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By ENDANG DARMAWAN

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Validation of Isoniazid for Therapeutic Drug Monitoring in Human Plasma by High-Performance Liquid Chromatography

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Abstract. Isoniazid is one of anti-tuberculosis agent which can cause hepatotoxicity. However, not all of the TB patients and health providers can recognize early symptoms of antituberculosis-induced hepatotoxicity. Thus, the Therapeutic Drug Monitoring needs to be performed to monitor the hepatotoxicity symptoms. The aim of this study is to establish the validity of the Isoniazid assay method using High-Performance Liquid Chromatography from human plasma. We recruited 6 healthy subjects for this validation study. The validation was performed using Shimadzu HPLC system with a model AT LC20 LC 10AT pump, detector SPD 20A and LC solution software. We used C18 column shim-pack VP-ODS (250 mm x 4.6 mm, id 5µm) as well as other tools such as centrifuges, vortex, appliance glass (Pyrex IWAKI) and other supporting tools. Chemicals and solvents was used from Merck Germany. Isoniazid standard compounds were obtained from SIGMA. Our study has been approved by National Ethics Committee of Health Research. Our study shows that the method applied in HPLC has the good linearity ($r = 0.998$) with coefficient of variance (CV) of system appropriateness test is 0.61% and the equation of linearity was $y=8756.87x+27724.82$. The value of Limit of Detection (LoD) and Limit of Quantification (LOQ) were 1.517µg/ml and 4.597 µg/ml, respectively. The precision on the concentration of 0.5; 5.0; 15.0 ug/ml are 5.24%, 0.79%, 2.83%, respectively for intraday precision and 4.86%; 2.18%; 2.01%, respectively for interday precision. The recoveries on the particular concentrations are 100.79%; 108.91%; 92.19%, respectively for intraday recovery and 101.73%; 99.63%; 82.75%, respectively for interday recovery. This validation method is a good alternative for the application of TDM in monitoring the treatment of TB patients' clinical practice

Keywords: Validation, isoniazid, therapeutic, monitoring

BACKGROUND

Currently, Indonesia is in the 5th rank of the highest tuberculosis prevalence over the world [1]. Since 1995, WHO introduced the Directly Observed Treatment Shortcourse (DOTS) program, and this program has been proven as effective in controlling the tuberculosis disease. The mortality rate decrease about 45% and the tuberculosis prevalence decrease about 42%. However, the incidence of tuberculosis is still high [1,2].

Anti-tuberculosis agents which used in the treatment of tuberculosis are isoniazide, rifampicin, ethambutol and pyrazinamide. One of the most popular side effect due to the use of the medications are hepatotoxicity [3,4]. Isoniazid is the most possible drug caused hepatotoxicity due to its toxic metabolite [5].

The incidence of hepatotoxicity due to the use of antituberculosis was 20% [6]. Due to the long duration of treatment of tuberculosis, we need to do the monitoring of hepatotoxicity symptoms and provide the appropriate treatment [7]. One of the method to do the monitoring is Therapeutic Drug Monitoring (TDM) which is one part of

the pharmaceutical care services [8]. TDM may help the physician to understand about the complexity treatment of tuberculosis and also help the physician to decide about the dose or treatment recommended after having the results of TDM [9].

Isoniazid or Isonicotinic acid Hydrazide (INH) is a white or colorless crystals or white crystalline powder and odorless. It is slowly affected by air and light and is easily soluble in water, slightly soluble in ethanol, sparingly soluble in chloroform and in ether. INH is well absorbed in the gastrointestinal after oral administration and is distributed throughout the body. Its peak concentration in serum is depend on the dose administered. The peak serum levels achieved within 1-3 hours after oral administration and the effective concentration was found 24 hours after the drug is swallowed. The average half-life in fast acetylators reaches nearly 80 minutes, while the value of hours is typical for slow acetylators [10].

This study objective is to establish the validity of the Isoniazid assay method using High-Performance Liquid Chromatography from human plasma

METHODS

Subjects

We recruited 6 healthy subjects as volunteer of blood plasma sample. The blood was taken 2 hour after the antituberculosis agents were taken by the patients. We gave the informed consent to the subject and asked them to signature the informed consent form as the agreement for participation in this study. Our study has been approved by the National Ethic Committee of Health Research.

Reference Compound and Reagents

Chemicals and solvents used from Merck Germany, among others: methanol pro analysis (pa) (3906609839), Diethyl ether pa, Trichlor solution of acetic acid (TCA) 10% pa (3639707643), pa Acetonitrile (1632730213) and aquabidest. The isoniazid as standard compounds was obtained from SIGMA.

Chromatographic Conditions

This study used a Shimadzu HPLC system with a model of AT LC20 LC 10AT pump, detector SPD 20A and LC solution software. The column used was a C18 column shim-pack ODS (250 mm x 4.6 mm, id 5µm). Mobile phase consisted of *Aquabidest* : *Acetonitrile* (97:3)% with flow rate 1 mL/ minute, *UV detection* at a wavelength of 262 nm, retention time 15 minute and inject volume 20 µL.

Preparation of plasma standards and samples

The standard samples were prepared from 450 µl of plasma, then added by 50 µl validation solution to make 5.00; 50.00 and 150.00 µg/mL. The solution concentrations for linearity were 0.5; 1.00; 3.00; 5.00 and 15.00 µg/mL. The solution concentrations for accuracy, intraday and interdayrecision were 0.5; 5.0; 15.00 µg/mL.

The 1000 mL of 10% TCA was added onto each the concentration solutions then follow these procedures such as; 20 seconds of vortex for with a maximum speed and 10 minutes of centrifuged at a speed of 13000 rpm, temperature of 4°C. The supernatant was poured into the tube and added by 3 mL of ether. The extraction was performed using these procedures; 20 seconds of vortex and 10 minutes of centrifuged at 4000 rpm. Finally, the 20 mL water phase was injected into the HPLC [11,12].

Validation Method

The validation method of INH using HPLC was modified from the previous studies [11,12]. We used HPLC due to this instrument has high selectivity and sensitivity and it also can perform the analysis with small concentration of sample using UV adsorben detector [13].

System Suitability

The system suitability was examined from the one point of 20 µL solution from standard curve which was injected into HPLC. We replicated this method for six times. From the chromathograph we defined the coefficient of variance (CV) of retention time and wide of area ratio [14,15].

Linearity

The equation of linier regression: $y = bx+a$ described the association between INH concentration and area wide ratio of chromatograph or the high of chromatograph peak. The calibration curve was used to defined the sample concentration. [14,15].

LoD and LoQ

The value of LoD and LoQ were defined from the equation and standar of deviation (SD). LoD was defines as signal and noise ratio (S/N) with the 3:1 comparison. The LoQ was defined using 10:1 comparison of S/N. [14,15]

Accuracy

The recovery describes the value of accuracy. The Area Under the Curve (AUC) of isoniazid was processed by linear regression equation to get the actual levels. The value of recovery can be seen from the average % of recovery or actual levels divided theoretical level and multiplied by 100%. The value of good recovery was 80-120%. [14,15]

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Intra-day and Inter-day precision

Intra-day precision values can be seen from the % of coefisien of variance (CV) of each replication. The method met the requirements of precision if the value of the average % CV was <15%. [14,15]

RESULTS

The results of this study are listed in Table 1. The suitability system shows the CV T_R and CV peak area are 0,26% and 0,61% respectively. The equation for regression linier of 0.5; 1; 3; 5; 10; dan 15 $\mu\text{g/mL}$ concentration is $y = 8756.873x + 27724.816$, with $r = 0,998$. The regression linier can be seen in Graph 1. The LoD and LoQ met the criteria of FDA and ICH [14,15] which are 1.571 $\mu\text{g/mL}$ and 4.597 $\mu\text{g/mL}$, respectively.

TABLE 1. Results of validation measurement for INH using HPLC

Parameter	Required Criteria*	Results
System suitability	$CV \leq 2\%$	$CV T_R = 0,26\%$ $CV \text{ peak area} = 0,61\%$
linearity	$r \geq 0,99$	$r = 0,998$ $y = 8756.873x + 27724.816$
LOD	$S/N \approx 3:1$	1,517 $\mu\text{g/mL}$
LOQ	$S/N \approx 10:1$	4,597 $\mu\text{g/mL}$

CV = coefisien of variance; T_R = Retention Time; r = Coefficient of correlation; LOD = Limit of Detection; LOQ = Limit of Quantification. *Adopted from [14]; [15]

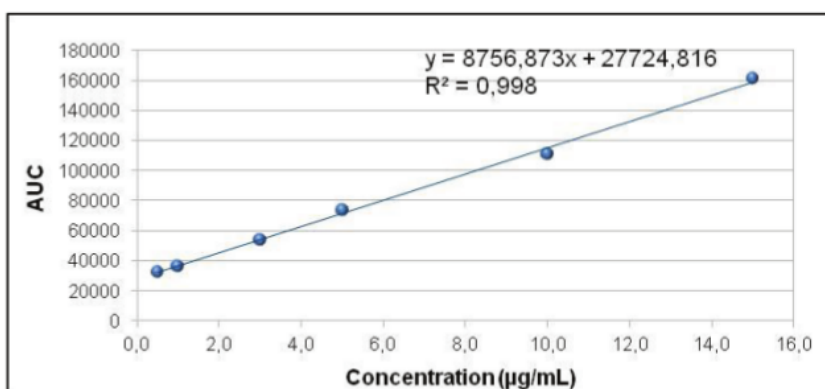


FIGURE 1. Linearity of INH concentration and AUC

Table 2 shows the results of accuracy and precision. The accuracy of of this method are shown by % recovery of intraday and interday sampel for 0.5; 5.0; and 15.0 $\mu\text{g/mL}$ concentration, which are 100.79%, 108.91%, and 92.19%, respectively and 101.73%, 99.63%, and 82.75% , respectively. The intraday precision is defined as CV (%) from the concentration of 0.5; 5.0 and 15.0 $\mu\text{g/mL}$ are 5.24%, 0.79% and 2. 83%, respectively. Furthermore,

the interday precision on those particular concentration are 4.86%, 2.18%, and 2.01% respectively.

TABLE 2. Results of precision and accuracy

Concentration (µg/mL)	Mean ± SD (µg/mL)	Precision (CV %)	Accuracy (R %)	Required criteria
Intraday (n = 5)				
0,5	0,50 ± 0,03	5,24%	100,79%	Precision = CV ≤ 15% Accuracy = 80-120%
5	5,45 ± 0,04	0,79%	108,91%	
15	13,83 ± 0,39	2,83%	92,19%	
Interday (n = 5)				
0,5	0,51 ± 0,02	4,86%	101,73%	
5	4,98 ± 0,11	2,18%	99,63%	
15	12,41 ± 0,25	2,01%	82,75%	

DISCUSSION

Our study found that the method used in HPLC is valid and reliable. HPLC has high selectivity and sensitivity, also it can measure the component of sample until nanogram limit using uv deector. Plasma has many components which may affect the isoniazide concentration measurement. Thus, using HPLC is appropriate method to measure isoniazide concentration [13]. The validation which defined in this study were system suitability, linearity, LoD and LoQ, accuracy and precision [8].

The suitability system was performed before the validation procedures to assure that HPLC can work appropriately during the analysis [14,16]. This suitability system test assures that the chromatography has good reproducibility and resolution for the analysis [17]. Linearity was performed to understand the association between the real concentration of isoniazide and the instrument response [16,18].

Accuracy was defined to assure that the method used in the analysis can show the concentration of sample is almost similar to the real concentration [14]. The precision was performed to assure that the repeated measurement has similar results of concentration [16,18].

Previous study about rifampicin validation method for TDM using stirbar-sorptive extraction/HPLC-UV showed that the method could be applied in real situation due to the similar effectivity to liquid-liquid extraction/HPLC-UV. The intraassay and interassay precision and accuracy were performed in the concentration less than 25.0 microgram/mL and had the results of less than 10%. Other parameters also met the criteria for validation [19]. However, previous other study used Hydrophylic Interaction Liquid Chromathography (HILIC). The benefit of using HILIC was the ion-pair reagent of mobile phase was free detected. This method could be applied in the TDM method, due to the precision and accuracy were less than 10% and the limit of quantification was 4 ng/ml for isoniazid and rifampicin [21]. In the HPLC method, the modification of mobile phase component is required due to the character of the drugs. In this study, isoniazid has hydrophilic character thus we only used acetonitrile and aquabidest as the mobile phase.

The other method for TDM of antituberculosis is matrix-assisted laser desorption/ionization source equipped with tandem time-of-flight (MALDI-TOF/TOF) mass spectrometry. This method was introduced by Sirgel et al and presented that the minimal concentration detected using this method was until 0.08 pmol/microL [20]. The qualitative method of rifampicin and isoniazid ingestion also performed in previous study using non-invasive method. This procedure presented that the qualitative method was as effective as the method used in HPLC [21].

Many methods have been performed in the previous studies with valid results of antituberculosis TDM method. However, we need to consider the instrument which is available in our country such as HPLC-UV. Regarding to the budget in performing the TDM method, it is not possible to do the TDM in all tuberculosis patients. We need to make the priority for patients selection such as; ineffective treatment and patients with multidrug resistance of antituberculosis.

CONCLUSION

According to the results it can be concluded that the use of HPLC has met the criteria of accuracy, precision, suitability of the system, linearity, LoD and LoQ. Such methods have validity in accordance with the required Anonymous (2001) and Anonymous (2005) so that the validation method can be used to establish the levels of INH in the plasma in the handling of patients with tuberculosis as well as an effort to monitor the effectiveness of treatment in the activities of Therapeutic Drug Monitoring (TDM).

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