INTRODUCTION

There is always a constant and continues challenge in the standardization and quality control of the ayurvedic and polyherbal formulations in the determination of active constituents of each ingredient in the polyherbal formulation which requires optimal separation techniques of biomarkers with the high resolution and least interferences from each other. Also there is an urgent need of scientific evidence and clinical validation with chemical, biological standardization and preclinical data[1]. Recognition of the medical and health benefits of herbal medicines with health claim is growing worldwide and one of the challenges in the acceptance of herbal medicines is the lack of standardization.[2] The quality of herbal medicine, i.e., the profile of the constituents in the final product has implications in efficacy and safety, due to the complex nature of the chemical constituents of plant-based drugs. Modern analytical techniques are increasing to overcome these problems of separation, identification and determination of the active constituents for such polyherbal formulations. The advancement in the chromatographic and spectroscopic techniques made it possible to determine the active constituents in a mixture with comparatively

ABSTRACT

The US patented polyherbal formulation for the prevention and management of Type II diabetes and its vascular complications was used for the present study. The formulation consists of roots of Salacia species, leaves of Lagerstroemia parviflora and fruit rind of Garcinia indica. The use of reversed phase C18 HPLC column was used and eluted with isocratic mobile phase of acetonitrile and phosphoric acid buffer solution enabled the efficient separation of chemical markers within 20min. Validation of the method was performed in order to demonstrate its selectivity, accuracy, precision, repeatability and recovery. All calibration curve shows good linear correlation coefficients (r² > 0.995) within tested ranges. Three markers in this polyherbal formulation were quantified were Mangiferin (1.53% w/w), Ellagic acid (0.9655 w/w), Hydroxycitric acid (5.3% w/w). Intra and inter day RSDs of retention times and peak areas were less than 3%. The recoveries were between 95% and 102.5%. In conclusion a method has been developed for the simultaneous quantification of three markers in this polyherbal formulation. The established RP-HPLC method was simple, precise and accurate and can be used for the quality control of the raw materials as well as formulations.

Keywords: Polyherbal formulation, Mangiferin, Ellagic acid, Hydroxycitric acid, RP-HPLC

Development and validation of a RP-HPLC method for the simultaneous determination of Mangiferin, Ellagic acid and Hydroxycitric acid in polyherbal formulation

Ananth Kumar Kammalla1, Mohan Kumar Ramasamy1, Agarwal Aruna2, Dubey GP3 and Ilango Kaliappan1*

1Interdisciplinary School of Indian System of Medicine, SRM University, Kattankulathur-603203, Tamil Nadu, India
2National Facility for Tribal & Herbal Medicine, Institute of Medical sciences, Banaras Hindu University, Varanasi, India
3Faculty of Ayurveda, Institute of Medical sciences, Banaras Hindu University, Varanasi, India

Submission Date: 12-1-2014
Accepted Date: 24-3-2014

*Corresponding author.
Prof Dr Kaliappan Ilango, Dean,
Interdisciplinary School of Indian System of Medicine (ISISM),
SRM University, Kattankulathur-603203,
Kancheepuram (Dt), Tamil Nadu, India.
Tel: 91-44-27455818; Fax: 91-44-47432342.
E-mail: ilangok67@gmail.com

DOI: 10.5530/pj.2014.3.4
little clean-up, particularly methods using high performance liquid chromatography (HPLC) with reverse phase column are most appropriate for the analysis of multiple constituents present in the herbal preparations.

A polyherbal formulation consists of *Salacia rosashushhii* (Hippocrateaceae), *Salacia oblonga* (Hippocrateaceae), *Garcinia indica* (Guttiferae), *Lagstroemia parviflora* (Lythraceae) for the prevention and management of Type-II Diabetes mellitus and its associated vascular complications was developed and patented by Dubey et al.\[3\] from SRM University, TamilNadu, India. In our present investigation we have developed a simple optimized and validated RP-HPLC method for the standardization of this polyherbal formulation. Three chemical markers were selected for the quantification and one was from each medicinal herb used as raw materials, Mangiferin for Salacia species,\[4\] Ellagic acid for *Lagstroemia parviflora*\[5\] and Hydroxycitric acid for *Garcinia indica*.\[6\] The chemical structures of the compounds are depicted in Fig 1. The pharmacological actions of these markers were extensively studied and proved to have various pharmacological activities such as antimutagenic, antidiabetic,\[7\] antiviral, antioxidant\[8\] anticancer\[9\] and weight reduction.\[10\]

Although many approaches including thin layer chromatography\[11\] High performance liquid chromatography with UV-Vis,\[12–14\] PDA or MS/MS\[15\] have reported to analyze the contents of compounds mentioned above individually or collectively in two or more. However, there were no reports on simultaneous determination of these three compounds in a polyherbal formulation by RP-HPLC-PDA detection.

**Figure 1.** Chemical structures of compounds
In the present study a simple, practical and cost effective HPLC method with isocratic elution mode for the quality control of herbal formulation was proposed and successfully applied for first time. The method was validated on the basis of its sensitivity, linearity, precision, accuracy, limit of detection (LOD) and Limit of Quantification (LOQ) according to the International Conference on Harmonization (ICH) guidelines.[10] The proposed method can be used to determine the contents of Mangiferin, Ellagic acid and Hydroxycitric acid in any polyherbal formulation.

MATERIALS AND METHODS

Chemicals, reagents and materials

Polyherbal formulation was purchased from M/s Varansi Bio research Pvt. Ltd. Mangiferin, Ellagic acid and Hydroxycitric acid was purchased from the Sigma Aldrich (MO,USA), Chennai as a gift sample. HPLC grade Acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Water was obtained from ultra-purified Milli-Q Biocel (Millipore). All solvents and samples were filtered through MILLEX FG (Millipore), 13mm, 0.2μM, nonsterile membrane sample filter paper before injecting into system.

APPARATUS AND CHROMATOGRAPHIC CONDITIONS

The analyses were performed on a Shimadzu LC-20AD HPLC system equipped with Rheodyne 7725 injection valve furnished with 20μL loop, an SPD-M20A photodiode array detector and Labsolutions software. Separation was carried out using a Phenomenex C<sub>18</sub> column (250mm × 4.6mm i.d., 5μm pore size). The column was optimized and maintained at 28ºC throughout analysis and detection wavelength was set at 254nm for Mangiferin, Ellagic acid and 220nm for Hydroxycitric acid.

SAMPLE PREPARATION

The 20 intact polyherbal formulation composition capsules were weighed and powder was remained empty gelatin capsule was weighed to calculate the average weight equivalent to 100mg of *Salacia oblonga* and extracted three times with 100 mL methanol. Mixed standard stock solution was prepared by accurately (1.0mg/mL) weighing three markers i.e., Mangiferin, Ellagic acid and Hydroxycitric acid and dissolved in acetonitrile water (1:1). The working standard solution was prepared by diluting the mixed standard solution with the same to a series of proper concentrations to construct calibration curve. The standard stock and working solutions were all stored at 4ºC until use.

CALIBRATION

The contents of the markers were determined using a calibration curve established with six dilutions of each standard at concentrations ranging from 2 to 32μg/mL and each concentration was measured in triplicate. The corresponding peak areas were plotted against the concentrations of the markers injected. The linearity was evaluated by linear regression analysis which was calculated by least square regression method. Before injecting solutions, the column was equilibrated for at least 30 min with mobile phase flowing through system. Peak identification was achieved by comparison of both the retention time (R<sub>t</sub>) and UV absorption spectrum with those obtained for standards. The reference substances employed to construct the calibration curves were Mangiferin, Ellagic acid and Hydroxycitric acid.

VALIDATION PARAMETER

The method was validated according to ICH guidelines for linearity, precision, accuracy, selectivity, LOD and LOQ (16). Selectivity was checked using an extract of polyherbal formulation and mixture of standard solution of markers by the method in the concentration range of 2 to 32μg/mL. The accuracy of the proposed method was determined by a recovery study, carried out by adding standard markers to the extract of polyherbal formulation. The samples were spiked with three different amounts of standards prior to extraction. The spiked samples were extracted in triplicate and analyzed under the previously established optimal conditions. The obtained average contents of the target compounds were used to calculate the spiked recoveries. Precision was determined by repeatability and inter-day and intraday reproducibility experiments. A standard solution containing three markers was six times, the mean amount and standard deviation (SD) value of each constituent was calculated. The LOD and LOQ of marker compounds were calculated at signal to noise (S/N) ratio of approximately 3:1 and 10:1 respectively.
RESULTS AND DISCUSSION

Optimization of HPLC chromatographic conditions

Optimum chromatographic conditions were obtained after running different mobile phase with reversed phase C\textsubscript{18} column. Many different gradient systems of mobile phases were tried to achieve the separation of peaks. Phosphate buffer solution was preferred over other mobile phases because it resulted in improved separation. Selecting 254nm for Mangiferin, Ellagic acid and 220nm for Hydroxycitric acid as the detection wavelength resulted in an acceptable response and enables the detection of all three compounds used in this study and column was maintained at 28ºC throughout analysis with flow rate of 0.5 mL/min with acetonitrile. HPLC fingerprint for polyherbal formulation was developed and elution was carried out at a flow rate of 0.5mL/min with acetonitrile as solvent A and buffer solution (0.03% v/v Phosphoric acid) as solvent B using isocratic elution at the ratio of 45:55 respectively. Each run was followed by a 10 min wash with 10% acetonitrile.

QUANTIFICATION OF MARKERS PRESENT IN POLYHERBAL FORMULATION

The three markers were found in formulation and they were quantified with respect to Mangiferin (1.56 ± 0.02) Ellagic acid (0.91 ± 0.07) and Hydroxycitric acid (5.33 ± 0.22). The chromatographs of a mixture of Mangiferin, Ellagic acid and Hydroxycitric acid and chromatogram of polyherbal formulation were shown at Fig. 2 – Fig. 8 and Polyherbal formulation showed complete separation of three markers. The results obtained are shown in Table 3.
VALIDATION OF METHOD

The HPLC method was validated by defining the selectivity, linearity, accuracy, precision, LOD and LOQ. For qualitative purposes, the method was evaluated by taking into account the precision in the retention time and selectivity of marker compounds eluted. A high repeatability in the retention time was obtained for standards and extracts even at high concentration. For quantitative purpose, linearity, accuracy, precision, LOD and LOQ were evaluated. LOD and LOQ values were 2.53µg/mL and 7.66µg/mL for Mangiferin, 4.62µg/mL and 14.01µg/mL for Ellagic acid and 1.38µg/mL and 4.21µg/mL for Hydroxycitric acid respectively. Linear correlation was obtained between peak area and concentration of three markers in the range of 2 to 32µg/mL. Values of the regression coefficients ($r^2$) of the markers were higher than 0.99 thus confirming the linearity of the methods (Table 1), the high recovery values (95-103%) indicated a satisfactory accuracy. Relative standard deviation of all the parameters was less than 3.5% for the degree of repeatability, indicating the high repeatability of the developed method (Table 2). The low coefficient of variation values of intraday and interday precision revealed that the method is precise (Table 4). Therefore, this HPLC method can be regarded as selective, accurate and precise.

CONCLUSION

The results indicate that this polyherbal formulation contains a number of markers that may be responsible for its pharmacological activity. The developed HPLC method will assist in the standardization of this polyherbal formulation using biologically active chemical markers. The developed HPLC method for simultaneous determination of Mangiferin, Ellagic acid and Hydroxycitric acid from polyherbal formulation is accurate, precise, reproducible and repeatable, which are currently the subject of further investigation, apart from those standards studied. With growing demand for herbal drugs and increased belief in the usage of herbal medicine, the development of a standardization tool will help in maintaining quality of herbal products

CONFLICTS OF INTEREST

All authors have none to declare

Table 1. Regression parameter, Linearity, limit of detection (LOD) and limit of quantification (LOQ) of proposed HPLC method

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration range (µg/mL)</th>
<th>$R_t$ (min)</th>
<th>Wavelength (nm)</th>
<th>Regression equation</th>
<th>$R^2$</th>
<th>LOD (µg/mL)</th>
<th>LOQ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mangiferin</td>
<td>2–32</td>
<td>4.76 ± 0.05</td>
<td>254</td>
<td>$y = 31170x - 29273$</td>
<td>0.99</td>
<td>2.53</td>
<td>7.66</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>2–32</td>
<td>6.09 ± 0.01</td>
<td>254</td>
<td>$y = 7255.2x + 5704.9$</td>
<td>0.98</td>
<td>4.62</td>
<td>14.01</td>
</tr>
<tr>
<td>Hydroxy citric acid</td>
<td>2–32</td>
<td>13.49 ± 0.11</td>
<td>220</td>
<td>$y = 22592x - 9919.5$</td>
<td>0.99</td>
<td>1.38</td>
<td>4.21</td>
</tr>
</tbody>
</table>

Table 2. Repeatability and recovery study for the three markers in Polyherbal formulation

<table>
<thead>
<tr>
<th>Compound</th>
<th>Content (mg/g)</th>
<th>Added amount (mg)</th>
<th>Recorded amount (mg)</th>
<th>Recovery (%)</th>
<th>RSD(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mangiferin</td>
<td>1.56 ± 0.02</td>
<td>1</td>
<td>2.48 ± 0.19</td>
<td>97.78 ± 1.43</td>
<td>1.46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>3.61 ± 0.11</td>
<td>101.40 ± 3.31</td>
<td>3.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>5.83 ± 0.06</td>
<td>104.91 ± 1.22</td>
<td>1.16</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>0.91 ± 0.07</td>
<td>0.5</td>
<td>1.28 ± 0.07</td>
<td>91.67 ± 1.43</td>
<td>1.46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>1.86 ± 0.20</td>
<td>110.53 ± 2.95</td>
<td>2.66</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>2.36 ± 0.05</td>
<td>100.00 ± 2.40</td>
<td>2.44</td>
</tr>
<tr>
<td>Hydroxy citric acid</td>
<td>5.33 ± 0.22</td>
<td>3</td>
<td>8.53 ± 0.05</td>
<td>102.81 ± 0.69</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>11.66 ± 0.15</td>
<td>103.24 ± 1.35</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9</td>
<td>14.83 ± 0.30</td>
<td>103.72 ± 2.13</td>
<td>2</td>
</tr>
</tbody>
</table>

Figure 8. HPLC chromatogram of Polyherbal formulation at 220 nm.
Table 3. Quantification of Mangiferin, Ellagic acid and Hydroxy citric acid

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Amount of Mangiferin (% w/w)</th>
<th>Amount of Ellagic acid (% w/w)</th>
<th>Amount of Hydroxy citric acid (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyherbal formulation</td>
<td>1.56 ± 0.02</td>
<td>0.91 ± 0.07</td>
<td>5.33 ± 0.22</td>
</tr>
</tbody>
</table>

Table 4. Precision of the Intra-day and Inter-day HPLC measurement for Mangiferin, Ellagic acid and Hydroxy citric acid in Polyherbal formulation

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Intraday</th>
<th>Inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Content (% w/w)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>Mangiferin</td>
<td>1.51 ± 0.02</td>
<td>1.32</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>0.91 ± 0.01</td>
<td>1.05</td>
</tr>
<tr>
<td>Hydroxy citric acid</td>
<td>5.33 ± 0.05</td>
<td>0.93</td>
</tr>
</tbody>
</table>

ACKNOWLEDGEMENTS

The authors are wish to express their gratitude to the Department of Science and Technology, Government of India, India for providing Financial assistance to carry out the research.

REFERENCES