Simultaneous determination of first-line anti-tuberculosis drugs and one metabolite of isoniazid by liquid chromatography/tandem mass spectrometry in patients with human immunodeficiency virus-tuberculosis coinfection

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ABSTRACT
The incidence rate of tuberculosis (TB) in patients with human immunodeficiency virus (HIV) infection is 26 times higher than that in other patients. Patients with both infections require long-term combination therapy, which increases therapy complexity and might lead to serious adverse reactions and drug-drug interactions. To optimize therapy for patients with HIV and TB coinfection, we developed an ultra-high-performance liquid chromatography/tandem mass spectrometry (UHPLC-MS/MS) method to simultaneously quantify four anti-tuberculosis drugs and one isoniazid (INH) metabolite. Blood samples (n = 32) from 16 patients with HIV and TB coinfection were collected. Plasma protein precipitation with acetonitrile was followed by a hydrazine reaction between INH and cinnamaldehyde (CA) to produce phenylhydrazine (CA-INH) and dilution with heptafluorobutyric acid. The separation was performed on an Acquity UHPLC HSS T3 1.8 μm column (2.1 x 100 mm, Waters) with a mobile phase consisting of 10 mmol/L ammonium formate (pH = 4) in water (solvent A) and 0.1 % formic acid in methanol (solvent B) in a gradient elution. The compounds were detected using a positive multiple reaction monitoring model. INH, acetyl-INH (AC-INH), rifampicin (RIF), ethambutol (EMB), and pyrazinamide (PZA) showed good linear relationships in their quantitative ranges, with lower limits of quantification of 48, 192, 200, 96, and 480 ng/mL, respectively. The inter- and intraday precision was within 15 %, and the accuracy was between 85 % and 115 %. The mean plasma concentrations of INH, AC-INH, RIF, EMB, and PZA in patients were 1990.23 (24 – 16 600), 863.06 (96 – 2880), 3507.05 (229 – 9800), 808.10 (149 – 2130), and 18 838.33 (240 – 34 800) ng/mL, respectively. The plasma concentrations detected in the 16 patients were lower than the targeted concentrations in HIV-negative TB patients. In summary, we developed a simple UHPLC-MS/MS method for simultaneous quantification of first-line TB drugs, and successfully applied it for therapeutic drug monitoring in patients with HIV and TB coinfection. This method will facilitate monitoring of TB drugs in the future.

1. Introduction

With the widespread application of antiretroviral therapy, HIV is no longer the most deadly infectious disease. However, a high rate of virological failure (10–20 %) is still observed in patients receiving this therapy [1, 2, 3]. In addition, patients with acquired immune deficiency syndrome (AIDS) often suffer from a variety of opportunistic infectious diseases, of which tuberculosis (TB) is the most common. The incidence rate of TB in patients with AIDS is 26 times higher than that in other patients [4]. In patients with TB, HIV coinfection is present in 3.8 %–72.3 % [5, 6] of cases. Patients with both infections require long-term combination therapy, which can lead to poor compliance, multidrug resistance, and serious adverse reactions, such as liver dysfunction and thrombocytopenia [7, 8], and even the need to change the antiretroviral

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treatment protocol [9, 10]. Furthermore, there are potential drug-drug interactions in the treatment of AIDS complicated with TB [11, 12]. Moreover, the area under the curve (AUC) for first-line drugs for treating patients with HIV-TB co-infection is lower than that in patients with TB alone [13]. Therefore, individualized treatment is very important for patients with HIV-TB coinfection.

At present, the dominant first-line anti-TB drugs are isoniazid (INH), rifampicin (RIF), ethambutol (EMB), and pyrazinamide (PZA) [14]. For INH, the major active metabolite is acetyl-INH (AC-INH), which can also be monitored for INH dose adjustment [15]. Many studies have addressed plasma concentration monitoring for anti-TB drugs [8, 16, 17, 18]. Furthermore, because multidrug combined therapy is necessary for TB, a method to simultaneously monitor several anti-TB drugs in human plasma is needed. Several liquid chromatography (LC) or LC-tandem mass spectrometry (LC-MS/MS) methods for the measurement of first-line anti-TB drug levels in plasma have been reported [15, 19, 20, 21, 22, 23, 24, 25]. However, these methods have disadvantages, such as long chromatographic separation time [25], poor retention of INH, and partial separation of AC-INH and INH on reversed-phase columns [25].

In this study, we aimed to develop an ultra-high-performance liquid chromatography/tandem mass spectrometry (UHPLC-MS/MS) method to retain and separate four first-line anti-TB drugs and AC-INH on a reversed-phase column for the monitoring of plasma drug concentrations in patients with HIV-TB co-infection.

2. Materials and methods

2.1. Chemicals and reagents

Isoniazid-d4 (INH-d4), N-demethyl-rifampicin-d8 (N-demethyl-RIF-d8), ethambutol-d4 (EMB-d4), AC-INH, and acetyl isoniazid-d4 (AC-INH-d4) were purchased from Toronto Research Chemicals Inc. (Toronto, Canada). INH and EMB were obtained from the United States Pharmacopeia (Rockville, MD, USA), RIF from Dr. Ehrenstorfer (Augsburg, Germany), PZA from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and pyrazinamide-d3 (PZA-d3) from TLC PharmaChem Inc. (Newmarket, Canada). HPLC-grade methanol and acetone were purchased from ANPEL Laboratory Technologies (Shanghai, China), formic acid (FA) from Anaqua Chemicals Supply Company (Wilmington, Delaware, USA), and cinnamaldehyde (CA), vitamin C, and heptfluorobutyric acid (HFBA) from Sigma-Aldrich Company (Darmstadt, Germany). Ammonium formate (NH₄FA) was purchased from Honeywell (Charlotte, NC, USA), Blank plasma was collected from healthy volunteers, and the protocol was approved by Shanghai Public Health Clinical Center (2016-A020-01). Ultrapure water was produced in the laboratory using a Millipore system (Millipore, Bedford, MA, USA). Blank plasma was collected from healthy volunteers, and informed consent was obtained from all volunteers.

2.2. Volunteer enrollment and sample collection

The experimental protocol was approved by the Ethics Committee of the Shanghai Public Health Clinical Center (No. 2020-S177-02), and informed consent was obtained from all volunteers.

2.2.1. Healthy enrollment

Six healthy volunteers were enrolled, and 20 mL blood was collected from each individual into potassium ethylenediaminetetraacetic acid (EDTA-K₂)-anticoagulation tubes. The volunteers were selected according to the following criteria.

Inclusion criteria: age of 18–65 years, no HIV and TB infection, no TB drugs taken, and willingness to participate in the study.

Exclusion criteria: patients with cancer, pregnant, and breast-feeding.

2.2.2. Patient enrollment

This study was conducted at the Shanghai Public Health Clinical Center, Shanghai, China. Hospitalized adult HIV/TB co-infected patients (n = 16) were enrolled from May to October 2020 according to the following criteria. Inclusion criteria: no less than 18 years old, HIV and TB co-infected, have taken TB drugs for at least seven days, and willing to participate in the study. Exclusion criteria: patients with cancer, pregnant, and breast-feeding.

Two blood samples (2 mL each) were collected at random time points from each patient in EDTA-K₂-anticoagulant tubes. After centrifugation, plasma was collected and frozen at −40 °C for further usage.

2.3. LC-MS/MS conditions

2.3.1. Optimizing mass spectrometry condition

The Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA) coupled to an AB Sciex Triple Quad 5500 mass spectrometer (Applied Biosystems/AB SCIEX, Boston, MA, USA) was used for sample analysis. Quantification was achieved by multiple reaction monitoring in positive ion mode. The mass spectrometer was operated using the following settings: ion spray voltage, 5.5 kV; collision gas, medium; curtain gas, 40 psi; ion source gas 1 and ion source gas 2, 60 psi; entrance potential, 10 eV; collision cell exit potential, 13 eV. The declustering potential and collision energy (eV) were optimized as shown in Table 1.

2.3.2. Optimizing sample preparation and chromatography separation conditions

To improve hydrophobicity of INH, we derivatized INH with cinnamaldehyde. To improve the retention of highly polar compounds

| Compound (IS) | Precursor ion (m/z) | Product ion (m/z) | Declustering potential (eV) | Collision energy (eV) |
|--------------|--------------------|------------------|-----------------------------|-----------------------|
| CA-INH       | 252.1              | 121.0            | 60                          | 30                    |
| (CA-INH-d4)  | 256.1              | 125.1            | 70                          | 20                    |
| AC-INH       | 180.1              | 138.2            | 90                          | 20                    |
| (AC-INH-d4)  | 184.2              | 142.1            | 90                          | 20                    |
| RIF          | 823.4              | 791.4            | 53                          | 23                    |
| (N-demethyl-RIF-d8) | 817.3     | 785.5            | 40                          | 23                    |
| EMB          | 205.1              | 116.1            | 70                          | 12                    |
| (EMB-d4)     | 209.1              | 120.2            | 57                          | 12                    |
| PZA          | 124.0              | 81.0             | 60                          | 25                    |
| (PZA-d3)     | 127.0              | 84.0             | 75                          | 25                    |

*IS, internal standard; CA-INH, isoniazid (INH) + cinnamaldehyde (CA); CA–INH-d4, INH-d4 + CA; AC-INH, acetyl isoniazid; AC-INH-d4, acetyl isoniazid-d4; RIF, rifampicin; N-demethyl-RIF-d8, N-demethyl-rifampicin-d8; EMB, ethambutol; EMB-d4, ethambutol-d4; PZA, pyrazinamide; PZA-d3, pyrazinamide-d3.
Figure 1. Reaction between isoniazid (INH) and cinnamaldehyde (CA) (A), and the chromatography profiles of INH (B) and CA-INH (C) CA-INH, the derivatization production of INH and CA. The peak was highlighted by asterisk (*).

Figure 2. Electrospray product ion spectra (MS) of the five analytes (A) and their internal standards (B) A1, A2, A3, A4, and A5 show the peaks for the product ion spectra (MS) of CA-INH, AC-INH, RIF, EMB, and PZA, respectively. B1, B2, B3, B4, and B5 show those for the corresponding internal standards. AC-INH, acetyl isoniazid; CA-INH, isoniazid + cinnamaldehyde; INH, isoniazid; RIF, rifampicin; EMB, ethambutol; PZA, pyrazinamide.
in the chromatography column, we added ion-pairing reagent (HFBA) to the sample diluted solution. After optimizing the method for sample preparation, we compared the elution solution (B) of acetonitrile (ACN) and methanol, and solution A of 0.1% FA water, 5 mM NH₄FA, 5 mM NH₄FA (pH 4.0), 10 mM NH₄FA, and 10 mM NH₄FA (pH 4.0). Two kinds of spiked plasma standards with concentration of upper limit of quantification (ULOQ) and lower limit of quantification (LLOQ) were used. Chromatographic separation was performed on an Acquity UPLC HSS T3 1.8 μm column (2.1 × 100 mm, Waters) at a flow rate of 0.35 mL/min with HPLC gradient of 0–0.5 min 2% B, 1.5–2.5 min 80% B, and 3.0–4.0 min 2% B. The column temperature was 35 °C. For each sample, 5.0 μL was injected with a total running time of 4.0 min per sample.

2.4. Standard solutions and ISs

Analytes and their ISs were accurately weighed and dissolved separately in 90% methanol (methanol:water 90:10, v/v) to obtain stock solutions with a concentration of 1 mg/mL. Then, the stock solutions for INH, RIF, EMB, PZA, and AC-INH were mixed and diluted with 50% methanol to obtain a working solution (named S1) with the following concentrations: INH 60, AC-INH 240, RIF 250, EMB 120, and PZA 600 μg/mL. Then, S1 was diluted with 50% methanol to obtain a series of working solutions. In total, seven concentrations of working standards were prepared, including INH at 60 000, 54 000, 24 000, 9600, 2400, 960, and 480 ng/mL; AC-INH at 240 000, 216 000, 96 000, 38 400, 9600, 3840, and 1920 ng/mL; RIF at 250 000, 225 000, 100 000, 40 000, 10

Figure 3. The affected chromatograms of CA-INH, AC-INH, and EMB with or without CA and HFBA addition. A. without CA and HFBA (marked as (CA(-)+HFBA(-))); B. without CA and with HFBA (marked as (CA(-)+HFBA(+))); C. with CA and without HFBA (marked as (CA(+)+HFBA(-))); D. with CA and HFBA (marked as (CA(+)+HFBA(+))). AC-INH, acetyl isoniazid; CA-INH, isoniazid + cinnamaldehyde; EMB, ethambutol; CA, cinnamaldehyde; HFBA, heptafluorobutyric acid. The peaks of each compound were highlighted by asterisks (*).
000, 4000, and 2000 ng/mL; EMB at 120 000, 108 000, 48 000, 19 200, 4800, 1920, and 960 ng/mL; and PZA at 600 000, 540 000, 240 000, 96000, 24 000, 9600, and 4800 ng/mL. Four types of quality control (QC) working solutions were prepared in the same way at concentrations of 48 000, 19 200, 1200, and 480 μg/mL for INH; 200 000, 80 000, 5000, and 2000 μg/mL for RIF; 192 000, 76 800, 4800, and 1920 μg/mL for AC-INH; 96 000, 38 400, 2400, and 960 μg/mL for EMB; and 480 000, 192 000, 12 000, and 4800 μg/mL for PZA. The working standards were combined with blank plasma at 10-fold dilutions to obtain calibration standards.

Stock solutions of ISs were also combined and diluted with 50 % methanol to obtain the working IS solution with INH-d4 (5 μg/mL), AC-INH-d4 (10 μg/mL), N-demethyl-RIF-d8 (100 μg/mL), EMB-d4 (6 μg/mL), and PZA-d3 (250 μg/mL). All solutions were stored at −40 °C.

2.5. Sample preparation

Calibration standards or patient plasma (50 μL) were mixed with 20 μL of IS working solution, 20 μL of 0.5 % vitamin C, and 300 μL of acetonitrile. After vortexing for 3 min, the mixtures were centrifuged at 4 °C and 12 000 rpm for 10 min. The supernatant (40 μL) was mixed with 4 % CA (40 μL of each) to produce the hydrazone between INH and CA. After vortexing and allowing the sample to stand at room temperature (20–25 °C) for 30 min, 360 μL of diluent (20 % acetonitrile, 0.1 % FA, and 2 % HFBA) was added. After vortexing again, 5 μL of this mixture was injected into the UHPLC for analysis.

2.6. Method validation

The method validation was followed the US Food and Drug Administration guidelines.

2.6.1. Specificity

Three types of plasma samples (blank plasma, spiked calibration standard, and patient plasma) were prepared and analyzed as described in section 2.4. The specificity of the method was verified by comparing the chromatogram profiles of these three sample types.

2.6.2. Limits of detection and quantification (LOD and LOQ)

The LOD was estimated as the concentration level resulting in a peak area of three times the baseline noise measured in blank plasma extracts at the retention times of the analytes, and the LOQ was determined as the limit of quantification at which analytes/signals were 10. Six replicates were prepared.

2.6.3. Calibration curves

The linear ranges of the five compounds were INH 48–6000 ng/mL, AC-INH 192–24 000 ng/mL, RIF 200–25 000 ng/mL, EMB 96–12 000 ng/mL, and PZA 480–60 000 ng/mL. The lower limit of quantitation (LLOQ) was defined as the lowest concentration of the calibration standards with a precision <20 % and accuracy within ±20 %. The x-coordinate was the concentration of the analyte, and the y-coordinate was the area ratio of the analyte to the internal standard peak. The calibration curve was obtained by linear regression with weighted least squares (weighted factor 1/ρ²).
2.6.4. Inter- and intraday accuracy and precision

Four types of QC samples were prepared with six replicates for each: high (HQC), medium (MQC), low (LQC), and the LLOQ. The samples were processed with the same sample preparation method, and three analytical batches were continuously assessed to obtain the inter- and intraday precision and accuracy.

2.6.5. Extraction recovery and matrix effect

The extraction recovery and matrix effect were evaluated at three QC levels (HQC, MQC, and LQC) with six replicates. The extraction recovery and matrix effect were obtained by calculating the peak area ratio for the spiked sample to the spiked extraction sample, and for the spiked extraction sample to the solvent-substituted sample at the same nominal concentration, respectively.

2.6.6. Stability

The stability of analytes in plasma was investigated with the HQC and LQC samples (6 replicates for each) at room temperature for 4 h, at 4 °C for 4 h, in the autosampler for 24 h after sample processing, for three freeze-thaw cycles from −40 °C to room temperature, and for long-term storage for 30 d at −40 °C.

2.7. Application of the UHPLC-MS/MS method

The validated UHPLC-MS/MS method was applied for the simultaneous determination of first-line anti-TB drugs (INH, RIF, EMB, and PZA) and AC-INH in patients with HIV-TB coinfection.

2.8. Statistical analysis

Data are presented as means and range or scatter plots showing individual values. Plasma analyte concentrations below the quantifiable limit were recorded as 1/2 LLOQ [26].

3. Results

3.1. LC-MS method development and validation

3.1.1. LC-MS method

To improve the retention of INH (soluble in water) in the column and better separate INH and AC-INH, INH was reacted with CA to produce the hydrazone (Figure 1A). INH and INH-d4 were detected to have a pair of ions at m/z of 252.1–121.0 (CA-INH, Figure 2 A1) and 256.1–125.1 (CA–INH–d4, Figure 2 B1), respectively. The other four analytes and their ISs were hydrogen adduct ions ([M + H]+). Typical MS profiles are shown in Figure 2 A2–A5 for the four analytes, and Figure 2 B2–B5 for their ISs.

After derivation of INH with CA, the retention time of INH was increased with intensity improving significantly (Figure 3). Furthermore, with the addition of HFBA, AC-INH was eluted as a single peak, and the retention time of EMB was increased to 2.1 min from less than 1 min (Figure 3).

After optimizing the sample preparation conditions, we compared the mobile phase B (ACN or methanol containing 0.1% FA) (Figure 4) and found that AC-INH could retain poorly, and CA-INH and RIF had multiple peaks in the column using ACN containing 0.1% FA. Then we
fixed B (methanol containing 0.1% FA), compared aqueous mobile A, and found that 10 mM NH₄FA (pH = 4.0) was the best for the elution of AC-INH and RIF (Figure 5) within insignificant difference for the other compounds. The retention times of CA-INH, AC-INH, RIF, EMB, and PZA were approximately 2.5, 1.7, 2.6, 2.1, and 1.8 min, respectively (Figure 6). As shown in Figure 6, all analytes were retained and separated well. No interfering endogenous substances were detected in the blank plasma, which demonstrates that the specificity of this method is acceptable.

### 3.1.2. LC-MS method validation

The LOD of AC-INH, PZA, EMB, CA-INH, and RIF, were 1/3 of the LLOQ with signal to noise (S/N) ratios of about 3.5, 6.3, 43.6, 9.9, and 3.9, respectively (Table 2). The LOQ was equal to LLOQ.

#### Table 2. The limit of detection (LOD) by the UHPLC-MS/MS method.

| Analytes | No. 1 | No. 2 | No. 3 | No. 4 | No. 5 | No. 6 | Average |
|----------|-------|-------|-------|-------|-------|-------|---------|
| AC-INH   | 4     | 3.8   | 3.1   | 3.9   | 4     | 2.1   | 3.5     |
| PZA      | 3.5   | 7     | 8.3   | 5.9   | 9.3   | 3.8   | 6.3     |
| EMB      | 57    | 45.5  | 35.4  | 57.8  | 27.3  | 38.3  | 43.6    |
| CA-INH   | 5     | 10.5  | 10    | 10.2  | 9.5   | 14.1  | 9.9     |
| RIF      | 5     | 3.5   | 3     | 4.6   | 4     | 3.5   | 3.9     |

No. 1 to 6 represent six replicates.

#### Table 3. Inter- and intraday precision and accuracy for the analytes in human plasma.

| Analytes | Concentration (ng/mL) | Intra-day | Inter-day |
|----------|-----------------------|-----------|-----------|
|          | Measured concentration (ng/mL) | Precision (%) | Accuracy (%) | Measured concentration (ng/mL) | Precision (%) | Accuracy (%) |
| INH      | 48                    | 45.93     | 7.18      | 95.71     | 45.28 | 6.91 | 94.36    |
|          |                       | 120       | 118.83    | 3.17      | 98.98     | 121.50 | 4.01 | 101.28 |
|          |                       | 1920      | 2003.33   | 3.62      | 104.36    | 1956.67 | 4.88 | 101.89 |
|          |                       | 4800      | 5003.33   | 3.18      | 104.22    | 4874.44 | 3.24 | 101.59 |
| RIF      | 200                   | 207.67    | 11.68     | 103.80    | 189.78    | 12.11  | 94.91    |
|          |                       | 500       | 532.50    | 6.71      | 106.49    | 524.06  | 7.65  | 104.83 |
|          |                       | 8000      | 8831.67   | 4.38      | 110.40    | 8210.56 | 7.27  | 102.64 |
|          |                       | 20000     | 21633.33  | 7.37      | 108.22    | 20200   | 7.14  | 100.99 |
| EMB      | 96                    | 86.68     | 8.43      | 90.30     | 84.85     | 6.81   | 88.38    |
|          |                       | 240       | 236.33    | 4.17      | 98.36     | 239.11  | 4.69  | 99.61    |
|          |                       | 3840      | 4023.33   | 3.24      | 104.77    | 3900    | 4.19  | 103.89 |
|          |                       | 9600      | 10030     | 4.70      | 104.58    | 9650    | 7.27  | 100.55 |
| PZA      | 480                   | 482.67    | 8.14      | 100.54    | 457.78    | 7.43   | 95.34    |
|          |                       | 1200      | 1238.33   | 2.67      | 103.22    | 1226.11 | 5.07  | 102.16 |
|          |                       | 19200     | 19566.67  | 3.29      | 101.92    | 19288.89 | 2.81  | 100.46 |
|          |                       | 48000     | 47816.67  | 1.80      | 99.61     | 47927.78 | 2.80  | 99.85    |
| AC-INH   | 192                   | 201.33    | 11.63     | 104.91    | 188.56    | 10.21  | 98.23    |
|          |                       | 480       | 476.17    | 5.32      | 99.20     | 480.50  | 5.70  | 100.13 |
|          |                       | 7680      | 8061.67   | 5.29      | 104.20    | 7829.44 | 4.42  | 101.94 |
|          |                       | 19200     | 19300     | 4.16      | 100.51    | 19188.89 | 2.97  | 99.96    |

* The QC samples from top to bottom are the LLOQ, LQC, MQC, and HQC.
CA-INH, AC-INH, RIF, EMB, and PZA showed linear relationships in the ranges of 480–6000 ng/mL, 192–24 000 ng/mL, 200–25 000 ng/mL, 96–12 000 ng/mL, and 48–60 000 ng/mL, respectively. The inter- and intraday precision for the HQC, MQC, LQC, and LLOQ were all less than 15.00 %, and the accuracy ranged from 85.00% to 115.00% (except for the LLOQ) (Table 3).

### 3.1.3. Extraction recovery and matrix effect

The ranges of the extraction recovery and matrix effect for all analytes were 84.24%–99.91% and 88.23%–103.24%, respectively (Table 4).

### 3.1.4. Stability

The stability of INH, AC-INH, RIF, EMB, and PZA in human plasma was investigated. The analytes were stable under different storage conditions with deviations within ±15% (Table 5).

### 3.2. Clinical information and drug concentrations in patient samples

The validated UHPLC-MS/MS method was successfully applied to detect the plasma drug concentrations of INH, AC-INH, RIF, EMB, and PZA in patients with HIV-TB co-infection. In this study, 16 patients were enrolled, including 15 men and 1 woman, with an average age of 46.69 (27–72) years. Two samples were taken at random time points from each patient; therefore, 32 samples were collected. The mean plasma concentrations of INH, AC-INH, RIF, EMB, and PZA were 1990.23 (24–16 600), 863.06 (96–2880), 3507.05 (229–9800), 808.10 (149–2130), and 18 838.33 (240–34 800) ng/mL, respectively. The detailed clinical information and drug concentrations are shown in Table 6 and Figure 7.

### 4. Discussion

The main reason for the poor treatment outcomes in patients with drug-susceptible TB may be the lower-than-expected plasma concentrations of first-line anti-TB drugs [17, 27], especially in patients with HIV-TB co-infection [17]. As noted by Daskapan, HIV affects the Cmax and AUC of anti-TB drugs; for example, HIV reduces the AUC of RIF and Cmax of INH [28, 29, 30]. In patients with TB, Cmax values of daily doses in HIV-positive patients were 20% lower than those in HIV-negative patients [31]. More than 60% of patients have low Cmax for RIF and EMB [32].

To improve the clinical effect, therapeutic drug monitoring (TDM) has been widely performed to optimize drug doses in patients infected with TB, especially with HIV-TB [33, 34, 35, 36]. To perform TDM, several methods have been developed to quantify drug concentration, including gas chromatography (GC) [37], GC-MS [31], high performance liquid chromatography [38, 39], and LC-MS/MS [14, 40]. However, most of them have disadvantages, such as long analysis time [14, 25, 38] (as long as 26 min) [38] and poor chromatograph retention [19, 23, 40, 41].

### Table 4. Extraction recovery and matrix effect for the analytes in human plasma.

| Analytes | Concentration (ng/mL) | Extraction recovery | Matrix effect |
|----------|-----------------------|---------------------|--------------|
|          |                       | Mean (%) RSD (%)    | Mean (%) RSD (%) |
| INH      | 120                   | 94.18 3.78          | 94.91 2.86    |
|          | 1920                  | 97.20 2.17          | 92.39 3.23    |
|          | 4800                  | 95.94 3.00          | 88.23 4.06    |
| AC-INH   | 480                   | 84.24 3.40          | 95.53 4.18    |
|          | 7680                  | 97.41 2.64          | 99.66 3.05    |
|          | 19 200                | 94.15 3.41          | 102.4 5.26    |
| RIF      | 500                   | 99.59 9.87          | 97.36 4.53    |
|          | 8000                  | 98.66 3.35          | 95.89 3.26    |
|          | 20 000                | 99.91 3.11          | 98.05 2.16    |
| EMB      | 240                   | 93.83 2.44          | 98.02 3.08    |
|          | 3840                  | 93.83 1.19          | 99.86 3.71    |
|          | 9600                  | 93.36 4.53          | 94.92 2.58    |
| PZA      | 1200                  | 97.46 3.33          | 97.38 3.42    |
|          | 19 200                | 98.60 2.49          | 98.61 3.58    |
|          | 48 000                | 97.52 2.78          | 101.36 2.08   |

* The QC samples from top to bottom are the LQC, MQC, and HQC.

### Table 5. Stability of analytes in human plasma.

| Analytes | Concentration (ng/mL) | Stability (RSD %) |
|----------|-----------------------|-------------------|
|          |                       | Short-term (4 h at room temperature) | Short-term (4 h at 4 °C) | Long-term (30 d at -40 °C) | 24 h at auto-sampler (4 °C) | Three freeze-thaw |
| INH      | 120                   | 5.27             | 7.81            | 10.01            | 10.59            | 10.81            |
|          | 4800                  | 7.55             | 7.29            | 7.69             | 5.84             | 9.25             |
| RIF      | 500                   | 11.67            | 7.07            | 13.14            | 4.38             | 11.27            |
|          | 20 000                | 8.74             | 5.14            | 8.75             | 6.84             | 8.06             |
| EMB      | 240                   | 3.54             | 4.73            | 5.84             | 3.04             | 4.90             |
|          | 9600                  | 6.77             | 6.95            | 6.53             | 6.72             | 6.34             |
| PZA      | 1200                  | 1.62             | 2.70            | 2.25             | 2.03             | 3.18             |
|          | 48 000                | 2.00             | 3.46            | 3.24             | 2.28             | 2.33             |
| AC-INH   | 480                   | 5.79             | 8.17            | 4.75             | 2.71             | 5.88             |
|          | 19 200                | 5.08             | 7.72            | 3.32             | 5.04             | 6.06             |

* The top and bottom QC samples are the LQC and HQC, respectively.
For example, in a previous study, the retention and peak shape of EMB were not favorable [19]. INH and AC-INH have weak retention and cannot be separated very well [23] or have a longer retention time [25]. In this study, INH was derivatized to phenylhydrazone by CA, which significantly increased its hydrophobicity. The reaction between INH and CA is simple, and only requires incubation at room temperature for about 30 min. Furthermore, to improve the retention of strong polar compounds, HFBA was added to the dilution solution. In a previous study, HFBA (0.02%) was added to the mobile phase [40], which might affect the detection of negatively ionized compounds. Therefore, this effect will be less notable in our method than in the reported method [40]. Furthermore, RIF might be self-oxidized to rifampicin quinone [42]. In this work, vitamin C was used to prevent the oxidation of RIF into benzoquinone [43, 44].

The developed method was successfully used to detect plasma drug concentrations in patients with HIV-TB coinfection. The average concentrations detected in this study were much lower than the target concentrations for INH (3–6 μg/mL), RIF (8–24 μg/mL), PZA (20–60 μg/mL), and EMB (2–6 μg/mL) [34]. The results from our study are in good agreement with a previous report indicating that RIF and EMB have a significantly lower C_{max} in HIV/TB than in TB alone [13]. The C_{max} of

![Figure 7. Measured plasma concentrations of INH, AC-INH, RIF, EMB, and PZA in patients with HIV-TB coinfection. AC-INH, acetyl isoniazid; INH, isoniazid; RIF, rifampicin; EMB, ethambutol; PZA, pyrazinamide.](image-url)

| No. | Age (y) | Sex | Weight | GFR (mg/mL) | Cr (μmol/L) | ALT (U/L) | AST (U/L) | Concentration (ng/mL) |
|-----|---------|-----|--------|-------------|-------------|-----------|-----------|-----------------------|
|     |         |     |        | INH*        | AC-INH*     | RIF       | EMB       | PZA*                  |
| 1   | 36      | F   | 40     | 157.579     | 36.38       | 7         | 12        | 24 ND ND 187 6340     |
| 2   | 28      | M   | 45     | 198.124     | 46.29       | 11        | 14        | 319 96 ND 347 7570    |
| 3   | 39      | M   | 50     | 164.959     | 51.27       | 15        | 9         | ND ND ND 7190 1740 ND |
| 4   | 40      | M   | 50     | 125.781     | 64.85       | 37        | 30        | ND ND ND 6050 1890 ND |
| 5   | 27      | M   | 60     | 271.573     | 24.03       | 8         | 15        | ND ND ND 3660 429 ND  |
| 6   | 42      | M   | 53.7   | 103.846     | 75.91       | 22        | 20        | ND ND ND 16600 2110 22 |
| 7   | 50      | M   | 45     | 311.587     | 28.41       | 8         | 20        | ND ND ND 70.7 ND ND |
| 8   | 35      | M   | 40     | 318.622     | 29.67       | 23        | 27        | ND ND ND 2120 768 607 |
| 9   | 56      | M   | 60     | 155.643     | 50.82       | 41        | 37        | 45.5 848 1320 794 31900 |
| 10  | 68      | M   | 60     | 190.895     | 41.15       | 43        | 43        | 24 491 713 595 28900  |
| 11  | 51      | M   | 52     | 219.322     | 38.38       | 13        | 20        | 12100 2880 3280 667 34800 |
| 12  | 72      | M   | 62     | 91.702      | 76.9        | 21        | 22        | 6590 1610 ND 1230 20000 |
| 13  | 35      | M   | 60     | 155.401     | 51.05       | 18        | 21        | 406 248 9310 450 18700 |
| 14  | 36      | M   | 48     | 127.198     | 65.11       | 40        | 38        | 864 753 4160 1080 12500 |
| 15  | 68      | M   | 60     | 149.492     | 50.86       | 10        | 7         | 308 96 4720 237 9100  |
| 16  | 64      | M   | 58     | 163.294     | 50.09       | 38        | 26        | 24 ND ND ND ND ND |
|     | Average | 46.7 | 52.7 | 181.56 | 51.05 | 21.92 | 22.56 | 1990.23 863.06 3507.05 808.10 18838.33 |
| Min | 27      | 40   | 91.702 | 24.03 | 7 | 7 | 24 | 96 | 229 | 149 | 240 |
| Max | 72      | 62   | 318.622 | 76.9 | 43 | 43 | 16600 | 2880 | 9800 | 2130 | 34800 |

ND: not detected. GFR, glomerular filtration rate; Cr, plasma creatinine; ALT, Alanine aminotransferase; AST, aspartate aminotransferase. * For the concentration less than LLOQ, 1/2 LLOQ was recorded, with 24 ng/mL for INH, 96 ng/mL for AC-INH, and 240 ng/mL for PZA.
EMB in HIV-positive patients was 20% lower than that in HIV-negative patients [31]. Furthermore, some concentrations detected in this work were very low, which might be due to the random sampling. The trough concentrations were much lower than the peaks [41], with that of INH being less than 0.25 μg/mL [45] and that of RIF being 0.6 (0.1–10.3) μg/mL [46]. The lower drug concentrations might also be due to intra and inter-individual variability in patients. As shown in Table 5, 87.5% of the patients had a GFR higher than the normal range of 80–120 mL/min. All patients had creatinine clearance rates of less than 80–120 mL. The ALT and AST levels in all patients were normal (less than 50 U/L). In summary, variabilities in the concentrations of TB drugs might not only be due to the interaction between antiretrovirals and TB drugs, but also intra and inter-individual variability (such as in Cr and GFR). In the future, it is necessary to perform PK/PD study in large sample clinical trials.

5. Conclusion

In this study, we developed a UHPLC-MS/MS method to simultaneously quantify first-line anti-TB drugs and a metabolite of INH (AC-INH). This method was successfully applied to monitor plasma drug concentrations in patients with HIV and TB. The concentrations in patients with HIV-TB coinfection were lower than those in patients with TB who were HIV-negative according to a previous report [34]. One limitation of this study is that the samples were collected at random times. Further, well-designed TDM studies with a large cohort must be performed.

Declarations

Author contribution statement

Yaru Xing: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Lin Yin; Xiaoqin Le; Jun Chen; Lin Zhang: Performed the experiments; Analyzed and interpreted the data.

Yingying Li: Analyzed and interpreted the data.

Hongzhou Lu: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Lijun Zhang: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article supplementary material referred to in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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