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SMALL-VOLUME ELECTROCHEMICAL DETECTOR FOR MICROCOLUMN LIQUID CHROMATOGRAPHY

Y. HIRATA, P.T. LIN, M. NOVOTNÝ and R.M. WIGHTMAN*

Department of Chemistry, Indiana University, Bloomington, Ind. 47405 (U.S.A.)

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SUMMARY

A submicroliter electrochemical detector for liquid chromatography has been designed, using pressure-annealed pyrolytic graphite technology. The analytical performance of this detector was studied in connection with a reversed-phase packed microcapillary column at very low flow-rates. Although the miniaturized version of the electrochemical detector is less sensitive, a direct analysis of a number of urinary metabolites in 0.1—1.0 μl samples is feasible.

INTRODUCTION

Several recent communications have demonstrated the viability of capillary liquid chromatography (LC) [1—6]. Capillary LC is of great interest because of its potential for achieving a greater resolving power than conventional high-performance liquid chromatography (HPLC). Capillary LC and microbore HPLC [6—8] are also attractive in that consumption of the mobile phase is dramatically reduced; flow-rates employed in capillary LC are typically 0.5—5 μl/min. These extremely low flow-rates require that small-volume detectors be designed and this has been achieved for UV absorbance [7] and spectrofluorometric detection [5]. The chief limitation of capillary LC is that extremely small sample volumes must be used. Despite the great resolving power of the columns, this limitation requires that pre-concentration steps or very sensitive detectors be employed for analysis of trace components in complex mixtures. In this communication we report the use of electrochemical detectors with capillary LC.

Liquid chromatography with electrochemical detection has become a widely accepted technique. Detection of separated compounds with carbon electrodes has found a special utility in the analysis of body fluids and tissue samples for the determination of tyrosine and tryptophan metabolites [9]. The success of

*To whom correspondence should be addressed.

electrochemical detection arises from its sensitivity; changes in concentration of 10^{-8} *M* can readily be determined in flowing streams with carbon electrodes. Several different types of carbon materials have been evaluated for use as detectors for liquid chromatography [10—13]. One of these, pressure-annealed pyrolytic graphite (PAPG) [14], is an ideal material for designing a microelectrochemical cell since it is extremely flat and will not deform into the extremely small channel required for use with microcapillary columns.

We describe here performance of a low-volume electrochemical cell (approximately 0.1 μl) which responds in a linear fashion to changes in concentration at the flow-rates employed in capillary LC. Because of the reduced area of the electrode and the lower flow-rates employed, the detector is less sensitive than an electrochemical cell designed for use with conventional HPLC columns (approximately 2-μl volume). Nevertheless, the electrode is sufficiently sensitive to detect directly a number of compounds in human urinary samples without preconcentration. In addition, the response of the electrochemical detector is compared to the miniaturized fluorescence and absorbance detectors of comparable volume and is found to be far superior in this application.

EXPERIMENTAL

Electrochemical cells

The design of the conventional electrochemical detector is identical to that described previously [14]. In the low-volume cell, the dimensions were reduced to provide suitable operation with the extremely low flow-rates employed with microcapillary columns. The low-volume cell is illustrated in Fig. 1. The cell body is constructed from Lucite, and the two halves are held together by four bolts. The lower half contains a 0.16-cm deep groove, 0.95 cm wide, which extends the length of the block. A copper plate, which serves as a connection to the working electrode, is placed on the floor of the groove, and a 0.95 cm × 1.9 cm piece of pressure-annealed pyrolytic graphite (Union Carbide, Parma, Ohio, U.S.A.) is placed on top of the copper so that the basal plane will be exposed to the solution. The graphite extends approximately 0.5 mm above the top of the lower block. A polyethylene spacer is placed on top of the graphite. A hole cut in the spacer, which coincides with the inlet and outlet openings of the upper half, determines the length (0.30 cm) and width (0.10 cm) of the electrochemical cell. The spacer thickness is 50 μm, giving a cell volume of 0.15 μl. Polyethylene spacers do not compress as much as PTFE spacers, and thus, are preferable in this application. The inlet for the cell is fabricated by silver soldering three concentric stainless-steel tubes. The inner tube has an I.D. of 0.01 cm, and the outer tube is a 20-gauge needle (0.084 cm). The soldered tubes ensure that the inlet assembly is sufficiently rigid for manipulation. The inlet tube is press fit into the upper Lucite block and sanded so that the lower surface of the block is flat. The inlet is connected to the microcapillary column via Teflon capillary tubing. The outlet is a 0.064-cm hole bored in the Lucite block which leads to a compartment containing the saturated calomel reference and platinum wire auxiliary electrode. The entire cell is connected to a potentiostat which applies a constant potential and provides a voltage output proportional to the electrolysis current (Bioanalytical Systems, West Lafayette, Ind., U.S.A.).

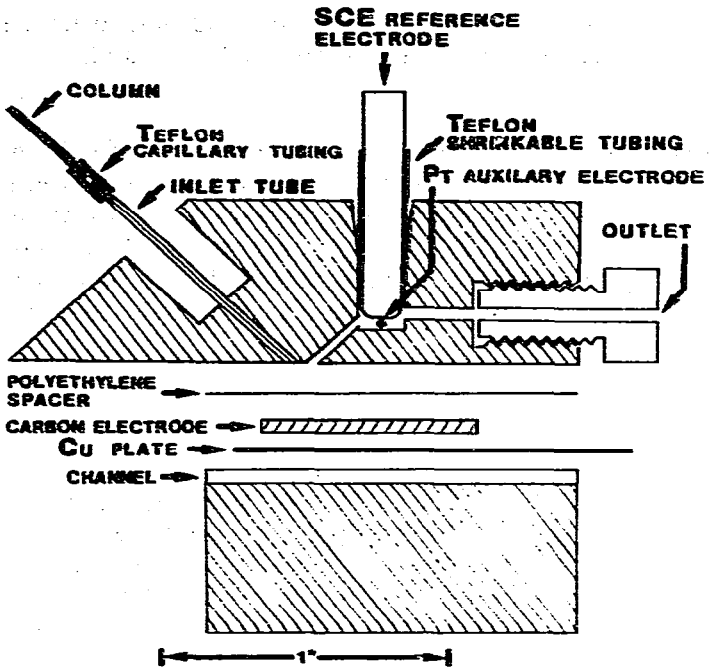

Fig. 1. Schematic diagram of the low-volume electrochemical cell.

The properties of pressure-annealed pyrolytic graphite have been previously discussed [14]. For sensitive and reproducible detection, the surface must be anodically oxidized before use. Complete electrolysis of the graphite is not possible with the low-volume cell since the auxiliary electrode does not oppose the working electrode (the miniature dimensions of the cell make this type of construction difficult). Therefore, the graphite is oxidized in a conventional flow electrochemical cell, and then incorporated in the low-volume cell.

Chromatographic system

Fabrication of microcapillary columns was identical to that described previously [4]. The packed columns contained 30-μm porous silica particles (LiChrosorb SI-100, Merck, Rahway, N.J., U.S.A.), and were drawn on a commercial glass drawing machine. The particles in the drawn column were reacted with octadecyltriethoxysilane (PCR Research Chemicals, Gainesville, Fla., U.S.A.). Reversed-phase columns based on packed alumina particles were also investigated; the resulting chromatograms exhibited skewed peaks for the oxidizable components in whole urine samples and, thus, this approach was not pursued.

The chromatographic system has also been previously described [5]. The pump was a Varian Model 8500 high-pressure syringe pump. In addition to the electrochemical detector, a UV monitor (Varian, Chicago, Ill., U.S.A.) operated at 254 nm and a spectrofluorometric detector (Schoeffel FS970 fluorometer, Westwood, N.J., U.S.A.) were employed. Both had an internal volume of approximately 0.1 μl. These detectors preceded the electrochemical detector in

dual detector applications. The "stop-flow" sampling method was used for sample injection [5]. Sample injection volumes varied from 0.1 to 1.0 μl.

Chemicals

Buffers for liquid chromatography were prepared from reagent grade chemicals dissolved in water that was distilled from basic permanganate and filtered through a 0.8-μm filter. Compounds for use as standards were from Sigma (St. Louis, Mo., U.S.A.); Regis (Morton Grove, Ill., U.S.A.); or Aldrich (Milwaukee, Wisc., U.S.A.). Urine samples from normal males were acidified with perchloric acid, centrifuged, and frozen until use. They were then directly applied to the column.

RESULTS AND DISCUSSION

Cell evaluation

Recently, Weber and Purdy [15] have developed an analytical expression for the current from an electrochemical cell of the type employed here as a liquid chromatographic detector. This expression relates the current to the cell dimensions, flow-rate, and concentration for an electrochemical cell that has the geometry of a rectangular channel and which operates under the conditions of laminar flow and at "infinite" potential. The carbon electrode forms the floor of the channel, and the height (b) and width (W) of the channel are determined by the polyethylene spacer. Opposing the electrode is either the auxiliary electrode or an inert material containing the inlet and outlet tubes. In the cells described here, the inlet and outlet tubes determine the length (L) of the channel. Since PAPG is an unusual electrode material, and since our cell geometry permits some electrolysis in the regions of non-laminar flow, we have evaluated the utility of this expression to describe the current from our conventional volume (≈ 2 μl) detector.

To determine whether amperometric or coulometric conditions obtain, the following expression should be evaluated:

$$r = \frac{DWL}{Ub} \tag{1}$$

where D is the diffusion coefficient (cm²/sec) and U is the volume flow-rate (cm³/sec). For $r \ll 0.3337$, the expression derived by Weber and Purdy [15] reduces to an equation previously given by Posey and Meyer [16]:

$$I = 1.467\, nFC^{\circ} \left(\frac{DLW}{b}\right)^{2/3} U^{1/3} \tag{2}$$

where I is the Faradaic current (A), n is the number of Faradays per mole, F is the Faraday constant, C° is the concentration (moles/ml), and the cell dimensions are given in cm. For the large-volume cell, good agreement with this equation is obtained. A deaerated 10^{-6} M dopamine solution in pH 5.2 citrate—acetate buffer was pumped through various electrochemical cells at an applied potential of 0.8 V. Cells with electrode areas of 0.40 and 0.32 cm² were evaluated with spacers of 50 μm, 75 μm, 100 μm, 125 μm and 150 μm at several different

flow-rates from 0.04 to 3.8 ml/min. (The areas are geometric areas, the spacer thicknesses are from the manufacturers' specifications, and the flow-rates were determined separately for each experiment.) A plot of the resulting 56 current measurements in the form log I versus log $[UA^2/b^2]$ gives a slope of 0.3666, a correlation coefficient of 0.9971, and an intercept in good agreement with the known concentration and estimated diffusion coefficient.

The low-volume cell with an electrode area of 0.03 cm^2 and a 50-μm spacer has a value of r greater than 0.3337 at the flow-rates employed in capillary LC. These dimensions are in part determined by the mechanical limitations of construction of a small cell. Under these conditions Weber and Purdy [15] have shown that the current is given by

$$I = nFUC^{\circ}[1 - 0.3992 \exp\{2.505(0.3337 - r)\}] \quad (3)$$

Operation of the electrochemical detector where this equation applies is usually undesirable since the detector becomes more sensitive to changes in flow-rate and also because the sample is entirely decomposed precluding further analysis of the sample. For the low-volume cell used in this work, the response is given in Fig. 2. For this cell, the logarithmic plot gives a slope of 0.318 rather than

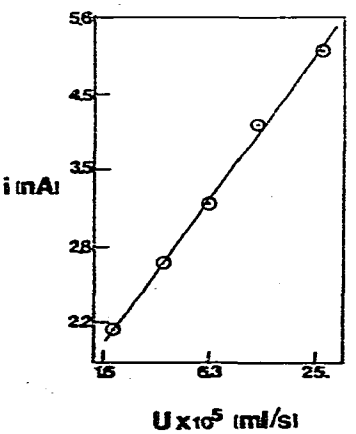

Fig. 2. Logarithmic plot of the amperometric response of the low-volume electrochemical cell as a function of flow-rate. Dopamine (10^{-6} M) in pH 5.2 citrate—acetate buffer was continuously flowed past the detector, and the increase in current over the residual current was determined.

the expected slope of 1 with a correlation coefficient of 0.9992. Although the current response of the low-volume cell is less than expected from eqn. 3, the reduced dependence of the current on flow-rate is advantageous. With this cell coulometric behavior is not observed until very slow flow-rates (70% electrolysis of 1 μl/min). Three different factors may be the cause of this discrepancy between theory and experiment. First, the distance required to obtain laminar flow occupies a significant portion of the length of the electrode with the low-volume cell. Second, the large diameter of the outlet tube increases the effective width of the channel over the last 20% of the cell length to an extremely large value. Third, the anodic oxidation of the carbon may not entirely acti-

vate the carbon surface; it has already been shown that an unoxidized basal plane of PAPG is a very inefficient electrode [14].

Since the conventional and low-volume cells are operating under entirely different flow-rates, direct comparison of the detector responses is difficult. Using typical values in eqn. 2 for the electrode area, spacer thickness, and flow-rate for each cell, the current from the low-volume cell should be 10% of the conventional cell for the same concentration. In fact, our detector is approximately three times less sensitive than theory predicts, so the output current from the small cell is $\approx$ 3% of the conventional cell when both are operating under usual conditions. Although the absolute current is less, the noise level of the current from the low-volume cell is also much reduced since it has a smaller surface area [10]. A comparison of signal-to-noise ratios with the two electrodes at $1 \cdot 10^{-4}$ ml/sec for the low-volume cell and $1 \cdot 10^{-2}$ ml/sec for the large cell indicates that the detection limit is reduced by approximately 10 for the low-volume cell.

Although construction of a low-volume cell with a platinum auxiliary electrode located opposite the working electrode is sufficiently difficult to preclude routine use, we have evaluated the flow-rate dependence of such a cell under conditions where $r \gg 0.337$. Under these conditions the current is flow-rate independent at very slow flow-rates for a reversible compound [15]:

$$I = nFUC(0.2671 + \frac{DLW}{Ub}) \quad (4)$$

and, indeed this is the case for the low-volume cell with 10^{-6} *M* dopamine in citrate—acetate buffer (pH 5.2) at flow-rates up to $3 \cdot 10^{-5}$ ml/sec.

Chromatographic detection

The low-volume electrochemical cell has been employed as a detector for microcapillary liquid chromatography in an investigation of the separation of acidic and neutral metabolites of tyrosine and tryptophan in human urine. During the course of this investigation, detectors were found to remain operable for about three weeks. Failure of the detector was evidenced by a high background current which results from leaking of the mobile phase under the polyethylene spacer.

Fig. 3 shows the separation of a 0.1 *M* acetic acid solution containing methoxyhydroxyphenyl glycol (MHPG), *p*-hydroxyphenylacetic acid (PHPAA), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA) and vanillic acid (VA), each at a concentration of $1.6 \cdot 10^{-4}$ *M*. Four of these compounds can readily be identified in human urine; only MHPG is unresolved (Fig. 4). The urinary sample that was used to obtain the chromatogram in Fig. 4 was only acidified to prevent air oxidation of the phenolic compounds. A sample overload of 1 μl of acidified urine decreased somewhat the column efficiency as compared to standards where typically 0.1-μl samples were introduced (compare Figs. 3 and 4). The sample was not a 24-h collection, nor was it hydrolyzed, but it gives an outstanding example of the combined resolution and sensitivity that the microcapillary LC—electrochemical method provides.

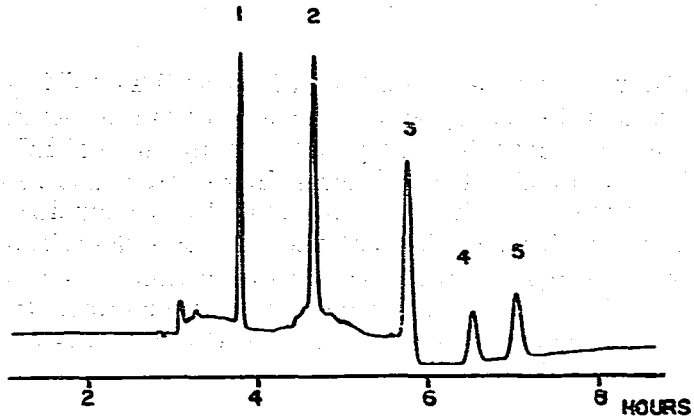

Fig. 3. Chromatogram of 0.3 μl of a solution containing $1.6 \cdot 10^{-4}$ *M* MHPG (1), PHPAA (2), 5-HIAA (3), HVA (4), and VA (5). Conditions: 60 m reversed-phase column, 0.2 *M* acetate buffer (pH 4.0), flow-rate of 1 μl/min, E_{app} = 1.0 V, 20 nA full scale.

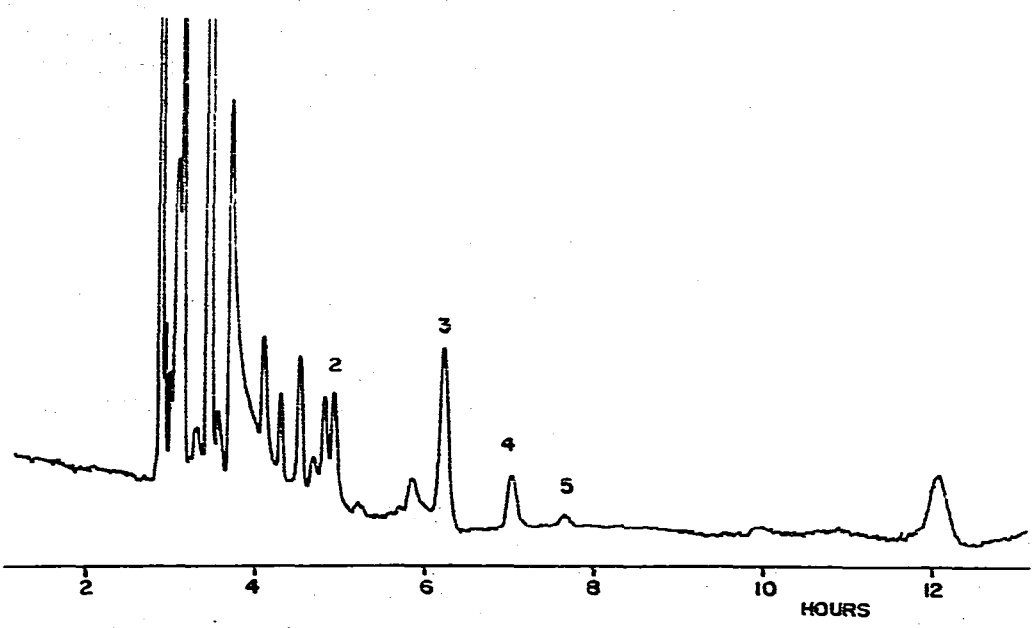

Fig. 4. Chromatogram of 1.0 μl of human urine, acidified to pH 2. Resolved components include PHPAA (2), 5-HIAA (3), HVA (4), and VA (5). Conditions as in Fig. 3 except 3 nA full scale.

Neither the fluorescence nor the UV detector were found sufficiently sensitive to provide any information on the eluting compounds in the urine samples. The electrochemical detector has been compared to the UV detector in the separation of 10^{-2} *M* standards; comparison of the chromatograms showed that band broadening is not introduced by interfacing the electrochemical detector to the column.

CONCLUSIONS

Electrochemical detection of compounds separated by microcapillary LC is a sensitive method for detecting oxidizable compounds. Because of the reduced dimensions of the cell and the lower flow-rates compared to conventional LC, the detector has a detection limit approximately 10 times higher than a large-volume cell. Nevertheless, the high column efficiency coupled with the sensitivity and selectivity of the detector provide a useful technique for the identification of compounds in a complex biological fluid. Sample pretreatment is not required, and, thus, the analysis scheme is much simpler than other methods. For the compounds discussed in this paper, the electrochemical method is far more sensitive than fluorescence or UV detection.

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