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Simultaneous detection of thiols and disulfides by capillary electrophoresis–electrochemical detection using a mixed-valence ruthenium cyanide-modified microelectrode

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Abstract

Thiols and disulfides are separated and detected by capillary electrophoresis–electrochemical detection using a mixed-valence ruthenium cyanide-modified microelectrode. A carbon fiber array microelectrode was employed to maximize the signal-to-noise ratio. Detection limits for glutathione disulfide, cystine and homocystine were 2.5, 1.3 and 1.1 fmol, respectively. The response for cystine was linear over two orders of magnitude with a correlation coefficient of 0.992. The long-term stability and overall reproducibility of the electrode were investigated and found to be highly dependent on the cation concentration in the electrophoretic buffer. The selectivity of this technique for disulfides was demonstrated by the detection of cystine in the urine of a patient with kidney stones.

1. Introduction

There is currently a great deal of interest in the detection of oxidized and reduced thiols in biological and chemical systems. Thiols and disulfides play important roles in drug metabolism and protein synthesis, as well as having been used as radio-protective agents and antibiotics. Examples of important thiols and their corresponding disulfides include glutathione (GSH), glutathione disulfide (GSSG), cysteine and cystine. Glutathione plays an important role in drug metabolism and toxicity. The appearance of high levels of GSSG in tissue have been shown to be related to oxidative stress. Cysteine is an amino acid which plays a critical role in protein synthesis and structure. High levels of cystine in the urine are indicative of kidney dysfunction.

The determination of disulfides is a particularly challenging analytical problem because most have no distinguishing chromophores, and the thiol group is no longer available for derivatization. One approach is to separate the thiols and disulfides into fractions, and then chemically reduce the disulfide to its corresponding thiol. The resulting thiol in each fraction is then determined colorimetrically following reaction with Ellman's reagent, 3,3'-dithiobis(6-nitrobenzoic acid) [1]. This wet chemistry is tedious, although relatively sensitive. Utilizing this chemistry, disulfides have also been detected following separation by liquid chromatography using a two-stage solid-phase

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postcolumn reactor [2]. Disulfides have also been determined by liquid chromatography–electrochemical detection (ED) using a dual gold–mercury amalgam electrode [3]. In this case, the disulfides are reduced electrochemically prior to detection of the thiol at the second electrode.

Modified electrodes have been used for the detection of a wide variety of analytes including thiols, carbohydrates and amines [4,5]. In 1989, Cox and Gray [6] reported a new type of modified electrode based on a mixed-valence (mv) ruthenium oxide cross-linked with cyanide. This electrode has been employed for the detection of insulin, cystine and methionine following flow injection analysis [4]. More recently, Kennedy et al. [7] utilized the mv RuCN-modified electrode for direct detection of insulin in pancreatic B cells.

Capillary electrophoresis (CE) is a powerful tool for separation of a wide range of analytes, including both large and small molecules [8,9]. Thiols and disulfides have been separated by using CE prior to UV detection [10]. However, this method of detection yields poor detection limits as a results of the small optical pathlength characteristic of CE (typically less than 100 μm). In addition, the wavelength employed is 200 nm, which limits selectivity in biological matrices. Previously, we demonstrated the use of modified electrodes for ED in CE [11,12]. Since this method of detection is based on a reaction occurring at an electrode surface, the limits of detection are not compromised by the small dimensions characteristic of microcolumn-based separation systems. CE–ED has been extensively applied for the analysis of catecholamines in single cells and for the detection of amino acids and pharmaceuticals in microdialysate samples [13–17].

In this paper, the separation and detection of thiols and disulfides by CE–ED is reported. A carbon fiber array microelectrode modified with a ruthenium-containing inorganic film is employed as the working electrode. The selectivity of the method is demonstrated by the determination of cystine in urine.

2. Experimental

2.1. Chemicals and solutions

L-Cystine, D,L-homocystine and oxidized glutathione were obtained from Sigma (St. Louis, MO, USA) and used as received. Stock solutions of disulfides were prepared daily in 1 *M* HCl to a final concentration of 10 m*M*. They were then immediately diluted in 10 m*M* HCl, 10 m*M* sodium phosphate, pH 2.8, to the appropriate concentration level. Solutions were stored at 4°C until use. All solutions were made with NANOpure water (Sybron-Barnstead, Boston, MA, USA) and passed through a 0.2-μm pore size membrane filter prior to CE.

2.2. Apparatus

The construction of the basic CE system has been described elsewhere [11]. However, in this case end column detection was employed, as described by Huang et al. [18]. A 28 cm × 360 μm O.D. × 20 μm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was employed in all separations. The run buffer was 10 m*M* sodium phosphate, 10 m*M* HCl, pH 2.8. Samples were injected using a laboratory-built pressure-injection system. Using the continuous fill mode, the injection volume was calculated to be 440 pl by recording the time required for the sample to reach the detector.

A BAS LC-4C amperometric detector (Bioanalytical Systems, West Lafayette, IN, USA) was used for amperometric detection in a conventional three-electrode mode. A Pt auxiliary and Ag/AgCl reference electrode were used in all studies. Electropherograms were recorded with a Model BD-41 dual-pen strip chart recorder (Kipp and Zonen, Netherlands). The detector potential was set at +850 mV unless otherwise indicated.

Cyclic voltammetry was carried out with a Model CySy-1 computerized electrochemical analyzer (Cypress Systems, Lawrence, KS, USA) in a three-electrode system cell with an

Ag/AgCl reference electrode and a platinum wire auxiliary electrode.

2.3. Construction of microelectrodes

Carbon fiber array microelectrodes were prepared as follows: a bundle (15–20) of 10-μm-diameter carbon fibers (Amoco Performance Products, Greenville, SC, USA) was carefully inserted into a 3 cm piece of fused-silica capillary of 150 μm I.D. × 360 μm O.D., until they protruded approximately 0.5 cm from one end of the capillary and approximately 1 cm out of the opposite end of the capillary. The latter was bonded to a length of copper wire using silver epoxy (Ted Pella, Redding, CA, USA). At the detection end, the carbon fibers (0.5 cm) were cut off along the cross-section of the capillary, which was then filled with epoxy (Miller-Stephenson, Danbury, CT, USA). Once cured, the capillary end was gently polished on fine emery paper until a smooth surface was achieved, with the cross-sections of carbon fibers being clearly observed. The electrode was then ultrasonicated in acetone and NANOpure water for several minutes. A single carbon fiber disk electrode was also made and used for comparison. The same preparation procedure was employed, except that a single 33-μm-diameter carbon fiber was placed in a 50 μm I.D. × 360 μm O.D. fused-silica capillary.

The modification of microelectrodes was performed according to previously reported methods [6,7,19]. Briefly, the electrode was cycled between 500 and 1100 mV vs. Ag/AgCl at a scan rate of 50 mV/s for a total of 50 cycles in a deoxygenated plating solution of 2 m*M* $RuCl_3$, 2 m*M* $K_4Ru(CN)_6$ and 0.5 *M* KCl, which had been adjusted to pH 2.0 with 1 *M* HCl. The initial and final potentials were 500 mV. Following the modification procedures, the electrode was removed from the plating solution, rinsed thoroughly with distilled water, and allowed to air dry for ca. 20 min.

Since problems were encountered with grounding of the Nafion joint at pH values below 3 (presumably due to protonation of the Nafion film), end-column detection was employed. This method, which has been described in detail by Huang et al. [18], mandates the use of capillaries of relatively small internal diameter (20 μm or less) to minimize background current due to the electrophoretic current. In this work, a "wall-jet" design similar to that previously described was used [11].

2.4. Sample preparation

Human urine samples were immediately diluted with the run buffer (1:10 dilution), filtered (2-μm pore) and directly injected onto the CE system.

3. Results and discussion

3.1. Cyclic voltammetry

A cyclic voltammogram of the modified electrode in the supporting electrolyte (solid curve) and in a solution of glutathione disulfide (dashed curve) is presented in Fig. 1. The electrode exhibited only a single redox couple in 10 m*M*

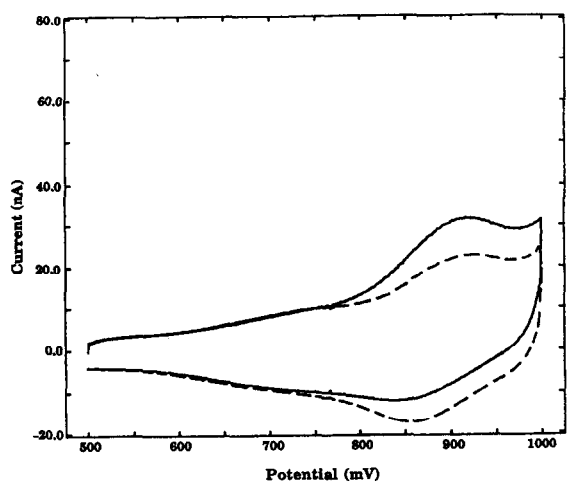

Fig. 1. Cyclic voltammogram of 10^{-4} *M* oxidized glutathione (GSSG) using the mv RuCN-modified carbon fiber array microelectrode in 10 m*M* sodium phosphate–10 m*M* HCl, pH 2.8; scan rate, 50 mV/s. Curve 1 shows the surface waves of the electrode in blank electrolyte.

sodium phosphate buffer, pH 2.8, with the anodic and cathodic peak potentials (E_{pa} and E_{pc}) around 900 and 850 mV, respectively. It has been suggested that these surface waves can be attributed to the reversible oxidation of Ru^{3+} [20,21]. The electrocatalytic oxidation of GSSG using this electrode is apparent in Fig. 1. There is an increase in the oxidation peak current upon addition of the analyte, without any obvious shift in the peak potential. This behavior is characteristic of a typical heterogeneous catalytic oxidation electrode process [5]. As is the case with other modified electrodes containing mixed-valence inorganic films [20–22], the voltammetry was found to be dependent on the cation concentration. However, the voltammetric behavior of the electrode was not dependent on the type of cation used in the supporting electrolyte.

3.2. End-column CE–ED system using a carbon fiber array microelectrode

For these studies, a disk-type microelectrode was employed. Compared to the single carbon fiber electrode, it is much easier to handle and operate and also to align with the separation capillary since it is sealed in a capillary of exactly the same outer diameter as the separation capillary [11]. In addition, its surface can be easily renewed by polishing. This attribute is particularly important for the reproducible modification of the microelectrode. One of the primary advantages of using an array electrode is that it is possible to obtain the higher current responses characteristic of a macroelectrode with these low background currents. Carbon fiber array electrodes have been used effectively as detectors for liquid chromatography [23,24] and flow injection analysis [25]. In all of these applications, the carbon fiber array electrodes have been reported to exhibit low background currents and good reproducibility. In addition, microarray electrodes have been shown to be useful for measurements at extreme positive potentials [26] and have been used in the construction of peroxidase-modified amperometric biosensors for the electrocatalytic reduction of hydrogen peroxide [27]. All of these factors prompted us to investigate the applicability of carbon fiber array electrodes for CE–ED. Therefore, a modified carbon fiber array microelectrode was used for all subsequent work.

Separation of $5 \cdot 10^{-5}$ *M* each of homocystine, cystine and oxidized glutathione by CE–ED using the carbon fiber array microelectrode-based detector is shown in Fig. 2. All of these homodisulfides were resolved and were easily detected at the micromolar level. Detection limits were 2.5, 3.0 and 6.2 μM for homocystine, cystine and oxidized glutathione, respectively ($S/N = 3$). Based on an injection volume of 440 pl, the mass detection limits correspond to 1.1, 1.3 and 2.5 fmol. The response of cystine was examined over the concentration range of $5 \cdot 10^{-6}$ to 10^{-4} *M*. Linear regression analysis yielded a slope of 0.027 nA/μM with a correlation coefficient of 0.992.

The stability of the detector response for cystine was examined using a pressure injection flow-through (FIA) system in the same capillary. A comparison of the response of the electrode in two different buffers, one containing 150 m*M* Na^+ and the other 10 m*M* Na^+, is shown in Fig. 3A. These data show that both the sensitivity and stability of electrode response are much greater in 150 m*M* sodium phosphate buffer than in 10 m*M* sodium phosphate–10 m*M* HCl at the same pH. This is not unexpected considering the

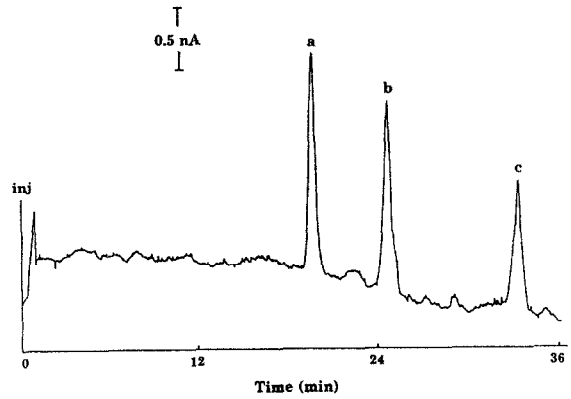

Fig. 2. Electropherogram of $5 \cdot 10^{-5}$ *M* each of homocystine (a), cystine (b) and GSSG (c). Buffer, 10 m*M* sodium phosphate–10 m*M* HCl, pH 2.8. Separation voltage, 20 kV. Detection potential, 850 mV vs. Ag/AgCl.

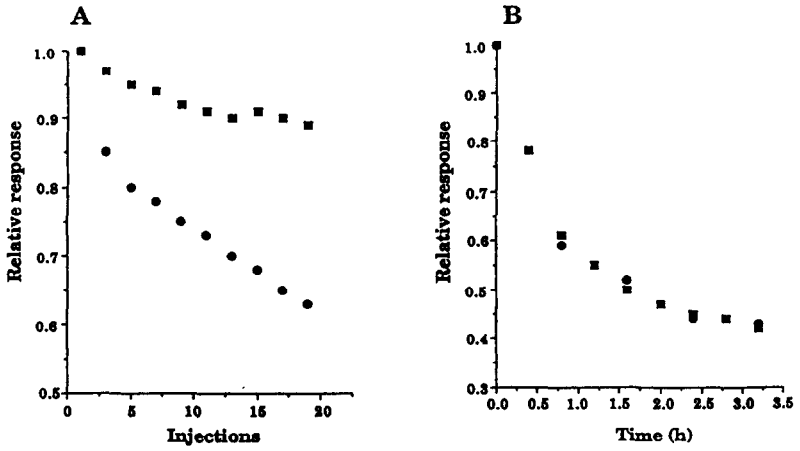

Fig. 3. (A) Relative current response for $5 \cdot 10^{-5}$ *M* cystine at the modified electrode using FIA; carrier phase in FIA is (■) 150 m*M* sodium phosphate, pH 2.8 and (●) 10 m*M* sodium phosphate–10 m*M* HCl, pH 2. Detection potential was fixed at 850 mV vs. Ag/AgCl. (B) Effect of injection frequency of cystine on detector response under CE conditions; run buffer 10 m*M* sodium phosphate; (■) injection of cystine every 15 min and (●) injection of cystine every 45 min.

requirement for a high cation concentration by the mv RuCN-modified electrode to facilitate both the electrochemical process of the deposited film and the catalytic oxidation of disulfides [6,19].

The detector stability under CE–ED conditions is shown in Fig. 3B. It was observed that the current response decreased gradually over time, and that the magnitude was essentially independent of the number of sample injections. This indicates that the reduction in response is not due to passivation of the electrode surface but to a gradual loss of catalytic activity of the film. This observation is in agreement with previous reports in which it has been suggested that the film catalyzes the oxidation of disulfides through the transfer of oxygen as well as electrons, thereby producing a non-passivating product [19].

From all of these observations, it was concluded that the decrease in detector response resulted from the continuous loss of electrochemical activity of the modified electrode due to the use of a buffer solution of inadequate cation concentration. This is confirmed by the observation of the baseline and background noise level in blank run buffer. As can be seen from Fig. 2, the electrode was relatively noisy, and exhibited a baseline drift. These same phenomena were observed in a FIA–ED system using the same buffer conditions. The noise level and baseline drift could be markedly reduced in the FIA–ED by increasing the sodium concentration in the mobile phase to 150 m*M*.

It is clear that the performance of the electrode for the catalytic oxidation of disulfides in the CE–ED system would be significantly improved if a buffer containing a high (> 150 m*M*) concentration of cation were used. Unfortunately, a buffer solution of extremely high conductivity is impractical for the present CE–ED system because the high electrophoretic current causes losses in efficiency due to Joule heating and the generation of high background in the electrochemical detector. It was found that the detector response stabilized after 90 min at 850 mV vs. Ag/AgCl in the 10 m*M* sodium phosphate run buffer. Under these conditions, eight successive injections of 10^{-6} *M* cystine produced a R.S.D. value of 8.7% for peak current measurement.

The stability of the electrode can be improved by decreasing the time period at which the detection potential is applied. Only 40% of the initial response for $5 \cdot 10^{-5}$ *M* cystine remained after 4 h of continuous operation of the working electrode in the CE–ED system. On the other hand, the electrode gave 65% of the initial

current response in the same time period, as long as it was held at open circuit between sample injections. The reproducibility of electrode regeneration was also investigated. Responses for $5 \cdot 10^{-5}$ *M* cystine at ten individually modified electrode surfaces using the same electrode (with the surface being renewed by polishing) resulted in a R.S.D. of 13.2%. The catalytic current was measured for the first sample injection after the electrode had been subjected to the applied potential for approximately 30 min.

3.3. Analytical applications

Simultaneous detection of thiols and disulfides

Thiols such as cysteine and reduced glutathione (GSH) are also detected by this modified electrode. An electropherogram showing the simultaneous detection of 10^{-5} *M* GSH and $5 \cdot 10^{-5}$ *M* GSSG obtained with the CE–ED system is shown in Fig. 4A. This is the first report of the simultaneous electrochemical detection of thiols and disulfides by CE. An analytical application is shown in Fig. 4B for the determination of contaminating GSSG in commercially available GSH (Sigma). That GSH from Sigma contains a small amount of the homodisulfide GSSG has been previously demonstrated by CE using UV detection [10]. Calculations by external standard method revealed that there was about 5% of the contaminant present in the GSH sample.

Determination of urinary cystine

The urinary concentrations of cystine in patients with kidney stones are excessively high because the diminished renal tubular resorption of this compound increases its concentration in urine [28]. Cystine is poorly soluble in acid urine and when its concentration exceeds its solubility, precipitation of both crystals and stones in the urinary tract results.

The application of the CE–ED system with a mv RuCN-modified electrode for the analysis of urinary cystine from a kidney stone patient was performed in order to evaluate its performance in real sample analysis. As shown in Fig. 5, a well-defined cystine peak can be clearly iden-

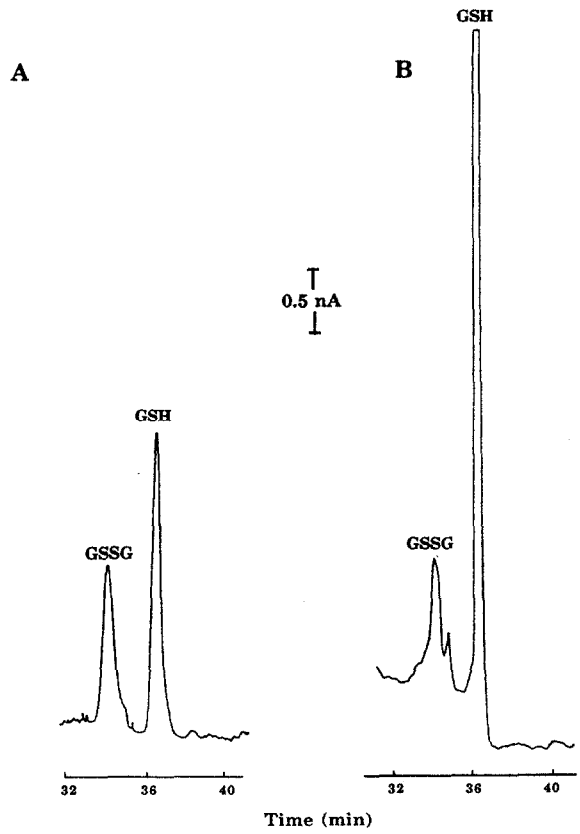

Fig. 4. (A) Electropherograms of $5 \cdot 10^{-5}$ *M* GSSG and 10^{-5} *M* GSH; (B) Electropherogram of GSH sample from Sigma, concentration: 10^{-4} *M*. Conditions as in Fig. 2.

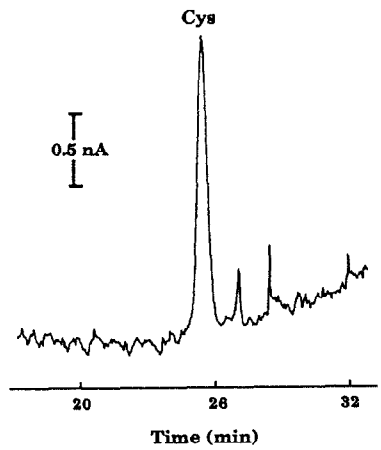

Fig. 5. Electropherogram of a urine sample (1:10 dilute) from a subject with kidney stones. The peak corresponds to $6 \cdot 10^{-4}$ *M* cystine. Conditions as in Fig. 2.

tified from the background of the urine. The concentration of cystine in this urine sample was calculated as approximately 0.6 m*M*. No cystine was detectable in urine obtained from healthy volunteers.

4. Conclusions

A modified electrode based on a mv RuCN film has been evaluated for the simultaneous detection of thiols and disulfides by CE. An end-column CE–ED system using a microarray disk carbon fiber electrode was employed. The divergence in the requirements of buffer (or cation) concentration between CE and ED is still a limiting factor in improvement of the sensitivity and reproducibility of the detection system. Possible solutions to this problem are under investigation.

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