

# OPTIMISATION AND VALIDATION OF HPLC METHOD FOR SIMULTANEOUS QUANTIFICATION OF RIFAMPICIN, ISONIAZID, PYRAZINAMIDE, AND ETHAMBUTOL HYDROCHLORIDE IN ANTI-TUBERCULOSIS 4-FDC TABLET

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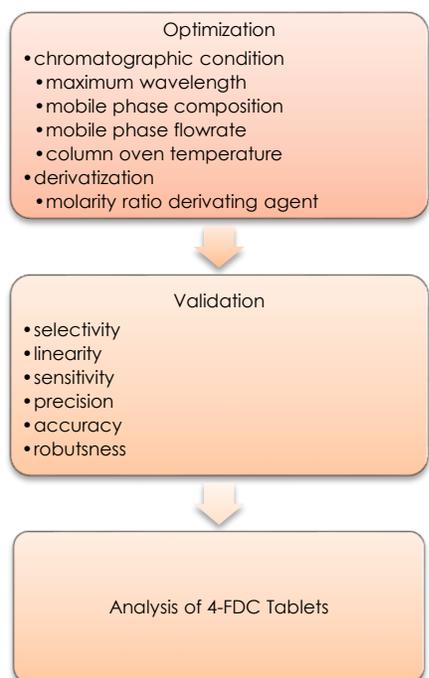
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## Graphical abstract



## Abstract

High-performance liquid chromatography (HPLC) method has been developed and validated for the simultaneous quantification of four components, namely rifampicin (RIF), isoniazid (INH), pyrazinamide (PYR), and ethambutol hydrochloride (ETM), contained in anti-tuberculosis drugs in fixed dose combination tablet (4-FDC). In order to increase the sensitivity of EMB, the pre-column derivatization technique with phenethyl isocyanate (PEIC) was carried out. The separation was accomplished using Waters Symmetry C8 (250× 4.6 mm i.d.; 5 µm) at 30°C. The mobile phase used was a mixture of acetonitrile and 20 mM phosphate buffer solution (pH 6.8) containing triethylamine and delivered at 1.5 mL/minute using gradient elution. The UV detector was set at 210 nm. The method was validated in terms of selectivity, linearity, accuracy, precision, detection limit, quantification limit, and robustness according to International Conference on Harmonization (ICH). The optimized method is successfully used for quantitative analysis of RIF, INH, PYR and ETM in 4-FDC tablets. The level of these drugs in 4-FDC tablets were in accordance to that specified in Indonesian pharmacopeia.

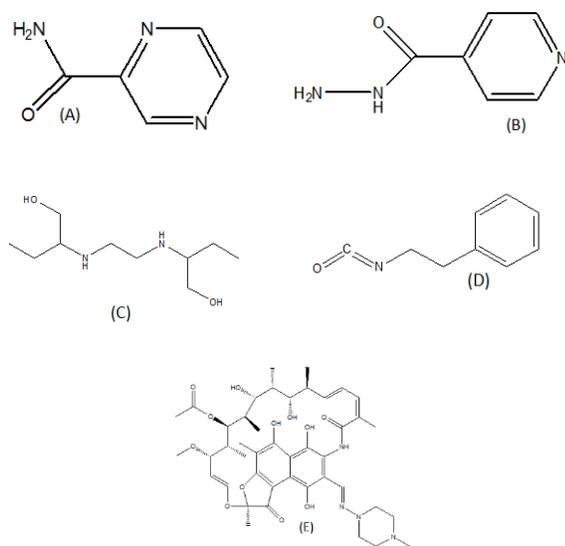
**Keywords:** HPLC, rifampicin, isoniazid, pyrazinamide, ethambutolhydrochloride, 4-FDC tablet

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## 1.0 INTRODUCTION

Tuberculosis (TB) remains a major global health problem to millions of people every year and is a major cause of death, especially in the African countries. The latest report in 2012 showed that there were 8.6 million of new TB cases and 1.3 million ended in death [1]. During the treatment of TB, the World Health Organization (WHO) has recommended the use of fixed-dose combination (FDC) because it can help patient adherence and prevent the medicine resistance to accelerate the success of TB control programs [2].

The advance in pharmacology and pharmaceuticals have contributed to create a tablet comprising a combination of several kinds of anti-TB medicines without disturbing the bioavailability of those drugs in one fixed dose, known as fixed dose combination (FDC). One of the FDC types available in the market is the combination of rifampicin (RIF), isoniazid (INH), pyrazinamide (PYR), and ethambutol (ETM), commonly called as 4-FDC tablet. The combination of these medicines (4-FDC) has potent bactericide and has a low toxicity; therefore, it can be used as the first choice for the treatment of TB [2]. In order to assure the quality of 4-FDC tablet, it is necessary to develop analytical method for the simultaneous analysis of drugs contained in 4-FDC (RIF, INH, PYR and ETM). The chemical structures of RIF, INH, PYR and ETM are shown in Figure 1.



**Figure 1** The chemical structures of pyrazinamid (A), isoniazid (B), ethambutol hydrochloride (C), phenethyl isocyanate (D), and rifampicin (E)

WHO recommended the use of high performance liquid chromatography (HPLC) for analysis of TB 4-FDC tablet using 2 separate systems in which RIF is set apart due to its stability consideration [3]. In addition, USP XXXII also determined 4-FDC tablet using HPLC with 2 different systems, in which ETM was determined using

different condition from that of PYR, INH and RIF due to the weak absorption response of ETM in UV detector [4]. The quantitative analysis of drugs using 2 separate systems needs a long time and is not suitable for routine analysis, as a consequence, some efforts have been made to use HPLC in one system for determination of PYR, INH, RIF, and ETM in 4-FDC tablet simultaneously.

Some analytical methods have been developed for analysis of anti-TB drugs, namely HPLC with fluorescence detector [5], HPLC with pre-column derivatization [6], HPLC assay using UV detector after pre-column derivatization [7], high performance thin layer chromatography [8], capillary electrophoresis with electrochemiluminescence detection [9], capillary zone electrophoresis [10], ion-pair liquid chromatography using UV detector [11], atomic absorption spectrophotometry [12], and near infrared spectroscopy combined with multivariate calibration [13].

Due to the limited study on the simultaneous assay of these drugs (PYR, INH, RIF, and ETM) in 4-FDC, then it is necessary to have an alternative method for quantitative assay of 4-FDC simultaneously. Therefore, the objective of this study was to develop HPLC method using UV-vis detector for simultaneous analysis of four components in 4-FDC tablet. The developed method involved derivatization technique using phenethyl isocyanate (PEIC) [7].

## 2.0 MATERIALS AND METHOD

### 2.1 Chemicals and Reagents

The reference standards of rifampicin (RIF), isoniazid (INH), pyrazinamide (PYR) and ethambutol hydrochloride (ETM) were of reference standard of Indonesian Pharmacopoeia and were obtained from the National Agency of Drug and Food Control, Republic of Indonesia. The chemicals and reagents used were of pro-analytical grade. The solvents used were of HPLC grade. The tablet dosage form was obtained from pharmacy in Denpasar, Bali, Indonesia.

### 2.2 Liquid Chromatography Condition

The chromatographic experiments were performed with a HPLC system consisting of two pumps (LC 20AD Prominence, Shimadzu, Japan), a SPD M20A Diode Array Detector set at 210 nm (Shimadzu, Japan), and temperature control device was maintained at 30°C. The data acquisition was achieved with LC-Solution (Shimadzu, Japan). The automatic load sample injections were carried out with 20 µL sample loop. The HPLC separations were performed on a Waters Symmetry C8 (250 × 4.6 mm I.D.; 5 µm) stainless steel column. The optimization of chromatographic system is done by determining the ratio of mobile phase composition. The mobile phase used was acetonitrile (A) and phosphate buffer 20 mM (pH 6.8) containing 1.5 mL of triethylamine (TEA) per 1 L of buffer

solution (B) [14]. Then, the flow rate of mobile phase was optimized. To determine UV detector wavelength, the absorption spectra were scanned from 190 - 400 nm using a HPLC system connected to a Diode Assay Detector (Shimadzu, Japan).

### 2.3 Preparation of Standard Solution

The stock solutions of RIF, INH, PYR and PEIC were prepared by accurately weighing of each reference standards and were dissolved in acetonitrile to get stock solution with concentrations of 0.6, 0.3, 1.6 and 1.96 mg/mL, respectively. The stock solution of EMB (1.1 mg/mL) was prepared in acetonitrile containing 1% TEA. The working standards were prepared by diluting each stock solution with acetonitrile to get the final concentrations of 72 µg/mL (RIF), 36 µg/mL (INH), 192 µg/mL (PYR), and 132 µg/mL (ETM).

### 2.4 Derivatization

The derivatization procedure was performed as follows: in test tube, 1 mL of each working or sample solution was added with 0.215 mL of PEIC 1.96 mg/mL. Subsequently, the mixture was mixed for 10 min in a swing bed at room temperature. The organic solvent was evaporated under a nitrogen stream. The residue was reconstituted with 1 mL of methanol: phosphate buffer (75:25 v/v). The solution was filtered through 0.45 µm millipore, and 20 µL of solution was injected into HPLC system.

### 2.5 Method Validation

Method validation was performed according to guideline in International Conference on Harmonization (1996) by determining selectivity, linearity, sensitivity, precision, accuracy, and robustness [15]. The selectivity assay was done by comparing retention times of each single standard, mixed standards, and components on the tablet. The calculation of similarity values of the mixed standards and components on 4-FDC tablet was also performed. The linearity study was done by varying the concentration of each stock standards. The calibration curve was obtained by plotting the concentration of each drugs (RIF, INH, PYR, and ETM) with its peak area. The sensitivity assay was performed by calculating limit of detection (LoD) and limit of quantization (LoQ) based on signal to noise ratio of 3: 1 and 10: 1 for LoD and LoQ, respectively. The precision of the developed method was assessed using repeatability (intraday precision) and intermediate precision (interday precision) assays and expressed by relative standard deviation (% RSD) values. The accuracy of analytical method was carried out by standard addition method and expressed as recovery percentage at 3 specific ranges, namely 80, 100, and 120% from target of analyte [15]. The robustness test was performed by varying parameter of mobile phase flow rate of ± 0.1 mL/min, gradient/slope time of ± 20%, and hold time of ± 20% [16]. The differences among

parameter values were tested statistically using one way analysis of variance (One way-ANOVA).

### 2.6 Analysis of 4-FDC Tablet

Ten tablets were weighed and homogeneously grounded to fine powders. The powder equivalent to 15 mg RIF, 7.5 mg INH, 40 mg PYR and 27.5 mg ETM was accurately weighed, placed in a 25 mL volumetric flask, and diluted to volume with acetonitrile (containing 1% TEA). The sample was ultrasonicated and then filtered through a 0.45 µm millipore filter to obtain clear solution. After being derivatized, derivatives were determined using HPLC as described above.

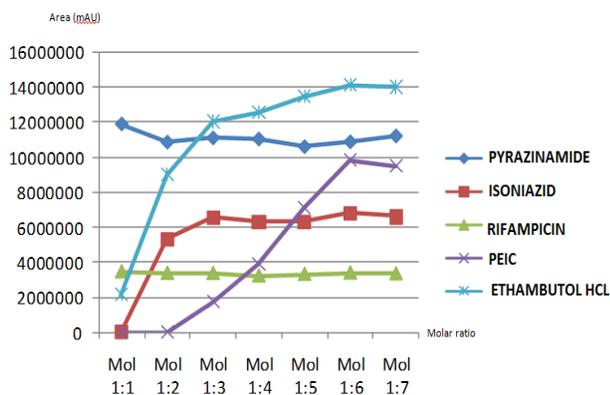
## 3.0 RESULTS AND DISCUSSION

### 3.1 Optimization of the Chromatographic Condition and Derivatization

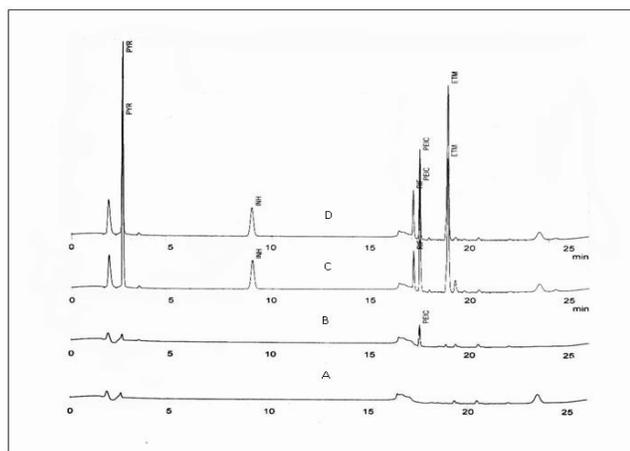
During the optimization, the mobile phase used was acetonitrile (A) and phosphate buffer pH 6.8 (B) using isocratic system with a ratio of A: B of 10:90, 30:70, 50:50, 70:30, and 90:10 v/v. Using this condition, RIF, INH-PEIC, PYR, and ETM-PEIC were not eluted in the same system. ETM has a high polarity, therefore, it could not be separated using reverse phase column. The derivatization of ETM with PEIC enables EMB-PEIC to have chromophoric groups, as a consequence, the derivatized products could be detected in UV detector. Besides, the polarity of ETM-PEIC was reduced so it can be stuck in such a way on a reverse phase column. For this reason, the gradient system was exploited to elute RIF, INH-PEIC, PYR, and ETM-PEIC simultaneously. The gradient system used was acetonitrile (A) and phosphate buffer 20 mM (pH 6.8) containing 1.5 mL TEA for each 1 L (B). The gradient program is (A : B) 22:78 v/v at 0 min, and linear gradient up to 12 min, changed to 60:40 for 1 min, and this ratio was held to 22 min. The composition of the mobile phase was returned to its original composition (22:78) for 1 min. The system was held for 2 min before injection.

The next optimization was performed on mobile phase flow rate. The optimal result in terms of peak resolution was achieved at 1.5 mL/min, and the column oven temperature was 30°C. While, the optimum UV detection is set at 210 nm. The condition of derivatization process was also optimized by comparing the molarity of derivatizing agent required in the reaction. Phenethyl isocyanate (PEIC) is known to have strong reactivity to primary amine and hydroxyl groups [17]. During derivatization of 4-FDC tablet, PEIC would react with primary amine group in INH and hydroxy group in ETM. The derivatization can increase the sensitivity of ETM response during UV detection and increase the hydrophobicity of the analyte, therefore, analytes can be more retained in reverse phase column [7]. The result showed that the optimum condition during derivatization of ETM with PEIC was achieved with the molarity ratio of ETM: PEIC of 1:6, as

shown in Figure 2. This result is consistent with that reported by Wang *et al.* [7]. The representative chromatogram obtained using the optimized condition was shown in Figure 3.



**Figure 2** Effect of the molar ratio of ethambutol hydrochloride (ETM) to phenethyl isocyanate (PEIC) on the derivatization



**Figure 3** Chromatograms of the PEIC-derivatives-FDC: (A) solvent, (B) PEIC, (C) mix-reference standards, (D) sample 4-FDC tablet. PYR = pyrazinamid, INH = isoniazid, ETM = ethambutol hydrochloride, PEIC = phenethylisocyanate RIF = rifampicin

### 3.2 Validation of Analytical Method

The selectivity test showed that the evaluated components (RIF, INH-PEIC, PYR, and ETM-PEIC) in 4-FDC tablet had good separation with a resolution value ( $R_s$ ) > 2.0 [16]. The retention time as well as the chromatogram profile of four drugs in FDC tablet was similar to that of the mixed standard references. The peak purity test using diode array detector showed that the peak purity is close to 1 (> 0.95), indicating that there was no interference in peak purity [18], from tablet additives, impurities, degraded compounds, and excess derivatizing agents. Table 1 listed the results of linearity test for the relationship between analytes

concentration and peak height. The correlation coefficient ( $r$ ) value of > 0.999 was achieved [19]. The method also produced %y-intercept value < 2.0 %, which indicated that errors in regression can be negligible [19]. From the values of  $r$  and %y-intercept, it can be concluded that the developed method are linear over the concentration ranges as listed in Table 1.

**Table 1** The linearity test of pyrazinamid (PYR), isoniazid (INH), rifampicin (RIF) and ethambutol (EMB)

Comp	Regression equation	r	%y-intercept	Range ( $\mu\text{g/mL}$ )
PYR	$y=4.22 \times 10^7 + 1.26 \times 10^5$	0.9999	0.73	16.94-250.94
INH	$y=9.23 \times 10^7 - 4.41 \times 10^4$	0.9997	1.18	7.20-81.77
RIF	$y=2.20 \times 10^7 - 1.20 \times 10^4$	0.9993	1.54	24.35-121.75
EMB	$y=6.66 \times 10^7 - 8.80 \times 10^4$	0.9999	1.09	10.29-164.64

**Table 2** Precision studies of pyrazinamid (PYR), isoniazid (INH), rifampicin (RIF) and ethambutol (EMB) using intra-day and intermediate precision tests

Comp	RSD values (%)	
	Intra-day	Inter-day (intermediate precision)
PYR	0.50	0.28
INH	1.02	0.54
RIF	0.84	0.18
EMB	0.62	0.02

**Table 3** Robustness test result of the method

Comp	Significance level from test during Robustness test *		
	Flow	Gradient Time	Hold Time
PYR	0.729	0.418	0.449
INH	0.812	0.497	0.660
RIF	0.322	0.941	0.058
EMB	0.584	0.201	0.143

\*) Significance level was declared at 0.05

**Table 4** Analytical results of pyrazinamid (PYR), isoniazid (INH), rifampicin (RIF) and ethambutol (EMB) in samples of 4-FDC tablet (n = 6)

Spl	Compounds*							
	PYR		INH		RIF		ETM	
	RS level (%)	RS D (%)						
Bran d A	106.59	0.50	103.55	1.02	105.57	0.84	107.10	0.62
Bran d B	95.65	0.99	99.02	1.27	105.31	1.19	107.06	0.67

\*]pyrazinamid (PYR), isoniazid (INH), rifampicin (RIF) and ethambutol (EMB) tablets contain not less 90,0 percent and not more than 110,0 percent of the labeled amounts of rifampicin ( $C_{43}H_{58}N_4O_{12}$ ), isoniazid ( $C_6H_7N_3O$ ), pyrazinamide ( $C_5H_5N_3O$ ), and ethambutol hydrochloride ( $C_{10}H_{24}N_2O_2 \cdot 2HCl$ ) [4].

The precision tests demonstrated that %RSD value for repeatability and intermediate precision were of 0.5 – 1.02% for intra-day precision, and 0.02-0.54% for intermediate precision (Table 2). The RSD values are lower than those required by Horwitz function (RSD <2.0%) [16]. The accuracy test was determined using standard addition method by spiking 80, 100 and 120% of target values into samples. The results of recovery percentage of RIF, INH-PEIC, PYR, and ETM-PEIC in 4-FDC obtained was in the range of 98.96 % -101.43%. These values are in the range of those specified in AOAC(98 - 102%) [16]. The robustness test, as determined by one way analysis of variance, showed no significant difference ( $P > 0.05$ ), indicating that the method is robust enough to be influenced by small variation in the analytical method conditions (the variation of mobile phase flow rate, gradient/slope time and hold time) [16]. The robustness results of RIF, INH-PEIC, PYR, and ETM-PEIC in 4-FDC tablet were compiled in Table 3. The limit of detection (LoD) values obtained are 5.08, 2.16, 4.92, and 3.08  $\mu\text{g/mL}$  for RIF, INH-PEIC, PYR, and ETM-PEIC, respectively. The values of limit of quantification (LoQ) are 16.94, 7.20, 16.39, and 10.27 for RIF, INH-PEIC, PYR, and ETM-PEIC, respectively.

The developed and validated method is further used for the quantitative analysis of RIF, INH, PYR, and ETM in 4-FDC tablets. The content of the evaluated tablets for each drug as shown in Table 4. The levels of RIF, INH-PEIC, PYR, and ETM-PEIC in 4-FDC tablet meet the monograph specification, i.e. in the range of 90 – 110% from the specified levels.

## 4.0 CONCLUSION

The reversed HPLC has been successfully used for simultaneous assay of four components namely rifampicin, isoniazid, pyrazinamide and ethambutol hydrochloride in 4-FDC tablet. The developed method showed acceptable precision and accuracy with good linearity (%RSD < 1.09 %, %recovery 98.96 % -101.43 %, and  $r > 0.999$ ). The advantages of this method include 4-FDC assay simultaneously, fast, and simple; thus, this method can be used for routine analysis of 4-FDC tablets.

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