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METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANIOUS ESTIMATION OF ETHAMBUTOL AND ISONIAZID BY USING RP-HPLC

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ABSTRACT: A simple, Accurate, precise method was developed for the simultaneous estimation of the Ethambutol and Isoniazid in Tablet dosage form. Chromatogram was run through Std Agilent 150 x 4.6 mm, 5µ. Mobile phase containing Buffer Potassium dihydrogen ortho phosphate: Acetonitrile taken in the ratio 55:45%v/v was pumped through column at a flow rate of 1 ml/min. Temperature was maintained at 30°C and UV detection was observed at 260 nm. Retention time of Ethambutol and Isoniazid were found to be 2.365min and 2.956 min. %RSD of the Ethambutol and Isoniazid were and found to be 0.3 and 0.7 respectively. %Recovery was obtained as 99.55% and 99.41% for Ethambutol and Isoniazid respectively. LOD, LOQ values obtained from regression equations of Isoniazid Ethambutol and Isoniazid were 0.370, 1.122 and 0.128, 0.388 respectively. Regression equation of Isoniazid is y = 7472.x + 3927, and y = 7779.x + 6691 of Ethambutol. Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

Keywords: Isoniazid, Ethambutol, RP-HPLC, Mobile phase, Retention time.

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1. INTRODUCTION

Ethambutol is an oral chemotherapeutic agent which is specifically effective against actively growing microorganisms of the genus Mycobacterium, including M. tuberculosis. Ethambutol inhibits RNA synthesis and decreases tubercle bacilli replication. Nearly all strains of M.

tuberculosis and M. kansasii as well as a number of strains of MAC are sensitive to ethambutol. [1-10]

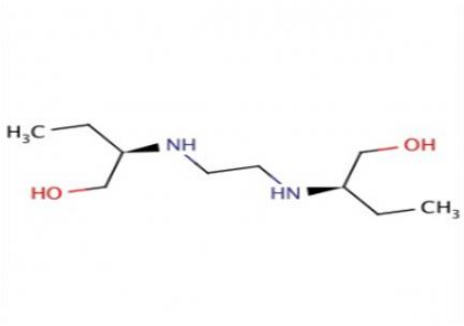

Fig 1: Chemical structure of Ethambutol

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. Once activated, isoniazid inhibits the synthesis of mycoloic acids, an essential component of the bacterial cell wall. At therapeutic levels isoniazid is bacteriocidal against actively growing intracellular and extracellular Mycobacterium tuberculosis organisms. [11-14]

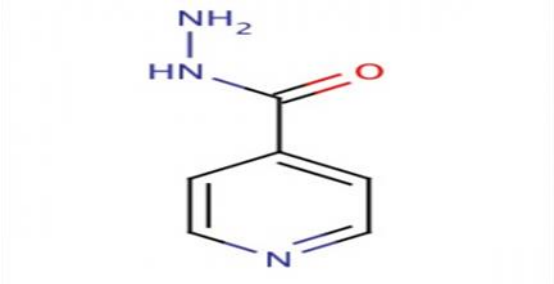

Fig 2: Chemical Structure of Isoniazid

A comprehensive survey of literature reveals that few analytical methods have been reported for the estimation of Ethambutol and Isoniazid in individually and combined dosage forms like spectrophotometry, HPLC, bio analytical methods and stability indicating methods. [15-21] From the past decades there is no method has been reported for the simultaneous estimation of Ethambutol and Isoniazid in bulk and combined dosage form. The present attempt was made to develop simple, precise, accurate, robust and cost effective RP-HPLC method for the simultaneous estimation of Ethambutol and Isoniazid. The developed method was validated according to ICH guidelines. [22-25]

2. MATERIALS AND METHODS

Materials:

Isoniazid and Ethambutol pure drugs (API), Combination Isoniazid and Ethambutol tablets (*MYCONEX 600*), Distilled water, Acetonitrile, Phosphate buffer, Methanol, Potassium dihydrogen ortho phosphate buffer, Ortho-phosphoric acid. All the above chemicals and solvents are from Rankem.

Instruments:

WATERS HPLC 2695 SYSTEM equipped with quaternary pumps, Photo Diode Array detector and Auto sampler integrated with Empower 2 Software. UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2 mm and 10mm and matched quartz cells integrated with UV win 6 Software was used for measuring absorbances of Isoniazid and Ethambutol solutions. Electronic balance, pH meter and Ultra sonicator also used in this study.

Methods:

Diluent: Based up on the solubility of the drugs, diluent was selected, Acetonitrile and Water taken in the ratio of 50:50

Preparation of Standard stock solutions:

Accurately weighed 7.5mg of Isoniazid, 15mg of Ethambutol and transferred to 25ml flasks and 3/4 th of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (300µg/ml of Isoniazid and 600µg /ml Ethambutol).

Preparation of Standard working solutions (100% solution): 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (30µg/ml of Isoniazid and 60µg/ml of Ethambutol)

Preparation of Sample stock solutions:

5 tablets were weighed and the average weight of each tablet was calculated, then the weight equivalent to 1 tablet was transferred into a 100ml volumetric flask, 50ml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC filters (3000µg/ml of Isoniazid and 6000µg/ml of Ethambutol)

Preparation of Sample working solutions (100% solution):

1ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (300µg/ml of Isoniazid and 600µg/ml of Ethambutol).

Method Validation

The developed method was validated according to ICH guidelines. The validation was performed by different parameters like specificity, linearity, precision, accuracy, quantification limits, robustness and system suitability.

3. RESULTS AND DISCUSSION

Optimized Chromatographic Conditions

Mobile phase : 55% KH_2PO_4 : 45% Acetonitrile

Flow rate : 1ml/min

Column : Agilent C18 (150mm x 4.6, 5µm)

Detector wave length : 260nm

Column temperature : 30°C

Injection volume : 10µL

Run time : 6 min

Diluent : Water and Acetonitrile in the ratio 50:50

Results : Both peaks have good resolution, tailing Factor, theoretical plate count and resolution.

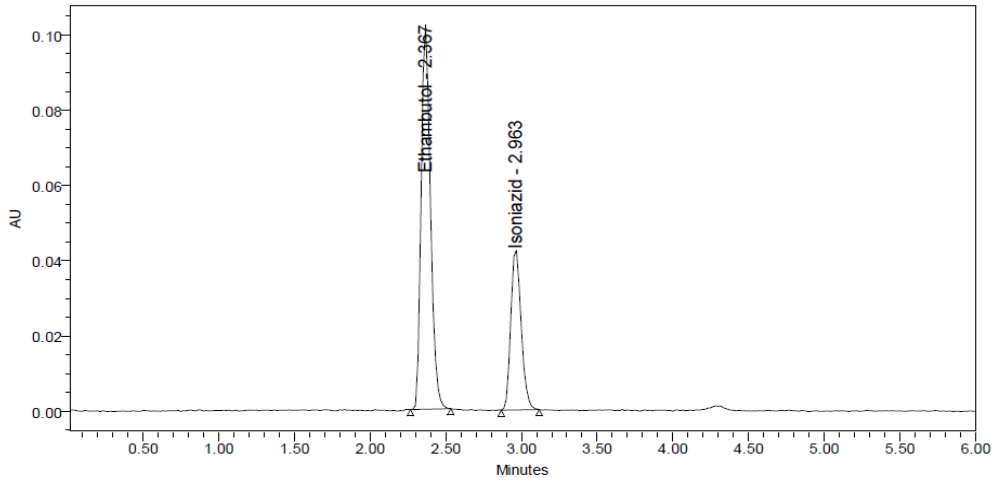

Fig 3: Optimized chromatogram for Ethambutol and Isoniazid

Observation: Ethambutol and Isoniazid were eluted at 2.367 min and 2.963 min respectively with good resolution. Plate count and tailing factor was very satisfactory, so this method was optimized and to be validated.

System Suitability:

All the system suitability parameters were within the range and satisfactory as per ICH guidelines. According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits.

Table 1: System suitability parameters for Ethambutol and Isoniazid

S no	Ethambutol			Isoniazid			
Inj	Rt(min)	USP Plate Count	Tailing	Rt(min)	USP Plate Count	Tailing	Resolution
1	2.365	6599	1.19	2.955	8969	1.18	4.9
2	2.365	6683	1.19	2.956	9030	1.19	4.9
3	2.365	7236	1.18	2.957	9111	1.17	4.9
4	2.365	7188	1.18	2.958	9130	1.17	5.0
5	2.365	7058	1.18	2.961	9262	1.15	4.9
6	2.365	6869	1.18	2.961	9084	1.15	4.9

Specificity:

Retention times of Ethambutol and Isoniazid were 2.365 min and 2.956 min respectively. We did not found and interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

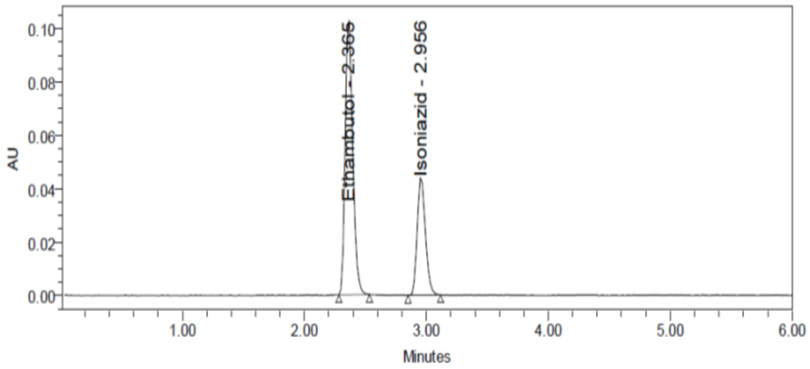

Fig 4: Standard Chromatogram

Linearity:

Six linear concentrations of Isoniazid (7.5-45µg/ml) and Ethambutol (15-90µg/ml) were injected in a duplicate manner. Average areas were mentioned above and linearity equations obtained for Isoniazid was y = 7472.x + 3927 and of Ethambutol was y = y = 7779.x + 6691. Correlation coefficient obtained was 0.999 for the two drugs.

Table 2: Linearity table for Ethambutol and Isoniazid.

Isoniazid		Ethambutol	
Conc (µg/mL)	Peak area	Conc (µg/mL)	Peak area
0	0	0	0
7.5	60200	15	124893
15	119232	30	246742
22.5	176208	45	359102
30	225055	60	475639
37.5	287386	75	584972
45	336353	90	706174

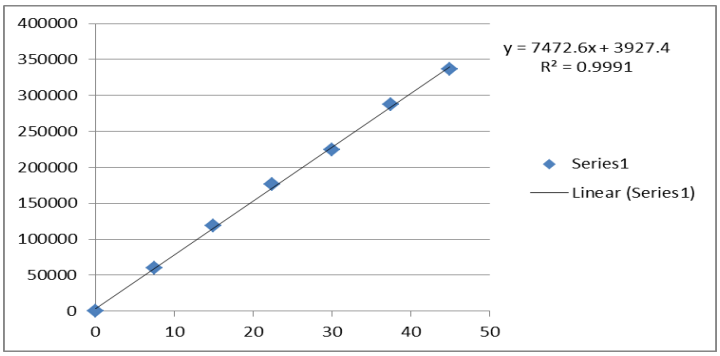

Fig 5: Calibration curve of Isoniazid

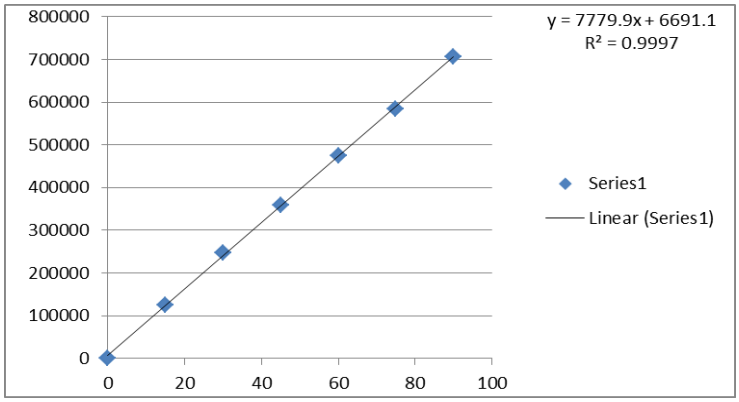

Fig 6: Calibration curve of Ethambutol

Precision:

From a single volumetric flask of working standard solution six injections were given and the obtained areas were mentioned above. Average area, standard deviation and % RSD were calculated for two drugs. % RSD obtained as 0.3% and 0.7% respectively for Ethambutol and Isoniazid. As the limit of Precision was less than "2" the system precision was passed in this method.

Table 3: Precision results for Ethambutol and Isoniazid

S. No	Area of Ethambutol	Area of Isoniazid
1.	473123	223849
2.	471226	226185
3.	469620	226473
4.	470679	226886
5.	469233	223249
6.	472270	225463
Mean	471025	225351
S.D	1503.1	1483.1
%RSD	0.3	0.7

Accuracy:

Three levels of Accuracy samples were prepared by standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 99.55% and 99.41% for Ethambutol and Isoniazid respectively.

Table 4: Accuracy results of Isoniazid

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	15	14.857	99.05	99.41%
	15	14.863	99.09	
	15	14.869	99.13	
100%	30	29.815	99.38	
	30	29.807	99.36	
	30	29.778	99.26	
150%	45	44.764	99.47	
	45	44.877	99.73	
	45	45.083	100.18	

Table 5: Accuracy results of Ethambutol

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	30	29.771	99.24	99.55%
	30	29.800	99.33	
	30	29.782	99.27	
100%	60	59.737	99.56	
	60	59.447	99.08	
	60	59.882	99.80	
150%	90	89.837	99.82	
	90	90.083	100.09	
	90	89.752	99.72	

LOD & LOQ:

The sensitivity of the proposed method for measurement of Ethambutol and Isoniazid were estimated in terms of Limit of Detection (LOD) and Limit of Quantification (LOQ).The LOD and LOQ were calculated by using the slope and SD of response (intercept).The mean slope value and SD of response were obtained after plotting six calibration curves.

Table 6: Sensitivity results for Ethambutol and Isoniazid

Molecule	LOD	LOQ
Isoniazid	0.370	1.122
Ethambutol	0.128	0.388

Robustness:

Robustness conditions like Flow minus (0.9ml/min), Flow plus (1.1ml/min), mobile phase minus (60B:40A), mobile phase plus (50B:50A), temperature minus (25°C) and temperature plus (35°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit.

Table 7: Robustness data for Ethambutol and Isoniazid.

S.no	Condition	%RSD of Isoniazid	%RSD of Ethambutol
1	Flow rate (-) 0.9ml/min	0.7	0.5
2	Flow rate (+) 1.1ml/min	0.5	0.1
3	Mobile phase (-) 60B:40A	0.8	0.8
4	Mobile phase (+) 50B:50A	0.2	0.8
5	Temperature (-) 25°C	0.5	0.6
6	Temperature (+) 35°C	0.7	0.8

Assay:

Cadila pharmaceuticals, bearing the label claim Isoniazid 300mg, Ethambutol 600mg. Assay was performed with the above formulation. Average % Assay for Isoniazid and Ethambutol obtained was 100.56 and 99.74% respectively.

Table 8: Assay Data of Ethambutol

S.no	Standard Area	Sample area	% Assay
1	473123	468501	99.36
2	471226	472791	100.27
3	469620	469007	99.47
4	470679	471693	100.04
5	469233	470651	99.82
6	472270	471602	100.02
Avg	471025	470708	99.83
Stdev	1503.1	1665.9	0.35
%RSD	0.3	0.4	0.4

Table 9: Assay Data of Isoniazid

S.no	Standard Area	Sample area	% Assay
1	223849	223755	99.19
2	226185	228185	101.16
3	226473	222532	98.65
4	226886	223425	99.05
5	223249	225620	100.02
6	225463	224445	99.50
Avg	225351	224660	99.59
Stdev	1483.1	2013.3	0.89
%RSD	0.7	0.9	0.9

Forced degradation Study:

Degradation studies were performed with the formulation and the degraded samples were injected. Assay of the injected samples was calculated and all the samples passed the limits of degradation.

Table 10: Degradation Data of Ethambutol

S.No	Degradation Condition	% Drug Degraded	Purity Angle	Purity Threshold
1	Acid	2.38	0.252	0.322
2	Alkali	3.47	0.175	0.299
3	Oxidation	9.43	0.235	0.334
4	Thermal	4.97	0.217	0.347
5	UV	1.21	0.211	0.339
6	Water	0.266	0.341	0.288

Table 11: Degradation Data of Isoniazid

S.No	Degradation Condition	% Drug Degraded	Purity Angle	Purity Threshold
1	Acid	10.58	0.356	0.540
2	Alkali	6.43	0.210	0.282
3	Oxidation	7.76	0.555	0.631
4	Thermal	8.95	0.407	0.579
5	UV	1.94	0.341	0.530
6	Water	0.50	0.450	0.632

4. CONCLUSION

A simple, Accurate, precise method was developed for the simultaneous estimation of the Ethambutol and Isoniazid in Tablet dosage form. Retention time of Ethambutol and Isoniazid were found to be 2.365min and 2.956 min. %RSD of the Ethambutol and Isoniazid were and found to be 0.3 and 0.7 respectively. %Recovery was obtained as 99.55% and 99.41% for Ethambutol and Isoniazid respectively. LOD, LOQ values obtained from regression equations of Isoniazid Ethambutol and Isoniazid were 0.370, 1.122 and 0.128, 0.388 respectively. Regression equation of Isoniazid is y = 7472.x + 3927., and y = 7779.x + 6691. of Ethambutol. Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this research are available within the article.

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CONFLICT OF INTEREST

Authors have no conflict of interest.

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