Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 5, 2014

Original Article

IDENTIFICATION, QUANTIFICATION AND VALIDATION OF β-SITOSTEROL FROM *HOLOPTELEA INTEGRIFOLIA* (Roxb.) PLANCH USING HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY METHOD

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Received: 12 Mar 2014 Revised and Accepted: 02 Apr 2014

ABSTRACT

Objective: The Present study was designed to develop a new simple, precise, rapid and selective high-performance thin-layer chromatographic (HP TLC) method for the determination of beta-sitosterol in methanolic leaf extract of *holoptelea integrifolia* (Roxb.) Planch.

Methods: As per ICH guidelines we have applied different concentrations of beta-sitosterol as standard on HPTLC plates for the quantification of beta sitosterol from the plant extract. Concentration of standard beta-sitosterol was 0.1µg/µl

Results: The retention factor of beta-sitosterol was 0.38. Linearity was obtained in the range of 100 ng-500 ng for beta sitosterol. The developed and validated HPTLC method was employed for beta-sitosterol in methanolic leaf extract of *holoptelea integrifolia* (Roxb.) Planch for Standardization of the content of the marker. Satisfactory recoveries of 85.0 -86.0 % were obtained for beta-sitosterol.

Conclusion: The results obtained in validation assays indicate the accuracy and reliability of the developed HPTLC method for the quantification of beta-sitosterol in methanolic leaf extract of *holoptelea integrifolia* (Roxb.) Planch.

Keywords: *holoptelea integrifolia* (Roxb.) Planch, Beta-sitosterol, HPTLC

INTRODUCTION

Nature has blessed mankind with a treasure of medicinal plants. Natural products have always remained a profile source for the discovery of new drugs and are used since Vedic period [1].

Ayurveda has been a vibrant system of health care in India and has been practiced since 6000 years but growth as an industry has commenced only a few years back. India's share in the global exports of herbal medicines is at around 10 per cent only which is low. Therefore, there is a need to transform Ayurveda into a dynamic, scientifically validated and proof based industry which takes its roots from rich knowledge base of old tradition and solemn [2-4].

It is necessary to develop methods for rapid, precise and accurate identification and estimation of active constituents or marker compound/s as the qualitative and quantitative target to assess the authenticity and inherent quality [5,6].Through various analytical techniques like TLC, HPLC and HPTLC we can ascertain the presence of these compounds 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, user friendly, rapid and cost effective [7]. Thus, HPTLC is most widely used at industrial level for routine analysis of herbal medicines. *Holoptelea Integrifoila* belongs to the family ulmaceae commonly called as Indian Elm and commonly used in India by the tribal people for it's medicinal properties. The mucilaginous bark is boiled and the juice squeezed out and applied to rheumatic swellings[8]. In traditional system of medicine, bark and leaves of *Holoptelea Integrifoila* used as bitter, astringent, acrid, thermogenic, anti-inflammatory, digestive, carminative, laxative, anthelmintic, depurative, repulsive, urinary astringent and in rheumatism [9,10]. The plant *Holoptelea integrifolia* is used traditionally for the treatment of inflammation, gastritis, dyspepsia, colic, intestinal worms, vomiting, wound healing, leprosy, diabetes, haemorrhoids, dysmenorrhoea and rheumatism[11,12]. By considering the demand of this plant, there is a need of simple and rapid analytical method.

for the manufacturer of plant based medicines. Thus, the objective of the present work was to Identify, Quantify and validate a High Performance Thin Layer Chromatography method For estimation of the biomarker beta-sitosterol present in Holoptelea Integrifolia (Roxb.) planch.

MATERIALS AND METHODS

Materials

Standard and reagents

β-sitosterol (purity 98%), was purchased from Sigma-Aldrich Chemie GmbH (Aldrich, Division, Steinbeim, Germany). Ethyl acetate, toluene, glacial acetic acid, methanol and sulphuric acid used in the present research work were of HPLC grade and were procured from E. Merck Mumbai, India.

Plant material

The leaves of *Holoptelea Integrifolia* (Roxb.) planch were collected in the Month of August from the agricultural fields of Tirunelveli district, Tamil Nadu, India. The plant was identified and leaves of *Holoptelea Integrifolia* were authenticated and confirmed from Dr.V.Chelladurai, Research Officer, Botany, C.C.R.A.S. (Retired), Govt of India by compairing morphological features (leaf and stem arrangement, flower /inflorescence arrangement, fruit and seed morphology etc.). The leaves of *Holoptelea Integrifolia* (Roxb.) planch ., were dried in a preset oven at 45°C, and powdered using motor and pestle and then sieved through BSS mesh size 85 and stored at 25°C, in an airtight container.

HPTLC instrumentation

Chromatographic conditions

The sample solutions were spotted in the form of bands of width 8.0 mm with a Camag microlitre syringe on precoated silica gel aluminium plate 60F254 (20 cm × 10 cm with 250 µm thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai) using a Camag Linomat V (Switzerland). The plates were prewashed by methanol and activated at 120°C for 20 min

prior to chromatography. A constant application rate of 1.0 µl/s was employed and space between two bands was 5 mm. The slit dimension was kept at 6.0mm × 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 acetate - glacial acetic acid, in the volume ratio of 8:2:0.2. (v/v) and 20 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, Mut tenz, Switzerland) saturated with filter paper whatman no :1 in the mobile phase.

The optimized chamber saturation time for mobile phase was 20 min at room temperature (25^0 C ± 2) at relative humidity of 60% ± 5. The length of chromatogram run was 8.0 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 IV in the reflectance absorbance mode at 540 nm and operated by Win CATS software (1.4.6 Camag) with the help of tungstant lamp. Subsequent to the development; TLC plate was dipped in Anisaldehyde sulphuric acid reagent followed by drying in oven at 110°C. 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.

Preparation of standard solutions of β-sitosterol

Prepration of Standard stock solutions concentration (1.0µg/µl) was prepared in 10 mL standard volumetric flask, by dissolving 10.0 mg of accurately weighed beta sitosterol, in about 5.0 mL of methanol, followed by vortex and finally making up the volume of solution to 10.0 mL, with methanol. 1.0 mL of the above stock solution was diluted to 10.0 mL, with methanol to give standard solution of beta-sitosterol, with concentration of 0.1µg/µl.

Sample solutions

Sample solutions were prepared by transferring 250 mg of extract into a 5.0 mL methanol was taken in a dry, clean stoppered test tube (capacity 20 mL). To the above stoppered test tube, 10mL of methanol was added and the test tube was shaken at 20 rpm, on a rotary shaker at room temperature (28°C±2°C) for 24 hrs. The contents of tube were then filtered through Whatman filter paper No. 41. The filtrate obtained was used as sample solution for the further HPTLC analysis

Method validation

The proposed HPTLC method was validated according to the International Conference on Harmonization guidelines [13-22]. All measurements were performed in triplicates.

Linearity

The linearity of the method was tested by applying standard β-sitosterol solution from, 100 ng to 500 ng to silica gel alumina plates using above chromatographic condition. The densitograms were recorded and the peak areas of β-sitosterol for each applied concentration were noted. The response factors were calculated for each concentration of β-sitosterol by dividing peak areas by corresponding concentration of beta-sitosterol. The results indicated in Table 1.0, show that within the concentration range indicated, there was a good correlation between mean peak area and concentration of beta-sitosterol.

Concentration µg/ml.

Limit of detection (LOD) and Limit of quantitation (LOQ)

The limit of detection was determined at a signal to noise ratio of 3:1. The limit of quantitation was determined at a signal to noise ratio of 10:1. The LOD and LOQ values obtained are listed in Table 1.

Calibration graph

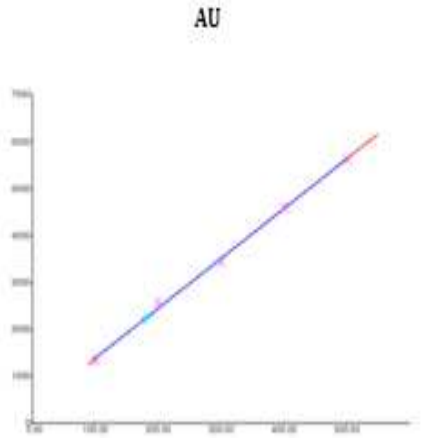

Fig. 1: Calibration curve of beta-sitosterol

Table 1: Results of Method validation parameters for the estimation of β-sitosterol by the proposed HPTLC Method

Parameter	Results
Linear range (g/mL)	35.00 to 100.00
Correlation coefficient (r)	0.9989
LOD (g/mL)	35.00
LOQ (g/mL)	100.0
Instrumental precision	0.482 % R.S.D.
Intra-assay precision	0.462 % R.S.D.
Intermediate precision	0.643% R.S.D.

Table 2: Results of Recovery of beta sitosterol from the leaves of *Holoptelea Integrifolia* (Roxb.) planch .

level	Wt of the sample	Wt of std added (µg)	Mean amount of beta sitosterol found (µg/g)	Percentage recovery (%)
0	1.003	0	0.3081	85.33%
0.32	1.002	0.32	0.3068	
0.40	1.003	0.40	0.3046	

Where, Sample : *Holoptelea Integrifolia* (Roxb.) planch, Mean amount of betasitosterol found in 1.0 g of sample = 0.37 mg

Precision studies

Precision of the method was evaluated by repeatability (intra-day) and instrumental precision. Each level of precision was investigated by the instrumental precision was studied by analyzing the standard solution of β-sitosterol of concentration 500 ng,in ten replicates, of *Holoptelea Integrifolia* (Roxb.) planch respectively. The results expressed as % R.S.D. of peak area of β-sitosterol, are listed in Table 1. The results indicate that the method is precise and reproducible.

Accuracy Studies

The accuracy of the method was established by performing recovery experiments, using the standard addition method, at three different levels. About 500mg of powdered leaves of *Holoptelea Integrifolia* (Roxb.) planch was accurately weighed into each of the four stoppered test tubes. Known amounts (0.00 µg, 0.32 µg and 0.40 µg) of powdered β-sitosterol standard were added in solution form to each of the stoppered test tubes respectively and 10.00 mL, methanol was added to each test tube.

The stoppered test tubes were then shaken at 20 rpm on rotary shaker for overnight at room temperature (28°C ± 2°C). The contents of the test tube were filtered separately and each solution was analyzed seven times, under optimized chromatographic conditions. Value of percentage recovery for β-sitosterol was determined. The results of the recovery experiment are given in Table 2. Figure no 5 The value of percentage recovery of β-sitosterol is 85.33 indicating good accuracy of the method.

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.

Robustness

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..

System suitability

System suitability was carried out to verify that the resolution and reproducibility of the system were acceptable for the analysis. System suitability test was carried out by applying 5µl of standard solution of β- sitosterol solution of concentration 500ng on TLC plate in five replicates and analyzing under specified chromatographic conditions.

The parameters used to determine system suitability were repeatability of peak areas and retention factor of β-sitosterol for replicate analysis. The values of mean peak area of β-sitosterol, retention factor (Rf value) of β-sitosterol was found to be 5371.668 and 0.38 respectively with % R.S.D. value is 2.58.

Application of validated method for the quantification of β-sitosterol from *Holoptelea Integrifolia* (Roxb.) planch

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.

Amount of β-sitosterol present in the sample solution was determined from the calibration curve by using the peak area of β sitosterol in the sample solution. To ascertain the repeata ility of the method, the assay experiment was repeated seven times. Mean amount of β- sitosterol in dried seed powder from Holoptelea Integrifolia (Roxb.) planch was found to be 179.10 ng **.**

Solution stability

The stability of standard β-sitosterol solution was determined by comparing the peak areas of β- sitosterol solution, of concentration 500ng at different time intervals, for a period of minimum 48 hrs, at room temperature. The results showed that the peak area of β-sitosterol almost remained unchanged (% R.S.D. was less than 3) over a period of 48 hrs, and no significant degradation was observed within the given period, indicating the stability of standard solution of β-sitosterol, for minimum 48 hrs.

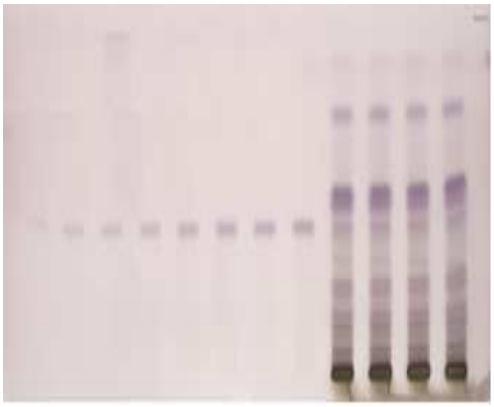

Fig. 2: High Performance Thin Layer Chromatography Separation of Standard β-sitosterol and Methanolic Extract of Dried leaves Powder of *Holoptelea Integrifolia* (Roxb.) planch .

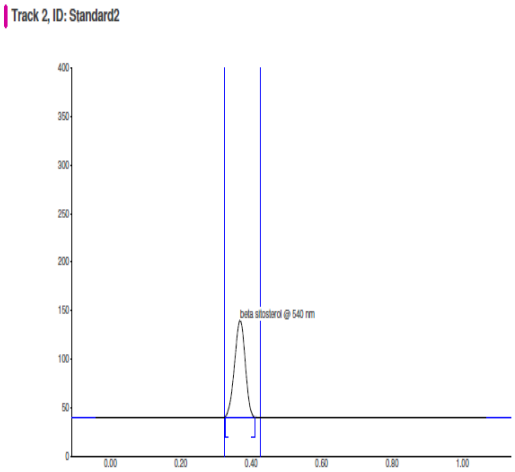

Fig 3: High Performance Thin Layer Chromatographic Determination of β-sitosterol A TypicalChromatogram of Standard β-sitosterol solution

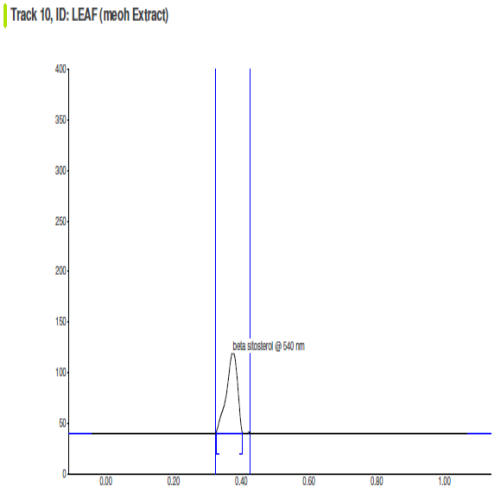

Fig 4:High Performance Thin Layer Chromatography Determination of β-sitosterol A Typical Chromatogram Obtained from Methanolic Extract of Dried leaves Powder of *Holoptelea Integrifolia* (Roxb.) planch .

Fig. 5: Recovery

RESULTS AND DISCUSSION

The identity of peak of β-sitosterol in sample solution was confirmed by comparing its Rf value with that of standard β-sitosterol (**0.38).** A good linear relationship was obtained for β-sitosterol, in the concentration range of 350ng to 100ng with correlation coefficient of 0.9986. The average percent recovery of β- sitosterol at three levels was 85.33 (Table 2). A simple, rapid, accurate and convenient method was developed for the first time, for estimation of beta-sitosterol by HPTLC. This method can be used to standardize different Ayurvedic medicines containing *Holoptelea Integrifolia* (Roxb.) planch . This developed and validated HPTLC method can be used to determine batch to batch variations and routine analysis by herbal manufacturers of *Holoptelea Integrifolia* (Roxb.) planch . formulations. Thus, these analytical standardization techniques facilitate manufacturers to market their plant based medicines with defined content of respective bioactives and to ensure its quality.

CONCLUSION

A new HPTLC method has been developed, validated and used for the quantification of β- sitosterol from the methanolic extract of dried leaves powder of *Holoptelea Integrifolia* (Roxb.) planch . The HPTLC method for the determination of β- sitosterol was validated in terms of linearity, precision, accuracy, system suitability, sample stability. The developed HPTLC technique is precise, specific, accurate, stability indicating and can be used for the routine quality control analysis and quantitative determination of β-sitosterol from *Holoptelea Integrifolia* (Roxb.) planch .

ACKNOWLEDGEMENT

The authors wish to thank Mr. Prashant S. Hande, Application Specialist and Anchrom Lab, Anchrom Test Lab Pvt. Ltd. Mulund (E), Mumbai. 400081 for his excellent and generous help for analyzing the HPTLC data.

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