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DETERMINATION OF CYSTEINE AND GLUTATHIONE IN PLASMA AND BLOOD BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION USING A CHEMICALLY MODIFIED ELECTRODE CONTAINING COBALT PHTHALOCYANINE

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SUMMARY

An assay procedure utilizing amperometric detection at carbon paste electrodes containing cobalt phthalocyanine has been developed for the determination of cysteine and glutathione in blood and plasma samples following preliminary separation by reversed-phase liquid chromatography. The electrocatalytic acitivity of these chemically modified electrodes permitted optimum response at a potential of +0.75 V vs. Ag/AgCl, several hundred millivolts lower than that required at conventional carbon electrodes. A detection limit of less than 4 pmol was obtained for each compound with minimal sample preparation. In case of fouling of the electrode surface by sample constituents the cobalt phthalocyanine electrode surface could be renewed with a variability of less than 10% and an equilibration time of less than 10 min.

INTRODUCTION

Recently, we have demonstrated that incorporation of cobalt phthalocyanine (CoPC) into an otherwise conventional carbon paste mixture can produce activated electrode response toward numerous solutes which undergo electro-oxidation at unmodified carbon electrodes only at a substantial overvoltage [1, 2]. This enhanced response, which has been observed for compounds including hydrazine, thiols, and several keto-acids, consists of large concentration-dependent currents at potentials lower than those usually required for the oxidation of these compounds. In particular, we have shown that the performance of the CoPC-containing chemically modified electrodes (CMEs) is sufficiently stable and reproducible to permit their use in analytically oriented

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applications such as flow injection and liquid chromatography detection. Up to this point, however, the usage of these electrodes has been restricted to studies involving the analysis only of standard laboratory-prepared samples. Thus, it is of particular interest to examine the performance of CoPC-containing CMEs in more realistic analysis situations with sample matrices commonly encountered in practice.

Accordingly, in this work, we describe an application involving the use of CoPC-containing carbon paste CMEs for the determination of the sulfhydryl compounds cysteine and glutathione in whole blood and plasma. Several different electrode systems have previously been utilized for the electrochemical detection (ED) of these types of compounds following liquid chromatography (LC). These include carbon paste [3], glassy carbon [4], gold [5], mercury [6–9], and mercury–gold amalgam [10, 11], all of which have been reported to allow detection limits in the 3–15 pmol range. Differences lie in the nature of the oxidation process involved in the electrode process, the detector potential required, and the overall ease of system operation. For the carbon electrodes which depend on the direct oxidation of the thiols, high positive potentials on the order of +1.0 V vs. Ag/AgCl (or higher) were required for optimum detection [3]. For the mercury-based electrodes which perform the detection indirectly by oxidation of the mercury itself to produce a mercury–thiol complex, the use of much lower potentials was possible. This served to enhance the selectivity of the detection considerably but was accompanied by some decrease in convenience due to the formation and break-in of the amalgam surface [11] and occasional rapid deterioration in electrode response [10]. The assay reported here, which analogously employs the CoPC CME as an electrocatalytic sensing electrode for LC–ED, provides an alternative approach for thiol detection. The strengths of the CME approach include excellent sensitivity and improved selectivity compared to conventional carbon electrodes. In addition, the ease and reproducibility with which fresh carbon paste CME surfaces can be generated make the approach especially attractive with blood and plasma matrices prone to causing electrode fouling and deactivation.

EXPERIMENTAL

Chemicals

Glutathione (reduced form), cysteine hydrochloride, and homocysteine were obtained from Sigma, and CoPC from Eastman Kodak. All chemicals were used as received without further purification.

Electrodes

Unmodified carbon paste was prepared by thoroughly hand-mixing 5 g of graphite powder (Fisher Scientific) with 3 ml of Nujol oil (McCarthy Scientific, Fullerton, CA, U.S.A.). Modified paste was made in the same manner except that 0.01 g of CoPC was mixed with the graphite powder before addition of the Nujol.

Apparatus

The equipment used for cyclic voltammetry, flow injection, and liquid

chromatography were essentially the same as described previously [2]. In all cases, potentials were measured against an Ag/AgCl reference electrode..

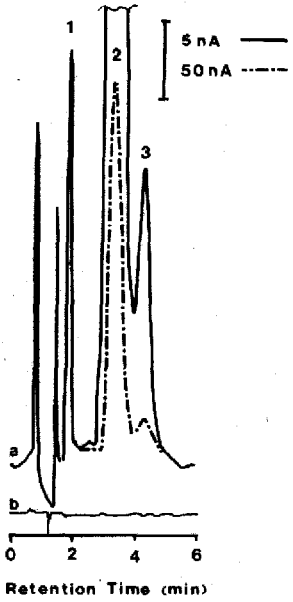
Hydrodynamic voltammograms were obtained via flow injection by recording the response of the working electrode placed in the conventional thin-layer cell configuration ordinarily employed in LC—ED but with only a short length of narrow stainless-steel tubing inserted (in place of the column) between the injector and detector. A pre-column (5 cm × 4 mm) packed with 40-μm pellicular C_{18} particles was placed before the injector in order to minimize flow fluctuations. Liquid chromatography experiments were performed with a 3 cm × 4.6 mm Perkin Elmer C_{18} column containing 3-μm spherical particles; a Brownlee Labs. (Santa Clara, CA, U.S.A.) C_{18} guard cartridge was inserted between the injector and the analytical column. For both flow injection and liquid chromatography, the mobile phase was methanol—potassium dihydrogen phosphate (5:95) adjusted to pH 2.4 with orthophosphoric acid. In addition, a 2.5 m*M* concentration of sodium octane sulfonate ion pairing agent was also maintained. The injection volume was always 20 μl, and the flow-rate was 1.0 ml/min at 25°C.

Sample preparation

A procedure similar to that recommended by Rabenstein and Saetre [6] was employed. Blood was collected in EDTA vacutainers, and a portion was centrifuged to provide plasma. A 700-μl volume of 1.0 g/l EDTA (disodium salt) and 200 μl of 20% (v/v) orthophosphoric acid were placed into each 75 × 12 mm polyethylene sample tube and centrifuged thoroughly. A 100-μl volume of blood or plasma was then added, and the contents were thoroughly mixed. The resulting suspension was injected into the chromatograph through a Cameo Nylon filter (Micron Separations, Honeoye Falls, NY, U.S.A.) used to prevent injection of particulates. Standard solutions of cysteine, homocysteine, and glutathione were treated in the same manner except for the filtration.

RESULTS AND DISCUSSION

The electrochemical behavior of numerous sulfhydryl compounds at CoPC-containing carbon paste CMEs has already been described in some detail [2]. In general, cyclic voltammograms obtained for these species under the relatively acidic conditions best suited for their reversed-phase chromatography exhibited irreversible peak-shaped oxidation waves between +0.7 and +0.9 V vs. Ag/AgCl. For the endogenous sulfhydryls of interest here, anodic peak potentials at pH 2.4 were: +0.70 V for cysteine, +0.73 V for homocysteine, and +0.82 V for glutathione. At unmodified carbon electrodes, virtually no redox activity can be observed for the same compounds at these potentials. Rather, applied potentials well in excess of +1.0 V vs. Ag/AgCl were required to produce comparable current levels. As in our earlier work, the sulfhydryl currents at the carbon paste CMEs appeared in the potential region corresponding to the Co(II)PC/Co(III)PC redox process [12, 13] and have therefore been attributed to the catalytic oxidation of the thiols by Co(III)PC produced directly at the electrode surface. As expected for such mediated CME processes [14] the observed currents were directly proportional to the sulfhydryl concentration



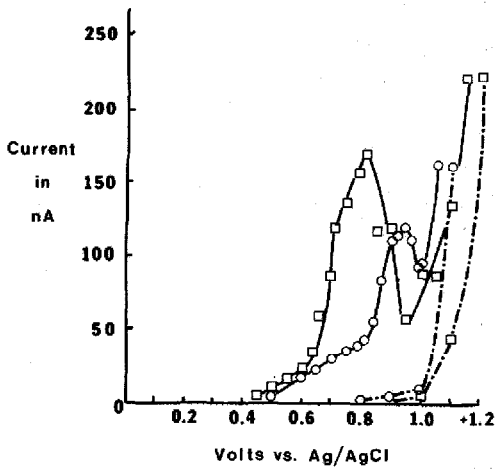


Fig. 1. Chromatogram of $1 \cdot 10^{-5}$ *M* cysteine (1), $1 \cdot 10^{-5}$ *M* glutathione (2), and $1 \cdot 10^{-5}$ *M* homocysteine (3) at a CoPC electrode (a) and at unmodified carbon paste electrode (b). Electrode potential: +0.75 V vs. Ag/AgCl. Mobile phase: methanol—0.05 *M* phosphate buffer pH 2.4 (5:95) with 2.5 m*M* octane sulfonate. Flow-rate 1.0 ml/min.

Fig. 2. Hydrodynamic voltammograms of cysteine (□) and glutathione (○) at CoPC carbon paste electrode (—) and at unmodified electrode (—·—). Same mobile phase and flow-rate as in Fig. 1.

and to the square root of the potential scan rate employed (for scan rate values less than 600 mV/s). This behavior is exactly analogous to that previously reported for CoPC both when the modifier was solubilized by sulfonation of the phthalocyanine ring system [15—18] and when it had been adsorbed onto a solid graphite surface [19]. However, our interest has focused on the carbon paste approach because the ease with which this type of CME surface is initially formed and subsequently regenerated makes its use especially well suited for analytical and chromatographic applications. Fresh catalytically active CoPC-containing carbon paste surfaces can be generated within minutes with a 5—10% reproducibility in current response simply by removing the exposed outer layer and smoothing on a fresh surface [1, 2].

The utility of CoPC CMEs for LC—ED of sulfhydryl species such as cysteine and glutathione is illustrated in Figs. 1 and 2 in which chromatographic data obtained with the CMEs is shown alongside that obtained using conventional carbon paste electrodes. Chromatograms (see Fig. 1) recorded at an applied potential of +0.75 V for a laboratory-prepared mixture of cysteine, homocysteine, and glutathione exhibited well formed peaks only for the CoPC CME. Because the concentrations employed were selected so as to approximate the normal levels of these species in plasma and whole blood, the glutathione concentration was intentionally adjusted to be 100-fold higher than that of the others. The hydrodynamic voltammograms, constructed from a series of flow injection experiments performed at different applied potentials and shown in

Fig. 2, further delineate the enhanced response of the CMEs compared to the conventional electrodes. The latter provide a usable response only in very high potential regions subject to considerably larger backgrounds and more likely interferences. The hydrodynamic voltammogram for homocysteine was essentially the same as that shown for cysteine.

Calibration curves for cysteine and homocysteine standards, treated by the same procedure as described in Experimental for blood samples, were linear over the range 5—200 pmol injected. Least-squares regression analysis for concentrations in this range gave for cysteine current = 46.3 pA/pmol · C + 509 pA and for homocysteine current = 47.6 pA/pmol · C — 630 pA. Correlation coefficients of 0.999 and coefficients of variation (n = 4) of 4.20% or less were obtained from both. Minimum detectable quantities (signal-to-noise ratio of 2) were 2.7 and 2.2 pmol, respectively. What appeared to be column overload effects due to the higher natural concentration of glutathione prevented the response for this analyte to be linear over the expected physiological range of 0.2 to 20 nmol injected. But the glutathione calibration curve (Fig. 3), although leveling off somewhat at higher concentrations, still permitted quantitation to be made. The detection limit for glutathione was 3.7 pmol injected.

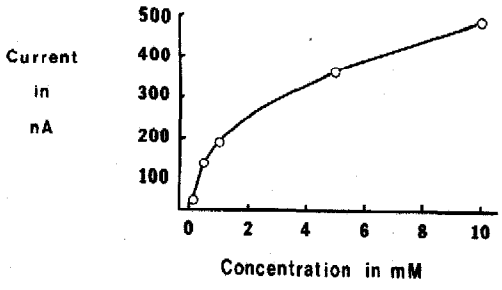


Fig. 3. Calibration curve for glutathione.

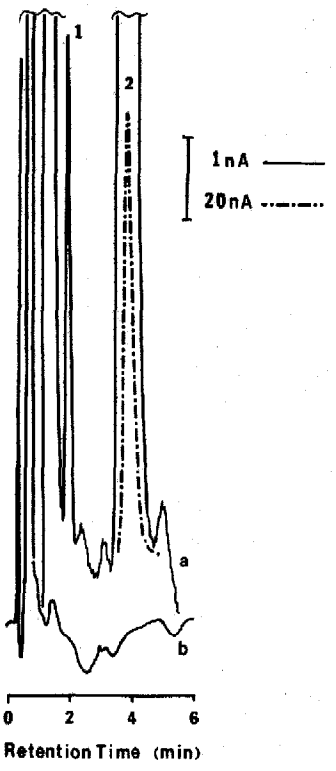


Fig. 4. Chromatograms of prepared blood samples at CoPC-modified electrode (a) and unmodified electrode (b). Same mobile phase, flow-rate, and potential as in Fig. 1. Peaks: 1 = cysteine; 2 = glutathione.

TABLE I

CYSTEINE AND GLUTATHIONE LEVELS IN PLASMA AND BLOOD

Determinations were performed in triplicate.

Subject	Plasma cysteine concentration (mean ± S.D.) (μM)	Blood glutathione concentration (mean ± S.D.) (mM)
1	1.83 ± 0.05	1.01 ± 0.06
2	7.26 ± 0.07	0.84 ± 0.1
3	5.33 ± 0.05	0.74 ± 0.03
4	7.78 ± 0.01	1.59 ± 0.08
5	10.36 ± 0.08	0.92 ± 0.03

A typical chromatogram obtained for a whole blood sample collected from a healthy volunteer and prepared as above is shown in Fig. 4. Shown are the responses obtained at E = +0.75 V both for a CoPC CME and for an unmodified carbon paste surface. As expected from the above discussion, peaks possessing the same retention as cysteine and glutathione were clearly resolved from the sample background at the modified electrode only. No response for homocysteine, which exists primarily in the oxidized disulfide form [20], was observed at either electrode. Chromatograms of plasma samples were virtually the same in appearance except that, as expected, little or no glutathione was evident. The occasional observation of trace amounts of glutathione in plasma samples was probably attributable to sample hemolysis that occurred prior to centrifugation. Table I lists the results of triplicate analyses performed on blood and plasma samples obtained from five healthy volunteers. The cysteine and glutathione levels determined via the CME assay procedure compare well with those reported previously by liquid chromatography with mercury-based electrochemical detection [8, 9].

In previous work where the CoPC-containing carbon paste electrodes were used only with standard deionized water solutions of sulfhydryl compounds, electrode response was quite stable under chromatographic conditions as long as the applied potential was kept below +0.80 V vs. Ag/AgCl during detection [2]. At more positive potentials, continued injections of sulfhydryl solutions resulted in a progressive and largely irreversible loss of electrocatalytic response. However, when the potential was limited to less than +0.80 V, only a very gradual decrease was observed with 85—90% of the initial current levels still maintained even after 8 h of continuous chromatography and more than 100 separate injections. Not surprisingly, analysis of blood and plasma samples resulted in a somewhat more rapid deterioration in electrode response. In the present work, sequential injection of twenty or more prepared blood samples caused the observed chromatographic peak currents to decrease to some 75—80% of the initially observed levels even at an applied potential of +0.75 V. A similar rate of electrode deactivation was also observed for unmodified electrodes on exposure to the blood or plasma sample matrix and thus does not represent a problem peculiar to the CoPC electrocatalysis scheme. Rather, it

is most likely due to a general surface fouling effect not uncommonly encountered in electrochemical studies of physiological matrices. In any case, because new CME surfaces can be regenerated with a variation of roughly 5% and the equilibration time for fresh surfaces is less than 10 min, the surface deactivation is actually a less severe problem here than for most other electrode systems.

In general, the performance of the CoPC-containing carbon paste CMEs for cysteine and glutathione determination seems to be at least comparable to that of other carbon and mercury-based electrode systems. The detection limits of less than 3 pmol found for the CMEs are equal to or less than those of previously reported assays [3—11] which, in all cases, are well below what is routinely required for quantitation of normal physiological levels of the compounds. The electrocatalytic nature of the CME enables lower potential operation (and therefore somewhat enhanced selectivity) compared to unmodified carbon electrodes. At the same time, electrode fabrication, equilibration, and surface renewal characteristics appear preferable to those of the mercury electrode systems. In any case, this work demonstrates that the use of CoPC CMEs is clearly suitable for analysis performed in complex sample matrices such as blood and plasma without the need for elaborate sample treatment procedures.

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REFERENCES

1 K.M. Korfhage, K. Ravichandran and R.P. Baldwin, Anal. Chem., 56 (1984) 1514.
2 M.K. Halbert and R.P. Baldwin, Anal. Chem., 57 (1985) 591.
3 I. Mefford and R.N. Adams, Life Sci., 23 (1978) 1167.
4 I.C. Shaw, A.E.M. McClean and C.H. Boult, J. Chromatogr., 275 (1983) 206.
5 K. Freuzig and J. Frank, J. Chromatogr., 218 (1981) 615.
6 D.L. Rabenstein and R. Saetre, Anal. Chem., 49 (1977) 1036.
7 R. Saetre and D.L. Rabenstein, Anal. Chem., 50 (1978) 276.
8 R. Saetre and D.L. Rabenstein, Anal. Biochem., 90 (1978) 684.
9 D.L. Rabenstein and R. Saetre, Clin. Chem., 24 (1978) 1140.
10 R.F. Bergstrom, D.R. Kay and J.G. Wagner, J. Chromatogr., 222 (1981) 445.
11 L.A. Allison and R.E. Shoup, Anal. Chem., 55 (1983) 8.
12 L.D. Rollman and R.T. Iwamoto, J. Am. Chem. Soc., 90 (1968) 1455.
13 V.R. Shepard and N.R. Armstrong, J. Phys. Chem., 83 (1979) 1268.
14 R.W. Murray, in A.J. Bard (Editor), Electroanalytical Chemistry, Vol. 13, Marcel Dekker, New York, 1984, p. 191.
15 N.N. Kundo and N.P. Keier, Russ. J. Phys. Chem., 42 (1968) 707.
16 J. Dolansky, D.M. Wagnerova and J. Veprek-Siska, Coll. Czech. Chem. Commun., 41 (1976) 2326.
17 A. Skorobogaty and T.D. Smith, J. Mol. Catal., 16 (1982) 131.
18 W.M. Brouner, P. Piet and A.L. German, Polymer Bull., 8 (1982) 245.
19 J. Zagal, C. Fierro and R. Rozas, J. Electroanal. Chem., 119 (1981) 403.
20 J.A. Schneider, K.H. Bradley and J.E. Seegmiller, J. Lab. Clin. Med., 71 (1968) 122.