Optimization and validation of high-performance liquid chromatography method for analyzing 25-desacetyl rifampicin in human urine

Lily*, L Laila2 and B E Prasetyo2

1Master of Biomedical Sciences, Faculty of Medicine, Universitas Sumatera Utara, Medan 20155, Indonesia
2Department of Pharmaceutical Technology, Faculty of Pharmacy, Universitas Sumatera Utara, Medan 20155, Indonesia
*Corresponding author: lucky_chocho@yahoo.com

Abstract. A selective, reproducibility, effective, sensitive, simple and fast High-Performance Liquid Chromatography (HPLC) was developed, optimized and validated to analyze 25-Desacetyl Rifampicin (25-DR) in human urine which is from tuberculosis patient. The separation was performed by HPLC Agilent Technologies with column Agilent Eclipse XDB-C18 and amobile phase of 65:35 v/v methanol: 0.01 M sodium phosphate buffer pH 5.2, at 254 nm and flow rate of 0.8ml/min. The mean retention time was 3.016minutes. The method was linear from 2–10μg/ml 25-DR with a correlation coefficient of 0.9978. Standard deviation, relative standard deviation and coefficient variation of 2, 6, 10μg/ml 25-DR were 0-0.0829, 0-3.1752, 0-0.0317%, respectively. The recovery of 5, 7, 9μg/ml25-DR was 80.8661, 91.3480 and 111.1457%, respectively. Limits of detection (LoD) and quantification (LoQ) were 0.51 and 1.7μg/ml, respectively. The method has fulfilled the validity guidelines of the International Conference on Harmonization (ICH) bioanalytical method which includes parameters of specificity, linearity, precision, accuracy, LoD, and LoQ. The developed method is suitable for pharmacokinetic analysis of various concentrations of 25-DR in human urine.

1. Introduction
Rifampicin is an antituberculosis drug for pulmonary and extra-pulmonary tuberculosis. Experimental measurements of the time course of biliary excretion and elimination of rifampicin in urine would be useful in better determining the clearance terms.[1] Rifampicin is metabolized in the liver to an active metabolite, deacetyl rifampicin and undergoes enterohepatic recycling.[2]

Figure 1. Chemical structure of 25-DR [3].
Desacetyl rifampicin is synonymous with 25-O-Deacetyl-3 - [(E) - [(4-methyl-1-piperazinyl) imino] methyl] rifamycin; 25-Deacetylrifampicin (25-DR); 25-Desacetylrifampin; 25-O-Deacetylrifampicin; 25-O-Desacetylrifampin; Deacetylrifampin; Desacetylrifampicin; Desacetylrifampin [4], with molecular formula C$_{41}$H$_{56}$N$_{4}$O$_{11}$ and molecular weight 780.9.[3]

Methods of measuring drugs in biologic media are increasingly important problems related to bioavailability and bioequivalence studies, new drug development, drug abuse, clinical pharmacokinetics and drug research are highly dependent on accurately measured drugs in biological fluids.[5] Very few HPLC methods have been developed for the quantification of rifampicin and its metabolite, 25-DR in the plasma and urine.[6]

Several HPLC procedures have been reported for quantitative estimation of rifampicin and its metabolite, in serum/plasma or urine, but many of these methods suffer from limitations such as lengthy and tedious procedures, high plasma/serum sample volumes required, large quantities of solvents involved, sometimes very complex mobile phase composition.[7] The present study was carried out by modifying HPLC method of Panchagnula et al. to achieve an optimal and valid condition based on the validity of bioanalytical methods [8] so as to be directly applicable to analysis 25-DR in the human urine.

2. Materials and Methods

2.1. Materials

Standard 25-DR (purity of 98%) Santa Cruz® was purchased from PT. Biosains Medika Indonesia. Methanol(MeOH) Merck® of HPLC grade was purchased from PT. Multi Medika Laboratory. Syringe Filters Whatman® PVDF Ø 13 mm, Pore Size (PS) 0.5μm purchased from CV. Smart Chemical Indonesia. Vial HPLC 2ml was purchased from PT. BercaNiagaMedika. The chemicals, Sodium dihydrogen phosphate Merck®, Ortho Phosphoric Acid 85%, and Anhydrate Disodium phosphate were of analytical grade.

2.2. Instrument HPLC

Instrument HPLC Agilent Technologies 1220 Infinity LC VL has system configuration G4290C, serial number DECB00113, made in Germany. Components include anisocratic pump, dual-channel gradient pump (with degasser), manual injector, autosampler, column oven, and detector. The configuration comes with at least one pump, one injection system, one detector and includes Agilent Instrument Utilities Software.[9]

2.3. Method of work

The research was conducted at the Research Laboratory of the Faculty of Pharmacy, University of Sumatera Utara.

2.3.1. Preparation of Standard Stock Solutions 25-DR. The stock of 25-DR 1mg/ml was dissolved with 1ml MeOH. The standard concentration solution of 25-DR 1000μg/ml was obtained.

2.3.2. Preparation of Calibration Working Solution 25-DR. The standard stock solution of 25-DR 1000μg/ml was diluted with MeOH to obtain the final concentration calibration working solution 25-DR 2, 4, 6, 8, 10μg/ml, that desired in the calibration range and was found sufficient to provide detectable signals 25-DR at the lowest concentration level. Each concentration prepared in sixuplicate. Vortex (Boeco®) for 15 seconds. Centrifuge (Velocity 18R®) at 10,000rpm for 15 minutes. The solution is taken with a3ml syringe and filtered with syringe filter, directly inserted into the HPLC vial. Sonication (Branson®) for 15 minutes and ready to be injected into HPLC.
2.3.3. Preparation of Quality Control (QC) Solution 25-DR Intraday and Interday. The 2 sets simplicity of each final concentration 2, 6, 10 μg/ml QC solution 25-DR intraday and interday was prepared by mixing stock solution 25-DR 1000 μg/ml with MeOH. 1 set as an interday stored in a freezer -20°C for further processing the next day. Intraday was avortex for 15 seconds. Centrifuged at 10,000 rpm for 15 minutes. The solution is taken and filtered directly into the HPLC vial. Sonication for 15 minutes then ready to be injected into HPLC.

2.3.4. Preparation of Sample Recovery 25-DR. The final concentration 5, 7, 9 μg/ml of 25-DR recovery sample was prepared by applying a stock solution of 25-DR 1000 μg/ml into 500 µl blank human urine.

2.4. The validity of the quantitative KCKT method
Parameters validity of the bioanalytical method is the specificity, linearity, calibration range, accuracy, precision, LoD, and LoQ.[8]

2.4.1. Selectivity. Selectivity should be demonstrated proven by using at least 6 human blank urine samples and evaluated for interference. The absence of interfering components is accepted.[10]

2.4.2. Linearity. Linearity is determined by injecting a standard solution at a concentration range [11], a minimum of 5 concentrations should be used [12], in this study 5ml, 2-10 μg/ml, simplicity. The parameter of the calibration curve is slope and intercept must be linear.[10] The calibration curve obtained by plotting the peak height with the concentration, and the linear coefficient correlation ($r^2$) was determined by calculation of regression calibration graph.[11]

2.4.3. Precision. Precision should be shown for low, medium and high QC samples.[10] The precision of this method is demonstrated as intraday and interday. Precision must be measured using a minimum three concentrations within the range of concentrations sample [13] in sixplicates.[11] Precision is expressed as standard deviation (SD) [8], relative standard deviation (RSD) [12], the coefficient of variation (CV).[10] The precision specified at each concentration level should not exceed 20% of the CV.[13]

2.4.4. Accuracy. Accuracy should be reported as a percent recovery by an assay from the number of known analytic in the sample.[8] Minimum of three concentrations in the recommended concentration range.[13] The recovery sample was spike 25-DR for a final concentration of 5, 7, 9 μg/ml and injected simplicity. The minimum specific range for assay of a substance or finished product (drug) is normally from 80–120% of the test concentration.[8]

2.4.5. LoD and LoQ. LoD is the smallest number of analyses in a detectable sample.[8] LoQ is the lowest concentrations of the analyses in a sample that can be determined quantitatively with appropriate precision and accuracy.[12] The calculation equation of LoD = (3.3×δ)/S, and LoQ = (10×δ)/S, where δ = standard deviation of the response, and S = the slope of the calibration curve of the linear regression equation.[8]

2.4.6. Data Analysis. Acquisition and processing of HPLC data are performed by Agilent Technology Revision Software: Rev. B.04.03 (16).

3. Result and Discussion

3.1. Optimization of HPLC Method
HPLC method to determine 25-DR in human urine is modified method of Panchagnula R. et al., 1999. Spiking of 25-DR in blank urine eluted well with complete peak resolution in the course of
chromatogram for 10 min. The optimum conditions of the mobile phase are at 65:35 v/v of MeOH: 0.01 M sodium phosphate buffer (pH adjusted to 5.2 with 2% of orthophosphoric acid). The mobile phase was filtered with a membrane filter of cellulose nitrate Whatman® Ø 47 mm, PS0.45 μm under vacuum Whatman® and gas is removed by sonicated in an ultrasonic bath Kudos® for 15 minutes before use. The mobile phase is pumped at a flow rate of 0.8 ml/min during analysis, at room temperature. The detector is installed at a wavelength of 254 nm. Chromatographic separation and quantification created on column Agilent Eclipse XDB-C_{18} (id. 4.6x150 mm, 5 µm), PN 993967-902, SN USKH058674, LN B 09116, and Made in the USA. The volume of injected sample is 5 μl. Based on the chromatographic conditions outlined above, 25-DR has a mean retention time (RT) of 3.016 minutes.

3.2. Method Validity
3.2.1. Selectivity. This method has a high selectivity for 25-DR because there is no interference peak of endogenous compounds shows at RT from any of the sixplicate of evaluated samples urine blank (Figure 2).
3.2.2. **Linearity.** Linear regression of the calibration plot shows a good linear relationship between peak height and concentration 25-DR in the range 2-10μg/ml. The linear regression equation observed as: \( Y = 0.7625 X - 0.375 \) and \( r^2 = 0.9978 \), indicating the instrument respond well to 25-DR.

\[
Y = 0.7625 X - 0.375 \\
r^2 = 0.9978
\]

**Figure 3.** A calibration curve with concentration range of 25-DR 2, 4, 6, 8, 10μg/ml.

3.2.3. **Precision.** Precision QC 25-DR intraday and interday is <5%, indicating the method can be repeated and reused. Intraday and interday precision are determined (Table 1).

| Concentration 25-DR (μg/ml) | SD Intraday | RSD% Intraday | SD Interday | RSD% Interday |
|-----------------------------|-------------|---------------|-------------|---------------|
| 2                           | 0.0718      | 1.2378        | 0.0718      | 0.0124        |
| 6                           | 0.0718      | 1.2378        | 0.0718      | 0.0144        |
| 10                          | 0.0677      | 0.6911        | 0.0677      | 0.0069        |

**Table 1.** Intraday and interday precision.

3.3.4. **Accuracy.** Percentage recovery accuracy of spiking 25-DR 5, 7 and 9μg/ml was 80.8661%, 91.3480%, and 111.1457%, respectively, indicating the effectiveness of extraction techniques.
3.3.5. LoD and LoQ. LoD and LoQ are determined separately based on the SD of the response and slope of a calibration curve for determination of 5 concentrations sixplicate. LoD and LoQ for 25-DR were 0.51 μg/ml and 1.7 μg/ml, respectively, indicating the sensitivity of the method analysis for determination 25-DR in human urine samples.

4. Conclusions
HPLC method of quantification of 25-DR in human urine successfully modified from the Panchagnula R. etal., 1999. The analysis follows the guidelines validity bioanalytical method of the ICH. The performance of the method is acceptable, and the results of the analysis are reliable. The modified method can be very useful for routine analysis of the sample because it has a short duration with a total time of run of 10 minutes per sample, which is important for a large number of samples, requires no long sample processing, and requires less urine volume (500 μl). This HPLC method is selective, reproducibility, effective, sensitive, simple and fast, so it is suitable for pharmacokinetic research, bioavailability or 25-DR bioequivalence studies in human urine.

References
[1] Lyons M A, Reisfeld B, Yang R S H and Lenaerts A J 2013 A physiologically based pharmacokinetic model of rifampin in mice Antimicrob. Agents Chemother. 57 1763–71
[2] Duraimuthumani G and Karthick S P 2014 A high performance liquid chromatographic assay of rifampin in plasma of non HIV-infected tuberculosis patients Int. J. Curr. Res. Chem. Pharma. Sci. 1 90–8
[3] Santa Cruz Biotechnology, Inc. 25-Desacetyl Rifampicin (CAS 16783-99-6) (Dallas)
[4] Toronto Research Chemicals 25-Desacetyl Rifampicin (Canada)
[5] Sridharan D, Thenmozhi A and Sundaranandavalli S 2010 Bioanalytical method development and validation of atenolol in human plasma by LCMS Asian J. Pharm. Clin. Res. 3 92–4
[6] Kumar A K H, Chandra I, Geetha R, Chelvi K S, Lalitha V and Prema G 2004 A validated high-performance liquid chromatography method for the determination of rifampicin and desacetylrifampicin in plasma and urine Indian J. Pharmacol. 36 231–3
[7] Panchagnula R, Sood A, Sharda N, Kaur K and Kaul CL 1999 Determination of rifampicin and its main metabolite in plasma and urine in presence of pyrazinamide and isoniazid by HPLC method J. Pharm. Biomed. Anal. 18 1013–20
[8] International Conference on Harmonization Harmonized Tripartite Guideline 2005 Validation of analytical procedures: text and methodology Q2 (R1) (European Union, Japan and USA) pp 1–13
[9] Agilent Technologies, Inc. 2015 User manual agilent 1220 Infinity LC (Germany)
[10] European Medicines Agency 2009 Guideline on validation of bioanalytical methods (UK) pp 1-17
[11] Memon A H and Memon N 2017 Development and validation of a simple and sensitive RP-HPLC method for determination of rifampicin in bulk and tablets Sindh Univ. Res. Jour. (Sci Ser.) 49 213–8
[12] Ethiopian Food, Medicine & Healthcare Administration & Control Authority 2014 Good manufacturing practice guideline for pharmaceutical products (Ethiopia) pp 60–1
[13] United States Food and Drug Administration 2013 Guidance for industry bioanalytical method validation revision 1 (US) pp 4–10