HPTLC method Development & Validation for quantification of Markers of Dhatrinisha churna

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ABSTRACT

Introduction: Dhatrinisha churna has been traditionally used in the Ayurvedic system of medicine and by traditional medical practices of India to treat hyperlipidemia. A sensitive, reliable, selective, precise and accurate densitometric High Performance Thin Layer Chromatography (HPTLC) fingerprinting method has been developed for the simultaneous determination and quantification of Curcumin and Ellagic acid in Dhatrinisha churna. Method: Quantification of Curcumin and Ellagic acid were done by developed densitometric HPTLC method. Validation of method performs in order to demonstrate its selectivity, accuracy, precision, repeatability and recovery study. Result: All calibration curves showed good linear correlation coefficients ($r^2 > 0.997$) within the tested ranges. The chromatogram of Dhatrinisha churna was quantified with respect to Curcumin (1.072% w/w) and Ellagic acid (0.867% w/w). Intra-and inter-day RSDs of retention times and peak areas were less than 1.92%. The recoveries were between 96.60 and 101.40%. Conclusion: A method has been developed for the simultaneous quantification of two markers in Dhatrinisha churna. The proposed HPTLC method was found to be simple, précised and accurate and can be used for the quality control of the raw materials as well as formulations.

Key words: Dhatrinisha churna; Curcumin; Ellagic acid; HPTLC

INTRODUCTION

Herbal medicine has been enjoying renaissance among the customers throughout the world. However, one of the impediments in the acceptance of the ayurvedic medicines is the lack of standard quality control profiles. The quality of herbal medicine i.e. the profile of the constituents in the final product has implication in efficacy and safety. Due to the complex nature and inherent variability of the chemical constituents of plant-based drugs, it is difficult to establish quality control parameters. To overcome these problems modern analytical techniques are expected to help in circumventing this problem.[1,2]

From such polyherbal formulations separation, identification and estimation of chemical components is very difficult.[3] Literature survey revealed that the above mentioned marker compound have various pharmacological properties. The rhizome and roots of *Curcuma* species are frequently used in cosmetics and spas for skin nourishment. Pharmacological study reveals its various medical activities such as antioxidant, promotion of blood circulation to remove blood stasis and treatment of cancer. The antioxidants are claimed biological active in protecting the body, the skin collagen and elastic tissue against damaging by reactive oxygen species.[4,5] Gallic acid and ellagic acid are hydrolysable tannins and present in a rich variety of plants like in tea, red wine, fruits, beverages and various medicinal plants. Gallic acid and ellagic acid are hydrolysable tannins and present in a rich variety of plants like in tea, red wine, fruits, beverages and various medicinal plants. Gallic acid is known to have antiinflammatory, antimutagenic, anticancer and antioxidant activity.[6,7] Ellagic acid has been found to exhibit antimutagenic, antiviral, anticancer, antitumor and antioxidant properties, along with whitening of the skin.[8,9]

The advances in chromatographic techniques made it possible to quantify the chemical constituents in a mixture with comparatively little clean-up using high performance thin layer chromatography (HPTLC).[10] Present study deals with development and validation of methods for quantification of some of the important marker compounds viz. Curcumin and Ellagic acid in Dhatrinisha churna.

Dhatrinisha churna is an Ayurvedic preparation mentioned in the *Chikisthasthan*, Chapter-II, Slock-8 of ayurvedic literature *Susrut Sambita and Chater-6, Slock-26, 772 of Ayurvedic literature Charak Sambita* for the treatment of Hyperlipidemia. Dhatrinisha churna has been also used by...
traditional medical practices of India to treat hyperlipidemia. It consists of the mixture of the fine powder of the dried rhizome of Haridra (Curcuma longa Linn., F.- Zingiberaceae) and dried fruit of Amalaki (Emblica officinalis Gaertn. Syn. Phyllanthus emblica Linn. F. – Euphorbiaceae). Traditionally it is widely used for the treatment of hyperlipidemia and in diabetes. Curcumin and Ellagic acid are the main active markers present in the Haridra and Amalaki respectively and are mainly responsible for their aid pharmacological action.

In the present investigation, we have developed simple, optimized and validated HPTLC method for the standardization of Dhatrinisha churna. Two chemical markers were selected, one from each medicinal herbs used as raw materials. The method was validated on the basis of its selectivity, linearity, precision, accuracy, limit of detection and limit of quantification according to ICH requirements.

MATERIALS AND METHODS

Plant Materials

Individual components of Dhatrinisha churna were procured from a Yucca enterprise, Mumbai, Maharashtra and authenticated by comparison with herbarium specimens. The drugs were cleaned, dried and powdered separately and passed through 40 # sieve. These both powders were mixed well in equal proportion uniformly.

HPTLC method development

Optimum chromatographic conditions were obtained after running different mobile phase. Many different mobile phase and scanning wavelength were tried for the best separation of peaks. Chromatography was performed at 25 ± 2°C, relative humidity 40%, on 10 cm × 10 cm aluminum foil HPTLC plates coated with 0.2 mm layers of silica gel 60F254 (E. Merck). Solutions (5 μL) were applied to the plates as bands 6 mm wide by use of a CAMAG (Muttenz, Switzerland) Linomat V sample applicator equipped with a 100 μL syringe (Hamilton, Bonaduz, Switzerland). Linear ascending development to approximately 80 mm from the point of application was performed with toluene : ethyl acetate : formic acid (16: 14: 1: 4 v/v) as mobile phase in a CAMAG 20 cm × 10 cm × 4 cm glass twintrough chamber previously saturated with mobile phase vapor for 25 min. After development, the plate was dried in hot air oven at 105°C for 5 min. Densitometric scanning in absorbance mode at 330 nm was then performed by use of a CAMAG TLC scanner-III linked to Wincats software (V 1.4.3.6336). The slit dimensions were 5.00 mm × 0.45 mm and the scanning speed 20 mm s⁻¹. For calibration, stock solutions of Curcumin and Ellagic acid of different concentration (100-600 ng/band) were applied to the HPTLC plate to prepare a calibration plot and to check for reproducibility. Peak areas were recorded and calibration plots were prepared by plotting average peak area against concentration. Peak area and amount of standards data were treated by linear least-square regression analysis.

Method Validation

The method was validated according to ICH guideline for linearity, precision, accuracy, selectivity, limit of detection and limit of quantification. Selectivity was checked using an extract of Dhatrinisha churna and a mixture of standards in order to optimize separation and detection. Linearity of the method was performed by analyzing a standard solution of markers by the proposed method in the concentration range 100-600 ng/spot. The accuracy of the proposed method was determined by a recovery study, carried out by adding standard markers in the Dhatrinisha churna. The samples were spiked with three different amounts of standard compounds 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 as the actual values in order to calculate the spike recoveries. Precision was determined by repeatability and interday and intraday reproducibility experiments of the proposed method. A standard solution containing two markers was injected six times; Dhatrinisha churna was also extracted six times to evaluate the repeatability of the extraction process. The mean amounts and standard deviation (SD) value of each constituent were calculated. The Limit of Detection (LOD) and Limit of Quantification (LOQ) of markers compounds were calculated at signal-to-noise ratio of approximate 3:1 and 10:1 respectively.

RESULT

Method development and Quantification

TLC densitometric methods were developed using HPTLC for the quantification of two marker compounds from Dhatrinisha churna. Solvent systems were optimized to achieve best resolution of the marker compounds from the other components of the sample extracts. Several methods tried to separate out markers from extract such as Choloform: Acetic acid: Methanol (80: 5: 15), Ethyl acetate: Formic acid: Acetic acid (100: 11: 11) etc. Of the various solvent system tried, the one containing toluene: ethyl acetate: methanol: formic acid (16: 14: 1: 4) gave best resolution of curcumin (Rf = 0.74) and ellagic acid (Rf = 0.51) in the presence of other compounds in the sample extract and enabled the quantification of marker compounds. The amount of markers found in Dhatrinisha churna were
Method Validation

The HPTLC method was validated by defining the selectivity, linearity, accuracy, precision, limits of detection and limit of quantification. For qualitative purposes, the method was evaluated by taking into account the precision in the Rf and selectivity of marker compounds. A high repeatability in the Rf time was obtained for both, standards and extracts even at high concentration. For quantitative purpose linearity, accuracy, precision LOD and LOQ were evaluated. LOD and LOQ values for Curcumin 24 ng/spot and 36 ng/spot & for Ellagic acid 25 ng/spot and 72 ng/spot respectively. Linear correlation was obtained between peak area and concentration of two markers in the range of 100-600 ng/spot. Values of the regression coefficients (r²) of the markers were higher than 0.99, thus confirming the linearity of the methods. The high recovery values (96.60-101.40%) indicated a satisfactory accuracy. Relative standard deviation of all the parameters was less than 2.5% for the degree of repeatability, indicating the high repeatability of the proposed method. The low coefficient of variation values of intraday and interday precision reveals that the proposed method is precise. The result of validation parameters were shown in Table 1-3.

Table 1: Regression Parameter, Linearity, Limit of Detection (LOD) and Limit of Quantification (LOQ) of the Proposed HPTLC Method for Dhatrinisha churna

<table>
<thead>
<tr>
<th>Markers</th>
<th>Conc. range (ng/spot)</th>
<th>Rf *</th>
<th>Regression equation</th>
<th>R²</th>
<th>LOD</th>
<th>LOQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>100-600</td>
<td>0.74 ± 0.03</td>
<td>Y = 47.296 + 0.85X</td>
<td>0.999</td>
<td>24</td>
<td>36</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>100-600</td>
<td>0.51 ± 0.04</td>
<td>y = 21063x + 733.6</td>
<td>0.997</td>
<td>25</td>
<td>72</td>
</tr>
</tbody>
</table>

*Mean ± SD (n=6)

Table 2: Repeatability and Recovery Tests for the Two Markers in Dhatrinisha Churna

<table>
<thead>
<tr>
<th>Markers</th>
<th>Contents * (mg/g)</th>
<th>Added amount (mg)</th>
<th>Recorded amount * (mg)</th>
<th>Recovery rate * (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>10.72 ± 0.04</td>
<td>5</td>
<td>15.55 ± 2.04</td>
<td>96.60 ± 2.04</td>
<td>2.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>20.667 ± 0.71</td>
<td>99.50 ± 0.71</td>
<td>2.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>25.930 ± 1.55</td>
<td>101.40 ± 1.55</td>
<td>2.30</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>8.67 ± 0.02</td>
<td>4</td>
<td>12.558 ± 1.08</td>
<td>97.20 ± 1.08</td>
<td>1.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>16.774 ± 2.30</td>
<td>101.30 ± 2.30</td>
<td>1.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>20.662 ± 0.71</td>
<td>99.93 ± 0.71</td>
<td>2.12</td>
</tr>
</tbody>
</table>

*Mean ± SD (n=3)

1.072 ± 0.04% w/w (curcumin) and 0.867 ± 0.02% w/w (Ellagic acid) respectively. The HPTLC chromatogram obtained for Markers and Dhatrinsha chturna are shown in Figure 1 & 2 respectively.
Therefore, this HPTLC method can be regarded as selective, accurate and precise.

**CONCLUSION**

The results indicate that Dhatrinisha churna contains a number of markers that may be responsible for its therapeutic activity. The developed HPTLC method will assist in the standardization of Dhatrinisha churna using biologically active chemical markers. The proposed HPTLC methods for simultaneous estimation of Curcumin and Ellagic acid from Dhatrinisha churna seems to be accurate, precise, reproducible and repeatable. Dhatrinisha churna also contained a number of other constitute, which are currently the subject of further investigation, apart from those standards studied. With the growing demand for herbal drugs and with increased belief in the usage of herbal medicine, this standardization tool will help in maintaining the quality and batch to batch consistency of this important Ayurvedic preparation.

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