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Research Article

Stability Indicating Assay Method (Siam) For the Estimation of Ozenoxacin Using RP-HPLC Method

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A B S T R A C T

In this study, accurate, precise, simple and economical stability indicating RP-HPLC method was developed and validated for the estimation of ozenoxacin. As per ICH guidelines, chromatographic separation was achieved by isocratic elution on HPLC (model-Agilent 1200 infinity II Hplc system). Instrument equipped with column Eclipse XDB (C18X150X4.6mm,5µ) was used, at flow rate of 1.0ml/min, with ratio 10mM at pH 3 : methanol 52:48 % V/V at 250nm wavelength and column over temperature 25°c, injection volume was 20 µl, run time was 8 min. This method has found to be validated and the obtained results from that this developed method suitable for routine analysis.

Keywords: Ozenoxacin, isocratic elution, model-Agilent 1200 infinity II HPLC system, 250nm wavelength

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INTRODUCTION

Analytical Chemistry[1]

Analytical chemistry involves separation, identification and determination of the relative amount of the component in a sample matter. Quality is always associated with accuracy and reproducibility other criteria can be cost, speed and information. Analytical monitoring of pharmaceutical product or of specific ingredients within the product is necessary to ensure the safety and efficacy throughout the shelf life, including storage, distribution and use etc.

Analytical technique play important role in maintaining and assuring the quality of substance. The reliability, utility, accuracy, interception and specificity of the measurement are responsibilities of analytical chemist. In general terms pharmaceutical analysis comprises, those procedures necessary to determine the identity, strength, quality and purity of drugs and chemicals.

The discipline of analytical chemistry consists of

a) Qualitative analysis

b) Quantitative analysis

Qualitative analysis deals with the identification of elements, ions, or compounds present in the sample while quantitative analysis deals with the determination of how much of one or more constituent(s) are present.

Selection of Analytical Method [2, 3]

First stage in the selection or development of method is to establish what is to be measured and how accurately it should be measured. Unless one has series of methods at hand to assess quality of the product, validation program may have limited validity. The selected method must have the following parameters:

1. As simple as possible.
2. Most specific.
3. Most productive, economical and convenient.
4. As accurate and precise as required.
5. Multiple sources of key component (reagents, columns, TLC plates) should be avoided.
6. To be fully optimized before transfer for validation of its characteristics such as accuracy, precision, sensitivity, robustness, ruggedness etc.

Most commonly used method in HPLC[4]

a) Normal phase chromatography

Normal phase HPLC was the first kind of HPLC chemistry used and separates based on polarity. This method uses a polar stationary phase and a non-polar mobile phase and is used when the analyte of interest is fairly polar in nature. The polar analyte associates with and is retained by the polar stationary phase. Adsorption strengths increase with increase in analyte polarity, and the interaction between the polar analyte and the polar stationary phase (relative to the mobile phase) increases the elution time. The interaction strength not only depends on the functional groups in the analyte molecule, but also on steric factors and structural isomers. Use of more polar solvents in the mobile phase will decrease the retention time of the analyte while more hydrophobic solvents tend to increase retention time. Particularly polar solvents in a mixture tend to deactivate the column by occupying the stationary phase surface. This is somewhat particular to normal phase because it is most purely an adsorptive mechanism (the interactions are with a hard surface rather than a soft layer on a surface).

Mechanism: Retention by interaction of the stationary phase polar surface with polarparts of the sample molecules.

1. **Stationary phase**: It is a bonded siloxane with polar functional group like SiO_2,Al_2O_3, -NH_2, -CN, -NO_2, -Diol.
2. **Mobile phase**: Non-polar solvents like heptane, hexane, cyclohexane, chloroform,ethyl ether, dioxane.
3. **Application**: Separation of non-ionic, non-polar to medium polar substances.
4. **Sample elution Order**: Least polar components are eluted first.

B) Reverse phase chromatography

Reversed phase HPLC (RP-HPLC) consists of a non-polar stationary phase and a moderately polar mobile phase. One common stationary phase is silica which has been treated with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}.

The retention time is therefore longer for molecules which are more non-polar in nature, allowing polar molecules to elute more readily. Retention time is increased by the addition of polar solvent to the mobile phase and decreased by the addition of more hydrophobic solvent. Reversed phase chromatography is so commonly used that it is not uncommon for it to be incorrectly referred to as "HPLC" without further specification.

RP-HPLC operates on the principle of hydrophobic interactions which result from repulsive forces between a relatively polar solvent, the relatively non-polar analyte and the repulsive non-polar stationary phase. The driving force in the binding of the analyte to the stationary phase is the decrease in the area of the non-polar segment of the analyte molecule exposed to the solvent.

Mechanism: Retention by interaction of the (stationary phase) non-polar hydrocarbonchain with non-polar parts of sample molecules.

1. **Stationary phase**: It is bonded siloxane with non-polar functional groups like n-octadecyl (C-18) or n-octyl (C-8), ethyl, phenyl, -$(CH_2)_n$-diol, -$(CH_2)_n$-CN.
2. **Mobile Phase**: Polar solvents like methanol, acetonitrile, water or buffer (sometimeswith additives of THF or dioxane).
3. **Applications**: Separation of non-ionic and ion forming non-polar to medium polarsubstances (carboxylic acids hydrocarbons).
4. **Sample elution order**: Most polar components are eluted first.

Components of the HPLC system

High performance liquid chromatography consists of following major components

a) HPLC gradient mixers

b) HPLC pumps

c) HPLC columns

d) HPLC detectors.

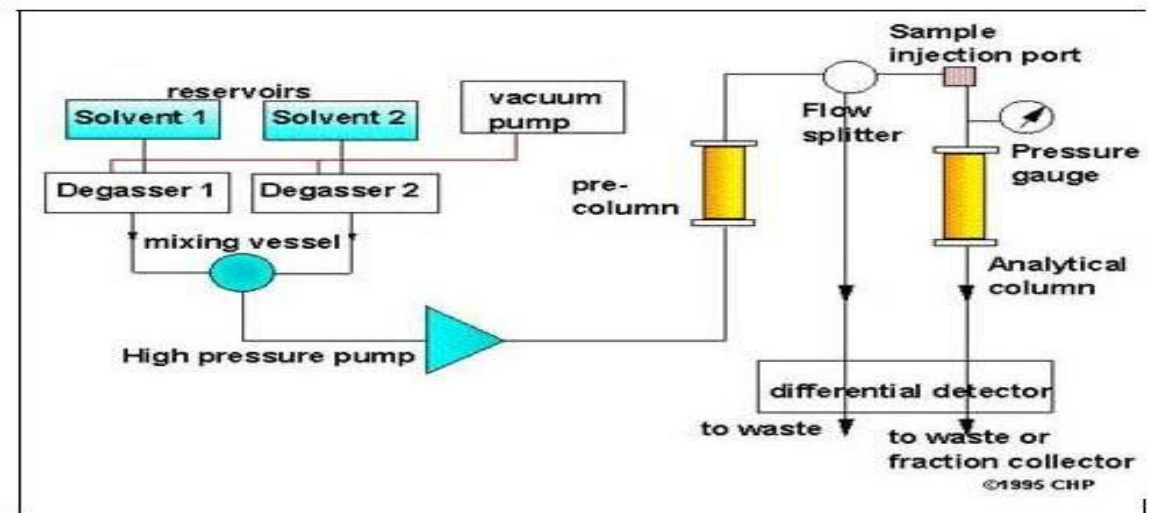

Figure 1: Schematic diagram of HPLC instrument.

a) HPLC gradient mixers

HPLC gradient mixers provide a very precise control of solvent composition to maintain a reproducible gradient profile. This can be complicated in HPLC by the small elution volumes required by many systems. It is much more difficult to produce a constant gradient when mixing small volumes than when mixing large volumes. For low-pressure systems, it requires great precision in the operation of the miniature mixing valves used and low dispersion flows throughout the mixer. For multi-pump high-pressure systems, it requires a very precise control of the flow rate while making very small changes of the flow rate.

b) HPLC pumps

Because of the small particles used in modern HPLC column packing, modern LC pumps need to operate reliably and precisely at pressures of 10,000 psi or at least 6,000 psi. To operate at these pressures and remain sensibly inert to the wide variety of solvents used HPLC pumps usually have sapphire pistons, stainless steel cylinders and return valves fitted with sapphire balls and stainless steel seats. For analytical purposes, HPLC pumps should have flow rates that range from 0 to 10 ml/min, but for preparative HPLC,

flow rates in excess of 100 ml/min may be required. It is extremely difficult to a very constant flow rate at very low flow rate.

Pump Pressure

Pumps vary in pressure capacity, but their performance is measured on their ability to yield a consistent and reproducible flow rate. Pressure may reach as high as 6000 lbf/inch^2 (~40 MPa, or about 400 atmospheres). Modern HPLC systems have been improved to work at much higher pressures and therefore be able to use much smaller particle sizes in the columns (< 2 micrometers). These "Ultra High Performance Liquid Chromatography" systems or UHPLCs can work at upto 15,000 lbf/inch^2 (-100 MPa or about 1000 atmospheres).

c) HPLC columns

Column is often referred to as the heart of the HPLC separation process. HPLC columns are packed with very fine particles (usually a few microns in diameter) to attain the low dispersion that give the high plate counts expected of modem HPLC. LC columns, in general, achieve their separation by exploiting the different intermolecular forces between the solute and the stationary phase and those between the solute and the mobile phase. The column will retain those substances that interact more strongly with the stationary phase than those that interact more strongly with the mobile phase.

C18 and C8 HPLC columns

1. Classic reversed-phases for all general purpose applications.
2. Excellent peak shape and efficiency compared to competitive columns.
3. Classic reversed-phase retention and selectivity.
4. C18 is generally more retentive than the C_8
5. Various factors that govern the retention of component are as follows**:**

Internal diameter

The internal diameter (i.d.) of an HPLC column is a critical aspect that determines of analyte that can be loaded onto the column and also influences sensitivity. Larger columns are usually seen in industrial applications such as the purification of drug product for later use. Low i.d. columns have improved sensitivity and lower solvent consumption at the expense of loading capacity.

1. Larger i.d. columns (over 10 mm) are used to purify usable amount of materials because of their large loading capacity.
2. Analytical scale columns (4.6 mm) have been the most common type of column though smaller columns are rapidly gaining popularity. They are used in traditional quantitative analysis of samples and often use a UV-VIS absorbance detector.
3. Narrow-bore columns (1-2 mm) are used for applications when morr sensitivity is desired either with special UV-VIS detectors, fluorescence detectors or with other detection methods like liquid chromatography-mass spectrometry.

4. Capillary columns (under 0.3 mm) which are used almost exclusively with alternative detection means such as mass spectrometry. They are usually made from fused silica capillaries, rather than the stainless steel tubing that larger columns employ.

Particle size

Most traditional HPLC is performed with the stationary phase attached to the outside of small spherical silica particles (very small beads). These particles come in a variety of sizes with 5µm beads being the most common. Smaller particles generally provide more surface area and better separations, but the pressure required for optimum linear velocity increases by the inverse of the particle diameter cubed. This means that changing to particles that are half as big in the same size of column will double theperformance, but increase the required pressure by a factor of eight. Larger particles are more often used non-HPLC applications such as solid-phase extraction.

Pore size

Many stationary phases are porous to provide greater surface area. Small pores provide greater surface area while larger pore size has better kinetics especially for larger analyte. For example a protein which is only slightly smaller than a pore might enter the pore but not easily leave once inside.

d) HPLC detectors

The function of the detector in HPLC is to monitor the mobile phase as it emerges from the column. HPLC detectors use the same detection principles with extra care being given to the small solute elution volumes that result from the combination of high column efficiencies with small volumes. In order to give an accurate chromatographic profile the detector sampling (cell) volume must be a small fraction of the solute elution volume. If the detector volume were larger than the elution volume then peaks that appeared are with flat tops as the whole peak would be resident in the detector at the same time. This means that as column volume decrease and system efficiencies increase the volume of the detector cell must also decrease. This is of course at odds for the requirement of detector to maintain high sensitivity, as this is usually dependant on having a larger cell volume. Again, this requires very careful design of modem detectors.

Types of detectors

1. **Ultraviolet detector**

   a) Most widely used.

   b) Principle-Absorption of UV visible light as the eluent from the column is passed through a small flow cell held in radiation beam.

   c) Suitable for Gradient elution.

2. **Fluorescence detector**

   a) Principle-Enable fluorescent compounds present in mobile phase to be detected by passing the column eluent through a cell irradiated with ultraviolet light and measuring any resultant fluorescent radiation.

   b) Very sensitive and selective.

3) **Refractive index detector**

   a) Principle-These are differential refractometer which respond to change in the bulk property of the refractive index of the solution of the component in the mobile solvent system.

   b) It is less sensitive.

4) **Electrochemical detector**

   a) Principle- These are based on standard electrochemical principles involving Zmperometry, voltametry and polarography.

   a) These are very sensitive for substances that are electro active, i.e. those that undergo oxidation or reduction at a suitable potential.

5) **Photodiode array detector (PDA)**

A photodiode array (PDA) is a linear array of discrete photodiodes on an integrated circuit (IC) chip. For spectroscopy it is placed at the image plane of a spectrometer to allow a range of wavelengths to be detected simultaneously. In this regard it can be thought of as an electronic version of photographic film. Array detectors are especially useful for recording the full UV-visible absorption spectra of samples that are rapidly passing through a sample flow cell, such as in an HPLC detector.

Method development in HPLC

Methods for analyzing drugs by HPLC can be developed, provided one has knowledge about the nature of the sample namely, its molecular weight, polarity, ionic character, pKa values and the solubility parameter. An exact recipe for HPLC method development cannot be provided because method development involves considerable trial and error procedures. The most difficult problem usually is where to start, what type of column is worth trying with what kind of mobile phase.

The water soluble active phamaceutical ingredients is further differentiated as ionic or non ionic which can be separated by reverse-phase. Similarly, the organic soluble API can be classed as polar and non-polar and equally separated by reverse phase. In some cases the non-polar API may have to be separated using adsorption or normal phase HPLC, in which mobile phase would be non-polar organic solvent.

Steps involved in development of HPLC method

a) Literature survey

Here a detailed account of all analytical methods developed for the drug is collected to avoid duplication of the method developed. Details about the structure of the drugs ad their physicochemical properties are also collected.

b) Selection of chromatographic method

First, reversed phase should be tried. If not successful, normal phase should be taken into consideration. For ion exchange or ion pair chromatography, first ion suppression by pH control and then reversed phase chromatography should be tried.

c) Selection of stationary phase

By studying the polarity of sample and mobile phase use of a stationary phase of different polarity should be done to

achieve successful separation.

d) Selection of column

The HPLC column is the heart of the method, critical for the separation. The column must possess the selectivity, efficiency and reproducibility to provide good separation. Commonly used reversed phases are C_{18} (octadecyl silane, $USPL_1$), C_8 (octyl silane, $USPL_7$) phenyl ($USPLI_{11}$) and cyano ($USPL_{18}$). They are chemically different bonded phases and demonstrate significant changes in the selectivity using the same mobile phase. For example, a C_8 phase (reversed phase) can prove to be time saving over a C_{18} as it does not retain analyte as strongly as the C_{18} phase. For normal phase applications cyano phases are the most versatile. C_{18} (250 x 4.6mm) column are more often used in the laboratory. These columns are able to resolve a wide variety of compounds due to their selectivity and high plate counts.

e) Selection of mobile phase

If the sample contains ionic or ionizable compounds, then use of a buffered mobile phase is recommended to ensure the reproducible results. Under unfavorable circumstances, pH changes as little as 0.1 pH units can have a significant effect on the separation. On the other hand properly used buffer allows controlling the pH easily.Buffer works best at the pKa of its acid. At this pH, the concentration of the acidic form and the basic form of the buffering species is equal and thus the buffering capacity maximal. Phosphate has two pKa values in the range of interest for silica-based chromatography one at pH-2 and the other at pH-7. The pKa of the acidic buffer is 4.75. Citrate has three pKa values: 3.08, 4.77 and 6.40. Between citrate and phosphate buffers, the entire pH range useful for silica chromatography can be covered. In many cases, sonophillic interactions cause tailing, mostly due to ion-exchange interaction. This can reduced or suppressed by the use of amine-based buffers or by using acidic mobile phases, or a combination thereof whenever buffers or other mobile phase activities are used, the solubility of the mobile phase is checked. This is especially true for gradient applications. Acetonitrile is the preferred organic modifier in reverse-phase-chromatography.

Validation of analytical techniques

Introduction to validation

Validation is a proof that a process works and this must be done using scientific ad statistical principles. This is done to establish process capability and to confirm end acceptability. Validation determines process variables and the acceptable limits for these variables and accordingly sets up appropriate in process controls, which specifies alert and action levels.

ICH guideline **Q2 (R1)** Method Validation Parameters are as follows:-

a) Accuracy

b) Precision

c) Specificity

d) Linearity

e) System suitability

f) Ruggedness

g) Robustness

a) Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness.

It is measured as the % of analyte recovered by assay or by spiking samples in a blind study. Accuracy should be established across the specified range (that is, line of working range) of the analytical procedure. For the assay of the drug substance, accuracy measurements are made by comparison of the results with the analysis of a standard reference material or to compare the results obtained from a second well-characterized independent procedure, the accuracy of which is stated and/or defined.

ICH Guidelines Q2(R1) recommend assessment of accuracy at three levels covering the specified range (i.e. three concentration levels and three replicates at each level of the total analytical procedure). The data should be reported as the percent recovery of the known amount added or as the difference between the mean and true values with confidence intervals.

The % recovery was then calculated by using formula.

$$\% \text{ Recovery} = \frac{(A - B)}{C} \times 100$$

Where,

A: % Total amount of drug estimated

B: % Amount of drug found on preanalyzed basis

C: % Amount of pure drug added.

b) Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous samples. It is expressed as standard deviation or coefficient of variation.

Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Repeatability: Repeatability expresses the precision under the same operating conditionsover a short interval of time. It is also termed as intra-assay precision.

Intermediate precision: Intermediate precision expresses within-laboratories variations:different days, different analysts, different equipment, etc.

Reproducibility: Reproducibility expresses the precision between laboratories(collaborative estudies, usually applied to standardization of methodology).

c) Specificity

It is the ability to assess unequivocally the analyte in the presence of components which may he expected to be present in the sample under consideration. This might include degradants, impurities, matrices, excipients etc.

This definition has the following implications:

i. Identification: to ensure the identity of an analyte.

ii. Purity Tests: to ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, i.e. related substances test, heavy metals, residual solvents content, etc.

iii. Assay (content or potency): to provide an exact result which allows an accurate statement on the content or potency of the analyte in a sample.

e) Linearity

The linearity of an analytical procedure is its ability to obtain test results that are directly proportional to the concentration of analyte in the sample within a given range. A linear relationship should be evaluated across the range of the analytical procedure. It may be demonstrated directly on the drug substance (by dilution of a standard stock solution) and/or separate weighing of synthetic mixtures of the drug product components, using the proposed procedure.

Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods.

f) System suitability

System suitability is a Pharmacopoeial requirement and is used to verify whether the resolution and reproducibility of the chromatographic system is adequate for analysis to be done. The tests were performed by collecting data from five replicate injections of standard solutions.

g) Ruggedness

Ruggedness is the degree of reproducibility of the results obtained under a variety expressed as % RSD. The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under variety conditions such as different laboratories and different days etc.

h) Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Perform experiments by changing conditions such as temperature (±5 °C), change in wavelength (±2 nm), ionic strength of buffers and level of additives to mobile phase.

EXPERIMENTALWORK AND RESULTS

Preparation of Standard Solutions

Stock standard solution of Ozenoxacin

Accurately weighed 10.0 mg of drug Ozenoxacin was dissolved and diluted to 10 ml in 0.1N HCl in a volumetric flask (conc. 1 mg/ml).

Working standard solution of Ozenoxacin

1.0 ml of above stock standard solution was diluted to 10.0 ml with mobile phase (Methanol: 10mM (pH 3)) (48:52% v/v) in a 10.0 ml volumetric flask (conc.100µg/ml). 1.0 ml of this solution was further diluted to 10.0 ml with mobile phase in a 10 ml volumetric flask to give 10µg/ml concentration.

Preparation of Phosphate Buffer Solution (pH 8)

Weigh accurately 0.142gm of sodium di-phosphate & transfer to a 100ml of volumetric flask containing water for HPLC, and then made up the volume up to the mark with water for HPLC.

Preparation of sample solution

Accurately weighed quantity of 1g cream (equivalent to 10mg) of Ozenoxacin was transferred to 10 ml chloroform in a 10 ml volumetric flask. The content was sonicated for 10 min.

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 8 ml of 0.1 NHCI, & transferred to 10 ml volumetric flask. The beaker was washed with 2 portions each of 1ml of 0.1N HCl and washing were added to adjust volume to 10ml with 0.1N HCl. 1.0 ml solution 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 and the solution was filtered through Nylon filter (0.45µ).

Determination of wavelength for detection of Ozenoxacin

The working standard solution of Ozenoxacin (10µg/ml in mobile phase) was scanned in the range of 200-400 nm against solvent blank in diode array detector and spectrum was recorded. UV spectrum shows λmax at 250.0 nm and 293.0 nm, out of which 250 nm was selected as wavelength of detection for HPLC study. The UV spectra of Ozenoxacin is depicted in fig.1

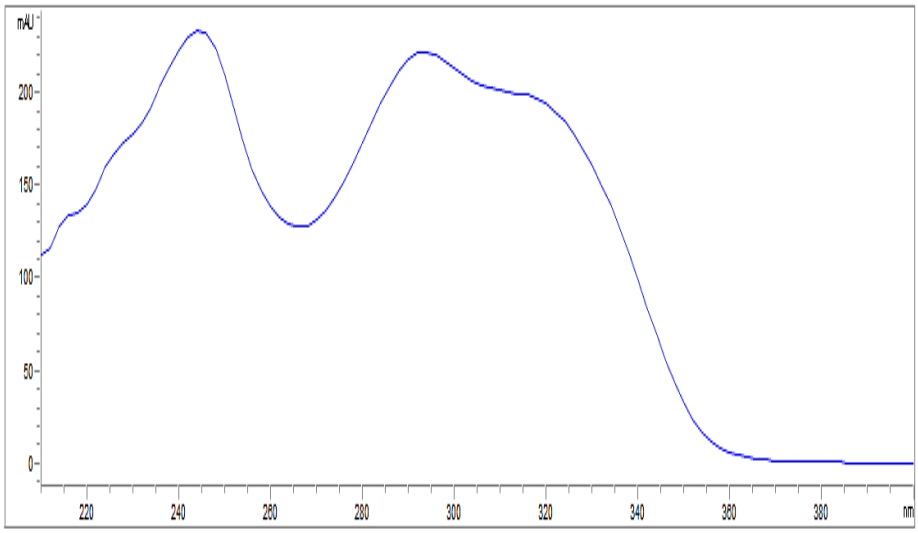

Figure 2: UV-Spectrum of Ozenoxacin in 0.1N HCl

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 3.

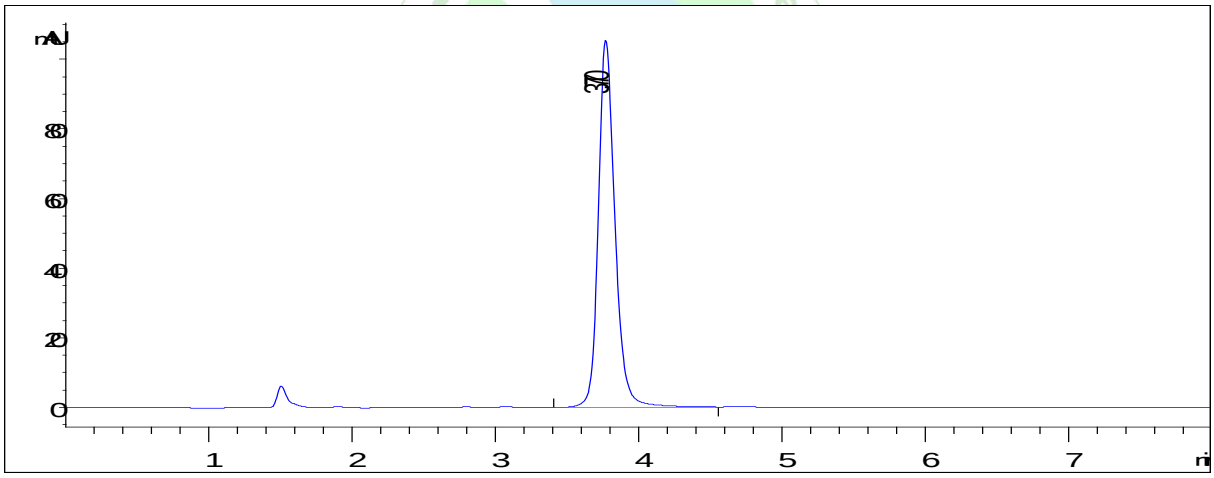

Figure 3: HPLC chromatogram of standard drug Ozenoxacin

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.1.

Table 1: 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**

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.

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.2

Table 2: 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**

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 figure 4 and Table 3 shows the results of linearity study.

Table 3: Results of Study of linearity of Ozenoxacin

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

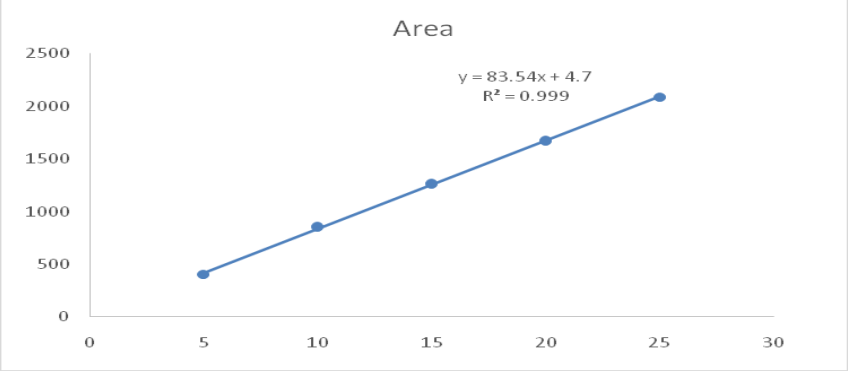

Figure 4: Study of linearity of Ozenoxacin

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 4.

Table 4: 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

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 the results of recovery study are shown in Table 5.

Table 5: 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**

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 6.

Table 6: 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

Ruggedness

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

Inter-day study

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

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.

Different Analysts

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

Table 7: 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**

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 8: LOD and LOQ results of Ozenoxacin

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

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.9.

Table 9: 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

Assay of cream sample

Accurately weighed quantity of 1g cream (equivalent to 10mg) of Ozenoxacin was transferred to 10 ml chloroform in a 10 ml volumetric flask. The content was sonicated for 10 ml. 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 (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 HCl and washing were added to adjust volume to 10ml with 0.1 N HCI. 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 solution was filtered through Nylon filter (0.45µ). Chromatograms of standard and sample solutions were recorded under optimized conditions and the drug content was calculated.

Table 10: Result of assay of Ozenoxacin sample solution

Sr. no.	Wt. of 1% cream (mg)	Peak Area	Amt. of drug Estimated (mg)	% Label claim
1	1102	854.25	10.03	100.31
2	1042	845.08	9.92	99.23
3	1066	849.22	9.97	99.71
4	1023	844.58	9.91	99.17
5	1050	838.12	9.84	98.41
6	1125	860.62	10.10	101.05
Mean				**99.64**
Standard deviation				**0.932**
% Relative standard deviation				**0.935**

SUMMARY AND DISSCUSION

As per the ICH guidelines and regulatory authorities' worldwide, it has become mandatory to establish stability indicating assay method (SIAM) for the drug substance (DS) and drug product (DP) to generate the stability data. SIAM is the validated quantitative analytical method that can detect the changes with time in the chemical, physical, or microbiological properties of the DS and DP, and that are specific so that the contents of active ingredient, degradation products, and other components of interest can be accurately measured without interference. In the present project work, successful attempts have been made to develop SIAM for the estimation of Ozenoxacin.

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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.

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