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Validation of second derivative spectrophotometry method for determination of isoniazide, pyrazinamide and rifampicin in combined pharmaceutical doses form

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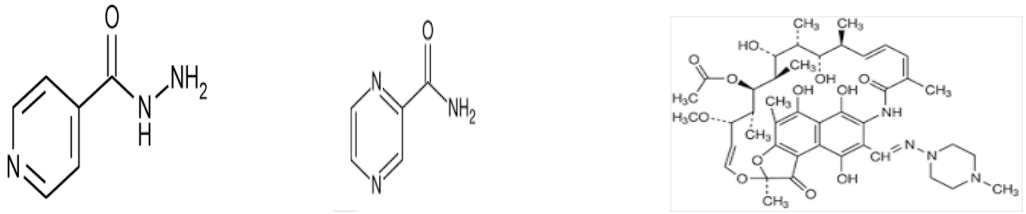
ABSTRACT

A second derivative spectrophotometric method was validation for determination of ternary mixtures of Isoniazide (INH), Pyrazinamide (PRZ), Rifampicin (RIF) that available in fixed dose combination (FDC) of antituberculosis. The aim the research to test the validation of second derivative spectrophotometry method for determination of INH, PRZ and RIF combinations in commercial tablets.The determination of INH, PRZ and RIF can use the second derivative spectrophotometry method with zero crossing technique and use 0.1N HCl as a solvent, and measured at Δλ 2 nm with wavelength of 302.40 nm for INH, and 299.80 nm for RIF, measured at Δλ 16 nm with wavelength of 253.60 nm for PRZ.. The validation test in commercial tablets showed that the recovery percentage for INH are 99.88% wirh Relative Standard Deviation (RSD) = 1.318%, PRZ are 99.84% with RSD = 1.39%, and RIF are 100.95% with RSD = 0.415%. The proposed of second derivative spectrophotometry method was validated and successfully applied for the assay of INH, PRZ and RIF in combined pharmaceutical doses form.

Keywords: Isoniazid, Pyrazinamide, Rifampicin, Derivative Spectrophotometry, Validation.

INTRODUCTION

Isoniazide (INH) is one of the effective anti-tuberculosis drµgs. The use of INH is usually combined with other antituberculosis drµgs such as Rifampin and Pyrazinamide. Assay using HPLC, where the mobile phase is a solution of 4.4 grams of sodium docusate P in P 600 mL of methanol, add 400 ml of water, adjust to pH 2.5 with sulfuric acid 2 N [1]. **Pyrazinamide (PRZ)** is rather difficult to dissolve in water and difficult to dissolve ethanol, ether and chloroform. Identification using ultraviolet spectrum showed maximum absorption at a wavelength of approximately 268 nm differ not more than 3.0% [1]. **Rifampicin (RIF)** is rather difficult to dissolve in water; easily soluble in chloroform; soluble in ethyl acetate and methanol. RIF assay using HPLC, where the mobile phase used is the water mixture: acetonitrile P: a solution of 1.0 M phosphate buffer-citric acid, 0.5 M sodium perchlorate [1].



a **b** **c**

Figure 1. Chemical structure of (a) INH, (b) PRZ, (c) RIF [1]

One of the main challenges facing analytical chemists is the spectrophotometry determination of two or more compounds in the same sample without preliminary separation. Resolving the overlapped spectra of multicomponent mixtures dosage forms whether ternary or more as mixtures was rather a difficult task [2]. Derivative spectrophotometry, a well established analytical technique which is frequently used in the contemporary analysis of drµgs in mixtures when the spectral classic bands of components are overlapped [3].

Researchers have conducted research using derivatives spectrophotometry method to establish the levels of binary and ternary mixtures , that is the zero-crossing method [3,4,5,6,7]. Althoµgh this method is rarely used for the determination of ternary mixtures, but in the application at the higher derivatives , determination of ternary mixtures can be done . This method is faster and simpler [8].

The aim of this work is to validation a second derivative spectrophotometry method for determination of this ternary mixture in tablet dosage form using 0.1 N HCL as solvent.

MATERIALS AND METHODS

Apparatus The spectrophotometric measurements were carried out on a Shimadzu UV-1800 spectrophotometer. The absorbance spectrum were measured using 1 cm quartz cells. For the derivative method, The absorbance spectrum were recorded on the same spectrophotometer, with 1 cm quartz cells and supported with UV-Probe 2:34 software.

Materials and reagents All materials and reagents were analytical reagent grade. Materials used are 0.1N HCl, Rifampicin (Shenyang Antibiotic Manufacturer), INH (Indonesian Reference Substance), Pyrazinamide (Asean Reference Substance), 3-FDC tablet (containing 400 mg pyrazinamide, rifampicin 150 mg and INH 75 mg).

Preparation of standard solutions Stock solutions containing 50 mg/mL INH, 50 mg/mL PRZ and 50 mg/mL RIF were prepared in 0.1N HCl. Further dilutions were done using 0.1N HCl as described under construction of calibration graphs.

Preparation of calibration graphs Different aliquouts of the standard solution of INH, PRZ and RIF, was transferred into 50 ml volumetrric flask. The solutions were then completed to the volume with 0.1N HCl, so that the final concentration for INH were 5.0 mg/mL; 7.5 mg/mL, 10.0 mg/mL; 12.5 mg/mL and 15.0 mg / mL, PRZ were 6.0 mg/mL; 7.5 mg/m, 9.0 mg/mL, 10.5 mg/mL, 12.0 mg/mL and RIF were 6.0 mg/mL, 9.0 mg/mL mg/mL, 12.0 mg/mL, 15.0 mg/mL and 18 mg/ml. The absorption spectrum of each solution was recorded within the wavelength range of 200-400 nm and stored.

Maximum Absorbance Spectrum
Pipette 2.2 mL INH, 1.2 mL PRZ and 2.7 mL RIF. Absorbance was measured at a wavelength of 200-400 nm.

Derivatives Absorbance Spectrum
Prepared Absorbance spectrum from INH standard solution with a concentration of 5.0 µg/mL, 7.5 µg/mL, 10.0 µg/mL, 12.5 µg/mL, and 15.0 µg/mL PRZ standard solution with a concentration of 6.0 µg/mL, 7.5 µg/mL, 9.0 µg/mL, 10.5 µg/mL and 12 µg/mL and RIF standard solution with a concentration of 6.0 µg/mL, 9.0 µg/mL, 12.0 µg/mL, 15.0 µg/µmL, and 18.0 µg/mL at a wavelengthof 200-400 nm, the absorbance spectrum is transformed into the first and second derivatives with $\Delta\lambda$ 2 nm, 4 nm, 8 nm, 16 nm.

Determination of Zero Crossing
Determination of the zero crossing is obtained by overlapping the absorbance spectrum of each derivative in a range of concentration of the solution. Each substance's zero crossing shown by the wavelength that has zero absorbance at various concentrations.

Determination of Wavelength Analysis
Created INH solution with a concentration of 7.5 µg/mL, PRZ solution with a concentration of 12 µg/mL, RI Fsolution with a concentration of 9 µg/mL, and a ternary mixture solution of 7.5 µg/mL INH, 12 µg/mL PRZ and 9 µg/mL RIF. The four solutions was measured their absorbance at a wavelength of 200-400 nm, and then transformed into an first and second derivatives absorption spectrum of each single substances and a ternary mixture PRZ solution of INH and RIF. The second derivative absorbance spectrum of single substances and ternary solution mixture was overlapped. The selected wavelengths is a wavelengths where absorption of single two substances provide a value of zero while the other compound and Ternary mixture similar to or exactly the same.

Preparation and Determination Calibration Curve Linearity

Created INH standard solution with a concentration of 5.0 µg/mL, 7.5 µg/mL, 10.0 µg/mL, 12.5 µg/mL and 15 µg/mL, then measured the second derivative absorbance (Δλ = 2 nm), PRZ standard solution with a concentration of 6.0 µg/mL; 7.5 µg/mL, 9.0 µg/mL, 10.5 µg/mL µg/mL, and 12.0 µg/mL, then measured the second derivative absorbance (Δλ = 16 nm), RIF standard solution with a concentration of 6.0 µg/mL; 9.0 µg/mL; 12.0 µg/mL; 15.0 µg/mL, and 18.0 µg/mL, then measured the second derivative absorbance (Δλ = 2 nm) in predetermined wavelength analysis. Conducted analysis of the relationship between concentration and absorbance values thus obtained by linear regression equation y = ax + b. Based on the value of absorbance at a wavelength analysis, calculate the limit of detection and the limit of quantitation [9,10]. Determination of the limits of detection and the limit of quantitation can use the formula:

$$SD = \sqrt{\frac{\sum (y - yi)^2}{n - 2}}$$

$$LOD = \frac{3 \times SD}{slope}$$

$$LOQ = \frac{10 \times SD}{slope}$$

Explanation
SD = Deviation Standard
LOD = Limit of Detection
LOQ = Limit of Quantitation [9.10].

Determination of INH, RIF PRZ mixture in Tablet

Twenty 3-FDC tablet weighed and crushed homogeneous. Powder weighed equivalent to 50 mg PRZ, put in a 100 ml flask, added approximately 50 mL of 0.1N HCl, sonicated for 10 minutes, then added 0.1 N HCl until the line mark. Shaken and filtered (the first few mL of filtrate discarded). Pipette 1.2 mL filtrate into a flask of 50 mL, 0.1 N HCl is added to the line mark. Detection by using UV spectrophotometric at a wavelength of 200-400 nm analysis.

Method of Validation

The proposed method has been extensively validated in terms of linearity, accuracy, precision. The accuracy of the method was determined by calculating the recovery of INH, PRZ and RIF by the standard addition method [9,10].

RESULTS AND DISCUSSION

Determination of the Maximum Absorbance Spectrum

Determination of maximum absorbance spectrum performed at a wavelength of 200-400 nm. Measurements for INH at a concentration of 11 mg/mL, PRZ at a concentration of 6.5 mg/ mL, and RIF at a concentration of 13.5 mg/mL. Based on the research results, obtained the maximum wavelength INH at 266,40nm. PRZ at 268.60 nm, and RIF at 228.20 nm

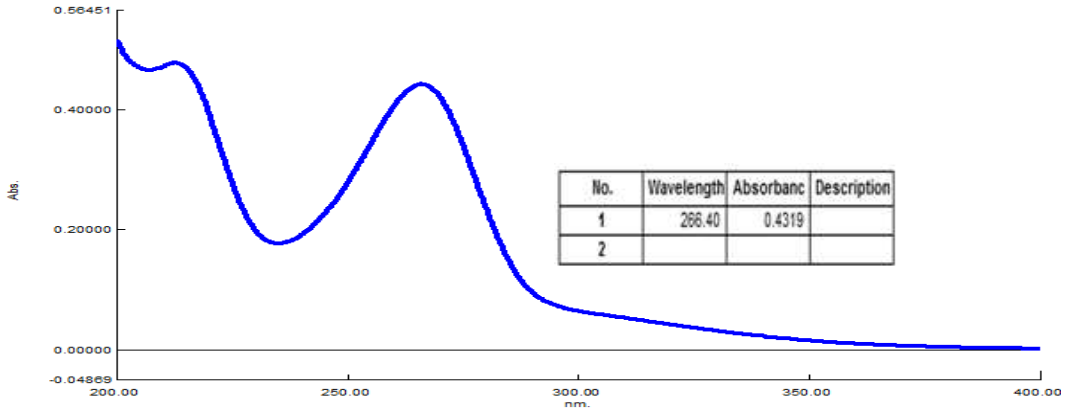


Figure 2. The maximum absorbance spectrum of 11µg / mL INH

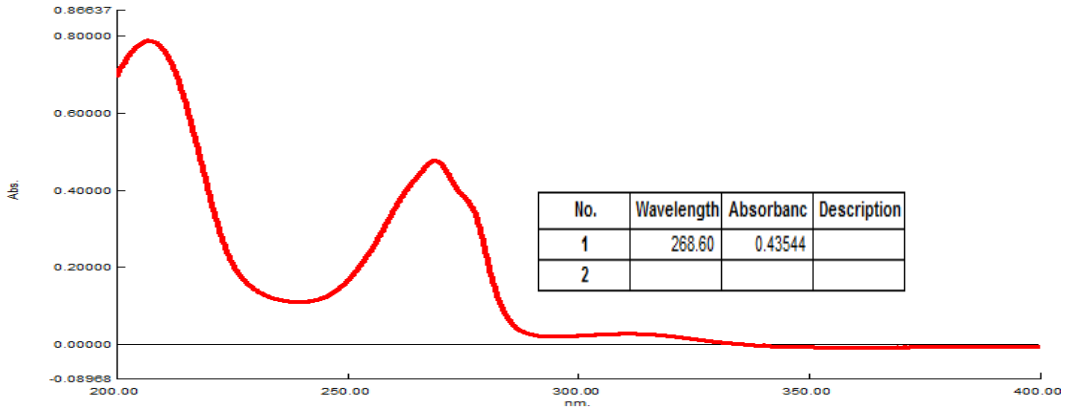


Figure 3. The maximum absorbance spectrum of 6.5 µg/ml PRZ

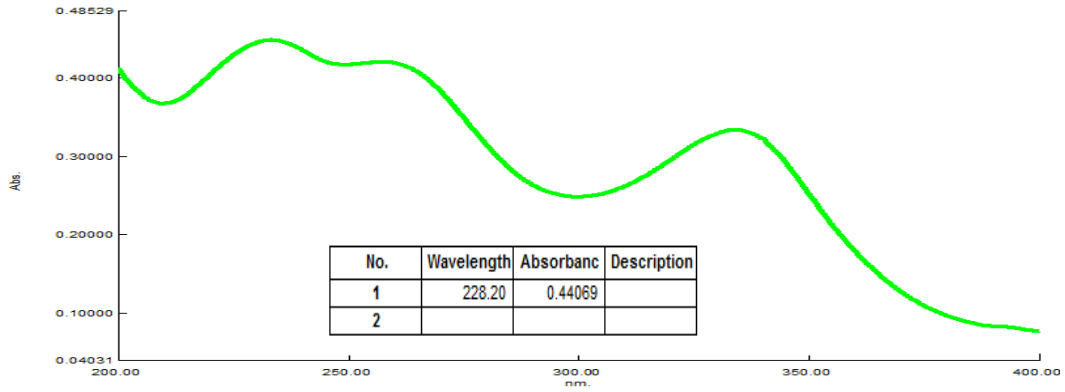


Figure 4. The maximum absorpbance spectrum of 13.5 µg/ml RIF

Determination of Absorbance Spectrum

The results of the determination of the absorbancr spectrum made for solution 7.5 µg/mL INH, solution of 12 µg/mL PRZ, and solution of 9 µg/mL RIF, then made absorbance spectrum at a wavelength of 200 -400 nm. As for the comparison of the content of INH: PRZ: RIF is 2.5: 4: 3.

Determination of Zero crossing of First Derivatives Absorbance

Absorbance spectrum of INH concentration of 7.5 µg/mL, PRZ concentration of 12 µg/mL and RIF a concentration of 9 µg/mL is transformed into the first derivative absorbance spectrum with Δλ 2 nm, 4 nm, 8 nm and 16 nm. The determination result of zero crossing of the first derivative absorbance obtained by overlapping the first derivative absorbance spectrum of each substances. Zero crossing in the first derivative spectrum of each substances is indicated by the wavelength which has a zero absortion. INH, PRZ and RIZ overlap absorption spectrum can be seen in Figure 5.

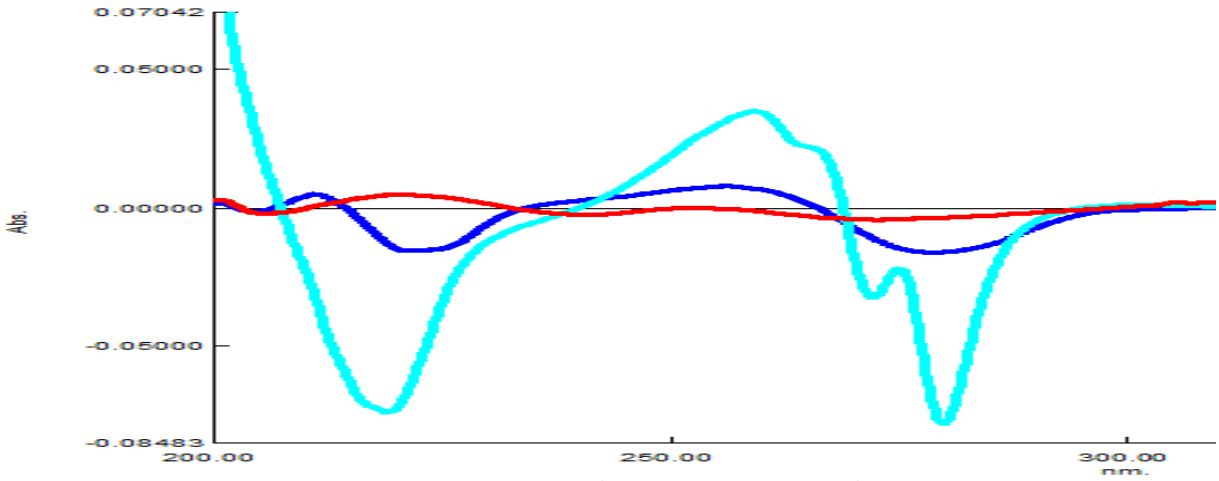


Figure 5. Overlap absorption spectrum of INH, PRZ and RIP on the first derivatives

Based of the results shown in the figure 5 above, it can be seen that the overlap of the first derivative absorption of INH, PRZ, and RIF zero crossing is not obtained. So it can not be performed determination of each substance then continued on the second derivative.

Determination of Zero Crossing on the second derivative absorbance

The determination results of the second derivative absorbance spectrum is made by first making the absorption spectrum from INH solution with a concentration of 7,5 µg/mL, solution PRZ with a concentration of 12 µg/mL and RIF solution with a concentration of 9 µg/mL at a wavelength of 200-400 nm. The obatined absorance spectrum transformed into a second derivative absorbance spectrum with Δλ 2 nm, 4 nm, 8 nm and 16 nm. The second derivatives absorbance spectrum of each of these substances was overlapped. The overlapping INH, RIF, PRZ and the second derivative absorbance can be seen in Figure 6 and Figure 7.

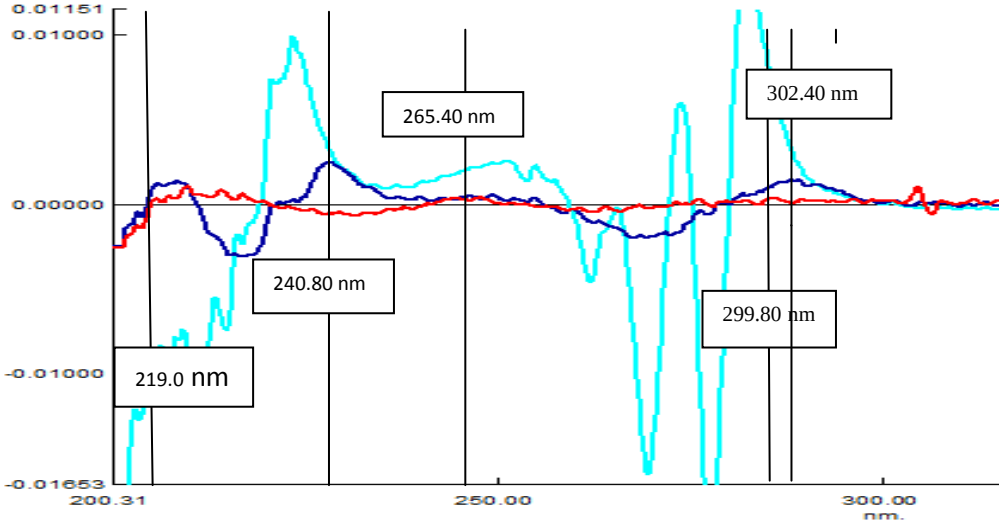


Figure 6. The overlapping second derivative absorbance spectrum Δλ 2 for INH, PRZ and RIF

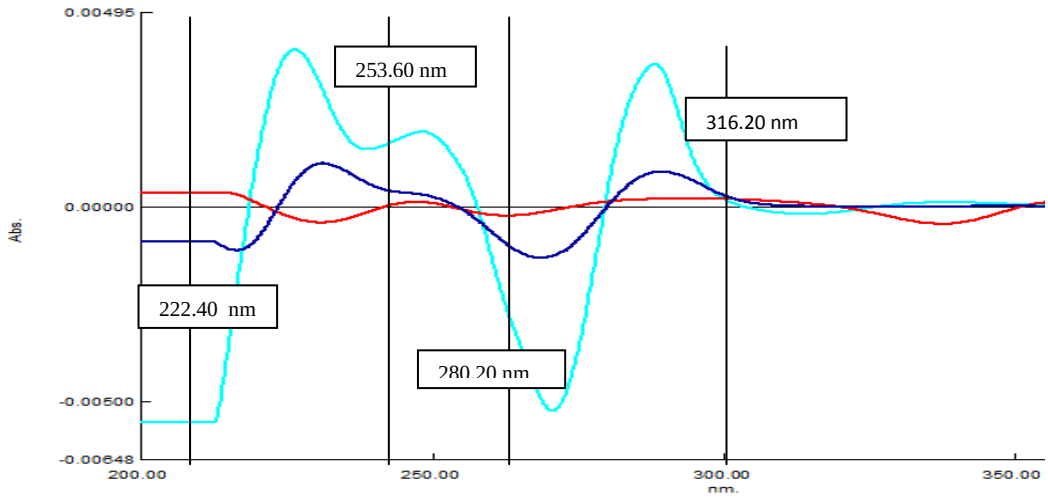


Figure 7. The overlapping second derivative absorbance spectra Δλ 16 for INH, PRZ and RIF

Based of Figure 6 it can be seen from the overlapping absorbance INH, PRZ and RIF obtained a result of zero crossing in the second derivative absorbance with Δλ 2 at a wavelength of 219.00 nm, 265.40 nm and 302.40 nm to INH. Meanwhile, at a wavelength of 240.80 nm for PRZ and at a wavelength of 299.80 to RIF.

Based of Figure 7 from the overlapping absorbance INH, PRZ and RIF obtained a result of zero crossing in the second derivative absorbance with Δλ 16 at a wavelength of 222.20 nm, 253.60 nm 280.20 nm and 316.40 nm for PRZ. The result of the zero crossing of INH, PRZ and RIF can be usefor to assay the mixture of three substances.

Determination of Wavelength Analysis INH, PRZ and RIF

Determination of the wavelength analysis is done by making the INH solution of 7.5 µg/mL, PRZ solution of µg/mL, RIF solution of 9 µg/mL and mixed solution of 7.5 µg/mL INH, 12 µg/mL PRZ, and 9 µg/mL RIF. Created the first derivative absorbance spectrum of each solution 7.5 µg/mL INH, 12 µg/mL PRZ, 9 µg/mL RIF, and the mixture of 7.5 µg/mL INH, 12 µg/mL PRZ, 9 µg/mL RIFand then overlap. The same prosedure was done for the second derivative spectrum. Determination of the wavelength analysis of absorption spectrum on each derivative is done by observing aertain wavelengths where absorption of a single two compounds provide a value of zero while the other substance and mixtures three identical or exactly the same. INH, PRZ, and RIF wavelength spectrum analysis can be seen in Figure 8, Figure 9 and Figure 10.

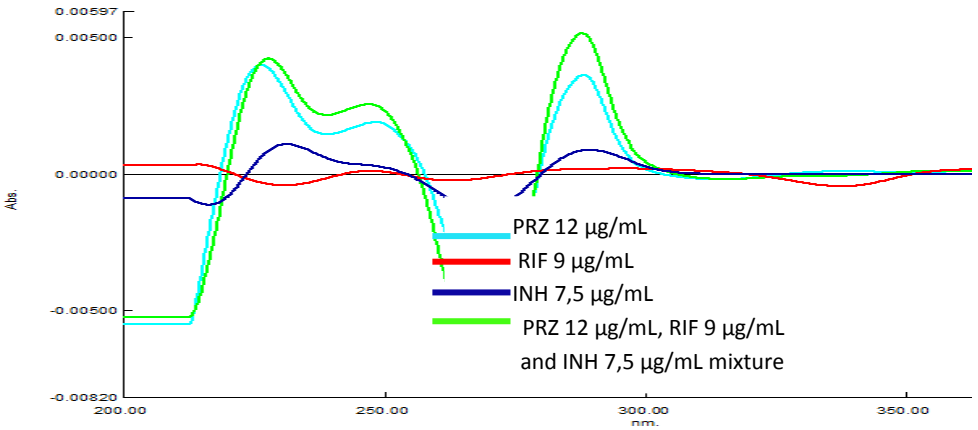


Figure 8. Wavelength Analysis of PRZ with λ 253.60 nm

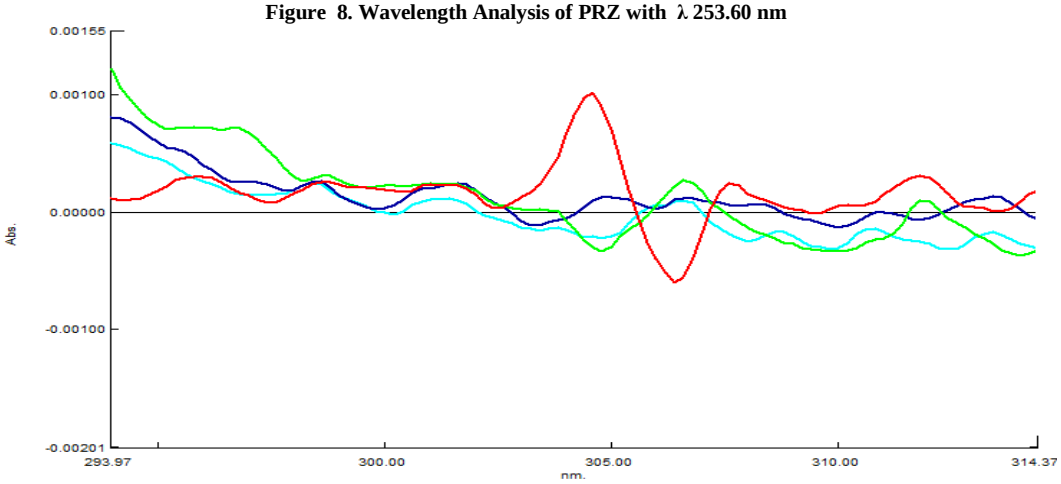


Figure 9. Wavelength Analysis of RIF with λ 299.80 nm

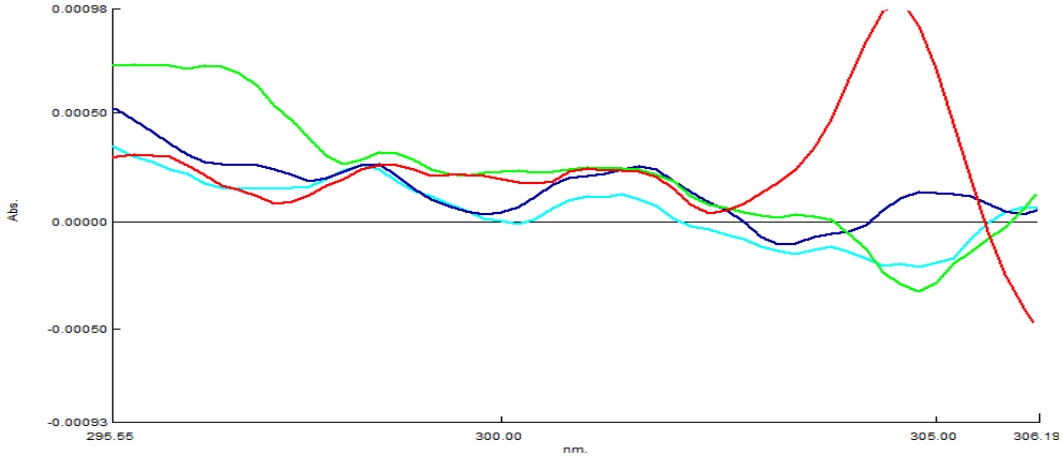


Figure 10 Wavelength Analysis of RIF λ = 302.40 nm

Based on Figure 8, Figure 9 and Figure 10, obtained wavelengths that can be used for the determination of INH, PRZ and RIF mixtur. Determination of the PRZ component used the second derivatives at Δλ 16, while for RIF and INH components used second derivatives at Δλ 2. It is known based on wavelength selection analysis for each derivative. Wavelength analysis is obtained by determining the *zero crossing* for INH, PRZ and RIF

Wavelength analysis is determined by overlapping the absorbance spectrum of each derivative INH, PRZ, RIF in single form and Mixture of INH, PRZ and RIF. Wavelengthsis determinde where the absorbance of the two compounds give zero value while the absorbance of the other compounds and ternary mixture of INH, PRZ and RIF about the same or exactly the same. At first derivative absorbance spectrum, the wavelengths analysis of INH, RIF PRZ and can not be found.

Therefore, the second derivative absorbance spectrum at Δλ 2, the wavelength analysis of RIF and INH can be found. But wavelength analysis for PRZ can not be found. PRZ wavelengths for analysis can be found on the second derivative absorbance spectrum at Δλ 16.

Based on the results of the second derivative absorbance spectrum Δλ 2, it is known that the zero crossing of PRZ at a wavelength of 299.80 nm, zero crossing for RIF at a wavelength of 299.80 nm and zero crossing INH at a wavelength of 219.00 nm, 265.40 nm and 302.40 nm. While based on the second derivative absorbance spectrum Δλ 16, it is known that the *zero crossing* for PRZ is at a wavelength of 222.40 nm, 253.60 nm, 280.20 nm and 316.40 nm. After the second derivative absorption spectrum of the three substances and the mixture of three components overlapped, wavelength analysis for PRZ at 253.60 nm in the second derivative absorbance spectrum at Δλ 16, while for RIF at 299.80 nm and INH at 302.40 in the second derivative absorbance spectrum Δλ 2.Wavelength and absorbance analysis on the second derivative at Δλ 2 and Δλ 16 can be seen in Table 1 and Table 2.

Table 1 Analysis Wavelength and Absorbance on Second Derivatives with Δλ 2

Wavelength (nm)	Absorbance			
	PRZ 12 µg/mL	RIF 9 µg/mL	INH 7.5 µg/ mL	Mixture of INH,PRZ and RIF
219.00	-0.0003	-0.0001	-0.0027	- 0.0025
240.80	0.0013	0.0000	0.0003	0.0019
265.40	-0.0002	-0.0004	-0.0014	- 0.0022
299.80	**0.0000**	**0.0002**	**0.0000**	**0.0002**
302.40	**-0.0000**	**0.0000**	**0.0001**	**0.0001**

Table 2. Analysis Wavelength and Absorbance on Second Derivatives with Δλ 16

Wavelength (nm)	Absorbance			
	PRZ 12 µg/mL	RIF 9 µg/mL	INH 7.5 µg/ mL	Mixture of INH,PRZ and RIF
222.40	0.0030	- 0.0001	- 0.0003	0.0022
253.60	**0.0013**	**- 0.0000**	**0.0000**	**0.0013**
280.20	0.0006	0.0001	0.0001	0.0013
316.40	- 0.0002	0.0000	0.0000	-0.0002

Based on Table 1 and Table 2 the obtained wavelengths analysis of PRZ, RIF and INH used is 253.60 nm, 299.80 nm and 302.40 nm, respectively. Determination of wavelength analysis is based on the value of the fourth absorbance solution at these wavelengths.

Based on the results of the second derivative absorbance spectrum at a wavelength Δλ 16 253.60nm, the absorbance value of RIF and INH is zero, while the absorbance value for PRZ and Mixture solution of INH, PRZ and RIF have the same absorption value of 0.0013, so for PRZ, the wavelength analysis is at 253.60 nm.

Based on the results of the second derivative absorpbance spectrum at Δλ 2, the RIF wavelengths analysisused was 299.80 nm as at this wavelength, the absorbance value for PRZ and INH is zero, while for RIF and mixture solution of INH, PRZ and RIF have the same absorption value, were 0.0002. The INH wavelength analysis used was 302.40 nm because at this wavelength, the absorbance value for PRZ and RIF is zero, while for INH and Mixture solution of INH, PRZ and RIF have the same absorption value, were 0.0001. The of the mixture can be done without prior separation, because the wavelength of the zero crossing of each compound are not the same [11].

When the ternary mixture has a zero crossing wavelength more than one, then the chosen wavelength analysis is the zero crossing wavelength that the absorbance partner and the mixture is exactly the same, because this wavelength can selectively measure the abosrbance of it's compound partner and has the biggest absorption. In the biggest absorption, the absorbance is more stable, so the error of analysis can be minimized [11].

Detection Limit and Quantitation Limits

The detection limit and quantitation limit were calculated from the regression equation obtained from the calibration curve. The limit of detection of INH I s1.4080 µg/mL, RIF is 0,1424 µg/mL and PRZ is 1.2560 ug/mL and limit of quantitation of INH, PRZ and RIF is 4.6934 µg/mL 1,4280 µg/mL and 4.1867 µg/mL. It shows that the determination of PRZ with a concentration of 12 mg/mL, RIF with concentration of 9 mg/mL and INH with a concentration of 7.5 mg/mL can be detected and measure by second derivative spectrophotometric with zero crossing method.

The limit of detection is the lowest concentration of the analyte in the sample that can still be detected. The limit of quantitation was defined as the lowest analyte concentration in a sample that can still meet the careful and thoroµgh criteria [10].

Assay of INH, RIF PRZ and Tablet Preparation

Assay of INH, RIF PRZ in each tablet contains 75 mg INH, 400 mg PRZ and 150 mg INH. The assay of INH, PRZ and RIF at each dosage is 7.5 mg/mL INH,12 mg/mL PRZ and 9 mg/mL RIF.The achievement of the comparative test can be done by an addition of the standard solution 4.51 µg/mL for RIF from standard solution of RIF and 5.25 µg/mL for INH from standard solution of INH, so that it can be measured by derivative spectrophotometric method. The absorbance spectrum results is transformed into a second derivative absorbance spectrum with $\Delta\lambda$ 2 and $\Delta\lambda$ 16 nm. Based on the determined absorbance spectrum of INH, RIF PRZ at the wavelength analysis that has been obtained previously, That is wavelengths 253.60 nm, 299.80 nm and 302.40 nm. The assay of INH, RIF PRZ in the Tablet with the trade name of 3-FDC after analyzed statistically in table 3.

Table 3. Contents of INH and RIF PRZ in Tablets 3-FDC

No.	Samples	Amount (mg)	Requirements (mg)
1	INH	75.96-77.745	67.5 – 82.5
2	PRZ	394.92 to 416.44	360 - 440
3	RIF	144.81 to 154.29	135 - 165

It can be seen in Table 3, the contents of INH, RIF PRZ in the tablet Rimcure ® meets the requirements of the United States Pharmacopoeia XXX[12].

Validation test

Accuracy Test Results

Accuracy test with the parameter of the recovery percentage is performed using 3-FDC tablets bystandard addition method in a range in the sample. In this case, three specific ranges used was 80%, 100% and 120%, which is composed of 70% and 30% of raw samples [9,10].

Table 4 Results of recovery of INH, RIF PRZ by standard addition method on a 3-FDC tablet.

Specific range %	Reacquisition PRZ (%)	Reacquisition RIF (%)	Reacquisition INH (%)
80	101.99	99.13	98.87
	101.57	101.41	100.26
	101.15	100.58	97.99
100	99.42	100.59	101.42
	99.76	100.92	101.76
	99.08	100.25	101.09
120	98.25	100.99	99.47
	98.53	101.26	99.75
	98.82	101.53	10 0:03
Mean (% *recovery*)	99.84	100.74	10 0:07
Standard Deviation (*SD*)	1.39	.7347	1.22
Relative Standard Deviation (*RSD*) (%)	1.39	0.7	1.22

Based of Table 3 shows that the average percentage recovery obtained for PRZ is 99.84%, for RIF is 100.74%,for INH is 100.07%. The obtained accuracy value show that this method qualifies validation requirements (accuracy value requirements is 98% -102%) [9,10].

Precision Test Value

Precision test is a parameter that indicates a closer analysis which done in several repetitions. Precision test is done by calculation of the relative standard deviation. Based on calculation data on the assay of INH, PRZ and RIF obtained relative standard deviation of 1.39% for PRZ, 0.7% for RIF and 1.22% for INH. The results relative standard deviation for INH, PRZ and RIF meet the requirements, namely ≤2% [10].

CONCLUSION

1. Derivative spectrophotometric method with zero crossing can be used to establish the levels of ternary mixtures of INH, PRZ and RIF.
2. The assay of ternary mixtures of INH, RIF, PRZ and the 3-FDC tablets meet the requirements of USP XXX (2007).
3. Validation test performed on 3-FDC tablet that the second derivative spectrophotometric method validation meets the requirements.
4.

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