Development of HPTLC method for estimation of piperine, guggulsterone E and Z in polyherbal formulation

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A B S T R A C T

Aim: Triphala guggul a polyherbal tablet formulation is used for sinusitis, allergies, boils, constipation, piles, high cholesterol, rheumatism, mal-absorption, purgative and as blood purifier. In the present study an attempt has been made to develop a simple, precise, rapid and cost-effective high performance thin-layer chromatographic (HPTLC) method for quantitative estimation of piperine, guggulsterone E and Z in Triphala guggul formulation.

Method: The different batches of formulation were prepared in laboratory by using authenticated raw material and were subjected to various physical and chemical evaluations. Then the prepared formulation and three commercial formulations were investigated for the qualitative and quantitative estimation of mentioned constituents. The methanolic extract of all formulations were quantified by using HPTLC studies. Linear regression data for the calibration curves of standards viz. piperine, guggulsterone E and Z showed a good linear relationship over a concentration range of 0.06–0.14 µg/spot, 2.5–17.5 µg/spot, 5–30 µg/spot respectively with the correlation coefficient of 0.99085, 0.99847, 0.9990 respectively and thus exhibits good linearity between concentration and area. The content of guggulsterone E (14.68 %w/w, 13.05 %w/w, 6.36 %w/w, 14.36 %w/w); guggulsterone Z (31.81 %w/w, 26.95 %w/w, 11.62 %w/w, 23.86 %w/w); and piperine (0.068 %w/w, 0.015 %w/w, 0.321 %w/w, 0.0375 %w/w) were found in TF, TN, TM, TP prepared and three marketed formulation respectively.

Conclusion: The proposed HPTLC method was found to be rapid, simple and linear for quantitative estimation of piperine, guggulsterone E & Z in different formulations and extracts.

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1. Introduction

Herbal medicine has been enjoying renaissance among the customers throughout the world. However, one of the impediments in the acceptance of the Ayurvedic formulation is the lack of standard quality control profile. Although, considerable work is being done to evaluate herbs for their quality, safety and efficacy, there is a need of a well-defined specific method for routine analysis of herbal raw materials and formulations with regard to constituents responsible for its efficacy. Development of methods for analysis of plant products poses difficulty, due to their unknown chemical profile, complex nature and inherent variability of the chemical constituents. It is therefore difficult to establish quality control parameter for plant based drugs due to which unregulated sales of adulterated and spurious drugs are observed.

‘Triphala guggul’ is a traditional Ayurvedic herbal formulation consisting of the dried fruits of three medicinal plants, Terminalia chebula (Combretaceae), Terminalia belerica (Combretaceae) and Emblica officinalis (Euphorbiaceae), these are combined with Commiphora mukul (Burseraceae) and Piper longum (Piperaceae) for the treatment of sinusitis, allergies, boils, constipation, piles, high cholesterol, mal-absorption and as a purgative, blood purifier, anti-inflammatory and anti-rheumatic.5 The pharmacopoeia standards in Ayurvedic or herbal pharmacopoeia appear inadequate to ensure the quality of plant drugs or their formulations.

In the present study, an attempt has been made to develop a simple, rapid and accurate HPTLC method for estimation of piperine as well as guggulsterone E and Z in marketed formulations of Triphala guggul tablet. These constituents are considered to be...
the active components and can be considered as marker compounds. The method developed was also used for chemical fingerprint analysis.

2. Materials and methods

2.1. Equipment and chromatographic condition

A CAMAG HPTLC system equipped with a sample applicator Linomat IV using 100 µl syringe and connected to a nitrogen tank; twin trough plate development chamber; CAMAG TLC scanner-3 with winCATS software. Each HPTLC plate precoated, silica gel G 60 F254 size 20 x 10 cm accommodated twenty tracks of standards and samples, applied according to following settings: bandwidth 4 mm; distance between bands 5 mm; The plates were developed to 8 cm in a twin trough glass chamber, saturation time 30 min, scanning mode Absorbance/Reflectance; temperature 20 ± 5 °C and separation technique ascending.

2.2. Chemicals

Standard piperine (Sigma Aldrich), guggulsterone E and Z (Yucca Enterprises, Mumbai), precoated silica gel G 60 F254 TLC aluminium plates (20 x 20 cm, 0.2 mm thick) (Merck Ltd. Germany) and AR grade chemicals were used. The marketed samples were purchased from local market of Nagpur, Maharashtra.

2.3. Drugs

T. chebula (Hrida), T. belerica (Baheda), E. officinalis (Amla), P. longum (Pimpli) and C. mukul (Guggul) authenticated crude drugs were procured from Natural Remedies, Bangalore. The raw material was subjected to physical and chemical evaluation. The formulation was prepared in laboratory in different batches as per the formula given in the Bhaishajya Ratnavali. The three batches of formulated were subjected to the pharmacopoeial evaluation of tablets. The marketed samples were procured from local market of Nagpur, Maharashtra. These formulations were subjected to the pharmacopoeial evaluation of tablets. The three batches of marketed formulations from three different manufacturers in India namely, Formulation 1 Triphala guggul by Unza Pharmaceutical (batch C-21 [TM1], C-26 [TM2], D-17 [TM3]), Formulation 2 Triphala guggul by Baidyanath (batch 329 [TN1], 356 [TN2], 357 [TN3]), Formulation 3 Triphala guggul, by Ayurvedic Rasashala (batch 030035 [TP1], 051052 [TP2], 050302 [TP3]) were procured from local market of Nagpur, Maharashtra. These formulations were subjected to the pharmacopoeial evaluation of tablets. The batches which pass the evaluation test were used for qualitative and quantitative study using HPTLC.

2.4. HPTLC method for estimation of phytoconstituents

2.4.1. Preparation of standard guggulsterone E solution

A stock solution of 0.5 mg/ml was prepared by dissolving 5 mg of guggulsterone E in methanol and volume was made to 10 ml and different amounts 5, 10, 15, 20, 25, 30, 35 µl were applied in triplicate on TLC plates, using a Camag Linomat IV sample applicator.

2.4.2. Preparation of standard guggulsterone Z solution

A stock solution of 1 mg/ml was prepared by dissolving 10 mg of guggulsterone Z in methanol and volume was made to 10 ml and different amounts 5, 10, 15, 20, 25, 30 µl were applied in triplicate on TLC plates.

2.4.3. Preparation of standard piperine solution

A stock solution of 0.1 mg/ml was prepared by dissolving 1 mg of piperine in methanol and volume was made to 10 ml and 6, 8, 10, 12, 14 µl were applied in triplicate on TLC plates.

2.4.4. Sample preparation

The sample was weighed (250 mg); sonicated with methanol for 25 min, filtered through Whatmann filter paper and volume was made to 10 ml in volumetric flask. 5 µl (for estimation of guggulsterone E & Z) and 10 µl (for estimation of piperine) of prepared and marketed formulations samples were applied on plate in 4 mm band with the help of Linomat IV applicator and developed under the same conditions as described for the standards.

2.5. Calibration curve

Calibration curve was constructed according to requirement of ICH guidelines. Each concentration was applied to a plate (20 x 10 cm) in triplicates of 4 mm band length with a distance of 5 mm between each two bands. The distance from the plate side edge was 10 mm and from the bottom of the plate was also 10 mm. The application rate was 15 µl/s, standard zones were quantified by linear densitometric scanning using Camag TLC scanner. Deuterium lamp was utilized as a source of radiation. Evaluation was done using linear regression analysis via peak areas and calibration curve was prepared by plotting peak area vs. concentration applied.

2.6. Method validation

2.6.1. Linearity

The linearity of the HPTLC method was evaluated by analysing a series of different concentrations of the standards (guggulsterone E, Z and piperine), where each concentration was applied in triplicate. Linear regression data for the calibration curves of standards guggulsterone E and Z, piperine showed a good linear relationship.

2.6.2. Specificity

The specificity of the method was determined by analysing the drug standard and test samples. The peak for test sample was confirmed by comparing its Rf and spectrum with those of the standard.

2.6.3. System precision

The system precision was assessed by determination of six different concentrations of standards each applied in triplicate.

2.6.4. Method precision (repeatability)

Repeatability (precision) was determined by repeated analysis of standard samples using the same equipment, same analytical procedures and same laboratory and on the same plate. Repeatability of measurement was determined by spotting 10 µl of standard drug solution on TLC plate, after development spot was scanned six times without changing position. The % RSD was determined.

2.6.5. Limits of detection and quantitation

Limit of detection and limit of quantitation were validated based on signal to noise ratio where the minimum concentration at which the standard solutions can be reliably detected was recorded. Limits of detection (LODs) and limits of quantization (LOQs) were calculated using the expressions 3σ/n and 10σ/s, respectively, in which σ is intercept standard deviation and s is the slope of calibration curve.

2.6.6. Recovery study

The accuracy of proposed method was evaluated by addition of standard drug solution to pre-analysed tablet sample solution at three different concentration levels at 50, 100, and 150% of linearity. This parameter shows the proximity between the experimental values and the real ones.
2.6.7. Robustness

By introducing small changes in the mobile phase composition, mobile phase volume and duration of mobile phase saturation, the effects on the results were examined. Robustness of the method was done in triplicate at a concentration level of 10 μg/spot for guggulsterone E, Z and 0.10 μg/spot for piperine.

2.6.8. Sample analysis

The developed method can be applied in determination of guggulsterone E & Z as well as piperine in polyherbal formulations. Three batches of laboratory formulation were prepared by using authentic raw material and three batches of three different marketed companies were selected and analysed by the proposed method.

3. Results

Standard guggulsterone E (Rf: 0.19, Fig. 1), guggulsterone Z (Rf: 0.27, Fig. 2) and piperine (Rf: 0.62, Fig. 3) showed single peak in HPTLC chromatogram. Calibration curve was prepared by plotting peak area vs. concentration applied. The linear regression data for the calibration curves of standards guggulsterone E, Z and piperine showed a good linear relationship over a concentration range of 2.5–17.5 μg/spot, 5–30 μg/spot, 0.06–0.14 μg/spot respectively with the correlation coefficient of 0.99847, 0.9990 and 0.99085 respectively and linear regression equation was found to be: \( y = 195.317 + 94.585x \), \( y = 332.423 + 101.518x \), \( y = 5417.084 + 38873x \) respectively, where \( y \) is the spot area and \( x \) is the concentration of the analyte. It was observed that other constituents present in the formulations did not interfere either with the peak of guggulsterone E, Z and piperine therefore the method was specific. The spectrum of respective standards and respective spots present in the samples was found to be similar or overlapped (Fig. 4). The proposed HPTLC methods were validated for intra and interday variations. The values of percent relative standard deviations (RSDs) were found to be 0.80, 1.17, 0.69 (interday) 1.22, 2.25, 0.22 (intraday) for guggulsterone E, Z and piperine respectively which indicate that the method was precise. Limit of detection and limit of quantitation where the minimum concentration at which the standards solution can be reliably

Fig. 1. (a) HPTLC scan densitogram (b) Calibration curve of standard guggulsterone E.

Fig. 2. (a) HPTLC scan densitogram (b) Calibration curve of standard guggulsterone Z.
detected was recorded as 1.07 μg/spot, 1.54 μg/spot, 0.12 μg/spot and minimum concentration at which the analyte can be reliably quantified was found to be 3.28 μg/spot, 4.68 μg/spot, 0.36 μg/spot for guggulsterone E, guggulsterone Z and piperine respectively (Table 1). To study the accuracy of the method, recovery studies were performed at three different concentration levels at 50, 100, and 150% of linearity. The recovery ranged from 98.80 to 101.05%, 98.69 to 101.81%, 97.75 to 102.33% for guggulsterone E, Z and piperine respectively (Table 2) indicating that the proposed HPTLC method was accurate. All the batches of prepared and marketed formulation were subjected to analysis by optimized HPTLC method. Table 3 shows the percentage of guggulsterone E, Z and piperine present in the all formulations. The above study reflects that the percentage of guggulsterone E and Z is much less in formulation TM as compared to other formulations and percentage of piperine is much less in formulation TN, even it shows variation in percentage of above mentioned constituents in different batches.

### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Guggulsterone E</th>
<th>Guggulsterone Z</th>
<th>Piperine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (μg/spot)</td>
<td>2.5–17.5 μg/spot</td>
<td>5–30 μg/spot</td>
<td>0.06–0.14 μg/spot</td>
</tr>
<tr>
<td>Detection wavelength (λ&lt;sub&gt;max&lt;/sub&gt;)</td>
<td>242 nm</td>
<td>242 nm</td>
<td>343 nm</td>
</tr>
<tr>
<td>Rf value</td>
<td>0.19</td>
<td>0.27</td>
<td>0.62</td>
</tr>
<tr>
<td>Regression equation</td>
<td>y = 195.317 + 94.585x</td>
<td>y = 332.423 + 101.518x</td>
<td>y = 5417.084 + 38873x</td>
</tr>
<tr>
<td>Slope (b)</td>
<td>94.585</td>
<td>101.518</td>
<td>38873</td>
</tr>
<tr>
<td>Intercept (a)</td>
<td>195.317</td>
<td>332.423</td>
<td>5417.084</td>
</tr>
<tr>
<td>Correlation coefficient (R&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>0.99847</td>
<td>0.999</td>
<td>0.99085</td>
</tr>
<tr>
<td>Limit of detection (μg/spot)</td>
<td>1.07</td>
<td>1.54</td>
<td>0.12</td>
</tr>
<tr>
<td>Limit of quantitation (μg/spot)**</td>
<td>3.28</td>
<td>4.68</td>
<td>0.36</td>
</tr>
<tr>
<td>Intraday precision (RSD, %)**</td>
<td>1.22%</td>
<td>2.25%</td>
<td>0.22%</td>
</tr>
<tr>
<td>Interday precision (RSD, %)**</td>
<td>0.80%</td>
<td>1.17%</td>
<td>0.69%</td>
</tr>
</tbody>
</table>

**Average of six determinations.
Table 2
Results of recovery study.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Initial amount (µg/spot)</th>
<th>Amount added (µg/spot)</th>
<th>Amount of marker added in formulation (µg/spot)</th>
<th>Amount recovered(%)</th>
<th>Recovery ± SD(%)</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guggulsterone E</td>
<td>18.4</td>
<td>50%</td>
<td>9.2</td>
<td>98.80</td>
<td>0.10</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>18.4</td>
<td>100%</td>
<td>18.4</td>
<td>101.05</td>
<td>0.14</td>
<td>0.39</td>
</tr>
<tr>
<td>Guggulsterone Z</td>
<td>46.4</td>
<td>50%</td>
<td>23.2</td>
<td>99.24</td>
<td>0.15</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>46.4</td>
<td>100%</td>
<td>46.4</td>
<td>98.69</td>
<td>0.59</td>
<td>0.65</td>
</tr>
<tr>
<td>Piperine</td>
<td>0.02</td>
<td>50%</td>
<td>0.01</td>
<td>101.81</td>
<td>1.01</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>100%</td>
<td>0.02</td>
<td>102.33</td>
<td>0.0052</td>
<td>1.70</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>150%</td>
<td>0.03</td>
<td>97.75</td>
<td>0.0081</td>
<td>2.08</td>
</tr>
</tbody>
</table>

*Average of three determinations.

Table 3
Percentage of piperine, guggulsterone E and Z in Triphala guggul formulation.

<table>
<thead>
<tr>
<th>Types of sample</th>
<th>Guggulsterone E</th>
<th>Guggulsterone Z</th>
<th>Piperine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total area included in peak</td>
<td>%w/w</td>
<td>Total area included in peak</td>
</tr>
<tr>
<td>TF1</td>
<td>1889.27</td>
<td>14.32</td>
<td>4790.8</td>
</tr>
<tr>
<td>TF2</td>
<td>1967.45</td>
<td>14.92</td>
<td>5206.8</td>
</tr>
<tr>
<td>TF3</td>
<td>1944.76</td>
<td>14.8</td>
<td>5149.7</td>
</tr>
<tr>
<td>TM1</td>
<td>1439.07</td>
<td>1.05</td>
<td>1838.7</td>
</tr>
<tr>
<td>TM2</td>
<td>1299.38</td>
<td>9.35</td>
<td>2028.9</td>
</tr>
<tr>
<td>TM3</td>
<td>1224.6</td>
<td>8.7</td>
<td>1953.9</td>
</tr>
<tr>
<td>TN1</td>
<td>2537.74</td>
<td>15.11</td>
<td>4565.9</td>
</tr>
<tr>
<td>TN2</td>
<td>1930.35</td>
<td>14.68</td>
<td>3716.6</td>
</tr>
<tr>
<td>TN3</td>
<td>1304.48</td>
<td>9.38</td>
<td>2976.5</td>
</tr>
<tr>
<td>TP1</td>
<td>1808.27</td>
<td>13.68</td>
<td>3134.8</td>
</tr>
<tr>
<td>TF2</td>
<td>1970.34</td>
<td>15.02</td>
<td>3592.1</td>
</tr>
<tr>
<td>TF3</td>
<td>1964.74</td>
<td>14.4</td>
<td>3362</td>
</tr>
</tbody>
</table>

4. Discussion

The HPTLC method optimized in this work for the quantification of guggulsterone E, Z and piperine is simple, rapid, accurate, reproducible and sensitive. It can be useful to analyze a wide variety of guggul and piperine containing products. The method was established taking requirements of high precision and economy into consideration. The validation parameters for the developed method were the specificity, calibration curve, precision (repeatability), recovery and accuracy. The method resulted in a sharp, symmetrical, and well resolved peak. The spectrum of standard and sample shown end to tail well overlapping. Linear regression data for the plot confirmed the good linear relationship and the resulting equation was operational in the concentration range of 2.5–17.5 µg/spot for guggulsterone E, 5–30 µg/spot for guggulsterone Z, 0.06–0.14 µg/spot for piperine. The optimized method was found precise and accurate.

The wide variations in the labelled content in the marketed formulations were observed. This shows that Ayurvedic formulations are not standardized and thus obviously lead to marked differences in the therapeutic efficacy of the formulations, when administered. Hence, the newly developed HPTLC method could be proposed for routine quality control process for the estimation of guggulsterone E, Z and piperine in formulations. It can be adopted to standardize the product inprocess and the content of guggulsterone E, Z and piperine can be altered during its formulation stage, thus ensuring desired therapeutic efficacy of the herbal product. This would also minimize or avoid the batch-to-batch variation. This content of research will also be useful in reckoning the precise shelf life and stability of the respective formulations having these phytoconstituents. These types of analytical protocols following sophisticated modern techniques are important for worldwide acceptance and to bring the herbal formulations at par with allopathic formulations. It also serves as a rapid and specific tool in the herbal research, thereby, allowing the manufacturers to set quality standards and specifications so as to seek marketing approval from regulatory authorities for therapeutic efficacy, safety and shelf life of herbal drugs.

Conflicts of interest

All authors have none to declare.

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