Development and validation of liquid chromatography–mass spectrometric method for simultaneous determination of moxifloxacin and ketorolac in rat plasma: application to pharmacokinetic study

B. Raju, M. Ramesh, Roshan M. Borkar, Raju Padiya, Sanjay K. Banerjee and R. Srinivas

ABSTRACT: A highly sensitive, selective and rapid liquid chromatography–electrospray ionization mass spectrometry (LC-MS) method has been developed and validated for simultaneous determination of moxifloxacin (MFX) and ketorolac (KTC) in rat plasma. Gemifloxacin (GFX) was used as an internal standard (IS). A simple protein precipitation method was used for the extraction of analytes from rat plasma. Effective chromatographic separation of MFX, KTC and GFX was achieved on a Kromasil C18 column (4.6 mm, 5 µm) using a mobile phase consisting of acetonitrile–10 mM ammonium acetate (pH 2.5)–0.1% formic acid (50:25:25) in an isocratic elution, followed by detection with positive ion electrospray ionization mass spectrometry using target ions of [M + H]+ at m/z 402 for MFX, m/z 256 for KTC and m/z 390 for GFX in selective ion recording mode. The method was validated over the calibration range of 5–100 ng/mL for MFX and 10–6000 ng/mL for KTC. The method demonstrated good performances in terms of intra- and inter-day precision (0.97–5.33%) and accuracy (93.91–101.58%) for both MFX and KTC, including lower and upper limits of quantification. The recoveries from spiked control samples were >75% for MFX and >79% for KTC. The matrix effect was found to be negligible and the stability data were within acceptable limits. Further, the method was also successfully applied to a single-dose pharmacokinetic study in rats. This method can be extended to measure plasma concentrations of both drugs in human to understand drug interaction and adverse effects.

Keywords: moxifloxacin; ketorolac; LC-MS method; simultaneous determination; pharmacokinetic study

Introduction

Moxifloxacin (1-cyclopropyl-7-(5,5)-2, 8-diazabicyclo (4.3.0)-non-8-yl-6-fluoro-8-methoxy-1,4-dihydro-4-oxo-3-quinoline carboxylic acid hydrochloride, MFX) is a synthetic fourth-generation broad-spectrum fluoroquinolone antibiotic. It acts by inhibiting DNA gyrase, a type II topoisomerase and topoisomerase IV, which are involved in DNA replication and metabolism (Keating and Scott, 2004; Drlica and Zhao, 1997). MFX at higher concentration may increase the risks of QTc interval prolongation, cardiac arrhythmias, peripheral neuropathy, tendon rupture, tendonitis, central nervous system stimulation and convulsions (Chiba et al., 2004; US-FDA, 2009). Similarly, Ketorolac tromethamine ([rac]-5-benzoyl-3,2-H-pyrrolo [1,2a] pyrrole-1-carboxylic acid), a nonsteroidal anti-inflammatory drug, is indicated for short-term management of moderate to severe pain and shows a high incidence of side effects like gastric bleeding. Concerns about the high incidence of reported side effects led to restriction in its dosage and maximum duration of use. Recently, the US Food and Drug Administration (FDA) has approved an intranasal formulation of ketorolac tromethamine (Sprix Nasal Spray) for short-term management of moderate to moderately severe pain requiring analgesia at the opioid level. The combination of ketorolac with moxifloxacin is extensively used for the treatment of post-operative inflammation and infection following cataract surgery (Durrie et al., 2007). This combination can also be used for other purposes. However, the concomitant administration of a nonsteroidal anti-inflammatory drug and MFX may increase the risks of central nervous system stimulation and convulsions (Sarro and Sarro, 2001). To correlate the adverse effect with drug concentration, there is a need to develop a sensitive, rapid and selective method for simultaneous determination of MFX and ketorolac (KTC) in plasma.

Several chromatographic methods have been reported for the determination of MFX in biological fluids with UV and fluorescence detection (Djurdjевич et al., 2006; Kumar and Ramachandran, 2009;...
All these existing methods involve an expensive fluorescence detector, special analytical columns, a gradient elution program with ion pairing agents and tedious time consuming liquid–liquid extraction (Djurdjevic et al., 2006; Chan et al., 2006; Nguyena et al., 2004; Liang et al., 2002; Lemoine et al., 2000; Xu et al., 2010). These increase the cost and complexity of the method, even though sensitivity of the fluorescence detector is much higher than that of a UV detector. Similarly to MFX, HPLC and LC-MS methods are available in the literature for the determination of KTC and its enantiomers in plasma, which involve liquid–liquid extraction and long chromatographic run times (Wu and Massey, 1990; Chaudhary et al., 1993; Vakily et al., 1995; Jones and Bjorksten, 1994; Diaz-Perez et al., 1994; Patri et al., 2011). In the very few LC-MS methods that have been reported for the determination of MFX in plasma, expensive cartridges are utilized to process the plasma sample (Vishwanathan et al., 2002; Pranger et al., 2010; Vu et al., 2011). Although all the methods described possess good sensitivity, none were designed for simultaneous determination of MFX and KTC for drug monitoring in plasma utilizing simple sample preparation technique and short chromatographic run time. Therefore, the present study was designed to develop a rapid and sensitive LC-MS assay method for simultaneous determination of MFX and KTC in rat plasma. The same method can be extended to measure the plasma concentration of both drugs in human to understand adverse effects, drug metabolism and drug interactions.

Experimental

Chemicals and reagents

Pure MFX, KTC and gemifloxacin (GFX) were gift samples from Symed Laboratories, Hyderabad, India. HPLC-grade acetonitrile and methanol used in the present study were purchased from Merck (Mumbai, India).

![Figure 1](image-url)  
**Figure 1.** Positive ion ESI-MS spectra of (a) moxifloxacin (MFX; m/z 402) (b) ketorolac (KTC; m/z 256) and (c) gemifloxacin (GFX; m/z 390).
and used without further purification. Water was purified by using a Milli-pore Milli-Q plus purification system (Millipore Corp., Bedford, MA, USA). Ammonium acetate and analytical reagent grade formic acid and acetic acid used in the present study were purchased from S.D. Fine Chemicals (Mumbai, India). All other chemicals were of analytical grade.

Animals

All animal experiments were undertaken with the approval of Institutional Animal Ethical Committee of Indian Institute of Chemical Technology (IICT), Hyderabad. Male Sprague–Dawley rats (200–250 g) were purchased from the National Institute of Nutrition, Hyderabad, India. The animals were housed in BIOSAFE, an animal quarantine facility of IICT, Hyderabad, India. The animal house was maintained at temperature 22 ± 2 °C with relative humidity of 50 ± 15% and 12 h dark/light cycle. The animals were fasted for 12 h prior to pharmacokinetic study. Six Sprague–Dawley rats with similar weight and age were utilized to carry out the present pharmacokinetic study to reduce the variation of drug concentration in plasma.

Instrumentation

LC-MS equipment and conditions. The HPLC analysis was performed on an Agilent 1200 series HPLC instrument (Agilent Technologies, USA)
Preparation of stock, working solutions and IS

The stock solutions of MFX and KTC were prepared by dissolving the reference standards in methanol. By using this stock solution, serial dilutions were made to prepare the primary aliquots of MFX and KTC in methanol-water (50:50, v/v) for calibration curve and quality control (QC) samples. Similarly, stock solution of 1 mg/mL of IS in methanol was also prepared and diluted with the same diluents to prepare working solution containing a concentration of 50 ng/mL.

Preparation of calibration curve and quality control samples

A 10 µL aliquot of each primary aliquot was spiked in 190 µL of blank K-3 EDTA rat plasma to yield calibration curve samples ranging from 5.00 to 100 ng/mL for MFX and from 10.0 to 6000 ng/mL for KTC. Similarly, low, medium and high QC samples (LQC, MQC and HQC) were prepared independently at three different concentration levels of 15 (LQC), 40 (MQC) and 75 ng/mL (HQC) for MFX; and 30 (LQC), 2500 (MQC) and 4500 ng/mL (HQC) for KTC. Blank plasma sample and zero samples were also prepared and analyzed. All the stock solutions were stored at 0–4 °C for further use.

Sample preparation

A simple protein precipitation method was used for the extraction of analytes in rat plasma. To an aliquot of 200 µL plasma in an Eppendorf tube, 200 µL of IS solution was added followed by 600 µL of acetonitrile. The mixture was thoroughly vortexed and then centrifuged at 5000 rpm for 15 min to settle down the precipitate. Aliquots of 200 µL of supernatant fluid were taken into HPLC vials. Only 10 µL aliquots of sample solution were injected into LC-MS system.

Method validation

The bioanalytical method was validated according to FDA guidelines (US Food and Drug Administration, 2001).

Specificity and selectivity. The specificity and selectivity of the method were investigated by screening analysis of six individual blank rat plasma and spiked plasma samples. Two other blank plasma samples containing an IS concentration of 50.0 ng/mL were also prepared and tested for interference. Each blank sample was tested for omission of endogenous interference at the retention times of analytes and IS.

Linearity and sensitivity. The linearity of the method was evaluated by using five calibration standards over a calibration range of 5.0–1000 ng/mL for MFX and 10.0–60000 ng/mL for KTC in rat plasma. The calibration curves were established by plotting peak area ratios of calibration standards to the IS vs the nominal concentrations of analytes. The linearity was measured by calculating the linearity and sensitivity of the method using the following equation:

\[ \text{Linearity (CV, %)} = \left( \frac{\text{standard deviation of the concentration}}{\text{mean concentration}} \right) \times 100. \]

Accuracy (\%) = (standard deviation of the concentration/mean concentration) \times 100.

Precision (\%) = (coefficient of variation) = (standard deviation/mean concentration) \times 100.

Table 1. Determination (n = 6) of intra- and inter-day precision and accuracy of MFX and KTC

<table>
<thead>
<tr>
<th>QC Sample</th>
<th>QC</th>
<th>Theoretical Concentration (ng/mL)</th>
<th>Mean Estimated Concentration (ng/mL)</th>
<th>Precision (CV, %)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LQC</td>
<td>5</td>
<td>5.08 ±0.27</td>
<td>5.33 ±0.28</td>
<td>2.96</td>
<td>99.61</td>
</tr>
<tr>
<td>MQC</td>
<td>10</td>
<td>14.42 ±0.42</td>
<td>14.29 ±0.34</td>
<td>2.85</td>
<td>98.65</td>
</tr>
<tr>
<td>HQC</td>
<td>30</td>
<td>72.09 ±0.88</td>
<td>72.43 ±0.71</td>
<td>1.02</td>
<td>96.94</td>
</tr>
<tr>
<td>LQC</td>
<td>5</td>
<td>5.01 ±0.16</td>
<td>5.01 ±0.16</td>
<td>1.77</td>
<td>99.65</td>
</tr>
<tr>
<td>MQC</td>
<td>10</td>
<td>14.64 ±0.26</td>
<td>14.64 ±0.26</td>
<td>1.02</td>
<td>96.71</td>
</tr>
<tr>
<td>HQC</td>
<td>30</td>
<td>72.35 ±0.90</td>
<td>72.35 ±0.90</td>
<td>1.02</td>
<td>96.71</td>
</tr>
</tbody>
</table>

**Table 1.** Determination (n = 6) of intra- and inter-day precision and accuracy of MFX and KTC.
Simultaneous determination of moxifloxacin and ketorolac

Table 2. Stability data of MFX and KTC in rat plasma (n = 6)

<table>
<thead>
<tr>
<th>Quality control</th>
<th>Autosampler stability⁶</th>
<th>Bench-top stability⁷</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean concentration ± SD</td>
<td>Percentage change</td>
</tr>
<tr>
<td>MFX</td>
<td>14.79 ± 0.35</td>
<td>−0.91</td>
</tr>
<tr>
<td>LQC</td>
<td>74.24 ± 0.66</td>
<td>−0.70</td>
</tr>
<tr>
<td>HQC</td>
<td>28.88 ± 0.56</td>
<td>−1.04</td>
</tr>
<tr>
<td>KTC</td>
<td>4426.55 ± 62.20</td>
<td>−0.88</td>
</tr>
</tbody>
</table>

⁶Kept at autosampler temperature (4 °C) for 24 h.
⁷Exposed at ambient temperature (25 °C) for 8 h.
⁸After three freeze–thaw cycles.
⁹Stored at −80 °C for 30 days.

the calibration curve was determined using linear regression analysis and also evaluated by its correlation coefficient, slope and intercept. The lower limit of quantification (LLOQ) was determined as the lowest concentration of analytes with coefficient of variation (CV) not exceeding 20% and accuracy in the range of 80–120%.

Precision and accuracy. Intra- and inter-day precision and accuracy were investigated by determining QC samples at four different concentrations for six replicates. The precision of the method was determined by CV and accuracy was evaluated by recovery. The acceptable limit of CV was <20% for the LLOQ, and ≤15% for LQC, MQC and HQC as per FDA guidelines. The accuracy was calculated as percentage difference in mean value of the observed concentration and nominal concentrations of QC samples.

Recovery and matrix effect. The extraction recovery of MFX and KTC in rat plasma was evaluated by comparing peak area ratios of analytes to calibrants. The extraction recovery of MFX and KTC was determined using linear regression analysis and also evaluated by its correlation coefficient, slope and intercept. The lower limit of quantification (LLOQ) was determined as the lowest concentration of analytes with coefficient of variation (CV) not exceeding 20% and accuracy in the range of 80–120%.

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Preparation of samples. Plasma samples containing MFX and KTC at the concentrations of 60 and 10.0 ng/mL, respectively, were prepared. These samples were diluted to 2-fold and 4-fold the original concentration using blank plasma. The dilution integrity was determined by recalculating the concentrations of these diluted samples, whose CV should be ≤15%.

To evaluate the carry-over effect, blank aqueous sample, blank extracted plasma sample and ULOQ extracted plasma samples were prepared. Alternatively, one blank aqueous sample followed by aqueous ULOQ sample, extracted blank plasma sample and extracted ULOQ plasma samples were injected. Carry-over should be ≤20% of response of the mean extracted LLOQ samples and ≤5% of the response of the extracted IS samples.

Stability. The stability of analytes was determined under different storage conditions and evaluated at LQC and HQC levels. The benchtop and long-term stability of plasma samples were analyzed at ambient temperature (25 °C) for 8 h, and −80 °C for 30 days, respectively.

Autosampler stability was carried out at 4 °C for 24 h. For freeze–thaw stability, samples were stored at −20 °C and thawed at room temperature every 24 h. The extracted samples were determined after three freeze–thaw cycles. The obtained results of stability samples were compared with the nominal concentrations of the analytes at 0 h. The plasma samples were considered as stable if the mean percentage changes are within the acceptance criteria of ±10%.

Results and discussion

LC-MS method development

The separation of analytes and IS from endogenous substances was tested on a Kromasil C18 column (100 × 4.6 mm, 5 μm) with different mobile phases comprising different buffers such as ammonium formate, ammonium acetate with various organic solvents like methanol and acetonitrile. During this process, severe tailing was observed for MFX owing to its amphoteric property and silanolic interaction (Djurdjevic et al., 2006). The tailing problem was solved by adjusting the pH of the mobile phase. Thus, the method was optimized with a mobile phase consisting of acetonitrile (A), 10 mM ammonium acetate buffer (pH 2.5; solvent B) and 0.1% formic acid (solvent C; 50:25:25, v/v) in an isocractic elution mode. The flow-rate of the mobile phase was 0.5 mL/min, the column temperature at 25 °C and the injection volume was 10 μL. The ESI source conditions were also optimized to get good signal intensity and sensitivity even at very low concentrations of analytes. The optimized conditions were as described above.

Method validation

Specificity and selectivity. The chromatograms showed no significant interference around the retention times of analytes and IS in drug-free rat plasma samples. The retention times of MFX, KTC and IS were 2.2, 6.0 and 3.1 min, respectively. Representative chromatograms of blank plasma, blank plasma spiked with IS, blank plasma spiked with MFX, KTC and 2 h post-dose rat plasma samples are shown in Fig. 2.

Linearity and lower limit of quantification. The calibration data were analyzed by linear least-square regression analysis. The calibration curve exhibited a good linearity between peak area ratios and concentrations of analytes with a mean correlation coefficient, r² (± SD) of 0.998 (± 0.0010) for MFX and 0.998 (± 0.0011) for KTC. Each calibration concentration was back-calculated with the help of a calibration equation. The LLOQ of MFX and KTC were 5.0 and 10.0 ng/mL, respectively.
**Table 2.** Continued

<table>
<thead>
<tr>
<th>Bench-top stability&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Freeze–thaw stability&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Long-term stability&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Percentage change</strong></td>
<td><strong>Mean concentration ± SD</strong></td>
<td><strong>Percentage change</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CV</td>
</tr>
<tr>
<td>–2.44</td>
<td>13.89 ± 0.09</td>
<td>0.69</td>
</tr>
<tr>
<td>–0.44</td>
<td>69.72 ± 1.78</td>
<td>2.56</td>
</tr>
<tr>
<td>–2.63</td>
<td>27.79 ± 0.16</td>
<td>0.60</td>
</tr>
<tr>
<td>–1.93</td>
<td>4196.55 ± 62.3</td>
<td>1.48</td>
</tr>
</tbody>
</table>

**Precision and accuracy.** The intra- and inter-day precisions for MFX and KTC were 1.02–5.33% and 0.97–4.48%, respectively. The accuracy was 96.1–101.5% for MFX and 93.9–97.49% for KTC. Data for assay precision (%CV) and accuracy (percentage recovery) were within the acceptable limits (Table 1).

**Extraction recovery and matrix effect.** The extraction recovery of MFX and KTC was found to be >75 and >79%, respectively. The recovery of IS was >89%. The matrix factor was 0.88–1.05 (LQC) and 0.91–1.06 (HQC) for MFX, and 0.86–0.99 (LQC) and 0.89–1.03 (HQC) for KTC. These results indicate that there was no endogenous interference in the quantification of analytes.

**Dilution test and carry-over effect.** The percentage accuracies of six replicates of 2- and 4-fold dilutions for MFX and KTC were within 85–115% of their nominal concentrations. The CVs for both 2- and 4-fold dilutions were less than 10% for both MFX and KTC. No carry-over effect was observed at the retention times of MFX, KTC, and IS.

**Stability.** Bench-top stability was carried out on storage of samples at ambient temperature (25 °C) for 8 h; the mean percentage change of analytes in plasma was less than ±10% from their nominal concentrations. The mean percentage changes of analytes in plasma during three freeze–thaw cycles were within the acceptance limits. The analytes were stable at 4 °C for 24 h in the autosampler (autosampler stability) and at −80 °C for 30 days (long-term stability; Table 2).

**Pharmacokinetic study**

The validated method was successfully applied to investigate the pharmacokinetic study in adult male Sprague–Dawley rats (n = 6) in which plasma concentrations of analytes were determined up to 24 h. After fasting for 12 h, rats were orally administered with MFX and KTC in saline through gavage of 10 and 6 mg/kg doses, respectively. Blood samples (300 μL) were collected at 0.25, 0.50, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, and 24.0 h, thoroughly vortexed and centrifuged at 5000 rpm for 15 min at 4 °C; and plasma was collected and stored at −20 °C until analysis. The mean plasma concentration time profiles of MFX and KTC are shown in Fig. 3. The pharmacokinetic parameters were estimated using noncompartmental analysis with Kinetica 2000 software (version 3.0). The maximum plasma concentration ($C_{\text{max}}$) and time at which the concentration reached the maximum ($T_{\text{max}}$) for MFX were found to be 419318 ± 6.10 ng/mL and 30 min, respectively. While $C_{\text{max}}$ and $T_{\text{max}}$ for KTC were 2500 ± 392.93 ng/mL and 60 min, respectively. The plasma concentration–time curve from 0 h to the last measurable concentration (AUC<sub>0–L</sub>) and area under plasma concentration–time curve from 0 h to infinity (AUC<sub>0–∞</sub>) for MFX were 331.28 ± 35.86 and 3303.56 (ng h/mL), respectively. The terminal half-life ($t_{1/2}$) was found to be 10.37 ± 0.81 h for MFX and 3.91 ± 0.45 h for KTC.

**Conclusions**

The method reported here is the first LC-MS quantitative assay for simultaneous determination of MFX and KTC in rat plasma and it was also successfully applied to investigate a single-dose pharmacokinetic study in rats. The method was specific, precise and accurate, and provided a satisfactory linearity and LLOQ. The method allows for a much higher sample throughput owing to short chromatographic run time, and simple and low-cost sample preparation method. The same method can be extended to measure plasma concentrations of both drugs in human to understand drug metabolism, drug interaction and adverse effects of moxifloxacin and ketorolac.

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Simultaneous determination of moxifloxacin and ketorolac

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