SIMULTANEOUS ASSAYS OF SULFAMETHOXAZOLE AND TRIMETHOPRIM IN SUSPENSION DOSAGE FORM By THREE ANALYTICAL METHODS OF UV SPECTROPHOTOMETRY

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ABSTRACT

The research goal is to apply and validate of the ratio of the absorption method (RA), Area Under Curve Method (AUC) and Chemometric spectrophotometry methods (CMS) for simultaneous assays of Sulfamethoxazole and Trimethoprim combined in a suspension. Measurements of absorption for RA method in the wavelength at 249 nm and 258 nm, the AUC method absorption measurement in the wavelength at 257-267 nm for Sulfamethoxazole and wavelength 283-293 nm for Trimethoprim and absorption measurements to for Sulfamethoxazole and Trimethoprim in CMS methods performed on a wavelength in the range of 230-310 nm with intervals of 2 nm. The RA method has linearity (3.3-8.9) µg/ml, accuracy = 99.89%, and precision = 0.20% for Sulfamethoxazole and linearity = (9.2-25.2) µg/ml, accuracy = 101.28%, and precision = 0.71% for Trimethoprim, while the AUC method has an accuracy of 99.34%, and precision = 0.44% for Sulfamethoxazole, and accuracy = 100.05%, precision = 1.16% for Trimethoprim whereas Chemometric method has an accuracy = 100.69%, precision = 0.43% for Sulfamethoxazole and accuracy = 101.25%, precision = 0.43% for Trimethoprim. The three analytical methods of spectrophotometry have validation requirements according to ICH guidelines (2005) and the level of Sulfamethoxazole and Trimethoprim met Indonesian pharmacopeia (2014) requirements.

Keywords: Three Methods, Spectrophotometry, Sulfamethoxazole, Trimethoprim, Validation.

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INTRODUCTION

Cotrimoxazole is a combination of sulfamethoxazole (SMZ) and trimethoprim (TMP), the combination has good bactericidal activity by inhibiting two-stage nucleic acid biosynthesis and synergistic effects and reducing the danger of resistance compared to trimethoprim or sulfamethoxazole mono-therapy singly.^{1,2} Assay of SMZ and TMP in a single form can be determined with an ultraviolet spectrophotometry method where the SMZ has a maximum absorption in the wavelength at 257 nm and TMP in the wavelength at 287 nm.³⁻⁵

Some combination of the spectrophotometric method with mathematical software, among others: chemometric methods, intersection methods, q-absorbance ratio spectrophotometric method, the area under a curve, multivariate calibration and mean centering ratio spectra such as to the determination of betamethasone valerate and neomycin sulfate, prednisolone and 5-aminosalicylic acid, carvedilol and hydrochlorothiazide; paracetamol, ibuprofen, and caffeine, etilefrine HCl with chlorpheniramine.⁶⁻¹⁷ The research goal is to application and validation of, AUC method, CMS methods, and RA method for simultaneous assays of SMZ and TMP combined in suspension with methanol as a solvent.

EXPERIMENTAL

Material and Methods

Ultraviolet-visible spectrophotometer (Shimadzu 1800, Japan), a set of Personal Computers (PC) equipped with UV-Probe 2.42 software, Minitab version 16.2.4.4 software (Toshiba, Japan) Methanol E. Merck From PT Rudang Jaya, Medan, Indonesia, standard of SMZ, standard of TMP from Health

Department of Indonesian Government, raw material of SMZ and TMP from Kimia Farma Plant Production, Indonesia, Co-trimoxazole suspension produced locally from PT Sanbe Farma Bandung Indonesia.

Preparation of Stock and Working Solution

Carefully weighed 25 mg of SMZ and TMP respectively, then put each into a volumetric flask 50 ml, dissolved with 50% methanol solvent and filled to the line mark, then piped 5 ml each into a volumetric flask 25 ml, diluted with 50% methanol to the line mark, so that each solution is obtained with a concentration of 100 µg/ml.¹⁵

Preparation of a Spectrum Absorption Maximum for SMZ and TMP

Pipette 0.61 ml of SMZ solution and 1.72 ml of TMP solution, then inserted each into the volumetric flask 10 ml, diluted with 50% methanol until the line mark, to obtain a solution of 6.1 mg/ml SMZ and 17.2 µg/ml TMP, and then each measured absorption in the wavelength at 200-400 nm.^{3,15-17}

Validation Method

The three analytical methods of UV Spectrophotometry for simultaneous assays of SMZ and TMP was validated as per ICH guidelines.¹⁸

Accuracy Test

The Accuracy test method is a range of 80%, 100%, 120%. The following is the formula for percent recovery.¹⁸⁻²⁰

$$\text{Percentage recovery} = \frac{C_F - C_A}{C_A^*} \times 100\% \quad (1)$$

Note:

CF=Concentration after addition

CA= Sample concentration before the addition

CA⁺=Concentration of the standard substances added.

Precision

Precision is expressed by the RSD of the data series. To search for RSD using the following formula:¹⁷

$$RSD = \frac{SD}{X} \times 100\% \quad (2)$$

Note:

RSD=Relative standard deviation

SD=Standard deviation

X=Average data.

Linearity, LOD, and LOQ

Linearity is the analysis of the relationship between concentration and absorption for each substance so that a linear regression equation and its correlation value are obtained.¹⁸⁻²⁰

According to the absorption at the analysis of wavelength, LOD and LOQ are counted as below:

$$SD = \sqrt{\frac{\sum (Y - \bar{Y})^2}{n - 2}} \quad (3)$$

$$LOD = \frac{3.3 \times SD}{Slope} \quad (4)$$

$$LOQ = \frac{10 \times SD}{Slope} \quad (5)$$

Note:

Y=Amplitude; a=Slope; X=Concentration (µg/ml); b=Constant; SD = Standard Deviation ; Slope= a

Preparation of Sample Suspension

Pipette 0.62 ml of a drug suspension, then quantitatively on a 50 ml volumetric flask. Dissolved with 50% methanol, then homogenized with a sonicator for 15 minutes. Filtered, pipette 5 ml of the filtrate and transfer to a 25 ml volumetric flask, filled with 50% methanol to the line marked and then piped 0.61 ml filtrate and transfer to 10 ml volumetric flask, added 1.6 ml of TMP solution and added 4 drops of

NH₄OH to pH 10, then added with 50% methanol to mark line to obtain a solution containing 6.1 µg/ml SMZ and 17.2 µg/ml TMP.¹⁵

The Ratio of Absorption Methods

Construction the Point of Intersection and the Maximum Wavelength Selected

The absorption spectrum at the intersection point is obtained by overlapping the maximum absorption curve of the SMZ and TMP solution so that it can determine the intersection wavelength and the selected maximum wavelength.⁷

Preparation the Calibration Curve of the RA Method

The ratio of the maximum absorption spectra was obtained from the concentration, and get the regression line equation. The RA method was validated by calculating accuracy, linearity, precision, LOD, and LOQ.¹⁸⁻²⁰

Assays of Sample

The suspension preparation solution is measured absorption in the wavelength at 200-400 nm, and calculated by UV Probe software 2.42 with a divisor of 17.2 µg / ml TMP for SMZ and 6.1 µg / ml SMZ as a divisor for TMP, and the formula to obtain the value is below:⁷⁻¹¹

$$Ca: \frac{Qm-Qy}{Qx-Qy} \times \frac{A}{ax1} \quad (6)$$

$$Cb: \frac{Qm-Qx}{Qy-Qx} \times \frac{A}{ay1} \quad (7)$$

$$Qm = \frac{A2}{\frac{A1}{ax2}} \quad (8)$$

$$Qx = \frac{ax2}{ax1} \quad (9)$$

Note:

A = Absorption at intersection wavelength

a1 and a2 = Absorptivity x and y at intersection wavelength

Ca = Concentration SMZ

Cb = Concentration TMP

A2 = Absorption of the sample solution at the maximum wavelength of a component (λ 2)

A1 = Absorption of sample solutions at intersection wavelengths of two components (λ 1)

ax2 = Absorption of the SMZ working solution at the maximum wavelength of one component (λ 2)

ax1 = Absorption of an SMZ working solution at intersection wavelengths of two components (λ 1)

ay2 = Absorption of the TMP working solution at the maximum wavelength of one component (λ 2)

ay1 = Absorption of a TMP working solution at intersection wavelengths of two components (λ 1).

Area Under the Curve Method

The AUC spectrum absorptions of SMZ and TMP

The absorption spectrum of 6.1 µg/ml for SMZ, and the TMP absorption spectrum at concentration is 17.2 µg/ml are overlapped, so the spectrum area is obtained at wavelengths of 257-267 nm for SMZ and 283-293 nm for TMP, then the area values are calculated with a divisor of 17.2 µg/ml TMP for SMZ and 6.1 µg/ml SMZ as a divisor for TMP.¹⁶⁻¹⁷

Preparation of Calibration Curve

The value AUC of SMZ spectrum at 257-267 nm is plotted at a concentration of (3.3, 4.7, 6.1, 7.5, 8.9) µg / ml and value AUC of TMP spectrum at 283-293 nm are plotted at a concentration of (9.2, 13.2, 17.2, 21.15, 25.1) µg / ml to obtain a regression equation. The AUC method was validated with accuracy, linearity, precision, LOD, and LOQ.¹⁸⁻²⁰

Determination of SMZ and TMP in the Suspension Preparation.

The sample solution containing 6.1 µg/ml of SMZ and 17.2 µg/ml of TMP, and measure the absorption in the wavelength at 200-400 nm, then made AUC at spectrum (257-267) nm for SMZ and at (283-293) nm

for TMP and calculated by UV Probe software 2.42 with a divisor of 17.2 µg/ml TMP for SMZ and 6.1 µg/ml SMZ as a divisor for TMP, and calculated with the following equation¹⁶⁻¹⁷:

$$AUC_{\lambda_1 - \lambda_2} = X_{\lambda_1 - \lambda_2}^M bC_{\lambda_1 - \lambda_2}^M + X_{\lambda_1 - \lambda_2}^N bC_{\lambda_1 - \lambda_2}^N \quad (10)$$

$$AUC_{\lambda_3 - \lambda_4} = X_{\lambda_3 - \lambda_4}^M bC_{\lambda_3 - \lambda_4}^M + X_{\lambda_3 - \lambda_4}^N bC_{\lambda_3 - \lambda_4}^N \quad (11)$$

Note:

$X_{\lambda_1 - \lambda_2}$ = AUC _{$\lambda_1 - \lambda_2$} concentration in µg/ml,

$X_{\lambda_3 - \lambda_4}$ = AUC _{$\lambda_3 - \lambda_4$} / concentration in µg/ml

AUC _{$\lambda_1 - \lambda_2$} and AUC _{$\lambda_3 - \lambda_4$} = Value of AUC for each component in a mixture

$X_{\lambda_1 - \lambda_2}^M$ and $X_{\lambda_3 - \lambda_4}^M$ = each absorptivity value from SMZ

$X_{\lambda_1 - \lambda_2}^N$ and $X_{\lambda_3 - \lambda_4}^N$ = each absorptivity value from TMP

b = thickness of cuvette (1 cm)

C^M and C^N = concentration of component SMZ and TMP

Chemometric Methods

Preparation An Absorption Spectrum

The absorption spectrum at the concentration of working solution in 6.1 µg / ml SMZ and 17.2 µg / ml TMP are measured at wavelengths of 230-310 nm.

Construction of Calibration Curves

The Partial Least Squares value of SMZ and TMP absorption spectrum in the wavelength at 230-310 nm is plotted with the concentration to get the regression equation.

Determination of SMZ and TMP Levels in Suspension Preparations

The sample solution containing 6.1 mg/ml of SMZ and 17.2 mg/ml of TMP, and measure the absorption spectrum of the partial least squares chemometric methods in the wavelength at 200-310 nm with intervals of 2 nm. The calculated absorption value UV Probe 2.42 software with a divisor of 17.2 µg/ml TMP for SMZ and 6.1 µg/ml SMZ as a divisor for TMP.^{6,21-22}

RESULTS AND DISCUSSION

Determination of the Absorption Spectrum

The single-component of SMZ and TMP absorption spectrum curves are overlapped. The results in the overlapping absorption spectrum of SMZ and TMP (Fig.-1).

Based on Fig.-1, that the conventional ultraviolet spectrophotometry method cannot be used for determination of SMZ and TMP content in the mixture because the spectrum of the two substances overlaps, then the RA, AUC and CMS methods are applied to determine the content of a mixture can be done.

Method of Validation

The third method for assay spectrophotometry of SMZ and TMP validated by the validation parameters: accuracy, precision, detection limit and limits the quantity.¹⁶⁻¹⁸ In this study, the validation test was carried out by the standard addition method (Table-1).

Based on Table-1 above, it can be seen that the test results of validation parameters for the three methods have met the validation requirements of ICH guidelines 2015 so that all three methods have been valid for the assay of SMZ and TMP in the suspension.¹⁸⁻²⁰

Result of RA Methods

Determination of intersection points by overlapping the maximum absorption curve of two substances. Based on Fig.-2, It's can be obtained that SMZ and TMP produce Intersection points at 2 points, namely at wavelengths of 249 nm and 271.8 nm, but in this study an intersection point is used in the wavelength at 249 nm because it approaches the maximum absorption that meets the Lambert-Beer law and that point occurs before reaching the maximum wavelength of the two substances⁴⁻⁵.

Fig.-1: Absorption Spectra Overlap of 6.1 µg/ml SMZ and 17.2 µg/ml TMP

Table-1: Validation Method Test of RA Methods, AUC, and CMS Methods

Parameter	SMZ			TMP		
	RA	AUC	CMS	RA	AUC	CMS
Spectrum absorption	249 nm	257-267 nm	230-310 nm	249 nm	283-293 nm	230-310 nm
Regression equation	$y = 0.063x - 0.003$	$y = .0232x - 0.005$	$y = 0.0711x + 0.007$	$y = 0.023x - 0.004$	$y = 0.0102x - 0.005$	$y = 0.0252x + 0.004$
R^2	0.9997	0.9978	0.9999	0.9995	0.9980	0.9999
Accuracy(%)	99.89	99.34	100.69	101.28	100.05	101.25
Precision (%)	0.20	0.44	0.44	0.71	1.16	0.43
LOD (µg/mL)	0.2664	0.5164	0.0986	1.1744	1.7212	0.2143
LOQ (µg/mL)	0.8087	3.7247	0.3288	3.5588	12.4158	0.7142

Fig.-2: Overlapping Results and Intersection Points

Determination of SMZ and TMP levels in Suspension Preparations

Determination performed an absorption spectrum at 249 nm and an absorption maximum of SMZ and TMP at 258 nm and 288 nm and the results of the calibration curve (Fig.-3). Based on the Fig.-3, it is shown that the SMZ has a regression equation at 249 nm is $Y = 0.063 X - 0.003$ and at 258 nm is $Y = 0.071 X + 0.0000$ and has the same r^2 value at 0.9999 as well as TMP has a regression equation at 288 nm is $Y = 0.025 X + 0.0000$ which and at λ 249 nm is $Y = 0.022 X + 0.004$ which also has the same r^2 value at 0.9999, that means the RA method has good linearity. The calculated concentration of SMZ and TMP component is obtained by the formula the following mathematical equation:

$$C_x = \frac{Q_m - Q_y}{Q_x - Q_y} X \frac{A}{a \times 1} \quad (12)$$

$$C_y = \frac{Q_m - Q_x}{Q_y - Q_x} X \frac{A}{a \times 1} \quad (13)$$

The calculation results were obtained levels of SMZ and TMP in suspension preparations (Table-2).

Fig.-3: RA Calibration Curve of (a) SMZ at 258 nm and 249 nm and (b) TRI at 288 nm and 249 nm

Result of the AUC Method

The AUC method is a method of conducting the assay with measuring and calculating the absorption area under the curve of the two wavelengths, at before and after the maximum wavelength of a drug component.

Selected by Wavelength Area for AUC Method

The wavelength of the SMZ and TMP analysis in the wavelength range for SMZ ($\lambda = 257-267$) nm and TMP ($\lambda = 283-293$) nm. The selected wavelength range is to provide linearity with the value of the correlation coefficient value of ≤ 1 . It means this method has an excellent method because it has linearity ≤ 1 (Table 1) and the following equation calculated the component:

$$A_1(\lambda_1 - \lambda_2) = a(\lambda_1 - \lambda_2) C_S + a(\lambda_1 - \lambda_2) C_T \quad (1)$$

$$A_2(\lambda_3 - \lambda_4) = a(\lambda_3 - \lambda_4) C_S + a(\lambda_3 - \lambda_4) C_T \quad (2)$$

Note:

A_1 = AUC value of mixture at range $\lambda = 257 - 267$ nm

A_2 = AUC value of mixture at range $\lambda = 283 - 293$ nm

C_S = concentration SMZ

C_T = concentration TMP

The AUC results of each drug at the maximum absorption of SMZ and TMP can be the calculation of the concentration of components with the following equation:

$$A_1 = 0.0252 C_S - 0.0083 C_T \quad (3)$$

$$A_2 = 0.00377 C_S + 0.01017 C_T \quad (4)$$

Then the use of equation 1 and equation 2 above can be calculated the values of C_S and C_T with mathematical solutions.

The Determination Result of SMZ and TMP Levels in Suspension Preparation

The sample has been prepared, then measured in the wavelength at 200-400 nm. The absorption spectrum of the samples obtained is a mixture of SMZ and TMP spectrums to get Area Under Curve values in the area 257-267 for SMZ and TMP divisor and 283-293 for TMP with SMZ divisor (Fig.-4), and calculated using the regression equation (Table.-1), and statistical calculations (Table.- 2). The levels of SMZ and TMP were obtained in the suspension form, and after performing the statistical calculation (Table-2) is $99.94 \pm 1.3696\%$ for SMZ and $99.03 \pm 1.5749\%$ for TMP and fulfill the requirements in the Indonesian Pharmacopoeia 5th edition.⁴

Result of Chemometrics Methods

Partial least squares (PLS) are one type of multivariate calibration of the chemometric method used to process data because it is able to produce a calibration model with a good predictive ability for large amounts of data. Regression analysis from SMZ and TMP can be seen in Table-1 and Fig.-5.

The absorption spectrum of the sample is measured to get 41 sets of data absorption and with prepared as by a calibration model for data of partial least square value of chemometric, value and get 41 sets of data

absorption of with prepared as by a calibration model. The selected wavelength range is the range provides of the best linear relationship between the PLS and concentration as indicated by the correlation coefficient ≤ 1 (Table-1). The concentration where the absorbance obtained fulfill the law of Lambert-beer in the range of 0.2-0.6 and calculated levels obtained then performed statistical calculations²².

Fig.-4: Curve Absorption of AUC For (a) SMZ at (257-267) nm and (b) TMP at (283-293) nm

Fig.-5: Curve of PLS Response Plot of (a) SMZ and (b) TMP at 230-310 nm with Interval 2 nm

Result of Statistic Calculation of RA, AUC and CMS Methods

The result of calculation statistic each of SMZ and TMP content can be seen in Table-2 below:

Table-2: Statistic Calculation of SMZ and TMP

Component (%)	RA	AUC	CMS	Requirements
SMZ (%)	99,64± 0,28	99.94 ± 0.37	98,66 ± 0,0105	90- 110
TMP (%)	96,80 ± 0,74	99.03±0.57	98,12 ± 0,0051	90 -110

Based on Table-2, can be seen that the three analytic methods of ultraviolet spectrophotometry have levels that meet requirements according to the 4th edition of the Indonesian pharmacopeia monograph. It means that the three methods can use for the determination simultaneous of SMZ and TMP mixture in a suspension dosage form.

CONCLUSION

The spectrophotometry method by ratio absorption methods, the area under curve method, and chemometric method can be applied for the determination of the levels of SMZ and TMP in suspension preparations because they have fulfilled the validation requirements with accuracy, precision, linearity, LOD, and LOQ parameters. The ratio absorption methods, the area under curve method, and chemometric method, although have a difference in procedures, regression equation, and wavelength measurements, but the levels obtained appropriate the requirements. The ratio absorption methods, the area under curve

method, and chemometric method is a simple, practical, easy to implement, and economical method by using efficient mathematical software to determination sulfamethoxazole and trimethoprim mixture.

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