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Rapid Determination of Dopamine and Its Metabolites During *in vivo* Cerebral Microdialysis by Routine High Performance Liquid Chromatography With Electrochemical Detection[1]

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Objective To determine dopamine and its metabolites during *in vivo* cerebral microdialysis by routine high performance liquid chromatography with electrochemical detection. **Methods** Microdialysis probes were placed into the right striatum of Wistar rat brains and perfused with Ringer's solution at a rate of 1.5 μL/min. A reverse phase HPLC with electrochemistry was used to assay DA, DOPAC, and HVA after cerebral microdialysates were collected every 20 minutes from awake and freely moving rats. In order to identify the reliability of this method, its selectivity, linear range, precision and accuracy were tested and the contents of DA, DOPAC, and HVA in rat microdialysates were determined. **Results** The standard curve was in good linear at the concentration ranging from 74 nmol/L to 1.5 μmol/L for DOPAC (r^2= 0.9996), from 66 nmol/L to 1.3 μmol/L for DA (r^2=1.0000) and from 69 nmol/L to 1.4 μmol/L for HVA (r^2=0.9992). The recovery of DOPAC (0.30, 0.77, 1.49 μmol/L), DA (0.26, 0.69, 1.32 μmol/L), and HVA (0.27, 0.71, 1.37 μmol/L) was 82.00±1. 70%, 104.00±4.00%, 98.70±3.10%; 92.30±1.50%, 105.30±2.30%, 108.00±2.00%; 80.00±7.80%, 107.69±8.00%, and 108.66±3.10%, respectively at each concentration. Their intra-day RSD was 3.3%, 3.4%, and 2.5%, and inter-day RSD was 4.2%, 2.3%, and 5.6%, respectively. The mean extracellular concentrations of DOPAC, DA, and HVA in rat brain microdialysates were 10.7, 2.4, and 9.2 μmol/L (n=6), respectively. **Conclusion** The findings of our study suggested that the simple, accurate and stable method can be applied to basic researches of diseases related to monoamines neurotransmitters by cerebral microdialysis in rats.

Key words: Dopamine (DA); 3,4-dihydroxyphenylacetic acid (DOPAC); Homovanillic acid (HVA); Cerebral microdialysis; High performance liquid chromatography; Electrochemical detection

INTRODUCTION

Dopamine (DA), an important neurotransmitter in the central nervous system, plays a critical role in the course of some neurodegenerative diseases and neurological disorders. For example, abnormal loss of DA rich neurons may lead to Parkinson's disease[1], which is becoming increasingly popular in the elderly people. Therefore, monitoring DA and its metabolites has been attractive to neuroscientists, and rapid and precise methods detecting these elements are of particular interest.

Over the past few years, several techniques such as high performance liquid chromatography (HPLC) with native fluorescence detection[2], HPLC with precolumn derivatization and fluorescence detection[3], HPLC with post-column fluorogenic derivatization and chemoluminescence detection[4], and capillary electrophoresis with laser-induced fluorescence and intensified charge-coupled device detection[5] in assaying biogenic amines have been reported. However, HPLC with electrochemical detection (ECD) remains a method of choice. In this aspect, emphases have been laid on the improvement of ECD. Poly (*para*-aminobenzoic acid) modified electrode[6] and chemically modified electrode based on ring-disk electrode as the dual electrochemical detector[7] are two relatively successful attempts. But there still exist some shortcomings, e.g. complicated pretreatment, high cost, low sensitivity or time consumption.

The present study aimes at rapidly determining DA and its two metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) by routine HPLC-ECD, equipped with an amperometric detector. The method

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described here has the characteristics of relatively high sensitivity, high precision and short time for analysis without modification and thus can be used in most research laboratories.

MATERIALS AND METHODS

Chemicals and Reagents

Dopamine hydrochloride (DA, ＞99%), 3, 4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 1-heptanesulfonic acid sodium salt (HSA), triethylamine (TEA, ≥99) were purchased from Sigma (St. Louis, MO, USA). Acetonitrile (ACN, HPLC grade) was purchased from Fisher (New Jersey, USA). Phosphorate acid (PA) and EDTA tetrasodium salt were obtained from Guoyao Group Co., Ltd (Shanghai, China). Ringer's fluid was prepared in our laboratory. Deionized water of at least 18.2 MΩ cm specific resistance was used to prepare all the solutions.

Standards Preparation

Stock solutions of DA (0.5 mmol/L), DOPAC (0.6 mmol/L) and HVA (0.5 mmol/L) were prepared in 0.1 mol/L perchloric acid ($HClO_4$) and aliquots were stored at -70℃ for a period not exceeding 3 months. Calibration standards, with the concentration ranged from 66 nmol/L to 1.3 μmol/L for DA, 74 nmol/L to 1.5 μmol/L for DOPAC and 69 nmol/L to 1.4 μmol/L for HVA, were freshly prepared by successive dilution of the stock solutions with mobile phase and stored at 4℃ before using.

Sample Collection

Sample collection was carried out through a microdialysis system including mainly the MD-1001 Baby Bee Syringe Drive and MD-1020 Bee Hive Controller (Bioanalytical Systems, Inc., West Lafayette, USA). A microdialysis probe (MAB/6; O.D. 0.6 mm, membrane length 4 mm, cut-off 15 000 Da, Microbiotech AB, Stockholm, Sweden) was inserted into the right striatum of awake and freely moving rats (coordinates: AP + 0.2 mm; ML -3.0 mm; DV -3.5 mm from bregma) and perfused with Ringer's solution at a rate of 1.5 μL/min. The Ringer's solution contained 125 mmol/L NaCl, 3.3 mmol/L KCl, 2.4 mmol/L Mg_2SO_4, 1.25 mmol/L KH_2PO_4, 1.85 mmol/L $CaCl_2$. Microdialysates were collected at intervals of 20 minutes in vials containing 5 μL of 0.1 μmol/L ascorbic acid in order to prevent DA and its metabolites from oxidation. The samples were kept at -70℃ immediately after collection.

Probe Recovery

Before the probe was placed into the rat striatum, it was put in a mixed standard solution containing DA 1.3 μmol/L, DOPAC 1.5 μmol/L, and HVA 1.4 μmol/L. The same perfusate, flow rate, collection times and other conditions described before were used here to determine the probe recovery, which was expressed as the ratio of the peak areas of DA, DOPAC and HVA measured in microdialysates *in vitro* to that measured in the mixed standard solution. The probe recoveries for DA, DOPAC and HVA were 30.3%, 25.8%, and 25.6%, respectively.

Chromatographic Conditions

A Shimadzu (Kyoto, Japan) LC-10ADvp HPLC system equipped with a binary pump, Rheodyne 7725i injector, CTO-10A column oven and L-ECD-6A amperometric detector was used to optimize the chromatographic conditions. Separations were performed by a Hypersil GOLD C_{18} column (ODS, 150×4.6 mm i.d., 5 μm, Thermo, UK). The column and detector were housed in the compartment of CTO-10A column oven at 40℃. The analytes were detected at an oxidation potential of 0.75 V vs *in situ* Ag/AgCl reference electrode. The isocratic mobile phase (pH=2.1) consisted of 8.65 mmol/L HSA, 0.35% TEA, 0.4% PA, 6.25% ACN and 0.26 mmol/L EDTA tetrasodium salt and was delivered at a flow rate of 1.0 mL/min. The injected sample volume was 20 μL and the total analysis time was 15 minutes.

RESULTS

Linearity

The linearity was evaluated with the calibration curves plotted with the calibration standards containing DA, DOPAC, and HVA in the concentration ranges described before. The peak areas *vs* the concentrations of DA, DOPAC, and HVA had good linear relationships within the studied ranges, and the linear regression equation was expressed as: y=64.569x-6.3534 (r^2=1.0000); y=97.259x-5.8029 (r^2=0.9996); and y=68.446x-3.2864 (r^2=0.9992), respectively, where y refers to peak areas and x refers to the concentrations.

Selectivity

Under the same chromatographic conditions, the representative chromatograms of the blank Ringer's solution, the standard Ringer's solution and the rat cerebral microdialysates are shown in Fig.1. No other peaks which may interfere with the analytes were present. The retention time of DA, DOPAC, and HVA was 6.72, 9.05, and 12.94 minutes, respectively.

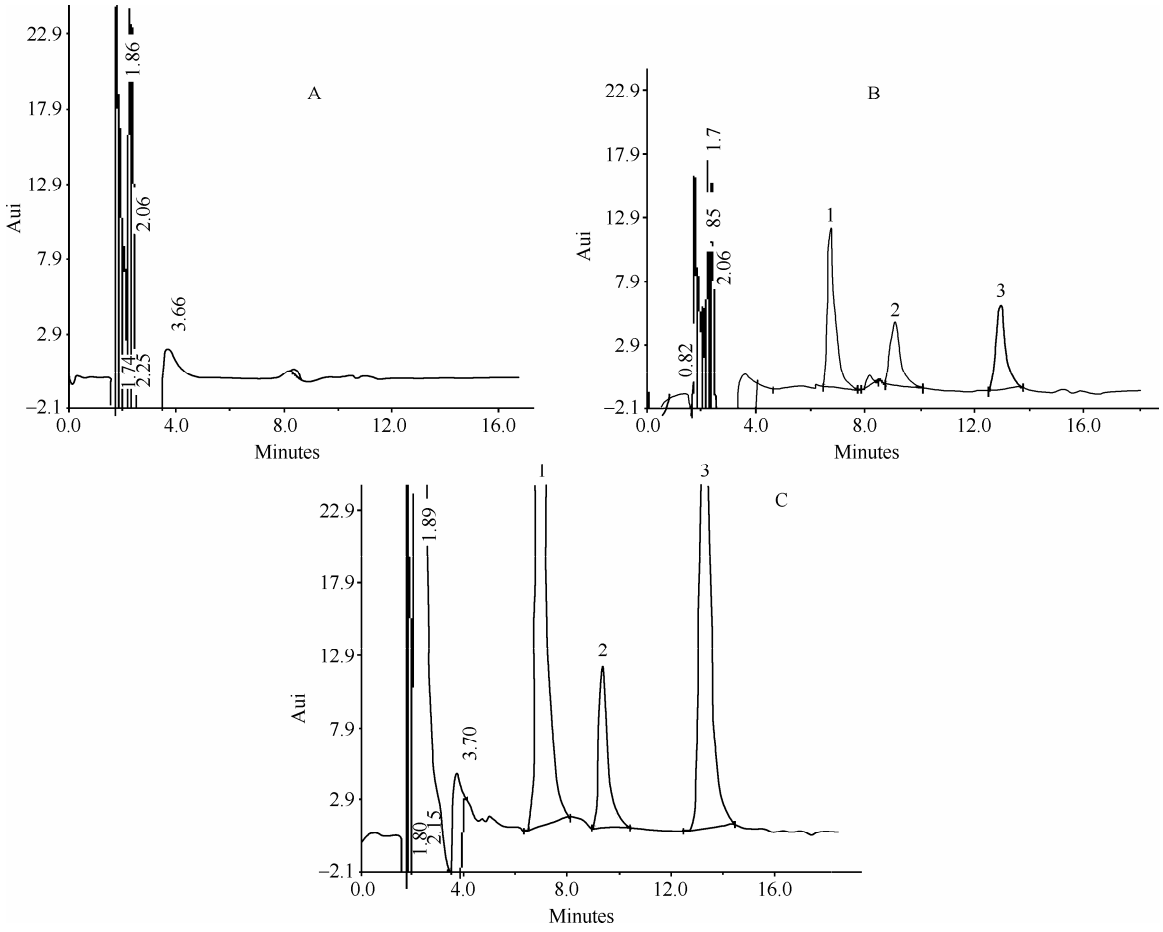

FIG. 1. Chromatograms of the blank Ringer's solution **(A)**, the standard Ringer's solution (1. DOPAC 1.49 μmol/L; 2. DA 1.32 μmol/L; 3. HVA 1.37 μmol/L) **(B)** and the rat cerebral microdialysate (1. DOPAC 10.52 μmol/L; 2. DA 2.43 μmol/L; 3. HVA 9.06 μmol/L) **(C)**.

Accuracy and Precision

The accuracy of the methods was evaluated with the recovery. The standards of DA, DOPAC and HVA (0.05, 0.13, and 0.25 μg/mL, respectively) were added into the blank mobile phase in order to obtain the recovery, which were calculated by the ratios of the experimentally measured concentrations to those actually added.

For the determination of the precisions, repeated injections of standard solutions were carried out 5 times in the first day and once a day in the 5 consecutive days. The intra-day and inter-day precision was expressed as the relative standard deviations (% RSD.) of the corresponding peak areas. Both the recovery and precision are shown in Table 1.

TABLE 1

Results of Accuracy for DA, DOPAC, HVA, and Intra-day and Inter-day RSD Tests

	Concentration (μmol/L)	Recovery (%, $\bar{x} \pm s$, n=5)	Intra-day RSD (%, n=5)	Inter-day RSD (%, n=5)
DOPAC	0.30	82.00±1.70		
	0.77	104.00±4.00	3.3	4.2
	1.49	98.70±3.10		
DA	0.26	92.30±1.50		
	0.69	105.30±2.30	3.4	2.3
	1.32	108.00±2.00		
HVA	0.27	80.00±7.80		
	0.71	107.69±8.00	2.5	5.6
	1.37	108.66±3.10		

Sensitivity

The limit of detection (c_{LOD}) was calculated as that of the analyte concentration (c_A) resulting in a signal (S) 3-fold higher than the standard deviation of the noise (σ_{noise}), which has been described elsewhere[8]:

$$c_{LOD} = \frac{3\sigma_{noise}}{S} c_A$$

and the σ_{noise} was determined as 0.2 × peak-to-peak noise. The c_{LOD} of DA, DOPAC and HVA was 74, 66, and 69 p*M*, respectively. According to Laszlo, the limit of quantification(c_{LOQ}) could be calculated as c_{LOQ}=2.9 c_{LOD}, and thus the c_{LOQ} for DA, DOPAC, and HVA was 214, 191, and 200 p*M,* respectively.

Quantification of DA, DOPAC, and HVA in Rat Striatal Brain Microdialysates

The method mentioned above was used to quantify DA, DOPAC, and HVA in rat brain microdialysates. Since the method of external standard was used in this experiment, *in vitro* probe recoveries were adopted to quantify them. The *in vivo* concentrations of DA, DOPAC and HVA were computed approximately by the following equation:

$$C_{in\ vivo} = c_{measured} / probe\ recovery_{\ in\ vitro}$$

and their mean extracellular concentrations were 10.7, 2.4, and 9.2 μmol/L (n=6) respectively. Taking into account simultaneous determination of DA, DOPAC, and HVA and the difficulty in the application of *in vivo* probe recovery, this method was feasible and could reflect the true concentration of DA or its two metabolites inside the rat striatal extracellular fluid.

DISCUSSION

The method of HPLC-ECD has been widely used in the determination of bioamines and their metabolites in *in vivo* cerebral microdialysates of rats. Though some improved methods are available, modification and derivation or dual electrodes are needed. The aim of this protocol is to develop a method for simultaneous detection of bioamines and their metabolites by routine and isocratic HPLC-ECD. The results of this study indicate that this highly sensitive analytic technique can determine these materials in rat brain microdialysates. All the tests can be completed within 15 minutes.

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