Original Article

A conventional HPLC-MS method for the simultaneous determination of ofloxacin and cefixime in plasma: Development and validation

Abstract

Objective: A simple, rapid, and sensitive high performance liquid chromatography-mass spectrometry (HPLC-MS) method was developed and validated for the simultaneous determination of ofloxacin (OFL) and cefixime (CEF) in human plasma using the moxifloxacin as internal standard.

Methodology: Analytes were separated using an Agilent LCMS system equipped with a Zorbax eclipse XBD C_{18} column (150 mm × 4.6 mm i.d., 5 µm) and using a mobile phase consisting of a mixture of acetonitrile, methanol and 0.5% formic acid in a ratio of 23:10:67% v/v and flow rate was set at 0.6 mL/min. Plasma samples were extracted using the protein precipitation with acetonitrile and analyzed by positive ion mode.

Results: The linearity of the proposed method was investigated in the concentration range of 4-500 ng/mL (r = 0.9996) for OFL and 40-6000 ng/mL (r = 0.9998) for CEF. The lower limits of quantification were 4 ng/mL and 40 ng/mL for OFL and CEF respectively, which reach the level of both drugs possibly found in human plasma. Further, the reported method was validated as per the ICH guidelines and found to be well within the acceptable range.

Conclusion: The proposed method is simple, rapid, accurate, precise, and appropriate for pharmacokinetic and therapeutic drug monitoring in the clinical laboratories.

Key words:
Cefixime, HPLC-MS, ofloxacin, plasma, validation

Introduction

Ofloxacin (OFL) ((±)-9-fluro-2, 3 dihydro-3-methyl-10 [4-methyl-1-piperazynyl-7-oxo-7H-pyrido [1, 2, 3-de]-1, 4-benzoxacine-6-carboxilic acid [Figure 1a]) is one of the most frequently used fluorinated quinolone antibiotics.[1] It is potent 3rd generation fluorinated quinolone antibiotic and mechanism of action is belived to at bacterial Deoxyribonucleic acid gyrase and topoisomerase IV.[2]

Cefixime (CEF) ([6R, 7R]-7-[[2-[2-amino-1,3-thiazol-4-yl]-2 [carboxymethyloxyimino] acetyl]amino]-3-ethenyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid [Figure 1b]). It is effective against bacteria causing infection of the ear, throat, urinary tract, gonorrhea, and pneumonia.[3,4] CEF is also used for the treatment of multidrug-resistant enteric fever and pharyngitis in children. It is the best oral antibiotic for switch therapy due to its very good efficacy and safety profile and an inexpensive nature.

A combination of OFL and CEF is available in the market, which is highly active against typhoid fever, urinary and respiratory tract infections, noscomial infections, soft-tissue, and intra-abdominal infections caused by bacteria.[5-7] Two analytical methods namely HPLC[8] and HPTLC[9] are reported for the simultaneous determination of OFL and CEF in pharmaceutical preparations, but for the simultaneous estimation of OFL and CEF in human plasma has not been reported so far. On the other hand, reported methods for the determination of OFL and CEF were in

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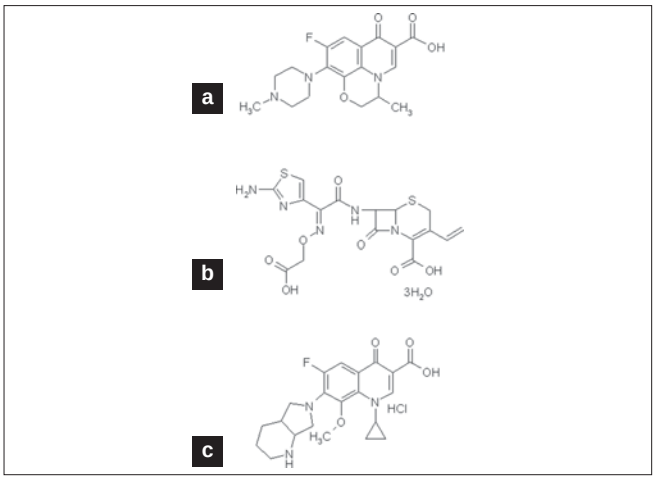

Figure 1: Structures of ofloxacin (a) cefixime trihydrate (b) and moxifloxacin (c)

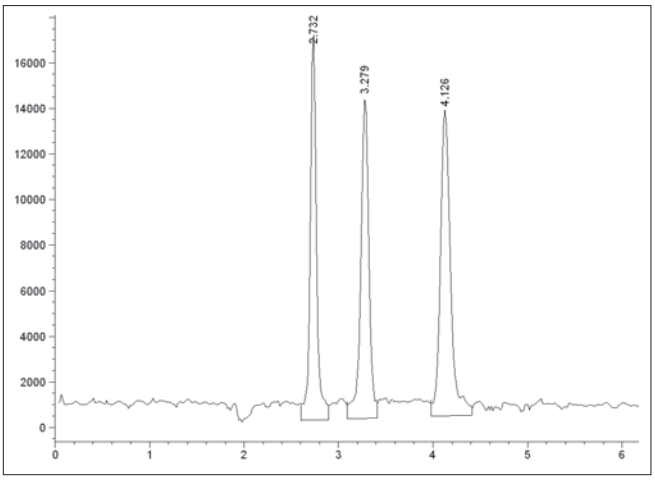

Figure 2: Chromatogram of ofloxacin, cefixime and moxifloxacin in scan mode

single or with other drugs in pharmaceutical preparations and biological fluids. Some of the other reported methods were spectrophotometry,[10] fluorometry,[11-13] HPLC,[14-23] liquid chromatography tandem mass (LC-MS/MS)[24,25] and capillary electrophoresis.[26-30] Further, most of the reported methods for the determination of OFL and CEF in biological fluids involve tedious sample preparation procedures (liquid/liquid or solid phase extraction), low extraction yields and low sensitivity. Recently, a determination method using the LC-tandem mass spectrometry (LC-MS/MS) has been reported for the estimation of CEF.[24] However, the MS-MS detector needs to be delicately set and LC-MS/MS equipment is very expensive. HPLC method with Ultraviolet detection has been reported for the determination of CEF in serum samples with the different sensitivities (200 ng/mL[15] and 50 ng/mL[4]). However, the analytical run time was found to be much high (>10 min). Furthermore, a very low concentration of OFL determination was achieved using HPLC fluorescent detector.[17] However, fluorescent detector can't be used in our method because CEF is not fluorescent. To bypass these difficulties, we have developed more conventional procedures for determining OFL and CEF using the LC coupled with the mass spectrometry (LC-MS). This assay is simple and robust, as well as sufficiently sensitive for pharmacokinetic studies. Hence, in the present study, simple analytical method was developed and validated for simultaneous determination OFL and CEF in human plasma. Newly develop method could be used for pharmacokinetic and therapeutic drug monitoring.

Experimental

Chemicals and reagents

In this study, analytical grade chemicals and reagents were used. OFL, CEF, and the internal standard moxifloxacin (MOX), [Figure 1c] were received as gift samples. Acetonitrile, methanol, formic acid, acetic acid, and ammonium acetate were purchased from Sigma (St Louis, MO, USA).

Stock solutions (10 μg/mL) of OFL, CEF, and MOX were prepared in the methanol. These solutions were diluted with mobile phase for further use. The drug-free human plasma was spiked with the above solutions for the determination of recovery, precision, accuracy, and limits of detection and quantitation. All standard solutions were covered with aluminum foil to protect from the light and stored at 4°C until used.

HPLC-MS instrumentation and conditions

The Agilent LC-MS system consisted of Quat pump, an auto injector, degasser with Agilent Chem station data module (Agilent 1200 series, Germany) and Quadrupole LC-MS 6120 detector. Zorbax eclipse C_{18} (150 mm × 4.6 mm i.d, 5 μm) reverse phase analytical column was selected to separate active ingredients. The mobile phase was prepared by mixing acetonitrile, methanol and an aqueous formic acid solution (0.5%) in a ratio of 23:10:67 respectively and the mobile phase was filtered through a 0.45 μm nylon filter just before use. Isocratic HPLC was performed by setting the flow-rate at 0.6 mL/min and temperature at 25°C. The analyte responses were recorded by monitoring the eluate by MS detector. Area integration, peak area measurement, calculations, and the plotting of the chromatograms were performed by Agilent Chemdraw program. Time of analysis was 5 min. The analytes were ionized by an electrosprey ionization (ESI) source in positive ion mode. The following source conditions were used for the ionization of analytes: nebulizing gas (N_2) 10 l/min; drying gas (N2) temperature 250°C; electrospary probe (capillary) voltage 3900 V; skimmer voltage 90V; cone voltage 4. First data acquisition was performed with scan mode (200-500) to know the molecular ion peaks of all the three compound. [Figure 2]. Further study was performed by selected ion monitoring (M + H) + for OFL at m/z 362.0, CEF at m/z 453.8 and internal standard (IS) at m/z 402.0.

Preparation of stock and standard solutions

Stock solutions of OFL, CEF, and MOX were prepared by dissolving accurately weighed amounts of each reference compound in methanol to yield concentrations of 10 μg/mL. These stock solutions were stored at 4°C and thawed on the day of analysis. The required different concentration

solutions were prepared by diluting the above solutions in the mobile phase.

Preparation of plasma samples

Drug free blood samples were collected from healthy human volunteers, spiked with suitable volumes of the standard solutions of OFL and CEF followed by MOX and centrifuged for 5 min at 8,000 rpm. 200 µL of plasma was transferred into an eppendorf micro centrifuge tube and 400 µL of acetonitrile was added to precipitate proteins. The suspension was properly mixed using a vortex mixer (Stuart Scientific, UK) for 5 min and centrifuged at 13,000 rpm for 10 min (Eppendorf Centrifuge, USA). Then, clear supernatant was filtered using syringe filter and 20 µL was injected into the HPLC system.

Procedures

Calibration, linearity

On a daily basis calibration standards in plasma were prepared by spiking 200 µL of blank drug free human plasma with appropriate volumes of standard solutions of both drugs to get final concentrations of 4, 50, 100, 200, 400, 500 ng/mL for OFL and 40, 100, 500, 2000, 4000, 6000 for CEF. The standard MOX solution was added to all the above solution to get a constant concentration of 500 ng/ml in each solution. The calibration samples were subjected to the above sample preparation procedure.

In order to determine the linearity of the method, spiked standard samples at six concentrations over the range 4-500 ng/mL of OFL and 40-6000 ng/mL of CEF with 500 ng/mL of IS were prepared. The analysis was performed in three separate analytical runs for three sets of above solutions. Calibration curves were constructed by the peak-area ratios of the analyte to the IS versus the nominal standard concentration adopting least-squares linear regression. The concentrations of the unknown samples were calculated using the linear regression equation. The sensitivity of this LCMS method was examined by the measurement of the lower limit of detection (LOD) and limit of quantitation (LOQ). The LOD and LOQ of the method were determined by the signal-to-noise ratio using the equations 3 S/N and 10 S/N respectively.

Specificity and selectivity

The specificity of the method was evaluated by analyzing processed blank drug free human plasma and spiked with OFL, CEF, and IS. The matrix effect was scrutinized by evaluating the response (peak area) of OFL and CEF in the reconstituted solution of plasma (spiked OFL and CEF into the blank plasma, $n = 5$) with that of the standard solution at the same nominal concentration. Plasma samples were prepared by adopting the procedure described in the sample preparation section.

Precision and accuracy

Inter and intraday precision values were estimated by assaying control plasma containing three different concentrations of 10, 100, 400 ng/mL of OFL and 100, 2000, and 4000 ng/mL of CEF. The relative standard deviation (RSD) was obtained by performing the experiment 5 times on 1 day and repeating for three separate days. Accuracy of the method was investigated as the percentage of supposed concentration.

Recovery

The absolute recoveries of OFL, CEF, and IS from plasma at above three concentrations were determined by injecting plasma samples and standard solutions into the chromatographic system. Peak area ratios of the analytes after extraction of plasma samples were compared with those of direct injection of standard solutions. The differences between the peak areas of these mixtures correspond to the ion suppression. The recoveries were determined in triplicate.

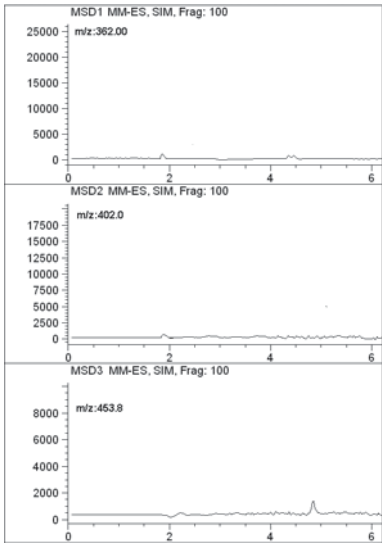

Figure 3: Selected ion monitoring chromatograms of blank human plasma

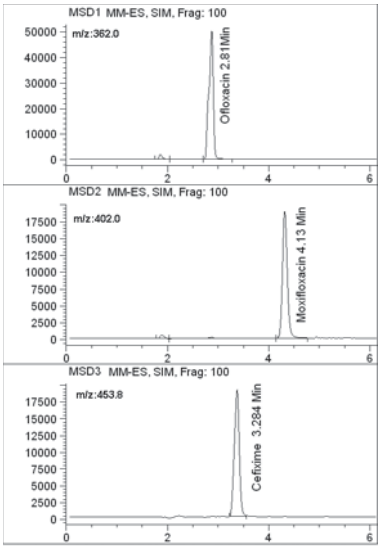

Figure 4: Selected ion monitoring chromatograms of human plasma spiked with ofloxacin (500 ng/mL), moxifloxacin (500 ng/mL) and cefixime (4000 ng/mL)

Table 1: Intraday and interday precision and accuracy for ofloxacin and cefixime

Expected (ng/mL)	Intraday (*n*-5)		Interday (*n*-15)		Accuracy (%)	Recovery (%)	Quotient*(%) (Ion.Supp %)
	Measured (ng/mL)	RSD (%)	Measured (ng/mL)	RSD (%)			
Ofloxacin							
10	9.81±0.53	5.40	10.16±0.72	7.09	98.4±7.7	90.7±8.1	95.4 (4.6)
100	99.82±2.8	2.81	98.09±6.61	6.74	99.2±5.5	95.2±3.1	93.8 (6.2)
400	392.69±12.95	3.30	396.05±18.9	4.77	98.6±4.6	93.4±5.0	96.4 (3.6)
Average					98.6±5.9	93.1±5.4	
Cefixime							
100	98.38±3.02	3.07	99.07±4.39	4.43	99.1±4.8	93.1±4.5	95.6 (4.5)
2000	1962.39±80.23	4.09	2041.28±104.83	5.14	99.4±2.4	90.5±5.1	92.9 (7.1)
4000	3942.73±171.09	4.34	3922.21±199.4	5.08	98.3±5.3	94.3±4.8	91.3 (8.7)
Average					98.9 ±4.0	92.6±4.8	

*Peak height ratios between a spiked solution of prepared blank serum and a solution without sample preparation at the same concentrations, RSD: Relative standard deviation

Table 2: Stability of ofloxacin and cefixime trihydrate in human plasma

Ofloxacin spiked concentration (ng/mL)	10	100	400
Measured concentration (ng/mL)			
Freeze and thaw stability			
Mean ± SD	9.88±0.3	98.02±2.9	393.21±15.2
RE (%)	−2.2	−1.2	−1.7
Post-preparative stability (24 h at room temperature)			
Mean ± SD	10.27±0.2	98.1±3.8	391.65±16.6
RE (%)	2.7	−1.9	−2.08
Stability for 15 days at −20°C			
Mean ± SD	9.75±0.3	97.3±3.2	395.5±20.8
RE (%)	−2.5	−2.7	−1.12
Cefixime trihydrate spiked concentration (ng/mL)	100	2000	4000
Measured concentration (ng/mL)			
Freeze and thaw stability			
Mean ± SD	98.6±2.2	1965.7±43.9	3945.9±135.2
RE (%)	−1.4	−1.71	−1.35
Post-preparative stability (24 h at room temperature)			
Mean ± SD	101.2±4.6	1958.1±69.8	3853.5±116.6
RE (%)	1.27	−2.1	−3.65
Stability for 15 days at −20°C			
Mean ± SD	97.31±2.8	1949.3±57.2	3909.8±110.8
RE (%)	−2.69	−2.53	−2.25

RE: Relative error, SD: Standard deviation

Stability studies

Three replicates of samples at each of 10, 100, 400 ng/mL of OFL and 100, 2000, and 4000 ng/mL of CEF concentrations were used to assess the stability of OFL and CEF in human plasma under a different storage circumstances: three cycles of freezing (−20°C) thawing (25°C) stability, post-preparative stability (room temperature for 24 h) and long-term storage stability at−20°C for 15 days. Samples were concluded to be stable at verity of experimental conditions if the average deviations were within ± 15% of the actual valve.

Results and Discussion

Method optimization

Different mobile phase was screened to achieve favorable separation and a mixture of acetonitrile, methanol, and water was found to be optimal. Ammonium acetate, formic acid, and acetic acid additives were explored for separation with good resolution and for appropriate ionization of analytes. It was found that ammonium acetate ionized the OFL but failed to ionize CEF completely. However, formic acid and acetic acid showed good ionization of both the drugs, with formic acid modifier showed increased sensitivity and maintained sharp and symmetrical peaks for both analytes. The percentage of formic acid in water was studied and 0.5% was found to be optimal. The mobile consisting of acetonitrile:Methanol:Water with 0.5% formic acid in the ratio 23:10:67% demonstrated good linearity.

The OFL, CEF, and IS were analyzed by MS in ESI positive ion mode. The negative ion mode was also tested, but the positive ion mode sensitivity was higher than that of negative ion mode. The ESI revealed better signals for the protonated molecules of OFL, CEF, and MOX compared to atmospheric pressure chemical ionization (APCI). The chromatogram of the mass spectrum in scan mode gave most sensitive ions of (M + H) + at 90 eV for OFL, CEF, and IS. The most sensitive ions were (M + H) +, hence, the quantitative analysis was performed in selected ion monitoring (SIM) mode for OFL at 362.0 m/z, CEF at 453.8 m/z and IS at 402.0 m/z. The analyte peaks were confirmed by matching their retention times and mass spectra with solutions of standards.

Method validation

The correlation coefficients obtained (>0.999) confirmed that the calibration curves were linear over the range of 4-500 ng/mL for OFL and 40-6000 ng/mL for CEF in the

human plasma. The regression equations constructed from the calibration curves were $y = 5.19\ x10^{-3} \times 2.87x10^{-3}$ and $y = 6.34x10^{-3} \times - 2.38x10^{-3}$ with a correlation coefficient (r value) of 0.9996 and 0.9998 for OFL and CEF, respectively, where "y" represents the ratios of OFL and CEF peak areas to that of IS. And "x" represents the plasma concentration of OFL and CEF. The lower limit of quantitation (LLOQ) was 4 and 40 ng/mL and the LOD was 1.31 ng/mL and 13.4 ng/mL for OFL and CEF, respectively.

Figure 3 show selected-ion recording chromatograms acquired from an extract of the drug free-plasma sample. Figure 4 show chromatograms acquired from a spiked plasma sample containing 500 ng/mL of OFL, 4000 ng/mL of CEF and 500 ng/mL of MOX (IS). Under expressed chromatographic conditions, retention times were 2.81 min for OFL, 3.28 min for CEF and 4.13 min for MOX. Assays performed on drug-free human plasma succeeded to show no interfering peaks during the interested intervals of the retention times.

The results of intra and inter-day precision and accuracy are tabulated in the Table 1. The RSD was calculated and the results indicate that tested samples satisfy the requirements of biological analysis.

The mean recoveries of OFL and CEF from spiked human plasma were 93.1 ± 5.4% and 92.63 ± 4.8%, respectively. The recovery of the IS was 94.2 ± 5.0% at the concentration of 500 ng/mL. These results showed that this method accomplish the high degree of reproducibility and accuracy. The ion suppression was found in the range of 3.6-8.1% [Table 1], which was relatively small, hence complicated sample preparation like liquid-liquid extraction or solid phase extraction was not used.

The results of the stability experiments indicate that there was no significant difference in plasma samples after three freeze-thaw cycles. In extracts, the analytes were stable after storage at room temperature for 24 h, and the plasma samples were also stable after storage at −20°C at least for 2 weeks [Table 2].

Conclusions

The objective of the present work was achieved by developing a specific, rapid, sensitive, and inexpensive LCMS method for determination of OFL and CEF in plasma and validated. Further, the simple sample preparation method was developed and the analytes were separated on a reversed phase column with MS detection. The specificity test showed that no additional peaks due to endogenous substances were observed that would interfere with OFL and CEF. In addition, the high accuracy, precision, recovery, and low detection limits allow to use the newly developed a procedure for pharmacokinetic and clinical studies.

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