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Research Article

Ion-Pair Chromatography for Simultaneous Analysis of Ethionamide and Pyrazinamide from Their Porous Microparticles

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Abstract. Ethionamide (ETA) and pyrazinamide (PZA) are considered the drugs of choice for the treatment of multidrug-resistant tuberculosis. Current methods available in the literature for simultaneous determination of ETA and PZA have low sensitivity or involve column modifications with lipophilic cations. The aim of this study was to develop a simple and validated reversed-phase ion-pair HPLC method for simultaneous determination of ETA and PZA for the characterization of polymeric-based porous inhalable microparticles in *in vitro* and spiked human serum samples. Chromatographic separation was achieved on a Phenomenex C_{18} column (250 mm×4.6 mm) using a Shimadzu LC 10 series HPLC. The mobile phase consisted of A: 0.01% trifluoroacetic acid in distilled water and B: ACN/MeOH at 1:1 *v/v*. Gradient elution was run at a flow rate of 1.5 mL/min and a fixed UV wavelength of 280 nm. The validation characteristics included accuracy, precision, linearity, analytical range, and specificity. Calibration curves at seven levels for ETA and PZA were linear in the analytical range of 0.1–3.0 μg/mL with correlation coefficient of r^2>0.999. Accuracy for both ETA and PZA ranged from 94 to 106% at all quality control (QC) standards. The method was precise with relative standard deviation less than 2% at all QC levels. Limits of quantitation for ETA and PZA were 50 and 70 ng/mL, respectively. There was no interference from either the polymeric matrix ions or the biological matrix in the analysis of ETA and PZA.

KEY WORDS: ethionamide; HPLC; microparticles; pyrazinamide; tuberculosis.

INTRODUCTION

Tuberculosis (TB) is one of the most frequent and important infectious diseases that cause morbidity and death (1,2). Current clinical treatment for drug-susceptible tuberculosis which ranges from 6–12 months includes frontline drugs such as rifampicin (RFP), isoniazid (INH), pyrazinamide (PZA), ethambutol, streptomycin, and *p*-amino salicylic acid. The drugs are initially given in a combination of three to four for a few months since the susceptibility of the organism is not certain, and subsequently, the dose is reduced. Resistance to one or more frontline drugs is a result of incomplete adherence to chemotherapy, errors in the case management, and HIV pandemic (3–5). Mycobacterium TB strains can be resistant to either isoniazid or rifampicin or both isoniazid and rifampicin. Strains that are resistant to both isoniazid and rifampicin are known as multidrug-resistant (MDR) strains and are the causative agents of MDR tuberculosis (6). MDR-TB treatment involves the use of second-line drugs that have limited efficacy and are not suitable for short-course treatment. MDR-TB patients in whom treatment failed have high risk of death (7–10). When the MDR-TB strain is resistant to INH and RFP, initial treatment is the combination of ethionamide (ETA), fluoroquinolone, another bacteriostatic drug such as ethambutol, PZA, and aminoglycoside (kanamycin, amikacin, or capreomycin) for 3 months or until sputum purulence conversion. The combination of ETA, ofloxacin, and another bacteriostatic drug (cycloserine) is recommended for at least 18 months during continuation phase (5,11). PZA, a frontline drug, is recommended for MDR-TB since it is effective in acidic medium (bacilli inside macrophages), and also, resistance to PZA is not easy to acquire nor prove (5). Inhaled therapy is the most promising for the treatment of tuberculosis. However, for an efficient bactericidal activity against the mycobacteria, drugs need to be encapsulated into a suitable drug delivery system to sustain their effects and ensure their rapid uptake into macrophages that harbor mycobacterium TB (12).

Microparticles are particulate systems with diameter ranges from 0.1 to 200 μm that can be formulated with biodegradable or nonbiodegradable polymers (13). Within the microparticles, the

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drug is uniformly dispersed throughout the polymeric matrix. Polymeric microspheres are widely used in drug delivery due to their ability to encapsulate a wide range of drugs, biocompatibility, high bioavailability, and sustained release characteristics (14). Poly (DL-lactide-co-glycolide) (PLGA) is widely employed for their preparation due to its biodegradation into compatible metabolite monomers, lactic acid and glycolic acid (15). Yang *et al.* formulated large porous PLGA microparticles with large geometric diameters and small aerodynamic diameters to avoid agglomeration and increase the efficiency of inhalable formulation of porous microparticles. Porous or hollow microparticles aggregate less and disaggregate more easily under shear forces which results in higher aerosolization efficiency (16). Regarding its potential benefits and compliance in anti-TB patients, porous inhalable PLGA microparticles were considered in this study as an efficient inhalable drug delivery system for a binary anti-TB therapy encompassing ETA and PZA. The proposed first part of the study was to develop an efficient chromatographic method for the simultaneous determination of both drugs in *in vitro* and spiked human serum samples. Another study is in progress that not only focuses on the application of quality by design in the formulation and optimization of their microparticles but also tests their clinical performance in volunteers. Scarce information was found in the literature regarding the simultaneous determination of both drugs. The published chromatographic methods for the quantitation of each drug were based on the mass spectroscopy which is expensive for the routine analysis (17,18).

Reversed-phase ion-pair chromatography with perfluorinated carboxylic acids as an ion-pair agent has been used for determination of amino acids and peptides (19–21). Ion-pairing agents such as trifluoroacetic acid (TFA) would increase the affinity of the positively charged solute for the hydrophobic stationary phase as opposed to phosphoric acid. It was reported that TFA could be used as a mobile phase stabilizer and an effective ion-pair reagent to control retention and selectivity (22). The aim of the current study was to develop a validated reversed-phase ion-pair chromatography method for the simultaneous analysis of two anti-TB drugs, namely ETA and PZA (Fig. 1), for the characterization of their porous PLGA microparticles in *in vitro* and spiked human serum samples. TFA was employed as an ion-pairing agent for the simultaneous separation and further elution of the two drugs.

METHOD AND MATERIALS

Materials

Ethionamide, pyrazinamide, and perchloric acid were purchased from Sigma-Aldrich (MO, USA). HPLC-grade acetonitrile, methanol, tetrahydrofuran, dimethyl sulfoxide, and polyvinyl alcohol (PVA) (molecular weight (Mwt) of 40 kDa) were purchased from Fisher Scientific (NJ, USA). TFA and acetaminophen USP were purchased from EM Sciences (NJ, USA) and City Chemical Corporation (NJ, USA), respectively. PLGA 50:50 was purchased from Durect Corporation (AL, USA). Millipore™ ultrapure water was used for the preparation of mobile phase and sample working standards. Pooled drug-free human serum type AB was purchased from MP Biomedicals (OH, USA).

Instrumentation and Chromatographic Conditions

Shimadzu HPLC system equipped with dual LC-10ADvp pumps, DGU-14A degasser, SPD-10ADvp UV–vis detector, SIL-10ADvp autosampler, and SCL-10Avp system controller was used. Data were collected using Class VP v5.0 software. The mobile phase was composed of A: 0.01% TFA in water and B: ACN/MeOH (50:50). Gradient elution run was carried out on a Phenomenex Luna C18 column (250 mm×4.6 mm, 5 μm) with Zorbax C18 (17×1 mm, 5 μm) guard column at a flow rate of 1.5 mL/min. The gradient run was started with 100% A and 0% B then 2% B at 1 min, 70% B at 16 min through 18 min, and 2% B at 20 min through 25 min (end of the run). The wavelength was fixed at 280 nm, and injection volume was 20 μL.

Preparation of Standard Solutions

Standard stock solutions of ETA, PZA, and acetaminophen (internal standard) were prepared in amber-colored volumetric flasks by dissolving 100 mg of each drug in 100 mL ACN/MeOH (50:50) with the aid of stirring for 10 min to obtain a final concentration of 1 mg/mL each. Calibration and system suitability standard solutions were prepared by diluting the above standard solution.

System Suitability Standard

System suitability standard solutions were prepared daily by diluting the stock solution with the mobile phase to obtain the working system suitability standard concentration of 1 μg/mL. System suitability was determined from six replicates of the working solution before sample analyses. The USP acceptance criteria was relative standard deviation (RSD) less than 2% for peak area, theoretical plates >3,000, USP tailing factor ≥0.5 and ≤2.0, and capacity factor (k') greater than 2.0 (23).

Method Validation

Linearity and Range

Standard calibration curves were constructed with seven calibrators over a concentration range of 0.1–3.0 μg/mL for both ETA and PZA using acetaminophen as an internal standard (IS) at a concentration of 1.5 μg/mL. The analytical range was established by demonstrating the accuracy and precision at both the lowest and highest concentrations of the calibration range.

Accuracy and Precision

Accuracy and precision of the method were determined by analyzing the quality control (QC) standard samples at three concentrations of both ETA and PZA (0.1, 1.2, 3.0 μg/mL). The method precision was established by five replicate injections of three QC levels for the intraday precision and on three successive days for the intermediate precision. Precision and accuracy were expressed as RSD and percent recovery, respectively, at three standards for 3 days.

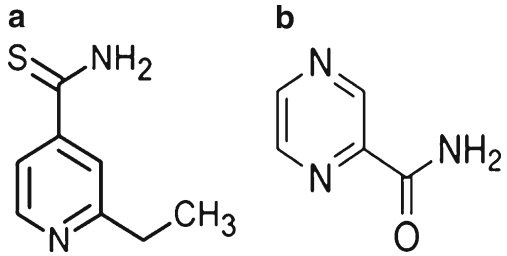

Fig. 1. Chemical structures of **a** ethionamide and **b** pyrazinamide

Limit of Quantitation and Limit of Detection

Concentration at the signal-to-noise ratio (SN) of 10:1 was the limit of quantitation (LOQ), and SN of 3:1 was the limit of detection (LOD), estimated from the following formula:

$$LOQ = \frac{C \times SN}{SN_M}$$

where C is the concentration being evaluated, SN is the signal-to-noise ratio of 10:1 for LOQ and 3:1 for LOD, and SN_M equals H/N_D, where SN_M is the calculated signal-to-noise ratio, H is the peak height, and N_D is the measured noise height.

Specificity

The ability of the developed method to separate both drugs from the *in vitro* samples during the development of porous microparticles was investigated. Porous microparticles were prepared by modification of the double emulsion method described by Yang *et al.* (16). Briefly, a specified amount of PLGA polymer (50:50) was dissolved in a mixture of dichloromethane and methanol as the organic phase. The internal aqueous phase was a solution of the effervescent salt ammonium bicarbonate (NH_4HCO_3) in distilled water. Two milliliters of the above solution was added dropwise to the organic phase followed by homogenization at 4,000 rpm for 6 min. The resultant w/o emulsion was added dropwise to 1% PVA solution while homogenizing at 5,000 rpm for 8 min. The double emulsion (w/o/w) formed was thus stirred at 900 rpm using a paddle stirrer (R1376, IKA Works Inc., NC, USA) for 3 h followed by centrifugation at 15,000 rpm for 30 min to harvest the formed microparticles. The porous microparticles were then washed with 10 mL of distilled water to remove the adsorbed free drug and PVA then dried under vacuum overnight. Drug loadings were performed by dissolving the specified amount of the two drugs in the primary aqueous phase. The matrix factor was determined by both extracting the two drugs from the formed microparticles and the free drug solutions after harvesting and washings. For efficient extraction of both drugs, the loaded porous microparticles were dissolved in 100 mL of tetrahydrofuran and dimethyl sulfoxide (1:1 *v/v*) solution by stirring followed by homogenization and vortexing. The above solution was spiked with IS. This solution was diluted to a system suitability working concentration of 1 μg/mL with the mobile phase. Matrix factor percent RSD was determined from six runs of microparticle drug extracts from the microparticles using the following equation:

$$Matrix\ factor(\%) = \frac{Response\ factor\ in\ presence\ of\ matrix\ ions}{Response\ factor\ in\ absence\ of\ matrix\ ions} \times 100$$

The matrix effect for the determination of both drugs in spiked human serum samples was also investigated. Sample preparation to determine the specificity of the method to quantitate both drugs in human serum was performed as described by Watabe *et al.*, with some modifications (24). Briefly, 100 μL of 6% (*w/v*) perchloric acid was added to 200 μL of human serum spiked with ETA, PZA, and IS. This mixture was vortexed for 1 min followed by addition of

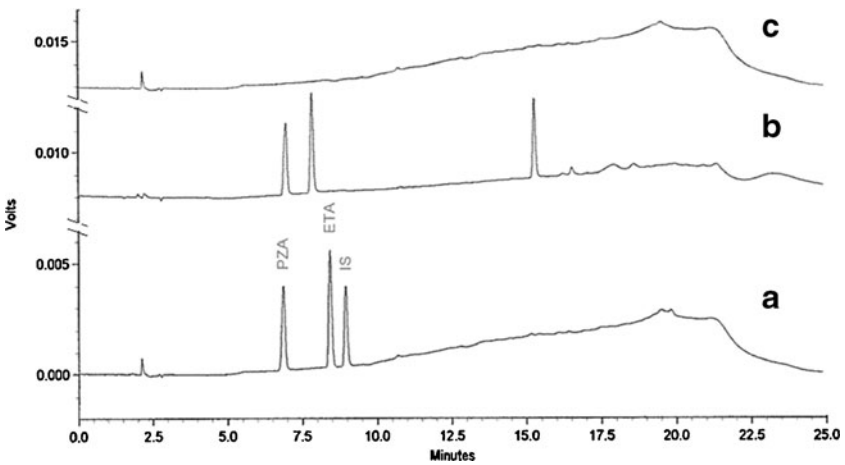

Fig. 2. Chromatograms of *A* system suitability standard of 1 μg/mL ETA and PZA, *B* system suitability standard of 1 μg/mL ETA and PZA, and *C* blank run after sample analysis

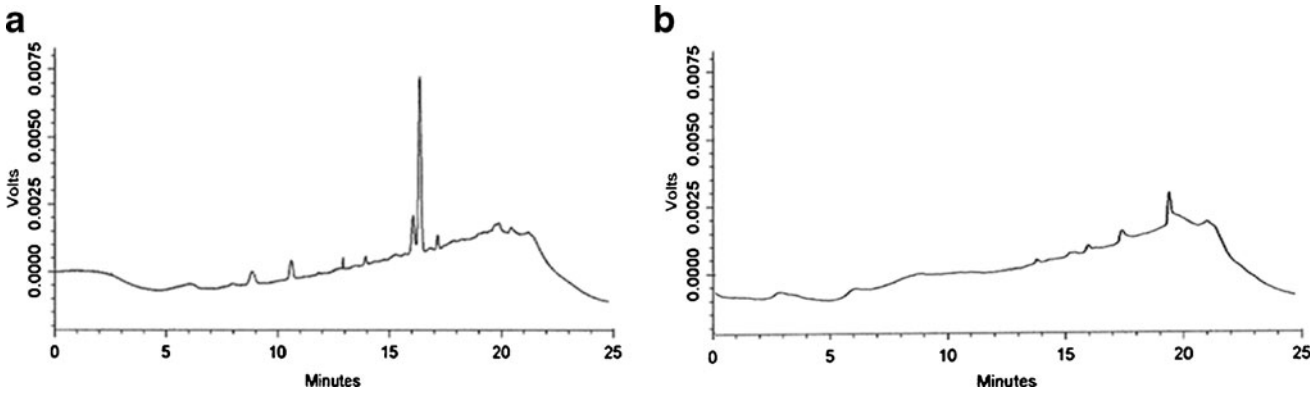

Fig. 3. Chromatograms showing **a** the ghost peaks with a null injection (without injecting sample or blank) due to mobile phase contamination and **b** a decrease in ghost peaks by optimizing a fresh mobile phase to A: 0.01% TFA in distilled water and B: ACN/MeOH (50:50)

100 μL methanol. The mixture was then centrifuged at 17,000 rpm for 30 min, and the supernatant collected was diluted as required and used for specificity determination as described with the *in vitro* samples.

Robustness

Robustness examines the effect that operational parameters have on the analysis results (25). Robustness shows the reliability of an analysis with respect to deliberate variations in method parameters. To determine the robustness of the method, the elution parameters were varied individually by adjusting the mobile phase pH (±0.2), detector wavelength (±5 nm), organic phase percentage of the mobile phase (±5%), and HPLC pump flow rate (±0.1 mL/min).

RESULTS AND DISCUSSION

Method Development and Optimization

The available methods for the simultaneous determination of ETA and PZA have some limitations such as the high LOQ (26,27) and the use of lipophilic cations to modify the stationary phase that can deteriorate the HPLC column for other applications (28). The main objective of this study was to develop a simple reversed-phase ion-pair HPLC method for simultaneous analysis of both drugs from the *in vitro* and spiked human serum samples during characterization of their porous microparticles. Because PZA is more hydrophilic than ETA, gradient elution was preferred over isocratic to avoid the elution of PZA in the void volume or the peak shape deterioration for ETA. High baseline drift was a common problem with high-pressure binary gradient elution method. With a steep gradient, there was either an increase in baseline drift or a decrease in resolution between ETA and PZA. The gradient elution of 2% organic phase at 1 min to 70% organic phase at 16 min resulted in a lower baseline drift and improved resolution between the two compounds. Compared to *o*-phosphoric acid as an ion-pairing agent, TFA increased not only the resolution between compounds of interest but also the sensitivity to detect both ETA and PZA (Fig. 2, traces A and B). As indicated from the clear chromatogram after sample blank run, there was no significant carryover or overretention of the two compounds (Fig. 2, trace C). The mobile phase was prepared daily as mobile phase contamination was one of the primary reasons for the ghost peaks in gradient elution (29). It was observed that the incidence of ghost peaks decreased significantly with the use of fresh mobile phase. In addition, there was no interference from the ghost peaks in the analysis of compounds of interest (Fig. 3).

Table I. System Suitability of 1 μg/mL of Both ETA and PZA (n=6)

Parameters	Specifications[a]	Day 1	Day 2	Day 3
ETA system suitability standard, 1 μg/mL				
Capacity factor (k')	>2.0	3.22	3.21	3.21
Capacity factor (RSD)	≤2.0	0.067	0.067	0.053
Area (% RSD)	≤2.0	1.25	1.27	1.54
Plates (column)	>3,000	35,236	34,456	35,962
USP tailing	(0.5–2.0)	1.11	1.11	1.11
Pyrazinamide system suitability, 1 μg/mL				
Capacity factor (k')	>2.0	2.46	2.42	2.43
Capacity factor (RSD)	≤2.0	0.093	0.14	0.094
Area (% RSD)	≤2.0	0.69	1.03	1.45
Plates (column)	>3,000	12,982	13,037	16,995
USP tailing	(0.5–2.0)	0.87	0.90	1.02

Standard deviations of the listed values did not exceed 2%
ETA ethionamide, *RSD* relative standard deviation, *USP* United States Pharmacopeia
[a] Acceptance criteria per the USP 35/NF 30 specifications

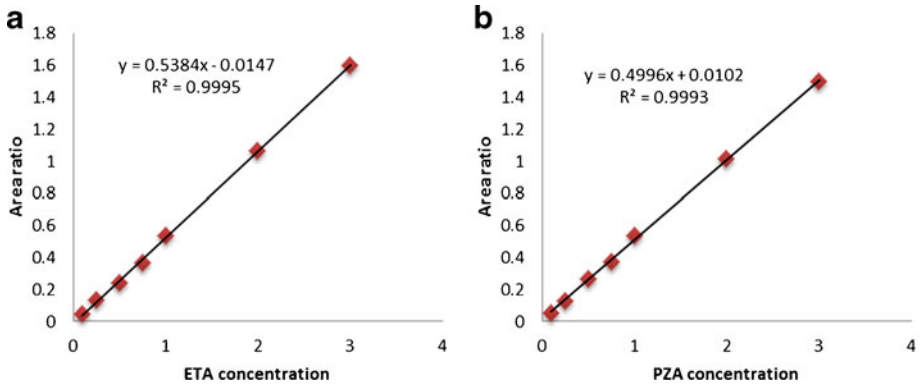

Fig. 4. Calibration curves of **a** ETA and **b** PZA for analytical range of 0.1–3.0 μg/mL

Table II. Parameters and Linearity Data of Both ETA and PZA Calibration Curves

Standard curves	Range, μg/mL	Calibrators	Slope	*Y*-intercept	R^2
ETA calibration					
Day 1	0.1–3.0	7	0.5384	−0.0147	0.9995
Day 2	0.1–3.0	7	0.5394	−0.0134	0.9994
Day 3	0.1–3.0	7	0.5391	−0.0142	0.9995
PZA calibration					
Day 1	0.1–3.0	7	0.4996	0.0102	0.9993
Day 2	0.1–3.0	7	0.4989	0.0103	0.9995
Day 3	0.1–3.0	7	0.4992	0.0104	0.9994

ETA ethionamide, *PZA* pyrazinamide

The detection sensitivity of the developed method to ETA increased significantly by the addition of methanol to the organic phase. The optimum absorptivity was observed with a 1:1 *v*/*v* acetonitrile to methanol as the organic compartment of the mobile phase. It is worthy to note that increasing the methanol percentage in the organic phase more than 50% resulted in peak shape deterioration of both compounds.

System Suitability

The system suitability test verifies that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be conducted (30,31). Six replicates of system suitability standard 1 μg/mL of both PZA and ETA were injected for three successive days. All critical parameters tested (k', peak area RSD, retention time RSD, column plates, tailing, *etc.*) met the USP acceptance criterion on all days (Table I). Area RSD was less than 2% on all 3 days for both drugs (Table I). Both ETA and PZA passed the USP tailing and capacity factor criteria with values <2 and >2, respectively. Adequate resolution of >3 between PZA and ETA peaks ensured the specificity of the method to analyze both compounds.

Linearity and Analytical Range

Linearity of the method was established by preparing standard calibration curves over the analytical range of 0.1–3.0 μg/mL for both ETA and PZA (Fig. 4). This narrow but

Table III. Accuracy of Both ETA and PZA (n=5)

Sample	0.1 μg/mL	1.2 μg/mL	3.0 μg/mL
Ethionamide			
Day 1	106.97%	102.48%	101.09%
Day 2	101.21%	106.45%	100.44%
Day 3	98.82%	103.57%	97.44%
Pyrazinamide			
Day 1	104.71%	100.43%	99.11%
Day 2	106.45%	104.67%	99.66%
Day 3	94.61%	102.42%	97.44%

Accuracy was expressed as the percent recovery of QC samples. Standard deviations of the listed values did not exceed 2%

Table IV. Precision of Both ETA and PZA (Percent RSD, n=5)

Sample	0.1 μg/mL	1.2 μg/mL	3.0 μg/mL
Ethionamide			
Day 1	0.83	1.04	0.72
Day 2	1.48	1.33	0.41
Day 3	1.41	0.41	0.33
Pyrazinamide			
Day 1	0.39	0.92	1.08
Day 2	1.55	1.17	0.97
Day 3	1.76	1.12	1.11

Standard deviations of the listed values did not exceed 2%

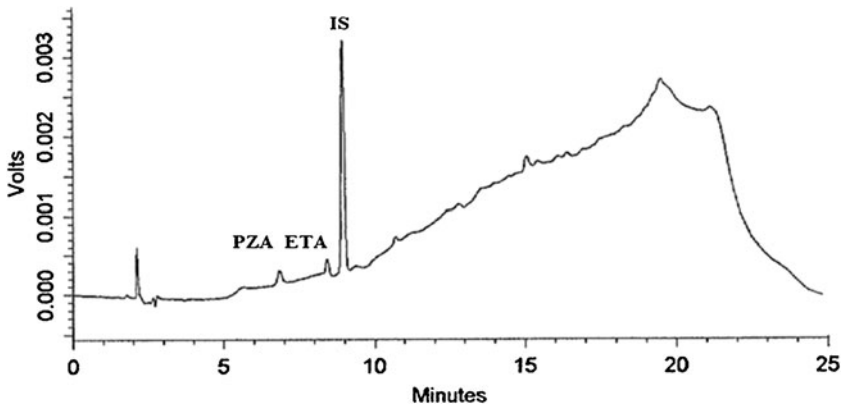

Fig. 5. Chromatogram of simultaneous ETA and PZA elution at their LOQ concentrations

chromatographically necessary analytical range was first a result of ETA sensitivity where, below 0.1 μg/mL, sensitivity was lost, and secondly, above 3 μg/mL results in trace amounts of ETA sticking on the column, causing the excessive need to wash the column with mobile phase. The results in Table II show a linear correlation between analyte peak area and the concentration of the drug over the analytical range with $R^2 \geq 0.999$.

Accuracy and Precision

Accuracy and precision were established across the analytical range for both compounds. The intra-and interday accuracy and precision were calculated using multiple injections of the QC samples. Results for the intraday accuracy and precision of PZA and ETA are summarized in Tables III and

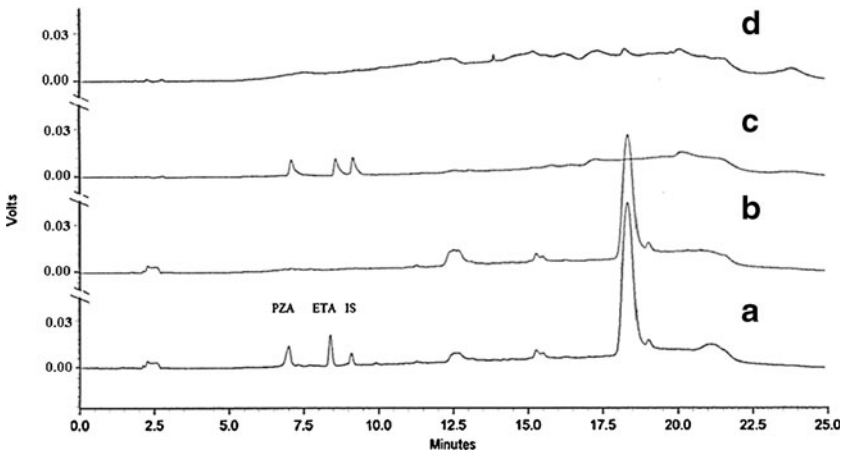

Fig. 6. Chromatograms of *A* drugs' extract from destruction of drug-loaded PLGA porous microparticles with ETA, PZA, and IS; *B* extract of drug-free PLGA porous microparticles; *C* drugs' extract from human serum loaded with both ETA and PZA at a concentration of 1 μg/mL; and *D* extract of drug-free human serum

IV, respectively. The accuracy across the analytical ranged from 94.6 to 107% for all QC samples. Despite being within the acceptance limits, the matrix effect resulted in accuracy of more than 100% in the higher QC samples.

Limit of Quantitation and Limit of Detection

LOQs determined by estimating signal-to-noise ratio of 10:1 for ETA and PZA were 50 and 70 ng/mL, respectively (Fig. 5). Area RSD for six runs of both the compounds at the LOQ concentrations was <3%. LODs determined by estimating signal-to-noise ratio of 3:1 for ETA and PZA were 15 and 21 ng/mL, respectively.

Specificity

Specificity was established by observing no peaks in the blank chromatograms and no coeluting peaks in the drugs' loaded samples shown in Fig. 6. Additionally, the Shimadzu Class VP peak purity software was used to evaluate diode array spectral data of the chromatographic peaks and determined that no impurities were present or coeluting with both drugs. The validated method was used to assess its potential utility not only to characterize the porous microparticles loaded with both drugs but also to analyze spiked human serum samples for the determination of both drugs in human blood. Direct determination of the drugs' loadings was done by extraction from their formulation in biodegradable PLGA-based microparticles. The extract was diluted with mobile phase and injected onto the HPLC using the described validated method. Representative chromatograms for ETA and PZA are shown in Fig. 6 (trace A and B). The chromatogram showed that there was no interference from the microparticle components with the peaks of both drugs. The mean matrix factors for six runs of the drugs' extract from destruction of drug-loaded PLGA porous microparticles with ETA and PZA were 0.95 and 0.99, respectively. The matrix factor percent RSD for six runs of ETA and PZA were 1.28% and 1.21%, respectively. The drug-loaded blood sample was utilized to assess the potential of the analytical method to qualitatively separate both drugs from the biological matrix as a surrogate for evaluating the bioavailability of both drugs after extravascular administration. For this purpose, drug-loaded human serum was diluted with mobile phase and injected on the HPLC using the described method. Representative chromatograms for both drugs are shown in Fig. 6 (traces C and D). The chromatograms show that there was no coelution of both drugs with any of the biological matrix components. Moreover, the mean matrix factors for six runs of the drugs' extract from human serum loaded with both ETA and PZA at concentrations of 1 μg/mL were 1.02 and 0.97 with RSD values of 1.02% and 2.27%, respectively. Further formulation screening *in vitro* and *in vivo* studies are in progress for optimizing the formulation parameters for higher drug loadings and bioavailability using the validated analytical method.

Robustness

The robustness of the method was evaluated by making small adjustments to the operating parameters of the system suitability procedure. The obtained data (not shown) indicated that the method could handle small deliberate changes to the operating conditions while maintaining its accuracy, precision, and reproducibility. All robustness data remained <5% RSD for each adjusted parameter with the exception of increased pH from nominal to high. For example, the capacity factor (k') of ETA elution changed from 3.2±0.06 to 3.1±0.04 by varying the pH of the mobile phase from 2.8 to 3.2, respectively. Variability increased to 3.1% RSD due to sensitivity to increasing pH. As a result, none of the adjustments caused a significant change ($p<0.05$) in resolution between ETA system suitability standard and PZA, peak area, theoretical plates, and/or USP tailing factor.

CONCLUSION

Insufficient therapeutic levels of ETA and PZA in patients diagnosed with MDR-TB can lead to death or cases of extensive drug-resistant tuberculosis. A validated analytical method would be helpful for the therapeutic drug monitoring and can be also applied for simultaneous pharmacokinetic studies of ETA and PZA. In this prospect, a simple and efficient gradient reverse-phase ion-pair HPLC method has been developed and validated according to USP category I. The proposed method was accurate, precise, sensitive, specific, and linear in the range specified. Moreover, it can be successfully used for determination of ETA and PZA from both the *in vitro* and spiked human serum samples of their porous PLGA microparticles and similar formulations with PLGA matrix.

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