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Research Article

VALIDATED HPTLC METHOD FOR SIMULTANEOUS QUANTIFICATION OF GYMNEMAGENIN AND GALLIC ACID IN HERBAL DOSAGE FORM

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ABSTRACT

The research work was carried out to develop and validate new, rapid, precise and robust high performance thin layer chromatographic method for concurrent quantitative determination of gymnemagenin and gallic acid in selected herbal formulation with densitometric detection. Separation was attained on Merck aluminium HPTLC plates precoated with silica gel 60 F_{254}. The solvent system which is optimaized contained toluene: ethyl acetate: methanol: acetic acid: formic acid (10.4: 4: 4: 0.4: 0.3, v/v/v/v). Developed plates were derivatized by 5% sulphuric acid reagent followed by heating at 110°C for 4 min in a preheated oven followed by scanning at 456 nm in reflectance-absorbance mode. The Rf (Retention factor) was found to be 0.58±0.02, for gymnemagenin and 0.41±0.02, for gallic acid. Results were found to be linear over a range of 200-1000 ng band^{-1} and 80-240 ng band^{-1} for gymnemagenin and gallic acid respectively. The proposed HPTLC method was validated according to ICH Q2 (R1) guideline. The proposed HPTLC method can be applied for quality-control testings to quantitative analysis of gymnemagenin and gallic acid simultaneously in selected marketed formulation.

Keywords: Gymnemagenin, Gallic acid, HPTLC, Simultaneous quantification, ICH.

1. INTRODUCTION

Gymnemic acid; a triterpenoid saponins isolated from plant *Gymnema sylvestre* is liable for its anti-diabetic activity [1]. A Gymnemic acid (Fig. 1) is a common aglycone of gymnemagenin, produced after serial acid and base hydrolysis [2].

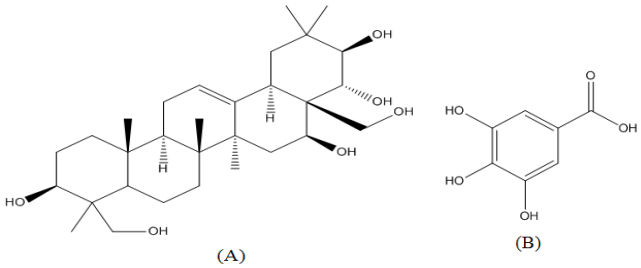

Fig. 1: Structures of (A) Gymnemagenin, (B) Gallic acid

Gallic acid is having cardio-protective, antioxidant activity astringent activity, and anti-inflammatory activity, chemically it is 3, 4, 5 trihydroxybenzoic acid [3, 4]. Literature survey showed that gymnemagenin was analyzed by various chromatographic techniques [5-11]. For estimation of gallic acid few HPTLC [12-16], HPLC [17-21] methods have been reported. No desitometric method available for the simultaneous estimation of these two markers in selected herbal formulation. Hence, the aim of the present research work was to develop and validate simple, precise, accurate densitometric method for the concurrent quantification of gymnemagenin and gallic acid in polyherbal formulation selected for the study.

2. EXPERIMENTAL

2.1. Material and Chemicals

All the chemicals used in the research work were of analytical grade and procured from Merck Specialities Private Limited (Mumbai, India) and used without further purification. The HPTLC silica gel 60 F_{254} plates were purchased from E. Merck (Darmstadt, Germany. Standard markers gymnemagenin and gallic acid were purchased from Natural Remedies, Bangalore, India and from Merck Specialities Private Limited, Mumbai, India, respectively. D.B.T. Tablet, the polyherbal

preparation used in the research was procured from the native market.

2.2. Instrumentation and Chromatographic Conditions

HPTLC was performed on 20 cm × 10 cm aluminum plates precoated with Silica gel 60 F_{254} (E. Merck, Darmstadt, Germany). Samples and standards were applied to the plates as 6 mm bands, 15 mm apart, 8 mm from the bottom and left edge of the plate by use of a CAMAG (Muttenz, Switzerland) Linomat V sample applicator equipped with a 100 µL Hamilton syringe. Plates were developed in mobile phase toluene: ethyl acetate: methanol: acetic acid: formic acid (10.4: 4: 4: 0.4: 0.3, v/v/v/v). Twin trough glass chamber (Camag Muttenz, Switzerland) 20 cm×10 cm equilibrated for 15 min with mobile phase used in linear ascending developmentat room temperature. The chromatographic run was 8 cm, then plate was subjected to air dry. In order to derivatize developed plate, it was dipped into 5% sulphuric acid reagent, heated in a preheated oven at 110°C for 4min. 5.00 mm was the slit dimension whereas width was set to 0.45 mm, a scanning rate of 10 mm/s were employed. Camag TLC scanner III was used for densitometric at 456nm, software used was win CATS version 1.4.4.

2.3. Preparation of Standard Stock Solutions

Standard stock solutions of gymnemagenin and gallic acid were prepared separately by dissolving 10mg each accurately weighed in 10mL methanol. From the above solution, 1mL of solution was further diluted to 10mL with methanol to obtain a solution of 100 μg /mL.

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 superimposed (Fig.2). As both marker compounds showed significant absorbance at 456nm, the wavelength was selected for further analysis.

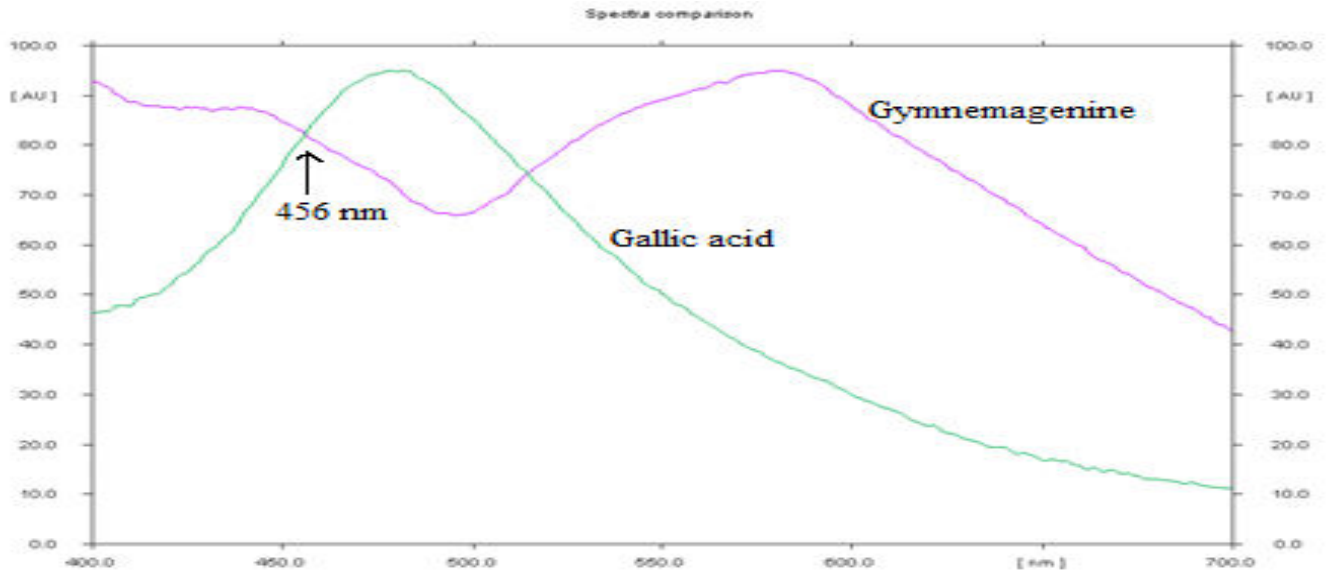

Fig. 2: Overlain visible spectra of gymnemagenin and gallic acid

2.5. Preparation of Sample Solutions

Sample preparation was carried out separately as common method is not useful for maximum extraction of both markers.

2.5.1. Sample Preparation of Gymnemagenin

Tablet polyherbal formulation was selected for the study and subsequently twenty tablets were precisely weighed and their average weight was calculated. Fine powder equivalent to ten tablets was subjected to reflux in 2N methanolic HCl (50%, 50mL). Reflux was carried out for 2 h and then filtered. The precipitate was obtained by adding filtrate to ice cold water then refluxed in 50mL of 2% methanolic KOH for 2 h. The cooled mixture was diluted with double distilled water then subjected to the extraction with ethylacetate. The separated ethyl acetate layer was dried over anhydrous sodium sulphate and then evaporated. The resulting residue was reconstituted with 10mL methanol.

2.5.2. Sample Preparation of gallic acid

Powder equivalent to 10 tablets was taken in volumetric flask having capacity of 100 mL. 70mL methanol was added and then ultrasonicated for 1 h for maximum extraction of markers followed by volume adjustment with methanol. Resulting filtered solution was diluted suitably and used for further analysis.

2.6. Assay Validation

Validation of the developed method was performed as per the International Conference on Harmonization guidelines ((ICH) Q2 (R1)) for following parameters [22].

2.6.1. Linearity and Range

The linearity of the method was assessed by the analysis of standard solutions of the gymnemagenin and gallic acid at six concentrations in the range of 200-1000 ng band^{-1}and 80-240 ng band^{-1}, respectively. The linearity was calculated by linear least-squares regression analysis for generation of calibration curve. The regression equation with intercept, slope, and coefficient of correlation was calculated. The study was conducted in six replicates (Fig. 2).

2.6.2. Sensitivity

Sensitivity of the method was checked by determining the LOD i.e. limit of detection and LOQ i.e. limit of quantitation. The LOD and LOQ were calculated through formula $3.3 \times Sy.x/S$ and $10 \times Sy.x/S$, respectively, where S is the slope of the linearity plot and $Sy.x$ is the standard deviation of residuals from line.

2.6.3. Specificity

In specificity studies, marker solutions of gymnemagenin and gallic acid and the marketed sample solutions were applied on an HPTLC plate and the plate was developed then derivatized, and scanned as described above. The peak purity (98%) of standard gymnemagenin and gallic acid was assessed by comparing the spectra at three points comprising peak start, peak apex, and peak end. Peak purity of the sample was determined by comparing overlaid spectra of the gymnemagenin and gallic acid in sample and standard chromatograms.

2.6.4. Precision Studies

Precision was confirmed by intra- and interday variation studies. The intra-assay precision of the developed methodology was evaluated by analysing hexa replicates at concentration of Gymnemagenin (800 ng band^{-1}) and gallic acid (160 ng band^{-1}) on the same day. Interassay precision was assessed in three different laboratory days at the same levels. The data generated was estimated and results stated as (%) RSD.

2.6.5. Accuracy Studies

The accuracy of the method was assessed by spiking preanalyzed samples with known amounts of standard gymnemagenin and gallic acid solution and then reanalysed by the HPTLC method. At three different concentration levels (80, 100, 120%), the spiking was done and average percent recovery at each concentration levels was calculated. Concentrations were estimated in hexaplicate.

2.6.6. Robustness Studies

The robustness of the optimized method was studied by introducing small deliberate changes in experimental conditions and results examined. Factors varied were amount of mobile phase (±5%), mobile phase (toluene) composition (±0.1mL), time from band application to development of chromatogram (+10 min), and time from chromatography to scanning (+15 min). Single factor was varied at a time, to study the effect. 200 ng band^{-1} concentration was used for both gymnemagenin and gallic acid for this study. The standard deviation of peak areas and % relative standard deviation were calculated for each variable factor.

2.6.7. Solution Stability

The stability of gymnemagenin and gallic acid standard solutions (200 ng band^{-1}) was tested at room temperature for 0, 6, 12, 24, and 48 h of storage. The stability of the solutions was determined by comparing peak areas at each time point against freshly prepared standard marker solutions.

3. RESULTS AND DISCUSSION

Several herbal formulations are effective in their use, but they lack in standardization process, so there is a necessity to develop suitable analytical methods for such formulations. HPTLC is more widely used than other chromatographic methods in case of natural product analysis. In the current work, an attempt has been made to develop and validate novel, fast, accurate, precise, and robust HPTLC method for concurrent quantification of gymnemagenin and gallic acid in the polyherbal formulation. Results achieved indicate the reliability of the proposed HPTLC method.

3.1. Optimization of HPTLC Method

To obtain the desired Rf value range (0.2-0.8), minimum resolution (Rs≥ 1.5), various solvent systems containing various ratios of n-hexane, ethanol, methanol, toluene, dichloromethane, ethylacetate, water and acetone were tried. Finally, the solvent system composed of toluene: ethyl acetate: methanol: acetic acid: formic acid (10.4: 4: 4: 0.4: 0.3, v/v/v/v) was selected for attaining well separated peaks. The wavelength used for detection and quantitation was 456 nm. The retention factors for gymnemagenin was found to be 0.58±0.02 and for gallic acid 0.41±0.02 (Fig. 3).

3.2. HPTLC Method Validation

3.2.1. Linearity and Range

By plotting marker concentration against peak areas obtained, linear relationship was observed. The results were observed to be linear over a range of 200-1000 ngband^{-1} for gymnemagenin and 80-240 ng band^{-1} for gallic acid (Table 1).

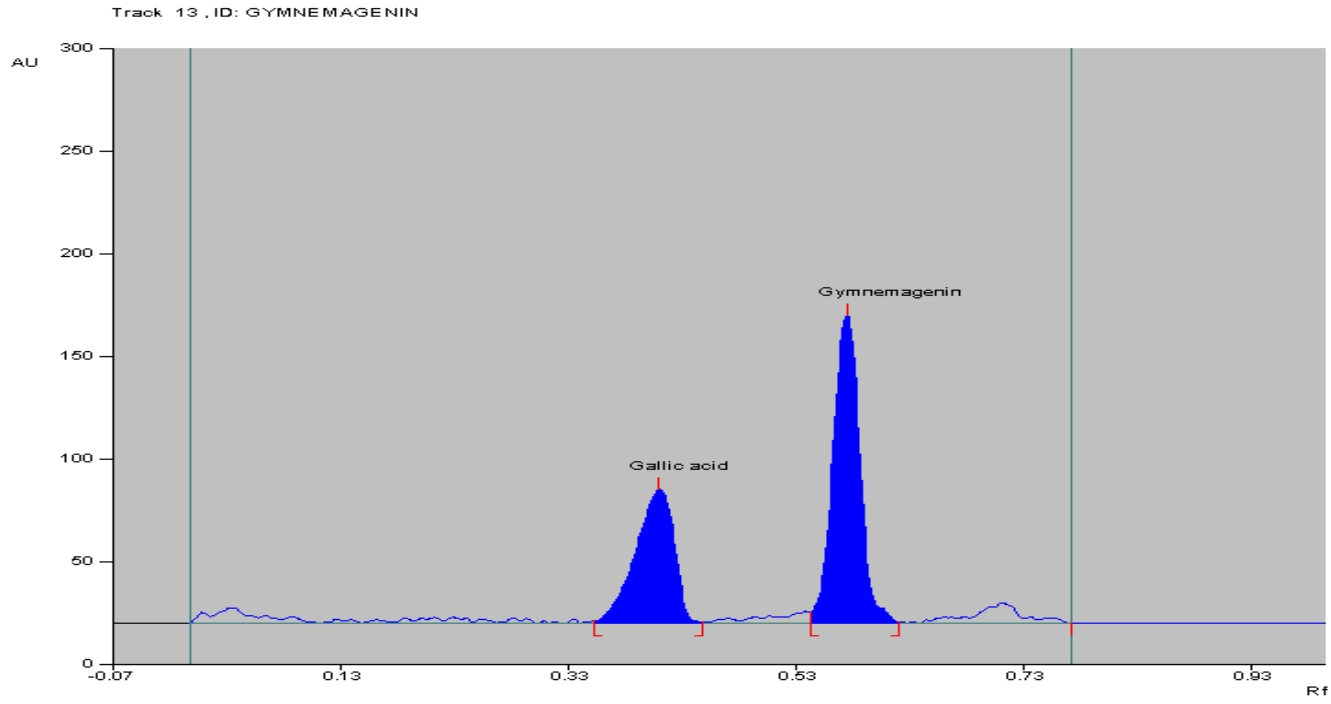

Fig. 3: Densitogram obtained from mixed standard solution of gymnemagenin and gallic acid scanned at 456 nm

Table 1: Data of calibration curve

Parameters	Gymnemagenin	Gallic acid
Linearity	200-1000 ng band^{-1}	80-240 ng band^{-1}
R^2	0.999	0.999
slope	2.44	11.46
Intercept	739.9	-377.6
Confidence limit of scope	2.304-2.592	10.85-12.07
Confidence limit of intercept	644.5-835.3	-481.40- -273.85
S_{yx}^2	24.74	21.04

(n=6)

3.2.2. Sensitivity

The LOD and LOQ for gymnemagenin and gallic acid were found to be 33.35 and 6.05 ng band^{-1} and 101.08 and 18.35 ng band^{-1}, respectively, representing good sensitivity of the HPTLC method (Table 2).

Table 2: LOD and LOQ of Gymnemagenin and gallic acid

Parameter	Gymnemagenin	Gallic acid
LOD	33.35 ng band^{-1}	6.05 ng band^{-1}
LOQ	101.08 ng band^{-1}	18.35 ng band^{-1}

3.2.3. Specificity

The peak purity for gymnemagenin and gallic acid was assessed by comparing visible spectra acquired at the start, apex and end positions showing r(S,M) = 0.999, 0.998 and r(M, E) = 0.999, 0.998, respectively. Peak purity data indicated that peaks obtained for gymnemagenin and gallic acid were pure.

3.2.4. Precision

Both intra- and interday precision studies showed RSD less than 2% indicating good precision of the method (Table 3).

3.2.5. Accuracy

Recoveries of 99.55-100.72% for gymnemagenin and 99.16-101.65% for gallic acid indicate that the planned simultaneous HPTLC method is found to be reliable for analysis of selected herbal dosage form. (Table 4).

3.2.6. Robustness Studies

Robustness was checked after small intentionally done variations in the analytical method parameters. There is no major change in the peak areas due to the modifications in the operational conditions (%RSD < 2) which indicate robustness of the method (Table 5).

Table 3: Precision Study

Marker compound	Intraday -interday		
	Concentration	Percent obtained	% RSD
Gymnemagenin	800 ng band^{-1}	99.06/98.74	0.62/0.72
Gallic acid	160 ng band^{-1}	98.52/99.34	0.60/0.88

(n=6)

Table 4: Recovery Study

Drug	Amount taken	Amount added	Total Amount	Amount found	% recovery ± % RSD
Gymnemagenin	400	320	720	722.6	100.36±0.24
	400	400	800	805.8	100.72±0.18
	400	480	880	876.1	99.55±0.22
Gallic acid	100	80	180	178.5	99.16±0.14
	100	100	200	203.3	101.65±0.31
	100	120	220	222.1	100.95 ±0.26

(n=3)

Table 5: Robustness study

Parameter	Mean peak area ± SD		% RSD	
	Gymnemagenin	Gallic acid	Gymnemagenin	Gallic acid
Mobile phase composition±0.1mL	2744.4±4.3	1450.5±8.3	0.65	0.48
Amount of mobile phase±5%	2739.8±6.2	1438.9±6.4	0.48	0.75
Time from band application to chromatography+10min	2753.6±3.7	1447.2±4.8	0.82	0.62
Time from chromatography to scanning + 10min	2748.2±5.1	1454.6±3.9	0.34	0.46

(n=6)

3.2.7. Solution Stability

Solution Stability of standard solution of gymnemagenin and gallic acid was evaluated at room temperature for 48h. The %RSD was found to be below 2.0% which indicates that both standard and sample solution were stable at room temperature upto 48h.

3.3. Analysis of marketed herbal Formulation

Analysis was done on marketed herbal dosage form to ensure the method validity. Six replicate determinations were performed. The percent content was found to be 0.0425% and 0.186% for gymnemagenin and gallic acid, respectively in marketed herbal formulation (Table 6).

Table 6: Analysis of marketed herbal formulation

Name of the formulation	Content (%)	
	Gymnemagenin	Gallic acid
D.B.T. Sugar Digestive Tablets	0.0425	0.186

4. CONCLUSION

Results of analysis indicate the reliability of the proposed HPTLC method. Both intra- and interday precision studies showed RSD less than 2% indicating good precision. Both standard and sample solutions were stable at room temperatureup to 48h as the % RSD was found to be below 2.0%. Peak purity data indicated that peaks obtained for gymnemagenin and gallic acid were pure. Hence, in the present research work, attempt has been made to develop and validate new, precise, accurate, and robust HPTLC of gymnemagenin and gallic acid for simultaneous quantification in the selected herbal dosage form formulation.

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Conflict of interest

Authors declare that we have no conflicting interest in this research.

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