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Research Article

VALIDATION OF HPTLC METHOD FOR QUANTITATIVE ESTIMATION OF L-DOPA FROM *MUCUNA PRURIENS*

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ABSTRACT
In the present study, high performance thin-layer chromatographic method was developed and validated for the quantification of L-Dopa from *Mucuna pruriens* (Black-coloured seeds). TLC aluminium plates precoated with silica gel were used as the stationary phase in a twin see through glass chamber saturated with n-butanol: glacial acetic acid :water 4:1:1 (v/v), as the mobile phase, performed at room temperature (25 ± 2°C). The Rf value of L-Dopa was found to be 0.39. Linearity was found to be in the concentration range 100–1200 ng/spot. The linear regression analysis of calibration plots showed good linear relationship between peak area and peak height (r^2 = 0.996). The method was validated for precision, recovery, robustness, specificity, detection and quantification limits. The limits of detection and quantification were 0.010 and 3.14 ng/ spot, respectively. The recovery was 100.5 %. Statistical analysis of data proves that the method is reproducible for the analysis of L-Dopa.
Keywords: FTIR, HPTLC, L-Dopa, *Mucuna pruriens,* Germplasm, ICH guidelines.

INTRODUCTION

Now-a-days the search for drugs from natural sources have gaining significant contribution in clinical, pharmacological research, because of vast majority of plant system have found to contain diverse array of phytochemical isoforms that they possess. India has very long, safe and continuous usage of many herbal drugs in the traditional medicinal systems viz., Ayurveda, Yoga, Unani, Siddha, Homeopathy and Naturopathy[1]. Since ancient times a number of Indian traditional plants have been used globally. There are many references to Indian medicinal plants and in trade a number of historical documents. Indian medicinal plants provide rich source of phytochemical compounds that are all showing health protective effects at various levels.

Mucuna pruriens Linn, commonly known as “cowhage” or “velvet bean” or “Athmaguptha” in India, and climbing legume endemic in India and other parts of the tropics including America’s and South Africa. The different parts of the plant such as seeds, leaves and roots have been used for the treatment of various diseases. The seed of velvet bean is an important natural source of L-Dopa which has been to have reported anti Parkinson’s, aphrodisiac, antidiabetic, antitumor ant neuroprotective activities [2, 3, 4, 5].Under the species *Mucuna pruriens* two varieties are existing i.e., *Mucuna pruriens var pruriens* (itching bean) and *Mucuna pruriens var utilis* (velvet bean)[6,7].The seeds of *Mucuna pruriens var pruriens,* collected from the pods covered with itching trichromes are documented to contain high levels of L-Dopa than the other varieties [8]. Further more, it has also been documented that the concentration of L-Dopa varying with different agro climatic regions, generations and across the equatorial plane.

Though there is dearth information available on the variability and methods to determine L-Dopa, viz titrimetric method[9], UV assay[10]; HPLC methods of determination[11]; reversed phase high performance liquid chromatography[12, 13] are time consuming and laborious. However, the information on comparative evaluation of L-Dopa content in different germplasm collected at various agro ecological regions has not been documented. In this connection, the present paper not only describes a simple, specific, accurate, precise HPTLC determination in seeds extracts but also compares with standard and in formulation(s).

MATERIALS AND METHODS

Standard L-Dopa was purchased from Sigma. All chemicals and reagents used were of analytical grade and were purchased from Merck Chemicals, India.

Collection of Seeds

Black-coloured seed germplasm of velvet bean were collected from Valanadu District, Kerala and Trinelveli District, Tamil Nadu bordering Western Ghats, India. The collected seeds were thrashed to remove any foreign material and they were washed with tap water followed by rinsing with distilled water. The shade dried seeds samples were powdered in Willey Mill at 60mesh size. The powdered samples of velvet bean stored in air tight containers at refrigerated condition until further usage.

Extraction of L-Dopa from The Seeds

Powdered samples of both the germplasm velvet bean were initially defatted with different solvents such as chloroform, petroleum ether, methanol and water by cold percolation method. After three days the extract was filtered using Whatmann No. 1 filter. Then they were concentrated in vacuum evaporator and freezed at -20°C and stored at the same until usage. The standard was re-crystallized to get off-white colored pure L- Dopa. The extracted L-Dopa was characterized by HPTLC and FTIR.

HPTLC Techniques for Extracted Samples

The samples were spotted in the form of bands with a Camag microlitre syringe on a pre-coated silica gel plates 60 F254 (10cm x10cm with 0.2 mm thickness, E. Merck, Darmstadt, Germany) using a Camag Linomat V Automatic Sample Spotter (Muttenz, Switzerland). The plates were pre-washed by methanol and activated at 60°C for 5 min prior to chromatography. The plate was developed in a solvent system (6.0 ml) of *n*-butanol–acetic acid–water (4.0 : 1.0 : 1.0) in a CAMAG glass twin-through chamber (10±10 cm) previously saturated with the solvent for 30 min (temperature 25±2 °C, relative humidity 40%). The development distance was 8 cm. Subsequent to the scanning, TLC plates were air

dried and scanning was performed on a Camag TLC scanner III in absorbance mode at 280 nm and operated by Win Cats software 4.03 version.

Calibration Curve of Standard L-Dopa

A stock solution of L-dopa (1mg/ml) was prepared by dissolving an accurately weighed 10 mg of L-dopa standard in 5 ml of anhydrous formic acid and volume was made up to 10 ml with methanol in a volumetric flask. Standard working solutions were prepared by diluting stock solution with methanol in the concentration range 10—120µg/ml. Standard solution (10µl) was spotted on the TLC plate to obtain final concentration range of 100—1200 ng/spot. Each concentration was spotted six times on the TLC plate.

Method validation

Precision

Repeatability of the sample application and measurement of peak area were carried out by spotting six replicates of the same spot (600ng/spot of L-dopa) and was expressed in terms of percent relative standard deviation (%RSD). The intra- and inter- day variation for the determination of L-dopa was carried out at five different concentration levels of 200,400,600,800 and 1000ng/spot.

Robustness

Robustness of the method was checked by introducing small changes in mobile phase composition, mobile phase volume, duration of mobile phase saturation and activation of prewashed TLC plates with methanol and the effects on result were examined. Robustness was done in triplicate at a concentration level of 600ng/spot and the %R.S.D of peak areas was calculated.

Limit of Detection and Limit of Quantification

In order to estimate the limit of detection (LOD) and limit of quantification (LOQ), the signal to noise ratio was determined. LOD was considered as 3:1 and LOQ as 10:1.

Recovery Studies

The pre-analyzed samples were spiked with 20, 40, 60, 80 and 100% of the standard L-dopa and the mixtures were reanalyzed by proposed method. The method was repeated for six times. This was done for check of recovery of L-dopa at different levels in the formulation.

Ruggedness

A solution of concentration 1000 ng/spot was prepared and analyzed on day 0 and after 6, 12, 24, 48 and 72 h. Data were treated for %R.S.D to assess ruggedness of the method.

Specificity

The specificity of the method was ascertained by analyzing the standard drug and seed extract. The seed extract of L-dopa was confirmed by comparing Rf values and spectra of the standard. The peak purity of L-dopa was assessed by comparing the spectra at three different levels like peak start (S), peak apex (M) and peak end (E) positions of the spot.

FTIR of L-Dopa Extracted From Seeds

To determine the concentration of L-Dopa content in seed extracts, the extract isolates were characterized by FTIR.

RESULT AND DISCUSSION

HPTLC method was developed and validated for the determination of L-Dopa in *Mucuna pruriens*. The mobile phase composition was n-butanol: glacial acetic acid: water (4:1:1) and it showed a well defined peak of R_f value 0.39 (Fig: 1&1a). This has been validated using the reports[14,] and in comparison to the reported method, our method requires simple solvent systems to separate the L- Dopa from the extract (Table-1).

Calibration Curve

Linearity was estimated using standard 10 - 120µg/ml of L-Dopa (Fig: 2). Calibration equation and correlation coefficient was analyzed using least square linear regression analysis of peak area. Correlation coefficient was found to be (r^2) 0.996 in the concentration range of 100 – 1200ng/spot with respect to the peak area (Fig: 2a)

Method Validation

HPTLC method was validated on the basis of precision, robustness, LOD and LOQ, recovery studies, ruggedness and specificity.

Precision

The relative standard deviations for repeatability of sample application and repeatability of measurement of peak area were found to be 0.093 and 0.016 respectively. The measurement of the peak area at five different concentrations levels of 200, 400, 600, 800 and 1000ng/spot. The precision measurement of peak area (intra and inter day precision) and instrumental variations for different concentration levels were found to be < 1% of %RSD which indicates the excellence of the method precision of (Table -2).

Robustness

The robustness was performed by introducing small deliberate changes in the mobile phase concentration (±2) the corresponding RSD values were obtained and it was indicated that the method was robust.

LOD and LOQ

The LOD was found to be 0.01 ng/spot and LOQ was found to be 3.14 ng / spot.

Specificity

Comparing the spectra at peak start, peak apex and peak end position of the spot assessed the peak purity of L- Dopa. Good correlation (r^2=0.996) was obtained between standard and sample spectra of L – Dopa(Table-4)..

Accuracy (% Recovery)

The sample was spiked with a known amount of standard and the percent ratio between the required and expected concentrations were calculated to be, 20, 40, 60, 80 and 100% (Table-3). The accuracy and reproducibility for the quantification of L –Dopa from seed extract after spiking was found to be in a range of 99.7 – 100.5 %.

CONCLUSION

The Rf values of L – Dopa obtained here can be used in the estimation of the compound. Thus we conclude that HPTLC technique is an accurate, specific, precise and robust method for the determination and quantification of L – Dopa in the seed extract. Statistical analysis of data proves that the method is reproducible for the analysis of L-Dopa.

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Table: 1. Linear regression of calibration curve for the estimation of L-Dopa*

Linearity range (ng)	100-1200ng
Correlation coefficient(r^2)	0.996
Instrumental precision (CV%)(n=5)	0.95
Slope (95% confidence limit)	3216.1
Confidence limit of slope (95% confidence limit	2879.4-2972.8
Intercept	1265.7
Specificity	specific
Confidence limit of intercept (95% confidence limit)	1242.8-1279.26

* All the values are replications of six time analysis

Table: 2. Intra and inter day precision of HPTLC Method (n=6)

Concentration of L-Dopa(ng)	Intra-day precision			Inter-day precision		
	Mean area	S.D(±)	% RSD	Mean area	S.D(±)	% RSD
200	5490.773	5.15	0.093	5418.667	8.08	0.149
400	9437.77	4.53	0.048	9566.767	11.92	0.124
600	10897.55	1.99	0.018	12066.3	1.94	0.016
800	13589.02	3.50	0.025	13790.53	10.36	0.075
1000	15301.46	3.03	0.019	14377	12.4	0.086

Table: 3. Recovery Studies (n=6)

Excess drug added to analyte (%)	Theoretical content(ng)	Amount found(ng)	Recovery (%)	% RSD
0	200	202.1	100	0.761
20	550	551.86	100.5	0.365
40	750	751.67	100	0.126
60	1000	1002.53	100	0.143
80	1250	1252.15	99.7	0.114
100	1500	1502.73	99.87	0.095

Table: 4. Method Validation parameters for estimation of L-Dopa

Parameter	Data
Linearity range (ng)	100-1200ng/spot
Correlation coefficient	0.9967
Limit of detection	0.01006
Limit of quantitation	3.14
Accuracy(% n=6)	100
Precision(% RSD)	0.95
Inter day(n=6)	0.149
Intraday(n=6)	0.018
Robustness	Robust
Specificity	Specific

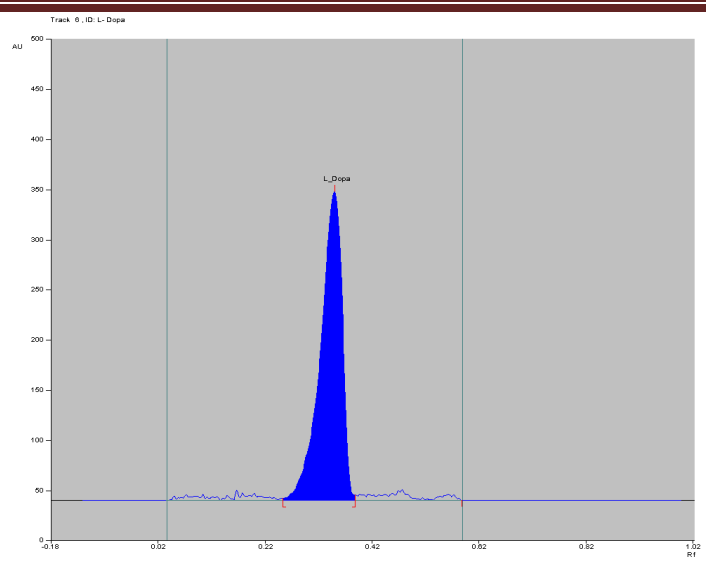

Fig: 1. HPTLC chromatogram of standard (L-Dopa)

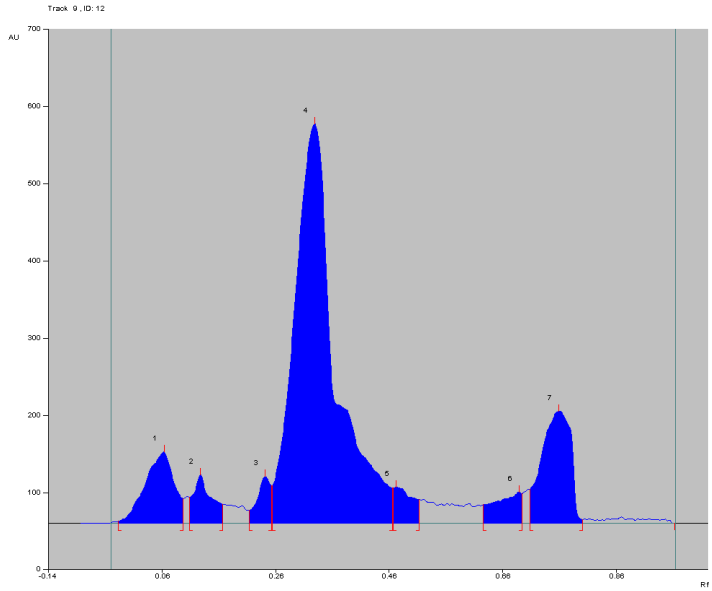

Fig: 1(a). Chromatogram of velvet bean (Black -Coloured seed)

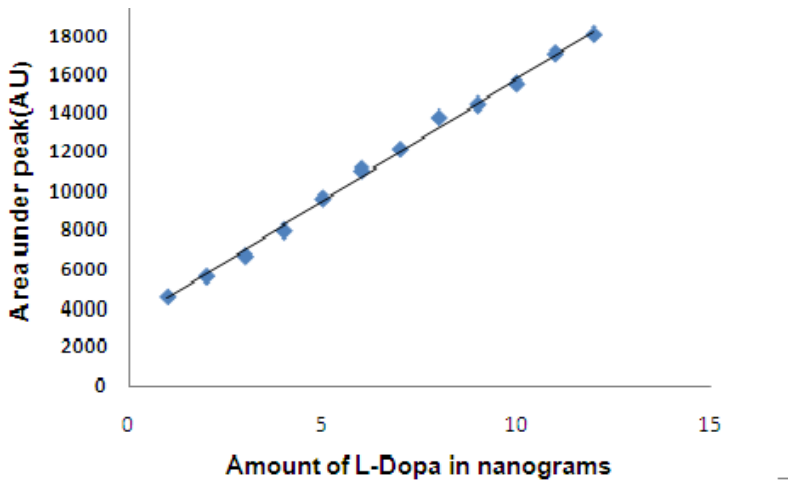

Fig. 2. Calibration curve for the estimation of L-Dopa

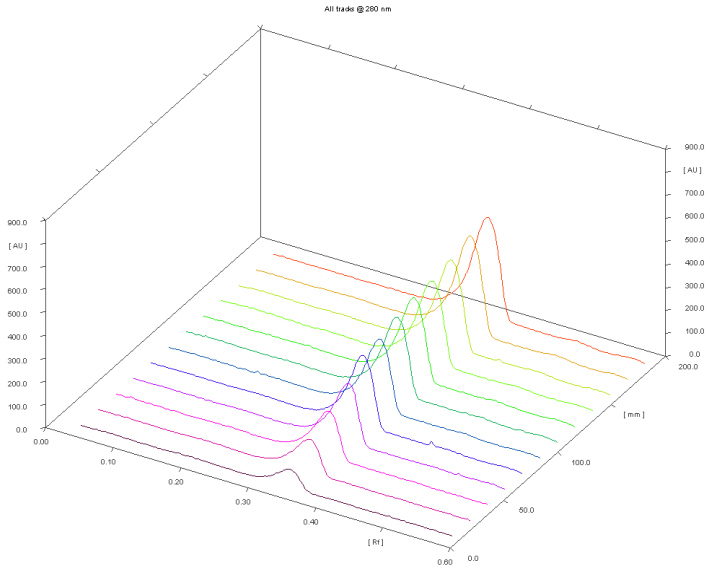

Fig: 2a. Linearity range of Standard (L-Dopa) by HPTLC analysis

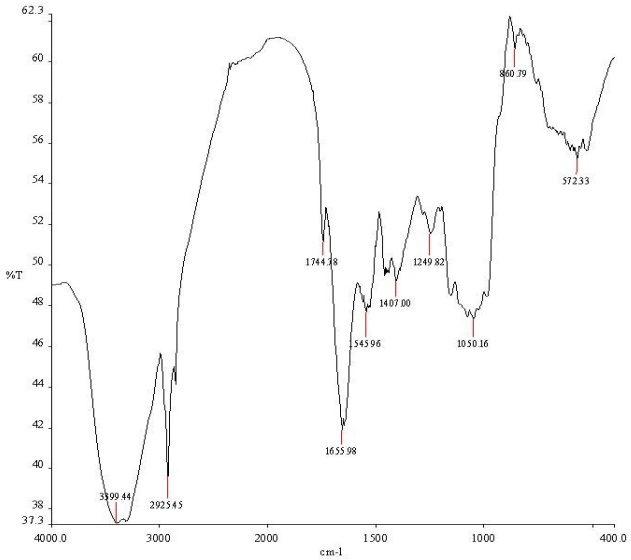

Fig: 3. FTIR analysis of velvet bean (Black -coloured seed)

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