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MICROCYLINDER ELECTRODES AS SENSITIVE DETECTORS FOR HIGH-EFFICIENCY, HIGH-SPEED LIQUID CHROMATOGRAPHY

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SUMMARY

A carbon fiber microcylinder electrode ($r = 3.5$ μm) is used as a detector for high-efficiency and high-speed liquid chromatography. The microcylinder has a detection volume of a few picoliters, and can be placed directly at the outlet frit of the column. With proper positioning equipment, the electrode can be placed at the region of the outlet frit where the separation efficiency is highest. Such selective sampling results in greatly increased measured efficiency over a conventional electrochemical detector when short (4 cm) columns of conventional diameter (3.2 mm) are used. The microcylinder detector is sensitive and subpicomole detection limits are obtained in less than 30 s for norepinephrine. The need for expensive positioning equipment is eliminated by mounting the electrode into a fitting which can be mated directly with the column.

INTRODUCTION

In situations such as microdialysis experiments where large numbers of chemically labile samples are collected in a short time, very rapid separation and analysis of these samples is desirable. In order to achieve these high-speed separations, high-efficiency columns with small volumes are required[1–5]. In addition to the decreased analysis time, columns with small volumes result in reduced mobile phase consumption and increased sensitivity because the solutes are not diluted to the extent they would be in a conventional column. However, in order to realize the potential of high-speed separations, detectors with very low detection volumes and very rapid response times are required. In this paper we report the use of 4 cm × 3.2 mm I.D. analytical cartridge columns to achieve rapid separations while meeting the detector requirements with carbon-fiber electrodes.

Microelectrodes have several advantages that make them ideal detectors for such high-speed separations. As a consequence of their small dimensions (μm),

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detection volumes are very small. The diffusion layer (δ) for a microcylinder electrode under quasi-steady-state conditions is given by

$$\delta = r_0 \ln [2(D_m t)^{\frac{1}{2}}/r_0]$$

where r_0 is the electrode radius (3.5 μm in this experiment), D_m is the diffusion coefficient for the analyte (*ca.* $5 \cdot 10^{-6}$ cm^2 s^{-1}) and t is the time of the experiment[6]. For a 5-s peak, the dimension of the solution sampled is *ca.* 15 μm, thus, for a 50-μm long cylindrical electrode, the detection volume is 35 pl. Additionally, the microelectrode can be directly mated with the column outlet, eliminating any extra-column band broadening from connecting tubing. This advantage has been exploited by Jorgensen and others in capillary liquid chromatography[7–13]. If the microelectrode is much smaller than the outlet of the column, the region of highest efficiency can be selectively sampled, increasing the measured efficiency[14]. Noise for electrochemical detectors is related to the electrode capacitance which is directly proportional to the area[15–17]. Therefore, even though small electrodes result in small currents, less noise is expected as well. The low capacitance also leads to a rapid detector response time. Finally, microelectrodes are less sensitive to flow variations than conventional electrochemical detectors[18].

In this paper we demonstrate that single carbon-fiber electrodes are useful for the detection of catecholamines following rapid separation. Liquid chromatography with electrochemical detection has become the major method for the determination of catecholamines and related compounds[19–23]. The combination of the spatial selectivity and high sensitivity of the carbon-fiber microcylinder with high-speed chromatography increases the usefulness of this technique. Here we report the determination of trace amounts of catecholamines in under 1 min.

EXPERIMENTAL

Chromatographic system

A syringe pump (Model μLC-500, Isco, Lincoln, NE, U.S.A.) was used to deliver the mobile phase to the system through a pneumatically actuated loop injector with a 10-μl internal loop (Model 3XL, Scientific Systems, State College, PA, U.S.A.). An old 4-cm column placed before the loop injector was used as an in-line filter. Analytical cartridge columns with 3-μm RP-18 packing were obtained from Brownlee Labs. (Santa Clara, CA, U.S.A.). The detector was either a carbon-fiber microcylinder electrode placed directly at the exit frit of the column with a micropositioner[14], or a microcylinder electrode permanently mounted into a plastic fitting (see below). For comparison, a commercially available thin-layer amperometric detector was employed with a 5-ml spacer (Model TL8A, Bioanalytical Systems, West Lafayette, IN, U.S.A.) and connected to the column with 4 cm of 0.01-in. I.D. tubing.

An IBM personal computer with a Labmaster board (Scientific Solutions, Solon, OH, U.S.A.) was used to collect data and to open and close the loop injector. In order to inject a symmetrical bolus of solute, the loop injector was returned to the "load" position before the entire sample passed through the loop. The injection time was adjusted so that 7 μl were injected regardless of flow-rate. Chromatographic

figures of merit were evaluated with statistical moments using the exponentially modified Gaussian model[24].

The potentiostat used was of local design and construction. In order to measure subpicoampere currents required for low concentrations with the microcylinder electrode an AD 310J varactor bridge operational amplifier (Analog Devices, Norwood, MA, U.S.A.) was used in the current-to-voltage convertor. The feedback resistor was a 1 GΩ, 1% precision resistor, and the potentiostat time constant was 180 ms. For curent measurements with the thin-layer detector, a potentiostat with a 1 MΩ feedback resistor and 35 ms time constant was used. Detection limits were defined as signal-to-noise (S/N) = 3. Measurements were taken at 0.7 or 0.8 V *vs.* the saturated sodium chloride electrode (SSCE).

Electrodes

Microelectrodes were prepared by aspirating carbon fibers into glass capillaries and pulling the glass around the fibers with a capillary puller (Narisige, Tokyo, Japan). The fibers were then sealed with epoxy (Epon 828 with metaphenylenediamine as crosslinking agent, Miller-Stephenson, Canbury, CT, U.S.A.)[6]. Electrodes were either *ca.* 50 or 100 μm in length with a nominal radius of 3.5 or 5 μm, respectively. The electrodes were electrochemically pre-treated daily by applying a potential of 1.8 V *vs.* SSCE for 30 s.

Permanently mounted microcylinder

The center of a Mini-tight fitting (Upchurch Scientific, Oak Harbor, WA, U.S.A.) was enlarged to accommodate a microcylinder electrode (1.2 mm O.D.) with a drill bit large enough to allow passage of eluent around the electrode walls (Fig. 1). A hole perpendicular to the fitting axis was drilled from the fitting grip to the center hole into which was inserted a 1/16-in. tubing to serve as the drain to waste. Perpendicular to this, another hole was drilled into which the platinum wire auxiliary electrode was placed. The central hole was expanded from the top of the fitting to the perpendicular exit hole so that tubing with an I.D. equal to the electrode O.D. could be

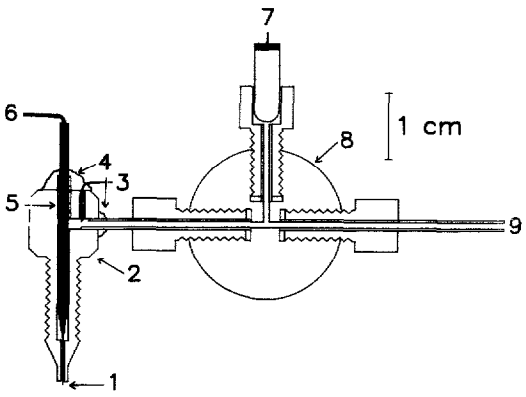

Fig. 1. Permanently mounted microcylinder detector: 1 = microcylinder carbon-fiber working electrode; 2 = minitight end fitting; 3 = platinum wire auxiliary electrode; 4 = clear silicone sealant; 5 = guide/seal tube; 6 = hook-up wire for working electrode; 7 = SSCE reference electrode; 8 = tee; 9 = tubing to waste.

placed. This tubing served both as a guide for inserting the electrode into the fitting and as a seal, forcing flow out of the exit hole. Electrodes and tubing were held in place and sealed with clear silicone sealant (General Electric, Waterford, NY, U.S.A.). The reference electrode was placed in a tee through which the eluent flowed.

Reagents

All chemicals were used as received from commercial sources. Except where noted, the mobile phase consisted of 0.1 m*M* disodium EDTA, 1.0 m*M* octyl sodium sulphate, and 0.2 *M* monobasic sodium phosphate. All solutions were prepared in doubly distilled, deionized water and filtered with 0.45-μm paper (Gelman Sciences, Ann Arbor, MI, U.S.A.). Samples were stored in 0.1 *M* perchloric acid and dilutions were carried out so that the final perchloric acid concentration was 0.001 *M*. Test compounds included norepinephrine (NE), epinephrine (E), and 3,4-dihydroxybenzylamine (DHBA). Bovine adrenal medullary chromaffin cells were used as a test system for biological applications of this system. The cells were prepared and cultured using published procedures[25].

RESULTS AND DISCUSSION

Column characterization

The calculated and the experimentally determined radial concentration distributions of the 4-cm column are shown in Fig. 2. As before[14] a large deviation of the experimental from the theoretical concentration distribution is observed, indicative of an uneven distribution of solutes onto the column. However, there is less evidence of channeling at this column than at the 10-cm column studied previously.

Similar to the findings reported for the 10-cm column, the efficiency for the 4-cm column is greatest at the column center (h = 1.9) and decreases substantially as the wall is approached (h = 4.2 at 1.0 mm from the center). Also, the retention time is 5% greater near the column wall than at the column center. Most significantly, the regions

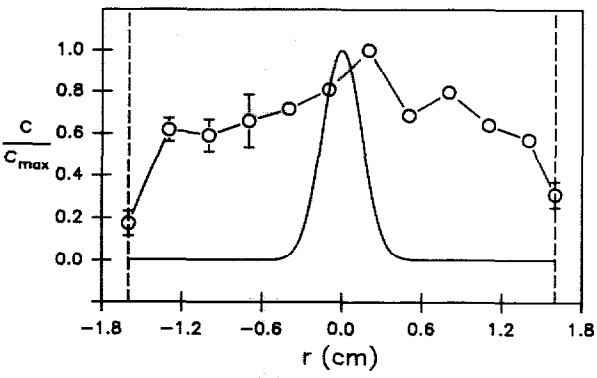

Fig. 2. Radial concentration distribution (normalized by maximal concentration, c_{max}) of DHBA on 4-cm column. Open circles, experimentally determined distribution; solid line, calculated concentration distribution for point injection (σ_r = 0.15 mm). The walls of the column are denoted by the vertical dashed lines. Flow-rate, 0.4 ml/min.

of highest efficiency, fastest flow, and highest concentration occur near the same radial position.

As the electrode was raised above the column frit, the observed efficiency at the center of the 4-cm column was nearly constant for a distance of 0.6 mm. Thus, as with a 10-cm column[14], a 0.6-mm fiber could be used without loss of efficiency. With a longer fiber, however, the increase in electrode area and the accompanying electrochemical noise have been found to offset the advantage of increased faradaic current.

The dependence of the reduced axial plate height on the reduced linear velocity for a 4-cm column is shown in Fig. 3. The open circles are the response of the centrally located microcylinder electrode and the closed circles are the response of the thin-layer detector. The data show that the thin-layer detector is clearly unsatisfactory at optimum chromatographic flow-rates because it acts as a mixing chamber. The optimum reduced velocity of *ca.* 2.6 using the microcylinder electrode (corresponding to a volume flow-rate of 0.2 ml min^{-1}) falls within the expected range ($2 < v < 10$)[28]. The minimum reduced axial plate height of 3.2 observed using the microcylinder electrode indicates that the column is well packed at its center.

Fast, high-efficiency chromatography

To investigate the possibility of achieving fast separations with high efficiency, a reduced linear velocity of 20 was chosen as a reasonable compromise between separation time and efficiency. This velocity corresponds to a flow-rate of 0.6 ml min^{-1}, and the column volume is passed in *ca.* 13 s. The maximum pressure required at this relatively moderate flow-rate for the columns studied is *ca.* 1600 p.s.i. (Note that this includes the pressure drop across the inline filter.) Therefore, the conditions required for these separations are easily attained with simple equipment.

Fig. 4A shows the separation of 70 pmol of each of eight compounds, with baseline resolution of all (except DHBA and homovanillic acid) in less than 50 s. The feature on the dopamine peak is unique to this test mixture and is observed to vary with radial position. For comparison, the chromatogram of the same mixture on the same column with the thin-layer detector is shown in Fig. 4B.

Conventional detectors sample the entire eluate exiting the column, giving a weighted average of the individual efficiencies across the column outlet[14]. The microelectrode, however, can be placed at the region of the column where the efficiency is highest, discarding the fraction of the eluate in which the solute is most dispersed.

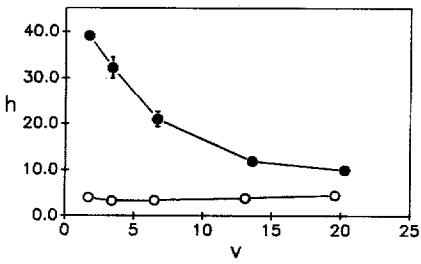

Fig. 3. Dependence of reduced axial plate height on reduced linear velocity at the center of the outlet of a 4-cm column for 70 pmol NE. (○) microcylinder electrode; (●) thin-layer electrochemical detector.

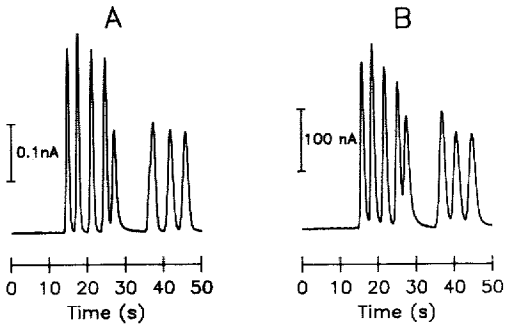

Fig. 4. Chromatogram of 70 pmol each of L-3,4-dihydroxyphenylalanine, 3,4-dihydroxyphenylacetic acid, epinephrine, DHBA, homovanillic acid, dopamine, metanephrine, and epinine (in order of elution) with (A) centrally located microcylinder electrode, (B) thin-layer electrochemical detector. Flow-rate, 0.6 ml min^{-1}.

Shown in Table I are the improvements in measured efficiency for a centrally located microelectrode over a thin-layer amperometric detector for three different compounds. The improvement is especially dramatic at this moderate linear velocity due to the dead volume of the thin-layer amperometric detector. When compared to the results previously reported for the 10-cm column[14], it is apparent that the microelectrode provides greater increase in efficiency over the thin-layer detector in applications which involve short retention times.

Analytical use of the microcylinder

It is because of their extremely small size relative to the chromatographic column that microcylinders can be used to probe spatial heterogeneities at the column outlet. This is also the reason low detection limits can be obtained even though only a very small fraction of the eluate is sampled by the electrode. For electrodes of all size, the amount of noise generated is proportional to the electrode area, yet the current signal for microelectrodes is proportional only to the radius of the electrode[15]. The signal-to-noise ratio, then, increases with decreasing electrode size. With the ability to measure very small current (subpicoampere) therefore comes the ability to detect small amounts of material (subpicomole).

As shown in Fig. 5, subpicomole detection limits can be obtained using microcylinders at the center of the outlet of 4-cm columns (open squares). Comparable

TABLE I

COMPARISON OF NUMBER OF THEORETICAL PLATES (PER METRE) FOR A CENTRALLY LOCATED MICROCYLINDER ELECTRODE AND A THIN-LAYER ELECTROCHEMICAL DETECTOR

Column, 4 cm × 3.2 mm I.D., flow-rate, 0.6 ml min^{-1}.

Compound	*Microcylinder*	*Thin layer*	*Increase (%)*
NE	172 000	55 300	210
E	176 000	61 600	190
DHBA	171 000	56 400	200

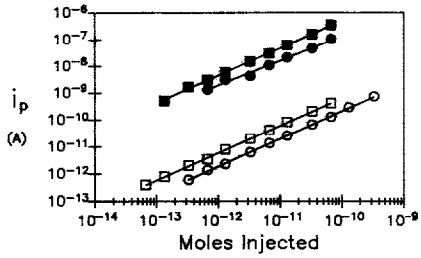

Fig. 5. Calibration curve for NE. Squares, 4-cm column; circles, 10-cm column; filled symbols, thin-layer amperometric detector; open symbols, centrally located microcylinder electrode.

detection limits are also obtained with the thin-layer detector (filled squares). Detection limits with the 4-cm column are nearly an order of magnitude lower than with a 10-cm column of the same diameter[14] because the solute is diluted to a lesser extent as it elutes. Additionally, the efficiency and concentration maxima occur at the same radial position for the 4-cm column, unlike the 10-cm column where the concentration and efficiency maxima are widely separated[14].

An example of the utility of this technique is shown in Fig. 6. Here catecholamines released from bovine chromaffin cells following 5-min nicotine stimulation have been determined with the system described in this paper (Fig. 6A) and compared to a conventional separation system consisting of a thin-layer electrochemical detector and a 25-cm reverse-phase column (Biophase, Bioanalytical Systems, West Lafayette, IN, U.S.A.) (Fig. 6B). The 4-cm column allows separation and quantitation within 30 s whereas separation of the same mixture on the 25-cm column requires 6 min. As a consequence of the small internal volume of the 4-cm column, injections of large amounts of acid (0.1 *M* $HClO_4$ as is typically used to lyse the cells) can have detrimental effects on the peak shapes of eluting compounds. It has been found that this effect can be avoided when volumes less than 7 μl of the sample are injected onto the column.

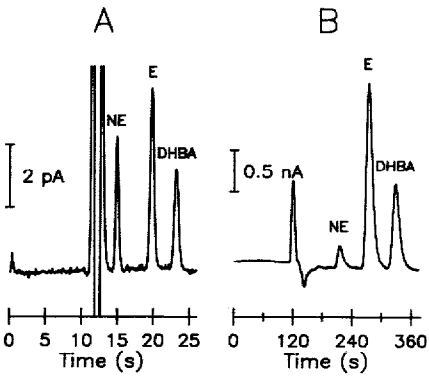

Fig. 6. Separation of total catecholamines in bovine chromaffin cells following 5-min nicotine stimulus: (A) 4-cm column with centrally located microelectrode, flow-rate = 0.6 ml min^{-1}; (B) 25-cm column flow-through electrochemical detector, flow-rate = 1.15 ml min^{-1}, mobile phase 0.1 *M* citric acid–10% methanol–0.6 m*M* sodium octyl sulfate–0.1 m*M* disodium EDTA, adjusted to pH 4.2 with NaOH.

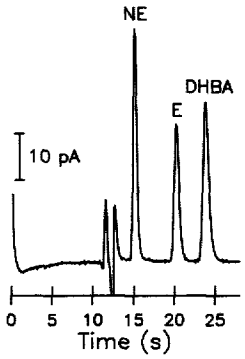

Fig. 7. Chromatogram of 7 pmol each of NE, E, and DHBA with permanently mounted microcylinder electrode. Flow-rate, 0.6 ml min^{-1}.

Permanently mounted microcylinder

The positioning equipment required to make the measurements described in this paper is costly and not available in many laboratories. Therefore, it is desirable to combine the advantages offered with the microcyliner with the ease of a one-piece detector. Such a detector is shown in Fig. 1. A microelectrode is permanently mounted inside a Mini-tight connector that has been drilled out to accommodate moble-phase flow past the electrode barrel. The eluent then flows by the auxiliary electrode and the reference electrode to waste. The electrode extends past the Mini-tight connector so that the tip of the electrode is suspended just above the frit at the center of the column. Recall that the electrode can be placed 0.6 mm from the column frit without significant losses in measured efficiency. Solutes that elute near the edge of the column, where efficiency is less, do not reach the electrode surface, and, thus, do not contribute to the band broadening observed at this detector.

Fig. 7 shows a chromatogram recorded using the permanently mounted microcylinder electrode. The efficiency evaluated in plates per metre was twice that found with the conventional detector. This detector out-performs the thin-layer detector because of the low detection volume and selective placement of the microcylinder. However, the efficiency is not as good as measured with the electrode positioned with micromanipulators. This is because the permanently mounted electrode cannot be placed exactly at the position where the efficiency is the highest.

CONCLUSIONS

The major advantages of coupling microcylinder electrodes with liquid chromatography are spatially selective sampling and the extremely small dead volume of the electrode. Yet even though a very small fraction of the eluate is sampled, subpicomole detecton limits can be attained because of the low noise of the electrode. This means that when microelectrodes are coupled with short columns and relatively fast flow-rates, complex mixtures can be separated in less than 1 min. The need for expensive positioning equipment can be eliminated by permanently mounting the microelectrode into an end-fitting that easily can be coupled with any liquid chromatography column. With smaller columns and better control of the separation,

even faster high-efficiency separations are possible. In addition, electrochemical techniques used with conventional electrodes (pulsed techniques, multiple electrodes, and reductive potentials, etc, see ref. 29 for an overview) may be used with the miniaturized detector to extend these advantages to analytes other than catecholamines.

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