Development and validation of RP-HPLC method for Estimation of Rosuvastatin and Ezetimibe in bulk and pharmaceutical dosage form

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Abstract- A precise and robust method was developed method for the estimation of Rosuvastatin and Ezetimibe in bulk and pharmaceutical dosage form. The Method used Agilent 1260 Infinity II model HPLC with DAD detector and Agilent Poroshell EC-120 C18 column (150 mm × 4.6 mm, 4 µm). The Mobile phase combination used was 0. .1% Trifluoroacetic acid, Methanol and Acetonitrile [30:40:30]. Flow rate at 1 ml/min and wavelength at 232 nm with run time of 6 minutes. The retention time of Rosuvastatin (RSV) and Ezetimibe (EZB) peaks was at 2.34 and 2.99 minutes, respectively. The developed method was validated according to ICH Q2 (R1) guidelines. The instrument precision for RSV & EZB had a %RSD of 0.16% and 0.12%, respectively. The Intra & Inter day precision for RSV & EZB had a %RSD of 0.50% and 0.55%, respectively. Method was linear and accurate for concentration range of 80-120 µg/ml and 40-60 µg/ml for RSV & EZB respectively, with regression coefficient of 0.999 for both RSV & EZB and % RSD for accuracy for RSV at 80%, 100% and 120% was found to be 0.18%, 0.16% and 0.07%, respectively; and for EZB at 80%, 100% and 120% was found to be 0.18%, 0.08% and 0.12% respectively. The LOD & LOQ for RSV are 0.35 µg/ml and 1.06 µg/ml respectively and the LOD & LOQ for EZB are 0.10 µg/ml and 0.29 µg/ml respectively.

Keywords: Rosuvastatin, Ezetimibe, RP-HPLC, Robustness, %RSD, Precision, LOD, LOQ, Accuracy, linear.

1. INTRODUCTION

A combination of rosuvastatin and ezetimibe, in conjunction with a healthy diet, is utilized in order to reduce levels of LDL cholesterol (bad cholesterol) in the blood. Additionally, it is utilized either on its own or in conjunction with other medications for the treatment of homozygous familial hypercholesterolemia (HoFH). A statin, also known as an HMG-CoA reductase inhibitor, is rosuvastatin, and ezetimibe is a cholesterol absorption inhibitor on the other hand.

One of the strongest HMG-CoA reductase inhibitors, rosuvastatin can decrease LDL-C by 55% [1]. Other positive effects on the cholesterol panel include a 6% increase in HDL-C, a 15% or larger decrease in TG, and a decrease in atherosclerotic plaque cholesterol [2]. Rosuvastatin has antioxidant, endothelial, and anti-inflammatory properties [3,4]. Rosuvastatin's hydrophilicity reduces myopathy and rhabdomyolysis and allows it to be taken at any time of day [5]. Few drug–drug interactions occur because only 10% of the drug is converted by Cytochrome P450 enzymes and 90% is eliminated by biliary mechanisms. Ezetimibe, the only medication in its class, inhibits NPC1L1, reducing cholesterol absorption by up to 67% and LDL-C by 15–20% [6]. HDL-C rises 3% without affecting TG. Ezetimibe plus statin cut high-sensitivity CRP 10% more than statin monotherapy and reduce inflammation [7]. Ezetimibe has few medication interactions like rosuvastatin because it is glucuronidated [8].

Commercially available rosuvastatin/ezetimibe combinations are 10/10 mg, 20/10 mg, and 40/10 mg [9]. The lipid panel can be changed with a lower dose of each drug due to their complementing processes. When statins lower lipids by reducing liver cholesterol synthesis, the body increases cholesterol absorption, which can reduce statin efficacy. Ezetimibe blocks cholesterol absorption, enhancing statins' LDL-C-lowering effects.

The IUPAC name of Rosuvastatin is (E,3R,5)-7-[4-(4-fluorophenyl)-2-[methyl(methylsulfonyl)amino]-6-propan-2-ylpyrimidin-5-yl]-3,5-dihydroxyhept-6-enoic acid [10].

The IUPAC name of Ezetimibe is (3R,4S)-1-(4-fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)azetidin-2-one [11].

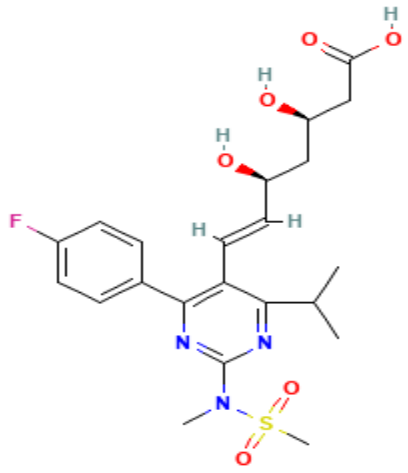


Figure 1: Structure of Rosuvastatin [10]

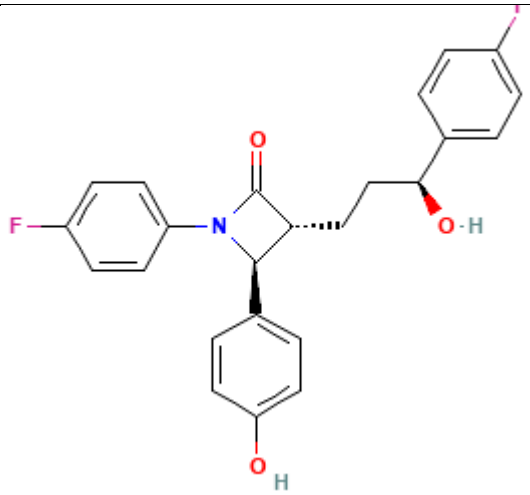


Figure 2: Structure of Ezetimibe [11]

According to the literature review [12-27], there was few Liquid Chromatography analysis for Simultaneous estimation of CEF & AVB in Combination pharmaceutical dosage form. So, current study was planned for development and validation of method developed for Rosuvastatin & Ezetimibe.

Table 1: Quality Target Profile for HPLC Method development

Parameter	Limits
Theoretical Plates	Not less than 2000
Asymmetry	Not More than 2.0 (Fairly at 1.0)
Tailing Factor	Not More than 2.0 (Fairly at 1.0)
Run time	Not More than 20 minutes
Resolution	Not Less than 2.0

2. MATERIAL AND METHOD

2.1. Chemicals and Reagents

A complimentary samples of Rosuvastatin and Ezetimibe were made available by Aadhaar Life Sciences Pvt. Ltd. In India, Qualigens was the supplier of the HPLC-grade acetonitrile and Methaol that was purchased. A grade of Trifluoroacetic acid that was of AR quality was acquired from Merck in India. A water supply was provided via the internal Milli-Q system. All of the weighing was carried out on NABL scales that had been calibrated. The production of the samples was carried out with the use of the analytical balance and Type A glassware.

2.2. Instrumentation

The instrument used for development and validation was an Agilent 1260 Infinity II equipped with a quaternary pump and DAD detector. Software from Agilent called Openlab Ezchrom was used. Wet chemistry was conducted using the Labman ultrasonicator and the Aczet analytical balance.

2.3. HPLC Method Development

2.3.1. The table 2 and 3 describes trials done during the development phase with the results and observations.

Table 2: Method Development for Rosuvastatin & Ezetimibe HPLC

Trial No.	Mobile Phase	Ratio	Diluent	Column	Wavelength
1	0.1% Trifluoroacetic Acid : Methanol	50-50	0.1% Trifluoroacetic Acid : Methanol (50:50)	Agilent Poroshell EC-120 C18 (150 x 4.6 mm, 4µ)	250
2	0.1% Trifluoroacetic Acid : Methanol Acetonitrile	40-50-10	0.1% Trifluoroacetic Acid : Methanol (50:50)	Agilent Poroshell EC-120 C18 (150 x 4.6 mm, 4µ)	232
3	0.1% Trifluoroacetic Acid : Methanol Acetonitrile	30-40-30	0.1% Trifluoroacetic Acid : Methanol (50:50)	Agilent Poroshell EC-120 C18 (150 x 4.6 mm, 4µ)	232

Table 3: Method development results of Rosuvastatin and Ezetimibe

Trial No.	Rosuvastatin				Ezetimibe			
	RT	TP	Asymmetry	Resolution	RT	TP	Asymmetry	Resolution
1.	No peak observed							
2.	4.13	10321	1.69	0.00	7.59	11988	1.25	15.76
3.	2.34	8352	1.36	0.00	3.00	10278	1.26	5.98

Following all of the aforementioned tests, it was discovered that the peak of highest absorption occurred at a wavelength of 232 nm. The diluent was maintained at a constant ratio of 50-50 0.1% Trifluoroacetic acid to Methanol throughout all of the trials. Throughout all of the tests, the Agilent Poroshell EC-120 C18 (150 x 4.6 mm, 4 micron) was consistently utilized. In accordance with the quality target profile that had been established in advance for the development work, the conditions for trial 3 were finalized, and individual Standard was executed in order to validate the retention times. As can be seen in figure 3, the chromatograms of the method development were displayed.

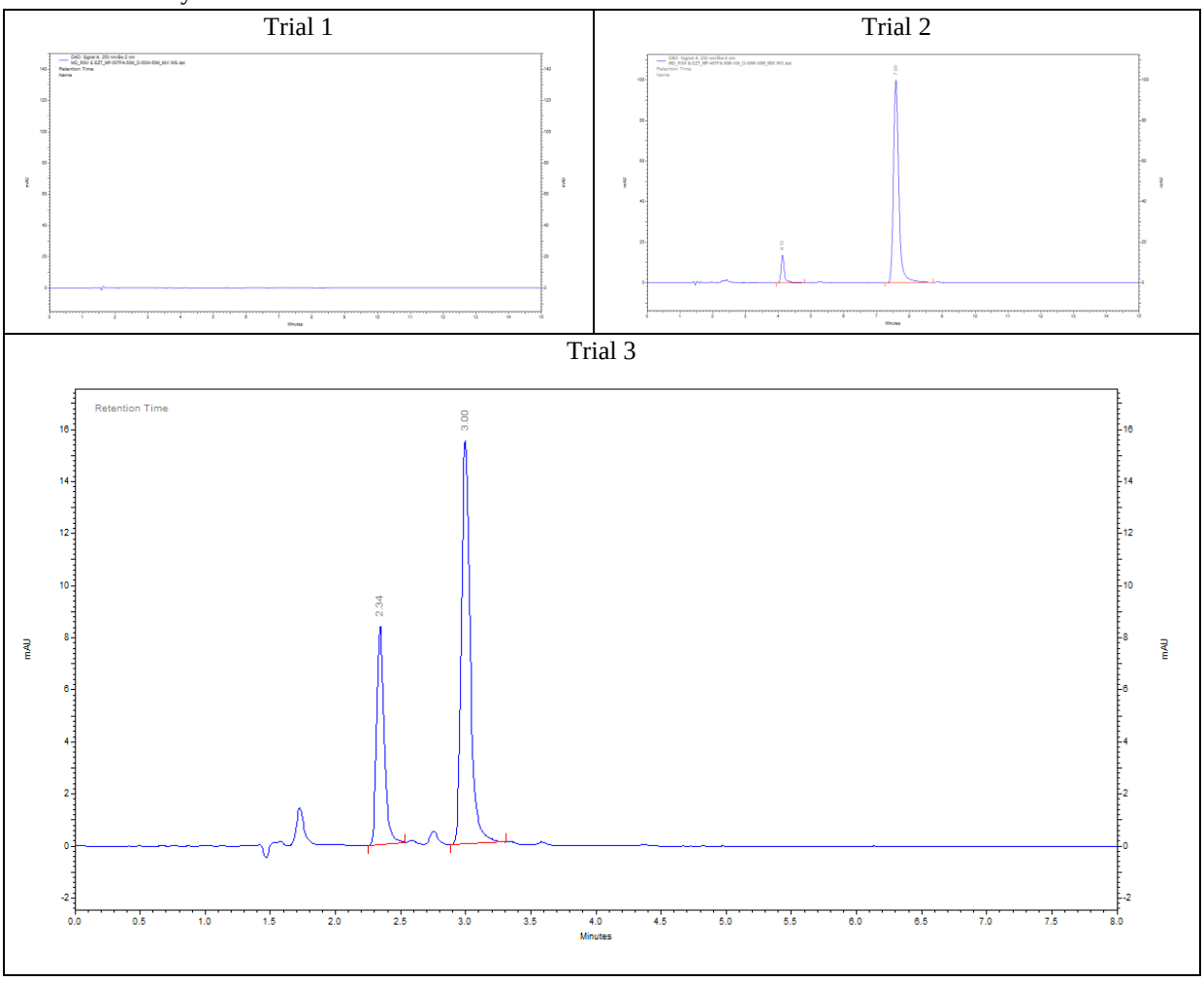


Figure 3: Method Development Trials

2.3.2. Final Chromatographic Conditions:

Table 4: Final Chromatographic Condition

Parameter	Condition
HPLC Instrument	Agilent 1260 Infinity II
Column	Agilent Poroshell EC-120 C18 (150 mm x 4.60 mm,4µm)
Wavelength	232 nm
Mobile Phase	Mobile Phase A –0.1% Trifluoroacetic acid: 30% Mobile Phase B – Methanol: 40% Mobile Phase C – Acetonitrile : 30%
Diluent	0.1% Trifluoroacetic acid : Methanol (50:50) v/v
Run time	6 minutes
Injection Volume	10 micro liters
Flow Rate	1.0 ml/min
Column oven Temperature	30°C (± 2°C allowed by Robustness)

2.3.3. Preparation of Mobile Phase

Preparation of 0.1% Trifluoroacetic acid

Take 800 ml of water using graduated cylinder. Pipette out 1 ml of Trifluoroacetic acid and add this to measured water, mix well then adjust the volume to 1000 ml using water .

Mobile Phase: 30%- 0.1% Trifluoroacetic acid: 40% Methanol: 30% Acetonitrile

Mix separately measured 300 ml of 0.1% Trifluoroacetic acid and 400 ml of Methanol and 300 ml of Acetonitrile into a suitable container. Filter the mobile phase through 0.45 µm nylon membrane filter. Briefly sonicate to degas.

2.3.4. Preparation of Diluent

Mix separately measured 500 ml of Trifluoroacetic acid and 500 ml of Methanol into a suitable container and mix well. Mixture is to be filtered through 0.45 µm nylon membrane filter. Briefly sonicate to degas.

2.3.5. Preparation of Standard Solution

A. Working Standard:

1. Rosuvastatin Standard Stock Solution-I (RSSS-I):
   i. Initially Prepare a Standard Stock Solution (RSSS-I) of by adding10 mg of Rosuvastatin in 10 ml volumetric flask & add 5 ml diluent, mix for 2 minutes and make the volume to 10 ml with diluent. Further dilute 1 ml of above solution to 10 ml with diluent. (Conc. Of Rosuvastatin= 100 µg/ml).
2. Ezetimibe Standard Stock Solution-I(ESSS-I):
   i. Then prepare a Standard Stock Solution (ESSS-I) of Ezetimibe by adding 5mg in 10 ml volumetric flask & add 5 ml diluent, mix for 2 minutes and make the volume to 10 ml with diluent. Further dilute 1 ml of above solution to 10 ml with diluent. (Conc. of Ezetimibe= 50µg/ml).
3. Then add 1.0 ml of RSSS-I &1.0 ml ESSS-I in 10 ml volumetric flask and add 5 ml diluent and vortex and make up the volume with diluent.(Conc. of Rosuvastatin=10µg/ml& Ezetimibe =5µg/ml).

B. Preparation of Sample for Assay

1. Rozavel EZ were used as marketed product .
2. Weigh powder equivalent to 10 mg of Rosuvastatin and 5 mg of Ezetimibe and transfer to 100 ml volumetric flask & add 85 ml diluent, mix for 5 minutes and make the volume to 100 ml with diluent. (Conc. of Rosuvastatin = 100 µg/ml and Ezetimibe = 500 µg/ml).
3. Then add 1.0 ml of above stock solution in 10 ml volumetric flask and add 5 ml diluent and vortex and make up the volume with diluent (Conc. of Rosuvastatin= 10 µg/ml & Ezetimibe = 5 µg/ml).

2.4. Method validation

2.4.1. Specificity

The preparation of individual injections of Rosuvastatin and Ezetimibe, with concentrations of 10 µg/ml and 5 µg/ml, respectively, was carried out, and by analyzing the Retention Time, peaks were observed. Injection of blank was performed to guarantee that there would be no interference from the blank peak with the primary analyte peaks.

2.4.2. System Suitability

For the purpose of determining whether or not the system was suitable, a series of tests was carried out first. According to the ICH guideline system, the theoretical plate count, tailing factor, and resolution are all found to be within the acceptable parameters specified by the system.

2.4.3. Accuracy

To determine the accuracy of a technique, one must examine how closely its test findings correspond to the actual value. In the recovery studies, three distinct concentration levers were evaluated. At each level, three replicate injections were performed and the amount of drug present, the percentage of recovery, and the related standard deviation were calculated.

2.4.4. Repeatability

The degree of concordance that exists between the findings of individual tests is something that determines the analytical precision. An examination was performed on multiple samples of a uniform sample. After preparing a single sample in accordance with the instructions, six injections were done from the same sample and checked to ensure that the system was suitable. Instrument precision was done as Instrument precision (how good the instrument execute back to back replicate injection of similar concentration).

2.4.5. Linearity

The capacity of an analytical method to produce results that are proportionate to analyte concentrations within a certain range is referred to as the methodological linearity of the method.

When determining linearity, there were five different sets of standard solutions that were utilized. The regression equation was established by plotting the peak area against the concentration of the standard solution on the calibration curve. This allowed for the development of the equation. For the purpose of determining the slope, intercept, and correlation coefficient, the least-squares method was utilized.

2.4.6. LOD and LOQ

The limit of detection (LOD) and the limit of quantification (LOQ) are terms that indicate the capability of the method to detect and quantify the smallest amount of analyte, respectively. Calculating the LOD and LOQ required the use of the standard deviation and the slope of the regression line, which were both determined by the following equations.

2.4.7. Robustness

The Robustness was performed changing the column temperature by ± 2˚C and Wavelength by ± 2 nm.

Table 5: Robustness Trials

Condition	Increased	Normal	Decreased
Column Oven Temperature	32˚C	30˚C	28˚C
Wavelength	234 nm	232 nm	230 nm

2.4.8. Inter-day & Intraday Precision:

To determine the stability of the solution for intraday precision, the prepared working standard was analyzed in the morning and in the evening, and the percentage of relative standard deviation (RSD) was computed. The identical solution was injected on the second day, and the results of the intraday precision and percent relative standard deviation were compared with the data from the morning.

3. RESULTS AND DISCUSSION

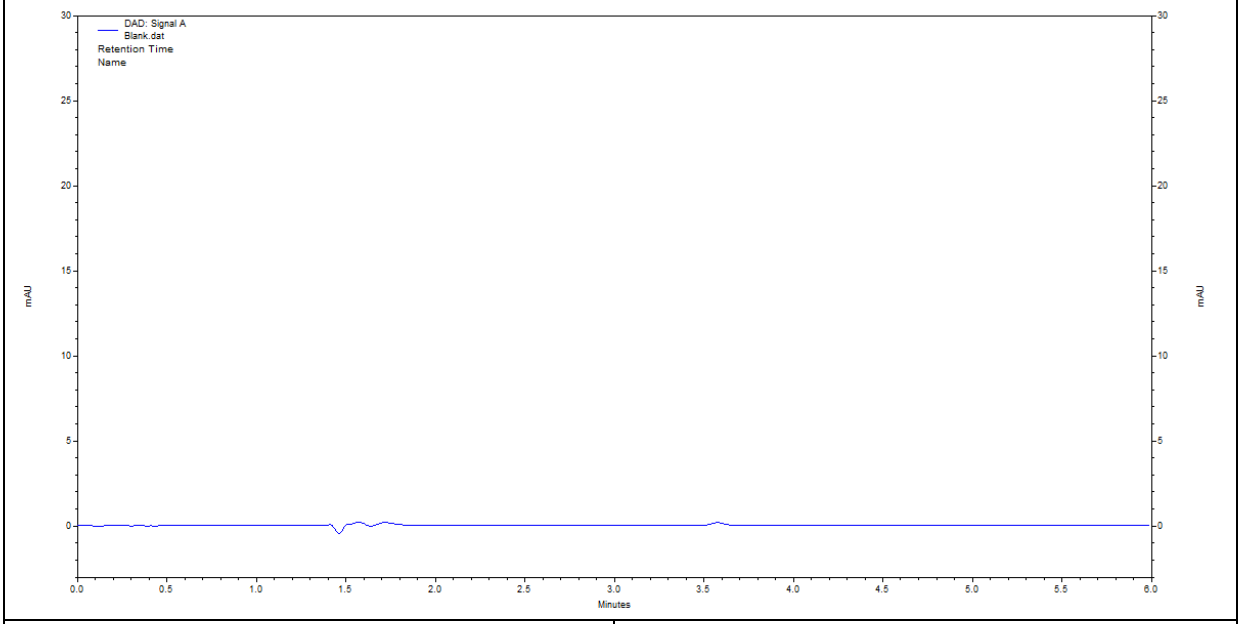
3.1. Specificity

Specificity was performed to check if there was any interaction between the peaks from blank or the APIs.

Table 6: Specificity results of Rosuvastatin and Ezetimibe

Sample	Rosuvastatin			Ezetimibe		
	RT	Area	% Assay	RT	Area	% Assay
Rosuvastatin	2.34	70714	-	-	-	-
Ezetimibe	-	-	-	2.99	148245	-
MIX WS	2.34	70637	-	2.99	148031	-
Drug Product	2.34	69984	99.08	2.99	147475	99.62

a. Diluent



b. Rosuvastatin WS | c. Ezetimibe WS

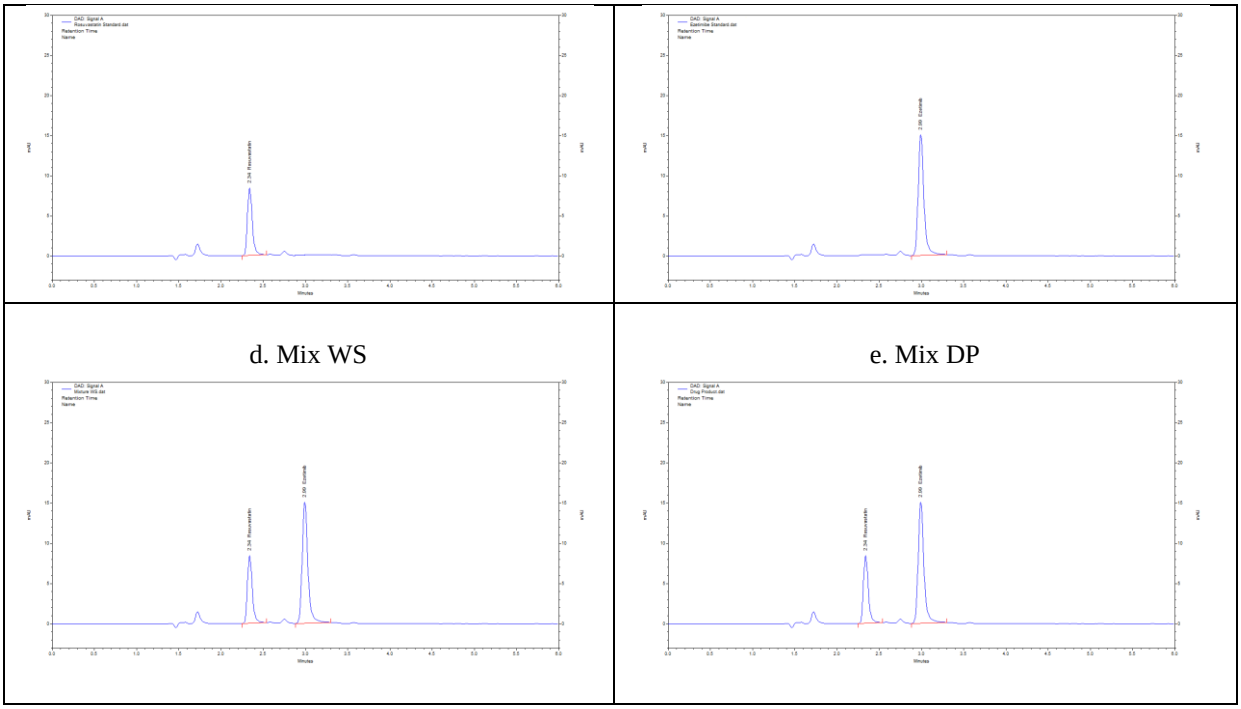


Figure 4: Chromatogram ID a]Diluent, b] Rosuvastatin c] Ezetimibe, d] Mixture Working Standard e]Drug Product of RSV& EZB.

3.2. Instrument Precision and System suitability

The HPLC Instrument was tested for its suitability to perform the validation. Based on the limits mentioned in table 1, the equipment was found to be suitable for continuing the validations. Instrument precisions of both the drugs were performed after system suitability and the reported data in below shows the relative standard deviation for Instrument precision of RSV & EZB are 0.16% and 0.12% respectively. This %RSD shows the method is very much precise with respect to multiple sample preparation for same concentration. The data is shown in table 7-.9.

Table 7: System suitability for Rosuvastatin

Rosuvastatin				
Reps	RT	Asymmetry	Theoretical Plates	Resolution
Rep 1	2.34	1.27	8320	0.00
Rep 2	2.34	1.25	8411	0.00
Rep 3	2.34	1.22	8247	0.00
Rep 4	2.34	1.24	8194	0.00
Rep 5	2.34	1.29	8085	0.00
Rep 6	2.34	1.21	8386	0.00
Avg	2.34			
STDEV	0.00			
RSD	0.00			

Table 8: System suitability for Ezetimibe

Ezetimibe				
Reps	RT	Asymmetry	Theoretical Plates	Resolution
Rep 1	2.99	1.28	10246	5.92
Rep 2	2.99	1.24	10379	5.92
Rep 3	2.99	1.21	10199	5.92
Rep 4	2.99	1.29	10287	5.92
Rep 5	2.99	1.20	10310	5.92
Rep 6	2.99	1.24	10142	5.92
Avg	2.99			
STDEV	0.00			
% RSD	0.00			

Table 9: Instrument precision of Rosuvastatin and Ezetimibe

Repeatability		
Sample ID	Peak Area	
	Rosuvastatin	Ezetimibe
100% Rep 1	70637	148031
100% Rep 2	70581	147919
100% Rep 3	70799	148147
100% Rep 4	70678	148079
100% Rep 5	70459	148394
100% Rep 6	70604	147955
AVG	70626	148088
STDEV	112.38446	171.2866
% RSD	0.16	0.12

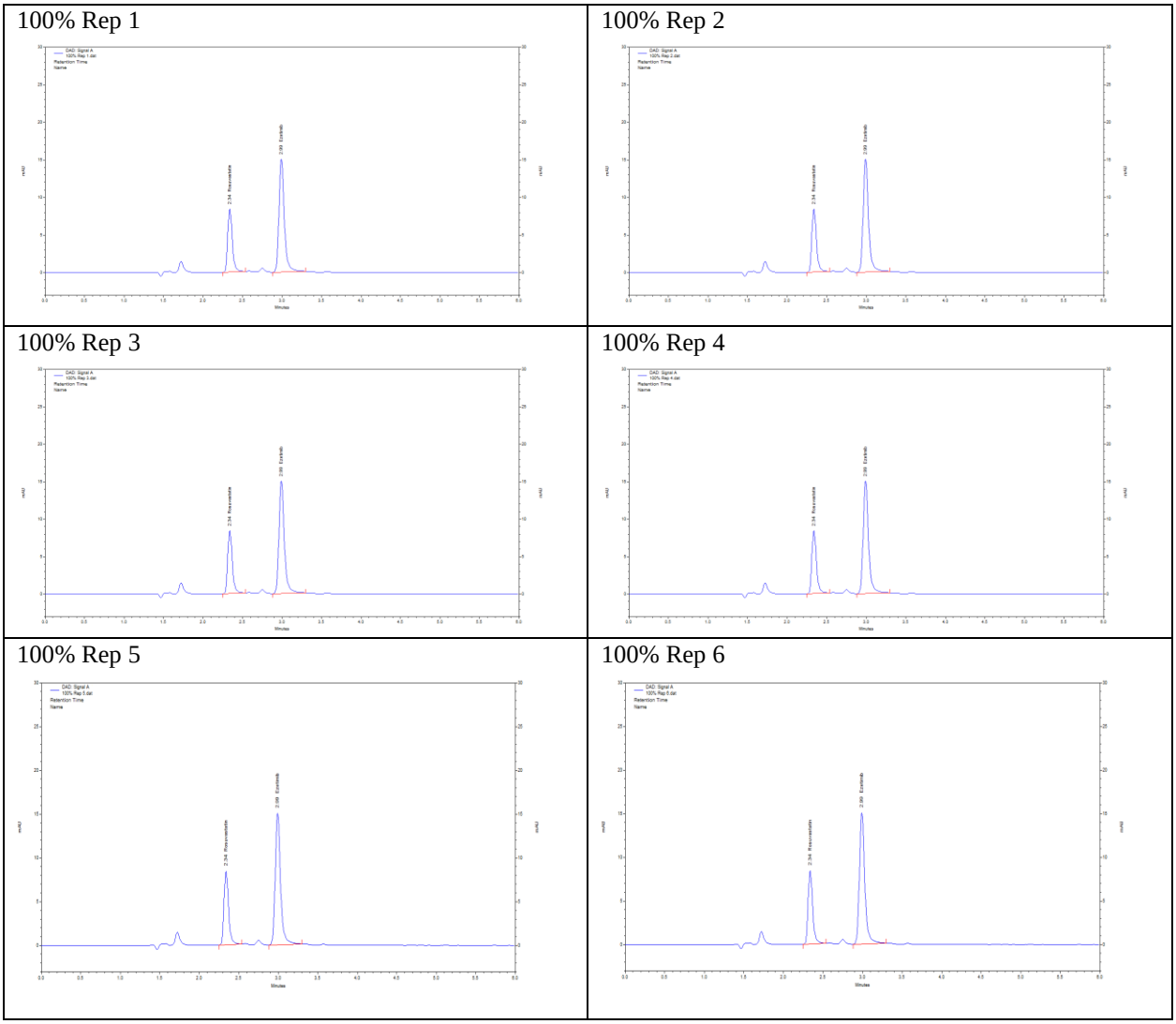


Figure 5: Instrument Precision Rosuvastatin & Ezetimibe

3.3. Linearity of Rosuvastatin & Ezetimibe

Linearity was performed at different levels. The graph plotted between peak area and concentration showed linearity with correlation coefficient as shown in table below. The linearity data in shown in table 10 and graph in figure 6.

Table 10: Linearity data of RSV & EZB

Rosuvastatin			Ezetimibe		
% Level	Conc (ug/ml)	Area	% Level	Conc (ug/ml)	Area
80	8	56653	80	4.0	118681
90	9	63196	90	4.5	132904
100	10	70637	100	5.0	148031
110	11	77763	110	5.5	163091
120	12	84902	120	6.0	177838
R^2= 0.999			R^2= 0.999		

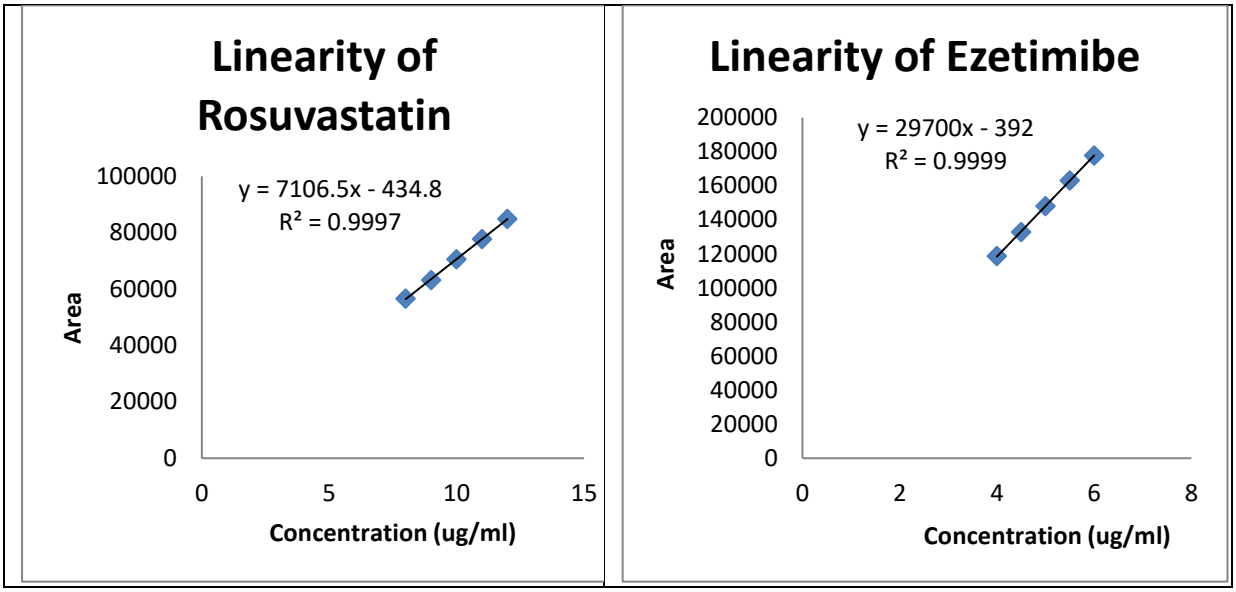


Figure 6: Linearity graph of Rosuvastatin and Ezetimibe

3.4. LOD and LOQ for Rosuvastatin and Ezetimibe

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) were determined for RSV & EZB. The results of analysis are shown in table 11.

Table 11:LOD and LOQ for RSV & EZB

Name	LOD (µg/ml)	LOQ (µg/ml)
Rosuvastatin	0.35	1.06
Ezetimibe	0.10	0.29

The LOD and LOQ were significantly low, implying the method to be very efficient in determining low concentration of drug. This value of LOD and LOQ can be used during cleaning validation in industry which can help companies know if the manufactured vessel or equipment is free from APIs stains.

3.5. Accuracy

Accuracy for RSV was performed in triplicates and it was observed that the method was accurate for the range 80%, 100% and 120%. The relative standard deviation for 80%, 100% and 120% were 0.18%, 0.16% and 0.07% respectively. The accuracy determined the methods ability to analyses different concentration of drug in solution accurately. The accuracy data is shown in table 12.

Table 12: Accuracy data for Rosuvastatin

Rosuvastatin		
Std Wt. (mg)	% Purity	Stock Conc. (ug/ml)
10	99.7	997.00

Std Area	70626

Sample ID	Reps	Spiked Conc (ug/ml)	Area	Amount Recovered (ug/ml)	% Recovery	AVG	STDEV	%RSD
80%	Rep 1	79.76	56653	79.97	100.27	100.22	0.179922	0.18
	Rep 2		56514	79.78	100.02			
	Rep 3		56712	80.06	100.37			
100%	Rep 1	99.70	70637	99.72	100.02	100.07	0.1603	0.16
	Rep 2		70581	99.64	99.94			
	Rep 3		70799	99.94	100.24			
120%	Rep 1	119.64	84902	119.85	100.18	100.12	0.067452	0.07
	Rep 2		84874	119.81	100.14			
	Rep 3		84792	119.70	100.05			

Accuracy for EVB was performed in triplicates and it was observed that the method was accurate for the range 80%, 100% and 120%. The relative standard deviation for 80%, 100% and 120% were 0.18%, 0.08% and 0.12%

respectively. The accuracy determined the methods ability to analyses different concentration of drug in solution accurately. The accuracy data is shown in table 13.

Table 13: Accuracy data for Ezetimibe

Ezetimibe		
Std Wt. (mg)	% Purity	Stock Conc. (ug/ml)
5	99.7	498.50

Std Area	148088

Sample ID	Reps	Spiked Conc (ug/ml)	Area	Amount Recovered (ug/ml)	% Recovery	AVG	STDEV	%RSD
80%	Rep 1	39.88	118681	39.95	100.18	99.97	0.184925	0.18
	Rep 2		118264	39.81	99.83			
	Rep 3		118356	39.84	99.90			
100%	Rep 1	49.85	148031	49.83	99.96	99.96	0.076985	0.08
	Rep 2		147919	49.79	99.89			
	Rep 3		148147	49.87	100.04			
120%	Rep 1	59.82	177838	59.86	100.07	99.96	0.118722	0.12
	Rep 2		177417	59.72	99.84			
	Rep 3		177652	59.80	99.97			

3.5. Inter and Intraday Precision

Intra and inter day precision study was performed and reported the % RSD change in peak area of the APIs at different time points. The acceptance criteria is to have %RSD of peak area <2%. The Results are given in Table 14.

Table 14: Intra & Interday Precision of RSV & EZB

Intra Day precision					
Day 1	Sample ID	Rosuvastatin		Ezetimibe	
		Area	Assay	Area	%Assay
Morning	WS	70637	-	148031	-
	DP	69984	99.08	147475	99.62
Evening	WS	68245	-	148241	-
	DP	67025	98.21	146224	98.64

Inter Day precision					
Day	Sample ID	Rosuvastatin		Ezetimibe	
		Area	Assay	Area	%Assay
Day 2	WS	67445	-	146327	-
	DP	66254	98.23	144452	98.72
		% RSD	0.50	% RSD	0.55

3.6. Robustness

Robustness is done to check how deviating the method is with respect to its critical parameters. All over the world, the equipment is calibrated before use, but to know if the method is robust, changes were done in column temperature and Wavelength as shown in table 15 and 16.

Table 15: Robustness study - Change in Column temperature

Column Oven Temp Change							
Condition	Sample	Rosuvastatin			Ezetimibe		
		RT	Area	Assay	RT	Area	Assay
28℃	WS	2.34	71226	-	2.99	150123	-
	DP	2.34	70584	99.10	2.99	149421	99.53
30℃	WS	2.34	70637	-	2.99	148031	-
	DP	2.34	69984	99.08	2.99	147475	99.62
32℃	WS	2.34	69547	-	2.99	146228	-
	DP	2.34	68845	98.99	2.99	145903	99.78

Table 16: Robustness study - Change in Wavelength

Wavelength (nm)							
Condition	Sample	Rosuvastatin			Ezetimibe		
		RT	Area	Assay	RT	Area	Assay
230	WS	2.34	71220	-	2.99	155281	-
	DP	2.34	70549	99.06	2.99	154095	99.24
232	WS	2.34	70637	-	2.99	148031	-
	DP	2.34	69984	99.08	2.99	147475	99.62
234	WS	2.34	71344	-	2.99	149345	-
	DP	2.34	70696	99.09	2.99	148525	99.45

Hence, the method was found to be robust with a small change in column temperature and change in wavelength. There was no significant change in Retention time, or Area of replicate injection.

CONCLUSION

In this research article, a precise and accurate method was developed based on method developed technique for estimation of RSV& EZB in bulk drugs and formulation by RP-HPLC technique. The developed method was validated for accuracy, precision and robustness. The proposed methods were found to be appropriate due to its simplicity, reliability, sensitivity, rapidness and selectivity for detection at very low concentrations. Validation data demonstrates that, these methods are accurate, precise, simple and economic and can be used in the routine analysis of Rosuvastatin and Ezetimibe in various formulations.

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