Selective Determination of Levodopa in the Presence of Vitamin B₆, Theophylline and Guaifenesin Using a Glassy Carbon Electrode Modified with a Composite of Hematoxylin and Graphene/ZnO

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An electrode has been developed based on using a composite of hematoxylin/graphene/ZnO nanocomposite to modify a glassy carbon electrode (GCE). The electrode (HGGCE) was tested and found to be applicable for the voltammetric analysis of levodopa in the presence of vitamin B₆, theophylline and guaifenesin using a 0.1 M phosphate buffer solution (PBS) pH 7 as the solvent. The HGGCE was used as the working electrode in cyclic voltammetry (CV) and square wave voltammetry (SWV) studies on the electrochemical behavior of levodopa at its surface. The results showed a dramatic enhancement in the oxidation current of levodopa and a shift in its oxidation potential towards more negative potentials as opposed to identical tests using bare GCE as the working electrode. The studies showed that the increase in the oxidation current has two linear profiles in two concentration ranges of 0.05 – 90.0 and 90.0 – 1000.0 μ M. The detection limit of SWV analysis using the modified electrode was determined to be 0.03 μ M ($S/N = 3$). Further advantages of the methods based on HGGCE include the simple modification procedure of the electrode, as well as its excellent sensitivity and reproducibility. The modified electrode was eventually found to be applicable to the determination of mixtures of levodopa, vitamin B₆, theophylline and guaifenesin in real samples.

Keywords Levodopa, vitamin B₆, theophylline, guaifenesin, hematoxylin, graphene/ZnO nanocomposite

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Introduction

The brain's failure in producing enough levels of dopamine causes Parkinson's disease (PD), which is a serious degenerative disease of the central nervous system. The age of Parkinson's onset is generally around 60 and the chances of developing PD greatly increase with age. However, in about 5 to 10% of PD patients, experience the condition develops before age 50.¹⁻³ PD leads to tremors, muscular stiffness, difficulty in performing simple tasks like walking and talking, or impaired balance.⁴⁻⁶ Dopamine cannot be simply taken as pills, since it cannot easily pass through the blood/brain barrier, and hence other compounds should be used for treating the condition. One of the most common drugs used for treating PD is levodopa (3-(3,4-dihydroxyphenyl)-L-alanine), which forms dopamine after crossing the blood/brain barrier. Other medications are different from levodopa in that they merely prevent or slow dopamine's breakdown or simply mimic it. Long term administration of levodopa, on the other hand, can lead to gastritis, paranoia, schizophrenia and/or dyskinesia. These side effects have been

found to be reduced by vitamin B₆, but vitamin B₆ also reduces the efficiency of levodopa.⁷⁻⁹

The term vitamin B₆ or pyridoxine is used to refer to a wide group of rather distinct water-soluble bioactive compounds, which act as an important enzymatic co-factor needed for over 140 bioreactions and play a key role in tolerance against abiotic stress. The presence of OH and NH₂ groups in the structure of vitamin B₆ gives it the ability to directly react and scavenge peroxy radicals.¹⁰⁻¹² Pyridoxine is further essential for the metabolism of red blood cells, and also influences the effective function of the nervous and immune systems. The vitamin can also cause normocytic, microcytic, or sideroblastic anemia as a result of impaired heme synthesis.

Further to these, and given that taking high doses of pyridoxine to avoid the side effects of levodopa can lower levodopa's main effect, administration of some drugs such as theophylline is necessary to help avoid using high levels of vitamin B₆.^{13,14} Theophylline is a derivative of methyl xanthine and can be found in soft drinks, many foods and natural products like cocoa, tea and chocolate, and can reportedly relax bronchial muscles, help with the secretion of gastric acid and stimulate the central nervous system.¹⁵⁻¹⁷ The compound is a common medication for treating the conditions associated with chronic asthma. The effective concentration of theophylline is in the

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range of 5 – 20 µg/mL, above which the compound can lead to symptoms ranging from arrhythmia, heartburn, tachycardia and cardiac arrest to fever dehydration, insomnia, anorexia, respiratory arrest and even coma.^{18–20}

Another drug that is used together with the above is guaifenesin or glyceryl guaiacolate, which help relax the chest and throat congestion due to the common cold, infections, or allergies, making coughing out easier.^{21–24}

In consideration of the above, there is a need to develop fast, simple, and accurate procedures for the analysis of these compounds both in pharmaceutical preparations and human body fluids. Among the various techniques used to this end, electrochemical methods have proven to offer remarkable advantages,^{25–28} in terms of sensitivity, simplicity, relatively fast analysis and low costs. However, the limitation with using most solid electrodes for the simultaneous analysis of a combination of these four drugs is the serious overlapping of their oxidation potentials. One of the approaches used for overcoming such limitations of conventional electrodes and resolving the overlap of the oxidation signals is the modification of the electrode surfaces.^{29–32}

Hematoxylin (7,11b-dihydroindeno[2,1-c]chromene-3,4,6a,9,10-(6H)-pentol), a natural dye obtained from the heartwood of *Haematoxylum campechianum*,³³ has four aromatic hydroxyl groups and conjugated π orbitals, which allow it to act as an electron or hydrogen donor. Some recent research has been focused on evaluating the electrochemical properties of electrodes modified with hematoxylin,³⁴ and the results have shown hematoxylin to be a proper redox mediator for the electrocatalysis of biologic compounds. Oxidizing hematoxylin using oxidizing agents has been reported to convert it to hematein.³⁵

Many recent reports have focused on graphene as a modifying agent, with various advantages like high electrical conductivity and theoretical specific surface area (2630 m² g⁻¹), good thermal conductivity (~5000 W m⁻¹ K⁻¹), as well as high Young's modulus (~1.0 TPa) and intrinsic mobility (200000 cm² V⁻¹ s⁻¹).^{36–41} Yet the negligible energy storage capacity of graphene seriously limits its extensive use, and many researchers have consequently focused on preparing graphene inorganic composites, as a means for enhancing its properties. In these composites the graphene sheet acts as a conductive support for inorganic species, e.g. the metal oxides which in turn provide further pseudo-capacitance and avoid the restacking of the sheets.^{42–44} One of the metal oxides that can be used for preparing such composites is ZnO particles or nanoparticles which offer high electrical conductivity, high thermal stability, large band gap (3.37 eV at room temperature), high excitation binding energy (60 meV), a large isoelectric point (IEP) (~9.5) and negligible toxicity, in addition to being widely available and cheap.^{45–49}

Given that reviewing the literature did not reveal any publications on the simultaneous electrocatalytic determination of levodopa, vitamin B₆, theophylline and guaifenesin using a modified electrode, the present research was focused on developing a modified glassy carbon electrode for this purpose. The analytical behavior of the developed electrode was evaluated in the analysis of levodopa in the presence of vitamin B₆, theophylline and guaifenesin and the electrode was eventually used for determining the mentioned compounds in real samples. Also, this work resulted in better dynamic range, detection limit or selectivity compared to most other studies reported previously.

Experimental

Apparatus and chemicals

The measurements were performed on an Autolab potentiostat/galvanostat (PGSTAT 302 N, Eco Chemie, the Netherlands), while the experimental parameters were controlled using General Purpose Electrochemical System (GPES) software. In addition to the working electrode, which was the modified GCE, the three-electrode cell further included an Ag/AgCl/KCl (3.0 M) reference electrode and a platinum wire as the auxiliary. The pH measurements were performed on a Metrohm 710 pH meter.

The solutions used for the experiments were freshly prepared. Levodopa, vitamin B₆, theophylline, guaifenesin and the other chemicals used were of analytical grade and were procured from Merck chemical company (Darmstadt, Germany). Orthophosphoric acid and its salts were used to prepare the buffer solutions with pH values in the range of 2.0 – 9.0. Hematoxylin was obtained from Sigma Aldrich Co. (USA) and graphene/ZnO nanorods were prepared as described elsewhere.⁴⁰

Modifying the GCEs

The first step started with polishing the GCEs using slurry of 0.05 µm Al₂O₃ in water, followed by the electrochemical activation of the surface of the electrodes in 0.1 M sodium bicarbonate solution in potential range of –1.1 to 1.7 V using cyclic voltammetry at 100 mV s⁻¹. Next 5 µL of graphene/ZnO nanocomposite suspension (0.01 g/L mL in water) was distributed over the activated GCE surface. Eventually, to precipitate the hematoxylin on the surface of the GGCE, the assembly was placed in a 1.0 M solution of hematoxylin. It was modified by 10 cycles of potential scan rate between 0.4 and 0.5 V at 50 mV s⁻¹.

For comparison also hematoxylin modified glassy carbon electrode (HGCE) was also, prepared as discussed before without graphene/ZnO nanocomposite. Activated glassy carbon was prepared as discussed before without graphene/ZnO nanocomposite and hematoxylin in 0.1 M sodium bicarbonate solution in potential range of –1.1 to 1.7 V using cyclic voltammetry at 100 mV s⁻¹ and a bare glassy carbon electrode (GCE) was prepared without graphene/ZnO nanocomposite and hematoxylin.

Preparing the real samples

Ten milliliters of each urine sample was taken from the original sample stored in a refrigerator and centrifuged at 2000 rpm for 15 min, and then the supernatant was filtered with a 0.45 µm filter. Then known volumes of the solution were taken and diluted in a 25-mL flask using PBS (pH 7.0). Eventually the samples were spiked with known quantities of levodopa, vitamin B₆, theophylline and guaifenesin.

Blood serum samples were filtered and centrifuged before being diluted using PBS (pH 7.0), and were finally spiked with the same analytes as the urine samples.

Results and Discussion

Assessing the HGGCE

To evaluate the electrochemical properties of the HGGCE, cyclic voltammetry studies were performed using one of the modified electrodes in a PBS (pH 7.0) solution. The results included well-defined and reproducible CVs, with the anodic peak occurring at 190 and the cathodic peak at 150 mV (vs. Ag/AgCl/KCl (3.0 M)) and a peak separation potential

Fig. 1 CVs of HGGCE in 0.1 M PBS (pH 7.0) at scan rate 10 mV s⁻¹ in the absence (a) and presence of 0.1 mM levodopa (b). (c) Under conditions (a) and (d) under conditions (b) for a HGCE. (e – g) Under conditions (b) for activated, graphene modified and bare GCE, and finally (h) under conditions (a) for bare GCE.

($\Delta E_p = (E_{pa} - E_{pc})$) of 40 mV. The ΔE_p was higher than the value of $59/n$ mV associated with reversible systems.⁴⁹ This suggested that the redox reaction of the hematoxylin couple in an aqueous system at the surface of HGGCE is quasi reversible in nature.⁴⁹

Electrocatalytic oxidation of levodopa

The reactions of levodopa and hematoxylin are pH-dependent and naturally optimizing the pH of the test solutions constitutes a critical step in obtaining optimal electrocatalytic oxidation results. To perform the optimization, levodopa solutions were prepared using 0.1 M PBS solutions with pH values in the range of 2.0 to 9.0. These solutions were studied by cyclic voltammetry using the HGGCEs as the working electrodes. The results showed that the optimal pH for the electrocatalytic oxidation of levodopa is the neutral pH, as reflected by the gradual enhancement in the anodic peak current, and the parallel decrease in the cathodic peak current. As a result, the rest of the studies were performed under neutral pH 7.0 conditions.

The results of CV studies on the electrochemical oxidation of a 0.1 mM levodopa under neutral pH using HGGCE (curve b), HGCE (curve d), activated GCE (curve e), graphene modified GCE (curve f) and bare GCE (curve g) are given in Fig. 1. The curves clearly show that the anodic peak potential (APP) for the oxidation of levodopa at the surface of HGGCE (curve b) and HGCE (curve d) is around 190 mV, while the APP values are 380 and 240 mV, respectively, in the case of the graphene modified GCE (curve f) and activated GCE (curve e). With the bare GCE, the APP is around 450 mV as seen in curve g, which indicates that oxidation of the analyte at the surface of bare GCE is actually negligible under the experimental conditions. In the case of the graphene-modified GCE (curve f) and activated GCE (curve e), the peaks indicate an acceptable level of the reaction but at more positive potentials as opposed to the studies performed using HGGCE or HGCE as the working electrodes (curves c and d).

It can easily be concluded that the most efficient electrocatalytic effect on the oxidation of levodopa occurs in the case of HGGCE

where APP of levodopa shifted by about 190 and 50 mV toward the negative values as compared with the graphene-modified and activated GCEs (curves f and e) respectively. Also the electrooxidation of the analyte at the HGGCE (curve b) gave rise to remarkably enhanced anodic peak current as opposed to HGCE (curve d), clearly showing that the interaction of ZnO nanocomposite and hematoxylin definitely improves electrooxidation of levodopa. The CV acquired with an HGGCE working electrode in a 0.1 M PBS (pH 7.0) in the absence of levodopa includes a well-behaving redox reaction (curve a) and upon adding 0.1 mM of levodopa, the anodic peak current is considerably enhanced (curve b), indicating the strong electrocatalytic effect. Further, in the case of using the HGGCE as working electrode in the CV study of a 0.1 M PBS (pH 7.0), a very low peak current was observed (curve c). Also a pair of well-defined redox waves can also be observed for the hematoxylin film attached to graphene. Compared to the bare electrode, while taking account the porous interfacial layer of the ZnO nanocomposite modified GCE, hematoxylin can penetrate through the conductive porous channels onto the electrode more easily, leading to higher sensitivity. Consequently, ZnO nanocomposite can be considered as a new material for immobilization of hematoxylin for electron transfer reactions.⁴⁹

Based on these results, we propose an EC' catalytic mechanism,⁴⁹ shown in Scheme 1, to describe the electrochemical oxidation of levodopa at HGGCE. In this scheme, levodopa is oxidized in the catalytic (C) reaction by the oxidized form of hematoxylin produced at the electrode surface *via* an electrochemical (E) reaction.

As to the effect of scan rate on the electrooxidation reaction, the HGGCEs were used in linear sweep voltammetry (LSV) studies and the results are given in Fig. 2. As shown by the results in Fig. 2, the APP shifted to more positive values upon increasing scan rate. This indicates that the electrochemical reaction faces kinetic limitations. Further, the plot of peak height (I_p) vs. the square root of scan rate ($v^{1/2}$) was a linear curve in the scan rate window of 2 – 100 mV s⁻¹, which suggests that when applying sufficient overpotentials the reaction becomes diffusion rather than surface controlled (Fig. 2A).⁴⁹ The plot of the scan rate-normalized current ($I_p/v^{1/2}$) vs. scan rate (Fig. 2B) also proved to be similar to that of a typical EC process.⁴⁹

Chronoamperometric studies

Different levodopa solutions in PBS (pH 7.0) were also used in chronoamperometric tests using HGGCEs at 250 mV and the results are illustrated in Fig. 3. It is known that for electroactive materials the electrochemical currents observed under mass transport-limited conditions follow Cottrell's equation.⁴⁹ Best fit I vs. $t^{-1/2}$ curves plotted based on experimental data obtained for different levodopa solutions are illustrated in Fig. 3A, and the slopes of these lines were next plotted vs. levodopa concentration and the results are given in Fig. 3B. Using the slopes and Cottrell's equation, the mean value for the diffusion coefficient (D) of the analyte was determined to be 1.12×10^{-5} cm²/s.

Calibration curve

The results of SWV studies on different levodopa solutions are illustrated in Fig. 4 as a plot of peak current vs. levodopa concentration. The curve was found to be linear over two concentration windows ranging from 0.05 to 90.0 μM and 90.0 to 1000.0 μM, with respective slopes of 0.0664 and 0.0274 μA μM⁻¹. The difference in the slopes for the calibration curves is due to the different activity of the electrode surface with low

Scheme 1 Electrocatalytic oxidation of levodopa at the HGGCE.

Fig. 2 LSVs of HGGCE in 0.1 M PBS (pH 7.0) containing 20.0 μM levodopa at various scan rates; numbers 1 – 12 correspond to 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mV s^{-1} , respectively. Insets: (A) Variation of anodic peak current vs. $v^{1/2}$. (B) Variation of the scan rate-normalized current ($I_p/v^{1/2}$) with scan rate.

Fig. 3 Chronoamperograms obtained at HGGCE in 0.1 M PBS (pH 7.0) for different concentrations of levodopa. The numbers 1 – 6 correspond to 0.0, 0.1, 0.25, 0.5, 0.75 and 1.0 mM of levodopa. Insets: (A) Plots of I vs. $t^{1/2}$ obtained from chronoamperograms 2 – 6. (B) Plot of the slope of the straight lines against levodopa concentration.

and high concentrations of the analyte. In the lower levodopa concentration, due to a high number of active sites (in relation to the total number of the analyte molecules), the slope of the first calibration curve is high. While in the higher levodopa concentration, due to decreasing active sites (in relation to the total number of analyte molecules, mainly at the surface of the electrode), the slope of the second calibration decreased, too. The lower sensitivity (slope) in the second section was attributed to the kinetic limitation. The limit of detection (3σ) was also determined to be 0.03 μM . These values are comparable with values reported by other research groups for electrocatalytic oxidation of levodopa at the surface of chemically modified electrodes by other mediators (Table 1).

Determination of levodopa in the presence of vitamin B₆, theophylline and guaifenesin

The capability of the modified electrode to simultaneously analyze levodopa, vitamin B₆, theophylline and guaifenesin, as a novel quality for any modified electrode reported so far, was also evaluated. This was performed by concurrently altering the concentrations of each of these species in their mixtures and recording the corresponding SWVs. The results included well-defined anodic peaks at 160.0, 680.0, 980.0 and 1180.0 mV which were attributed to levodopa, vitamin B₆, theophylline and guaifenesin, respectively. These observations (Fig. 5) supported the fact that the modified electrode is capable of simultaneously determining these analytes.

Fig. 4 SWVs of HGGCE in 0.1 M (pH 7.0) containing different concentrations of levodopa. Numbers 1 – 10 correspond to 0.05, 0.1, 0.5, 1.0, 5.0, 10.0, 30.0, 50.0, 70.0 and 90.0 μM of levodopa. Insets: (A) SWVs of HGGCE in 0.1 M (pH 7.0) containing different concentrations of levodopa. Numbers 11 – 19 correspond to 100.0, 200.0, 300.0, 400.0, 500.0, 600.0, 700.0, 800.0 and 1000.0 μM of levodopa. (B) The plots of the electrocatalytic peak current as a function of levodopa concentration in the range of 0.05 – 90.0 μM and 90.0 – 1000.0 μM .

Fig. 5 SWVs of HGGCE in 0.1 M PBS (pH 7.0) containing different concentrations of levodopa + vitamin B₆ + theophylline + guaifenesin in μM , from inner to outer: 200.0 + 450.0 + 350.0 + 175.0, 300.0 + 750.0 + 550.0 + 300.0, 400.0 + 950.0 + 700.0 + 375.0, 550.0 + 1200.0 + 900.0 + 475.0, and 650.0 + 1500.0 + 1100 + 600.0, respectively. Insets: (A) plot of I_p vs. levodopa concentrations, (B) plot of I_p vs. vitamin B₆ concentrations, (C) plot of I_p vs. theophylline concentrations and (D) plot of I_p vs. guaifenesin concentrations.

Table 1 Comparison of some electrochemical procedures used in the determination of levodopa

Electrode	Linear range/ μM	Detection limit/ μM	Ref.
Graphite screen printed electrode modified with graphene oxide/ZnO nanorods	1.0 – 1000.0	0.45	40
Carbon paste electrode modified with ZnO nanorods and 5-(4'-amino-3'-hydroxy-biphenyl-4-yl)-acrylic acid	0.1 – 70.0	0.035	50
Carbon paste electrode modified with ZnO nanoparticles/multi-walled carbon nanotube	5.0 – 500.0	0.08	51
Glassy carbon electrode modified with ZnO nanowire arrays and three-dimensional graphene foam	0.05 – 20.0	0.0005	52
Glassy carbon electrode modified with fullerene-functionalized carbon nanotubes	0.5 – 2000.0	0.035	53
Gold screen printed electrode	99.0 – 1200.0	68.0	54
Ferrocene modified carbon nanotubes paste electrode	2.0 – 50.0	1.2	55
Carbon paste electrode modified with trinuclear ruthenium ammine complex incorporated in NaY zeolite	120.0 – 10000.0	85.0	56
Glassy carbon electrode modified with carbon nanotubes and chitosan	2.0 – 220.0	0.6	57
Carbon paste electrode modified with dysprosium nanowire	0.01 – 1.0	0.004	58
Glassy carbon electrode modified with hematoxylin/graphene/ZnO nanorods	0.05 – 1000.0	0.03	This work

Even in the presence of these species the sensitivity of SWV analyses on levodopa using the modified electrode was $0.0286 \mu\text{A } \mu\text{M}^{-1}$ and did not reveal considerable changes, the sensitivity value is very close to that observed in single samples, *i.e.* $0.0274 \mu\text{A } \mu\text{M}^{-1}$. The results clearly indicate that the oxidation of each compound is independent from the others when using the modified electrode.

Interference studies

The effects of the presence of different potentially interfering compounds on the determination of levodopa were also evaluated under optimal conditions. The compounds were chosen from those commonly present in pharmaceutical levodopa formulations and/or biological fluids and included L-lysine, glucose, uric acid, L-asparagine, L-serine, L-threonine, L-proline, L-histidine, L-glycine, L-tryptophan, L-phenylalanine,

lactose, tyrosine, saccharose, fructose, benzoic acid, methanol, ethanol, urea, caffeine, Mg^{2+} , Al^{3+} , NH_4^+ , F^- , SO_4^{2-} and S^{2-} . For the purpose of this study, the tolerance limit was defined as the highest concentration of each interfering substance, which led to errors of less than $\pm 5\%$, in the response observed for levodopa. None of the tested compounds was found to lead to serious interferences in the analysis of levodopa.

Analysis of real samples

The electrode was also used to determine levodopa, vitamin B₆, theophylline and guaifenesin in human blood serum and urine, and the observations are given in Table 2, where the reproducibility has been expressed in terms of the mean relative standard deviation (RSD). The results are indicative of satisfactory recoveries for all of the tested species.

Table 2 The application of HGGCE for simultaneous determination of levodopa, vitamin B₆, theophylline and guaifenesin in urine and human blood serum samples ($n = 5$)

Sample	Spiked				Found				Recovery, %				RSD, %			
	Levodopa	Vitamin B ₆	Theophylline	Guaifenesin	Levodopa	Vitamin B ₆	Theophylline	Guaifenesin	Levodopa	Vitamin B ₆	Theophylline	Guaifenesin	Levodopa	Vitamin B ₆	Theophylline	Guaifenesin
Human blood serum	0	0	0	0	—	—	—	—	—	—	—	—	—	—	—	—
	5.0	40.0	42.0	45.0	4.9	41.2	42.3	45.2	98.0	103.0	99.5	100.4	2.4	3.2	1.8	2.7
	10.0	50.0	52.0	55.0	10.1	49.1	54.1	54.8	101.0	98.2	103.0	99.6	1.7	2.7	2.2	3.1
	15.0	60.0	62.0	65.0	15.5	59.8	62.8	64.1	103.3	99.7	100.5	98.6	3.4	1.9	2.9	2.3
	20.0	70.0	72.0	75.0	19.8	70.2	70.8	77.8	99.0	100.3	97.7	103.7	1.9	2.2	3.2	2.7
Urine	0	0	0	0	—	—	—	—	—	—	—	—	—	—	—	—
	7.5	35.0	40.0	47.5	7.7	34.8	38.9	47.9	102.7	99.4	97.2	100.8	3.2	1.9	2.3	1.9
	12.5	45.0	50.0	57.5	12.3	46.5	50.5	57.3	98.4	103.3	101.1	99.6	3.1	2.7	2.8	2.9
	17.5	55.0	60.0	67.5	17.1	55.2	59.2	69.5	97.7	100.4	98.7	103.0	1.7	2.2	3.1	2.3
	22.5	65.0	70.0	77.5	22.6	63.5	72.5	77.3	100.4	97.7	103.6	99.7	2.2	1.8	2.9	3.4

All concentrations are in μM .

Conclusions

The work was aimed at developing a sensitive and selective electrochemical method for the analysis levodopa to achieve enhanced peak currents. As opposed to the response obtained for bare glassy carbon electrodes, the response obtained using the modified electrode was found to be greatly enhanced, and the electrode revealed excellent merits in terms of lower detection limit, and enhanced sensitivity and selectivity. Using the electrode in square wave voltammetric analysis of levodopa in bulk form and pharmaceutical formulations was found to produce satisfactory results. This was also true for its application in the SWV analysis of levodopa determination in the presence of vitamin B₆, theophylline and guaifenesin, and also the analysis of levodopa, vitamin B₆, theophylline and guaifenesin in real samples with rather complex matrices.

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