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LC-MS/MS method for simultaneous quantification of the first-line anti-tuberculosis drugs and six primary metabolites in patient plasma: Implications for therapeutic drug monitoring

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ABSTRACT

The pharmacokinetic profiling of drug substances and corresponding metabolites in the biological matrix is one of the most informative tools for the treatment efficacy assessment. Therefore, to satisfy the need for comprehensive monitoring of anti-tuberculosis drugs in human plasma, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed and validated for simultaneous quantification of first-line anti-tuberculosis drugs (ethambutol, isoniazid, pyrazinamide, and rifampicin) along with their six primary metabolites. Simple single-step protein precipitation with methanol was chosen as the most convenient sample pre-treatment method. Chromatographic separation of the ten analyte mixture was achieved within 10 minutes on a reverse-phase C8 column using mobile phase gradient mode. The multiple reaction monitoring mode (MRM) was used for analyte detection and quantification in patient samples. The chosen quantification ranges fully covered expected plasma concentrations. The method exhibited acceptable selectivity; the within- and between-run accuracy ranged from 87.2 to 113.6%, but within- and between-run precision was between 1.6 and 14.9% (at the LLOQ level CV < 20%). Although the response of the isonicotinic acid varied depending on the matrix source (CV 21.8%), validation results proved that such inconsistency does not affect the accuracy and precision of results. If stored at room temperature plasma samples should be processed within 4 h after collection, temporary storage at −20 °C up to 24 h is acceptable due to stability issues of analytes. The developed method was applied for the patient sample analysis (n = 34) receiving anti-tuberculosis treatment with the first-line drugs.

1. Introduction

Tuberculosis (TB) is an airborne infectious disease caused by *Mycobacterium tuberculosis*. Despite the current medical development, an estimation of 10 million new cases and 1.4 million deaths in 2019 proves that TB remains a global health problem [1]. The cure rate of 85% is reached using a 6-month standardized treatment regimen consisting of four first-line anti-tuberculosis drugs (ethambutol (ETB), pyrazinamide (PZA), rifampicin (RIF) and, isoniazid (IZN)) [1,2]. However, 15% of patients experience a relapse or develop a drug-resistant form of the disease and require treatment prolongation with costly and more toxic second-line drugs. Poor treatment adherence, nutrition, comorbidities, drug-drug interactions, and even the patient's genotype may contribute to inter-individual differences in drug exposure among TB patients [3–10]. The pharmacokinetic profiling of drug substances in various biological matrices is an efficient approach to investigate

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pharmacokinetic variability and to evaluate drug efficiency and the outcome of the therapy. At the individual level, interpretation of a patient's pharmacokinetic profile as a part of therapeutic drug monitoring (TDM) program allows to assess the dosing regime and decide whether a transition from standardized to personalized treatment strategy could improve treatment outcome [3,10–13].

The liquid chromatography-tandem mass spectrometry (LC-MS/MS) is the most utilised analytical technique for the TDM in research laboratories and has become the method of choice in the clinical setting. In the past years, numerous reverse-phase (RP) and hydrophilic interaction liquid chromatography (HILIC) based methodologies have been developed and validated to analyse different combinations of the first-line anti-tuberculosis drugs in clinical samples (Table 1) [14–24]. The selection of appropriate separation technique highly depends on the available technical solutions and the ability to adjust chromatographic conditions. For example, *Gao et al.* [16] performed chromatographic separation on the Zorbax-SB C18 column, but *Zhou et al.* [17] used Intersil HILIC to separate the same combination of four first-line anti-tuberculosis drugs. In contrast, *Wang et al.* [24] reported that ETB was weakly retained on C18 chromatographic columns, but the use of Acquity BEH HILIC column resulted in issues with RIF and its derivate peak shapes. The best results were achieved with CAPCELL PAK-ADME – a chemically modified RP column [24]. *Hee et al.* [15] and *Song et al.* [23] also both employed modified C18 chromatographic columns specifically designed to retain hydrophilic compounds such as acetylisoniazid and isoniazid.

Regarding sample preparation techniques, the majority of the authors had chosen single-step protein precipitation with organic solvents (e.g., acetonitrile, methanol) for plasma sample preparation due to the balance between easiness of sample handling and sufficient quality of plasma extracts that are compatible with LC-MS/MS system.

As the metabolite profiling is essential for the correct establishment of pharmacokinetic profile and has to be viewed in context with the parent drug, a single method is needed to quantify both the first-line anti-tuberculosis drugs and their primary metabolites. Only a few of the published methods incorporate primary metabolites (Table 1). Although the method reported by *Sundell et al.* [19] comprises the broadest spectra of analytes within a single LC-MS/MS method, it lacks such important primary metabolites as pyrazine-2-carboxylic acid (the active form of PZA) and hepatotoxic 5-hydroxypyrazine-2-carboxylic acid. On the other hand, as illustrated in Table 1, the quantification ranges used by various methods broadly cover the subtherapeutic concentration range of the drug substances but, in some cases, lack the middle and upper range of clinically expected plasma concentrations described in the literature [3]. Routine sample dilution to fit the calibration curves and a combination of multiple LC-MS/MS methods to obtain pharmacokinetic data for all compounds of interest is time-consuming, requires large sample volumes and complex technical solutions.

Therefore, this study aimed to develop and validate a single LC-MS/MS method for simultaneous determination of all four first-line anti-tuberculosis drugs and their six primary metabolites in human plasma. The relevance of the developed method was confirmed by clinical sample analysis of TB patients undergoing treatment with first-line anti-tuberculosis drugs.

2. Materials and methods

2.1. Chemicals and materials

5-hydroxypyrazine-2-carboxylic acid (5OHPZ2A; purity: 98%), acetylisoniazid (ACIZN; purity: 98%), and 25-desacetylrifampicin (25DRIF; purity: 94%) were supplied from Carbosynth (Berkshire, UK). Pyrazinamide (PZA; purity: 98%), ethambutol (ETB; purity: 98%), isoniazid (IZN; purity: 98%), isonicotinic acid (IZNAC; purity: 99%), pyrazine-2-carboxylic acid (PZ2A; purity: 99%), rifampicin (RIF; purity: 99%) were from Alfa Aesar (Kandel, Germany). 5-hydroxypyrazinamide (5OHPZA; purity: 97%) was purchased from Cymit (Barcelona, Spain), phenformin hydrochloride (IS; analytical standard, purity: 98%) was from Sigma-Aldrich (Schnelldorf, Germany). Formic acid (FA; MS grade) and dimetylsulfoxide (DMSO; HPLC grade) were from Sigma-Aldrich (Schnelldorf, Germany). Methanol (MeOH; HPLC grade) was purchased from Merck (Darmstadt, Germany). Water was purified by Milli-Q Plus water purification system (Millipore, Molsheim, France). Commercial human plasma (anticoagulant: ethylenediaminetetraacetic acid (EDTA), Innovative Research, Novi, MI, USA) and blank human plasma samples from national biobank (section 2.6.) were used for the development and validation of the assay.

2.2. UPLC-MS/MS instrumentation and conditions

An Acquity UPLC H-Class chromatographic system (Waters, Milford, MA, USA) coupled to a XEVO TQ-S tandem mass spectrometer (Waters, Milford, MA, USA) was used for analysis. The chromatographic separation was achieved on the Waters Acquity UPLC BEH C8 column (2.1 × 75 mm; 1.7 µm). The other columns tested were: Waters Acquity UPLC BEH HILIC (2.1 × 50 and 100 mm; 1.7 µm), Acquity UPLC BEH Amide (2.1 × 50 and 100 mm; 1.7 µm), and Acquity UPLC BEH C18 (2.1 × 50 mm; 1.7 µm). The analyte mixture consisting of ten compounds was separated using a gradient elution at a flow rate of 0.2 mL/min. The mobile phase consisted of 0.1% FA solution (A) and methanol (B). The gradient mode was as follows: 0–1 min, 99% A; 1–2.5 min, 99 → 2% A; 2.5–4.5 min, 2% A, then equilibration to the initial conditions for 5.5 min. The total run time was 10 min. The volume of injection was 1 µL. The autosampler and the column temperature was maintained at 5 °C and 30 °C, respectively. The calibration standards were run in triplicate but all samples in duplicate.

The mass spectrometer was operated in positive electrospray ionisation mode (ESI+) with the capillary voltage of 3.0 kV. The source temperature was set to 140 °C, the desolvation gas (N_2) temperature was kept at 600 °C at a flow rate of 1000 L/h, and the cone gas (N_2) flow rate was 200 L/h. The multiple reaction monitoring mode (MRM) was used for detection and quantification of the analytes. The precursor-to-product ion transitions and MRM parameters specified for each compound and IS are listed in Table 2. Data acquisition and analysis were performed using MassLynx software and TargetLynx module (version 4.1., Waters, Milford, MA, USA).

2.3. Preparation of stock solutions, calibration standards, and quality control samples

The stock solutions of the analytes were prepared in DMSO to a final concentration of 10 mg/mL (RIF, 25DRIF, IZN, ACIZN, IZNAC, and ETB) or 2 mg/mL (PZA, PZ2A, 5OHPZA, 5OHPZ2A). The internal standard was dissolved in the mobile phase (0.1% FA solution) to reach a final concentration of 1 mg/mL. The stock solutions were stored at −20 °C.

The highest calibration standard was prepared by spiking an appropriate volume of stock solutions of the analytes into blank plasma. The prepared calibration standard was serially diluted with blank plasma to obtain six more standards for the construction of the calibration curves. The calibration ranges for all analytes are given in Table 3. Quality control (QC) samples were prepared at the lower limit of quantification (LLOQ), low (LQC), medium (MQC), and high (HQC) concentrations.

2.4. Sample preparation

The following single-step protein precipitation with methanol was used to prepare calibration standards, QC samples, and clinical samples. An aliquot of plasma sample (50 µL) was mixed with 450 µL of freshly prepared internal standard solution in methanol (10 µg/mL) and centrifuged at 10000 rpm for 10 min at 5 °C. Then, 100 µL of the

Table 1
Overview of main characteristics of the published methods offering simultaneous analysis of the first-line anti-tuberculosis drugs with or without primary metabolites and comparison to the method developed in this study.

Reference	Analytes	Quantification range (μg/mL)	Sample volume (V, μL)	Sample preparation technique	LC column	Analysis time, min
[14]	PZA	0.10–30.0	200	LLE	Agilent	4.0
	PZ2A	0.03–9.00			Zorbax Eclipse XBD-C18	
	5OHPZ2A	0.002–0.60			(4.6 × 100 mm; 3.5 μm)	
[15]	RIF	0.025–50.0	20	SPE	Agilent	6.0
	25DRIF	0.0025–5.00			Zorbax SB-aq	
	IZN	0.005–10.0			(4.6 × 50 mm; 5 μm)	
	IZNAC	0.0125–5.00				
	ACIZN	0.0125–5.00				
[16]	PZA	0.20–4.00	100	PP	Agilent	8.5
	IZN	0.08–2.00			Zorbax SB-C18	
	ETB	0.0002–1.00			(2.1 × 100 mm; 3.5 μm)	
	STM	2.00–200				
	RIF	0.20–4.00				
[17]	PZA	0.004–4.00	100	PP	GL Sciences	<2.0
	IZN	0.004–4.00			Intersil HILIC	
	ETB	0.0005–0.50			(2.1 × 75 mm; 3 μm)	
	STM	0.01–16.0				
	RIF	0.004–4.00				
[18]	PZA	0.31–39.2	100	PP	GL Sciences	3.5
	IZN	0.077–9.80			Intersil HILIC	
	ETB	0.0015–1.96			(2.1 × 150 mm; 3 μm)	
	RIB	0.0015–1.96				
	RIF	0.0038–4.90				
[19]	PZA	0.32–40.0	200	LLE at neutral and acidic conditions	GL Sciences	4.0
	IZN	0.08–10.0			Intersil HILIC	
	ETB	0.04–5.00			(2.1 × 75 mm; 3 μm)	
	RIF	0.20–25.0				
	25DRIF	0.04–5.00				
	IZNAC	0.08–10.0				
	ACIZN	0.04–5.00				
	5OHPZA	0.06–7.50				
[20]* Group 1	PZA	2.00–100	100	PP	Waters	9.0
					Atlantis HILIC	
					(2.1 × 150 mm; 3 μm)	
[20]* Group 2	IZN	0.10–5.00	50	PP	Waters	13.0
	ETB	0.10–5.00			Atlantis dC18	
	RIF	0.20–10.0			(2.0 × 150 mm; 3 μm)	
[21]	PZA	1.00–100	500	PP	Phenomenex	8.0
	IZN	0.10–10.0			Gemnini C18	
	ETB	0.02–5.00			(4.6 × 150 mm; 4.6 μm)	
	RIF	0.20–20.0				
	ACIZN	0.10–10.0				
[22]	PZA	2.00–80.0	10	Ultrafiltration	Waters	2.5
	ETB	0.20–8.00			Atlantis T3 C18	
	IZN	0.20–8.00			(2.0 × 100 mm; 3 μm)	
[23]	PZA	5.00–80.0	50	Two-step PP	YMC Co.	3.0
	IZN	0.50–8.00			Hydrosphere C18	
	ETB	0.50–8.00			(2.0 × 50 mm; 3 μm)	
	RIF	5.00–80.0				
	25DRIF	N/D**				
	ACIZN	N/D				
[24]*	PZA	0.005–50.0	100	PP	Shiseido	7.0
	IZN	0.005–7.50			CAPCELL PAK-ADME	
	ETB	0.001–5.00			(2.1 × 50 mm; 3 μm)	
	RIF	0.005–7.50				
This study***	PZA	1.17–75.0	50	PP	Waters	10.0
	IZN	0.16–10.0			Acquity UPLC BEH C8	
	ETB	0.16–10.0			(2.1 × 75 mm; 1.7 μm)	
	RIF	0.47–30.0				
	25DRIF	0.47–30.0				
	IZNAC	0.16–10.0				
	ACIZN	0.16–10.0				
	5OHPZA	1.17–75.0				
	5OHPZ2A	2.34–75.0				
	PZ2A	1.17–75.0				

STM – streptomycin; RIB – rifabutin; IZN – isoniazid; IZNAC – isonicotinic acid; ACIZN – acetylisoniazid, RIF – rifampicin; 25DRIF – 25-desacetylrifampicin; PZA – pyrazinamide; PZ2A – pyrazine-2-carboxylic acid; 5OHPZA – 5-hydroxypyrazinamide; 5OHPZ2A – 5-hydroxypyrazine-2-carboxylic acid; ETB – ethambutol; LLE – liquid-liquid extraction; SPE – solid phase extraction; PP – protein precipitation; *quantification ranges are showed only for the first-line anti-tuberculosis drugs; **N/D – not determined; ***method developed in this study.

Table 2
Optimized mass spectrometric parameters used for detection of the analytes and IS in the MRM mode and corresponding retention times of the analytes and IS.

Compound	*Precursor ion (m/z)*	*Fragment ion (m/z)*	*Cone voltage (V)*	*Collision energy (eV)*	*Retention time* (min)
IZN	*138.3*	*121.0*	*40*	*10*	*1.09*
IZNAC	*124.3*	*79.9*	*30*	*16*	*1.20*
ACIZN	*180.3*	*121.1*	*60*	*18*	*1.32*
RIF	*821.4*	*789.3*	*30*	*14*	*4.57*
	823.4	*791.3*	*30*	*14*	*4.71*
25DRIF	*781.4*	*399.2*	*50*	*15*	*4.62*
PZA	*124.3*	*80.9*	*20*	*12*	*3.62*
PZ2A	*125.1*	*80.9*	*20*	*6*	*2.94*
5OHPZA	*140.1*	*122.9*	*30*	*8*	*2.06*
5OHPZ2A	*141.1*	*54.8*	*50*	*15*	*1.71*
ETB	*205.4*	*116.0*	*40*	*18*	*0.83*
IS	*206.2*	*104.9*	*30*	*20*	*4.10*

IZN – isoniazid; IZNAC – isonicotinic acid; ACIZN – acetylisoniazid, RIF – rifampicin; 25DRIF – 25-desacetylrifampicin; PZA – pyrazinamide; PZ2A – pyrazine-2-carboxylic acid; 5OHPZA – 5-hydroxypyrazinamide; 5OHPZ2A – 5-hydroxypyrazine-2-carboxylic acid; ETB – ethambutol; IS – phenformin (internal standard).

Table 3
Calibration range and LLOQ for the first-line anti-tuberculosis drugs and their primary metabolites.

Compound	*Calibration range (μg/mL)*	*LLOQ (μg/mL)*	r^2
IZN	*0.16–10.0*	*0.16*	*0.995*
IZNAC	*0.16–10.0*	*0.16*	*0.991*
ACIZN	*0.16–10.0*	*0.16*	*0.9992*
ETB	*0.16–10.0*	*0.16*	*0.9998*
RIF	*0.47–30.0*	*0.47*	*0.996*
25DRIF	*0.47–30.0*	*0.47*	*0.989*
PZA	*1.17–75.0*	*1.17*	*0.998*
PZ2A	*1.17–75.0*	*1.17*	*0.997*
5OHPZA	*1.17–75.0*	*1.17*	*0.993*
5OHPZ2A	*1.17–75.0*	*2.34*	*0.996*

supernatant was transferred to a glass vial with 900 μL of mobile phase A (0.1% FA solution), briefly vortexed, and used for analysis.

The IS solution in methanol (10 μg/mL) and the mobile phase A used for the sample dilution were stored at 5 °C before use.

2.5. Method validation

The validation procedure of the developed method was performed according to the guidelines of European Medicines Agency on bioanalytical method validation [25].

2.5.1. Selectivity

The selectivity of the method was evaluated by comparing the signal intensity in the blank sample with that in the sample containing IS and analytes at the LLOQ level, both prepared in duplicate from six individual sources of the matrix. The analyte signal intensity in the blank sample should be less than 20% of the analyte signal at the LLOQ level. The IS signal intensity in blank sample should not exceed 5% of that in analytical sample.

2.5.2. Calibration curve and lower limit of quantification

The calibration ranges for PZA, RIF, IZN, ETB, and corresponding metabolites (Table 3) were chosen, based on the therapeutic range of the particular drug [3]. Generally, seven nonzero calibration standards were prepared in blank plasma and analysed in triplicate in three separate analytical runs. The seven-point calibration curve for each analyte was constructed by plotting the peak area against the analyte concentration in the calibration standard and applying an appropriate weighting factor ($1/x$ or $1/x^2$). Since the IS was used for system stability monitoring during an analytical run, absolute calibration was applied to calculate the analyte concentration. The back-calculated concentrations of the calibration standards should be within 15% of the nominal concentration (at the LLOQ level – 20%).

The LLOQ was considered the lowest analyte concentration in the sample that can be measured with a certain degree of accuracy (within 20% of the nominal concentration) and precision (coefficient of variation, CV $\leq$ 20%). At the LLOQ level, the signal-to-noise ratio should be at least 5.

2.5.3. Accuracy and precision

The within-run accuracy and precision were demonstrated by analysis of five replicates of QC samples per concentration level at four concentration levels (LLOQ, low, medium, and high). Data from three analytical runs performed in three consecutive days were used to ascertain between-run accuracy and precision.

The accuracy was expressed as the difference between the measured and nominal concentrations of the QC samples. Assessing within- and between-run accuracy, the concentration of QC samples should be within 15% of the nominal concentration, except for the LLOQ level, where 20% is acceptable.

The precision was defined as the variance between replicate samples and expressed as the coefficient of variation (CV, %). Evaluating within- and between-run precision, the CV should not exceed 15% (at the LLOQ level CV $\leq$ 20%).

2.5.4. Matrix effect

The matrix effect was determined at two concentration levels (LLOQ and high) using matrix factor (MF). The MF was the ratio between the analyte peak area in the post-extraction spiked samples and that of reference sample prepared in 0.1% FA solution. The post-extraction spiked samples were prepared using blank plasma from six different individuals. The samples were prepared in duplicate. The CV of MF calculated from six different plasma sources should be within 15%.

2.5.5. Stability

The analyte stability in human plasma was investigated at different storage conditions by analysing QC samples at the LQC and HQC levels. For short-term stability, samples were stored on a benchtop at room temperature for 1 and 4 h before processed. The long-term stability was assessed after QC sample storage at −20 °C for 24 h, 7 days, and 3 months, respectively. The freeze-thaw stability was evaluated after subjecting QC samples to 3 freeze-thaw cycles from the freezer (−20 °C) to room temperature in a seven-day period. All QC samples were prepared in duplicate. For the autosampler stability, calibration standards were reanalysed after 24 h of storage in the autosampler (5 °C). The stability of analyte stock solutions was evaluated after stock storage at −20 °C for 2 months, while the stability of the IS stock solution was assessed after storage at −20 °C for 14 days. All analytes were quantified against the freshly prepared calibration standards. The stability was calculated from the reference sample concentration and expressed as a percentage of the nominal concentration. Stability should be within 15% of the nominal concentration.

2.5.6. Carry-over

Sample-to-sample carry-over was assessed by injecting the blank sample immediately after three consecutive injections of the highest calibration standard. Following the highest calibrator, the signal of the blank sample should be less than 20% of the analyte signal at LLOQ level and 5% of the IS signal.

2.5.7. Dilution integrity

The dilution integrity was demonstrated using samples containing analytes at a concentration two times the highest calibration standard. The samples were diluted before analysis with blank plasma in the ratio

1:5 and 1:10. In total, five replicates per dilution factor were prepared and analysed. The dilution of the study samples should not affect the accuracy and precision of the results; the accuracy and precision should be within 15%.

2.6. Collection of clinical samples

Blank human plasma samples (anticoagulant: EDTA) from healthy volunteers were received from the national biobank Genome Database of Latvian population [26] and used to validate the developed method. The clinical applicability of the reported method was assessed by analysing human plasma samples from newly diagnosed otherwise healthy pulmonary TB patients (n = 34) admitted to the Riga East University Hospital, Centre of Tuberculosis and Lung Diseases. All patients were undergoing WHO-recommended TB treatment regimen [2] and were receiving ETB, PZA, RIF, and IZN for ten days before sample collection. Blood samples were collected pre-dose (0 h) and 2 h, 6 h after drug administration into vacutainers with EDTA. The samples were then immediately centrifuged at 4000 rpm (3488*g*) for 15 min at 4 °C to separate the plasma. The separated plasma was collected and stored at −70 °C until analysis.

The study was approved by the Central Medical Ethics committee of Latvia (approval No: 01-29.1/1), the Ethics Committee of Riga East University Hospital (approval No: 24-A/15), and the Ethics Committee of Riga Stradins University (approval No: 6-3/1/6).

3. Results and discussion

3.1. Optimisation of UPLC-MS/MS conditions

Both HILIC and RP modes were tested for separation of the analytes. In the experiments conducted, the mobile phase organic constituent was acetonitrile, methanol, or isopropanol. Mobile phase additives (i.e., FA, ammonium acetate, ammonia hydroxide, acetic acid) were used to improve the peak shape and retention times for better separation of the analytes. The representative chromatograms are shown in (Supplementary Fig. 1).

HILIC separation mode was tried on Acquity UPLC BEH HILIC and Acquity UPLC BEH Amide columns. The analytes of interest were weakly retained on the BEH HILIC column and eluted within 3 min, even using 100 mm column. *Sundell et al.* [19] achieved optimal results under similar conditions, but, in our hands, observed peak overlapping due to early analyte elution was unacceptable. The use of the BEH Amide column allowed to achieve sufficient retention for all analytes. The prolongation of retention times for polar analytes on amide columns, in comparison to chemically unmodified HILIC stationary phases, was confirmed earlier and might be explained by differences in dominating type of interactions between the stationary phase and analytes [27]. However, RIF, 25DRIF, and ETB yielded poor peak shapes and attempts to improve that by addition of ammonium acetate (10–50 mM) to the mobile phase as suggested by the other authors [17,18] caused severe ionisation suppression of PZ2A and 5OHPZ2A. *Priyanka et al.* [14] experimentally proved that the ionisation of acidic PZA metabolites is either sensitive to mobile phase pH changes. In our hands, the analysis on the BEH Amide column was possible only in two mobile phase systems with opposite pH. Therefore, the optimisation of the chromatographic conditions on this column was discontinued.

Acquity UPLC BEH C18 and BEH C8 columns were applied in RP mode. The most polar analytes (ETB, IZN, and IZNAC) eluted in void volume on the BEH C18 column. Application of highly aqueous initial mobile phase composition (up to 99% water) did not result in sufficient retention of these analytes. The published data [15,23,24] suggested that the use of chemically modified C18 columns could facilitate chromatographic separation of the analytes. However, considerably better results were achieved on the unmodified BEH C8 column. The shorter alkyl chains are less hydrophobic and thus enhanced retention of polar constitutes at similar conditions. The further adjustment of chromatographic conditions (e.g., mobile phase composition, flow rate) was directed towards the separating overlapping peaks – IZN and metabolites, 5OHPZ2A and 5OHPZA. An increase in column temperature would again result in shortening of retention times and for this reason, was not considered. The mobile phase gradient (0.1% FA solution (A) and methanol (B); 99 → 2% A) at a flow rate of 0.2 mL/min was suitable for the separation of the analyte mixture (Fig. 1). At the given conditions, ionisation issues or peak splitting were not observed. The sensitivity of the developed method allowed us to reduce the injection volume to 1 μL. If the injection volume was increased, peaks tended to broaden, and column overload was expected. Although the analytes were separated in less than 5 min, the total run time was extended to 10 min for system equilibration.

ESI+ mass spectra was acquired for all analytes. The most abundant fragments were chosen from product ion spectra to set appropriate MRM transitions. Precursor-product ion pairs and optimised MRM parameters for ten analytes and IS are shown in Table 2.

Optimised chromatographic conditions prevented peak overlapping and enabled accurate quantification of the analytes. Due to the formation of rifampicin quinone, two RIF peaks were observed, as reported in the literature [28]. Thereby, two ion-transitions were used for accurate rifampicin quantification.

3.2. Sample preparation

Literature reports the application of liquid-liquid extraction (LLE), solid-phase extraction (SPE), and protein precipitation (PP) techniques for sample pre-treatment (Table 1). Preliminary experiments of LLE with ethyl acetate resulted in low recovery for PZ2A and 5OHPZ2A, and interferences with unseparated plasma components for the other analytes were observed. The introduction of polar acidic pyrazinamide metabolites in the analyte mixture required sample fractionation to achieve optimal recovery for acidic and basic compounds, as suggested by the other authors [14,19]. Similarly, the HybridSPE^{TM}-Phospholipid Ultra cartridge (Supelco) retained both polar analytes due possible Lewis acid-base interaction on the surface of the stationary phase under chosen conditions. Then, single-step PP with methanol and acetonitrile (with or without FA or ammonium hydroxide additive) were investigated. The PP with methanol without the use of any additives successfully reduced the excessive amount of endogenous matrix components in the final sample and provided consistent results, as shown in Section 3.3.4. The applied PP method was simple, fast, and cost-effective, allowing high-throughput analysis of study samples.

3.3. Method validation

3.3.1. Selectivity

In the MRM chromatograms shown in Fig. 2 representing the blank plasma sample and the first calibration standard containing IS and analytes at LLOQ level, it is seen that no potentially interfering signals at the expected retention time of the analytes and IS appear in the individual MRM channels of the blank plasma sample. If comparing the analytical signal of blank plasma samples to their equivalents with IS and analytes at LLOQ level, it was found that the signal intensity of 5OHPZ2A at LLOQ level is lower than expected, and the signal of blank matrix in this MRM channel comprised 25% of the analyte signal.

3.3.2. Calibration curve and lower limit of quantification

The seven-point calibration curves were obtained by plotting the peak area versus analyte concentration in the corresponding calibration standard. The results confirmed a linear analytical response in calibration ranges given in Table 2 with the correlation coefficients (r^2) in the range of 0.989–0.9998 when weighting factors 1/x or $1/x^2$ applied (Table 3). The back-calculated concentrations for the calibration standards were 86.3–108.7% (at the LLOQ level 83.7–106.1%)

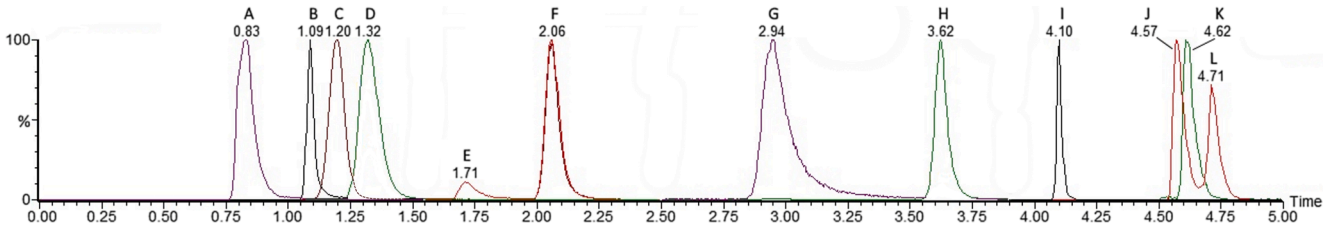

Fig. 1. Overlay plot of MRM chromatograms (seventh calibration standard, STD7). The chromatographic separation of the analyte mixture was achieved on BEH C8 column within 5 min. The total run time was extended to 10 min for system equilibration. A – ETB (0.83); B – IZN (1.09); C – IZNAC (1.20); D – ACIZN (1.32); E – 5OHPZ2A (1.71); F – 5OHPZA (2.06); G – PZ2A (2.94); H – PZA (3.62); I – IS (4.10); J, L – RIF (4.57, 4.71); K – 25DRIF (4.62).

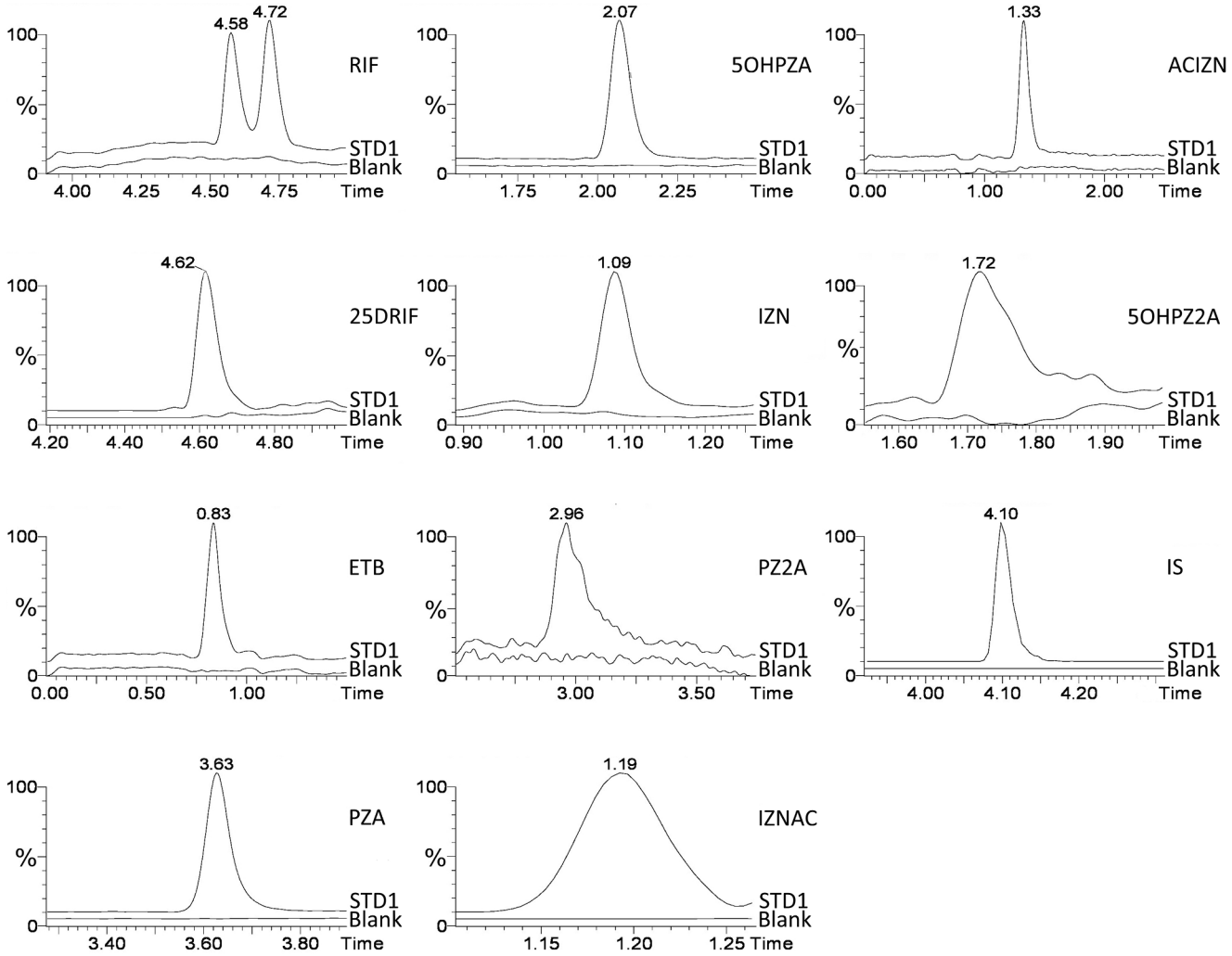

Fig. 2. Representative MRM chromatograms of the blank plasma sample (Blank) and the first calibration standard (STD1), containing analytes at LLOQ level and IS.

(Supplementary Table 1).

The LLOQ for all analytes was set at a concentration of the lowest calibration standard where the CV between replicate samples was in the range 0.3–12.9% but signal-to-noise ratio $\geq$ 5. Considering the low signal intensity of 5OHPZ2A at the LLOQ level, it was decided to set the second level calibration standard as LLOQ, where the signal-to-noise ratio was >5.

The chosen quantification ranges for all first-line anti-tuberculosis drugs correspond to the theoretically expected plasma concentrations [3] and are broader than those reported in the majority of previous studies (Table 1). The quantification ranges for metabolites were derived from the respective quantification range of the parent drug. As a consequence of expanding quantification ranges, the LLOQ values tend to be slightly higher. However, quantitation below the therapeutic window still is possible with a sufficient degree of accuracy and precision.

3.3.3. *Accuracy and precision*

The within- and between-run accuracy and precision were evaluated at four concentration levels (LLOQ, LQC, MQC, and HQC). The results are given in Table 4. The within-run accuracy in all concentration levels was between 87.2 and 113.6%. The CV between replicate samples (n = 5) at all concentration levels was 1.6–14.9%, except for LLOQ level, where 16.6% for IZNAC and 17.3% for 25DRIF complied with the

Table 4
The within- and between-run accuracy and precision for the first-line anti-tuberculosis drugs and their primary metabolites in human plasma.

Compound	QC level	Nominal concentration (μg/mL)	Within-run		Between-run	
			Accuracy (%)	Precision (CV, %)	Accuracy (%)	Precision (CV, %)
IZN	LLOQ	0.16	108.0	12.8	101.9	8.7
	LQC	0.40	98.7	11.5	97.0	7.4
	MQC	4.00	92.9	6.8	94.5	7.4
	HQC	8.00	99.0	9.2	95.5	9.6
IZNAC	LLOQ	0.16	95.2	16.6	97.2	12.1
	LQC	0.40	105.4	8.6	101.7	8.3
	MQC	4.00	94.9	8.4	97.7	6.8
	HQC	8.00	97.5	5.4	99.6	4.7
ACIZN	LLOQ	0.16	94.1	5.3	91.1	6.8
	LQC	0.40	93.7	4.0	97.4	5.2
	MQC	4.00	91.1	3.9	96.3	4.3
	HQC	8.00	90.2	3.7	92.5	5.3
ETB	LLOQ	0.16	109.3	4.8	99.3	7.6
	LQC	0.40	104.3	5.4	97.0	5.9
	MQC	4.00	97.1	5.8	04.0	5.2
	HQC	8.00	96.2	4.7	94.4	5.2
RIF	LLOQ	0.48	113.6	6.1	109.3	5.6
	LQC	1.20	104.7	3.2	103.5	5.4
	MQC	12.0	107.1	3.1	104.6	3.5
	HQC	24.0	101.4	3.3	100.8	4.7
25DRIF	LLOQ	0.48	100.7	10.0	98.7	13.3
	LQC	1.20	91.1	12.5	97.5	10.0
	MQC	12.0	92.5	6.0	98.0	6.0
	HQC	24.0	102.5	4.5	97.1	6.4
PZA	LLOQ	1.20	96.4	11.2	93.7	8.4
	LQC	3.00	99.0	9.3	100.2	6.6
	MQC	30.0	93.3	9.4	96.7	5.6
	HQC	60.0	89.5	9.2	91.7	6.4
PZ2A	LLOQ	1.20	95.5	9.5	93.8	11.0
	LQC	3.00	103.1	7.1	102.4	7.3
	MQC	30.0	96.9	3.8	98.3	3.8
	HQC	60.0	94.0	3.3	93.5	4.7
5OHPZA	LLOQ	1.20	99.1	7.9	93.4	7.9
	LQC	3.00	107.3	5.9	103.9	5.3
	MQC	30.0	97.2	4.4	97.8	3.8
	HQC	60.0	89.4	4.5	91.4	5.2
5OHPZ2A	LLOQ	1.20	88.0	14.9	92.2	11.5
	LQC	3.00	105.4	9.9	102.2	7.6
	MQC	30.0	102.9	5.8	99.3	4.4
	HQC	60.0	96.3	5.1	93.3	4.8

requirements. The between-run accuracy and precision were calculated as the average of three analytical runs performed on three consecutive days and ranged 91.1–109.3% and 3.5–13.3%, respectively.

3.3.4. Matrix effect

Matrix effect was assessed at LQC and HQC concentration levels using MF calculated for six different matrix sources. The mean MF for most of the analytes was in the range of 0.9–1.1, thus exhibiting a minimal contribution of the matrix to ion suppression or enhancement (Table 5). The MF < 0.5 for ETB and IZN indicated significant ion suppression by unseparated matrix components. The response of IZNAC at the LQC level varied depending on the matrix source; the CV between six different matrix sources was 21.8%. However, the results of linearity, accuracy, and precision confirmed that observed inconsistency did not affect the quantification of the IZNAC.

3.3.5. Stability

The obtained results of analyte stability in human plasma at various storage conditions are summarized in Table 6. Accordingly, all tested compounds were found to be stable in the human plasma samples at room temperature up to 1 h, but after 4 h, a significant reduction in 25DRIF, IZN, and IZNAC concentrations was observed. The samples were stable when stored at −20 °C for 24 h; storage time extension to 7 days resulted in the IZN and IZNAC concentration decrease by more than 15%. Moreover, the plasma sample exposure to three freeze-thaw cycles in 7 days contributed to the degradation of RIF and 5OHPZ2A. The long-

Table 5
The calculated matrix factors (MF) for the analytes and IS at the low and high QC levels.

Compound	QC level	MF (mean ± SD; n = 6)	CV (%)
IZN	LQC	0.4 ± 0.03	6.3
	HQC	1.3 ± 0.1	6.6
IZNAC	LQC	0.6 ± 0.1	21.8
	HQC	0.8 ± 0.1	11.9
ACIZN	LQC	1.1 ± 0.1	5.1
	HQC	1.1 ± 0.04	3.5
ETB	LQC	0.4 ± 0.02	3.8
	HQC	0.5 ± 0.02	3.9
RIF	LQC	1.1 ± 0.1	6.6
	HQC	0.8 ± 0.04	5.0
25DRIF	LQC	1.3 ± 0.2	14.2
	HQC	1.1 ± 0.2	17.5
PZA	LQC	1.0 ± 0.02	1.7
	HQC	1.0 ± 0.01	0.9
PZ2A	LQC	1.1 ± 0.1	6.0
	HQC	1.0 ± 0.03	2.5
5OHPZA	LQC	0.9 ± 0.1	9.2
	HQC	1.0 ± 0.1	8.2
5OHPZ2A	LQC	1.0 ± 0.04	3.7
	HQC	1.0 ± 0.02	2.2
IS	LQC	0.7 ± 0.1	7.3
	HQC	0.8 ± 0.05	6.6

Table 6
Stability of the analytes in human plasma at various storage conditions.

Compound	QC level	Stability (%)					
		Stored at RT* for:		Stored at −20 °C for:			Freeze-thaw (3 cycles, −20 °C, 7 days)
		1 h	4 h	24 h	7 days	3 months	
IZN	LQC	102.9	82.2	87.2	82.8	56.4	73.1
	HQC	89.9	93.0	100.3	84.0	64.7	93.1
IZNAC	LQC	93.1	78.1	94.3	91.0	95.4	89.1
	HQC	90.3	72.3	98.9	75.0	80.3	91.8
ACIZN	LQC	95.7	88.8	84.7	98.5	80.6	86.6
	HQC	95.2	95.0	98.6	94.3	91.3	101.7
RIF	LQC	95.4	89.9	90.9	97.0	94.4	75.8
	HQC	94.5	94.3	92.7	94.8	90.3	87.5
25DRIF	LQC	95.6	89.3	89.5	94.5	81.3	92.4
	HQC	89.4	76.7	93.6	86.3	77.7	94.8
PZA	LQC	97.8	94.2	86.9	90.8	112.1	90.3
	HQC	95.4	92.9	99.4	96.8	101.7	98.3
PZ2A	LQC	92.0	94.5	88.2	93.7	116.5	86.6
	HQC	91.5	91.6	98.3	93.0	103.6	97.3
5OHPZA	LQC	98.8	92.8	84.9	97.3	148.6	87.6
	HQC	94.5	90.9	96.4	92.5	138.5	93.7
5OHPZ2A	LQC	94.2	86.1	89.7	98.4	83.7	76.5
	HQC	93.5	90.6	99.3	92.9	80.6	87.0
ETB	LQC	98.1	97.3	88.5	105.0	103.9	94.3
	HQC	100.0	96.2	98.7	98.2	100.9	100.8

*RT – room temperature

Table 7
Concentration of the first-line anti-tuberculosis drugs and six primary metabolites in TB patient plasma (n = 34) pre-dose (0 h), 2 and 6 h after drug administration.

Compound	Measured concentration (μg/mL, mean ± SD; n = 34)			Therapeutic range 2 h post-dose [3] (μg/mL)
	Pre-dose (0 h)	2 h post-dose	6 h post-dose	
IZN	0.14 ± 0.11	2.79 ± 1.61	1.39 ± 0.88	3–6
IZNAC	0.08 ± 0.09	0.38 ± 0.20	0.39 ± 0.15	N/A*
ACIZN	0.23 ± 0.22	1.38 ± 1.05	1.58 ± 0.88	N/A
RIF	0.17 ± 0.42	2.31 ± 2.59	2.63 ± 1.70	8–24
25DRIF	0.00 ± 0.00	0.05 ± 0.13	0.07 ± 0.12	N/A
PZA	9.52 ± 7.37	38.4 ± 16.8	36.1 ± 12.3	20–60
PZ2A	4.36 ± 3.82	8.77 ± 4.89	11.4 ± 4.85	N/A
5OHPZA	1.07 ± 0.88	2.38 ± 1.09	3.22 ± 1.06	N/A
5OHPZ2A	3.09 ± 2.85	3.29 ± 2.45	5.40 ± 3.00	N/A
ETB	0.44 ± 0.28	2.68 ± 1.60	1.94 ± 0.86	2–6

N/A* – not applicable.

term stability test at −20 °C for 3 months caused substantial degradation of the analytes. Similar findings regarding the limited plasma stability of IZN, RIF and their metabolites were previously mentioned by the other authors [18,21,22]. Based on these results, it is recommended that plasma samples should be processed within 4 h after collection. If necessary, temporary storage at −20 °C up to 24 h is acceptable.

The IZNAC and 25DRIF were found to be unstable in the autosampler (stability 71.6–81.3%), as the stability of the other analytes ranged from 86.1 to 100.1% and complied with the requirements. The IS stock solution remained unchanged for 14 days at −20 °C, whereas the 5OHPZA stock solution was the only exhibiting a concentration reduction of more than 15% when stored at −20 °C for 2 months.

3.3.6. Carry-over

The signal of the blank plasma sample following the highest calibration standard ranged from 0.9 to 20.3%. The IS signal in the corresponding MRM channel was not detected at all. As a result, it was assumed that significant carry-over between samples did not occur.

3.3.7. Dilution integrity

Dilution integrity was demonstrated by analysing the samples containing analytes above HQC before 5- and 10-fold dilution. When performing analysis of processed samples, it was found that the CV between replicate samples (n = 6) was between 3.6 and 10.3%. In contrast, the accuracy for ETB and IZN was between 78.4 and 83.7%, but for the remaining analytes was within 15% and met the acceptance criteria. The obtained results suggest that study samples with analyte concentration above HQC can be diluted before sample preparation, apart from cases when ETB and IZN exceed the upper limit of quantification.

3.4. Clinical application

The validated method was applied to the human plasma samples from otherwise healthy pulmonary TB patients (n = 34) subjected to the TB treatment regimen with four first-line anti-tuberculosis drugs 10 days before sample collection. Concentrations of four drug substances and six metabolites were measured before drug administration (0 h), 2 and 6 h after receiving a single dose of anti-tuberculosis drugs. The mean concentrations are given in Table 7. Overall, the measured plasma concentrations were within the analytical range of the proposed method. It was possible to determine baseline concentrations before drug intake and estimate whether plasma concentrations confirm the therapeutic window reported in the literature [3]. The observed subtherapeutic plasma concentrations of the RIF (<8 μg/mL) and IZN (<3 μg/mL) 2 h after drug intake in the study population were consistent with the previous findings and can be explained with pharmacokinetic variability among TB patients [28–31]. The auto-induction capacity of RIF over the period of treatment tends to reduce its exposure, whereas IZN plasma concentration mostly depends on the N-acetyltransferase 2 (NAT2) enzymatic activity [10,32,33]. The full interpretation of the study results will be published in a separate paper.

To date, this is the first report on the LC-MS/MS method that allows determining the full primary metabolite profile of anti-tuberculosis drugs in human plasma within a single run. Therefore, the developed method can be employed in population-scale studies to enhance the knowledge of the pharmacokinetics of anti-tuberculosis drugs and for the therapeutic drug monitoring purposes. The offered method can be either employed in clinical practice, as the obtained pharmacokinetic data would justify the need for transition from standardized to individualized TB therapy.

4. Conclusions

The validation results confirmed the performance of the reported method, thus guaranteeing the quality of the results. The main advantages are the requirement of small sample volume for analysis, simple sample pre-treatment procedure, and the ability to analyse all four first-line anti-tuberculosis drugs and all six primary metabolites within a single run to enhance the utility of pharmacokinetic studies. Besides, the chosen quantification ranges fully cover clinically expected plasma concentrations, thereby reducing the need for sample dilution before the pre-treatment procedure. The clinical sample analysis gave an insight into the method's clinical applicability, where the aim was to determine TB drug exposure in the study population. The obtained results highlight the importance of future studies addressing issues related to the efficacy of TB treatment.

CRediT authorship contribution statement

Agnija Kivrane: Writing – original draft, Validation. **Solveiga Grinberga:** Methodology, Project administration, Supervision. **Eduards Sevostjanovs:** Methodology, Validation. **Viktorija Igumnova:** Conceptualization, Resources. **Ilva Pole:** Clinical sample collection. **Anda Viksna:** Clinical sample collection. **Dace Bandere:** Project administration, Supervision, Funding acquisition. **Alvils Krams:** Project administration. **Andra Cirule:** Project administration. **Osvalds Pugovics:** Project administration, Supervision. **Renate Ranka:** Project administration, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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