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Capillary electrophoresis of neurotransmitters with amperometric detection at melanin-type polymer-modified carbon electrodes

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Abstract

This paper reports on the advantages of combining the separation capability of capillary electrophoresis using a novel home-made electrochemical cell adapted to commercially available capillary electrophoresis equipment with the permselective properties of melanin-type polymer-modified carbon electrodes as amperometric detectors. The excellent permselective properties of the melanic polymer have allowed a large improvement on the quantification of dopamine, epinephrine and norepinephrine. A linear response was obtained in the range 8–200 pg of dopamine with sensitivities between 3- and 5-times higher than those at the bare carbon electrodes and detection limits at submicromolar level (41 fmol in the volume injected). The effect of the carbon electrodes size, and capillary electrophoresis parameters on the analytical performance is also discussed.
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Keywords: Capillary electrophoresis; Neurotransmitters; Melanin; Polymer; Modified electrodes; Carbon electrodes

1. Introduction

Capillary electrophoresis (CE) becomes an attractive method for liquid phase separations owing to high separation efficiency, low sample consumption and relatively short analysis times [1]. Regarding CE detectors, the UV-absorbance ones have been the most frequently used in commercial capillary electrophoresis instruments although the detection limits are relatively high due to the short optical path lengths. Lower detection limits can be achieved by laser induced fluorescence detection. However, the laser equipment is rather expensive and in most of the cases a sample derivatization, which complicates the analytical process, is necessary to make, particularly if small volumes of samples have to be analyzed.

An alternative to the conventional spectroscopic detection systems, are the electrochemical methods [2,3] that presents the advantages of allowing a high sensitivity and the possibility of miniaturization without compromising the detection. In addition, electrochemical detection can be used to quantify a broad range of important analytes, owing to the variety of electrode materials and electrochemical processes that can be used for the detection. The success in the electrochemical detection is strongly dependent on the choice of the working electrode materials. Different electrodes have been used, carbon [4–7], sol–gel [8], platinum [9], gold [10], nickel [11] and copper [12]. Chemically modified electrodes [6,7,9,13], in general based on the incorporation of a catalyst or a redox mediator, have extended the applicability of capillary electrophoresis with electrochemical detection to compounds that otherwise could not be detected electrochemically. Most of the electrodes used in CE electrochemical detection are based on carbon materials owing to their low background, low cost, high stability and resistance to passivation [4,6,7].

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Polymer-modified electrodes have been largely used in the area of electroanalysis to improve electrocatalytic properties, to increase the stability and reproducibility of the electrode response, to improve molecular recognition, preconcentration and permselective properties [14–16].

The melanin-type polymer electrogenerated from L-dopa oxidation have demonstrated to possess excellent permselective properties towards anionic species [17]. Applications of this type of polymer-modified electrode for the amperometric detection of dopamine using flow injection analysis have been reported [18].

In this paper, we described a wall-jet amperometric detector based on the use of melanin-type polymer-modified carbon electrodes combined with a novel home-made electrochemical cell adapted to commercial available capillary electrophoresis equipment. To our knowledge, the application of such melanin-type polymer carbon electrode in a capillary electrophoresis system has not been reported.

The goal of this work is to combine the separation capability of CE with the sensitivity and versatility of electrochemical techniques to develop a more sensitive methodology for neurotransmitters quantification, based on the properties of the melanin-type polymer that makes it possible the use of high ascorbic acid concentrations to amplify the neurotransmitters oxidation signal.

2. Experimental

2.1. Reagents

L-(+)-Ascorbic acid was purchased from Panreac (Madrid, Spain). 3,4-Dyhydroxyphenyl acetic acid (dopac), 3-hydroxytyramine (dopamine), L-β-3,4-dihydroxyphenylalanine (L-dopa), 4-[1-hydroxy-2-(methylamino)-ethyl]-1,2-benzene diol (epinephrine) and arterenol (norepinephrine) were from Sigma (Madrid, Spain). All stock solutions were prepared before starting each set of experiments and stored under refrigeration away from the light. Diluted solutions were prepared just before use from the stock solutions. All other chemicals were of analytical-reagent grade. Water for preparation of solutions was purified with a Milli-Q Milli-RO water system (ρ = 18 MΩ) (Millipore, Spain). Buffers and samples were sonicated for 5 min and microfiltered through a 0.45 μm MFS-13 filters (Advantec MFS, Inc., USA).

2.2. Apparatus and electrodes

Electrochemical measurements were performed in the three electrode mode using an electrochemical analyzer BAS 100B connected to a 386/PC computer. Data storage and conversion to a "txt" files were performed by BASCOM 2.21 software (BAS, West Lafayette, USA). Silver and platinum wires were employed as pseudo-reference and counter electrodes, respectively. All potentials given in this work were referred to that reference system. All experiments were carried out at room temperature by applying the desired operating potential. Currents were allowed to reach a stable baseline prior to the amperometric monitoring.

Graphite disk electrodes with 300 and 500 μm diameter were prepared by threading a pencil lead (Pentel, Japan) through a 20 mm length PTFE tube with an adequate ID and OD of 1.6 mm. Glassy carbon electrodes (Goodfellow, 1 mm diameter) were prepared in the same way. One end was inserted into a pipette tip and filled with carbon paste (prepared by mixing graphite powder Acheson #38 (Fischer Scientific, Madrid, Spain) and mineral oil (Aldrich Chemical, Madrid, Spain), in a 70:30 w/w proportion). The final electrical contact was established with a copper wire. Then this side was sealed with non-conductive cyanoacrilate glue Loctite 401 and polyolefin primer Loctite 770 (Loctite, Spain). The other extreme was polished either on a weighing paper, in the case of graphite disk electrodes or on emery paper (600), followed by emery paper (BAS) and fine grades of alumina slurries 0.3 and 0.05 μm (Buehler) in the case of glassy carbon disk electrodes. The electrodes were exhaustively rinsed with water after each polishing step.

The melanin-type polymer-modified glassy and graphite carbon electrodes were prepared as it was previously described [17]. Briefly, the polymer was electrogenerated from a 3×10^{-3} M L-dopa solution in a 0.050 M phosphate buffer pH 7.4 by applying 1 V for 60 min. Once the polymer was obtained, the electrode was washed with water and cycled in the same supporting electrolyte between −400 and 800 mV at 100 mV/s (five cycles).

Capillary electrophoresis experiments were carried out with a SpectraPHORESIS 100 (Thermo Quest Corporation, Spain). Data acquisition and processing were accomplished using a 486/PC equipped with two channels and a ChromCard software package (Thermo Quest Corporation, Spain). No variation was introduced in the original commercial set up. A 59 cm fused silica column with a 2 cm Nafion tubing decoupler [19] was used for electrophoretic separations with electrochemical detection. This column has an ID of 75 μm and an OD of 365 μm and was supplied by Supelco, cat. no. 77500 (Bellefonte, USA). At the beginning of each day, the capillary was conditioned by flushing for 5 min with 1 M NaOH, 5 min with 0.1 M NaOH, 5 min with purified water and 5 min with the separation buffer. Between runs, the capillary was rinsed consecutively with water and the separation buffer for 6 min. The resulting analysis time for every run was 15 min. Samples were introduced by hydrodynamic mode for 0.5 s (the injected volume being 41 nL).

The electrochemical cell used in this work was prepared in our lab and it was recently described [5]. This electrochemical device used in connection with commercial capillary electrophoresis system allowed us to obtain a good performance for analytes with reversible or irreversible electrochemical behavior in a simple way and without needing complicated precision apparatus. No distortion on analytical signals was observed and stable baselines were obtained in all measurements [5,20].

Amperometric detection at a constant potential was performed with a BAS LC-4C amperometric detector (West Lafayette, IN) using an electrochemical cell configuration similar to that previously described [5].

3. Results and discussion

The advantages of using melanin-type modified carbon electrodes for the electrochemical detection of neurotransmitters previous CE separation are reported in the following sections. Fig. 1 shows cyclic voltammograms for 1×10^{-3} M ascorbic acid (A), and 5×10^{-5} M dopamine (B), epinephrine (C) and norepinephrine (D) obtained at bare graphite disk electrode (dotted lines) and at a melanin-type polymer-modified graphite disk electrode (solid lines). As expected, the film effectively rejects negatively charged species such ascorbic acid (pK_1 = 4.1). In contrast, the oxidation of the cationic neurotransmitters resulted in peak currents even larger than those obtained at the bare graphite disk electrode, due to a more efficient electron transfer at the polymer-modified surface, as it was previously reported [17]. We evaluated the possible preconcentration of the cationic neurotransmitters at the melanic polymer immobilized at the electrode and under our working conditions this effect was negligible (not shown).

Hydrodynamic voltammograms (HDVs) for 1×10^{-5} M dopamine, norepinephrine, epinephrine and 5×10^{-4} M ascorbic acid were also performed at melanin-type polymer-modified glassy carbon and graphite disk electrodes. A similar behavior was observed for the two carbon electrodes. In the case of the neurotransmitters, potentials higher than 500 mV, the currents reach a maximum although at potentials higher than 700 mV the signal becomes noisy (not shown). Therefore, based on the HDVs results the selected working potential was 700 mV, where the signal-to-noise ratio is the highest. It is important to mention that, as expected, no signal was obtained in the hydrodynamic voltammograms for ascorbic acid at the melanin-type polymer-modified electrodes. The behavior of dopac, the main metabolite of dopamine in brain, was also evaluated and since the molecule is negatively charged under the working conditions, no oxidation current was observed in the whole studied range.

The electropolymerization time demonstrated to be an important variable. Different times, between 5 and 120 min were evaluated. In all cases, for polymerization times shorter than 5 min the rejection of the ascorbic acid was not significant. The best signal was obtained in all cases for electropolymer-

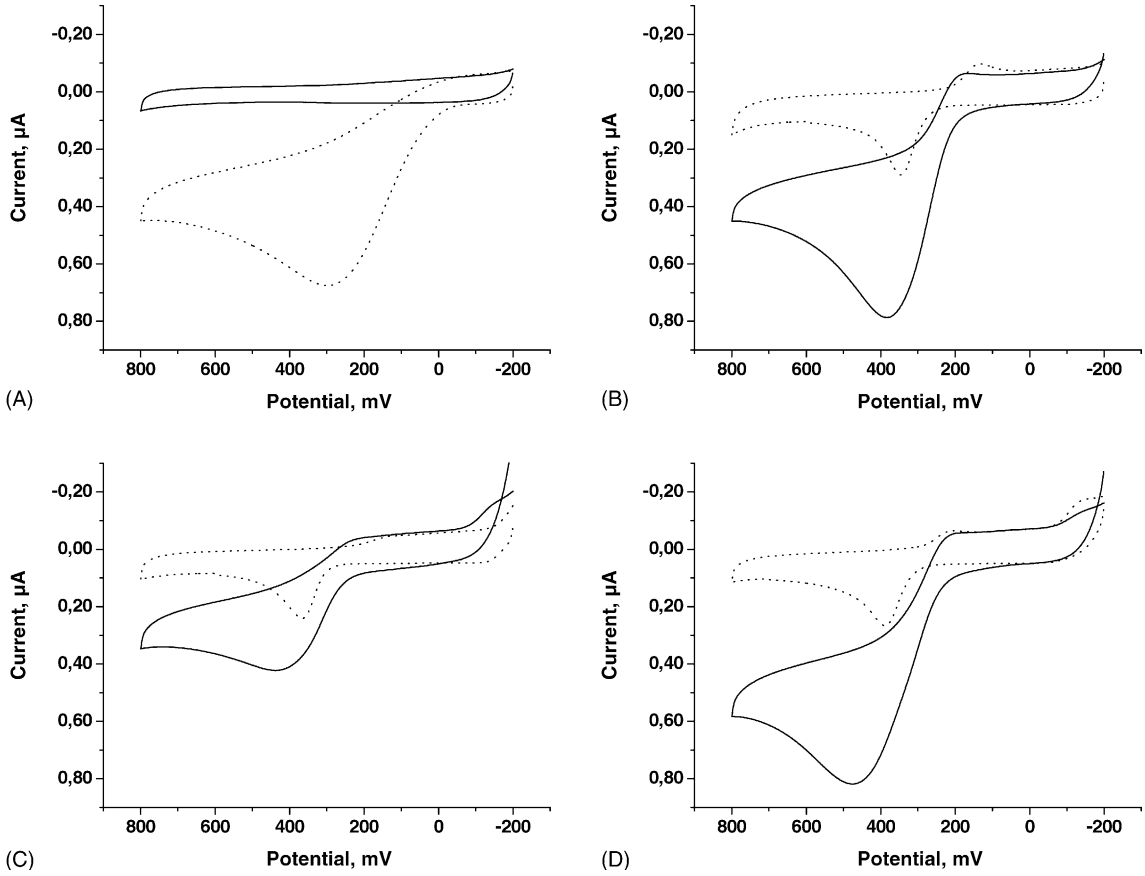

Fig. 1. Cyclic voltammograms of 1×10^{-3} M ascorbic acid (A) and 5×10^{-5} M dopamine (B), epinephrine (C) and norepinephrine (D) in a 0.050 M phosphate buffer solution at pH 7.4, at a 500 μm bare graphite disk electrode (dotted lines) and 500 μm coated graphite disk electrode (solid lines). Scan rate 50 mV/s. First scan in positive direction.

ization times higher than 50 min, being 60 min the optimum to obtain the most reproducible response.

The feasibility to electropolymerize L-dopa in the CZE electrochemical cell was also evaluated using different polymerization times. For this study, a 0.050 M phosphate buffer solution pH 7.4 containing 3×10^{-3} M L-dopa was flushed across the capillary column in order to make a renewable flow solution in the electrode surface. The best compromise between sensitivity and selectivity was obtained for times higher than 120 min. Therefore, since the time necessary to get a polymer with the best permselective properties is twice longer than that for obtaining it in a conventional electrochemical cell, all subsequent polymerizations were performed outside the CE electrochemical cell.

This electrochemical device allowed the use of normal-sized electrodes and commercial CZE instruments to detect neurotransmitters. Glassy carbon electrodes (Ø = 1 mm) or graphite disk electrodes (Ø = 300 and 500 μm) allowed us to obtain a high signal-to-noise ratio, low detection limit, excellent stability, and high mass transfer rates, making the operation simpler than that using a carbon fiber electrode.

It is widely known that ascorbic acid reacts with the oxidation product of catecholamines (quinones) reducing it to the corresponding catecholamine. Based on the excellent permselective properties of the melanic polymers, it is possible to use high concentration of ascorbic acid in the measurement solution to amplify the oxidation current of the neurotransmitters improving, thus, their quantification. Therefore, the key of this methodology is the use of the melanic polymer as a barrier that allows the use of ascorbic acid in the detection solution to improve the sensitivity of the oxidation of dopamine, epinephrine and norepinephrine.

Therefore, since ascorbic acid was used to amplify the oxidation of neurotransmitters at the surface of the electrode, the CE response of the electrode in the presence of ascorbic acid was evaluated. The study was carried out using a 75 μm ID capillary column filled with 0.030 M phosphate buffer solution pH 5.6 and a separation voltage of 15 kV. The electrochemical measurements were performed in a cell filled with a 0.050 M phosphate buffer solution pH 7.4 while applying a potential of 700 mV as detection potential.

Fig. 2 shows the electropherograms for 1×10^{-5} M dopamine under different conditions. Fig. 2A depicts the response of dopamine at the bare GCE using a phosphate buffer solution containing 1×10^{-3} M ascorbic acid. A large and noisy background signal was obtained as a consequence of the electrooxidation of 1×10^{-3} M ascorbic acid. Under these conditions, no oxidation signal for dopamine was observed. When the measurement solution was phosphate buffer without ascorbic acid, a small dopamine oxidation signal was observed (Fig. 2B). Fig. 2C shows the electropherogram obtained for 1×10^{-5} M dopamine at a melanin-type polymer-modified glassy carbon electrode in phosphate buffer solution containing 1×10^{-3} M ascorbic acid. As expected, a dopamine oxidation signal almost four times higher than that at glassy carbon without the polymer is obtained due to the

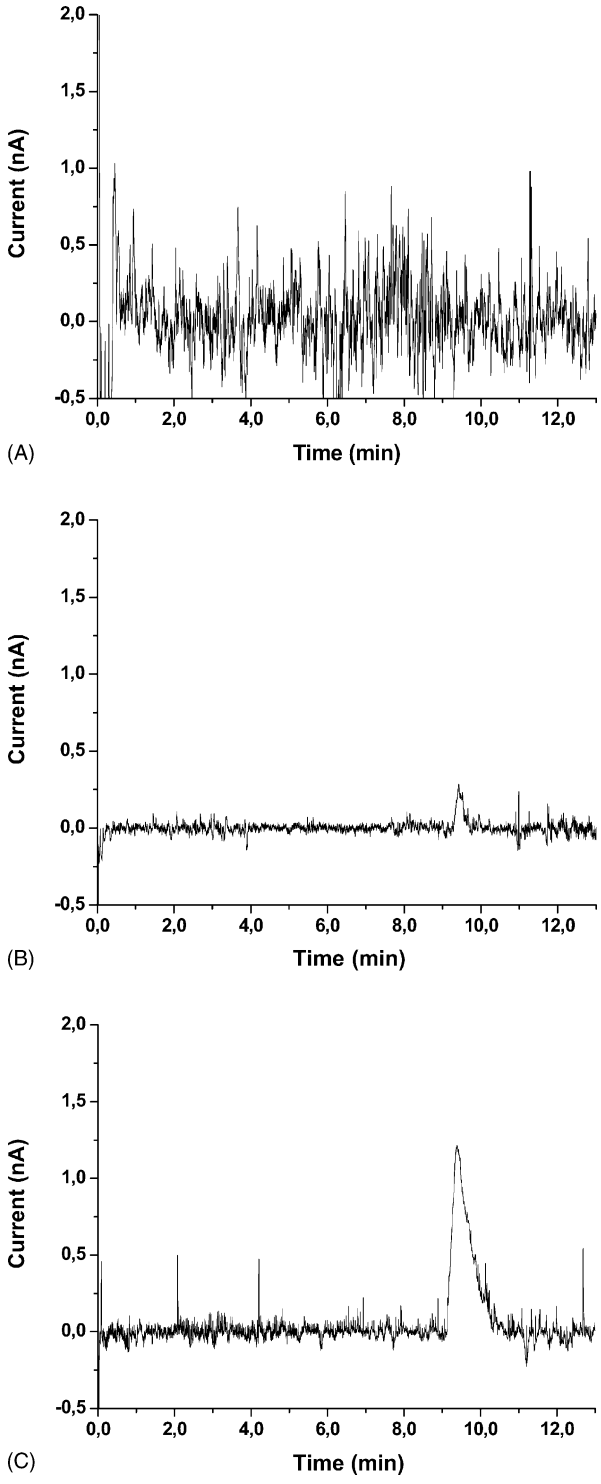

Fig. 2. Electropherograms with electrochemical detection for 1×10^{-5} M dopamine at: (A) a bare glassy carbon electrode in 0.050 M phosphate buffer solution pH 7.40 containing 1×10^{-3} M ascorbic acid, (B) a bare glassy carbon electrode in 0.050 M phosphate buffer solution pH 7.40 without ascorbic acid and (C) at a melanin-type polymer-modified glassy carbon electrode in buffer solution containing 1×10^{-3} M ascorbic acid. Conditions: buffer solution in the detection cell 0.050 M phosphate buffer at pH 7.4, detection potential 700 mV. Capillary length 75 cm. Separation buffer: 0.030 M phosphate, pH 5.6. Separation voltage: 15 kV. Hydrodynamic sample introduction for 0.5 s. Electropolymerization conditions as in the text.

amplification of dopamine oxidation mediated by ascorbic acid and only possible due to the presence of the melanic polymer. Similar effect was observed in the case of the other positively charged molecules such as epinephrine and norepinephrine (not shown).

The effect of pH and concentration of the buffer on the electrophoretical separation of neurotransmitters was evaluated in order to select the optimum separation conditions. Since the physico-chemical properties of these neurotransmitters are very similar, the direct determination of mixtures of these compounds is a dare task. However, when the capillary electrophoresis separation is used, the electroactive neurotransmitters can be separated according to the charge-to-mass at the separation pH. Considering both, the resolution effect and the appearance time that decreases at higher pHs we selected a borate buffer solution pH 9.6 to separate the neurotransmitters object of this work. As it is widely known, the concentration of the separation buffer plays an important role in the migration rate of the compounds. Borate concentrations higher than 0.040 M produced a considerable increase in the migration time of neurotransmitters. Therefore, we selected a 0.040 M borate buffer solution pH 9.6 to separate the neurotransmitters of interest, dopac and ascorbic acid.

The effect of the applied voltage on the analytical performance was also studied between 5 and 25 kV. Although the resolution improved when increasing the voltage from 5 to 20 kV, the selected running voltage for the subsequent studies was 15 kV (22 μA) in order to avoid the Joule's heating effect and get analysis times sufficiently short (4 min). It is important to mention that no interference was observed in the detection cell when this voltage was applied.

Different capillary lengths were also tested. In order to decrease the analysis time the length was reduced. A capillary length of 59 cm demonstrated the best compromise between time and resolution. Under these conditions the three neurotransmitters were separated and detected in times lower than 4 min.

Another important parameter is the gap distance between the disk electrode and the capillary outlet in the electrochemical device. To minimize the diffusional and convective broadening of the analyte zone, the gap distance should be as short as possible; to avoid smaller peak height and poorer detection limits [21]. Based on the sensitivity and reproducibility of the signals, we selected a 100 μm gap column-electrode distance. The RSD evaluated for a 500 μm electrode, rise from a value of 5.9% (n = 6) for 100 μm distance to 15.9% (n = 7) for 50 μm. Other small distances were also evaluated but the reproducibility of the analytical signal was poor.

The size of the electrodes is another important parameter in CE with electrochemical detection [22–24]. It is known that the best performance, is obtained when the working electrode diameter is higher than the capillary column inner diameter. The separation of the neurotransmitters was evaluated by using melanin-type modified graphite electrodes of 500 and 300 μm. Fig. 3 shows the electropherograms for the separation of dopamine, epinephrine and norepinephrine

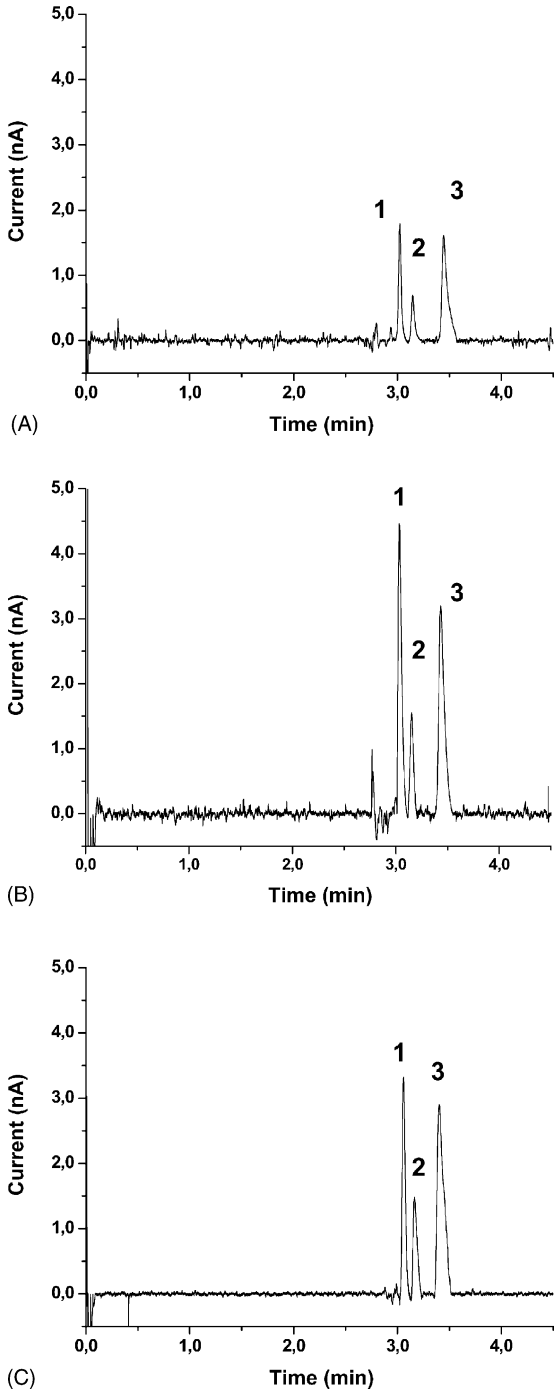

Fig. 3. Electropherograms of 1×10^{-5} M dopamine (1), epinephrine (2) and norepinephrine (3) obtained using electrodes with different diameters: graphite disk electrodes 300 μm (A) and 500 μm (B), and glassy carbon electrode 1 mm (C). Conditions: buffer solution in the detection cell 0.050 M phosphate buffer at pH 7.4 containing 1×10^{-3} M ascorbic acid. Separation buffer: 0.040 M borate buffer at pH 9.6. Other conditions as in Fig. 2.

using different size electrodes and a gap distance column-electrode of 100 μm. Fig. 3A shows the amperometric response for 1×10^{-5} M neurotransmitters using a 300 μm graphite electrode. Well-defined signals were obtained for the three neurotransmitters. Fig. 3B shows the response obtained

at a 500 μm graphite electrode modified with the melanic polymer. In this case there is an increase in the signal due to a better collection of the solution coming from the column since the electrode is a little wider than the column. For comparison it is also shown the response obtained at a mm glassy carbon electrode modified with the melanic polymer (Fig. 3C). We can observe in Fig. 3C that the peak signal is smaller and slightly broader than that obtained at 500 μm electrodes. This behavior could be explained due to the difference between the inner diameter of the capillary column and electrode size. We described above, that the size of the electrode and the gap distance between electrode and column are very important operational parameters. In our study we observe that for distances column-electrode of 100 μm and electrodes with diameters higher than 750 μm the signal becomes smaller and broader. Therefore, a 500 μm graphite electrode was selected for all subsequent studies.

The reproducibility of the neurotransmitters quantification using the melanin-type polymer-modified carbon electrodes in combination with capillary electrophoresis as well as the stability of the modified electrodes were evaluated by successive injections of neurotransmitters. Fig. 4 shows the oxidation currents for 1×10^{-5} M of dopamine, epinephrine and norepinephrine obtained at a melanin-type polymer-modified electrode using a detection potential of 700 mV. For series of 20 analysis (with a total of 300 min), the RSD were 3.2, 3.6 and 5.7% for dopamine, epinephrine and norepinephrine, respectively. These results demonstrated the high reproducibility of the overall assay and the stability of the polymer-modified electrode. Higher analysis times presented the inconvenience of a slight electrode poisoning and a loose of the polymeric matrix, producing a decrease in the signal. For instance, after 450 min analysis, the decrease in the current for dopamine is around 80%. It is also important to mention that for analysis time higher than 450 min the permselectivity of the polymeric electrode practically disappeared and, consequently, the signal for the neurotransmitters largely decreases.

A set of standard mixture solutions of the three neurotransmitters ranging from 1×10^{-6} to 2.5×10^{-5} M was tested to determine the linearity of this detection method. Table 1 summarized the regression equation, correlation coefficient and detection limit according to the $3s_b/m$ criterion, where m is the slope of the calibration curve and s_b the standard deviation. We can observe that the detection limit for dopamine in the case of polymeric electrode was three times higher than those obtained at the bare carbon disk electrode. Fig. 5 shows two electropherograms under the best separation and detection conditions for the same concentration of neurotransmitters, ascorbic acid and dopac. Huge signals for ascorbic acid and dopac were obtained at bare graphite disk electrode (Fig. 5A). On the contrary, a large decrease for ascorbic acid and dopac oxidation signals was obtained at the polymer-coated graphite disk electrode (Fig. 5B). These results demonstrate that the melanin-type polymer-modified graphite carbon electrode is suitable as an amperometric detector for capillary electrophoresis, allowing a reproducible and more sensitive quantification of mixtures containing dopamine, epinephrine and norepinephrine.

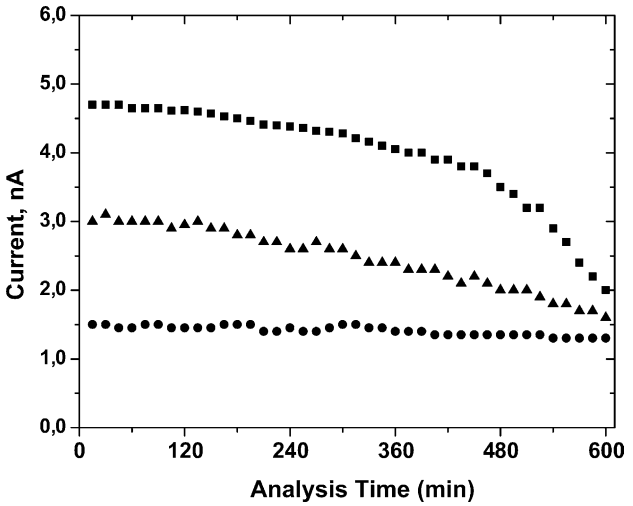

Fig. 4. Evolution of the electrochemical signal in capillary electrophoresis for 1×10^{-5} M dopamine (■), epinephrine (●) and norepinephrine (▲) in a coated carbon disk electrode with the analysis time. The total time of each analysis is 15 min. Other conditions as in the text.

Table 1
Analytical characteristics of the capillary zone electrophoresis with electrochemical detection method for the neurotransmitters determination

Electrode	Compound	Regression equation[a]	Correlation coefficient (n = 9)	Detection limit (μM)[b]	RSD[c] (%)	
					Peak current	Migration time
Bare carbon disk	Dopamine	$y = 0.1 + 154x$	0.998	2.7	3.2	1.7
	Epinephrine	$y = 0.0 + 131x$	0.990	1.3	3.5	1.9
	Norepinephrine	$y = -0.1 + 147x$	0.990	1.7	3.4	2.2
Coated carbon disk	Dopamine	$y = -0.1 + 464x$	0.9990	0.9	3.1	1.8
	Epinephrine	$y = 0.0 + 170x$	0.998	1.0	3.7	1.8
	Norepinephrine	$y = 0.0 + 316x$	0.9992	0.8	3.0	1.9

[a] Where y is the peak current in nA and x is the neurotransmitter in mM.
[b] Detection limit according to the $3s_b/m$ criterion, m is slope of the calibration curve and s_b the standard deviation.
[c] Ten injection in the same capillary (10 mM of each neurotransmitters).

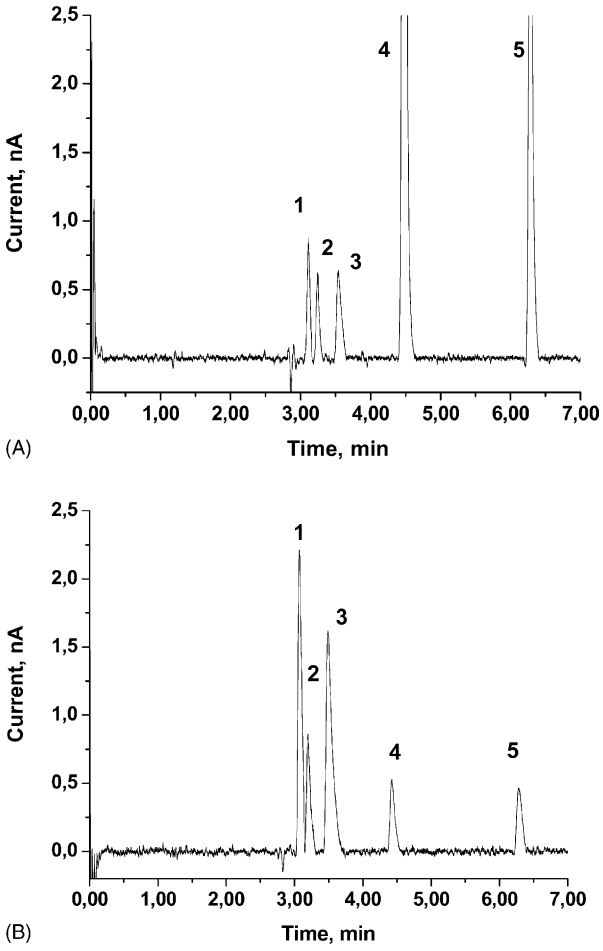

Fig. 5. Electropherograms for the capillary zone electrophoresis with electrochemical detection of 5×10^{-6} M dopamine (1), epinephrine (2), norepinephrine (3), 1×10^{-3} M ascorbic acid (4) and 5×10^{-5} M dopac (5) under optimal conditions. Bare carbon disk electrode (A) and coated carbon disk electrode (B). Optimal conditions as in the text.

4. Conclusions

The results obtained in this work demonstrate that the melanin-type polymer-modified graphite carbon electrodes can be used as amperometric detector for capillary electrophoresis. An important improvement, in contrast to bare carbon electrodes, in the sensitivity for the determination of dopamine, epinephrine and norepinephrine was obtained using the carbon electrodes modified with the melanic polymer. The electrode demonstrated to be stable and reproducible enough to be used as a CE electrochemical detector. The success in the combination of capillary electrophoresis and the new amperometric detector open the doors to the development of new methodologies for the determination of other electroactive compounds.

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