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MARKER BASED STANDARDIZATION OF COMMERCIAL FORMULATIONS AND EXTRACTS CONTAINING BETA-SITOSTEROL D- GLUCOSIDE USING HPTLC

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ABSTRACT
A new simple, precise, rapid and selective high-performance thin-layer chromatography (HPTLC) method has been developed for determination of beta-sitosterol-D-glucoside in Karela (*Momordica charantia* Linn.) extracts as well as in plant based formulations containing Ashwagandha (*Withania somnifera* L. Dunal.)
The mobile phase consisted of toluene: ethyl acetate: formic acid (5: 5: 0.5) (v/v) and TLC plates precoated with $60F_{254}$ silica gel were used as the stationary phase. Detection was carried out densitometrically using a UV detector at 540 nm after spraying with Anisaldehyde – Sulphuric acid Reagent (ASR). The retention factor of beta-sitosterol-D-glucoside was 0.47. Linearity was obtained in the range of 1.0 to 3.5 µg per spot for beta sitosterol -D-glucoside. The method was précise and accurate as the RSD was < 2 %. The method was found to be sensitive, selective and robust.
The developed and validated HPTLC method was employed for standardization of methanol and petroleum ether extracts of Karela and seven different formulations containing Ashwagandha for the content of the beta-sitosterol-D-glucoside..

KEYWORDS: Karela, Ashwagandha, *Momordica charantia*, *Withania somnifera*, Beta-sitosterol-D-glucoside, HPTLC

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INTRODUCTION

Ayurveda emphasis the relationship between man and plants throughout the development of human culture. Herbal medicines as the major remedy in traditional system of medicine have been used in medical practices since antiquity. The practice continues today because of its biomedical benefits as well as place in cultural beliefs in many parts of world and have made a great contribution towards maintaining human health.[1]

In olden times, Vaidyas used to treat patients on individual basis, and prepare drug according to the requirement of the patient. But the scenario has changed now; herbal medicines are being manufactured on the large scale in Pharmaceutical units, where manufacturers come across many problems such as availability of good quality raw material, authentication of raw material, availability of standards, proper standardization methodology of single drugs, extracts and formulation, quality control parameters etc.[2]

It is necessary to develop methods for rapid, precise and accurate identification and estimation of active constituent/s or marker compound/s as the qualitative and quantitative target to assess the authenticity and inherent quality[3-4]. Through various analytical techniques like TLC, HPLC and HPTLC we can ascertain the presence of these compound/s in plants and also quantify them. HPTLC offers many advantages over other chromatographic techniques such as unsurpassed flexibility (esp. stationary and mobile phase), choice of detection wavelength, user friendly, rapid and cost effective[5]. Thus, HPTLC is most widely used at industrial level for routine analysis of herbal medicines.

Beta sitosterol D- glucoside is a phytosterol and it is present in *Momordica charantia* Linn. as well as in *Withania somnifera* (L.) Dunal and many other plants. Beta sitosterol D- glucoside has many pharmacological activities like androgenic, antiadenomic, anticancer, antiedemic, antiinflammatory etc[6-13]. This compound is biologically active and thus it can be considered as a biomarker. So far there is no analytical method reported for estimation of beta sitosterol D- glucoside in plant products. Therefore, there is a need of simple and rapid analytical method for the manufacturers of Ayurvedic medicines using beta sitosterol D- glucoside as marker compound. Thus, the objective of the present work was to develop and validate a High Performance Thin Layer Chromatography method for estimation of beta-sitosterol-D-glucoside in various herbal products.

MATERIALS AND METHODS

Solvents and chemicals

Standard beta sitosterol-D-glucoside and commercial methanol and petroleum ether extracts of *Momordica charantia* Linn. were supplied by Amsar Pvt. Ltd., India as a gift sample. Traditional formulations containing Ashwgandha such as galenicals –Arishta (two brands), powders- Churna (two brands), tablet form- Vati, and even modern dosage forms such as Capsules and Tablets were procured from the local market. All chemicals and reagents used were of analytical grade and purchased from Rankem and S. D. Fine Chemicals, India.

HPTLC instrumentation

The sample solutions were spotted in the form of bands of width 8 mm with a Camag microlitre syringe on precoated silica gel aluminum plate $60F_{254}$ (20 cm × 10 cm with 250 µm thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai) using a Camag Linomat V applicator (Switzerland). The plates were pre-washed by methanol and activated at 60°C for 5 min prior to chromatography. The slit dimension was kept at 5mm × 0.45 mm and 10 mm/s scanning speed was employed. The slit bandwidth was set at 20 nm, each track was scanned thrice and baseline correction was used. The mobile phase consisted of toluene: ethyl actetate: formic acid (5: 5: 0.5) (v/v) and 15 ml of mobile phase was used per chromatography. 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 optimized chamber saturation time for mobile phase was 30 min at room temperature (25 °C ± 2) at relative humidity of 60% ± 5. The length of chromatogram run was 8 cm. Subsequent to the scanning, TLC plates were dried in a current of air with the help of an air dryer. Densitometric scanning was performed with Camag TLC scanner III in the reflectance-absorbance mode at 540 nm after spraying with Anisaldehyde – Sulphuric acid reagent (ASR) and operated by Win CATS software (1.3.0 Camag).

Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light. Evaluation was carried out by comparing peak areas with linear regression.

Standard solutions

Stock solutions of reference standard beta-sitosterol-D-glucoside standard were prepared by dissolving 10.0 mg of beta-sitosterol-D-glucoside in 10 mL methanol, yielding stock solution of concentration = 1 mg mL^{-1}. From this, 0.1, 0.3, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 µ L of solution were applied using LINOMAT 5 applicator with the band width of 8 mm., which gave different concentration ranging 0.1-3.5 µg / spot respectively.

Sample solutions

For *Momordica charantia* Linn Extracts

2g of methanolic and petroleum ether extract each was transferred to 100 ml volumetric flask containing 50 mL of methanol and petroleum ether (40-60°C) respectively and the extract was macerated on a shaker for 24 hrs at room temperature. The volume was madeup to 100 mL with respective solvents and then filtered. 1.0 mL of this extract was diluted to a 10 mL with methanol and petroleum ether (40-60°C) respectively.

For Ashwagandha vati

2 g of powdered vati was transferred to 100 mL volumetric flask containing 50 mL of methanol and the mixture was macerated on a shaker for 24 hrs at room temperature. The volume was madeup to 100 mL with methnaol and then filtered. 1.0 mL of this extract was diluted to a 10 mL with methanol.

For Ashwagandha Chruna

Two brands of churna namely Brand I and Brand II were analyzed.

2 g of chruna (each brand) was transferred to 100 mL volumetric flask containing 50 mL of methanol and the mixture was macerated on a shaker for 24 hrs at room temperature. The volume was madeup to 100 mL with methnaol and then filtered. 1.0 mL of this extract was diluted to a 10 mL with methanol.

For Ashwagandha Capsule

Sample solutions of capsule formulation were prepared same as that of vati by transferring 2 g of capsule contents.

For Ashwagandha Tablet

Sample solutions of tablet formulation were prepared by transferring 2 g of powdered tablet to 100 mL volumetric flask containing 50 mL of methanol and the following the procedure was same as that of vati.

For Ashwagandharishta

Two brands of ashwagandharishta namely Brand A and Brand B were analyzed and the sample preparation was as follows:

10 mL of ashwagandharishta (each brand) was evaporated to dryness. 2 g of residue was dissolved in 100 mL methanol in a volumetric flask. 2.5 mL of this extract was diluted to 10 mL with methanol.

A constant application rate of 2.0 µl/s was employed for all the sample solutions.

ASSAY VALIDATION

The proposed HPTLC method was validated according to the International Conference on Harmonization (ICH) guidelines [14-22]. All measurements were performed in triplicates.

Calibration studies

Linearity was evaluated in the range of 0.5 - 3.5 µg/ spot for beta sitosterol-D-glucoside. Peak area versus concentration was subjected to least square linear regression analysis and the slope, intercept and correlation coefficient for the calibration were determined. Limit of detection (LOD) and limit of quantitation (LOQ) were determined from the calibration curve using the following expressions: $3\sigma/S$ and $10\sigma/S$, where σ is the standard deviation and S is the slope of the calibration curve.

Precision studies

Precision of the method was evaluated by repeatability (intra-day) and instrumental precision. Each level of precision was investigated by three sequential replicates of injections of beta sitosterol-D-glucoside at concentrations of 1.5, 2.0 and 2.5 µg / spot.

Accuracy studies

In order to evaluate the validity of the proposed method, accuracy was evaluated through the percentage recoveries of known amounts of beta sitosterol-D-glucoside added to solutions of extracts and all commercial products. The analyzed samples were spiked with 80, 100 and 120 % of 2.5µg of beta sitosterol-D-glucoside standard solution. Accuracy was calculated from the following equation:
[(spiked concentration − mean concentration)/spiked concentration] × 100.

Robustness

For the determination of the robustness of method, chromatographic parameters, such as mobile phase composition and detection wavelength, were intentionally varied to determine their influence on the retention time and quantitative analysis.

Stability studies

Stability of the sample solutions was tested after 24, 48 and 72 hours after preparation and storage at 4.0°C and 25.0°C separately. Stability was assessed by comparing the chromatographic parameters of the solutions after storage with the same characteristics of freshly prepared solutions.

RESULTS

Method optimization

The proposed method gave very good separation and resolution of the standard beta sitosterol-D-glucoside (R_f value = 0.47) as indicated in **(Fig. 1).**

Method validation

Linearity, limit of detection and limit of quantitation

Under the above described experimental conditions, linear correlation between the peak area and applied concentration was observed in the concentration range of 0.5 - 3.5 µg / spot of beta-sitosterol-D-glucoside. The correlation coefficient of beta sitosterol-D-glucoside was found to be 0.998. The peak area (y) is proportional to the concentration of beta-sitosterol-D-glucoside (x) following the regression equation y = 0.5326x + 4.4055 **(Fig 2)**. The experimentally derived LOD and LOQ of beta sitosterol-D-glucoside were determined to be 0.10 and 0.30 µg /spot respectively.

Precision

Precision data on repeatability (intra-day) and instrumental variation for three different concentration levels are summarized in **Table 1**. Precision studies showed R.S.D. less than 1%, indicating a sufficient precision.

Accuracy

All the samples of extracts and formulations were spiked with the known amount of standard, and the percent ratios between the recovered and expected concentrations were calculated. Desirable recoveries of 97.07-109.52 % beta-sitosterol-D-glucoside indicate that the proposed HPTLC method is reliable for the quantification of beta-sitosterol-D-glucoside in two Karela extracts and seven Ashwagandha formulations (**Table 2)**.

Robustness

The mobile phase composition was altered by ± 2 % changes in the ratio of ethyl acetate and formic acid and also in chamber saturation time. No changes were observed in retention time and peak shape. Thus the method was found to be robust.

Analysis of Ashwagandha formulations

Validity of the proposed method was applied to standardization for methanol and petroleum ether extracts of Karela and both traditional and modern dosage forms viz. Ashwagandha Vati, Arishta Brand I and Brand II, Churna Brand A and Brand B, Tablet and Capsule. The shape of the peaks was not altered by other substances present in the matrix. The percent content of beta-sitosterol-D-glucoside for the two extracts of Karela and all the seven formulations of Ashwagandha are indicated in **Table 3**.

Stability studies

Stability of beta-sitosterol-D-glucoside in the sample solutions was evaluated to verify whether spontaneous degradation occurred within 3 days. The results were calculated as the percentage of non-degraded content of beta-sitosterol-D-glucoside standard at the 21, 48, 72 hours. All extracts and

formulations showed more than 98 % of non degraded content i.e, less than 5% degradation at both investigated temperature (**Table 4, 5**).

DISCUSSION

The simple, précise, accurate and reproducible HPTLC method was successfully developed and validated for analysis of herbal products containing beta-sitosterol-D-glucoside. This method enabled to detect and quantified the amount of beta-sitosterol-D-glucoside as least as 0.343% to as high as 10.736 % in two different plant products.

Petroleum ether extract of Karela contains higher amount of beta-sitosterol-D-glucoside compared to methanol extract and Ashwagandha formulations. The Ashwagandha formulations showed good measureable amount of beta-sitosterol-D-glucoside using this method.

This method can be employed for analysis of various herbal products such as extracts and formulations containing beta-sitosterol-D-glucoside. The method can be applied by the herbal manufacturers to estimate Beta-sitosterol-D-glucoside in their products as routine quality control method and to keep a check on to the batch to batch variations.

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Table 1: Precision Studies for Beta-sitosterol-D-glucoside

	Concentration (µg/spot)	(%RSD)
Intra- day precision	1.5	0.19
	2.0	0.18
	2.5	0.07
Inter- day precision	1.5	0. 08
	2.0	0.10
	2.5	0.11

Table 2: Recovery studies for Beta-sitosterol-D-glucoside in various herbal products

Extracts & Formulations	Amount added (% of 2.5 µg)	Recovery ± S.D. (%)
Methanol extract of Karela	80	109.52 ±0.12
	100	109.49 ±0.33
	120	109.11 ±0.58
Petroleum ether extract of Karela	80	101.46 ±0.54
	100	101.10 ±0.14
	120	101.84 ±0.41
Vati	80	105.77 ±0.02
	100	105.01 ±0.54
	120	105.15 ±0.17
Capsules	80	99.82 ±0.56
	100	99.73 ±0.94
	120	99.05 ±0.05
Tablets	80	97.33 ±019
	100	96.99 ±0.11
	120	97.07 ±0.23
Churna (Brand A)	80	109.08 ±0.09
	100	109.29 ±0.63
	120	109.14 ±0.58
Churna (Brand B)	80	107.22 ±0.22
	100	107.12 ±0.44
	120	107.49 ±0.56
Arishta (Brand I)	80	101.08 ±0.98
	100	101.65 ±0.45
	120	101.58 ±0.66
Arishta (Brand II)	80	103.33 ±0.12
	100	103.29 ±0.86
	120	103.07 ±0.52

Table 3: Percent Content of Beta-sitosterol-D-glucoside in various herbal products

Extracts & Formulations	Percent Content ± S.D. (%)
Methanol extract of Karela	8.108±0.87
Petroleum ether extract of Karela	10.736±0.10
Vati	0.670±0.05
Tablet	0.343 ±0.71
Capsule	0.351±0.68
Churna (Brand A)	0.748±0.25
Churna (Brand B)	0.698±0.97
Arishta (Brand I)	0.541 ±0.81
Arishta (Brand II)	0.582 ±0.16

Table 4: Stability Studies of Beta-sitosterol-D-glucoside in formulations in various herbal products

Extracts & Formulations	Temperature					
	4°C			25°C		
	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
Methanol extract of Karela	99.86	99.23	98.11	98.49	98.11	98.01
Petroleum ether extract of Karela	99.90	99.81	98.69	98.61	98.06	98.70
Vati	99.01	98.97	98.07	98.89	98.09	98.01
Tablet	99.27	98.89	98.37	99.03	98.72	98.03
Capsule	99.89	99.01	98.59	98.61	98.06	98.70
Churna (Brand A)	99.61	99.13	98.35	98.92	98.37	98.11
Churna (Brand B)	99.54	99.00	98.14	98.82	98.39	98.17
Arishta (Brand I)	99.81	99.02	98.72	99.09	98.73	98.51
Arishta (Brand II)	99.67	98.15	98.88	99.86	98.55	98.19

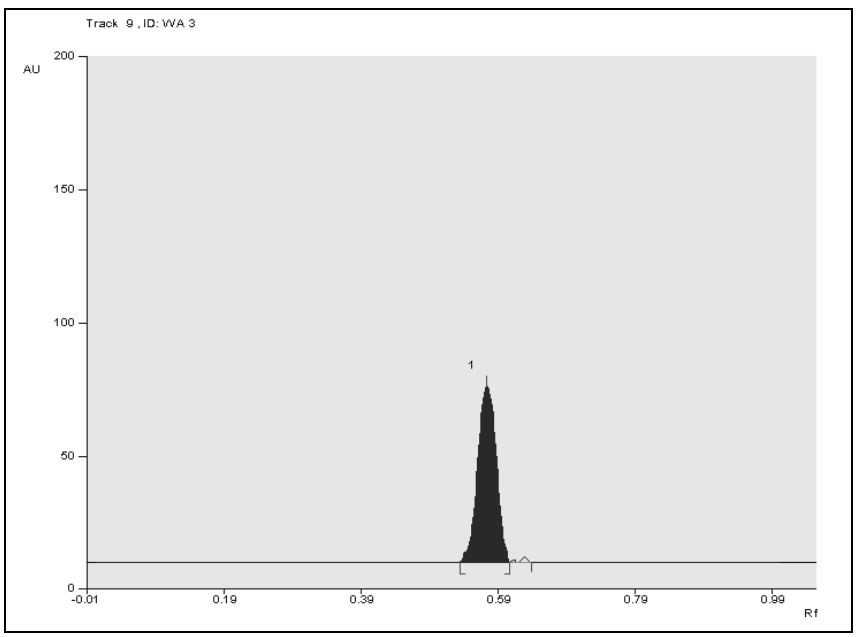

Figure 1: Chromatogram of standard Beta-sitosterol-D-glucoside

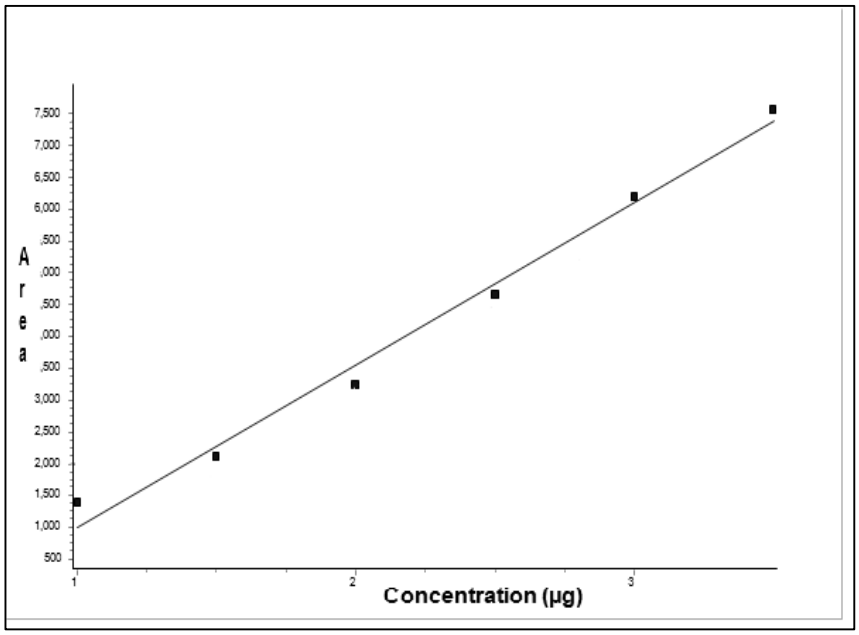

Figure 2: Calibration curve of Beta-sitosterol-D-glucoside

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