Development and Validation of a Derivative Spectrophotometric Method for Simultaneous Multicomponent Determination of Levodopa, Carbidopa and Entacapone

Zolfagharifar, Mohammad; Hariri, Roshanak; Souri, Effat; Barazandeh Tehrani, Maliheh*+

Department of Medicinal Chemistry, Faculty of Pharmacy and Pharmaceutical Sciences Research Center ,Tehran University of Medical Sciences, Tehran, I.R. IRAN

ABSTRACT: *In this study entacapone, levodopa, and carbidopa, were determined with high precision in the presence of each other. UV-Vis spectroscopy was used as an easy and low-cost technique for the analysis and the derivative spectrophotometric method was applied for the elimination of absorption interferences. For this purpose, the derivative spectra of each compound were studied separately, and zero crossing points were determined for each of them. The zero-crossing points in which the absorption was observed only for one compound were found and evaluated for quantitative analysis. Calibration curves were drawn from the second and third derivative signals for each compound and the linear range was determined. The method was linear in the range of 1-5 µg/mL for levodopa, 0.25-1.7 µg/mL for carbidopa, and 2-14 µg/mL for entacapone. The accuracy and precision of the proposed method were evaluated by within-day and between-day tests (CV < 1.56% and error < 1.7%) and finally, these drugs were determined in pharmaceutical dosage forms by the developed method.*

KEYWORDS*: Levodopa; Carbidopa; Entacapone; Derivative spectroscopy; Simultaneous determination.*

INTRODUCTION

Parkinson's Disease (PD) is one of the most common neurodegenerative disorders that can eventually cause a person to have many movement problems. In this disease, with the decrease of dopamine in the neurons of the substantia nigra, various symptoms such as bradykinesia, resting tremor, cogwheel rigidity, etc. appear. The incidence of this disease is related to age, and statistics show that 1% of people over the age of 60 have Parkinson's disease, and it is predicted that 1.2 million people will suffer from this disease by 2030 [1]. Regarding the importance of Parkinson's disease and its negative effects on the patient's life, several efforts have been performed for the treatment of this disease. For this purpose, a number of drugs such as levodopa, carbidopa, and entacapone have introduced

**To whom correspondence should be addressed.*

+ E-mail: barazand@sina.tums.ac.ir

1021-9986/2023/9/3012-3018 7/$/5.07

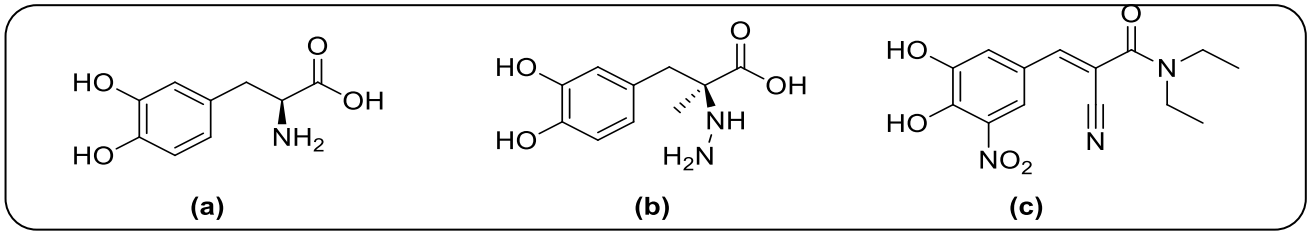


Fig. 1: Chemical structure of (a) levodopa, (b) carbidopa and (c) entacapone

and are used for treatment. Levodopa, (2S)-2-amino-3-(3,4-dihydroxyphenyl) propanoic acid (Fig. 1a), is the precursor of dopamine, norepinephrine, and epinephrine [2]. Carbidopa (Fig. 1b), (2S)-3-(3,4-dihydroxyphenyl)-2-hydrazinyl-2-methylpropanoic acid, is another drug which is prescribed for the treatment of Parkinson's disease. Carbidopa inhibits the peripheral metabolism of levodopa which leads to a greater proportion of levodopa to cross the blood–brain barrier and affect the central nervous system [3]. Therefore, by using levodopa and carbidopa simultaneously, the patient can benefit from better therapeutic effects and fewer side effects [4].

Entacapone (Fig. 1c), (*E*)-2-cyano-3-(3,4-dihydroxy-5-nitrophenyl)-*N*,*N*-diethylprop-2-enamide, is a selective and reversible peripheral catechol-O-methyltransferase (COMT) inhibitor. Catechol-O-methyltransferase is an enzyme which involved in the metabolism of dopamine and levodopa [5], so for higher and sustained plasma levels of levodopa, entacapone is administered in association with levodopa and carbidopa in pharmaceutical formulations such as Stalevo® [6]. Regarding the significant role of levodopa, carbidopa and entacapone in the treatment of Parkinson's disease, determination of the mentioned drugs are important and therefore it's of the great interest among several research teams. Although there are some reports describing individual determination of entacapone, levodopa and carbidopa in biological fluids [6-9] or in tablet forms, there are just a few methods for quantification of these drugs in combination [10-12], but they are complicated and expensive. Also literature survey revealed some HPLC methods for combination product analysis [13-16]. Methods based on HPLC, compared to UV, suffer from a number of disadvantages such as complicated instruments, difficult procedures and expensive solvents for the determination. On the other hand, UV- visible spectroscopy is an appropriate method for analytical applications for the determination of drugs because of the large number of its advantages such as low cost, easy and available method, high speed, precision, and accuracy [17, 18], but sometimes, due to the overlap of the spectrums in this method, it is necessary to use the derivative spectroscopy to solve this problem [19-23]. In addition, levodopa, carbidopa, and entacapone have not been determined in the presence of each other by derivative spectrophotometric method until now. Therefore in this study, we report a novel and facile method to achieve this purpose.

EXPERIMENTAL SECTION

Chemicals

Levodopa, carbidopa, entacapone, and sodium hydroxide were purchased from Sigma Aldrich. All the solvents, such as methanol, were obtained from Sigma Aldrich (HPLC Grade, ≥99.9%).

Apparatus

UV-visible absorption spectra were recorded using a double-beam Shimadzu Spectrophotometer Model 160, with 1.0 cm quartz cells.

Preparation of stock and working solutions

For preparation of the standard stock solution of levodopa (20 μg/mL), carbidopa (5 μg/mL) and entacapone (40 μg/mL), drug powder of each compound was dissolved in 0.5 M NaOH solution. Finally, each solution was diluted 10 times, with the same solvent, to prepare working solutions for 0-4 th order spectra. All the solutions were kept at room temperature.

Preparation of solution mixture of drugs to determine the best wavelength in zero-crossing points

For preparation of a solution with fix concentration of entacapone, to three 10 mL volumetric flasks, were added 1 mL of levodopa solution (20 μg/mL), 1mL of carbidopa solution (5 μg/mL) and 0.5, 1 and 2 mL of entacapone (40 μg/mL).

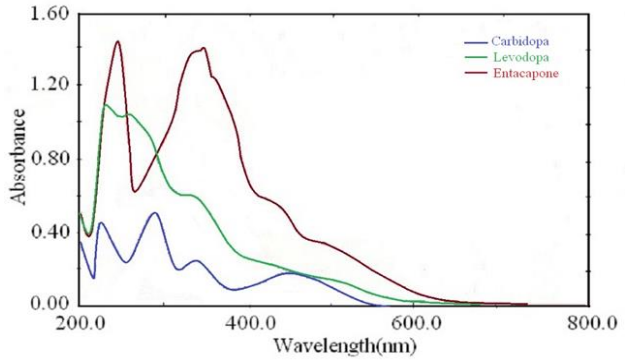


Fig. 2: Zero order spectra of levodopa (a), carbidopa (b) and entacapone (c)

Then, 0.5 M NaOH solution weas added to reach the final volume of 10 mL for each solution.

The same procedure was performed to prepare solutions with fixed concentrations of levodopa or carbidopa and variable concentrations of the other drugs.

Preparation of solution mixture of drugs for calibration curves

For drawing the calibration curves, 3 sets of the solutions were prepared for each drug. In each set, 10 solutions were prepared by constant concentrations of 2 drugs, and variable concentrations of the third one as analyte. The variable concentrations in each set for entacapone, levodopa and carbidopa were 2-14, 1-5 and 0.25-1.7 µg/mL, respectively.

Pharmaceutical formulation

Stalevo® tablets (containing 100 mg levodopa, 25 mg carbidopa and 200 mg entacapone) were manufactured by Novartis Pharma AG, Switzerland and purchased from a local pharmacy.

Analysis of pharmaceutical dosage form

For the determination of levodopa, carbidopa and entacapone in commercial products, the contents of 5 caplet of Stalevo® were dissolved in 20 mL of 0.5 M NaOH solution. The mixture was sonicated for 10 minute and then the precipitates were filtered off, and the remained solution was used for the analysis. The concentration level of the solution was 1000 µg/mL, 250 µg/mL and 2000 µg/mL based on levodopa, carbidopa and entacapone respectively. The standard sample was prepared by dissolving 1 mg of levodopa, 0.25 mg of carbidopa and 2 mg of entacapone in 10 mL of 0.5 M NaOH solution.

RESULTS AND DISCUSSIONS

Optimization conditions

To find the suitable solvent, it was considered that carbidopa and levodopa were soluble in methanol and entacapone was soluble in water. Using methanol–water (50:50) all the three drugs were dissolved, but complete overlap was seen for levodopa and carbidopa. Best results were obtained by using 0.5 M NaOH solution as solvent. Stable absorption spectra were obtained after 20 minute exposure of drugs to 0.5 M NaOH solution and it remains stable up to 120 minute.

According to Fig. 2, carbidopa, levodopa and entacapone maximum absorption are in the same range, and because of this coverage, the zero order spectra of these drugs are not suitable for their simultaneous determination. Hence, using derivative spectrophotometry is necessary for simultaneous measurement of these drugs. Therefore, the derivative spectra (1-4 th order) were prepared and applied for the analysis in the interval of 250 to 700 nm.

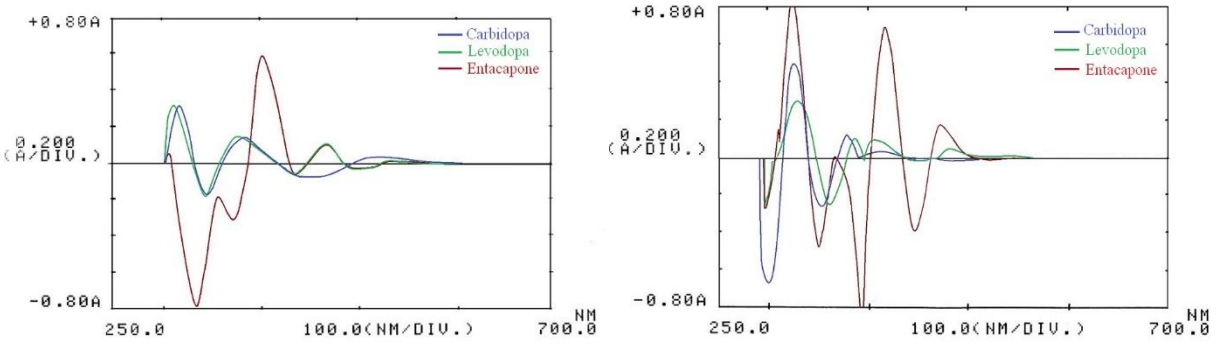
According to zero crossing points (Table 1), the third derivative of levodopa shows absorption at 338 nm ($\Delta\lambda$=24.5) and 585.5 nm ($\Delta\lambda$=31.5), while carbidopa and entacapone had no absorption in these points (Fig. 3b). Afterwards, the linear correlation between different concentrations of levodopa (1-5 µg/mL) in presence of constant concentration of two other drugs (carbidopa 0.5 µg/mL and entacapone 4 µg/mL), was studied and it was observed that the amplitude at 338 nm showed the best correlation. Ergo, this wavelength was chosen for levodopa determination. Similar studies were carried out for the second to fourth derivative spectra of two other drugs (Table 1). Eventually, the wavelengths of 446 nm (^{2}D, $\Delta\lambda$=31.5) for carbidopa (Fig. 3a) and 473.5 nm (^{3}D, $\Delta\lambda$=24.5) for entacapone (Fig. 3b), which showed the best linear correlation, were chosen to optimize this method.

Calibration curve of levodopa, carbidopa and entacapone in the presence of two other drugs

Synthetic solutions containing variable concentrations of levodopa between 1-5 µg/mL in the presence of fixed concentration of carbidopa (0.5 µg/mL) and entacapone (4 µg/mL) were applied to obtain the calibration curves. Calibration curves were plotted seven times, and statistical calculations for the determination of the line equation and correlation coefficient were done. Slop, SD of slop, SD

Table 1: Zero crossing points for entacapone, levodopa and carbidopa in 0.5 M NaOH

Derivative degree	Entacapone (λ nm)	Levodopa (λ nm)	Carbidopa (λ nm)
^{2}D , Δλ=20		461	461
	540	540	
^{2}D , Δλ=24.5	535	535	
		463	463
^{2}D , Δλ=28	541, 496	541, 496	
		586.5, 464	586.5, 464
^{2}D , Δλ=31.5	446, 537.5	446, 537.5	
		471.4, 597	471.4, 597
^{3}D , Δλ=24.5	338		338
		473.5	473.5
^{3}D , Δλ=31.5	585.5		585.5
		432.5	432.5
^{4}D , Δλ=18		399, 465	399, 465
^{4}D , Δλ=21		404, 449, 495.5	404, 449, 495.5
^{4}D , Δλ=24		459	459
^{4}D , Δλ=27		523	523



(a) (b)

Fig. 3: Derivative spectra of lev(odopa, carbidopa and entacapone: a) second order spectra (Δλ = 31.5); b) third order spectra (Δλ = 24.5.

of intercept and RSD were calculated, which proves that there is a linear correlation between concentration and absorption in the investigated wavelength range. Table 2 shows the statistical analysis of the experimental data. The calibration curves were linear between 1-5 µg/mL with a high correlation coefficient (R^2 = 0.999). The regression equation was Y=0.142X+0.072.

The regression equation for the calibration curve of carbidopa in the presence of levodopa (2 µg/mL) and entacapone (4 µg/mL) was Y= 0.087 X+ 0.011. The high value of the correlation coefficient (0.998) indicates the good linearity of the calibration graph (Table 2).

The calibration curve for entacapone in the presence of levodopa (2 µg/mL) and carbidopa (0.5 µg/mL) was linear between 2-14 µg/mL. The regression equation was Y=0.085 X+ 0.004 with correlation coefficient of 0.999 and RSD% of slope was 0.763 for nine experiments, which indicates the good repeatability of the calibration graph (Table 2).

Accuracy and Precision

To evaluate the within-day and between-day accuracy and precision, synthetic mixtures of levodopa, carbidopa and entacapone in four different concentration values were prepared and the derivative values at specified wavelengths were measured. The relative standard deviation values for three replicate determinations were less than 1.5% that indicates reasonable repeatability of the proposed method (Table 3).

Table 2: Statistical data of calibration curves of levodopa, carbidopa and entacapone (n=7)

Parameters	Levodopa[a]	Carbidopa[b]	Entacapone[c]
	$^3D_{338.0}$ (Δλ=24/5)	$^2D_{446.0}$ (Δλ=31/5)	$^3D_{473.5}$ (Δλ=24/5)
Linearity range	1-5 μg/mL	0.25-1.7 μg/mL	2-14 μg/mL
Regression equation	Y=0.142X+0.072	Y=0.087 X+0.011	Y=0.085 X+ 0.004
SD of slope	1.46×10^{-3}	7.72×10^{-4}	6.51×10^{-4}
RSD of slope (%)	1.024	0.890	0.763
SD of intercept	8.15×10^{-3}	1.45×10^{-3}	7.99×10^{-3}
Correlation coefficient	0.999	0.998	0.999
LOQ	0.103	0.090	0.760
LOD	0.034	0.030	0.250

[a] in the presence of entacapone (4 μg/mL) and carbidopa (0.5 μg/mL)

[b] in the presence of entacapone (4 μg/mL) and levodopa (2 μg/mL)

[c] in the presence of levodopa (2 μg/mL) and carbidopa (0.5 μg/mL)

Table 3: Accuracy and precision data for simultaneous determination of levodopa[a], carbidopa[b] and entacapone[c]

Added (μg/mL)	Within-day (n = 3)			Between-day (n = 9)		
	Found (μg/mL)	CV (%)	Error (%)	Found (μg/mL)	CV (%)	Error (%)
Levodopa[a]						
$^3D_{338.0}$ (Δλ=24/5)1/000	0.988± 0.002	0.171	-1.164	0.991± 0.010	1.042	-0.927
2.000	2.008± 0.015	0.738	0.421	2.010± 0.017	0.843	0.516
4.000	3.994± 0.002	0.053	-0/148	4.010± 0.028	0.689	0.218
5.000	4.965± 0.003	0.054	-07/705	4.964± 0.028	0.556	-0/722
Carbidopa						
$^2D_{446.0}$ (Δλ=31/5) 0/250	0.251±0.003	1.064	0.442	0.248± 0.003	1.384	-0.768
0.500	0.503± 0.007	1.556	0.664	0.505± 0.008	1.557	1.063
1.000	1.007± 0.013	1.243	0.733	1.007± 0.008	0.839	0.670
1.700	1.695±0.013	0.771	-0.275	1.682±0.013	0.766	-1.009
Entacapone[c]						
$^3D_{473.5}$ (Δλ=24/5) 2/000	1.966± 0.002	0.119	-1.703	1.976±0.027	1.345	-1.180
6.000	6.027± 0.016	0.265	0.457	6.088± 0.071	1.161	1.462
10.000	10.073±0.058	0.573	0.733	10.084± 0.038	0.378	0.844
14.000	13.973±0.098	0.700	-0.193	13.996± 0.114	0.816	-0.026

[a] in the presence of entacapone (4 μg/mL) and carbidopa (0.5 μg/mL)

[b] in the presence of entacapone (4 μg/mL) and levodopa (2 μg/mL)

[c] in the presence of levodopa (2 μg/mL) and carbidopa (0.5 μg/mL)

Determination of levodopa, carbidopa and entacapone in commercial product

Levodopa, carbidopa and entacapone were determined in commercial caplet Stalevo® manufactured by Novartis according to the derivative spectroscopy procedure. The results obtained from this method (Table 4) were compared with previously reported HPLC method [16]. Each method was repeated four times. The findings confirmed the accuracy and reliability of the new derivative spectroscopy method for measuring these drugs. As shown in Table 4, using the two-tailed t-test and F-test method, it was revealed that no significant difference was observed between the results obtained from the proposed method and the reported HPLC method (p-value>0.05) [16].

Table 4: Comparison of the developed method with the reference method for the determination of Stalevo® tablets (n=4)

Compound	Label claimed (mg)	Found(mean ± sd)				Statistical Tests*
		Proposed method		HPLC method		
		mean±sd	Recovery (%)	mean±sd	Recovery (%)	
Levodopa	100.00	98.12±0.62	98.85	101.58±1.25	101.58	t = 0.007 F = 0.180
Carbidopa	25.00	25.59±0.91	102.00	25.86±2.9	103.44	t = 0.889 F = 0.034
Entacapone	200.00	196.97±2.11	98.49	193.62±5.76	96.81	t = 0.166 F = 0.064

*Theoretical values of t and F at p = 0.05 are 3.18 and 9.27 respectively

CONCLUSION

In this study, a novel method for the simultaneous determination of levodopa, carbidopa and entacapone was introduced based on derivative spectrophotometry. Spectrophotometry is a facile and low cast method which could be a suitable alternative technique for time consuming or expensive methods, for determination of drugs. The results from within-day and between-day tests indicated that the proposed method is an accurate, precise, and economic method for the determination of these drugs (CV < 1.56% and error < 1.7%). Another advantage of this method is that there is no need for any prior separation or using organic solvents for sample preparation. Because of the short time needed for sample preparation, the method is appropriate for determination of multiple samples. Levodopa, carbidopa, and entacapone were all recovered at 98.85%, 102.00%, and 98.49%, respectively, using the derivative spectrophotometric method in comparison to the HPLC method (101.58, 103.44, and 96.81, respectively). It appears that this new approach might be a suitable replacement for previously reported methods (p-value > 0.05) and be used for quick analysis of active ingredients in quality control samples.

Acknowledgment

This study was part of a M.D. thesis (grant no. 97-02-33-38758) supported by Tehran University of Medical Sciences (https://research.tums.ac.ir).

Received : Nov. 15, 2022 ; Accepted : Feb. 04, 2023

REFERENCES

[1] Liu, Z., Ma S., Recent Advances in Synthetic α-Glucosidase Inhibitors, Chem Med Chem., **12(11)**: 819-829 (2017).

[2] Saeedi M., et al., Heterocyclic Compounds: Effective α-Amylase and α-Glucosidase Inhibitors, *Current Topics in Medicinal Chemistry*, **17(4)**: 428-440 (2017).

[3] Armstrong M.J., Okun M.S., Diagnosis and Treatment of Parkinson Disease: A Review *Jama*, **323(6)**: 548-560 (2020).

[4] Jankovic Tan J.EK., Parkinson's Disease: Etiopathogenesis and Treatment, *Journal of Neurology, Neurosurgery & Psychiatry*, **91(8)**: 795-808 (2020).

[5] Fabbri M., Ferreira J.J., Rascol O., COMT Inhibitors in the Management of Parkinson's Disease, *CNS Drugs*, 1-22 (2022).

[6] Liao X., et al., Levodopa/Carbidopa/Entacapone for the Treatment of Early Parkinson's Disease: A Meta-Analysis, *Neurological Sciences*, **41(8)**: 2045-2054 (2020).

[7] Jiang R., et al., Determination of Levodopa by Chromatography-Based Methods in Biological Samples: A Review, *Analytical Sciences*, **38(8)**: 1009-1017 (2022).

[8] Paim, C., et al., Stability-Indication LC Determination of Entacapone in Tablets, *Chromatographia*,. **65(9)**: 595-599 (2007).

[9] Salimian M., Sohrabi M.R., Mortazavinik S., Application of Net Analyte Signal and Principal Component Regression for Rapid Simultaneous Determination of Levodopa and Carbidopa in Commercial Pharmaceutical Formulation and Breast (Human) Milk Sample Using Spectrophotometric Method, *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, **283**: 121741 (2022).

[10] Abdel-Ghany M.F., et al., Investigation of Different Spectrophotometric and Chemometric Methods for Determination of Entacapone, Levodopa and Carbidopa in Ternary Mixture, *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, **171**: 236-245 (2017).

[11] Nigović B., New Approach on Sensitive Analysis of Pimavanserin, Levodopa and Entacapone Based on Synergistic Effect Of Graphene Nanoplatelets and Graphitized Carbon Nanotubes in Functionalized Polymer Matrix, *Electrochimica Acta*, **439**: 141700 (2023).

[12] Burmaoğlu R.E., Sağlık Aslan S., , A rapid Liquid Chromatography/Tandem Mass Spectrometry Method for Simultaneous Determination of Levodopa, Carbidopa, Entacapone and Their Six Related Compounds in Film-Coated Tablets, Rapid Communications in Mass Spectrometry, **34(12)**: e8782 (2020).

[13] Wollmer E., Klein S., Development and Validation of a Robust and Efficient HPLC Method for the Simultaneous Quantification of Levodopa, Carbidopa, Benserazide and Entacapone in Complex Matrices, *Journal of Pharmacy & Pharmaceutical Sciences*, **20**: 258-269 (2017).

[14] Subramanian V.B., et al., A simple High-Performance Liquid Chromatography Method Development for Carbidopa and Levodopa Impurities: Evaluation of Risk Assessment Before Method Validation by Quality by Design Approach, *Separation Science Plus*, **3(11-12)**: 530-539 (2020).

[15] Bulduk İ., Gökçe S., Development and Validation of a Effective and Reliable HPLC Method for the Quantification of Levodopa and Carbidopa in Pharmaceutical Formulations, *Hacettepe Journal of Biology and Chemistry*, **49(4)**: 413-422 (2021).

[16] Bhatnagar P., et al., Stability Indicating HPLC Method for Simultaneous Estimation of Entacapone, Levodopa and Carbidopa in Pharmaceutical Formulation, *J. Chromatogr. Sep. Tech.*, **6(304)**: 2 (2015).

[17] Alibakhshi M., Sohrabi M.R., Davallo M., Using Chemometrics Methods for Determination of Aripiprazole and Quetiapine as Antipsychotic Drugs in Pharmaceutical Mixture and Biological Fluid by Spectrophotometry Method Based on Continuous Wavelet Transform and Multivariate Calibration, *Iranian Journal of Chemistry and Chemical Engineering (IJCCE)*, **41(6)**: 1870-1882 (2022).

[18] Heydari S., Comparison of Two Liquid-Liquid Microextractions for the Detection of Crocin in the Saffron and Biological Samples Using UV-Vis Spectrophotometry, I*ranian Journal of Chemistry and Chemical Engineering (IJCCE)*, **37(1)**: 99-108 (2018).

[19] GhadimLoozadeh S., Sohrabi M.R., Fard H.K., Development of Rapid And Simple Spectrophotometric Method for the Simultaneous Determination of Anti-Parkinson Drugs in Combined Dosage form Using Continuous Wavelet Transform and Radial Basis Function Neural Network, *Optik*, **242**: 167088 (2021).

[20] Pagani A.P., CABEZóN M.A., IBáñEZ G.A., Simultaneous Kinetic-Spectrofluorometric Determination of Levodopa and carbidopa Using Partial Least-Squares Regression, *Analytical Sciences*,. **25(5)**: 633-638 (2009).

[21] Souri, E., et al., Development of a Rapid Derivative Spectrophotometric Method for Simultaneous Determination of Acetaminophen, Diphenhydramine and Pseudoephedrine in Tablets, *Iranian Journal of Pharmaceutical Research (IJPR)*, **14(2)**: 435 (2015).

[22] Passos M.L. Saraiva M.L.M., Detection in UV-Visible Spectrophotometry: Detectors, Detection Systems, and Detection Strategies, *Measurement*, **135**: 896-904 (2019).

[23] Verma G., Mishra M., Development and Optimization of UV-Vis Spectroscopy-A Review, *World Journal of Pharmaceutical Research*, **7(11)**: 1170-1180 (2018).