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ANALYTICAL METHOD VALIDATION REPORT FOR ASSAY OF ROSUVASTATIN AND BEMPEDOIC ACID BY HPLC

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ABSTRACT
Analytical Method Development and Validation for Bempedoic acid and Rosuvastatin in bulk and Combined Dosage Form by RP-HPLC. New method was established for simultaneous estimation of Bempedoic acid and Rosuvastatin by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Bempedoic acid and Rosuvastatin by using Inertsil C18 (4.6mm ×250mm, 5µm particle size), flow rate was 1.0 ml/min, mobile phase ratio was (55:45% v/v) Methanol: Phosphate buffer pH 4.8 (pH was adjusted with ortho phosphoric acid), detection wavelength was 282nm. The instrument used was WATERS Alliance 2695 separation module, Software: Empower 2, 996 PDA detector. The retention times were found to be 1.688mins and 3.282mins. The % purity of Bempedoic acid and Rosuvastatin was found to be 99.86%. The system suitability parameters for Bempedoic acid and Rosuvastatin such as theoretical plates and tailing factor were found to be 7586, 1.69 and 6235 and 1.58, the resolution was found to be 10.85. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study of Bempedoic acid and Rosuvastatin was found in concentration range of 100µg-500µg and 30µg-70µg and correlation coefficient (r2) was found to be 0.999 and 0.999, % recovery was found to be 100.112% and 100.16%, %RSD for repeatability was 0.1702 and 0.043 respectively. The precision study was precise, robust, and repeatable. The LOD value was found to be 2.1µg/ml and 1.28µg/ml, and LOQ value was 6.3µg/ml and 3.84µg/ml for Bempedoic acid and Rosuvastatin respectively. Hence the suggested RP-HPLC method can be used for routine analysis of Bempedoic acid and Rosuvastatin in API and Pharmaceutical dosage form.

KEYWORDS: Bempedoic acid and Rosuvastatin, Method Development, Validation, Accuracy, Precision.

INTRODUCTION
Rosuvastatin is an HMG-CoA reductase inhibitor used to lower lipid levels and reduce the risk of cardiovascular disease including myocardial infarction and stroke. Rosuvastatin, also known as the brand name product Crestor, is a lipid-lowering drug that belongs to the statin class of medications, which are used to lower the risk of cardiovascular disease and manage elevated lipid levels by inhibiting the endogenous production of cholesterol in the liver.[1] More specifically, statin medications competitively inhibit the enzyme hydroxy methyl glutaryl-coenzyme A (HMG-CoA) Reductase, which catalyzes the conversion of HMG-CoA to Mevalonic acid and is the third step in a sequence of metabolic reactions involved in the production of several compounds involved in lipid metabolism and transport including cholesterol, low-density lipoprotein (LDL) (sometimes referred to as "bad cholesterol"), and very low-density lipoprotein (VLDL).[2] Prescribing of statin medications are considered standard practice following any cardiovascular events and for people with a moderate to high risk of development of CVD, such as those with Type 2 Diabetes. The clear evidence of the benefit of statin use coupled with very minimal side effects or long term effects has resulted in this class becoming one of the most widely prescribed medications in North America.[3] The IUPAC name of Rosuvastatin is (E, 3R, 5S)-7-[4-(4-fluoro phenyl)-2-[methyl (methyl sulfonyl) amino]-6-propan-2-yl pyrimidin-5-yl]-3, 5-

dihydroxy hept-6-enoic acid. The Chemical Structure of Rosuvastatin is shown in following figure-1.

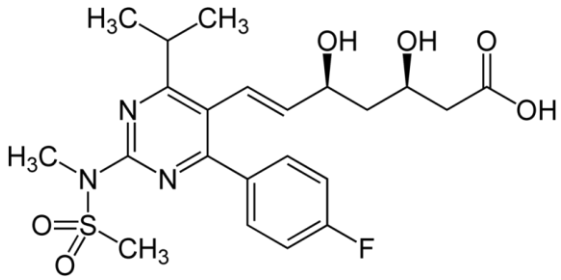

Fig. 1: Chemical Structure of Rosuvastatin.

Bempedoic acid is a drug used in conjunction with lifestyle modification and/or other agents for the treatment of refractory hypercholesterolemia. High levels of LDL cholesterol (LDL-C) are a major risk factor for cardiovascular events.[4] Caused by genetic mutations or lifestyle factors, hypercholesterolemia can significantly reduce quality of life and increase the risk of mortality from cardiovascular disease. About 1 in 4 patients, or 15 million Americans with elevated LDL-C, are insufficiently managed with maximally tolerated statin therapy alone, requiring additional treatment for hypercholesterolemia. Bempedoic acid is first-in-class adenosine triphosphate-citrate lyase (ACL) inhibitor used once a day for reducing LDL cholesterol levels in statin-refractory patients.[5] It was developed by Esperion Therapeutics Inc. and approved by the FDA on February 21, 2020. A combination product of Bempedoic acid and ezetimibe was approved on February 26, 2020 for increased control of LDL cholesterol levels in patients experiencing refractory elevations despite previous statin treatment. Bempedoic acid is indicated to reduce the risk of myocardial infarction and coronary revascularization in adults who are unable to take recommended statin therapy and have established cardiovascular disease or are at high risk of a cardiovascular event.[6] It is also indicated as an adjunct to diet, with or without other LDL-C lowering therapies, to reduce LDL-C in adults with primary hyperlipidemia, including heterozygous familial hypercholesterolemia (HeFH). Bempedoic acid in combination with ezetimibe is also indicated for the same. The IUPAC name of Bempedoic acid is 8-hydroxy-2, 2, 14, 14-tetra methyl penta decanedioic acid. The Chemical Structure of Bempedoic acid is shown in figure-2.

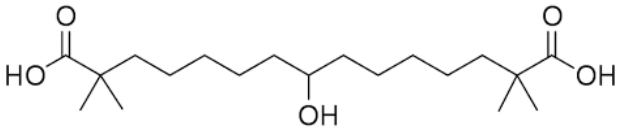

Fig. 2: Chemical Structure of Bempedoic acid.

MATERIALS AND METHODS

Table 1: Instruments and Equipments.

S. No	Instruments	Software	Model	Company
1	HPLC	Empower 2	Alliance 2695 separation Module. 996 PDA detector.	Waters
2	Weighing Balance	N/A	XEX 200	Sartorius
3	Sonicator	N/A	SE60US	Labman

Table 2: Chemicals and Reagents.

S. No	Chemical	Brand names
1	Water for HPLC	LICHROSOLV (MERCK)
2	Methanol for HPLC	LICHROSOLV (MERCK
3	Acetonitrile for HPLC	Merck

Wavelength Selection

UV spectrum of 10 µg/ml Rosuvastatin and Bempedoic acid in methanol was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 240 nm. At this wavelength both the drugs show good absorbance.[7]

Preparation of Phosphate buffer (pH 5.0)

To prepare phosphate buffer solution, by adding 6.8gm of phosphate buffer in a 1000ml water. Adjust this solution to pH 5.0 by using sodium hydroxide.

Preparation of Mobile Phase

Mix a mixture of phosphate buffer 300 ml (30%) and 700 ml Acetonitrile (70%) and degas in ultrasonic water bath for 5 minutes. Filter through 4.5 µ filter under vacuum filtration.[8]

Diluents Preparation

Phosphate buffer: Acetonitrile (30:70) ratio.

Preparation of the Bempedoic Acid & Rosuvastatin Standard & Sample Solution

Standard Solution Preparation

Accurately weigh and transfer 90 mg of Bempedoic acid and 20 mg of Rosuvastatin working standard into a 25 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Sample Solution Preparation
Accurately weigh and transfer of equivalent tablet powder of 90 mg of Bempedoic acid and 20 mg of Rosuvastatin (330 mg) into a 25 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock Solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure
Inject 20 µL of the standard, sample into the chromatographic system and measure the areas for Bempedoic acid and Rosuvastatin peaks and calculate the %Assay by using the formulae.[9]

Analytical Method Validation
Validation is a process of establishing documented evidence which provide a high degree of assurance that specific activity will consistently produce a desired result or product meeting its predetermined specification and quality characteristics.[10]

System Suitability
System suitability is the evaluation of the components of an analytical system to show that the performance of a system meets the standards required by a method. A system suitability evaluation usually contains its own set of parameters.[11] For chromatographic assays, these may include tailing factor, resolution, precision, capacity factor time and theoretical plates.

Accuracy
Preparation of Standard Stock Solution
Accurately weigh and transfer 90 mg of Bempedoic acid and 20 mg of Rosuvastatin working standard into a 25 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.[12]

Preparation Sample Solutions
For Preparation of 50% Solution (With respect to target Assay concentration)
Accurately weigh and transfer 45 mg of Bempedoic acid and 10 mg of Rosuvastatin working standard into a 25 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

For Preparation of 100% Solution (With respect to target Assay concentration)
Accurately weigh and transfer 90 mg of Bempedoic acid and 20 mg of Rosuvastatin working standard into a 25 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.[13] (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

For Preparation of 150% Solution (With respect to target Assay concentration)
Accurately weigh and transfer 135 mg of Bempedoic acid and 30 mg of Rosuvastatin working standard into a 25 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure
Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions. Calculate the Amount found and Amount added for Bempedoic acid & Rosuvastatin and calculate the individual recovery and mean recovery values.[14]

Acceptance Criteria
The %RSD for each level should not be more than 2.

Precision
Repeatability
Preparation of Stock Solution
Accurately weigh and transfer 90 mg of Bempedoic acid and 20 mg of Rosuvastatin working standard into a 25 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure
The standard 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.

Ruggedness
To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day.[15]

Preparation of Stock Solution
Accurately weigh and transfer 90 mg of Bempedoic acid and 20 mg of Rosuvastatin working standard into a 25 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with

diluent.

Procedure
The standard solutions prepared in the precision were injected on the other day, 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. The % RSD for the area of five standard injections results should be not more than 2%

Linearity
Preparation of Stock Solution
Accurately weigh and transfer 90 mg of Bempedoic acid and 20 mg of Rosuvastatin working standard into a 25 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.[16] (Stock solution)

Preparation of Level – I
0.1 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – II
0.2 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – III
0.3 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – IV
0.4 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – V
0.5 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Procedure
Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.[17]

Acceptance Criteria: Correlation coefficient should be not less than 0.999.

Limit of Detection
The detection limit is determined by the analysis of samples with known concentration of analyte and by establishing that minimum level at which the analyte can reliably detected.[18]

Limit of Quantitation
The quantification limit is generally determined by the analysis of sample with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.[19]

Robustness
The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results.[20]

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

A. The flow rate was varied at 0.9 ml/min to 1.1ml/min
Standard solution 108 ppm of Bempedoic acid & 24 ppm of Rosuvastatin were prepared and analysed using the varied flow rates along with method flow rate.

On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10%.

B. The Organic Composition in the Mobile phase was varied from 50% to 50%
Standard solution 108 ppm of Bempedoic acid & 24 ppm of Rosuvastatin were prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method.

On evaluation of the above results, it can be concluded that the variation in 10%. Organic composition in the mobile phase affected the method significantly. Hence it indicates that the method is robust even by change in the Mobile phase ± 10.

RESULTS AND DISCUSSION

Analytical Method Development
Optimized Chromatographic Conditions
Table 3: Shows Optimized Chromatographic Conditions.

Equipment	High Performance Liquid Chromatography equipped with Auto Sampler and PDA detector
Column	Spursil C18 (250*4.6mm, 5 µm)
Flow rate	1.0 mL/ min
Wavelength	240 nm
Injection Volume	20 µl
Column temperature	Ambient
Run time	10 min

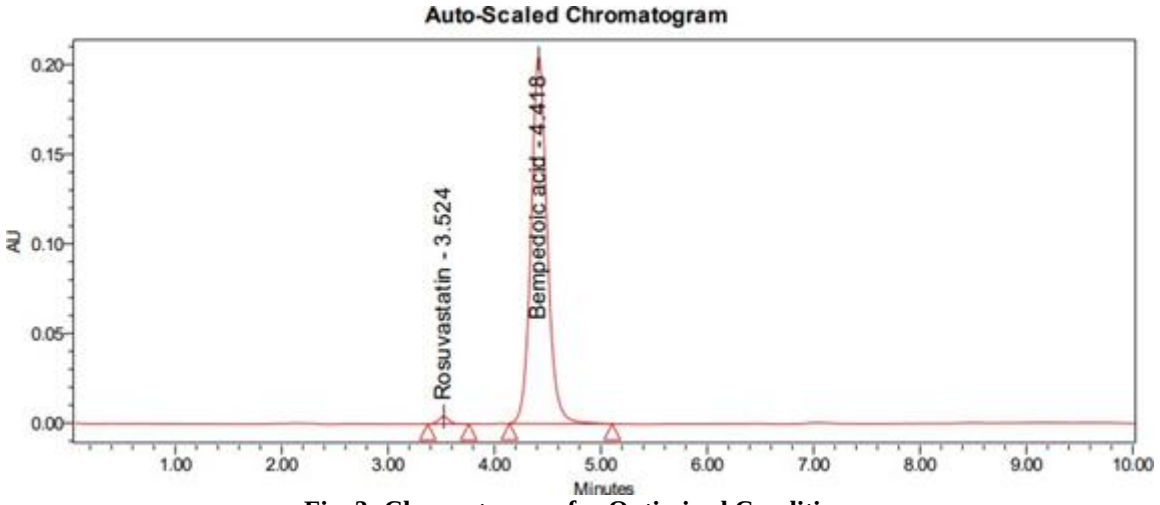

Fig. 3: Chromatogram for Optimized Condition.

Method validation

As per ICH guidelines, the method validation parameters checked were linearity, precision, accuracy, limit of detection and limit of quantification.[21]

Accuracy

To ensure the reliability and accuracy of the method recovery studies were carried out by standard addition method. A known quantity of pure drug was added to preanalysed sample and contents were reanalyzed by the proposed method and the percent recovery was reported.[22] The results were given in table 4.

Table 4: Accuracy Observation of Bempedoic acid.

% Concentration (at Specification Level)	Peak Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	1055764	45	45.08	100.17	**100.33**
100%	2132744	90	91.06	101.17	
150%	3151028	135	134.53	99.65	

Table 5: Accuracy Observation of Rosuvastatin.

% Concentration (at specification Level)	Peak Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	13883	10	10.26	102.58	100.74
100%	27022	20	19.97	99.84	
150%	40483	30	29.91	99.71	

Observation

The accuracy studies were shown as % recovery for Bempedoic acid and Rosuvastatin at 50%, 100% and 150% the limits of % recovery should be in range of 98-102%.

The results obtained for Bempedoic acid and Rosuvastatin were found to be within the limits. Hence the method was found to be accurate.

The limits of % recovery of drugs were 98-102 % and from the above results it indicates that the commonly used excipients present in the pharmaceutical formulation do not interfere in the proposed method.[23]

Precision

The repeatability of the method was verified by calculating the % RSD of three replicate injections of 100% concentration (90µg/ml of Bempedoic acid and 20µg/ml of Rosuvastatin) on the same day and for intraday precision % RSD was calculated from repeated studies.[24] The results were given in table 6.

System Precision

Table 6: Observation of System Precision.

Injection	Area for Bempedoic acid	Area for Rosuvastatin
Injection-1	2128651	27340
Injection-2	2141733	27340
Injection-3	2084645	27497
Injection-4	2125632	27287

Injection-5	2135815	27442
Injection-6	2149902	27310
Average	2127730	27369.33
Standard Deviation	22867.51	81.97
%RSD	1.0	0.3

Acceptance Criteria

- In the precision study %RSD was fond to be less than 2%.
- For precision studies 5 replicated injections of Bempedoic acid and Rosuvastatin formulation was performed.
- %RSD was determined for peak areas of Bempedoic acid and Rosuvastatin.
- The acceptance limits should be not more than 2% and the results were found to be within the acceptance limits.[25]

Ruggedness

Ruggedness is the research of the effect of external circumstances on the approach. To evaluate the robustness of the offered strategy, elements were purposely varied. These influences included system variance, diverse analysis, and atmospheric changes.[26] Two different analysts prepared the test solution according to the test method and injected six doses of test solution into the HPLC system at a flow rate of 1.0 ml/min.

Table 7: Observation of Ruggedness Day1.

Injection	Area for Bempedoic acid	Area for Rosuvastatin
Injection-1	2041733	27340
Injection-2	2084645	27497
Injection-3	2025632	27287
Injection-4	2008618	27287
Injection-5	2035815	27310
Injection-6	2052672	26910
Average	2041519	27271
Standard Deviation	25890.91	194.0
%RSD	0.7	1.2

Acceptance Criteria

- %RSD of five different sample solutions should not more than 2.

Linearity

Analytical method linearity is defined as the ability of the method to obtain test results that are directly proportional to the analyte concentration, within a specific range. The mean peak area obtained from the HPLC was plotted against corresponding concentrations to obtain the calibration graph. The results of linearity study (Figure 4 and 5) gave linear relationship over the concentration range of 36 - 180 µg/ml for Bempedoic acid and 8 - 40 µg/ml for Rosuvastatin respectively.[27] From the regression analysis, linear equations were obtained: y = 19659x – 12261 for Bempedoic acid and y = 742.28x + 6565.6 and the goodness-of-fit (r2) were found to be 0.9991 & 0.9993 for Bempedoic acid and Rosuvastatin respectively, indicating a linear relationship between the concentration of analyte and area under the peak.[28] The Linearity data were shown in tables-8 and 9.

Table 8: Linearity Observation of Bempedoic acid.

S. No.	Linearity Level	Concentration	Area
1	I	36	679688
2	II	72	1398189
3	III	108	2166875
4	IV	144	2784702
5	V	180	3525027
Correlation Coefficient			0.999

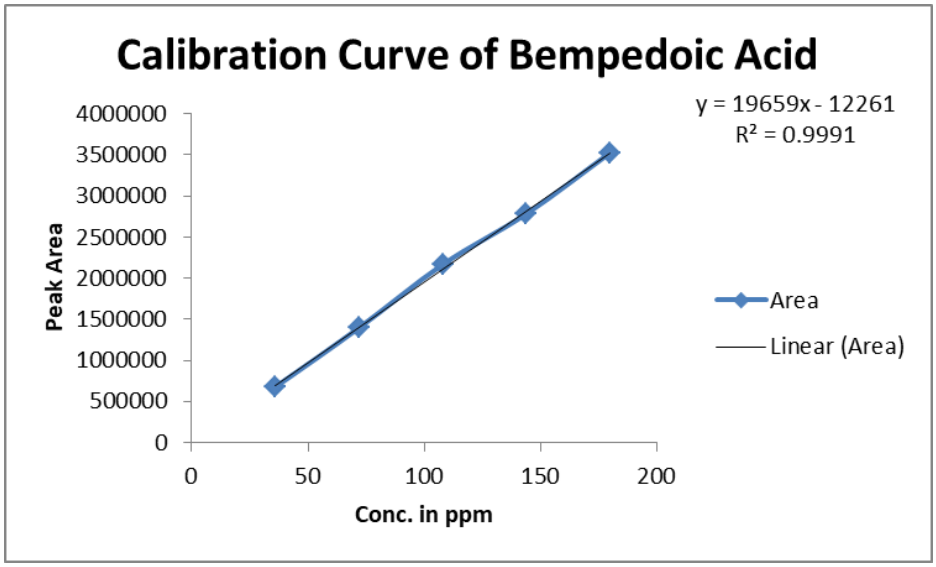

Fig. 4: Calibration Curve of Bempedoic acid.

Table 9: Linearity Observation of Rosuvastatin.

S. No	Linearity Level	Concentration	Area
1	I	8	12562
2	II	16	18542
3	III	24	24297
4	IV	32	29952
5	V	40	36548
Correlation Coefficient			0.999

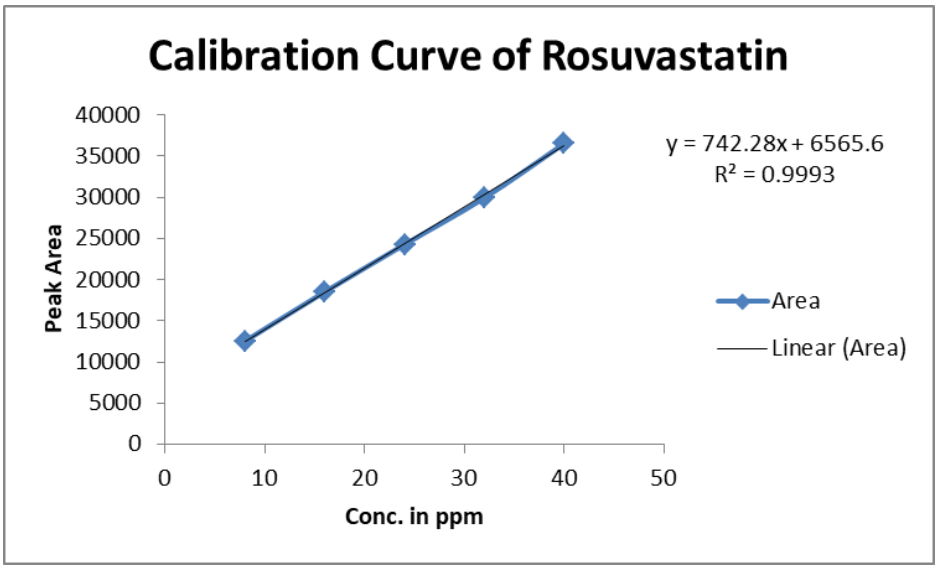

Fig. 5: Calibration Curve of Rosuvastatin.

The linearity range was found to be 36-180 and 08-40µg/ml for both Bempedoic acid and Rosuvastatin respectively. Calibration curve was plotted and correlated Co-efficient for both the drugs found to be 0.999.

Robustness

The robustness was performed for the flow rate variations from 0.9 ml/min to 1.1ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Rosuvastatin and Bempedoic acid.[29] The method is robust only in less flow condition and the method is robust even by change in the Mobile phase ± 5%. The standard and samples of Rosuvastatin, Bempedoic acid were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count.

Variation in Flow rate

Table 10: Flow rate Observation of Bempedoic acid (Robustness).

S. No.	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.9	4565.01	1.09
2	1	4402.39	1.12
3	1.1	4525.26	1.08

Results for actual flow rate have been considered from assay standard.

Table 11: Flow rate Observation of Rosuvastatin (Robustness).

S. No.	Flow Rate (ml/min)	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
1	0.9	5705.32	1.01	3.59
2	1	6959.54	1.01	3.60
3	1.1	5402.20	1.08	3.66

On evaluation of the above results, it can be concluded that the variation in flow rate not affect the method significantly.[30]

Variation in Organic Composition

Table 12: System Suitability Results Bempedoic acid (Robustness).

S. No.	Change in Organic	System Suitability Results	
	Composition in the Mobile Phase	USP Plate Count	USP Tailing
1	10% less	3796.37	1.06
2	*Actual	4402.39	1.12
3	10% more	4246.82	1.06

Table 13: System Suitability Results Rosuvastatin (Robustness).

S. No.	Change in Organic Composition in the Mobile Phase	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
1	10% less	5384.81	1.02	2.85
2	*Actual	6959.54	1.01	3.60
3	10% more	4785.72	1.01	3.55

Acceptance Criteria

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

SUMMARY AND CONCLUSION

A simple, Accurate, precise method was developed for the simultaneous estimation of the Bempedoic acid and Rosuvastatin in bulk and tablet dosage form. Chromatogram was run through DIKMA Spursil C_{18} column (4.6 mm x 250mm, 3.0 µm). Mobile phase containing phosphate buffer 300 ml (30%) and 700 ml Acetonitrile (70%) was pumped through column at a flow rate of 1ml/min. Temperature was maintained at Ambient. Optimized wavelength for Bempedoic acid and Rosuvastatin was 240 nm. Retention time of Bempedoic acid and Rosuvastatin were found to be 4.418 min and 3.524 min. The % purity of Bempedoic acid and Rosuvastatin was found to be 100.57% and 100.15 % respectively. The system suitability parameters for Bempedoic acid and Rosuvastatin such as theoretical plates and tailing factor were found to be 4402.39, 6959.54, 1.12 and 3.60. The linearity study for Bempedoic acid and Rosuvastatin correlation coefficient (r2) was found to be 0.999 and 0.999, % mean recovery was found to be 100.33 % and 100.74%, %RSD for repeatability was 1.0 and 0.3, % RSD for intermediate precision was 0.7 and 1.2 respectively. The precision study was precise, robust and repeatable. The results of study showed that the proposed RP-HPLC method is a simple, accurate, precise, rugged, robust, fast and reproducible, which may be useful for the routine estimation of Bempedoic acid and Rosuvastatin in bulk and tablet dosage form.

The Developed HPLC method was validated and it was found to be simple, precise, accurate and sensitive for the simultaneous estimation of Bempedoic acid and Rosuvastatin in its bulk and tablet dosage form. Hence, this method can easily and conveniently adopt for routine quality control analysis of Rosuvastatin and Bempedoic acid in its bulk and tablet dosage form.

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