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Simultaneous Determination of Pyrazinamide, Rifampicin, Ethambutol, Isoniazid and Acetyl Isoniazid in Human Plasma by LC-MS/MS Method

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ABSTRACT

Monitoring of anti-tuberculosis drug concentrations in human plasma is very important in the treatment of TB disease. In this study, we described a method that can rapidly and simultaneously measure the plasma concentrations of four anti-tuberculosis drugs (isoniazid, rifampicin, pyrazinamide, and ethambutol) and one major metabolite (acetylisoniazid) using high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS). To adjust for degradation and losses during sample preparation, Diltiazem hydrochloride was used as an internal standard. Samples were prepared by using protein precipitation with methanol. Four drugs and one metabolite were simultaneously separated by using the high-performance liquid chromatography system with Gemini C18 column and a mobile phase consisting of MeOH and Ammonium acetate 5mM, pH 3.5. The method was found to have high selectivity. Precision estimated by the coefficient of variation was <15% for all drugs. The linear range of the calibration curve for PZA 1.0–100 µg/mL; RIF 0.2–20 µg/mL; INH 0.1–10 µg/mL; AcINH 0.1–10 µg/mL; and EMB 20–5000 ng/mL. The lower limit of quantification was 1 µg/mL; 0.2 µg/mL; 0.1 µg/mL; 0.1 µg/mL and 20 ng/mL, respectively. The results of validation for specificity/selectivity, precision, accuracy, linearity, limit of quantification, recovery, and stability show that the method meets the requirements of a bioanalytical method. Our method may serve well for routine therapeutic monitoring of the first-line anti-TB drugs in patient plasma.

INTRODUCTION

Tuberculosis (TB) is a major public health issue in Vietnam. Vietnam is ranked 12th among the 22 highest TB burden countries with an estimated incidence of 140 new cases per 100,000 in 2014 (Hoa *et al.*, 2010). National TB Control Programme (NTP) data indicates that 6 months of first-line month therapy for new TB patients results in 10% treatment failures or post-treatment relapses. TB treatment is very long and complex, unfortunately, many patients fail treatment even though they have fully susceptible TB infections that should, in theory, respond to the treatment (Mukherjee *et al.*, 2004). The treatment of TB consists of combinatorial regimens of three

or four first-line drugs to prevent resistance such as isoniazid, rifampicin, pyrazinamide, and ethambutol. Even there are the majority of tuberculosis patients who respond to a standardized short course of treatment, but it has been reported that a small group of patients has a poor response to treatment (Mirsaeidi *et al.*, 2018). These patients have low serum drug concentrations, leading to clinical failure or relapse. For patients who failure to standard therapy and presented low drug concentrations, the treatment could be successfully improved by adjusting dose regimens (Alsultan *et al.*, 2014). Therefore, it is necessary to have an efficient method to measure the standard antituberculosis drug concentrations to facilitate early screening of therapeutic failure. Several studies reported the analytical method for measurement of anti-tuberculosis drug concentrations in human plasma, but there are a few methods that could simultaneously measure first-line drugs and their major metabolites. Mukherjee *et al.*, have studied four drugs, including isoniazid, rifampicin,

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pyrazinamide, and ethambutol in children to correlate the plasma concentrations of these drugs with clinical outcome of therapy (Mukherjee *et al.*, 2015). The LC-MS/MS method may be the most appropriate one for measurement of anti-TB drug concentration in human plasma which has been reported (Song *et al.*, 2007, Um *et al.*, 2007). However, previous LC-MS/MS methods developed could only measure a limited number of anti-TB drugs, then leading time-consuming to determine all four drugs. In the present study, we developed a simple and rapid method for the simultaneous measurement of plasma concentrations of four first-line drugs (pyrazinamide, rifampicin, isoniazid, ethambutol) and acetylisoniazid, one major metabolite using LC/MS/MS to apply for therapeutic drug monitoring.

MATERIALS AND METHODS

Chemicals and reagents

Standard of Pyrazinamide, Rifampicin, Isoniazid, Ethambutol hydrochloride and Diltiazem hydrochloride from NIDQC; Acetyl Isoniazid from Canada; Methanol, Ammonium acetate (Merck, Germany); All blank plasma sources were collected from healthy volunteers.

Sample preparation

- Stock solutions of PZA (2000 µg/mL), RIF (400 µg/mL), INH (400 µg/mL), AcINH (500 µg/mL) and EMB (200 µg/mL) in methanol were made.

- Working standard solutions of 200 µg/mL PZA, 40 µg/mL RIF, 20 µg/mL INH, 20 µg/mL AcINH and 10 µg/mL EMB in plasma were prepared from the stock solutions.

- A calibration curve consists of a blank sample, a zero sample and eight non-zero plasma standards spiked with PZA, RIF, INH, AcINH, EMB and IS.

- Three quality control (QC) concentration levels: LQC (Low-quality control), MQC (Medium-quality control), HQC (High-quality control) and LLOQ (Lower limit of quantification) were constructed from separate stock standard solutions (Table 1). Sample preparation and sample treatment procedure were protected from light.

Table 1: QC samples in plasma.

Sample	Concentration				
	PZA (µg/mL)	RIF (µg/mL)	INH (µg/mL)	AcINH (µg/mL)	EMB (ng/mL)
LQC	3	0.6	0.3	0.3	60
MQC	50	10	5	5	2500
HQC	80	16	8	8	4000
LLOQ	1.0	0.2	0.1	0.1	20

Internal standard (IS) stock solution (Diltiazem hydrochloride) was prepared at 250 µg/mL in methanol. IS working solution was prepared by diluting the IS stock solution in methanol: water (1:1, v/v) to get the concentration of 50 µg/mL.

Sample preparation

To 0.5 mL of plasma sample, add 50 µL of IS (Diltiazem hydrochloride) working solution, vortex for 5 seconds. And then 1.5 mL of MeOH was added and followed by vortexing for 10 seconds. 250 µL of water was also added and mixed well for 5 seconds. Centrifuge at 9000 rpm/min for 5 minutes. Pipette the clear supernatant and inject into the LC-MS/MS system.

Analytical method validation

The analysis was performed on a triple quadrupole LC-MS/MS (Waters-USA) with a Surveyor MS pump (Acquity H class QSM) and autosampler (Acquity H class FTN). The mass spectrometer was a TSQ Quantum Access Max mass spectrometer. The autosampler tray temperature was set at 10°C. The high-performance liquid chromatography (HPLC) system was coupled to a quadrupole mass spectrometer. The HPLC components were chromatographic column: Gemini C18; 150 * 4.6 mm; 4.6 µm; Guard Column: C18; 4 × 3 mm; Column temperature 40°C; Detector: Xevo TQD. The mobile phase containing MeOH: and Ammonium acetate 5 mM, pH 3.5. The elution gradient was shown in Table 2. Injection volume: 1 µL; The method had a run time of 8 min. The mass spectrometric conditions were shown in Table 3.

Table 2: Gradient program of the mobile phase.

Time (min)	Flow rate (µL/min)	MeOH (%)	Ammonium acetate 5 mM pH 3.5 (%)
0.0	600	60	40
2.0	600	60	40
2.5	700	90	10
5.0	700	90	10
6.0	600	60	40
8.0	600	60	40

The validation was performed based on the US Food and Drug Administration (FDA) guidelines (Health *et al.*, 2017) and European Medicines Agency (EMA) guidelines (Agency, 2011) and parameters included were selectivity and specificity, linearity, accuracy and precision, matrix effects, recovery, carry over, dilution integrity and stability in human plasma.

System suitability test (SST)

We analyzed MQC sample in plasma following the analytical procedure for system suitability test. Injected this sample with six replicates. All chromatograms and peak parameters were recorded.

Selectivity

Selectivity was assessed by comparing chromatograms and peak responses of 06 blank plasma samples collected from 6 different volunteers with 06 standard samples spiked with IS and PZA, RIF, INH, AcINH, EMB at LLOQ level (1.0 µg/mL; 0.2 µg/mL; 0.1 µg/mL; 0.1 µg/mL and 20 ng/mL, respectively) in 6 above blank plasma sources, respectively.

Table 3: Mass spectrometric conditions.

Parameters \ Analytes	Pyrazinamide	Rifampicin	Isoniazid	Acetyl Isoniazid	Ethambutol	Diltiazem (IS)
Ion source	ESI (+)	ESI (+)	ESI (+)	ESI (+)	ESI (+)	ESI (+)
Capillary voltage (kV)	3.5	3.5	3.5	3.5	3.5	3.5
Cone voltage (V)	30	44	34	34	34	20
Desolvation temperature (°C)	500	500	500	500	500	500
Desolvation gas (L/H)	800	800	800	800	800	800
Cone gas (L/H)	20	20	20	20	20	20
Collision energy (V)	14	18	13	20	14	12
Precusor ion (Dalton)	124.00	823.46	138.00	180.00	205.16	415.10
Product ion (Dalton)	81.00	791.49	121.00	121.00	116.13	178.00

Matrix effects

The matrix effects were assessed at LQC and HQC levels by comparing chromatograms and peak area of following samples: 06 blank plasma samples collected from six different volunteers; standard samples containing IS and PZA, RIF, INH, AcINH, EMB at LQC and HQC concentration in each above matrix; six standard samples containing IS and PZA, RIF, INH, AcINH, EMB at LQC and HQC concentration in the solvent mixture (methanol and water). Determine the matrix factor: MF_{PZA}, MF_{RIF}, MF_{INH}, MF_{AcINH}, MF_{EMB}, MF_{IS} and the ratio: MF_{PZA}/MF_{IS}, MF_{RIF}/MF_{IS}, MF_{INH}/MF_{IS}, MF_{AcINH}/MIS and MF_{EMB}/MF_{IS}.

Carry-over

The carry-over was analyzed as follows:
Prepared samples:
- Six blank human plasma samples;
- Six standard samples in human plasma at LLOQ concentration; six standard samples in human plasma at ULOQ (Upper limit of quantification) concentration. Then these samples were extracted following the procedure. Injected 06 LLOQ samples firstly and then injected a blank sample after each ULOQ sample, alternately. Record all the chromatograms and peak area.

Calibration curve and linearity range

Evaluated the concentration range of PZA: 1.0 µg/mL–100 µg/mL; RIF: 0.2 µg/mL–20 µg/mL; INH: 0.1 µg/mL–10 µg/mL; AcINH: 0.1 µg/mL–10 µg/mL and EMB: 20 ng/mL–5000 ng/mL. Each calibration curve includes 8 standard samples. Analyze these samples following the procedure. Record all the chromatograms and peak area. From the experimental data, choose the most appropriate weighting factor from the popular weighting models: 1, $1/x$, $1/x^2$, $1/x^{1/2}$. Determine the accuracy of each standard sample.

Determination of lower limit of quantification

The lower limit of quantification (LLOQ) is the lowest concentration on the calibration curve to demonstrate that the developed analytical method is specific/selective, precise and accurate. LLOQ was assessed by preparing six blank human plasma samples; six blank plasma spiked with PZA, RIF, INH, AcINH and EMB at concentration of about: 1.002 µg/mL; 0.199 µg/mL; 0.105 µg/mL; 0.106 µg/mL; 20.0 ng/mL; respectively and IS; one calibration curve with eight standard samples in human plasma. Recorded all the chromatograms and peak responses and determined the accuracy and precision of the LLOQ samples.

Intra-day and inter-day accuracy and precision

Intra-day precision and accuracy were evaluated at 4 concentrations: LLOQ, LQC, MQC, and HQC. Each concentration was repeated six replicates. Inter-day accuracy and precision have evaluated the accuracy and precision of the analytical method on five different analytical days and determine inter-day precision at each QC level and LLOQ. Requirements for method is intra-day accuracy and precision was the Mean ± SD accuracy at each QC level should be within 85%–115% of the nominal concentration, except for the LLOQ which should be 80%–120% of the nominal concentration; and intra-day precision at each QC level should not exceed 15% of the CV% value, except for the LLOQ where it should not exceed 20% of the CV% value.

Recovery

The extraction recovery was evaluated by determining the recovery of the analytical method at 3 concentration levels: LQC, MQC, and HQC in six replicates. The recovery of the drugs should not exceed 100%, but the extent of recovery of the drugs and the internal standard should be consistent with each other (Zhou *et al.*, 2013).

Dilution integrity

When the concentrations of the drugs are higher than the upper limit of quantification (ULOQ) of the calibration curves, the sample should be diluted. Dilution integrity experiments were performed with three LDC, MDC and HDC samples. Dilute these samples 2 times by blank plasma. Prepare a calibration curve and QC samples in plasma. Analyze the above samples following the analytical procedure and determine the concentrations, accuracy, and precision of diluted samples. Dilution integrity was achieved if the mean accuracy should be 85–115% of the nominal concentration and the precision should not exceed 15% of the CV.

Stability

Autosampler stability of processed plasma samples was evaluated at LQC and HQC concentrations. These samples were extracted following the proposed procedure and stored in autosampler at 10°C. One part of the processed sample volume was injected immediately to determine the initial concentration. The remaining sample volume was still stored in autosampler

until it was analyzed to determine the concentration after 20 hours.

      Short-term stability of plasma samples was evaluated at LQC and HQC concentrations. The QC samples were stored at room temperature for 2, 4 hours. Determined the concentration of those samples. The stability samples were analyzed against with the calibration curve.

      Long-term stability of plasma samples was also evaluated at LQC and HQC concentrations which were stored at $-70^{\circ}C \pm 5^{\circ}C$. Determined the concentration of those samples after 5 days and 18 days.

RESULTS AND DISCUSSION

      Choosing a positive or negative ion mode in the LC-MS/MS method depends on the molecular structure of the analytes.

In the molecular structure of all compounds PZA, RIF, EMB, INH, and AcINH, there is an amino group–a functional group containing N atom and an unused free pair of electrons capable of accepting H^+ to become positively charged ion, therefore PZA, RIF, INH, ACINH, and EMB were better detected in the positive ion mode. Furthermore, previous studies also used all positive ion mode (Prahl *et al.*, 2016). The product ion spectrum of the $[M^+H]^+$ ion of PZA showed a major fragment ion at *m/z* 81, of RIF, major fragment ions were observed at *m/z* 792 due to the loss of neutral CH_3OH, of INH showed a major fragment ion at *m/z* 121 due to loss of neutral NH_3, and AcIHN showed the same fragment ion at *m/z* 121, and for EMB the major fragment ions were at *m/z* 116, and diltiazem hydrochloride had fragment ion at *m/z* 178 as showed in Figure 1 (Molden *et al.*, 2003, Song *et al.*, 2007).
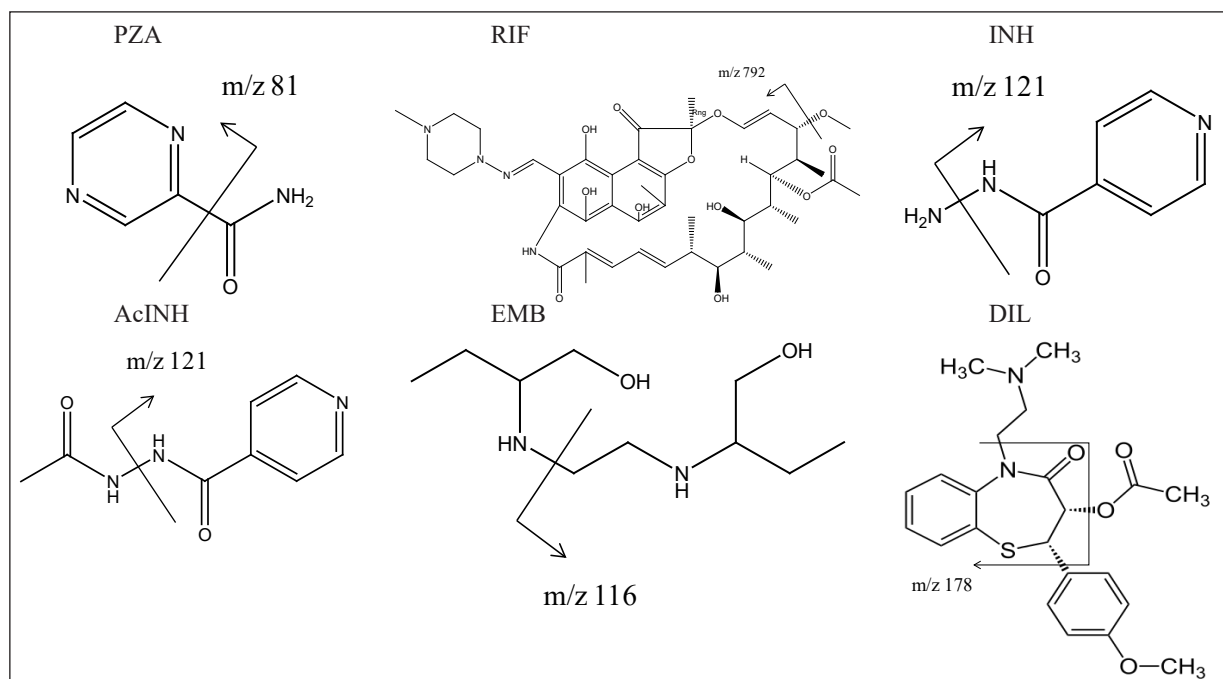
Fig. 1: Chemical structures and product ions of the four tuberculosis drugs (isoniazid, rifampicin, pyrazinamide, and ethambutol) examined, one metabolite (acetylisoniazid), and internal standards (Diltiazem hydrochloride).

System suitability test

      Our results showed the method had a system suitability test because:

      - The peak of analytes (PZA, RIF, INH, AcINH, and EMB) and IS was symmetrical and identifiable;

      - Repeatability of the retention time of analytes and IS was not exceeded 1.0% of the CV% value.

      - Repeatability of analytes, IS peak area and each analyte/IS peak area ratio did not exceed 5.0% of the CV% value.

      The results were presented in Figure 2.

Selectivity

      The method was found to have high selectivity because Peak of PZA, RIF, INH, AcINH, EMB and IS was symmetrical and identifiable; At the retention time of PZA, RIF, INH, AcINH, and EMB, peak response of each LLOQ sample was at least 5 times the response of respective blank plasma sample; At the

retention time of IS, peak response of each LLOQ sample was at least 20 times the response of respective blank plasma sample. Chromatogram blank plasma sample and blank plasma sample spiked with IS and PZA, RIF, INH, AcINH, EMB standard at LLOQ concentration are presented in Figure 3 and Figure 4. The analyzed method is based on the LC-MS/MS technique, which is a highly selective and specific analytical method. The quantitative principle of the method is based on the recognition of the mass number of both the product ion and precursor ion. Therefore, if the two analytes have different mass number of product ion and precursor ion, they will be independently identified and quantified without being confused. In the method, the precursor/product ion of metabolites should have a different mass number of precursor/product ion of analytes. In this study, for PZA: 124/81 Dalton; metabolite: Pyrazinoic acid: 125.0→80.9 Dalton; 5 hydroxy pyrazinoic: 141.0→81.0 Dalton; for RIF: 823.46/791.49 Dalton; metabolite: 25-0-desacetylrifampin: 781.1-749.1 Dalton; for

INH: 138.00/121.00 Dalton; metabolite: AcINH: 180.00/121.00 Dalton; and for EMB: 205.16/116.13 Dalton.

Matrix effect

The use of internal standards compensates for unexpected matrix effects. The calculated CV% ($n = 6$) of the internal standard normalized matrix factor is presented in Table 4. The calculated CV% of the internal standard normalized matrix factor was not exceeding 15% of the CV% value.

Carry-over

No carry-over was observed for PZA, RIF, INH, AcINH, and EMB based on the retention time of PZA, RIF, INH, AcINH and EMB, the Mean ± SD peak area of LLOQ sample was at least 5 times the response of blank sample and at the retention time of

IS, the Mean ± SD peak area of LLOQ sample was at least 20 times the response of blank sample.

Calibration curve

The accuracy of standards was within 85%–115% of nominal concentration for all eight calibration standards of PZA, RIF, INH, ACIH, and EMB, then the calibration curve of PZA, RIF, INH, ACIH, and EMB consisted of all eight calibration standards. From the experimental data, the weighting factor of 1/x2 was chosen. Results of experimental calibration curves with the weighting factor of 1/x2 and calibration equations of the standard curves were presented in Figure 5. PZA, RIF, INH, AcINH and EMB showed an acceptable linear response in the range 1.0–100 µg/mL; 0.2–20 µg/mL; 0.1–10 µg/mL; 0.1–10 µg/mL; 20–5000 ng/mL, respectively.
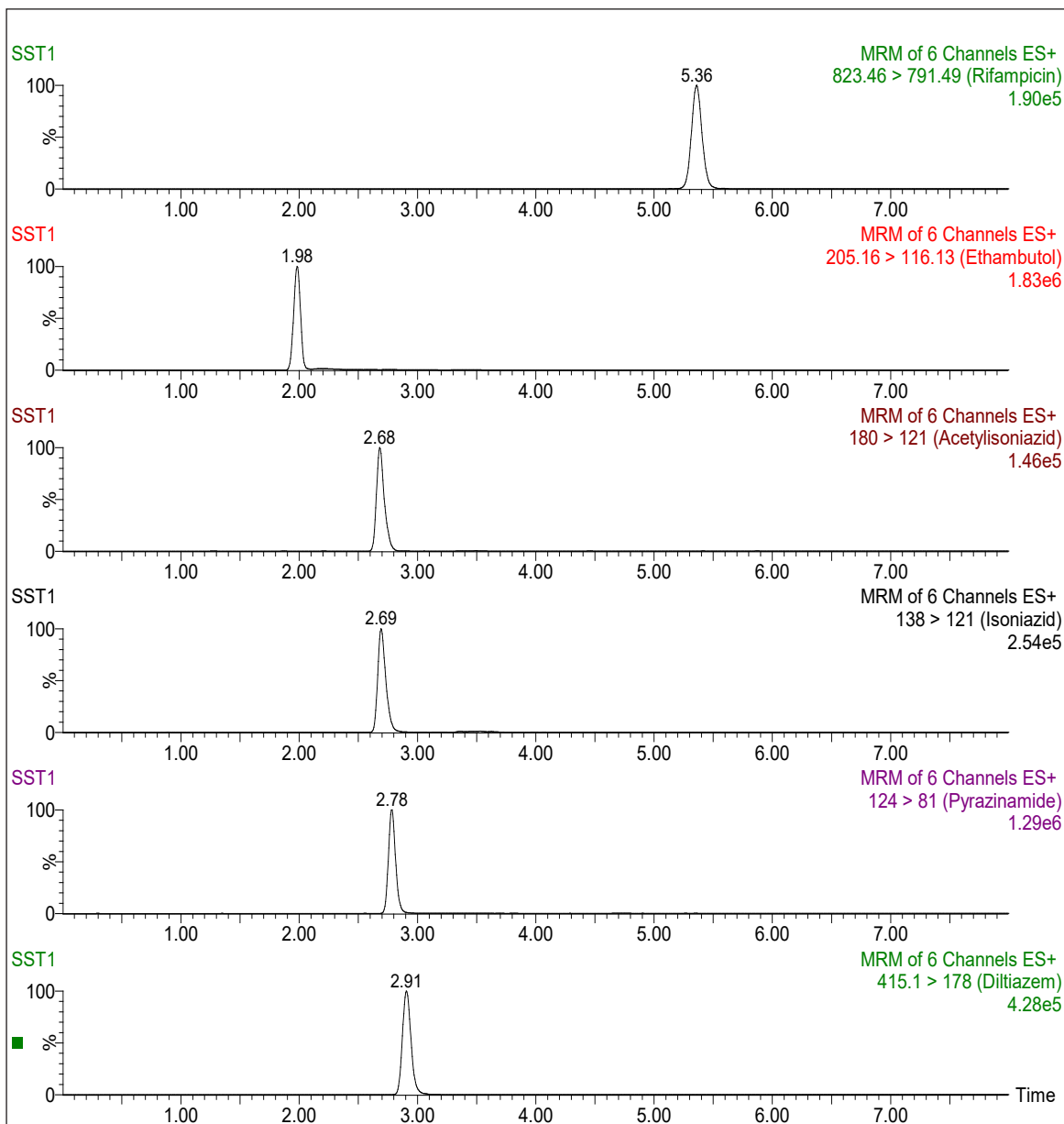

Fig. 2: Chromatogram of blank plasma spiked with IS and PZA, RIF, INH, AcINH and EMB standards at MQC concentration.

Lower limit of quantification

Requirements for LLOQ sample was: at the retention time of PZA, RIF, INH, AcINH and EMB, response of the separate LLOQ sample should be at least 5 times the response of blank sample respective; and accuracy of the LLOQ sample should be 80%–120% of the nominal concentration; and precision should not exceed 20% of the CV% value. Results were presented in Table 5. Our methods showed the LLOQ for all compounds was below the recommended normal ranges. LLOQ of PZA was 1.0 µg/mL; of RIF 0.2 µg/mL; of INH 0.1 µg/mL; of AcINH 0.1 µg/mL and of EMB 20 ng/mL.

Table 4: Results of matrix effect- PZA, RIF, INH, AcINH, and EMB.

	PZA		RIF		INH		AcINH		EMB	
	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
	MF_{PZA}/MF_{IS}	MF_{PZA}/MF_{IS}	MF_{RIF}/MF_{IS}	MF_{RIF}/MF_{IS}	MF_{INH}/MF_{IS}	MF_{INH}/MF_{IS}	MF_{AcINH}/MF_{IS}	MF_{AcINH}/MF_{IS}	MF_{EMB}/MF_{IS}	MF_{EMB}/MF_{IS}
Mean ± SD	0.588 ± 0.08	0.590 ± 0.06	0.616 ± 0.07	0.647 ± 0.07	1.117 ± 0.12	1.128 ± 0.16	1.185 ± 0.18	1.123 ± 0.13	0.433 ± 0.06	0.409 ± 0.04
CV (%)	12.9	10.4	11.5	11.1	10.7	13.9	15.0	11.3	13.9	10.4
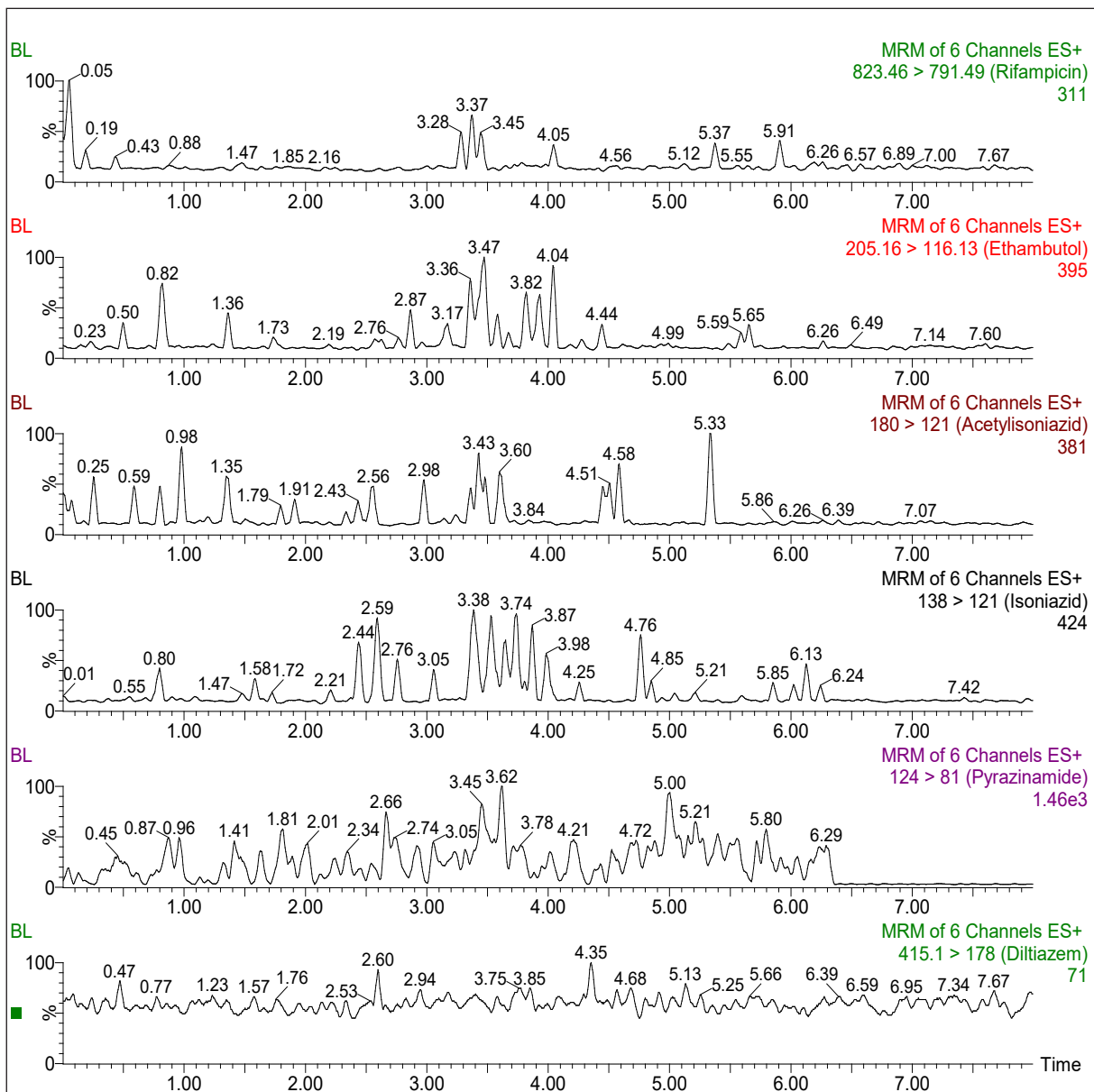
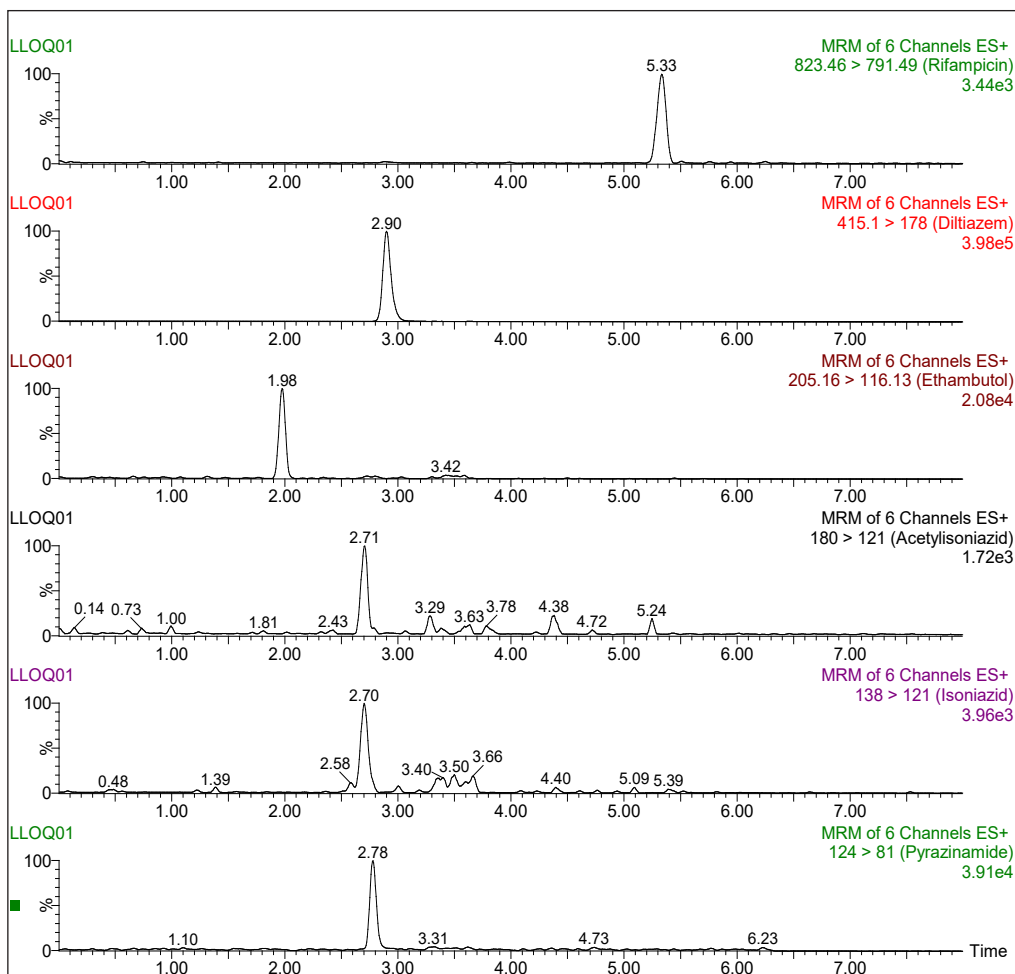**Fig. 3:** Chromatogram of blank plasma sample.

Fig. 4: Chromatogram of blank plasma sample spiked with IS and PZA, RIF, INH, AcINH, and EMB standard at LLOQ concentration.

Table 5: Accuracy and precision at LLOQ level: PZA, RIF, INH, AcINH, and EMB.

	PZA	RIF	INH	AcINH	EMB
Accu. (%)	105.8 ± 9.5	102.0 ± 6.3	99.9 ± 15.4	100.6 ± 10.5	90.4 ± 10.1
CV (%)	9.0	6.2	15.5	10.5	11.1

Table 6: Intra-day accuracy and precision PZA, RIF, INH, AcINH, and EMB.

PZA			
	LLOQ	LQC	MQC	HQC
Accu.(%)	102.5 ± 10.1	93.2 ± 1.8	90.5 ± 3.8	91.8 ± 1.8
CV (%)	9.9	1.9	4.2	2.0
RIF			
	LLOQ	LQC	MQC	HQC
Accu.(%)	118.1 ± 11.8	101.6 ± 3.7	98.4 ± 2.9	100.4 ± 3.0
CV (%)	10.0	3.6	3.0	3.0
INH			
	LLOQ	LQC	MQC	HQC
Accu.(%)	97.2 ± 13.1	104.0 ± 5.7	101.7 ± 5.0	98.4 ± 3.0
CV (%)	13.4	5.5	4.9	3.0
AcINH			
	LLOQ	LQC	MQC	HQC
Accu.(%)	90.9 ± 10.0	97.3 ± 13.0	98.5 ± 4.3	98.2 ± 3.2
CV (%)	11.0	13.4	4.4	3.2
EMB			
	LLOQ	LQC	MQC	HQC
Accu.(%)	100.6 ± 18.9	88.9 ± 4.4	90.1 ± 3.2	89.6 ± 1.5
CV (%)	18.8	4.9	3.5	1.7

Intra-day accuracy and precision

Our method was found to be acceptably precise and accurate as the intra-day precision were <15% (Table 6). In this study, we used Diltiazem hydrochloride as internal standards. We found that the use of internal standards was necessary in order to obtain the desired accuracy and CV%. RIF and INH are labile compounds and the use of internal standards helps to compensate for the degradation during sample preparation.

Inter-day precision

The method was also found to be acceptably precise and accurate as inter-day precision were <15% (Table 7) as the requirements for Inter-day precision was the inter-day precision at each QC level should not exceed 15% of the CV% value, except for the LLOQ where it should not exceed 20% of the CV% value.

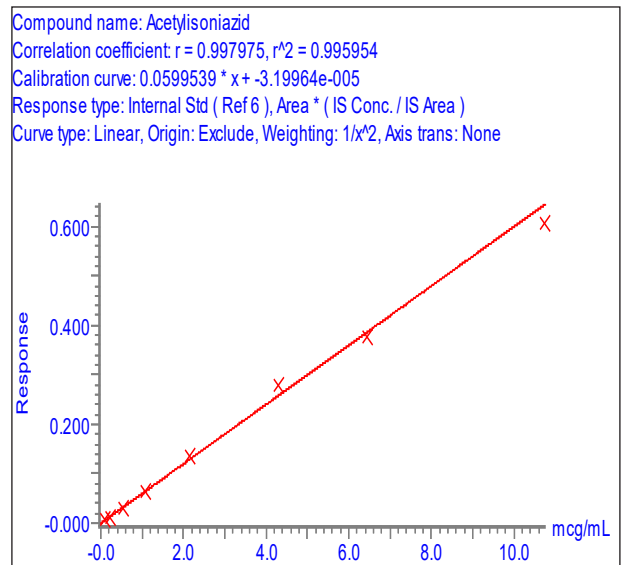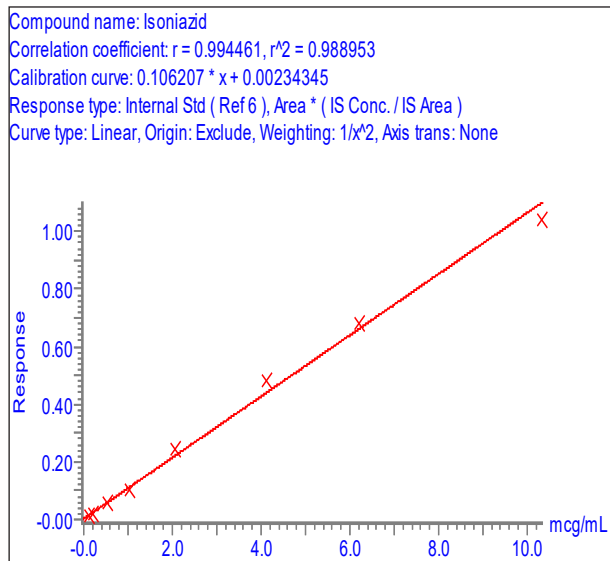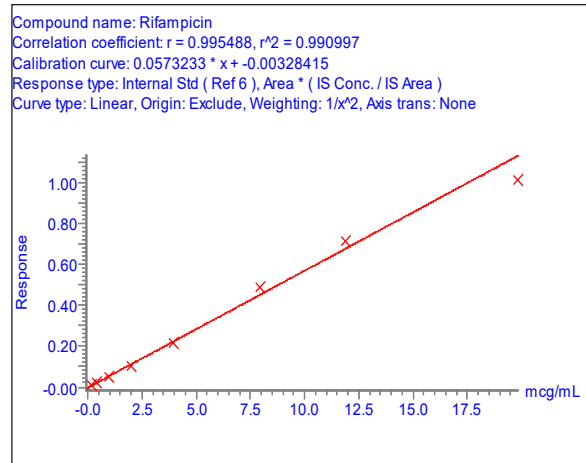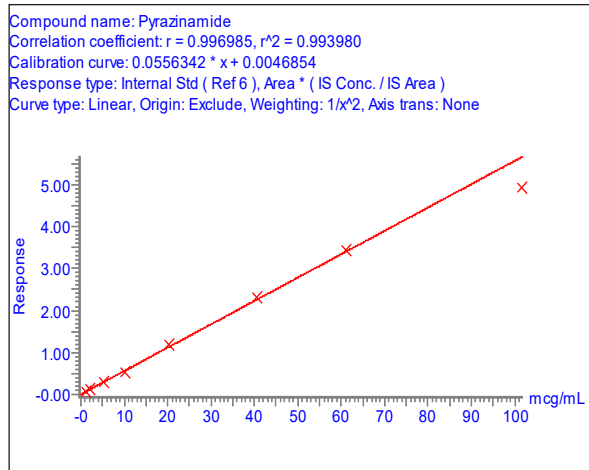**Fig. 5:** Calibration curves with the weighting factor of $1/x^2$- PZA, RIF, INH, ACIH and EMB.

Table 7: Inter-day precision PZA, RIF, INH, AcINH and EMB (*n* = 5).

PZA				
	LLOQ	**LQC**	**MQC**	**HQC**
Accu. (%)	102.1 ± 9.2	101.4 ± 8.3	100.6 ± 7.2	100.3 ± 6.5
CV (%)	9.0	8.1	7.2	6.4
RIF				
	LLOQ	**LQC**	**MQC**	**HQC**
Accu. (%)	101.1 ± 14.3	94.2 ± 8.1	95.9 ± 4.9	99.0 ± 6.2
CV (%)	14.2	8.6	5.1	6.2
INH				
	LLOQ	**LQC**	**MQC**	**HQC**
Accu. (%)	98.5 ± 14.0	100.0 ± 9.6	100.1 ± 8.0	99.0 ± 7.0
CV (%)	14.2	9.6	8.0	7.1
AcINH				
	LLOQ	**LQC**	**MQC**	**HQC**
Accu. (%)	101.5 ± 14.6	105.0 ± 12.0	104.2 ± 7.1	104.0 ± 5.8
CV (%)	14.4	11.4	6.8	5.5
EMB				
	LLOQ	**LQC**	**MQC**	**HQC**
Accu. (%)	103.0 ± 18.1	97.7 ± 10.1	96.3 ± 7.1	92.8 ± 4.7
CV (%)	17.6	10.3	7.3	5.0

Our method has LLOQ (PZA: 1.0 µg/mL; RIF: 0.2 µg/mL; INH: 0.1 µg/mL; AcINH: 0.1 µg/mL and EMB: 20 ng/mL) lower than other methods, and linear range was larger (PZA 1.0–100 µg/mL; RIF 0.2–20 µg/mL, INH 0.1–10 µg/mL, and EMB 20–5000 ng/mL) as compared with previous methods: Prahl's method: PZA: 4–80 µg/mL, RIF: 0.75–30 µg/mL, INH: 0.5–10 µg/mL, EMB: 0.25–10 µg/mL (Prahl *et al.*, 2016); Sang Hoon Song's method: PZA: 0.4–291.2 µg/mL, RIF: 1.1–320 µg/mL, INH: 1.1–320 µg/mL, and EMB: 0.07–17.5 µg/mL (Song *et al.*, 2007) and Sturkenboom's method: INH: 0.2–8 mg/L; EMB: 0.2–8 mg/L; PZA: 2–80 mg/L (Sturkenboom *et al.*, 2015). Furthermore, the total analysis time in our method was short, about 8 minutes, can detect and quantify exactly the concentration of 5 active analytes and the internal standard.

Recovery

The method was found to have the good recovery. The results were shown in Table 8.

Extraction method

Due to the physical and chemical properties of PZA, RIF, INH, EMB, and AcINH (water-soluble compounds), most authors used extraction method as protein precipitation. In our method, we have developed a simple, easy-to-implement, economic, environmental and human-friendly method. We used only one step of protein precipitation with 100% MeOH, while other authors have used the method more complex, more time-consuming and more extraction steps. Prahl *et al.*, have used two steps in their method. The first step they precipitated protein with 50% MeOH and stored at –20ºC for 1 h. The second step was followed by centrifugation to eliminate precipitation, and then continued precipitated with acetonitrile. (Prahl *et al.*, 2016). Sang Hoon Song also extracted sample by two steps: the first step with 50% MeOH and second step used MeOH (Song *et al.*, 2007). Our method also showed that recovery rates of the studied compounds were relatively high (over 60% and nearly 95%) and stable (recovered rates at different levels of LQC, MQC, and HQC were not more than 15%) (Table 8).

Table 8: Recovery of IS, PZA, RIF, INH, AcINH, EMB.

	93.5		
Recovery (%)			
PZA			
	LQC	MQC	HQC
Recovery (%)	94.5	88.5	83.7
RIF			
	LQC	MQC	HQC
Recovery (%)	65.6	65.1	63.5
INH			
	LQC	MQC	HQC
Recovery (%)	63.7	64.6	59.4
AcINH			
	LQC	MQC	HQC
Recovery (%)	79.5	76.1	69.2
EMB			
	LQC	MQC	HQC
Recovery (%)	84.9	77.6	74.2

Dilution integrity

Concentrations of anti-TB drugs in human plasma have been reported vary in a wide range. Some samples may have a concentration higher than the ULOQ of the calibration curves. To validate the dilution integrity of the method, the accuracy and precision were assayed using three QC samples spiked with each drug at concentrations higher than the ULOQ with additional series of dilutions. Our data showed that the mean accuracy was in the range of 85–115% of the nominal concentration and the precision was not exceeded 15% of the CV for all five drugs (Table 9). The results suggest that if samples have drug concentrations higher than upper of the calibration curve, sample dilution could be performed for reanalysis.

Table 9: Results of dilution integrity for PZA, RIF, INH, AcINH, and EMB.

PZA			
	LDC	**MDC**	**HDC**
Accu. (%)	106.4 ± 6.1	107.6 ± 3.7	98.2 ± 2.8
CV (%)	5.7	3.4	2.9
RIF			
	LDC	**MDC**	**HDC**
Accu.(%)	103.8 ± 6.8	105.3 ± 4.6	100.3 ± 5.1
CV (%)	6.5	4.4	5.1
INH			
	LDC	**MDC**	**HDC**
Accu.(%)	107.5 ± 8.8	112.7 ± 3.8	104.2 ± 5.7
CV (%)	8.2	3.4	5.5
AcINH			
	LDC	**MDC**	**HDC**
Accu.(%)	102.0 ± 10.7	107.1 ± 2.9	98.1 ± 3.8
CV (%)	10.5	2.7	3.9
EMB			
	LDC	**MDC**	**HDC**
Accu.(%)	112.6 ± 4.5	105.1 ± 2.6	98.8 ± 3.1
CV (%)	4.0	2.5	3.1

Stability

Autosampler stability

The autosampler stability test showed no significant bias between fresh and stored samples based on the difference between the concentration of the stored processed samples and that of the freshly processed samples should not exceed 15%, and CV% of determinations of samples at each concentration should not exceed 15%. The results are presented in Table 10.

Short-term stability

The criterion for short-term stability is the difference between the concentration of the stored plasma samples with that of freshly prepared samples should not exceed 15%. CV% of determinations of samples at each concentration should not exceed 15%. The results were presented in Table 11. Short-term stability test showed that INH and AcINH in human plasma were stabled after 2 hours but was unstable after 4 hours stored at room temperature.

Long-term stability

The requirement for long-term stability is the difference between the concentration of the stored samples and that of freshly prepared samples should not exceed 15% and the CV% of determinations of samples at each concentration should not exceed 15%. The results were presented in Table 12. Long-term stability test showed that all samples in human plasma were stabled after 18 days stored at –70°C ± 5°C.

Concentration monitoring of PZA, RIF, AcINH, INH,

and EMB has been attracted many researchers. Julie *et al.*, also have developed a method for simultaneous quantification of pyrazinamide, rifampicin, isoniazid, and ethambutol by liquid chromatography/tandem mass spectrometry. In that study, they used isotopically labeled compounds as internal standards. As compared with our method, ours has wide linear range of the calibration curve than theirs, such as in their method, for PZA 4–80 µg/mL, for RIF 0.75–30 µg/mL, for INH was 0.5–10 µg/mL, for EMB 0.25–10 µg/mL and meanwhile in our method was for PZA 1.0–100 µg/mL;, RIF 0.2–20 µg/mL, INH 0.1–10 µg/mL, and EMB 20–5000 ng/mL (Prahl *et al.*, 2016). Sang Hoon Song *et al.*, also have developed a method to measure concentrations of four anti-tuberculosis drugs (isoniazid, rifampicin, pyrazinamide, and ethambutol) and two major metabolic ratios (acetylisoniazid/isoniazid and 25-desacetylrifampicin/rifampicin). Their method showed the linearity range for PZA, RIF, INH, and EMB was 0.4–291.2, 1.1–320, 0.07–18.6, and 0.07–17.5 µg/mL, respectively (Song *et al.*, 2007). Marieke *et al.* also developed a method to quantify isoniazid, pyrazinamide, and ethambutol in serum using isotope-labeled isoniazid-D4 and ethambutol-D4 as internal standards. In that method, the range of quantification for isoniazid and ethambutol was 0.2–8 mg/L; and for pyrazinamide 2–80 mg/L (Sturkenboom *et al.*, 2015). Compared with previous methods, our method was effective to determine plasma concentrations of PZA, RIF, INH, AcINH, and EMB, and can be used to monitor drug concentrations in TB treatment.

Table 10: Results of autosampler stability: PZA, RIF, INH, AcINH, and EMB.

	LQC Freshly processed	LQC After 20 hours/10ºC	HQC Freshly processed	HQC After 20 hours/10ºC
PZA				
CV (%)	3.8	4.8	3.3	5.0
Variation (%)		−0.7		−6.6
RIF				
CV (%)	4.0	5.0	3.1	5.2
Variation (%)		14.6		0.5
INH				
CV (%)	6.5	12.0	5.1	5.1
Variation (%)		−12.7		−11.7
AcINH				
CV (%)	8.4	8.4	2.9	4.2
Variation (%)		9.0		2.6
EMB				
CV (%)	5.5	7.1	2.4	5.5
Variation (%)		−3.5		−6.4

Table 11: Results of short-term stability of plasma samples: PZA, RIF, INH, ACINH, and EMB.

	LQC Freshly processed	HQC Freshly processed	LQC After 2 hours/ room temperature	HQC After 2 hours/ room temperature	LQC After 4 hours/ room temperature	HQC After 4 hours/ room temperature
PZA						
CV (%)	3.7	3.5	10.0	4.3	5.8	2.7
Variation (%)			13.0	5.6	13.6	0.5
RIF						
CV (%)	4.6	4.8	7.8	4.8	7.8	4.9
Variation (%)			13.4	1.6	14.8	−7.3
INH						
CV (%)	9.4	1.1	12.0	3.7	7.0	1.8
Variation (%)			5.1	−8.2	−11.2	−18.4*
AcINH						
CV (%)	8.0	1.6	10.2	5.0	7.6	3.8
Variation (%)			10.4	−14.9	−7.8	−24.1*
EMB						
CV (%)	6.0	3.5	5.7	5.5	6.7	3.8
Variation (%)			14.0	4.8	14.1	−2.4

*: unstable

Table 12: Results of long-term stability of plasma samples: PZA, RIF, INH, ACINH, and EMB.

Conc. (µg/mL)	LQC Freshly processed	HQC Freshly processed	LQC After 5 days	HQC After 5 days	LQC After 18 days	HQC After 18 days
PZA						
CV (%)	8.5	7.7	6.2	4.2	5.0	4.4
Variation (%)			−7.3	−9.6	4.6	7.9
RIF						
CV (%)	6.5	8.7	8.5	4.9	6.4	5.3
Variation (%)			−6.7	−9.4	−4.5	−9.3
INH						
CV (%)	5.9	6.1	3.9	3.0	4.6	4.3
Variation (%)			−8.6	−3.4	−5.7	−6.0
AcINH						
CV (%)	9.7	7.6	6.2	3.6	3.6	3.5
Variation (%)			−8.7	−0.5	−5.0	−3.0
EMB						
CV (%)	6.6	4.9	2.6	3.0	6.2	3.9
Variation (%)			−14.3	−9.9	−14.5	−6.5

CONCLUSIONS

We have shown a rapid, simple, selective, and sensitive LC-MS/MS method for the simultaneous analysis of PZA, RIF, INH, AcINH, and EMB in human plasma. The results of validation for specificity/selectivity, precision, accuracy, linearity, limit of quantification, recovery, and stability showed that the method meets the requirements of a bioanalytical method. Our method may serve well for routine therapeutic monitoring of the first-line anti-TB drugs in patient plasma.

ABBREVIATION

AcINH: Acetyl Isoniazid;
EMB: Ethambutol;
INH: Isoniazid;
PZA: Pyrazinamide;
RIF: Rifampicin;
DIL: Diltiazem hydrochloride;
MF: matrix factor;
LQC: low-quality control;
MQC: medium quality control;
HQC: high-quality control.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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