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# APPLICATION OF HPLC FOR SIMULTANEOUS DETERMINATION OF ISONIAZID, RIFAMPICIN AND PYRAZINAMIDE IN A FIXED DOSE FORMULATION

**Bijoy Kumar Panda[1], Manoj Dikkatwar[1], Sathiyanarayanan L*[2]**

[1]*Department of Clinical Pharmacy, Poona College of Pharmacy, Bharati Vidyapeeth (Deemed to be University), Erandwane, Pune, Maharashtra, India*

[2]*Department of Pharmaceutical Chemistry, Poona College of Pharmacy, Bharati Vidyapeeth (Deemed to be University), Erandwane, Pune, Maharashtra, India*

**Corresponding author: l.satyanarayan@bharatividyapeeth.edu*

## ABSTRACT

A precise, accurate, sensitive and robust RP-HPLC method was developed and validated for Isoniazid (INH), Rifampicin (RIF) and Pyrazinamide (PYZ) in fixed-dose combination (FDC) antitubercular pharmaceutical dosage form. Chromatographic analysis was performed on a 250 × 4.6 mm I.D. C18column packed with 5 mm-in-size particles applying gradient elution with a mobile phase composed of 20 mM monobasic sodium phosphate buffer (pH 7) and acetonitrile (B). A:B ratio was 48:52 v/v for the initial 5 min, and then it was maintained at 96:4 v/v; the flow rate was 1 ml/min. UV detection was performed at 264 nm. The total run time was 20 min. The retention time was found to be 5.43 min, 7.31 min and 17.52 min for INH, PYZ and RIF respectively. The method was validated with respect to linearity, accuracy, precision, specificity and sensitivity in accordance with ICH guidelines. Limits of detection were of 0.063, 0.036 and 0.059 µg/ml and limits of quantification were of 0.19, 0.11 and 0.18 µg/ml for isoniazid, pyrazinamide and rifampicin respectively. High recovery and low coefficients of variance confirmed the suitability of the method for the simultaneous analysis of the three considered drugs.

**Keywords:** Isoniazid, Pyrazinamide, Rifampicin, Fixed Dose Combination, HPLC

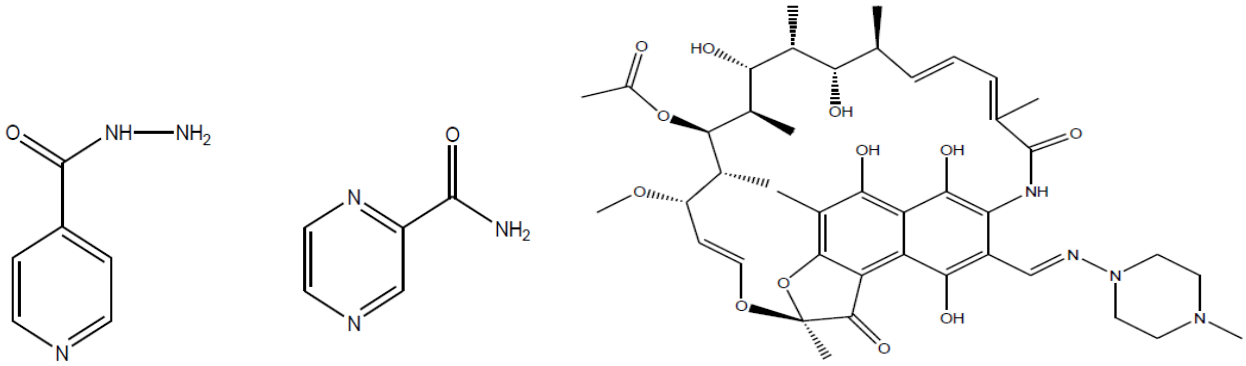
## 1. INTRODUCTION

Tuberculosis (TB) is an infectious disease caused predominantly by *Mycobacterium tuberculosis* and among the leading causes of mortality in worldwide [1]. India accounts for 1/5 of the global TB burden. In 2015, WHO reported that there were an estimated 10.4 million incident TB cases in which 62% of these cases were of male and 90% of cases were of adults. Six countries such as India, Indonesia, China, Nigeria, Pakistan and South Africa were of accounted for 60% of the global total TB cases [2].

The main goals of treatment are rapid killing of bacteria and prevent recurrence of disease. *M. bacterium* tuberculosis grows very slowly and require multiple drug over a long period of time due to their complex cell wall structure. Isoniazid, Rifampicin, Pyrazinamide and Ethambutol in Fig. 1 are considered as first-line anti-tubercular agents.

The directly observed treatment short course (DOTS) contains six or eight months regimen that consist of two months treatment (intensive phase) of Isoniazid, Rifampicin, Pyrazinamide and Ethambutol followed by four month regimen (continuous phase) of Isoniazid and Rifampicin in FDC'S in which all drugs in one tablet [3]. This improves the patient's medication adherence, easy to administer the drug, less chance of the prescribing error and improved drug supply. There are several issues in implementation of DOTS therapy which shows poor therapeutic plasma drug concentration level leads to poor response to disease [4].

In view of the fact that the most of drugs have various physicochemical properties, combining different drugs ensure the multi-targeting of *M. tuberculosis*. It is important to consider the safety, efficacy and the quality requirements for FDC products [5, 6]. The quality requirements include stability, assay and identification testing as well as the determination of degradation products and related substances. Nevertheless, serious matter has been stirring on the utility of these products due to quality problems [7, 8]. INH, PYZ and RIF have been determined by high-performance liquid chromatography from pharmaceutical formulations.



Isoniazid Pyrazinamide Rifampicin

**Fig. 1: Chemical structure of Isoniazid (INH), Pyrazinamide (PYZ) and Rifampicin (RIF)**

However, many of these methods suffer from limitations such as complex and tedious procedures, lack of reproducibility, time consuming, use of sophisticated instruments and cumbersome process [8-12]. These two studies have not use C18 columns for separation [9, 10], whereas, studies that have used C18 columns have reported time consuming process [8-12]. One study reported less time consuming process but mobile phase ratio used were different and cumbersome [11]. Thus, there was need of suitable analytical technique for the simultaneous estimation of Isoniazid, Pyrazinamide and Rifampicin in tablet formulation which could serve as basic for stability studies and can be used for plasma estimation. Keeping, in view of this an attempt was made to develop a simple, precise, accurate and sensitive HPLC method for the simultaneous estimation of Isoniazid, Pyrazinamide and Rifampicin in pharmaceutical solid dosage forms.

## 2. MATERIAL AND METHODS

### 2.1. Chemicals

Working standards of Rifampicin (RIF), Isoniazid (INH) and Pyrazinamide (PYZ) were gifted from the Lupin Research Park. (Aurangabad, India). HPLC grade Acetonitrile was procured from Sigma Aldrich, (India). Liquid Chromatography grade water was obtained by the double distillation and purification through Milli- Q water purification system. A pharmaceutical FDC of Anti TB drug was purchased from the local pharmacy for evaluation. Phosphate buffer solution was prepared by dissolving 0.11 g of monobasic sodium dihydrogen phosphate in 1000 ml of HPLC grade water and pH 7 was adjusted by using triethylamine (HPLC grade).

### 2.2. Instrumentation and chromatographic conditions

Liquid chromatography system consists of preparative HPLC (JASCO, Tokyo, Japan) with UV-detector (UV 2075) and pumps (PU 2087, PU 2080). Separation was performed on LC column (Thermo Scientific, C18 (250×4.6mm, 5 µm size). Data acquisition and analysis were carried out using Borwin/HSS 2000 software (LG 1580-04; JASCO). The mobile phase A consisted of phosphate buffer: acetonitrile (96:4) v/v and B consist of 100% acetonitrile. The HPLC equipment was operated at ambient temperature. The flow rate of mobile phase was maintained at 1 ml/min. Detection was carried out at λ max 264 nm and the injection volume was 20µl.

### 2.3. Preparation of stock and working standard solution

Stock solution containing 1 mg/ml of INH and PYZ was prepared in mobile phase A and 1 mg/ml of RIF prepared in 100% acetonitrile in 100 ml volumetric flask. For RIF solution amber colored volumetric flask was used and 0.5 mg/ml ascorbic acid was added to prevent the degradation of rifampicin during sample preparation due to exposure of light. Working standard solutions were prepared by diluting above both stock solutions in mobile phase A to produce 100 µg/ml concentrations of INH, PYZ and RIF.

### 2.4. Sample preparation of Fixed Dose Combination Tablets

Twenty tablets of Akurit-4® (labeled to contain 75 mg Isoniazid, 400 mg of Pyrazinamide, 150 mg of Rifampicin and 275 mg of Ethambutol, Lupin Ltd.) were weighed and finely powdered. Powder equivalent to 10 mg of INH was taken into the 100 ml amber colored flask. 20

ml Acetonitrile was added and sonicated for 10 min to dissolve rifampicin (RIF) completely as it was getting partially dissolved with mobile phase A. But, a study conducted by Chellini *et al.* [13] had instructed to dissolve with 50 ml of mobile phase A. Solution was filtered through the membrane filter and the volume was made up to 100 ml with mobile phase A from which 20µl was injected into the HPLC system for analysis.

### 2.5. Validation parameters

Validation was performed following the ICH Q2A guidelines for single laboratory validation of methods of analysis [14]. The method was validated as regards to its linearity, precision (within- and between-day), accuracy, robustness and sensitivity.

### 2.6. Linearity

From standard stock solution, a series of dilution were made in the range of 20-100 µg/ml for INH, PYZ and RIF from which 20 µl was injected into the HPLC system. Calibration standards were run before and after the samples; both sets of standard peak areas were used to calculate the linear regression equation as well as the coefficient of determination. The calibration curve for each standard was obtained by plotting a graph of mean peak areas of that standard against the corresponding concentrations. Blank samples were included with each set. Six calibration curves constructed on six separate days were analyzed to evaluate the linearity of each calibration curve. The calibration curve was constructed by weighted (1/y) least-squares linear regression analysis. The calibration curves were described by the following linear equation: $y = mx \pm c$, where y is the analytes area and x is the concentration (µg/ml). The slope, intercept and correlation coefficient were calculated for each standard curve. Unknown concentrations were calculated from the equation of the calibration curve.

### 2.7. Precision (Repeatability)

The repeatability was evaluated by the three replicate for each drug at concentration of 100 µg/ml for INH, PYZ and RIF. By observing the peak area % RSD was calculated and which determine the repeatability of method.

### 2.8. Accuracy

The accuracy of method was determined by the recovery studies. To determine the recovery of the method, three standard solutions with low, intermediate and high concentrations (levels) were analyzed. The percentage recovery was performed by three determinations and was calculated by the relationship between the experimental concentration (Cexp) and the theoretical concentration (Cteo) expressed as percentage using the following equation: (Cexp/ Cteo) x 100.

### 2.9. Robustness

The robustness of the developed method was studied by evaluating the effect of small but deliberate variations in chromatographic conditions. The parameters studied were flow rate and mobile phase composition. By evaluating the retention time and peak area observed and % RSD calculated which should not be more than 2%.

## 3. RESULT AND DISCUSSION

### 3.1. HPLC method development and optimization

Selection of appropriate wavelength was necessary to determine all 3 drug component simultaneously in single HPLC run. To determine the appropriate wavelength the working standard solution of INH, PYZ and RIF in mobile phase A were scanned over the range of 200-400 nm. By observing the overlain spectra of all the three drugs INH, PYZ and RIF gave high signals at 270, 272 and 260 nm respectively. Therefore the common wavelength selected was 264 nm. It was observed that there was no interference from the mobile phase or baseline disturbance at 264 nm. Moreover, the response and intensity of INH, RIF and PYZ was found to be good, so 264 nm was finally selected for the further analytical method development.

The main problem in developing HPLC method for simultaneous estimation of anti-tubercular drug in single run was selection of appropriate mobile phase because of the large difference in molecular weight and polarity of drugs. For elution of RIF high percentage of organic solvent was required and for INH and PYZ more aqueous phase required. Therefore, initial trials were performed to optimize the mobile phase composition by taking the solvent like methanol, acetonitrile and phosphate buffer in different ratios in order to get good separation, sharp peak without tailing and without interference of the excipients.

After taking several isocratic run for each drug due to wide polarity difference we choose the gradient elution technique. Mobile phase A composition contains 20 mM monobasic sodium phosphate buffer (pH 7) and Acetonitrile (96:4 v/v) and Mobile phase B was 100% Acetontrile. The flow rate was chosen 1.0 ml/min and

detection wavelength was 264 nm at which all drugs give better response. From the obtained chromatogram it was concluded that the gradient elution was necessary and gradient flow program. Gradient elution was carried out as follows:

100% mobile phase A was first held for 10 min, then mobile phase B was raised up to 52% in 10.8 min, mobile phase B was held at this level until 20 min, and at 20.1 min, mobile phase

A was switched back to 100% until 25 min (re-equilibration).

The retention time was found to be 5.43 min, 7.31 min and 17.31 min for INH, PYZ and RIF respectively.

### 3.2. Method Validation

The method was validated according to International Conference on Harmonization guideline for validation of analytical procedures [14].

### 3.3. System suitability

System suitability test (SST) was performed to ensure that the developed method is adequate to perform in chromatographic system. Retention time (RT), Tailing factor (T), theoretical plates (N) and resolution were evaluated for triplicate injection of drug sample at a concentration of 100 ppm. There were no interferences on the INH, PZA and RIF peaks due to the components of the samples. INH, PYZ, and RIF were eluted at 5.43 min, 7.31 min and 19.31, respectively (Fig. 2). All the three peaks were well separated from the others.

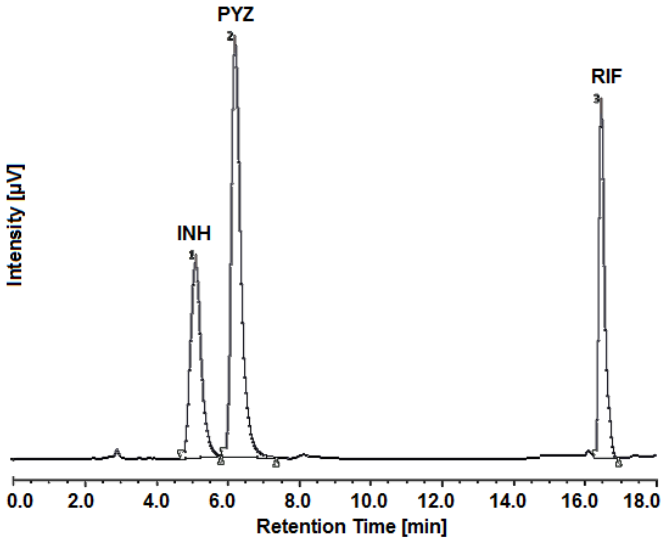



**Fig. 2: HPLC chromatogram of INH, PYZ, and RIF at 264 nm by using 20 mM monobasic sodium phosphate buffer (pH 7) and acetonitrile (96:4 v/v) as a mobile phase**

The SST is an integrated part of the analytical method and it ascertains the suitability and effectiveness of the operating system. The results of the SST are reported in Table 1.

**Table 1: System suitability test results (SST)**

| Parameters | Drugs | | |
|---|---|---|---|
| | INH | PYZ | RIF |
| Retention time | 5.43 | 7.31 | 19.31 |
| Tailing factor | 0.59 | 1.81 | 1.83 |
| Theoretical plate | 3750 | 8432 | 12077 |
| Resolution | - | 2.093 | 7.53 |

### 3.4. Linearity

The linear regression equation describing the obtained calibration plots for all drugs and which shows that correlation coefficient greater than 0.997 and showed linear response over the concentration range of 20-100 µg/ml. The linear regression equations for each drug are as follows:

INH: $y=437.9x + 12333$, $(n=3, r^2 = 0.999)$

PYZ: $y = 919.7x + 43468$, $(n=3, r^2 = 0.998)$

RIF: $y = 458.8x + 11396$, $(n=3, r^2 = 0.998)$

Where y is the response (peak area) and x is the concentration.

### 3.5. Precision

The method was found to be precise and % RSD value was less than 2 which is recommended in ICH guideline. The results of repeatability studies are shown in Table 2.

**Table 2: Repeatability study results**

| Parameter | INH | PYZ | RIF |
|---|---|---|---|
| Concentration (µg/ml) | 100 | 100 | 100 |
| % RSD | 1.091 | 1.408 | 1.446 |

### 3.6. Accuracy

The accuracy of the method was evaluated using three concentrations with low, intermediate and high concentrations of the calibration at 80%, 100%, and 120% for INH, PZA, and RIF. The % recoveries of INH, PZA, and RIF ranged from 98.66 to 99.62%, 99.20 to 100.12 and 99.39-100%, respectively (Table 3). The accuracy results demonstrated that the results of the mean tests were close to the true concentrations of analytes. The acceptance criteria given in ICH for recovery of the accuracy is within 98 - 102%.

**Table 3: Accuracy of Isoniazid, Pyrazinamide and Rifampicin (INH, PYZ and RIF)**

| Sample | Parameters | | | | | |
|---|---|---|---|---|---|---|
| | Level | Sample conc. (µg/ml) | Amount added (µg/ml) | Total conc. | % Recovery | % RSD |
| INH | 80% | 7.5 | 6.0 | 13.5 | 99.25% | 0.25 |
| | 100% | 7.5 | 15.0 | 15.0 | 98.66% | 1.02 |
| | 120% | 7.5 | 16.5 | 16.5 | 99.62% | 0.63 |
| PYZ | 80% | 40 | 32 | 72 | 99.86% | 0.86 |
| | 100% | 40 | 40 | 80 | 100.12% | 1.50 |
| | 120% | 40 | 48 | 88 | 99.20% | 0.46 |
| RIF | 80% | 15 | 12 | 27 | 100% | 0.87 |
| | 100% | 15 | 15 | 30 | 99.63% | 1.65 |
| | 120% | 15 | 18 | 33 | 99.39% | 0.52 |

**Table 4: Robustness results of Isoniazid, Pyrazinamide and Rifampicin (INH, PYZ and RIF)**

| Factors | levels | Retention time | | | % RSD | | |
|---|---|---|---|---|---|---|---|
| | | INH | PYZ | RIF | INH | PYZ | RIF |
| Flow rate (ml/min) | | | | | | | |
| 0.8 | -0.2 | 5.75 | 7.90 | 16.67 | 0.26 | 0.63 | 0.26 |
| 1.0 | 0 | 5.43 | 7.31 | 17.52 | 0.95 | 0.71 | 0.67 |
| 1.2 | +1.2 | 3.91 | 6.66 | 18.66 | 0.10 | 1.58 | 0.10 |
| % Acetonitrile proportion in mobile phase (v/v) | | | | | | | |
| 3 | -1 | 5.90 | 7.62 | 17.01 | 1.11 | 1.32 | 0.86 |
| 4 | 0 | 5.43 | 7.31 | 17.52 | 0.98 | 0.87 | 0.12 |
| 5 | +1 | 4.88 | 6.95 | 19.21 | 0.46 | 0.32 | 0.27 |

### 3.7. Robustness

It was observed that there were no marked changes in the chromatograph obtained by the slightly changing in flow rate (±0.2 ml/min) and % acetonitrile proportion in mobile phase (±1%).The low values of %RSD for each of drug proposed that during all deliberate variations, assay value of test preparation (MQC) was not affected and it was in accordance with that of actual (Table 4). Hence, the newly developed analytical method was considered to be robust.

### 3.8. Selectivity

Selectivity of method is checked by injecting mixture of drugs into the HPLC system. The sharp peaks of INH, PYZ and RIF were obtained at retention time of 5.43, 7.31 and 17.52 min, respectively. The same retention times prove the selectivity of column. Chromatogram was as shown in Fig. 3.

This method is better because one wavelength was used for determination of all the three anti-tubercular drugs in FDC tablet dosage form compared to a study [12] where operating wavelengths were variable.

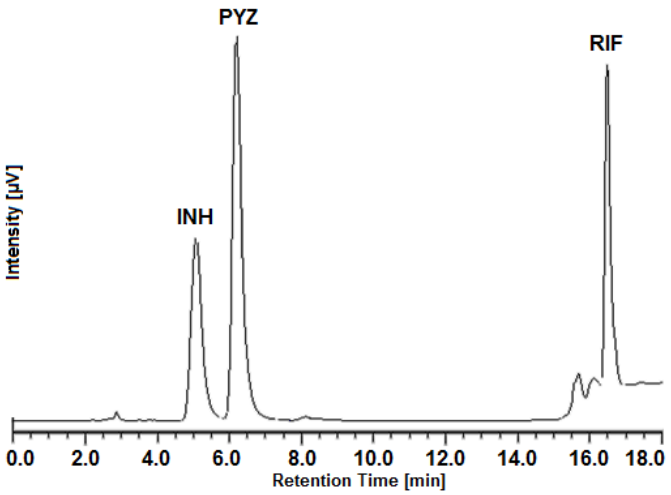



**Fig. 3: HPLC chromatogram of INH, PYZ, and RIF at 264 nm by using 20 mM monobasic sodium phosphate buffer (pH 7) and acetonitrile (96:4 v/v) as a mobile phase in fixed-dose combination**

Our method had a shortest run time so less time consuming compared to studies [8, 12] where run time was 40 min and 31.4 min respectively. Compared to the study [13] carried out by Chellini *et al*, the method adopted by them was validated by carrying out separation at different wavelength (264nm) on preparative HPLC (Jasco). The method did not provide reproducibility on this instrument at the defined wavelength for separation. The method may be applied for bioanalytical procedure as the peaks have appeared at a considerable retention time where very less possibility of interference of plasma peaks exists compared to other studies [11-13, 15].

## 4. CONCLUSION

A new reversed-phase HPLC method was developed and validated for simultaneous analysis of INH, PYZ and RIF in pharmaceutical formulation. It has been shown that the method was simple, linear, accurate, repeatable, selective and robust. The main advantage of the study was that the mobile phase preparation is simple and UV detector used in the experiment which is available in all small scale laboratories and the entire analysis was done at single wavelength in relatively short duration as compared to previously described methods. The proposed method may be suitable for analysis of first line Anti-Tubercular drugs due to the desirable results and can be widely employed for routine quality control analysis of these drugs in pharmaceutical formulations.

## 5. ACKNOWLEDGEMENTS

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