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Development and Validation of High Performance Liquid Chromatography Mass Spectrometry Method for Determination of Rifampicin, Isoniazid and Pyrazinamide from Tablet Preparation

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Abstract: High performance liquid chromatography is a separation system with high speed and efficiency. The research results showed that the optimum conditions for high performance liquid chromatography mass spectrometry method can be used to analyze a mixture of rifampicin, isoniazid and pyrazinamide. C_{18} column as stationary phase and mass spectrometry as a detector in selected ion monitoring mode. Optimum conditions obtained were type of mobile phase mixture 0.1% formic acid solution in water and 0.1% formic acid solution in methanol with flow rate of 0.5 mL/min, the ratio of mobile phase 90%:10% up to 3 minutes, and then converted into the ratio of mobile phase 30%:70% at 3.1 minutes and maintained until 10 minutes and the column oven temperature 35^{o}C. These optimum conditions have good separation results. It can be seen from the resolution and the selectivity.The optimum conditions of high performance liquid chromatography mass spectrometry method is used for the determination of mixture of rifampicin, isoniazid and pyrazinamide in tablet preparation and meet the requirements of the method validation test. The method validity testing included the accuracy test with recovery percentage (% recovery) parameter, the precision test with relative standard deviation parameter, the specificity test, the limit of detection test, the limit of quantitation test and the linearity test.

Keywords: Rifampicin, Isoniazid, Pyrazinamide, High Performance Liquid Chromatography Mass Spectrometry, Method Development.

Introduction

Tuberculosis is still a major health problem in the world. Various parties try to work together to fight it[1]. The drugs used in the treatment of tuberculosis can be divided into two categories: primary anti-tuberculosis and secondary anti-tuberculosis. The primary anti-tuberculosis have higher efficacy and better safety than secondary anti-tuberculosis. Primary anti-tuberculosis drugs are rifampicin, isoniazid, pyrazinamide and ethambutol[2]. Rifampicin, isoniazid and pyrazinamide is a combination of anti-tuberculosis drugs. Tablet dosage form in combination of rifampicin, isoniazid and pyrazinamide can be seen in the market; it is also contained in a single component dosage form[3]. Which according to the Law of the Republic of Indonesia number 36 year 2009 on health of article 105 paragraph 1 that the form of pharmaceutical drugs dosage form and pharmaceutical raw materials must be eligible Pharmacopoeia Indonesia or other standard books. In

addition, monitoring of drug therapy (therapeutics drug monitoring (TDM)) needs to be done to find out that the drug levels in the body is in the therapeutic range[4]. Methods of analysis for rifampisin, isoniazid and pyrazinamide by high performance liquid chromatography tandem mass spectrometry have been reported, but isoniazid and pyrazinamide still overlap when combined in a single chromatogram[5].

Therefore, the purpose of this study is to develop methods for the analysis of a mixture of rifampicin, isoniazid and pyrazinamide by high performance liquid chromatography mass spectrometry. This research will be carried out the optimization of high performance liquid chromatography mass spectrometry method which is expected to be used for the analysis of a mixture of rifampicin, isoniazid and pyrazinamide. Optimizations performed on a type of mobile phase mixture, the composition of mobile phase, mobile phase flow rate and the column oven temperature. Expected high-performance liquid chromatographic mass spectrometry method developed can be used as an alternative method to the hospital to assay mixture of rifampicin, isoniazid and pyrazinamide from human plasma simultaneously and as an alternative method for the Food and Drug Supervisory Agency (BPOM) and the drug industry to assay preparations containing a mixture of rifampicin and/or isoniazid and/or pyrazinamide simultaneously.

Materials And Methods

Equipments

The equipment used in the study include: double distilled water (Aquatron), water purifier (Elga), set of high performance liquid chromatography mass spectrometry (Agilent) (Table 1.), vacuum pump (Boeco and Gast), holder and membrane filters (Whatman), analytical balance (Boeco), infrared spectrophotometer (Shimadzu) and other glassware (Oberoi and Iwaki).

Table 1. Set of high performance liquid chromatography mass spectrometry (Agilent).

Part	Type
Degasser	G1379B
Bin Pump	G1312A
Automatic Load Sample	G1329A
Thermostatted Column Compartment	G1316A
C_{18}Columns (30 mm × 4,6 mm) 5 µm	XDB–C_{18}
Quadrupole Liquid Chromatography Mass Spectrometry	G6120A

Reagents and Standards

Reagents and Standards used in this study include: methanol high performance liquid chromatography grade (E-Merck), formic acid (E-Merck), acetic acid (E-Merck), rifampicin standard (PT. Indofarma), isoniazid standard (PT. Indofarma), and pyrazinamide standard (PT. Indofarma).

Preparation of Reagents

0.1% formic acid solution in double distilled water that has been purified.

0.5 mL of formic acid (E-Merck) put into 1000 mL measuring glass, then add double distilled water that has been purified up to 500 mL. Subsequently the mixture was stirred and filtered with a cellulose nitrate membrane filter 0.2 µm. The result is 0.1% formic acid solution in double distilled water that has been purified.

0.1% formic acid solution in methanol

0.5 mL of formic acid (E-Merck) put into 1000 mL measuring glass, then add methanol up to 500 mL. Subsequently the mixture was stirred and filtered with a membrane filter politetraflouroethylene (PTFE) 0.5 µm. The result is 0.1% formic acid solution in methanol.

1% acetic acid solution in double distilled water that has been purified.

5 mL of acetic acid (E-Merck) put into 1000 mL measuring glass, then add double distilled water that has been purified up to 500 mL. Subsequently the mixture was stirred and filtered with a cellulose nitrate membrane filter 0.2 µm. The result is 1% acetic acid solution in double distilled water that has been purified.

1% acetic acid solution in methanol

5 mL of acetic acid (E-Merck) put into 1000 mL measuring glass, then add methanol up to 500 mL. Subsequently the mixture was stirred and filtered with a membrane filter politetraflouroethylene (PTFE) 0.5 µm. The result is 1% acetic acid solution in methanol.

Preparation of rifampicin solution

Carefully weighed 500 mg rifampicin standard (PT. Indofarma), put into a 50 mL volumetric flask, added 25 mL of methanol, sonicated for 5 minutes until dissolved, diluted with double distilled water that has been purified to the mark line and shaken to obtain a solution with a concentration of rifampicin standards (PT. Indofarma) 10000 µg/mL.

Preparation of isoniazid solution

Carefully weighed 500 mg isoniazid standard (PT. Indofarma), put into a 50 mL volumetric flask, added 25 mL of methanol, sonicated for 5 minutes until dissolved, diluted with double distilled water that has been purified to the mark line and shaken to obtain a solution with a concentration of isoniazid standards (PT. Indofarma) 10000 µg/mL.

Preparation of pyrazinamide solution

Carefully weighed 500 mg pyrazinamide standard (PT. Indofarma), put into a 50 mL volumetric flask, added 25 mL of methanol, sonicated for 5 minutes until dissolved, diluted with double distilled water that has been purified to the mark line and shaken to obtain a solution with a concentration of pyrazinamide standards (PT. Indofarma) 10000 µg/mL.

Preparation of a mixture of rifampicin, isoniazid and pyrazinamide

Rifampicin solution, isoniazid solution, and pyrazinamide solution each pipetted 0.8 mL, 0.2 mL and 2 mL, mixed into 10 mL volumetric flask, then diluted with a mixture of methanol and water 50%:50% to the mark line and shaken. Subsequently the mixture was pipetted 5 mL, and then put into 10 mL volumetric flask, diluted with a mixture of methanol and water 50%:50% to the mark line and shaken. The resulting mixture pipetted 0.2 mL and put into 5 mL volumetric flask, then diluted with a mixture of methanol and water 50%:50% to the mark line and shaken to produce a solution with a concentration of rifampicin standard 16 µg/mL, the concentration isoniazid standard 4 µg/mL and the concentration of pyrazinamide standard 40 µg/mL. The mixture solution was filtered through a polytetraflouroethylene membrane filter (PTFE) 0.2 µm. Then, the filtrate solution is filled into the sample vial, sonicated for 15 min and 2.5 µL injected into a high performance liquid chromatographic system via an automatic injector. Detection using mass spectrometric detector in the positive ionization type detection with selected ion monitoring mode (selected ion monitoring (SIM)). Ion mass spectrometry was monitored in positive ion that has a mass 823.4 for rifampicin standard, 138.1 for isoniazid standard, 124.0 for pyrazinamide standard and recorded the chromatogram.

Preparation of sample

20 tablets were weighed and crushed until homogeneous. Weighed powder equivalent to 75 mg of isoniazid (150 mg rifampicin, pyrazinamide 400 mg), powder was added to a 100 mL volumetric flask, dissolved and diluted with a solvent to mark the line and shaken. Solution was filtered (the first few ml of the filtrate discarded). Subsequently 1 mL of the filtrate was taken and put into a 100 mL volumetric flask, diluted with a solvent to mark lines and shaken. The mixture solution was filtered through a polytetraflouroethylene membrane filter (PTFE) 0.2 µm.Then, the filtrate solution is filled into the sample vial, sonicated for 15 min and 2.5 µL injected into a high performance liquid chromatographic system via an automatic injector. Detection using mass spectrometric detector in the positive ionization type detection with selected ion monitoring mode (selected ion monitoring (SIM)). Ion mass spectrometry was monitored in positive ion that has a mass 823.4 for rifampicin standard, 138.1 for isoniazid standard, 124.0 for pyrazinamide standard and recorded the chromatogram.

Analysis Procedure

Identification of rifampicin standard, isoniazid standard and pyrazinamide standard by fourier transform infrared spectrophotometer (fourier transform infrared (FTIR))

Identification test carried out using fourier transform infrared spectrophotometer (fourier transform infrared (FTIR)), by: weighing rifampicin standard, isoniazid standard and pyrazinamide standard each 2 mg and 200 mg potassium bromide. Then each standard and potassium bromide is put into the mortar, crushed until homogeneous. Furthermore each of the substances analyzed with fourier transform infrared spectrophotometer (fourier transform infrared (FTIR)) with wavenumber range 4000-500 cm^{-1} and recorded the infrared spectrum. Infrared spectra obtained were compared with the standard infrared spectra from the literature.

Qualitative Analysis of Rifampicin, Isoniazid and Pyrazinamide in Tablet Preparations

Qualitative analysis of a tablet containing a mixture of rifampicin, isoniazid and pyrazinamide done by analyzing the mass of positive ions of each peak is detected. Qualitative analysis was also carried out by comparing the retention time of the tabletpreparation containing a mixture of rifampicin, isoniazid and pyrazinamide with rifampicin raw retention time (PT. Indofarma), raw isoniazid (PT. Indofarma) and raw pyrazinamide (PT. Indofarma).

Preparation of high performance liquid chromatography mass spectrometry instrument

Nitrogen generator tool is turned on until ready to generate nitrogen gas used for mass spectrometry detectors. Then the tools of high performance liquid chromatography mass spectrometry is enabled, by: mass spectrometry detector is turned on, set a positive detection, left for a while until the conditions of mass spectrometry into a vacuum condition, run the pump on the type of mobile phase mixture, ratio of mobile phase, mobile phase flow rate and the column oven temperature is set at a predetermined condition, then the mobile phase is passed to obtain a stable pressure, which indicates a high performance liquid chromatography mass spectrometry system has been stabilized and ready for analysis.

Determination of optimum conditions for high performance liquid chromatography mass spectrometry instrument

High performance liquid chromatography conditions varied to obtain optimum results for the analysis. Chromatographic conditions were varied:

- **Type of mobile phase mixture**.
  - Double distilled water (which has been purified, filtered through a cellulose nitrate membrane filter 0.2 μm and sonicated for 30 min) and methanol (which is filtered through a polytetrafluoroethylene membrane filter (PTFE) 0.5 μm and sonicated for 30 minutes).
  - 0.1% formic acid solution in double distilled water that has been purified (after being mixed and then filtered through a cellulose nitrate membrane filter 0.2 μm and sonicated for 30 minutes) and 0.1% formic acid solution in methanol (after being mixed then filtered through a polytetrafluoroethylene membrane filter (PTFE) 0.5 μm and sonicated for 30 minutes).
  - 1% acetic acid solution in double distilled water that has been purified (after being mixed and then filtered through a cellulose nitrate membrane filter 0.2 μm and sonicated for 30 minutes) and 1% acetic acid solution in methanol (after being mixed then filtered through a polytetrafluoroethylene membrane filter (PTFE) 0.5 μm and sonicated for 30 minutes).

- **Composition of mobile phase.**

  Each type of mobile phase mixture performed the optimization at various ratio of mobile phase: 10%:90%, 30%:70%, 50%:50%, 70%:30%, 90%:10%.

- **Mobile phase flow rate**.

  The type of mobile phase mixture and composition of mobile phase that provides the best chromatograms (at a mobile phase flow rate of 0.5 mL/min) performed the optimization on mobile phase flow rate: 0.3 mL/min, 0.5 mL/min and 0.7 mL/min.

- **Column Oven Temperature.**

  The type of mobile phase mixture, the composition of mobile phase and mobile phase flow rate that provides the best chromatogram (at a column oven temperature of 35°C) to be optimized in the column oven temperature: 30°C, 35°C and 40°C.

Validation Procedure

Accuracy

Accuracy performed by standard addition method. The method of standard additions can be done by measuring the recovery percentage (% recovery) at 3 specific range, such as: 80%, 100% and 120%. Where in each specific ranges used 70% of the sample (analyte) were analyzed and 30% raw to be added. Then mix the sample (analyte) and analyzed by the same procedure as the samples. Recovery percentage (% recovery) can be calculated using the following formula:

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

Description:
C_F = concentration of the samples obtained from the measurement
C_A = real sample concentration
C_A^* = concentration of analyte is added.

Precision

Precision expressed as standard deviation (SD) or relative standard deviation (RSD) of a set of data. Relative standard deviation (RSD) can be calculated using the following formula:

$$RSD = \frac{SD}{\overline{X}} \times 100\%$$

Description:
RSD =relative standard deviation
SD = standard deviation
$\overline{X}$ = data average.

Linearity

Rifampicin solution, isoniazid solution, and pyrazinamide solution each pipetted 7.5 mL, 3.75 mL and 20 mL, mixed into 100 mL volumetric flask, then diluted with a mixture of methanol and water 50%:50% to the mark line and shaken. Subsequently the mixture was pipetted 5 mL, and then put into 10 mL volumetric flask, diluted with a mixture of methanol and water 50%:50% to the mark line and shaken. The resulting mixture pipetted 0 mL, 0.0125 mL, 0.05 mL, 0.1 mL, 0.2 mL, 0.4 mL, 0.8 mL and 3.2 mL and put into 5 mL volumetric flask, then diluted with a mixture of methanol and water 50%:50% to the mark line and shaken to produce a solution with a concentration of rifampicin standard 0 µg/mL, 0.9375 µg/mL, 3.75 µg/mL, 7.5 µg/mL, 15 µg/mL, 30 µg/mL, 60 µg/mL and 240 µg/mL, the concentration isoniazid standard 0 µg/mL, 0.46875 µg/mL, 1.875 µg/mL, 3.75 µg/mL, 7.5 µg/mL, 15 µg/mL, 30 µg/mL and 120 µg/mL and the concentration of pyrazinamide standard 0 µg/mL, 2.5 µg/mL, 10 µg/mL, 20 µg/mL, 40 µg/mL, 80 µg/mL, 160 µg/mL and 640 µg/mL. The mixture solution was filtered through a polytetraflouroethylene membrane filter (PTFE) 0.2 µm. Then, the filtrate solution is filled into the sample vial, sonicated for 15 min and 2.5 µL injected into a high performance liquid chromatographic system via an automatic injector. Detection using mass spectrometric detector in the positive ionization type detection with selected ion monitoring mode (selected ion monitoring (SIM)). Ion mass spectrometry was monitored in positive ion that has a mass 823.4 for rifampicin standard, 138.1 for isoniazid standard, 124.0 for pyrazinamide standard and recorded the chromatogram.

Specifity

The method of high performance liquid chromatography mass spectrometry has had acceptable accuracy, the method of high performance liquid chromatography mass spectrometry can also be automatically entered as a method specific criteria.

Limit of Detection and Limit of Quantitation

Limit of detection (LOD) and limit of quantitation (LOQ) can be calculated using the following formula:

$$LOD = \frac{3{,}3 \times S_y}{b}$$

$$LOQ = \frac{10 \times S_y}{b}$$

Description:
LOD = limit of detection
LOQ = limit of quantitation
S_y = residual standard deviation
b = slope of the calibration curve.
S_y value can be calculated by the formula:

$$S_y = \sqrt{\frac{\sum(Y_i - Y_i^*)^2}{(n - 2)}}$$

Description:
Y_i = response at X_i concentrations
Y_i^* = response calculated from the regressionat X_i concentration.
n = number of treatments.

Results And Discussions

Identification of rifampicin standard, isoniazid standard and pyrazinamide standard by fourier transform infrared spectrophotometer (fourier transform infrared (FTIR))

Identification test for rifampicin standard, isoniazid standard and pyrazinamide standard performed using an infrared spectrophotometer (fourier transform infrared) in the wavenumber range 4000-500 cm^{-1}. Infrared spectra obtained were compared with the standard infrared spectra from the literature[6]. Infrared spectrum for rifampicin standard, isoniazid standard and pyrazinamide standard can be seen in Figure 1., Figure 2. and Figure 3. below.

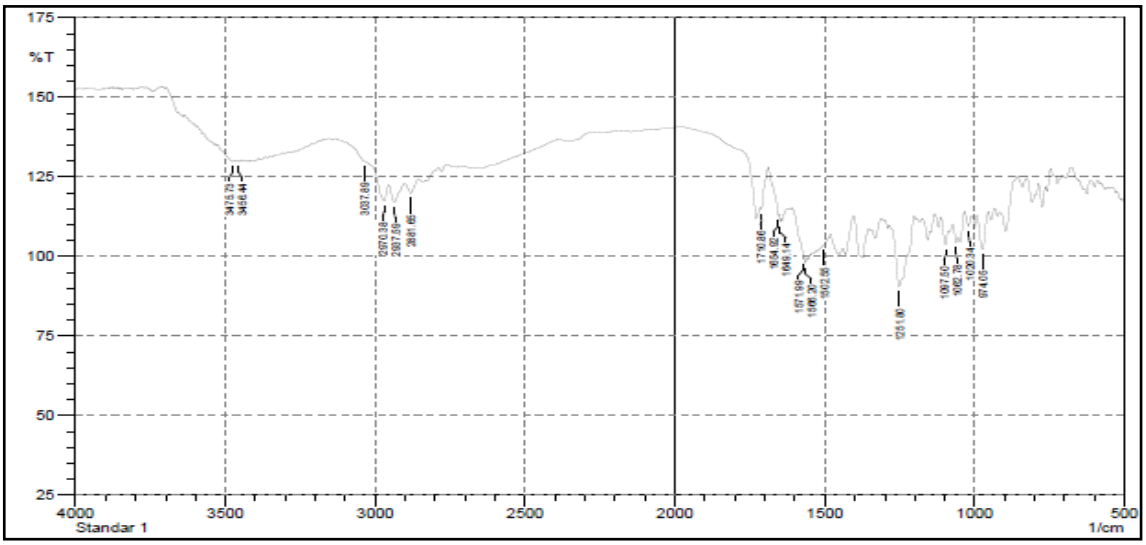


Figure1. Infrared spectrum for rifampicin standard.

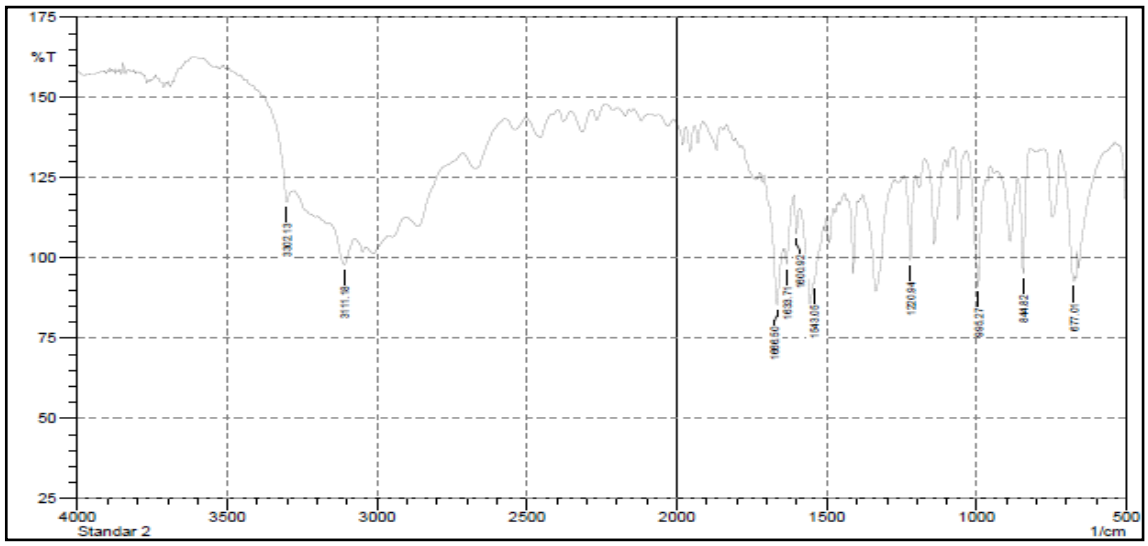


Figure2. Infrared spectrum for isoniazid standard.

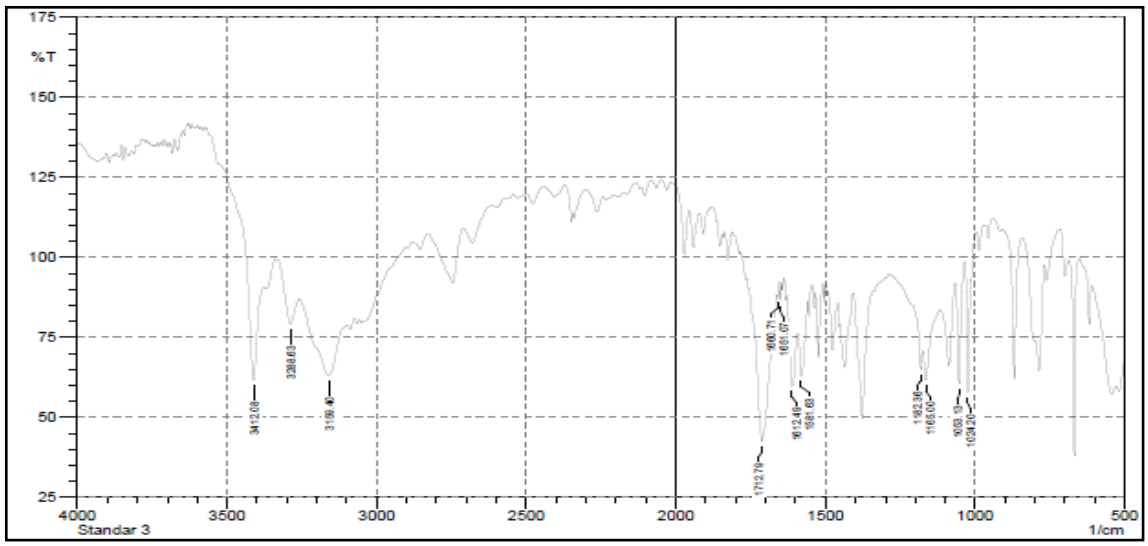


Figure3. Infrared spectrum for pyrazinamide standard.

Fingerprint identification, functional groups and bonds between atoms of the rifampicin standard, isoniazid standard and pyrazinamide standard compared to the literature[7] [8] [9] can be seen in Table 1., Table 2. and Table 3.

Table 2. The results of the fingerprint, the functional groups and bonds between atoms of rifampicin standard.

Fingerprint, functional groups and bonds between atoms	**Wavenumber of rifampicin standard**
Fingerprint	974,05; 1062,78 and 1566,20 cm^{-1}
C−N	1020,34 and 1251,8 cm^{-1}
C−O	1097,50 cm^{-1}
Benzen	1502,55 and 1649,11 cm^{-1}
C═C	1571,99 cm^{-1}
C═N	1654,92 cm^{-1}
C═O	1710,86 cm^{-1}
C−H Alifatic	2881,65; 2937,59 and 2970,38 cm^{-1}
C−H Aromatic	3037,89 cm^{-1}
O−H	3456,44 cm^{-1}
N−H	3475,73 cm^{-1}

Table 3. The results of the fingerprint, the functional groups and bonds between atoms of isoniazid standard.

Fingerprint, functional groups and bonds between atoms	Wavenumber of isoniazid standard
Fingerprint	677,01; 844,82 and 1543,05 cm^{-1}
C−N	995,27 and 1220,94 cm^{-1}
C═C	1600,92 cm^{-1}
C═N	1633,71 cm^{-1}
C−H Aromatic	1666,50 cm^{-1}
N−H	3111,18 cm^{-1}

Table 4. The results of the fingerprint, the functional groups and bonds between atoms of pyrazinamide standard.

Fingerprint, functional groups and bonds between atoms	Wavenumber of pyrazinamide standard
Fingerprint	1024,20; 1165,00; 1581,63 and 1651,07 cm^{-1}
C−N	1053,13 and 1182,36 cm^{-1}
C═C	1612,49 cm^{-1}
C═N	1660,71 cm^{-1}
C═O	1712,79 cm^{-1}
C−H Aromatic	3159,40 cm^{-1}
N−H	3288,63 and 3412,08 cm^{-1}

Based on fingerprint identification, functional groups and bonds between atoms, wavenumber obtained for rifampicin standard, isoniazid standard and pyrazinamide standard similar to the wavenumber found in the literature[7] [8] [9]. Infrared spectrum of the data obtained can be concluded that the identified standard is rifampicin, isoniazid and pyrazinamide.

Determination of optimum conditions for high performance liquid chromatography mass spectrometry instrument

High performance liquid chromatography conditions varied to obtain optimum results of the analysis. Chromatographic conditions were varied:

- **The type of mobile phase mixture and composition of mobile phase.**

  - Optimization of conditions for high performance liquid chromatography using a type of mobile phase mixture double distilled water (which has been purified, filtered through a cellulose nitrate membrane filter 0.2 μm and sonicated for 30 min) and methanol (which is filtered through a polytetrafluoroethylene membrane filter (PTFE) 0.5 μm and sonicated for 30 minutes) with a ratio of mobile phase 10%:90%, 30%:70%, 50%:50%, 70%:30%, 90%:10% and the mobile phase flow rate 0.5 mL/min, that rifampicin can only be eluted with a ratio of mobile phase 10%:90% and 30%:70%. While for isoniazid and pyrazinamide can be eluted in the entire mobile phase composition. However, isoniazid and pyrazinamide can not separate well on the whole mobile phase composition, so it is not good used for analysis.
  - Optimization of conditions for high performance liquid chromatography using a type of mobile phase mixture 0.1% formic acid solution in double distilled water that has been purified (after being mixed and then filtered through a cellulose nitrate membrane filter 0.2 μm and sonicated for 30 minutes) and 0.1% formic acid solution in methanol (after being mixed and then filtered through a polytetrafluoroethylene membrane filter (PTFE) 0.5 μm and sonicated for 30 minutes) with a ratio of mobile phase 10%:90%, 30%:70%, 50%:50%, 70%:30%, 90%:10% and mobile phase flow rate 0.5 mL/min, that rifampicin also can only be eluted with a ratio of mobile phase 10%:90% and 30%:70%. While for isoniazid and pyrazinamide also be eluted in the entire mobile phase composition. However, isoniazid and pyrazinamide can only be separated (as indicated by the resolution of greater than 2) in the ratio of mobile phase 90%:10%. In the composition of mobile phase 90%:10% rifampicin can not be eluted, so the need for gradient elution system was applied to analyze rifampicin, isoniazid and

pyrazinamide simultaneously. Where initially the system is run on a composition of the mobile phase 90%:10% until isoniazid and pyrazinamide eluted (and separated) and converted into the ratio of mobile phase 30%:70% and maintained until rifampicin eluted.

- Optimization of conditions for high performance liquid chromatography using a type of mobile phase mixture 1% acetic acid solution in double distilled water that has been purified (after being mixed and then filtered through a cellulose nitrate membrane filter 0.2 µm and sonicated for 30 minutes) and 1% acetic acid solution in methanol (after being mixed and then filtered through a polytetrafluoroethylene membrane filter (PTFE) 0.5 µm and sonicated for 30 minutes) with a ratio of mobile phase 10%:90%, 30%:70%, 50%:50%, 70%:30%, 90%:10% and mobile phase flow rate 0.5 mL/min, that rifampicin also can only be eluted with a ratio of mobile phase 10%:90% and 30%:70%. While for isoniazid and pyrazinamide also be eluted in the entire mobile phase composition. However, isoniazid and pyrazinamide can only be separated (as indicated by the resolution of greater than 2) in the ratio of mobile phase 90%:10%, but isoniazid has the shape of chromatograms that are not sharp (low theoretical plates), so it is not good used for analysis.

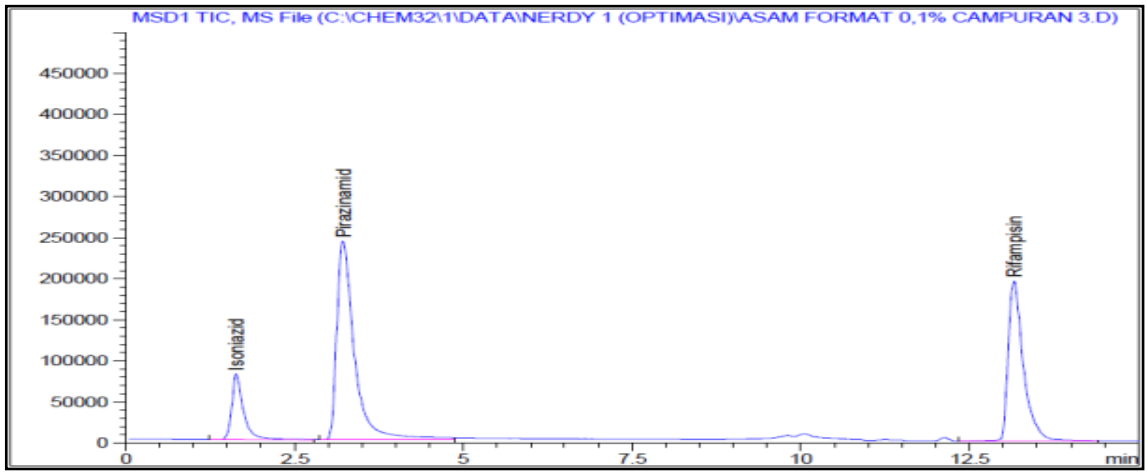
Based on the data obtained, type of mobile phase mixture of the selected mobile phase was 0.1% formic acid solution in double distilled water that has been purified (after being mixed and then filtered through a cellulose nitrate membrane filter 0.2 µm and sonicated for 30 minutes) and 0.1% formic acid solution in methanol (after being mixed and then filtered through a polytetrafluoroethylene membrane filter (PTFE) 0.5 µm and sonicated for 30 minutes). While the ratio of mobile phase used was a gradient elution system, where initially the system is run on a mobile phase composition 90%:10% until isoniazid and pyrazinamide eluted (and separated) and converted into a ratio of mobile phase 30%:70% and maintained until rifampicin eluted.

- **Mobile phase flow rate**.

Mobile phase flow rate 0.3 mL/min, the ratio of mobile phase 90%:10% known that the 4.5 minutes isoniazid and pyrazinamide was eluted (and separated) so it is converted into a ratio of mobile phase 30%:70% in minutes 4.6 and retained until rifampicin eluted (up to minute 15). Changes in mobile phase composition on the mobile phase flow rate 0.3 mL/min can be seen in Table 5. below. Mobile phase flow rate 0.3 mL/min give the resolution and selectivity for isoniazid and pyrazinamide were 4.65 and 1.97; resolution and selectivity for pyrazinamide and rifampicin is 24.97 and 4.09. Theoretical plates for rifampicin is 19252; theoretical plates for isoniazid is 610; theoretical plates for pyrazinamide is 961. Tailing factor is 1.722 for rifampicin; tailing factor is 1.582 for isoniazid; tailing factor is 1.906 for pyrazinamide. Chromatograms on mobile phase flow rate 0.3 mL/min can be seen in Figure 4. below.

Table 5. Changes in mobile phase composition on the mobile phase flow rate 0.3 mL/min.

Time (minute)	Mobile Phase A	Mobile Phase B	Elution
0	90	10	Balance
0-4,5	90	10	Isocratic
4,5-4,6	90→30	10→70	Linear Gradien
4,6-15	30	70	Isocratic



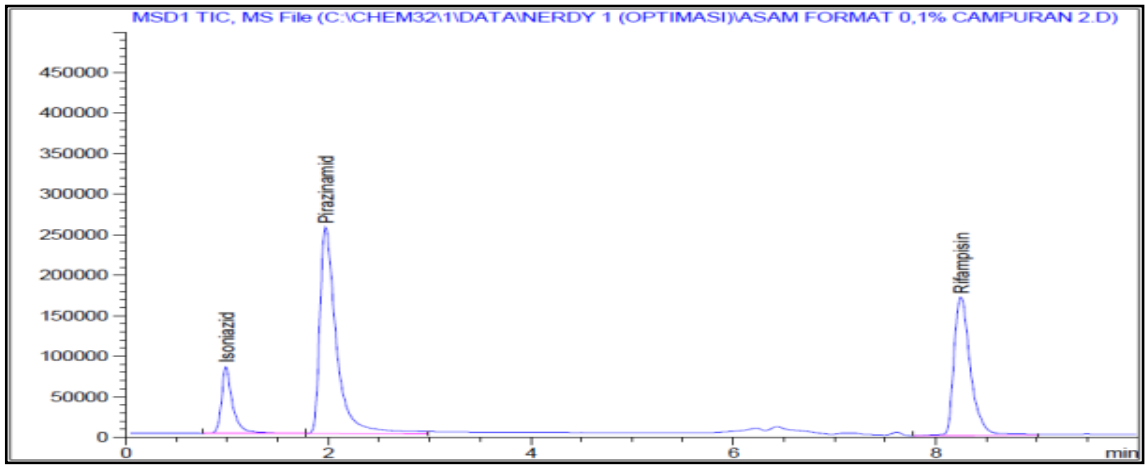
RetTime [min]	k'	Area	Height	Symm.	Width [min]	Plates	Resolution	Selectivity
1.637	-	9.72826e5	8.03033e4	0.53	0.1559	610	-	-
3.220	-	4.45617e6	2.42013e5	0.47	0.2445	961	4.65	1.97
13.160	-	3.07650e6	1.94949e5	0.58	0.2232	19252	24.97	4.09

Figure4. Chromatograms on mobile phase flow rate 0.3 mL/min.

Mobile phase flow rate 0.5 mL/min, the ratio of mobile phase 90%:10% known that the 3 minutes isoniazid and pyrazinamide was eluted (and separated) so it is converted into a ratio of mobile phase 30%:70% in minutes 3.1 and retained until rifampicin eluted (up to minute 10). Changes in mobile phase composition on the mobile phase flow rate 0.5 mL/min can be seen in Table 6. below. Mobile phase flow rate 0.5 mL/min give the resolution and selectivity for isoniazid and pyrazinamide were 4.52 and 1.99; resolution and selectivity for pyrazinamide and rifampicin is 23,11 and 4.17. Theoretical plates for rifampicin is 13883; theoretical plates for isoniazid is 522; theoretical plates for pyrazinamide is 910. Tailing factor is 1.474 for rifampicin; tailing factor is 1.520 for isoniazid; tailing factor is 1.886 for pyrazinamide. Chromatograms on mobile phase flow rate 0.5 mL/min can be seen in Figure 5. below.

Table 6. Changes in mobile phase composition on the mobile phase flow rate 0.5 mL/min.

Time (minute)	Mobile Phase A	Mobile Phase B	Elution
0	90	10	setimbang
0-3	90	10	isokratik
3-3,1	90→30	10→70	gradien linear
3,1-10	30	70	isokratik



RetTime [min]	k'	Area	Height	Symm.	Width [min]	Plates	Resolution	Selectivity
0.991	-	6.42532e5	8.27363e4	0.57	0.1021	522	-	-
1.976	-	2.93427e6	2.55005e5	0.47	0.1541	910	4.52	1.99
8.248	-	1.94154e6	1.70729e5	0.66	0.1648	13883	23.11	4.17

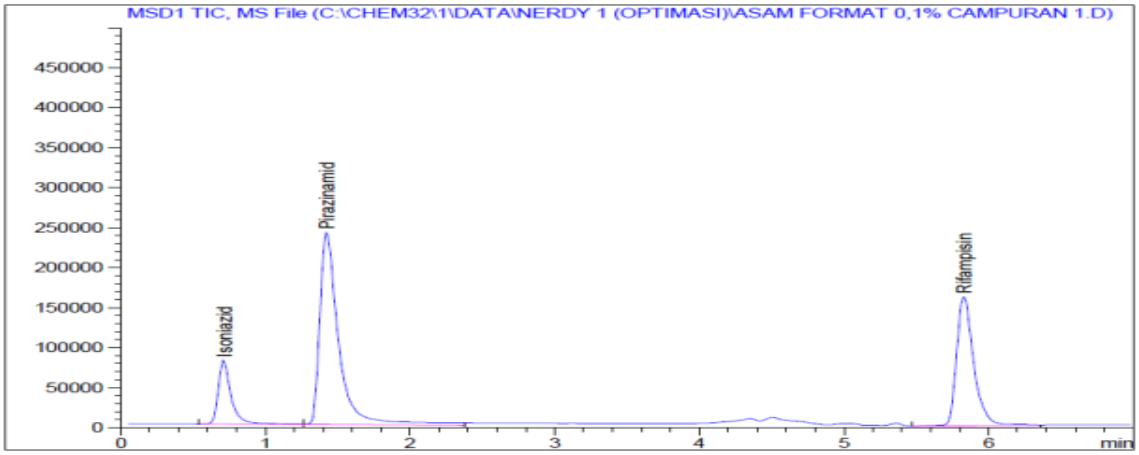
Figure 5. Chromatograms on mobile phase flow rate 0.5 mL/min.

Mobile phase flow rate 0.7 mL/min, the ratio of mobile phase 90%:10% known that the 2 minutes isoniazid and pyrazinamide was eluted (and separated) so it is converted into a ratio of mobile phase 30%:70% in minutes 2.1 and retained until rifampicin eluted (up to minute 7). Changes in mobile phase composition on the mobile phase flow rate 0.7 mL/min can be seen in Table 7. below. Mobile phase flow rate 0.7 mL/min give the resolution and selectivity for isoniazid and pyrazinamide were 4.04 and 2.00; resolution and selectivity for pyrazinamide and rifampicin is 21.45 and 4.09. Theoretical plates for rifampicin is 13278; theoretical plates for isoniazid is 388; theoretical plates for pyrazinamide is 751. Tailing factor is 1.406 for rifampicin; tailing factor

is 1.478 for isoniazid; tailing factor is 1.778 for pyrazinamide. Chromatograms on mobile phase flow rate 0.7 mL/min can be seen in Figure 6. below.

Table 7. Changes in mobile phase composition on the mobile phase flow rate 0.7 mL/min.

Time (minute)	Mobile Phase A	Mobile Phase B	Elution
0	90	10	Setimbang
0-2	90	10	Isokratik
2-2,1	90→30	10→70	gradien linear
2,1-7	30	70	Isokratik



RetTime [min]	k'	Area	Height	Symm.	Width [min]	Plates	Resolution	Selectivity
0.712	-	4.96279e5	7.98543e4	0.60	0.0850	388	-	-
1.424	-	2.18011e6	2.39724e5	0.49	0.1223	751	4.04	2.00
5.829	-	1.34286e6	1.61522e5	0.67	0.1191	13278	21.45	4.09

Figure 6. Chromatograms on mobile phase flow rate 0.7 mL/min.

Resolution of the three conditions (mobile phase flow rate 0.3 mL/min, 0.5 mL/min and 0.7 mL/min) greater than 1.5, which indicates that the separation is completely separate. But in the mobile phase flow rate 0.5 mL/min has a most optimum total resolution value; where the difference in total resolution at the mobile phase flow rate 0.5 mL/min to the total resolution at the mobile phase flow rate 0.3 mL/min lower than the difference in total resolution at the mobile phase flow rate 0.5 mL/min to the total resolution at the mobile phase flow rate 0.7 mL/min.

Selectivity of the three conditions (mobile phase flow rate 0.3 mL/min, 0.5 mL/min and 0.7 mL/min) greater than 1; which indicates that the chromatographic system has the ability to separate or distinguish different analytes. But in the mobile phase flow rate of 0.5 mL/min have a most optimum total selectivity value; where the total selectivity value on the mobile phase flow rate 0.5 mL/min greater than the total selectivity value on the mobile phase flow rate 0.3 mL/minutes and the total selectivity value on the mobile phase flow rate 0.7 mL/min.

Tailing factor of three conditions (mobile phase flow rate 0.3 mL/min, 0.5 mL/min and 0.7 mL/min) has a value which is almost adjacent. But in the mobile phase flow rate of 0.5 mL/min had the most optimum tailing factor; where the difference in tailing factor on the mobile phase flow rate 0.5 mL/min to the tailing factor on the mobile phase flow rate 0.7 mL/min lower than the difference in tailing factor on the mobile phase flow rate 0.5 mL/min to the tailing factor on the mobile phase flow rate 0.3 mL/min.

Analysis time and theoretical plates of the three conditions (mobile phase flow rate 0.3 mL/min, 0.5 mL/min and 0.7 mL/min) have different values. But on the mobile phase flow rate 0.5 mL/min had the most optimum conditions; where the analysis time for the mobile phase flow rate of 0.5 mL/min is 10 minutes and considerable theoretical plates. Time analysis on the mobile phase flow rate of 0.5 mL/minute for 10 minutes only 3 minutes different with the analysis time on the mobile phase flow rate 0.7 mL/minute for 7 minutes. But theoretical plates on the mobile phase flow rate 0.5 mL/min greater than the theoretical plate on the mobile phase flow rate of 0.7 mL/min. Theoretical plates on the mobile phase flow rate 0.3 mL/min greater than the

theoretical plates on the mobile phase flow rate 0.5 mL/min. However, the analysis time on the mobile phase at a flow rate 0.3 mL/minute is 15 minutes different 5 minutes with the analysis time on the mobile phase flow rate of 0.5 mL/minute that is 10 minutes. Based on the data obtained, the mobile phase flow rate of the selected mobile phase was 0.5 mL/min.

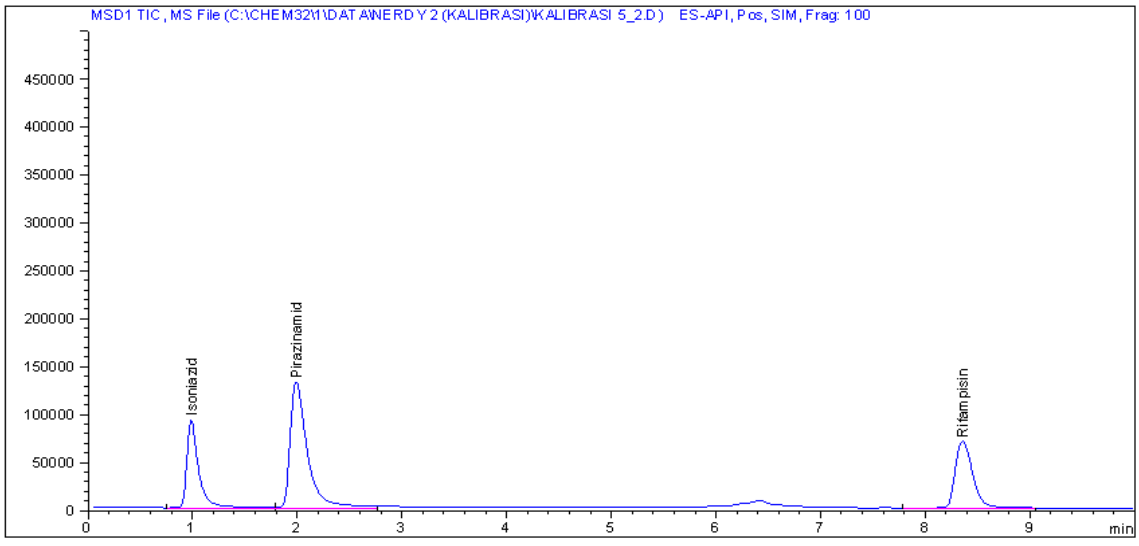
- **Column Oven Temperature**.

Optimization of the column oven temperature, then made variations of the column oven temperature at three different conditions (30°C, 35°C and 40°C). In all three conditions showed no difference in the resolution, selectivity, tailing factor, retention time and theoretical plates. Selected column oven temperature optimum condition was 35°C, as referring to a study conducted by Dionex[10], in 2010; in which all three components were analyzed at 35°C.

Qualitative Analysis of a tablet containing a mixture of rifampicin, isoniazid and pyrazinamide done by analyzing the mass of the positive ions of each peak is detected. From the analysis of the mass of the positive ions with mass spectrometry detection with the type of scanning note that in the tabletpreparations containing a mixture of rifampicin, isoniazid and pyrazinamide peaks were found at the time of 8,447 minutes with ion mass 823.4, at the time of 0.988 minutes with ion mass 138.1 and at the time 2.015 minutes with ion mass124.0. From the analysis results can also be shown that in the preparation of tablets containing a mixture of rifampicin, isoniazid and pyrazinamide contained rifampisin that the molecular mass of 822.4, isoniazid that the molecular mass of 137.1, and pyrazinamide that the molecular mass of 123.0.

Qualitative analysis was also carried out by comparing the retention time of the tabletpreparations containing a mixture of rifampicin, isoniazid and pyrazinamide with retention time of raw rifampicin (PT. Indofarma), raw isoniazid (PT. Indofarma) and raw pyrazinamide (PT. Indofarma). Retention time for the raw rifampicin (PT. Indofarma), raw isoniazid (PT. Indofarma) and raw pyrazinamide (PT. Indofarma) is at the time of 8.317 minutes, 0.989 minutes and 1.993 minutes. Retention time for rifampicin, isoniazid and pyrazinamide in tablet preparations containing a mixture of rifampicin, isoniazid and pyrazinamide was at 8.447 minutes, 0.988 minutes and 2.015 minutes.

Chromatogram data analysis results of mixture of raw rifampicin (PT. Indofarma), raw isoniazid (PT. Indofarma) and raw pyrazinamide (PT. Indofarma), as well as data from mass spectrometry detection by detecting the type of scanning of each detected peak can be seen in Figure 7. below, and chromatogram analysis results of tabletpreparations containing a mixture of rifampicin, isoniazid and pyrazinamide and outcome data detection with mass spectrometry detection type of scanning of each detected peak can be seen in Figure 8. below.



Peak #	RetTime [min]	Type	Width [min]	Area	Area %	Name
1	0.989	VV	0.1140	7.23396e5	23.4918	Isoniazid
2	1.993	VV	0.1727	1.51675e6	49.2556	Pirazinamid
3	8.357	VV	0.1842	8.39203e5	27.2526	Rifampisin

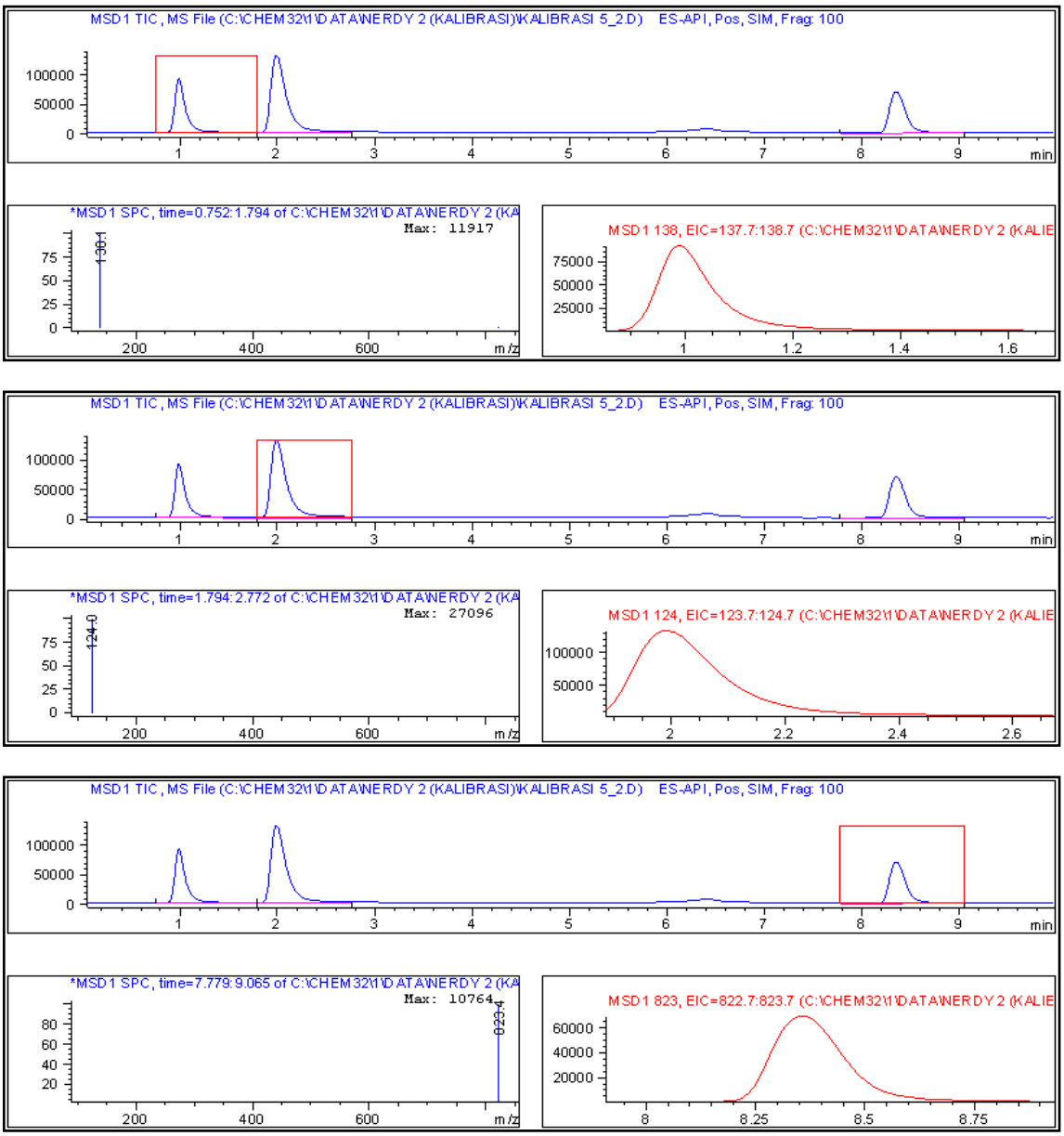
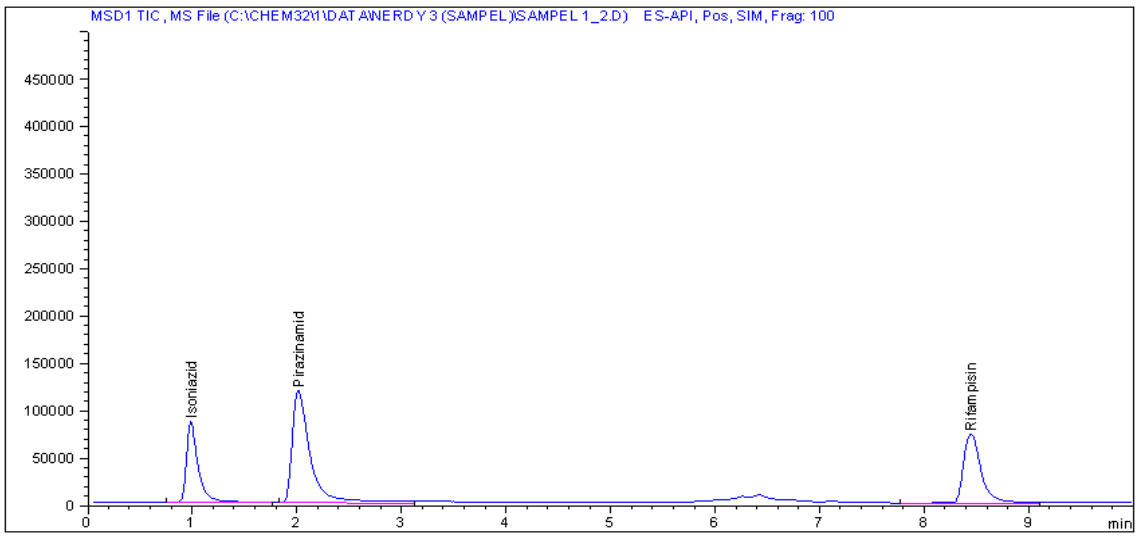


Figure 7. Chromatogram data analysis results of mixture of raw rifampicin (PT. Indofarma), rawisoniazid (PT. Indofarma) and raw pyrazinamide (PT. Indofarma), as well as data from mass spectrometry detection by detecting the type of scanning of each detected peak.



Peak #	RetTime [min]	Type	Width [min]	Area	Area %	Name
1	0.988	VV	0.1155	6.85996e5	22.6268	Isoniazid
2	2.015	VV	0.1840	1.45119e6	47.8658	Pirazinamid
3	8.447	VV	0.1872	8.94605e5	29.5075	Rifampisin

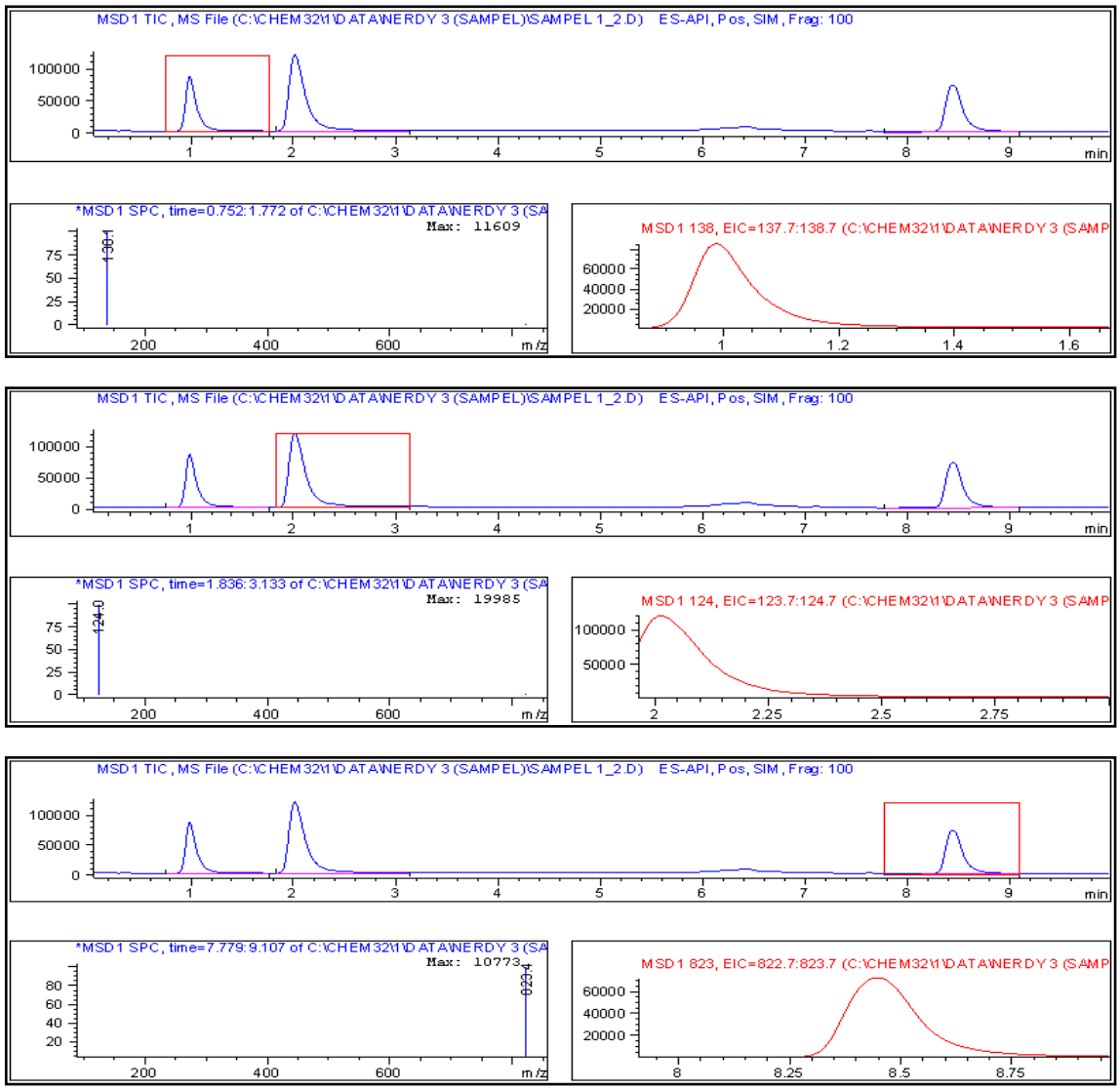


Figure 8. Chromatogram analysis results of tablet preparations containing a mixture of rifampicin, isoniazid and pyrazinamide and outcome data detection with mass spectrometry detection type of scanning of each detected peak.

The results of the assay mixture of rifampicin, isoniazid and pyrazinamide from tablet preparations were determined simultaneously can be seen that the tablet preparations were analyzed meet the requirements of the U.S. Pharmacopeia 30th edition (United States Pharmacopoeia) in 2007. Data calculation results for mixture of rifampicin, isoniazid and pyrazinamide from tablet preparation after a statistical test can be seen in Table 9. below.

Table 8. Data calculation results for mixture of rifampicin, isoniazid and pyrazinamide from tablet preparation after a statistical test.

Name	Rifampisin	Isoniazid	Pirazinamid
Tablet Preparations	107,9261% ± 0,6121%	96,5796% ± 0,9125%	101,0941% ± 3,0178%

Test accuracy is done by measuring the recovery percentage (% recovery) at 3 specific range, such as: 80%, 100% and 120%. Recovery percentage (% recovery) area data of test resultsfor raw rifampicin (PT. Indofarma), raw isoniazid (PT. Indofarma) and raw pyrazinamide (PT. Indofarma) with the standard addition method are given in Table 9.

Table9. Recovery percentage (% recovery) area data of test results for raw rifampicin (PT. Indofarma), raw isoniazid (PT. Indofarma) and raw pyrazinamide (PT. Indofarma) with the standard addition method are given in Table 9.

Specific Ranges	Area			Recovery Percentage (% Recovery)		
	Rifampisin	Isoniazid	Pirazinamid	Rifampisin	Isoniazid	Pirazinamid
80%	704804	561099	1165450	99,0861%	100,3355%	98,9266%
	703984	557936	1171180	98,6521%	98,4022%	100,7453%
	705467	562566	1164640	99,4370%	101,2322%	98,6695%
100%	872764	696768	1436660	99,6135%	101,1290%	100,8103%
	873940	696431	1436990	100,1114%	100,9642%	100,8941%
	869115	697634	1427280	98,0685%	101,5525%	98,4286%
120%	1041420	825651	1691270	100,2106%	98,8928%	98,5536%
	1041680	827941	1689120	100,3024%	99,8259%	98,0986%
	1043170	825837	1693850	100,8281%	98,9686%	99,0995%
Recovery Percentage (% Recovery)				99,5900%	100,1448%	99,3585%

Accuracy testing obtained recovery percentage (% recovery) for raw area rifampicin (PT. Indofarma), raw isoniazid (PT. Indofarma) and raw pyrazinamide (PT. Indofarma) with the standard addition method on a variety of specific ranges (such as: 80%, 100% and 120%) respectively were 99.5900%, and 99.3585% 100.1448%.

Precision test obtained relative standard deviation (RSD) values for the raw rifampicin (PT. Indofarma), raw isoniazid (PT. Indofarma) and raw pyrazinamide (PT. Indofarma) respectively are 0.8818%, 1.1672% and 1.1374%.

The method of high performance liquid chromatography mass spectrometry has had acceptable accuracy, so the method of high performance liquid chromatography mass spectrometry can also be automatically entered as a method specific criteria.

The results of the calibration curve linearity test for raw rifampicin (PT. Indofarma), raw isoniazid (PT. Indofarma) and raw pyrazinamide (PT. Indofarma) in a mixture of water and methanol (50%: 50%) at various concentrations obtained a linear relationship between the concentration of raw rifampicin (PT. Indofarma), raw isoniazid (PT. Indofarma) and raw pyrazinamide (PT. Indofarma) in a mixture of water and methanol (50%: 50%) with the area. The correlation coefficient of raw rifampicin (PT. Indofarma), raw isoniazid (PT. Indofarma) and raw pyrazinamide (PT. Indofarma) in a mixture of water and methanol (50%: 50%) respectively 1.0000, 1.0000 and 0.9999. Regression equation for raw rifampicin (PT. Indofarma), raw isoniazid (PT. Indofarma) and raw pyrazinamide (PT. Indofarma) in a mixture of water and methanol (50%: 50%) respectively is y = 52483,6987 × x + 37945,9823, y = 90890,8408 × x + 24913,7740 and y = 32818,9728 × x + 110283,8065. Chromatogram results of detection of mixture of raw rifampicin (PT. Indofarma), raw isoniazid (PT. Indofarma) and raw pyrazinamide (PT. Indofarma) in a mixture of water and methanol (50%: 50%) at various concentrations for linearity testing seen in Figure 9. below. Data area for raw rifampicin (PT. Indofarma), raw isoniazid (PT. Indofarma) and raw pyrazinamide (PT. Indofarma) in a mixture of water and methanol (50%: 50%) at various concentrations for linearity testing respectively can be seen in Table 10., Table 11. and Table 12. below. Standard calibration curve for raw rifampicin (PT. Indofarma), raw isoniazid (PT. Indofarma) and raw pyrazinamide (PT. Indofarma) in a mixture of water and methanol (50%: 50%) at various concentrations for linearity testing in a row can be seen in Figure 10., Figure 11. and Figure 12. below.

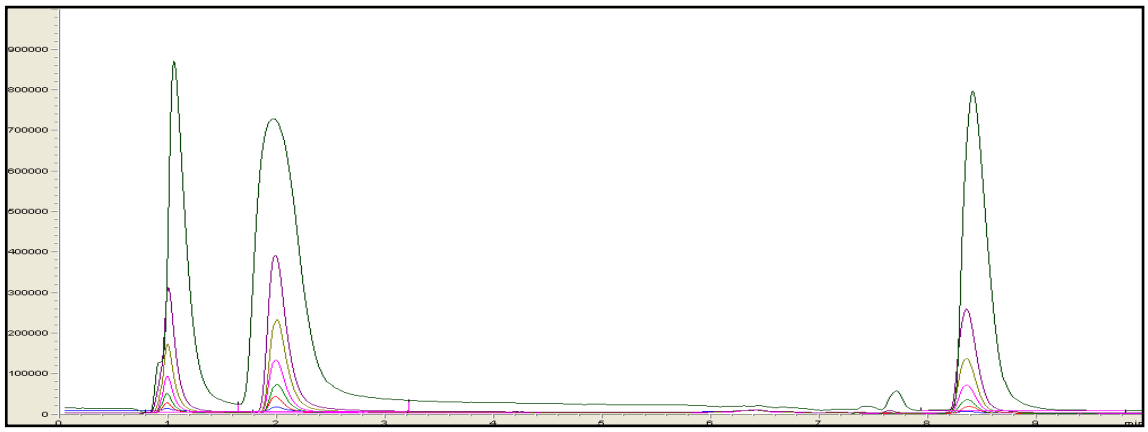


Figure 9. Chromatogram results of detection of mixture of raw rifampicin (PT. Indofarma), raw isoniazid (PT. Indofarma) and raw pyrazinamide (PT. Indofarma) in a mixture of water and methanol (50%: 50%) at various concentrations.

Table 10. Data area for raw rifampicin (PT. Indofarma) in a mixture of water and methanol (50%: 50%) at various concentrations.

Data	X (Concentration / µg/mL)	Y (Area / A)
1	0	0
2	0,9375	108836
3	3,75	228043
4	7.5	415787
5	15	839203
6	30	1637070
7	60	3191750
8	240	12629400

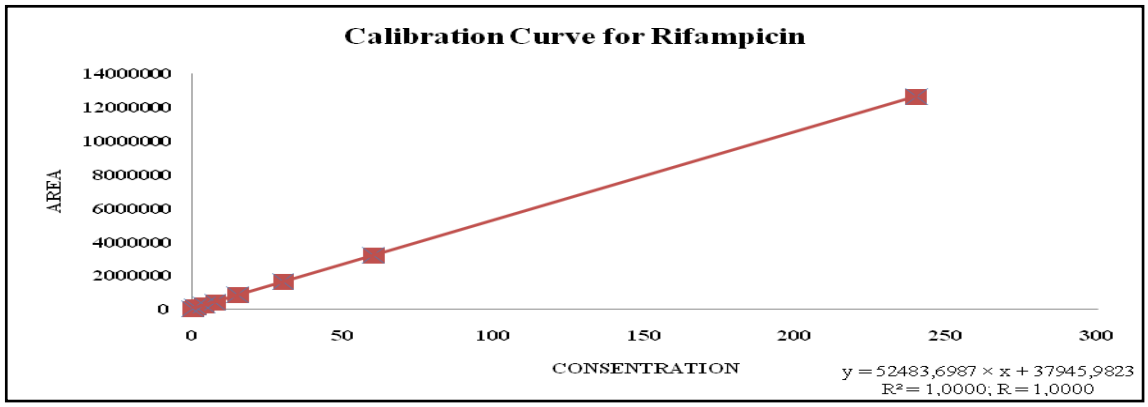


Figure 10. Standard calibration curve for raw rifampicin (PT. Indofarma) in a mixture of water and methanol (50%: 50%) at various concentrations.

Table 11. Data area for raw isoniazid (PT. Indofarma) in a mixture of water and methanol (50%: 50%) at various concentrations.

Data	X (Concentration / µg/mL)	Y (Area / A)
1	0	0
2	0,4688	50085,3
3	1,875	190658
4	3,75	369797
5	7,5	723396
6	15	1405030
7	30	2768380
8	120	10924500

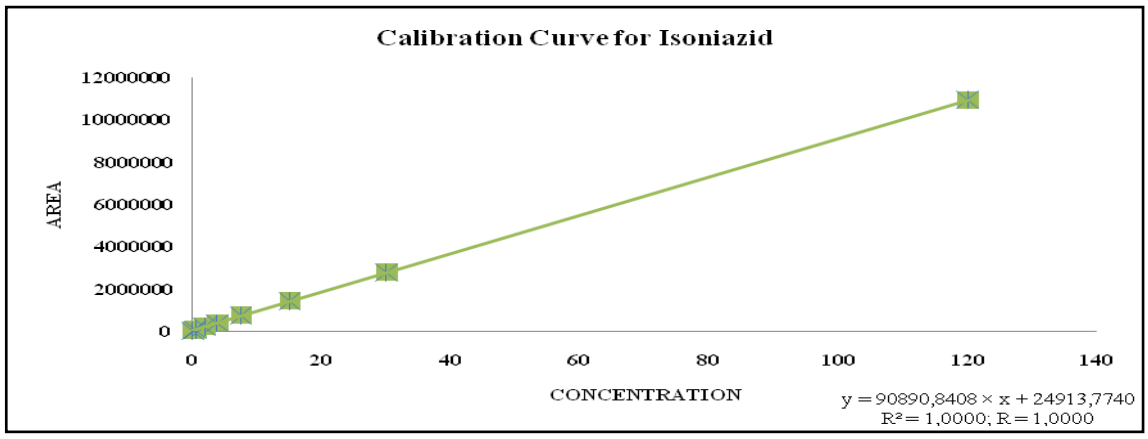


Figure 11. Standard calibration curve for raw isoniazid (PT. Indofarma) in a mixture of water and methanol (50%: 50%) at various concentrations.

Table 12. Data area for raw pyrazinamide (PT. Indofarma) in a mixture of water and methanol (50%: 50%) at various concentrations.

Data	X (Concentration / µg/mL)	Y (Area / A)
1	0	0
2	2,5	115230
3	10	438326
4	20	813366
5	40	1516750
6	80	2915390
7	160	5223980
8	640	21119300

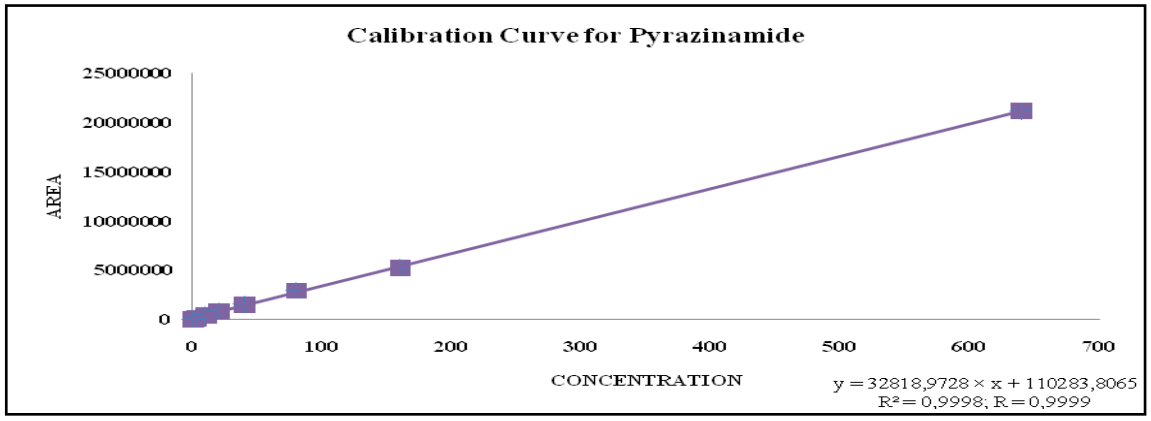


Figure 12. Standard calibration curve for raw pyrazinamide (PT. Indofarma) in a mixture of water and methanol (50%: 50%) at various concentrations.

Limits of detection for raw rifampicin (PT. Indofarma), raw isoniazid (PT. Indofarma) and raw pyrazinamide (PT. Indofarma) in a mixture of water and methanol (50%: 50%) respectively were 0.5013 mg / mL, 0.2260 mg / mL and 4.1103 mg / mL, while the limit of quantitation for raw rifampicin (PT. Indofarma), raw isoniazid (PT. Indofarma) and raw pyrazinamide (PT. Indofarma) in a mixture of water and methanol (50%: 50%), respectively, also is 1.5190 mg / mL, 0.6848 mg / mL and 12.4555 mg / mL.

Conclusions

High performance liquid chromatography mass spectrometry method has been developed, so it can be used for the analysis of a mixture of rifampicin, isoniazid and pyrazinamide. Which first performed optimization of mobile phase (type of mobile phase mixture, composition of mobile phase, mobile phase flow

rate and column oven temperature) to obtain the optimum conditions of high-performance liquid chromatographic mass spectrometry for analysis of a mixture of rifampicin, isoniazid and pyrazinamide.

Optimum conditions obtained were type of mobile phase mixture 0.1% formic acid solution in double distilled water that has been purified (after being mixed and then filtered through a cellulose nitrate membrane filter 0.2 µm and sonicated for 30 minutes) and a 0.1% formic acid solution in methanol (after being mixed and then filtered through a polytetrafluoroethylene membrane filter (PTFE) 0.5 µm and sonicated for 30 minutes) with a mobile phase flow rate 0.5 mL/min, the ratio of mobile phase 90%:10% up to 3 minutes, with isoniazid and pyrazinamide was eluted (and separated), then converted into a ratio of mobile phase to 30%:70% at 3.1 minutes and maintained until rifampicin eluted (up to 10 minutes) and the column oven temperature of 35^{o}C.

The optimum conditions of high performance liquid chromatography mass spectrometry were used for the assay mixture of rifampicin, isoniazid and pyrazinamide from tablet preparations meets the requirements of the validation method test. The validity testing of the method with the accuracy test by recovery percentage (% recovery) parameters, the precision testby relative standard deviation (RSD)parameters, the specificitytest, the limits of detectiontest, the limit of quantitation test and the linearity test.

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