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DEVELOPMENT AND VALIDATION OF UV SPECTROPHOTOMETRIC METHOD AND RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF EZETIMIBE AND FLUVASTATIN IN SYNTHETIC MIXTURE

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ABSTRACT

An accurate, precise and reproducible UV-spectrophotometric methods and liquid chromatographic assay method were developed and validated for the determination of Ezetimibe and Fluvastatin in synthetic mixture. Spectrophotometric estimation was done by derivative spectroscopic method and methanol as solvent. In this method λmax for Ezetimibe and Fluvastatin were selected at 245 nm and 270nm. RP-HPLC analysis was carried out using Thermo C-18 column (4.6 x 250mm, 5µ particle size) and mobile phase composed of Acetonitrile : water pH 3.3 (60:40% v/v)at a flow rate of 1.0 ml/min and chromatogram was recorded at 235 nm. Linearity was evaluated over the concentration range of 1 -6 µg/ml and 8-48 µg/ml for Ezetimibe and Fluvastatin in UV spectrophotometric and in RP-HPLC method Linearity was evaluated over the concentration range of 1-6 µg/ml and 8-48 µg/ml for Ezetimibe and Fluvastatin (the value of r^2 = 0.9985 and r^2= 0.9992 found were by UV method for Ezetimibe and Fluvastatin and the value of r^2 = 0.9989 and r^2= 0.9993 found were by RP-HPLC method for Ezetimibe and Fluvastatin). The developed methods were validated according to ICH guidelines and values of accuracy, precision and other statistical analysis were found to be in good accordance with the prescribed values therefore the both methods can be used for routine monitoring of Ezetimibe and Fluvastatin in the assay of Synthetic mixture of both drugs.

INTRODUCTION:

Ezetimibe is selective cholesterol absorption inhibitor. It is an anti-hyperlipidemic agent. It is used in treatment of Hypercholesterolemia. It inhibits the absorption of cholesterol and decreasing delivery of intestinal cholesterol to the liver. It is metabolized into its glucuronide in liver and small intestine which prevent absorption of cholesterol. Fluvastatin is HMG-CoA reductase inhibitor.3-Hydroxy 3-methyl glutaryl coenzyme A (HMG-COA) reductase is responsible for converting of HMG-CoA to mevalonate, the rate – limiting step in cholesterol biosynthesis. It is

used to reduce cholesterol levels and prevent cardiovascular disease. It decreases low density lipoprotein (LDL) cholesterol. From the literature survey, it was observed that various methods are reported for analysis of Ezetimibe and Fluvastatin individually as well as in combination with other drugs. But no analytical method has been reported for analysis of Ezetimibe and Fluvastatin synthetic mixture. A successful attempt has been made to estimate two drugs simultaneously by First order derivative Spectrophotometric method.

MATERIAL AND METHODS:

➢ **Instruments**

- UV Visible Spectrophotometer: Shimadzu model 1800
- Digital analytical weighing balance: Wenser DAB-220
- Melting point apparatus
- IR spectrophotometer: Model- Miracle-10, single reflection ATR accessory-8300, shimadzu

➢ **Chemicals and Materials:**

- Ezetimibe and Fluvastatin were as a gift sample supplied by (Torrent Pharmaceuticals, Ahmedabad) and (Intas Pharmaceuticals, Ahmedabad).
- Methanol (Aventor Performance Material, India) (AR Grade)

❖ **UV Spectrophotometric method:**

❖ **Identification of pure API:**

❖ **Melting point determination:**

- Melting point of Ezetimibe API and Fluvastatin API has been determined by open capillary method using Melting point apparatus in which the Ezetimibe and Fluvastatin were filled in Capillary tubes and kept in the Melting point apparatus.
- **Solubility determination:**
  The solubility study of Ezetimibe and Fluvastatin were determined by taking 10 mg of both drug in 10 ml volumetric flasks, add the required quantity of solvent and shaken for few minutes.

U.V Spectrophotometric Method:

First-order derivative method for Ezetimibe and Fluvastatin

➢ **Experimental work**

Instruments and Apparatus

- UV Visible Spectrophotometer: Shimadzu model 1800
- Digital Analytical balance – Wensar DAB – 220
- Sonicator- Equitron
- Volumetric Flask- 10,50,100 ml (Borosilicate)
- Measuring Cylinder- 10,50,100 ml (Borosilicate)

Chemical and Reagents

- Ezetimibe API (Torrent Pharmaceuticals, Ahmedabad)
- Fluvastatin API (Intas Pharmaceuticals, Ahmedabad)
- Methanol (Avantor Performance Material, India)

Spectrophotometric conditions

- Mode: Absorption (scanning)
- Scan Speed: Medium
- Wavelength Range: 200-400nm
- Initial Baseline Correction: Methanol (AR grade)

Preparation of standard stock solution

Preparation of standard stock solution of Ezetimibe (100µg/ml)

- Accurately weighed Ezetimibe (10mg) was transferred in to a 100ml volumetric flask, dissolved in Methanol and diluted to the mark with same solvent to obtain a standard stock solution (100µg/ml).

Preparation of working standard solution of Ezetimibe (1-6µg/ml)

- From the above 100µg/ml stock solution pippeted out 0.1ml, 0.2ml, 0.3ml, 0.4ml and 0.5ml and 0.6ml of solution and transferred to 10ml of volumetric flask and made up the volume up to 10ml with Methanol to produce concentration 1,2,3,4,5 and 6µg/ml respectively.

Preparation of standard stock solution of Fluvastatin (100µg/ml)

- Accurately weighed Fluvastatin (10mg) was transferred in to a 100ml volumetric flask, dissolved in Methanol and diluted to the mark with same solvent to obtain a standard stock solution (100µg/ml).

Preparation of working standard solution of Fluvastatin (8-48µg/ml)

- From the above 100µg/ml stock solution pippeted out 0.8ml, 1.6ml, 2.4ml, 3.2ml, 4ml and 4.8ml of solution and transferred to a 10ml volumetric flask and made up the volume up to 10ml with Methanol to produce concentration 8, 16, 24, 32, 40, and 48µg/ml respectively.

Procedure of selection of wavelength:

0.2 ml standard stock solution of Ezetimibe (100 µg/ml) and 1.6 ml standard stock solution of Fluvastatin (100 µg/ml) was transfer in 10 ml volumetric flask and dilute up to mark with Methanol to get the 2 µg/ml of Ezetimibe and 16 µg/ml of Fluvastatin. Each solution was scanned in the range 200 – 400 nm.

The Spectra are converted to First Order Derivative. The zero crossing point (ZCP) of Ezetimibe was found to be 245 nm and ZCP of Fluvastatin was found to be 270 nm. Hence, these wavelengths 245 nm and 270 nm were selected as analytical wavelengths.

Preparation of Calibration Curve

Calibration curve for Ezetimibe:

An aliquots of stock solution of Ezetimibe (100 µg/ml) 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 ml were pipette out in 10 ml volumetric flask and was made up to the mark with Methanol which will give 1, 2, 3, 4, 5 and 6 µg/ml solution was prepared and absorbance was measured at 270 nm in U.V. Spectrophotometer.

Graph of Absorbance vs. Concentration (µg/ml) was plotted

Calibration curve for Fluvastatin:

An aliquots of stock solution of Fluvastatin (100 µg/ml) 0.8, 1.6, 2.4, 3.2, 4.0 and 4.8 ml were pipette out in 10 ml volumetric flask and was made up to the mark with Methanol which will give 8, 16, 24, 32, 40 and 48 µg/ml solution was prepared and absorbance was measured at 245 nm in U.V. Spectrophotometer.

Graph of Absorbance vs. Concentration (µg/ml) was plotted

METHOD VALIDATION: [9]

The developed method was validated with respect to linearity, accuracy, precision, limit of detection and limit of quantification in accordance with the ICH guideline.

LINEARITY & RANGE (n=6):

The linearity of Ezetimibe and Fluvastatin was taken to be in the range of 1-6 µg/ml and 8-48 µg/ml respectively. Calibration curve of Absorbance Vs Concentration was plotted and from that slope, intercept, correlation coefficient and regression line equation for Ezetimibe and Fluvastatin was constructed.

PRECISION:

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

Precision may be considered at three levels:

Intermediate (Intraday) precision, reproducibility (Interday precision), repeatability.

1) Intraday precision (n=3):

Solutions containing 1, 2, 3 µg/ml of Ezetimibe and 8, 16, 24 µg/ml of Fluvastatin were analyzed three times on the same day and % RSD was calculated.

2) Interday Precision (n=3):

Solutions containing 1, 2, 3 µg/ml of Ezetimibe and 8, 16, 24 µg/ml of Fluvastatin were analyzed three different successive days and % RSD was calculated.

3) Repeatability (n=6):

Solutions containing 2 µg/ml of Ezetimibe and 16 µg/ml of Fluvastatin were analyzed for six times and % R.S.D was calculated. % R.S.D was not more than 2%.

LIMIT OF DETECTION (LOD):

Limit of Detection can be calculated using following equation as per ICH guidelines.

$$LOD = 3.3 \times (\sigma / S)$$

Where, σ = standard deviation of the Y intercept of calibration curve

S = Mean slope of the corresponding calibration curve.

LIMIT OF QUANTIFICATION (LOQ):

Limit of Quantification can be calculated using following equation as per ICH guidelines.

$$LOQ = 10 \times (\sigma / S)$$

Where, σ = standard deviation of the Y intercept of calibration curve

S = Mean slope of the corresponding calibration curve.

ACCURACY (RECOVERY STUDY) (n=3):

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. Accuracy of the developed method was confirmed by doing recovery study as per ICH guideline at three different concentration levels 50%, 100%, 150% and the values were measured for Ezetimibe (**2 µg/ml**) and Fluvastatin (**16 µg/ml**). This performance was done in triplicate.

Preparation of sample solution:

- The synthetic mixture of Ezetimibe and Fluvastatin was prepared in ratio of 1:8
- Accurately weighed equivalently weight of Ezetimibe (10 mg) and Fluvastatin (80 mg) and transferred in 100 ml volumetric flask and made up to mark with methanol allow to sonicated.
- 0.2 ml from the above solution was pipetted out. This solution was filtered through whatman filter paper. The filtrate was diluted to the mark with methanol. The mixture contains 2 µg/ml of Ezetimibe and 16 µg/ml of Fluvastatin.

ASSAY:

Preparation of Synthetic Mixture: [5]

The synthetic mixture of Ezetimibe and Fluvastatin was prepared in ratio of 1:8

- Common excipients, Microcrystalline cellulose, Polyvinyl pyrrolidone, Magnesium stearate, Lactose, Talc along with the drug Ezetimibe 10 mg and Fluvastatin 80 mg.
- Accurately weighed equivalent weight of Ezetimibe (10mg) and Fluvastatin (80mg) and transferred in 100 ml volumetric flask and make up to half mark with methanol. This solution was sonicated and made up to mark with methanol.
- This solution was filtered through Whatman filter paper. The mixture contains 100 µg/ml of Ezetimibe and 800 µg/ml of Fluvastatin.

Preparation of Sample Solution:

- Accurately 0.2 ml of the above solutions was pipetted out into 10 ml volumetric flask and the volume was adjusted up to the mark with methanol. Final

concentration of Ezetimibe was 2 µg/ml and Fluvastatin was 16 µg/ml.

RESULT AND DISCUSSION:

- **Selection of wavelength for Ezetimibe and Fluvastatin**
- To determine the wavelength for measurement Ezetimibe (2 µg/ml) and Fluvastatin (16 µg/ml) solutions were scanned between 400-200 nm. Absorbance maximum were obtained at their λmax 245 nm and 270 nm for Ezetimibe and Fluvastatin respectively.

RP-HPLC Method

Experimental work:

Instrument and apparatus:

- Shimadzu HPLC (LC- 2010 –CHT) Instrument [software Lab solution]
- Column-Thermo C-18 (250×4.6 mm, 5 µm)
- Digital Analytical Balance – Wensar DA 13–220 (India)
- pH meter (Systronic India)
- Sonicator – Equitron (India)
- Volumetric flask – 10, 50 and 100 (Borosilicate)
- Pipettes – 1, 2, 5 and 10 ml (Borosilicate)
- Beaker – (Borosilicate)

Chemicals and Materials:

- Acetonitrile- Fisher India Ltd. (HPLC grade)
- Methanol- Fisher India Ltd. (HPLC grade)
- Water- Astron Chemical India. (HPLC grade)
- OPA (10% Ortho Phosphoric Acid) – Krishna Chem Industry
- Ezetimibe (Torrent Pharmaceuticals, Ahmedabad)
- Fluvastatin (Intas Pharmaceuticals, Ahmedabad)

5.2 Selection of Detection Wavelength:

The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. Absorbance maximum was obtained was at 235 nm. So, 235 nm was selected for detection of Ezetimibe and Fluvastatin in synthetic mixture.

5.3 Mobile phase selection:

- Various composition and pH of mobile phase were tried and changed for optimization. After number of trial experiments, it was established that the mobile phase ACN: water (pH 3.3 adjusts with Ortho phosphoric acid) (60:40 % v/v) shows good peak shape and resolution. The pKa value for Ezetimibe and Fluvastatin is 9.73 and 4.5 respectively.
- **Preparation of 10% Ortho phosphoric acid**

  10% Ortho phosphoric acid was prepared by diluting 1.33 ml of concentrated Orthophosphoric acid (70%) in 10 ml HPLC grade water.

5.4 Chromatographic condition:

- Column: Thermo C-18 (250 mm × 4.6 mm, 5 µm)
- Mobile phase: ACN: Water (pH 3.3 adjusts with Ortho phosphoric acid)

  (60:40 % v/v)
- Flow rate: 1 ml/min

- Run time: 10 min
- Detection wavelength: 235 nm
- Detector: U.V Detector
- Injection volume: 10 µl

5.5 Preparation of standard stock solution:

- Ezetimibe (100 µg/ml):

  Accurately weighed Ezetimibe (10 mg) was transferred to a 100 ml volumetric flask, dissolved and diluted to the mark with acetonitrile to obtain a standard stock solution (100 µg/ml).

- Fluvastatin (100 µg/ml):

  Accurately weighed Fluvastatin (10 mg) was transferred to a 100 ml volumetric flask, dissolved and diluted to the mark with acetonitrile to obtain a standard stock solution (100 µg/ml).

- **Method Validation**
- The developed method was validated with respect to specificity, selectivity, linearity, accuracy, precision, limit of detection and limit of quantification, robustness, assay in accordance with the ICH guideline

➢ **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.

➢ **Linearity & Range**

- The linearity of Ezetimibe and Fluvastatin was found to be in the range of 1-6µg/ml and 8-48 µg/ml respectively.

Preparation of Calibration Curve

- An aliquots of stock solution of Ezetimibe (100 µg/ml) 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 ml and Fluvastatin (100 µg/ml) 0.8, 1.6, 2.4, 3.2, 4, 4.8 ml was pipette out in 6 different 10ml volumetric flask, dissolved and diluted with mobile phase upto the mark to obtain concentration 1, 2, 3, 4, 5, 6 µg/ml of Ezetimibe and 8, 16, 24, 32, 40, 48 µg/ml of Fluvastatin.10µl of each solution were injected into HPLC system and analyzed. Calibration curve was obtained by plotting peak area (µV.s) vs. Concentration (µg/ml). Linearity of both the drugs was checked in term of slope, intercept and correlation coefficient

- **Precision**

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: Intermediate (Intraday) precision, reproducibility (Interday precision), repeatability.

1. **Intraday Precision (n=3**): Solution containing 1, 2, 3 µg/ml of Ezetimibe and 8, 16, 24 µg/ml of Fluvastatin were analyzed three times on the same day and %RSD was calculated.

2) Interday Precision (n=3): Solution containing 1, 2, 3 µg/ml of Ezetimibe and 8, 16, 24 µg/ml of Fluvastatin were analyzed three different successive days and %RSD was calculated.

3) Repeatability (n=6): Solutions containing 2 µg/ml of Ezetimibe and 16 µg/ml of

Fluvastatin were analyzed for six times and %RSD was calculated. %RSD was not more than 2%.

- **Limit of Detection (LOD):**

  Limit of Detection can be calculated using following equation as per ICH guidelines.

  $LOD = 3.3 \times (\sigma/S)$

  Where, σ = standard deviation of the Y intercept of calibration curve

  S = Mean slope of the corresponding calibration curve.

- **Limit of Quantification (LOQ):**

  Limit of Quantification can be calculated using following equation as per ICH guidelines.

  $LOQ = 10 \times (\sigma/S)$

  Where, σ = standard deviation of the Y intercept of calibration curve

  S = Mean slope of the corresponding calibration curve

- **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. Accuracy of the developed method was confirmed by doing recovery study as per ICH guideline at three different concentration levels 50%, 100%, 150% and the values were measured at all wavelengths for Ezetimibe (2 µg/ml) and Fluvastatin (16 µg/ml). This performance was done in triplicate. The amount of Ezetimibe and Fluvastatin were calculated at each level % recoveries by measuring the peak area and evaluating using specific equation.

- **Robustness**

  The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variation in method parameters and provides an indication of its reliability during normal usage. It should show the reliability of an analysis with respect to deliberate variation in method parameter.

- In case of liquid chromatography, examples of typical variations are:

- Influence of variations of pH in mobile phase;

- Influence of variation in mobile phase composition;

- Different columns

- Flow rate

- **System suitability tests**

  System suitability tests are an integral part of liquid chromatography. They are used to verify that resolution and reproducibility of chromatography system are adequate for the analysis to be done. The tests include Resolution (R), Column efficiency (N) and Tailing factor (T).

- **Assay:**

- **Preparation of Synthetic Mixture of Ezetimibe and Fluvastatin:**

- Accurately weighed powder equivalent to10mg Ezetimibe and

80mg Fluvastatin from the prepared synthetic mixture and transferred in 100 ml volumetric flask, dissolved and make up to the mark with acetonitrile. This solution was sonicated and filtered. The mixture contains 100 µg/ml of Ezetimibe and 800 µg/ml of Fluvastatin.

- **Preparation of Sample Solution:**
- From the above synthetic mixture solution 0.2 ml was pipetted out into 10 ml volumetric flask and made up to the mark the with mobile phase to obtain final concentration of Ezetimibe 2 µg/ml and Fluvastatin 16 µg/ml.

Result and Discussion

Selection of Elution Mode

- Reverse phase chromatography was chosen because of its recommended use for ionic and moderate to non-polar compounds. Reverse phase chromatography is not only simple, convenient but also performs better in terms of efficiency, stability and reproducibility.
- C_{18} column was selected because it is least polar compare to C_4 and C_8 columns. C_{18} column allows eluting polar compounds more quickly compare to non-polar compounds.
- In addition to this UV detector is used which allows easy detection of the compounds in UV transparent organic solvents. Hence, C_{18} (250×4.6mm) column of 5µm particles packing was selected for separation of Ezetimibe and Fluvastatin. Isocratic mode was chosen due to simplicity in application and robustness with respect to longer column stability.

Selection of Detection Wavelength:

- The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. Absorbance maximum was obtained was at 235 nm. So, 235 nm was selected for detection of Ezetimibe and Fluvastatin.
- **Chromatography**
  The mobile phase Acetonitrile: Water pH-3.3 (60:40%v/v) was selected because it was found to ideally resolve the peaks with retention time 6.58 min and 8.61 min for Ezetimibe and Fluvastatin, respectively. Thermo C_{18} (250×4.6 mm, 5 µm) column was used for separation of Ezetimibe and Fluvastatin with flow rate of 1.0 ml/min.
- **System suitability Parameters**

The retention time, resolution, tailing factor and number of theoretical plates are shown in table. The values obtain demonstrated the suitability of the system for the analysis of these drugs in combination.

Method Validation:

SPECIFICITY:

- It was prove by comparing the chromatogram of mobile phase, standard solution and test preparation solution to that there was no peak of mobile phase and no any interference of excipients with the peak of Ezetimibe and Fluvastatin.
- **LINEARITY**
  The linearity of Ezetimibe and Fluvastatin was found to be 1- 6µg/ml and 8-48µg/ml, respectively.

Table 1:- Melting Point of Ezetimibe and Fluvastatin

S.No	Drug	Reported melting point	Observed melting point
1	Ezetimibe	164-166°C	164-167°C
2	Fluvastatin	194-197°C	194-198°C

- **IR spectral determination:**

A) **Ezetimibe**

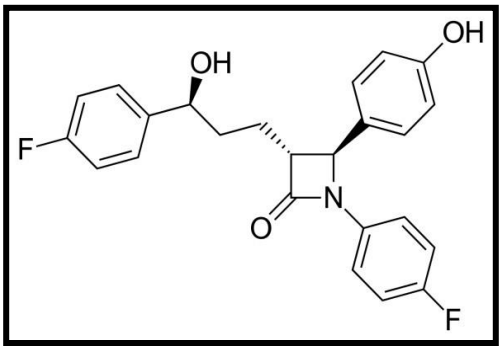

Fig 1 Structure of Ezetimibe

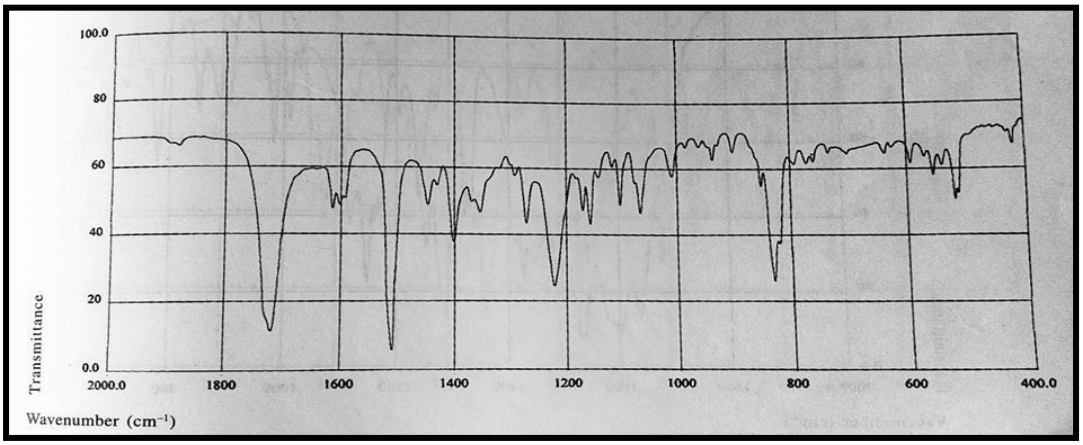

Fig 2 Ezetimibe Reference IR spectra

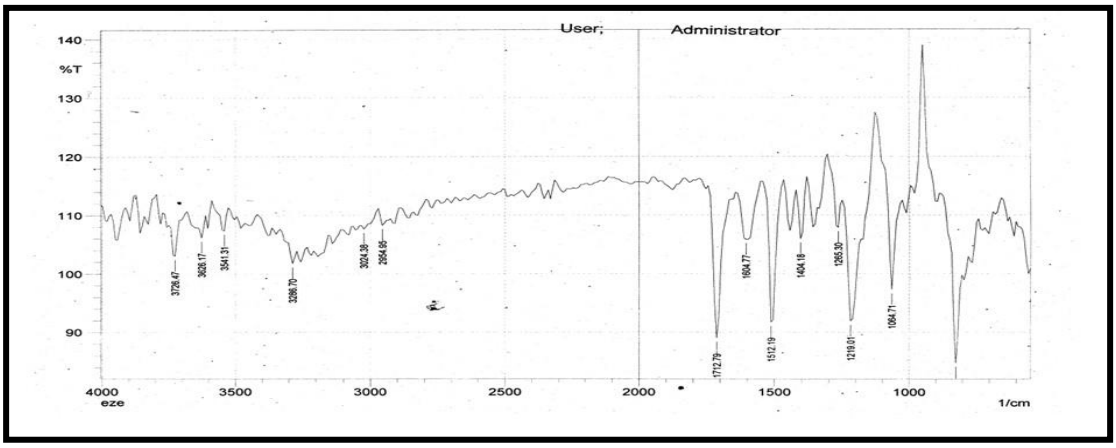

Fig 3 Sample IR Spectra of Ezetimibe

TABLE 2:- Interpretation of FT-IR spectra of Ezetimibe

S. No.	Functional group Characteristic	Standard Absorption (cm^{-1})	Observed Absorption (cm^{-1})
1	C-F	1400-1000	1265.3
2	C=C	1600-1450	1512.19
3	O-H	3550-3200	3286.7
4	C=O	1750-1680	1712.79
5	C-N	1350-1000	1219.01
6	C-H	3040-3010	3029.38

B) Fluvastatin

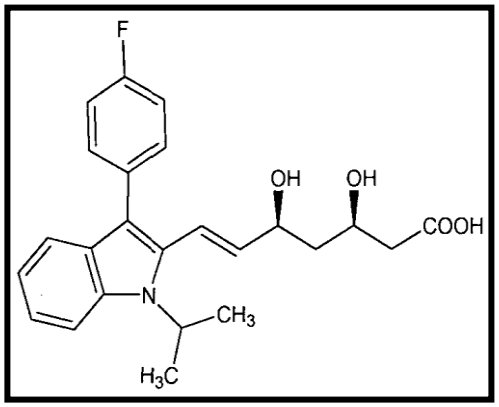

Fig 4 Structure of Fluvastatin

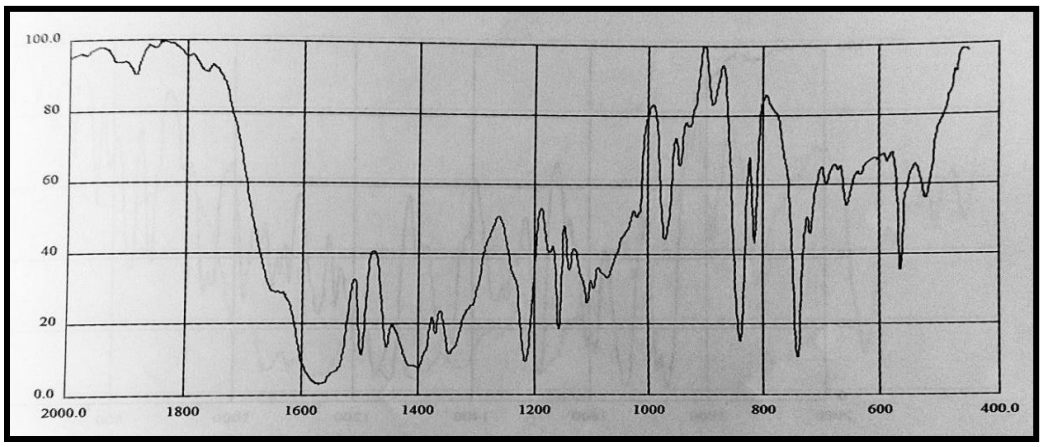

Fig 5 Fluvastatin Reference IR spectra

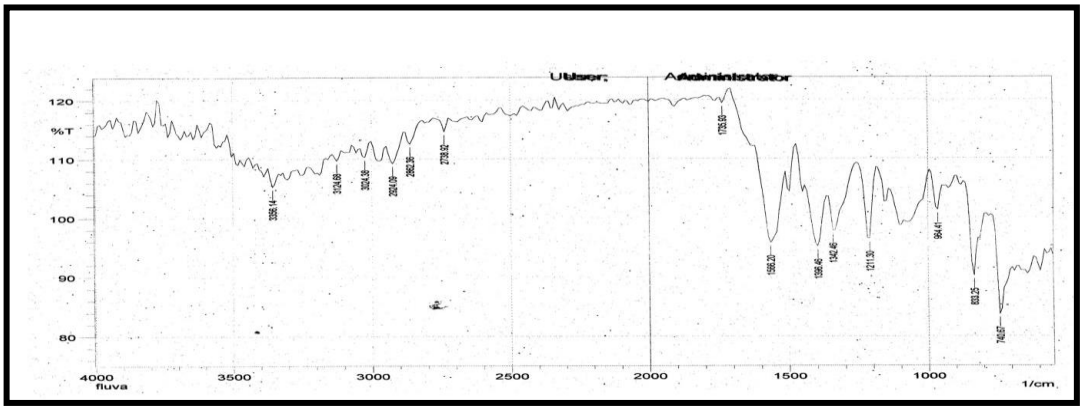

Fig 6 Sample IR Spectra of Fluvastatin

TABLE 3:- Interpretation of FT-IR spectra of Fluvastatin

S.No.	Functional group Characteristic	Standard Absorption (cm^{-1})	Observed Absorption (cm^{-1})
1	C-F	1400-1000	1396.46
2	C=C	1600-1450	1566.2
3	O-H	3550-3200	3356.14
4	C-H	3040-3010	3024.38
5	C-N	1350-1000	1211.3
6	C=O	1750-1680	1735.93

- **UV Identification:**

Table 4 Wavelength of Ezetimibe and Fluvastatin

Drug	Reported λmax (Methanol)	Observed λmax (Methanol)
Ezetimibe	230nm	230nm
Fluvastatin	305nm	304nm

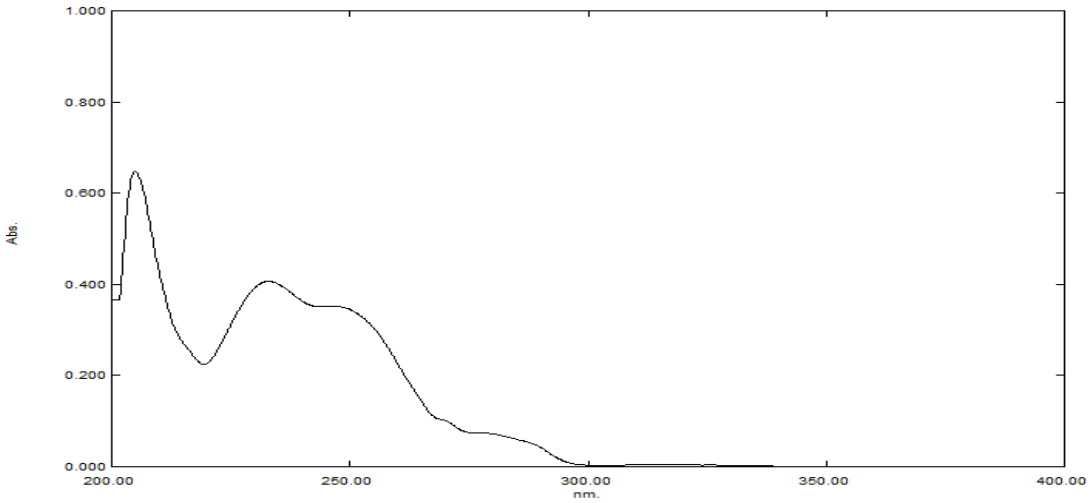

Fig 7 UV Spectra of Ezetimibe (2µg/ml)

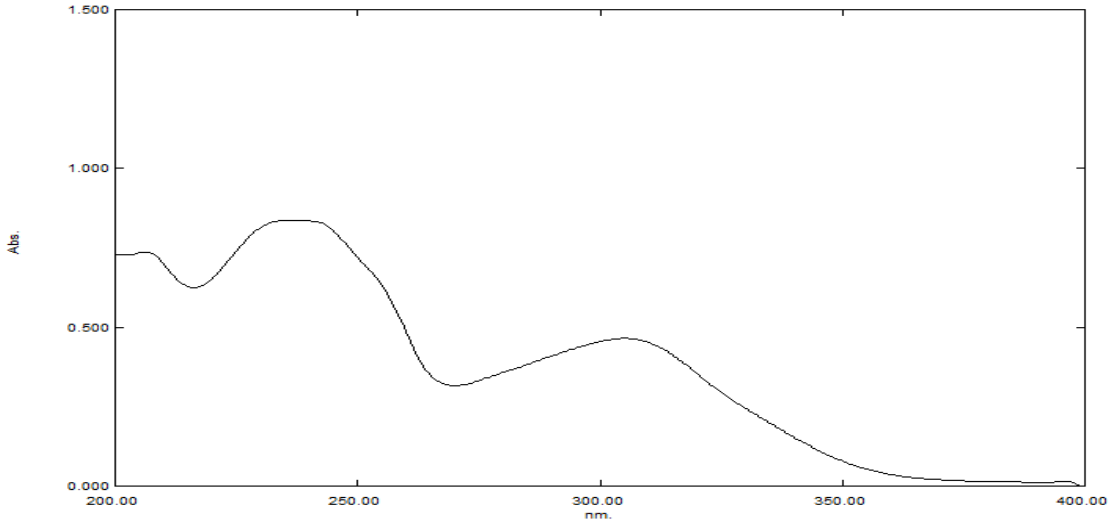

Fig 8 UV Spectra of Fluvastatin (16µg/ml)

Table 5:- Solubility of Ezetimibe and Fluvastatin

Solvent	Ezetimibe	Fluvastatin
Water	Insoluble	Soluble
Methanol	Soluble	Soluble

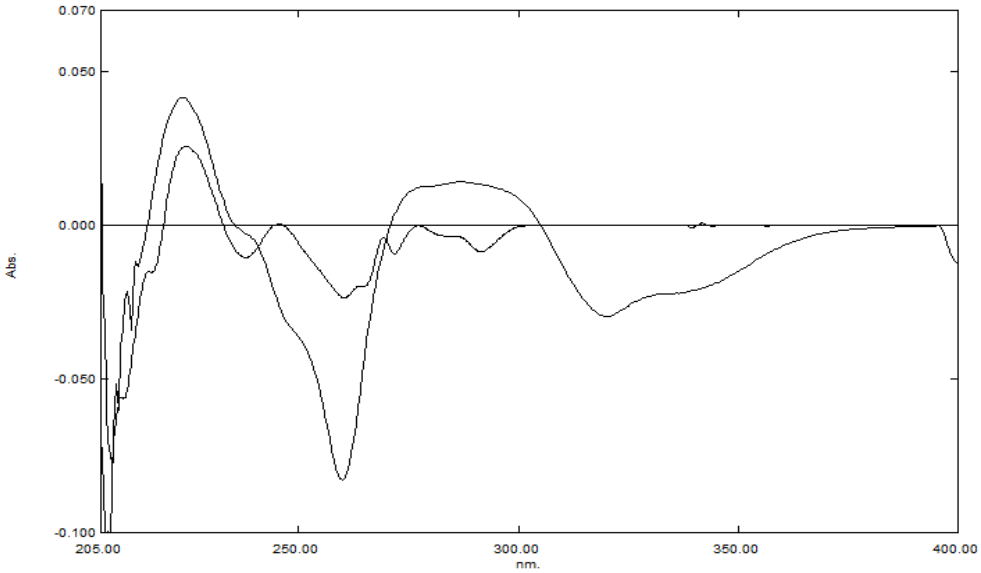

Fig 9: Zero crossing point of Ezetimibe at 245 nm (2µg/ml) and Fluvastatin at 270 nm (16µg/ml)

LINEARITY AND RANGE:

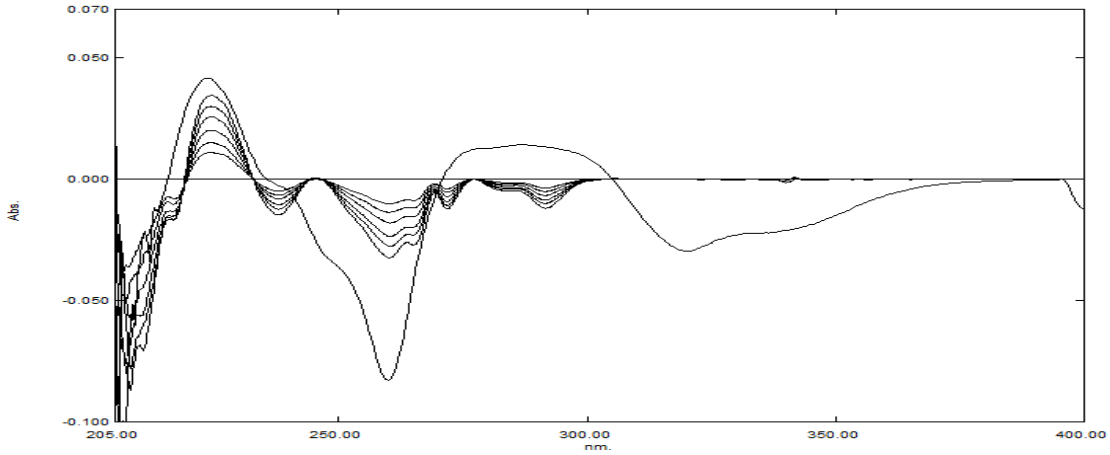

Fig 10: Linearity of 1st Derivative spectra of Ezetimibe (270 nm)

TABLE: 6 Linearity data of Ezetimibe

Concentration (µg/ml)	Mean Absorbance ± SD (n=6)	%RSD
1	|-0.0063|± 0.00010	1.643
2	|-0.0092|± 0.00012	1.310
3	|-0.0123|± 0.00013	1.118
4	|-0.0161|± 0.00016	0.993
5	|-0.0194|± 0.00017	0.886
6	|-0.0225|± 0.00019	0.860

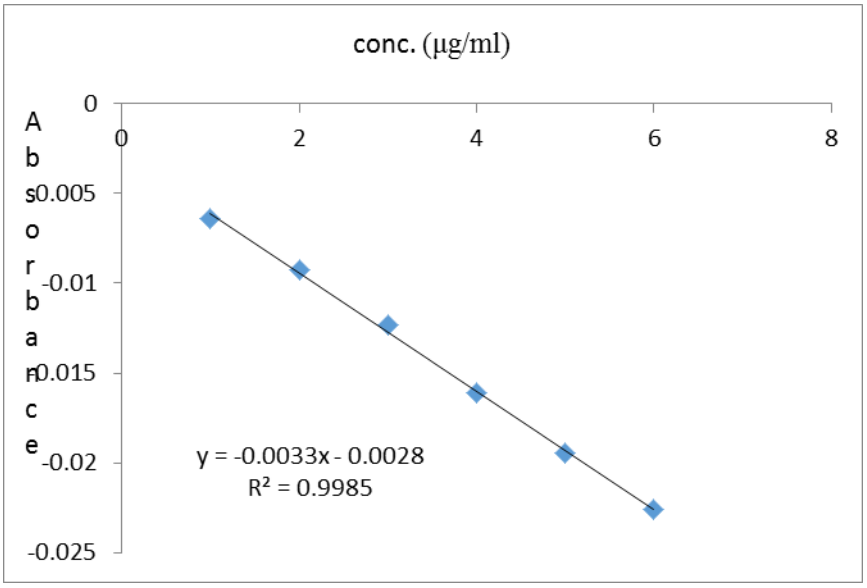

Fig 11 Calibration Curve of Ezetimibe (1-6 µg/ml)

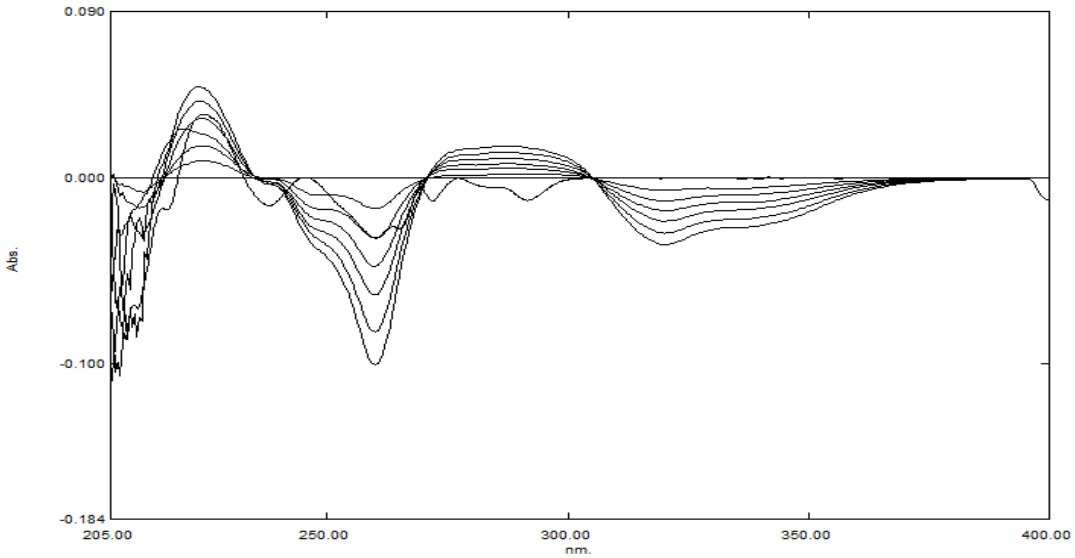

Fig 12: Linearity of 1st Derivative spectra of Fluvastatin (245 nm)

TABLE: 7 Linearity data of Fluvastatin

Concentration (µg/ml)	**Mean Absorbance ± SD (n=6)**	**%RSD**
8	|-0.0123|± 0.00017	1.416
16	|-0.0216|± 0.00025	1.191
24	|-0.0294|± 0.00027	0.929
32	|-0.0394|± 0.00030	0.779
40	|-0.0475|± 0.00034	0.733
48	|-0.0560|± 0.00036	0.652

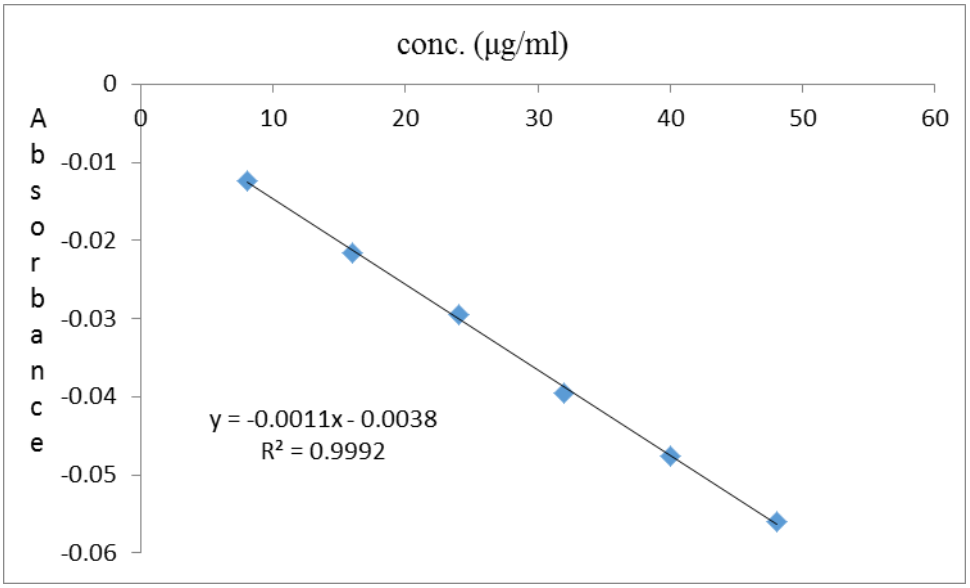

Fig 13: Calibration Curve of Fluvastatin (8-48 µg/ml)

➢ **PRECISION:**

TABLE 8: Precision study of Ezetimibe

Ezetimibe		
Intraday Precision of Ezetimibe		
Conc. (µg/ml)	**Mean Absorbance ± SD (n = 3)**	**% RSD**
1	|-0.0068|± 0.00011	1.689
2	|-0.0097|± 0.00010	1.030
3	|-0.0126|± 0.00011	0.911
Interday Precision of Ezetimibe		
1	|-0.0063|± 0.00011	1.813
2	|-0.0098|± 0.00017	1.767
3	|-0.0128|± 0.00017	1.353
Repeatability of Ezetimibe		
Conc. (µg/ml)	**Mean Absorbance ± SD (n = 6)**	**% RSD**
2	|-0.0094|± 0.00010	1.109

TABLE 9: Precision study of Fluvastatin

Fluvastatin		
Intraday Precision of Fluvastatin		
Conc. (µg/ml)	**Mean Absorbance ± SD (n = 3)**	**% RSD**
8	|-0.0122|± 0.00015	1.245
16	|-0.0215|± 0.00020	0.966
24	|-0.0294|± 0.00025	0.854
Interday Precision of Fluvastatin		
8	|-0.0124|± 0.00017	1.396
16	|-0.0216|± 0.00026	1.224
24	|-0.0295|± 0.00030	1.016
Repeatability of Fluvastatin		
Conc. (µg/ml)	**Mean Absorbance ± SD (n = 6)**	**% RSD**
16	|-0.0217|± 0.00022	1.050

- **LOD AND LOQ**

TABLE: 10 LOD and LOQ for Ezetimibe and Fluvastatin

Parameter	**Ezetimibe**	**Fluvastatin**
LOD (µg/ml)	0.1169	0.3987
LOQ (µg/ml)	0.3542	1.2083

- **ACCURACY**

TABLE 11: Recovery study

Name of Drug	**%Level of recovery**	**Amount Taken (µg/ml)**	**Amount Added (µg/ml)**	**Total Amount (µg/ml)**	**Amount Recoverd (µg/ml)**	**% recovery (n=3) ± SD**
Ezetimibe	50	2	1	3	2.96	98.66±0.0208
	100	2	2	4	3.95	98.74±0.0251
	150	2	3	5	4.97	99.44±0.0321
Fluvastatin	50	16	8	24	23.80	99.16±0.0264
	100	16	16	32	31.75	99.35±0.0351
	150	16	24	40	39.81	99.54±0.0404

TABLE: 12 Analysis of synthetic mixture

Name of Drug	Amount in Synthetic Mixture Taken (µg/ml)	Amount Found (µg/ml)	% Assay (n=3) ± SD
Ezetimibe	2	1.97	98.56 ±0.0360
Fluvastatin	16	15.86	99.15 ±0.0416

TABLE: 13 Summary of validation parameter

Parameter	Ezetimibe	Fluvastatin
Wavelength (nm)	270	245
Beer's Law Limit	1-6 µg/ml	8-48 µg/ml
Regression equation (y = mx +c)	y = -0.0033x - 0.0028	y = -0.0011x - 0.0038
Correlation Coefficient (r^2)	0.9985	0.9992
Intraday Precision (%RSD, n=3)	0.91-1.68	0.85-1.24
Interday Precision (% RSD, n=3)	1.35-1.81	1.09-1.39
Repeatability (% RSD, n=6)	1.109	1.050
Accuracy (% Recovery,n=3)	98.66-99.44	99.16-99.54
LOD (µg/ml)	0.1169	0.3987
LOQ (µg/ml)	0.3542	1.2083
Assay	98.56%	99.15%

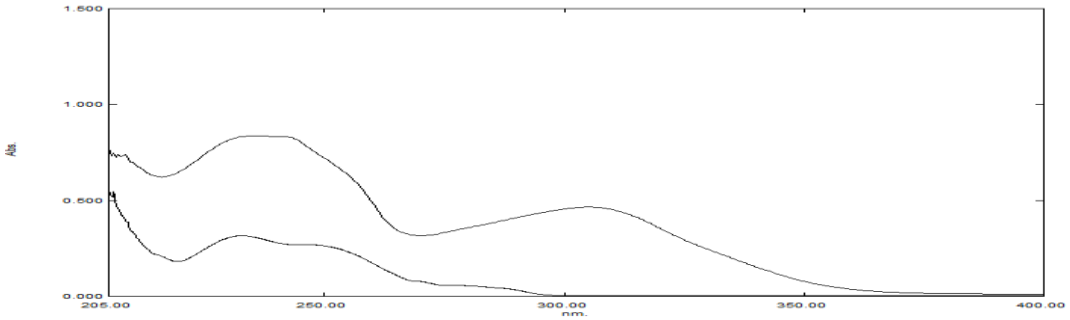

Figure 14 zero order spectra of Ezetimibe (2 µg/ml) and Fluvastatin (16 µg/ml) in methanol

Optimization of Chromatographic conditions:

Table 7.1: Mobile phase optimization trial for Ezetimibe and Fluvastatin

Trial	Mobile Phase	Ratio (v/v)	Remark
1	ACN : Water (pH= 3.5)	70:30	Less resolution was obtained
2	ACN : Water (pH= 3.5)	65:35	Less resolution was obtained
3	ACN : Water (pH= 3.5)	62:38	Less resolution was obtained
4	ACN : Water (pH= 3.5)	60:40	Less resolution was obtained
5	ACN : Water (pH= 3.3)	60:40	Good resolution, with sharp peak Retention Time Ezetimibe- 6.5 min Fluvastatin- 8.6 min

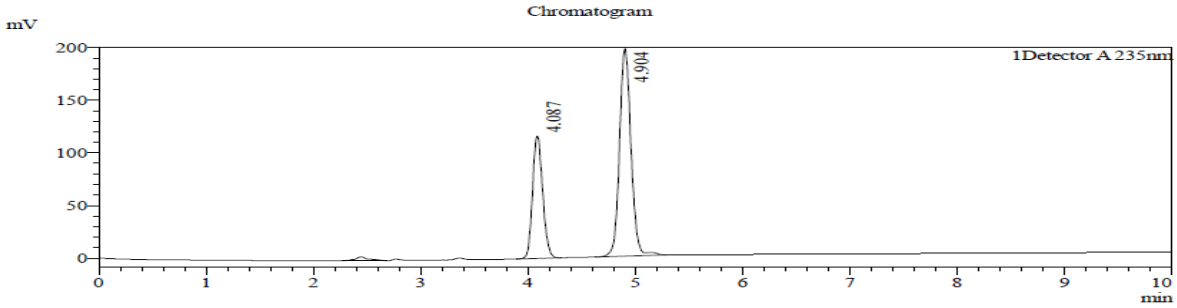

Fig 15 Chromatogram of Ezetimibe and Fluvastatin in ACN: Water (pH 3.5) (70:30%v/v)

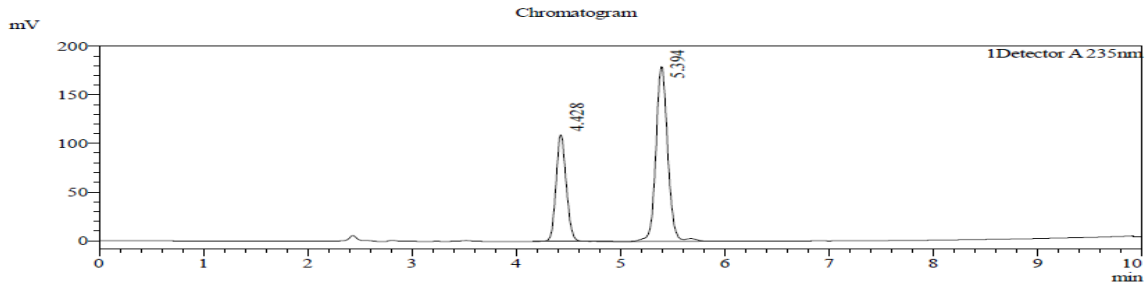

Fig 16 Chromatogram of Ezetimibe and Fluvastatin in ACN: Water (pH 3.5) (65:35%v/v)

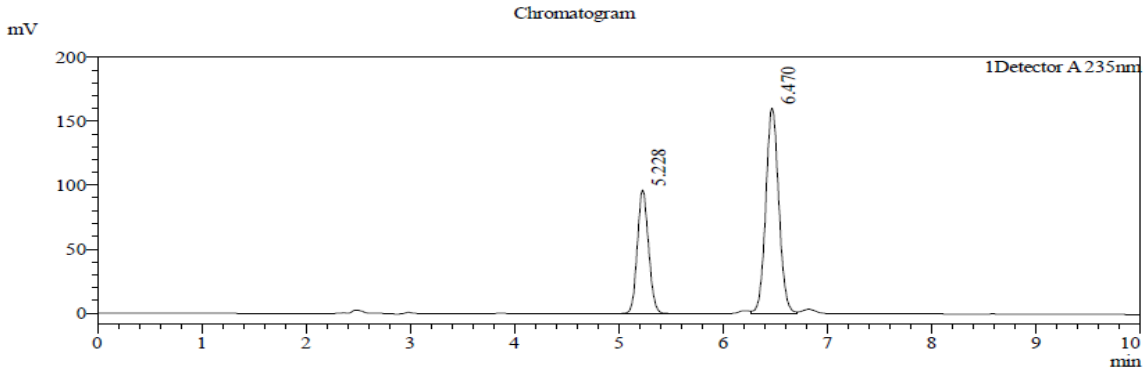

Fig 17 Chromatogram of Ezetimibe and Fluvastatin in ACN: Water (pH 3.5) (62:38%v/v)

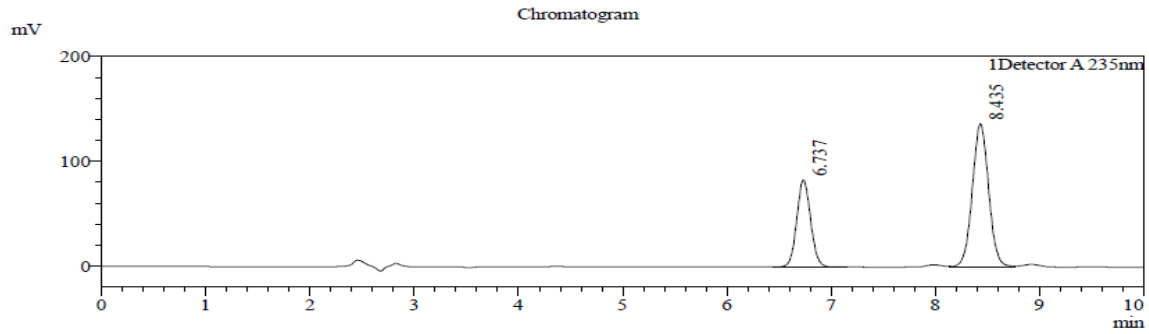

Fig 18 Chromatogram of Ezetimibe and Fluvastatin in ACN: Water (pH 3.5) (60:40%v/v)

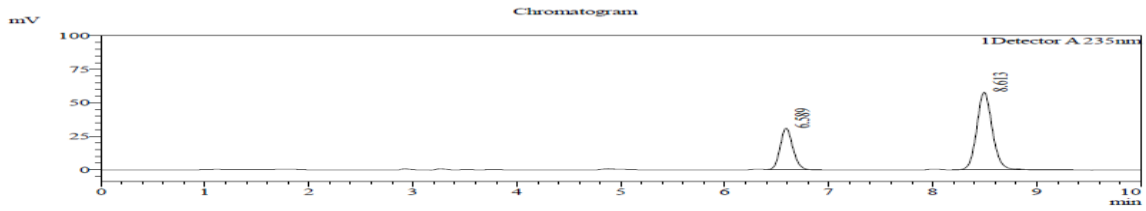

Fig 19 Chromatogram of Ezetimibe (2 µg/ml) and Fluvastatin (16 µg/ml) in ACN: Water (pH 3.3) (60: 40 %v/v)

Table 14: System suitability parameter

Parameter	Retention Time	Tailing Factor	Number of Theoretical Plate	Resolution
Ezetimibe	6.589	1.134	84266	-
Fluvastatin	8.613	1.112	99565	7.436

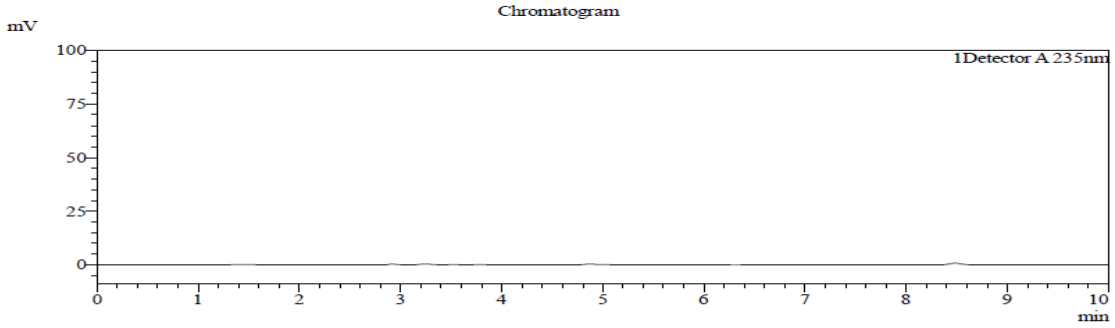

Fig 20: Chromatogram of Blank in ACN: Water (pH 3.3) (60: 40 %v/v)

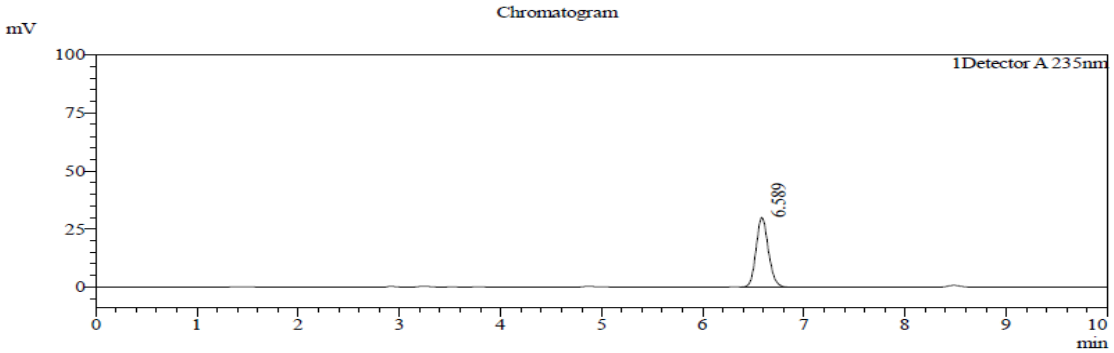

Fig 21: Chromatogram of Ezetimibe (2 µg/ml) in ACN: Water (pH 3.3) (60: 40 %v/v)

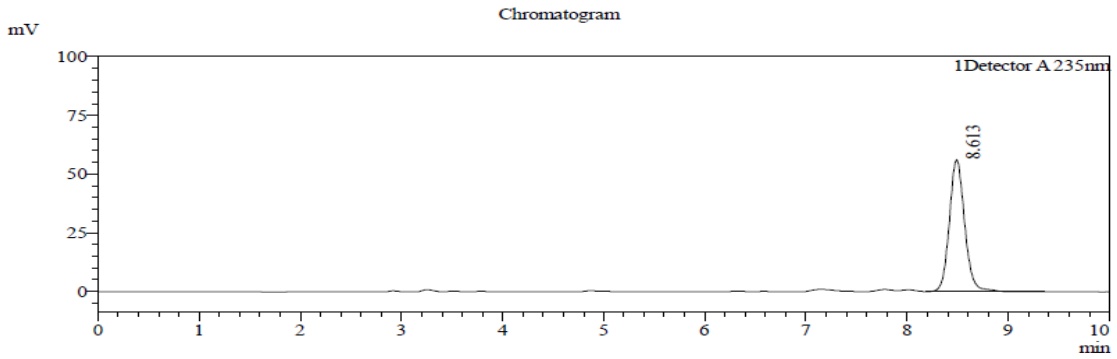

Fig 22: Chromatogram of Fluvastatin (16 µg/ml) in ACN: Water (pH 3.3) (60: 40 %v/v)

- **LINEARITY**

The linearity of Ezetimibe and Fluvastatin was found to be 1- 6µg/ml and 8-48µg/ml,respectively.

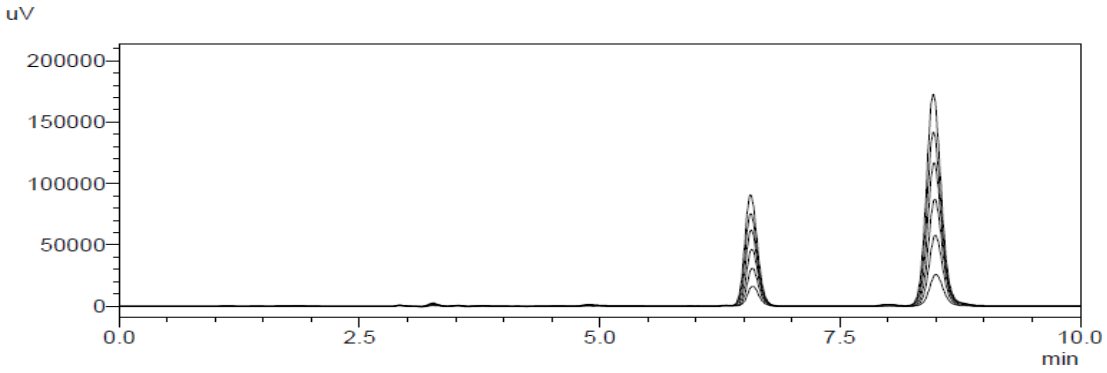

Fig 23: Overlay chromatogram of Ezetimibe and Fluvastatin in ACN: Water (pH 3.3) (60: 40 %v/v)

TABLE 15: Linearity data of Ezetimibe

Concentration (µg/ml)	Mean Peak Area (µV*sec) ± SD (n=6)	%RSD
1	138356 ± 1006.51	0.7274
2	268079 ± 1623.04	0.6054
3	410600 ± 2398.83	0.5842
4	556680 ± 2416.24	0.4340
5	686430 ± 2545.84	0.3708
6	853136 ± 2705.57	0.3171

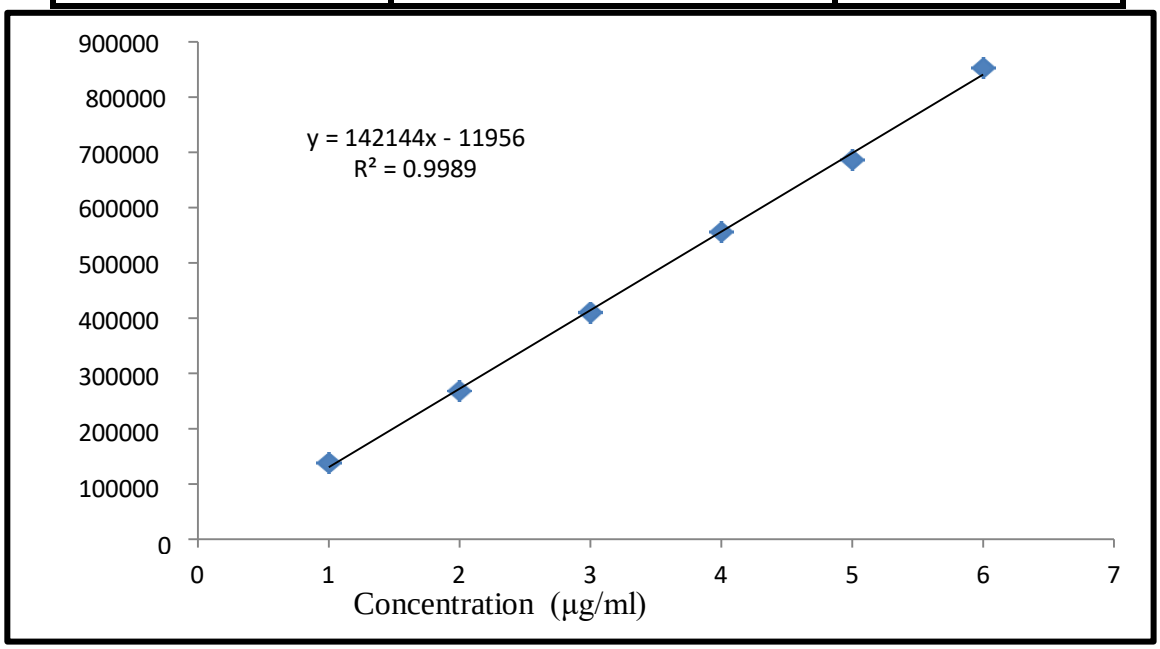

Fig 24: Calibration Curve of Ezetimibe (1-6 µg/ml)

TABLE 16: Linearity data of Fluvastatin

Concentration (µg/ml)	Mean Peak Area (µV*sec) ± SD (n=6)	%RSD
8	267931 ± 2159.96	0.8061
16	605436 ± 4344.19	0.7175
24	931562 ± 5929.91	0.6365
32	1251983 ± 7311.09	0.5839
40	1550617 ± 8061.51	0.5198
48	1925339 ± 8431.53	0.4379

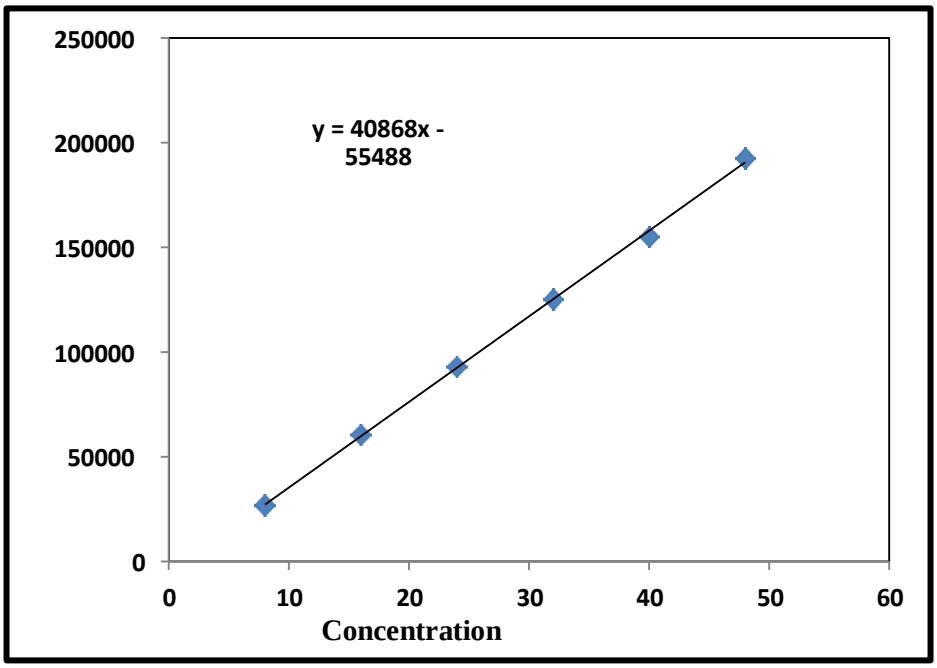

Fig 25: Calibration Curve of Fluvastatin (8-48 µg/ml)

- **PRECISION**

TABLE 17: Precision study of Ezetimibe

Ezetimibe		
Intraday Precision of Ezetimibe		
Conc. (µg/ml)	**Mean Peak Area (µV*sec) ± SD (n = 3)**	**% RSD**
1	139028 ± 907.98	0.6530
2	266819 ± 1558.35	0.5840
3	408838 ± 1770.13	0.4329
Interday Precision of Ezetimibe		
1	138563 ± 994.65	0.7178
2	267436 ± 1699.62	0.6355
3	409182 ± 2032.87	0.4968
Repeatability of Ezetimibe		
Conc. (µg/ml)	**Mean Peak Area (µV*sec) ± SD (n = 6)**	**% RSD**
2	268046 ± 1601.01	0.5972

TABLE 18: Precision study of Fluvastatin

Fluvastatin		
Intraday Precision of Fluvastatin		
Conc. (µg/ml)	**Mean Peak Area (µV*sec) ± SD (n = 3)**	**% RSD**
8	266943 ± 1917.56	0.7183
16	606834 ± 3881.94	0.6397
24	923116 ± 4625.67	0.5010
Interday Precision of Fluvastatin		
8	267043 ± 2175.67	0.8147

16	606368 ± 4673.54	0.7707
24	924033 ± 5202.91	0.5630
Repeatability of Fluvastatin		
Conc. (µg/ml)	**Mean Peak Area (µV*sec) ± SD (n = 6)**	**% RSD**
16	605453 ± 4189.78	0.6920

- **ACCURACY**

TABLE 19: Recovery study

Name of Drug	%Level of recovery	Amount Taken (µg/ml)	Amount Added (µg/ml)	Total Amount (µg/ml)	Amount Recoverd (µg/ml)	% Recovery ± SD (n=3)
Ezetimibe	50	2	1	3	2.98	99.37 ± 0.2250
	100	2	2	4	3.98	99.58 ± 0.2451
	150	2	3	5	4.99	99.84 ± 0.3629
Fluvastatin	50	16	8	24	23.85	99.36 ± 0.2857
	100	16	16	32	31.89	99.62 ± 0.3781
	150	16	24	40	39.91	99.71 ± 0.4738

- **LOD and LOQ**

TABLE: 20: LOD and LOQ Data

Parameter	Ezetimibe	Fluvastatin
LOD (µg/ml)	0.0438	0.2785
LOQ (µg/ml)	0.1330	0.8441

- **ASSAY**

TABLE 21: Analysis of synthetic mixture

Name of Drug	Amount Taken (µg/ml)	Amount Found (µg/ml)	% Assay ± SD (n=3)
Ezetimibe	2	1.99	99.56 ± 0.3356
Fluvastatin	16	15.96	99.77 0.4452

- **ROBUSTNESS**

Table 22: Robustness data of Ezetimibe and Fluvastatin

Condition	Variation	Ezetimibe	Fluvastatin
		% Assay ± SD (n=3)	% Assay ± SD (n=3)
Detection wavelength (235 ±2nm)	233 nm	98.39 ± 0.5230	98.52 ± 0.2128
	235 nm	**99.54 ± 0.3547**	**99.78 ± 0.4573**
	237 nm	98.60 ± 0.2343	98.63 ± 0.5392
Change in Mobile Phase composition ACN: Water (pH 3.3) (60:40 ±2v/v)	58:42	98.42 ± 0.3774	98.33 ± 0.2663
	60:40	**99.61 ± 0.2809**	**99.48 ± 0.3601**
	62:38	98.36 ± 0.5196	98.28 ± 0.4064

TABLE: 23: Summary of Validation Parameter

Parameter	Ezetimibe	Fluvastatin
Linearity Range	1-6 µg/ml	8-48 µg/ml
Regression equation (y = mx +c)	y = 142144x - 11956	y = 40868x – 55488
Correlation Coefficient (r^2)	0.9989	0.9993
Intraday Precision (%RSD, n=3)	0.4329 - 0.6530	0.5010 - 0.7183
Interday Precision (% RSD, n=3)	0.4968 - 0.7178	0.5630 - 0.8147
Repeatability (% RSD, n=6)	0.5972	0.6920
Accuracy (% Recovery, n=3)	99.37 - 99.84	99.36 - 99.71
LOD (µg/ml)	0.0438	0.2785
LOQ (µg/ml)	0.1330	0.8441
%Assay (n=3)	99.56	99.77

CONCLUSION

Simple, rapid, accurate and precise RP-HPLC and UV spectrophotometric methods have been developed and validated for the routine analysis of Ezetimibe and Fluvastatin in synthetic mixture. Both methods are suitable for the simultaneous determination of Ezetimibe and Fluvastatin in multi component formulation without interference of each other. The amount found from the proposed methods were found in good agreement with the label claim of the formulation. Also the value of standard deviation and coefficient of variation calculated were satisfactorily low, indicating the suitability of the proposed methods for the routine estimation of combination dosage forms.

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REFERENCES:

1. Rang H., Dale M., Ritter J., Flower R. Rang Dale's pharmacology; 6th Edn; Elsevier Publication, 2007, pp 285-291.
2. Roger W., Cate W., Clinical Pharmacy and Therapeutics; 4th Edn; Elsevier Publication, 2007, pp 346-366.
3. Winfried Marz," Fluvastatin combination with ezetimibe in patients with high cardiovascular risk", Clinical Trail.gov, August **2017.**
4. Maryadele NJ., The Merck Index an Encyclopedia of chemicals drugs and biological; 14th Edn; Merck Research Laboratories, UK, 2006, pp 668,722
5. Rowe RC., Sheskey PJ., Quinn ME. Handbook of Pharmaceutical Excipients; 6th Edn; Pharmaceutical press, London, 2009, pp 129-133, 210-214, 404-407, 663-666, 728-730.
6. Smith., Introduction to Ultraviolet (U.V.) Spectroscopy; pp 597-599.
7. Skoog DA., Hollar JA., Nieman TA., Principle of Instrumental Analysis; 5th Edn; Thomsan Asia Pvt. Ltd, pp 300-328, 725-744.
8. Owen T., Fundamentals of UV-visible Spectroscopy; Hewlett-Packard publication, pp 38-45.
9. ICH Q2 (R1), 2005, Validation of Analytical Procedure: Text and Methodology, International Conference on Harmonization, IFPMA, Geneva, Switzerland.
10. Beckett AH., Stenlake JB., Practical Pharmaceutical Chemistry, 4thEdn; CBS Publication, 2001 part 1, pp 87.
11. Willard HH., Merritt LL Jr., Dean JA., and Settle FJ Jr., Instrumental Method of Analysis, 7th Edn, CBS Publisher & Distributors, New Delhi, pp 177-178, 580-582,640.