ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF LAWSONE IN POLYHERBAL FORMULATION

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Abstract: A simple, rapid, specific, linear, accurate, precise and robust RP-HPLC method has been developed for estimation of Lawsone. The chromatographic separation was achieved on Chromolith® high resolution RP-18 endcapped (100 mm x 4.6 mm) (Merck Pvt. Ltd.) column using Water : Methanol (50: 50 v/v), pH 2 adjusted using 0.02 M Trifluoro Acetic Acid as mobile phase at 267 nm. Linearity was found to be 10-50 μg/ml for Lawsone. The correlation coefficient was found to be 0.999 for Lawsone. The %RSD for precision was found to be less than 2% and the % recovery was found to be between 98-102 %. The proposed method was found to be simple and sensitive for the routine quality control application of Lawsone in Polyherbal Formulation.

Keywords: Lawsone, Lawsonia inermis, Henna, HPLC Method, Method Validation, Polyherbal Formulation

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INTRODUCTION

Glamorization and trying to look attractive and adorable are a part of the human nature. Among women, the tradition of coloring their hands, feet, eyes, lips, cheeks and hair has persisted in every age and era. Among the natural aids to beautification, “Henna” has tenaciously maintained its popularity from ancient times beyond memory.

This tropical shrub has been popularly used for coloring the hands, feet and hair since time immemorial. In Henna, nature has generally packed both medicinal and commercial advantage, its popularity is not confined to any country or religion but has traveled for Arabia, Africa, China, Indo-Pak subcontinent and rest of the world. While medicinally the leaves and aerial parts of *Lawsonia inermis* L. are frequently used as a herbal remedy for an array of human disorders including wounds, ulcers, cough, bronchitis and jaundice, especially by female along with other herbal medicines.¹

Henna consists of dried leaves of *Lawsonia inermis* Linn. (Fam. Lythraceae); a small, elegant bush with fragrant flowers, cultivated and naturalised all over the country.² It consists of Lawsons (coloring matter), lacoumarin and laxathones. Other constituents are 5-10% gallic acid, esculatin, scopolutin, white resin, sugars, tannin, fats, mucilage, traces of alkaloid and xanthones.³,⁴

![Figure 1: Chemical Structure of Lawsone](image)

The principle coloring compound of Henna is “Lawsone”, a red-orange colored compound present in dried leaves in a concentration of 1-1.5 % w/w. It is mainly responsible for the colorant property of Henna leaves.⁵ Chemically, it is called as 2-hydroxynaphthalene-1,4-dione having Molecular Formula C₁₀H₆O₃, Molecular Weight 174.15 g/mol and Melting Point 195-196°C.⁶ It is insoluble in water at 0.2%, soluble in ethanol, methanol, ethyl glycol and dimethyl formamide.⁷ Lawsone is proposed to be used as a non-oxidising hair colouring agent at a maximum concentration of 1.5% (typical concentration 1.26%) in the finished cosmetic product.
Henna is an important but a controversial drug in market in Indo-Pak subcontinent. Due to the adulteration and use of other species as source of henna powder in trade, the drug has become adulterated. In view of the extent of adulteration attached to this drug, it was deemed necessary to study the market samples to ascertain their botanical identity.¹

Literature survey revealed that the UV Spectrophotometric method, High Performance Liquid Chromatography (HPLC) and High Pressure Thin Layer Chromatography (HPTLC) methods are available for estimation of Lawsone in *Lawsonia inermis* extracts. But the HPLC method has not been reported for Lawsone estimation in polyherbal formulation.

**MATERIALS AND METHOD**

**Reagents and Chemicals**

HPLC grade of methanol (Finar Ltd.), Extrapure Trifluoro acetic acid (Finar Ltd.) and water were used for analysis. The standard of Lawsone was obtained from AUM Research Laboratories, Gandhinagar, India. Purity and structure of standard compound were confirmed by melting point determination and Infrared spectral analysis. All market samples were analysed with this method.

**Equipments**

Analytical HPLC 3000 system equipped with Quaternary Pump (P 3000 EDC analyze HPLC Pan system) connected to UV 3000 Detector and Clarity Software were used. Shimadzu analytical balance, Ultrasonicator and pH meter were used for this study.

**Standard preparation**

Standard stock solution (2000μg/ml) was prepared in methanol which is used as diluent. Weighed accurately 20mg of Lawsone standard into 10 ml volumetric flask and dissolved in diluent with intermediate sonication, diluted to volume with diluent and mixed. Further diluted the resulting solution to achieve a concentration of 200 μg/ml.

**Sample Preparation from Polyherbal formulation**

Accurately weighed powder of Polyherbal formulation, equivalent to 5 mg of Lawsone was taken in 100 ml of volumetric flask, to it was added 100 ml of Methanol : Water (50:50) mixture, and was put aside for 1 hour. After 1 hour content was extracted by filtering it. The residue was collected and air dried. The extract was dissolved in 50 ml of Chloroform and partition was done with 50 ml of Water. Chloroform layer was collected from the separating funnel. Chloroform layer was properly air dried. The above extract was
dissolved in 25 ml of volumetric flask with Methanol and diluted up to mark to obtain 200 μg/ml solution of Lawsone from Polyherbal formulation. A sample solution was injected under chromatographic condition and peak area was measured and % assay was calculated from regression equation. Result was an average of 3 determinations.

**Chromatographic conditions**

Mobile phase was composed of Water : Methanol (50:50), pH was adjusted to 2 by using 0.02M Trifluoro Acetic Acid. Detection of Lawsone was achieved by using a UV detector at 267 nm. Merck Chromolith® high resolution RP-18 endcapped (100 mm × 4.6 mm) column was used for analysis and Methanol was used as diluent.

**System Suitability Test Parameters**

It was performed by applying six replicates of Lawsone standard solution of 30 μg/ml concentration and verifying percentage relative standard deviation (RSD) of peak areas.

**METHOD VALIDATION:**

The proposed method was validated according to the International Conference on Harmonization (ICH) guidelines.

**Linearity**

Calibration curve of Lawsone consists of different concentrations of Lawsone (200 μg/ml) ranging from 10-50 μg/ml. The solutions were injected automatically through auto sampler (20 μl) and chromatograms were recorded. Then calibration curve was constructed by plotting peak area against concentration of the drug.

**Precision**

Precision of the method was verified by repeatability, intraday and interday precision studies. Repeatability precision were performed by repeated analysis of the solution containing 30 μg/ml of Lawsone six times. Intra-day precision was performed by analysis of the solution containing 10, 30 and 50 μg/ml solution of Lawsone on the same day. Inter-day precision was performed on these three concentrations on three different days. Peak areas were expressed in terms of standard deviation (S.D.) and relative standard deviation (%RSD).
Specificity

The chromatogram of blank was compared with those acquired from Lawsone standards, correlation in terms of interference at retention time and peak area was evaluated to indicate the specificity of method.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ were estimated from the set of 3 calibration curves used to determine method linearity. The LOD and LOQ were calculated as;

$$\text{LOD} = 3.3 \times \frac{\text{SD}}{\text{Slope}}$$

$$\text{LOQ} = 10 \times \frac{\text{SD}}{\text{Slope}}$$

Where, SD = the standard deviation of Y- intercept of 3 calibration curves.

Slope = the mean slope of the 3 calibration curves.

Accuracy (recovery study)

The accuracy of the method was determined by calculating recovery of Lawsone at different spiked level by standard addition method. Standard Lawsone solutions were added at three different levels (50, 100,150 %). At each level three determinations were performed. The amounts of standard recovered were calculated in terms of % recovery and % RSD for Polyherbal Formulation.

Robustness

Robustness of the method was determined by small, deliberate changes in flow rate, and detection wavelength.

Typical changes include flow rate changed to 1.0 ± 0.2 ml/min and detection wavelength changed to 267 ± 2 nm.

Quantitative analysis of Lawsone in Polyherbal Formulations

The proposed RP-HPLC method was applied to analyze Lawsone in Polyherbal Formulation. Sample solution of 32 µg/ml Lawsone was injected under chromatographic condition and peak areas was measured and % assay were calculated from regression equation.
RESULTS AND DISCUSSION

Chromatographic condition was optimized to achieve sharp and symmetrical peak of Lawsone, shown in Table 1. The plot of peak area versus respective concentrations of Lawsone was found to be linear in the concentration range of 10-50 μg/ml with correlation coefficient 0.9995. The linearity of calibration graph and adherence of the system to beer’s law validated by determining correlation coefficients which is shown in the Figure 3. The LOD was found to be 0.55 μg/ml and the LOQ was found to be 1.68 μg/ml for Lawsone [Table 3]. The %RSD value for repeatability, Intraday and Interday precision was found to be less than 2 (n=6), the results obtained are shown in Table 4. The chromatogram of blank is shown in the Figure 5, good correlation (in terms of $t_R$ and area) indicates the specificity of method. The method was found to be robust as the results were not significantly affected by slight variation in Flow rate and Detection Wavelength. The average recoveries of the Lawsone was in the range of 98-102% [Table 5]. The developed method was applied on polyherbal formulation; there was no interference from other components present in formulation. Result is shown in Table 6.

Table 1: Chromatographic Condition

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Optimized Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Merck Chromolith® C18 column (100 mm × 4.6mm)</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>Water : Methanol (50:50), pH was adjusted to 2 by using 0.02M Trifluoro Acetic Acid</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1 ml/min</td>
</tr>
<tr>
<td>UV Detection</td>
<td>267 nm</td>
</tr>
<tr>
<td>Injection</td>
<td>20 μl</td>
</tr>
<tr>
<td>Pressure</td>
<td>1000-2000 PSI</td>
</tr>
<tr>
<td>Temperature</td>
<td>Ambient</td>
</tr>
</tbody>
</table>
Figure 2: Chromatogram of Lawsone standard (30 µg/ml)

Table 2: System suitability test parameters of Lawsone

<table>
<thead>
<tr>
<th>System Suitability Parameters</th>
<th>Lawsone</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention Time (min) ± SD*</td>
<td>4.67 ± 0.07</td>
<td>1.61</td>
</tr>
<tr>
<td>Tailing Factor (T_f) ± SD*</td>
<td>1.002 ± 0.01</td>
<td>0.59</td>
</tr>
<tr>
<td>Number of Theoretical Plates (N) ± SD*</td>
<td>2319 ± 39.04</td>
<td>1.68</td>
</tr>
</tbody>
</table>

*Results are mean of six determinations

Table 3: Calibration Curve, Limit of Detection and Limit of Quantitation.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Lawsone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity Range*</td>
<td>10-50 µg/ml</td>
</tr>
<tr>
<td>Correlation Co-efficient</td>
<td>0.9995</td>
</tr>
<tr>
<td>Slope</td>
<td>1017.6</td>
</tr>
<tr>
<td>Intercept</td>
<td>10937</td>
</tr>
<tr>
<td>LOD*</td>
<td>0.55 µg/ml</td>
</tr>
<tr>
<td>LOQ*</td>
<td>1.68 µg/ml</td>
</tr>
</tbody>
</table>

*Results are mean of three determinations
Figure 3: Calibration curve of Lawsone (10-50 µg/ml)

\[ y = 1017.6x + 10937 \]
\[ r^2 = 0.9995 \]

Figure 4: Chromatogram of Calibration curve for Lawsone (Overlay)

Table 4: Results of Precision of proposed method

<table>
<thead>
<tr>
<th>Conc. (µg/ml)</th>
<th>Intraday* %RSD</th>
<th>Interday* %RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1.15</td>
<td>1.25</td>
</tr>
<tr>
<td>30</td>
<td>1.22</td>
<td>1.22</td>
</tr>
<tr>
<td>50</td>
<td>1.07</td>
<td>0.99</td>
</tr>
<tr>
<td>Average</td>
<td>1.14</td>
<td>1.15</td>
</tr>
</tbody>
</table>

*Results are mean of three determinations*
CONCLUSION

Ayurvedic and herbal formulations are Polyherbal in nature. Considering the widespread use of Lawsone in plant based medicines, a HPLC method was developed for estimation of Lawsone in presence of other plant constituents. The present study shows that the method developed for the estimation of Lawsone was simple, rapid, specific, linear,
accurate, precise and robust. Hence, above said method can be successfully applied for routine quality control analysis and estimation of Lawsone in Polyherbal formulation.

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