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# VALIDATION OF HPLC METHODS FOR DETERMINATION OF ISONIAZID, RIFAMPICIN, PYRAZINAMIDE, AND ETHAMBUTOL IN A FIXED-DOSE COMBINATION ANTITUBERCULOSIS

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**Abstract:** Simple HPLC methods using two HPLC systems for the quantification of isoniazid (INH), pyrazinamide (PZA), rifampicin (RIF) and ethambutol (EMB) in a fixed-dose combination (FDC) antituberculosis were developed and validated. The chromatographic separation of INH, PZA, and RIF was carried out using Inertsil ODS-3 5µm, C18 (length 150 mm; inner diameter 4.6 mm), UV detection at 254 nm, and a gradient system composed of acetonitrile (A) and 3% acetonitrile in 10 mM monobasic potassium phosphate buffer pH 6.8 (B) at a flow rate of 1.5 mL/min. The gradient profile was (A:B) 0:100 v/v for 5 min, then a linear gradient to 50:50 v/v allowed to maintain at (A:B) 50:50 v/v for 15 min, then return to 0:100 v/v for 1 min, and finally re-equilibrate for another 5 min before the next injection. The retention time of INH, PZA, and RIF was 2.9, 4.0 and 10.4 min, respectively. The limit of quantification of the method for INH, PZA, and RIF was 1.07, 1.74 and 1.13 µg/mL, respectively. The separation of EMB was performed using Hypersil BDS 5 µm, C18 (4.6 mm x 250 cm), UV detection at 254 nm, using an elution isocrartic procedure. The mobile phase mixture composed of 2.0 mM of copper sulfate ($CuSO_4.5H_2O$), 3 g/L sodium hexanesulfonate (pH4.5) in distilled water and tetrahydrofuran, 75:25 (v/v) at a flow rate of 0.4 mL/min. The retention time of EMB was 14.92 min. The limit of quantification of the method for EMB was 4.28 µg /mL.

**Keywords:** antituberculosis, isoniazid, pyrazinamide, rifampicin, ethambutol

**บทคัดย่อ:** วิธีวิเคราะห์หาปริมาณยาไอโซไนอาซิด (INH) ยาพัยราซินาไมด์ (PZA) ยาไรแฟมปิซิน (RIF) และยาอีแธมบิวทอล (EMB) ในยาต้านวัณโรคชนิดยาเม็ดรวมหลายขนาน โดยใช้วิธี HPLC อย่างง่าย 2 ระบบ ในการแยก INH, PZA และ RIF ใช้คอลัมน์ชนิด Inertsil ODS-3 5 ไมโครเมตร C18 (ความยาว 150 มิลลิเมตร; เส้นผ่านศูนย์กลาง 4.6 มิลลิเมตร) ตรวจวัดโดยยูวีวิสิเบิลที่ 254 นาโนเมตร ระบบเฟสเคลื่อนที่ใช้ คือ ระบบการปรับอัตราส่วน ประกอบด้วย อะซิโตไนไตรล์ (A): 3% อะซิโตไนไตรล์ ใน 10 มิลลิโมล โมโนเบซิก โพแทสเซียม ฟอสเฟต บัฟเฟอร์ pH 6.8 (B) อัตราการไหล 1.5 มิลลิลิตร/นาที โดยเริ่มต้นที่ A: B (0:100) เป็นเวลา 5 นาที และปรับความเข้มข้นเป็น A:B (50:50) 15 นาที และปรับความเข้มข้นเป็น A: B (0:100) 1 นาที และสุดท้ายปรับให้เข้าสู่สมดุลในเวลา 5 นาที ก่อนฉีดครั้งถัดไป โดย INH, PZA และ RIF แยกออกมาที่เวลา 2.9, 4.0 และ 10.4 นาที ตามลำดับ ค่าปริมาณต่ำสุดที่สามารถวิเคราะห์ได้อย่างถูกต้องของ INH, PZA, และ RIF เท่ากับ 1.07, 1.74 และ 1.13 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ ในการแยก EMB ใช้คอลัมน์ Hypersil BDS 5 ไมโครเมตร, C18 (เส้นผ่านศูนย์กลาง 4.6 มิลลิเมตร; ความยาว 250 มิลลิเมตร) ตรวจวัดโดยยูวีวิสิเบิล ที่ 254 นาโนเมตร ระบบไอโซเครติก ประกอบด้วย 2.0 มิลลิโมลาร์ คอปเปอร์ ซัลเฟต ($CuSO_4.5H_2O$) และ 3 กรัม/ลิตร โซเดียม เอกเซนซัลโฟเนท (pH4.5) ในสารละลายน้ำ และ เททราไฮโดรฟูแรนในอัตราส่วน 75:25 อัตราการไหล 0.4 มิลลิลิตร/นาที โดย EMB ออกมาที่เวลา 14.92 นาที ค่าปริมาณต่ำสุดที่สามารถวิเคราะห์ได้อย่างถูกต้องของ EMB เท่ากับ 4.28 ไมโครกรัมต่อมิลลิลิตร

**คำสำคัญ: ยาต้านวัณโรค, ไอโซไนอาซิด, พัยราซินาไมด์, ไรแฟมปิซิน, อีแธมบิวทอล**

## INTRODUCTION

One of the great solicitude of infectious diseases is Tuberculosis (TB) that is the mainspring by *Mycobacterium tuberculosis,* which is classified in the Mycobacteriaceae family. Statistically, it killed more people than other microbial pathogens. TB is an airborne infectious disease that has increasingly become a global public health problem. Even though, the lung is the majority infection site, other organ systems are also susceptible (Kochi, 1997). During the 18th and 19th centuries, Tuberculosis have reached epidemic proportions in Europe and North America earning the designation, "Captain Among these Men of Death" and commenced to abate. The curative methods in both the prevention and treatments of this epidemic have been improved significantly. However, most developing countries particularly, in Africa, Asia and Latin America, tuberculosis has been remaining as a noted cause of mortality and morbidity (Herfindal *et al.*, 1992).

Mycobacteria are acid-fast, non-spore forming, non-motile and non-encapsulated bacilli. They are anaerobes, which grow appropriately at human body temperature and obliging slow growth rate (requiring 2-6 weeks for growth on agar). The genus *Mycobacterium* contains various species, including pathogenic and saprophytic. *Mycobacterium leprae*, is subject for leprosy, *M. avium*, *M. bovis* and *M. africanum*, are responsible for a small fraction of the tuberculosis infections. In fact, the most considerable strict pathogen is the tubercle bacilli called *M. tuberculosis* (Henry, 1993). TB bacteria can live in the person without construction loaded with diseased or no symptoms if the immune system of an individual person is capable to control the contagion. This is often referred as latent TB infection. People with latent TB infection cannot disseminate TB bacteria to others (Coetzee and Manomed, 1996). However, if this bacterium becomes active in the body and multiply, some person will promote symptoms (active TB). Persistent cough which progresses over weeks and sputum production usually occur with pulmonary infection. Other symptoms such as tightness or chest pain and acute or recurrent pleuritic pain are also related to pulmonary infection. Meningitis is presented as a mild headache if the bacilli target the central nervous system (CNS). White blood count is usually normal, but tends to be lower than average. Gross hematuria, dysuria, frequency, and flake pain are all part of the extrapulmonary tuberculosis symptoms (Wells *et al.*, 2007).

TB bacteria increase very slowly and divide occasionally therefore, management usually has to be extended for six months to insure inactive bacteria are stream and the enduring is wretch. People with respiratory TB are generally not catching after two weeks of handling. TB composed is fully curable by treating with correct drugs and suitable time. Several antibiotics indigence to be taken over a number of months to prevent resistance developing to the TB drugs (Kochi, 1997).

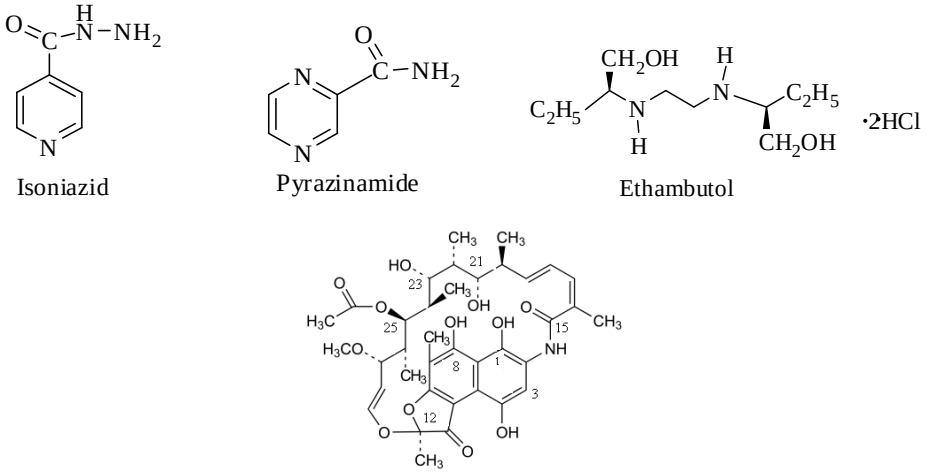


**Figure 1.** Structures of isoniazid (INH), pyrazinamide (PZA), ethambutol (EMB) and rifampicin (RIF)

World Health Organization (WHO) has estimated that one third of the world's population is now corrupted with TB, 9.4 million new tuberculosis cases and a total of 1.7 million deaths annually (WHO, 2008). Promoting a universal treatment programs, the Global Plan to Stop TB 2006-2015 was launched by WHO (2006) and the principal target was to lower of prevalence and mortification due to TB by 50% as procure to 1990. For these reasons, WHO strongly recommended the use of fixed-dose combination (FDC) for the treatments of tuberculosis. The combination available consists of isoniazid (INH), pyrazinamide (PZA), rifampin (RIF), and ethambutol (EMB) (Figure 1).

Since most drugs have dissimilar physical and chemical properties, conbining different drugs ensure the multi-targeting of *M. tuberculosis*. It is important to consider the safeness, efficacy, and the quality requirements for FDC products. The quality requirements include stability, assay and identification testing, as well as the determination of degradation products and related substances. Nevertheless, serious matter has been stirring on the utility of these products due to quality problems (Wells *et al.*, 2007; Calleri *et al.*, 2002). The methods for the determination of INH (isonicotinic acid hydrazide), PZA (pyrazine carboxylamide), RIF 3-[(4-methyl-1-piperazinyl) iminomethyl]-rifamycin SV), and EMB (ethambutol hydrochloride) in pharmaceutical formulations and biological samples have been reported. Examples include high-performance liquid chromatography (Calleri *et al.*, 2002; Khunawar and Rind, 2002; Glass *et al.*, 2007; Huan, *et al.*, 2012; Mariappan *et al.*, 2000; Prema, *et al.*1984; USP 32, Wanna-impikul and Lapponampai., 2005), liquid chromatography/tandem mass spectrometry (Koegelenberg *et al.*, 2013; Song *et al.*, 2007), high-performance thin-layer chromatography (Shewiyo *et al.*, 2012), single multiplex allele-specific polymerase chain reaction (PCR) assay (Yang *et al.*, 2005), micellar electrokinetic chromatography using UV detection (Acedo-Valenzuela *et al.*, 2002; Faria *et al.*, 2010), multivariate spectrophotometric calibration method (Héctor and Alejandro, 1999), colorimetric analysis (Martin *et al.*, 2006), non-aqueous titration (BP 2012), spectrofluorimetric method (Maaboud *et al.*, 2013) and UPLC-UV method (Nguyen *et al.*, 2008; Singh *et al.*, 2014 ). However, many of these methods suffer from limitations such complex and tedious procedures.

## MATERIALS AND METHODS

INH, PZA, RIF and EMB HCl reference standards were purchased from Sigma-Aldrich (St. Louis, MO). Potassium dihydrogen phosphate was of analytical grade and purchased from

Merck (Darmstadt, Germany). Acetonitrile HPLC grade (J.T.Baker, USA) was used to prepare mobile phase. Methanol and tetrahydrofuran (HPLC grade) was purchased from Mallinckrodt Baker (Paris, KY). Copper sulfate, sodium hexanesulfonate and phosphoric acid were from Fluka, Buchs, Switzerland. Ultrapure water was obtained from a MilliQ Millipore system (Millipore, Bedford, MA). FDC tablets were purchased from a local manufacturer. The label claim for A was to contain 60 mg of INH, 300 mg PZA, 120 mg of RIF, and 225 mg EMB HCl.

### *Analysis of INH, PZA, and RIF*

#### Preparation of standards and sample

Standard of INH, PZA, and RIF were prepared by dissolving 16.5 mg of INH, 82.5 mg of PZA, and 33 mg of RIF in 10 mL ultrapure water and 40 mL methanol. Transferred 5 mL of each stock solution into a 50 mL volumetric flask and diluted with methanol to get final concentrations of 33 µg/mL INH, 165 µg/mL PZA, and 66 µg/mL RIF (working solution). All solutions mentioned above were kept at 4 ºC before use.

#### Sample preparation

Twenty tablets were weighed and finely powdered. An accurately weighed powder sample corresponding to the average weight equivalent to 132 mg INH, 660 mg of PZA, and 264 mg of RIF was transferred to a 100 mL volumetric flask followed by the addition 20 mL of ultrapure water. The solution was sonicated for 10 min at room temperature and then made up to volume with methanol. This solution was filtered through a 0.45 µm polytetrafluoroethylene (PTFE) syringe filter. 2.5 mL of the filtrate was transferred to 100 mL volumetric flask and diluted to volume with methanol.

#### Chromatographic condition

The study was performed by using a HPLC with UV detector for chromatographic separation of compounds. The C18 Inertsil ODS-3 column (150 x 4.6 mm, 5 µm) was used. The 20-µL injection volumes, flow rate of 1.5 mL/min, column temperature at 30°C were used. The detection wavelength of INH, PZA, and RIF were 254 nm. The data were processed using Chromeleon 6.30 software (Dionex). The chromatographic separation was achieved using a gradient system (Table 1).

**Table1** The gradient system used in chromatographic separation

| Time (min) | Ratio (acetonitrile: 3%acetonitrile in 10 mM $KH_2PO_4$ buffer pH 6.8) |
|---|---|
| 0-5 | 0:100 |
| 5-6 | 50:50 |
| 6-20 | 50:50 |
| 20-21 | 0:100 |
| 21-25 | 0:100 |

#### Method Validation

Validation was performed following the ICH Q2A guidelines for single laboratory validation of methods of analysis. The method was validated as regards its specificity, linearity, accuracy, precision (within- and between-day), and sensitivity.

To determine the accuracy of the method, three standard solutions with low, intermediate and high concentrations (levels) were analyzed. The percentage recovery was performed by three determinations and was calculated by the relationship between the

experimental concentration (Cexp) and the theoretical concentration (Cteo) expressed as percentage using the following equation: (Cexp/ Cteo) x 100.

To evaluate the within- and between-day precision, three replicates of three standard solutions at low, medium, and high concentrations were assayed on the same day and three consecutive days, respectively.

The calibration curves were studied at five concentrations including the range of 25.6 – 38.4 µg/mL INH, 128.0 – 192.0 µg/mL PZA, and 53.0 – 79.5 µg/mL RIF. Each solution was injected in three replicates on the same day and the linearity was evaluated by linear regression analysis which was performed without applying any type of mathematical transformation or data weighing. A regression equation of the calibration curve was calculated by least square linear regression ($r^2 \geq 0.999$).

Limit of detection (LOD) and limit of quantitation (LOQ) were determined using calibration curve method according to ICH guidelines.

$$LOD = 3.3 \times \frac{SD}{S}$$

And

$$LOQ = 10 \times \frac{SD}{S}$$

where $SD$ = the standard deviation of the y-intercept
$S$ = the average of the slope

***Analysis of EMB***

**Preparation of standard and sample**

Standard of EMB HCl was prepared by dissolving 50 mg in 100 mL mobile phase. Transferred 10 mL of stock solution into a 50 mL volumetric flask and diluted with mobile phase to get final concentrations of 100 µg/mL EMB (working solution). This solution mentioned above was kept at 4 ºC before use.

**Sample preparation**

Twenty tablets were weighed and finely powdered. An accurately weighed powder sample corresponding to the average weight equivalent to 5 mg EMB was transferred to a 50 mL volumetric flask followed by the addition 25 mL of mobile phase. The solution was sonicated for 10 min at room temperature and then made up to volume with mobile phase. This solution was filtered through a 0.45 µm polytetrafluoroethylene (PTFE) syringe filter.

**Chromatographic condition**

The study was performed by using a HPLC with UV detector for chromatographic separation of compounds. The Hypersil BDS C18 column (250 X 4.6 mm, 5 µm) was used. The 10-µL injection volumes, flow rate of 0.4 mL/min, column temperature at 30°C were used. The detection wavelength of EMB was 254 nm. The chromatographic separation of EMB was obtained by using a mixture of 2 mM of copper sulfate ($CuSO_4.5H_2O$) and 3 g/L sodium hexanesulfonate in 750 mL distilled water (adjust to a pH of 4.5 with 10% v/v phosphoric acid solution), adding 250 mL of tetrahydrofuran, followed by isocratic elution as the mobile phase. The data were processed using Chromeleon 6.30 software (Dionex).

**Method validation**

The reliability of the purpose method was validated according to the ICH guidelines for single laboratory validation of methods of analysis.

The linearity of the method was examined by analyzing a series of EMB at concentrations of 80-120 µg/mL in mobile phase. Each standard solution at different concentration was carried out in triplicate (n=3).

The accuracy was assessed in terms of the recovery (%). Repeatability and intermediate precision at three different concentration levels with low, intermediate and high concentrations (80%, 100%, 120%) as described in method validation of INH, PZA, and RIF.

## RESULTS AND DISCUSSION

### *Analysis of INH, PZA, and RIF*

By using the developed sample preparation and validated chromatographic system, there were no interferences on the INH, PZA and RIF peaks due to the components of the samples. INH, PZA, and RIF were eluted at 2.98, 4.06, and 10.40 min, respectively (Figure 2). All the three peaks were well separated from the others. The SST is an integrated part of the analytical method and it ascertains the suitability and effectiveness of the operating system. The results of the SST are reported in Table 2.

**Table 2** System suitability results

| Sample | Number of threoretical plate (N) | RSD% of the area | Tailing factor | Retention time (min) |
|---|---|---|---|---|
| INH | 3443 | 0.98 | 1.11 | 2.98 |
| PZA | 3918 | 0.44 | 1.02 | 4.06 |
| RIF | 29928 | 0.33 | 1.08 | 10.40 |

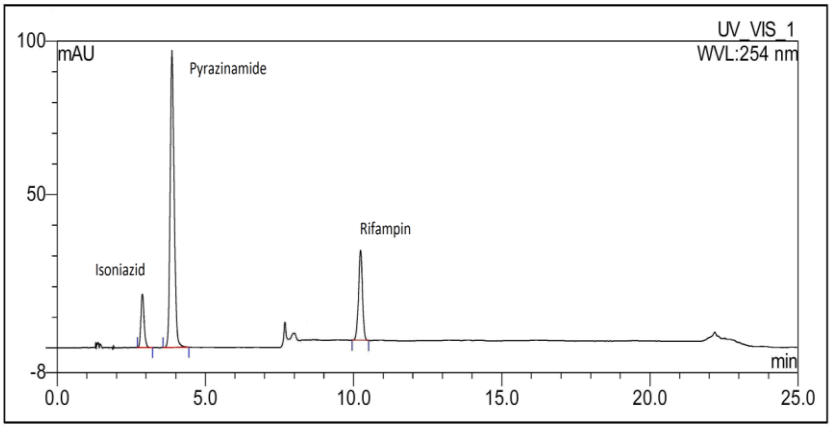


**Figure 2** HPLC chromatogram of 33 µg/mL INH, 165 µg/mL PZA, and 66 µg/mL RIF at 254 nm by using acetonitrile (A) : 3% acetonitrile in 10 mM $KH_2PO_4$ pH 6.8 (B) gradient a mobile phase

Linearity data of INH, PZA, and RIF are shown in Tables 3. The concentration ranges were: INH from 25.6 – 38.4 µg/mL, PZA from 128.0 – 192.0 µg/mL and RIF from 53.0 – 79.5 µg/mL. The linear equation for INH was y = 0.0840x-0.1364, PZA was y = 0.0899x+0.0995, while the equation for RIF was y = 0.1022x+0.0557. The linear regression analysis obtained by plotting the peak area of the three analytes vs concentration showed excellent correlation coefficients (correlation coefficient greater than 0.9997), and the linearity data are reported in Table 3.

**Table 3** Linear regression and statistical analysis

| Sample | Concentration (µg/mL) | Slope | Intercept | Coefficient of Determination ($r^2$) |
|---|---|---|---|---|
| INH | 25.6 – 38.4 | 0.0840 | – 0.1364 | 0.9997 |
| PZA | 128.0 – 192.0 | 0.0899 | 0.0995 | 1.0000 |
| RIF | 53.0 – 79.5 | 0.1022 | 0.0557 | 0.9998 |

The limits of quantification (LOQ) for INH, PZA, and RIF of the method were 1.07, 1.74, and 1.13 μg/mL, respectively. The limits of detection (LOD) for INH, PZA, and RIF of the method were 0.35, 0.57 and 0.37 μg/mL, respectively.

The accuracy of the method was evaluated using three concentrations with low, intermediate and high concentrations of the calibration at 25.6 – 38.4 μg/mL, 128.0 – 192.0 μg/mL, and 53.0 – 79.5 for INH, PZA, and RIF. The % recoveries of INH, PZA, and RIF ranged from 98.31 to 101.79%, 98.22 to 101.87%, and 98.45-100.70%, respectively (Table 4). The accuracy results demonstrated that the results of the mean tests were close to the true concentrations of analytes. The acceptance criteria given in ICH for recovery of the accuracy is within 98 - 102%.

**Table 4** Accuracy of INH, PZA, and RIF

| Sample | Concentration (μg/mL) | %Recovery (Cexp/ Cteo)x 100 | S.D. |
|---|---|---|---|
| **INH** | 25.6 | 100.07 – 100.99 | 0.46 |
| | 32.0 | 98.31 – 101.42 | 1.57 |
| | 38.4 | 101.70 – 101.79 | 0.05 |
| **PZA** | 128.0 | 101.75 – 101.87 | 0.06 |
| | 160.0 | 98.22 – 100.25 | 1.02 |
| | 192.0 | 100.36 – 101.82 | 0.74 |
| **RIF** | 53.0 | 98.45 – 100.61 | 1.22 |
| | 66.2 | 100.56 – 100.70 | 0.07 |
| | 79.5 | 100.25 – 100.60 | 0.17 |

The precision was evaluated at three different concentrations in three replicates for INH, PZA, and RIF (25.6 – 38.4 μg/mL, 128.0 – 192.0 μg/mL, and 53.0 – 79.5 μg/mL). For within-day precision, the % coefficients of variation (C.V.) for INH, PZA, and RIF ranged from 0.14 to 0.36%, respectively. The values of C.V. for the between-day precision of the assay for INH, PZA, and RIF were from 0.09 to 0.34 %, respectively (Tables 5 and 6). This value was in the acceptance criteria (not greater than 2%) of ICH.

**Table 5** Repeatability (within-day precision)

| Sample | Concentration (μg/mL) | Mean response ± S.D. | C.V. (%) |
|---|---|---|---|
| Within-day | | | |
| INH | 25.6 | 25.98 ± 0.09 | 0.34 |
| | 33.0 | 33.51± 0.06 | 0.20 |
| | 38.4 | 38.90 ± 0.12 | 0.32 |
| PZA | 128.0 | 127.45± 0.36 | 0.28 |
| | 165.0 | 164.44± 0.23 | 0.14 |
| | 192.0 | 191.42 ± 0.32 | 0.16 |
| RIF | 53.0 | 53.47± 0.14 | 0.26 |
| | 66.2 | 67.01± 0.14 | 0.21 |
| | 79.5 | 80.71± 0.29 | 0.36 |

**Table 6** Reproducibility (between-day precision)

| Sample | Concentration (µg/mL) | Mean response ± S.D. | C.V. (%) |
|---|---|---|---|
| Between-day | | | |
| INH | 25.6 | 25.97 ± 0.08 | 0.29 |
| | 33.0 | 33.52± 0.12 | 0.34 |
| | 38.4 | 38.88 ± 0.12 | 0.30 |
| PZA | 128.0 | 127.18± 0.25 | 0.19 |
| | 165.0 | 164.80± 0.25 | 0.15 |
| | 192.0 | 190.76 ± 0.41 | 0.22 |
| RIF | 53.0 | 53.47± 0.14 | 0.26 |
| | 66.2 | 67.16± 0.06 | 0.09 |
| | 79.5 | 80.98± 0.18 | 0.23 |

Determination of pharmaceutical formulation analysis

The quantification 4-FDC (A) was performed by external standard procedure. Sample of fixed dose tablets were analyzed using the validated method. Recovery data from the study are reported in Table 7. Overall average recovery yields for ISN, PZA, and RIF were 99.23%, 99.97% and 100.69%, respectively.

**Table 7** Sample quantification results

| Compound | Mean recovery ± SD % | RSD% |
|---|---|---|
| INH | 99.23 ± 1.02 | 1.03 |
| PZA | 99.97 ± 1.56 | 1.57 |
| RIF | 100.69 ±0.14 | 0.13 |

***Analysis of EMB***

The chromatographic separation of EMB was obtained by using a mixture of 2 mM of copper sulfate ($CuSO_4.5H_2O$) and 3 g/L sodium hexanesulfonate in 750 mL distilled water (adjust to a pH of 4.5 with 10% v/v phosphoric acid solution), adding 250 mL of tetrahydrofuran, followed by isocratic elution as the mobile phase at a flow rate of 0.4 mL/min. By using the developed sample preparation and validated chromatographic system, there were no interferences on the EMB peaks due to the components of the samples. EMB was eluted at 14.75 min (Figure 3). The SST is an integrated part of the analytical method and it ascertains the suitability and effectiveness of the operating system. The results of System suitability of EMB are reported in Table 8

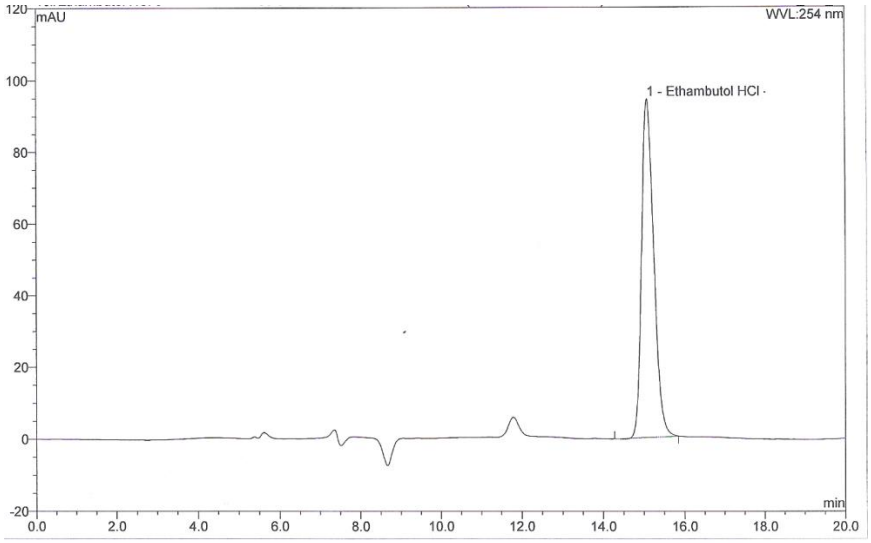


**Figure 3** HPLC chromatogram of 100 µg/mL ethambutol at 254 nm using tetrahydrofuran: a mixture of 2 mM of copper sulfate ($CuSO_4.5H_2O$) and 3 g/L sodium hexanesulfonate in distilled water pH of 4.5 (25: 75) as a mobile phase

**Table 8** System suitability of EMB

| Standard | System suitability | | |
|---|---|---|---|
| | Number of theoretical plate (N) | Tailing factor (asymmetric peak) ($T_f$) | Precision of retention time (%RSD) |
| EMB | 3443 | 1.11 | 0.98 |

Calibration curve parameter for EMB was shown in Table 9. Each standard solution analysis at 80, 90, 100, 110, 120 µg/mL was carried out in triplicate (n=3). The range of EMB was linear with regression of 80-120 µg/mL. The linear equation of EMB was y = 0.2738x – 0.1748 with $r^2$ of 0.9994. The result of the regression was obtained by plotting the peak areas of the analyte vs concentrations.

**Table 9** Linear regression and statistical analysis

| Sample | Concentration (µg/mL) | Slope | Intercept | Coefficient of Determination ($r^2$) |
|---|---|---|---|---|
| EMB | 80.0 – 120.0 | 0.2738 | – 0.1748 | 0.9994 |

The LOQ and LOD for EMB of the method were 4.29 and 1.415 µg/mL, respectively.

The accuracy of the method was evaluated using three concentrations with low, intermediate and high concentrations of the calibration at 80.0 – 120.0 µg/mL. The % recoveries of EMB ranged from 98.46 to 100.34%, (Table 10). The accuracy results demonstrated that the results of the mean tests were close to the true concentrations of analyte.

**Table 10** Accuracy of EMB

| Sample | Concentration (µg/mL) | % Recovery (Cexp/ Cteo)x 100 | C.V. (%) |
|---|---|---|---|
| EMB | 80.0 | 98.46– 99.62 | 0.59 |
| | 100.0 | 99.43–100.34 | 0.47 |
| | 120.0 | 98.97– 99.80 | 0.44 |

The precision was evaluated at three different concentrations in three replicates for EMB (80.0 – 120.0 μg/mL). For within-day precision, the % coefficients of variation (C.V.) for EMB ranged from 0.17 to 0.37%, respectively. The values of C.V. for the between-day precision of the assay for EMB were from 0.24 to 0.31%, respectively (Table 11).

**Table 11** Repeatability (within-day precision and between-day precision)

| Sample | Concentration (μg/mL) | Mean response ± S.D. | C.V. (%) |
|---|---|---|---|
| Within-day | | | |
| EMB | 80.0 | 80.52 ± 0.30 | 0.37 |
| | 100.0 | 100.65 ± 0.23 | 0.23 |
| | 120.0 | 120.78 ± 0.20 | 0.17 |
| Between-day | | | |
| EMB | 80.0 | 80.60 ± 0.25 | 0.31 |
| | 100.0 | 100.75± 0.20 | 0.20 |
| | 120.0 | 120.90± 0.29 | 0.24 |

Determination of pharmaceutical formulation analysis

The quantification 4-FDC (A) was performed by external standard procedure. Recovery data from the study are reported in Table 13. Recovery yield for EMB was 99.95%.

**Table 12** Sample quantification result

| Compound | Mean recovery ± SD % | RSD% |
|---|---|---|
| EMB | 99.95±0.47 | 0.47 |

A simple HPLC for the quantification of INH, PZA, RIF, and EMB in a fixed-dose combination antituberculosis was confirmed. The method was validated according to published ICH Q2A guidelines and manifested good achievement. The results also proved that no matrix effects were detected. The LOQ of 1.07, 1.74, 1.13, and 1.415 μg/mL for INH, PZA, RIF, and EMB, respectively, in this study was higher than the LOQ of 0.05-1.0 μg/ml in blood samples using HPLC/MS/MS (Song *et al.*, 2007), the LOQ of 0.019, 0.088, 0.546, and 0.42 μg/ml in sample dosage forms using spectrofluorimetric method (Maaboud et al 2013), and the LOQ of 0.24, 0.40, 0.60, and 0.30 μg/mL for INH, PZA, RIF, and EMB, respectively, using by pre-column derivatization (Huan, *et al.*, 2012). This method used less solvent consumption, less time or labor intensive for evaporation step prior to analysis. However, HPLC/UV method by pre-column derivatization with phenethyl isocyanate (PEIC) was not available for large numbers of samples, this method required long incubation period of PEIC used in the process as the derivatizing agent which this solvent is highly cost (Huan, *et al.*, 2012). It is commonly accepted that LC-MS-MS facilitates the injection of low sample volume and MS-MS detection is more sensitive than UV detection; nevertheless, the cost of instrument hinders the implementation of the technique in some laboratories.

The leading problem in the advancement of this method for the contemporaneous analysis of INH, PZA, and RIF was to find a suitable combination of mobile phase to separate the components. Calleri et al (2002), reported that the optimal pH of the phosphate buffer for separate was found to be 3.5 but the C18 column was not fortitude in this pH if compare to use at the pH 6.8 in our system.

An isocratic HPLC-UV method has been developed for determination of EMB in the presence of other anti-TB drugs. The proposed method is simple accurate and precise. The method is suitable for using in routine analysis of pharmaceutical dosage forms. EMB had

been assayed using a non-aqueous titration method, which is a time consuming procedure (BP 2012). The sensitivity and reproducibility of non aqueous titration was a comparatively poor measure. The fact that EMB does not have any chromophore to absorb UV light as well as low retention on reversed-phase columns limited the application of HPLC-UV method for its determination, several methods have been developed HPLC with fluorescence detection, CE with electrochemiluminescence detection, ion-pair reversed phase liquid chromatography with UV detection. However, it can form a complex with cupric ions. Thus, this methodology is highly useful to assess the determination of EMB in pharmaceutical preparations in the presence of other anti-TB drugs. The present method has the advantages of being simple and one of the preferable choices.

As a result, the two methods could be used for the quantification of the 4-FDC anti-tuberculosis constituents in pharmaceutical formulations. Although, the high LOQ values for the determination of INH, PZA, RIF, and EMB that achieved in this study was due to the fact that the old generation of UV-VIS detector was employed, it was accuracy and sufficient to determine. For these reasons, the present study describes the simple method and UV detector for the analysis of INH, PZA, RIF, and EMB in a fixed-dose combination antituberculosis.

## CONCLUSION

Four FDC outputs were prosperously determined by applying two methods, i.e. one for analyzing INH, PZA and RIF, and a second for analyzing EMB hydrochloride. The developed reversed HPLC provides a convenient and efficient method for separation and determination of INH, PZA and RIF in combined dosage from and represents a progress in respect to existing procedures for its simplicity and selectivity. The results of validation showed that this proposed method is linear, precise, accurate, selective and can be employed for the assay of INH, PZA, RIF and EMB in dosage form. Obviously, the present method offer many attractive advantages, being simple, the analytical equipment here used is fairly basic and integral part of almost any analytical laboratory.

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