Method Development and Validation for Fingolimod by HPLC/UV in Immediate-Release Oral Capsule and Study the Effect of Excipients on Solubility and Dissolution Behavior

Abstract
A simple, selective, linear, precise, and accurate RP-HPLC method was developed and validated for the Quantitation of Fingolimod Hydrochloride from bulk and formulations. Chromatographic separation was achieved isocratically on Zorbax Plus C 8 column (250×4.6 mm, 5μ particle size) using a mobile phase, Acetonitrile and Di-butyl ammonium phosphate buffer in the ratio of 45:55 v/v. The flow rate was 2.0 ml/min and effluent was detected at 198nm and 100μl of sample was injected. Linearity was observed in the concentration range of 0.224 - 1.68 μg/ml. The percentage RSD for precision and accuracy of the method was found to be less than 2%. The method was validated according to the ICH guidelines with respect to specificity, linearity, accuracy, precision and robustness. The method developed can be used for the routine analysis of Fingolimod hydrochloride. The effect of excipients on the dissolution of Fingolimod was studied in different dissolution media.

Keywords: Fingolimod; Sclerosis; Hydrochloride; Modulating; Analysis

Abbreviations: RP: Reverse Phase; HPLC: High-Performance Liquid Chromatography; UV: Ultra Violet; LC: Liquid Chromatography; MS: Mass Spectrometry; DAD: Diode Array Detectors; LOD: Limit of Detection; LOQ: Limit of Quantification; RSD: Reflex Sympathetic Dystrophy; DSC: Differential scanning Calorimetry; FT-IR Infrared Spectroscopy

Introduction
Fingolimod (2-amino-2-[2-(4-octylphenyl) ethyl] propane-1,3-diol hydrochloride) is an immune modulating drug, it is used mainly for multiple sclerosis. It reduces the rate of relapses in relapsing-remitting multiple sclerosis by over half, but has serious adverse effects (Figure 1) [1-3]. This paper includes composed of two parts. The first part focuses on Quantitative determination of Fingolimod hydrochloride using RP-HPLC-UV in the concentration of dissolution instead of LC/MS in Fingolimod HGC.

The second part is a study of Fingolimod hydrochloride dissolution profile of Fingolimod hydrochloride in different media compared with reference product and the effect of excipients on the dissolution

Instrumentation, Reagents and Chemicals
Agilent 1260 HPLC system equipped with Zorbax Plus C8, 250x4.6mm, 5μm column, DAD detector N-dibutyl amine, orthophosphoric acid of analytical Grade, water and acetonitrile (HPLC grade) Fingolimod hydrochloride Working standard.

Procedure
Preparation of buffer solution (Di-butyl ammonium phosphate)
Add 8.3 ml Orthophosphoric acid to 20 ml N-dibutyl amine while stirring and addition of water to 100 ml then adjust to pH 2.5 then complete the volume to exactly 1000 ml purified water.

Mechanism of detection
Di-butyl ammonium phosphate is ion-pair reagent which used as mobile phase to attract to Fingolimod hydrochloride molecules that result in demolishing tailing in Fingolimod peak and increase UV absorbance of whole molecule hence it increase method sensitivity up to 200 mg/ml (Figure 2).

Chromatographic condition
Fingolimod was eluted in Zorbax Plus C8, 250×4.6mm, 5μm column using a mobile phase mixture of buffer and acetonitrile in the ratio of 55:45 % v/v at temperature 30°C. The lambda max of the drug in mobile phase was 198nm, so column outlet was monitored at 198nm. The injection volume was 100μl, flow rate 2.0 ml/min and the total runtime was 5 min.

Standard solution preparation
Dissolve accurately weighed 28mg Fingolimod Hydrochloride in to a 250 ml volumetric flask, add about 150 ml of solvent (Acetonitrile: water 1:1) then sonicate for about 10 min then complete to volume with the same solvent , then dilute 1.0 ml of Solution to 100 ml with the mobile phase (Figure 3).
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Construction of Calibration Curve

The standard Fingolimod Hydrochloride solution was further diluted in 100 ml volumetric flask to obtain various concentrations ranging from 0.224 to 1.68μg/ml. From this each standard solutions 100μL was injected in to the HPLC system. The chromatograms were recorded. The concentrations of the Fingolimod Hydrochloride in μg/ml is taken in X axis and peak area of the individual concentrations of standard solution were taken in Y axis. The calibration graph was plotted (Figure 4) (Table 1) [4,5].

Figure 1: Structure of Fingolimod (2-amino-2-[2-(4-octylphenyl) ethyl] propane-1, 3-diol hydrochloride).

Figure 2: Structure of Dibutylammonium phosphate.

Figure 3: Fingolimod Hydrochloride HPLC peak.

Figure 4: Linearity Curve of Fingolimod hydrochloride.
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Table 1: Parameters for Fingolimod Hydrochloride.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>0.224 - 1.68</td>
</tr>
<tr>
<td>Slope</td>
<td>300.09</td>
</tr>
<tr>
<td>Intercept</td>
<td>8.54</td>
</tr>
<tr>
<td>R2</td>
<td>0.9992</td>
</tr>
</tbody>
</table>

Limit of Quantitation and Limit of Detection

LOQ and LOD were calculated using HPLC Agilent Chemstation to find the ratio of signal to noise 10:1 and 3.3:1 for LOQ and LOD respectively. The limit of Quantitation is found to be 112 mg/ml while the limit of detection is 56 mg/ml.

Table 2: Measurement of accuracy by standard addition method.

<table>
<thead>
<tr>
<th>Working Concentration Percent</th>
<th>Working Concentration (mc/ml)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>80%</td>
<td>0.896</td>
<td>101.79%</td>
</tr>
<tr>
<td>100%</td>
<td>1.12</td>
<td>99.54%</td>
</tr>
<tr>
<td>120%</td>
<td>1.344</td>
<td>98.53%</td>
</tr>
</tbody>
</table>

Dissolution Profile

The formula of Fingolimod capsule contains only mannitol and magnesium stearate [6]. Dissolution is performed in three standard media using apparatus 1 (Basket) at 100 RPM at 37.5 °C and the volume of media is 500 ml and the sample was directly injected to HPLC apparatus (Figure 5) (Table 3) [7].

Table 3: Dissolution parameters Fingolimod capsule contains only mannitol and magnesium stearate.

<table>
<thead>
<tr>
<th>Time (Min)</th>
<th>Test Release</th>
<th>Reference Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>86.72</td>
<td>88.42</td>
</tr>
<tr>
<td>10</td>
<td>96.83</td>
<td>93.36</td>
</tr>
<tr>
<td>15</td>
<td>100.05</td>
<td>96.09</td>
</tr>
<tr>
<td>30</td>
<td>100.27</td>
<td>99.73</td>
</tr>
</tbody>
</table>

Figure 5: Dissolution profile for Fingolimod capsule contains only mannitol and magnesium stearate.

Precision

To demonstrate agreement among results, a series of measurements are done with Fingolimod hydrochloride ten replicate injections of the specific standard at various time intervals on the same day were injected into the chromatograph and the value of %RSD was found to 1.26%. In inter-day precision same standard was injected on different days and the found %RSD was 1.68% [4,5].

Accuracy

Accuracy was done by recovery study using standard addition method, known amount of standard Fingolimod into pre-analyzed samples and subjected to proposed HPLC method. The results of recovery studies are shown in (Table 2) [4,5].
The dissolution of the drug in pH 2 gave in media the more than 85% in the first 5 min. While in acetate media pH 4.5 the dissolution rate decreased and showed more than 85% in 30 min but it is less than 80% after 15 min, the dissolution in acetate media was repeated after removal of magnesium stearate from the capsule to find out that dissolution rate exceed 85% in the first 15 min (Table 4) (Figure 6).

On the other hand the dissolution does not exceed 25% after 30 min in phosphate media pH 6.8 and these results are very similar to reference drug of Novartis.

However it was observed that the dissolution of Fingolimod Hydrochloride doubled to reach 50% after 30 min after removal of magnesium stearate in the formula (Table 5) (Figure 7).

Magnesium stearate in acidic media has no effect due to high solubility of Fingolimod hydrochloride in acidic media.

**Figure 6:** Dissolution profile for Fingolimod capsule in the absence of magnesium stearate with different pH (2.0&4.5) by using HPLC.

**Figure 7:** Dissolution profile for Fingolimod capsule in the absence of magnesium stearate with pH: 6.8 by using HPLC.

**Table 4:** Dissolution parameters for Fingolimod capsule in the absence of magnesium stearate with different pH (2.0&4.5) by using HPLC.

<table>
<thead>
<tr>
<th>Time</th>
<th>Test (No Magnesium Stearate) Release</th>
<th>Test Release</th>
<th>Reference Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>79.20</td>
<td>32.25</td>
<td>97.51</td>
</tr>
<tr>
<td>10</td>
<td>81.94</td>
<td>54.95</td>
<td>53.33</td>
</tr>
<tr>
<td>15</td>
<td>92.75</td>
<td>72.96</td>
<td>69.80</td>
</tr>
<tr>
<td>30</td>
<td>95.25</td>
<td>97.51</td>
<td>100.04</td>
</tr>
</tbody>
</table>
Table 5: Dissolution parameters for Fingolimod capsule in the absence of magnesium stearate with pH: 6.8 by using HPLC.

<table>
<thead>
<tr>
<th>Time (Min)</th>
<th>Test Without Mg Stearate Release</th>
<th>Test Release</th>
<th>Reference Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>10.51</td>
<td>8.56</td>
<td>3.74</td>
</tr>
<tr>
<td>10</td>
<td>22.85</td>
<td>13.59</td>
<td>19.18</td>
</tr>
<tr>
<td>15</td>
<td>22.85</td>
<td>16.66</td>
<td>24.68</td>
</tr>
<tr>
<td>30</td>
<td>50.41</td>
<td>21.1</td>
<td>25.23</td>
</tr>
</tbody>
</table>

Influence of Magnesium Stearate on Fingolimod Hydrochloride

During dissolution it was observed that removing magnesium stearate from the formula rises the rate of dissolution especially in acetate and phosphate media therefore IR test and Differential scanning Calorimetry (DSC) to determine if the interaction between drug and magnesium stearate is hydrophobic effect or hydrogen bonding [8].

FT-IR scanning of Fingolimod and Magnesium stearate mixture show that the peak of O-H at 3400 cm⁻¹ in Fingolimod FT-IR Spectrum has been diminished and the strength of C=O peak in magnesium stearate FT-IR spectrum at 1650 cm⁻¹ has decreased in mixture sample spectrum [9] (Figure 8-11).

This interaction has been confirmed by performing DSC for Fingolimod hydrochloride and magnesium stearate mixture which show large endothermic peak of the mixture magnesium stearate and Fingolimod hydrochloride compared with each individual material indicating the presence of hydrogen bonding. On the other hand, hydrophobic effect is always accompanied with decrease in heat absorbance (Figure 12) [10,11].

![Figure 8: IR spectrum of Fingolimod HCL](image1)

![Figure 9: IR spectrum of Magnesium stearate.](image2)
Conclusion

The developed RP-HPLC method was validated and the system suitability studies were performed and all parameters combined with the simplicity and ease of operation ensures that the validated method can successfully be used for routine analysis of Fingolimod hydrochloride in bulk and capsule dosage formulation. Although magnesium stearate has influence on Fingolimod hydrochloride solubility, it has no effect on its bioavailability.

References


8. Is magnesium stearate safe to use as a supplement excipient?

