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METHOD DEVELOPMENT AND VALIDATION OF DRUG COMBINATION IN A FORMULATION OF ISONIAZID, RIFAMPICIN AND PIPERINE BY RP-HPLC METHOD

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ABSTRACT

A simple, isocratic reverse phase high pressure liquid chromatographic method has been validation and method development of new drug combination. The method was carried out on an Waters xterra reverse phase C 18 (250 mm x 4.6 mm I.D; particle size 5 µm) column with a mobile phase consisting of methanol and ortho phosphoric acid (pH was adjusted to 2.2 by using hydrochloric acid) in the ratio of 40:60 at a flow rate 1.0 mL/min. Detection was carried out at λ 282 nm. The retention times of Isoniazid , Rifampicin and Piperine were found to be 2.513, 3.625 and 5.316 min respectively. The developed method was validated in terms of accuracy, precision, limit of detection/quantitation, linearity and robustness. The proposed method is highly sensitive, precise, accurate and easy to perform and successfully applied for the estimation of these drugs in bulk and capsule dosage form.

KEYWORDS: Methanol, Ortho phosphoric, Isoniazid, Rifampicin Piperine, HPLC, and Validation.

INTRODUCTION

Analytical chemistry may be derived as the science and art of determining the composition of material in terms of the elements of compounds contained. By means of analytical techniques both qualitative analysis (the presence or absence of one or more elements) and quantitative analysis (how much amount is present) can be done. Pharmaceutical analysis may be defined as the application of analytical procedures used to determine the purity, safety and quality of drugs and chemicals. It includes both qualitative and quantitative analysis of drugs and pharmaceutical substances start from bulk drugs to the finished dosage form.[1]

High Performance Liquid Chromatography (HPLC)[2]

HPLC was introduced commercially in 1969 and since then it has undergone extensive modifications and innovation which lead to its emergence as the foremost analytical tool for quantitative analysis. HPLC is a type of liquid chromatography that employs a liquid mobile phase and a very finely divided stationary phase. In order to obtain a satisfactory flow rate liquid must be pressurized to a few thousands of pounds per square inch.

For the present study, the drugs Isoniazid, Rifampicin and Piperine were selected for their estimation. The HPLC method was considered the choice of estimation, since this method is the most powerful of all chromatographic and other separative methods. The HPLC method has enabled analytical chemist to attain great success in solving his analytical problems. The HPLC is the method of choice in the field of analytical chemistry, since this method is precise, accurate and linear.

Isoniazid (Laniazid, Nydrazid), also known as isonicotinylhydrazine (INH), is an organic compound that is the first-line medication in prevention and treatment of tuberculosis.[3] Rifampicin or rifampin is a bactericidal antibiotic drug of the rifamycin group. It is a semisynthetic compound derived from *Amycolatopsis rifamycinica*.[4] Piperine is the alkaloid responsible for the pungency of black pepper and long pepper, along with chavicine (an isomer of piperine). It has also been used in some forms of Traditional medicine and as an insecticide.[5]

METHODOLOGY

Preparation of Reagents[6]

Preparation of 0.1N Orthophosphoric Acid Buffer

Mix of 6.7 ml of phosphoric acid with 50.0 ml of a 4 percent solution of dilute sodium hydroxide solution and dilute to 1000.0 ml with HPLC grade water and PH is adjusted to 2.2.

Preparation of Mobile Phase
A Combination of Methanol (50%) and 0.1N Orthophosphoric Acid buffer (PH2.2) (50%) was mixed and degassed in ultrasonic water bath for 5 minutes finally filtered through 0.45µ membrane filter. This prepared solution was used as mobile phase.

Diluent
HPLC grade water was used as diluent.

Standard Stock Solution Preparation
Weighed and transferred 200mg of Rifampicin working standard 300mg of Isoniazid working standard and 10mg of Piperine working standard into 100 mL volumetric flask, added 50 mL of diluent and sonicate to dissolve and dilute to volume with diluent.

Preparation of Standard Solution
Weighed and transferred 200mg of Rifampicin working standard 300mg of Isoniazid working standard and 10mg of Piperine working standard into 100 mL volumetric flask, added 50 mL of diluent and sonicate to dissolve and dilute to volume with diluent. From this solution 5ml was taken into a 25ml volumetric flask and made up to the mark with diluent.

Preparation of Sample Solution
Finely grind preweighed 20 tablets. Transfer grinded sample quantitatively equivalent to 200mg of Rifampicin 300mg of Isoniazid and 10mg of Piperine into 100mL volumetric flask, added 20mL of diluent, sonicate to dissolve for 20 minutes and diluted to volume with diluent. Further filter the solution through filter paper.

Selection of Chromatographic Methods
The proper selection of methods depends upon the nature of the sample (ionic or ionisable or neutral molecule) its molecular weight and stability. The drugs selected are polar, ionic and Reversed phase chromatography can be used because of its simplicity and suitability.

Optimized Method
Chromatographic Conditions
Mobile Phase: 0.1N OPA:Methanol (60:40), Column Temperature: 30oC, Flow Rate: 1mL/min, λmax: 282.1 nm, Injection Volume: 10µL, Run Time: 7 min and Detector: Waters 2998 Photo Diode Array detector. Inference: Sharp peaks at 2.541min (INH) and 3.679min (RFP) and 5.360min (PIP) were eluted and they were well separated at lower concentration. Mode of Operation: Isocratic. Stationary Phase: Waters xterra C18 column (250 mm x 4.6 mm I.D; particle size 5 µm).

Validation of Developed RP-HPLC Method[6]
As per the International Conference on Harmonization (ICH) guidelines the method validation parameters such as specificity, linearity, precision, accuracy, limit of detection/quantitation and robustness were optimized,

System Suitability
The standard I solution was injected one time and standard II solution was injected 5 times.

Acceptence Criteria
Tailing factor for the peaks due to Isoniazid, Rifampicin and Piperine in Standard solution Should not be more than 2. Theoretical plates for the Isoniazid, Rifampicin and Piperine peaks in Standard solution Should not be less than 2500.

Accuracy
The accuracy of the method shall be demonstrated through determination on samples in three concentrations from 50% (300.0µg/mL for INH; 200.0µg/mL for RFP; 10.0µg/mL for PIP), 100% (600.0µg/mL for INH; 400.0µg/mL for RFP; 20.0µg/mL for PIP), and 150% (900.0µg/mL for INH; 600.0µg/mL for RFP; 30.0µg/ml fog PIP), (Three replicates from 100% and six replicates from 50% & 100%) of the theoretical concentrations employed as per the usual procedure and the chromatograms were recorded.

Acceptance Criteria
The % Recovery for each level should be between 97.0 to 103.0%.

Precision
Weighed and transferred 200mg of Rifampicin working standard 300mg of Isoniazid working standard and 10mg of Piperine working standard into 100 mL volumetric flask, added 50 mL of diluent and sonicate to dissolve and dilute to volume with diluents. The sample solution was injected for six times and measured the area for all six injections in HPLC.

Acceptance Criteria
The % RSD for the area of six standard injections results should not be more than 2%.

Limit of Detection (LOD)
From the above preparation 1ml of solution is transferred to 10ml of volumetric flask and the volume made with the diluent.

Limit of Quantitation (LOQ)
From the above preparation 0.5ml of solution is transferred to 10ml of volumetric flask and the volume made with the diluent.

Linearity
Injected each level into the chromatographic system and measured the peak area. Ploted a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculated the correlation coefficient.

Acceptance Criteria
Correlation coefficient should be not less than 0.999.

Specificity
The blank, placebo, standard I and standard II solutions were injected. Blank, placebo and standard I were injected one time and the standard II was injected 5 times.

Robustness
As part of the Robustness, deliberate change in the Flow rate, Temperature Variation were made to evaluate the impact on the method.

Sample solution was injected and analysed using the varied flow rates (1±0.2) along with method flow rate and sample solution was injected and analysed using the varied temperatures (350C±5).

ASSAY
Sample and standard was injected into the chromatographic system and measured the areas for INH, RFP and PIP peaks and calculated the % Assay by using the formulae.

Calculation: (For Isoniazid, Rifampicin and Piperine)

$$\frac{AT}{AS} X \frac{WS}{DS} X \frac{DT}{WT} X \frac{P}{LC} X \frac{Avg.wt}{100} X\ 100$$

Where:
AT = average area of sample. WT = Weight of sample taken in mg.
AS = average area of standard. DS = Dilution factor of standard solution.
WS = Weight of working standard taken in mg. DT = Dilution factor of sample solution.
P= Percentage purity of working standard. LC = label claim of INH, RFP and PIP

RESULTS AND DISCUSSION

Optimized method
It was performed on Waters xterra C18 (250 mm x 4.6 mm I.D; particle size 5 µm) with a mobile phase composition of 0.1N OPA: Methanol in the ratio of 60:40 at a flow rate of 1 mL/min. 10 µL of sample was injected and the analytes are monitored at 282.1 nm. The run time was 7 min.

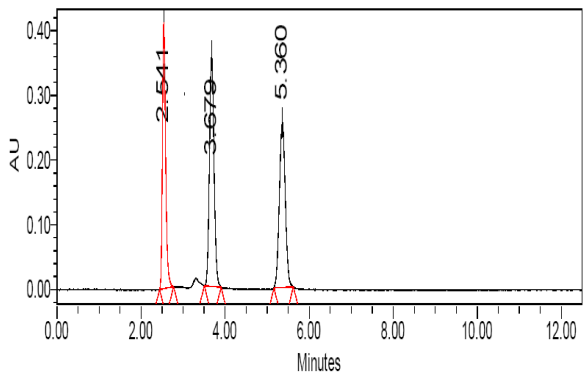

Fig 1: Typical Chromatogram of INH, RFP and PIP.

ASSAY: The % assay can be calculated by using the formula,

$$\frac{AT}{AS} X \frac{WS}{DS} X \frac{DT}{WT} X \frac{P}{LC} X \frac{Avg.wt}{100} X\ 100$$

Potency of INH: 99.1%
Potency of RFP: 99.7%
Potency of PIP: 99.1%

Table 1: Assay results.

S. No.	Sample Weight	Sample Area (INH)	% Assay of INH	Sample Area (RFP)	% Assay of RFP	Sample area (PIP)	% Assay of PIP
1	640.00	2282283	99	2771510	98	2655290	99
2	640.00	2288324	99	2783859	99	2654018	98
3	640.00	2311239	100	2798255	99	2651483	98
4	640.00	2278057	99	2784180	99	2659025	99
5	640.00	2297412	100	2793067	99	2657838	99
6	640.00	2291463	100	2786174	99	2654328	99
Avarage Assay:			100		99		99
STD			0.51		0.32		0.10
%RSD			0.52		0.33		0.10

System suitability

Table 2: System suitability parameters.

Parameter	INH	RFP	PIP
Retention time	2.541	3.679	5.360
Resolution	-	6.60	7.14
Theoretical plates	5505	5366	6917
Tailing	1.33	1.10	1.04

Accuracy
The mean percentage recoveries for INH, RFP and PIP were found to be 100%, 99% and 99% respectively. The high percentage recovery indicates that the proposed method was highly accurate.

Precision

Table 3: Precision results for INH.

	SampleName	Inj	Name	RT	Area
1	PRECISION1	1	ISONIAZID	2.511	2294901
2	PRECISION2	1	ISONIAZID	2.511	2292197
3	PRECISION3	1	ISONIAZID	2.507	2291629
4	PRECISION4	1	ISONIAZID	2.510	2295093
5	PRECISION5	1	ISONIAZID	2.510	2291463
6	PRECISION6	1	ISONIAZID	2.509	2296459

Table 4: Precision results for PIP.

	SampleName	Inj	Name	RT	Area
1	PRECISION1	1	PIPERINE	5.280	2655290
2	PRECISION2	1	PIPERINE	5.281	2654018
3	PRECISION3	1	PIPERINE	5.281	2651483
4	PRECISION4	1	PIPERINE	5.282	2659025
5	PRECISION5	1	PIPERINE	5.281	2657838
6	PRECISION6	1	PIPERINE	5.282	2654328

Table 5: Precision results for RFP.

	SampleName	Inj	Name	RT	Area
1	PRECISION1	1	RIFAMPICN	3.603	2783194
2	PRECISION2	1	RIFAMPICN	3.604	2788165
3	PRECISION3	1	RIFAMPICN	3.603	2788479
4	PRECISION4	1	RIFAMPICN	3.602	2784285
5	PRECISION5	1	RIFAMPICN	3.601	2789021
6	PRECISION6	1	RIFAMPICN	3.603	2780055

The % RSD values of INH, RFP and PIP were found to be 0.6, 0.4 and 0.4 respectively. The low % RSD values (below 2) indicates that the method was Precise.

Limit of Detection (LOD)

The LOD values for INH, RFP and PIP were 7.129, 23.763 and 36.276 respectively.

Limit of Quantitation (LOQ)

The limit of detection and limit of quantitation were performed and the results indicates the sensitivity of the method.

Linearity

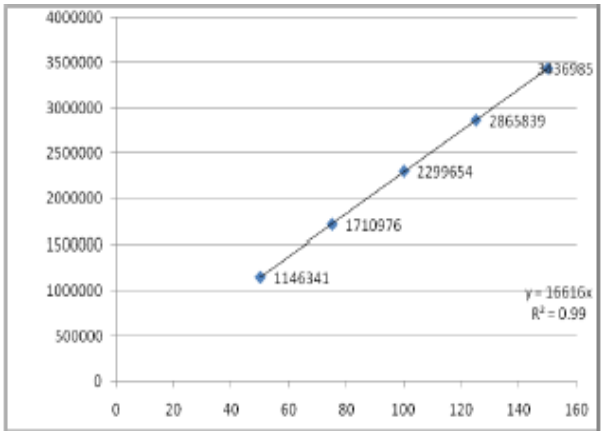

Fig 2: Calibration curve of INH.

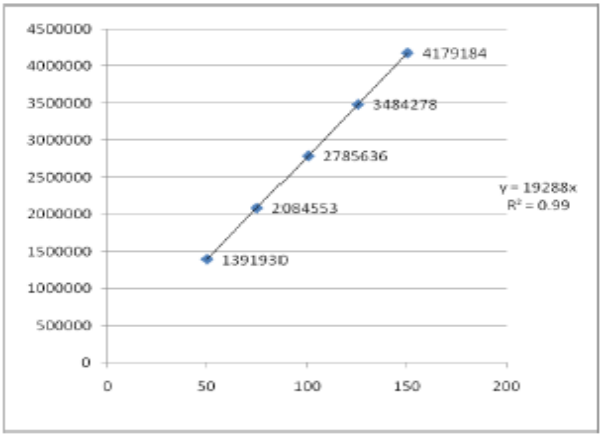

Fig 3: Calibration curve of RFP.

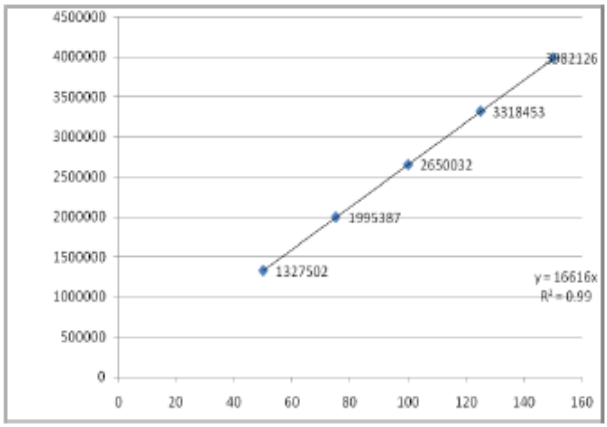

Fig 4: Calibration curve of PIP.

Table 6: Regression equation of linearity plots and coefficient of correlation.

Drug	Conc.(µg/ml)	Equation of regression line	R^2
INH	300-900	Y = 16616x	0.999
RFP	200-600	Y = 19288x	0.9990
PIP	10-30	Y = 16616x	0.99

From the above data it was observed that Quantitative linearity was obeyed in the concentration range of 300-900, 200-600 and 10-30 µg/mL of INH, RFP and PIP respectively. The regression equations of concentration over their peak areas were found to be Y = 16616x (R2 = 0.999), Y = 19288x (R2 = 0.9990) and Y = 16616x (R2 = 0.99) for INH RFP and PIP respectively where Y is the peak area and x is concentration of drugs (µg/mL).

Robustness

Robustness of the method was performed by varying the flow rate of mobile phase.

Table 7: pH of buffer and buffer concentration.

	SampleName	Inj	Name	RT	Area	USP Resolution	USP Tailing	USP Plate Count	s/n
1	FLOW1	1	ISONIAZID	3.118	2875370		1.270	6972	329.225
2	FLOW2	1	ISONIAZID	2.511	2232197		1.292	5296	342.805
3	TEMP1	1	ISONIAZID	2.507	1351629		1.325	5146	174.066
4	TEMP2	1	ISONIAZID	2.510	2235093		1.300	5230	288.136

Table 8: Robustness for INH.

	SampleName	Inj	Name	RT	Area	USP Resolution	USP Tailing	USP Plate Count	s/n
1	FLOW1	1	RIFAMPICN	4.476	3488277	7.157	1.110	6531	276.080
2	FLOW2	1	RIFAMPICN	3.604	2708165	2.461	1.126	5355	295.120
3	TEMP1	1	RIFAMPICN	3.603	1628479	2.026	1.136	5271	151.340
4	TEMP2	1	RIFAMPICN	3.602	2694285	0.551	1.123	5448	247.523

Table 9: Robustness for RFP.

	SampleName	Inj	Name	RT	Area	USP Resolution	USP Tailing	USP Plate Count	s/n
1	FLOW1	1	PIPERINE	6.588	3268717	8.067	1.026	8003	195.067
2	FLOW2	1	PIPERINE	5.281	2554018	7.257	1.046	6991	218.040
3	TEMP1	1	PIPERINE	5.281	1551483	7.402	1.036	6682	111.741
4	TEMP2	1	PIPERINE	5.282	2569025	7.293	1.046	6858	182.904

From the above data it was found the asymmetric factor was less than 2.0 and theoretical plates were more than 2000 for INH, RFP and PIP peaks, which illustrates the good robustness of the developed method.

Specificity

Specificity is the method performed for checking the excipients and solvent effect on the developed method by injecting them seperately.

Table 10: Results for specificity.

	Specificity	INH		RFP		PIP	
S.No.	Sample Name	RT	Area	RT	Area	RT	Area
1	Blank	-	-	-	-		
2	INH Standard	2.513	2280879				
3	RFP Standard			3.625	2807755		
4	PIP Standard					5.316	2670249
5	Mixed Standard	2.513	2288324	3.616	2783859	5.296	2662716
6	Placebo						
7	Sample	2.513	2297412	3.606	2793067	5.281	2665772

CONCLUSION

The developed HPLC method was applied for simultaneous estimation of Isoniazid, Rifampicin and Piperine in Pharmaceutical dosage forms. No interfering peaks were found in the chromatogram indicating that excipients used in capsule formulations didn't interfere with the estimation of the drugs by the proposed HPLC method. Thus, it concludes a simple, sensitive, precise and accurate RP-HPLC method was developed and validated for the simultaneous estimation of Isoniazid, Rifampicin and Piperine in bulk and pharmaceutical dosage form.

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