Development and Validation of a HPTLC Method for Simultaneous Densitometric Analysis of Gymnemagenin and 18ß-Glycyrrhetinic Acid in Herbal Drug Formulation

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ABSTRACT
A new, simple high performance thin layer chromatographic (HPTLC) method for simultaneous determination of gymnemagenin and 18ß-glycyrrhetinic acid in herbal drug formulation has been developed and validated. There are no reported methods for simultaneous HPTLC analysis of gymnemagenin and 18ß-glycyrrhetinic acid in herbal drug formulation. Hence an attempt has been made to develop accurate, precise densitometric HPTLC method for simultaneous estimation of these biomarkers in a marketed herbal formulation (Diabecon DS tablets). Marketed herbal formulation was subjected to acid hydrolysis followed by base hydrolysis (to obtain gymnemagenin) and extraction with ethyl acetate. 18ß-Glycyrrhetinic acid was obtained after acid hydrolysis followed by extraction with chloroform. The separation was carried out on Merck aluminum plates precoated with silica gel 60 F254 using toluene: ethyl acetate; methanol 3:6:1 (v/v/v) as mobile phase. The separated spots were derivatized with modified vanillin-sulphuric acid reagent and scanned at 651 nm. The retention factor for gymnemagenin and 18ß-glycyrrhetinic acid were found to be 0.29 ± 0.01 and 0.69 ± 0.01 respectively. The method was validated with respect to linearity, accuracy, precision, in accordance with ICH guidelines. The calibration curve was found to be linear over a range of 100 –1500 ng per spot for gymnemagenin and 200-1800 ng per spot for 18ß-glycyrrhetinic acid. The method has been successfully applied for the analysis of marketed herbal drug formulation. The content of gymnemagenin and 18ß-glycyrrhetinic acid was found to be 0.1469 % and 0.8983 %, respectively. The proposed method was found to be accurate, precise, rapid and can be used for quantitative analysis of this marketed herbal formulation in quality-control laboratories.

Key words: Gymnemagenin, 18ß-glycyrrhetinic acid, HPTLC, simultaneous estimation.

INTRODUCTION
Gymnemic acid is a group of triterpenoid saponins isolated from Gymnema sylvestre which is responsible for its anti-diabetic action[1]. Gymnemagenin is a common aglycone of gymnemic acids, produced after acidic and basic hydrolysis[2]. Chemically gymnemagenin is 3ß, 16ß, 21ß, 22α, 23, 28-hexahydroxy-olean-12-ene[3].

18ß-Glycyrrhetinic acid is an aglycone of glycyrrhizin possesses antihyperglycemic action on streptozotocin induced diabetic rats[4]. Extensive literature survey reveals that few HPTLC[5, 6, 7] and HPLC[8] methods have been reported for estimation of gymnemagenin. HPTLC[9, 10] and HPLC[11, 12] methods have also been reported for estimation of 18ß-glycyrrhetinic acid individually and in combination with other marker compounds. To the best of our knowledge no reports were found for simultaneous estimation of gymnemagenin and 18ß-glycyrrhetinic acid by HPTLC method. Gymnemagenin has poor absorption in UV regions[5]. It is therefore necessary to develop methods for rapid, precise and accurate identification and estimation of active constituent/s or marker compound/s as the qualitative and quantitative target to assess the authenticity and inherent quality[13, 14]. HPTLC is most widely used at industrial level for routine analysis of herbal medicines[15].

Hence the objective of this work was to develop and validate simple, accurate, and reproducible procedure for the simultaneous HPTLC analysis of gymnemagenin and 18ß-glycyrrhetinic acid in herbal drug formulation.

MATERIALS AND METHODS
Solvents and chemicals
Standard gymnemagenin (Lot. No: T11G058) and 18ß-glycyrrhetinic acid (Lot. No: T10K024) were procured from Natural Remedies Bangalore, India. Herbal drug formulation used in this study was Diabecon DS tablets (B. No: AA21011, Himalaya, India) were purchased from the local market. All chemicals and reagents used were of analytical grade and purchased from Merck specialties Pvt. Ltd. (Mumbai, India). Double distilled water was used in the present work.

Instrumentation and Chromatographic Conditions
The sample solutions were spotted on precoated silica gel aluminium plate 60F254 (20 cm × 10 cm with 250 µm thickness; E. Merck, Darmstadt, Germany) in the form of bands of 6 mm width with a Hamilton syringe (100 µL) using a Camag Linomat V (Switzerland) sample applicator. The plates were pre-washed with methanol, activated in an oven at 105°C for 20 min, then left to cool to room temperature. The slit dimension was kept at 5mm × 0.45 mm and 10 mm/s scanning speed was employed. Plates were then developed, at constant temperature, with 20 mL mobile phase consisting of toluene: ethyl acetate: methanol 3:6:1 (v/v/v). Linear ascending development was carried out in 20 cm x 10 cm twin trough glass chamber (Camag, Mut tenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 25 min at room temperature (25 ± 2°C) at relative humidity of 60 ± 5%. Based on the current literature survey, several derivatization reagents has been examined such as 5% sulphuric acid, anisaldehyde sulphuric acid, but modified vanillin-sulphuric acid reagent shows promising and reproducible results with chromophore stability for...
observed that both drugs showed considerable absorbance at 651 nm (Fig. After chromatographic development and derivatization process bands were
methanol to get a solution of concentration 100 µg/mL. These stock solutions
Preparation of standard stock solutions
Standard stock solutions of pure drugs were prepared separately by
dissolving 10 mg of each drug in 10 mL of methanol to get concentration of
1000 µg/mL. From this 1 mL of solution was further diluted to 10 mL with
methanol to get a solution of concentration 100 µg/mL. These stock solutions
were used for further studies.
Selection of detection wavelength
After chromatographic development and derivatization process bands were
scanned over the range of 400-700 nm and the spectra were overlain. It was
observed that both drugs showed considerable absorbance at 651 nm (Fig.
Hence 651 nm was selected as the wavelength for detection.

For gymnemagenin
Based on literature survey several trials were taken for hydrolysis of marketed formulation to obtain free form of gymnemagenin. The method
given by Trivedi et. al. was slightly modified to obtain the optimum amount of
gymnemagenin. For analysis of the tablet dosage form, twenty tablets
were weighed and their average weight was calculated. The tablets were
finely powdered and powder equivalent to two tablets (2082 mg) was
refluxed for two hours in 2 N 50% methanolic HCl, filtered and filtrate was
added in ice cold water to obtain precipitate which was refluxed for 2 h in 50
mL of 2% methanolic KOH. The mixture was cooled, diluted with water
and extracted with ethyl acetate. Ethyl acetate layer was separated, dried
over anhydrous sodium sulphate and evaporated. The residue was
reconstituted in 25 mL methanol.

For 18ß-glycyrrhetinic acid
The method given by Rathee et. al. was slightly modified to obtain the
optimum amount of 18ß-glycyrrhetinic acid. Since glycyrrhetinic acid is
present in bound form in the drug, the drug was subjected to acid hydrolysis.
For analysis of the tablet dosage form, twenty tablets were weighed and
and their average weight was calculated. The tablets were finely powdered and
powder equivalent to five tablets (5205 mg) was hydrolyzed with 2N
aqueous hydrochloric acid (100 mL) under reflux on a heating mantle for 2
hours at 100°C. The extract was filtered through Whatman I filter paper
and the marc was washed with minimum amount of double distilled water
(~ 10 mL) and filtered. The combined filtrates were pooled together to a
separating funnel and further extracted with chloroform (50 mL × 3). The
CHCl3 extracts were dried over anhydrous sodium sulphate, concentrated
and the volume was made up to 25 mL with methanol.

Assay validation
The proposed method was optimized and validated as per the International
Conference on Harmonization (ICH) guidelines[16]. All measurements were
performed in triplicates.

Precision studies
Set of three different concentrations in three replicates of mixed standard
solutions of gymnemagenin (400, 500 and 600 ng/spot) and 18ß-
glycyrrhetinic acid (800, 1000 and 1200 ng/spot) were prepared. All the
solutions were analyzed on the same day in order to record any intra-day
variations in the results. For inter-day variation study, three different
concentrations of the mixed standard solutions in linearity range were
analyzed on three consecutive days.

Accuracy studies
In order to evaluate the validity of the proposed method, accuracy was
evaluated through the percentage recoveries of known amounts of mixture
gymnemagenin and 18ß-glycyrrhetinic acid added to solutions of marketed
herbal formulation. Marketed herbal formulation was spiked with the known
amount of standards, and the percent ratios between the recovered and
expected concentrations were calculated. The analyzed samples were spiked
with 80, 100 and 120 % of 0.5 µg gymnemagenin and 0.5 µg of 18ß-
glycyrrhetinic acid standard solutions. Accuracy was calculated from the
following equation:

\[
\text{[(spiked concentration - mean concentration) / spiked concentration] × 100.}
\]

Robustness studies
The effect of small, deliberate variation of the analytical conditions on the
peak areas and retention factor of the drugs were examined. Factors varied
were mobile phase composition and detection wavelength. One factor at a
time was changed to study the effect. The robustness of the method was

RESULTS AND DISCUSSION

HPTLC method optimization

Different mobile phases containing various ratios of toluene, n-hexane, ethanol, methanol, ethyl acetate, and acetone were tried. Finally the mobile phase consisting of toluene: ethyl acetate: methanol (3: 6: 1, v/v) was selected for obtaining well resolved peaks. The optimum wavelength for detection and quantitation used was 651 nm. The retention factors for gymnemagenin and 18β-glycyrrhetinic acid were found to be 0.29 ± 0.01 and 0.69 ± 0.01, respectively. Representative densitogram obtained from a mixed standard solution of gymnemagenin and 18β-glycyrrhetinic acid is shown in Fig.2.

Linearity, limit of detection and quantitation

The results were found to be linear in a range of 100 -1500 ng / spot for gymnemagenin and 200-1800 ng / spot for 18β-glycyrrhetinic acid. The correlation coefficients (r) for the plots were 0.99991 for gymnemagenin and 0.99972 for 18β-glycyrrhetinic acid. The calibration plots obtained for gymnemagenin and 18β-glycyrrhetinic acid is shown in Fig.3 and Fig.4. The LOD and LOQ for gymnemagenin and 18β-glycyrrhetinic acid was found to be 26.50 and 80.31 ng and 51.88 and 157.23 ng, respectively.

Accuracy

As shown in Table 1, satisfactory recoveries of 98.2 -101.3 % and 99.4 -102.7 % for gymnemagenin and 18β-glycyrrhetinic acid, respectively which indicate that the proposed simultaneous densitometric method is reliable for the quantification of gymnemagenin and 18β-glycyrrhetinic acid in this marketed herbal formulation.

Analysis of marketed herbal formulation

Validity of the proposed method was applied to standardization for herbal tablet dosage forms viz. Diabecon DS tablets. The shape of the peaks was not altered by other substances present in the matrix. The percent content of both viz., gymnemagenin and 18β-glycyrrhetinic acid in marketed herbal formulation was found to be 0.1469 % and 0.8983 %, respectively.

Robustness studies

Robustness of the method checked after deliberate alterations of the analytical parameters showed that areas of peaks of interest and retention factor remained unaffected by small changes of the operational parameters (% RSD < 2). The summary of validation parameters of proposed method are given in Table 2.

Table 1: Results of recovery studies.

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Amount added</th>
<th>% Recovery ±S.D.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gymnemagenin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18β-Glycyrrhetinic acid</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>DSM tablets</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>600</td>
<td>600</td>
<td>600</td>
</tr>
</tbody>
</table>

*Average of three determinations.

Table 2: Summary of validation parameters of proposed method.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Gymnemagenin</th>
<th>18β-Glycyrrhetinic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (ng/band)</td>
<td>100-1500</td>
<td>200-1800</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.99991</td>
<td>0.99972</td>
</tr>
<tr>
<td>LOD (ng/band)</td>
<td>26.50</td>
<td>51.88</td>
</tr>
<tr>
<td>LOQ (ng/band)</td>
<td>80.31</td>
<td>157.23</td>
</tr>
<tr>
<td>Accuracy (% Recovery)</td>
<td>98-101</td>
<td>99-102</td>
</tr>
<tr>
<td>Precision (%RSD)</td>
<td>0.12-0.23</td>
<td>0.14-0.19</td>
</tr>
<tr>
<td>Intraday (n=3)</td>
<td>0.16</td>
<td>0.18-0.32</td>
</tr>
<tr>
<td>Interday (n=3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*LOD =Limit of detection.; LOQ =Limit of quantitation.; RSD = Relative standard deviation.; n = Number of determinations
CONCLUSION
The validated HPTLC method employed proved to be simple, fast, accurate, precise and robust and thus can be used for routine analysis of gymnemagenin and 18β-glycyrrhetinic acid in this herbal tablet formulation.

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