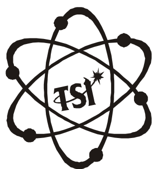

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A rapid densitometric method for simultaneous quantification of two biologically active compounds in *Vernonia cinerea* whole plant powder using HPTLC

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ABSTRACT

In present study, an attempt has been made to develop a simultaneous HPTLC method of quantitative determination of β-Sitosterol and Lupeol in *Vernonia cinerea* whole plant powder. Chromatography was performed on silica gel 60 F_{254} HPTLC plate, with toluene: methanol, 88:12 (v/v), as mobile phase. After development, plates were treated with Lieberman-Burchard Reagent, detection and quantification were performed by densitometry at 366 nm in fluorescence mode. The method was validated in terms of its linearity, limits of detection and quantification and precision following standard protocols. β-Sitosterol and Lupeol was found to be linear in the range of 15.0μg mL^{-1}-35.0μg mL^{-1} and 45.0μg mL^{-1} - 105.0μg mL^{-1} respectively. Average content of β-Sitosterol and Lupeol in whole plant powder of *Vernonia cinerea* was found to be 0.4896±0.00035 mg and 1.4882 ± 0.0002 mg respectively. The method was found to be simple, precise, accurate, specific and sensitive and can be used for routine quality control of herbal raw materials and for the quantification of these compounds in plant materials.

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KEYWORDS

HPTLC;
Vernonia cinerea;
β-Sitosterol;
Lupeol.

INTRODUCTION

Herbal medicine has been enjoying renaissance among the customers throughout the world. However, one of the implements in the acceptance of the Ayurvedic or Siddha formulations is the lack of standard quality control profile[1]. The quality of herbal medicine that is 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 the plant based drugs, it is difficult to establish quality control parameters and modern analytical techniques are excepted to help in circumventing this problem[2]. Standardization of herbal formulations in terms of quality of raw materials, manufacturing practices, and composition is important to ensure quality and optimum levels of active principles for their bio-potency. Recently, the concept of marker-based standardization of herbal drugs is gaining momentum. Identification of major and unique compounds in herbs as markers and development of analytical methodologies for monitoring them are the key steps involved in marker-based standardization. HPTLC has recently emerged as a preferred analytical tool for fingerprints and quantification of

marker compounds in herbal drugs because of its simplicity, sensitivity, accuracy, suitability for high throughput screening, etc.[3].

Vernonia cinerea Linn. (Fam. Asteraceae); an erect, rarely decumbent, branched herb, 12-75cm high, found throughout India ascending to an altitude of 1800m. (known as Sahadevi in Sanskrit). It is considered to posse's anti-inflammatory, anthelmintic, diuretic activity, and it is used against skin diseases[4]. HPTLC methods, hitherto, has not been reported for simultaneous estimation of β-sitosterol and Lupeol from *Vernonia cinerea.* In this paper development and validation of a HPTLC method for the quantitative analysis β-sitosterol and Lupeol is reported. The proposed method has been validated as per ICH guidelines[5,6].

MATERIALS AND METHODS

Plant material

Whole plant of *Vernonia cinerea* Linn. was collected from Matunga (Mumbai) region of India during the flowering season. It was authenticated from Blatter Herbarium, St.Xavier's College, Mumbai, India. After collection, the whole plant was washed with water thoroughly to remove soil particles, dust and extraneous matter. The plant material was drained to remove excess of water by spreading over filter paper for 6 h in shade away from sunlight. The plant material was then placed in an oven at 45±5°C and allowed to dry for 4 days. Immediately after drying, it was powdered using an electrical mixer-grinder and sieved through a BSS mesh No. 80 sieve and stored in airtight Pearlpet® containers at 25°C. The containers were labeled with details such as date of collection, weight of powder, time of collection and the season of collection.

Chemicals

All the chemicals used in the experiments were of analytical grade. Reference standard β-sitosterol and Lupeol (purity 98%) were procured from Sigma Aldrich (GERMANY).

TLC conditions

Spotting device: Linomat IV sample applicator; CAMAG (Muttenz, SWITZERLAND); Syringe: 100µl Hamilton (Switzerland); TLC Chamber: Glass twin trough chamber; CAMAG; Densitometer: TLC Scanner in conjunction with CATS software; CAMAG; HPTLC plates: $20\times10cm^2$, 0.2mm thickness precoated with silica gel 60 F_{254}: E. Merck and Solvent System: toluene: methanol (88:12, v/v).

Sample preparation

1000mg of whole plant material of *Vernonia cinerea* Linn. was extracted with 10ml of methanol. The mixture was vortexed for 1 min and it was kept overnight for extraction. It was filtered through whatmann filter paper no. 1 and filterate obtained was subjected to TLC for simultaneous quantitation of β-sitosterol and Lupeol.

Preparation of standard solution

The stock solutions of β-sitosterol and Lupeol (1000µg ml^{-1}) each were prepared separately in methanol. The stock solution were quantitatively transferred to give a solution of appropriate concentration range of β-sitosterol (15.0µg mL^{-1}-35.0µg mL^{-1}) and lupeol (45.0µg mL^{-1}-105.0µg mL^{-1}) respectively. Standard solutions were prepared by dilution of the stock solution.

Calibration curves

Standard solution (10µl) of β-sitosterol (15.0µg mL^{-1}-35.0µg mL^{-1}) and Lupeol (45.0µg mL^{-1}-105.0µg mL^{-1}) were applied in triplicate on precoated silica gel 60 F_{254} HPTLC plates (E.Merck) of uniform thickness of 0.2mm. The plates were developed in a solvent system of toluene: methanol (88:12, v/v) in CAMAG twin trough chamber up to a distance of 8.5cm. After development, the plate was dried in air, the plate was derivatized in Liebermann-Burchard reagent and heated for 10 minutes at 105±2°C. The plate was scanned at 366nm using fluorescence- reflectance mode by CAMAG Scanner 2 and Wincats software for β-sitosterol and Lupeol. The peak areas were recorded. Respective calibration curves were prepared by plotting peak area versus concentration of β-sitosterol and Lupeol applied.

Simultaneous quantitation of β-sitosterol and Lupeol from whole plant powder of Vernonia cinerea Linn

Sample solutions (10µl) were applied in triplicate

on a precoated HPTLC plates with CAMAG Linomat spotter. The band width was 7mm and the space between two bands was 8mm. The plate was developed and scanned at 366nm after derivatization in Liebermann- Burchard reagent. The peak areas and absorption spectra were recorded. The amount of β-sitosterol and Lupeol in the sample was calculated using the respective calibration curves.

Method validation

The method was validated for precision, repeatability accuracy. Precision of the method was checked by repeated scanning (n=5) of the same spot of β-sitosterol and Lupeol seven times each. The repeatability of sample application and measurement of peak area were expressed in terms of %CV. Variability of the method was studied by intra-day precision and inter-day precision. In order to estimate limit of detection (LOD) and limit of quantitation (LOQ), blank methanol was spotted to determine signal to noise ratio. LOD was considered as 3:1 and LOQ as 10:1. Accuracy of the method was tested by performing recovery studies at three different levels (0%, 50%, and 100% addition)[8].

RESULTS AND DISCUSSION

In the present study, we quantitate two marker compounds viz. β-sitosterol and Lupeol in whole plant powder of *Vernonia cinerea* Linn. by HPTLC densitometric method using silica gel HPTLC. The developed method was validated as per the ICH guidelines (TABLE 1-3). Mobile phase composition was optimized by introducing small changes in the composition of toluene: and methanol. It was found that β-sitosterol and Lupeol resolved well at R_F 0.42 and 0.59 (Figure 1) respectively in the solvent system of toluene: methanol (88:12, v/v). from other components of the sample extracts.

Linearity

A good linearity was achieved in the concentration ranges of 15.0μg mL^{-1}-35.0μg mL^{-1} for β-sitosterol and 45.0μg mL^{-1}-75.0μg mL^{-1} for Lupeol. The regression equations and correlation coefficient for the reference were y=65.76x+90.42, R^2=0.9996 for β-sitosterol and y=0.0031x-0.003, R^2=0.9989 respectively (TABLE 1).

Instrumental precision and intra-day and inter-day precision

Instrumental precision was checked by repeated scanning of the same spot of and β-sitosterol and Lupeol five times each. Standards of β-sitosterol were spotted both at intra-day (spotting each concentration three times within 24 hour) and inter-day (spotting each concentration three times during 3 days intervals) interval to check the precision. The results are expressed as %RSD.

Recovery

The recovery was used to evaluate the accuracy of the method. The present recovery was calculated. Recovery studies at three different levels were done on *Vernonia cinerea* Linn. by accurately spiked with various concentrations of reference solutions just prior to the extraction. The percentage recovery at three different levels for β-sitosterol was found to be 100.20±1.11% and 100.02±0.67 % for Lupeol. The results were shown in (TABLE 2).

Limit of detection and limit of quantitation

The LOD and LOQ were found to be 5 and 10μg ml^{-1} for β-sitosterol while 35 and 40μg ml^{-1} for Lupeol respectively.

Quantitative determination

All samples were extracted, as described above and analyzed by HPTLC. The content of each compound was determined by the corresponding regression equation and results are summarized in TABLE 3. The result indicated β-sitosterol and Lupeol were detected in *Vernonia cinerea* Linn. The chromatographic overlay of standards along with plant material is represented in (Figure 2). Whole plant powder of *Vernonia cinerea* Linn. was found to contain 0.4896±0.00035 mg of β-sitosterol and 1.4882±0.0002 mg of Lupeol. (TABLE 3).

CONCLUSION

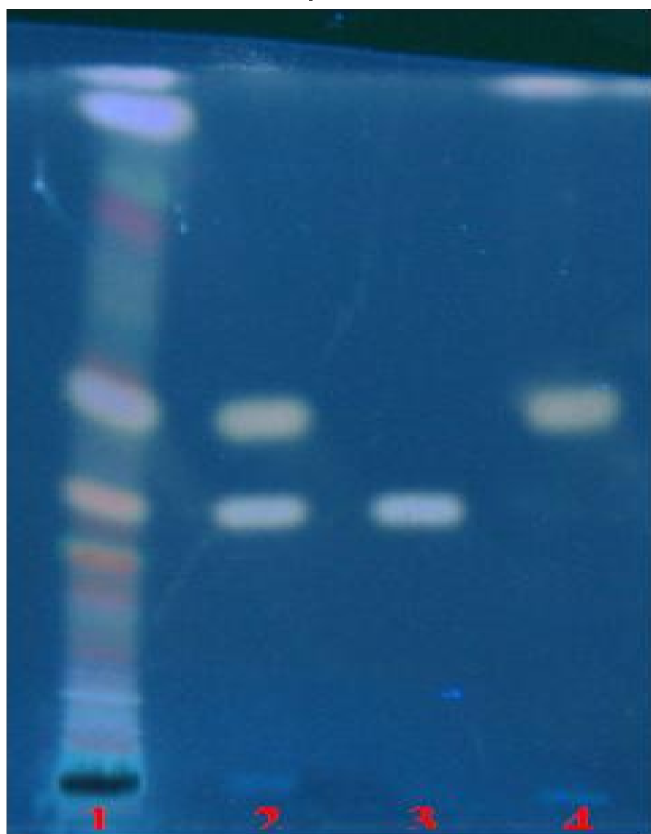
The application of a simple, rapid and accurate HPTLC method for the quantitation of β-sitosterol and Lupeol in whole plant powder of *Vernonia cinerea* Linn.

TABLE 1 : Method validation parameter for the quantitation of β-sitosterol and Lupeol

Parameters	β-sitosterol	Lupeol
Linearity range[µg mL^{-1}]	15.0 - 35.0	45.0 - 105.0
Slope (m)[a]	65.76	21.75
Intercept(c)[a]	90.42	211.68
Correlation coefficient (R)	0.9996	0.9989
LOD[µg mL^{-1}]	5.0	35.0
LOQ[µg mL^{-1}]	10.0	40.0
Intraday precision (n=3 COV)	0.82 %	0.97 %
Interday precision (n=3 COV)	1.14 %	1.03 %
System Suitability	0.74%	0.96%

[a]of the equation y=mx+c, where y is peak area, m is the slope, x is the concentration and c is the intercept.

Figure 1 : Chromatographic plate of whole plant powder of *Vernonia cinerea* Linn with β-sitosterol and Lupeol



Track 1: Whole plant powder of *Vernonia cinerea* Linn
Track 2: β-sitosterol and Lupeol
Track 3: β-sitosterol
Track 4: Lupeol

The method was validated to track the active principles in the complex mixture of herbal ingredients. The method could be extended for the marker-based standardization of other herbal product containing β-sitosterol and Lupeol. The method was found to be simple, precise, accurate, specific and sensitive and can be used for routine quality control of herbal raw materials and for the quantification of these compounds in plant materials.

TABLE 2 : Recovery study of β-sitosterol and Lupeol from whole plant of powder of *Vernonia cinerea* Linn

	Level	Conc. of std [µg mL^{-1}]	Amt of std found	SD	%COV	%Recovery
β-sitosterol	0	25	24.8219	0.1113	0.4484	99.2879
	10%	27.5	27.3137	0.0518	0.1898	99.3227
	20%	30	30.1899	0.0567	0.1878	100.6333
	30%	32.5	33.0971	0.0816	0.2473	101.5685
					Mean	100.2031
Lupeol	0	75	75.0717	0.2067	0.2753	100.0956
	10%	82.5	82.1317	0.1597	0.1945	99.5536
	20%	90	90.7414	0.2939	0.3239	100.8238
	30%	97.5	97.3744	0.3046	0.3128	99.8712
					Mean	100.0860

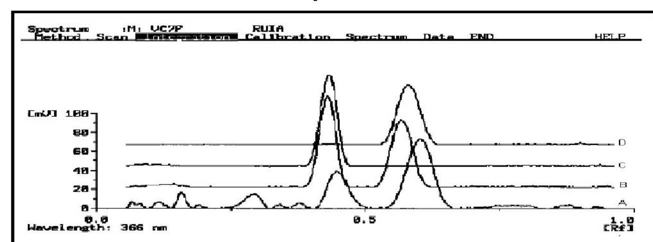
*Mean±SD, n= 3

TABLE3 : Marker compounds quantified from whole plant of powder of *Vernonia cinerea* Linn

Sample Tested	Content of Marker compound* in mg	
	β-sitosterol	Lupeol
Whole plant of powder of *Vernonia cinerea* Linn with β-sitosterol and Lupeol.	0.4854±0.00035	1.4882±0.0002

*Mean ± SD, n= 3

Figure 2 : Chromatographic overlay of whole plant powder of *Vernonia cinerea* Linn with β-sitosterol and Lupeol



A: Methanolic extract of plant powder
B: Mixture of β-Sitosterol and Lupeol
C: Standard β-Sitosterol
D: Standard Lupeol

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