Journal of Chromatography B, 877 (2009) 1698–1704

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

A rapid and robust liquid chromatography/tandem mass spectrometry method for simultaneous analysis of anti-tuberculosis drugs—Ethambutol and pyrazinamide in human plasma

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ARTICLE INFO

Article history:
Received 3 December 2008
Accepted 14 April 2009
Available online 18 April 2009

Keywords:
Ethambutol
Pyrazinamide
Human plasma
Liquid chromatography
Mass spectrometry

ABSTRACT

Ethambutol and pyrazinamide are two first-line anti-tuberculosis drugs. Though they are normally combined for the treatment, their highly different polarity complicates simultaneous liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis of these two drugs in human plasma with decent peak shape and retention. Here we report a rapid and robust LC/MS/MS method for the simultaneous determination of these two drugs in human plasma. Human plasma samples, together with the isotopically labeled internal standards were extracted using protein precipitation, and then separated on a Chromolith SpeedROD RP-18e column and detected with mass spectrometry. The mobile phase is 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in methanol. Addition of trifluoroacetic acid in the mobile phases was found to be able to improve peak shape as well as to increase the retention of ethambutol, thus being able to analyze these two drugs at the same time with both drugs having decent peak shape and enough retention on a C_{18} column. An atmospheric pressure chemical ionization interface was chosen to reduce ion suppression from sample matrix components and provide high sensitivity. The standard curve range was 10.0–5000 ng/mL for ethambutol and 50.0–25,000 ng/mL for pyrazinamide using a plasma sample volume of 50.0 μL. This method has a very short run time of 3.8 min. The method has been fully validated, and <15% relative standard deviation was obtained for both analytes.

1. Introduction

Tuberculosis remains one of the most common infectious diseases, which caused death of millions of people each year [1,2]. Tuberculosis is normally treated with combination therapy due to rapid development of resistance when single drug is used [2]. Ethambutol and pyrazinamide, together with isoniazid and rifampicin, are first-line drugs for tuberculosis treatment. These four drugs are normally combined in the first few months treatment to avoid development of resistance. Then the number is usually reduced to two drugs for the remainder of the treatment based on drug sensitivity testing that is usually available by this time in the course. Ethambutol and pyrazinamide combination is also one of the recommended treatments for latent tuberculosis infection [3].

A number of quantitative methods for the determination of anti-tuberculosis drugs in biological matrices have been reported [4–8]. However, many of the reported methods have limitations such as low sensitivity or long chromatographic run times. For the combination of ethambutol and pyrazinamide with or without other drugs, it is desirable to develop an analytical method to allow both drugs to be quantified simultaneously in human plasma.

The purpose of this project was to develop a liquid chromatography/tandem mass spectrometry method for the simultaneous quantitation of ethambutol and pyrazinamide in human plasma. The method was expected to have a short run time, thus enabling efficient analysis of large number of plasma samples obtained for pharmacokinetics studies, or for therapeutic drug monitoring with combinational drug therapy containing ethambutol and pyrazinamide.

2. Experimental

2.1. Materials and reagents

Ethambutol dihydrochloride and pyrazinamide were purchased from Sigma–Aldrich (St. Louis, MO). The internal standards, ethambutol-d_4 dihydrochloride and pyrazinamide-d_3, were from Covance in-house synthesis (Covance, Madison, WI). Acetonitrile (HPLC grade), formic acid (96%) and trifluoroacetic acid (1-mL ampule) were all purchased from Sigma–Aldrich (St. Louis, MO). Methanol (HPLC grade) was purchased from Fisher Scientific (Fair Lawn, NJ). Water was purified by a Barnstead system.

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doi:10.1016/j.jchromb.2009.04.023

2.2. Instrumentation

The high-performance liquid chromatography (HPLC) system consists of a Shimadzu SIL-HTc autosampler, LC-10ADVP solvent delivery system and a column heater (Columbia, MD). The mass spectrometer was an Applied Biosystems MDS Sciex (Toronto, Canada) API-4000 triple quadrupole mass spectrometer. An atmospheric pressure chemical ionization (APCI) source was used. Data was collected and processed using Sciex Analyst 1.1 data collection and integration software.

2.3. Chromatographic conditions

The HPLC column was a Chromolith SpeedROD RP-18e (50 mm × 4.6 mm) from Merck (Darmstadt, Germany). Column temperature was held at 25 °C. The mobile phase A was 0.1% trifluoroacetic acid in water, and mobile phase B was 0.1% trifluoroacetic acid in methanol. The gradient was as follows: 2% mobile phase B was first held for 0.5 min, then mobile phase B was raised up to 15% in 1.7 min, at 2.21 min, mobile phase B was adjusted to 85% and held at this level until 2.9 min, at 2.91 min, mobile phase B was switched back to 2% until 3.8 min. The flow rate was 1.00 mL/min. A typical injection volume was 5 μL.

2.4. MS/MS detection

Precursor ions for analytes and internal standards were determined from mass spectra obtained by teeing-in the neat solution of each individual compound into an LC stream and then into the API-4000 mass spectrometer. Using APCI source, the mass spectrometer was operated in the positive ionization mode. With Q1 full scan, protonated molecular ions of all compounds were observed. Each of the precursor ions were subjected to collision-induced dissociation to determine the resulting product ions. The determined multiple reaction monitoring (MRM) transitions were 205 → 116 for ethambutol, 124 → 81 for pyrazinamide, 209 → 120 for ethambutol-d_4, and 127 → 84 for pyrazinamide-d_3. Fig. 1A and B shows the fragmentation of ethambutol and pyrazinamide, respectively. In these two figures, the parent masses and their fragmentation patterns are clearly shown.

Interface independent parameters and heated nebulizer parameters were optimized during the infusion of a solution of all compounds with HPLC mobile phase. These were (arbitrary units if not specified) CAD: 8, Gas 1: 30, CUR: 30, CE: 23 V for ethambutol and ethambutol-d_4 and 26 V for pyrazinamide and pyrazinamide-d_3, current: 3 mA, ionization source temperature: 500 °C, and dwell time: 150 ms. Unit resolution was used for both Q1 and Q3.

2.5. Preparation of standards and quality control samples

Stock standard solutions for ethambutol and pyrazinamide were prepared in duplicate and compared using the appropriate weight for the lot purity, moisture, and salt correction. The calibration standards and the quality control (QC) samples were prepared from separate stock standard solutions. Stock standard solutions of ethambutol of 2.50 mg/mL and pyrazinamide of 12.5 mg/mL were prepared in 50% methanol in water. Intermediate working solutions (ethambutol/pyrazinamide) of 125/625, 100/500, 62.5/313, 20.0/100, 5.00/25.0 and 2.00/10.0 were prepared from one set of stock standard solutions and were used for calibration standard preparation. Intermediate standard solutions of 250/1250, 40.0/200 and 2.50/12.5, prepared from another set of stock standard solutions, were for quality control samples preparation. They were all prepared in 50% methanol in water by dilution from the stock standard solutions. The working calibration standard human plasma samples were prepared by spiking corresponding

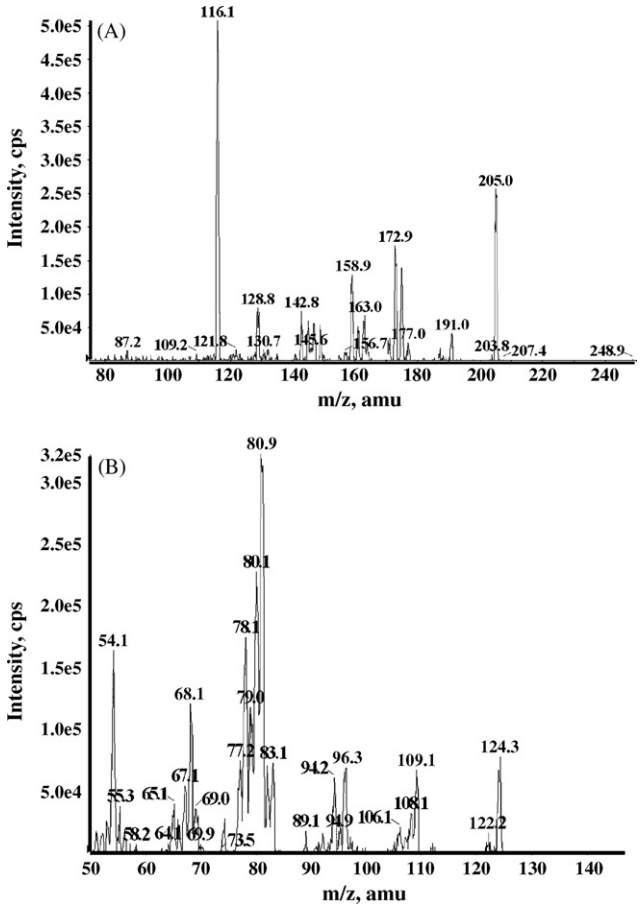

Fig. 1. Product ion mass spectra of ethambutol (A), and pyrazinamide (B).

intermediate standard solutions of 125/625, 100/500, 62.5/313, 20.0/100, 5.00/25.0, and 2.00/10.0 μg/mL into blank human plasma to provide concentrations (ethambutol/pyrazinamide) of 5000/25,000, 4000/20,000, 2500/12,500, 800/4000, 200/1000, 70.0/350, 20.0/100 and 10.0/50.0 ng/mL. The quality control plasma samples were prepared at 25,000/125,000, 3750/18,800, 400/2000, 30.0/150 and 10.0/50.0 ng/mL (ethambutol/pyrazinamide) by spiking human plasma with the second sets of standard stock solutions, which were for dilution QC preparation, and the intermediate standard solutions of 250/1250, 40.0/200 and 2.50/12.5 μg/mL. For the spiking, typically, the intermediate standard solutions of 20.0–200 μL were spiked into 5.00 mL of human plasma. Internal standard stock solutions of 100 μg/mL of ethambutol-d_4 and pyrazinamide-d_3 each were prepared in methanol. Working internal standard solution was prepared in 1.25% formic acid in acetonitrile containing 125 ng/mL of each of ethambutol-d_4 and pyrazinamide-d_3.

2.6. Sample preparation

The sample pre-treatment is based on protein precipitation. 50.0 μL of blank matrix, control-0 (blank with internal standard), QC samples and calibration standard samples were acidified with 50.0 μL of 5% formic acid aqueous solution, after vortex-mixing, 400 μL of 1.25% formic acid in acetonitrile were added to all blank matrix samples, 400 μL of intermediate internal standard solution was added to all other samples. This mixture was then vortex-mixed vigorously for ~2 min, and then centrifuged at 3000 rpm at room temperature for approximately 5 min. 100 μL of the supernatant was transferred and dried down under nitrogen stream at 45 °C.

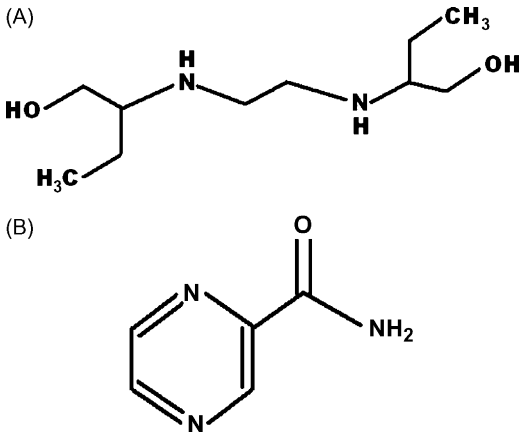

Fig. 2. Chemical structure of ethambutol (A) and pyrazinamide (B).

The residue was then reconstituted with 400 μL of 0.1% formic acid in methanol:water (5:95, v:v).

2.7. Validation procedure

The whole validation is based on Covance Bioanalytical Method Validation SOP.

2.7.1. Calibration curve and linearity

The eight-point calibration curve was constructed by plotting peak area ratio of ethambutol and pyrazinamide to their corresponding internal standard versus ethambutol and pyrazinamide concentrations. A linear regression with weighing factor of $1/x^2$ was applied. The concentrations of the standards were then back-calculated. Standards with back-calculated concentrations of 85.0–115.0% of their theoretical values were considered acceptable.

2.7.2. Intra-assay and inter-assay precision and accuracy

To evaluate intra-assay and inter-assay precision and accuracy, three consecutive batches were done. Each batch contained a freshly prepared calibration curve and six replicates of QC-LLOQ, low QC, mid QC and high QC. Dilution QC of six replicates were also included in one batch to evaluate impact of the dilution process to precision and accuracy to ensure that dilution of study samples will not affect accuracy and precision. Dilution QC concentration was at five times the upper limit of the standard curve. They were diluted 10× into calibration curve with blank matrix. By Covance SOP, these three consecutive batches should pass all criteria. Failure of any batch will fail the whole validation. Intra-assay precision was calculated by obtaining the relative standard deviation (RSD) of the six replicates of each QC level, and intra-assay accuracy was calculated by averaging the accuracies of six replicates of each QC level against the fresh curve. For inter-assay, precision was calculated by obtaining the RSD of all 18 replicates at each QC level from all three batches, accuracy was obtained by averaging the accuracies of all 18 replicates at each QC level from all three batches. Acceptable accuracy and precision were ≤15.0% accuracy and ≤15.0% RSD at every concentration level except for the LLOQ where ≤20.0% accuracy and ≤20.0% RSD were acceptable.

For QCs used for validation, Covance SOP does not specify whether they should be freshly prepared or can also be stored. In practice, both freshly prepared QCs and stored QCs can be used. For stored QCs, they were typically used within two weeks of storage. Due to usage of stored QCs, the subsequent long-term matrix stability should cover the storage of this period of time. In some cases when client's SOPs were used, the SOP specified QCs were then used.

2.7.3. Selectivity

Blank samples from six different lots of blank matrix were processed without internal standard to evaluate presence of interfering peaks. Interfering peaks at the retention time of interest should not exceed 20.0% of LLOQ. At maximum, one in six lots is allowed to have more than 20.0% of LLOQ interference.

These blank matrix lots were also separately spiked at mid QC levels for both ethambutol and pyrazinamide in the same way as regular mid QC preparation, and single replicate of each QC lot were

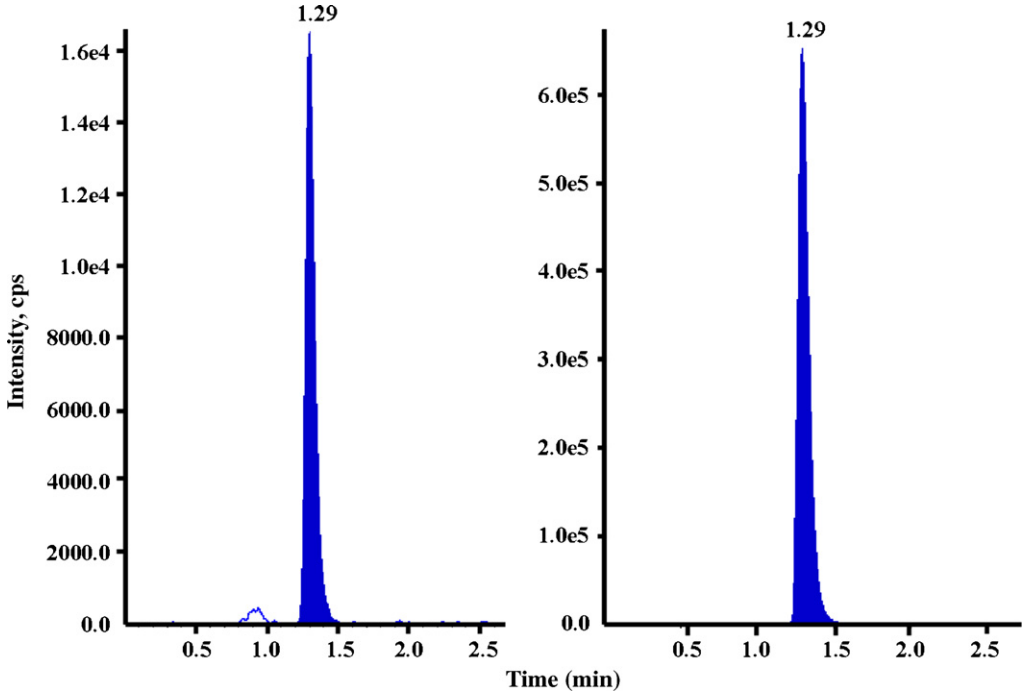

Fig. 3. Representative chromatogram of ethambutol (70.0 ng/mL) (left) and its internal standard (right) in human plasma.

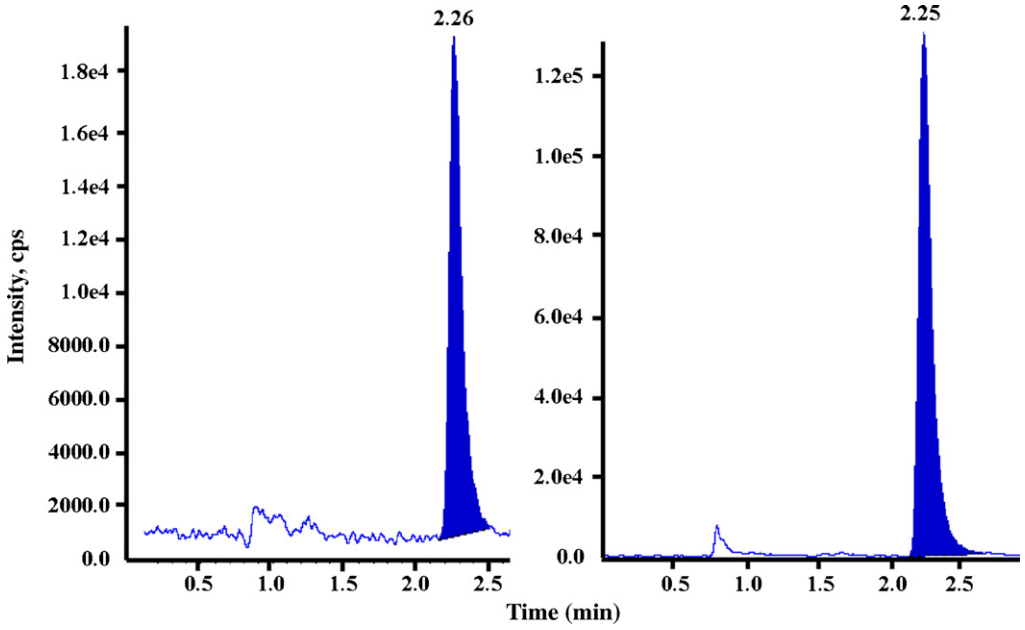

Fig. 4. Representative chromatogram of pyrazinamide (350 ng/mL) (left) and its internal standard (right) in human plasma.

extracted as regular QCs. Regular QC acceptance criteria also apply to the selectivity QCs.

2.7.4. Recovery

To evaluate the loss of drugs and/or internal standards during sample preparation, additional blank matrix samples were also extracted without addition of internal standard. At reconstitution step during extraction, instead of adding reconstitution solutions, three solutions containing all drugs and internal standards at concentrations that mimic the concentrations of low QC, mid QC and high QC in their final extracts were added. Three replicates for each QC level were prepared. The drugs and internal standards area counts from these samples were compared with corresponding precision/accuracy QCs of six replicates to evaluate any loss of either drugs or internal standards. No acceptance criteria were applied to this parameter, but it is preferable to observe consistent recovery for all three QC levels.

2.7.5. Matrix effect

To evaluate matrix effect, blank matrix samples from six different lots were processed without internal standard. At the reconstitution step during extraction, instead of using reconstitution solution, a neat solution was added. The neat solution was prepared by spiking both compounds and their internal standards in the reconstitution solution at concentrations that mimic the final concentrations of both compounds and their internal standards in the final extract for mid QC. These samples refer to post extraction spike samples. Water was aliquotted in triplicates and extracted and reconstituted in the same way as the above post extraction spike samples. These samples refer to pure solution samples. The area counts for all the compounds from the post extraction spike samples were then compared with the area counts from the pure solution samples to evaluate the matrix effect. Matrix effect is calculated as follows: mean peak area from the post extraction spike samples subtracts the mean peak area from the pure solution samples, then divided by mean peak area from the pure solution samples and multiplied by 100. Positive value indicates percent enhancement, and negative value indicates percent suppression.

2.7.6. Stability

Stability of both drugs in different matrices and under different conditions was evaluated. The detailed tests are described below.

Room temperature matrix stability was assessed by leaving low and high QC samples at room temperature for 41 h before extraction. Freeze/thaw matrix stability was assessed by repeatedly freezing/thawing low and high QC samples for three cycles before extraction. The initial freezing was at least 24 h before first thawing. The second and third freezings were at least 12 h. Each thawing step was around 1 h at room temperature. For both room temperature and freeze/thaw matrix stability evaluation, six replicates of each

Table 1
Precisions and accuracies of calibration standards of ethambutol in human plasma from three validation batches.

Analysis group	Theoretical concentration (ng/mL)							
	10.0	20.0	70.0	200	800	2500	4000	5000
	Measured concentration (ng/mL)							
001	10.6	17.3	74.8	201	781	2590	4070	4880
002	9.69	20.7	76.1	FC	809	2510	3900	4590
003	9.85	20.1	73.2	222	832	2510	3740	4370
n	3	3	3	2	3	3	3	3
Mean	10.0	19.4	74.7	212	807	2540	3900	4610
RSD (%)	4.9	9.3	1.9	NA	3.2	1.8	4.2	5.6
Accuracy	100.0	97.0	106.7	106.0	100.9	101.6	97.5	92.2

FC: failed acceptance criteria.

Table 2
Precisions and accuracies of calibration standards of pyrazinamide in human plasma from three validation batches.

Analysis group	Theoretical concentration (ng/mL)							
	50.0	100	350	1000	4000	12500	20000	25000
	Measured concentrations (ng/mL)							
001	50.3	99.5	340	1040	3710	13100	21000	24300
002	49.2	103.0	350	FC	3990	12700	20100	24100
003	52.7	87.6	350	1130	4350	12900	18900	21700
n	3	3	3	2	3	3	3	3
Mean	50.7	96.7	347	1090	4020	12900	20000	23400
RSD (%)	3.5	8.3	1.7	NA	8.0	1.6	5.3	6.2
Accuracy	101.4	96.7	99.1	109.0	100.5	103.2	100.0	93.6

FC: failed acceptance criteria.

QC level were tested, and these two tests were included in a precision and accuracy batch. To evaluate processed-sample re-injection reproducibility, a precision and accuracy batch, after initial injection that passed all criteria, the batch was then stored at 2–8 °C for 73 h and re-injected. Long-term frozen matrix stability was evaluated by storing low mid and high QC samples at both −10 to −30 °C and −60 to −80 °C storage conditions for 56 days before extraction. Six replicates of each QC level, a freshly prepared calibration curve as well as two replicates of freshly prepared low, mid and high QC samples that served as batch acceptance QCs were used for long-term frozen matrix stability evaluation. Stability of stock standard solutions and intermediate standard solutions was also evaluated by comparing the stored stock and intermediate standard solutions to freshly prepared stock and intermediates solutions.

3. Results and discussions

3.1. Liquid chromatography separation

During method development, it was observed that ethambutol and pyrazinamide showed distinctively different chromatographic behavior. For example, on a C_{18} column, when using widely used mobile phase components like formic acid, water, methanol, acetonitrile etc., ethambutol showed very weak retention on this type of column, causing it being eluted into the injection salt valley, causing potential ion suppression. In addition, it showed serious peak tailing. Pyrazinamide showed decent peak shapes as well as decent retention. From the chemical structure as shown in Fig. 2A and B, it can be seen that ethambutol is extremely polar, this should explain why it did not have much retention on a C_{18} column and caused the peak tailing. In the end, still with a C_{18} column, trifluoroacetic acid was added in the mobile phase, a decent peak shape as well as stronger retention was then obtained for ethambutol. This peak shape improvement and increase of retention could be interpreted in two ways: first, addition of trifluoroacetic acid might suppress the ionization of the hydroxyl groups on the silica column, thus reduced the polar interaction between ethambutol and the negatively charged hydroxyl groups on the silica, resulting in a better peak shape, and second, trifluoroacetic acid in the mobile phase might serve as an ion-pairing agent, which paired with ethambutol and increased its retention on C_{18} column. With addition of trifluoroacetic acid in the mobile phases, both ethambutol and pyrazinamide can be analyzed on a C_{18} column simultaneously. The typical chromatograms for ethambutol and pyrazinamide are shown in Figs. 3 and 4.

Table 3
Precisions and accuracies of quality control samples for ethambutol in human plasma.

Analysis group	Statistics	Theoretical concentration (ng/mL)			
		10.0	30.0	400	3750
001	*n*	6	6	6	6
	Intra-assay mean	9.04	27.3	427	4050
	RSD (%)	14.7	8.8	5.2	4.0
	Accuracy (%)	90.4	91.0	106.8	108.0
002	*n*	6	6	6	6
	Intra-assay mean	11.4	30.6	416	3950
	RSD (%)	8.7	8.6	5.2	7.8
	Accuracy (%)	114.0	102.0	104.0	105.3
003	*n*	6	6	6	6
	Intra-assay mean	10.4	31.0	432	3440
	RSD (%)	11.6	3.7	2.8	5.0
	Accuracy (%)	104.0	103.3	108.0	91.7
Overall	*n*	18	18	18	18
	Inter-assay mean	10.3	29.6	425	3810
	RSD (%)	14.5	9.0	4.5	9.1
	Accuracy (%)	103.0	98.7	106.3	101.6

Table 4
Precisions and accuracies of quality control samples for pyrazinamide in human plasma.

Analysis group	Statistics	Theoretical concentration (ng/mL)			
		50.0	150	2000	18800
001	*n*	6	6	6	6
	Intra-assay mean	44.1	129	1950	18800
	RSD (%)	8.8	5.6	6.3	3.4
	Accuracy (%)	88.24	86.0	97.5	100.0
002	*n*	6	6	6	6
	Intra-assay mean	53.8	140	1860	18200
	RSD (%)	5.8	5.5	5.8	8.5
	Accuracy (%)	107.6	93.3	93.0	96.8
003	*n*	6	6	6	6
	Intra-assay mean	54.2	144	2280	16100
	RSD (%)	10.2	5.7	1.4	4.2
	Accuracy (%)	108.4	96.0	114.0	85.6
Overall	*n*	18	18	18	18
	Inter-assay mean	50.7	138	2030	17700
	RSD (%)	12.4	7.0	10.1	8.6
	Accuracy (%)	101.4	92.0	101.5	94.1

Table 5
Precisions and accuracies of dilution QCs for ethambutol and pyrazinamide in human plasma.

Analysis group	Statistics	Theoretical concentration (ng/mL)	
		Ethambutol, 25000	Pyrazinamide, 125000
001	*n*	6	6
	Intra-assay mean	24600	135000
	RSD (%)	2.9	2.7
	Accuracy (%)	98.4	108.0

Table 6
Recoveries at low, mid and high QC levels for ethambutol, ethamtubol-d_4, pyrazinamide and pyrazinamide-d_3 in human plasma.

QC levels	Statistics	Ethambutol	Ethamtubol-d_4	Pyrazinamide	Pyrazinamide-d_3
Low	RSD (%)[a]	5.4/5.9	3.0/1.2	10.5/2.0	8.7/1.7
	Recovery (%)	119.9	106.5	44.8	47.2
Mid	RSD (%)[a]	4.0/2.9	2.7/1.6	3.8/0.2	3.0/0.1
	Recovery (%)	102.2	96.0	39.9	41.0
High	RSD (%)[a]	2.8/2.1	3.9/2.5	3.5/0.2	3.9/0.6
	Recovery (%)	99.5	95.7	50.2	45.9
Overall	Recovery (%)	107.2	99.4	45.0	44.7

[a] RSD: RSD of six replicates of extracted QC samples/RSD of three replicates of recovery samples.

Table 7
Matrix effect for ethambutol, ethamtubol-d_4, pyrazinamide and pyrazinamide-d_3 in human plasma.

Sample type	Post extraction spike	Pure solution	Post extraction spike	Pure solution
Compound	Ethambutol		Ethambutol-d_4	
RSD (%)[a]	2.9	2.8	2.4	1.7
Matrix effect (%)	15.4		13.1	
Compound	Pyrazinamide		Pyrazinamide-d_3	
RSD (%)[a]	5.2	0.9	5.0	0.8
Matrix effect (%)	−6.4		−6.2	

[a] RSD for post extraction spike is from six different matrix lots with one replicate of each, and RSD for pure solution is from three replicates.

Trifluoroacetic acid is well known for its ion suppression. To evaluate its effect on ethambutol and pyrazinamide, the chosen trifluoroacetic acid mobile phases were compared with formic acid mobile phases, which have exactly the same solvent and same percentage of acid in the mobile phases. Under same liquid chromatography and mass spectrometry conditions, compared to formic acid, trifluoroacetic acid showed ~7% enhancement for ethambutol and ~27% enhancement for pyrazinamide.

Under the optimized HPLC and MS conditions, ethambutol and pyrazinamide were baseline separated with retention times of 1.26 and 2.26 min, respectively.

3.2. Calibration curve and linearity

For three consecutive batches, the calibration curves showed an overall accuracy of 92.2–106.7% with RSD of less than 10%. The detailed results are shown in Tables 1 and 2. The applied ranges for ethambutol and pyrazinamide, together with the dilution QC levels, fully cover the clinical concentration levels of around 200–1000 ng/mL for ethambutol and 1000–100,000 ng/mL for pyrazinamide, which were obtained from a cohort study by McIlleron et al. [9].

3.3. Precision and accuracy

Tables 3 and 4 show the inter- and intra-assay precision and accuracy. The method was found to be highly accurate and precise. For ethambutol, accuracy of 90.4–114.0% and precision of 2.8–14.7% RSD for intra-assay, and accuracy of 98.7–106.3% and precision of 4.5–14.5% RSD for inter-assay were obtained for all QC levels including LLOQ. For pyrazinamide, accuracy of 86.0–114.0% and precision of 1.4–10.2% RSD for intra-assay, and accuracy of 92.0–101.5% and precision of 7.0–12.4% RSD for inter-assay were obtained for all QC levels including LLOQ.

Table 5 shows the precision and accuracy of dilution QCs for both ethambutol and pyrazinamide. Accuracy of 98.4% with an RSD of 2.9% for ethambutol and accuracy of 108.0% with an RSD of 2.7% for pyrazinamide were obtained.

Table 8
Stability of ethambutol and pyrazinamide in human plasma under different conditions.

Statistics	Ethambutol			Pyrazinamide		
	Low QC	Mid QC	High QC	Low QC	Mid QC	High QC
		Three freeze/thaw matrix stability				
n	6		6	6		6
RSD (%)	4.1	NA	3.1	2.9	NA	2.4
Accuracy (%)	103.0		91.2	98.0		87.8
		41 h room temperature matrix stability				
n	6		6	6		6
RSD (%)	5.8	NA	1.8	3.9	NA	0.9
Accuracy (%)	106.0		92.8	95.3		87.8
		56 days −10 to −30 °C matrix stability				
n	6	6	6	6	6	6
RSD (%)	3.8	2.1	0.9	4.1	2.2	1.8
Accuracy (%)	108.3	110.0	103.7	89.3	96.0	89.4
		56 days −60 to −80 °C matrix stability				
n	6	6	6	6	6	6
RSD (%)	2.4	2.3	1.1	3.1	0.8	1.9
Accuracy (%)	108.3	109.3	102.1	90.0	95.5	88.8

3.4. Selectivity

In all the tested six lots of blank matrix samples, the retention regions of ethambutol, pyrazinamide and their internal standards were all free of significant interference peaks. For selectivity QCs, accuracy of 96.5–114% with an RSD of 5.8% was obtained for ethambutol while 102.8–118% accuracy and 5.0% RSD were seen for pyrazinamide.

3.5. Recovery

As shown in Table 6, overall recovery of 107.2% for ethambutol, 99.4% for ethambutol-d_4, 45.0% for pyrazinamide and 44.7% for pyrazinamide-d_3 were obtained. Both compounds show consistent recovery results for all three QC levels. Pyrazinamide showed much lower recovery compared to ~100% for ethambutol. Since it is consistent at all three QC levels for both drug and internal standard, no further investigation was done for the cause.

3.6. Matrix effect

As shown in Table 7, matrix effect of 15.4% for ethambutol, 13.1% for ethambutol-d_4, −6.4% for pyrazinamide and −6.2% for pyrazinamide-d_3 were observed. All six matrix lots showed very similar matrix effect for both analytes and their corresponding internal standards.

3.7. Stability

Stability of ethambutol and pyrazinamide in human plasma under different conditions were evaluated. The detailed results are shown in Table 8. As seen from the table, three freeze/thaw cycles, 41 h room temperature storage, 56 days storage at −10 to −30 °C, 56 days storage at −60 to −80 °C, and 73 h processed-sample re-injection reproducibility have been established. In addition, 58 days stability for stock standard solutions and 54 days for intermediate standard solutions stored at 2–8 °C were established. All of these demonstrate the robustness and ruggedness of the method.

4. Conclusions

Addition of trifluoroacetic acid in mobile phase greatly improved the peak shape as well as increased the chromatographic retention of ethambutol, enabling simultaneous analysis with pyrazinamide together on a C_{18} column. Thus, a rapid and robust LC/MS/MS method has been developed for these two anti-tuberculosis drugs in human plasma. This method has been fully validated and can be directly applied to patient sample analysis.

Acknowledgement

The author would like to thank Covance Organic Synthesis lab for providing the internal standards for both ethambutol and pyrazinamide.

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