SIMULTANEOUS ESTIMATION AND DEGRADATION STUDIES OF ATORVASTATIN, EZETIMIBE AND FENOFIBRATE BY RP-HPLC USING QBD APPROACH

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Abstract:

This investigation aimed to develop and validate a simultaneous method for the determination of atorvastatin, ezetimibe and fenofibrate by RP-HPLC and its degradation studies. The analytical method was carried out using Waters X-Bridge C18 250 x 4.6 mm i.D., 5 µm. A 3^2 factorial design was applied to simultaneously analyze the independent variables and response. Batch optimization was done on the basis of numerical optimization and Derringer Desirability approach, respectively. The optimized mobile phase consists of acetonitrile: water: methanol having a ratio of 60:10:30v/v/v was performed at a flow rate of 1ml.min. The pH of the mobile phase was adjusted up to 3 with orthophosphoric acid. The detection carried out at a observed wavelength of 233 nm. Assay for the marketed formulation was carried out and % assay for atorvastatin, ezetimibe and fenofibrate was found to be 100.1 %. 99.9% and 99.9% respectively. Different degradation studies were carried out using 0.1N HCl, 0.1N NaOH, 1%H_2O_2, photo degradation and thermal degradation. The method was validated according to ICH guidelines which include accuracy, precision, specificity, linearity, and analytical range.

Keywords: Atorvastatin, ICH Guidelines, Ezetimibe, Fenofibrate, Degradation studies.

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1. Introduction

Atorvastatin(3R,5R)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-propan-2-ylpyrrol-1-yl]-3,5 dihydroxy heptanoic acid which is a competitive inhibitor of 3-hydroxy-3-methyl-glutaryl- CoA reductase (HMG-CoA) which is an early rate limiting step in cholesterol biosynthesis(1). The inhibition of synthesis leads to consumption of intracellular cholesterol, which increases the expression of low-density lipoprotein (LDL) receptor on hepatocytes resulting in a fall in serum LDL cholesterol concentration to about 40% and high systemic disappearance of LDL cholesterol(2). Atorvastatin is white to off white crystalline powder having molecular formula $C_{33}H_{35}FN_2O_5$ and having molecular weight 558.64g/mol(3).It belongs to category of Antihyperlipidemic which is having pka of 4.30 and log P of 4.22(4). Atorvastatin belongs to BCS class II which is having less solubility high permeability(5). In Indian Pharmacopoeia, reported λ_{max}at 246 nm and atorvastatin freely soluble in methanol; slightly soluble in ethanol (95%) & very slightly soluble in water(6). Chemical structure of Atorvastatin is shown in **Figure 1**.

Ezetimibe (3R, 4S)-1-(4-fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4hydroxyl phenyl) azetidin-2-one(7).Ezetimibe is an selective cholesterol absorption inhibitor, which is an potent and selectively prevents absorption of cholesterol from dietary and biliary sources by prevention transport of cholesterol through the intestinal wall(8). Ezetimibe having molecular formula and molecular weight of $C_{24}H_{21}F_2NO_3$ and 409.4g/mol(9). Ezetimibe belongs to Antihyperlipidemic category which is having pka of 4.39 and log P 9.66(10). Ezetimibe belongs to category of BCS class II which is low solubility high permeability(11). As per Indian pharmacopoeia reported λ_{max}233 nm and freely soluble in methanol, ethanol and insoluble in water as reported(12).Chemical structure of Ezetimibe is shown in **Figure 2**.

Fenofibrate (FEN), chemically 2-[4-(4-chlorobenzoyl) phenoxy] - 2-methyl-propanoic acid 1-methylethyl ester, is a lipid- regulating agent(13). It is a white solid having molecular formula and molecular weight $C_{20}H_{21}ClO_4$and 360.8 g/mol having melting point of 79–82˚C(13). It belongs to category of Antihyperlipidemic and BCS class i.e. low solubility and high permeability, having pka -4.9 and log P 4.80(14, 15). It is insoluble in water and the reported $\lambda_{max}$is 286 nm and having solubility in methanol and ethanol and insoluble in water is official given in USP and BP(16).Chemical structure of Fenofibrate is shown in **Figure 3**.

The "QbD" initiative was created by the Food and Drug Administration Office of Generic Drugs with the goal of "incorporating quality into pharmaceutical products to ultimately ensure the safety of patients." Adopting the AQbD technique is a quality- and time-efficient solution for creating novel analytical procedures (17,18). The QbD is a systematic approach to development that starts with established objectives and emphasises product and process understanding and process control, based on sound science and quality risk management, according to the International Conference on Harmonisation (ICH) Q8(R2) (19,20).
All manufactured items in the pharmaceutical industry must be of the highest quality to guarantee the least possible harm to patients. To make sure that products fulfil particular criteria, researchers, producers, and developers use a variety of technical equipment and analytical techniques, including LC, during the creation process.

The Box-Behnken Design (BBD) is a useful tool for streamlining the method development for the QbD approach (21) . Retention time, peak form, peak area, symmetry, tailing factor, number of theoretical plates, and relative retention time are some of the variables that may have an impact on the critical variable of the chromatographic separation and were the focus of this study (22). Based on the Ishikawa (fishbone) diagram gathered from the literature survey, the most crucial factors were chosen. In order to estimate these medications simultaneously using the RP-HPLC technology and the QbD methodology, success has been achieved. The suggested techniques were improved and verified in accordance with ICH Q2(R2) criteria (23,24).

2. Materials and methods

Experimental work
Atorvastatin was purchased from La Pharma, Ludhiana, Punjab, Ezetimibe and Fenofibrate were also purchased fromArkle Healthcare Pvt. Ltd. Chandigarh respectively. Acetonitrile, methanol, Tetrahydrofuran (HPLC grade) was purchased from the Rankem (New Delhi, India), HPLC grade water was collected from the purification systems ELix 03 (Millipore, USA) in our institutional water plant. 0.45 micrometre Millipore membrane filter and syringe filter were purchased from the local market. The pharmaceutical dosage form used in the study was containing the 10mg of atorvastatin; ezetimibe and

the fenofibrate were 10mg and 160mg respectively. Tablets were obtained from the local market name as Fibator EZ manufactured by Sun Pharma.

Chromatographic conditions

Analysis was carried out using Waters HPLC which is equipped with UV-Vis detector (2489) having empower software. Initially a number of mobile phase combinations was tried to separate atorvastatin and ezetimibe using the C_{18} column. C_{18} column act as stationary phase also helps in improving resolution. Column used for the estimation of drugs was Waters X-Bridge C_{18} column having dimensions (250µm×4.6mm i.d, 5µm). Detector was carried out 233nm. The injection volume was 20µL and run time was 6.5min. Isocratic mobile phase which consists of acetonitrile: water: methanol having ratio 60:10:30 v/v/v at pH 3.0. pH of the mobile phase was adjusted using diluted orthophosphoric acid. Flow rate was 1.0 mL/min at 2589-2595 psi. Mobile phase was filtered through 0.45µm membrane filter and degassed before use.

Preparation of Mobile Phase

Measured volume of acetonitrile, water and methanol was taken in volumetric flask. For adjusting the pH of prepared mobile phase 0.1% orthophosphoric acid was dissolved in 100 mL of ultra-pure water. pH was adjusted to 3.0 using diluted orthophosphoric acid. Mobile phase was filtered using membrane filter and degassed before use.

Preparation of Standard solution

Stock solutions were prepared by dissolving 10 mg of atorvastatin and 10 mg of ezetimibe in 10 mL of solvent which consists of methanol and water in the ratio 8:2 in the 10mL volumetric flask. Solutions were sonicated before use. For atorvastatin and ezetimibe, final concentration of stock solution was 1000µg/mL. Further dilutions were made from the stock solution and same diluents were used for adjusting the volume.

Preparation of Sample Solutions

Sample solution of Atorvastatin and ezetimibe was prepared using the marketed formulation. Marketed formulation Avas-Ez containing 10 mg of Atorvastatin and 10 mg of Ezetimibe were weighed and crushed into fine powder. Quantity of powder was equivalent to weight of one tablet that was accurately weighed. Weighed amount was dissolved in solvent system in the volumetric flask. Sample solution was sonicated and filtered using syringe filter. Final concentration of the sample was same as standard solution.

Method Development using QbD Approach

For simultaneous method optimization, a full factorial 3^2 design was applied to simultaneously evaluated the independent variables and responses. From the preliminary trials, Acetonitrile volume (X1), Water volume (X2) and flow rate (X3) was taken into consideration and theoretical plates (Y1) and resolution (Y2) was taken as responses. Method validation and optimization was done on the basis of ANOVA and Derringer Desirability approach, respectively. Numerical and graphical optimization was done on the basis of Grid search approach.

3. Results

Firstly, the different trials were carried on the API of the drugs to find the better mobile composition for developing an RP-HPLC method, this would be possible by changing the composition of the mobile phase, and this helps to develop a sensitive and accurate and précised HPLC method. According to the United States Pharmacopeia (USP) guideline, the system suitability test was fulfilled by studying few parameters like theoretical plate, retention time, peak area, capacity factor, asymmetry factor, etc. A factorial response methodology was applied by taking acetonitrile volume (X1), water volume (X2) and flow rate was taken as independent variables and theoretical plates and resolution was taken as response variables. Model selection and validation was performed and suitable analysis was done for optimization and method batch analysis.

Method Optimization using QbD Approach

A 3^3 full factorial design with two centre points was selected, taking into account the number of variables i.e.3 to be researched. A8experiment design grid was generated upon considering 3 continuous factors namely Acetonitrile Volume (X1), Water Volume (X2) and Flow rate (X3). Sample solution prepared by each of the experiment was executed and the Resolution (Y1) and Theoretical Plates (Y2) were selected as a response. Different mathematical models were validated and screened and found 2 factor interaction (2FI) was observed to the validated mathematical model. The fit summary analysis was performed for the variables and found best correlated with predicted R^2 was found to be 0.973. The -1 and +1 level for each factor is listed in. (**Table No.1)**

Analysis of Variance and Response Surface Analysis

The ANOVA results indicated that the independent variables have produced significant effect on

response (p <0.0001), thus implies that the significancy effect of predictive mathematical model on the desired responses. The p value of Y1 and Y2 was observed to be p<0.0008 and 0.0009, respectively. The underlining observations implying that the predicted mathematical model 2FI was best fitted and produced significant effect on the response.Resolution is defined as difference between separation of two peaks in a fast and completely target mode through a column and its serves as a key HPLC performance indicator. By dividing the difference in peak retention durations by the average peak width, resolution is calculated.Response analysis revealed that shows that X1and X2 has significant effect on response Y1 (**Figure 4A-B**) with 2FI as a contributed model. The observations of polynomial equations also support the predicted observations from response analysis. From the response surface analysis, it is also evident that Flow rate (X3) has direct positive effect on Y2 within the studied range (**Figure 4C**). Increase in X1 and X3 variables has yielded a higher Y2 (**Figure 4D-E**). However, lower the flow rate produces quadratic approximation to the response (**Figure 4F**). A maximum desirability of 0.807 was achieved by the combined analysis. ANOVA of independent variables and response terms are given in. (**Table No.2)** The corresponding polynomical equations was written below:

Retention Time =+3.46*A--1.250*B-0.026-0.2*C-0.12*AB-0.11* AC-0.026* BC (1)

Theoretical Plates: = +27.22*A-8.322*B+9.372*C+27.32*AB-82.32*AC-55.32*BC (2)

Optimization by Desirability Function

After assigning the goals, the response has been analyzed and proposed significancy of the mathematical model has been validated. After simultaneously assigning the goals, the optimized trial containing 60% acetonitrile, 10% water and 30% methanol with a flow rate of 1ml/min produced highest desirability of 0.803 **Figure 5**. The predicted response of the optimized batches has been analyzed by the software and the net observation has been showing the percent error less than <0.5% which indicated that the optimized trial was best fitted by the software and selected for further validation procedures.

Method Validation

The developed RP-HPLC method was validated according to ICH guideline parameters. The entire identification followed by the separation of Atorvastatin, Ezetimibe and Fenofibrate by the RP-HPLC technique was obtained with no interference **Figure 6** which corroborates the specificity of the developed method.

At optimized concentration of solvents and instrumental conditions, the linearity curve of the HPLC method for the atorvastatin, ezetimibe and fenofibrate standard solution in methanol from the stock solution individually. The linearity curve was followed in the concentration range of 10 to 50 µg/ml for Atorvastatin and Ezetimibe respectively and for the fenofibrate the concentrations were 160 to 200µg/ml.

The results confirmed that the concentration range in which the standard curve of linearity was performed has good reproducibility as per. (**Table No.3)**

Validation Parameters

Linearity-Calibration curve of the atorvastatin, ezetimibe and fenofibrate were plotted using the concentration verses the peak area. The five concentrations were taken as 10-50µg/mL for atorvastatin and ezetimibe respectively, and the concentration curve for Fenofibrate were 160-200 µg/mL as shown (**Figure 7, 8 and 9**).

Precision- In a precision method, it can be performed by the different ways; firstly it would be précised on the basis of system as well as the method precision. In a system precision includes the evaluation of the system by injection the same concentration of sample six five times and in the method, it involves the method evaluation by injecting the five preparations of the sample having the same concentration. Similarly, the intermediate precision of the method was checked by injection in the replicate sample of injection of the solution for the 6 times on the same day as the intraday precision study of drugs, similarly as in the interday precision the different analyst analysed the method on another day, the chromatogram was recorded and thus it represents the Intermediate precision. Mean peak area and the percentage relative standard deviation (RSD) were calculated. From the data obtained, the developed RP/HPLC method was found to be précised as per the system and the method precision as shown in the **(Table No.4 (a, b, c))** and (**Table No.5 (a, b, c))** shows the intermediate precision of Atorvastatin, Ezetimibe and Fenofibrate.

Robustness- Robustness is an analytical procedure is a measure of its capacity to remain unaffected by

small, but deliberate variations in method parameters. It was observed that the variations like flow rate of mobile phase, wavelength does not have any significant effect on the method performance, which demonstrated that the developed RP-HPLC method is robust. (**Table No.6 (a, b, c))** shows the Robustness of atorvastatin, ezetimibe and fenofibrate at different flow rates and (**Table No.7 (a, b, c))** shows the Robustness of atorvastatin, ezetimibe and fenofibrate at different wavelengths.

Detection and Quantification limits- The limits of detection and the limit of quantification of Atorvastatin were 0.017 and 0.349, for ezetimibe were 0.050 and 0.234, similarly for the Fenofibrate the LOD and LOQ were 0.048 and 0.510 respectively.

Assay

The assay for marketed formulation was performed to evaluate the purity of Atorvastatin, ezetimibe and fenofibrate in the formulation, and the percentage assay of the drug was calculated. The obtained results are shown in the (**Table No.8).**

Assay = Peak area of the tablet solution / Peak area of standard solution ×100

Stability studies

Stability studies for Atorvastatin, Ezetimibe and Fenofibrate was performed by preparing the test concentration of 10 µg/mL for atorvastatin and ezetimibe respectively and for the fenofibrate the concentration was 160 µg/mL.

Degradation studies

1) **Acid Hydrolysis-** For acid hydrolysis, Atorvastatin, ezetimibe and fenofibrate 1mg/mL solution were taken respectively, and then exposed to 0.1N HCl for overall 0h at the room temperature at this condition degradation is observed and all the three drugs were not found to be stable. Degradation behaviour with 0.1N HCl of Atorvastatin, Fenofibrate and Ezetimibe is shown in **Figure 10 (a) (b) (c)**.
2) **Alkaline Hydrolysis-** For alkaline hydrolysis, Atorvastatin, ezetimibe and fenofibrate, 1mg/mL solution were exposed to the 0.1N NaOH for 0h at room temperature at this condition degradation observed, that will decrease the peak area and the drug was found to be unstable.Degradation behaviour with 0.1N NaOH of Atorvastatin, Fenofibrate and Ezetimibe is shown in **Figure 11 (a) (b) (c)**.
3) **Oxidative degradation-** For studying the oxidative stress on the drugs Atorvastatin, Ezetimibe and Fenofibrate, firstly the 1mg/mL solution was taken and exposed to 1% H_2O_2at0h, at this condition degradation observed for both the drugs.Degradation behaviour with 1% $H_20_2$of Atorvastatin, Fenofibrate and Ezetimibe is shown in **Figure 12 (a) (b) (c)**.
4) **Photo degradation-** For studying the photo degradation study of the drugs like Atorvastatin, ezetimibe and fenofibrate, firstly the drugs were exposed under the sunlight and then the analysis was done under the different two-time intervals by injecting the sample at the 0hrs.Photo degradation behaviour of Atorvastatin, Fenofibrate and Ezetimibe is shown in **Figure 13 (a) (b) (c)**.
5) **Thermal Degradation-** Thermal degradation behaviour of Atorvastatin, Fenofibrate and Ezetimibe is shown in **Figure 14 (a) (b) (c)**.

4. Discussion

This study was aimed to develop and validate a method using RP-HPLC for the simultaneous estimation of drugs i.e. atorvastatin, ezetimibe and fenofibrate in marketed formulation. Marketed formulation that was used in performing experimental work was Fibator EZ manufactured by Sun Pharma ,consists of 10mg atorvastatin, 160mg fenofibrate and 10 mg ezetimibe and the percentage assay was also calculated for the formulation using RP-HPLC. Different validation parameters according to ICH guidelines were also performed to check the precision, linearity of the method in the marketed formulation. Stability and degradation studies was also performed to check the effect of different solvents and other chemical substances on marketed formulation and also to determine the suitability of the process. All the results have been calculated using the RP-HPLC method.

5. Conclusion

In this present work a new simple, linear, precise, accurate and robust RP- HPLC method has been developed and validated for the estimation of atorvastatin, ezetimibe and fenofibrate in the pharmaceutical dosage form. Linearity curve for the atorvastatin and ezetimibe were 10-50µg/mL respectively, similarly for the fenofibrate the linearity was in the range of 160-200µg/mL Validation procedure revealed that %RSD is less than 2. With this developed method it is revealed that a simple, précised and accurate new RP-HPLC method was developed and validated for the quantitative determination of the drugs in the tablet dosage form. The method was validated according to ICH [Q2R1]

guidelines that are followed by the parameters like Accuracy, Precision, Linearity, LOD & LOQ and Robustness.

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Conflict of interest
The authors declare that there is no conflict of interest.

6. References

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Figures with Legends

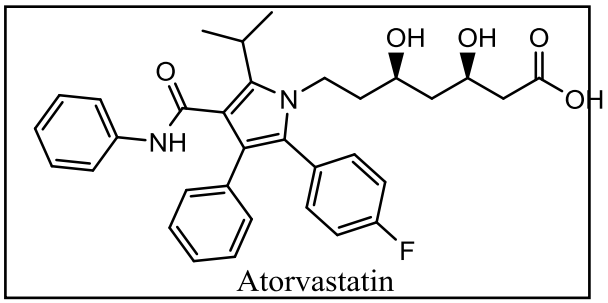

Figure 1. Chemical structure of Atorvastatin

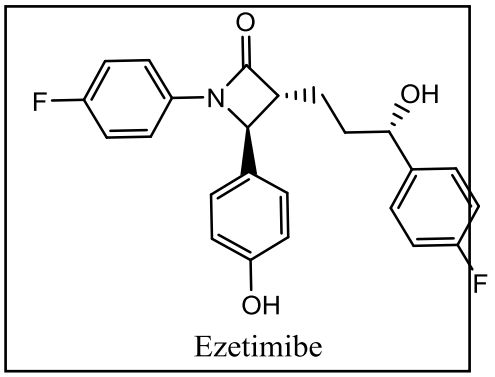

Figure 2. Chemical structure of Ezetimibe

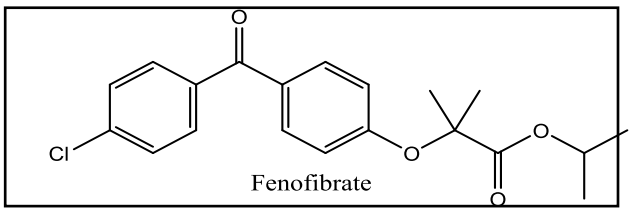

Figure 3. Chemical structure of Fenofibrate

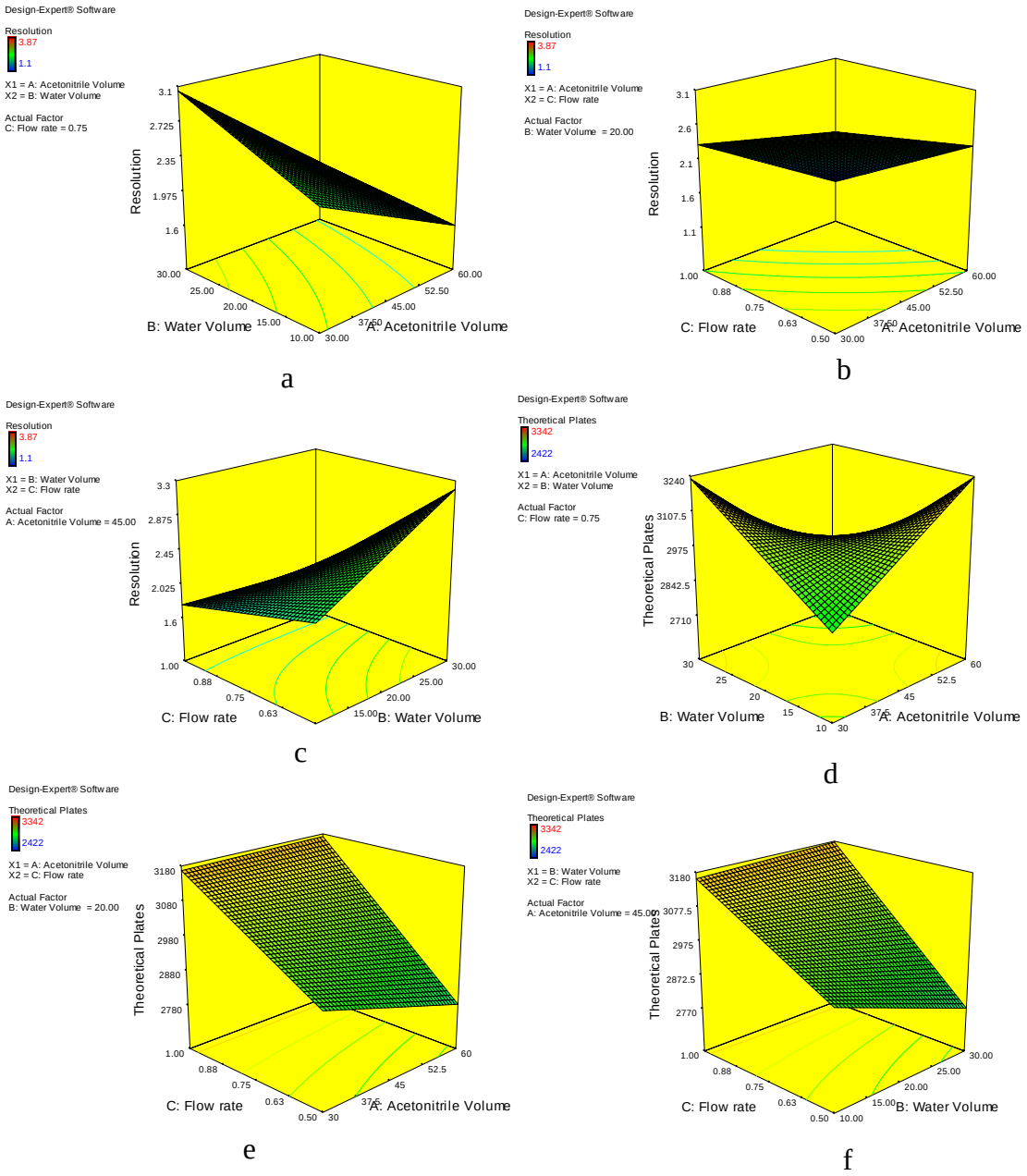

Figure 4: Response surface analysis depicted the possible interactions of a), b) and c) on Y1 and d), e) and f) on Y2

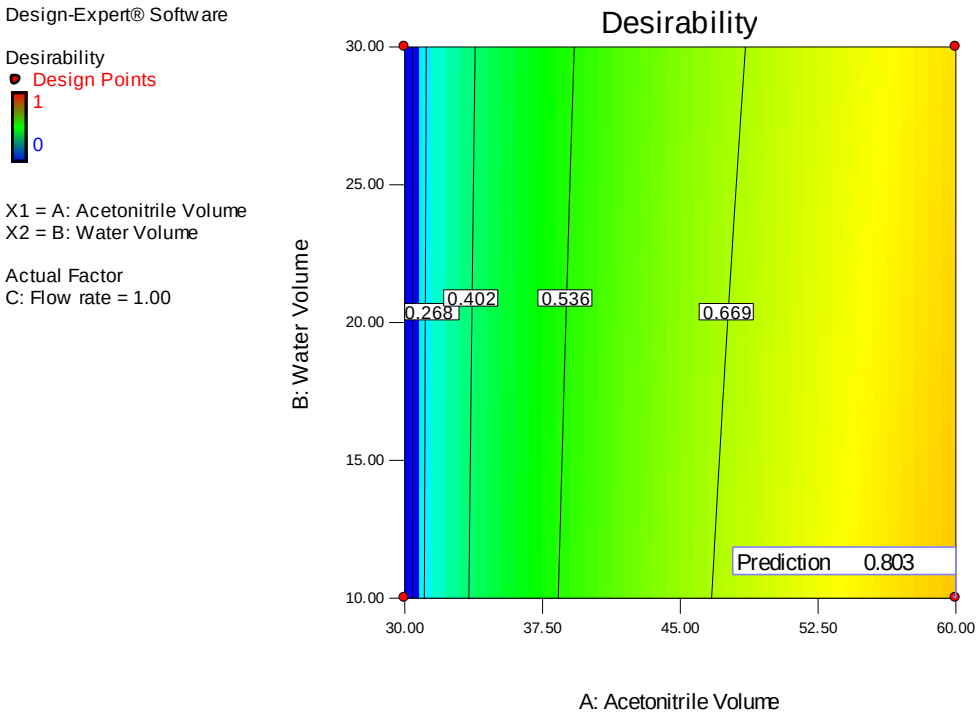

Figure 5.

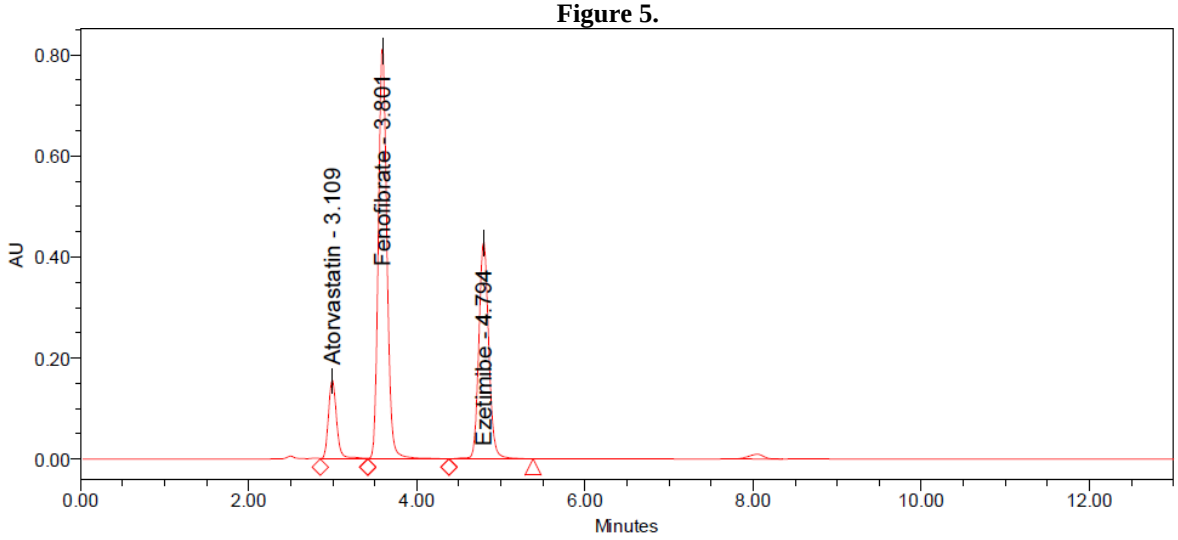

Figure 6. Chromatogram of Sample solution with no interferences

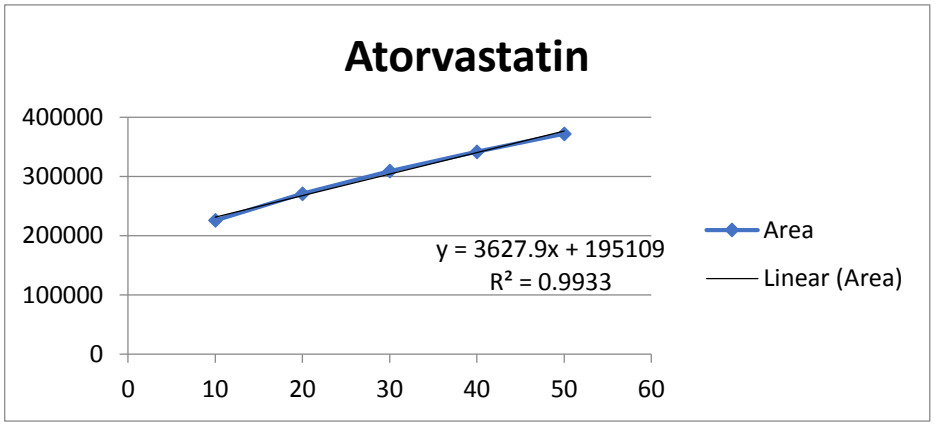

Figure 7.Linearity curve of Atorvastatin

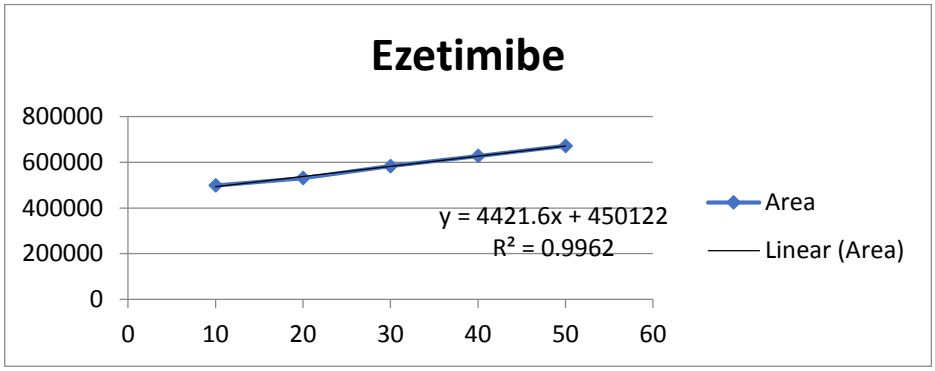

Figure 8.Linearity curve of Ezetimibe

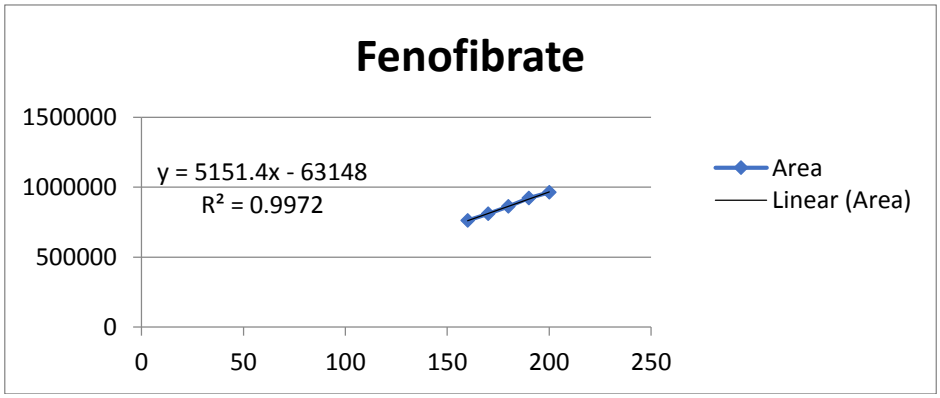

Figure 9.Linearity curve of Fenofibrate

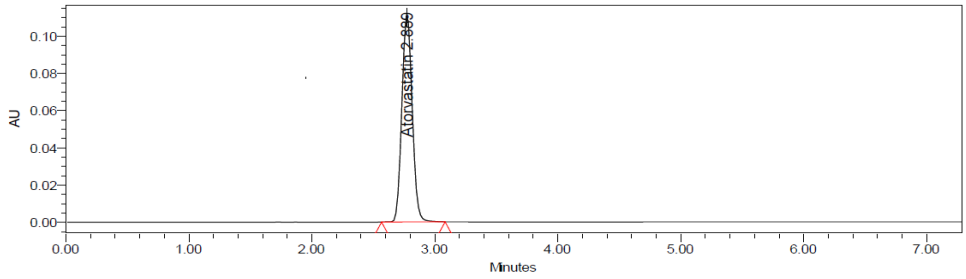

Figure 10 (a). Degradation behaviour of Atorvastatin with 0.1N HCl

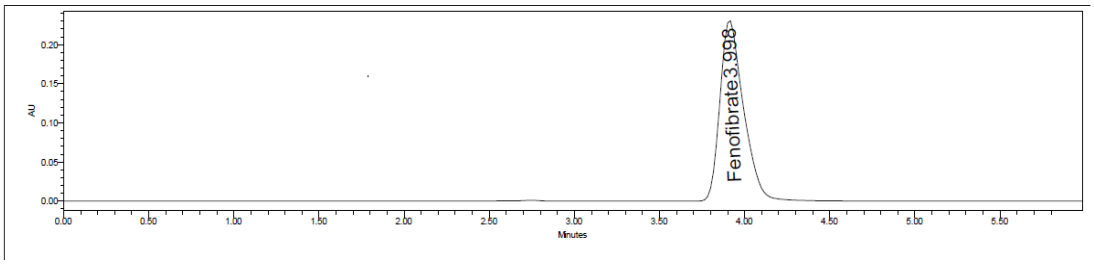

Figure 10 (b). Degradation behaviour of Fenofibrate with 0.1N HCl

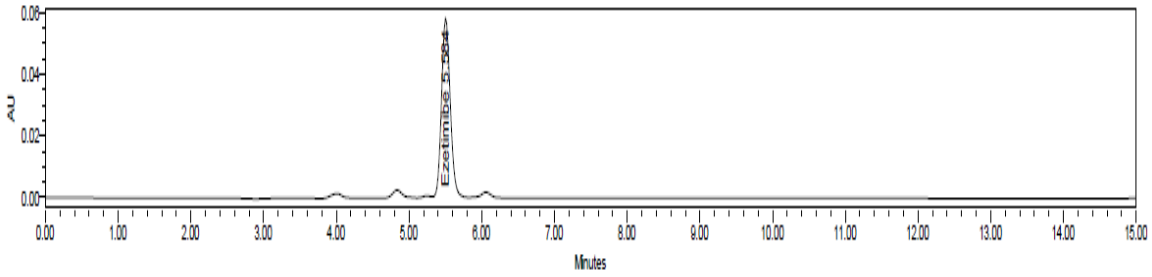

Figure 10 (c). Degradation behaviour of Ezetimibe with 0.1N HCl

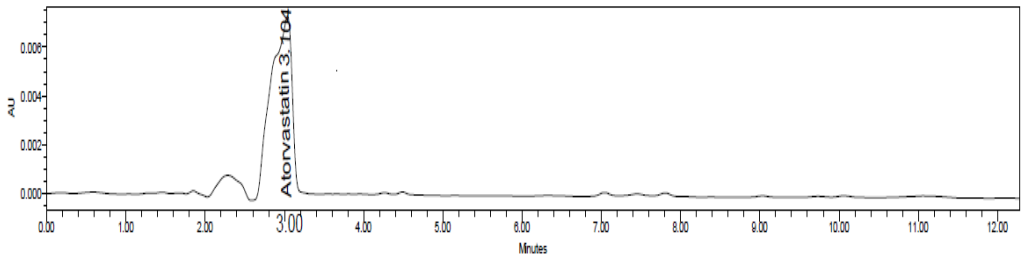

Figure 11(a). Degradation behaviour of Atorvastatin with 0.1N NaOH

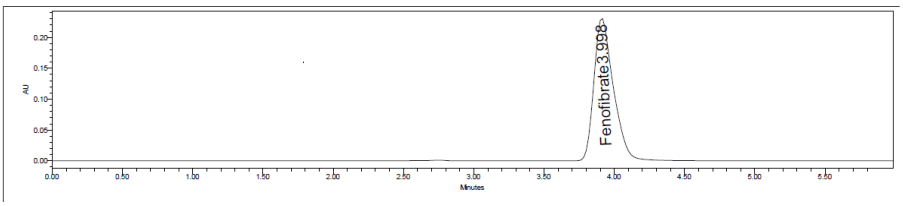

Figure 11 (b). Degradation behaviour of Fenofibrate with 0.1N NaOH

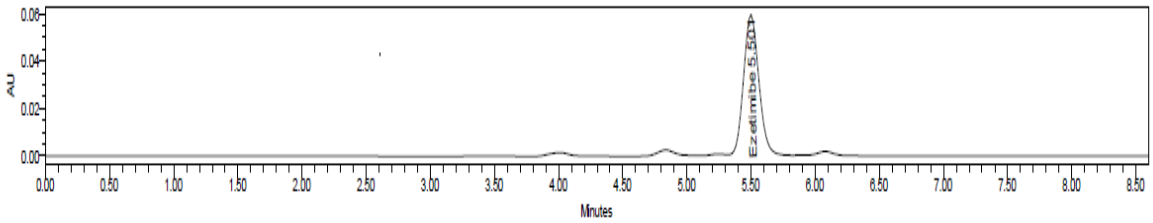

Figure 11 (c). Degradation behaviour of Ezetimibe with 0.1N NaOH

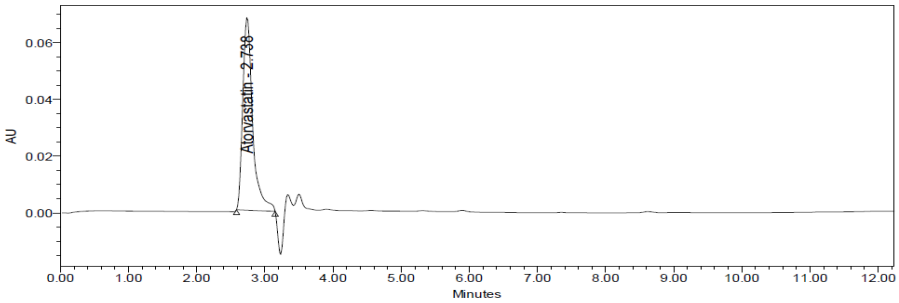

Figure 12 (a). Degradation behaviour of Atorvastatin with 1% H_20_2

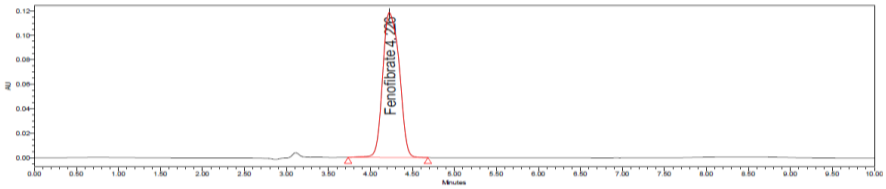

Figure 12 (b). Degradation behaviour of Fenofibrate with 1% H_20_2

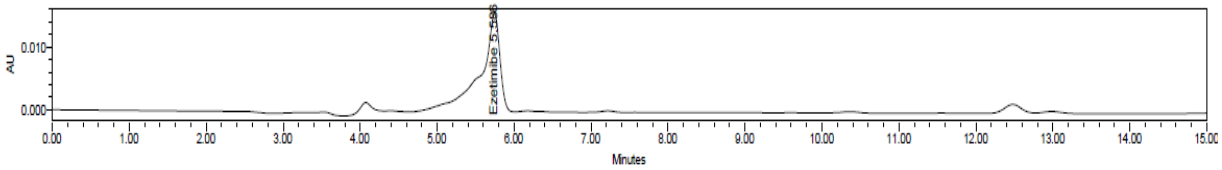

Figure 12 (c). Degradation behaviour of Ezetimibe with 1% H_20_2

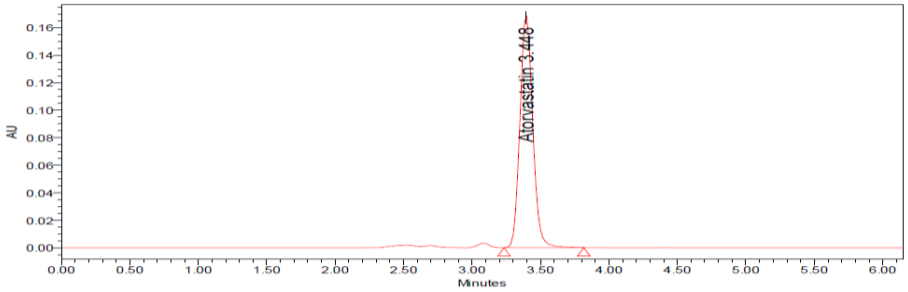

Figure 13 (a). Photo degradation behaviour of Atorvastatin

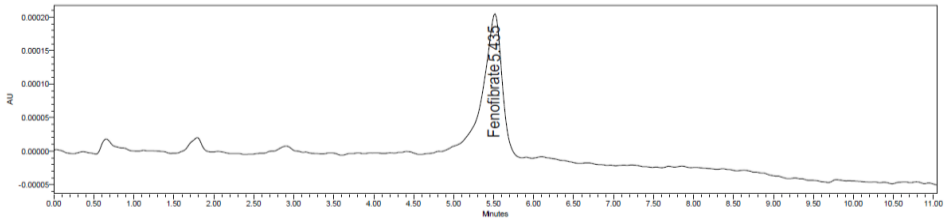

Figure 13 (b). Photo degradation behaviour of Fenofibrate

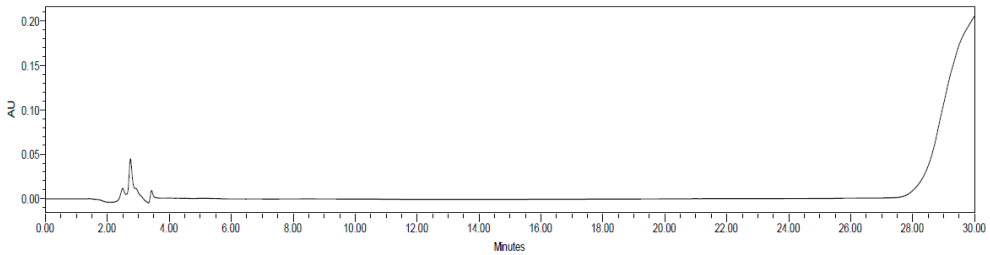

Figure 13 (c). Photo degradation behaviour of Ezetimibe

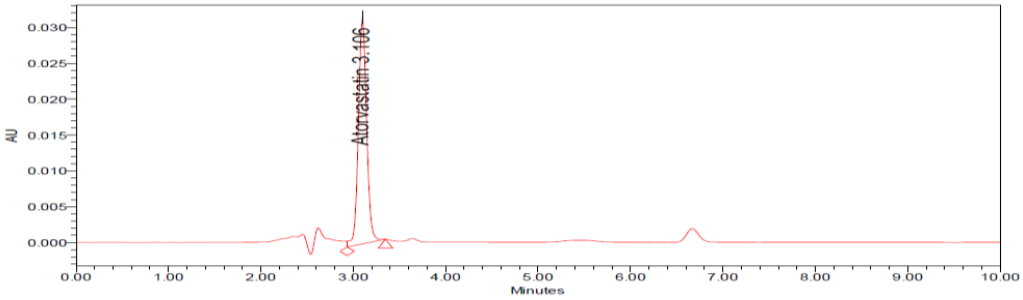

Figure 14 (a). Thermal degradation behaviour of Atorvastatin

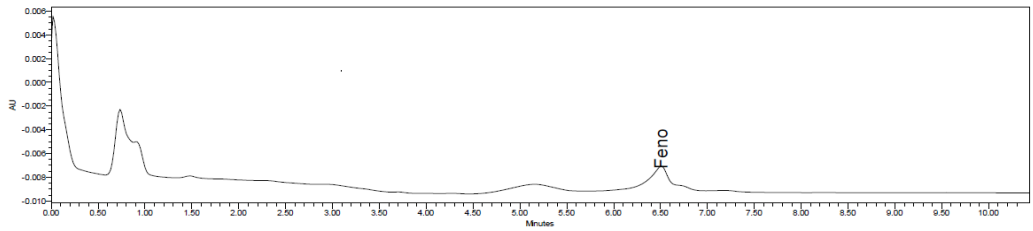

Figure 14 (b). Thermal degradation behaviour of Fenofibrate

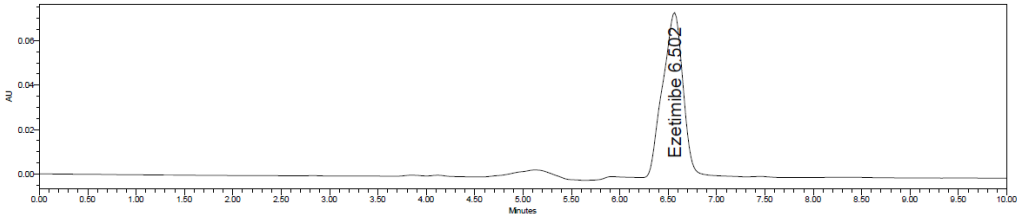

Figure 14 (c). Thermal degradation behaviour of Ezetimibe

Tables with Legends

Table No.1: Box-Behnken design for the study of three experimental factors in coded and actual levels with experimental results

			Factor 1	**Factor 2**	**Factor 3**	**Response 1**	**Response 2**
Std	Run	Block	A:Acetonitrile Volume	B:Water Volume	C:Flow rate	Resolution Time	Theoretical Plates
			mL	mL	mL/min	min	HETP
6	1	Block 1	60.00	10.00	1.00	1.12	3332
8	2	Block 1	60.00	30.00	1.00	1.1	3012
3	3	Block 1	30.00	30.00	0.50	3.87	3120
7	4	Block 1	30.00	30.00	1.00	2.24	3342
4	5	Block 1	60.00	30.00	0.50	2.53	2422
1	6	Block 1	30.00	10.00	0.50	2.32	2722
5	7	Block 1	30.00	10.00	1.00	2.42	2993
2	8	Block 1	60.00	10.00	0.50	2.09	3143

Table No.2: Analysis of variance (ANOVA) of independent variables and response terms

Analysis of variance							
Response	**Source**	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**	**Inference**
Resolution	**Model**	**0.38743**	**6**	**16.31212**	**0.327222**	**< 0.0001**	**Significant**
	X1-Acetonitrile Volume	0.38732	1	0.98622	0.277322	< 0.0001	
	X2-Water Volume	5.42122	1	17.3203	0.287732	< 0.0001	

	X3-Flow rate	1.76883	1	0.76288	1.9363545	0.1874	
	Residual	0.29388	2	0.293283			
	Lack of Fit	**0.918272**	**2**	**0.53292**	**13.54608**	**0.3051**	**Not significant**
	Pure Error	0.01072	4	0.003282			
	Cor Total	20.3224	17				
Theoretical Plates	**Model**	**9837.58**	**6**	**74010.27**	**1.277629**	**0.0006**	**Significant**
	X1-Acetonitrile Volume	10804.807	1	8978.80	2.72744	0.0009	
	X2-Water Volume	2022.75	1	10804.75	8.361363	0.0001	
	X3-Flow rate	4.6014	1	12.19101	1.2864374	0.6091	
	Residual	6.279332	2	52.74416			
	Lack of Fit	**4411.402**	**2**	**14.13410**	**0.1695476**	**0.9117**	**Not significant**
	Pure Error	0.29823	4	3.73017			
	Cor Total	14.28832	16				

Table No.3: Linearity data for Atorvastatin, Ezetimibe and Fenofibrate

Atorvastatin		**Ezetimibe**		**Fenofibrate**	
Concentration (µg/mL)	**Peak area**	**Concentration (µg/mL)**	**Peak area**	**Concentration (µg/mL)**	**Peak Area**
10	225989	10	499052	160	762171
20	270951	20	531449	170	809491
30	309081	30	583648	180	863064
40	341688	40	627688	190	922568
50	372013	50	672013	200	963201

Table No.4(a):System and Method Precision for Atorvastatin

System Precision		**Method Precision**	
Concentration(µg/mL)	**Peak Area**	**Concentration(µg/mL)**	**Peak Area**
10	228429	10	224540
10	229045	10	223002
10	229001	10	221315
10	229049	10	223204
10	228328	10	223414
10	229828	10	221921
Mean	228946.7	Mean	222899.3
Standard Deviation	491.6925	Standard Deviation	1043.015
%RSD	0.214	%RSD	0.467

Table No.4(b):System and Method Precision for Ezetimibe

System Precision		**Method Precision**	
Concentration (µg/mL)	**Peak Area**	**Concentration(µg/mL)**	**Peak Area**
10	499052	10	499140
10	498512	10	496215
10	499002	10	496210

10	498125	10	497856
10	497510	10	497862
10	498520	10	497561
Mean	498453.5	Mean	497474
Standard Deviation	526.7832	Standard Deviation	1021.63
%RSD	0.105	%RSD	0.205

Table No.4(c): System and Method Precision for Fenofibrate

System Precision		**Method Precision**	
Concentration(µg/mL)	**Peak Area**	**Concentration(µg/mL)**	**Peak Area**
160	762174	160	761487
160	763145	160	763845
160	764581	160	768459
160	763124	160	762145
160	764125	160	769451
160	767581	160	761012
Mean	764121.7	Mean	764399.8
Standard Deviation	1728.227	Standard Deviation	3350.255
%RSD	0.226	% RSD	0.438

Table No.5 (a): Intermediate precision of Atorvastatin

Intraday Precision		**Interday Precision**	
Concentration(µg/mL)	**Area**	**Concentration(µg/mL)**	**Area**
10	228429	10	224529
10	229045	10	225045
10	229001	10	221274
10	227149	10	222449
10	228328	10	223428
10	229828	10	222588
Mean	228630	Mean	223218.8
Standard Deviation	823.613	Standard Deviation	1282.455
% RSD	0.360	% RSD	0.574

Table No.5 (b): Intermediate precision of Ezetimibe

Intraday Precision		**Interday Precision**	
Concentration(µg/mL)	**Area**	**Concentration(µg/mL)**	**Area**
10	499521	10	497214
10	498561	10	499321
10	498723	10	495623
10	499863	10	498563
10	497581	10	496851
10	499741	10	494621
Mean	498998.3	Mean	497032.2
Standard Deviation	800.8626	Standard Deviation	1604.554
% RSD	0.160	% RSD	0.322

Table No.5(c): Intermediate precision of Fenofibrate

Intraday Precision		**Interday Precision**	
Concentration (µg/mL)	**Area**	**Concentration (µg/mL)**	**Area**
160	762584	160	762984
160	764581	160	764105
160	768917	160	763981

160	762147	160	762410
160	769841	160	765014
160	762891	160	763059
Mean	765160.2	Mean	763592.2
Standard Deviation	3088.267	Standard Deviation	864.817
% RSD	0.403	% RSD	0.113

Table No.6 (a): Robustness of Atorvastatin at the FR of 0.8, 1 and 1.2mL/min.

At 0.8mL/min.		**At 1.0mL/min.**		**At 1.2mL/min.**	
Concentration (µg/mL)	**Area**	**Concentration (µg/mL)**	**Area**	**Concentration (µg/mL)**	**Area**
8	378124	10	383324	12	391258
8	378451	10	383314	12	390147
8	378142	10	383327	12	390124
Mean	378239	Mean	383321.7	Mean	390509.7
Standard Deviation	150.086	Standard Deviation	5.557777	Standard Deviation	529.2349
%RSD	0.039	%RSD	0.001	%RSD	0.135

Table No.6 (b):Robustness of Ezetimibe at the FR of 0.8, 1 and 1.2mL/min.

At 0.8mL/min.		**At 1.0mL/min.**		**At 1.2mL/min.**	
Concentration (µg/mL)	**Area**	**Concentration (µg/mL)**	**Area**	**Concentration (µg/mL)**	**Area**
8	268941	10	284513	12	295874
8	268471	10	284987	12	298541
8	268394	10	284612	12	295368
Mean	268602	Mean	284704	Mean	296594.3
Standard Deviation	241.7616	Standard Deviation	204.1519	Standard Deviation	1391.915
%RSD	0.090	%RSD	0.071	%RSD	0.469

Table No.6(c): Robustness of Fenofibrate at the FR of 0.8, 1 and 1.2mL/min.

At 0.8mL/min.		**At 1.0mL/min.**		**At 1.2mL/min.**	
Concentration (µg/mL)	**Area**	**Concentration (µg/mL)**	**Area**	**Concentration (µg/mL)**	**Area**
120	421587	160	521479	190	581244
120	421784	160	521784	190	581743
120	421587	160	521684	190	581423
Mean	421652.7	Mean	521649	Mean	581470
Standard Deviation	92.866	Standard Deviation	126.951	Standard Deviation	206.409
%RSD	0.022	%RSD	0.024	%RSD	0.035

TableNo.7(a):Robustness of Atorvastatin at different wavelength 244nm, 246nm, 249nm

Wavelength 244nm		**Wavelength 246nm**		**Wavelength 249nm**	
Concentration (µg/mL)	**Area**	**Concentration (µg/mL)**	**Area**	**Concentration (µg/mL)**	**Area**
10	201417	10	383334	10	141258
10	201459	10	383104	10	142562
10	201358	10	383314	10	140254
Mean	201411.3	Mean	383250.7	Mean	141358

Standard Deviation	41.427	Standard Deviation	104.029	Standard Deviation	944.88
%RSD	0.020	%RSD	0.027	%RSD	0.668

Table No.7(b): Robustness of Ezetimibe at different wavelength 230nm, 233nm, 236nm

At wavelength 230		**At wavelength 233**		**At wavelength 236**	
Concentration (µg/mL)	**Area**	**Concentration (µg/mL)**	**Area**	**Concentration (µg/mL)**	**Area**
10	145982	10	284513	10	120147
10	145329	10	284712	10	120356
10	145289	10	284651	10	120398
Mean	145533.3	Mean	284625.3	Mean	120300.3
Standard Deviation	317.675	Standard Deviation	83.243	Standard Deviation	109.770
%RSD	0.218	%RSD	0.029	%RSD	0.091

Table No. 7(c):Robustness of Fenofibrate at different wavelength 283nm, 286nm, 289nm

At wavelength283		**At wavelength 286**		**At wavelength 289**	
Concentration (µg/mL)	**Area**	**Concentration (µg/mL)**	**Area**	**Concentration (µg/mL)**	**Area**
160	401258	160	521479	160	310258
160	401896	160	521784	160	310256
160	401785	160	521687	160	310874
Mean	401646.3	Mean	521650	Mean	310462.7
Standard Deviation	278.307	Standard Deviation	127.23	Standard Deviation	290.857
%RSD	0.069	%RSD	0.024	%RSD	0.093

Table No.8: Assay of marketed formulation

Drug	**Peak Area of Standard**	**Peak Area of Sample**	**Label claim(mg)**	**Amount found (mg)**	**Percentage assay**
Atorvastatin	383334	383849	10	10.01	100.1
Ezetimibe	284513	284484	10	9.99	99.9
Fenofibrate	521479	520982	160	159.84	99.9