Validation of pyrazinamide in human plasma using Hplc-Uv for therapeutic drug monitoring

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Validation of Pyrazinamide in Human Plasma Using Hplc-Uv for Therapeutic Drug Monitoring

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Abstract. Currently, Indonesia is in the 5th rank of the highest tuberculosis prevalence over the world. The treatment of tuberculosis is going complicated due to the side effect experienced by the patients. The four combination of antituberculosis agent used in minimally 6 months of treatment could stimulate the hepatotoxicity as the one of the dominant side effect in tuberculosis treatment. Thus, it is important to do the Therapeutic Drug Monitoring (TDM) to optimize the tuberculosis treatment. This study is aimed to validate the TDM of pyrazinamide in human plasma using High Performance Liquid Chromatography-UV. We recruited 6 TB patients in the validation of pyrazinamide study. The C18 column shim-pack VP-ODS (250 mm x 4.6 mm, id 5μm) and aquabidest-acetonitrile as mobile phase were applied in this study. We used Shimadzu HPLC system with a model AT LC20 LC 10AT pump, detector SPD 20A and LC solution software. We performed the analysis for linearity, system appropriateness, accuracy and recovery to develop the validation method. This study has been approved by National Ethics Committee of Health Research. Our study shows that the linearity is good with value of r^2> 0.99 and the equation y = 16740.876x - 2953.615. The CV TR and CV peak area for system suitability are 1.46% and 0.29%, respectively. The LoD and LoQ value are 2.532 and 7.672 μg/mL, respectively. The accuracy on the concentration of 1.00, 8.00, 60.00 ug/ml are 108.80 %, 92.57 % and 100.98 %, respectively for intraday accuracy and 103.18 %, 92.44%, and 94.94%, respectively for interday accuracy. Furthermore, the precision on the concentration of 1.00, 8.00, 60.00 ug/ml are 1.17%, 3.57%, 3.32%, respectively for intraday precision and 3.66%, 1.37% and 1.59%, respectively for interday precision. In conclusion, the method which we applied in this study was sensitive and reliable for routine TDM of pyrazinamide.

Keyword: HPLC, Validation, Pyrazinamide, Tuberculosis

BACKGROUND

Tuberculosis is still being one of communicable diseases which can cause of death. In 2013, around 9.0 million people were still experiencing tuberculosis and 1.5 million people died due to it. The rapid diagnosis and effective treatment could decrease the mortality and prevalence of tuberculosis [1]. In developed country, even though the government has adopted the Direct Observed Treatment Shortcourses (DOTS) from WHO, the burden of tuberculosis is still high [1].

Isoniazid, Rifampicin, Ethambutol and Pyrazinamide are four anti-tuberculosis agents which should be used as first line treatment of tuberculosis for minimally 6 month duration of treatment. These combination may cause drug interaction and adverse drug reaction.

Pirazinamide is derivative of nicotinic acid which has similar structure with isoniazid. It well absorbed orally and widely distributed throught the body. Its mechanism as anti-tuberculosis is bactericidal and sterilizers working in acid medium, like machrophages and sites of acute inflammation. Pyrazinamidase is an enzyme which converts pyrazinamide into pyrazinoic acid. Pyrazinoic acid can decrease the intracellular pH which finally inactivated some enzymes [2,3].

Previous study about pharmacokinetic parameters of first-line antituberculosis showed that the treatment failure was depend on the pharmacokinetic parameters. The decrease of AUC of pyrazinamide, isoniazid and rifampicin may cause the poor long-term outcome [4]. Long duration of tuberculosis treatment may cause side effect. The most side effect experience by patients and were responsible on the poor adherence of tuberculosis treatment were hepatitis, dyspepsia, exanthema and athralgia. The major side effects of pyrazinamide are exanthema, hepatotoxicity and gastrointestinal disturbances. Thus, to increase patients' adherence during the tuberculosis treatment, the side effect monitoring should be closely done. Furthermore, patients should be well informed about these side effect and the medication to overcome the side effect.However, due to the complex treatment of tuberculosis, such as the potential of drug interaction and averse drug effect, the Therapeutic Drug Monitoring (TDM) can be done as one method to optimize the therapeutic effect and to minimize the adverse effect [5]. TDM also can be applied to define the slow response of patients and the resistance to the antituberculosis drugs. Thus, it is important to develop the TDM method in the country with high burden of tuberculosis disease. *This study is aimed to validate the TDM of pyrazinamide in human plasma using HPLC-UV.*

METHOD

Subjects

We recruited six adult tuberculosis patients of Public HealthCenter in Yogyakarta. The blood sample was drown from cubiti venous as much as 5 mL 2 hours after the patients taking the antituberculosis drug. We gave the informed consent to the patients and asked them to signature the informed consent form as the participants. This research has been aproved by National Ethic Commitee of Health Research.

Reagents

This research used HPLC Shimadzu LC20 AT with LC 10AT pump, SPD 20A detector and LC solution software. The column C18 shim-pack VP-ODS (250 mm x 4.6 mm, id 5 μm) was used in this instruments with other glass instruments (IWAKI pyrex).

The chemiclas used were from Merck (Germany), such as; methanol pro analysis (pa, 3906609839), diethyl ether (pa), 10% of trichlor acetic acid (TCA), acetonitryl (pa, 1632730213) and aquabidest. The pyrazinamide as standar compund was from BP/USP/COS.DIFC CO., LTD, Korea.

Sample Preparation

The standard curve was defined using 450 μL of plasma added by 50 μL pyrazinamide solution with concentration of 10.0; 80.0 and 600.0 μg/mL. The concentration of standard curve for linearity were 1.0; 2.0; 4.0; 8.0; 16.0; 30.0; and 60.0 μg/mL. The concentration of standard curve for accuracy, intraday precision and interday precision were 1.0; 8.0 and 60.0 μg/mL. The 1000 μL of TCA 10% was added into the solution then followed the next procedures; 20 minutes of vortex with maximal speed, 10 minutes of centrifuge with 13000 rpm and 4°C of temperature. The 3 mL of ether was added into the supernatan for extraction process. This technique was aimed to separate pyrazinamide from other medications. The 20 mL of water solution of extraction process was injected into HPLC [6,7]

System Suitability Test.

The system suitability was defined by injected the *20* μL of one point of standard curve into HPLC system. This procedures were replicated for 6 times and the coefficient of variance was defined from retention time and wide of area ratio [8,9].

Linearity.

The Area Under the Curve (AUC) of pyrazinamide was assesed to get the linear regression equation; y=bx+a with R>0.99. The AUC showed the association between ration of pyrazinamide concentration and area wide or the high of peak of chromatography [8,9].

Limit of Detection (LoD) and Limit of Quantification (LoQ).

The value of LoD and LoQ were define using the linear regression equation and Standard of Deviation (SD). The ration of signal to noise (S/N) with comparison of 3;1 was defined as LoD and the 10:1 comparison of S/N was defined as LoQ [8,9].

Accuracy

The accuracy was examined from recovery. The value of recovery (%) was computed from actual concentration of pyrazinamide divided by theoritical concentration of pyrazinamide and was counted in %. The criterion met accuracy with acceptance criteria required by USFDA was 80-120% [8,9].

Intraday and Interday precision

The precision intraday could be seen from coefficient of variation (CV,%) of each replication of standard curve. The required CV for precision is less than 15% [8,9].

Instrumental parameters

HPLC	: Shimadzu
Stationary phase	: Shim-pack VP-ODS (C18) 250 x 4,6 mm Column
Mobile phase	: Aquabidest : Acetonitrile (97:3)%
Flow rate	: 1 mL/minute
Detector	: UV 262 nm
Retention time	: 15 minute
Injected volume	: 20 μL

RESULTS

Our study defined that the method used in the validation procedures of pyrazinamide can be applied in clinical setting. Table 1 shows the results of system suitability test, linearity, LoD and LoQ. All the parameters met the requirement for validation test.

TABLE 1. System suitability test, Linearity, LoD and LoQ of Pyrazinamide using HPLC

| Parameter	Required Criteria*	Results
System Suitability	$CV \leq 2\%$	CV T_R = 1,46% CV peak area = 0,28%
Linearity	$r \geq 0,99$	r = 0,999 y = 16740.876x - 2953.615
LoD	$S/N \approx 3:1$	2,532 µg/mL
LoQ	$S/N \approx 10:1$	7,672 µg/mL

CV = *Coefficient of Variation*; T_R = *Retention Time*; r = coefficient of correlation; LOD = *Limit of Detection*; LOQ =*Limit of Quantification*
* Adopted from [8]; [9]

The graph 1 presents the regression analysis results in system suitability test. System suitability was performed to determine the effectivity of system when the method was applied. The results of system suitability were met the required criteria which was less than 2%. The linearity was shown in graph 1 with the R= 0.999 and the equation was y=16740,876x+2953.615.
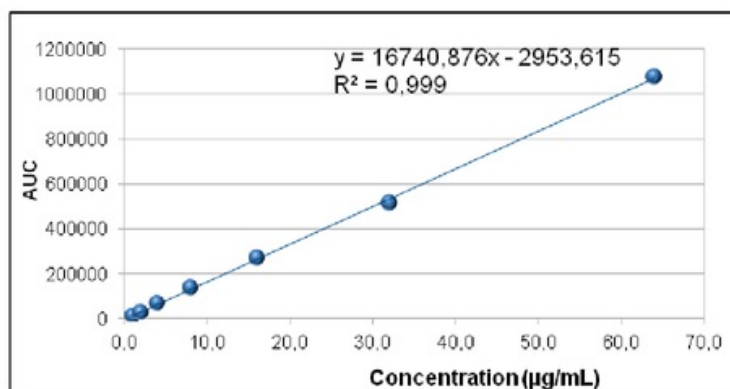
GRAPH 1. Linier regression between pyrazinamide concentration and AUC

The value of LoD and LoQ were established to understand the lowest concentration of sample which had good accuracy and precision. Both of the LoD and LoQ had the value less than 3:1 and 10:1, respectively.

The precision and accuracy results are liste in Table 2. The intraay and interday accuracy and precision were met the required criterion which are CV < 15% and value between 80-120%, respectively. Table 2 shows the precision and accuracy of TDM method of pyrazinamide in HPLC.

TABLE 2. Precision and Accuracy of Pyrazinamide TDM using HPLC

Concentration (µg/mL)	Intraday (n = 6)			Interday (n = 6)			Required Criteria*
	Mean ± SD (µg/mL)	Precision (CV %)	Accuracy (R %)	Mean ± SD (µg/mL)	Precision (CV %)	Accuracy (R %)	
1	1,09± 0,01	1,17%	108,80%	1,03± 0,04	3,66%	103,18%	Precision :
8	7,41± 0,26	3,57%	92,57%	7,39± 0,10	1,37%	92,44%	CV ≤ 15%
60	60,59 ± 2,01	3,32%	100,98%	56,96 ± 0,91	1,59%	94,94%	Accuracy : 80-120%

*: US FDA acceptance criteria for accuracy and precision

DISCUSSION

In general, we present that the method for pyrazinamide TDM using HPLC is valid and reliable. All the assay met the criteria involved in FDA and ICH. It means that this method can be applied in daily practice to understand the pharmacokinetic parameters of pyrazinamide which mostly related to the poor outcome of tuberculosis treatment. Previous study in tuberculosis patients with HIV infection showed that pyrazinamide peak concentration was low due to HIV infection [10]. Of the previous studies about TDM validation of antituberculosis, the HPLC-UV was the most valid and reliable instrument to do the TDM.

One method which was used for pyrazinamide TDM was HPLC-diode array detector (HPLC-DAD) in two studies. The two studies showed that the method used in validation of rifampicin, isoniazide, ethambutol and pyrazinamide TDM in one pharmaceutical formulation met the requirement of linearity, precision and accuracy [11,12]. One of the method used mobile of monobasic sodium phosphate buffer with triethylamine and acetonitrile [12]. This method which is used in these previous studies was recommended due to its simplicity and accuracy [11].

The other method for pyrazinamide validation was in the formulation of ethionamide and pyrazinamide [13]. This study used reversed phase ion pair of HPLC for simultaneous detection of pyrazinamide and ethionamide and the mobile phase was trifluoroacetic acid in distilled water and acetonitrile:methanol. The concentration range of ethionamide and pyrazinamide was 0.1-3.0µg/mL and met the requirements for linearity, Accuracy, precision and specifity [13]. The difference of mobile phase component to our study is due to the lipophilic and hydrophilic character of the antituberculosis. Our study only tests pyrazinamid which is more hydrophilic than ethionamide, thus we only need to us acetonitrile:aquabidest as the mobile phase. This technique is selective for pyrazinamide, because there is no overlapping retention time with other medications.

The previous study about TDM of antituberculosis is using electro spray tandem mass-spectrometry [14]. However, this method is quiet expensive to be applied in clinical setting in Indonesia. Due to the limitation of sophisticated instruments in our country, the method using HPLC/UV is the most possible method which could be applied in the clinical settings. To understand the pharmacokinetic parameter of antituberculosis drugs during the treatment is important due to the high prevalence of tuberculosis and the incidence of multi-drug resistance and HIV infection in Indonesia.

CONCLUSION

We conclude that the method to do TDM of pyrazinamide using HPLC is valid and can be used in clinical setting.

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