HPLC Method for the Simultaneous Analysis of Fluoroquinolones and Oxazolidinones in Plasma

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A chromatographic method was implemented and validated for the simultaneous determination of antimicrobials proposed for the treatment of mycetoma: three fluoroquinolones: ciprofloxacin, moxifloxacin and sparfloxacin; two oxazolidinones: DA-7157 (DA2; torezolid) and its prodrug DA-7218 (DA1). Separation of analytes was achieved on an Atlantis dC18 column (150 × 4.6 mm i.d., 5 μm particle size) with a mobile phase composed of acetonitrile and trifluoroacetic acid 0.1% (v/v) using a gradient program. Total running time was 30 min. Quantification of sparfloxacin was carried out using a DAD at 278 nm; the oxazolidinones DA1 and DA2 and the quinolones ciprofloxacin and moxifloxacin were analyzed by fluorescence with an excitation wavelength of 292 nm and an emission wavelength of 525 nm. Intraday precision was in the range of 3.2 and 14.1%. Linearity range was from 0.3 to 10 μg/mL for sparfloxacin using DAD detector, and from 0.2 to 10 μg/mL for ciprofloxacin, 0.3 to 10 μg/mL for DA2, 0.4 to 10 μg/mL for DA1 and 0.04 to 10 μg/mL for moxifloxacin with fluorescence detector. Acetonitrile was used to precipitate proteins from plasma. Recoveries at low, medium and high concentration were between 80 and 120%. Limits of quantification were between 0.04 and 0.4 μg/mL in plasma. The method can be applied for individual or simultaneous determination of the antimicrobials in plasma.

Introduction

Mycetoma is a chronic subcutaneous infectious disease characterized by the tumefaction of the region affected and the production of cavitary abscesses which drain a seropulent secretion containing the etiologic agent. In Mexico, 98% of the total cases are produced by actinomycetes and ~86% are produced by Nocardia brasiliensis (1). The highest cure rates (70%) have been obtained with the use of sulfamethoxazole (Sx)–trimethoprim (T). In our dermatology service, amikacin has been added to the Sx–T combination to treat severe cases of mycetoma or those cases involving subjacent organs, obtaining a cure rate of ~95% in a series of 52 patients. However, in some cases, the use of these antimicrobials carries the risk of side effects, or the development of bacterial resistance, making necessary the search for new therapeutic alternatives. Others antimicrobials have been tested in vivo to have therapeutic alternatives for the treatment, including oxazolidinones and fluoroquinolones (2, 3). Most therapeutic schemes for bacterial infections involve only one antimicrobial; however, in the case of infections by actinobacteria such as tuberculosis, nocardiosis and mycetoma, with long periods of treatment, multi-therapy is often used in order to prevent failure due to formation of resistant bacteria to any of antimicrobial: the possibility of generating a double or triple mutant resistant is compared with a single antimicrobial. Combinations of fluoroquinolones and oxazolidinones are suggested for this purpose.

Several methods have been reported for quantifying the different fluoroquinolones in serum, plasma and other matrices (4–11). Most of them used high-performance liquid chromatography (HPLC) with ion pair reagent (7–11). Previous to this work we published an analytical HPLC method to quantification of garenoxacin, in combination of other antibiotics in plasma and tissue of actinomyctoma. The separation was carried with satisfactory results without using ion pair reagent (12).

On the other hand, oxazolidinones are a new, unique class of synthetic antibacterial agents effective against many Gram-positive bacteria, including aerobic pathogenic actinomycetes of the genera Mycobacterium, Nocardia and Actinomadura. Linezolid, the first oxazolidinone on the market, has been proved to be active in animal models, as well as in clinical trials with patients infected with Nocardia spp. (13). Torezolid (DA-7157, DA2), another oxazolidinone, also was shown to be active in vitro against several Gram-positive species, with the observation of a level of activity superior to that of linezolid (14). The DA2 compound is produced by the metabolism of a highly hydrophilic prodrug, DA-7218 (DA1). In plasma, there are several reports about the determination of linezolid, all of them use HPLC/UV–vis or HPLC/MS (15–17). The concentrations of DA1 and DA2 in plasma samples were analyzed using a similar HPLC method developed in our laboratory using a fluorescence detector (18), with aim to probe the activity of the prodrug, DA1 to inhibit the production of experimental lesions in BALB/c mice.

Since these antibiotics may provide an alternative for treatment of mycetoma, and has been thought its possible administration in combination or individually, the purpose of this project was to adapt a method by HPLC with UV and fluorescence detection for the simultaneous determination of moxifloxacin, ciprofloxacin, sparfloxacin, DA1 and DA2 (structures are shown in Supplementary data, Figure S1) in mouse plasma, which has been used as a model to probe activity in vivo. Although some of these antibiotics have been determined simultaneously, for example ciprofloxacin and moxifloxacin (7, 10), linezolid and moxifloxacin (16) and DA2 and DA1 (18), there are no reports for the determination of these fluoroquinolones and oxazolidinones together. The results will be used to correlate plasma levels with the treatment given.
Experimental

Reagents
Ciprofloxacin, moxifloxacin, sparflloxacin and trifluoroacetic acid (TFA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). DA1DA1 and DA2 were supplied by the Research Laboratory of Dong-A Pharmaceutical Company (Yongin, South Korea). To assay the selectivity of the proposed method, Sx and T were included. Both were Sigma-Aldrich products.

Acetonitrile HPLC grade was purchased from Fisher Scientific (Houston, TX, USA). Deionized water was purified by a Milli-Q System (Millipore Co., MA, USA). The mobile phases were filtered through a 0.45-μm Nylon filter (Waters Corporation, Milford, MA, USA) before use. Standard solutions were prepared in methanol HPLC grade and filtered through Nylon acrodisks (Waters Co., Millford, MA, USA).

Equipment and chromatographic conditions
The chromatographic separation was achieved using a Waters 2690 Alliance liquid chromatograph with diode array detector 996 (DAD) and fluorescence detector 474. An Atlantis dC18 column 150 × 4.6 mm i.d., with 5 μm particle size (Waters) was used. Samples were eluted with a mobile phase consisting of 0.1% TFA in water (solvent A) and acetonitrile (solvent B); the gradient used is shown in Table I. The flow rate of the mobile phase was 1.0 mL/min. The injection volume was 10 μL. Detection and quantification of Torezolid and its metabolite and quinolones, ciprofloxacin, moxifloxacin and sparfloxacin were by UV–vis at a wavelength of 278 nm and fluorescence; the fluorescence intensities were measured at an excitation wavelength of 292 nm and an emission wavelength of 525 nm. Total running time was 30 min.

Standard solutions
Ten milliliters of the standard stock solutions of each antibiotic were prepared in methanol at a concentration of 1 ng/mL and stored at −20°C. From these solutions, standard stock mixtures from each antibiotic at concentrations ranging from 10 to 100 μg/mL were prepared and stored at −20°C for 30 days; from these dilutions, the working aqueous solutions (working standards) of the drugs were prepared weekly to yield final concentrations of 0.1, 0.5, 2, 5 and 10 μg/mL.

Sample treatment
Drug-free plasma pools from Balb/C mice samples were frozen at −20°C. Before the assay treatment, frozen samples were thawed to room temperature (~22°C).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>TFA 0.1%</th>
<th>Acetonitrile</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
<td>40</td>
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<td>15</td>
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<tr>
<td>25</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>27</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>30</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

Plasma treatment
To 50 μL of the thawed plasma, 150 μL acetonitrile was added to precipitate proteins, the mixture was vortexed for 5 s and centrifuged for 5 min at 5304 × g, and the supernatant was filtered through 0.45-μm Nylon filters (Waters). Filtrates were received into 150 μL inserts.

Method validation
Linearity, limits of detection and quantification
Calibration curves were constructed at six concentration levels: 0.1, 0.5, 2, 5 and 10 μg/mL for all tested antibiotics. The area values were plotted against the concentration and calibration plots were constructed using an external standard method. Curves were constructed from triplicate runs.

To take into consideration the matrix effect, calibration curves were constructed in plasma free of antimicrobials. Limits of detection (LOD) were the minimum concentration that produces a response that can differentiate from the noise (ratio signal/noise > 3). The LOQ were calculated as the minimum concentration calculated with suitable precision and accuracy (19).

Precision
The precision of the system and method was evaluated by means of the coefficients of variation (CVs) of peak areas. CVs of the system were calculated by means of injection of three working mixtures at 0.4, 2 and 10 μg/mL, each in triplicate. Intraday precision of the method in plasma were determined by assaying three spiked samples at 0.4, 2 and 10 μg/mL. All samples were processed in triplicate. Interday precision was calculated by working aliquots of the same samples on different days (n = 5). These samples were also injected 8 and 24 h after their preparation to test their stability.

Recovery
Recoveries were evaluated by adding to 50 μL of the plasma pool the adequate volume from the stock solution of the antibiotic mixture to achieve 0.4, 2 and 10 μg/mL. The procedure for the sample preparation was the same as that previously described. Results were calculated from the calibration curves by means of the external standard method. The procedure was repeated three times.

Selectivity
Selectivity was tested by examining seven different samples of unspiked plasma free of antibiotics to confirm that the signals originating from the matrix did not interfere with the signals from the analytes. Identification of each compound in the spiked samples was made by the retention times as well as by comparison of the DAD spectra from the chromatographic signals with those of the respective standards. Additionally, Sx and T were added to the plasma and analyzed to demonstrate that the signals were not overlapped with those of the antibiotics included in this method.

Stability
To determine the stability of the analytes in the matrix, the plasma pool was spiked with the standard mixture to achieve a
concentration of 2.0 μg/mL for each antimicrobial; 50 μL fractions were stored at –20 °C for 1 month. Each week a sample was thawed to record a measurement. Results were evaluated using control graphs constructed with the results obtained from the precision experiment. Also, the stability of each sample was tested 10 and 24 h after their preparation maintained at room temperature.

**Results**

**Chromatographic separation**

Representative chromatograms of a mixture of the seven antimicrobial standards at 1 μg/mL are shown in Figure 1. For DAD (1A), the monitoring wavelength was 278 nm and for the fluorescence detector (1B) λ<sub>ex</sub> 292 and λ<sub>em</sub> 525 nm were used. The complete elution of the antimicrobials was obtained in the first 15 min.

Figure 2A shows the UV chromatogram of plasma spiked with the standard mixture at 5 μg/mL. Figure 2B shows the same chromatogram with fluorescence detection. Retention time and resolution of antimicrobials are showed in Table II.

**Method validation**

Tables III and IV show the results for the validation of the method in plasma.

![Figure 1. HPLC chromatograms of the standard mixture of the seven antimicrobial at 1 μg/mL: (A) detection at 278 nm and (B) fluorescence detection (λ<sub>ex</sub> 292 nm, λ<sub>em</sub> 525 nm). Elution order is: T, ciprofloxacin (C), DA1, sparfloxacin (S), moxifloxacin (M), Sx and DA2. Separation conditions are described in the text.](image1)

![Figure 2. HPLC chromatograms of plasma spiked with the standard mixture at 5 μg/mL. (A) Detection at 278 nm and (B) fluorescence detection (λ<sub>ex</sub> 292 nm, λ<sub>em</sub> 525 nm). Elution order is T, ciprofloxacin (C), DA1, sparfloxacin (S), moxifloxacin (M), Sx and DA2. Separation conditions are described in the text.](image2)
Validation Parameters of the Method Used for the Analysis of the Antimicrobials in Plasma

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Linear range (μg/mL)</th>
<th>RF area/ concentration</th>
<th>CV of RF</th>
<th>Correlation coefficient ($r^2$)</th>
<th>LOD (μg/mL)</th>
<th>LOQ (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin (F)</td>
<td>0.2–10</td>
<td>4.66 × 10^4</td>
<td>11.5</td>
<td>0.991</td>
<td>0.05</td>
<td>0.20</td>
</tr>
<tr>
<td>Ciprofloxacin (UV)</td>
<td>0.2–10</td>
<td>5.41 × 10^4</td>
<td>12.2</td>
<td>0.995</td>
<td>0.05</td>
<td>0.20</td>
</tr>
<tr>
<td>DA1 (UV)</td>
<td>0.4–10</td>
<td>5.28 × 10^4</td>
<td>7.2</td>
<td>0.995</td>
<td>0.10</td>
<td>0.40</td>
</tr>
<tr>
<td>DA1 (F)</td>
<td>0.4–10</td>
<td>5.49 × 10^4</td>
<td>7.9</td>
<td>0.995</td>
<td>0.10</td>
<td>0.40</td>
</tr>
<tr>
<td>Sparfloxacin (UV)</td>
<td>0.3–10</td>
<td>1.86 × 10^4</td>
<td>9.5</td>
<td>0.999</td>
<td>0.10</td>
<td>0.30</td>
</tr>
<tr>
<td>Moxifloxacin (UV)</td>
<td>0.20–10</td>
<td>3.51 × 10^4</td>
<td>9.2</td>
<td>0.997</td>
<td>0.05</td>
<td>0.20</td>
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<tr>
<td>Moxifloxacin (F)</td>
<td>0.04–10</td>
<td>1.76 × 10^4</td>
<td>12.3</td>
<td>0.998</td>
<td>0.01</td>
<td>0.04</td>
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<tr>
<td>DA2 (UV)</td>
<td>0.3–10</td>
<td>1.63 × 10^4</td>
<td>12.2</td>
<td>0.999</td>
<td>0.08</td>
<td>0.30</td>
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<tr>
<td>DA2 (F)</td>
<td>0.3–10</td>
<td>1.57 × 10^4</td>
<td>12.0</td>
<td>0.992</td>
<td>0.08</td>
<td>0.30</td>
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</table>

F, fluorescence detection; UV, UV detection.

Table IV

Validation Parameters of the Method Used for the Analysis of the Antimicrobials

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Concentration (μg/mL)</th>
<th>% Recovery</th>
<th>CV intraday</th>
<th>CV interday</th>
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<tr>
<td>Ciprofloxacin</td>
<td>0.4</td>
<td>103</td>
<td>10.4</td>
<td>15.8</td>
</tr>
<tr>
<td>2</td>
<td>107</td>
<td>7.2</td>
<td>13.5</td>
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<tr>
<td>10</td>
<td>107</td>
<td>5.3</td>
<td>14.4</td>
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</tr>
<tr>
<td>DA1</td>
<td>0.4</td>
<td>111</td>
<td>5.7</td>
<td>9.1</td>
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<tr>
<td>2</td>
<td>96</td>
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<td>99</td>
<td>8.0</td>
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<tr>
<td>Sparfloxacin</td>
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<tr>
<td>Moxifloxacin</td>
<td>0.4</td>
<td>111</td>
<td>14.1</td>
<td>15</td>
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<tr>
<td>2</td>
<td>107</td>
<td>7.8</td>
<td>8.0</td>
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<td>10</td>
<td>112</td>
<td>2.4</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>DA2</td>
<td>0.4</td>
<td>114</td>
<td>13.8</td>
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</tr>
<tr>
<td>2</td>
<td>108</td>
<td>5.7</td>
<td>9.5</td>
<td></td>
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<tr>
<td>10</td>
<td>106</td>
<td>6.1</td>
<td>8.0</td>
<td></td>
</tr>
</tbody>
</table>

The response factors (RFs) were calculated as the relationship between response and concentration. A linear response implies CVs equal to or <15% in the RFs. In this study, CVs resulted between 7.2% for DA1 and 12.3% for MX. Table III shows the linear range, the RF, the correlation coefficient of the linear equation ($r^2$), LOD and LOQ obtained for each antimicrobial.

Precision and accuracy

The precision for both the system and the method was evaluated. Method precision was determined by using plasma samples spiked and treated at three different concentration levels: low, medium and high. The CVs obtained for all antimicrobials were <15%. Recoveries at low, medium and high concentration were between 80 and 120% (Table IV).

Stability

The results of the stability study showed that plasma spiked with antimicrobials was stable up 1 month if stored frozen at –20°C with no evidence of decomposition. Treated samples at room temperature must be analyzed within 10 h of the treatment, because after 24 h the signals showed lower intensities by up to 50%.

Selectivity

Analytical signals from the plasma matrix appeared at retention times different from the retention times of the antimicrobials before 8 min (Fig. 3); none interference in the UV–vis and fluorescence chromatograms was observed.

Applicability

To evaluate the effectiveness of the proposed method, plasma samples obtained from mice treated with the antimicrobials were analyzed.

Figure 4 shows a chromatogram of the plasma of a mouse treated with moxifloxacin. All the other antimicrobials were determined alone and in mixtures (Sx–T, DA1 and DA2). The results obtained showed the applicability of the method.

Discussion

In this paper, we developed a method for the determination of five antimicrobials with proven activity against N. brasiliensis (2, 3, 12, 13), three fluoroquinolones: ciprofloxacin, moxifloxacin and sparfloxacin; two new oxazolidinones: DA2 (torezolid) and its produrg DA1. These antimicrobials could be used as alternative treatment for actinomycetoma.

In 2012, Sousa et al. (20) conducted an extensive review of the analytical methods for the determination of fluoroquinolones in different biological and pharmaceutical matrices; in this review, several liquid chromatographic methods are discussed including advantages and applicability. For oxazolidinones, there are reports on the determination of linezolid in plasma, all using HPLC/UV–vis or HPLC/MS (14–17). To our knowledge, there are no reports for determination of these fluoroquinolones and oxazolidinones together. Our method allows the simultaneous determination of these antimicrobials in plasma.

For the development of the method, an Atlantis dC18 column was selected. Previous to this work an evaluation of different chromatographic systems was made (12). The endcapped Atlantis dC18 column, which exhibits better retention of polar compounds with good peak shapes, permitted the separation of all the antimicrobials without using the ion pair reagent. The use of tetra-butyl ammonium phosphate in the elution solvent as an ion pair agent has been recommended to analyze ionic compounds including several quinolones, as garenoxacin and gatifloxacin (7–9). However, the ion pair reagents present several inconvenient as it required longer conditioning times for the column; another disadvantage is the short life of the column; for these reasons, this system was discarded.

With the gradient program used, all components eluted within the first 15 min. After this time, the column was washed with
Figure 3. HPLC-UV chromatograms ($\lambda = 278$ nm). (A) A plasma pool of untreated mice (B) plasma pool spiked with a standard mixture of 0.1 $\mu$g/mL of each antibiotic.

Figure 4. HPLC chromatograms of plasma samples from mice treated with (A) moxifloxacin (10.78 min), (B) sparfloxacin (10.52 min) and (C) DA1 (10.13 min), peak at 12.12 min corresponding to DA2.
100% acetonitrile and subsequently was conditioned with initial composition before next injection, this was important to maintain the reproducibility of results.

The validation of the chromatographic method was carried out according to FDA guidelines (19). The higher values of LOD were for DA1, with the lower RFs. Using the DAD detector, a good response of the majority of antimicrobials was obtained at 278 nm. The lowest LOQ were for the moxifloxacin analyzed using fluorescence detection, with a sensitivity five times greater than UV as show in Table III. Ciprofloxacin, DA1 and DA2 presented RFs similarly in UV and fluorescence. Sparfloxacin presented fluorescence but the answer was very low, because it was quantified solely by UV–vis. The LOQs for moxifloxacin and ciprofloxacin are higher than other reported (20); however, this method allows the simultaneous determination of all of these antimicrobials, and the results were satisfactory considering the doses usually administered and levels expected in plasma.

As show in Table IV, the intra- and interday precision (CV) were <15%. Recoveries at low, medium and high concentration were between 80 and 120%. Results of precision and recovery for peak areas were according to FDA guidelines. Peak identification in each chromatogram was achieved by means of tR as well as spectral analysis. Analytical signals from the plasma matrix appeared at retention times different from the retention times of the antimicrobials (before 8 min, Fig. 3); none interference in the UV–vis and fluorescence chromatogram was observed. Furthermore, signals of Sx and T, the two most commonly used antibiotics in the treatment of actinomycetoma, presented tR different, so, if any fluoroquinolone or an oxazolidinone is used in combination with Sx or T, interference will not occur. Amoxicillin is a beta-lactam antibiotic, highly polar, that has also been proposed and used in the treatment of actinomycetoma. Under similar chromatographic conditions (12) elutes before T and does not interfere with the antibiotics analyzed in this work.

Taken in account results of stability, the samples can be stored for 1 month at −20°C. Moreover, once the plasma has been prepared for the analysis (protein precipitation), the samples are stable within the first 10 h.

The effectiveness of the proposed method was evaluated by testing samples of mouse plasma that was treated with these antimicrobials (alone or in combination.) The chromatograms showed signals which could be identified (absorption spectrum) and quantified without interferences.

Conclusions

A method was developed and validated for the quantification of ciprofloxacin, moxifloxacin, sparfloxacin, DA-7157 and DA-7268 in plasma from mice. The HPLC method is easy to perform and has adequate precision and accuracy. This study demonstrates the applicability of the method in a mouse model.

Supplementary data

Supplementary data are available at Journal of Chromatographic Science online.

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