A high-throughput LC–MS/MS method for simultaneous determination of isoniazid, ethambutol and pyrazinamide in human plasma

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Rationale: Tuberculosis (TB) remains a challenging global infectious disease, mainly affecting the lungs. First-line anti-TB drugs play a crucial role in slowing down the rapid spread of TB. In addition, the patient might benefit from therapeutic drug monitoring since it has become an accepted clinical tool for optimizing TB treatment.

Methods: A simple and sensitive liquid chromatography/tandem mass spectrometry method was developed to monitor the plasma level of isoniazid, ethambutol and pyrazinamide in plasma samples. A one-step extraction procedure using an Ostro™ plate was applied, and extracts were analyzed by gradient elution followed by detection on a mass spectrometer by multiple reaction monitoring mode.

Results: The analytes were separated within 4.2 min and over the concentration range of 0.2–10 μg/ml for isoniazid and ethambutol and 1–65 μg/ml for pyrazinamide. The method was successfully validated according to the European Medicine Agency guideline for the selectivity, linearity and lower limit of detection, precision and accuracy, matrix effect, extraction recovery, carryover, dilution integrity and stability, and applied for quantification of analytes in clinical samples from TB patients.

Conclusions: The presented method allows sensitive and reproducible determination of selected anti-TB drugs with advantages such as low sample volume requirement, short run time of analysis, one-step sample preparation procedure with capabilities for phospholipids removal, and a low quantification limit as well as a high degree of selectivity.

1 INTRODUCTION

Cough-driven spread of Mycobacterium tuberculosis is the main transmission route of this tuberculosis (TB)-causing agent. Despite the COVID-19 pandemic, the World Health Organization still ranks TB among the most common causes of death worldwide.1 The illness affects the respiratory tract in so-called pulmonary TB and the extrapulmonary form of the disease is recognized in M. tuberculosis infection sites other than the lungs. In the Slovak Republic, a low TB burden country, the incidence of both drug-susceptible and drug-resistant forms of TB remains relatively stable.2,3
First-line anti-TB drugs comprising oral forms of isoniazid, ethambutol, pyrazinamide, rifampicin, rifabutin and rifapentine derivatives are useful in drug-susceptible TB treatment regimes. Slow response to treatment, and more or less severe adverse drug reactions (ADRs) might occur, or patient compliance may cause a problem. Thus, the standard 6-month first-line therapy course in drug-sensitive M. tuberculosis strains is often extended. Low plasma levels of certain drugs have been associated with TB relapses and therapy failures, poor overall treatment outcomes and acquired microbial resistance. Changes in pharmacokinetic parameters caused by comorbidities, concomitant medications and intra-individual differences are more likely to result in drug resistance. The patient might benefit from therapeutic drug monitoring (TDM) since this has become an accepted clinical tool for optimizing TB treatment and overall management. In dose adjustments, clinicians should also consider severe ADRs, e.g., central and peripheral neurotoxicity, hepatotoxicity and ocular toxicity for isoniazid, pyrazinamide and ethambutol, respectively. TDM helps in these adjustments to maximize the therapeutic dose to the most effective and simultaneously least toxic concentration.

Nowadays, the combination of liquid chromatography with tandem mass spectrometry (LC–MS/MS) presents a powerful and sensitive technique to perform TDM on a large number of samples. Several LC–MS/MS methods have been published for simultaneously quantifying anti-TB drugs in human plasma. However, these methods lack good separation and/or peak shape, and suffer from disadvantages like long run time, large sample volume, low sensitivity, and time-consuming and strenuous sample preparation.

The scope of the present study was to develop and validate a sensitive and high-throughput LC–MS/MS method suitable for clinical and toxicological TDM of isoniazid, ethambutol and pyrazinamide (Figure 1). A 96-well plate format enabling protein precipitation and phospholipid removal in one step was chosen for sample processing. In addition, stable isotope-labelled analytes were used as internal standards (ISs) helped to reduce matrix effects and to improve the method's accuracy. The method was validated according to the European Medicines Agency (EMA) guideline for bioanalytical methods validation and was used to determine the plasma level of anti-TB drugs of interest in a real group of patients, focusing on actual plasma concentration and the manifestation of ADRs.

2 MATERIALS AND METHODS

2.1 Chemicals and reagents

Reference standards of isoniazid (purity 98%), ethambutol dihydrochloride (purity 98%), pyrazinamide (purity 98%), isoniazid-d4 (purity 98%, isotopic purity 99.3%) and ethambutol-d4 dihydrochloride (purity 98%, isotopic purity 99.1%) were purchased from Toronto Research Chemicals. Acetonitrile, methanol, water, ammonium formate and formic acid were LC–MS grade and were obtained from Honeywell Riedel-de Haën. Analyte-free human plasma (blank plasma) with potassium EDTA as an anticoagulant was obtained from healthy and untreated volunteers (men and women aged 25–30 years).

2.2 Instrumentation and LC–MS/MS conditions

The XEVO TQ-S triple quadrupole mass spectrometer system (Waters) was equipped with a multimode ionization source (ESCI®) and operated in electrospray ionization (ESI) mode. Detection was achieved using positive ESI, and ion source parameters were set up as

| Compound name  | MRM transitions (m/z) | Cone voltage (V) | Collision energy (eV) | Dwell time (ms) |
|----------------|----------------------|------------------|-----------------------|----------------|
| Isoniazid      | 138.08 > 79.07       | 20               | 20                    | 41             |
|                | 138.08 > 121.13      | 20               | 14                    | 41             |
| Ethambutol     | 205.15 > 44.20       | 70               | 22                    | 40             |
|                | 205.15 > 116.17      | 70               | 14                    | 40             |
| Pyrazinamide   | 124.08 > 53.97       | 40               | 20                    | 40             |
|                | 124.08 > 81.01       | 40               | 16                    | 40             |
| Isoniazid-d4   | 142.05 > 124.97      | 20               | 14                    | 41             |
| Ethambutol-d4  | 209.16 > 120.02      | 65               | 18                    | 40             |

Underlined MRM transitions (m/z) are used as quantifier ions.
follows: capillary voltage 2.0 kV, source offset 50 V and ion source temperature 150°C. The desolvation gas was nitrogen at a flow rate of 1000 L/h and temperature 550°C. To prevent contamination of the sample cone, nitrogen was used as the cone gas at a flow rate of 150 L/h. The mass spectrometer was operated in a multiple reaction monitoring (MRM) experiment using a unit mass resolution (0.75 Da). Argon at a flow rate of 0.15 ml/min was used as the collision gas. Collision energies and cone voltages were individually optimized for each analyte and its respective IS (Table 1). Data were acquired using MassLynx software, version 4.2 (Waters), calibrated and quantified by TargetLynx software (Waters).

The Acquity UPLC® system (Waters) includes a binary gradient pump, an autosampler with a flow-through needle design (SM-FTN), and a column thermostat coupled with a mass spectrometer. Chromatographic separation was achieved on an Acquity UPLC® HSS T3 (50 × 2.1 mm, 1.8 μm) column fitted with an in-line filter (0.2 μm). The column temperature was set at 35°C while the temperature of the autosampler was maintained at 8°C. The separation of analytes was done using a gradient elution program over 4.2 min with 10 mM ammonium formate in water (pH 6.4, mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 0.4 ml/min (Table 2) and an injection volume of 2 μl.

2.3 Preparation of calibration standards and quality control samples

Stock solutions for isoniazid, ethambutol and pyrazinamide were prepared from their reference standards in methanol. Afterwards, a series of seven working solutions was prepared by diluting the appropriate volume of stock solutions with methanol/water (50:50, v/v) to get a concentration range of 4–200 μg/ml for isoniazid and ethambutol, and 20–1300 μg/ml for pyrazinamide. Moreover, four working solutions were prepared for quality control (QC) samples using independently prepared stock solutions. The working solution for the ISs was prepared using the reference standard in methanol. All the solutions were stored at −20°C.

The calibration standards and QC samples were made by spiking 950 μl of blank plasma with 50 μl of the respective working solution. In this way, seven calibration standards were prepared at final concentrations of 0.2, 0.5, 1.5, 3, 5, 7.5 and 10 μg/ml for isoniazid and ethambutol, and 1, 2.5, 10, 20, 35, 50 and 65 μg/ml for pyrazinamide. QC samples were prepared at four concentration levels (lower limit of quantification [LLOQ], low, medium and high): 0.2, 0.7, 4 and 8 μg/ml for isoniazid and ethambutol, and 1, 3, 25 and 55 μg/ml for pyrazinamide. The calibration and QC samples were freshly prepared immediately before analysis.

2.4 Sample preparation procedure

Patients’ plasma samples, calibration standards and QC samples were processed as follows. A 100-μl aliquot of plasma sample and 20 μl of IS working solution were pipetted into the Ostro™ 96-well plate (Waters), which was placed on the 2-ml collection plate. First, the mixture was homogenized by vortex-mixing for 2 min at 650 rpm. Subsequently, 300 μl of cold 1% formic acid in acetonitrile was forcefully and rapidly pipetted into the wells and mixed by aspirating 10 times using an eight-channel pipette. After mixing, the plate was placed onto an Otto SPEcialist positive pressure manifold (Waters) and nitrogen at 30 psi was applied for 2 min. Afterwards, a 10-μl aliquot of elutes was transferred into a new 2-ml collection plate, 1 ml of water was added to every well, and the plate was shaken at 600 rpm for 5 min then analyzed.

2.5 Method validation

The validation parameters were selectivity, carryover effect, linearity, LLOQ, precision and accuracy, matrix effect, extraction recovery, dilution integrity and the stability of isoniazid, ethambutol and pyrazinamide in human plasma following the EMA guideline for the validation of bioanalytical methods.23

The method’s selectivity was assessed by monitoring and comparing the quantification MRM transitions of each analyte and IS in blank human plasma from six different sources to blank plasma samples spiked with analytes at LLOQ to check the possible interference at the peak regions. Selectivity was guaranteed if the interference due to endogenous substances was <20% and <5% of the mean peak response of each analyte and corresponding IS, respectively.

The carryover effect was evaluated by comparing an extract of blank plasma injected immediately after the highest calibration standard was injected in triplicate. The extract of blank plasma should demonstrate no significant response at the retention times for analytes and ISs.

Three calibration curves were prepared using seven calibration points in the concentration range of 0.2–10 μg/ml for isoniazid and ethambutol, and 1–65 μg/ml for pyrazinamide on three separate days. Calibration curves were constructed by plotting peak area ratios (analyte/respective IS) against nominal plasma concentrations. Isoniazid-d₄ was used as an IS for creating a calibration curve of both
isoniazid and pyrazinamide, and ethambutol-d₄ was used as an IS for ethambutol. Linear weighted least-squares analysis was performed, and a weighting factor of 1/x² was used. A coefficient of determination (R²) >0.98 was expected for all calibration curves. The lowest calibration point on the calibration curve was considered as the LLOQ if the signal-to-noise (S/N) ratio was higher than 10. Furthermore, the peak of the LLOQ sample should be separated with reproducible accuracy and precision of less than ±20%. The upper limit of quantification (ULOQ) was considered the highest calibration point.

The intraday precision and accuracy were evaluated for each analyte in six replicates at four QC levels (LLOQ, low, medium and high concentration levels) within 1 day on the same analytical run. In contrast, interday precision and accuracy were assessed from the analysis of QC samples on three consecutive days. The coefficient of variation (CV, %) was used to estimate precision, and accuracy was represented by a percentage of the nominal concentration (%). The CV and accuracy should be within 20% at LLOQ and 15% for the low, medium and high QC levels.

The matrix effects were evaluated by comparing the peak areas obtained from eight different blank plasma samples. The extracted plasma samples were spiked with standard solutions of analytes at two QC levels (low and high) as well as IS solution and compared to pure reference standard solution in water at equivalent concentrations. The normalized IS matrix factors were calculated by dividing the matrix factor of the analyte by the matrix factor of the corresponding IS. The CV of the normalized IS should not be greater than 15%.

The extraction recoveries from plasma for each analyte were calculated by comparing the peak areas of QC samples at two levels (low and high) to that of the blank sample extracted with analytes at the same concentration. The extraction recoveries were investigated in plasma samples obtained from eight different sources. Dilution integrity was verified by spiking blank plasma in six replicates at a concentration two times higher than the ULOQ for each analyte. Subsequently, these highly concentrated plasma samples were diluted 2-fold with blank plasma before extraction and analyzed with calibration standards prepared on the same day. Accuracy and precision within ±15% were set as acceptance criteria.

The stability of isoniazid, ethambutol and pyrazinamide in plasma was confirmed by analyzing six replicates of spiked plasma samples at two concentration levels (low and high QC) under different storage conditions. The short-term stability was tested after the exposure of the samples at room temperature for 12 h. Next, freeze–thaw stability was tested after three freeze–thaw cycles from –20°C to room temperature. Long-term stability was assessed after storage of spiked samples at –20°C for 2 months. Finally, postprocessing stability was evaluated after 48 h of storage in the autosampler at 8°C. The accuracies were expressed as absolute values of the differences between stability samples and freshly prepared samples. The measured concentrations of analytes in stability samples were compared against freshly prepared QC samples, and stability was considered when accuracy and precision were within ±15%.

2.6 | Method application

The validated method was used to quantify antituberculotics in plasma samples collected from patients with TB. The Ethics Committee approved the study protocol of the Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava (Slovakia). Nine TB patients (three females, six males) were included in the study. All patients were treated with isoniazid, ethambutol and pyrazinamide for at least 2 weeks. The blood samples were drawn at two time points to monitor the 2- and 6-h postdose concentrations. Blood was collected in a tube with potassium EDTA as an anticoagulant, centrifuged at 2500 × g for 15 min at room temperature and stored at –20°C until analysis.

3 | RESULTS AND DISCUSSION

3.1 | Method development and LC–MS/MS optimization

Several LC–MS/MS-based methods have been developed to quantify first-line anti-TB drugs, including isoniazid, ethambutol and pyrazinamide, in human plasma,12–22 but not all of them are suitable for application in routine and high-throughput TDM. The main limitations of these studies are poor chromatographic separation of analytes, large sample volume requirements, inadequate concentration range, laborious sample preparation and thus low throughput of analysis. Here, we present the development of the LC–MS/MS method that effectively separates all analytes of interest with a wide quantification range.

The method development started with the optimization of MS parameters as well as fragmentation conditions for each analyte and IS. The optimization was completed using a mass spectrometer internal fluidic pump with analytes dissolved in methanol/water (50:50, v/v). Finally, considering the chemical structure of all analytes, the positive ESI mode was selected. For all analytes, the protonated ions [M + H]⁺ were observed at m/z 138.08 for isoniazid, m/z 205.15 for ethambutol and m/z 124.08 for pyrazinamide. Furthermore, the best detector response for all analytes was observed at 2.0 kV and 550°C for capillary voltage and desolvation temperature, respectively. Subsequently, collision-induced fragmentation was used to produce fragments of analytes, and the MS/MS parameters were optimized by observing the maximal response of the product ions. The optimized collision energies and cone voltages are presented in Table 1. The same procedure was done for ISs. The product ion spectra with the proposed fragmentation pattern are shown in Figure S1.

In the next step, we optimized and evaluated chromatographic conditions to separate all analytes, reach a short analysis time and get symmetry peaks. Thus, three UHPLC columns with different modifications of the octadecylsiline (C18) stationary phase were tested, including the Acquity UPLC HSS T3, Acquity UPLC BEH C18, Premier C18AX and two HILIC-based columns, namely CORTECS UPLC HILIC and BEH Amide (Figure S2). In addition, combinations of
several mobile phases, buffers and additives were evaluated. The best chromatographic conditions with reasonable retention and resolution were achieved using the Acquity UPLC HSS T3 column with 10 mM ammonium formate in water as mobile phase A and acetonitrile as mobile phase B. The separation was carried out using a gradient elution program (Table 2) and the total analysis time was 4.2 min. Retention times of analytes were as follows: 0.69 min for isoniazid, 0.91 min for pyrazinamide and 1.19 min for ethambutol. Representative chromatograms of blank plasma, blank plasma spiked with analytes of interest at LLOQ and study sample are shown in Figures 2, S3 and S4. During the separation, neither the fronting nor tailing of peaks was detected.

As one of our aims was to develop a high-throughput method suitable for routine use, an Ostro™ 96-well plate was chosen for sample preparation. This plate presents a pass-through method for the simultaneous removal of plasma proteins and phospholipids using a simple protocol. This sample extraction method provides consistent recovery, eliminates matrix effects caused by phospholipids, can be done in a short time and reduces the possibilities for errors during sample preparation (e.g., transfer between centrifuge tubes). Our previous study showed that the efficacy of phospholipid removal is more than 95%. 24

Compared with previously published studies,12–22 our method provides a short analysis time and an efficient clean-up sample procedure which prolongs the column lifetime. The quantification range is enough to quantify selected drugs after their therapeutical doses. Moreover, only 100 μl of the plasma sample is required for analysis and a one-step extraction procedure was used for sample pretreatment. The novelty of our method is implementing a 96-well format for sample preparation, which increases the speed of sample preparation, eliminates errors during sample manipulation and provides high throughput. Thus, our method is suitable for routine TDM of isoniazid, ethambutol and pyrazinamide.

3.2 | Method validation

During the validation, no significant interferences were observed in extracts from blank plasma samples at the retention time of analytes and their corresponding ISs (Figure 2). The interference responses were lower than 5% of the LLOQ sample for each analyte and less than 0.1% of the corresponding ISs. In addition, no carryover was detected in blank extracts injected after triplicate injections of the highest calibration standard.

The linearity of calibration curves was assessed, and all curves were linear over the concentration range of 0.2–10 μg/ml for isoniazid and ethambutol, and 1–65 μg/ml for pyrazinamide. The accuracies and precisions of all calibrators were under 6% and 5%, respectively, for all analytes. The means (n = 3) of regression equations obtained by least-squared regression were $y = 0.000238819x - 0.00108195 (r^2 = 0.997)$ for isoniazid, $y = 0.0000323215x + 0.000454305 (r^2 = 0.998)$ for pyrazinamide and $y = 0.000109214 + 0.00035931 (r^2 = 0.998)$ for ethambutol, where y represents the peak-area ratio of an analyte to IS and x represents the concentration of the analyte. Detailed information about linearity validation is shown in Table S1 and Figure S5.

Table S1 shows complete data for linearity validation. The LLOQ was established for each analyte as the lowest point of the calibration curve. The precisions and accuracies of LLOQ samples were acceptable, with CV values <5% and accuracies within 96.1%–107.8% for the analytes. The S/N ratio of LLOQ for each analyte was much higher than 10.

**FIGURE 2** Representative chromatograms of blank plasma (A), blank plasma spiked with a standard solution at the lower limit of quantification (B), and plasma samples obtained from the patient (C). The retention times for isoniazid, ethambutol and pyrazinamide are 0.69, 1.19 and 0.91, respectively. The x axis represents retention time (min) and the y axis represents the relative intensity (%) [Color figure can be viewed at wileyonlinelibrary.com]
The method’s intra- and interday precision and accuracy were examined by analyzing QC samples at four concentration levels. As shown in Table 3, the intra- and interday precisions were lower than 9%, and accuracies were within 92.1%–105.5%. We can conclude that the method demonstrates excellent precision, accuracy and reproducibility for the simultaneous quantification of isoniazid, ethambutol and pyrazinamide in human plasma samples.

Matrix effects and extraction recoveries were evaluated using two different QC levels (low and high), and drug-free plasma samples from eight individuals were used. The average matrix effects of all analytes expressed as IS-normalized matrix factors were lower than 5% (Table S2). Validation data showed negligible ion suppression or enhancement from the rat plasma. The mean extraction recoveries of analytes in human plasma samples varied from 68.9% to 76.4% (Table S2). Recoveries were reproducible among the different sources of plasma samples, and recoveries of corresponding ISs were similar to analytes. It can be concluded that matrix effects and extraction recoveries do not limit our method.

To reanalyze samples over the ULOQ, the dilution integrity was verified. First, blank plasma samples were spiked with working solution at concentration twice higher than ULOQ. These highly concentrated samples were subsequently diluted 2-fold with blank plasma and analyzed. Precisions (CVs) were <5.1%, and accuracies ranged from 95.7% to 102.9% for all analytes (data not shown). Our results prove dilution integrity, thus samples over calibration curve ranges can be reanalyzed after the appropriate dilution.

The results of stability validation proved that isoniazid, ethambutol and pyrazinamide are stable under different storage conditions in human plasma (Table 4). The data indicate the stability of all analytes at room temperature for 12 h, after long-term storage at −20°C for 2 months, after three freeze-thaw cycles and after 48 h in the sample manager at 8°C. Moreover, the stability of analytes was assessed in whole blood, therefore we can conclude that all analytes were stable for 2 h in whole blood before centrifugation.

### TABLE 3 Intra- and interday accuracies and precisions of isoniazid, ethambutol and pyrazinamide in human plasma

| Analyte  | Nominal concentration (μg/ml) | Intraday (overall mean, n = 6) | Interday (overall mean, n = 18) |
|----------|-----------------------------|-------------------------------|---------------------------------|
|          | Measured concentration (μg/ml) | Accuracy (%) | Precision (CV, %) | Measured concentration (μg/ml) | Accuracy (%) | Precision (CV, %) |
| Isoniazid | 0.2 | 0.188 | 94.2 | 2.3 | 0.209 | 104.8 | 8.9 |
|          | 0.7 | 0.648 | 92.6 | 1.8 | 0.712 | 101.7 | 7.8 |
|          | 4 | 3.753 | 93.8 | 1.1 | 3.935 | 98.4 | 5.3 |
|          | 8 | 8.176 | 102.2 | 1.2 | 8.387 | 104.8 | 2.2 |
| Ethambutol | 0.2 | 0.201 | 100.6 | 4.0 | 0.211 | 105.5 | 4.7 |
|          | 0.7 | 0.656 | 93.9 | 1.3 | 0.684 | 97.8 | 3.5 |
|          | 4 | 3.759 | 94.0 | 2.6 | 3.851 | 96.3 | 3.6 |
|          | 8 | 8.051 | 100.7 | 1.9 | 8.099 | 101.2 | 0.8 |
| Pyrazinamide | 1 | 0.989 | 98.9 | 4.0 | 0.982 | 98.3 | 1.5 |
|          | 3 | 2.761 | 92.1 | 3.4 | 2.844 | 94.8 | 4.3 |
|          | 25 | 25.191 | 100.8 | 5.0 | 24.686 | 98.7 | 5.6 |
|          | 65 | 56.120 | 102.0 | 2.3 | 55.707 | 101.3 | 1.6 |

### TABLE 4 Results of stability validation of isoniazid, ethambutol and pyrazinamide in human plasma under different storage conditions (n = 6)

| Analyte  | Nominal concentration (μg/ml) | Short-term stability | Freeze–thaw stability | Long-term stability | Post-processing stability |
|----------|-----------------------------|----------------------|------------------------|---------------------|--------------------------|
|          | Accuracy (%) | CV (%) | Accuracy (%) | CV (%) | Accuracy (%) | CV (%) | Accuracy (%) | CV (%) |
| Isoniazid | 0.7 | 1.8 | 3.6 | 0.3 | 2.7 | 2.8 | 4.4 | 3.9 | 1.6 |
|          | 8 | 3.5 | 3.0 | 1.7 | 2.8 | 2.2 | 3.8 | 8.5 | 2.5 |
| Ethambutol | 0.7 | 4.8 | 3.1 | 1.7 | 2.0 | 5.6 | 3.7 | 1.4 | 4.1 |
|          | 8 | 2.5 | 3.2 | 4.9 | 2.1 | 7.1 | 4.0 | 1.2 | 6.2 |
| Pyrazinamide | 5 | 6.8 | 3.1 | 8.3 | 3.8 | 7.4 | 4.0 | 4.2 | 3.7 |
|          | 55 | 1.4 | 5.0 | 10.0 | 2.6 | 6.7 | 3.7 | 5.0 | 3.3 |
The measured plasma concentrations of antituberculotics in TB patients

| Patient no. (gender) | BMI | Isoniazid (µg/ml) | Ethambutol (µg/ml) | Pyrazinamide (µg/ml) |
|---------------------|-----|------------------|--------------------|---------------------|
|                     |     | Dose (mg/kg) | 2 h postdose | 6 h postdose | Dose (mg/kg) | 2 h postdose | 6 h postdose | Dose (mg/kg) | 2 h postdose | 6 h postdose |
| 1 (F)               | 15.8| 7.7       | 4.1 | 3.0 | 20.5 | 1.7 | 1.3 | 25.6 | 29.7 | 20.4 |
| 2 (F)               | 17.8| 5.1       | 3.8 | 1.4 | 20.5 | 1.8 | 0.9 | 25.6 | 25.6 | 16.0 |
| 3 (M)               | 29.1| 3.6       | 4.0 | 1.7 | 14.3 | 2.1 | 1.2 | NA   | ND   | ND   |
| 4 (M)               | 19.8| 5.0       | 4.0 | 1.6 | 20.0 | 1.9 | 1.1 | 25.0 | 30.6 | 22.1 |
| 5 (M)               | 17.2| 5.1       | 3.4 | 2.0 | 20.3 | 2.7 | 1.2 | 25.4 | 40.8 | 31.9 |
| 6 (F)               | 30.1| 4.0       | 1.6 | 1.1 | 10.7 | 2.4 | 2.1 | 26.6 | 42.6 | 23.2 |
| 7 (M)               | 16.1| 5.9       | 1.1 | 0.7 | 23.5 | 1.8 | 1.4 | 29.4 | 29.0 | 11.2 |
| 8 (M)               | 22.1| 4.4       | 3.6 | 2.0 | 17.7 | 1.9 | 1.7 | 22.1 | 31.0 | 23.0 |
| 9 (M)               | 26.7| 5.1       | 5.3 | 1.7 | 15.2 | 1.1 | 1.0 | 25.3 | 35.2 | 24.0 |

The standard and recommended \( c_{\text{max}} \) values are 3–6 µg/ml for isoniazid, 2–6 µg/ml for ethambutol and 20–60 µg/ml for pyrazinamide. BMI, body mass index; F, female; M, male.

### 3.3 Method application

The developed and fully validated LC–MS/MS method was applied to quantify all the analytes in plasma samples from TB patients. All patients were treated with isoniazid, ethambutol and pyrazinamide for at least 2 weeks. The steady-state concentrations should therefore have been achieved considering the biological half-times of the respective drugs. The patients had been taking antituberculotics to cover the recommended daily dose of 4–6 mg/kg for isoniazid, 15–20 mg/kg for ethambutol and 20–30 mg/kg for pyrazinamide (except for one patient who did not take pyrazinamide). In TDM of first-line antituberculotics, the recommendation is to analyze 2- and 6-h postdose samples. The first one sample is used to evaluate the peak concentrations of drugs (\( c_{\text{max}} \)) and the second allows us to estimate the rate and completeness of absorption. The standard and recommended \( c_{\text{max}} \) values are 3–6 µg/ml for isoniazid, 2–6 µg/ml for ethambutol and 20–60 µg/ml for pyrazinamide.\(^{25}\) The measured concentrations of plasma samples in our study are summarized in Table 5. The 2-h postdose concentrations were in the range of 1.1–5.3 µg/ml for isoniazid, 1.1–2.7 µg/ml for ethambutol and 25.6–42.6 µg/ml for pyrazinamide. Based on recommended \( c_{\text{max}} \) values, the measured concentrations in two patients for isoniazid and six patients for ethambutol were below the expected range. On the other hand, none of the patients reached concentrations higher than recommended. Moreover, our data show the typical pattern for 2- and 6-h postdose samples: the concentrations after 2 h were significantly higher than those after 6 h so there is no delayed absorption of drugs. We can therefore conclude that the implementation of TDM in TB treatment might be beneficial and help individualized drug dosing, especially in patients with suspected lower plasma concentrations. Finally, we did not analyze rifampicin as one of the first-line TB treatments due to autooxidation to rifampicin quinone. It is essential to prevent this autooxidation process immediately after sample drawing as well as during sample preparation and analysis. Unfortunately, we could not prevent this degradation process during the method development, and thus we have not included rifampicin in our study.

### 4 CONCLUSIONS

A simple and high-throughput LC–MS/MS method for simultaneous quantification of isoniazid, ethambutol and pyrazinamide in human plasma was developed and validated. The presented method has several significant advantages compared to previously published methods, such as low sample volume (100 µl), short run time of analysis, one-step sample preparation procedure with capabilities for phospholipids removal, and a low quantification limit as well as a high degree of selectivity. The method was successfully validated in compliance with EMA guidelines for selectivity, linearity and LLOQ, precision and accuracy, matrix effect, extraction recovery, carryover, dilution integrity and stability under different conditions. Furthermore, the method was applied to quantifying first-line anti-TB drugs in plasma samples of real, treated patients with TB. TDM of anti-TB drugs in combination with clinical or bacteriological examinations might be helpful for the successful management of patients with TB.

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### CONFLICTS OF INTEREST

The authors have declared no conflict of interest.

### PEER REVIEW

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DATA AVAILABILITY STATEMENT
The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.

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