METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF GALLIC ACID AND PIPERINE IN HERBAL EXTRACT AND POLYHERBAL FORMULATION BY HPTLC

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ABSTRACT

This article gives a simple, rapid and accurate High performance thin layer chromatographic method for determination of Gallic acid and Piperine in developed herbal formulation. Merck TLC aluminium sheets of silica gel G60 F254 with the thickness of 200µm was used to carry out the separation. Toluene: ethyl acetate: formic acid(5:3.5:0.5 v/v/v), was used as mobile phase. Analysis of the compounds was carried out by densitometry in the absorbance mode at 290 nm. The selected mobile phase gave the well defined peak at the Rf value of 0.20±0.03 and 0.50 ±0.03 for gallic acid and piperine respectively. Method was then validated for accuracy, linearity, precision, specificity, robustness, limit of detection (LOD) and limit of quantification (LOQ) as per ICH guidelines. The linear regression analysis data for the calibration plots showed good linear relationship with the regression coefficient (r²) of 0.994 and 0.993 for the gallic acid and piperine respectively, in the concentration range of 200-900ng/spot. The limit of detection and limit of quantification were 0.72 and 2.18 ng/spot, respectively for gallic acid and 36.88 and 111.78 ng/spot, respectively for piperine. In conclusion, statistical analysis of data showed that the method is precise, selective and reproducible for estimation of both compounds. Hence the developed method can be applied in routine quality control analysis for identification and quantification of gallic acid and piperine in their extracts and developed herbal formulation.
INTRODUCTION

Emblica Officinalis or Phyllanthus emblica, Commonly known as Indian gooseberry or Amla, from the family Euphorbiaceae. It is most important plant in the Indian traditional system of medicine. It is traditionally used for treatment of various diseases such as ulcers, heart disease, diarrhoea, haemorrhage, heart disease, liver disorders, diabetes, dysentery etc. [1,2] It also has some important properties such as antioxidant[1][2][3], immunomodulatory[1], anticancer [1,2] Gastroprotective, [4] anti-hyperlipidemic, [5] hypolipidemic [5] antiatherogenic, [5] anti microbial. [6,7] Emblica officinalis fruits contain gallic acid, ellagic acid, quercetin, kaempferol, emblicanin, flavanoids, glycosides and proanthocyanidines. [1]

Piper longum , commonly known as pippali, belongs to family piperaceae. Drug consist of dried spikes of Piper longum. It has been used as therapeutic agent in treatment of various pathological condition. It shows some medicinal and pharmacological activities such as, immunomodulatory activity [8] stimulant effect, anti asthmatic activity, hepatoprotective activity, antimicrobial activity [9,10] and enhancement of bioavailability [11,12] Piperine is the principle pungent substance in pepper species. [13]

Gallic acid and piperine are constituents present in the extract of emblica officinalis and piper longum, which can be used as markers for their HPTLC simultaneous determination. So far no method for simultaneous estimation of gallic acid and piperine in extract and developed formulation by HPTLC method is found in literature So, the present work aims to develop and validate HPTLC method for simultaneous estimation of gallic acid and piperine in plant extract and developed herbal formulation.

![Figure 1: Structure of Gallic acid](image1.png)  ![Figure 2: Structure of Piperine](image2.png)
MATERIALS AND METHODS

Reagents and Materials
Analytical grade methanol, toluene, ethyl acetate and formic acid were purchased from SD Fine Chemicals, Mumbai, India, Standard Gallic acid was procured from Research-Lab Fine Chem. industries, and piperine was procured from Sigma Aldrich.

Plant Material
Fruits of *Piper longum* and *Emblica Officinalis* were purchased from Ayurvedic medicinal dealer, Shree Ganesh Aushadi Bhandar, 229, Kalbadevi Road, Mumbai and were authenticated by Dr. H.M. Pandit, Department of Botany, Khalsa College, Matunga, Mumbai. Specimen sample was stored in their lab and voucher number was taken.

Preparation of Extracts
The fruits of *Emblica officinalis* and *Piper longum* were dried and powdered. Individual drug powder, each weighing 50g of *Emblica officinalis* and *Piper longum* were extracted using ethanol and methanol as respectively. Extraction was carried out using hot continuous percolation method in soxhalet apparatus. Both extracts were then filtered evaporated and dried under reduced pressure with rotary evaporator to get it in dried powdered form.

Preparation of Standard Solutions
Gallic acid and piperine stock solutions (1000 µg/ml) were prepared by dissolving accurately weighed 100mg of each standard in 100ml of methanol and were sonicated for 10 mins.

Preparation of Sample Solutions
Extract solution: Accurately weighed 100 mg of each extract was dissolved in methanol and final volume was made up to 100 ml to get stock solution containing 1000 µg/ml each. The solutions were filtered through 0.45 µ membrane filter (Millipore) this solution was further used for HPTLC assay.

Polyherbal gel formulation : For estimation of the content of gallic acid and piperine in developed formulation, accurately weighed 100 mg gel was mixed with methanol with vigorous shaking and sonicated for 10 mins on Ultrasonic Bath. The solution was filtered through 0.45 µ membrane filter (Millipore) and the filtrate was used for the further analysis.
**Instrumentation and Chromatographic Conditions**

Both the drugs i.e. gallic acid and piperine showed absorbance at 290 nm. Hence 290 nm was selected as analytical wavelength. TLC aluminium plates (10 × 10) percolated with, silica gel 60F$_{254}$ of 200 µm in thickness (Merck, Mumbai, India) was used as stationary phase. Standard solutions of markers and samples were applied to the plates as bands with band width of 6.00 mm, 10.00 mm from bottom to the same chromatographic plate by using CAMAG (Muttenz, Switzerland) Linomat 5 Sample Applicator equipped with 100µl Hamilton syringe. The slit dimensions was set to 5×0.30 nm. Ascending development to a distance of 90 mm was performed at room temperature (28±2°C), with Toluene: Ethyl acetate: Formic acid in the ratio of 5:3.5:0.5 (v/v/v) as mobile phase in Camag glass Twin-trough chamber which was previously saturated with mobile phase for 20 mins. After development the plated were dried and scanned at 290 nm with CamagTLCScanner3 using the deuterium lamp with winCAT software.

**Calibration Curve for Gallic Acid and Piperine**

Serial dilutions were prepared to get concentration range of 0.1-0.9 µg/ml for both Gallic acid and Piperine. Aliquot of each above solutions were applied in triplicate on TLC plate to obtain concentration in the range of 100-900 ng/spot for both the standards. Peak area of each band was recorded. Calibration curves was obtained by plotting area vs. concentration of gallic acid and piperine and was treated for linear least square regression analysis.

**Method Validation**

The optimized HPTLC method was validated with respect to following parameters in accordance with ICH Q2 (R1) guidelines.

**Linearity and Specificity**

Standard stock solutions of the Gallic acid and piperine were diluted to prepare linearity standard solutions in the concentration range of 0.1-0.9 µg/ml each. Calibration curve was obtained by plotting peak vs. concentration of analyte. Specificity of the method was determined by means of complete separation of standards in presence of other excipients commonly present in formulation.

**Precision**

The precision was determined by performing intraday and interday assay of standard solutions at three levels of concentrations (100, 200, 300 ng/spot for gallic acid and 400, 500,
600 ng/spot for piperine) in triplicates, The samples were analysed, three times on same day for intraday precision and three times on different days for interday precision.

**Limit of Detection and Limit of Quantification (LOD And LOQ)**

LOD and LOQ were determined by using following formula,

\[
LOD = 3.3\sigma/S; \quad LOQ = 10\sigma/S,
\]

where \(\sigma\) is standard deviation of the response and S is slope of the calibration curve.

**Accuracy**

Accuracy of the proposed method was determined by using recovery of drug at three different levels using standard addition method. In 10 µl of extract solution known amounts of gallic acid and piperine standard (80%, 100%, 120%) were added by spiking on the same plate in triplicate. Mean recovery was calculated and compared with the expected results.

**Robustness**

Robustness was determined by making small and deliberate changes in chromatographic conditions like mobile phase composition (±2 ml of major component) and saturation time (± 2min ). the robustness of the method was determined at two concentration levels (300 and 400 ng/spot).

**RESULT AND DISCUSSION**

**Selection of Analytical Wavelength**

The overlay UV spectra of both drugs showed isoabsorptive point at 290 nm , hence 290 nm was selected as wavelength for analysis.(Figure 3)

![Figure 3: Overlay UV Spectra of Gallic acid and Piperine.](image)
Optimization of Chromatographic Conditions

The experimental conditions for HPTLC such as wavelength detection and mobile phase composition were optimized to provide accurate, reproducible and precise results for determination of gallic acid and piperine. The mixed standard stock solution containing 100 µg/ml OF gallic acid and piperine was spotted on TLC plate and it was developed in different solvent systems. Good resolution and sharp peaks with minimum tailing was obtained using Mobile phase consisting Of Toluene : Ethyl Acetate : Formic acid in the ratio of 5: 3.5: 0.5 (v/v/v). Gallic acid and piperine was satisfactorily resolved with Rf value of 0.21± 0.03 and 0.52 ±0.03 respectively.(Fig 4)

Figure 4 : Chromatogram Of Standard Gallic acid , Piperine and Mixture of both.

Linearity

Linearity of the developed method was studies by plotting calibration curves of gallic acid and piperine at different concentrations levels in triplicates ranging from 0.1 - 0.9µg each. The linearity regression co-efficient (r²) value found to be 0.994 and 0.993 for gallic acid and piperine respectively. The results are given in Table 1.
Specificity

It was observed that other constituents present in the extract did not interfere with the peaks of gallic acid and piperine. Thus the proposed method was proved to be specific. The spectra of standard Gallic acid corresponded with amla extract and standard piperine corresponded with pippali extract is shown in Fig 6A and 6B respectively.

Table 1 : Linear regression data for calibration plot for Gallic acid and Piperine(n=3)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Gallic acid</th>
<th>Piperine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (ng/spot)</td>
<td>100-900</td>
<td>100-900</td>
</tr>
<tr>
<td>Equation</td>
<td>( y = 7.9285x + 700.83 )</td>
<td>( y = 14.397x + 3009 )</td>
</tr>
<tr>
<td>Correlation coefficient (( r^2 )) ±SD</td>
<td>0.9942 ± 0.000635085</td>
<td>0.9937 ± 0.002452</td>
</tr>
<tr>
<td>Slope ± SD</td>
<td>7.9285± 0.047435887</td>
<td>14.397± 0.324935</td>
</tr>
<tr>
<td>Intercept ± SD</td>
<td>700.83± 1.730347</td>
<td>3009 ± 160.9306683</td>
</tr>
</tbody>
</table>

Figure 5: Calibration Curve of (A) Gallic acid , (B) Piperine.

![Calibration Curve](image)

Figure 6: (A) Overlay Spectra of Standard Gallic acid and Gallic acid From Extract (B) Overlay Spectre of Standard Piperine and Piperine From Extract
Figure 7 shows chromatogram of placebo gel containing commonly used gel exipients. There were no interfering peaks at R_f values of gallic acid and piperine.

![Figure 7: Chromatogram Of Placebo gel.](image)

**Precision**

Intraday precision is used to describe the variations in method, at three different concentration levels within same day (Table 2), interday precision is for variation between different days (Table 3). The % RSD for Precision of the method was found to be less than 2%.

**Table 2: Intraday Precision Results**

<table>
<thead>
<tr>
<th>Levels</th>
<th>Gallic Acid</th>
<th></th>
<th></th>
<th>Piperine</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration (ng/spot)</td>
<td>200</td>
<td>400</td>
<td>600</td>
<td>200</td>
<td>400</td>
</tr>
<tr>
<td>Peak Area</td>
<td>Session 1</td>
<td>2311.1</td>
<td>3841.9</td>
<td>5297.3</td>
<td>5874.3</td>
<td>8824.5</td>
</tr>
<tr>
<td></td>
<td>Session 2</td>
<td>2341.9</td>
<td>3881.2</td>
<td>5427.6</td>
<td>5894.2</td>
<td>8901.3</td>
</tr>
<tr>
<td></td>
<td>Session 3</td>
<td>2298.1</td>
<td>3911.6</td>
<td>5317.9</td>
<td>5866.9</td>
<td>8866.7</td>
</tr>
<tr>
<td>Average</td>
<td>2317.03</td>
<td>3878.23</td>
<td>5347.6</td>
<td>5878.47</td>
<td>8864.17</td>
<td>11564.9</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>22.49</td>
<td>34.95</td>
<td>70.04</td>
<td>14.12</td>
<td>38.46</td>
<td>55.15</td>
</tr>
<tr>
<td>%RSD</td>
<td>0.9708</td>
<td>0.9010</td>
<td>1.3098</td>
<td>0.2402</td>
<td>0.4339</td>
<td>0.4768</td>
</tr>
</tbody>
</table>
Table 3: Interday Precision Results

<table>
<thead>
<tr>
<th>Levels</th>
<th>Gallic Acid</th>
<th>Piperine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration (ng/spot)</td>
<td>200</td>
</tr>
<tr>
<td>Peak Area</td>
<td>Day 1</td>
<td>2279.5</td>
</tr>
<tr>
<td></td>
<td>Day 2</td>
<td>2240.1</td>
</tr>
<tr>
<td></td>
<td>Day 3</td>
<td>2256.8</td>
</tr>
<tr>
<td>Average</td>
<td>2258.8</td>
<td>3859.6</td>
</tr>
<tr>
<td>%RSD</td>
<td>0.8755</td>
<td>0.8101</td>
</tr>
</tbody>
</table>

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

The LOD and LOQ were found to be 0.72, 2.18 ng/spot and 36.88, 111.78 ng/spot for gallic acid and piperine respectively.

Accuracy

Results of accuracy are given in table no.4.

Table 4: Accuracy Results

<table>
<thead>
<tr>
<th>Drug</th>
<th>% Level</th>
<th>Initial amount (ng/spot)</th>
<th>Spiked amount (ng/spot)</th>
<th>% Recovery ± SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>80</td>
<td>500</td>
<td>400</td>
<td>99.84111111 ±0.2160504</td>
<td>0.216394232</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>500</td>
<td>500</td>
<td>98.79533333 ±0.6862946</td>
<td>0.694663014</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>500</td>
<td>600</td>
<td>99.36030303 ±1.1794936</td>
<td>1.187087397</td>
</tr>
<tr>
<td>Piperine</td>
<td>80</td>
<td>500</td>
<td>400</td>
<td>100.5385 ±0.517921</td>
<td>0.515147055</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>500</td>
<td>500</td>
<td>98.67333 ±0.657599</td>
<td>0.666440357</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>500</td>
<td>600</td>
<td>99.37818 ±1.484893</td>
<td>1.494183902</td>
</tr>
</tbody>
</table>

Robustness

Table 5: Robustness results

<table>
<thead>
<tr>
<th>Mobile phase composition</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gallic acid 200 (ng/spot)</td>
</tr>
<tr>
<td></td>
<td>Gallic acid 400 (ng/spot)</td>
</tr>
<tr>
<td></td>
<td>Piperine 200 (ng/spot)</td>
</tr>
<tr>
<td></td>
<td>Piperine 400 (ng/spot)</td>
</tr>
</tbody>
</table>
Results (Table 5) showed that the developed method was robust for small but deliberate changes in mobile Phase composition and saturation time. The % RSD for the peak areas for parameters under study was less than 2 % which indicated that the developed method was robust.

**Analysis Of Plant Extract and Gel Formulation**

The developed method was applied for detection and quantification of gallic acid in *Emblica officinalis* and piperine in *Piper longum*. The samples of gel was analysed successfully by the proposed method. The gallic acid content in *Emblica officinalis* extract was found to be 0.87% w/w and piperine content in *Piper longum* extract was found to be 0.95% w/w.

**CONCLUSION**

The proposed HPTLC method was found to be simple, accurate, precise, specific, robust and economic. This method can be successfully used for routine analysis of Gallic acid and Piperine in raw materials, extracts, and pharmaceutical formulations without any interference. The method can be extended to study the degradation of gallic acid and piperine under different stress conditions as per recommendations of ICH guidelines.

**ACKNOWLEDGEMENT**

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**REFERENCES**


