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Development and Validation of HPLC Method for Simultaneous Determination of Three Constituent in 4-FDC Tablet by Pre-Column Derivatization

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ABSTRACT

RP-HPLC method has been developed and validated for assay to three of four constituents in anti-tuberculosis 4-FDC tablet simultaneously: rifampicin, isoniazid and pyrazinamid. To increase UV absorbance of the three components, phenyl isothiocyanate (PITC) is used in pre-column derivatization. Derivatization parameters were optimized and the results are molar ratio of the analytes to PITC, 1:40 and time for vortex processing is 60 seconds. Derivatization result was separated using stationary phase Phenomenex Luna C_{18}, 5 µm (250 x 4.6 mm). Phosphate buffer 8 mM pH 4.8 and acetonitrile were used as mobile phase with gradient system, flow rate 1.0 mL/minute and detector UV 254 nm. This method was validated with some parameters: selectivity, linearity, precision, accuracy, LOD, LOQ and stability. This method cannot be performed on a compound of ethambutol because its reaction produced two derivatives. So this method can only be applied to assay to three constituent in anti-tuberculosis 4-FDC tablet simultaneously.

Keywords: HPLC, pre-column derivatization, rifampicin, isoniazid, pyrazinamid

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INTRODUCTION

Tuberculosis is an infectious disease caused by *Mycobacterium tuberculosis.* Based on data from World Health Organization (WHO) in 2014, there were an estimated 9.6 million incident cases of tuberculosis (TB), among of them: 5.4 million cases of male patients; 3.2 million of female and 1.0 million of children. In 2014, the area of south-east Asia and western pacific region recorded approximately 58 % TB cases. Indonesia, India and China had the largest number of cases (10 %, 23 % and 10 % of global total, respectively). It is now estimated that there are about 1 million new TB cases per year in Indonesia, twice the previously estimated level [1].

TB treatment requires 3 to 5 types of drugs simultaneously depend on the patient's condition. The most effective first-line anti-TB drug, rifampicin, became available in the 1960s. The currently recommended treatment for new cases of drug-susceptible TB is a six-month regimen of four first-line drugs: rifampicin (RIF), isoniazid (INH), pyrazinamid (PYR) and ethambutol (EMB). These drugs can be administered as a single dose or in the form of fixed-dose combination (FDC). WHO and The International Union against Tuberculosis and Lung Disease (IUATLD) recommended FDC formulations of the essential anti-tuberculosis drugs as one further step to ensure adequate treatment of patients [1, 2]. The chemical structures of RIF, INH, PYR and EMB are shown in Figure 1.

In a clinical experiment comparing a FDC tablet with regimen of the same drugs given as separate pill, showed no difference in treatment outcome or side effects. The use of FDC tablet can reduce the number of pills taken daily at the stage of intensive treatment, so the patient convenient will be increase and the potential of medical errors became

decrease [3]. Tablet of anti-TB as substitute a single drug administration creates new problem in terms of determination of each constituent in order to ensure the quality and security of drugs.

Some of assay methods for constituent contained in 4-FDC tablet were done using several methods. According to USP 36th [4], the simultaneous quantification of four constituent in 4-FDC tablet was done using two systems. The first system was used to separate rifampicin, isoniazid and pyrazinamid simultaneously and the second system was used for ethambutol (because ethambutol cannot absorb ultraviolet ray) [5]. These assay methods use various instruments such as HPTLC [6, 7], LC-MS [8] and HPLC/UV [9]. Nowadays, there has been developed a new simple and rapid method for simultaneous quantification of four constituent in 4-FDC tablet using phenyl ethyl isocyanate (PEIC) as pre-column derivative with HPLC/UV [10, 11].

Phenyl ethyl isocyanate (PEIC) is isocyanate derivative that can react with primary and secondary amine groups and hydroxyl groups. So these compounds can be used in pre-column derivatization process. This PEIC together with ethambutol will react to form new compounds that can absorb UV rays [10, 11]. Another cyanate derivative that also reacts with primary and secondary amines groups and hydroxyl groups is phenyl isothiocyanate (PITC) [12].

PITC was first used in 1949 as peptide sequence by Edman [12]. The chemical structure of PITC is shown in Figure 1. PITC is currently widely used as derivate compound of amino acids and peptides. Some journals that used PITC as derivate in assay of drug compounds by using HPLC are determination of tranexamic acid in human serum [13] and assay of glucosamine in raw materials, dosage forms and plasma [14]. Based on some of these facts, the simultaneous assay of rifampicin, isoniazid, pyrazinamid and ethambutol was performed using HPLC with UV detector and PITC as pre-column derivate.

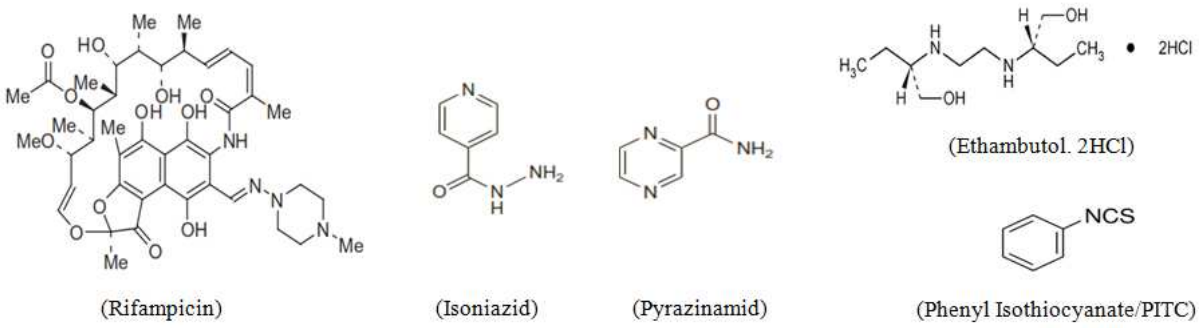

Figure 1 The chemical structures of rifampicin (RIF), isoniazid (INH), pyrazinamid (PYR), ethambutol (EMB) and Phenyl Isothiocyanate (PITC)

MATERIALS AND METHODS

Chemicals and Reagents

Rifampicin, isoniazid, pyrazinamid and ethambutol references were of reference standard of Indonesian Pharmacopeia and were obtained from National Agency of Drug and Food Control, Republic of Indonesia. Phenyl isothiocyanate (PITC) was purchased from Sigma-Aldrich. Acetonitrile and methanol were of HPLC reagent grade, potassium dihydrogenphospate and triethylamine from Merck. The 4-FDC tablet dosage form was obtained from pharmacy in Pekanbaru.

Instruments

The chromatographic experiments were performed with a HPLC system consisting of two pumps (LC 20AD, Shimadzu, Japan), a SPD M20A Diode Array Detector set at 254 nm (Shimadzu, Japan), and temperature control device was maintained at 30 ^{o}C. The data acquisition was achieved with LC-Solution (Shimadzu, Japan). The automatic load sample injection was carried out with 50 µL sample loop. The HPLC separation was performed on a Phenomenex Luna C_{18}, 5 µm (250 x 4.6 mm I.D.) stainless steel column.

Preparation of Standard Solution

Stock solution of RIF, INH, PYR and PITC were prepared by dissolving accurately weighed reference substances or chemicals in acetonitrile to get final concentration of 0.6 mg/mL; 0.3 mg/mL; 1.6 mg/mL and 11.09 mg/mL, respectively. The EMB stock solution (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 0.06 mg/mL (RIF); 0.03 mg/mL (INH); 0.16 mg/mL (PYR) and 0.11 mg/mL (EMB). All solution mentioned above were kept at 4 ^{o}C before use.

Pre-Column Derivatization
The derivatization procedure was performed as follows: in test tube, 1 ml of each working solution or sample solution was added with 0.39 ml of PITC 11.09 mg/mL. The mixture was mixed for 1 min using vortex and then the organic solvent was evaporated under a stream of nitrogen at 50 ^{o}C temperature on water bath. The residue was reconstituted with 2.0 mL of methanol : phosphate buffer 8 mM pH 9.9 (75:25 v/v). The solution was filtered through 0.45 µm Millipore and 50 µL of solution was injected into HPLC system.

Chromatographic Conditions
The separation of analytes was conducted with gradient system. The elution process was carried out with a mobile phase of phosphate buffer 8 mM pH 4.8 (A) and acetonitrile (B). The gradient profile of mobile phase was (A:B) 100:0 (v/v) at 0 min, then a linear gradient to 70:30 (v/v) for 5 min and it turned to 20:80 (v/v) for 15 min remain in linear gradient. The solvent composition was returned to 100:0 (v/v) over 10 min before the next injection. To determine the optimal ultraviolet wavelength, absorption spectra were scanned from 190 nm to 400 nm using HPLC system connected with a Diode Array Detector (Shimadzu, Japan). The analytes were measured at 254 nm because acetonitrile as mobile phase gave low absorbance at this wavelength.

Analysis of Rifampicin, Isoniazid and Pyrazinamid in Pharmaceutical Formulation
Twenty tablets were weighed to determine the average tablet weight and homogenously grounded to fine powders. The powder tablet equivalent to 15 mg isoniazid was weighed and placed in a 50 mL volumetric flask and diluted to volume with acetonitrile (containing 1 % TEA). The sample was stirred by ultrasonic to dissolve and filtered through a 0.45 µm Millipore filter to obtain clear solution. The filtrate was dilute with acetonitrile by 10 times for subsequent process. After derivatization process, derivatives were determined using standard solution as references.

Validation
When the chromatographic and the experimental conditions were established, method validation was performed following International Conference on Harmonization (ICH) specification. According to guideline in ICH Q2B, validation of analytical method for single laboratory must fulfill several parameters such as selectivity, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ) and robustness [15].

The selectivity assay was done by comparing retention times of each single standard, mixed standard and outstanding example of pharmaceutical dosage form (4-FDC tablet). The linearity was done by varying the concentration of each stock standard. Intra-day precision (repeatability) and inter-day precision (intermediate precision) assay were evaluated in six replicates of standard solution at low concentration on the same day and three consecutive days. The accuracy of analytical method was carried out by standard addition method and expressed as recovery percentage at three specific ranges (80 %, 100 % and 120 %). LOD and LOQ were determining using external calibration curve method. The robustness test was performed by varying timing for vortex processing.

RESULT AND DISCUSSION

Derivatization conditions
Derivatization aims to increase the detect ability of the analyte and, sometimes to achieve the addition of a chromatographic handle onto the analyte molecule. Pre-column derivatization are using simple instrument and cost less than post-column derivatization. One example of typical reagent for pre-column derivatization is PITC. Derivatization with PITC employs UV detection. PITC derivatives are fairly stable at room temperature, reacts with a wide variety of primary amines, secondary amines and hydroxyl groups [12].

The derivatization process using Edman reagent produced phenylthiocarbamyl (PTC) derivative compounds separated by RP-HPLC with the solvent used was acetonitrile [16]. Based on this process, acetonitrile was chosen as reaction solvent for the derivatization process.

The molar ratios of analytes to PITC start from 1:4 to 1:44. At this experimental, different amounts of PITC were allowed to react with the 4-FDC at optimized conditions. Figure 2 shows product of derivatization process between PITC with various molar ratios of analytes. Peak area of the resulting product increased with increasing ratio molar of analyte to PITC. When peak area provides constant value, the reaction was considered perfect. So, the amount of PITC that was reacted at the constant condition was chosen (In this study, equivalently, 0,39 mL of 11,09 mg/mL PITC).

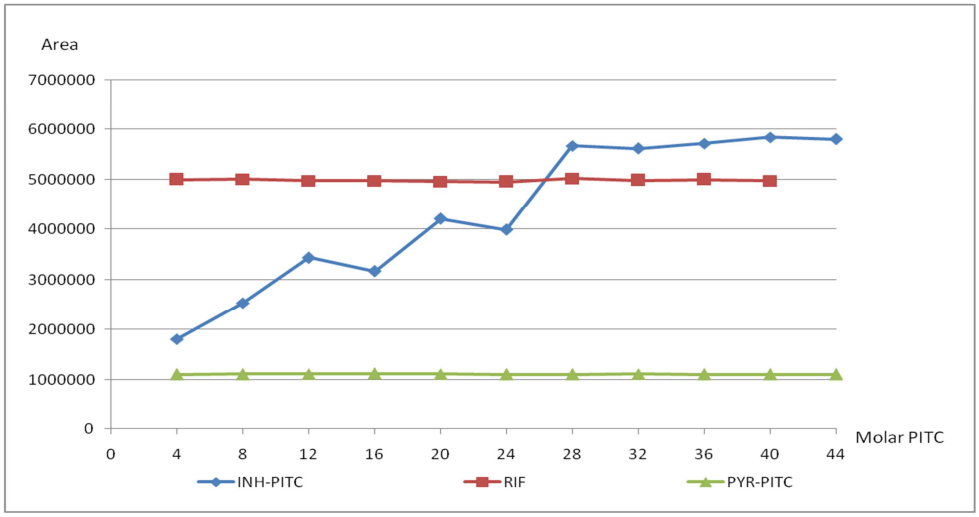

Figure 2 Effect of the molar ratio of isoniazid (INH), pyrazinamid (PYR) and rifampicin (RIF) to phenyl isothiocyanate (PITC)

The reaction between PITC with four constituent in 4-FDC tablet were assisted by vortex for a few second. To find out how long the optimal time needed for this reaction, it has been conducted the experiment on three different variations of time: 15 seconds, 30 seconds and 60 seconds. Figure 3 appears that at the time of 60 seconds, peak area of each product derivatization result in high value. The result of derivatization process would then dry under nitrogen gas stream to remove residual of PITC [16].

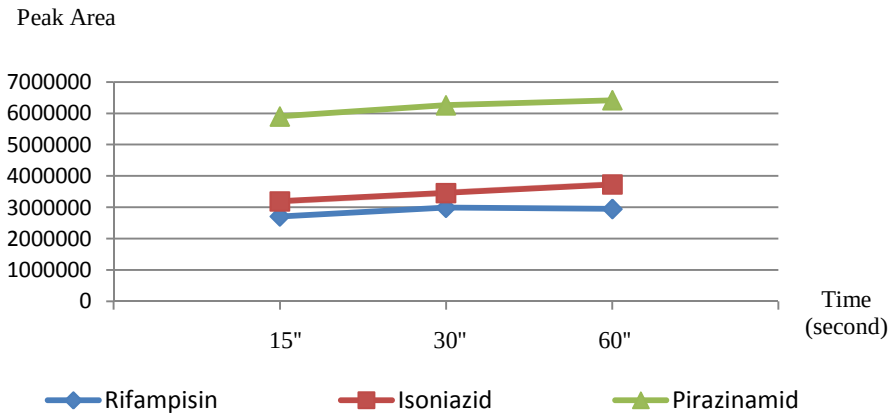

Figure 3 Effect of vortex processing time for peak area of RIF-PITC, INH-PITC and PYR-PITC

Chromatography Conditions

Chemical modification may modify compounds in order to provide absorption in the wavelength range UV or VIS, with high sensitivity and selectivity using HPLC. At this optimization process, the mobile phase used was a mixture of 8 mM phosphate buffer pH 4,8 (A) with acetonitrile (B), using gradient system. At the time of eluted, RIF, INH-PITC and PYR-PITC were not eluted on the same system. The peak of RIF will appear at 16th minutes; INH-PITC at 13th minutes and PYR-PITC at 9th minutes. For ethambutol, after derivatization process will form two different compounds. The first compound will appear at 12th minutes (EMB-PITC1) and second compound at 22nd minutes (EMB-PITC2). The formations of two types of these compounds were caused ethambutol besides having secondary amine groups also have hydroxyl groups. So PITC reacts with both these groups [5].

Based on the structure of INH, it was known that there is one primary amine group and one secondary amine group. After react with PITC, INH will form INH-PITC that having a shape of spectrum and retention time differs from INH. For RIF, despite it has a secondary amine group and several hydroxyl groups but it does not react with PITC. Chromatogram of RIF standard identical with RIF that reacted with PITC and retention times is the same. As for PYR, after react with PITC, it will form PYR-PITC derivatives, where these derivatives will be out in 9th minutes, which is different from PYR standard which came out in 3.3rd minutes. Chromatogram for each analyte is shown in Figure 4. Each analytes was measured absorbance at a wavelength of 254 nm, because at this wavelength

acetonitrile as the mobile phase will give the smallest absorption and does not interfere with the uptake of each analytes. The spectra of RIF, INH-PITC, PYR-PITC, EMB-PITC1 and EMB-PITC2 were shown in Figure 5.

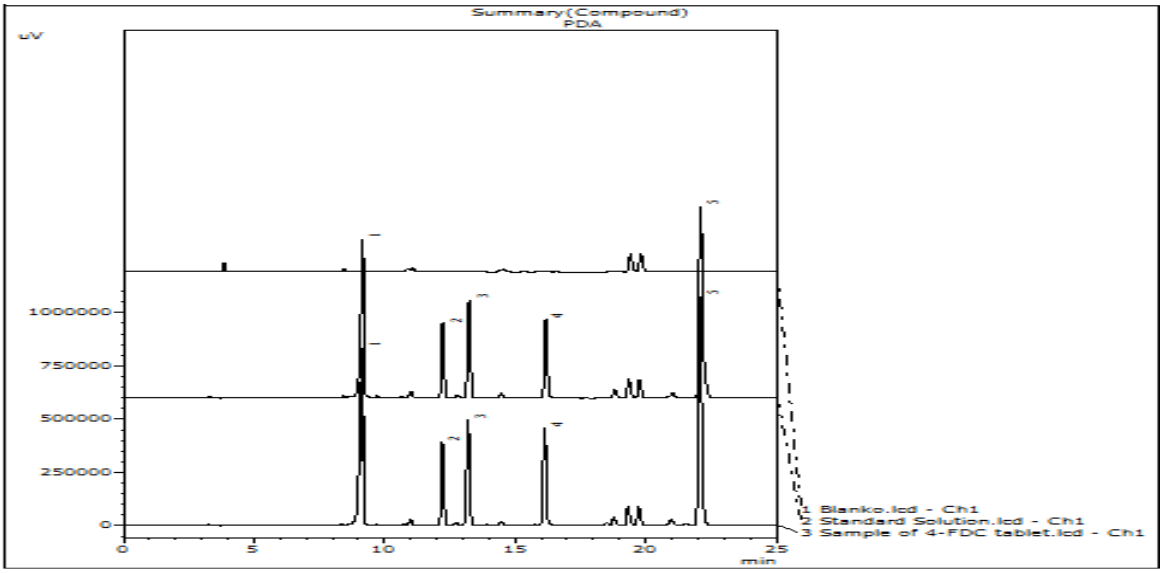

Figure 4 Chromatograms of the PITC-FDC: Blank, Working solution, Sample solution
Peaks: 1. PYR-PITC; 2. EMB-PITC1; 3. INH-PITC; 4. RIF; 5. EMB-PITC2

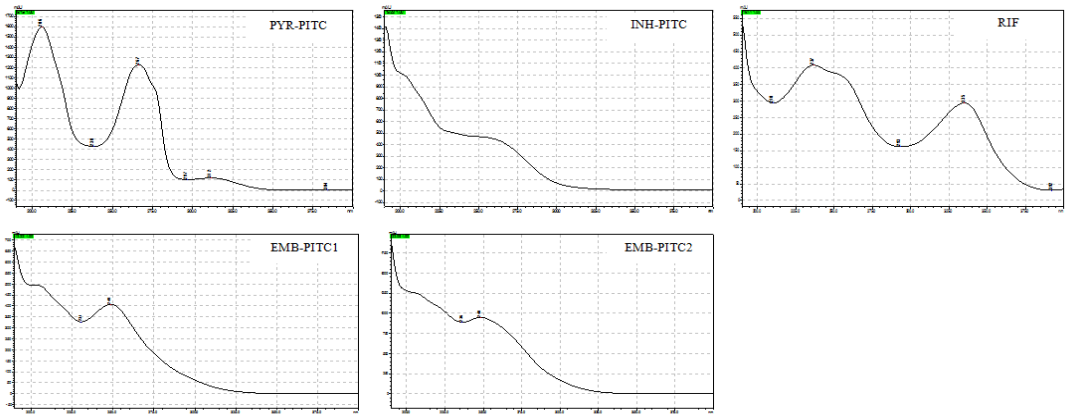

Figure 5 Spectra of PYR-PITC, EMB-PITC1, INH-PITC, RIF, and EMB-PITC2

Validation parameters of optimized method

After development and optimization of the methodology, some validation parameters, such as selectivity, linearity, precision, accuracy, limit of detection, limit of quantification and stability were determined. Based on the result of selectivity test, it is known that the RIF, INH-PITC and PYR-PITC have a good resolution (Rs > 2.0). The retention time of each compound contained in a sample of 4-FDC tablets compared with the retention time of the standard solution are the same. The peak purity test using DAD detector showed that the three components of 4-FDC tablet (RIF, INH-PITC and PYR-PITC) have a purity is close to 1 (> 0.999), suggesting that each peak is not disturbed by impurities.

The linearity test was performed to each standard solution at the optimal conditions. Based on the linearity test, data were summarized in Table 1. The LOD and LOQ values were obtained from an external calibration curve of each standard solution. This value is greater that the study conducted by Wang, *et al* [11] which used phenyl ethyl isocyanate as pre-column derivate.

Table 1 Parameters of validation

Compound	Regression equation	r	Range of concentration (µg/mL)	n	SD	LOD (µg/mL)	LOQ (µg/mL)	% RSD	
								Intra-day	Inter-day
Rifampicin	Y = 503687,93x-54241,67	0,9996	10-90	9	43822,22	2,87	8,70	3,58	4,12
Isoniazid	Y = 124032,74x-486744,71	0,9996	10-70	7	87757,59	2,33	7,08	1,75	2,03
Pyrazinamid	Y = 37335,94x-610060,33	0,9995	20-200	10	77186,13	6,82	20,67	3,99	7,00

Precision test was performed on each standard solution (RIF, INH and PYR), % RSD for intra-day precision and inter-day precision obtained are shown in Table 1. Table 1 shows the value of intra-day precision ranged between 1.75 – 3.99 % and inter-day precision values ranged between 2.03 – 7.00 %. These values are high when compared with the results of Wang *et al* [11] who received grades of precision intra-day and inter-day respectively is 0.78 – 0.98 % and 0.81 – 1.86 %. However, according to the AOAC requirements [17], a precision value is acceptable for the analyte of 0.0001 % was 7.3 (% RSD). The high value of inter-day precision was due to the instability of RIF, INH-PITC and PYR-PITC in the storage that reduces the area of each peak.

In the accuracy test (Table 2), the recovery values are in the range of 98.71 – 108.66 %, on condition of acceptance by AOAC [17]. This value is obtained from the accuracy test using addition method with range of concentration are 80 %, 100 % and 120 % with each of the ranges contains about 70 % of the samples and the 30 % standard solution. The assays on 4-FDC anti-TB tablet circulating on Pekanbaru city tested using this method, the results obtained levels of RIF, INH and PYR each was 107.27 %, 104.10 % and 104.38 %.

Table 2 Accuracy of the method

Compound	Range of Accuracy		
	80%	100%	120%
	% Recovery	% Recovery	% Recovery
Rifampisin	105,29	108,66	103,26
Isoniazid	105,43	102,01	100,27
Pirazinamid	99,74	98,71	107,48

CONCLUSION

Assay of three constituents in anti-tuberculosis 4-FDC tablet can be performed simultaneously by using phenyl isothiocyanate (PITC) as pre-column derivatization. PITC as pre-column derivatization in this method cannot be used to assay of EMB because of the reaction between PITC with EMB will produce two different compounds. So the method is used only to quantification of RIF, INH and PYR compounds simultaneously using HPLC instrument.

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