International Journal for Multidisciplinary Research (IJFMR)
E-ISSN: 2582-2160 ● Website: www.ijfmr.com ● Email: editor@ijfmr.com

To Study Force Degradation of Ozenoxacin Using RP-HPLC

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ABSTRACT

In this study a simple, robust and accurate RP-HPLC was developed and validated for the force degradation study of ozenoxacin as per ICH guidelines. Chromatographic separation and elution of peak was achieved by isocratic elution on agilant – 1260 infinite II HPLC system. An analysis C18X150X4.6mm 5mm, eclipse XDB column was used at flow rate 1ml/min in the mobile phase ratio methanol: 10 mM (adjusted pH -3 in the ratio of 42:52 %v/v at wavelength 250 nm and the temperature 25ºc. The force degradation study was performed on ozenoxacin under acidic, basic, oxidation, thermal, photodegradation,. The result obtained from the experiments was proved that the developed method suitable for routine analysis.

Keywords: Ozenoxacin, Agilant – 1260 Infinite II HPLC System, Force Degradation, Chromatographic separation

INTRODUCTION

Stability Indicating Assay Method (SIAM)

The active pharmaceutical ingredients (API) in the formulation, processing and storage may expose to variety of environmental condition like heat, humidity, light, etc. and may undergo degradation. This would lead the contamination of product with its degradation products, thus adversely affecting therapeutic efficacy and safety of drug products (DP). Therefore the stability-studies of API and its formulation is an utmost important aspect of formulation development so as to minimize its degradation and also to establish appropriate storage condition**.**

Regulatory authorities mainly emphasize on stability of drug in dosage form, as it exposed to various environmental conditions. The term drug stability refers to extent to which API and DP retain same properties and characteristics present at the time of manufacturing, within specific limits, throughout the period of storage. Hence it is necessary to develop a method that can establish shelf life of drug product, determine the level of certain specifications and set controlled limits for different chemical entities of drugs, and to support stability of drug used in clinical and non-clinical studies. Such method in pharmaceutical analysis is known as stability indicating assay method (SIAM)[(1)]. The knowledge gained from stress testing can be useful for-

1. the development of stable formulation and appropriate packaging design,
2. controlling of manufacturing and processing parameters,
3. identification and isolation of toxic degradants during API synthesis,
4. recommendation of appropriate storage conditions and shelf life determination, and
5. designing and interpreting environment studies as the degradation of the drug the environment will

often be similar degradation observed during stress-testing studies.

The pharmaceutical industry is required to establish the identity and purity of all marketed drug products. Drug regulatory authorities and International Conference on Harmonization (ICH) recommended that the impurities should be isolated and characterized in drug substances and drug products, when present at the threshold levels. The identification of process related impurities and degradation products can provide insight to production of impurities and degradation mechanism.

The ICH guidelines explicitly require, conducts of forced decomposition studies under a variety of conditions like extreme pH, light, oxidation, dry heat, etc. and separation of drug from degradation products. A large number of such SIAM are reported for single ingredient drug products, however most of them fall short in meeting the current regulatory requirements.

The various ICH guidelines have been incorporated to carry the effect of regulation. The ICH guideline Q1A on Stability Testing of New Drug Substances and Products[(2)] emphasizes on testing the changes which occur during storage and are likely to influence quality, safety, and/or efficacy. It is also mentioned that forced decomposition studies at temperatures in 10 ºC increments above the accelerated, extremes of pH and oxidative and photolytic conditions should be carried out on API, so as to establish the inherent stability characteristics and degradation pathways to support the suitability of the proposed analytical procedures. The ICH guidelines Q3B entitled

Impurities on New Drugs Products‟ emphasizes on documented evidence for validation of analytical procedures and suitability of the method for detection and quantitation of degradation products[(3)]. The ICH guidelines Q6A, which provide note for guidance on specification[(4)], also mention the requirement of stability-indicating assay under universal tests/criteria for both API and DP. The ICH guideline Q5C deals with Stability Testing of Biotechnological/ Biological Products[(5)].

Unfortunately, none of the ICH guidelines provide an exact definition of a Stability Indicating Method. Elaborate definition of Stability Indicating Methodology is provided by US-FDA stability guidelines[(6)] of 1987. The new US-FDA draft guidelines[(7)] of 1998, have recommended the major changes with respect to (i) the requirement ofvalidation, and (ii) the analysis of degradation products and other components, apart from the active ingredients(s). The definition in the draft guideline of 1998 reads as: *"validatedquantitative analytical methods that can detect the changes with time in the chemical, physical, or microbiological properties of drug substance and drug product, and that are specific so that the contents of active ingredient, degradation products, and other components can be accurately measured without interference."*

Even the United States Pharmacopoeia (USP) has a requirement listed under stability studies in manufacturing‟, which says that samples of the products should be assayed for potency by the use of a Stability-Indicating Assay Method.[(8)]

Stability Testing: Regulatory Considerations

The current regulatory guidance governing forced degradation studies of pharmaceuticals are extremely general. They itemize broad principles and approaches with few practical instructions. There is no single document that comprehensively addresses issues related to stress studies such as objectives, timing, selection of stress conditions, and extent of degradation[(9)]. As the ICH guidelines reflect the current inspectional tendencies, they carry the de facto force of regulation. The ICH guideline Q1A on Stability Testing of New Drug Substances and Products[(10)] emphasizes that the testing of those attributes which are susceptible to change during storage and are likely to influence quality, safety and /or efficacy must

be done by validated stability-indicating testing methods. The ICH guideline Q3B entitled „Impurities in New Drug Products" emphasizes on providing documented evidence that analytical procedures are validated and suitable for the detection and quantitation of degradation products[11]. It is also required that analytical methods should be validated to demonstrate that impurities unique to the new drug substance do not interfere with or are separated from specified and unspecified degradation products in the drug product. The ICH guideline Q6A, which provides note for guidance on specification[12] also mentions the requirement of stability-indicating assays under Universal Tests/criteria for both drug substances and drug products. The same is also a requirement in the guideline Q5C on Stability Testing of Biotechnological/Biological Products.[13]

Strategy for Stress Studies

Stability studies should include testing of those attributes of the drug substance that are susceptible to change during storage and are likely to influence quality, safety, and/ or efficacy. The testing should cover, as appropriate, the physical, chemical, biological, and microbiological attributes. Validated stability indicating analytical procedure should be applied. Whether and to what extent replication should be performed will depend on the results from validation studies.[20]

The overall strategy for developing a stability indicating method using stress testing has been expressed in Fig. 1. These studies are typically performed on both the drug substance and the formulated product.[21]

Fig. 1: Overall strategy for the prediction, identification, and control of stabilityrelated issues

Intrinsic Stability

ICH defines stress testing as an investigation of the "intrinsic stability" characteristics of the molecule. The concept of "intrinsic stability" is critically based on following aspects:

- Conditions leading to degradation (Degradation mechanisms)
- Rates of degradation (Relative or Otherwise)
- Structures of the major degradation products
- Pathways of degradation

Once these four areas have been investigated and understood, stability related issues can be identified or predicted.

Conditions Leading to Degradation (Degradation Mechanisms)

Reynolds *et al*. [22] described stress testing should include conditions that examine specifically for four main pharmaceutically relevant degradation mechanisms: (a) hydrolytic, (b) oxidative, (c) thermolytic, and (d) photolytic. The potential for these degradation pathways should be assessed in DS and DP (and/or drug - excipient mixtures). These mechanisms can be assessed in a systematic way by exposure to stress conditions of heat, humidity, photo stress (UV and VIS), oxidative conditions, and aqueous conditions across a broad pH range. [23]

Hydrolytic degradation

Drug degradation that involves reaction with water is called hydrolysis. As water is present at significant levels in many drugs (e.g., hydrates), in many excipients, and even at normal atmospheric conditions, it is not surprising that hydrolysis is a common degradation problem. Hydrolysis is affected by pH, buffer

salts, ionic strength, solvent, and other additives such as complexing agents, surfactants, and excipients. (24)

Hydrolysis reactions are typically acid or base catalyzed. Acidic, neutral, and basic conditions should therefore be employed in order to induce potential hydrolytic reactions. This is especially important when the compound being tested has an ionizable functional group(s) and can exist in different ionization states under relevant aqueousconditions. It is particularly important to be sure to test hydrolysis at unique protonation states, unless there are a large number of ionizable functional groups as is often the case with peptides and proteins. In such cases, a practical approach is to simply expose the sample to a wide pH range in defined increments (e.g., 1 pH unit).

A major challenge in designing hydrolytic stress tests is compound solubility. Many small molecule drugs are not soluble in water at the concentrations typically used for analytical evaluation (i.e., 0.1 - 1 mg/ml) across the entire pH range. Thus, either a slurry/suspension must be used to examine the hydrolytic stability of a compound or a co-solvent must be added to facilitate dissolution under the conditions of low solubility. The two most commonly used co-solvents are acetonitrile and methanol. Because methanol has the potential of participating in the degradation chemistry (e.g., acting as a nucleophile to react with electrophilic sites or intermediates in the degradation pathways), it should be used with caution (especially under acidic conditions) if the compound being tested contains a carboxylic acid, ester, or amide as these groups may react with methanol. Acetonitrile is generally regarded as an inert solvent and is typically preferable to methanol in hydrolytic stress-testing studies. However, acetonitrile is not completely inert and can participate in the degradation reactions leading to artifactual degradation results. For example, acetonitrile is known to contribute to base-catalyzed epoxidation reactions in the presence of peroxides. Acetonitrile will also degrade, in the presence of base (e.g., pH 13) and/or acid (e.g., pH 1) under elevated temperatures, to detectable levels of acetamide and/or acetic acid, which can show up as early eluting peaks (when monitoring at low wavelengths) on RP-HPLC. In the presence of radicals [e.g., generated during prolonged sonication as part of the analytical workup or in the presence of free radical initiators such as 2, 2-azobisisobutyronitrile (AIBN)], acetonitrile can be oxidized to small amounts of formyl cyanide that will readily react with nucleophiles (such as amines), resulting in a "formylation" reaction. Other co-solvents recommended for hydrolytic studies are shown in Table No. 3 (25)

Table No.3: Organic co-solvent that have been used for stress testing studies

Acidic pH	Neutral pH	Basic pH
Acetonitrile [a]	Acetonitrile [a]	Acetonitrile [a]
DMSO	NMP(N-Methylpyrrolidone)	DMSO
Acetic Acid	-	Glyme[a] (Glycol ethers)
Propionic Acid	-	Diglyme (Glycol diethers)
-	-	p-Dioxane

[a] Volatile solvent : may evaporate at higher temperatures

Co-solvents have potential to affect the degradation rates and pathways. The degradation reactions and rates involved will depend on a variety of factors such as the dielectric constant, solvent polarity, ionic strength, whether or not the solvent is protic or aprotic, the surface energy (i.e., of the solid—liquid interface in a slurry/ suspension), etc. For example, a degradation reaction involving acid-catalyzed

hydrolysis with a cationic intermediate or a polarized transition state will be facilitated by a solvent with a high dielectric constant, and the addition of a co-solvent that reduces the effective dielectric constant will reduce the rate of such a reaction. Solvation of a compound in an aqueous co-solvent mixture may involve formation of a "solvent cage" of the more non-polar solvent around the compound, potentially leading to some protection from hydrolysis. Solvent composition can also affect tautomeric states of molecules, which in turn can affect both degradation rates and pathways. The effective pH of an aqueous solution will also change upon addition of a co-solvent, which can both affect the degradation rate and change the degradation pathway(s) e.g., by facilitating different protonation states.[26]

In conclusion, testing of the hydrolytic susceptibility of a DS should involve exposure to acidic, neutral, and basic conditions in the pH range of 1-13, preferably in 100% aqueous conditions. Elevated temperatures with an upper limit of 70˚C are recommended for accelerating the hydrolytic reactions. Higher temperatures can be used, but the risk of non-Arrhenius behavior increases significantly and leads to unpredictable degradation pathways. The longest recommended time period for stressing at the highest temperature is 2 weeks, although longer times can certainly be used if desired.

Oxidative degradations

Oxidative reactions along with hydrolysis are the two most common mechanisms of drug degradation. Oxidative drug degradation pathways reactions are typically autoxidative, which is radical initiated. Radical-initiated reactions start with an initiation phase involving the formation of radicals (rate-limiting step), followed by a propagation phase and eventually a termination phase. The nature of oxidative reaction is complex. Oxidative intermediates are often thermally unstable and may decompose via alternate pathways at elevated temperatures. Increase in temperature, therefore, may not lead to predictable changes in degradation rates, and the observed oxidative rates and pathways may be different than those observed at lower temperatures. In solution, oxidative rates and pathways may be dependent on the dissolved oxygen concentration. Thus, the reaction rate in solution may actually be reduced at higher temperatures because of the decrease in oxygen content of the solvent. This may be partially overcome by bubbling oxygen or air through the solution while heating or by storing the solution under oxygen in an airtight vessel with high pressure (at least a few atmospheres). The susceptibility to oxidative degradation can be studied in solution using a radical initiator (e.g., AIBN, 40˚C, up to 1 week) and exposure to hydrogen peroxide (e.g., 0.3% hydrogen peroxide, up to 1 week at room temperature, in the dark) in separate studies. As both of these oxidative susceptibility studies are in solution, it may be useful to control the pH such that all relevant protonation states of the drug are tested. The oxidative tests could be carried out at 1 pH unit above and below of the compound being tested. Room temperature storage is sufficient for the hydrogen peroxide test. The use of higher temperatures (e.g., >30˚C) with hydrogen peroxide should be done with caution because the O-O bond is a weak bond that will readily cleave at elevated temperatures to form hydroxyl radicals, a much harsher oxidative reagent. The use of transition metals [e.g., copper (II) and iron (III) at 1-5 mM, 1-3 days] is also recommended for evaluation of oxidative susceptibility.[28]

Thermolytic degradation

Thermolytic degradation is usually thought of as degradation caused by exposure to temperatures high enough to induce bond breakage, that is, pyrolysis. Thus, any degradation mechanism that is enhanced at elevated temperatures can be considered a "thermolytic pathway".

Thermolytic pathways may lead to hydrolysis/dehydration, isomerization/epimerization, decarboxylation, rearrangements and some kinds of polymerization reactions. Hydrolytic reactions are actually a subset of thermolytic pathways. The ICH Stability guideline suggests studying the effect of temperatures in 10°C increments above the accelerated temperature test condition (i.e., 50 °C, 60 °C, etc.). It is not clear why the guideline suggests 10˚C increments, but it may be related to the importance of understanding whether or not any degradation (in the solid state) mechanism change as a result of increasing temperature. Studies with such temperature increase would be useful for constructing Arrhenius plots to allow prediction of degradation rates in the solid state at different temperatures. Waterman and Adami [29] have asserted that the relative humidity under which a solid drug product is stored is a critical variable when attempting to use the Arrhenius relationship. He showed evidence that degradation rate of formulated products (with pathways involving hydrolytic or oxidative degradation) often hold to the Arrhenius relationship if the relative humidity is held constant at the different elevated temperatures. Based on the literature and kinetic considerations, temperature up to 70 °C (at high and low humidity) should provide a rapid, reasonably predictive assessment of the solid-state degradation pathways and relative stabilities of most drug substances at lower temperatures.

Photolytic degradation

Photolytic degradation is the degradation that results from exposure to ultraviolet or visible light in the wavelength range of approximately 300-800 nm. Exposure to radiation at wavelengths <300nm is not needed because a pharmaceutical compound would not experience such exposure during its life cycle. For photolytic degradation to occur, radiation must be absorbed either by the drug substance or by the formulation. Photo degradation rates are therefore directly dependent on the amount of incident radiation and on the amount of radiation that is absorbed by the compound or the formulation. It is important to remember that a compound may undergo photolytic degradation even if it does not itself absorb radiation in the UV or visible region. This can only happen if there is some additional agent in the formulation, intentionally or adventitiously present, that facilitates absorption.

The ICH photostability guideline (Q1 B) refers to both forced degradation studies (stress testing) and confirmatory testing. As confirmatory photostability testing is designed to be a part of the definitive, formal stability testing, it can be thought of as being analogous to an accelerated stability study. Thus, the minimum recommended exposure outlined in Q1B (i.e., 1.2 million lux-hr visible and 200W-hr/m^2 UV) is not the exposure recommended for forced degradation studies. In fact, there is no mention of recommended exposures for forced degradation studies and the design is left open. A photo exposure in the range of three to 10 times the confirmatory exposure seems a reasonable amount of photostress for forced degradation studies, remembering that photo degradation, of the compound being studied, beyond 20-30% would not be necessary or desired. It should be remembered that photo degradation products formed under stress conditions (i.e., "potential" photo degradation products) may not always be observed under confirmatory conditions. Such differences may be exacerbated by the use of different photon sources for stress testing and confirmatory studies.[30 , 31]

The stress decision trees

The decision trees are constructed for investigating different types of stress conditions for a new drug substance. The general approach taken in the construction of these flow charts is that the new drug is assumed to be labile in nature and, accordingly, it is subjected to stress conditions given for labile

substances. Dependent upon the results, decision is taken on whether to increase or decrease the strength of the reaction conditions. The increase or decrease, if required, is done step-wise and those stress conditions are accepted wherever a sufficient decomposition is obtained.

Depending upon the information of degradation chemistry, the following six classes can be identified. (32)

- Extremely labile
- Very labile
- Labile
- Stable
- Very stable

Category of Drug	Time of exposure	Temperature	Extent of decomposition
Practically stable	5 days	Refluxing	None
Very stable	2 days	Refluxing	Sufficient
Stable	1 day	Refluxing	Sufficient
Labile	12 h	Refluxing	Sufficient
Very labile	8 h	40°C	Sufficient
Extremely labile	2 h	25°C	Sufficient

- Practically stable

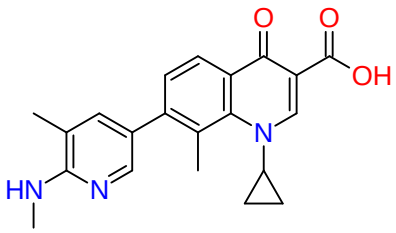
Table No.4: Classification system for acidic or alkaline

Category of Drug	Strength of acid/alkali	Time of exposure	Temperature	Extent of decomposition
Practically stable	5 N	2 days	Refluxing	None
Very stable	2 N	1 day	Refluxing	Sufficient
Stable	1 N	12 h	Refluxing	Sufficient
Labile	0.1 N	8 h	Refluxing	Sufficient
Very labile	0.01 N	8 h	40°C	Sufficient
Extremely labile	0.01 N	2 h	25°C	Sufficient

Table No.5: Classification system for hydrolysis under neutral conditions

DRUG PROFILE OF OZENOXACIN(35)

OZENOXACIN



Structure

IUPACName:1-cyclopropyl-8-methyl-7-[5-methyl-6-(methylamino) pyridin-3-yl]-4-oxoquinoline-3-carboxylic acid

Molecular formula: $C_{21}H_{21}N_3O_3$

Molecular weight: 363.4 g/mol

PK Value:A- [Strongest acidic 6.1]

B- [Strongest basic 6.77]

Description: Pale yellow crystalline solid powder

Solubility:

Soluble- 0.1 N HCL, 0.1N NaOH

Insoluble- Acetonitrile, water, methanol

Slightly soluble- Chloroform

Category: Antimicrobial non-fluorinated quinolone Antibiotic

LITERATURE REVIEW:

Santhosh Paudel*et al.* (2023) [36]have reported the determination of Ozenoxacin in API and pharmaceutical dosage form. Chromatogram was run through Azilent C18 150 x 4.6 mm, 5µm. Mobile phase containing 0.1N NH_2PO_4: Acetonitrile taken in the ratio 60:40was pumped through column at a flow rate of 1.0ml/min. Temperature was maintained at 30°C. Optimized wavelength selected was 314.0nm. Retention time of Ozenoxacin was found to be 2.429 min.

Amarnath Reddy Ramireddy*et al.* (2022)[37]have reported simultaneous estimation of Ozenoxacin and Benzoic Acid in a pharmaceutical cream formulation, according to theICH guidelines. Chromatographic separation was achieved by gradient elution, on RP-HPLC Instrument, equipped with column C8 (150 mm × 4.6 mm, 5 µm particle size) using Ultra Violet (UV) detector at 235 nm wavelength, by using Mobile Phase A: triethylamine, trifloroacetic acid and water (1:1:1000) and Mobile Phase B: methanol and Diluent: water, acetonitrile and triethylamine (500:500:1), at flow rate 1 ml/min, injection volume of 20 µL, column oven temperature 45°Cand run time: 15 min.Forced degradation of Ozenoxacin cream 1% w/w formulation was performed and found that validated method has stability indicating potential that needs to be further studied.

Benjamin Santos *et al.*(2014)[38]have reportedin vitro percutaneous absorption and metabolism of Ozenoxacin in excised human skinIn vitrostudies using excised human skin samples were conducted to evaluate the percutaneous absorption and skin metabolism of Ozenoxacin. The formulations studied were 1% ointment, 1% cream and 2% cream. Permeation assays met the conditions for infinite dose experiments. In all but one case, Ozenoxacin concentrations in receptor fluid samples of Franz diffusion cells were below the limits of quantification (0.04 µg/ml) by liquid chromatography/mass spectrometry/electrospray ionization at the designated time points. Across all four absorption studies, ≤ 0.015% of the applied Ozenoxacin dose permeated through the skin over the course of 24 or 48 h. Ethnic origin had noinfluence on absorption. Ozenoxacin at concentrations of 7, 35 and 70 µM was metabolically stablein the presence of freshly prepared human skin discs

RATIONALE

The literature survey has revealed that, no stability indicating assay method has been reported for the estimationof Ozenoxacin in bulk drug in pharmaceutical formulation.In the present project, attempt was

made todevelop stability indicating assay method using RP-HPLC for the specific estimation of Ozenoxacin in bulk and pharmaceutical formulation. The developed method aimed to be a novel, simple, sensitive, rapid, accurate andreproducible analytical method.

EXPERIMENTAL WORK

Chromatographic conditions

Different solvent and buffers of different pH were tried by permutation and combination toobtain adequate retention of the drug. Finally, mixture of Methanol and 10mM phosphate buffer (adjusted to pH 3.0 with dilute phosphoric acid) in the ratio of 48:52% v/v was found to yield satisfactory retention time of Ozenoxacin at 3.77 min, with sharp symmetrical peak and well resolved from all thedegradation products. One of the chromatograms of standard solution of Ozenoxacin is depicted in Figure 16.

Thefollowingchromatographicconditionsweremaintainedthroughoutthemethoddevelopment.
Instrument : Agilent 1260 HPLC system
Column : Eclipse XDB C18 column (150mm x 4.6 mm i.d, 5 µm).
Mobile Phase : Methanol and 10mM phosphate buffer (pH3) in the ratio of (42:52% v/v)
Detection wavelength: 250 nm
Flow rate : 1 ml/min.
Temperature : 25℃
Injection volume: 20µl

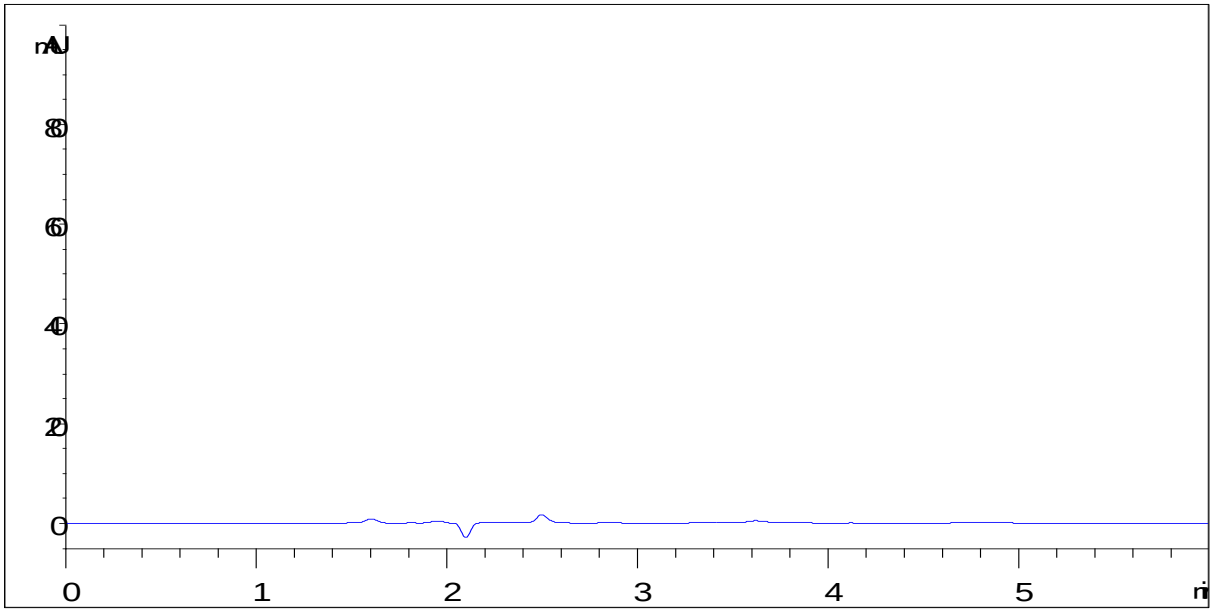


Fig. 15: HPLC chromatogram of blank

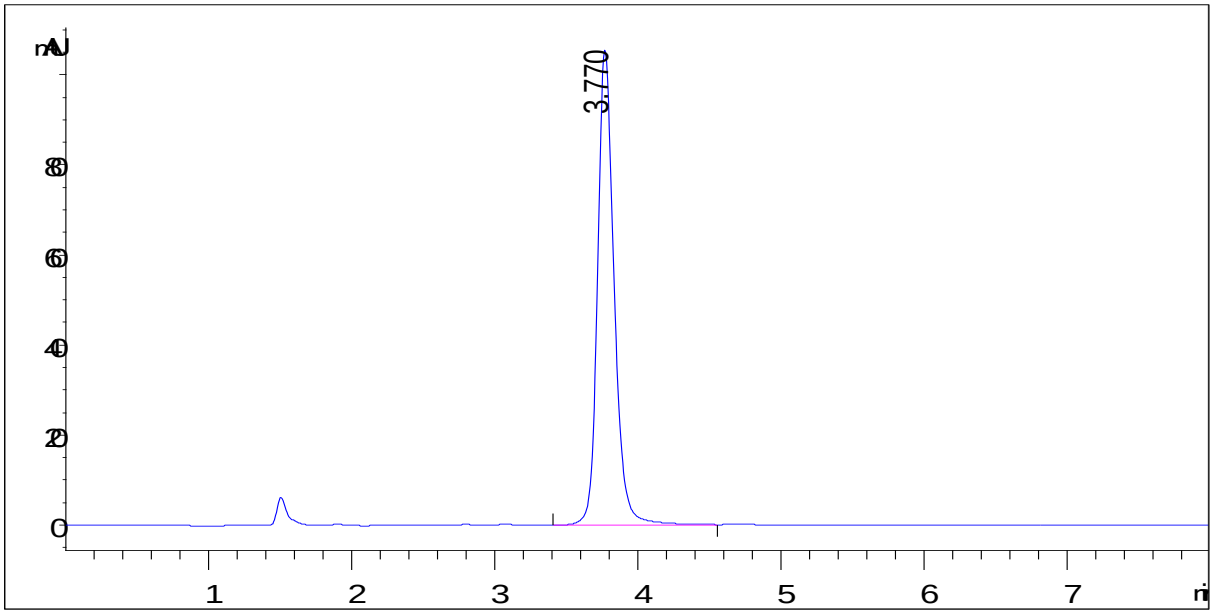


Fig. 16: HPLC chromatogram of standard drug Ozenoxacin

7.7 Forced Degradation study of Ozenoxacin (API)

The forced degradation study was carried out on API to ascertain the intrinsic stability of drug in different stress conditions. The forced degradation study was initiated using 1mg/ml of drug in different stress conditions like hydrolysis at acidic, and basic pH, oxidation with H_2O_2, photolysis and dry heat (thermal). The samples were withdrawn periodically like 1hr, 3hr, 5hr, 8hr and 24hr diluted appropriately with mobile phase to give 10µg/ml concentration. Small amount of 0.1N HCl may be use as cosolvent for solubility of drug.Stress study samples were analysed by set. HPLC method and % drug degradation was calculated using standard solution. The forced degradation study was stopped at point where 5-20% of drug was found to degrade compared to standard.

7.7.1 Acid hydrolysis

Ozenoxacin (10.0 mg) was dissolved in adequate amount of 0.1 N HCl in 10.0 ml volumetric flask, remaining volume was made up to the mark with aqueous 0.1 N HCl (concentration 1 mg/ml). This 10.0 ml solution was kept at room temperature for 24 h.

7.7.2 Base hydrolysis

Ozenoxacin (10.0 mg) was dissolved in adequate amount of 0.1 N NaOH in 10.0 ml volumetric flask, remaining volume was made up to the mark with aqueous 0.1 N NaOH (concentration 1.0 mg/ml). This 10.0 ml solution was kept at room temperature for 5 h.

In case of acid and base hydrolysis, samples were neutralized appropriately prior to dilution.

7.7.3. Oxidative degradation

Ozenoxacin (10.0 mg) was dissolved in adequate amount of 0.1N HCl in 10.0 ml volumetric flask, remaining volume was made up to the mark with 0.1N HCl (concentration 1 mg/ml). 1.0 ml of this solution was further diluted to 10.0 ml with 3% H_2O_2 and it was kept at room temperature in dark place for 3 h.

All the above stress sample were diluted appropriately with mobile phase to give 10µg/ml concentration.

7.7.4 Thermal degradation

A sufficient quantity of Ozenoxacin (about 100mg) was uniformly spread in a covered Petri-dish and kept in oven at 100°C for 24 h. 10.0mg sample was withdrawn and dissolved & diluted appropriately with 0.1N HCl (conc. 1mg/ml). Further dilution was made with mobile phase (conc. 10µg/ml).

7.7.5 Photo degradation

A sufficient quantity of Ozenoxacin (about 100mg) was uniformly spread in a covered Petri-dish and kept in sunlight. 10mg sample was withdrawn periodically on 1, 3, 5 and 8 days.

In case of thermal as well as photo degradation, samples were dissolved & diluted appropriately with 0.1N HCl (conc. 1mg/ml). Further dilution was made with mobile phase (conc. 10µg/ml).

Finally, the prepared stressed samples of Ozenoxacin were analyzed in HPLC under optimized chromatographic conditions and chromatograms were recorded. HPLC chromatograms of forced degradation samples of Ozenoxacin are depicted in Figure 17-21.

Table No. 15: Force degradation study of Ozenoxacin by HPLC

Sample and condition of exposure	Area	% Estimation
0.1 N HCl (At room temp for 24 h)	809.88	95.09
0.1 N NaOH (At room temp 5 h)	803.35	94.33
3% H_2O_2 (At room temp for 3 h)	804.44	94.46
Thermal (At 100℃ for 24 h)	693.03	81.37
Sunlight (day 8)	784.65	92.13

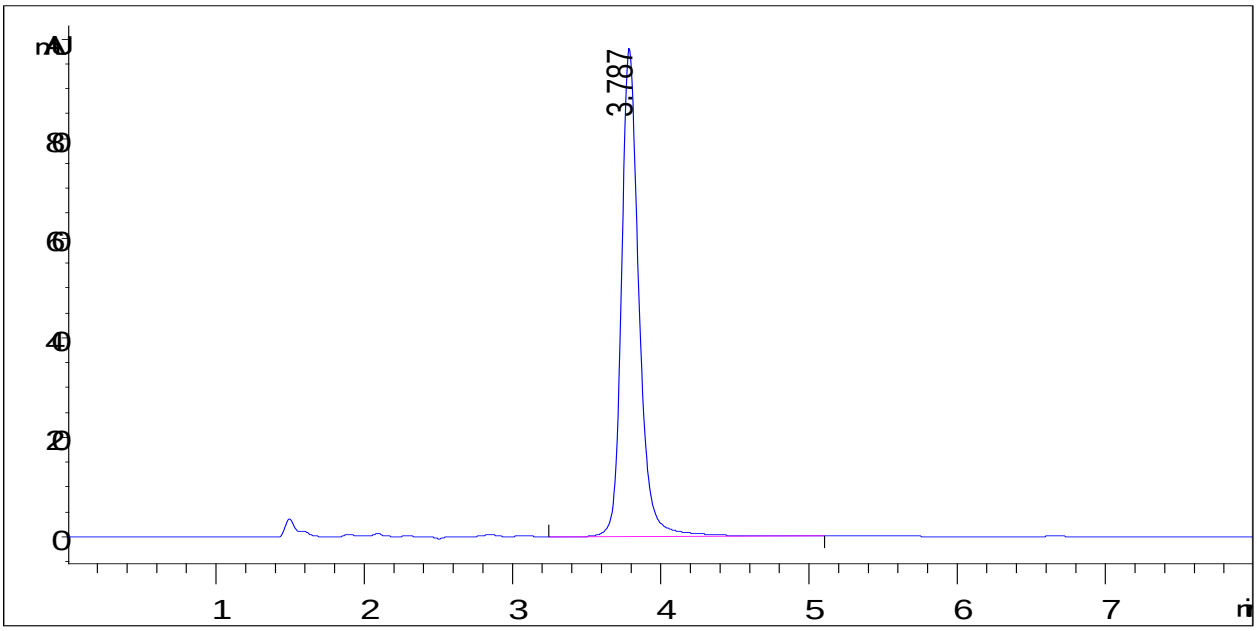


Fig. 17: HPLC chromatogram of stress sample of Ozenoxacin in 0.1 N HCl (At room temp for 24 Hrs)

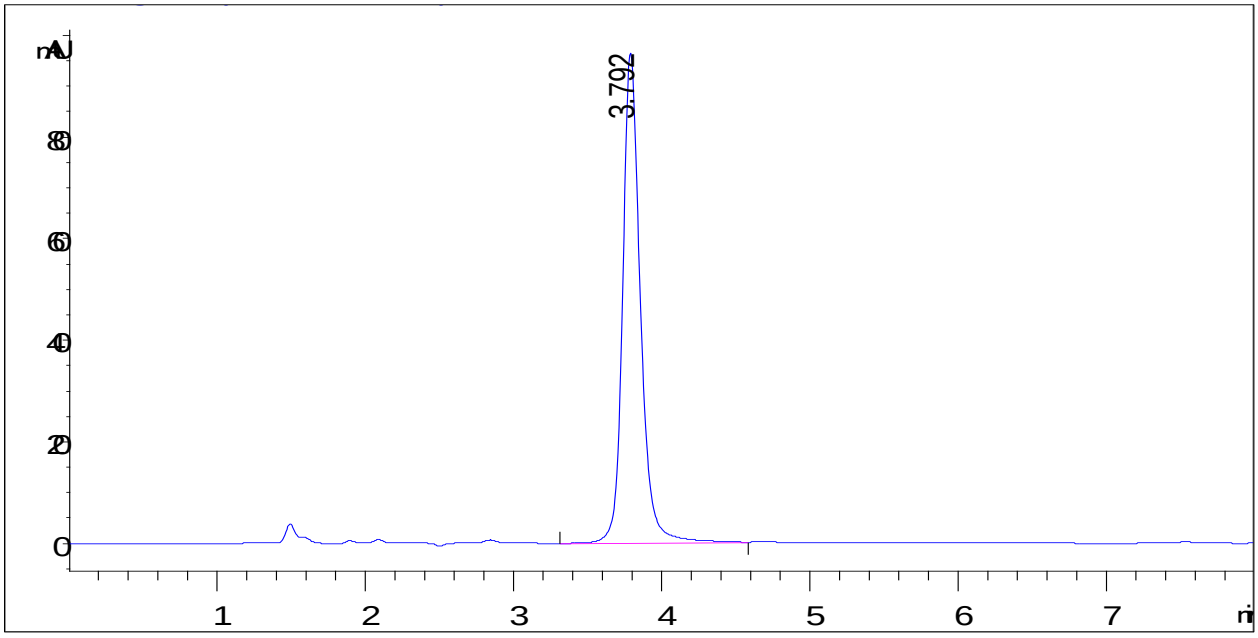


Fig. 18: HPLC chromatogram of stress sample of Ozenoxacin in 0.1 N NaOH (At room temp for 5 Hrs)

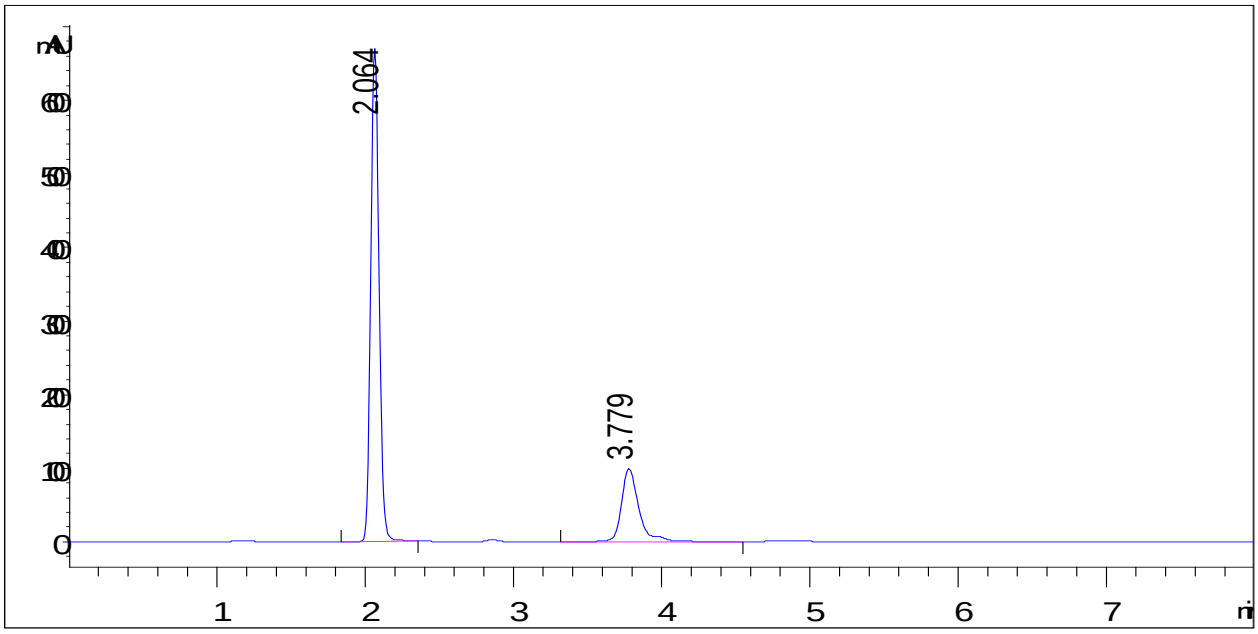


Fig. 19: HPLC chromatogram of stress sample of Ozenoxacin in 3% H_2O_2 (At room temp for 3 Hrs)

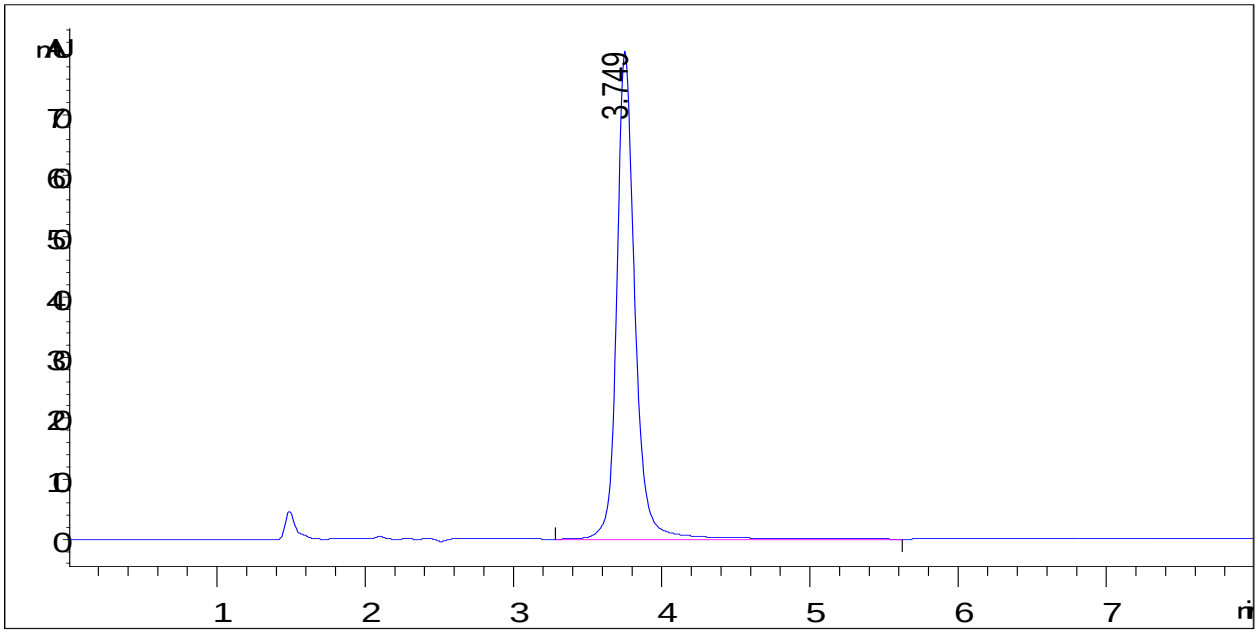


Fig. 20: HPLC chromatogram of stress sample of Ozenoxacin in Sunlight (for 8 days)

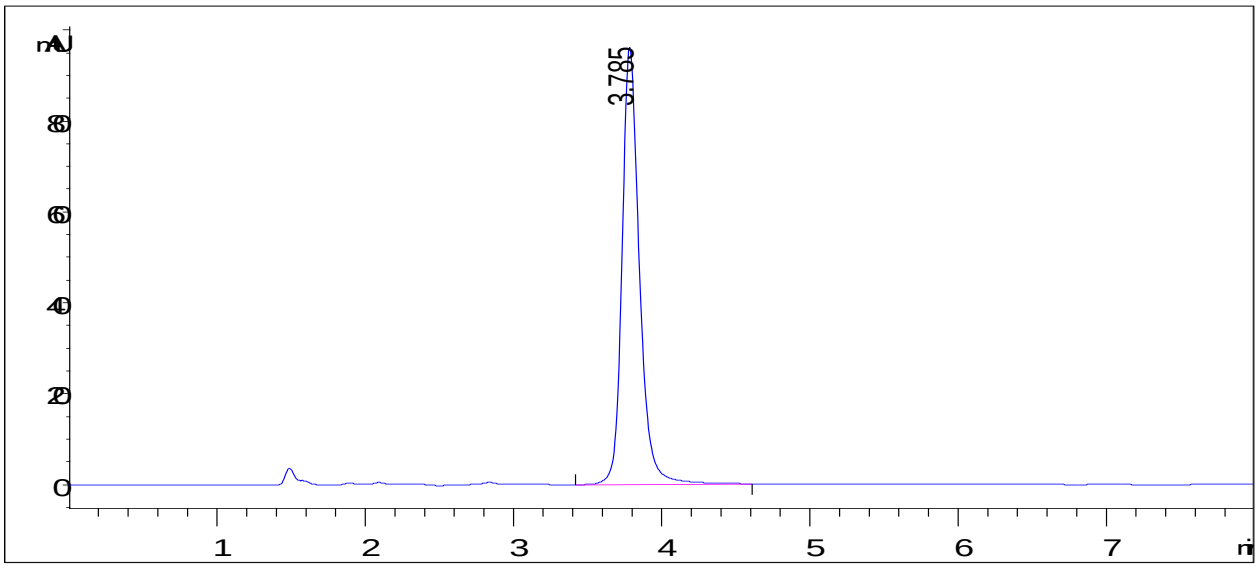


Fig. 21: HPLC chromatogram of stress sample of Ozenoxacin at 100°C (for 1 day)

7.8 Stability test of Standard and Sample Solutions

Stability of working standard and sample solutions of Ozenoxacin was studied by injecting solution at different time intervals to maximum for 24 hrs. The results are shown in table no.16.

Table no. 16: Study of Stability of standard and sample solutions

Time (Hr)	Area	
	Standard solution	Sample solution
0	853.23	845.56
1	857.89	840.39
3	852.07	852.31

5	854.18	844.18
8	846.67	836.43
24	828.54	832.78
Mean	**848.76**	**841.94**
±SD	**10.553**	**6.961**
%RSD	**1.243**	**0.826**

7.9. Validation of proposed method

According to the guidelines of ICH Q2 (R1) all the parameters as discussed below were analyzed and validated accurately following the procedure of the proposed method.

7.9.1. System suitability test parameters

For system suitability test parameters, six replicate injections of working standard solution of Ozenoxacin (10µg/ml each) were injected and analyzed under optimized chromatographic conditions. The results of system suitability test parameters study are depicted in Table No. 17.

Table No. 17: Results of System suitability test parameters study of Ozenoxacin

Sr. No	Retention Time (min)	Capacity Factor(k')	Area	Symmetry	Plates
1	3.729	0.99	855.23	0.73	5382
2	3.708	0.98	850.57	0.73	5229
3	3.72	0.99	858.73	0.72	5351
4	3.755	1.01	843.81	0.73	5328
5	3.771	1.02	854.85	0.73	5277
6	3.772	1.02	846.48	0.75	5234
Mean	**3.74**	**1.00**	**851.61**	**0.73**	**5300.16**
±SD	**0.027**	**0.017**	**5.701**	**0.009**	**63.300**
%RSD	**0.728**	**1.719**	**0.669**	**1.343**	**1.194**

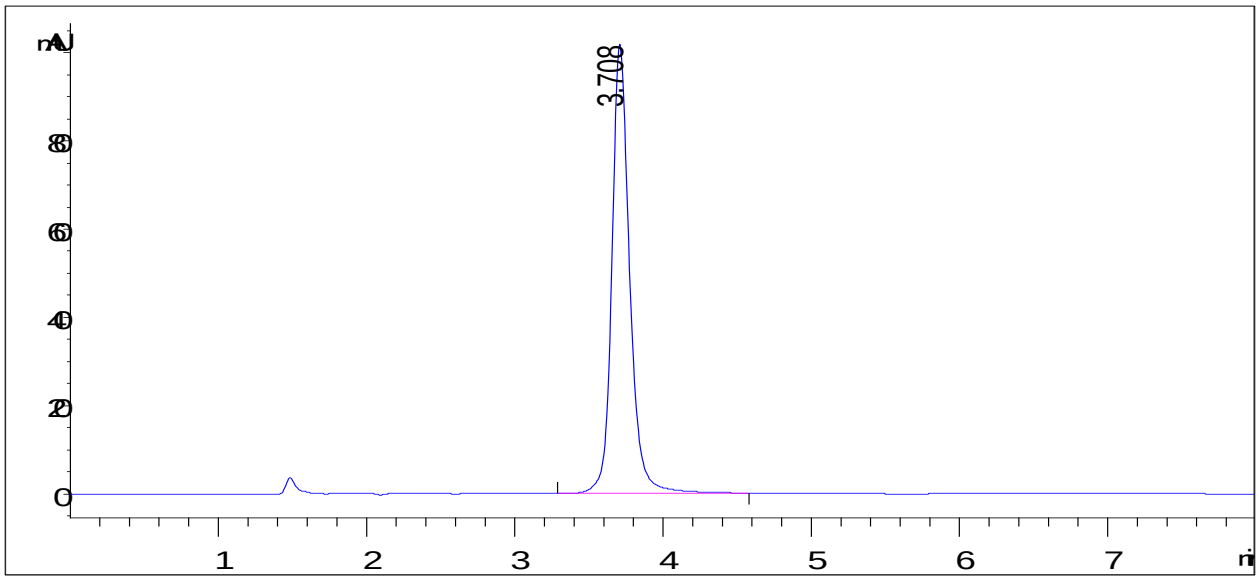


Fig 22: HPLC Chromatogram of system suitability study.

7.9.2 Linearity:

1.0 ml of standard solution of Ozenoxacin was transferred in a 10.0 ml of volumetric flask and the volume was made up to the mark with mobile phase to give 100µg/ml concentration of Ozenoxacin. five aliquot portions of this solutions (0.5 ml, 1.0 ml,1.5ml, 2.0 ml, 2.5 ml,) was further diluted separately to 10.0 ml with mobile phase to give concentration range of 5-25µg/ml. All the solutions were analyzed using the standard chromatographic conditions and the responses were measured as peak areas. The calibration curve was obtained by plotting peak area vs concentration (graph) shown in fig. no. 23 and Table No.18 shows the results of linearity study.

Table no. 18: Results of Study of linearity of Ozenoxacin

Sr.No.	Conc.(µg/ml)	PeakArea
1	5	406
2	10	856
3	15	1267
4	20	1675
5	25	2085

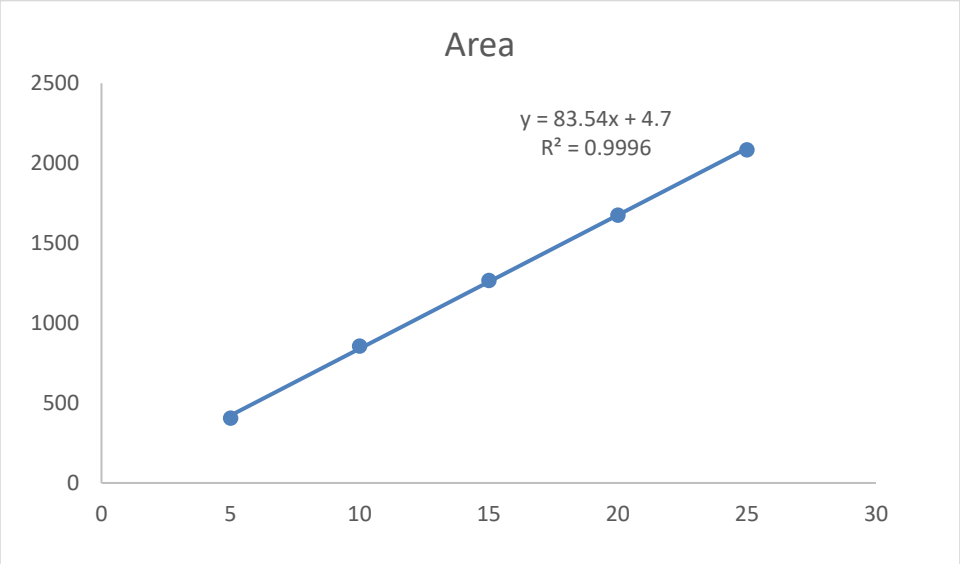


Fig. 23: Study of linearity of Ozenoxacin

7.9.3 Precision

The working sample solution of Ozenoxacin (10 µg/ml concentration) was used for the comparison with sample solutions by area normalization method. An accurately weighed Six quantities of 1gram (1%) cream equivalent to 10 mg of Ozenoxacin was transferred to different 10 ml volumetric flasks and dissolved in adequate quantity of chloroform using ultra sonication for 10 minutes. The above solution was transferred to 100ml separating funnel with rinsing of 10 ml phosphate buffer (pH8) (in 2-3 portion). Both the chloroform & aqueous layer were mixed with vigorous shaking & the solution was allowed to stand for 5 min to separate the layers. The chloroform layer was separated in a 50 ml beaker. The aqueous layer was washed by 1-2 portion each of 2ml chloroform (if required). Collect the all-chloroform fraction in beaker & allowed to evaporate at room temperature. The aqueous layer was

discarded. The residue after chloroform evaporation obtained was dissolved in about 8ml of 0.1 N HCl, & transferred to 10 ml volumetric flask. The beaker was washed with 2 portions each of 1ml of 0.1N HCI and washing were added to adjust volume to 10ml with 0.IN HCl. Pipette out 1.0 ml filtrate was diluted to 10 ml with mobile phase (Methanol:10mM phosphate buffer (pH 3) (48:52%v/v) in a 10 ml volumetric flask. 1.0 ml of this solution was further diluted to 10ml mobile phase in a 10 ml volumetric flask. The solutions were filtered by Nylon filter (0.45µ) and analyzed using the optimized chromatographic conditions. The results of precision study are shown in Table No. 19.

Table no 19: Results of Study of precision of Ozenoxacin

Sr. No.	Wt.of1% Cream (mg)	Peak Area	Amt.ofdrug Estimated (mg)	% label claim
1	1005	873.92	10.26	100.98
2	986	865.41	10.16	101
3	997	868.83	10.20	102
4	979	847.01	9.94	99
5	984	856.59	10.05	100
6	990	851.18	9.99	99
Mean				**100.33**
Standard deviation				**1.208**
%Relative standard deviation				**1.204**

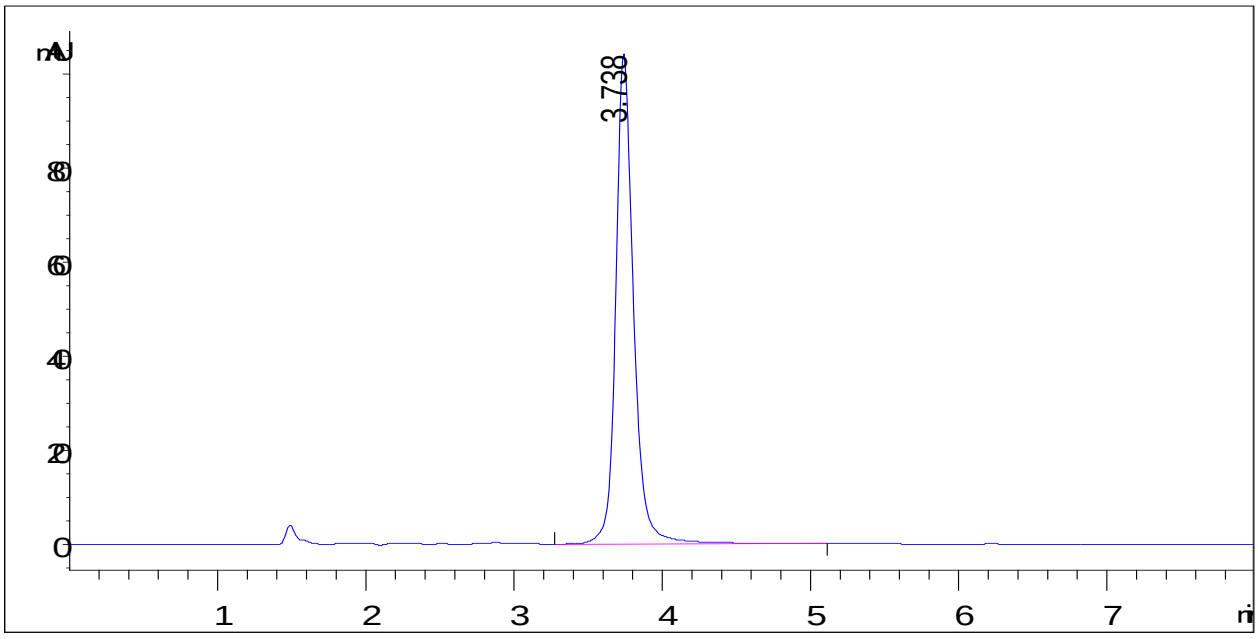


Fig 24: HPLC Chromatogram of Precision study.

7.9.4 Accuracy

To ascertain the accuracy of the proposed methods, recovery study was carried out by standard addition method at 80%, 100%, and 120% of the test concentration. Accurately weighed quantity of Ozenoxacin cream equivalent to about 500 mg of Ozenoxacin was transferred individually to nine triplicate 10.0 ml

volumetric flasks. Standard Ozenoxacin (in powder form) was added as 3.0 mg (80%) and 5.0 mg (100 %) and 7.0 mg (120%) except. The content was sonicated for 10 min. It was then transferred to 100ml separating funnel with rinsing of 10 ml phosphate buffer (pH8) (in 2-3 portion). Both the chloroform & aqueous layer were mixed with vigorous shaking & the solution was allowed to stand for 5 min to separate the layers. The chloroform layer was separated in a 50 ml beaker. The aqueous layer was washed by 1-2 portion each of 2ml chloroform (if required). Collect the all-chloroform fraction in beaker & allowed to evaporate at room temperature. The aqueous layer was discarded. The residue after chloroform evaporation obtained was dissolved in about 8ml of 0.1 N HCl, & transferred to 10 ml volumetric flask. The beaker was washed with 2 portions each of 1ml of 0.1N HCI and washing were added to adjust volume to 10ml with 0.1 N HCl. Pipette out 1.0 ml filtrate was diluted to 10 ml with mobile phase (Methanol:10mM phosphate buffer (pH 3) (48:52%v/v) in a 10 ml volumetric flask. 1.0 ml of this solution was further diluted to 10ml mobile phase in a 10 ml volumetric flask. The solutions were filtered by Nylon filter (0.45µ) and analyzed using the optimized chromatographic conditions. The recovery study in concentration 80%-120% are depicted in figure 25-28 and the results of recovery study are shown in Table no.20.

Table no. 20: Results of recovery study of Ozenoxacin

Level of addition	**Wt. of 1% Cream (mg)**	**Wt. of API (mg)**	**Peak Area**	**Amt. of Drug Estimated (mg)**	**Amt. of Drug Recovered (mg)**	**% Drug Recovered**
80%	493	3	672.02	7.89	2.96	98.66
	512	2.82	677.51	2.83	2.83	100.35
	493	3.14	692.98	3.20	3.20	101.91
100%	503	4.9	849.36	4.94	4.94	100.81
	481	5.04	830.29	4.93	4.93	97.81
	486	5.09	844.67	5.05	5.05	99.21
120%	512	7.10	1037.99	7.06	7.06	99.43
	516	7.00	1037.15	7.01	7.01	100.14
	508	7.13	1043.72	7.17	7.17	100.56
					MEAN	**99.87**
					±SD	**1.232**
					%RSD	**1.234**

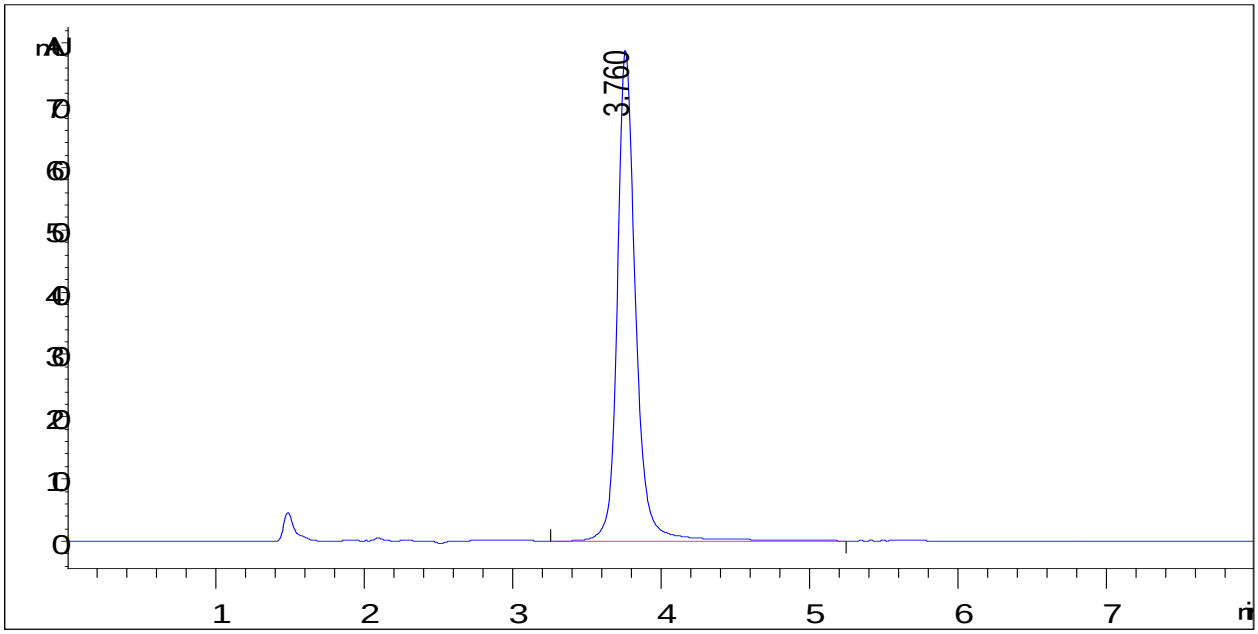


Fig. 25: HPLC chromatogram of recovery study of Ozenoxacin sample at 80%

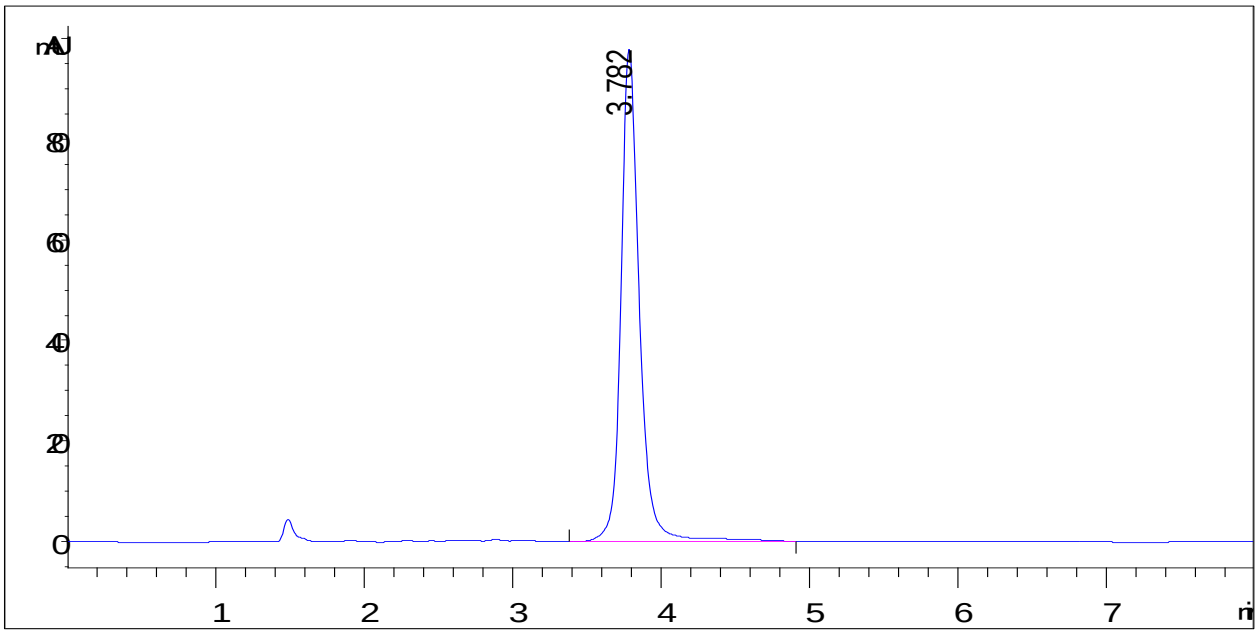


Fig. 26: HPLC chromatogram of recovery study of Ozenoxacin sample at 100%

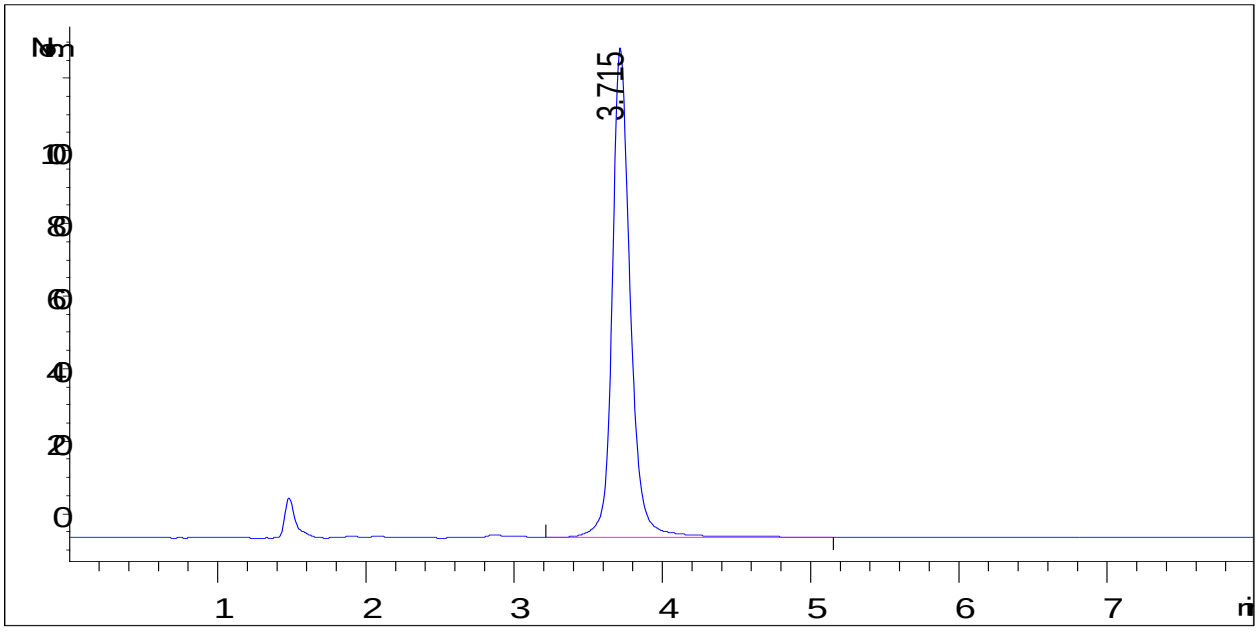


Fig. 27: HPLC chromatogram of recovery study of Ozenoxacin sample at 120%

7.9.5 Robustness

The robustness method was studied by varying the chromatographic condition by making a small deliberate change in the detection wavelength by ±5 nm, change in flow rate by 0.1ml/min, change in mobile phase composition by ±2 % v/v and change in column temp. (+5°C) the chromatograms were recorded. The results of robustness study for Ozenoxacin are shown in Table No.21 and figure from 28-35.

Table no. 21: Results of robustness study of Ozenoxacin

Sr No.	Parameter	Optimized Condition	Used Condition	Peak Area	Retention Time	Plate Count	Peak Symmetry
1.	Flow rate (±0.1ml/min)	1 ml/min	0.9	845.78	4.337	6360	0.76
			1.1	862.45	3.371	5203	0.75
2.	Detection wavelength (±5nm)	250 nm	245	861.57	3.795	5683	0.77
			255	834.60	3.794	5508	0.76
3.	Mobile phase composition (±2v/v)	Methanol: 10 mm Ammonium acetate (pH3) (48:52% v/v)	46:54	842.44	3.380	5456	0.77
			50.50	845.99	4.198	5659	0.74
4.	Column temperature (±5°C)	25°C	20°C	854.85	3.771	5577	0.73
			30°C	846.48	3.772	5608	0.75

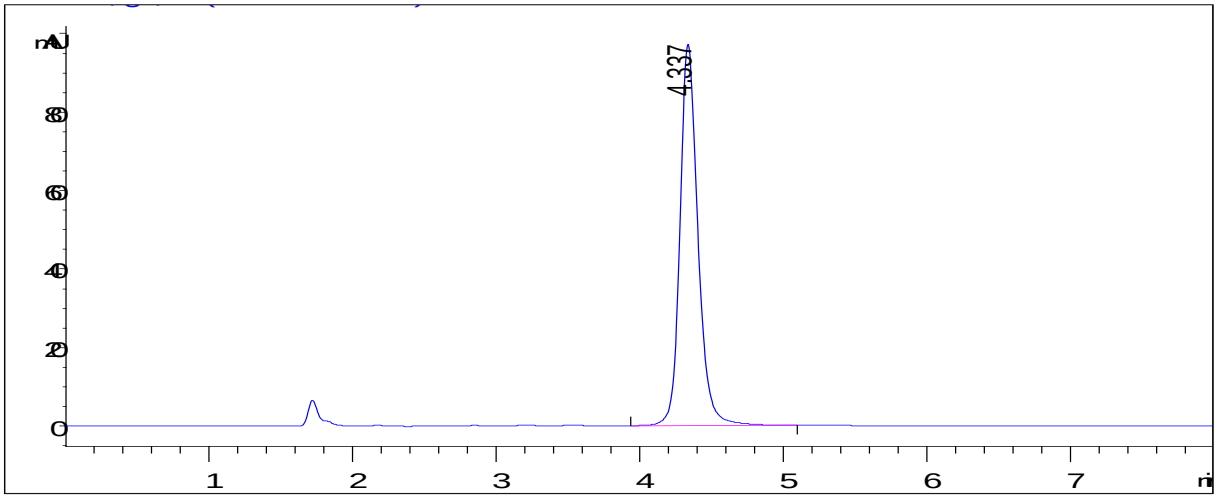


Fig. 28: HPLC chromatogram of robustness at flow rate 0.9 ml/min

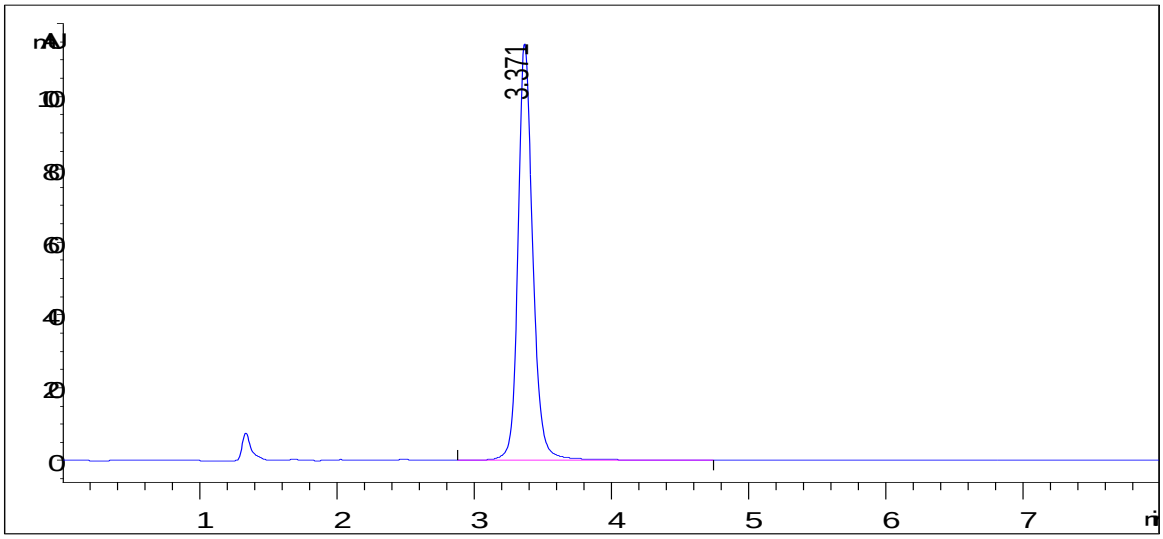


Fig. 29:HPLC chromatogram of robustness at flow rate 1.1 ml/min

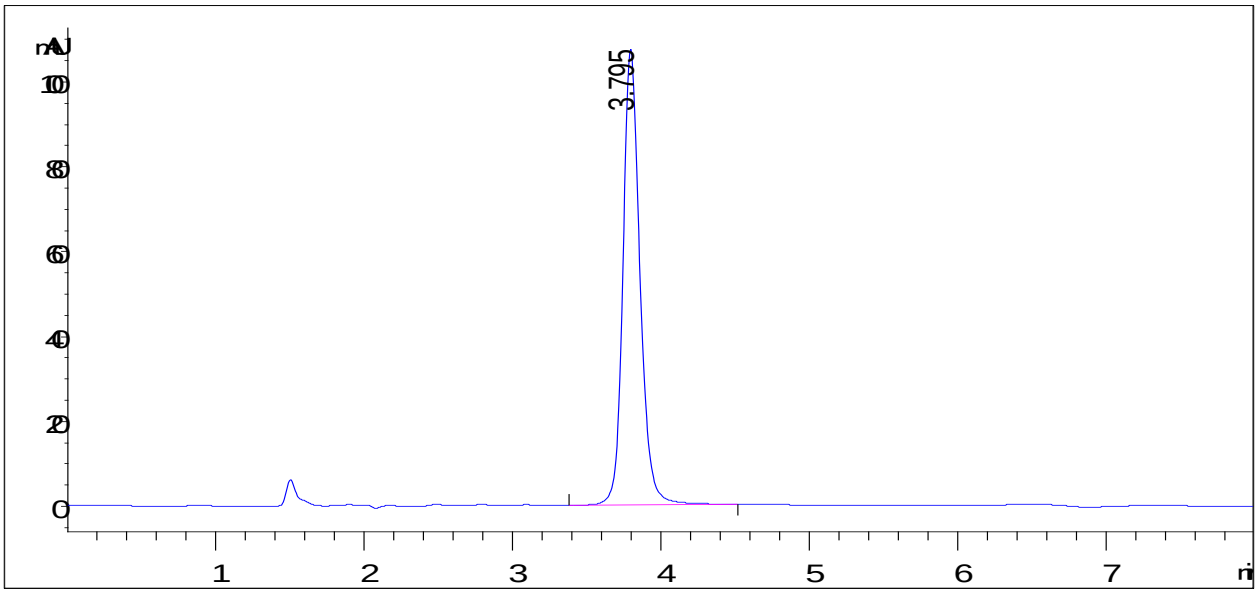


Fig. 30: HPLC chromatogram of robustness at wavelength 245 nm

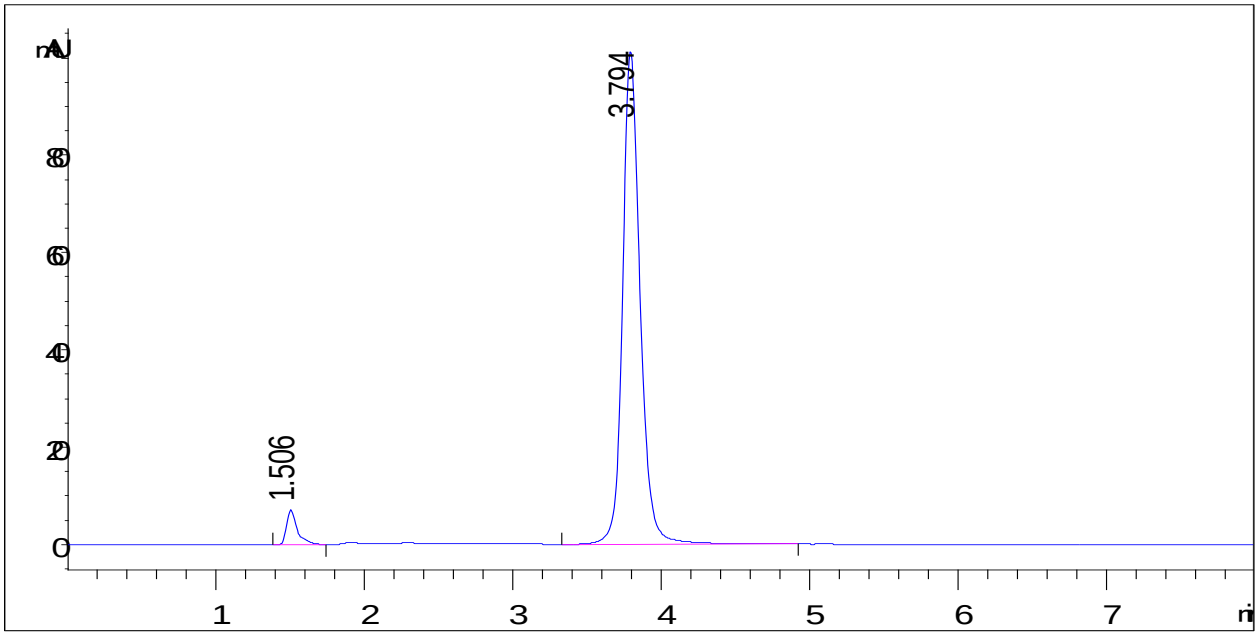


Fig. 31: HPLC chromatogram of robustness at wavelength 255 nm

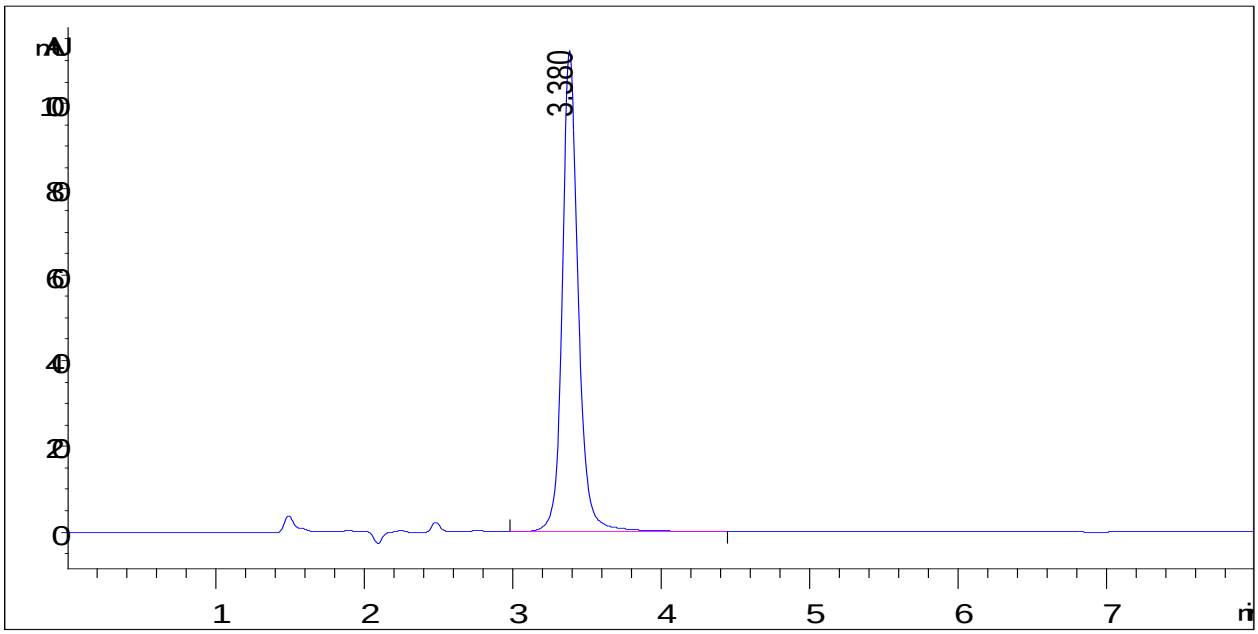


Fig. 32: HPLC chromatogram of robustness at mobile phase 46:54%v/v

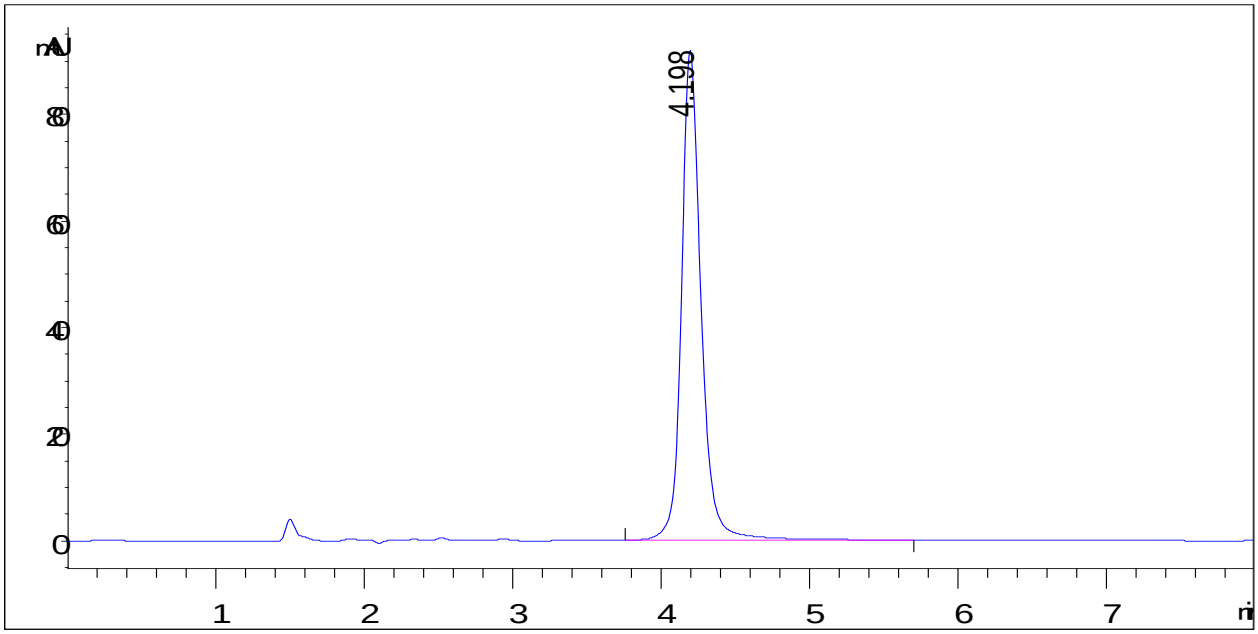


Fig. 33: HPLC chromatogram of robustness at mobile phase 50:50%v/v

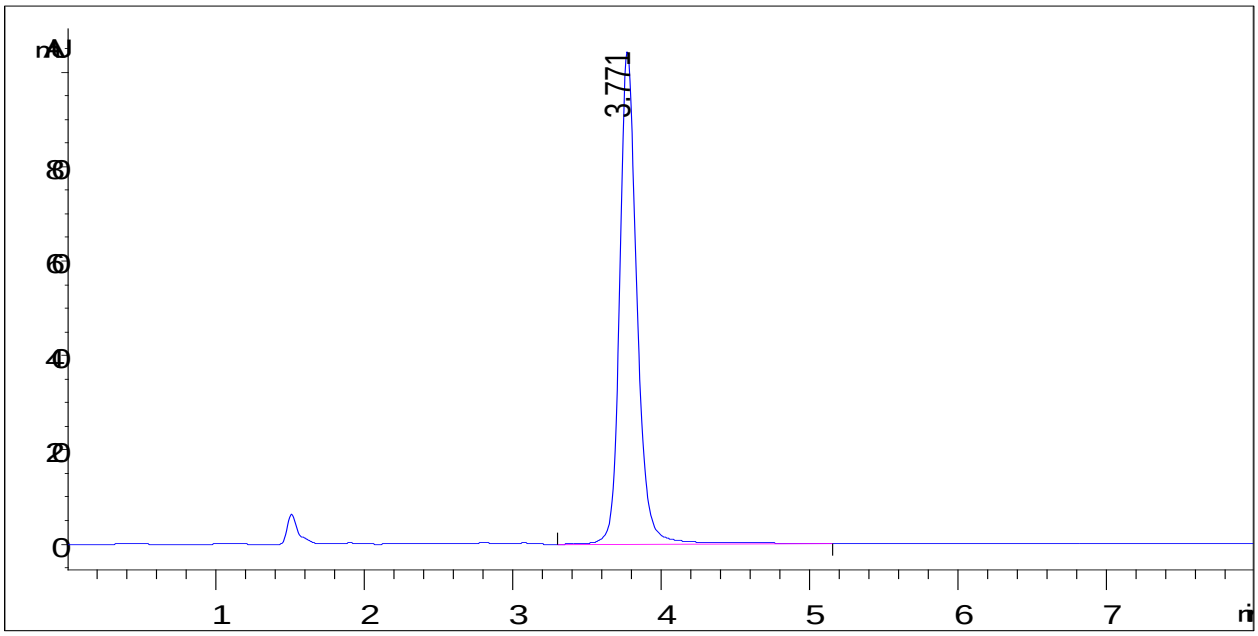


Fig. 34: HPLC chromatogram of robustness at column temp. 20°C

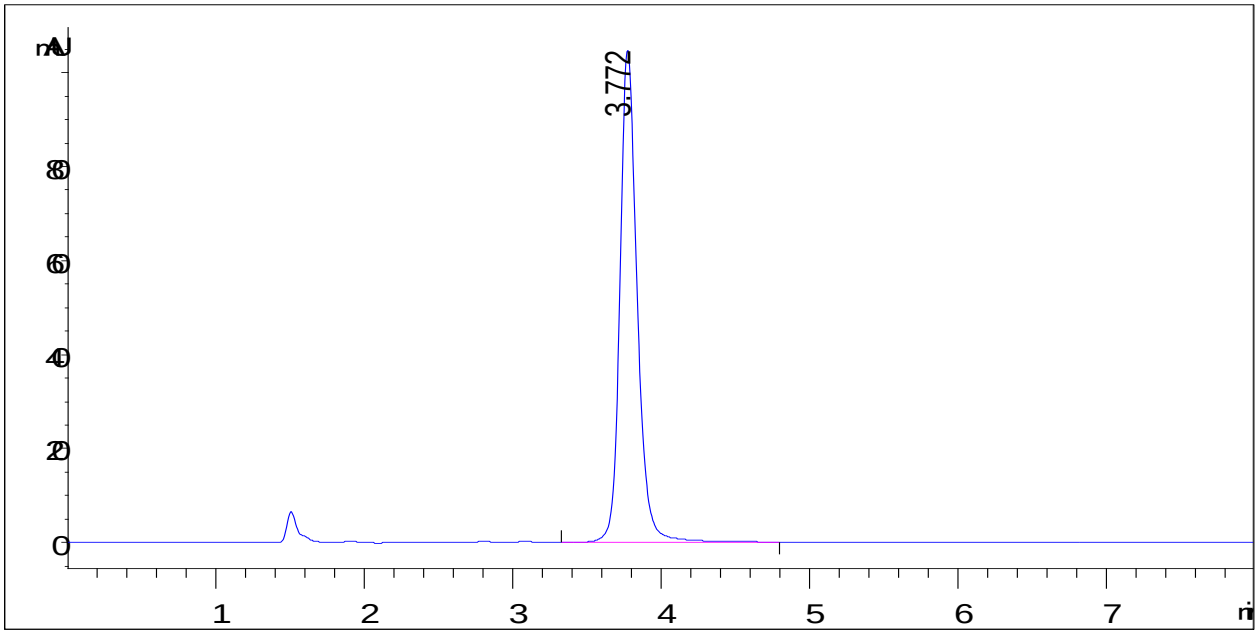


Fig. 35: HPLC chromatogram of robustness at column temp. 30°C

7.9.6. Ruggedness

The study of ruggedness conditions was ascertained on the basis of three different conditions.

7.9.6.1 Inter-day study

The study was performed by replicate estimation of same sample of cream formulation on three different days by proposed method.

7.9.6.2 Intra-day study

The study was performed by replicate estimation of same sample of cream formulation on same day at three different intervals by proposed method.

7.9.6.3 Different Analysts

The study was performed by replicate estimation of same sample of cream formulation by three different analysts by proposed method.

Table No. 22: Results of Ruggedness study for Ozenoxacin

Sr. No.	% Drug estimation		
	Intra Day	Inter Day	Different Analyst
1	99.94	100.38	100.05
2	99.93	97.47	100.30
3	100.21	100.73	99.42
Mean	**100.02**	**99.52**	**99.92**
±SD	**0.158**	**1.789**	**0.453**
%RSD	**0.158**	**1.798**	**0.453**

7.9.7. LOD and LOQ

LOD and LOQ for Ozenoxacin were evaluated by injecting a series of solutions duly diluted with known concentrations. Based on the response and slope of regression equation,the LOD and LOQ were

calculated by using formula.

(1) LOD=3.3 (SD)/S, and

(2) LOQ=10(SD)/S

- The LOD of drug was found to be **0.5903µg/ml**
- LOQ was found to be **1.7888µg/ml**

Table no. 23: LOD and LOQ results of Ozenoxacin

Limit of Detection (LOD)	0.5903 µg/ml
Limit of Quantitation (LOQ)	1.7888 µg/ml

7.9.8. Specificity Study

Working standard solution of Ozenoxacin was freshly prepared (10µg/ml concentration) and used for comparison of results by peak area normalization method. Accurately weighed quantity of 1% cream equivalent to about of 10 mg Ozenoxacin were transferred to six different 10.0 ml volumetric flasks. The samples were then exposed to stress conditions. The solutions were then analyzed in similar manner as described under estimation of Ozenoxacin in cream formulation. The results of specificity study are shown in Table No.24 and the HPLC chromatograms of specificity sample of Ozenoxacin was shown in figure 36-40.

Table no. 24: Results of specificity study of Ozenoxacin

Standard and condition of exposure	Area	% labelled claim
0.1 N HCl (At room temp for 24 Hrs)	818.61	96.12
0.1 N NaOH (At room temp for 5 Hrs)	821.32	96.44
3% H_2O_2 (At room temp for 3 Hrs)	803.55	94.35
Thermal (At 100°C for 1 day)	753.15	88.43
Sunlight (for 8 days)	815.28	95.73

The HPLC chromatograms of specificity study of Ozenoxacin cream are as follows:

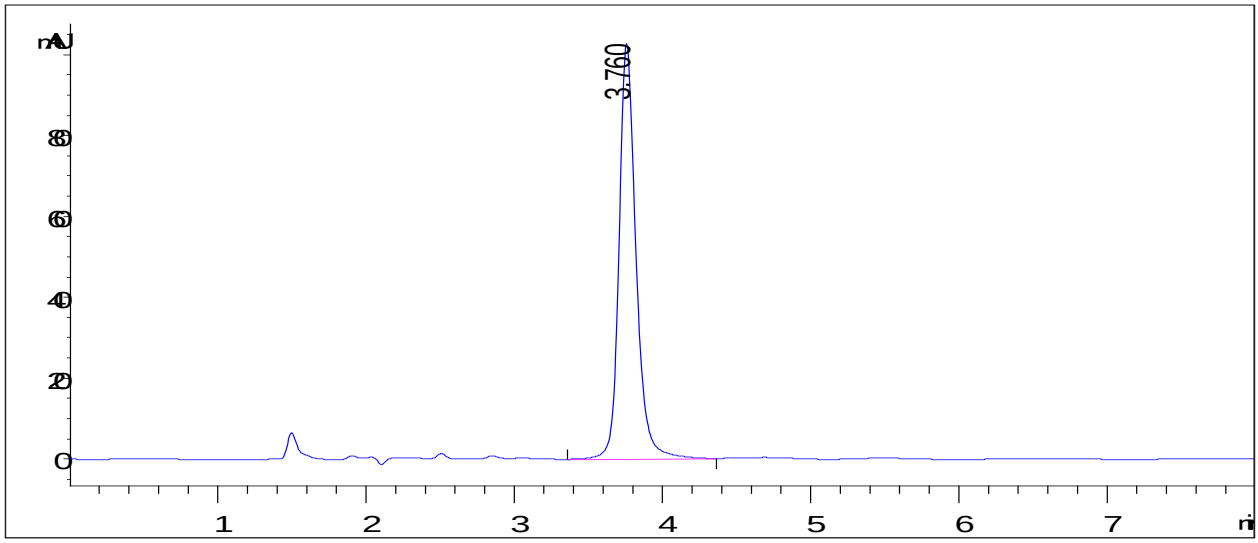


Fig. 36: HPLC chromatogram of stress sample of Ozenoxacin cream in 0.1 N HCl (At room temp for 24 Hrs)

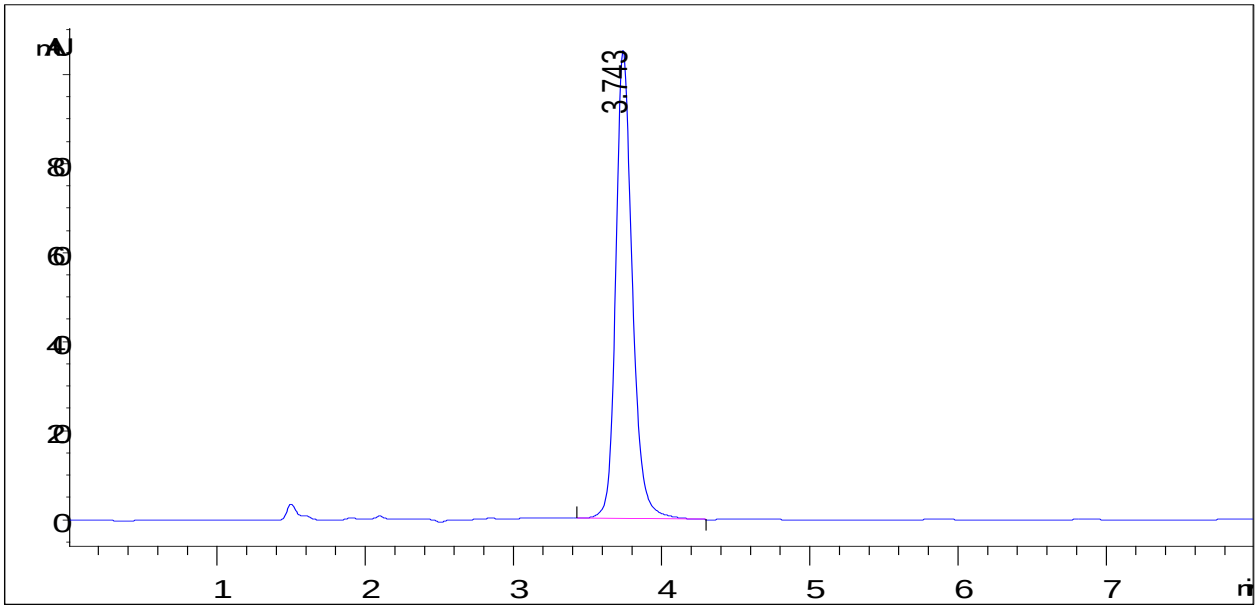


Fig. 37: HPLC chromatogram of stress sample of Ozenoxacin cream in 0.1 N NaOH (At room temp for 5 Hrs)

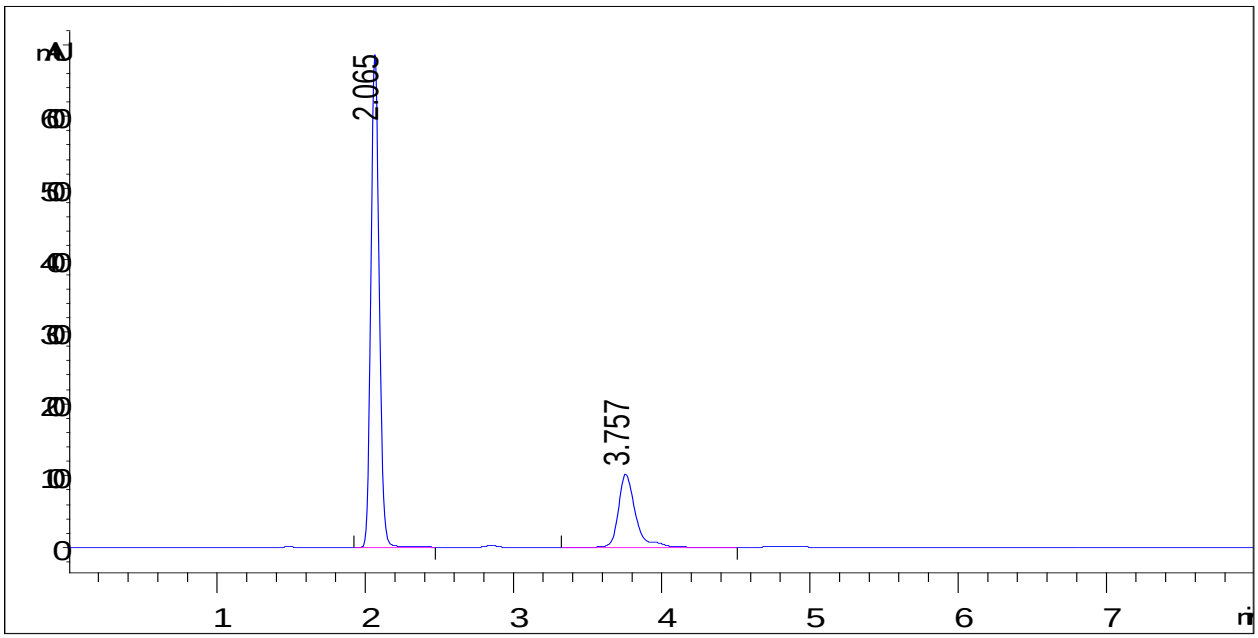


Fig. 38: HPLC chromatogram of stress sample of Ozenoxacin cream in 3% H_2O_2 (At room temp for 3 Hrs)

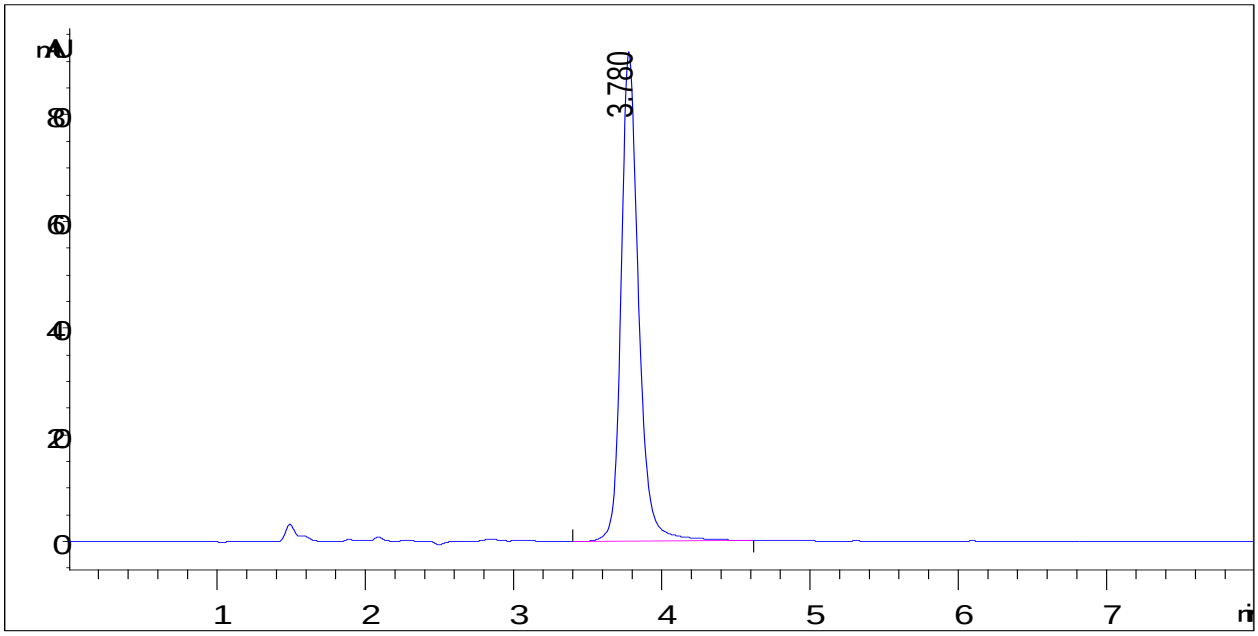


Fig. 39: HPLC chromatogram of stress sample of Ozenoxacin cream at 100°C (for 1 day)

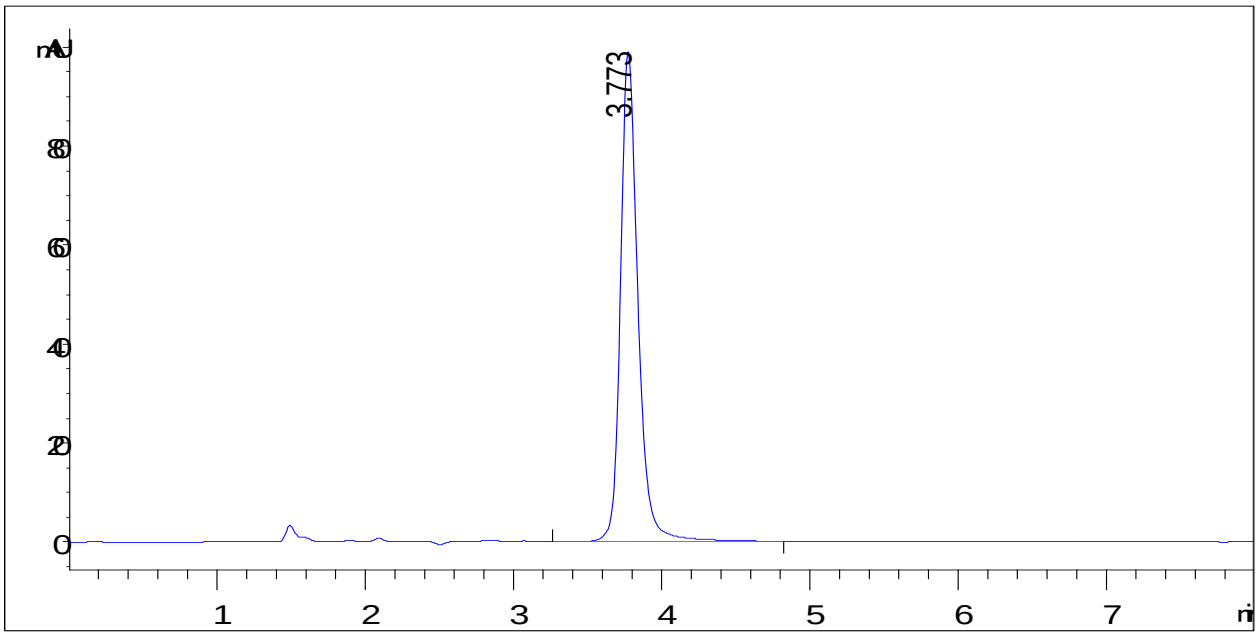


Fig. 40: HPLC chromatogram of stress sample of Ozenoxacin cream in Sunlight (for 8 days)

CONCLUSION

In the present project work, a stability indicating RP-HPLC method was developed and validated for the estimation of Ozenoxacin in cream formulation. The forced degradation study carried out on drug showed significant degradation products generated under the various conditions of exposure. All the degradation products are well resolved from parent drug under the optimized chromatographic conditions.

Moreover, the method is in true sense can be said to be specific stability indicating assay method for Ozenoxacin due to its capability to estimate the drug content unequivocally free of interference from its degradation products.

The validation of method indicates that the method is simple, precise, accurate, rugged, and reasonably specific for the estimation of Ozenoxacin in pharmaceutical formulations.

The proposed RP-HPLC method can be adopted for estimation of Ozenoxacin in routine quality control in the pharmaceutical industries.