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Development and validation of ezetimibe using RP-HPLC

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Abstract
A simple, rapid and sensitive RP-HPLC and method was developed and validated for estimation of Ezetimibe by using in bulk and tablet dosage form. Chromatography was carried out by using C18,5µ (250×4.6) mm phenomenex column as a stationary phase with mobile phase containing a mixture of potassium dihydrogen phosphate and disodium hydrogen phosphate (Ph-7): Acetonitrile in the ratio of 25:75 v/v as mobile phase. The flow rate was 1ml/min. The effluent was monitored at 232nm with the retention time 3.8 mins of drug. Calibration curve was plotted within the range of 6-10µg/ml for ezetimibe and correlation was found to be 0.999. The accuracy range was found to be 98-102%. The %RSD values for all parameters was found to be 2 for. The proposed method can be useful for routine determination of Ezetimibe in pharmaceutical dosage form and the method is validated according to ICH guidelines.

Keywords: ezetimibe, RP-HPLC, and ICH guidelines

1. Introduction
Ezetimibe is a Dietary Cholesterol Absorption inhibitor. It is an azetidinone derivative and Anticholesteremic Agent that inhibits the intestinal sterol absorption. It is used to reduce total LDL, cholesterol level, Apolipoproteins B in the treatment of Hyperlipidemias. Ezetimibe appears to interact physically with cholesterol transporters at the brush borders of the small intestine and inhibits the clearance of cholesterol and related phytosterols. As a result, ezetimibe causes a decrease in the level of blood cholesterol or an increase in the clearance of cholesterol from the blood stream [1]. A literature survey revealed that a few UV spectroscopic methods, and chromatographic methods like HPLC have been reported for the estimation of Ezetimibe in pharmaceutical formulation. But their method has longer retention time for HPLC. The present studies involve development of RP-HPLC using simple mobile phase containing acetonitrile and buffer for quantitative estimation of ezetimibe in Bulk and tablet dosage forms which requires. The developed method was validated as per ICH guidelines.

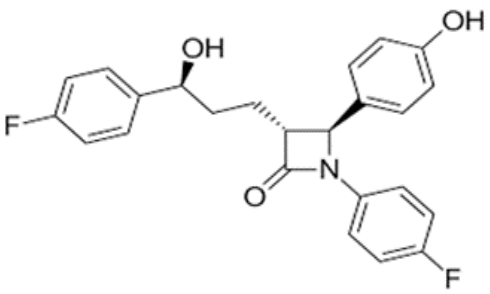

Fig 1: Chemical structure of Ezetimibe

2. Materials and method
2.1 Materials
Qualified standards and samples of Ezetimibe were obtained from Aurobindo Pharmaceutical pvt. ltd, and Lupin pvt. ltd. Analytical/HPLC graded chemicals and solvents used were obtained from different manufacturers methanol, acetate, acetonitrile, potassium dihydrogen phosphate, disodium hydrogen phosphate buffer were obtained from S D Fine Chem Limited, Thermo Fishers Scirntific India

2.2 Method
Instrumentation: Quantitative analysis was performed on isocratic high-performance liquid chromatography system of Shimadzu model LC-20AD and pumps connected with UV detector. The data acquisition was performed by LC solution software. UV spectrophotometer of Elico model 210 and performed. Sonicator Labotech, Analytical balance Con tech, pH meter Elico.

Developed Method
A series of ezetimibe solution ranging from 6-10µg/ml (RP HPLC), of standard ezetimibe solutions was transferred into series of 10ml calibrated flasks and all were made up to the mark with Dual phosphate buffer pH 7.0and the absorbance was measured at 232nm against the blank. A calibration curve was constructed for ezetimibe by plotting absorbance versus concentration. A representative UV spectrum and calibration curve in phosphate buffer pH 7 are represented in Fig.3 and Fig.2, respectively. The optical characteristics such as Beer's law limit, molar absorptivity was calculated and summarized in Table 2. Regression equation, correlation coefficient, slope and intercept are also in Table 10.

Separation was carried out using a phenomenex C18 reverse phase column of 250×4.6mm 5µm particle size at ambient temperature. The mobile phase consisted of acetonitrile and phosphate buffer in the ratio of 75:25 v/v with flow rate of 1ml/min. was eluted. The retention time was found to be 3.8 minutes, Standard chromatogram of ezetimibe was shown in figure-5 and detection was carried out at 232nm using UV-visible detector.

2.3 Preparation of Solutions

2.3.1 Preparation of dual phosphate buffer pH (7)

Measured accurately 0.05gms of Potassium dihydrogen phosphate and 0.030gms of anhydrous disodium hydrogen phosphate and transferred to 100ml volumetric flask and to this added 100ml of HPLC grade water and adjusted the pH-7 by anhydrous disodium hydrogen phosphate, and Filtered through 0.45µ membrane filter.

2.3.2 Preparation of Mobile Phase

Mobile phase was prepared by Mixing 500 volume of pH 7 buffer and 500 volumes of Acetonitrile and degassed the mixture before use.

2.3.3 Preparation of standard stock solution (1000µg/ml)

Accurately weighed and powdered 10 tablets, and transferred a quantity of tablet powder equivalent to 10mg EZETIMIBE into 10ml volumetric flask. Added 6ml diluents and sonicated for 30 minutes with occasional shaking and, diluted to volume with diluents. i.e., (1000µg/ml)

2.3.4 Preparation of Primary standard solution (100µg/ml)

Pipette out 1ml from the sample stock solution then transfer into the 10ml volumetric flask. Add diluent to make up the volume. i.e., (100µg/ml)

2.3.5 Preparation of Secondary standard (10µg/ml)

1ml each of sample stock solutions were pipetted from the above sample stock solution into a 10-mL volumetric flask and diluted up to the mark with diluent.

2.3.6 Preparation of sample stock solution (1000µg/ml)

Accurately weighed and powdered 10 tablets, and transferred a quantity of tablet powder equivalent to 10mg EZETIMIBE into 10ml volumetric flask. Added 6ml diluents and sonicated for 30 minutes with occasional shaking and, diluted to volume with diluents. i.e., (1000µg/ml)

2.3.7 Preparation of Primary sample solution (100µg/ml)

Pipette out 1ml from the sample stock solution then transfer into the 10ml volumetric flask. Add diluent to make up the volume. i.e., (100µg/ml)

2.3.5 Preparation of Secondary sample (10µg/ml)

1ml each of sample stock solutions were pipetted from the above sample stock solution into a 10-mL volumetric flask and diluted up to the mark with diluent.

System suitability

Preparation of STD 10µg/ml: Pipette 1ml of solution from primary stock solution into 10ml volumetric flask and make up the volume up to the mark.

- Standard 8µg/ml solutions were injected six times as per test method and chromatograms were recorded.

Linearity

Preparation of std 2 µg/ml

1.0 ml were pipetted from the above secondary standard solution into a 10ml volumetric flask and diluted up to the mark with diluent.

Preparation of std 4µg/ml

2 ml were pipetted from the above secondary standard solution into a 10ml volumetric flask and diluted up to the mark with diluent.

Preparation of std 6µg/ml

3 ml were pipetted from the above secondary standard solution into a 10ml volumetric flask and diluted up to the mark with diluent.

Preparation of std 8µg/ml

4 ml were pipetted from the above secondary standard solution into a 10ml volumetric flask and diluted up to the mark with diluent.

Preparation of std 10µg/ml

1ml of standard stock solutions were pipetted from the above primary standard stock solutions into a 10-mL volumetric flask and diluted up to the mark with diluent.

Accuracy

Procedure

- **Preparation of standard 8µg/ml:** 4 ml were pipetted from the above standard solution into a 10-mL volumetric flask and diluted up to the mark with diluent.
- **Preparation of Sample solution of 8µg/ml:** 4 ml were pipetted from the above sample solution into a 10-mL volumetric flask and diluted up to the mark with diluent.
- **Preparation of sample 7.2µg/ml:** Pipette 3.2ml from secondary stock solution of Ezetimibe and transferred in to 10ml volumetric flask and dilute up to mark with diluents.
- **Preparation of 80%solution (7.2 µg/ml):** Pipette 3.2ml of standard solution and 4ml of sample solution and transfer into 10ml volumetric flask and make up the volume up to mark with mobile phase.
- **Preparation of 100% solution (8 µg/ml):** Pipette 4ml of standard solution and 4ml of sample solution and transfer into 10ml volumetric flask and make up the volume up to mark with mobile phase.
- **Preparation of 120% solution (8.8 µg/ml):** Pipette 4.8ml of standard solution and 4ml of sample solution and transfer into 10ml volumetric flask and make up the volume up to mark with mobile phase.

Sample solutions were prepared in triplicate for each level and analyzed as per test method. The individual % recovery, % average recovery and % RSD for recovery at each level were calculated and the results are found to be within limit.

Precision

Procedure

- **Preparation of 8ug/ml sample solution:** Pipette 4ml from secondary sample stock solution of EZETIMIBE and transferred into a 10-ml volumetric flask and diluted up to the mark with the mobile phase.

Robustness

Procedure

- **Preparation of 8ug/ml sample solution:** Pipette 4ml from secondary sample stock solution of EZETIMIBE and transferred into a 10-ml volumetric flask and diluted up to the mark with the mobile phase.
- **Change in flow rate (± 0.1 ml/min):** A study was conducted to determine the effect of variation in flow rate. The system suitability parameters were evaluated at the flow rate of 1.1 ml/min. and 0.9 ml/min. Chromatograms were recorded and listed below.
- **Change in mobile phase ratio (±.2):** A study to establish the effect of variation in mobile phase composition was conducted. Mobile phases were prepared by changing the volume of each component in mobile phase by absolute ±2%. The System suitability parameters were evaluated with the above mobile phases.
- **Change in pH (± 0.2):** A study to establish the Effect of variation in pH of buffer in mobile phase was conducted. Mobile phases were prepared with buffer having different pH between 6.8 and 7.2 System suitability parameters were evaluated by using the above mobile phases.

2.4 Validation parameters

2.4.1 System suitability

System suitability is defined as "the checking of a system, before or during analysis of unknowns, to ensure system performance." System suitability criteria may include such factors as plate count, tailing factor, retention and resolution.

2.4.2 Specificity

Specificity is the ability to asses unequivocally the analyte in the presence of components which may be expected to be present. Typically, these might include impurities, degradants, matrix, etc.

2.4.3 Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

2.4.4 Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision may be considered at three levels repeatability, intermediate precision and reproducibility

i) **Repeatability:** Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed as intra –assay precision.
ii) **Intermediate precision:** Intermediate precision expresses with in – laboratories variations: different days, different analyte, different equipment etc.
iii) **Reproducibility:** Reproducibility expresses the precision between laboratories

2.4.5 Limit of detection

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value

2.4.6 Limit of quantification

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantization limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and /or degradation products.

2.4.7 Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

2.4.8 Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

2.4.9 Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

2.5 Development Trials

Trial -1

Diluent	**:** Mobile phase
Column	**:** phenomenex
Mobile phase	**: (**pH-5) Acetonitrile: Acetate buffer (50:50)
Injection volume	**:** 20ul
Flow rate	**:** 1.0ml/min
Detection wavelength	**:** 232nm
Retention time	: 6.8mins
Inference	**:** Theoretical plate is not with in the limit

Trail -2

Diluent	**:** Mobile phase
Column	**:** Phenomenex
Mobile phase	**:** (pH 6.8) acetonitrile: phosphate buffer (80:20)
Injection Volume	**:** 20µL

Flow rate : 1.0ml/min
Detection wavelength: 232nm
Retention time : 12.5mins
Inference : The retention time is very high. Buffer is changed.

Trail -3
Diluent : Mobile phase
Column : Phenomenex (250 x 4.6mm) 5µ
Mobile phase : (pH 4) Acetonitrile: phosphate buffer (70:30)
Injection Volume : 20 µl
Flow rate : 1.0 ml/ min
Detection wavelength: 232nm
Retention time : 6.5mins
Inference : More column washing is required for the column to get stabilized, peak is assymetric.

Trail-4
Diluent : Mobile phase
Column : Phenomenex (250 x 4.6mm) 5µg
Mobile phase : (pH 7) Acetonitrile: phosphate buffer (70:30)
Injection Volume : 20 µl
Flow rate : 1.0 ml/ min
Detection wavelength: 232nm
Retention time : 4.3mins
Inference : To reduce the retention time, mobile phase ration is changed.

2.6 Optimized chromatogram
Diluent : Mobile phase
Column : Phenomenex (250 x 4.6mm) 5µg
Mobile phase : **(**pH 7) Acetonitrile: phosphate buffer (75:25)
Injection Volume : 20 µl
Flow rate : 1.0 ml/ min
Detection wavelength: 232nm
Retention time : 3.8mins

3. Results and Discussion of RP-HPLC

Results of RP-HPLC
System suitability

Table 1: Results of system suitability

S.NO	RT	Area	Theoretical plate	Tailing factor
1	3.828	446270	8222.976	1.286
2	3.820	461998	8324.106	1.286
3	3.825	460772	8370.590	1.326
4	3.821	460682	8245.482	1.269
5	3.835	460682	8252.501	1.312
6	3.832	460969	8226.748	1.300
MEAN	3.827	458539		
SD		233.98		
%RSD		0.05%		

Linearity

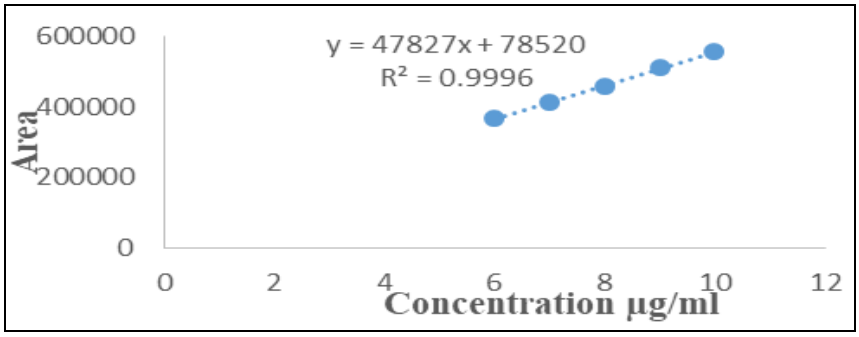

Fig 2: Calibration plot of Ezetimibe

Fig 3: Spectrum of Ezetimibe

Table 2: Results of Linearity

Concentration	Retention time	Peak Area	Theoretical plate	Tailing factor
6µg/ml	3.832	460969	8226.760	1.300
7µg/ml	3.818	367069	8274.370	1.306
8µg/ml	3.838	510755	8617.781	1.328
9µg/ml	3.818	367069	8274370	1.306
10µg/ml	3.827	562253	9155.460	1.283

Accuracy

Table 3: Results of Accuracy

Sample ID	Concentration(µg/Ml)		Area	% Recovery	Avg. % Recovery	SD	% Rsd
	Amount of Pure Drug	Amount of Sample					
80%	3.2µg/Ml	4µg/Ml	365147	99%	99.4%	2069.5	0.5%
80%	3.2µg/Ml	4µg/Ml	368852	100.2%			
80%	3.2µg/Ml	4µg/Ml	364003	98.9%			
100%	4µg/Ml	4µg/Ml	457959	99.5%	99.3%	677.7	0.1%
100%	4µg/Ml	4µg/Ml	459455	98.8%			
100%	4µg/Ml	4µg/Ml	459327	99.7%			
120%	4.8µg/Ml	4µg/Ml	547608	99.2%	99.8%	2143.5	0.3%
120%	4.8µg/Ml	4µg/Ml	551208	99.8%			
120%	4.8µg/Ml	4µg/Ml	554620	100.4%			

Precision

Table 4: Results of Intraday precision

S. No	Concentration	Retention Time	Area	Assay
1	8ug/ml	3.828	446270	100.2%
2	8ug/ml	3.820	461998	99.90%
3	8ug/ml	3.825	460772	99.88%
4	8ug/ml	3.821	460682	99.84%
5	8ug/ml	3.838	460545	99.95%

6	8ug/ml	3.832	460969	99.70%
	MEAN	3.827	458539	
	SD	0.68	233.98	
	%RSD	0.001%	0.05%	

Table 5: Interday Precision

S. No	Concentration	Retention Time	Area	Assay
1	8 ug/ml	3.817	462031	100.23%
2	8 ug/ml	3.816	460058	99.71%
3	8 ug/ml	3.817	461540	100.10%
4	8 ug/ml	3.832	463425	100.59%
5	8 ug/ml	3.821	467150	101.57%
6	8 ug/ml	3.811	466719	101.45%
	MEAN	3.819	463487	
	SD	0.0529	2631.18	
	%RSD	1.3%	0.5%	

Robustness

Table 6: Results of Change in Flow rate

S.NO	1.1mi/min			0.9ml/min		
	Rt	AREA	% ASSAY	Rt	AREA	% ASSAY
1	3.777	461523	100.1%	3.936	460269	99.77%
2	3.774	460751	99.89%	3.944	462728	10041%
3	3.774	464149	100.78%	3.939	464941	100.99%
MEAN	3.775	462141	-	3.9396	462646	-
SD	0.0041	1159.31	-	0.0032	1908.2	-
%RSD	0.03%	0.2%	-	0.008%	0.41%	-

Table 7: Results of Change in Mobile phase ratio

S.NO	73:27			77:23		
	Rt	AREA	% ASSAY	Rt	AREA	%ASSAY
1	3.951	460830	99.92%	3.773	460095	99.7%
2	3.912	463081	10050%	3.775	461855	10018%
3	3.919	464893	100.98%	3.774	462635	100.39%
MEAN	3.9273	462934	-	3.774	461528	-
SD	0.0167	1659.9	-	0.001	1062.36	-
%RSD	0.4%	0.3%	-	0.02%	0.2%	-

Table 8: Results of Change in pH

S.NO	pH-6.98			pH-7.02		
	Rt	AREA	% ASSAY	Rt	AREA	% ASSAY
1	3.958	466286	101.34%	3.768	466600	101.4%
2	3.950	1462473	100.34%	3.762	466397	101.3%
3	3.950	462570	100.37%	3.777	464951	100.9%
MEAN	3.9526	46377	-	3.769	469582	-
SD	0.0037	1775.0	-	0.1634	734.1	-
%RSD	0.09%	0.3%	-	0.1%	0.1%	-

Assay of pharmaceutical formulation

Table 9: Results of Assay

Concentration (µg/ml)	Area
6µg/ml	367069
7µg/ml	411473
8µg/ml	459990
9µg/ml	510387
10 µg/ml	556745
SAMPLE	461998(100.1%)

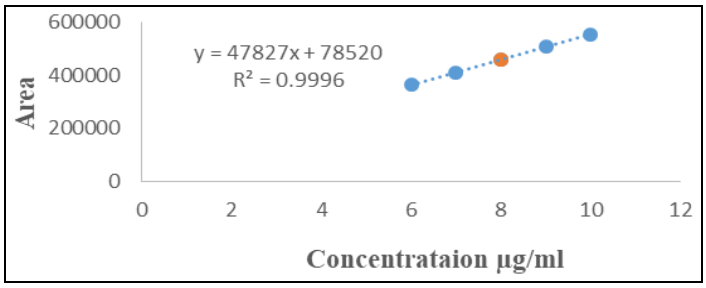

Fig 4: Assay graph of Ezetimibe

Limit of detection and limit of quantification

Table 10: Results of Limit of Detection and Limit of Quantification

Sample	Intercept	Slope	LOD	LOQ
EZETIMIBE	47827	78520	3.3 x σ/S= 3.3x3682.89/48231 =0.25ug/ml	10 x σ/S = 10x3682.89/48231 =0.076ug/ml
	47860	78702		
	49006	70800		
Mean	48231	76007		
S. D	3682.89			

5. Conclusion

The developed method was validated as per the International Conference on Harmonization ICH (Q2B) Guidelines, and was found to be applicable for routine quantitative analysis of Ezetimibe by RP-HPLC tablet and bulk dosage dosage forms. This method has been found to be better than previously reported methods, due to its high sensitivity. Hence above method can be used for routine analysis of tablets of Ezetimibe without any interference.

Table 11: Comparison of previous methods and present RP-HPLC method

Parameter	Previous method	Present RP-HPLC method
Retention time	4.5-14.5mins	3.8mins
Linearity	2ug/ml-250ug/ml	6µg/ml-10ug/ml
LOD	5-286.77ug/ml	0.25ug/ml
LOQ	3.2-869.01ug/ml	0.07ug/ml
Precision (% RSD)	0.92%-1.18%	0.3%-0.3%
Accuracy (% recovery)	98%-100%	99.8% - 100.4%

Table 12: Results of RP-HPLC

S.no	Parameter	RP-HPLC
1.	Wave length	232nm
2.	Linearity	6µg/ml-10ug/ml
3.	Correlation coefficient	0.999
4.	LOD	0.25ug/ml
5.	LOQ	0.07ug/ml
6.	Precision(%RSD)	0.1%
7.	Accuracy (%recovery)	99.2%-100.4%

6. Reference

1. https://pubchem.ncbi.nlm.nih.gov/compound/Ezetimibe#section=Top
2. www.pharmatutor.org/pharma-analysis/analytical-aspects-of-uv-visible-spectroscopy/electromagnetic-radiation.htmlwww.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf
3. www.drugbank.ca/drugs/DB00973
4. Akmar SK, Lata Kothapalli, Asha Thomas, Sumitra Jangam, Deshpande AD. For Reverse phase high performance liquid chromatography method for estimation of Ezetimibe in bluk and pharmaceutical formulations. International Journal of Pharm Tech Research. 2007; 69(5)-697.
5. Baokar shrikrishna b.1, erande r.S.2 and shaikh s.G.2 on for analytical method development and validation for estimation of ezetimibe from tablet dosage form by using rp-hplc International Journal of Research in Pharmaceutical and Biomedical Sciences, 2017; 2(2).ISSN:2229-3701.
6. Baby P, Sudha lakshmi, Ramchandran D, Rambabu CR. For spectrophotometric determination of ezetimibe, E-Journal of Chemistry, 2009; 7(1):101-104.ISSN:0973-4945.
7. Chandra Raju, Alam MD Naushad, Sharma Keshav Dutt, Singh Sujeet, Kumar Sanjay, Verma Daleep 17/07/13 for Develop a new rp-hplc method and validation for quantitative drug content determination of ezetimibe from marketed formulated tablets.
8. Ghanshyam Panjwani, Ragvendra Shetty, Gautham Shenoy and Dinesh Dhamecha. For Development and Validation of a new Reversed-phase HPLC Method for the Determination of Ezetimibe in Pharmaceutical Dosage forms, Indian Journal of Pharmaceutical Education and Research, 2013; 47(1).