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# AN AMPEROMETRIC DETECTOR FOR USE WITH SMALL-BORE LIQUID CHROMATOGRAPHIC COLUMNS

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## SUMMARY

A miniaturized electrochemical flow cell for amperometric detection in liquid chromatography has been designed to meet the requirement of low volumetric dispersion when using small-bore columns. The volume of the thin-layer cell is in the range 10–50 nl, depending on the spacer dimensions, with the electrode area being about 1 $mm^2$. The cell is constructed from glass and designed in such a way that the working electrode can be mounted without alterations to its surface state.

The flow-rate dependence, contribution to peak dispersion and linear dynamic range were investigated. After separation on a 250 × 1 mm I.D. column, a detection limit for dopamine of about 0.15 pg was achieved with a linear dynamic range of $10^6$. The variance of the extra-column dispersion was determined to be less than 0.3 $\mu l^2$, including injector and connecting capillaries. The applicability of the detector is illustrated with the detection of dopamine and its major metabolites, dihydroxyphenylacetic acid and homovanillic acid, and of Met- and Leu-enkephalin in samples of biological origin.

## INTRODUCTION

Recent advances in the development of small-bore liquid chromatographic columns have given a tremendous impetus to the miniaturization of liquid chromatography (LC)[1]. At present, chromatographic systems utilizing columns of 1 mm I.D., commonly referred to as microbore columns, are commercially available from several manufacturers. The advantages of such LC systems include low mobile phase consumption, high mass sensitivity in detection and small injection volumes. However, a prerequisite for the optimal use of miniaturized columns is injection and detection systems designed to keep extra-column band broadening sufficiently small[1]. In addition, a detection system with a very high sensitivity is required for trace analytical applications[2].

Electrochemical detection in combination with liquid chromatography (LCEC) has developed to become a valuable tool in organic trace analysis, owing to

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the selectivity and high sensitivity achievable. Since determination of catecholamines in the picogram range was reported in 1973[3], LCEC techniques have been applied successfully to numerous other analytical problems[4].

Amperometric detection lends itself to miniaturization and detectors with a sub-microlitre cell volume have been described by several workers[5–10]. Although an increased mass sensitivity has been reported for these detectors, the detection limits in concentration units are far above those normally reported for conventional LCEC. More recent reports, however, have indicated the possibility of using electrochemical detectors in combination with miniaturized columns to obtain a high concentration sensitivity[11,12].

In this paper, a miniaturized amperometric detector, compatible with 1 mm I.D. columns, is described. Limits of detection demonstrated are in the low nanomolar range, using a 1-$\mu$l injection volume. A recently introduced pyrolytic carbon film electrode[13] is used as the working electrode. This electrode is characterized by a low differential capacity (2–7 $\mu$F/cm$^2$)[14], which is one prerequisite for low noise operation in amperometric detectors[15]. The electrode material cannot, however, be polished without alterations to its surface state, and for this reason the cell has been designed in such way that the working electrode can be mounted with intact surface properties.

## EXPERIMENTAL

### *Detector*

The electrochemical detector cell is illustrated schematically in Fig. 1. A glass capillary (borosilicate glass, Veridia, France), 5 mm O.D. and 0.15 mm I.D., which serves as the main body of the detector, was heated over a flame and bent to an approximate right-angle, and then ground with silicon carbide paper to expose the capillary holes (see Fig. 1). The distance between the inlet and outlet was about 3 mm, with no residual capillary channel remaining in between. During grinding and also during the subsequent polishing step, involving an aqueous slurry of 1 $\mu$m alumina, the glass capillary was firmly fixed in a plastic holder. The polished surface

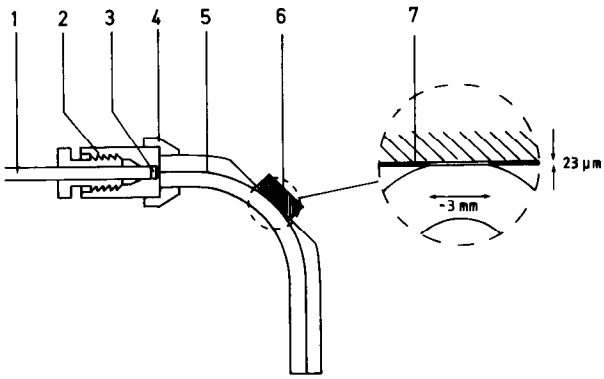


Fig. 1. Amperometric detector. (1) Microbore column; (2) Valco LDV union; (3) 2-$\mu$m stainless-steel frit; (4) epoxy resin; (5) glass capillary, 0.15 mm I.D.; (6) working electrode; (7) Mylar spacer, 23 $\mu$m. A close-up of the thin-layer region is shown in the circle (not to scale).

was next coated with a film of tin(IV) oxide[16] and a connecting lead was fixed to its rim with silver epoxy. This arrangement served as the auxiliary electrode. The spacer was a 23-$\mu$m polyester film (Mylar type A, DuPont) with a 3 × 0.4 mm duct, which was cut under microscope using a scalpel.

The detector was assembled by firmly securing the working electrode to the tin(IV) oxide-coated glass body using a special screw-clamp with the spacer sandwiched in between. This allowed the mounting of the working electrode without altering its surface state. For this study, a previously described[10] pyrolytic carbon film electrode (PCFE) was used. The volume of the thin-layer cell was less than 30 nl, with the exposed working electrode area being about 1.2 $mm^2$.

The cell body was connected to the column with a Valco LDV union, which was cut in half (see Fig. 1), polished and butted against the end of the detector body (polished in the same way as described above). A capillary wire was used to ensure the correct positioning, and the connection was fixed first with a fast-setting epoxy and then finally secured using a cover of slow-setting quartz powder-filled epoxy (Araldite CW 1248 KS). During use, the outlet of the cell was immersed in a beaker (containing the mobile phase) in which a saturated calomel reference electrode (SCE) was positioned. Reported potential values are given *versus* this reference half-cell. The detector assembly and column were housed in a protective sturdy metal box, which served as a Faraday cage. The injector was firmly secured to the outside of the box.

The potentiostat was of conventional design and constructed from National LF 356 operational amplifiers. A built-in second-order active low-pass filter with a cut-off frequency of 0.14 Hz was used to reduce noise. Currents were monitored with a Radiometer strip-chart recorder. For evaluation of extra-column dispersion, the cut-off frequency of the active filter was changed to 3 Hz and a Nicolet Model 1090A digital oscilloscope was used to record the output currents.

*Chromatographic equipment*

A uniform flow was delivered by an ISCO Model 314 metering pump (with a Nitronic 50 stainless-steel cylinder and a Hastelloy C stainless-steel plunger). Samples were injected with an electrically controlled four-port Valco injector (CFSV-4 UHPe, 0.2 $\mu$l) or a Rheodyne Model 7520 injector (0.2 or 1 $\mu$l). A conventional pressure gauge (Alltech, Arlington Heights, IL, U.S.A.) was inserted in a tee-configuration with the outlet of the pump. An in-line 2 $\mu$m steel screen filter preceded the injector. Two different 250 × 1 mm I.D. columns were used, one packed with Partisil 10 ODS-3 Micro-B (Whatman, Clifton, NJ, U.S.A.) and the other with Spherisorb S5 ODS-2 (Scantec, Partille, Sweden). Although a 1/16 in. O.D. column can be connected directly to the injector and detector as depicted in Fig. 1, two short (about 40 mm) pieces of 0.13 mm I.D., 1/16 in. O.D. stainless-steel capillary tubing (cut at right-angles and with polished endings) were used for the connections to allow more flexibility. For determination of steady-state currents a Rheodyne 7120 injector equipped with a 1.5-ml sample loop was employed.

*Reagents*

All buffer components were of analytical-reagent grade. Methanol (p.a., Merck, Darmstadt, F.R.G.) and acetonitrile (LiChrosolv, Merck) were used as or-

ganic modifiers. Sodium octanesulphonate (Fluka, Buchs, Switzerland) was used as an ion-pair reagent. Standard 10 m*M* solutions of hydroquinone, dopamine (DA), dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA), all from Sigma, were prepared in 0.1 *M* perchloric acid and diluted to the desired concentration prior to use. Enkephalin standards and rat striatum extracts were gifts from the National Defence Research Institute (FOA 4, Umeå, Sweden). Distilled water, further purified with a Millipore Milli-Q filtration system, was used for all solutions. The mobile phases were filtered through a 0.45-$\mu$m Millipore filter prior to use.

## RESULTS AND DISCUSSION

### *Detector characterization*

*Flow-rate dependence.* The magnitude of the current response of an electrochemical detector is a function of certain operating conditions, *e.g.*, solution velocity, solute concentration and cell dimensions. Equations relating the mass-transport-limited steady-state current to several operational parameters at a channel electrode have been theoretically derived[17–19]. A pure $F_v^{1/3}$ ($F_v$ = volumetric flow-rate) dependence is obtained if the coulometric yield, $Q$, is less than 10%[20]. However, owing to the low flow-rates used in microbore chromatography, higher values of $Q$ are often encountered and a more complex behaviour is then observed. Roosendaal and Poppe[19] derived approximate equations, which, within a relative error of less than 3%, can be used over the entire flow-rate range.

In order to verify that the theoretical relationships are valid for the present detector, the flow-rate dependence of the steady-state current was studied. The equations proposed by Roosendaal and Poppe[19] were used to calculate the theoretical curve. In their approach they use the dimensionless parameter $\xi = LDW/F_v b$ (where

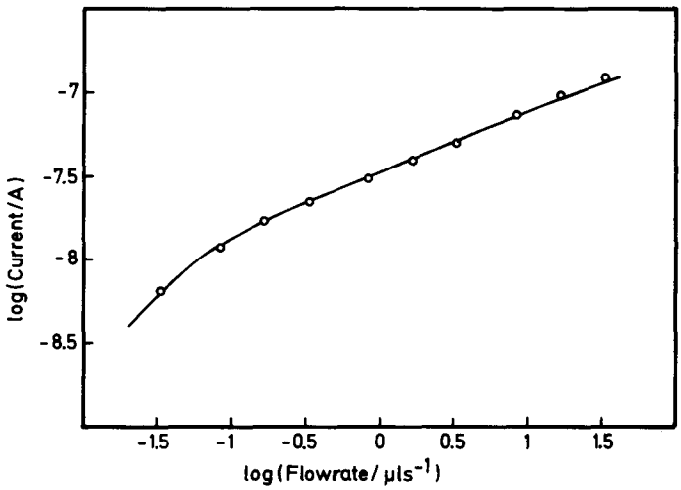


Fig. 2. Flow-rate dependence of steady-state current obtained with 1 $\mu$*M* hydroquinone in 0.1 *M* phosphate buffer (pH 6.8). Experimentally obtained data; solid line, calculated using the equations given by Roosendaal and Poppe[19]. The parameters used in the calculation are given in the text.

$L$ is the electrode length and $W$ its width, $F_v$ is the volumetric flow-rate, $b$ is the channel height and $D$ is the diffusion coefficient for the electroactive species). For $\xi$ less than 0.01,

$$Q = 1.467\xi^{2/3} \quad (1)$$

and for $\xi$ values above 0.01

$$Q = 1 - \sum_{i=1}^{3} a_i \exp(-\lambda_i^2 \xi_i) \quad (2)$$

The values of $a_i$ and $\lambda_i$ can be found elsewhere[19]. The steady-state current is easily calculated when the flow-rate and depolarizer concentration are known. The following parameters were used in the calculation presented in Fig. 2: $b$ = 0.023 mm, $L$ = 3 mm, $W$ = 0.4 mm and $D$(hydroquinone) = $0.84 \cdot 10^{-3}$ mm$^2$/s[18].

The experimental results were obtained by injecting a large volume of 1 $\mu M$ hydroquinone in a flow of 0.1 $M$ phosphate buffer (pH 6.8). The detector was connected to the injector with a short piece of PTFE tubing (0.15 mm I.D.). The volume injected, 1.5 ml, was sufficiently large to yield a steady-state signal at all of the flow-rates studied (2–2000 $\mu$l/min). Current blanks were obtained by injecting the buffer alone. The detector potential was set to +1.00 V *vs.* SCE to ensure that the current was mass-transport limited. The flow-rates were checked by measuring the time required to fill a microsyringe and proved to be within 1% of the nominal settings.

In Fig. 2, experimental data are compared with the theoretical predictions. A fairly close fit is obtained, showing that the proposed equations describe the $i$–$F_v$ relationship for the present detector. At a flow-rate of 50 $\mu$l/min, commonly used with 1 mm I.D. columns, the coulometric efficiency found was about 19%. Hence the current response, at these low flow-rates, deviates from a pure $F_v^{1/3}$ dependence (see Fig. 2), in agreement with the results of Weber[20].

*Extra-column band broadening.* On reducing the column diameter from 4.6 to 1.0 mm, the volume of the eluted peaks will decrease 20-fold, assuming the same column length and efficiency[21]. This implies that the extent of the extra-column band broadening has to be sufficiently small in order to preserve the small peak band widths obtained with miniaturized columns.

The variance of the extra-column band broadening was measured by connecting the detector to the Rheodyne injector with 40 × 0.13 mm I.D. capillary tubing. Measuring the variance in this way provides an estimate of the total extra-column dispersion of the chromatographic system. Injections of a 100 $\mu M$ hydroquinone solution were made and the corresponding peaks were recorded on a digital oscilloscope. From the recordings the peak widths at half-height ($W_{\frac{1}{2}}$) were measured and the peak variances were calculated using the equation[22]

$$\sigma^2 = \left(\frac{W_{\frac{1}{2}}}{2.354}\right)^2 \quad (3)$$

During these measurements the cut-off frequency for the active filter was changed to 3 Hz because of the short elution time in the experiment. The results obtained with the 0.2- and 1-$\mu$l loops at different flow-rates are shown in Table I. For example, at a flow-rate of 20 $\mu$l/min the peak variance is less than 0.15 $\mu$l$^2$ with the 0.2-$\mu$l loop.

TABLE I

EXTRA-COLUMN VARIANCES AT DIFFERENT FLOW-RATES

The Rheodyne 7520 injector was used to inject 0.1 m*M* hydroquinone and from the peaks obtained the width at half-height was used to calculate the variance. The detector was connected to the injector with a 40 × 0.13 mm I.D. stainless-steel capillary. Three measurements were made at each flow-rate and the reported results are the ranges obtained.

| *Sample loop volume (μl)* | *Flow-rate (μl/min)* | *Peak variance (μl²)* |
|---|---|---|
| 0.2 | 20 | 0.14–0.15 |
| | 50 | 0.28–0.30 |
| | 100 | 0.31–0.33 |
| 1 | 20 | 0.35–0.38 |
| | 50 | 0.62–0.67 |
| | 100 | 0.69–0.75 |

This can be compared with a value of less than 0.2 $\mu l^2$ at 20 μl/min, which has been reported elsewhere[23] for the dispersion contribution by the same injector, including 50 × 0.13 mm I.D. capillary tubing. Hence the measured variance is due mainly to the injector, which implies that the detector cell alone shows an extra-column band broadening compatible with even further miniaturized columns.

*Linear dynamic range.* Unless proper precautions are taken, the linear dynamic range for an amperometric detector can be restricted by a significant uncompensated resistance of the electrochemical cell. Normally this problem is avoided by placing the auxiliary electrode in the close vicinity of the working electrode. However, this is difficult when designing miniaturized cells, but has been achieved in the presented detector design by coating the glass capillary with a tin(IV) oxide film, which serves as an auxiliary electrode.

To confirm that the uncompensated cell resistance is negligible, the linear dynamic range was determined with solutions of hydroquinone in the range 20 n*M*–10 m*M*. The mobile phase was the same as stated above and an injection volume of 0.2 μl was used. The potential was set to +0.50 V, close to where the wave of the hydrodynamic voltamogram reached the plateau. Too high a cell resistance would cause deviations from linearity at the higher concentrations. The linearity of the response was evaluated from the slope of a log *i vs.* log *C* plot. A linear dependence was found ($R^2 > 0.9999$) with a slope of 1.01. Hence a linear dynamic range of at least $10^6$ is obtained and the uncompensated resistance in the cell is sufficiently low not to cause any problem even when currents in the μA range are encountered.

*Repeatability.* By loading the 1-μl sample loop manually with 10 μl of a 10 μ*M* hydroquinone solution, a peak-height relative standard deviation of 0.5% ($n = 10$) was obtained. This can be regarded as satisfactory, considering the small sample loop volume and the manual injection procedure. In this instance the solute was dissolved in the mobile phase (the same as used in the previous experiment). However, when water was used as the solvent, irreproducible results were obtained and serious peak tailing was observed. One likely explanation of this behaviour is the viscosity difference between water and the mobile phase, and the difference in

diameter between the sample loop and outlet port, which makes it difficult to fill or empty the sample loop completely when loading or injecting, respectively.

*Ruggedness.* A major problem encountered during this study was to accomplish a reliable means for mounting the working electrode, which is a prerequisite for the system performance illustrated in this paper. Owing to the small dimensions of the flow cell, the positioning of the spacer must be accurately controlled. This can be accomplished by using a microscope during the mounting. However, the problem is to maintain this spacer position when securing the working electrode. A slight displacement may cause partial blockage of inlet or outlet, which results in high noise levels and/or background currents and also leakage in some instances. In addition, susceptibility to entrapment of air bubbles is enhanced if the spacer is incorrectly positioned. This is a well known cause of inferior performance. Unfortunately, the only solution was to dismount the cell and replace the spacer, which is a fairly time-consuming process. However, once a satisfactory mounting had been achieved, the cell performance illustrated in this paper was maintained for extended periods of time.

*Chromatographic applications*

*Biogenic amines.* Much of the analytical LCEC work concerns the measurement of biogenic monoamines and their metabolites in different biological samples. Fig. 3 shows a chromatogram of a perfusate sample obtained after intracerebral

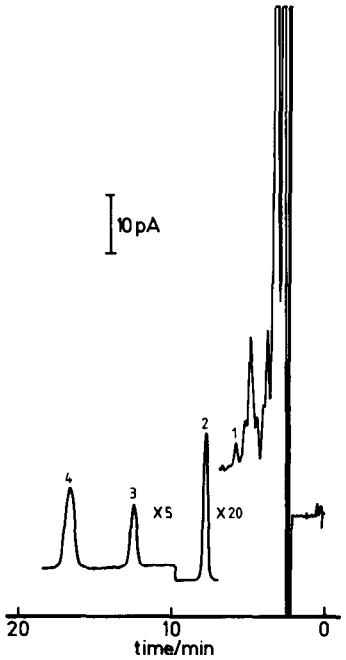


Fig. 3. Separation of dopamine and monoamine metabolites in a rat brain dialysis sample on a 250 × 1 mm I.D. Spherisorb S5 ODS-2 column. Mobile phase: 0.15 *M* $NaH_2PO_4$–0.5 m*M* sodium octanesulphonate–0.1 m*M* EDTA–20% methanol (pH 4.0); 50 μl/min. Detection at +0.70 V *vs.* SCE. Injection volume: 1 μl. Components: 1 = dopamine, 0.57 pg; 2 = dihydroxyphenylacetic acid, 111 pg; 3 = 5-hydroxyindoleacetic acid, 46 pg; 4 = homovanillic acid, 45 pg.

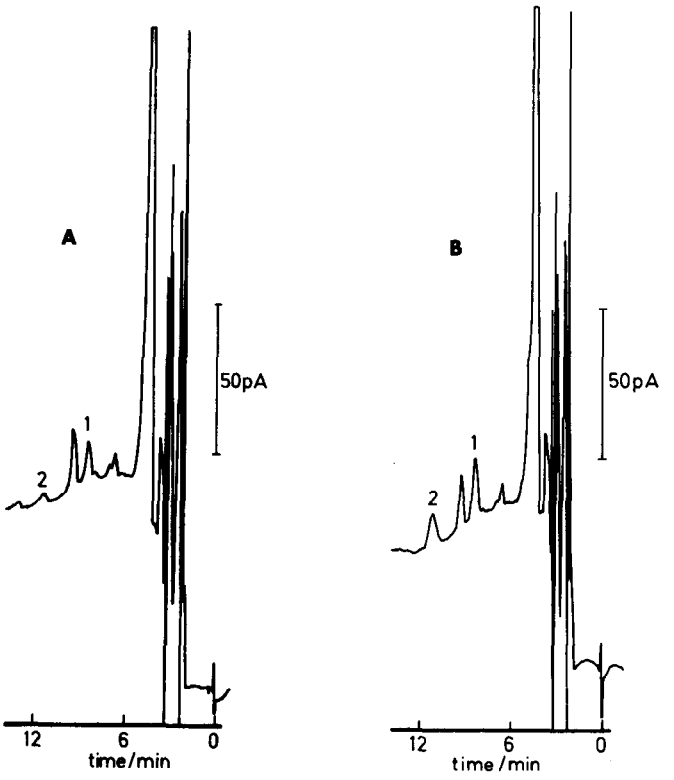


Fig. 4. (A) Separation of enkephalins in an ethanolic rat striatum extract on a 250 × 1 mm I.D. Partisil 10 ODS-3 column. Mobile phase: 25 m*M* phosphate buffer (pH 6.9)–12.5% methanol–12.5% acetonitrile; 50 μl/min. Detection at +1.10 V *vs.* SCE. Injection volume: 0.2 μl. Components: 1 = Met-enkephalin; 2 = Leu-enkephalin. (B) Same as (A), but with 20 pg of both enkephalins added to the sample.

dialysis[24] of the nucleus accumbens of an awake rat. The chromatographic separation was accomplished using a mobile phase consisting of 0.15 *M* $NaH_2PO_4$, 0.5 m*M* sodium octanesulphonate, 0.1 m*M* EDTA and 20% (v/v) methanol (pH 4.0). The potential was set to +0.70 V and an injection volume of 1 μl was used. The dopamine peak corresponds to a concentration of 3.7 n*M* (0.57 pg), which is a typical level in this kind of sample. The limit of detection for dopamine, defined as twice the peak-to-peak noise level, is estimated to be less than 0.15 pg (1 n*M* using a 1-μl injection volume). This level is about the same as that found using conventional LCEC.

*Enkephalins.* The two pentapeptides Met- and Leu-enkephalin can be monitored by amperometric detection owing to the presence of the electrochemically active tyrosine residue[25], although the electrode potential required, about +1.10 V, will cause the background current to rise substantially. In particular, most glassy carbon materials are known to exhibit poor long-term stability as regards both background current and sensitivity when operated at potentials above +1 V. A mobile phase consisting of acetonitrile–methanol–25 m*M* phosphate buffer (pH 6.9) (25:25:150, v/v) was employed to effect the separation. In this instance the use of EDTA was ruled out owing to its inherent electroactivity at high positive potentials. Passivation

of steel surfaces exposed to the mobile phase was found to be necessary in order to minimize interferences from leaking iron(II) ions[26]. Fig. 4A shows a chromatogram obtained after injection of a rat striatum extract. The ethanolic extract was diluted with an equal volume of mobile phase prior to injection. Fig. 4B shows a chromatogram of the same diluted extract spiked with a standard solution of enkephalins. The amount added was 20 pg of each enkephalin. The extract had been stored at −20°C for several months prior to analysis and some degradation had probably occurred. In spite of that, it is clearly seen that the enkephalins can be determined at physiological levels in an injected volume of only 0.2 μl. The chromatograms also show that the detector behaves well even at a potential of +1.10 V; in fact, the noise level at this potential is almost the same as that at +0.70 V, *i.e.*, about 0.5–0.7 pA peak-to-peak. Further, during this study the detector was operated continously for almost 2 months at potentials above +1.0 V with stable background currents and without any decrease in sensitivity for the enkephalins.

## CONCLUSIONS

In many biomedical applications, the amount of sample is limited and the high mass sensitivity achievable in miniaturized LC is an obvious asset. In this work, however, the samples used for the two applications illustrated were intended for analysis by conventional LC methods and only small portions of the samples were used. In order to exploit the microbore system fully, the sampling should be adapted to the smaller volume of sample required. This further reduction in sample size should pave the way for new possibilities in biomedical research.

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