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Separation of cyclic nitroxide free radicals and their redox forms with dual microelectrochemical detection

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Abstract

A gradient elution method with conventional electrochemical detection was developed to separate four analogs of the nitroxide 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) free radical in less than 3 min. The corresponding hydroxylamines and oxoammonium ions of these free radicals were separated using the identical chromatographic method. A dual microelectrochemical detector was constructed that allowed for simultaneous anodic and cathodic detection of all three redox forms. This detector was superior to a conventional electrochemical detector because it could provide immediate information about the oxidation state of the nitroxide, it nearly eliminated post-column band broadening, and it was less susceptible to changes in oxygen concentration of the mobile phase during gradient elutions.

Keywords: Detection, LC; Microelectrochemical detection; 2,2,6,6-Tetramethyl-1-piperidinyloxy free radical; Nitroxide free radicals

1. Introduction

Cyclic nitroxides are stable free radicals that are useful probes of biomolecules and membranes as well as participants in a variety of important biochemical reactions [1]. One important class of biological reactions that these compounds undergo are redox reactions. As shown in Fig. 1, the free radical can be readily oxidized to an oxoammonium ion or reduced a hydroxylamine. The amount of nitroxide in a particular oxidation state can yield important information about the oxidizing or reducing ability of the nitroxides chemical environment [1]. The free radicals are metabolized by cells at different rates following malignant transformation [2,3]. For this reason, the ability to monitor the metabolism of nitroxides may lead to improved strategies for early detection of cancer. The oxoammonium ion can protect cells from damage by catalyzing the dismutation of toxic superoxide ions [4]. Additionally, hydroxylamines are permeable to the lipid bilayer of cell membranes and can act as electron shuttles to compounds which are encapsulated in a liposome and would otherwise be inaccessible to reducing agents [5,6].

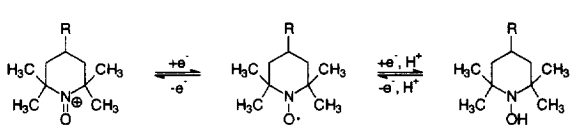

Fig. 1. Electrochemical reactions of a cyclic nitroxide free radical (center) to form the oxoammonium ion (left) and the hydroxylamine (right).

A method that is able to monitor all three redox

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forms of the nitroxide would be valuable for following the redox reactions of nitroxides. The predominant method used to monitor reactions of the nitroxides is electron paramagnetic resonance (EPR). However, only the free radical form of the nitroxide is paramagnetic, so EPR measurements are limited to monitoring changes in concentration of the free radical. No information about the other redox forms of the nitroxide is obtained. The free radical and oxoammonium ion forms of the nitroxide absorb ultraviolet radiation, but the hydroxylamine typically has a very small molar absorptivity [7]. Reactions of nitroxides have been investigated with electrochemical methods [8], but it is difficult to distinguish redox states directly.

If the redox forms of the nitroxide are first separated with liquid chromatography, electrochemical detection can be used because the free radical and the oxoammonium ion can be reduced, while the free radical and the hydroxylamine can be oxidized. Although it is not possible for all three redox forms to exist in solution (the hydroxylamine and the oxoammonium ion proportionate to form the free radical), it is desirable to have a method that can screen solutions for the presence of all three redox forms. However, to detect all three redox forms in a single chromatographic run, simultaneous oxidations and reductions must be recorded. This can be achieved with parallel dual electrochemical detection where the potential of one electrode is set to oxidize the free radical and the hydroxylamine and the other is set to reduce the free radical and the oxoammonium ion.

Dual thin-layer electrochemical detectors have been commercially available for some time, and their advantages over single-electrode detectors are well-documented [9]. Detectors constructed from multiple microelectrodes have flow-independent response, very low background noise and can record current at several different potentials simultaneously [10–13]. Additionally, if a microelectrode detector is directly mated to the column outlet, the elimination of connecting tubing combined with the ultra-low detection volume (<1 nl) nearly eliminates post-column band broadening [14]. Two groups have realized these advantages in capillary electrophoresis using a dual on-column microelectrode detector [15,16]. An additional benefit of using microelectrodes in conventional liquid chromatography is that the microelectrode can be placed at the column center, where separation efficiency is highest [17]. A dual microelectrode detector directly mated to the center of a conventional liquid chromatography column, therefore, should be an excellent detector for nitroxides and their redox forms.

The objective of this work is to develop a method capable determining the redox states of different nitroxides from a chemical or biological system. First, a method is developed to separate a mixture of the free radicals of TEMPO, 4-hydroxyTEMPO, 4-aminoTEMPO and 4-carboxyTEMPO. This same method can be used to separate a mixture of one of these free radicals and its redox forms. Next, it is shown that a dual microcylinder detector placed in an inexpensive plastic fitting can be used for simultaneous anodic and cathodic detection while nearly eliminating post-column band broadening. This, combined with the position of the detector at the center of the outlet frit, results in 2-channel chromatograms with significantly less band broadening than obtained at a conventional electrochemical detector.

2. Experimental

2.1. Chromatographic system

The chromatographic system consisted of two LC-600 gradient elution pumps (Shimadzu, Kyoto, Japan), a PEEK manual injection port with a 20 μl loop (Rheodyne, Cotati, CA, USA), a 30×4.5 mm C_8 cartridge column with 5 μm particles (Applied Biosystems, Foster City, CA, USA), and either a commercial thin-layer detector or a dual microelectrochemical detector (described in Section 2.2). Because nitroxides can be reduced by stainless steel [18], PEEK tubing and fittings were used throughout the chromatographic system. An EI-400 bipotentiostat (Ensman Instrumentation, Bloomington, IN, USA) was used for potential control and current amplification for all chromatographic experiments. The data was recorded on a personal computer equipped with a Labmaster data acquisition card (Scientific Solutions, Solon, OH, USA) using locally

written software. All separations were carried out at room temperature.

Mobile phase A consisted of 0.2 *M* sodium phosphate monobasic, adjusted to pH 3.0 with phosphoric acid, and 10%, v/v acetonitrile. Mobile phase B differed only in the concentration of acetonitrile (40%). A flow-rate of 1.0 ml/min was used for all experiments. For isocratic separations, 90% A and 10% B was used, yielding an effective acetonitrile concentration of 13%. For gradient elutions, the gradient consisted of a linear change from 10% B at injection to 100% B at 1.2 min. Following each separation, the column was equilibrated for 7 min before injecting another sample.

2.2. Electrochemical methods

The conventional electrochemical detector was a TL-5 thin-layer cell (Bioanalytical Systems, W. Lafayette, IN, USA) with a single glassy-carbon working electrode (r=1.5 mm). The flow channel (volume ≈10 μl) was formed by a 0.005-in. (1 in.=2.54 cm) (12.7 μm) thick gasket sandwiched between two blocks, one containing the working electrode and the other containing inlet and outlet ports. The outlet tubing from the flow cell was made of stainless steel and served as the auxiliary electrode. The block containing the working electrode had an additional port (downstream from the working electrode) to which the reference electrode reservoir was connected. When necessary, the working electrode was polished with 1 μm alumina. No further chemical or electrochemical treatments were used.

Fig. 2 shows a schematic diagram of the dual microcylinder detector. The detector was prepared by placing single carbon fibers with radii of 5 μm (Thornell P-55, Amoco Performance Products, Atlanta, GA, USA) in each side of a thick-septum theta glass capillary (World Precision Instruments, Sarasota, FL, USA). Theta glass, named for its resemblance to the Greek letter theta, is a single barrel capillary that has an electrically insulating glass septum running down its entire length. For the thick septum glass used here, the outer wall is 0.2 mm thick, the septum is 0.5 mm thick and the outer diameter of the capillary is 1.5 mm. The theta glass capillary was then pulled in a pipet puller (Sutter

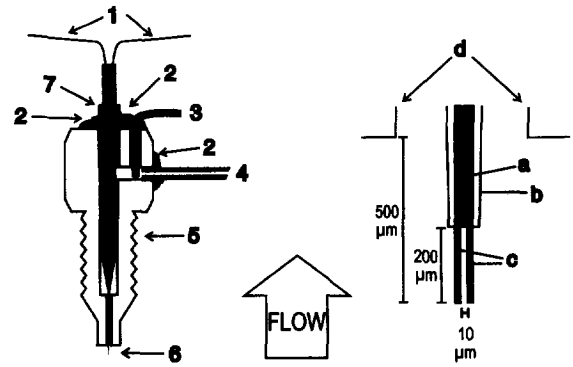

Fig. 2. Dual microcylinder detector: (1) hook-up wires; (2) epoxy sealant; (3) platinum wire auxiliary electrode; (4) exit tubing leading to reference electrode; (5) minitight fitting; (6) dual microcylinder working electrode. The right-hand portion of the figure shows an enlarged detail of the dual microcylinder electrode: (a) septum of the theta glass capillary; (b) outside wall of the theta glass capillary; (c) carbon-fiber cylinder electrodes; (d) minitight fitting.

Instruments, Novato, CA, USA) and carefully placed inside a Minitight fitting (Upchurch Scientific, Oak Harbor, WA, USA) that had been prepared as described previously [14]. Briefly, the center hole was enlarged to accommodate the electrode and a hole perpendicular to the fitting axis was drilled to the center to serve as a waste drain. An additional hole was drilled perpendicular to the waste hole, and a platinum wire auxiliary electrode was epoxied in place (TorrSeal epoxy, Varian Vacuum Products, Lexington, MA, USA). Next, the pulled capillary was inserted into the central hole using a piece of PTFE tubing (I.D.=1.5 mm) as a guide. The tip of the capillary was allowed to extend beyond the end of the fitting and then the assembly was placed under a microscope. Next, the tip of the pulled capillary was cut so that approximately 200 μm of each carbon fiber extended beyond the glass insulator. The distance between the parallel carbon-fiber electrodes was approximately 10 μm. The gap between the fibers and the glass insulator was filled with epoxy (Epon 828, Shell Oil, Houston, TX, USA). After electrical connection was made between each carbon fiber and a hook-up wire with mercury, the electrode was epoxied (TorrSeal) in the fitting so that the carbon fibers extended beyond the tip of the fitting by less than 500 μm. The completed detector was then screwed directly into the column outlet and the

waste drain was screwed into a tee containing the reference electrode. The carbon fiber electrodes were used without chemical or electrochemical treatment.

Bulk electrolysis was carried out at pH 7 (phosphate buffer) in a 75-ml glass cell using a reticulated vitreous carbon mesh electrode (Bioanalytical Systems). The platinum wire auxiliary electrode was isolated from the bulk solution by a porous glass frit. A commercial voltammetric analyzer (BAS-50W, Bioanalytical Systems) was used for all cyclic voltammetry and bulk electrolysis. Potentials for bulk electrolysis were selected from cyclic voltammograms of each nitroxide. Bulk oxidations were performed at a potential 0.15 V more positive than the anodic peak potential, and reductions were done at 0.1 V more negative than the corresponding cathodic potential. Loss of the free radical was confirmed by recording the EPR and UV–Vis spectra before and after electrolysis. The electrolyses were run until a current ratio of 1% of original was achieved for oxidations and 5% for reductions. This difference is due to the increased background currents recorded during the reductions. Where specified, solutions were partially electrolyzed by stopping the electrolysis before completion. All solutions were stored at 4°C.

A commercial Ag/AgCl reference electrode (Bioanalytical Systems) was used for all experiments.

2.3. Reagents

All water was distilled and deionized. The free radical forms of the TEMPO analogs were purchased from Aldrich (Milwaukee, WI, USA) and stored in a dessicator below 0°C. All other reagents were used as received from commercial sources.

3. Results and discussion

3.1. Method optimization

Choosing a suitable column for the separation of TEMPO and its analogs is complicated by the difference in polarities among these free radicals. Columns of conventional length with C_{18} and C_8 stationary phase result in at least 1-h retention times for TEMPO, even at elevated organic modifier concentrations. Elution times for TEMPO in C_1, C_2 and amine columns are much lower, but the remaining more polar analogs cannot be resolved. The final choice of a 3-cm long C_8 column allows for resolution of the free radicals studied in under 3 min after separation conditions are optimized.

The first separation parameter optimized was organic modifier concentration. Acetonitrile was chosen as the modifier because of strong interaction between TEMPO and the stationary phase. The elution time of TEMPO is much more sensitive to changes in acetonitrile concentration at pH 7 than the other three polar analogs. Even with a short column TEMPO is retained indefinitely on the stationary phase with no added acetonitrile. As the acetonitrile concentration approaches 40%, TEMPO elutes within 3 min. The oxoammonium ion of TEMPO, however, is not soluble above 25% acetonitrile. For this reason, a linear gradient from 13% to 40% acetonitrile over the first 1.2 min is chosen for all separations involving TEMPO. In samples with no TEMPO, isocratic conditions with 13% acetonitrile are chosen.

Of the structural analogs in this study, only the retention times of 4-carboxyTEMPO and 4-aminoTEMPO are significantly affected by changes in mobile phase pH. The pK_a values of these nitroxides are 4.12 and 8.97 [19], respectively, so at neutral pH both exist as ions. As the pH of the mobile phase is lowered, a larger fraction of 4-carboxyTEMPO is present in the protonated form, resulting in a longer elution time. An optimum pH of 3 is selected for all analyses because at this pH all four analogs are completely separated under both gradient and isocratic conditions.

In order to find the optimal potential for anodic and cathodic detection, hydrodynamic voltammograms were recorded. Fig. 3 shows a plot of normalized peak height as a function of detector potential for the four free radicals. In this figure, oxidations are represented as negative (down) current and reductions are represented as positive (up) current. The data shows that for all four free radicals, anodic detection is more sensitive than cathodic detection. Best sensitivity is obtained at +1.0 V for oxidations and −0.8 V for reductions. Fig. 3 also shows that 4-aminoTEMPO and 4-hydroxyTEMPO can be de-

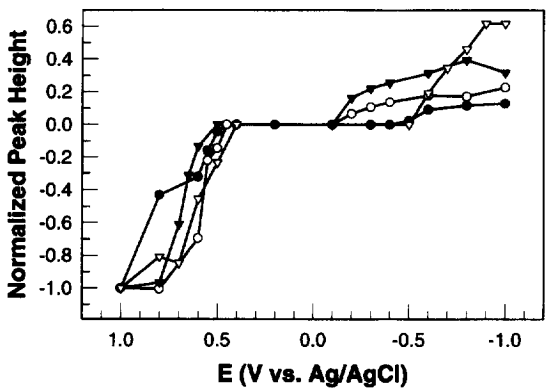

Fig. 3. Hydrodynamic voltammograms for TEMPO (●), 4-hydroxyTEMPO (○), 4-aminoTEMPO (▼) and 4-carboxyTEMPO (▽). For comparison purposes, the data for each compound has been normalized to the peak height at +1.0 V. Data points are the average peak height for three chromatograms.

tected at potentials less negative than that at which oxygen reduction occurs (about −0.6 V). This may be especially useful in conditions where gradient elutions with cathodic detection are necessary.

Shown in Fig. 4 are chromatograms for a mixture of the four free radicals with anodic (A, +1.0 V) and cathodic (B, −0.8 V) detection using the gradient elution. At this pH (3.0), the cationic 4-amino-TEMPO elutes first, followed by the neutral species 4-hydroxyTEMPO, 4-carboxyTEMPO and TEMPO.

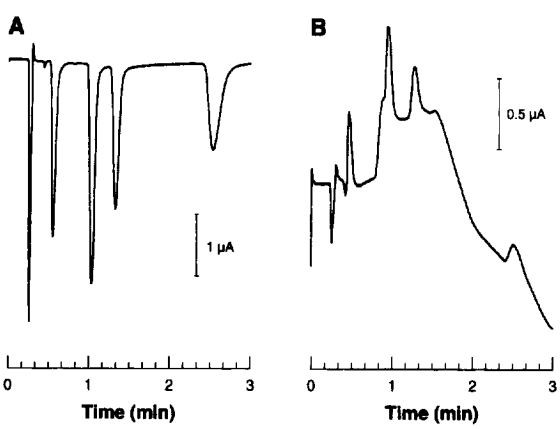

Fig. 4. Separation of the nitroxide free radicals (10 nmol each) using the gradient program with (A) anodic thin-layer detection at +1.0 V and (B) cathodic thin-layer detection at −0.8 V. The radicals, in order of elution, are 4-aminoTEMPO, 4-hydroxy-TEMPO, 4-carboxyTEMPO and TEMPO. The first peak at 0.18 min is the void peak.

The difference in sensitivity between anodic and cathodic detection is readily apparent from the difference in peak size. An additional factor that affects the sensitivity of the cathodic detection is the background reduction of oxygen. The changing baseline in Fig. 4B arises from differences in oxygen concentration between the mobile phase reservoirs. Although both reservoirs are sparged and pressurized with helium, oxygen can diffuse into the mobile phase through the plastic connecting tubing.

3.2. Separation of redox forms

Separate chromatographic runs are required for the oxidized solutions and the reduced solutions of the free radical since these redox forms cannot coexist in solution. Chromatograms of the electrolysis solution following reduction of the free radical to the corresponding hydroxylamine are shown in Fig. 5. Gradient elution is used only for TEMPO (Fig. 5A). For each solution, spectra obtained by EPR and UV–Vis spectroscopy are consistent with the formation of the hydroxylamine. In the chromatogram of each solution, an attenuated peak for the free radical is observed and a new peak appears for the hydroxylamine at a shorter retention time than the free radical.

Fig. 6 shows chromatograms with cathodic electrochemical detection for solutions that have been subjected to oxidative bulk electrolysis. As before, gradient elution is used only for TEMPO (Fig. 6A). Each chromatogram shows a peak for the free radical, and at least one additional peak. Peak 3 is a system peak that is observed in all cathodic chromatograms and comes from the reduction of oxygen dissolved in the sample. This peak is absent from chromatograms recorded after injections of deoxygenated samples.

The chromatogram of oxidized TEMPO in Fig. 6A exhibits no distinct reduction peak for the oxoammonium ion. The small peaks just past the void peak may arise from the oxoammonium ion of TEMPO. Electrochemical and spectroelectrochemical analysis of this ion shows that it is unstable but reverts back to the free radical [7,20]. The chromatogram of oxidized 4-hydroxyTEMPO in Fig. 6B also exhibits no peak for the oxoammonium ion. This is consistent with results obtained by electrochemistry and EPR

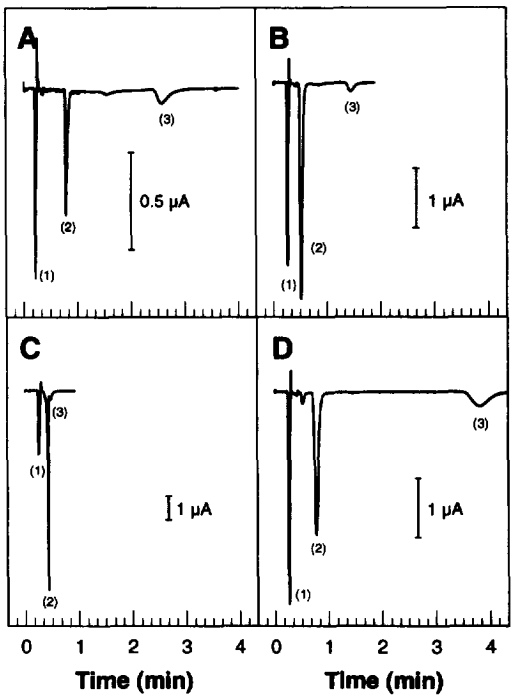

Fig. 5. Separation of the hydroxylamine and free radical redox forms of (A) TEMPO, (B) 4-hydroxyTEMPO, (C) 4-amino-TEMPO and (D) 4-carboxyTEMPO with anodic thin-layer detection at +1.0 V. For each chromatogram, peak (1) is the void peak, peak (2) is the hydroxylamine and peak (3) is the free radical. The gradient program was used for TEMPO; all other chromatograms were recorded under isocratic conditions with 10% acetonitrile in pH 3.0 phosphate buffer. Each hydroxylamine was prepared by bulk electrolysis of a 0.5 m*M* solution of the corresponding free radical.

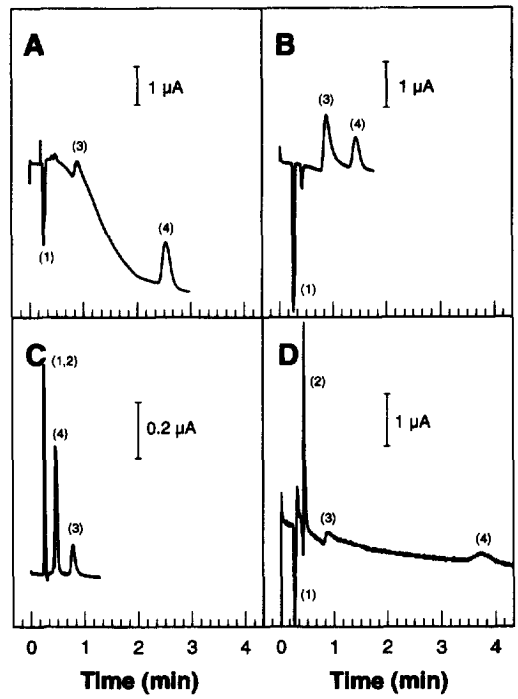

Fig. 6. Separation of the oxoammonium ion and free radical redox forms of (A) TEMPO, (B) 4-hydroxyTEMPO, (C) 4-amino-TEMPO and (D) 4-carboxyTEMPO with cathodic thin-layer detection at −0.8 V. For each chromatogram, peak (1) is the void peak, peak (2) is the oxoammonium ion, peak (3) is a system peak and peak (4) is the free radical. Each oxoammonium ion was prepared by bulk electroysis of a 0.5 m*M* solution of the corresponding free radical.

that indicate the oxoammonium ion of 4-hydroxy-TEMPO is unstable but does not revert back to the free radical. The inverse peak observed just after the void volume is of unknown origin. Although the peak direction indicates oxidation, it is unlikely that a species would be oxidized at such a negative potential (−0.8 V).

The oxoammonium ion of 4-aminoTEMPO has a +2 charge. As shown by the chromatogram of oxidized 4-aminoTEMPO in Fig. 6C, no new peak is observed for this electrolysis solution. The direction of the void peak, however, is opposite that observed for the other oxidized nitroxide solutions. The dication produced by the oxidation, therefore, likely elutes with the void volume and undergoes a 2-electron reduction, resulting in the observed cathodic peak.

Fig. 6D shows the chromatogram of oxidized 4-carboxyTEMPO. Electrochemical experiments have shown that this oxoammonium ion is relatively stable and only slowly reverts to the free radical [20]. This chromatogram shows a large cathodic peak for the oxoammonium ion just after the void volume and a small peak for the free radical. This behavior is consistent with that expected for a stable oxoammonium ion.

3.3. Dual microelectrochemical detection

Fig. 7 shows a chromatogram of the free radical mixture recorded at a dual microcylinder electrode with one electrode set at +1.0 V and the other at

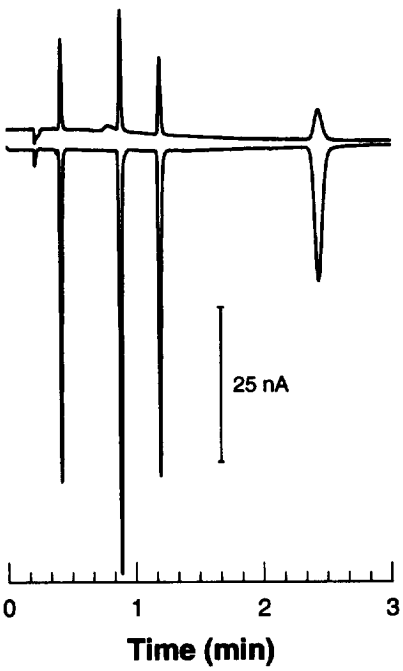

Fig. 7. Separation of the nitroxide free radicals (10 nmol each) using the gradient program with simultaneous anodic (+1.0 V) and cathodic (−0.8 V) microelectrochemical detection. Elution order is identical to that described in Fig. 4.

−0.8 V. All conditions except detector are identical to those of Fig. 4, but the data was acquired in a single run. As with the thin-layer detector, anodic current is plotted in the negative (down) direction and cathodic current is plotted in the positive (up) direction. Immediately apparent is the dramatically improved peak shape. This improvement in peak shape comes from the very small volume of the detector (<1 nl) and the fact that the detector is placed at the exit frit of the column, so no connecting tubing is required.

Another reason that the peak shape is improved is that the electrode only samples the central fraction of the eluant. It is known that when solutes reach the column wall, greater dispersion occurs because of interactions with the wall. Knox et al. have shown that this wall effect can be avoided when

$$\frac{(d_c - 2r_w d_p)^2}{Ld_p} \geq 16\left(\frac{B}{v} + C\right) \quad (1)$$

where d_c is the column diameter, r_w is the number of packing material diameters that this effect extends from the wall (ca. 30), d_p is the diameter of the packing material, L is the length of the column and v is the reduced velocity (ud_p/D_m, where u is the linear velocity of the mobile phase, and D_m is the diffusion coefficient of the solute in the mobile phase) [21]. For packed columns, $B \approx 1.4$ and $C \approx 0.06$ [21]. Under the conditions of this study, the left-hand term greatly exceeds the right-hand term, and so no wall effect is expected. Although in the present study the electrode position could not be changed, it is known that in the type of columns used for this work, even weakly-retained solutes reach the column wall [17]. This deviation from the theory was attributed to non-ideal injection of solutes. Therefore, by placing the detector at the column center, only the central portion of the eluant, where efficiency is highest, is sampled. This selective sampling increases the observed efficiency of the separation.

While there is no doubt that the peaks recorded with the dual microelectrode detector are narrower than those recorded with the conventional detector, it is worthwhile to discuss possible reasons for the broad peaks recorded with the conventional detector. One reason might be chemical interactions between the nitroxides and the glassy carbon electrode. However, we have previously shown that none of these nitroxides or their redox products exhibit adsorption at glassy carbon, and only 4-amino-TEMPO exhibits a very weak adsorption at carbon-fiber electrodes [8]. Another source of band-broadening might be the large injection volume. The injection volume used was 20 μl, compared to a detection volume of 10 μl for the conventional detector. Additionally, the design of the conventional flow cell may contribute to excessive band broadening. Injections of samples with the column removed exhibit broad, tailing peaks, likely arising from poor flow patterns in the detector itself. The detector used here is of an older design (≈15 years old) and has been superseded by cells with more efficient flow patterns.

Also apparent from the chromatograms in Fig. 7 is the smaller void peak and the lower susceptibility to changes in oxygen concentration at the carbon-fiber electrode. The sensitivity of carbon electrodes to many compounds is a function of the chemical or

electrochemical pretreatment of the electrode [22]. Cyclic voltammograms of oxygen in phosphate buffer show that the overpotential for oxygen reduction is greater at an untreated carbon-fiber electrode ($E_{1/2} \approx -0.75$ V) than at a glassy carbon electrode ($E_{p/2} \approx -0.50$ V). At the cathodic detection potential ($E = -0.8$ V), therefore, the rate of oxygen reduction at the glassy carbon electrode is at its maximum value. This results in a higher current for oxygen reduction (relative to the nitroxide free radical reductions) at the glassy carbon electrode than at the carbon-fiber electrode.

An example of the utility of the dual microcylinder detector is shown in Fig. 8. Here partially electrolyzed solutions are analyzed using the dual electrochemical detector. With the dual electrode, the peak for the free radical can be immediately identified because it shows simultaneous anodic and cathodic peaks. An oxoammonium ion exhibits a cathodic peak only, and a hydroxylamine shows an anodic peak only. Fig. 8A shows the chromatogram for partially oxidized 4-carboxyTEMPO. The dual peak (4) for the free radical occurs at 3.5 min, and a cathodic peak (2) for the oxoammonium ion occurs at about 0.5 min. The small anodic peak that occurs

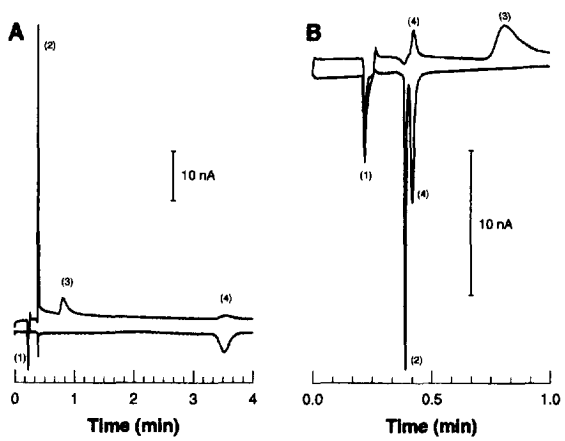

Fig. 8. Chromatograms of partially electrolyzed solutions of cyclic nitroxide free radicals recorded at the dual microelectrochemical detector. (A) Oxidized 4-carboxyTEMPO. Peak (1) is the void peak, peak (2) is the oxoammonium ion, peak (3) is a system peak and the peaks at (4) are the free radical. (B) Reduced 4-amino-TEMPO. Peak (1) is the void peak, peak (2) is the hydroxylamine, peak (3) is a system peak and peak (4) is the free radical. Each solution was prepared by bulk electrolysis of a 0.5 m*M* solution of the corresponding free radical.

simultaneously with the cathodic oxoammonium peak is probably due to diffusional communication between the electrodes.

At microcylinder electrodes, the diffusion layer, δ, is given by the equation

$$\delta = r_0 \ln \frac{2\sqrt{D_m t}}{r_0} \quad (2)$$

where r_0 is the radius of the electrode and t is time [23]. For the peaks with widths of 1 s, the width of the diffusion layer is 11 μm ($r_0 = 5$ μm, $D_m \approx 5 \times 10^{-6}$ cm^2 s^{-1}). Therefore, at the spacing of electrodes used here (ca. 10 μm), the hydroxylamine formed at the cathodic electrode can diffuse back to the anodic electrode and be oxidized back to the oxoammnoium ion. This type of diffusional feedback has been used previously at interdigitated microelectrodes (width = 5 μm, spacing = 5 μm) to enhance the sensitivity of a liquid chromatography detector [24]. The magnitude of the effect in the present detector, and hence the sensitivity, could be increased by using even more closely-spaced electrodes and/or slower chromatographic flow-rates.

Fig. 8B shows a chromatogram for partially reduced 4-aminoTEMPO. As before, the peak for the free radical (4) can be immediately recognized by the simultaneous anodic and cathodic peaks. The hydroxylamine (peak 2) elutes just before the free radical. Baseline resolution between the free radical peak and the hydroxylamine peak is nearly achieved with the microelectrode detector.

The dual microelectrode detector is surprisingly rugged and reproducible. Although the carbon fibers are not chemically or electrochemically treated, their response during the course of the study was stable and reproducible. Once the electrode is in place, it is protected from physical damage or breakage by the column end fitting. Because cartridge columns are used, the electrode can remain in the fitting when the column is replaced. When not in use, the electrode was stored in air without noticeable change in its response. The dual detectors used over the course of the study were not cleaned or treated in any manner. Because of the relative ease of preparation of the electrode, the low cost of materials involved (<$20 US), and because the cylinder electrodes cannot be polished, we recommend that the electrode be dis-

carded upon contamination or loss of a reproducible response.

4. Conclusions

Electrochemical detection is well-suited for the analysis of nitroxide free radicals and their redox forms. Anodic detection is best for detection of the free radical and hydroxylamine forms of the nitroxides. Cathodic detection, although it can be used for detection of the free radicals, is needed only for detection of the oxoammonium ions of TEMPO, 4-aminoTEMPO and 4-carboxyTEMPO. These results also show that, based on order of elution, the free radical form of the nitroxides studied is the least polar, the hydroxylamine is of intermediate polarity, and the oxoammnoium ion is most polar.

Using a dual microelectrochemical detector improves the versatility of this chromatographic method. In addition to the improved peak shape obtained with the microelectrode, the redox form of the nitroxide can be determined readily. The free radical can be immediately identified by the simultaneous anodic and cathodic peaks. The hydroxylamine or the oxoammonium ion also can be identified by the direction of the peak; a cathodic peak indicates presence of the oxoammonium ion and an anodic peak indicates presence of the hydroxylamine. Because only one chromatographic run is required to determine which oxidation states of the nitroxide are present, this ability for simultaneous detection combined with the rapid time scale of the separation make the method particularly useful for studying solutions of questionable stability.

The dual microcylinder detector described in this work has several advantages to previous multiple microelectrode detectors. First, the detector is designed to be easily coupled directly to the column, and therefore no connecting tubing is required. Second, because of their larger size, the sensitivity of microcylinders is superior to that of the disk microelectrodes used previously for most studies [14]. Third, the configuration is readily adaptable to use redox cycling to enhance the sensitivity of the detector. The degree of diffusional interaction between the electrodes can be controlled by varying the distance between electrodes. Finally, the detector construction is simple and the materials required are inexpensive. This low cost virtually makes the detector disposable.

The dual microelectrochemical detector described here should be readily adapted to capillary chromatography of the nitroxides. Microelectrode detectors are well-suited for use in capillary chromatography [25] and the ability to do microscale separations of the nitroxides and simultaneously determine their oxidation states should be of great interest for following redox reactions of nitroxides in biological studies. Additionally, at the slow flow-rates typically used in capillary liquid chromatography, the time term in Eq. (2) would be large. Therefore, increased diffusional feedback between the parallel carbon fibers in capillary chromatography should enhance the sensitivity of this detector.

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