Simultaneous estimation of Atorvastatin and Ezetimibe Results and discussion

Chapter 7

7.1 Reagents and Pharmaceutical preparations

Atorvastatin (ATOR) and Ezetimibe (EZET) were kindly supplied by Biocon India Ltd, India and Torrent Pharma, India and certified to contain 99.3% and 99.8% purity respectively. The drugs are used without further purification. All the solvents used in spectrophotometric analysis were of spectroscopic and HPLC grade. Modlip-EZ and Lipikind-EZ tablets of Torrent and Mankind respectively were claimed to contain 10 mg of ATOR and 10 mg of EZET are used in analysis.

7.2 Procedure

Standard solutions of ATOR and EZET

It was used stock solutions of ATOR and EZET 1 mg mL^{-1} in methanol. The working solutions were 0.04 mg mL^{-1} prepared by transferring 2.0 mL from respective stock solution to a 50 mL volumetric flask and completing to volume with methanol and acetonitrile: methanol (75:25, v/v) for spectrophotometric and HPLC methods respectively. The 0.1 μg μL^{-1} working solutions each of ATOR and EZET in methanol were prepared respectively for HPTLC analysis.

Preparation of mobile phase

- HPLC; acetonitrile : methanol (75:25,v/v)
- HPTLC; chloroform : methanol : toluene (4 : 3 : 5, v/v)

Pharmaceutical sample solution

Twenty Modlip-EZ and Lipikind-EZ tablets of Torrent Pharmaceuticals and Mankind Pharma respectively were claimed to contain 10 mg of ATOR and 10 mg of EZET

were weighed accurately and powdered. An amount of the powder equivalent to 10 mg ATOR and 10 mg EZET (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 1:10 with mobile phase and methanol for HPLC and chemometric methods respectively. All the proposed spectrophotometric, RP-HPLC and HPTLC methods were applied and the concentration of each component in both the formulations was determined.

Spectrophotometric methods

Iso-absorptive method (Q - Absorbance method) (Q-ANAL)

A calibration set containing seven dilutions each of ATOR (4-22 μg mL^{-1}) and EZET (4-24 μg mL^{-1}) was prepared in methanol and UV spectra (Fig. 7. 1 and 7. 2) were recorded in the wavelength range between 210-350 nm versus solvent blank.

Chemometric calibration

A calibration set of 25 samples was prepared in methanol, applying a multilevel multifactor design in which five levels of concentrations of ATOR and EZET introduced The levels were in the range of 4-22 mg mL^{-1} and 4-24 mg mL^{-1} for ATOR and EZET respectively as shown in Table 7. 1. UV spectra were recorded in the wavelength range 221-290 nm versus solvent blank and digitized absorbance was recorded at 1 nm intervals (Fig. 7. 6). The computation was made in R-software

environment. CLS, ILS, PCR and PLS algorithms were applied to the UV absorption data matrix to determine their concentration of title ingredients.

Chromatographic methods

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 EZET 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. 7. 3, 7. 5 (a)). Similarly EZET 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 EZET was in the range of 4-24 μg mL^{-1} (Fig. 7. 3, and 7. 5 (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 ($28^0C \pm 2$). 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 7. 5).

HPTLC calibration

Different volume of standard mixture (ATOR 0.1 μg μL^{-1} + EZET 0.1μg μL^{-1}) 2, 4, 6, 8, 10 and 12 μL injection $spot^{-1}$ were made to obtain a concentration range 100-1200 ng $spot^{-1}$ each of ATOR and EZET respectively. The above solutions were spotted in three replicate on TLC plate. Densitometric scanning was performed in the absorbance mode at 235 nm for the estimation of ATOR and EZET (Fig. 7. 4 and 7. 7). The data of peak area versus drug concentrations were treated by polynomial regression mode (Table 7. 5).

Table 7. 1 Composition of the concentration (calibration) set

Mixture Number	Concentration (μg mL^{-1})	
	ATOR	EZET
1	4.0	4.0
2	8.0	4.0
3	12.0	4.0
4	16.0	4.0
5	22.0	4.0
6	4.0	8.0
7	8.0	8.0
8	12.0	8.0
9	16.0	8.0
10	22.0	8.0
11	4.0	12.0
12	8.0	12.0
13	12.0	12.0
14	16.0	12.0
15	22.0	12.0
16	4.0	20.0
17	8.0	20.0
18	12.0	20.0
19	16	20.0
20	22.0	20.0
21	4.0	24.0
22	8.0	24.0
23	12.0	24.0
24	16.0	24.0
25	22.0	24.0

Preparation of binary mixtures for spectrophotometric and HPLC predictions

Applying multilevel multifactorial design in which four level concentrations of ATOR and EZET within the stated range were introduced and prepared 16 synthetic binary mixtures of titled ingredients as shown in Table 7. 2.

Table 7. 2 Composition of binary mixture for predictions

Mixture No.	Concentration ($\mu g\ mL^{-1}$)	
	ATOR	EZET
1	4.0	4.0
2	10.0	4.0
3	14.0	4.0
4	22.0	4.0
5	4.0	10.0
6	10.0	10.0
7	14.0	10.0
8	22.0	10.0
9	4.0	18.0
10	10.0	18.0
11	14.0	18.0
12	22.0	18.0
13	4.0	20.0
14	10.0	20.0
15	14.0	20.0
16	22.0	24.0

7.3 Results and discussion

Spectrophotometric methods

7.3.1Q - Absorbance method (Q- ANAL)

The method involves the formation of Q-Absorbance equation at 239 nm (iso-absorptive point) and 247 nm (λ_{max} of ATOR) using methanol as solvent (Fig. 7. 1).

Selection of analytical wavelengths for Q- Absorbance method: Pure drug sample of ATOR and EZET, which were separately dissolved in methanol to give two solutions of 8 μg mL^{-1} and scanned between wavelength ranges of 200-350 nm. From the overlain spectra of both dugs (Fig. 7. 2) wavelength 239 nm (iso-absorptive point) and 247 nm (λ_{max} of ATOR) were selected for formation of Q-Absorbance equation. For calibration curve, working stock solution of ATOR and EZET were appropriately diluted to obtain concentration range of 4-22 μg $mL^{-1}$of ATOR 4-24 μg mL^{-1} for EZET. The absorbance of ATOR and EZET at 239 nm and 247 nm were measured and calibration curve were plotted (Table 7. 3). 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 formulations.

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

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

Where C_{EZET} and C_{ATOR} are the concentrations of EZET and ATOR respectively. A_1 is absorbance of sample at isobestic point (239nm), ax_1 is absorptivity of EZET at 239 nm. Q_x is ratio of absorptivity of EZET at 247 nm to absorptivity at 239 nm. Q_y is ratio of absorptivity of ATOR at 247 nm to absorptivity at 239 nm. Q_m is ratio of absorbance of samples (binary mixtures) at 247 nm to absorbance of sample at 239

nm. The ratio absorptivity values Q_x and Q_y were found to be 0.9503 and 1.1.067 respectively.

Figs.7. 1 and 7. 2 shows the zero- order spectra of ATOR and EZET as well as their corresponding binary mixture in methanol. As shown, ATOR exhibits absorption maxima at 247 nm while EZET shows maxima at 232 nm. The spectra of ATOR and EZET were overlapped in the region of their absorption maxima. For this reason to solve overlapped spectra, Q – Absorbance spectrophotometric method along with four chemometric calibrations using the zero-order spectra were applied.

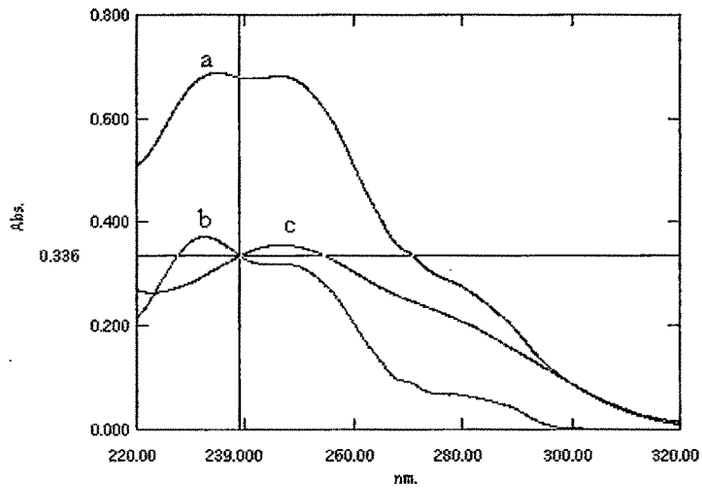

Fig. 7. 1 Zero-order absorption spectra a) 8 µg mL^{-1} of ATOR, b) 8 µg mL^{-1} of EZET and c) their mixture in methanol representing the isobestic point at 239nm

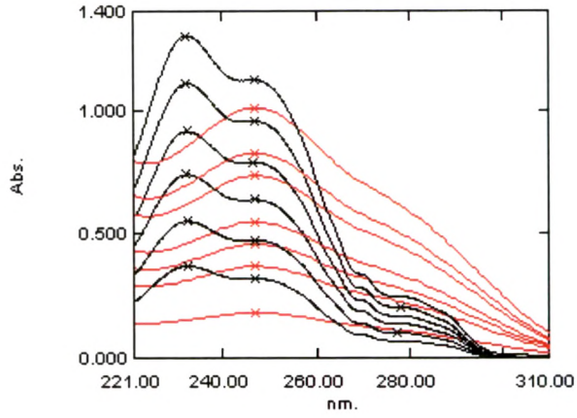

Fig. 7. 2 Overlay zero-order absorption spectra of standard dilutions of ATOR and EZET used in calibration

Q – Absorbance spectrophotometric method permits the determination of components in mixtures at wavelengths corresponding to a isobestic point (239 nm) and λ_{max} of ATOR (247 nm). The values at these points permit better sensitivity and accuracy.

Table 7. 3 Analytical data of the calibration graphs for determination of ATOR and EZET by Q-ANAL and HPTLC method

Parameters	Q-ANAL	
	ATOR	EZET
Wavelength (nm)	239 & 247	
Linearity range (µg mL^{-1})	4-22	4-24
Intercept (a)	0.0003 & 0.065	0.037 & 0.009
Standard deviation of the intercept (S_a)	0.003 & 0.001	0.005 & 0.00
SE of Intercept[1]	0.0235 & 0.002	0.002 & 0.005
Slope (b)	0.0347 & 1.238	1..452 & 0.997
Standard deviation of the slope (S_b)	0.003 & 0.007	0.132 & 0.054
SE of slope	0.006 & 0.038	0.058 & 0.037
Correl. coeff	1 & 1	0.9998 & 0.9999
RSE[2]	0.0021 & 0.001	0.0067 & 0.0048

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

Table 7. 4 Results obtained for the determination of ATOR and EZET in the different synthetic mixtures by using the Q-ANAL method

Mixture number	ATOR		EZET	
	Added (μg mL^{-1})	Recovery (%)	Added (μg mL^{-1})	Recovery (%)
1	4	100.9	4	99.1
2	4	101.2	8	97.6
3	4	100.6	16	98.9
4	4	100.8	24	98.2
5	8	100.5	4	98.6
6	8	101.7	8	101.8
7	8	99.9	16	99.1
8	8	101.3	24	100.9
9	16	101.8	4	102.0
10	16	101.6	8	100.0
11	16	99.4	16	100.9
12	16	102.7	24	100.8
13	22	101.2	4	100.9
14	22	100.9	8	101.3
15	22	101.2	16	101.4
16	22	101.6	24	99.6
	$\bar{x}$:	101.083		100.06
	SD:	0.77	SD:	1.36
	RSD:	0.76	RSD:	1.36

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

7.3.2 Chemometric techniques

Chemometric techniques are other methods gaining wide application for the resolution of the drug mixtures. A calibration set of 25 laboratory prepared binary mixtures within the stated range were prepared. The UV absorbance data was obtained by measuring the absorbances in the region of 221 - 290 nm (Fig. 7. 6). By using the correlation between calibration concentrations and its absorbance data, the chemometric calibrations were calibrated within the CLS, PCR and PLS algorithms. The numerical results of calibrations are shown in Table 7. 6.

The quality of multicomponent analysis is dependent on the wavelength range and spectral mode used. 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 before 220nm and after 290 nm were not used because both ATOR and EZET did not comprise significant linearity at the concentrations used in this region, any absorbance data beyond this region would have introduced a significant amount of noise, thereby decreasing the precision of estimation of title ingredients. 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 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 Table 7. 6 and 7. 7.

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 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 mean squares error of prediction (MSEP) was calculated (Table 8). 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. 7. 8 and 9) indicates factor two is 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 concentration and the PRESS (Prediction Error Sum of Squares) was calculated. It was given by the equation, and values were indicated in Table 7.6.

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

7.3.3 RP-HPLC and HPTLC methods

The HPLC and HPTLC mobile phase acetonitrile: methanol (75:25, v/v) and chloroform : methanol : toluene (4 : 3 : 5) was selected respectively. Because it was found that these mobile phases ideally resolves the peaks with retention time (R_t) 2.09 min and 2.63 min for ATOR and EZET respectively in HPLC (Fig. 7. 3) and retention factor (R_f) 0.34 and 0.42 for ATOR and EZET respectively in HPTLC (Fig. 7. 4).

7.4 Validation of analytical method

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 ATOR and EZET(in triplicates) was carried out by proposed Q- ANAL, HPLC and chemometric methods. The mean recoveries, % errors and the relative standard deviations of prediction sets were computed and indicated in Table 7. 4 (Q-ANAL) and Table 7. 9 (HPLC and chemometrics). 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 of CLS PCR and PLS against the predicted concentrations. The residuals appear randomly distributed around zero, indicating good prediction ability of the model (Fig. 7. 10, 11 and 12).

Linearity

The linearity of the proposed RP-HPLC , Q-ANAL and chemometric methods for determination of ATOR and EZET was evaluated by analysing a series of different

concentrations of standard drug. In this study seven concentrations were chosen, ranging between 4-22 μg mL^{-1} of ATOR and 4-24 μg mL^{-1} of EZET. Similarly in HPTLC linearity was evaluated by analysing a series of different concentrations of standard drug ranging between 100-1200 ng for each of ATOR and of EZET (Fig. 7. 7). 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 (Table 7.5).

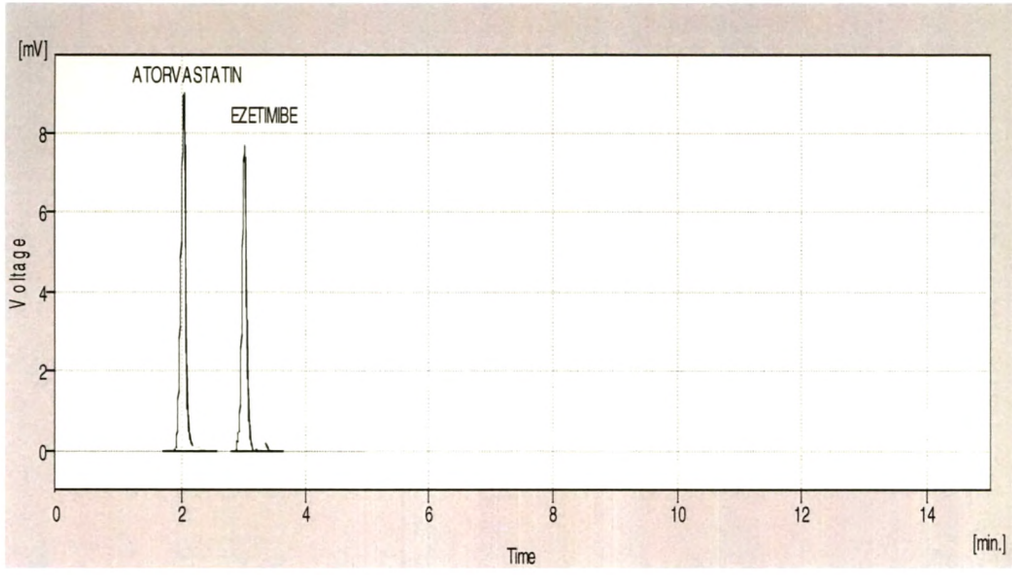

Fig. 7. 3 HPLC chromatogram showing retention time (R_t) of 12 μg mL^{-1} of ATOR (2.040 min) and 8 μg mL^{-1} of EZET (3.010 min) in laboratory-prepared mixture

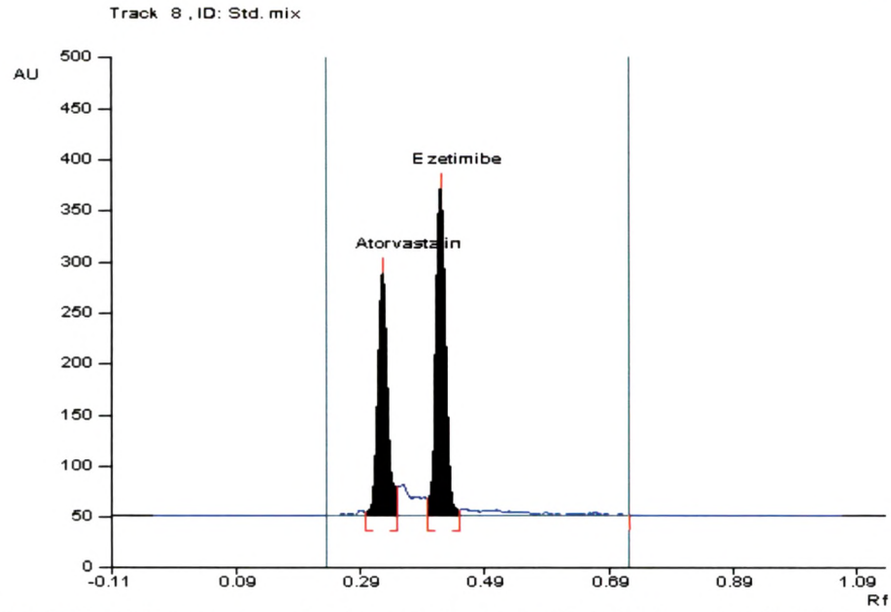

Fig. 7. 4 HPTLC chromatogram showing retention factor (R_f) of 800 ng of ATOR (0.34) and (b) 1000 ng of EZET (0.42) in laboratory-prepared mixture

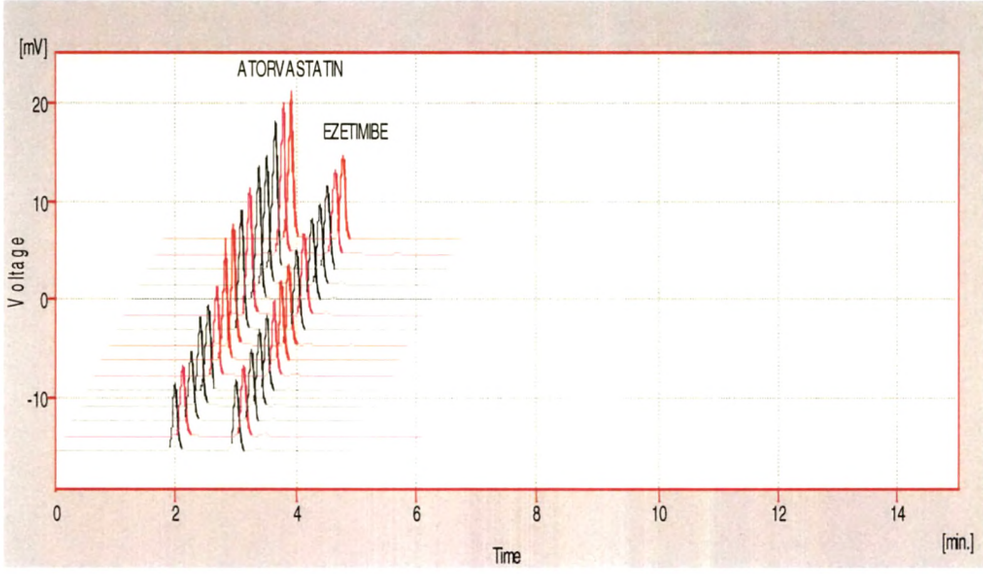

Fig. 7. 5 (a) HPLC 3-Dimensional chromatograms set of five standard dilutions of ATOR (in triplicate) using 8 µg mL^{-1} of EZET as internal standard

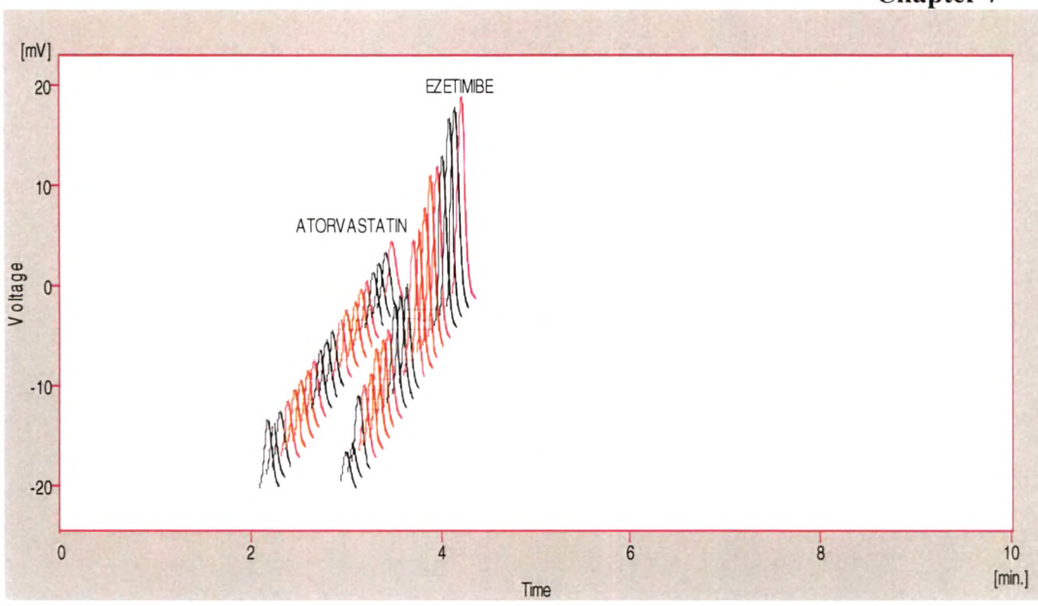

Fig. 7. 5 (b) HPLC 3-Dimensional chromatograms set of seven standard dilutions of EZET (in triplicate) using 8 µg mL^{-1} of ATOR as internal standard

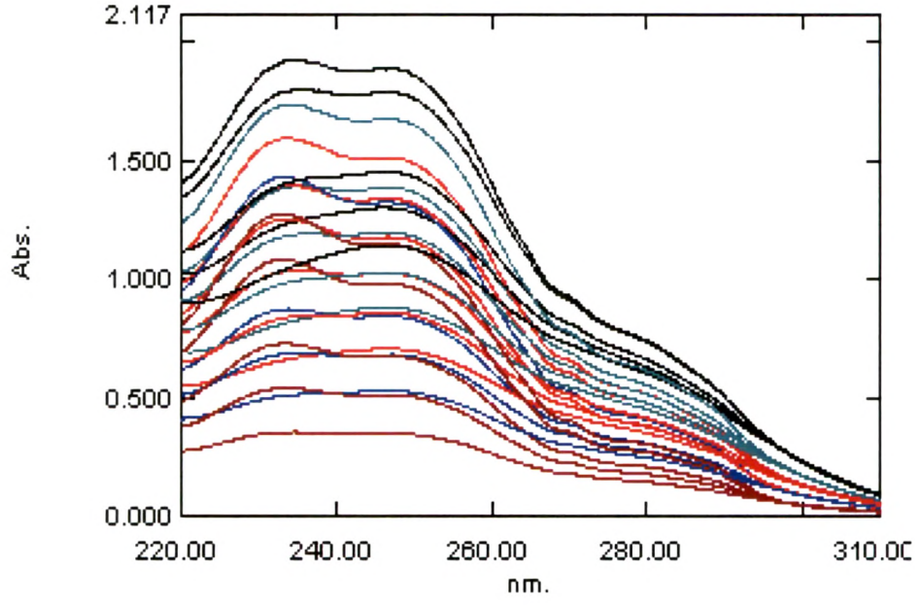

Fig. 7. 6 Zero-order overlay absorption spectra of different level of binary mixtures of ATOR and EZET in methanol and used for calibration

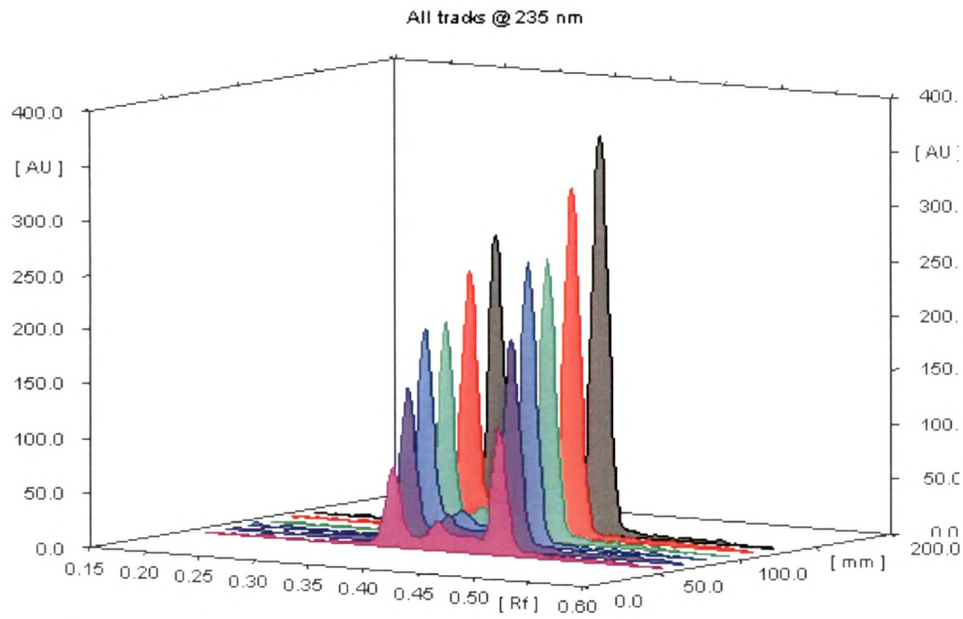

Fig. 7. 7 HPTLC 3-Dimensional chromatograms set of six standard dilutions of ATOR and EZET for calibrations

Table 7. 5 Characteristic parameters of the calibration equations for the proposed HPLC and HPTLC methods for simultaneous determination of ATOR and EZET

Parameters	HPLC		HPTLC	
	ATOR	EZET	ATOR	EZET
Calibration range (µg mL^{-1})	4 - 22	4 - 24	0.1-1.2	0.1-1.2
Detection limit (µg mL^{-1})	0.005×10^{-2}	0.027×10^{-2}	0.0024×10^{-2}	0.0047×10^{-2}
Quantitation limit (µg mL^{-1})	0.018×10^{-2}	0.089×10^{-2}	0.0079×10^{-2}	0.01551×10^{-2}
Regression equation (Y)[a]				
Slope (b)	41.76	54.058	2.347	1.957
Standard deviation of the slope (S_b)	1.5×10^{-3}	1.70×10^{-2}	0.194	0.009
Relative standard deviation of the slope (%)	0.5409	0.699	1.54	1.46
Intercept (a)	27.356	28.918	186.29	208.63
Standard deviation of the intercept (S_a)	0.000710	0.000372	1.99	1.67
Correlation coefficient	0.9984	0.9852	0.9999	0.9977
Theoretical plates	2846	6197	NA	NA
Symmetry factor	0.903	1.115	NA	NA
Resolution	6.359		NA	NA

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

Table 7. 6 Statistical parameters of chemometric methods in calibration step

Component	CLS			ILS			PCR				PLS			
	SEC	RSE	R^2	SEC	RSE	R^2	SEC	PRESS	RSE	R^2	SEC	PRESS	RSE	R^2
ATOR	0.197	1.4056	0.998				0.0370	0.0316	0.2632	0.9986	0.0370	0.0316	0.2632	0.998
EZET	0.238	1.6958	0.999				0.0226	0.0117	0.1448	0.9994	0.0225	0.0117	0.1448	0.999

Table 7. 7 Statistical parameters of chemometric methods in prediction step

Component	CLS				ILS				PCR				PLS			
	SEP	a	b	r	SEP	a	b	r	SEP	a	b	r	SEP	a	b	r
ATOR	0.225	0.124	1.005	0.999					0.195	0.095	1.002	0.999	0.199	0.117	1.002	0.999
EZET	0.302	0.342	1.012	0.999					0.321	0.084	1.013	0.999	0.324	0.084	1.013	0.999

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

Table 7. 8 MSEP values of ATOR and EZET determined during PCR and PLS cross validation calibration step

PCR	1 comps	2 comps	3 comps	4 comps	5 comps	6 comps	7 comps	8 comps	9 comps	10 comps	11 comps	12 comps	13 comps	14 comps	15 comps
ATOR	0.4180	0.0013	0.0014	0.0015	0.0016	0.0016	0.0015	0.0015	0.0015	0.0016	0.0016	0.0015	0.0016	0.0022	0.0022
EZET	0.6899	0.0005	0.0004	0.0003	0.0004	0.0004	0.0004	0.0004	0.0004	0.0004	0.0004	0.0004	0.0004	0.0003	0.0004
PLS															
ATOR	0.4191	0.0013	0.0015	0.0017	0.0017	0.0021	0.0022	0.0025	0.0026	0.0026	0.0026	0.0026	0.0025	0.0025	0.0025
EZET	0.6913	0.0005	0.0004	0.0004	0.0004	0.0005	0.0004	0.0004	0.0004	0.0005	0.0005	0.0006	0.0006	0.0006	0.0006

16 comps	17 comps	18 comps	19 comps	20 comps	21 comps	22 comps
0.0023	0.0026	0.0026	0.0026	0.0028	0.0026	0.0025
0.0004	0.0004	0.0004	0.0004	0.0004	0.0007	0.0006
0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025
0.0006	0.0006	0.0006	0.0006	0.0006	0.0006	0.0006

Table 7. 9 Recovery results in prediction from Zero- order spectra for ATOR and EZET in synthetic mixtures by proposed RP-HPLC and chemometric techniques

Recovery (%)										Error%									
HPLC		CLS		ILS		PCR		PLS		HPLC		CLS		ILS		PCR		PLS	
ATOR	EZET	ATOR	EZET	ATOR	EZET	ATOR	EZET	ATOR	EZET	ATOR	EZET	ATOR	EZET	ATOR	EZET	ATOR	EZET	ATOR	EZET
97.1	96.7	95.8	94.0	94.9	102.6	99.7	97	99	95.8	-2.9	-3.3	-4.2	-6.0	-5.1	2.6	-0.3	-3	-1	-3.7
96.3	96.3	98.3	93.6	96.4	101.8	100	95.4	99.6	98.3	-3.7	-3.7	-1.7	-6.4	-3.6	1.8	0.0	-4.7	-0.4	-4.4
98.9	95.2	99.3	93.7	98.7	98.8	98.8	95.3	98.5	99.3	-1.1	-4.8	-0.7	-6.3	-1.3	-1.2	-1.2	-4.8	-1.5	-4.5
100.2	97.4	100	92.7	101.3	94.9	98.8	94.1	98.6	100	0.2	-2.6	0.0	-7.3	1.3	-5.1	-1.2	-5.9	-1.4	-5.9
101.8	94.1	101.2	93.3	104.9	98.2	100.2	94.2	101	101.2	1.8	-5.9	1.2	-6.7	4.9	-1.8	0.2	-5.8	1	-5.8
98.1	97.7	98.0	98.2	95.5	96.0	99.6	101.9	101	98.0	-1.9	-2.3	-2.0	-1.8	-4.5	-4.0	-0.4	1.9	1	7.3
96.2	92.4	94.6	93.3	96.3	97.3	97.4	100.2	97.5	94.6	-3.8	-7.6	-5.4	-6.7	-3.7	-2.7	-2.6	0.2	-2.5	-2.7
96.8	98.7	96.1	93.9	97.5	96.3	98.4	97.6	98.4	96.1	-3.2	-1.3	-3.9	-6.1	-2.5	-3.7	-1.6	-2.4	-1.6	-3.3
95.8	94.7	94.8	93.9	97.1	98.3	97.7	97.8	97.7	94.8	-4.2	-5.3	-5.2	-6.1	-2.9	-1.7	-2.3	-2.2	-2.3	-3.8
95.9	97.5	96.9	94.1	98.7	104	98.8	96.8	98.6	96.9	-4.1	-2.5	-3.1	-5.9	-1.3	4	-1.2	-3.2	-1.4	-3.9
95.2	95.8	95.5	93.7	98.9	102.8	98.1	96.9	98.1	95.5	-4.8	-4.2	-4.5	-6.3	-1.1	2.8	-1.9	-3.1	-1.9	-4.4
97.1	95.2	94.8	93.9	98.8	96.1	98.1	97.1	97.9	94.8	-2.9	-4.8	-5.2	-6.1	-1.2	-3.9	-1.9	-2.9	-2.1	-4.5
97.9	97.1	94.2	94.2	101.4	98.4	97.8	97.4	97.9	94.2	-2.1	-2.9	-5.8	-5.8	1.4	-1.6	-2.2	-2.6	-2.1	-4.2
99.1	98.2	94.1	100.2	97.8	102.9	99.2	97.5	97.5	94.1	-0.9	-1.8	-5.9	-0.2	-2.2	2.9	-0.8	-2.5	-2.5	-4.4
95.8	101.2	94.1	100.1	99.4	98.4	99.2	97.9	98.2	94.1	-4.2	1.2	-5.9	-0.1	-0.6	-1.6	-0.8	-2.1	-1.8	-3.8
$\bar{x}$ 97.48	96.54	96.5	94.8	98.5	99.1	98.8	97.1	98.6	96.5										
RSD:1.90	2.19	2.4	2.6	2.6	3	0.9	2.1	1.1	2.4										

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

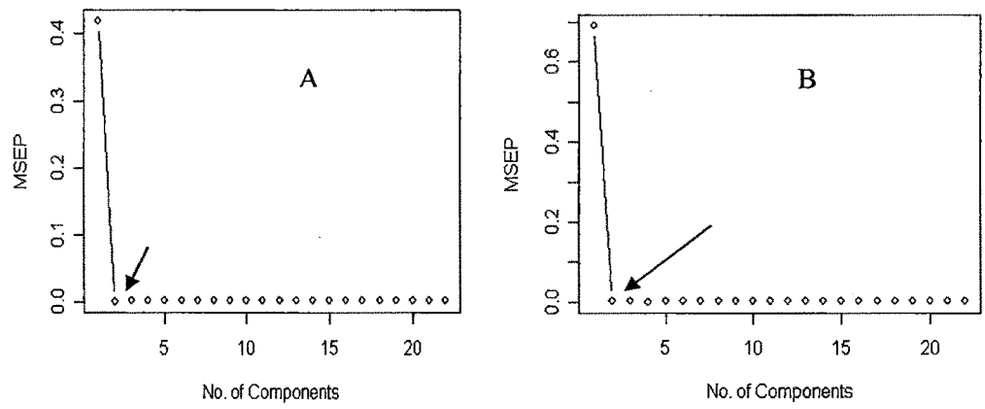

Fig. 7. 8 MSEP plots of a calibration set obtained using leave-one-out validation of PCR-model for A) ATOR and B) EZET in zero-order absorbtion data

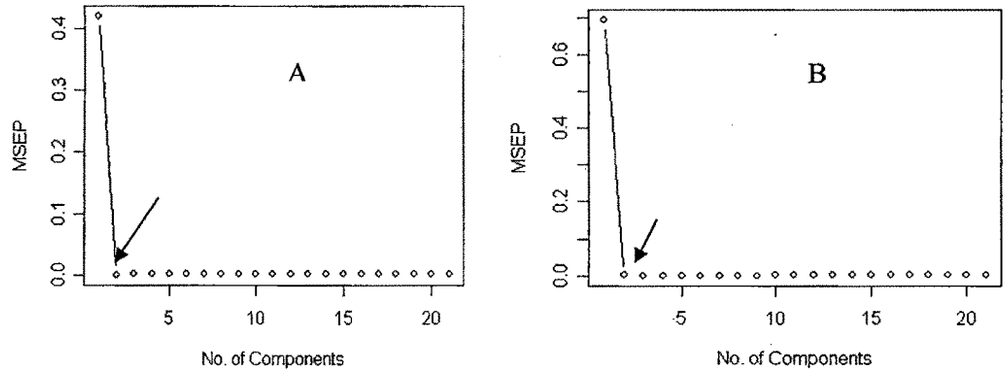

Fig. 7. 9 MSEP plots of a calibration set obtained using leave-one-out validation of PLS-model for A) ATOR and B) EZET in zero-order absorbtion data

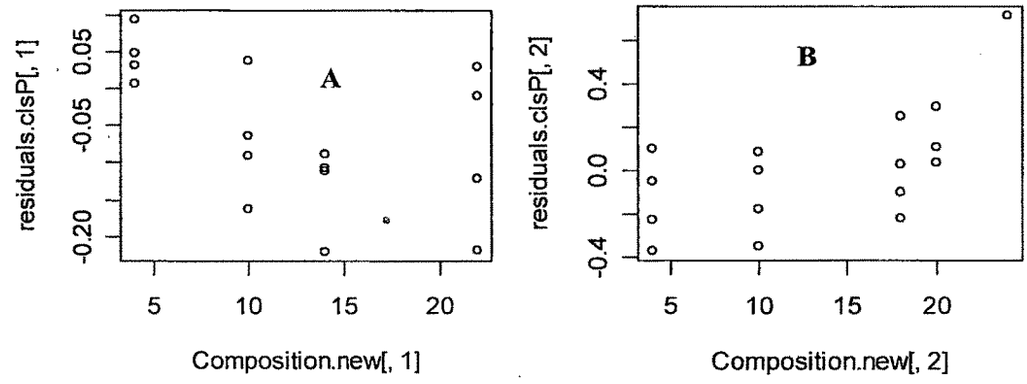

Fig. 7. 10 Plot of concentrations residuals (residuals.clsP) of CLS against the predicted concentrations (Composition.new) of A) ATOR and B) EZET in prediction set

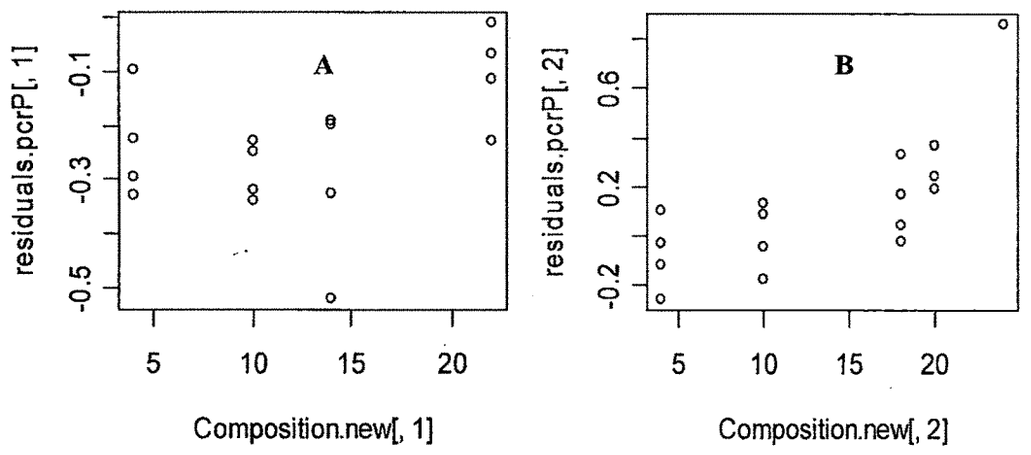

Fig. 7. 11 Plot of concentrations residuals (residuals.pcrP) of PCR against the predicted concentrations (Composition.new) of A) ATOR and B) EZET in prediction set

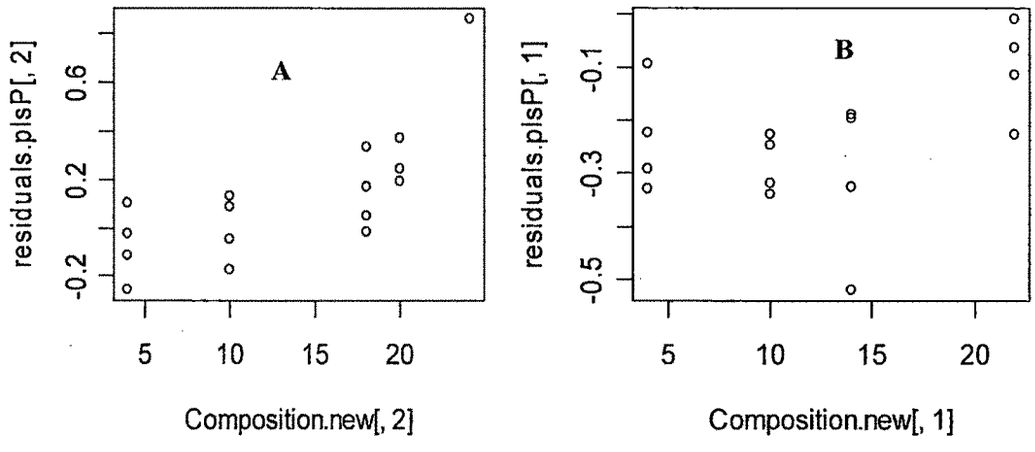

Fig. 7. 12 Plot of concentrations residuals (residuals.plsP) of PLS against the predicted concentrations (Composition.new) of A) ATOR and B) EZET in prediction set

Range

The calibration range of the proposed Spectrophotometric, RP-HPLC and HPTLC 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)^2 of the commercial pharmaceutical formulations (

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 methods and the response obtained was plotted against the initial unknown concentration set at 0. And Chemometric and Q-ANAL recoveries were also determined. The results obtained are compared with expected results. The excellent mean recoveries and standard deviation (Table 7.10) suggested good accuracy of the proposed methods and no interference from formulations excipients.

Table 7. 10 Application of standard addition technique for analysis of ATOR and EZET in Modlip-tablet formulation

ATOR & EZET		ATOR							EZET						
		% recovery ± SD[a]							% recovery ± SD[a]						
Claimed	Added	HPLC	HPTLC	CLS	ILS	PCR	PLS	Q-ANAL	HPLC	HPTLC	CLS	ILS	PCR	PLS	Q-ANAL
10	0	100.19± 0.68	97.85 ±0.85	101.13 ±0.48	98.35 ±0.35	101.41 ±0.344	101.40 ±0.34	96.32±1.34	99.1 ± 0.05	97.69 ± 0.97	97.1 ± 0.04	99.46 ± 0.32	9.74± 0.69	96.22±0.34	102.1±0.52
10	4	98.38 ±1.36	103.86 ±2.14	99.32 ±1.62	107.36 ±2.64	101.55 ±1.53	101.55 ±1.53	96.87±1.99	98.35 ± 0.51	100.38.± 0.46	96.55 ± 0.51	101.38.± 0.91	98.24± 0.58	100.43±0.49	106.7±0.46
10	8	101.46 ±0.50	96.45 ±0.43	100.18 ±0.51	97.95 ±0.33	100.71 ±0.31	101.90 ±0.94	96.59±2.14	94.25 ± 0.51	101.89 ± 0.56	95.65 ± 0.71	100.79 ± 0.83	100.18± 0.57	97.36±1.67	104.3±0.46
10	10	99.45 ±1.63	106.34 ±2.64	98.92 ±1.72	105.39 ±2.34	100.51 ±1.56	101.95 ±1.54	97.35±2.04	96.83 ± 0.71	99.63 ± 0.62	98.23 ± 0.31	98.33 ± 0.42	100.4± 0.56	100.39±2.04	97.4±0.28
10	12	100.33 ±0.36	98.45 ±1.58	101.93 ±0.46	99.48 ±1.38	100.82 ±1.94	101.38 ±0.84	98.36±1.64	99.63± 0.18	99.44± 0.46	98.28± 0.38	100.49± 0.38	98.36± 0.47	101.47±1.99	94.9±0.28

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

Table 7. 11 Precision study results of prepared binary mixture

Validation parameter	HPLC			Chemometric				Q-ANAL	HPTLC	
	% RSD			% RSD				% RSD	% RSD	
Repeatability[a]	Peak area	Peak asymmetry	Retention time	CLS	PCR	PLS	ILS		Peak area	Retention factor
EZET	0.834	0.5934	0.205	1.31	1.417	1.338	0.75	2.12	0.419	0.219
ATOR	1.133	0.8826	0.96614	1.46	0.615	0.621	1.44	2.06	1.673	0.3601
Intermediate precision[b]										
EZET	1.228	0.8702	0.6940	1.34	1.383	1.791	1.71	1.64	0.528	0.4243
ATOR	1.307	0.7238	0.0739	1.36	0.793	0.594	1.187	1.12	1.513	0.9157

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

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

Analytes solution and mobile phase stability

Stability of EZET 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 for 48h. No interfering substances were found.

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 and % recovery RSD in Q-ANAL and chemometric methods for each title ingredient.

The repeatability (within-day in triplicates) and intermediate precision (for 3 days) was carried out at three concentration levels for each compound. The obtained results within and between days trials are in acceptable range indicating good precision of the proposed methods (Table 7.11).

Specificity

1 HPTLC chromatogram was obtained using diluents, mobile phase, placebo, ATOR standard, EZET standard, ATOR + EZET + diluents and ATOR + EZET +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, Q-ANAL and chemometric methods.

Robustness

The robustness of the proposed HPLC method was assessed for peak asymmetric and peak resolution factor (Table 7.12) 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 factor (Table 7.13) 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 [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 (Table 7. 5). In spectrophotometric methods the standard deviation of blank determinations and slope values of calibration equation. The LOD and LOQ values 0.047 μg mL^{-1} and 1.550 μg mL^{-1} respectively for ATOR and 0.063 μg mL^{-1} and 0.2097 μg mL^{-1} EZET was found.

Table 7.12 Robustness of chromatographic method

Parameter	Peak asymmetry		Resolution between ATOR and EZET
	ATOR	EZET	
Flow rate (mL min^{-1})			
0.9	0.902±0.01	1.198±0.034	6.298±0.21
1.0	0.893±0.02	1.125±0.032	6.359±0.36
1.1	0.832±0.06	1.186±0.042	6.354±0.32
Acetonitrile % in mobile phase			
72	0.878±0.22	1.124±0.031	6.397±0.32
75	0.893±0.02	1.125±0.032	6.359±0.36
78	0.843±0.14	1.199±0.042	6.312±0.31
Change in detection wavelength			
236 nm	0.898±0.04	1.198±0.034	6.392±0.22
239 nm	0.893±0.02	1.125±0.032	6.359±0.36
242 nm	0.812±0.02	1.123±0.012	6.399±0.24

- Average of three experiments

Table 7. 13 Robustness of HPTLC method

Parameter	Peak area ± % RSD		Retention factor	
	ATOR	EZET	ATOR	EZET
Mobile phase toluene composition				
4	2647±0.032	3847±0.031	0.37±0.01	0.47±0.19
5	2996±0.034	3875±0.012	0.34±0.02	0.46±0.21
6	2722 ± 0.024	3883 ± 0.013	0.30±0.09	0.41±0.23
Change in detection wavelength				
232	2625 ± 0.021	3722 ± 0.011	0.35±0.07	0.47±0.12
235	2996±0.034	3875±0.012	0.34±0.02	0.46±0.21
238	2875 ± 0.032	3737 ± 0.012	0.33±0.11	0.43±0.14
Chamber saturation time (in min)				
08	2891 ± 0.052	3798 ± 0.19	0.37±0.07	0.48±0.29
10	2996±0.034	3875±0.012	0.34±0.02	0.46±0.21
12	2856 ± 0.016	3903 ± 0.015	0.36±0.03	0.42±0.23

- Average of three experiments

Application of the developed method for analysis of commercial formulations

Applicability of the proposed method was tested by analyzing the commercially available tablet formulations Modlip-EZ of Torrent was claimed to contain 10 mg of ATOR and 10 mg of EZET.

No published method has been reported for simultaneous determination of these binary components in formulations. So the results of the proposed Q-ANAL, 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 7. 14 and 7. 15) denote no significant difference in the results achieved by the proposed methods.

Table 7. 14 Results obtained for the Lorilip tablets by using HPLC, HPTLC and chemometric calibrations

Formulation (LIPIKIND tablets)	% recovery						
		Chemometric methods					
	HPLC	CLS	ILS	PCR	PLS	Q-ANAL	HPTLC
ATOR							
mean[a] ±SD[b]	99.02±1.38	96.37±0.01	96.99±0.00	98.12±0.05	98.23±0.67	96.37±1.28	99.84±0.13
F		5.31	4.96	4.88	4.97	5.39	5.99
t		2.01	2.29	2.09	2.15	1.09	2.27
EZET							
mean[a] ±SD[b]	97.32±0.19	102.13±0.06	103.02±0.069	104.19±0.16	104.38±0.19	100.2±0.12	96.49±0.05
F		5.09	4.36	4.21	4.29	5.97	6.29
t		2.01	1.96	1.26	1.37	2.11	2.28

(label claim: 10 mg of ATOR and 10 mg EZET per LIPIKIND tablet)

a, Mean recovery value of five determinations for each method, b, Standard deviation

($n_1 = n_2 = 5$), Theoretical values for *t* and F at P = 0.05 are 2.31 and 6.39 respectively

Table 7. 14 Results obtained for the Lorilip tablets by using HPLC, HPTLC and chemometric calibrations

Formulation (LIPIKIND tablets)	% recovery						
		Chemometric methods					
	HPLC	CLS	ILS	PCR	PLS	Q-ANAL	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	99.78±1.67	0.89
F		2.16	2.97	4.17	4.69	5.39	2.92
t		1299	2.11	1.15	1.78	2.19	1.28
EZET	100.2	99.0 ±	101.21±	100.1 ±	101.4 ±		104.43±
mean[a] ±SD[b]	± 0.31	0.99	0.08	0.17	0.44	96.37± .94	0.43
F		4.37	3.53	4.92	2.91	5.96	4.19
t		1.98	2.06	1.46	1.74	2.30	1.37

(label claim: 10 mg of ATOR and 10 mg EZET per LIPIKIND 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