Charecterization of biologically prepared silver nanoparticles of *Erythroxylum monogynum* by UV – Visible Spectrophotometer, Transmission Electron microscope and X-ray Diffraction Technique.

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Abstract:

Silver nanoparticles prepared from various plant sources are known to contain alot of properties which are most beneficial to human society in various ways.Charecterization of silver nano particles of *Erythroxylum monogynum* is very much necessary to know the properties of them.The techniques like the UV-Vis spectrophotometer,Transmission electron microscopy and X-ray diffraction (XRD) measurement techniques used to determine the variuos parameters like shape,size,structure,stability, lattice pattern/;, crystallographic phases of materials prepared etc. The properties depended on the parameters of the nanoparticles.

Key words : Silver nanoparticles, *Erythroxylum monogynum*, UV-Vis spetrophotometer, Transmission elctron microscopy, X-ray diffraction (XRD) measurement.

Introduction :

Silver nanoparticles are very tiny particles of silver and have great applications in different fields of various sciences. The wide range of applications of silver nano particles are due to the specificity in properties of physical, chemical as well as biological sciences (Ingle et.al.,2008; Pal et.al., 2007). Nanoparticles contains elctrical, thermal, optical,and biological properties (Gurunathan S et.al.,2015 ; Li WR et.al.,2010;Mukherjee P et.al., 2001).Nanoparticles of silver are less toxic to animals when compared with the arificial fungicides, however the toxicity due to nanoparticles may show in algae, plants, and fungi which may show some effects positively (Sondi and Salopek-Sondi 2004). In the field of medicine the nanoparticles contains a large number of applications practically (Pauwels E et.al., 2008).They are prepared by using various combinations of biosources and silver compounds. Nanoparticles properties are based on aspect ratio of crystal size, density and morphology (Anandan K et.al.,2011 ; Laokul P et.al., 2011). Erythroxylum monogynum silver nanoparticles are prepared and were charecterized by the various techniques to know the vaious parametres of the particles. The techniques like UV-Vis Spectrophotometer ,Transmission electron microscopy,X-RD studies prformed.

UV-Vis Spectrophotometer Analysis :

Ultra Violet- Visible spectroscopy is easy, sensitive, Specific for Various types of nano Particles, requires only less time duration for analysis of measurement, and finally almost required no calibration for characterization of particle in the state of suspensions of colloid. (Huang XH et.al.,2007 ; Leung AB et.al., 2006).This is used to examine the shape and size of nano particles especially in the aqueous suspensions.Silver nanoparticle absorption based on size of particle chemical surroundingsand the dielectric state . [Link.s et.al 2003;Noginov MA et.al., 2007]

UV-vis spectroscopy is also applied to monitor the production and to know the stability of AgNPs (Sastry M et.al., 1998).AgNPs have specific properties optically which make them potentially interact with perticular wavelengths of the light. Along with this, the UV-vis spectroscopy is speed, very easy, most simple, very responsive, specific for different types of AgNps, it measures within only a very short time interval, and finally there is no calibration requiredfor charecterisation of the particle of suspensions especially for the colloids (Huang J et.al.,2007).In Silver nanoparticles the bands like valence band and the conduction band and exist very close to together where the electrons freely moves .The surface plasmon resonance (SPR) property was shown by these free electrons and the absorption band,is because of the electron collective oscillation of AgNps in resonance along with the waves of light(Das, R.Et.al., 2009 ; Kreibig, U et.al .,1995; Taleb A et.al 1998). The absorption of Silver nanoparticles depends on the parameters like size of the particle dielectric medium, and surroundings of chemical origin.The peak which we observe here is due to the surface Plasmon and is very well noted for different metal particles of nano size with ranging capacity from 2-100 nm (Henglein A). In the current work supernatant containing AgNps were diluted with the water which is deionised in the ratio of 1:1 and the need measure the absorbance by using a UV-Vis spectrophotometer (Thermo scientific-EVOLUTION 201).This was equipped with quartz cells which are matching and the resolution of about 1nm from 200-800nm.The control used is Milli-Q water. The spectroscopic analysis studies were performed at the room temperature reaction.

Transmission Electron Microscopy (TEM) Analysis :

Transmission Electron Microscopy is a important, mostly used, technique to characterize the nanomaterials, in order to obtain size of particle, morphology as well as distribution. (Lin PC et.al.,2014 ; Joshi M et.al.,2008 ;Williams DB et.al .,2009).Transmission electron microscope (TEM) is the most widely used technique to characterize metal nanoparticles and it was utilised to see the shape and size of nanoparticles of the silver. AgNPs samples for Transmission Electron Microscope grids were synthesised by solution sonication for about a time period of five minutes and keeping a few drops on the 300 mesh of copper which was carbon –coated grid and water evaporated completely under the lamp and operated at an voltage acceleration of 200 kilo volts by utilizing the EM 2000 Fx-II, TEM (Transmission electron microscope), this is 200KV HRTEM from the JEOL from the Japan, for to sample charecterisation after the usual alignment procedures. *In-situ* Camera of LCD (Liquid crystal display) is used to record those pictures.

X-ray diffraction (XRD) measurement Analysis :

Xray diffraction measurement technique are using to describe the identification and as well bulk nanoparticles[AgNPs], samples in forensic,geochemical,industrial catrgories. (Yazdian N et.al.,2013 ; Khan A et.al.,2013 ; Dolatmoradi A et.al 2013 ; Aghili SE et.al 2012)This technique analyse molecular as well as crystalline structure of both molecular and crystal structures and also dimension of biologically synthesized AgNPs with h, k, l values.

For XRD analysis, the sample containing silver nanoparticles was prepared by drop-coating onto glass substrates and dried at 45°C in a vacuum drying oven. Vacuum-dried AgNps were utilized for to analyse the powder X-ray diffraction.The dried sample was placed on analytical tape and the paterns of X-ray diffraction were noted in mode of scanning on an analytical instrument called X`pert PRO PAN runned at 40 Kilo Volts.

The current of the 30 milli Ampere with the Copper potassium radiation. The intensities of the diffraction were noted from 5.0170° - 81.9730, in the 2θ angles, and particles size (L) of the silver was calculated using formula called Debye–Scherrrer's equation.

$$L = \frac{0.9\lambda}{\beta\cos\theta}$$

where, λ is wavelength for X-ray, β is the full width and half maximum and θ is the Bragg's angle.

Results :

UV-Vis spectroscopy

The absorbance spectroscopy is a rapid way to obtain data of silver nanoparticle solution. The charecterisation of silver nanoparticles done by UV-Vis spectroscopy, one of the most commonly used methods silver nano particle characterization of structure. In the biological method of AgNPs production shows the electromagnetic waves absorption in the visible range because of its property called optical resonance which occurs because of its collective electron to and fro movements, mixed with the light of incidance gives the UV-Vis spectra of the ninty six hours old AgNPs synthesized from *Erythroxylum monogynum* .The peak of the above spectra was found at

440nm and this peak was due to Surface Plasmon Resonance (SPR) property of silver nanoparticles. Surface plasmon resonance (SPR) is defined by Link and El-Sayed (2003) as "the electric field of an incoming light wave which induces a polarization of the electrons with respect to the more heavier ionic core of a nanoparticle of spherical shape". SPR is easily and rapidly measured by UV absorbance and peak widths and can indicate particle size and distribution from band widths and the λ peak maxima absorbance. This phenomenon complies with Mie's theory for spherical particles. This method, however, has a narrow working size range of 2-20 nm, although the upper limit could be as large as 50-80 nm (Van Hyning and Zukoski 1998; Link and El-Sayed 2003).

The spectra recorded at various reaction times and observed higher intensity in absorption spectra of silver solution with the time period resulting the generation of increased number of nano particles of silver in the solution; this is similar with the findings (Zhang *et al.*, 2011). The generated aqueous solution of silver nano particles showed the strong absorption at the band between the range 410 to 440 nm which is a typical absorption band of spherical nano particles of silver because of their their surface Plasmon.

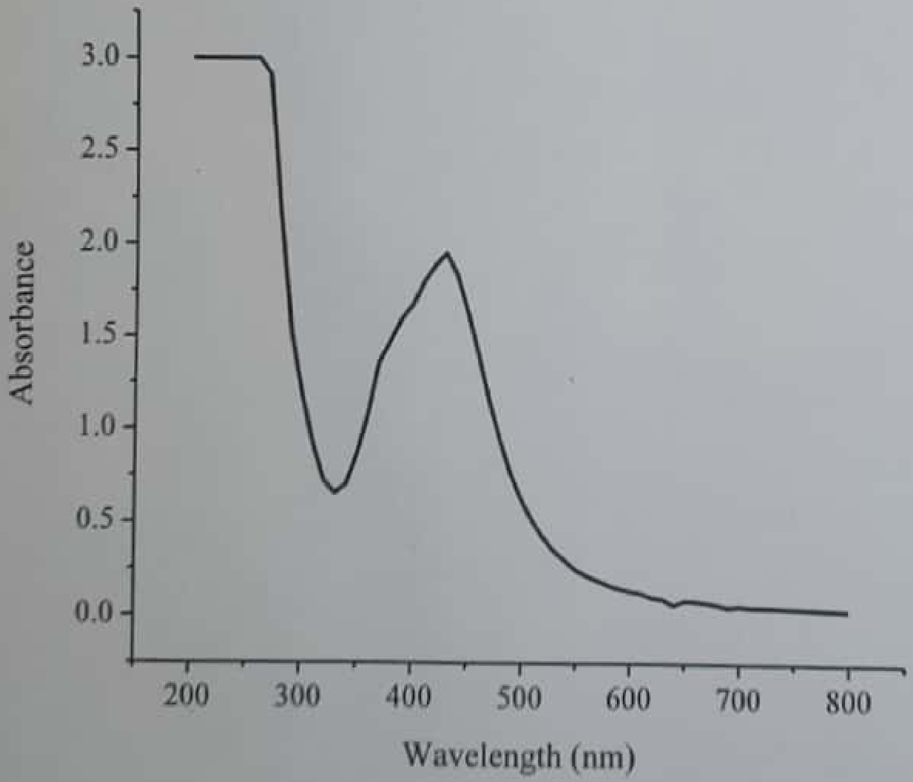
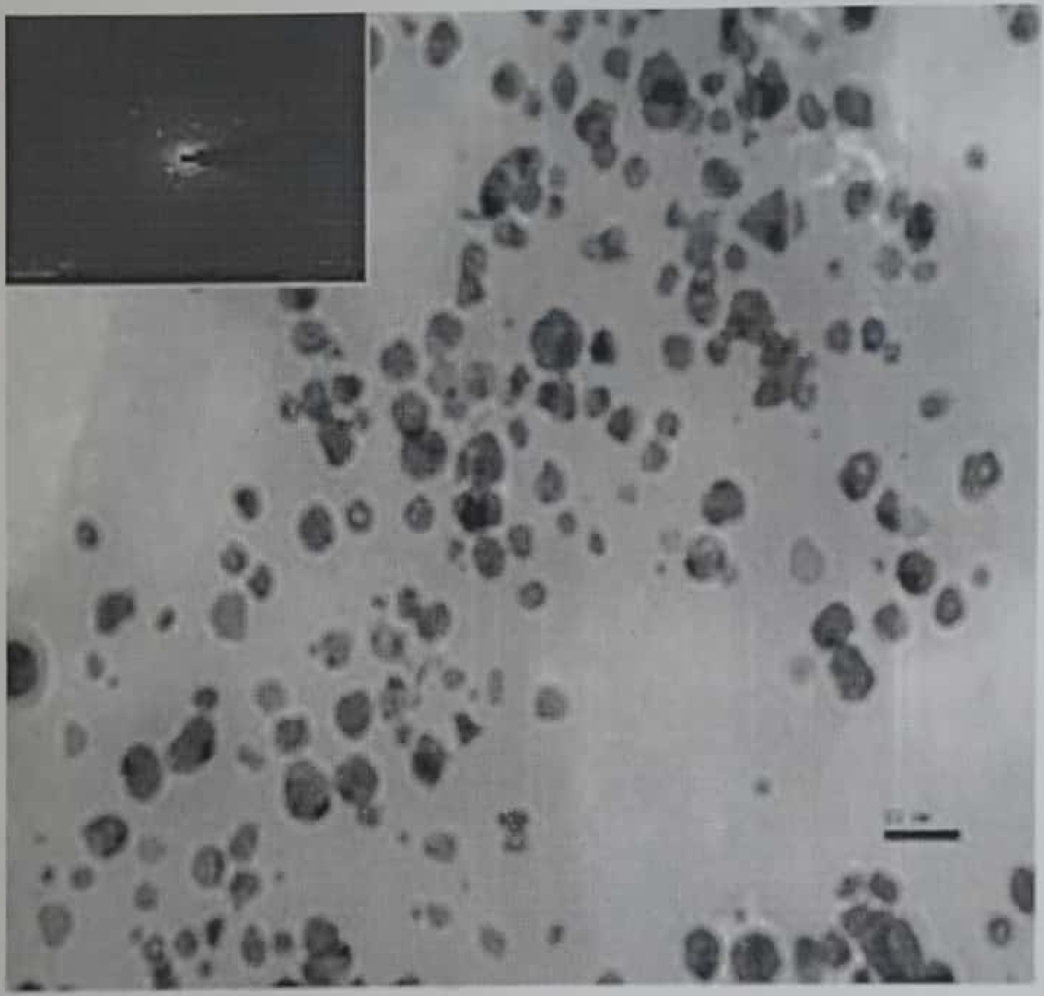


Figure 4.1.0 : UV-Vis spectra of silver nanoparticles showing maximum absorbance at 440nm

Transmisssion Electron Microscopy Results :

The well known method for imaging solid materials at atomic resolution is Transmission Electron Microscopy (TEM). The method was used to see the shape as well as size of Ag NPs. TEM image of produced nanoparticles of silver from the results it was observed that most of the AgNPs contains the circular shape. The image of the TEM individual AgNPs as well as a number of aggregates with the size ranging fifteen to twenty nano meters and also electron diffraction at high resolution indicating specific planes of silver nano particles.



Transmisssion Electron Microscopy – Analysis image

X-ray diffraction measurement- Results :

The crystalline nature of Ag nanoparticles was confirmed from the X-ray diffraction analysis.
Following Figure shows the XRD pattern with the diffraction peaks at 34.0728, 52.2141 64.5005, 77.0516 corresponding to the planes (111),(200),(220),(311).which were in agreement with the face centered cubic crystal structure of metallic silver. The high intense peak for FCC materials is generally agreement with the face centered cubic crystal structure of metallic silver. The high intense peak for FCC materials is generally (111) reflection, which was observed in the sample. The peaks intensity reflected the high degree silver nanoparticles crystallinity. The diffraction peaks are broad which shows that the crystallite size is very small.

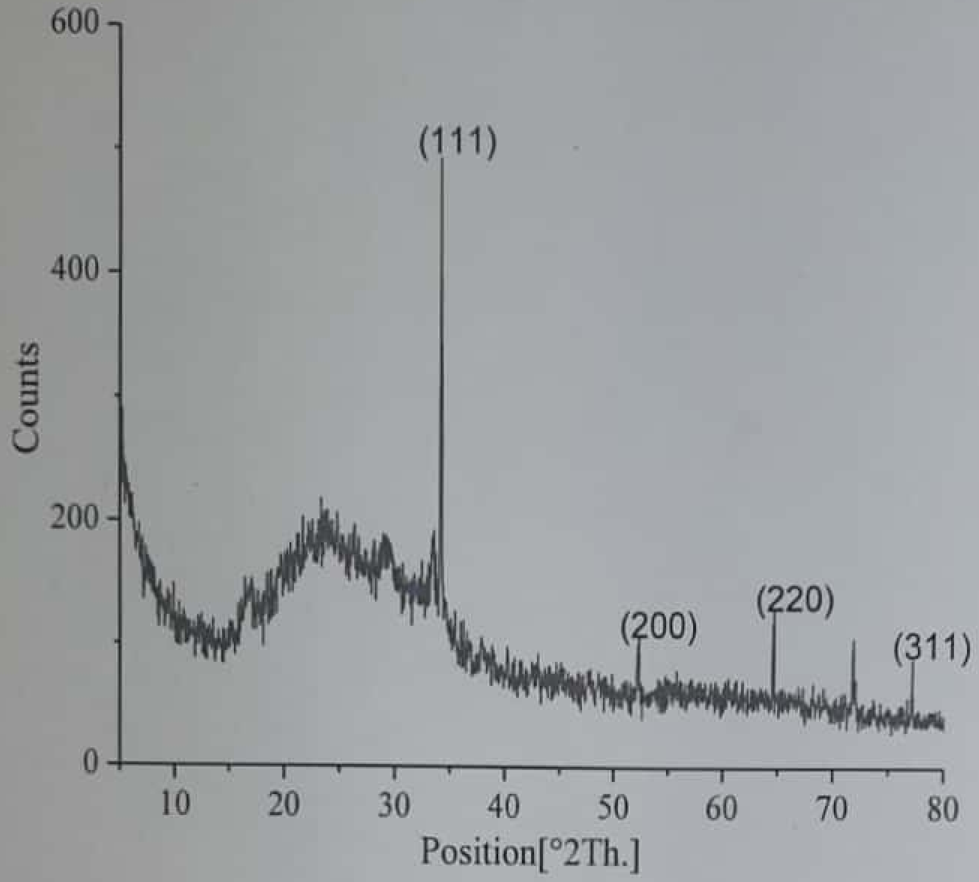


Figure 4.1.4 : X-ray diffraction pattern of prepared silver nanoparticles

X-Ray Diffraction Peak List of Silver Nanoparticles

Pos. [°2Th.]	Height [cts]	FWHM [°2Th.]	d-spacing [Å]	Rel. Int. [%]
33.4040	111.71	0.4697	2.68251	8.96
34.0728	384.56	0.1299	2.63137	30.86
52.2141	33.48	0.2273	1.75193	2.69
64.5005	45.21	0.4180	1.44353	7.19
71.7811	52.29	0.2376	1.31397	4.20

Conclusion : The results from UV –Visible spectrophotometer gave the strong absorption at the bands between the range of 410 to 440 nm which shows a typical absorption band of spherical nano particles because of their their surface Plasmon. The image of the TEM individual silver nanoparticles as well as a number of aggregates with the size ranging fifteen to twenty nano meters and also electron diffraction at high resolution indicating specific planes of silver nano particles. The high intense peak for FCC materials is generally (111) reflection, which was observed in the sample. The peaks intensity reflected the high degree silver nanoparticles crystallinity. The diffraction peaks are broad which shows that the crystallite size is very small.

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Papers Published in Journals:

S. No.	Title of the paper with author names	Name of the Journal	Publication status
1	Simple HPLC method for the determination of Cefixime, Ofloxacin and Linezolid in solid dosage forms Manchuru Vanaja and J. Sreeramulu.	International Journal of Research in Pharmacy and Chemistry, 2018, 8(4), 530-545.	April 2018
2	Paliperidone determination by using HPLC method in blood plasma samples – Stability indicating method Manchuru Vanaja and J. Sreeramulu.	International Journal of Pharmacy and Biological Sciences, 2019, 9(1), 357-368.	January 2019
3	Stability indicating HPLC method for the determination of Darunavir in blood plasma samples Manchuru Vanaja and J. Sreeramulu.	Rasayan Journal of Chemistry, 2019, 12 (2), 839-848.	June 2019

4	Characterization of biologically prepared Silver Nanoparticles of Erythroxylum monogynum by UV-visible spectrophotometer, Transmission Electron microscope and X-ray Diffraction technique. Manchuru Vanaja and M.Dhanunjay	International Journal of Research and Analytical Reviews (IJRAR) Volume 6	June-2019
5	Novel and stability indicating HPLC method for Ezetimibe, Rosuvastatin, Atorvastatin in tablets form Manchuru Vanaja and J. Sreeramulu.	Caribbean journal of Science and Technology-2020,8(1)001-024	Feb-2020
6.	Microwave assisted Synthesis, Molecular Docking Studies And Antibacterial Activity Of 4,6-Bis-(2-(Aryl)Pyrazolo[1,5-A]Pyrimidin-7-Yl)Benzene-1,3-Diols And 4,6-Bis-(1-(Aryl)-1h-Pyrazol-3-Yl) Benzene-1,3-Diols Manchuru Vanaja[1*] and Palle Malleswarareddy[2*]	Heterocyclic Letters Vol. 12| No.3||May-July|2022 ISSN : (print) 2231–3087 / (online) 2230-9632	July-2022

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Research Article

SIMPLE HPLC METHOD FOR THE DETERMINATION OF CEFIXIME, OFLOXACIN AND LINEZOLID IN SOLID DOSAGE FORMS

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ABSTRACT

Cefixime, Ofloxacin and Linezolid three drugs can be used to treat bacterial infections. Each drug works with different mechanism. Stability indicating RP- HPLC method was developed for Cefixime, Ofloxacin and Linezolid quantification in tablet dosage form. RP-HPLC method was validated with precision, specificity, accuracy, ruggedness, robustness and linearity parameters. Liquid chromatographic conditions are mobile phase A: 0.5M KH_2PO_4 in HPLC grade water and mobile phase B: Acetonitrile, Agilent make Zorbax SB-C18, 100 x 4.6mm, 5µm, 280 nm, 1.0ml/min, 25 min (gradient program: mobile phase B at 0min 5%, 5min 5%, 10 min 16%, 14 min 16%, 17 min 34%, 20 min 5% and 25 min 5%. All validation results shown the accuracy results and % RSD for test area, %assay values were also within the limits. This HPLC method can be used to analyze the regular product quality control purpose.

Keywords: Cefixime, Ofloxacin, Linezolid, Tablets dosage form, Method development and validation.

INTRODUCTION

Cefixime is belongs to cephalosporins and it is an anti-biotic medicinal product. Cefixime fights for bacterial infections[1]. Cefixime is used to treat Gonorrhea, urinary and respiratory track bacterial infections and middle ear infection[2]. Cefixime inhibits the bio-synthesis of cell walls. Cefixime medicinal product was approved in United States in 1989. Brand names of this drug product are Suprax, Taxim O, Texit, Cef-3, Denvar, 3-C and Zifi. Side effects of Cefixime are headache, vomiting, diarrhea, abdominal pain, bloating, vaginal fungal infection, intestinal infection[3].

Ofloxacin is an antibiotic and used to treat bacterial infections like cellulitis, pneumonia, urinary tract infections, plague and prostatitis[4]. USFDA was approved this antibiotic drug in 1985 and world health organization listed as essential medicines[5]. Most frequent side effects are vomiting, diarrhea, insomnia, headache, dizziness, nausea and itching[5].

Linezolid is an antibacterial oxazolidinone class drug product and used to treat pneumonia and skin infections. But, linezolid cannot work for colds, flu and other viral infections[6]. Safe antibiotic if prescribed for short period and common side effects are diarrhea, rash, nausea and headache[7-8].

Chemical structures of Cefixime, Ofloxacin and linezolid were represented in figure-1. Table-1 represented the marketed combination products of Cefixime, Ofloxacin and linezolid.

Cefixime and Ofloxacin are available in the market in solid dosage form with multiple strengths. Ofloxacin and Linezolid are available in solid dosage combination form.

Literature survey reveals the few reported methods on HPLC, LCMS, TLC methods for the determination of cefixime-ofloxacin, cefixime-linezolid[9-14]. Naga M *et al.,* (2017), Ghimire S *et. al.,* (2018) and Hassouna ME *et. al.,* (2017), Prabhu S *et.al.,* (2010) were reported the RP-HPLC methods to determine cefixime-ofloxacin separately and cefixime-linezolid separately. There is no reported method to determine the three components by using simple HPLC method. Hence, the necessity of the HPLC method requirement was understood. The objective of this present research work is to develop a stability indicating HPLC method for the determination of three active components such as Cefixime, Ofloxacin, and Linezolid in combination solid dosage forms.

MATERIALS AND METHODS

MATERIALS

The Chromatographic system consisted of 1100 agilent separation module which provides quaternary solvent, 100 vial capacity, column heater and cooler module, VWD UV detector. In this research authors were tried with different makes of HPLC columns were checked but eventually, Agilent Zorbax SB-C18, 4.6x100 mm, 5 µm particle size was suitable. Cefixime, Ofloxacin, Linezolid standard materials were obtained from Aptuit Laurus Laboratroies Hyderabad.

MOBILE PHASE AND SOLUTIONS PREPARATION

Preparation of mobile phase A

Measured, 1000ml distilled water with class-A measuring cylinder and add 6.8 g of KH_2PO_4 resulting solution was degassed with 0.45µ filter paper.

Mobile Phase-B

HPLC grade Acetonitrile was used. Measured volume was sonicated for 5minutes using the sonicator and filtered using the vacuum pump.

Diluent solution

Put 250 mL of mobile phase A, 250 mL of Acetonitrile into a 1000ml beaker. The resulting solution was mixed for some time to get homogeneous dilution solution.

HPLC conditions

Column: Agilent Zorbax SB- C18, 100 x 4.6mm, 5µm
Flow rate : 1.0 mL/minute
Detection : 280 nm
Injection Volume : 20 µL
Column temperature : 30°C
Analysis time : 25 minutes

Mobile Phase Elution Gradient Program

Standard solution

50 mg of Cefixime standard, 50 mg Ofloxacin standard and 150 mg of Linezolid were weighed accurately with calibrated analytical balance and transferred into a 100 mL volumetric flask. 50 mL of diluent was added to dissolve the contents and mixed well. Remaining volume was filled and mixed. 1.0ml of this solution was pipetted and transferred in to 50 ml class A volumetric flask and diluted with diluent.

Preparation of Cefixime and Ofloxacin Sample Solution

Randomly selected 20 tablets and weighed individually and calculated the average weight of one tablet and prepared the fine powder. Equivalent to 50 mg of Cefixime and Ofloxacin tablets powder was weighed and transferred into 100 mL volumetric flask. 50 ml of diluent was added and dissolve the content by using hand shake and sonication for 10 minutes. Further volume was diluted with diluent. Stock solution was filtered with what man filter. 1 mL of the above solution was transferred into a 50 mL volumetric flask and diluted.

Preparation of Cefixime and Linezolid Sample Solution

Randomly selected 20 tablets and weighed individually and calculated the average weight of one tablet and prepared the fine powder. Equivalent to 50 mg of Cefixime and 150 mg Linezolid tablets powder was weighed and transferred into 100 mL volumetric flask. 50 ml of diluent was added and dissolve the content by using hand shake and sonication for 10 minutes. Further volume was diluted with diluent. Stock solution was filtered with whatman filter. 1 mL of the above solution was transferred into a 50 mL volumetric flask and diluted.

% component (Cefixime, Ofloxacin and Linezolid) value calculation formula

$$\frac{\text{Tarea X Tweight X 1 X 100 X 50 X Label claim X Potency}}{\text{Sarea X 100 X 50 X Sweight X 1X Tablet weight X 100 X 100}}$$

In the above calculation formula, Tarea is Peak area from sample preparation; Sarea is Average peak area from standard solution; Tweight is weight of standard taken in mg; Sweight is the weight of standard solution.

RESULTS AND DISCUSSION

HPLC method optimization

Three components standard materials (Cefixime, Ofloxacin and linezolid) were analysed for solubility study, UV spectroscopic study and pKa estimation. UV absorbance was represented in figure-2 for Cefixime, Ofloxacin and linezolid. UV spectrums confirmed the wavelength absorbance values and based on the absorbance of three components, UV wavelength was measured at 280 nm.

Solubility results reveals that Cefixime has high polarity and Ofloxacin has medium polarity and linezolid has less polar than other two components. Based on the understanding of the literature published methods, development trails were initiated with acetate buffer and methanol composed mobile phase. Less carbon C8 250 mm column was used, 280 nm, 20µL injection volume, 30°C column oven temperature was used.

Development trial-1

Conditions

1. 0.25 M ammonium acetate used as buffer; 2. Buffer and Acetonitrile mixed in the ratio of 28:72 v/v, isocratic elution; 3. Intertsil C8 250x4.6mm,5µ column; 4. Flow rate 1.0ml/min, 30°C column temperature, 280 nm; 20µL injection volume.

Observation

All three peaks were eluted but Cefixime peak shape was eluted near void volume of the column with poor peak shape. Further optimization carried out by changing the HPLC column and gradient program. Development trial mixed sample chromatogram was represented in figure 3.

Development trial-2

Conditions

1. 0.5M of ammonium acetate used as mobile phase A; 2. Acetonitrile used as mobile phase B; 4. Intertsil ODS-3 250x4.6mm,5µ column; 5. 1.0ml/min flow rate, 40°C column oven temperature, wavelength 280 nm; 6. Gradient program at 0 min 20% mobile phase B, at 8 min 20%, at 15 min 70%, at 22 min 70%, at 23 min 20% and at 27 min 20%; 7. Diluent: mobile phase A and B 50:50 v/v.

Observation

All three peaks were eluted after the column void volume but blank interference was observed. Blank interference should be minimized with mobile phase buffer and gradient program. Development trial mixed sample chromatogram was represented in figure 4.

Development trial-3

Conditions

1. 0.5M of KH_2PO_4 used as mobile phase A; 2. Acetonitrile used as mobile phase B; 4. Zorbax C18 100x4.6mm,5µ column; 5. 1.0ml/min pump mobile phase flow rate, 40°C column oven temperature, wavelength 280 nm; 6. Gradient program at 0 min 5% mobile phase B, at 5 min 5%, at 10 min 16%, at 14 min 16%, at 17 min 40% 20 min 5% and at 25 min 5%; 7. Diluent: mobile phase A and B 50:50 v/v.

Observation

All three peaks were eluted with good peak shape and no interference was observed at all three product peaks. Slight modification required for linezolid peak early elution. Development trial mixed sample chromatogram was represented in figure 5.

Method validation

Optimized HPLC method procedure was evaluated with method validation parameters such as precision, linearity, specificity, accuracy, ruggedness and robustness. % RSD for replicate standard solutions and replicate test solutions were calculated, linearity correlation coefficient was evaluated, recovery %RSD was evaluated.

System suitability

System suitability was evaluated with freshly prepared standard solutions. Five replicate standard solution injections were performed and calculated the %RSD for retention time and peak area. Other parameters theoretical plates and tailing factor were measured. Peak purity of three components was checked. System suitability results were tabulated in table-3. Blank, placebo and standard solution chromatograms were represented in figure-6, 7 and 8. %RSD values were within the limit 2.0%.

Precision

Precision also called as repeatability. Precision parameter was performed with six replicate test solutions preparations. Six replicate solutions were injected in to the HPLC system. Peak area, %RSD results were calculated and tabulated in table-4. Test solution of cefixime and Ofloxacin, cefixime and linezolid were represented in figure-9 and 10. Precision results were satisfactory and %RSD values were below 2.0%.

Specificity

Specificity parameter is used to evaluate the interference from blank, placebo, known and stress study un-known impurities. Stress studies acid, base, peroxide, thermal and UV light conditions were evaluated. Figure-11 to 20 represented the all stress studies chromatograms for cefixime-ofloxacin and cefixime-linezolid test samples. Table-5, 6 and 7 represented the stress study conditions and results. Results were satisfactory and all un-known impurities were separated and have no interference with products.

Linearity

Linearity parameter was evaluated with standard solution by preparing five different concentrations. Linearity levels are 50%, 75%, 100%, 125% and 150% concentrations. All five linearity solutions were injected into the HPLC system and calculated the correlation coefficient values. Correlation coefficient was calculated for concentration versus peak area. Results were tabulated in table-8 and linearity solutions overlay chromatogram was represented in figure-21 and linearity graphs were represented in figure-22 to 24. Results were satisfactory, correlation coefficient values were above 0.999.

Accuracy

Accuracy was evaluated to establish the recovery of the components. Different concentration of active components was added to the placebo (constant concentration for all accuracy levels). Accuracy levels 50%, 75%, 100%, 125% and 150% were evaluated. 50% and 150% were performed with six replicate preparations and remaining concentration levels were three replications. Accuracy recovery and %RSD were calculated and tabulated in table-9. % recovery results were between 97% to 103% and %RSD values were below 2.0%.

Ruggedness

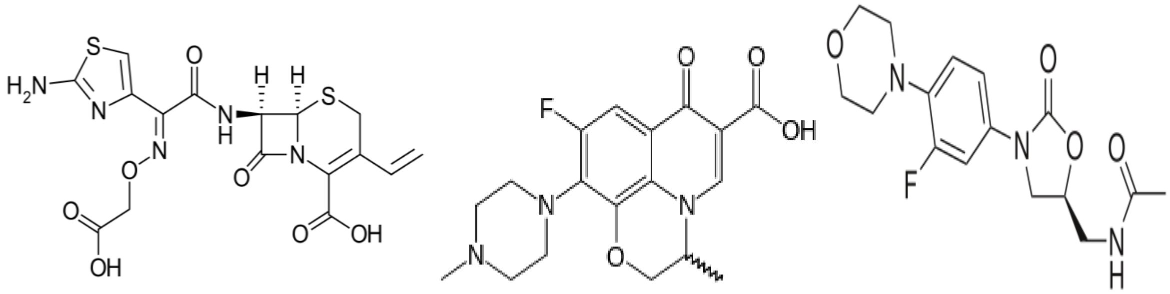
Sample solutions were used to perform ruggedness of the HPLC method. Precision test samples 1 and 2 were used to perform solution stability at room temperature and refrigerator storage conditions. Post analysis of precision 1 and 2 samples were kept at room temperature and refrigerator conditions. Analysis was performed at day-1 and day 3. Samples assay values were calculated and % assay difference found below 2.0%. Results were tabulated in table-10.

Robustness

Robustness of the method was evaluated by changing the chromatographic conditions like mobile phase flow rate, column oven temperature. System suitability was conducted to check the variation changes and results were satisfactory. Retention time, area %RSD, theoretical plates and tailing factor results were tabulated in table-11.

CONCLUSION

Stable and rugged HPLC method was developed for the quantitative determination of Cefixime, Ofloxacin and Linezolid in solid dosage form. Cefixime-Ofloxacin is available in tablet combination dosage form and Cefixime and Linezolid also available in tablets dosage form. Optimized method was evaluated with precision, linearity, specificity, ruggedness and robustness validation parameters. %RSD for area (not more than 2.0%), % recovery (between 97% - 103%), % of degradation, Correlation coefficient (not less than 0.999) and variation change difference (mobile phase flow rate, column oven temperature) were evaluated and results were satisfactory.



Cefixime **Ofloxacin** **Linezolid**

Fig. 1: Chemical structures of Cefixime, Linezolid and Linezolid

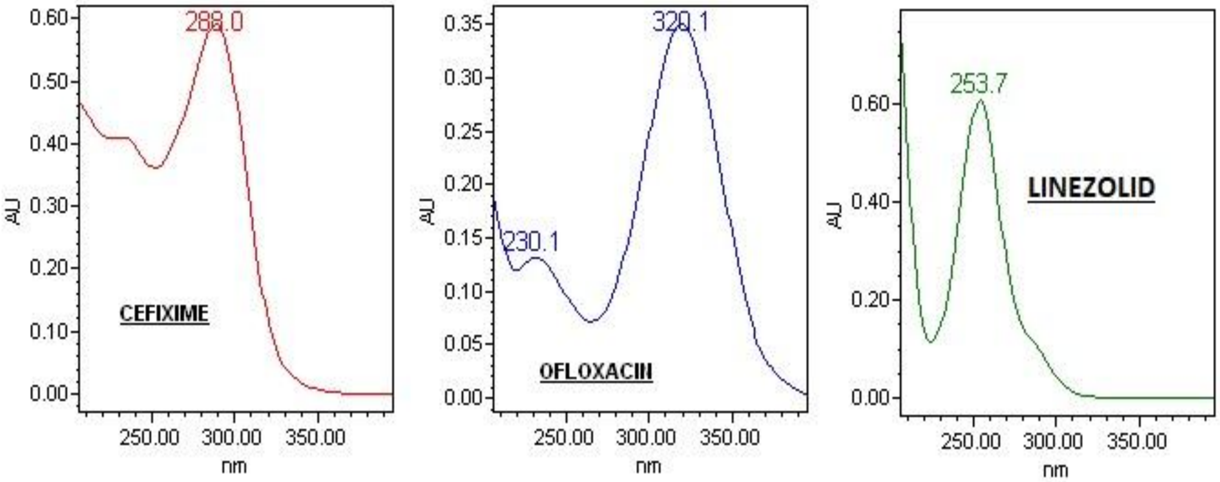


Fig. 2: UV spectrum of Cefixime, Ofloxacin and Linezolid

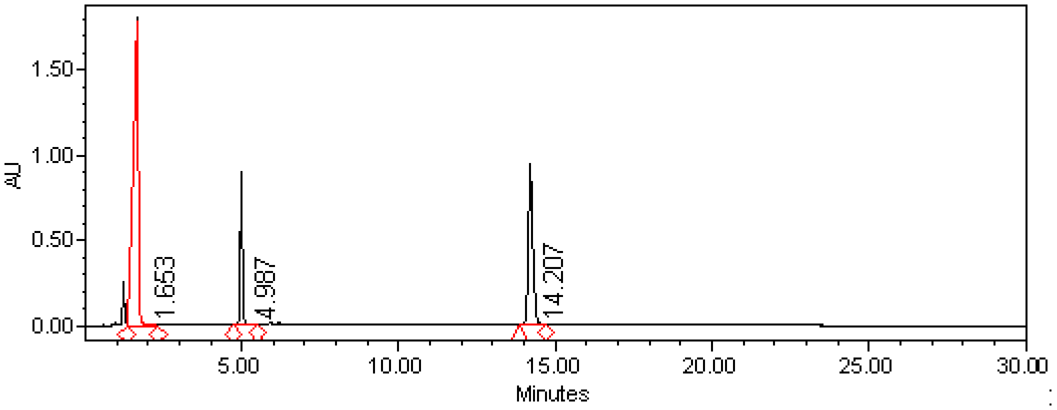


Fig. 3: Method development trial-1 chromatogram

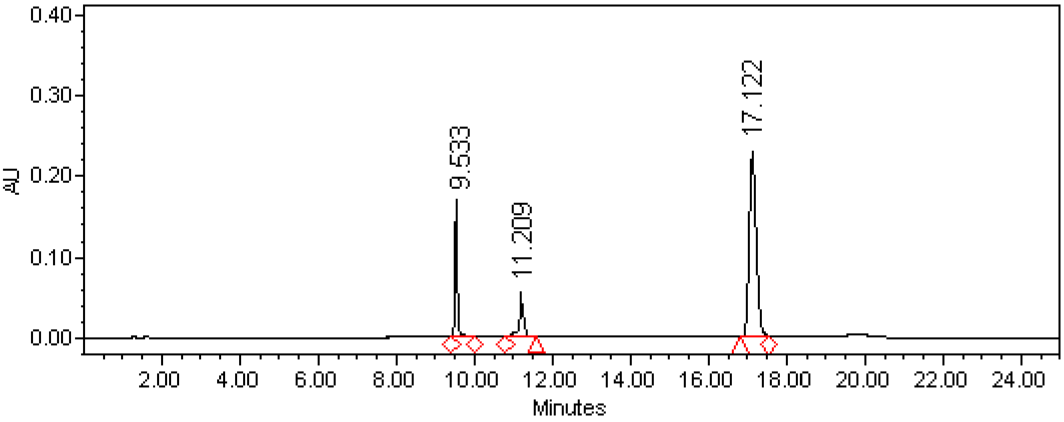


Fig. 4: Method development trial-2 chromatogram

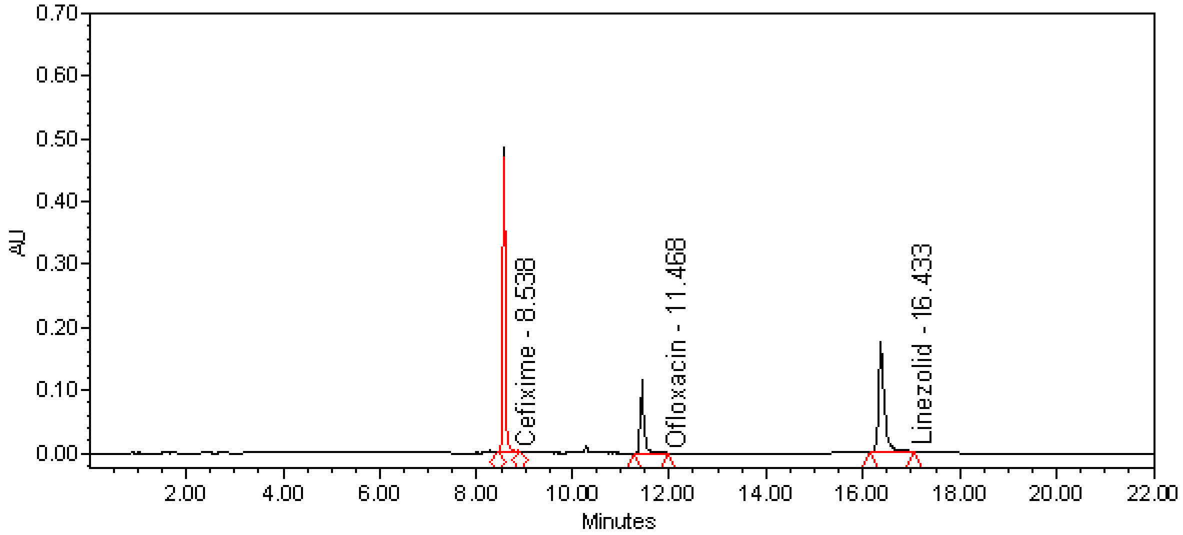


Fig. 5: Method development trial-3 chromatogram

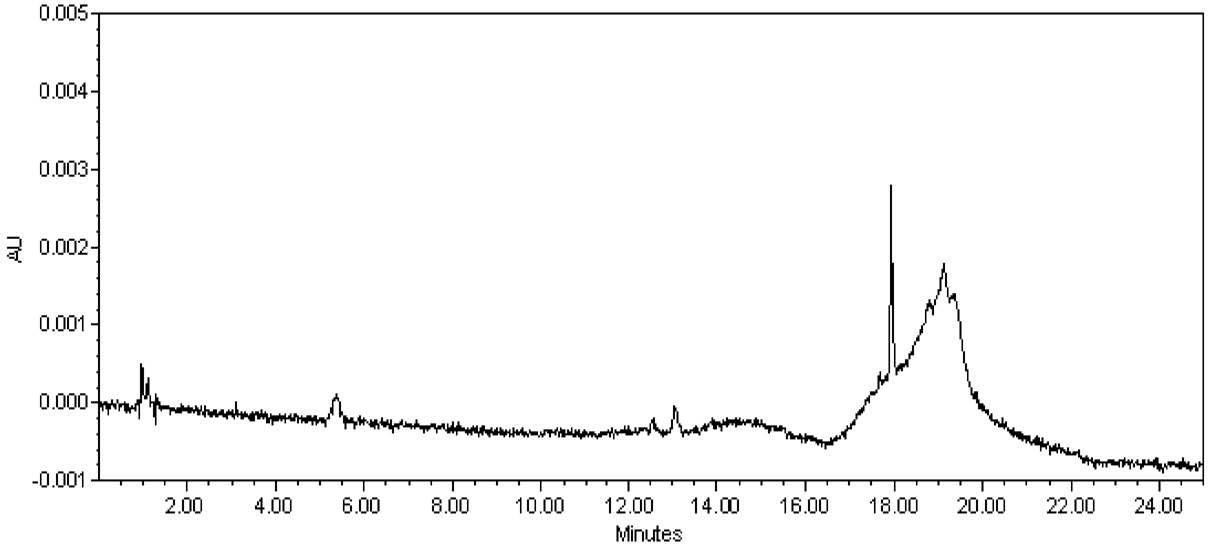


Fig. 6: Blank Chromatogram

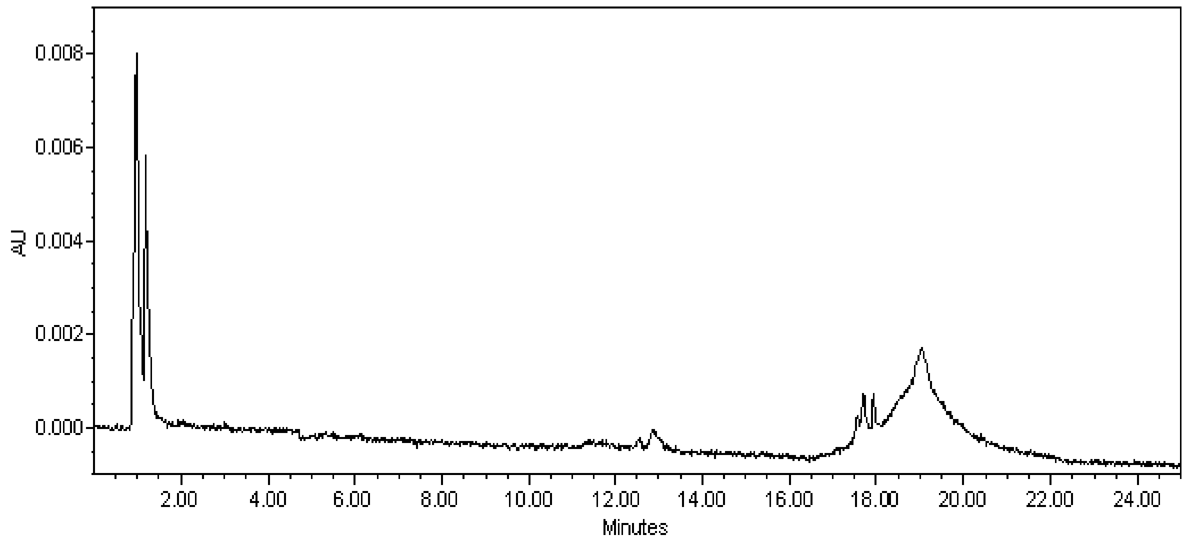


Fig. 7: Placebo Chromatogram

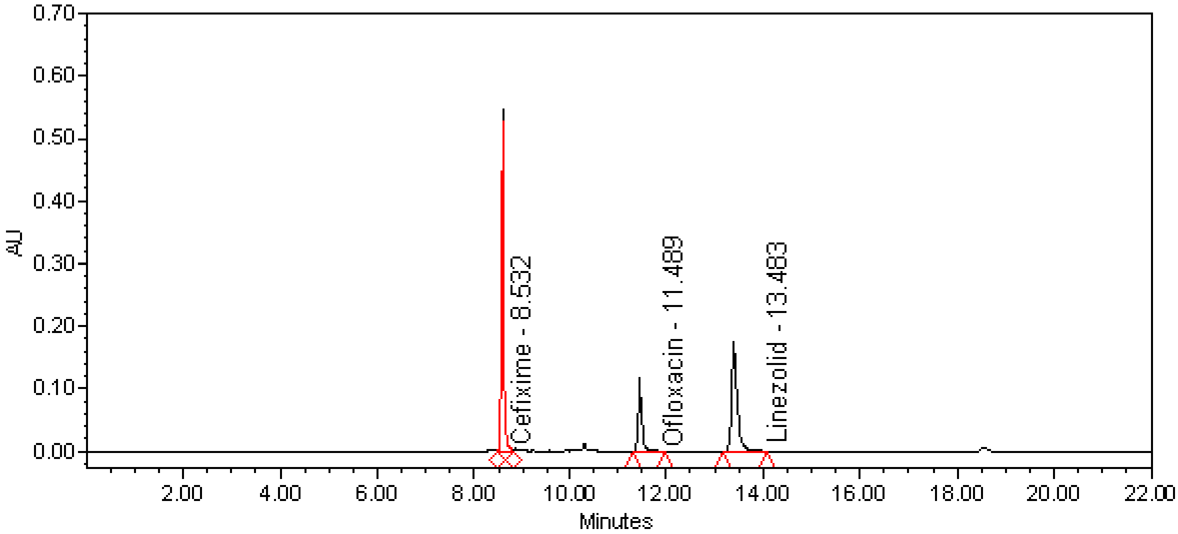


Fig. 8: Standard solution Chromatogram

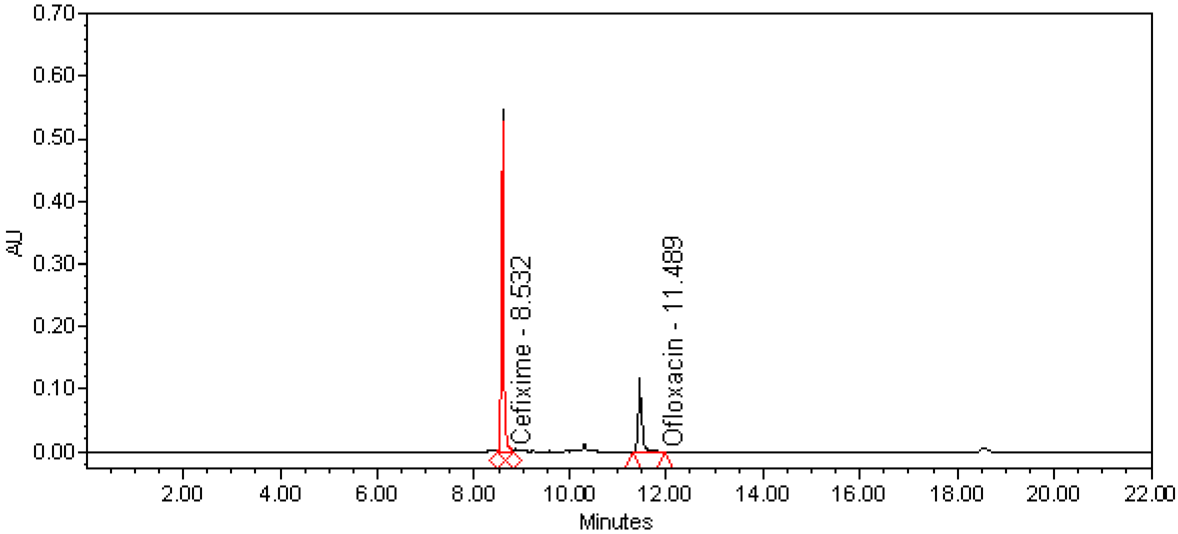


Fig. 9: Cefixime and Ofloxacin test sample chromatogram

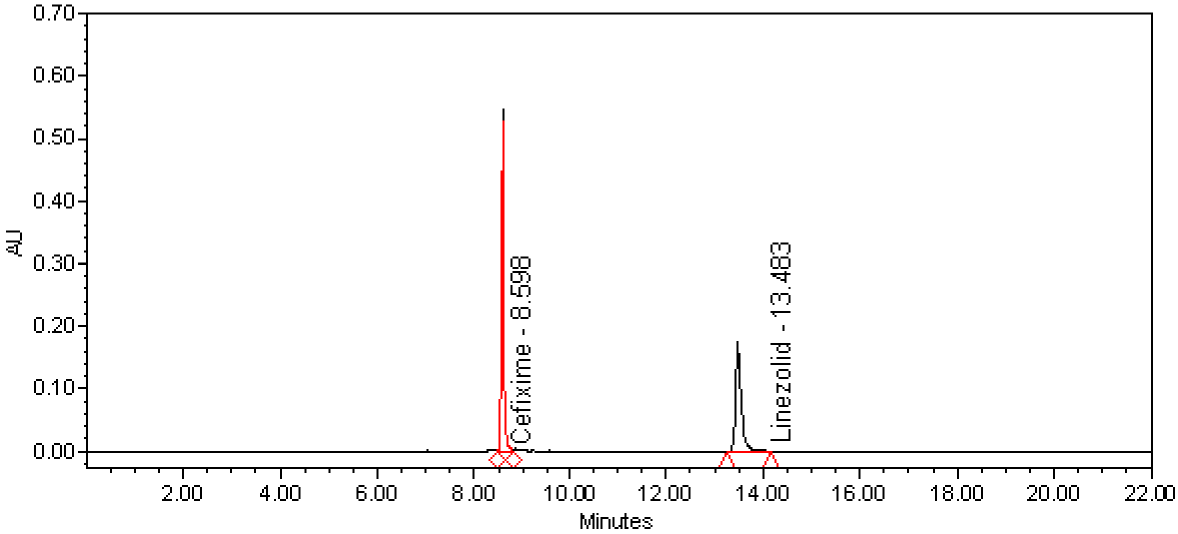


Fig. 10: Cefixime and Linezolid sample chromatogram

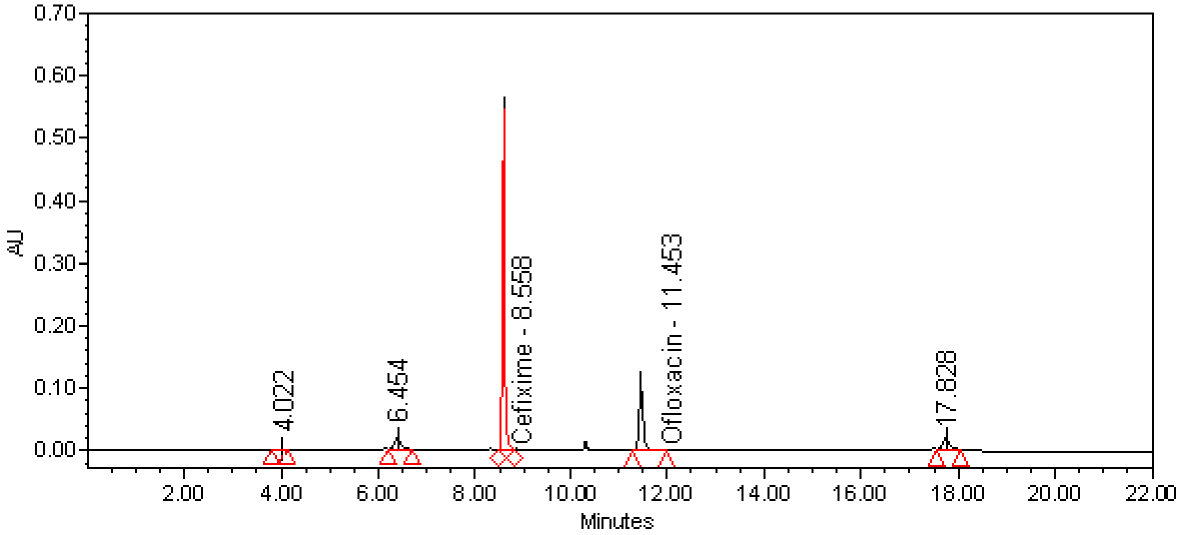


Fig. 11: Cefixime and Ofloxacin Acid stress study chromatogram

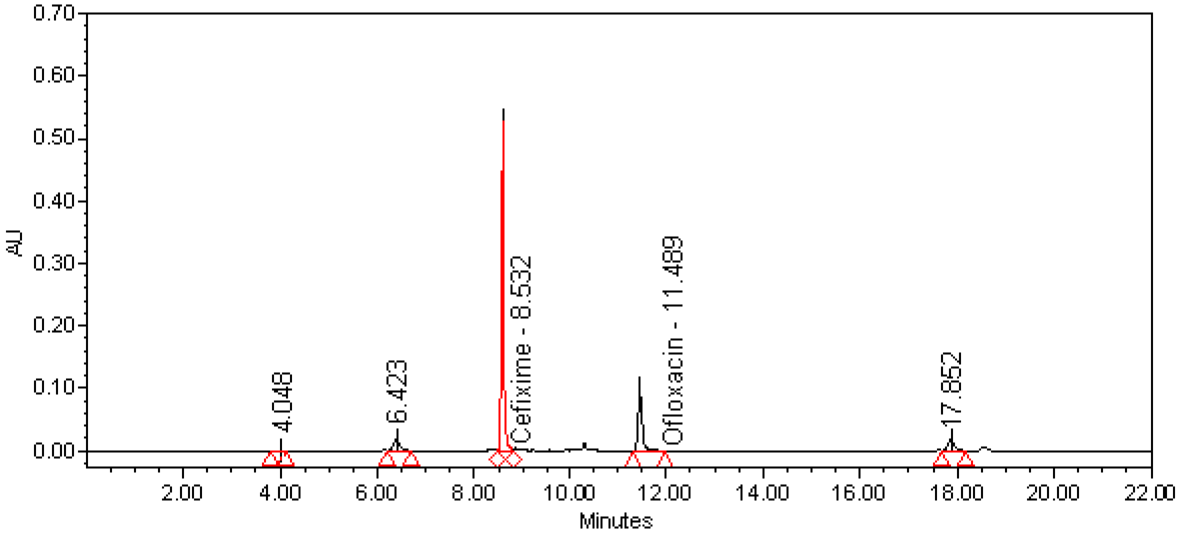


Fig. 12: Cefixime and Ofloxacin Base stress study chromatogram

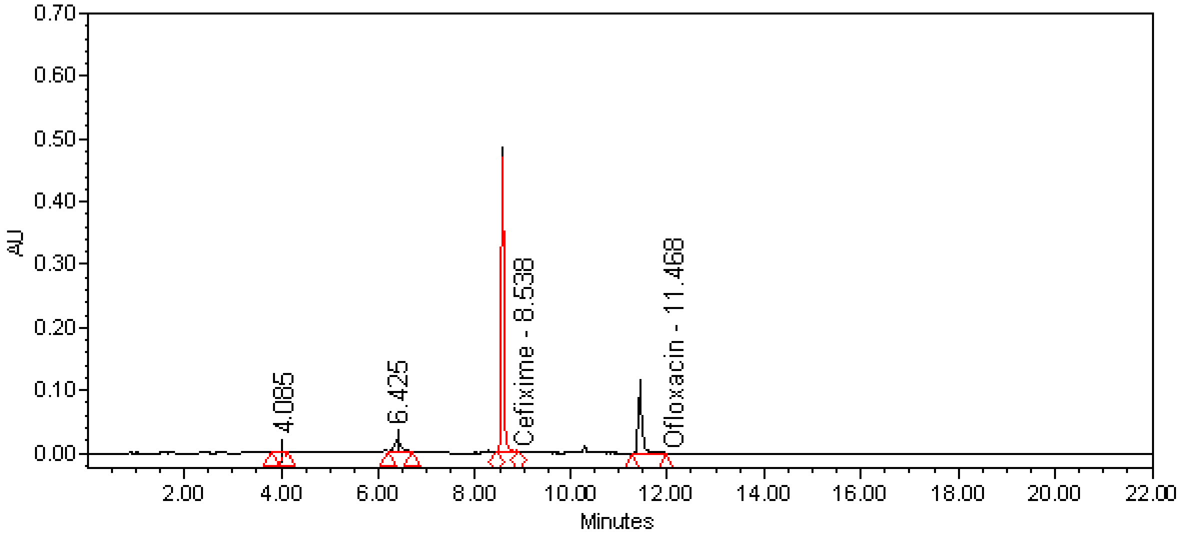


Fig. 13: Cefixime and Ofloxacin Peroxide stress study chromatogram

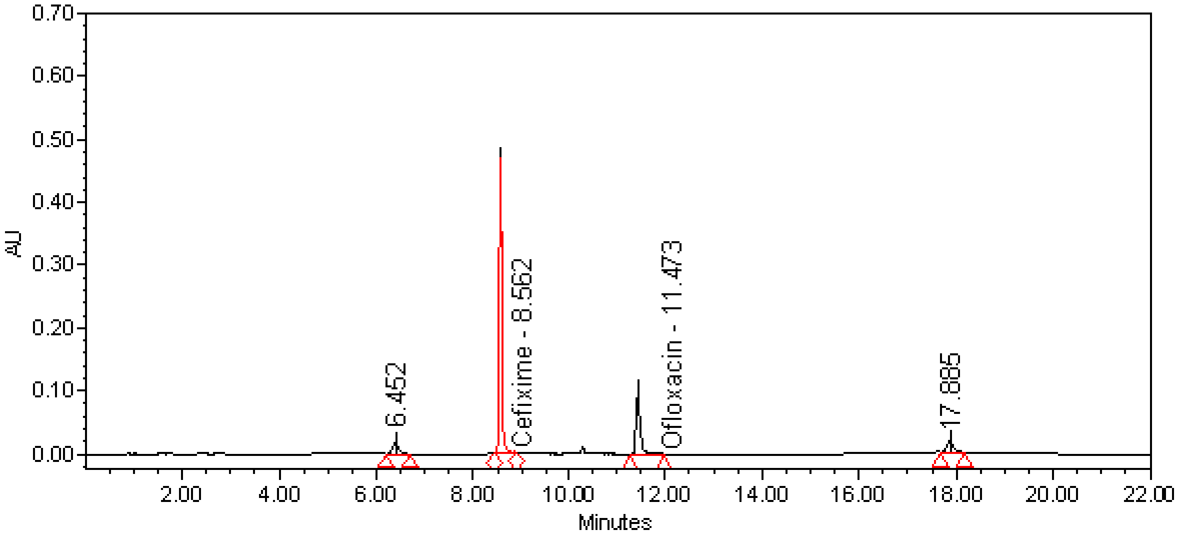


Fig. 14: Cefixime and Ofloxacin Thermal stress study chromatogram

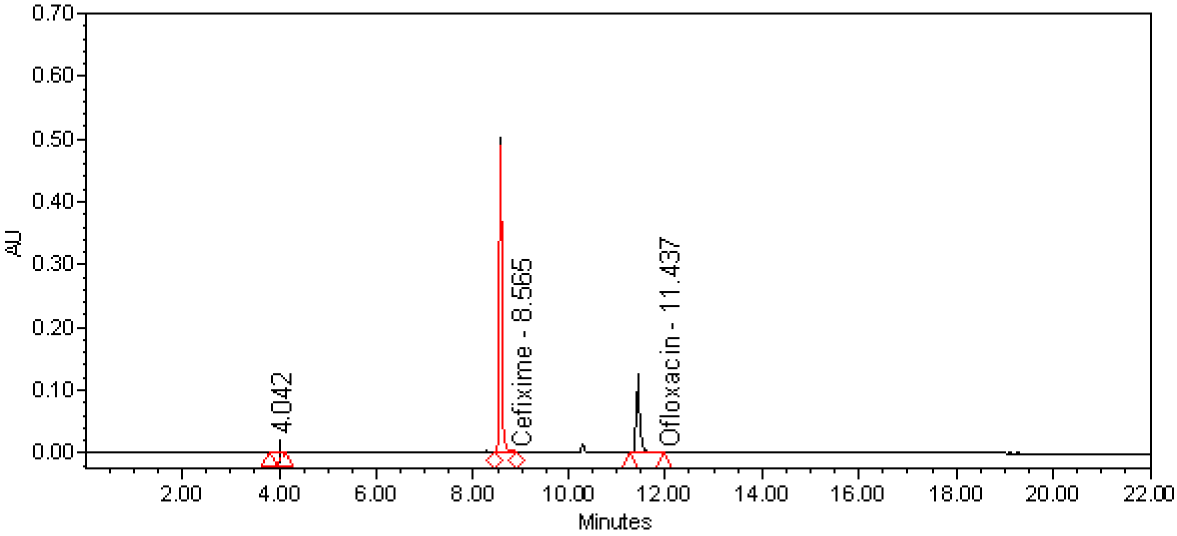


Fig. 15: Cefixime and Ofloxacin UV stress study chromatogram

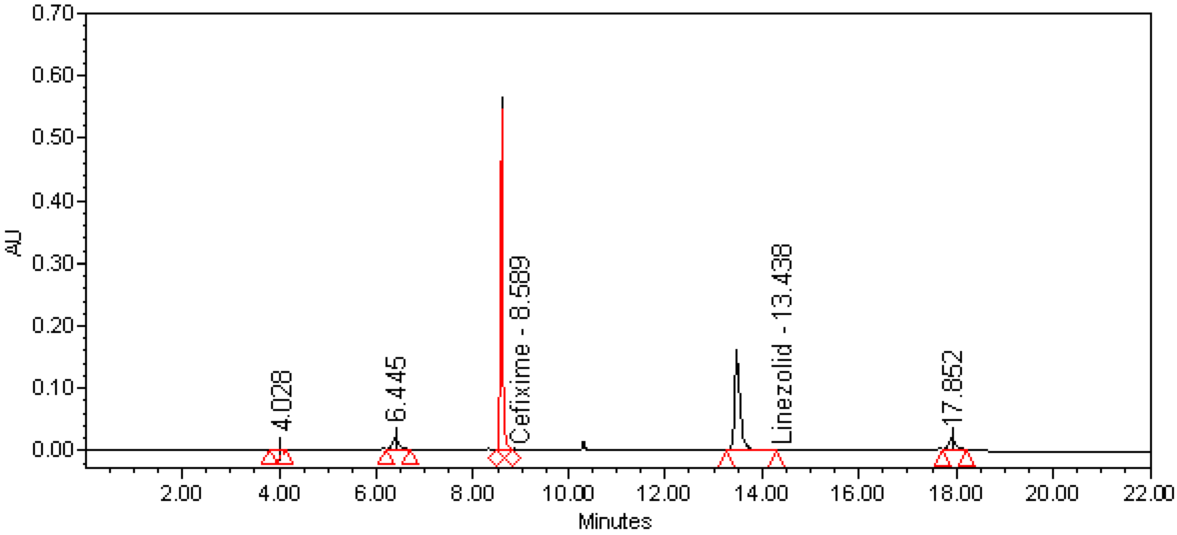


Fig. 16: Cefixime and Linezolid Acid stress study chromatogram

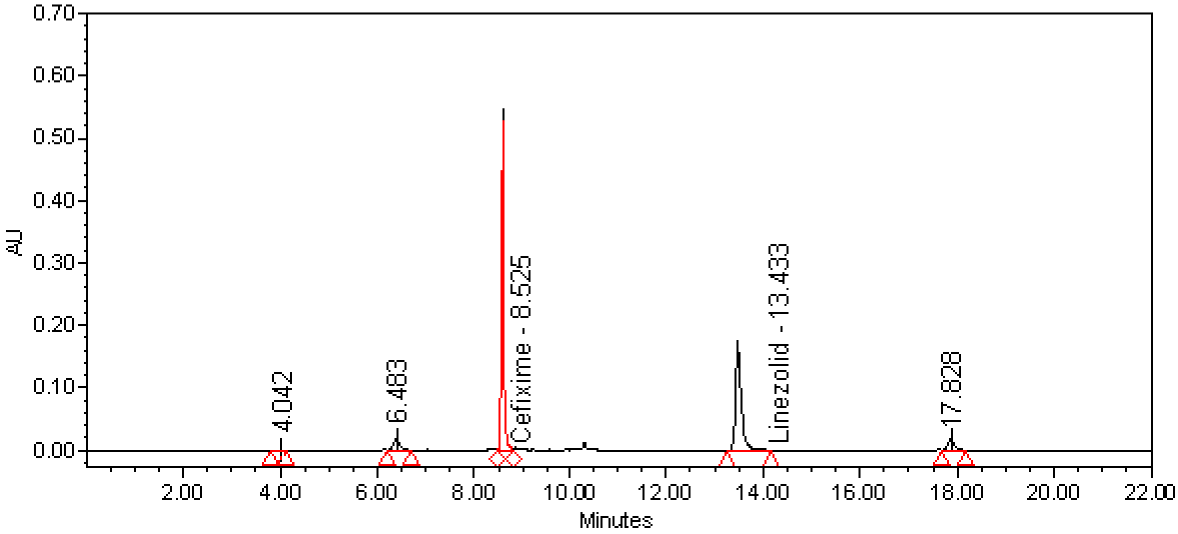


Fig. 17: Cefixime and Linezolid Base stress study chromatogram

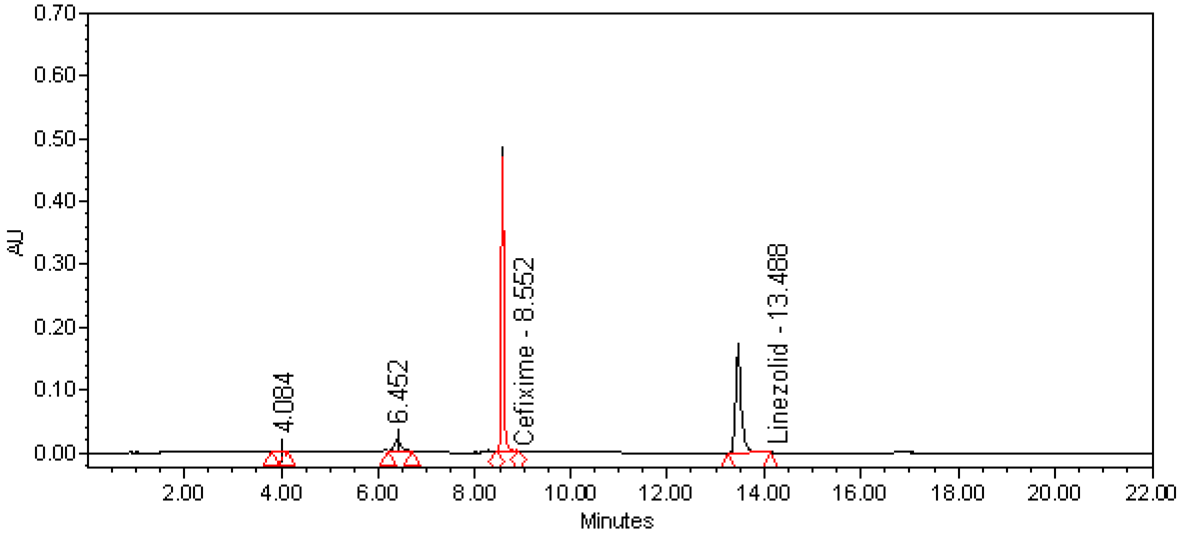


Fig. 18: Cefixime and Linezolid Peroxide stress study chromatogram

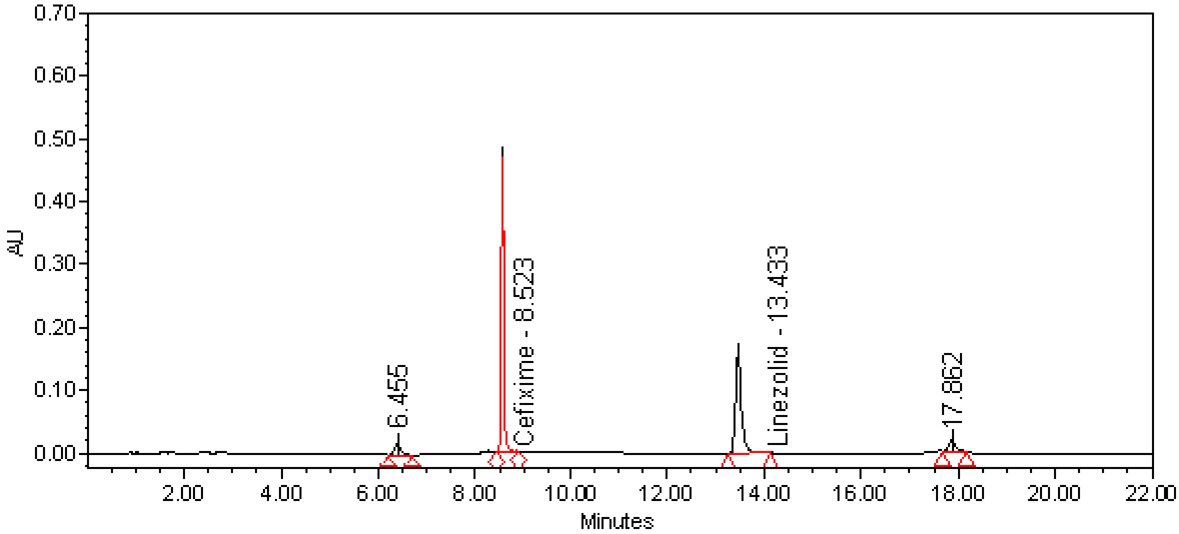


Fig. 19: Cefixime and Linezolid Thermal stress study chromatogram

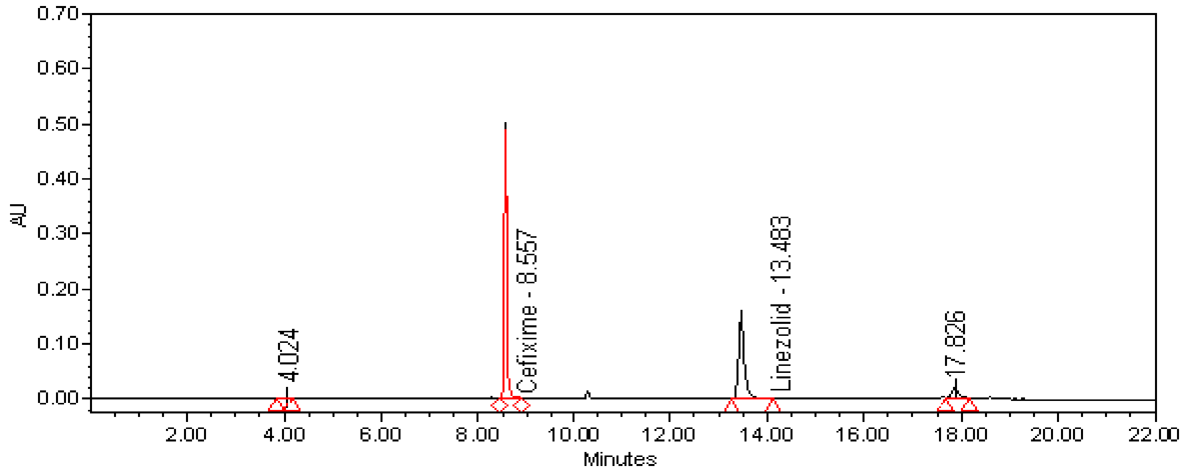


Fig. 20: Cefixime and Linezolid UV stress study chromatogram

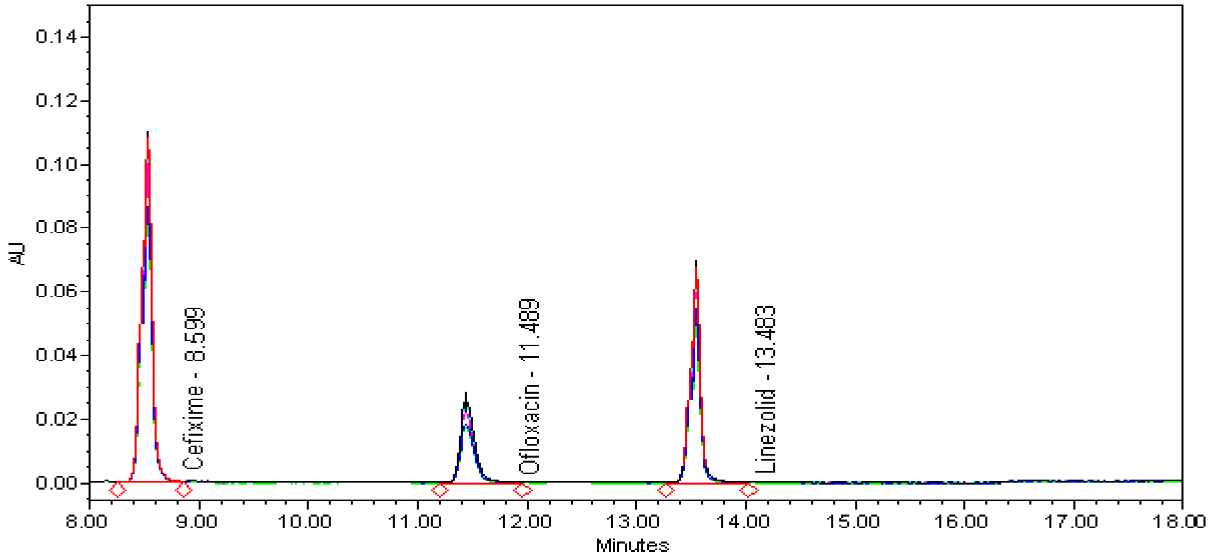


Fig. 21: Linearity overlay chromatogram

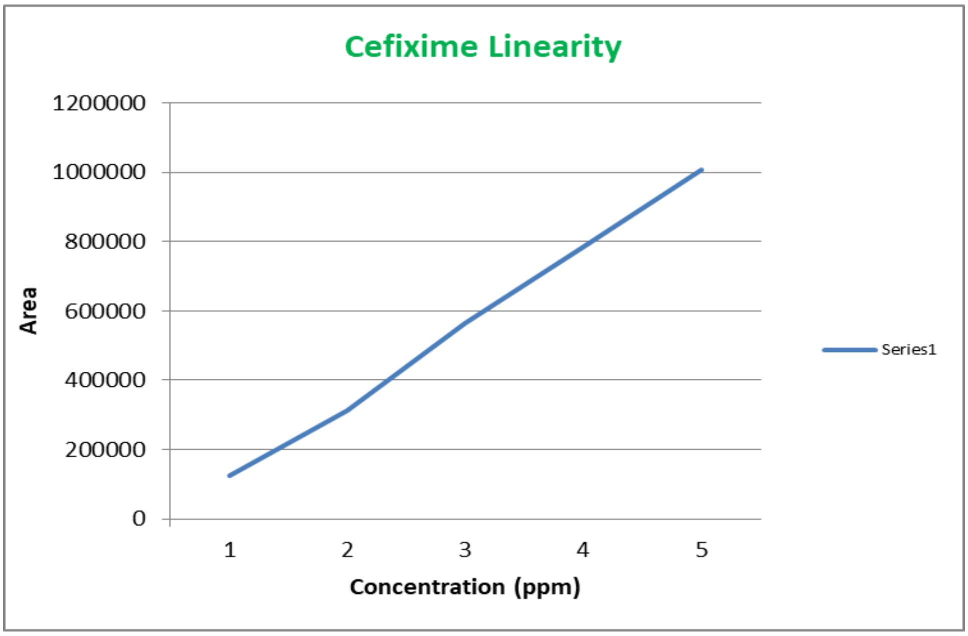


Fig. 22: Cefixime Linearity graph

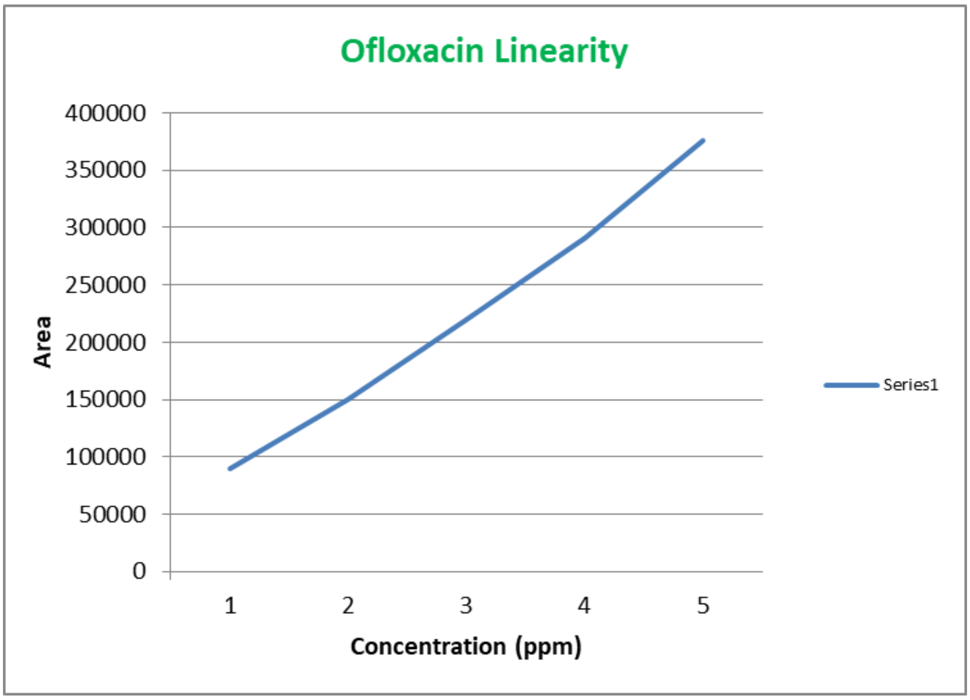


Fig. 23: Ofloxacin Linearity graph

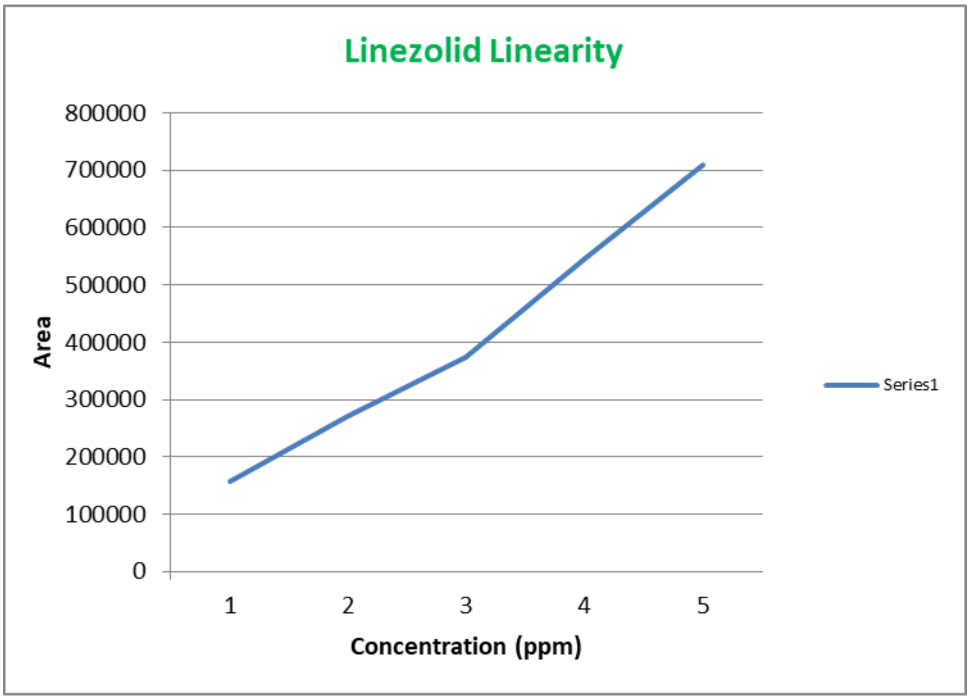


Fig. 24: Linezolid Linearity graph

Table 1: Marketed medicinal products

Brand name	Company name	Composition
EUROX-O tablets	Health guard	Cefixime 200 mg and Ofloxacin 200 mg Cefixime 100 mg and Ofloxacin 100 mg
MILIXIM-O tablets	Glenmark	
MYCEF PLUS tablets	Amro Pharma	
OFCEF tablets	JB chemicals	
RINTAX PLUS tablets	Octane biotech	
LINCEF tablets	Alkem	Cefixime 200 mg, Linezolid 600 mg
Linezonix –CF	Phoenix	
Lizomac-CX	Macleods	
Morbicef-L	Intra labs	

Table 2: Gradient program

Time (Minutes)	Mobile phase-A (%v/v)	Mobile phase-B (%v/v)
0.00	95	5
5.00	95	5
10.00	84	16
14.00	84	16
17.00	66	34
20.00	95	5
25.00	95	5

Table 3: System suitability results

Injection	Retention time (min)			Area		
	Cefixime	Ofloxacin	Linezolid	Cefixime	Ofloxacin	Linezolid
1.	8.53	11.48	13.48	566504	216345	371025
2.	8.52	11.48	13.47	567125	216314	370152
3.	8.53	11.49	13.49	567314	216781	370145
4.	8.54	11.48	13.47	559987	214987	371025
5.	8.53	11.49	13.47	561046	215164	370146
%RSD	0.08	0.05	0.07	0.63	0.37	0.13
	Theoretical plates			Tailing factor		
1.	5342	5468	5497	1.2	1.1	1.2
2.	5216	5900	5682	1.1	1.3	1.2
3.	5415	6102	5637	1.3	1.2	1.3
4.	5701	5803	5429	1.2	1.4	1.1
5.	5634	5269	5498	1.4	1.2	1.2
Average	5461	5708	5548	1.24	1.24	1.20
Peak purity Results						
Active component		Purity angle	Purity threshold		Peak purity Results	
Cefixime		0.330	0.433		Pass	
Ofloxacin		0.161	0.389		Pass	
Linezolid		0.111	0.256		Pass	

Table 4: Precision and intermediate results

S.No.	Precision % Assay				Intermediate precision % Assay			
	Cefi.	Oflo.	Cefi.	Line.	Cefi.	Oflo.	Cefi.	Line.
1	99.8	101.2	100.6	101.3	101.3	101.2	101.0	100.6
2	101.2	100.4	101.3	100.8	100.5	100.7	100.6	101.0
3	100.6	101.2	100.4	100.4	100.6	100.2	100.4	101.3
4	100.1	99.9	99.9	100.8	99.6	101.0	100.8	100.8
5	99.9	100.1	100.3	101.4	100.6	100.7	99.9	100.4
6	100.8	101.6	100.8	101.3	100.3	100.1	100.5	100.1
Average	100.4	100.73	100.5	101	100.48	100.65	100.5	100.7
% RSD	0.54	0.65	0.47	0.49	0.55	0.37	0.25	0.42

Table 5: Specificity stress study conditions

Cefixime Ofloxacin sample	Cefixime Linezolid sample
Acid stress/1N-60°C/60 minutes	Acid stress/1N-60°C/60 minutes
Base Stress/1N- 60°C/2 hrs	Base Stress/1N- 60°C/2 hrs
Peroxide stress/3%- 60°C/1 hrs	Peroxide stress/3%- 60°C/1 hrs

Thermal (80°C for 6 hrs)	Thermal (80°C for 6 hrs)
UV energy of 200-watt hrs/^{2}m	UV energy of 200-watt hrs/^{2}m

Table 6: Specificity Results

Stress condition	Cefixime			Ofloxacin			Linezolid		
	Purity angle	**Purity threshold**	**Pass/ fail**	**Purity angle**	**Purity threshold**	**Pass/ fail**	**Purity angle**	**Purity threshold**	**Pass/ fail**
Acid	0.332	0.412	Pass	0.113	0.251	Pass	0.121	0.236	Pass
Base	0.315	0.421	Pass	0.116	0.261	Pass	0.131	0.250	Pass
Peroxide	0.264	0.484	Pass	0.132	0.269	Pass	0.125	0.253	Pass
Thermal	0.269	0.428	Pass	0.223	0.278	Pass	0.120	0.243	Pass
UV	0.289	0.451	Pass	0.120	0.236	Pass	0.196	0.238	Pass

Table 7: Specificity results

Peak RT (min)	Cefixime and Ofloxacin samples degradation				
	Acid	**Base**	**Peroxide**	**Thermal**	**UV**
4.1	1.45	1.48	1.36	NA	1.40
6.4	1.61	1.50	1.42	1.41	NA
17.8	1.30	1.43	NA	1.46	NA
Cefixime and Linezolid samples degradation					
4.2	1.43	1.46	1.39	NA	1.40
6.4	1.29	1.38	1.40	1.42	NA
17.8	1.38	1.42	NA	1.40	1.39

Table 8: Linearity results

Linearity level	Cefixime		Ofloxacin		Linezolid	
	Conc.	**Area**	**Conc.**	**Area**	**Conc.**	**Area**
50%	5.21	125910	5.17	89910	15.48	155910
75%	7.52	313236	7.49	150236	22.59	270236
100%	10.10	565504	10.04	219345	28.9	375025
125%	12.45	785681	12.48	290681	37.8	545681
150%	15.2	1005610	15.15	375610	47.13	710610
Correlation coefficient.	0.9993		0.9992		0.9994	

Table 9: Accuracy samples preparations and recovery results

Recovery level	Sample Prepn.	Cefixime Recovery		Ofloxacin Recovery		Linezolid Recovery	
		% Recovery	**Mean recovery/ %RSD**	**% Recovery**	**Mean recovery/% RSD**	**% Recovery**	**Mean recovery/% RSD**
50%	1	99.6	100.31 /0.55	100.3	100.30/ 0.47	100.2	100.40/0.51
	2	101.2		99.7		101.3	
	3	100.3		101.0		100.4	
	4	100.5		100.3		99.9	
	5	99.9		100.6		100.0	
	6	100.4		99.9		100.6	
75%	1	100.8	101.06 /0.30	100.7	100.36/ 0.35	100.1	100.60/0.46
	2	101.0		100.0		100.7	
	3	101.4		100.4		101.0	
100%	1	99.9	100.26 /0.40	100.6	100.63/ 0.35	100.4	100.46/0.60
	2	100.2		101.0		101.1	
	3	100.7		100.3		99.9	
125%	1	101.0	100.43/ 0.55	100.6	100.63/ 0.35	100.3	100.06/0.21
	2	100.4		100.3		100.0	
	3	99.9		101.0		99.9	
150%	1	100.3	100.48 /0.37	99.9	100.16/ 0.44	100.4	100.28/0.32
	2	101.0		100.3		99.9	
	3	100.7		101.0		100.3	
	4	100.6		100.1		100.8	
	5	100.4		99.9		100.0	
	6	99.9		99.8		100.3	

Table 10: Sample solution stability results

Room Temperature								
Time interval	**Cefixime-Ofloxacin sample**				**Cefixime-Linezolid sample**			
	Cefixime		**Ofloxacin**		**Cefixime**		**Linezolid**	
	% Assay	**% Diff.**	**% Assay**	**% Diff.**	**% Assay**	**% Diff.**	**% Assay**	**% Diff.**
Initial-1	99.8	NA	101.2	NA	100.6	NA	101.3	NA
Intial-2	101.2		100.4		101.3		100.8	
Day-1	100.2	0.4	100.4	0.8	100.9	0.3	100.3	1.0
Day-1	100.4	0.8	100.8	0.4	100.0	1.3	100.6	0.2
Day-3	101.1	1.3	101.0	0.2	100.4	0.2	100.0	1.3
Day-3	100.5	0.7	100.6	0.2	100.6	0.7	100.5	0.3

Table 11: Flow rate variation, temperature variation system suitability results

Variation	**Robust Parameters**		**RT (min)**	**5 inj. Area %RSD**	**USP Plate Count avg.**	**USP Tailing avg.**
Flow variation	Actual (1.0ml/min)	Cefi.	8.53	0.32	5681	1.12
		Oflo.	11.41	0.25	5490	1.01
		Line.	13.46	0.21	5389	1.10
	Low (0.9ml/min)	Cefi.	8.63	0.40	5709	1.30
		Oflo.	11.56	0.34	6100	1.41
		Line.	13.64	0.29	6081	1.13
	High (1.1ml/min)	Cefi.	8.32	0.32	5937	1.10
		Oflo.	11.34	0.41	5890	1.15
		Line.	13.40	0.29	5687	1.31
Column oven temp.	Low 25°C	Cefi.	8.62	0.31	5909	1.25
		Oflo.	11.54	0.28	6012	1.01
		Line.	13.52	0.43	6081	1.15
	High 35°C	Cefi.	8.29	0.40	5964	1.12
		Oflo.	11.35	0.36	5937	1.32
		Line.	13.40	0.30	6106	1.30

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STABILITY INDICATING DETERMINATION OF DARUNAVIR WITH HPLC IN BLOOD PLASMA SAMPLES

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ABSTRACT

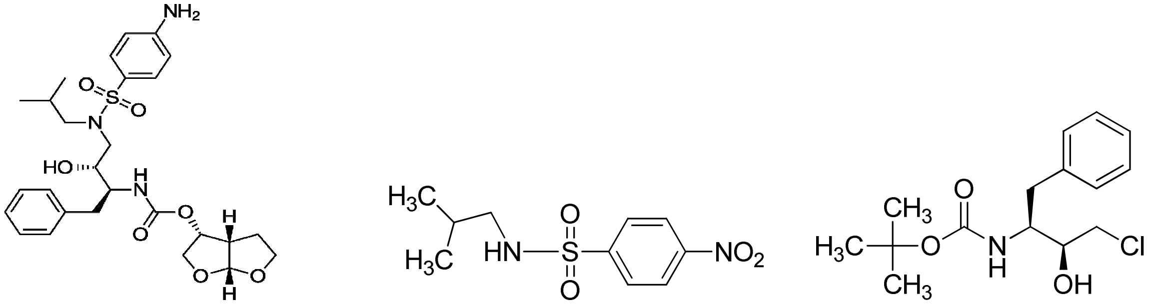
Darunavir is an HIV/ AIDS treatment drug product. Darunavir is used with other medicines line cobicistat or ritonavir. The present objective is to develop and validate the HPLC method for the determination of darunavir in the blood plasma sample. Mobile Phase composed for pH 8.10 buffer (K_2HPO_4), Methanol and acetonitrile in the ratio of 488:162:350 v/v/v. The diluent is a mixture of water and acetonitrile 40:60 v/v. X-Bridge C18 5μm 4.6x250mm column, wavelength 262 nm, injection volume 20μL, flow rate 1.3 ml/min and column oven temperature 35°C were used. Method validation was performed and results were within the acceptable limits. Results confirmed that the developed method is stability indicating and can be applied to analyze the blood plasma samples.

Keywords: Darunavir, blood plasma samples, tablets dosage form, method validation, antiretro viral drug product.

INTRODUCTION

Darunavir is used as an antiretroviral medication to control HIV / AIDS. This drug is recommended to use with other antiretroviral medicines. Darunavir was innovated by Tibotec at University of Illinois at Chicago[1-2]. It can be used to control after a needle stick injury. Darunavir is approved in the USA in the year of 2006 and it is listed in WHO essential medicines. Darunavir drug is available in Darunavir/ Cobicistat, Darunavir/ Ritonavir combination single pill[3-4]. Darunavir is well tolerated by patients however it has side effects like high blood sugar, diabetes, muscle pain, tenderness, abdominal pain, and constipation, vomiting and headache[5-6]. Darunavir is available in ethanolate form and in the market, it is available in tablets dosage form with 75 mg, 150 mg and 600 mg.

Present research work objective was to develop a simple, stability indicating HPLC method for the quantification of darunavir in blood plasma and urine samples. Two known synthetic impurities were considered for the HPLC method. Chemical structure of darunavir and its impurities were represented in Fig.-1.



Darunavir **Impurity-1** **Impurity-2**

Fig.-1: Chemical Structure of Darunavir and its Impurities

Darunavir: [(3aS,4R,6aR)-2,3,3a,4,5,6a-hexahydrofuro[2,3-b]furan-4-yl] N-[(2S,3R)-4-[(4-aminophenyl)sulfonyl-(2-methylpropyl)amino]-3-hydroxy-1-phenylbutan-2-yl]carbamate

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Impurity-1: N-isobutyl-4-nitrobenzenesulfonamide
Impurity-2: Tert-butyl ((2S,3S)-4-chloro-3-hydroxy-1-phenylbutan-2-yl)carbamate
Literature survey was performed and reported methods by HPLC/ MS are, Takahashi M (2007)[7], Patel BN (2012)[8], Reddy BR (2012)[9], Babu GR (2013)[10], Satyanarayana L (2011)[11], Goldwirt L (2007)[12]. FT-IR method by Kogawa AC (2013)[13]. Main objective of this research work was to develop a simple, stability indicating HPLC method for the determination of darunavir in blood plasma and urine samples.

EXPERIMENTAL

Reagents

Di-potassium hydrogen phosphate anhydrous AR grade, Ortho phosphoric Acid AR grade, Acetonitrile HPLC Gradient Grade, Methanol HPLC Gradient Grade, Water for HPLC or equivalent.

Preparation of Dilute Orthophosphoric Acid

Diluted 1 mL of Orthophosphoric acid to 10 mL with water and mixed well.

Preparation of Buffer Solution (pH 3.0)

Weighed and transferred accurately about 4.35 g Di-potassium hydrogen phosphate anhydrous into 1000 mL water, sonicated to dissolve and mixed well. pH adjusted to 3.0 ± 0.05 with dilute Orthophosphoric acid. Mixed well and filtered through a 0.45µ membrane filter.

Preparation of Mobile Phase

4.35 g Dipotassium hydrogen phosphate anhydrous into 1000 mL water, sonicate to dissolve and mix well. Adjust pH to 8.10 ± 0.05 with dilute Orthophosphoric acid. pH 8.10 buffer, Methanol and acetonitrile in the ratio of 488:162:350 v/v/v respectively.

Preparation of Diluent

Prepare a mixture of Water and Acetonitrile in the ratio of 40:60 **v/v** respectively.

Chromatographic Conditions

Column: X-Bridge C18 5µm 4.6x250mm
Wavelength: 262 nm
Injection volume: 20µL
Flow rate: 1.3 ml/min
Column oven temperature: 35°C

Preparation of Diluted Standard Solution

Weighed and transferred accurately 40mg of Darunavir standard into a 100 mL volumetric flask. 70 mL of diluent added and sonicated to dissolve and mix well. 10 mL of this solution pipetted into 100 mL volumetric flask and made up the volume to 50 mL with diluent and mixed well.

Preparation of Sample Solution

Sample powder equivalent to 40 mg of darunavir weighed and transferred to 100 mL volumetric flask. 70 mL diluent added and sonicated for 30 minutes with intermittent shaking. Diluted up to the mark with diluent and mixed well. 10 ml of solution pipetted and diluted to 100 ml with diluent.

Bioanalytical Sample

The liquid-liquid extraction procedure was used to prepare plasma sample. Darunavir tablets 300mg were used; equivalent to 300mg of Darunavir test sample was spiked into 10ml plasma and stored at 2°C for 1 day. Stored spiked solutions were pull-out from the freezer and allowed reach room temperature. A 0.5ml aliquot was transferred to 10.0 mL polypropylene centrifuge tubes. 5.0 mL of ethyl acetate was used to get the two layers and extraction was completed. Centrifuge tubes were kept on vibramax unit for 15 min. Final solutions were centrifuged at 5000 rpm for 5 min at 4°C. 1ml of the supernatant solution was transferred into polypropylene tubes and evaporated at 40°C. The dried residue was dissolved in 200µL of the mobile phase. Dilution was performed to reach 40ppm concentration. Samples, 20µL by volume, were then injected into the column and analyzed by HPLC on the same day to avoid any degradation. The column temperature oven was maintained at ambient temperature.

RESULTS AND DISCUSSION

Method Development

Literature survey and analytes nature were understood and initial method development were started with phosphate buffer and acetonitrile in the ratio of 60:40; inertsil C18 250mm, 4.6mm; 1.0ml/min flow rate; 20µL injection volume. Impurity-1 and 2 were eluted along with darunavir. Three components UV absorbance was scanned from 200 to 400 nm to select the maximum UV absorbance wavelength. Based on the UV absorbance spectrum for three analytes 267 nm was selected. Figure-2 represented the UV spectrum for darunavir and its impurities. Later we changed the mobile phase ratio with methanol and buffer pH also studied to get the high resolution between darunavir and impurities. Finally, pH8.1 buffer, acetonitrile and methanol mobile phase gave satisfactory results. Final method individual chromatograms were shown in Fig.-3 to 5.

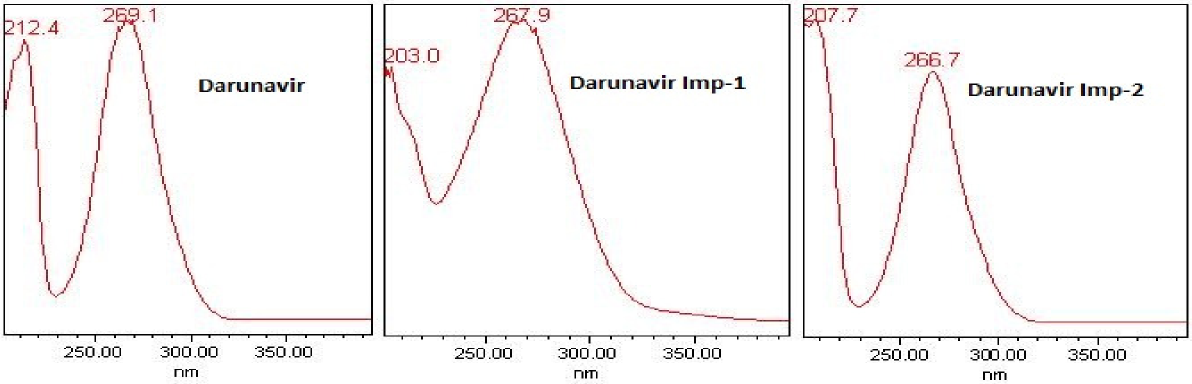

Fig.-2: UV Spectrum Darunavir and its Impurities 1 and 2

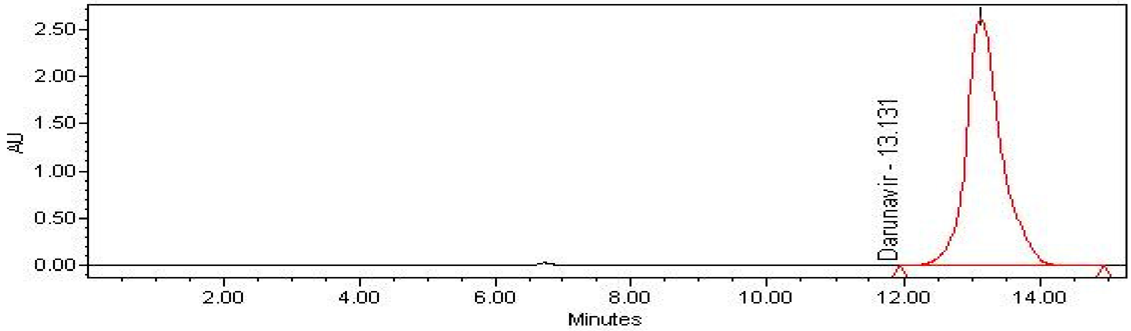

Fig.-3: Darunavir chromatogram

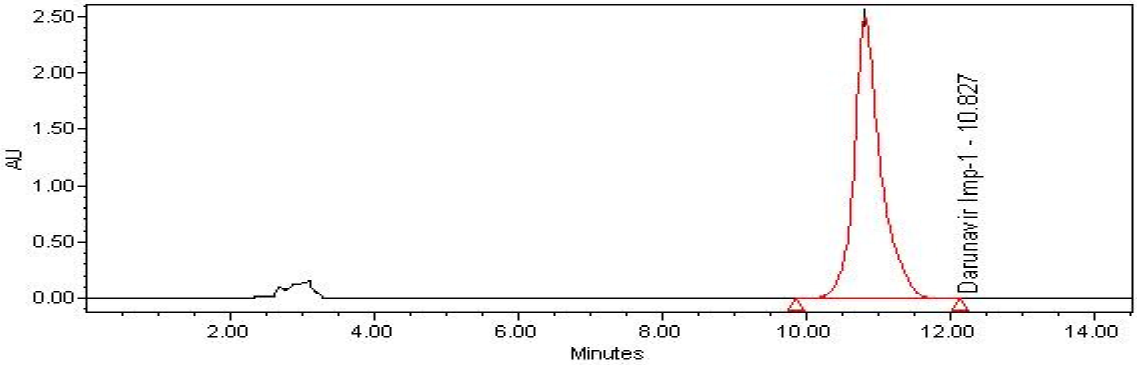

Fig.-4: Darunavir Impurity-1 Chromatogram

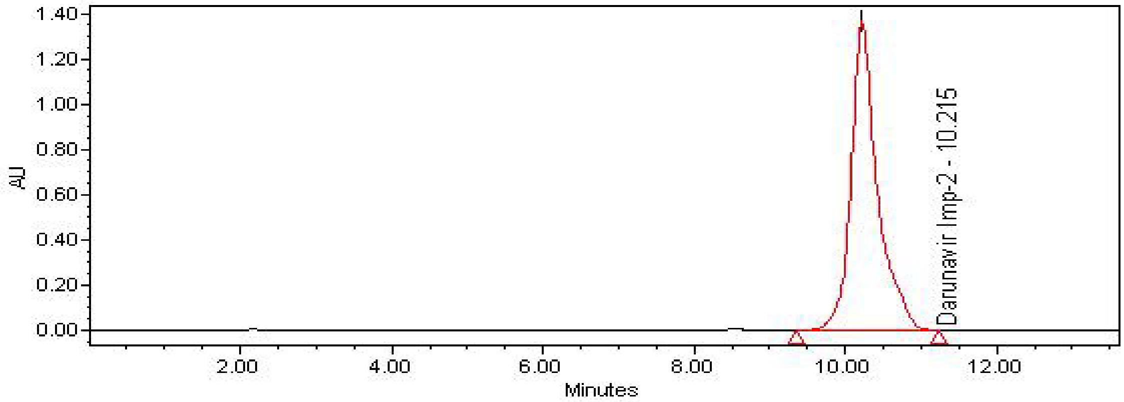

Fig.-5: Darunavir Impurity-2 Chromatogram

System Suitability

Final method was performed to confirm the method suitability and instrument suitability. FigureS-6, 7 and 8 represented the diluent, excipients solution and standard solution 5 injections. Table-1 represents the system suitability results.

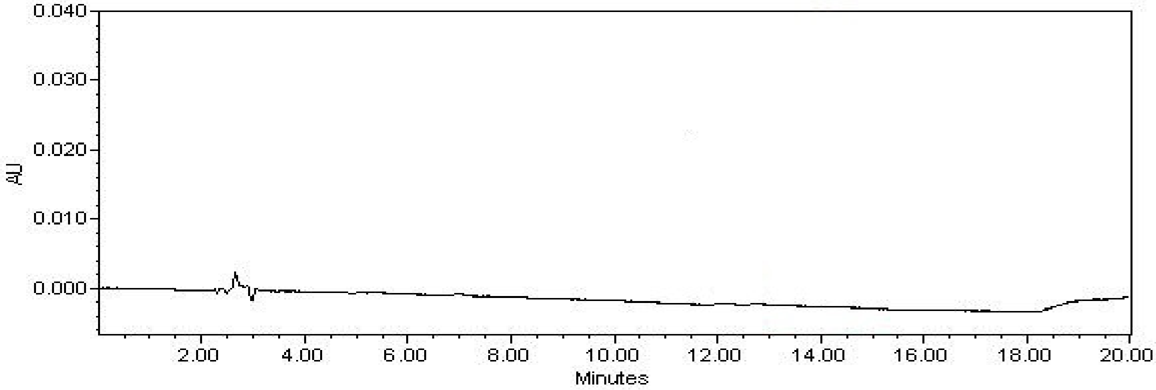

Fig.-6: Diluent Chromatogram

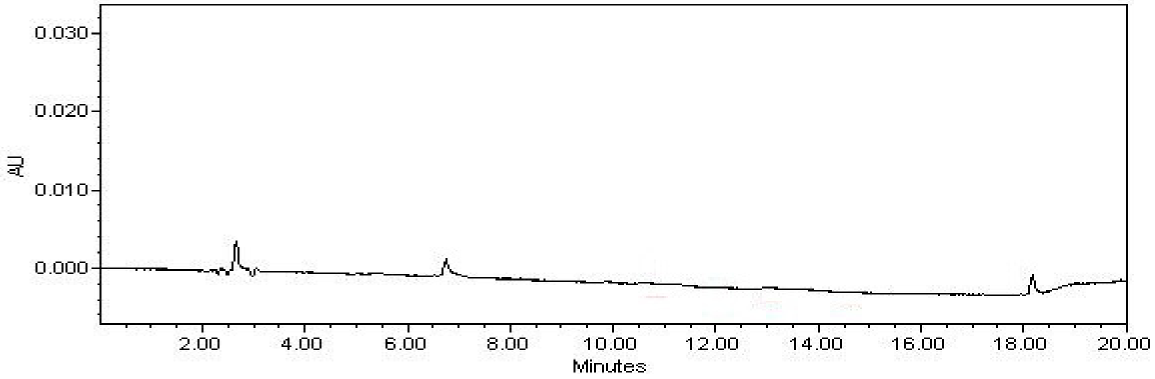

Fig.-7: Excipients Chromatogram

Table-1: System Suitability Results

Injection	Darunavir		
	Retention time (min)	Area	Tailing factor
1.	14.01	1545122	1.18
2.	14.08	1542314	1.15
3.	14.07	1531524	1.14
4.	14.06	1551241	1.16
5.	14.00	1543214	1.17
%RSD	0.25	0.46	NA

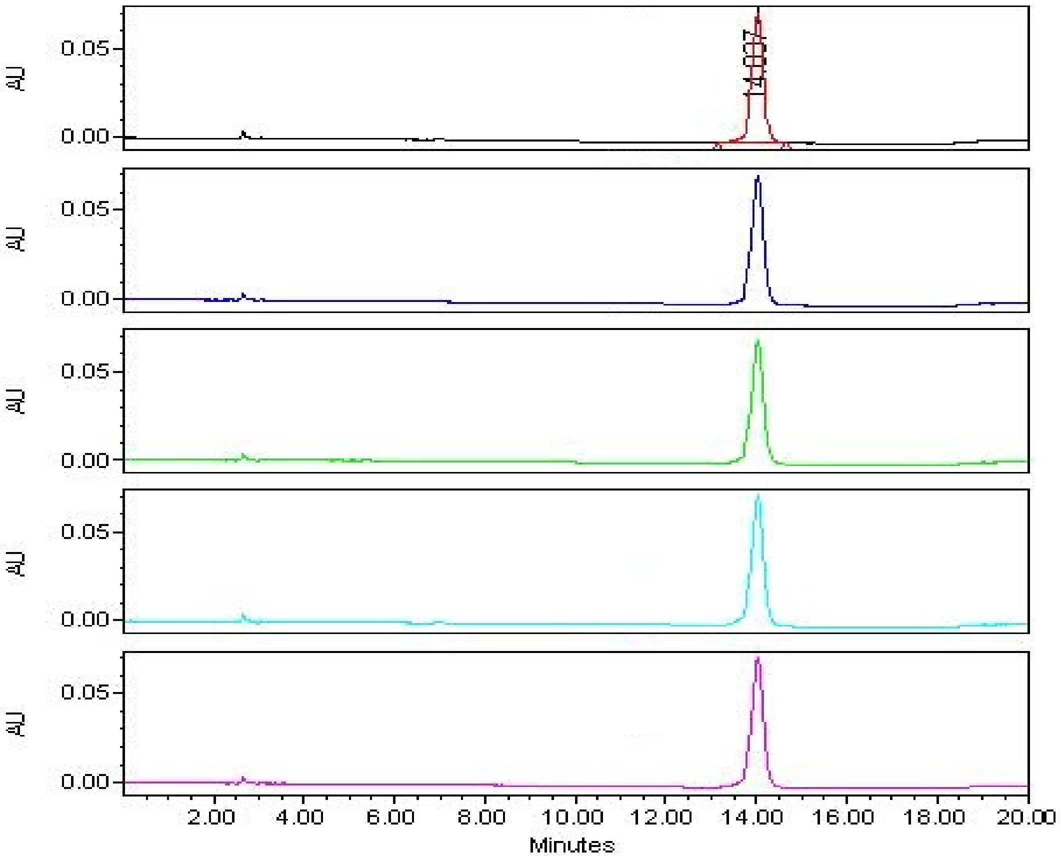

Fig.-8: Five Replicate Standard Solution Injections

Method validation was performed to confirm the method stability indicating property with specificity, precision, accuracy, ruggedness, robustness and linearity.

Specificity

Specificity was evaluated for the interference of blank, placebo and known impurities 1 and 2. Forced degradation studies were performed for acid, base, peroxide, thermal, water and UV light. Blank and placebo and known impurities-1 and 2 were well separated and no interference was observed at the retention time of darunavir. Figure-9 t14 represented the force degradation chromatograms. Table-2 represented the force degradation results and peak purity values.

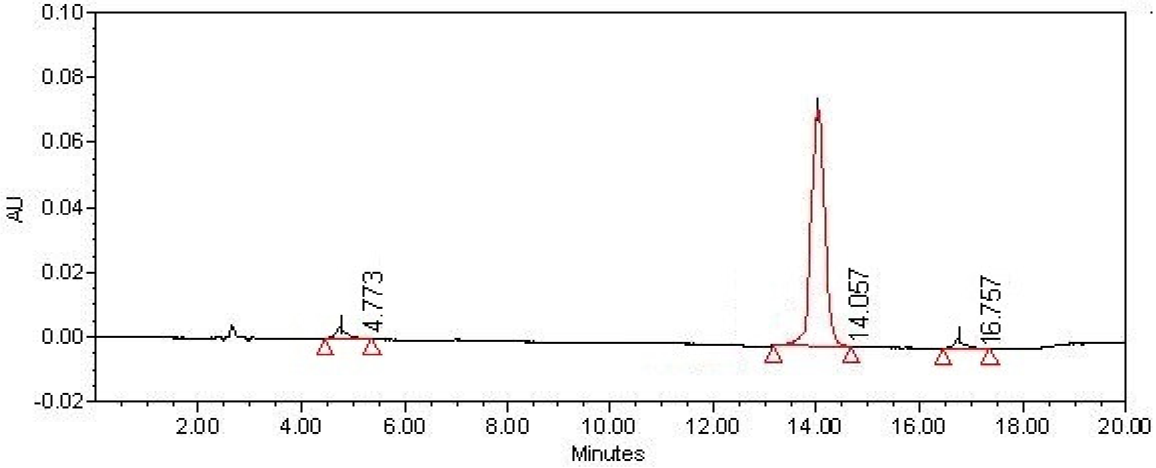

Fig.-9: Acid Degradation Sample Chromatogram

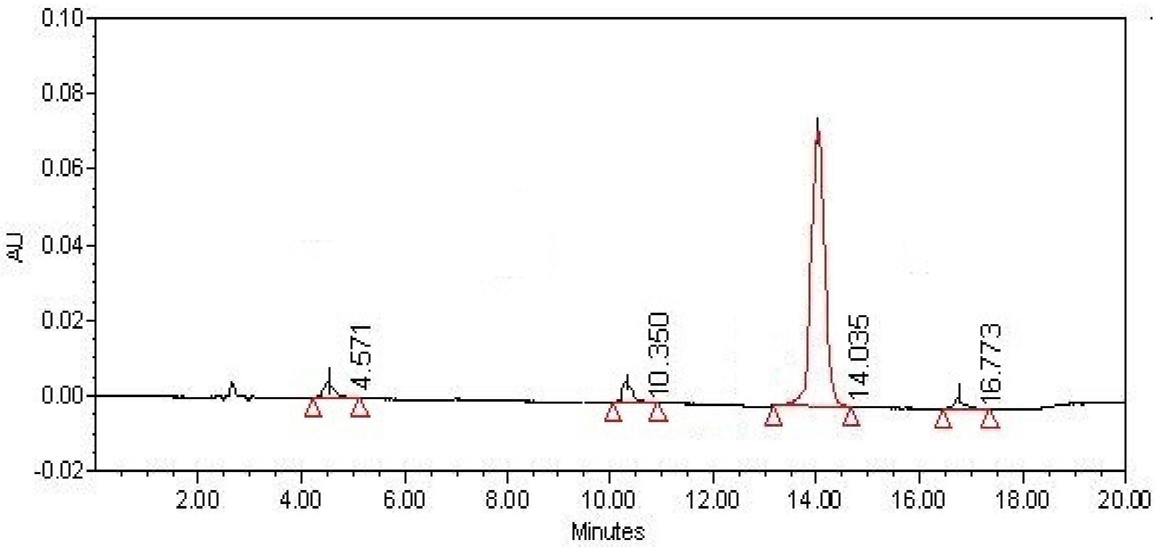

Fig.-10: Base Degradation Sample Chromatogram

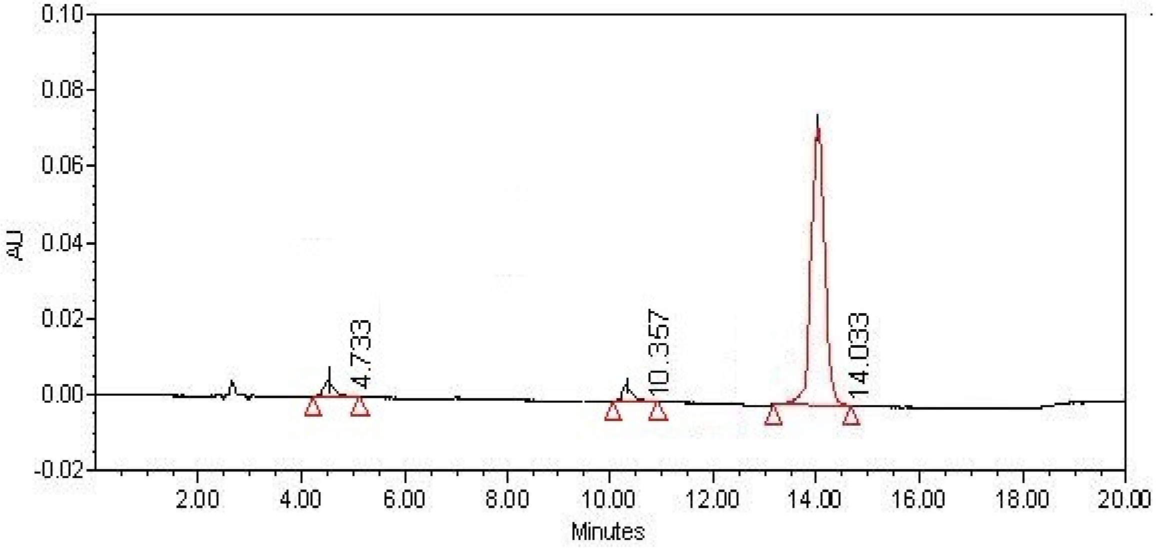

Fig.-11: Peroxide Degradation Sample Chromatogram

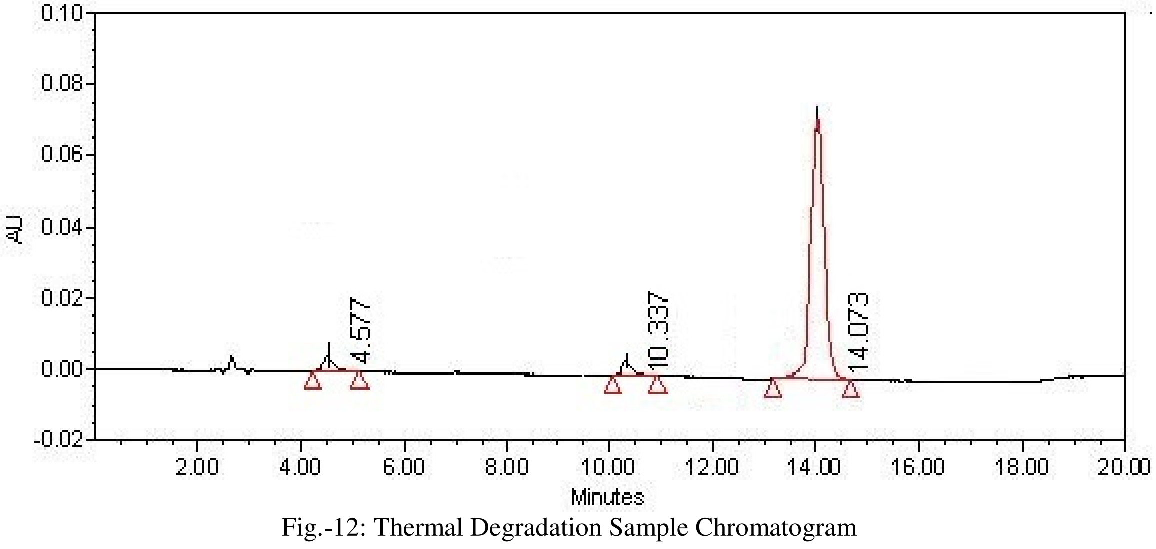

Fig.-12: Thermal Degradation Sample Chromatogram

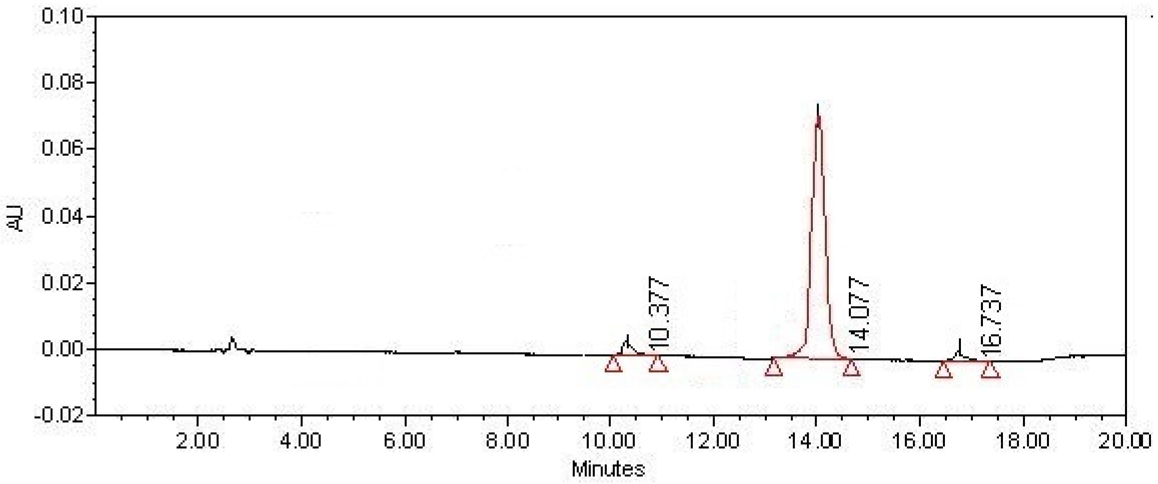

Fig.--13: UV/Visible Degradation Sample Chromatogram

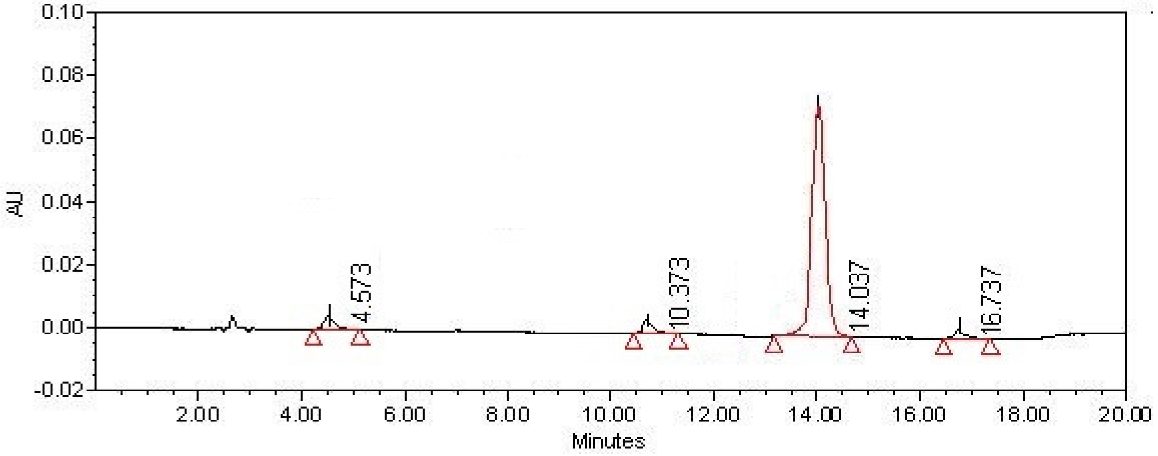

Fig.-14: Water Degradation Sample Chromatogram

Table-2: Stress Study Results

S. No.	Degradation Condition	Darunavir Assay (%)	Purity Angle	Purity Threshold	Purity Flag	Interference
1.	Acid degradation (5N HCl, 1hr, 60°C)	97.04	0.204	0.391	NO	NO
2.	Base degradation (5N NaOH, 1hr, 60°C)	96.98	0.313	0.306	NO	NO
3.	Peroxide degradation (3% H_2O_2, 1hr, RT)	97.89	0.241	0.440	NO	NO
4.	Water degradation (Water, 6hr, 60°C)	96.20	0.288	0.443	NO	NO
5.	Thermal degradation (24hr, 80°C)	97.25	0.276	0.430	NO	NO
6.	UV/ visible light (UV light 200 watt hr/sq. meter) (Visible light 1.2 million lux hrs)	96.80	0.228	0.420	NO	NO

Linearity

Linearity was performed with five different concentration levels with freshly prepared solutions. Concentration levels 20 ppm to 60 ppm was performed. Linearity results were tabulated in below Table-3 and linearity overlay chromatograms were represented in Fig.-15.

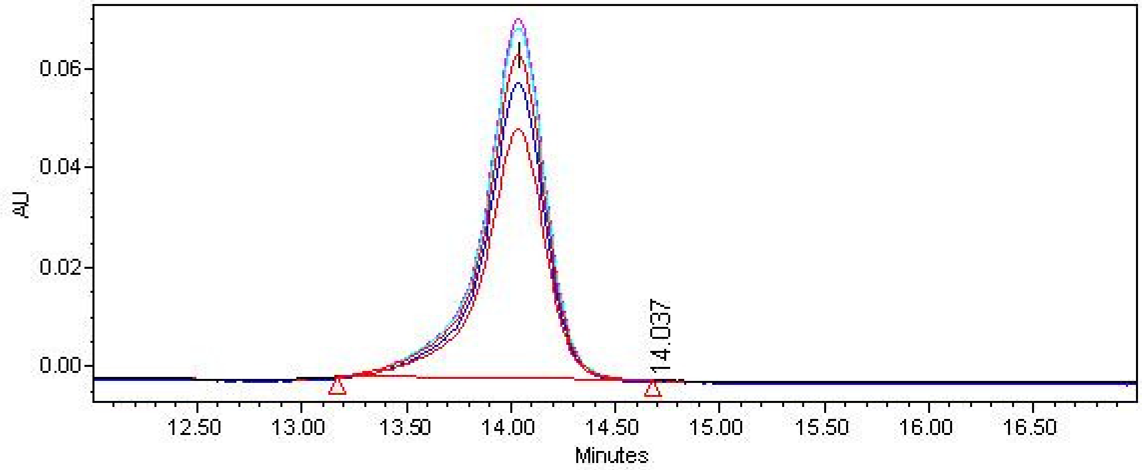

Fig.-15: Linearity Solutions Chromatograms Overlay View.

Table-3: Linearity Results

Linearity Level	1	2	3	4	5	Coefficient of Correlation
Conc. (µg/ml)	20	30	40	50	60	0.9993
Area	652580	113521	1625122	2215401	2681051	

Precision

Method and system precision were performed with 6 replicate test solution preparations. Test solutions were prepared as per the test procedure mentioned in the material and methods section. %RSD and % assay were calculated and reported in table-4. %RSD is within the acceptable limit i.e. 2.0 and % assay values are between 98% and 102%.

Table-4: Precision and Intermediate Precision Results

Precision Results								
S. No.	1	2	3	4	5	6	Average	%RSD
% Assay	99.63	99.67	99.89	99.84	98.99	99.19	99.55	0.33
Intermediate Precision								
% Assay	99.87	99.28	99.59	99.67	99.82	99.68	99.69	0.22

Accuracy

Accuracy was evaluated with 3 different concentration levels. A known concentration of sample was spiked to plasma sample and recovery studies were performed. Below Table-5 represented the accuracy results.

Table-5: Accuracy Results

Darunavir Accuracy													
µg /mL	Added	20.12			40.23						60.31		
	Recov.	20.16	20.61	20.31	40.20	40.15	40.19	40.31	40.29	40.31	60.19	60.21	60.19
% Recovery		100.20	101.34	100.94	99.93	99.80	99.90	100.20	100.15	100.20	99.80	99.83	99.80
% Recovery Mean		100.82			100.02						99.81		

Ruggedness

The ruggedness of the final method was checked with precision test samples. Precision sample-1 and 2 were stored at room temperature and refrigerator. Solutions stability was established for 3 days at room temperature and 5 days for refrigerator conditions. % difference in the assay of darunavir and tailing factor, %RSD was evaluated. Results were tabulated in Table-6.

Table-6: Ruggedness Results

Time in Day	Bench Top Stability Test Solution				Tailing Factor	%RSD	Bench Top Stability Standard Solution
	Test-1	Test-2	Difference				Similarity factor
			Test-1	Test-2			
Initial	99.63	99.67	NA	NA	1.10	0.5	NA
Day-1	99.29	99.35	0.34	0.32	1.16	1.8	1.10
Day-3	99.23	99.20	0.40	0.47	1.13	1.0	1.06
	Refrigerator Stability Test Solution						Refrigerator Stability Standard Solution
Initial	99.63	99.67	NA	NA	1.13	0.5	NA
Day-3	99.59	99.45	0.04	0.22	1.15	1.0	1.05
Day-5	99.29	99.31	0.34	0.36	11	0.2	1.03

Robustness

The ruggedness of the method was determined by verifying the chromatographic conditions such as flow rate, column temperature, mobile phase pH, and acetonitrile and methanol ratio. Tailing factor and %RSD for 5 standard solutions peak area were calculated and result acceptable (tailing factor not more than 2.0 and %RSD is 2.0%). Results were listed in Table-7 Filter verification was performed for PVDF and NYLON filters. Results were tabulated in Table-8.

Table-7: Results of Effect of Variations

Condition	Flow Rate			Column Temperature		
	1.1 ml/min	1.3 ml/min	1.5 ml/min	30°C	35°C	40°C
Tailing Factor	1.1	1.1	1.1	1.1	1.1	1.1
% RSD for 5 Inj.	0.7	1.4	1.2	0.7	0.6	1.4
	Mobile Phase pH			M.P. Acetonitrile Ratio		
	pH 7.9	pH 8.1	pH 8.3	100%	90%	110%
Tailing Factor	1.1	1.1	1.1	1.1	1.1	1.1
% RSD for 5 Inj.	0.7	1.4	1.5	0.7	0.4	1.0
	M.P. Acetonitrile Ratio					
Tailing Factor	100%	1.3	90%	1.6	110%	1.3
% RSD for 5 Inj.		0.6		0.8		0.9

Table-8: Filter Variability Results

	Centrifuged		Nylon Filter				PVDF Filter			
	% Assay		% Assay		% Difference		% Assay		% Difference	
	Spl-1	Spl-2	Spl-1	Spl-2	Spl-1	Spl-2	Spl-1	Spl-2	Spl-1	Spl-2
Darunavir	0.381	0.380	0.377	0.377	0.004	0.003	0.383	0.384	0.002	0.004

CONCLUSION

Darunavir estimation in blood plasma sample was performed using RP-HPLC stability indicating method. HPLC method was developed by using a simple mobile phase with pH 8.1. Mobile phase composed of water, acetonitrile and methanol (488:162:350 v/v/v). X-Bridge C18 5µm 4.6x250mm column, 262 nm wavelength, 20µL injection volume, 1.3 ml/min flow rate and column heater 35°C were applied. Method validation was performed with precision, accuracy, ruggedness, linearity, specificity and robustness. Results were within the acceptable limits such as tailing factor not more than 2.0, %RSD not more than 2.0%, linearity correlation coefficient 0.999 and % recovery 98% to 102%. The optimized method can be used to determine darunavir in plasma extraction samples.

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MICROWAVEASSISTED SYNTHESIS, MOLECULAR DOCKING STUDIES AND ANTIBACTERIAL ACTIVITY OF 4,6-BIS-(2-(ARYL)PYRAZOLO[1,5-*A*]PYRIMIDIN-7-YL)BENZENE-1,3-DIOLS AND 4,6-BIS-(1-(ARYL)-1*H*-PYRAZOL-3-YL) BENZENE-1,3-DIOLS

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Abstract: Bis-pyrazolo[1,5-*a*] pyrimidine and bis-pyrazole derivatives**(3a-d& 4a-d)**were synthesized under conventional heating and microwave irradiation methodsfromcyclization of3-Dimethylamino-1-[5-(3-dimethylamino-acryloyl)-2,4-dihydroxy-phenyl]-propenone **(2)** with 5-aminopyrazoles, and aryl hydrazines respectively. The structures of the title compounds were elucidated on the basis of their spectral data and elemental analyses and all the compounds**(3a-d & 4a-d)** were screened for *invitro*antibacterial activity.Docking studies performed for all the compounds **(3a-d & 4a-d)** with Glucosamine-5-phosphate synthase residues and all the results were reported.

Keywords:*Bis-pyrazolo[1,5-a]pyrimidine;bis-pyrazole;bis-enaminones;microwave irradiation; antibacterial activity; molecular docking.*

1. Introduction

The pyrazolo[1,5-*a*]pyrimidine derivatives attracted much attention because of their applications as anti-mycobacterial,[i]antidiabetic,[ii]kinase[iii] and phophodiestrase[iv] inhibitors, and also for their valuable antiangiogenic,[v]fungicidal,[vi]cytotoxic,[vii]antitubercular,[viii] antimicrobial and anthelmintic activities.[ix]Many pyrazole derivatives have been reported to possess diverse pharmacological activities such as antiinflammator,[x]antimicrobial,[xi,xii]antihypertensive,[xiii] etc. Bis-heterocyclic compounds are gain increased interest in the recent past as the dimeric analogues have proven to be having better and potential biological activity than the corresponding monomer. The bis-heterocyclic molecules were also shown to exhibit such diverse pharmacological activities such as antimicrobial,[xiv-xvi]antifungal,[xvii] anti-inflammatory,[xviii] anti-viral[xix] and cytotoxicity.[xx-xxv] Synthesis of bis-pyrazolo[1,5-*a*]pyrimidine andbis-pyrazolenot seem to have been reported so far. Recently, our research work has been directed to the synthesis ofbis-pyrazolo[1,5-*a*]pyrimidine and bis-pyrazole derivatives.In the course of our investigations, we found that 3-dimethylamino-1-[5-(3-dimethylamino-acryloyl)-2,4-dihydroxy-phenyl]-propenone**(2)**is a highly versatile and useful building block for the synthesis of bis-pyrazolo[1,5-*a*]pyrimidine and bis-pyrazole derivatives**(3a-d & 4a-d)**.

On the other hand, microwave irradiation has gained the attention of chemists during the last few decades due to its unique advantages, such as shorter reaction times, cleaner reaction products, higher yields and better selectivity's, being a valuable alternative to accomplish more efficient syntheses of a variety of organic compounds.In this way, targeting the preparation of the mentioned nitrogen containing heterocycles, we report herein the synthesis of bis-pyrazolo[1,5-*a*]pyrimidine and bis-pyrazole derivatives**(3a-d & 4a-d)**under microwave irradiation.

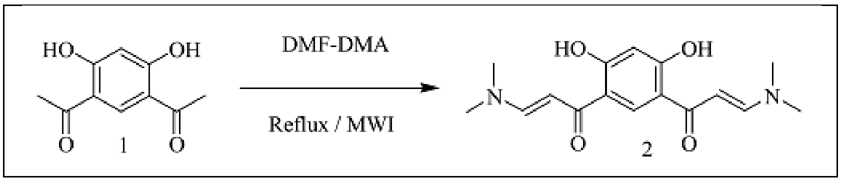
2. Experimental

All the chemicals were purchased from Aldrich and Fluka. Melting points were determined in open capillary tubes and uncorrected. The purity of the compounds was checked by TLC using precoated silica gel plates 60_{254}(Merck).Microwave reactions were carried out in the milestone multi SYNTH microwave system. IR (KBr) spectra were recorded on a Shimadzu FT-IR-8400s spectrophotometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on Bruker Avance II 300 MHz instrument using tetramethylsilane as an internal standard. Mass spectra were measured on a GCMS-QP 1000 EX mass spectrometer. Elemental analysis was determined by using a Thermo Finnigan CHNS analyzer.

2. i. Synthesis Of 3-Dimethylamino-1-[5-(3-Dimethylamino-Acryloyl)-2,4-Dihydroxy-Phenyl]-Propanone(2).

Conventional heating method: A mixture of dimethylformamide-dimethylacetal (DMF-DMA) (1.18 mL, 10 mmol) was and 4,6-diacetyl resorcinol **(1)** (1.0 g, 5 mmol) was refluxed for 4 hr at 120°C. The reaction progress checked by TLC, after completion of the reaction, the mixture was cooled to room temperature poured into methanol and resulting precipitate was filtered, dried to give crude product 3-dimethylamino-1-[5-(3-dimethylamino-acryloyl)-2,4-dihydroxy-phenyl]-propenone **(2)** was recrystallized from benzene to give yellow crystals with 65% yield.

Microwave irradiation method: A solution ofdimethylformamide-dimethylacetal (DMF-DMA) (1.18 mL, 10 mmol) and 4,6-diacetyl resorcinol **(1)** (1.0 g, 5 mmol) weretaken in a quartz tube and inserted into Teflon vial with screw capped and then subjected to microwave irradiation at 160 W for 4 min. within 30 sec intervals. The reaction progress checked by TLC, after completion of the reaction cooled to room temperature and poured into methanol and resulting precipitate was filtered, dried to give crude product, recrystallized from benzene to afford 3-dimethylamino-1-[5-(3-dimethylamino-acryloyl)-2,4-dihydroxy-phenyl]-propenone **(2)** yellow crystals with 84% yield.



Scheme-1: Condensation of 4,6-diacetyl resorcinol with DMF-DMA

2. ii. Synthesis Of Bis-Pyrazolo[1,5-*A*] Pyrimidine and Bis-Pyrazole Derivatives (3a-D & 4a-D)

Conventional heating method: A mixture of 3-dimethylamino-1-[5-(3-dimethylamino-acryloyl)-2,4-dihydroxy-phenyl]-propenone **(2)** (1.0 g, 3 mmol) and 5-amino pyrazoles (6 mmol)/aryl hydrazines (6 mmol) in glacial acetic acid were refluxing for 4-5 hr at 110°C. Progress of the reaction was monitored by TLC. After completion of the reaction, the reaction mixture was poured in ice water, filtered the residue, dried over vacuum and recrystallized from chloroform to give yellow crystals of 4,6-bis-(2-(aryl)pyrazolo[1,5-*a*]pyrimidin-7-yl)benzene-1,3-diols/4,6-bis-(1-(aryl)-1*H*-pyrazol-3-yl)benzene-1,3-diols **(3a-d & 4a-d)**.

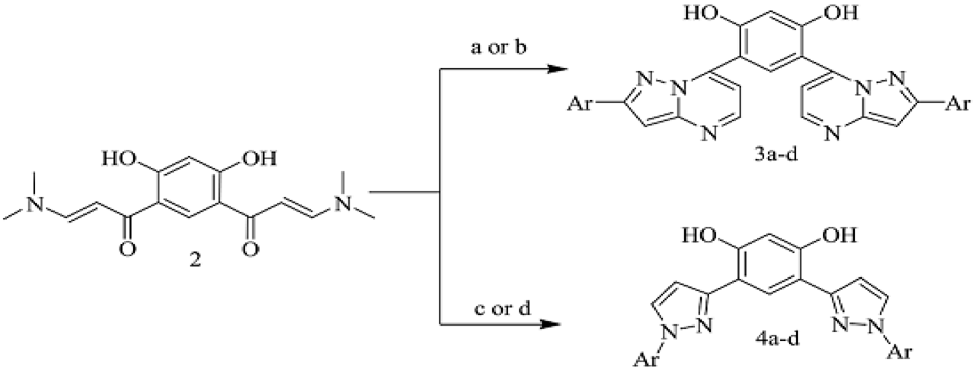
Microwave irradiation method: A mixture of 3-dimethylamino-1-[5-(3-dimethylamino-acryloyl)-2,4-dihydroxy-phenyl]-propenone **(2)** (1.0 g, 3 mmol) and 5-amino pyrazoles (1.03 g, 6 mmol) / aryl hydrazines (0.732 g, 6 mmol) in glacial acetic acid as catalytic amount was taken in a quartz tube and inserted into Teflon vial with screw capped and then subjected to microwave irradiation for 4-5 min at 160 W within 30 sec intervals. After the completion of reaction, the reaction mixture was poured in ice water, filtered the residue, dried over vacuum and recrystallized from chloroform to give yellow solid crystals of 4,6-bis-(2-(aryl)pyrazolo[1,5-*a*]pyrimidin-7-yl)benzene-1,3-diols/4,6-bis-(1-(aryl)-1*H*-pyrazol-3-yl)benzene-1,3-diols **(3a-d & 4a-d)**.

3. Biological Properties:

The newly synthesized bis-pyrazolo[1,5-*a*] pyrimidine and bis-pyrazole derivatives **(3a-d & 4a-d)** were screened for their antibacterial activity against gram negative bacteria *viz. Escherichia coli*, *Proteus mirabilis*strains and gram-positive bacteria *viz. Staphylococcus aureus, Bacillus subtilis* strains at three concentrations *i.e.* 200, 100 and 50 µg using ditch dilution methods. The test organism was atwohour culture of *Escherichia coli, Proteus mirabilis, Staphylococcus aureus* and *Bacillus subtilis* incubated and grown in peptone-water medium (temp-37°C). DMSO was used as solvent control which did not show any zone of inhibition. Muller-Hilton agar medium was used as culture medium. The culture plates were incubated at 37°C for 24 hrs. The newly synthesized compounds were screened for their antibacterial activity against gram negative bacteria *viz. Escherichia coli, Proteus mirabilis*strains and gram-positive bacteria viz. *Staphylococcus aureus, Bacillus subtilis* with three concentrations *i.e.* 200, 100 and 50 µg.

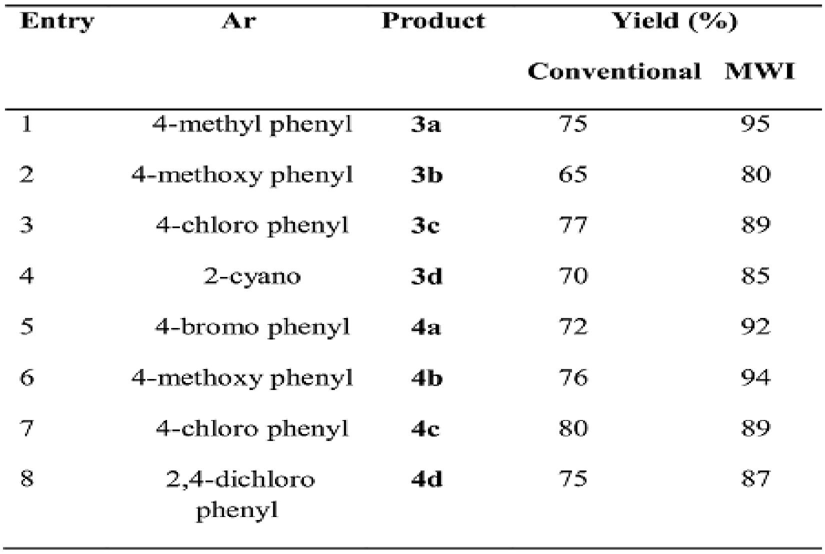
4. Results and Discussions

Compounds**(4a-d)** were synthesized by cyclizationkey intermediate of 3-Dimethylamino-1-[5-(3-dimethylamino-acryloyl)-2,4-dihydroxy-phenyl]-propenone**(2)**with suitable aryl hydrazines. The bis-pyrazole derivatives**(4a-d)** have been obtained in good yields in the presence of acid catalyst. The treatment of 3-Dimethylamino-1-[5-(3-dimethylamino-acryloyl)-2,4-dihydroxy-phenyl]-propenone**(2)**with 5-aminopyrazoles in acidic condition afforded the bis-pyrazolo[3,4-*d*]pyrimidine derivatives**(3a-d)**.The key intermediate of bis-enaminone**(2)**which, were obtained from the N,N-dimethylformamide dimethyl acetal (DMF-DMA)with 4,6-diacetyl resorcinol**(1)**.



a) 5-amino pyrazoles, gla. acetic acid, reflux, **b)** 5-amino pyrazoles, gla. acetic acid, MWI, **c)** aryl hydrazines, gla. acetic acid, reflux, **d)** aryl hydrazines, gla. acetic acid, MWI

Scheme-2: Various steps involved in cyclisation of bis-enaminones.



Entry	Ar	Product	Yield (%)	
			Conventional	MWI
1	4-methyl phenyl	**3a**	75	95
2	4-methoxy phenyl	**3b**	65	80
3	4-chloro phenyl	**3c**	77	89
4	2-cyano	**3d**	70	85
5	4-bromo phenyl	**4a**	72	92
6	4-methoxy phenyl	**4b**	76	94
7	4-chloro phenyl	**4c**	80	89
8	2,4-dichloro phenyl	**4d**	75	87

Table 1. Yields of bis-enaminones through cyclization.

The structures of the compounds **(3a-d & 4a-d)** were confirmed on the basis of IR, ^{1}H-NMR, ^{13}C-NMR and mass spectral data. The IR spectrums showed the absence of the peaks of C=O and presence of common characteristic absorption peaks for C=N at ~1620 cm^{-1}.The ^{1}H-NMR spectrum of **3a** displayed four doublets signals at δ 6.35, 7.74, 7.08 and 7.42 ppm represent for pyrazole and bromophenyl, respectively, and the other aromatic protons appeared as two singlet at δ 6.46 and 7.24. In case **4a** ^{1}H-NMR spectrum shows two doublet signals at δ 8.26 and 8.58 ppmrepresents pyrimidine ring protons, singlet signal at δ 6.80 represents for pyrazole protons, and the other aromatic protons appeared at aromatic region 6.36-7.25**.**The ^{13}C-NMR spectrums data showed the absence of C=O peak and the presence of common characteristic absorption peak as they are consistent with the proposed structure. The mass spectrum compounds **(3a-d&4a-d)**displayedtheirion peaks, which consistent with their molecular formulas.

4.1. Spectral Data:

***Compound* (2):** m.p. 210 °C;Anal. Calcd. for $C_{16}H_{20}N_2O_4$C, 63.14; H, 6.62; N, 9.20. Found: C, 63.32; H, 6.66; N, 9.35; IR (KBr, cm^{-1}): 3417, 1636, 1279, 1106, 1021 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-*d*$_6$, *δ* / ppm): 2.45 (12H, *s*, 4 x CH_3), 5.78 (2H, *d*, α-olefinic protons), 6.12 (1H, *s*, aromatic), 7.80 (2H, *d*, β-olefinic protons), 8.12 (1H, *s*, aromatic), 11.62 (2H, *s*, 2 x OH); ^{13}C-NMR (75 MHz, DMSO-*d*$_6$, *δ* / ppm): δ 45.1, 89.2, 103.6, 115.6, 130.6, 155.3, 168.1, 189.4; MS (*m/z*, (relative abundance, %)): 305 (M+H, 100).

***Compound* (3a).**m.p. 249°C;Anal. Calcd. for $C_{32}H_{24}N_6O_2$: C, 73.27; H, 4.61; N, 16.02. Found: C, 73.25; H, 4.63; N, 16.12; IR (KBr, cm^{-1}): 3394, 1619, 1532, 1278; ^{1}H-NMR (300 MHz, $CDCl_3$, *δ* / ppm): 2.35 (6H, *s*, 2 x CH_3), 6.36 (1H, *d*, aromatic), 6.80 (2H, *s*, pyrazoleprotons), 7.10-7.25 (9H, *m*, aromatic), 8.26 (2H, *d*, pyrimidine protons), 8.52-8.61 (2H, *m*, pyrimidine protons), 11.02 (1H, *s*, 2 x OH); ^{13}C-NMR (75 MHz, $CDCl_3$, *δ* / ppm): δ

24.6, 99.7, 111.4, 115.6, 118.4, 128.4, 128.7, 129.6, 130.1, 138.8, 146.2, 148.2, 149.5, 152.5, 157.0;MS (*m/z,* (relative abundance, %)):525 (M+H, 100).

***Compound* (3b).**m.p. 257^{o}C;Anal. Calcd. for $C_{32}H_{24}N_6O_4$: C, 69.06; H, 4.35; N, 15.10. Found: C, 69.12; H, 4.42; N, 15.18; IR (KBr, cm^{-1}): 3403, 1620, 1530, 1305; ^{1}H-NMR (300 MHz, $CDCl_3$, δ / ppm): 3.84 (6H, *s,* OCH_3), 6.24 (1H, *d,* aromatic), 6.89 (2H, *s,* pyrazoleprotons), 7.02-7.40 (9H, *m,* aromatic), 7.86 (2H, *d,* pyrimidine protons), 8.48 (2H, *d,* pyrimidine protons), 11.02 (2H, *s,* 2 x OH); MS (*m/z,* (relative abundance, %)):557 (M+H, 100%);

***Compound* (3c).** m.p. 187^{o}C;Anal. Calcd. for $C_{30}H_{18}Cl_2N_6O_2$C, 63.73; H, 3.21; N, 14.86. Found: C, 63.62; H, 3.02; N, 14.78; IR (KBr, cm^{-1}): 3399, 1620, 1532, 1275; ^{1}H-NMR (300 MHz, $CDCl_3$, δ / ppm): 6.25 (1H, *d,* Ar-H), 6.86-7.32 (6H, *m,* pyrazoleprotons&aromatic), 7.39 (4H, *d,* aromatic), 7.86 (2H, *d,* pyrimidine protons), 8.39 (1H, *s,* aromatic), 8.52 (2H, *d,* pyrimidine protons), 10.18 (2H, *s,* 2 x OH); MS (*m/z,* (relative abundance, %)):565 (M+H, 100).

***Compound* (3d).**m.p. 215^{o}C;Anal. Calcd. for $C_{32}H_{24}N_6O_2$: C, 60.91; H, 2.56; N, 28.42. Found: C, 60.71; H, 2.48; N, 28.36; IR (KBr, cm^{-1}): 3248, 1667, 1536, 1273; ^{1}H-NMR (300 MHz, $CDCl_3$, δ / ppm):6.58 (2H, *s,* pyrazoleprotons), 6.83 (1H, *d,* aromatic), 7.88 (2H, *d,* pyrimidine protons), 8.42 (1H, *s,* aromatic), 8.56 (2H, *d,* pyrimidine protons), 10.46 (1H, *s,* 2 x OH);MS (*m/z,* (relative abundance, %)):395 (M+H, 100);

***Compound* (4a).**m.p. 220^{o}C;Anal. Calcd. for $C_{26}H_{22}N_4O_4$: C, 52.20; H, 2.92; N, 10.15. Found: C, 52.35; H, 2.98; N, 10.26; IR (KBr, cm^{-1}): 3138, 1623, 1547, 1297, 1266; ^{1}H-NMR (300 MHz, $CDCl_3$, δ / ppm): 5.64 (2H, *bs,* 2 x OH), 6.35 (2H, *d,* pyrazoleprotons), 6.46 (1H, *s,* aromatic), 7.08 (4H, *d,* aromatic), 7.24 (1H, *s,* aromatic), 7.42 (4H, *d,* aromatic) 7.74 (2H, *d,* pyrazoleprotons); ^{13}C-NMR (75 MHz, $CDCl_3$, δ / ppm): 158.5, 156.5, 139.6, 133.6, 129.4, 126.0, 124.8, 120.6, 113.4, 109.4, 103.6;MS (*m/z,* (relative abundance, %)):550 (M+H, 100);

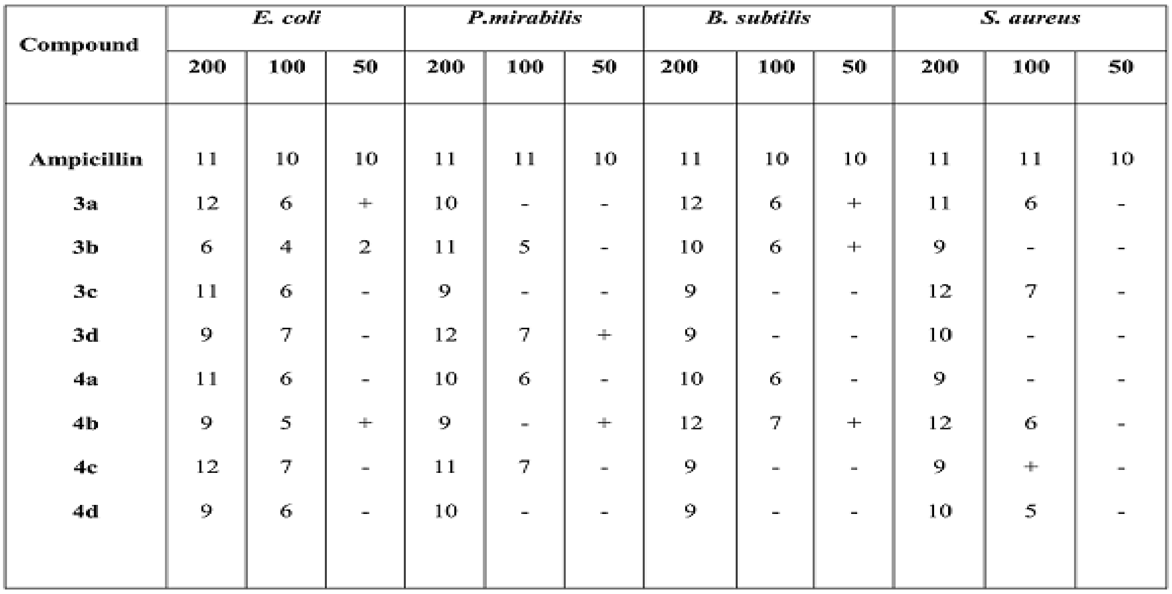
***Compound* (4b).**m.p. 201^{o}C;Anal. Calcd. for $C_{24}H_{16}Br_2N_4O_2$: C, 68.71; H, 4.88; N, 12.33. Found: C, 68.84; H, 4.98; N, 12.44;IR (KBr, cm^{-1}): 3135, 1623, 1515, 1300, 1249; ^{1}H-NMR (300 MHz, $CDCl_3$, δ / ppm):3.85 (6H, *s,* 2 x OCH_3), 6.18 (1H, *d,* Ar-H), 6.76 (2H, *d,* pyrazoleprotons), 6.95-7.02 (4H, *m,* aromatic), 7.04-7.18 (4H, *m,* aromatic), 7.52 (2H, *d,* pyrazoleprotons), 8.48(1H, *s,* aromatic); MS (*m/z,* (relative abundance, %)):455 (M+H, 100).

***Compound* (4c).**m.p. 182^{o}C; Anal. Calc. for $C_{24}H_{16}Cl_2N_4O_2$: C, 62.22; H, 3.48; N,12.09. Found: C, 62.16; H, 3.36; N, 12.04; IR (KBr, cm^{-1}): 3104, 1628, 1532, 1266, 1227; ^{1}H-NMR (300 MHz, $CDCl_3$, δ / ppm): 5.54 (2H, *bs,* 2 x OH), 6.45 (2H, *d,* pyrazoleprotons), 6.65 (1H, *s,* aromatic), 7.08 (4H, *d,* aromatic), 7.52 (4H, *d,* aromatic) 7.82 (2H, *d,* pyrazoleprotons), 7.86 (1H, *s,* aromatic); MS (*m/z,* (relative abundance, %)):463 (M+H, 100).

***Compound* (4d)**m.p. 197^{o}C; Anal. Calc. for $C_{24}H_{14}Cl_4N_4O_2$: C, 54.16; H, 2.65; N, 10.53. Found: C, 54.20; H, 2.72; N, 10.60; IR (KBr, cm^{-1}): 3118, 1625, 1526, 1297, 1266; ^{1}H-NMR (300 MHz, $CDCl_3$, δ / ppm): 5.54 (2H, *bs,* 2 x OH), 6.45 (2H, *d,* pyrazoleprotons), 6.65 (1H, *s,* aromatic), 7.08 (4H, *d,* aromatic), 7.52 (4H, *d,* aromatic) 7.82 (2H, *d,* pyrazoleprotons), 7.86 (1H, *s,* aromatic); MS (*m/z,* (relative abundance, %)):533 (M+H, 100).

Out of these concentrations chosen for antibacterial activity, the best result was obtained with 200 µg and hence this was optimum concentration. Compounds **3d** and **4a** were exhibited maximum activity against *E. coli* at 200µg/disc. **3b, 3c, 4c**and **4d** showed moderate activity and **3a** and **4b** did not exhibit significant activity against *E. coli.* In case of *P. mirabilis* compound **3a** and**4d** showed maximum activity at 200µg/disc, **3d, 3b** and **4b** exhibited moderate activity, where as **3c, 4a**and **4c** were found to be inactive. In case of *B. subtilis* compound **3b**and**4a** showed maximum activity at 200µg/disc, **3d** and **4b** exhibited moderate activity, where as **3c, 3d, 4c**and**4d** were found to be inactive. In case of *S. aureus* compound

3band**4c** showed maximum activity at 200µg/disc, **3c, 3d** and **4a** exhibited moderate activity, where as **3a, 4b** and **4d,** were found to be inactive as shown in **Table-2**.



Compound	*E. coli*			*P.mirabilis*			*B. subtilis*			*S. aureus*		
	200	100	50	200	100	50	200	100	50	200	100	50
Ampicillin	11	10	10	11	11	10	11	10	10	11	11	10
3a	12	6	+	10	-	-	12	6	+	11	6	-
3b	6	4	2	11	5	-	10	6	+	9	-	-
3c	11	6	-	9	-	-	9	-	-	12	7	-
3d	9	7	-	12	7	+	9	-	-	10	-	-
4a	11	6	-	10	6	-	10	6	-	9	-	-
4b	9	5	+	9	-	+	12	7	+	12	6	-
4c	12	7	-	11	7	-	9	-	-	9	+	-
4d	9	6	-	10	-	-	9	-	-	10	5	-

Table-2: Antibacterial activity of bis-pyrazolo[1,5-*a*] pyrimidine and bis-pyrazole derivatives

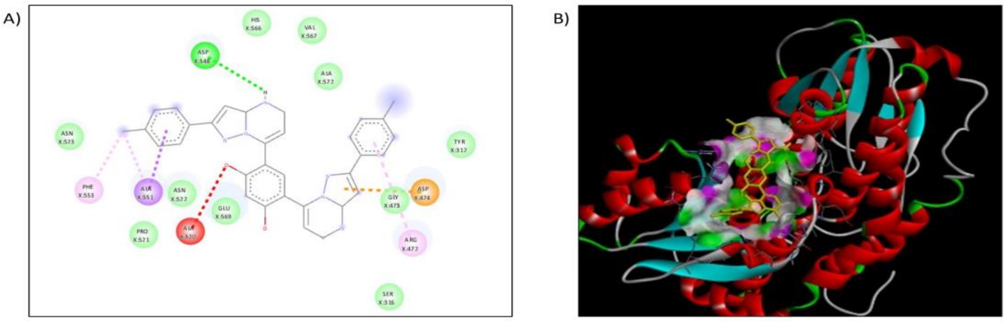
5. Docking Studies

AutoDock vina with PyRx-Virtual screening tool[xxvi]was used to carry out the docking studies. Crystal structure of Glucosamine-6-phosphate synthase (PDB ID:2VF5) of EColi was retrieved from the RCSB PDB Database. Protein preparation was carried out using Swiss PDB Viewer by removing co crystallized ligand and adding missing residues.

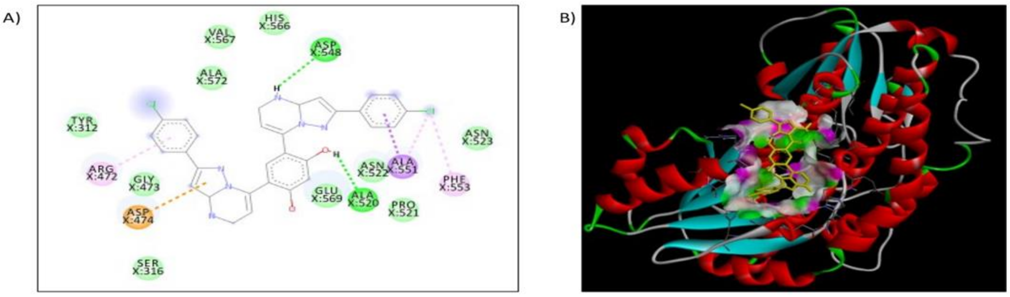
MarvinsketchfromChemAxon was used for drawing the ligands and minimized, which were clustered using the BIOVIA Discovery Studio Visualizer 2020. The ligand files were saved as SD files. Then the clustered ligands were imported to PyRx software and energy minimization was conducted and converted to PDBQT files. The protein was also loaded to PyRx software and prepared by adding hydrogens, removing water molecules and minimizing energy. Grid attributes were selected based on the bound co-crystallized ligand with dimensions X:11, Y: 21, Z: 12. The ligands in the PDBQT files were docked to the protein using the PyRx virtual screening tool. The docking scores were tabulated in Table-3.

COMPOUNDS	DOCKING SCORES(KCAL/MOL)
3a	**-10.1**
3b	**-9.1**
3c	**-10**
3d	**-9.3**
4a	**-10**
4b	**-8.6**
4c	**-8.9**
4d	**-8.3**
Ampicillin	**-8.2**

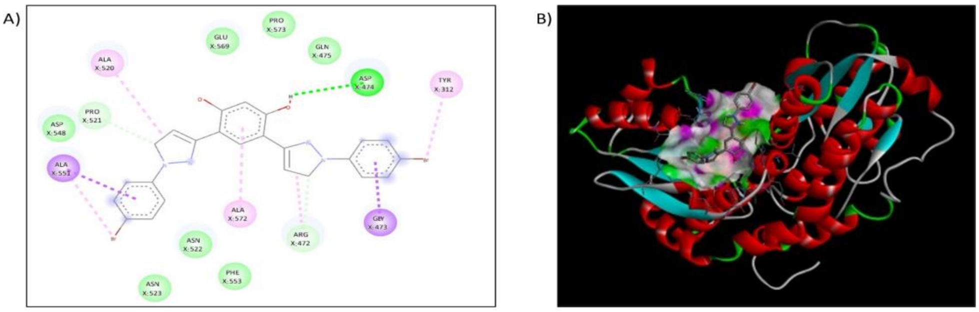
Table:3 Docking scores of newly bis-pyrazolo[1,5-*a*] pyrimidine and bis-pyrazole derivatives.



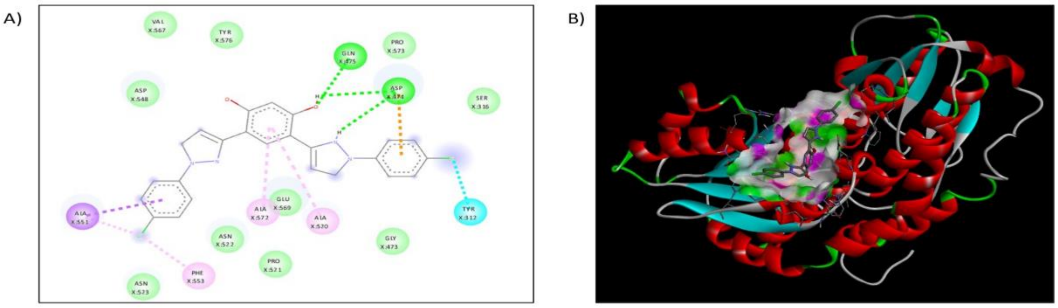
Fig(1): A) Shows hydrogen bond interaction (Green dotted lines) of compound 3a with Glucosamine 6 -phosphate synthase residues ASP548 and pi -alkyl interaction (pink color dotted lines) with PHE553, ARG472 along with Pi -anion with ASP474 and pi -sigma interaction with ALA551.
B) Shows Glucosamine 6-phosphate synthase with 3a surface image



Fig(2): A) Shows hydrogen bond interaction (Green dotted lines) of compound 3c with Glucosamine 6 -phosphate synthase residues ASP548, ALA520 and pi-alkyl interaction (pink color dotted lines) with PHE553, ARG472 along with Pi-anion with ASP474 and pi -sigma interaction with ALA551.
B) Shows Glucosamine 6-phosphate synthase with 3c surface image



Fig(3): A) Shows hydrogen bond interaction (Green dotted lines) of compound 4a with Glucosamine 6 -phosphate synthase residues ASP474 and pi -alkyl interaction (pink color dotted lines) with ARG472,TYR312,ALA520 along with pi -sigma interaction with ALA551,GLY473.
B) Shows Glucosamine 6-phosphate synthase with 4a surface image



Fig(4): A) Shows hydrogen bond interaction (Green dotted lines) of compound 4c with Glucosamine 6 -phosphate synthase residues ASP474,GLN475 and pi -alkyl interaction (pink color dotted lines) with ALA572,ALA520,PHE553 along with pi -sigma interaction with ALA551 and Halogen interaction with TYR312.
B) Shows Glucosamine 6 -phosphate synthase with 4c surface image

6. Conclusion

In conclusion, we have described an efficient synthesis of bis-pyrazolo[1,5-*a*] pyrimidine and bis-pyrazole derivatives under microwave irradiation. The process proved to be a simple, environmentally friendly with high yields and high rate of acceleration was achieved. From the docking studies it was clear that all the compounds are showing interactions at the binding site. Compounds **3a** and **4b** showedmaximum activity against all bacterial strains in all concentrations.

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Microwaveassisted Synthesis, Molecular Docking studies and Antibacterial Activity of 4,6-Bis-(2-(Aryl)Pyrazolo[1,5-*a*]Pyrimidin-7-yl)Benzene-1,3-diols and 4,6-Bis-(1-(Aryl)-1*h*-Pyrazol-3-Yl) Benzene-1,3-diols

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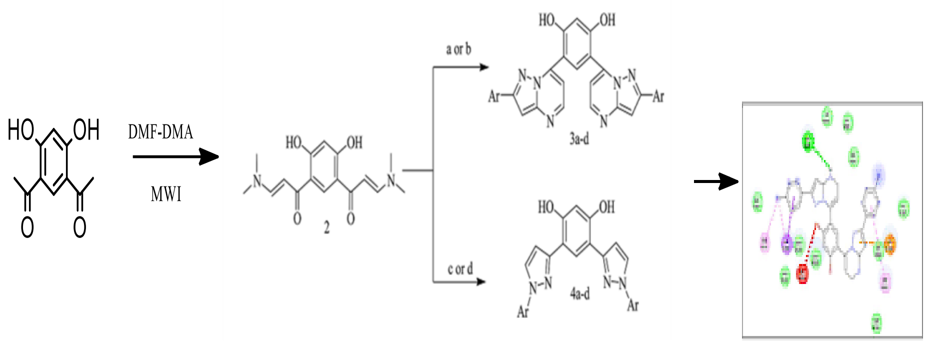
Bis-pyrazolo[1,5-*a*]pyrimidine and bis-pyrazole derivatives**(3a-d & 4a-d)**were synthesized under conventional heating and microwave irradiation methodsfromcyclization of 3-Dimethylamino-1-[5-(3-dimethylamino-acryloyl)-2,4-dihydroxy-phenyl]-propenone**(2)**with 5-aminopyrazoles, and aryl hydrazines respectively. The structures of the title compounds were elucidated on the basis of their spectral data and elemental analyses and all the compounds**(3a-d & 4a-d)** were screened for *invitro*antibacterialactivity.Docking studies performed for all the compounds **(3a-d & 4a-d)** with Glucosamine-5-phosphate synthase residues and all the results were reported.



Microwave Assisted Synthesis, Molecular Docking studies of 4,6-Bis-(2-(Aryl) Pyrazolo[1,5-a] Pyrimidin-7-yl) Benzene-1,3-diols and 4,6-Bis-(1-(Aryl)-1h-Pyrazol-3-Yl) Benzene-1,3-diols

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Paliperidone Determination by Using HPLC Method in Blood Plasma Samples-Stability Indicating Method

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Abstract

Paliperidone product is used to control schizoaffective disorder in adults. In the market 3mg, 6mg, 9mg and 12mg strength tablet dosage form is available. Regular used strength tablets are 3mg and 6mg. Objective was to develop a simple HPLC method to evaluate the qualitative and quantitative quantity of the Paliperidone with known impurities separation. Blood plasma samples were prepared and injected into the HPLC. Simple HPLC method was developed and validated the method. *Buffer:* 2.1g of TBAHS in 100 mL of HPLC grade water, *Mobile phase:* Buffer, Acetonitrile 90:10 v/v, Zorbax SB C18 100 x 4.6 mm, 3.5 µm, flow rate 1.0 mL/min, 275 nm wavelength, 10 µL injection volume, column temperature 40°C were used. Run time 20 min was performed. Method validation was performed with precision, linearity, accuracy, ruggedness, robustness and specificity (interference and force degradation). Optimized method can be used to determine the Paliperidone in tablets dosage form.

Keywords

Paliperidone, Schizoaffective disorder, Dopamine antagonist, HPLC method development and validation.

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INTRODUCTION

Paliperidone is a dopamine antagonist and 5HT2A antagonist. The chemical name is (±)-3-[2-[4-(6-fluoro-1, 2benzisoxazol-3-yl)-1-piperidinyl] ethyl]-6, 7, 8, 9-tetrahydro-9-hydroxy-2-methyl-4Hpyrido [1, 2-a] pyrimidin-4-one. Molecular formula is $C_{23}H_{27}FN_4O_3$ and molecular weight is 426.49 g/mol. Paliperidone is sparingly soluble in 0.1N HCl and methylene chloride; practically insoluble in water, 0.1N NaOH, and hexane; and slightly soluble in N, N-dimethylformamide. It is an antipsychotic class drug product. Paliperidone is used for the treatment of schizophrenia and schizoaffective disorder. Paliperidone is used for mental/ mood disorders, medication can decrease hallucinations and increase the more clearly and positively thinking ness. Paliperidone side effects are tachycardia, somnolence, insomnia, headache, hyperprolactinaemia and sexual dysfunction.

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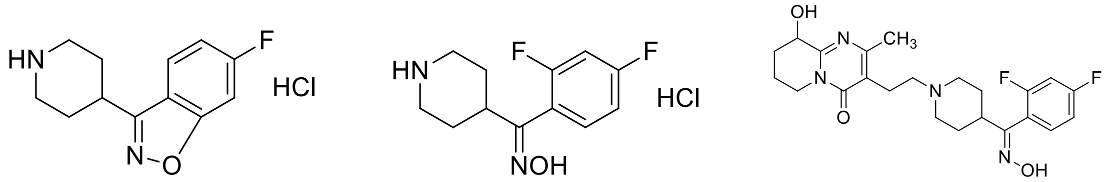
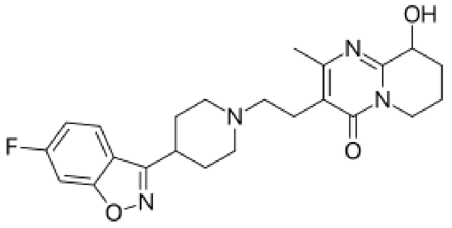


Impurity-1 Impurity-2 Impurity-3



Paliperidone

Figure-1: Chemical structure of the Paliperidone and its impurities.

Impurities Chemical names:

Impurity-1: 6-fluoro-3-(piperidin-4-yl) benzo[d]isoxazole hydrochloride

Impurity-2: (2, 4-difluorophenyl) (piperidin-4-yl) methanone oxime hydrochloride

Impurity-3: (Z)-3-(2-(4-((2,4-difluorophenyl)(hydroxyimino)methyl)piperidin-1-yl)ethyl)-9-hydroxy-2-methyl-6,7,8,9-tetrahydro-4H-pyrido[1,2-a]pyrimidin-4-one.

A complete literature survey was conducted to understand the literature and publications. Few methods were reported on HPTLC (Patel RB, 2010) [7], extraction HPLC method (SL Mendez, 2014) [8], paliperidone and its related impurities method (Bindu KH, 2010) [9] and (Jadhav SA, 2011) [10], (Rao KN 2013) [11] and few methods reported for enantiomers (Locatelli I, 2009)[12] and (Swarnalatha G, 2014) [13]. Based on the literature understanding there is no method reported to determine the paliperidone and its impurities for the determination of the blood plasma samples. Our objective of this study was to develop a simple and stability indicating HPLC method for the quantification of the paliperidone in blood plasma samples.

MATERIAL AND METHODS

Reagents:

Analytical reagent grade Tetrabutyl ammonium hydrogen sulphate (TBAHS), HPLC grade water and methanol were used.

HPLC Instrument:

Waters Alliance 2695 separations module equipped with gradient elution capability, 2487 UV detector and an auto sampler. Empower work station data handling system.

Chromatographic conditions:

Zorbax SB C18 100x4.6 mm, 3.5µm equivalent column, flow rate 1.0 mL/min, 275 nm wavelength, 10 µL injection volume, column temperature 40°C were used. Run time 20 min was performed.

Mobile phase:

Buffer: 2.1g of TBAHS weighed and transferred into a beaker containing 100 mL of HPLC grade water and mixed to dissolve. Filtered through 0.45 micron or finer porosity membrane filter.

Mobile phase: Buffer, Acetonitrile 90:10 v/v.

Diluent: Mobile Phase.

Standard solution:

Weighed accurately 50.0 mg of Paliperidone a working standard into a 100 mL clean, dry volumetric flask, added 60mL of diluent and sonicated to dissolve. Made up to volume with diluent and mixed.

Sample solution:

12.5 mg of Paliperidone into 25mL volumetric flask, 10 mL of diluent added and mixed for 20 min further diluted and mixed. Allowed settling the solution and filtered the clear supernatant solution using a 0.45µm syringe filter.

Plasma sample solution preparation:

Blood plasma samples were prepared with extraction process. Lquid-liquid extraction process was applied. 100mg was spiked in to 10ml plasma and stored for 1day (24 Hours). For processing, the stored spiked samples were withdrawn from the freezer and allowed to thaw at room temperature. An aliquot of 500µL was transferred to prelabeled 10.0 mL polypropylene centrifuge tubes. Extraction solvent, 5.0 mL of ethyl acetate, was then added to extract the drug. The samples were then kept on a vibramax unit and vortexed for 15 min. Samples were then centrifuged at 5000 rpm for 5 min in a refrigerated centrifuge (4°C). Supernatant solution, 1 mL was then transferred into pre-labeled polypropylene tubes and was allowed to evaporate to dryness under nitrogen at constant temperature of 40°C. The dried residue was then dissolved in 200µL of mobile phase and transferred into shell vials containing vial inserts for analysis. Dilution was performed to reach 500ppm concentration.

System suitability:

1. Tailing factor should be NMT 2.0 for the Paliperidone standard peak.
2. Theoretical plates corresponding to Paliperidone peak should NLT 5000.
3. The %RSD for five replicate standard injections should be NMT 2.0%.

RESULTS AND DISCUSSION

METHOD DEVELOPMENT:

Literature published methods were reviewed and understood the Paliperidone chemical and physical properties. Three impurities were evaluated and those three impurities are forming during synthesis and tablets formulations. All three known impurities were evaluated to understand the UV maximum absorbance. All three impurities and Paliperidone have 230nm maximum absorbance so 230nm was selected to perform the analysis. Figure-2 represented the UV spectrums for all four analytes.

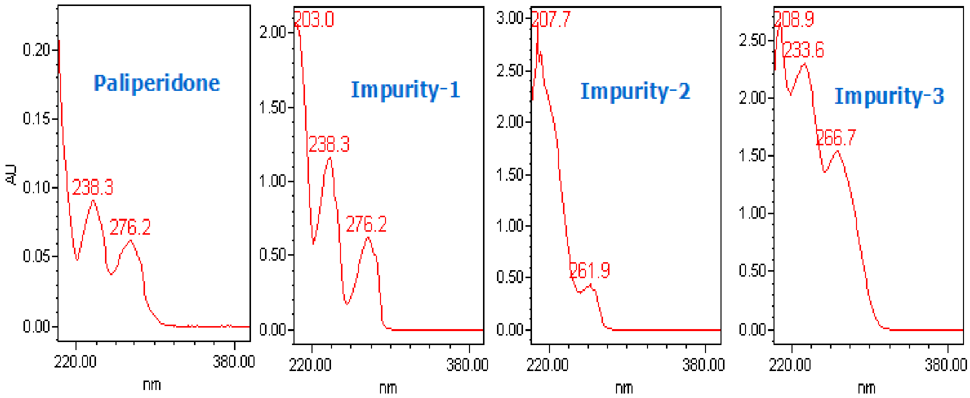


Figure-2: UV spectrums for Paliperidone and its impurities

Initial HPLC method development was performed with ammonium acetate buffer and acetonitrile as organic modifier. Experimental trials were discussed.

Experiment-1: mobile phase: 0.03M ammonium acetate in water as buffer and acetonitrile 60:40v/v; diluent: water and acetonitrile 60:40v/v; column YMC pack C18 150*4.6mm, 3µm; flow rate 1.0ml/min; 230 nm wavelength; 50µL injection volume; 40°C column oven temperature; sample solution 1.0mg/ml and standard solution 0.2% concentration with respect to sample solution.

Results: Paliperidone was eluted at 3 min and other impurities were eluted very near. Base line noise was observed. Ionic pair buffer salt may give separation

Experiment-2: mobile phase: 2g TBAHS (tetra butyl ammonium hydrogen sulphate) in 100 ml water as buffer and acetonitrile 60:40v/v; diluent: water and acetonitrile 60:40v/v; column YMC pack C18 150*4.6mm, 3µm; flow rate 1.0ml/min; 230 nm wavelength; 50µL injection volume; 40°C column oven temperature; sample solution 1.0mg/ml and standard solution 0.2% concentration with respect to sample solution.

Results: Paliperidone was eluted at 4 min. Three impurities were eluted very early about 2min with poor peak shape.

Experiment-3: mobile phase: 2g TBAHS (tetra butyl ammonium hydrogen sulphate) in 100 ml water as buffer and methanol 60:40v/v; diluent: water and acetonitrile 60:40v/v; column Zorbax SB C18 100*4.6mm, 3µm; flow rate 1.0ml/min; 230 nm wavelength; 50µL injection volume; 40°C column oven temperature; sample solution 1.0mg/ml and standard solution 0.2% concentration with respect to sample solution.

Results: Paliperidone was eluted at 6.5 min, impurity-1 eluted at 9.7min, impurity-2 eluted at 2.39min and impurity-3 eluted at 2.4min. Three impurities were well separated and have good peak shape. Each analytes Paliperidone and its impurities 1, 2 and 3 chromatograms were represented in Figure-3 to 6.

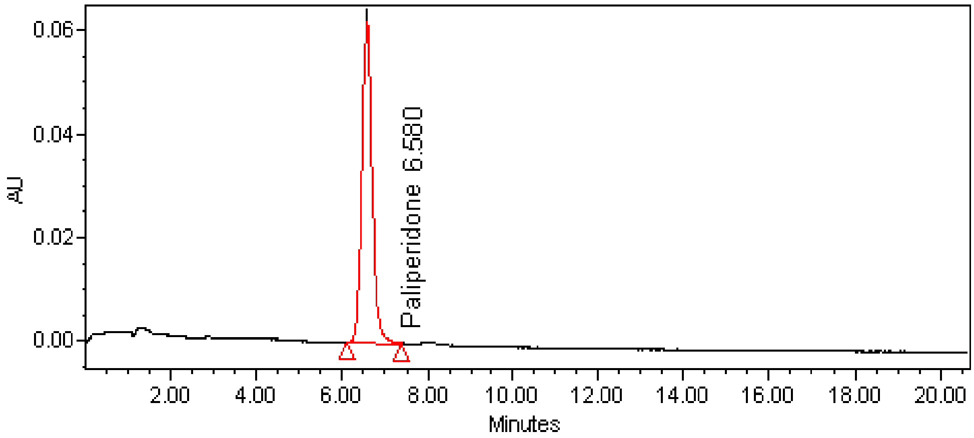


Figure-3: Paliperidone chromatogram

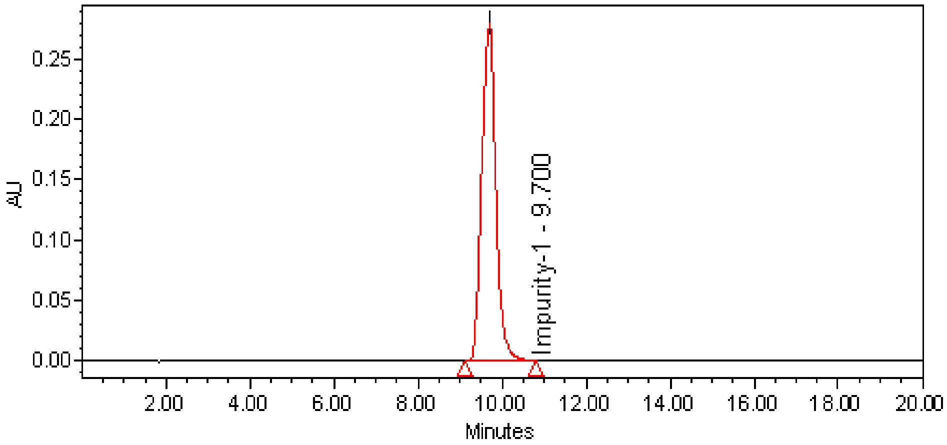


Figure-4: Impurity-1 Chromatogram

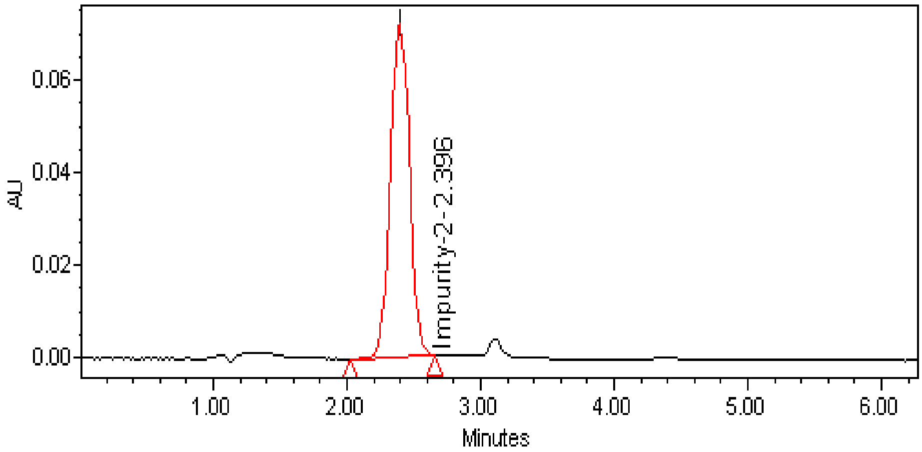


Figure-5: Impurity-2 Chromatogram

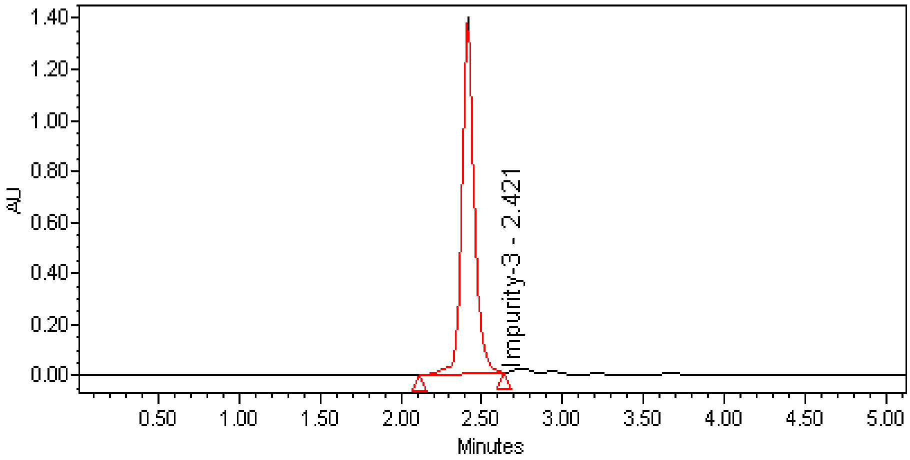


Figure-6: Impurity-3 Chromatogram

Specificity:
Specificity experiments were performed with acid, base, peroxide, thermal, UV light, humidity and water hydrolysis stress degradation studies. Interference between known and unknown impurities was evaluated and found that there is no interference. % of assay for all stress studies were reported in table-1. Stress studies chromatograms were represented in figure-7 to 14.

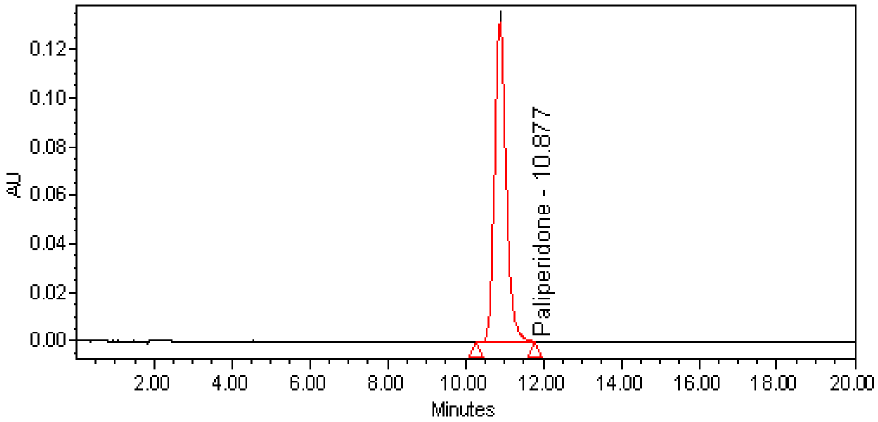


Figure-7: As such test solution as such 6 mg tablets chromatogram

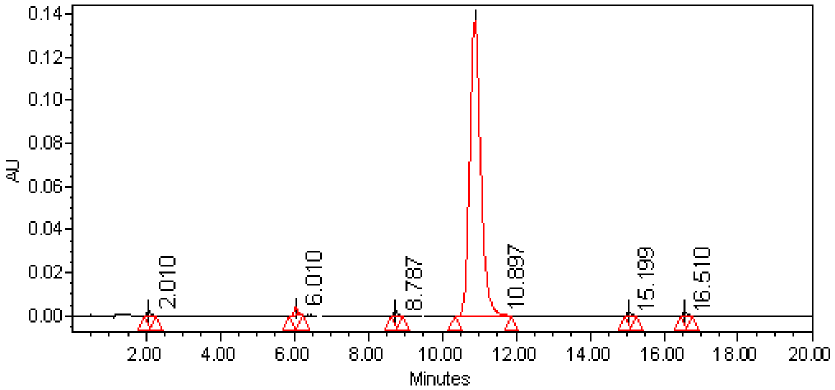


Figure-8: Acid degradation sample chromatogram

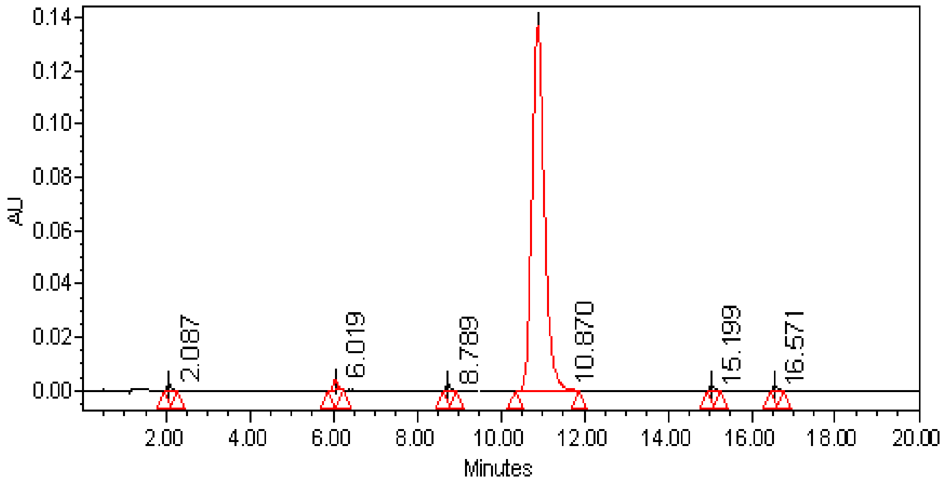


Figure-9: Base degradation sample chromatogram

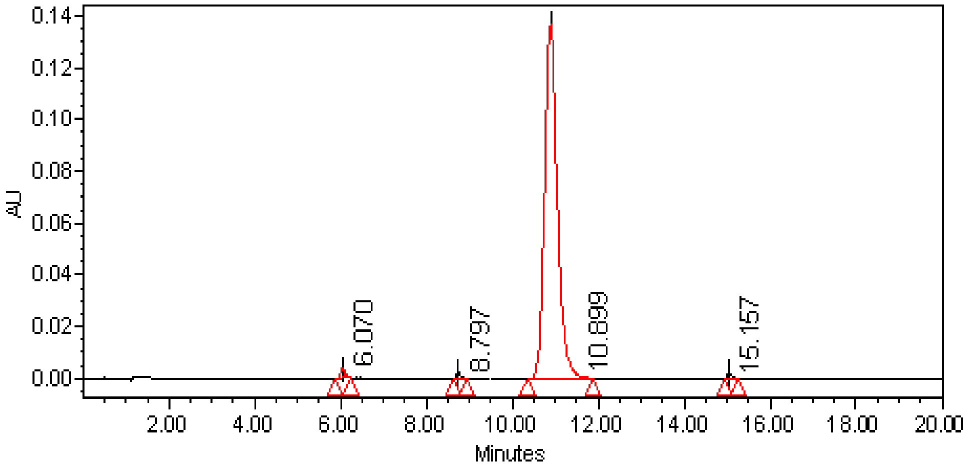


Figure-10: Peroxide degradation sample chromatogram

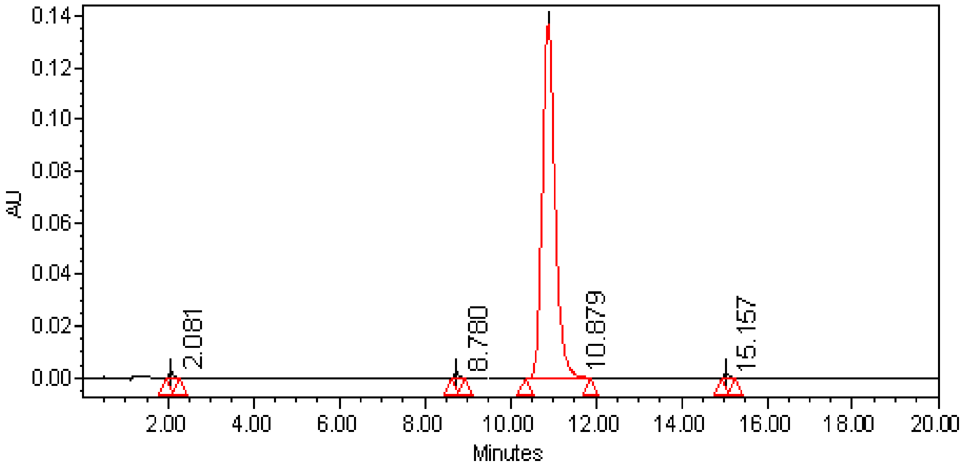


Figure-11: Thermal degradation sample chromatogram

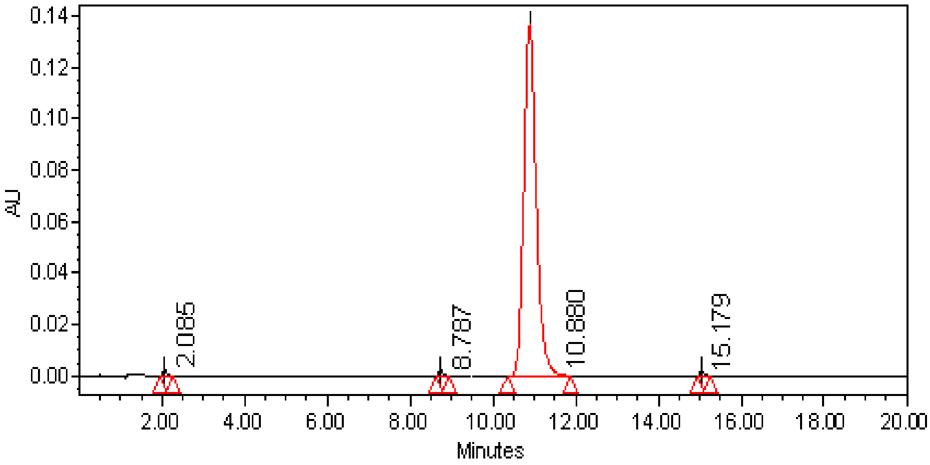


Figure-12: UV degradation sample chromatogram

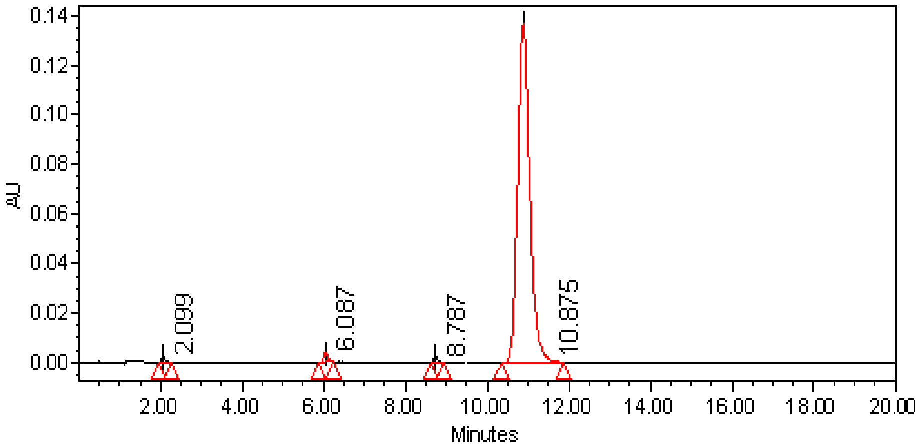


Figure-13: Water hydrolysis sample chromatogram

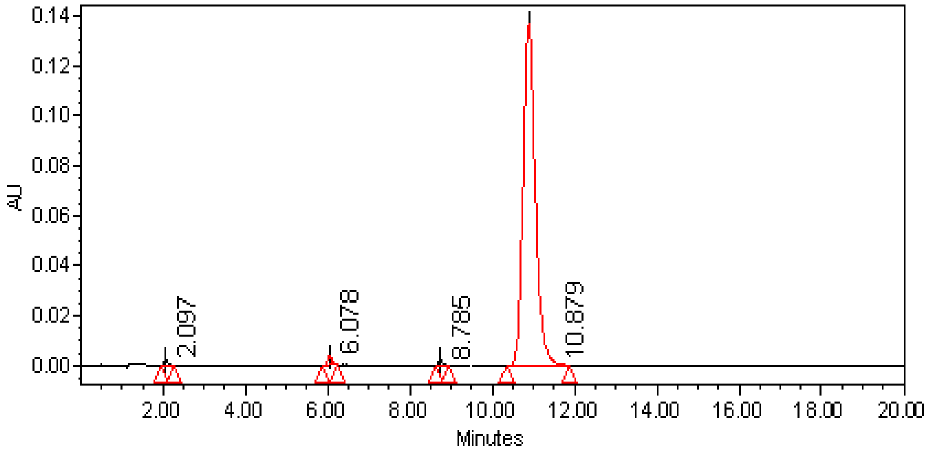


Figure-14: Humidity degradation sample chromatogram

Table-1: Stress study results

S. No.	Degradation condition	Paliperidone % of assay	Purity Angle	Purity Threshold	Peak purity
1.	Test as such	99.6%	0.073	0.255	Pass
2.	Acid (5N HCl, 1hr, 60°C)	94.2%	0.071	0.309	Pass
3.	Base (5N NaOH, 1hr, 60°C)	93.4%	0.146	0.283	Pass
4.	Peroxide (3% H_2O_2, 1hr, RT)	94.8%	0.084	0.243	Pass
5.	Water (Water, 6hr, 60°C)	94.6%	0.094	0.255	Pass
6.	Thermal (24hr, 80°C)	95.6%	0.073	0.255	Pass
7.	UV/ visible light (UV light 200 watt hr/sq. meter) (Visible light 1.2 million lux hrs)	93.9%	0.089	0.289	Pass
8.	Humidity (90%RH, 25°C, 7hr)	94.8%	0.068	0.216	Pass

Precision:

Method precision was performed for both the tablets strength 3mg, 6mg, 9mg and 12 mg. Six replicate test samples were performed freshly, analysed on HPLC and calculated the assay values and % RSD for six replicate preparations. Precision results confirmed the method repeatability and reproducibility. System suitability was evaluated with five replicate solutions. Blank, placebo and standard solutions chromatograms were represented in figure-15 to 17. Figure-18 represented the five replicate standard injections. Intermediate precision was performed with different HPLC instrument; different analyst and different lot column. Intermediate precision results were satisfactory. Precision and intermediate precision results were compared and found to be acceptable. Table-2 represented the precision and intermediate precision results.

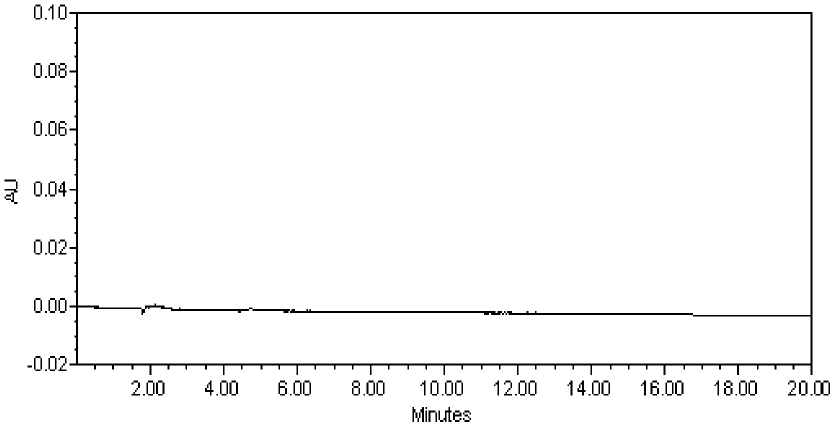


Figure-15: Blank Chromatogram

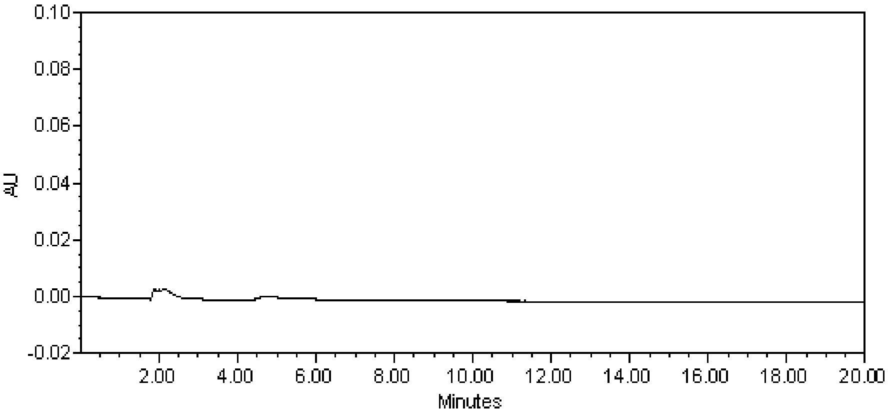


Figure-16: Placebo Chromatogram

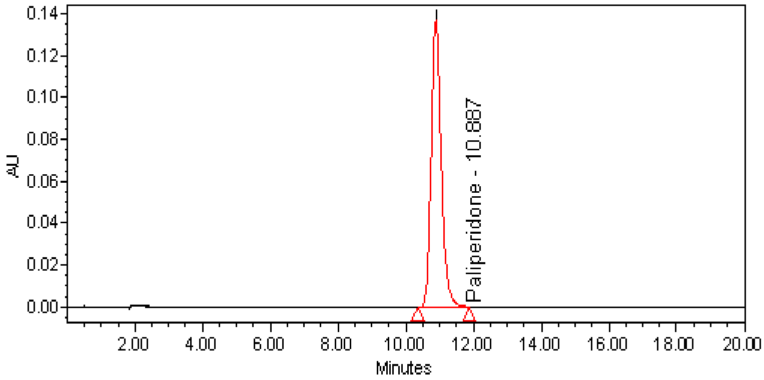


Figure-17: Standard chromatogram

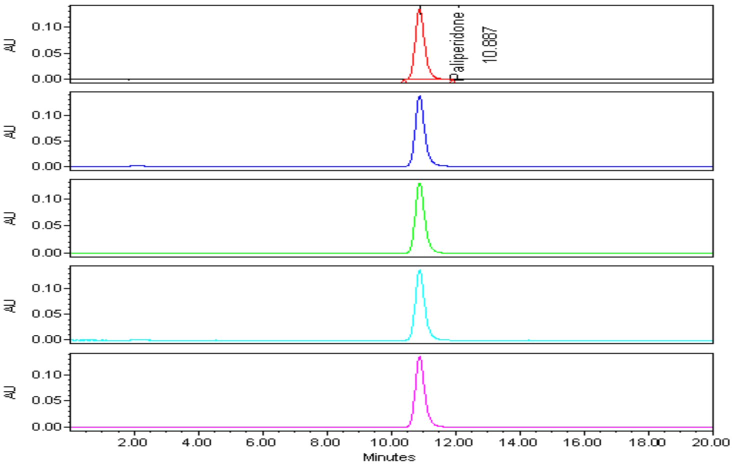


Figure-18: Five replicate standard solution overlay chromatogram

Table-2: Precision and intermediate precision results

Paliperidone	1	2	3	4	5	6	Average	%RSD
Precision results								
Assay (%)	101.25	100.12	99.79	101.21	99.68	100.58	100.43	0.68
Intermediate precision								
Assay (%)	100.16	100.25	99.87	100.15	99.88	100.21	100.08	0.16

Linearity:

Method linearity was performed to confirm the method linearity range. Five different concentration linearity levels were prepared with the standard material. 50% to 150% of standard and test solution concentrations were coved and confirmed the linearity correlation coefficient value. Correlation coefficient value 0.9993 was observed and linearity plot was drawn. Table-3 represented the Paliperidone linearity results. Overlay chromatograms were represented in figure-19 and linearity plot was shown in figure-20.

Table-3 Paliperidone Linearity results

Linearity level	1	2	3	4	5	Corr. of coeffi.
Conc. (µg/ml)	244	311	490	620	763	
Area	1254080	1669805	2561244	3356413	4191864	0.9993

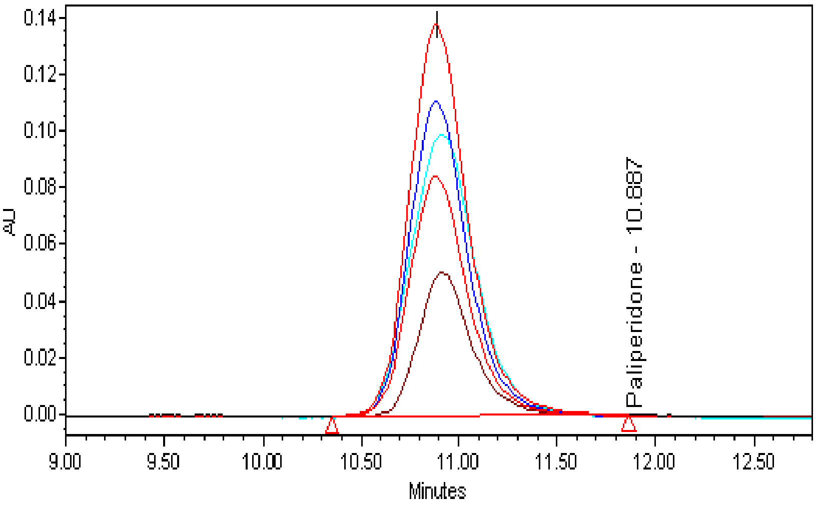


Figure-19: Linearity over lay chromatogram

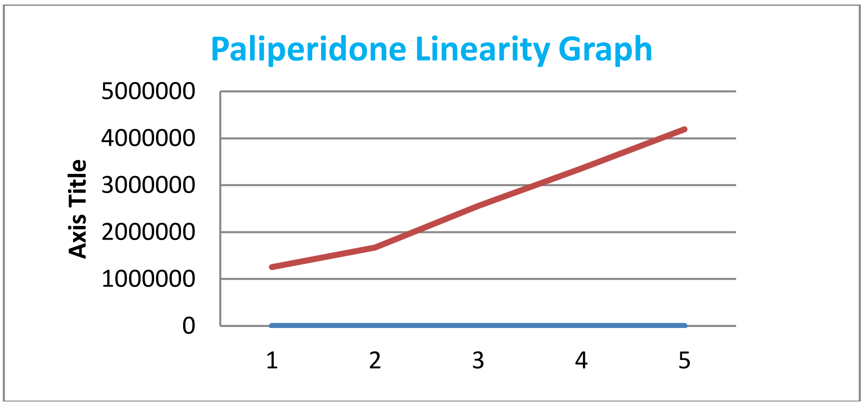


Figure-20: Linearity Graph

Accuracy:
Accuracy of the method was evaluated with three different concentration levels 50%, 100% and 150% of the test concentration. Higher concentration 150% level was prepared for six replicates and other accuracy levels were three replicates to confirm the method accuracy. % recovery was calculated and found to be within the acceptable limits (limit: 98% to 103%). Accuracy results were tabulated in 4.

Table-4: Accuracy results

	Paliperidone accuracy results												
µg /mL	Added	248			506			750					
	Recov.	243	246	251	509	510	516	742	749	751	748	743	758
% recov. (%)		97.98	99.19	101.21	100.59	100.79	101.98	98.93	99.87	100.13	99.73	99.07	101.07
Mean (%)		99.46			101.12			99.80					

Ruggedness:
Ruggedness was performed to confirm the test solutions and standard solution stability in room temperature and refrigerator storage conditions. Two precision test samples were used to study the storage stability. Day-0, 1, 3 were evaluated at room temperature storage samples and day-0, 3 and were evaluated at refrigerator storage conditions. Standard solutions were injected and systems suitability parameters tailing factor and %RSD were

evaluated. Table-5 represented the ruggedness results.

Table-5: Ruggedness results

Time in day	Bench top stability test solution				Tailing factor	%RSD	Bench top stability standard solution
			Difference				Similarity factor
	Test-1	Test-2	Test-1	Test-2			
Initial	101.25	100.12	NA	NA	1.3	1.3	NA
Day-1	100.16	100.92	1.09	0.8	1.4	1.5	0.99
Day-3	99.69	101.10	1.56	0.98	1.1	1.4	1.0
	Refrigerator stability test solution						Refrigerator stability standard solution
Initial	101.25	100.12	NA	NA	1.4	1.3	NA
Day-3	101.65	100.31	0.4	0.19	1.2	1.5	0.99
Day-5	100.89	100.96	0.36	0.84	1.1	1.2	1.01

Robustness:

Robustness was evaluated with analytical method chromatographic conditions. Mobile phase flow rate, column oven temperature and mobile phase organic solvent ratio were studied and reported the system suitability results. Table-6 represented the robustness results. Filter variations were analysed with PVDF and NYLON types. Filter variation results confirmed that there is no difference in the filter change. Filter validation results were tabulated in table-7.

Table-6: Results of Effect of variations

Condition	Flow rate			Column temperature		
	0.8 & 1.2 ml/min	0.6 & 1.0 ml/min	1.0 & 1.4 ml/min	30°C	25°C	35°C
Tailing factor	1.3	1.6	1.3	1.3	1.5	1.2
% RSD	0.52	0.58	0.49	0.39	0.42	0.49
	Mobile phase organic solvent ratio					
	pH 6.0	pH 5.8	pH 6.2			
Tailing factor	1.2	1.6	1.3			
% RSD	0.46	0.52	0.58			

Table-7: Filter Variability results

Centrifuged		Nylon filter				PVDF filter			
% assay		% assay		% Difference		% assay		% Difference	
Spl-1	Spl-2	Spl-1	Spl-2	Spl-1	Spl-2	Spl-1	Spl-2	Spl-1	Spl-2
100.64	101.01	100.31	100.69	0.33	0.32	100.63	100.89	0.01	0.12

CONCLUSION

Paliperidone medicinal product is used to treat schizophrenia and schizoaffective disorder. It is more effective than Haloperidol, Quetiapine and aripiprazole. Simple HPLC method was developed and validated to evaluate the qualitative and quantitative of Paliperidone in tablets dosage forms 3mg, 6mg, 9mg and 12 mg strengths. *Buffer:* 2.1g of TBAHS 100 mL of HPLC grade water; *Mobile phase:* Buffer, Acetonitrile 90:10 v/v, Zorbax SB C18 100x4.6 mm, 3.5µm column, flow rate 1.0 mL/min, 275 nm wavelength, 10 µL injection volume, column temperature 40°C were used. Run time 20 min. method validation was performed to confirm the each parameter such as precision, linearity, accuracy, ruggedness, robustness, specificity. Method validation results precision assay values (98% to 102%), %RSD for six replicates, linearity correlation co-efficient value not less than 0.999 were confirmed the method repeatability and reproducibility. Specificity (blank, placebo and impurities interference and force degradation) confirmed the stability indicating nature. Method can be used to study the Paliperidone in tablets dosage form.

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Novel and stability indicating HPLC method for Ezetimibe, Rosuvastatin, Atorvastatin in tablets form

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Abstract:

Rugged and robust HPLC method was developed for assay determination of Ezetimibe, Rosuvastatin, and Atorvastatin in tablet dosage form. These three drugs used to treat the human body bad and good cholesterol management in blood. Ezetimibe and rosuvastatin are available in the market in tablets dosage form; Ezetimibe and atorvastatin combinations also available in the market. 0.05 M KH_2PO_4 buffer was used as mobile phase A and acetonitrile is used as mobile phase B. Gradient program was used as eluent, 30% of mobile phase B at 0 min; 30 % at 5 min; 42% at 8 min; 40 % at 12 min; and 30 % at 16 min and 30 % at 20 min. Agilent make Zorbax SB C18 150*4.6 mm, 5 µ HPLC column was used. 20 µL injection volume, 20 min runtime, 1.0 ml/min flow rate, 230 nm and 50°C column oven temperature were applied for analysis. Mobile phase A and B were mixed in the ratio of 50:50 v/v and used as diluent. All three analytes were eluted with high resolution and the retention time of ezetimibe 15.3 min, rosuvastatin 9.0 min and atorvastatin 17.1 min. method validation was performed as per ICH quality guidance. Results were achieved with accuracy and precision. Hence, the developed and validated method was applicable for routine drug product manufacturing quality evaluation.

Keywords: Ezetimibe, Rosuvastatin, Atorvastatin, HPLC,
Method development, Method validation

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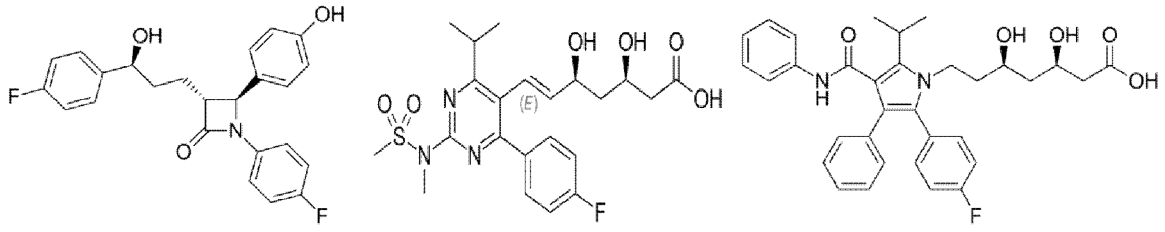
Introduction:

Ezetimibe controls the absorption of cholesterol and decreasing the release of intestinal cholesterol to the liver. Ezetimibe (EZE) is [(3R,4S)-1-(4-fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)- 2-azetidinone]. It manages the cholesterol absorption (primary hypercholesterolemia). It inhibits the absorption of biliary and dietary cholesterol from small intestine without influencing absorption of fat soluble vitamins, triglycerides and bile acids.[1-2] 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. [3-4]

Atorvastatin is used to treat anti-hyperlipidemic (lipid lowering) class of drug which reduces the amount of fatty or lipid substances such as cholesterol and triglycerides from the body. Chemical name of atorvastatin is 7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-(propan-2-yl)-1H-pyrrol-1-yl]-3,5-dihydroxyheptanoate**,** calcium salt (2:1) trihydrate**.** Figure-1 represented the chemical structures of ezetimibe, Atorvastatin, rosuvastatin.

Rosuvastatin calcium is chemically, bis[(E)-7[4-(4-fluorophenyl)-6-isopropyl-2-[methyl (methyl-sulphonyl)amino]pyrimidin-5-yl](3R,5S)-3,5-dihydroxyhept-6-enoic acid] calcium salt). It belongs to a statins family, which are employed to lower hypercholesterolemia and related conditions and to prevent cardiovascular diseases. It increases the number of hepatic low density lipoprotein receptors involved in the catabolism of LDL and also inhibits hepatic synthesis of very low density lipoprotein. [5-8]



Ezetimibe Rosuvastatin Atorvastatin

Figure-1: Chemical structure of Ezetimibe, Atorvastatin and Rosuvastatin

Literature survey was performed for these three analytes determination for single HPLC method but there is no single method was reported. Few methods were reported for ezetimibe and rosuvastatin determination by using HPLC, UV spectrophotometric methods.[9-11] Ezetimibe and atorvastatin combination product also have very few reported methods. [12-17]

The main objective of this research work was to develop an single, accurate and rugged HPLC method to determine ezetimibe, rosuvastatin and atorvastatin in pharmaceutical drug products.

Materials and Method:

Chemicals and reagents:

HPLC standard acetonitrile and methanol were purchased from Qualigens fine chemicals, Mumbai, India. Distilled, 0.45 µm filtered water used for HPLC analysis. Sd. fine chem analytical grade KH_2PO_4 salt was purchased and used for the preparation of mobile phase. Milli-Q water was used for the analysis.

Chromatographic conditions:

Agilent make HPLC systems and carry win UV spectrophotometer were used for this research work. Zorbax SB C18, 150×4.6 mm, 5 µm HPLC column was used (agilent make). 1.0 ml/min flow rate, 420 µL injection volume and 50°C column oven temperature were applied. UV absorbance was measure at 230 nm.

Mobile phase A:

6.8 g of KH_2PO_4 buffer salt was weighed accurately and transferred in to 1000 ml beaker, mixed well. Sonication was performed to dissolve the contents. Resulting solution was degassed with and filtered through 0.45µm Millipore membrane filter and sonicated.

Mobile phase B:

HPLC grade acetonitrile was used as mobile phase B, degassed through 0.45µm. Millipore membrane filter and sonicated for few minutes.

Diluent:

Mobile phase A and B were mixed in the ratio of 50:50 v/v and degassed through 0.45µ filter.

Standard Solution:

40 mg of each standard material such as rosuvastatin, ezetimibe and Atorvastatin was weighed accurately and transferred in to a 100 mL volumetric flask and dissolved in 50mL of diluent and sonicated to dissolve the contents. Further, volume was filled with diluent solution. From the above stock solution 5 mL aliquot was pipetted in to a 50mL volumetric flask and dissolved in the solvent and made up to the mark with the diluent.

Ezetimibe and Atorvastatin test sample solution:

The contents of twenty ezetimibe and Atorvastatin tablets were taken and finely powdered. A mass equivalent to 56mg of each ezetimibe and Atorvastatin was transferred to a 100mL volumetric flask and dissolved in 50mL of the diluent. The solution was kept for sonication for 15min. The solution was made up to the mark with the diluent and filtered through a 0.45µ membrane filter. 5mL aliquot of the above solution was transferred to a 50mL volumetric flask and diluted to the mark with diluent.

Ezetimibe and rosuvastatin test sample solution:

The contents of twenty ezetimibe and rosuvastatin tablets were taken and finely powdered. A mass equivalent to 56mg of each ezetimibe and rosuvastatin was transferred to a 100mL volumetric flask and dissolved in 50mL of the diluent. The solution was kept for sonication for 15min. The solution was made up to the mark with the diluent and filtered through a 0.45µ membrane filter. 5mL aliquot of the above solution was transferred to a 50mL volumetric flask and diluted to the mark with diluent.

Assay Calculation:

% of Assay=

$$\frac{\text{Ta X Tw X 5 X 100 X 50 X Tweight X Spotency}}{\text{Sa 100 X 50 X Sw X 5 X Label claim X 100}} \text{ x 100}$$

Ta= Peak area in test solution
Sa= Peak area in standard solution
Tw= Sample weight used for test solution preparation
Sw= Standard weight used for standard solution preparation
Tweight= Tablets average weight
Label claim= drug content in one tablet
Spotency= Standard material potency

Results and Discussion:

Method Optimization:

Ezetimibe, Atorvastatin, rosuvastatin standard materials are available in solid stable form and can be stored at room temperature. Solubility of three ingredients was evaluated with different solvents like different pH value buffers, acetonitrile and methanol. Solubility study results shown that, all the three components have solubility with mixture of buffer, methanol and acetonitrile. Further UV spectral studies were performed by using Agilent makes carry 60 UV/ Visible spectrophotometer. Three ingredients were prepared with 2 ppm concentration to perform the UV spectral analysis. UV spectrum was scanned from 200 to 400 nm. Figure-2 to 4 represented the UV spectrum of ezetimibe, Atorvastatin and rosuvastatin. Based on the UV spectrum results we have selected 230 nm to measure the analytes.

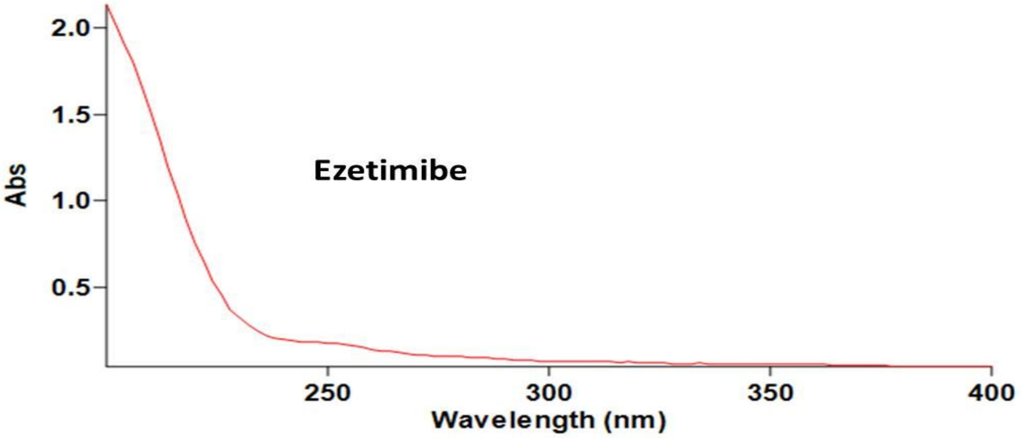


Figure-2: Ezetimibe UV spectrum

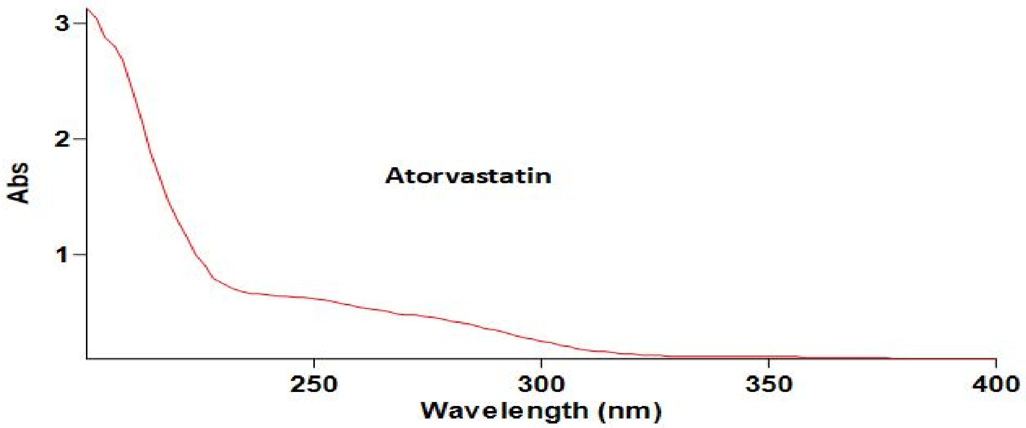


Figure-3: Atorvastatin UV spectrum

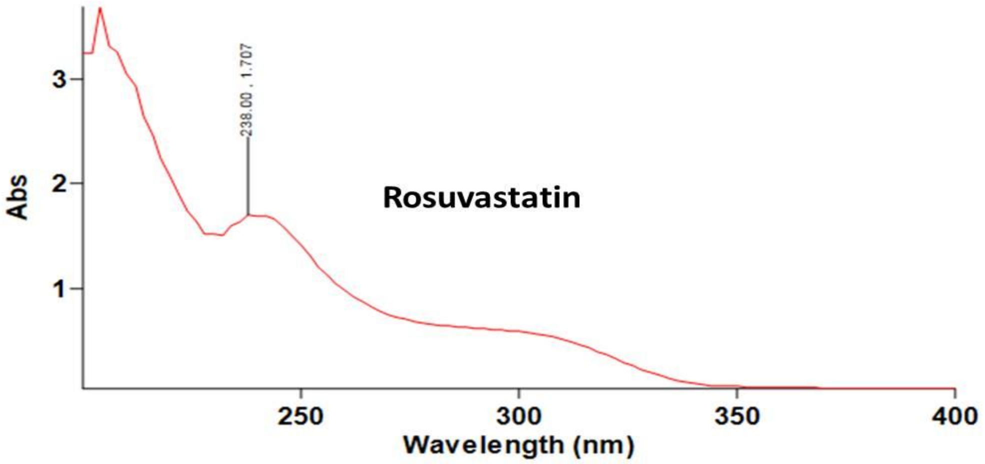


Figure-4: Rosuvastatin UV spectrum

Method development experiment-1:

Conditions:

Buffer: 0.5 ml orthophosphoric acid and 0.5 ml tri-ethyl amine transferred in to 1000 mL of water and mixed well to dissolve. Adjusted the pH value to 3.5 with tri-ethyl amine and filtered the solution through 0.45µm membrane filter and degassed. Mobile Phase A: Buffer; Mobile Phase B: Analytical grade acetonitrile. Isocratic elution: Mobile phase A: Mobile phase B 55:45 v/v, Column: Intertsil C8, 250 x 4.6 mm, 5µm; Flow rate: 1.0 mL/min Column Temperature: Ambient; Volume of Injection: 20µL; Wave Length: 230 nm; Run Time: 35 min. Diluent: Mixed 500 mL of water and 500 mL of Acetonitrile in the ratio of 50:50% v/v and degassed. Preparation of Standard Solution: Individual sample solutions were prepared with 250 ppm concentration with diluent solution.

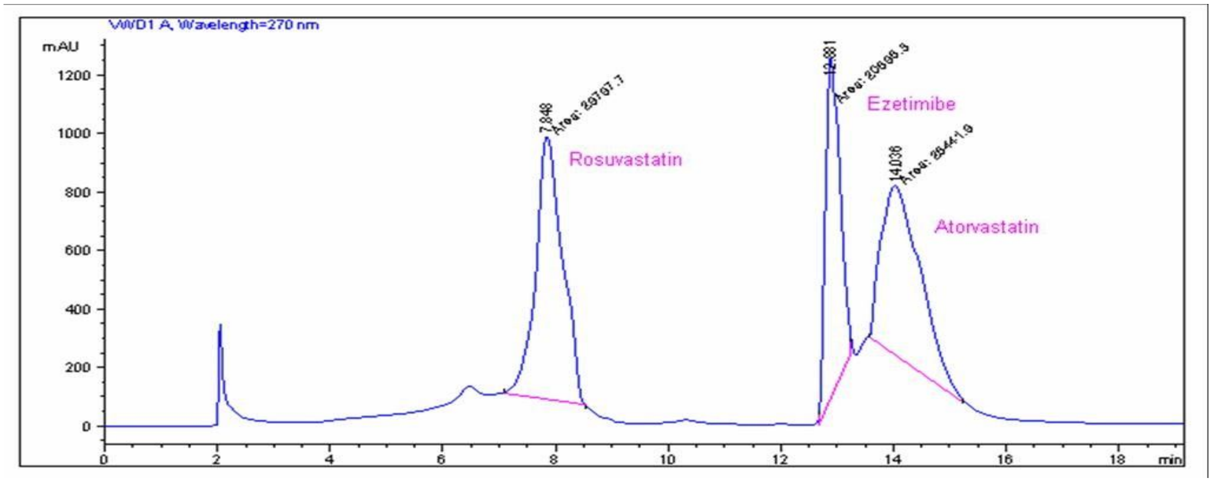


Figure-5: Method development trial-1 chromatogram

Observation: Three standard materials were eluted (rosuvastatin 7.8 min; ezetimibe 12 min and atorvastatin 14min) at but Atorvastatin and ezetimibe were eluted closely. Further experiments shall be carried out with salt buffer to optimize retention time and peak shape. Figure-5 represented the method development trail chromatogram.

Method development experiment-2:

Conditions:

Buffer: 0.7 g of ammonium acetate salt was weighed accurately and transferred in to one liter milli-Q water and mixed well to dissolve. Adjusted the pH value to 3.0 with acetic acid and filtered the solution through 0.45µm membrane filter and degassed. Mobile Phase A: Buffer; Mobile Phase B: Analytical grade acetonitrile; elution: Mobile phase A: Mobile phase B 60:40 v/v; Column: Intertsil C8, 250 x 4.6 mm, 5µm; Flow rate: 1.0 mL/min; Column Temperature: Ambient; Volume of Injection: 20µL; Wave Length: 230 nm; Run Time: 35 min; Preparation of diluent: 500 mL of water and 500 mL of Acetonitrile were mixed and degassed. Standard Solution: Individual sample solutions were prepared with 250 ppm concentration with diluent solution.

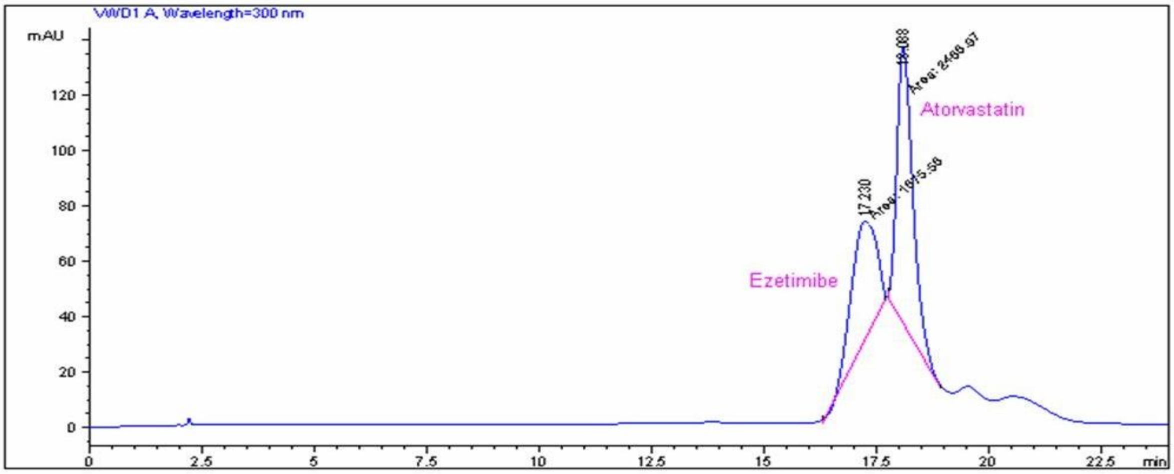


Figure-6: Method development trial-2 chromatogram

Observation: ezetimibe and atorvastatin were co-eluted. Further experiments shall be carried out by changing the buffer salt to achieve good peak shape and different retention time for each analyte. Figure-6 represented the method development trial chromatogram.

Method development experiment-3:

Buffer solution: 3.4 g of KH_2PO_4 salt was weighed accurately and transferred in to 1000 mL of water and mixed well to dissolve. Filtered the solution through 0.45µm membrane filter and degassed. Mobile Phase A: Buffer; Mobile Phase B: Analytical grade acetonitrile. Gradient program was applied to separate the ezetimibe and atorvastatin.

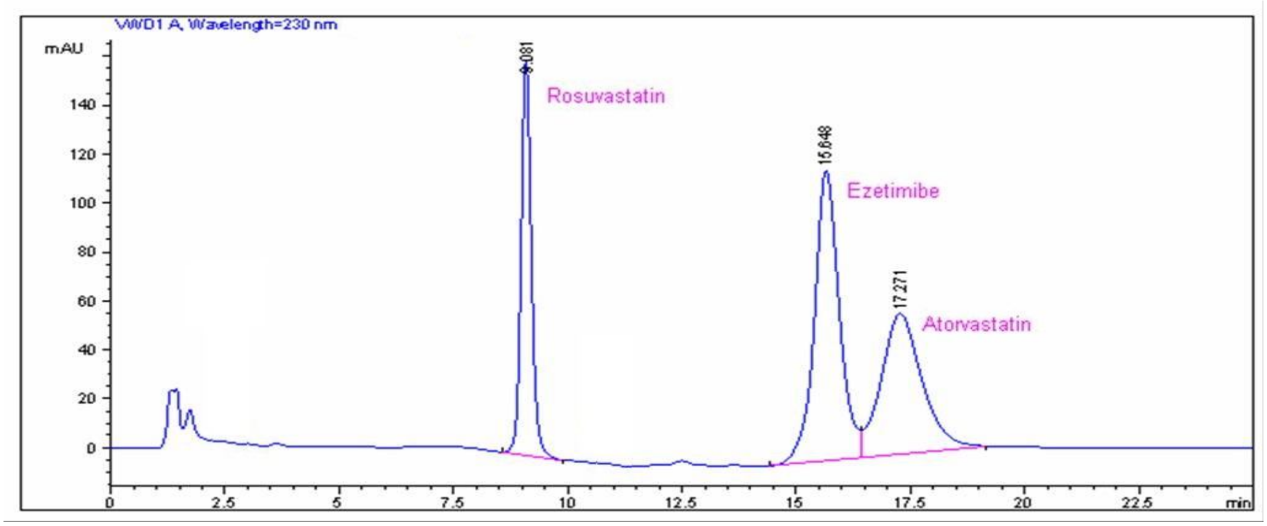


Figure-7: Method development trial-3 chromatogram

Observation: Three standard materials were eluted but resolution between ezetimibe and atorvastatin was low. Figure-7 represented the method development trial chromatogram.

Method development experiment-4:

Buffer solution: 6.8 g of KH_2PO_4 salt was weighed accurately and transferred in to 1000 mL of water and mixed well to dissolve. Filtered the solution through 0.45µm membrane filter and degassed. Mobile Phase A: Buffer and B: acetonitrile; Gradient elution: mobile phase B 27% at 0 min; 32% at 4 min; 45% at 8 min; 58% at 12 min; 27% at 16 min and 27% at 20 min. other chromatographic conditions were applied as like development trial 3.

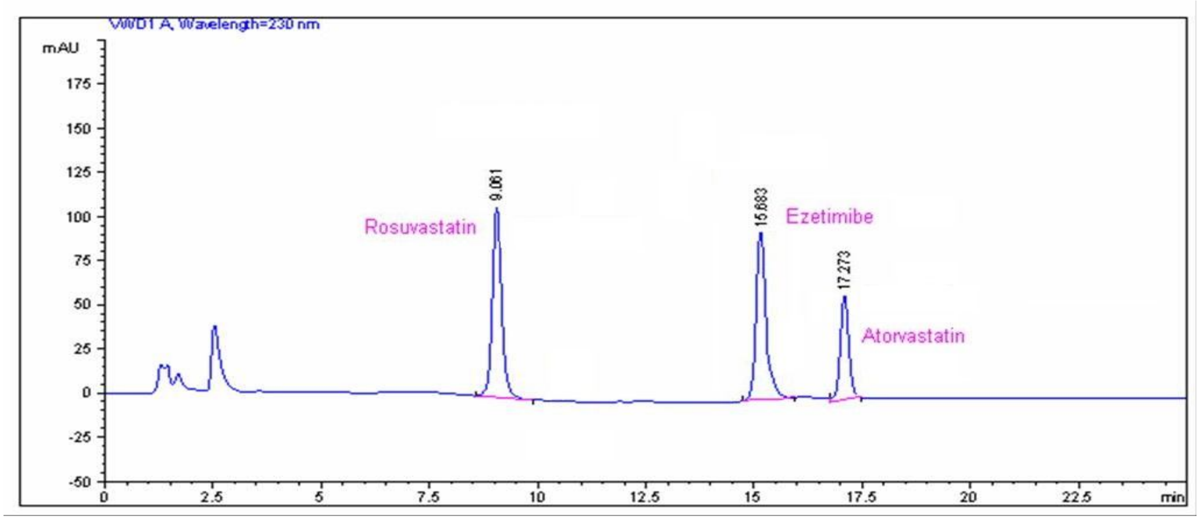


Figure-8: Method development trial-4 chromatogram

Observation: Three analytes were eluted with good peak shape. Hence, this optimized method has been considered as final method. Further, method validation can be performed. Figure-8 represented the mixed sample chromatogram.

Method validation:

Method validation was performed as per ICH (international council for harmonization of technical requirements for pharmaceutical for human use), USFDA guidance. All parameters such as precision, linearity, specificity, accuracy, ruggedness, robustness were performed. Method validation results are meeting the acceptable limits which are specified in the guidance documents.

Specificity:

Specificity was performed to check the interference from the diluent, placebo and stress study conditions. Acidic, base, peroxide, thermal, photolytic and water stress conditions were applied on drug product. Ezetimibe and rosuvastatin test samples and ezetimibe and atorvastatin samples were stressed separately. Freshly prepared stress samples were injected into the HPLC. Stress studies results were tabulated in table 1 and 2. Figure 9 to 20 were represented the force degradation studies chromatograms. Stress study conditions were listed below,

Ezetimibe and Rosuvastatin tablets Stress study conditions:

- Acid Hydrolysis : 0.5 N HCl at 55°C for 10 hours
- Base Hydrolysis : 1N NaOH at 55°C for 15 hours
- Oxidation (10% H_2O_2): at 30 °C for 6 hours
- Photolytic : UV-light (200 watts hr / m^2)
- Heat : at 55°C for 18 hours
- Water Hydrolysis : at 55°C for 10 hours

Ezetimibe and Atorvastatin tablets Stress study conditions:

- Acid Hydrolysis : 0.5N HCl at 55°C for 12 hours
- Base Hydrolysis : 1N NaOH at 55°C for 6 hours
- Oxidation (10% H_2O_2): at 30 °C for 12 hours
- Photolytic : UV-light (200 watts hr / m^2)
- Heat : at 55°C for 12 hours
- Water Hydrolysis: at 55°C for 10 hours

Table-1: Stress study results.

Active Name	**1. Force degradation % assay results**					
	Acid	**Base**	**Peroxide**	**UV**	**Thermal**	**Water**
Ezetimibe and rosuvastatin tablets						
Ezetimibe	91.8	92.9	93.6	92.8	91.8	94.8
Rosuvastatin	92.6	92.1	92.8	92.1	93.0	93.8
Ezetimibe and atorvastatin tablets						
Ezetimibe	90.2	92.6	92.3	95.1	92.0	94.0
Atorvastatin	91.1	93.5	93.9	92.6	93.4	94.9

Table-2: Stress study unknown peaks data

RT	**2. Force degradation % area**					
	Acid	**Base**	**Peroxide**	**UV**	**Thermal**	**Water**
Ezetimibe and rosuvastatin tablets						
2.9	2.1	2.4	ND	ND	1.9	2.9
6.7	2.0	1.9	2.3	2.7	2.1	2.1
21.0	ND	ND	1.5	1.6	1.6	1.4
Ezetimibe and atorvastatin tablets						
2.9	2.9	2.1	ND	ND	ND	ND
6.7	1.2	1.0	2.4	1.4	ND	ND
21.0	1.2	ND	1.6	0.9	1.1	1.0

*ND= Not Detected

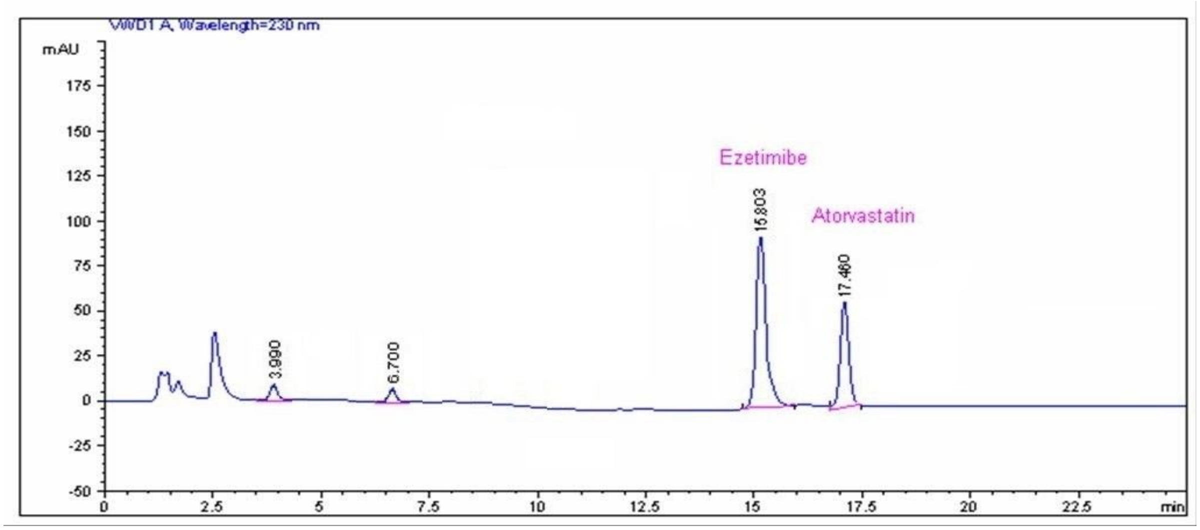


Figure-9: Ezetimibe and Atorvastatin acid degradation chromatogram

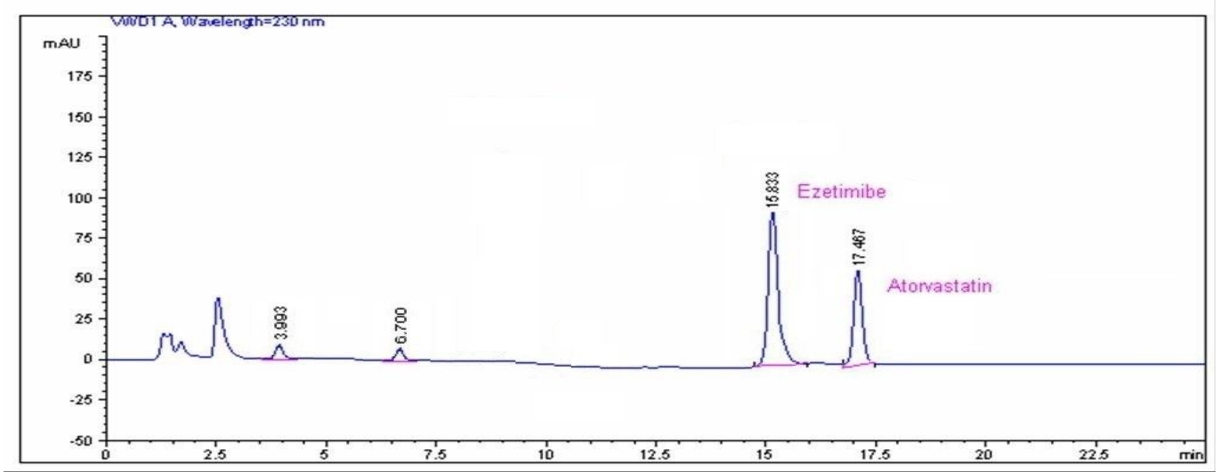


Figure-10: Ezetimibe and Atorvastatin base degradation chromatogram

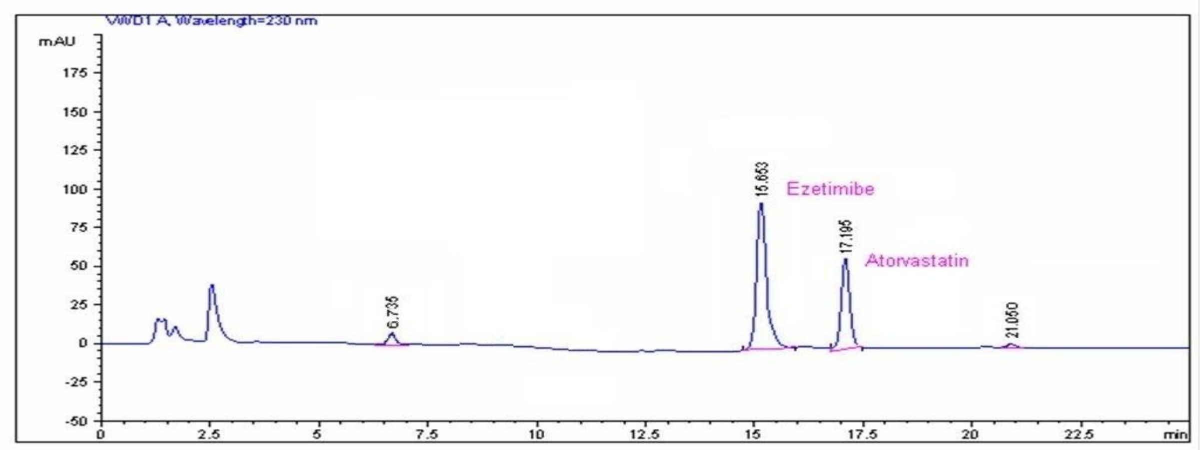


Figure-11: Ezetimibe and Atorvastatin peroxide degradation chromatogram

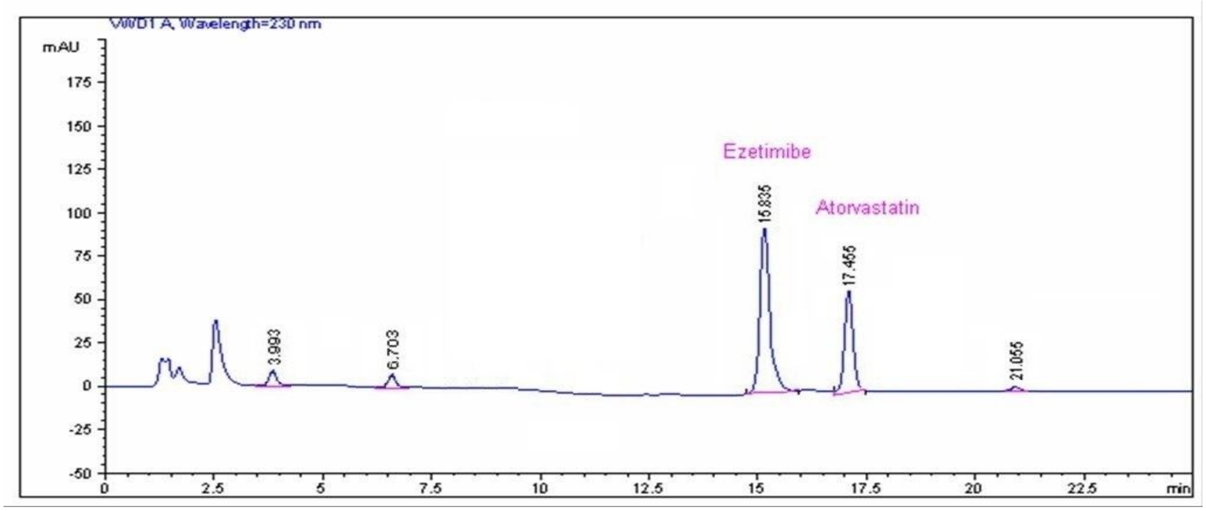


Figure-12: Ezetimibe and Atorvastatin thermal degradation chromatogram

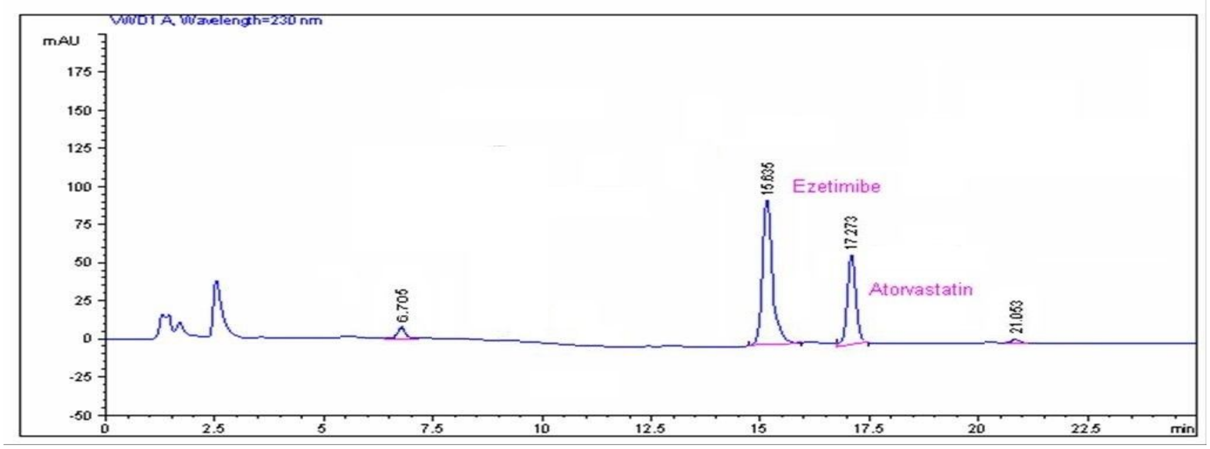


Figure-13: Ezetimibe and Atorvastatin UV degradation chromatogram

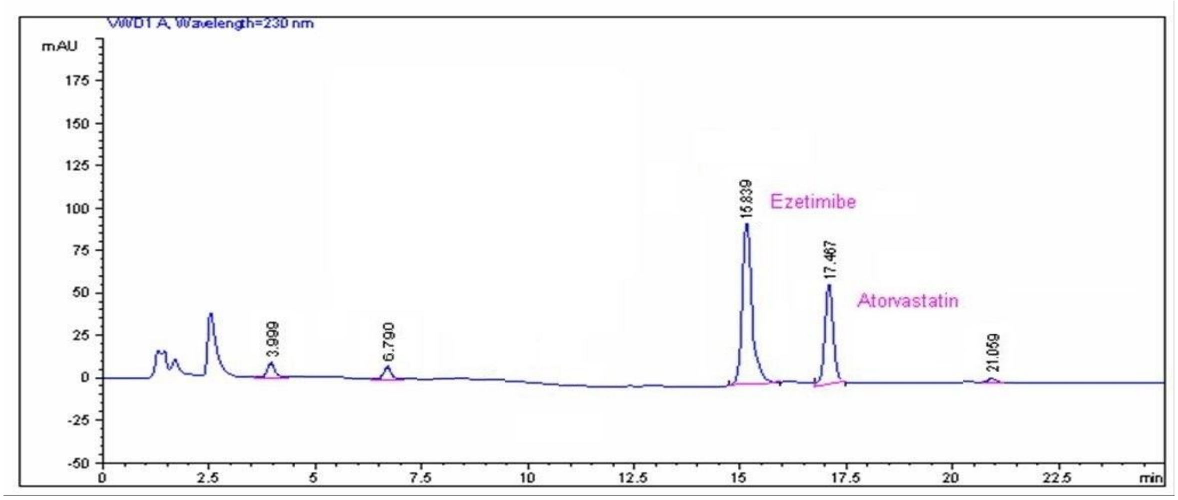


Figure-14: Ezetimibe and Atorvastatin water degradation chromatogram

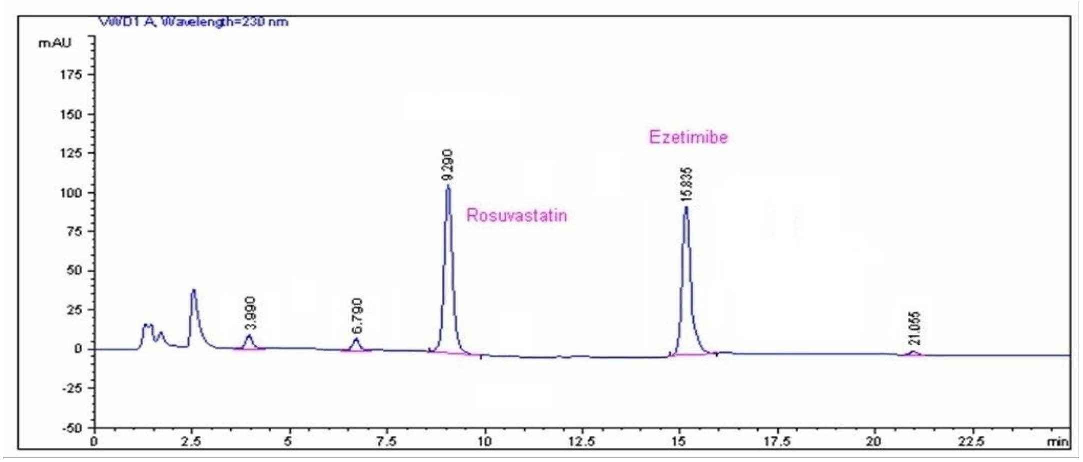


Figure-15: Ezetimibe and Rosuvastatin acid degradation chromatogram

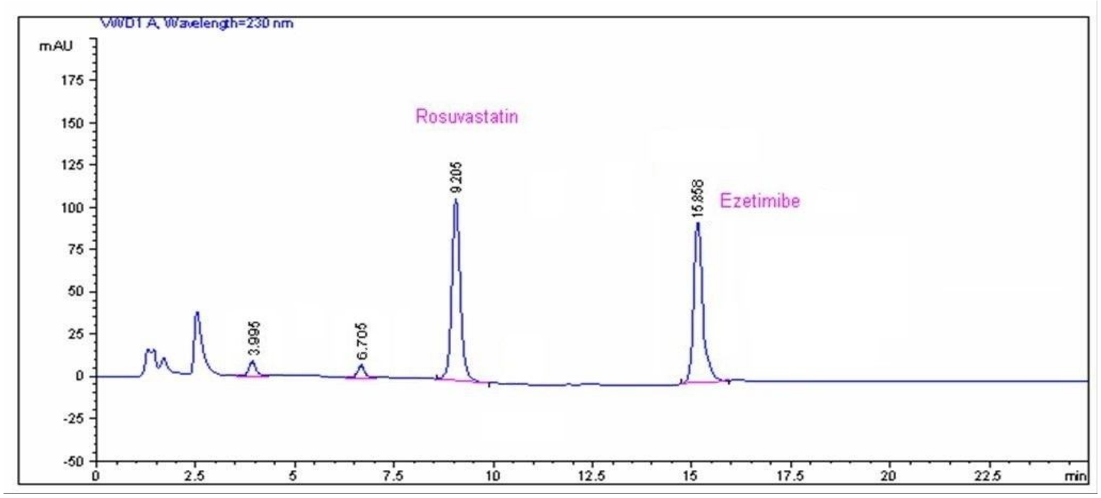


Figure-16: Ezetimibe and Rosuvastatin base degradation chromatogram

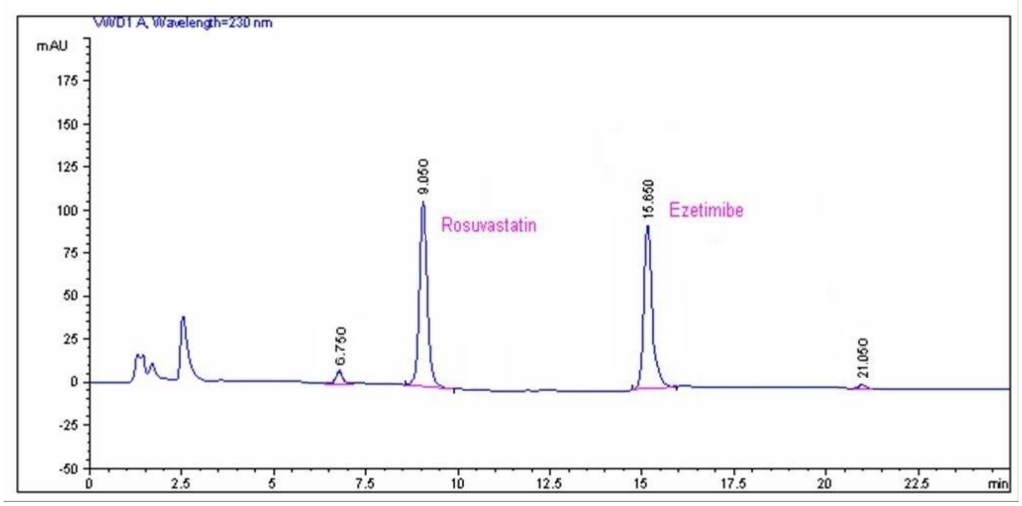


Figure-17: Ezetimibe and Rosuvastatin peroxide degradation chromatogram

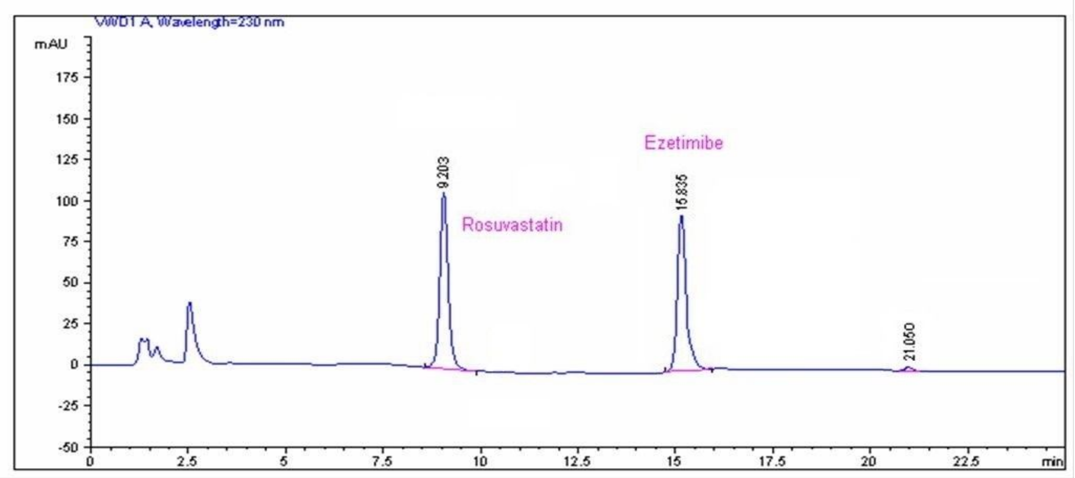


Figure-18: Ezetimibe and Rosuvastatin thermal degradation chromatogram

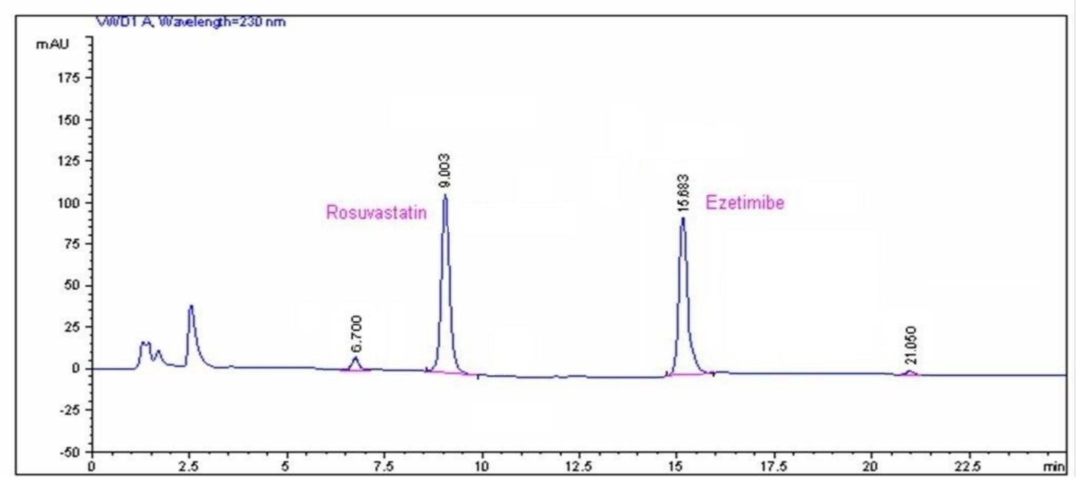


Figure-19: Ezetimibe and Rosuvastatin UV degradation chromatogram

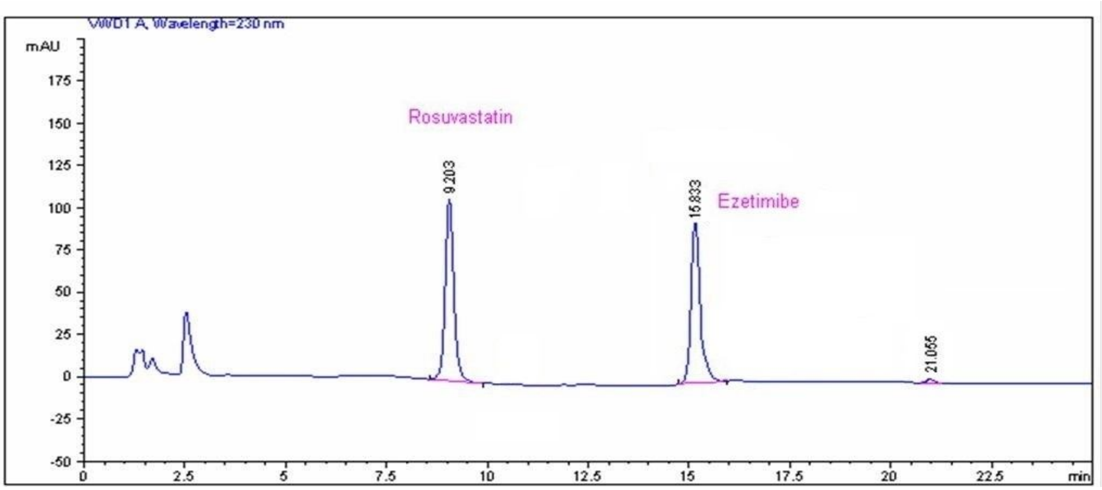


Figure-20: Ezetimibe and Rosuvastatin water degradation chromatogram

Precision:

Precision was performed for system precision with 5 replicate standard injections and method precision with 6 replicate sample preparations. Ezetimibe and rosuvastatin tablets were used to prepare the ezetimibe, rosuvastatin sample solution. Ezetimibe and Atorvastatin tablets were used to prepare ezetimibe, Atorvastatin sample solution. Figure-21 to 27 represented the blank, placebo sample, standard solution and test sample chromatograms. Table 3 and 4 summarized the system suitability and precision results.

Table-3: System suitability Results:

System Suitability parameter (five replicate injections)	**Observations**		
	Rosuvastatin	**Ezetimibe**	**Atorvastatin**
Retention time (min)	9.0	15.5	17.2
Tailing factor (avg.)	1.09	1.3	1.01
%RSD (5 replicates)	1.22	1.11	1.16

Table-4: Method Precision Results:

Active Name	**Precision sample preparation (% content)**						**%RSD**
	1	**2**	**3**	**4**	**5**	**6**	
Rosuvastatin	100.16	99.86	99.68	100.58	100.61	101.25	0.57
Ezetimibe	99.18	100.25	99.76	101.21	100.56	101.31	0.82
Ezetimibe	101.25	101.29	100.98	100.36	100.28	100.64	0.44
Atorvastatin	100.29	100.28	100.25	101.29	101.21	101.52	0.59

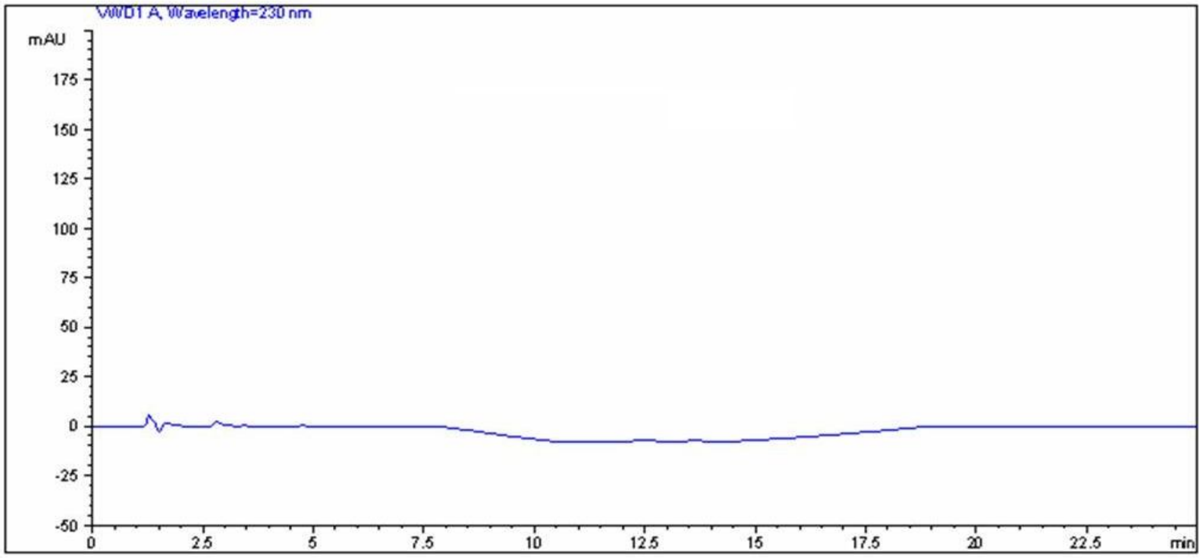


Figure-21: Blank Chromatogram

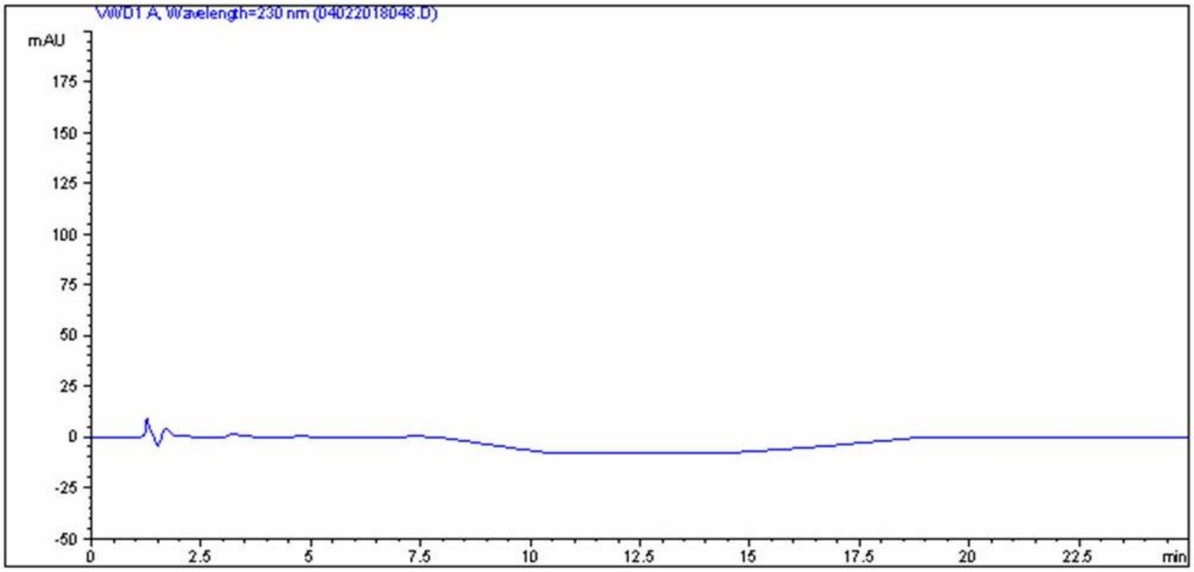


Figure-22: Placebo Chromatogram

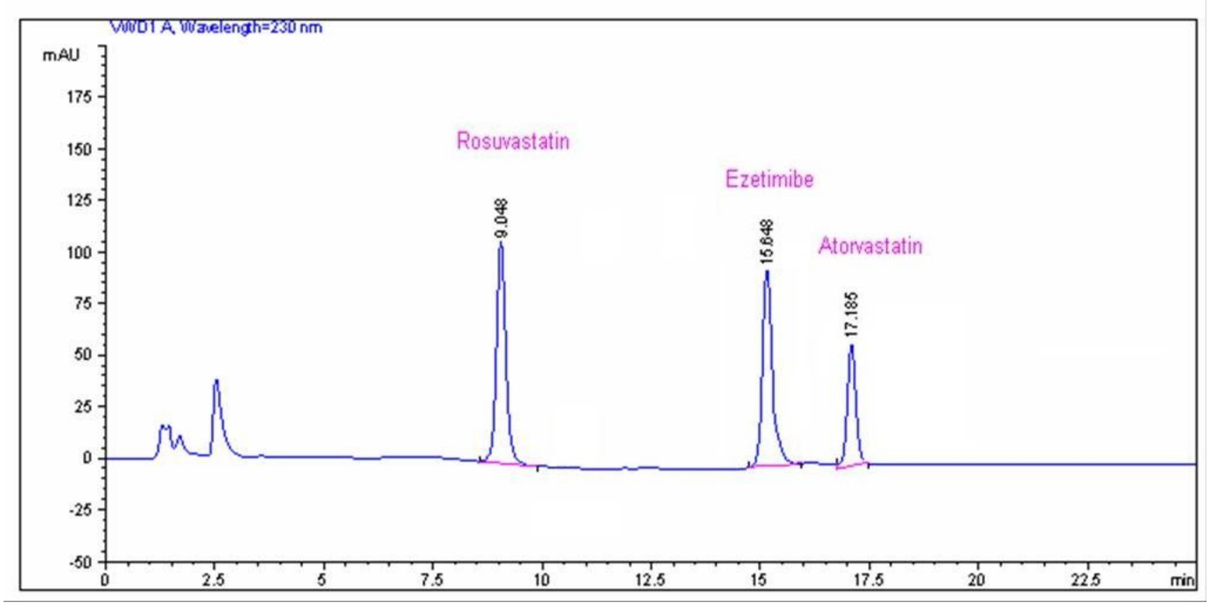


Figure-23: Standard-1 Chromatogram

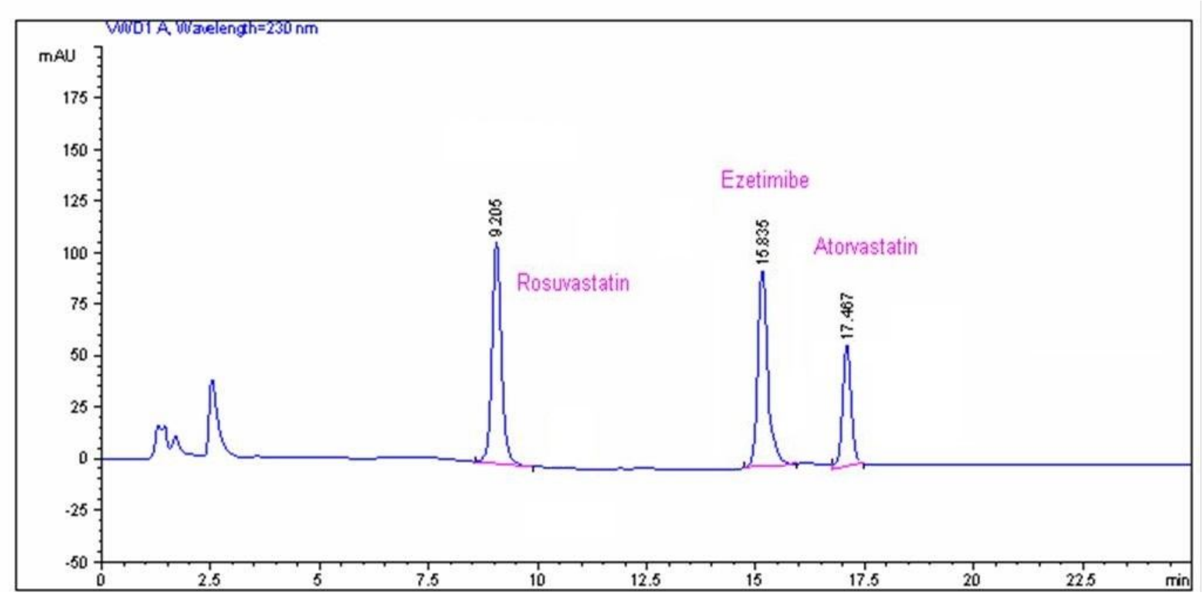


Figure-24: Standard-2 Chromatogram

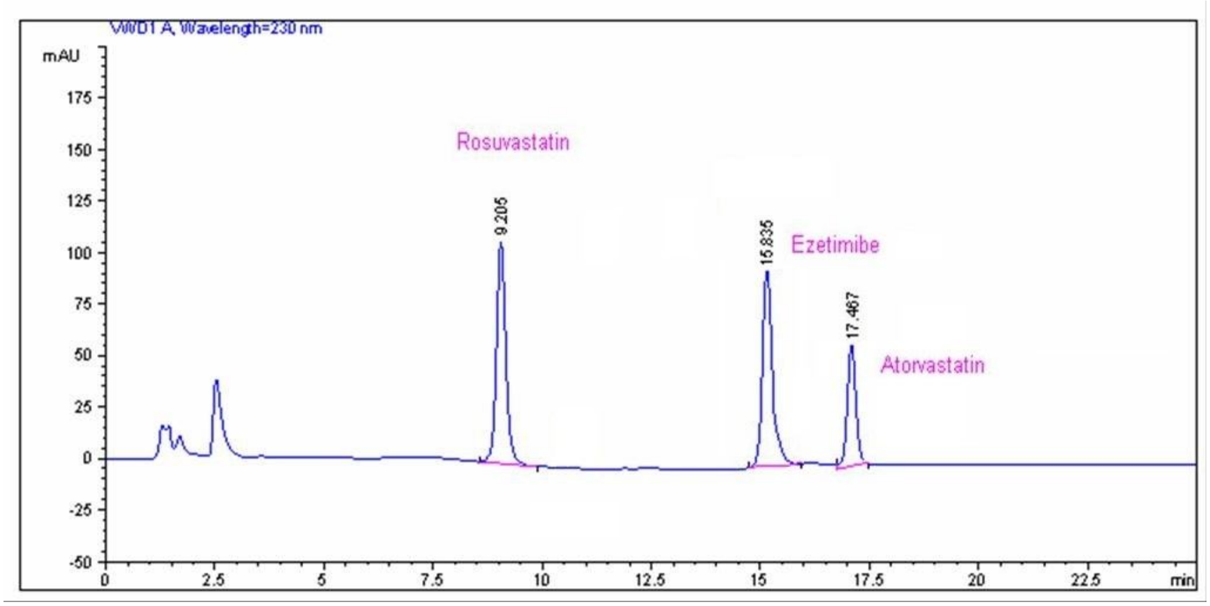


Figure-25: Standard-3 Chromatogram

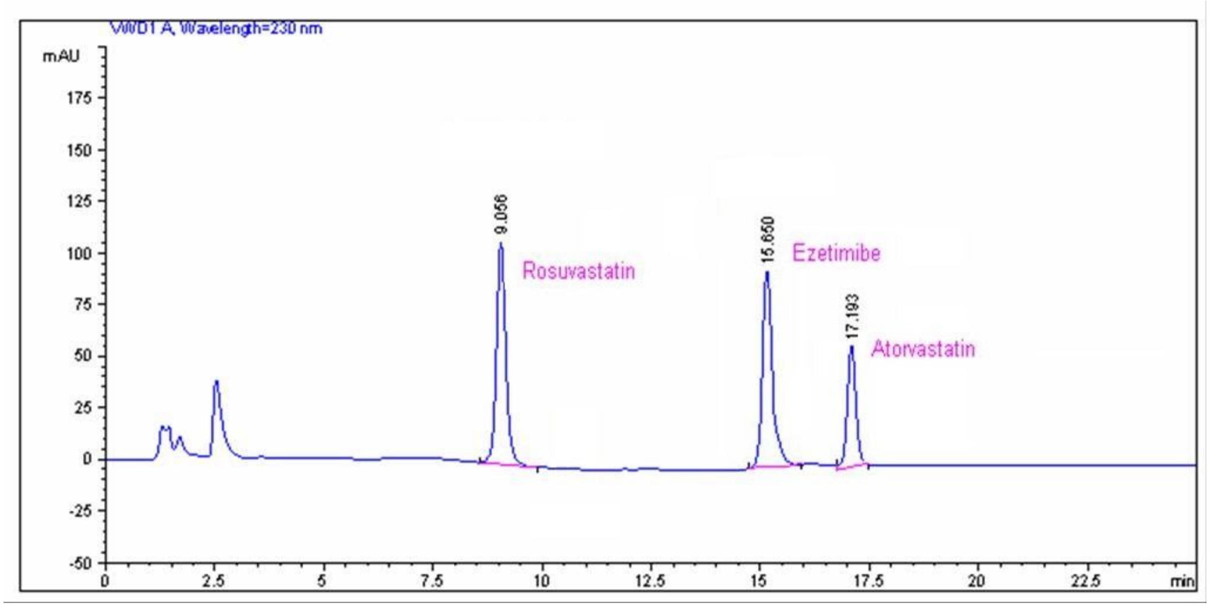


Figure-26: Standard-4 Chromatogram

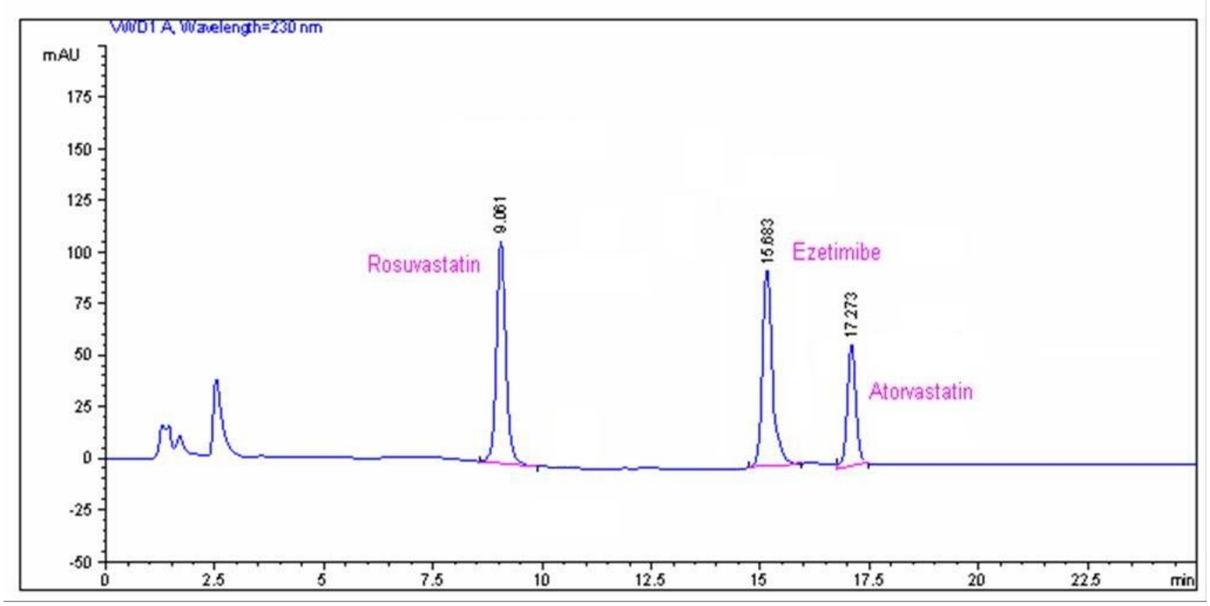


Figure-27: Standard-5 Chromatogram

Linearity:

Linearity was performed with five different concentration levels. Linearity solutions were prepared 50%, 75%, 100 %, 125% and 150% of solutions. These samples were prepared as per the finalized method. Linearity chromatograms were repsented in figure-28. Table-5 represented the linearity results. Figure-29 to 31 shown the linearity graph for rosuvastatin, ezetimibe and atorvastatin.

Table-5: Linearity Results

Linearity Level	**Rosuvastatin**		**Ezetimibe**		**Atorvastatin**	
50%	28	753	28	1342	28	2811
75%	42	1173	42	1965	42	4460
100%	56	1600	56	2653	56	5950
125%	70	1933	70	3223	70	7405
150%	84	2379	84	3928	84	8775
Correlation Coefficient	0.9991		0.9995		0.9994	

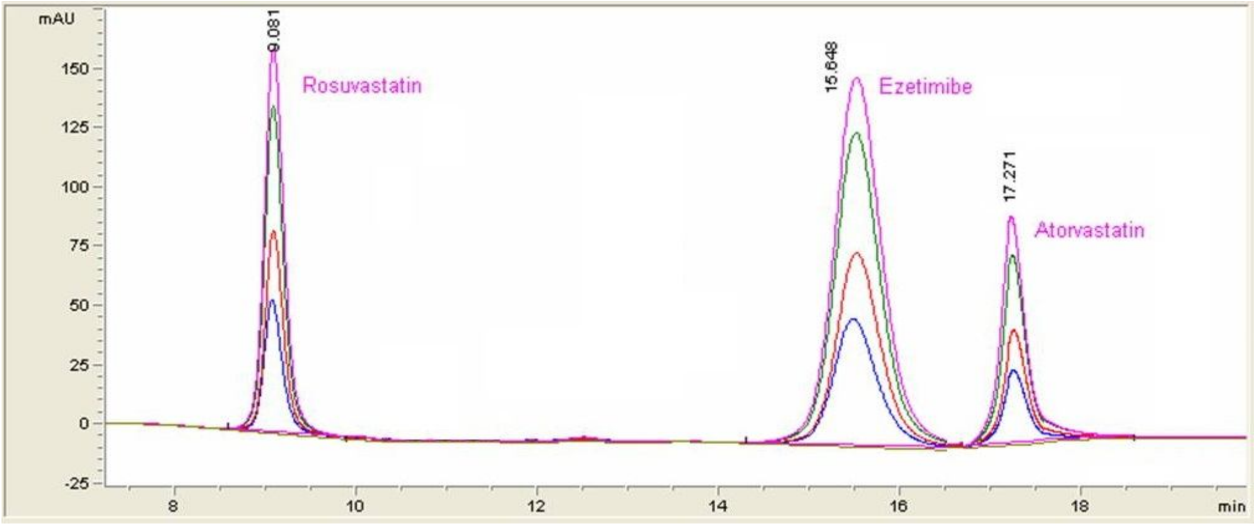


Figure-28: Linearity solutions overlay chromatogram

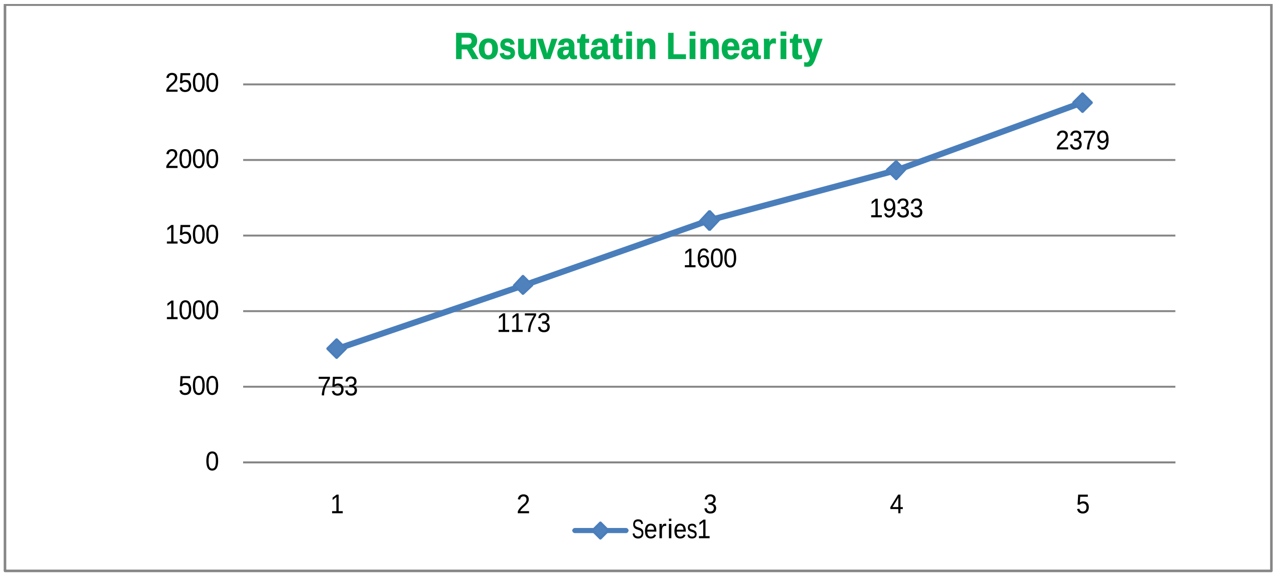


Figure-29: Rosuvastatin Linearity graph

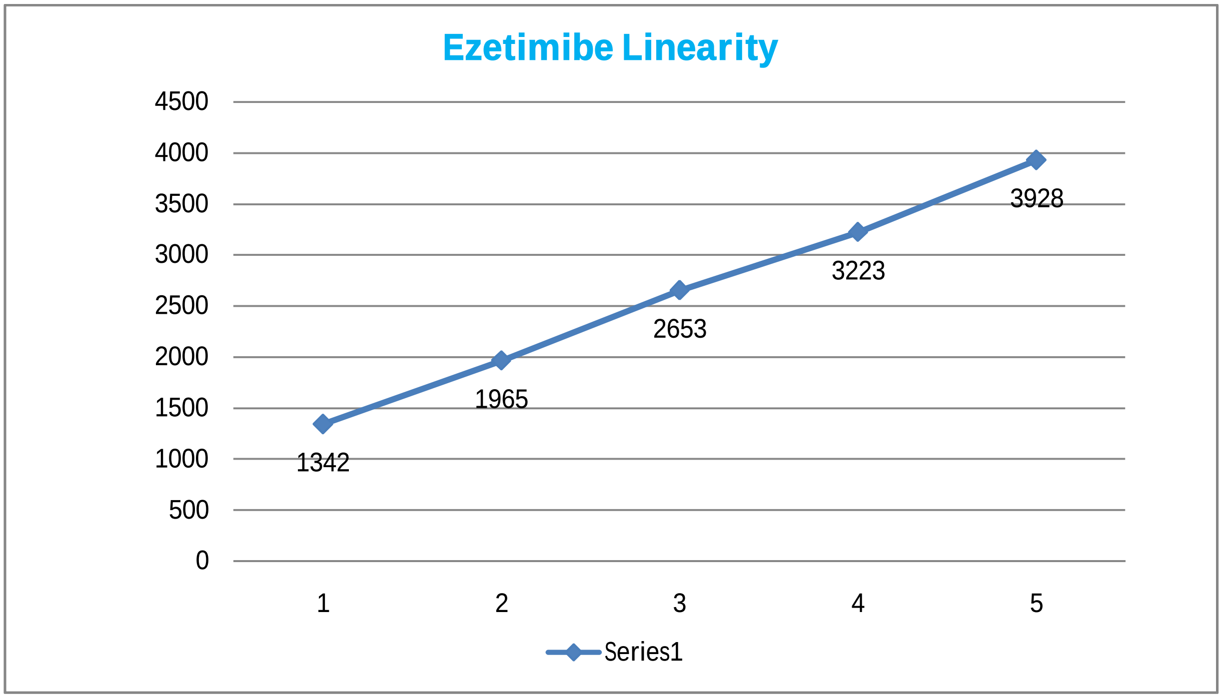


Figure-30: Ezetimibe linearity graph

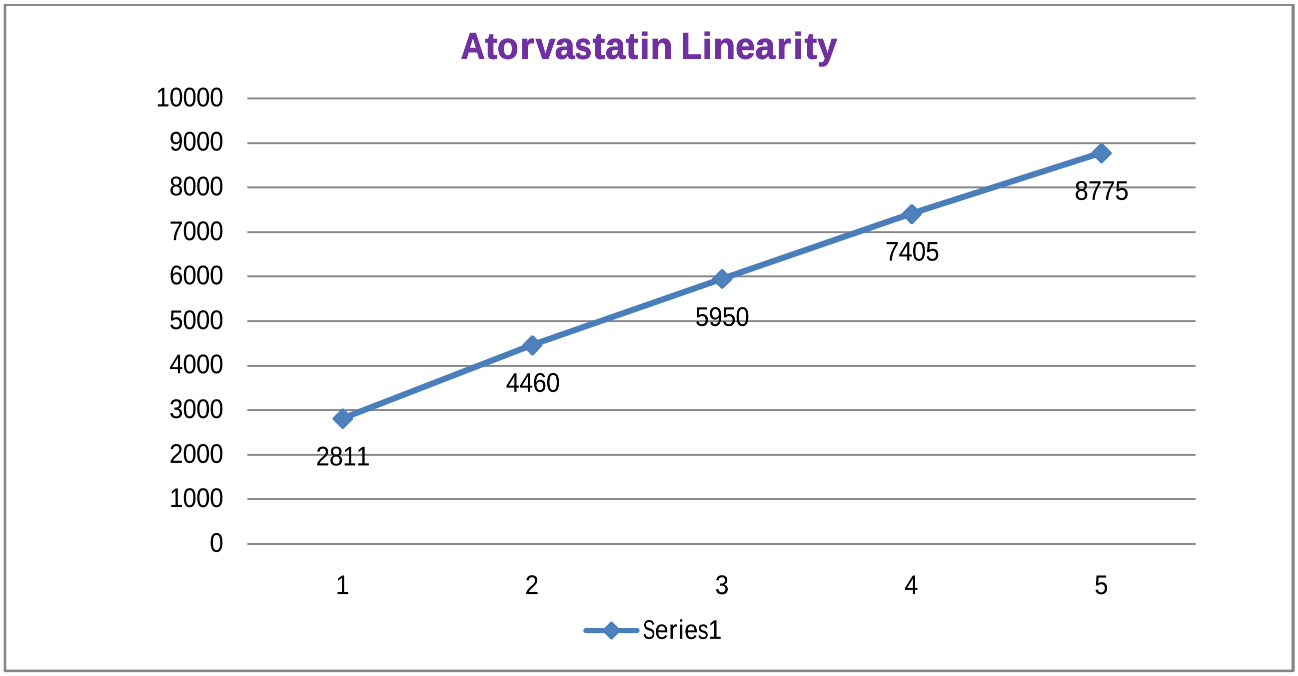


Figure-31: Atorvastatin Linearity Graph

Accuracy:

Accuracy of the method was determined on three concentration levels by recovery experiments. The recovery studies were carried out by spiked placebo recovery method and the percentage recoveries with standard deviations were calculated. Accuracy results were tabulated in table 6. Method was found to be accurate.

Table-6: Accuracy results

(a) Accuracy results			
Level		***Rosuvastatin***	
50%	28.01	27.19	97.07
	28.04	27.87	99.39
	28.07	28.13	100.21
100 %	56.15	55.87	99.50
	56.17	56.25	100.14
	55.89	56.21	100.57
150 %	84.18	84.81	100.75
	83.89	86.12	102.66
	84.52	84.56	100.05
Ezetimibe			
50%	27.89	27.71	99.35
	28.14	28.14	100.00
	28.65	28.53	99.58
100	56.17	56.54	100.66

%	56.52	56.58	100.11
	56.24	56.17	99.88
150 %	84.87	84.36	99.40
	84.56	84.57	100.01
	84.31	84.39	100.09
Atorvastatin			
50%	28.14	28.18	100.14
	28.61	27.81	97.20
	28.15	28.65	101.78
100 %	56.54	56.18	99.36
	56.27	56.80	100.94
	56.19	56.94	101.33
150 %	84.81	84.37	99.48
	84.26	84.15	99.87
	84.57	84.60	100.04

Ruggedness:

Freshly prepared samples were analysed as part of precision parameter. The same samples were stored at room temperature for three days and analysed at day 1 and day-3. Ruggedness was performed with day-1 sample and day 3 sample. Both time intervals results are complying with the specified limits (not more than 2.0% of assay). Table-7 presented the ruggedness results.

Table-7: Solution stability of Assay samples

Duration	**Sample solution-1**		**Sample solution-2**	
	Actual	**% variation**	**Actual**	**% variation**
Rosuvastatin				
Initial	101.54	NA	100.56	NA
Day-1	100.92	0.6	100.32	0.2
Day-3	100.26	1.3	100.34	0.2
Ezetimibe				
Initial	100.68	NA	99.98	NA
Day-1	99.89	0.79	101.00	1.02
Day-3	101.60	0.92	100.06	0.08
Atorvastatin				
Initial	100.14	NA	100.65	NA
Day-1	99.98	0.16	100.31	0.34
Day-3	101.00	0.86	99.87	0.78

Robustness:

Robustness parameter was performed with flow variation, column oven temperature variation and filter validation. Flow rate was checked with 0.8ml/min and 1.2ml/min; column oven temperature evaluated at 45°C and 55°C and filter validation was performed with centrifuged and PVDF filter paper. Results were listed in table 8 and 9.

Table-8: Robustness results

S.No.	Parameter		**Rosuvastatin**		**Ezetimibe**		**Atorvastatin**	
			Tailing factor	%RSD (5 inj.)	Tailing factor	%RSD (5 inj.)	Tailing factor	%RSD (5 inj.)
1	Flow rate (mL/min)	0.8	1.0	0.63	1.1	0.45	1.1	0.61
2		1.2	0.9	0.59	1.0	0.16	1.0	0.54
3	Temp.(°C)	45	1.1	0.14	1.2	0.61	0.9	0.16
4		55	1.0	1.0	0.9	0.56	1.1	1.13

Table-9: Effect of 0.45 µm PVDF filters on standard solution

S. No.	**Standard solution**	**Rosuvastatin**		**Ezetimibe**		**Atorvastatin**	
		% Assay(w/w)	**% of difference**	**% Assay(w/w)**	**% of difference**	**% Assay(w/w)**	**% of difference**
1	Centrifuged	100.90	NA	104.16	NA	100.77	NA
1	0.45 µm PVDF filter	100.63	0.27	101.16	3.00	100.17	0.60

Conclusion:

Simple, high resolution and accurate cost effective RP-HPLC method has been developed for estimation of ezetimibe, Rosuvastatin and Atorvastatin in tablet dosage forms. Optimized method was evaluated with all validation parameters such as precision, accuracy, linearity, specificity, ruggedness and robustness. Method has no interference with placebo and diluent. Six replicate test samples assay value %RSD values were 2.0%, linearity correlation coefficient value was below 0.999 and accuracy recovery %RSD was 97% to 103%. The proposed method is simple, fast, accurate and precise for the simultaneous quantification three ingredients in tablets dosage form. The proposed method can be used for the routine analysis.

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