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ABSTRACT

Treatment of multidrug-resistant tuberculosis (MDR-TB) is challenging due to high treatment failure rate and adverse drug events. This study aimed to develop and validate a simple LC-MS/MS method for simultaneous measurement of five TB drugs in human plasma and to facilitate therapeutic drug monitoring (TDM) in MDR-TB treatment to increase efficacy and reduce toxicity. Moxifloxacin, levofloxacin, prothionamide, pyrazinamide and ethambutol were prepared in blank plasma from healthy volunteers and extracted using protein precipitation reagent containing trichloroacetic acid. Separation was achieved on an Atlantis T3 column with gradient of 0.1% formic acid in water and acetonitrile. Drug concentrations were determined by dynamic multiple reaction monitoring in positive ion mode on a LC-MS/MS system. The method was validated according to the United States' Food and Drug Administration (FDA) guideline for bioanalytical method validation. The calibration curves for moxifloxacin, levofloxacin, prothionamide, pyrazinamide and ethambutol were linear, with the correlation coefficient values above 0.993, over a range of 0.1–5, 0.4–40, 0.2–10, 2–100 and 0.2–10 mg/L, respectively. Validation showed the method to be accurate and precise with bias from 6.5% to 18.3% for lower limit of quantification and −5.8% to 14.6% for LOW, medium (MED) and HIGH drug levels, and with coefficient of variations within 11.4% for all levels. Regarding dilution integrity, the bias was within 7.2% and the coefficient of variation was within 14.9%. Matrix effect (95.7%–112.5%) and recovery (91.4%–109.7%) for all drugs could be well compensated by their isotope-labelled internal standards. A benchtop stability test showed that the degradation of prothionamide was over 15% after placement at room temperature for 72 h. Clinical samples (n = 224) from a cohort study were analyzed and all concentrations were within the analytical range. The signal of prothionamide was suppressed in samples with hemolysis which was solved by sample dilution. As the method is robust and sample preparation is simple, it can easily be implemented to facilitate TDM in programmatic MDR-TB treatment.

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1. Introduction

As one of the major infectious diseases, tuberculosis (TB) was responsible for 1.45 million deaths worldwide in 2018 [1]. Considered a global public health crisis by the United Nations [2], new multidrug-resistant or rifampicin-resistant tuberculosis (MDR/RR-TB) cases were estimated to be 500,000 in 2018 [1], with an annual increase of over 20% between 2009 and 2016 [3]. As a middle-income country with high TB burden, China harbors the second largest number of MDR/RR-TB cases globally [1]. The treatment success rate for MDR-TB, defined as TB with resistance to at least rifampicin and isoniazid, was far below the rate for drug-susceptible TB (56% vs 85%) [1]. Indeed, most of the recommended second-line drugs are believed to be less effective against TB and more toxic than first-line drugs [1,4–6].

In 2019, the World Health Organization (WHO) treatment guidelines for MDR/RR-TB were updated [4], and kanamycin and capreomycin were excluded from the list of recommended MDR-TB drugs due to their association with poorer outcomes and impairment of kidney function and hearing. Instead, drugs such as linezolid and bedaquiline are now recommended for all MDR-TB patients [4], although they are not readily used in most Chinese regions because of the high costs and out-of-pocket expenditures. According to national MDR-TB treatment guideline [7], the commonly used standardized drug regimen for MDR-TB is a 6-month intensive phase using pyrazinamide, kanamycin (amikacin, capreomycin), levofloxacin (moxifloxacin), cycloserine (PAS, ethambutol) and prothionamide, followed by an 18-month consolidation phase using pyrazinamide, levofloxacin (moxifloxacin), cycloserine (PAS, ethambutol) and prothionamide. An individual drug regimen should be designed when drug susceptibility testing results for second-line drugs are available. In Chinese MDR-TB designated hospitals, moxifloxacin was frequently used to replace levofloxacin due to comparable effectiveness and less phototoxicity [8,9]. Similar to linezolid and bedaquiline, cycloserine is frequently replaced by ethambutol due to the high costs, routinely paid for by patients themselves in China [10]. As for the new TB drugs, such as delamanid and pretomanid, they are still not available in most settings in China.

The unavailability of new TB drugs in China means there is an urgent need to optimize MDR-TB treatment based on the currently recommended drug regimens. Therapeutic drug monitoring (TDM) has been recommended to ensure adequate drug concentrations for efficacy and also to avoid toxicity, thus improving the MDR-TB treatment [4,11,12]. To guide TDM, the area under the drug concentration–time curve/minimum inhibitory concentration (AUC/MIC) is generally believed to be the best pharmacokinetic/pharmacodynamic (PK/PD) indices predicting efficacy for moxifloxacin, levofloxacin, pyrazinamide and ethambutol [11], although the indices have scarce clinical validation and are mainly defined by hollow fiber infection models [13–16]. As for prothionamide, there is no information on the best PK/PD index and clinical threshold. Based on the available literature on ethionamide [17], AUC/MIC is assumed to be the best index for prothionamide since these two drugs are closely linked. However, most laboratories still collect blood samples at 2 and 6 h after oral intake of the drugs and have not yet moved to AUC/MIC guided TDM [18].

Implementing TDM in programmatic MDR-TB treatment in China requires a multi-analyte assay that is fit for purpose, hence it may differ from multi-analyte assays in other settings. Several LC-MS/MS methods for second-line drugs have been reported [19–25], but only three of them were designed to measure three or more second-line drugs simultaneously. A research group from Korea developed an LC-MS/MS method for simultaneous quantification of nine second-line drugs in human plasma and a separate method for dried blood spots, but the sample preparation was complex due to a multi-step operation of acidification and neutralization [23,25]. In the assay by Kim et al., 20 TB drugs were divided into two groups based on their chemical properties, and were extracted and analyzed differently, causing an increased workload [24]. As ionized polar compounds, aminoglycosides cannot be clearly separated from other polar interference peaks on reversed phase columns [26], a repurposed commercial immunoassay might be a better option to implement their TDM [27] rather than developing a separate method on a hydrophilic interaction chromatographic (HILIC) column [28]. In this study, we aimed to develop and validate an LC-MS/MS method with simple sample preparation for simultaneous determination of moxifloxacin, levofloxacin, prothionamide, pyrazinamide and ethambutol, a method which can minimize the workload and turnaround time and can be easily implemented in other laboratories to facilitate the clinical practice of TDM in MDR-TB treatment in China.

2. Materials and methods

2.1. Chemicals and reagents

The reference standards of moxifloxacin, levofloxacin, prothionamide, pyrazinamide and ethambutol, as well as their internal standards of

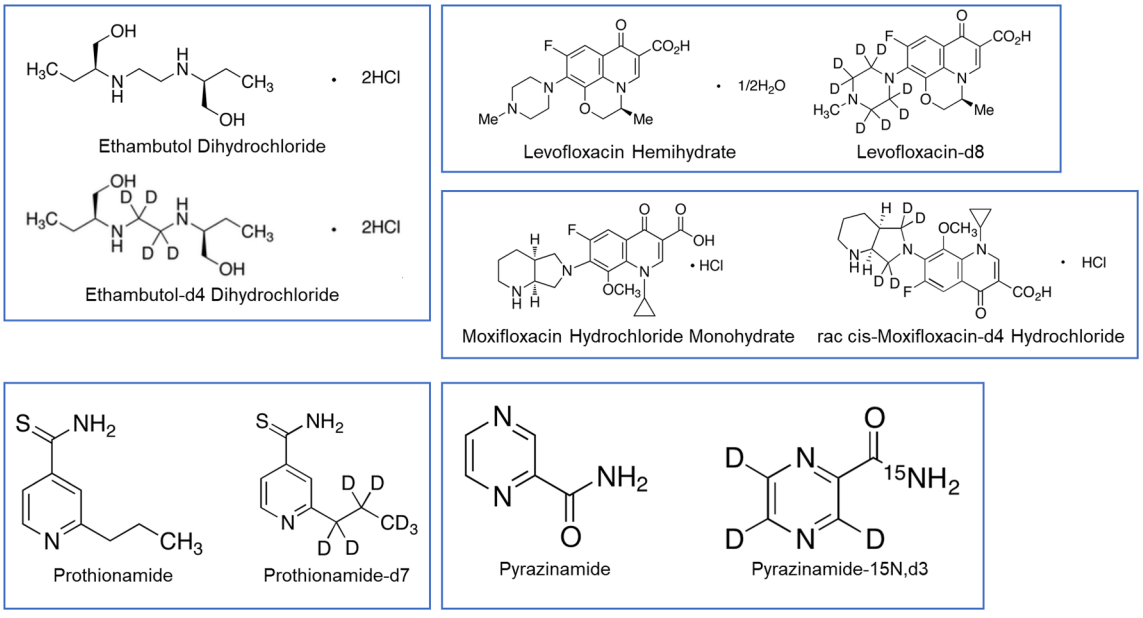

Fig. 1. Structures of the analytes under study and their corresponding internal standards.

moxifloxacin-d4, levofloxacin-d8, prothionamide-d7, pyrazinamide-^{15}N,d3 and ethambutol-d4, were purchased from the Toronto Research Chemicals Co. (North York, ON, Canada) (Fig. 1). Methanol, acetonitrile, trichloroacetic acid (TCA), and formic acid were HPLC or ACS grade and were obtained from Sigma Chemical Co. (St Louis, MO, USA). Ultrapure water was prepared by Millipore Milli Q water purification system (Merck KGaA, Darmstadt, Germany). Eighteen healthy volunteers who had not taken the aforementioned antibiotics during the previous 3 months were enrolled in China. Each had 10 mL of blank human plasma taken and stored at −80 °C.

2.2. Standard solutions

The stock solutions for moxifloxacin, levofloxacin, prothionamide, pyrazinamide and ethambutol were prepared at 1, 4, 2, 10, 2 mg/mL, respectively. Two batches of stock solutions were prepared separately for the subsequent preparation of calibration (CAL) standards and quality control (QC) samples. All internal standard stock solutions were prepared at 1 mg/mL. Moxifloxacin, moxifloxacin-d4, levofloxacin, levofloxacin-d8, prothionamide and prothionamide-d7 stock solutions were prepared in 50% methanol and 50% water. Pyrazinamide, ethambutol and their respective internal standards were dissolved in ultrapure water. All stock solutions were stored at −20 °C before use.

Concentrations of the CAL standards and QC samples in this study were decided on the basis of a previous review [29], and were listed in Table 1. CAL standards and QC samples were prepared by spiking stock solution of moxifloxacin, levofloxacin, prothionamide, pyrazinamide and ethambutol to the blank human plasma, of which the amount did not exceed 4% of the total volume. CAL standards at different concentrations were prepared separately rather than using serial dilution. All CAL standards and QC samples were made freshly, one day prior to the validation, since a previous study indicated that they remained stable at −80 °C for at least four weeks [24]. The samples were divided into small portions and were stored at −80 °C, except for those used for evaluation of benchtop stability (20 °C) and stabilities at 4 °C and −20 °C. Stock solutions of internal standards were mixed and diluted in water to produce working solution with final concentrations of 2.5, 20, 5, 50 and 5 mg/L for moxifloxacin-d4, levofloxacin-d8, prothionamide-d7, pyrazinamide-^{15}N, d3 and ethambutol-d4, respectively. The precipitation reagent used in this study was 20% TCA in methanol–acetonitrile (20:80, v/v).

2.3. Sample preparation

For sample preparation, 100 μL human plasma were mixed with 100 μL working solution of internal standards in Eppendorf polypropylene tubes. Subsequently, 400 μL precipitation reagent were added. After vortexing for 3 min, the tubes were centrifuged at 4 °C for 10 min at 11,000 × g in an Allegra 64R Centrifuge coupled with a 45° fixed-angle rotor (Beckman Coulter, Indianapolis, IN, USA). Then 200 μL aliquots of the supernatants were transferred into screw cap autosampler vials made of transparent glass, and loaded into the autosampler. Finally, 1 μL of the supernatant was injected into the LC-MS/MS system.

2.4. LC-MS/MS conditions

The assay was developed and validated on an Agilent 1260 Infinity LC system, consisting of a G1312B binary pump, a G1367E autosampler, a G1330B thermostat, and a G1316A thermostatted column compartment, coupled with an Agilent 6430 triple-quadrupole mass spectrometer (Agilent, Santa Clara, CA, USA). The temperature of the autosampler was set at 4 °C. The chromatographic separation was performed on an Atlantis T3 column (2.1 × 100 mm, 3 μm; Waters Co., Milford, MA, USA), and temperature was set at 30 °C. The mobile phase was a gradient of 0.1% formic acid in water (solvent A) and 100% acetonitrile (solvent B), with a constant flow rate of 0.3 mL/min (Table 2). The total running time was 10.50 min for each injection.

Mass spectrometric analysis was carried out in positive ion mode and dynamic multiple reaction monitoring (MRM) with a gas temperature of 350 °C, a gas flow of 9 L/min, a nebulizer pressure of 30 psi and a positive capillary voltage of 2,000 V. Mass transitions and relevant mass spectrometer conditions for all drugs and internal standards are summarized in Table 1. Integration of peak height and peak area, as well as data analysis were performed using Agilent MassHunter software version B.06.00 (Agilent, Santa Clara, CA, USA).

2.5. Validation of the assay

With reference to the 2018 United States' Food and Drug Administration (FDA) guideline for bioanalytical method validation [30], selectivity, specificity, accuracy, precision, linearity, matrix effect, recovery, dilution integrity, carryover and stabilities under different conditions were validated. The blank plasma from six different healthy volunteers were individually used to evaluate the effect of endogenous substances by preparing blank plasma samples with and without adding internal standards. The absence of interfering substances was confirmed where the responses were < 20% of the lower limit of quantification (LLOQ) for each drug and < 5% for their internal standards. Carryover was evaluated by sequential injection of pretreated LLOQ-QC, upper limit of quantification (ULOQ)-QC samples and blank plasma, and the values should be within 20% of LLOQ. Accuracy and precision (within- and between-run) were measured by using 15 replicated QC samples for each concentration level, i.e. LLOQ, LOW, medium (MED) and HIGH levels, on three consecutive days (five replicated QC samples at each concentration level per day), and were calculated by the calibration curve generated by freshly prepared CAL

Table 1
The mass spectrometer conditions and concentrations of calibration and quality control samples.

Analyte	Mass transition (*m/z*)		Fragmentor (V)	CE (eV)	CAV (V)	Calibration concentrations (mg/L)	QC sample concentrations (mg/L)				
	Parent	Product					LLOQ	LOW	MED	HIGH	> ULOQ
Ethambutol	205.2	116.1	90	10	1	0.2, 0.4, 1, 2, 4, 6, 8, 10	0.2	0.7	5	8	25
Ethambutol-d4	209.2	120.1	90	10	1						
Pyrazinamide	124.1	81.1	100	16	2	2, 4, 10, 20, 40, 60, 80, 100	2	7	50	80	200
Pyrazinamide-15 N,d3	128.1	84.1	110	16	2						
Prothionamide	181.0	154.0	120	18	8	0.2, 0.4, 1, 2, 4, 6, 8, 10	0.2	0.7	5	8	25
Prothionamide-d7	188.1	161.1	130	20	8						
Levofloxacin	362.2	318.2	120	16	8	0.4, 1, 2, 5, 10, 20, 40	0.4	0.7	20	32	100
Levofloxacin-d8	370.1	326.2	130	16	8						
Moxifloxacin	402.2	384.2	140	20	4	0.1, 0.2, 0.5, 1, 2, 4, 5	0.1	0.2	2.5	4	12.5
Moxifloxacin-d4	406.2	388.2	140	20	5						

CE: collision energy; CAV: cell accelerator voltage; QC: quality control; LLOQ: lower limit of quantification; MED: medium; > ULOQ: above the upper limit of quantification (five-time dilution by pooled blank plasma before sample preparation).

Table 2
Chromatographic conditions (gradient).

Time (min)	A (%)[a]	B (%)[b]	Flow (ml/min)
0.00	100	0	0.3
0.01	95	5	0.3
1.50	95	5	0.3
5.50	60	40	0.3
8.50	60	40	0.3
8.51	100	0	0.3
10.50	100	0	0.3

[a] Mobile phase A: 0.1% formic acid in water.
[b] Mobile phase B: 100% acetonitrile.

standards each day. The linearity was assessed based on the 7-point, $1/x^2$ weighted calibration curves for moxifloxacin and levofloxacin, and the 8-point, 1/x weighted calibration curves for prothionamide, pyrazinamide and ethambutol. The weighting function was used to improve the performance of calibration curves at LLOQ and LOW levels, especially when a wide calibration range was used [31]. Inter-assay variability of calibration curves was evaluated on four consecutive days, consisting of three days on accuracy and precision tests and one day on stability tests. To evaluate accuracy and precision, bias to nominal concentration and coefficient of variation (CV) were calculated per run and the values should be within 20% for LLOQ and 15% for the other QC levels.

To evaluate recovery and matrix effect, three sets of solutions (A, B and C) were prepared in quintuplicate at LOW, MED and HIGH levels, and were measured in a single run. In this study, sets A, B and C represented the extract of the spiked matrix, the spiked extract of the blank matrix and the spiked extraction solution, respectively. The calculation of recovery was made by dividing the peak area of A by the peak area of B, while matrix effect was calculated by dividing the peak area of B by the peak area of C. An additional test on matrix effect and recovery was performed in accordance to the European Medicines Agency guideline on bioanalytical method validation [32]. In brief, working solution of internal standards was added for compensation and all calculations were performed using the response ratio of analyte and internal standard. Procedures to determine dilution integrity were similar to accuracy and precision tests. In brief, three portions of QC samples, prepared at levels above the ULOQ, were diluted five times by

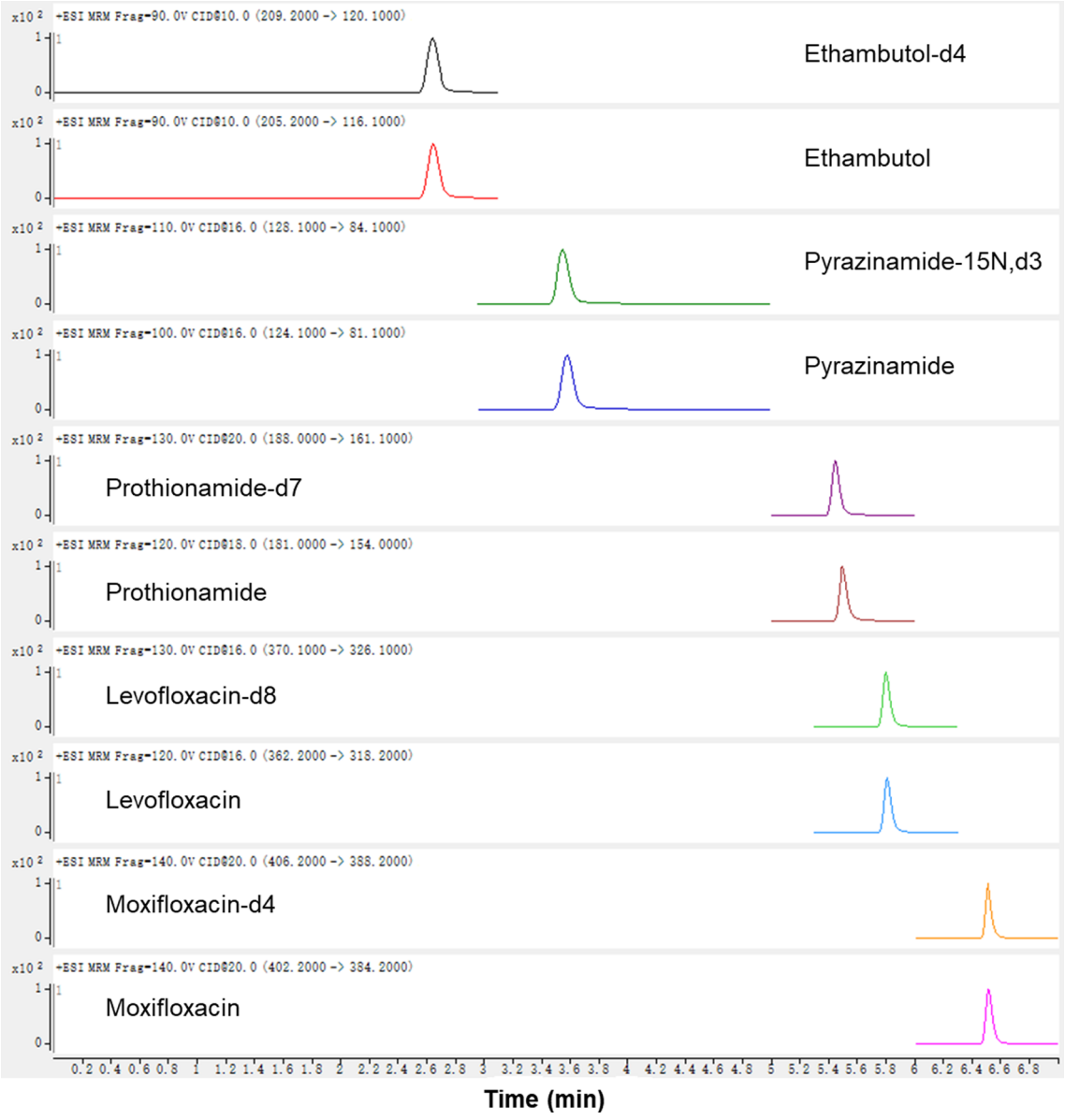

Fig. 2. Representative LC-MS/MS dynamic multiple reaction monitoring chromatograms for each drug compound and internal standard.

pooled blank plasma and measured in quintuplicate on three consecutive days. The bias and CV were not allowed to exceed 15%.

The stability tests, including autosampler stability, freeze and thaw stability, short-term and one-month storage stability, were performed for each drug using QC samples at LOW and HIGH levels. The autosampler stability was tested by reinjecting pretreated QC samples after placing them in the autosampler for 24 and 48 h. Stability was also tested after three cycles of freeze and thaw, storage in ambient (20 °C) for 6 and 72 h, in 4 °C and −20 °C for 72 h, as well as in −20 °C and −80 °C for 1 month. All stability tests were done in five replicates per concentration and a deviation above 15% of the nominal value was considered as unstable.

2.6. Measurement of clinical samples

A registered cohort study (ClinicalTrials.gov, NCT02816931) was conducted in Xiamen, China to enroll the MDR-TB patients and the study protocol has been previously published [33]. In brief, MDR-TB patients were enrolled before initiation of second-line anti-TB treatment. Patients took moxifloxacin and levofloxacin once daily at doses of 0.4 and 0.5 g. Prothionamide was given three times a day at doses of 0.2 g for patient weight < 50 kg and 0.25 g for patient weight ≥ 50 kg. The drug dose for pyrazinamide was 0.4 g three times a day. Ethambutol was given once daily at doses of 0.75 g for patient weight < 50 kg and 1.0 g for patient weight ≥ 50 kg. After two weeks in-patient treatment, venous blood samples were collected from patients via a venous catheter at pre-dose, and at 1, 2, 4, 6, 8 and 10 h after witnessed intake of second-line drugs. Blood samples were immediately centrifuged at 1,600 × g for 10 min and the upper layer of plasma was stored at −80 °C within an hour of sample collection. The method validated in this study was used to measure the drug concentrations in the collected clinical samples.

3. Results

3.1. Method development

Optimized MS conditions for each compound are listed in Table 1. TCA was added to the precipitation reagent as an ion-pairing reagent to achieve a satisfactory retention time for ethambutol. After adding a flushing step into the gradient from 5.50 to 8.50 min (Table 2), the carryover for moxifloxacin, levofloxacin, prothionamide, pyrazinamide and ethambutol was reduced to insignificant levels. Representative LC-MS/MS chromatograms for each compound are shown in Fig. 2. The mean retention times for moxifloxacin, levofloxacin, prothionamide, pyrazinamide and ethambutol were 6.50, 5.80, 5.50, 3.55 and 2.65 min, respectively.

3.2. Method validation

3.2.1. Selectivity and carryover

The blank plasma collected from 18 healthy volunteers were individually tested for endogenous substances and no interfering peaks were observed at the retention time of study drugs and internal standards. Results of carryover test showed that blank injections following ULOQ level had peaks well below LLOQ level for all study drugs (15.6%, 9.6%, 1.2%, 0.0% and 1.0% for moxifloxacin, levofloxacin, prothionamide, pyrazinamide and ethambutol, respectively). The bias for successive injections of LLOQ level after ULOQ level ranged from −2.4% to 3.8%.

3.2.2. Linearity, accuracy and precision

The calibration curves for moxifloxacin, levofloxacin, prothionamide, pyrazinamide and ethambutol were linear over a range of 0.1–5, 0.4–40, 0.2–10, 2–100 and 0.2–10 mg/L respectively, and all the coefficients of determination (R^2) were above 0.993. Inter-assay variability of calibration curves is shown in Table 3. The bias calculated

Table 3
Calibration curve, accuracy, precision and dilution integrity (n = 5).

Compound	Inter-assay variability of calibration curves (n = 4)				Nominal conc. (mg/L) [a]	Accuracy (% bias)	Precision (% CV)	
	Slope (SD)	Intercept (SD)	R^2 (SD)	Weight			Within-run	Between-run
Ethambutol	0.229 (0.007)	−0.014 (0.001)	0.998 (0.001)	1/x	0.2	18.3	1.9	2.1
					0.7	2.5	1.7	2.2
					5	−2.9	3.1	4.1
					8	−5.8	3.4	4.6
					25	−3.2	5.6	12.0
Pyrazinamide	0.032 (0.001)	−0.017 (0.003)	0.997 (0.002)	1/x	2	17.0	1.8	3.8
					7	8.0	2.2	1.3
					50	6.5	3.3	3.9
					80	2.4	2.9	3.1
					200	5.3	5.5	13.7
Prothionamide	0.343 (0.031)	0.017 (0.006)	0.998 (0.001)	1/x	0.2	9.1	2.2	3.0
					0.7	9.6	2.3	11.4
					5	6.6	2.4	3.4
					8	3.9	3.3	6.3
					25	−2.9	4.3	9.2
Levofloxacin	0.062 (0.006)	−0.007 (0.001)	0.996 (0.002)	$1/x^2$	0.4	6.5	1.9	7.4
					0.7	−2.0	3.7	6.8
					20	4.7	2.8	4.9
					32	0.0	3.8	5.8
					100	3.2	5.4	14.9
Moxifloxacin	0.747 (0.046)	−0.019 (0.003)	0.993 (0.002)	$1/x^2$	0.1	13.3	2.4	3.7
					0.2	4.7	4.1	3.4
					2.5	14.6	3.1	4.2
					4	7.2	4.2	10.3
					12.5	7.2	4.9	9.3

R^2: coefficient of determination; SD: standard deviation; CV: coefficient of variation.

[a] Nominal concentration from top to bottom: the presented lower limit of quantification, low, medium, high and above the upper limit of quantification levels for each drug.

in comparison with the nominal concentration for the five drugs ranged from 6.5% to 18.3% at LLOQ level and −5.8% to 14.6% at LOW, MED and HIGH levels. Within-run precision for the study drugs ranged from 1.8% to 2.4% at LLOQ level and 1.7% to 4.2% at LOW, MED and HIGH levels, while between-run precision ranged from 2.1% to 7.4% at LLOQ level and 1.3% to 11.4% at the other three levels. All the results met the acceptance criteria stated in the FDA guideline, i.e. within 20% for LLOQ level and 15% for LOW, MED and HIGH levels. For levels above the ULOQ, the bias in reference to the nominal concentration, the within- and between-run precision ranged from −3.2% to 7.2%,4.3% to 5.6% and 9.2% to 14.9%, respectively.

3.2.3. Matrix effect and recovery

Matrix effect and recovery of the five drugs were determined in pooled blank plasma. As shown in Table 4, significant ion enhancement exceeding 15% was observed for moxifloxacin (116.6% to 124.9%) and the recovery for ethambutol at LOW level was 120.7%. The use of internal standards perfectly compensated the ion enhancement and recovery, whereas the compensated matrix effect and recovery ranged from 95.7% to 112.5%, and 91.4% to 109.7%, respectively.

3.2.4. Stability

Stabilities of moxifloxacin, levofloxacin, prothionamide, pyrazinamide and ethambutol under different test conditions were determined using 2 concentrations of QC samples in 5 replicates. As shown in Table 5, autosampler stability was assessed to be at least 48 h for all drugs after sample preparation. Freeze and thaw stability was assessed to be acceptable for all drugs for at least 3 cycles. The stabilities of moxifloxacin, levofloxacin, pyrazinamide and ethambutol were assessed to be acceptable for at least 72 h under room temperature (20 °C), 4 °C and −20 °C. As for prothionamide, it was found to be stable for at least 6 h at room temperature but decreased more than 15% after placement at room temperature for 72 h, both at LOW and HIGH levels. The stabilities for all drugs were acceptable for at least one month when stored at −20 °C and −80 °C.

3.3. Clinical measurement

In total, 32 MDR-TB patients were eligible for inclusion in the pilot study and 224 blood samples were collected after two weeks of MDR-TB treatment. The median age was 32.5 (interquartile range, 25.3–43.8) years and 43.8% were male. The median weight was 51.0 (interquartile range, 46.0–56.3) kg and 34.4% of patients were below 50 kg. Chest X-ray results showed that patients with unilateral infiltration, bilateral infiltration, single cavity and multiple cavities accounted for 31.3%, 65.6%, 28.1% and 46.9%, respectively. Comorbidities were generally uncommon, but two patients had diabetes mellitus type 2 and one patient had concurrent pneumoconiosis. Overall, moxifloxacin, levofloxacin, prothionamide, pyrazinamide and ethambutol were measured in 168, 49, 203, 210 and 126 clinical samples, respectively. The concentrations for moxifloxacin, levofloxacin, prothionamide, pyrazinamide and ethambutol ranged between 0.12 and 3.85, 0.43–6.43, 0.21–3.89, 2.31–50.80 and 0.22–2.86 mg/L, respectively. All concentrations were within the defined analytical range, indicating fitness for clinical practice.

4. Discussion

In this study, we developed and validated a simple LC-MS/MS method for simultaneous determination of moxifloxacin, levofloxacin, prothionamide, pyrazinamide and ethambutol in human plasma using their corresponding isotope-labelled internal standards. TCA was added into the precipitation reagent as an ion-pairing reagent to achieve retention time for high-polar analytes. The LC-MS/MS method was validated and results showed good selectivity, specificity, accuracy and precision. Simple sample preparation and instrumentation setting enables the method to be used for drug monitoring in laboratories equipped with mass spectrometry with limited sensitivities, facilitating the application and generalization of TDM in Chinese programmatic MDR-TB treatment.

Ion suppression, a phenomenon generally occurring among compounds with high polarity when a reversed phase column is used [34,35], was observed for ethambutol. As previous studies have indicated, the use of an HILIC column to replace the reversed phase columns might be a solution to this problem [28,36]. However, it was difficult to achieve good peak shapes for all study compounds using a HILIC column in our preliminary tests. To control ion suppression, in the subsequent test we added TCA to the precipitation reagent to achieve a satisfactory retention time for ethambutol. As a commonly used protein precipitant [37], TCA also acted as an ion-pairing reagent [26]. However, adding TCA directly to the mobile phase has been found to be a contamination risk of the ion source, causing the suppression of signal [38,39]. Therefore, we chose to avoid these problems by adding TCA to the sample during the sample preparation. The amount of TCA was optimized to ensure that all ions were fully paired without causing signal suppression.

Carryover for moxifloxacin has also been reported in previous studies, with some failing to solve this problem [19,40]. In our study, after repeating the gradient several times in the same run, the peaks for moxifloxacin and levofloxacin could be observed until the fourth

Table 4
Matrix effect and recovery (n = 5).[a]

Compound	Nominal conc. (mg/L)[b]	Guidelines[c]	LOW concentration		MEDIUM concentration		HIGH concentration	
			ME (CV)	RE (CV)	ME (CV)	RE (CV)	ME (CV)	RE (CV)
Ethambutol	0.7, 5, 8	Absolute	100.5 (1.6)	120.7 (2.5)	104.5 (2.9)	102.0 (6.0)	101.5 (2.6)	95.7 (3.9)
		Compensated	96.7 (0.8)	109.7 (2.3)	100.1 (0.3)	103.8 (2.8)	95.7 (0.7)	100.9 (3.4)
Pyrazinamide	7, 50, 80	Absolute	93.7 (2.2)	100.3 (2.4)	99.7 (2.7)	88.1 (4.2)	100.2 (3.7)	88.9 (5.0)
		Compensated	98.2 (1.5)	101.3 (1.4)	101.9 (1.2)	98.3 (4.2)	99.0 (1.2)	102.3 (4.3)
Prothionamide	0.7, 5, 8	Absolute	89.6 (2.8)	93.3 (3.9)	100.3 (3.5)	90.2 (2.5)	99.1 (0.6)	90.7 (3.2)
		Compensated	105.5 (1.7)	100.9 (3.0)	112.5 (0.5)	102.2 (1.6)	109.5 (0.8)	91.4 (2.6)
Levofloxacin	0.7, 20, 32	Absolute	101.1 (3.8)	98.4 (3.0)	106.9 (3.3)	91.0 (2.7)	105.3 (1.5)	98.2 (3.4)
		Compensated	100.6 (1.9)	101.5 (2.5)	101.3 (0.6)	108.8 (3.8)	100.2 (1.2)	106.9 (3.6)
Moxifloxacin	0.2, 2.5, 4	Absolute	116.6 (2.5)	105.3 (4.8)	124.9 (5.3)	93.2 (6.8)	119.3 (3.4)	96.5 (4.0)
		Compensated	102.7 (2.0)	108.6 (3.5)	102.9 (1.0)	104.5 (2.2)	97.4 (0.8)	102.5 (3.2)

ME: matrix effect; RE: recovery; CV: coefficient of variation.

[a] All data are presented in percentage

[b] Nominal concentrations for each compound are shown in the order of LOW, MEDIUM and HIGH levels.

[c] "Absolute" indicated the results were obtained according to the United States' Food and Drug Administration 2018 guideline for bioanalytical method validation while "compensated" indicated the results were obtained according to the European Medicines Agency guideline on bioanalytical method validation 2012.

Table 5
Stability results under different conditions (n = 5).

Stability	Condition	QC level	Ethambutol		Pyrazinamide		Prothionamide		Levofloxacin		Moxifloxacin	
			CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)	CV (%)	Bias (%)
Autosampler stability (4 °C)	After 24 h in autosampler	LOW	2.4	2.7	2.5	7.1	1.8	8.7	2.1	−3.6	2.8	7.7
		HIGH	4.1	−2.6	4.6	4.4	3.7	−2.8	4.5	4.6	4.5	13.1
	After 48 h in autosampler	LOW	3.2	3.1	2.5	9.6	2.7	1.6	2.8	−2.2	2.6	3.7
		HIGH	4.2	−1.8	5.1	5.1	3.0	−5.7	4.1	7.1	5.0	12.3
Freeze and thaw stability	After 1st cycle at −80 °C	LOW	1.5	1.7	1.1	6.7	1.0	4.0	0.9	−3.6	2.8	2.1
		HIGH	0.9	−4.8	0.9	3.3	0.9	−3.4	0.8	3.5	0.7	9.8
	After 2nd cycle at −80 °C	LOW	1.3	1.8	1.6	8.4	1.8	5.4	1.7	−3.6	1.6	2.2
		HIGH	0.9	−4.3	1.0	4.0	0.6	−3.8	1.0	3.6	1.8	7.9
	After 3rd cycle at −80 °C	LOW	0.6	1.0	0.8	6.5	0.5	4.3	1.7	−4.2	2.4	1.8
		HIGH	2.1	−3.7	1.7	4.9	1.3	−2.8	1.0	2.3	2.8	7.8
Benchtop stability	After 6 h at ambient (20 °C)	LOW	3.1	7.7	3.7	13.3	3.9	7.2	3.9	1.2	4.2	8.1
		HIGH	2.4	2.7	2.7	11.9	2.1	3.3	2.6	10.3	1.6	14.9
	After 72 h at ambient (20 °C)	LOW	2.8	−1.4	2.2	5.8	3.5	–23.9	3.3	−8.1	4.2	−4.3
		HIGH	1.7	−7.2	1.3	2.5	0.8	−17.2	1.3	−4.0	1.5	0.7
Short term stability	After 72 h at 4 °C	LOW	0.8	1.0	0.7	8.8	1.9	0.3	1.9	−7.2	2.4	−3.1
		HIGH	4.8	−3.9	4.6	7.3	4.4	−4.4	4.7	−1.6	4.7	4.3
	After 72 h at −20 °C	LOW	2.2	0.2	1.6	6.9	2.6	5.7	2.5	−7.5	2.8	−2.6
		HIGH	3.4	−5.5	4.0	5.2	1.7	−4.7	3.8	−4.9	3.0	1.8
One-month stability	After 1 month at −20 °C	LOW	4.4	1.9	3.0	8.4	2.8	2.2	4.8	−2.9	5.6	3.7
		HIGH	1.5	−4.3	1.7	5.3	0.9	−2.8	1.5	2.8	1.3	7.4
	After 1 month at −80 °C	LOW	3.6	1.7	2.4	9.5	2.8	4.0	3.1	−4.3	2.7	1.0
		HIGH	0.6	−3.1	1.2	5.8	0.9	−1.2	0.5	3.8	0.4	8.3

QC: quality control; CV: coefficient of variation.

gradient cycle. It indicated a typical memory effect influencing the column, hence an additional flushing step in the gradient was necessary [41]. With an optimized gradient, carryover for moxifloxacin and levofloxacin could be reduced to insignificant levels at 0.1 and 0.4 mg/L, respectively. Notably, after an injection of ULOQ level, the bias of following successive injections of LLOQ level was observed to be marginal.

Several issues were encountered when measuring prothionamide in clinical samples. Firstly, matrix effect on prothionamide varied between subjects but its internal standard corrected well for that; secondly, the signal of prothionamide was suppressed in the samples with severe hemolysis, where dilution was found to be an effective solution [42]; thirdly, the benchtop stability test showed that the degradation of prothionamide in plasma was over 15% both at LOW and HIGH levels after placement at room temperature for 72 h, indicating the need of prompt storage at −80 °C. Significant degradation of prothionamide in room temperature has also been reported in a previous study [24]. However, our benchtop stability test showed that prothionamide was stable at room temperature for at least 6 h, which should provide enough time for sample preparation in a single analytical run.

The benefits of using TDM for second-line TB drugs have been previously reported. In the Netherlands, TDM was performed to adjust doses for several drugs to reduce toxicity during MDR-TB treatment and contributed to the high success rate of the treatment [43,44]. A retrospective study in China found that a lower concentration of pyrazinamide was associated with longer time to culture conversion [45]. Another study in Virginia retrospectively measured the drug concentrations of second-line drugs in MDR-TB patients and observed common individual pharmacokinetic variabilities [46]. Thus, quantification of moxifloxacin, levofloxacin, prothionamide, pyrazinamide and ethambutol in a single assay with simple sample preparation and low requirement for mass spectrometry capabilities, is rational and practical for TDM implementation in China. The use of this multi-analyte assay will help us better understand the variability in drug exposure of second-line drug concentrations in Chinese MDR-TB patients in relation to treatment response and adverse drug effects.

This study has several limitations. Firstly, we didn't include all second-line drugs available in Chinese programmatic MDR-TB treatment into the assay but only those frequently used. Secondly, the aminoglycoside class of antibiotics and capreomycin were not included due to difficulties in clear separation from other polar interference peaks on reversed phase columns due to their high polarity. Considering the accessibility of commercial kits for aminoglycoside [27] and the exclusion of capreomycin from the recommended MDR-TB drug list [4], the need to develop a separate assay for these drugs is less urgent. Thirdly, cycloserine was not included due to its high cost, routinely paid for by patients themselves in China. Ethambutol was included in lieu of cycloserine, as it is commonly used in MDR-TB regimens.

5. Conclusion

In conclusion, we developed and validated a simple LC-MS/MS method for simultaneous measurement of moxifloxacin, levofloxacin, prothionamide, pyrazinamide and ethambutol in human plasma using their isotope-labelled internal standards. This method can be applicable for drug monitoring in routine clinical practice in China, and for future clinical studies to explore the added value of TDM.

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CRediT authorship contribution statement

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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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2020.122397.

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