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Validated stability indicating RP-HPLC method for simultaneous determination of atorvastatin, fenofibrate and folic acid in bulk and pharmaceutical dosage form

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ABSTRACT

The main aim of the present research work was to develop a new simple, precise, selective, accurate and rapid reverse phase high performance liquid chromatographic stability indicating method had been developed and validated for simultaneous quantitative determination of Atorvastatin, Fenofibrate and Folic acid in bulk and pharmaceutical dosage form. The chromatographic separation was achieved with Agilent XDB C18 150×4.6 mm, 5µm particle size column. The optimized mobile phase consisting of pH 3.3 Phosphate buffer: Acetonitrile (30:70 %v/v). The flow rate was 1.0 mL/min and eluents were detected at 280 nm using PDA detector. The retention time of Folic acid, Atorvastatin, Fenofibrate were found to be 2.507, 3.202 and 6.005 respectively. The percentage recoveries for Folic acid, Atorvastatin, Fenofibrate were found to be in the range of 100.26 -100.69 %, 100.01 – 100.81 % and 100.05 – 100.97 %. The calibration curve was constructed between peak area vs concentration and demonstrated good linear in the range of 5 -30 µg/ml for Atorvastatin, 80-480 µg/ml for Fenofibrate and 2.5-15 µg/ml for Folic acid. Degradation studies were studied for Atorvastatin, Fenofibrate and Folic acid under various stress conditions such as acid hydrolysis, base hydrolysis, oxidation, thermal, photochemical and UV. All the degradation peaks were resolved effectively using developed method with different retention times. The developed method was validated according to ICH guidelines. As the method could effectively separates the degradation products from active ingredient, it can be used for routine analysis of drug both in bulk and pharmaceutical dosage form.

Key words: Atorvastatin; Fenofibrate; Folic acid; RP-HPLC; Degradation; Validation; ICH-guidelines; stability indicating

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INTRODUCTION

Atorvastatin [1,2] (ATN) is (β R, δ R)-2-(4-fluorophenyl)-β,δ-dihydroxy-5-(1-methyl ethyl)-3-phenyl-4-((phenylamino)carbonyl)-1H-pyrrole-1-hepatonoicacid, a Hydroxymethylglutaryl-CoA Reductase Inhibitors. As HMG-CoA reductase is responsible for converting HMG-CoA to mevalonate in the cholesterol biosynthesis pathway. It is given in a combination with a novel drug of Fenofibrate and many other drugs. Fenofibrate [3-4], (FNF) is 2-[4-(4-chlorobenzoyl)phenoxy]-2-methyl-propanoic acid, 1-methylethyl ester. It is indicated for the treatment of hypercholesterolemia and mixed dyslipidemia. Folic acid [5] (FOA) is (2S)-2-[(4-{[(2-amino-4-oxo-1,4-dihydropteridin-6-yl)phenyl)formamido]pentanedioic acid, is a vitamin B complex, dietary supplements. The

folic acid congeners are transported across cells by receptor-mediated endocytosis synthesize purine and thymidylate nucleic acids. The drugs prescribed individually, as well as multicomponent dosage forms available in the market. The chemical structures of Atorvastatin, Fenofibrate and Folic acid are shown in

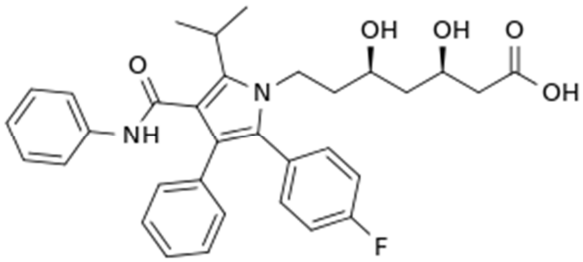


Figure 1: Chemical Structure of Atorvastatin

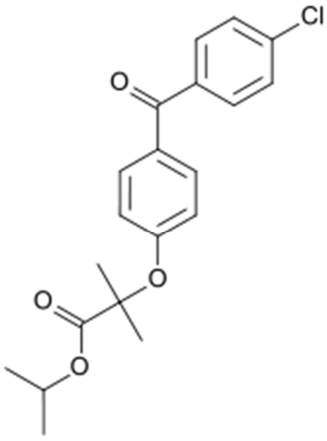


Figure 2: Chemical Structure of Fenofibrate

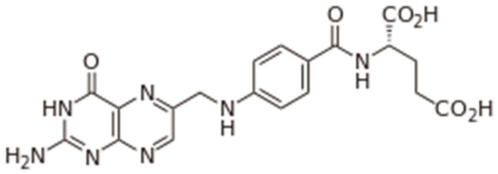


Figure 3: Chemical Structure of Folic acid

Literature survey revealed few analytical methods for the quantitative determination of Fenofibrate and Vinpocetine by liquid chromatography [6], HPTLC methods [7] and Atorvastatin in combination with other drugs by RP-HPLC [8-12], UV Spectrophotometric methods [13-14]. Similarly there are few analytical methods for estimation of Fenofibrate and folic acid in combination with other drugs [15-18]. An RP-HPLC method is also found for the estimation of Atorvastatin, Fenofibrate and folic acid. On the contrary to the best of our knowledge, there is no official method for the stability-indicating simultaneous estimation of Atorvastatin, Fenofibrate and Folic acid by RP-HPLC in bulk and tablet dosage form. The disadvantages of the reported HPLC method are lack of stability indicating. The retention time of the active analytes and total runtime in the reported method is more than the proposed method which leads to a longer runtime for a single sample. The preparation of buffer and maintenance of pH of the buffer makes the method cumbersome. Furthermore the reported method does not reported the stability indicating. Due to the above facts, we planned to develop and validate a simple, rapid, economical, precise and accurate stability-indicating simultaneous determination of Atorvastatin, Fenofibrate and Folic acid in bulk drug and pharmaceutical dosage form. The new method is capable of separating all three active analytes present in the dosage form.

MATERIALS AND METHODS

Chemicals and solvents:
Atorvastatin, Fenofibrate and Folic acid were obtained as gift samples from Spectrum Laboratories Limited, Hyderabad, India. The commercial Pharmaceutical tablets of Lorisk tablets containing 10 mg of Atorvastatin, 160 mg of Fenofibrate, and 5 mg of Folic acid respectively (manufactured by Grandix Pharmaceuticals) were procured from local pharmacy. Potassium dihydrogen phosphate – AR grade (Merck), Orthophosphoric acid- HPLC grade (Fisher Scientific), Acetonitrile-HPLC grade (Merck), Methanol – HPLC grade (Merck), Sodium hydroxide-GR grade (Merck), Hydrochloric acid (Merck), Hydrogen peroxide (Merck) and water for HPLC- Milli-Q grade.

Instrumentation:
The chromatographic separations were performed using HPLC-Waters alliance (Model-2695) consisting of an in-built auto sampler, a column oven and 2996 PDA detector. The data was acquired through Empower-2-software. The column used was Agilent XDB C18 150×4.6 mm, 5µm. Meltronics sonicator was used for enhancing dissolution of the compounds. Elico pH meter was used for adjusting the pH of buffer solution. All weighing was done on Sartorius balance (model AE-160).

Chromatographic conditions:
The mobile phase consists of phosphate buffer pH 3.3: Acetonitrile in the ratio of 30:70 % v/v. The mobile phase was pumped from solvent reservoir in the ratio of 30:70 % v/v to the column in the flow rate of 1.0 ml/min whereas run time set was 8 min. The separation was performed on Agilent XDB 150mm x 4.6mm, 5µm column and the column was maintained the temperature of 30°C and the volume of each injection was 10 µL. Prior to injection, the column was equilibrated for at least 30 min with mobile phase flowing through the system. The eluents were monitored at 280 nm.

Preparation of phosphate buffer pH 3.3 solution:
Accurately weighed 1.36gm of potassium dihydrogen orthophosphate in 900 mL with HPLC grade water, dissolve and pH adjusted to 3.30 with dilute phosphoric acid and finally make up the volume with water to 1000 mL and filtered through 0.45 µm membrane filter and sonicate to degas.

Preparation of mobile phase:
Phosphate Buffer pH 3.3 and Acetonitrile taken in the ratio 30:70 % v/v mixed well and sonicate to degas.

Preparation of diluent:
Water and methanol in the ratio of 50:50 %v/v

Preparation of standard solution:
(20 µg/ml Atorvastatin, 320 µg/ml Fenofibrate and 10 µg/ml Folic acid)
Accurately Weighed and transferred 5 mg of Atorvastatin, 80 mg of Fenofibrate and 5 mg of Folic acid working Standards into a individual 25 mL, 25 mL and 50 mL clean dry volumetric flasks, add 3/4th volume of methanol, sonicated for 10 minutes and make up to the final volume with diluent. From the above stock solution, 1 mL was pipette out into 10 mL volumetric flask and then make up to the final volume with diluent and thus we have (20 µg/ml Atorvastatin, 320 µg/ml Fenofibrate and 10 µg/ml Folic acid).

Preparation of sample solution:
20 tablets were weighed and calculate the average weight of each tablet then the weight equivalent to 1 tablet was transferred into a 50 mL volumetric flask, 3/4th volume of methanol added and sonicated for 30 min, further the volume made up with diluent and filtered through 0.45µm nylon filter. From the filtered solution 1ml was pipette out into a 10 ml volumetric flask and made up to volume with diluent.

Label Claim: 10mg of Atorvastatin + 160mg of Fenofibrate + 5mg of Folic acid

Method development and optimization:
The present investigation was aimed to develop a simple, precise and accurate HPLC method to estimate Atorvastatin, Fenofibrate and Folic acid by using RP-HPLC C_{18} column. Initially different mobile phase compositions were tried and finally the mobile phase was optimized with pH 3.3 phosphate buffer and acetonitrile in

proportions of 30:70%v/v. With the above mobile phase a good resolution between Folic acid and Atorvastatin; Atorvastatin and Fenofibrate was achieved with a reasonably short runtime of 10 minutes. UV detection was carried out at 280 nm, all the analytes showed good absorbance at this wavelength. The retention time of Folic acid, Atorvastatin and Fenofibrate was found to be 2.507, 3.202 and 6.005 min, respectively. The peak shapes of all the analytes were symmetrical and the asymmetry factor was lesser than 2.0.

Validation of Proposed method:

The developed method was validated as per the ICH (International Council on Harmonization) guidelines with respect to System suitability, Precision, Specificity, Forced degradation studies, Linearity, Accuracy, Limit of detection and Limit of quantification [18].

Linearity:

Aliquots of 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 mL were taken from stock solution of concentration 0.02 mg/ml Atorvastatin, 0.32 mg/ml Fenofibrate, 0.01 mg/ml Folic acid and then diluted with diluent up to mark. Such that the final concentrations were in the range 5ppm-30ppm for Atorvastatin, 80ppm-480ppm for Fenofibrate and 2.5ppm-15ppm for Folic acid. Volume of 10µl of each sample was injected in three times for each concentration level and calibration curve was constructed by plotting the peak area versus drug concentration. A linear relationship between peak response vs. concentration was observed in the range of study. The observations and calibration curve were shown in **Table 1** and Figure 4, 5, 6.

Table-1: Linearity data

S.No	% level	ATN (ppm)	ATN mean Area	FFE (ppm)	FFE mean area	FCA (ppm)	FCA mean area
1	25	5.00	73582	79.92	1352125	2.50	80165
2	50	9.99	155216	159.84	2702088	5.00	168541
3	75	14.99	233295	239.76	3900136	7.49	243491
4	100	19.98	312220	319.68	5200051	9.99	322909
5	125	24.98	385671	399.60	6603631	12.49	402071
6	150	29.97	452863	479.52	7994941	14.99	485293

Figure-4: Linearity of Atorvastatin:

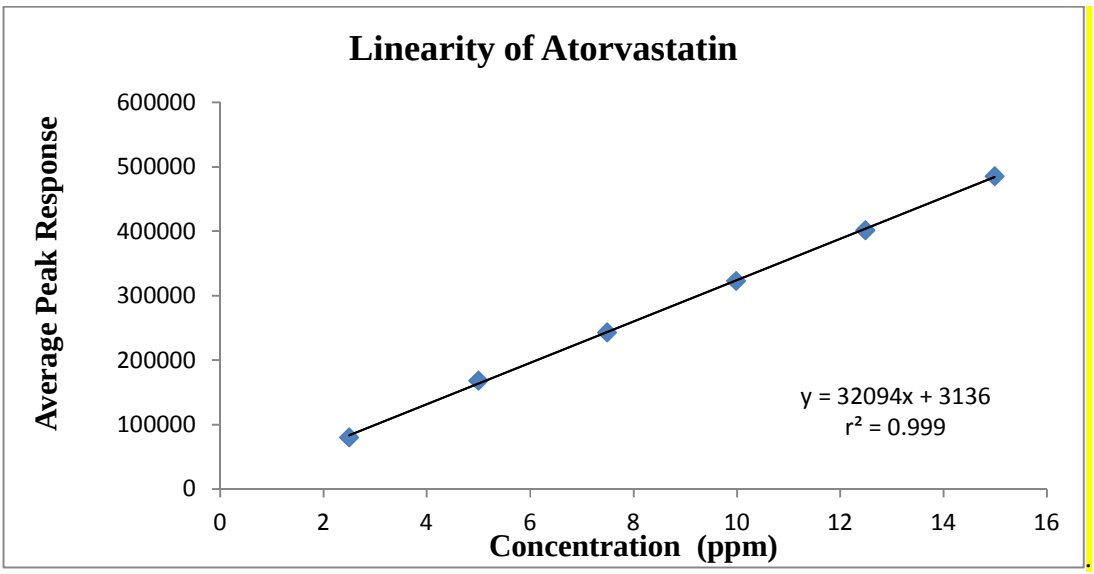


Figure-5: Linearity of Fenofibrate:

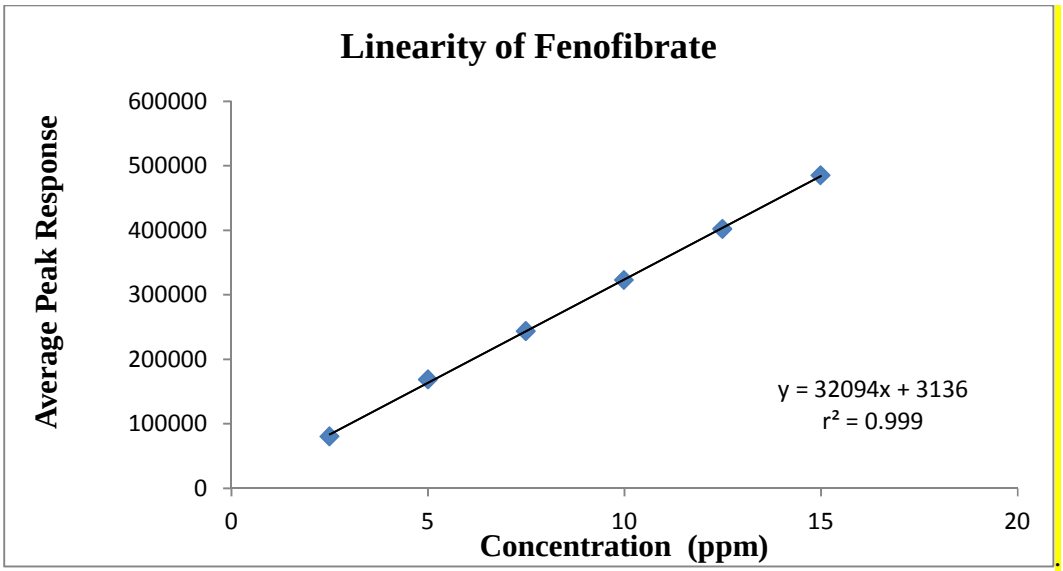
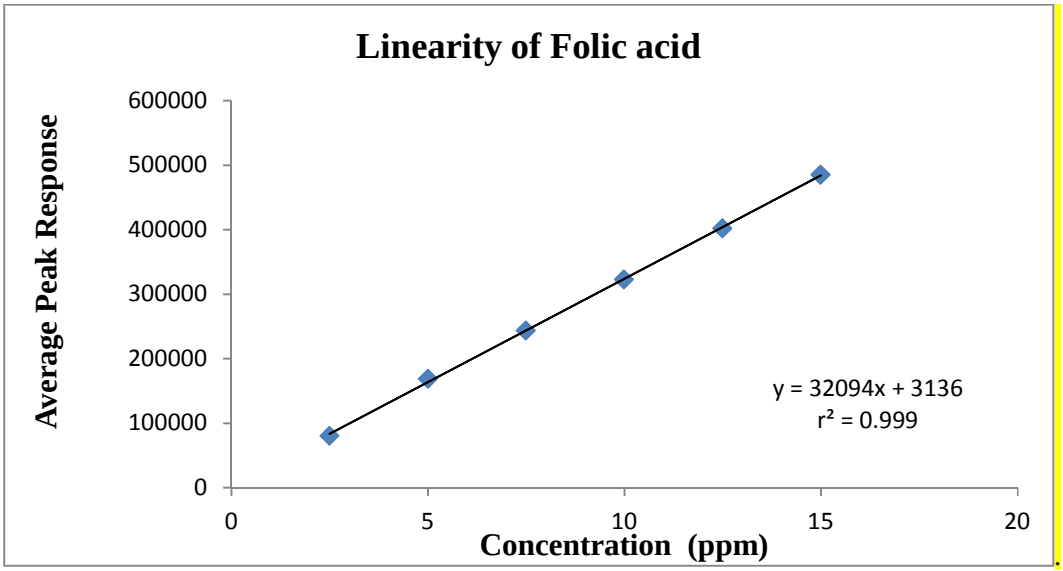


Figure-6: Linearity of Folic acid:



Optimized Chromatographic conditions and system suitability parameters for proposed HPLC method for Atorvastatin, Fenofibrate and Folic acid

Parameter		**Chromatographic conditions**
Instrument	**:**	Waters 2695, High performance Liquid Chromatography
Flow rate	**:**	1 ml/min
Column	**:**	Agilent XDB C_{18}, 150 x 4.6 mm, 5µ.
Detector wave length	**:**	280nm
Column temperature	**:**	30°C
Injection volume	**:**	10µL
Run time	**:**	8 min
Diluent	**:**	water and methanol (50:50%v/v)

Mode of separation : Isocratic mode

System precision:
Precision is the measure of closeness of the data values to each other for a number of measurements under the same analytical conditions. Standard solution of Atorvastatin (20µg /ml), Fenofibrate (320µg/ml) and Folic acid (10µg/ml) were prepared as per procedure and injected for 6 times. Results for responses are shown in **Table 2**.

Table-2: System precision data

INJECTIONS	Peak Response		
	Atorvastatin	Fenofibrate	Folic acid
1	307613	5289085	322372
2	305034	5290326	320968
3	307782	5298694	322862
4	307684	5271659	322532
5	306887	5298043	320082
6	307147	5320309	322070
AVG	307025	5294686	321814
S.D	1034.5	15902.8	1068.9
%RSD	0.3	0.3	0.3

Precision:
Method precision and intermediate precision study of Atorvastatin, Fenofibrate and Folic acid were carried out by estimating corresponding responses for 6 times on the same day and on consecutive days by different HPLC by different analyst for the concentration of Atorvastatin (20µg /ml), Fenofibrate (320µg/ml) and Folic acid (10µg/ml). The percent relative standard deviation (%RSD) was calculated which was within the acceptable criteria of not more than 2.0. The results were shown in **Table 3**.

Table-3: Precision results
Table-3A: Method precision data

Sample Preparations	%Assay		
	Atorvastatin	Fenofibrate	Folic acid
Sample-1	102.8	99.8	101.3
Sample-2	98	101.3	101.5
Sample-3	101.1	100.3	101.1
Sample-4	101.6	101	101.8
Sample-5	100.7	100.1	99.5
Sample-6	98.3	100.0	101.5
AVG	100.4	100.4	101.3
S.D	1.89	0.60	0.98
%RSD	1.89	0.60	0.96

Table-3B: Intermediate precision data

Sample Preparations	%Assay		
	Atorvastatin	Fenofibrate	Folic acid
Sample-1	100.1	99.8	98.8
Sample-2	98.6	97.9	100.1
Sample-3	100.5	98.7	98.8
Sample-4	99.7	99.3	98.5
Sample-5	99.9	98.5	101.5
Sample-6	99.5	99.4	99.7
AVG	99.7	98.9	99.6
S.D	0.65	0.69	1.14
%RSD	0.65	0.70	1.13

Accuracy:
To determine the accuracy in sample preparation method of standard additions was made for measuring the recovery of the drugs. A fixed amount of sample was taken and standard drug was added at 50%, 100% and 150% levels. The results were analyzed and the results were found to be within the limits. The accuracy was expressed as the percentage of the respective active analytes recovery and also plotted linearity graph between the average amounts

added and average amount recovered for all the active analytes and found to be linear for all the concentration levels. The results and calibration curves were shown in **Table 4** and Figure 7, 8, 9.

Table-4: Accuracy results

%level	Atorvastatin			Fenofibrate			Folic Acid		
	Amount Added (µg/ml)	Amount recovered (µg/ml)	%Recovery	Amount Added (µg/ml)	Amount recovered (µg/ml)	%Recovery	Amount Added (µg/ml)	Amount recovered (µg/ml)	%Recovery
50	10	10.02	100.19	160	160.35	100.22	5	5.03	100.69
50	10	10.05	100.51	160	160.07	100.05	5	5.02	100.42
50	10	10.06	100.58	160	160.26	100.16	5	5.03	100.55
100	20	20.16	100.81	320	321.70	100.53	10	10.07	100.66
100	20	20.06	100.31	320	321.09	100.34	10	10.04	100.40
100	20	20.00	100.01	320	323.10	100.97	10	10.05	100.54
150	30	30.20	100.67	480	481.59	100.33	15	15.08	100.52
150	30	30.24	100.80	480	481.99	100.42	15	15.04	100.26
150	30	30.14	100.47	480	483.06	100.64	15	15.09	100.64
Average			100.48	Average		100.41	Average		100.52
SD			0.27	SD		0.28	SD		0.14
RSD			0.27	RSD		0.28	RSD		0.14

Figure-7: Accuracy Linearity of Atorvastatin

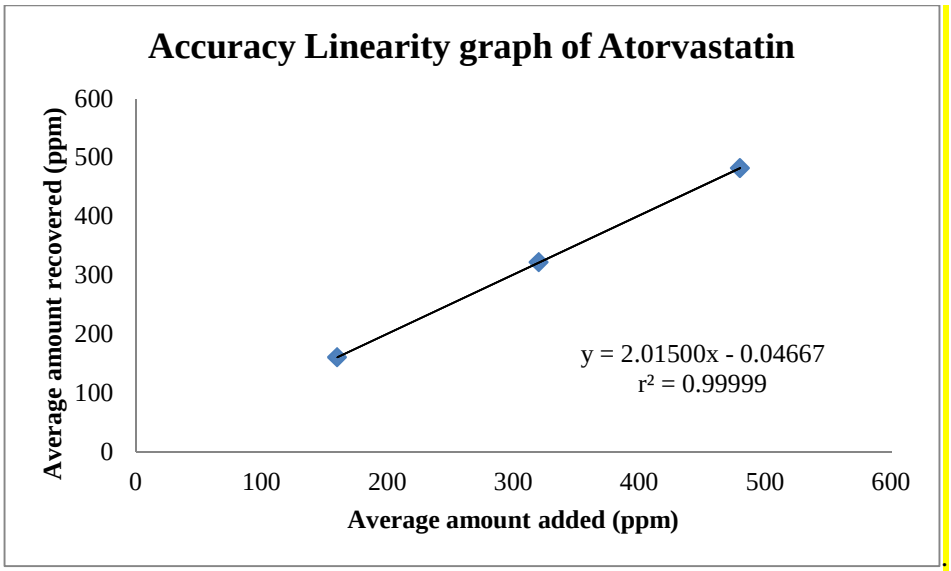


Figure-8: Accuracy Linearity of Fenofibrate

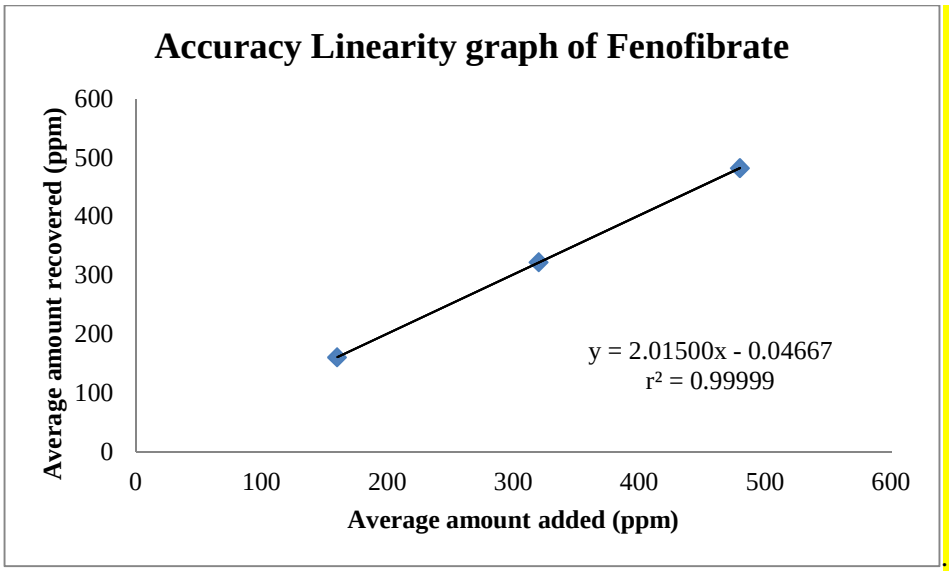


Figure-9: Accuracy Linearity of Folic acid

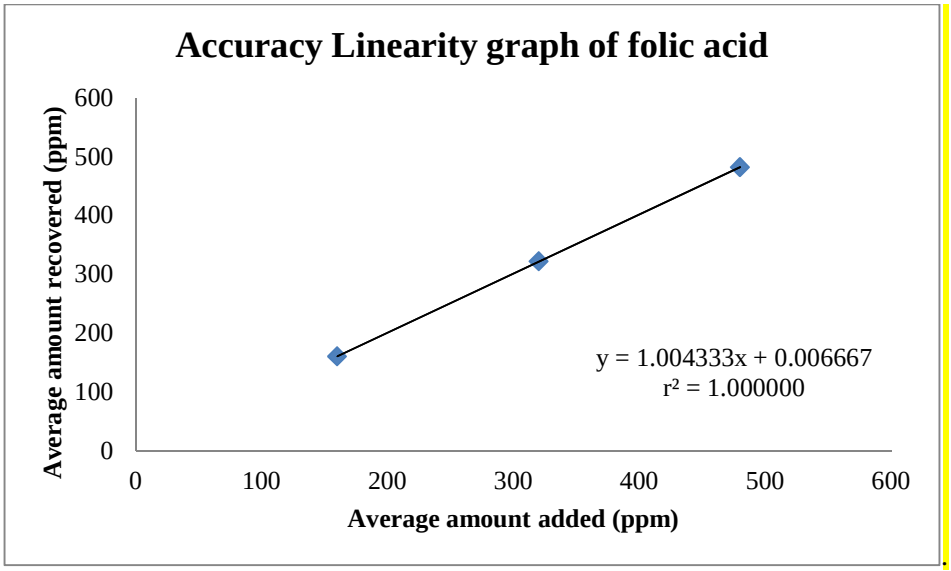


Table-5: Limit of Detection and Limit of Quantification

S.No.	Analyte Name	Signal to Noise ratio values	
		LOD	LOQ
1	Atorvastatin	3.3	10.2
2	Fenofibrate	3.2	10.1
3	Folic acid	3.1	9.7

Limit of Detection and Limit of Quantification:

LOD and LOQ were calculated using the waters empower software for signal to noise ratio method. Limit of Detection and Limit of Quantification were found to be 0.12 µg/ml and 0.36 µg/ml respectively for Atorvastatin, 0.20 µg/ml and 0.60µg/ml respectively for Fenofibrate and 0.06 µg/ml and 0.19 µg/ml respectively for Folic acid. Observed all obtained signal to noise ratio values for LOD and LOQ were found to be within the acceptance criteria as per ICH guidelines. The results were shown in **Table 5** and Figure 10, 11.

Figure 10: Typical Chromatogram of Limit of Detection

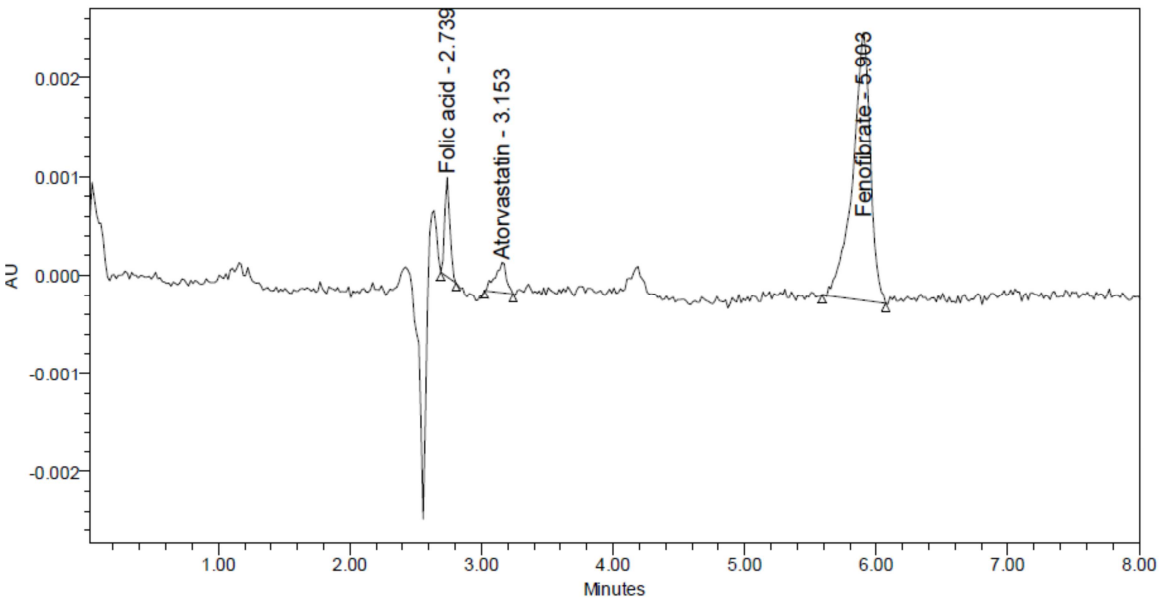
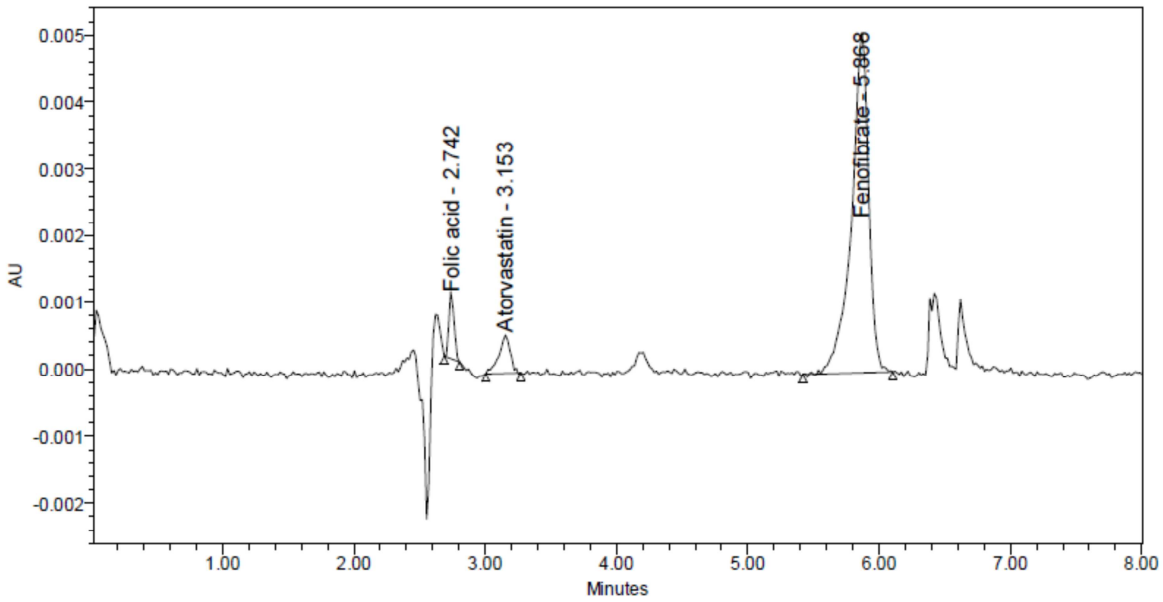


Figure 11: Typical Chromatogram of Limit of Quantitation



Robustness:

Robustness was carried by varying three parameters from the optimized chromatographic conditions such as making small changes in flow rate (±0.1ml/min), mobile phase composition (±5%) and column temperature (±5ºC). It was observed that the small changes in these operational parameters did not lead to drastic changes of retention time of the peak of interest, plate count (NLT 2000), tailing factor (NMT 2.0), resolution (NLT 2.0) and the %RSD for six replicate injections (NMT 2.0) were found to be within the acceptance criteria. The degree of reproducibility of the results proven that the method is robust. The results were shown in **Table 6** (6A, 6B, 6C) and Figure 12 to 18.

Table-6: Robustness results

Table-6A: Robustness results for Atorvastatin

S. No.	Parameter	As such method	Used	%RSD for peak area(n=6)	Retention Time	Plate count	Tailing Factor
1	Flow Rate (±0.1 ml/min)	1.0 ml/min	0.9 ml/min	1.9	3.495	8828	1.01
			1.0 ml/min	0.3	3.154	8666	0.98
			1.1 ml/min	0.4	2.871	8320	0.99
2	Column Temperature (±5ºC)	30ºC	25ºC	0.6	3.113	8148	0.99
			30ºC	0.3	3.154	8666	0.98
			35 ºC	1.6	2.271	3258	1.05
3	Mobile phase composition	Buffer:ACN 30:70 %v/v	35:65	0.6	3.113	8150	0.99
			30:70	0.3	3.154	8666	0.98
			25:75	0.7	3.191	9229	0.99

Table-6B: Robustness results for Fenofibrate:

S. No.	Parameter	As such method	Used	%RSD for peak area(n=6)	Retention Time	Plate count	Tailing Factor
1	Flow Rate (±0.1 ml/min)	1.0 ml/min	0.9 ml/min	0.3	6.482	11324	0.99
			1.0 ml/min	0.3	5.901	10749	1.01
			1.1 ml/min	0.4	5.309	10902	0.98
2	Column Temperature (±5ºC)	30ºC	25ºC	0.4	5.615	11424	0.99
			30ºC	0.3	5.901	10749	1.01
			35 ºC	1.1	5.308	10894	0.98
3	Mobile phase composition	Buffer:ACN 30:70 %v/v	35:65	0.3	5.615	11431	0.98
			30:70	0.3	5.901	10749	1.01
			25:75	0.4	6.096	11021	0.98

Table-6C: Robustness results for Folic acid:

S. No.	Parameter	As such method	Used	%RSD for peak area(n=6)	Retention Time	Plate count	Tailing Factor
1	Flow Rate (±0.1 ml/min)	1.0 ml/min	0.9 ml/min	1.5	2.764	3632	1.08
			1.0 ml/min	0.3	2.487	3480	1.06
			1.1 ml/min	0.4	2.654	8320	0.96
2	Column Temperature (±5ºC)	30ºC	25ºC	0.6	2.510	3139	1.11
			30ºC	0.3	2.487	3480	1.06
			35 ºC	0.6	2.871	8333	0.99
3	Mobile phase composition	Buffer:ACN 30:70 %v/v	35:65	0.4	2.510	3026	1.32
			30:70	0.3	2.487	3480	1.06
			25:75	0.8	2.483	3634	1.25

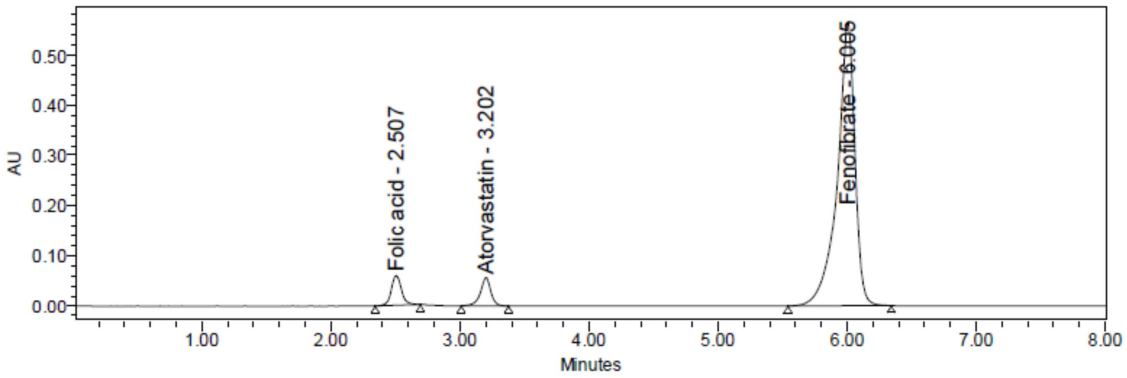


Figure 12: Typical Chromatogram of Standard

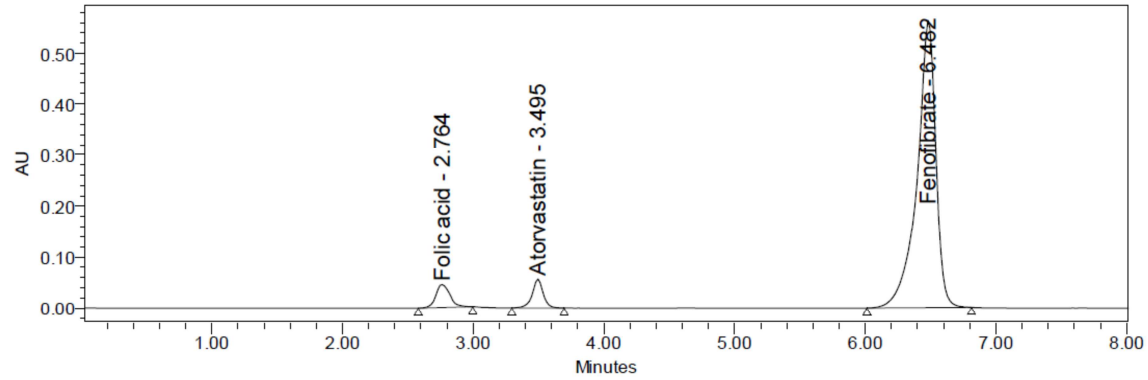


Figure 13: Typical Chromatogram of flow minus

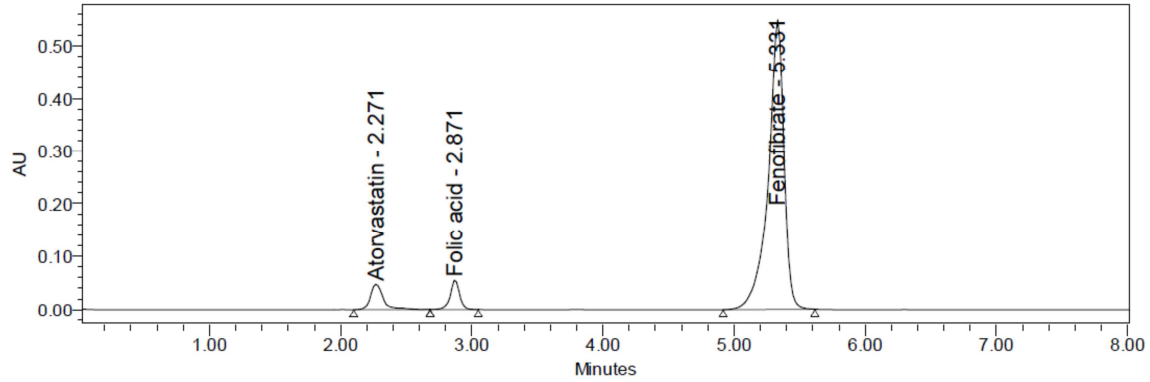


Figure 14: Typical Chromatogram of flow plus

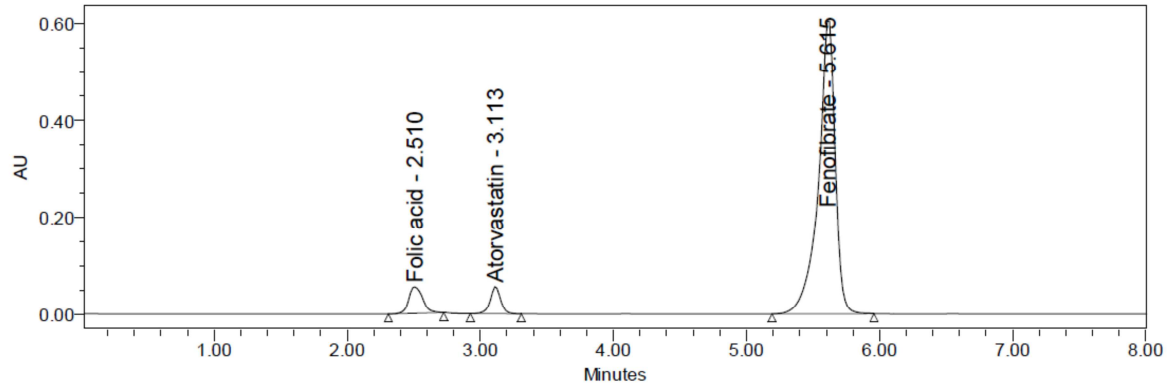


Figure 15: Typical Chromatogram of Column temperature minus

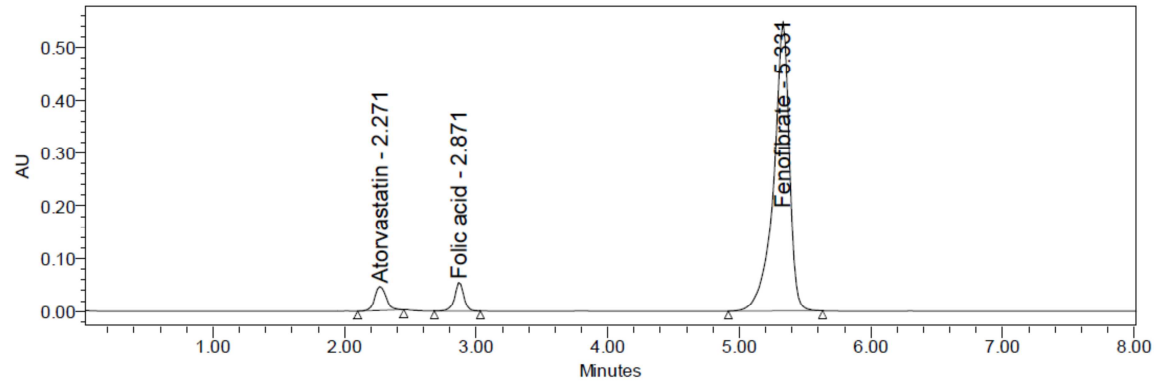


Figure 16: Typical Chromatogram of Column temperature plus

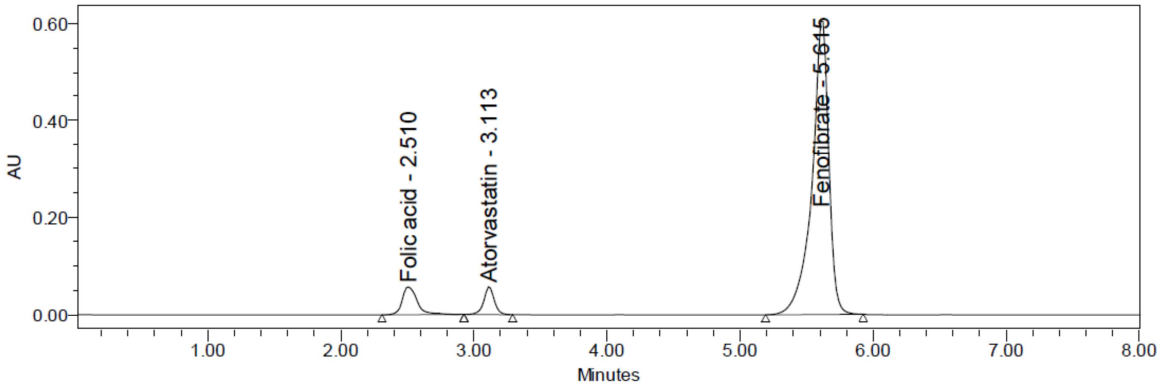


Figure 17: Typical Chromatogram of mobile phase composition minus

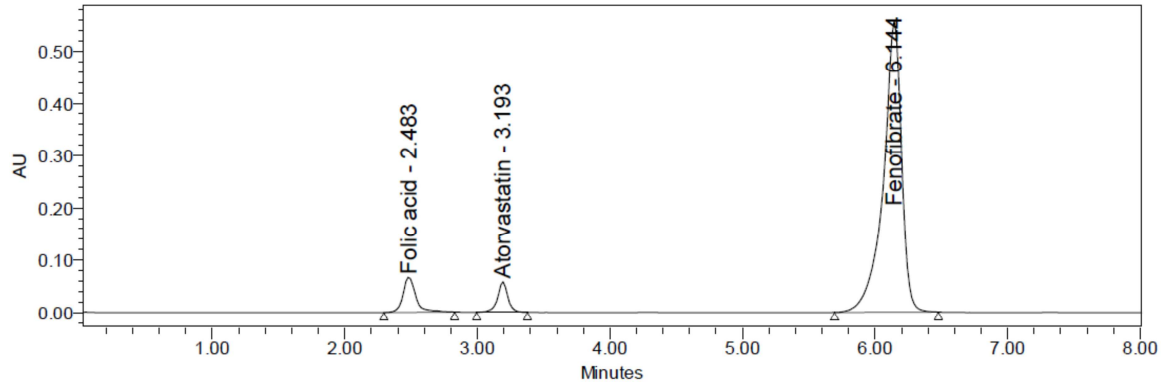


Figure 18: Typical Chromatogram of mobile phase composition plus

System suitability:

The system suitability was determined by making six replicate injections from freshly prepared standard solutions. The observed RSD values were well within usually accepted limits (≤2%). Theoretical plates, tailing factor and resolution of Folic acid, Atorvastatin and Fenofibrate were determined and found to be well within the acceptable limits. The results summarized in **Table 7**.

Table-7: System suitability results

System suitability parameters	Result			Acceptance criteria
	FOA	ATN	FNF	
Retention Time	2.49	3.15	5.90	For information
% RSD for area count of six replicate injection of standard	0.33	0.34	0.30	NMT 2.0
Tailing factor	1.1	1.0	0.9	NMT 2.0
Theoretical plates	3480	8666	10749	NLT 2000
Resolution	N/A	3.5	10.5	NLT 2.0

Specificity:

The specificity of the method was performed by injecting blank solution (without any sample) and then a drug solution of 10µl injected into the column, under Optimized chromatographic conditions to demonstrate the separation of three molecules Folic acid, Atorvastatin and Fenofibrate from any of the impurities, if present. As there was no interference of impurities and also no change in the retention time, the method was found to be specific. The chromatograms of blank, placebo and sample solution was shown in Figure 19, 20, 21.

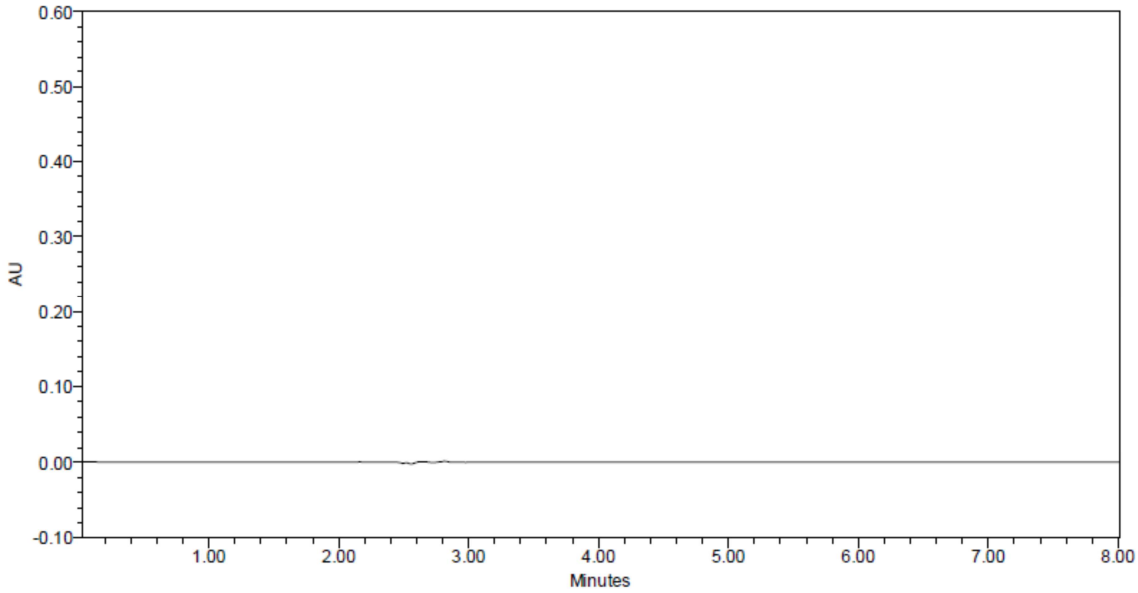


Figure 19: Typical Chromatogram of Placebo

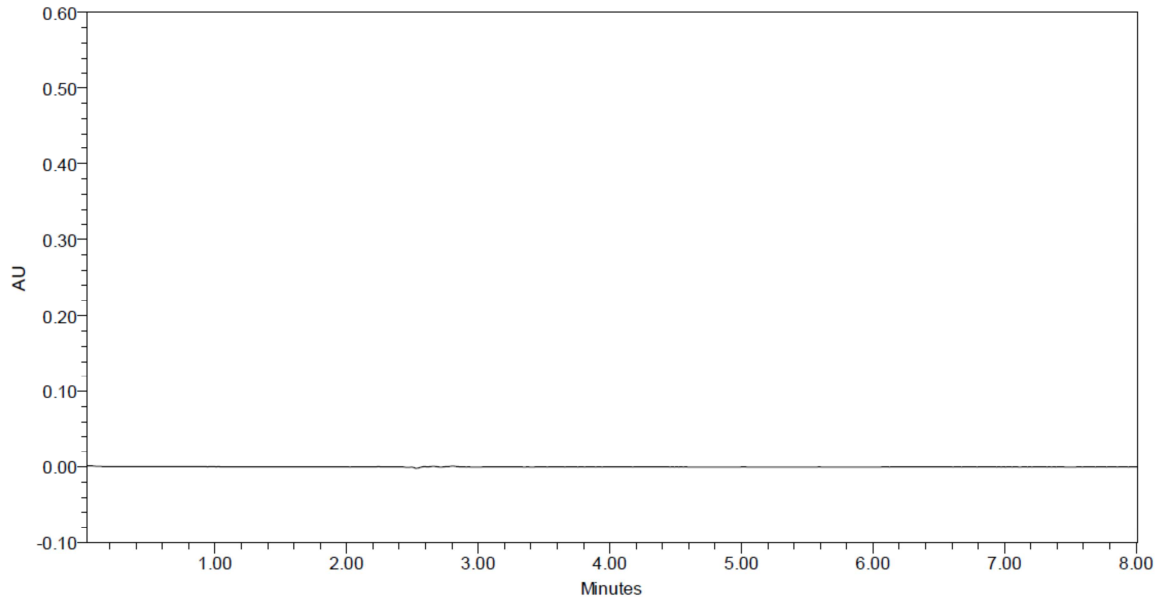


Figure 20: Typical Chromatogram of Blank

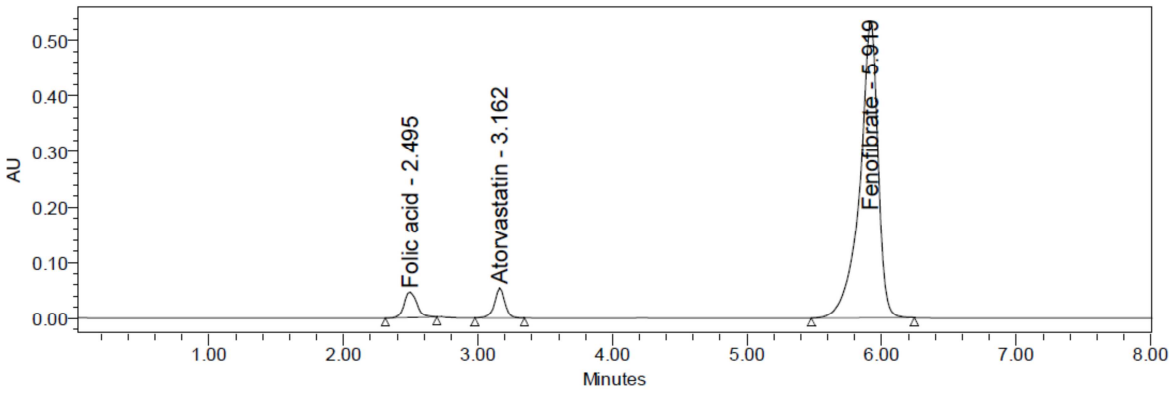


Figure 21: Typical Chromatogram of Sample

Stability of analytical solution:

To find out the stability of the proposed method for the assay of formulation containing Atorvastatin, Fenofibrate and Folic acid was analyzed by the developed method. The stability of the solutions under study was established by keeping the solution at room temperature for 48 hours. The results indicate no significant change in assay values indicating stability of each active analyte in the solvent used during analysis. The results are recorded in **Table 8**.

Table-8: Results for Solution stability

% Assay results calculated against the freshly prepared standard solution			
Sample at Time (hour)	**% Assay results for each active analytes**		
	Atorvastatin	**Fenofibrate**	**Folic acid**
0^{th} hour	100.1	99.8	101.3
24^{th} hour	99.7	99.7	101.0
48 hour	99.9	99.9	99.8
Mean	99.9	99.8	100.7
Standard deviation	0.20	0.10	0.79
(%) Relative standard deviation	0.20	0.10	0.79

Forced Degradation Studies:
Forced degradation studies were performed to demonstrate the optimized method is stability indicating. To prove the method which can be able to measure accurately active pharmaceutical ingredient in presence of degradants which are expected to be formed during different types of degradations applied to the drug sample.

For forced degradation analysis, aliquots of stock were separately treated with 1ml of 2N HCl (Acid stability), 1ml of 2N NaOH (Alkaline stability), 1ml of 20% H2O2 (Oxidative degradation), exposure of sample drug solution at 105ºC for 6 hrs (dry heat degradation), photo stability degradation (exposure of drug at 200 watt hours/m^2) and neutral degradation (refluxing with water at 60ºC for 6 hours. Stability of these samples was compared with fresh sample on the day of analysis. The HPLC chromatograms of degraded products show no interference at the respective peaks and the individual analytes peak purity values found to be within the acceptable limits, hence the method was specific and stability indicating. The chromatograms were shown in figures 22 to 27 and the results were shown in **Table 9** (9A, 9B, 9C). The detailed degradation for each condition is as follows:

Oxidation:
To sample stock solution of Atorvastatin, Fenofibrate and Folic acid, 1 ml of 20% hydrogen peroxide (H_2O_2) was added. The solution was kept for 30 min at 60^0C and cooled to room temperature and finally made up to volume with diluent. For HPLC study, the resultant solution was diluted to obtain 20µg/ml, 320µg/ml and 10µg/ml of all components and 10 µl of sample solution was injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid Degradation Studies:
To sample stock solution of Atorvastatin, Fenofibrate and Folic acid, 1mL of 2N Hydrochloric acid was added and refluxed for 30mins at 60^0C and cooled to room temperature and neutralized with 1 mL of 2N sodium hydroxide solution and finally made up to volume with diluent. For HPLC study, the resultant solution was diluted to obtain 20µg/ml, 320µg/ml and 10µg/ml of all components and 10 µl of sample solution was injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation Studies:
To sample stock solution of Atorvastatin, Fenofibrate and Folic acid, 1mL of 2N sodium hydroxide solution was added and refluxed for 30mins at 60^0C and cooled to room temperature and neutralized with 1 mL of 2N Hydrochloric acid solution and finally made up to volume with diluent. For HPLC study, the resultant solution was diluted to obtain 20µg/ml, 320µg/ml and 10µg/ml of all components and 10 µl of sample solution was injected into the system and the chromatograms were recorded to assess the stability of sample.

Dry Heat Degradation Studies:
The sample stock solution was placed in oven at 105^0C for 6 hours to study dry heat degradation and after dry heat cooled to room temperature. For HPLC study, the resultant solution was diluted to obtain 20µg/ml, 320µg/ml and 10µg/ml of all components and 10 µl of sample solution was injected into the system and the chromatograms were recorded to assess the stability of sample.

Photo Stability studies:
The photochemical stability of the drug was also studied by exposing the sample stock solution to UV Light by keeping the beaker in UV Chamber for 7days or 200 Watt hours/m^2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 20µg/ml, 320µg/ml and 10µg/ml of all components and 10 µl of sample solution was injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral Degradation Studies:
Stress testing under neutral conditions was studied by refluxing the sample stock solution in water for 6hrs at a temperature of 60ºC. For HPLC study, the resultant solution was diluted to obtain 20µg/ml, 320µg/ml and 10µg/ml of all components and 10 µl of sample solution was injected into the system and the chromatograms were recorded to assess the stability of sample.

Table-9 Forced Degradation Studies
Table-9A: Forced Degradation study results for Atorvastatin

S. No.	Degradation condition	%Assay	%Degradation	Purity Angle	Purity Threshold	Purity Flag
1	Controlled sample	100.1	--	0.301	1.348	No
2	Acid Degradation	94.3	5.8	0.644	0.712	No
3	Base Degradation	96.2	3.9	0.656	0.752	No
4	Peroxide Degradation	93.2	6.9	4.097	9.575	No
5	Thermal Degradation	99.6	0.5	0.483	0.744	No
6	UV Degradation	98.2	1.9	1.864	3.642	No
7	Water Degradation	99.4	0.7	0.177	0.351	No

Table-9B: Forced Degradation study results for Fenofibrate

S. No.	Degradation condition	%Assay	%Degradation	Purity Angle	Purity Threshold	Purity Flag
1	Controlled sample	99.8	---	0.110	0.292	No
2	Acid Degradation	95.3	4.5	0.069	0.254	No
3	Base Degradation	96.3	3.5	0.069	0.253	No
4	Peroxide Degradation	97.2	2.6	0.254	5.319	No
5	Thermal Degradation	93.2	6.6	0.116	0.296	No
6	UV Degradation	96.2	3.6	0.061	0.251	No
7	Water Degradation	97.3	2.5	0.093	0.252	No

Table-9C: Forced Degradation study results for folic acid

S. No.	Degradation condition	%Assay	%Degradation	Purity Angle	Purity Threshold	Purity Flag
1	Controlled Sample	101.3	---	0.121	0.395	No
2	Acid Degradation	94.3	7.0	6.399	9.639	No
3	Base Degradation	96.6	4.7	0.759	1.187	No
4	Peroxide Degradation	94.2	7.1	1.607	2.177	No
5	Thermal Degradation	97.3	4.0	5.047	7.363	No
6	UV Degradation	98.4	2.9	7.917	9.144	No
7	Water Degradation	97.6	3.7	2.510	5.532	No

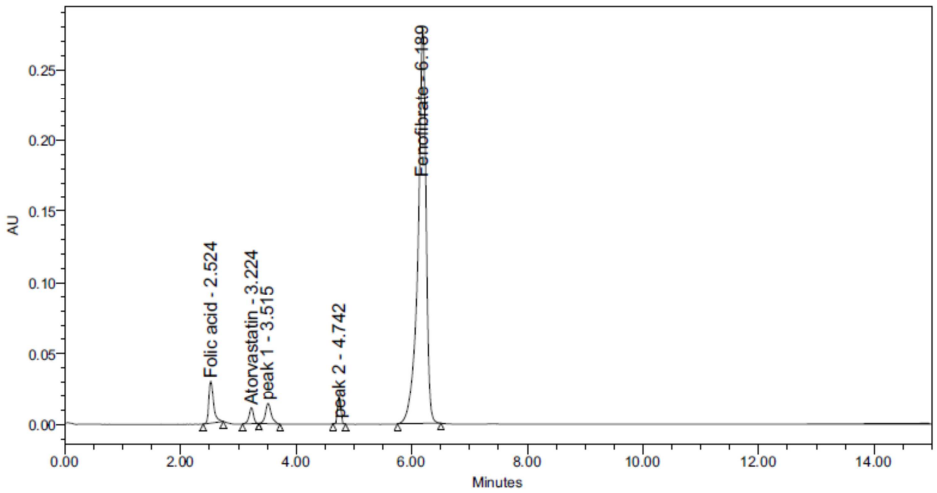


Figure22: Typical chromatogram of Acid Degradation sample

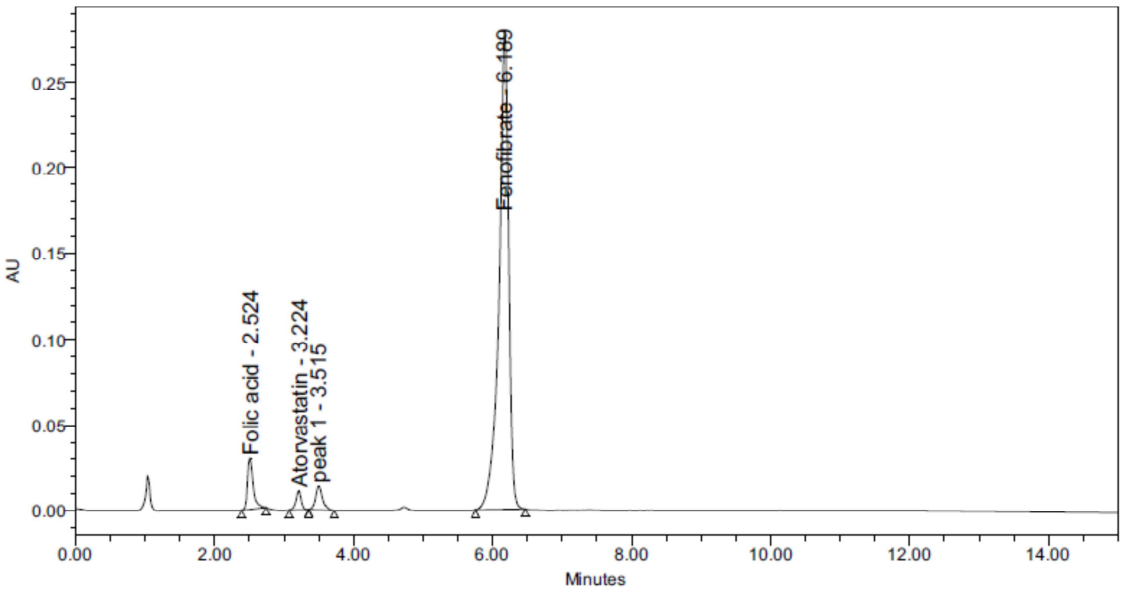


Figure 23: Typical chromatogram of Base Degradation sample

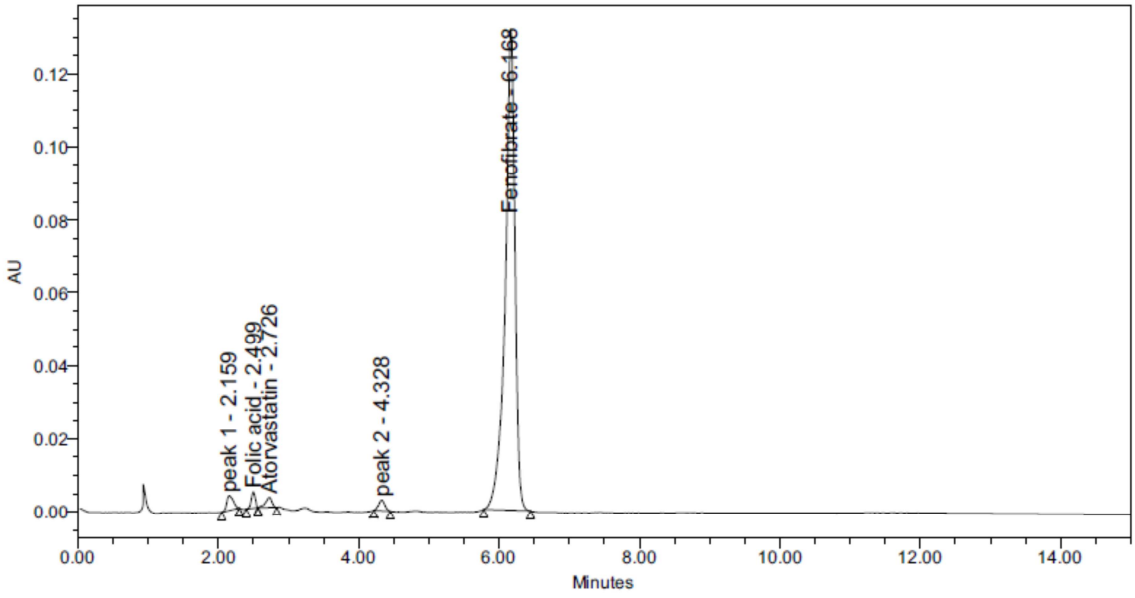


Figure 24: Typical chromatogram of Peroxide Degradation sample

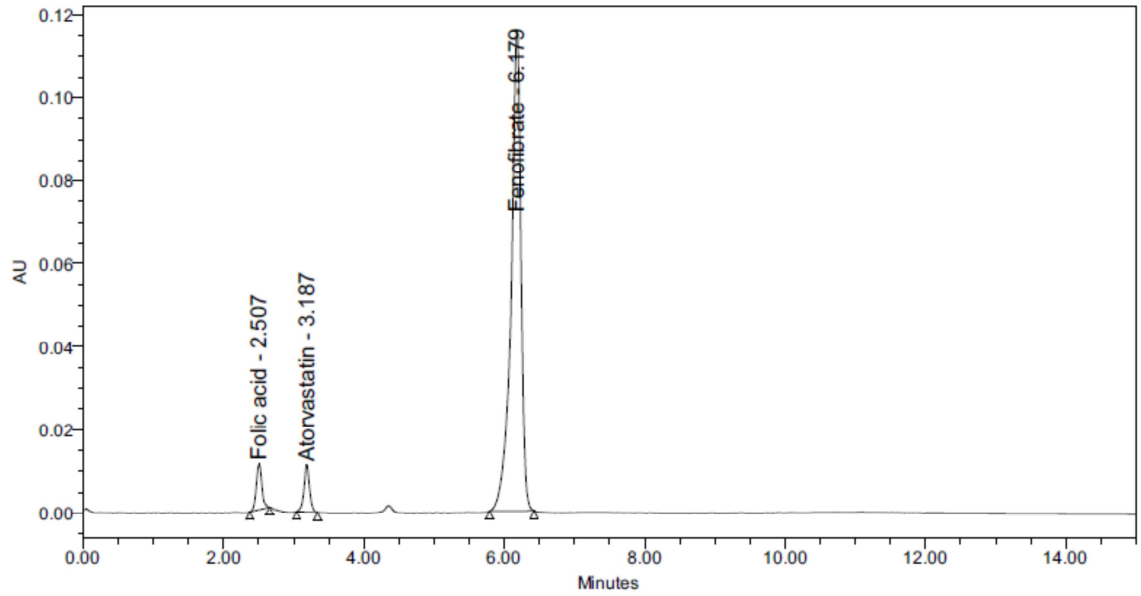


Figure 25: Typical chromatogram of Thermal Degradation sample

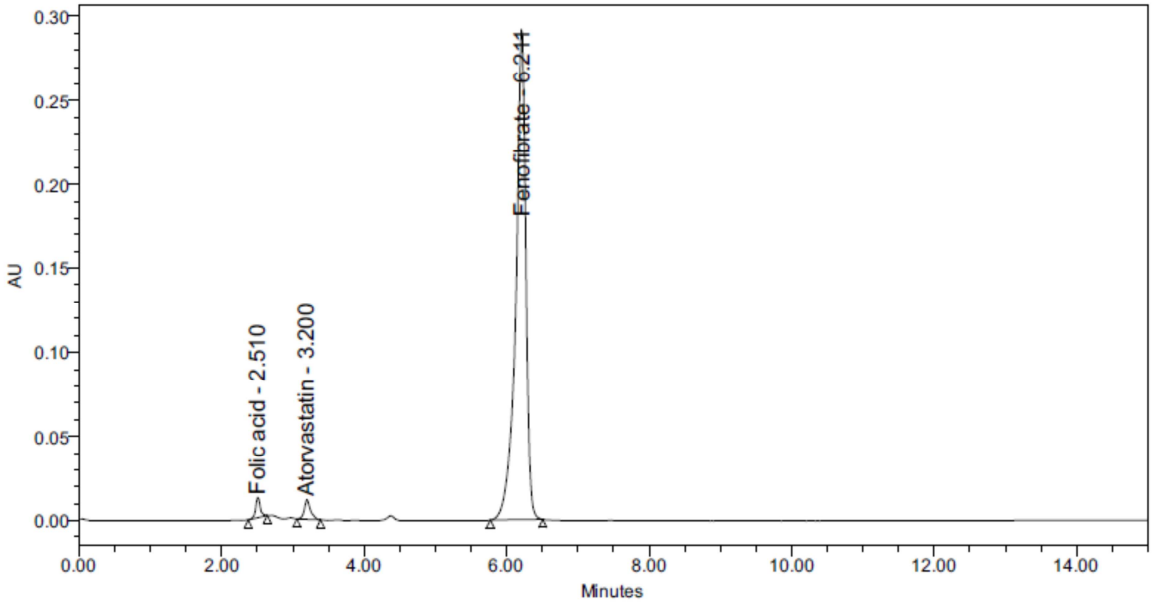


Figure 26: Typical chromatogram of UV Degradation sample

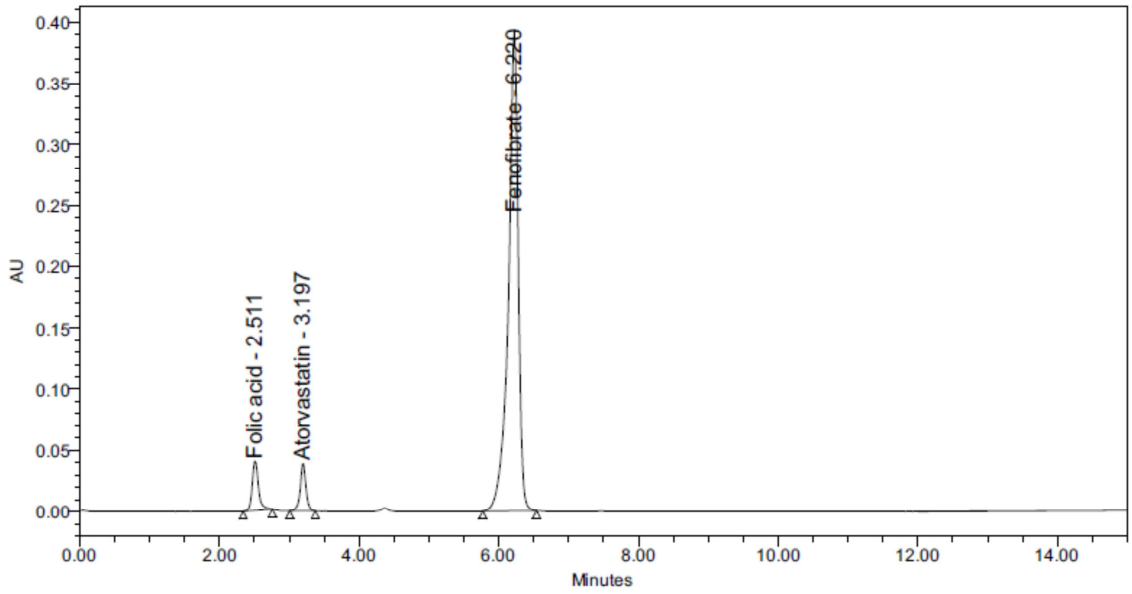


Figure 27: Typical chromatogram of Water degradation sample

RESULTS AND DISCUSSION

The main aim for development of chromatographic method was to get reliable method for quantification of Atorvastatin, Fenofibrate and Folic acid from bulk and pharmaceutical dosage form and which will be applicable for the degradation products also. Different chromatographic conditions were employed for the analysis of the Atorvastatin, Fenofibrate and Folic acid in both bulk and pharmaceutical dosage form. Finally the analysis was performed by using Phosphate Buffer pH 3.30: Acetonitrile in the ratio of 30:70 %v/v at a flow rate 1.0 ml/min. Samples were analyzed at 280 nm at an injection volume of 10 µL and separation was carried by using Agilent XDB-C_{18}, (150 x 4.6 mm, 5µ) column. The retention time and tailing factor were calculated. The retention time of Folic acid, Atorvastatin and Fenofibrate were found to be 2.507, 3.202 and 6.005 respectively. The proposed column was selected which gave a sharp and symmetrical peak with 1.06 tailing factor and theoretical plates of 3480 for Folic acid, 0.98 tailing factor and theoretical plates of 8666 for Atorvastatin and 1.01 tailing factor and theoretical plates of 10749 for Fenofibrate. The resolution between Folic acid and Atorvastatin is 3.5 and the resolution between Atorvastatin and Fenofibrate is 10.5 and found to be within the acceptable limit (not less than 2.0). The calibration curve was linear over the concentration range of 5-30 µg/ml for Atorvastatin, 80-480 µg/ml for Fenofibrate and 2.5-15 µg/ml for Folic acid. Six different concentrations of Atorvastatin, Fenofibrate and Folic acid in the given range were prepared and injected into HPLC. The linearity of the method was statistically confirmed. RSD values for accuracy and precision studies obtained were less than 2.0% which revealed that developed method was accurate and precise. The analytical solution is found to be stable up to 48 hours at room temperature. Hence, the analytical method is validated and can be used for routine analysis and for stability study.

Forced degradation studies concluded that all the degrading peaks obtained during degradation were well resolved from the main drugs i.e. Atorvastatin, Fenofibrate and Folic acid. And the peak purity was passed i.e. purity angle was less than purity threshold as per waters Empower-2 software. Hence the method is found to be stability indicating.

Therefore proposed validated stability indicating method was successfully applied to determine Atorvastatin, Fenofibrate and Folic acid in Bulk and Pharmaceutical dosage form.

CONCLUSION

The developed method is rapid, accurate, simple and selective and proved to be stability indicating for the simultaneous estimation of Atorvastatin, Fenofibrate and Folic acid in Bulk and pharmaceutical dosage form. The sample preparation is simple, the analysis time is short and the elution is by isocratic method. To our present

knowledge, no attempts have yet been made to estimate this multidrug mixture by stability indicating analytical procedure. All the active ingredients were profitably resolved with good resolution and quantified. The retention time of Folic acid, Atorvastatin and Fenofibrate were found to be 2.507, 3.202 and 6.005 minutes respectively. The validation parameters like system suitability, linearity, accuracy, robustness, specificity and solution stability, limit of detection and limit of quantization were found to be within the acceptance limits. The excipients of the commercial sample analyzed did not interfere in the analysis, which proved the specificity of the method for these drugs. Forced degradation studies of different conditions shows that all the degradants were well resolved from these main drug peaks and able to quantify the Atorvastatin, Fenofibrate and Folic acid in presence of degradants, diluent and excipients which proved that the method is found to be stability indicating. Hence the proposed method can be conveniently adopted for the routine quality control analysis in the bulk and combined formulations.

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