Development and Validation of RP-HPLC method for quantification of Loturine from Polyherbal formulation containing Symlocos Racemosa (Roxb).

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Received 08 October 2012; accepted 09 November 2012

Abstract

Background: Lodhra (Symlocos racemosa Roxb.) is one of the imperative plants in Ayurveda reported to posses aphrodisiac property. The Loturine is one of the important markers of Lodhra.

Aim: To develop simple, rapid, accurate RP-HPLC methods for estimation of Loturine from Polyherbal Formulation containing Lodhra extract.

Methods and Materials: A simple RP-HPLC method was developed for estimation and quantitative determination of Loturine from extract and formulation. For this analysis the scanning wavelength was set on 288 nm. The % of Loturine content in extract and formulation were determined. The validation was done as per ICH guidelines.

Statistical Analysis: The RSD and Correlation coefficient (r2) were calculated by standard formulas.

Results: The Loturine was satisfactorily resolved at Rr 5.3 minutes. The linear regression analysis data for the calibration plots showed good linear relationship with r2 = 0.9992. The % RSD values for system precision, method precision and intermediate precision was found to 0.72 %, 0.89 % and 1.22 % respectively.

Conclusion: Statistical analysis of the data showed that the method is reproducible and selective for the determination of Loturine from Lodhra extract and formulation.

Keywords: Loturine, Symlocos racemosa, HPLC, Quantitation.

1. Introduction

Traditionally herbal formulations have been used by the human society to prevent and treat the various diseases. Herbal medicines are in great demand in developing as well as developed countries for the primary health care because of their wide range of medicinal activities. Lodhra (Symlocos racemosa Roxb.) is one of the important plants reported to posses aphrodisiac property. It is reported to have the phosphodiesterase inhibitor activity and can be used in erectile dysfunction [1, 2]. As per Ayurvedic literature Lodhra is also useful for erectile dysfunction [3]. Unani medicine uses it as an emmenagogue and aphrodisiac. It is widely used not only for gynecological disorders but is also used as a potent remedy for inflammation and cleaning of the uterus [4]. Its decoction is also used for the treatment of bowel complaints and ulcers [5]. Medicinally bark is useful in eye diseases, blood purification, leprosy, dropsy and liver complaints [4]. A large number of chemical compounds belonging to different classes such as alkaloids, diterpenoid lactones, glycosides, phenolics compounds are reported in Lodhra. Bark contains flavonal glucosides like symplocoside, symposium, leucopolargonidin 3-glucoside, ellagic acid, flavonol glycoside like rhamnetin 3-digalactoside, triterpenoids like 19α-hydroxyarjunolic acid-3,28-O-bis-β-glucopyranosides, 19α-hydroxyasiatic acid-3,28-O-bis-β-glucopyranosides, betulin, Oleanolic acid, β-sitosterol and α-amyrin [6]. Apart from these chemical constituents the bark mainly contains alkaloids Loturine, isoloturine and harmarne [7, 8]. Many formulation of Lodhra are also reported in Ayurveda like “Rodharasava”, “Pusyanuga Churna”, “Gangadhara Churna” etc. [9]. Standardization of herbal formulations in terms of quality of raw materials, manufacturing practices, and composition is important to ensure quality and optimum levels of active principles for their bio-potency. Recently, the concept of marker-based standardization of herbal drugs is getting hold of impetus. Identification of unique compounds in herbs as markers and development of analytical methodologies for monitoring them are the key steps involved in marker-based standardization. As per literature review, although Lodhra is an important plant in Ayurveda, still very few analytical methods like HPTLC of (-) epiafzelechin [10] β-sitosterol [11] and...
Ellagic acid [12] are reported and no analytical method has been reported for estimation of Loturine from formulation containing Lodhra extract. Hence in this article, the attempts has been made to standardize the polyherbal formulation containing Lodhra extract by developing RP-HPLC method for the estimation and quantitation of Loturine as chemical marker. The developed analytical method was validated as per ICH guidelines.

2. Experimental

2.1. Plant Material

The water extract of bark of Lodhra (Batch No LODHRA/PCI/JUNE2012) was procured from Phytoconcentrate, Ahmedabad, India.

2.2. Standard Preparation

The stock solution of the standard Loturine (100 μg mL⁻¹) was prepared and further diluted to form 25 μg mL⁻¹ solution. This solution was used as standard solution.

2.3. Capsule formulation

A hard gelatin Capsule formulation containing 120 mg of Piper betel (Leaf) extract (Plantex, Vijaywada, India. Batch No-P9121826), 10 mg of Crocus sativus (Stigma) extract (Plantex, Vijaywada, India. Batch No-P9111753), 25 mg of Myristica fragrans (Fruit) extract (Plantex, Vijaywada, India. Batch No-P9030350), 80 mg of Symlocos racemosa. (Bark) extract (Phytoconcentrate, Ahmedabad, India. Batch No- LODHRA/PCI/JUNE2012), as actives; and microcrystalline cellulose BP (RanQ Remedies Pvt. Ltd. Sinner, India), methyl hydroxybenzoate BP (Alta Lab Ltd., Khopoli, India), propyl hydroxybenzoate BP (Alta Lab Ltd., Khopoli, India), purified talc BP (Nilkanth, Jodhpur, India), magnesium stearate BP (Amithi Drugs, Ahmedabad, India) and colloidal anhydrous silica BP (Evonik Degussa Corp, New Jersey, USA) was developed and prepared in-house.

2.4. Chemicals

HPLC grade solvents like acetonitrile, triethylamine and methanol were obtained from Merck Ltd, India. Standard Loturine [Potency 98 % Product no.103276] was purchased from Sigma, Bangalore, India.

Table 1 Mobile phase gradient program

<table>
<thead>
<tr>
<th>Time (Minutes)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>8</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>14</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>15</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>20</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

2.5. Chromatographic Conditions for HPLC

High Performance Liquid Chromatography was performed with Waters 2695 Alliance system with a 2996 photodiode array detector (PDA). Loturine was separated on a reverse-phase 288 mm × 4.6 mm, 5μ, Zorbax SB C-18 column (Agilent). The mobile phase was prepared by taking 50 mM of Ammonium Acetate in water and adjust its pH 8.7 with triethylamine (solvent A) and acetonitrile (solvent B). The mobile phase was degassed and filtered through 0.45-µm filter before use. The gradient program was used given in Table 1. The mobile phase flow rate was 1 mL min⁻¹. Before the first injection, the column was saturated for 30 minutes with the initial mobile phase. The column temperature was maintained at 30°C. The injection volume was kept 10 μL. The PDA was set at 288 nm to acquire the chromatogram. The Loturine was identified by comparing the retention time and spectra obtained from sample and standard solutions. The present work was performed in an air-conditioned room maintained at 25°C.

2.6. Preparation of calibration curve

The stock solution of the standard Loturine (100 μg mL⁻¹) was diluted to obtain seven different concentrations (12.5 – 37.5 μg mL⁻¹) for the preparation of calibration curve and these were injected into the system. These samples were analyzed by the method as described under chromatographic conditions. The peak areas were recorded and calibration curve was prepared by plotting average peak area against concentration of Loturine. The data of peak areas against concentration was treated by linear least regression analysis.

2.7. Preparation of Test Solution of Extract

100 mg of Lodhra bark extract was accurately weighed and added to 15 mL methanol. It was allowed to sonicate for 15 minutes. The final volume was adjusted up to 20 mL and the solution was filtered through whatman filter paper no 41. This resulting solution was used as test solution of extract.

2.8. Preparation of Test Solution of Capsule Formulation

20 Capsule of polyherbal formulation were weighed, powder was separated from capsule shell and average filled content was determined. The powder of the formulation equivalent to 100 mg of Lodhra extract was accurately weighed and added to 15 mL methanol. It was allowed to sonicate for 15 minutes. The final volume was adjusted up to 20 mL and the solution was filtered through whatman filter paper no 41. This resulting solution was used as test solution of formulation.

2.9. Validation of HPLC Method

International Conference on Harmonization (ICH) guidelines (CPMP/ICH/381/95; CPMP/ICH/281/95) were followed for the validation of the analytical procedure. The proposed HPLC method was validated in terms of precision, specificity, linearity, accuracy, solution stability and robustness of the sample application on the in-house capsule formulation as per the guidelines [13].

2.9.1. Precision

The precision of the system was carried out by six replicate injections from the same vial of standard at the analytical concentration and was expressed in terms of percent relative standard deviation, % RSD (Acceptance criterion: % RSD should not be more than 2.0%). Six different samples of the polyherbal formulation were analyzed for method precision. The percent assay of Loturine and % RSD was calculated (Acceptance criterion: % RSD should not be more than 2.0%). The intermediate precision was carried out on two different systems for six different samples by two different analysts. The Loturine content and % RSD was calculated (Acceptance criterion: % RSD should not be more than 2.0%).
2.9.2. Specificity
The specificity of the method was ascertained by analyzing diluent, standard, extract samples and formulation equivalent concentration and placebo (the placebo for Lodhra extract was prepared by using the same composition mentioned under capsule formulation, except the Lodhra extract) to examine the interference of diluent and placebo with analyte peak. The specificity of the method was studied by assessment of peak purity of Loturine using Waters empower software and diode array detector (Acceptance criterion: peak purity should pass) and represented in terms of purity angle, purity threshold and purity flag.

2.9.3. Accuracy
The recovery study was carried out by spiking known amount of standard Loturine in placebo for formulation at 80%, 100% and 120% of working concentration. The percentage recovery was calculated against respective level (Acceptance criterion: % recovery should be in the range of 98%-102%).

2.9.4. Solution stability
The sample solution was prepared as per the proposed method and subjected to stability study at room temperature for 24 hrs. The sample solution was analyzed at initial and at different time intervals up to 24 hrs. The change in peak area response of Loturine in sample solution with respect to time was calculated as absolute percent difference against initial response.

2.9.5. Robustness
The robustness of the method was determined by slight deviation in the method parameters. The parameters selected were deviation in the wavelength, column temperature, flow rate and mobile phase composition of organic solvent. The retention time of Loturine was determined and %RSD with system suitability parameters was observed.

2.9.6. Assay of Loturine from extracts and formulation
The percent content of Loturine in Lodhra bark extract and formulation was determined as per the method described under chromatographic conditions.

Figure 1 Chromatogram of standard Loturine

3. Results
3.1. Chromatographic Study
The composition of mobile phase in HPLC method was optimized by testing different solvent compositions of varying polarity and the best results were obtained by using present method which produces highly symmetrical peaks showing good resolution between Loturine and other peaks. The scanning wavelength selected was 288 nm for Loturine. At this wave length the Loturine showed optimum response [Figure 1-4]. Peak purity was assessed by comparison of overlay spectra of standard and test peak at the start, apex and end was found satisfactory [Figure 5]. The Loturine was satisfactorily resolved with R value about 5.3 minutes.

Figure 2 Chromatogram of extract of Lodhra Bark

Figure 3 Chromatogram of Polyherbal formulation

Figure 4 Spectra of Loturine

Figure 5 Purity Spectra of Loturine for Specificity
3.2. Calibration curve
The calibration curve of peak area against concentration was linear in the range 12.5-37.5 µg mL⁻¹ for Loturine. The calibration line was represented by linear equation \( y = 22488x + 65767 \) [Figure 6], where \( y \) is response and \( x \) is amount. For this equation the correlation coefficient, \( r^2 \), was 0.9992 [Table 2].

![Figure 6 Linearity Graph for Loturine](image)

**Table 2** Method validation parameters for quantitation of Loturine

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Parameters</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Specificity</td>
<td>Specific</td>
</tr>
<tr>
<td>2</td>
<td>Linearity (correlation coefficient)</td>
<td>0.9992</td>
</tr>
<tr>
<td>3</td>
<td>System precision. (% RSD) (n=6)</td>
<td>0.72</td>
</tr>
<tr>
<td>4</td>
<td>Method Precision. (% RSD) (n=6)</td>
<td>0.89</td>
</tr>
<tr>
<td>5</td>
<td>Intermediate precision. (% RSD) (n=6)</td>
<td>1.22</td>
</tr>
<tr>
<td>6</td>
<td>Solution stability</td>
<td>Stable</td>
</tr>
<tr>
<td>7</td>
<td>Regression equation</td>
<td>( y = 22488x + 65767 )</td>
</tr>
</tbody>
</table>

3.3. Precision
The values of system precision, method precision and intermediate precision are given against sample application and scanning of peak area and expressed in terms of %RSD. The values were found to be 0.72%, 0.89% and 1.22% respectively for system precision, method precision and intermediate precision, the % RSD values depicted in **Table 2** showed that the proposed method provides acceptable level of system precision, method precision and intermediate precision.

3.4. Specificity
The peak purity of Loturine was assessed by comparing their respective spectra at peak start, peak apex, and peak end positions of the peak from standard and extracts and formulation [Figure 4, 5]. Purity angle and Purity threshold was found to be 0.761 and 2.846 respectively with no purity flag [Table 6].

3.5. Recovery
The recovery study was carried out by spiking known amount of standard Loturine in placebo at 80%, 100% and 120% of working concentration and found to be 99.11%, 98.90% and 99.14% as depicted in **Table 4**.

3.6. Solution stability
The absolute percentage difference for the area of Loturine in extract and formulation solution to that of initial response does not exceed an acceptable limit upto 24 hours of preparation at room temperature indicating the stability of the sample solution.

3.7. Robustness
The given method was optimized by doing robustness. The peak area for each analyte was calculated for each parameter and % RSD was found to be less than 2%. The values of %RSD as shown in **Table 3** indicated better robustness of the method [Table 3].

**Table 3:** Robustness of the method

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Parameters for HPLC</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wavelength</td>
<td>0.71</td>
</tr>
<tr>
<td>2</td>
<td>Column Temperature</td>
<td>0.62</td>
</tr>
<tr>
<td>3</td>
<td>Flow Rate</td>
<td>1.15</td>
</tr>
<tr>
<td>4</td>
<td>Mobile Phase Gradient</td>
<td>1.29</td>
</tr>
</tbody>
</table>

3.8. Assay of Loturine
The % content of Loturine was found to be 0.57 and 0.56 %w/w from Lodhra bark extract and polyherbal formulation and reported in **Table 5**.

HPLC is a powerful analytical technique for the herbal drug analysis. This method has accuracy, sensitivity, reproducibility, resolution and automation. The method also provides nanogram sensitivity, adequate linearity and repeatability [14].

The method reported here is rapid and suitable for the quantitation of Loturine from the bark extract of Lodhira and formulation containing Lodhra.

**Table 5** Determination of % Loturine from extract and formulation

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Analysis of Sample</th>
<th>Assay (%w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Water extract of bark of Lodhira</td>
<td>0.57</td>
</tr>
<tr>
<td>2</td>
<td>Polyherbal formulation</td>
<td>0.56</td>
</tr>
</tbody>
</table>

**Table 6** Specificity Parameters

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Standard</th>
<th>Purity Angle</th>
<th>Purity Threshold</th>
<th>Purity Flag</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Loturine</td>
<td>0.761</td>
<td>2.846</td>
<td>No Flag found *</td>
</tr>
</tbody>
</table>

No Flag found* means no interference in Loturine peak.
4. Discussion
HPLC is an authoritative analytical technique for the herbal drug analysis. This method has accuracy, sensitivity, reproducibility, resolution and automation. The method also provides nanogram sensitivity, adequate linearity and repeatability [14]. The method is precise for sample application and measurement of peak areas with low values of % R.S.D. suggested an excellent precision of the method. The results obtained after recovery study showed the method is accurate for the analysis of Loturine from Lodhra bark extract and polyherbal formulation containing Lodhra extract. The method reported here is rapid and suitable for the estimation and quantitation of Loturine from Lodhra bark extract and polyherbal formulation containing Lodhra extract. Loturine can easily be quantified in the presence of other constituents from the extract of Lodhra bark and polyherbal formulation without compromising the accuracy.

5. Conclusion
The proposed HPLC method is simple, rapid, specific and accurate techniques to quantify the Loturine in the presence of other constituents from the formulation without compromising the accuracy. Method validation proves that method is selective and reproducible for the estimation of Loturine from extract and polyherbal formulations.

References:

Source of support: Nil; Conflict of interest: None declared