THE SIMULTANEOUS QUANTIFICATION OF RIFAMPICIN AND ISONIAZID IN PATIENTS WITH TUBERCULOSIS APPLIED TO VOLUMETRIC ABSORPTIVE MICROSAMPLING DEVICES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Objective: Rifampicin and isoniazid are the main tuberculosis treatment regimens requiring blood level measurement to optimize the treatment process. This study aims to analyze rifampicin and isoniazid quantitatively in volumetric absorptive microsampling (VAMS) prepared from a small volume of TB patients using HPLC.

Methods: Analytes on the VAMS tip were extracted using 1000 ml of acetonitrile containing 10 µg/ml of cistolazol as an internal standard. Analytical separation was performed on the C-18 column at 40 °C with a mobile phase mixture of 50 mmol ammonium acetate buffer pH 5.0-acetonitrile-methanol (40:30:30), flow rate 0.5 ml/min. The analysis was carried out with the calibration curve over a range of 1.0–30 µg/ml for rifampicin and 0.4–20 µg/ml for isoniazid.

Results: Analyte analysis in 21 patients showed that the measured value of rifampicin was 3.39–16.77 µg/ml, and isoniazid was 2.63–10.43 µg/ml at 2 h post-dose. 53.8% of patients had low blood concentrations in at least one of the drugs, 28.5% of the patients were in the therapeutic range, and 23.81% had a high blood concentration of isoniazid alone.

Conclusion: The concentration of rifampicin and isoniazid in 21 tuberculosis patients varied. Dose adjustment is needed because most patients had low blood concentrations of one of the drugs, and a limited number had a high blood isoniazid concentration alone. Only some patients simultaneously had plasma concentrations within the target range of the drugs. This method was valid and reliably utilized for therapeutic drug monitoring of antituberculosis.

Keywords: Isoniazid, Rifampicin, TDM, Tuberculosis, VAMS

INTRODUCTION

Tuberculosis (TB) is a severe disease developed from Mycobacterium tuberculosis that remains one of the most prevalent causes of mortality worldwide. Approximately 1.6 million people were infected, with 1.6 million deaths in 2021. Indonesia has become the third-largest contributor to global TB cases among the top 30 TB burden countries, accounting for 9.2% of all TB cases [1]. The global TB burden remains strongly linked to hazardous living conditions, HIV co-infection, the emergence of drug-resistant TB, and poor treatment outcomes [2]. The poor treatment outcomes are caused by many factors, including low blood drug concentrations [3]. The drugs commonly prescribed in the treatment of TB are rifampicin and isoniazid. The bactericidal action and post-antibiotic effect of rifampicin occurred at blood concentrations of 8–24 µg/ml [4]. Isoniazid blood concentrations are suggested to be 3-6 µg/ml to provide the therapeutic effect [5]. Therefore, determining blood drug concentrations is essential to therapeutic drug monitoring to improve outcomes during therapy.

Generally, the determination of drug concentrations involves blood samples acquired by venipuncture. Despite being regarded as the gold standard, this conventional technique is invasive and has several limitations, such as requiring a particular storage condition, managed shipments, and huge sample volumes. Micro-sampling techniques such as DBS and VAMS have been developed to overcome the disadvantages of conventional sampling techniques. This method acknowledges a smaller volume of blood samples, safe handling, inexpensive shipping, room temperature storage, and minimal invasiveness, increasing patient comfort. However, the dried blood spot (DBS) technique has a drawback in that the influence of different hematocrit levels (HCT) will affect spot size, sample homogeneity, drying time, and analyte recovery [6].

Another micro-sampling technique, volumetric absorptive micro-sampling (VAMS), can minimize the effect of hematocrit on DBS. The porous hydrophilic tip of VAMS has been designed to absorb a fixed sample volume [6]. Previous studies have successfully reported the utilization of VAMS in various therapeutic drug monitoring activities, such as imatinib mesylate [7], clozapine [8], and phenylalanine [9]. However, applying the VAMS method to analyze rifampicin and isoniazid from tuberculosis patients has never been reported. The analytical method in this study operated on high-performance liquid chromatography (HPLC) with a PDA detector because the method is more economical than LC-MS/MS, which has been used in previous studies [10-12]. This study aims to quantitatively analyze rifampicin and isoniazid in TB patients applied to volumetric absorptive microsampling through a valid and reliable HPLC method.

MATERIALS AND METHODS

Materials

Rifampicin (US Pharmacopeia, USA), isoniazid (Sigma-Aldrich, USA), cistolazol as an internal standard (Sigma-Aldrich, USA), volumetric absorptive microsampling (NeoteryxTM, USA), acetonitrile and methanol (HPLC grade), ammonium acetate (Merck, Germany), aquabidest (Ikapharmindo, Indonesia), and human whole blood (Indonesian Red Cross).

Instrument

The high-performance liquid chromatography system was conducted using the LC-20AD Shimadzu series equipped with a pump, degasser, an autosampler, photodiode array detector (Waters, 2996), C-18 column (Waters, SunfireTM 5 µm; 250 mm x 4.6 mm), evaporator (Turbo Vaap LV), pH meter (EUTECH), ultrasonicator (Elmasonic), microcentrifugator (centrifuge 16M), vortex (Maxi mix II), and micropipette Eppendorf (Socorex).
Chromatographic analysis was performed using a C-18 column (5 µm; 250 mm x 4.6 mm) with a temperature of 40 °C. The mobile phase contained 0.05 M of ammonium acetate buffer, pH 5.0, acetonitrile-methanol (40:30:30) under isocratic elution conditions with the flow within 0.5 ml/min. A volume of 20 ml was used as the injection volume, and 261 nm was the detection wavelength.

Sample preparation
Sample preparation of VAMS was prepared by dipping the Mitra® tip in the spiked whole blood with the appropriate concentration and drying it for an hour. Mitra® tips were removed and put in a microtube. The extraction process was performed using a protein precipitation technique by adding 1 ml of acetonitrile and 50 µl of internal standard 10 µg/ml into the sample. It was sonicated at 30 °C for 15 min, vortexed for 2 min, and centrifuged for 5 min at 10,000 rpm. The supernatant was pipetted as much as 850 µl and evaporated under nitrogen at 40 °C for 20 min. The dried extract was reconstituted in 200 µl of methanol. The mixture was homogenized with a 10 s vortex and a 5 min sonication. A total of 20 µl aliquots were injected into the HPLC system.

Method validation in volumetric absorptive micro-sampling
Method validation in this study referred to the US Food and Drug Administration (FDA) guidance on bioanalytical method validation. The full validation of the analytical method in volumetric absorptive micro-sampling was performed in terms of parameters, selectivity, carry-over, the lower limit of quantification (LLOQ), linear calibration curve, accuracy, precision, dilution integrity, and stability test. In addition, the recovery test was also completed. The validation was carried out with the coefficient of variation (CV) and the relative difference (% diff) requirement of <20% for LLOQ and 15% for the other validated concentration [13].

Selectivity and carry-over
The selectivity test was performed by determining LLOQ and blank samples from six different sources. Carry-over was evaluated by analyzing the blank after the upper limit of quantification (ULOQ) concentration was analyzed. It was carried out in five replicates. The blank interference response at the retention time of the analyte should be <20% of the LLOQ response and should not exceed 5% of the internal standard response to qualify both for the selectivity and carry-over tests [13].

Calibration curve
The calibration curve measured three replicates of blank, zero, and six concentration levels ranging from 1-30.0 µg/ml and 0.4-29 µg/ml for rifampicin and isoniazid, respectively. The linear equation used to recalculate the concentration of calibration standards was constructed by plotting the peak area ratio (PAR) of the IS versus the concentration of the analytes. The calibration curve should be 15% of theoretical concentrations in each validation run, except at LLOQ, which should be 20% [13].

Precision and accuracy
The precision and accuracy test assessed four level concentrations (LLOQ, OCL, QCM, and QCH) on the same day (within-run) and different days (between-run) on five replicates of each. The requirement for within- and between-run precision (CV) was <15%, and the accuracy was ±15% of nominal concentrations, except for LLOQ, which was ±20% [13].

Recovery
The recovery was carried out by comparing the response of the extracted sample and blank spiked with the analyte post-extraction at three level concentrations (QCL, QCM, and QCH). It was evaluated three times. The reproducible was qualified with a CV value not exceeding 15% [13].

Dilution integrity
Dilution integrity was tested for five replicates of the higher ULOQ concentration (2x QCH), serially diluted to ½ and 1/2% of the concentration. The acceptance criteria for dilution integrity were accuracy and precision (CV) within ±15% [13].

Stability
The stability test analyzed QCL and QCH in VAMS samples and the standard solution of rifampicin, isoniazid, and cilostazol, each with three replicates. The VAMS samples were stored at room temperature, and the standard solutions were at 4 °C. The stability of VAMS samples was analyzed at 0, 6, and 24 h for short-term stability and on days 7, 14, and 30 for long-term stability. The standard solutions were analyzed on days 7, 14, and 30. The accuracy at each level should be ±15% [13].

Ethical approval
This study has been accepted for ethical approval by the ethics committee at dr. Chashbullah Abdulmajid General Hospital Bekasi No. 012/KEPK/RScM/V/2022.

Application of the method
All patients were determined based on the propriety of inclusion criteria, including those diagnosed with pulmonary tuberculosis at dr. Chashbullah Abdulmajid Hospital. The patients received the rifampicin and isoniazid regimen in a fixed dose combination and were 18–50 y old during blood collection. Blood samples from the patients were collected 2 and 6 h after administration from the fingertips and absorbed in 30 µl of Mitra® VAMS. The tips were stored in the Mitra® clamshell at room temperature and put with a desiccant until analysis was conducted. The sample tips that were going to be analyzed were taken off the handle and put into 1000 µl of acetonitrile with 10 µg/ml IS added. The extraction procedure followed the sample preparation described previously. The concentrations of rifampicin and isoniazid were calculated using daily calibration curves, and the quality control samples were added at each analytical run to provide data validity.

RESULTS AND DISCUSSION
Method validation in VAMS
Selectivity and carry-over
The analytical method was validated to ensure that it was selective, sensitive, accurate, reproducible, and suitable for analyzing the samples. The method was found to have high selectivity because no interference peaks were detected in the retention time of each analyte. The blank response is shown in fig. 1. The retention time for considered analytes was 2.55 min for isoniazid, 12.41 min for rifampicin, and 10.93 min for the internal standard. The result showed no interference from the endogenous components or cross-interference between analytes and the IS under the assay conditions.

Fig. 1: Chromatogram of a blank sample
The carry-over was calculated as the peak area observed in the blank expressed as a percentage of the mean peak area determined in the same run for the lowest calibration standard. The carry-over test results met the requirement that the mean interference response at the retention time of rifampicin was less than 2.53%, isoniazid was less than 1.71%, and chloramphenicol as an internal standard was less than 0.14%. These indicated that the previous assay with the highest concentration would not influence the current assay.

**Calibration curve**

The lower limit of quantitation (LLLOQ) was 1.0 and 0.4 μg/ml for each rifampicin and isoniazid, demonstrating satisfactory sensitivity for this method. Rifampicin at a concentration of 1.0 μg/ml produced an accuracy value (%diff) of 3.97% to 13.06% with a CV value of 6.40%. At the same time, isoniazid at a concentration of 0.4 mg/ml obtained a %diff value between 0.51% and 18.43% with a CV value of 6.40%. The linearity was determined graphically by plotting the back-calculated concentration versus the theoretical concentration. The calibration curve conducted the linear regression $y = 0.0295x - 0.0073$ for rifampicin and $y = 0.046x + 0.0204$ for isoniazid. The correlation coefficient (R²) of each rifampicin and isoniazid was 0.9975 and 0.9907, indicating that the instrument response and analyte concentration have a linear relationship. The method produced well-defined results proportional to the analyte concentration within the specified range since all the back-calculated concentrations were<15% of the theoretical concentrations and<20% for LLLOQ.

**Accuracy, precision, and recovery test**

This method was sufficiently precise and accurate since all the QC, QCM, and QCH samples were less than 15% and the LLLOQ was beneath 20% over three consecutive, independent runs. The accuracy and precision within and between-run results were summarized in table 1. The results for all calibrator samples emphasize the robustness of this method for measuring blood rifampicin and isoniazid applied in VAMS.

The recovery of the extraction of the VAMS device is an important aspect to be evaluated because it may affect the level of the analyte measuring process in the patient. The recovery test was calculated from the area ratio of the extracted analyte to the analyte spiked after extraction. The spiked post-extraction could be a 100% reference point reflecting the VAMS extraction recovery. It is shown in table 1 that each analyte in each calibrator level had a high recovery value (≥ 90%) with a CV of ≤ 15%. This data indicated that the extraction process in this study was optimal and reproducible.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>QC</th>
<th>Cons (µg/ml)</th>
<th>Accuracy (%diff)</th>
<th>Precision (%CV)</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Within-run (n=5)</td>
<td>Between-run (n=15)</td>
<td>Within-run (n=5)</td>
<td>Between-run (n=15)</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>LLLOQ</td>
<td>0.4</td>
<td>-6.69 to 17.09</td>
<td>-12.49 to 13.75</td>
<td>3.08</td>
</tr>
<tr>
<td></td>
<td>QCL</td>
<td>1.2</td>
<td>-13.48 to 5.31</td>
<td>-13.48 to 13.28</td>
<td>8.90</td>
</tr>
<tr>
<td></td>
<td>QCM</td>
<td>10.0</td>
<td>-12.70 to 6.87</td>
<td>-12.70 to 13.36</td>
<td>2.80</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>QCL</td>
<td>1.50</td>
<td>-13.54 to 11.45</td>
<td>-13.36 to 13.56</td>
<td>6.81</td>
</tr>
<tr>
<td></td>
<td>QCH</td>
<td>1.0</td>
<td>-17.76 to 16.70</td>
<td>-17.76 to 17.09</td>
<td>9.94</td>
</tr>
<tr>
<td></td>
<td>QCM</td>
<td>3.0</td>
<td>-10.87 to 1.38</td>
<td>-12.44 to 7.40</td>
<td>5.63</td>
</tr>
<tr>
<td></td>
<td>QCH</td>
<td>15.0</td>
<td>-0.37 to 9.40</td>
<td>-11.68 to 13.13</td>
<td>2.31</td>
</tr>
<tr>
<td></td>
<td>QCH</td>
<td>22.5</td>
<td>-5.36 to 11.73</td>
<td>-14.77 to 11.73</td>
<td>6.59</td>
</tr>
</tbody>
</table>

**Dilution integrity and stability**

Dilution integrity had to be established to ensure accurate measurement for samples with concentrations above the upper limit of the standard curve. The dilution integrity preparing five replicates of VAMS with a concentration above LLLOQ (2xQCL) then diluted to half and quarter dilutions resulted in the % diff value ranging from 13.24% to 9.56% for isoniazid and from 14.75% to 12.49% for rifampicin. The CV values were less than 0.10% and 10.20% for isoniazid and rifampicin, respectively. This data suggested that samples with concentrations higher than the standard curve upper limit could be diluted with a blank matrix without affecting the final calculated concentration.

The results of the storage stability of stock solution showed a %diff ranging from 14.30 to 2.01% for all analytes in methanol at room temperature (25 °C) for 24 h and in the refrigerator (4 °C) for a month. The results of the rifampicin and isoniazid stability tests on VAMS also showed good stability because the values of %CV and %diff were less than 15% for all control samples. The stability of the analytes in VAMS has been demonstrated during a room-temperature storage period of up to a month with desiccant and protection from light. All stability test results met the FDA requirements, indicating that each step taken during sample preparation, processing, analysis, and even the storage conditions of the VAMS used will not affect the concentration of the analyte. Therefore, the VAMS technique is suitable for collecting blood samples from TB patients to monitor drug concentrations.

**Analysis of study samples**

Monitoring rifampicin and isoniazid concentrations aims to evaluate the current dosing, which can help determine individually antituberculosis dose regimen. Inappropriate dosage is one of the drug-related problems in adult TB patients [14]. Adjustment doses could improve the antituberculosis treatment outcome by maximizing the therapeutic effect and minimizing its toxicity. A total of 21 patients had signed informed consent before the sampling and analysis process. The characteristics of the patient are summarized in table 2. All samples were obtained at 2 and 6 h after the administration due to the variability of oral absorption. The 2 h post-dose concentrations of isoniazid and rifampicin are usually the most informative due to the Cmax occurring. Unfortunately, too low 2 h values do not characterize delayed absorption or malabsorption. Thus, 6 h post-dose was collected to differentiate between these two scenarios. The value also provides information regarding eliminating drugs with short half-lives, such as rifampicin and isoniazid [15].

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female, n (%)</td>
<td>8 (38.1)</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>13 (61.9)</td>
</tr>
<tr>
<td>Age, years, mean (SD)</td>
<td>30 (10.7)</td>
</tr>
<tr>
<td>weight, kg, mean (SD)</td>
<td>47.2 (7.6)</td>
</tr>
<tr>
<td>Daily drug dose, mg/day, median</td>
<td>450</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>450</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>450</td>
</tr>
<tr>
<td>2 h post-dose concentration, µg/ml median (IQR)</td>
<td>7.41 [5.1-10.7]</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>5.80 [4.3-6.6]</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>2.94 [2.6-5.1]</td>
</tr>
<tr>
<td>6 h post-dose concentration, µg/ml median (IQR)</td>
<td>0.66 [0.5-1.6]</td>
</tr>
</tbody>
</table>

At 2 h post-dose, 66.67% and 47.62% of patients had concentrations in the therapeutic range of isoniazid and rifampicin, respectively. 23.81% of patients had a high isoniazid level, with the highest being on patient SN03 at 10.43 µg/ml. This result is slightly higher than a study conducted in Bali, which found that 16.7% of patients had...
isoniazid levels above the therapeutic range [16]. The isoniazid level in the upper therapeutic range might be associated with slow acetylation. The rate of acetylation of isoniazid significantly alters its blood concentrations, in which the slow acetylator patients have higher levels of isoniazid than intermediate and rapid acetylation [17]. It might be responsible for the increased risk of adverse reactions such as hepatotoxicity since isoniazid can bind to liver proteins and cause immune-mediated liver injury. In Indonesia, 26.2% of 172 patients experienced major adverse reactions to antituberculosis, 60% of which were drug-induced hepatitis [18].

In contrast, none of the patients had a rifampicin concentration level above the therapeutic range, but most patients (52.38%) had a low concentration. The subtherapeutic of isoniazid was found in 9.52% of patients, with the lowest on patient SN11 of 2.63 µg/ml. In the previous study, low levels of both isoniazid and rifampicin were also identified in 34% out of 60 patients [19]. Low drug levels are associated with an unfavorable clinical response, the acquisition of drug resistance, and treatment failure [20, 21]. Hence, the adjustment of the dose should be projected individually. Blood sampling 6 h after administration showed the highest of 7.88 and 2.99 µg/ml while the lowest of 1.17 and 0.41 µg/ml for rifampicin and isoniazid, respectively. The results confirmed that none of the patients experienced delayed absorption since no one had a high concentration of rifampicin and isoniazid at 6 h post-dose. The results of the analysis are demonstrated in fig. 3 and 4.

![Fig. 3: Isoniazid concentration in 2-and 6 h post-dose of 21 patients](image1)

![Fig. 4: Rifampicin concentration in 2-and 6-h post-dose of 21 patients](image2)

Based on these results, 52.38% of patients had low blood concentrations in at least one of the drugs for both at 2 and 6 h post-dose. Those might be identified as malabsorption cases. Rifampicin and isoniazid dosages can be adjusted to maintain therapeutic effects and prevent toxicity. The results are quite interesting, even though a limited number of subjects were used. Using more subjects with a cohort study will provide more comprehensive information regarding follow-up therapy to improve the success of antituberculosis therapy. However, this study can complement several other studies on using rifampicin and isoniazid in Indonesia [16, 22]. The use of VAMS in this study gives more advantages that provide patient comfort due to its minimal invasiveness and the small volume needed. Moreover, no conversion factors are needed since no significant differences exist in determining the drugs in plasma and microsampled [23, 24]. It can be concluded that the method is reliable and effective for therapeutic drug monitoring of rifampicin and isoniazid.

**CONCLUSION**

The volumetric absorptive micro-sampling technique was utilized to analyze rifampicin and isoniazid concentrations in 21 tuberculosis patients. 52.38% of patients had low blood concentrations in at least one of the drugs, indicating that a treatment dose adjustment is needed. 28.57% of the patients were in the therapeutic range, and 23.81% had a high blood concentration of isoniazid. This method was valid and reliably utilized for therapeutic drug monitoring.

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Nil

**AUTHORS CONTRIBUTIONS**

All authors have contributed equally.

**CONFLICT OF INTERESTS**

The authors declare no conflict of interest.

**REFERENCES**


