Received: 01-08-2013; Accepted: 05-08-2013

“METHOD DEVELOPMENT AND VALIDATION OF ANTI-TUBERCULAR DRUGS IN FIXED DOSE FORMULATION BY RP-HPLC TECHNIQUE”

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KEYWORDS:
Ethambutol Hydrochloride, INH, RP-HPLC, fixed dose combinations.

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ABSTRACT

A reverse phase HPLC method has been developed for the simultaneous determination of Ethambutol Hydrochloride (ETH) and Isoniazid (INH) by using C18 Thermo Hypersil ODS, (250 X 5.4 mm X 4.5µ) column and the mobile phase was prepared by mixing methanol: ammonium acetate buffer (pH-7.03) in the ratio of (50:50). Retention times of ETH and INH were 2.01 min and 7.0 min respectively. This method shows to be linear ($t^2>0.99$), precise (RSD<2%), accurate (recovery of (98-102)% of ETH and (98-102)% of INH), specific and robust. LOD and LOQ of ETH found to be 0.190ppm and 0.635ppm; for INH it was found to be 0.569ppm and 1.89ppm.
INTRODUCTION:
INH is chemically known as pyridine-4-carboxyhydrazide[1]. ETH is chemically known as (S,S)-N,N-ethylenbis(2-aminobutan-1-ol)dihydrochloride[2]. ETH (800mg) and INH (300mg) acts as mycolic acid synthesis inhibitors [3]. It is a single dose anti tubercular regimen. It is official in Indian Pharmacopoeia. Highly sensitive, selective and rugged RP-HPLC method would be very useful for the estimation of ETH and INH in pharmaceutical formulations. Literature survey reveals need for simultaneous estimation of drugs in combined dosage forms. Few methods were reported by HPLC [4-6], UV-Spectrophotometry[7-9], Electroanalytical methods[10]. The purpose of this study was to develop sensitive, simple, precise, accurate and rugged method for Simultaneous estimation of ETH and INH in bulk and combined dosage form.

Fig:1 Structure of INH and ETH

MATERIALS AND METHODS
Apparatus:
Separation and estimation was carried out using HPLC (Waters India Pvt Ltd) equipped with PDA detector, column used in experiment was C18 Thermo Hypersil ODS, (250 X 5.4 mm X 4.5μ) analytical balance used was LABINDIA, Digital pH meter LABINDIA-PHNA. The mobile phase was prepared by mixing methanol: ammonium acetate buffer (pH-7.03) in the ratio of (50:50) was filtered and degassed. Injection volume is 10μL and the detection was at 276nm.

Reagents and solutions:
Pure sample of ETH and INH and other reagents such as acetonitrile, methanol, double distilled water (HPLC grade) and ammonium acetate of AR grade were used.

Preparation of standard drug solutions
Accurately about 80mg of the ETH and 31.5mg INH pure drug was weighed and transferred into two separate 50ml clean, dry volumetric flask and add 20ml of water; sonicated for 20 minutes and diluted with water.
Preparation of sample drug solution:
Tablet powder equivalent to 800 mg of ETH was transferred into a 100 ml of volumetric flask and added 50ml of water and sonicated for 30 minutes and made up the volume with water. Further dilute 5ml of the above solution to 25 ml in a volumetric flask and diluted with water.

Marketed Formulation:

<table>
<thead>
<tr>
<th>Brand name</th>
<th>Content</th>
<th>Mfg. Company</th>
<th>Batch. No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combunex</td>
<td>ETH &amp; INH (800mg &amp; 300mg Respectively)</td>
<td>LUPIN Ltd</td>
<td>J110643</td>
</tr>
</tbody>
</table>

Selection of Wavelength

Appropriate dilution was prepared using standard stock solution of 100µg/ml of ETH and INH. Both the solution were scanned over range of 400-200nm, using medium scan speed. The absorption maxima ($\lambda_{max}$) of ETH was 288 nm and absorption maxima ($\lambda_{max}$) of INH was 263nm. Isobestic point of the combination of ETH and INH was found to be 276nm.

Fig: 2 Isobestic Point of ETH and INH

Preparation of mobile phase

The mobile phase was prepared by mixing methanol: ammonium acetate Buffer (pH-7.03) in the ratio of (50:50), it was filtered and degassed.

Selection of mobile phase for method Optimization and experimental condition:

Several trials were performed to obtain optimized condition for RP-HPLC method by altering the mobile phase and with different ratio. Other parameters were also altered like temperature, flow rate. Finally the mobile phase for optimized condition was selected and given follows.
Assay:
Assay of marketed tablet formulation containing ETH (800mg) and INH (300mg) was performed by preparing the sample solutions as described earlier in the preparation of the sample. The assay of the commercial sample was calculated by comparing the areas of standard and sample peaks. The assay of marketed formulation Combunex was found to be within the limit; the chromatogram is shown in Fig 4.
This optimized method was validated for linearity, accuracy, precision and specificity as per ICH guidelines [11].

Linearity:
The linearity of calibration curves (analyte to peak area ratio vs concentration) in pure solution was checked over the concentration ranges of about 50-150% (Assay concentration in mcg/ml) for ETH and INH. The total eluting time was less than 10min. The regression line relating to standard concentrations of drug using regression analysis, the calibration curves were linear in the studied range and equations of the regression analysis were obtained: y = 165907x + 8307, $R^2$ =0.999 for ETH and y = 11298x +800.7, $R^2$ =0.999 for INH.

Accuracy:
Accuracy of the method was determined by recovery experiments at spiked levels of 50%, 100%, 150%. The recovery studies were carried out three times, the percentage recovery and percentage relative standard deviation were calculated.

Precision:
The precision of the analytical method was studied by analysis of multiple sampling of homogeneous sample.

Specificity:
A solution containing a mixture of tablet was prepared using sample preparation procedure and injected into the system, to evaluate possible interfering peaks.
RESULTS AND DISCUSSION

Validation:
System suitability tests were carried out on freshly prepared standard solution and all the parameters are within limit. Results were shown in table No.3

TABLE NO: 3 System suitability Data

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Ethambutol HCl (± %RSD)</th>
<th>INH (± %RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention Time</td>
<td>2.01±0.23</td>
<td>7.0±0.14</td>
</tr>
<tr>
<td>Theoretical plate</td>
<td>9936±0.18</td>
<td>12509±0.48</td>
</tr>
<tr>
<td>Tailing Factor</td>
<td>1.1436±0.14</td>
<td>1.1254±0.23</td>
</tr>
</tbody>
</table>

± %RSD = Percentage Relative Standard Deviation.

The method was linear in the concentration range of 4-24µg/ml for ETH and 4-24µg/ml for INH, with a correlation coefficient of 0.999 for ETH and 0.999 for INH.

Fig: 5 Linearity plot of ETH and INH

The study of accuracy of the developed method has been done. The recovery was found in the range of 99-102% for ETH and 100-102% for INH shown in Table.4; indicating the accuracy of method and the % RSD of ETH and INH is 0.056 and 0.34 respectively.
TABLE: 4 ACCURACY DATA OF THE ANALYSIS OF ETH AND INH

<table>
<thead>
<tr>
<th>Concentration of Spiked level (%)</th>
<th>Amount Std added µg/ml</th>
<th>Total amount found µg/ml</th>
<th>% Recovery µg/ml</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETH</td>
<td>INH</td>
<td>ETH</td>
<td>INH</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>400</td>
<td>150</td>
<td>398.7</td>
<td>149.6</td>
</tr>
<tr>
<td>100</td>
<td>800</td>
<td>300</td>
<td>799.5</td>
<td>299.5</td>
</tr>
<tr>
<td>150</td>
<td>1200</td>
<td>450</td>
<td>1198.3</td>
<td>449.3</td>
</tr>
</tbody>
</table>

TABLE: 5 Validation parameter data of ETH and INH

<table>
<thead>
<tr>
<th>Validation Parameters</th>
<th>ETH</th>
<th>INH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rt</td>
<td>2.01</td>
<td>7.0</td>
</tr>
<tr>
<td>Run Time</td>
<td>10 min</td>
<td>10 min</td>
</tr>
<tr>
<td>Asymmetry</td>
<td>1.1436</td>
<td>1.1254</td>
</tr>
<tr>
<td>Theoritical Plates</td>
<td>9936</td>
<td>12509</td>
</tr>
<tr>
<td>LOD</td>
<td>0.190ppm</td>
<td>0.569ppm</td>
</tr>
<tr>
<td>LOQ</td>
<td>0.635ppm</td>
<td>1.89ppm</td>
</tr>
<tr>
<td>Linearity</td>
<td>R²=0.999</td>
<td>R²=0.999</td>
</tr>
<tr>
<td>Precision</td>
<td>(0.66)</td>
<td>(1.7)</td>
</tr>
<tr>
<td>% RSD &lt; 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recovery</td>
<td>98-102%</td>
<td>98-102%</td>
</tr>
</tbody>
</table>

CONCLUSION:
The Proposed RP-HPLC method is suitable for simultaneous determination of ETH and INH in Fixed Dose Combinations (FDCs) without any interferences form each other. The accuracy of the methods was assessed by recovery studies at three different levels. The method was found to be precise as indicated by the repeatability analysis, showing % RSD less than 2. All the parameters for both the drugs met the criteria of ICH guidelines for method validation. The developed method may be recommended for routine and QC analysis of the investigated drugs to provide simple, accurate and reproducible quantitative analysis for the determination of determination of ETH and INH in combined formulation.

ACKNOWLEDGEMENTS
The authors are grateful to principal and management, Krupanidhi College of Pharmacy for their continuous support and encouragement and for providing the necessary facilities.

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