Uluslararası Tarım Araştırmalarında Yenilikçi Yaklaşımlar Dergisi
International Journal of Innovative Approaches in Agricultural Research 2023, Vol. 7 (2), 208-219
https://doi.org/10.29329/ijiaar.2023.568.5

Original article

Development and Validation of Analytical Methods for Quantification of L-Dopa; Application to Plant Materials

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Abstract

An essential neurotransmitter used to treat nervous system dysfunction like Parkinson's disease is L-dopa. The amount of L-dopa in plant materials has been measured in this study using chromatographic and spectrophotometric methods that are user-friendly, straightforward, quick, and affordable. Utilizing conventional instrumental parameters, high-performance liquid chromatography and spectrophotometry methods were established. In accordance with ICH guidelines, these analytical methods were validated for linearity, accuracy, precision, and robustness. The chromatographic procedure made use of an Agilent Extend C18 (250×4.6 mm, 5 µm) column. 0.1% trifloroacetic acid solution and acetonitrile (92/8, v/v) were used as mobile phase. It was run in isocrotic mode and the flow rate was 1 mL min-1. The retention time of L-dopa was determined as 3.85 minutes. L-dopa was identified using the spectrophotometric method, which involved measuring the solutions' absorbance at a wavelength of 280 nm. In spectrophotometric analysis, ultra-pure water as a solvent gave sufficient molar absorptivity at a λmax of 280 nm. The results showed that spectrophotometric and chromatographic methods were linear, accurate, precise, robust, and the percent recovery was within standard limits. No statistically significant distinction existed between the methods within the 95% confidence interval ($p<0.05$). The developed methods can be used to quantify L-dopa in plant materials for routine analysis and have been found to be very efficient.

Keywords: L-Dopa, Quantification, Chromatography, Spectrophotometry, Validation.

Received: 07 February 2023 * **Accepted:** 22 March 2023 * **DOI:** https://doi.org/10.29329/ijiaar.2023.568.5

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INTRODUCTION

L-Dopa is an amino acid analog that belongs to the catecholamine class (Figure 1). It is a precursor to the neurotransmitters dopamine and norepinephrine, which function as messengers in brain regions responsible for emotion and movement. The main motor symptoms of Parkinson's disease are currently treated with it, which is thought to be the most effective dopaminergic drug. It is now thought to be the most effective dopaminergic treatment for Parkinson's disease's basic motor symptoms. The most common neurodegenerative movement disorder worldwide is Parkinson's disease. (Balestrino & Schapira, 2020; Khan et al., 2021). When midbrain neuronal cells die, dopamine, which is essential for physiological motor control, cannot be synthesized, and Parkinson's disease develops. Parkinson's Disease symptoms can be reduced by improving dopamine levels in the brain (Balestrino& Schapira, 2020). L-Dopa pharmacological treatment is based on its conversion to dopamine to increase bioavailability at the peripheral synaptic level. Here, the amino acid aromatic decarboxylase enzyme converts L-Dopa to dopamine (Hall & Church, 2020). After a while, the pharmaceutical efficacy starts to decline. After a half-life of 50 to 90 minutes, serious side effects such as motor fluctuations, orthostatic hypotension, hallucinations, and dyskinesias emerge. All of these factors have led to the development of L-Dopa extended-release formulations in order to increase bioavailability and decrease negative effects (Hall & Church, 2020; Rezak, 2007; Nutt, 2008; Tizabi et al.,2021; Poewe & Antonini, 2015; Müller, 2015).

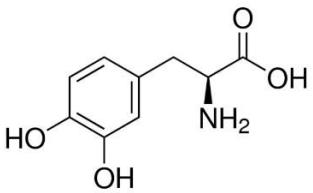

Figure 1. Chemical structure of L-Dopa

L-Dopa is chemically synthesized in a process that requires advanced technologies and expensive chemicals are used in its synthesis (Valdés, 2004). L-Dopa is also found in natural plant materials. Plant-based L-Dopa production has several advantages over chemical methods, including lower costs and a pure product. L-Dopa, which comes from plant sources, also lessens side effects and helps the disease advance more slowly. Several Fabaceae plants contain high levels of L-Dopa (Sushama et al., 2013). The highest concentration of L-Dopa is found in the genus Mucuna. The broad bean plant also contains a significant amount of L-Dopa. L-Dopa and its metabolites can either be insufficient or excessive, which can have an impact on how the body controls important functions. As a result, the content of L-Dopa in all plant matrices intended for human consumption must be monitored.

By using high performance liquid chromatography HPLC-UV, different analytical techniques have been used to identify L-Dopa in various plant materials (Baranowska & Płonka, 2015; Siddhuraju & Becker, 2001; Goyoaga et al., 2008; Renna et al., 2020; Etemadi et al. 2018; Randhir et al., 2002; Yang et al., 2001; Dhanani et al., 2015; Bulduk & Topal, 2020; Polanowska et al., 2019; Kasture et al., 2014; Rathod & Patel, 2014; Patil et al., 2015; Singh et al. 2010). Liquid chromatography-mass spectrometry/mass spectrometry LC MS/MS (Pavón-Pérez et al., 2019; Abdel-Sattar et al., 2021; Varga & Varga, 2014), UV spectrophotometry (Vadivel & Biesalski, 2012; Rahmani-Nezhad et al., 2018), High-performance thin-layer chromatography (HPTLC) (Aware et al., 2017), Capillary electrophoresis with ultra-violet detection CE-UV (Chen et al., 2005), Electrochemical methods (Kalachar et al., 2011; Mwatseteza & Torto, 2007; Arvand et al., 2016; Rwei et al., 2018), and Nuclear Magnetic Resonance NMR (Fernandez-Pastor et al., 2019).

However, there is no standardized method for determining L-Dopa. For this reason, new, fast and sensitive analytical methods have been tried to be developed for the determination of L-Dopa in plant materials. It was validated according to ICH guidelines to evaluate the reproducibility and broad applicability of the developed method.

This study's purpose was to develop and validate analytical procedures for measuring the amount of L-Dopa present in plant materials using liquid chromatography and UV spectrophotometry methods.The results of these methods were statistically compared using analysis of variance (ANOVA). In addition, their reliability and applicability were evaluated by focusing on routine quality control analyzes. Over the last two decades, some progress has been made in the development of sensitive and selective extraction and detection methods for the clear identification of L-Dopa in plant matrices, particularly *Vicia faba L.* However, a major challenge in determining L-Dopa is to quantitatively elucidate the compound of interest from the matrix. The use of effective extraction methods may enhance the use of natural products containing significant amounts of this active substances in Parkinson's therapeutic interventions. Liquid-solid extraction, ultrasound-assisted extraction, and microwave-assisted extraction techniques are the most commonly used, but the various proposed procedures still have some discrepancies in terms of extraction solutions, time, and temperature values.time and temperature values. As a result, advances have been made in the analysis of L-Dopa; however, more effort is required to establish applicable analytical protocols for routine determinations of this compound.

MATERIALS and METHODS

Reagents and materials

L-Dopa (USP) Reference Standard, trifluoroacetic acid and acetonitrile (HPLC grade) were obtained from Sigma-Aldrich Chemie GmbH. The ultrapure water used in the experimental studies was produced using the Millipore water purification system (Bedford, MA, USA).

In this study, *Vicia faba L.* grown in the fields of Faculty of Agriculture and Natural Sciences (Uşak University, Turkiye) was used. The plant's various organs were divided up and dried for 15 days in a dark room for 15 days. The dried material was crushed in a mortar to a fine powder.

Analytical instruments and conditions

HPLC analizleri, dörtlü bir pompa, otomatik örnekleyici, ultraviolet dedektörü (UV) ve ChemStation yazılımdan oluşan bir Agilent 1260 sistemi üzerinde gerçekleştirilmiştir. The column used was a Extend C18 (250 mm × 4.6 mm i.d.; 5 µm particle size) from Agilent, maintained at 30 C. UV detection was performed at 280 nm for peak identification. The mobile phase consisted of 0.1 % trifluoroacetic acid and acetonitrile (92:8, v/v), at a flow rate of 1 mL min^{-1}. The injection volume was 10 µL.

Ultraviolet-visible spectrophotometric analyses were carried out using a Shimadzu UV 1800 spectrophotometer with UV-Probe software and quartz cuvettes. L-Dopa was quantified at a wavelength of 280 nm using ultrapure water as a blank.

Preparation of standard and sample solutions

Exactly 25 mg of the L-Dopa reference standard was precisely weighed and transferred into a 50 mL measuring flask to prepare L-Dopa stock standard solution. It was dissolved by adding 20 mL of ultrapure water and then volume was completed to 50 mL with ultrapure water. The standard solutions were prepared at 6 different concentrations in the range of 5-30 µg mL^{-1} by diluting with ultrapure water from the stock standard solution.

Vicia faba L. extract was prepared using leaves, flowers, and seeds of *Vicia faba L.* plant collected from Uşak (Turkey). L-Dopa is an amino acid that dissolves well in water. Therefore, ultrapure water was used as solvent in L-Dopa extraction. The extracts were made by mimicking the home brewing settings. Exactly 500 mg of ground dried sample was weighed and transferred to a 100 mL beaker and flask. It was added to 50 ml of boiled ultrapure water and left to infuse for 10 minutes. The mixture was filtered through white band filter paper following the extraction procedure.

Validation of analytical methods

The improved analytical methods were validated comprehensively using the methodologies indicated in ICH guidelines Q2(R1) (Goyoaga et al., 2008).

Linearity

Stock standard solutions containing 500 µg mL^{-1} L-Dopa in ultrapure water were prepared in triplicate. Aliquots of these solutions were diluted in ultrapure water, to six different concentrations, corresponding to 5, 10, 15, 20, 25, and 30 µg mL^{-1} of L-Dopa. Calibration curves were created by plotting concentration versus peak area for the chromatographic method, and concentration versus absorbance plot for the spectrophotometric method. The least squares method of regression analysis was applied to the calibration data.

Precision

The intra-day precision was evaluated by analyzing six sample solutions (n = 6), at 100% of the test concentration (25 µg mL^{-1}), using spectrophotometric and chromatographic methods. Similarly, the inter-day precision was evaluated on three consecutive days (n = 18). L-Dopa contents and the relative standard deviations (R.S.D.) were calculated.

Accuracy

The L-Dopa reference standard was precisely weighed and added to three different concentrations of plant sample solutions (18, 24, and 30 µg mL^{-1}). Samples at each concentration were prepared in triplicate, analyzed by developed analytical methods, and recovery percentages were determined.

Specificity

A sample solution with a concentration of 25 µg mL^{-1} was prepared using the sample preparation procedure and analyzed with the developed analytical methods. The UV spectra of this solution was recorded in the range of 200-800 nm for the spectrophotometric examination, in order to analyze the existence of probable interfering bands at 280 nm. Furthermore, the spectral purity of L-Dopa peaks in chromatograms produced with sample solutions was analyzed using the UV spectra recorded by the ultraviolet-visible detector.

Detection and quantitation limits of analytical methods

Standard solutions diluted in the range of 0.10-2.00 µg mL^{-1} were prepared from the stock standard solution. It was analyzed in decreasing concentrations with the developed analytical methods. For the chromatographic method, the limit of detection (LOD) was defined as the concentration at which the signal-to-noise ratio was 3, and the limit of quantification (LOQ) was the concentration at which the signal-to-noise ratio was 10. In the spectrophotometric method, the limits of detection (LOD) and

quantification (LOQ) were determined by evaluating the absorbance values of diluted solutions, UV spectra and relative standard deviation of the measured values.

The L-Dopa contents of the sample solutions were determined using both methods and the results were statistically compared using the ANOVA test and the Tukey multiple comparison test applied at the 0.05 significance level.

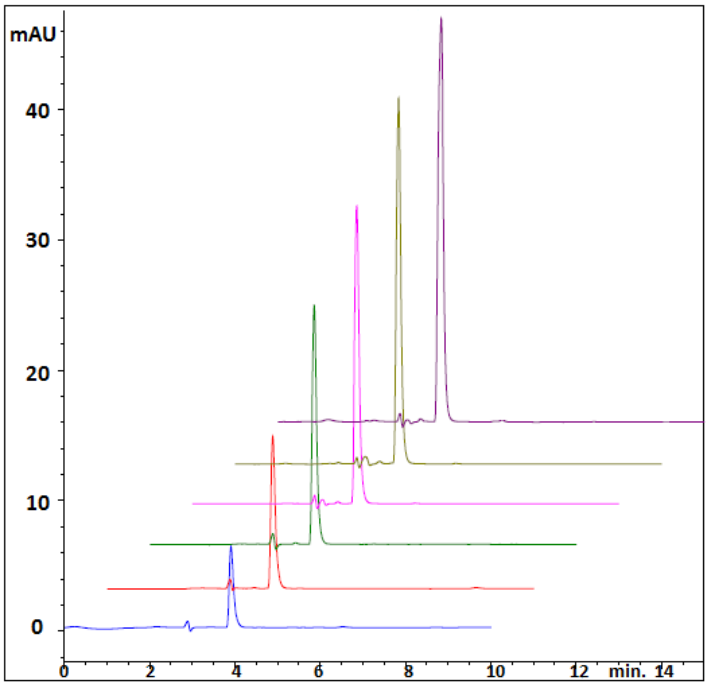

Figure 2. Overlap chromatogram obtained for an L-Dopa standart solutions (5- 30 µg mL^{-1}), using Agilent Extend C18 column (250 mm × 4.6mm i.d.; [5 m particle size) at 30 C and mobile phase composed of 0.10% trifluoroacetic acid and acetonitrile (92:8, v/v), at a flow rate of 1.0 mL min^{-1}. Detection were performed at 280 nm.

RESULTS and DISCUSSION

Acetonitrile proved to be a more suitable organic solvent than methanol during the development of the chromatographic method in terms of the retention time of L-Dopa. Acetonitrile showed a better organic solvent than methanol with respect to the retention time of L-Dopa during the chromatographic method development stage, Trifluoroacetic acid was used to acidify the mobile phase in order to make sure that L-Dopa was completely ionized and had a suitable peak shape. Therefore, using a mobile phase pH 2.0 and a C_{18} column, sufficient peak symmetry (tailing factor = 1.02) and short run time (3.85 min) were achieved. The overlap chromatogram of L-Dopa standard solutions is presented in Figure 2. When evaluated in the 200-400 nm range of the L-Dopa spectrum (Fig. 3), a wavelength of 280 nm was chosen

for detection because L-Dopa provides sufficient molar absorptivity. This wavelength was more selective in terms of interfering compounds or solvents in the samples.

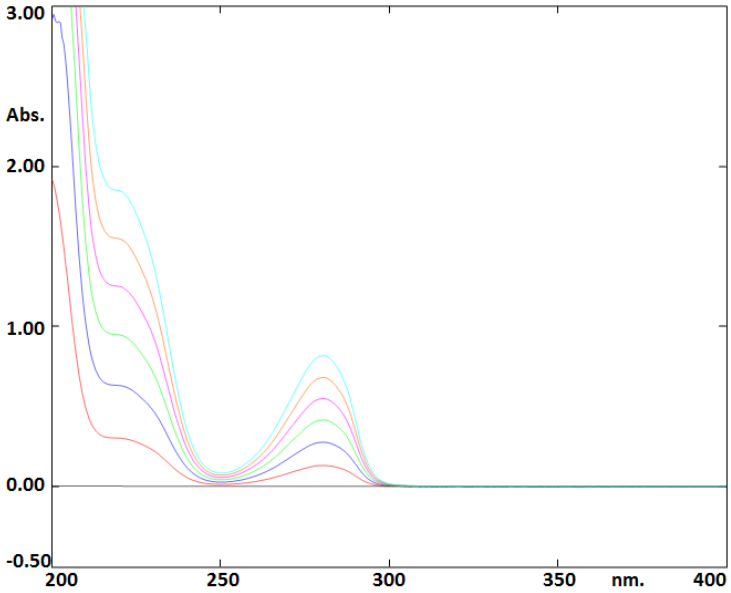

Fig. 3. Overlap spectrum of L-Dopa standart solutions (5-30 µg mL^{-1}), in ultrapure water.

Table 1. Overview of the linearity data obtained for L-Dopa by the spectrophotometric and chromatographic methods

Regression parameters	**Spectrophotometric method**	**Chromatographic method**
Regression coefficient (r^2)	0.9997	0.9999
Slope±S.E.	0.027±0.0001	8.77±0.10
Intercept± S.E.	0.015±0.0014	1.21±1.15
R.S.E. (%)	0.30	0.25
Concentration range (µg mL^{-1})	5-30	5-30
Number of points	6	6

S.E: Standard error, R.S.E: Relative standard error.

Validation of analytical methods

Peak area in the chromatographic method and absorbance in the spectrophotometric method were evaluated as the response. L-Dopa concentrations and analytical technique responses were found to be linearly related. Table 1 shows the regression analysis results using the least squares method. High correlation coefficients (r^2) values were obtained for both analytical methods. A random pattern of regression residues was discovered, and no substantial divergence from linearity was seen in the tested range. Table 2 displays the precision data collected for the developed methods. The analytical methods developed have an R.S.D. values of less than 2.0%, which gives good precision. When compared to the spectrophotometric method, the chromatographic method produced more precise results.

The standard addition method was used to assess the accuracy of the analytical methods. Both analytical methods showed average recoveries close to 100% and showed sufficient accuracy (Table 2).

Peak purities higher than 99.0% were obtained for L-Dopa in the chromatograms of the sample solutions and it was observed that other impurities did not elute with the main peak in the evaluation of the specificity of the chromatographic method. For the schromatographic method, no inhibitory peaks were observed at the retention time of L-Dopa in the chromatogram of the sample solution. For the spectrophotometric method, no absorption band was observed at 280 nm in the spectrum obtained from the sample solution. As a result, it was discovered that the method's detection wavelength was specific for detecting L-Dopa.

The LOD and LOQ values for the chromatographic method were determined as 0.50 and 1.50 g mL^{-1}, respectively, based on signal-to-noise ratios of 3 and 10. It was possible to determine the absorption band of the L-Dopa standard solution with a concentration of 0.70 µg mL-1 in the spectrophotometric method. The absorbance value corresponding to this concentration was determined as 0.035. As a result, this concentration was chosen as the detection limit. The quantification limit of the spectrophotometric method was defined as the lowest concentration providing sufficient precision and absorbance value, and this value was determined as 2.30 µg mL^{-1}. The soluson absorbance value at the quantification limit was determined as 0.076.

According to the results obtained (Table 2), the chromatographic method proved to be a more sensitive method and allowed measuring L-Dopa at concentrations about one and a half times lower than the spectrophotometric method.

Table 2. Validation parameters of the analytical methods

Validation parameters	**Spectrophotometric method**	**Chromatographic method**
Precision (Intra-day, *n*=6, R.S.D. %)	0.45	0.34
Precision (Inter-day, *n*=18, R.S.D. %)	0.63	0.47
Accuracy (Average recovery, *n*=9, %)	99.33	99.84
LOD/LOQ (µg mL^{-1})	0.70/2.30	0.50/1.50

Table 3. L-Dopa contents in the organs of *Vicia faba L.*

The organs	**L-Dopa contents (n=6, mg g. $^{-1}$ d.w.)**	
	Spectrophotometric method	**Chromatographic method**
Laeaf	34.95±0.21	35.50±0.16
Flowers	53.65±0.12	53.70±0.08
Seed	7.95±0.34	8.25±0.22

S.D: Standard deviation.

Analysis of L-Dopa in plant material

L-Dopa in plant material was analyzed using validated chromatographic and spectrophotometric methods (Table 3). At a confidence level of 0.05, the ANOVA test indicated a statistically significant difference between the findings obtained for plant material using the different methods. Turkey's multiple comparison tests demonstrated that, for plant material analysis, the means obtained by chromatographic and spectrophotometric were statistically equivalent ($p > 0.05$). A higher mean L-Dopa content was found when the chromatographic method was applied to the plant samples. The presence of biological substances in the plant matrix can interfere with the analysis, leading to different values. It has been observed that the chromatographic analysis technique is a more sensitive and selective approach and can be successfully used for the quantification of L-Dopa in biological matrices. However, we cannot leave aside the time and cost of analysis. The spectrophotometric method is obviously cheaper and easier to use than the chromatographic method and requires a shorter analysis time. The development and validation of straightforward and trustworthy procedures are crucial for securing L-Dopa because it is widely used as a potent antiparkinsonian medication.

Conclusion

In this study, two different analytical techniques (spectrophotometric and chromatographic) were developed for the quantification of L-Dopa in plant materials. Impurities from plant materials did not adversely affect analytical methods. Chromatographic and spectrophotometric methods showed to be adequate methods to quantify L-Dopa in plant materials. The chromatographic method presented more reliable results than the spectrophotometric method and was more accurate. The spectrophotometric method is obviously cheaper and easier to use than the chromatographic method and requires a shorter analysis time. Because these methods are quick and easy to use, they can be successfully applied to quality control analyses aimed at quantifying and identifying L-Dopa in plant products. These techniques can be used effectively in quality control analyses for the detection and quantification of L-Dopa in herbal products because they are straightforward and quick.

ADDITIONAL STATEMENT

Author contrubution rates

The authors contributed equally to the study.

The text confirming adherence to the ethical standards for research and publication

IJIAAR's research and publication ethics principles were followed throughout the article's process.

Conflict of interest declaration

There is no potential conflict of interest in this study.

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