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# A stability indicating RP-HPLC method development for determination of ezetimibe in tablet dosage form

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**ABSTRACT**

*A reliable and sensitive isocratic stability indicating RP-HPLC method has been developed and validated for assay of Ezetimibe in tablets and for determination of content uniformity. An isocratic separation of Ezetimibe was achieved on Zorbax SB C18 (250mm x 4.6mm), 5 µm particle size columns with a flow rate of 1 ml/min and using a UV detector to monitor the eluate at 232nm. The mobile phase consisted of 0.02N ortho phosphoric acid: acetonitrile (20:80 v/v).The drug was subjected to oxidation, hydrolysis, photolysis and thermal degradation. All degradation products in an overall analytical run time of approximately 6 min with the parent compound Ezetimibe eluting at approximately 3.5 min. Response was a linear function of drug concentration in the range of 1-10 µg/ml ($r^2$ = 0.9993). Accuracy (recovery) was between 100.80. Degradation products resulting from the stress studies did not interfere with the detection of Ezetimibe and the assay is thus stability-indicating.*

**3. Keywords:** Content uniformity, method validation, Ezetimibe, stability- indicating

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## INTRODUCTION

Ezetimibe [1-2} inhibits the absorption of cholesterol, decreasing the delivery of intestinal cholesterol to the liver. Ezetimibe (EZE) is [(3*R*,4*S*)-1-(4-fluorophenyl)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-2-azetidinone] . It is a selective cholesterol absorption inhibitor used in the treatment of primary hypercholesterolemia. It inhibits the absorption of biliary and dietary cholesterol from small intestine without affecting absorption of fat soluble vitamins, triglycerides and bile acids. After oral administration, Ezetimibe is metabolized into its glucuronide in the liver and small intestine, which is also active in prevention of absorption of cholesterol. Ezetimibe does not have significant pharmacokinetic interactions with other lipid lowering drugs as it does not influence the activity of cyotochrome P450 [3].

A detailed survey of analytical literature for Ezetimibe revealed few methods based on a variety of techniques such as UV-spectrophotometry [4]. Liquid chromatographic-tandem mass spectrometric (LC-MS/MS) methods have been developed for the estimation of ezetimibe in biological fluids [5-8]. HPLC is often the first choice for developing an analytical method as compared till date, none of the reported analytical procedure describes a simple, satisfactory

and validated HPLC method for studying the effect of stress on pharmaceutical dosage forms as well as for assay and determination of content uniformity of Ezetimibe in tablet dosage forms.

The objective of this work was to develop a simple, precise, reliable and rapid stability-indicating liquid chromatographic analytical method for assay of Ezetimibe and for determination of the content uniformity of a tablet formulation, to validate the method in accordance with ICH guidelines [9]. The method was successfully used for the assay of Ezetimibe and determination of the content uniformity in the tablet formulation. Determination of content uniformity is nowadays, an important test included in USP 30 [10]. Since there is no literature report of a validated analytical method for determination of the content uniformity of Ezetimibe in pharmaceutical dosage forms (10 mg) this was performed in the current work.

## MATERIALS AND METHODS

Ezetimibe reference standard (label claim 99.8% pure) was provided by Ranbaxy Pharmaceuticals Ltd., Gurgaon India. Tablets of Ezetimibe, Ezedoc,, with a 10 mg label claim, manufactured by Lupin Pharmaceutical Pvt. Ltd., India were procured from a local pharmacy. HPLC grade acetonitrile, water, methanol and sodium phosphate acid were obtained from Merck India Limited, Mumbai, India. Analytical grade hydrochloric acid, sodium hydroxide pellets and hydrogen peroxide solution 30% (v/v) were obtained from Ranbaxy Fine Chemicals, New Delhi, India and 0.45 µm nylon membrane filter was obtained from Pall Life Sciences, Mumbai.

### Chromatography

The chromatographic system used to perform development and validation of this assay method consisted of an perkin elemer series 200 LC pump, and turbochrom series 200 UV\VIS detector (Perkin elmer). Chromatographic analysis was performed on Zorbax SB C18 (250mm x 4.6mm), 5 µm particle size) column. Separation was achieved using a mobile phase consist of 0.02N ortho phosphoric acid: acetonitrile (20:80 v/v) solution at a flow rate of 1 ml/min. The eluent was monitored using UV detector at a wavelength 232 nm. The column was maintained at ambient temperature and injection volume of 20 µl was used. The mobile phase was filtered through 0.45 µm filter prior to use.

### Preparation of stock, standard and test solutions:

Stock solution 40 µg/ml of Ezetimibe reference standard was prepared by transferring 10 mg, accurately weighed, into a 25 ml volumetric flask and adding 20 ml 0.02N ortho phosphoric acid: acetonitrile (20:80 v/v). The mixture was sonicated for 2 min to dissolve the Ezetimibe and the solution was then diluted to volume with the same solvent mixture. Standard solution 10µg/ml was prepared by diluting 2.5 ml standard stock solution to 10 ml, in a volumetric flask, with the same solvent mixture.

To prepare stock solution 40 µg/ml for assay, 20 tablets were weighed and mixed. An aliquot of powder equivalent to the weight of 1 tablet was accurately weighed and transferred to 25ml volumetric flask. 20 ml of 0.02N ortho phosphoric acid: acetonitrile (20:80 v/v) was added to the flask and the mixture was sonicated for 10 min with normal hand shaking. The contents of the flask were then left to return to room temperature and diluted to volume with the same solvent mixture. This solution (10 ml) was filtered through a 0.45-µm nylon syringe filter.

To prepare test solution (10 µg/ml) for assay 2.5 ml test stock solution was transferred to 10 ml volumetric flask and diluted to volume with 0.02N ortho phosphoric acid: acetonitrile (20:80 v/v).

### Method validation

In the developed method, by using same concentration of analyte for the assay and for determination of content uniformity, method could be validated simultaneously except for determination of precision. The specificity of the method was evaluated to ensure there was no interference from placebo components (prepared in solution) or from products resulting from forced degradation.

### Linearity

Eight solutions were prepared containing 1, 2, 4, 6,8 and 10 µg/ml of Ezetimibe concentrations, of the test solution. Each solution was injected in duplicate. Linearity was evaluated by linear-regression analysis.

### Precision

System precision was evaluated by analyzing the standard solution five times and method precision (repeatability)

was evaluated by assaying six sets of test samples prepared for assay determination and ten sets of samples prepared for determination of content uniformity, all on the same day (intra-day precision). System precision and method precision were also determined by performing the same procedures on a different day (inter-day precision), and by another person under the same experimental conditions (intermediate precision).

**Accuracy**

Accuracy was assessed by determination of the recovery of the method at three different concentrations (corresponding to 80, 100 and 120% of test solution concentration) by addition of known amounts of standard to placebo preparation. For each concentration, three sets were prepared and injected in duplicate.

**Robustness**

The robustness of the method was evaluated by assaying test solutions after slight but deliberate changes in the analytical conditions. The factor chosen for this study were the mobile phase composition (0.02N ortho phosphoric acid: acetonitrile 20:80 v/v) and (25:75,v/v). The Robustness was determined by injecting duplicate injections of standard and three-sample solutions in single at each different condition with respect to control condition. Robustness of the method was checked by varying the instrumental conditions such as flow rate (±10%), organic content in mobile phase ratio (±2%), wavelength of detection (±5nm), column oven temperature (±5˚C) and change in pH of buffer (±0.2). Sample solution was injected in each condition and assayed for Ezetimibe. Robustness of the method is indicated by the overall RSD value between the data of set-1 and data at each variable condition. (Acceptance criteria: Over all RSD should not be more than 2%).

**Solution stability**

12 sample solutions of the Ezetimibe (6µg/ml) was prepared and kept at room temperature. It was analyzed initially and at different time intervals. As the cumulative RSD up to 780 min meets the acceptance criteria, it is concluded that sample is stable in analytical solution for at least 13 hr at room temperature. (Acceptance criteria: Cumulative RSD should not be more than 2 %.)

**System suitability**

The suitability of the chromatographic system was tested before each stage of validation. Five replicate injections of standard preparation were injected and asymmetry, number of theoretical plates and relative standard deviation of peak area were determined.

**Forced degradation studies**

To perform the forced degradation study 50 mg drug was subjected to acidic, alkaline, oxidizing, thermal and photolytic conditions. For acidic degradation the drug was heated under reflux with 0.1 M HCl at 80º for 2 h and the mixture was neutralized. For alkaline degradation the drug was treated with 0.1 M NaOH at 80º for 2 h and the mixture was neutralized. For degradation under oxidizing conditions the drug was heated under reflux with (30%, v/v) $H_2O_2$ at 80º for 2 h. For thermal degradation the powdered drug was exposed at 70º for 48 h. For photolytic degradation the powdered drug was exposed to sunlight for 48 h. The placebo was also subjected to the same stress conditions to determine whether any peaks arose from the declared excipients. After completion of the treatments the solutions were left to return to room temperature and diluted with solvent mixture to furnish 50 µg/ml solutions. The purity of the drug peak obtained from the stressed sample was measured UV detector. shows the chromatogram of untreated drugs in tablet solution.

**Determination of Ezetimibe in dosage forms:**

The content of twenty tablets were taken and weighed. Powder equivalent to Simvastatin 10 mg was accurately weighed and transferred to a 25 ml volumetric flask and 20 ml of mobile phase was added to the same and flask was sonicated for 5 min. The flask was shaken, and the volume was diluted to the mark with the same mixture. The above solution was filtered using Whatman filter paper No.1. Appropriate volume of the aliquot was transferred to a 10 ml volumetric flask and the volume was made up to the mark with mobile phase to obtain 10 µg/ ml of Ezetimibe. The solution was sonicated for 10 min. The solution was injected at above chromatographic conditions and peak areas were measured. The quantification was carried out by keeping these values to the straight line equation of calibration curve.

## RESULTS AND DISCUSSION

In this work an analytical HPLC method for assay and determination of content uniformity of Ezetimibe in a tablet formulation was developed and validated. The basic chromatographic conditions were designed to be simple and easy to use and reproduce and were selected after testing the different conditions that affect HPLC analysis, for example column, aqueous and organic components of the mobile phase, proportion of mobile phase components, detection wavelength, diluents and concentration of analyte. The on Zorbax SB C18 (250mm x 4.6mm), column was used because of its advantages of high resolving capacity, better reproducibility, low-back pressure, and low tailing. The proportion of the mobile phase components was optimized to reduce retention times and enable good resolution of Ezetimibe.

A detection wavelength of 232nm was selected after scanning the standard solution over the range 200-400 nm by use of the UV detector. Detection at 232 nm resulted in good response and good linearity. The chromatogram shown in figure 1.

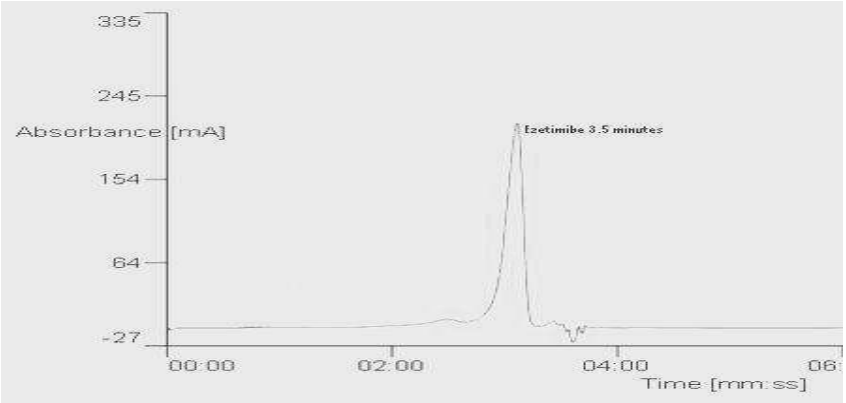



**Figure 1: Chromatogram of the standard solution of Ezetimibe**

After development of the analytical method, it was validated in accordance with ICH and USP guidelines. This furnished evidence the method was suitable for its intended purpose. To determine linearity a calibration graph was obtained by plotting Ezetimibe concentration against peak area. Linearity was good in the concentration range 1-10 µg/ml. The regression equation was y= 62737x+1335 where x is the concentration in µg/ml and y is the peak area in absorbance units; the correlation coefficient was 0.9993. The results were shown in table 1 and figure 2.

**Table 1: Data for calibration curve for ezetimibe**

| Sample ID | Conc. (µg/mL) | Area Counts (µV*sec) | | |
|---|---|---|---|---|
| | | Inj#1 | Inj#2 | Mean |
| **L-1** | 1 | 122337 | 122357 | 122347 |
| **L-2** | 2 | 164266 | 164226 | 164246 |
| **L-3** | 4 | 259117 | 259017 | 259067 |
| **L-4** | 6 | 341897 | 341727 | 341812 |
| **L-5** | 8 | 426252 | 426852 | 426552 |
| **L-6** | 10 | 510110 | 506550 | 506330 |
| | | | **Slope** | 42880 |
| | | | **Intercept** | 81846 |
| | | | **r** | 0.9993 |

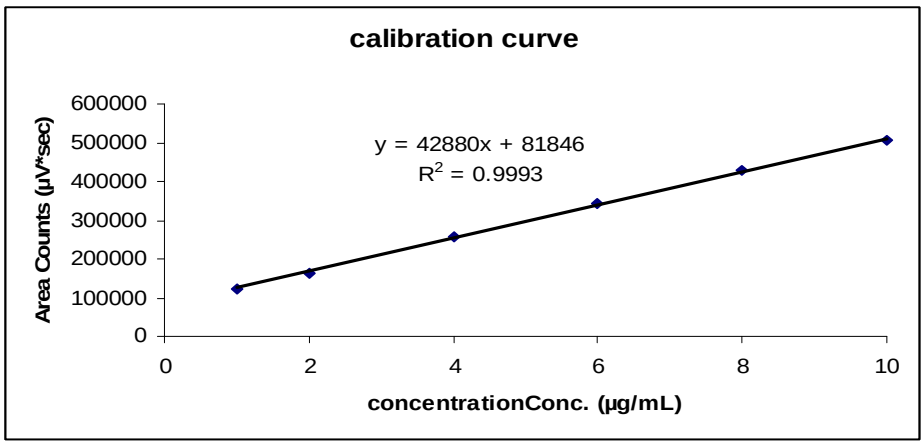
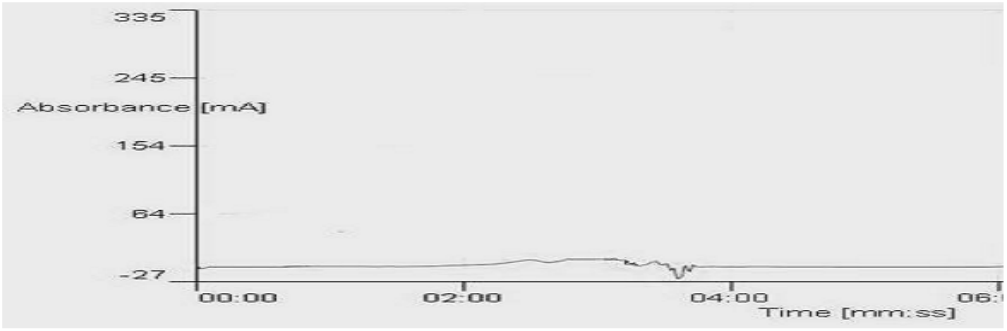
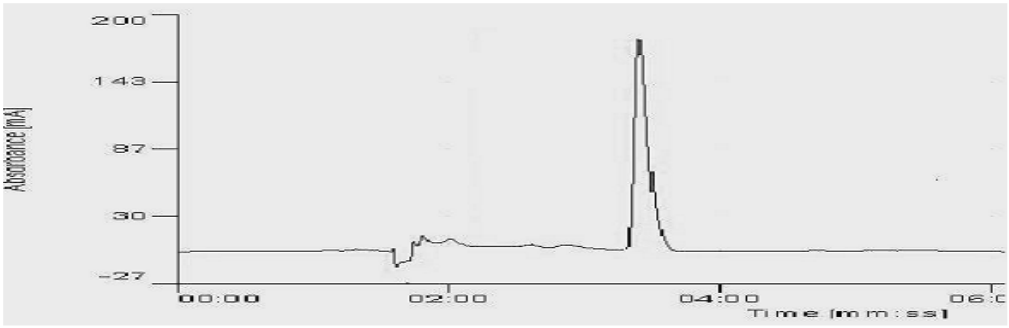



**Figure 2: Calibration curve for Ezetimibe**

The specificity of the method was determined by checking for interference with the drug from placebo components. The chromatograms show the specificity of the method in figure 3.




Chromatogram of placebo (specificity)




Chromatogram of sample (specificity)

**Figure 3: Specificity study of Ezetimibe**

The specificity of the method was also evaluated by checking the peak purity of the analyte peak during the forced degradation study. The peak purity of the Ezetimibe peak under different stress conditions was 1.00, which is satisfactory and indicates there was no interference with the analyte peak from degradation products.

The accuracy of the method was assessed by determination of recovery for three concentrations of Ezetimibe (4, 5, and 6µg/ml) were added to a placebo preparation and the amount of Ezetimibe recovered, in the presence of placebo

interference, was calculated. The mean recovery of Ezetimibe was 100.09 %, which is satisfactory and result was shown in table 2.

**Table 2: Accuracy for Ezetimibe**

| Sample | Mean area counts | Amt. Recovered (mg) | Actual amt. Added (mg) | % Recovery |
|---|---|---|---|---|
| **80%-Rec-1** | 265191 | 10.05 | 10 | 100.5 |
| **80%-Rec-2** | 266007 | 10.14 | 10 | 101.4 |
| **80%-Rec-3** | 266327 | 10.17 | 10 | 101.7 |
| **100%-Rec-1** | 324474 | 9.84 | 10 | 98.4 |
| **100%-Rec-2** | 325115 | 9.94 | 10 | 99.4 |
| **100%-Rec-3** | 326664 | 9.95 | 10 | 99.5 |
| **120%-Rec-1** | 388250 | 10.07 | 10 | 100.7 |
| **120%-Rec-2** | 389536 | 10.12 | 10 | 101.2 |
| **120%-Rec-3** | 381307 | 9.89 | 10 | 98.9 |
| **Mean ± SD** | | | | **100.19 ± 1.17** |

**Table 3: Summary of Validation Parameters**

| PARAMETERS | OBSERVATION |
|---|---|
| **SPECIFICITY** | No Interference was found w.r.t. excipients |
| **LINEARITY (R)**[5] | .9993 |
| **RANGE** | 70 – 130 % of test concentration |
| **PRECISION (RSD)*** | |
| a) Repeatability (n=6) (system precision) | 0.58 |
| b) Intermediate Precision (inter-analyst) (n=6) | 1.66 |
| c) Method Precision (n=6) | 0.54 |
| **ACCURACY (% Recovery)**** | 99.81 ± 1.084 |
| **STABILITY IN ANALYTICAL SOLUTION** | Stable |
| **STRESS DEGRADATION** | The Peaks were **Pure** without any interference |
| **ROBUSTNESS (Overall RSD)***** | Less than 2% |
| a) Change in Wavelength | 1.03 |
| ● 237 nm | 1.58 |
| ● 227nm | |
| b) Change in Flow rate | 0.34 |
| ● 1.1ml/min | 0.64 |
| ● 0.9 ml/min | |
| c) Change in Organic Conc. | 0.40 |
| ● - 2% | 0.45 |
| ● + 2% | |
| d) Change in Column Temp. | 1.24 |
| ● 25°C | 0.29 |
| ● 35°C | |
| e) Change in pH of Buffer | 0.27 |
| ● 2.8 | 0.54 |
| ● 3.2 | |
| **RUGGEDNESS(Overall RSD)***** | 1.66 |

*Acceptance Criteria: RSD ≤ 2 %. ** Acceptance mean recovery: for 80 – 120 %.*
**** Acceptance Criteria: RSD ≤ 2 %*

The mean values of method precision (repeatability) were RSD 0.58 Intermediate precision was established by determining the overall (intra-day and inter-day) method precision For intermediate precision, overall assay value (n=12) was RSD 1.66, and method precision was RSD 0.64. The robustness of the method was assessed by assaying test solutions under different analytical conditions deliberately changed from the original conditions. For each different analytical condition the standard solution and test solution were prepared separately. The result obtained from assay of the test solution was not affected by varying the conditions and was in accordance with the true value.System suitability data were also found to be satisfactory during variation of the analytical conditions.

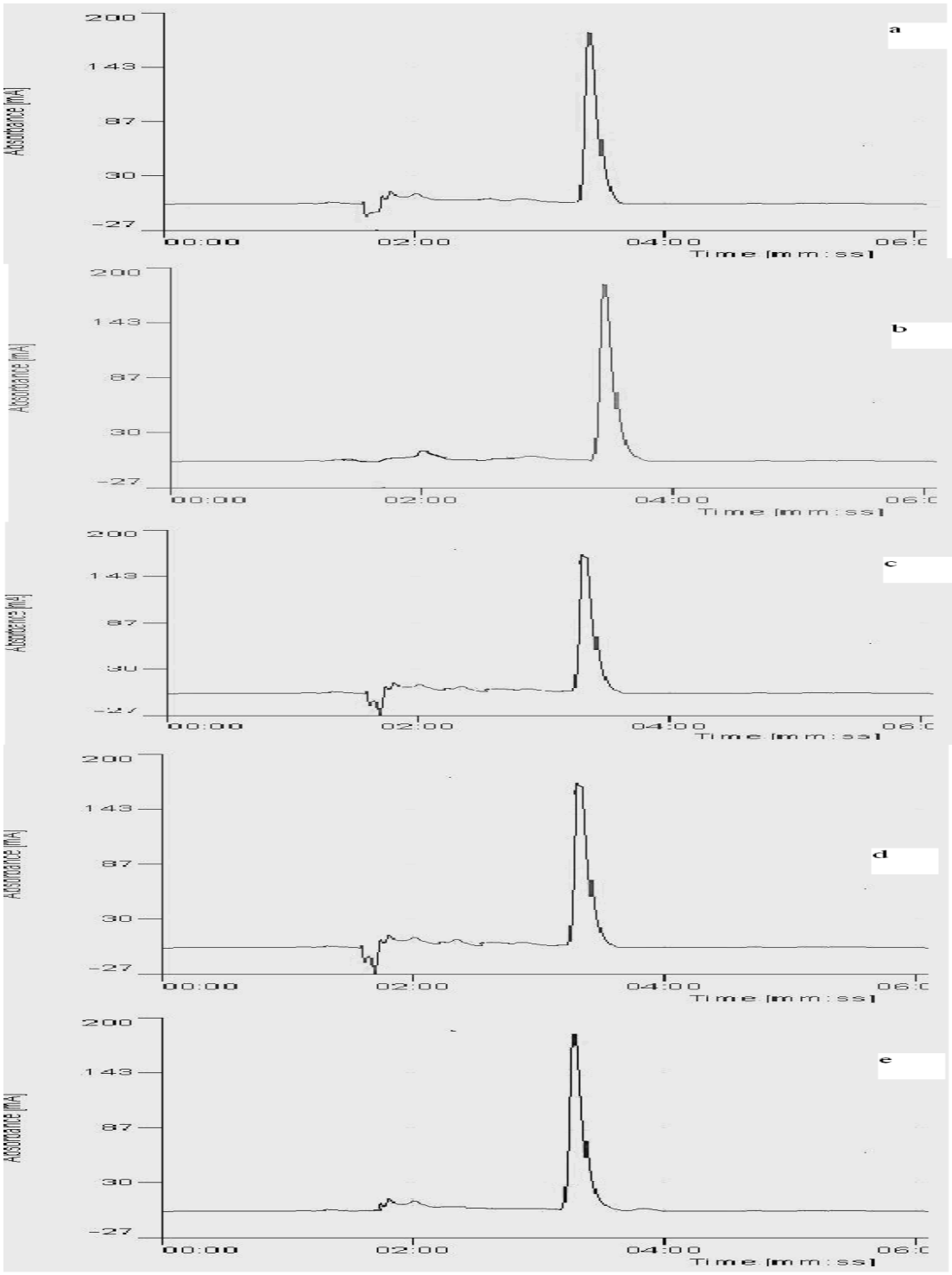



**Figure 4: Chromatogram of forced degradation study. Chromatogrphic profiles of rosuvastatin tablets after subjecting them to (a) acidic, (b) alkaline, (c) oxidative, (d) thermal and (e) photolytic degradation.**

The analytical method therefore remained unaffected by slight but deliberate changes in the analytical conditions. Result was shown in table 4. During study of the stability of stored solutions of standards and test preparations for

assay determination the solutions were found to be stable for up to 13 h. Assay values obtained after 13 h were statistically identical with the initial value without measurable loss. Result was shown in table 3.

Major degradation up to 10.71 % occurred under acidic conditions Figure 3a. Under alkaline conditions the drug was degraded by approximately 1.06% Figure 3b. The drug was approximately 1.37% degraded under oxidizing conditions Figure 3c. The drug was degraded 8.25% under thermal condition Figure 3d and 2.49% degradation occurred under photolytic conditions Figure 3e and table 4.

**Table 4: Degradation study for Ezetimibe**

| Sample | Ezetimibe Area counts (µv*sec) | Ezetimibe | |
|---|---|---|---|
| | | Assay mg | Percent Degradation |
| **Sample (1 N HCl, )** | 317865 | 9.13 | 10.71 |
| **Sample (1 N NaOH, l)** | 375122 | 9.90 | 1.06 |
| **Sample ($H_2O_2$ 30%, )** | 366802 | 9.97 | 1.37 |
| **Sample Thermal Deg. (105˚C/1hr)** | 315449 | 8.23 | 8.25 |
| **Sample Photolytic Deg. (2600 Lux/24 hr)** | 367514 | 9.75 | 2.49 |

The assay value for the marketed formulation was found to be within the limits, as listed in Table 5. The low RSD value indicated the suitability of the method for routine analysis of Ezetimibe in pharmaceutical dosage forms.

**Table 5: Assay of Ezetimibe in Tablet**

| Label claim 10mg | Area Counts (µV*sec) | Assay (mg) | % Assay |
|---|---|---|---|
| **1** | 229452 | 9.99 | 99.90 |
| **2** | 231682 | 10.09 | 100.9 |
| **3** | 228244 | 9.90 | 99.90 |
| **Mean** | 230567 | 10.04 | 100.4 |
| **SD** | 1744 | 0.095 | 0.577 |
| **RSD** | **0.756** | **0.946** | **0.575** |

## CONCLUSION

The intensive approach described in this manuscript was used to develop and validate a liquid chromatographic analytical method that can be used for both assay and determination of content uniformity of Ezetimibe in a pharmaceutical dosage form. Degradation products produced as a result of stress did not interfere with detection of Ezetimibe and the assay method can thus be regarded as stability indicating. This HPLC method for assay and determination of content uniformity of Ezetimibe in a tablet formulation was successfully developed and validated for its intended purpose. The method was shown to specific, linear, precise, accurate, and robust. Because the method separates Ezetimibe and all the degradation products formed under variety of stress conditions it can be regarded as stability indicating. Because there is no pharmacopeial method for assay and determination of content uniformity of Ezetimibe in pharmaceutical dosage forms, this method is recommended to the industry for quality control of drug content in pharmaceutical preparations.

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