Research Article

Quantification of Gymnemagenin and β-Sitosterol in Marketed Herbal Formulation by Validated Normal Phase HPTLC Method

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1. Introduction

Gymnemic acid is isolated from Gymnema sylvestre which is responsible for its antidiabetic action [1]. Gymnemagenin is an aglycone of gymnemic acids, produced after acidic and basic hydrolysis [2]. Literature survey reveals that few HPLC [2] and HPTLC [3–6] methods have been reported for estimation of gymnemagenin. β-Sitosterol is a waxy, white phytosterol [7] with antidiabetic and antioxidant potential [8]. HPTLC [9–14] and HPLC [7, 15, 16] methods have also been reported for analysis of β-sitosterol either individually or in combination with other markers. HPTLC is widely used in industry for routine analysis of herbal medicines [9]. Though individual analytical methods are available for gymnemagenin and β-sitosterol, no HPTLC method is available for their concurrent analysis. In this prospective, the research study was undertaken to develop and validate new, rapid, accurate, precise, robust, and sensitive procedure for quantification of gymnemagenin and β-sitosterol in a polyherbal formulation.

2. Experimental

2.1. Materials and Chemicals. Standard gymnemagenin and β-sitosterol were procured from Natural Remedies (Bangalore, India). Herbal drug formulation used in the research study was D.B.T. Sugar Digestive tablets (Sharangdhar Pharmaceuticals Pvt. Ltd., Pune, India) and were purchased from the local market. Double distilled water was used in the present work. Analytical grade chemicals and reagents were used in the study and purchased from Merck Specialities Pvt. Ltd. (Mumbai, India). Precoated silica gel 60 F254 aluminium HPTLC plates were purchased from E. Merck, Darmstadt, Germany.
2.2. Instrumentation and Chromatographic Conditions. HPTLC plates were prewashed with methanol and activated at 120°C for 15 min prior to chromatographic analysis. The sample solutions were applied by spray-on technique on HPTLC plate in the form of bands of 6 mm width, located 8 mm from bottom and 15 mm apart with a Camag syringe (100 μL), under a continuous stream of nitrogen using a Camag Linomat V (Switzerland) sample applicator.

A constant application rate of 150 nL s⁻¹ was used during analysis. The slit dimension was kept at 5 mm × 0.45 mm with a scanning speed of 10 mm/s. HPTLC plates were then developed with 20 mL mobile phase consisting of toluene: ethyl acetate: methanol (6.5: 2.5: 1.4, v/v/v). Linear ascending development was used in 20 cm × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the solvent system. The optimized chamber saturation time for solvent system was 15 min at room temperature (25 ± 2 °C) and relative humidity of 60 ± 5%. Based on the literature survey [5, 10] several derivatizing agents have been examined such as modified vanillin-sulphuric acid reagent and anisaldehyde sulphuric acid, but 5% sulphuric acid reagent gave good and reproducible results with chromophore stability of 25 min and hence was selected for derivatization. It was prepared by adding 5 mL concentrated sulphuric acid in approximately 70 mL methanol in 100 mL volumetric flask with thorough mixing followed by final volume adjustment with methanol. The length of chromatographic run was 80 mm. After chromatography, plates were dried in an air current. Developed plates were dipped into 5% sulphuric acid reagent, heated at 110°C for 4 min in a preheated oven. Densitometric scanning was done in reflectance-absorbance mode at 423 nm within 25 min after derivatization using Camag TLC Scanner III with winCATS software (Version 1.4.4).

2.3. Preparation of Standard Stock Solutions. Standard stock solutions of gymnemagenin and β-sitosterol were prepared separately by dissolving 10 mg each in 10 mL methanol to get concentration of 1000 μg/mL. From this 1 mL of solution was further diluted to 10 mL with methanol to obtain a solution of 100 μg/mL. These standard stock solutions were used for further analyses.

2.4. Selection of Detection Wavelength. After HPTLC development and derivatization, bands were scanned over the range of 400–700 nm and the spectra were overlain. Both marker compounds showed considerable absorbance at range of 400–700 nm and the spectra were overlain. Both peak purity of gymnemagenin and β-sitosterol was assessed by comparing the visible spectra of markers at peak start, peak apex, and peak end positions of the band, that is, r (start, middle) and r (middle, end).

2.5. Preparation of Sample Solutions. It was observed that common method is not applicable for complete extraction of both gymnemagenin and β-sitosterol; hence sample preparation was performed separately.

2.5.1. Sample Preparation for Gymnemagenin. The published method [3] was slightly modified to obtain the optimum amount of gymnemagenin. For analysis of the marketed formulation, twenty tablets were accurately weighed and their average weight was determined. The tablets were finely powdered and tablet powder equivalent to ten tablets was refluxed for 2 h in 2 N methanolic HCl (50%, 50 mL) and filtered and the filtrate was added in ice cold double distilled water to obtain precipitate which was refluxed for 2 h in 50 mL of 2% methanolic KOH. After cooling, the mixture was diluted with double distilled water and extracted with ethyl acetate and the ethyl acetate layer was separated followed by drying over anhydrous sodium sulphate and evaporated. The obtained residue was reconstituted in 10 mL methanol.

2.5.2. Sample Preparation for β-Sitosterol. For analysis of β-sitosterol in tablet dosage form, powder equivalent to ten tablets was taken in 100 mL volumetric flask containing approximately 70 mL methanol and sonicated for 1 h to ensure complete extraction of β-sitosterol followed by final volume adjustment with methanol. This solution was filtered through Whatman number 1 filter paper and used for further analyses.

2.6. Assay Validation. The developed densitometric method was optimized and validated as per the International Conference on Harmonization guidelines (ICH) Q2 (R1) for linearity, range, LOD, LOQ, specificity, precision, and accuracy [17].

2.6.1. Linearity and Range. Linearity was evaluated by applying stock solutions on the HPTLC plate in the range of 100–1200 ng band⁻¹ and 200–1200 ng band⁻¹ for gymnemagenin and β-sitosterol, respectively. Peak area versus concentration was subjected to least square linear regression analysis. To prove linearity, residual analysis was also performed along with correlation coefficient (Figure 2). The study was conducted in six replicates.

2.6.2. Sensitivity. Sensitivity was determined by establishing the limit of detection (LOD) and limit of quantitation (LOQ). They were calculated as $3.3 \times S_\sigma /S$ and $10 \times S_\sigma /S$, respectively, where $S_\sigma$ is the standard deviation of residuals from line and S is the slope of the linearity plot.

2.6.3. Specificity. In specificity studies, gymnemagenin and β-sitosterol marker solutions and the marketed sample solutions were applied on an HPTLC plate and the plate was developed, derivatized, and scanned as described above. The peak purity of gymnemagenin and β-sitosterol was assessed by comparing the visible spectra of markers at peak start, peak apex, and peak end positions of the band, that is, r (start, middle) and r (middle, end).

2.6.4. Precision Studies. Precision was verified by intra- and interday variation studies. Both intra- and interday precision studies were conducted in six replicates. Gymnemagenin (200 ng band⁻¹) and β-sitosterol (300 ng band⁻¹) were prepared and analyzed on the same day in order to record any intraday variations in the results. For interday variation study, the above-mentioned drug sample concentration was
analyzed on three consecutive days. The % relative standard deviation was taken as a measure of precision.

2.6.5. Accuracy Studies. In accuracy studies, marketed formulation was spiked with known amount of standards (standard addition method). The samples were spiked with 80, 100, and 120% of 500 ng band\(^{-1}\) of both gymnemagenin and \(\beta\)-sitosterol standard solutions. The percent ratios between the recovered and expected concentrations were estimated in hexaplicate.

2.6.6. Robustness Studies. The effect of small, deliberate variation of the analytical conditions on the peak areas of the drugs was examined. Factors varied were mobile phase (toluene) composition (±0.1 mL), amount of mobile phase (±5%), time from band application to chromatographic development (+10 min), and time from chromatography to scanning (+15 min). One factor was varied at a time, to study the effect. The robustness of the HPTLC method was studied six times at a concentration of 200 ng band\(^{-1}\) for both gymnemagenin and \(\beta\)-sitosterol. The standard deviation of peak areas and % relative standard deviation (%RSD) were calculated for each variable factor.

2.6.7. Solution Stability. The stability of gymnemagenin and \(\beta\)-sitosterol standard solutions (600 ng band\(^{-1}\)) was tested after 0, 6, 12, 24, and 48 h of storage at room temperature. The stability of the solutions was determined by comparing peak areas at each time point against freshly prepared standard markers.

3. Results and Discussion

Many herbal formulations are effective, but they lack in standardization, and hence there is a need to develop suitable analytical methods for such formulations. In case of natural product analysis, HPTLC is more widely used than other chromatographic methods. In the present work, an attempt has been made to develop and validate new, fast, precise, accurate, and robust HPTLC method for concurrent quantification of gymnemagenin and \(\beta\)-sitosterol in the polyherbal formulation. Results obtained indicate the reliability of the proposed densitometric method.

3.1. Optimization of HPTLC Method. To obtain the desired \(R_f\) value range (0.2–0.8), minimum resolution (Rs ≥ 1.5), different solvent systems containing various ratios of toluene, dichloromethane, n-hexane, ethanol, methanol, water, ethyl acetate, and acetone were tried. Finally, the solvent system composed of toluene:ethyl acetate:methanol (6.5:2.5:1.4, v/v/v) was selected for obtaining well separated peaks. The wavelength used for detection and quantitation was 423 nm. The retention factors for gymnemagenin and \(\beta\)-sitosterol were found to be 0.27 ± 0.02 and 0.78 ± 0.02, respectively (Figure 1).

3.2. HPTLC Method Validation

3.2.1. Linearity and Range. Linear relationship was observed by plotting marker concentration against peak areas obtained. The results were found to be linear over a range of 100–1200 ng band\(^{-1}\) and 200–1200 ng band\(^{-1}\) for gymnemagenin and \(\beta\)-sitosterol, respectively (Table 1).

| Parameters                  | Gymnemagenin | \(\beta\)-Sitosterol |
|-----------------------------|--------------|----------------------|
| Linearity range (ng band\(^{-1}\)) | 100–1200     | 200–1200             |
| \(r^2\)                     | 0.999        | 0.998                |
| Slope                       | 3.617        | 4.528                |
| Intercept                   | 645.660      | 371.630              |
| Confidence limit of slope\(^a\) | 3.484–3.749  | 4.183–4.872          |
| Confidence limit of intercept\(^a\) | 547.738–743.582 | 121.295–621.958    |
| \(S_{y.x}\)\(^b\)          | 34.899       | 72.132               |

\(^a\)95% confidence limit. \(^b\)Standard deviation of residuals from line.
3.2.2. Sensitivity. The LOD and LOQ for gymnemagenin and β-sitosterol were found to be 31.84 and 52.56 ng band⁻¹ and 96.48 and 159.30 ng band⁻¹, respectively, indicating good sensitivity of the HPTLC method.

3.2.3. Specificity. The peak purity for gymnemagenin and β-sitosterol was assessed by comparing visible spectra acquired at the start (S), apex (M), and end (E) of the peak obtained from the scanning of band; that is, \( r(S, M) = 0.999, 0.998 \) and \( r(M, E) = 0.999, 0.998 \), respectively. Peak purity data showed that peaks obtained for gymnemagenin and β-sitosterol were pure.

3.2.4. Precision. As recommended by ICH guidelines, both intra- and interday precision studies showed RSD < 2%, indicating good precision (Table 2).

3.2.5. Accuracy. Recoveries of 101.26–102.11% for gymnemagenin and 99.88–102.51% for β-sitosterol indicate that the proposed simultaneous densitometric method is reliable for the estimation of gymnemagenin and β-sitosterol in the marketed herbal formulation used in the study (Table 3).

3.2.6. Robustness Studies. Robustness of the densitometric method was checked after deliberate alterations of the analytical parameters (Table 4). It showed that areas of peaks of interest remained unaffected by small changes of the operational parameters (%RSD < 2) which indicate robustness of the method.

3.2.7. Solution Stability. Stability of standard solution of gymnemagenin and β-sitosterol was evaluated at room temperature for 48 h. The percentage relative standard deviation

Table 2: Intra- and interday precision of the HPTLC method (n = 6).

| Marker compound | Concentration | Intra/interday concentration obtained | % RSD |
|-----------------|---------------|--------------------------------------|-------|
| Gymnemagenin    | 200           | 198.8/198.4                           | 0.25/0.28 |
| β-Sitosterol    | 300           | 300.6/299.3                           | 0.32/0.30 |

*Concentration in ng band⁻¹; RSD is the relative standard deviation.

Table 3: Results of recovery studies (n = 6).

| Drug           | Amount taken | Amount added | Amount recovered ± S.D | % Recovery ± % RSD |
|----------------|--------------|--------------|------------------------|-------------------|
| Gymnemagenin   | 500          | 400          | 405.24 ± 1.86          | 101.31 ± 0.458    |
|                | 500          | 500          | 505.85 ± 1.71          | 101.77 ± 0.338    |
|                | 500          | 600          | 612.66 ± 1.68          | 102.11 ± 0.274    |
| β-Sitosterol    | 500          | 400          | 410.04 ± 1.74          | 102.51 ± 0.424    |
|                | 500          | 500          | 499.4 ± 1.01           | 99.88 ± 0.202     |
|                | 500          | 600          | 609.42 ± 1.36          | 101.57 ± 0.223    |

*Concentration in ng band⁻¹; RSD is the relative standard deviation.
was found to be below 2.0% indicating that both standard and sample solutions were stable up to 48 h at room temperature.

### 3.2.8. Analysis of Marketed Polyherbal Formulation

Validity of the proposed HPTLC method was applied for standardization of herbal tablet dosage in six replicate determinations. The percent content of gymnemagenin and β-sitosterol in marketed herbal formulation was found to be 0.0405% and 0.1377%, respectively.

### Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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