Original Article

Stability Indicating Method Development and Validation for the Simultaneous Estimation of Ethambutol and Isoniazid in Bulk and Pharmaceutical Dosage form by using RP-HPLC

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INTRODUCTION

Ethambutol (commonly abbreviated EMB or simply E) is a medication primarily used to treat tuberculosis. It is usually given in combination with other tuberculosis drugs, such as isoniazid, rifampicin and pyrazinamide.

Isoniazid, also known as isonicotinylhydrazide (INH), is an antibiotic used as a first-line agent for the prevention and treatment of both latent and active tuberculosis. It is effective against mycobacteria, particularly Mycobacterium tuberculosis.

A simple, accurate, precise method was developed for the simultaneous estimation of the Isoniazid and Ethambutol in Tablet dosage form. Chromatogram was run through kromasil. Mobile phase containing Buffer and Acetonitrile taken in the ratio 58:42 was pumped through column at a flow rate of 1 ml/min. Buffer used in this method was 0.1% OPA solution. Temperature was maintained at 30°C. Optimized wavelength for Isoniazide and Ethambutol was 220nm. Retention time of Isoniazide and Ethambutol were found to be 2.430 min and 2.989 min. % RSD of the Isoniazide and Ethambutol were found to be 0.9 and 0.8 respectively. % assay was obtained as 99.72% and 100.21% for Isoniazide and Ethambutol respectively. LOD, LOQ values are obtained from regression equations of Isoniazide and Ethambutol were 1.56 ppm, 0.07 ppm and 4.72 ppm, 0.21 ppm respectively. Regression equation of Isoniazide is y = 2462x + 20382, and y = 5037x + 89279. Of Ethambutol

Key Words: Isoniazide, Ethambutol, RP-HPLC.
In the proposed work, attempt shall be made to develop a
ew HPLC method for simultaneous estimation of Isoniazide and Ethambutol to develop a validated method
according to ICH guidelines. To apply validated method for the estimation of Isoniazide and Ethambutol in
pharmaceutical formulation

2. MATERIALS AND METHODS

Isoniazide and Ethambutol, Combination Isoniazide and Ethambutol capsules, distilled water, acetonitrile, phosphate
buffer, ammonium acetate buffer, glacial acetic acid, methanol, potassium dihydrogen phosphate buffer, tetra
hydrofuran, tri ethyl amine, ortho-phosphoric acid etc.

Instruments:
HPLC instrument used was of WATERS HPLC 2965
SYSTEM with Auto Injector and PDA Detector. Software
used is Empower 2. UV-VIS spectrophotometer PG
Instruments T60 with special bandwidth of 2mm and 10mm
and matched quartz was be used for measuring absorbance
for Isoniazide and Ethambutol solutions.

Preparation of buffer: Buffer:
1 ml of Ortho phosphoric acid was taken in a 1000ml of
volumetric flask add about 900ml of milli-Q water added
degas to sonicate and finally make up the volume with
water, pH was adjusted by triethylamine to 3.8

Standard Preparation:
(300µg/ml Isoniazid& 600µg/ml Ethambutol) Accurately
Weighed and transferred 30mg&60mg of Isoniazid and
Ethambutol working Standards into a 10ml and 10ml clean
dry volumetric flask respectively, add 5ml and 5ml of
diluent, sonicated for 30 minutes and make up to the final
volume with diluents. From the above stock solutions, 1ml
was pipette out in to a 10ml volumetric flask and then make
up to the final volume with diluent.

Sample Preparation:
5 tablets were weighed and calculate the average weight of
each tablet then the weight equivalent to 1 tablet was
transferred into a 100 ml volumetric flask, 70ml of diluent
added and sonicated for 30 min, further the volume made up
with diluent and filtered. From the above stock solutions, 1ml
was pipette out in to a 10ml volumetric flask and then make
up to the final volume with diluent.

Linearity:
Linearity solutions are prepared such that 0.25ml, 0.5ml,
0.75ml, 1ml, 1.25ml, 1.5ml from the Stock solutions
Isoniazide and Ethambutol are taken in to 6 different
volumetric flasks and diluted to 10ml with diluents to get
75ppm, 150ppm, 225ppm, 300ppm, 375ppm, 450ppm of
Isoniazide and 150ppm, 300ppm, 450ppm, 600ppm,
750ppm, 900ppm of Ethambutol

Accuracy:
Standard Preparation:
(300µg/ml Isoniazid& 600µg/ml Ethambutol) Accurately
Weighed and transferred 30mg&60mg of Isoniazid and
Ethambutol working Standards into a 10ml and 10ml clean
dry volumetric flask respectively, add 5ml and 5ml of
diluent, sonicated for 30 minutes and make up to the final
volume with diluents. From the above stock solutions, 1ml
was pipette out in to a 10ml volumetric flask and then make
up to the final volume with diluent.

Preparation of 50% Spiked Solution:
weight equivalent to 600mg of tablet powder was transferred
into a 100 ml volumetric flask, 50ml of diluent added and
sonicated for 30 min, further the volume made up with
diluent and filtered. 1ml from each standard stock solution
was pipette out and taken into a 10ml volumetric flask to
that 1ml of filtered Accuracy 100% Sample stock solution
was spiked and made up with diluents.

Preparation of 100% Spiked Solution:
weight equivalent to 1200mg of tablet powder was transferred
into a 100 ml volumetric flask, 50ml of diluent added and
sonicated for 30 min, further the volume made up with
diluent and filtered. 1ml from each standard stock solution
was pipette out and taken into a 10ml volumetric flask to
that 1ml of filtered Accuracy 100% Sample stock solution
was spiked and made up with diluents. Preparation of
150% Spiked Solution: weight equivalent to 1800 mg of
tablet powder was transferred into a 100 ml volumetric flask,
50ml of diluent added and sonicated for 30 min, further
the volume made up with diluent and filtered. 1ml from each
standard stock solution was pipette out and taken into a 10ml
volumetric flask to that 1ml of filtered Accuracy 100% Sample stock solution was spiked and made up with diluents.

METHOD DEVELOPMENT
Trials were done by changing columns and Mobile phases
and were reported below.

TRIAL: 1
Column Used : ODS 250 x 4.6 mm, 5µ.
Mobile phase : Water: Acetonitrile (50:50A)
Flow rate : 1ml/min
Wavelength : 220nm
Temperature : 30 C
Injection Volume : 10 1

Fig 1: Trial 1 chromatogram

Observation: Etambutol peak was eluted but isoniazide
peak was not eluted. So further trail was carried out.

TRIAL: 2
Column Used : ODS 250 x 4.6 mm, 5µ.
Mobile phase : Buffer: Acetonitrile (50:50A)
Buffer : 0.01N KH2PO4 solution
Flow rate : 1ml/min
Wavelength: 220nm
Temperature: 30°C
Injection Volume: 10 μl

Observation: Resolution was not passed. So further trials are carried out.

**TRIAL: 3**
Column Used: Kromasil 250 x 4.6 mm, 5 μ.
Mobile phase: buffer: Acetonitrile (70:30A)
Buffer: 0.1% OPA
Flow rate: 1 ml/min
Wavelength: 220nm
Temperature: 30°C
Injection Volume: 10 μl

Observation: Isoniazide peak shape was not good so trial was carried out.

**TRIAL: 4**
Column Used: Kromasil 250 x 4.6 mm, 5 μ.
Mobile phase: buffer: Acetonitrile (60:40A)
Buffer: 0.1% OPA
Flow rate: 1 ml/min
Wavelength: 220nm
Temperature: 30°C
Injection Volume: 10 μl

Observation: peak shape was not good. So further trial was carried out.

**TRIAL: 5**
Column Used: Kromasil 250 x 4.6 mm, 5 μ.
Mobile phase: buffer: Acetonitrile (60:40A)
Buffer: 0.1% OPA
Flow rate: 1 ml/min
Wavelength: 220nm
Temperature: 30°C
Injection Volume: 10 μl

Observation: peak shape was good. So further trial was carried out.

**OPTIMIZED METHOD**
Drugs were eluted with good retention time, resolution; all the system suitable parameters like Plate count and Tailing factor were within the limits.

Column Used: Kromasil 250 x 4.6 mm, 5 μ.
Buffer: 0.1% OPA
Mobile phase: buffer: Acetonitrile (58:42A)
Flow rate: 1.0 ml/min
Diluent: water:acn: 50:50
Wavelength: 220nm
Temperature: 30°C
Injection Volume: 10 μl

Observation: peak shape and retention time is good.

**3. RESULTS AND DISCUSSION**

1. System suitability: All the system suitability parameters are within range and satisfactory as per ICH guidelines

System suitability studies of Isoniazide and Ethambutol method

<table>
<thead>
<tr>
<th>Property</th>
<th>Isoniazide</th>
<th>Ethambutol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time (tt)</td>
<td>2.00 min</td>
<td>2.90 min</td>
</tr>
<tr>
<td>Theoretical plates (N)</td>
<td>785 ± 65.48</td>
<td>6500 ± 63.48</td>
</tr>
<tr>
<td>Tailing factor (T)</td>
<td>1.33 ± 0.117</td>
<td>1.73 ± 0.17</td>
</tr>
</tbody>
</table>

Fig 7: Chromatogram of blank
2. Linearity: Six Linear concentrations of Isoniazide (75-450ppm) and Ethambutol (150-900ppm) are prepared and injected. Regression equation of the Isoniazide and Ethambutol are found to be, 
\[ y = 2462x + 20382 \] and 
\[ y = 5037x + 89279 \] and regression coefficient was 0.999.

3. Precision:
Intraday precision (Repeatability): Intraday Precision was performed and % RSD for Isoniazide and Ethambutol were found to be 0.9% and 0.8% respectively.

Inter day precision: Inter day precision was performed with 24 hrs time lag and the %RSD Obtained for Isoniazide and Ethambutol were 1.4% and 0.9%.
Fig 18: Accuracy 150% Chromatogram of Isoniazide and Ethambutol
5. LOD: Limit of detection was calculated by std deviation method Isoniazide and Ethambutol and LOD for Isoniazide and Ethambutol were found to be 1.56 and 0.07 respectively.

Fig 19: LOD Chromatogram of Isoniazide and Ethambutol
6. LOQ: Limit of Quantification was calculated by std deviation method Isoniazide and Ethambutol and LOQ for Isoniazide and Ethambutol were found to be 4.72 and 0.21 respectively.

Fig 20: LOQ Chromatogram of Isoniazide and Ethambutol

Summary

<table>
<thead>
<tr>
<th>Parameters</th>
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<th>Ethambutol</th>
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<tr>
<td>Retention time</td>
<td>2.430 min</td>
<td>2.989 min</td>
</tr>
<tr>
<td>%RSD</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>% Assay</td>
<td>99.72%</td>
<td>100.21%</td>
</tr>
<tr>
<td>LOD</td>
<td>1.56ppm</td>
<td>0.07ppm</td>
</tr>
<tr>
<td>LOQ</td>
<td>4.72ppm</td>
<td>0.8ppm</td>
</tr>
</tbody>
</table>

Regression equation of Isoniazide is $y = 2462x + 20382$, and $y = 5037x + 89279$ Of Ethambutol. Retention times are 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.

5. REFERENCES

Conflict of Interest: None
Source of Funding: Nil