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Research article

# Simultaneous content analysis of Rifampicin, Isoniazid and Pyrazinamide in tablet dosage form by spectrophotometry ultraviolet with area under curve method

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**Key words:** AUC, Rifampicin, Isoniazid, Pyrazinamide, Validation.

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**Abstract**

The goal of this research was to use spectrophotometry specifically area under curve (AUC) method for simultaneous determination of rifampicin (RFP), isoniazid (INH) and pyrazinamide (PRZ) on tablets. This method can be used to determine the level of a single compound or mixture of two substances with no separation of first and derivative spectrum. This method uses two wavelength regions. Area under the curve where spectra of both the substance of spectrum overlap is the selected wavelength region to determine the levels of both substances. The AUC method begins by calculating the level of each spectrum with various concentrations at each the range absorptionspectrum. Absorption spectrum range (248-258) nm for RFP, (260.2-270.2) nm for INH and ranges (271.8 – 281.8) nm for PRZ with methanol as solvent. Absorption spectrum range (248.8-258.8) nm for RFP, (261.2-271.2) nm for INH and ranges (272.8 – 282.8) nm for PRZ with methanol and methanol phosphate buffer pH 6 as solvent. The spectrophotometry UV with AUC method can be used for simultaneous determination of RFP, INH and PRZ mixture. The validation test results can be described as test results that have good linearity, accuracy and precision.

## Introduction

The standard therapy of tuberculosis in general use anti tuberculosis (AT) such as isoniazid, rifampicin, ethambutol, streptomycin, and pyrazinamide which is the primary drug group [1]. One combination of AT are effective and often used in tuberculosis therapy is a combination of three drugs such as isoniazid, rifampicin, and pyrazinamide [2].

The combination of these drugs provide the advantage of a good therapy, because improving adherence to treatment and reduce the risk of resistance, treatment costs and errors in treatment. However, the combination of these drugs provide a new challenge for the pharmaceutical industry with respect to the development of new methods of analysis in the determination of the levels of drug [3]. Some researchers have done a simultaneous determination of RFP, INH and PRZ mixture by HPLC method, a derivative spectrophotometric method and simultaneous equation. The UV spectrophotometric AUC method was developed for RFP, INH and PRZ which was accurate, precise and selective [4-6].

Rifampicin [RFP] (Figure 1 a) is chemically known as 5, 6, 9, 17, 19, 21-hexahydroxy-23-methoxy-2, 4, 12, 16, 18, 20,22-heptamethyl-8-[N-(4-methyl-1-piperazinyl) formimidoyl]-2, 7-(epoxypentadeca[1,11,13] trienimino)-naphtho[2,1-b]furan-1,11(2H)-dione 21-acetate. It is a bactericidal on the growth phase and m. leprae, both outside as well as inside cells, the deadly germs that also dormant phase.

Isoniazid [INH] (Figure 1 b) is chemically known as Isonicotinic acid hydrazide acid. It is bactericidal to rapidly dividing mycobacteria, but is bacteriostatic if the mycobacteria are slow growing.

Pyrazinamide [PRZ] (Figure 1 c) is chemically known as Pyrazine-2-carboxamide. It is bactericidal or bacteriostatic, depending on pH and important antimycobacterial drug used in the contemporary short course therapy of tuberculosis [7].

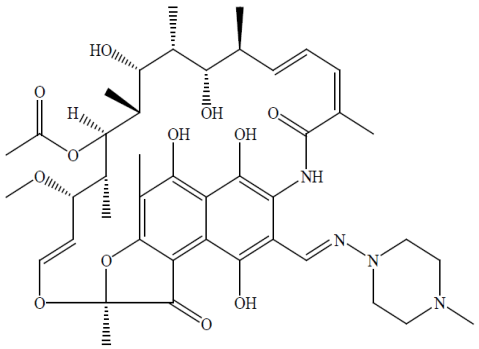


**Figure 1a. Chemical Structure of Rifampicin [RFP].**

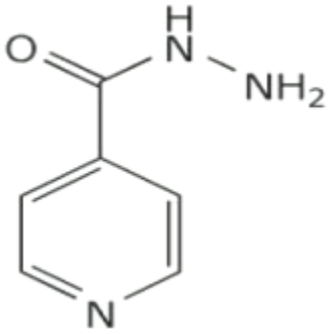

**Figure 1b. Chemical Structure of Isoniazid [INH].**

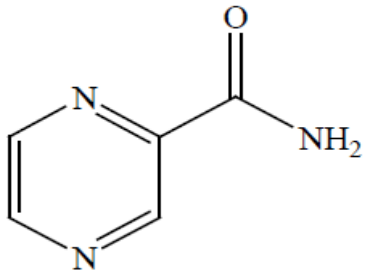

**Figure 1c. Chemical Structure of Pyrazinamide [PRZ].**

Recently, RFP has been marketed in combination with INH and PRZ in tablet dosage form [Pro TB 3 Kid®tablets] is intended for oral administration for the treatment of tuberculosis. Rifampicin, isoniazid and pyrazinamide in literature survey reveal that few methods are available for the simultaneous analysis of RFP, INH and PRZ combination. Many techniques for determination of RFP, INH and PRZ have been reported in pharmaceutical formulations and biological samples. Therefore, the aim of this work was directed to the development of simple, sensitive, selective and validated spectrophotometric method for the simultaneous determination of RFP, INH and PRZ in their combined dosage form.

## Material and methods

### Apparatus

UV visible spectrophotometer Shimadzu 1800 was used to measure spectra. The solvent used was methanol and methanol phosphate buffer pH 6 for the assay and connected to personal computer loaded with UV-Probe 2.34 software.

### Pure drugs

Rifampicin [99.0%], Isoniazid [99.0%], Pyrazinamide [99.96%], all were gifted from Kimia Farma Plant Bandung, Sumatera Utara, Indonesia.

### Reagents and materials

The reagents used during analysis include Methanol [E-Merck], Double distilled water [PT. Ika Pharmindo], Sodium hydroxide [E-Merck] 0.1 N, Potasium dihydrogen phosphate [E-Merck] 0.1, Rifampicin (Batch No. RFP/R-186/10, Mfg. Date: 27 October 2018, Exp. Date: 27 October 2022), (Batch No. INH/I-187/09, Mfg. Date: 12 April 2018, Exp. Date: 13 April 2022), (Batch No. PYZ/P-188/11, Mfg. Date: August 2017, Exp. Date: July 2022) were used and obtained as a gift samples from Kimia Farma Plant Bandung, Sumatera Utara, Indonesia.

### Preparation of stock solutions

50mg weighed quantities of PRZ, INH and RFP powder was transferred to 50ml volumetric flask separately. methanol absolute and methanol dapar phosphate pH 6 absolute were transfer to volumetric flask for preparation of primary stock solution. Pipette out aliquots from primary stock in 25 ml volumetric flasks to get secondary stock of 100 µg/mL PRZ, 49.98µg/mL INH and 33.32µg/mL RFP. Pipette out 0.9 ml of secondary stock solution in separate 10 ml volumetric flasks and make the volume with methanol absolute and methanol dapar phosphate pH 6 absolute to get concentrations 9 µg/mL PRZ, 4.40 µg/mL INH and 2.99 µg/mL RFP.

### Preparation maximum wavelength spectrum

Scan final stock solutions of RFP, INH and PRZ in UV spectrophotometer between 200-400nm, using diluents as a blank for maximum absorption wavelength.

### The validation test for area under curve method

As per ICH guideline the method is validated and following parameters were evaluated

### Accuracy

Accuracy tests were done using the standard addition methods, which made three concentrations of analyte samples with a specific range of 80%, 100%, and 120% [8].

The formula for percent recovery:

% recovery = (amount of substance recovered ÷ amount of substance originally taken) × 100

### Precision

The determination of precision is based on the relative standard deviation (RSD) value 2%.
RSD is formulated as follows:

$$RSD = \frac{SD}{X} x\ 100\%$$

Where
SD = sample standard deviation
X = sample mean

### Linearity

The linearity states are obtained tests in accordance of concentrations analyte. The coefficient of relations is

used to determine the linearity of an analytical method [9].

**Limit of detection (LOD) and Limit of quantitation (LOQ)**

The LOD and LOQ calculations are performed by using below formula.

$$LOD = \frac{3.3 \text{ x SD}}{\text{slope}}$$

$$LOQ = \frac{10 \text{ x SD}}{\text{slope}}$$

**Area under curve method**

**Preparation of absorption spectrum of RFP, INH and PRZ**

Pipette out 12.0 µg/mL RFP, 10.0 µg/mL INH and 9 µg/mL PRZ each and measured their absorption spectrum and then used a UV probe 2.42 software to calculate the area values in the 248-258 nm for RFP, 260.2 – 270.2 nm for INH and 271.8-281.8 nm areas for PRZ with methanol as solvent. The area value in the 248.8 – 258.8 nm for RFP, 261.2 – 271.2 nm for INH and 272.8 – 282.8 nm area for PRZ with methanol dapar phosphate pH 6 as solvent [10-13].

**Preparation of calibration curve for area under curve method**

The pipetted aliquots of (6, 9, 12, 15, 18) µg/mL for RFP, (5, 7.5, 10, 12.5, 15) µg/mL for INH and (6, 7.5, 9, 10.5, 12) µg/mL for PRZ are used to get the regression equation, respectively.

**Assays procedure for tablet by area under curve method**

Twenty tablets were weighed and finely powder. A weighed of the tablet powder containing 50 mg PRZ and transferred to a 50 ml volumetric, dissolved with methanol and methanol dapar phosphate pH 6. Then pipette out 0.9 mL sample solution and add 0.75 mL stock solution (7.501 µg/mL) of RFP for standard addition and add 0.70 mL stock solution (7.001 µg/mL) of INH for standard addition [9]. Then measured absorption spectrum was created in the area of 248-258 nm for RFP, 260.2 – 270.2 nm for INH and 271.8-281.8 nm areas for PRZ with methanol as solvent. The area value in the 248.8 – 258.8 nm for RFP, 261.2 – 271.2 nm for INH and 272.8 – 282.8 nm area for PRZ with methanol dapar phosphate pH 6 as solvent at the AUC method [14-16].

**Results and discussion**

**Absorption spectrum of RFP, INH and PRZ**

Preparation of absorption spectrum from RFP, INH and PRZ with UV spectrophotometry using methanol and methanol dapar phosphate pH 6 solvent obtained UV spectrum showed absorption of wavelengths for 239.6 nm for RFP, 261.4 nm for INH and 268.6 nm for PRZ with methanol as solvent and 237.6 nm for RFP, 259.4 nm for INH and 268.8 nm for PRZ with methanol dapar phosphate pH 6. Absorption spectrum curves can be seen in Figures 1-8.

The figure 1-6 showed the maximum absorption of RFP, INH and PRZ and it is 239.6 nm, 261.4 and 268.6 nm with methanol as solvent and 237.6 nm, 259.6 nm, 268.8 nm with methanol dapar phosphate pH 6 as solvent.

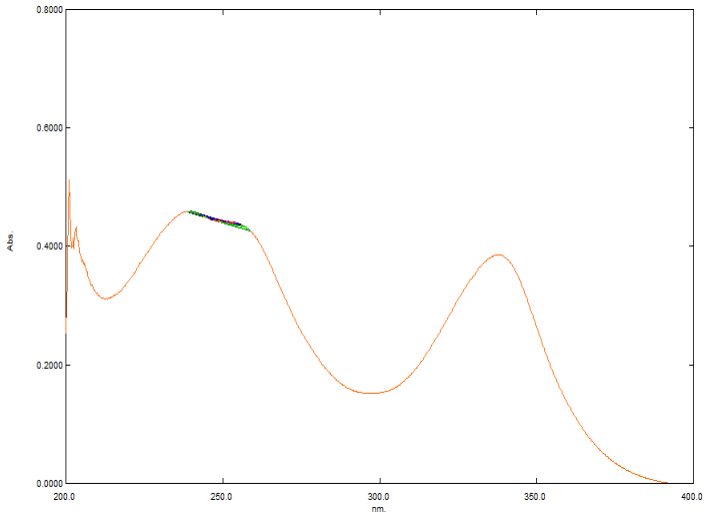


**Figure 1. RFP absorption spectrum with methanol as solvent.**

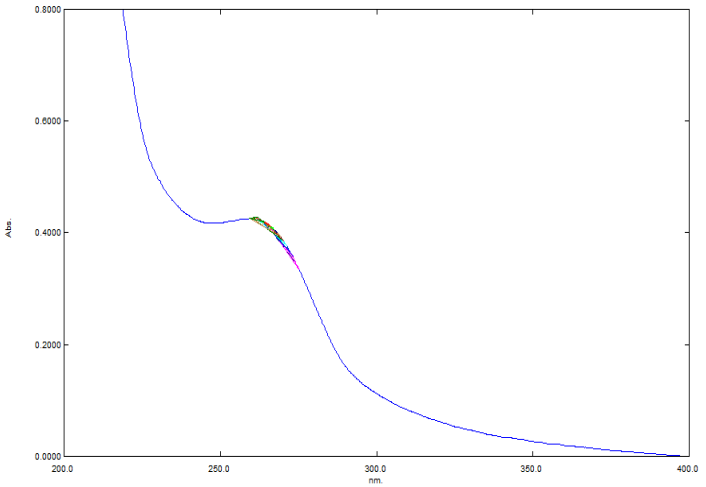


**Figure 2. INH absorption spectrum with methanol as solvent.**

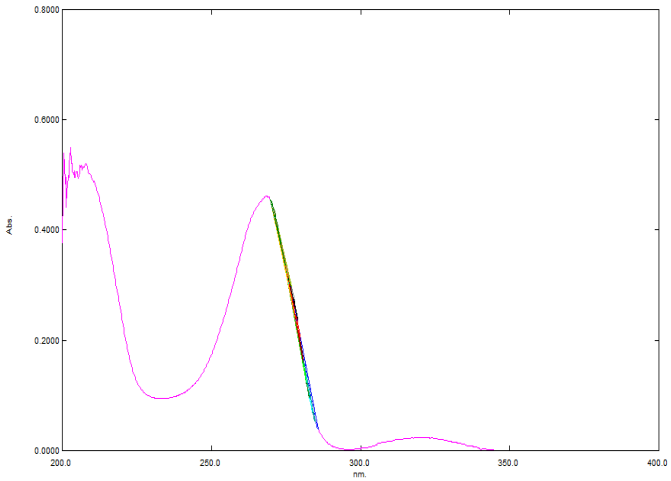


**Figure 3. PRZ absorption spectrum with methanol as solvent.**

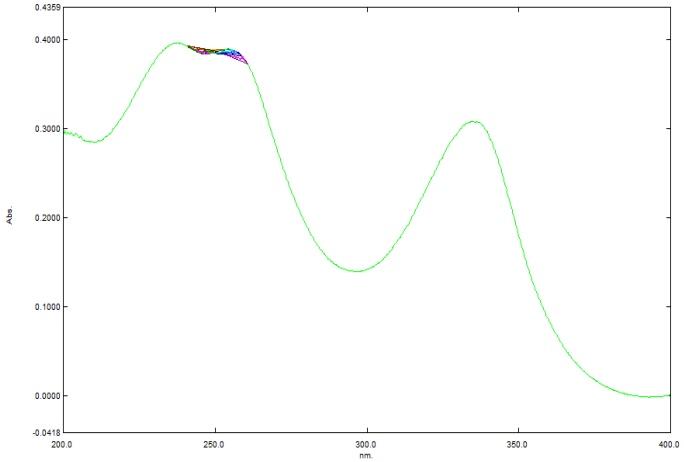


**Figure 4. RFP absorption spectrum with methanol dapar phosphate pH 6 as solvent.**

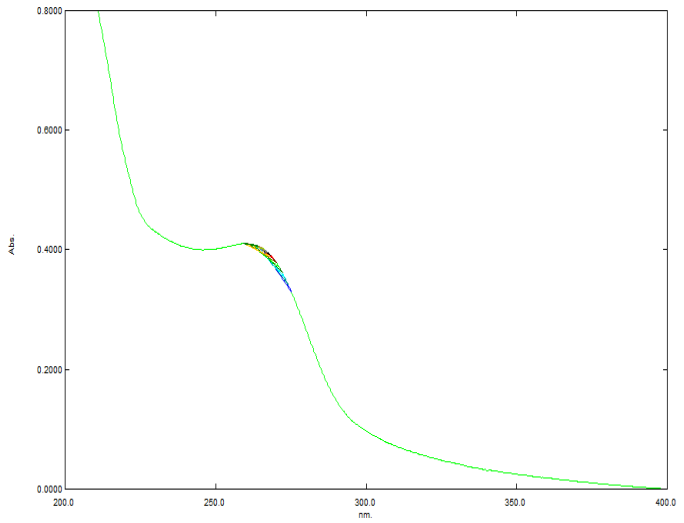


**Figure 5. INH absorption spectrum with methanol dapar phosphate pH 6 as solvent.**

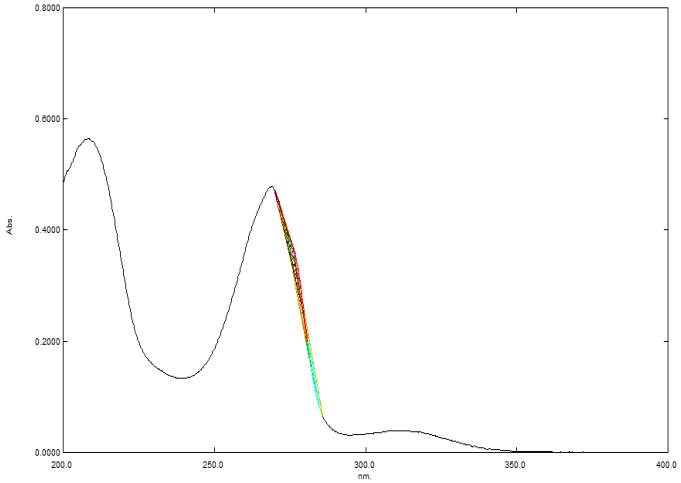


**Figure 6. PRZ absorption spectrum with methanol dapar phosphate pH 6 as solvent.**

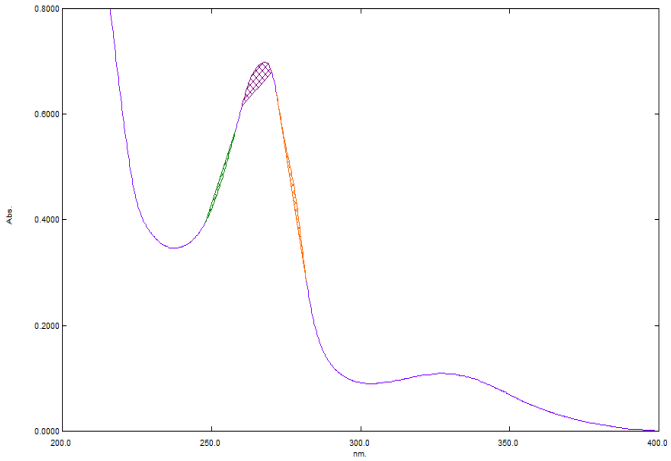


**Figure 7. RFP, INH, PRZ absorption mixture spectrum with methanol as solvent.**

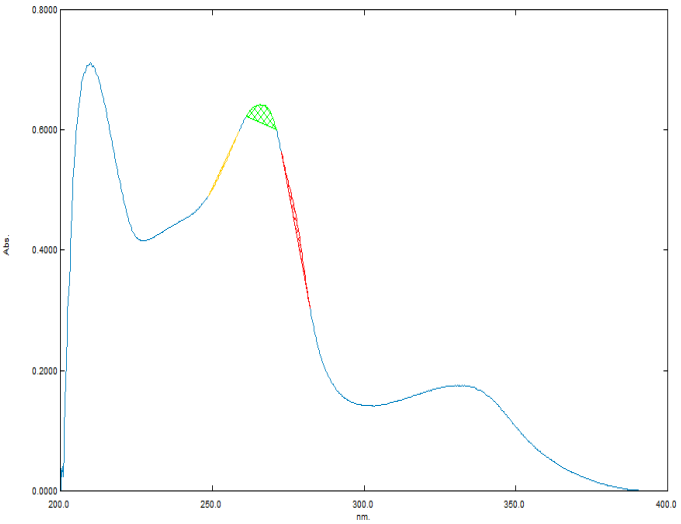


**Figure 8. RFP, INH, PRZ absorption mixture spectrum with methanol dapar phosphate pH 6 as solvent.**

The AUC method begins by calculating the level of each spectrum with various concentrations at each range of absorption spectrum. Based on figure 7 and figure 8 it can be seen the absorption spectrum range (248-258) nm for RFP, (260.2-270.2) nm for INH and ranges (271.8 – 281.8) nm for PRZ with methanol as solvent. Absorption spectrum range (248.8-258.8) nm for RFP, (261.2-271.2) nm for INH and ranges (272.8 – 282.8) nm for PRZ with methanol dapar phosphate pH 6 as solvent. According to this result, the spectrophotometry UV with AUC method can be used for simultaneous determination of RFP, INH and PRZ mixture.

**Table 1. Wavelength Analysis of RFP, INH and PRZ with methanol as solvent.**

| Component | Wavelength | Δλ | Linearity |
|---|---|---|---|
| **Rifampicin** | 245-250 | 5 | 0.9611 |
| | 246-251 | 5 | 0.9841 |
| | **248-258** | **10** | **0.9969** |
| | 249-259 | 10 | 0.9953 |
| **Isoniazid** | 264.2-269.2 | 5 | 0.9865 |
| | 265.2-270.2 | 5 | 0.9885 |
| | 259.2-269.2 | 10 | 0.9923 |
| | **260.2-270.2** | **10** | **0.9979** |
| **Pyrazinamide** | 274.8-279.8 | 5 | 0.9953 |
| | 275.8-280.8 | 5 | 0.9941 |
| | 270.8-280.8 | 10 | 0.9947 |
| | **271.8-281.8** | **10** | **0.9961** |

**Table 2. Wavelength Analysis of RFP, INH and PRZ with methanol buffer phosphate pH 6 as solvent.**

| Component | Wavelength | Δλ | Linearity |
|---|---|---|---|
| **Rifampicin** | 243.8-248.8 | 5 | 0.9839 |
| | 244.8-249.8 | 5 | 0.9879 |
| | 247.8-257.8 | 10 | 0.9927 |
| | **248.8-258.8** | **10** | **0.9978** |
| **Isoniazid** | 262.2-267.2 | 5 | 0.9984 |
| | 263.2-268.2 | 5 | 0.9910 |
| | 260.2-270.2 | 10 | 0.9971 |
| | **261.2-271.2** | **10** | **0.9988** |
| **Pyrazinamide** | 272.8-277.8 | 5 | 0.9943 |
| | 273.8-278.8 | 5 | 0.9989 |
| | 271.8-281.8 | 10 | 0.9986 |
| | **272.8-282.8** | **10** | **0.9991** |

## Determination of RFP, INH and PRZ by the AUC method

The absorption spectrum of a mixture of the RFP, INH and PRZ spectra has obtained the value of AUC in the area (248-258) nm for RFP, (260.2-270.2) nm for INH and (271.8-281.8) nm for PRZ with methanol as solvent. AUC in the area (248.8-258.8) nm for RFP, (261.2-271.2) nm for INH and (272.8-282.8) nm for PRZ with methanol buffer phosphate pH 6 as solvent then the concentration is calculated using a regression equation to obtain the level of the RFP, INH and PRZ.

**Table 3. Levels of Rifampin, INH and Pirazinamid in the preparation of tablets of AUC method.**

| Drug | Methanol (mg) | Methanol buffer phosphate pH 6 (mg) | Claims in label (mg) | Drug Content Found (mg) |
|---|---|---|---|---|
| Rfiampicin | 75.08±1.10 | 74.54±0.54 | 75 | 67.5-82.5 |
| Isoniazid | 50.15±0.20 | 50.40±1.47 | 50 | 45-55 |
| Pyrazinamide | 156.28±1.09 | 154.53±0.81 | 150 | 135-165 |

Calculation of the levels of rifampin, isoniazid and pyrazinamide statistically with the distribution table t with a 99% confidence level with a value of $\alpha = 0.01$, n = 6; DK = 5, the distribution table of the retrieved value t = tables 4.0321. The data is rejected if t counts ≥ t table or t count ≤ t –table as shown in Table 4.

$$t\ count = \left[\frac{(x - \bar{X})}{SD/\sqrt{n}}\right]$$

## Validation Test

The production of valid test results must be achieved for chemical analysis activities. The validation test results can be described as test results that have good linearity, accuracy and precision. Validation parameters for this method are shown in Table 5.

**Table 4. Calculation of the levels rifampicin, isoniazid and pyrazinamide statistically with methanol and methanol buffer phosphate as solvent of AUC method.**

| Drug | Methanol (%) | Methanol buffer phosphate pH 6 (%) |
|---|---|---|
| Rifiampicin | 100.11±1.10% | 99.86±0.87% |
| Isoniazid | 100.30±0.20% | 100.80±1.47% |
| Pyrazinamide | 104.19±1.09% | 103.02±0.81% |

**Table 5. Validation of area under curve method.**

| Parameter | Rifampicin | | Isoniazid | | Pyrazinamide | |
|---|---|---|---|---|---|---|
| Methods | Methanol | Methanol buffer phosphate pH 6 | Methanol | Methanol buffer phosphate pH 6 | Methanol | Methanol buffer phosphate pH 6 |
| Linearity | 0.9969 | 0.9978 | 0.9974 | 0.9988 | 0.9961 | 0.9991 |
| LOD | 1.9492 | 1.7144 | 1.3002 | 1.0788 | 1.4006 | 0.6195 |
| LOQ | 5.9066 | 5.1951 | 3.9402 | 3.2693 | 4.2445 | 1.8772 |
| Accuracy | 100.46% | 100.81% | 100.83%. | 100.71% | 100.42% | 100.72% |
| Precision | 0.66% | 0.90% | 0.51% | 0.79%. | 0.57% | 0.93% |

Based on table 5 it can be seen the research has a good validation method for simultaneous determination of the RFP, INH and PRZ because all parameters of validation test have according to the validation requirements on ICH guideline, this means these methods have met the validation requirements.

## Linearity

The value of the linearity value of the correlation coefficient is described well in area under curve method is almost entirely a number approaching one that indicates that there is a relationship or an excellent correlation between concentration with absorbance values. It also indicates that increasing concentration then any absorption value will increase.

## Accuracy

Accuracy is the parameter that is done by the method of adding raw on a certain range of the sample, and then both of them measured, calculated added raw returns it receives back, or test are often referred to with the retrieval test. In this case the three specific ranges in use are 80%, 100% and 120% in which the composition is composed of 70% and 30% raw samples. Accuracy values obtained showed that this method qualifies validation methods (value terms accuracy is 98%-102%).

## Precision

Precision is a parameter that indicates the results of nearly analysis are done within a few repetitions. Precision suggests that these methods deliver the results to each other though tested in some of the replication. Precision parameter values to be reflected in the resulting, from results obtained area under curve method qualified validation (3.9%).

AUC can be used for the determination of the levels of a binary mixture, can eliminate the step of derivatives and the creation of spectrum ratio as well as the calculation of the value of the mean centered so that this method is more efficient, faster, and simpler analysis of the work medicinal preparations

## Limit of detection (LOD) and limit of quantification (LOQ)

The limit of Detection (LOD) is defined as the lowest concentration of analytes in a sample can still be detected. LOD is most commonly used in chemical analysis. LOD is the levels of analytes gives the response of response blank solution (yb) plus 3 raw blank solution (3Sb). While the LOQ is defined as the lowest concentration of analytes in a sample can be determined with precision and accuracy that is acceptable on the operational condition of the methods used [17].

Area Under Curve (AUC) can be used to determine the level of binary mixture, can eliminate derivative steps so that this method is more efficient, faster, and simpler in carrying out analysis of drug preparations. However, this method also has the disadvantage that it is difficult to obtain an analysis, wavelength for each substance to be analyzed and it is not clear that the spectrum of the two substances in the mixture to be analyzed is completely separated, because the determination of concentration only uses the AUC value and equation regression [18-19].

## Conclusion

Method of ultraviolet spectrophotometry in Area Under Curve (AUC) method can be used to set the levels of rifampin, Isoniazid and pyrazinamide in tablet dosage form simultaneously and qualified validation methods

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