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HPTLC FINGERPRINTING AND QUANTIFICATION OF B SITOSTEROL IN EXTRACT AND POLYHERBAL FORMULATIONS

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ABSTRACT

A new, simple, sensitive, precise and robust high-performance thin layer chromatographic (HPTLC) method was developed for the estimation of **β sitosterol** in herbal extracts and polyherbal formulation. Analysis of **β sitosterol** was performed on TLC aluminium pre-coated plates with silica gel 60F-254 as stationary phase. Linear ascending development was carried out in twin trough glass chamber saturated with mobile phase consisting of Toluene: ethyl acetate: formic acid (5:2:1 v/v/v) for **β sitosterol** at room temperature (25±2^{o}C). After development the plate was derivatized with 20% ethanolic H_2SO_4. Camag TLC scanner III was used for spectrodensitometric scanning and analysis of plate in absorbance mode at 364 nm for **β sitosterol**. The system was found to give compact spots for **β sitosterol** (R_f 0.42). The data for calibration plots showed good linear relationship with $r2$ = 0.9984 in the concentration range of 60–360 ng for **β sitosterol**. According to international conference on harmonization (ICH) guidelines the present method was validated for precision, repeatability and recovery. The limits of detection and quantification were determined. Statistical analysis of the data showed that the method is reproducible and selective for estimation of **β sitosterol**.

KEYWORDS: High-performance thin layer chromatography; Method validation; **β sitosterol**; Polyherbal formulations.

1. INTRODUCTION

Herbal medicine has been enjoying revitalization among the customers throughout the world. However, one of the impediments in the acceptance of the ayurvedic/herbal medicines is the lack of standard quality control profiles. The quality of herbal medicine i.e. the profile of the constituents in the final product has implication in efficacy and safety. Due to the complex

nature and inherent variability of the chemical constituents of plant-based drugs, it is difficult to establish quality control parameters. To overcome these problems modern analytical techniques are expected to help in circumventing this problem.[1,2]

The dried fruit of ***Anethum Sowa*** *from the* Family: **Apiaceae** is an annual herb cultivated through India chiefly in Punjab, Uttar Pradesh, Gujarat, Maharashtra, Assam and West Bengal.[3] The literature survey revealed for the variety of therapeutic actions of ***Anethum Sowa*** including antifungal, antibacterial, a Insecticidal, ovicidal, antioxidant, **hypoglycemic activity.[4–8]** The principle substance is carvone and **β sitosterol**. (Fig.1) It also contain dihydrocarvone, d-limonene, d-phellandrene, pinene, dill-apiol, quarcetin, protein, carbohydrate, minerals, fat, fiber.[9]

Certain herbal extracts and polyherbal formulations containing active constituents were standardized by high-performance thin layer chromatography (HPTLC) method. HPTLC method was reported to be the most suitable for the estimation of active constituents of extracts, plant species (raw material) and polyherbal formulations. Therefore, an attempt has been made to develop accurate, specific, repeatable and reproducible HPTLC method for the determination of **β sitosterol** in herbal extracts and Polyherbal formulation.

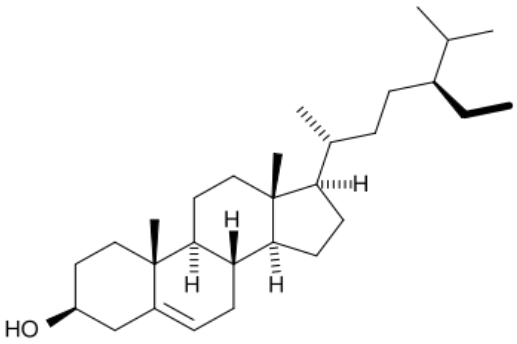

Fig. no. 1: Structure of β sitosterol.

2. EXPERIMENTAL

2.1. Materials

Standard **β sitosterol** was purchased from Indus extract, Mumbai. Spry dried aqueous extracts of *Anethum sowa* were obtained from Prashant Pharmaceuticals (ISO 9001 herbal unit) Rajpipla, Gujarat, India. All chemicals including solvents were of analytical grade from E. Merck, India. The HPTLC plates Silica 60F254 (20 cm x 20 cm) were purchased from E. Merck (Darmstadt, Germany).

2.2. Instrumentation and chromatographic conditions

The samples were spotted in the form of bands of 6 mm with Camag microlitre syringe on pre-coated silica gel aluminium Plate 60F-254 (20 cm ×10 cm) with 200 µm thickness (E. Merck, Germany) using a Camag Linomat V (Switzerland) sample applicator. A constant application rate of 150 nL s−1 was employed and space between two bands was 14 mm. The slit dimension was kept at 2mm×0.45mm and 20 mm s−1 scanning speed was employed. The composition of mobile phase is Toluene: ethyl acetate: formic acid (5:2:1 v/v/v) for **β sitosterol**. Linear ascending development was carried out in a twin trough glass chamber saturated with mobile phase. The optimized chamber saturation time for the mobile phase was 22 min for **β sitosterol** at room temperature (25±2^{0}C). The length of chromatogram run was 80 mm. Then the plate was allowed to dry at room temperature. Derivatized the plate with 20% ethanolic H_2SO_4 reagent by dipping for 5 s and dried the plate for 5 min at 45^{0}C in an oven. The separated bands on the HPTLC plates were scanned over the wavelength of 364nm.

2.3. Calibration curve of β sitosterol

A stock solution of **β sitosterol** 200 µg/ml in chloroform was prepared. Different volumes in the range 0.2 – 1.2 µl of **β sitosterol** were applied on TLC plate to obtain concentration of 60 - 360 ng of **β sitosterol**.(Fig. no. 2).

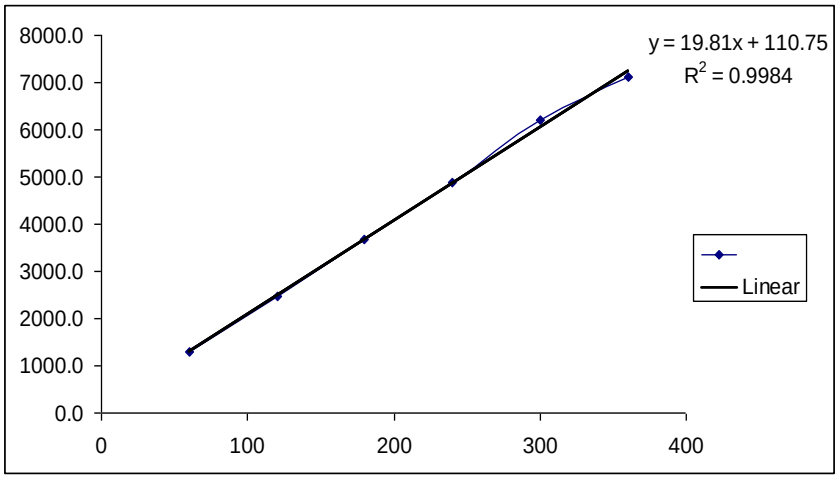

Fig 2: Calibration Curve for β Sitosterol.

Y= 19.81 X + 110.75

Coefficient of correlation = 0.9984;

Slope = 19.81; Intercept =110.75

2.4. Method validation

2.4.1. Instrumental precision and inter-day and intra-day precision

Precision of the method was determined as intra-day and inter-day variations. Intra-day variations was determined by analyzing 120, 140, 200 ng/spot of standard solution of β sitosterol for three times on the same day. Inter-day precision was determined by analyzing 120, 140, 200 ng/spot of standard solution of β sitosterol for three consecutive days over a period of a week.

Table 1: Intra- and Inter-day precision study.

Marker compounds	Concentration (ng/spot)	Intra-day precision*	Inter-day precision*
β sitosterol	180	1.21	0.87
	240	0.83	0.50
	300	0.62	0.53

* Relative standard déviation (% CV, n=3)

2.4.2. Accuracy (recovery study)

Recovery experiments were performed at three different levels i.e. 80, 100 and 120 %. To the pre-analysed sample solutions; a known amount of standard drug solution of MAG was over spotted at three different levels. The chromatogram was developed and scanned as discussed in instrumentation condition.

Table 2: Recovery study.

Marker compounds	Amount present in the sample, (ng)	Amount added (ng)	Amount found (ng)	Recovery (%)	Average recovery (%)
β sitosterol	20	16	36.07**±0.31**	101.14	100.31±1.27
	20	20	40.14**±0.40**	101.25	
	20	24	43.54**±0.13**	98.56	

*Mean ± standard deviation (SD, n=3)

2.4.3. Sensitivity, limit of detection and limit of quantification

The sensitivity of measurements of **β Sitosterol** by the use of the proposed method was estimated in terms of the Limit of Detection (LOD) and Limit of Quantitation (LOQ). The LOD and LOQ were calculated using equation LOD = 3.3 x N/B and LOQ = 10 x N/B, where, 'N' is standard deviation of the peak areas of the drugs (n = 3), taken as a measure of noise, and 'B' is the slope of the corresponding calibration curve. The linearity equation was

found to be Y = 11.444x + 8.08. The LOD and LOQ for β sitosterol for were found to be 3.89 ng and 11.79 ng, respectively [where, N =, B = 11.444].

2.4.4. Repeatability

Repeatability of sample application was assessed by spotting 0.6 µL contain 120 ng/spot of standard β sitosterol for on TLC plate in triplicate; develop and scanned as described in instrumentation and chromatographic condition.

2.4.5. Specificity

The specificity of the method was ascertained by analyzing the standard drug and extract. The spot for β sitosterol in the sample was confirmed by comparing the R_f value and spectra of the spot with that of the standard. The peak purity of β sitosterol was assessed by comparing the spectra at three different levels, viz. peak start (S), peak apex (M), peak end (E), position of the spot.

Table 3: Method validation parameters of marker compounds by HPTLC.

Parameters	β sitosterol
Instrumental precision (% CV)(n=3)	0.45
Repeatability	1.29
Limit of detection (ng/spot)	5.80
Limit of quantification (ng/spot)	18.07
Specificity	Specific
Linearity (Correlation of coefficient) Range (ng/spot)	**0.9984** 60-360

2.5. Analysis of β sitosterol in herbal extracts

50 mg aqueous extract of *A. Sowa* was transferred in 10 ml volumetric flask and volume make up with 10 ml methanol. Both resulting solution was filtered using Whatmann filter paper 41. An appropriate volume of 5 µL aqueous extract was spotted on TLC plate. The analysis was repeated in triplicate.

2.6. Analysis of β sitosterol in polyherbal formulation

Polyherbal formulation (Tablet) prepared using the combination of aqueous extract of *T. terrestris, G. glabra, and Anethum sowa,* all these extract added in fixed quantity and increase the bulk by adding excipients like flavoring agent, color, binding agent etc. and this polyherbal formulation analyzed with the marker compound β sitosterol. To determine the

content of β sitosterol in polyherbal formulation; require amount of formulation was weighed and crushed into fine powder. Then it was transferred to 10 ml volumetric flask containing 10 ml of methanol. Resulting solution was filtered using Whatmann filter paper 41. An appropriate volume 10 µL was spotted for analysis of β sitosterol, The concentration was determined by linear regression equation. The analysis was repeated in triplicate.

Table 4: Marker compounds quantified by HPTLC from aqueous extract and poly herbal formulation.

Sr. no.	Marker compounds	Aqueous extract (%)	Poly herbal formulation (%)
1.	β sitosterol	0.754	0.164

3. RESULTS AND DISCUSSION

3.1. Selection of mobile phase

The standard solution and the test solution were spotted on HPTLC plates and different solvents as well as combination of solvents have been tried to get a good separation and stable peak. The optimized mobile phase for β sitosterol was Toluene: ethyl acetate: formic acid, (5:2:1) (*v/v/v*) was selected for estimation of the drug by HPTLC method, which gave good resolution with R_f = 0.42 (β sitosterol) (Fig. 5.) and Well defined spots were obtained when the chamber was saturated with mobile phase for 22 min at room temperature.

3.2. Calibration curves

The present HPTLC method for estimation of β sitosterol showed a good correlation coefficient (*r*2 = 0. **0.9984**) in the concentration range of 60–360 ng /spot with respect to the peak area. The mean value (±S.D.) of slope and intercept were **19.81** & **110.75**. No significant difference was observed in the slope of standard curves (ANOVA, $P > 0.001$).

3.3. Method validation

The real goal of validation process is to determine limits of allowed variability for the conditions needed to run the method. The accuracy of the method was determined by recovery experiments. The recovery studies were carried out three times and the percentage recovery were calculated and presented. From the data obtained, recoveries of added standard drugs were found to be accurate (Table 2). Three repeated standard solutions were made and response factors of drug peaks and %R.S.D. were calculated and presented. From the data obtained, the developed HPTLC method was found to be precise. The calibration curves were plotted using the response factors versus concentration of standard solution. These data

demonstrate that the methods have adequate sensitivity to the concentrations of the analytes. The LOD and LOQ of the developed method were determined by injecting progressively low concentrations of the standard solutions using the developed methods. The LOD is the smallest concentration of the analyte that gives a measurable response. The LOD and LOQ were found to be 5.80 ng and 18.07 ng [where, N = 35.34, B = 11.444] which indicate adequate sensitivity of the method. The LOD and LOQ values determined are affected by the separation conditions, i.e., HPTLC plates, reagent; instrumentation and detection wavelength and data system solvents other than AR grade solvent can result in large changes in single to noise ratio due to base line noise and drift (Table 3). The precision (Table 1) of the methods were studied by carrying out experiments by changing conditions. It was observed that there were no marked changes in the chromatograms. The values obtained demonstrated the suitability of the system for the analysis of the above drug system suitability parameters might fall within ±3% standard deviation range, during routine performance of the method.

3.4. Estimation of β sitosterol in herbal extracts and in polyherbal formulations

Spot at R_f 0.42 was observed in the chromatogram for β sitosterol in extracts and polyherbal formulation respectively. The content of β sitosterol in aqueous extract and poly herbal formulation were found to be 0.754 % and 0.164% (Table 4).

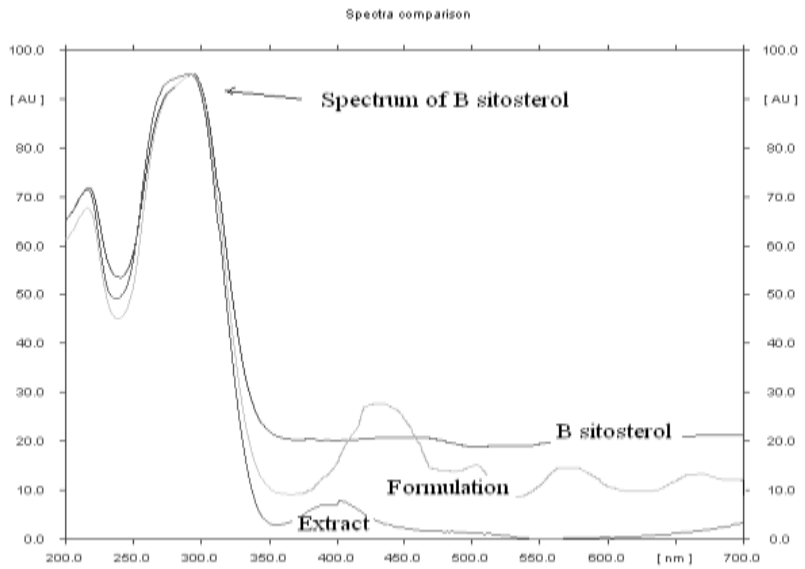

Fig. 3: Comparative spectral study of β Sitosterol, *A. Sowa* extract and polyherbal formulation.

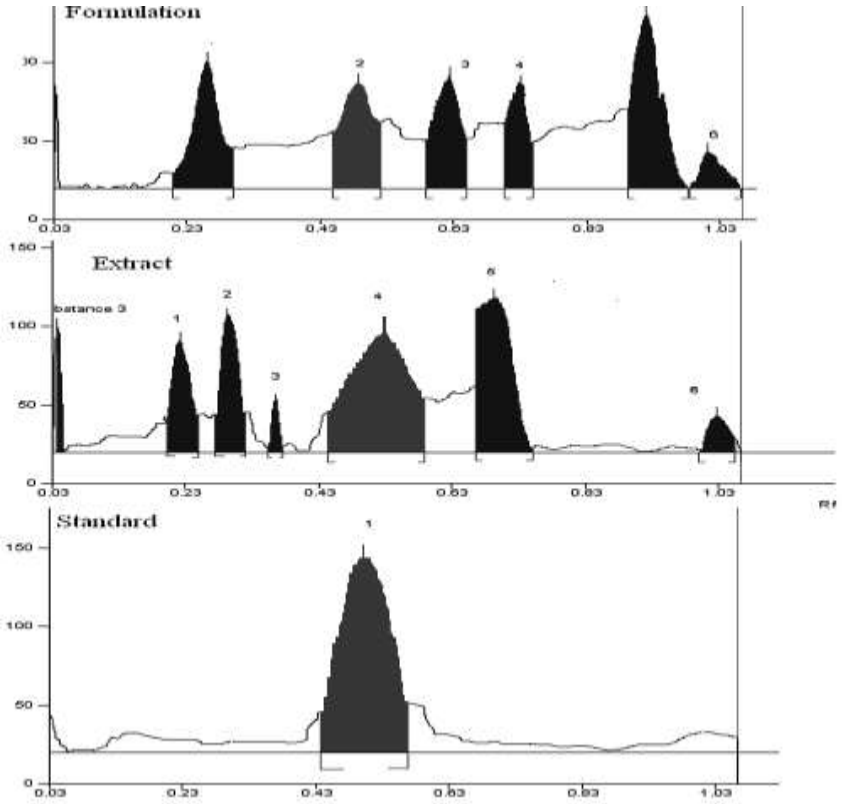

Fig. 4: Comparative HPTLC densitogram of standard β Sitosterol at R_f-0.42 With *A. Sowa* extract and Polyherbal formulation at 364 nm.

4. CONCLUSION

From the above studies, it can be concluded that HPTLC technique can be successfully used for estimation of β sitosterol in extracts and polyherbal formulations. The developed HPTLC method for this estimation of β sitosterol is simple, accurate, linear and rapid. Statistical analysis proves that the method is reproducible and selective for the analysis of β sitosterol.

CONFLICT OF INTEREST

Nil.

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