Simutaneous estimation of Atorvastatin and Fenofibrate Methodology, Results and discussion

Chapter 5

5.1 Reagents and Pharmaceutical prepaarations

Atorvastatin calcium (ATOR) and Fenofibrate (FENO) were kindly supplied by Biocon India Limited, India and Dr. Reddy's India and certified to contain 99.3% and 99.7% purity respectively. The drugs are used without further purification. All the solvents used in spectrophotometric analysis were of analytical reagent grade. Lorilip Table 5.ts batch number I.P 5022 of Unichem Laboratories Ltd., India, were claimed to contain 10 mg of ATOR and 200 mg of FENO are used in analysis.

5.2 Procedure

Standard solutions of ATOR and FENO

1. ATOR stock solution: 1 mg mL^{-1} in methanol
2. ATOR working solution: a. 0.04 mg mL^{-1} in methanol, prepared by transferring 2.0 mL from stock ATOR to a measuring flask 50 mL and completing to volume with methanol and acetonitrile: methanol (73:27, v/v) for spectrophotometric and HPLC methods respectively.

b. 0.002 mg mL^{-1} in methanol prepared by transferring 2.5 mL from (a) to a measuring flask 50 mL and volume completed with methanol and acetonitrile: methanol (73:27, v/v) for spectrophotometric and HPLC methods respectively.

3. FENO stock solution: 1 mg mL^{-1} in methanol
4. FENO working solution: 0.04 mg/ml in methanol prepared by transferring 2.0 ml from stock FENO to a measuring flask 50 ml and completing to volume with methanol and acetonitrile: methanol (73:27, v/v) for spectrophotometric and HPLC methods respectively.
5. The 0.1μg μL^{-1} and 0.05 μg μL^{-1} working solutions of ATOR and FENO in methanol were prepared respectively for HPTLC analysis.

Preparation of mobile phase

- HPLC; acetonitrile : methanol (73:27,v/v)
- HPTLC; chloroform : methanol : toluene (4 : 3 : 6, v/v)

Pharmaceutical sample solution

Twenty Lorilip Tablets (batch number I.P 5022) of Unichem Laboratories Ltd. (Pondichery, India,) which were claimed to contain 10 mg of ATOR and 200 mg of FENO, were weighed accurately and powdered. An amount of the powder equivalent to 10 mg ATOR and 200 mg FENO (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 1:100 with methanol and HPLC mobile phase for spectrophotometric and HPLC determinations respectively. 4 µg mL^{-1} concentration of ATOR was added as standard addition. All the proposed spectrophotometric, HPLC and HPTLC methods were applied and the concentration of each component in formulation 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 FENO (2-20 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 FENO were introduced. The levels were in the range of 4-22 and 2-20 μg mL^{-1} for ATOR and FENO respectively as shown in Table 5. 1. UV spectra 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 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 ATOR working solutions were taken in different volumetric flasks and 8 μg mL^{-1} of FENO was added to each flask as internal standard and diluted with mobile phase such that the final concentration of ATOR were in the range of 4-22 μg mL^{-1} (Fig. 5. 8a). Similarly FENO 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 FENO was in the range of 2-20 μg mL^{-1} (Fig. 5. 8(a)). 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 5. 9).

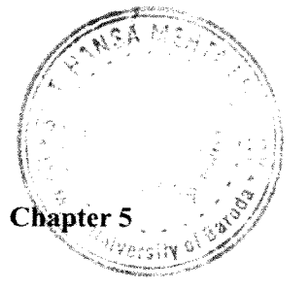


Table 5. 1 Composition of the concentration (chemometric training) set

Mixture Number	Concentration ($\mu g\ mL^{-1}$)	
	ATOR	FENO
1	4.2	2
2	4.2	6
3	4.2	10
4	4.2	16
5	4.4	2
6	4.4	6
7	4.4	10
8	4.4	16
9	4.4	20
10	4.5	2
11	4.5	6
12	4.5	10
13	4.5	16
14	4.5	20
15	4.8	2
16	4.8	10
17	4.8	16
18	4.8	20
19	5	2
20	5	6
21	5	10
22	5	16
23	5	20

HPTLC calibration

Different volume of standard mixture (ATOR 0.05 $\mu g\ \mu L^{-1}$ + FENO 0.025 $\mu g\ \mu L^{-1}$) 2, 4, 6, 8 and 10 µL injection $spot^{-1}$ were made to obtain a concentration range 100-1000 ng $spot^{-1}$ and 50- 500 ng $spot^{-1}$ of ATOR and FENO respectively. The above solutions were spotted in three replicate on TLC plate. Densitometric scanning was performed in the absorbance mode at 285 nm for the estimation of ATOR and FENO (Fig. 5. 7 and 9). The data of peak area versus drug concentrations were treated by polynomial regression mode (Table 5. 9).

Preparation of binary mixtures for spectrophotometric and HPLC predictions

Applying multilevel multifactorial design in which four level concentrations of ATOR and FENO within the stated range were introduced and prepared 16 synthetic binary mixtures of titled ingredients as shown in Table 5. 2.

Table 5. 2 Composition of binary mixture for predictions

Mixture No.	Concentration (µg mL^{-1})	
	ATOR	FENO
1	4.2	10
2	4.4	10
3	4.6	10
4	4.8	10
5	5	10
6	4.5	2
7	4.5	4
8	4.5	6
9	4.5	8
10	4.5	10
11	4.5	12
12	4.5	14
13	4.5	16
14	4.5	18
15	4.2	20

5.3 Results and discussion

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

5.3.1 Simultaneous equation method (SEM)

A calibration set containing seven dilutions each of ATOR (4-22 µg mL^{-1}) and FENO (2-20 µg mL^{-1}) was prepared in methanol and UV spectra were recorded in the

wavelength range 210-350 nm versus solvent blank. The overlay absorption spectra of standard solutions ATOR and FENO are shown in the Fig. 5. 1 and 2.

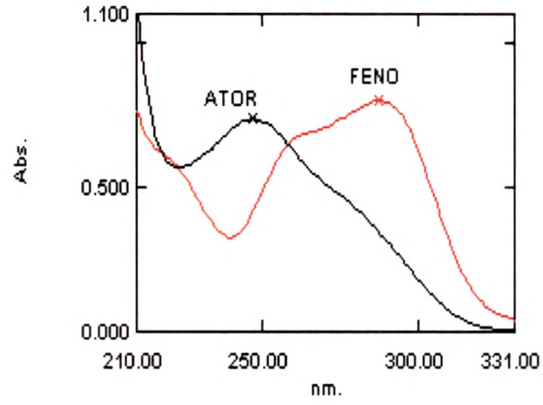


Fig. 5. 1 Zero-order overlay absorption spectra of 16 µg mL^{-1} of ATOR and 16 µg mL^{-1} of FENO in methanol

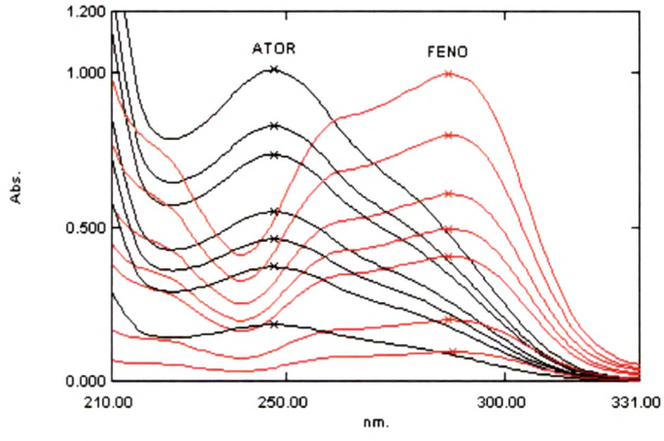


Fig. 5. 2 Overlay Zero-order absorption spectra for standard dilutions of ATOR and FENO

Absorbences of the above standard solutions of ATOR and FENO were measured at two wavelengths 247 and 287nm, 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 FENO were prepared as shown in Table 5. 2. Absorbences of binary mixture solutions were measured at 247 and 287nm. The concentration of each component of the binary mixture was calculated by using simultaneous equation.

$$C_{FENO} = \frac{Am_{287} * E_{FENO247} - Am_{247} * E_{FENO287}}{E_{ATOR287} * E_{FENO247} - E_{ATOR247} * E_{FENO287}}$$

$$C_{ATOR} = \frac{Am_{247} * E_{ATOR287} - Am_{287} * E_{ATOR247}}{E_{ATOR287} * E_{FENO247} - E_{ATOR247} * E_{FENO287}}$$

C_x = concentration of x,

Am_n= absorbance of sample solution at 'n' nm,

E_{xn} = absorptivity of x at 'n' nm,

For simultaneous determination of ATOR and FENO in their binary mixture a simultaneous equation method was successfully developed. Standard solutions of ATOR and FENO were prepared to determine their absorptivity values at two selected λmax 247 nm and 287 nm. The absorptivity values ± SD at 247 and 287 nm for both the drugs were $E_{FENO247}$ = 254.80 ± 6.36, $E_{FENO287}$ = 494.40 ± 3.99, $E_{ATOR247}$ = 458.922 ± 2.23 and $E_{ATOR287}$ = 217.36 ± 1.38.

5.3.2 Q - Absorbance method (Q- ANAL)

The method involves the formation of Q-Absorbance equation at 258 nm (iso-absorptive point) and 287 nm (λ_{max} of FENO) using methanol as solvent.

Selection of analytical wavelengths for Q- Absorbance method: Pure drug sample of ATOR and FENO, which were separately dissolved in methanol to give two solutions of 16 μg mL^{-1} and scanned between wavelength ranges of 200-350 nm. From the overlain spectra of both dugs (Fig. 5. 3) wavelength 258 nm (iso-absorptive point) and 287 nm (λ_{max} of FENO) were selected for formation of Q-Absorbance equation. For calibration curve, working stock solution of ATOR and FENO were appropriately diluted to obtain concentration range of 4-22 μg $mL^{-1}$of ATOR 2-20 μg mL^{-1} for

FENO. The absorbance of ATOR and FENO at 258 nm and 287 nm were measured and calibration curve were plotted. The absorptivities ($A_{1\%}^{1cm}$) of each drug at both the wavelengths were determined.

The absorbance and absorptivity values at these wavelengths were substituted in following equation to obtain the concentration of laboratory prepared binary mixtures and in formulation.

$$C_{ATOR} = \left(\frac{\text{Qm - Qy}}{\text{Qx - Qy}} \right) * \frac{A_1}{ax_1}$$

$$C_{FENO} = \frac{A_1}{ax_1} - C_{ATOR}$$

Where C_{ATOR} and C_{FENO} are the concentrations of ATOR and FENO respectively. A_1 is absorbance of sample mixtures at isobestic point (258 nm), ax_1 is absorptivity of ATOR at 258 nm. Q_x is ratio of absorptivity of ATOR at 287 nm to absorptivity at 258 nm. Q_y is ratio of absorptivity of FENO at 287 nm to absorptivity at 258 nm. Q_m is ratio of absorbance of samples (binary mixtures) at 287 nm to absorbance of sample at 258 nm. The ratio absorptivity values Q_x and Q_y were found to be 0.5244 and 1.235 respectively.

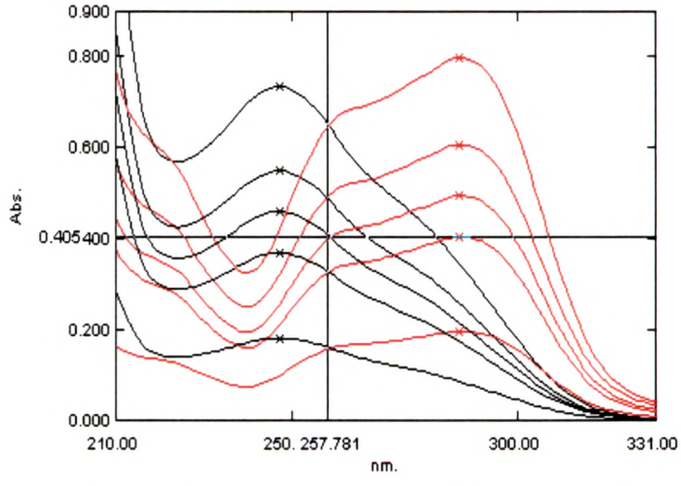


Fig. 5. 3 Overlay zero-order absorption spectra of standard dilutions of ATOR and FENO representing the isobestic point at 257.781 nm (258 nm)

5.3.3 First derivative Zero-crossing method (FDZC)

A calibration set of seven dilutions each of ATOR and FENO within a stated range was prepared and UV spectra were recorded and converted the same to first derivative spectra in the wavelength range 210-350 nm versus solvent blank. The first derivative overlay spectra of standard solutions of ATOR and FENO are shown in the Fig. 5. 4 (a) and 4 (b).

Preparation of calibration curve

The absorption spectra of working standard solutions of ATOR and FENO were recorded between 210-350 nm in triplicates and stored in the memory of the instrument. The first derivative of the working standard solutions were traced 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 247 nm that of FENO crosses zero at 239 nm and 287 nm. The amplitudes at 239 nm and 287 nm were plotted against the respective concentrations of ATOR. It was found that 287 nm shows the good linearity for the determination of ATOR. The method shows good linearity in the range of 4 to 22 μg mL^{-1} for ATOR. Similarly the amplitudes at 225 nm and 247 nm were plotted against the respective concentrations of FENO. It was found that 247 nm shows the good linearity for the determination of FENO. The method shows good linearity in the range of 2 to 20 μg mL^{-1} for FENO.

Spectrophotometric first derivative zero crossing method was successfully developed for simultaneous determination of ATOR and FENO 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 5. 3. Methanol was selected as solvent, 287 nm was selected for the determination of ATOR as the first derivative spectra of FENO shows zero amplitude (zero cross) at 287 nm. Similarly 247 nm was selected for the determination of FENO as the first derivative spectra of ATOR shows zero amplitude (zero cross) at 247 nm.

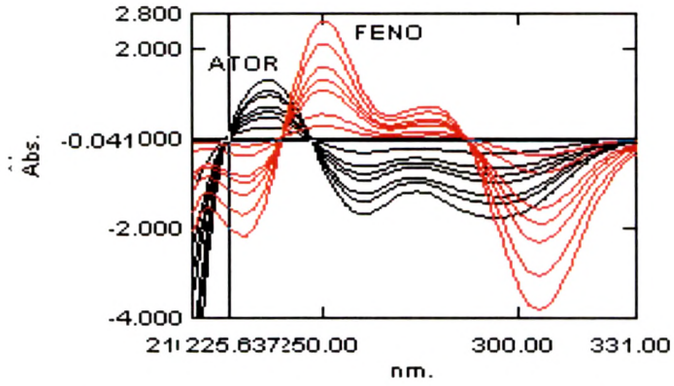


Fig. 5. 4 (a) Overlay first derivative absorption spectra of standard dilutions of ATOR and FENO represents the zero crossing of ATOR at 225 nm and 247nm

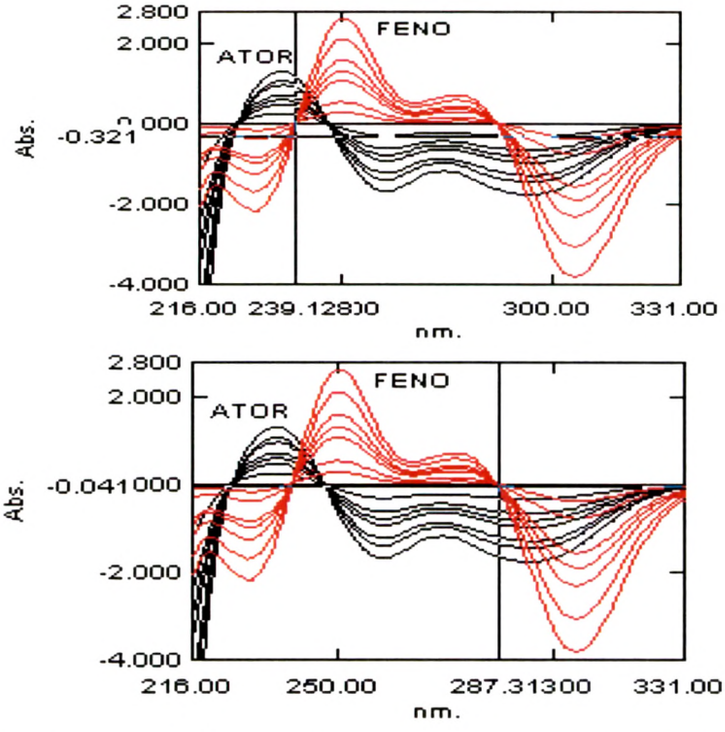
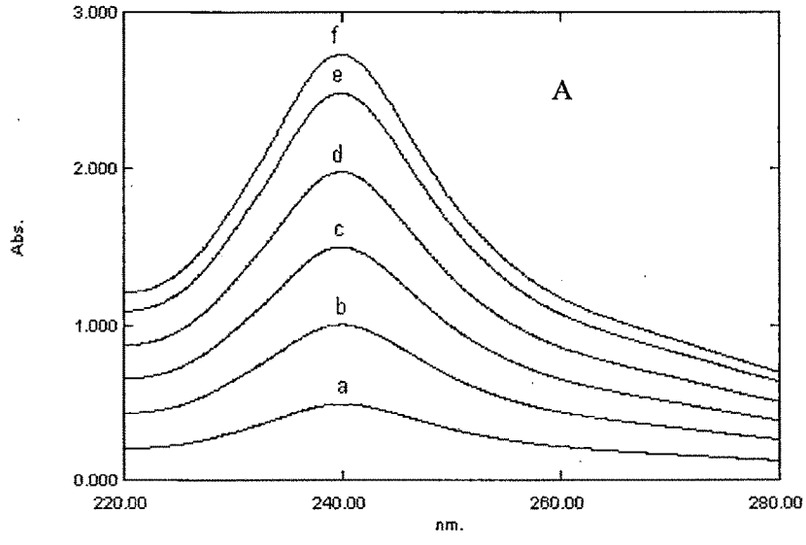


Fig. 5. 4 (b) Overlay first derivative absorption spectra of standard dilutions of ATOR and FENO represents the zero crossing of FENO at 239 nm and 287nm

5.3.4 Ratio spectra first derivative spectrophotometry (RFD)

The ratio spectra of different ATOR standards at increasing concentration in methanol were obtained by dividing each with the stored spectrum of the standard solution of 16 μg mL^{-1} FENO by computer aid as divisor spectra; these ratio spectra are shown in Fig. 5. 5A. The first derivatives (^{1}DD) of this spectrum traced with interval of $\Delta\lambda$ = 8 nm are illustrated in Fig. 5. 5B. As seen in Fig. 5. 5B, one minimum (247.5 nm) and one maximum (232.5 nm) exist and we found that both were suiTable 5. for determination of ATOR in ATOR and FENO mixtures. The wavelength of 247.5 nm was selected for the determination of this compound in the assay of synthetic mixtures and Table 5.ts, due to its lower RSD values and more suiTable 5. mean recovery compared with other wavelength. For the determination of FENO, the ratio spectra of different FENO standards at increasing concentrations in methanol, obtained by dividing each with stored spectrum of the standard solution of 12 μg mL^{-1} of ATOR as divisor spectra by computer aid, are demonstrated in Fig. 5. 6A. The first derivatives (^{1}DD) of this spectrum traced with intervals of $\Delta\lambda$ = 8 nm are illustrated in Fig. 5. 6B. As seen in Fig. 5. 6B, there exist one minimum (229 nm) and one maximum (254 nm) and in this also both were suiTable 5. for the determination of FENO in FENO and ATOR mixtures. The peak at wavelength 254 nm was selected because of its lower RSD and more suiTable 5. mean recoveries.



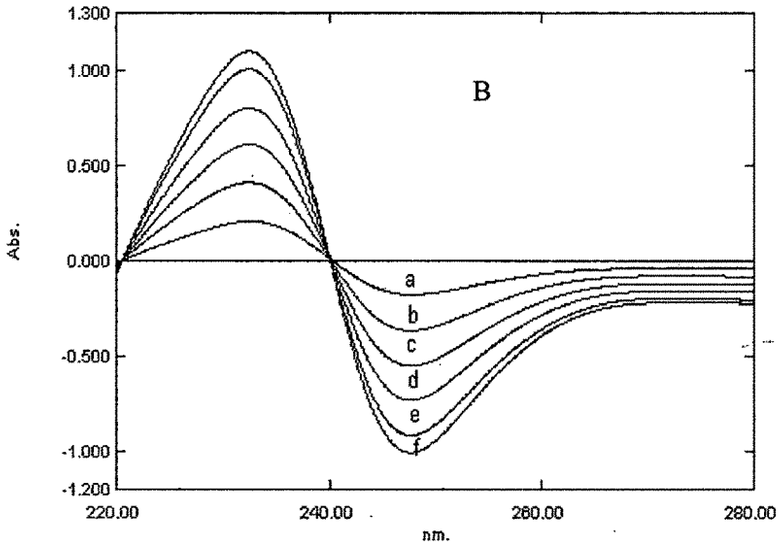
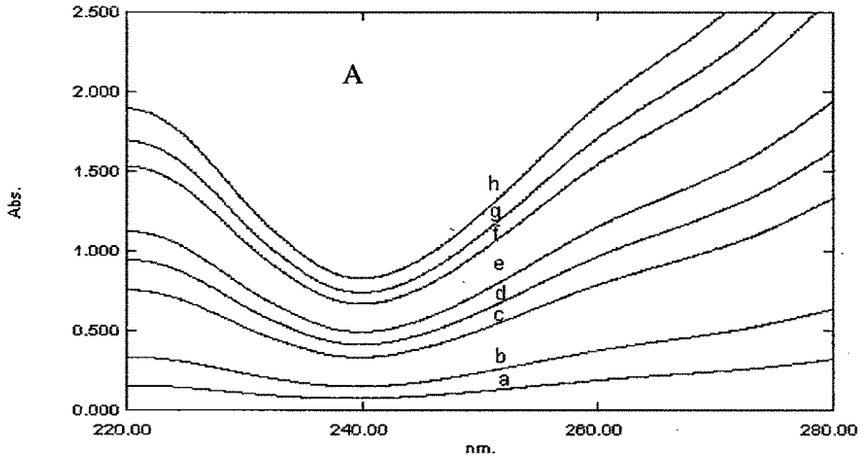


Fig. 5. 5 (a) Ratio spectra (A) and first derivative of the ratio spectra (B) of a) 4 µg mL^{-1} b) 8 µg mL^{-1} c) 12 µg mL^{-1} d) 16 µg mL^{-1} e) 20 µg mL^{-1} f) 22 µg mL^{-1} solution of ATOR in methanol when 16 µg mL^{-1} of FENO in methanol used as divisor ($\Delta\lambda$ = 8 nm), scaling factor =10



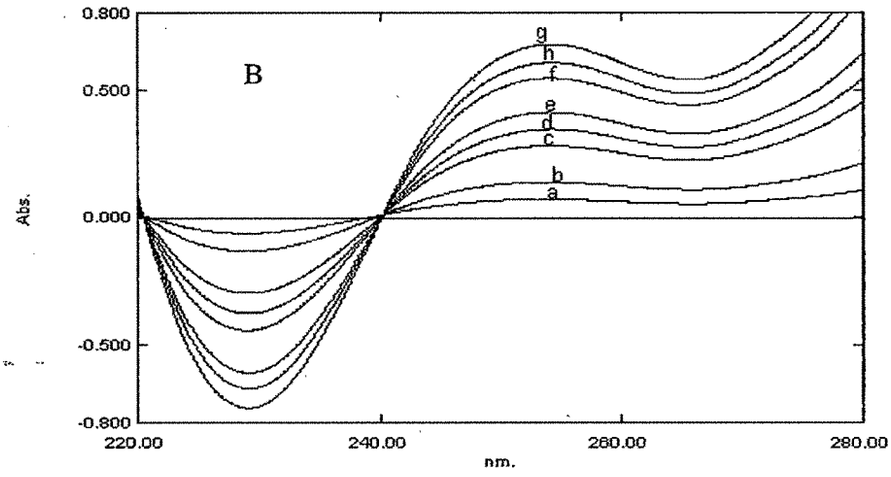


Fig. 5. 5 (b) Ratio spectra (A) and first derivative of the ratio spectra (B) of a) 2 μg mL^{-1} b) 4 μg mL^{-1} c) 8 μg mL^{-1} d) 10 μg mL^{-1} e) 12 μg mL^{-1} f) 16 μg mL^{-1} g) 18 μg mL^{-1} h) 20 μg mL^{-1} solution of FENO in methanol when 12 μg mL^{-1} of ATOR in methanol used as divisor ($\Delta\lambda$ = 8 nm), scaling factor =10

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 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 the main instrumental parameter: the standard spectrum of 12 μg mL^{-1} of ATOR and 16 μg mL^{-1} of FENO was considered as divisor for the determination of FENO and ATOR in mixtures respectively.

Calibration graphs were established from analytical signals measured at 247.5 nm for standards containing 4 - 22 μg mL^{-1} of ATOR and at 254 nm for standards containing 2 – 20 μg mL^{-1} of FENO, corresponding to maxima and minima in the absence of each other. All the analytical parameters are illustrated in Table 5. 3.

Table 5. 3 Analytical data of the calibration graphs for the determination of ATOR and FENO by spectrophotometric method

	SEM		Q- ANAL		FDZC		RFD	
Parameters	ATOR	FENO	ATOR	FENO	ATOR	FENO	ATOR	FENO
Wavelength (nm)	247 & 287		258 & 287		287	247	247.5	254
Linearity range ($\mu g\ mL^{-1}$)	4-22	2-20	4-22	2-20	4-22	2-20	4-22	2-20
Intercept (a)	0.0003 & 0.0045	0.0069 & 0.0076	0.0007 & 0.0011	0.0069 & 0.0048	0.0005	0.003	0.0012	0.0061
SE of Intercept[1]	0.011 & 0.015	0.256 & 0.019	0.0023 &	0.054 & 0.098	0.0563	0.001	0.0022	0.0032
Slope (b)	0.0459 & 0.987	0.0268 & 0.587	0.0414 & 0.0216	0.041 & 0.0502	0.9114	0.945	0.0459	0.0335
SE of slope	0.003 & 0.005	0.005 & 0.026	0.001 &	0.009 & 0.058	0.012	0.065	0.0001	0.0002
Correl. coeff	1 & 1	0.999 & 0.999	0.9996 & 0.9999	0.9995 & 0.9997	0.9999	0.9998	1	0.999
RSE[2]	0.0036 & 0.0037	0.0023 & 0.035	0.0018 & 0.0984	0.0069 & 0.0563	0.0078	0.0054	0.0022	0.0043

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

The proposed method was successfully applied for the determination of the two drugs in laboratory-prepared mixtures.

5.3.5 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 5. 2) samples in various concentrations of ATOR and FENO (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 5. 4. Their numerical values were completely accepTable 5. 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 FENO within the range of 4 - 22 $\mu g\ mL^{-1}$ and 2 – 20 $\mu g\ mL^{-1}$ respectively with lowest co-relation co-efficient, intercept and slope (Table 5. 3).

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 FENO was as shown in Table 5. 5 (a) and 5 (b).

Accuracy

Accuracy of the proposed methods was determined by performing recovery study in triplicates from previously analyzed formulation and from laboratory prepared synthetic mixture by standard addition method at four and three levels respectively. 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 5. 6a and 6b).

Table 5. 4 Prediction results for ATOR and FENO in synthetic mixtures by proposed spectrophotometric techniques

Mixture added		Recovery (%)								Error%							
		SEM		Q - ANAL		FDZC		RFD		SEM		Q - ANAL		FDZC		RFD	
ATOR	FENO	ATOR	FENO	ATOR	FENO	ATOR	FENO	ATOR	FENO	ATOR	FENO	ATOR	FENO	ATOR	FENO	ATOR	FENO
4.2	10	100.8	98.6	101.3	98.4	102.4	99.4	101.3	98.5	0.8	-1.4	1.3	-1.6	2.4	-0.6	1.3	-1.5
4.4	10	101.3	98.9	101.7	99.4	101.6	98.1	101.6	98.2	1.3	-1.1	1.7	-0.6	1.6	-1.9	1.6	-1.8
4.6	10	101.7	97.5	100.9	99.7	101.7	98.4	100.0	99.4	1.7	-2.5	0.9	-0.3	1.7	-1.6	0	-0.6
4.8	10	103.4	98.3	102.8	98.2	101.6	98.6	102.7	98.2	3.4	-1.7	2.8	-1.8	1.6	-1.4	2.7	-1.8
5	10	100.9	97.2	101.3	98.7	103.5	98.4	102.9	98.2	0.9	-2.8	1.3	-1.3	3.5	-1.6	2.9	-1.8
4.5	2	101.9	99.1	103.6	98.4	102.5	99.3	100.8	101.3	1.9	-0.9	3.6	-1.6	2.5	-0.7	0.8	1.3
4.5	4	102.6	98.3	102.7	99.3	101.6	100.2	99.8	97.7	2.6	-1.7	2.7	-0.7	1.6	0.2	-0.2	-2.3
4.5	6	103.5	99.2	102.7	99.7	102..3	100.2	101.3	100.4	3.5	-0.8	2.7	-0.3	2.3	0.2	1.3	0.4
4.5	8	100.7	100.4	101.7	100.2	101.6	99.4	101.3	101.1	0.7	0.4	1.7	0.2	1.6	-0.6	1.3	1.1
4.5	10	98.6	100.6	102.6	99.4	102.7	99.5	101.8	100.0	-1.4	0.6	2.6	-0.6	2.7	-0.5	1.8	0
4.5	12	103.5	98.3	103.4	100.4	103.4	100.5	97.4	100.0	3.5	-1.7	3.4	0.4	3.4	0.5	-2.6	0
4.5	14	102.4	100.5	101.6	98.3	102.4	102.6	102.7	100.8	2.4	0.5	1.6	-1.7	2.4	2.6	2.7	0.8
4.5	16	101.5	99.2	101.5	99.9	103.5	101.1	103.2	100.7	1.5	-0.8	1.5	-0.1	3.5	1.1	3.2	0.7
4.5	18	102.5	98.4	100.5	98.6	102.5	98.7	103.2	101.3	2.5	-1.6	0.5	-1.4	2.5	-1.3	3.2	1.3
4.5	20	103.9	101.7	101.4	98.9	100.8	99.8	103.7	101.2	3.9	1.7	1.4	-1.1	0.8	-0.2	3.7	1.2
	$\bar{x}$	101.94	99.08	101.98	98.9	102.16	99.61	101.6	99.8								
	RSD	1.377	1.252	0.903	0.727	0.809	1.197	1.622	1.321								

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

Table 5. 5 (a) Intra day precision for determination of ATOR and FENO

Mixture No.	% RSD							
	SEM		Q-ANAL		FDZC		RFD	
	ATOR	FENO	ATOR	FENO	ATOR	FENO	ATOR	FENO
1	1.236	0.945	1.458	1.0340	1.329	1.98	2.01	1.001
2	1.345	1.356	1.274	10851	1.123	0.935	1.6535	2.11
3	0.958	0.960	1.065	1.486	0.142	1.222	0.5045	0.601
4	. 0.899	1.123	0.596	0.695	1.221	1.021	0.233	0.251
5	1.348	1.101	0.653	1.549	1.311	1.13	0.206	1.301
Average of % RSD	1.1572	1.097	1009	1.169	1.0252	1.2576	0.9214	1.0528

*** Average of three experiments in a day.

Table 5. 5 (b) Inter day precision for determination of ATOR and FENO

Mixture No.	% RSD							
	SEM		Q-ANAL		FDZC		RFD	
	ATOR	FENO	ATOR	FENO	ATOR	FENO	ATOR	FENO
1	1.65	1.001	1.154	1.024	1.547	1.007	1.547	1.057
2	1.254	1.058	1.584	1.640	0.910	0.941	1.584	1.742
3	0.928	0.921	1.028	1.521	0.196	1.058	0.356	0.2596
4	0.952	1.654	0.528	0.683	1.348	0.962	0.2394	0.2654
5	1.158	1.569	0.689	1.254	1.358	1.548	0.461	1.592
Average of % RSD	1.184	1.240	0.996	1.224	1.071	1.103	0.848	0.983

*** Average of three experiments in three different days.

Table 5. 6a Application of standard addition technique for analysis of FENO and ATOR in Lorilip Tablets

Serial No	ATOR						FENO					
	Concentration in $\mu g\ mL^{-1}$		% recovery ± SD[a]				Concentration in $\mu g\ mL^{-1}$		% recovery ± SD[a]			
	Claimed	Added	SEM	Q -ANAL	FDZC	RFD	Claimed	Added	SEM	Q -ANAL	FDZC	RFD
1	0.5+ 4[☼]	0	96.25± 0.721	100.43± 0.780	96.25± 0.721	100.43± 0.780	10	0	94.15± 0.621	101.13± 0.580	95.25± 0.621	101.33± 0.670
2	0.5+ 4[☼]	4	94.80± 0.69	98.75± 0.78	94.80± 0.69	98.75± 0.78	10	2	96.10± 0.66	97.65± 0.68	93.70± 0.59	98.65± 0.38
3	0.5+ 4[☼]	8	99.875± 0.68	101.78± 0.86	99.875± 0.68	101.78± 0.86	10	4	98.75± 0.78	99.58± 0.86	98.87± 0.56	102.68± 0.76
4	0.5+ 4[☼]	12	99.57± 0.566	102.3± 0.564	99.57± 0.566	102.3± 0.564	10	8	102.57± 0.466	101.4± 0.464	99.47± 0.166	101.2± 0.464
5	0.5+ 4[☼]	16					10	10				

[☼] Standard addition of ATOR , * average of three experiments, [a] sd standard deviation.

Table 5. 6b. Application of standard addition technique for analysis of FENO and ATOR in certified reference material

Serial No	% Standard addition	ATOR				FENO			
		% recovery ± SD[a]				% recovery ± SD[a]			
		SEM	Q-ANAL	FDZC	RFD	SEM	Q-ANAL	FDZC	RFD
1	80	97.82 ±0.7	102.67 ± 1.4	103.57 ± 0.76	103.75 ± 1.92	97.63 ±0.9	98.61 ± 0.9	101.22 ± 0.86	103.53± 0.01
2	100	100.64 ± 1.85	98.66 ± 1.1	101.75 ± 0.82	100.91 ± 0.24	102.1 ± 0.15	97.59 ± 1.5	103.95 ± 0.22	101.99± 0.24
3	120	104.20 ± 0.12	96.24 ± 0.98	99.42 ± 2.2	101.62 ± 1.72	102.3 ± 0.17	102.15±0.92	96.66 ± 1.2	97.79± 1.22

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

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 FENO 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 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 5. 7 LOD and LOQ

Results observed	ATOR	FENO
*SD	0.01376	0.0433
LOD ($\mu g\ mL^{-1}$)	0.272	0.090
LOQ ($\mu g\ mL^{-1}$)	0.3672	0.128

* Standard deviation of intercepts of five calibration curves

Application of the proposed methods for Tablet analysis

Applicability of the method was tested by analyzing the commercially available formulations containing the binary mixture of ATOR and FENO. 0.4 $\mu g\ mL^{-1}$ constant standard addition was chosen for ATOR. The values of % recovery from formulation as shown in the Table 5. 8 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 FENO from their binary mixture formulation.

Table 5. 8 Results obtained for the Lorilip tablets by using the Spectrophotometric methods

Methods	ATOR (Label claim= 10mg per table t) Mean[a] ± SD[b]	FENO (Label claim= 200mg per tablet) Mean[a] ± SD[b]
SEM	(9.6) ± 4.19	193 ± 3.90
Q-ANAL	(9.7) ± 3.95	199 ± 2.30
FDZC	(9.6) ± 2.72	198 ± 2.24
RFD	(9.8) ± 2.86	194 ± 2.36

Mean[a], mean value of five determinations (n=5) for each method; SD[b], Standard deviation; Values in paranthesis correspond to the parameters calculated after accounting for the actual values for ATOR that is without standard addition

5.4 Results and discussion

Chromatography, HPTLC and Chemometric methods

The HPLC and HPTLC mobile phase acetonitrile: methanol (73:27, v/v) and chloroform : methanol : toluene (4 : 3 : 6, v/v) was selected respectively. Because it was found that these mobile phases ideally resolves the peaks with retention time (R_t) 1.931 min and 3.94 min for ATOR and FENO respectively in HPLC (Fig. 5. 6) and retention factor (R_f) 0.19 and 0.76 for ATOR and FENO respectively in HPTLC (Fig. 5. 7).

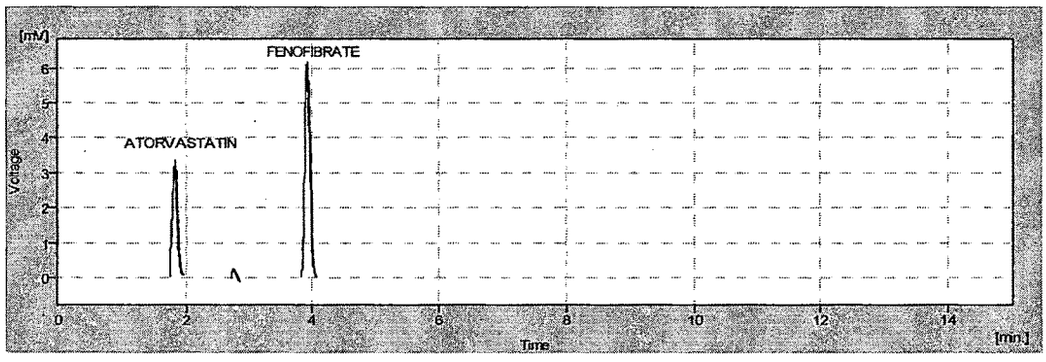


Fig. 5. 6 HPLC chromatogram showing retention time (R_t) of 8 µg mL^{-1} of ATOR (1.93 min) and 16 µg mL^{-1} of FENO (3.94 min) in laboratory-prepared mixture

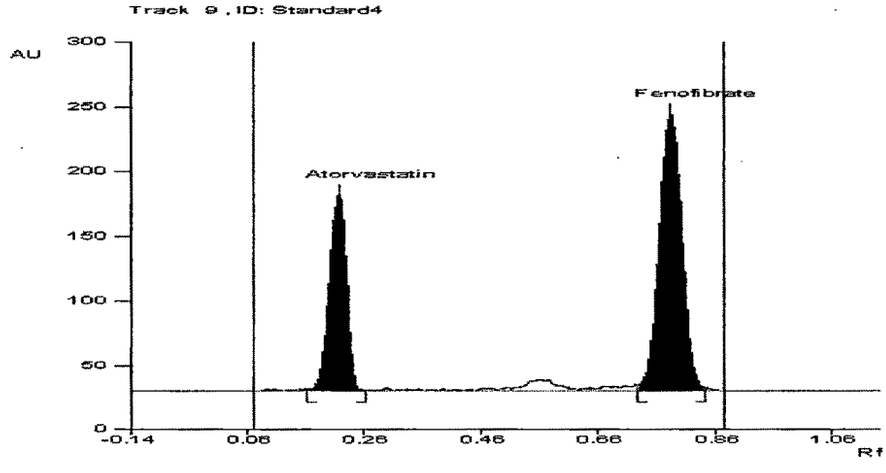


Fig. 5. 7 HPTLC chromatogram showing retention factor (R_f) of 500 ng of ATOR (0.19) and (b) 250 ng of FENO (0.76) in laboratory-prepared mixture

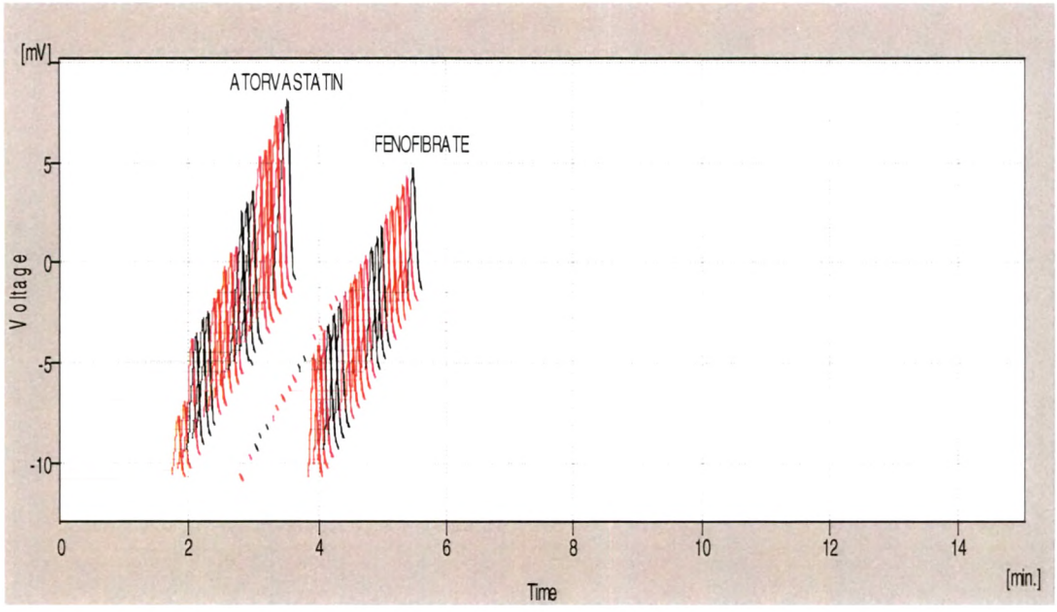


Fig. 5. 8 (a) HPLC 3-Dimensional chromatograms set of seven standard dilutions of ATOR (in triplicate) using 8 µg mL^{-1} of FENO as internal standard

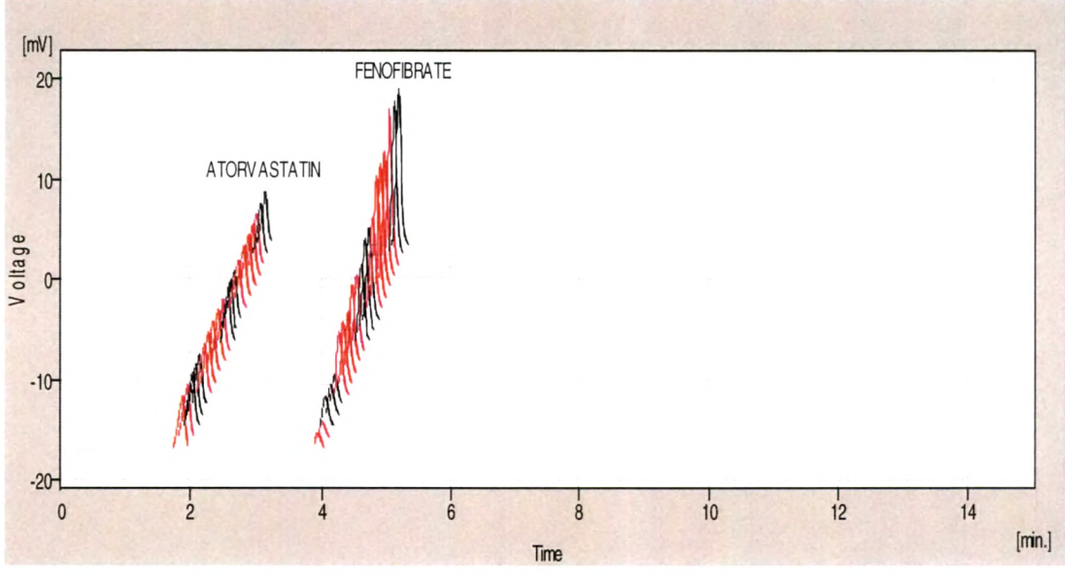


Fig. 5. 8 (b) HPLC 3-Dimensional chromatograms set of seven standard dilutions of FENO (in triplicate) using 8 µg mL^{-1} of ATOR as internal standard

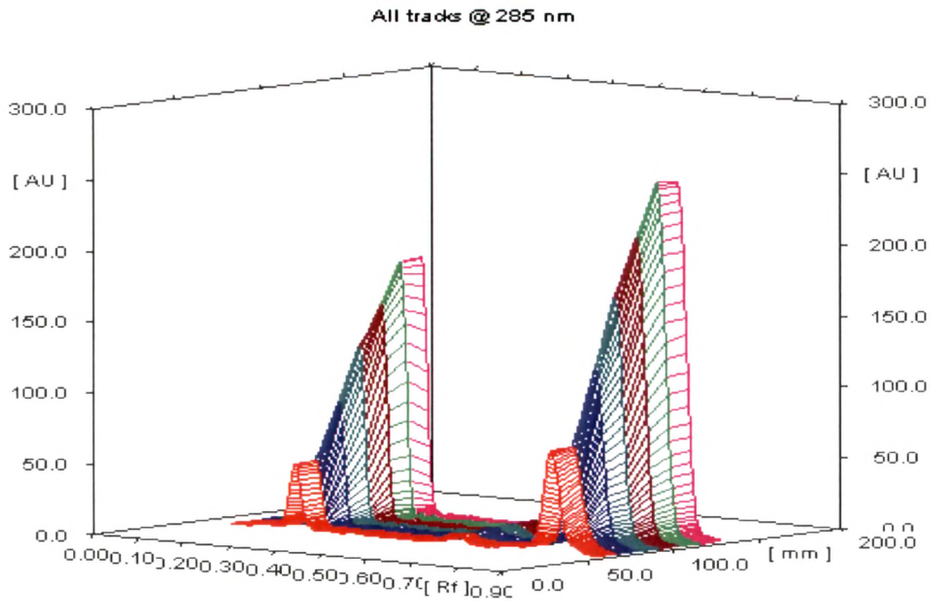


Fig. 5. 9 HPTLC 3-Dimensional chromatograms set of six standard dilutions of ATOR and FENO for calibration

Table 5. 9. Characteristic parameters of the calibration equations for the proposed HPLC and HPTLC methods for simultaneous determination of ATOR and FENO

Parameters	HPLC		HPTLC	
	ATOR	FENO	ATOR	FENO
Calibration range (μg mL^{-1})	10 - 100	2 - 16	0.1-1	0.05 -0.5
Detection limit (μg mL^{-1})	0.005×10^{-2}	0.027×10^{-2}	0.0049×10^{-2}	0.0037×10^{-2}
Quantitation limit (μg mL^{-1})	0.018×10^{-2}	0.089×10^{-2}	0.0169×10^{-2}	0.0129×10^{-2}
Regression equation (Y)[a]				
Slope (b)	0.3468	2.3137	8.080	29.384
Standard deviation of the slope (S_b)	1.7×10^{-3}	1.90×10^{-2}	1.76	1.77
Relative standard deviation of the slope (%)	0.449	0.744	1.365	2.195
Intercept (a)	0.0239	0.3775	27.442	1095.43
Standard deviation of the intercept (S_a)	0.000650	0.000346	23.46	16.63
Correlation coefficient	0.9999	0.9999	0.9996	0.9993
Theoretical plates	2372	7818	NA	NA
Symmetry factor	1.050	1.389	NA	NA
Resolution	4.992		NA	NA

a $Y = a + bC$, where C is the concentration of compound in μg mL^{-1} and Y is the peak area.

Simutaneous estimation of Atorvastatin and Fenofibrate
Methodology, Results and discussion

Chapter 5

Chemometric techniques are other methods gaining wide application for the resolution of the drug mixtures. A 23 calibration set of standard binary mixtures was randomly prepared as mixtures of ATOR and FENO in the possible composition in methanol in the concentration range illustrated in Table 5. 1. The U V absorbance data was obtained by measuring the absorbances in the region of 221 - 300 nm. By using the correlation between calibration concentration and its absorbance data, the chemometric calibrations were calibrated within the CLS, ILS, PCR and PLS algorithms.

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 310 nm were not used because ATOR has no absorbance at the concentrations used in this region any absorbance data beyond 310 nm would have introduced a significant amount of noise, thereby decreasing the precision of ATOR 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 80 experimental points per spectrum, as spectra were digitized at 1 nm intervals.

Statistical parameter

The predictive ability of a 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 drug, C_i^{Found} is the predicted concentration of drug and n the total number of the synthetic mixtures. The numerical values are quoted in respective Table 5.s 10 and 11.

Selection of optimum number of factors for PCR and PLS

For PCR and PLS methods, a number of 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 regression applied. The predicted concentrations were then compared with the actual ones for each of the calibration samples and root 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 (Fig. 5. 12 and 13) indicates factor three and four were optimum by PCR and PLS respectively for the estimation of title drugs. At the selected principal components of PCR and PLS the concentrations of each sample was then predicted and compared with known concentration and the PRESS (Prediction Error Sum of Squares) was calculated. It was given by the equation, and values were indicated in Table 5. 10.

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

5.5 Validation of analytical method

To check the validity (predictive ability) of the calibration models, the simultaneous analysis of the prediction set containing each of 15 samples in various concentrations of ATOR and FENO(in triplicates) was carried out by HPLC and chemometric methods. The mean recoveries, % errors and the relative standard deviations of prediction sets were computed and indicated in Table 5. 12. Their numerical values were completely acceptable. because of their good recoveries and hence found satisfactory for the validation.

Linearity

The linearity of the proposed HPLC, HPTLC and chemometric methods for determination of ATOR and FENO was evaluated by analysing a series of different concentrations of standard drug. In this study seven concentrations were chosen, ranging between 4-22 $\mu g\ mL^{-1}$ of ATOR and 2-20 $\mu g\ mL^{-1}$ of FENO. Similarly in HPTLC linearity was evaluated by analysing a series of different concentrations of standard drug ranging between 200-1000 ng of ATOR and 50-500 ng of FENO (Fig. 5. 9). Each concentration was repeated three times and obtained information on the variation in peak area response and absorbances at stated wavelength region in HPLC, HPTLC and chemometric methods respectively. The linearity of the calibration graphs of proposed methods was validated by the high value of correlation coefficient, slope and the intercept.

Range

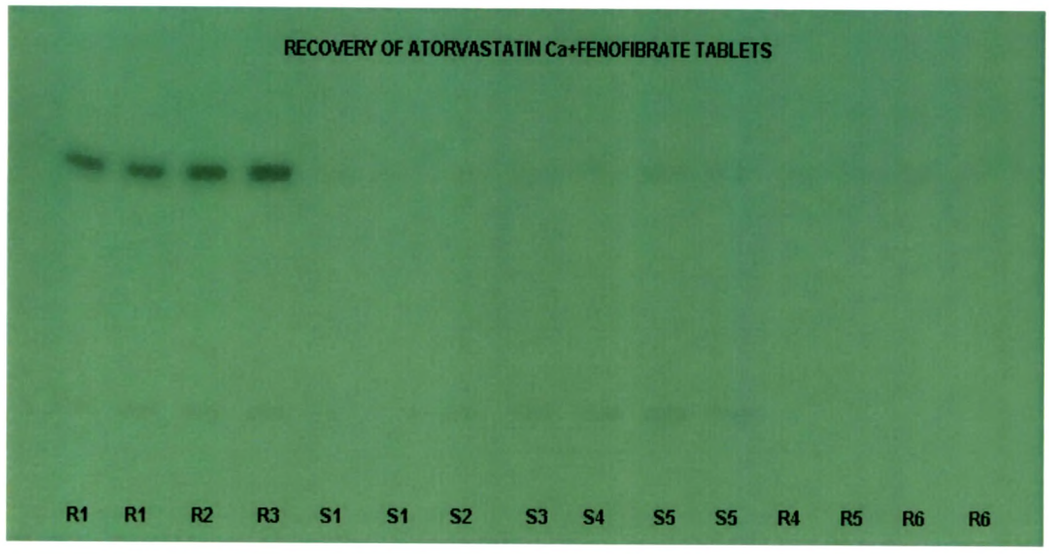
The calibration range of the proposed methods was established through wide consideration of the practical range necessary, according to each ingredient concentration present in pharmaceutical products of different manufacturers.

Accuracy

The study was performed by increasing standard addition of known amounts of studied drugs to an unknown concentration (constant volume)^2 of the commercial pharmaceutical formulations (Standard addition access the effect of a sample matrix changes the analytical sensitivity of the method).

A constant volume of the unknown solution is added to each of five 10 mL volumetric flasks. Then a series of increasing volumes of working standard solutions are added. Finally, each flask is made up to the mark with solvent and mixed well. The concentration of the working standard solutions added should be chosen to increase the concentration of the unknown by minimum 30% in each succeeding flask. The resulting mixtures were analysed by the proposed HPLC and HPTLC (Fig. 5. 10) methods and the response obtained was plotted against the initial unknown concentration set at 0. And chemometric recoveries were also

determined. The results obtained are compared with expected results. The excellent mean recoveries and standard deviation (Table 5. 13 (a) and 13 (b)) suggested good accuracy of the proposed methods and no interference from formulations excipients.



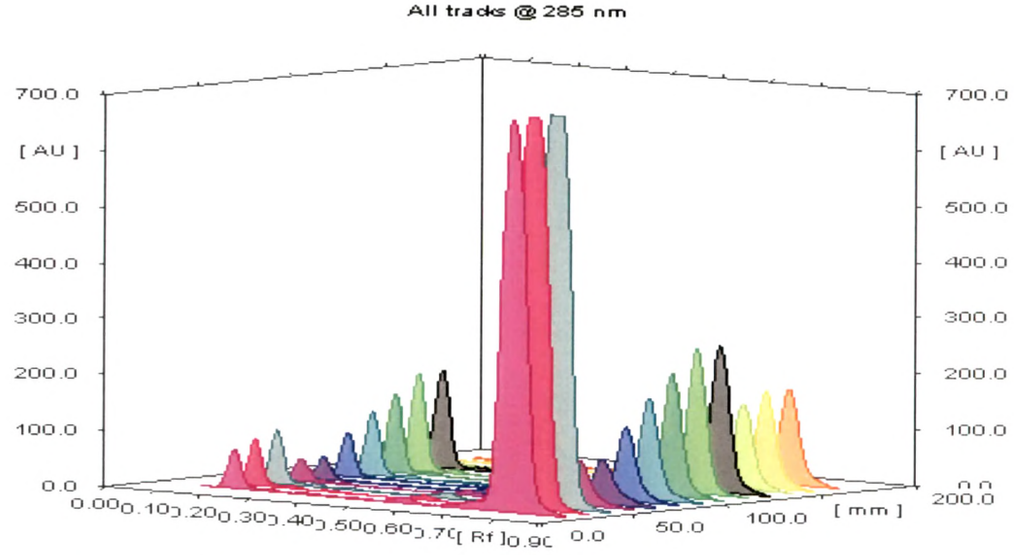


Fig. 5. 10 Image @ 285 nm and 3-Dimensional HPTLC chromatogram of ATOR and FENO used for recovery studies

Table 5. 10 Statistical parameters of chemometric methods in calibration step of zero-order spectra

Component	CLS	ILS	PCR			PLS		
	SEC	SEC	SEC[a]	PRESS[b]	RSE[c] (%)	SEC	PRESS	RSE (%)
ATOR	0.132	0.000	0.195	0.838	0.0456	0.212	0.989	0.065
FENO	0.163	0.057	0.021	0.010	0.1221	0.019	0.008	0.123

a Standard error of calibration, *b* Predicted error sum of squares, *c* Relative standard error of calibration of single component.

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

Table 5. 11 Statistical parameters of chemometric methods in the validation step

Component	CLS				ILS				PCR				PLS			
	SEP	a	b	r	SEP	a	b	r	SEP	a	b	r	SEP	a	b	r
ATOR	0.191	-1.860	1.376	0.947	0.138	-2.660	1.572	0.972	0.068	-0.188	1.029	0.977	0.080	-0.503	1.097	0.970
FENO	0.705	-0.079	0.948	0.999	0.160	0.080	1.002	0.999	0.398	-0.059	0.973	0.999	0.526	0.074	0.951	0.999

a, intercept; b, slope; r, correlation coefficient

Table 5. 13 (a) Application of standard addition technique for analysis of FENO and ATOR in Lorilip tablets

Serial No	ATOR						FENO					
	Concentration in µg mL^{-1}		% recovery ± SD[a]				Concentration in µg mL^{-1}		% recovery ± SD[a]			
	Claimed	Added	CLS	ILS	PCR	PLS	Claimed	Added	CLS	ILS	PCR	PLS
1	0.5+ 4[☼]	0	97.19 ± 0.75	99.56 ± 0.89	96.25± 0.72	99.57± 0.56	10	0	96.12 ± 0.07	98.56 ± 0.23	93.70± 0.59	95.25± 0.62
2	0.5+ 4[☼]	4	95.85 ± 0.81	99.75± 0.81	94.80± 0.69	98.75± 0.78	10	2	96.75 ± 0.61	100.76.± 0.81	97.65± 0.68	101.13± 0.58
3	0.5+ 4[☼]	8	98.345 ± 0.85	102.89 ± 0.73	99.875± 0.68	101.78± 0.86	10	4	97.45 ± 0.81	101.29 ± 0.63	99.58± 0.86	98.87± 0.56
4	0.5+ 4[☼]	12	98.54 ± 0.82	101.1 ± 0.48	100.43± 0.78	102.3± 0.56	10	8	99.43 ± 0.21	99.11 ± 0.58	101.4± 0.46	99.47± 0.16
5	0.5+ 4[☼]	16	96.31 ± 0.32	97.2 ± 2.18	96.7 ± 1.98	96.9 ± 1.99	10	10	98.37 ± 1.56	96.33 ± 1.67	98.7 ± 2.01	98.91 ±2.09

[☼] standard addition of ATOR , * average of five experiments, [a] sd standard deviation.

Table 5. 13 (b) Application of standard addition technique for analysis of FENO and ATOR in Lorilip tablets

Serial No	ATOR				FENO			
	Concentration in µg mL^{-1}		% recovery ± SD[a]		Concentration in µg mL^{-1}		% recovery ± SD[a]	
	Claimed	Added	HPLC	HPTLC	Claimed	Added	HPLC	HPTLC
1	0.5+ 4[☼]	0	96.32 ± 0.97	93.45 ± 2.36	10	0	97.59 ± 1.97	98.36 ± 2.11
2	0.5+ 4[☼]	4	98.35 ± 1.67	96.45 ± 1.57	10	2	96.57 ± 2.01	92.18 ± 1.97
3	0.5+ 4[☼]	8	99.36 ± 0.97	99.46 ± 0.97	10	4	98.34 ± 1.64	97.67 ± 1.58
4	0.5+ 4[☼]	12	102.94 ± 1.67	106.49 ± 1.94	10	8	97.59 ± 1.62	98.26 ± 1.49
5	0.5+ 4[☼]	16	107.36 ± 1.36	105.59 ± 1.94	10	10	99.33 ± 0.99	99.65 ± 0.87

[☼] standard addition of ATOR , * average of five experiments, [a] sd standard deviation.

Table 5. 12 Recovery results in prediction from Zero- order spectra for ATOR and FENO in synthetic mixtures by proposed RP-HPLC and chemometric techniques

Recovery (%)										Error%									
HPLC		CLS		ILS		PCR		PLS		HPLC		CLS		ILS		PCR		PLS	
ATOR	FENO	ATOR	FENO	ATOR	FENO	ATOR	FENO	ATOR	FENO	ATOR	FENO	ATOR	FENO	ATOR	FENO	ATOR	FENO	ATOR	FENO
96.7	95.1	95.8	94.0	94.9	102.6	99.7	97	99	95.8	3.3	4.9	-4.2	-6.0	-5.1	2.6	-0.3	-3	-1	-3.7
97.8	92.5	98.3	93.6	96.4	101.8	100	95.4	99.6	98.3	2.2	7.5	-1.7	-6.4	-3.6	1.8	0.0	-4.7	-0.4	-4.4
98.2	94.6	99.3	93.7	98.7	98.8	98.8	95.3	98.5	99.3	1.8	5.4	-0.7	-6.3	-1.3	-1.2	-1.2	-4.8	-1.5	-4.5
100	93.4	100	92.7	101.3	94.9	98.8	94.1	98.6	100	0	6.6	0.0	-7.3	1.3	-5.1	-1.2	-5.9	-1.4	-5.9
102.3	92.6	101.2	93.3	104.9	98.2	100.2	94.2	101	101.2	-2.3	7.4	1.2	-6.7	4.9	-1.8	0.2	-5.8	1	-5.8
97.1	99.2	98.0	98.2	95.5	96.0	99.6	101.9	101	98.0	2.9	0.8	-2.0	-1.8	-4.5	-4.0	-0.4	1.9	1	7.3
95.5	91.2	94.6	93.3	96.3	97.3	97.4	100.2	97.5	94.6	4.5	8.8	-5.4	-6.7	-3.7	-2.7	-2.6	0.2	-2.5	-2.7
97.8	94.7	96.1	93.9	97.5	96.3	98.4	97.6	98.4	96.1	2.2	5.3	-3.9	-6.1	-2.5	-3.7	-1.6	-2.4	-1.6	-3.3
94.1	93.8	94.8	93.9	97.1	98.3	97.7	97.8	97.7	94.8	5.9	6.2	-5.2	-6.1	-2.9	-1.7	-2.3	-2.2	-2.3	-3.8
96.7	98.5	96.9	94.1	98.7	104	98.8	96.8	98.6	96.9	3.3	1.5	-3.1	-5.9	-1.3	4	-1.2	-3.2	-1.4	-3.9
98.4	96.8	95.5	93.7	98.9	102.8	98.1	96.9	98.1	95.5	1.6	3.2	-4.5	-6.3	-1.1	2.8	-1.9	-3.1	-1.9	-4.4
96.8	94.5	94.8	93.9	98.8	96.1	98.1	97.1	97.9	94.8	3.2	5.5	-5.2	-6.1	-1.2	-3.9	-1.9	-2.9	-2.1	-4.5
98.2	96.3	94.2	94.2	101.4	98.4	97.8	97.4	97.9	94.2	1.8	3.7	-5.8	-5.8	1.4	-1.6	-2.2	-2.6	-2.1	-4.2
100.1	97.2	94.1	100.2	97.8	102.9	99.2	97.5	97.5	94.1	-0.1	2.8	-5.9	-0.2	-2.2	2.9	-0.8	-2.5	-2.5	-4.4
95.3	100.2	94.1	100.1	99.4	98.4	99.2	97.9	98.2	94.1	4.7	-0.2	-5.9	-0.1	-0.6	-1.6	-0.8	-2.1	-1.8	-3.8
$\bar{x}$ 97.66	95.37	96.5	94.8	98.5	99.1	98.8	97.1	98.6	96.5										
RSD:2.11	2.73	2.4	2.6	2.6	3	0.9	2.1	1.1	2.4										

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

Analytes solution and mobile phase stability

Stability of FENO 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 (Fig. 5.11) using same mobile phase and spectrophotometric methods at 6h interval and all the solutions were found to be stable. for 72h. No interfering substances were found.

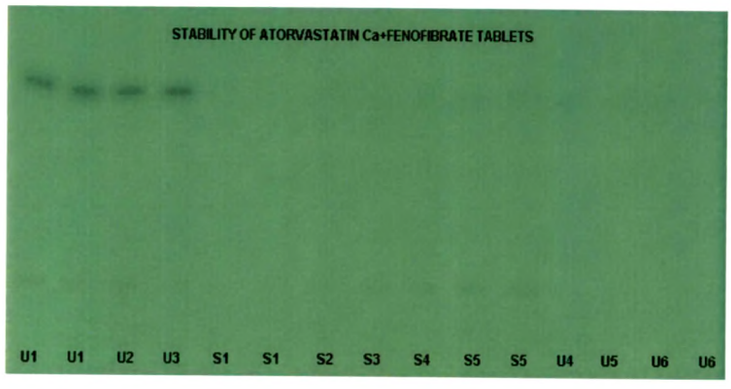


Fig. 5. 11 Image @ 285 nm of ATOR and FENO used for stability studies

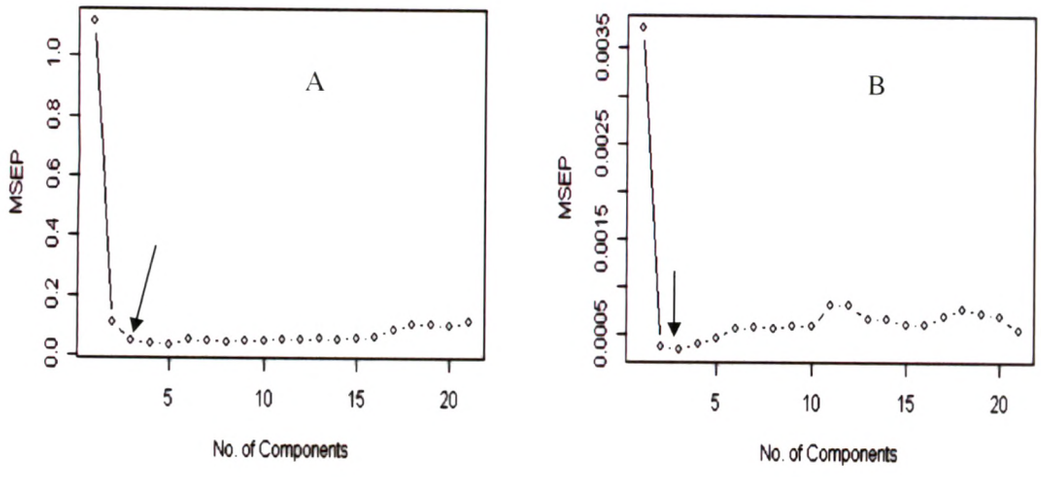


Fig. 5. 12 Representation of MSEP values generated from calibration by PCR A) for ATOR B) for FENO

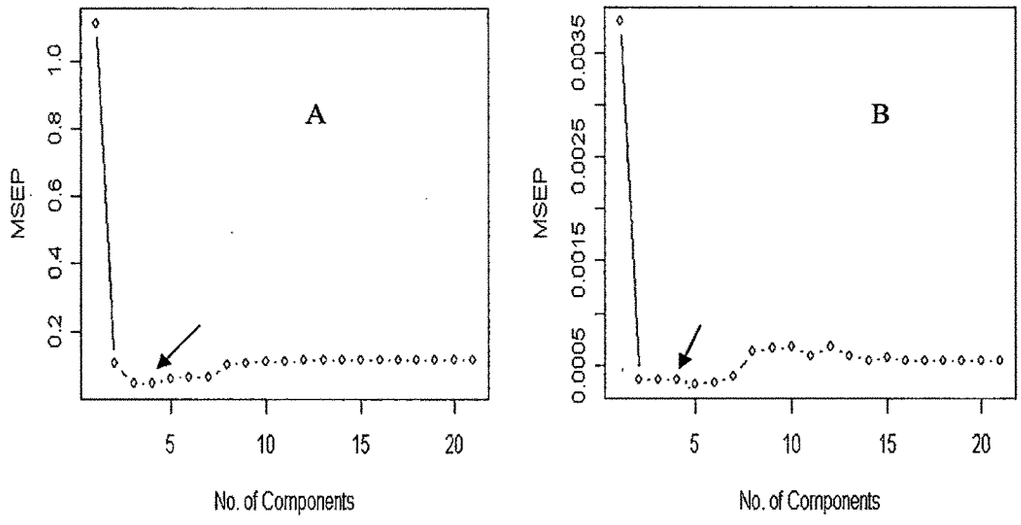


Fig. 5. 13 Representation of MSEP values generated from calibration by PLS A) for ATOR B) for FENO

Precision (Method reproducibility)

Method reproducibility was demonstrated by repeatability and intermediate precision measurements of peak area, peak asymmetry and retention time parameters of HPLC, peak area, retention factor parameters of HPTLC (Fig. 5. 14) and % recovery RSD in chemometric methods for each title ingredient.

The repeatability (within-day in triplicates) 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 5. 14).

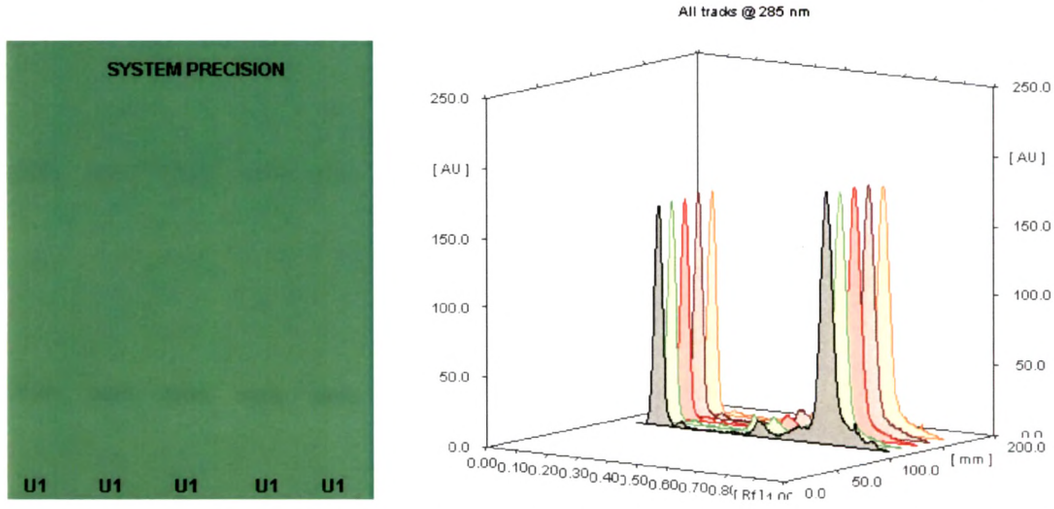


Fig. 5. 14 Image @ 285 nm and 3-Dimensional HPTLC chromatogram of ATOR and FENO demonstrating system precision

Table 5. 14 Precision study results of prepared binary mixture

Validation parameter	HPLC			Chemometric				HPTLC	
	% RSD			% RSD				% RSD	
Repeatability [a]	Peak area	Peak asymmetry	Retention time	CLS	PCR	PLS	ILS	Peak area	Retention factor
FENO	0.734	0.5634	0.105	2.085	1.8144	1.834	0.634	0.314	0.215
ATOR	1.093	0.2876	0.90654	2.162	0.3159	0.3269	1.193	1.173	0.3104
Intermediate precision[b]									
FENO	1.521	0.7707	0.4948	2.446	1.0867	1.5971	1.411	0.421	0.3248
ATOR	1.737	0.7508	0.0937	1.987	0.9346	0.2906	1.627	1.113	0.9137

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

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

Specificity

A representative three dimensional HPTLC chromatogram (Fig. 5.15) was obtained using diluents, mobile phase, placebo, ATOR standard, FENO standard, ATOR + FENO + diluents and ATOR + FENO +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 HPLC and Chemometric methods.

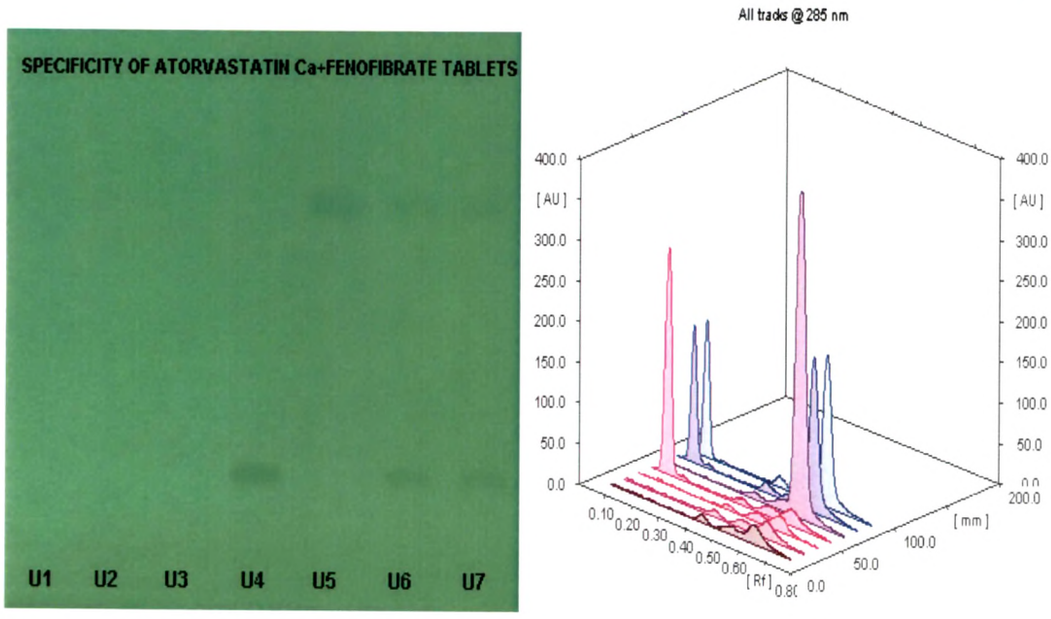


Fig. 5. 15 Image @ 285 nm and 3-Dimensional HPTLC Chromatogram of diluents, mobile phase, placebo, ATOR standard, FENO standard, ATOR + FENO + diluents and ATOR + FENO +diluents + placebo in specificity studies

Robustness

The robustness of the proposed HPLC method was assessed for peak asymmetric and peak resolution factor (Table 5. 15) by purposely altering the HPLC conditions:

- Mobile phase organic content (± 3%)
- Mobile phase flow rate (± 0.1)
- Detection wavelength (± 3)

Similarly robustness of the proposed HPTLC method was assessed for peak area and retention time (Table 5. 16) 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.

Table 5. 15 Robustness of RP-HPLC method

Parameter	Peak asymmetry		Resolution between ATOR and FENO
	ATOR	FENO	
Flow rate (mL min^{-1})			
0.9	1.02±0.01	0.98±0.034	1.98±0.31
1.0	0.93±0.023	1.24±0.032	2.7±0.21
1.1	1.32±0.04	1.86±0.032	2.4±0.12
Acetonitrile % in mobile phase			
70	0.78±0.12	1.24±1.21	1.97±0.42
73	0.93±0.032	1.24±0.021	2.7±0.013
76	1.43±0.94	0.99±0.32	1.12±0.41
Change in detection wavelength			
254 nm	0.98±0.034	0.98±0.034	1.92±0.02
257 nm	0.93±0.021	1.24±0.031	2.7±0.01
260 nm	1.12±0.02	1.23±0.02	1.99±0.04

- Average of three experiments

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

The limit of detection (LOD) and limit of quantification (LOQ) are calculated according to ICH [3] recommendations where the approach based on the signal-to-noise ratio. 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. The results are given in Table 5. 9.

Table 5. 16 Robustness of HPTLC method

Parameter	Peak area ± % RSD		Retention factor	
	ATOR	FENO	ATOR	FENO
Mobile phase toluene composition				
5	3512±0.012	3414±0.03s1	0.17±0.04	0.87±0.29
6	3492 ± 0.068	3268 ± 0.214	0.19±0.13	0.76±0.31
7	3522 ± 0.04	3283 ± 0.006	0.20±0.09	0.81±0.33
Change in detection wavelength				
282	3625 ± 0.001	3422 ± 0.001	0.25±0.07	0.77±0.32
285	3492 ± 0.068	3268 ± 0.214	0.19±0.13	0.76±0.31
288	3275 ± 0.002	3237 ± 0.002	0.23±0.11	0.73±0.34
Chamber saturation time (in min)				
08	3091 ± 0.062	3298 ± 0.09	0.17±0.17	0.77±0.29
10	3492 ± 0.068	3268 ± 0.214	0.19±0.13	0.76±0.31
12	3456 ± 0.06	3303 ± 0.025	0.16±0.	0.72±0.33

- Average of three experiments

Application of the developed method for analysis of commercial formulation

Applicability of the proposed method was tested by analyzing the commercially available Table 5.t formulation Lorilip Table 5.ts labeled to contain 10 mg of ATOR and 200 mg of FENO. Further more, it was demonstrated that in formulation ATOR levels were low compared to FENO, ranging 1:20. In final dilutions ATOR level remains lower than the linearity range of this compound. The levels of ATOR in the prepared samples are raised by standard addition in its final concentration within the calibration linear range. The amount of standard addition was kept as low as possible to minimize error of prediction for the actual ATOR levels. 0.4 $\mu g\ mL^{-1}$ constant standard addition was chosen for ATOR.

No published method has been reported for simultaneous determination of these binary components in formulations. So the results of the proposed CLS, ILS, PCR, PLS and HPTLC 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 5. 16) denote no significant difference in the results achieved by the proposed methods.

Table 5. 16 Results obtained for the Lorilip tablets by using HPLC, HPTLC and chemometric calibrations

Formulation (Lorilip tablets)	% recovery					
		Chemometric methods				
	HPLC	CLS	ILS	PCR	PLS	HPTLC
ATOR	99.8 ±	99.4 ±	97.85±	99.3 ±	99.3 ±	101.32±
mean[a] ±SD[b]	0.46	1.31	1.76	0.98	0.93	0.89
F		1.99	2.01	1.95	1.08	1.98
t		1.13	1.97	2.67	4.09	2.12
EZET mean[a]	100.2	99.0 ±	101.21±	100.1 ±	101.4 ±	104.43±
±SD[b]	± 0.31	0.99	0.08	0.17	0.44	0.43
F		0.41	0.43	0.63	2.21	2.41
t		4.63	1.23	3.32	1.1	1.99

(label claim: 10 mg of ATOR and 200 mg FENO 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

Reference:

(1) Miller, J. N. *Analyst* **1991**, *116*, 3.

(2) Rubinson, K. A. *Chemical Analysis, Little*; Brown & Co.: Boston, 1987.

(3) Yokohama 2006.