

Development and Validation of an HPTLC - Densitometric Method for Determination of Levodopa in seeds of *Mucuna pruriens* and its capsule dosage form

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Abstract

A simple, sensitive, selective HPTLC - densitometric method was developed and validated for the determination of Levodopa in seeds of *Mucuna pruriens* and its herbal dosage form. Analysis of Levodopa was carried out on TLC plates pre-coated with silica gel 60F₂₅₄ as stationary phase. Linear ascending development of the plate was done in a pre-saturated twin trough glass chamber. The mobile phase consisted of n-butanol – glacial acetic acid – water (5:1:4, v/v/v) at room temperature (25 ± 2° C). Camag TLC scanner III was used for spectrodensitometric scanning and analysis was done in absorption mode at 280nm. The system gave compact spot for Levodopa (R_f value of 0.39 ± 0.04). The polynomial regression analysis data for the calibration plots showed correlation coefficient $r = 0.999$ in concentration range 100 – 1000 ng/ spot with respect to peak area. According to International Conference on Harmonization (ICH) guidelines, the method was validated for precision, recovery, robustness and ruggedness. The limits of detection and quantification were determined. The statistical data analysis showed that the method is reproducible and selective for estimation of Levodopa.

Keywords:

Mucuna pruriens; Levodopa; HPTLC - Densitometric method; Method Validation; Dosage form

1. Introduction

Mucuna pruriens from family fabaceae, an annual, half woody, twinning herb with long cylindrical branches found in the foothills of the Himalayas, plains of several eastern, southern and middle parts of India and in Central and South America [1]. From the ancient times, the different parts of this plant like seeds, leaves and roots have been used for treatment of many diseases. The seeds of *Mucuna pruriens* containing Levodopa as an important constituent, have been reported for Anti – Parkinson's activity [2-6], Aphrodisiac activity [7-8], Antidiabetic activity [9-13], Hypoglycemic and Hypocholesterolemic activity [14], Antitumor activity [15], Antioxidant activity [16-18], Neuroprotective activity [19], Learning and memory enhancing effect [20], Antivenom activity [21-22], Anti inflammatory activity [23] etc. Phytochemical investigation revealed the presence of tryptamine, 5-HT, mucunine, mucunadine, prurienine and prurieninine [24-25].

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Several analytical methods have been reported for the determination of Levodopa based on non – aqueous titration [26-27], UV [27], HPLC [28-29] and HPTLC [30-31] in formulations and also in plant extracts. The present work includes not only the estimation of Levodopa in herbal dosage form but also isolation and quantitative estimation of Levodopa in the crude drug of *Mucuna pruriens*. The proposed method was validated in compliance with ICH guidelines [32].

2. Experimental

2.1. Materials

Standard Levodopa was procured from Sun Pharmaceuticals, Vadodara, India. Seeds of *Mucuna pruriens* were collected from the local supplier and were assessed biologically by the Department of Botany. All chemicals and reagents used were of analytical grade and were purchased from Merck Chemicals, India.

2.2 Isolation of L-DOPA from the seeds [33]

The seeds of *Mucuna pruriens* were dried in shade and powdered in a mechanical grinder. Then it was passed through sieve no. 20. The seed powder was macerated with 1% aqueous acetic acid containing 0.1% sodium sulfide for 6hrs at 60°C. It was filtered and concentrated to half volume by rotary vacuum evaporator. Then the concentrate was freeze-dried to get the crystals of Levodopa. The crystals of Levodopa were re-crystallized to get off-white colored pure Levodopa. The isolated Levodopa was qualitatively characterized by FTIR and LC-MS (Figure 7 and 8).

2.3. Instrumentation and Chromatographic techniques

The samples were spotted in the form of bands of width 6mm with a Camag microlitre syringe on pre-coated silica gel plate 60F₂₅₄ (20 cm × 10 cm) with 200µm thickness (E.Merck, Germany) using a Camag Linomat V sample applicator (Switzerland). Space between two bands was 10mm. The slit dimension was kept at 6.00mm × 0.45mm and 20mm/sec scanning speed was employed. The composition of mobile phase was n-butanol – glacial acetic acid – water (5:1:4, v/v/v). Linear ascending technique was used for development for TLC plates in a twin trough glass chamber saturated with mobile phase. The chamber was previously saturated with the solvent system for 30mins at room temperature (25 ± 2° C) at relative humidity (45 ± 5%). The length of chromatogram run was 80mm. Subsequent to the development, TLC plates were air dried. Densitometric scanning was performed on Camag TLC scanner III in the absorbance mode at 280nm. The source of radiation utilized was a deuterium lamp.

2.4. Calibration curve of Levodopa

A stock solution of Levodopa (100µg/ml) was prepared in distilled water. Different volumes of stock solution 1, 2, 4, 6, 8, 10µl were spotted on TLC plate to obtain concentration of 100, 200, 400, 600, 800 and 1000ng/spot of Levodopa. The data of peak areas were plotted against the corresponding concentration.

2.5. Method validation

2.5.1. Precision

Repeatability of the sample application and measurement of peak area were carried out by spotting six replicates of the same spot (400ng/spot of Levodopa) and was expressed in

terms of percent relative standard deviation (%R.S.D). The intra- and inter- day variation for the determination of Levodopa was carried out at three different concentration levels of 200, 400 and 600ng/spot.

2.5.2. 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 pre-washed TLC plates with methanol, the effects on result were examined. Robustness was done in triplicate at a concentration level of 400ng/spot and the %R.S.D of peak areas was calculated.

2.5.3. 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.

2.5.4. Recovery studies

The pre-analyzed samples were spiked with 80, 100 and 120% of the standard Levodopa and the mixtures were reanalyzed by proposed method. The method was repeated for six times. This was done for check of recovery of Levodopa at different levels in the formulation.

2.5.5. Ruggedness

A solution of concentration 1000 ng spot⁻¹ was prepared and analyzed on day 0 and after 6, 12, 24, 48 and 72 hrs. Data were treated for %R.S.D to assess ruggedness of the method.

2.5.6. Specificity

The specificity of the method was ascertained by analyzing the standard drug and the isolate. The isolated Levodopa was confirmed by comparing R_f values and spectra of the spot with that of standard. The peak purity of Levodopa 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.

2.6. Analysis of Levodopa in herbal isolate

The isolated Levodopa was qualitatively characterized by FTIR and LC-MS/MS. To determine the content of Levodopa in herbal isolate, 10mg of isolate was dissolved in 10ml of distilled water. Successive dilutions were done to get a solution of concentration 100 ng μL^{-1} . Then 10 μL of sample was applied on TLC plate. The analysis was repeated for six times.

2.7. Analysis of Levodopa in marketed herbal formulation

To determine the content of Levodopa in marketed formulation a single composition capsule of *Mucuna pruriens* was chosen. The content of one capsule was dissolved in 100ml of distilled water for 24hrs, with occasional shaking. After 24 hrs, it was kept at 60°C for 2-3 hrs, filtered on a Whatman no.41 filter paper. 1mL was taken and diluted with methanol to get 100 ng μL^{-1} . 4 μL sample was applied on the TLC plate followed by development and scanning. The analysis was repeated for six times.

3. Results and Discussion

3.1. Development of the optimum mobile phase

The TLC procedure was optimized with a view to quantify the herbal isolate. Initially n-butanol – glacial acetic acid – water in varying ratios was tried. The mobile phase n-butanol – glacial acetic acid – water (5:1:4, v/v/v) gave a sharp and well defined peak at $R_f = 0.39$ (Figure 1). Well defined spots were obtained when the chamber was saturated with mobile phase for 30min at room temperature.

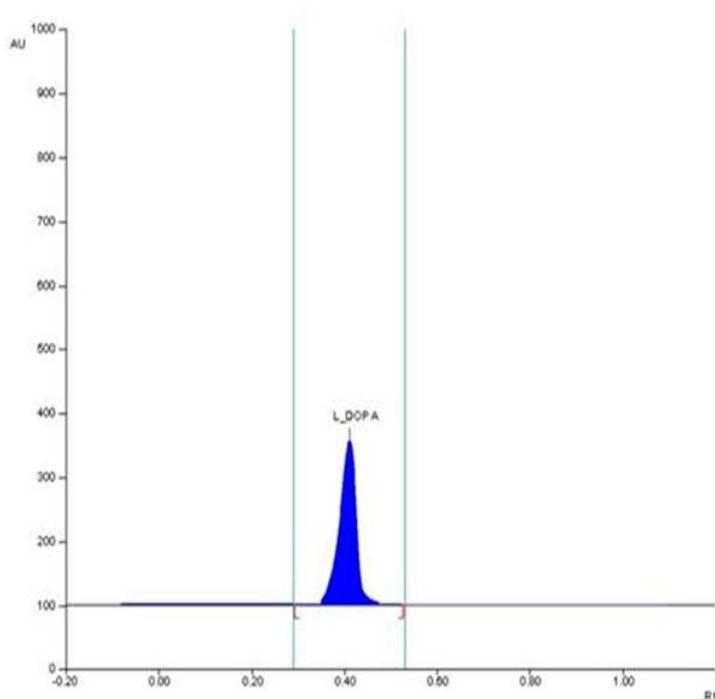


Fig.1. Chromatogram of Standard L –DOPA (1000ng/spot), mobile phase: n-butanol – glacial acetic acid – water (5:1:4, v/v/v)

3.2. Calibration curve

The developed HPTLC method for estimation of Levodopa showed a good correlation coefficient (r) 0.999 in concentration range of 100 – 1000ng/spot with respect to the peak area.

3.3. Method validation

The measurement of peak area at three different concentration levels showed good values of % R.S.D. (< 1%) for inter –and intra – day variation, which suggested an excellent precision of the method (Table 1). The low values of S.D and %R.S.D. obtained after small changes in experimental conditions indicated robustness of the method (Table 2). Detection limit and quantification limit with signal- to- noise ratio of 3:1 and 10:1 were found to be 3.52 and 9.14ng respectively, which indicates the adequate sensitivity of the method.

Table 1. Intra- and Inter- day precision (n = 6)

Amount (ng/spot)	Intra-day precision			Inter-day precision		
	Mean area	S.D.	%R.S.D.	Mean area	S.D.	%R.S.D.
200	1585.83	1.53	0.096	1538.56	1.67	0.108
400	3168.39	1.72	0.054	3215.37	1.99	0.061
600	4798.27	1.47	0.030	4682.80	1.85	0.039

Table 2. Robustness of the HPTLC method (n = 3, 400ng/spot)

Parameters	S.D. of peak area	%R.S.D.
(1) Mobile phase composition (n – butanol – glacial acetic acid - water)	1.59	0.048
(2) Mobile phase volume (15, 20 and 25ml)	1.48	0.045
(3) Duration of saturation (20, 30 and 40mins)	1.69	0.052
(4) Activation of pre-washed TLC plates	1.34	0.042

The proposed method when used for extraction and quantification of Levodopa in the formulation, the recovery was found 99.39 – 100.475% as listed in Table 3. Low %R.S.D. value of 0.0492 between the peak area values proved the ruggedness of the method indicating Levodopa is stable during the extraction procedure as well as during the analysis. The peak purity of Levodopa 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. Good correlation ($r = 0.999$) was obtained between the standard and sample overlain spectra of Levodopa (Fig. 2)

Table 3: Recovery studies (n = 6)

Excess drug added to analyte (%)	Theoretical content (ng)	Amount found (ng)	Recovery (%)	%R.S.D.
0	400	398.76	99.69	0.110
80	600	602.85	100.475	0.264
100	800	795.14	99.39	0.147
120	1000	998.60	99.86	0.098

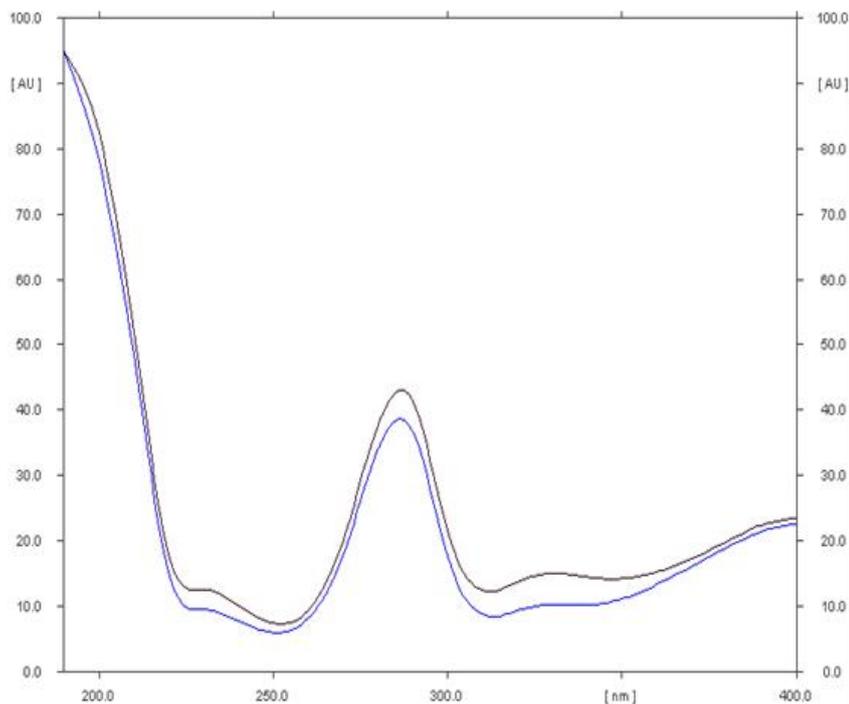


Fig. 2. Spectral comparison of standard L-DOPA and herbal isolate (— L-DOPA, — herbal isolate)

3.4. Estimation of L-DOPA in herbal isolate

A single and prominent spot at $R_f = 0.39$ was found in the chromatogram of the isolated Levodopa from seeds (Fig. 3). The total Levodopa content was found to be 4.826 % (w/w) in the seeds.

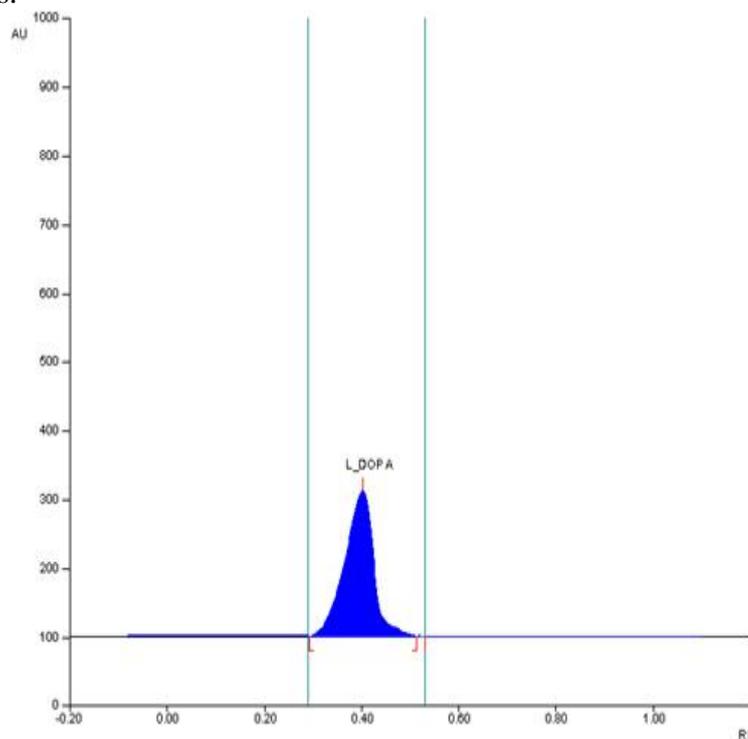


Fig. 3. Chromatogram of Isolated L –DOPA from seeds (1000ng/spot), mobile phase: n-butanol – glacial acetic acid – water (5:1:4, v/v/v)

3.5. Estimation of Levodopa in marketed herbal formulation

A single spot at 0.43 was observed in the chromatogram of Levodopa extracted from herbal capsules (Fig. 4 and Fig. 5). The % of Levodopa found to be 8.44% and 7.48% for two different batches and was well within the limits (Label claim 5-10%). A good overlain spectrum was found between the standard and the Levodopa extracted from the formulations (Fig. 6).

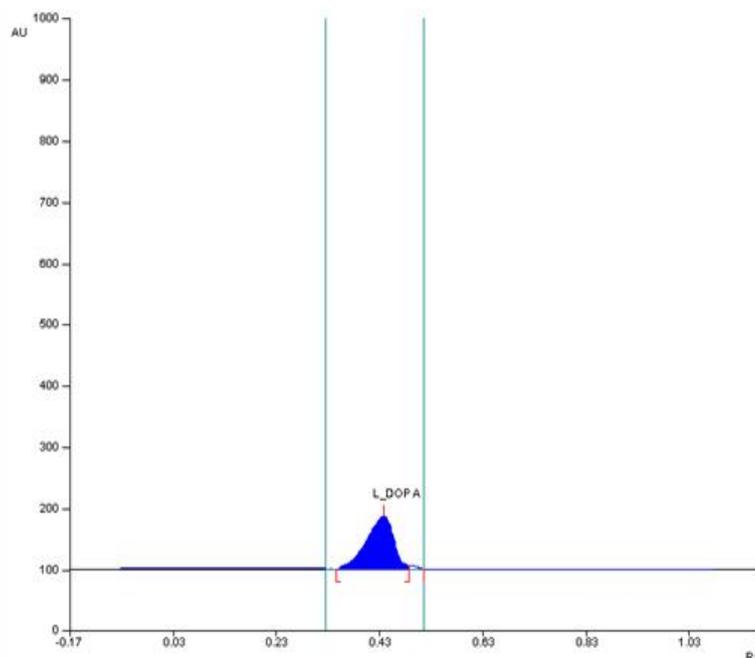


Fig. 4. Chromatogram of L-DOPA extracted from herbal single component formulation (1st batch), mobile phase: n-butanol – glacial acetic acid – water (5:1:4, v/v/v)

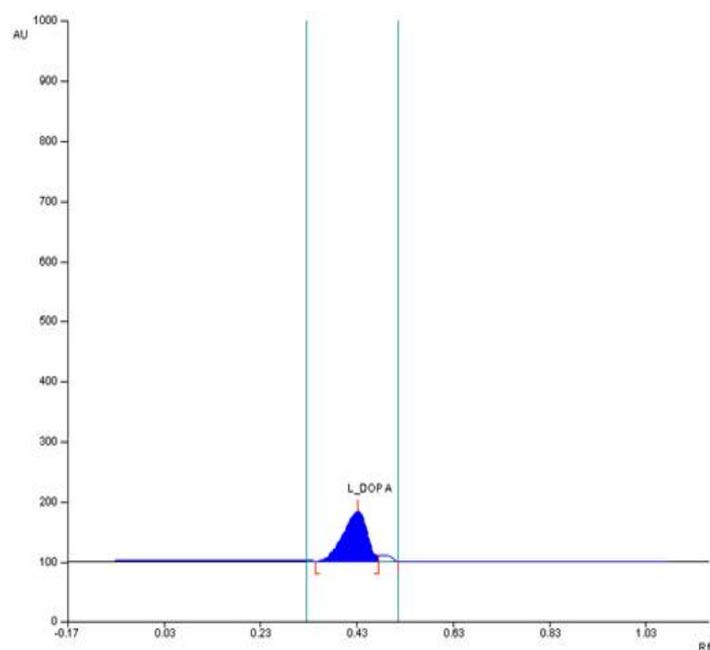


Fig.5. Chromatogram of L-DOPA extracted from herbal single component formulation (2nd batch), mobile phase: n-butanol – glacial acetic acid – water (5:1:4, v/v/v)

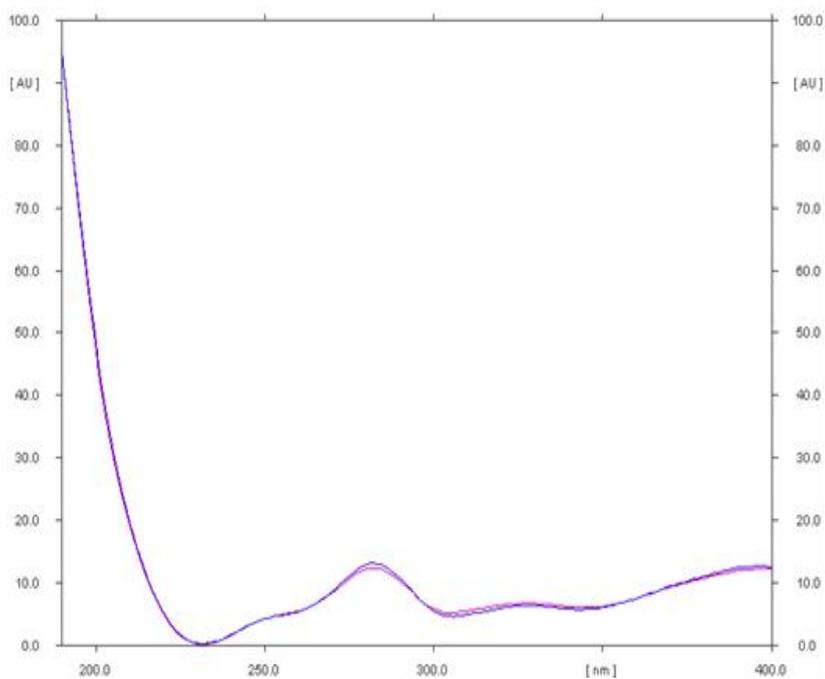


Fig. 6. Spectral comparison of standard L-DOPA and L-DOPA extracted from herbal single component formulation (— L-DOPA, — L-DOPA isolated form formulation)

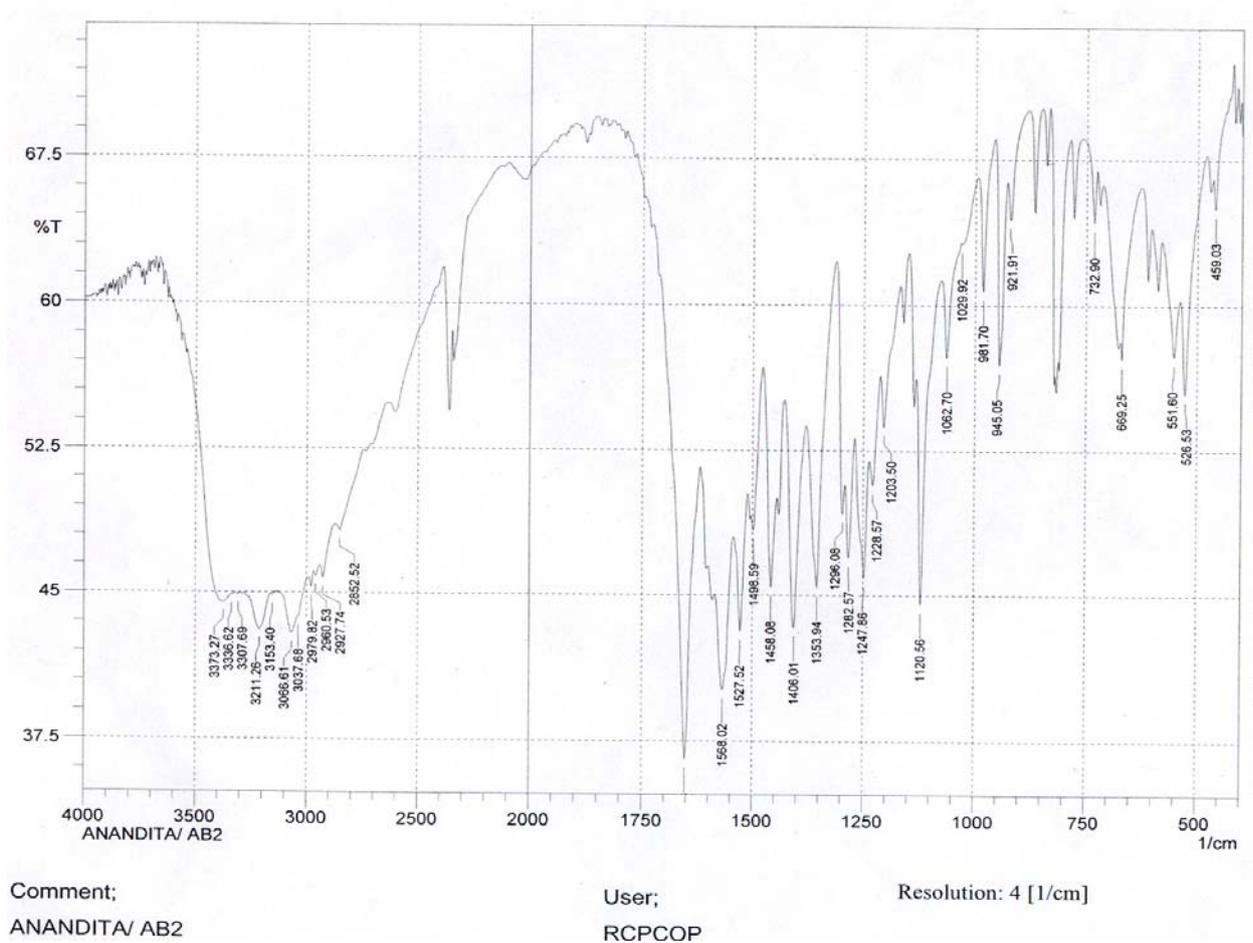


Fig. 7. FTIR of isolated Levodopa

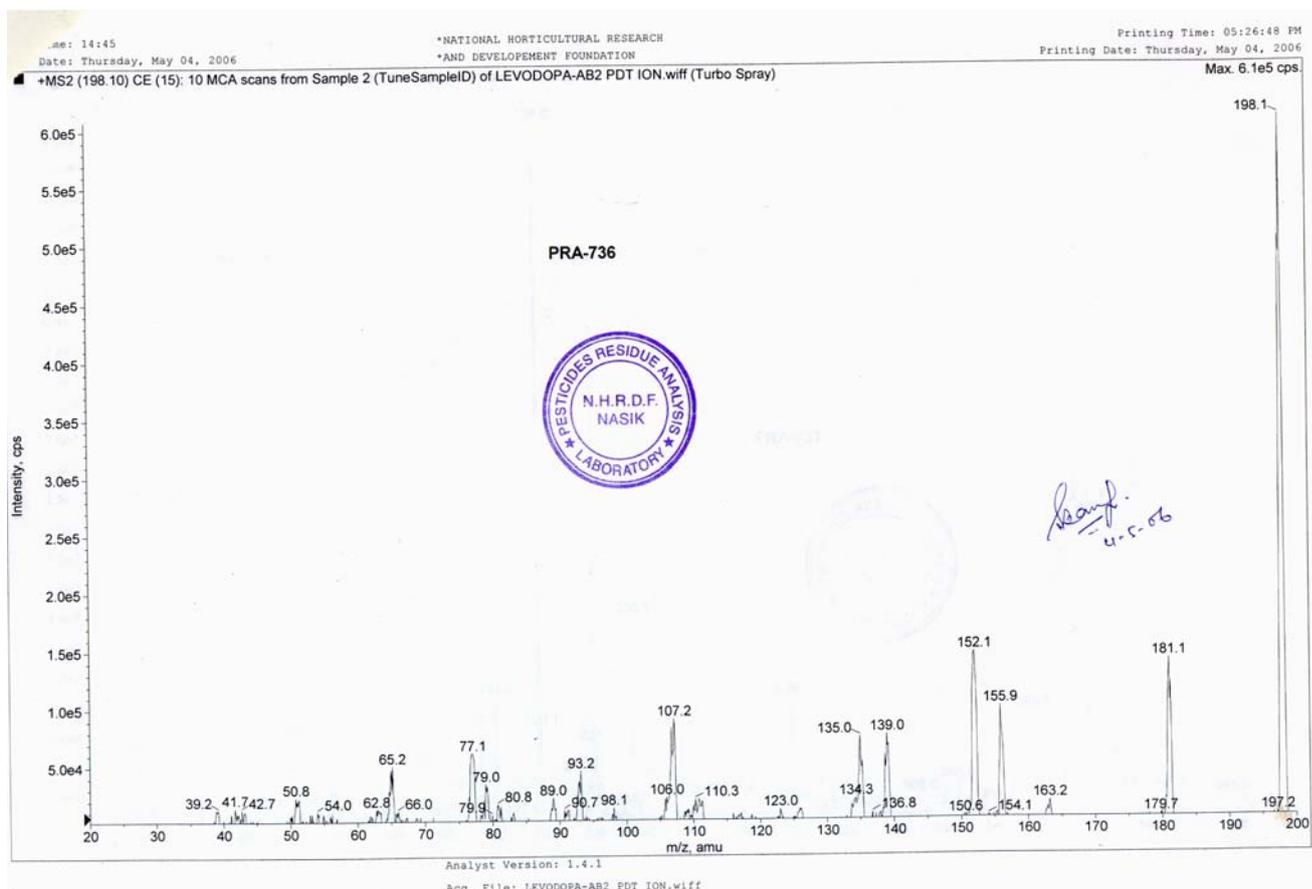


Fig. 8. LC – MS of isolated Levodopa

4. Conclusion

The developed HPTLC technique is an accurate, precise, specific and robust for the determination of L-DOPA both in herbal extract and marketed formulation. Statistical analysis proves that the method is reproducible and selective for the analysis of Levodopa. Further the proposed method can be used for degradation of Levodopa under stressed condition as per ICH guidelines.

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