Simultaneous estimation of Atorvastatin and Amlodipine
Methodology, Results and discussion

Chapter 6

6.1 Reagents, Standards and Pharmaceutical preparations

Pure samples of Atorvastatin calcium (ATOR) and Amlodipine besylate (AMLO) were kindly supplied by Biocon India Limited, India and Torrent Pharma, India and were certified to be 99.3 and 99.8% pure respectively. The drugs are used without further purification. All the solvents used in spectrophotometric and HPLC analysis were of analytical reagent grade. Caduet and Lipikind-AM tablets of Pfizer Pharmaceuticals, and Mankind Pharma Pvt. Ltd., respectively were claimed to contain 10 mg of ATOR and 5 mg of AMLO are used in analysis.

6.2 Procedure

Standard solutions of ATOR and AMLO

It was used stock solutions of ATOR and AMLO 1 mg mL^{-1} in methanol. The working solutions were 0.04 mg mL^{-1} prepared by transferring 2.0 mL from respective stock solution to a 50 mL volumetric flask and completing to volume with methanol. 0.2 μg μL^{-1} and 0.1 μg μL^{-1} working solutions of AMLO and ATOR in methanol were prepared respectively for HPTLC analysis.

Preparation of mobile phase

- HPTLC; ethyl acetate : 1,4-dioxane : methanol : 25% ammonia (10 : 1 : 3 : 1)

Prepared laboratory mixtures

In measuring flasks 10 mL, aliquot volumes of ATOR and AMLO from their corresponding working solutions were transferred accurately to prepare mixtures containing different ratios of the two drugs.

Preparation of pharmaceutical sample solution

Twenty Caduet and Lipikind-AM tablets of Pfizer Pharmaceuticals, and Mankind Pharma Pvt. Ltd., respectively were claimed to contain 10 mg of ATOR and 5 mg of AMLO were weighed accurately and powdered. An amount of the powder equivalent to 10 mg ATOR and 5 mg AMLO (content of one tablet) was dissolved in 60 mL of methanol. The solution was sonicated for 10 min and filtered into a 100 mL volumetric flask through 0.45μ nylon membrane filter. The residue was washed 3 times with 10 mL of methanol, and then the volume was completed to

100 mL with the same solvent. This solution was diluted to 2:10 with methanol and proposed spectrophotometric and with further dilutions HPTLC methods were applied and the concentration of each component in both the formulations was determined.

Spectrophotometric method (Ratio first derivative method)

Calibration sets

A calibration set containing seven dilutions each of ATOR (4-22 μg mL^{-1}) and AMLO (4-24 μg mL^{-1}) was prepared in methanol and UV spectra were recorded in the wavelength range between 210-350 nm versus solvent blank.

Chemometric calibration

A calibration set of 23 synthetic binary mixtures was prepared in methanol applying a multilevel multifactor design in which different levels of concentrations of ATOR and AMLO were introduced. The levels were in the range of 4-22 and 4-24 μg mL^{-1} for ATOR and AMLO respectively as shown in Table 5. 1. Zero-order and first derivative UV spectra (Fig. 6. 5 (a) and 6. 5 (b)) were recorded in the wavelength range 210-350 nm versus solvent blank and digitized absorbance was recorded at 1 nm intervals. The computation was made in R-software environment. CLS, ILS, PCR and PLS algorithms were applied to the UV absorption data matrix of these binary mixtures.

HPTLC calibration

Different volumes of standard mixture (ATOR 0.1 μg μL^{-1} + AMLO 0.2 μg μL^{-1}) 2, 4, 6, 8,10,12 and 14 μL injection $spot^{-1}$ were made to obtain a concentration range 100-1400 ng $spot^{-1}$ and 200- 2400 ng $spot^{-1}$ of ATOR and AMLO respectively. The above solutions were spotted in three replicate on TLC plate. Densitometric scanning was performed in the absorbance mode at 254 nm for the estimation of ATOR and AMLO (Fig. 6. 6 (a) and 6. 6 (b)). The data of peak area versus drug concentrations were treated by polynomial regression mode (Table 5. 2).

Table 6. 1 Composition of the concentration (calibration) set

Mixture No.	Concentration ($\mu g\ mL^{-1}$)	
	ATOR	AMLO
1	4	4
2	4	8
3	4	16
4	4	20
5	4	24
6	8	4
7	8	8
8	8	16
9	8	20
10	8	24
11	12	4
12	12	8
13	12	16
14	12	20
15	12	24
16	16	4
17	16	8
18	16	16
19	16	20
20	16	24
21	22	4
22	22	8
23	22	16

Preparation of binary mixtures for RFD and chemometric predictions

Various concentrations of synthetic mixtures containing ATOR and AMLO within the stated range were introduced and prepared for predictions by proposed ratio first derivative spectrophotometric and chemometric methods.

Spectral characteristics of ATOR and AMLO

Aliquot portions equivalent to 12 μg mL^{-1} each of ATOR and AMLO in methanol were taken separately. Zero-order and first derivative UV absorption spectra of both solutions and one binary mixture were recorded (Fig. 6. 1a and 6. 1b).

Procedure

Ratio spectra first derivative spectrophotometry (RFD)

The ratio spectra of different ATOR standards at increasing concentration in methanol obtained by dividing each with the stored spectrum of the standard solution of 12 μg mL^{-1} AMLO by computer aid as divisor spectra was shown in Fig. 6. 2A. The first derivative (^{1}DD) of this spectrum traced with smoothing factor ($\Delta\lambda$=8) nm and scaling factor (=10) are illustrated in Fig. 6. 2B. As seen in fig 2B there exist one minimum (296.1nm) and one maximum (281nm) and found that both were suitable for determination of ATOR in ATOR and AMLO mixture. The wavelength 296.1 nm selected for the determination of this compound in the assay of synthetic mixtures, tablets, due to its lower R.S.D values and more suitable mean recovery compared with other wavelengths. For the determination of AMLO, the ratio spectra of different AMLO standards at increasing

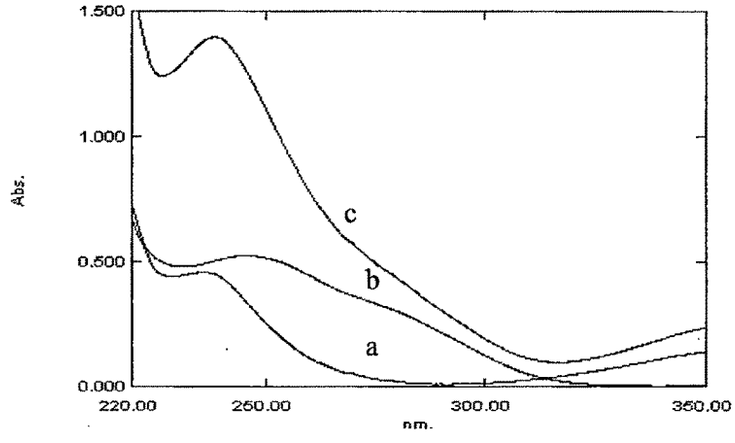


Fig. 6. 1 (a) Zero-order absorption spectra: a) 12 μg mL^{-1} of AMLO, b) 12 μg mL^{-1} of ATOR and c) their mixture in methanol

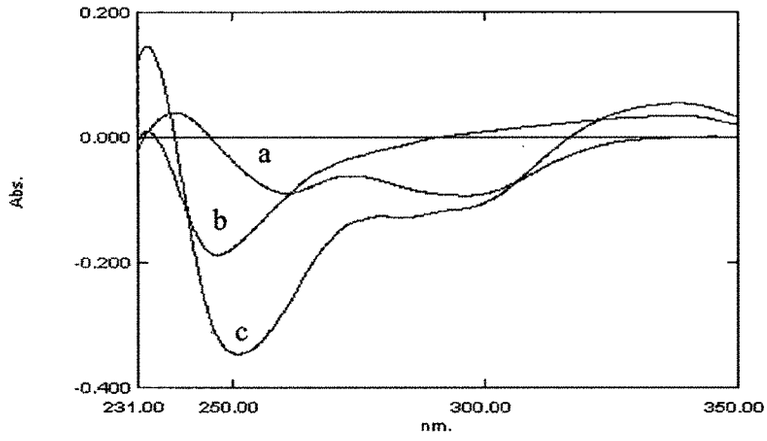


Fig. 6. 1 (b) First derivative absorption spectra: a) 12 μg mL^{-1} of ATOR, b) 12 μg mL^{-1} of AMLO, and c) their mixture in methanol

concentrations in methanol obtained by dividing each with stored spectrum of the standard solution of 8 μg mL^{-1} of ATOR as divisor spectra by computer aid was demonstrated in Fig. 6. 3A. The first derivative (^{1}DD) of this spectrum traced with interval of $\Delta\lambda$=8 nm and scaling factor (=10) are illustrated in Fig. 6. 3B. As seen in fig 6. 3B there exist one minimum (247 nm) and this is found suitable for the determination of AMLO in AMLO and ATOR mixture.

6.3 Results and discussion

Figs. 6.1 (a) and 6. 1 (b) shows the zero- order and first derivative spectra of ATOR and AMLO as well as their corresponding binary mixture in methanol. As shown, ATOR exhibits absorption maxima at 246.4 nm while AMLO shows maxima at 236.4 nm. The spectra of ATOR and AMLO were overlapped in the region of their absorption maxima. For this reason to solve overlapped spectra, ratio spectra first derivative spectrophotometry and four chemometric calibrations using the zero-order and first derivative spectra were separately applied.

Ratio first derivative methods were used for analysis of mixtures with overlapped spectra. This method permits the determination of components in mixtures at wavelengths corresponding to a maximum or minimum. The values at these points permit better sensitivity and accuracy. The main instrumental parameters that affect the shape of the derivative ratio spectra are the wavelength scanning speed,

the concentration of divisor spectra, smoothing ($\Delta\lambda$) and scaling factor. The effects of these parameters were studied and fast scanning speed, smoothing factor ($\Delta\lambda$=8), scaling factor (=10) was selected. Divisor concentration is main instrumental parameter, the standard spectra of 8 μg mL^{-1} of ATOR and 12 μg mL^{-1} of AMLO was considered as divisor for the determination of AMLO and ATOR in mixtures respectively.

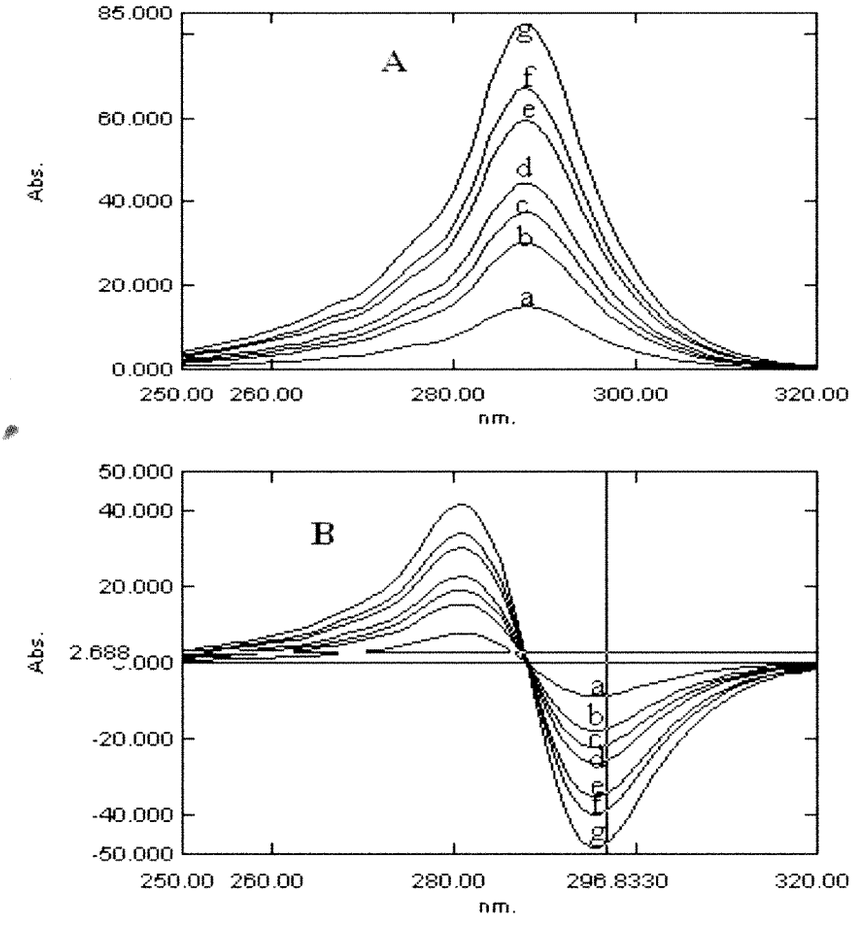


Fig. 6. 2 Ratio spectra (A) and first derivative of the ratio spectra (B) of a) 4 μg/mL b) 8 μg mL^{-1} c) 10 μg mL^{-1} d) 12 μg mL^{-1} e) 16 μg mL^{-1} f) 18 μg mL^{-1} g) 22 μg mL^{-1} solution of ATOR in methanol when 12 μg mL^{-1} of AMLO in methanol used as divisor ($\Delta\lambda$ = 8 nm), scaling factor =10

Calibration graphs were established from analytical signals measured at 247.5 nm for standards containing 4-22 μg mL^{-1} of ATOR and at 236.4 nm for standards containing 4-24 μg mL^{-1} of AMLO corresponding to maxima and minima in the absence of each other. All the analytical parameters are illustrated in Table 6. 2.

The proposed method was successfully applied for the determination of the two drugs in laboratory prepared 16 synthetic binary mixtures. Recoveries and relative standard deviations results of RFD and chemometric methods are given in Table 6. 3 and Tables 6. 6 (a) 6. 6 (b) respectively are found satisfactory.

A critical evaluation of all the proposed method was performed by statistical analysis of the data, where slopes, intercepts and correlation coefficients of RFD and HPTLC methods were shown in Table 6. 2 and chemometric methods in Table 6. 4 (a) and 6. 4 (b) respectively. The selectivity of the proposed method was also assessed by the analysis of synthetic mixtures, where satisfactory results were obtained over the stated calibration range.

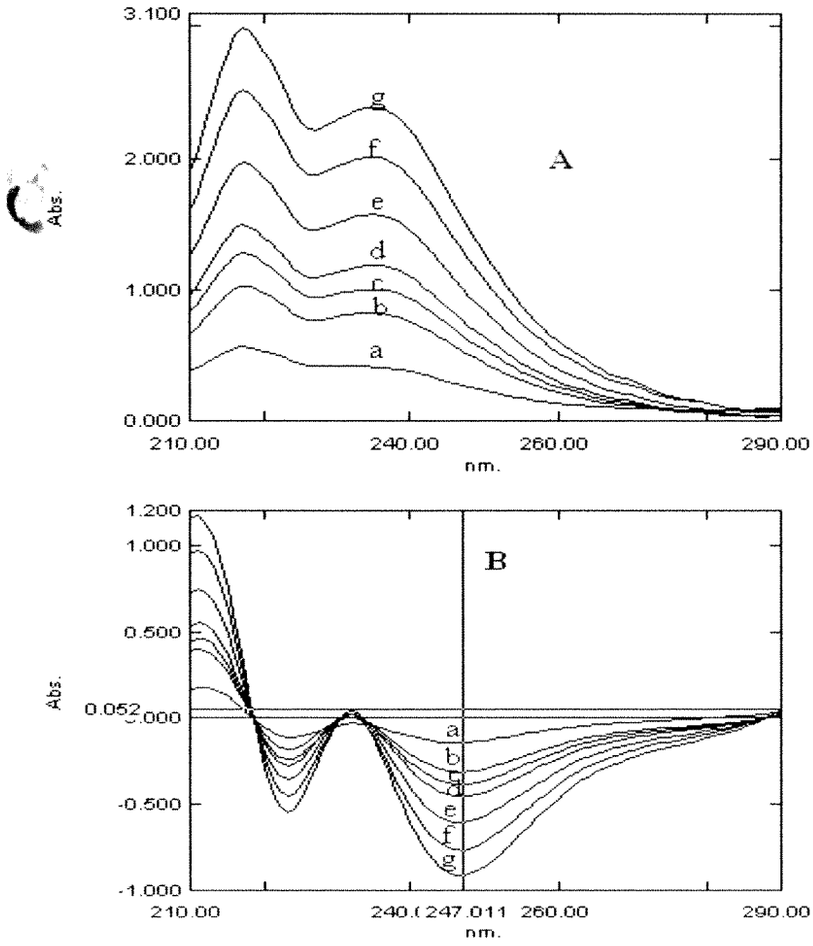


Fig. 6. 3 Ratio spectra (A) and first derivative of the ratio spectra (B) of a) 4 µg/mL b) 8 µg mL^{-1} c) 10 µg mL^{-1} d) 12 µg mL^{-1} e) 16 µg mL^{-1} f) 20 µg mL^{-1} g) 24 µg mL^{-1} solution of AMLO in methanol when 08 µg mL^{-1} of ATOR in methanol used as divisor ($\Delta\lambda$ = 8 nm), scaling factor =10

Table 6. 2 Analytical data of the calibration graphs for determination of ATOR and AMLO by RFD and HPTLC method

Parameters	RFD		HPTLC	
	ATOR	AMLO	ATOR	AMLO
Wavelength (nm)	247.5	236.4	254	
Linearity range (μg mL^{-1})	4-22	4-24	0.1-1.4	0.2-2.4
Intercept (a)	0.0003	0.0042	223.94	396.67
Standard deviation of the intercept (S_a)	0.100	0.005	1.65	2.57
SE of Intercept[1]	0.0363	0.021	0.0024	0.0037
Slope (b)	0.0459	0.0326	1.928	2.109
Standard deviation of the slope (S_b)	0.135	0.165	0.111	0.046
SE of slope	0.012	0.065	0.0001	0.0002
Correl. coeff	1	0.9995	0.9997	0.9989
RSE[2]	0.0064	0.0034	0.0042	0.0076

[1]Standard error, [2]Residual standard error

Multivariate calibration

Chemometric techniques are other methods gaining wide application for the resolution of the drug mixtures. A calibration set of 23 samples was randomly prepared by multilevel factorial design in which five levels of concentrations of ATOR and AMLO were introduced. The levels were in the range of 4 - 22 and 4 - 24 μg mL^{-1} for ATOR and AMLO respectively, and illustrated in Table 6. 1. The UV absorbance data was obtained by measuring the absorbances in the region of 221 - 310 nm. By using the correlation between calibration concentrations and its absorbance data, the chemometric calibrations were calibrated within the CLS, ILS, PCR and PLS algorithms. The numerical results of calibrations are shown in Tables6. 4 (a) and 6. 4 (b).

Table 6. 3 Results obtained for the determination of ATOR and AMLO in the different synthetic mixtures by using the ratio spectra derivative method

Mixture	ATOR		AMLO	
number	Added (μg mL^{-1})	Recovery (%)	Added (μg mL^{-1})	Recovery (%)
1	4	100.9	4	98.1
2	4	101.1	8	97.1
3	4	101.0	16	99.1
4	4	101.5	24	99.2
5	8	101.9	4	97.6
6	8	101.4	8	102.5
7	8	98.9	16	98.4
8	8	102.3	24	101.7
9	16	100.5	4	101.0
10	16	102.6	8	101.0
11	16	98.9	16	101.3
12	16	101.3	24	101.8
13	22	102.2	4	101.3
14	22	101.5	8	102.3
15	22	102.2	16	100.4
16	22	100.6	24	98.6
	$\bar{x}$:		$\bar{x}$:	
	SD:	1.07	SD:	1.76
	RSD:	1.06	RSD:	1.76

$\bar{x}$, mean value; SD, Standard deviation; RSD, Relative standard deviation

The quality of multicomponent analysis is dependent on the wavelength range and spectral mode used calibration set chosen and calibration range. All the information present in the sample target should be present in the calibration data set. It has been one of the main drawbacks in development studies of multivariate method. Except ILS the remaining CLS, PCR and PLS techniques are designated as full spectrum computational procedures, thus wavelength selection is seemingly unnecessary, and so all available wavelengths are often used. Stepwise multiple linear regressions have been used for the selection of frequencies in ILS. However, measurements from spectral wavelengths that are not informative in a

model will degrade performance. Hence amplitudes after 280 nm were not used because AMLO has no absorbance at the concentrations used in this region any absorbance data beyond 280 nm would have introduced a significant amount of noise, thereby decreasing the precision of AMLO estimation and predictive ability of the model. Original and reconstructed spectra of the calibration matrix were compared in order to select the range of wavelengths. The region, which is best, reconstructed also considered. This entailed using 60 experimental points per spectrum, as spectra were digitized at 1 nm intervals.

Statistical parameter

The predictive ability of a calibration model in chemometric methods can be defined in various ways. The most general expression is the standard error of calibration (SEC) and prediction (SEP), which is given by the following equation;

$$\text{SEP (SEC)} = \sqrt{\frac{\sum_{i=1}^{N}(C_i^{Added} - C_i^{Found})^2}{n}}$$

Here C_i^{Added} is the added concentration of drugs, C_i^{Found} is the predicted concentration of drugs and n is the total number of the synthetic mixtures. The numerical values are quoted in Tables 6. 4 (a), 6. 4 (b), 6. 5 (a) and 6. 5 (b).

Selection of optimum number of factors for PCR and PLS

For PCR and PLS methods, 23 calibration spectra were used for the selection of the optimum number of factors by using the cross validation technique. This allows modelling of the system with the optimum amount of information and avoidance of overfitting or underfitting. The cross-validation procedure consisting of systematically removing one of a group of training samples in turn and using only the remaining ones for the construction of latent factors and applied regression. The predicted concentrations were then compared with the actual ones for each of the calibration samples and mean squares error of prediction (MSEP) was calculated. The MSEP was computed in the same manner each time a new factor was added to the PCR and PLS model. The selected model was that with the fewest number of factors such that its MSEP values were not significantly greater than that for the model, which yielded the lowest MSEP. A plot of MSEP

values against number of components (Figs. 6. 8, 9, 10 and 11) indicates that factors 3 and 2 were optimum in zero order and first derivative spectra respectively for the estimation of principle ingredients by PCR and PLS. At the selected principal components of PCR and PLS the concentrations of each sample were then predicted and compared with known concentration and the PRESS (prediction error sum of squares) was calculated. It was given by this equation, and values are indicated in Tables 6. 5 (a) and 6. 5 (b).

$$\text{PRESS} = \sum_{i=1}^{n} \left(C_i^{Added} - C_i^{Found} \right)^2$$

6.4 Validation of the developed methods

To check the validity (predictive ability) of the chemometric calibration models, the simultaneous analysis of the prediction set containing 22 samples of various concentrations (in triplicates) of ATOR and AMLO was carried out by considering zero-order and first derivative spectra and the results are given in Tables 6. 6 (a) and 6. 6 (b). Similarly 16 binary sets are used in RFD method. The values of the mean percent errors corresponding to RFD were shown in Table 6. 3. CLS, ILS, PCR and PLS calibrations for the synthetic mixtures were completely acceptable because of their smallest values and hence found satisfactory for the validity of all calibration methods.

Linearity

The linearity of the proposed RFD and chemometric methods for determination of ATOR and AMLO was evaluated by analysing a series of different concentrations of standard drug. In this study seven concentrations were chosen, ranging between 4-22 μg mL^{-1} of ATOR and 4-24 μg mL^{-1} of AMLO. Similarly in HPTLC linearity was evaluated by analysing a series of different concentrations of standard drug ranging between 100-1400 ng of ATOR and 200-2400 ng of AMLO (Fig. 6. 7). Each concentration was repeated three times and obtained information on the variation in peak area response in HPTLC at 254 nm and absorbances at stated wavelength region for RDF and chemometric methods were recorded. The

linearity of the calibration graphs of proposed methods was validated by the high value of correlation coefficient, slope and the intercept.

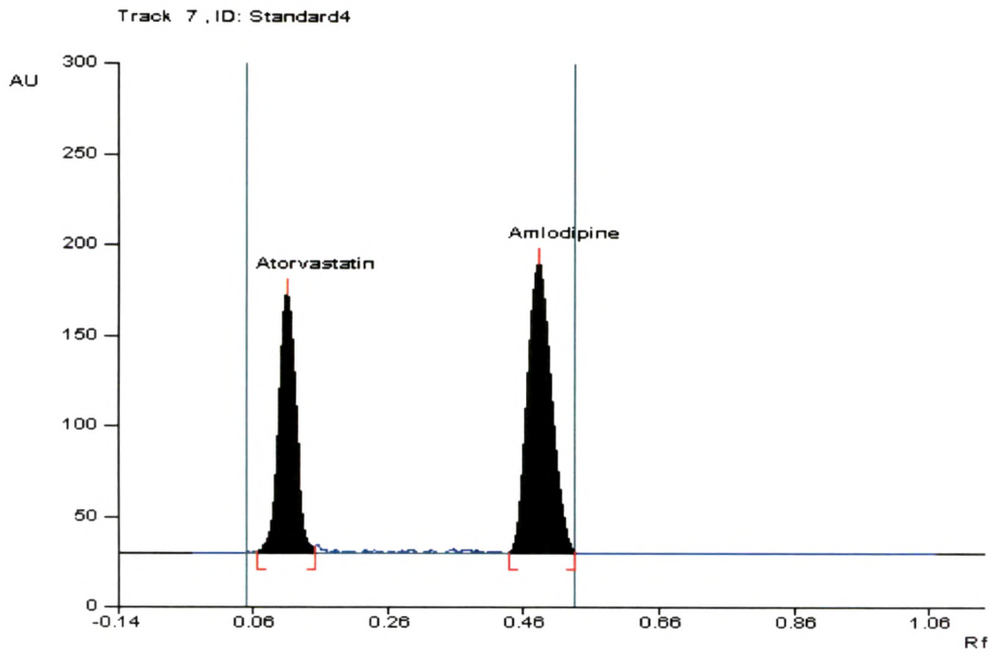
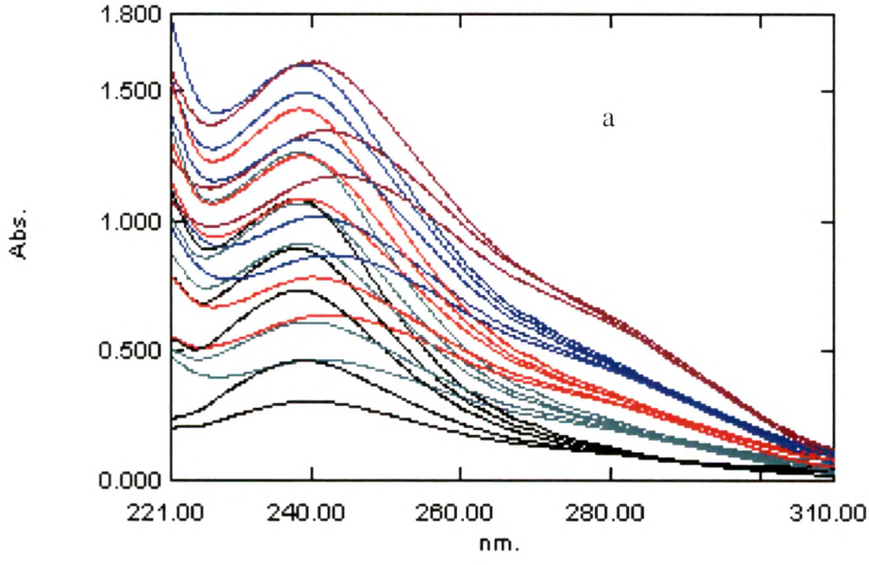


Fig. 6. 4 HPTLC chromatogram showing Retention factor (R_f) of 800 ng of ATOR (0.14) and (b) 1600 ng of AMLO (0.52) in laboratory prepared mixture



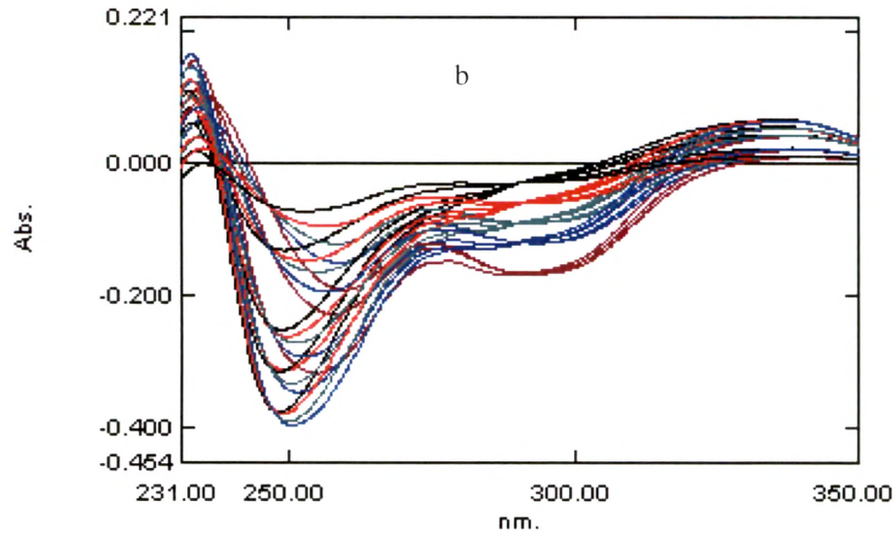


Fig. 6. 5 (a) Zero-order and (b) first derivative overlay absorption spectra of different level of binary mixtures of ATOR and AMLO in methanol and used for calibration

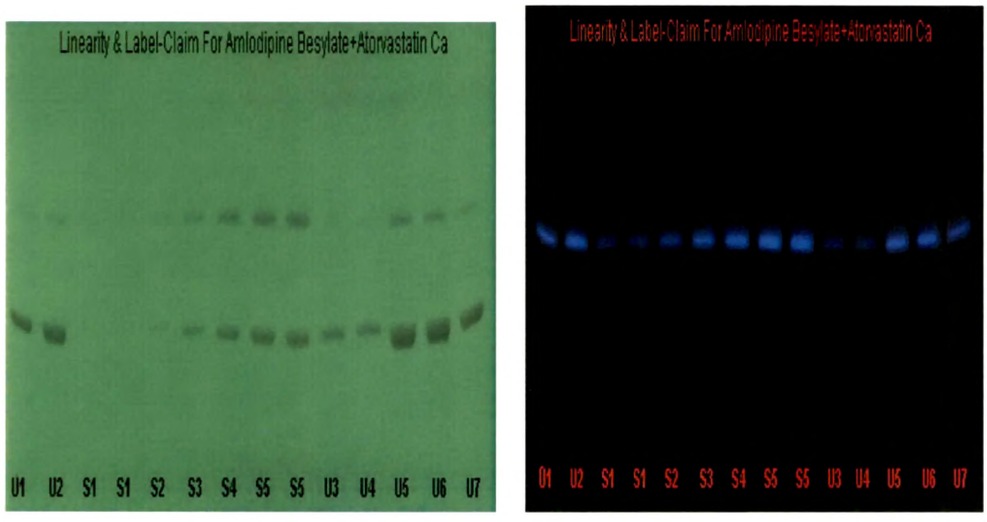


Fig. 6. 6 (a) HPTLC scanned UV-image at 254 nm and fluorescent images of standard dilutions of ATOR and AMLO for calibration

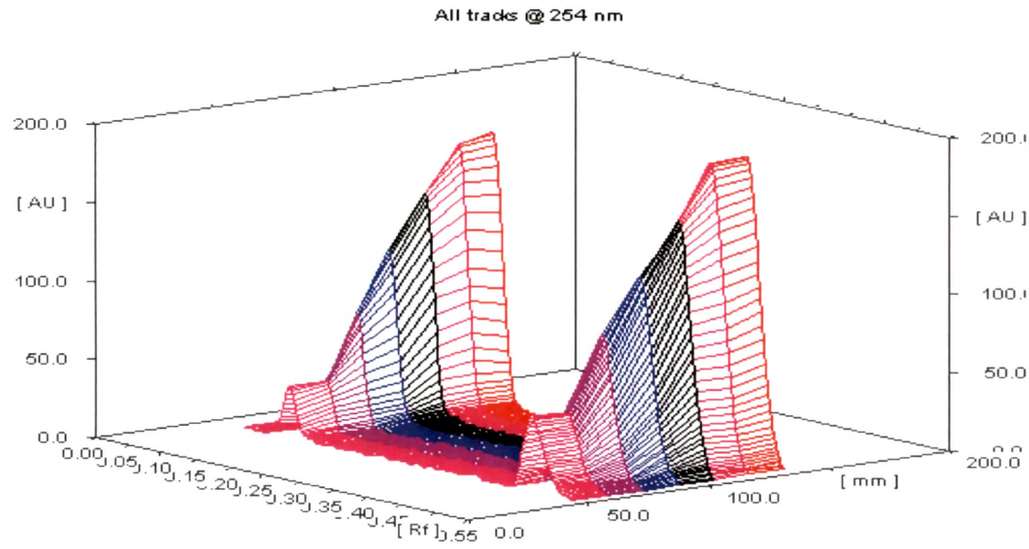


Fig. 6. 6 (b) HPTLC 3-Dimensional chromatograms set of six standard dilutions of ATOR and AMLO for calibration

Table 6. 4 (a) Statistical parameters of chemometric methods in calibration step of zero-order spectra

Component	CLS	ILS	PCR			PLS		
	SEC	SEC	SEC	PRESS	RSE[a] (%)	SEC	PRESS	RSE
ATOR	0.2020	2.08e-06	0.019	0.0080	0.1441	0.0185	0.0075	0.1403
AMLO	0.2438	2.54e-07	0.0179	0.0071	0.1127	0.0182	0.0073	0.1145

Table 6. 4 (b) Statistical parameters of chemometric methods in calibration step of First derivative spectra

Component	CLS	ILS	PCR			PLS		
	SEC	SEC	SEC	PRESS	RSE[a] (%)	SEC	PRESS	RSE
ATOR	0.2423	0.1489	0.0193	0.0082	0.1463	0.0193	0.0082	0.1463
AMLO	0.2577	0.0743	0.0215	0.0101	0.1351	0.0215	0.0102	0.1351

a, Relative standard error of calibration of single component

$$\mathrm{RSE}^{a}(\%) = \sqrt{\frac{\sum_{i=1}^{N}\left(C_i^{\,Added} - C_i^{\,Found}\right)^2}{\sum_{i=1}^{N}\left(C_i^{\,Added}\right)^2}} \times 100$$

Table 6. 5 (a) Statistical parameters of chemometric methods in prediction step of Zero-order spectra

Component	CLS				ILS				PCR				PLS			
	SEP	*a*	*b*	*r*	SEP	*a*	*b*	*r*	SEP	*a*	*b*	*r*	SEP	*a*	*b*	*r*
ATOR	0.157	0.055	1.000	0.999	0.178	0.100	0.996	0.999	0.110	0.052	1.000	0.999	0.111	0.047	1.000	0.999
AMLO	0.247	0.255	0.981	0.999	0.203	0.006	1.002	0.999	0.149	0.053	0.998	0.999	0.149	0.053	0.998	0.999

a, Intercept; *b*, slope; *r*, correlation coefficient.

Table 6. 5 (b) Statistical parameters of chemometric methods in prediction step of First derivative spectra

Component	CLS				ILS				PCR				PLS			
	SEP	*a*	*b*	*r*	SEP	*a*	*b*	*r*	SEP	*a*	*b*	*r*	SEP	*a*	*b*	*r*
ATOR	0.205	0.191	1.019	0.999	0.400	0.091	1.022	0.999	0.122	0.084	0.998	.0999	0.121	0.084	0.998	0.999
AMLO	0.199	0.173	0.985	0.999	0.307	0.106	1.002	0.999	0.153	0.019	0.997	0.999	0.153	0.020	0.997	0.999

a, Intercept; *b*, slope; *r*, correlation coefficient.

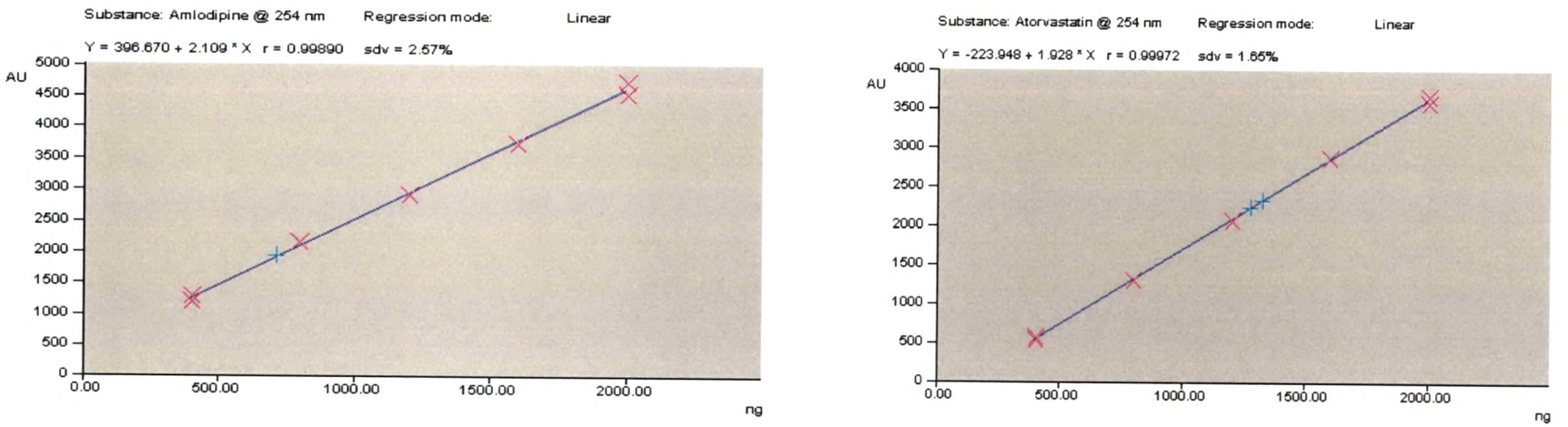


Fig. 6. 7 HPTLC linearity of standard dilutions of ATOR and AMLO for calibration

Table 6. 6 (a) Recovery results in prediction from Zero- order spectra for ATOR and AMLO in synthetic mixtures by proposed chemometric techniques

Mixture added		Recovery (%)								Error%							
		CLS		ILS		PCR		PLS		CLS		ILS		PCR		PLS	
ATOR	AMLO	ATOR	AMLO	ATOR	AMLO	ATOR	AMLO	ATOR	AMLO	ATOR	AMLO	ATOR	AMLO	ATOR	AMLO	ATOR	AMLO
4	4	98.83	109.78	100.75	100.00	100.67	98.85	100.61	98.88	-1.17	9.78	0.75	0.00	0.67	-1.15	0.61	-1.12
4	8	98.31	104.82	99.25	96.13	100.65	100.28	100.53	100.29	-1.69	4.82	-0.75	-3.88	0.65	0.28	0.53	0.29
4	16	110.24	100.18	106.25	98.94	99.53	98.90	99.34	98.88	10.24	0.18	6.25	-1.06	-0.47	-1.10	-0.66	-1.12
4	20	104.36	100.65	103.00	99.80	99.97	100.01	99.84	100.00	4.36	0.65	3.00	-0.20	-0.03	0.01	-0.16	0.00
4	24	98.37	100.76	100.50	101.08	100.94	100.56	100.86	100.56	-1.63	0.76	0.50	1.08	0.94	0.56	0.86	0.56
8	4	100.74	106.96	99.75	100.50	101.23	97.85	101.22	97.87	0.74	6.96	-0.25	0.50	1.23	-2.15	1.22	-2.13
8	8	101.69	103.58	98.75	102.00	101.28	100.24	101.30	100.25	1.69	3.58	-1.25	2.00	1.28	0.24	1.30	0.25
8	16	101.98	100.89	99.88	99.00	101.23	100.11	101.20	100.11	1.98	0.89	-0.13	-1.00	1.23	0.11	1.20	0.11
8	20	101.78	97.81	98.25	99.05	102.04	97.58	102.12	97.59	1.78	-2.19	-1.75	-0.95	2.04	-2.42	2.12	-2.41
8	24	100.07	99.33	102.13	100.13	101.62	99.48	101.66	99.49	0.07	-0.67	2.13	0.13	1.62	-0.52	1.66	-0.51
12	4	99.94	103.44	100.50	97.50	100.44	96.41	100.41	96.42	-0.06	3.44	0.50	-2.50	0.44	-3.59	0.41	-3.58
12	8	99.63	100.84	100.50	102.13	100.07	98.61	100.06	98.61	-0.37	0.84	0.50	2.13	0.07	-1.39	0.06	-1.39
12	16	98.55	98.61	101.50	98.25	99.09	98.34	99.05	98.34	-1.45	-1.39	1.50	-1.75	-0.91	-1.66	-0.95	-1.66
12	20	100.20	99.64	101.67	100.20	101.03	100.11	101.04	100.11	0.20	-0.36	1.67	0.20	1.03	0.11	1.04	0.11
12	24	100.20	98.95	100.00	99.79	100.68	99.64	100.68	99.64	0.20	-1.05	0.00	-0.21	0.68	-0.36	0.68	-0.36
16	4	99.96	102.82	100.69	102.75	100.15	98.31	100.12	98.32	-0.04	2.82	0.69	2.75	0.15	-1.69	0.12	-1.68
16	8	100.32	102.70	102.81	100.13	100.54	100.91	100.47	100.91	0.32	2.70	2.81	0.13	0.54	0.91	0.47	0.91
16	16	100.24	99.86	100.13	102.38	100.37	100.46	100.36	100.46	0.24	-0.14	0.13	2.38	0.37	0.46	0.36	0.46
16	20	101.45	99.28	102.31	100.30	101.86	100.21	101.86	100.21	1.45	-0.72	2.31	0.30	1.86	0.21	1.86	0.21
16	24	98.89	98.13	99.69	101.33	99.40	99.24	99.43	99.25	-1.11	-1.87	-0.31	1.33	-0.60	-0.76	-0.57	-0.75
22	4	101.58	95.86	99.32	100.75	100.43	97.06	100.46	97.03	1.58	-4.14	-0.68	0.75	0.43	-2.94	0.46	-2.97
22	8	99.67	98.38	98.59	102.38	99.43	99.42	99.45	99.41	-0.33	-1.62	-1.41	2.38	-0.57	-0.58	-0.55	-0.59
22	16	100.62	97.75	100.23	102.75	100.66	99.42	100.70	99.42	0.62	-2.25	0.23	2.75	0.66	-0.58	0.70	-0.58
	$\bar{x}$	100.76	100.91	100.71	100.31	100.57	99.21	100.55	99.21								
	RSD:	2.5	3.2	1.8	1.7	0.8	1.2	0.8	1.2								

$\bar{x}$, Mean recovery value; RSD, Relative standard deviation,

Table 6. 6 (b) Recovery results in prediction from First derivative spectra for ATOR and AMLO in synthetic mixtures by proposed chemometric techniques

Mixture added		Recovery (%)								Error%							
		CLS		ILS		PCR		PLS		CLS		ILS		PCR		PLS	
ATOR	AMLO	ATOR	AMLO	ATOR	AMLO	ATOR	AMLO	ATOR	AMLO	ATOR	AMLO	ATOR	AMLO	ATOR	AMLO	ATOR	AMLO
4	4	91.55	103.95	97.15	92.05	100.39	97.33	100.39	97.33	-8.45	3.95	-2.85	-7.95	0.39	-2.67	0.39	-2.67
4	8	93.05	102.55	97.30	99.39	100.85	100.18	100.85	100.18	-6.95	2.55	-2.70	-0.61	0.85	0.18	0.85	0.18
4	16	98.74	100.47	100.15	100.72	103.24	99.96	103.24	99.96	-1.26	0.47	0.15	0.72	3.24	-0.04	3.24	-0.04
4	20	99.11	100.62	100.00	98.93	102.59	100.30	102.59	100.30	-0.89	0.62	0.00	-1.08	2.59	0.30	2.59	0.30
4	24	99.93	100.53	100.15	99.10	101.99	100.59	102.00	100.59	-0.07	0.53	0.15	-0.90	1.99	0.59	2.00	0.59
8	4	97.55	104.33	100.99	93.95	101.29	98.40	101.29	98.40	-2.45	4.33	0.99	-6.05	1.29	-1.60	1.29	-1.60
8	8	98.81	102.48	102.48	98.16	101.49	99.81	101.49	99.81	-1.19	2.48	2.47	-1.84	1.49	-0.19	1.49	-0.19
8	16	100.35	100.81	102.34	100.45	101.51	100.20	101.51	100.20	0.35	0.81	2.34	0.45	1.51	0.20	1.51	0.20
8	20	100.35	97.75	103.61	97.79	101.13	97.40	101.13	97.40	0.35	-2.25	3.61	-2.21	1.13	-2.60	1.13	-2.60
8	24	101.42	99.26	103.76	100.30	101.03	99.47	101.03	99.47	1.42	-0.74	3.76	0.30	1.03	-0.53	1.03	-0.53
12	4	98.74	100.87	101.03	92.45	100.26	96.94	100.26	96.94	-1.26	0.87	1.03	-7.55	0.26	-3.06	0.26	-3.06
12	8	98.95	100.00	99.88	99.84	100.02	98.89	100.02	98.89	-1.05	0.00	-0.12	-0.16	0.02	-1.11	0.02	-1.11
12	16	99.34	98.42	100.88	99.50	99.12	98.39	99.12	98.39	-0.66	-1.58	0.88	-0.50	-0.88	-1.61	-0.88	-1.61
12	20	101.99	99.44	101.98	99.93	101.21	99.67	101.21	99.67	1.99	-0.56	1.97	-0.08	1.21	-0.33	1.21	-0.33
12	24	102.31	98.94	101.93	99.03	100.86	99.38	100.86	99.38	2.31	-1.06	1.93	-0.97	0.86	-0.62	0.86	-0.62
16	4	99.52	101.81	100.30	95.23	100.20	99.41	100.20	99.41	-0.48	1.81	0.30	-4.78	0.20	-0.59	0.20	-0.59
16	8	100.27	102.81	101.04	101.23	100.22	102.13	100.22	102.13	0.27	2.81	1.04	1.22	0.22	2.13	0.22	2.13
16	16	101.35	100.05	101.01	99.06	100.62	100.33	100.62	100.32	1.35	0.05	1.01	-0.94	0.62	0.33	0.62	0.32
16	20	103.11	99.46	102.64	101.43	101.94	100.25	101.94	100.25	3.11	-0.54	2.64	1.43	1.94	0.25	1.94	0.25
16	24	100.72	98.29	100.05	100.48	99.36	99.23	99.36	99.23	0.72	-1.71	0.05	0.47	-0.64	-0.77	-0.64	-0.77
22	4	100.90	98.18	102.32	103.45	100.79	98.85	100.79	98.85	0.90	-1.82	2.32	3.45	0.79	-1.15	0.79	-1.15
22	8	99.80	99.16	101.65	101.81	99.35	99.55	99.35	99.56	-0.20	-0.84	1.65	1.81	-0.65	-0.45	-0.65	-0.44
22	16	101.65	98.40	102.86	99.99	100.70	99.53	100.70	99.53	1.65	-1.60	2.86	-0.01	0.70	-0.47	0.70	-0.47
		99.54	100.37	101.11	98.88	100.88	99.40	100.88	99.40								
	RSD:	2.70	1.90	1.70	2.90	1.00	1.20	1.00	1.20								

$\bar{x}$, Mean recovery value; RSD, Relative standard deviation

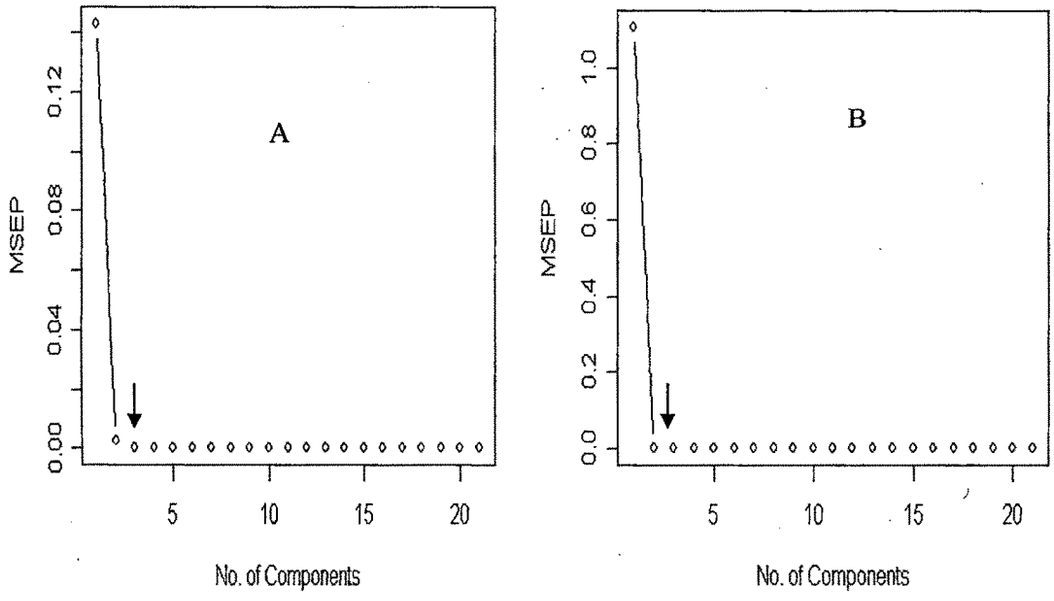


Fig. 6. 8 MSEP plots of a calibration set obtained using leave-one-out (LOO) cross validation of PCR-model for A) ATOR and B) AMLO in zero-order absorption data

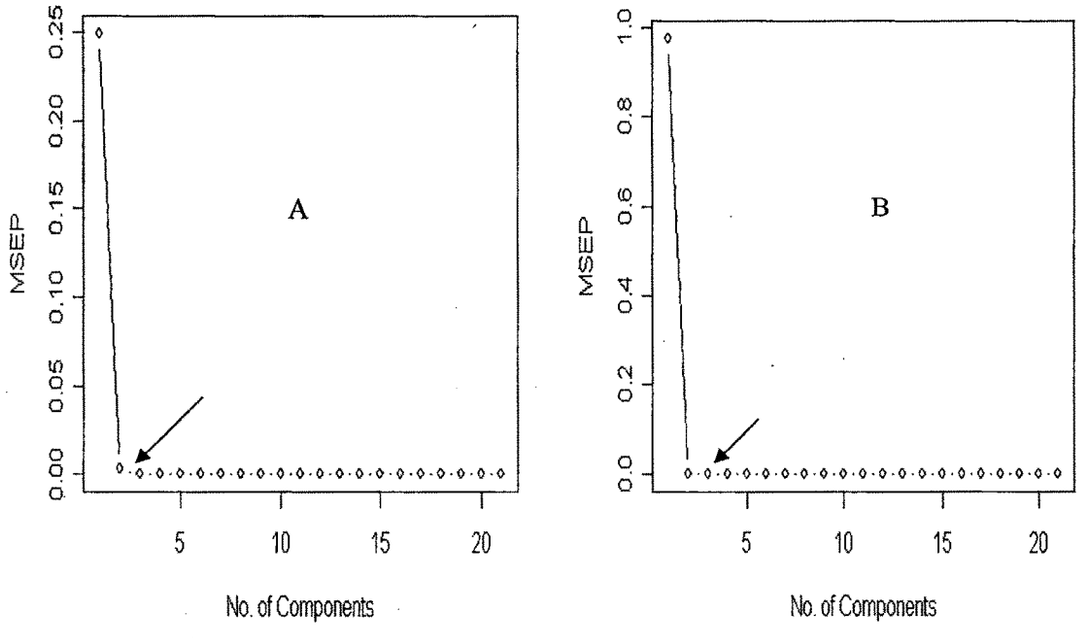


Fig. 6. 9 MSEP plots of a calibration set obtained using leave-one-out (LOO) cross validation of PLS-model for A) ATOR and B) AMLO in zero-order absorption data

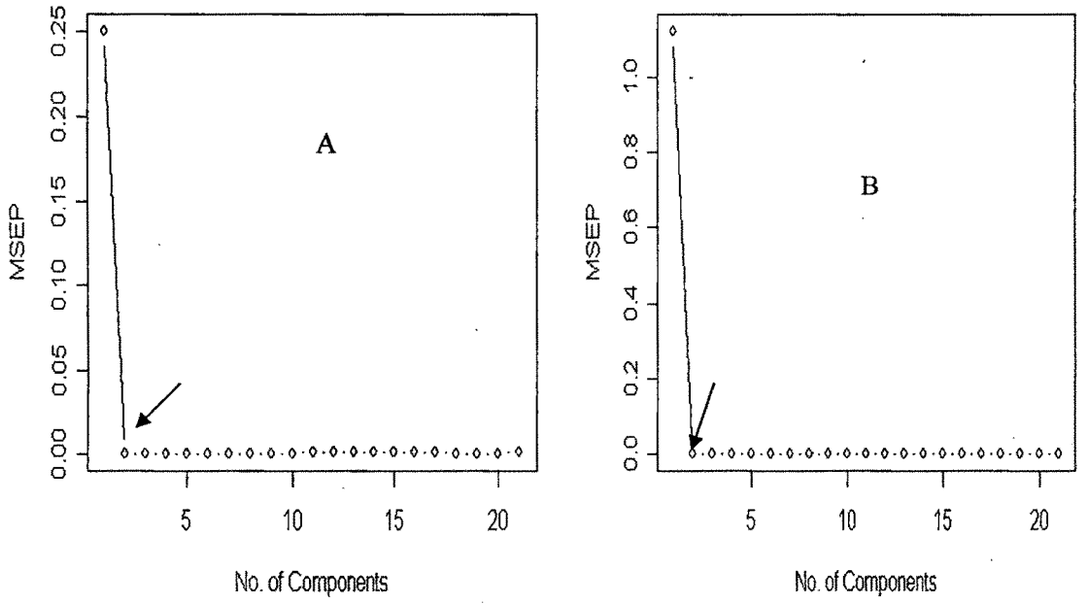


Fig. 6. 10 MSEP plots of a calibration set obtained using leave-one-out (LOO) cross validation of PCR-model for A) ATOR and B) AMLO in first derivative absorption data

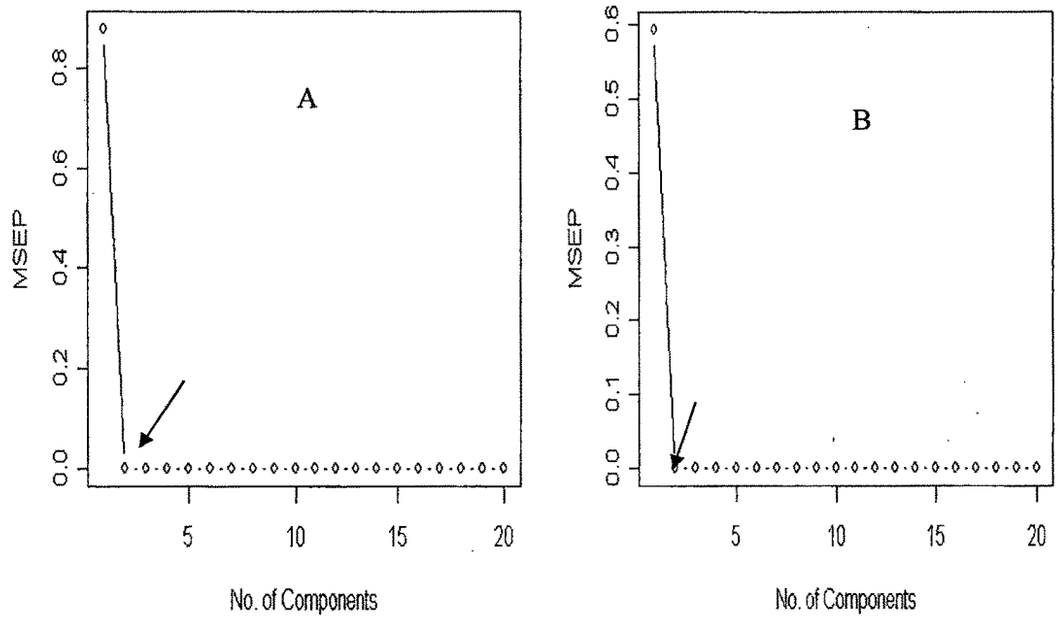


Fig. 6. 11MSEP plots of a calibration set obtained using leave-one-out (LOO) cross validation of PLS-model for A) ATOR and B) AMLO in first derivative absorption data

Range

The calibration range of the proposed RFD, HPTLC and chemometric methods was established through wide consideration of the practical range necessary, according to each ingredient concentration present in pharmaceutical products of different manufacturers.

Recovery

The interference of excipients and tablet additives for two drugs was tested for the application of proposed methods to commercial tablet formulation, and no interference was observed according to the experimental results of RFD, HPTLC and chemometrics with both zero-order and first derivative spectra. For this reason, the standard solutions of pure drugs corresponding to tablet content were added to the tablet solutions and analysed. In this case, mean recovery and relative standard deviation of CLS, ILS, PCR and PLS calibrations were found satisfactory for the validity of the proposed methods (Table 6. 7 (a) and 6. 7 (b)).

In HPTLC the binary mixture of the sample was applied on TLC plate to obtain the concentration of 500, 600 and 700 ng of ATOR, 250, 300 and 350 ng of AMLO were flanked between standards and chromatogram was recorded (Fig. 6. 12). The results revealed that the amounts of ATOR and AMLO in their mixture can be determined without prior separation and without any interference from formulation excipients.

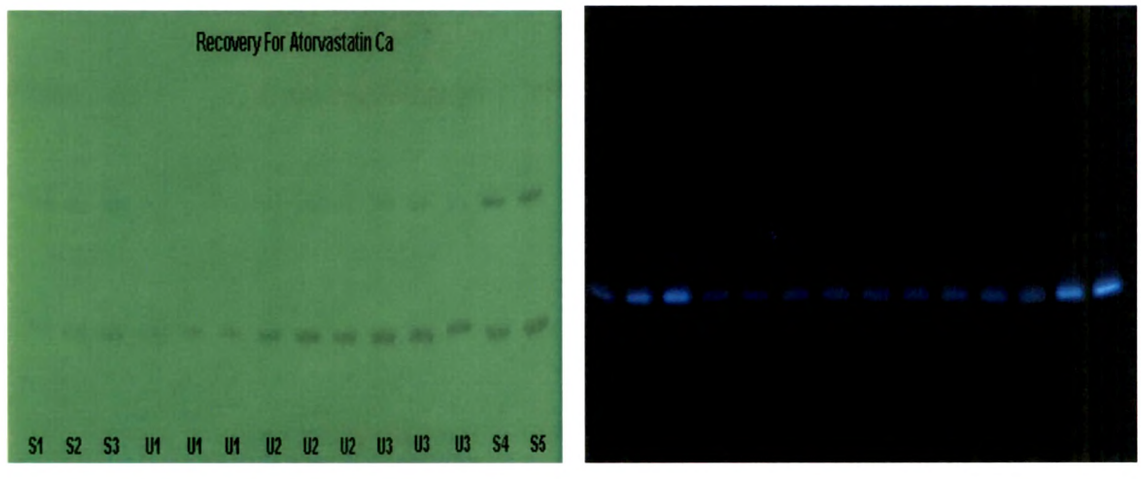


Fig. 6. 12 (a) HPTLC scanned UV-image at 254 nm and fluorescent images of standard and sample dilutions of ATOR and AMLO for recovery studies

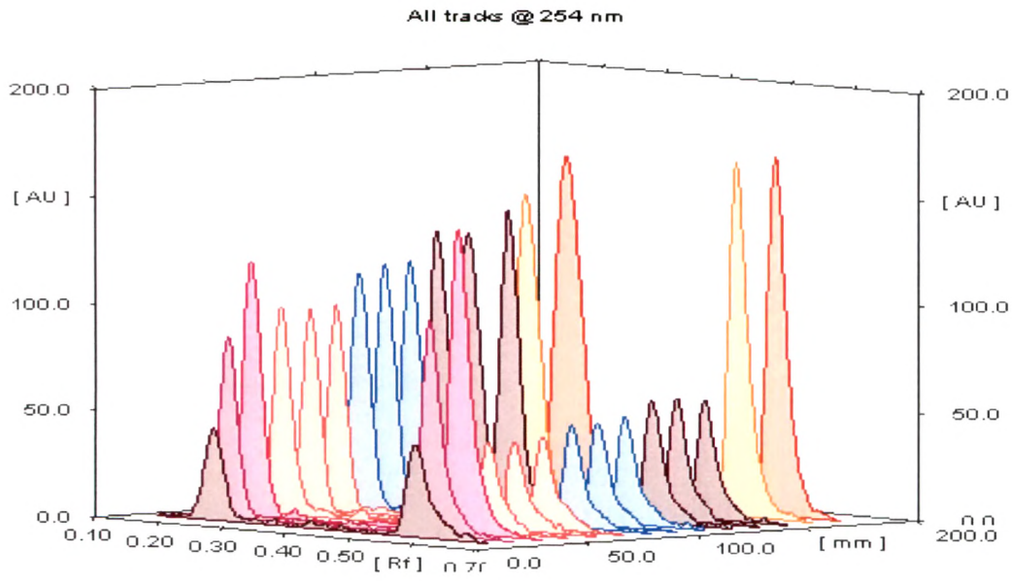


Fig. 6. 12 (b) 3-Dimensional HPTLC chromatogram of ATOR and AMLO binary mixtures used for recovery studies

Table 6. 7 (a) Results obtained for Accuracy studies by using proposed chemometric methods

Methods	Zero order spectra				First derivative spectra			
	CLS	ILS	PCR	PLS	CLS	ILS	PCR	PLS
ATOR	101.13	98.35	101.41	101.4	104.1	104.12	104.26	104.26
mean[a] ± RSD[b]	± 0.489	± 0.356	± 0.3448	± 0.3446	± 1.033	± 0.349	± 1.0183	± 1.0182
AMLO	99.32	107.36	101.55	101.55	102.51	107.97	103.61	103.61
mean[a] ± RSD[b]	± 1.626	± 2.641	± 1.5337	± 1.5336	± 0.255	± 0.798	± 1.6635	± 1.6638

a, mean recovery value of five determinations for each method

b, Relative standard deviation

Table 6. 7 (b) Results obtained for Accuracy studies by using proposed RDF and HPTLC methods

Methods	RFD	HPTLC
ATOR mean[a] ± RSD[b]	100.91 ± 0.389	103.71 ± 1.053
AMLO mean[a] ± RSD[b]	98.99 ± 1.746	101.11 ± 0.273

Specificity

A representative three dimensional HPTLC chromatogram (Fig. 6.13) was obtained using diluents, mobile phase, placebo, ATOR standard, AMLO standard, ATOR + AMLO + diluents and ATOR + AMLO +diluents + placebo demonstrating the high degree of selectivity and that the peak of interest is attributed only to analytes, no endogenous interference was observed at the retention time of analytes. Similarly no interference was observed in RFD and chemometric methods.

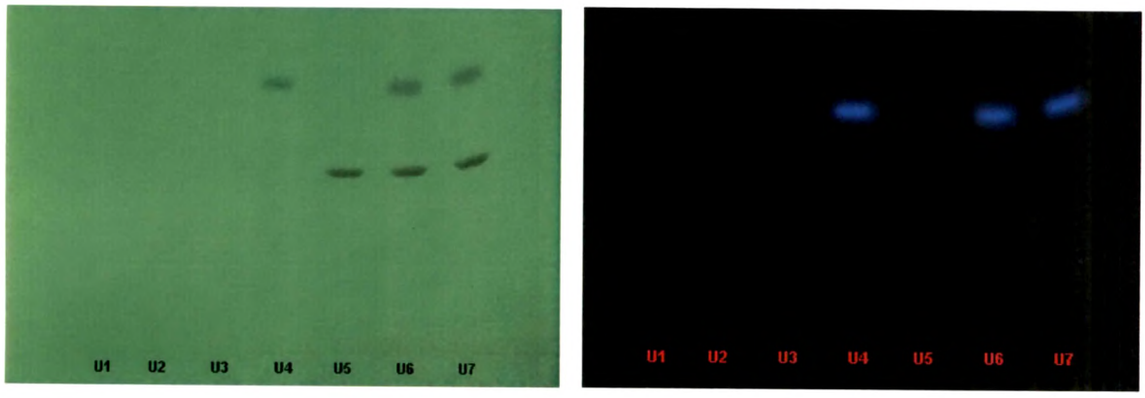


Fig. 6. 13 (a) HPTLC scanned UV-image at 254 nm and fluorescent images of diluents, mobile phase, placebo, AMLO standard, ATOR standard, ATOR + AMLO + diluents and ATOR + AMLO +diluents + placebo in specificity studies

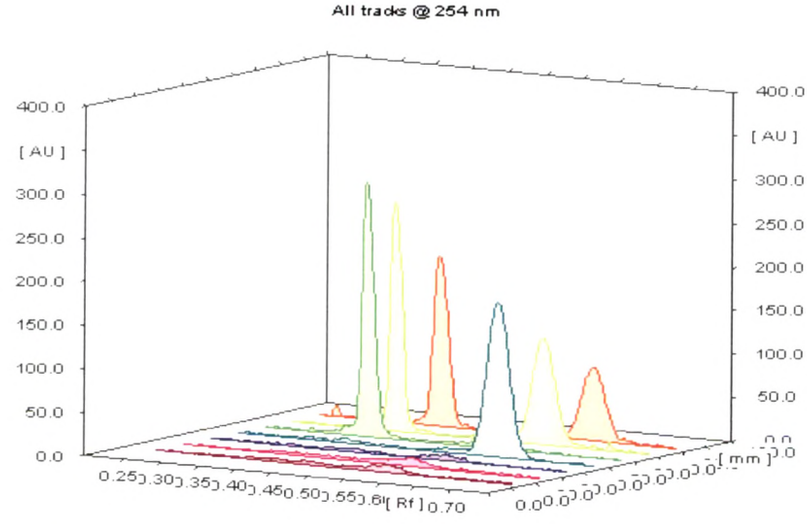


Fig. 6. 13 (b) 3-Dimensional HPTLC chromatogram of diluents, mobile phase, placebo, AMLO standard, ATOR standard, ATOR + AMLO + diluents and ATOR + AMLO +diluents + placebo in specificity studies

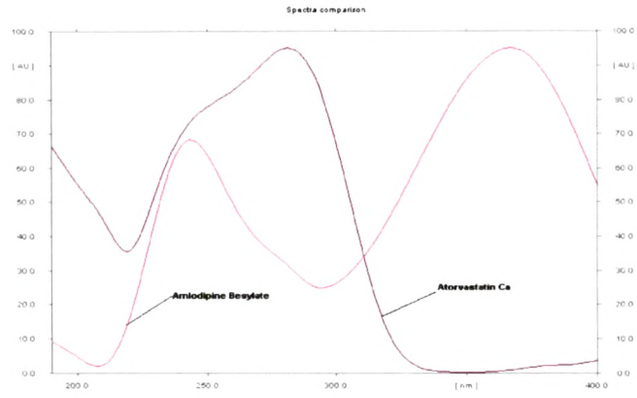


Fig. 6. 14 Overlay absorption spectra of ATOR and AMLO scanned on TLC plate at 254 nm

Precision (Method reproducibility)

Method reproducibility was demonstrated by repeatability and intermediate precision measurements of peak area, retention factor parameters of HPTLC (Fig. 6. 15) and % recovery RSD in RFD and chemometric methods for each title ingredient.

The repeatability (within-day in triplicates) and intermediate precision (for 3 days) was carried out at three concentration levels for each compound. The obtained results within and between day's trials are in acceptable range indicating good precision of the proposed methods (Table 6. 8).

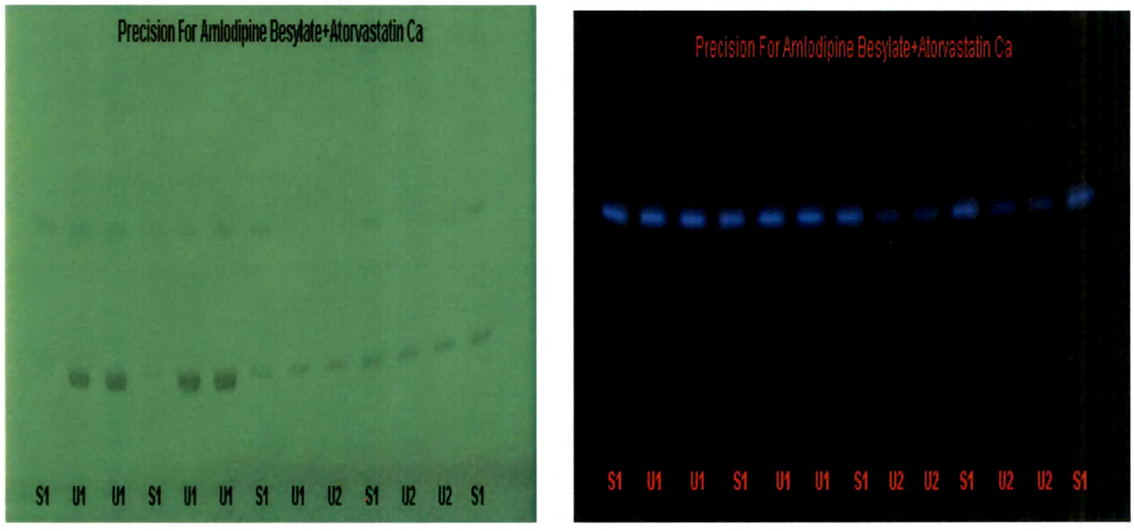


Fig. 6. 15 (a) HPTLC scanned UV-image at 254 nm and fluorescent images of standard and sample dilutions of ATOR and AMLO for precision studies

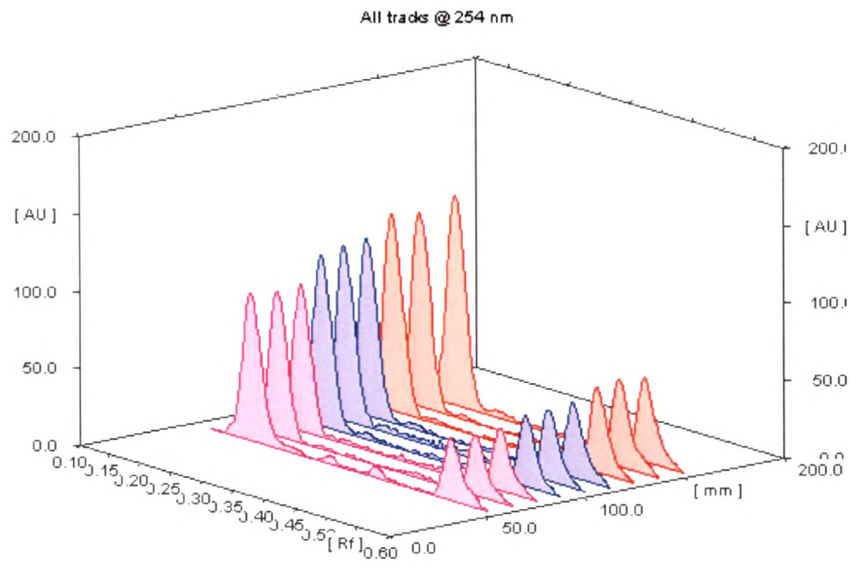


Fig. 6. 15 (b) 3-Dimensional HPTLC chromatogram of 500, 600 and 700 ng spot^{-1} of ATOR and 250, 300 and 350 ng spot^{-1} AMLO binary mixtures used for precision studies

Table 6. 8 Precision study results of prepared binary mixture

Validation parameter	RFD % RSD	Chemometric Zero-order % RSD				Chemometric Ist derivative % RSD				HPTLC % RSD	
Repeatability[a]		CLS	ILS	PCR	PLS	CLS	ILS	PCR	PLS	Peak area	R_f
AMLO	0.974 ±0.0	0.736±0.00	0.529 ±0.01	0.109 ±0.03	0.946 ±0.04	2.17± 0.03	1.714± 0.04	1.654± 0.03	0.91± 0.03	1.376 ±0.03	0.927±0 .04
ATOR	1.365 ±0.00	1.037±0.03	0.199 ±0.04	0.918 ±0.09	0.913 ±0.03	2.242 ±0.03 6	0.357± 0.06	0.397± 0.04	0.698 ±0.03	1.389 ±0.04	0.947±0 .04
Intermediate precision[b]											
AMLO	1.627 ±0.03	1.499±0.04	0.787 ±0.03 1	0.498 ±0.04 1	0.910 ±0.01 8	2.346 ±0.08	1.036± 0.027	1.529± 0.036	0.932 ±0.01 4	0.783 ±0.03	0.899±0 .01
ATOR	0.935 ±0.02 1	1.767±0.02	0.729 ±0.03 6	0.099 ±0.04 8	1.027 ±0.01 8	1.939 ±0.03	0.989± 0.01	0.289± 0.02	1.128 ±0.04	0.982 ±0.02	1.837±0 .02

[a] Repeatability, three replicates of three concentration levels within-day.

[b] Intermediate precision, three replicates of three concentration levels between-days (3-days).

Robustness

The robustness of the proposed HPTLC method was assessed for peak area and retention factor (Table 6. 9) by purposely altering the HPTLC conditions:

- Composition of ethyl acetate in mobile phase (± 1%)

- Detection wavelength (± 3)
- Chamber saturation time (± 2)

In spectrophotometric methods Double-beam Shimadzu (Japan) UV-vis Spectrophotometer (model UV-1700 and 1601) were used to access the robustness. The digital absorbances recorded by both the instruments did not have significant effect on the determination of title drugs.

Analytes solution and mobile phase stability

Stability of ATOR and AMLO in solutions within linear concentration was studied by keeping the solutions at room temperature for seven days during validation process of proposed methods. Content of both ingredients was checked by proposed HPTLC method using same mobile phase and spectrophotometric methods at 6h interval and all the solutions were found to be stable for seven days. No interfering substances were found.

Table 6. 9 Robustness of HPTLC method

Parameter	Peak area ± % RSD		Retention factor	
	ATOR	AMLO	ATOR	AMLO
Mobile phase ethyl acetate composition				
9	2765±0.045	2875±0.046	0.12±0.04	0.50±0.06
10	2847±0.068	2994±0.059	0.14±0.02	0.52±0.03
11	269 ±0.043	2858±0.036	0.13±0.01	0.54±0.05
Change in detection wavelength				
251	2586±0.051	2732±0.031	0.11±0.03	0.50±0.04
254	2847±0.068	2994±0.059	0.14±0.02	0.52±0.03
257	2755±0.052	2637±0.042	0.13±0.01	0.53±0.02
Chamber saturation time (in min)				
08	2609 ±0.062	2980±0.049	0.13±0.02	0.55±0.04
10	2847±0.068	2994±0.059	0.14±0.02	0.52±0.03
12	2366±0.056	2703±0.025	0.15±0.03	0.53±0.02

- Average of three experiments

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

The LOD and LOQ are calculated according to ICH recommendations where the approach based on the S.D of the response of blank and the slope of the calibration curve. LOD and LOQ for spectrophotometric methods were found to be 0.31 μg mL^{-1} and 0.36 μg mL^{-1} for ATOR and 0.87 μg mL^{-1} and 1.14 μg mL^{-1} for AMLO respectively. Similarly for HPTLC (Fig. 6. 16)

method LOD and LOQ were found to be 0.040 µg mL^{-1} and 0.06 µg mL^{-1} for ATOR and AMLO respectively.

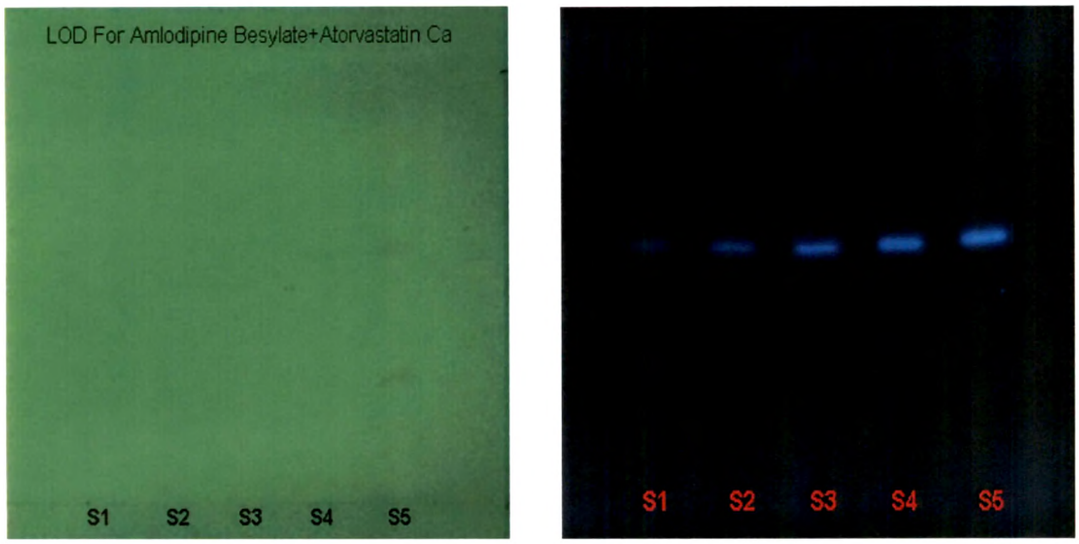


Fig. 6. 16 (a) UV 254 nm and fluorescent images of ATOR and AMLO for LOD and LOQ studies

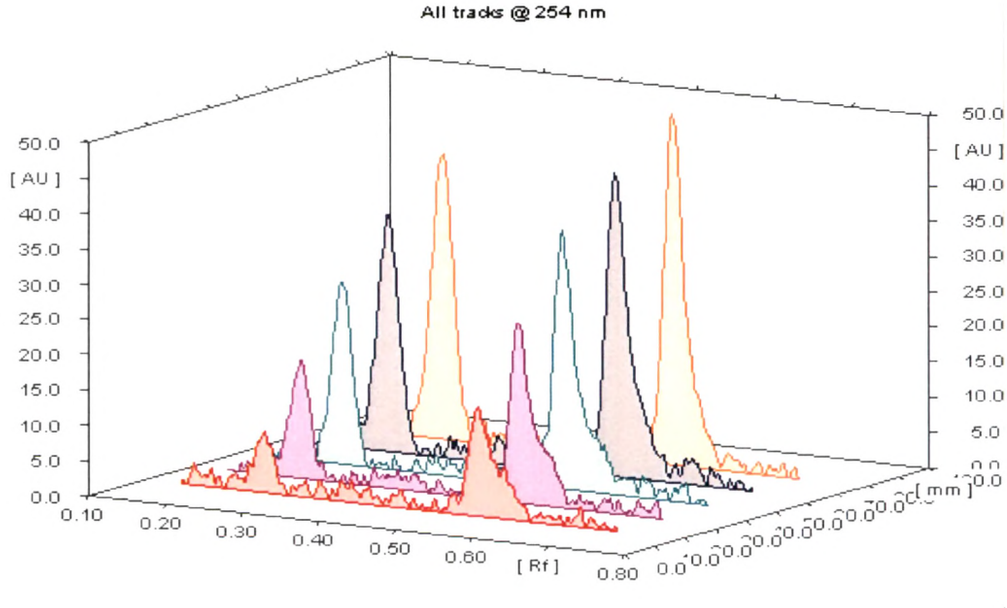


Fig. 6. 16 (b) 3-Dimensional HPTLC chromatogram of ATOR and AMLO binary mixtures used for LOD and LOQ studies

Application of proposed methods for pharmaceutical formulations

Twenty Caduet and Lipikind tablets were weighed accurately and powdered. An amount of the powder equivalent 10 mg ATOR and 05 mg AMLO (content of one tablet) was dissolved in 60 mL of methanol. The solution was sonicated for 10 mins, filtered into 100 mL volumetric flask through 0.45µ nylon membrane filter. The residue was washed three times with 10 mL of solvent then the volume was completed to 100 mL with the same solvent. This solution was further diluted to desired working range with methanol. All the proposed RFD, chemometric and HPTLC methods were applied to the solutions. The experimental results of the proposed methods on commercial tablet formulation were presented in Table 6. 10 (a) and 6. 10 (b). The results of all methods were very close to each other as well as to the label value of commercial pharmaceutical formulations.

In addition the results obtained by proposed methods were statistically compared between HPTLC methods at the 95% confidence level with the aid student't-test and F-tests. The calculated t and F values never exceed the theoretical t- and F- values, at 0.05 level of significant difference. Therefore, these statistical tests denote no significant difference in the results achieved by RFD, HPTLC and the four chemometric methods on zero-order and first derivative spectra.

Table 6. 10 (a) Comparison of results obtained for the LIPIKIND tablets by using chemometric calibrations and HPTLC method

Methods	Chemometric methods: Zero order spectra				RFD	Chemometric methods: First derivative spectra				HPTLC
	CLS	ILS	PCR	PLS		CLS	ILS	PCR	PLS	
ATOR mean[a] ± SD[b]	101.13 ± 0.495	100.17 ± 0.659	100.01 ± 0.773	100.78 ± 0.377	100.78 ± 0.376	100.9 ± 0.684	100.68 ± 0.634	101.06 ± 0.608	101.06 ± 0.609	100.39 ± 0.334
F	1.172	3.066	4.212	4.208	4.32	4.236	4.236	3.308	3.308	
t	1.254	1.8146	0.59	0.581	2.11	1.484	0.895	2.134	2.141	
AMLO mean[a] ± SD[b]	99.52 ± 1.521	102.16 ± 1.397	101.74 ± 1.531	101.74 ± 1.531	101.81 ± 1.645	100.77 ± 0.973	101.15 ± 1.159	101.15 ± 0.891	101.51 ± 0.891	101.83 ± 0.722
F	1.17	1.386	1.1545	1.1545	5.32	1.815	2.5768	1.5251	1.5247	
t	2.28	0.366	0.068	0.0683	2.01	1.963	1.1132	0.6267	0.626	

(label claim: 10 mg of ATOR and 5 mg AMLO per tablet)
a, Mean recovery value of five determinations for each method, b, Standard deviation
($n_1 = n_2 = 5$), Theoretical values for *t* and F at P = 0.05 are 2.31 and 6.39 respectively

Table 6. 10 (b) Comparison of results obtained for the Caduet tablets by using chemometric calibrations and HPTLC method

Methods	Chemometric methods: Zero order spectra				RFD	Chemometric methods: First derivative spectra				HPTLC
	CLS	ILS	PCR	PLS		CLS	ILS	PCR	PLS	
ATOR mean[a] ± SD[b]	98.37 ± 0.32	96.34 ± 0.67	100.52 ± 0.68	100.78 ± 0.63	96.33 ± 0.876	98.07 ± 0.92	96.14 ± 0.57	102.32 ± 0.61	102.75 ± 0.99	100.39 ± 0.33
F	1.142	3.52	4.55	4.58	4.29	1.177	3.59	4.27	4.38	
t	1.33	1.79	0.67	0.72	2.18	1.03	1.99	1.68	1.94	
AMLO mean[a] ± SD[b]	96.3 ± 1.88	96.7 ± 1.54	101.97 ± 1.73	102.38 ± 1.79	105.10 ± 1.51	98.3 ± 1.01	98.9 ± 1.50	104.3 ± 1.59	104.28 ± 1.79	101.83 ± 0.72
F	1.19	1.35	1.19	1.153	4.01	4.38	4..98	5.16	5.44	
t	2.08	1.36	2.06	2.09	2.50	2.09	1.88	2.29	2.24	

(label claim: 10 mg of ATOR and 5 mg AMLO per tablet)
a, Mean recovery value of five determinations for each method, b, Standard deviation
($n_1 = n_2 = 5$), Theoretical values for *t* and F at P = 0.05 are 2.31 and 6.39 respectively