Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 5, Issue 1, 2013

Research Article

HPTLC METHOD DEVELOPMENT AND VALIDATION FOR DENSITOMETRIC ANALYSIS OF 18β-GLYCYRRHETINIC ACID AND β-SITOSTEROL IN POLYHERBAL DRUG FORMULATION

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Received: 04 Nov 2012, Revised and Accepted: 19 Dec 2012

ABSTRACT

This paper reports new, accurate and precise high performance thin layer chromatographic (HPTLC) method for densitometric determination of 18β-glycyrrhetinic acid and β-sitosterol in herbal drug formulation (Madhuveer Liquid). Marketed herbal formulation was suitably diluted with methanol and ultrasonicated for 1 h (to extract β-sitosterol). Acid hydrolysis of marketed herbal formulation followed by extraction with chloroform gives 18β-glycyrrhetinic acid. The analysis was carried out on aluminum plates precoated with silica gel 60 F_{254} using toluene: ethyl acetate: methanol (14.3:3.8:1.9, v/v/v) as mobile phase. Developed HPTLC plate was subjected to post chromatographic derivatization with 5% sulphuric acid reagent followed by heating at 110°C for 6 min in a pre-heated oven. Derivatized plate was scanned at 429 nm. The retention factor for 18β-glycyrrhetinic acid and β-sitosterol were found to be 0.32 ± 0.01 and 0.71 ± 0.01, respectively. The calibration curve was linear over a range of 150-2000 ng $spot^{-1}$ for 18β-glycyrrhetinic acid and 200-1500 ng $spot^{-1}$ for β-sitosterol. The proposed HPTLC method was applied for the analysis of marketed formulation. The % content of 18β-glycyrrhetinic acid and β-sitosterol was found to be 0.3777 % and 0.0437 %, respectively. The HPTLC method was validated in accordance with ICH guidelines. The proposed method was found to be precise, robust, accurate and rapid for quantitative estimation of 18β-glycyrrhetinic acid and β-sitosterol in marketed herbal formulation.

Keywords: 18β-Glycyrrhetinic acid, β-Sitosterol, HPTLC, Densitometric estimation, Validation

INTRODUCTION

Aglycone of glycyrrhizin, 18β-glycyrrhetinic acid possesses antihyperglycemic action on streptozotocin induced diabetic rats [1]. Extensive literature survey reveals that few HPTLC [2-5] and HPLC [6,7]methods have been reported for estimation of 18β-glycyrrhetinic acid. β-Sitosterol is white, waxy phytosterol [8] responsible for reduction of cholesterol level in plasma [9] and improves liver function activity (GDP, GOP) [10]. HPTLC [11-15] and HPLC [8,16,17] methods have also been reported for analysis of β-sitosterol either individually or in combination with other marker compounds. Over the past decade HPTLC has been successfully used in the analysis of pharmaceuticals, plant constituents, and biomacromolecules [18]. A major advantage of HPTLC is its ability to analyze several samples simultaneously using a small quantity of mobile phase, thereby reducing the time and cost of analysis [19,20]. No reports were found for simultaneous analysis of 18β-glycyrrhetinic acid and β-sitosterol by HPTLC method.

Hence the present study was undertaken to develop and validate simple, accurate, precise and robust analytical procedure for simultaneous estimation of 18β-glycyrrhetinic acid and β-sitosterol in herbal drug formulation by HPTLC.

MATERIALS AND METHODS

Solvents and chemicals

Standard 18β-glycyrrhetinic acid and β-sitosterol were procured from Natural Remedies Bangalore, India. Herbal drug formulation used in this study (Madhuveer Liquid, Manbro Pharma Pvt. Ltd., Thane, India) was purchased from the local market. All chemicals and reagents used in the study were of analytical grade and purchased from Merck specialties Pvt. Ltd. (Mumbai, India). Double distilled water was used in the present research study.

HPTLC Instrumentation and Chromatographic conditions

The sample solutions were applied on pre-washed and activated precoated silica gel aluminium HPTLC plate $60F_{254}$ (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 slit dimension was kept at 5mm × 0.45 mm and 10 mm/s scanning speed was employed. HPTLC plate was then developed, at constant temperature, with 20 mL mobile phase consisting of toluene: ethyl acetate: methanol (14.3:3.8:1.9, v/v/v). Linear ascending development was carried out in 20 cm x 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The 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 derivatizing agents has been examined such as 10% sulphuric acid, vanillin-sulphuric acid reagent, anisaldehyde sulphuric acid but 5% sulphuric acid reagent showed promising and reproducible results with chromophore stability for 25 min and hence was selected for derivatization. It was prepared by mixing 5 mL conc. sulphuric acid in approximately 70 mL methanol in 100 mL volumetric flask and final volume was adjusted with methanol. The length of chromatogram run was 8 cm. After chromatography, developed plate was dried in a current of air and plate was dipped into 5% sulphuric acid reagent, heated at 110°C for 06 min in a pre-heated oven. Densitometric scanning was performed within 10 min after derivatization process using Camag TLC scanner III with winCATS software version 1.4.4 in the reflectance mode at 429 nm.

Preparation of standard stock solutions

Standard stock solutions of pure marker compounds 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 give a solution of 100 µg/mL. These stock solutions were used for further studies.

Selection of detection wavelength

After chromatographic development and derivatization, bands were scanned over the range of 400-700 nm and the spectra were overlain. It was observed that both markers showed considerable absorbance at 429 nm (Fig1) and hence was selected for analysis.

Construction of calibration plots

Linearity was evaluated in the range of 150-2000 ng $spot^{-1}$ for 18β-glycyrrhetinic acid and 200-1500 ng $spot^{-1}$ for β-sitosterol. For preparation of calibration plots 1.5, 2.5, 6, 11 and 20 µL of 18β-glycyrrhetinic acid standard solution (100 µg/mL) and 2 , 3, 7, 8, 10 and 15 µL β-sitosterol (100 µg/mL) were applied to the plates which were then chromatographed and scanned as described above. Peak area versus concentration was subjected to least square linear

regression analysis and the intercept, slope and correlation coefficient for the calibration were determined. Residual analysis was performed to ascertain linearity. Limit of detection (LOD) and limit of quantitation (LOQ) were calculated as 3.3 σ/S and 10 σ/S, respectively. Where S is the slope of the calibration plot and σ is the standard deviation of the response (y-intercept).

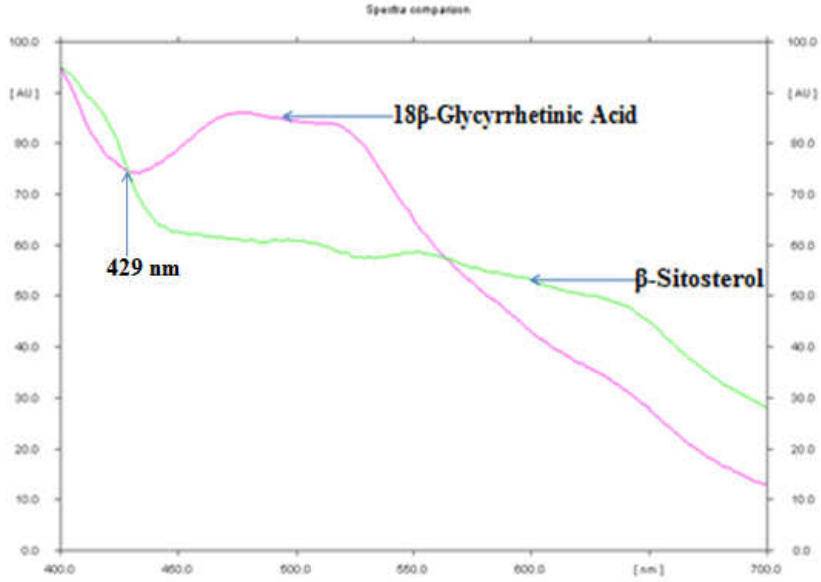

Fig. 1: Overlain visible spectra of 18β-glycyrrhetinic acid and β-Sitosterol

Preparation of sample solutions

It was found that single method is not applicable to obtain these two marker compounds, hence sample preparation for 18β-glycyrrhetinic acid and β-sitosterol was performed separately. Madhuveer Liquid is an aqueous extract formulation and was used for HPTLC analysis.

For 18β-glycyrrhetinic acid

The published method[2]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 (200 mg *Glycyrrhiza glabra* extract) was hydrolyzed with 2N aqueous hydrochloric acid (100 mL) under reflux for 2 h. The hydrolyzed 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 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.

For β-sitosterol

For analysis of β-sitosterol 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 I filter paper and used for further analysis.

Assay validation

The proposed method was optimized and validated as per the International Conference on Harmonization (ICH) guidelines [21]. All measurements were performed in triplicates.

Precision studies

Set of three different concentrations in three replicates of mixed standard solutions of 18β-glycyrrhetinic acid (200, 400 and 600 ng $spot^{-1}$) and β-sitosterol (300, 400 and 700 ng $spot^{-1}$) were prepared. All the solutions were analyzed on the same day in order to record any intra-day variations in the results. Inter-day precision study involves analysis of three different concentrations of the mixed standard solutions in linearity range on three consecutive days.

Specificity

To confirm specificity of the method, 18β-glycyrrhetinic acid and β-sitosterol standard solutions and the sample solution were applied to a HPTLC plate and the plate was developed, derivatized and scanned as described above. The peak purity of 18β-glycyrrhetinic acid and β-sitosterol was assessed by comparing the spectra of marker compounds at peak start, peak apex and peak end positions of the spot i.e., r (start, middle) and r (middle, end).

Accuracy studies

Accuracy was evaluated through the percent recoveries of known amounts of mixture of 18β-glycyrrhetinic acid and β-sitosterol added to solutions of marketed herbal formulation. Marketed herbal formulation was spiked with the known amount of standard markers and the percent ratios between the recovered and expected concentrations were determined. The analyzed samples were spiked with 80, 100 and 120 % of 500 ng $spot^{-1}$ of both 18β-glycyrrhetinic acid and β-sitosterol. 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 markers were examined. Factors varied were detection wavelength and mobile phase composition. One factor at a time was changed to study the effect. The robustness of method was checked at concentration of 500 ng $spot^{-1}$ for both 18β-glycyrrhetinic acid and β-sitosterol, respectively.

RESULTS AND DISCUSSION

HPTLC method optimization

Different mobile phases containing various ratios of acetone, n-hexane, ethanol, methanol, toluene, ethyl acetate, acetone and water were tried. Finally the mobile phase consisting of toluene: ethyl acetate: methanol (14.3:3.8:1.9, v/v/v) was selected for which gave well resolved peaks. The optimum wavelength selected for detection and quantitation was 429 nm. The retention factors for 18β-glycyrrhetinic acid and β-sitosterol were found to be 0.32 ± 0.01 and 0.71 ± 0.01, respectively (Fig 2).

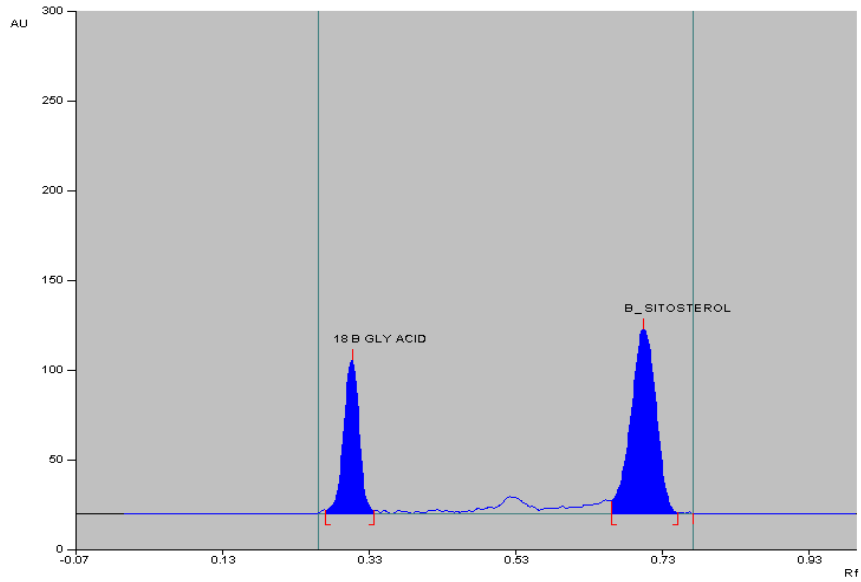

Fig. 2: Densitogram obtained from mixed standard solution of 18β-glycyrrhetinic acid and β-sitosterol

HPTLC method validation

Linearity, limit of detection and limit of quantitation

The results were found to be linear in the range of 150 - 2000 ng spot^{-1} for 18β-glycyrrhetinic acid and 200 - 1500 ng spot^{-1} for β-sitosterol. The correlation coefficients (r) for the plots were 0.99983 for 18β-glycyrrhetinic acid and 0.99936 for β-sitosterol. The peak area (y) is proportional to the concentration of 18β-glycyrrhetinic acid following the regression equation y = 1.981x + 351.4 (Fig 3) and for β-sitosterol y = 2.688x + 402.6 (Fig 4). The LOD and LOQ for 18β-glycyrrhetinic acid and β-sitosterol were found to be 45.94 and 38.37 ng spot^{-1} and 139.22 and 116.28 ng spot^{-1}, respectively.

Precision

Intra-day variation, as RSD (%), was in the range of 0.10 - 0.13 for 18β-glycyrrhetinic acid and 0.12 - 0.18 for β-sitosterol. Inter-day variation, as RSD (%) was found to be in the range of 0.12 - 0.16 for 18β-glycyrrhetinic acid and 0.15 - 0.19 for β-sitosterol. Precision studies for 18β-glycyrrhetinic acid and β-sitosterol showed RSD less than 2 %, indicating a good precision.

Specificity

Assessment of peak purity of 18β-glycyrrhetinic acid and β-sitosterol by comparing the spectra of marker compounds at peak start, peak apex and peak end positions of the spot was checked, r (start, middle) = 0.999, 0.998 and r (middle, end) = 0.9993, 0.9991, respectively. Good correlation was also obtained between markers and sample spectra of 18β-glycyrrhetinic acid and β-sitosterol.

Accuracy

Results of the accuracy shows recoveries of 98.04 - 102.67 % and 98.37 - 101.96 % for 18β-glycyrrhetinic acid and β-sitosterol, respectively indicating the reliability of the proposed densitometric method for the quantitation of 18β-glycyrrhetinic acid and β-sitosterol in the marketed herbal formulation (Table 1).

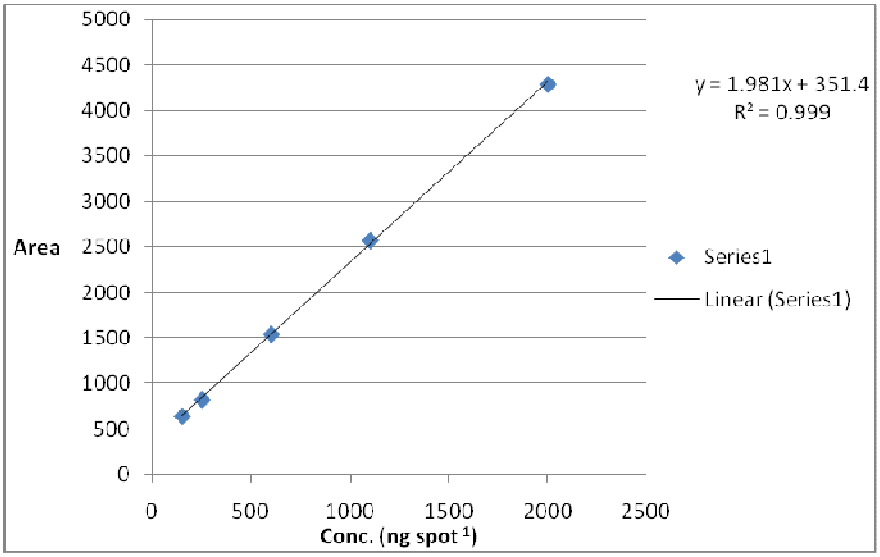

Fig. 3: Linearity graph of 18β-glycyrrhetinic acid

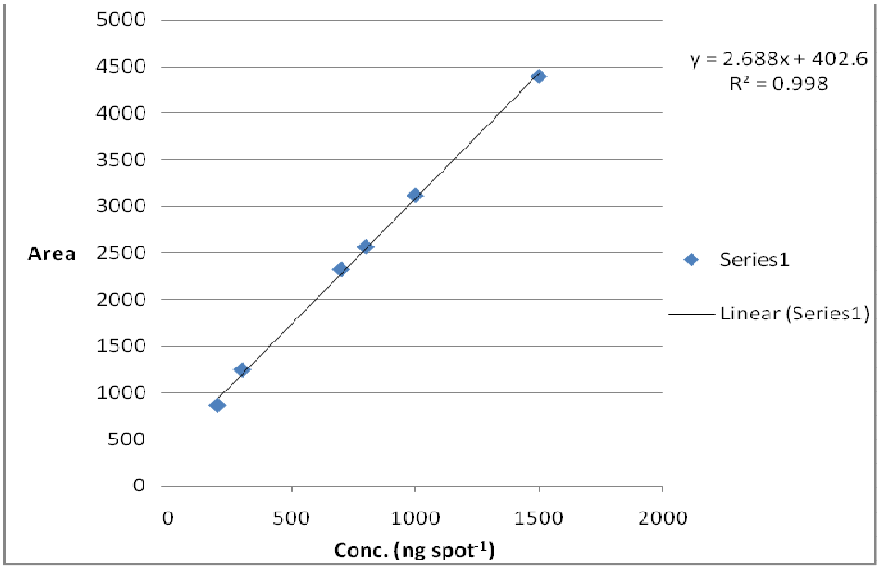

Fig. 4: Linearity graph of β-sitosterol

Table 1: Results of recovery studies

Formulation	Amount added		% Recovery ± S.D.*	
	18β-glycyrrhetinic acid (ng spot^{-1})	β-sitosterol (ng spot^{-1})	18β-glycyrrhetinic acid	β-sitosterol
Madhuveer Liquid	400	400	98.04 ±0.93	101.96 ± 1.04
	500	500	99.40 ± 0.34	98.80 ±1.96
	600	600	102.67 ± 0.53	98.37 ±0.20

*Average of three determinations

Analysis of marketed herbal formulation

Proposed validated method was applied to standardization for herbal formulation viz. Madhuveer Liquid. The shape of the peaks was not altered by other substances present in the marketed formulation. The percent content of 18β-glycyrrhetinic acid and β-sitosterol in marketed herbal formulation was found to be 0.3777 % and 0.0437 %, respectively.

Robustness studies

Robustness of the proposed method checked after deliberate alterations of the analytical parameters indicated 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 2: Summary of validation parameters of proposed method

Parameters	18β-glycyrrhetinic acid	β-sitosterol
Linearity range (ng spot^{-1})	150-2000	200-1500
Correlation coefficient (r)	0.99983	0.99936
LOD (ng spot^{-1})	45.94	38.37
LOQ (ng spot^{-1})	139.22	116.28
Accuracy (% Recovery)	98.04 -102.67	98.37 -101.96
Precision (% RSD)		
Intra day (n = 3)	0.10- 0.13	0.12 - 0.18
Inter day (n = 3)	0.12 - 0.16	0.15-0.19
Robustness	Robust	Robust
Specificity	Specific	Specific

LOD = Limit of detection.

LOQ = Limit of quantitation.

RSD = Relative standard deviation.

n = Number of determinations

CONCLUSION

The validated HPTLC method employed was found to be simple, fast, accurate, precise, robust and thus can be used for routine analysis of 18β-glycyrrhetinic acid and β-sitosterol in this liquid herbal formulation.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to University Grants Commission (UGC), New Delhi, India, for financial assistance to carry out research work under the scheme of Special Assistance Programme (SAP) of Departmental Research Support (DRS) Phase II. Thanks are also extended to Anchrom Enterprises Pvt. Ltd, Mumbai, India, for providing technical support.

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