



## Research Article

## Determination of Rifampicin, Isoniazid, Pyrazinamide and Ethambutol Hydrochloride in 4FDC Tablet by FTIR Spectrophotometry in Combination with Multivariate Calibration

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## ABSTRACT

This study was aimed to develop a fast, inexpensive, simple, and not involving hazardous reagent for the determination of rifampicin (RIF), isoniazid (INH), pyrazinamide (PZA) dan ethambutol hydrochloride (ETH) in 4FDC tablet based on infrared spectrophotometry in combination with chemometrics of partial least square (PLS) calibration. The analytical procedure involves three steps, namely calibration, validation and sample assay. The multivariate calibration model is evaluated by determining the precision and accuracy parameters, as suggested by IUPAC. The results showed that the optimized wavenumber range for the determination of RIF was the combined wavenumbers of 433 – 1552  $\text{cm}^{-1}$  and 1756 – 3412  $\text{cm}^{-1}$ , the wavenumber of 433 – 873  $\text{cm}^{-1}$  was selected for quantification of INH, 1714 – 2756  $\text{cm}^{-1}$  for PZA, and 1552 – 2970  $\text{cm}^{-1}$  for ETH. The predicted residual error sum of square (PRESS) values of RIF, INH, PZA and ETH contents were 0.00392%, 0.011724%, 0.007604%, and 0.003368%, respectively. The root mean square error of prediction (RMSEP) values obtained for analysis of RIF, INH, PZA and ETH were 0.01979%, 0.03424%, 0.027575% and 0.018351%, respectively. The values of coefficient of determination ( $R^2$ ) for the relationship between actual values of these drugs and calculated values of these drugs using FTIR spectroscopy were 0.990, 0.977, 0.992 and 0.998 for RIF, INH, PZA and ETH, respectively. The developed method was successfully applied for the determination of 4FDC tablet. The contents of RIF, INH, PZA and ETB in 4FDC tablets were in agreement with those specified in monograph of Indonesian Pharmacopeia.

**Keywords:** infrared spectrophotometry, rifampicin, isoniazid, pyrazinamide, ethambutol hydrochloride, partial least square.

### 1. Introduction

World Health Organization (WHO) reported that the incidence rate of tuberculosis (TB) in Indonesia was 185 per 100.000 population with the morbidity rate was 27 per 100.000 population every year [1]. Indonesia belong to the five countries with the largest number of tuberculosis incident cases in 2012, after India, China and South Africa [2]. In order to decrease the tuberculosis incidence, WHO and International Union against Tuberculosis and Lung Disease (IUATLD) have recommended the use of 4FDC (Fixed-Dose Combination) tablets for TB treatment. 4FDC contains

150 mg rifampicin (RIF), 75 mg isoniazid (INH), 400 mg pyrazinamide (PZA) and 275 mg ethambutol hydrochloride (ETH) [3]. The chemical structure of these drugs were presented in Figure 1.

Many techniques for determination of 4FDC have been reported in pharmaceutical formulations and biological samples. These methods are ultraviolet-visible spectrophotometric with multivariate calibration [4–7], polarography [8], High Performance Liquid Chromatography (HPLC) with UV detector [9, 10, 11], High Performance Thin Layer Chromatography (HPTLC) [12], HPLC with colorimetric detection in plasma and urin

[13] and by HPLC in plasma sample [14]. Infrared spectrophotometry in combination with multivariate calibration have been developed for the determination the contents of RIF, INH and PZA without ETH in tablet combination [15–17] and for determining dissolution testing of RIF, INH and PZA [18]. Currently, 4FDC monograph have used two HPLC systems with ultraviolet detector to quantitatively analyze the four constituents (RIF, INH, PZA and ETH), as listed in National Formulary USP30-NF25 [19] and The International Pharmacopoeia 4<sup>th</sup> edition [20]. However these methods are impractical, so that the development of analytical methods in one system is highly needed.

Most of the analytical methods used for analysis of 4-FDC is costly and time consuming due to the sample preparation. Additionally, the analytical method indicated that the sample should be analyzed immediately after the sample preparation, because of the high unstability of certain active agent (rifampicin) in the solution. Hence, this method faced unsatisfactory to be used as a routine method for quality control of product. Because of this reason, Fourier Transform Infrared (FTIR) has already developed to be an alternative quantitative method for determination of RIF, INH, PZA and ETH in 4FDC tablet. Currently, with the development of computer software and multivariate analysis, infrared spectrophotometry is widely used to simultaneously analyze the multicomponent drugs [21]. This study aimed to develop a fast, inexpensive, simple, and not involving hazardous reagent for the determination of RIF, INH, PZA and ETH using infrared spectrophotometry in combination with multivariate calibration of partial least square (PLS). Study of chemometrics-assisted infrared spectrophotometry based on PLS is conducted in three phases, namely calibration, validation and unknown sample assay [22].

## 2. Materials and Methods

### 2.1 Material and Samples

The reference standards of rifampicin (RIF), isoniazid (INH), pyrazinamide (PZA) and ethambutol hydrochloride (ETH) used for the calibration modelling were of reference standard of Indonesian Pharmacopoeia and obtained from the National Agency of Drug and Food Control, Republic of Indonesia. Potassium bromide with infrared spectroscopy grade was used for the preparation of sample powders. The different commercially available tablet samples containing RIF, INH, PZA and ETH as an active ingredient were obtained from National Agency of Drug and Food Control, district of Medan, Indonesia.

### 2.2 Instrumentation and Software

The measurements of FTIR spectra of calibration and validation samples as well as commercial samples were done using Shimadzu IR-Prestige 21 with DRS 8000 application. All measurements were recorded in the wavenumber range of 4700-400  $\text{cm}^{-1}$  averaging 45 scans at a resolution of 4  $\text{cm}^{-1}$ . The spectrum of each standard as well as sample was ratioed against a fresh background spectrum recorded from KBr powder.

### 2.3 Preparation of calibration and validation samples

Two set of standard mixtures containing of RIF, INH, PZA and ETH in the concentration range between 0.01 and 1.0 mg were selected randomly and prepared in KBr to formulate powders with the total weight of 100 mg. A set of 10 standard mixtures as calibration set samples and the other set of 10 standard mixtures as validation set of validation samples was prepared. The mixture composition of RIF, INH, PZA and ETH used in calibration and validation samples was listed in Table 1 and Table 2, respectively. Each mixture were recorded using FTIR spectrophotometer in the wavenumber range of 4700-400  $\text{cm}^{-1}$  averaging 45 scans, and at a resolution of 4  $\text{cm}^{-1}$ .

### 2.4 Sample preparation

The study used two different commercially drug of 4FDC. Twenty tablets of each kind of samples were taken, weighed, and finely powdered. An accurately 150 mg sample mixture (drugs and KBr) was prepared. Then the samples were scanned in wavenumber of 4700 – 400  $\text{cm}^{-1}$  averaging 45 scans at a resolution of 4  $\text{cm}^{-1}$ . All determinations were performed in three replicates for each kind of samples.

### 2.5 Statistical analysis

The spectral absorbance data were exported to MS excel to facilitate processing data using Minitab software version 16 (Minitab Corp., USA). Some parameter criteria namely coefficient of determination ( $R^2$ ), root mean square error of calibration (RMSEC), root mean square error of prediction (RMSEP), root mean square error of cross validation (RMSECV) and predicted residual sum of square (PRESS) were evaluated using Minitab version 16.

## 3. Result and Discussion

Figure 2 is FTIR spectra of the mixture of rifampicin (RIF), isoniazid (INH), pyrazinamide (PZA) and ethambutol hydrochloride (ETH) in the calibration set samples (A) and in the validation set samples (B). The first step for analysis of RIF, INH, PZA and ETH is the optimization of wavenumber range capable of providing the highest values of coefficient of determination ( $R^2$ ) and the lowest values of errors. With the aid of multivariate calibration of partial least square (PLS), all wavenumbers in FTIR spectra can be used for quantitative analysis, however, measurements from spectral wavelengths that are non-informative in the model can degrade the performance of calibration model [23]. Therefore, it is suggested to appropriately select the wavenumbers.

Based on these criteria, finally the combined wavenumber region of 433 – 1552  $\text{cm}^{-1}$  and 1756 – 3412  $\text{cm}^{-1}$  was used for quantitative analysis of RIF, while the wavenumber of 433 – 873  $\text{cm}^{-1}$  was used for analysis INH. The wavenumber of 1714 – 2756  $\text{cm}^{-1}$  is suitable for quantification of PZA contents, and ETH contents was preferred to be determined at wavenumber of 1552 – 2970  $\text{cm}^{-1}$ . Using these wavenumber regions, all 4FDC components have RMSEC values close to 0 and  $R^2$  close

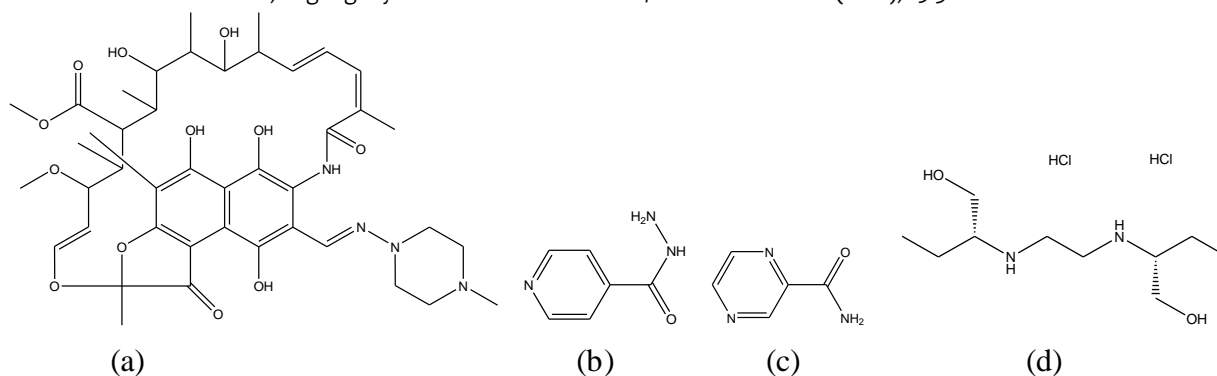


Figure 1. Chemical structure of rifampicin (a), isoniazid (b), pyrazinamide (c) and ethambutol hydrochloride (d).

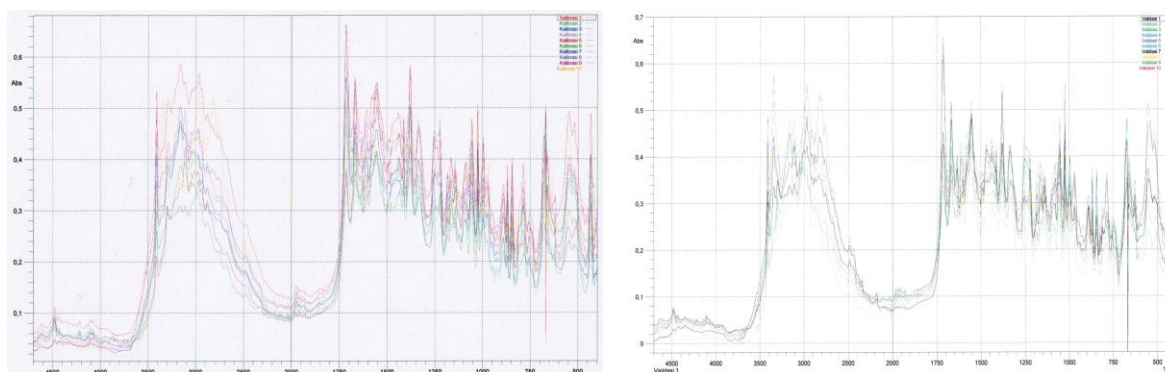


Figure 2. Group of Infrared spectra of rifampicin, isoniazid, pyrazinamide and ethambutol hydrochloride in calibration set samples (A) and validation set samples (B)

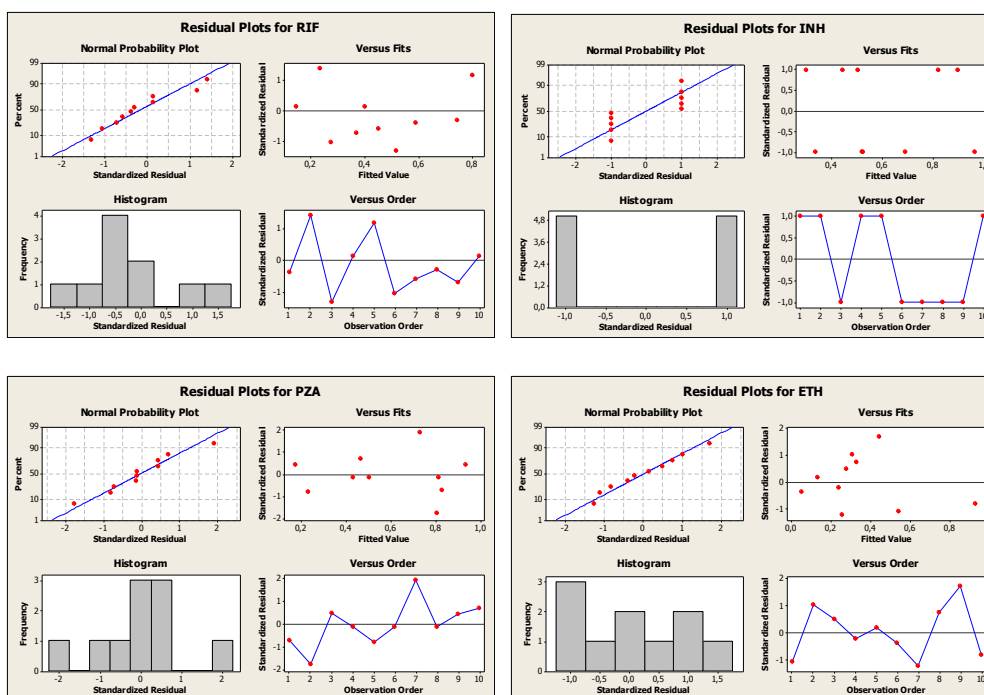


Figure 3. Residual plots of rifampicin (A), isoniazid (B), pyrazinamide (C), and ethambutol hydrochloride (D) from PLS leave one out cross validation.

to 1, for the relationship between actual values of RIF, INH, PZA, and ETH with FTIR-partial least square predicted values, except PZA having  $R^2$  value of 0.987. These results indicated that the calibration model generated using those wavenumber regions are satisfactory.

During the modelling using multivariate calibration, the over-fitting of the regression model can occur. Over-fitting can be understood that the model generates an optimistic model in the calibration data set, but the model would not perform well on other data sets with similar material [24].

Table 1. The composition of calibration set samples consisting of rifampicin, isoniazid, pyrazinamide and ethambutol hydrochloride.

No	Rifampicin (mg)	Isoniazid (mg)	Pyrazinamide (mg)	Ethambutol HCl (mg)
1	5.86	8.06	8.12	5.31
2	2.36	4.37	7.56	3.12
3	5.14	3.32	1.85	2.84
4	1.44	4.96	8.09	2.38
5	8.02	8.81	2.04	1.33
6	2.73	5.14	4.99	0.45
7	4.51	6.79	7.85	2.35
8	7.40	9.46	4.25	3.40
9	3.66	5.16	9.51	4.70
10	3.99	2.96	4.77	9.29

Table 2. The composition of validation set samples consisting of rifampicin, isoniazid, pyrazinamide and ethambutol hydrochloride.

No	Rifampicin (mg)	Isoniazid (mg)	Pyrazinamide (mg)	Ethambutol HCl (mg)
1	5.37	3.46	1.74	7.32
2	6.98	5.62	0.93	5.02
3	4.63	7.9	9.54	0.99
4	6.25	6.87	8.58	1.27
5	3.17	7.03	7.9	4.42
6	2.3	5.04	4.42	1.11
7	4.16	7.48	3.75	3.54
8	2.68	9.82	2.58	2.38
9	4.99	8.23	1.45	1.58
10	1.53	2.57	7.58	9.91

Table 3. The PRESS, RMSECV and R<sup>2</sup> values obtained during cross validation for analysis of rifampicin (RIF), isoniazid (INH), pyrazinamid (PZA) and ethambutol (ETH).

Drugs	R <sup>2</sup>	PRESS	RMSECV	Principle components
RIF	0.98555	0.005962%	0.02442%	7
INH	0.99984	0.000077%	0.00278%	8
PZA	0.971489	0.018716%	0.04326%	3
ETH	0.975001	0.013725%	0.03705%	5

Therefore, the calibration model should be validated. In this study, the validation of calibration model was performed using cross validation with leave one out technique and external validation using external samples which are different from the calibration samples.

Cross-validation using "leave-one out" technique means that the calibration samples is left out from PLS model, and the remaining samples are used to make PLS model. Furthermore, the removed sample is calculated using the new developed PLS model. This procedure was repeated; leaving each calibration sample out in turn. Then, the difference between the actual and predicted value for each sample is calculated. The sum of the squares of these differences is called the predicted residual error sum of squares (PRESS). Besides, root mean square errors of cross validation (RMSECV) and coefficient determination (R<sup>2</sup>) during cross validation for the relationship between actual and

FTIR calculated values for these four drugs were also computed. Table 3 compiled the PRESS, RMSECV and R<sup>2</sup> values obtained during cross cross validation for RIF, INH, PZA and ETH. The residual plots of rifampicin (A), isoniazid (B), pyrazinamid (C) and ethambutol HCl (D) resulted from PLS leave one out cross validation is shown in Figure 3.

The analytical results of external validation from validation set samples for RIF are PRESS value of 0.00392%, RMSEP value of 0.01979% and R<sup>2</sup> of 0.990; for INH, PRESS value of 0.011724%, RMSEP value of 0.03424% and R<sup>2</sup> value of 0.977; for PZA, PRESS value is 0.007604%, with RMSEP value of 0.027575% and R<sup>2</sup> of 0.992; and for ETH, the PRESS, RMSEP, and R<sup>2</sup> values are 0.003368%, 0.018351, and 0.998, respectively. The results assay of commercially tablet sample are RIF (101.26 ± 6.09%), INH (100.84 ± 4.19%), PZA (106.71 ± 1.42%) and ETA (102.99 ± 5.19%) for sample A, and for sample B, the contents obtained are 105.60 ± 2.97%

(RIF),  $102.12 \pm 2.61\%$  (INH),  $95.30 \pm 3.05\%$  (PZA) and  $101.44 \pm 1.76\%$  (ETH). Based on the results, it can be concluded that FTIR spectrophotometry combined with multivariate calibration of PLS can be used as an alternative method to assay RIF, INH, PZA and ETH in 4FDC tablet without separation step.

#### 4. Conclusion

FTIR spectroscopy in combination with partial least square at the optimized wavenumber regions has been used for the determination of rifampicin, isoniazid, pyrazinamide, and ethambutol hydrochloride in tablet combination 4FDC. The developed method has acceptable validation parameter and is successfully applied for content assay of commercial 4FDC tablet.

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