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Research Article

A HIGHLY SPECIFIC, SENSITIVE AND HIGH THROUGHPUT METHOD FOR THE DETERMINATION OF OFLOXACIN IN HUMAN PLASMA BY LIQUID CHROMATOGRAPHY COUPLED WITH TANDEM MASS SPECTROMETRY

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ABSTRACT

A suitable, highly specific and sensitive liquid chromatography tandem mass spectrometric method has been developed and validated for the determination of Ofloxacin from 100µL of human plasma by Protein Precipitation. Ciprofloxacin was used as an internal standard. Quantified by the transition, 362.200→261.420, 332.205→282.500 for Ofloxacin and Ciprofloxacin respectively and detected by TSQ Quantum Ultra triple quadrupole mass spectrometer. Detection was carried out by using ESI source in positive polarity. Chromatographic separation of analyte and internal standard were carried out by reverse phase C18 column at the flow rate of 0.500mL/min with mobile phase of Acetonitrile: 0.02% Formic acid (60:40) v/v. The assay of Ofloxacin was linear over the range of 20.133 ng/mL to 2001.800 ng/mL with a precision of ≤0.45 % and ≤6.88 % respectively, Mean extraction recovery obtained was 89.84%. Samples were stable at room temperature for 6 hrs and also stable at four freeze-thaw cycle. The aim is to develop a suitable, highly specific, and sensitive analytical method for the quantitation of Ofloxacin in the low nanogram range in human plasma.

Keywords: Ofloxacin; LC-MS/MS; Validation; Human Plasma

INTRODUCTION

Ofloxacin is a broad spectrum quinolone antibiotic. Chemically, it is [9-Fluoro-2,3-dihydro-3-methyl-10- (4-methyl-1-piperazinyl) -7-oxo-7H-pyrido [1,2,3-de] -1,4-benzoxacine-6-carboxylic acid [1], is mainly used for the treatment of infections (respiratory tract, kidney, skin, soft tissue, Urinary tract infection), urethral and cervical gonorrhoea [2]. Its molecular formula is $C_{18}H_{20}FN_3O_4$ having a molecular weight of 361.37. g/mol [1]. It is sparingly soluble in aqueous solutions with pH 7 and freely soluble in aqueous solutions with pH above 9 [3]. Literature survey revealed that a number of methods are available for quantitative estimation of ofloxacin by using techniques like Spectrophotometric [4-5], High Performance Liquid Chromatography (HPLC) [6-7] ,High Performance Thin Layer Chromatography (HPTLC) [8] and Liquid Chromatography tandem mass spectroscopy (LCMS/MS) [9]. So far it is well known that there is no high throughput method for the determination of Ofloxacin in human plasma. Authors propose a highly specific, sensitive and high throughput analysis method with very good analyte recovery and also with less matrix effect. The suitability of this method for the quantitative determination of ofloxacin was assessed and established by validation. Ofloxacin acts on DNA gyrase and toposiomerase IV, enzymes which, like human topoisomerase, prevents the excessive super coiling of DNA during replication or transcription. By inhibiting their function, the drug thereby inhibits normal cell division [2].

The current article describes a validated LC-MS/MS method for estimation of Ofloxacin, using 0.02% Formic acid buffer and Acetonitrile in the ratio of 40:60%v/v. The column used was Zorbax C18, (50x 4.6mm, 3.5µ) with flow rate of 0.5 ml/min. In this context, authors present a simple, fast, and sensitive analytical method for determination of ofloxacin in human plasma using HPLC coupled with tandem mass spectrometry.

MATERIALS AND METHODS

Materials and Reagents

Ofloxacin and the internal standard Ciprofloxacin is purchased from sigma. Methanol (HPLC grade), Acetonitrile (HPLC), Formic acid (GR grade) were obtained from Merck and Milli-Q-Water was collected from In-house.

Instruments

The Liquid chromatography coupled with tandem Mass Spectrometer (LC-MS/MS) system consists of a Finnigan Surveyor aAutosampler, Surveyor LC Pump Plus solvent delivery system and a column Oven (Thermo Electron Corporation) used for ion separation. Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer was used for ion detection. An Electron Spray Ionization (ESI) source was used. Data was collected and processed using LC Quan Version. 2.5.6 Data collection and integration software.

Chromatographic Condition

The Liquid Chromatographic separations were carried out by using Zorbax, C18, 50X4.6 mm, 3.5µ column (Agilent). Column temperature was held at 30°C. Auto sampler tray temperature was 10°C. Mobile phase is composed of Acetonitrile: 0.02% Formic acid (60:40) v/v with flow rate of 0.500 mL/min and the run time is 3.00 min. A typical injection volume of 10.0µL was injected into the mass spectrometer.

MS/MS Detection

Precursor ions for analyte and internal standard were determined from mass spectra obtained by the TSQ mass spectrometer. TSQ mass Spectrometer includes an electronically-controlled, integrated syringe pump. The Mass spectrometric conditions for Ofloxacin and the internal standard were optimized by separate infusion into the MS at a flow rate of 10µL/min constantly while adjusting MS parameters to achieve maximal intensity. Electro-spray ionization in positive ion mode (ESI+ve) was used for ionization and selective reaction monitoring (SRM) mode was chosen for detection. The optimized precursor ions pairs were m/z 362.200→261.420 for Ofloxacin and 332.205→282.500 for Ciprofloxacin (Refer was shown in Figure 1. and 2.). The optimized MS parameters were as follows: Ion Spray voltage: 5000volt, Sheath gas pressure: 50psi, Auxiliary gas pressure: 12psi, Capillary temperature: 320°C. Collision Pressure: 1.5psi. Peak areas were automatically integrated using LC Quan Version 2.5.6 (Thermo Corporation).

Preparation of Calibration standards and quality control samples

The calibration standards and the quality control (QC) samples were prepared from separate stock standard solutions. The concentration of Ofloxacin used for preparing calibration standard and quality control was 1001.899 µg/mL were prepared in Methanol. The Spiking solutions of calibration standards and quality control concentrations were prepared in Acetonitrile: 0.02% Formic acid

(60:40) %v/v. The calibration standards in human plasma samples were prepared from corresponding spiking solutions into blank human plasma to provide concentrations range between 20.133 ng/mL to 2001.800 ng/mL. The Quality control samples were prepared from corresponding spiking solutions in human blank plasma to attain the concentration of 20.239 ng/mL (Lower Limit of Quantification Quality Control), 50.597 ng/mL (Lower Quality Control), 903.512 ng/mL (Middle Quality Control) and 1701.530 ng/mL (Higher Quality Control) for Ofloxacin. For the spiking typically, the spiking solutions volume of 20µL were spiked into 980µL of human blank plasma. The Internal standard stock solution 1001.023 µg/mL of Ciprofloxacin was prepared in methanol. Working internal standard solution 1.001 µg/mL was prepared in Acetonitrile: 0.02% Formic acid (60:40) %v/v.

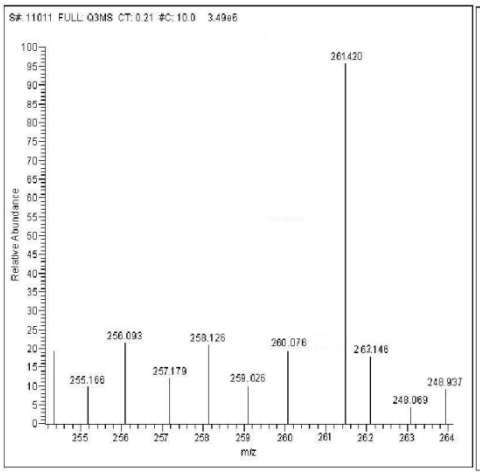

Figure 1: Product ion of Ofloxacin.

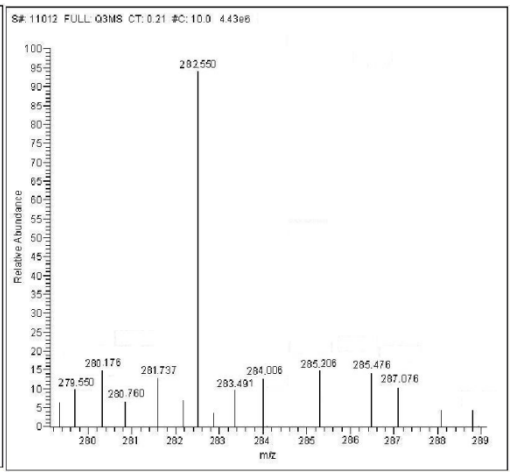

Figure 2: Product ion of Ciprofloxacin

Sample Extraction

An aliquot of 0.100ml of plasma sample was taken into a clean RIA vial. After addition 50 µl of Internal Standard (1.001 µg/ml) samples were vortexed well for approximately 10 seconds. For extraction of Ofloxacin from the plasma samples 1.5ml of Acetonitrile was added and vortexed in a Vibromax for 10 minutes at 2500rpm. Followed by extraction, the samples were subjected to centrifugation at 4500rpm for 5 minutes at 4ºC and 0.500ml of the supernatant was evaporated till dryness under a stream of Nitrogen gas. Finally, the residue was reconstituted with 0.500ml of mobile phase and 10 µl of the sample volume were injected into LCMS/MS.

Validation

Carry over

Carry over has not been found by this method.

Selectivity and Specificity

Blank human plasma from six different lots (including one Haemolysed and one Lipimic) were processed without Analyte and internal standard. And with the same six lots, LLOQ level is processed to evaluate the presence of any interference at the retention time of Analyte and Internal standard.

Matrix factor

Evaluated the matrix factor at low and high quality control concentrations, to ensure that the precision, selectivity and sensitivity are not compromised due to a change in matrix. Matrix factor can be termed as the quantitative measurement of the matrix effect. Aqueous mixtures of internal standard and analyte at concentrations representing 100% extraction of internal standard and analyte at low and high QC concentrations were prepared as Reference Samples**.** Processed duplicate 6 different lots of blank matrices (from six individuals, including, one Haemolysed and one Lipimic). Evaporated Samples were reconstituted with reference sample; it is compared with respective aqueous reference sample prepared in mobile phase.

Calibration Curve and Linearity

The nine-point calibration curve was constructed by plotting, peak area ratio of Ofloxacin to their corresponding internal standard versus Ofloxacin concentrations. A linear regression with weighing factor of linear $1/x^2$ was applied.

Within the batch and between the batch accuracy and precision

Within the batch precision and accuracy were determined by analysis of six replicates of each QC sample (n = 6) at LLOQ QC, LQC, MQC and HQC concentration levels extracted with a set of standards in one batch. The same procedure was repeated on different day with new samples to determine between the batch precision and accuracy.

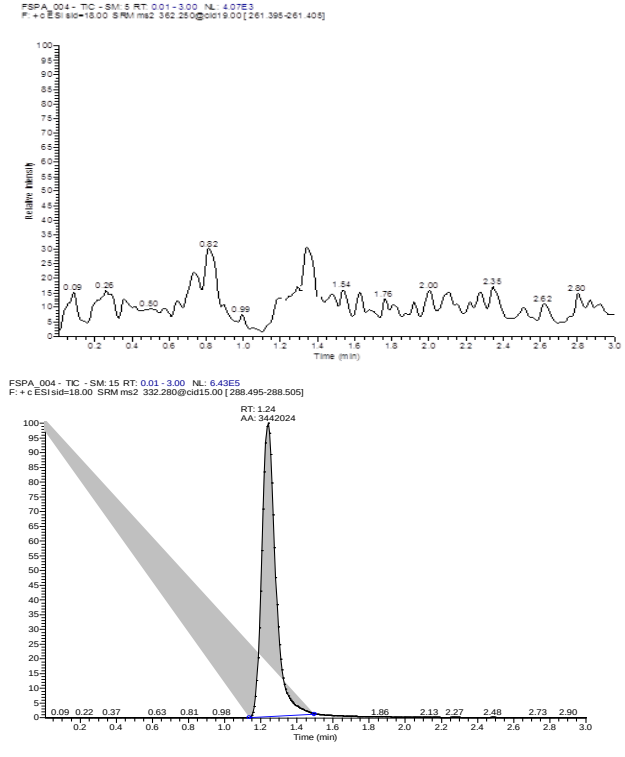

Figure 3: Blank + IS

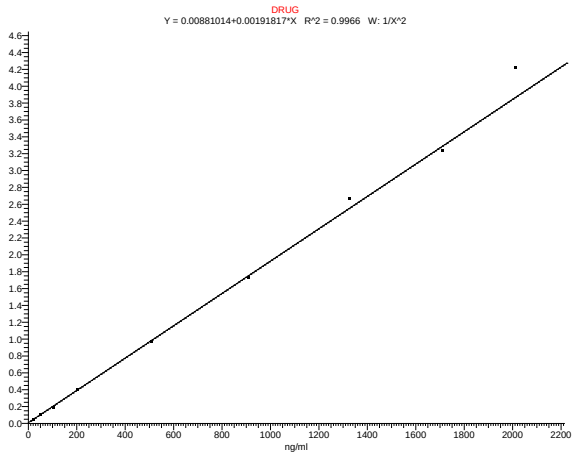

Figure 4; Linearity curve

Recovery

Recovery is carried out to evaluate the loss of drugs and/or internal standards during sample extraction. The drugs and internal standards area counts from extracted QC samples were compared with corresponding QC's reference sample to evaluate any loss of either drugs or internal standards. No acceptance criteria were applied to this parameter, but it is preferable to observe consistent recovery for all three QC levels except LLOQ QC.

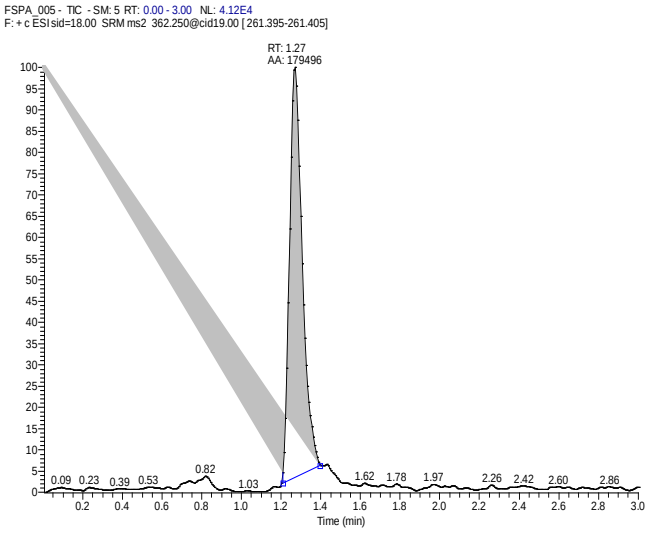

Figure 5: Standard 1

Stability

Stability of both drugs in different matrices and under different conditions was evaluated. The detailed tests are described below. Stability was assessed by comparing the mean concentration of the stored QC samples with the mean concentration of freshly prepared QC samples. Drug stability in pooled human blank plasma is a function of the storage conditions, the chemical properties of the drug and the matrix. The following tests were performed to evaluate the stability, Short-term and Long-term Stock solution stability, Bench top stability, Freeze and thaw stability, Autosampler stability, Wet Extract Stability, Dry extract stability, Long Term stability In Matrix.

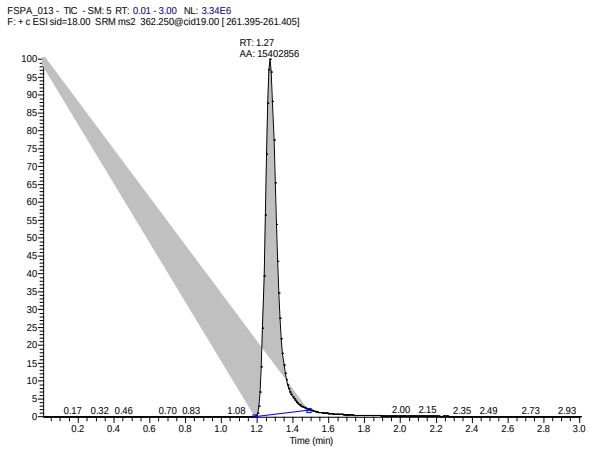

Figure 6: Standard 9

RESULT AND DISCUSSION

Method development

The main objective was to develop and to validate a suitable, highly specific, and sensitive analytical method for the quantitation of Ofloxacin in the low nanogram range in human plasma. During MS tuning, it was found that Ofloxacin were better detected in the positive ion mode.

In the optimization of chromatographic condition, during method development, Ofloxacin had a problem in peak shape in most of the mobile phases, which was reported as earlier [6-9]. Finally a mobile phase composed of Acetonitrile: 0.02% Formic acid (60:40) v/v was found to provide better results. A very low quantification level of 20.133 ng/mL was achieved, which was found to be lower than that of the reported LCMS/MS methods [9]. During the optimization of extraction procedure, it was observed that the matrix effect was not being eliminated by using Liquid-Liquid Extraction methods. This may be because of the reasons like less extraction recoveries, experienced with different solvents. Solid phase extraction was the better alternative when compared with Liquid-Liquid Extraction and protein precipitation. But, if either Liquid -Liquid Extraction or protein precipitation can resolve the issues like matrix effect and recovery, they will be a better alternative to the Solid-phase extraction procedures. This will help the researchers to avoid the cost constraints. Hence the authors propose an extraction procedure by using protein precipitation with matrix effect being eliminated because of good extraction recoveries (Refer Tables 1, 2 and 4). Ciprofloxacin was used as an internal standard. It is known that the shorter column gives shorter analysis time. Different types of shorter column were tried during the method development, but most of the columns are exhibiting more matrix effect as the drug is eluting in the void volume. Finally better results were obtained with Zorbax, C18, 50X4.6 mm, 3.5µ column, where in analytes were not eluted in the void volume and thus matrix effect is being eliminated. The run time is 3.00 min, which is very lower than the methods reported earlier [9]. As per reported literatures, there is no high throughput LCMS/MS method for the analysis of ofloxacin [9]. Researchers were successful in providing an extraction procedure with less matrix effect, better repeatability and good peak shape. More over, the entire procedure is very simple and precise owing to other advantages over cost and time. In the proposed method, relative matrix effect was not found. The method developed was novel, rapid, and selective and high-throughput as LCMS/MS was used for the determination of Ofloxacin.

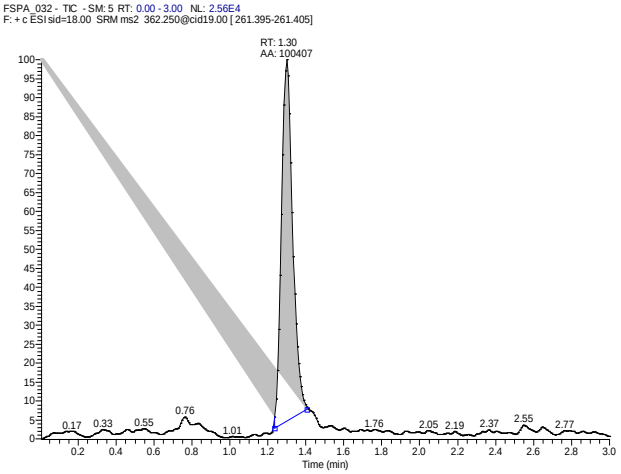

Figure 7: LLOQ QC

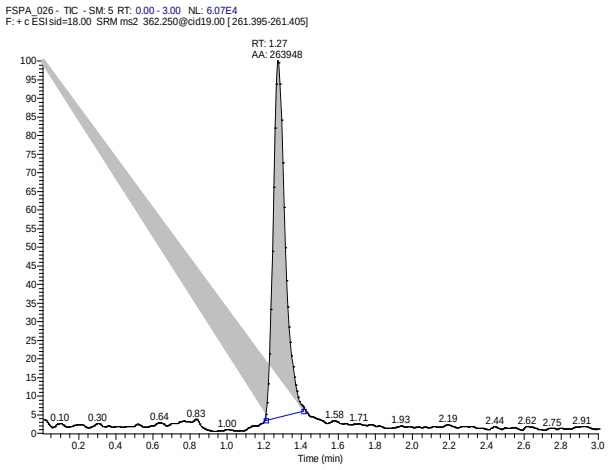

Figure 8: LQC

Validation

Carry over

Carry over has not been found by this method.

Selectivity and Specificity

No interference from the blank plasma at the retention time of the Analyte and Internal standard.

Matrix Effect

Observed % CV for matrix Effect is 6.62 % and 3.34 % for LQC, 3.05 % and 2.67 % for HQC for Ofloxacin and Internal standard respectively. The detailed results are shown in Tables 1 and 2. All six matrix lots showed very similar matrix effect for both analyte and their corresponding internal standard.

Table 1: Matrix Effect for analyte

	Drug-Ofloxacin			
S.No	**Aqueous Sample**		**Post Extracted Sample**	
1	2173823	48915	2257992	49936
2	2151182	52251	2458198	54422
3	2108587	57310	2362500	59575
4	2100986	48864	2304363	50709
5	2119987	50772	2284647	55482
6	2044280	45319	2342498	52320
Mean	2116474.2	50571.8	2335033.0	53740.7
%CV	2.12	7.98	3.05	6.62

Table 2: Matrix Effect for IS

S.No	**Drug-Ciprofloxacin**			
	Aqueous Sample		**Post Extracted Sample**	
	HQC	LQC	HQC	LQC
1	570637	502462	574527	512108
2	584484	526690	597003	520917
3	547342	500219	604491	495754
4	560711	527306	570539	513147
5	571645	497977	565194	481538
6	551176	495527	586675	527649
Mean	564332.5	508363.5	583071.5	508518.8
%CV	2.47	2.88	2.67	3.34

Calibration Standard and Linearity

For four consecutive batches, the calibration curves showed an overall accuracy of 97.66 % - 103.82 % with % CV of 0.45 % - 6.88 % for Ofloxacin. Detailed results are shown in Table 3. The Calibration Standards met the acceptance criteria.

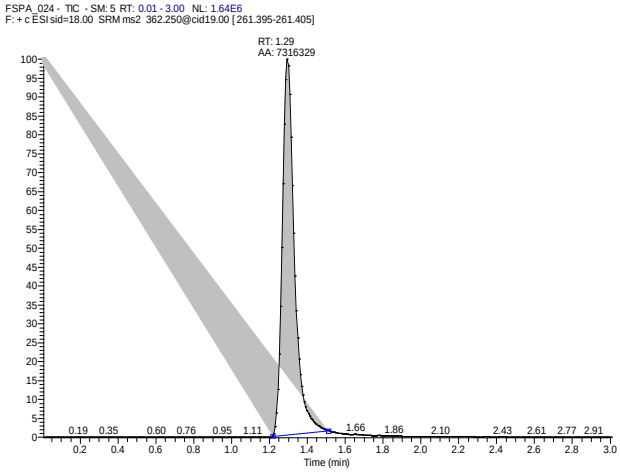

Figure 9: MQC

Table 3: Accuracy and Precision of Calibration Standards for Ofloxacin

	Theoretical concentration (ng/mL)								
Nominal Conc.	**20.133**	**50.585**	**101.170**	**202.339**	**505.848**	**903.278**	**1318.686**	**1701.530**	**2001.800**
Batch 01	20.248	50.467	89.547	203.681	481.231	956.326	1354.218	1745.859	2008.314
Batch 02	19.894	49.687	100.981	199.475	498.957	905.351	1321.957	1725.245	2011.212
Batch 03	20.680	47.745	105.745	201.698	487.258	925.125	1342.901	1709.031	1991.012
Batch 04	21.123	49.981	99.547	197.984	508.651	964.381	1410.485	1821.012	2004.938
Mean	20.486	49.470	98.955	200.710	494.024	937.796	1357.390	1750.287	2003.869
Precision	2.60	2.41	6.88	1.25	2.47	2.93	2.79	2.83	0.45
Accuracy	101.75	97.80	97.81	99.19	97.66	103.82	102.94	102.87	100.10

Table 4: Stability of Ofloxacin

Parameter	Bench top		Auto Sampler		Wet Extract		Dry Extract		Freez Thaw	
QC level	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
Precision	2.38	4.15	2.55	5.72	1.66	2.99	1.96	2.28	1.04	2.30
% Stability	103.7	100.58	99.06	97.53	99.91	97.79	99.69	95.34	100.26	95.16

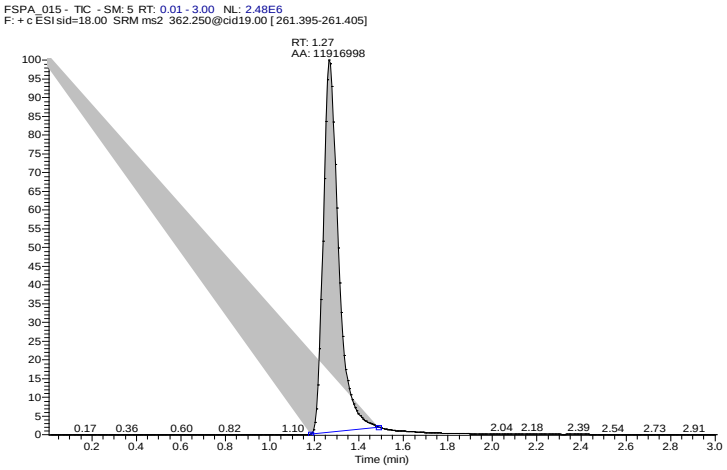

Figure 10: HQC

Accuracy and Precision

Table 5: show within the batch and between the batch accuracy and precision for the Ofloxacin. The method was found to be highly accurate and precise for Ofloxacin. RSD for within the batch and between the batch assay were obtained for all QC levels including LLOQ QC were met the acceptance criteria.

Table 5: within the batch and between the batch Accuracy and Precision for Ofloxacin

Analytes	Ofloxacin	
Parameter	Within the Batch	Between the Batch
Accuracy (%)	98.00-111.27	101.98-105.37
Precision	1.39-9.62	2.40-8.71

Recovery

Table 6: show the overall recovery of 89.84 % for Ofloxacin, and 98.26 % for Ciprofloxacin were obtained. Ofloxacin and Ciprofloxacin shows consistent recovery results for all three QC levels.

Table 6: Recovery of Analyte and IS

Recovery (%)		
QC Level	Ofloxacin	Ciprofloxacin
LQC	90.78	96.42
MQC	91.27	100.36
HQC	87.49	98.00
Mean Recovery	89.84	98.26
%CV	2.29	2.02

Stability

Stability of Ofloxacin in human plasma under different conditions was evaluated. The detailed results are shown in Table 4: as seen from the table, four freeze/thaw cycles, 6 h room temperature storage and 6 hrs autosampler stability, 6 hrs dry extract stability and 6 hrs stock solution stability at room temperature has been established. In addition, 8 days stability for standard stock solutions and wet extract stability shown for 6 hrs were established. All of these demonstrate the ruggedness of the method.

CONCLUSION

A suitable, highly specific and sensitive LC-MS/MS method has been described for the determination of Ofloxacin in human plasma. This method is linear over the range of 20.133 ng/mL to 2001.800 ng/mL for Ofloxacin. Using Zorbax, C18 (50x4.6mm), 3.5µ column (Agilent), the chromatographic elution step is undertaken in a short time with high resolution. The total run time is 3.00 min. Hence, this method is useful for the high-throughput analysis of subject samples. Expected recoveries were observed in the present processing technique for all three QC levels. There is no relative matrix effect in our quantitative analysis. The values obtained from system suitability demonstrated the suitability of the system for the analysis of Ofloxacin.

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