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DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF RIFAMPICIN AND ISONIAZID IN BULK AND TABLET DOSAGE FORM

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Isoniazid, Rifampicin, HPLC method, ICH Gui

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ABSTRACT: A new HPLC method has been developed and validated for the simultaneous estimation of Rifampicin and Isoniazid, offering a reliable and efficient approach for their analysis. The method employs a mobile phase composed of pH 3.5 phosphate buffer, methanol, and water in a 45:30:25 ratio. It demonstrates a linear response for both drugs within specific concentration ranges, allowing for accurate quantification. Detection is performed at 239 nm, with retention times of 2.8 minutes for Rifampicin and 3.7 minutes for Isoniazid. Accuracy studies have shown excellent recovery rates within ICH guidelines, ensuring the reliability of the results. Notably, the method exhibits high precision with RSD values within acceptable limits, indicating its reproducibility. It has also been found to be simple, rapid, robust, and reproducible, making it well-suited for routine use. This validated HPLC method holds significant potential for both pharmaceutical and clinical applications, enabling accurate and efficient analysis of Rifampicin and Isoniazid in bulk and tablet dosage forms.

INTRODUCTION: Rifampicin is an antibiotic used to treat numerous types of mycobacterial infections including Mycobacterium avium complex, leprosy, and in conjunction with other antibacterials to treat latent or active tuberculosis. A semisynthetic antibiotic generated from Streptomyces mediterranei. It has a broad antibacterial range, including effectiveness against numerous types of Mycobacterium. In sensitive organisms it inhibits DNA-dependent RNA polymerase activity by creating a stable compound with the enzyme. It thereby suppresses the beginning of RNA synthesis. Rifampin is antibacterial and operates on both intracellular and extracellular organisms [1].

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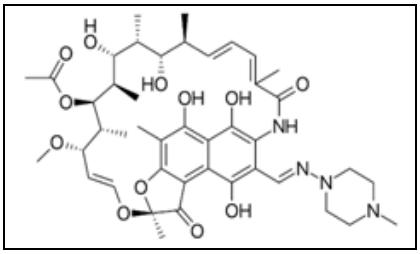

FIG. 1: STRUCTURE OF RIFAMPICIN

Isoniazid is an antibiotic used to treat mycobacterial infections; most typically usage in combination with other antimycobacterial medicines for the treatment of active or latent tuberculosis. Antibacterial agent used largely as a tuberculostatic. It remains the therapy of choice for tuberculosis [2].

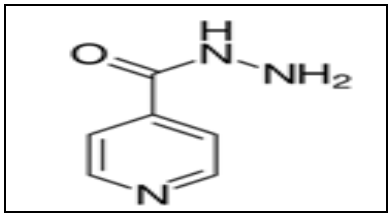

FIG. 2: STRUCTURE OF ISONIAZID

MATERIALS AND METHOD:

Instruments: The chromatographic method was performed Analytical Technologies HPLC system coordinated with a variable wavelength programmable UV identifier and a Rheodyne injector outfitted with 20µl fixed circle. An opposite stage Cosmosil C18 (250mm x 4.6ID, Particle size: 5 micron) was utilized. Wenser High Precision Balance Model: PGB 100 electronic equilibrium were utilized for Spectrophotometric judgments and gauging purposes individually [3].

Reagents and Chemicals: Rifampicin and Isoniazid was procured from Pharma Tech Solutions. HPLC grade Acetonitrile and water were acquired from Merck specialities private restricted, Mumbai.

Chromatographic Conditions: Cosmosil C18 (250mm x 4.6ID, Particle size: 5 micron) was utilized for the chromatographic method at wavelength of 239 nm. pH 3.5 Phosphate buffer: Methanol: Water (45:30:25) was chosen as mobile phase for elution and the same solvent was utilized in the preparation of standard and sample solutions [4]. The elution was checked by infusing the 20µl and the flow rate was changed in accordance with 0.8 ml/min.

Preparation of Standard Stock Solutions: Accurately Weighed and transferred 9 mg of Rifampicin and 6 mg of Isoniazid working Standards into a 100ml clean dry volumetric flask, add 3/4th volume of diluent, sonicated for 5 minutes and make up to the final volume with diluents. and the final concentration of Rifampicin is 90 µg/mL and 60 µg/mL is of Isoniazid. The working standard solutions of these drugs were obtained by appropriate dilution of the respective stock solution with mobile phase [5].

Preparation of Mobile Phase A (pH 3.5 Phosphate Buffer): Dissolve 68.0 g of potassium dihydrogen phosphate in water and dilute to 1000.0 ml with the same solvent. Adjust the pH 3.5 with o-phosphoric Acid. Mobile phase was filtered through 0.45µm membrane filter and degassed by sonication for 20 min [6].

Preparation of Mobile Phase B: 100 % Methanol

Selection of Mobile Phase: Standard solutions of Rifampicin (45µg/mL) and Isoniazid (30µg/mL) were injected into the RP-HPLC system and run in different solvent systems[7]. Different mobile phases systems like Phosphate buffer and methanol were initially tried in the isocratic mode in order to determine the best conditions.

HPLC Method Development:

Optimisation of RP-HPLC Method: The HPLC technique was designed for the simultaneous measurements of Rifampicin and Isoniazid. Different mobile phases were gone after for the process optimisation, nevertheless adequate retention periods, hypothetical plates and high resolution were found with pH 3.5 Phosphate buffer: Methanol: Water (45:30:25) utilizing Cosmosil C18 (250mm x 4.6ID, Particle size: 5 micron) using gradient technique [8].

TABLE 1: OPTIMIZED CHROMATOGRAPHIC CONDITIONS

Mobile phase	pH 3.5 Phosphate buffer: Methanol: Water (45:30:25)
Selection of column	Cosmosil C18 (250mm x 4.6mm ID, Particle size: 5 µm)
Injection volume	20 µL
Flow rate	0.8 ml/min
Column temperature	Room Temperature
Detection wavelength	239 nm
Run Time	6.0 minutes
Retention time	Rifampicin (2.8 min) and Isoniazid (3.7 min)

Validation of RP-HPLC Method: Validation of the optimized RP-HPLC method was performed in accordance with the ICH Q2 (R1) guidelines [9].

Linearity: Test solutions of different concentration were injected separately, and the chromatograms were recorded [10].

A series of test preparations of Rifampicin and Isoniazid were prepared by taking 1 ml - 5 ml from the stock solution containing Rifampicin (450µg/mL) and Isoniazid (300µg/mL) respectively in five 10 ml volumetric flask and final volume make up to the mark with mobile phase. A 20 µl volume of each concentration was injected into HPLC, three times under the optimized chromatographic conditions [11].

Accuracy: Samples are prepared normally covering 50 % to 150 % of the nominal sample preparation concentration. These samples are analyzed and the recoveries of each are calculated.

Precision: Intraday precision study was carried out by preparing test solution of same concentration and analyzing it at three different times in a day [12]. The same procedure was followed for two different days to determine interday precision [13]. The result was reported as % RSD.

Limit of Quantitation (LOQ) & Limit of Detection (LOD): The LOD and LOQ were analysed from the slope(s) of the calibration curve and the standard deviation (SD) of the peak areas using the formula [14]:

LOD = 3.3 s/s and LOQ = 10 s/s

Robustness: Robustness was assessed by modifying the chromatographic conditions such as mobile phase composition, detection wavelength, flow rate, and so on, and the % RSD should be supplied [15]. Small alterations were tolerated under the ideal circumstances, and the method's resilience was established. Individual variations of ± 2 nm in detecting wavelength and ± 0.1 ml/min in flow rate were tried. In triplicate, solutions of 100% test concentration with the required adjustments in the optimal circumstances were injected into the system [16].

Ruggedness: Ruggedness is the research of the effect of external circumstances on the approach. To evaluate the robustness of the offered strategy, elements were purposely varied. These influences included system variance, diverse analysis, and atmospheric changes [17]. Two different analysts prepared the test solution according to the test method and injected three doses of test solution into the HPLC system at a flow rate of 0.8 ml/min [18].

Assay of Marketed Formulation: 20 tablets of marketed formulation (AKT-2) of Lupin Pharmaceutical were taken, weighed individually, and crushed into fine powder. Average weight of tablet sample was weighed and transferred to 1000 mL volumetric flask & diluent was added to make up the volume. Sonicate for 10 min with occasional swirling [19]. The above solution was filtered through 0.45µm membrane filter. The prepared stock solution is of 450 µg/ml of Rifampicin and 300 µg/ml of Isoniazid. For Analysis 3 ml solution was withdrawn and diluted up to 10 ml and injected into system [20].

System Suitability: To verify the system, procedure, and column performance, system suitability features were studied. Six times a standard solution of Rifampicin and Isoniazid was injected into the system, and system suitability properties were analyzed [21].

RESULT AND DISCUSSION:

Linearity: It was clarified from the analytical method linearity as the ability of the method to obtain test results that are directly proportional to the analyte concentration, within a specific range [22]. The peak area obtained from the HPLC chromatograph was plotted against corresponding concentrations to obtain the calibration graph. Isoniazid was found to be linear in the concentration range of 6-30 µg/ml and Rifampicin is in the range of 9-45 µg/ml [23].

TABLE 2: SUMMARY OF RESULTS OF LINEARITY

Sr. no.	Rifampicin		Isoniazid	
	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area
1	9	689341	6	320145
2	18	1325580	12	633982
3	27	2038458	18	910452
4	36	2798401	24	1189301
5	45	3476598	30	1463089

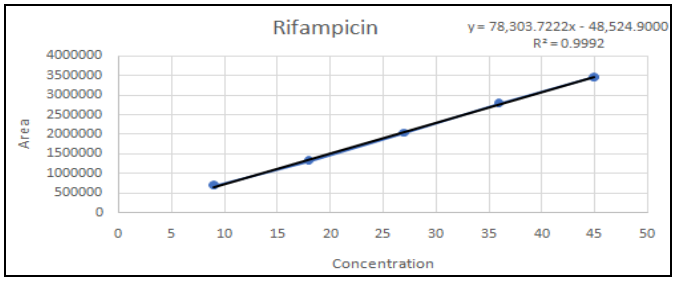

FIG. 3: CALIBRATION CURVE FOR RIFAMPICIN

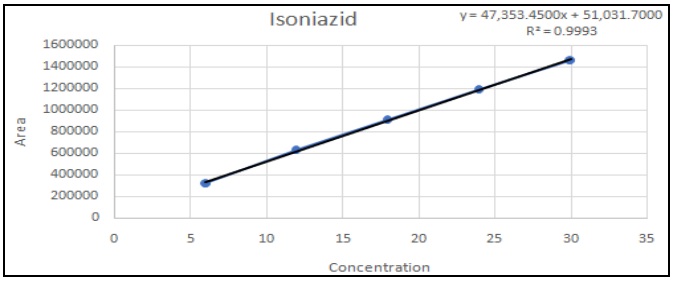

FIG. 4: CALIBRATION CURVE FOR ISONIAZID

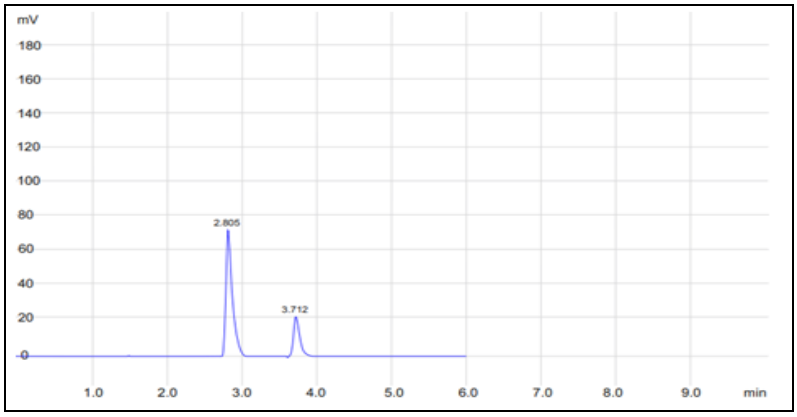

FIG. 5: CHROMATOGRAPH OF RIFAMPICIN AND ISONIAZID

Accuracy: The accuracy of the method determines the closeness of results obtained by that method to the true value. From the results of accuracy testing, it was showed that the method is accurate within the acceptable limits [24].

The % RSD is calculated for the Rifampicin and Isoniazid and all the results are within limits. Acceptable accuracy was within the range and not more than 2.0% RSD [25].

TABLE 3: STATISTICAL VALIDATION FOR ACCURACY OF RIFAMPICIN

Level of addition	% Mean recovery*	SD	% RSD
50%	99.18	0.65	0.65
100%	100.30	0.64	0.64
150%	99.59	0.70	0.70

TABLE 4: STATISTICAL VALIDATION FOR ACCURACY OF ISONIAZID

Level of addition	% Mean recovery*	SD	% RSD
50%	100.09	0.49	0.49
100%	100.99	0.40	0.40
150%	99.79	1.15	1.15

Precision: Intraday and interday precision assures the repeatability of test results. The % RSD found was below 2 for both Rifampicin and Isoniazid.

TABLE 5: DATA FOR INTRADAY PRECISION OF RIFAMPICIN

Sr. no.	Conc. (µg/mL)	Area	Mean	SD	%RSD
1	9	678554			
2	9	689564	688919.67	10058.99	1.46
3	9	698641			
4	27	2032657			
5	27	2025465	2024537.00	17243.08	0.85
6	27	2015489			
7	45	3457971			
8	45	3456892	3460969.33	6151.41	0.18
9	45	3468045			

TABLE 6: DATA FOR INTERDAY PRECISION OF RIFAMPICIN

Sr. no.	Conc. (µg/mL)	Area	Mean	SD	%RSD
1	9	698654			
2	9	681870	689855.33	8421.51	1.22
3	9	689042			
4	27	2035454			
5	27	2030145	2029915.33	5657.00	0.28
6	27	2024147			
7	45	3487645			
8	45	3478604	3487133.00	8284.87	0.24
9	45	3495150			

TABLE 7: DATA FOR INTRADAY PRECISION OF ISONIAZID

Sr. no.	Conc. (µg/mL)	Area	Mean	SD	%RSD
1	0.5	987014			
2	0.5	996317	987260.67	8935.55	0.91
3	0.5	978451			
4	1.5	2681402			
5	1.5	2671841	2672085.00	18394.86	0.69
6	1.5	2663012			
7	2.5	4410215			
8	2.5	4425148	4419068.33	7843.38	0.18
9	2.5	4421842			

TABLE 8: DATA FOR INTERDAY PRECISION OF ISONIAZID

Sr. no.	Conc. (µg/mL)	Area	Mean	SD	%RSD
1	0.5	987452			
2	0.5	978420	988138.33	10079.04	1.02
3	0.5	998543			
4	1.5	2678131			
5	1.5	2681041	2685191.00	9816.57	0.37
6	1.5	2696401			
7	2.5	4412545			
8	2.5	4420236	4415935.33	3925.48	0.09
9	2.5	4415025			

Robustness: Robustness was studied by different deliberate variations in the chromatographic conditions i.e. Change in flow rate and wavelength. From robustness study % RSD was found to be within limit of 2 % for the Rifampicin and Isoniazid [23]. Hence, it is robust and complies per ICH guidelines [26].

TABLE 9: DATA FOR ROBUSTNESS STUDY OF RIFAMPICIN AND ISONIAZID

Sr. no.	Parameter	Condition	Rifampicin				Isoniazid			
			Area	Mean	SD	%RSD	Area	Mean	SD	%RSD
1	Change in Flow	0.7	2035641	2033745	6550	0.32	2686626	2682514	9686	0.36
2	rate (ml/min)	0.8	2026456				2671450			
3		0.9	2039140				2689465			
1	Change in	237	2036648	2033368	5966	0.29	916840	915847	4879	0.53
2	Wavelength (nm)	239	2026481				920154			
3		241	2036975				910548			

Ruggedness: Ruggedness was studied by different analysts. From robustness study % RSD was found to be within limit of 2 % for the Rifampicin and Isoniazid. Hence it is complying as per ICH guidelines.

TABLE 10: DATA FOR RUGGEDNESS STUDY OF RIFAMPICIN AND ISONIAZID

Sr. no.	Analyst	Rifampicin				Isoniazid			
		Area	Mean area*	SD	% RSD	Area	Mean area*	SD	% RSD
1	Analyst-I	2035647	2031736	7057	0.35	913504	918101	4431	0.48
		2023589				922347			
		2035971				918452			
2	Analyst-II	2035641	2034333	5046	0.25	906647	913353	5809	0.64
		2028761				916845			
		2038597				916567			

Specificity: Excipients and impurities were not interacting with the standard drugs. Hence the method is specific.

TABLE 11: DATA FOR SPECIFICITY STUDY OF RIFAMPICIN AND ISONIAZID

Drug	Drug conc. (µg/ml)	Excipients (µg/ml)	Total conc. (µg/ml)	Area	Mean	SD	%RSD

Rifampicin	9	18	27	681545			
	9	18	27	670124	676969.67	6039.50	0.89
	9	18	27	679240			
	18	18	36	1312507			
	18	18	36	1309426	1315993.67	8842.53	0.67
	18	18	36	1326048			
	27	18	45	2023564			
	27	18	45	2035670	2031627.33	6983.06	0.34
	27	18	45	2035648			
Isoniazid	6	12	18	326968			
	6	12	18	320945	325851.00	4453.82	1.37
	6	12	18	329640			
	12	12	24	640344			
	12	12	24	635668	638168.00	2354.78	0.37
	12	12	24	638492			
	18	12	30	908965			
	18	12	30	910246	912150.67	4454.64	0.49
	18	12	30	917241			

% Assay of Marketed Formulation: The % Assay of (AKT-2) marketed formulation of Lupin Pharmaceutical was calculated.

TABLE 12: DATA OF % ASSAY OF MARKETED FORMULATION

Sr. no.	Drug	Area of Sample	Area of Standard	% Assay
1	Rifampicin	2012546	2038458	98.73
2	Isoniazid	908248	910452	99.76

System Suitability Parameters: System suitability parameters were measured to verify the system, method and column performance. Standard solution of Rifampicin and Isoniazid was injected in to the system for six times and system suitability parameters were checked [27].

TABLE 13: SYSTEM SUITABILITY PARAMETER

Sr. no.	Isoniazid			Rifampicin		
	Retention Time (min)	Theoretical plates	Asymmetry Factor	Retention Time (min)	Theoretical plates	Asymmetry Factor
1	2.824	9055	1.07	3.902	10244	1.09
2	2.814	9562	1.08	3.809	11048	1.09
3	2.798	9922	1.08	3.964	10663	1.08
4	2.814	9823	1.07	4.075	11546	1.1
5	2.862	9716	1.08	3.811	10696	1.09
6	2.81	9736	1.07	3.904	10967	1.1
Mean			1.08			1.09
SD			0.01			0.01
%RSD			0.51			0.69

Summary: The Rifampicin was found to be linear in the concentration range of 9-45 µg/ml and Isoniazid is 6-30 µg/ml. From Accuracy study % recovery of Rifampicin was found in the range of 98.72-100.98% and Isoniazid is 98.77-101.45% which is in the limits accordingly the ICH guidelines [28]. Intraday and Interday precision assures that % RSD was within limits of ICH guidelines i.e., NMT 2 for both Rifampicin and Isoniazid. Limit of detection and limit of Quantitation of Rifampicin is 0.24µg/ml – 0.72µg/ml and Isoniazid is 0.06 µg/ml – 0.19 µg/ml respectively [29]. Robustness was studied by deliberate variation i.e., change in Flow rate and change in Wavelength which was within 2 % of RSD as per ICH guidelines.

The ruggedness study gives results within the limits of 2% in which variation in Analyst was studied30. The % assay of AKT-2 was found to be Rifampicin (98.73%) and Isoniazid (98.76%).

CONCLUSION: The proposed chromatographic method for determining Rifampicin and Isoniazid from pure and dosage forms was found to be simple, precise, accurate, rapid, and specific. The mobile phase employed for method development is relatively easy to make and affordable likewise.

The sample recoveries in the formulation were giving excellent results. This approach is inexpensive and run time is comparatively short which permits speedy analysis among all the created methods and consequently, all the investigated validation parameters provided acceptable results with appropriate correlation co-efficient and lower % RSD as per the ICH criteria. The discovered approach may be applied by industry for quantitative simultaneous measurement of Rifampicin and Isoniazid as bulk and in tablet dosage form.

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CONFLICT OF INTREST: The authors declare no conflict of interest.

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