Development and Validation of a Quantification Method for L-Dopa in Plants and Pharmaceutical Materials

Bitkilerde ve Farmasötik Materyallerde L-Dopa için Analiz Metodunun Geliştirilmesi ve Validasyonu

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ABSTRACT

L-Dopa is an antiparkinsonian drug and is used per oral application. A reliable liquid chromatographic method for determination of L-Dopa in leaves, flowers and seeds of *Vicia faba* **L.** grown in Turkish habitats and pharmaceutical dosage forms has been developed. The analysis has been carried out using Ace C18 (5 µm, 4.6 x 250 mm,) column, and the separation was carried out using a mobile phase consisting of 50 mM potassium dihydrogen phosphate (pH 2.3) at a flow rate of 1.2 mL min^{-1} with UV detection at 280 nm. The method has been validated according to the acceptance criteria of the ICH guidelines. The method demonstrated good linearity ($R^2 > 0.999$) over the assayed concentration range, good intra-day and inter-day precision. The detection limit (LOD) and the quantification limit (LOQ) values were determined as 1.70 and 5.13 µg mL^{-1} for L-Dopa. Developed method has been successfully used for the determination of L-Dopa in pharmaceutical formulations and plant materials.

Key Words
L-Dopa, HPLC-UV, method, calidation, plant material.

ÖZ

L-Dopa antiparkinson ilaçtır ve oral uygulama için kullanılır. Türk habitatlarında yetiştirilen *Vicia faba* L.'nin yapraklarında, çiçeklerinde ve tohumlarında ve farmasötik dozaj formlarında L-Dopa' nın belirlenmesi için güvenilir bir sıvı kromatografik metod geliştirilmiştir. Analizler Ace C18 (5 µm, 4.6 x 250 mm,) kolon kullanılarak yapıldı ve ayırma, 1.2 mL min^{-1} akış hızında 50 mM potassium dihydrogen phosphate (pH 2.3) içeren bir mobil faz kullanılarak 280 nm de UV deedektör ile gerçekleştirildi. Metod, ICH yönergelerinin kabul kriterlerine göre valide edilmiştir. Metod, analiz edilen konsantrasyon aralığında iyi doğrusallık ($R^2 > 0.999$), gün içi ve günler arası kesinlik gösterdi. L-Dopa için teşhis limiti (LOD) ve tayin limiti (LOQ) değerleri 1.70 ve 5.13 µg mL^{-1} olarak belirlenmiştir. Geliştirilen metod, ilaç formülasyonlarında ve bitki materyallerinde L-Dopa' nın belirlenmesi için başarıyla kullanılmıştır.

Anahtar Kelimeler
L-Dopa, HPLC-UV, metod, validasyon, bitki materyali.

INTRODUCTION

During the last few years, new medicines have become available for treating Parkinson's disease. Nevertheless, L-Dopa has been regarded as a gold standard treatment for motor symptoms since the administration of dopamine supplementation [1–3]. L-Dopa is a natural precursor of dopamine and is used as a prodrug, as it may cross the brain-blood barrier, contrary to dopamine. The chemical properties of L-Dopa have been given in Table 1 [4].

Long-term use of L-Dopa leads to motor problems and variations such as the 'wearing-off' condition characterized by moments without the benefits of therapy and moments with its advantages but with additional dyskinesia. Research has shown that high L-Dopa doses have also been associated with patient dyskinesia [5-7]. Therefore, it is recommended that doses of L-Dopa be modified according to the patient's individual needs based on clinical response and adverse event profile [1]. Several analytical methods for the determination of L-Dopa and its metabolites in biological matrices have been identified in the literature using high-performance liquid chromatography and various detection techniques such as electrochemical detection [8–16], tandem mass spectrometry (MS-MS) [17–22], and fluorescence [23, 24]. Most of the above-mentioned methods have a common low sensitivity limitation and time consuming chromatographic separation.

Therefore, the purpose of this study was to develop and validate a robust and efficient HPLC method to determine the amount of L-Dopa in drug formulations in a pharmaceutical laboratory for the routine quality control study. The pharmaceutical industries use the time-consuming LC method and various mobile phases for different dosage forms of drugs. But with the system being developed, the cost and time required to adjust mobile phases could be avoided, as only one mobile phase could be used for all drugs and their combinations. The method should be applied from drug release experiments with prototypes and marketed fixed-dose combinations and to determine the number of samples under compendial testing conditions. Hence the method needs to be robust against matrix effects caused by formulation excitements.

MATERIALS and METHODS

Reagents

All of the chemicals were used without further purification in this study. L-Dopa standard was acquired from Sigma-Aldrich (Istanbul, Turkey). Pharmaceutical tablets were bought from local pharmacies. Potassium dihydrogen phosphate, orthophosphoric acid, and triethylamine have been purchased from Merck Ltd. Ultrapure water was obtained using a Milli-Q System with conductivity lower than $0.05\ \mu S\ cm^{-1}$. All other chemicals were of analytical reagent grade.

Determination of λ_{max}

$20\ \mu g\ mL^{-1}$ standard solution of L-Dopa in ultrapure water was scanned on a UV spectrophotometer (Shimadzu UV-1800 spectrophotometer). λmax was obtained from the UV spectrum of L-Dopa.

Chromatographic Conditions

HPLC analysis was performed on an Agilent 1260 HPLC system (Agilent Technologies, USA), equipped with binary pump, auto sampler, vacuum degasser, thermostatted column compartment, UV detector, and Chems-

Table 1. The chemical properties of L-Dopa.

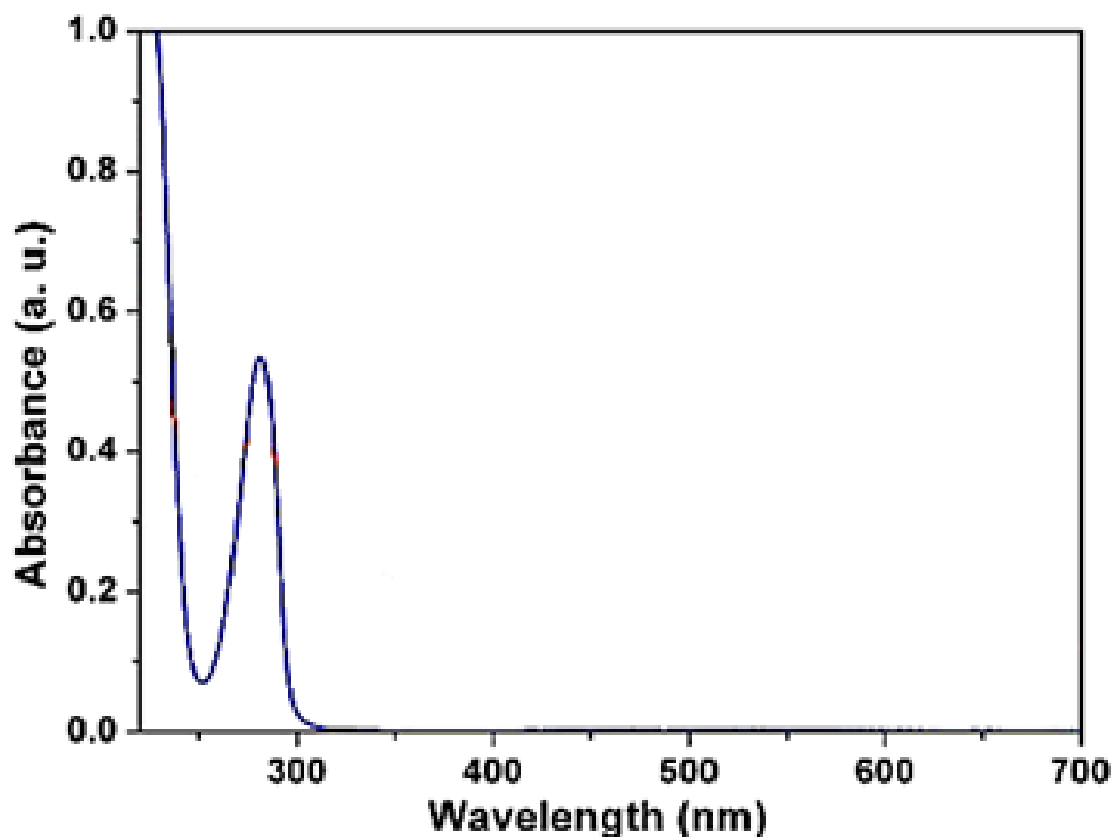| The chemical properties	Value
3 Structure of molecules	
The formula of molecular:	$C_9H_{11}NO_4$
The weight of the molecules ($g\ mol^{-1}$):	197.19
logP:	0.05
pK_a:	pK_a (Strongest Acidic): 1.65 pK_a (Strongest Basic): 9.06

Figure 1. Designed target molecule compared with econazole.

tation software. Chromatographic analysis was carried out on a column Ace 5 C18 (250 x 4.6 mm, 5 μm). The mobile phase was 50 mM potassium dihydrogen phosphate (pH 2.3). For buffer solution: 6.80 g potassium dihydrogen phosphate was accurately weighed. The volume was completed in liters by dissolving in deionized water. 5 ml of triethylamine was added. The pH of the obtained solution was adjusted to 2.3 by adding orthophosphoric acid. The mobile phase was filtered through a 0.45 μm membrane filter and degassed before use. Chromatographic analysis was carried out at a temperature of 30 °C using a flow rate of 1.2 mL min^{-1} and a run time of 10 min. The injection volume was 20 μL, and the wavelength was set at 280 nm. The mobile phase has been used as a diluent and blank preparation.

Preparation of Standard Solutions
The standard of 100.00 mg L-Dopa was accurately weighed and transferred to a 100 mL volumetric flask (Class A), and mixed with 35 mL of deionized water, dissolved in an ultrasonic bath until a clear solution was obtained, equilibrated to room temperature (~24 °C)

and, completed to volume with deionized water. This prepared solution was called as stock standard solution. Standard solutions were prepared by diluting from stock standard solution with ultrapure water so that the final L-Dopa concentration was 20, 40, 60, 80 and 100 μg mL^{-1}.

Preparation of Sample
L-Dopa is an amino acid that dissolves well in water. Ten of tablets were weighed and crushed in mortar and ground into fine powder. Powder samples equal to ~50 mg of L-Dopa were placed in a 500 mL volumetric flask, diluted with 200 mL of deionized water and dissolved in an ultrasonic bath for 30 minutes. It was equilibrated at room temperature (~24 °C) and, completed to volume with deionized water. All samples were filtered through a 0.45 μm Millipore Millex-HV filter before injection.

1 g of the leaves, flowers and fruits of the dried, ground *Vicia faba* L. is carefully weighed separately and 50 ml of boiling deionized water is added. It was extracted in an ultrasonic bath with duration of 15 min. It was filtered

through white filter paper. The extracts were stored in the fridge until analysis.

Validation Procedure

L-Dopa solution, 20 μg mL^{-1} in deionized water, was tested by the UV spectrophotometer. λmax was determined as 280 nm from the UV spectrum of L-Dopa (Figure 1).

The validation study was conducted in accordance with the recommendations for validation of the International Conference on Harmonization [25, 26]. Parameters analyzed include linearity, accuracy, precision, the limit of detection (LOD), limit of determination (LOQ), specificity, the suitability of the system and robustness. During the method validation, parameters were studied as the specificity, linearity, accuracy, precision and suitability of the system, robustness, detection limit (LOD) and quantification limit (LOQ).

The new HPLC method was primarily designed for measuring high drug loads and the release of drugs. However, the method should also be applied in routine testing as much as possible, for instance for testing pharmaceutical formulations. For the second reason, the method must ensure that the drug is properly identified within a broad range of concentrations. A specific concentration range was selected for the validation procedure based on those considerations. Accordingly, the concentration range for L-Dopa was selected as 20-100 μg mL^{-1} for method validation.

The specificity which is an essential part of the validation of the method has been assessed as follows: First, a certain amount of L-Dopa was dissolved in a known amount of ultra-pure water and a stock standard solution was prepared at the concentration of 1000 mg L^{-1}. Different concentrations of standard solutions were prepared by dilution from prepared stock standard solution with ultra-pure water. Then, the impact of excipients used in the manufacture of the selected marketed dosage forms on the proper assessment of L-Dopa peak was screened. After sample analysis, the chromatograms were assessed for peak area and excipient interference at retention periods of L-Dopa.

The linearity of the analytical method was determined by preparing and injecting standard solutions in the range of 20 to 100 μg mL^{-1} of L-DOPA solution. The calibration curve was formed by drawing the peak area against the concentration of L-Dopa and the regression equation slope and intercept point was calculated. Each solution was injected six times.

The accuracy and recovery of the method were determined by calculating recoveries by the spiking method. A known amount of standard solutions of 10, 40, 120 μg mL^{-1} were spiked to a known amount of sample solutions (40 μg mL^{-1}). The amount was estimated by the regression equation of the calibration curve.

The precision has been assessed on the method's repeatability and reproducibility. The intra-day repeatability has been assessed on the same day by determining the relative standard deviation (RSD %) of the areas obtained from the injection of six standard solutions replicates (L-Dopa concentration, 40 μg mL^{-1}). The same standard was injected one time per day during a period of six nonconsecutive days in the reproducibility assessment (interday repeatability).

The detection limit (LOD) of the drug is a characteristic value for the precision of the method in which the corresponding compound is only measurable, whereas the quantitation limit (LOQ) is the lowest concentration with acceptable linearity, accuracy, and certainty. Linearity range was satisfactory for analyses of L-Dopa in pharmaceuticals and plant materials. In this method, the LOD and LOQ levels were calculated by standard deviation of the calibration curve.

The suitability of the system was assessed by determining the tailing factor and the reproducibility of peak areas and retention times. Additionally, for this analyte, calibration curves were established and linearity checked.

Various method parameters were tested to assess the reliability and robustness of the proposed HPLC method. The self-imposed parameters limits set for these parameters have been inspired by other publications and our experience. We studied parameters as follows: The flow of the mobile phase (± 0.10 mL min^{-1}), column temperature (± 5°C), buffer concentration (± 0.01 M) and pH value (± 0.10) for the mobile phase. It was investigated these parameters by injecting a series of dilutions with three individual standards for lowest, medium and highest concentrations of L-Dopa in triplicate. The robustness of the system has been measured by absolute average recovery, RSD (precision) and R^2 of the calibration curves that resulted.

RESULTS and DISCUSSION

Development of the method
Several preliminary studies were conducted to optimize the chromatographic conditions for the quantification of L-Dopa. Mobile phases consisting of several buffer systems were tried at the beginning of the study, they could not meet the required system parameters.

A phosphate buffer (pH of 2.30) was chosen as a mobile phase based on these criteria. Furthermore, the chosen eluents have been designed for solvent pH and ionic resistance. The mobile phase was 0.050 mM potassium dihydrogen phosphate (pH: 2.3). For buffer solution: 6.80 g potassium dihydrogen phosphate was accurately weighed. The volume was completed in liters by dissolving in deionized water. In order to decrease the tailing of L-Dopa peak 0.5% triethylamine (TEA) was used as ion pairing agent in the mobile phase. 5 ml of triethylamine was added. The pH of the obtained solution was adjusted to 2.3 by adding orthophosphoric acid.

HPLC analysis was carried out on a column Ace 5 C18 (250 x 4.6 mm, 5 μm). Appropriate volume for injection and column temperature were selected and the initial isocratic program was also modified step by step. Mobile phase running at different flow rates (0.5-1.5 mL min^{-1}) and pH ranges were tested. Chromatographic analysis was conducted at 30°C with a flow rate of 1.2 mL min^{-1} and a run time of 10 min. The injection amount was 20 μL, and ultraviolet measurements were at 280 nm. The mobile phase was used as a blank and diluent preparation.

The sample solution was analyzed for 60 minutes to ensure that there were no matrix components remaining in the column for much longer under the specified conditions. However, continuing the analysis after 10 minutes will increase both analysis time and cost. Overlapping peaks were not observed to overlap in samples from sample analyses injected into the system consecutively with 10 minutes of analysis time. Due to all these, the analysis time was determined as 10 min.

Specificity
The new method's specificity was evaluated by first analyzing blank solvents/media, and then samples
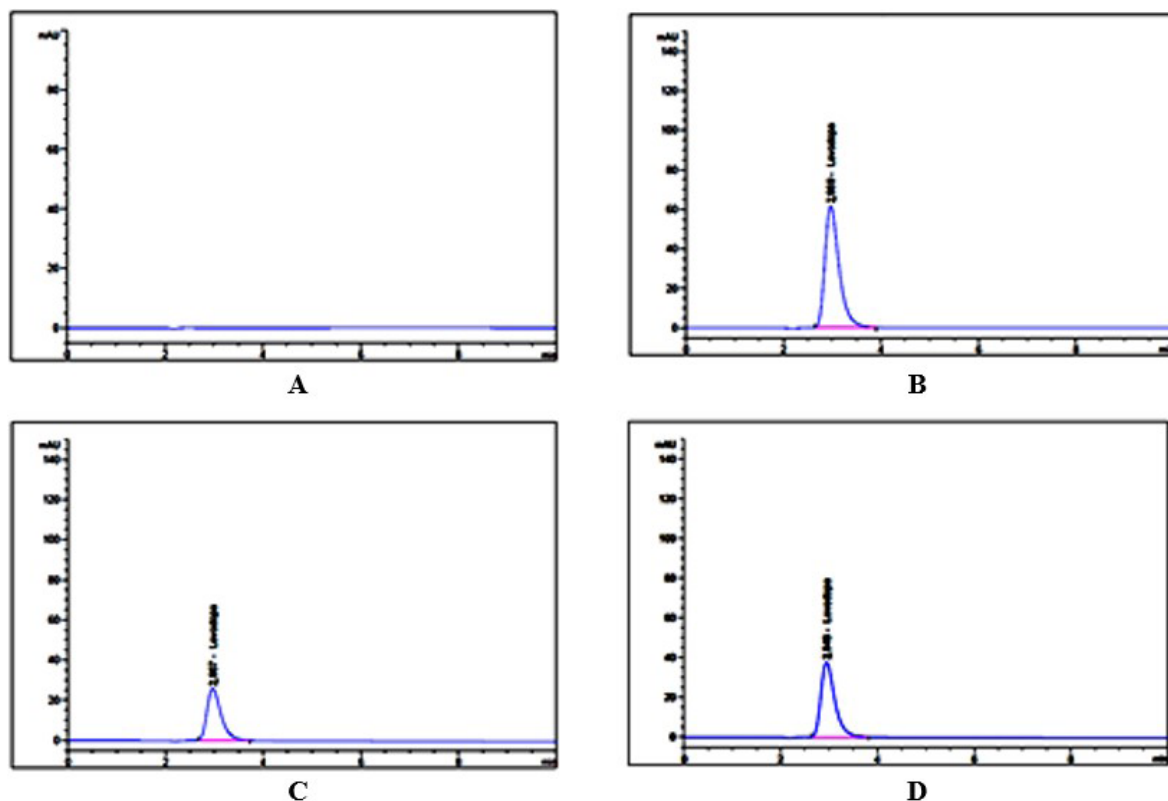

Figure 2. Chromatogrames A. Blank B. L-Dopa standard solution. C. Pharmaceutical sample. D. Plant material.

containing a single combination of drugs or drugs. The process showed good separation of the compound in standard solutions by chromatography (L-Dopa reference material dissolved in ultrapure water). No peak interference was observed between the analytes with blank media, buffer components or excipients of the marketed dosage formulations. Interference studies were shown by injection of blank, sample and standard solution. No drug excipient interactions were observed. There was no peak at the retention time of L-Dopa (Figures 2A and 2B). This indicates that the blank and drug excipients did not interfere. The retention time was 2.9495 min for L-Dopa. The chromatogram of the drug sample and extract of the plant material is presented in Figure 2C, 2D.

Linearity

The solutions for the linearity test were prepared from the solution of L-Dopa at five different levels of concentration ranging from 20–100 µg mL^{-1}. The calibration curve was created by plotting the substance area versus the concentration. The correlation coefficients, slopes and y-intercepts of the calibration plots were obtained and reported in Figure 3. The mean linear regression equation of the calibration curves was calculated as y = 12.419 x + 2.153 (r^2 = 0.9999). The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated according to the following equation: LOD or LOQ = κSDa/b, where 10 for LOQ and κ= 3 for LOD, SDa represents the standard deviation of the intercept, and b represents the slope. The LOD was 1.70 µg mL^{-1} and the LOQ was determined as 5.13 µg mL^{-1}.
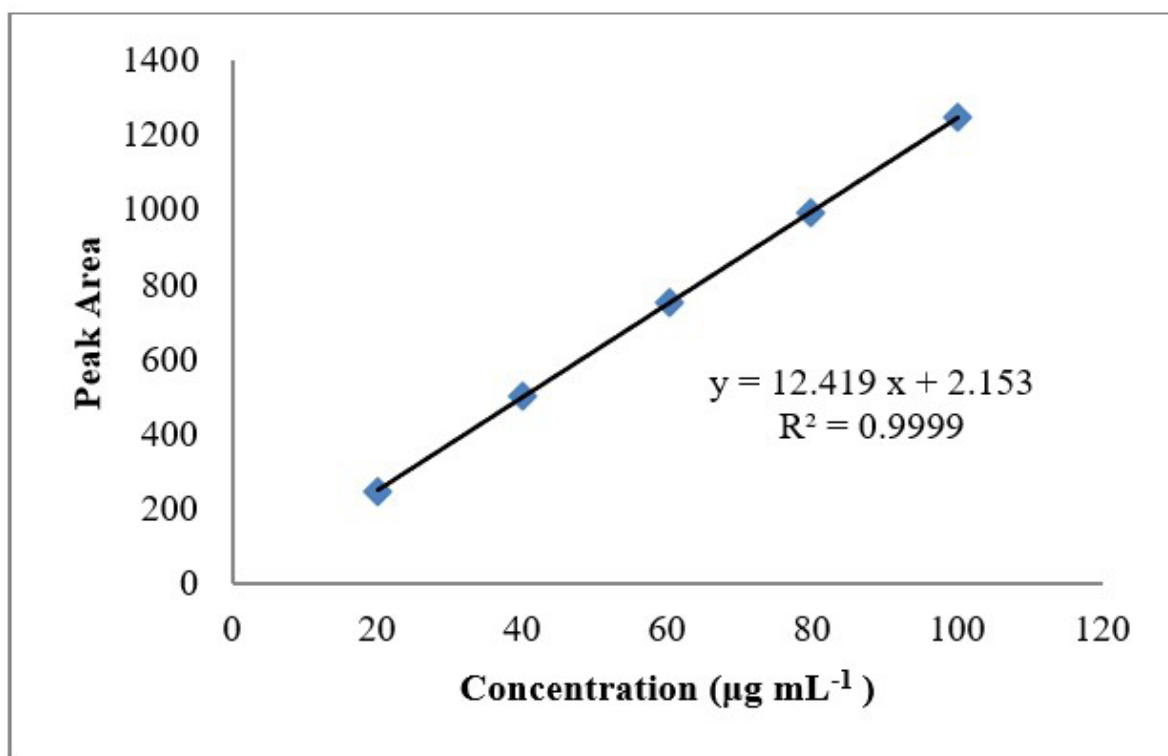

Figure 3. Calibration curve for L-Dopa standard solutions (at 280 nm).

Table 2. Summary of validation data of developed HPLC-UV method

Parameter	Value
RT (min).	2.9495
Linear range ($\mu g\ mL^{-1}$)	20 – 100
Linear regression equation	y=12.419x+2.153
Recovery %	100.32
Correlation Coefficient (R^2)	0.9999
Lack of Fit	
F	3.14
p	0.555
SE of Intercept	5.8312
SD of Intercept	13.2215
LOD/LOQ ($\mu g\ mL^{-1}$)	1.70/5.13

Accuracy

The standard addition technique was carried out to prove the accuracy of the proposed method. After adding a certain amount (40 $\mu g\ mL^{-1}$) of pure sample solution to the 10, 40, 120 $\mu g\ mL^{-1}$ concentrations of standard L-Dopa solutions, the analysis were performed. The percentage recoveries for the drug ranged from 100.08–100.41%. The results of the recovery study have been presented in Table 3.

Precision

The precision was expressed as relative standard deviation (RSD% = SD/mean×100). The relative standard deviation (RSD) of the calibration standards (n = 6) for intra-day precision (repeatability) and inter-day (intermediate) precision were 1.19% and 3.80%, respectively (Table 4).

In our case the presented HPLC method establishes that RSD % values less than 5 that are acceptable and below 10 % (for reproducibility). Our data is suitable our method for validation requirements.

Table 3. Accuracy of the HPLC Method.

Nominal value L-Dopa ($\mu g\ mL^{-1}$)	Spiked amount ($\mu g\ mL^{-1}$)	Measured amount[a] ($\mu g\ mL^{-1}$) (Mean±S.D.[b])	Recovery (%)	RSD (%)
40	10	50.04±0.06	100.08	0.12
	40	80.27±0.37	100.34	0.46
	120	160.65±0.84	100.41	0.53

[a]Five independent analyses.
[b]Standard deviation

Table 4. Precision of the HPLC Method.

Compound	Repeatability(n=6)		Reproducibility (n=6)	
	Mean±SD	RSD, %	Mean±SD	RSD, %
L-Dopa	40.07±0.48	1.19	40.78±1.55	3.80

SD: Standard deviation; RSD: Relative standard deviation

System suitability

Primary parameters to evaluate system suitability such as symmetry factor, retention factor were determined for the lowest (40 µg mL^{-1}), medium (60 µg mL^{-1}) and highest (80 µg mL^{-1}) concentrations for this analytes and were listed in Table 5. L-Dopa had excellent peak symmetry. In addition, the peaks of the analytes showed consistently low variability in peak areas and retention times. Calibration curve coefficient in this study was above 0.999, which indicates that the method was suitable for samples with simple or rather complex matrices.

Robustness

The robustness study data show that the linearity, absolute mean recovery, and precision of the developed method remain unaffected by small changes in critical method parameters. The corresponding results have been shown in Table 6. Temperature variations, flow rate, ionic strength, and pH value did not affect this analyte's recovered amount. The absolute mean drug recovery for all substances was between 99% and 101% and the level of RSD was less than 1.00%.

Table 5. Results for system suitability

System suitability parameters	Std. Solution Conc. µg mL^{-1}		
	40	60	80
Symmetry factor	0.6597	0.6530	0.6576
Peak areas (% RSD)	0.1685	0.1527	0.1432
Retention times (% RSD)	0.0597	0.0536	0.0521

Table 6. Results for robustness

Robustness Parameters	Parameter Value	Abs. Mean Recovery %	RSD %
Mobile phase flow rate	1.10 mL min^{-1}	100.34	0.12
	1.30 mL min^{-1}	99.88	0.05
Column temperature	25 0C	99.76	0.07
	35 0C	100.21	0.18
Buffer concentration	0.040 M	100.86	0.93
	0.060 M	99.91	0.64
pH value	2.20	100.12	0.71
	2.40	99.75	0.63

Table 7. L-Dopa contents of flowers, leaf and seed

Sample	Flowers±SD	Leaf±SD	Seed±SD
L-Dopa mg g^{-1}	52.21±0.07	34.45±0.14	7.68±0.18

SD: Standard deviation

Determination of L-Dopa content in different parts of *Vicia faba* L.

L-Dopa contents of flowers, leaf and seed were analyzed by validated HPLC method were presented in the table 7.

Determination of L-Dopa in the pharmaceutic formulations

The proposed method was applied in the pharmaceutical forms to quantitatively evaluate L-Dopa. The obtained results were in the agreement with the results specified in the approved labeled content of L-Dopa (Table 8).

Table 8. Determination of L-Dopa in tablets by the proposed methods (n=5).

	Label claim[a] (mg/per tablet)	Mean±S.D	Recovery (%)	RSD (%)
Proposed method	250	250.38±1,35	100.15	0.54

aSINEMET® 250 mg Tablet

Conclusions

A HPLC method for an effective analysis of L-Dopa in plant material and pharmaceutical formulations was developed and validated. There was no significant difference in the statistical comparison of the assay results for L-Dopa in tablet dosage forms and plant material by this method. The method allows a fast and robust quantification without any interference caused by formulation excipients or ingredients. The findings of the tablet dose study according to the analytical method are reliable, reproducible, and are in good agreement with drug label claims. Therefore this method can be used successfully in tablet dosage forms and plant materials for routine analysis of L-Dopa.

Acknowledgments

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