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Short Communication

High-performance liquid chromatography of sulfur-containing amino acids and related compounds with amperometric detection at a modified electrode

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ABSTRACT

Organic disulfides generally are not oxidized at bare electrodes under conditions that are suited to routine amperometric detection, and thiols are typically oxidized in a manner that leads to partial blockage of the surface. Modification of a carbon electrode with a film of Ru(III,IV) oxide stabilized with cyano-cross-links permits the amperometric detection of cystine, cysteine, glutathione, methionine, and glutathione disulfide under conditions compatible with their chromatographic separation on a strong cation-exchange column. Detection limits of 0.2–0.6 μM and linear dynamic ranges of at least 1–50 μM were obtained. The electrode was stable for at least 11 days with a pH 1 citrate, phosphate mobile phase.

INTRODUCTION

Electrochemical detectors are attractive for high-performance liquid chromatography (HPLC) because of their sensitivity and wide linear dynamic range. A limiting factor is that many species which have standard potentials in the range that is appropriate for redox at an electrode are not significantly electroactive at usual surfaces although they are redox active in homogeneous solution. In some cases the difference in behavior is due to slower charge transfer kinetics at electrode surfaces than in homogeneous solution. A second factor is adsorption of an intermediate of the electrochemical reaction that blocks further electrolysis.

Modification of electrode surfaces by immobilizing reversible redox couples thereon provides a route to permit amperometric detection of many of these species. For example, in the study described herein, we use a glassy carbon electrode modified by electrodeposition of a 1–5 monolayer film of a mixed-valence ruthenium oxide polymer cross-linked with cyanide, mvRuCN [1,2]. The Ru-species present at 0.92 V *vs.* a silver–silver chloride electrode is a sufficiently strong oxidizing agent to react with species such as As(III) [1], cysteine [3], glutathione [3], cystine, insulin [4] and

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thiocyanate [5]. These species are not electrochemically oxidized at bare surfaces in a manner that is suitable for analytical methodology. Quantitative results are obtained at the mvRuCN electrode because the current which flows as a result of re-oxidation of the ruthenium is directly proportional to the concentration of the analyte in solution over a wide range.

Another means of overcoming the above-stated limitations of bare electrodes is to step the potential to values that sequentially generate a reactive oxide layer on a surface of Pt or Au which promotes electrolysis and then electrochemically restore the surface to its initial state [6,7]. This pulsing program is repeated for each point displayed in the recorded chromatogram. An example of the applicability of this pulsed detector which is related to the present study is the elimination of the passivation of a Pt surface during the oxidation of thiourea and related compounds [8]. The product of the two-electron oxidation of thiourea is an adsorbed dimer that blocks further charge transfer at a bare electrode. By generating a reactive oxide of the electrode metal, the dimer is further oxidized, and electrode activity is retained.

The first report of application of a modified electrode to amperometric detection in a flow system involved the determination of hydrazine [9]. A carbon paste electrode into which a metallophthalocyanine was mixed was used as a detector for flow-injection analysis and HPLC. Baldwin and co-workers applied this electrode to several other analytes including thiols [10], oxalic acid and α-keto acids [11] and carbohydrates [12]. The practical application of the metallophthalocyanine-loaded carbon paste electrodes is limited by lack of long-term stability without pulsing the potential. We reported the determination of nitrite by flow-injection amperometry at a platinum electrode modified with a film of quaternized poly(4-vinylpyridine) which was impregnated with a redox mediator, hexachloroiridate; however, this complex-loaded ionomer also was not sufficiently stable for practical application [13].

Prahbu and Baldwin recently discussed the relative merits of a modified electrode at constant potential and a classical electrode used in the pulsed mode as electrochemical detectors for carbohydrates [14], which are not generally electroactive at constant potential. Using a copper-based chemically modified electrode [14], 1.2 pmol of glucose was determined by HPLC, and the electrode showed less than 10% loss of sensitivity after 5 h. The useful lifetime of the modified electrode was 2–3 days under constant-potential application. The merits of this system relative to pulsed amperometric detectors for carbohydrates were stated as superior detection limits and compatibility with the most common instrumentation for electrochemical detection in HPLC (that is, current measurement at constant applied potential).

The amperometric detector based on the mvRuCN electrode, which is used in the present study, also operates in a true potentiostatic mode. It yields sensitivities at the pmol level in flow systems [3–5] and is stable for periods up to three months in solutions which are optimized for electrochemical measurements [5]. However, prior to the present study it had not been demonstrated that the mvRuCN electrode retains these characteristics under conditions that are compatible with separation of mixtures by HPLC. In this article, conditions are described that permit both HPLC separation and amperometric detection at the mvRuCN electrode for a set of analytes which are important in bioanalytical chemistry. These compounds are not all detected by any other reported electrode at constant potential although certain thiols are directly oxidized at carbon electrodes [15] and are detected with a gold amalgam electrode by their promotion of the oxidation of mercury [16].

EXPERIMENTAL

The liquid chromatographic system consisted of a dual-piston pump, which was taken from a Dionex 2010i ion chromatograph; a 100-μl loop injector; a 250 × 4.6 mm I.D. Partisil SCX (10 μm) strong cation-exchange analytical column (Alltech), and a Bioanalytical Systems (BAS) electrochemical detector. This detector comprised an MF 1000 glassy carbon electrode which was modified as described below, an MF 2020 silver–silver chloride reference electrode and an MF 1018 stainless-steel block as the auxiliary electrode. A BAS CV 37 potentiostat was used to apply the potentials and measure the currents, which were monitored *vs.* time on an Esterline Angus Speed Servo II strip chart recorder. All chromatograms were recorded using the amperometric detector, modified as described below, with an applied potential of 0.92 V *vs.* silver–silver chloride.

Unless otherwise noted, the chemicals were ACS reagent grade and were used without further purification. The $RuCl_3 \cdot 3H_2O$ was obtained from Pfaltz and Bauer, and the $K_4Ru(CN)_6 \cdot 3H_2O$ was from Alpha Products. The L-cysteine hydrochloride hydrate (99%) and glutathione (reduced form), 98%, were from Aldrich. L-Cystine and methionine in comparable purities were obtained from Sigma, and the oxidized form of glutathione was from Boehringer Mannheim. The cysteine and oxidized form of glutathione were stored in a desiccator at 4°C. The water used was house-distilled and further purified by passing it through a Barnstead NANOpure II system.

The cystine stock solution was prepared in dilute NaOH. Serial dilutions were made using the mobile phase. Stock solutions of methionine and other individual compounds were made immediately before use by dissolving the compound in the mobile phase solution. All mobile phases were made up fresh each day. Prior to use all mobile phases were filtered through a 0.45-μm Metricel membrane filter (Gelman) and were deaerated with oxygen-free nitrogen for 1 h.

The electrodes were prepared by electrochemical deposition of a mixed-valence ruthenium oxide film that was stabilized with cyano-cross-links [1,2]. Prior to surface modification, the glassy carbon electrodes were polished successively using 1-μm, 0.3-μm and 0.05-μm alumina (Mark V Laboratory, East Granbury, CT, U.S.A.) on a metallographic polishing cloth (Buehler, Evanston, IL, U.S.A.) with deionized water as the lubricant. The electrodes were thoroughly rinsed and sonicated using deionized water (sonication time, 10 min). Freshly prepared electrodes were assembled in a flow cell which was filled with a plating solution containing 2 m*M* $RuCl_3$, 2 m*M* $K_4Ru(CN)_6$ and 0.5 *M* KCl with the pH adjusted to 2.0 using HCl. The glassy carbon indicator was then cycled between 500 mV and 1100 mV *vs.* Ag/AgCl at a scan rate of 50 mV/s for a total of forty cycles. The initial and final potentials were 500 mV. The cell was then filled at open circuit with the mobile phase and stored in this condition until needed.

RESULTS AND DISCUSSION

The analytes selected were cysteine (CSH), cystine (CSSC), glutathione (GSH), the oxidized (disulphide) form of glutathione (GSSG) and methionine (MET). The initial chromatographic experiments were based on conditions reported by Eggli and Asper [17] and by Werkhoven-Goewie *et al.* [18]. The eluents were citric acid (0.090 *M*)

TABLE I

HPLC OF GSH, CSH AND MET IN CITRATE–PHOSPHATE BUFFERS WITH AMPEROMETRIC DETECTION AT A MODIFIED ELECTRODE

$V_{1/2}$ = half width volume; k' = capacity factor; correlation coefficients (r^2) and standard deviations of slopes are based on six replicate experiments; calibration data were not obtained in the pH 2.6 buffer at 0.3 ml/min.

Analyte	pH	Flow-rate (ml/min)	k'	$V_{1/2}$ (ml)	Conc. range (μM)	Slope (nA/μM)	r^2
GSH	2.6	1.0	1.6	0.4	1–100	5.7 ± 0.2	0.998
CSH	2.6	1.0	1.7	0.4	1–100	9.4 ± 0.2	0.999
MET	2.6	1.0	2.8	0.8	1–200	3.9 ± 0.1	0.999
GSH	2.6	0.3	1.6	0.14	–	–	–
CSH	2.6	0.3	1.6	0.14	–	–	–
MET	2.6	0.3	2.7	0.32	–	–	–
GSH	2.2	0.3	1.8	0.16	1–100	7.7 ± 0.1	0.999
CSH	2.2	0.3	1.9	0.16	1–100	9.9 ± 0.2	0.999
MET	2.2	0.3	3.3	0.35	2–200	3.9 ± 0.1	0.999

and Na_2HPO_4 (0.022 M) buffers at pH 2.6 and 2.2, respectively. With a subset of GSH, CSH and MET, alone and in mixtures, chromatograms were obtained at 1.0 and 0.3 ml/min on the Partisil SCX column. In addition, calibration curves were obtained for each of these analytes. The results are summarized in Table I.

In these experiments, mixtures of GSH and CSH were not resolved, but the data in Table I demonstrate that the detector response was sensitive (*ca.* 4–10 nA/μM) and was directly proportional to concentration over a wide range. With the pH 2.6 buffer and a flow-rate of 1.0 ml/min, the detection limits, which were taken as the concentration that yielded a signal of three times the background noise, were 0.25 μM, 0.15 μM and 0.45 μM for GSH, CSH and MET, respectively.

Changing the mobile phase to 18.6 mM ammonium citrate–60.7 mM H_3PO_4 at pH 2.4 [17] did not result in separation of GSH and CSH on the Partisil SCX column. Hence, the effect of pH of the citric acid–phosphate system on the chromatographic behavior was further investigated. A a result, a mobile phase of 0.04 M citric acid–0.1 M Na_2HPO_4 at pH 2.0 was identified as suitable for the resolution of a mixture of

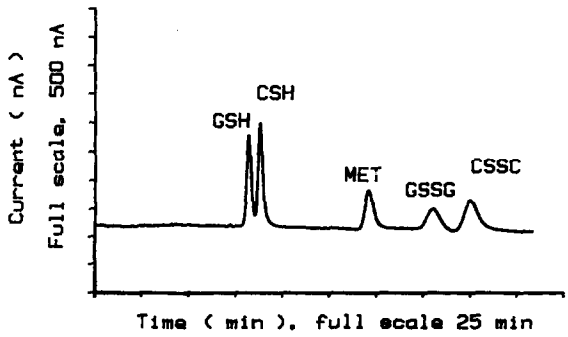

Fig. 1. HPLC of a five-component mixture with a pH 2.0 citrate–phosphate buffer as the mobile phase and amperometric detection at the mvRuCN electrode at 0.92 V *vs.* Ag/AgCl.

GSH, CSH, CSSC, GSSG and MET (Fig. 1) on this Partisil SCX column. All further work on the performance of the mvRuCN eletrode as an amperometric detector for these compounds was performed with this HPLC eluent.

The reproducibility of the detector response was investigated by injecting seven samples of a mixture of GSH, CSH and MET, where each concentration was 25 μM. The respective peak currents were 178 $\pm$ 1, 223 $\pm$ 4 and 93 $\pm$ 2 nA. Calibration curves were linear for all of the investigated analytes, as shown in Table II.

To test the stability of the detector in the selected mobile phase, chromatograms were obtained on a single preparation of the modified electrode over an eleven-day period. The total working time was about 50 h. Calibration curves for GSH, CSH and MET were obtained each day using the concentration range 1–100 μM. The results are summarized in Table III. Although the data are statistically constant over this period, the useful lifetime of that particular electrode preparation did not extend beyond eleven days. For example, on day 13, the slopes of calibration curves for GSH, CSH and MET decreased to 4.8, 6.0 and 2.9 nA/μM, respectively.

The detectability of the amperometric detector based on the mvRuCN electrode was compared to other reported detectors for the selected compounds. For example, HPLC experiments on GSH and CSH with amperometric detection have been performed using the oxidation of mercury as the anodic response [16,19,20]. With a Zorbax ODS column (5 μm, 250 $\times$ 4.6 mm), an eluent of 0.05 M trichloroacetate (1% CH_3OH) at pH 2.0, and a 100-μl injection volume, the detection limits for GSH and CSH were 1.8 and 1.2 pmol [20], respectively, compared to 18 pmol GSH and 15 pmol CSH in the present study. The electrode areas were the same in both cases. Differences in the chromatographic system may account, in part, for the poorer detection limits in the present study. A post-column ligand-exchange detection system [18] also provided detection limits that are superior to those with the mvRuCN detector for GSH, CSH, MET and CSSC. The following detection limits were reported in ref. 18 for GSH, CSH, MET and CSSC, respectively (values from the present study are in parenthesis): 16 (18), 3.2 (15), 20 (38) and 9.6 (42) pmol.

We are continuing to work toward optimizing the mvRuCN electrode as the indicator in an amperometric detector (constant potential) for HPLC. A factor that is somewhat deleterious to the detection limit is the high background that results because

TABLE II

HPLC CALIBRATION CURVES FOR SELECTED THIOLS AND DISULFIDES WITH AMPEROMETRIC DETECTION AT THE MODIFIED ELECTRODE IN A pH 2.0 CITRATE–PHOSPHATE MOBILE PHASE

Analyte	Range (μM)	Slope (nA/μM)	S.D. of slope (nA/μM, n = 6)	r^2	LOD[a] (μM)
GSH	1–100	6.9	0.2	0.999	0.18
CSH	1–100	8.7	0.1	0.999	0.15
MET	1–200	3.6	0.1	0.999	0.38
GSSG	1–50	2.0	0.1	0.999	0.63
CSSC	1–50	3.0	0.1	0.999	0.42

[a] LOD (limit of detection) = concentration that yields a current of three times the background noise.

TABLE III

TEST OF THE STABILITY OF THE MODIFIED ELECTRODE IN A pH 2.0 CITRATE–PHOSPHATE MOBILE PHASE

General conditions are same as those in Table II.

Day	Calibration curve slope (nA/μM)		
	GSH	CSH	MET
1	7.1	8.9	3.7
2	7.0	8.9	3.6
3	7.1	8.9	3.7
4	7.1	8.7	3.6
5	7.1	8.6	3.6
6	7.0	8.6	3.6
7	7.0	8.4	3.7
8	6.8	8.2	3.6
9	7.0	8.4	3.8
10	6.9	8.0	3.6
11	6.5	7.8	3.6

this surface catalyzes the oxidation of water (Fig. 1). Variation of the formulation of the modifier may permit the use of a lower applied potential which perhaps would result in a decrease in background without compromising the other performance characteristics of this electrode. But another important factor is that the HPLC system that we assembled is unlikely to duplicate state-of-the-art systems in overall stability and, therefore, detectability.

CONCLUSION

The described modified electrode permits the determination of GSH, CSH, MET, GSSG and CSSC in the μM range by HPLC with amperometric detection at constant potential. This set of analytes is not electroactive at other surfaces. The electrode is stable in a mobile phase suitable for the HPLC separation of these compounds for about ten days without cleaning or reactivation.

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