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Original Article

A VALIDATED ISOCRATIC RP-HPLC METHOD FOR CONCURRENT ESTIMATION OF GYMNEMAGENIN, GALLIC ACID AND 18B-GLYCYRRHETINIC ACID IN POLYHERBAL FORMULATION

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ABSTRACT

Objective: To develop and validate a simple, precise, selective, and accurate reversed phase high performance liquid chromatography method for concurrent analysis of gymnemagenin, gallic acid and 18β-glycyrrhetinic acid in polyherbal formulation.

Methods: The chromatographic separation was achieved on a Thermo Synchronis C_{18}, 5 µm, 250 × 4.6 mm i. d. analytical column. The mobile phase comprised of methanol: water (88: 12, v/v), pH 3.1 adjusted with orthophosphoric acid. The flow rate was kept at 0.8 mL min^{-1}. Quantitation was achieved with UV detection at 218 nm, based on peak area.

Results: The retention time for gallic acid, gymnemagenin, and 18β-glycyrrhetinic acid was found to be 3.08, 4.15, and 10.30 min, respectively. Validation of the RP-HPLC method was performed as per International Conference on Harmonization (ICH) Q2 (R1) guideline. The proposed method showed good linearity in the range of 100-1000 µg mL^{-1} for gymnemagenin, 2.5-50 µg mL^{-1} for gallic acid and 50-500 µg mL^{-1} for 18β-glycyrrhetinic acid. The % content of gymnemagenin, gallic acid and 18β-glycyrrhetinic acid in the marketed formulation was found to be 0.1320, 0.2129 and 0.2799 %, respectively.

Conclusion: The proposed method can be useful in the quality control of gymnemagenin, gallic acid and 18β-glycyrrhetinic acid in polyherbal formulation.

Keywords: Gymnemagenin, Gallic acid, 18β-glycyrrhetinic acid, Isocratic HPLC, ICH.

INTRODUCTION

Gymnemic acid belongs to triterpenoid saponins class and is isolated from *Gymnema sylvestre* which is responsible for its anti-diabetic activity [1]. A common aglycone of gymnemic acids is gymnemagenin (Figure 1), produced after sequential acid and base hydrolysis [2]. Gymnemagenin is 3β, 16β, 21β, 22α, 23, 28-hexahydroxy-olean-12-ene [3]. Gallic acid is 3, 4, 5 trihydroxy benzoic acid and possess astringent activity, anti-inflammatory, cardio-protective, antioxidant activity and are proven to show beneficial effects on human health [4, 5]. Chemically, 18β-glycyrrhetinic acid (Figure 1) is 3 β-Hydroxy-11-oxo-12-oleanen-30-oic acid, an aglycone portion of glycyrrhizin which is responsible for antihyperglycemic action on streptozotocin induced diabetic rats [6]. Literature survey showed that gymnemagenin was analyzed by HPLC [2], HPTLC [7-12] and HPLC–ESI–MS/MS [13] methods. Few HPTLC [14-18], HPLC [19-22] and HPLC/DAD/ESI-MS [23] methods have been reported for estimation of gallic acid. 18β-Glycyrrhetinic acid was analyzed individually and in combination with other marker compounds by some HPLC [24-26]and HPTLC [27-31]methods. No reports were found for simultaneous quantification of gymnemagenin, gallic acid and 18β-glycyrrhetinic acid by HPLC method. Hence the objective of the research work was to develop and validate simple, precise, robust and accurate RP-HPLC method for the concurrent quantification of gymnemagenin, gallic acid and 18β-glycyrrhetinic acid in polyherbal formulation.

Experimental

Solvents and chemicals

Standard marker gymnemagenin, 18β-glycyrrhetinic acid was purchased from Natural Remedies, Bangalore, India and gallic acid from Merck Specialities Private Limited, Mumbai, India. Polyherbal formulation (Madhuveer Liquid) used in the study was purchased from the local market. HPLC grade reagents and chemicals were used in the study and purchased from Merck Specialities Private Limited, Mumbai, India. Double distilled water filtered through 0.45 µ filter paper was used in the research work.

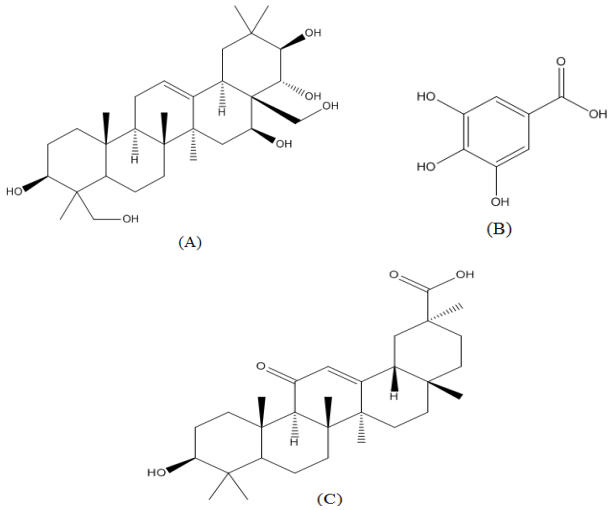


Fig. 1: Chemical structures of (A) Gymnemagenin, (B) Gallic acid, and (C) 18β-Glycyrrhetinic acid

RP-HPLC Instrumentation and chromatographic conditions

The HPLC system (Jasco corporation, Tokyo, Japan) consisting of Jasco PU-2080 plus and PU-2087 plus intelligent pump along with manual injector (20 µL loop capacity) and UV- 2075 plus UV/VIS detector. ChromNAV control center 1.08.03 (Build 4) version software was used during the study. The chromatographic separation was achieved on Thermo Synchronis C_{18} analytical column (250×4.6 mm i. d., 5 µm) at 218 nm wavelength. The mobile phase comprised of methanol: water (88:12, v/v), pH 3.1, adjusted

with orthophosphoric acid. The flow rate was set to 0.8 mL min^{-1}. The ultrasonicator used in the study was Toshcon SW-4.5. All materials were weighed on Mettler Toledo A B207-5 balance. The volumetric glasswares of 'A' grade were used throughout the study.

Preparation of standard stock solutions

Standard stock solutions of markers were prepared separately by dissolving 10 mg of each marker in 10 mL methanol to get concentration of 1000 µg $mL^{-1}$and used for further analysis.

Selection of detection wavelength

To obtain UV spectrum, 5 µL solution (in triplicate) of all phytoconstituents were applied on HPTLC plate and subjected to densitometric scanning over a range of 200-400 nm. Densitometric spectra obtained were overlain which showed that all phytoconstituents have reasonable absorption at 218 nm. Hence it was selected as the detection wavelength (Figure 2) for analysis.

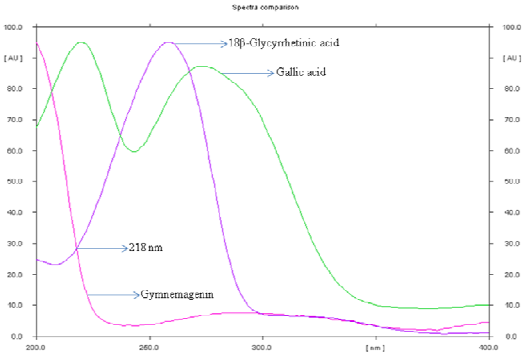


Fig. 2: Overlain UV spectrum of gymnemagenin, gallic acid and 18β-glycyrrhetinic acid

Construction of calibration plots

For preparation of calibration plots, standard solution of gymnemagenin (1000 µg mL^{-1}) was suitably diluted separately to obtain concentrations of 100, 200, 400, 600, 800, 1000 µg mL^{-1}. Gallic acid (1000 µg mL^{-1}) was diluted separately to obtain concentrations of 2.5, 5, 10, 20, 40, 50 µg $mL^{-1}$and 18β-glycyrrhetinic acid (1000 µg mL^{-1}) was diluted separately to obtain concentrations of 50, 100, 200, 300, 400, 500 µg mL^{-1}. Peak area versus concentration of the drug was plotted to obtain calibration plot. Linearity was evaluated in the range of 100-1000 µg mL^{-1} for gymnemagenin, 2.5-50 µg mL^{-1} for gallic acid and 50-500 µg mL^{-1} for 18β-glycyrrhetinic acid.

Preparation of analytical samples

It was found that single method is not applicable for complete extraction of all these markers. Hence sample preparation for gymnemagenin, gallic acid and 18β-glycyrrhetinic acid was performed, separately.

Sample preparation for gymnemagenin

Reported method [7] was slightly modified to obtain the optimum amount of gymnemagenin. For analysis of the marketed formulation, 100 mL liquid formulation was refluxed for 2 h in 2 N methanolic HCl (50 %, 100 mL), 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 10 mL methanol and used with suitable dilutions for further analysis.

Sample preparation for gallic acid

For analysis of gallic acid in liquid formulation, 10 mL liquid was taken in 100 mL volumetric flask containing approximately 70 mL methanol and ultrasonicated for 1 h to ensure complete extraction of drug followed by final volume adjustment with methanol. Resulting solution was filtered through Whatman filter paper no. 1 and used with suitable dilutions for further analysis.

Sample preparation for 18β-glycyrrhetinic acid

The published method[27] was slightly modified to obtain the optimum quantity of 18β-glycyrrhetinic acid. Since glycyrrhetinic acid is present in bound form in the drug, the drug was subjected to acid hydrolysis. For analysis of the liquid formulation, 10 mL liquid formulation was hydrolyzed with 2N aqueous hydrochloric acid (100 mL) under reflux for 2 h.

The hydrolyzed extract was filtered through Whatman filter paper no. 1 and the marc was washed with minimum amount of double distilled water (~10 mL) and filtered. The combined filtrates were pooled together in a separating funnel and extracted with chloroform (3×50 mL). The combined $CHCl_3$ extracts were dried over anhydrous sodium sulphate, concentrated and the volume was made up to 10 mL with methanol.

Assay validation

The proposed RP-HPLC-UV method was optimized and validated as per the International Conference on Harmonization [(ICH) Q2 (R1)] recommendations for accuracy, precision, linearity, robustness, and system suitability [32].

Linearity and Range

Linearity was performed by injecting stock solutions in the range of 100-1000 µg mL^{-1} for gymnemagenin, 2.5-50 µg mL^{-1} for gallic acid and 50-500 µg mL^{-1} for 18β-glycyrrhetinic acid. Peak areas obtained were processed and calibration curves were generated by Microsoft Excel software. To prove linearity, residual analysis was also performed along with correlation coefficient. Each standard solution of six different concentrations was injected in six replicates and chromatographed using the chromatographic conditions mentioned above.

Sensitivity

Sensitivity of the proposed RP-HPLC method was illustrated by determination of the limit of detection (LOD) and limit of quantitation (LOQ). As per ICH recommendations, the standard deviation of the response and the slope of the calibration plots were used to determine detection and quantification limits.

Specificity

The specificity of the proposed RP-HPLC method was estimated by analyzing the standard marker and sample. Peaks for gymnemagenin, gallic acid and 18β-glycyrrhetinic acid were confirmed by comparing the retention time. Excipients present in the herbal formulation did not interfere with the peaks of gymnemagenin, gallic acid and 18β-glycyrrhetinic acid.

Precision studies

In order to judge the quality of the proposed HPLC method, precision was determined. The precision of the proposed HPLC method was verified by intra-day and inter-day precision studies. Intra-day precision was performed by analysis of single concentration in six replicates of mixed standard solutions of gymnemagenin (200 µg mL^{-1}), gallic acid (10 µg mL^{-1}) and 18β-glycyrrhetinic acid (200 µg mL^{-1}) which were prepared on the same day. Intermediate precision was performed by repeating analysis on three consecutive days. The peak areas were recorded and percentage relative standard deviation (% RSD) was calculated.

Accuracy studies

Accuracy studies were carried out to study the suitability and reliability of the proposed method. Accuracy studies were carried out in triplicate by standard addition method. Accuracy was determined through the percentage recoveries of known amounts of mixture of gymnemagenin, gallic acid and 18β-glycyrrhetinic acid added to solutions of marketed polyherbal formulation.

The samples were spiked with 80, 100 and 120 % of gymnemagenin (200 µg mL^{-1}), gallic acid (10 µg mL^{-1}) and 18β-glycyrrhetinic acid (100 µg mL^{-1}) standard solutions. The percent ratios between the recovered and expected concentrations were estimated.

Robustness studies

The effects of small, deliberate variation of the analytical conditions on the peak areas of the drugs were examined. The robustness of the proposed chromatographic method was performed at a concentration of 200 µg mL^{-1} for gymnemagenin, 10 µg mL^{-1} for gallic acid and 200 µg mL^{-1} for 18β-glycyrrhetinic acid. The standard deviation of peak areas and % RSD were calculated for each variable parameter.

Analytical solution stability

The stability of gymnemagenin (200 µg mL^{-1}), gallic acid (10 µg mL^{-1}) and 18β-glycyrrhetinic acid standard solutions (200 µg mL^{-1}) was performed after 0, 6, 12, 24 and 48 h of storage at room temperature. Solution stability was determined by comparing peak areas at each time point against freshly prepared solutions of standard markers.

System suitability

System suitability is essential for the assurance of the quality performance of the HPLC system. It was studied by taking the % RSD of retention time, resolution, peak asymmetry and theoretical plates of the five injections of gymnemagenin, gallic acid and 18β-glycyrrhetinic acid using developed method.

RESULTS AND DISCUSSION

HPLC method optimization

During the optimization of the proposed RP-HPLC method, different HPLC columns, mobile phases of various compositions of acetonitrile, water, methanol, potassium dihydrogen phosphate, sodium dihydrogen phosphate buffer with different molarities and different pH were tried. Finally the mobile phase consisting of methanol: water (88: 12, v/v), pH 3.1, adjusted with orthophosphoric acid was selected as it gave well resolved peaks. The column used was Thermo Synchronis C_{18} analytical column (250×4.6 mm i. d., 5 µm) and a flow rate of 0.8 mL min^{-1}. The optimum wavelength for detection and quantitation used was 218 nm. Average retention time for gallic acid, gymnemagenin, and 18β-glycyrrhetinic acid were found to be 3.08, 4.15 and 10.30 min, respectively (Figure 3).

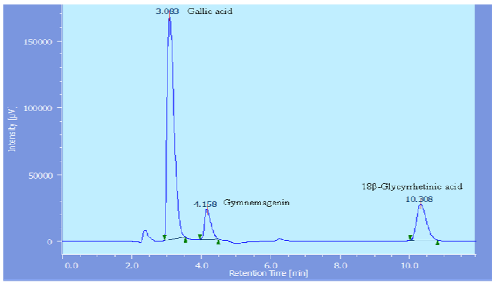


Fig. 3: Representative chromatogram obtained from a mixed standard solution of gymnemagenin, gallic acid and 18β-glycyrrhetinic acid

HPLC method validation

Linearity and Range

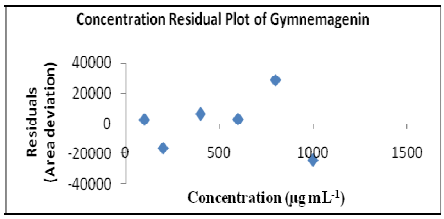
The results were found to be linear (Table 1) in the range of 100-1000 µg mL^{-1} for gymnemagenin, 2.5-50 µg mL^{-1} for gallic acid and 50-500 µg mL^{-1} for 18β-glycyrrhetinic acid.

To ascertain linearity, residual analysis was performed (Figure 4). Slope was significantly different from zero. Residual analysis (the differences between the measured and the calculated values) is the non-numerical test [33, 34]. Only a residual plot without any tendency proves the linearity of the calibration [35, 36].

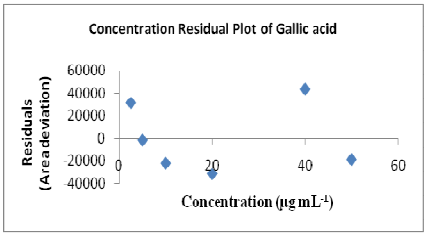
Table 1: Linear regression data for the calibration curves (n = 6).

Parameters	Gymnemagenin	Gallic acid	18β-Glycyrrhetinic acid
Linearity range (µg mL^{-1})	100-1000	2.5-50	50-500
r^2	0.999	0.999	0.999
Slope	2662	150150	10905
Intercept	16659	165050	-64828
95 % Confidence limit of slope	2588.553-2737.277	147964.499-152336.813	10549.005-11260.943
95 % Confidence limit of intercept	-28470.958-61789.906	104312.344-225786.764	-172847.221- 43191.939
Sy.x[a]	18684.250	30874.490	44720.709

n = Number of determinations; r = Coefficient of correlation; [a]Standard deviation of residuals from line.



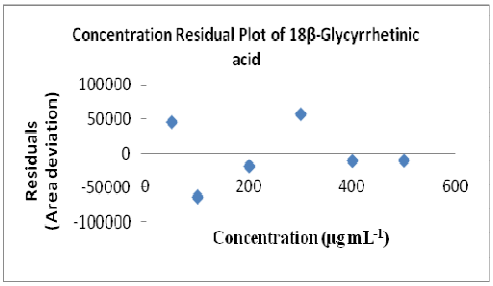
(A)



(B)

Sensitivity

The LOD and LOQ for gymnemagenin, gallic acid and 18β-glycyrrhetinic acid were found to be 23.15, 0.67, 13.53 µg mL^{-1} and 70.16, 2.05, 41.00 µg mL^{-1}, respectively, indicating good sensitivity of the proposed HPLC method.



(C)

Fig. 4: Concentration Versus Residual Plot of (A) Gymnemagenin (B) Gallic acid and (C) 18β-glycyrrhetinic acid.

Table 2: Intra and inter day precision of the HPLC method (n=6)

Marker compound	Actual concentration [a]	Intra/Inter day	
		Concentration obtained [a]	% RSD
Gymnemagenin	200	198.3/198.5	0.92/1.08
Gallic acid	10	9.86/9.91	1.02/0.99
18β-Glycyrrhetinic acid	200	197.3/197.9	1.18/1.24

[a] μg mL^{-1}; RSD = Relative standard deviation

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

Drug	Amount taken[a]	Amount added[a]	Amount found[a]± SD	% Recovery ± % RSD
Gymnemagenin	200	160	356.1 ± 3.29	98.92 ± 0.92
	200	200	392.7 ± 4.01	98.17 ± 1.02
	200	240	434.8 ± 4.14	98.82 ± 0.95
Gallic acid	10	08	17.7 ± 0.21	98.63 ± 1.21
	10	10	19.7 ± 0.22	98.76 ± 1.15
	10	12	22.1 ± 0.23	100.53 ± 1.07
18β-Glycyrrhetinic acid	100	80	177.6 ± 2.07	98.68 ± 1.17
	100	100	199.8 ± 2.11	99.94 ± 1.05
	100	120	216.5 ± 2.14	98.41 ± 0.99

n = Number of determinations; [a] μg mL^{-1}; SD = Standard deviation; RSD = Relative standard deviation

Specificity

It was found that, the base line did not show any significant noise and there were no other interfering peaks around the retention time of gymnemagenin, gallic acid and 18β-glycyrrhetinic acid, indicating specificity of the proposed chromatographic method.

Precision

The developed RP-HPLC method was found to be precise (Table 2), with % RSD values for repeatability and intermediate precision studies below 2 % as recommended by ICH Q2 (R1) guideline.

Accuracy

Satisfactory recoveries for gymnemagenin, gallic acid and 18β-glycyrrhetinic acid were obtained (Table 3), which indicate that the proposed chromatographic method is reliable for the simultaneous quantification of selected markers in this herbal formulation.

Analysis of marketed herbal formulation

Validity of the proposed RP-HPLC-UV method was applied to standardization of herbal dosage form in six replicate determinations. The percent content of gymnemagenin, gallic acid and 18β-glycyrrhetinic acid in marketed herbal formulation was found to be 0.1320, 0.2129 and 0.2799 %, respectively.

Robustness studies

As shown in Table 4, peak areas of the selected phytoconstituents remained unaffected (% RSD < 2), indicating robustness of the RP-HPLC method.

Analytical solution Stability

Solution stability of gymnemagenin, gallic acid and 18β-glycyrrhetinic acid was estimated at room temperature for 48 h. Low percentage relative standard deviation (below 2.0 %), indicated that both standard and sample solution was stable up to 48 h at room temperature.

System suitability

Higher number of theoretical plates (≥ 2000), peak symmetry (≤ 2), high resolution between the peaks (≥ 2.0), and proper retention time indicated suitability of the proposed HPLC method for quantification of gymnemagenin, gallic acid and 18β-glycyrrhetinic acid (Table 5).

Table 4: Robustness study of gymnemagenin, gallic acid and 18β-glycyrrhetinic acid (n = 6, 200 μg $mL^{-1}$for both gymnemagenin and 18β-glycyrrhetinic acid, 10 μg $mL^{-1}$for gallic acid)

Parameter varied	Mean peak area ± SD			% RSD		
	Gymnemagenin	Gallic acid	18β-Glycyrrhetinic acid	Gymnemagenin	Gallic acid	18β-Glycyrrhetinic acid
Mobile phase (Methanol) composition (± 1%)	535921.3 ± 7454.96	1679256 ± 24181.67	2080372 ± 28105.46	1.39	1.44	1.35
Buffer pH (± 0.1)	534592 ± 5665.95	1651777 ± 20410.16	2102480 ± 25198.43	1.05	1.23	1.19
Elution flow rate (± 0.1 mL min^{-1})	533178.3 ± 6665.53	1648889 ± 17594.19	2086502 ± 25865.17	1.25	1.06	1.23
Detection wavelength (± 2 nm)	534417 ± 6084.84	1662451 ± 20063.34	2109117 ± 22993.62	1.13	1.20	1.09

n = Number of determinations; SD = Standard deviation; RSD = Relative standard deviation

Table 5: System suitability parameters of chromatogram for gallic acid, gymnemagenin and 18β-glycyrrhetinic acid

Parameters	Proposed HPLC method					
	Gallic acid	% RSD	Gymnemagenin	% RSD	18β-Glycyrrhetinic acid	% RSD
Retention time (min)	3.08	0.93	4.15	0.68	10.30	1.22
Peak asymmetry	1.41	0.90	1.43	0.83	1.28	0.88
Theoretical plates	2144	0.80	2536	0.71	6388	0.90
Resolution ± % RSD	3.09 ± 0.40		14.55 ± 0.54			

RSD = Relative standard deviation

CONCLUSION

The validated HPLC method employed proved to be simple, rapid, precise, accurate, robust and thus can be intended for routine analysis of gymnemagenin, gallic acid and 18β-glycyrrhetinic acid in the herbal formulation used in the study.

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CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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