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A Validated Liquid Chromatographic Method for the Determination of Rifampicin and Isoniazid in Pharmaceutical Formulations

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Authors' contributions

This work was carried out in collaboration between all authors. Authors BHB and PVVS designed the study, wrote the protocol. Author MKK analyses of the study like method development and validation study, performed the experimental process and author JKK managed the literature searches, wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

A simple RP-HPLC method with more accuracy and precission was developed for determination of Rifampicin and Isoniazid simultaneously. A mobile phase consisted of methanol, acetonitrile and water in the ratio 60:20:20(v/v) was used, at a flow rate of 1 mL/min. The wavelength of detection was 254 nm and the column used was Kromasil C18, (250 x 4.6 nm, 5 µm). The retention time for rifampicin and isoniazid are 3.42 and 7.43 min respectively. A concentration range of 40-100 µg/mL was studied to test the linearity of the developed method. The correlation coefficient (r^2) of regression was found to be 0.998 which is almost equal to 1. The LOQ was 1.75 µg/mL for both rifampicin and isoniazid and the LOD was 0.5 µg/mL for both rifampicin and isoniazid. The mean percentage recovery of rifampicin was 99.56% and 99.83% for isoniazid respectively.

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Keywords: Rifampicin; isoniazid; RP-HPLC; pharmaceutical formulation; simultaneous estimation.

1. INTRODUCTION

Rifampicin (Fig. 1), a complex semi synthetic macro cyclic antibiotic derived from *streptomyces mediterranei* is a member of the rifamycin class of antibiotics used for the treatment of tuberculosis and other infectious diseases [1]. Chemically it is known as (12Z, 14E, 24E)- (2S, 16S, 17S, 18R, 19R, 20R, 21S, 22R, 23S) - 1,2 - dihydro- 5, 6, 9, 17, 19 -pentahydroxy, 23 - methoxy- 2, 4, 12, 16, 18, 20, 22 heptamethyl -8- (4-methylpiperazin -1 yliminomethyl) -1, 11 - dioxo 2, 7 (epoxypentadeca -1, 11, 13 trienimino) naphtha [2,1-b] furan -21-yl acetate. Isoniazid (4-Pyridinecarboxylic acid, hydrazide) (Fig. 2), the hydrazide of Isonicotinic acid is a synthetic analog of pyridoxine [2] and it is a common drug used for the treatment of tubercolosis. It is chemically it is known as is onicotinohydrazide. It is widely used together with rifampicin, ethambutol and pyrazinamide among others, for the chemotherapy of tubercolosis.

A survey of literature revealed that there are only two high performance liquid chromatographic [3,4] methods reported for the simultaneous estimation of rifampicin and isoniazid in pharmaceutical dosage forms. However, there are some methods [5-9] for the determination of titled drugs with other combinations like ethambutol and pyrazinamide.

1.1 Objective

In the present work an attempt was made to develop a simple, precise and accurate HPLC method for the simultaneous estimation of rifampicin and isoniazid in pharmaceutical dosage forms.

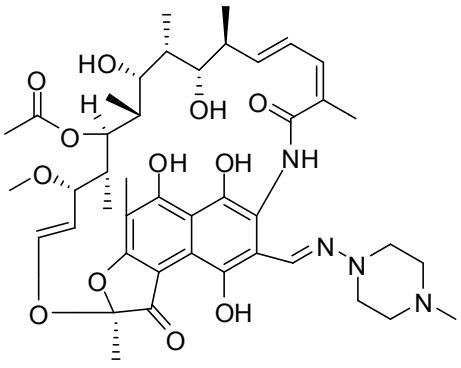


Fig. 1. Rifampicin

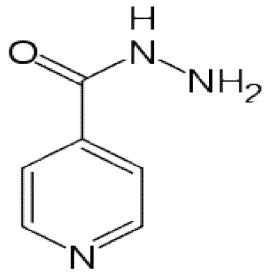


Fig. 2. Isoniazid

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Methanol and water of HPLC grade were produced from Merck specialties Pvt. Ltd., Mumbai. A working standard of rifampicin and isoniazid was provided by Vivimed laboratories, Hyderabad.

2.2 Equipment

Chromatographic separation was performed on a PEAK chromatographic system equipped with LC-P7000 isocratic pump; Rheodyne injector with 20 µL fixed volume loop, variable wavelength programmable UV detector UV7000 and the output signal was monitored and integrated by PEAK Chromatographic Software version 1.06. Teccomp UV-2301 double beam UV-Visible spectrophotometer was used to carry out spectral analysis and the data was recorded by Hitachi software. Sonicator (1.5L) Ultrasonicator was used to sonicating the mobile phase and samples. Standard and sample drugs were weighed by using Denver electronic analytical balance (SI-234) and pH of the mobile phase was adjusted by using Systronics digital pH meter.

2.3 Year of Experiment: 2014

2.3.1 Site

Department of Chemistry, Acharya Nagarjuna University, Nagarjunanagar, Guntur 522510, Andhra Pradesh (India).

2.4 Chromatographic Conditions

Kromasil C18 (250 mm length, 4.6 mm diameter and 5 µ particle size) column was used for the

separation; a mobile phase of methanol, acetonitrile and water in the ratio 60:20:20 was at pH 4.8. The mobile phase was filtered through 0.45 µm membrane filter paper and delivered at a flow rate of 1 mL/min and the wavelength of detection was at 254 nm. The injection volume was 20 µL, and the total run time 8 min. The resulting chromatogram was shown in Fig. 3.

2.5 Preparation of Standard Solutions

10 mg of the standard drug Rifampicin and Isoniazid was weighed accurately and was dissolved in 10 mL methanol separately to get a concentration of 1000 µg/mL of both Rifampicin and Isoniazid separately. It was sonicated to dissolve completely. Then it was filtered through membrane filter paper. This standard stock solution used to prepare necessary concentrations to construct calibration curve by proper dilution.

2.6 Preparation of Sample Solution

Twenty tablets of R-CINEX (RIF-100 mg, INZ-100 mg) taken and powdered. A quantity of powder equivalent to 10 mg of Rifampicin and Isoniazid taken and transferred in to 100 mL light resistant flask and made up to the required volume by using mobile phase. The solution was filtered through 0.45 µm filter. From the filtrate 15 mL was taken separately in a 25 mL volumetric flask and make up to the mark with mobile phase to get a sample solution concentration of 60 µg/mL of both rifampicin and isoniazid.

2.7 Method Development

2.7.1 Selection of the mobile phase

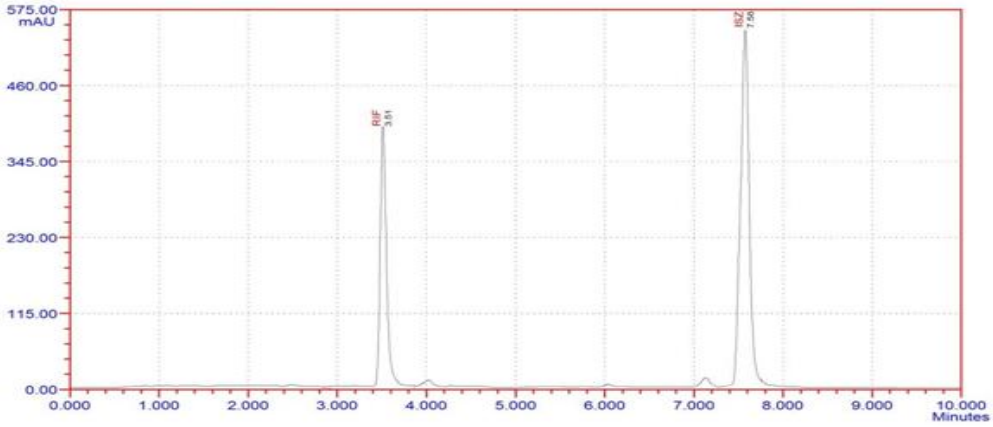
Several systematic trials were performed to optimize the mobile phase. Different solvents like methanol, water and acetonitrile in different ratios; different pH values of the mobile phase ratios by using different buffer solutions in order to get sharp peak and base line separation of the components and without interference of the excepients. Satisfactory peak symmetry, resolved and free from tailing was obtained in mobile phase Methanol: Acetonitrile: water 60:20:20(v/v) in isocratic condition for both rifampicin and isoniazid.

2.7.2 Selection of the mobile phase flow rate

Flow rates of the mobile phase were changed from 0.5–1.2 mL/min for optimum separation. A minimum flow rate as well as minimum run time gives the maximum saving on the usage of solvents. It was found from the experiments that 1.0 mL/min flow rate was ideal for the successful elution of the analyte.

2.7.3 Detection wavelength

The spectrum of diluted solutions of the Rifampicin and Isoniazid methanol was recorded



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1	RIF	3.513	40736	263685.0	42.105	0.76	5871
2	ISZ	7.580	53646	362564.2	57.895	1.17	25070
	Sum:		94382	626249.2	100.0000		

Fig. 3. Standard chromatogram of rifampicin (t_R 3.42 min) and isoniazid (t_R 7.43 min)

separately. The absorption spectrum of Rifampicin and Isoniazid obtained by scanning the mixture sample on UV spectrophotometer in UV region (200-400 nm) in spectrum mode showed that the drug has maximum absorbance at 254 nm. Analysis was carried out by adjusting the UV detector of the HPLC system at 254 nm.

2.7.4 Choice of stationary phase

Preliminary development trials have performed with octadecyl columns with different types, configurations and from different manufacturers. Finally, the expected separation and pattern of peak was succeeded by using analytical Kromasil C-18 column with 250 x 4.6 mm internal diameter and 5 µm particle size.

2.8 Method Validation

The validation of the method was carried out in terms of parameters like linearity, precision, accuracy, limit of detection, limit of quantification, specificity and robustness.

2.9 Linearity

The linearity of the method was tested over a concentration range of 40 to 100 µg/mL of rifampicin and isoniazid. 20 µL of each concentration was injected in triplicate into the HPLC system. The response was read at 254 nm and the corresponding chromatograms were recorded. The regression value of the plots was computed by least square regression method.

2.10 Precision

The precision of the method was determined by inter day and intraday studies, solutions of standard and sample were repeated thrice in a day and percentage relative standard deviation (%RSD) was calculated.

2.11 Accuracy

The accuracy of the method was determined by recovery experiments. The recovery studies were performed by the regular addition method. At 50%, 100%, 150% level, the percentage recovery was calculated. For both the drugs, recovery was performed in the same way. The recovery studies were performed in triplicate.

2.12 Robustness

Robustness of the method was studied by making slight changes in chromatographic conditions, such as mobile phase ratio, pH and wavelength of detection.

2.13 System Suitability and Specificity

The system suitability and specificity was established by analyzing the number of theoretical plates, retention time and tailing factor.

2.14 Limit of Detection and Limit of Quantification

The limit of detection (LOD) is the smallest concentration of the analyte that gives the measurable response. LOD was calculated using the following formula:

$$\mathrm{LOD} = \frac{3.3 \text{ x standard deviation}}{\text{Slope of calibration curve}}$$

The limit of quantification (LOQ) is the smallest concentration of the analyte, which gives a response that can be accurately quantified. LOQ was calculated using the formula:

$$\mathrm{LOQ} = \frac{10 \text{ X standard deviation}}{\text{Slope of calibration curve}}$$

3. RESULTS AND DISCUSSION

All the validation parameters for the proposed method were determined according to Conference on Harmonization (ICH) guidelines [10-12]. Several systematic trials were performed to optimize the mobile phase by taking the solvents methanol, water and acetonitrile in different ratios and different pH values of the mobile phase ratios by using different buffer solutions in order to get sharp peak and base line separation of the components and without interference of the excepients. Satisfactory peak symmetry, resolved and free from tailing was obtained in mobile phase methanol: acetonitrile: water 60:20:20(v/v) in isocratic condition for both rifampicin and isoniazid.

The separation of rifampicin (Rt.3.42 min) and isoniazid (Rt. 7.43 min.) for the selected mobile phase methanol: acetonitrile: water 60:20:20 (v/v) is much prominent. A variation in the structure of the two drugs was also an additional advantage in the separation of the drugs [3] [Shah Y]. The present developed method was free of buffer so, it cannot halm the column for usage of longer periods also compared to earlier method [3]. Thus the proposed method for the simultaneous estimation of rifampicin and

isoniazid is more efficient, simple and accurate to determine the above combination in pharmaceutical formulations.

A system suitability test was applied to representative chromatograms for various parameters. A graph was constructed covering a concentration range 40-100 ppm. The calibration curve was obtained for a series of concentration in the range of 40-100 ppm and it was found to be linear. The data of regression analysis of the calibration curves are shown in Table 2. Low values of standard deviation denoted very good repeatability of the measurement. The method was found to be accurate and precise, as indicated by recovery studies and % RSD is less than 1.2 [13,14].

3.1 Linearity

The linearity of the method was tested over a concentration range of 40 to 100 µg/mL of rifampicin and isoniazid. Peak area (A) and concentration (c) of each drug substance was subjected to regression analysis to calculate the correlation coefficients. A correlation coefficient r^2=0.998 for both rifampicin and isoniazid of the regression was found to be almost equal to 1. Linearity results were presented in Table 1 and graph in Fig. 4.

3.2 Precision

The intraday %RSD of rifampicin and isoniazid were found to be 0.78% and1.02%, respectively. The inter day %RSD of rifampicin and isoniazid were found to be 1.14% and 0.84% respectively, shown in Tables 2 and 3. The values of %RSD within a day, day to day variation <1.15% proves that the method is precise.

3.3 Accuracy

The accuracy of the method was determined by recovery experiments. The recovery studies were performed by the regular addition method. At 50%, 100%, 150% level, the percentage recovery was calculated and presented in Table 4. Recovery was found to be within the range of 99.56% to 99.83%, which indicates the accuracy of the method. For both the drugs, recovery was performed in the same way. The recovery studies were performed in triplicate. The results of recover studies were found to be satisfactory.

3.4 Robustness

Robustness of the method was studied by making slight changes in chromatographic conditions, such as mobile phase ratio, pH and wavelength of detection (Table 5). It was observed that there were no marked changes in the chromatograms obtained. So the developed RP-HPLC method was robust.

3.5 System Suitability and Specificity

The parameters of system suitability study and specificity shown in Table 6 demonstrates the suitability and specificity of the system for the analysis of these drugs in combination.

3.6 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ values of rifampicin and isoniazid have shown in Table 7 supports the sensitivity of the developed method.

Table 1. Results of linearity

S. no	Concentration in µg/mL	Rifampicin peak area	Isoniazid peak area
1	40	188618	239978
2	50	228555	314185
3	60	264514	364586
4	70	316942	434707
5	80	359161	513514
6	90	395739	571384
7	100	454061	646800
	Slope	4454.3	6466.74
	Intercept	3122.864	10443.6
	Correlation coefficient	0.998	0.998

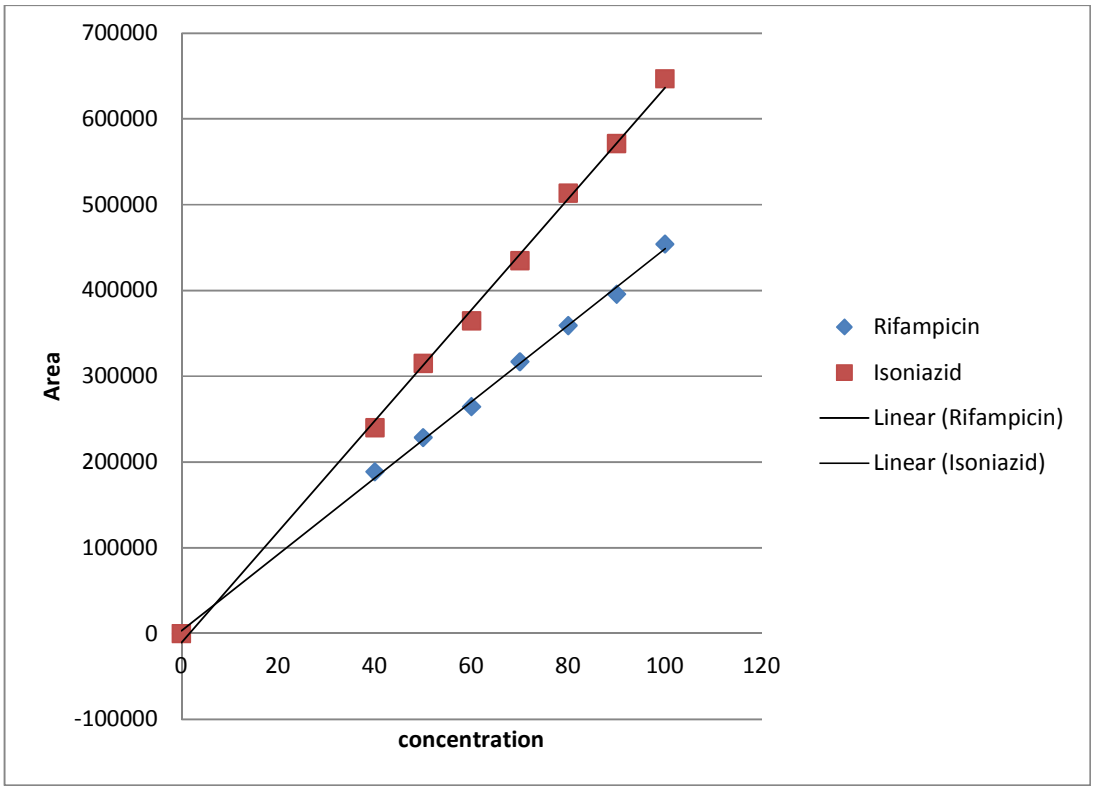


Fig. 4. Linearity of rifampicin and isoniazid

Table 2. Results of precision study (Intra-day)

S. no	Concentration in µg/mL	Rifampicin peak area	Isoniazid peak area
1	60	267346	368769
2	60	264058	361144
3	60	262198	360698
4	60	264624	369091
5	60	266447	362495
6	60	267389	363251
		RSD:0.78	RSD:1.02

RSD = Relative standard deviation

Table 3. Results of precision study (Inter-day)

S. no	Concentration in µg/mL	Rifampicin peak area	Isoniazid peak area
1	60	252458	378290
2	60	259025	374219
3	60	259617	370119
4	60	260282	373081
5	60	256733	370426
6	60	259602	375855
	Mean area	257952.8	373665
		RSD:1.14	RSD:0.84

RSD = Relative standard deviation

Table 4. Accuracy data of the developed method

% of recovery				Rifampicin		Isoniazid	
	Target conc., (µg/mL)	Spiked conc., (µg/mL)	Final conc., (µg/mL)	Conc., obtained Mean±SD	% of assay Mean±SD	Conc., obtained Mean±SD	% of assay Mean±SD
50%	40	20	60	60.43±0.673	100.7±1.1	60.13±0.59	100.23±0.98
100%	40	40	80	78.89±0.295	98.61±0.3	80.01±1.00	100.02±1.26
150%	40	60	100	99.36±0.868	99.3±0.8	99.24±0.61	99.24±0.61

SD = Standard Deviation of three replicate experiments

Table 5. Results of robustness

Condition	Rifampicin mean area	% difference	Isoniazid mean area	% difference
Standard	264514		364586	
Mp Changes-1 M:A:W (65:17.5:17.5 v/v)	262839	-0.63	364970	0.10
Mp Changes-2 (55:22.5:22.5 v/v)	268558	1.52	365391	0.22
WL Changes-252	261300	-1.21	360261	-1.18
WL Changes- 256	266077	0.59	364819	0.063
pH -4.6	261429	-1.1	361749	-0.77
pH-5.0	260922	-1.3	365674	0.29

MP: Mobile Phase, WL: Wavelength

Table 6. System suitability and specificity

Drug	Concentration (µg/mL)	R. time	Area	TP	TF	Resolution
Rifampicin	60	3.42 min	264515	7598	0.75	……..
Isoniazid	60	7.43 min	364586	35105	1.09	25.2

Table 7. Limit of detection and limit of quantification

Parameter	Rifampicin	Isoniazid
LOD	0.5 µg/mL	0.5 µg/mL
LOQ	1.75 µg/mL	1.75 µg/mL

4. CONCLUSION

A simple RP-HPLC method with more accuracy and precession was developed for simultaneous determination of rifampicin and isoniazid. The retension times and quality of separation of the two drugs was good and more over the mean percentage recoveries were good. The LOQ was 1.75 µg/mL for both rifampicin and isoniazid and the LOD was 0.5 µg/mL for both rifampicin and isoniazid. Hence the proposed method is applicable for the determination of rifampicin and isoniazid in pharmaceutical formulations.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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