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Highly sensitive chromatographic assay for dopamine determination during in vivo cerebral microdialysis in the rat

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Abstract

A highly sensitive, yet simple, isocratic high-performance liquid chromatographic (HPLC) assay with electrochemical detection (ED) for the determination of extracellular dopamine (DA) in brain microdialysates is presented. The method makes possible the detection of less than 100 p*M* (less than 1 fmol on column) and the quantitation of 200 p*M* (2 fmol on column) of DA with the use of a narrow-bore rather than capillary or microbore column. Analysis is feasible within an 11-min run-time, and thus is suitable for the relatively short sampling intervals used in microdialysis experiments. In the calibration range of 0.2 to 10 n*M*, the method has excellent linearity and precision, with intra-day relative standard deviations (RSD) of 0.5–2.4% and between-day RSD of 2.1–4.3%.

Keywords: Dopamine

1. Introduction

The development in the 1980s of in vivo cerebral microdialysis as a method for the sampling of extracellular fluid in targeted brain areas has made it possible to study the regulation of certain neurotransmitters in the central nervous system. The introduction of microdialysis was a direct consequence of the availability of very sensitive assays for biogenic amines based on electrochemical detection, and the use of highly efficient reversed-phase chromatographic columns for their separation.

Although techniques such as in vivo voltammetry [1], liquid chromatography (LC) with native fluorescence detection [2], LC with post-column fluorogenic derivatization and chemoluminescence detection [3], and even capillary electrophoresis with laser-induced fluorescence and intensified charge-coupled device detection [4] have been developed to quantify very low concentrations of dopamine, the method of choice remains HPLC with electrochemical detection. In order to achieve high sensitivity in assaying dopamine, both amperometric [5,6] and coulometric [7–9] detectors have been employed.

The need to detect ever decreasing amounts of biogenic amines, in very small dialysate volumes, has led to another development—miniaturization in LC–ED. Thus, microbore columns are often used in combination with amperometric detectors [10–12], while coulometric cells, due to their large internal

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volume, prevent the use of columns with diameters of 2 mm or less. Moreover, the use of smaller working electrodes in amperometric cells can lead to a better signal-to-noise ratio in spite of a lower signal. By combining capillary LC and micro flow-cells, the detection limit for some biogenic amines has reached the attomole level [13].

The method we describe for the detection and quantitation of DA in rat brain microdialysates achieves high sensitivity, high precision, and a relatively short analysis time without the challenges of miniaturization and could be more readily carried out in any research laboratory. Although there are many reports in the methods section of the biomedical literature on high-sensitivity DA analysis, most of them are only summary descriptions and fail to provide validation data.

2. Experimental

2.1. Materials

Dopamine hydrochloride (>99%), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindole-3-acetic acid (5-HIAA), 4-hydroxy-3-methoxyphenylacetic acid (HVA) and EDTA disodium salt dihydrate were purchased from Sigma (St. Louis, MO, USA). *Ortho*-phosphoric acid 85% (HPLC grade), perchloric acid 70% (ACS grade), sodium hydroxide (K^+<0.02%, ACS grade) and 1-octanesulfonic acid (OSA) sodium salt monohydrate were purchased from Fluka (Milwaukee, WI, USA). Citric acid monohydrate (certified ACS) and methanol (Optima grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Artificial cerebrospinal fluid (CSF) was obtained from Harvard Apparatus (Holliston, MA, USA), and helium (ultra high purity) was purchased from Matheson Tri-Gas (Parsippany, NJ, USA). Deionized water of at least 17.8 MΩ cm specific resistance was used to prepare all the solutions.

2.2. Methods

2.2.1. Preparation of standard curves and sample collection

A 10^{-3} *M* stock solution of DA was prepared in deionized water and aliquots were stored at −80 °C for a period not exceeding 3 months. Calibration standards were prepared freshly every time by successive dilution of the stock solution with artificial CSF containing 0.015 *M* $HClO_4$, and were refrigerated at 4 °C until injection. The concentration of the standards ranged from 0.2 to 50 n*M*. The composition of the artificial CSF was the following: Na^+ 150 m*M*, K^+ 3 m*M*, Ca^{2+} 1.4 m*M*, Mg^{2+} 0.8 m*M*, PO_4^{3-} 1.0 m*M* and Cl^- 155 m*M*. Ten μl of the calibration standards were promptly injected and peak-heights were plotted versus the amount of injected DA by linear regression.

Cerebral microdialysis was performed in awake, freely moving rats using CMA/12 microdialysis probes (CMA/Microdialysis, North Chelmsford, MA, USA) with membranes that measured 2 mm in length and 0.5 mm in diameter. The probes were perfused with artificial CSF at a rate of 2 μl/min. Microdialysates were collected at 15 min intervals in vials containing 5 μl of $HClO_4$ 0.1 *M* in order to minimize oxidation. The samples were injected immediately following collection.

2.2.2. Chromatographic conditions

The HPLC system used for the quantitation of DA consisted of a GBC model LC1120 isocratic pump (GBC Scientific Equipment Pty Ltd, Dandenong, Australia), a Rheodyne model 9725i injector with a 20-μl PEEK sample loop (Rheodyne, Rohnert Park, CA, USA), a PEEK pulse dampener, and an INTRO amperometric detector (Antec, Leyden, The Netherlands). The electrochemical flow cell was a confined wall-jet configuration model (VT-03, Antec) with a 3-mm glassy carbon working electrode. The cell was mounted with a 25-μm spacer and a salt bridge Ag/AgCl reference electrode, with the cell potential set to +375 mV. The signal from the current–potential converter (the integrator output) of the detector was filtered with a LINK (Antec) low-pass in-line noise filter, set at a 30-s peak-width, and was integrated by a computerized data acquisition system using WinChrom chromatography software (GBC Scientific Equipment). Chromatographic separation was achieved on a Princeton SPHER C_{18} column, 100 mm×2.1 mm I.D., with a 5-μm particle size and a 100-Å pore size (Princeton Chromatography, Cranbury, NJ, USA). The flow cell, injector, pulse dampener and the analytical column were incorporated in the Faraday-shielded oven compartment of

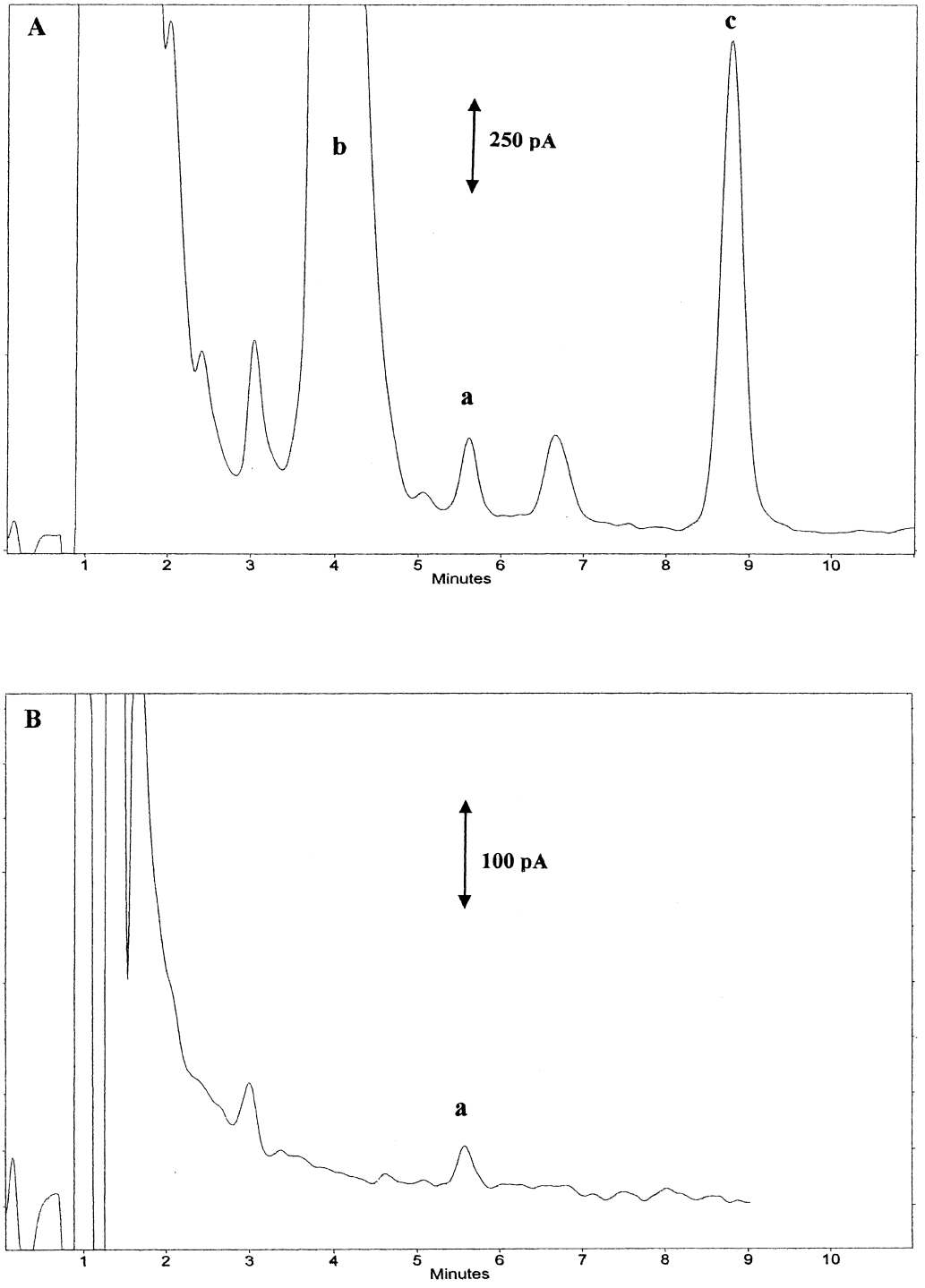

Fig. 1. Elution profile of a microdialysate sample from the rat nucleus accumbens (A) and of a 200 p*M* (2 fmol on column) dopamine calibration standard (B). Peaks: a, dopamine; b, DOPAC; c, 5-HIAA.

Table 1
Validation data for the chromatographic assay

Absolute amount of DA injected (fmol)	2	10	20	40	100
Between-day precision ($n=6$)	4.3	2.2	2.1	2.6	2.3
Within-day precision ($n=6$)	2.4	0.6	0.6	0.5	0.7

The values represent coefficients of variation (C.V.%) for the peak-heights.

the integrator and the temperature was maintained at 30 °C. The mobile phase consisted of an ion-pairing phosphate–citrate buffer (pH 4.5) and 6% methanol. The buffer was made up of H_3PO_4 50 m*M*, citric acid 50 m*M*, EDTA (disodium salt dihydrate) 40 mg/l, OSA (sodium salt monohydrate) 65 mg/l, and the pH was adjusted to 4.5 with NaOH solution. The mobile phase was sparged with helium and was delivered at a flow-rate of 0.27 ml/min. The injected sample volume was 10 μl and the total analysis time was 11 min.

3. Results and discussion

In Fig. 1 the separation of different compounds in a microdialysate sample from the rat nucleus accumbens (A) and the chromatogram of a dopamine calibration standard (B) are shown. Calibration curves were constructed in the range of 0.2 to 10 n*M* (2 to 100 fmol of DA injected) and were consistently linear with correlation coefficients higher than 0.999. A typical regression equation is: $y = 172.36x + 6.88$ ($r^2 = 0.9999$). Moreover, the linearity of the response was tested up to 50 n*M* (500 fmol on column) with the same result. The method proved to have high precision as illustrated in Table 1 by the coefficients of variation for the peak-heights. In order to determine between-day precision, data from six consecutive calibrations were used. These calibration curves exhibited a slope of 172.62±1.71 and an intercept of 14.63±7.63 (mean±S.E.).

The concentration detection limit c_{LOD} was calculated as the analyte concentration (c_A) that results in a signal (S) three times higher than the standard deviation of the noise (σ_{noise}):

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

with σ_{noise} determined as 0.2× peak-to-peak noise. Under the chromatographic conditions described the c_{LOD} for dopamine was 60 p*M*. Expressed as an

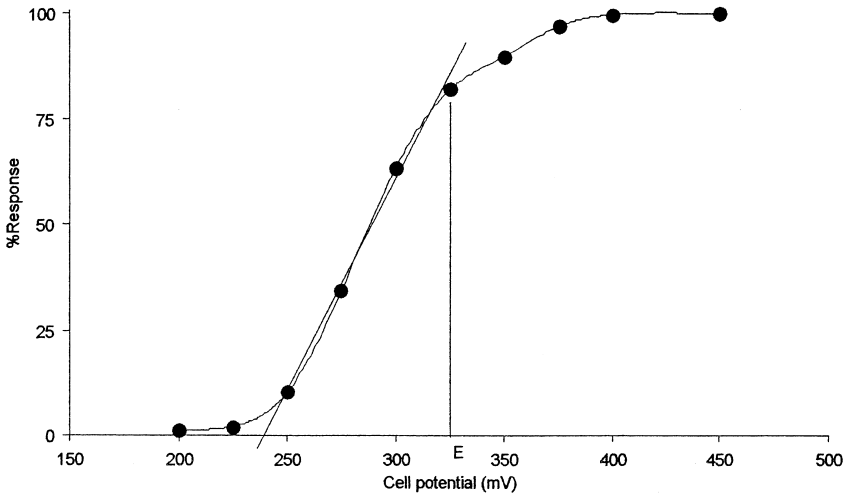

Fig. 2. Hydrodynamic voltammogram of dopamine at the glassy carbon electrode of the electrochemical flow cell. At (E) the electrochemical signal becomes diffusion limited.

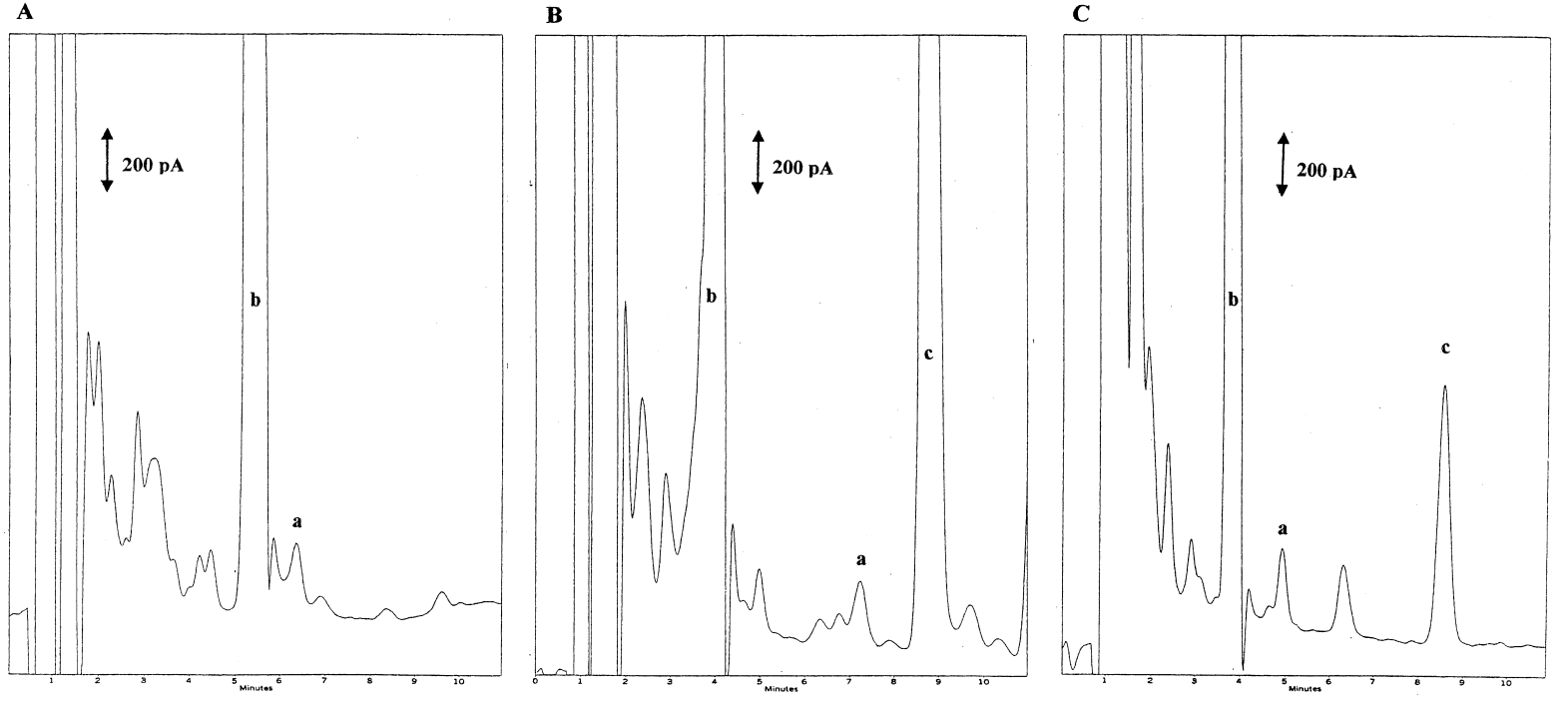

Fig. 3. Chromatograms of rat nucleus accumbens microdialysate samples obtained under different pH values and ion-pairing agent concentrations: (A) pH = 3.1, OSA = 100 mg/l, (B) pH = 4.5, OSA = 100 mg/l, (C) pH = 4.5, OSA = 50 mg/l. Peaks: a, dopamine; b, DOPAC; c, 5-HIAA.

absolute amount, the limit of detection was 0.6 fmol with a 10-μl injection volume. As previously described [14], the limit of quantitation (c_{LOQ}), calculated as $c_{LOQ} = 2.9c_{LOD}$, was 175 p*M*.

In order to achieve high sensitivity and selectivity, and relatively short analysis time, the cell potential, the ion-pairing agent concentration, and the pH of the mobile phase were optimized. Since we were interested in analyzing only the dopamine, a cell potential of 375 mV was employed. The hydrodynamic voltammogram in Fig. 2 demonstrates that the electrochemical signal of DA becomes diffusion limited at 325 mV. However, our choice of a working potential higher than this value was made based on the need for higher sensitivity and less concern for selectivity, since we achieved a good resolution of the DA peak by optimizing the mobile phase composition. Furthermore, by working at 375 mV, late-eluting peaks like HVA were oxidized only to a very small degree and thus the total run time shortened. This allowed the injection of each microdialysate sample as soon as it was collected—a requirement in studies where the initiation of the drug administration is dependent on reaching a stable baseline level of DA. In such studies, monitoring of the dopamine level in real time is necessary, precluding the option to store the samples for later analysis.

The pH values evaluated were between 3 and 6. In this range the retention time of the biogenic amines is only slightly pH-dependent, while their acid metabolites (DOPAC, 5-HIAA, HVA) elute in a significantly pH-dependent manner. By increasing the pH within the specified range, these metabolites will be less retained on the column. On the other hand, the retention times of the amines are particularly dependent upon the ion-pairing agent concentration, such that increasing concentrations of the agent will prolong the retention times of the amines. Concentrations ranging from 50 to 100 mg/l of OSA (sodium salt monohydrate) were tested. The effect of pH and the ion-pairing agent concentration on the relative retention times of dopamine and its acid metabolites is illustrated by the chromatograms in Fig. 3.

By choosing a pH value of 4.5, we ensured the elution of all acid metabolites in a rather short run time, while our choice of ion-pairing agent concentration provided a very good resolution of the DA from DOPAC, 5-HIAA, and other endogenous compounds. Under these chromatographic conditions, serotonin has a retention time of ~15 min and would not interfere with the DA determination in a subsequent run.

This method compares favorably with HPLC–ED methods of high sensitivity found in the literature [7,10,15], while having the advantage of a simple set-up, such as a narrow-bore chromatographic column, manual injector, and a single working electrode electrochemical flow cell. The possibility of further increasing the detection sensitivity could be explored by using a smaller (2 mm) diameter working electrode that might improve the signal-to-noise ratio.

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