ISSN 0975-6299

Vol 2/Issue 2/Apr-Jun 2011

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE

ANALYTICAL CHEMISTRY

QUANTITATION OF β-SITOSTEROL FROM *Celastrus paniculatus* Willd. USING VALIDATED HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY METHOD

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ABSTARCT

A sensitive high-performance thin-layer chromatographic method has been established for quantitation of β-sitosterol from dried seed powder of *Celastrus paniculatus* Willd. Chromatographic separation was performed on TLC alumina plates precoated with silica gel $60F_{254}$ as the stationary phase with toluene-ethyl acetate-glacial acetic acid (6.0:1.5:0.1v/v/v) as the mobile phase. The plates were scanned densitometrically in the reflectance-absorbance mode at 580 nm. The method was validated for linearity, precision, limit of detection (LOD), limit of quantitation (LOQ), and accuracy. Linearity was found to be in the range of 40.00µg/mL to 100.00µg/mL with value of correlation coefficient (r) = 0.9986. Instrumental precision, repeatability and intermediate precision were determined to evaluate the precision of the method. The accuracy of the method was established by evaluating percent recovery of β-sitosterol at three different levels and it was found to be 98.52.

ISSN 0975-6299 Vol 2/Issue 2/Apr-Jun 2011

KEYWORDS

β-sitosterol; *Celastrus paniculatus* Willd; HPTLC; Method validation.

1. INTRODUCTION

Celastrus paniculatus Willd. (Family-Celastraceae) commonly known as Malkanguni and Jyotishmati, is an important Indian medicinal forest climber growing in sub–Himalayan tracts up to 2000m in Central India, Western ghats, Eastern Ghats extending to Rajmahal Hills in Bihar and Orrisa[1].

In Indian traditional system of medicine *Celastrus paniculatus* Willd. is used as an appetizer, laxative, emetic aphrodisiac, brain tonic and used for treatment of cough, asthma, gout and headache[2]. The oil extracted from the seeds has a tranquilizing effect, besides being a central muscle relaxant, anti-emetic, anti-ulcerogenic and adaptogen with memory enhancing properties[3]. The chemical constituents present in seeds of *Celastrus paniculatus* are β-sitosterol, linolenic acid, palmitic acid, linoleic acid and β-amyrin [4,5].

β-sitosterol, the principal phytosterol appears to have important immunomodulatory[6] and anti-inflammatory[7] activities in human and animal physiology. TLC method for identification of β-sitosterol from plants like *Cynodon dactylon* (Linn.) Pers.[8] and HPTLC method for its quantitation from *Caesalpinia bonduc* (Linn.) Roxb.[9] has been reported in literature. However, HPTLC method for quantitation of β-sitosterol from *Celastrus paniculatus* Willd. has not been reported in literature. In the present research work, a simple, sensitive and accurate High Performance Thin Layer Chromatographic method has been developed and validated for the quantitation of β-sitosterol from seeds of *Celastrus paniculatus* Willd.

2. EXPERIMENTAL METHODS

2.1. Materials

2.1.1. 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. Distilled water used was purified with Sartorius (Arium 61315, made in USA) water purification unit.

2.1.2.. Plant material

The seeds of *Celastrus paniculatus* Willd., were collected from wild plants found in Keshav Srushti, Mumbai, India and were authenticated from Botanical Survey of India (BSI), Pune, India. The seeds of *Celastrus paniculatus* Willd., were dried in a preset oven at 45 ± 5ºC, and powdered using motor and pestle and then sieved through BSS mesh size 85 and stored at 25°C, in an airtight container.

2.1.2.1 Preparation of standard solutions of β-sitosterol

Stock solution of β-sitosterol(1000.0 µg/mL), 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.

5.0 mL of the above stock solution was diluted to 10.0 mL, with methanol to give standard solution of beta-sitosterol, with concentration of 500.0µg/mL. The aliquots (0.2 mL to 2.8 mL) of 500.0 µg/mL solution of beta-sitosterol, were transferred to 10.0 mL volumetric flasks and the volume of each flask was made upto 10.0 mL, with methanol, to obtain the working standard solutions of beta-sitosterol, in the

concentration range of 10.0µg/mL to 140.00 µg/mL.

2.1.2.2. Preparation of sample solutions

Accurately weighed, 1.002g dried seed powder of *Celastrus paniculatus* Willd., 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 (28ºC ± 2ºC) for 12 hrs. The contents of tube where then filtered through Whatman filter paper No. 41. The filtrate obtained was used as sample solution for carrying out the experiment.

2.3. Instrumentation

2.3.1. Chromatographic conditions

The samples were spotted in the form of bands of width 6mm and 12.2mm apart with a Camag microlitre syringe on precoated silica gel aluminium Plate 60 F_{254} having 20 cm × 10 cm dimensions (E. Merck, Darmstadt, Germany) using a Camag Linomat IV sample applicator (Muttenz, Switzerland).

The mobile phase consisted of toluene - ethyl acetate - glacial acetic acid, in the volume ratio of 6:1.5:0.1. Linear ascending development was carried out in 20 × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 20 min at room temperature. The length of chromatogram run was 80mm. Subsequent to the development; TLC plate was sprayed with methanol - sulphuric acid reagent followed by drying in oven at 110ºC.

Densitometric scanning was performed on Camag TLC scanner III in the reflectance–absorbance mode at 580 nm and was operated by CATS software (V 3. Camag).

3. Method validation

3.1. Linearity

The linearity of the method was tested by applying standard β-sitosterol solution from 10.00µg/mL to 140.00µg/mL 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.

3.2. 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.

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

Parameter	Results
Linear range (n = 5) µg/mL	40.00 to 100.00
Correlation coefficient (r)	0.9986
LOD µg/mL	10.00
LOQ µg/mL	40.00
Instrumental precision % R.S.D. (n=10)	0.38
Intra-assay precision % R.S.D. (n=3)	0.44
Intermediate precision % R.S.D. (n=3)	0.54

3.3. Precision

The method was validated in terms of instrumental precision intra assay precision, and intermediate precision.

The instrumental precision was studied by analyzing the standard solution of β-sitosterol of concentration 60.00µg/mL, in ten replicates, in the chromatographic system under the specified conditions. The intra assay precision method was studied by analyzing three different concentration of sample solution i.e methanolic extract of dried seed powder of *Celastrus paniculatus* Willd. in triplicates. The Intermediate precision of the method was evaluated by analyzing the sample solution in triplicates on three different days, in the chromatographic system, under the specified conditions.

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.

3.4. 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 10µL of standard solution of β-sitosterol solution of concentration 60.0µg/mL 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 (R_f value) of β-sitosterol was found to be 3022.27 and 0.49 respectively with % R.S.D. value less than 2

3.5.Application of validated method for the quantitation of β-sitosterol from Celastrus paniculatus Willd.

The quantitation of β-sitosterol was done using above validated HPTLC method. 10µL of the sample solution was applied as bands in seven replicates, on the TLC plate, with a Camag Linomat IV sample applicator and analysed using the optimized chromatographic conditions.

The identity of peak of β-sitosterol in the sample solution was confirmed by

comparing the retention factor (R_f) value of the sample with that of the standard solution of β-sitosterol having R_f value as 0.49 (Figure 1). A Typical Chromatogram of Standard β-sitosterol under above mentioned chromatographic condition is represented in Figure 2. A Typical Chromatogram of plant extract showing the peak of β-sitosterol is represented in Figure 3.

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 repeatability of the method, the assay experiment was repeated seven times. Mean amount of β-sitosterol in dried seed powder from *Celastrus paniculatus* Willd. was found to be 0.37 mg/g.

Figure 1

High Performance Thin Layer Chromatography Separation of Standard β-sitosterol (A) and Methanolic Extract of Dried Seed Powder of Celastrus paniculatus Willd. (B)

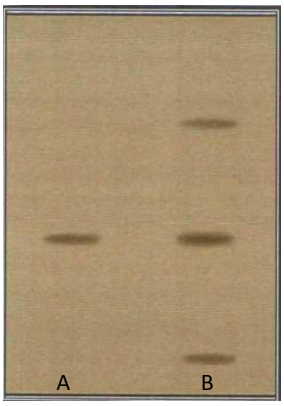


Figure 2

High Performance Thin Layer Chromatographic Determination of β-sitosterol A Typical Chromatogram of Standard β-sitosterol solution

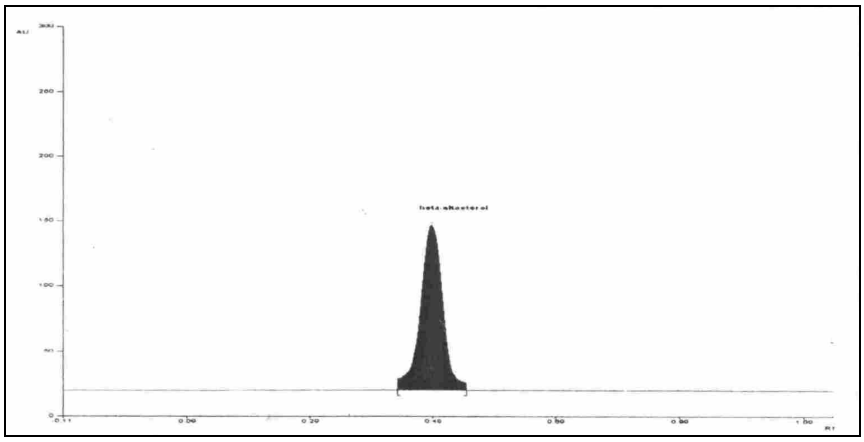
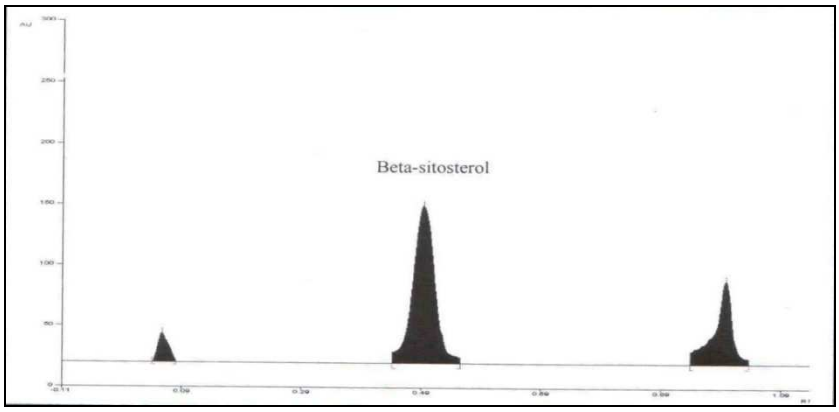


Figure 3
High Performance Thin Layer Chromatography Determination of β-sitosterol A Typical Chromatogram Obtained from Methanolic Extract of Dried Seed Powder of Celastrus paniculatus Willd.



3.6. Accuracy

The accuracy of the method was established by performing recovery experiments, using the standard addition method, at three different levels. About 1.00g of powdered roots of *Celastrus paniculatus* Willd. was accurately weighed into each of the four stoppered test tubes. Known amounts (0.00 µg, 1 µg, 2 µg and 3 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^{o}C $\pm$ 2^{o}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 then determined. The results of the recovery experiment are given in Table 2. The value of percentage recovery of β-sitosterol is 98.52, indicating good accuracy of the method.

Table 2
Recovery of β-sitosterol from the root bark of Celastrus paniculatus Willd.

Level	Wt of sample * (g)	Wt of std added (µg)	Mean amount of β-sitosterol found (mg/g)**	Percent recovery [%]
0	1.002	0	0.3751	
1	1.005	1	0.3826	98.52
2	1.004	2	0.3965	

 # Mean amount of β-sitosterol found in 1.0g of sample = 0.37 mg
** Sample: Dried root Celastrus paniculatus Willd.*
** * Mean amount of β-sitosterol found (n = 7)*

3.7. Solution stability

The stability of standard β-sitosterol solution was determined by comparing the peak areas of β-sitosterol solution, of concentration 70.0µg/mL, 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 2) 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.

4. RESULTS AND DISCUSSION

The identity of peak of β-sitosterol in sample solution was confirmed by comparing its R_f value with that of standard β-sitosterol (0.49). A good linear relationship was obtained for β-sitosterol, in the concentration range of 40.00µg/mL to 100.00µg/mL, with correlation coefficient of 0.9986. The average percent recovery of β-sitosterol at three levels was 98.52 (Table 2).

TLC method reported in literature[8] was used for detection of β-sitosterol from *Cynodon dactylon* (Linn.) Pers. and HPTLC method reported in literature[9] was for quantitation of β-sitosterol from seeds of *Caesalpinia bonduc* (Linn.) Roxb. The mobile phase used in the present research work for quantitation of β-sitosterol from methanolic dried seed powder extract of *Celastrus paniculatus* Willd. is also relatively simpler as compared to the mobile phase used in the above reported method[9].

5. CONCLUSION

A new HPTLC method has been developed validated and used for the quantitation of β-sitosterol from the methanolic extract of dried root powder *Celastrus paniculatus* Willd. 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 and stability-indicating and can be used for the routine quality control analysis and quantitative determination of β-sitosterol from *Celastrus paniculatus* Willd.

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