INTRODUCTION

Chromatography is a powerful separation tool that is used in all branches of science, and is often the only means of separating components from complex mixtures. It is mostly implemented in science subjects such as chemistry and life sciences, especially in biochemistry. Chromatography comes from the Greek words, chroma meaning ‘color’ and graphein meaning ‘to write’. Chromatography can be analytical or preparative, based on the main objective for which it has been followed. Analytical chromatography makes use of only a small amount of a mixture and determines the components of that particular mixture. Depending on the techniques used in chromatography, the process is broadly classified as, Adsorption chromatography, Partition chromatography. New types of chromatography developed during the 1930s and 1940s made the technique useful for many separation processes. Chromatography technique was developed substantially as a result of the work of Archer John Porter Martin and Richard Laurence Millington Synge during the 1940s and 1950s. They established the principles and basic techniques of partition chromatography, and their work encouraged the rapid development of several chromatographic methods like, Paper Chromatography, Gas Chromatography, High Performance Liquid Chromatography. Advances are continually improving the technical performance of chromatography, allowing the separation of increasingly similar molecules. Chromatography is used as a technique to separate the additives, vitamins, preservatives, proteins and amino acids. Some other uses are in the detection of drugs or medications in the urine. It is used by pharmaceutical companies to prepare large amounts of pure materials that are further required in making medicines. Also, it is used to check the presence of any contamination in the manufactured compounds. It is also very popular in forensic science for investigative purposes.
MATERIALS AND METHODOLOGY:

Materials:

For the determination of Bortezomib in tablet dosage form by HPLC method the following Chemicals were used such as Methanol, Distilled water, Acetonitrile, Formic acid.

Method Development

Chromatographic trails for estimation of Bortezomib by RP-HPLC.

Table 1: Trial 1: Chromatographic trails for estimation of Bortezomib by RP-HPLC

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>Methanol: water (50:50 v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>C18 Phenomenex Luna (250x4.6 mm;5µ)</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.0 ml/min</td>
</tr>
<tr>
<td>Temperature</td>
<td>Ambient</td>
</tr>
<tr>
<td>Wavelength</td>
<td>280 nm</td>
</tr>
<tr>
<td>Injection volume</td>
<td>20 µl</td>
</tr>
<tr>
<td>Run time</td>
<td>10 min</td>
</tr>
<tr>
<td>Retention time</td>
<td>7.6min</td>
</tr>
<tr>
<td>Inference</td>
<td>Broad peak and peak shape is not good</td>
</tr>
</tbody>
</table>

Table 2: Trial 2: CMG showing trial-1 chromatogram

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>Acetonitrile : water (50:50 v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>C18 Phenomenex Luna (250x4.6 mm;5µ)</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.0 ml/min</td>
</tr>
<tr>
<td>Temperature</td>
<td>Ambient</td>
</tr>
<tr>
<td>Wavelength</td>
<td>280 nm</td>
</tr>
<tr>
<td>Injection volume</td>
<td>20 µl</td>
</tr>
<tr>
<td>Run time</td>
<td>10 min</td>
</tr>
<tr>
<td>Retention time</td>
<td>6.3min</td>
</tr>
<tr>
<td>Inference</td>
<td>Broad peak and peak shape is not good</td>
</tr>
</tbody>
</table>

Preparation of the optimized mobile phase:

Preparation of 0.1% formic acid

Weigh 0.1ml of formic acid and transferred to 100 ml volumetric flask, dissolved in sufficient quantity of water and then diluted to the mark with water ¹.

Preparation of mobile phase

Preparation of mobile phase by using Methanol and water in the ratio of Acetonitrile : 0.1% formic acid (50: 50 v/v). The mobile phase was filtered through 0.45 um membrane filter paper. After filtration it was ultra sonicated for 10 minute on ultra sonicator ².

Preparation of stock solution of Bortezomib

API of Bortezomib (10mg) accurately weighed and transferred to 10 ml volumetric flask, dissolved in mobile phase. The solution contains 1000ug/ml of Bortezomib. The solution was filtered through 0.45 um membrane filter paper and firs few drops of filtrate were discarded ³.

Preparation of sample solution:

Take 25 mg equivalent tablet powder of GSF and dilute up to 25 ml with Acetonitrile : 0.1% formic acid (50: 50 v/v). Sonicate it for 10 minutes. Filter the solution through Whatmann filter paper no. 41. This solution was used as sample solution ⁴.

Procedure:

20 µL of the blank, standard and sample was injected in to the chromatographic system and areas for the Bortezomib peaks were used for calculating the % assay by using the formulae ⁵.

System suitability

- Tailing factor for the peaks due to Bortezomib in standard solution should not be more than 1.5.
- Theoretical plates for the Bortezomib peaks in standard solution should not be less than 1.5.
Analytical Method Validation

Validation parameters
1. Specificity
2. Linearity
3. Range
4. Accuracy
5. Precision
   i. Repeatability
   ii. Intermediate precision
6. Limit of Detection
7. Limit of Quantitation
8. Robustness

1. Specificity
   In the case of assay, demonstration of assay specificity is required to show that the procedure is unaffected by the impurities or excipients. Specificity of an analytical method indicates that the analytical method is able to measure accurately and specifically the analyte of interest without any interference from blank. So here, the specificity was determined by the comparison of the chromatograms of
   a. Blank (mobile phase)
   b. Standard sample solutions of Bortezomib
   c. Sample solution of Bortezomib

Acceptance criteria:
Chromatogram of standard and sample should be identical with near Retention time.

2. Linearity

Preparation of solution:
API of Bortezomib (10mg) accurately weighed and transferred to 10 ml volumetric flask, dissolved in sufficient quantity of methanol and then diluted to the mark with mobile phase.

Preparation of level – 1 (20µg/ml of Bortezomib )
From the standard solution (SS) 0.2 ml was taken in to 10 ml volumetric flask and diluted up to mark with methanol.

Preparation of level – 2 (40µg/ml of Bortezomib )
From the standard solution (SS) 0.4 ml was taken in to 10 ml volumetric flask and diluted up to mark with diluents.

Procedure:
Each level was injected in to the chromatographic system and peak area was measured. Plot a graph of peak area versus concentration (on x -axis concentration and on y axis peak area) and the correlation was calculated.

Acceptance criteria
Correlation coefficient should be not less than 0.9994.

3. Range

Based on precision, linearity and accuracy data it can be concluded that the assay method is precise, linear and accurate in the range of 20-120 µg/ml of Bortezomib respectively.

4. Accuracy

The accuracy of the test method is demonstrated by % of recovery. Accuracy was performed in three different three concentration levels and injected three times (Like 50%, 100%, and 150%). The observations are mentioned below.

Preparation of sample solutions

Preparation of 50% solution (with respect to target assay concentration)
From the stock solution 0.4ml was taken in to 10ml volumetric flask and diluted up to the mark with diluents.

Preparation of 100% solution (with respect to target assay concentration)
From the stock solution 0.8 ml was taken in to 10ml volumetric flask and diluted up to the mark with diluents.

Procedure:
The standard solutions of accuracy 50%, 100% and 150% were injected in to chromatographic system. Calculate the amount found and amount added for Bortezomib and calculate the individual % recovery and mean % recovery values.

Acceptance criteria
% Recovery at each spike level shall be not less than 98.0 and not more than 102.0.

5. Precision

i. Repeatability

Preparation of solution
From the stock solution 0.8 ml was taken in to 10ml volumetric flask and diluted up to the mark with methanol.

Procedure
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 to be within the specified limits.

Acceptance criteria
The % RSD for the area of five standard injections results should not be more than 2.

ii. Intermediate precision/Ruggedness
To evaluate the intermediate precision (also known as ruggedness) of the method, precision was performed on different day by using different make column of same dimensions.

Preparation of solution
From the stock solution 0.8 ml was taken in to 10ml volumetric flask and diluted up to the mark with methanol.

Procedure
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 to be within the specified limits.

Acceptance criteria
The % RSD for the area of five sample injections results should not be more than 2%.

6. Limit of detection (LOD)

LOD’s can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (s) at levels approximating the LOD according to the formula. The standard deviation of the response can be determined based on the standard deviation of y- intercepts of regression lines.

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Formula: $\text{LOD} = 3.3 \times \frac{SD}{S}$

Where

SD – Standard deviation (SD)  
S – Slope

Acceptance criteria
The values should not more than 3 for LOD solution.

7. Limit of quantification (LOQ)
LOQ’s can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (s) according to the formula. Again, the standard deviation of the response can be determined based on the standard deviation of y- intercepts of regression lines.

Formula: $\text{LOD} = 10 \times \frac{SD}{S}$

Where

SD – Standard deviation (SD)  
S – Slope

Acceptance criteria
The values should not more than 10 for LOQ solution.

8. Robustness
As Part of the robustness, deliberate change in the flow rate, mobile phase composition was made to evaluate the impact on the method

a) The flow rate was varied at ± 10%. Standard solution 40 µg/ml of Bortezomib was prepared and analysed using the varied flow rates along with method flow rate.

b) The Temperature was varied (± 5°C) Standard solution 40 µg/ml of Bortezomib was prepared and analysed using the varied flow rates along with method flow rate.

RESULTS AND DISCUSSION
Validation Report:

Specificity:
Specificity by Direct comparison method
There is no interference of mobile phase, and placebo with the analyte peak and also the peak purity of analyte peak which indicate that the method is specific for the analysis of analytes in their dosage form.

Table 3: Specificity Data

<table>
<thead>
<tr>
<th>S.No</th>
<th>Peak Name</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Blank</td>
<td>Nil</td>
</tr>
<tr>
<td>2</td>
<td>Placebo</td>
<td>Nil</td>
</tr>
<tr>
<td>3</td>
<td>Standard</td>
<td>$R_t$: 5.3 min  (\lambda_{max}: \text{nm})</td>
</tr>
</tbody>
</table>

System suitability: System suitability test was an integral part of method development and has been used to ensure adequate performance of the chromatographic system.

Table 4: Results of System Suitability

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Result</th>
<th>Acceptance Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time (Rt)*</td>
<td>5.3 min</td>
<td>More than 2</td>
</tr>
<tr>
<td>Resolution factor*</td>
<td>NA</td>
<td>--</td>
</tr>
<tr>
<td>Number of theoretical plates (N)*</td>
<td>3652</td>
<td>More than 2000</td>
</tr>
<tr>
<td>Tailing factor (T)*</td>
<td>1.32</td>
<td>Less than 2</td>
</tr>
</tbody>
</table>

* Number of injections: 6 replicates

Results for intraday and inter day precision

Table 5: Intraday and Inter day precision

<table>
<thead>
<tr>
<th>S.No</th>
<th>Intraday precision Area</th>
<th>Inter day precision Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>244125</td>
<td>242145</td>
</tr>
<tr>
<td>2</td>
<td>242451</td>
<td>241245</td>
</tr>
<tr>
<td>3</td>
<td>244512</td>
<td>248596</td>
</tr>
<tr>
<td>4</td>
<td>242010</td>
<td>245487</td>
</tr>
<tr>
<td>5</td>
<td>241201</td>
<td>242403</td>
</tr>
<tr>
<td>6</td>
<td>254125</td>
<td>251593</td>
</tr>
<tr>
<td>Mean</td>
<td>244737.3</td>
<td>245244.8</td>
</tr>
<tr>
<td>Std Dev</td>
<td>4353.645</td>
<td>3769.877</td>
</tr>
<tr>
<td>%RSD</td>
<td>1.778905</td>
<td>1.537189</td>
</tr>
</tbody>
</table>

Table 6: Linearity

<table>
<thead>
<tr>
<th>S. No</th>
<th>Concentration (µg/mL)</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>68451</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>121021</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>184241</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>241520</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>304512</td>
</tr>
<tr>
<td>6</td>
<td>120</td>
<td>365412</td>
</tr>
</tbody>
</table>

Accuracy
Accuracy of the method was determined by Recovery studies [Table 7]. To the formulation (pre analyzed sample), the reference standards of the drugs were added at the level of 50%, 100% [Figure 6, 7].

Linearity and range

Figure 4: Chromatogram showing Linearity 20 µg/mL

Figure 5: Chromatogram showing Linearity 40 µg/mL
Table 7: Results of accuracy

<table>
<thead>
<tr>
<th>Spiked Concentration (μg/mL)</th>
<th>Peak area</th>
<th>Amount added (μg/mL)</th>
<th>Amount Found (μg/mL)</th>
<th>Recovery</th>
<th>% Mean Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>50%</td>
<td>121021</td>
<td>40.01</td>
<td>40.2496</td>
<td>100.5989</td>
<td>101.54</td>
</tr>
<tr>
<td></td>
<td>120041</td>
<td></td>
<td>39.92367</td>
<td>99.78423</td>
<td></td>
</tr>
<tr>
<td></td>
<td>125412</td>
<td></td>
<td>41.70998</td>
<td>104.2489</td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td>241520</td>
<td>80.02</td>
<td>80.3256</td>
<td>100.3819</td>
<td>101.37</td>
</tr>
<tr>
<td></td>
<td>248745</td>
<td></td>
<td>82.72852</td>
<td>103.3848</td>
<td></td>
</tr>
<tr>
<td></td>
<td>241452</td>
<td></td>
<td>80.30298</td>
<td>100.3536</td>
<td></td>
</tr>
</tbody>
</table>

LOD & LOQ:

Table 8: Results of LOD & LOQ:

<table>
<thead>
<tr>
<th>S.NO</th>
<th>Parameter</th>
<th>Slope</th>
<th>Standard Deviation</th>
<th>Value (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Limit of Detection</td>
<td>5978</td>
<td>4353</td>
<td>2.402</td>
</tr>
<tr>
<td>2</td>
<td>Limit of Quantification</td>
<td></td>
<td></td>
<td>7.281</td>
</tr>
</tbody>
</table>

Robustness:
**CONCLUSION:**

The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study for Bortezomib was found in concentration range of 20µg-120µg/ml and correlation coefficient ($r^2$) was found to be 0.9994, % recovery was found to be 101.37%, % RSD for repeatability was 1.778, % RSD for intermediate precision was 1.537 respectively. The precision study was precise, robust, and repeatable. LOD value was 2.6402, and LOQ value was 7.281 respectively. Hence the suggested RP-HPLC method can be used for routine analysis of Bortezomib in API and pharmaceutical dosage form.

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**REFERENCES:**


