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In vivo determination of the monoamine neurotransmitters in rat brain by liquid chromatography with a thioctic acid/iridium oxide–palladium modified electrode

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Abstract

The fabrication and application of a electrochemical detector (ED) with the thioctic acid (TA)/iridium oxide (IrO_2)–palladium (Pd) chemically modified electrode (CME) for liquid chromatography (LC) were described. The electrochemical behavior of dopamine (DA) and ascorbic acid (AA) at the TA/IrO_2–Pd CME were investigated by differential pulse voltammetry (DPV). It was found that the CME could be permselective to DA cations and exile the AA anions, which could be used to determine the monoamine neurotransmitters and avoid the interference of AA in LC. In liquid chromatography with electrochemical detection (LC–ED), DA, norepinephrine (NE), 5-hydroxytryptamine (5-HT), epinephrine (E) and 3,4-dihydroxyphenylactic acid (DOPAC) were determined at the CME. The linear ranges of five analytes were over three orders magnitude and the limits of detection were 0.05 pmol for DA, 0.05 pmol for NE, 0.05 pmol for E, 0.10 pmol for 5-HT and 1.00 pmol for DOPAC. The application of this method coupled with microdialysis sampling for the in vivo determination of the monoamines in rat brain was also investigated.

Keywords: Thioctic acid/iridium oxide–palladium modified electrode; Neurotransmitter; LC–ED; Microdialysis sampling

1. Introduction

The monoamine neurotransmitters, such as dopamine (DA), norepinephrine (NE), and 5-hydroxytryptamine (5-HT) are well known as the important neurotransmitters. They are released from the brain neurons to extracellular fluids and play important roles in various biological, pharmacological and physical processes [1]. Furthermore, many diseases are related to the changes of the monoamine neurotransmitter concentrations. For example, in Parkinson's and other similar diseases, there is extensive damage to neural cells which creates deficiencies of dopamine [2,3]. Hence, in order to realize and control the physiological and pharmacological roles of these monoamine neurotransmitters, it is important to develop the method that can effectively separate and determine these compounds.

The determination of these monoamine neurotransmitters have been carried out by using spectrophotometer [4], fluorescence [5], chemical luminescence [6], pseudopolarography [7], voltammetry [8], capillary

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electrophoresis [9] and liquid chromatography [10]. Some of these methods have been used in biological matrices and pharmaceuticals to determine the monoamine neurotransmitters. However, because the monoamine neurotransmitters are absence of spectrum group, the applications of fluorometric or other photometric methods for the determination of them were limited.

Liquid chromatography (LC) is the most commonly employed method for analysis of complex samples in vivo and in vitro [11]. LC with electrochemical detection (ED) has been successfully used to simultaneously determine many important analytes in biological system [12,13]. Significant advantages, such as simplicity, rapidity and high sensitivity, have been achieved by combining ED with HPLC. However, there still have two difficulties on in vivo determination of some interesting matters in the brain tissue. One is that many substances present in brain can foul the electrode surface and the column. The other is that the neurotransmitters interested are present in very low concentrations, while many other electroactive species, such as ascorbic acid (AA), are present at high concentrations and interfere with the determination of the interested substances.

A lot of efforts have been spent to resolve the difficulties. For the first problem, sampling by microdialysis can be used to resolve it. As an in vivo sampling technique, microdialysis has been extensively used in neuroscience, pharmacology and physiological [14,15]. It can prevent the interference of large molecules, mainly proteins, in the biological matrix. The dialysates can be injected into LC and analyzed.

For the second problem, a promising approach for enhancing the sensitivity and selectivity is through the use of chemically modified electrode (CME). The CME has received tremendous attention for their applications in neuroscience, pharmacology and physiological area [16,17]. Recently, there has been much interest in a kind of CME which is based on the electrodeposition of metal or metal oxide on insert surface, such as ultrafine palladium particles supported on graphitic substrates [18], dispersed in carbon paste matrices [19], or metal and metal oxide electrodeposited on Pt electrode [20]. These CMEs have been wildly applied for the determination of many important analytes in biological systems. However, when determine the neurotransmitters in vivo, the coexisting electroactive species such as AA which has a high concentration in brain can cause a serious interference. In order to enhance the selectivity of the CME, a variety of discriminative coatings based on their different transport properties can be used to resolve the problem. One of the most well-known modifiers is Nafion (a perfluorosulfonated ionomer), which was commonly used to select cations and repel anions with its negatively charged sites [21,22]. However, the pratical utility of Nafion film-based electrode has the disadvantages of slow response time, low diffusion coefficients and memory effects [23,24]. These disadvantages limited the application of Nafion as a electrode modifier. Recently, thioctic acid (TA) has been investigated widely as an electrode modifier because the organsulfur of TA are known to bind strongly at a variety of metals or metal oxides, and the hydrophilic head groups of TA also provide negatively charged sites under a certain pH value on the electrode surface. The electrode modified by TA can be used to determine the cations and repel the anions with a good selectivity and high sensitivity. Several applications based on the properties of the terminal groups have been reported [25,26].

In this paper, we prepared a TA/IrO_2–Pd modified electrode on determination of the neurotransmitters in vivo. The applications of the iridium oxide modified electrode have been reported on the determination of pH [27], H_2O_2 [28], SO_2 [29], and CO_2 [30], etc. However, to the best of our knowledge, there is no report on the determination of monoamine neurotransmitters by using the IrO_2–Pd modified electrode. In the experiment, the IrO_2 and Pd were electrodeposited on a carbon electrode by cyclic voltammetry (CV), and the TA was modified on the layer of IrO_2–Pd. Significant advantages have been achieved by combining electrocatalytic function of the IrO_2–Pd with the charge-exclusion and pre-concentration features of TA. When the CME was used as the amperometric detector for liquid chromatography, the TA/IrO_2–Pd CME showed a good stable, reproducible response to the monoamine neurotransmitters and their metabolites, and the CME can eliminated the interference of AA. The application of this method coupled with microdialysis sampling for the in vivo determination of the monoamine neurotransmitters in rat brain was satisfactory.

2. Experimental

2.1. Regents

Dopamine (DA), epinephrine (E), norepinephrine (NE), 5-hydroxytryptamine (5-HT), 3,4-dihydroxyphenylacetic acid (DOPAC) and ascorbic acid (AA) were analytical grade and purchased from Sigma (USA), $(NH_4)_3IrCl_6$ was analytical grade and came from Johnson Matthey Chemicals Limited (England), $PdCl_2$ was analytical grade and came from Aldrich (USA), thioctic acid was analytical grade and came from E. Merck Company (Switzerland). All buffer components were analytical reagents or better quality. Double-distilled deionized water was used for all solutions. Prior to use, all solutions were degassed with purified nitrogen for 20 min.

2.2. Apparatus

Electrochemical experiments were performed with a CHI832 electrochemical system (CHI Co., USA). The three-electrode system consisted of a TA/IrO_2–Pd CME or glass carbon (GC) electrode as working electrode, a saturated calomel electrode as reference electrode and a gold wire electrode as counter electrode.

Liquid chromatographic experiments were conducted on a model 510 pump and a U6K injector (Waters Assoc., USA). The injection volume was 20 μl. The column was Zorbax C_8 (4.6 mm × 25 cm) (Du Pont, USA). The detector consisted of a laboratory-made thin-layer cell and a CH1 Potentiostat (Jiangsu Electrochemical Instruments Works, Jiangsu, China). The working electrode was a TA/IrO_2–Pd CME or GC electrode. The mobile phase was 0.2 mol/l phosphate buffer (pH = 6.0) and which was delivered at a constant flow rate of 1.0 ml/min. All the experiments were performed at room temperature and the pH value was calibrated with pH meter (Horiba Ltd., Japan).

Microdialysis was accomplished by using a CMA 101 microdialysis pump (CMA Microdialysis AB, Stockholm, Sweden) and a CMA 12 microdialysis probe (dialysis length: 3 mm, diameter: 0.24 mm, BAS Company, Japan). The probe was perfused with the Ringer solution [31] (147 mmol/l Na^+, 4.0 mmol/l K^+, 2.2 mmol/l Ca^{2+}) at a flow rate of 1.0 μl/min.

2.3. Electrode preparation

Prior to its modification, the GC electrode surface was polished with 0.5 μm alumina on a polishing micro-cloth and rinsed with deionized water. Subsequently, it was ultrasonicated thoroughly with acetone, NaOH solution (50% (w/w)), HNO_3 (1:1 (v/v)) and doubly distilled water.

2.4. Electrodeposition of iridium oxide and palladium

The electrodeposition of iridium oxide and palladium was conducted according to the literature [32]. The pretreated GC electrode was cycled in a buffer solution containing 0.10 mmol/l $PdCl_2$, 0.20 mmol/l $(NH_4)_3IrCl_6$ and 0.20 mol/l Na_2SO_4 over the potential range from +0.90 to −0.30 V. The electrodeposition of iridium oxide and palladium was finished after 15 cycles. Then, the IrO_2–Pd CME was rinsed with doubly distilled water and absolute ethanol for several times.

2.5. Modification of TA on IrO_2–Pd modified electrode

The IrO_2–Pd modified electrode was immersed in an ethanol solution containing 0.1% TA for 30 min and allowing the solvent to evaporate in air.

2.6. In vivo microdialysis experiment

Animal care was accordance with the Guide for the Care and Use of Laboratory Animal (NIH Publication No. 86-23, 1985, Bethesda, MD). Experiments were performed on Sprague–Dawley (SD) male rats weighing 200–250 g. The animals were anesthetized by 20% urethane (1.25 g/kg) and placed in a strereotaxic frame. The hole was drilled in the skull and then the microdialysis probe was implanted in the striatum. The microdialysis probe was stereotaxically implanted into the striatum at coordinates 0.2 mm posterior to the bregma, 4.0 mm lateral from midline and the middle of the probe 4.5 mm below the dura [33]. The microdialysis samples were collected at the microdialysis rate of 1.0 μl/min every 25 min and analyzed on the TA/IrO_2–Pd CME in LC–ED. The dialysates samples collected over the first 60 min were discarded to

allow recovery from the acute effects of the surgical procedure.

3. Results and discussion

3.1. Electrodeposition of IrO_2 and Pd by cyclic voltammetry

Fig. 1 shows the electrodeposition of IrO_2 and Pd with cyclic voltammetry on a GC electrode in a solution containing 0.10 mmol/l $PdCl_2$, 0.20 mmol/l $(NH_4)_3IrCl_6$ and 0.20 mol/l Na_2SO_4. In the process of electrodeposition with a potential range from +0.90 to −0.30 V (versus SCE), the increase of the baseline current near 0.37 V was indicated the film formation. A pair of redox waves were observed at E_{pa} of 0.66 V and E_{pc} of 0.16 V (E_{pa} and E_{pc} represented the anodic and cathodic peak potential, respectively), which was due to the $IrCl_6^{2-}/IrCl_6^{3-}$ couple. As studies previously [34], in neutral and basic media both $IrCl_6^{2-}$ and $IrCl_6^{3-}$ were converted slowly to various hydrated oxides and eventually formed precipitates. During the process of film formation in Fig. 1, $IrCl_6^{3-}$ was converted into an oxide at the primary step followed by a transformation to other oxides such as

$$Ir_2O_3 + H_2O = 2IrO_2 + 2H^+ + 2e$$

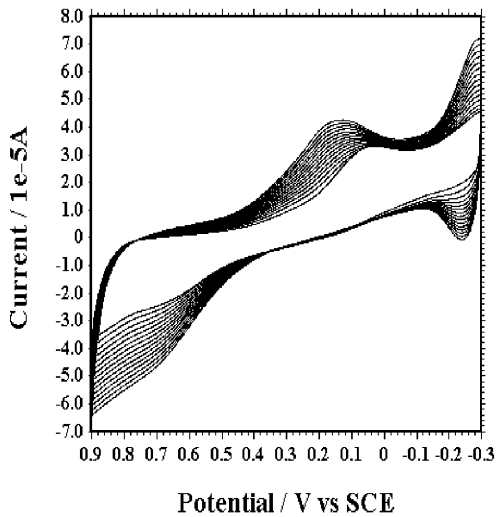


Fig. 1. The electrodeposition of IrO_2–Pd at the glass carbon electrode in a solution containing 0.10 mmol/l $PdCl_2$, 0.20 mmol/l Na_2IrCl_6, and 0.20 mol/l Na_2SO_4.

The role of Pd^{2+} was to provide a metallic layer on the glass carbon, which could catalyze the electrolytic generation of hydrogen and thereby formed a basic environment on the electrode surface. In addition, the modified electrode in the presence of Pd^{2+} was more stable in acid or neutral solutions [32].

3.2. DPV of DA and AA on GC electrode and TA/IrO_2–Pd CME

Fig. 2 shows the differential pulse voltammogram of DA and AA on the bare GC electrode and the TA/IrO_2–Pd CME. On the GC electrode, AA and DA both showed oxidative peaks at 0.305 and 0.206 V, respectively. On the TA/IrO_2–Pd CME, there was no obvious oxidative peak for AA. However, DA showed an oxidative peak at 0.110 V. Compared with the data on the bare GC electrode, the peak current of DA increased and its overpotential decreased on the CME, while the current response of AA decreased clearly on the TA/IrO_2–Pd CME. There were two reasons for the phenomena. Firstly, the amine group of DA is positively charged, whereas, the hydroxyl next to the carbonyl group of AA is negatively charged in pH 6.0 buffer solution. The result showed that the TA modified electrode could effectively exclude the negative AA ions from the electrode but accumulated the positive DA species. Secondly, the apparent increase of oxidation current of DA at the TA/IrO_2–Pd CME was based on the catalytic effect of the IrO_2 and Pd. According to the research of Cox and Jaworski [28], the electrode process could be described as follows (Scheme 1).

3.3. Relationship between the TA modified time on IrO_2–Pd electrode and the current response of DA and AA

Fig. 3 shows the relationship between the modified time of TA and the responses of DA and AA at the CME. The modified time of TA had important effect on the permselective transport for the DA cations and against the AA anions. When the modified time of TA was short, the TA was also less closely packed on the electrode. Then the sensitivity of the TA/IrO_2–Pd CME for DA was higher than that of the bare GC electrode because of the catalysis of IrO_2–Pd. Meanwhile, the selectivity of the CME for DA with respect

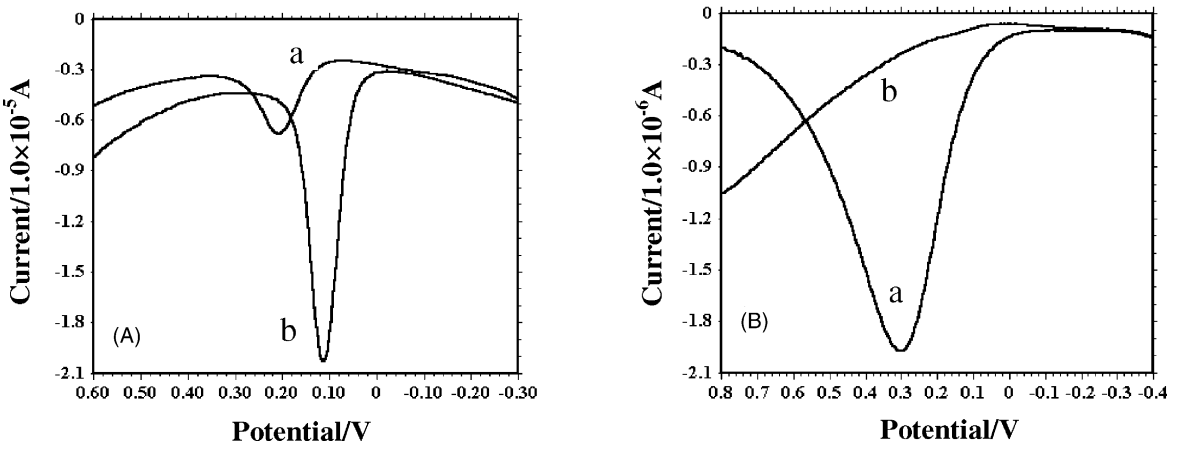
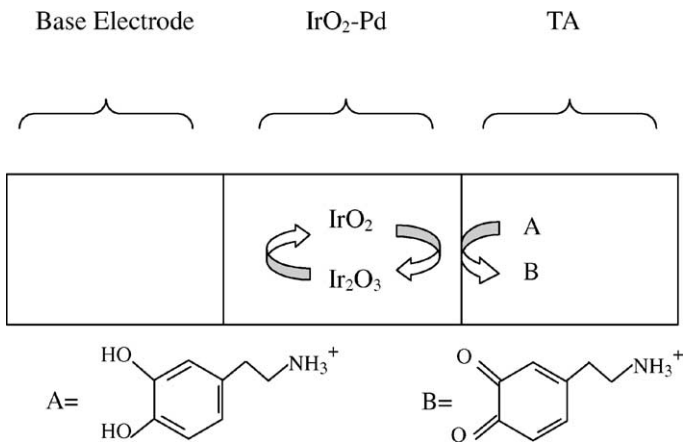


Fig. 2. Differential pulse voltammogram of DA and AA at the bare GC (a) electrode and the TA/IrO_2–Pd CME (b). (A) Containing 1.0×10^{-4} mol/l DA; (B) containing 1.0×10^{-4} mol/l AA. Electrolyte: 0.20 mol/l phosphate solution (pH = 6.0).

to AA was higher than that of the bare GC electrode. When prolonged the modified time of TA, the electrode was closely packed with TA, then the substantial decrease in sensitivity and the increase in selectivity of the CME for DA could be observed. This could be explained by the fact that when the modified time of TA was short, the DA cations could conveniently penetrate onto the electrode surface and were catalyzed by the IrO_2–Pd, then the electrochemical response increased. If the modified time of TA was too long, the CME had a good selectivity for DA. But because the DA cations were difficult to reach the IrO_2–Pd layer, the sensitivity of the CME for DA decreased. In order to get the better selectivity and sensitivity, the selected modified time of TA was 30 min.

3.4. pH effect of mobile phase

The pH value of mobile phase has a great effect on the separation and determination of these neurotransmitters and their metabolites in LC–ED. At pH < 5, although the retention time was short, the peaks of



Scheme 1. Electrode reaction process of DA on the TA/IrO_2–Pd CME.

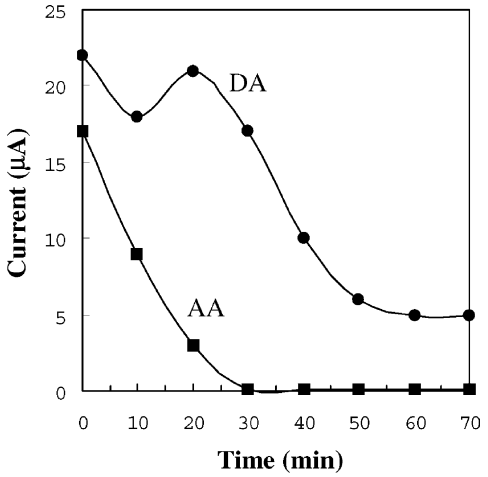


Fig. 3. The relationship between the modified time of TA and the current responses of 1.0×10^{-4} mol/l DA and AA at the IrO_2–Pd electrode in the pH 6.0 phosphate buffer solution.

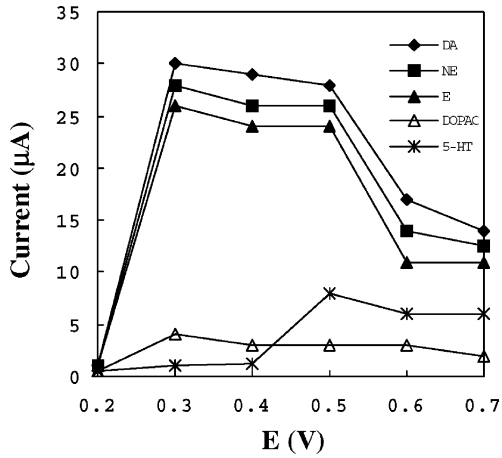


Fig. 4. Hydrodynamic voltammograms of a mixture of 1.0×10^{-4} mol/l DA, NE, E, DOPAC, and 5-HT at the TA/IrO_2–Pd CME in LC–ED. Column, Zorbax C_8 (4.0 mm × 25 cm); injection volume: 20 μl; mobile phase: 0.2 mol/l phosphate solution; pH = 6.0; flow rate: 1.0 ml/min.

DA, E, and NE had a little overlap. At pH > 6.5, the NE, DA, DOPAC, and 5-HT had tailed peaks and they could not reach the baseline separation. At pH = 6.0, all the neurotransmitters and their metabolites had good responses and separation. Moreover, because most of the neurotransmitters and their metabolites are cations under the pH = 6.0 and the –COOH group of TA is negatively charged. The CME can enhance the responses of the cations and reduce the interference of AA ($pK_a = 4.1$), so the selected pH value of the experiment was 6.

3.5. Hydrodynamic voltammetry (HDV)

Hydrodynamic voltammetry is a suitable method to select the appropriate potential applied on HPLC–ED. In this study, a standard solutions of each of the five analytes was repetitively injected while the HPLC–ED operating potential was increased from 0 to 0.6 V in 0.1 V increments. Fig. 4 is the hydrodynamic voltammogram of the mixed sample containing 1.0×10^{-4} mol/l DA, NE, E, 5-HT, and DOAPC on the TA/IrO_2–Pd CME. When the applied potential was larger than +0.20 V, the current responses of DA, NE, E, DOPAC increased apparently, and reached the maximum value at +0.30 V. But the current response of 5-HT was still very low. When the potential increased continuously, the current response of 5-HT increased quickly and reached the maximum value at +0.50 V. When the potential increased larger than +0.60 V, all the current responses decreased obviously. Moreover, if the applied potential was larger than +0.60 V, the baseline current also became high and the other substances maybe responded on the electrode. In order to obtain the best selectivity and signal/noise ratio, +0.50 V was chosen as the optimum detection potential.

3.6. Linearity and detection limits

To determine the linearity for DA, NE, E, 5-HT, and DOPAC on the TA/IrO_2–Pd CME in LC–ED, a series of mixed standard solutions of these analytes ranging from 0.5×10^{-8} to 5.0×10^{-4} mol/l were tested. The ranges of the linear relationships observed between currents and concentrations were over three orders of magnitude and all the coefficients were more than 0.999. The analytical data for the analytes were shown in Table 1.

The reproducibility was estimated by making repetitive injection (eight times) of a standard solution containing 50 μmol/l mixture for the five analytes under the same condition every 30 min. The relative standard deviation of the peak currents were found to be 1.2% for DA, 1.3% for NE, 1.7% for E, 1.6% for 5-HT, and

Table 1
Analytical data of the five analytes at the LC–ED[a]

Compounds	Linear range (mol/l)	Detection limit (pmol)	Regression equation ($Y = bX + a$)[b]	Correlation coefficient
DA	0.5×10^{-8} to 5.0×10^{-4}	0.05	$Y = 4.564X - 0.7647$	0.9998
NE	0.5×10^{-8} to 5.0×10^{-4}	0.05	$Y = 2.734X + 0.3881$	0.9995
E	0.5×10^{-8} to 5.0×10^{-4}	0.05	$Y = 9.310X + 0.050$	0.9998
5-HT	1.0×10^{-8} to 5.0×10^{-4}	0.1	$Y = 4.553X + 0.2380$	0.9999
DOPAC	1.0×10^{-7} to 5.0×10^{-4}	1.0	$Y = 5.343X + 0.2198$	0.9995

[a] LC–ED conditions as in Fig. 5.
[b] Where Y and X represent the peak current (nA) and the concentration of the analytes (μmol/l), respectively.

2.1% for DOPAC. Hence, the currents were stable and reproducible on the TA/IrO_2–Pd CME.

3.7. Different liquid chromatograms of monoamine neurotransmitters on GC electrode and TA/IrO_2–Pd CME

Fig. 5 are the liquid chromatograms of AA, NE, E, DA, DOPAC, and 5-HT on the bare GC electrode and on the TA/IrO_2–Pd CME. From the chromatograms, the neurotransmitters of NE, E, DA, 5-HT, and DOPAC had good responses on bare GC electrode and on TA/IrO_2–Pd CME. But the chromatographic peak of NE was interfered by the peak of AA on the bare GC electrode. While on the TA/IrO_2–Pd CME, the response of AA was eliminated and did not interfere with the determination of NE in LC–ED. Moreover, the current responses of DA, NE, E, and 5-HT on the TA/IrO_2–Pd CME were much larger than those on the bare GC. It could be ascribed to that the permselectivity of TA/IrO_2–Pd CME increased the current responses of cations, such as DA, NE, E, 5-HT, and reduced the current response of AA anion on the TA/IrO_2–Pd CME. So the TA/IrO_2–Pd CME showed good selectivity and sensitivity for the determination of most neurotransmitters. However, the current response of DOPAC on the TA/IrO_2–Pd CME had little lower than that on the bare GC electrode. This was because DOPAC is also an anion in pH 6.0 buffer

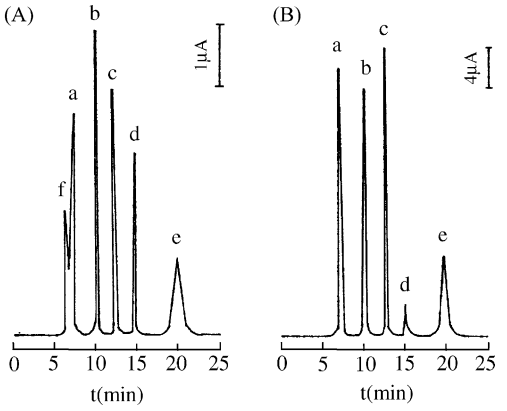


Fig. 5. Chromatograms of 1.0×10^{-4} mol/l: (a) NE; (b) E; (c) DA; (d) DOPAC; (e) 5-HT; and (f) AA, at (A) the bare GC electrode and (B) the TA/IrO_2–Pd CME. Applied potential: +0.50 V; other conditions are as in Fig. 4.

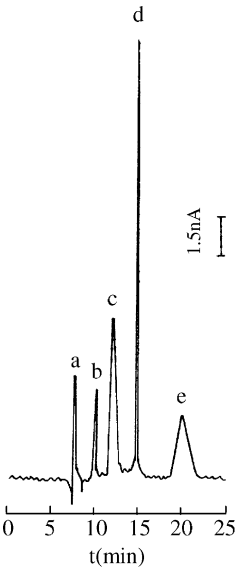


Fig. 6. Chromatograms of the monoamine neurotransmitters and their metabolites: (a) NE; (b) E; (c) DA; (d) DOPAC; and (e) 5-HT, at the striatum of the rat brain obtained by LC–ED coupled with microdialysis sampling. Other conditions as in Fig. 5.

Table 2
Content of the monoamine neurotransmitters and their metabolites in the striatal dialysates of the rat brain[a]

DA (10^{-8} mol/l)	NE (10^{-8} mol/l)	E (10^{-8} mol/l)	5-HT (10^{-8} mol/l)	DOPAC (10^{-8} mol/l)
Striatum				
4.36 ± 0.056	2.98 ± 0.035	2.82 ± 0.048	6.05 ± 0.072	112 ± 2.32

[a] The values shown are calculated from the calibration curves and are mean of $n = 3$ in each case. LC–ED conditions as in Fig. 5.

solution. The negative charged TA/IrO_2–Pd CME suppressed the response of DOPAC on the CME. But because the DOPAC has a higher concentration than DA, NE, E, and 5-HT in rat brain, and perhaps the hydrophilic interaction between DOPAC and the TA/IrO_2–Pd CME is favorable for the permeation of DOPAC [35], the TA/IrO_2–Pd CME is suitable for the determination of the monoamine neurotransmitters and their metabolites in real samples and could effectively remove the interference of AA present in LC–ED.

3.8. In vivo experiment

Fig. 6 shows the chromatogram of monoamine neurotransmitters and their metabolites at the striatum of rat brain using the microdialysis sampling. From the chromatogram, DA, NE, E, 5-HT, and DOPAC showed clear peak current responses, but AA did not show obvious peak current response. Therefore, although AA has a much higher concentration than DA in rat brain, it did not interfere with the determination of monoamine neurotransmitters and their metabolites. The microdialysis relative recovery was found to be 52.8% for DA, 47.6% for NE, 48.2% for E, 51.8% for 5-HT, and 56.5% for DOPAC under the microdialysis rate of 1.0 μl/min. The concentrations of analytes in brain microdialysate were calibrated according to the literature [36]. The related analytical results were given in Table 2. The concentrations of DA, NE, E, 5-HT, and DOPAC detected were within the normal range [37–39]. All these results suggested that the TA/IrO_2–Pd CME was very reliable and sensitive to determine the neurotransmitters and their metabolites in rat brain.

4. Conclusion

In this paper, the TA/IrO_2–Pd CME was prepared and used as the amperometric detector for liquid chromatography to determine the monoamine neurotransmitters. All the differential pulse voltammetry (DPV) and liquid chromatographic experiments showed that the TA/IrO_2–Pd CME effectively responded to monoamine cations and exiled the ascorbic acid anion, which could be used in the determination of neurotransmitters and removed the interference of AA. In LC–ED, the TA/IrO_2–Pd CME showed good and stable current response to DA, NE, E, 5-HT, and DOPAC. The linear ranges of five analytes were over three orders of magnitude and the limits of detection were 0.05 pmol for DA, 0.05 pmol for NE, 0.05 pmol for E, 0.10 pmol for 5-HT, and 1.00 pmol for DOPAC. The application of this method coupled with microdialysis sampling for the in vivo determination of the monoamine neurotransmitters and their metabolites in rat brain were satisfactory.

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