Method for simultaneous analysis of nine second-line anti-tuberculosis drugs using UPLC-MS/MS

Minje Han1,2, Sun Hee Jun1, Jae Ho Lee3,4, Kyoung Un Park1,2, Junghan Song1,2 and Sang Hoon Song2,5*

1Department of Laboratory Medicine, Seoul National University Bundang Hospital, Gyeonggi-do, South Korea; 2Department of Laboratory Medicine, Seoul National University College of Medicine, Seoul, South Korea; 3Department of Internal Medicine, Seoul National University Bundang Hospital, Gyeonggi-do, South Korea; 4Department of Internal Medicine, Seoul National University College of Medicine, Seoul, South Korea; 5Department of Laboratory Medicine, Seoul National University Hospital, Seoul, South Korea

*Corresponding author. Tel: +82-2-2072-3326; Fax: +82-2-747-0359; E-mail: cloak21@snu.ac.kr

Received 27 November 2012; returned 14 January 2013; revised 15 March 2013; accepted 27 March 2013

Objectives: Therapeutic drug monitoring (TDM) of anti-tuberculosis (TB) drugs is beneficial for patients responding slowly to treatment and those with multidrug-resistant TB. We used ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) to develop a rapid method for simultaneously measuring the blood concentrations of nine second-line anti-TB drugs: streptomycin, kanamycin, clarithromycin, cycloserine, moxifloxacin, levofloxacin, para-aminosalicylic acid, prothionamide and linezolid.

Methods: Serum samples were extracted with acidified methanol and neutralized with NaOH. A Waters Acquity HSS T3 column and gradients of ammonium formate and acetonitrile in 0.1% formic acid were used for UPLC separation. Drug concentrations were determined by multiple reaction monitoring in positive ion mode, and assay performance was evaluated. We applied this method to TDM, analysing random serum samples from 85 patients treated with second-line drugs.

Results: Sample preparation using acidified methanol extraction followed by neutralization yielded good recovery and ionization efficiency, with chromatographic separation achieved within 3 min per sample. Within-run and between-run precisions were 1.7%–7.5% and 1.7%–12.4%, respectively, at concentrations representing low and high levels for the nine drugs. Lower limits of detection and quantification were 0.025–0.5 and 0.25–5.0 µg/mL, respectively. Linearity was acceptable at five concentrations for each drug. No ion suppression was observed at the retention time for most compounds, except for streptomycin, kanamycin and cycloserine, which were eluted close to the void volume of the column. In a limited pilot study, all quantifiable human samples had values within the validated assay ranges.

Conclusions: The performance of our MS/MS detection technique was generally acceptable. The method provided rapid, sensitive and reproducible quantification of nine second-line anti-TB drugs and should facilitate drug monitoring during treatment.

Keywords: tandem mass spectrometry, multiplex analysis, therapeutic drug monitoring

Introduction

Approximately one-third of the world’s population has been infected with Mycobacterium tuberculosis and 1.7 million people died of the resulting disease in 2009. The global incidence rate continues to fall, but this rate of decrease is slower than desired. In Korea, the incidence of tuberculosis (TB) is high, reaching nearly 50,000 people per year. Treatment for TB is based on first-line anti-TB drugs, including isoniazid, rifampicin, pyrazinamide and ethambutol.

The emergence and spread of multidrug-resistant (MDR) TB and extensively drug-resistant (XDR) TB make the situation more complex, and warrant the development of more effective treatment protocols. Recently, the WHO produced guidelines for the programmatic management of drug-resistant TB. According to the guidelines, at least four second-line anti-TB drugs should be included for effective treatment during the intensive treatment phase. This regimen should include pyrazinamide, a fluoroquinolone, a parenteral agent, ethionamide (or prothionamide) and cycloserine. In situations where cycloserine cannot be used, para-aminosalicylic acid (PAS) has been recommended. Group 5 drugs, as classified by the WHO, such as clofazimine, linezolid, amoxicillin/clavulanate, thioacetazone, imipenem/clastatin and clarithromycin, which have unclear efficacy against M. tuberculosis and many adverse effects, may be used but are not included among those drugs that constitute the standard regimen.
Therapeutic drug monitoring (TDM) constitutes individualized drug dosing guided by drug plasma concentrations, an approach that can improve responses to drug treatment. To date, TDM for first-line anti-TB drugs has been shown to be of potential use for the treatment of active TB. More recently, clinicians have begun to use TDM in a limited number of studies in which second-line anti-TB drugs have been shown to improve the management of MDR-TB.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is a powerful technique that enables the simple, specific and multiplex quantification of drugs. It has also been used for TDM of first-line anti-TB drugs. In terms of second-line anti-TB drugs, several methods have been employed to measure individual drugs using LC-MS/MS. However, to the best of our knowledge, there have been no reports on the simultaneous measurement of second-line anti-TB drugs.

In this study, we developed and evaluated the performance of simple, rapid and validated methods for the simultaneous measurements of nine commonly used second-line anti-TB drugs in Korea by using ultra performance liquid chromatography (UPLC)-MS/MS.

Materials and methods

Chemicals and reagents

The methanol and water (J.T. Baker, Phillipsburg, NJ, USA) used in these studies were of HPLC grade. Formic acid, ammonium formate, HCl, NaOH, streptomycin, dihydrostreptomycin, kanamycin, gentamicin, clarithromycin, cycloserine, muscimol, moxifloxacin, levofloxacin, linezolid, lomefloxacin, PAS, 4-aminobenzoic acid, prothionamide and ethionamide were purchased from the Sigma Chemical Co. (St Louis, MO, USA). Dihydrostreptomycin, gentamicin, roxithromycin, muscimol, lomefloxacin, 4-aminobenzoic acid and ethionamide were used as internal standards (ISs).

Standard solutions and ISs

All standards stock solutions were prepared at 1 mg/mL. Stock solutions of streptomycin, dihydrostreptomycin, kanamycin, gentamicin, cycloserine, moxifloxacin, levofloxacin and 4-aminobenzoic acid were prepared in distilled water. Stock solutions of clarithromycin, roxithromycin, linezolid and PAS were prepared in 80% methanol in water. Muscimol was dissolved in 50% methanol in water, and lomefloxacin, prothionamide and ethionamide in 100% methanol.

Stock solutions were stored at −70°C prior to use. Standard stock solutions of all the drugs listed above were combined and serially diluted in 50% methanol to produce working standards. Calibration curves were constructed with five concentrations of each standard (streptomycin, kanamycin, cycloserine and PAS at 5, 10, 20, 50 and 100 μg/mL; clarithromycin and prothionamide at 0.5, 1, 2, 5 and 10 μg/mL; and moxifloxacin, levofloxacin and linezolid at 1, 2, 4, 10 and 20 μg/mL). Three quality control samples of low, medium and high concentrations (10 and 50 μg/mL for streptomycin, kanamycin, cycloserine and PAS; 1 and 5 μg/mL for clarithromycin and prothionamide; and 2 and 10 μg/mL for moxifloxacin, levofloxacin and linezolid) were prepared by spiking stock solutions into blank serum.

Stock solutions of dihydrostreptomycin, gentamicin, roxithromycin, muscimol, lomefloxacin, 4-aminobenzoic acid and ethionamide were combined and diluted in 50% methanol to produce the working IS solution, which contained 100 μg/mL dihydrostreptomycin, gentamicin, roxithromycin and 4-amino benzolic acid, 10 μg/mL muscimol and 20 μg/mL lomefloxacin and ethionamide. For streptomycin, dihydrostreptomycin was used as the IS; for kanamycin, gentamicin was used; for clarithromycin, roxithromycin was used; for cycloserine, muscimol was used; for moxifloxacin, levofloxacin and linezolid, lomefloxacin was used; for PAS, 4-aminobenzoic acid was used; and for prothionamide, ethionamide was used.

Sample preparation

For sample preparation, serum samples and controls (50 μL of each) were mixed well with 5 μL of 3 M HCl, 50 μL of 50% methanol and 50 μL of IS. After vortexing for 1 min, mixtures were centrifuged for 2 min at 13000 rpm. For calibration, 50 μL aliquots of each standard solution were placed into tubes containing 50 μL of blank serum, 5 μL of 3 M HCl and 50 μL of IS. After vortexing for 1 min, mixtures were centrifuged for 2 min at 13000 rpm. Then 90 μL aliquots of the supernatants obtained from graphic separation were transferred into tubes containing 4 μL of 1 M NaOH and 90 μL of 100% methanol, and these mixtures were centrifuged for 5 min at 13000 rpm. Finally, 100 μL aliquots of these supernatants were transferred to a 96-well microplate and loaded into the autosampler of the UPLC system.

LC-MS/MS conditions

We revised the conditions and procedures that we had previously reported for multiplex determination of first-line anti-TB drugs. Samples were analysed using a Waters ACQUITY UPLC system (Waters, Watford, UK) using an HSS T3 column (50.0×2.1 mm, 1.8 μm; Waters, Watford, UK). A VanGuard Pre-Column BEC C18 (1.7 μm; Waters, Watford, UK) was used as a guard column. The flow rate was 200 μL/min. The mobile phase was a gradient of a mixture of 10 mM ammonium formate in 0.1% formic acid (solvent A) and acetonitrile in 0.1% formic acid (solvent B). The gradient profile used was (A:B) 80:20 v/v for 0.7 min, followed by a linear gradient to 50:50 v/v until 1.0 min, maintained until 2.0 min and returned to 80:20 v/v at 2.2 min. The total running time was 3.0 min. A volume of 10 μL of each sample was injected onto the HSS T3 column, and chromatographic separation was performed at room temperature for 3 min. A Waters Xevo TQ MS tandem mass spectrometer (Waters, Manchester, UK) was operated using the following settings: capillary voltage, 3.5 kV; cone voltage, 20–50 V; collision energy, 7–32 eV, depending on analytes (Table 1); and collision gas, argon at 3.0×103 mbar. Quantification was achieved by selected reaction monitoring (SRM) in positive ion mode. Integration of peak area and data analysis were both performed using MassLynx 4.1 software (Waters, Milford, MA, USA).

Validation of the assay

Within-run and between-run precisions, linearity, lower limits of detection (LLODs), lower limits of quantification (LLOQs) and matrix effects were evaluated for each drug. Within-run precisions were determined by 10 replicated analyses of low, medium and high concentrations for 10 consecutive days. The passing criteria were defined as the coefficient of variation (CV) within ±20% for the low concentrations and ±15% for the medium and high concentrations. Linearity was evaluated as recommended by the CLSI (formerly the NCCLS). In brief, blank sera and sera spiked with high-concentration standard solutions were mixed in different proportions to produce three solutions containing intermediate concentrations. Each was then analysed in quadruplicate. Inter-assay calibration variability data were evaluated on five consecutive days with concentrations of 5.0–100 μg/mL for streptomycin, kanamycin, cycloserine and PAS; 0.5–10 μg/mL for clarithromycin and prothionamide; and 1.0–20 μg/mL for moxifloxacin, levofloxacin and linezolid.

The LLOD was defined as the lowest concentration with a signal-to-noise ratio of >3.0, and the LLOQ was defined as the lowest concentration with a precision <20% and an accuracy within ±20%.
drugs had reached a constant steady state. All drugs were given simultaneously to collect the patients’ blood contained EDTA anticoagulant.

twice a day for PAS, 750 mg daily for levofloxacin, 11; 500–1000 mg daily for kanamycin, 9; 500–1000 mg daily for linezolid, 21; 500–750 mg daily for levofloxacin, n = 30; 3.3 g three times a day or 6.6 g twice a day for PAS, n = 14; 125 mg three times a day or 250 mg twice a day for prothionamide, n = 16; 600 mg daily for linezolid, n = 1. The tube initially used to collect the patients’ blood contained EDTA anticoagulant. Some patients took two or more drugs simultaneously. The duration of dosing differed between individuals, but samples were taken when the drugs had reached a constant steady state. All drugs were given simultaneously and samples were randomly collected from 0.5 to 15 h after administration. The assay was either performed within 4 h of sampling or sera were frozen immediately after harvesting and stored at −70 °C until required for analysis. We determined the peak concentration ranges for each drug.

This research was approved by the Seoul National University Bundang Hospital Institutional Review Board (No. B-1204-150-304). As this study used the residual samples, informed consent from the patients was exempted.

### Pharmacokinetic analysis for several anti-TB drugs

To determine the pilot steady-state pharmacokinetic parameters of several anti-TB drugs, serial bottles of blood were collected from two patients. Patient 1 was administered 1000 mg of streptomycin once a day, two packages (6.6 g) of PAS twice a day and 500 mg of prothionamide twice a day. Patient 2 was administered 1000 mg of streptomycin once a day, two packages of PAS twice a day and 250 mg of prothionamide twice a day. Drug concentration was analysed by ABBOTTBASE software (Abbott Laboratories, IL, USA). The maximum plasma concentration (C_{max}), time to reach C_{max} (t_{max}), elimination rate constant (k_e) and elimination half-life (t_{1/2}) were estimated.

### Results

#### Method development for UPLC-MS/MS

Quantification was performed in SRM mode, with a dwell time of 0.015 s for each compound. The best MS/MS conditions and SRM transitions are shown in Table 1.

We evaluated different LC columns, mobile phase compositions and flow rates to optimize peak shapes and separation conditions. Drug compounds and ISs were fully separated using UPLC with an HSS T3 column (50.0×2.1 mm, 1.8 μm). The eluant used was also optimized for compound separation within 3 min by changing the gradient composition. Lower initial concentrations of the organic phase (20%) led to the elution of the more polar compounds, streptomycin, dihydrostreptomycin, kanamycin, gentamicin, cycloserine and muscimol. A subsequent rapid gradient change to a higher organic component (50%) was used to elute the non-polar compounds, clarithromycin, roxithromycin, moxifloxacin, levofloxacin, linezolid, lomefloxacin, PAS, 4-aminobenzoic acid, prothionamide and ethionamide. Representative UPLC-MS/MS SRM chromatograms for each drug compound and IS are shown in Figure 1.

#### Linearity and inter-assay calibration variability

Linearity was evaluated as recommended by the CLSI. Streptomycin, kanamycin, clarithromycin, cycloserine, moxifloxacin, levofloxacin, linezolid, PAS and prothionamide produced linear responses over the following ranges: 0–100 (R^2 = 0.9997), 0–100 (R^2 = 0.9987), 0–10 (R^2 = 0.9995), 0–100 (R^2 = 0.9996), 0–20 (R^2 = 0.9923), 0–20 (R^2 = 0.9980), 0–20 (R^2 = 0.9982), 0–100 (R^2 = 0.9932) and 0–10 (R^2 = 0.9974) μg/mL, respectively. We generated calibration curves for nine anti-TB drugs at six concentrations. Inter-assay calibration variability data obtained over concentrations of 5–100 μg/mL for streptomycin, kanamycin, cycloserine and PAS, 0.5–10 μg/mL for clarithromycin and prothionamide, and 1–20 μg/mL for moxifloxacin, levofloxacin and linezolid on five consecutive days showed a linear and reproducible curve in the observed analytical ranges (Table 2).

### Pilot application to TDM

Evaluation for the extraction methods was performed by comparing acid treatment with acid treatment followed by neutralization. Signal intensity changes after treatment were estimated for each drug.

Ion suppression was initially evaluated by the post-column infusion model method. In addition, the quantitative assessment of the ‘absolute’ versus ‘relative’ matrix effects was performed for the compounds and their ISs, which showed suspicious ion suppressions in post-column infusion methods. The mean absolute matrix effect (%) was examined by comparing the MS/MS response (peak areas) obtained from the serum sample with the MS/MS response of the same analyte present in the mobile phase. The presence of a relative matrix effect was also assessed by direct comparison of the MS/MS responses (peak areas) of an analyte spiked into serum samples originating from five different sources (two from normal controls, one from diabetes patients and one from heart disease patients). The variability in these responses, expressed as CVs (%), was considered as a measure of the relative matrix effect.

#### Table 1. MS/MS conditions and drug SRM transitions

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cone voltage (V)</th>
<th>Collision energy (eV)</th>
<th>SRM transition m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomycin</td>
<td>50</td>
<td>32</td>
<td>582.6→263.2</td>
</tr>
<tr>
<td>Dihydrostreptomycin (IS)</td>
<td>50</td>
<td>32</td>
<td>584.6→263.2</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>25</td>
<td>25</td>
<td>485.5→163.1</td>
</tr>
<tr>
<td>Gentamicin (IS)</td>
<td>28</td>
<td>22</td>
<td>478.6→157.1</td>
</tr>
<tr>
<td>Cycloserine</td>
<td>20</td>
<td>7</td>
<td>103.0→75.1</td>
</tr>
<tr>
<td>Muscimol (IS)</td>
<td>20</td>
<td>10</td>
<td>115.0→98.1</td>
</tr>
<tr>
<td>Prothionamide</td>
<td>32</td>
<td>19</td>
<td>181.0→154.1</td>
</tr>
<tr>
<td>Ethionamide (IS)</td>
<td>29</td>
<td>18</td>
<td>167.0→140.0</td>
</tr>
<tr>
<td>PAS</td>
<td>24</td>
<td>21</td>
<td>154.1→119.0</td>
</tr>
<tr>
<td>4-Aminobenzoic acid (IS)</td>
<td>25</td>
<td>13</td>
<td>138.0→94.1</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>35</td>
<td>20</td>
<td>362.3→318.2</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>35</td>
<td>22</td>
<td>402.4→384.3</td>
</tr>
<tr>
<td>Linezolid</td>
<td>30</td>
<td>18</td>
<td>338.3→296.2</td>
</tr>
<tr>
<td>Lomefloxacin (IS)</td>
<td>33</td>
<td>17</td>
<td>352.2→308.2</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>30</td>
<td>20</td>
<td>748.8→590.6</td>
</tr>
<tr>
<td>Roxithromycin (IS)</td>
<td>30</td>
<td>22</td>
<td>837.9→679.6</td>
</tr>
</tbody>
</table>

**Pilot application to TDM**

After collecting the residual patient samples after routine laboratory tests, serum concentrations of the anti-TB drugs were determined in patients treated using standard dosing as a second-line treatment (500–1000 mg daily for streptomycin, n = 11; 500–1000 mg daily for kanamycin, n = 9; 250–500 mg twice a day for clarithromycin, n = 34; 250–500 mg twice a day for cycloserine, n = 25; 400 mg daily for moxifloxacin, n = 21; 500–750 mg daily for levofloxacin, n = 30; 3.3 g three times a day or 6.6 g twice a day for PAS, n = 14; 125 mg three times a day or 250 mg twice a day for prothionamide, n = 16; 600 mg daily for linezolid, n = 1). The tube initially used to collect the patients’ blood contained EDTA anticoagulant. Some patients took two or more drugs simultaneously. The duration of dosing differed between individuals, but samples were taken when the drugs had reached a constant steady state. All drugs were given simultaneously and samples were randomly collected from 0.5 to 15 h after administration. The assay was either performed within 4 h of sampling or sera were frozen immediately after harvesting and stored at −70 °C until...
Multiplexed anti-TB drug analysis by UPLC-MS/MS

Precision, LLOD and LLOQ
The within-run precisions (CVs) of the nine drugs were 2.2%–6.3% at the low concentration, 2.3%–4.6% at the medium concentration and 1.7%–7.5% at the high concentration. Between-run precisions were 3.4%–10.4% at the low concentration, 2.7%–12.4% at the medium concentration and 1.7%–11.0% at the high concentration (Table 2). All precisions were within the passing criteria and showed acceptable performance. To determine LLODs and LLOQs, pooled sera were spiked with known concentrations of the nine drugs in 50% methanol. LLODs for the nine drugs were 0.025–0.5 μg/ml and LLOQs were 0.25–5.0 μg/ml, depending on the drug being tested (Table 2). These values are sufficient for routine drug monitoring because these LLOQs are much lower than therapeutic ranges.

Ion suppression
Evaluation of matrix effects was performed using ion suppression testing. A variable ion suppression region (solvent front) was observed between 0.4 and 0.7 min in the obtained chromatogram, but no ion suppression was observed at the retention time for most compounds, except streptomycin, kanamycin and cycloserine (Figure 2). Therefore, we performed the quantitative assessment of ion suppression for three compounds and their ISs. The absolute matrix effects for streptomycin, dihydrostreptomycin, kanamycin, gentamicin, cycloserine and muscimol were 63.6%, 65.5%, 91.1%, 93.6%, 120.2% and 105.2%, respectively. The variability values (CVs) calculated from the response of the five different sources were also acceptable (12.2% for streptomycin, 11.2% for dihydrostreptomycin, 7.6% for kanamycin, 15.9% for gentamicin, 4.8% for cycloserine and 2.1% for muscimol), which suggested that the relative matrix effect was not significant.

Assays for blood samples
Blood samples were randomly taken 0.5–15 h after administering standard oral or intramuscular doses of the drugs. Drug serum concentrations were considerably different: streptomycin, 5.0–38.0 μg/ml (n = 11); kanamycin, 5.0–37.7 μg/ml (n = 9); clarithromycin, 0.5–4.3 μg/ml (n = 34); cycloserine, 5.0–49.6 μg/ml (n = 25); moxifloxacin, 1.0–4.7 μg/ml (n = 21); levofloxacin, 1.0–12.0 μg/ml (n = 30); PAS, 5.0–51.4 μg/ml (n = 14); prothionamide, 0.5–4.5 μg/ml (n = 16); and linezolid, 4.3 μg/ml (n = 1). 

Pilot pharmacokinetic analysis
For patient 1, the C_{max}, T_{max}, k_e and t_1/2 of streptomycin were 23.1 μg/ml, 1 h, 0.098 h^{-1} and 7.0 h, those of PAS were 49.2 μg/ml, 1 h, 0.25 h^{-1} and 2.8 h, and those of prothionamide were 7.4 μg/ml, 2 h, 0.16 h^{-1} and 4.3 h. For patient 2, the C_{max}, T_{max}, k_e and t_1/2 of streptomycin were 15.5 μg/ml, 2 h, 0.090 h^{-1} and 7.7 h, those of PAS were 58.5 μg/ml, 0.5 h, 0.26 h^{-1} and 2.6 h.

Figure 1. Representative UPLC-MS/MS SRM chromatograms for nine anti-TB drugs together with seven ISs: streptomycin with dihydrostreptomycin (IS); kanamycin with gentamicin (IS); cycloserine with muscimol (IS); prothionamide with ethionamide (IS); PAS with 4-aminobenzoic acid (IS); levofloxacin, moxifloxacin and linezolid with levofloxacin (IS); and clarithromycin with roxithromycin (IS). MRM, multiple reaction monitoring; ES, electrospray.
Table 2. Inter-assay variability of calibration curves, within-run and between-run precision, LLOD and LLOQ

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inter-assay variability of calibration curves (n = 5)</th>
<th>Precision, CV (%)</th>
<th>LLOQ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inter-assay variability (n = 5)</td>
<td>LLOD, concentration (µg/mL)</td>
<td>accuracy (bias)</td>
</tr>
<tr>
<td></td>
<td>observed range (µg/mL)</td>
<td>nominal concentration (µg/mL)</td>
<td>within-run</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>5–100 0.0028 (0.0026, 0.0030) –0.0005 (–0.0012, 0.0003) 0.9988 (0.9973, 1.0004)</td>
<td>14.8 27.6 82.7</td>
<td>0.5</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>5–100 0.0071 (0.0059, 0.0083) –0.0061 (–0.0089, –0.0032) 0.9993 (0.9987, 0.9999)</td>
<td>14.9 29.3 89.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>0.5–10 0.3841 (0.2121, 0.5561) 0.0361 (0.0046, 0.0676) 0.9949 (0.9906, 0.9991)</td>
<td>0.0361</td>
<td>0.5</td>
</tr>
<tr>
<td>Cycloserine</td>
<td>5–100 0.0043 (0.0030, 0.0055) –0.0005 (–0.0062, 0.0052) 0.9981 (0.9963, 1.0000)</td>
<td>12.4 23.8 86.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>1–20 0.0439 (0.0226, 0.0652) –0.0062 (–0.0170, 0.0046) 0.9979 (0.9963, 0.9995)</td>
<td>3.1 7.1 19.3</td>
<td>0.05</td>
</tr>
<tr>
<td>Levofoxacin</td>
<td>1–20 0.0686 (0.0419, 0.0954) –0.0267 (–0.0423, –0.0119) 0.9945 (0.9917, 0.9972)</td>
<td>2.5 5.4 16.6</td>
<td>0.1</td>
</tr>
<tr>
<td>Linezolid</td>
<td>1–20 0.0943 (0.0613, 0.1273) –0.0024 (–0.0612, 0.0565) 0.9971 (0.9940, 1.0002)</td>
<td>4.1 8.9 15.6</td>
<td>0.05</td>
</tr>
<tr>
<td>PAS</td>
<td>5–100 0.0250 (0.0190, 0.0309) –0.0346 (–0.1187, 0.0494) 0.9962 (0.9943, 0.9981)</td>
<td>15.1 35.2 95.9</td>
<td>0.5</td>
</tr>
<tr>
<td>Prothionamide</td>
<td>0.5–10 0.0735 (0.0636, 0.0833) 0.0058 (–0.0083, 0.0200) 0.9996 (0.9994, 0.9999)</td>
<td>2.2 4.7 8.3</td>
<td>0.05</td>
</tr>
</tbody>
</table>
and those of prothionamide were 2.6 µg/mL, 3 h, 0.62 h⁻¹ and 1.1 h. The pharmacokinetic parameters of the two patients show similar results not only compared with each other, taking into consideration the different doses of prothionamide administered, but also with other reports.²⁷

**Discussion**

TDM has been used in the treatment of a variety of conditions to ensure optimal dosing that maximizes therapeutic benefits while minimizing toxicity. For many years, TDM for first-line anti-TB drugs has been recognized as an important tool for optimizing TB treatment.⁶,²⁷–³⁰ However, in terms of second-line TB drugs, only a limited number of studies have been performed for some drugs.⁵,¹⁰,¹²,¹³ To perform the pharmacokinetic studies and TDM effectively, validated methods for quantifying plasma drug concentrations are mandatory. The goal of this study was to set up a simple, rapid, multiplexed and validated UPLC-MS/MS method. Our method was rapid, with approximately 10 min of sample preparation time and 3 min of UPLC-MS/MS running time. Assay performance, including linearity, precision and sensitivity, was satisfactory for TDM. To the best of our knowledge, this is the first description of a validated method for the simultaneous measurement of nine commonly used second-line anti-TB drugs.

A successful method required the quantification of two distinct classes of compounds: polar compounds, such as streptomycin, dihydrostreptomycin, kanamycin, gentamicin, cycloserine and muscimol, and relatively non-polar compounds, such as clarithromycin, roxithromycin, moxifloxacin, levofloxacin, linezolid, lomefloxacin, PAS, 4-aminobenzoic acid, prothionamide and ethionamide. By changing the gradient composition of the mobile phase from the initial concentrations of the organic phase that were low (20%) to concentrations of the organic component that were higher (50%), we were successful in achieving separation of the compounds except for the early co-eluted ones, streptomycin, kanamycin and cycloserine. With this method, initial elution of the more polar compounds was followed by elution of the non-polar compounds, all within 3 min. Peak tailings of the chromatogram were observed for the non-polar compounds, such as PAS, levofloxacin and moxifloxacin, but these have minor effects on the quantification.

In terms of sample preparation, we simply used protein precipitation with acidified methanol followed by supernatant neutralization and dilution. Aminoglycosides are polar, necessitating careful handling during the sample preparation step, since a significant amount of non-specific binding to glass and plastic was found to produce inconsistent results.¹¹ Pre-treating the sample with acid was essential for a good recovery of aminoglycosides from the samples. However, in our study, acid treatment yielded negative effects on ionization efficiency for most of the other drugs. Therefore, we neutralized the extracted supernatants by adding NaOH, which resulted in an increase in signal intensities for all the compounds except polar compounds such as streptomycin, kanamycin and cycloserine (Table 3). Nevertheless, signal intensities for those drugs were acceptable.

Ion suppression is the most significant source of error in MS, particularly when a stable isotope cannot be used as an IS. From an analysis of post-column infusion methods²⁵ to evaluate protracted

![Figure 2. Infusion chromatograms to assess the matrix effects of streptomycin, kanamycin and cycloserine.](image-url)
ionization effects, we found that matrix effects could be associated with the compounds streptomycin, kanamycin and cycloserine. Because they eluted close to the void volume on an HSS T3 column, matrix components might have possibly interfered with the compound signal. However, quantitative assessment\(^{26}\) of the ‘absolute’ versus ‘relative’ matrix effect for streptomycin, kanamycin and cycloserine, along with their corresponding ISs (dihydrostreptomycin, gentamicin and muscimol, respectively), indicated a similar pattern of matrix effects when comparing the drugs to their ISs. Thus, these structurally analogous ISs and modified chromatographic conditions increased the likelihood that the analytes and IS would co-elute, resulting in a similar degree of ion suppression for both the analytes and IS. Therefore, even though the method we devised was not ideal in terms of ion suppression, assay performances, such as accuracy, precision and LOQ, were found to be sufficient for routine TDM.

The benefits of TDM of second-line anti-TB drugs have recently been studied. The conclusion was that TDM of linezolid might improve safety outcomes associated with long-term treatment in adult patients,\(^{11}\) whereas TDM of moxifloxacin could have a significant impact on clinical decisions, particularly in patients with XDR-TB.\(^{12}\) Furthermore, individualized dosing of linezolid\(^{10}\) or moxifloxacin\(^ {13}\) based on limited sampling was determined to be feasible. According to the WHO guidelines,\(^ {8}\) at least four drugs should be used for MDR-TB patients. Therefore, multiplex monitoring of more than four drugs is required for the effective and comprehensive TDM of second-line anti-TB drugs. Our revised method enables multiplex monitoring and may be a very effective routine in the clinical setting. We have applied our methods to a limited number of samples from patients who have been treated with any one of a number of second-line anti-TB drugs. The concentrations of each drug exhibited variability in the trough to peak levels, most probably caused by random sampling times. The main purpose of this is to show that the method works in a real-life situation. Using the method we devised, we will in the future perform pharmacokinetic studies to determine the exact pharmacokinetic parameters for individual second-line anti-TB drugs.

Recently, drug monitoring by sampling dried blood spots was purported to be very useful in remote rural areas because of its sample stability and easy logistics.\(^ {9,18,32,33}\) Judging from the LOQ values obtained in this study, the method we devised may be sufficiently sensitive to permit quantification of drug concentrations in dried blood spots. In the future, we will apply this method to dried blood spots and evaluate its performance, which should eventually enable easy and multiplex monitoring of second-line anti-TB drugs.

One limitation of this study related primarily to the small number of patients recruited for the pilot application of the method we devised for TDM. We were also limited in that we could not use stable isotopes for ISs, despite the fact that the ISs we used were structurally analogous to the test compounds. No correlation study was carried out between the measured levels of drugs and the effects on patients, the success or adverse reactions of the treatment, and we propose this as the next step of validation of the findings.

In conclusion, we describe a UPLC-MS/MS technique that was developed to simultaneously measure serum concentrations of nine second-line anti-TB drugs. The method we devised allows for the simple, rapid, sensitive and reproducible quantification of second-line anti-TB drugs, and offers a viable approach for prospective pharmacokinetic studies, high throughput and the simultaneous TDM of second-line anti-TB drugs.

### Funding

This study was supported by internal funding.

### Transparency declarations

None to declare.

### References

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