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Research Article

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Simultaneous quantification of gymnemagenin and 18β-glycyrrhetinic acid in herbal drug formulation by validated RP-HPLC method

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ABSTRACT

A new, rapid, accurate, and precise reverse phase high performance liquid chromatographic (RP-HPLC) method for simultaneous quantification of gymnemagenin and 18β-glycyrrhetinic acid in herbal drug formulation has been developed and validated. To obtain gymnemagenin, polyherbal tablet formulation was subjected to acid hydrolysis followed by base hydrolysis and extraction with ethyl acetate. 18β-Glycyrrhetinic acid was obtained after acid hydrolysis of polyherbal formulation followed by extraction with chloroform. 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 comprising of methanol: water, pH 2.8, adjusted with orthophosphoric acid (92:08, v/v). The flow rate was set to 0.8 mLmin^{-1}. The retention time for gymnemagenin and 18β-glycyrrhetinic acid was found to be 3.82 and 7.15 min, respectively. Validation of the HPLC method was carried out as per International Conference on Harmonization (ICH) Q2 (R1) guidelines. The calibration curve was found to be linear over a range of 50 - 1000 µgmL^{-1} for gymnemagenin and 50 - 500 µgmL^{-1} for 18β-glycyrrhetinic acid. The method has been applied for the analysis of marketed formulation. The content of gymnemagenin and 18β-glycyrrhetinic acid was found to be 0.2040 and 0.7431 %, respectively.

Keywords: Gymnemagenin, 18β-glycyrrhetinic acid, RP - HPLC, simultaneous quantification, ICH.

INTRODUCTION

Gymnemic acid belongs to triterpenoid saponins 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]. 18β-Glycyrrhetinic acid (Figure 1) is an aglycone portion of glycyrrhizin responsible for antihyperglycemic action on streptozotocin induced diabetic rats [4]. Literature survey showed that gymnemagenin was analyzed by HPLC [2], HPTLC [5-10] and HPLC–ESI–MS/MS [11] methods. 18β-Glycyrrhetinic acid was estimated individually and in combination with other marker compounds by some HPLC [12, 13] and HPTLC [14-18] methods. No reports were found for simultaneous quantification of gymnemagenin and 18β-glycyrrhetinic acid by HPLC method.

The nature of the sample/compound, solubility and its molecular weight decides the proper selection of the stationary phase [19]. High performance liquid chromatography is one of the most important techniques used in the pharmaceutical industry. [20-22]

Hence the objective of the research work undertaken was to develop and validate simple, robust, precise and accurate HPLC method for the simultaneous quantification of gymnemagenin and 18β-glycyrrhetinic acid in polyherbal formulation used in the study.

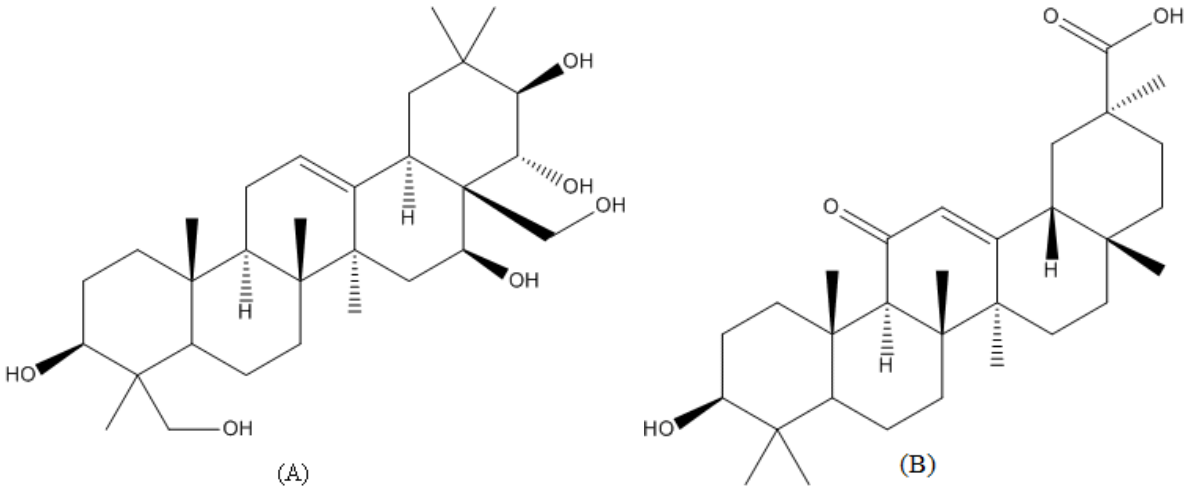


Figure 1. Chemical structures of (A) Gymnemagenin and (B) 18β-glycyrrhetinic acid

EXPERIMENTAL SECTION

Solvents and chemicals:

Standard marker gymnemagenin and 18β-glycyrrhetinic acid were purchased from Natural Remedies, Bangalore, India. Polyherbal formulation used in the study was Diabecon DS tablets, were purchased from the local market. All reagents and chemicals used in the study were of HPLC grade and purchased from Merck Specialities Pvt. Ltd. (Mumbai, India). Double distilled water filtered through 0.45 µ filter paper was used in the work.

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 per injection) and UV - 2075 plus UV/VIS detector. ChromNAV control center 1.08.03 (Build 4) version software was used for analysis. The chromatographic analysis was carried out on Thermo Synchronis C_{18} analytical column (250 × 4.6 mm i.d., 5 µm) at a 218 nm wavelength. The mobile phase composition was methanol: water, pH 2.8, adjusted with orthophosphoric acid (92:08, v/v) with 0.8 $mLmin^{-1}$ flow rate. The ultrasonicator used in the study was Toshcon SW - 4.5. All weighings were performed on Mettler Toledo A B207 – 5 balance. The volumetric glasswares of 'A' grade were used throughout the research work.

Preparation of standard stock solutions:

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

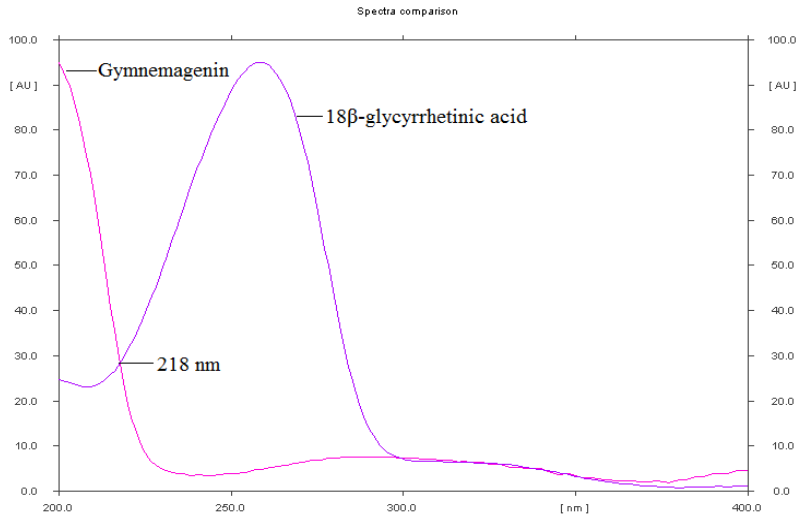


Figure 2: Overlain UV spectrum of Gymnemagenin and 18β-Glycyrrhetinic acid

Selection of detection wavelength:
To obtain UV spectrum, 5 µL solution (in triplicate) of both markers 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 both markers have reasonable absorption at 218 nm. Hence it was selected as the detection wavelength (Figure 2).

Construction of calibration plots:
Linearity was evaluated in the range of 50 - 1000 µgmL^{-1} for gymnemagenin and 50 - 500 µgmL^{-1} for 18β-glycyrrhetinic acid. For preparation of calibration plots, standard solution of gymnemagenin (1000 µgmL^{-1}) was suitably diluted separately to obtain concentrations of 50, 200, 400, 600, 1000 µgmL^{-1} and 18β-glycyrrhetinic acid (1000 µgmL^{-1}) was diluted separately to obtain concentrations of 50, 100, 200, 400, 500 µgmL^{-1}. Peak area versus concentration of the drug was plotted to obtain calibration plot.

Preparation of sample solutions:
It was found that for complete hydrolysis of glycosides to yield these two biomarkers, single method is not applicable, hence sample preparation for gymnemagenin and 18β-glycyrrhetinic acid was performed separately.

For gymnemagenin:
Several trials were taken to obtain free form of gymnemagenin by hydrolysis of marketed formulation. Reported method [5] was slightly modified to obtain the optimum amount of gymnemagenin. For analysis of the marketed formulation, twenty tablets were weighed and their average weight was estimated. The tablets were finely powdered and powder equivalent to ten tablets 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 10 mL methanol.

For 18β-glycyrrhetinic acid:
Published method [14] was modified to obtain the optimum amount of 18β-glycyrrhetinic acid. Marketed formulation was subjected to acid hydrolysis to obtain free form of 18β-glycyrrhetinic acid. Twenty tablets were weighed, their average weight was calculated. The tablets were powdered and powder equivalent to five tablets was hydrolyzed with 2N aqueous hydrochloric acid (100 mL) under reflux 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 chloroform extracts were dried (anhydrous sodium sulphate), concentrated, and the volume was made up to 25 mL with methanol.

Assay validation:
The proposed RP-HPLC method was optimized and validated as per the International Conference on Harmonization guidelines [(ICH) Q2 (R1)] for accuracy, precision, linearity, robustness, and system suitability [23].

Linearity and Range:
Linearity was performed by injecting stock solutions in the range of 50 - 1000 µgmL^{-1} for gymnemagenin and 50 - 500 µgmL^{-1} for 18β-glycyrrhetinic acid. Peak area obtained versus concentration was subjected to least square linear regression analysis. To prove linearity, residual analysis was also performed along with correlation coefficient. Each standard solution of five 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 as $3.3 \times S_{y.x} / S$ and $10 \times S_{y.x} / S$, respectively. Where, S is the slope of the linearity plot and $S_{y.x}$ is the standard deviation of residuals from line.

Specificity:
The specificity of the proposed RP-HPLC method was estimated by analyzing the standard marker and sample. Peaks for both gymnemagenin 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 and 18β-glycyrrhetinic acid.

Precision studies:
The precision of the developed RP-HPLC method was verified by intra-day and inter-day precision. Intra-day precision was performed by analysis of single concentration in six replicates of mixed standard solutions of gymnemagenin (200 μgmL^{-1}) and 18β-glycyrrhetinic acid (200 μgmL^{-1}) which were prepared on the same day. Intermediate precision was performed by repeating studies on three consecutive days. The peak areas were recorded and percentage relative standard deviation (RSD) was estimated.

Accuracy studies:
Accuracy studies were carried out by standard addition method in triplicates. Accuracy was evaluated through the percentage recoveries of known amounts of mixture of gymnemagenin and 18β-glycyrrhetinic acid added to solutions of herbal formulation and the percent ratios between the recovered and expected concentrations were calculated. Accuracy was calculated from the following equation:

[(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. Four factors, such as buffer pH (± 0.1), organic composition (methanol) of the mobile phase (± % 1), elution flow rate (± 0.1 $mLmin^{-1}$) and detection wavelength (± 2 nm) were varied. One factor at a time was changed to study the effect. The robustness of the RP-HPLC method was performed at a concentration of 200 μgmL^{-1} for both gymnemagenin and 18β-glycyrrhetinic acid.

Solution stability:
The stability of gymnemagenin and 18β-glycyrrhetinic acid standard solutions (200 μgmL^{-1}) was performed after 0, 6, 12, 24 and 48 h of storage at room temperature. Solution stability was estimated 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, peak asymmetry, theoretical plates, and resolution of the five injections of both standard markers (200 μgmL^{-1}) using developed method.

RESULTS AND DISCUSSION

HPLC method optimization:
Different HPLC columns, mobile phases of various compositions of methanol, acetonitrile, potassium dihydrogen phosphate, sodium dihydrogen phosphate buffer with different molarities, water and different pH were tried. Finally the mobile phase consisting of methanol: water (92:08, v/v), pH 2.8, 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 $mLmin^{-1}$. The optimum wavelength for detection and quantitation used was 218 nm. Average retention times ± standard deviation for gymnemagenin and 18β-glycyrrhetinic acid were found to be 3.82 ± 0.02 and 7.15 ± 0.03 min, respectively (Figure 3).

HPLC method validation:
Linearity and Range:
The results were found to be linear in a range of 50 - 1000 μgmL^{-1} for gymnemagenin and 50 - 500 μgmL^{-1} for 18β-glycyrrhetinic acid (Table 1).

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

Parameters	Gymnemagenin	18β-glycyrrhetinic acid
Linearity range (μgmL^{-1})	50 - 1000	50 - 500
Correlation coefficient (r)	0.999	0.999
Slope	2444	8144
Intercept	-33752	31388
95 % Confidence limit of slope	2384.956 - 2503.014	7921.772- 8368.008
95 % Confidence limit of intercept	-66750.4 - (-754.475)	-36470.5 - 99246.92
$S_{y.x}$[a]	11912.78	23515.26

[a] *Standard deviation of residuals from line.*

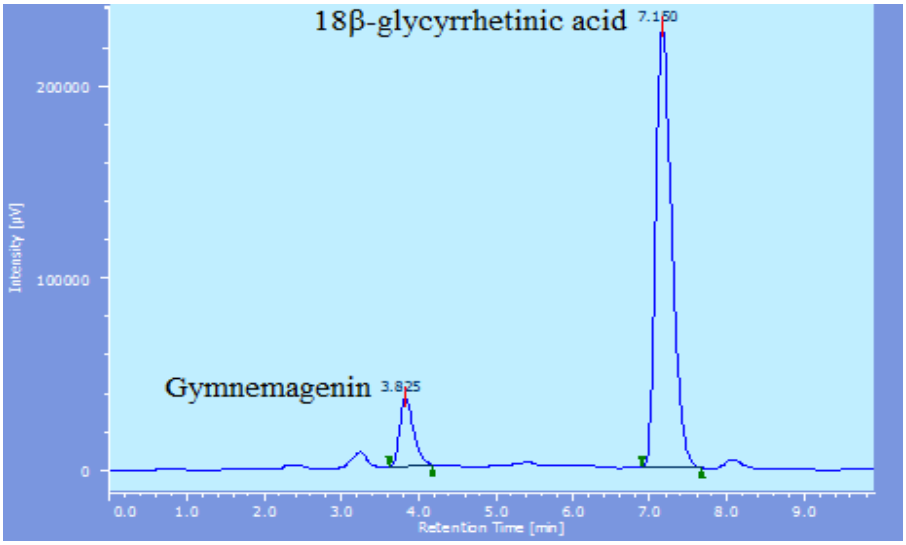
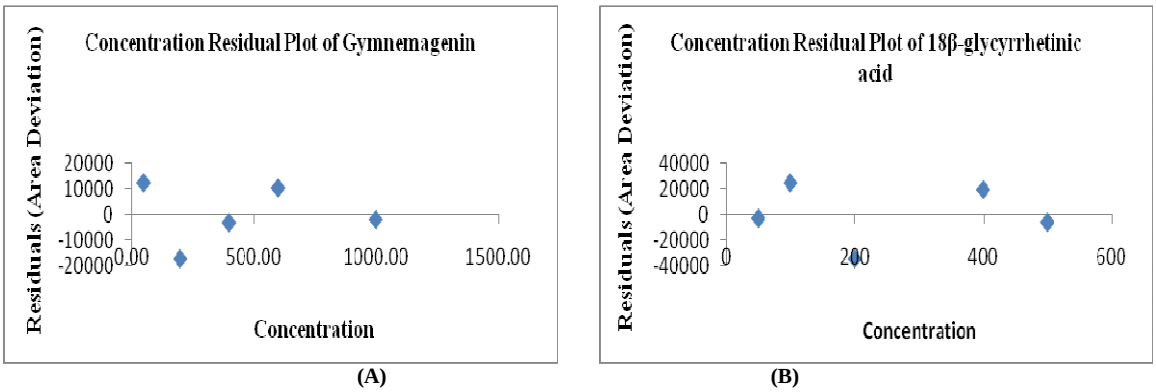


Figure 3: Representative chromatogram obtained from a mixed standard solution of gymnemagenin and 18β-glycyrrhetinic acid

To ascertain linearity, residual analysis was performed (Figure 4). Slope was significantly different from zero.



(A) **(B)**

Figure 4: Concentration Versus Residual Plot of (A) Gymnemagenin and (B) 18β-glycyrrhetinic acid

Sensitivity:

The LOD and LOQ for gymnemagenin and 18β-glycyrrhetinic acid were found to be 16.08 and 9.52 µgmL^{-1} and 48.74 and 28.87 µgmL^{-1}, respectively, indicating good sensitivity of the proposed RP-HPLC method.

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 and 18β-glycyrrhetinic acid, indicating specificity of the proposed RP-HPLC method

Precision:

Intra-day variation, as % RSD, was found to be in the range of 0.49 - 0.56 for gymnemagenin and 0.80 - 0.83 for 18β-glycyrrhetinic acid. Inter-day variation, as % RSD was found to be in the range of 0.52 - 0.63 for gymnemagenin and 0.81 - 0.87 for 18β-glycyrrhetinic acid, indicating a good precision (Table 2).

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	197.7/198.1	0.59/0.62
18β-glycyrrhetinic acid	200	196.8/197.5	0.86/0.89

[a] Concentration in µgmL^{-1}; RSD is the relative standard deviation

Accuracy

As shown in Table 3, satisfactory recoveries of 98.12 - 100.4 % and 98.66 - 101.3 % for gymnemagenin and 18β-glycyrrhetinic acid, respectively which indicate that the proposed RP-HPLC method is reliable for the concurrent quantification of gymnemagenin and 18β-glycyrrhetinic acid in this herbal tablet formulation (Table 3).

Table 3 Results of recovery studies (n=3)

Drug	Amount taken[a]	Amount added[a]	Amount found [a] ± S.D	% Recovery ± % RSD
Gymnemagenin	100	80	178.5 ± 1.29	99.16 ± 0.72
	100	100	198.2 ± 1.45	99.10 ± 0.73
	100	120	223.3 ± 1.41	101.50 ± 0.63
18β-glycyrrhetinic acid	100	80	179.2 ± 1.36	99.55 ± 0.75
	100	100	201.3 ± 1.50	100.65 ± 0.74
	100	120	216.4 ± 1.53	98.36 ± 0.70

[a] Concentration in μgmL^{-1}; RSD is the relative standard deviation

Analysis of marketed herbal formulation:

Validity of the proposed RP-HPLC method was applied to standardization of herbal tablet dosage form in six replicate determinations. The percent content of both viz., gymnemagenin and 18β-glycyrrhetinic acid in marketed herbal formulation was found to be 0.2040 % and 0.7425 %, respectively.

Robustness studies:

Robustness of the proposed RP-HPLC method was checked after small, deliberate changes of the analytical parameters (Table 4). It showed that peak areas of these two markers remained unaffected (% RSD < 2), indicating robustness of the method.

Table 4 Robustness testing (n = 6, 200 μgmL^{-1})

Parameter	SD of peak area		% RSD	
	Gymnemagenin	18β-glycyrrhetinic acid	Gymnemagenin	18β-glycyrrhetinic acid
Organic composition of the mobile Phase (Methanol; ± 1%)	1842.17	3554.57	0.42	0.40
Buffer pH (± 0.1)	1539.45	2382.14	0.35	0.27
Elution flow rate (± 0.1 mL min-1)	2254.71	3708.34	0.51	0.42
Detection wavelength (± 2 nm)	1455.30	5223.95	0.33	0.59

RSD is the relative standard deviation

Solution Stability:

Solution stability of gymnemagenin 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 (≥ 1), high resolution between the peaks (≥ 2.0), and proper retention time indicated suitability of the proposed RP-HPLC method for quantification of gymnemagenin and 18β-glycyrrhetinic acid (Table 5).

Table 5 System suitability parameters of chromatogram for gymnemagenin and 18β-glycyrrhetinic acid (200 μgmL^{-1})

Parameters	Proposed RP-HPLC method			
	Gymnemagenin	% RSD	18β-glycyrrhetinic acid	%RSD
Retention time (min)	3.82	0.71	7.15	0.49
Peak asymmetry	1.33	1.49	1.32	1.14
Theoretical plates	2311	1.43	5689	1.26
Resolution	9.56 ± 1.39			

RSD is the relative standard deviation

CONCLUSION

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

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