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Analytical Method Validation of Sulfamethoxazole in Human Plasma and Urine by HPLC-PDA

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Abstract

An analytical method for analyzing SMZ in human plasma and urine using high-performance liquid chromatography (HPLC) was developed. The separation was carried out on a Shim-pack GIST® C_{18} column (150 × 4.6 mm, 5 µm) with a temperature setting of 30°C. The mobile phase consisted of glacial acetic acid pH 2.5: methanol: acetonitrile (70:25:5, v/v/v) at a rate of 0.8 mL/min. Detection was done using a PDA detector in the range of λ190-800 nm, and quantification was carried out at λ265 nm. Plasma sample preparation used the protein precipitation method with acetonitrile as the precipitating solvent (1:3, v/v), while urine preparation used the liquid-liquid extraction method with 0.03 M H_2SO_4 and ethyl acetate. The developed method was proven selective, linear (r = 0.998) for plasma and (r = 0.996) for urine, accurate (%error ≤11.76% for LLOQ and ≤14.08% for concentrations above), precision (%RSD ≤4.52% for LLOQ and ≤4.48% for the concentrations above), sensitive with 0.7 µg/mL (plasma) and 0.17 µg/mL (urine). Stability tests were carried out to determine the shelf life of the samples under several conditions. The developed method is valid and suitable for pharmacokinetic studies of SMZ in human plasma and urine.

Keywords: Sulfamethoxazole; HPLC-PDA; Plasma; Urine; Bioanalytical method validation

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1 Introduction

Urinary tract infection (UTI) is the most common infection in Indonesia. UTI is an infectious disease characterized by microorganisms that can attack tissues in the urinary tract [1],[2]. UTI ranks fourth with the highest prevalence of infectious diseases after diabetic foot infections, typhoid, and sepsis [3].

Sulfamethoxazole (SMZ) is the first-line therapy in treating UTIs, which is still used in Indonesia [4],[5]. SMZ is a sulfonamide class of antibiotics that acts directly on folate synthesis in microbes.[6] In its use, SMZ is combined with trimethoprim (TMP), known as co-trimoxazole

[7]. This antibiotic was found to cause side effects, including hyperkalemia, especially in patients with impaired kidney function, elderly patients, diabetics, or AIDS [7],[8]. Therefore, it is necessary to conduct therapeutic drug monitoring (TDM) to optimize drug therapy and ensure patient safety [9].

Biological samples that can be used for TDM are blood plasma and urine. Plasma can form an equilibrium with body tissues describing the drug concentration in the blood [10]. Meanwhile, the main route of elimination of SMZ is through the kidneys, so the concentration of SMZ in urine is relatively large [11].

Some analytical methods that quantify SMZ include high-performance liquid chromatography (HPLC), spectrofluorometry, and UV spectrophotometry. However, spectrofluorometry tends to produce non-linear signals [12]. Whereas UV spectrophotometry is limited in selectivity and often produces overlapping spectra [13]. Therefore, the HPLC method can be an option for quantifying SMZ in plasma and urine [14]. In this study, we validated the SMZ analysis method in urine and blood using the HPLC method and solvent extraction with ethyl acetate. This study aimed to provide validated analytical methods for pharmacokinetic studies and therapeutic drug monitoring.

2 Materials and Methods

2.1 Material

The materials used are standard SMZ (Sigma-Aldrich, 99.8%) and TMP (Sigma-Aldrich, 99% purity); concentrated sulfuric acid (Merck); ethyl acetate pro-analysis (Merck); glacial acetic acid (Merck); sodium hydroxide (Merck); Methanol grade HPLC (Merck); Acetonitrile grade HPLC (Sigma Aldrich); sodium hydroxide pellets (Merck); ultrapure water or water purified from groundwater using Purelab Flex 3 (ELGA LabWater UK); Chromafil Xtra PTFE-syringe membrane 0.45 µm (Macherey-Nagel) and 0.45 µm cellulose nitrate filter membrane (GE Healthcare Life Sciences).

2.2 Instrument

Analysis with the chromatographic system was carried out using the HPLC method (LC-20AD Shimadzu) equipped with a pump (LC-20AD) and an autosampler (SIL-20ACHT). The stationary phase was a Shim-pack GIST® C_{18} column, and an oven column (CTO-20AC) set at 30°C. The mobile phase used included a combination of glacial acetic acid with a pH adjustment of 2.5 using 0.1 M NaOH: methanol: acetonitrile (70:25:5, v/v/v) which was active at a rate of 0.8 mL/min. The analytes were detected with a PDA detector (SPD-M20A 230V) at 265 nm [15]. The volume of the solution was taken using a 10-100 L and 100-1000 L micropipette (i-pipette). The pH of the mobile phase was measured with a digital pH meter (Mettler Toledo Seven Compact). The heating process was carried out with the help of a 500 mL beaker on a hotplate (IKA C-Mag HS 7).

2.3 Preparation of 0.03 M H_2SO_4 and standard stock solutions

A 0.03 M H_2SO_4 solution was prepared by diluting concentrated sulfuric acid with purified water. SMZ stock solution was prepared by dissolving a certain amount of reference standard with methanol to a concentration of 1000 mg/L.

2.4 Preparation of glacial acetic acid solution pH 2.5

A concentration of 1% glacial acetic acid in water was prepared by diluting glacial acetic acid with ultrapure water. The pH of glacial acetic acid solution was measured and adjusted in the range of 2.5 ± 0.1 with the addition of 0.1 M NaOH. The solution was filtered with a 0.45 µm cellulose nitrate membrane and equipped with a set of vacuum pumps.

2.5 Preparation of calibrator solutions and quality control samples

2.5.1 Plasma

The standard SMZ intermediate solution was prepared by adding a certain amount of stock solution to a 10 mL volumetric flask and adding methanol to the calibration limit to obtain the concentrations of 40, 80, 120, 180, 240, and 300 µg/mL. Ten microliters of each intermediate solution were added to 240 µL of plasma, thus obtaining a calibration curve concentration of 1.6, 3.2, 4.8, 7.2, 9.6, and 12 µg/mL. This concentration range was determined based on the pharmacokinetic range of SMZ in plasma [16]. Quality control

(QC) samples were prepared in the same way as the calibrator solution to obtain concentrations at the lower limit of quantification (LLOQ) level of 1.6 µg/mL, low (5 µg/mL), medium (6 µg/mL), and high (9 µg/mL).

2.5.2 Urine

The SMZ calibrator solution was prepared by adding stock solutions of 50, 100, 200, 400, 600, and 800 µL with a urine sample to obtain a final volume of 10 mL to obtain a calibration curve concentration of 5, 10, 20, 40, 60, 80 µg/mL. This concentration range was determined based on the pharmacokinetic range of SMZ in urine [17]. Quality control (QC) samples were prepared in the same way as the calibrator solution to obtain concentrations at the lower limit of quantification (LLOQ) level of 5 µg/mL, low (15 µg/mL), medium (35 µg/mL), and high (61 µg/mL).

2.6 Sample preparation

2.6.1 Plasma

Two hundred and fifty microliters of plasma SMZ samples were put in a microcentrifugation tube, and 750 µL of acetonitrile was added. The mixture was vortexed for 30 seconds until homogeneous and centrifuged at 10.000 rpm at 4°C for 10 minutes. The supernatant was taken using a syringe, filtered using a 0.45 µm filter membrane, and put into an autosampler vial to be analyzed by HPLC according to predetermined conditions.

2.6.2 Urine

The liquid-liquid extraction (ECC) technique used ethyl acetate to prepare urine samples. A total of 2.0 mL of SMZ solution in urine and blank urine was taken and transferred to a 15 mL test tube. The urine phase will be conditioned in an acidic environment by adding 1.0 mL of 0.03 M H_2SO_4. Then 2.0 mL of ethyl acetate is added to each test tube. After forming two completely separate phases, the ethyl acetate phase (upper layer) was taken and transferred to another test tube and then evaporated to dryness at a temperature of ±85°C. The remaining residue was reconstituted with 1 mL of methanol as solvent. The sample solution was filtered using a 0.45 µm membrane into an autosampler vial for further analysis by HPLC according to predetermined conditions.

2.7 Validation

The bioanalytical method was validated according to the European Medicines Agency (EMA) guidelines and the Food and Drug Administration (FDA). This research method uses selectivity, linearity, sensitivity, accuracy, precision, and stability to validate.

2.7.1 Selectivity

The selectivity test was carried out using the QC sample with the lowest concentration. The method is declared selective if the chromatogram peaks do not overlap and the resolution of the two peaks is well separated. If the chromatograms showed that the blank response is less than 20% of the analyte response at the LLOQ level, then the method is said to be selective [18],[19].

2.7.2 Linearity

The linearity test was measured using a standard solution of a calibration curve that had been prepared. The test can be said to meet if the correlation coefficient value (r) is 0.99 and the maximum %error is ±15% of the nominal concentration, except for LLOQ ±20% [18],[19],[20].

2.7.3 Sensitivity

LLOQ is the lowest concentration of the standard curve with five replications that meet the accuracy and precision requirements. Accuracy is expressed as %error of nominal concentration, while precision is defined as the coefficient of variation or relative standard deviation (RSD). The requirement for %error and %RSD values must be a maximum of ±20%. LOD is expressed as the lowest concentration of analyte that can still produce peaks on chromatography, and the results of six repetitions meet the requirements for the %RSD value of not more than 17% [19],[21].

2.7.4 Accuracy and precision

Testing of accuracy and precision is carried out within-run and between-run. Within-run is done on the same day, while between-run is done on three different days. The test was carried out using a QC sample solution with four concentration levels, and each concentration was replicated five times. Accuracy and precision must meet the requirements, namely

%error and %RSD are a maximum of ±15%, except for LLOQ ±20% [18],[19].

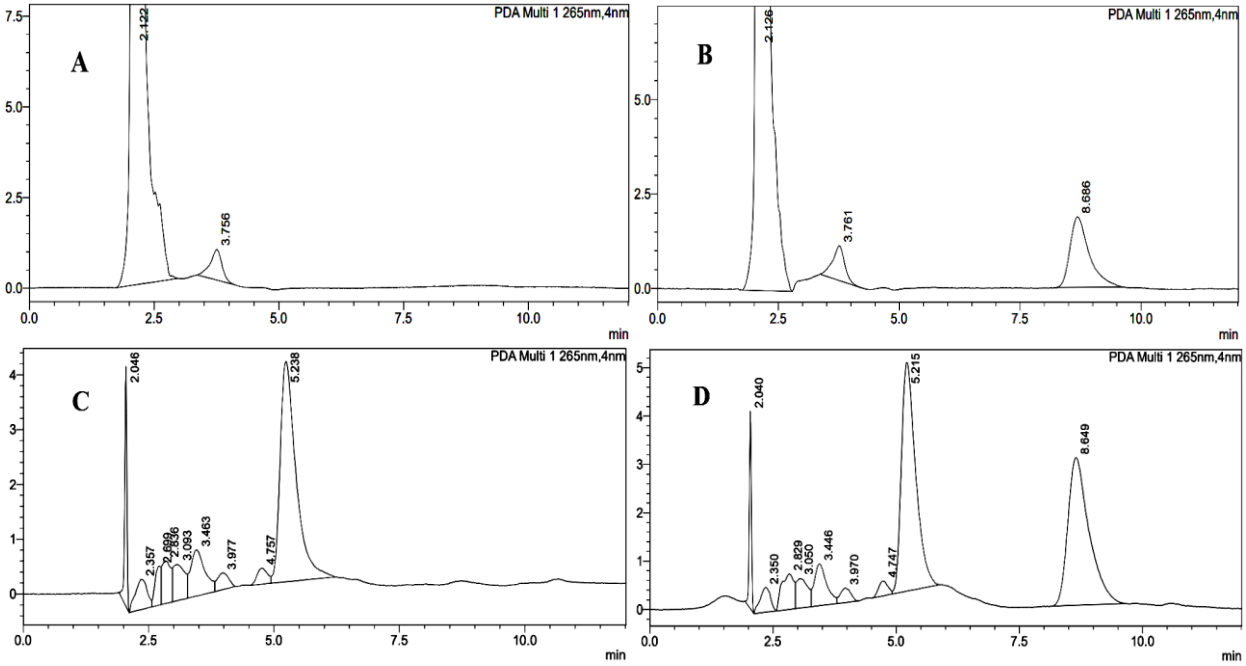
2.7.5 Stability

Stability testing aims to ensure that the analyte in the sample matrix in the sample preparation process and storage conditions during analysis do not change or damage. Stability tests were carried out, including stock stability tests (7 days, 4 °C), freeze-thaw (3 freeze-thaw cycles), short-term (24 hours, 25°C), medium-term (1 month, -20°C), and autosampler (24 hours) [18],[19].

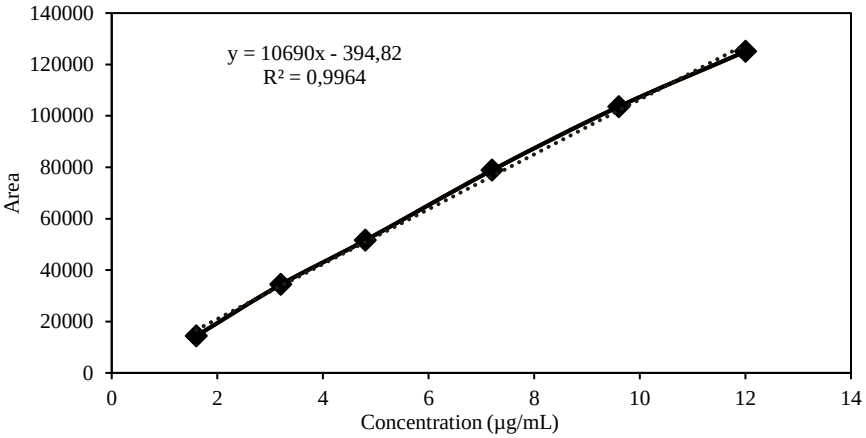
3 Result and Discussion

3.1 Method Development

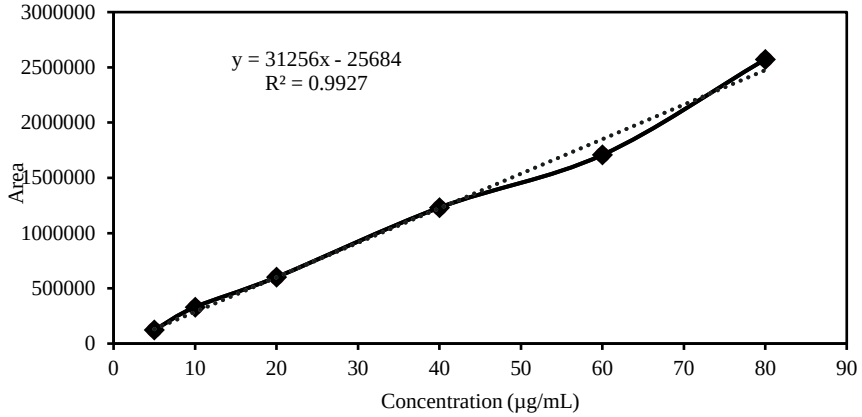
The method development of SMZ in this study was done to find a valid HPLC method for SMZ analysis. The optimization of the mobile phase and flow rate was performed. The optimum composition was found in a mixture comprised of 70:25:5 v/v/v glacial acetic acid (pH 2.5), methanol, and acetonitrile pumped at a flow rate of 0.8 mL/min. These conditions produced a good separation between SMZ and endogenous compounds. It also took place in a short time of less than 10 min.



Representative chromatogram of blank and spiked samples (SMZ in human plasma and urine). (a) blank human plasma, (b) SMZ in human plasma, (c) blank human urine, (d) SMZ in human urine. Mobile phase: glacial acetic acid (pH 2.5), methanol, and acetonitrile (70:25:5 v/v/v), Flow rate: 0.8 mL/min, Column: Shim-pack GIST® C18 (250 × 4.6 mm; 5 µm), Injection volume: 10 µL, and the UV detector was operated at 265 nm.



Calibration curve of sulfamethoxazole in plasma.



Calibration curve of sulfamethoxazole in plasma.

Table 1 Accuracy and precision of analysis method

Sample	Concentration (µg/mL)	Day	Within-run (n = 5)		Between-run (n = 15)	
			Error (%)	RSD (%)	Error (%)	RSD (%)
Plasma	1.6	1	6.08	4.43	7.54	4.52
		2	5.71	6.39		
		3	10.82	1.74		
	5	1	9.55	6.28	7.11	4.48
		2	4.91	7.12		
		3	6.88	0.04		
	6	1	9.71	3.23	10.72	3.98
		2	9.54	2.63		
		3	12.90	6.09		
	9	1	3.16	3.63	6.20	4.44
		2	2.71	3.62		
		3	12.74	6.06		
Urine	5	1	11.77	0.18	4.63	0.71
		2	1.25	1.59		
		3	0.88	0.37		
	15	1	9.41	0.31	4.79	0.38
		2	2.03	0.54		
		3	2.94	0.29		
	35	1	1.28	0.12	3.58	0.15
		2	4.70	0.22		
		3	4.75	0.11		
	61	1	1.43	0.33	5.65	0.51
		2	1.45	0.18		
		3	14.08	1.03		

3.2 Method Validation

3.2.1 Selectivity

The selectivity test was carried out by visual comparison between the chromatogram of the blank samples and the SMZ spiked samples. The test results showed that the SMZ peak could be separated well from the metabolites peak and other interferences at a retention time of about 8 minutes (Figure 1). This applies to sample preparation methods using liquid-liquid extraction and protein precipitation. Thus, the developed method was proved selective.

3.2.2 Linearity

The linearity test was carried out using a calibration curve standard solution made in the concentration range of 1.6-12 µg/mL for plasma and 5-80 µg/mL for urine. The relationship between concentration and response of SMZ in the form of peak area was shown from the calibration curve and the resulting regression equation. The regression equations obtained for the plasma and urine were y = 10690x - 394.82 (r = 0.998) and y = 31256x - 25684 (r = 0.996). Meanwhile, the % errors were 7.29% and 7.79% for plasma and urine, respectively. Thus, this method meets the requirements for linearity (r ≥ 0.99), and the %error is within ±15%.

3.2.3 Sensitivity

The sensitivity was assessed by determining the lower limit of quantification (LLOQ) and limit of detection (LOD). The LLOQ determined by this method is 1.6 µg/mL for plasma samples and 5 µg/mL for urine samples. At the LLOQ level, the %error and %RSD were 7.54% and 4.52% for plasma samples and 4.63% and 0.71% for urine samples. This result has met the existing acceptance criteria, which is in the range of ±20%. This shows that the concentrations of 1.6 µg/mL and 5 µg/mL are the lowest concentrations of SMZ in the sample that can still be quantified accurately and precisely. Thus, it could be said that the method used is quite sensitive to the pharmacokinetic range of SMZ.

The determination of the LOD value is based on visual observations. This method is done by making a sample solution at a specific concentration and diluting gradually until it can no longer be detected visually. The LOD were 0.7 µg/mL and 0.17 µg/mL for plasma and urine samples. The estimated LOD value was confirmed by injecting the SMZ solution prepared from the spiked samples at LOD for six repetitions so that the RSD value was 8.56% for plasma and 6.94% for urine. This result has fulfilled the %RSD requirement, which is 17% [21].

3.2.4 Accuracy and precision

Accuracy and precision tests were carried out with QC samples at concentrations of 1.6-9 µg/mL for plasma samples and 5-61 µg/mL for urine samples (Table 1). The average value of %error obtained for plasma and urine samples was 11.76% for LLOQ and 14.08% for concentrations above it. Meanwhile, the %RSD value obtained was 4.52% for LLOQ and 4.48% for concentrations above it. The within-run and between-run test results meet the requirements because the %error and %RSD obtained do not exceed 20% for LLOQ and 15% for concentrations above it [18],[19]. Thus, the method used is accurate and precise.

3.2.5 Stability

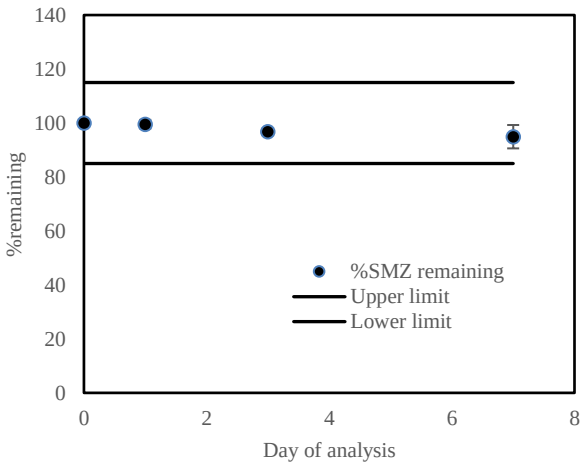


Figure 1 Stability of SMZ in stock solution under storage conditions for 7 days at 4°C.

The stability of SMZ in stock solution was evaluated. It showed good stability for seven days at 4°C (Figure 2) because the %remaining value was still in the 85-115% range based on the CI value (confidence interval, 90%). Meanwhile, the stability tests of SMZ in plasma and urine samples were carried out under four

different conditions (Table 2), consisting of freeze-thaw, short-term, medium-term, and autosampler tests. The %remaining values are also in the range of 85-115% so that they can be declared stable [18],[19].

Table 2 Stability study of SMZ in human plasma dan urine

Stability Test	SMZ remaining ($\bar{x}$ ± SD, %)	
	Plasma	Urine
Three cycles of freeze (-20°C) and thaw (25°C)	95.37 ± 3.55	89.37 ± 1.28
24 h after sample storage (25°C)	95.51 ± 5.78	98.63 ± 1.11
30 days after sample storage (-20°C)	90.20 ± 3.75	97.02 ± 1.53
24 h storage in autosampler	102.35 ± 1.17	98.61 ± 0.54

The developed method for SMZ analysis using the ECC method and protein precipitation combined with HPLC-PDA proved to meet the requirements of the bioanalytical method validation. Thus, this method can be continued for pharmacokinetic studies and therapeutic drug monitoring.

4 Acknowledgement

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5 Author contribution

The authors indicated in parentheses made substantial contributions to the following tasks of research: initial conception (D); design (D,A,K); provision of resources (D,A,K); collection of data (D,A,K); analysis and interpretation (D,A,K); writing and revision of paper (D,A,K).

6 Conflict of interest

There are no conflicts of interest.

7 Daftar Pustaka

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