Micellar electrokinetic capillary chromatography of vitamin B$_6$ with electrochemical detection

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ABSTRACT

A system for interfacing electrochemical detection with micellar electrokinetic capillary chromatography is demonstrated. This system couples the separation column to a short length of the same column material together with a section of porous graphite tubing which forms an electrically conductive joint. The joint is immersed in a buffer reservoir together with the ground electrode of the high-power source (15 kV). The reservoir is electrically insulated from the electrochemical cell containing the carbon fibre detector. This configuration effectively separates the detector from the high separation potential applied. Amperometric detection with micellar solutions is demonstrated for a mixture of B$_6$ vitamers on a 50 μm I.D. column. A detection limit of *ca.* 4 fmol is obtained. The linear dynamic range of the calibration plot is slightly over two orders of magnitude (from *ca.* 1 to 200 ppm).

INTRODUCTION

Zone electrophoresis in capillaries [capillary zone electrophoresis (CZE)] [1,2] is a rapidly developing field of research. CZE employs extremely high potential fields resulting in highly efficient separations of ionic solutes. In order to extend the advantages of CZE to neutral compounds, micellar electrokinetic capillary chromatography (MECC), first introduced by Terabe and co-workers [3,4], was developed. MECC employs buffers to which surfactants have been added at concentrations above their critical micelle concentrations. Separation is based on micellar solubilization and electrokinetic migration [4]. The two distinct phases, an aqueous phase and a micellar phase, present within the column, migrate at different velocities towards the electrode. As the electroosmotic flow of the aqueous phase is predominant, the micelles are transported towards the cathode, but exhibit a slower net velocity than the bulk aqueous phase. Thus non-ionic solutes appear to partition between the aqueous and micellar phases, which results in solute zone velocities between those of the two phases.

Although CZE has advanced rapidly, some limi-

tations in detection still exist. Owing to the small column dimensions and the extremely small zone widths, there is a need for on-column detection or detectors with very small effective volumes (less than a few nanolitres) in order to preserve the high efficiency of CZE. In addition, the detection technique must not disturb the potential field across the column. Based on these limitations, spectroscopic detectors capable of on-column detection before the cathodic reservoir have been used almost exclusively. Amongst these, laser-induced fluorescence [5,6] is the most sensitive on-column detection mode available for CZE and MECC, providing detection limits as low as sub-femtomoles [5]. However, a drawback of this detection mode is the need to derivatize most samples of interest. UV absorption, although more versatile, has poorer detection limits, generally in the picomole range or higher.

Electrochemical detection (ED) has been shown to be among the most sensitive methods available for capillary chromatography, with detection limits as low as 20 amol being reported [7]. In addition, ED allows the use of smaller diameter columns without losses in sensitivity [8].

Wallingford and Ewing [9,10] were the first to re-

port amperometric detection with CZE and MECC. Owing to the necessity to keep the detection end of the column in a buffer reservoir, electrochemistry, if performed in this reservoir, suffers from high noise levels due to the presence of a high-voltage electric field. To prevent this, the electrochemical detector was decoupled from the separation capillary by a small break in the capillary surrounded by a porous' glass capillary. This ensures that the applied potential drops across the joint, while electroosmotic flow forces the buffer and analyte past the joint to the carbon fibre working electrode positioned inside the end of the capillary. Recently, a new design of CZE–ED was reported by Huang *et al.* [11], in which the use of the aforementioned joint is no longer necessary. This is because the inside diameter of the separation capillary is so small (5 μm) that very little current is passed. With these systems, electrochemical detection of catecholamines, catechols and fluorescamine-labelled amino acids has been demonstrated with MECC [9–11].

Notwithstanding the work described, in general, MECC with ED has been largely unexplored. Our laboratory has recently demonstrated an alternative CZE–ED system that is based on the use of a porous graphite joint created in the column near the cathodic end. Ease of insertion of a carbon fibre electrode into the capillary is due mainly to the design of the electrochemical cell.

In this work, the sensitivity of the CZE–ED system was tested with norepinephrine. Application of the system to the separation and determination of vitamin B_6 and its vitamers was also investigated. Limits of detection, linearity and reproducibility data are presented for the vitamers.
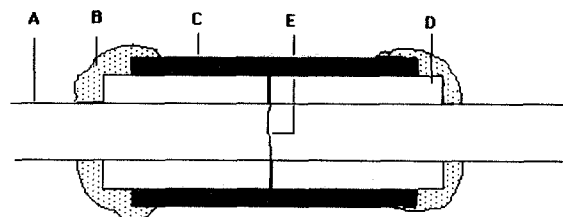

Fig. 1. Schematic diagram of porous graphite joint. A = Fused silica capillary; B = epoxy; C = graphite tube; D = PTFE tube; E = joint.

EXPERIMENTAL

Electrokinetic apparatus

Untreated fused-silica capillaries of 50 μm I.D. were obtained from J&W Scientific. The capillaries were filled with buffer and sodium dodecyl sulphate (SDS) solutions via a syringe. A high-voltage d.c. power supply was used to apply the potential field of 15 kV for separations. Injections were made at the anodic end of the capillary by siphoning from the sample solution at a higher level than the electrophoretic solution in which the other end of the tube was immersed.

Construction of the porous joint

A detailed diagram of the porous graphite joint is shown in Fig. 1. The surface of the polyimide-coated silica capillary, *ca.* 2.5 cm from one end, was lightly nicked with a sharp pen-knife. Gentle pressure applied to this region caused the column to break cleanly. A clean cut was also made at the centre of a 1.5-cm length of PTFE capillary (GL Sciences; O.D. 1.5 mm, I.D. 0.25 mm). Under a microscope, the PTFE capillaries were placed carefully over each end of the cut capillaries such that the joint could be re-formed easily. These two sections of the column were then positioned to form a clean joint in a 1-cm long porous graphite tube of 1.6 mm O.D. Slow-setting epoxy glue (Araldite; Ciba-Geigy) was applied to each end of the graphite segment and also along the region of capillary which was in contact with the PTFE capillary. After being allowed to cure at room temperature, the assembly was placed in a T-shaped glass container (Shown in Fig. 2). The two ends of the T-shaped glass container, containing the protruding capillaries, were sealed with epoxy so that the porous graphite joint and a region of the column were positioned inside the container filled with buffer.

After filling the coupled capillary with buffer, the anodic end was placed in a buffer reservoir. Platinum wires were placed in each reservoir so that a potential of 15 kV could be applied across the longer segment of the capillary (47.5 cm in length), which is termed the separation capillary.

Electrochemical detection

Electrochemical detection was performed with 7-μm diameter carbon fibres (Goodfellow) pro-
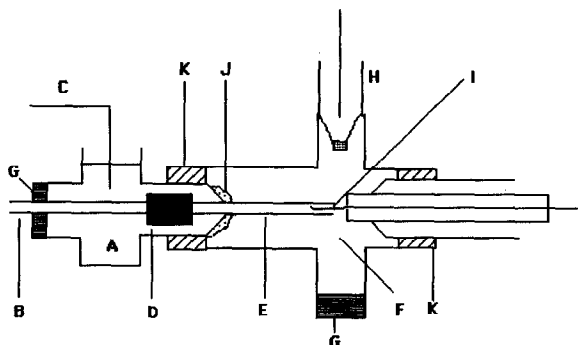
Fig. 2. Schematic diagram of the electrochemical cell. A = Buffer reservoir; B = separation capillary; C = platinum wire; D = graphite joint; E = detection capillary; F = electrolyte; G = stopper; H = reference electrode; I = carbon fibre working electrode; J = epoxy; K = ground-glass joint.

truding 1.0–1.5 mm from drawn glass capillaries as the working electrodes. The procedure for the construction of these electrodes is as follows. The carbon fibre (1.5–2.0 cm) was cemented onto the copper wire with silver-loaded epoxy (Epoxy Technology). This was then pulled through a drawn glass capillary such that *ca.* 0.5–1.0 cm of the fibre was extended from the tip of the capillary which was sealed with epoxy. The epoxy was allowed to cure at 70°C for 1 day. The carbon fibre was then cut so that *ca.* 1.0–1.5 mm was left exposed.

The electrochemical cell is shown in Fig. 2. The end of the detection capillary was positioned in the centre of the cell. The microelectrode was manipulated through the opposite end and into the detection capillary while viewing under a microscope. In operation, the detector was employed in a two-electrode mode. A sodium-saturated silver/silver chloride (Ag/AgCl) electrode was used as the second electrode. The cell was filled with electrolyte solution of 0.01 *M* phosphate buffer containing 10 m*M* SDS. The whole assembly was placed in an aluminium box which functions as a Faraday cage. Potential was applied between the reference and working electrodes using a waveform generator (Model PRR1; Hi-Tek Instruments). Oxidation currents were amplified and recorded with a Keithley Model 617 programmable electrometer and a Linear Instruments 1200 chart recorder, respectively.

Cyclic voltammetry

A conventional three-electrode voltammetric cell was used. An aqueous Ag/AgCl electrode, joined with a salt bridge, and platinum wire served as the reference and counter electrodes, respectively. Glassy carbon (3 mm diameter) was used as the working electrode. Cyclic voltammetry was performed with an EG & G Princeton Applied Research Model 264A polarographic analyser and voltammograms were recorded using a Graphtec W × 1100 recorder.

Chemicals

Sodium dihydrogenphosphate and sodium dodecyl sulphate were obtained from Fluka. The buffer solutions were prepared with water obtained from an Alpha Q water purification system (Millipore). Norepinephrine was purchased from Aldrich and pyridoxal, pyridoxol and pyridoxamine hydrochloride from Merck. Vitamin B$_6$ tablets containing 100 mg of pyridoxol hydrochloride per tablet as stated on the label were obtained from Blackmones.

Procedure for determination of volume injected

The anodic end of the capillary was immersed in the sample solution and lifted to an injection height of 8 cm and injection was performed for 5 s. The capillary was then immersed in the buffer reservoir at the same injection height. The solute plug was allowed to migrate in the tube by gravity and the migration time was measured.

Determination of vitamin B$_6$

All calibration standards of B$_6$ vitamers were prepared in the electrophoretic medium used. Solutions of these standards were stored at pH 4.60 in the dark and used for only 1–3 days.

The sample solution was prepared by dissolving one pulverized tablet in 20 ml of buffer followed by filtration through 0.7-μm Millipore filters. This stock sample was further diluted (1:250) with the electrophoretic medium before injection.

RESULTS AND DISCUSSION

Electrochemical detection interface

The heart of the MECC–ED system is the porous graphite joint created at the junction of the separation and detection capillaries. This porous conductive joint facilitates the isolation of the detector from

the high separation potential. The current arising from this separation potential is about 8 μA whereas the signal from the amperometric detector typically ranges from pico- to nanoamps. Therefore, if the detector is not isolated from the high electric field, small fluctuations in the separation current can amount to very high noise levels (maybe several orders of magnitude greater than the detector signal). For low-level current measurements as in this instance, the use of a two-electrode mode with the cell shielded from external electrical noise has been recommended [12].

The porous conductive joint was constructed easily by pushing the two capillaries through the graphite tube. With the gauge of the PTFE capillary, which forms a tight fit between the graphite tube and silica capillary, the separation and detection capillaries can be aligned easily within the porous graphite tube. When this coupler assembly is immersed in the buffer with the cathode, the separation potential can be applied selectively to the separation capillary. The strong electroosmotic flow generated in this column serves to force the solvent and analyte zones past the joint and through the second section to the detector.

In this work, a major concern with coupling the off-column detector to the capillary column is band broadening due to the graphite interface and the dead volume in the detection capillary. The band broadening aspect of the porous glass coupling system has been discussed in detail previously [9]. It was realized that the major sources of added band broadening stem from dispersion along the length of the detection capillary and the difficulty in attaining precision in the alignment of the two sections [10]. To overcome these problems, the detection capillary was kept as short as possible (not longer than 2.5 cm) in order to minimize band broadening. Further, good alignment was achieved by using tight-fitting tubings. The success of the coupling system was demonstrated by injecting norepinephrine (NE). The peak observed (Fig. 3) showed no indication of severe band broadening. The peak width for a similar length of column and migration time is comparable to those in previous work [10]. A detection limit (signal-to-noise ratio = 3) of the order of 0.4 fmol has been extrapolated for NE.

The currents (*ca.* 8 μA) flowing through the column due to the application of a 15-kV high

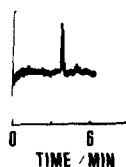Fig. 3. Electropherogram of 0.2 ppm of norepinephrine on a 50 μm I.D. column. 0.01 M phosphate buffer (pH 4.60) containing 10 mM SDS; separation potential, 15 kV (7.4 μA); electrode potential, 0.82 V *vs.* Ag/AgCl.

voltage were found to be in the same range as those observed with an MECC system without ED that had a similar length and diameter of column filled with buffer solution of similar concentration. This implies that the porous graphite joint in no way impedes the flow of current between the two ends of the high separation potential. Further, the graphite coupler is mechanically strong, which leads to ease of handling and manipulation in the T-shaped buffer reservoir. The use of ground-glass connections in the electrochemical cell (see the assembly in Fig. 2) also allows for simple dismantling of the set-up for cleaning and changing of solutions and ease of insertion of the carbon fibre working electrode.

One of the problems observed with this assembly is the deterioration of epoxy in buffer solutions. This deterioration was manifested in unacceptable noise levels due to leakage of current from the separation potential field. This problem can be alleviated by winding sealing film tightly over the epoxy. Finally, it is important to keep the graphite joint immersed in buffer to prevent it from drying out. It was noticed that if the joint was allowed to dry (for *e.g.*, in the oven), then on re-immersion in the buffer the detector could not be used for several hours owing to displacement of tiny air bubbles from the pores of the graphite tube which escaped through the detection capillary. However, once the air bubbles had been completely removed, this problem vanished.

Determination of vitamin B_6

Vitamin B_6 is not a single chemical entity but consists of pyridoxine (pyridoxol, PN), pyridoxamine (PM) and pyridoxal (PL), all of which are biologically equivalent. In the human body, pyridoxal and pyridoxamine, which are metabolites of pyridoxine, are precursors of important coenzymes.
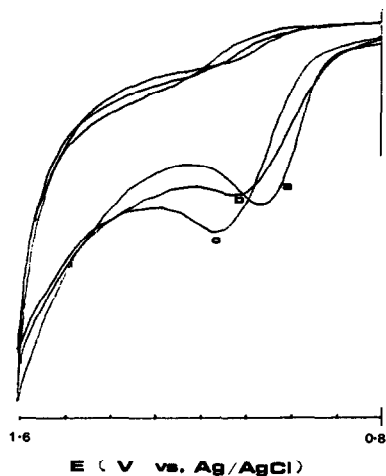
Fig. 4. Oxidation of $1 \cdot 10^{-3}$ M (a) PM, (b) PN and (C) PL in 0.01 M phosphate buffer containing 10 mM SDS at a carbon-fibre electrode. Reference electrode, Ag/AgCl; scan rate, 50 mV s^{-1}.

MECC has been employed to separate and determine B_6 vitamers in biological fluids [13] using laser-excited fluorescence detection. In this work, we report the use of ED, which allows better sensitivity.

The cyclic voltammograms of the three B_6 vitamers are shown in Fig. 4. They were obtained at the glassy carbon electrode in phosphate buffer solution containing 10 mM SDS and 0.1 M sodium perchlorate as supporting electrolyte. From Fig. 4, it was observed that the oxidation reactions were irreversible, with peak potentials at 1.06, 1.14 and 1.16 V for PM, PN and PL, respectively.

For the separation of the B_6 vitamers, a separation potential of 15 kV was applied, resulting in a current of 7.3 μA. Detection was effected by applying a potential of 1.20 V with respect to the Ag/AgCl (saturated NaCl) electrode. This potential is at least 40 mV past the peak potential for all the three compounds. From Fig. 5, it can be observed that this technique exhibits the selectivity and efficiency needed to separate the B_6 vitamers.

The limits of detection obtained in this work are given in Table I. They were determined by injecting 1 ppm solutions prepared from serial dilutions of the concentrated standards. The limits of detection were extrapolated to a signal-to-noise ratio of 3 and the results obtained were two orders of magnitude lower than those reported for a laser-excited fluorescence detector [13].
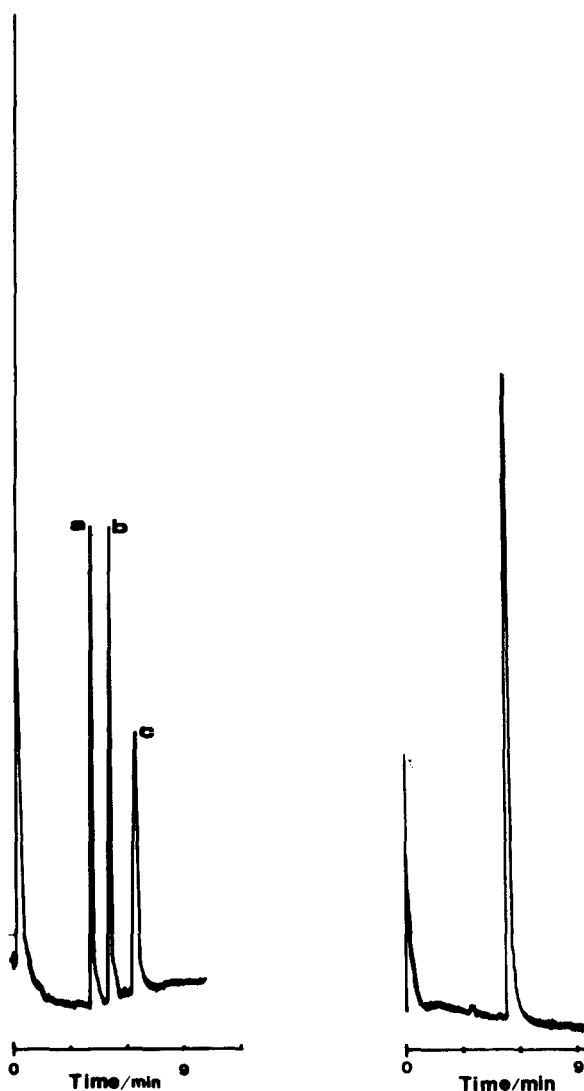
The quantities listed in Table I are injected

Fig. 5. Electropherogram of (a) PL, (b) PN and (c) PM. Electrode potential, 1.2 V vs. Ag/AgCl; other conditions as in Fig. 3.

Fig. 6. Electropherogram of a 250-fold dilution of extracted vitamin B_6. Electrode potential, 1.2 V vs. Ag/AgCl; other conditions as in Fig. 3.

amounts determined by the procedure given under Experimental. With the diameter of the capillary and the velocity of solute plug being known, the volume injected by gravity feed at this injection height can be calculated and subsequently the amount injected can be determined. The calculated volume of the sample plug injected in this manner

TABLE I

CALIBRATION DATA

Vitamer	DL^a (pg)	R.S.D.^{b} (%)	ρ^c
PN	1.0	3.9	0.999
PL	2.0	2.6	0.992
PM	1.0	4.1	0.996

^{a} Detection limit (based on a signal-to-noise ratio of 3; concentration of injected solution = 1 ppm).
^{b} Relative standard deviation (n = 3).
^{c} Correlation coefficient.

was *ca.* 2.4 nl, which is sufficiently small to preclude any overloading effects that can be detrimental to efficiency [14,15].

Table I also shows the reproducibility of the injections. These range from 2.6 to 4.1% relative standard deviation (R.S.D.), which is satisfactory. Calibration graphs for the three vitamers were obtained with good linearity (correlation coefficients from 0.992 to 0.999) over two orders of magnitude (from *ca.* 1 to 200 ppm). All experiments were performed in triplicate.

A chromatogram of the vitamin B$_6$ sample is shown in Fig. 6. The peak is that of pyridoxol hydrochloride (PN). This sample is a 1:250 dilution of the solution prepared by dissolving one tablet in 20 ml of micellar solution. The concentration of PN is *ca.* 19.7 ppm, as determined by standard addition. This corresponds to 98.3 mg (R.S.D. 0.98%) of PN in one tablet, which is close to that stated on the label (100 mg).

CONCLUSION

A simple, rugged, yet sensitive electrochemical detection system was developed for capillary electro-

phoresis. Satisfactory performance of the system was demonstrated with the determination of vitamin B$_6$. Preliminary results indicate that the MECC–ED system has the potential to achieve high sensitivity as a microanalytical method. Currently, our efforts are directed towards the optimization of the operating conditions of the system.

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