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**Article**

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# Development and Validation of a Simple High-Pressure Liquid Chromatography-Ultraviolet Detection Method for Simultaneous Quantitation of First-Line Anti-Tuberculosis Drugs in Formulations of Fixed-Dose Combination

**Sudha Vilvamani[†], Santhanamahalingam Mahalingam[†], Sruthi Nhavilthodi, Dharman Murugesan and Shanmugam Murugaiha Jeyakumar[*]**

Department of Clinical Pharmacology, ICMR-National Institute for Research in Tuberculosis, Chetpet, Chennai, Tamil Nadu 600031, India
[*]Author to whom correspondence should be addressed. Email: smjkumar@gmail.com, jeyakumar.sm@icmr.gov.in
[†]These authors contributed equally to this work.

**The current treatment protocol for drug-sensitive tuberculosis involves all four first-line anti-tuberculosis drugs: rifampicin, isoniazid, pyrazinamide and ethambutol hydrochloride in a single tablet, known as fixed-dose combination tablets. However, the analytical methods are scanty to test all these drugs simultaneously in a single run without any pre-sample process or using a simple method suitable for resource-limited settings. In this method, 50 mM potassium phosphate buffer containing 0.2% triethylamine (without pH adjustment) added with acetonitrile (98:2, v/v) was served as mobile phase A, while mobile phase B was 100% acetonitrile. All four drugs were separated within 10.3 min using a gradient mobile phase program in a C18 column (150 mm × 4.6 mm; 5 $\mu$m) and detected at two ultraviolet wavelengths (238 nm for rifampicin, isoniazid and pyrazinamide, and 210 nm for ethambutol hydrochloride). The method was selective, sensitive and linear with a correlation coefficient >0.999 with the acceptable precision and accuracy (<2% relative standard deviation) for all four drugs. In conclusion, the method is simple and it does not require any pH adjustment of the buffer/mobile phase, and within 11 min, the separation of all four drugs can be achieved. Overall, the method is suitable for quality testing of fixed-dose combination tablets in limited-resource settings.**

## Introduction

Tuberculosis (TB) is the second leading cause of death from infectious diseases, caused by a bacterial agent called *Mycobacterium tuberculosis*. According to the WHO Global Tuberculosis Report 2023, more than 7.5 million new cases of pulmonary TB and around 1.3 million deaths due to TB have been recorded worldwide during 2022, and majorly reported from countries with limited resource settings. To mitigate the spread of this communicable disease, the WHO constantly adopts and guides several strategies from time to time so that the TB elimination goal is achieved by 2030 (1). In this context, one of the strategies and recommendations introduced in the treatment protocol for drug-sensitive TB was the use of fixed-dose combination (FDC) tablets of first-line anti-TB drugs by replacing individual antibiotics to increase compliance and adherence to the treatment. The combination of four first-line drugs in a single tablet has overcome the limitations faced by various stakeholders involved in the TB elimination mission (WHO Report, 1999) (2). However, this has also posed a few challenges, such as testing of the formulations for their content uniformity and routine quality checking purposes, due to the non-availability of a single and simple method to simultaneously quantify all four first-line drugs. Way back in 1991, Gaitonde and Pathak (3) have reported a method for the estimation of isoniazid, pyrazinamide and rifampicin in combined dosage form using a reverse-phase liquid chromatography. Although few other methods can analyze some of these drugs in pharmaceutical formulations/FDC tablets, either individually or in combination (though not all), they involve pretreatment or derivatization of the parent compounds/drugs before analyzing by any analytical methods. Further, these methods are time-consuming, laborious and complex (4–8).

In 2002, Calleri *et al.* (9) have reported a simple and accurate high-pressure liquid chromatography (HPLC) method for the first time to determine isoniazid, rifampicin and pyrazinamide simultaneously in a pharmaceutical formulation. However, the method was devoid of ethambutol, the one of drugs in the FDC tablets. Later, Chellini *et al.* (10) reported a simple HPLC-ultraviolet (HPLC-UV) method to detect all four first-line drugs simultaneously in pharmaceutical formulations. By a thorough systematic approach, the authors could achieve the separation and quantification of all four drugs by employing dual wavelengths for the simultaneous detection (238 nm for rifampicin, isoniazid and pyrazinamide, 210 nm for ethambutol hydrochloride) and validated according to the international guidelines. As described previously, TB is highly prevalent in underdeveloped and developing countries, and several factors play an important role in achieving the TB elimination mission. Among many, the quality testing of pharmaceutical formulations/FDC is paramount, and for that, there is a need for a simple HPLC method to perform such assays even in limited-resource settings. In this context, we developed a simple HPLC-UV method to detect the first-line anti-TB drugs simultaneously and validated it according to the guidelines of the International Council for Harmonization

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(ICH), which may be suitable for quality testing of FDC tablets in programmatic settings with limited resources.

## Experimental

### Chemicals and reagents

Potassium dihydrogen phosphate (Monobasic; $KH_2PO_4$), dipotassium hydrogen phosphate (Dibasic; $K_2HPO_4$), triethylamine and pure compounds of rifampicin, isoniazid, pyrazinamide and ethambutol hydrochloride were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). HPLC-grade acetonitrile was obtained from Merck (Merck Life Science Private Ltd, Mumbai, India). HPLC columns and guard columns were from Merck (Merck KGaA, Darmstadt, Germany). FDC tablets (Macleods Pharmaceutical Ltd, Mumbai, India) were gifted for testing purposes.

### Chromatographic system

An HPLC system (Model: UFLC) with a photodiode array detector (SPD-M20A) (Shimadzu Corporation, Kyoto, Japan) consisting of a binary pump (LC-20-AD), degasser (DGU-20AS), temperature-controlled column oven (CTO-20A) and an auto sampler (SIL-20ACHT) was used for the analysis. The data acquisition was done with the Shimadzu's Lab Solution software (V. 5.92).

### Chromatographic conditions

Initially, different ratios and/or concentrations of acetonitrile and triethylamine in the potassium phosphate buffer (equimolar of monobasic and dibasic) were tried, in addition to variable gradient and time programming for the elution of all four drugs: rifampicin, isoniazid, pyrazinamide and ethambutol hydrochloride, using a reverse phase $C_{18}$ column (Purospher STAR RP-18 endcapped, 150 mm × 4.6 mm; 5 $\mu$m particle size) along with a guard column (30 mm × 4.6 mm; 5 $\mu$m particle size). Finally, the chromatographic separation was achieved with a gradient elution program consisting of two mobile phases (A and B), as described below, and the analysis was performed. 50 mM potassium phosphate buffer containing 0.2% triethylamine (the apparent pH of the mixed solvent was 7.16) was prepared and added with acetonitrile (98:2 v/v) and served as mobile phase A, while mobile phase B comprised 100% acetonitrile. The gradient program was set as, for the initial 7 min, 100% of mobile phase A, then the mobile phase B increased to 52% up to 11 min and then for the next 4 min 100% of mobile phase A for column equilibration. The total gradient run time was 15 min with a flow rate of 1.5 mL/min. The auto-sampler and column-oven temperatures were maintained at 4°C and 40°C, respectively, during the run. A sample volume of 25 $\mu$L was injected into an HPLC system, and the eluted compounds were detected at UV wavelengths of 238 nm (for rifampicin, isoniazid and pyrazinamide) and 210 nm (for ethambutol hydrochloride). A freshly prepared mobile phase was used for the analysis after filtering through a 0.2 $\mu$m membrane and degassing by ultrasonication.

### Preparation of stocks and working standards

The stock solution was prepared by accurately weighing and dissolving the pure compounds of rifampicin (10.0 mg), isoniazid (5.0 mg), pyrazinamide (28.0 mg) and ethambutol hydrochloride (20.0 mg) in 25 mL of mobile phase A in a volumetric flask to obtain the final concentration of 400, 200, 1120 and 800 $\mu$g/mL, respectively. The stock solution was sonicated for 10 min. From the stock solutions, working standards were prepared by making appropriate dilutions using the mobile phase A to get a desired concentration range (40, 80, 120, 160, 200 and 300 $\mu$g/mL for rifampicin, 20, 40, 60, 80, 100 and 150 $\mu$g/mL for isoniazid, 112, 224, 336, 448, 560 and 840 $\mu$g/mL for pyrazinamide and 80, 160, 240, 320, 400 and 600 $\mu$g/mL for ethambutol hydrochloride) for method validation.

### Method validation

The optimized method was validated according to the ICH guidelines Q2 (R1) (11) for linearity, sensitivity, accuracy, precision and robustness. In addition, the system suitability test was performed according to the US Pharmacopeia guidelines (12). The stock solutions were prepared afresh to test the validation parameters and performed in replicates.

#### System suitability test

The system suitability test was carried out using varying concentrations of rifampicin (80, 120, 160, 200 and 300 $\mu$g/mL), isoniazid (40, 60, 80, 100 and 150 $\mu$g/mL), pyrazinamide (224, 336, 448, 560, 840 $\mu$g/mL) and ethambutol hydrochloride (160, 240, 320, 400, 600 $\mu$g/mL) from three different stock solutions and evaluated peak area, retention time, theoretical plates and tailing factor for each drug.

#### Linearity

Initially, to check the linearity, varying concentrations of rifampicin (12, 16, 20, 32, 40, 80, 120, 160, 200, 300 and 400 $\mu$g/mL), isoniazid (6, 8, 10, 16, 20, 40, 60, 80, 100, 150 and 200 $\mu$g/mL), pyrazinamide (33.6, 44.8, 56, 89.6, 112, 224, 336, 448, 560, 840 and 1120 $\mu$g/mL) and ethambutol hydrochloride (24, 32, 40, 64, 80, 160, 240, 320, 400, 600 and 800 $\mu$g/mL) working standards were prepared and tested. Based on the correlation coefficients and slopes obtained from the calibration curves, six concentrations of each drug ranging between 40–300, 20–150, 112–840 and 80–600 $\mu$g/mL for rifampicin, isoniazid, pyrazinamide and ethambutol hydrochloride, respectively, were confirmed for its linearity.

#### Sensitivity and specificity

The sensitivity of the optimized method was measured in terms of limit of detection (LOD) and limit of quantification (LOQ). Both LOD and LOQ were calculated by the regression analysis of standards of six concentrations of each drug (using the standard deviation of the response and slope) according to the ICH guidelines (i.e., concentration that yields a signal-to-noise ratio of 3.3:1 for LOD and the lowest concentration that could be measured with a signal-to-noise ratio of 10:1 for LOQ).

To test the specificity of the method, mobile phase A (used for standard preparations) was injected as a blank to rule out any other compound eluting at the same retention time as these anti-TB drugs. Further, the peak purity index was obtained for each compound using Shimadzu's Lab Solution software (V. 5.92).

#### Accuracy

The method accuracy was done in a blinded manner, i.e., the spiking of standard solution with a known concentration was blinded to the person who performed the HPLC analysis. The standard solution spiked with four known/nominal

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concentrations of each drug (Low-1, Medium-2 and High-1) was analyzed using the optimized method parameters in three different stocks in replicates. Based on the obtained concentrations, the recovery was calculated and expressed as an accuracy in percentage.

### Precision

The precision was determined by evaluating interday and intraday variations in the obtained concentrations from the nominal/known concentrations of each drug spiked in the standard solutions and calculated as the relative standard deviation (RSD) in percentage for the obtained concentration.

### Robustness

The robustness was tested ($n = 3$) by making changes in the column-oven temperature (40°C and 32°C) and auto-sampler temperature (4°C and 15°C) in the optimized method. In addition, we tested a change in buffer pH to see whether it affects the response of these anti-TB drugs or not. For this purpose, we deliberately adjusted the pH of the 50 mM phosphate buffer containing 0.2% triethylamine to 7.0 using orthophosphoric acid (OPA) and then performed the HPLC analysis of five standard stocks ($n = 5$) as per the optimized condition.

### Ruggedness

The ruggedness of the method was evaluated ($n = 3$) by analyzing the same stock solution by different analysts and using triethylamine for the mobile phase from different make (Rankem, Avantor Performance Materials India Ltd, Thane, India).

### Stability

The stability of standard stocks was evaluated to demonstrate that the method is stability-indicating. For this purpose, three standard stocks were prepared, aliquoted and stored (protected from light) at room temperature (RT) of 4°C and −20°C. An aliquot from standard stocks of each testing condition (i.e., RT of 4°C and −20°C) was analyzed after 24 h, 48 h, 72 h and 1 week. The peak area/response was compared to those obtained with freshly prepared stocks/standards and expressed in percentage.

## Determination of FDC drugs in tablets

FDC tablets (containing 150 mg rifampicin, 75 mg isoniazid, 400 mg pyrazinamide and 275 mg ethambutol hydrochloride from Macleods; Batch No. ETN21055A) were weighed, crushed individually ($n = 3$) into powder and carefully transferred to a conical flask containing ∼75 mL of mobile phase A and mixed well. Then, the solution was sonicated for 10 min, filtered and transferred to a volumetric flask, and made up to 100 mL. From this stock, 1 in 10 dilution was made using mobile phase A to obtain the concentration of 150, 75, 400 and 275 $\mu$g/mL of rifampicin, isoniazid, pyrazinamide and ethambutol hydrochloride, respectively, and 25 $\mu$L was injected into the HPLC system for analysis. In addition, from the same batch of FDC strip, five tablets were weighed together and crushed into powder. From this, five portions were weighed and processed for analysis as described before (in penta-duplicate/pentaplicate); however, the concentration was adjusted to 300, 150, 800 and 550 $\mu$g/mL for rifampicin, isoniazid, pyrazinamide and ethambutol hydrochloride, respectively. Further, the peak purity index of these eluted compounds was obtained using Shimadzu's Lab Solution software (V. 5.92).

# Results

## Method development

The main objective of this work was to develop a simple HPLC method to measure all four first-line anti-TB drugs in formulations for low-resource program settings with acceptable accuracy and precision. During the initial phase of the testing, we used mobile phase A (50 mM potassium phosphate buffer) with or without acetonitrile at 2% and 4%. In the absence of acetonitrile in mobile phase A, the rifampicin peak did not elute separately, while the presence of acetonitrile helped in separating the rifampicin peak. Further, at the 2% level, the resolution was found to be better, and the tailing factor was <2.0. With this mobile phase A and acetonitrile (mobile phase B), a simple gradient program was developed after several trials to achieve the separation of each drug with a tailing factor of <2.0 and the elution of all drugs within 11 min (Figure 1).

## Method validation

### System suitability

According to the system suitability test performed under the optimized conditions, RSD of retention time, number of theoretical plates and tailing factors for all four drugs was within the acceptable limit of 2% (Table I).

### Linearity, sensitivity and specificity

The linearity of the method was determined for each drug using a calibration curve with six points in nine replicates ($n = 9$), as specified with the concentration range for each drug. Though we used very low to high concentrations initially, the response was found to be linear between 40–300 $\mu$g/mL for rifampicin, 20–150 $\mu$g/mL for isoniazid, 112–840 $\mu$g/mL for pyrazinamide and 80–600 $\mu$g/mL for ethambutol. The regression analysis of peak area/response for a given concentration of each drug for intraday and interday was performed and found that the correlation coefficient ($r^2$) was higher than 0.999 for all four drugs. The linearity data were subjected to residual error normality and homocedasticity tests and found that there were no statistical differences. The overall summary of the linear regression analysis is given in Table II.

In addition, from the regression analysis, the sensitivity of the method was calculated. The LODs of 4, 2, 11 and 6 $\mu$g/mL were for rifampicin, isoniazid, pyrazinamide and ethambutol hydrochloride, respectively, while the LOQs of the respective drugs were 12, 6.0, 32 and 18 $\mu$g/mL.

It was evident from the blank that there were no interfering peaks in the retention time of these drugs, which is suggestive of the specificity of the method (Figure 1). The peak purity index of rifampicin, isoniazid, pyrazinamide and ethambutol hydrochloride standards was 0.992, 1.0, 0.984 and 0.999, respectively, while it was 0.992, 1.0, 0.987 and 1.0, respectively, for the compounds analyzed from FDC tablets.

### Accuracy

The accuracy of the optimized method was calculated as the recovery percentage of the obtained concentration from the nominal/true concentration of all four drugs at four different concentrations (Low-1, Medium-2 and High-1) and summarized in Table III. Overall, the recovery of intraday and

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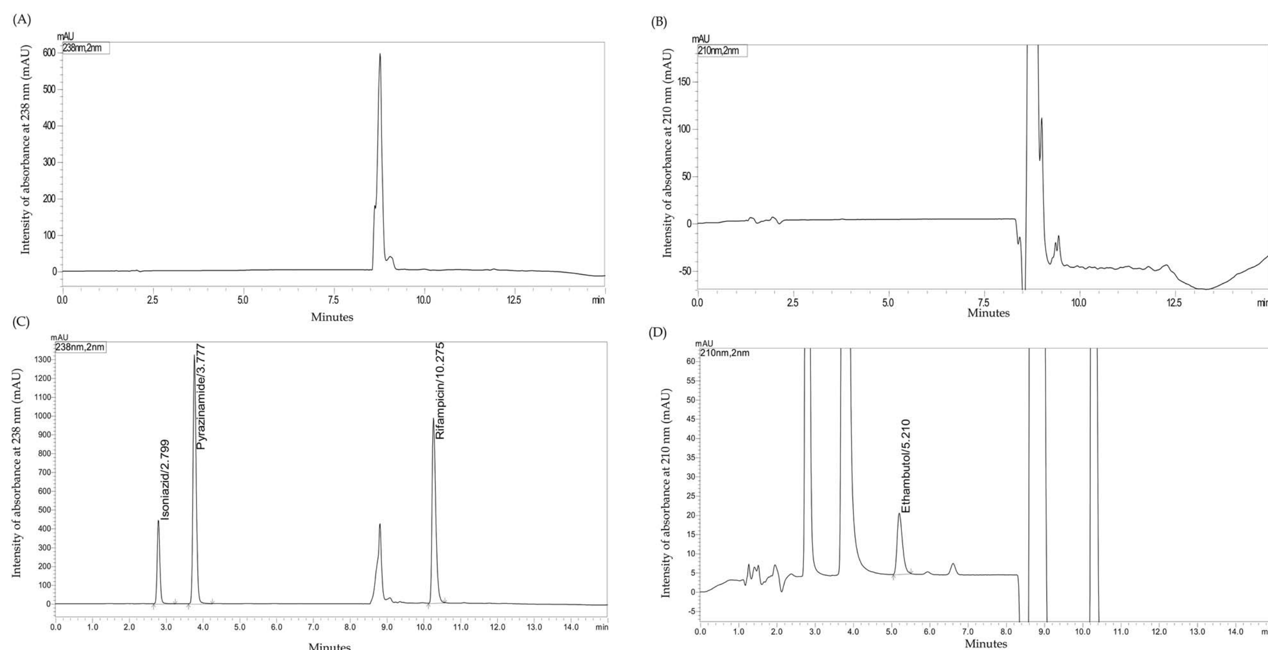


**Figure 1.** Chromatogram. Representative chromatograms of blank at 238 and 210 nm (A and B), and isoniazid, pyrazinamide and rifampicin detected at 238 nm (C) and ethambutol hydrochloride at 210 nm (D) of the standard solution under optimized conditions. mAU, milli-absorbance unit.

**Table I.** System Suitability Test

| | Retention time | | No. of theoretical plates | | Tailing factor | |
|---|---|---|---|---|---|---|
| | Mean | RSD% | Mean | RSD% | Mean | RSD% |
| Rifampicin (172 $\mu$g/mL) | 10.319 | 0.49 | 44,553 | 0.38 | 1.37 | 1.85 |
| Isoniazid (86 $\mu$g/mL) | 2.799 | 0.18 | 5529 | 0.64 | 1.16 | 0.60 |
| Pyrazinamide (482 $\mu$g/mL) | 3.776 | 0.23 | 7427 | 1.63 | 1.21 | 0.34 |
| Ethambutol hydrochloride (344 $\mu$g/mL) | 5.216 | 0.21 | 6002 | 0.73 | 1.40 | 0.73 |

Values are means of 3.0 standard stocks done in replicates. RSD, Relative standard deviation.

**Table II.** Summary of Linear Regression Analysis

| Linearity parameters | Rifampicin | Isoniazid | Pyrazinamide | Ethambutol hydrochloride |
|---|---|---|---|---|
| Concentration range ($\mu$g/mL) | 40–300 | 20–150 | 112–840 | 80–600 |
| No. of points used | 6 | 6 | 6 | 6 |
| Slope (m) ($\mu$g/mL) ± SD | 42,936 ± 517 | 27,841 ± 734 | 17,481 ± 150 | 482 ± 4 |
| Intercept (b) ± SD | −319,694 ± 105,178 | −45,328 ± 11,389 | −129,527 ± 33,204 | −294 ± 1115 |
| Correlation coefficient ($r^2$) | 0.9995 | >0.9999 | 0.9999 | >0.9999 |
| Residual normality (*P*-value) | 0.462 | 0.628 | 0.497 | 0.192 |
| Homocedasticity (*P*-value) | 0.722 | 0.628 | 0.127 | 0.948 |

Values are expressed as means ± SD of linear regression analysis from 9.0 standard stocks ($n = 9$). Shapiro–Wilk normality test and Breusch–Pagan test were used to calculate the residual normality and homocedasticity respectively (using R software; R-4.3.2). The *P*-value ≤ 0.05 considered statistically different.

interday analysis for rifampicin, isoniazid, pyrazinamide and ethambutol hydrochloride was 98.3%, 97.6%, 97.7% and 99.7% and 97.6%, 98.8%, 98.3% and 99.1%, respectively, with the RSD% lower than 2.0.

## Robustness and ruggedness

Further, the robustness of the optimized method was evaluated by deliberate changes in the auto-sampler and column-oven temperature and compared the response (peak area) with the optimized conditions. The peak response was not affected by the change in auto-sampler temperature to 15°C, instead of 4°C. However, the changing of the column-oven temperature from 40°C to 32°C affected the peak area (response) significantly at $P \leq 0.05$ level for all four drugs (Table IV). In addition, the deliberate change of pH in the buffer resulted in a significant reduction (86%) of the response of ethambutol hydrochloride, compared to the buffer used in the optimized condition; however, it remained comparable for the rest of the other drugs (Table V). The ruggedness of the method as evaluated by different analysts or changing the component of the mobile phase (triethylamine from different makes) suggests that these factors did not affect the peak response significantly (data not shown).

## Stability study

The data of the stability-indicating experiment are given in Figure 2, and considering the concentration variation of <2%, the concentrations of pyrazinamide and ethambutol

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**Table III.** Accuracy/Recovery Analysis of the Optimized Method

| | Nominal concentration (μg/mL) | Intraday (*n* = 3) | | | Interday (*n* = 3) | | |
|---|---|---|---|---|---|---|---|
| | | Obtained concentration (μg/mL) Means ± SD | Precision (%) | Accuracy (%) | Obtained concentration (μg/mL) Means ± SD | Precision (%) | Accuracy (%) |
| Rifampicin | 85 | 85 ± 0.2 | 0.21 | 98.6 | 81 ± 0.6 | 0.70 | 95.7 |
| | 150 | 150 ± 1.0 | 0.68 | 100.0 | 149 ± 0.3 | 0.22 | 99.5 |
| | 180 | 173 ± 0.3 | 0.19 | 96.3 | 174 ± 0.9 | 0.54 | 96.5 |
| | 270 | 266 ± 0.1 | 0.04 | 98.4 | 266 ± 0.8 | 0.32 | 98.6 |
| Isoniazid | 42.5 | 42 ± 0.6 | 1.44 | 97.6 | 42 ± 0.2 | 0.49 | 99.3 |
| | 75 | 73 ± 0.4 | 0.50 | 96.7 | 74 ± 0.1 | 0.15 | 98.7 |
| | 90 | 88 ± 0.2 | 0.18 | 97.4 | 88 ± 0.5 | 0.53 | 98.2 |
| | 135 | 133 ± 0.6 | 0.41 | 98.8 | 134 ± 0.6 | 0.46 | 99.0 |
| Pyrazinamide | 238 | 234 ± 3.6 | 1.51 | 98.4 | 238 ± 0.5 | 0.20 | 99.9 |
| | 420 | 403 ± 1.9 | 0.47 | 96.0 | 402 ± 0.3 | 0.07 | 95.8 |
| | 504 | 491 ± 0.1 | 0.18 | 97.5 | 495 ± 3.0 | 0.59 | 98.2 |
| | 756 | 747 ± 3.1 | 0.42 | 98.8 | 750 ± 4.6 | 0.61 | 99.1 |
| Ethambutol hydrochloride | 170 | 173 ± 2.8 | 1.62 | 101.5 | 171 ± 2.6 | 1.53 | 100.4 |
| | 300 | 292 ± 0.3 | 0.11 | 97.4 | 295 ± 0.4 | 0.13 | 98.2 |
| | 360 | 360 ± 1.4 | 0.39 | 100.0 | 354 ± 4.9 | 1.40 | 98.3 |
| | 540 | 539 ± 5.5 | 1.02 | 99.8 | 538 ± 1.8 | 0.34 | 99.6 |

Values are expressed as means ± SD of 3.0 standard stocks done in replicates

**Table IV.** Robustness of the Optimized Method—Change in Temperature

| | Peak area in % (*n* = 3) | | *P*-value |
|---|---|---|---|
| | Column-oven temperature | | |
| | 40°C | 32°C | |
| Rifampicin (180 μg/mL) | 100 ± 1.0 | 98 ± 1.0 | 0.002 |
| Isoniazid (90 μg/mL) | 100 ± 2.0 | 99 ± 1.0 | 0.050 |
| Pyrazinamide (504 μg/mL) | 100 ± 1.0 | 93 ± 1.0 | 0.004 |
| Ethambutol hydrochloride (360 μg/mL) | 100 ± 2.0 | 95 ± 1.0 | 0.026 |

Three standard stocks (*n* = 3) were prepared and tested for the robustness. The peak area was expressed in percentage with SD. The peak area of the optimized condition was considered 100% and calculated the peak area percentage of the tested temperature. The data were analyzed by paired sample *t*-test. The *P*-value ≤ 0.05 levels were considered significant.

**Table V.** Robustness of the Optimized Method—Change in pH

| | Peak area in % (*n* = 5) | | *P*-value |
|---|---|---|---|
| | Buffer | | |
| | Without pH adjustment | pH adjusted to 7.0 with OPA | |
| Rifampicin (172 μg/mL) | 100 ± 2.0 | 100 ± 1.0 | 0.536 |
| Isoniazid (86 μg/mL) | 100 ± 0.7 | 100 ± 0.4 | 0.327 |
| Pyrazinamide (482 μg/mL) | 100 ± 0.9 | 100 ± 0.6 | 0.338 |
| Ethambutol hydrochloride (344 μg/mL) | 100 ± 0.5 | 86 ± 0.9 | >0.0001 |

Five standard stocks (*n* = 5) were prepared and tested for the robustness. The peak area was expressed in percentage with SD. The peak area of the optimized condition without pH adjustment of buffer was considered 100% and calculated the peak area percentage of the tested condition (i.e., deliberate change in buffer pH). The data were analyzed by paired sample *t*-test. The *P*-value ≤ 0.05 levels were considered significant. OPA, Ortho phosphoric acid.

hydrochloride were stable at all the tested conditions, namely, RT, 4°C and −20°C till 1 week. However, the concentrations of rifampicin and isoniazid were stable up to 48 h when stored at −20°C.

### Analysis of FDC tablets

The testing of FDC tablets using this method showed that the mean drug content estimation either from the individual tablet assay (ranged between 95% and 103%) or from the combined tablets (ranged from 93.0% to 99.6%) was within the acceptable limits of 10% variation (i.e., neither <90% nor >110% of the stated/labeled amount) (Table VI).

## Discussion

Here, we developed a very simple HPLC-UV method, optimized the chromatographic conditions and validated some of the parameters, such as linearity, sensitivity, accuracy, precision and robustness, according to the ICH guidelines Q2 (R1) and system suitability test as per the USP. Accordingly,

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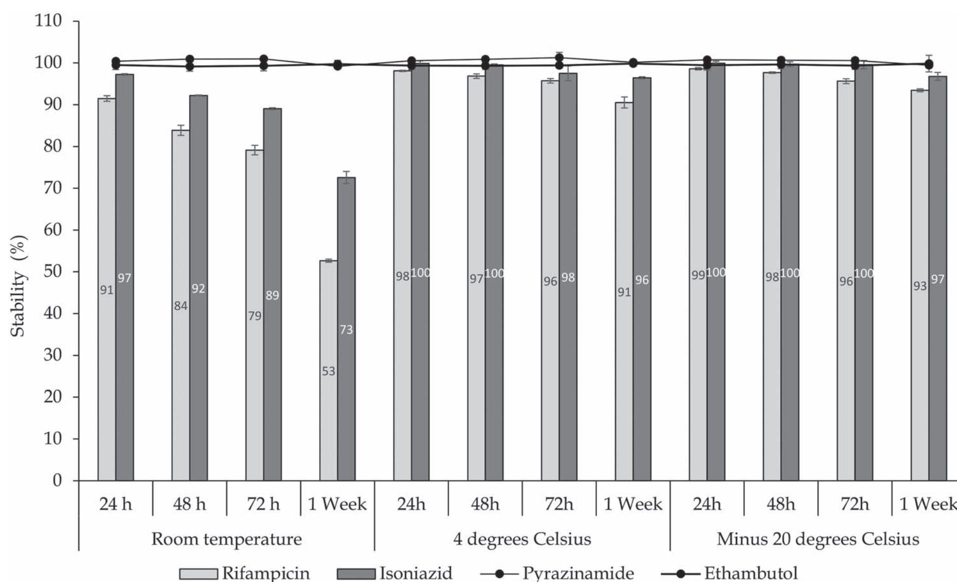


**Figure 2.** Stability experiment data. Three standard stocks ($n = 3$) were prepared and tested for stability at different experimental conditions, i.e., RT, 4°C and −20°C after 24 h, 48 h, 72 h and 1 week. The peak area/response was compared to those obtained with freshly prepared stocks/standards, and the stability was expressed in percentage with a standard deviation in the bar diagram.

**Table VI.** FDC Tablet Drug Content Assay Using the Optimized Method

| | Nominal concentration (μg/mL) | Individual FDC tablets ($n = 3$) | | Nominal concentration (μg/mL) | Five FDC tablets ($n = 5$) | |
|---|---|---|---|---|---|---|
| | | Obtained concentration (μg/mL) Means ± SD | Mean content (%) | | Obtained concentration (μg/mL) Means ± SD | Mean content (%) |
| Rifampicin | 150 | 146 ± 1.7 | 97 | 300 | 285 ± 4.9 | 95 |
| Isoniazid | 75 | 72 ± 2.2 | 96 | 150 | 140 ± 4.3 | 93 |
| Pyrazinamide | 400 | 412 ± 8.7 | 103 | 800 | 797 ± 11.1 | 100 |
| Ethambutol hydrochloride | 275 | 274 ± 2.8 | 100 | 550 | 539 ± 2.5 | 98.0 |

FDC tablets were individually crushed ($n = 3$) and assayed in replicates, or five tablets were crushed together and five portions were weighed and assayed as five replicates. Values are expressed as means ± SD and the mean content was expressed in percentage.

the data suggest the suitability of this HPLC-UV detection method to simultaneously quantify the first-line anti-TB drugs in pharmaceutical formulations, and the method requires fewer reagents and materials. Further, an overall run time of 11 min is required to complete the analysis of all four anti-TB drugs by this method. Earlier, several methods reported the quantification of anti-TB drugs in pharmaceutical products, but individually using different principles and/or techniques, including spectrophotometry and chromatography (13–15). In addition, some of the methods have reported the quantification of three anti-TB drugs simultaneously (5, 16). Nevertheless, the introduction of FDC tablets for the treatment of TB has thrived researchers to develop a suitable method to determine all four drugs in a single run, as this can reduce the turnaround time (for quality checking of formulations in bulk), cost, exposure to organic solvents, etc., to state a few. In 2012, Marcellos *et al.* (17) have reported a method for simultaneously analyzing first-line anti-TB drugs in tablets by spectrophotometry, and the results were comparable with capillary zone electrophoresis. However, according to the official compendia of the International Pharmacopoeia, HPLC-UV detection is considered a reference method for the content uniformity of pharmaceutical products for anti-TB drugs. Accordingly, the quantification of anti-TB drugs in the FDC tablets requires two separate HPLC methods, wherein one method is exclusively for the quantitation of rifampicin, while the other method is for the rest of the three drugs combined (18). Therefore, the quantification of all four drugs in the FDC tablets is cumbersome, expensive, laborious and less environmentally friendly (as both methods consume organic solvents, salts and acids to run an assay in HPLC).

Teixerira *et al.* (19) have reported a method for the simultaneous detection of rifampicin, isoniazid, pyrazinamide and ethambutol in FDC using near-infrared (NIR) spectroscopy technique-based multivariate calibration model. However, the authors have concluded that the results of the NIR-based model were not similar to that of the reference method, i.e., HPLC-UV detection (18). The earlier reported method of Gaitonde and Pathak (3) could simultaneously determine isoniazid, pyrazinamide and rifampicin in dosage form, excluding ethambutol, which is one of the major limitations of the method. Besides, it had other procedural disadvantages, such as the addition of clofazimine as an internal standard in the sample preparation and ion-pairing agents in the mobile phase (3). Shewiyo *et al.* (8) have developed a new reverse-phase high-performance thin layer chromatography (RP-HPTLC) method for detecting all four anti-TB drugs in the FDC tablets. However, this method needed two steps of

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processing for quantification, wherein rifampicin, isoniazid and pyrazinamide were detected at UV wavelengths, while ethambutol was derivatized before the detection by visible wavelength. Though, it is a simple method, derivatization of ethambutol and the technique involved; i.e., RP-HPTLC are some of the limitations of this new method. Previously, Franco *et al.* (20) have reported an ultra-HPLC (UHPLC) method for simultaneously analyzing all first-line drugs in the FDC tablets within a shorter run time (i.e., 4 min) with comparable results to a simple HPLC method. Although the method is simple and fast, the affordability of acquiring the UHPLC system and columns for low-resourced settings is restricted due to the high cost. Recently, several methods have emerged to measure these anti-TB drugs in other matrices (e.g., plasma and serum) using contemporary techniques, such as liquid chromatography with mass spectrometry detection (LC–MS), due to its advantages over other conventional detection systems (including photo-diode array detector) in terms of specificity, sensitivity, limited interference, low volume, high throughput, etc., (21–23). However, no such methods are available to measure these anti-TB drugs in FDC tablets to date.

As described in the earlier section, the method of Chellini *et al.* (10) is simple and can measure all four first-line drugs in a formulation using HPLC-UV detection without any derivatization or presample processing. Thus, this method had several advantages over earlier reported methods. However, unlike the adjustment of pH required for the monobasic phosphate buffer to 7.0 using OPA for the method of Chellini *et al.* (10), there is no requirement for adjusting the pH of 50 mM phosphate buffer in the present method. In addition, this method requires a smaller amount (50%) of the organic solvent, acetonitrile. Further, the analysis of FDC tablets by this method demonstrated that it is suitable for testing pharmaceutical formulations. Although, we could not test placebo FDC tablets, the peak purity was comparable to that of anti-TB drug standards and thus suggests no interfering compound at a given retention time. Notably, the method validation parameters indicate that the performance of this method is suitable for the analysis of all four first-line drugs with acceptable accuracy and precision (i.e., <2%). Nevertheless, the stability indicating data suggest that the stocks/standards have to be prepared every day if not stored at −20°C. Besides, triethylamine is a volatile compound, so the mobile phase has to be prepared afresh every day. Importantly, appropriate care and maintenance of the HPLC system and columns are required on a usage basis to avoid accumulation of salt deposits in the system components and columns due to the use of high salt concentrations in the buffer. These are a few limitations of this method. Overall, the present method is simple, easily adoptable and suitable for simultaneous measurement of four first-line anti-TB drugs in FDC tablets in low-resource settings.

## Conclusion

In conclusion, the current method is selective, sensitive and linear over high concentrations with a correlation coefficient >0.999. Further, the method shows the precision and accuracy of <2% RSD for all four first-line anti-TB drugs. The present method has a salient advantage over previous methods by circumventing the cumbersome benchwork in adjusting the pH of the buffer using OPA. In addition, the current method reduces the usage of acetonitrile by 50% in mobile phase A and completes the run time within 11 min to separate all four drugs. All these factors reduce the cost, usage of organic solvents and exposure to organic solvents (occupational hazards), which are salient attributes for an analytical procedure. Overall, the method is simple and easily adoptable, even for low-resource settings, to test the quality of FDC tablets containing first-line anti-TB drugs with acceptable accuracy and precision.

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## Author contributions

Sudha Vilvamani (Investigation), Santhanamahalingam Mahalingam (Investigation), Sruthi Nhavilthodi (Conceptualization, Investigation), Dharman Murugesan (Investigation), and Shanmugam Murugaiha Jeyakumar (Conceptualization, Formal analysis, Methodology, Writing—original draft, Writing—review & editing)

## Conflict of interest statement

None declared.

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