Simultaneous estimation of Atorvastatin and Ramipril
Results and discussion

Chapter 8

8.1 Reagents and Pharmaceutical preparations

Ramipril (RAMP) and Atorvastatin (ATOR) were donated by Dr. Reddy's Laboratories Limited (Hyderabad, AP, India) and Biocon, Ltd (Bangalore, KRN, India) certified to contain 99.6% and 99.92% purity respectively. The drugs were used without further purification. All the solvents used in analysis were of spectroscopic and HPLC grade (Ranbaxy Fine Chemicals Limited, New Delhi, India). Stator-R 2.5 tablets (label claim 2.5 mg RAMP and 10 mg ATOR) batch No. 6004 of Accent Pharma (Pondicherry, TN, India) and Rampitor*5 capsules (label claim 5 mg RAMP and 10 mg ATOR) batch No. AF 70027 of Atoz Life Sciences (Pondicherry, TN, India) was used in analysis.

8.2 Procedure

Standard stock and working solution for spectrophotometric and HPLC methods

1. ATOR stock solution: 1 mg mL^{-1} in methanol
2. ATOR working solution: 0.04 mg mL^{-1} in acetonitrile: 0.1M sodium perchlorate (70:30, v/v), prepared by transferring 2.0 mL from stock solution of ATOR to a measuring flask 50 mL and completing to volume with same solvent.
3. RAMP stock solution: 1 mg mL^{-1} in methanol
4. RAMP working solution: 0.04 mg mL^{-1} in acetonitrile: 0.1M sodium perchlorate (70:30, v/v) prepared by transferring 2.0 mL from stock solution of RAMP to a measuring flask 50 mL and completing to volume with same solvent.

Similarly the working solutions for HPTLC were 100 ng μL^{-1} for each ingredient prepared from respective stock solutions in a 10 mL volumetric flask and completing to volume with methanol.

Preparation of mobile phase

- **HPLC**

A solution of 0.1M sodium perchlorate was prepared in dissolving 14.046gms in 1000 mL of HPLC grade water. pH of the resulting solution was adjusted to 2.5 ± 0.2 by using 85% orthophosphoric acid. HPLC experiments were carried out using binary

pump. In one solvent reservoir acetonitrile and in another reservoir 0.1M sodium perchlorate was taken.

HPLC method

The mobile phase acetonitrile: 0.1M sodium perchlorate (pH 2.5) (70:30, v/v) was selected because it was found ideal to resolve the peaks with retention time (R_t) 2.275 ± 0.003 min and 3.178 ± 0.002 min for RAMP and ATOR respectively (Fig. 8. 5). Detection wavelength 210 nm was selected by scanning both standard ingredients over a wide range of wavelength 201 nm to 350 nm in spectrophotometer. The overlay UV-spectra of both the components and their binary mixture were shown (see Fig. 8. 1 (a)).

- **HPTLC**

Mobile phase consisting of chloform : methanol : ammonia (8:2:0.2, v/v/v) prepared to 20ml. Linear ascending development was carried out in twin trough glass chamber saturated with mobile phase at room temperature (25 ± 2°C). This mobile phase was found effective in resolving RAMP and ATOR with retention factor 0.45and 0.32 respectively (see Fig. 8. 9 (a) and 8. 9 (b)). Detection wavelength was 210 nm and 254 nm for RAMP and ATOR respectively.

Pharmaceutical sample solution

Twenty Stator-R tablets and Rampitor*5 capsules were weighed accurately. An amount of the powder equivalent to content of one unit of tablet and capsule was dissolved separately in 60 mL of methanol. The solutions were sonicated for 30 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 mobile phase, and then the volume was completed to 100 mL with the same solvent. These solutions were further diluted to 1:10 with mobile phase. The proposed Spectrophotometric, chemometric, RP-HPLC and HPTLC method was applied and the concentration of each component in both the formulations was determined.

Spectrophotometric methods

Calibration sets for simultaneous equation, first derivative zero-crossing and ratio first derivative methods

A calibration set containing seven dilutions each of ATOR (4-22 μg mL^{-1}) and RAMP (4-32 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 25 sample mixtures was prepared in acetonitrile: 0.1M sodium perchlorate (pH 2.5) (70:30, v/v), applying a multilevel multifactor design in which five levels of concentrations of RAMP and ATOR were introduced. The levels were in the range of 4-32 and 4-22 μg mL^{-1} for RAMP and ATOR respectively (Table 8.1). UV spectra were recorded in the wavelength range 201-270 nm versus solvent blank and digitised absorbances were recorded at 1 nm intervals (Fig. 8. 11). The computation was made in R-software environment. CLS, PCR and PLS algorithms were applied to the UV absorption data matrix of these binary mixtures to determine calibration equations.

HPLC calibration

The calibration study was carried out individually for both the ingredients at seven different concentration levels using either ingredient as internal standard during calibration of the other. Aliquots of standard RAMP working solutions were taken in different volumetric flasks and 8 μg mL^{-1} of ATOR was added to each flask as internal standard and diluted with mobile phase such that the final concentration of RAMP were in the range of 4-32 μg mL^{-1} (Fig. 8. 6 (a)). Similarly ATOR working solutions were taken in different volumetric flasks and 8 μg mL^{-1} of RAMP was added to each flask as internal standard and diluted with mobile phase such that the final concentration of ATOR was in the range of 4-22 μg mL^{-1} (Fig. 8. 6 (b)). All stock and working solutions were sonicated for 5 min, then filtered through a nylon membrane filter (0.45μ) prior to use. Triplicate 20 μL injections were made for each

concentration and chromatographed under specified condition at ambient temperature (28^0C). The peak area response ratio of the internal standard to pure analytes is determined beforehand and values obtained were plotted against corresponding concentrations. Regression analysis of the calibration data was then carried out (Table 8. 9).

HPTLC calibration

Different volume of working solution 2, 4, 6, 8, 10, and 12 μL $spot^{-1}$ of ATOR injection were made to obtain a concentration range 200-1200ng $spot^{-1}$. Similarly 2, 4, 6, 8, 10, 12 and 14 μL $spot^{-1}$ of RAMP was made to obtain a concentration range 200-1400ng $spot^{-1}$. The above solutions were spotted in three replicate on TLC plate. Densitometric scanning was performed in the absorbance mode at 210 nm and 254 nm for the estimation of RAMP and ATOR respectively Fig 8.10 (a) and 8.10 (b). The data of peak area versus drug concentrations were treated by polynomial regression mode (Table 8. 9).

Preparation of binary mixtures for spectrophotometric and HPLC predictions

Applying multilevel multifactorial design in which four level concentrations of ATOR and RAMP within the stated range were introduced and prepared 16 synthetic binary mixtures of titled ingredients as shown in Table 8. 2.

8.3 Results and discussion

Spectrophotometric methods (*simultaneous equation, first derivative zero-crossing and ratio first derivative methods*)

8.3.1 Simultaneous equation method (SEM)

A calibration set of seven dilutions each of ATOR (4-22 μg mL^{-1}) and RAMP (4-32 μg mL^{-1}) in acetonitrile: 0.1M sodium perchlorate (70:30 v/v) was prepared and UV spectra were recorded in the wavelength range 200-350nm versus solvent blank. The

overlay zero-order UV absorption spectra of standard solutions of ATOR and RAMP are shown in the Fig. 8.1 (a) and 8.1 (b).

Table 8.1 Concentration data for the different mixtures used in the calibration set and internal validation for the determination of RAMP and ATOR using chemometric methods

Mixture No.	Concentration ($\mu g\ mL^{-1}$)	
	RAMP	ATOR
1	4	4
2	4	8
3	4	12
4	4	16
5	4	22
6	8	4
7	8	8
8	8	12
9	8	16
10	8	22
11	16	4
12	16	8
13	16	12
14	16	16
15	16	22
16	24	4
17	24	8
18	24	12
19	24	16
20	24	22
21	32	4
22	32	8
23	32	12
24	32	16
25	32	22

Table 8.2 Composition of binary mixture for chromatographic and spectrophotometric predictions

Mixture No.	Concentration (μg mL^{-1})	
	RAMP	ATOR
1	4	4
2	4	8
3	4	16
4	4	22
5	8	4
6	8	8
7	8	16
8	8	22
9	24	4
10	24	8
11	24	16
12	24	22
13	32	4
14	32	8
15	32	16
16	32	22

Absorbencies of the above standard solutions of ATOR and RAMP were measured at two wavelengths 207 and 247nm, to get the absorptivity values at both wavelengths for both the drugs from the equation:

$$E = \frac{A}{bC} * 10000$$ Absorbance unit cm^{-1} gm 100 mL^{-1}

E = Absorptivity value, A = Absorbance, b = Path length of quartz cell (1 cm),
C = concentration in μg mL^{-1}

Binary mixture solutions for prediction of ATOR and RAMP were prepared as shown in Table 2. Absorbencies of binary mixture solutions were measured at 207 and 247nm. The concentration of each component of the binary mixture was calculated by using simultaneous equation.

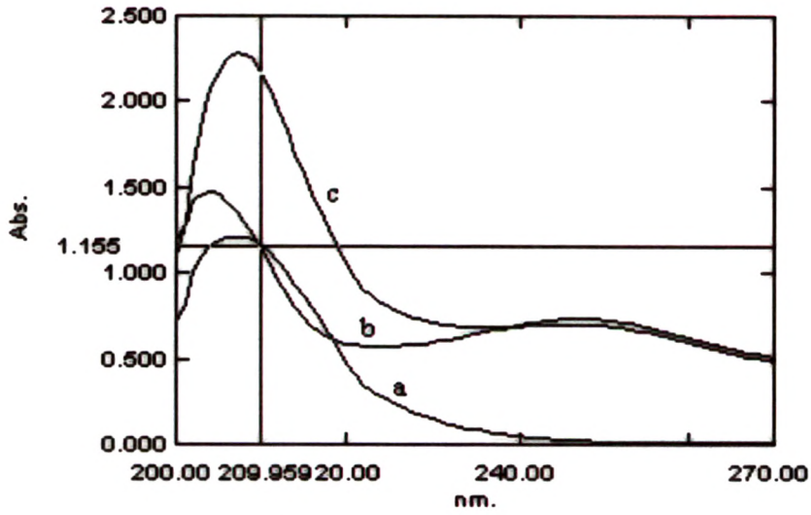

Fig. 8. 1 (a) Zero-order overlay absorption spectra: a) 32 µg mL^{-1} of RAMP, b) 16 µg mL^{-1} of ATOR and c) their binary mixture

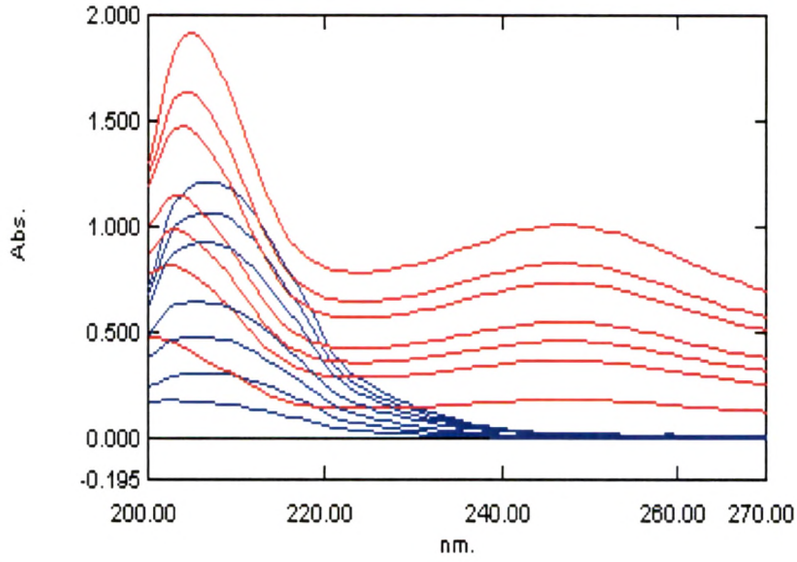

Fig. 8. 1 (b) Overlay zero-order absorption spectra for standard dilutions of ATOR and RAMP

$$C_{RAMP} = \frac{Am_{207} * E_{ATOR247} - Am_{247} * E_{ATOR207}}{E_{RAMP207} * E_{ATOR247} - E_{ATOR207} * E_{RAMP247}}$$

$$C_{ATOR} = \frac{Am_{207} * E_{RAMP247} - Am_{247} * E_{RAMP207}}{E_{RAMP247} * E_{ATOR207} - E_{ATOR247} * E_{RAMP207}}$$

C_x = concentration of x,
Am_n= Absorbance of Sample solution at 'n' nm,
E_{xn} = Absorptivity of x at 'n'

For simultaneous determination of ATOR and RAMP in their binary mixture a simultaneous equation method was successfully developed. Standard solutions of ATOR and RAMP were prepared to determine their absorptivity values at two selected λmax 247nm and 207 nm. The absorptivity values ± SD at 247 and 207 nm for both the drugs were found to be $E_{RAMP247}$ = 7.260 ± 1.351, $E_{RAMP207}$ = 390.852 ± 6.4195, $E_{ATOR247}$ = 458.922 ± 1.1535 and $E_{ATOR207}$ = 860.032 ± 10.252.

8.3.2 First derivative Zero-crossing method (FDZC)

A calibration set of seven dilutions each of ATOR (4-22 μg mL^{-1}) and RAMP (4-32 μg mL^{-1}) in acetonitrile : 0.1M sodium perchlorate (70:30 v/v) was prepared and UV spectra were recorded and converted the same to first derivative spectra in the wavelength range 200-350nm versus solvent blank. The first derivative overlay absorption spectra of standard solutions of ATOR and RAMP was shown in the Fig. 8.2.

Preparation of calibration curve

The absorption spectra of working standard solutions of ATOR and RAMP were recorded in the range of 200-350 nm in triplicates and stored in the memory of the instrument. The first derivative of the working standard solution were traced

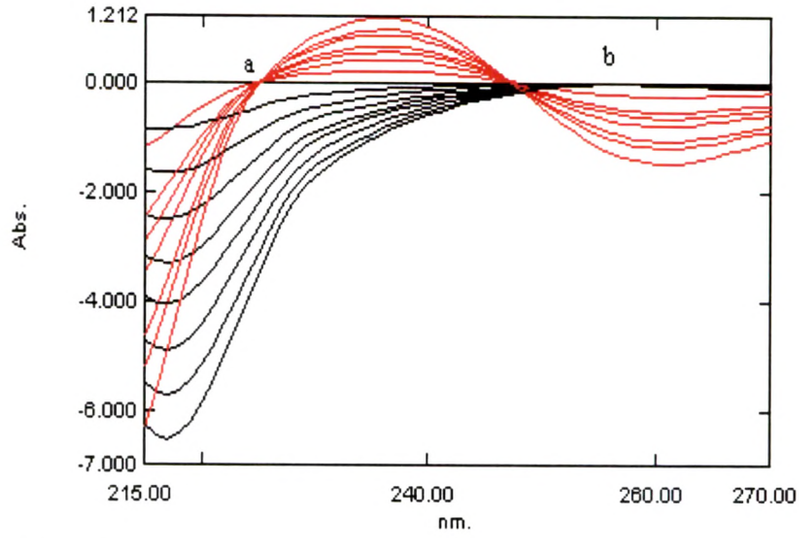

Fig. 8. 2 Overlay first derivative absorption spectra of standard dilutions of ATOR and RAMP, 'a' represents the zero crossing of ATOR at 225nm and 'b' represents the zero crossing of RAMP at 257nm.

with smoothing factor ($\Delta\lambda$ = 10) and scaling factor (=100) for determining the zero cross points for both the drugs. It was found that the first derivative spectrum of ATOR crosses zero at 225 nm and that of RAMP crosses zero at 257 nm. The amplitudes at 257 nm were plotted against the respective concentrations of ATOR. It was found that 257 nm shows the good linearity for the determination of ATOR. The method shows good linearity in the range of 4 - 22 $\mu g\ mL^{-1}$ for ATOR. Similarly the amplitudes at 225 nm were plotted against the respective concentrations of RAMP. It was found that 225 nm shows the good linearity for the determination of RAMP. The method shows good linearity in the range of 4 - 32 $\mu g\ mL^{-1}$ for RAMP (Fig. 8. 2).

Spectrophotometric first derivative zero crossing method was successfully developed for simultaneous determination of ATOR and RAMP from their binary mixture. The results obtained are discussed below.

Optimization and selection of method parameters

All the optimized method parameters are summarized in Table 8.3. Acetonitrile: 0.1M sodium perchlorate (70:30 v/v) was selected as solvent, 257 nm was selected for the determination of ATOR as the first derivative spectra of RAMP shows zero amplitude (zero cross) at 257 nm. Similarly 225 nm was selected for the determination of RAMP as the first derivative spectra of ATOR shows zero amplitude (zero cross) at 225 nm (Fig. 8. 2).

8.3.3 Ratio spectra first derivative spectrophotometry (RFD)

Ratio 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 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 (2800 nm min^{-1}), smoothing factor ($\Delta\lambda$ = 10) and scaling factor (=10) was selected. Divisor concentration is the main instrumental parameter; the standard spectrum of 10 µg mL^{-1} of ATOR and 16 µg mL^{-1} of RAMP was considered as divisor for the determination of RAMP and ATOR respectively in their mixture.

The first derivative ratio spectra of different ATOR standards at increasing concentrations in acetonitrile: 0.1M sodium perchlorate (70:30 v/v) was obtained by dividing each with the stored spectrum of the standard solution of 16 µg ml^{-1} of RAMP by a computer aid as divisor spectra. These ratio spectra are shown in Fig. 8.3 (A). The first derivative (1DD) of these spectra traced with interval of $\Delta\lambda$ = 10 nm and scaling factor (=10) are illustrated in Fig. 8. 3 (B). As seen in Fig. 8. 3 (B) two minima (267 nm and 296 nm) and two maxima (242 nm and 269nm) exist and we found that all the wavelengths were suitable for the determination of ATOR in ATOR and RAMP mixtures, but good linearity was obtained at 269 nm. The wavelength of 269 nm was selected for the determination of ATOR in the assay of synthetic

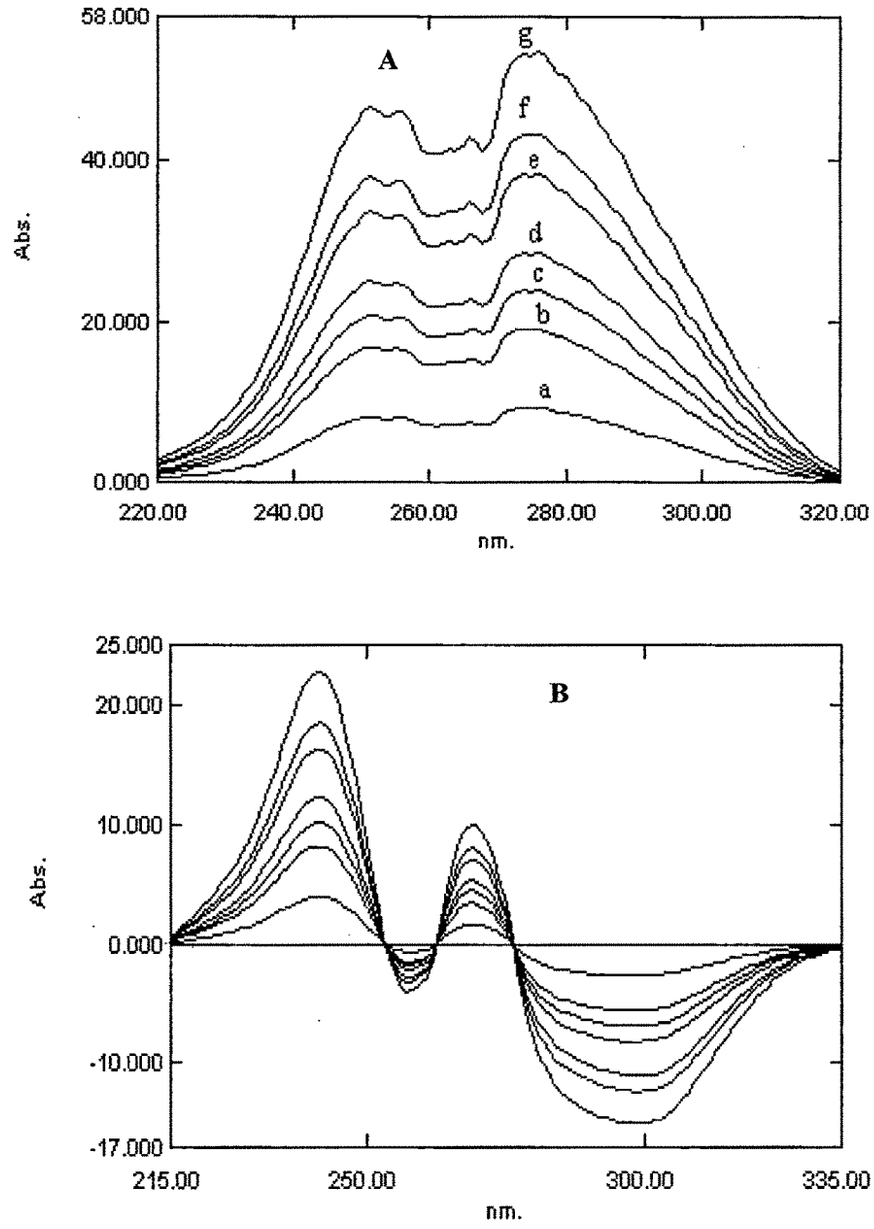

Fig. 8. 3 Ratio spectra (A) and first derivative of the ratio spectra (B): a) 4, b) 8, c) 10, d) 12, e) 16, f) 18, g) 22 µg mL^{-1} solution of ATOR when 16 µg mL^{-1} of RAMP used as divisor ($\Delta\lambda$ = 10 nm); scaling factor, 10

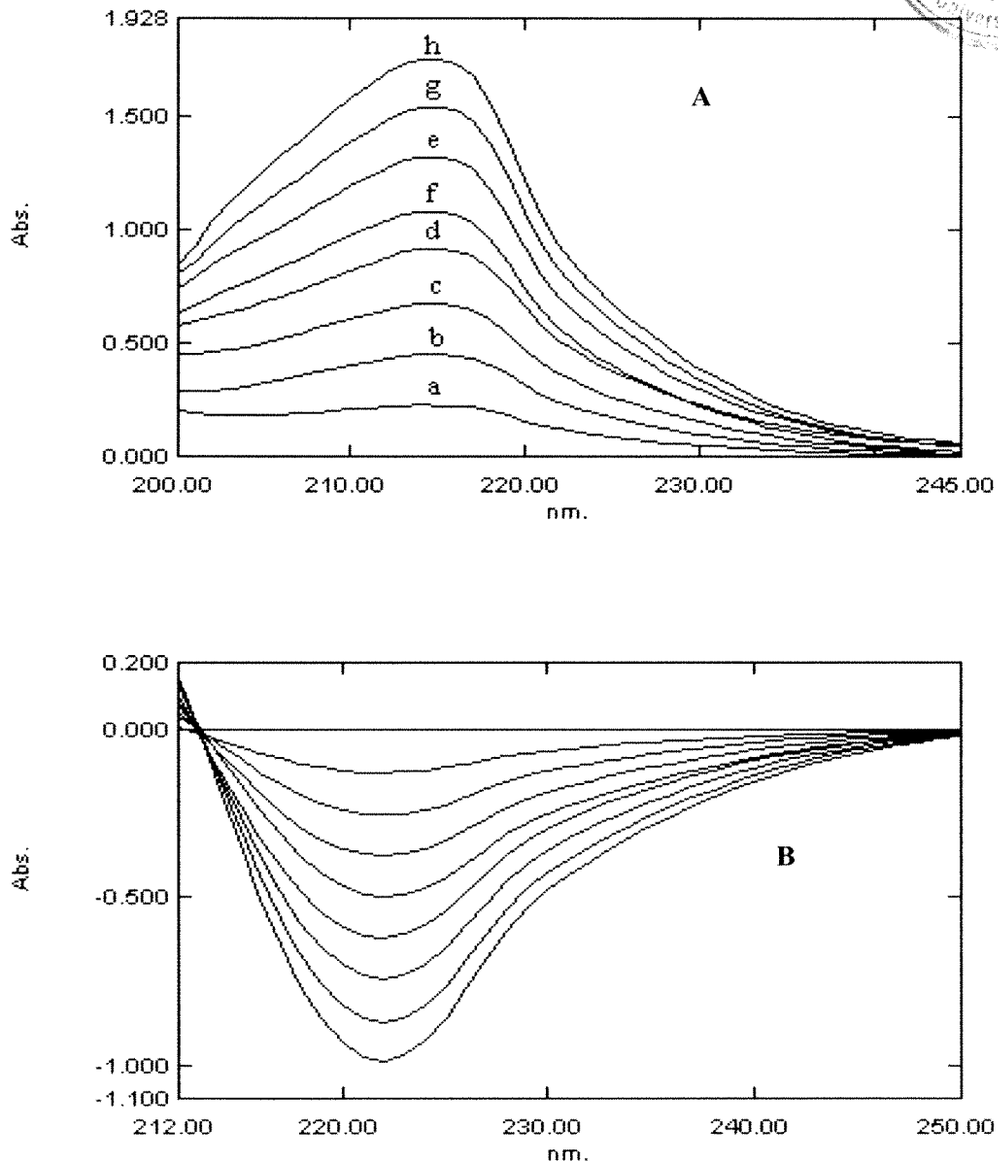

Fig. 8. 4 Ratio spectra (A) and first derivative of the ratio spectra (B): a) 4, b) 8, c) 12, d) 16, e) 20, f) 24, g) 28, h) 32 μg mL^{-1} solutions of RAMP when 10 μg mL^{-1} of ATOR used as divisor (Δλ = 10 nm); scaling factor 10

mixtures and commercial formulations, due to its lower RSD values and more suitable mean recovery compared with other wavelength. For the determination of

RAMP, the ratio spectra of different RAMP standards at increasing concentrations in acetonitrile: 0.1M sodium perchlorate (70:30, v/v) was obtained by dividing each with stored spectrum of the standard solution of 10 μg mL^{-1} of ATOR as divisor spectra by computer aid, are demonstrated in Fig. 8. 4 (A). The first derivatives (1DD) of this spectrum traced with intervals of $\Delta\lambda$ = 10 nm scaling factor (=10) are illustrated in Fig. 8. 4 (B). As seen in Fig 8.4 (B), there exist only one minimum (222 nm) with good linearity. And this wavelength was selected because of its lower RSD and more suitable mean recoveries for the determination of RAMP in ATOR and RAMP mixtures. Calibration graphs were established from analytical signals measured at 269 nm for standards containing 4 - 22 μg $mL^{-1}$of ATOR and at 222 nm for standards containing 4 - 32 μg $mL^{-1}$of RAMP, corresponding to maxima and minima in the absence of each other. All the analytical parameters are illustrated in Table 8.3. The proposed method was successfully applied for the determination of the two drugs in laboratory-prepared mixtures and in pharmaceutical formulations.

Table 8.3 Analytical data of the calibration graphs for the determination of ATOR and RAMP by spectrophotometric method

Parameters	SEM		SEM		FDZC		RFD	
	ATOR	RAMP	ATOR	RAMP	ATOR	RAMP	ATOR	RAMP
Wavelength (nm)	247		210		257	225	269	222
Linearity range (μg mL^{-1})	4 - 22	4-32	4 - 22	4-32	4 - 22	4-32	4 - 22	4-32
Intercept (a)	0.0146	0.0598	0.0165	0.054	0.0118	0.037	0.4574	0.0074
SE of Intercept[1]	0.0059	0.0152	0.005	0.011	0.017	0.002	0.0022	0.0032
Slope (b)	0.0398	0.984	0.0374	0.994	0.0618	0.110	1.285	0.1228
SE of slope	0.0024	0.0025	0.024	0.004	0.0064	0.0021	0.0001	0.0002
Correl. coeff	0.9996	0.9999	0.9997	0.9999	0.9996	0.9997	0.9996	0.9999
RSE[2]	0.0031	0.0057	0.008	0.0049	0.0023	0.0057	0.002	0.0043

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

8.4 Spectrophotometric method validation

To check the validity (predictive ability) of the calibration models, the simultaneous analysis of the prediction set containing each of 16 (Table 8.2) samples in various concentrations of ATOR and RAMP (in triplicates) was carried out by proposed spectrophotometric methods. The mean recoveries, % errors and the relative standard deviations of prediction sets were computed and indicated in Table 8.4. Their numerical values were completely acceptable because of their good recoveries and hence found satisfactory for the validation.

Linearity and range

All proposed spectrophotometric method showed good linearity for ATOR and RAMP within the range of 4 - 22 $\mu g\ mL^{-1}$ and 4 – 32 $\mu g\ mL^{-1}$ respectively with lowest co-relation co-efficient, intercept and slope (Table 8.3).

Accuracy

Accuracy of the proposed methods was determined by performing recovery study in triplicates from previously analyzed formulation at five concentration levels and from laboratory prepared synthetic mixture by standard addition method at three levels of concentration so that final concentration of both the drugs lies within the stated linearity range. The method showed % recovery ± SD for both the title ingredients indicating that the developed spectrophotometric method is accurate and is free from interference of excipients (Table 8.5a and 8.5b).

Precision

Inter day and intra day precision for proposed spectrophotometric methods were measured in terms of % RSD. The experiment was carried on laboratory prepared binary mixtures of title ingredients and repeated three times in a day for intra day (repeatability) and on three different days for inter-day precision (intermediate precision). The methods were found precise on intra day and inter day basis as the

average %RSD value for the determination of ATOR and RAMP was as shown in Table 8.6 (a) and 8.6 (b).

Robustness

Double-beam Shimadzu (Japan) UV-vis Spectrophotometer (model UV-1700 and 1601) were used to access the robustness. The average value of % RSD of the responses for determination of ATOR and RAMP less than 2 % reveals the robustness of the method.

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

The limit of detection (LOD) and limit of quantitation (LOQ) are calculated (Table 8.7) according to a formula given by Miller.[1] (LOD = 3 * SD/slope of calibration curve, LOQ = 10 * SD/slope of calibration curve and SD = Standard deviation of blank determinations.)

Table 8.4 Prediction results for ATOR and RAMP in synthetic mixtures by proposed spectrophotometric techniques

Mixture added		Recovery (%)						Error%					
		SEM		FDZC		RFD		SEM		FDZC		RFD	
ATOR	RAMP	ATOR	RAMP	ATOR	RAMP	ATOR	RAMP	ATOR	RAMP	ATOR	RAMP	ATOR	RAMP
4	4	101.5	99.4	101.8	98.1	102.48	101.81	1.50	-0.60	1.80	-1.90	2.48	1.81
8	4	100.2	97.5	100.4	97.4	102.62	102.98	0.20	-2.50	0.40	-2.60	2.62	2.98
16	4	99..9	98.7	102.1	99.1	100.32	102.90	-1.3	-1.30	2.10	-0.90	0.32	2.9
22	4	102.8	97.4	100.7	97.5	100.92	104.50	2.80	-2.60	0.70	-2.50	0.92	4.5
4	8	101.8	99.1	102.9	99.7	102.69	101.96	1.80	-0.90	2.90	-0.30	2.69	1.96
8	8	101.9	98.4	101.9	98.2	102.40	102.43	1.90	-1.60	1.90	-1.80	2.4	2.43
16	8	101.4	99.1	100.6	101.1	101.29	101.26	1.40	-0.90	0.60	1.10	1.29	1.26
22	8	101.8	98.9	100..8	101.1	101.5	101.59	1.80	-1.10	0.8	1.10	1.5	1.59
4	24	101.5	101.1	102.8	98.9	104.34	99.91	1.50	1.10	2.80	-1.10	4.34	-0.09
8	24	99.2	99.5	101.7	99.	102.86	100.12	-0.80	-0.50	1.70	-1.00	2.86	0.12
16	24	101.8	97.8	102.5	101.5	99.23	99.72	1.80	-2.20	2.50	1.50	-0.77	-0.28
22	24	101.4	101.5	101.9	101.7	102.03	99.97	1.40	1.50	1.90	1.70	2.03	-0.03
4	32	100.8	100.1	102.7	100.8	101.40	99.52	0.80	0.10	2.70	0.80	1.4	-0.48
8	32	100.7	99.5	100.4	99.4	102.28	99.73	0.70	-0.50	0.40	-0.60	2.28	-0.27
16	32	101.8	100.9	100.5	98.4	99.05	100.01	1.80	0.90	0.50	-1.60	-0.95	0.01
22	32	103.9	96.25	97.32	98.32	99.00	97.36	3.90	-3.75	-2.68	-1.68	-1.00	-2.64
	$\bar{x}$	101.50	99.07	101.35	99.39	101.53	100.99						
	RSD:	1.06	1.44	1.42	1.44	1.49	1.74						

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

Table 8.5 (a) Application of standard addition technique for analysis of RAMP and ATOR in Stator-R tablets tablets

Serial No	Concentration in µg mL^{-1}		Ramipril % recovery ± SD[a]			Concentration in µg mL^{-1}		Atorvastatin % recovery ± SD[a]		
	Claimed	Added	SEM	FDZC	RFD	Claimed	Added	SEM	FDZC	RFD
1	2.5	2.5☼	96.2 ± 0.64	97.3 ± 0.55	98.76 ± 0.74	10	0	104 ± 0.56	103 ± 0.64	97.19 ± 0.75
2	2.5	4	98.23± 1.4	96.22± 1.3	98.65± 0.74	10	2	983± 0.2	97.23± 0.24	95.85 ± 0.81
3	2.5	8	100.01 ± 0.62	98.11 ± 0.58	101.59 ±0.63	10	4	99.56 ± 0.53	98.65± 0.54	98.345 ± 0.85
4	2.5	16	98. 12± 0.86	99. 15± 0.78	100.1 ± 0.58	10	8	98.23±0.25	98.26±0.35	98.54 ± 0.82
5	2.5	24	98.24 ± 1.4	98.45± 1.32	98.36 ± 0.91	10	10	97.9 ± 0.89	98.7 ± 0.87	98.7 ± 0.75
6	2.5	28	97.2± 0.93	97.46± 0.95	98.48± 0.75	10	12	99.4 ± 0.13	99.66± 0.54	98.5 ± 0.05

* average of three experiments

[a] sd standard deviation

☼ 2.5 µg ml-1 of standard RAMP was added to raise the level to linear calibration range

Table 8.5 (b) Application of standard addition technique for analysis of RAMP and ATOR in certified reference material

Serial No	Concentration in µg mL^{-1}	Ramipril % recovery ± SD[a]			Atorvastatin % recovery ± SD[a]		
	Standard Addition	SEM	FDZC	RFD	SEM	FDZC	RFD
1	80	103.7 ± 1.19	102.6 ± 1.21	101.5 ± 0.43	98.5 ± 0.56	97.5 ± 0.63	101.4 ± 0.41
2	100	102.5 ± 0.75	102.6 ± 0.58	102.5 ± 0.74	97.4 ± 1.3	96.4 ± 1.25	101.7 ± 0.87
2	120	103 ± 0.21	103.2 ± 0.22	101 ± 1.5	99.5 ± 0.05	99.2 ± 0.13	98.8± 0.78

* average of three experiments, [a] sd standard deviation

Table 8. 6 (a) Intra day precision for determination of ATOR and RAMP

Mixture No.	% RSD					
	SEM		FDZC		RFD	
	ATOR	RAMP	ATOR	RAMP	ATOR	RAMP
1	1.12	2.67	1.11	1.96	2.25	1.05
2	0.87	2.11	0.78	1.98	1.86	2.27
3	1.19	0.33	0.98	0.33	0.50	0.60
4	0.45	2.03	0.45	1.98	0.30	0.25
5	0.57	0.98	0.43	0.85	0.41	1.32
Average of % RSD	0.84	1.62	0.75	1.42	1.06	1.10

*** Average of three experiments in a day.

Table 8. 6 (b) Inter day precision for determination of ATOR and RAMP

Mixture No.	% RSD					
	SEM		FDZC		RFD	
	ATOR	RAMP	ATOR	RAMP	ATOR	RAMP
1	2.27	3.88	1.584	2.541	1.8753	0.9901
2	1.41	1.14	1.351	1.2124	1.6662	3.9179
3	1.50	1.51	1.241	1.651	0.1840	1.1729
4	0.45	1.62	0.854	1.564	0.2620	0.7215
5	1.68	1.33	1.245	1.5521	0.4705	0.3978
Average of % RSD	1.46	1.90	1.255	1.7041	1.0645	1.0985

*** Average of three experiments in a day.

Table 8.7 LOD and LOQ

Results observed	ATOR	RAMP
*SD	0.001646	0.00049329
LOD ($\mu g\ mL^{-1}$)	0.004146	0.00132561
LOQ ($\mu g\ mL^{-1}$)	0.012564	0.00401701

* Standard deviation of intercepts of five calibration curves

Application of the developed method for analysis of commercial formulations

Applicability of the proposed method was tested by analyzing the commercially available tablet formulation Stator-R labeled to contain 2.5 mg of RAMP and 10 mg of ATOR and Rampitor*5 capsules labeled to contain 5 mg RAMP and 10 mg ATOR. The values of % recovery from formulation as shown in the Table 8.8 (a) and 8.8 (b) are found to be very close to each other as well as to the label value of commercial pharmaceutical formulation, which shows that the method is applicable for simultaneous determination of ATOR and RAMP from their binary mixture formulation. No published method has been reported for simultaneous determination of these binary components in formulations. Hence the proposed methods can be used for the routine determination of the titled ingredients in quality control laboratories.

Table 8.8 (a) Results obtained for the Rampitor capsules by using the Spectrophotometric methods

Methods	ATOR (Label claim= 10mg per tablet) Mean[a] ± SD[b]	RAMP (Label claim= 5mg per tablet) Mean[a] ± SD[b]
SEM	99.76 ± 0.55	103.73 ± 0.40
FDZC	99.65 ± 0.23	103.17 ± 0.959
RFD	98.76 ± 0.98	103.73 ± 0.40

Mean[a], mean value of five determinations for each method; SD[b], Standard deviation

Table 8.8 (b) Results obtained for the Stator-R tablets by using the Spectrophotometric methods

Methods	ATOR (Label claim= 10mg per tablet) Mean[a] ± SD[b]	RAMP (Label claim= 5mg per tablet) Mean[a] ± SD[b]
SEM	96.84 ± 0.74	96.83 ± 0.06
FDZC	96.94 ± 0.02	97.15 ± 0.065
RFD	98.33 ± 0.06	98.26 ± 0.021

Mean[a], mean value of five determinations for each method; SD[b], Standard deviation

8.5 Results and discussion

Chromatography, HPTLC and Chemometric methods

8.5.1Chromatography method

In order to effect the simultaneous elution of the two component peaks under isocratic conditions, the mobile phase composition was optimized after several trials with various organic solvents and buffers in different ratios. A satisfactory separation was obtained with a mobile phase consisting of acetonitrile: 0.1M sodium perchlorate (70:30, v/v) with pH adjusted to 2.5 ± 0.2 with orthophosphoric acid at a flow rate of 1.5 mL min^{-1}. Initial studies were performed while the effluent was monitored at 210 nm. The change in the wavelength of detection between the runs was performed to achieve better detector response. Both the components show reasonably good response at 210 nm. Under the described chromatographic conditions, the analyte peak was well defined, resolved and almost free from tailing with the retention time of 2.275 ± 0.003 min and 3.178 ± 0.002 min for RAMP and ATOR respectively (Fig. 8. 5). This allows determination of both drugs with reasonable responses for the two well resolved peaks with reliable results. For quantitative application linear calibration chromatograms were obtained with correlation coefficients better than 0.9999.

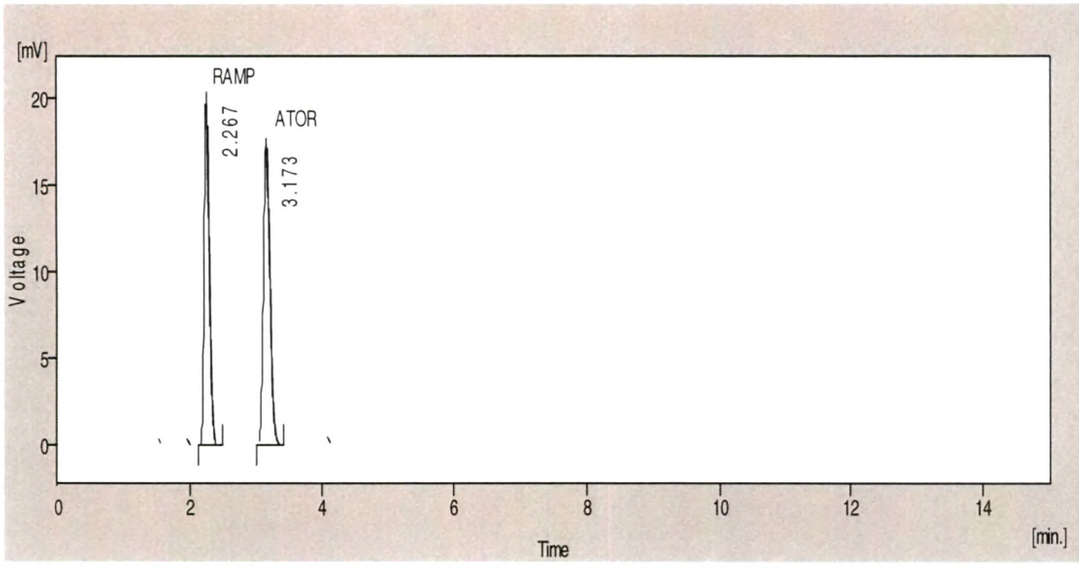

Fig. 8. 5 Chromatogram showing retention time (R_t) of (a) 32 µg ml^{-1} of RAMP (2.27min) and (b) 22 µg ml^{-1} of ATOR (3.17 min) in laboratory-prepared mixture

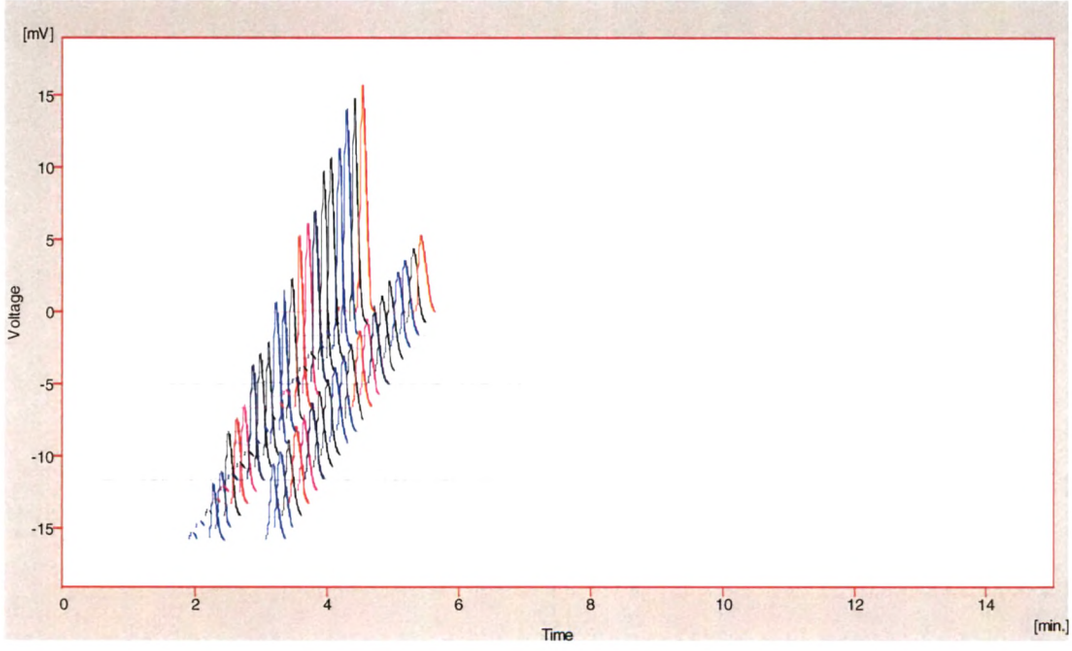

Fig. 8. 6 (a) HPLC 3-Dimensional chromatograms set of seven standard dilutions of RAMP (in triplicate) using 8 µg ml^{-1} of ATOR as internal standard

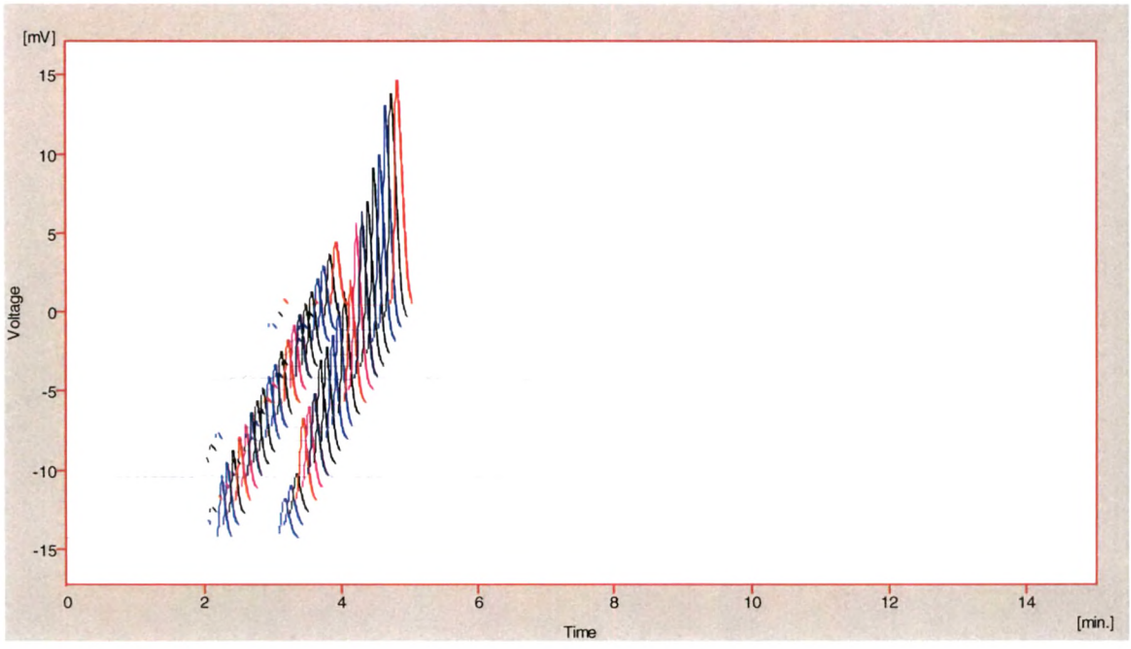

Fig. 8. 6 (b) HPLC 3-Dimensional chromatograms set of seven standard dilutions of ATOR (in triplicate) using 8 µg ml^{-1} of RAMP as internal standard

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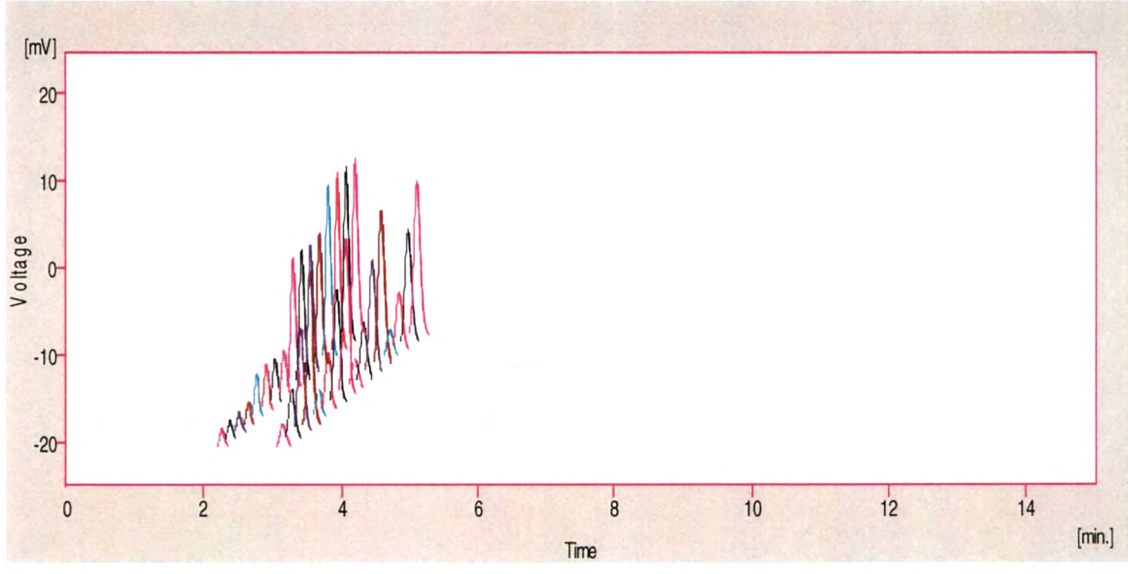


Fig. 8. 7 HPLC 3-Dimensional Chromatograms set of 16 binary mixtures of RAMP and ATOR prepared by four factorial designs for prediction

8.5.2 HPTLC method

The major advantage of HPTLC is that several samples can be run simultaneously using a small quantity of mobile phase unlike HPLC, thus lowering analysis time and cost per analysis. Mobile phase having pH 8 and above can be employed. Suspensions, dirty or turbid samples can be directly applied. It facilitates automated application and scanning in situ. HPTLC facilitates repeated detection (scanning) of the chromatogram with the same or different parameters. Simultaneous assay of several components in a multicomponent formulation is possible.

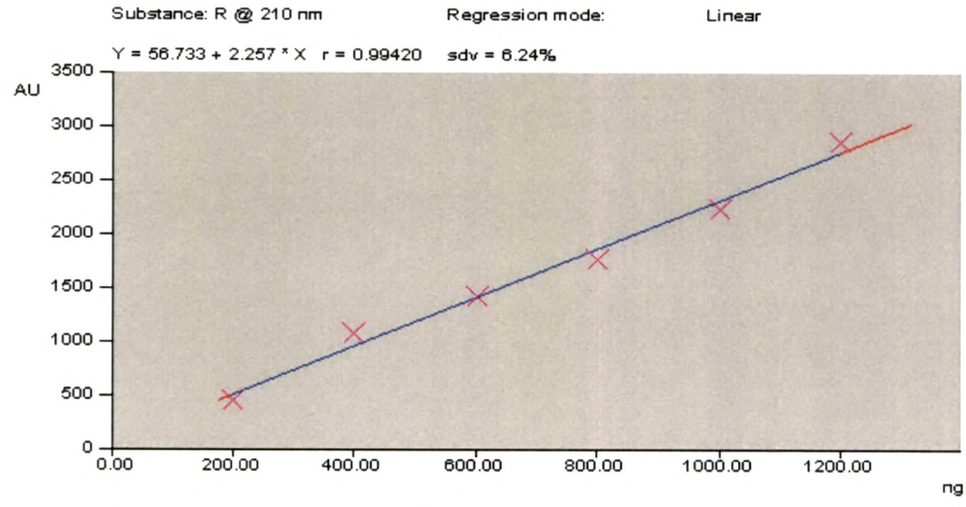


Fig. 8. 8 (a) Linearity curve of RAMP at 210 nm

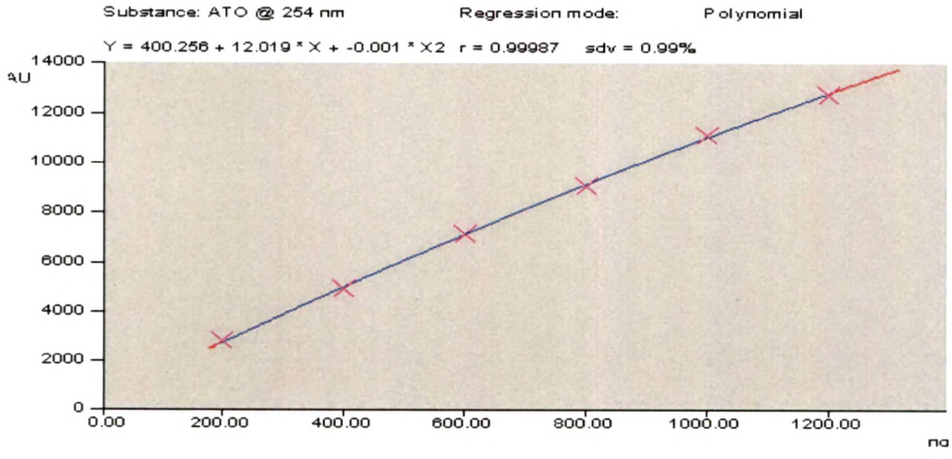


Fig. 8. 8 (b) Linearity curve of ATOR at 254nm

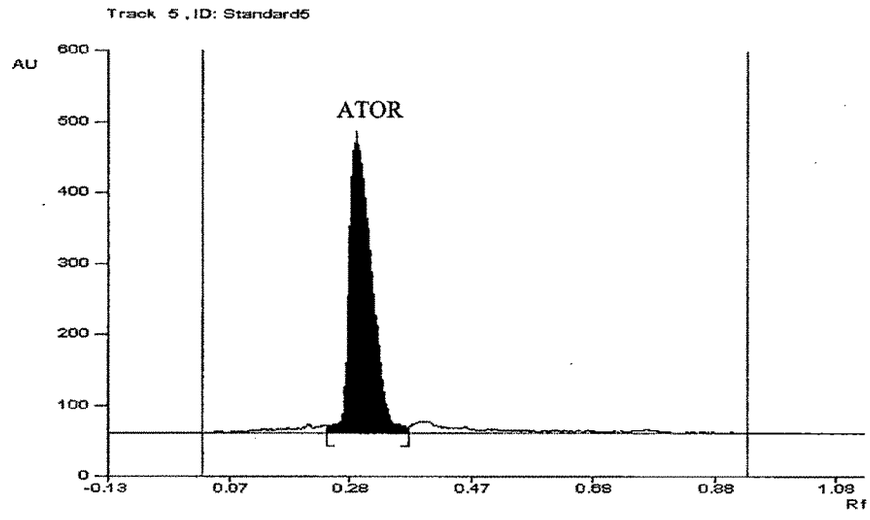


Fig. 8. 9 (a) HPTLC chromatogram of ATOR scanned at 254 nm showing retention factor 0.32

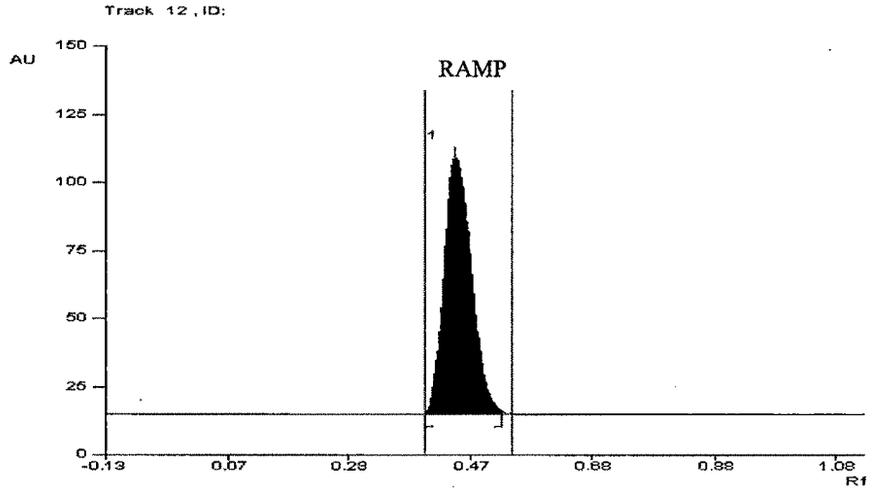


Fig. 8. 9 (b) HPTLC chromatogram of RAMP scanned at 210 nm showing retention factor 0.45

8.5.3 Chemometric methods

Chemometric techniques are gaining wide application for the resolution of the drug mixtures. A calibration set consisting of 25 binary mixtures prepared within the stated range was used. Electronic absorption spectra for the calibration samples shown in Table 8. 1 was recorded in the range 201-350 nm (Fig. 8. 11). The UV absorbance data was obtained by measuring the absorbances in the region of 201 - 270 nm and

then chemometric calibrations were calibrated within the CLS, PCR and PLS algorithms

The quality of multicomponent analysis is dependent on the wavelength range and spectral mode used. 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. However, measurements from spectral wavelengths that are not informative in a model will degrade performance. Hence amplitudes after 270 nm were not used because RAMP has no absorbance at the concentrations used in this region. Any absorbance data beyond 270 nm would have introduced a significant amount of noise, thereby decreasing the precision of RAMP estimation. Original and reconstructed spectra of the calibration matrix were compared in order to select the most informative wavelength range. The spectral region, which is best reconstructed, was considered. This entailed using 70 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,

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

Where C_i^{Added} the added concentration of drugs, C_i^{Found} is the predicted concentration of drugs and n the total number of the synthetic mixtures. The numerical values are quoted in respective Tables 8.11 and 8.13.

Selection of optimum number of factors for PCR and PLS

For PCR and PLS methods, a number of 25 calibration spectra were used for the selection of the optimum number of factors by using the cross validation technique. This allows modeling of the system with the optimum amount of information and avoidance of overfitting or underfitting. The cross-validation procedure (Leave-one-

out' (LOO)) consisting of systematically removing one of a group of calibration samples at a time and using the remaining ones for the construction of latent factors and regression was applied. 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 (Figure 8.12 and 8.13) indicates factor five was optimum for the estimation of title drugs by both PCR and PLS. At the selected principal components of PCR and PLS the concentrations of each sample was then predicted and compared with known concentrations and the PRESS (Prediction Error Sum of Squares) was calculated. It was given by the equation,

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

The values are indicated in Tables 8.11 and 8.13.

8.6 Validation of methods

To check the validity (predictive ability) of the calibration models, the simultaneous analysis of the prediction set containing each of 16 samples in various concentrations of RAMP and ATOR (in triplicates) was carried out by HPLC (Fig. 8. 7) and chemometric methods. The mean recoveries, % errors and the relative standard deviations of prediction sets were computed and indicated in Table 8.10. Their numerical values were completely acceptable because of their good recoveries and hence found satisfactory for the validation.

Another diagnostic test for chemometric methods with prediction sets was carried out by plotting the concentration residuals against the predicted concentrations. The residuals appear randomly distributed around zero, indicating good prediction ability of the model (Fig 8. 14).

Linearity

The linearity of the proposed HPLC (Fig. 8.6 (a) and 8.6 (b)) and chemometric methods for determination of RAMP and ATOR was evaluated by analysing a series of different concentrations of standard drug. In this study seven concentrations were chosen, ranging between 4-32 µg mL^{-1} of RAMP and 4-22 µg mL^{-1} of ATOR. Similarly in HPTLC six concentrations were choosen, ranging between 200-1200ng $spot^{-1}$ and 200-1400ng $spot^{-1}$ of ATOR and RAMP respectively (Fig 8.10 (a) and 8.10 (b)). Each concentration was repeated three times and obtained information on the variation in peak area response in HPLC, HPTLC and absorbances at stated wavelength region in chemometric methods respectively. The linearity of the calibration graphs of proposed chemometric, RP-HPLC and HPTLC methods were validated by the high value of correlation coefficient, slope and the intercept (Table 8.9).

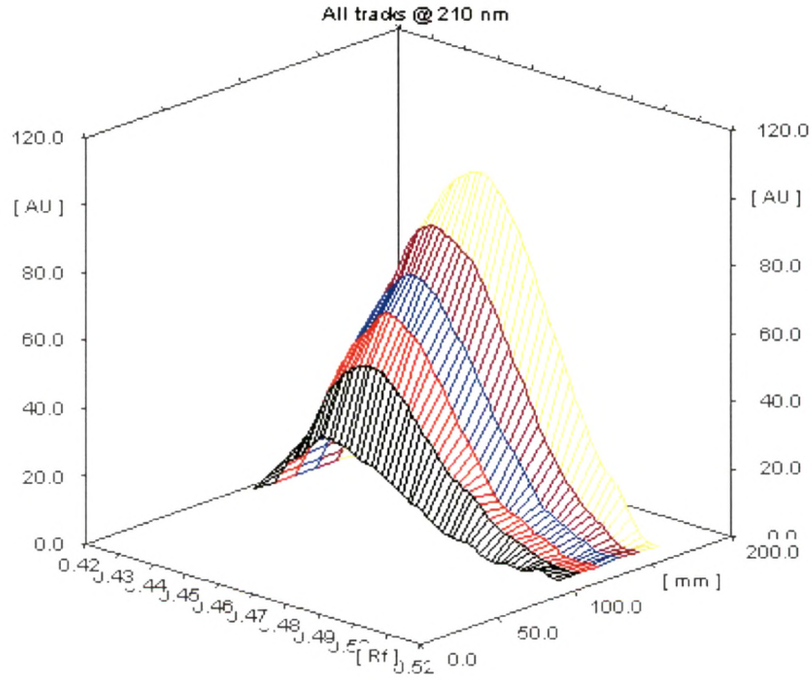


Fig. 8. 10 (a) Three-dimensional hiding wire frame image of the calibration HPTLC chromatograms for RAMP at 210 nm

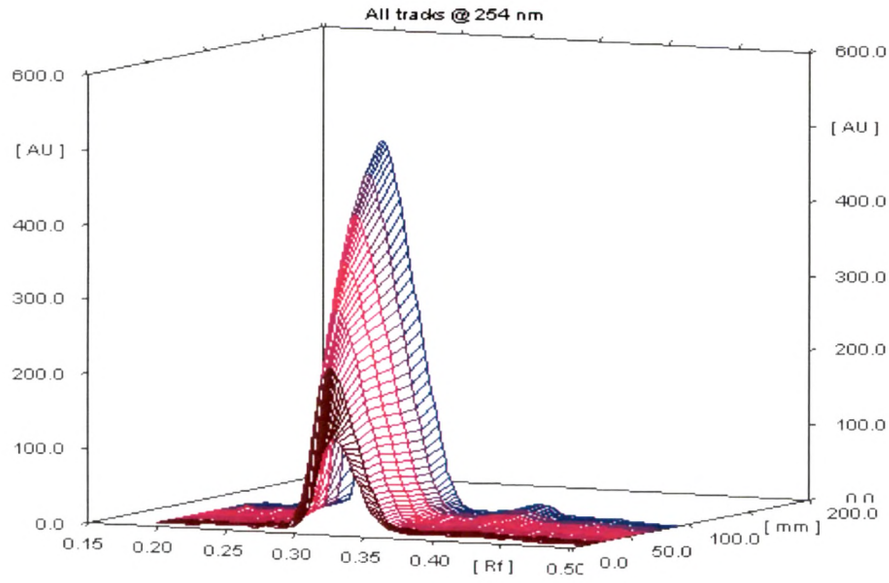


Fig. 8. 10 (b) Three-dimensional hiding wire frame image of the calibration HPTLC chromatograms for ATOR at 254 nm

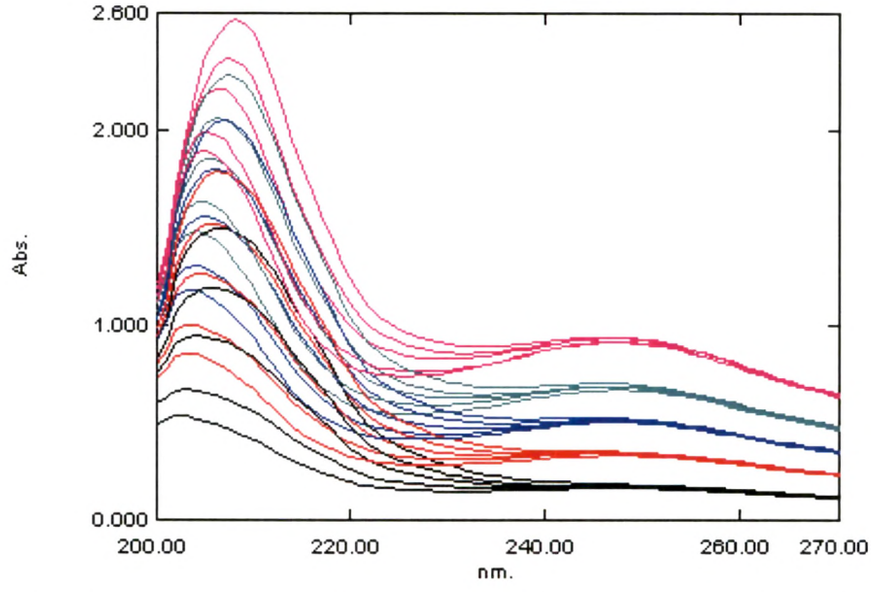
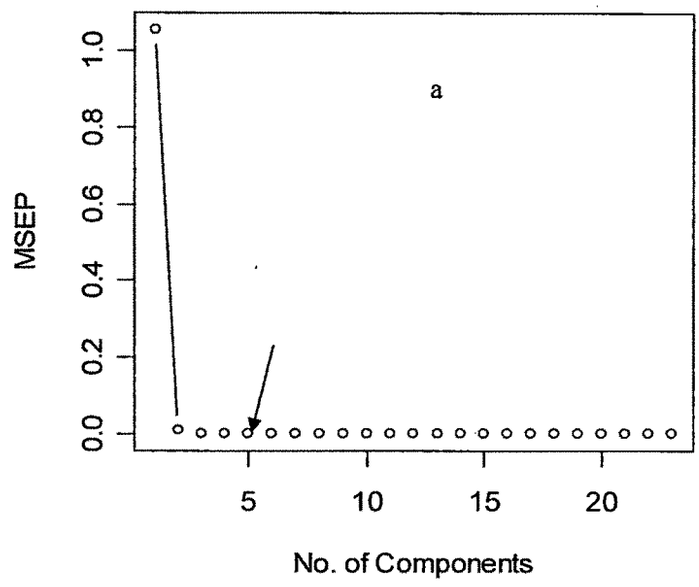


Fig. 8. 11 Zero order overlay absorption spectra of 25 laboratory prepared binary mixtures of ATOR and RAMP for chemometric calibration



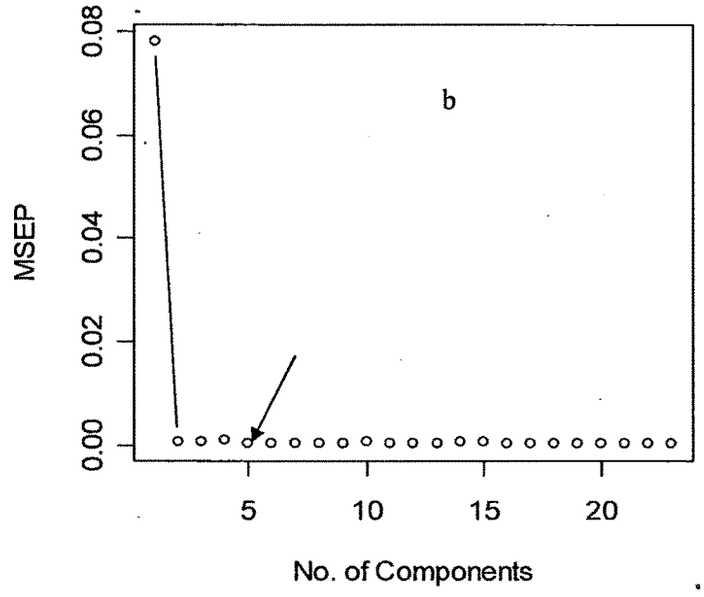
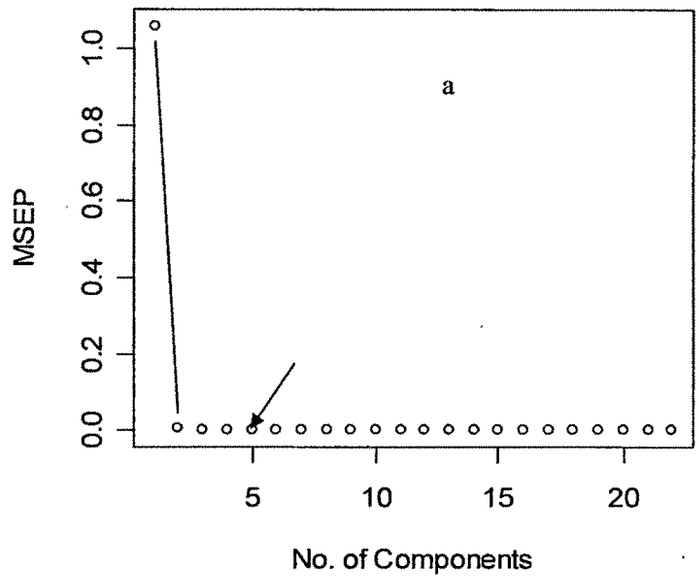


Fig. 8.12 MSEP plots of a calibration set obtained using leave-one-out (LOO) cross validation of PCR-model for a) RAMP and b) ATOR in zero-order absorption data



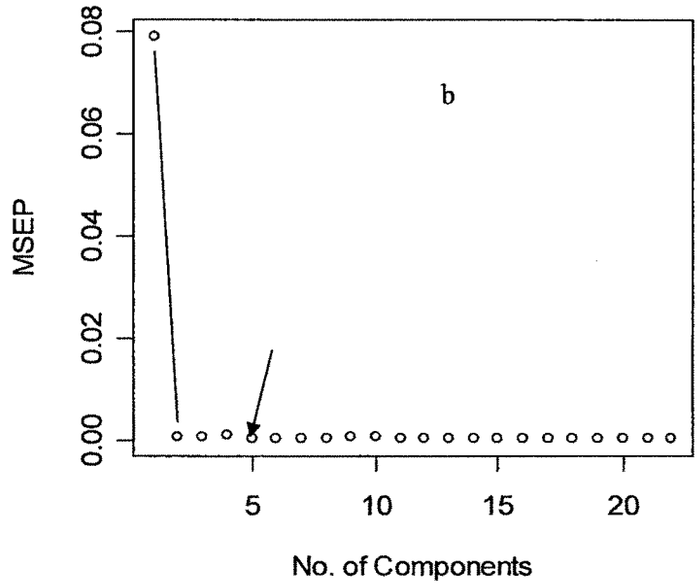


Fig. 8. 13 MSEP plots of a calibration set obtained using leave-one-out (LOO) cross validation of PLS-model for a) RAMP and b) ATOR in zero-order absorption data

Table 8. 9 Characteristic parameters of calibration equation for the proposed HPLC and HPTLC method for simultaneous determination of RAMP and ATOR

Parameters	HPLC		HPTLC	
	RAMP	ATOR	RAMP	ATOR
Calibration range (µg mL-1)	4 - 32	4 - 22	0.2-1.2	0.2 -1.4
Detection limit (µg mL-1)	1.78×10^{-2}	1.98×10^{-2}	0.0003×10^{-2}	0.0012×10^{-2}
Quantitation limit (µg mL-1)	5.93×10^{-2}	6.59×10^{-2}	0.0009×10^{-2}	0.0039×10^{-2}
Regression equation[r]				
Slope[b]	0.0715	0.2118	2.256	29.384
Standard deviation of the slope	0.0002	0.0035	2.76	0.99
Relative standard deviation of the slope (%)	0.2797	1.6531	1.365	2.195
Intercept[a]	0.0076	0.0196	56.733	1095.43
Standard deviation of the intercept	0.0024	0.0054	23.46	16.63
Relative standard deviation of the intercept (%)	31.9061	27.5840	0.9942	0.9998
Correlation coefficient	0.9999	0.9991	NA	NA
Theoretical plates	2888	3308	NA	NA
Symmetry factor	1.274	1.244	NA	NA
Resolution	5.408			

[r] $y = a + bc$, where c is the concentration of compound in µg mL^{-1} and y is the peak area

Range

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

Accuracy

The study was performed by standard addition of known amounts of studied drugs to an unknown concentration (constant volume) (Rubinson 1987) of the commercial pharmaceutical formulations (

The resulting mixtures were analysed by the proposed HPLC, HPTLC (Fig. 8. 15 (a) and 8.15 (b)) method and the response obtained was plotted against the initial unknown concentration set at 0 (Fig. 8. 13). And chemometric recoveries were also determined. The results obtained are compared with expected results. The excellent mean recoveries and standard deviation (Table 12 (a) and 12 (b)) suggested good accuracy of the proposed methods and no interference from formulations excipients.

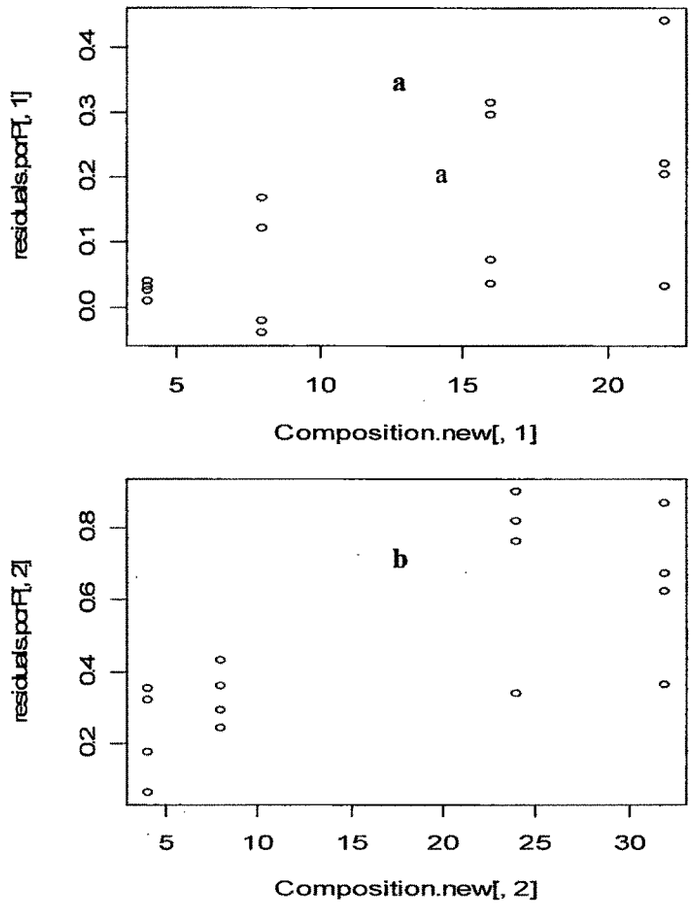


Fig. 8. 14 Plot of concentrations residuals (residuals.pcrP) against the predicted concentrations (Composition.new) of a) RAMP and b) ATOR in prediction set

Table 8.10 Recovery results in prediction for RAMP and ATOR in synthetic mixtures by proposed HPLC and chemometric techniques

Mixture added		Recovery (%)								Error %							
		HPLC		CLS		PCR		PLS		HPLC		CLS		PCR		PLS	
RAMP	ATOR	RAMP	ATOR	RAMP	ATOR	RAMP	ATOR	RAMP	ATOR	RAMP	ATOR	RAMP	ATOR	RAMP	ATOR	RAMP	ATOR
4	4	98.02	101.69	99.87	102.69	101.52	101.01	101.37	100.79	1.98	-1.69	0.13	-2.69	-1.52	-1.01	-1.37	-0.79
4	8	102.52	103.50	103.28	105.87	107.99	102.11	107.86	102.02	-2.52	-3.50	-3.28	-5.87	-7.99	-2.11	-7.86	-2.02
4	16	104.43	102.25	101.36	106.39	104.34	101.85	103.79	101.68	-4.43	-2.25	-1.36	-6.39	-4.34	-1.85	-3.79	-1.68
4	22	101.54	101.87	105.09	104.58	107.84	102.00	107.03	101.85	-1.54	-1.87	-5.09	-4.58	-7.84	-2.00	-7.03	-1.85
8	4	102.74	102.35	100.08	104.59	103.65	100.21	103.55	99.95	-2.74	-2.35	-0.08	-4.59	-3.65	-0.21	-3.55	0.05
8	8	102.10	103.33	100.03	102.11	103.05	101.51	102.91	101.32	-2.10	-3.33	-0.03	-2.11	-3.05	-1.51	-2.91	-1.32
8	16	105.01	101.68	102.54	98.89	105.40	101.96	105.44	101.97	-5.01	-1.68	-2.54	1.11	-5.40	-1.96	-5.44	-1.97
8	22	105.57	101.15	101.65	99.67	104.48	101.00	104.11	100.86	-5.57	-1.15	-1.65	0.33	-4.48	-1.00	-4.11	-0.86
24	4	101.87	98.89	99.32	103.54	101.41	100.63	101.37	100.26	-1.87	1.11	0.68	-3.54	-1.41	-0.63	-1.37	-0.26
24	8	104.65	99.92	99.99	101.68	103.75	99.72	103.75	99.69	-4.65	0.08	0.01	-1.68	-3.75	0.28	-3.75	0.31
24	16	104.64	101.00	98.98	98.37	103.17	100.45	103.20	100.49	-4.64	-1.00	1.02	1.63	-3.17	-0.45	-3.20	-0.49
24	22	103.19	102.61	100.03	97.98	103.40	100.93	103.36	100.87	-3.19	-2.61	-0.03	2.02	-3.40	-0.93	-3.36	-0.87
32	4	102.09	101.35	98.35	97.93	102.71	100.81	102.65	100.19	-2.09	-1.35	1.65	2.07	-2.71	-0.81	-2.65	-0.19
32	8	103.75	100.15	97.98	100.26	101.95	99.50	101.87	99.10	-3.75	-0.15	2.02	-0.26	-1.95	0.50	-1.87	0.90
32	16	104.05	101.06	99.89	103.58	102.10	100.22	102.23	100.51	-4.05	-1.06	0.11	-3.58	-2.10	-0.22	-2.23	-0.51
32	22	103.64	100.94	101.78	100.98	101.13	100.14	101.09	100.03	-3.64	-0.94	-1.78	-0.98	-1.13	-0.14	-1.09	-0.03
	$\bar{x}$	103.11	101.48	100.64	101.82	103.62	100.88	103.47	100.72								
	RSD[r]	1.77	1.20	1.86	2.75	1.98	0.82	1.87	0.86								

$\bar{x}$ mean recovery value.

[r] Relative Standard Deviation.

Table 8.11 Statistical parameters of chemometric methods in calibration step of Zero-order spectra

Component	CLS	PCR			PLS		
	SEC	SEC	PRESS	RSE[a] (%)	SEC	PRESS	RSE (%)
RAMP	1.5718	0.0136	0.0044	0.0679	0.0133	0.0042	0.0664
ATOR	0.2032	0.0203	0.0098	0.1432	0.0169	0.0068	0.1193

[a] Relative Standard Error of calibration of single component

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

Table 8.12 (a) Application of standard addition technique for analysis of RAMP and ATOR in Stator –R 2.5 tablets

Serial No	Ramipril							Atorvastatin						
	Concentration in µg ml^{-1}		% recovery ± SD[a]					Concentration in µg ml^{-1}		% recovery ± SD[a]				
	Claimed	Added	CLS	PCR	PLS	HPLC	HPTLC	Claimed	Added	CLS	PCR	PLS	HPLC	HPTLC
1	2.5	2.5☼	102 ± 0.78	100.3 ± 0.63	100.2 ± 0.49	98.2 ± 0.738	96.48±0.65	10	0	97 ± 0.68	103.6 ± 0.28	102 ± 0.78	104 ± 0.63	97.58±0.55
2	2.5	4	98.2 ± 1.2	98.2 ± 0.02	98 ± 0.01	103 ± 1.8	97.51±0.72	10	2	97.19 ± 1.2	100.3 ± 0.93	100.28 ± 0.8	99.2 ± 0.01	98.58±0.85
3	2.5	8	103 ± 0.09	99.3 ± 0.16	99.7 ± 0.19	103.2 ± 0.72	96.56±0.55	10	4	98.5 ± 0.19	106 ± 0.01	105.8 ± 0.09	100.5 ± 0.35	97.67±0.73
4	2.5	16	104.4 ± 0.01	102.5 ± 0.06	102.4 ± 0.12	102.8 ± 0.91	98.48±1.52	10	8	100.6 ± 0.09	98.9 ± 0.31	99.12 ± 0.46	102.4 ± 0.59	96.49±0.95
5	2.5	24	97.9 ± 1.3	98.3 ± 0.96	98.9 ± 0.87	100 ± 1.1	97.45±0.74	10	10	97.8 ± 0.95	100.5± 0.79	100.3 ± 0.92	99.9 ± 0.97	97.45±0.58
6	2.5	28	99.7 ± 0.06	100.2 ± 0.03	100.1 ± 0.05	97.1 ± 0.91	96.65±0.45	10	12	98.7 ± 0.23	98.5 ± 1.2	98.4 ± 0.37	99.2 ± 0.06	98.49±0.65

* average of three experiments, [a] sd standard deviation

☼ 2.5 µg ml-1 of standard RAMP was added to raise the level to linear calibration range

Table 8.12 (b) Application of standard addition technique for analysis of RAMP and ATOR in Rampitor*5 capsules

Serial No	Ramipril							Atorvastatin						
	Concentration in µg ml^{-1}		% recovery ± SD[a]					Concentration in µg ml^{-1}		% recovery ± SD[a]				
	Claimed	Added	CLS	PCR	PLS	HPLC	HPTLC	Claimed	Added	CLS	PCR	PLS	HPLC	HPTLC
1	5	0	97.3 ± 0.95	98.94 ± 0.23	99.1 ± 0.01	100.2 ± 0.08	97.58±0.55	10	0	96.7 ± 0.96	102.6 ± 0.34	102.2± 0.48	96.4 ± 0.73	96.48±0.65
2	5	4	96.9 ± 1.4	99.7 ± 0.92	99.6 ± 0.04	98.9 ± 0.07	98.58±0.81	10	2	97.28 ± 0.95	98.3 ± 0.43	99.12 ± 0.59	97.2 ± 0.76	97.51±0.72
3	5	8	96.7 ± 0.98	99.3 ± 0.36	99.6 ± 0.09	102.7 ± 0.19	97.67±0.73	10	4	98.9 ± 0.73	99.2 ± 0.00	99.78 ± 0.72	96.8 ± 0.53	96.56±0.55
4	5	16	98.97 ± 0.91	100.5 ± 0.53	102.7 ± 0.00	102.9 ± 0.83	96.49±0.95	10	8	95.4 ± 1.1	100.2 ± 0.09	99.99± 0.76	98.3 ± 1.2	98.48±1.52
5	5	20	96.9 ± 1.2	100 ± 0.01	100.3 ± 0.09	102 ± 0.01	97.49±0.89	10	10	98.8 ± 0.07	103.5 ± 0.03	102.3 ± 0.37	99.2 ± 0.00	97.48±0.85
6	5	24	99.2 ± 0.93	100.2 ± 0.02	100.1 ± 0.01	104.9 ± 0.07	98.4±0.86	10	12	96.7 ± 0.91	995 ± 1.1	100.7 ± 0.37	98.1 ± 0.02	98.45±0.70

* Average of three experiments

[a] SD standard deviation

Table 8.13 Statistical parameters of chemometric methods in prediction step of zero-order spectra

Component	CLS				PCR				PLS			
	SEP	*a*	*b*	*r*	SEP	*a*	*b*	*r*	SEP	*a*	*b*	*r*
RAMP	1.866	1.647	0.944	0.990	0.555	0.198	1.016	0.999	0.549	0.182	1.016	0.999
ATOR	0.354	0.482	0.984	0.999	0.187	0.023	1.011	0.999	0.176	0.034	1.011	0.999

[a] Intercept, [b] Slope, [r] Correlation coefficient

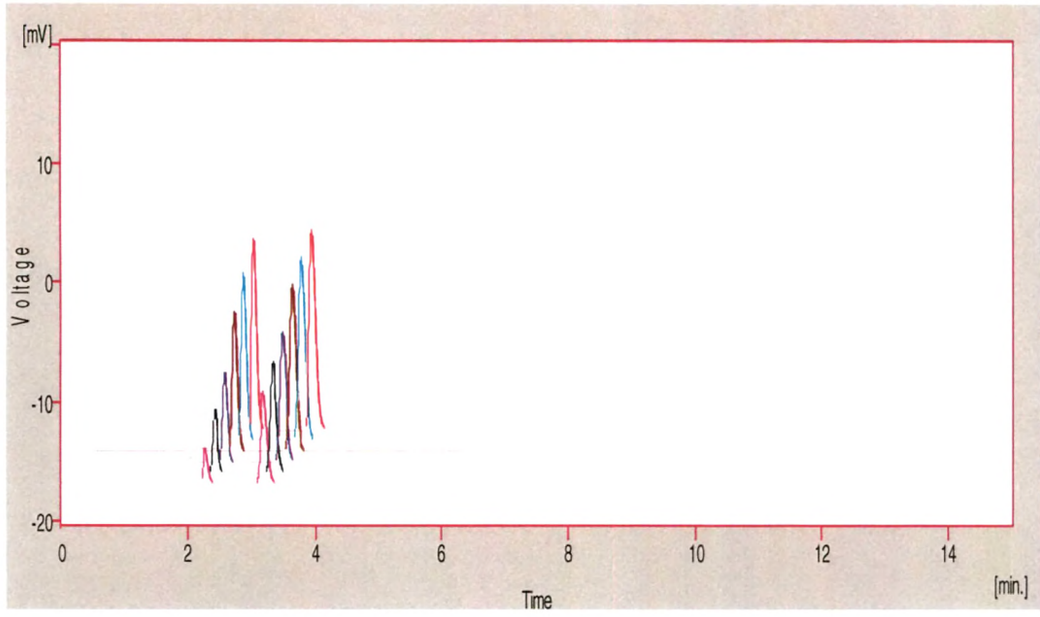


Fig. 8. 15 (a) HPLC 3-Dimensional chromatograms of RAMP and ATOR binary mixtures in Rampitor capsules used for accuracy studies

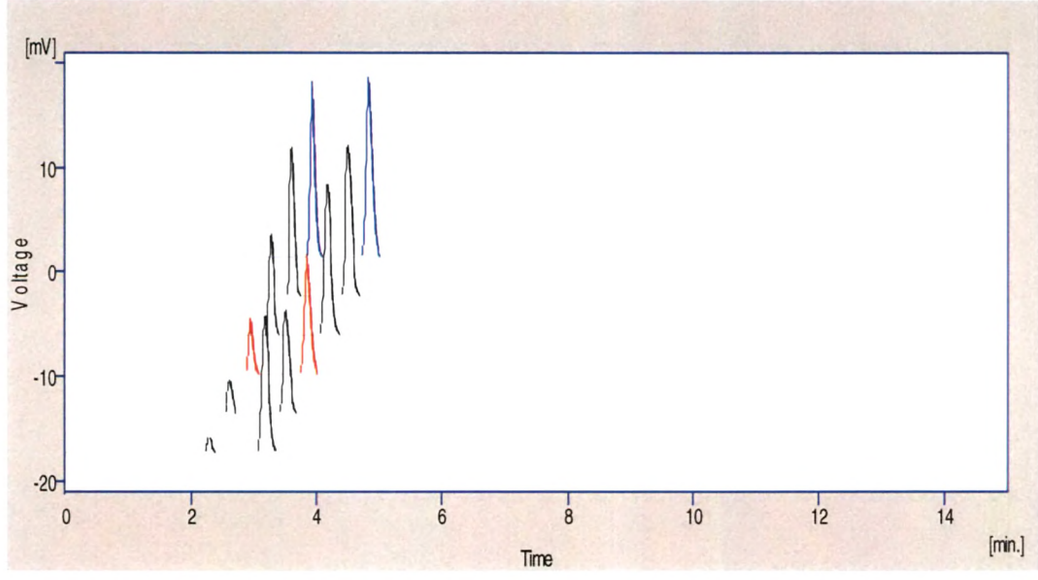


Fig. 8. 15 (b) HPLC 3-Dimensional chromatograms of RAMP and ATOR in Stator-R tablets used for accuracy studies

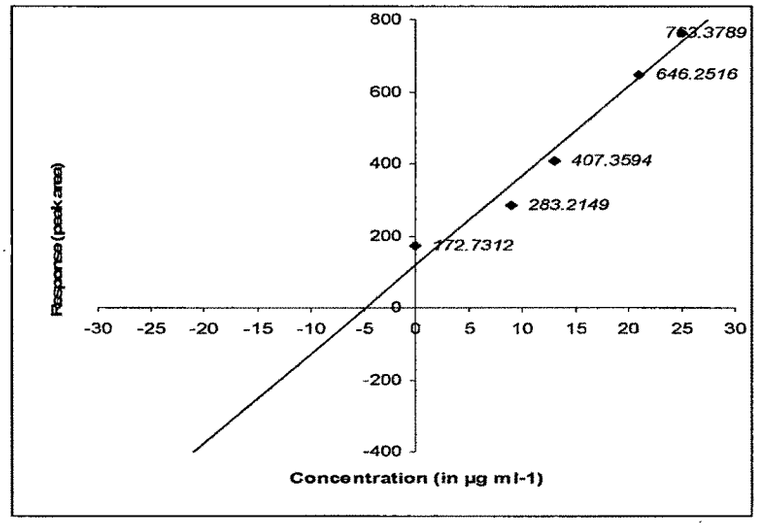


Fig. 8. 16 Plot of peak area versus concentration of RAMP with the initial concentration set at zero

Analyte solution and mobile phase stability

Stability of RAMP and ATOR in solutions within linear concentration was studied by keeping the solutions at room temperature for seven days during validation process. Content of both ingredients was checked by proposed HPLC, HPTLC method using same mobile phase and spectrophotometric methods at 6h interval and all the solutions were found to be stable (Fig. 8. 17).

Precision (Method reproducibility)

Method reproducibility was demonstrated by repeatability and intermediate precision measurements of % RSD of peak area, peak asymmetry and retention time parameters of HPLC and % recovery RSD in chemometric methods for each title ingredient.

The repeatability (within-day in triplicates) (Figure 8.18) and intermediate precision (for 3 days) was carried out at five concentration levels for each compound. The obtained results within and between days trials are in acceptable range indicating good precision of the proposed methods (Table 8.14).

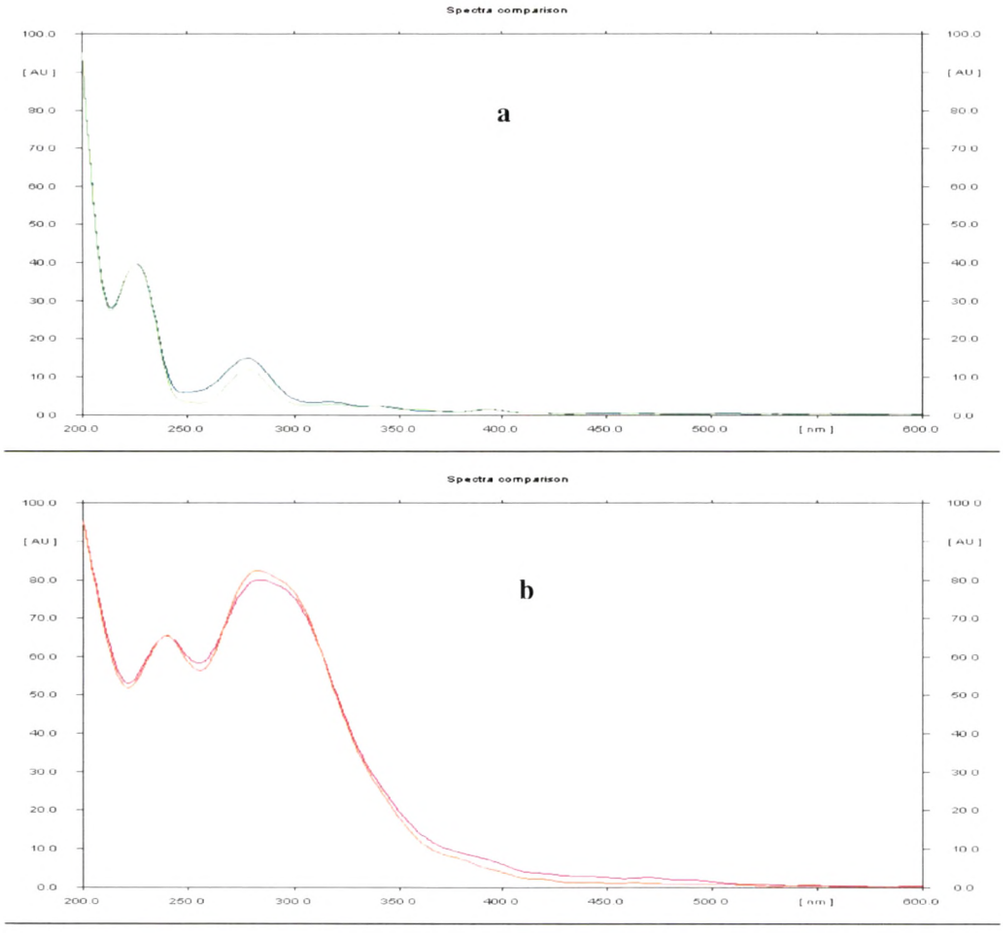


Fig. 8. 18 Spectra comparison of standard a) RAMP and b) ATOR in formulation in HPTLC during stability studies

Robustness

The robustness of the proposed HPLC method was assessed for peak asymmetric and peak resolution factor (Table 8.15 (a)) by purposely altering the HPLC conditions:

- Apparent pH of the mobile phase (± 0.3)
- Mobile phase organic content (± 3%)
- Mobile phase flow rate (± 0.2)
- Detection wavelength (± 2)

Table 8.14 Precision study results of prepared binary mixture

Validation parameter	HPLC % RSD			HPTLC % RSD	Chemometric % RSD		
Repeatability[a]	Peak area	Peak asymmetry	Retention time	Peak area	CLS	PCR	PLS
RAMP	0.734	0.563	0.105	0.0460	2.085	1.814	1.834
ATOR	1.093	0.287	0.906	0.0551	2.162	0.315	0.326
Intermediate precision[b]							
RAMP	1.521	0.770	0.494	0.0650	2.446	1.0867	1.597
ATOR	1.737	0.750	0.093	0.0625	1.987	0.9346	0.290

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

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

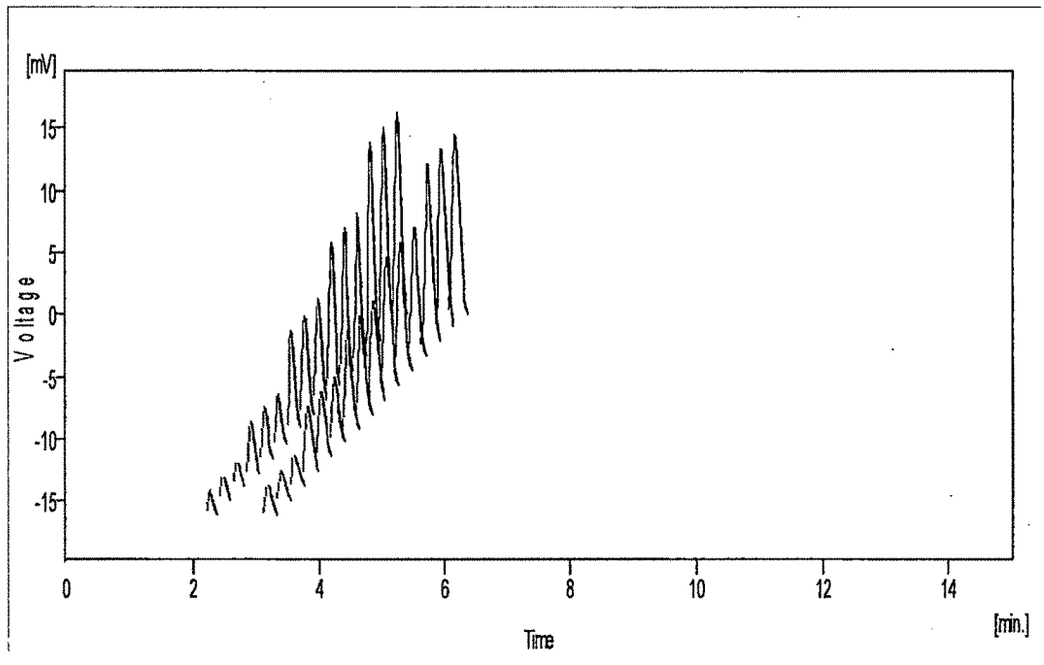


Fig. 8. 18 HPLC 3-Dimensional chromatograms of RAMP and ATOR binary mixtures used for Precision (repeatability) analysis

Similarly robustness of the proposed HPTLC method was assessed for peak area and retention factor of ATOR and RAMP (Table 8.15 (b)) by purposely altering the HPTLC conditions:

- Mobile phase toluene composition (± 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.

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

The limit of detection (LOD) and limit of quantification (LOQ) are calculated according to ICH (Validation of analytical procedures: text and methodology 2006) recommendations where the approach based on the signal-to-noise ratio. RP-HPLC and HPTLC chromatogram signals obtained with known low concentrations analytes were compared with the signals of blank samples. A signal-to-noise ratio 3:1 and 10:1 is considered for calculating LOD and LOQ respectively (Table 8.9).

Application of the developed method for analysis of commercial formulations

Applicability of the proposed method was tested by analyzing the commercially available tablet formulation Stator-R labeled to contain 2.5 mg of RAMP and 10 mg of ATOR and Rampitor*5 capsules labeled to contain 5 mg RAMP and 10 mg ATOR.

No published method has been reported for simultaneous determination of these binary components in formulations. So the results of the proposed HPTLC, CLS, PCR and PLS methods were statistically compared between results of proposed HPLC method at the 95% confidence level with the aid of Student's t-test and F-tests. The calculated t and F values never exceeded the theoretical t- and F- values, at 0.05

level of significant difference. The results of all methods were very close to each other as well as to the label value of commercial pharmaceutical formulations. Therefore, these statistical tests (Table 8.16) denote no significant difference in the results achieved by the proposed methods.

Table 8.15 (a) Robustness of RP-HPLC method

Parameter	Peak asymmetry		Resolution between RAMP and ATOR
	RAMP	ATOR	
Flow rate (mL min^{-1})			
1.4	1.284 ± 0.068	1.208 ± 0.059	5.310 ± 0.000
1.5	1.274 ± 0	1.24 ± 2.7× 10^{-16}	5.34 ± 0.05
1.6	1.249 ± 0.043	1.283 ± 0.006	5.348 ± 0.001
Acetonitrile % in mobile phase			
73	1.25 ± 0.001	1.22 ± 0.001	4.48 ± 0.006
70	1.274 ± 0	1.24 ± 2.7 × 10^{-16}	5.34 ± 0.05
67	1.275 ± 0.002	1.237 ± 0.002	5.942 ± 0.009
Change in pH			
2.8	1.279 ± 0.00	1.296 ± 0.062	5.084 ± 0.010
2.5	1.274 ± 0	1.24 ± 2.7× 10^{-16}	5.34 ± 0.05
2.2	1.286 ± 0.038	1.282 ± 0.038	4.951 ± 0.040
Change in detection wavelength			
209 nm	1.309 ± 0.062	1.298 ± 0.09	4.79 ± 0.03
210 nm	1.274 ± 0	1.24 ± 2.7× 10^{-16}	5.34 ± 0.05
211 nm	1.316 ± 0.06	1.303 ± 0.025	5.21 ± 0.076

* Average of three experiments

Table 8.15 (b) Robustness of HPTLC method

Parameter	Peak area ± % RSD		Retention factor	
	ATOR	RAMP	ATOR	RAMP
Mobile phase chloform composition				
7	2654 ± 0.68	2808 ± 0.59	0.31 ± 0.10	0.48 ± 0.615
8	2673 ± 0.45	2826 ± 0.57	0.32 ± 0.12	0.45 ± 0.19
9	2684 ± 0.58	2864 ± 0.49	0.34 ± 0.18	0.42 ± 0.15
Change in detection wavelength				
251 & 207	2694 ± 0.54	2899 ± 0.84	0.34 ± 0.12	0.41 ± 0.17
254 & 210	2673 ± 0.45	2826 ± 0.57	0.32 ± 0.12	0.45 ± 0.19
257 & 213	2689 ± 0.57	2807 ± 0.75	0.36 ± 0.17	0.44 ± 0.12
Chamber saturation time (in min)				
08	2653 ± 0.49	2848 ± 0.57	0.30 ± 0.20	0.41± 0.21
10	2673 ± 0.45	2826 ± 0.57	0.32 ± 0.12	0.45 ± 0.19
12	2649 ± 0.47	2854 ± 0.44	0.31 ± 0.18	0.43 ± 0.17

(n = 3), 200 ng $spot^{-1}$ of ATOR and 800 ng $spot^{-1}$ of RAMP

Table 8.16 Results obtained for the pharmaceutical samples by using HPLC, HPTLC methods and chemometric calibrations

Formulation	% recovery				
	HPLC	HPTLC	Chemometric methods		
			CLS	PCR	PLS
Stator-R tablets					
RAMP mean[a] ± SD[b]	99.8 ± 0.46	99.9±1.24	99.4 ± 1.31	99.3 ± 0.98	99.3 ± 0.93
F		1.89	1.99	1.95	1.08
t		1.16	1.13	2.67	4.09
ATOR mean[a] ± SD[b]	100.2 ± 0.31	98.7±0.85	99.0 ± 0.99	100.1 ± 0.17	101.4 ± 0.44
F		0.43	0.41	0.63	2.21
t		4.60	4.63	3.32	1.10
Rampitor Capsules					
RAMP mean[a] ± SD[b]	98.8 ± 0.46	98.8±1.09	99.2 ± 1.16	101.2 ± 0.38	101.3 ± 2.10
F		1.48	1.54	2.11	1.84
t		3.65	3.74	1.160	2.91
ATOR mean[a] ± SD[b]	99.3 ± 1.23	98.5±1.05	99.0 ±1.03	99.4 ± 0.93	100.48 ± 1.97
F		0.38	0.40	1.08	1.76
t		5.10	5.01	4.09	3.92

(Stator-R tablets label claim: 2.5 mg of RAMP and 10 mg ATOR per tablet)

(Rampitor capsules label claim: 5 mg of RAMP and 10 mg ATOR per capsule)

[a] Mean recovery value of five determinations for each method.

[b] Standard deviation.

(n1 = n2 = 5), Theoretical values for *t* and *F* at P = 0.05 are 2.31 and 6.39 respectively