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Research Article

**DEVELOPMENT OF NEW ANALYTICAL METHOD AND
VALIDATION OF SIMVASTATIN, EZETIMIBE IN PURE AND
PHARMACEUTICAL FORMULATION BY RP-HPLC**Badampudi Sam Benjamin*¹, Dr T.K.V. Kesav Rao, Dr CH.Prasad¹¹Department of Pharmaceutical Analysis, Pydah College of Pharmacy Patavala, Andhra University, Kakinada, Andhra Pradesh.

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Abstract:

A novel, precise, accurate, rapid and cost effective isocratic reverse phase high performance liquid chromatographic (RP-HPLC) method was developed, optimized and validated for the estimation of Netupitant (NET) and Palonosetron (PAL) in bulk and pharmaceutical dosage forms. The drugs were estimated using Phenomenex Gemini C18 (4.6mm×150mm, 5µm) particle size column. A mobile phase composed of tri ethylamine buffer and methanol in proportion of 32:68 v/v, at a flow rate of 1.0 ml/min was used for the separation. Detection was carried out at 248nm. The linearity range obtained was 30-70µg/ml for Netupitant and 10-50µg/ml for Palonosetron with retention times (Rt) of 3.297min and 5.405min for Netupitant and Palonosetron respectively. The correlation coefficient values were found to be 0.999 & 0.999. Precision studies showed % RSD values less than 2 % for both the drugs in all the selected concentrations. The percentage recoveries of Netupitant (NET) and Palonosetron (PAL) were found to be 100.1873% for Netupitant and 100.748% for Palonosetron respectively. The assay results of Netupitant (NET) and Palonosetron (PAL) were found to be 99.82%. The limit of detection (LOD) and limit of quantification (LOQ) were 2.6µg/ml and 7.8µg/ml for Netupitant and 3.4µg/ml 10.2µg/ml for Palonosetron respectively. The proposed method was validated as per the International Conference on Harmonization (ICH) guidelines. The proposed validated method was successfully used for the quantitative analysis of commercially available dosage form.

Keywords: Netupitant and Palonosetron, RP-HPLC, ICH Guidelines, Validation.**Corresponding author:****Badampudi Sam Benjamin,**

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INTRODUCTION:**Chromatography [2]:**

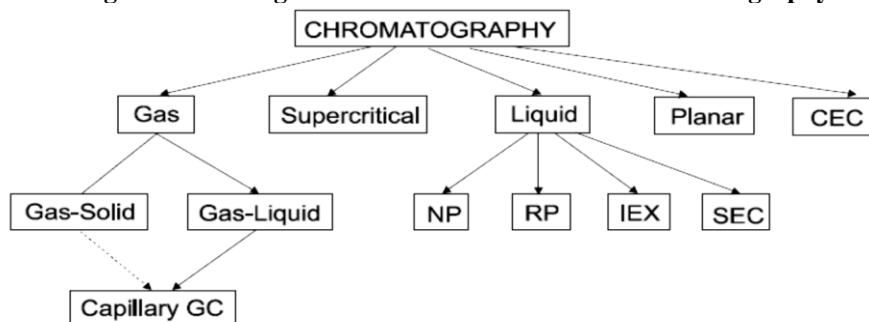
The chromatography was discovered by Russian Chemist and botanist *Micheal Tswett* (1872-1919) who first used the term chromatography (colour writing derived from Greek for colour – Chroma, and write – graphein) to describe his work on the separation of coloured plant pigments into bands on a column of chalk and other material such as polysaccharides, sucrose and insulin. “*Chromatography is a method in which the components of a mixture are separated on an adsorbent column in a flowing system*”. The adsorbent material, or stationary phase, first described by Russian scientist named Tswett in 1906, has taken many forms over the years, including paper, thin layers of solids attached to glass plates, immobilized liquids, gels, and solid particles packed in columns.

“Chromatography is a physical method of separation in which the component to be separated are distributed between two phases of which in stationary while other moves in a definite direction (IUPAC)”

Types of Chromatography:

The mobile phase could be either a liquid or a gas, and accordingly we can subdivide chromatography into Liquid Chromatography (LC) or Gas Chromatography (GC). Apart from these methods, there are two other modes that use a liquid mobile phase, but the nature of its transport through the porous stationary phase is in the form of either (a) capillary forces, as in planar chromatography (also called Thin-Layer Chromatography, TLC), or (b) electro osmotic flow, as in the case of Capillary Electro Chromatography (CEC)

Fig.No.1. Showing flow chart for classification of chromatography⁴

**Adsorption chromatography:**

Chromatography in which separation is based mainly on difference between the adsorption affinities of the sample components for the surface of an active solid. The analyte interact with solid stationary surface and are displaced with eluent for active sites on surface.

Partition chromatography:

This method results from a thermodynamic distribution of analytes between two liquid phases. On the basis of relative polarities of stationary and mobile phase, partition chromatography can be divided in to normal phase and reverse phase chromatography. In normal phase chromatography, the stationary phase bed is strongly polar in nature (e.g. Silica gel) and the mobile phase is non-polar (such as n-hexane or tetrahydrofuran). Polar sample are thus retained on polar surface of the column packing longer than polar material while in reverse phase chromatography, the stationary bed is non-polar (hydrophobic in nature, while the mobile phase is polar liquid, such as mixture of water and methanol or Acetonitrile. Here the more non polar the material is, the longer it will retain.

Size-exclusion chromatography:

This involves a solid stationary phase with controlled pore size. Solids are separated according to molecular size, with the large molecule unable to enter the pores eluted first.

Ion- exchange chromatography:

Involves a solid stationary phase with anionic or cationic groups on the surface to separation, HPLC and HPTLC methods have widely been exploited in pharmaceutical analysis because of its simplicity, precision, accuracy and reproducibility of result.

Solid-Phase Extraction [SPE]:

A sample preparation technique that uses LC principles to isolate, enriches, and/or purifies analytes from a complex matrix applied to a miniature chromatographic bed. *Offline* SPE is done with larger particles in individual plastic cartridges or in micro-elution plate wells, using low positive pressure or vacuum to assist flow. *Online* SPE is done with smaller particles in miniature HPLC columns using

higher pressures and a valve to switch the SPE column online with the primary HPLC column, or offline to waste, as appropriate. SPE methods use step gradients to accomplish bed conditioning, sample loading, washing, and elution steps. The goal is to remove matrix interferences and to isolate the analyte in a solution, and at a concentration, suitable for subsequent analysis.

Working Principle of HPLC:

The components of a basic High-Performance Liquid Chromatography [HPLC] system are shown in the simple diagram in figure 5. A reservoir holds the solvent [called the mobile phase, because it moves]. A high-pressure pump [solvent delivery system or solvent manager] is used to generate and meter a specified flow rate of mobile phase, typically millilitres per minute. An injector is able to introduce [inject] the sample into the continuously flowing mobile phase stream that carries the sample into the HPLC column.

The column contains the chromatographic packing material needed to effect the separation. This packing material is called the stationary phase because it is held in place by the column hardware. A detector is needed to see the separated compound bands as they elute from the HPLC column. The mobile phase exits the detector and can be sent to waste, or collected, as desired. When the mobile phase contains a separated compound band, HPLC provides the ability to collect this fraction of the elute containing that purified compound for further study. This is called preparative chromatography.

The detector is wired to the computer data station, the HPLC system component that records the electrical signal needed to generate the chromatogram on its display and to identify and quantitative the concentration of the sample constituents. Since sample compound characteristics can be very different, several types of detectors have been developed. For example, if a compound can absorb Ultra Violet light, a UV-absorbance detector is used. If the compound does not have either of these characteristics, a more universal type of detector is used, such as an Evaporative-Light-Scattering Detector [ELSD]. The most powerful approach is the use multiple detectors in series. For example, a UV and/or ELSD detector may be used in combination with a Mass Spectrometer [MS] to analyze the results of the chromatographic separation. This provides, from a single injection, more comprehensive information about an analyte. The practice of coupling a mass spectrometer to an HPLC system is called LC/MS.

MATERIALS AND METHOD:

HPLC WATERS Alliance 2695 separation module, Software: Empower 2, 996 PDA detector, pH meter- Lab India, Weighing machine- Sartorius, Volumetric flasks- Borosil, Pipettes and Burettes- Borosil, Beakers Borosil, Digital ultra sonicator- Lab man.

HPLC METHOD DEVELOPMENT:

Trails:

Preparation of standard solution:

Accurately weigh and transfer 10 mg of Simvastatin and Ezetimibe working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

Further pipette 2.25ml of the above Simvastatin and 0.45ml of the Ezetimibe stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure:

Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Mobile Phase Optimization:

Initially the mobile phase tried was Methanol: Water, Acetonitrile: Water with varying proportions. Finally, the mobile phase was optimized to Methanol: TEA buffer pH 4.8 in proportion 32:68 v/v respectively.

Optimization of Column:

The method was performed with various columns like C18 column, X- bridge column, Xterra. Phenomenex Gemini C18 (4.6mm×150mm, 5.0 µm) particle size was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

Optimized chromatographic conditions:

Instrument used :	Waters HPLC with auto sampler and PDA Detector 996 model.
Column :	Phenomenex Gemini C18 (4.6mm×150mm, 5.0 µm) particle size
Column temperature :	38°C
pH :	4.8
Mobile phase :	Methanol: TEA buffer pH 4.8 (32:68v/v)
Flow rate :	1ml/min
Wavelength :	248nm
Injection volume :	20µl
Run time :	7 min

Method validation**Preparation of mobile phase:****Preparation of mobile phase:**

Accurately measured 320ml (32%) of HPLC Methanol and 680ml of TEA buffer (68%) were mixed and degassed in a digital ultra sonicator for 15 minutes and then filtered through 0.45 μ filter under vacuum filtration.

Diluent Preparation:

The Mobile phase was used as the diluent.

RESULTS AND DISCUSSION:**Optimized Chromatogram (Standard)**

Column : Phenomenex Gemini C18 (4.6mm \times 150mm, 5.0 μ m) particle size

Column temperature : 38 $^{\circ}$ C

Wavelength : 248nm

Mobile phase ratio : Methanol: TEA buffer pH 4.8 (32:68v/v)

Flow rate : 1ml/min

Injection volume : 20 μ l

Run time : 7minutes

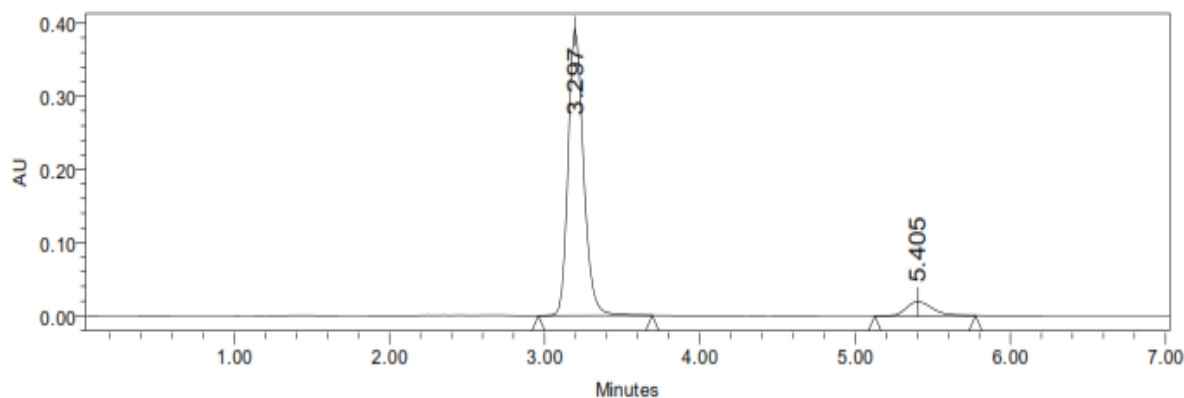


Fig: Optimized Chromatogram (Standard)

Table: Optimized Chromatogram (Standard)

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	USP Resolution
1	Simvastatin	3.297	859856	42569	1.24	7896	
2	Ezetimibe	5.405	5698	3652	1.36	6582	6.8

Observation: From the above chromatogram it was observed that the Simvastatin and Ezetimibe peaks are well separated and they shows proper retention time, resolution, peak tail and plate count. So it's optimized trial.

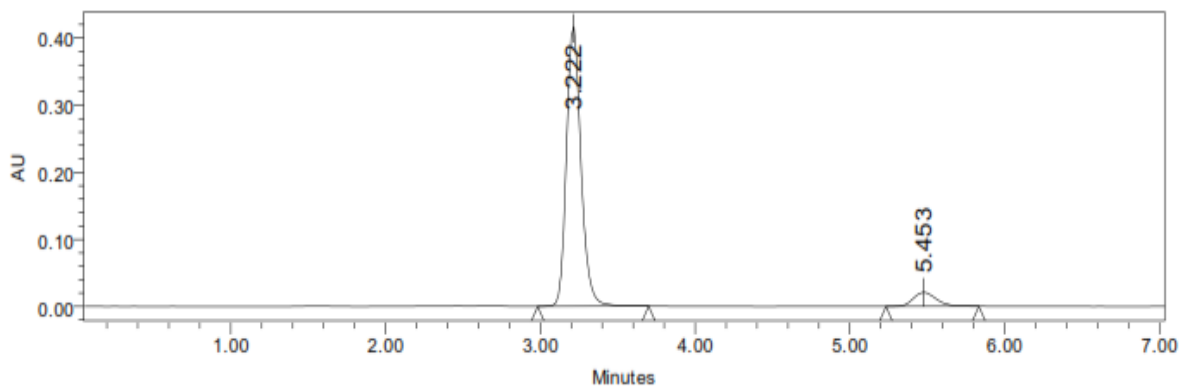
Optimized Chromatogram (Sample)

Figure-: Optimized Chromatogram (Sample)

Table-: Optimized Chromatogram (Sample)

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	USP Resolution
1	Simvastatin	3.222	865898	43659	1.26	7985	
2	Ezetimibe	5.453	5789	3785	1.38	6659	7.0

Acceptance Criteria:

- Resolution between two drugs must be not less than 2.
- Theoretical plates must be not less than 2000.
- Tailing factor must be not less than 0.9 and not more than 2.
- It was found from above data that all the system suitability parameters for developed method were within the limit.

System Suitability:**Table-: Results of system Suitability for Simvastatin**

S.No.	Peak Name	RT	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	USP Plate Count	USP Tailing
1	Simvastatin	3.200	859865	42568	7895	1.24
2	Simvastatin	3.248	859788	42587	7859	1.24
3	Simvastatin	3.299	857984	42659	7869	1.24
4	Simvastatin	3.297	854879	42875	7849	1.24
5	Simvastatin	3.297	857896	42487	7859	1.23
Mean			858082.4			
Std. Dev.			2024.409			
% RSD			0.235922			

Acceptance Criteria:

- %RSD of five different sample solutions should not more than 2.
- The %RSD obtained is within the limit, hence the method is suitable.

Table-: Results of System Suitability for Ezetimibe

S.No	Peak Name	RT	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	USP Plate Count	USP Tailing
1	Ezetimibe	5.413	5689	3659	6583	1.36
2	Ezetimibe	5.484	5687	3648	6592	1.37
3	Ezetimibe	5.405	5682	3698	6549	1.37
4	Ezetimibe	5.405	5649	3675	6571	1.36
5	Ezetimibe	5.409	5674	3649	6529	1.36
Mean			5676.2			
Std. Dev.			16.2696			
% RSD			0.286628			

Acceptance Criteria:

- %RSD of five different sample solutions should not more than 2.
- The %RSD obtained is within the limit, hence the method is suitable.

Assay (Standard):**Table-: Peak Results for Assay Standard****Simvastatin**

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Simvastatin	3.211	859785	42598	1.25	7856
2	Simvastatin	3.222	859865	42895	1.24	7859
3	Simvastatin	3.254	857849	42578	1.25	7869

Ezetimibe

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Resolution
1	Ezetimibe	5.414	5699	3685	1.36	6598	6.9
2	Ezetimibe	5.453	5687	3659	1.37	6537	6.9
3	Ezetimibe	5.424	5689	3649	1.36	6582	7.0

Assay (Sample):**Table-: Peak Results for Assay sample****Simvastatin**

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Simvastatin	3.297	865985	43659	1.26	7985
2	Simvastatin	3.294	865798	43875	1.26	7925
3	Simvastatin	3.295	865456	43659	1.27	7946

Ezetimibe

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Resolution
1	Ezetimibe	5.435	5789	3659	1.37	6659	6.9
2	Ezetimibe	5.417	5798	3684	1.38	6689	7.0
3	Ezetimibe	5.434	5749	3695	1.38	6648	6.9

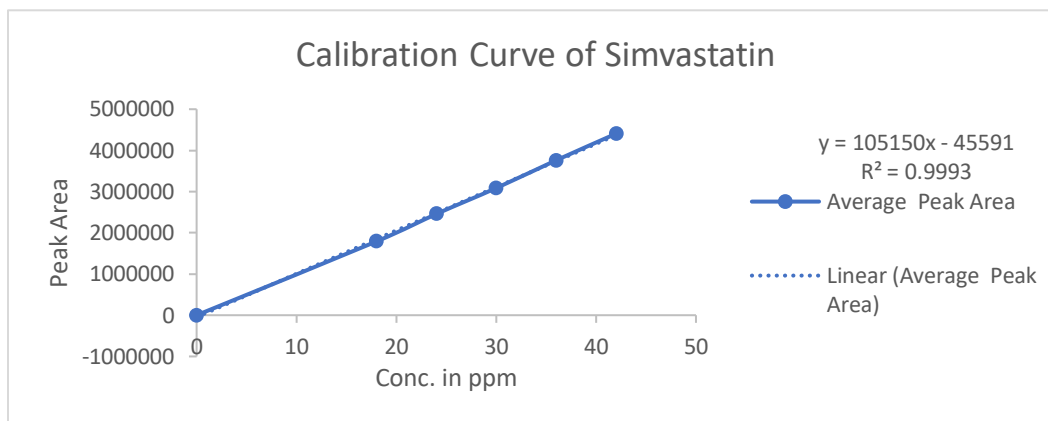
%ASSAY =

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

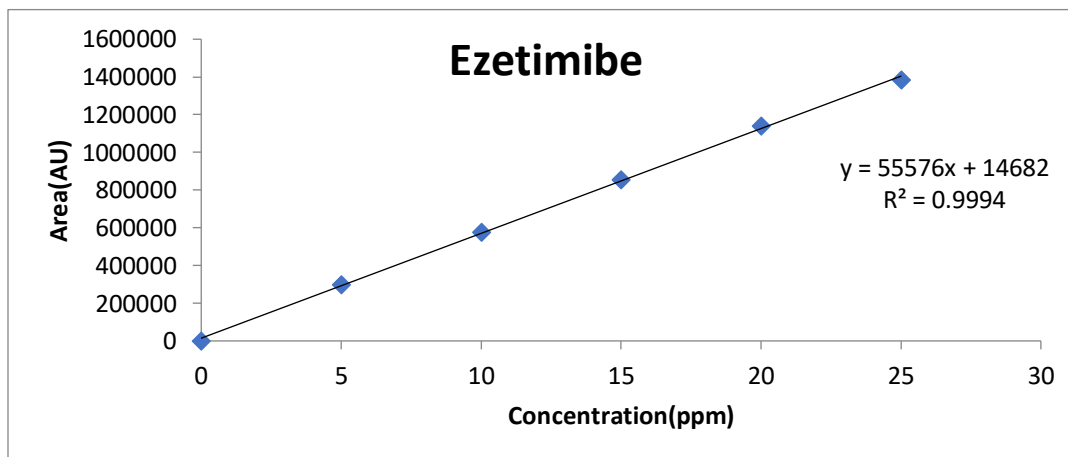
The % purity of Simvastatin and Ezetimibe in pharmaceutical dosage form was found to be 99.82%.

LINEARITY:**CHROMATOGRAPHIC DATA FOR LINEARITY STUDY: SIMVASTATIN:**

Concentration µg/ml	Average Peak Area
18	1789546
24	2456987
30	3085985
36	3759864
42	4406589

**Fig-: Calibration Curve of Simvastatin****EZETIMIBE**

Concentration µg/ml	Average Peak Area
10	2038
20	3859
30	5698
40	7489
50	9218

**Fig-: Calibration Curve of Ezetimibe**

REPEATABILITY

Table-: Results of Repeatability for Simvastatin:

S. No.	Peak name	Retention time	Area($\mu\text{V}\cdot\text{sec}$)	Height (μV)	USP Plate Count	USP Tailing
1	Simvastatin	3.213	859856	42659	7859	1.24
2	Simvastatin	3.253	857985	42598	7869	1.24
3	Simvastatin	3.297	856984	42587	7846	1.25
4	Simvastatin	3.215	856987	42569	7819	1.25
5	Simvastatin	3.254	859878	42894	7856	1.24
Mean			858338			
Std.dev			1454.222			
%RSD			0.169423			

Acceptance criteria:

- %RSD for sample should be NMT 2
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Table-: Results of repeatability for Ezetimibe:

S. No.	Peak Name	Retention time	Area($\mu\text{V}\cdot\text{sec}$)	Height (μV)	USP Plate Count	USP Tailing
1	Ezetimibe	5.441	5697	3659	6592	1.36
2	Ezetimibe	5.442	5689	3648	6539	1.36
3	Ezetimibe	5.409	5698	3692	6584	1.37
4	Ezetimibe	5.520	5639	3648	6579	1.36
5	Ezetimibe	5.424	5688	3689	6549	1.36
Mean			5682.2			
Std.dev			24.57031			
%RSD			0.432408			

Intermediate precision:

Table-: Results of Intermediate precision for Simvastatin

S.No.	Peak Name	RT	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	USP Plate count	USP Tailing
1	Simvastatin	3.211	868956	43659	7985	1.26
2	Simvastatin	3.211	869857	43985	7954	1.27
3	Simvastatin	3.210	865983	43879	7946	1.26
4	Simvastatin	3.212	866587	43865	7963	1.27
5	Simvastatin	3.211	864256	43875	7964	1.26
6	Simvastatin	3.297	868974	43562	7942	1.26
Mean			867435.5			
Std. Dev.			2167.095			
% RSD			0.249828			

Acceptance criteria:

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

Table-: Results of Intermediate precision for Ezetimibe

S.No.	Peak Name	RT	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	USP Plate count	USP Tailing
1	Ezetimibe	5.411	5785	3789	6659	1.37
2	Ezetimibe	5.410	5798	3758	6625	1.38
3	Ezetimibe	5.420	5766	3746	6649	1.38
4	Ezetimibe	5.423	5746	3795	6675	1.37
5	Ezetimibe	5.419	5782	3761	6653	1.38
6	Ezetimibe	5.409	5786	3752	6627	1.37
Mean			5777.167			
Std. Dev.			18.40018			
% RSD			0.318498			

Acceptance Criteria:

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

Table-: Results of Intermediate precision Day 2 for Simvastatin

S.No.	Peak Name	RT	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	USP Plate Count	USP Tailing
1	Simvastatin	3.211	845985	44585	8025	1.27
2	Simvastatin	3.233	847895	44895	8069	1.28
3	Simvastatin	3.244	848985	44758	8046	1.27
4	Simvastatin	3.297	847859	44548	8094	1.28
5	Simvastatin	3.297	845984	44865	8042	1.28
6	Simvastatin	3.202	847898	44254	8076	1.27
Mean			847434.3			
Std. Dev.			1201.345			
% RSD			0.141763			

Acceptance Criteria:

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

Table-: Results of Intermediate precision Day 2 for Ezetimibe

S.No.	Peak Name	RT	Area ($\mu\text{V}*\text{sec}$)	Height (μV)	USP Plate Count	USP Tailing
1	Ezetimibe	5.411	5898	3986	6852	1.39
2	Ezetimibe	5.410	5884	3955	6864	1.39
3	Ezetimibe	5.420	5863	3956	6829	1.40
4	Ezetimibe	5.405	5845	3945	6874	1.39
5	Ezetimibe	5.409	5896	3925	6829	1.39
6	Ezetimibe	5.463	5874	3962	6825	1.40
Mean			5876.667			
Std. Dev.			20.39281			
% RSD			0.347013			

Acceptance Criteria:

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

ACCURACY:**Table-: The accuracy results for Simvastatin**

% Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	451144.3	25	24.998	99.992%	100.1873%
100%	897248.3	50	50.104	100.208%	
150%	1344562	75	75.278	100.362%	

Acceptance Criteria:

- The percentage recovery was found to be within the limit (98-102%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

Table-: The accuracy Results for Ezetimibe

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	2895	15	15.084	100.560%	100.748%
100%	5685.333	30	30.282	100.940%	
150%	8449	45	45.335	100.744%	

Acceptance Criteria:

- The percentage recovery was found to be within the limit (98-102%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

Table-: Results for Robustness

Parameter used for sample analysis	Peak Area	Ezetimibe Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.1mL/min	5698	5.405	6582	1.36
Less Flow rate of 0.9mL/min	6452	6.250	6785	1.32
More Flow rate of 0.8mL/min	5254	4.863	6365	1.34
Less organic phase (about 5 % decrease in organic phase)	5487	6.196	6254	1.38
More organic phase (about 5 % Increase in organic phase)	5369	5.010	6298	1.33

Acceptance Criteria:

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

CONCLUSION:

High performance liquid chromatography is at present one of the most sophisticated tool of the analysis. The estimation of Simvastatin and Ezetimibe was done by RP-HPLC.

The TEA buffer was p^H 4.8 and the mobile phase was optimized with consists of Methanol: TEA buffer mixed in the ratio of 32:68 % v/v.

A Phenomenex Gemini C18 (4.6mm×150mm, 5.0 μ m) particle size or equivalent chemically bonded to porous silica particles was used as stationary phase.

The solutions were chromatographed at a constant flow rate of 1.0 ml/min. The linearity range of Simvastatin and Ezetimibe were found to be from 30-70 μ g/ml, 10-50 μ g/ml respectively. Linear regression coefficient was not more than 0.999, 0.999.

The values of % RSD are less than 2% indicating accuracy and precision of the method. The percentage recovery varies from 98-102% of Netupitant and Palonosetron. LOD and LOQ were found to be within limit.

The results obtained on the validation parameters met ICH and USP requirements. It inferred the method found to be simple, accurate, precise and linear.

The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision.

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