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CAPILLARY ELECTROPHORESIS WITH INDIRECT AMPEROMETRIC DETECTION

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SUMMARY

The use of indirect amperometric detection with capillary electrophoresis is demonstrated. The system consists of a porous glass coupler which allows amperometric detection at a carbon fiber electrode placed in the end of the capillary. 3,4-Dihydroxybenzylamine is added to the buffer system as a continuously eluting electrophore. Indirect amperometric detection in 9-μm I.D. capillaries provides detection limits as low as 380 attomole for the amino acid arginine. Finally, both direct and indirect amperometric detection can be accomplished simultaneously.

INTRODUCTION

Capillary electrophoresis (CE) is a highly efficient, small-volume separation technique that can be used to separate a wide range of biological compounds[1–4]. However, many biological compounds do not inherently possess the necessary characteristics for sensitive detection. Laser fluorescence and amperometric detection both provide subattomole detection limits and are among the most sensitive detectors available for CE[2,4–9]. Although these detection methods are very sensitive, selectivity is limited to those species which possess native fluorescence or are easily oxidized.

An area of great interest in CE is its use for separation and detection of substances removed from biological microenvironments. A microinjector[10] has been used to acquire samples from the cytoplasm of single nerve cells[4,8]. An important aspect of this experiment is the use of amperometry for sensitive detection of underivatized substances. We have developed a system which uses a porous glass coupler to allow electrophoresis in a "separation" capillary and an off-column amperometric detector in a "detection" capillary[7]. This system can provide subattomole detection limits for the easily oxidized catechols and indoles[9]; however, many solutes of interest are not easily oxidized and are, therefore, not detectable via this method.

Detectability can be provided by sample derivatization[1,2,5,6], but there are several drawbacks to this approach. The derivatization procedure can result in multiple products for peptides, derivatization is difficult with very small volume samples, and detection by this method is still far from universal. Indirect detection provides an alternative scheme that promises to be more universal. This detection

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scheme has been demonstrated with CE for the detection of bromide and several organic acids by indirect UV absorbance[11] and for native amino acids, nucleotides and nucleosides by indirect fluorescence[12,13]. In these experiments, a lower limit of detection of 50 amol was obtained for several native amino acids.

Previous reports of indirect detection in CE have centered on the detection of anions by use of UV absorbance or laser fluorescence[11–13]. In this paper, we demonstrate the use of indirect amperometry for the detection of several cationic amino acids and dipeptides. The advantages of indirect amperometric detection include sensitivity and instrumental simplicity while providing, in concept, detection of all ionic solutes.

EXPERIMENTAL

CE apparatus

The system used for electrochemical detection has been described by Wallingford and Ewing[7]. In this work, 5-μm and 10-μm diameter carbon fibers (Amoco Performance Products, Greenville, SC, U.S.A.) with an exposed length of 200–250 μm were used as the working electrodes for electrochemical detection. Detection was performed in the amperometric mode with a two-electrode configuration. The reference electrode was a sodium-saturated calomel electrode (SSCE). The low currents measured required that the detection end of the system be housed in a Faraday cage in order to minimize the effects of external noise sources. Untreated fused-silica capillaries having internal diameters of 26 and 9 μm and outer diameters of 260 and 370 μm, respectively, were obtained from Polymicro Technologies (Phoenix, AZ, U.S.A.). Injections were performed by electromigration[14].

Chemicals

2-Morpholinoethanesulfonic acid (MES) and 3,4-dihydroxybenzylamine (DHBA) were obtained from Sigma (St. Louis, MO, U.S.A.). All solutions were adjusted to the appropriate pH using dilute sodium hydroxide. Lysine (Lys), arginine (Arg), histidine (His), lysylphenylalanine (Lys-Phe), histidylphenylalanine (His-Phe), arginylleucine (Arg-Leu), histidylglycine (His-Gly), dopamine, norepinephrine, and epinephrine were used as received from Sigma.

RESULTS AND DISCUSSION

Indirect detection of amino acids

Indirect detection involves the addition of a detectable ionic component to the electrophoretic buffer. Zones of non-detectable analyte ions displace the added ions during zone migration. For analytes with the same charge as the detectable component, displacement of the detectable ions results in a lower level of the detector-active component, and negative peaks result. A schematic diagram outlining this process is shown in Fig. 1. Although this is a simplification and it is likely that many processes are occurring simultaneously, detectability is based on the properties of the detector-active component which results in a universal detection scheme.

Indirect electrochemical detection has been accomplished by addition of a cationic electrophore, DHBA, to the electrophoretic buffer. When the working

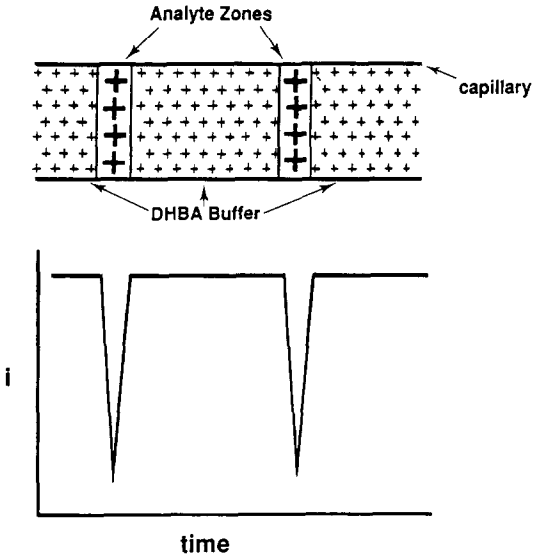
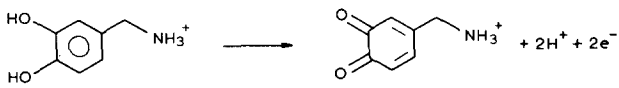


Fig. 1. Schematic diagram of cation displacement by migrating zones of cationic solutes.

electrode is held at a constant potential of +0.7 V *versus* the SSCE, the DHBA undergoes a two-electron two-proton transfer to give the corresponding orthoquinone as follows:



Continuous oxidation of this species, as it passes through the detector region, produces a constant background current. Due to the need to preserve charge neutrality, the cationic DHBA in the buffer is displaced by cationic analyte zones. As these cationic zones pass through the detector region, a lower level of oxidizable species is observed in these zones and the background current decreases. As a result, negative peaks are expected in the detection of cations.

Detection of several cationic native amino acids by indirect amperometry is demonstrated in Fig. 2. The electropherogram shows the separation of three non-electroactive amino acids obtained in a 26-μm I.D. capillary. Ethanol was added to the electrophoretic buffer in an attempt to change the zeta potential at the capillary surface and thus change the electroosmotic flow-rate. This, however, did not significantly enhance the resolution, although the peak tailing was decreased relative to a separation obtained without ethanol (not shown).

The first peak in the electropherogram represents a zone in which the DHBA is virtually absent. This displacement peak is present since all samples were prepared in buffer without the DHBA. A system peak, labelled S, is observed at a migration time corresponding to the rate of electroosmotic flow. The injected amounts of amino acids were 160, 160, and 150 fmol, respectively, for peaks A, B and C in an injection volume

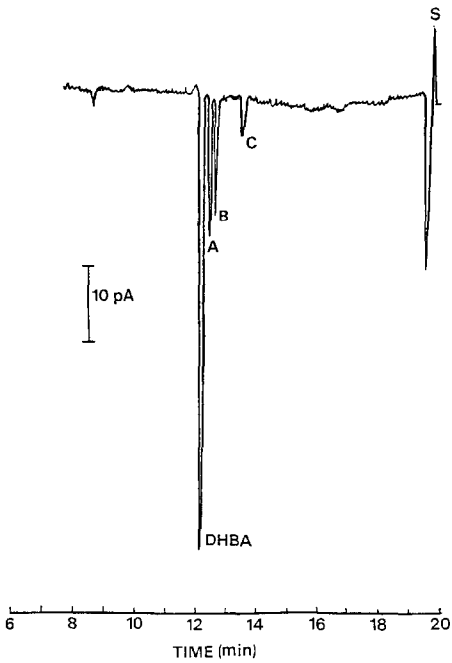


Fig. 2. Electropherogram of amino acids with indirect amperometric detection on a 26-μm I.D. capillary: buffer, 0.1 m*M* DHBA–0.025 *M* MES (pH 5.50)–10% (v/v) ethanol; 72.1-cm-long separation capillary; 0.6-cm detection capillary; injection by electromigration, 3 s at 15 kV; separation voltage, 15 kV; electrode potential, 0.7 V *vs.* SSCE. Peaks: A = Lys; B = Arg; C = His; S = system peak (electroosmotic flow velocity).

of 1 nl (based on the electroosmotic flow-rate). This corresponds to detection limits ranging from 14 to 43 fmol. To calculate the detection limit we used two times the peak-to-peak noise measured over a period of baseline equal to ten peak widths at half height[15]. Thus, detection limits reported are twice those that would be calculated using three times the root-mean-square noise.

Detection in narrow-bore capillaries

An important parameter in indirect detection is the dynamic reserve (DR)[16]. This dimensionless parameter is defined as the ratio of the background signal to the noise on that signal. Using indirect amperometric detection, we have found DR values ranging from 50 to 300. This wide range of DR values appears to be the result of differences in electrode response between detectors. These differences are believed to be due mainly to varying electrode area, but could be caused by cracks in the carbon fiber. Variations in the procedure used to seal the small electrodes with epoxy could also increase the surface area of the electrode. Electrochemical noise is thought to be governed, in part, by the exposed electrode area. The DR can be used to determine the minimum concentration (C_{lim}) that should theoretically be detectable under a given set of conditions. C_{lim} is defined as

$$C_{\mathrm{lim}} = \frac{C_{\mathrm{m}}}{(\mathrm{DR})(R)}$$

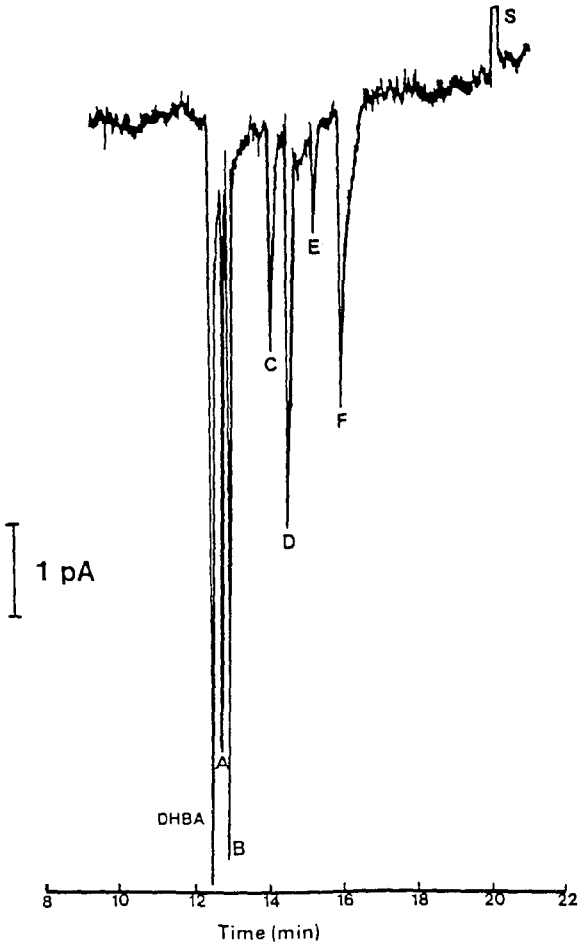


Fig. 3. Electropherogram of amino acids and peptides with indirect amperometric detection on a 9-μm I.D. capillary: buffer, 0.01 m*M* DHBA–0.025 *M* MES (pH 5.65); 97-cm separation capillary; 1.0-cm detection capillary; injection, 1 s at 20 kV; separation voltage, 20 kV. Peaks: A = 8.5 fmol Lys; B = 8.2 fmol Arg; C = 7.4 fmol His; D = 7.0 fmol Arg-Leu; E = 6.4 fmol His-Gly; F = 5.8 fmol His-Phe; S = system peak.

where C_m is the concentration of the electrophore and R is the displacement ratio, which takes into account the net charges of the electrophore and analyte ions[17]. Although the displacement ratio can only be roughly estimated for zone electrophoresis, this equation is useful because it predicts that lowering the electrophore concentration will lead to lower detection limits. Fig. 3 shows an electropherogram obtained for the separation of three non-electroactive amino acids and three non-electroactive dipeptides by using an electrophore concentration only one-tenth that used for the data shown in Fig. 2. In this separation, the numbers of theoretical plates for each peak were calculated from the peak half-widths and were found to be 355 000 for Lys, 169 000 for Arg, 69 000 for His, 161 000 for Arg-Leu, 228 000 for His-Gly, and 71 000 for His-Phe. The amount of each substance injected ranged from 8.5 fmol for Lys to 5.8 fmol for His-Phe in an injection volume of 52 pl. The detection

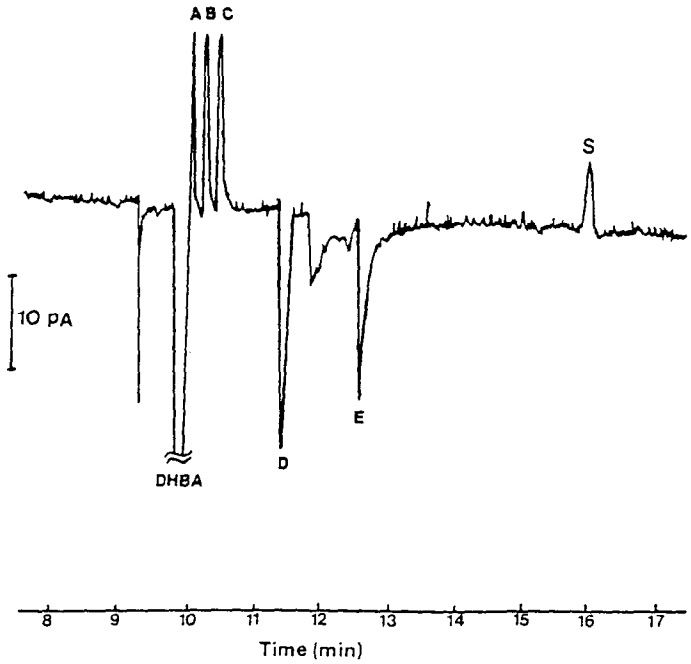


Fig. 4. Electropherogram of catecholamines and peptides with combined direct and indirect amperometric detection in a 9-μm I.D. capillary. Conditions are the same as for Fig. 3, except as follows: injection, 5 s at 25 kV; separation voltage, 25 kV. Peaks: A = dopamine; B = norepinephrine; C = epinephrine; D = Lys-Phe; E = His-Phe. The broad peak between peaks D and E is an unknown impurity in the Lys-Phe.

limit for Arg was 530 amol injected. Detection limits as low as 380 amol have been obtained for Arg with this system (not shown).

Simultaneous direct and indirect amperometric detection

An advantage of indirect amperometric detection is the ability to determine both electroactive and non-electroactive species simultaneously. Fig. 4 shows the separation of three easily oxidized catecholamines and two non-electroactive dipeptides in a 9-μm I.D. capillary. In this case, the detector is responding directly to the oxidation of separated catecholamines, and via the indirect mode for detection of the dipeptides. Total injections are 500 amol for each of the catecholamines, which coresponds to a detection limit of 74 amol when operated under these conditions. The amounts of dipeptides injected are 45 fmol for Lys-Phe and 37 fmol for His-Phe with corresponding detection limits of 5.2 and 5.5 fmol.

CONCLUSIONS

The results presented here demonstrate the utility of indirect amperometric detection with narrow-bore capillary electrophoresis. Separation efficiencies in the range from 69 000 to 355 000 theoretical plates are maintained with this method for the separation of relatively small molecules. A detection limit as low as 380 amol of Arg

has been achieved, and further buffer optimization could reduce this to the level of a few attomoles. In addition, amperometric detection has a distinct advantage over most other detectors in that it can easily be used in both the direct and indirect mode simultaneously. Combination of sensitive detection, instrumental simplicity and a universal nature make this a powerful approach to detection in capillary electrophoresis.

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