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Development and Validation of Simple and Stable LCMS Method for the Quantification of Potential Genotoxic Impurities in Ozenoxacin Pure Drug and its Commercial Preparations

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Abstract

Ozenoxacin is an antibiotic drug prescribed to treat various skin infections caused by various bacteria. Various chemical mechanisms such as stille coupling, Buchwald–Hartwig coupling, cyclization and saponification are involved during the process of synthesis of Ozenoxacin. In the process of synthesis, there is a possibility of the formation of related impurities and among them, some are genotoxic impurities. To date, in literature, there is no method reported for analysing Potential Genotoxic Impurities (PGIs) in Ozenoxacin and hence this study was initiated to develop an LCMS method for quantification of two genotoxic impurities of Ozenoxacin viz., nitroso impurity, ester impurity. The analytes were resolved on Alltima C18 column (150×4.6mm; 5 µm particle size) using 0.01 mM ammonium acetate at pH 4.8 and methanol in 80:20 (v/v) at 0.5 mL/min flow rate and 10 µ sample injection volume. The multiple reaction monitoring of the mass fragments confirms the parent ion at m/z of 364, 393 and 496 for Ozenoxacin, Nitroso and Ester impurity respectively with characteristic product ion at m/z 196. The method has a linearity range of 0.05 µg/mL to 1.0 µg/mL for three analytes with detection limits of 0.015, 0.011 and 0.015 µg/mL for Ester impurity, Ozenoxacin and Nitroso impurity respectively. The method was validated and produces acceptable results and can successfully separate the potential genotoxic impurities in spiked commercial samples. Based on the findings, it was concluded that this method can be practically useful for the identification and quantification of potential genotoxic impurities and may apply to the safe use of Ozenoxacin in clinical treatment.

Keywords: Cream Formulation, Ester Impurity, Genotoxic Impurities, LCMS Analysis, Nitroso Impurity, Ozenoxacin

1. Introduction

Ozenoxacin belongs to the quinolone class antibiotic drug prescribed for the treatment of impetigo caused by susceptible bacteria such as *Staphylococcus aureus* or *Streptococcus pyogenes* in adults as well as in 2 months above age paediatric patients[1]. It shows broad anti-microbial activity against both Gram-positive and Gram-negative organisms including the major pathogenic bacterium that causes superficial skin infections (*S. aureus*,

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S. epidermidis, and *S. pyogenes*) and acne vulgaris caused by *Propionibacterium acnes*[2]. It was very effective against the bacteria that develop resistance to fluoroquinolone antibiotics[3]. Ozenoxacin is active against some bacteria that have developed resistance to fluoroquinolone antibiotics[4]. Skin itching, dryness, redness, peeling and flaking are the side effects possible during the usage of Ozenoxacin.

Impurities in a pharmaceutical drug or drug product are the unwanted substances that remain active in active pharmaceutical ingredients or formulations[5]. These impurities arise from the materials used for the synthesis of drugs like precursor materials, solvents, reagents, and catalysts or may be derived as intermediate or by-products in the process of synthesis of drugs. Genotoxic impurities are the impurities that arise as intermediate or reactants in the process of synthesis of a drug and their structural alert related to genotoxicity[5]. Pharmaceutical regulatory organizations like the Food and Drug Administration, United States (US FDA), European Medicines Agency etc., recognise the effect of genotoxic impurities on human health and issued guidelines regarding the presence of genotoxic impurities in new drug formulations. The US FDA and ICH (International Council for Harmonisation) issued guidelines for detecting and quantification of genotoxic impurities in novel dosage forms[6,7]. However, controlling trace impurities in a pharmaceutical drug and drug product was a very tough assignment. Hence researchers are focusing on establishing various processes for minimization of impurities. The identification of the quantity of impurities present in any drug or drug product is the primary step for controlling the impurities. Various techniques were used for identifying and quantifying the trace level impurities in a pharmaceutical drug or the drug product[8].

The literature survey confirms that there is no analytical method available for the analysis of genotoxic impurities in Ozenoxacin. In literature, HPLC analytical methods were reported for the assay of Ozenoxacin in single[9,10] and combination with benzoic acid[2] in formulations. Hence the present study was intended to optimize and validate an accurate LCMS method for the analysis of Ozenoxacin and its potential genotoxic impurities such as nitroso impurity and ester impurity in pure drug and drug products. The molecular structure of Ozenoxacin and its genotoxic impurities in the study are given in Figure 1.

2. Materials and Methods

2.1 Instrumentation

The LCMS analysis was carried out on alliance 2695 LCMS system (Waters, Japan) equipped with triple quadrupole ZQ mass detector (LAA 1369, Waters, Japan), 0. 1-1500 µL sample injectable auto-injector. The analytes were separated on the C18 column of various configurations and the separated eluents were identified and characterized using Masslynx 4.2 (Waters, Japan) software. The mass detector was equipped with an electrospray ionization source operated in positive Multiple Reaction Monitoring (MRM) mode. The mass analyser was set with a 10 L/min flow of drying gas, 30 psi nebulizer pressure, 300°C gas temperature, and spray voltage of 5500 V.

2.2 Chemicals and Reagents

The Ozenoxacin active pharmaceutical ingredient with a purity of 99.32% and its two potential genotoxic impurities in the study such as Nitroso impurity and Ester impurity were procured from Oaknet Healthcare Pvt Ltd, Mumbai, Maharashtra. The pharmaceutical cream formulation containing 1% (w/w) of Ozenoxacin with brand Noxof was purchased from a local pharmacy. LiChropur™ reagents such as ammonium acetate, formic acid and HPLC grade chemicals such as methanol and acetonitrile were procured from Merck chemicals, Mumbai. Membrane filters (0.2 µm) were also purchased from Merck Chemicals, Mumbai.

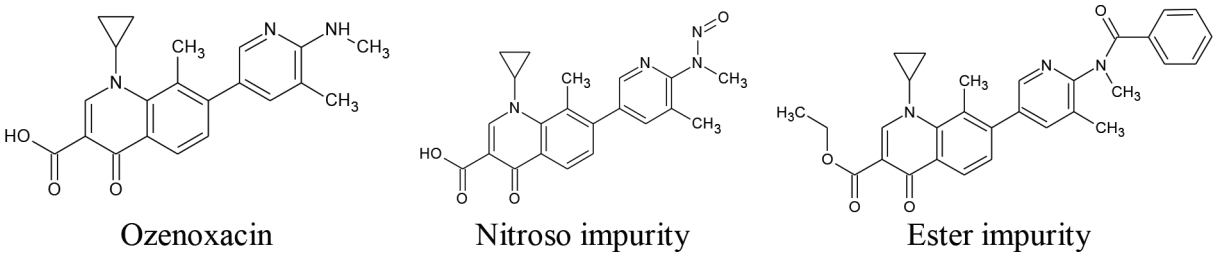


Figure 1. Molecular structure of Ozenoxacin and its genotoxic impurities in the study.

2.3 Preparation of Solutions

2.3.1 Standard Stock Solution

The standard stock solution of Ozenoxacin and its two potential genotoxic impurities such as Nitroso impurity and Ester impurity were prepared separately. 25 mg of Ozenoxacin, Nitroso impurity and Ester impurity were taken in 25 mL flask having 10 mL of methanol separately. The analystes were dissolved in solvent with the use of an ultrasonic bath sonicator for 2 minutes. The un-dissolved particles were removed by filtration using 0. 2µm filters separately. The volume of the flask was made up to the mark to obtain 1000 µg/mL standard solution of Ozenoxacin and its genotoxic impurities separately. The combined standard stock solution was prepared by mixing an equal volume of each analyte into a separate clean flask. The mixed standard solution was utilized for method optimisation and validation study.

2.3.2 Formulation Solution

An accurately weighed 1g cream formulation of Ozenoxacin was taken in a 100 mL flask having 50 mL Methanol. The Ozenoxacin and its impurities if any present were dissolved in solvent with the help of an ultrasonic bath sonicator for 5 minutes. The un-dissolved formulation particles and excipients were removed by filtration through 0.2 micron filters. The solution was filtered into a separate flask and the final volume in each flask was made up to the mark. The formulation solution containing 200 µg/mL equivalent of Ozenoxacin was obtained and the solution was used for evaluating the impurity content in the formulation sample.

2.4 Method Development

The guidelines issued by ICH were adopted for developing the analytical method for analysing Ozenoxacin and its potential genotoxic impurities such as Nitroso and Ester impurity[12]. The method development was performed by optimizing various analytical parameters and in each studied analytical condition, the pure solution having 1 µg/mL of Ozenoxacin, Nitroso impurity and Ester impurity was analysed. The method parameters like stationary phase, composition, pH and flow of mobile phase were changed. The chromatogram results in each studied condition were summarized to evaluate the acceptability of the method.

2.5 Method Validation

The method optimized for analysing Ozenoxacin and its Nitroso and Ester impurity was validated in terms of sensitivity, linearity, precision, reproducibility and stability as per ICH guidelines[13] and literature reported[14-17]. Further, the developed was studied for its applicability for assessing the genotoxic impurities in formulations.

3. Results and Discussion

3.1 Method Development

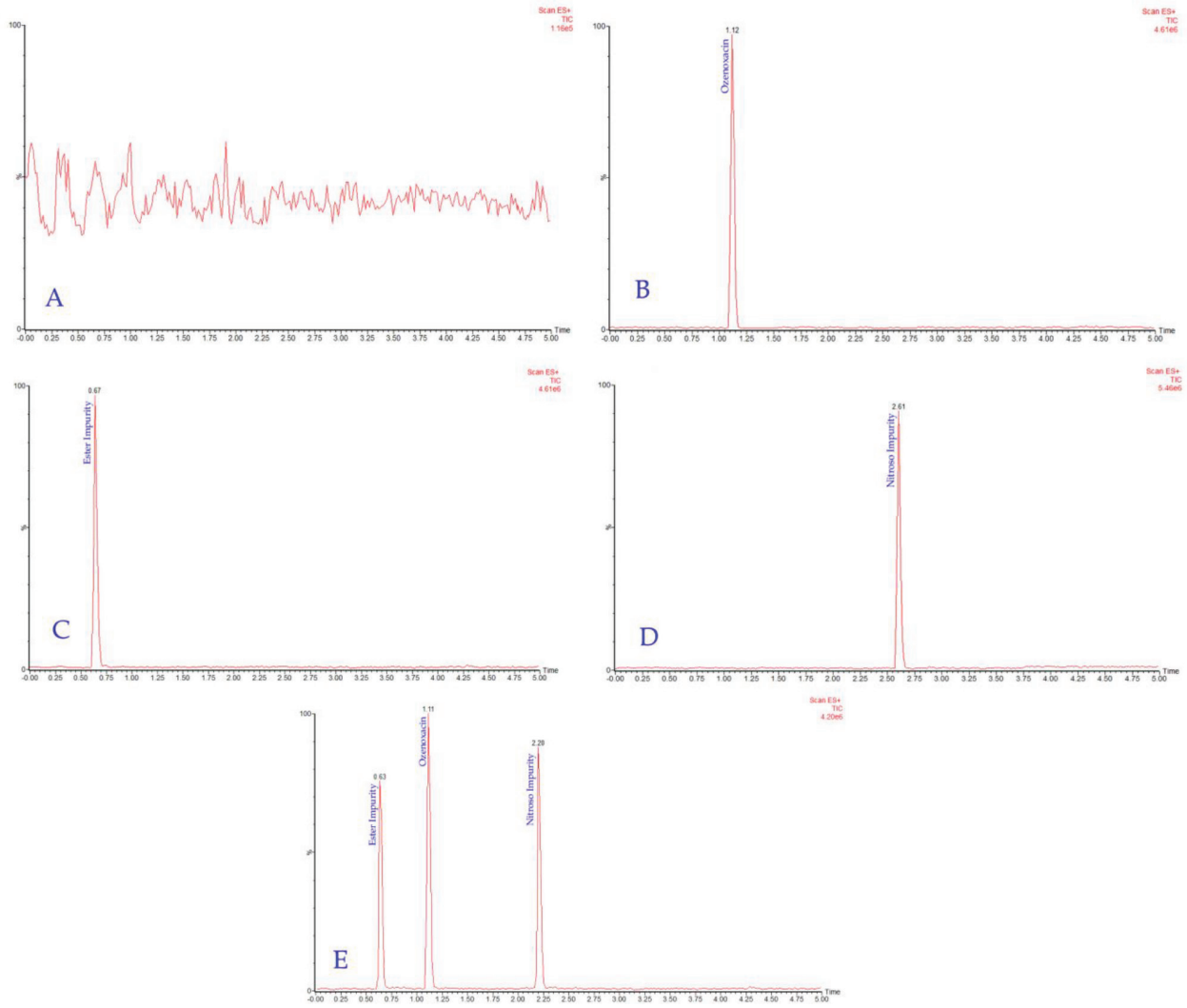
The present work intended to establish a reliable and sensitive LCMS method for the resolution and analysis of Nitroso and Ester impurities of Ozenoxacin. During the development of the method, the separation and detection of impurities at trace levels was prioritised. For this, different carbon-loaded C18 and C8 columns were studied for the separation of genotoxic impurities of Ozenoxacin. During the optimization of the mobile phase, various proportions of solvents such as Methanol, Ammonium acetate and Acetonitrile in different pH values were tested. The flow rate, column temperature and mass operating conditions were studied for the effective separation and detection of genotoxic impurities of Ozenoxacin at trace levels.

Based on the results achieved in each trial condition studied, it was confirmed that the analytes were effectively separated on Alltima C18 (150×4.6 mm; 5 µm particle size) column, 0.01 molar Ammonium acetate at pH 4.8 (using 0.0% Formic acid) and Methanol in the ratio of 80:20 (v/v) at 0.5 mL/min flow and 10 µ sample injection volume. As the molecular structures of Ozenoxacin, Nitroso and Ester impurity have different chemical structures, the mass operating conditions were individually optimized for the analytes in the study and Table 1 presents the mass operating conditions.

On analyzing the standard solution of Ozenoxacin and its impurities, the resultant chromatogram shows well-resolved peaks at 1.11, 0.63 and 2.20 minutes for Ozenoxacin, Ester impurity and Nitroso impurity respectively. The time of elution of the analytes in the individual analysis results shows a good correlation with the retention times observed in the combined standard solution whereas the blank analysis doesn't show any chromatographic detection. The mass fragmentation spectra of the peaks identified for Ozenoxacin and its genotoxic impurities clearly show the well-identified mass fragmentation peaks (Figure 2).

Table 1. Mass analyser conditions for detection of Ozenoxacin and its genotoxic impurities

S No	Analyte	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor (V)	Collision Energy (eV)	Electron Multiplier Voltage (V)	MS1 RES
1	Ozenoxacin	364	196	150	30	500	Wide
2	Nitroso impurity	393	196	130	20	500	Wide
3	Ester impurity	496	196	130	25	500	Wide



(A) Blank chromatogram. **(B)** Chromatograms obtained for standard solution analysis of Ozenoxacin. **(C)** Ester impurity. **(D)** Nitroso impurity. **(E)** and combined standard containing three analytes.

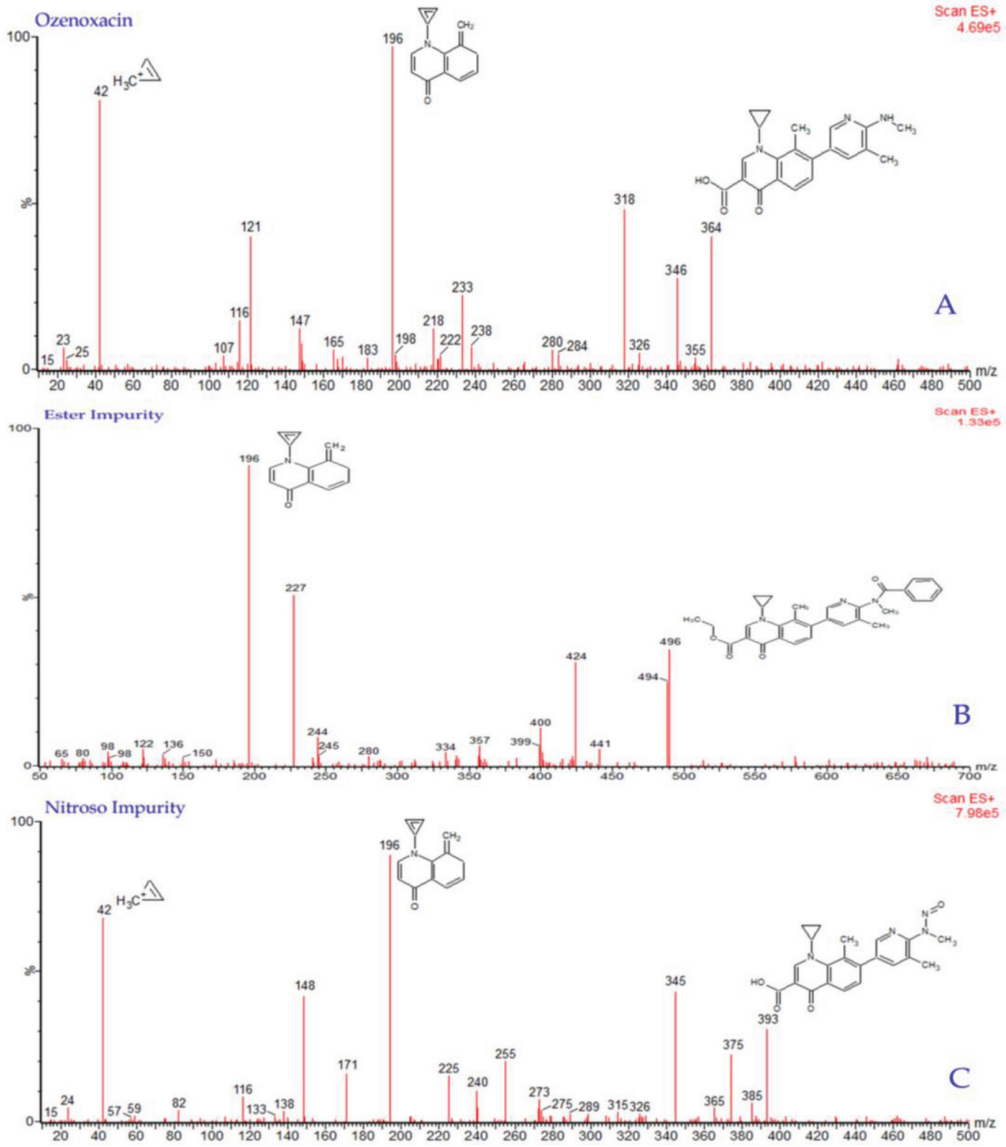
Figure 2. LCMS chromatograms of Ozenoxacin and its genotoxic impurities.

The mass fragmentation spectra of the peak identified at t_R 1.11 minutes show parent ion at m/z 364 and characteristic daughter fragments identified at m/z 196 and 42. The mass spectra of the peak correspond to Nitroso impurity identified at t_R 2.20 min showing parent ion at m/z 393 and daughter fragments identified at m/z 196 and 42. Whereas ester impurity identified at t_R 0.63 minutes shows parent and daughter ion fragments at m/z 496 and 196 respectively. The mass fragments were per the standard and no other detections in the mass spectra of both genotoxic impurities as well as standard. Hence it can be proved

the specificity of method for the resolution and analysis of Ozenoxacin and its genotoxic impurities in the study (Figure 3).

3.2 Method Validation

The method linearity was evaluated in various concentration levels for Ozenoxacin and its genotoxic impurities. The area response of the individual analyte was plotted with its prepared concentration for obtaining the linearity range. Based correlation coefficient of the obtained graphs, it was identified that the method produces accurate fit and well-correlated linearity identified in the concentration level of 0.05-1.0 µg/mL. The correlation coefficient and linear regression equations of the linear calibration curve were assessed by using least-squares linear regression analysis. The regression equation and correlation coefficient (R^2) obtained as y = 866647x + 84589 (R^2 = 0.9991), y = 636854x + 57035 (R^2 = 0.9994) and y = 732926x + 70070 (R^2 = 0.9992) for Ozenoxacin, Ester impurity and Nitroso impurity respectively. Table 2 represents the linearity results observed in the developed method.



(A) Mass fragmentation spectra obtained for standard solution analysis of Ozenoxacin. **(B)** Ester impurity. **(C)**; Nitroso impurity.

Figure 3. LCMS Mass fragmentation spectra of Ozenoxacin and its genotoxic impurities.

Table 2. Linearity results noticed validation of the method developed for quantification of genotoxic impurities of Ozenoxacin using LCMS/MS

S No	Concentration in µg/mL	Peak are Response Obtained		
		Ester Impurity	Ozenoxacin	Nitroso Impurity
1	0.05	81243.5	112328.9	96745.8
2	0.10	124251.3	167985.1	150762.3
3	0.15	155457.3	221458.3	188546.2
4	0.20	185469.8	264847.9	210693.1
5	0.25	222135.8	304515.7	251524.3
6	0.50	368153.5	525757.8	439852.3
7	1.00	695451.8	945187.3	801452.1

The spiked recovery experiment was conducted to evaluate method accuracy. Authentic concentration of the standard solution was spiked with three spiked levels (50, 100 and 150 %) and the solution was analysed in triplicates in the developed method. The percentage recovery in every injection and per cent, RSD in every recovery level for Ozenoxacin and its genotoxic impurities was assessed by correlating the area response of spiked solutions with its corresponding standard regression equation. Good recovery values in the limit of 98-102 and an acceptable percentage RSD of less than 2 were obtained for Ozenoxacin and its genotoxic impurities. Hence, it can be confirmed that the method was accurate. Accuracy study results are represented in Table 3.

The method precision was evaluated by analysing the pure solution containing 20 ng/mL concentration of each analyte in the developed method. The solution was analysed six times in intraday precision, and interday precision. The same solution was prepared and analysed by three different analytes for the evaluation of the robustness of the method. The same strength solution was examined by a 5 per cent change in the optimized conditions for evaluating the ruggedness. The method produced acceptable results in all the studied tests proving that the method was enough fit for the quantification of potential genotoxic impurities of Ozenoxacin. Table 3 summarises the results observed in the study.

The LOD (Detection Limit) and LOQ (Quantification Limit) concentrations of impurities as well as standard Ozenoxacin were assessed to evaluate the sensitivity of the developed method. The signal (n) to noise (n) ratio approach was assumed for evaluating method sensitivity and s/n of 3 and 10 was assumed as LOD and LOQ respectively. The LOD as obtained as 0.015, 0.011 and 0.015 µg/mL whereas LOQ was obtained as 0.050 µg/mL, 0.036 and 0.050 µg/mL respectively for Ester impurity, Ozenoxacin and Nitroso impurity. The reproducibility and repeatability of Ozenoxacin and its genotoxic impurities in the study were evaluated at LOQ concentration level. The LOQ level standard solution of Ozenoxacin and its genotoxic impurities was evaluated 6 times in one day and 6 times in three different days. The percentage RSD of resultant area responses was calculated and the achieved results were within the permissible limits confirming that the method has high reproducible and acceptable sensitive levels.

The solution stability of Ozenoxacin and its genotoxic impurities in the optimized method was determined by incubating the standard solution in an auto-sampler vial for 48 hours at 25°C. The solution was analysed periodically every 6 hours of time interval and per cent stability was evaluated by correlating the peak area obtained in each time interval with the freshly prepared standard solution of the same concentration level. On careful observation of results, it can be observed that there are no considerable variations were observed in the area response of standard and studied impurities during the stability study. There is no sight of additional compounds/detections identified within the run time confirming that the samples were stable up to 48 hours.

The formulation solution prepared from the cream formulation of Ozenoxacin was examined in the developed method for the determination of potential genotoxic impurities of Ozenoxacin in formulations. Similarly, the formulation sample spiked with a known quantity of impurities was also studied. The un-spiked sample chromatogram not show any peak corresponding

Table 3. Accuracy results noticed validation of method developed for quantification of genotoxic impurities of Ozenoxacin using LCMS/MS

S. No	Analyte	Level	Strength in µg/mL				% Recovery	% RSD
			Target	Spiked	Final	Recovered Mean±SD		
1	Ester impurity	50%	0.1	0.05	0.15	0.149±0.001	99.070±0.406	0.410
2		100%	0.1	0.1	0.2	0.198±0.001	98.940±0.279	0.282
3		150%	0.1	0.15	0.25	0.247±0.002	98.693±0.631	0.640
4	Ozenoxacin	50%	0.1	0.05	0.15	0.148±0.001	98.613±0.348	0.353
5		100%	0.1	0.1	0.2	0.199±0.002	99.327±0.769	0.774
6		150%	0.1	0.15	0.25	0.248±0.002	99.347±0.957	0.964
7	Nitroso impurity	50%	0.1	0.05	0.15	0.149±0.001	99.177±0.540	0.545
8		100%	0.1	0.1	0.2	0.199±0.002	99.360±0.933	0.939
9		150%	0.1	0.15	0.25	0.249±0.003	99.700±1.288	1.292

Table 4. The summary results noticed validation of the method developed for quantification of genotoxic impurities of Ozenoxacin using LCMS/MS

S No	Parameter	Results Observed		
		Ester Impurity	Ozenoxacin	Nitroso Impurity
1	Linearity range	0.05-1.0 µg/mL	0.05-1.0 µg/mL	0.05-1.0 µg/mL
2	% RSD Intraday Precision (n=6)	0.16	0.21	0.46
3	% RSD Interday Precision (n=6)	0.39	0.45	0.83
4	% RSD Ruggedness (n=6)	0.56	0.44	0.40
5	% Accuracy in 50% spiked level (n=3)	99.07	98.61	99.18
6	% RSD in 50% spiked level (n=3)	0.41	0.35	0.54
7	% Accuracy in 100% spiked level (n=3)	98.94	99.33	99.36
8	% RSD in 100% spiked level (n=3)	0.28	0.77	0.94
9	% Accuracy in 150% spiked level (n=3)	98.69	99.35	99.70
10	% RSD in 150% spiked level (n=3)	0.64	0.96	1.29
11	Per cent Change Robustness + change in composition of mobile phase - change in composition of mobile phase + change in pH of mobile phase - change in pH of mobile phase + change in column temperature - change in column temperature	 0.78 0.58 1.14 1.25 0.20 0.13	 0.20 0.03 0.58 0.36 0.48 0.43	 0.20 0.24 1.41 1.51 0.32 0.57
12	Per cent stability at 48 h (n=6)	96.25	98.12	97.08
13	LOD in µg/mL	0.015	0.011	0.015
14	LOQ in µg/mL	0.050	0.036	0.050
15	Per cent assay in formulation	Not detected	99.21	Not detected

to the impurities whereas the spiked sample chromatogram clearly shows the peaks corresponding to the genotoxic impurities in the study. Both the spiked and unspiked samples visualize the peaks corresponding to the Ozenoxacin. There is no detection of other impurities nor additional detections were noticed in the sample chromatogram suggesting that the method was fit for analysing the potential genotoxic impurities of Ozenoxacin. Table 4 presents the summarized results of this study.

The findings reported in this study were correlated with the methods reported in the literature. In the literature, no method was reported for quantification of genotoxic impurities of Ozenoxacin. Hence this study will be helpful for trace-level identification and quantification of trace-level genotoxic impurities of Ozenoxacin.

4. Conclusion

In this study, we presented a simple and sensitive LCMS approach that was capable of resolving, identifying and quantifying potential genotoxic impurities such as Ester and Nitroso impurities in Ozenoxacin bulk drug as well as in pharmaceutical cream formulations. The analytes were effectively resolved on Alltima C18 (150×4.6 mm; 5 µm particle size) column utilizing 0.01 molar ammonium acetate at pH 4.8 (using 0.0% formic acid) and methanol in 80:20 (v/v) at 0.5 mL/min flow as mobile phase and mass detector which was operated in MRM positive ion mode. The method validation was performed as per ICH norms and achieved results under acceptable levels. The method was linear in the 0.05-1.0 µg/mL concentration range and proved to be accurate, precise specific and stable. The method was very sensitive and can detect up to 0.015, 0.011 and 0.015 µg/mL respectively for Ester impurity, Ozenoxacin and Nitroso impurity. The samples and standards prepared in the developed method were confirmed to be stable for at least 48 hours. Based on the experimental findings, it was concluded that the method optimized could be significantly useful for the resolution and evaluation of Ester and Nitroso impurity in Ozenoxacin standards as well as formulations.

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