RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF BERBERINE IN BULK AND PHARMACEUTICAL DOSAGE FORMS

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Abstract: Berberine is a iso-quinoline alkaloid and is important phytochemical constituent of Berberis aristata. (berberidaceae.). An isocratic RP-HPLC method was developed for the estimation of Berberine in pharmaceutical dosage. WATERS HPLC Aquity system equipped with quaternary pumps, UV detector and Auto sampler integrated with Empower 2 Software Kromosil 250x4.6mm, 5μ and a mobile phase composed of 0.1%tri fluoro acetic acid:acetonitrile (70:30v/v). The flow rate was 1.0 ml/min and the analyses of column effluents were performed using UV-Visible detector at 344 nm. Retention time of Berberine was found as 5.003 min. This method has obeyed linearity over the concentration range of 2-12µg/ml and the regression coefficient obtained from linearity plot for Berberine was found as 0.997. %RSD of berberine was found to be 0.118 .%Recovery was obtained as 92-98. LOD, LOQ values obtained from regression equations are 0.488 µg/ml, 1.478 µg/ml respectively. Regression equation of berberine is y = 57.86.x+0.407.. Retention time was decreased and run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in industries.

Keywords: Berberine, RP-HPLC, VALIDATION

Introduction: The quality control of consumer products has become more challenging and demanding. The quality considerations of drugs are the most stringent among all consumer products. Several national and international agencies have prioritized the issue of assuring the quality of plant drugs. The effort of the World Health Organization is outstanding: over 20 years ago it first published Quality control methods for medicinal plant materials, which has been regularly updated and followed by a series of monographs on globally important medicinal plants. The quality of a plant product cannot be assured without assuring the quality of the raw material. Also required to ensure quality products are in-process control, quality control of the finished product, good manufacturing practice (GMP) controls and process validation. materials..

The WHO defines markers as “constituents of a medicinal plant material which are chemically defined and of interest for control purposes.” Markers serve to calculate the quantity of herbal substances or herbal preparations in the herbal medicinal product if the marker has been quantitatively determined in the herbal substance or herbal preparations. After a marker has been identified, it needs to be quantified or assayed in the test material for the purpose of quality control. Any of the major analytical techniques, including high performance liquid chromatography (HPLC), high performance thin layer chromatography (HPTLC), gas chromatography (GC), radioimmunoassay, ultraviolet or infrared spectrometry, and mass spectrometry, can be used to determine the quantities of marker in the test samples. Development of the assay method is followed by its validation..

Berberine is a iso-quinoline alkaloid and is a major constituent of the species Berberis aristata. (berberidaceae.). It is a yellow solid with a molecular weight 336.36. It belongs to the category Antipsychotic.
5,6-dihydro-9,10- dimethoxybenzo [g]-1, 3 benzodioxolo [5,6a] Quinolizium.

Its trade name was mother tincture of Berberis vulgaris, clear stone drops. Melting point was 145°C. Dosage forms are mother tincture - 30 ml; 100 ml. It is soluble in Methanol, acetonitrile, water. PKa was 2.47. Berberine has been tested and used successfully in experimental and human diabetes mellitus. Berberine has been shown to lower elevated blood glucose as effectively as metformin. The mechanisms of action include inhibition of aldose reductase, inducing glycolysis, preventing insulin resistant through increasing insulin receptor expression. A new study suggested Berberine may overcome insulin resistance via modulating key molecules in insulin signaling pathway, leading to increased glucose uptake in insulin-resistant cells.

Materials and method:
Electronics Balance-Denver, PIndia, Ultrasonicator-BVK enterprises, WATERS HPLC Aquity system equipped with quaternary pumps, UV detector and Auto sampler integrated with Empower 2 Software was used for LC peak integration and Data processing. UV spectrophotometer PG Instruments T60 with special band width of 2mm and 10mm and matched quartz cells integrated with UV-win 6 Software was used for measuring absorbance of berberine. The mobile phase used was 0.1% trifluro acetic acid, acetonitrole (70:30) 1ml/min. samples were analyzed at 344 nm detector wave length, and at an injection volume of 10 μL using Kromosil250x4.6mm, 5μ with run time of 6 min.

Method:
PREPARATION OF REQUIRED SOLUTIONS FOR ESTIMATION OF BERBERINE BY RP   HPLC

Preparation of mobile phase:
Here HPLC grade 500 mL acetonitrile was ultrsonicated for 20 minutes on ultrasonicator. Accurately measured out 0.5 mL of trifluoroacetic acid and transferred in to 500 mL volumetric flask. Volume was made up with water up to mark to got 0.1% TFA in water and the prepared solution ultrsonicated for 20 minutes on ultrasonicator.

Preparation of standard solution of Berberine:
About 10 mg of pure Berberine was accurately weighed and dissolved in 10 mL of mobile phase to get 1mg.mL⁻¹ stock solution. Working standard solution of Berberine was prepared with mobile phase. The final volume was made with the mobile phase. The standard solution was filtered through 0.45 μm nylon membrane filter and degassed by sonication.

Preparation of sample solution of Berberine:
1ml of prepared marketed formulation of mother tincture was taken into 10ml volumetric flask and the tincture was made to dissolve with mobile phase and made unto the mark with mobile phase. The resulting solution was filtered using Whatman Grade No.1 filter paper and degassed by sonication. This solution was further suitably diluted for chromatography.
Selection of detector wavelength

The UV-visible spectra of various diluted solutions of berberine in mobile phase were recorded using UV-visible spectrophotometer. The peak of maximum absorbance was observed at 344 nm. This wavelength was used for detection of Berberine.

METHOD VALIDATION:

Estimation of Berberine by Rp-hplc

Specificity
The effect of wide range of excipients and other additives usually present in the formulations of Berberine in the determinations under optimum conditions was investigated.

Preparation of standard stock solution for Berberine:
About 10 mg Berberine was weighed accurately. It was transferred to 10ml volumetric flask, dissolved and made up to the volume with mobile phase to obtain 1mg/ml solution.

Preparation of 10µg/ml solution of Berberine:
From the stock solution, 1ml was taken into 10ml volumetric flask and diluted and made up to the mark with mobile phase to obtain a concentration of 100µg/ml. From 100µg/ml solution, 1ml was taken into 10ml volumetric flask and diluted and made up to the mark with mobile phase to obtain a concentration of 10µg/ml.

Procedure

The specificity of the RP-HPLC method was established by injecting the mobile phase and placebo solution in triplicate and recording the chromatograms. The common Excipients have been added to the placebo solution and injected and tested.

Accuracy:
Accuracy is a measure of the closeness of test results obtained by a method to the true value. It is determined by applying the method to samples to which known amounts of analyte have been added. These should be analyzed against standard and blank solutions to ensure that no interference exists. The accuracy is then calculated from the test results as a percentage of the analyte recovered by the assay.

Preparation of Standard Solution (authentic):
Weigh accurately about 10mg of berberine and transfer to clean and dry 10ml volumetric flask and add diluents upto the mark then sonicate for 5min.

Preparation of Test Solutions:
Preparation of 25% Sample Solution:
1 ml of sample was taken and transfer to a 10 ml volumetric flask and make up to the mark with mobile phase and sonicate for 5 min. From this solution 1ml was taken and transfer to a 10ml volumetric flask and made up to the mark with mobile phase. From the above solution 1ml was taken and transferred to a 10ml volumetric flask and make up to the mark with mobile phase. Then sonicate for 5min.

Preparation of 50% Sample Solution:
2 ml of sample was taken and transfer to a 10 ml volumetric flask and make up to the mark with mobile phase and sonicate for 5 min. From this solution 1ml was taken and transfer to a 10ml volumetric flask and was made up to the mark with mobile phase. From the above solution 1ml was taken and transfer to a 10ml volumetric flask and make up to the mark with mobile phase. Then sonicate for 5min.

Preparation of 75% Sample Solution:
4 ml of sample was taken and transfer to a 10 ml volumetric flask and make up to the mark with mobile phase and sonicate for 5 min. From this solution 1ml was taken and transferred to 10ml volumetric flask and made up to the mark with mobile phase. From the above solution 1ml was taken and transferred to 10ml volumetric flask and make up to the mark with mobile phase. Then sonicate for 5min.

Acceptance: The mean % recovery at each level should not be less than 96% and should not be more than 103%.
Precision

Preparation of standard stock solution for Berberine.

About 10 mg Berberine was weighed accurately. It was transferred to 10ml volumetric flask, dissolved and made up to the volume with mobile phase.

Preparation of 10μg/ml solution of Berberine:

From the stock solution, 1ml was taken into 10ml volumetric flask and diluted and made up to the mark with mobile phase to obtain dilution with concentration 100 μg/ml. From 100 μg/ml solution, 1ml was taken into 10ml volumetric flask and diluted and made up to the mark with mobile phase to obtain a concentration of 10 μg/ml.

Procedure for estimation of Berberine:

Intraday and interday precision study of Berberine was carried out by estimating corresponding responses 3 times on the same day and on 3 different days for the concentration of 10 μg. The percent relative standard deviation (% RSD) was calculated which is within the acceptable criteria of not more than 2.0. The results for intraday and interday precision were presented in tables respectively.

Linearity:

Preparation of standard stock solution for Berberine:

About 10mg Berberine was weighed accurately. It was transferred to 10ml volumetric flask, dissolved and made up to the volume with mobile phase to obtain 1mg/ml solution. From the stock solution, 1ml was taken into 10ml and diluted with mobile phase and made up to the mark with mobile phase to obtain a concentration of 100 μg/ml.

Preparation of standard dilutions of Berberine:

From the 100 μg/ml solution, 0.2ml, 0.4ml, 0.6ml, 0.8ml, 1ml, 1.2ml were pipetted out in to 10ml volumetric flask and made upto the mark with mobile phase to obtain dilutions with concentrations 2μg/ml, 4μg/ml, 6μg/ml, 8μg/ml, 10μg/ml 12μg/ml respectively.

Procedure

Inject each concentration in to the chromatographic system and measure the peak area. Plot the graph of peak area on y axis versus concentration on x axis. Calculate the correlation coefficient.

Robustness:

Preparation of standard stock solution for Berberine: About 10 mg berberine was weighed accurately. It was transferred to 10ml volumetric flask, dissolved and made up to the volume with mobile phase to obtain 1mg/ml solution.

Preparation of 10μg/ml solution of Berberine:

From the stock solution, 1ml was taken into 10ml and diluted with mobile phase and made up to the mark with mobile phase to obtain a concentration of 100μg/ml. From 100μg/ml solution, 1ml was taken into 10ml and diluted with mobile phase and made up to the mark with mobile phase to obtain a concentration of 10μg/ml.

LOD sample Preparation: 0.25ml standard stock solutions was pipetted out and transferred to separate 10ml volumetric flasks and made up with diluents. From the above solutions 0.1ml each of beriberine, solution respectively were transferred to 10ml volumetric flask and made up with the same diluents.
**LOQ sample Preparation:** 0.25ml each from standard stock solutions was pipetted out and transferred to separate 10ml volumetric flask and made up with diluent. From the above solutions 0.3ml each of and solution respectively were transferred to 10ml volumetric flasks and made up with the same diluent.

**System suitability parameters**

The system suitability parameters were determined by preparing standard solutions of beriberine (100ppm) and the solutions were injected six times and the parameters like peak tailing, resolution and USP plate count were determined to check whether the results complies.

<table>
<thead>
<tr>
<th>System suitability parameters</th>
<th>Berberine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tailing factor (T)</td>
<td>1.344</td>
</tr>
<tr>
<td>Number of theoretical plate(n)</td>
<td>7448</td>
</tr>
<tr>
<td>Retention time (R_t)</td>
<td>5.123 mins</td>
</tr>
</tbody>
</table>

**Procedure**

**Assay of Berberine**

From the stock solution of standard and sample, 0.1 ml was pipette out in to 10ml volumetric flask individually. It was dissolved using mobile phase and made upto the mark with the same to get a solution of 10µg/ml of each. 20µl this standard and sample were injected three times separately in the chromatographic system. Measure the area of Berberine peaks and calculate % assay using the formula.

\[
\text{Assay } % = \left[ \frac{\text{AT}}{\text{AS}} \times \frac{\text{WS}}{\text{WT}} \times \frac{\text{DT}}{\text{DS}} \times \frac{\text{P}}{100} \right] \times 100
\]

Where:

- AT = Peak Area of berberine obtained with test preparation.
- AS = Peak Area of berberine obtained with standard preparation.
- WS = Weight of working standard taken in mg
- WT = Weight of sample taken in mg
- DS = Dilution of Standard solution
- DT = Dilution of sample solution
- P = Percentage purity of working standard

**Results and discussion**

An effort has been made to identify a Simple, Precise, Specific and Accurate method for estimation of Berberine in formulation by using RP-HPLC method.

During the selection of mobile phase several solvents were tried at various levels and finally selected mobile phase system was 0.1% tri fluoroacetic acid: Acetonitrile at ratio 70:30 at ambient temperature.

The concentration of (10µg/ml) of Berberine was prepared by using mobile phase. The above solution was scanned in the range of 200-400nm by using UV-VIS spectrophotometer with mobile phase as reference. After considering all the system suitability parameters, 0.1% tri fluoroacetic acid: Acetonitrile (70:30) was selected for analysis at optimized flow rate of 1.0 ml/min. The Retention time of Berberine was found to be 5.003 min as shown in the Fig 1.
The Linearity of Berberine was carried out at different concentrations ranging from 2-12µg/ml and correlation coefficient was found to be 0.997 which indicates that the concentration had given good linearity as shown in Table19. Accuracy was confirmed by Recovery Studies. The % recoveries of Berberine were found to be 99.57% and 96.88% which was in the acceptance limit of 95 to 103% as shown in Table 15, 16.

Table 1 Optimised Conditions

<table>
<thead>
<tr>
<th>optimised chromatographic conditions</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mode of separation</td>
<td>Isocratic elution</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>0.1% tri fluoro acetic acid: Acetonitrile (70 : 30 v/v)</td>
</tr>
<tr>
<td>Column</td>
<td>Kromosil 250x4.6mm, 5µ</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1 ml/min</td>
</tr>
<tr>
<td>Detector wavelength</td>
<td>344 nm</td>
</tr>
<tr>
<td>Injection volume</td>
<td>10µl</td>
</tr>
<tr>
<td>Oven temperature</td>
<td>Ambient</td>
</tr>
<tr>
<td>Run time</td>
<td>6 min</td>
</tr>
</tbody>
</table>

The Chromatogram of optimized method

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Fig No 1 Chromatogram of optimized method

Calibration curve of Berberine

\[ y = 57.863x + 0.4077 \]

\[ R^2 = 0.9979 \]
<table>
<thead>
<tr>
<th>s.no</th>
<th>Concentration (µg/ml)</th>
<th>Peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 µg/ml</td>
<td>121.559</td>
</tr>
<tr>
<td>2</td>
<td>4 µg/ml</td>
<td>243.534</td>
</tr>
<tr>
<td>3</td>
<td>6 µg/ml</td>
<td>334.81</td>
</tr>
<tr>
<td>4</td>
<td>8 µg/ml</td>
<td>445.206</td>
</tr>
<tr>
<td>5</td>
<td>10 µg/ml</td>
<td>582.214</td>
</tr>
<tr>
<td>6</td>
<td>12 µg/ml</td>
<td>705.786</td>
</tr>
</tbody>
</table>

**Table No 2 Linearity result of berberine**

Results: The correlation coefficient values were found to be within the acceptance limits for Berberine i.e. correlation coefficient is not less than 0.997.

**PRECISION**

Precision was studied to find out intra and inter day variations in the test methods of Berberine for the three times on the same day and different day. The %RSD values of Berberine for Precision was found to be 0.54 & 0.118 respectively as shown in the Table 3 which were in the acceptance limit of less than 2%.

<table>
<thead>
<tr>
<th>System precision</th>
<th>Method precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Injection sample</td>
<td></td>
</tr>
<tr>
<td>R&lt;sub&gt;t&lt;/sub&gt;</td>
<td>Area</td>
</tr>
<tr>
<td>Berberis aquifolium</td>
<td>5.13</td>
</tr>
<tr>
<td></td>
<td>5.11</td>
</tr>
<tr>
<td></td>
<td>5.10</td>
</tr>
<tr>
<td></td>
<td>5.09</td>
</tr>
<tr>
<td></td>
<td>5.08</td>
</tr>
<tr>
<td></td>
<td>5.09</td>
</tr>
</tbody>
</table>

**Table no 3 Results of precision**

**REPORT**: The results obtained indicate that the % RSD was found to be less than 2 % which was in the acceptance limits
Accuracy

<table>
<thead>
<tr>
<th>inject samples</th>
<th>spike level</th>
<th>area of peak</th>
<th>amount present (µg/ml)</th>
<th>amount recovered (µg/ml)</th>
<th>% recovery</th>
<th>mean recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Berberis Aquifolium</td>
<td>25%</td>
<td>642.312</td>
<td>12</td>
<td>11.09</td>
<td>92.41</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50%</td>
<td>871.822</td>
<td>14</td>
<td>15.06</td>
<td>100</td>
<td>96.88</td>
</tr>
<tr>
<td></td>
<td>75%</td>
<td>909.783</td>
<td>16</td>
<td>15.71</td>
<td>98.23</td>
<td></td>
</tr>
</tbody>
</table>

Table no 4  % Recovery data

REPORT: The mean recoveries of the drugs were found to be 96.88% under the acceptance criteria within the limit of 90 to 100%.

Specificity

The specificity was studied for the examination of the presence of interfering components, while the comparison of chromatograms there was no interference from placebo (Figure 15) with sample peak. They do not disturb the elution or quantification of Berberine; furthermore the well-shaped peaks also indicate the specificity of the method. Therefore, it was concluded that the method is specific. The specificity results are summarized in Table 5.

<table>
<thead>
<tr>
<th>Name of the solution</th>
<th>Retention time (Rt) minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase</td>
<td>No peaks</td>
</tr>
<tr>
<td>Placebo</td>
<td>No peaks</td>
</tr>
<tr>
<td>Berberine 10 µg./Ml</td>
<td>5.003 minutes</td>
</tr>
</tbody>
</table>

Table no 5 specificity data

Robustness

The Robustness of the method developed was validated by changing the flow Rate, Detection wavelength, and mobile phase has shown in Table 20. The selected flow rate, Detection wavelength and mobile phase gives good separation of the drug.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Parameter</th>
<th>Optimized</th>
<th>Used</th>
<th>Retention (Rt), min</th>
<th>Peak asymmetry</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Flow rate (± 0.2 mL/min)</td>
<td>1.0 mL/min</td>
<td>0.8 mL/min</td>
<td>5.286</td>
<td>1.344</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.2 mL/min</td>
<td>5.156</td>
<td>1.284</td>
</tr>
<tr>
<td>2.</td>
<td>Detection wavelength (± 5 nm)</td>
<td>344 nm</td>
<td>339 nm</td>
<td>5.082</td>
<td>1.118</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>349 nm</td>
<td>5.096</td>
<td>1.235</td>
</tr>
<tr>
<td>3.</td>
<td>Mobile phase composition (± 5 %)</td>
<td>60:40 v/v</td>
<td>55:45 v/v</td>
<td>5.125</td>
<td>1.118</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>45:55 v/v</td>
<td>5.241</td>
<td>1.474</td>
</tr>
</tbody>
</table>

Table no 6 Data of robustness
REPORT: The % RSD for asymmetry and Rt was found to be below 2% within the limit

LIMIT OF DETECTION

<table>
<thead>
<tr>
<th>s.no</th>
<th>Name of the drug</th>
<th>Limit of detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Berberine (µg/mL)</td>
<td>0.488 µg/ml.</td>
</tr>
</tbody>
</table>

Table no.7 Limit of detection of Berberine

LIMIT OF QUANTITATION

<table>
<thead>
<tr>
<th>s.no</th>
<th>Name of the drug</th>
<th>Limit of quantitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Berberine (µg/mL)</td>
<td>1.478 µg/ml.</td>
</tr>
</tbody>
</table>

Table no.8 Limit of Quantitation of Berberine

Assay of berberine

The tincture formulations were selected for analysis. The nominal concentration (100%) considered and 20µl of formulation was injected. The Assay percentages of Berberine present in the samples were found to be 99.61% and 109.87% as shown in Table 10.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Standard area</th>
<th>Sample area</th>
<th>Standard weight</th>
<th>Sample weight</th>
<th>Standard purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Berberine</td>
<td>705.786</td>
<td>775.346</td>
<td>10mg</td>
<td>11mg</td>
<td>99.75</td>
</tr>
</tbody>
</table>

Table no 9 Assay results of Berberine
All the above parameters combined with the simplicity and ease of operation ensures that the RP-HPLC method can be applied for Estimation of Berberine in routine analysis of the two drugs in tincture forms.

**Conclusion:**

An effort has been made to identify a Simple, Precise, Specific and accurate method for estimation of Berberine in formulation by using RP-HPLC method. During the selection of mobile phase several solvents were tried at various levels and finally selected mobile phase system was 0.1% tri fluoroacetic acid: Acetonitrile at ratio 70:30 at ambient temperature.

The concentration of (10µg/ml) of Berberine was prepared by using mobile phase. The above solution was scanned in the range of 200-400nm by using UV-VIS spectrophotometer with mobile phase as reference. After considering all the system suitability parameters, 0.1% tri fluoroacetic acid: Acetonitrile (70:30) was selected for analysis at optimized flow rate of 1.0 ml/min. The Retention time of Berberine was found to be 5.003 min as shown in the Fig .1.

The Linearity of Berberine was carried out at different concentrations ranging from 2-12µg/ml and correlation coefficient was found to be 0.997 which indicates that the concentration had given good linearity as shown in Table. Accuracy was confirmed by Recovery Studies. The % recoveries of Berberine were found to be 92.57% and 98.88% which was in the acceptance limit of 95 to 103% as shown in Table 2.

Precision was studied to find out intra and inter day variations in the test methods of Berberine for the three times on the same day and different day. The %RSD values of Berberine for Precision was found to be 0.33 & 0.54 respectively as shown in the Table 3 which were in the acceptance limit of less than 2%.

The specificity was studied for the examination of the presence of interfering components, while the comparison of chromatograms there was no interference from placebo with sample peak. They do not disturb the elution or quantification of Berberine; furthermore the well-shaped peaks also indicate the specificity of the method. Therefore, it was concluded that the method is specific. The specificity results are summarized in Table 5.

The Robustness of the method developed was validated by changing the flow Rate, Detection wavelength, and mobile phase has shown in Table 6. The selected flow rate, Detection wavelength and mobile phase gives good separation of the drug.

The tincture formulations were selected for analysis. The nominal concentration (100%) considered and 20µl of formulation was injected. The Assay percentages of Berberine present in the samples were found to be 99.61% and 109.87% as shown in Table 9. All the above parameters combined with the simplicity and ease of operation ensures that the RP-HPLC method can be applied for Estimation of Berberine in routine analysis.
References: