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RESEARCH ARTICLE

METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF ATORVASTATIN, FENOFIBRATE AND FOLIC ACID IN PHARMACEUTICAL DOSAGE FORM

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Abstract:
A simple, rapid, accurate and precise isocratic reverse phase high performance liquid chromatography (RP-HPLC) method has been developed for simultaneous estimation of atorvastatin, fenofibrate and folic acid in combined dosage form by using C_{18} column. Mobile phase consisting of a 40 volumes of mixed phosphate buffer (pH 6.0) : 60 volumes of acetonitrile and detection was carried out at 235 nm and the retention times of atorvastatin, fenofibrate and folic acid were found to be 4.407, 3.393and 7.450 min respectively. The developed method was validated as per ICH guideline for specificity, linearity, accuracy, and precision and system suitability. The new RP-HPLC method was successfully applied to marketed formulation without any interference from excipients.

Keywords: RP-HPLC, Atorvastatin, Fenofibrate and Folic acid, Validation.

Introduction

Atorvastatin is a Hydroxymethylglutaryl-CoA Reductase Inhibitors. As HMG-CoA reductase is responsible for converting HMG-CoA to mevalonate in the cholesterol biosynthesis pathway. IUPACname7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-(propan-2-yl)-1H-pyrrol-1-yl]-3,5 dihydroxyheptanoate (Fig.1). Its chemical formulais $C_{33}H_{34}FN_2O_5$with a molecular mass of 557.6319 g/mol[1].

Fenofibrate is a Hypolipidemic Agents Fenofibrate exerts its mechanism of action by activation of peroxisome proliferator activated receptor a (PPARa). IUPAC name propan-2-yl 2-{4-[(4-chlorophenyl) carbonyl] phenoxy}-2-methylpropanoate (Fig.2). Its chemical formula is$C_{20}H_{21}ClO_4$and molecular mass of 360.831[2].

Folic 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. IUPAC name (2S)-2-[(4-{[(2-amino-4-oxo-1,4-dihydropteridin-6-yl)phenyl)formamido]pentanedioic acid.(Fig.3). Itschemical formula is $C_{19}H_{19}N_7O_6$with a molecular mass of441.3975 g/mol[3].

Literature survey revealed some analytical methods for the quantitative determination of atorvastatin in combination with other drugs by RP- HPLC [4], UV Spectrophotometric method [5].Similarly there are few analytical methods[5-9]for estimation of fenofibrate and folic acid[10]in combination with other drugs. An RP-HPLC method [11] is also found for the estimation of

atorvastatin and fenofibrate but the aim of the present work is to develop a simple cost effective RP-HPLC method for simultaneous estimation of atorvastatin, fenofibrate and folic acid in bulk and pharmaceutical dosage form.

METHOD DEVELOPMENT:

Instrument:

Separation and quantitation was carried out by Shimadzu LC 20 AT VP HPLC on Inertsil ODS 3V (250x4.6mm) column. Samples were injected by using Hamilton syringe in to 20 µl fixed volume loop and chromatograms were integrated by Spinchrome software.

Chemicals and reagent:

Acetonitrile (HPLC grade), potassium di hydrogen phosphate, di potassium hydrogen phosphate and ortho phosphoric acid used were of AR grade purchased from Merck, Mumbai. Fenofibrate, atorvastatin and folic acid pure drugs obtained as gift samples from Aurobindo Pharmaceutical Ltd., Marketed product AFF (10 mg of atorvastatin+160mg fenofibrate + 5 mg of folic acid) was procured from local pharmacy.

Preparation of mobile phase:

Preparation of mixed phosphate buffer:

1.625 gm. of potassium di hydrogen phosphate (KH_2PO_4) and 0.3 gm of di potassium hydrogen phosphate (KH_2PO_4) was weighed and dissolved in 100ml of water and volume was made up to 1000ml with water. Adjust the pH to 6.0 using ortho phosphoric acid. The buffer was filtered through 0.45µ filters to remove all fine particles and gases.

A mixture of 40 volumes of Mixed Phosphate buffer pH 6.0: 60 volumes of acetonitrile was prepared. The mobile phase was sonicated for 10 min to remove gases.

Preparation of standard stock solution:

25mg of **atorvastatin, fenofibrate, folic acid** was weighed separately and transferred into separate 250ml volumetric flask and dissolved in methanol and then diluted up to the mark with methanol and prepared 10 µg /ml of solution by diluting 1ml to 10ml with methanol.

Selection of detection wavelength:

The wavelength of maximum absorption (λ_{max}) of the drug, 10 µg/ml solution of the drugs in methanol were scanned using UV-Visible spectrophotometer within the wavelength region of 200–400 nm against methanol as blank. The over laid spectrum of both the drug is given in Fig.4

Optimized chromatographic conditions:

Mobile phase : KH_2PO_4+K_2HPO_4: ACN
Ratio : 40:60v/v
Column : Inertsil ODS C_{18} (250×4.6mm× 5µ)
Wavelength : 235 nm
Flow rate : 1ml/min
pH : 6.0±0.05

METHOD VALIDATION:

The method was validated according to ICH guidelines with respect to linearity, specificity, precision, accuracy, system suitability.

System suitability studies:

For system suitability, six replicates of standard solutions of atorvastatin, fenofibrate and folic acid were injected and studied the suitability parameters like Plate number (N), Resolution (R), Tailing, and %RSD were studied with the help of standard chromatograms. The values obtained demonstrated the suitability of the system for the analysis of this drug combinations, system suitability parameters may fall below 2% standard deviation range during routine performance of the method. System suitability parameters were presented in table 1.

Linearity:

The linearity of the method was determined at five concentration levels. From each solution 10 µl is injected into the optimized chromatographic system. The calibration curves are constructed by plotting area against concentration of drugs (Fig.6, 7and 8). The slope and intercept value of calibration curves were y = 33.18x-16.57 for atorvastatin y=y=20.74x-165.7for fenofibrate and y=89.38x-0.031 for folic acid. Correlation coefficient was 0.996, 0.996 and 0.995 respectively. The linearity data for the drugs is presented in table 2.

Precision:

For precision the sample solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits and results are tabulated in table 3.

Ruggedness:

The ruggedness of the method was studied by the determining the analyst to analyst variation by performing the Assay by two different analysts. The results were tabulated in table 4.

Accuracy:

Accuracy of the method was determined by Recovery studies. The recovery studies were carried out by standard addition method at three different levels (80%, 100%, and 120%). The percentage recoveries were calculated and presented in the table 5,6and7. The percentage mean recoveries of fenofibrate, atorvastatin and folic acid were 99.47%, 99.16% and 99.78% respectively.

LOD and LOQ:
Limit of quantification and detection were predicted by plotting linearity curve for different nominal concentrations of fenofibrate, atorvastatin and folic acid. Relative standard deviation (σ) method was applied, the LOQ and LOD values were predicted using following formulas (a) and (b).

(a) LOQ = 10 σ / S
(b) LOD = 3.3 σ / S
Where σ = residual standard deviation of response
S = slope of the calibration curve

The LOD for this method was found to be 6.11µg/ml for fenofibrate and 0.31µg/ml and for atorvastatin, 0.06µg/ml for folic acid.

The LOQ for this method was found to be 18.51 µg/ml for fenofibrate , 0.95 µg/ml for atorvastatin, and 0.18µg/ml for folic acid.

Specificity:
Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. The chromatogram was taken by appropriate dilutions and the amount of each drug present in the sample mixture was determined and it was found that there is no interference with the analyte peak Fig.9 and 10.

Preparation of samples for Assay:

Preparation of Standard solution
Mixed standard stock solution (microgram/ml) was prepared by dissolving 160 mg of fenofibrate, 5 mg of folic acid and 10 mg of atorvastatin in sufficient mobile phase and diluted to 50 ml with mobile phase (stock). After that the solution was filtered by using 0.45-micron syringe filter and sonicated for 5 min. Further dilutions was made by adding 1 ml of stock solution to 10 ml of mobile phase to get 160 µg/mL of fenofibrate , 5 µg/mL of folic acid and 10µg/mL of atorvastatin. Standard solution was injected into the column and areas of each drug was recorded to calculate the amount of drug in the tablet.

Preparation of Sample solution

20 Tablets (each tablet contains fenofibrate-160 mg, atorvastatin-10 mg and folic acid-5 mg) were weighed and taken into a mortar crushed and uniformly mixed. Test stock solutions of 160µg/mL of fenofibrate, 5µg/mL of folic acid and 10 µg/mL of atorvastatin were prepared by dissolving required tablet powder in sufficient mobile phase. After that the solution was filtered using 0.45-micron syringe filter and sonicated for 5 min and diluted to 100ml with mobile phase. Further dilutions are prepared in 5 replicates of 160µg/mL of fenofibrate, 5µg/mL of folic acid and 10 µg/mL of atorvastatin was made by adding 1 ml of stock solution to 10 ml of mobile phase. Sample solution was injected into the optimized chromatographic system (n=5) and average area of each drug was recorded and substituted in the formula.

Calculation

The amount of the fenofibrate, folic acid and atorvastatin present in the formulation by using the formula given below, and results shown in table 7.

Where,

AS: Average peak area due to standard preparation
DT: Dilution of assay preparation
AT: Peak area due to assay preparation
WT: Weight of sample in assay preparation
WS: Weight of fenofibrate, folic acid and atorvastatinin mg

RESULTS AND DISCUSSION:
The method was developed by trial and error method. Several columns and mobile phases were tried but finally IntelsilC18 column was selected .Mobile phase consisting of a 40 volumes of mixed phosphate buffer of pH 6.0: 60 volumes of acetonitrile was found to be most suitable for separation of these drugs. System suitability parameters like retention time, resolution, tailing and plate count were shown uniformity and %RSD was less than 2% and the results are given in table 1 and from the obtained results we can say that the system is suitable for analysis. Linearity plot is obtained by taking concentrations on the X- axis and areas on the Y-axis and regression equations were found to be y = 33.18x-16.57 for atorvastatin y=20.74x-165.7 for fenofibrate and y=89.38x-0.031 for folic acid. Calibration curves are shown in Fig.6, 7 and 8.The precision of the proposed method was carried in terms of the repeatability and the %RSD values was found to be 1.06 for atorvastatin, 0.74 for fenofibrate and 0.66 for folic acid which reveal that the proposed method is precise. Precision studies were tabulated in table 3. The recovery values obtained shows that the method is accurate. Low LOD and LOQ values show that the method is sensitive. Method specificity was concluded by Fig.9and10 and those figures are atorvastatin, fenofibrate and folic acid standard chromatogram and other one is sample. It is observed from the above data diluent or excipient peaks are not interfering with the fenofibrate, atorvastatin and folic acid peaks. The assay values obtained are in good agreement with the label claim.

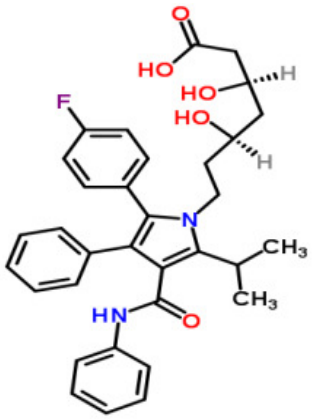

Fig 1: Structure of Atorvastatin

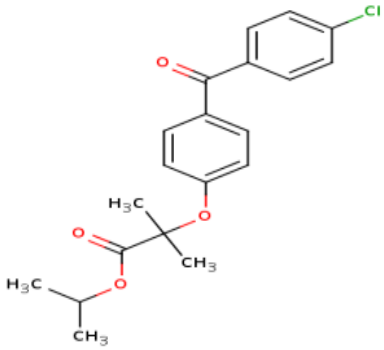

Fig 2: Structure of Fenofibrate

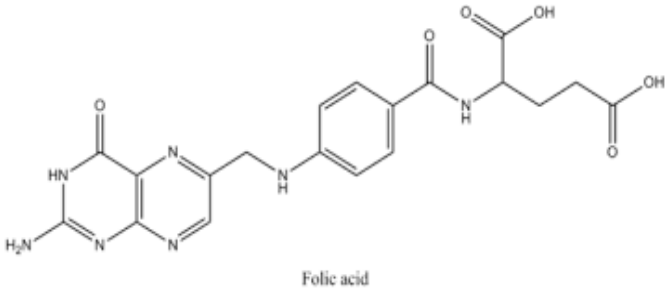

Fig 3: Structure of Folic acid

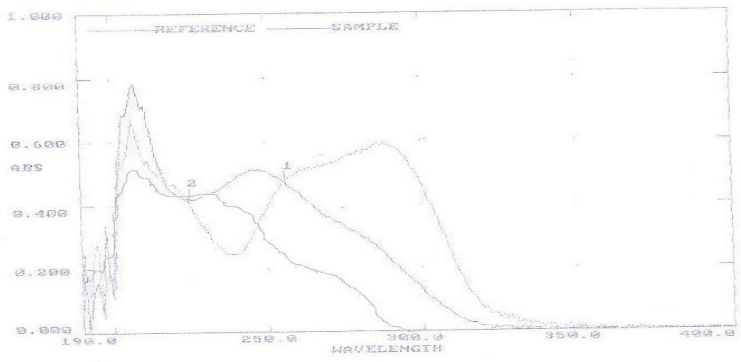

Fig4: Over laid spectrum of atorvastatin, fenofibrate and folic acid

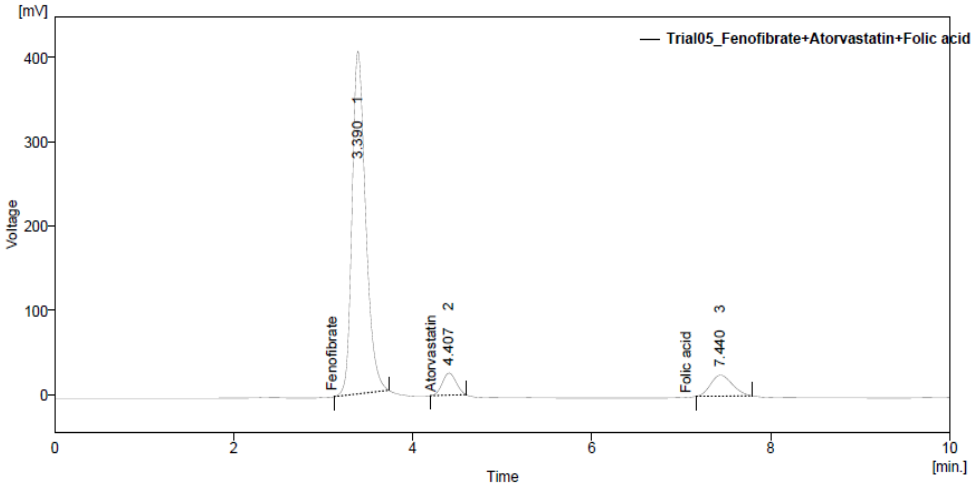

Fig 5: Typical Chromatogram of fenofibrate, folic acid and atorvastatin

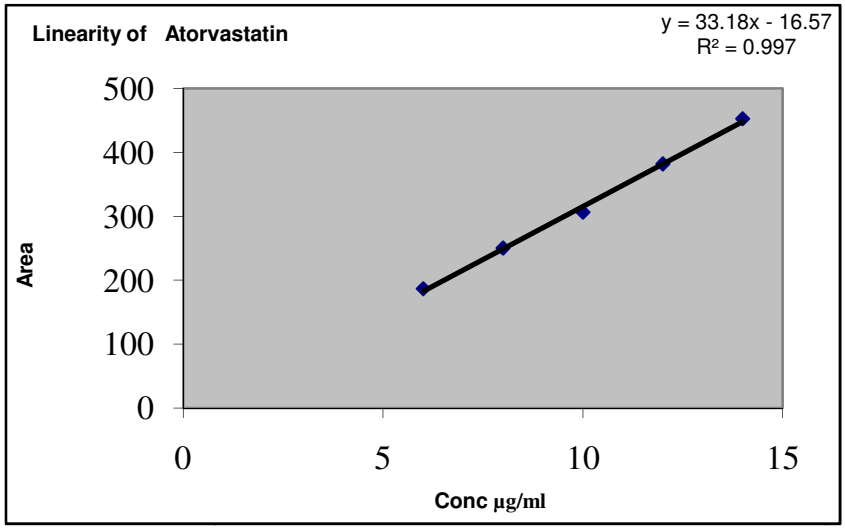

Fig.6: Linearity curve for atorvastatin

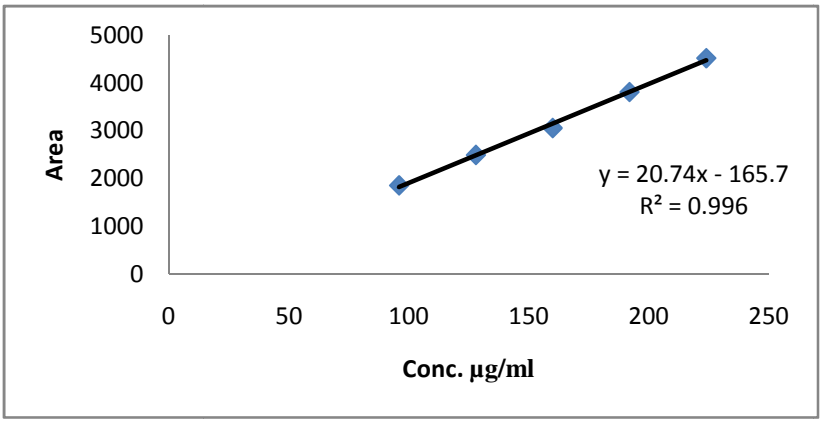

Fig. 7: Linearity curve for fenofibrate

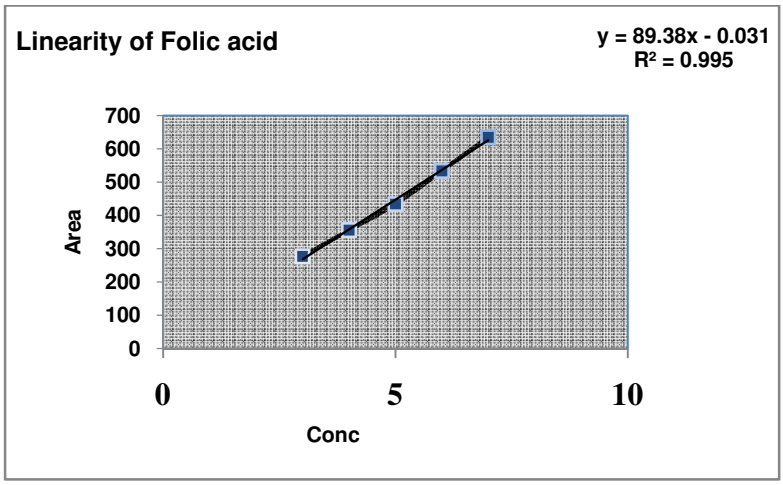

Fig. 8: Linearity curve for folic acid

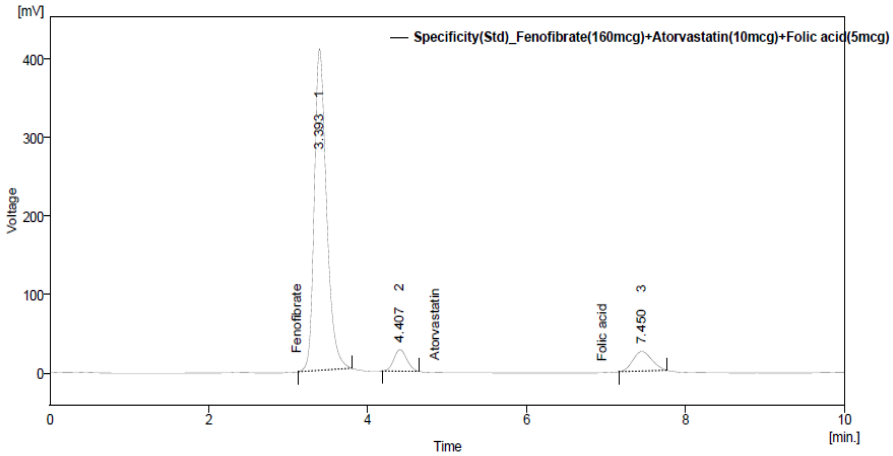

Fig 9: Standard chromatogram for atorvastatin , fenofibrate and folic acid

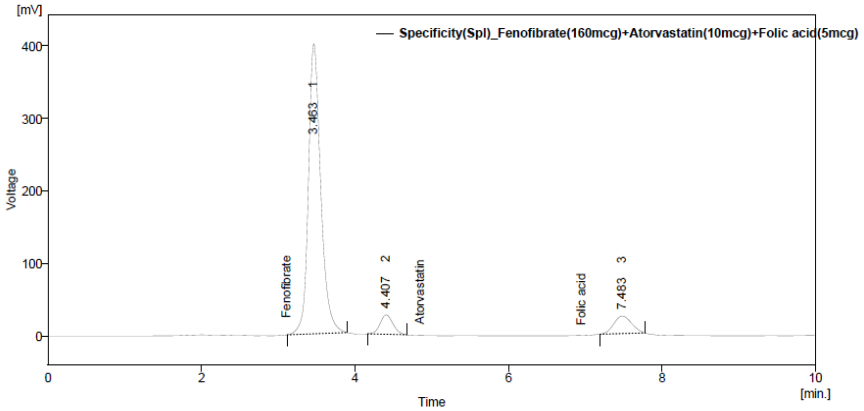

Fig 10: Sample chromatogram for atorvastatin , fenofibrate and folic acid

Table 1: System suitability parameters of the proposed method

S.No	Parameter	Fenofibrate	Atorvastatin	Folic acid
1	Retention time*	3.4018	4.407	7.450
2	Tailing Factor*	1.34	1.26	1.41
3	Peak area*	4401.823	323.910	434.524
4	Theoretical plates*	8897	4678	3982
5.	Resolution*	….	3.425	8.021
6.	%RSD of area under peak*			

Average of six determinations

Table 2: Linearity data of the proposed method

S.NO						
	Fenofibrate	**Atorvastatin**	**Folic acid**	**Fenofibrate**	**Atorvastatin**	**Folic acid**
1	96	6	3	1863.27	186.327	276.682
2	128	8	4	2499.69	249.969	355.477
3	160	10	5	3062.79	306.279	434.004
4	192	12	6	3815.98	381.598	533.759
5	224	14	7	4524.01	452.401	634.446
	Correlation coefficient			0.9974	0.9964	0.9959

Table3:Results for Method precision of Fenofibrate,Atorvastatinand Folic acid

S.NO	**Retention time (min)**			**Area**		
	3.393	4.407	7.450	4399.321	321.017	435.059
2	3.397	4.400	7.457	4411.932	327.285	431.134
3	3.407	4.403	7.450	4401.157	324.678	432.436
4	3.410	4.397	7.437	4446.486	321.628	437.173
5	3.407	4.403	7.450	4406.215	320.385	438.475
6	3.397	4.400	7.457	4345.829	328.465	432.844
Avg.	3.4018	4.402	7.450	4401.823	323.910	434.524
Std.dev	0.0070	0.003	0.007	32.429	342.7	2.888
%RSD	**0.21**	**0.08**	**0.10**	**0.74**	**1.06**	**0.66**

Table 4:Results for Ruggedness

Fenofibrate	**%Assay**	**Atorvastatin**	**%Assay**	**Folic acid**	**%Assay**
Analyst 01	97.73	Analyst 01	98.71	Analyst 01	97.66
Analyst 02	99.76	Analyst 02	100.83	Analyst 02	96.22
%RSD	1.45	%RSD	1.50	%RSD	1.05

Table5: Accuracy of Fenofibrate

Recovery level	Accuracy Fenofibrate					Average % Recovery
	Amount taken(mcg/ml)	Area	Average area	Amount recovered(mcg/ml)	%Recovery	
80 %	160	4539.822	4497.817	157.63	98.52	99.47%
	160	4532.307				
	160	4421.321				
100 %	192	5202.156	5296.465	194.59	101.35	
	192	5260.411				
	192	5426.827				
120 %	224	6249.806	6167.095	220.75	98.55	
	224	6024.208				
	224	6227.270				

Table 6: Accuracy of Atorvastatin

Recovery level	Accuracy Atorvastatin					Average % Recovery
	Amount taken(mcg/ml)	Area	Average area	Amount recovered(mcg/ml)	%Recovery	
80 %	10	310.213	308.539	9.87	98.74	99.16%
	10	311.768				
	10	303.636				
100 %	12	365.486	368.172	12.02	100.17	
	12	379.319				
	12	359.712				
120 %	14	443.330	438.857	13.80	98.58	
	14	437.930				
	14	435.311				

Table 7: Accuracy of Folic acid

Recovery level	Accuracy Folic acid					Average % Recovery
	Amount taken(mcg/ml)	Area	Average area	Amount recovered(mcg/ml)	%Recovery	
80 %	5	439.228	436.6127	4.9130	98.260	
	5	436.620				
	5	433.990				
100 %	6	522.234	530.08	6.106856	101.78	99.78%

	6	**550.046**				
	6	**517.960**				
120%	7	**624.907**	**618.418**	**6.951654**	**99.30935**	
	7	**604.657**				
	7	**625.690**				

Table 7: Assay results

Drug	Label claim (mg)	Amount obtained(mg)	% Recovery
Fenofibrate	160	159.98	99.98
Folic acid	5	5.01	100.2
Atorvastatin	10	9.99	99.90

CONCLUSION:

The proposed RP-HPLC method is found to be simple, precise, accurate and sensitive for the simultaneous estimation of atorvastatin, fenofibrate and folic acid in pharmaceutical dosage forms. Hence, this method can easily and conveniently adopt for routine quality control analysis ofatorvastatin, fenofibrate and folic acid and in its pharmaceutical dosage form.

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