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METHOD DEVELOPMENT AND VALIDATION FOR ETHAMBUTOL AND ISONIAZID BY RP-HPLC PROCESS

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Abstract:
A straightforward, Accurate, specific approach was created for the concurrent assessment of the Isoniazid and Ethambutol in Tablet dimension structure. Chromatogram was experienced Inertial ODS C185 m (4.6 x 250mm). Portable phase having Phosphate support and also Acetonitrile in the percentage of 30:70 was siphoned through segment at a stream pace of 1ml/min. Cradle made use of at pH 4.6. Temperature was stayed on top of at Ambient. Boosted regularity for Isoniazid as well as Ethambutol was 260nm. Maintenance period of Isoniazid and Ethambutol were considered as 2.395 minutes and also 3.906 min. The percentage merit of Isoniazid and Ethambutol was considered as 100.6 percentage and also 101.3 percentage independently. The framework appropriateness boundaries for Isoniazid and also Ethambutol, for example, theoretical plates and complying with element were deemed 1.3, 1012.4 and also 1.2, 1848.2 the objective was viewed as 9.0. The linearity research for Isoniazid and also Ethambutol was discovered in focus scope of 1µg-5µg as well as 100µg-500µg and also link coefficient (r2) was considered as 0.999 and also 0.999, percentage mean recuperation was considered as 100.1 percentage and 100.4 percentage, percentage RSD for repeatability was0.31 and 0.38, percentage RSD for middle of the road accuracy was 0.12 and 0.15 separately the accuracy study was precise, powerful and repeatable. LOD esteem was 2.94 as well as 3.03, and also LOQ esteem was 9.87 and 10.1 individually.

Keywords: *Isoniazid, Ethambutol, RP-HPLC*

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INTRODUCTION:

Ethambutol is indicated in combination with other anti-tuberculosis drugs in the treatment of pulmonary tuberculosis.[1] Ethambutol is commonly used in combination with isoniazid, rifampin, and pyrazinamide.[2] Ethambutol diffuses into *Mycobacterium* cells. Once inside the cell, ethambutol inhibits the arabinosyltransferases (embA, embB, and embC), preventing formation of the cell wall components arabinogalactan and lipoarabinomannan, and preventing cell division.[3] Decreased concentrations of arabinogalactan in the cell wall reduces the number of binding sites for mycolic acid, leading to the accumulation of mycolic acid, trehalose monomycolate, and trehalose dimycolate. Lipoarabinomannan is a component of a cell surface molecule involved in the interaction with host cells. Reduced levels of lipoarabinomannan may interfere with mycobacterial interaction with host cells.[4] IUPAC name (2S)-2-[(2-{[(2S)-1-hydroxybutan-2-yl]amino}ethyl)amino]butan-1-ol. Molecular formula $C_{10}H_{24}N_2O_2$. Molecular Weight 204.3.

Isoniazid is an antibiotic used to treat mycobacterial infections; most commonly use in combination with other antimycobacterial agents for the treatment of active or latent tuberculosis.[5] Isoniazid is a prodrug and must be activated by bacterial catalase. Specficially, activation is associated with reduction of the mycobacterial ferric KatG catalase-peroxidase by hydrazine and reaction with oxygen to form an oxyferrous enzyme complex.[6] Once activated, isoniazid inhibits the synthesis of mycoloic acids, an essential component of the bacterial cell wall.[7] At therapeutic levels isoniazid is bacteriocidal against actively growing intracellular and extracellular Mycobacterium tuberculosis organisms. Specifically, isoniazid inhibits InhA, the enoyl reductase from Mycobacterium tuberculosis, by forming a covalent adduct with the NAD cofactor.[8] It is the INH-NAD adduct that acts as a slow, tight-binding competitive inhibitor of InhA. IUPAC Name pyridine-4-carbohydrazide. Molecular Formula $C_6H_7N_3O$. Molecular Weight 137.13.

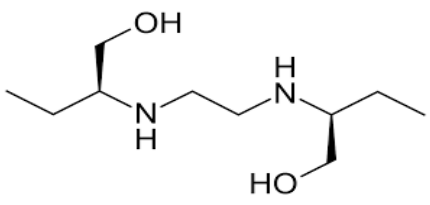

Figure 1: Structure of Ethambutol

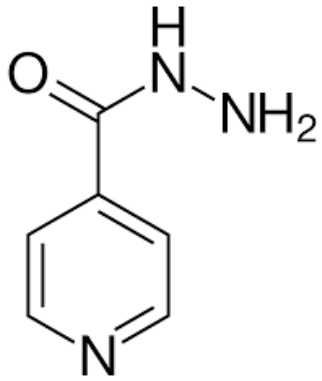

Figure 2: Structure of Isoniazid

The literature survey revealed that There are very few methods reported in the literature for analysis of Ethambutol and Isoniazid alone or in combination with other drugs in the pure form and pharmaceuticals formulations by RP-HPLC.[3-9] In view of the need for a suitable, cost-effective RP-HPLC method for routine analysis of Ethambutol and Isoniazid Simultaneous estimation of in pharmaceutical dosage form. Attempts were made to develop simple, precise, accurate and cost-effective analytical method for the estimation of Ethambutol and Isoniazid. The proposed method will be validated as per ICH guidelines. The objective of the proposed work is to develop a new, simple, sensitive, accurate and economical analytical method and validation for the Simultaneous estimation of Ethambutol and Isoniazid in pharmaceutical dosage form by using RP-HPLC. To validate the developed method in accordance with ICH guidelines for the intended analytical application i.e., to apply the proposed method for analysis of the drug in its dosage form.

MATERIALS AND METHODS:

Chemicals and Reagents: Ethambutol and Isoniazid were Purchased from market. NaH_2PO_4 was analytical grade supplied by Finerchem limited, Orthophosphoric acid (Merck), and Water and Methanol for HPLC (Lichrosolv (Merck).

Equipment and Chromatographic Conditions: The chromatography was performed on a Waters 2695 HPLC system, equipped with an auto sampler, UV detector and Empower 2 software. Analysis was carried out at 235 nm with column Inertsil ODS C185µm (4.6 x 250mm), dimensions at 25^0C temperature. The optimized mobile phase consists of Phosphate buffer and Acetonitril in the ratio of 30:70. Flow rate was maintained at 1 ml/min.

Preparation of solutions:

Preparation of buffer:

Weighed 6.8 grams of KH_2PO_4 was taken into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC water, adjusted the pH to 4.6 with ortho phosphoric acid.

Preparation of mobile phase:

A mixture of pH 4.6 Phosphate buffer 300 mL (30%), 700 mL of ACN (70%) are taken and degassed in ultrasonic water bath for 5 minutes. Then this solution is filtered through 0.45 µ filter under vacuum filtration.

The diluents:

The Mobile phase was used as the diluent.

Preparation of the individual Isoniazid standard preparation:

100 mg of working standard was accurately weighed and transferred into a 10 ml clean dry volumetric flask and about 2 ml of DMF is added. Then it is sonicated to dissolve it completely and made volume upto the mark with the diluant. (Stock solution). Further 10.0 ml from the above stock solution is pipette into a 100 ml volumetric flask and was diluted upto the mark with diluant.

Preparation of the individual Ethambutol standard preparation:

15 mg of Ethambutol working standard was accurately weighed and transferred into a 10ml clean dry volumetric flask and about 2ml of DMF is added. Then it is sonicated to dissolve it completely and made volume upto the mark with the diluant. (Stock solution). Further 10.0 ml from the above stock solution is pipette into a 100 ml volumetric flask and was diluted upto the mark with diluant.

Preparation of Sample Solution:

Accurately 10 tablets are weighed and crushed in mortar and pestle and weight equivalent to 100 mg of Isoniazid and 15 mg of Ethambutol (marketed formulation) sample into a 10mL clean dry volumetric flask and about 7mL of Diluents is added and sonicated to dissolve it completely and made volume upto the mark with the same solvent. (Stock solution) Further 3 ml of above stock solution was pipetted into a 10 ml volumetric flask and diluted upto the mark with diluant.

Procedure:

20µL of the standard, sample are injected into the chromatographic system and the areas for Isoniazid and Ethambutol peaks are measured and the %Assay are calculated by using the formulae.

METHOD:

The developed chromatographic method was validated for system suitability, linearity accuracy, precision, ruggedness and robustness as per ICH guidelines.

System suitability parameters: To evaluate system suitability parameters such as retention time, tailing factor and USP theoretical plate count, the mobile phase was allowed to flow through the column at a flow rate of 1.0 ml/min for 12 minutes to equilibrate the column at ambient temperature. Chromatographic separation was achieved by injecting a volume of 20 µL of standard into Inertsil ODS C185µm (4.6 x 250mm), the mobile phase of composition Sodium Phospahte buffer 3.5 pH and Acetonitrile (30:70) was allowed to flow through the column at a flow rate of 1.0 ml per minute. Retention time, tailing factor and USP theoretical plate count of the developed method are shown in table 1.

Assay of pharmaceutical formulation: The proposed validated method was successfully applied to determine Ethambutol and Isoniazid in their tablet dosage form. The result obtained for was comparable with the corresponding labeled amounts and they were shown in Table-2.

Validation of Analytical method:

Linearity: The linearity study was performed for the concentration of 100ppm to 500ppm and1ppm to 5ppm level. Each level was injected into chromatographic system. The area of each level was used for calculation of correlation coefficient. Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient. The resulte are shown in table 3.

Accuracy studies: The accuracy was determined by help of recovery study. The recovery method carried out at three level 50%, 100%, 150%. Inject the standard solutions into chromatographic system. Calculate the Amount found and Amount added for Ethambutol and Isoniazid and calculate the individual recovery and mean recovery values. The results are shown in table 4,5.

Precision Studies: precision was caliculated from Coefficient of variance for six replicate injections of the standard. The standard solution was injected for six times and measured the area for all six Injections in HPLC. The %RSD for the area of six replicate injections was found. The resulte are shown in table 6,7.

Ruggedness: To evaluate the intermediate precision of the method, Precision was performed on different day. The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found. The resulte are shown in table 8,9.

Robustness: As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method. The flow rate was varied at 0.8 ml/min to 1.2 ml/min. The resulte are shown in table 10,11,12,13

LOD and LOQ: The sensitivity of RP-HPLC was determined from LOD and LOQ. Which were calculated from the calibration curve using the following equations as per ICH guidelines. The resulte are shown in table 14.

LOD = 3.3σ/S and

LOQ = 10 σ/S, where

σ= Standard deviation of y intercept of regression line,

S = Slope of the calibration curve

RESULTS AND DISCUSSION:

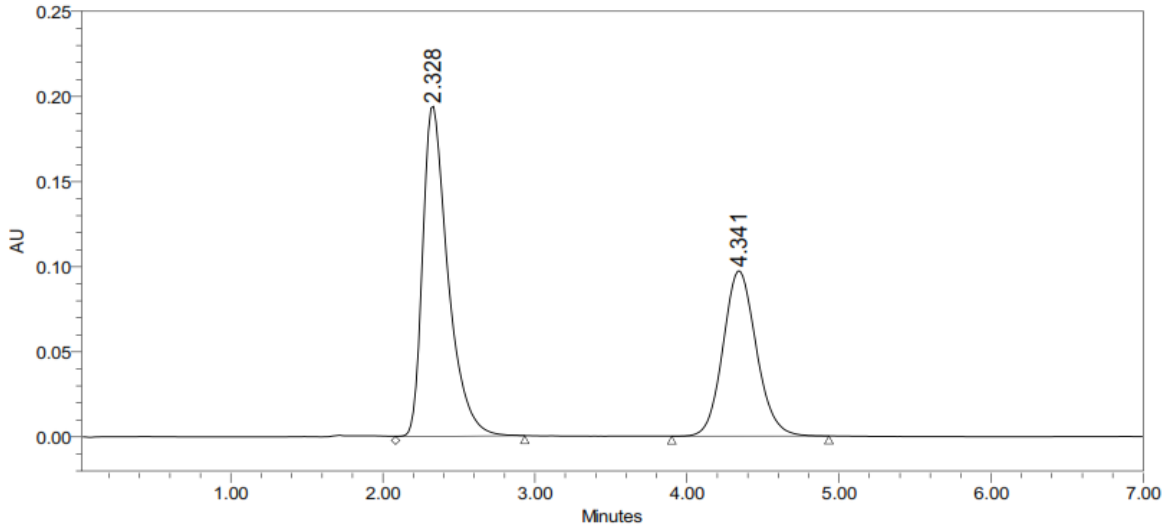

Figure 3: Standard chromatogram

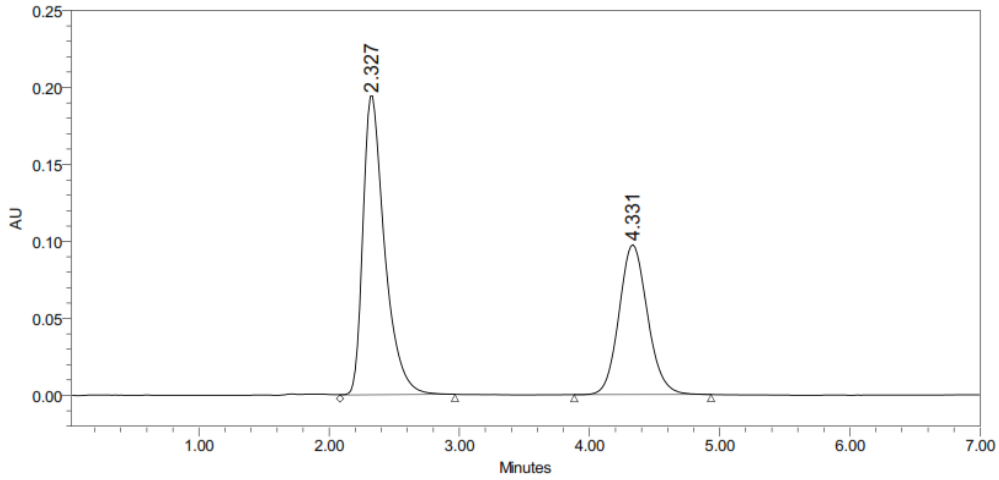

Figure 4: Sample chromatogram

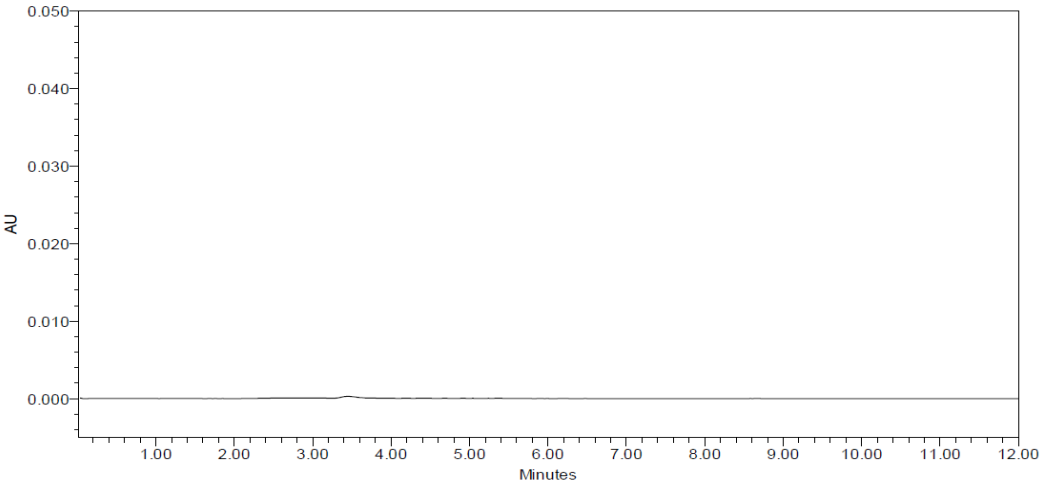

Figure 5: Blank chromatogram

Table 1: System suitability parameters

Parameters	Ethambutol	Isoniazid
Retention time	4.34	2.23
USP Plate count	2614	2632
USP Tailing	1.6	1.8

Table 2: Assay results for Ethambutol and Isoniazid

	Label Claim (mg)	% Assay
Ethambutol	15	101.3
Isoniazid	100	100.6

Table 3: Linearity results of Isoniazid and Ethambutol

S.NO	SAMPLE NAME	RT	AREA	HEIGHT	SAMPLE NAME	RT	AREA	HEIGHT
1	Linearty 1	2.309	1812101	145867	Linearty 1	4.304	1163273	74586
2	Linearty 2	2.322	2044373	176895	Linearty 2	4.323	1345955	87689
3	Linearty 3	2.324	2366122	206674	Linearty 3	4.214	1556574	101999
4	Linearty 4	2.336	2611248	228475	Linearty 4	4.524	1776565	117084
5	Linearty 5	2.340	2869662	259345	Linearty 5	4.218	1957821	129409

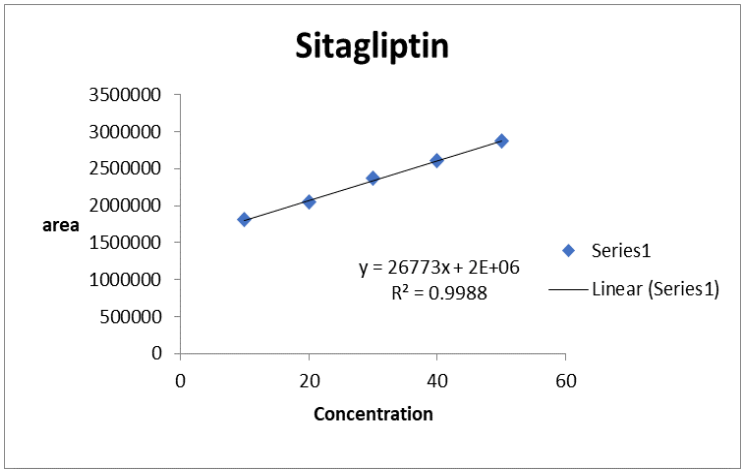

Figure 6: Linearity graph for Isoniazid

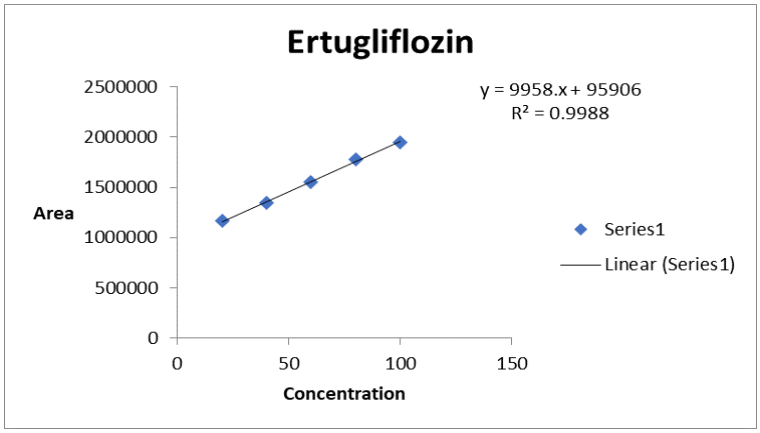

Figure 7: Linearity graph for Ethambutol

Table 4: Showing accuracy results for Ethambutol

%Concentration (at specification Level)	Area	Amount added(mg)	Amount found(mg)	% Recovey	Mean Recovery
50%	2331544	7.5	7.60	101.8%	100.4%
100%	3134597	15	14.8	99.9%	
150%	3917897	20	19.4	99.1%	

Table 5: Showing accuracy results for Isoniazid

%Concentration (at specification level)	Area Area	Amount Added(mg)	Amount Found(mg)	% Recovey % Recovery	Mean Recovery
50%	353757	50	50.8	101.3%	100.1%
100%	4734988	100	99.4	99.4%	
150%	5911698	150	148.9	99.2%	

Table 6: Precision results for Ethambutol

S.NO	Name	RT	Area	Height
1	Ethambutol	4.302	1401375	100174
2	Ethambutol	4.305	1401445	100068
3	Ethambutol	4.325	1402315	98415
4	Ethambutol	4.315	1404575	98155
5	Ethambutol	4.312	1408514	98144
Mean			1491354	
Std.dev			5882.5	
%RSD			0.38	

Table 7: Precision results for Isoniazid

S.NO	Name	RT	Area	Height
1	Isoniazid	2.320	2267519	196958
2	Isoniazid	2.341	2208588	197584
3	Isoniazid	2.356	2275569	195874
4	Isoniazid	2.344	2258841	194583
5	Isoniazid	2.325	2257967	194587
Mean			2254401	
Std.dev			6535.5	
%RSD			0.31	

Table 8. Ruggedness results of Ethambutol

S.NO	Name	RT	Area	Height
1	Ethambutol	4.302	1401375	95613
2	Ethambutol	4.305	1401442	95142
3	Ethambutol	4.325	1402312	95158
4	Ethambutol	4.315	1404673	95153
5	Ethambutol	4.312	1408512	95143
Mean			1455158	
Std.dev			2344.5	
%RSD			0.15	

Table 9. Ruggedness results of Isoniazid

S.NO	Name	RT	Area	Height
1	Isoniazid	2.325	2165319	186958
2	Isoniazid	2.315	2104788	187584
3	Isoniazid	2.356	2147469	185874
4	Isoniazid	2.325	2158641	184583
5	Isoniazid	2.331	218957	184587
Mean			219556	
Std.dev			2559	
%RSD			0.12	

Robustness results

Table 10: Flow variation results for Ethambutol

S.No	Flow Rate(ml/min)	System suitability results	
		USP Plate count	USP Tailing
1	0.8	1778.5	1.23
2	1.0	1547.2	1.2
3	1.2	1938.0	1.2

Table 11: Flow variation results for Isoniazid

S.No	Flow Rate(ml/min)	System suitability results	
		USP Plate count	USP Tailing
1	0.8	882.3	1.56
2	1.0	1244.0	1.1
3	1.2	968.2	1.6

Table 12: System suitability results for Ethambutol (Mobile phase)

S.No	Changein Organic Composition in the Mobile Phase	System suitability results	
		USP Plate count	USP Tailing
1	10% Less	1748.5	1.22
2	Actual	1548.2	1.2
3	10% More	1948.0	1.2

Table 13: System suitability results for Isoniazid (Mobile phase)

S.No	Changein Organic Composition in the Mobile Phase	System suitability results	
		USP Plate count	USP Tailing
1	10% Less	878.3	1.56
2	Actual	1234.0	1.1
3	10% More	969.2	1.6

Table 14: LOD, LOQ of Ethambutol and Isoniazid

Drug	LOD	LOQ
Ethambutol	3.03	10.1
Isoniazid	2.94	9.87

CONCLUSION:

The Developed HPLC method was validated and it was found to be simple, precise, accurate and sensitive for the simultaneous estimation of Ethambutol and Isoniazid in its pure form and in its pharmaceutical dosage forms. Hence, this method can easily and conveniently adopt for routine quality control analysis of Isoniazid and Ethambutol in pure and its pharmaceutical dosage forms.

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