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A UPLC-MS-MS method for the simultaneous quantification of first-line antituberculars in plasma and in PBMCs

Lorena Baietto, Andrea Calcagno, Ilaria Motta, Katia Baruffi, Viviana Poretti, Giovanni Di Perri, Stefano Bonora, Antonio D'Avolio

Abstract

Objectives TB is currently the second cause of death among patients affected with infectious diseases. Quantification of drug levels in plasma and in cells where Mycobacterium tuberculosis persists and grows may be useful in understanding the appropriateness of dosage regimens. We report a new and fully validated chromatographic method to quantify first-line antituberculars in plasma and PBMCs. The method was used for plasma and cell quantification of antituberculars in patients undergoing treatment with standard oral therapy.

Methods Ethambutol, isoniazid, pyrazinamide and rifampicin were extracted from plasma and PBMCs using two separate and optimized procedures; analysis was performed using UPLC coupled with a mass-mass detector system (UPLC-MS-MS). Antitubercular levels in patients were assayed at the end of the dosing interval (C_trough) and 2 h post-dose (C_max).

Results The method was accurate and precise. Recovery and the matrix effect were reproducible. While rifampicin intracellular concentrations were similar to plasma values (median intra-PBMC C_max = 7503 ng/mL versus median plasma C_max = 6505 ng/mL), isoniazid and pyrazinamide intracellular concentrations were lower than plasma values (median intra-PBMC C_max = 12 ng/mL versus median plasma C_max = 3258 ng/mL for isoniazid and median intra-PBMC C_max = 2364 ng/mL versus median plasma C_max = 26,988 ng/mL for pyrazinamide) and ethambutol intracellular concentrations were significantly higher than plasma values (median intra-PBMC C_max = 73,334 ng/mL versus median plasma C_max = 2244 ng/mL).

Conclusions The method was suitable for both therapeutic drug monitoring and for pharmacokinetic analysis. Should the clinical usefulness of measuring antitubercular drug intracellular concentrations be confirmed, this method could be useful to enhance the clinical application of intra-PBMC evaluation.

Introduction

In 2012 it was estimated that 8.6 million people worldwide developed TB and 1.3 million died with it; TB is considered the second cause of death among infectious diseases after HIV infection. Suboptimal drug concentrations (mostly in the plasma compartment) have been associated with delayed mycobacteria clearance, relapse and selection of drug-resistant strains.2-4

Macrophages play an important role in the control of Mycobacterium tuberculosis growth, spread and granuloma formation10 and immunodeficient patients are at higher risk of developing disseminated and extrapulmonary infections. As antibacterial activity is concentration-dependent, antitubercular drugs must reach appropriate levels at the site of action, i.e. inside infected macrophages.4 However, since resident macrophages are usually derived from circulating monocytes and since they are far more easily accessed, it may be relevant to measure antitubercular drugs in PBMCs.

We aimed to develop and validate a new chromatographic method to quantify ethambutol, isoniazid, pyrazinamide and rifampicin in plasma and PBMCs.

Materials and methods

Chemicals and reagents
Ethambutol, isoniazid, pyrazinamide, rifampicin, thymidine and 6,7-dimethyl-2,3-di(2-pyridyl)quinoxaline (QX) were purchased from Sigma-Aldrich (St Louis, MO, USA). Acetonitrile HPLC grade and methanol HPLC grade were purchased from VWR (Radnor, PA, USA). Formic acid was obtained from Sigma-Aldrich. HPLC grade water was prepared using a Milli-Q system coupled with a Synergy 185 system (Millipore, Billerica, MA, USA). Blank PBMCs were taken from buffy coats of healthy donors, kindly supplied by the Blood Bank of the Maria Vittoria Hospital (Turin, Italy).

**Equipment**

The chromatographic system used was an Acquity™ Ultraprformance Liquid Chromatography system (UPLC) (Waters, Milford, MA, USA) coupled with a Quadrupole Detector (TQD). An Acquity™ UPLC HSS T3 1.8 μm (2.1 × 150 mm) column (Waters), protected by an Acquity UPLC Column In-Line Filter (Waters), was used. MS-MS settings, cone voltages, collision energies and mass transitions are reported in Table S1 (available as Supplementary data at JAC Online).

**Stock solutions, standards and quality controls (QCs)**

Stock solutions of ethambutol, isoniazid and rifampicin were prepared in 100% methanol, a stock solution of pyrazinamide was prepared in water/methanol (50:50, v/v), a stock solution of thymidine was prepared in 100% water and a stock solution of QX was prepared in water/methanol (10:90, v/v).

An internal standard working solution (IS) was made by diluting thymidine and QX in water/methanol (50:50, v/v) at a concentration of 20 and 1 mg/mL, respectively. Calibration ranges and QC levels are reported in Table S2.

**Extraction procedure from plasma**

Two hundred microlitres of standard, QC and patient samples were added to 50 μL of IS and 400 μL of acetonitrile. After centrifugation at 20 000 g for 10 min at 4°C, supernatant was diluted 1:10 with water before injection into the UPLC system.

**Extraction procedure from PBMCs**

Standards, QCs and samples from patients, consisting of 1 mL of PBMCs at a concentration of 10×10⁶ cells/mL, were spiked with 50 μL of IS. Each sample was vortexed for 15 s and then sonicated in a water bath for 15 min at room temperature. Samples were centrifuged at 20 000 g for 10 min at 4°C, and supernatant was transferred to glass tubes. Supernatant was evaporated in a vacuum centrifuge at 60°C, reconstituted with 300 μL of water/acetonitrile (95:5, v/v) and injected into the UPLC system.

**Recruitment of patients, sampling and calculation of cell-associated drug concentrations**

Clinical samples were collected after obtaining written informed consent, according to local ethics committee indications (ASLTO2, protocol TB INTRA). The method was tested on patients who attended the Amedeo di Savoia Hospital (Turin). All subjects were over the age of 18 years and received standard oral dosages, given on an empty stomach, of ethambutol (20 mg/kg/day), isoniazid (5 mg/kg/day), pyrazinamide (20 mg/kg/day) and rifampicin (10 mg/kg/day). Samples were obtained after 4 weeks of treatment. Venous blood samples were collected at the end of the dosing interval (Cₜₐₕₐₜₗ) and 2 h post-dose (Cₘₐₓₚ). They were centrifuged (900 g for 15 min at 4°C) to separate the plasma. PBMCs were isolated from blood (28 mL) using BD Vacutainer™ CPT™ tubes. Tubes were centrifuged at 800 g for 15 min at 20°C. PBMCs were then washed twice in ice-cold 0.9% NaCl solution. Cell number and mean cellular volume (MCV) were determined using an automated cell counter (Z2™ Coulter Counter®, Beckman Coulter, Brea, CA, USA). Plasma samples and the resulting pellets of PBMCs, dissolved in 800 μL of water/methanol solution (30:70,
v/v), were stored at −80°C. The interval from blood sampling to PBMC storage was <1 h. PBMC-associated concentrations of antituberculars, expressed in ng/mL, were obtained using the following formula:

antitubercular amount (ng)/number of PBMCs × MCV (fl) × 10⁻¹².¹²⁻¹⁵

Results

Validation of the assay

The assay was validated in accordance with FDA guidelines (FDA, 2013 #16).

Representative chromatograms are reported in Figure S1.

Absence of interference from endogenous and exogenous compounds (antibacterials, antiretrovirals) was confirmed by the analysis of six different blank plasma and PBMC samples.

A quadratic forced through the zero calibration curve (mean \( r^2 > 0.99 \)) was used for all drugs assayed for both plasma and PBMCs. Intra- and inter-day accuracy (percentage error) and precision (percentage relative standard deviation) was <15% (\( n = 5 \); Table 1). The lower limit of quantification was considered the lowest point of the calibration curve (Table S2). The limit of detection (LOD) for the plasma method and the PBMC method was 58 and 2.93 ng/mL, respectively, for ethambutol, 58 and 0.391 ng/mL, respectively, for isoniazid, 68 and 0.391 ng/mL, respectively, for pyrazinamide and 117 and 0.976 ng/mL, respectively, for rifampicin.

Table 1. Average intra- and inter-day accuracy and precision obtained using the method of extraction from plasma and PBMCs described here

<table>
<thead>
<tr>
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<th>Mean accuracy (% error)</th>
<th>Mean precision (% RSD)</th>
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<tbody>
<tr>
<td>EMB INH PZA RIF</td>
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RSD, relative standard deviation; H, high; M, medium; L, low; EMB, ethambutol; INH, isoniazid; PZA, pyrazinamide; RIF, rifampicin.

Recovery was evaluated by calculating the ratio between peak area of extracted and un-extracted QCs (\( n = 5 \)).¹⁶ The matrix effect was assayed by calculating the ratio between peak area of un-extracted QCs and...
standards of the analytes present in the reconstitution solvent [water/acetonitrile (95:5, v/v)]. Results are reported in Table S3.

Antituberculars were found to be stable (degradation <20%) in stock solutions and in plasma at −80°C for 1 month and after three cycles of freezing at −80°C and thawing at room temperature (over 1 h).

**Absence of antitubercular loss during the PBMC washing procedure**

To assess whether the PBMC washing procedure leads to drug loss, 40 mL of washing supernatant was collected and quantified as reported above. We found that antitubercular levels in the washing supernatant were lower than the LOD and we can conclude that no drug loss happens during the PBMC washing procedure.

**Analysis of antitubercular concentrations in patients**

In Figure 1, plasma and PBMC-associated concentrations obtained from 15 subjects are reported. As reported in the literature, isoniazid and rifampicin had undetectable or very low plasma C_{trough} levels and ethambutol and pyrazinamide had low plasma C_{trough} levels. Plasma C_{max} values were within the target therapeutic range for the four drugs quantified. Isoniazid, pyrazinamide and rifampicin had undetectable or very low intracellular C_{trough} levels. Isoniazid and pyrazinamide seemed not to accumulate in PBMCs, rifampicin accumulated around 1-fold within PBMCs and ethambutol accumulated around 30-fold.

**Figure 1.**

Plasma and PBMC-associated concentrations of the four antituberculars obtained from 15 subjects.
Discussion

We developed and validated a new chromatographic method to simultaneously quantify first-line antituberculars in plasma and PBMCs. To date, only one study reported an assay to simultaneously quantify plasma concentrations of first-line antitubercular drugs using HPLC-MS-MS.\textsuperscript{17} Our method of extraction from plasma, compared with the previously published method, has the advantage of a faster extraction procedure using a single precipitation step with acetonitrile and direct injection into the UPLC system. The method described by Song \textit{et al.}\textsuperscript{17} involves two steps of deproteinization and a run time of 4 min. The longer time of the chromatographic run used in our method (6 min) allows better separation of analytes, reducing potential interferences from endogenous and exogenous compounds and reducing the matrix effect.

The only method published for antitubercular-associated PBMC concentrations was described by Hartkoorn \textit{et al.}\textsuperscript{18} and it only quantifies rifampicin. In our method, calibration curves and QCs used for the intracellular quantification of antituberculars were prepared in PBMCs, and recovery and the matrix effect were evaluated using PBMCs differently from what was observed in the method of Hartkoorn \textit{et al.},\textsuperscript{18} where the authors only used plasma as the matrix. Furthermore, we measured MCV (median MCV = 272 fl) instead of assuming a fixed MCV of 400 fl; this may be more accurate when estimating intracellular concentrations, reducing the probability of underestimating or overestimating drug levels in PBMCs.\textsuperscript{11,13-15,19} Rifampicin accumulation inside PBMCs was lower than that previously observed (intracellular:plasma ratio of 1 versus 1.8 in the work of Hartkoorn \textit{et al.}\textsuperscript{18}), but further studies are needed to confirm these findings.

High inter-patient variability was observed both in plasma and intracellular concentrations, thus supporting the use of therapeutic drug monitoring for optimizing the antitubercular drug pharmacokinetic profile. The reported intra-PBMC concentrations warrant further investigation in order to study the potential concentration-dependent efficacy or toxicity in patients treated for TB. This newly developed method, which allows the rapid and simultaneous quantification of first-line plasma and intra-PBMC antitubercular drug concentrations, may be a useful tool for tailoring antitubercular regimens in selected patients.

References