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High performance thin layer chromatographic method for quantification of β-sitosterol from *Vanda roxburghii* R.Br.

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ABSTRACT

The roots of Vanda roxburghii R.Br. (family Orchidaceae), known as 'Rasna' in the Ayurvedic system of medicine, are used to treat a variety of ailments, including rheumatism, dyspepsia, bronchitis, inflammations, diseases of the abdomen, hiccough and tremors and as an antipyretic agent. Chemically, V. roxburghii R.Br. has been reported to contain β-sitosterol, γ-sitosterol, heptacosane, octacosanol, acetyl tetracosylferulate, 17-β-hydroxy-14,20-epoxy-1-oxo-[22R]-3β-[O-β-D-glucopyranosyl]-5,24-withadienolide and melianin. Sterols are anti-inflammatory agents. β-sitosterol has been shown to possess anti-inflammatory and anti-pyretic properties. In this context, a method for quantitative estimation of β-sitosterol in the methanolic extract of the roots of Vanda roxburghii R.Br. has been developed. The HPTLC method used for the standardization was validated for the parameters like specificity, limits of detection and quantification, linearity, precision, accuracy and recovery. The content of β-sitosterol in the methanolic extract was found to be 0.016 %w/w.

Keywords: β-sitosterol, HPTLC, Methanolic extract, Standardization.

INTRODUCTION

Herbs are staging a comeback and herbal 'renaissance' is happening all over the globe. The herbal products today symbolise safety in contrast to the synthetics that are regarded as unsafe to human and environment. Among ancient civilisations, India has been known to be rich a repository of medicinal plants. In India, drugs of herbal origin have been used in traditional systems of medicines such as Unani and Ayurveda since ancient times [1]. A major lacuna in Ayurveda is the lack of drug standardisation, information and quality control. In the absence of pharmacopoeia data on the various plant extracts, it is not possible to isolate or standardise the active contents having the desired effects [2].

The roots of *Vanda roxburghii* R.Br. commonly called as Rasna, belonging to family *Orchidaceae* are widely used in Ayurvedic medicine for treatment of rheumatoid arthritis. It is also useful in dyspepsia, bronchitis, inflammations, diseases of the abdomen, hiccough and tremors and as an antipyretic agent [3]. *V. roxburghii* R.Br. has been reported to contain β-sitosterol (**Figure 1**), γ-sitosterol, heptacosane, octacosanol, acetyl tetracosylferulate [4], 17-β-hydroxy-14,20-epoxy-1-oxo-[22R]-3β-[O-β-D-glucopyranosyl]-5,24-withadienolide [5] and melianin [6]. Sterols are anti-inflammatory agents. β-sitosterol has been shown to possess anti-inflammatory and anti-pyretic properties [7].

Hence the quantification of β-sitosterol content in *V. roxburghii* R.Br. roots is done, which provides a therapeutically active chemical marker to standardize this drug.

This paper reports standardization of methanolic extract of roots of *V. roxburghii* R.Br. containing β-sitosterol as biomarker by HPTLC technique.

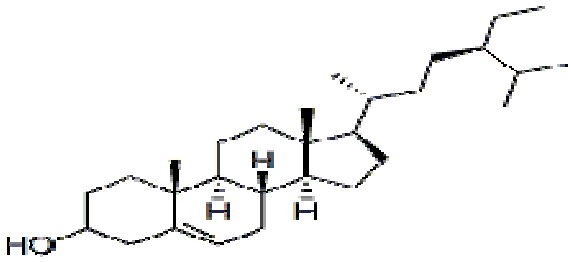

Figure 1: Structure of β-sitosterol.

MATERIALS AND METHODS

Materials

Dried roots of *V. roxburghii* R.Br. were procured from the local market in Mumbai and authenticated at Agharkar Research Institute, Pune. The voucher specimen (Ref. No. AHMA R 096) has been deposited in the institute for reference. Standard β-sitosterol was procured from Sigma Aldrich. Toluene, chloroform, methanol, sulphuric acid and ethanol used were of AR grade from S.D. Fine Chemicals.

Sample Preparation

The crude drug was air dried and pulverized to obtain a coarse powder. The powdered drug was defatted by extracting with petroleum ether (60-80^{0}C) followed by extraction with methanol using Soxhlet extractor. The extract obtained was concentrated by recovering the solvent by Rotary Flash Evaporator. The concentrated extract was then evaporated to dryness in vacuum oven at temperature not more than 50^{0}C. The dried extract was stored at 2-8^{o}C in refrigerator. 20 mg of the methanolic extract was accurately weighed, placed in 10ml volumetric flask, and was diluted with methanol upto the mark. This solution of methanolic extract of concentration 2 mg/ml was used for the experiment.

HPTLC

Chromatography was performed on aluminum-sheets precoated with silica gel 60 F_{254} HPTLC plates (Merck # BOL790330). Before use, the plates were prewashed with methanol, dried, and activated at 110ºC for 1 hour between two glass plates of larger dimensions to prevent deformation of the plates. Sample (20 µl) was applied to the plates, as 6-mm bands, 10 mm apart and 10 mm from the edge of the plates, by means of a Camag Linomat V sample applicator fitted with a 100 µl Hamilton syringe. After drying of the spots, the plates were developed to a distance of 80 mm in a Camag twin-trough chamber previously saturated with mobile-phase vapor for 20 min. The mobile phase was chloroform : toluene in the ratio 9.5 : 0.5 (v/v). After development, the plates were dried under current of air at room temperature and derivatized with freshly prepared Liebermann-Burchard reagent in a derivatization chamber for 20 seconds and dried at room temperature. After drying, plates were heated in an oven at 105ºC for 10 minutes before densitometric scanning. Densitometric evaluation of the plates was performed at λ = 366 nm, using a mercury lamp, with a Camag Scanner III in conjunction with winCATS Planar Chromatography Manager software, version 1.4.4.6337 for quantification. The scanning wavelength was 366 nm, the scanning speed 20 mm/s, the offset 10%, and the sensitivity (SPAN) was optimized automatically. Peak heights and peak areas were integrated for the entire track. Under these conditions, a proper separated peak for β-sitosterol was obtained at R_f = 0.21. The photographed chromoplate is shown in **Figure 2**. The overlay of the chromatograms obtained for standard β-sitosterol, methanolic extract of roots of *V. roxburghii* R.Br. and the spiked methanolic extract using the above procedure are given in **Figure 3.**

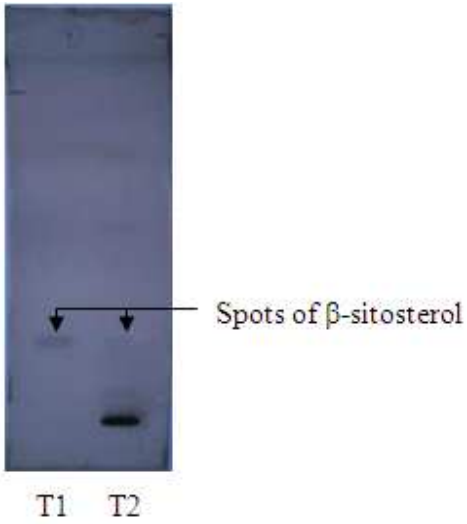

Figure 2: Photographed chromoplate after derivatization with Liebermann-Burchard reagent.

T1: standard β-sitosterol, T2: methanolic extract of roots of V. roxburghii R.Br.

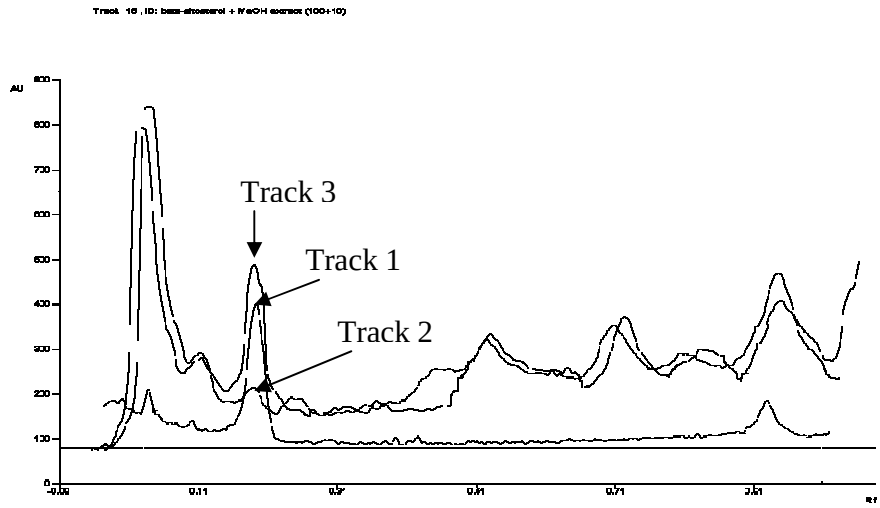

Figure 3: Superimposition of the HPTLC fingerprints of the chromatograms obtained for standard β-sitosterol, methanolic extract of roots of *V. roxburghii* R.Br. and the spiked methanolic extract.

The HPTLC plate was developed with chloroform : toluene in the ratio 9.5 : 0.5 (v/v), as mobile phase and detection was at λ = 366 nm. Tracks: 1, standard β-sitosterol; 2, methanolic extract of roots of *V. roxburghii* R.Br.; 3, spiked methanolic extract.

Validation of the HPTLC Method by Use of Calibration Standard of β-sitosterol

Specificity

A methanolic solution of reference standard of β-sitosterol (Sigma Aldrich Co., USA, 200 µg/ml, 10 µl) and methanolic extract solution in methanol (1mg/ml, 10 µl) was applied to a silica gel GF_{254} HPTLC plate and the plate was developed using the mobile phase and conditions described above. Under all the conditions mentioned above, a single spot of β-sitosterol at the R_f = 0.21. Peak purity tests were also performed for the spectra of the spot. Methanol (5 µl) was also spotted in parallel as a control. No additional peak was observed at the R_f of β-sitosterol.

Limits of Detection and Quantification using β-sitosterol

Reference standard solution (10 µg/ml) of β-sitosterol was chromatographed to determine the limits of detection and quantification. The limits of detection (LOD, n = 15, on the basis of the ratio of noise to peak height) and quantification (LOQ, n = 10, on the basis of the ratio of noise to peak-area) for β-sitosterol were determined by considering signal-to-noise ratio 3:1 and 10:1 for LOD and LOQ respectively. In densitometric scanning, the scanner automatically optimized the sensitivity on the basis of the highest concentration; accordingly, the corresponding limits of detection were fixed.

Linearity

In order to establish linearity, solution (200 µg/ml) of reference standard β-sitosterol was applied in triplicate to a HPTLC plate at six different concentrations 10, 20, 30, 40, 50 and 60 µl containing 2, 4, 6, 8, 10 and 12 µg respectively. The plate was developed, the spots were derivatized, and the detector response for the different concentrations was measured. The experiment was performed in triplicate and the mean was calculated. A graph was plotted of drug peak area against concentration of β-sitosterol (**Figure 4**).

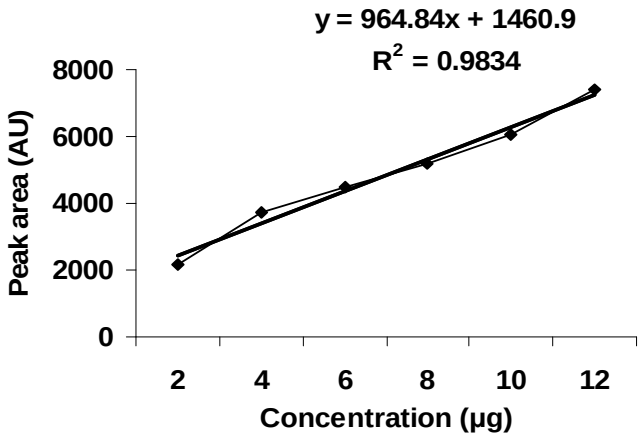

Figure 4: Linear regression calibration plot for β-sitosterol.

Precision

Intra-day and inter-day precision were determined by applying solutions 10, 20, 30, 40, 50 and 60 µl containing 2, 4, 6, 8, 10 and 12 µg respectively of β-sitosterol reference standard solution (200 µg/ml) to a HPTLC plate. The plate was then developed, derivatized and scanned densitometrically as described above and peak area was measured.

Accuracy and Recovery

The accuracy of the method at low, medium, and high concentrations was determined by sixfold replicate application and chromatography of 20 µl (4 µg), 40 µl (8 µg), and 60 µl (12 µg) of solution of β-sitosterol reference standard (200 µg/ml).

Reference standard solution (10 µl containing 200 µg/ml) of β-sitosterol was added to solution (100 µl containing 1000µg/ml) of the methanolic extract of roots of *V. roxburghii* R.Br. This was considered as the spiked methanolic extract. HPTLC analysis was performed for both, the spiked extract and solution (100 µl containing 1000 µg/ml) of the methanolic extract of roots of *V. roxburghii* R.Br. Recovery was calculated by use of the equation:

Recovery = [(A – B)/C] × 100
Note: A - quantity of β-sitosterol in the spiked extract
B - quantity of β-sitosterol in the methanolic extract without added standard
C - quantity of β-sitosterol added.

RESULTS AND DISCUSSION

An HPLTC method was established to quantify β-sitosterol in the methanolic extract of roots of *V. roxburghii* R.Br. The presence of β-sitosterol was verified by comparison of R_f = 0.21, by co-elution, and by comparison of the UV spectra obtained from the sample and the standard (**Figure 5**). The amount of β-sitosterol present in the methanolic extract was calculated from the calibration curve. The methanolic extract of the roots of *V. roxburghii* R.Br. was found to contain 0.016 %w/w of β-sitosterol.

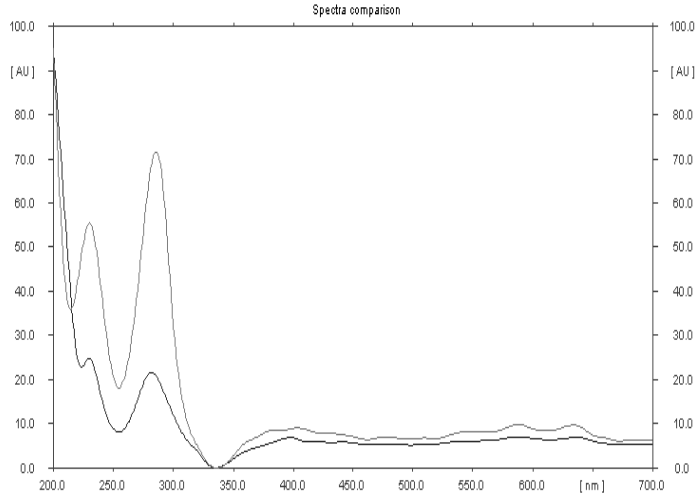

Figure 5: Superimposition of the UV spectra of β-sitosterol obtained from standard β-sitosterol and methanolic extract of roots of *V. roxburghii* R.Br.

Validation of the Method

Before use, plates were prewashed with methanol, dried, and activated to reduce error resulting from adsorption of volatile impurities from the environment, including the atmosphere. The specificity of the method was established by analysis of the methanolic extract of *V. roxburghii*, reference standard of β-sitosterol and control, i.e. methanol. Because no interfering peak originating from the control was observed on scanning at λ = 366 nm at the R_f value of β-sitosterol, the method was deemed to have been validated for specificity. Also, peak-purity test performed for the spectra of the spot did not reveal the presence of coeluting peaks at the R_f of β-sitosterol.

The limit of detection and quantification for β-sitosterol were found to be 20 ng (signal-to-noise ratio 3:1) and 40 ng (signal-to-noise ratio 10:1), respectively.

Plot of peak area against concentration of β-sitosterol was linear in the range of 2 to 12 µg with regression coefficient 0.9834.

Intra-day precision (RSD) for peak area ranged between 4.1 and 7.6 and inter-day precision (RSD) ranged between 3.9 and 8.8.

The determination of accuracy of the method at low (4 µg), medium (8 µg), and high (12 µg) concentrations resulted in relative standard deviations (RSD) of 7.7, 2.5 and 6.3. The accuracy of the method was also established by means of a recovery experiment. The mean recovery was close to 100 %, which indicates the accuracy of the method.

Table 1: Validation Summary.

Parameter	Result
Limit of Detection	20 ng
Limit of Quantification	40 ng
Linearity range	2 to 12 µg
Recovery	99.13 %

Table 2: Inter- and intra-day precision for quantification of β-sitosterol.

Concentration (µg)	Precision (RSD)	
	Intra-day (n = 6)	Inter-day (n = 6)
2	7.6	8.8
4	6.3	7.1
6	4.5	4.4
8	4.1	3.9
10	7.5	7.2
12	7.2	6.3

Table 3: Accuracy for quantification of β-sitosterol.

Concentration (µg)	RSD
4	7.7
8	2.5
12	6.3

CONCLUSION

A procedure for quantification of β-sitosterol, which can be used as biomarker in a validated HPTLC method, has been described in this paper. The HPTLC method was developed for the quantification of β-sitosterol in the methanolic extract of *V. roxburghii* R.Br. and validated for specificity, linearity, recovery and repeatability. The LOD and LOQ were also determined. This validated method enabled to determine the quantity of β-sitosterol – 0.016 % w/w in the methanolic extract of roots of *V. roxburghii* R.Br. It is suitable for the application of routine quality control analysis and quantification of β-sitosterol in the methanolic extract of roots of *V. roxburghii* R.Br.

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