USFDA-GUIDELINE BASED VALIDATION OF TESTING METHOD FOR RIFAMPICIN IN INDONESIAN SERUM SPECIMEN

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ABSTRACT

Regarding a new regulation from Indonesia FDA (Badan POM-RI), all new non patent drugs should show bioequivalence with the originator drug prior to registration. Bioequivalence testing (BE-testing) has to be performed to the people that represented of population to which the drug to be administrated. BE testing need a valid bio-analytical method for certain drug target and group of population. This research report specific validation of bio-analysis of Rifampicin in Indonesian serum specimen in order to be used for BE testing. The extraction was performed using acetonitrile while the chromatographic separation was accomplished on a RP 18 column (250 × 4.6 mm i.d., 5 µm), with a mobile phase composed of KH_2PO_4 10 mM-Acetonitrile (40:60, v/v) and UV detection was set at 333 nm. The method shown specificity compared to blank serum specimen with retention time of rifampicin at 2.1 min. Lower limit of quantification (LLOQ) was 0.06 µg/mL with dynamic range up to 20 µg/mL (R>0.990). Precision of the method was very good with coefficient of variance (CV) 0.58; 7.40 and 5.56% for concentration at 0.06, 5, 15 µg/mL, respectively. Accuracies of the method were 3.22; 1.94; 1.90% for concentration 0.06, 5 and 15 µg/mL respectively. The average recoveries were 97.82, 95.50 and 97.31% for concentration of rifampicin 1, 5 and 5 µg/mL, respectively. The method was also shown reliable result on stability test on freezing-thawing, short-term and long-term stability as well as post preparation stability. Validation result shown that the method was ready to be used for Rifampicin BE testing with Indonesian subject.

Keywords: *Rifampicin, Validation, USFDA-Guideline*

INTRODUCTION

Rifampicin (RIF) or (7*S*,9*E*,11*S*,12*R*,13*S*,14*R*,15*R*, 16*R*,17*S*, 18*S*,19*E*,21*Z*)-2,15,17,27,29 pentahydroxy-11-methoxy-3,7,12,14,16,18,22-heptamethyl-26-{(E)-[(4-methylpiperazin-1-yl)imino]methyl}-6,23-dioxo-8,30-dioxa-24 azat-etracyclo[23.3.1.1^{4,7}.0^{5,28}]triaconta-1(28),2,4,9,19,21, 25(29),26-octaen-13-yl acetate (Fig. 1) is a complex semisynthetic macrocyclic antibiotic derived from *Streptomyces mediterranei*. Rifampicin is a member of the rifamycin class of antibiotics used for the treatment of tuberculosis and other infectious diseases [1]. Tuberculosis remains a major health public problem and is the single most deadly infectious disease. It kills approximately two million people each year and therefore new formulations of rifampicin are being studied with the aim of improving the therapeutic index [2].

In order to characterise the pharmacokinetics of rifampicin after administration of these new formulations during preclinical studies, the drug levels in plasma as well as tissue samples need to be determined. It was therefore necessary to develop a sensitive, accurate and reproducible analytical method to analyze these samples. Several methods have been reported for RIF bianalysis [3-5] which mainly HPLC-UV based analysis.

At the same time the patent of this RIF has been expired lead to intention of many industrial companies to produce it with various formulations. One requirement to register of this type of drug is that the drug should have bioequivalent to the originator. The test proposed to check bioequivalence of drug is BE

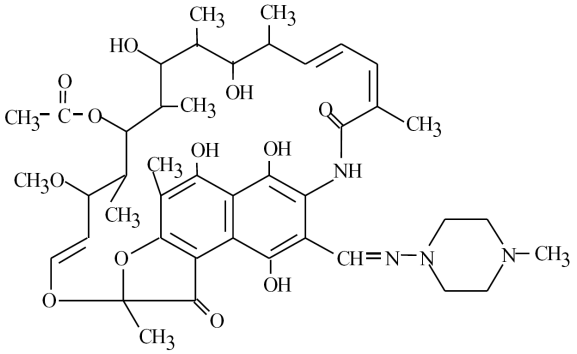

Fig 1. Chemical structure of rifampicin

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testing (Bioequivalence Testing). By the test, pharmacokinetic profiles of the drugs are compared. Due to individual or race specific drug pharmacokinetic profile, many national FDA required BE tested has to be performed to population from the country.

Indonesia FDA (Badan POM) has implemented BE testing regulation [6]. Therefore BE testing with Indonesia subject for new imitated drug such as Rifampicin is an obligation for pharmaceutical company that want to sell their product in Indonesia

BE employ tools of bioanalysis since blood serum is the main object of the testing. Several HPLC methods have been reported for the analysis of RIF in biological fluids. Most of the methods using reversed phase (RP) system with UV detection [3-5,7-10]. These methods have been reported used in bioequivalence test [11-14] but none of them using Indonesian sample. If the method wants to be applied for Indonesian BE-testing, the methods need to be validated with Indonesia subject as the sample of developing method is different from the one for the analysis. Beside that small modification of the method need to be validated, it is also one of the requirements set by Badan POM need to be validated [15].

The purpose of this study is to validate the method of analysis of the CIP using reversed phase HPLC and UV detection using Indonesian serum as sample. Validation is performed according to USFDA guideline for bioanalysis. The method to be validated was developed by Calleja et al. [7] where the details are described at experimental section. The result of this work can be used directly as protocol for BE testing of CIP for Indonesian.

EXPERIMENTAL SECTION

Materials

Blood serum was provided by Indonesian Red Cross Yogyakarta District. All chemicals were analytical grade. Rifampicin (RIF) standard was obtained from Calbiochem. The stock solution of RIF was prepared at concentration 200 ppm (20 mg RIF in 100 mL acetonitrile. All solvents (water and acetonitrile) were HPLC grade. Acetonitrile, dichloromethane, pentane and KH_2PO_4 were from Merck.

Instrumentation

Chromatography was performed with a high-performance liquid chromatograph (Knauer, Smartline series) equipped UV-Vis PhotoDiode Array detector set at 333 nm. The column was Lichrosper RP 18 column (250 × 4.6 mm i.d., 5 µm (Merck) at room temperature. The mobile phase composed of KH_2PO_4 10 mM-Acetonitrile (40:60, v/v) pumped with flow rate of 1.0 mL/min.

Procedure

Preparation of RIF from serum

One hundred µL of serum was added to 50 µL acetronitrile. The mixture was homogenized by vortex for 30 sec then added by 3 mL dichloromethane-*n*-pentane (1:1 v/v) then homogenized for 60 sec, centrifuge (bench top) at 3000 rpm for 5 min. The organic phase was moved to a new tube while extraction was repeated for aqueous phase. The results of extraction were combined and evaporated by nitrogen gas. The residue was then diluted by HPLC mobile phase.

Validation methods

Validation was performed based on USFDA Guideline for bioanalysis validation [16] using procedures as follow:

Selectivity test. Serum blank and RIF-fortified serum was analysis according to the preparation and HPLC procedure. The effect of the serum matrix to the RIF was investigated.

Calibration Curve (LLOQ, Lower Limit of Quantification, and Linear Dynamic range). Various concentration of RIF fortification was applied to the serum then subjected to the analysis using method to be validated. The detector respond of the HPLC at RIF retention time was investigated. LLOQ was RIF concentration when signal/noise of the detector equal to 5. Linear dynamic range was investigated start from LLOQ to 10 µg/mL concentration. Various concentration of RIF-fortified serum was analysed using the method. The linearity of the detector respond to the concentration was determined ($r \geq 0.999$).

Accuracy, Precession and Recovery. Precession was investigated from the coefficient of variance (CV) of 10 independent analyses using the methods. Three concentration was investigated which were LLOQ, 5 and 10 µg/mL, represented low, medium and high concentration respectively. Accuracy was investigated by measuring the fortified serum with low, medium and high concentration. The result of the analysis was compared to real value of the concentration. Recovery was investigated by measuring the recovery of the method at low, medium and high concentration with 5 independent analyses. The recovery was measured by comparing of the detection respond of RIF fortified serum with RIF standard solution at same concentration.

Stability Test. Various concentrations of the serum RIF fortified were subjected for freezing-thawing

stability, short-term stability (up to 24 h), long-term stability (up to 30 days) and post preparation stability.

RESULT AND DISCUSSION

Specificity of the method

Typical chromatograms are shown in Fig. 2. The retention time for RIF is 2.1 min and no interference from endogenous components or RIF metabolites is observed in serum. The baseline was relatively free from drift.

The result shows the methods has good specificity for RIF in serum. The retention time of RIF which is 2.1 min is very fast and seems promising in term of time for analysis. Previous reported methods resulted in retention time of RIF at 3.9 min [7] while different HPLC methods reported RIF retention time at 9-10 min from method developed by Kumar [8]. This method differs from validated method on the use of weaker mobile phase (KH_2PO_4 10 mM-acetonitrile (55:45, v/v)) although higher flow rate was applied (1.2 mL/min). All reports used sperical column except one report [9] used monolith column but unfortunaltely no information of the RIF retention time for this report. In term of time of analysis the validated method looks promising although employ sperical column rather than expensive monolith column which well known to has fast elution with high resolution.

Calibration Curve (LLOQ and Linear Dynamic range)

Although there is no peak at RIF retention time from blank sample, the detector respond was set as noise level. The signal to noise 5/1 was achieved with RIF concentration in serum 0.06 µg/mL which is concluded as LLOQ. This value is more than enough to be used for BE testing, based on the pharmacokinetic data of various formulation of RIF which has lowest concentration at approximately 1 µg/mL [11].

The linearity was verified from 0.06 to 20 µg/mL since the data of pharmacokinetic show that even administration of 600 mg, highest dose RIF available, never result peak concentration CIP in serum more than 12 ug/mL. The selected concentrations were 0; 0.06;0.1; 0.2; 0.4; 0.8; 1; 1.5; 2; 4; 5; 10; 15; and 20 µg/mL. The correlation coefficient between the peak-area of CIP and to concentration from 0-20 µg/mL was 0.9946 while higher correlation coefficient was obtained from 0-1.5 µg/mL (0.9976). The relation between peak area and concentration was demonstrated to be reproducible with CV of all concentration < 10% (n=5) meet the USFDA requirement (CV < 20% for LLOQ concentration and < 15% for other concentration) [16]. The result of linier dynamic range is in line with Calleja et al. who found

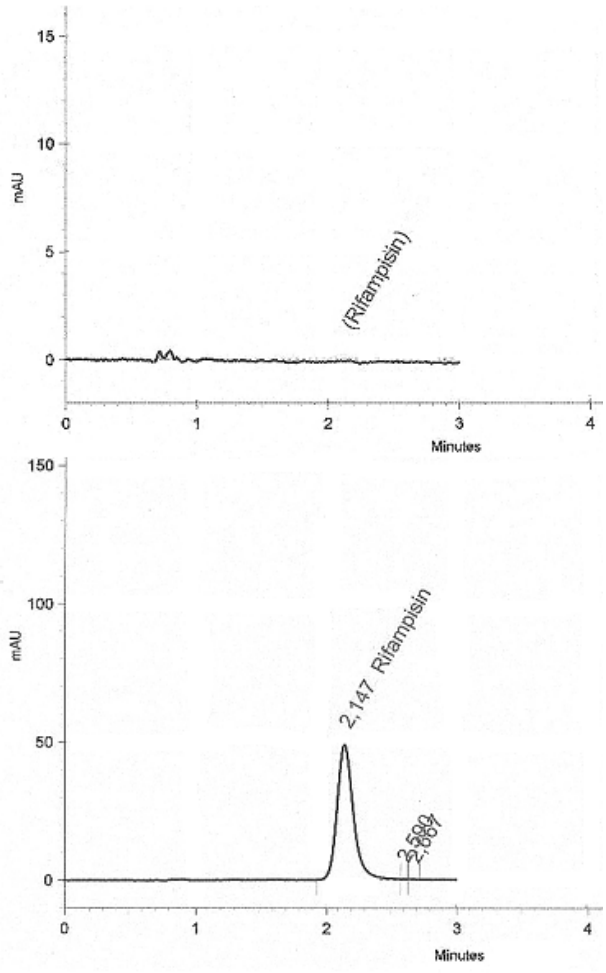

Fig 2. Chromatogram of blank serum (upper) and serum fortified with 2.5 ppm RIF(lower)

the linier dynamic range of this method from 0.1-50 µg/mL [7].

Accuracy, Precession and Recovery

The accuracy and precision were determined with five experiments for each concentration. The accuracy and precision are given in Table 1 and Table 2 respectively. Accuracy is defined as average difference of the result to the real value. According to USFDA acceptance criteria, the accuracy have to be < 20% at LLOQ concentration and < for other concentration. Result of accuracy test on Table 1 meets the USFDA acceptance criteria [16].

Precision is shown by coefficient of variance (CV). According to USFDA acceptance criteria, the accuracy has to be < 20% at LLOQ concentration and < 15% for other concentration. Result of precision test on table 2 meet the USFDA acceptance criteria [16].

Table 1. Accuracy of RIF method

Fortified concentration (µg/mL)	Concentration found (µg/mL)	Accuracy (%)
0.060	0.064	3.22
5.000	5.096	1.94
15.000	14.714	1.90

Table 2. Precision of RIF method

Fortified concentration (µg/mL)	Concentration found (µg/mL) ± SD	CV (%)
0.062	0.064 ± 0.0004	0.58
5.000	5.151 ± 0.381	7.39
15.000	14.836 ± 0.825	5.56

The recovery was also tested with five determinations on three concentrations, 1, 5 and 15 µg/mL. The recoveries for these concentrations are 97.82; 95.50 and 97.31% respectively. There is no value of recovery < 70% or > 120% at all experiments. These results also meet with USFDA criteria [16]. The recovery data obtained by this experiment was higher than the result obtained by Calleja el al. which was 83% using serum from rat as a matrix [7].

Stability

Stability test was performed to the sample with RIF concentration 2 and 15 µg/mL except for stock solution stability which was only performed to the 20 µg/mL concentration and post preparation stability which was performed to the 15 µg/mL concentration. RIF in serum looks stabile to freezing-thawing processes. After three s processes within 48 h, the average reduction of the concentration of both RIF-serum were less than 3% which almost the same with the precision of the method. The same result was obtained from short-term, stock solution stability and post preparation stability. The long-term stability was tested up to one month and the sample was still stable (concentration decrease is less than 3%).

CONCLUSION

The HPLC testing method for RIF in serum validated to the Indonesian serum present in the paper meet all criteria of USFDA bioanalysis guideline. The validated method is ready to be used as part of BE testing of RIF to Indonesian sample.

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