Therapeutic drug monitoring: how to improve moxifloxacin exposure in tuberculosis patients
Pranger, Anna

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Determination of moxifloxacin in human plasma, plasma ultrafiltrate, and cerebrospinal fluid by a rapid and simple liquid chromatography-tandem mass spectrometry method

A.D. Pranger, J.W.C. Alffenaar, A.M.A. Wessels, B. Greijdanus, and D.R.A. Uges

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Abstract

Moxifloxacin (MFX) is a useful agent in the treatment of multidrug-resistant tuberculosis (MDR-TB). At Tuberculosis Centre Beatrixoord, a referral center for tuberculosis in the Netherlands, approximately 36% of the patients have received MFX as treatment. Based on the variability of MFX AUC, the variability of in vitro susceptibility to MFX of M. tuberculosis, and the variability of penetration into sanctuary sites, measuring the concentration of MFX in plasma and cerebrospinal fluid (CSF) could be recommended. Therefore, a rapid and validated liquid chromatography-tandem mass spectrometry (LC-MS-MS) analysing method with a simple pre-treatment procedure was developed for therapeutic drug monitoring of MFX in human plasma and CSF. Because of the potential influence of protein binding on efficacy, we decided to determine both bound and unbound (ultrafiltrate) fraction of MFX. The calibration curves were linear in the therapeutic range of 0.05-5.0 mg/L plasma and CSF with CV in the range of -5.4% to 9.3%. MFX ultrafiltrate samples could be determined with the same method setup for analysis of MFX in CSF. The LC-MS-MS method developed in this study is suitable for monitoring MFX in human plasma, plasma ultrafiltrate, and CSF.
Introduction

Moxifloxacin (MFX) (Figure 1), one of the newer fluoroquinolones, has a broad spectrum of antimicrobial activity and is used for treating bacterial infections of the respiratory tract and soft tissues (1). *In vitro* and *in vivo* experiments showed bactericidal activity of MFX against *Mycobacterium tuberculosis* (*M. tuberculosis*) (1-3), which was high in comparison with other fluoroquinolones and is equal to or greater than isoniazid (1;3). Based on these results, MFX is more frequently introduced as a second-line agent in the treatment of tuberculosis (TB) in case there is resistance or intolerance to first-line agents like rifampicin, isoniazid, pyrazinamide, and ethambutol (4;5). At Tuberculosis Centre Beatrixoord, a referral centre for TB in the Netherlands, 36% of the patients have received MFX, which accounts for approximately 33 patients each year from 2006 to 2008. MFX seems also promising for the treatment of TB in sanctuary sites, like tuberculosis meningitis. Data from *in vivo* studies and humans suggest that MFX penetrates the cerebrospinal fluid (CSF) (4;6-9), although an inter- and intra-individual variable CSF penetration of MFX was observed (7-9).

Like other fluoroquinolones, the most predictive parameter for efficacy of treatment with MFX is the ratio of the area under the concentration curve (AUC) to the minimal inhibitory concentration (MIC) (10). This ratio is based on a study with an aerosol infection model of tuberculosis in BALB/c mice and can be used to estimate the adequate dose for treatment continuation or therapeutic drug monitoring (TDM) (10). Both the AUC and the MIC values can be variable. Because the AUC of MFX is subject to variability caused by drug-drug interaction of MFX with rifampicin (RIF), the AUC/MIC ratio can be influenced significantly (11;12). Measuring plasma concentrations can therefore be warranted in patients co-
medicated with RIF. Despite the high activity of MFX against *M. tuberculosis*, based on the methoxy group at the C-8 position (13;14), increased *in vitro* MIC's have been observed (15). A higher MIC has a major effect on the AUC/MIC ratio, limiting the use of MFX in a dosage of 400 mg once daily. A higher dosage might therefore be necessary in the treatment of multi-drug-resistant TB (MDR-TB). A daily dose of 600-800 mg is desirable to kill *M. tuberculosis* and prevent resistance (16). However, experience with long-term clinical use of this dose is still unavailable (16;17). As the efficacy of an antimicrobial agent is based on the unbound concentration of that drug, variability in protein binding can influence the AUC\textsubscript{unbound}/MIC ratio. Human data regarding this subject is lacking, but animal data shows intra-individual concentration-dependent variability in protein binding (10), and *in vitro* data shows that antimicrobial activity is influenced by plasma protein binding (18).

Considering the variability in MFX AUC, variability in *in vitro* susceptibility to MFX of *M. tuberculosis*, potential influence of protein binding on efficacy, and variability of penetration into sanctuary sites, a prospective observational pharmacokinetic study is mandatory to explore the effect on the treatment of TB with MFX as part of an anti-tuberculosis regimen. To this end, a simple and rapid method of analysis has to be developed for the determination of MFX (bound and unbound) in plasma and CSF, while complying with FDA guidelines.

The most commonly used method of analysis to determine MFX in human plasma or CSF uses high-performance liquid chromatography (HPLC) followed by fluorescent or UV detection (19-22). However, these methods require liquid-liquid extraction and are therefore time-consuming. To our knowledge, there is a paucity of data on methods of analysis using liquid chromatography-tandem mass spectrometry (LC-MS-MS) for the determination of MFX in human plasma or CSF. Only one method is described using solid-phase extraction (SPE) as sample pre-treatment procedure for the determination of MFX in plasma (23). As SPE is expensive and time-consuming, the objective of this study was to setup a simple, rapid, and validated LC-MS-MS method of analysis to determine MFX in human plasma, plasma ultrafiltrate, and CSF.
Experimental

Chemicals and reagents
MFX HCl was kindly provided by Bayer (Bay-12-8039, Leverkusen, Germany). The internal control, cyanoimipramine, was supplied by Roche (Woerden, the Netherlands). Acetonitrile and water for LC-MS were purchased from BioSolve (Valkenswaard, the Netherlands). The chemicals used, including methanol Lichrosolve and trifluoroacetic anhydride, are HPLC or analytical grade and were obtained from VWR (Amsterdam, the Netherlands). The precipitation reagent consisted of 0.04 mg/L cyanoimipramine dissolved in a mixture of methanol and acetonitrile (4:21, v/v). Pooled human plasma (ultrafiltrate) samples with EDTA as anticoagulant and pooled human CSF samples were made available according to the standard operating procedures of University Medical Center Groningen.

Preparation of stock solutions
Different stocks were prepared for the calibration standards and the quality control (QC) samples by dissolving MFX HCl in water. The stock solutions were diluted with the same solvent to obtain stock solutions for the validation of MFX in plasma and CSF at concentrations of 100 mg/L (calibration standards; Stock C and E) and 200 mg/L (QC samples; Stock D and F), respectively. The original stock solutions were diluted to working stock solutions at concentrations of 5 mg/L (calibration standards; Stock C1 and E1) and 10 mg/L (QC samples; Stock D1 and F1), respectively. For the QC samples of MFX in plasma ultrafiltrate, the same stock solutions were used as applied for the QC samples in plasma (Stock D and D1). The MFX concentration in the stock solutions was corrected for the drug hydrochloride.

Sample pre-treatment procedure for plasma ultrafiltrate
A volume of 500 μL controlled human plasma was transported into a Centrifree® Ultrafiltration device with an Ultracel® YM-T membrane for volumes of up to 1.0 mL (Millipore, Schwalbach, Germany). The devices were capped afterwards. The membrane had a hold-up volume of 10 μL and an active surface area equal to 0.92 m². The devices were placed in a 35º fixed angle Hettich EBA 21 rotor and spun at 1,640 x g for 20 min at room temperature (Sigma Aldrich, St. Louis, MO).

Preparation of calibration standards and QC samples
The calibration standards and QC samples were prepared by spiking controlled human EDTA plasma and human CSF with the appropriate amount of one of the eight prepared stock solutions. In the same way, QC samples of MFX in controlled human plasma
ultrafiltrate were prepared. All calibration and QC values were corrected for the drug hydrochloride. The theoretical concentrations are displayed in Table 1. A maximum of 5% of the final volume consisted of the added stock solution. The calibration standards and QC samples were prepared at day 1 of validation and stored at -20°C.

Table 1. Calibrations standards and Quality Control samples.

<table>
<thead>
<tr>
<th>Calibration standard*</th>
<th>Concentration (mg/L plasma)</th>
<th>Concentration (mg/L CSF)</th>
<th>Concentration (mg/L plasma ultrafiltrate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.050</td>
<td>0.051</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.100</td>
<td>0.102</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.250</td>
<td>0.256</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.500</td>
<td>0.511</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.000</td>
<td>1.022</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2.001</td>
<td>2.045</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>3.001</td>
<td>3.067</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>5.001</td>
<td>5.112</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Quality Control sample†</th>
<th>Concentration (mg/L plasma)</th>
<th>Concentration (mg/L CSF)</th>
<th>Concentration (mg/L plasma ultrafiltrate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLOQ</td>
<td>0.050</td>
<td>0.049</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>0.500</td>
<td>0.492</td>
<td>0.500</td>
</tr>
<tr>
<td>Med</td>
<td>2.481</td>
<td>2.438</td>
<td>2.501</td>
</tr>
<tr>
<td>High</td>
<td>4.962</td>
<td>4.876</td>
<td>5.002</td>
</tr>
</tbody>
</table>

*Stock solutions C/C1 (plasma) and E/E1 (CSF) were used to prepare the calibration standard.
†Stock solutions D/D1 (plasma) and F/F1 (CSF) were used to prepare quality control samples.
‡LLOQ = lower limit of quantitation.

Sample preparation
The samples were thawed until reaching room temperature. A volume of 750 μL precipitation reagents was added to 100 μL of each sample to promote protein precipitation (blank sample, calibration standard, QC sample) and vortex mixed for 1 min. The samples were then stored at -20ºC for at least 15 min to promote protein precipitation and finally centrifuged at 11,000 x g for 5 min. From the clear upper layer, 5 μL was injected in the LC-MS-MS system.

LC-electrospray ionization-MS-MS conditions
All experiments were performed on a Thermo Fisher (San Jose, CA) triple-quadrupole LC-MS-MS with a Finnigan Surveyor LC pump and an autosampler, which was set at a temperature of 20ºC. After sample preparation, 5 μL of the clear upper layer was injected on
a Thermo Electron HyPurity C18 analytical column (50 x 2.1 mm, 5-μm) (Interscience Breda, Breda, the Netherlands) for chromatographic separation. The column temperature was set at 20°C. The mobile phase had a flow of 300 μL/min and consisted of purified water, acetonitrile and an aqueous buffer (containing ammonium acetate 10 g/L, acetic acid 35 mg/L, and trifluoracetic anhydride 2 mL/L water), which was maintained constantly at 5% during the gradient (Table 2).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>A* (%)</th>
<th>B* (%)</th>
<th>C* (%)</th>
</tr>
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<tbody>
<tr>
<td>0.00</td>
<td>5</td>
<td>95</td>
<td>0</td>
</tr>
<tr>
<td>0.50</td>
<td>5</td>
<td>35</td>
<td>60</td>
</tr>
<tr>
<td>1.30</td>
<td>5</td>
<td>35</td>
<td>60</td>
</tr>
<tr>
<td>1.50</td>
<td>5</td>
<td>75</td>
<td>20</td>
</tr>
<tr>
<td>2.50</td>
<td>5</td>
<td>75</td>
<td>20</td>
</tr>
<tr>
<td>3.00</td>
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<td>95</td>
<td>0</td>
</tr>
<tr>
<td>3.50</td>
<td>5</td>
<td>95</td>
<td>0</td>
</tr>
</tbody>
</table>

*A = aqueous buffer; B = purified water; and C = acetonitrile.

The Finnigan TSQ Quantum Discovery mass selective detector (Thermo Scientific, Waltham, MA) was utilized for detection of positive ions (electrospray ionization) because of the presentation of amine and ketone groups in the MFX structure. These groups could be easily protonated (23). Selected reaction monitoring (SRM) was used as scanning mode. With a scan width of 0.5 m/z, the following transitions were measured: MFX m/z 402.0 to m/z 358.2 (collision energy 19 eV) and cyanoimipramine m/z 306.0 to m/z 218.0 (collision energy 39 eV). Ion spray voltage, sheath gas pressure, auxiliary gas pressure, and capillary temperature were set at 3,500 V; 35 arb (arbitrary units); 5 arb; and 350°C, respectively. Peak height integration of all components was achieved with Xcalibur software version 1.4. SRI (Thermo Fisher).

**Method validation**

The developed method was fully validated in accordance with the U.S. FDA’s *Guidance for Industry Bioanalytical Method Validation*. Selectivity, linearity, accuracy, precision, sensitivity, recovery, and stability are the seven criteria included in this guide. Precision was subdivided into within-run and between-run (24).
A single calibration curve was analyzed each day for three days. The calibration curves were constructed using a 1/x weighted linear regression of the ratios of the observed peak heights of MFX and the internal control cyanoimipramine against the spiked concentrations of the standards. The concentration of a sample of unknown concentration (patient samples) will then be determined based on one-point calibration (highest calibration point). QC samples were analyzed on three single days in fivefold. Selectivity was evaluated by processing and analyzing six different pools of controlled human plasma and human CSF. Sensitivity will be examined by respectively comparing the response of six lots of pooled human plasma and six lots of human CSF with the response of Lower Limit of Quantitation (LLOQ) samples. To evaluate interaction of MFX with other anti-tuberculosis drugs that can be co-medicated with MFX, a simultaneous determination of these agents with MFX will be done by using the developed method. A blank sample spiked with concentrations in the middle of the therapeutic range of ethambutol (5 mg/L), linezolid (10 mg/L), rifampicin (10 mg/L), and isoniazid (5 mg/L) will be used.

For three days consecutively, a sample with a concentration of 10 mg MFX/L plasma and a sample with a concentration of 10 mg MFX/L CSF were diluted ten times and then prepared in fivefold. Finally, each day, the five freshly prepared samples were measured.

Recovery was determined on three levels (low, med, high) in fivefold and measured by comparing the average peak height of the processed QC samples with the average peak height of the recovery samples. Recovery samples (low, med, high) were spiked in water instead of controlled human plasma or CSF (QC samples). A plasma or CSF sample after the precipitation procedure is comparable to water. The recovery was also processed for the internal control using the average peak height of the internal control.

Stability tests included three freeze-thaw cycles and storage stability. Storage stability consists of storage in the refrigerator (4°C), at room temperature (20-25°C), and after sample preparation in the autosampler (20°C). Stability at room temperature was measured with and without the influence of light because of the known light-sensitivity of MFX (25). Autosampler stability is included in connection with the opportunity to re-inject previously measured samples. All stability tests were performed on two levels (low and high) in fivefold for three days. Stability is defined as a change in concentration and should be ≤15%.

To investigate ion suppression or ion enhancement, simultaneous post-column infusion of a solution of MFX and cyanoimipramine and first injection of five samples precipitation reagents without internal control were followed by five samples of controlled human plasma.
A single calibration curve was analyzed each day for three days. The calibration curves were constructed using a 1/x weighted linear regression of the ratios of the observed peak heights of MFX and the internal control cyanoimipramine against the spiked concentrations of the standards. The concentration of a sample of unknown concentration (patient samples) will then be determined based on one-point calibration (highest calibration point). QC samples were analyzed on three single days in fivefold. Selectivity was evaluated by processing and analyzing six different pools of controlled human plasma and human CSF. Sensitivity will be examined by respectively comparing the response of six lots of pooled human plasma and six lots of human CSF with the response of Lower Limit of Quantitation (LLOQ) samples. To evaluate interaction of MFX with other anti-tuberculosis drugs that can be co-medicated with MFX, a simultaneous determination of these agents with MFX will be done by using the developed method. A blank sample spiked with concentrations in the middle of the therapeutic range of ethambutol (5 mg/L), linezolid (10 mg/L), rifampicin (10 mg/L), and isoniazid (5 mg/L) will be used.

For three days consecutively, a sample with a concentration of 10 mg MFX/L plasma and a sample with a concentration of 10 mg MFX/L CSF were diluted ten times and then prepared in fivefold. Finally, each day, the five freshly prepared samples were measured. Recovery was determined on three levels (low, med, high) in fivefold and measured by comparing the average peak height of the processed QC samples with the average peak height of the recovery samples. Recovery samples (low, med, high) were spiked in water instead of controlled human plasma or CSF (QC samples). A plasma or CSF sample after the precipitation procedure is comparable to water. The recovery was also processed for the internal control using the average peak height of the internal control.

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To investigate ions suppression or ion enhancement, simultaneous post-column infusion of a solution of MFX and cyanoimipramine and first injection of five samples precipitation reagents without internal control were followed by five samples of controlled human plasma (prepared with precipitation reagent without internal control), five samples of controlled human plasma (prepared with precipitation reagent with internal control cyanoimipramine), and two samples of the highest calibration point (5 mg/L), respectively. The same experiment was done with human CSF instead of plasma.

Results

Sample Carry Over
When high concentrations were analyzed during method development, sample carryover was observed. Sample carryover is defined as a residue of a previous sample in the current measurement (26-28). Carryover was greater than 0.05-0.1% in samples with a concentration higher than 0.50 mg/L. It was decided to re-inject patient samples with a measured concentration that was less than 0.50 mg/L. Prior to this re-injection, blank samples were injected (precipitation reagent) until no peak of MFX was observed. Five blank samples appeared to be enough to prevent sample carryover.

Internal control
As in our sample preparation, no extraction is preformed, and an internal control instead of an internal standard is suitable. Cyanoimipramine is used as internal control by our laboratory for all validation procedures of many drugs for TDM. As long as the results of the method validation comply with FDA guidelines for method validation, this compound can be used. The advantage of using a single agent in the laboratory is that only one precipitation reagent is available, and therefore, mistakes are prevented. Moreover, cyanoimipramine has never been used as drug in patients.

Ion suppression and ion enhancement
During post-column infusion, no ion suppression or ion enhancement was observed. There was no variation of baseline height between precipitation reagent (blank) and pooled human plasma with and without internal control, respectively.

Chromatography
The mean retention times of MFX and cyanoimipramine were 1.58 and 1.81 min, respectively. An example of the chromatographic results is shown in Figure 2.
Selectivity and sensitivity

There were no interfering peaks observed at the retention time of MFX or cyanoimipramine in the six lots of human plasma or CSF, respectively.

There also were no interfering peaks observed by simultaneous determination of ethambutol, linezolid, rifampicin, and isoniazid. The analyte response at the LLOQ is greater than five times the blank responses.
Linear regression

The calibration curves were linear over a range of 0.05 to 5.00 mg/L MFX, encompassing the therapeutic range of MFX in plasma and CSF for MDR-tuberculosis (16). Using a weight factor of $1/x$, the mean equations of the plasma (Eq.1) and CSF (Eq.2) standards were equal to

$$y = 0.7623x + 0.00062$$  \hspace{1cm} \text{Eq.1}

The mean correlation coefficient and determination coefficient were greater than 0.990, 0.999, and 0.998, respectively.

$$y = 0.4558x - 0.00158$$ \hspace{1cm} \text{Eq. 2}

The mean correlation coefficient and determination coefficient were greater than 0.990, 0.997, and 0.993, respectively. Variation coefficients (CV) of plasma and CSF calibration standards were measured in a range of -5.4 to 9.3%.

Concentrations above the calibration curve

The mean concentration of the diluted plasma samples was equal to 1.0 mg/L (undiluted: 10 mg/L) with a bias of 4%. The within-run and between-run CVs were 2.9% and 3.7%, respectively. The mean concentration of the diluted CSF samples was equal to 1.0 mg/L (undiluted: 10 mg/L) with a bias of -2%. The within-run and between-run CVs were 3.0% and 2.2%, respectively.

Accuracy and precision

The responses of the analyzed QC samples (LLOQ, low, med, and high) were statistically compared and calculated by using one-way ANOVA for each single concentration level. Results of these statistical calculations are shown in Table 3. All CVs correspond to the rules of the FDA, namely CV and bias <20% for the LLOQ and CV and bias <15% for concentration levels low, med, and high (24).

For this method, the mean LLOQ of the plasma samples was equal to 0.05 mg/L with an accuracy of 7.1% and an overall CV of 4.8%. The mean LLOQ of the CSF samples was equal to 0.05 mg/L with an accuracy of 2.8%, a within-run CV of 6.9%, and a between-run CV of 3.2%. In some cases, an F-test gave no significant difference in between-run and within-run precision. Thus, the overall CV would be noted without needing to be subdivided into between-run and within-run precision.
Chapter 3

Determination of the QC plasma ultrafiltrate samples with the method setup for determination of MFX in CSF seems possible according to the CV, all of which correspond to the previously mentioned FDA rules.

### Table 3. Results of accuracy and precision.

<table>
<thead>
<tr>
<th></th>
<th>LLOQ</th>
<th>Low</th>
<th>Med</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean concentration (mg/L)</td>
<td>0.05</td>
<td>0.54</td>
<td>2.60</td>
<td>5.10</td>
</tr>
<tr>
<td>Bias (%)</td>
<td>7.1</td>
<td>7.1</td>
<td>6.6</td>
<td>2.7</td>
</tr>
<tr>
<td>Within-run CV (%)</td>
<td>1.4</td>
<td>1.4</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Between-run CV (%)</td>
<td>1.6</td>
<td>1.6</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Overall CV (%)</td>
<td>4.8</td>
<td>3.7</td>
<td>2.1</td>
<td>1.9</td>
</tr>
<tr>
<td><strong>CSF</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean concentration (mg/L)</td>
<td>0.05</td>
<td>0.49</td>
<td>2.40</td>
<td>4.80</td>
</tr>
<tr>
<td>Bias (%)</td>
<td>2.8</td>
<td>-0.7</td>
<td>-1.1</td>
<td>-0.8</td>
</tr>
<tr>
<td>Within-run CV (%)</td>
<td>6.9</td>
<td>2.2</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>Between-run CV (%)</td>
<td>3.2</td>
<td>3.3</td>
<td>2.4</td>
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</tr>
<tr>
<td>Overall CV (%)</td>
<td>7.6</td>
<td>4.6</td>
<td>3.9</td>
<td>3.6</td>
</tr>
<tr>
<td><strong>Plasma ultrafiltrate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean concentration (mg/L)</td>
<td>0.49</td>
<td>2.70</td>
<td>5.40</td>
<td></td>
</tr>
<tr>
<td>Bias (%)</td>
<td>-1.0</td>
<td>7.3</td>
<td>7.3</td>
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<tr>
<td>CV (%)</td>
<td>4.7</td>
<td>2.5</td>
<td>2.0</td>
<td></td>
</tr>
</tbody>
</table>

### Recovery

The recoveries ranged from 114.2% to 124.8%, depending on the MFX concentration and the matrix type (Table 4). The internal control had a recovery of 96.5% and 100.2% for the plasma and CSF samples, respectively. All CVs were less than 15%.

### Stability

The results of the stability test are summarized in Table 5. The concentration of MFX will be viewed in comparison to the mean concentration of freshly prepared QC samples on two levels (low and high). After three freeze-thaw cycles, the stability of the QC samples (low and high) was not affected. Storage in the refrigerator for 120 h storage at refrigerator (4ºC), at room temperature, and after sample preparation in the autosampler (20ºC) did not affect...
stability of QC samples either (low and high). Exposure to or protection from light made no significant difference in stability of the QC samples for those 120 h.

**Table 4. Results of recovery.**

<table>
<thead>
<tr>
<th></th>
<th>Low</th>
<th>Med</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration (mg/L)</td>
<td>0.500</td>
<td>2.481</td>
<td>4.962</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>122.2</td>
<td>116.2</td>
<td>114.2</td>
</tr>
<tr>
<td>Within-run CV (%)</td>
<td>7.6</td>
<td>7.0</td>
<td>5.8</td>
</tr>
<tr>
<td><strong>CSF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration (mg/L)</td>
<td>0.492</td>
<td>2.438</td>
<td>4.876</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>124.8</td>
<td>122.7</td>
<td>124.8</td>
</tr>
<tr>
<td>Within-run CV (%)</td>
<td>11.2</td>
<td>9.7</td>
<td>10.2</td>
</tr>
</tbody>
</table>

**Table 5. Results of stability experiments (% bias).**

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th>CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Freezer-thaw 3 cycles</td>
<td>-2.1</td>
<td>-0.8</td>
</tr>
<tr>
<td>Autosampler (re-injection) 120 h</td>
<td>+0.2</td>
<td>-4.9</td>
</tr>
<tr>
<td>Refrigerator (4°C) 120 h</td>
<td>+3.1</td>
<td>+0.3</td>
</tr>
<tr>
<td>Room temperature (protection from light) 120 h</td>
<td>-0.3</td>
<td>-1.2</td>
</tr>
<tr>
<td>Room temperature (no protection from light) 120 h</td>
<td>-3.9</td>
<td>-5.0</td>
</tr>
</tbody>
</table>

**Clinical application**

This method was implemented in routine monitoring of MFX in TB patients suspected to have a low AUC/MIC ratio and in patients with TB meningitis. Figure 3 shows an MFX concentration time curve of a patient with TB meningitis receiving MFX in a dose of 400 mg once daily as a part of an anti-tuberculosis regimen. The MIC for MFX of the isolate was 0.125 mg/L. The AUC was 36.1 mg*h/L in plasma and 21.9 mg*h/L in CSF following a dose of MFX of 400 mg once daily. The AUC/MIC ratio was 289 for plasma and 175 for CSF. The fraction unbound was equal to 0.72; and as a result, the estimated AUC\_unbound/MIC was 208. Treatment was continued, and the patient was cured after nine months of treatment.
Figure 3. Typical patient curve in plasma and CSF. MFX plasma concentrations are represented by solid circles and CSF concentrations by open circles.

Discussion

The objective of this study was to setup a simple, rapid, and validated LC-MS-MS method of analysis for TDM of MFX in human plasma and CSF without time-consuming and expensive SPE. The method developed is fully validated based on the rules of the U.S. FDA’s Guidance for Industry Bioanalytical Method Validation (24).

For the SRM analysis, one transition was selected for both MFX and cyanoimipramine. The other transition for both target and internal control where of low stability and gave a low signal.

The slope of the calibration curve of MFX for plasma was different than that for CSF. Neither matrix showed ion enhancement or ion suppression during post-column infusion. An equal
difference between them was found without using an internal control for calculating the slopes. Sensitivity of the LC-MS-MS is more or less variable, which is acceptable as long as QC samples are analyzed in the same run.

The observed sample carryover, which is a common problem on LC-MS-MS (26-28), could not be eliminated entirely. By re-injection of a calculated amount <0.50 mg/L, this problem can be reduced to an acceptable level. However, in daily practice concentration levels are expected to be higher, and re-injection will not have to be applied frequently. As no interference was observed with other anti-tuberculosis drugs, the method of analysis was suitable for TDM in daily practice.

Because a plasma or CSF sample after the precipitation procedure is comparable with water, comparing of the average peak height of the QC samples in plasma or CSF with the average peak height of the QC samples in water corresponds with recovery in our developed LC-MS-MS method. However, a recovery of MFX of 114.2-124.8% for plasma and CSF is remarkable. The authors think that EDTA may be an explanation for this phenomenon as plasma obtained from sodium-heparin or lithium-heparin tubes resulted in a near 100% recovery. According to the FDA rules, the recovery of the analyte need not to be 100%, though. The recovery of the analyte and the internal control should be consistent, precise, and reproducible (24). In this case, the CVs comply with these guidelines.

Variability in protein binding has potential influence on efficacy of an antimicrobial agent as the unbound fraction of the drug can pass the membrane and interact with the bacteria. Determination of MFX in plasma ultrafiltrate seems necessary to explore the unbound drug AUC/MIC ratio. Based on the collection of ultrafiltrate, setup and validation of an individual method to determine MFX in plasma ultrafiltrate is very time-consuming. A matrix comparison showed that the fully validated method for determination of MFX in CSF was suitable to determine MFX in plasma ultrafiltrate. However, one must keep in mind that the collection of ultrafiltrate has to be reproducible. For example, pH (29) and temperature control (29;30) during ultrafiltration influence the percentage of bound/unbound drug.

The method of analysis developed is suitable for measuring MFX in plasma, CSF, and plasma ultrafiltrate as shown by the presented patient concentration time curve. The LLOQ of 0.05 mg/L is low enough to measure the unbound fraction of an MFX plasma sample obtained before the dose.
Chapter 3

Recently, MFX was recently compared with the anti-tuberculosis drug ethambutol in the initial treatment of tuberculosis in a randomized controlled trial (31). As MFX resulted in earlier sputum culture conversion compared to ethambutol, the position of this drug will probably expand. Considering the variability in MFX AUC, variability in in vitro susceptibility to MFX of M. tuberculosis, potential influence of protein binding on efficacy, and variability of penetration into sanctuary sites a simple, rapid, and validated LC-MS-MS method of analysis is mandatory to evaluate MFX in prospective studies and conduct TDM.

Conclusions

A simple, rapid, and validated LC-MS-MS method to analyze MFX in human plasma and CSF was developed. As no analytical interaction with other co-medication was observed, this method can be used for prospective pharmacokinetic studies and TDM of MFX in patients with tuberculosis.

Acknowledgement

The authors would like to thank Bayer for providing the MFX pure drug substance.
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References

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