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Electrochemical detection of catecholamines at sub-5 fg levels by redox cycling

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Abstract

Two simple modifications to a commercially available thin layer electrochemical detector cell permitted the attainment of ultra-low detection levels of two neurotransmitter catecholamines. An ESA model 5041 analytical cell was modified with a glassy carbon embedded ceramic composite electrode to allow the use of a thin 12 μm gasket. Also a capillary HPLC column was connected directly to the detector cell using a 30 μm i.d. fused silica capillary. These modifications permitted the extensive redox cycling of the electrochemically reversible catecholamines. The ensuing amplified analytical signal allowed the detector cell to achieve efficiencies of 1300%. This resulted in a mass limit of detection of 4 fg and a concentration limit of detection of 116 pM for dopamine with an S/N of 3.

Keywords: Electrochemical detection; Catecholamines; Neurotransmitters; Redox cycling; Low injection volumes

1. Introduction

Over the last 20 years electrochemical detectors (ECD) and high performance liquid chromatographic (HPLC) instrumentation have markedly improved. As a result, the limits of detection (LOD) achieved using HPLC-ECD have also greatly improved. The entire chromatographic system including the pump, injector, fittings, column, and detector must be considered when attempting to achieve the lowest LOD possible. Low dispersion injectors, proper fittings, efficient columns, and low volume ECDs all contribute to the performance of the whole chromatographic system. This work focuses primarily on detector cell design.

Because of low injection volumes, the best mass LOD is achieved using micro-bore and capillary HPLC. However, the low injection volumes used with these systems very often result in poor concentration LODs. When using small sample volumes the internal cell volume of the detector needs to be small to reduce band broadening. For very small injection volumes the flow-by or wall-jet amperometric cell designs are best suited because of the practicalities of manufacturing. For larger bore columns (≥ 2 mm i.d.) much of the band broadening observed is a result of the column. In this case, coulometric cells (ESA,

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Chelmsford, MA) offer excellent concentration LODs [1]. Using relatively large injection volumes (i.e. 20–40 μl) coulometric detectors can routinely achieve concentration LODs as low as 100 pM [2].

Small injection volumes are desired in applications where the volume of the sample is limited (e.g. neurochemical microdialysis experiments) or where samples are to be split between analytical systems. Under these circumstances the LOD is limited not only by the internal cell volume and design of the detector but also by the efficiency of the column. Smaller diameter columns (< 1 mm i.d.) have been difficult to pack reproducibly and can suffer from low column efficiencies. This creates band broadening and dilution of the analyte which results in poor LODs. In general, the efficiency of a column starts to decrease at around 1 mm i.d. [3].

One way to improve the system performance is to use a highly efficient, low-volume detector designed in such a way to promote redox cycling. Redox cycling detection is a means whereby the detector signal can be amplified—sometimes as much as an order of magnitude higher—as compared to that of normal detectors. First, this will only work with analytes that are electrochemically reversible under the conditions of the analysis. Second, the cell must be designed so that one electrode (the working electrode) oxidizes the analyte while a second electrode (e.g. a second working electrode or the counter electrode) reduces the analyte back to its original redox state. The amplification of the analytical signal depends on how many cycles between the working electrode and second electrode the analyte molecule undergoes. The measured analytical signal is the sum of the current generated from all the times each analyte molecule is oxidized and re-oxidized at the working electrode.

Research on redox cycling has previously been reported. For example Weber [4] used a cell called a parallel-opposed dual electrode cell (PODEC) [5]. A recent publication shows a flow-through recycling electrochemical cell made by microfabrication [6]. In the past, such detectors have suffered from high noise levels, significant band-broadening, a limited number of redox cycles and a failure to maintain uniform current and potential distributions in the cell [7]. Consequently, these cell designs with their HPLC systems resulted in only moderate gains in improving LODs of the studied analytes.

In the present study, a unique cell design coupled with capillary HPLC is used to achieve a low LOD using a cell design conceptually similar to the one reported by Weber [4]. A simple modification to a commercially available ECD cell resulted in considerable redox recycling. This design allows the analysis of very low volumes (< 1 μl) needed when using capillary HPLC columns.

The cell coupled with the low mobile phase flow rates (< 10 μl min^{-1}) used with capillary HPLC columns allows further recycling of the analyte between the working and counter electrodes. Using such a low flow rate as compared to the higher flow rates used in larger bore HPLC columns effectively permits the analyte to remain longer between the working and counter electrodes thus further enhancing the recycling effect. This subsequent amplification of the analytical signal provides an excellent method for achieving low concentration LODs for injection volumes < 1 μl.

2. Experimental

The HPLC system consisted of a model 580 pump, a model 5200 A single potentiostat ECD (1 nA V^{-1} current range, 5 s filter), a model 5041 analytical cell (of the thin layer amperometric type), a pulse damper, a D450 data station, and a degasser (all from ESA). A Rheodyne model 7520 injector (Rheodyne, Cotati, CA) with a 200 nl internal loop was used. Mobile phase was degassed on-line before being passed through the HPLC column (180 μm i.d. × 145 cm, Hypersil, C18 ODS 3 μm, LC Packings, San Francisco, CA). The analytical column was connected directly to the injector without the use of any connecting tubing. The output from the pump was split so that flow passed through both the analytical column and a second larger bore column (4.6 mm i.d. × 15 cm, Hypersil, C18 ODS 3 μm) placed in parallel. The effective split ratio was 1:650, which resulted in a flow rate of 2 μl

min^{-1} through the analytical column [8]. The flow rate was calibrated periodically by monitoring the void volume of the chromatogram. PEEK tubing was used pre-injector and post-detector cell in the system.

The standard model 5041 analytical cell (ESA) was modified by a commercial upgrade kit (model 5041 enhancement kit, ESA) which contains a glassy carbon electrode (electrode diameter of 0.32 cm) embedded in a composite ceramic material. The upgraded version of the model 5041 analytical cell is a three electrode ECD that has a working electrode placed parallel to the counter electrode. The working electrode assembly is made of glassy carbon embedded in a composite ceramic material. The ceramic material used in making the working electrode assembly is hard and does not deform under the pressure needed to seal it properly in the cell. Directly opposed to the working electrode is the counter electrode, which is a plate of stainless steel. Both the working and counter electrodes are highly polished and optically flat. Consequently, the ceramic working electrode and the stainless-steel counter electrode allow the use of spacer gaskets (Mylar, ESA) as thin as 12 μm. The gasket controls the distance between the working electrode and the counter electrode. A shorter distance between the working and counter electrode increases the recycling efficiency of the redox cycling process. The working electrode was seated in the cell using a 12 μm gasket with a torque force of 1.7 N-m (15 in-lb). The cell was further modified by connecting the output of the column directly to the inlet of the detector cell's flow channel using fused silica capillary tubing (30 μm i.d. × 25 cm, LC Packings) to further minimize dead volume.

All reagents (Aldrich, Milwaukee, WI) were analytical reagent grade. Water was purified using a Milli-Q (Millipore, Bedford, MA) system and exhibited a resistivity of 10^{18} MΩ-cm upon delivery. The water was further purified by passing it through a Sep-Pak C18 cartridge (Waters, Milford, MA). The mobile phase was: acetonitrile-sodium phosphate (25 mM)-triethylamine (0.72 μM)-ethylenediaminetetraacetic acid, disodium salt dihydrate (25 μM) (10:90 v/v) (pH 3.00). Norepinephrine hydrochloride, epinephrine free base and dopamine hydrochloride were dissolved in 12 mM hydrochloric acid to form a 1 mg ml^{-1} stock solution which was then stored at 4°C. The catecholamine stock solution was diluted in mobile phase as needed and used immediately.

Efficiencies were calculated in the following manner: A dopamine concentration of 10^{-6} g ml^{-1} of dopamine was injected. For a 200 nl injection this represented an injection of 200 pg 'on-column'. The output response of the chromatogram was current versus time. The dopamine peak was integrated which gave the charge or number of coloumbs consumed. This charge was compared to the theoretical charge based on 100% efficiency and 200 pg electrolyzed using the equation, $Q = nFN$ (where Q is charge, n is the number of electrons in the redox couple ($n = 2$ for the catecholamines used), F is Faraday's constant and N is the number of moles electrolyzed). The actual charge was divided by the theoretical charge to give the effective coulometric efficiencies.

3. Results and discussion

Fig. 1(A) shows a schematic of the 5041 analytical cell showing the placement of the working electrode parallel to the stainless steel counter electrode. A thin polymer gasket separates the working electrode from the counter electrode. The working electrode consists of a glassy carbon disk that is embedded and sealed tightly in a ceramic support. The counter electrode is made of 316 stainless steel. Both the stainless steel and ceramic material allow for a flat and parallel orientation which is critical for using thin gaskets. Gaskets as thin as 12 μm can be used with this cell. When using the 12 μm gasket the cell volume of the model 5041 analytical cell is 194 nl (not including the fused silica inlet tubing). Most commercially available cells use working electrodes fabricated with polymeric materials such as Teflon, Kel-F or PEEK. The use of polymeric materials in the electrode assembly greatly restricts how thin the gasket can be. Polymers readily distort or cold flow under pressure. This then limits how much force can be applied to the electrode assembly to seal the cell. If the gasket is too thin, then the

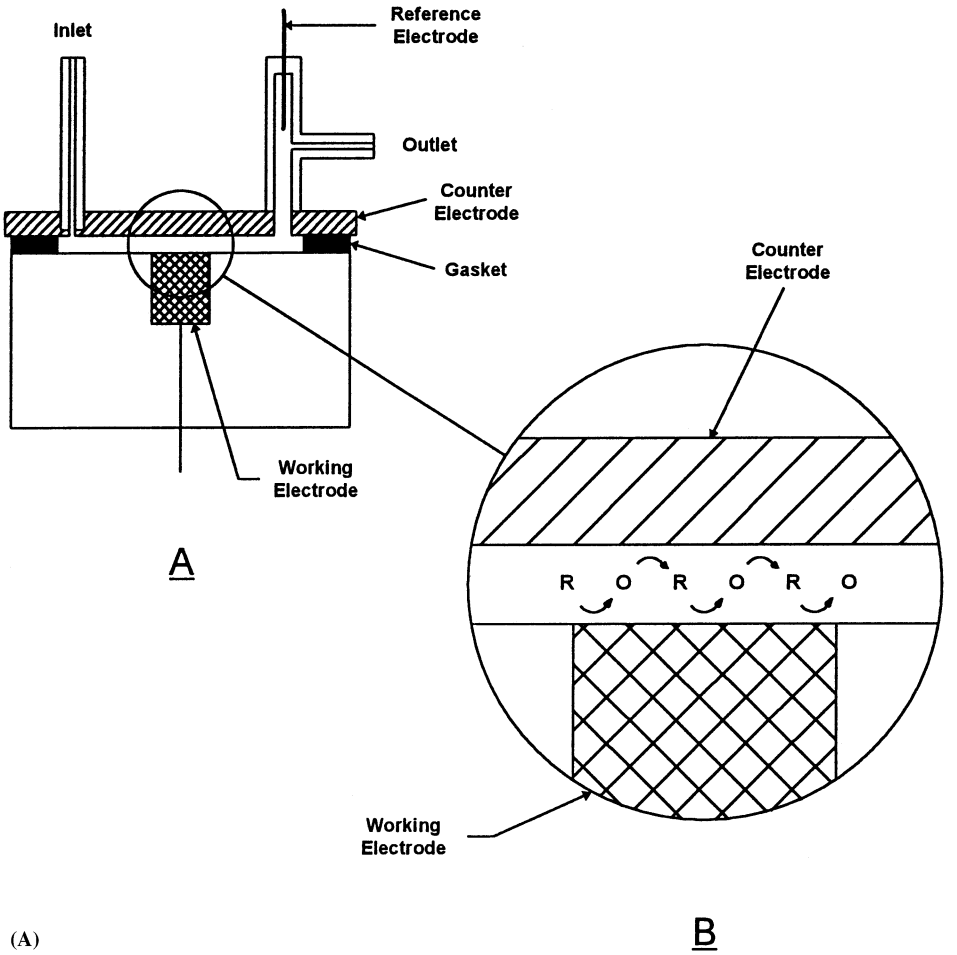

Fig. 1. (A) Schematic cut-away of ESA model 5041 analytical cell. (B) Working electrode detail showing redox cycling. As analyte R passes through the cell it is oxidized at the working electrode. The oxidized form of the analyte, O, can diffuse to the counter electrode and be reduced back to R, which can then be detected again at the working electrode.

working electrode can physically contact the other side of the flow chamber. This can cause an electrical short if the counter electrode is opposed to the working electrode or cessation of the mobile phase flow if the working electrode is opposed by an insulating medium.

Not much optimization was required. The system was set up with a flow splitter as described. For the column we used, typical flow rates were in the order of 1 μl min^{-1}. The flow was calibrated as described above until flow rates in the order of 1 μl min^{-1} were obtained. The best efficiencies were obtained with the thinnest gasket size (12 μm). Gaskets of this size are difficult to work with and it is very important that all dust and lint are removed from the cell. Some variability in the efficiencies were obtained as described above.

The reference electrode—made of palladium metal—is a maintenance free solid-state electrode which uses the α hydrogen–palladium couple. The reference electrode was isolated from the flow stream to eliminate potential artifacts that can result from high salt concentrations, such as in artificial cerebrospinal fluid, contacting the palladium reference electrode.

Fig. 1(B) shows the flow of an analyte R past a working electrode where the redox product O is generated. A thin-layer cell under normal operating conditions is very inefficient [9]. The flow across the electrode is so fast and the diffusion layer is so thin that only a few of the analyte R molecules will be oxidized. A number of factors influence the efficiency of the reaction of reactant R going to product O including the working electrode size (area), volume of the cell and velocity of flow through the cell. These variables can be adjusted with various consequences. For example, see Table 1.

As the analyte travels through the flow channel it is oxidized at the working electrode, re-reduced at the counter, re-oxidized at the working electrode, etc. Each cycle at the working electrode adds to the signal. This is shown schematically in Fig. 1(B). The number of cycles—or amplification of the signal—depends on the flow rate, the electrode size and width of the channel as well as the distance between the counter and working electrode.

Much of the noise in the background signal is due to instrumental fluctuations in the potential that then become coupled through the capacitance of the working electrode [10]. This is called 'non-faradaic' noise because the magnitude is a function of the capacitance (and hence the area) of the working electrode and no electrons actually cross the solution electrode interface [11]. Because the non-faradaic noise is not amplified, the signal to noise ratio is significantly enhanced in the supra-coulometric cell.

The compounds norepinephrine and dopamine were chosen for two reasons. First, they are representative of typical catecholamine neurotransmitters studied in microdialysis experiments where small sample volume is routine. And second, these three compounds are all electrochemically reversible in that they can be recycled between two electrodes yielding an amplified signal.

Fig. 2 shows a chromatogram for norepinephrine, epinephrine, and dopamine. From this chromatogram the limit of detection for norepinephrine and dopamine was determined to be 4 and 5 fg ($S/N = 3$), respectively.

In Fig. 2, note that epinephrine was not resolved from a large injection artifact. The mobile phase can be optimized for the resolution of the epinephrine, but this was not attempted in the present study. In dialysate samples this unresolved peak separation would not be an issue because epinephrine is not typically present in brain extra cellular space. As the sensitivity of the detector improves, system artifacts, which

Table 1
Detector cell changes and resulting outcome on performance

Adjustment made	Consequence
Increase working electrode area	Efficiency improved due to the greater probability that R will encounter the working electrode but the increase in noise will limit the overall sensitivity (signal/noise ratio). The volume of the cell will also increase leading to peak broadening.
Decrease velocity of analyte through cell	Efficiency improved due to the greater probability that R will encounter the working electrode but the chromatography will suffer. Peaks will be broader and overall analysis time will be prolonged. When one goes from a larger diameter column to a smaller diameter column the flow rate of the liquid decreases. This in turn decreases the velocity of the solution through the electrochemical detector in this instance. A decrease in velocity will serve to increase the efficiency of the detector while still maintaining the chromatography integrity.
Decrease cell volume	This will increase the probability that R will encounter the working electrode (less diffusion distance). However, for a given flow rate, a decrease in cell volume will lead to an increased velocity through the cell. The residency time of R will decrease and the electrolytic efficiency will decrease.

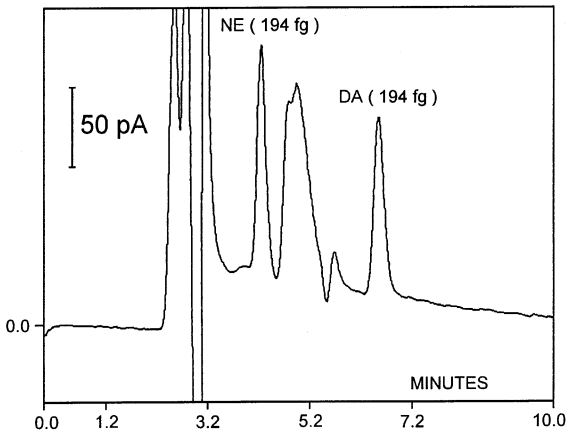

Fig. 2. Chromatogram showing 194 fg of norepinephrine, epinephrine, and dopamine on-column. Injection volume is 200 nl. The limit of detection is 4.1 fg for norepinephrine at an S/N of 3.

when less sensitive would not be observable, can now become problematic. Many of these artifact peaks are a result of the rotor seal material of the injector. A number of injectors were tried and the Rheodyne model 7520 injector consistently gave the best results; however, even with the 7520 injector artifacts remained. It should be noted that as the sensitivity of detectors are improved more demands are put on the performance of the other components.

Using a 2 μl min^{-1} flow rate and a 12 μm gasket, an electrochemical efficiency of 1300% was determined. The efficiencies calculated varied by as much as 20% from day to day. There are several reasons for this: capillary LC uses very small diameter columns and tubing. The way the flow rate was regulated using the flow splitter makes the flow-rate very sensitive to changes in temperature because the flow rate depends on the relative back-pressures on each branch of the flow splitter. The flow rate can therefore vary depending on the room temperature. The efficiencies vary with the flow rate accordingly. The higher the flow rate the lower the efficiencies. An experiment looking at response versus concentration showed the response to be linear over five orders of magnitude from 100 μM to 300 pM. In a log–log plot over five orders of magnitude the correlation coefficient was calculated to be 0.9999.

Fig. 3 shows a section of a chromatogram representing 18 fg of dopamine injected (0.2 μl of 464 pM dopamine solution). The calculated LOD at a S/N of 3 was determined to be 4 fg for the mass detection. This corresponds to 23 attomoles or 116 pM for the concentration limit of detection.

As mentioned in the introduction there is a recent publication describing a flow-through recycling electrochemical cell made by microfabrication [6]. In this work two parallel-opposed electrodes are separated by a 5–10 μm gap. Using flow rates of 50 nl min^{-1}, the authors obtained signal enhancements of over 60-fold due to redox cycling. Theoretical predictions estimated that one should be able to obtained enhancements of over 100-fold. In the work discussed in this paper we used a 12 μm gap and flow rates in the order of 1–2 μl min^{-1}. Therefore, it should be possible with better design and using lower flow rates to get even better signal enhancements than described in this paper.

4. Conclusion

Previous attempts to use redox cycling to enhance sensitivity have met with the problem of a limited number of redox cycles. By using a unique, commercially available analytical cell, this problem has been significantly reduced. The use of a fused silica capillary placed at the inlet of a commercially available cell has generated a detector ideally suited for capillary chromatography. A limit of detection of < 5 fg (0.2 μl of a 464 pM

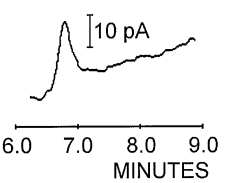

Fig. 3. Chromatogram of 17.6 fg of dopamine on-column. The limit of detection is 3.7 fg ($S/N = 3$). This corresponds to 23 attomoles or 116 pM limits of detection for a 200 nl volume which was injected on column.

solution) was obtained for dopamine with a linear dynamic range of over five orders of magnitude. The parallel placement of the working electrode and the counter electrode allows redox cycling of the substrate to occur so that the substrate's signal can be enhanced significantly beyond 100% efficiency. In this work we obtained efficiencies or signal enhancements up to 1300%.

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