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STABILITY-INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF OZENOXACIN AND ITS DEGRADATION PRODUCTS IN OINTMENT DOSAGE FORMS

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ABSTRACT

The present work was the development of a simple, efficient, and reproducible stability-indicating reverse-phase high performance liquid chromatographic (RP-HPLC) method for determination of Ozenoxacin and its degradation products. Including Alkaline Hydrolysis impurity ranging 11.01 %, Acid, Hydrolysis impurity ranging 11.61 % and Oxidation impurity ranging 14.76 % using ointment dosage form. The separation of Ozenoxacin and its degradation products in ointments was carried out on Kromasil C-18 (250 × 4.6 mm, 5 μm) column using Water. Flow rate was 0.7 mL min−1 with a column temperature of 35 ∘C and detection wavelength was carried out at 307 nm. The forced degradation studies were performed on Ozenoxacin ointment under acidic, basic, oxidative, thermal, humidity, and photolytic conditions. The degraded products were well resolved from the main active drug and also from known impurities within 1 hr. The method was validated in terms of specificity, linearity, LOD, LOQ, accuracy, precision, and robustness as per ICH guidelines. The results obtained from the validation experiments prove that the developed method is a stability-indicating method and suitable for routine analysis.

KEYWORDS: Ozenoxacin, Stability-Indicating RP-HPLC, Degradation impurity, Method Development, quinolone antibiotic.

INTRODUCTION

Ozenoxacin is a novel synthetic quinolone antimicrobial used for the treatment of impetigo. A 1% topical cream is approved for treatment of impetigo. Ozenoxacin is active against some bacteria that have developed resistance to fluoroquinolone antimicrobials. Appearance of ozenoxacin is White, Crystalline powder, its molecular Formula is 363.417, Molecular Formula of ozenoxacin is $C_{21}H_{21}N_3O_3$.Although the exposure response relationship for ozenoxacin after it has been applied topically has not yet been studied, a formal relationship is unlikely because systemic exposure of ozenoxacin following its topical application has been measured to be negligible. Ozenoxacin is a quinolone antibiotic drug. And, like most quinolones, ozenoxacin predominately executes its mechanism of action by entering into bacterial cells and acting to inhibit the bacterial DNA replication enzymes DNA gyrase A and topoisomerase IVAs DNA gyrase A and topoisomerase IV are essential to bacterial DNA replication activities including supercoiling, supercoil relaxation, chromosomal condensation, chromosomal decatenation and more, their inhibition is the principal action of ozenoxacin's mechanism and it has been demonstrated to be bactericidal against *S. aureus* and *S. pyogenes* organisms.[1,2,3]

In revers phase method stationary phase is nonpolar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. It works on the principle of hydrophobic interactions hence the more nonpolar the material is, the longer it will be retained.

Reversed-Phase HPLC is very widely applicable for small molecule analyses (M.Wt. < 2000 Da). A C18 or Octadecylsilane (ODS) column is usually the first choice for method development, although this may differ in certain industries where analytes are chemically very similar (little difference in hydrophobicity). Reversed phase HPLC successfully separates both polar and nonpolar neutral molecules with molecular weights below 2000 Daltons. Even homologs and benzologs can be baseline separated. Homologs may differ from their counterparts by only one carbon unit (methylene, CH_2). Reversed-phase HPLC can be extended to the separation of weak acids and bases with the addition of mobile phase modifiers and buffers to control ionization of the

sample molecules. Stronger acids and bases can be separated using an analog of reversed-phase HPLC - Ion Pair HPLC. Here, an additive is used to bind with the sample ion producing a neutral compound complex for separation. Alternatively stronger acids and bases have been analyzed alongside hydrophobic analytes using mixed-mode chromatography in recent times. Reversed-phase HPLC is a good choice for peptide and protein separations when short chain alkyl stationary phases are used. Here, analysis of compounds with a molecular weight above 2000 Daltons is possible. The separation of amines requires more attention, but can be easily accomplished by the use of additives, pH control, or the use of specially treated columns.[5,6]

MATERIAL AND METHODS

Selection and Procurement of Drug

Drug sample supplier

Table 1: Drug and Drug Supplier.

Name of Drug	Drug Supplier
Ozenoxacin	Swapnroop drug and pharmaceutical

List of reagents & chemicals used

Table 2: List of Reagents and Chemicals used.

Sr. No.	Name of chemicals	Manufacturer.
1.	Acetonitrile (HPLC grade)	Merck Ltd., India
2.	Methanol (HPLC)	Merck Ltd., India
3.	water (HPLC grade)	Merck Ltd., India
4.	0.05% OPA (HPLC grade)	Merck Ltd., India

Selection of formulation

From the literature survey and market survey we selected Maxide formulation for work.

Marketed Preparation

Table 3: List of brand names of combined formulations of Ozenoxacin.

Sr. No	Brand name	Formulation	Available strength	Address of manufacturer
1.	Ozepro	Cream	Ozenoxacin 1% W/W	Macleods pharma ltd

The marketed preparation was obtained from local market and is referred here after in this thesis by the name as such.

Selection of Analytical Technique

HPLC was selected as analytical technique for estimation of Ozenoxacin.

- **Instruments**

The analysis of the drug was carried out on Agilent Tech. Gradient System with Auto injector Equipped with Reverse Phase (Agilent) C_{18} column (4.6mm x 150mm; 5µm), a Quaternary Gradient (G130A) S.NO.DE 9180834) pump, a 20µl injection loop and UV (DAD) G13148 S.NO. DE71365875 Absorbance detector and running CHEMSTATION 10.1software.

- **Stock preparations**
  - Stock I : Standard Sample Preparation

Std .OZENOXACINE 5 mg in 10 ml Methanol = 1000 µgm/ml

  - Stock II : Tab solution Preparation

Take 5mg in 100 ml Methanol i.e. = 50 µgm/ml

- **For Accuracy Solution Preparations**
  - Take 10 µgm/ml Tab Solution For Accuracy,

80 % = 0.1 ml Tab Solution And Add 8µgm/ml Std And Make Up Vol 10 ml With Mobile Phase
100 % =0.1 ml Tab Solution And Add 10 µgm/ml Std And Make Up Vol 10 ml With Mobile Phase
120 %= 0.1 ml Tab Solution And Add12 µgm/ml Std And Std makes Up Vol 10 ml With Mobile Phase

Instruments and Equipments

Table 4: Instrument (HPLC) Details used during Method Development.

	Name of Instrument	Company Name
1	HPLC Instrument	Agilent with auto sampler(DAD Detector) (chem-station)
2	UV-Spectrophotometer	Analytical Technologies Limited
3	Column(C_8)	AgilentC_{18} (150mmX 4.6mm,5µm) :
4	pH meter	VSI pH meter(VSI 1-B)
5	Balance	WENSAR™ High Resolution Balance.
6	Sonicator	Ultrasonic electronic instrument

Experimental Work

Selection of Analytical Technique

HPLC was selected as analytical technique for estimation of Ozenoxacin

Instruments

The analysis of the drug was carried out on Agilent Tech. Gradient System with Auto injector, UV (DAD) & Gradient Detector. Equipped with Reverse Phase (Waters) C18 column (4.6mm x 150mm; 5µm), a 20µl injection loop and UV730D Absorbance detector and running chemstation 10.1 software.

Selection of stationary phase

The column used in this method C_{18}Waters the configuration of the column is 4.6 x 150mm, particle size 5 µm. C_{18} column gives high non polar retentively, symmetric peak shape, highly reproducible and stable ideal for HPLC method.

Solubility Studies

This study was carried out to find an ideal solvent in which drugs are completely soluble. Various solvents were tried for checking solubility of Ozenoxacin. From solubility studies it was concluded that of Ozenoxacin is freely soluble in Methanol and poorly soluble in water PH adjusted 0.05%Orthophosphoric Acid, Buffer pH 3.

Chromatographic conditions

The following chromatographic conditions were established by trial and error and were kept constant throughout the experimentation.

Table 5: chromatographic conditions (HPLC) details used during method Development.

1.	HPLC	Agilent Tech. Gradient System with Auto injector, UV Detector
2.	Software	chemstation 10.1
3.	Column	(Waters) C18 column (4.6mm x 150mm
4.	Particle size packing	5 µm
5.	Stationary phase	C-18 (Waters)
6.	Mobile Phase	Methanol: 0.05 % OPA 60 : 40
7.	Detection Wavelength	307 nm
8.	Flow rate	0.7 ml/min
9.	Temperature	Ambient
10.	Sample size	20 µl
11.	Ph	3
12.	Run Time	15 min
13.	Filter paper	0.45 µm

UV-VIS Spectrophotometer

UV-VIS Spectrophotometer was selected as analytical technique for estimation of Ozenoxacin. UV absorbance range of 200-400nm.

Instrument

Analytical Technologies® Limited UV-VIS Spectrophotometer is double beam, high seed scanning spectrophotometer, The instrument needs about 1minute for initialization. The light source used is Deuterium lamp of spectrophotometer, a computer is attached which helps in data processing and manipulation Quartz curette with path length 1cm was used.

Study on the selection of uv spectrum use in uv-vis spectrometer of Ozenoxacin :

Accurately weigh and transfer 5mg Ozenoxacin working standard into 10 ml Volumetric flask as about dilute Methanol prepared in completely and makes volume up to the mark with the same solvent to get 500µg/ml standard (stock solution) and 15 min sonicate to dissolve it and from the resulting solution 0.5ml was transferred to 10 ml volumetric flask and the volume was made up to the mark with Methanol**. (Fig No: 1)**

Study on the chromatographic conditions of Ozenoxacin

Accurately weigh and transfer 5mg Ozenoxacin working standard into 10 ml volumetric flask as about dilute Methanol prepared in completely and make volume up to the mark with the same solvent to get 500µg/ml standard (stock solution) and 15 min sonicate to dissolve it and from the resulting solution 0.1ml was transferred to 10 ml volumetric flask and the volume was made up to the mark with mobile phase Methanol:(0.05%OPA) Water solvent. The resulting 10µg/ml of solution was subjected to chromatographic analyses using mobile phases of different strengths with chromatographic conditions. Analytical column: Waters C18 Column (150mm x 4.6mm), 5µm particle size.

- Injection volume : 20µl
- Flow rate : 0.7 ml/min
- Detection : 307 nm
- Run Time : 15 min

➢ Following Mobile phase were tried:

Method Development of HPLC

- **List of Mobile Phase**

Table 06: Selection of mobile Phase.

Sr. No.	Mobile Phase
1.	0.1% (OPA) Water+ Methanol (10+90 % v/v)PH-3. Flow Rate 0.7 ml.C18 Column (150mm x 4.6mm), 5µm ,228nm/307 nm
2.	0.1% (OPA) Water+ Methanol (20+80 % v/v)PH-3. Flow Rate 0.7 ml.C18 Column (150mm x 4.6mm), 5µm ,307 nm
3.	0.1% (OPA) Water+ Methanol (30+70 % v/v)PH-3. Flow Rate 0.7 ml.C18 Column (150mm x 4.6mm), 5µm ,307 nm
4.	0.1% (OPA) Water+ Methanol (50+50 % v/v)PH-3. Flow Rate 0.7 ml.C18 Column (150mm x 4.6mm), 5µm ,307 nm
5.	Acetonitrile + 0.1% (OPA)water, (50:50 % v/v) PH-3 Flow Rate 0.7 ml. (C18 Column (150mm x 4.6mm), 5µm,307 nm
6.	Methanol+0.1 %(OPA) water, (60+40% v/v) PH-3 Flow Rate 0.7 ml. (C18 Column (150mm x 4.6mm), 5µm, 307 nm.

Preparation of Stock Standard Solution

Standard Solution Stock I: (Ozenoxacin)

Accurately weight and transfer 5 mg Ozenoxacin working standard into 10 ml volumetric flask as about diluents Methanol completely and make volume up to the mark with the same solvent to get 500µg/ml standard (stock solution) and 15 min Sonicate to dissolve it and the resulting stock solution 0.1ml was transferred to 10 ml volumetric flask and the volume was made up to the mark with mobile phase Methanol: (0.1%OPA)Water, prepared in (6ml MEOH: 4(0.1%OPA) ml WATER v/v)solvent .

Validation of method for analysis of Ozenoxacin:

The developed method was validated as per ICH guidelines.

Analytical Method validation

Analytical method validation was carried out as per ICH method validation guidelines Q2 (R1).

Linearity

Linearity of an analytical method is its ability to elicit test results that are directly or by a well-defined mathematical transformation, proportional to the concentration of analyst in samples within a given range, The Result are shown in; **(Table No 7)**

Determination

The linearity of the analytical method is determined by mathematical treatment of test results obtained by analysis of samples with analyst concentrations across the claimed range. Area is plotted graphically as a function of analyst concentration. Percentage curve fittings are calculated**.** The Result are shown in; **(Table No. 11 and Table No. 12)**

Preparation of standard stock solution for linearity

Average weight of Tablet sample (equivalent to 5 mg of Ozenoxacin) was weighed and transferred to 10 mL volumetric flask &diluents were added to make up the volume. Sonicated for 10 min with occasional swirling. 4 ml of this solution diluted upto 10 ml volumetric flask with diluents was added to make up the volume.

Table 07: Table for linearity.

Sr No.	Concentration (µg/mL)
	Ozenoxacin
1	5
2	10
3	15
4	20
5	25

Accuracy (recovery)

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. Accuracy may often the expressed as percent recovery by the assay of known added amounts of analyst.

The accuracy of an analytical method is determined by applying the method to analyzed samples, to which known amounts of analyst have been added. The accuracy is calculated from the test results as the percentage of analyst recovered by the assay, The Result are shown in; **(Table No: 13, 14)**

Preparation of standard stock solution

5 mg of Ozenoxacin working standards were weighed and transferred to 10 mL volumetric flask &diluents was added to make up the volume 0.1 ml of this solution diluted upto 10 ml with diluents.

Application of proposed method for analysis of Tablet formulation: Accuracy

The accuracy was determined by Ozenoxacin (equivalent to5 mg of Ozenoxacin) (80 %, 100 % and 120 % of the label claimed, respectively) to quantity equivalent to average weight of marketed Tablets. This powder mixture containing 5 mg of Ozenoxacin cream and then subjected to chromatographic analysis using the described method. The resulting mixtures were analyzed

in triplicates over three days. The % recovery of added drug was taken as a measure of accuracy**.**

Repeatability
Precision of the system was determined with the sample. Two replicates of sample solution containing 10µg/ml of Ozenoxacin were injected and peak areas were measured and %RSD was calculated it was repeated for two times result are shown in; **(Table No: 15)**

Precision
Precision of an analytical method is the degree of agreement among Individual test results when the procedure is applied repeatedly to multiple Samplings of a homogenous sample. Precision of an analytical method is usually expressed as standard deviation or relative standard deviation. Also, the results obtained were subjected to one way ANOVA and within-day mean square and between-day mean square was determined and compared using F-test.

Robustness
The mobile phase composition was changed in (±1 ml/ min^{-1}**)** proportion and the flow rate was of Methanol in the mobile phase composition (±1 ml/ min^{-1}**)** and the change in detection wavelength (±1 ml/ min^{-1}**)** and the effect of the results were examined and it was performed using 10µg/ml solution of Ozenoxacin in triplicate. The Result are shown in; **(Table No: 16)**

Detection Limit
Based on the S.D. of the response and the slope of calibration curve, the detection limit (DL) was calculated as,

$$DL = \frac{3.3\sigma}{S}$$

Were,
σ = the S.D. of the y-intercepts of regression lines.
S = the slope of the calibration curve.
The slope S may be estimated from the calibration curve and S.D. was used should be calculated from the y-intercepts of regression line in calibration curve.

Quantitation Limit
Based on the S.D. of the response and the slope of calibration curve, the quantitation limit (QL) was calculated as,

$$QL = \frac{10\sigma}{S}$$

Were,
σ = the S.D. of the y-intercepts of regression lines.
S = the slope of the calibration curve.
The slope S may be estimated from the calibration curve and S.D. was used should be calculated from the y-intercepts of regression line in calibration curve.

Analysis of marketed formulation
To determine the content of Ozenoxacin in marketed cream. 5 mg of Ozenoxacin cream was weighed with 10 mL Methanol. it was sonicated for 15 min. 4 mL of supernatant was then diluted up to 10 mL with mobile phase. The resulting solution was injected in HPLC and drug peak area was noted**.**

Regression equation was generated using peak areas of standard solutions. Using the regression equation and peak area of the sample the amount of Ozenoxacin in the sample was calculated. The amount of Ozenoxacin per ointment was obtained from the regression equation of the calibration curve as described in analysis of ointment formulation **(Table No.16.17).**

Degradation behaviour
Forced degradation studies of both the drugs namely Ozenoxacin were carried out individually and in under different stress conditions like acid hydrolysis, alkaline hydrolysis, hydrogen peroxide oxidation and photolysis. The results are shown in (**Table no: 18)**

Acid hydrolysis
The acid hydrolysis performed using 0.1N HCl at 70 ºC for 1 hr Ozenoxacin indicated degradation. The major degradation products for Ozenoxacin were observed at relative retention time (RRT) of 1 hr (11.61%); These impurities were also detected in the combination of Ozenoxacin. **(Fig no: 08)**

Alkaline hydrolysis
The alkaline hydrolysis condition was performed using 0.1N NaOH at 70 ºC for 1 hr and 2 hr Ozenoxacin. Degradation of Ozenoxacin was found to Ozenoxacin were observed at RRT of (11.01%). (**Fig no:09)**

Oxidation
In the oxidation condition with 3% H2O2 for 1 hr and 2 hr Ozenoxacin show any oxidative stress degradation peak in the chromatogram. The major degradation products for Ozenoxacin were observed at RRT of (14.76%) (**Fig no:10)**

Neutral
There was no major degradation observed for both Ozenoxacin and hence they were not sensitive to light at 70 ºC for 1hr. **(Fig no:11)**

RESULT AND DISCUSSION

Preliminary studies on Ozenoxacin
Melting point: The procured reference standard of Ozenoxacin was found to melt in the range of 255^{0}C respectively.

Solubility: The drug was found to be
- Soluble in Methanol,
- Soluble in water.

UV Spectroscopy
UV absorption of 10 µg/mL solution of Ozenoxacin in methanol was generated and absorbance was taken in the range of 200-400 nm. Λmax

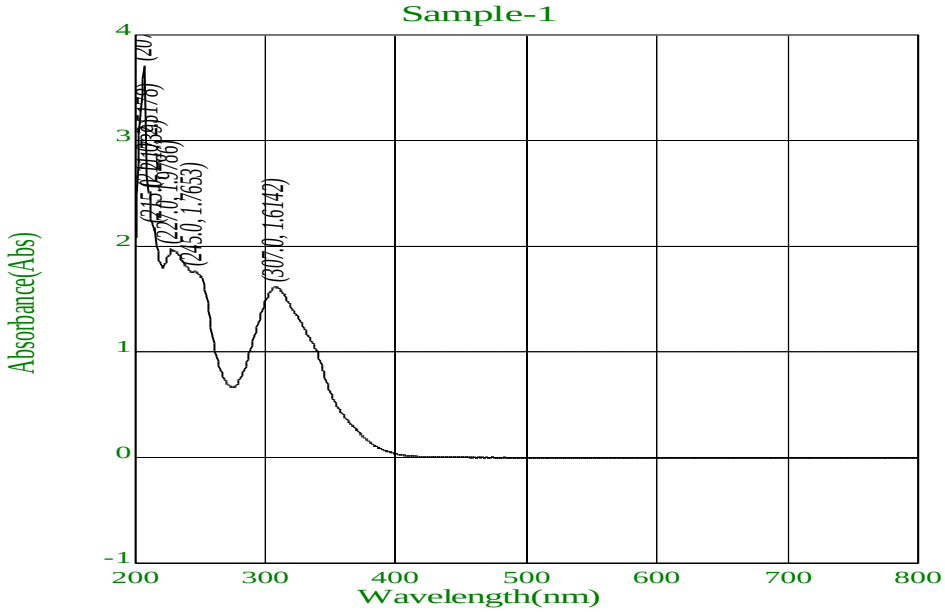

Fig. 1: Uv spectrum of Ozenoxacin.

Standard solutions were scanned in the range of 200-400nm ,against 10 ml methanol and volume make with methanol solvent system as reference Ozenoxacin in methanol was found to be 307 nm selected wavelength is 307 nm**(Figure No:1)**

Studies on the chromatographic behavior of Ozenoxacin.

After the selection of suitable mobile phase, it was then optimized for its reproducibility, sensitivity & accuracy. The optimized parameters for selected method are as below.

Table 8: Different Trials of Chromatographic Condition.

Fig. No.	Column used	Mobile phase, Flow Rate and Wavelength	Inj. Vol.	Observation	Conclusion
1	Agilent C18 (150 ×4.6mm, 5µ)	0.1% (OPA) Water+ Methanol (10+90 % v/v) PH-3. Flow Rate 0.7.307nm	20µl	Sharp Peaks were not obtained	Hence rejected
2	Agilent C18 (150 ×4.6mm, 5µ)	0.1% (OPA) Water+ Methanol (20+80 % v/v) PH-3. Flow Rate 0.7.307nm	20 µl	Sharp Peaks were not obtained	Hence rejected
3	Agilent C18 (150 ×4.6mm, 5µ)	0.1% (OPA) Water+ Methanol (30+70 % v/v) PH-3. Flow Rate 0.7.307nm	20 µl	Sharp Peaks were not obtained	Hence rejected
4	Agilent C18 (150 ×4.6mm, 5µ)	0.1% (OPA) Water+ Methanol (50+50 % v/v)PH-3. Flow Rate 0.7.307nm	20 µl	Sharp Peaks were not obtained	Hence rejected
5.	Agilent C18 (150 ×4.6mm, 5µ)	Acetonitrile + 0.1% (OPA)water, (50:50 % v/v) PH-3 Flow Rate 0.7 ml,307 nm	20 µl	Sharp Peaks were not obtained (HIGH TF)	Hence rejected
6.	Waters C18 (150 ×4.6mm, 2.5µ)	Methanol+ 0.1% (OPA)water(60:40% v/v) Flow Rate 0.7 ml ,307nm	20 µl	Sharp Peaks were not obtained	Sharp peak obtain so selected

Thus, from the above, it has been observed that, using mobile phase of Methanol+0.1% (OPA)water,(60:40 % v/v),PH3.,307nm, Flow rate 0.7 ml gave adequate retention at 3.712 min with good peak shape (Theoretical plates Ozenoxacin 5185).

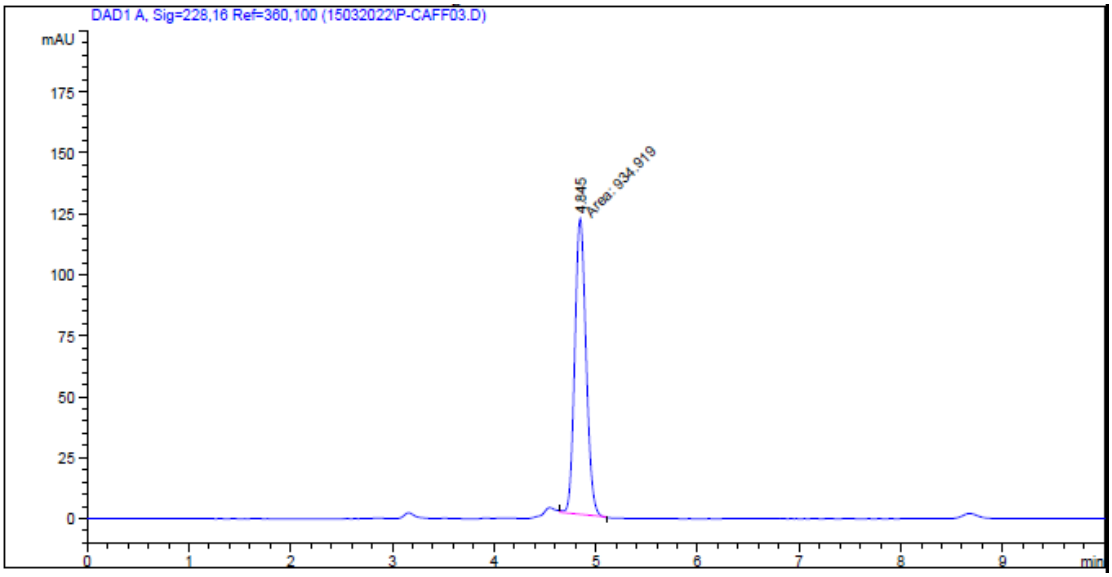

Fig. 02: Chromatogram of Final Trial 6.

Table 09: Result for Chromatogram of Final Trial 6.

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	4.845	934.9187	9556	0.87	0.0000

The final chromatographic conditions selected were as follow

- Analytical column: Waters C_{18} Column (150mm x 4.6mm, 5µm particle size).
- Injection volume: 20µl
- Flow rate: 0.7 ml/min
- Mobile phase: Methanol: water (60: 40 % V/V)
- Detection: 307nm
- Run Time: 15 min

Table 10: Result for standard Chromatogram of Ozenoxacin.

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	3.699	251.6111	5038	0.80	0.0000

Analytical of Method Validation

Linearity: From Ozenoxacin standard stock solution, different working standard solution (5-25µg/ml) were prepared in mobilephase 20µl of sample solution was injected into the chromatographic system using mixed volume loop injector. Chromatograms were recorded.

The area for each concentration were recorded (**Table No. 08**). The Calibration curves are shown in [**Fig. No.04**.]

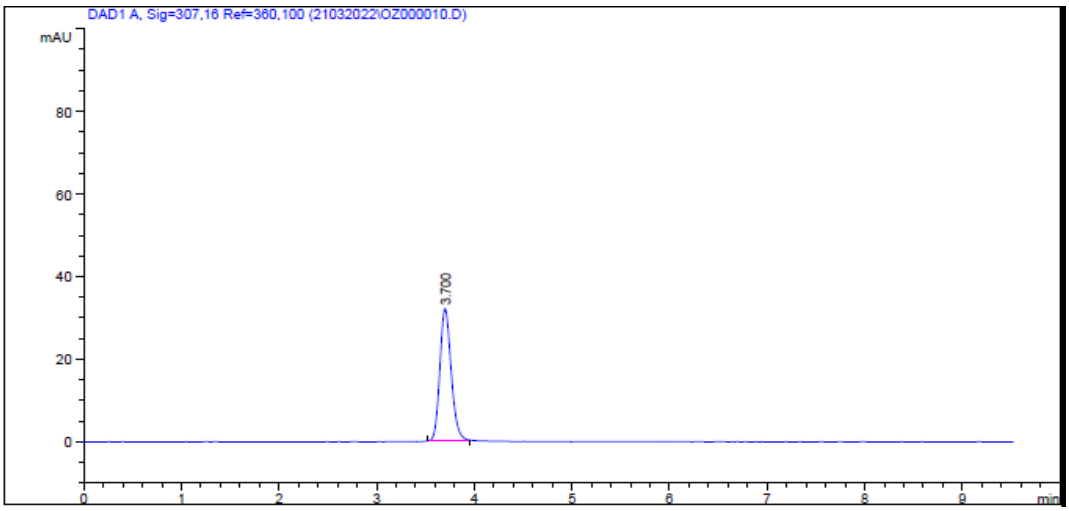

Fig. 03: Chromatogram of linearity.

Table 11: Linearity of Ozenoxacin.

Sr. No.	Concentration µg/ml	Area Ozenoxacin
1	5	65.94
2	10	125.23
3	15	188.93
4	20	252.75
5	25	321.34

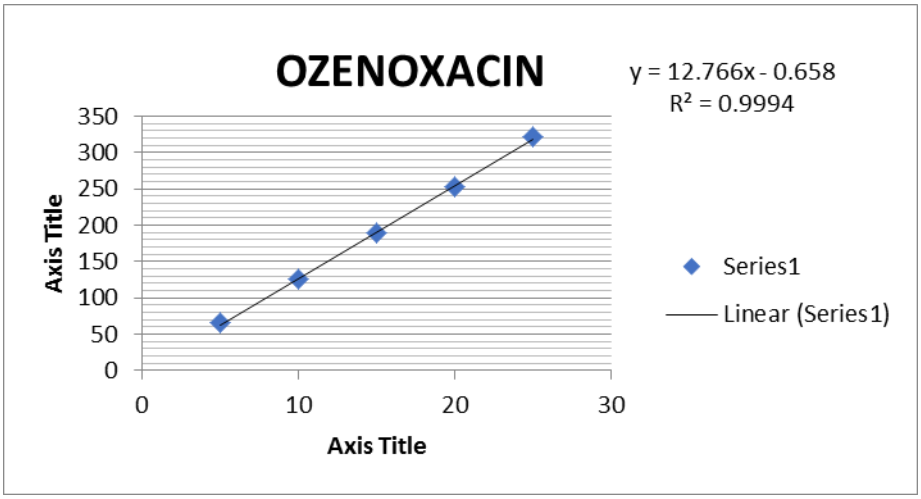

Fig.No.04. Calibration curve of Ozenoxacin

Table No 12. Regression equation data for Ozenoxacin.

Regression Equation Data Y=mx+c	
Slope(m)	12.76
Intercept(c)	0.658
Correlation Coefficient	0.999

Linearity of Ozenoxacin was observed in the range of 5-25 µg/ml. Detection wavelength used was 307nm. (**Table No. 07**)

The calibration curve yielded correlation coefficient (r^2) 0.999 & 0.999 for Ozenoxacin respectively. (**Table. No.08**)

Accuracy:- Recovery studies were performed to validate the accuracy of developed method. To pre analyzed Tablet solution, a definite concentration of standard drug (80%, 100%, and 120%) was added and then its recovery was analyzed (**Table No.09**). Statistical validation of recovery studies shown in (**Table No. 10**)

Table 13: Result of Recovery data for Ozenoxacin.

Drug	Sr No.	Level (%)	Amt. taken (µg/ml)	Amt. Added (µg/ml)	Area.Mean* ± S.D.	Amt. recovered Mean *±S.D.	%Recovery Mean *± S.D.
OZE	1	80%	5	4	9.05± 0.006	4.05± 0.06	101.17±0.16
	2	100%	5	5	10.07±0.04	5.07±0.04	101.49± 0.99
	3	120%	5	6	11.07±0.08	6.07±0.08	101.17± 1.39

***Mean of each 3 reading.**

Table 14: Statistical Validation of Recovery Studies Ozenoxacin.

Level of Recovery (%)	Mean % Recovery	Standard Deviation*	% RSD
80%	101.17	0.006	0.70
100%	101.49	0.049	0.48
120%	101.17	0.083	0.75

***Denotes average of three determinations.**

Accuracy of method is ascertained by recovery studies performed at different levels of concentrations (80%, 100% and 120%). The % recovery was found to be within 98-101% (**Table No. 09, 10**).

System suitability parameters: (Repeatability)
To ascertain the resolution and reproducibility of the proposed chromatographic system for estimation of Ozenoxacin system suitability parameters were studied. The result shown in below **(Table No.11)**

Result for Repeatability (SST)
Chromatogram System Suitability Results was found to be mean of five determinations were also satisfactory, hence the analytical method would be concluded that result shown in (**Table No :15)**

Table 15: Repeatability studies on Ozenoxacin.

Sr. No.	Concentration of Ozenoxacin(mg/ml)	Peak area	Amount found (mg)	% Amount found
1	10	132.580	10.26	102.60
2	10	130.579	10.24	102.58
		Mean	10.25	102.59
		SD	1.41	1.41
		%RSD	1.08	1.08

Repeatability studies Ozenoxacin was found to be, The %RSD was less than 2, which shows high percentage amount found in between 100% to 102% indicates the analytical method that concluded. **(Table No.15)**

Precision
The method was established by analysing various replicates standards of Ozenoxacin. All the solution was analyzed thrice in order to record any intra-day & inter-day variation in the result that concluded. The result obtained for intraday is shown in **(Table No. 16)** respectively.

Chromatogram of Precision

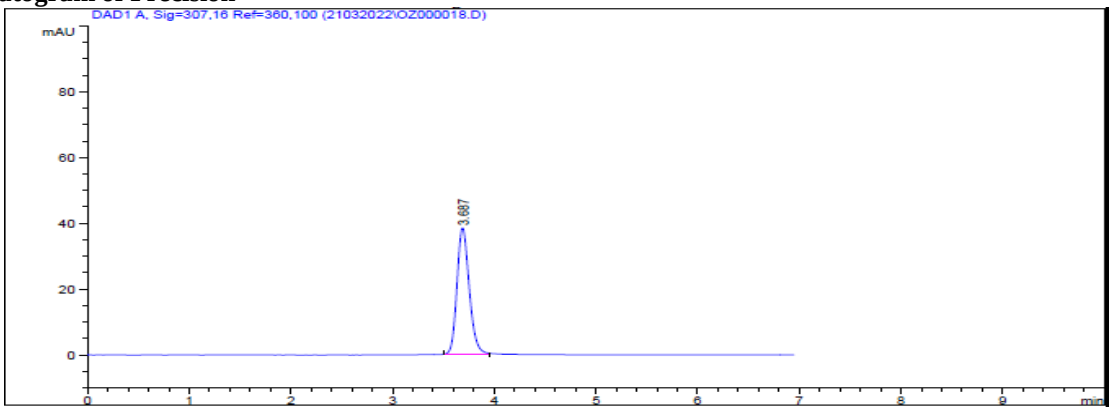

Fig. 05: Chromatogram of Precision.

Table 16: Result of Intraday and Inter day Precision for Ozenoxacin.

Conc^{n} (µg/ml)	Intraday Precision			Interday Precision		
	Mean± SD	%Amt Found	%RSD	Mean± SD	%Amt Found	%RSD
20	691.01±0.96	102.60	0.23	689.78±5.65	98.46	0.64
30	972.97±0.27	98.47	0.028	969.66±0.69	98.12	0.02
40	1313.45±0.49	101.09	0.037	1306.91±1.35	100.57	0.16

***Mean of each 3 reading**

Intraday and Inter day Precision for Ozenoxacin which shows the high precision %amount in between 98% to 100% indicates to analytical method that concluded.

Robustness
The Robustness of a method is its ability to remain unaffected by small deliberate changes in parameters. To evaluate the robustness of the proposed method, small but deliberate variations in the optimized method parameters were done. The effect of changes in mobile phase composition and flow rate, wavelength on retention time and tailing factor of drug peak was studied.

The mobile phase composition was changed in (±1 ml/min^{-1}**)** proportion and the flow rate was varied by of optimized chromatographic condition. The results of robustness studies. Robustness parameters were also found satisfactory; hence the analytical method would be concluded.

Flow Rate Chang 0.8 ml

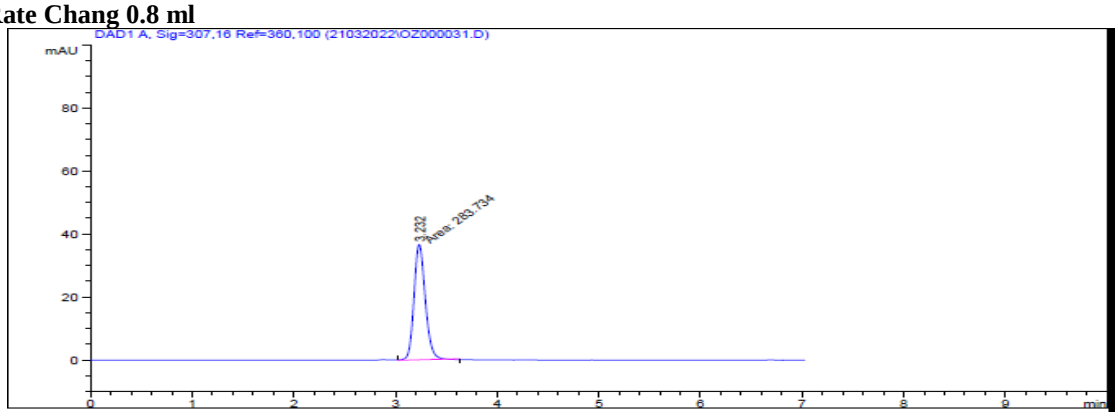

Fig. 06: Chromatogram of Flow rate change 0.8 ml.

Mobile phase composition Change: 61ml Meoh+ 0.05%(OPA)39ml Water
Wavelength Change 306 nm

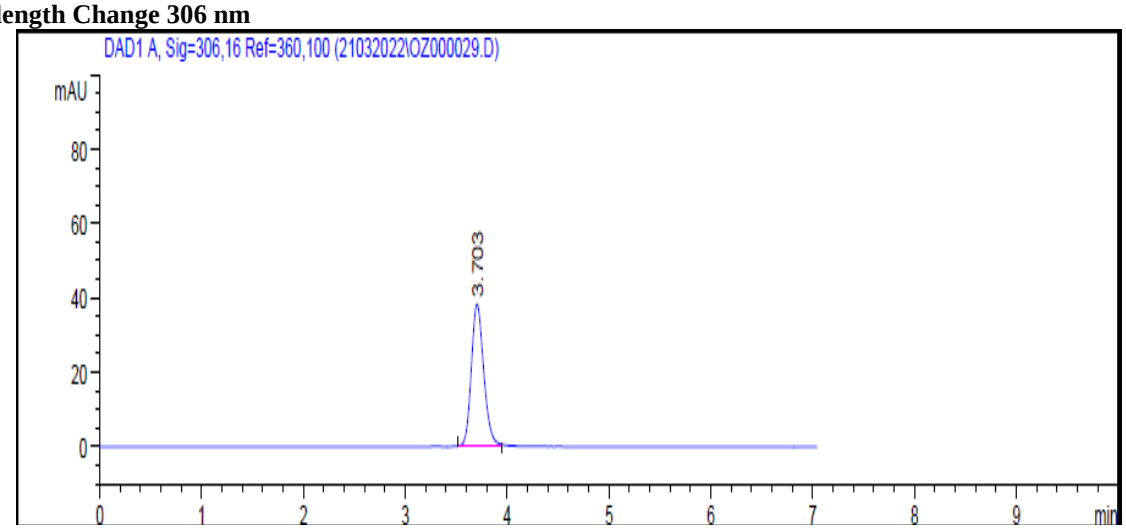

Fig. 07: Chromatogram of comp change wavelength change 306 nm.

Table No.17 Result of Robustness Study of Ozenoxacin.

Parameters	Conc.(µg/ml)	Amount of detected (mean ±SD)	%RSD
Mob-phase composition(61ml+39ml) Methanol + 0.05% (OPA)water	25	313.6±2.17	0.69
Mob-phase composition(59ml+41ml) Methanol + 0.05% (OPA)water	25	323.56±0.69	0.21
Wavelength change306nm	25	325.6±2.17	0.69
Wavelength Change 308nm	25	334.59±0.76	0.23
Flow rate change(0.6ml)	25	385.±1.18	0.30
Flow rate change(0.8ml)	25	284.03±0.42	0.15

Robustness Study of Ozenoxacin

The changes were doing flow rate (±1 ml/ min^{-1}), PH of mobile phase composition, and Wavelength. %RSD for peak area was calculated which should be less than 2%.the result shown in analytical method that concluded.(**Table No.23)**

Limit Detection

The LOD is the lowest limit that can be detected. Based on the S.D. deviation of the response and the slope The limit of detection (LOD) may be expressed as:

LOD = 3.3 (SD)/S

= 3.3 X 0.82 / 12.76

= 0.2133

Where, SD = Standard deviation of Y intercept

S = Slope

The LOD of Ozenoxacin was found to be 0.2133 (µg/mL) analytical methods that concluded.

Limit Quantification

The LOQ is the lowest concentration that can be quantitatively measured. Based on the S.D. deviation of the response and the slope,

The quantitation limit (LOQ) may be expressed as:

LOQ = 10 (SD)/ S

=10 X 0.82 / 12.76
= 0.6465

Where, SD = Standard deviation Y intercept
S = Slope
The LOQ of Ozenoxacin was found to be 0.6465 (µg/mL) analytical methods that concluded.

Analysis of Tablet formulation
Procedure
Weigh 20 Ozenoxacin Tablets and calculated the average weigh, accurately weigh and transfer the sample equivalent to 15.75 mg Ozenoxacin into 10 ml volumetric flask. Add about 10ml of diluents and sonicate to dissolve it completely and make volume up to the mark with diluent. Mix well and filter through 0.45 µm filter. Further pipette 0.3ml of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluents. (20 µg/ml). The simple chromatogram of test Ozenoxacin Shown in the amounts of Ozenoxacin per Tablet were calculated by extrapolating the value of area from the calibration curve. Analysis procedure was repeated five times with Tablet formulation. Tablet Assay for %Lable claim for %RSD Calculated.

Brand Name: Ozepro 5 gm
Take 5 mg in 100 ml methanol -------**50 *µgm/ml***
1) Take 4 from stock II. and make vol. with mobile phase 10 ML = 20 µgm/ml OZENOXACIN

Table.18. Analysis of marketed formulation.

Sr.no	Amount present in mg	Area(I)	Amount found in mg	% Label claim
	OZE	OZE	OZE	OZE
1	20	261.595	20.44961	102.25
2	20	260.913	20.39618	101.98
Mean	-	261.25	20.42	102.11
SD	-	0.482	0.038	0.189
%RsD	-	0.185	0.185	0.185

Analysis of marketed formulation were also % Label Claim was found to be 99-101% Satisfactory are concluded**.**

Tablet Assay for % Label Claim
Table 19: Tablet for %Label claim.

Sample	Label claimed	%Label claimed± SD	%RSD
Ozepro	Ozenoxacin 1% W/W	102.11± 0.18	0.18

Tablet Assay for % Label claim for were also was found to be 102.11% and %RSD are less than 2 satisfactory result that concluded. (**Table No15**).

Force of Degradation
Degradation behavior

Forced degradation studies of both the drugs namely Ozenoxacin were carried out individually and in combination under different stress conditions like acid hydrolysis, alkaline hydrolysis, hydrogen peroxide oxidation and photolysis.

Table 20: Degradation of different stress condition.

Sr no	Degradation parameter	%Degradation
1	Alkali DEG. 0. 1 N NAOH - AFTER 60 40mcg	11.01
2	Acid DEG.0. 1 N HCL- AFTER 60min 40mcg	11.61
3	3% H202 DEG AFTER 60min -40mcg	14.76
4	Neutral After 60 min -40mcg	0.87

Alkaline hydrolysis
The alkaline hydrolysis condition was performed using 0.1N NaOH at 70 ºC for 1hr Ozenoxacin. Degradation of Ozenoxacin. The major degradation products for Ozenoxacin were observed at 1 hr % degradation 11.61%.

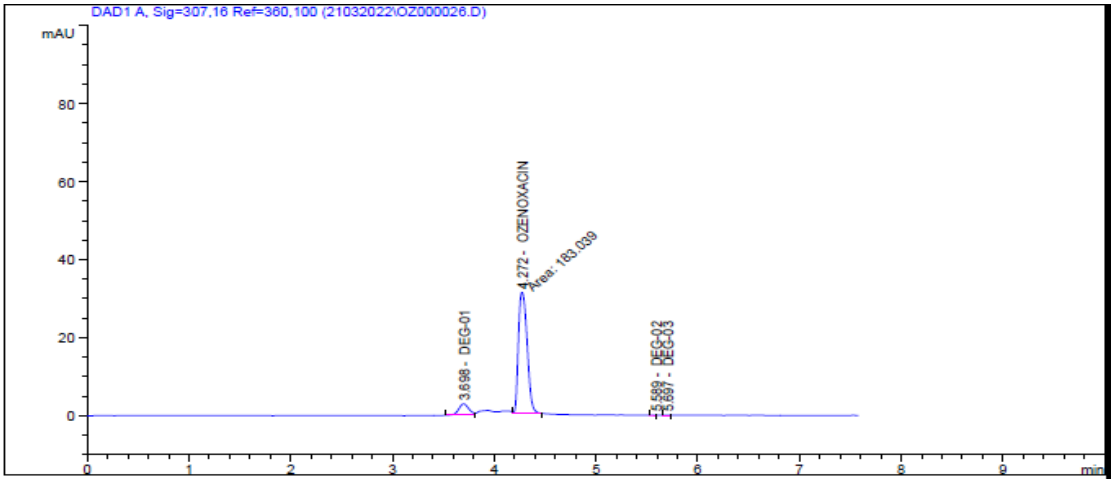

Fig. 08: Degradation for 0.1N NAOH at 60 min.

Acid hydrolysis

The acid hydrolysis performed using 0.1N HCl 1hr min for all three Ozenoxacin indicated degradation. The major degradation products for Ozenoxacin were observed at 1 hr % degradation 11.01%. The degradation product was observed at RRT of 1 hr. These impurities were also detected in Ozenoxacin.

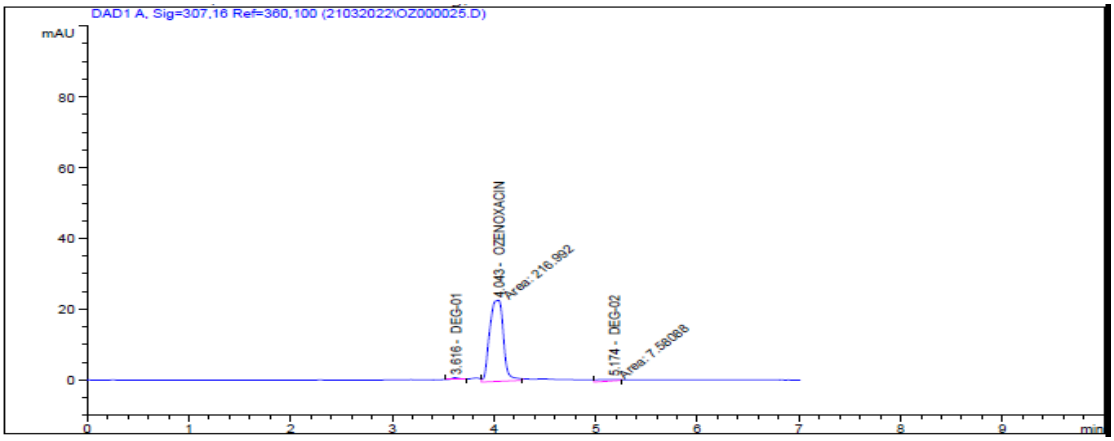

Fig. 09: Degradation for 0.1N HCL at 1 hr.

Oxidation

In the oxidation condition with 3% H_2O_2 for 1 hr, Ozenoxacin did not show any oxidative stress degradation peak in the chromatogram observed at 1 hr % degradation (1 hr for 14.76%).

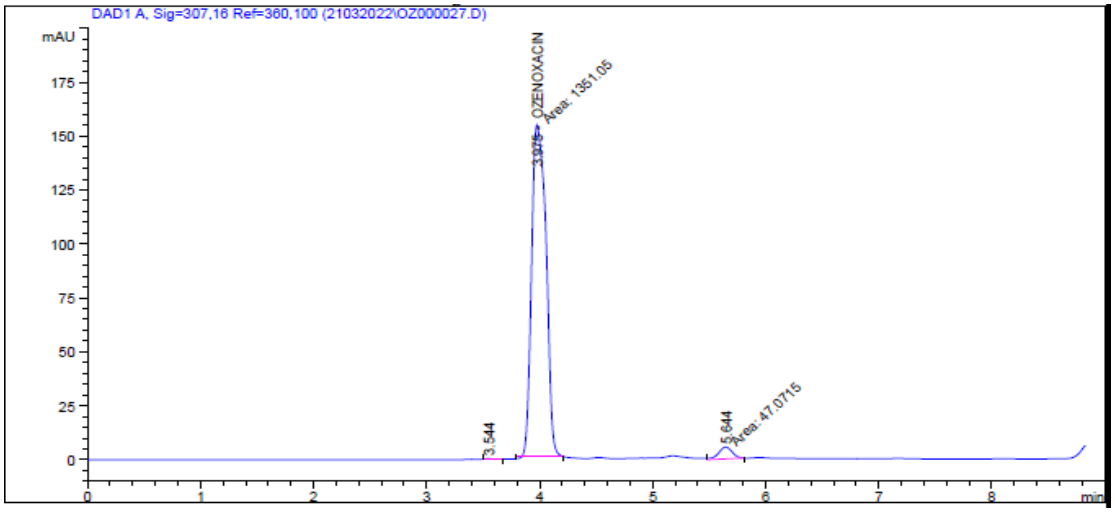

Fig 10: Degradation for 3% H2O2 at 1 hr.

Neutral Studies

There was no major degradation observed for both Ozenoxacin and hence they were not sensitive to light.

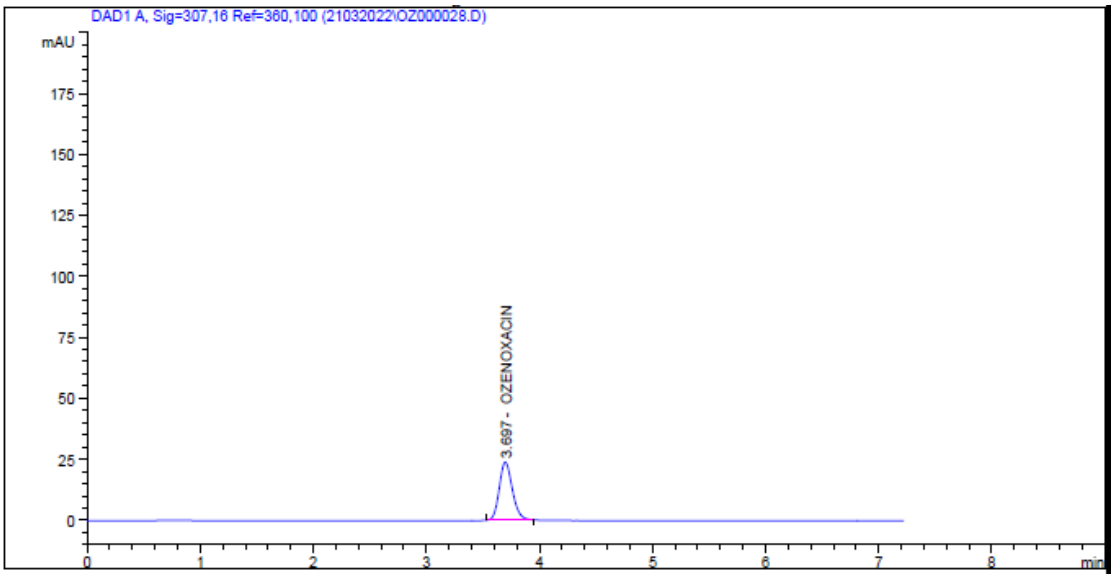

Fig. 11: Degradation for neutral at 1hr.

CONCLUSIONS

A novel RP-HPLC method was developed for the separation and quantification of Ozenoxacin and its related degradation impurities in its pharmaceutical dosage forms. Degradation behavior of Ozenoxacin was studied under various degradation conditions. Unknown degradation impurity of 14.76% was formed from Ozenoxacin in oxidation degradation and no degradation peaks were observed in other stress conditions. All the known degradation impurities and the unknown degradation impurities were well separated from Ozenoxacin revealing the stability indicating capability of the method. The developed method can be used for the quantification of related substances of Ozenoxacin in routine analysis.

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Conflict of Interest

The authors have declared no conflict of interest.

Abbreviations: RP-HPLC: Reverse Phase High performance liquid chromatography; OZE: Ozenoxacin. QbD: Quality by Design; ICH: International conference on harmonization; RSD: Relative standard deviation; SD: Standard deviation; LOD: Limit of detection; LOQ: Limit of Quantitation; Rf: Retention factor; SGLT2: Sodium-glucose co-transporter 2; API: Active pharmaceutical ingredient; UV: Ultraviolet.

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