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New dual electrochemical detector for microbore liquid chromatography Determination of dopamine and serotonin in rat striatum dialysates

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Abstract

A new type of liquid chromatographic (LC) dual thin-layer amperometric detector for the simultaneous measurement of trace levels of dopamine and serotonin in microdialysates is described. The concentrations of these analytes in rat dialysates are usually in the sub-nanomolar concentration range (typically, 0.10–5.00 pg in 5-μl dialysates). With this dual electrode, a glass-lined microbore column provides excellent sensitivity, selectivity, and separation. In addition, a three- to five-fold improvement in anodic current or cathodic responses over conventional dual electrodes in microbore LC can be achieved. Due to the irreversible electrochemical properties of some interference peaks, this dual electrode provides reliable measurement of dopamine based on the cathodic signal. The detection limit (signal-to-noise ratio=3) of this assay is 0.02 pg per injection for dopamine or serotonin. This new dual electrode allows the simultaneous measurements of basal dopamine and serotonin in rat striatum dialysates without the use of re-uptake inhibitors in perfusion medium.

Keywords: Microdialysis; Dopamine; Serotonin; 5-Hydroxytryptamine

1. Introduction

Microdialysis is a well-established modern technique for studying extracellular neurotransmitters or metabolites in the central nervous system [1–3]. The analysis requires the detection of low-picogram or even sub-picogram amounts of neurotransmitters in small volumes of dialysates [4–6]. Several studies have been carried out to lower detection limits in liquid chromatographic (LC) systems [7,8]. Alternatively, Auerbach et al. [9] collected large volume of dialysate (60 μl) to overcome the inadequate detection limit of their LC systems. However, neuronal release of serotonin (5-HT) and dopamine (DA) in these dialysates is often below the detection limits of conventional LC and at close to those of microbore LC in some circumstances [4,6,10,11]. This could lead to unreliable quantitation of 5-HT or DA in dialysates. In addition, this is particularly difficult for the determination of very low basal 5-HT levels in many studies because of its rapid metabolic inactivation and re-uptake [10]. Many studies included re-uptake inhibitors (such as citalopram or alaproclate) in the perfusion medium to increase

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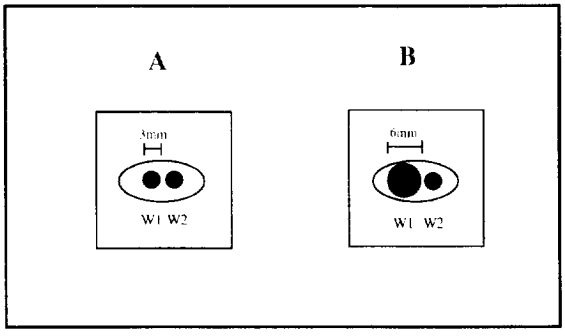

Fig. 1. Schematic of dual amperometric electrode construction of (A) a conventional and (B) the MF-1020 electrode.

5-HT levels in the dialysates to improve quantitative reliability [12,13]. However, Kreiss et al. [10] claimed that including an uptake inhibitor in the perfusion medium may not reveal accurate information about the control of endogenous 5-HT release.

The many advantages of dual-electrode LC–ED were described in our previous reports [14,15]. In the present study, a new dual amperometric cell (the MF-1020, BAS, as shown in Fig. 1) was investigated. Two working electrodes (a 6-mm diameter glassy carbon electrode upstream and a 3-mm electrode downstream) are embedded along one wall of the channel in the flow cell. The larger electrode surface area (four times larger when compared to a conventional 3-mm-diameter electrode) leads to higher current responses and sensitivity. The biomedical applications of this new detector in trace analyses of biogenic amines may shed light in the fields of neuroscience research. The analysis of trace level analytes with respect to analytical linearity, precision, and applications will also be discussed.

2. Experimental

Standard stock solutions of norepinephrine (NE), epinephrine (E), DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA), 3-methoxytyramine (3-MT) and 5-HT were prepared at a concentration of 2 ng/ml in 0.1 *M* perchloric acid and stored at −70°C in the dark and thawed in an ice bath prior to preparation of a standard mixture. Male Sprague-Dawley rats (300–350 g) were anaesthetized with urethane (1200 mg/kg, intrapeutoneally). A microdialysis probe (CMA/12, Carnegie Medicin, Stockholm, Sweden) was stereotaxically implanted into the striatum (AP 1.2 mm, ML 2.6 mm, DV 6.0 mm from bregma) using the rat brain in stereotaxic coordinates [24]. Dialysis probes were perfused with artificial cerebrospinal fluid (aCSF, containing 155 m*M* Na^+, 132 m*M* Cl^-, 1.1 m*M* Ca^{2+}, 2.9 m*M* K^+, 0.8 m*M* Mg^{2+}, pH 7.4 adjusted by CO_2) at 0.6 μl/min using a CMA/100 microinfusion pump. Dialysates were collected every 15 min in 5 μl of 0.1 *M* HCl containing 10^{-7} *M* ascorbic acid to preserve biogenic amines in a CMA/140 fraction collector. Dialysates (5 μl) were directly injected onto a microbore LC system with a dual potentiostat amperometric detector (BAS-4C and the MF-1020 electrode, Bioanalytical Systems, West Lafeyette, IN, USA) for the measurement of DA, 5-HT and their metabolites. Potentials for the anodic and the cathodic dual series working electrode were set at +0.75 V and +0.05 V with respect to a silver/silver chloride reference electrode, respectively [14,15]. Separation of these substances was achieved using a home-made glass-lined microbore column (10 cm× 1.0 mm I.D.) packed with 5-μm Inertsil-2 C_{18} particles (GL Sciences, Tokyo, Japan). The buffer consisted of 9.60 g monochloroacetic acid, 0.16 g sodium 1-octanesulfonate, 10 mg ethylenediaminetetraacetic acid, adjusted to pH 3.0 with 1 *M* sodium hydroxide. The final volume of the mixture was adjusted to 1 l with doubled distilled water. The mobile phase was prepared by mixing 50 ml acetonitrile and 950 ml phosphate buffer. The mixture was filtered through a 0.22-μm Nylon filter under reduced pressure and sparged by helium for 20 min. The flow-rate was 60 μl/min maintaining column pressure at ca. 12.4 MPa. The concentrations of biogenic amines and their metabolites in dialysates were calculated by determining each peak area ratio relative to the standard mixture. The identity of these peaks in the chromatogram was confirmed by their retention times, redox ratios, and a superimposed-alignment technique which was provided by Beckman (System Gold Data Analysis Software, Version 8.10).

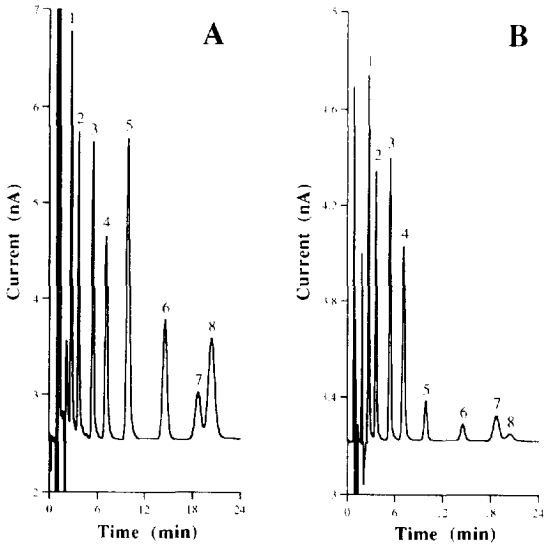

Fig. 2. Typical chromatograms of a standard mixture containing: (1) NE, 107 pg; (2) E, 101 pg; (3) DOPAC, 115 pg; (4) DA, 104 pg; (5) 5-HIAA, 106 pg; (6) HVA, 103 pg; (7) 3-MT, 109 pg and (8) 5-HT, 101 pg. (A) Anodic current; (B) cathodic current; applied potentials (vs. Ag/AgCl): anode (+) 0.75 V, cathode (+) 0.05 V.

3. Results and discussions

Fig. 2A and B show typical chromatograms of a standard mixture containing NE, E, DOPAC, DA, HVA, 5-HIAA, 3-MT and 5-HT. An analysis was completed within 24 min. In general, a glass-lined microbore column gave about 15–25% higher theoretical plates when compared to a stainless steel column with the same dimension and packing material. All analytes under study were well resolved. Calibration curves were obtained with standards (1–500 pg) prior to LC analysis of unknowns. The amounts of injected analyte was linearly related to the anodic or cathodic chromatographic areas obtained from standard mixtures. The correlations (r^2) for NE, E, DOPAC, DA and 3-MT in anodic and cathodic responses were linear ($r^2 \geq 0.999$). In contrast, the separation, detection limits and linear responses for 5-HIAA, HVA and 5-HT on the anode ($r^2 \geq 0.999$, in the range of 1–500 pg) were much better than those on the cathode ($r^2 \geq 0.997$, in the range of 10–500 pg). There were much smaller responses for 5-HIAA, HVA and 5-HT on the cathode than on the anode because of their electrochemical irreversibility. In general, the concentrations of NE, E, DOPAC, 3-MT and DA were determined from the cathodic chromatogram, whereas 5-HIAA, HVA and 5-HT were determined from the anodic chromatogram in the applications of the micro LC–ED system, unless there was co-elution with interfering peaks.

The precision and stability of the assays were tested using standard mixtures of various concentrations and a pooled microdialysate in 0.1 *M* HCl containing 10^{-7} *M* ascorbic acid (Table 1). The intra-assay variability was assessed with 25 replicates at 1-h intervals and expressed as coefficients of variation (C.V., %). In the standard mixture containing ca. 1 pg of each analyte, 5-HIAA and 5-HT exhibited higher C.V.s (6.52 and 5.84%, respectively) than the other analytes (≤4.6%) upon measurements.

Table 1
Anodic, cathodic responses (relative peak area) and redox ratios of biogenic amines and their metabolites at the MF-1020 and the conventional dual amperometric electrodes

	NE	E	DOPAC	DA	5-HIAA	HVA	3-MT	5-HT
MF-1020								
Anode	2280	1610	2350	2520	3050	2310	1860	2710
Cathode	754	500	745	879	156	175	588	154
Redox ratio	0.33	0.31	0.32	0.35	0.05	0.08	0.32	0.06
Conventional								
Anode	943	677	1010	1100	1030	838	700	900
Cathode	607	407	664	743	61	66	354	21
Redox ratio	0.64	0.60	0.66	0.67	0.06	0.08	0.51	0.02

However, 5-HIAA, HVA and 5-HT were not detectable in cathodic chromatograms. In the standard mixture containing ca. 5–50 pg of each analyte, all analytes exhibited satisfactory C.V.s (≤4.20%) for anodic measurements. The C.V. values for 5-HIAA, HVA, and 5-HT were acceptable in cathodic measurements. In general, the C.V. values for pooled dialysates were lower (<3%, except for that of 5-HIAA) than those of standard mixtures at about the same concentration ranges. This better precision in terms of C.V. values of dialysates may be due to the addition of 10^{-7} *M* ascorbic acid in the perfusate and some endogenous antioxidants in the dialysates. The inter-assay variabilities assessed with a standard mixture containing ca. 50 pg of each analyte during six consecutive working days were less than 4.52 and 10.8% in the anodic and cathodic measurements of all analytes. These C.V. values were much better than those obtained in our previous studies, especially at low picogram levels [14–17]. The detection limits (signal-to-noise ratio=3) of each analyte in the present assay was between 0.02–0.10 pg per injection.

Because the amperometric response is proportional to the electrode surface area, theoretically, the larger surface area of the MF-1020 electrode should provide 4-times the sensitivity when compared to the conventional electrode. However, the large electrode surface also slightly increases background noise and residual currents. Table 2 shows the cathodic and anodic responses and redox ratios of the MF-1020 and the conventional electrodes. In comparison with the conventional glassy carbon electrode, the MF-1020 electrode enhanced detection sensitivity 3–5-fold. In addition, this enhanced sensitivity in the upstream electrode also slightly increased the consecutive downstream responses. Recently, the volume of the amperometric flow-cell reduced to below 100 nl without difficulty using a thin spacer (16 μm). Hence, as a result of increased conversion efficiency in the present assay, the MF-1020 electrode and microbore LC provided signal enhancement beyond the chromatographic increase in peak concentration. Therefore, the overall detection limit of this system was lowered to the subfmol or even amol amounts for DA and 5-HT.

Fig. 3A and B show typical chromatograms of the microdialysate from a rat striatum. The retention

Table 2
Analytical precision of various standard mixtures and pooled dialysates on the intra (n=25) and inter-assay (n=6, in six consective working days) of the microbore LC–ED system using the MF-1020 electrode

	NE	E	DOPAC	DA	5-HIAA	HVA	3-MT	5-HT
Intra-assay								
ca. 1 pg								
Anode	3.35	4.11	2.41	1.84	6.52	2.83	3.45	5.84
Cathode	4.08	3.86	3.25	4.59	N.D.	N.D.	N.D.	N.D.
ca. 5 pg								
Anode	3.66	2.98	2.85	1.31	2.69	2.02	3.01	4.20
Cathode	3.23	3.07	2.46	2.71	7.71	6.60	2.63	N.D.
ca. 10 pg								
Anode	1.96	2.23	1.43	1.17	2.89	2.30	1.45	3.84
Cathode	1.80	1.92	1.40	2.36	5.86	4.28	2.44	11.9
ca. 50 pg								
Anode	1.01	0.85	0.66	0.78	1.55	2.59	0.79	1.03
Cathode	0.84	0.93	0.74	0.80	5.71	3.35	2.59	5.44
Pooled dialysates								
Anode	N.D.	N.D.	1.27	2.31	2.46	2.12	3.21	2.04
Cathode	2.58	N.D.	1.19	1.38	6.74	3.46	2.18	N.D.
Inter-assay								
ca. 50 pg								
Anode	2.01	2.56	3.27	2.10	2.03	2.41	1.76	4.52
Cathode	1.89	2.88	3.45	3.65	6.86	3.98	2.83	10.8

N.D., not detectable.

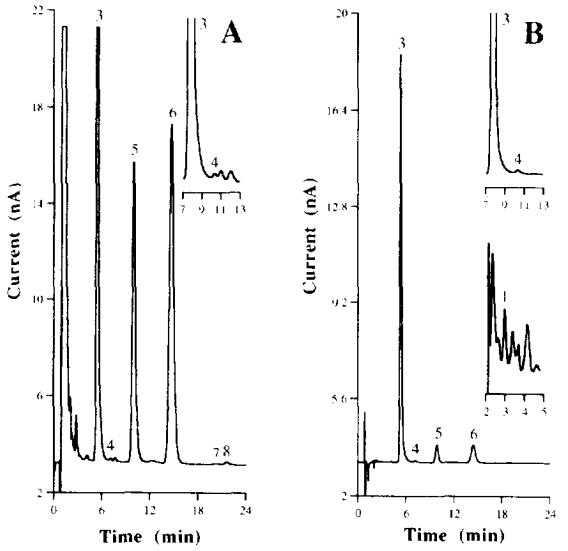

Fig. 3. Typical chromatograms of a striatal dialysate containing (1) NE, (3) DOPAC, (4) DA, (5) 5-HIAA, (6) HVA, (7) 3-MT and (8) 5-HT. (A) Anodic current; (B) cathodic current, applied potentials (vs. Ag/AgCl): anode (+) 0.75 V, cathode (+) 0.05 V.

time of each peak corresponding to NE, DOPAC, DA, HVA, 5-HIAA, 3-MT, and 5-HT in Fig. 3A and B was identical to that in Fig. 2A and B, respectively. In anaesthetized rats, 4 h after implantation of probe, basal concentrations (mean of four rats) in 5-μl dialysate obtained from rat striatum were 0.41 ng/ml for NE, 281.9 ng/ml for DOPAC, 1.09 ng/ml for DA and 1.10 ng/ml for 3-MT, 253.6 ng/ml for HVA, 0.58 ng/ml for 5-HT and 177.3 ng/ml for 5-HIAA. In this study, the DA and 5-HT concentrations increased 20- and 15-fold, respectively when HAL (0.05 mg/ml for 30 min) was perfused. These data were in agreement with those of other investigators [18,19]. Apparently, microbore LC with the MF-1020 electrode provides high sensitivity and low detection limit to enable the detection of basal DA and 5-HT. There were some interfering peaks (at ca. 11 and 12 min) close to the DA peak in the anodic chromatogram as shown in Fig. 3A. In our preliminary study, the peak shape of DA was asymmetric in our routine microdialysis experiment using a phosphate buffer mobile phase [14,15]. In addition, the redox ratios of DA ranging 0.09–0.14 were far below the authentic standard DA (redox ratio of ca. 0.35). After a fine tuning of mobile phase, three peaks, at least, were separated in the anodic chromatogram (Fig. 3A). Fortunately, these interfering peaks were dramatically diminished in the cathodic chromatogram as shown in Fig. 3B. A reliable assignment and measurement of the DA peak was thus achieved from the cathodic chromatogram. The redox ratio of DA was 0.32 in the rat striatum dialysate, which is very close to the redox value (0.35) of authentic standard. Although the responses were smaller, the cathodic chromatogram provides more reliable quantitation of DA content because of less interference. In general, clear DA peaks were obtained in the cathodic chromatograms in our routine microdialysis experiments. This might be due to the irreversibility of the contaminant(s). Hence, measurements of DA could be more reliably made from the cathodic chromatograms than from the anodic chromatograms which might contain unresolved peaks under varied chromatographic conditions. Occasionally, each peak was also verified by spiking with authentic standards to see if the addition increased the peak height without changing the retention time and peak shape. In addition, a superimposed-alignment technique is also used if chromatographic peaks were slightly different in elution times between runs or coeluted with other unknown interferences. These chromatograms were adjusted to align according to the differences between two selected peaks by the Beckman Gold Software system. Sometimes superimposition of these chromatograms is valuable in confirming these peaks. The measurement of biogenic amines in such small volumes and low detection limits has great analytical potential in microdialysis experiments.

4. Conclusion

It has become routine to isolate a few μl of dialysate from a living animal using in vivo microdialysis sampling technique [20–23]. Sample preconcentration is not practical with small volumes of dialysate. In addition, it is often necessary to determine amounts of individual compounds at or below the picogram range. Indeed, the vast majority of publications from laboratories active in microdialysis concern the analysis of DA and 5-HT by microbore LC–ED. Recently, microbore LC–ED has

become the method of choice for the determination of trace amounts of biogenic amines and their metabolites in dialysates. In the early studies, problems with the specificity and sensitivity of the analytical procedures might be responsible for the lack of agreement in the results of different groups.

Acknowledgments

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