A simple Simultaneous UV Spectrophotometric Method to Determine Isoniazid and Rifampicin Contents in One Combined Tablet

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**ABSTRACT**
Isoniazid and rifampicin are the first-line anti-tuberculosis drugs in the advanced phase. This study aimed at validating the method to determine the levels of isoniazid and rifampicin contents in one serve of combined tablet circulating at Pramuka market using simultaneous analysis of Ultraviolet Spectrophotometry with methanol as a solvent. The maximum wavelength of isoniazid and rifampicin obtained was 261 nm and 337 nm, respectively. The method used to confirm the validity here includes linearity, the limit of detection (LOD), limit of quantitation (LOQ), accuracy, and precision under the ICH guidelines. The results of the method validation for isoniazid and rifampicin were as follow: linearity ($R^2$) in the range 5-25 $\mu$g/mL showed about 0.9998 and 0.9997, the limit of detection (LOD) 0.4026 $\mu$g/mL and 0.4952 $\mu$g/mL, and the limit of quantification (LOQ) 1.3421 $\mu$g/mL and 1.6508 $\mu$g/mL respectively. Besides, the accuracy-test (% recovery) was in the range of 100.44% ± 0.41% to 107.26% ± 0.28%, while the precision test (% RSD) was less than 2%. The determination of the contents was carried out on samples purchased at Pramuka Market with predetermined criteria and obtained levels of active ingredients ranging from 92.774% ± 0.39% to 103.51% ± 0.41%. The method validation to determine the levels of isoniazid and rifampicin contents in one serve of combined tablet using simultaneous analysis of Ultraviolet (UV) Spectrophotometry with methanol as a solvent is recommended for preparation analysis at the market.

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**INTRODUCTION**
Tuberculosis (TB) is a contagious and infectious disease caused by bacteria called *Mycobacterium tuberculosis* which can attack various organs of the body, especially the lungs. TB is one of the top 10 diseases causing deaths in the world. In 2018, the largest number of new TB cases occurred in the Southeast Asia region (44%), followed by Africa (24%) and the Western Pacific (18%). Eight countries accounted for the two-thirds of new TB cases are India, China, Indonesia, Philippines, Pakistan, Nigeria, Bangladesh and South Africa (WHO, 2020).

The first-line anti-tuberculosis drugs are isoniazid (INH), IUPAC named pyridine-4-carbohydrazide (Figure 1), a Nicotinic acid derivative which is a synthetic analogue of pyridoxine with antimycobacterial activity (NCBI - Isoniazid, 2020). However, INH has never been used singly to treat active tuberculosis due to its rapidly developing resistance. It needs to be combined with other drugs, one of which is with rifampicin (RIF). RIF...
is a semisynthetic antibiotic compound which is derived from rifamycin (Figure 2) and produced by Streptomyces mediterranei fermentation (NCBI - Rifampicin, 2020). RIF, a broad-spectrum antibiotic, has a significant indication to treat pulmonary TB and other infectious diseases. Here, the combination of INH-RIF has been the most effective drug for TB for the past ten years (Sotgiu et al., 2015).

The need for the INH-RIF combination makes the pharmaceutical industry produce the drugs in one serve of the combined tablet. Such combination has the following advantages; not interfering with drug bioavailability, increasing compliance, reducing the risk of resistance, reducing treatment costs and minimizing errors in drug use (Tilinca et al., 2017). In Indonesia, two pharmaceutical industries produce one fixed-dose combination of INH-RIF.

Based on the preliminary observations, this drug combination is also sold at pharmacies available at Pramuka Market, which is a well-known pharmaceutical sales centre in East Jakarta and sells drugs at relatively low prices. Sadly, some pharmacies do not have a permit. A few years ago, outdated and illegal drugs were discovered (BPOM, 2020). To that end, the combination of INH-RIF tablet sold at the market needs to be determined as a form of quality control on drugs that have been circulating.

The existence of one combined INH-RIF tablet must be followed up by an analytical method to determine the levels of the two compounds in a single preparation or serve. Several methods to analyze and determine the INH-RIF levels in one serve of the combined tablet have been developed including LC-MS/MS (Prahle et al., 2016), High-Performance Liquid Chromatography (HPLC) (Zhou and Chen, 2010), FTIR combined with chemometrics (Kurniati et al., 2016), a volumetric sensor using carbon paste electrode (Hammam et al., 2004) and UV Spectrophotometry (Asadpour-Zeynali and Saeb, 2016).

INH and RIF are drugs that have groups of chromophores as compounds which can absorb radiation in the ultraviolet region. INH has maximum absorption in methanol solution at a wavelength of 263 nm, while RIF has a maximum absorption at a wavelength of 338 nm respectively (Indonesian Pharmacopoeia, 2014).

For this reason, the best method which is considered fast, simple, sensitive, inexpensive and green chemistry (using little solvent) is UV Spectrophotometry method. If the maximum wavelength of the two compounds is close, then the determination of the contents is carried out by simultaneous technique (Ashour et al., 2015).

In short, the validation method to determine the levels of INH-RIF combination tablets using UV spectrophotometry will be carried out simultaneously in this study. After finding out that the method is valid, determining the levels of INH-RIF purchased at pharmacies at Pramuka market which do not have a permit is carried out. Information about the results of this study will be needed by the public, especially patients using the INH-RIF combination drug in Jakarta.

MATERIALS AND METHODS

Instrument

The instruments used in this study were Ultraviolet-Visible Spectrophotometer (HITACHI U-2910, Japan) with a wavelength of 190-800 nm and analytical balance (AND GH-202, Japan). The statistical calculations were competed using Microsoft excel 2010 version.

Chemicals and Reagents

The materials used in this study were isoniazid and rifampicin (purchased from Sigma-Aldrich, Germany) and methanol (Sigma Aldrich).

Sampling

Tablets with a fixed-dose combination of INH-RIF (150 mg each) as samples were taken based on established criteria (purposive sampling). Samples were purchased at Pramuka Market in November 2019. The inclusion criteria in this study were pharmacies that did not have a license to practice, selling INH-RIF combined tablets with a concentration of 150 mg each, which were not expired in different batches.

Determination of Maximum Wavelength

A standard solution of INH, RIF and a combination of both with a concentration of 15 μg/ml was made, and the absorbance was measured at a wavelength of 200-400 nm.

Determination of Absorptivity

The INH and RIF solutions with concentrations of 5, 15 and 25 μg/ml were prepared, and the absorbance was measured at the maximum wavelengths of the INH and RIF. Absorptivity was calculated using the Lambert-Beer formula.

Making calibration curves and determining linearity

Standard solutions of INH and RIF with concentrations of 5, 10, 15, 20 and 25 μg/ml were prepared, and the absorbance was measured at the maximum wavelengths of each.
**Limit of Detection (LOD) and Limit of Quantification (LOQ)**

The calibration curve equation was used to calculate the LOD and LOQ. The standard deviation of the analytical response and the slope of the calibration curve method followed according to the equations, LOD = \(3.3 \sigma / S\) and LOQ = \(10 \sigma / S\), where \(\sigma\) is the standard deviation of the sample and \(S\) is the slope (ICH, 2005; FDA, 2015).

**Accuracy**

Accuracy was made by calculating the percentage of the recovery. The INH and RIF solutions with specific ranges of 80%, 100% and 120%, respectively, were measured as the percentage of the recovery. Measurements were made three times (ICH, 2005; FDA, 2015).

**Precision**

The precision test was measured using intra and inter-day variations (n = 6) with a concentration of 100%. Two different Intra-days were performed at 24-hour intervals. Here, Inter-day was carried out on different days for three consecutive days. The precision test was obtained using RSD (Relative Standard Deviation) parameters (ICH, 2005; FDA, 2015).

**Determination of Contents in the Samples**

20 INH-RIF combined tablets from the same batch were crushed into powder and carefully weighed to be equal to 150 mg of rifampicin. Samples were dissolved with methanol, filtered using filter paper, and diluted to 5 \(\mu g / ml\), and absorbance was measured at the maximum wavelengths of INH and RIF. The levels of the samples were calculated using the following formulas (Maleque et al., 2012).

\[
\begin{align*}
C_x &= \frac{A_2ax_2 - A_1ax_1}{ax_2ay_1 - ax_1ay_2} \\
C_y &= \frac{A_1ax_2 - A_2ax_1}{ax_2ay_1 - ax_1ay_2}
\end{align*}
\]

**Abbreviations**

- \(A_1\) = isoniazid and rifampicin absorbance at a wavelength of 337.0 nm
- \(A_2\) = isoniazid and rifampicin absorbance at a wavelength of 261.0 nm
- \(ax_1\) = isoniazid absorptivity at a wavelength of 261.0 nm
- \(ax_2\) = isoniazid absorptivity at a wavelength of 337.0 nm
- \(ay_1\) = rifampicin absorptivity at a wavelength of 261.0 nm
- \(ay_2\) = rifampicin absorptivity at a wavelength of 337.0 nm
- \(C_x\) = concentration of isoniazid
- \(C_y\) = concentration of Rifampicin

**RESULTS AND DISCUSSION**

**Maximum wavelength**

The maximum wavelengths of INH and RIF obtained are 261 and 337 nm respectively (according to the Indonesian Pharmacopoeia, the wavelengths are 263 and 338 nm). There is a 2 nm shift, and such a shift is considered appropriate. Overlay curves among INH, RIF and a combination of both can be seen in Figure 3. At the maximum wavelength of INH, the absorbance of RIF was found and vice versa. Therefore, the determination of INH and RIF levels can be completed using the simultaneous method (Asadpour-Zeynali and Saeb, 2016; Indonesian Pharmacopoeia, 2014; Ashour et al., 2015).
Table 1: Absorptivity value of Isoniazid dan Rifampicin

| Samples   | Maximum wavelength | n  | Absorptivity (L/g cm) |
|-----------|--------------------|----|-----------------------|
| Isoniazid | 261 nm (Isoniazid) | 3  | 0.0309 ± 0.0003 (ax1) |
|           | 337 nm (Rifampicin)| 3  | 0.0006 ± 0.0001 (ax2) |
| Rifampicin| 261 nm (Isoniazid) | 3  | 0.0389 ± 0.0005 (ay1) |
|           | 337 nm (Rifampicin)| 3  | 0.0337 ± 0.0006 (ay2) |

Table 2: The result of correlation coefficient (r), LOD and LOQ

| Sample      | Isoniazid          | Rifampicin         |
|-------------|--------------------|--------------------|
| Linier Regression | y=0.0306x+0.0058   | y=0.0335x+0.0040   |
| R^2         | 0.9998             | 0.9997             |
| LOD (µg/mL) | 0.4026             | 0.4952             |
| LOQ (µg/mL) | 1.3421             | 1.6508             |

Table 3: The result of the accuracy

| Sample | Conc  | N  | Recovery (%) |
|--------|-------|----|--------------|
| Isoniazid | 80%     | 3  | 100.64 ± 0.45 |
|         | 100%    | 3  | 100.44 ± 0.41 |
|         | 120%    | 3  | 105.80 ± 0.30 |
|         | 80%     | 3  | 100.53 ± 0.42 |
|         | 100%    | 3  | 100.78 ± 0.35 |
|         | 120%    | 3  | 107.26 ± 0.28 |
| Rifampicin | 100%    | 3  | 100.78 ± 0.35 |
|         | 120%    | 3  | 107.26 ± 0.28 |

Table 4: The result of precision

| Samples | Nominal concentration (mg) | Intra-day (n=3) | Interday (n=3) |
|---------|---------------------------|-----------------|----------------|
|         | Measured Concentration (mg) | Precision (% CV) | Measured Concentration (mg) | Precision (% CV) |
| Isoniazid | 50.05                      | 51.53 ± 0.99    | 1.94  | 51.27 ± 1.01 | 1.62  |
| Rifampicin | 50.10                      | 50.04 ± 0.60    | 1.20  | 50.07 ± 0.99 | 1.29  |

Table 5: The result of a commercial brand assay

| Sample | N | Tablet X1                  | Tablet X2       | Tablet Y1     |
|--------|---|---------------------------|-----------------|---------------|
| Isoniazid | 3 | 103.51 ± 0.41             | 92.77 ± 0.39    | 95.31 ± 0.51  |
| Rifampicin | 3 | 97.77 ± 0.35              | 95.99 ± 0.59    | 95.15 ± 0.91  |

Absorptivity

The determination of absorptivity aims at calculating the absorbance of INH, which is at the maximum wavelength of RIF as RIF is influenced by isoniazid absorbance and vice versa (Asadpour-Zeynali and Saeb, 2016). The absorptivity is obtained using the law of lambert beer. Besides, the determination is done by measuring the absorbance standard of INH and RIF at the two wavelengths, 261 nm (INH) and 337 nm (rifampicin) with three series of concentrations; low concentration (5 µg/ml), moderate concentration (15 µg/ml) and high concentrations (25 µg/ml). The use of the three concentrations is intended to represent the overall absorptivity concentration on the calibration curve. Here, the absorbance data on the measurement is used to calculate the absorptivity value. The results of the absorptivity are illustrated in Table 1.
Figure 3: Chromatogram of INH, RIF and INH: RIF (1:1) 15 μg/mL in methanol

Calibration Curve

A calibration curve is a statistical method used to determine the comparison of the effect of analyte levels with quantitative effects of tool response (Tilinca et al., 2017). The determination of the isoniazid and rifampicin calibration curves is performed to find a linear regression equation which accordingly can be used to search for a level where the absorbance has been measured. In this test, five series of concentrations were made, namely 5, 10, 15, 20 and 25 mg/mL according to the recommendations of ICH, 2005.

The results of the linear regression calculations for isoniazid and rifampicin were \( y = 0.0306x + 0.0058 \) and \( y = 0.0335x + 0.0040 \) respectively (Table 2). Based on the linear regression equation, a correlation coefficient \( R^2 \) was obtained to show linearity. The \( R^2 \)-value for isoniazid was 0.9998, and rifampicin was 0.9997 respectively. The increase in the concentration of the analyte was directly proportional to the absorbance so that the value of \( r \) approaches 1, which indicated that the linearity met the valid analysis criteria (Kurniati et al., 2016; Alekhya et al., 2020).

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD value is the smallest amount of analyte in a detectable sample that gives a significant response compared to blank. The LOD values for isoniazid and rifampicin were 0.4026 and 0.4952 mg/mL respectively (Table 2).

In the meantime, the LOQ value is the smallest quantity of analyte in a sample that can still meet the meticulous and thorough criteria. The values obtained in both limits were 1.3421 and 1.6508 mg/mL respectively. The Determination of LOD and LOQ was intended to ensure the ability of the method to determine the lowest limit that can be assessed qualitatively and quantitatively from the samples (Asadpour-Zeynali and Saeb, 2016).

Accuracy

The accuracy test in this study was carried out at the low (80%), moderate (100%) and high (120%) concentrations of analyte levels which are usually contained in the market (150 mg of isoniazid and 150 mg of rifampicin). It aimed at seeing the accuracy of the methods developed and studying the interference of excipient formulations (ICH, 2005; Begum et al., 2013). The percentage of the recovery obtained ranged from 100.44 ± 0.41% to 107.26 ± 0.28% (Table 3) in the range of 80-110%. Thus, the determination of the isoniazid and rifampicin levels with this method has accuracy under the requirements.

Precision

The precision test is the degree of concordance or closeness between the results of one test with another. In the meantime, precision value is a measure of the distribution of data around the median value. It is commonly written as a relative standard deviation (RSD) of repeated measurements (Zhou and Chen, 2010). In this study, the precision test was carried out at 100% (150 mg of isoniazid and 150 mg of rifampicin), with Intraday and Interday variations. In Intraday, for instance, the test was done two different times in one day, whereas in interday, it was conducted on different days for three consecutive days.

The purpose of the precision test with intraday and interday variations is to see the effect of time differences on sample repeatability (Alekhya et al., 2020). The results (Table 4) show that the precision test had met the requirements, with a value of % RSD <2%. The method in determining the isoniazid and rifampicin levels had good repeatability.

Based on the validity test, the determination of isoniazid and rifampicin levels simultaneously with the UV spectrophotometry method shows results that are under the requirements. Therefore, the method can be used to determine the isoniazid and rifampicin levels contained in the samples available at the market.

Determination of Contents in the Samples

After the analysis method was found valid, the determination of the level of fixed-dose combination tablets with isoniazid of 150 mg and rifampicin of 150 mg respectively was carried out on samples purchased from the Pramuka market.

Two drug brands obtained the tablets that have met the inclusion criteria; X and Y. Drug X had two different batch numbers, whereas drug Y had
one batch number. The results obtained are illustrated in Table 5. The determination of the levels ranges from 92.77% ± 0.39% to 103.51% ± 0.41% of the amount indicated on the label. The drugs purchased at Pramuka Market have fulfilled the requirements of having 90-110% levels (Indonesian Pharmacopoeia, 2014; Mohamed et al., 2015).

CONCLUSIONS

The determination to validate the method of combining isoniazid and rifampicin simultaneously using the Ultraviolet (UV) spectrophotometry has met the requirements. The isoniazid and rifampicin levels in one serve of combined tablet circulating at Pramuka market are in the range of 92.777% ± 0.39% to 103.51% ± 0.41%. Such levels have met the requirements set by the Indonesian Pharmacopoeia, 2014, mainly 90-110%.

Conflict of interest

The authors declare that they have no conflict of interest for this study.

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