Figure 7).

Application of both studied methods on pharmaceutical dosage form were demonstrated by preparing six replicate sample solutions of Caduet® tablets (10 mg- 10 mg) with good recoveries as shown in (Table 8).

The results obtained for the analysis of AM and AT for both methods were statistically compared with those obtained by applying the reported HPLC method. In statistical comparison between the reported method with the two methods developed resulted with no significant difference as shown in (Table 9).

Table 4: Accuracy expressed as % recovery of AM and AT by the proposed method

Sample	Taken (µg/mL)	Found(µg/mL)		Recovery %	
		AM	AT	AM	AT
1	5	4.98	4.90	99.60	98.10
2	7.5	7.47	7.45	99.60	99.30
3	10	10.04	9.85	100.40	98.50
4	12.5	12.74	12.39	101.90	99.10
5	15	14.98	15.03	99.90	100.22
			Mean ± S.D	100.28±0.962	99.04±0.8122
			RSD	0.959	0.820

Table 5: Regression and validation parameters for determination of AM and AT

Parameters	AM	AT
Linearity (Range)	2.5-17.5µg/mL	2.5 -17.5µg/mL
Intercept coefficient ±S.E	1.440 ±1.704	8.857± 0.2173
Slope coefficient ±S.E	46.984 ± 0.3215	42.043 ± 0.2173
Correlation coefficient (r) ± S.E	0.9999 ± 1.2×10^{-4}	0.9999 ± zero
LoD (µg/mL)	0.207	0.364
LoQ (µg/mL)	0.628	1.106
Precision:		
Repeatability	0.753	0.780
Intermediate precision	1.253	0.712

Table 6: System suitability parameters of analysis of AM and AT by the proposed HPLC method.

Parameters	AM	AT	Reference value
Resolution (Rs)	5.71	11.20	≥2
Relative retention time (α)	3.461	6.398	>1
Tailing factor (t)	0.85	0.97	=1
Height equivalent to theoretical plates (H=L/N)	0.005	0.002	The smaller the value ,the more efficient separation
Number of theoretical plates (N)	3468	7860	The higher the value, the more efficient separation

Table 7: Factors and levels applied to the robustness test by HPLC method

Factors	Units	Level (-1)	Level (+1)	Nominal
pH	-----	3.4	3.6	3.5
Methanol	%	73%	77%	75
Flow rate	mL/min	1	1.4	1.2
Wavelength	nm	238	240	239

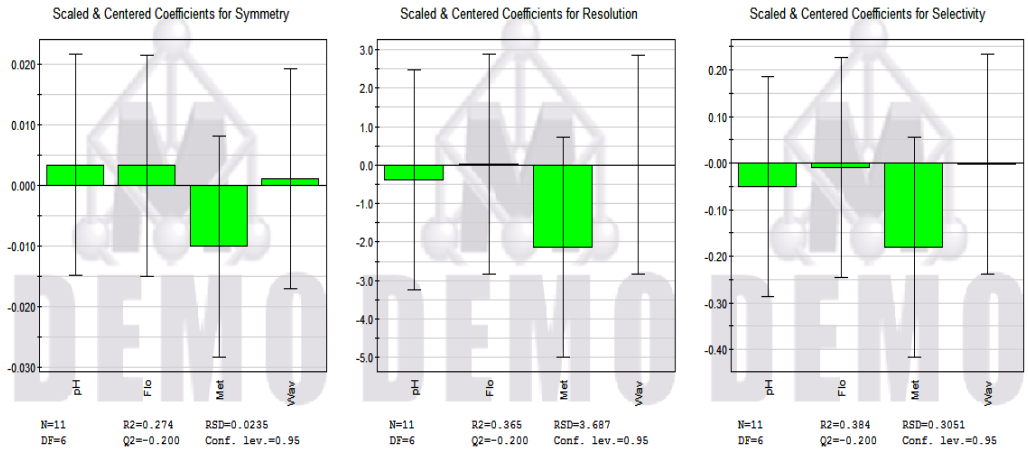


Fig. 7: The Coefficient plot of DOE for robustness testing of AM and AT that shows the factors influencing the chromatographic separation of the critical pair

Table 8: Application of the proposed methods to Caduet ® tablets (10mg-10mg)

Caduet® tablets claimed to contain 10mg AM and 10mg AT Batch no. 1172110	Methods	AM Found	AM Mean ± SD	AT Found	AT Mean ± SD
	PLS	9.89	98.98(0.85)	9.96	99.68(1.35)
	PCR	9.91	99.16(0.75)	9.96	99.60(1.40)
	RP-HPLC	9.88	98.82(0.62)	10.11	101.19(0.69)

Table 9: Statistical comparison of the results obtained by the proposed spectrophotometric and RP-HPLC methods and with the reported HPLC method [20] for the analysis of AM and AT in Caduet® tablets (10 mg-10mg).

Para-meters	PCR		PLS		HPLC		Reported[b] HPLC method	Reported[b] HPLC method
	AM	AT	AM	AT	AM	AT	AM	AT
Mean	99.16	100.00	99.00	100.00	99.00	101.19	99.01	101.00
SD	0.75	1.40	0.85	1.35	0.66	0.34	0.88	0.78
N	5	5	5	5	5	5	5	5
Variance	0.56	1.96	0.72	1.82	0.43	0.11	0.77	0.60
t-test (2.306) [a]	0.28	1.91	0.05	1.84	0.38	0.86		
F-test (6.388) [a]	1.36	3.18	1.06	2.98	1.73	5.06		

[a] The values in parentheses are the corresponding tabulated values at P< 0.05.

[b] HPLC method (ODS-3,(5µm,250mm×4.6mm, id. column) ,using acetonitrile: 0.025 M sodium dihydrogen phosphate 55:45 v/v and adjusted to pH 4.5 with phosphoric acid, flow rate 1 mL min^{-1}, 237 nm).

CONCLUSION

The proposed spectrophotometric and chromatographic methods showed several advantages regarding their simplicity, saving time and of less cost. The methods were applied successfully for the simultaneous determination of AM and AT in pharmaceutical preparation. RP-HPLC methods were validated via ICH guidelines and all results obtained were within acceptable range.

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