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Application of High Performance Liquid Chromatography to the Determination and Validation of Levodopa in *Mucuna Pruriens* L.

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ABSTRACT

The seeds of *Mucuna pruriens* L. were extracted with 40% ethyl-alcohol, 60% water with 1.5% glacial acetic acid for the detection of levodopa and some physio-chemical parameters. A sensitive, precise and accurate high-performance liquid chromatographic (HPLC) method has been developed for the quantitative estimation of levodopa in *Mucuna pruriens* extract. The method utilises sample preparation step followed by separation on a Phenomenex C_{18}, 250 x 4.6 mm, 5 µm particle size column, using 0.1 M KH_2PO_4 (pH 3.0 by ortho-phosphoric acid) as the mobile phase. Analysis of levodopa was carried out in the absorbance mode at 283 nm. The method was validated in terms of linearity, precision (inter and intraday), accuracy, limit of detection (LOD) and limit of quantification (LOQ). Linearity was observed in the range of 20-100 ppm with correlation coefficient of 0.9991. Detection limit was 5.6 ppm and quantification limit was 8.5 ppm. The repeatability of the method was found to be 0.49% and recovery values from 101.17 to 100.18% indicates best accuracy of the method. The results indicated that the extract obtained was 50% Levodopa content and 38% protein content. The proposed HPLC method was also found to be precise, specific, accurate and can be used for the identification and quantitative determination of levodopa in herbal extracts.

KEYWORDS

High-performance liquid chromatography, levodopa, method development, *Mucuna pruriens* L., validation.

INTRODUCTION

Mucuna pruriens L. (Leguminosae), commonly known as ‘‘the cowhage’’ or ‘‘velvet’’ bean; “kaunch” and ‘‘atmagupta’’ in India, is a climbing legume endemic in India and in other parts of the tropics including Central and South America. In Ayurvedic system of medicine, *M. pruriens* was used for the management of male infertility, nervous disorders and also as an aphrodisiac[10-12] its different preparations from the seeds are also used for the management of ageing, rheumatoid arthritis, diabetes, male infertility and nervous disorders.[1]

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Leaves are useful in ulcers, inflammation, cephalagia and general debility. The seeds in addition to levodopa, contains tryptamine, 5-hydroxytryptamine (5-HT), mucunine, mucunadine, prurienine and prurieninine.[4] It is also rich in fatty content.[8-11] *Mucuna pruriens* seed powder contains high amount of Levodopa, which is a neurotransmitter precursor and effective remedy for the relief in Parkinson’s disease.[6] L-DOPA is a metabolic precursor of dopamine that is capable of crossing the blood brain barrier. It is produced from L-tyrosine by trysosine hydroxylase and metabolized by *catechol-O-methyl transferase (COMT)*. In the brain L-DOPA is converted to dopamine. It is conventionally used to increase dopamine concentrations in the brain as a treatment for

Parkinson's disease and stroke recovery.[3] Nerve cells can use levodopa to make dopamine and replenish the brain's dwindling supply. Dopamine itself cannot be given because it doesn't cross the blood-brain barrier, the elaborate meshwork of fine blood vessels and cells that filters blood reaching the brain.

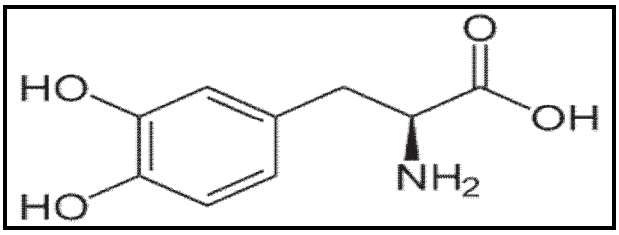

Figure 1: Levodopa (3,4 dihydroxyphenylalanine)

In India legumes constitute an important foodstuff and are the chief economic sources of proteins in the diets of economically weaker sections of population Now a day's research is being geared up to exploit the protein source from underutilized grain legume seeds. Underutilized species (both plant and animal) are those with a potential, not yet fully exploited, to contribute to food security and poverty alleviation.[11] among the different underutilized pulses, velvet bean is currently receiving global attention. The velvet bean, *Mucuna pruriens L.* pulse is a rich source of protein (20%), carbohydrate (65%), fat (15%) and several minerals.[12-13] The aim of this study was to develop 50% levodopa extract and its method and validation by HPLC.

MATERIAL AND METHODS

Plant Material

The seeds of *Mucuna pruriens* L. were purchased from Jharkhand, India and identified by our Taxonomist. A voucher specimen has been maintained at R&D Centre, Sanat Products Ltd., Sikandrabad, India. All other reagents were of HPLC grade or AR grade as per requirement. The active compound LevoDopa was purchased from ChromaDex (LGC Promochem, Bangalore, India).

Extraction Method

The air- dried seeds of *M. pruriens* were powdered and passed through 20 mesh sieve. The sieved material (100 g) was extracted with 400 ml 40% ethyl alcohol, 60% water with 1.5% glacial acetic acid at the temperature of 60-70°C for 1-2 h on a water bath. The material was filtered and marc was further refluxed three times with 300 ml ethyl alcohol (40%). Following this all the extracts were pooled together, concentrated up to 40% under vacuum using rota-vac (Heidolph, Schwalbach, Germany). The material was kept overnight for settle down. After that it filtered and the material was air-dried and use for assay.

Estimation of Physio-chemical Parameters

The protein content in *Mucuna* extract was determined by Kjeldahl method.[5] The extractive value in water (1% solution), pH, ash content (%) were estimated by the method of Ayurvedic Pharmacopoeia if India (API, 1999). Loss of drying was analysed by Karl Fisher (SpectraLab).

Preparation of Standard Solution

Standard solution of pure Levodopa was prepared by dissolving 2.0 mg in 20 ml (100 ppm) of 0.1N aqueous HCL in a volumetric flask (stock solution). For the determination of limit of detection (LOD) and limit of quantification (LOQ), 2 ml of the stock solution was diluted to 10 ml (20 ppm), 4 ml of the same stock solution was diluted to 10 ml (40 ppm), 6 ml of the same stock solution was diluted to 10 ml (60 ppm) for linearity study.

Preparation of Sample Solution

Approx. 50 mg grinded extract was taken and dissolved with 15 ml 0.1N aqueous HCL separately. The sample was sonicated for 20 min. After sonication the volume was made up to 50 ml with 0.1N aqueous HCL and filtered through 0.45 µm membrane filter.

High Performance Liquid Chromatography Chromatographic Conditions and Procedure

Levodopa was analysed by High Performance Liquid Chromatography (HPLC, Shimadzu, LC 2010A, Japan), Autosampler, UV-Detector. The data was acquired on the LC solution administrator data system (Japan). Phenomenex

C_{18} column (250 mm x 4.6 mm, 5 µm) (California, USA) and an isocratic mixture of 0.1M KH_2PO_4, pH 3.0 by ortho-phosphoric acid as a mobile phase. The mobile phase was filtered through 0.45 µm Millipore filter and degassed by sonication for 30 min. The flow rate was adjusted to 1.0 ml/min. Injection volume was adjusted to 20 µl and detection was made at 283 nm.

Calibration Curve

Five different concentrations of stock solution after dilution (20, 40, 60, 80, 100 ppm) with mobile phase were injected in triplicates. Regression equation and co-efficient of correlation (r^2) was derived (Table 1).

Method Validation

Validation of the analytical method was done according to the International Conference on Harmonization (ICH) guidelines16). The method was validated for linearity, precision, accuracy, LOD and LOQ.

Linearity

Linearity was determined by using five concentrations of the standard solution (20 -100 ppm). The calibration curve was obtained by plotting the absorbances versus the concentrations of the standard solution.

Table 1: Method validation parameters for the estimation of L-dopa by propose method

S.No.	Parameters	Values
1.	Instrumental precision	0.58
2.	(CV, %) (n = 7)	0.49
3.	Repeatability (CV, %)	8.5 ppm
4.	(n =5)	5.6 ppm
5.	Limit of quantification	0.9991
6.	(LOQ, ppm)	y = 14108x
7.	Limit of Detection	- 6171
	(LOD, ppm)	20-100
	Linearity (correlation coefficient)	
	Regression equation	
	Range (ppm)	

Table 2: Intra- and Inter-Day precision of proposed method

S. No.	Concentration (ppm)	Intra-day precision (RSD, %, n = 6)	Inter-day precision (RSD, %, n = 6)
1.	20	0.58	1.10
2.	40	0.62	0.69
3.	60	0.87	0.92
4.	80	0.94	0.83
5.	100	0.78	1.25

Table 3: Physiochemical analysis of *Mucuna pruriens* L. extract

S.No.	Parameters	Observations
1.	Description	Yellowish brown colour
2.	Levodopa content (%)	50%
3.	Protein Content (%)	38%
4.	Extractive Value in Water (1% Water)	85.46%
5.	Loss on Drying (%, at 105^0C)	2.8%
6..	Ash content (%)	3.86%

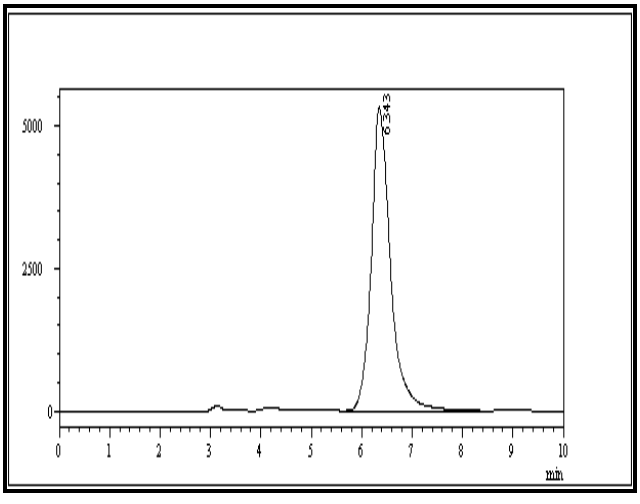

Figure 2: HPLC chromatogram of Reference Standard (Levodopa)

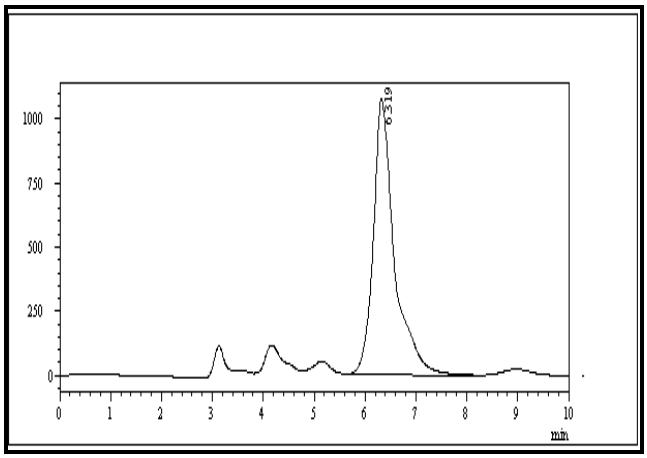

Figure 3: HPLC chromatogram of *Mucuna pruriens* L. extract

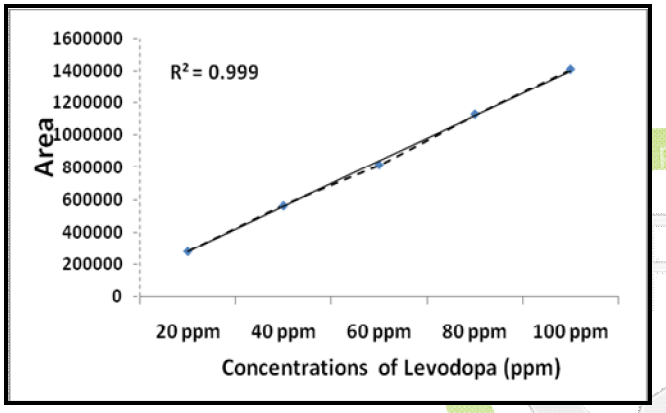

Figure 4: Linearity graph of different concentrations of *Mucuna pruriens* L. extract

Precision

The precision of the method was determined by analyzing 20, 40, 60, 80 and 100 ppm concentrations of Levodopa standard solution (n = 3) on the same day for intraday precision and on 3 different days for interday precision by the propose method. The precision was expressed as percent relative standard deviation (% RSD).

Accuracy

The accuracy of the method was tested by performing recovery studies at 3 levels of Levodopa reference standard (50%, 100%, and 150%). The average percentage recovery was estimated by applying values of peak area to the regression equations of the calibration graph. Three replicate samples of each concentration level were prepared.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

According to the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) recommendations, the approach based on the S.D. of the response and the slope were used for determining the limit of detection and limit of quantification.

RESULTS AND DISCUSSION

HPLC Analysis of Levodopa and Physio-chemical Parameters in Mucuna pruriens L. Extract

Mucuna pruriens was extracted with 40% ethyl alcohol, 60% water with 1.5 glacial acetic acid and observed that Levodopa was 50% and protein contents was 38% in *Mucuna* extract (Table 3). The extractive value (in 1% solution) was 85.46%, loss on drying was 2.8% and ash content was 3.86% in *Mucuna* extract L. (Table 3).

Development of HPLC Method

The method development and selection of a suitable mobile phase involved several trials because of the complexity of the chemical composition of the herbals and the affinity of the components towards various solvents. The proportions of the organic and aqueous phases were adjusted to obtain a rapid and simple assay method with reasonable run time, suitable retention time and sharp peak. Under optimized conditions HPLC with C_{18} column and UV detector at 283 nm using isocratic mixture of 0.1M KH_2PO_4 (pH 3.0 by ortho-phosphoric acid) as mobile phase results in well resolved symmetric peak for Levodopa. The total run time of Levodopa was found to be 10 min and the Levodopa appeared on chromatogram at 6.319 min in test sample (Fig 3). The retention time of reference standard (Levodopa) was observed to be 6.343 min (Fig 2). This indicates that the present HPLC method is rapid, easy and convenient. When the same drug solution was injected 6 times, the retention time of the peak was found to be same.

Method Validation

The method was validated for its linearity, accuracy, precision, limit of detection and limit of quantification. A good linear relationship was obtained within the concentration range of 20-100 ppm of Levodopa with a correlation coefficient (r^2) of 0.9991 (Fig 4).The representative linear equation was y = 14108x – 6171 (Table 1). The light intensity and concentration were subjected to lease square linear regression analysis to calculate the calibration equation and correlation coefficient. The linearity of calibration graph and adherence of the system to Beer's Law was validated by high value correlation coefficient. The intraday and interday precisions of Levodopa are given in Table 2. The results showed acceptable precision with RSD less than 2%. The percentage recovery of levodopa was found to be 101.17 to 100.18%, which indicates good accuracy of the method. The LOD and LOQ of Levodopa were 5.6 with an S/N ratio of 10:1 and 8.5 ppm, with an S/N ratio of 10:1 respectively (Table 1).

CONCLUSION

50% Levodopa extract was developed with the extraction of 40% ethyl alcohol, 60% water with 1.5% glacial acetic acid in *Mucuna* pruriens seeds. An analytical reverse-phase HPLC method was developed and validated for the determination of purity and assay of levodopa. The developed method is sensitive, simple, and accurate, and can be employed for monitoring the purity of levodopa in herbal extracts. The method has been proved to be selective and stable.

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