QUANTITATION OF LUPEOL OCTACOSANOATE IN *HEMIDESMUS INDICUS* R. Br. *R*OOT POWDER BY HPTLC

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ABSTRACT

A new, simple, sensitive, accurate and precise high-performance thin-layer chromatographic method for quantitation of Lupeol octacosanoate, a marker compound in Hemidesmus indicus was developed and validated. Lupeol octacosanoate is a unique compound found in Hemidesmus indicus root. This marker compound was isolated from the petroleum ether extract and identification was confirmed by using melting point and IR, UV, NMR spectroscopy. A petroleum ether extract of the root powder was chromatographed on silica gel 60F-254 plate with isopropyl alcohol: n- butanol (1:1, v/v) as mobile phase. The system was found to give compact spots for Lupeol octacosanoate (R_f 0.8 ± 0.04). Detection was performed by densitometric scanning in absorbance mode at 235 nm. The method was validated for linearity, accuracy, precision, limit of detection, limit of quantitation and specificity. The linear regression analysis data for the calibration plots for Lupeol octacosanoate showed good linear relationship with r^2 = 0.997, in the concentration range of 4000-12000 ng/spot. The limit of detection & limit of quantitation were 159.5 and 483.5 ng/spot. The average amount of Lupeol octacosanoate found in root powder was 36.5 mg/gm. This method can be used as quality control method for checking the purity of Hemidesmus indicus root powder & extract.

***Keywords**: Hemidesmus indicus, Lupeol octacosanoate, HPTLC, Quality control.*

INTRODUCTION

The health promotive, disease preventive and rejuvenation approach available in the Indian systems of medicine like 'Ayurveda' is gaining greater attention and popularity in many regions of the world. In India, many plants are reported to be used as a remedy for a number of diseases. The root of *Hemidesmus indicus* R.Br, locally called anantamul, is found throughout India, and belongs to family Asclepiadaceae. The roots of the plant are woody and have a sweet taste, with cooling effect, and are used in various ailments or diseases. It is a well-known drug in the Ayurveda system of medicine[1]. *Hemidesmus indicus* has been used in traditional medicine in biliousness, blood diseases, diarrhoea, respiratory disorders, skin diseases, syphilis, fever, bronchitis, asthma, eye diseases, epileptic fits in children, kidney and urinary disorders, loss of appetite, burning sensation and rheumatism[2]. Rootstock of this plant is woody and possesses a strong fragrance. Because of its typical aroma, these roots are being used as flavoring agent in 'sherbets' (sweet drinks). An unusual phenolic compound, 2-hydroxy-4-methoxybenzaldehyde is responsible for the fragrance in root organs[3]. Literature survey revealed that Lupeol octacosanoate (marker) is a unique compound found in *Hemidesmus indicus* root[4], apart from the bark of *Plumeria Obtusifoli*[5] and the stem of *Premna Fulva* (Verbenacene)[6]. HPTLC has been widely used as quality control tool for phytochemical evaluation of herbal drugs. In this work a precise and accurate HPTLC method has been developed for rapid determination of Lupeol octacosanoate in *Hemidesmus indicus* dried root powder. As this marker compound in unique to

the roots of *Hemidesmus indicus* , this method can be used as quality control to confirm identity of *Hemidesmus indicus* root powder. There is no reported HPTLC method for Lupeol octacosanoate.

EXPERIMENTAL

Reagents and standard used: All chemicals were AR grade were purchased from Sisco Research Laboratories Ltd; Mumbai (India).

Plant material: The root powder of *Hemidesmus indicus* was procured from local market.

Isolation of marker[4]:

Dried root powder of *Hemidesmus indicus* (70 gm) were exhaustively extracted with 500 ml petroleum ether in Soxhlet apparatus for 50 cycles and dark brown residue (3.7 gm) was obtained after evaporation of the solvent. The dark brown residue was subjected to column chromatography by using 100 gm of alumina and successive elution with petroleum ether, petroleum ether: benzene (4:1), benzene and benzene: diethyl ether mixture (1:1). Fractions were collected in test tubes. First three test tubes of petroleum ether fractions were concentrated to get solid substance and crystallization from n-hexane yielded white coloured lupeol octacosanoate (0.287 gm).

Instrumentation: Melting point of isolated compound was determined by open capillary & is uncorrected. Structural confirmation of the isolated compound was done by using UV, IR and NMR spectroscopy. FTIR spectra of the isolated compound was recorded using KBr on a Jasco FTIR V 460 plus spectrometer using Diffuse Reflectance Attachment and characteristic absorption signals are reported in cm^{-1}. UV spectrum was obtained using UV-Vis double beam spectrophotometer of make JASCO, model V-550 using n-hexane as a solvent. ^1H NMR spectra were recorded on Varian Mercury YH 300 (300 MHz FT NMR) spectrophotometer using TMS as an internal reference (Chemical shift represented in δ ppm).

High Performance Thin Layer Chromatography: Chromatographic separation was performed on Merck TLC plates precoated with silica gel 60 F_{254} (10 cm ×10 cm with 250 μm layer thickness) from E. Merck, Germany. The samples were applied onto the plates as a band with 6 mm width using Camag 100 microlitre sample syringe (Hamilton, Switzerland) with a Camag Linomat 5 applicator (Camag, Switzerland). Linear ascending development was carried out in a twin trough glass chamber (10 x 10 cm). Densitometric scanning was performed using Camag TLC scanner 3 at 235 nm and operated by winCATS software (V 1.4.2, Camag).

Preparation of Standard Stock Solution:

Standard stock solution of marker:10 mg of marker was weighed and dissolved in 10 ml of n-hexane to obtain 1000 μg/ml stock solution of marker.

Preparation of sample Solution: Sample solutions of *Hemidesmus indicus* root powder was prepared by accurately weighing 1 gm of powder and dispersing in 10 ml of petroleum ether. It was shaken for 2 hours using mechanical shaker. Then the solution was centrifuged at 1500 rpm for 20 minutes. The supernatant was used for quantitation.

Method Validation: This method was validated as per the ICH guidelines[7], the method validation parameters checked were linearity, accuracy, precision, limit of detection, limit of quantitation and specificity.

Preparation of calibration curves: From the final standard stock solutions of marker, a volume of 4-12 μL was spotted on the TLC plate to obtain final concentration in the range of 4000–12000 ng/spot. The solvent system, isopropyl alcohol: n- butanol (1:1, v/v) was the mobile phase used. Chromatogram was development in a twin trough glass chamber, using 20 minutes of

chamber saturation time. The length of chromatogram run was 80 mm. The developed plates were air-dried. Densitometric scanning was performed in the absorbance mode at 235 nm. The slit dimension was kept at 5 x 0.45 mm at scanning speed of 100 nm/s. After completion of scanning, peak areas of marker peak were noted. Peak areas were plotted against corresponding concentrations and least square regression analysis was performed to generate the calibration equation for marker (Table 1).The equation of the regression line is

$$y = 0.6187x + 1979 \ (r^2 = 0.\ 0.997)$$

Precision: The repeatability and intermediate precision of the method was demonstrated by intra-day and inter-day variation studies. In the intraday studies, 3 repeated measurements of standard and sample solutions were made in a day and percentage RSD values were calculated. In the inter day variation studies, 3 repeated measurements of standard and sample solutions were made on 3 consecutive days and percentage RSD values were calculated (Table 2).

Accuracy

For accuracy of method, recovery studies were carried out by applying the method to drug sample to which known amount of marker was added at level of 80, 100 and 120% (standard addition method). Three determinations were performed at each level, and the results obtained were compared with expected results.

Limit of Detection and Limit of Quantification

The detection limit (LOD) of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. LOD was calculated using the following formula

$$LOD = \frac{3.3 \times \text{Standard Deviation of the y-intercept}}{\text{Slope of calibration curve}}$$

The quantitation limit (LOQ) of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. LOQ was calculated using the following formula

$$LOQ = \frac{10 \times \text{Standard Deviation of the y-intercept}}{\text{Slope of calibration curve}}$$

Specificity: The specificity of the method was ascertained by analyzing standard isolated marker and the root powder. There were no interfering spots by the plant constituents at the R_f values of the marker. The absorption spectra of the resolved spots for the marker compound match with the spectra of the isolated standard marker indicating no interference by other plant constituents.

Determination of marker in dry powder: Dry root powder of *Hemidesmus indicus* (1gm) extracted in petroleum ether as described above and 3 µl volume of the extract were applied as bands to the plate. The plate was developed and scanned as described above and peak areas were recorded. The amount of marker was calculated using the calibration plot, considering the isolated marker to be 100% pure.

RESULTS AND DISCUSSION

Structural Elucidation of Isolated Compound:

Melting point of isolated compound was found 81-82^{0}C. This matches with reported value[4]. UV spectrum showed λ_{max}. at 235 nm and 270 nm. IR spectrum showed sharp peaks at 1740, 1640, 722, 2921, 1252 and 2875 cm^{-1}, while result obtained from ^{1}H NMR spectrum showed signals

at δ 0.83 (s, 3H), δ 0.85 (s, 9H), δ 0.97 (s, 3H), δ 1.06 (s, 3H), δ 1.24 (s, 50H), δ 1.67 (b, 3H), δ 4.55 (m, 3H). This matches the reported values for lupeol octacosanoate[4].

Optimization of Solvent System and Chromatographic conditions:

Chromatographic separation studies were carried out on the stock solution of marker. Initially the plates were spotted with marker (1000 µg/ml) and developed by linear ascending development using solvents like toluene, methanol, chloroform, isopropyl alcohol, dichloromethane, n-butanol, ethyl acetate, acetone, benzene etc. with chamber saturation. Based on the results of these initial chromatograms binary and ternary mixtures of solvents were tried to achieve optimum resolution. Benzene: ethyl acetate (9:1v/v), (7:3v/v), ethyl acetate: benzene (9:1 v/v), toluene: ethyl acetate (7:3 v/v),(9:1v/v), toluene: ethyl acetate: glacial acetic acid (7:2:1v/v/v), methanol :chloroform (7:3v/v), toluene: ethyl acetate : triethylamine (7:2:1v/v/v), methanol: chloroform: acetonitrile (4:2:4v/v/v), acetonitrile: methanol (1:1v/v), methanol: acetonitrile (1:1v/v) system was used to check the resolution. Methanol: chloroform: acetonitrile (4:2:4v/v/v), ethyl acetate: benzene (9:1v/v) showed good resolution and peak shape but R_f of marker peak was near 0.9. So, after these several trials, isopropyl alcohol: butanol (1: 1v/v) mixture was chosen as the mobile phase for analysis. It led to good resolution and peak shape was also acceptable (Figure 1). Other chromatographic conditions like chamber saturation time, run length, sample application rate and volume, sample application positions, distance between tracks, detection wavelength, were optimized to give reproducible R_f values, better resolution, and symmetrical peak shape for the marker.

Linearity: Marker showed good correlation coefficient when peak area of the resolved spot was plotted against concentration in the range of 4000-12000 ng/spot. Linearity was determined by evaluating five working standards. Table 1 summarizes Beer's law limit, linear regression equation and correlation coefficient for the method.

Precision: The proposed method was found to be precise as indicated by percent RSD (Relative Standard Deviation) not more than 1.5. The intra-day and inter-day precision results are shown in Table 2.

Accuracy: The proposed method when used for quantitation of marker from root powder after spiking with working standard afforded recovery 98.38%-101.7%.

Limit of Detection and Limit of Quantification

The limit of detection was found to be 159.5 ng/spot while the limit of quantitation was found to be 483.5 ng/spot.

Specificity: The specificity of the method was ascertained by analyzing standard isolated marker and the root powder. There were no interfering spots by the plant constituents at the R_f values of the marker. The absorption spectra of standard marker (R_f 0.8) and the corresponding spot present in root powder matched exactly, indicating no interference by the other plant constituents (Figure 2).

Determination of marker in dry powder:

The amount of marker found in dry powder was 36.5 mg/gm.

CONCLUSION

Lupeol octacosanoate which is present in *Hemidesmus indicus* R. Br. is one of the important marker compounds. This marker in present in roots of *Hemidesmus indicus* only. In this work a HPTLC method has been developed for quantitation of marker in dry root powder of *Hemidesmus indicus.* This method can be used as quality control method for *Hemidesmus indicus*. The validated HPTLC-densitometric method employed here proved to be simple, fast,

accurate, precise and sensitive, thus can be used for routine analysis of lupeol octacosanoate in *Hemidesmus indicus* root powder.

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Table-1: **Regression analysis of HPTLC response.**

Parameters	HPTLC Lupeol Octacosanoate
Wavelength (nm)[a]	235
Beer's Law Range (ng/spot)	4000-12000
Correlation coefficient (r^2)	0.997
Linear regression Equation[b] (y = mx + c)	
Slope (m)	0.6187
Intercept (c)	1979
No of Data Points	5

[a]is the Detection Wavelength for HPTLC method.
[b]with respect to y = mx + c, where y is the peak area and x is the concentration(ng/spot).

Table-2: Intra-day and Inter-day precision studies

Concentration (ng/spot)	% R.S.D	
	Intra-day	Inter-day
6000	0.38	0.46
8000	0.26	0.54
10000	0.34	0.35

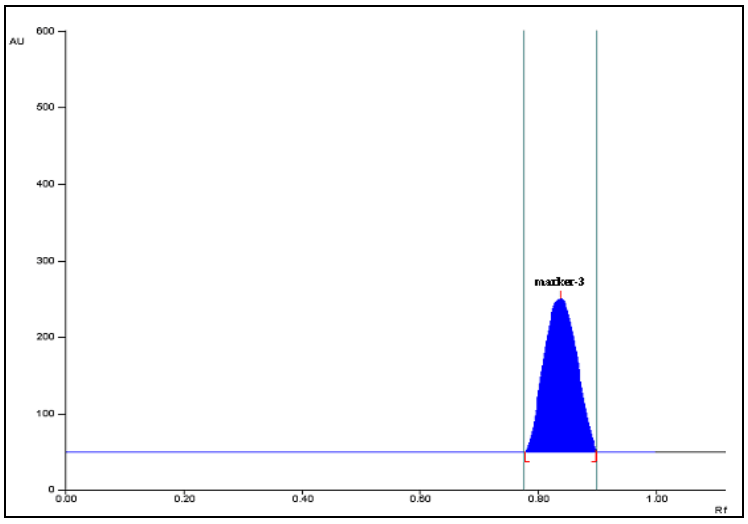

Fig.-1: A representative chromatogram of marker compound

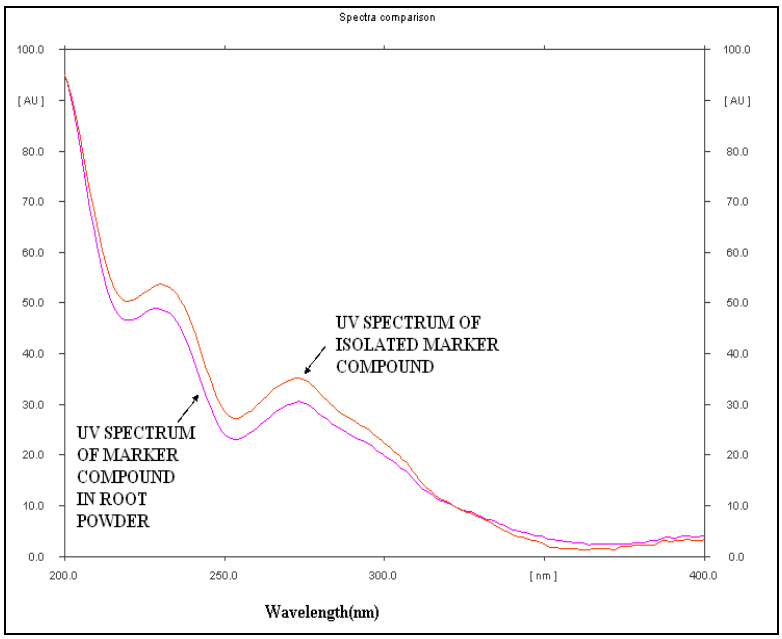

Fig.-2: Overlain spectra of standard lupeol octacosanoate and sample (at R_f 0.8 ± 0.04) in absorption mode in UV range, taken on the CAMAG TLC scanner 3

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Knowing trees, I understand the meaning of patience. Knowing grass, I can appreciate persistence.
-Hal Borland