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ORIGINAL PAPER

Determination of 2-Thiouracyl in Complex Samples by Liquid Chromatography with Electrochemical Detection

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Abstract

An assay procedure utilizing liquid chromatography and amperometric detection at a chemically modified electrode (CME) has been developed for the determination of 2-thiouracyl (2 ThU) in complex physiological samples. The electrocatalytic activity of the CME, which consisted of a glassy carbon surface coated with an electropolymerized cobalt phthalocyanine film, permitted optimum response at a potential of +0.75 V vs. Ag/AgCl, with a detection limit of 7 pmol for 2 ThU.

Keywords: Amperometric detection, 2-thiouracyl, modified electrode.

Introduction

Over the last two decades, the amperometric techniques have become widely accepted for analyte detection and quantitation in flow-injection analysis and liquid chromatography [1]. For example, liquid chromatography with electrochemical detection (LCEC) represents now a powerful method for the determination of thiols, phenols, catechols, aromatic amines and nitro compounds in complex physiological and environmental samples [2,3]. However, because the main requirement of the technique is that the analyte of interest undergoes oxidation or reduction at a comparatively low potential, many important but difficult-to-electrolyze species are not accessible to LCEC [4].

In the recent years, however, the use of chemically modified electrodes (CMEs) surface has expanded dramatically in chromatographic detection applications and numerous examples of analytical procedures enhanced significantly by the judicious application of CMEs have been reported [5,6]. One of the CME approaches that already has a great experimental deal of success is the use of electrocatalytic CMEs containing surface-confined redox mediators to enhance the response for solutes whose direct oxidation or reduction is not kinetically favored. For example, carbon paste or glassy carbon CMEs incorporating cobalt

phthalocyanine as modifier have been shown to enhance the detection of thiols, hydrazine, oxalic acid and carbohydrates [1,3].

The primary advantages cited in these reports have included greater resistance to electrode fouling, greater control of electrode selectivity and the activation of electrode response to new analytes that are electrolyzed either poorly or not at all at conventional unmodified electrodes surfaces [7].

In work that is particularly relevant to the 2-thiouracyl, CMEs containing incorporated cobalt phthalocyanine (CoPC) have been shown to carry out the electrocatalytic oxidation of thiols at low positive potentials well suited for selective HPLC based assays [8,9]. The electrocatalytic response of these CMEs derives from the ability of higher oxidation states of cobalt, generated electrochemically at modest potentials, to carry out thiol oxidation chemically under conditions comparatively free of back-ground currents and immune to most interferences. The CMEs employed have consisted either of carbon paste electrodes containing CoPC added manually to the paste mixture [10,11] or, more recently, glassy carbon electrodes covered with a polymer film produced by electro-oxidation of Co 4,4',4",4"'-tetraaminophthalocyanine [1,3]. This latter approach, in particular, yielded CMEs whose mechanical and chemical stability and broad solvent compatibility make them highly attractive for use in HPLC applications.

2-Thiouracyl and some related thioureas are receiving growing interest as selective melanoma (a dark pigmented malignant tumor) seekers. They are incorporated into growing melanin, apparently due to covalent binding to dopaquinone and the adduct is gradually tapped in the melanin polymer during its formation. To be clinically useful in melanoma scanning, 2-thiouracyl has been radioionicated and 5-iodo-2-thiouracyl (*ITU) was found to be localized in melanotic melanoma as selectively as thiouracil. Clinical trials with *ITU, for the detection of malignant melanoma, are in progress and the results are, so far, promising [12].

In further attempts to use the affinity of 2-thiouracyl for melanin-producing tissues in the design of drugs, active against malignant melanomas, guanidine-bridged adducts of the antraquinone drug daunorubicin with 2-thiouracyl were prepared [13].

The effect of 2-thiouracyl in correcting defects in folic acid function produced by vitamin B_{12} deficiency was studied. Addition of the thyroid inhibitor, thiouracyl, to a low methionine diet containing vitamin B_{12}, increased the oxidation of histidine to carbon dioxide and increased liver folate levels. It is possible that the effect of 2-thiouracyl in increasing folate function consists both in the effect of 2-thiouracyl in decreasing levels of methylenetetrahydrofolate reductase and also in its action in increasing S-adenosyl methionine which exerts a feed-back inhibition of this enzyme [14].

In this paper we describe a new application involving the use of the polymer CoPC electrodes for the determination of 2-thiouracyl (2 ThU) in complex samples. To our knowledge, there has been no previous report concerning electrochemical detection of 2-thiouracyl at either conventional or chemically modified electrodes. The assay procedure reported here, which employs the CoPC-CME as an electrocatalytic sensing electrode for liquid chromatography with electrochemical detection (LCED), provides an alternative approach for the direct detection of 2-thiouracyl with no derivatization and minimum sample treatment required. The strengths of the CME approach include its sensitivity and selectivity. In addition, the ease and reproducibility with which fresh CME surfaces can be generated make the approach very compatible with physiological sample matrices.

Materials and Methods

Reagents

2-Thiouracyl and uracyl were obtained from Aldrich Co., while glutathione and L-cysteine (reduced forms) were obtained from Sigma. All chemicals were used as received, without further purification. Co 4, 4', 4", 4'" tetraaminophthalocyanine was prepared according to the procedure of Achar et al. [15]. The solutions of 2-thiouracyl were prepared in phosphate buffer pH 3.0, ionic strength 0.05 M.

Electrodes

CMEs containing surface deposits of Co tetraaminophthalocyanine polymer (polyCoPC) were prepared according to the method reported by Li and Guarr [16] and fully described by Qi et al [1].

Appartus

The equipment used for cyclic voltammetry (CV), flow injection analysis (FIA) and HPLC was essentially the same as that described previously [3]. In all cases, potentials were measured against Ag/AgCl (3 M NaCl) reference electrode. HPLC effluents were also monitored at a wavelength of 280 nm with a Waters Associates Model 440 Absorbance Detector. Hydrodynamic voltammograms were obtained via FIA by recording the response of the working electrode placed in a conventional thin-layer cell configuration. For HPLC a 150 mm long, 4.1 mm i.d. Hamilton PRP-1 column containing reversed-phase poly(styrene-divinylbenzene) packing was inserted between the injector and detector. For all flow work, the mobile phase was 5% acetonitrile/95% 0.05 M potassium phosphate buffer adjusted to pH 3.0 with ortophosphoric acid. The flow rate was always 1.0 mL/min and the injection volume was 20 μL.

Results and Discussions

The electrochemical behavior of numerous thiol compounds at the polyCoPC-CME has already been described elsewhere in some detail [1,3]. In general, CVs obtained for these compounds under the mildly acidic conditions best suited for their reverse-phase chromatography exhibited irreversible peak-shaped oxidation waves at or above +0.5 V vs. Ag/AgCl. For 2-thiouracyl the behavior, shown in (**Figure 1**), was essentially the same, with a single anodic wave with a peak potential of +0.75 V at pH 3.0. Into the cathodic direction it observe some small reduction waves, whom intensities are a complex function of solution pH and of electrode aging.

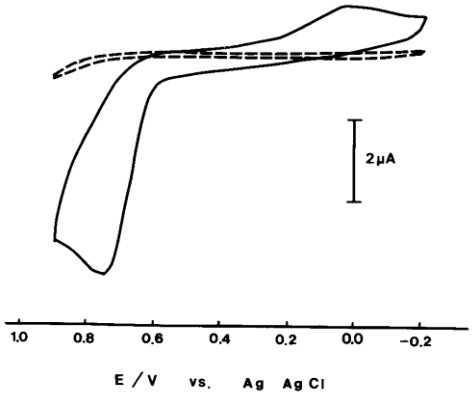

Figure 1. Cyclic voltammogram obtained for 0.5 mM 2 ThU in 0.050 M phosphate buffer (pH=3.0) at polyCoPC-CME. Shown as a dashed line is the voltammogram obtain at an unmodified electrode. Scan rate, 50 mV/s.

At unmodified glassy carbon electrodes, no redox activity at all can be observed for 2 ThU below +1.0 V. As in our earlier work [3], the thiol currents at the polyCoPC-CME appeared in the potential region corresponding to the Co(II)/Co(III) redox and therefore were attributed to the catalytic oxidation of the thiol by Co(III) PC produced directly at the electrode surface. As expected for such mediated CME processes, the observed currents were directly proportional to the 2 ThU concentration and to the square root of the potential scan rate employed (for scan rates up to 400 mV/s). The resulting catalytic currents (at +0.75 V) are direct proportional to the 2 ThU concentration, in the range 0.5 - 4.0 mM (**Figure 2**). In addition, the currents resulting from sulfhydryl group oxidation are controlled by diffusion, they presenting a linear dependency of square root of the swiping velocity of the potential, for swiping velocities up to 400 mV.

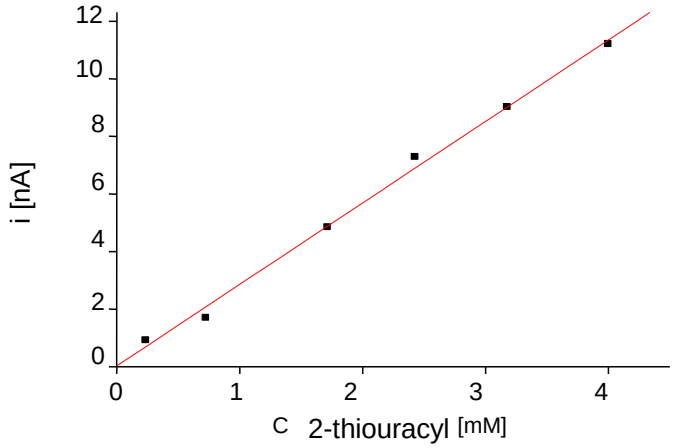

Figure 2. Calibration graph for 2 ThU.

CVs for 2 ThU were recorded in different solvent systems. In aqueous phosphate buffers from pH 2.5 to 8.0, the anodic wave associated with 2 ThU oxidation occurred at modest positive potentials at all pH values, but shifted to less positive potentials and decreased slightly in magnitude in more basic solutions. From (**Figure 3**), it can be observed that by pH decreasing (acidic media), the electrolytic process is shifting through more positively values of the potential. CVs obtained in nonaqueous solvents containing methanol or acetonitrile closely resembled those for purely aqueous media.

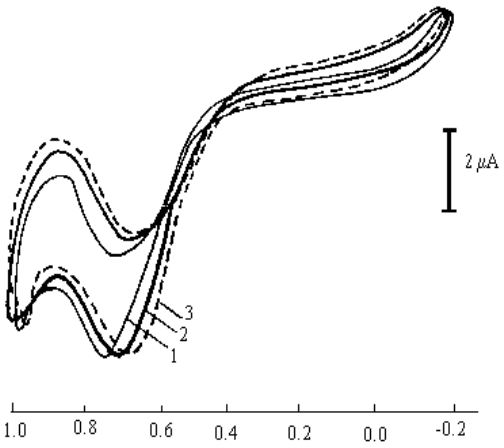

Figure 3. Influence of pH upon potential. (1) pH=2.5, (2) pH=5.5, (3) pH=8.0.

The CME - CoPC electrode can be kept in a 2 ThU solution under stirring or cycled in the potential range between -0.2 and 1.0 V for at least 10 hours without a considerable decrease in the catalytic activity. During this period of time, of 10 hours, over 60 experimental determinations were done with a 1 mM 2-thiouracyl solution. It was observed a decrease of the analytical signal of about 0.5% per hour.

On the basis of these observations, it appeared likely that LCED might be carried out for 2 ThU at low potentials and under a wide range of solvent conditions. In particular, the low applied potential permitted by usage of the CME should decrease the number and extent of possible interferences, thereby simplifying the sample treatment and chromatographic procedures required.

In (**Figure 4**) is presented the hydrodynamic voltammogram (HDV) for a 0.50 mM 2 ThU solution. Examination of this figure, constructed from a series of flow injection experiments at different applied potentials, shows that 2 ThU oxidation in the flow system was similar to that in CV, showing maximum current levels at approximately +0.75 V. As has been seen for other reported CoPC-catalyzed oxidations [1,3,10,11], the CME activity diminished at higher potentials and produced a peak-shaped HDV. On the basis of the HDV, it was apparent that the optimum potential for LCED detection of 2 ThU at polyCoPC-CMEs should fall between +0.70 and +0.80 V vs. Ag/AgCl. As all of the analytes studied here (L-cysteine, reduced glutathione, and 2 ThU) gave a good response at +0.75 V, this potential represented a reasonable choice for most work.

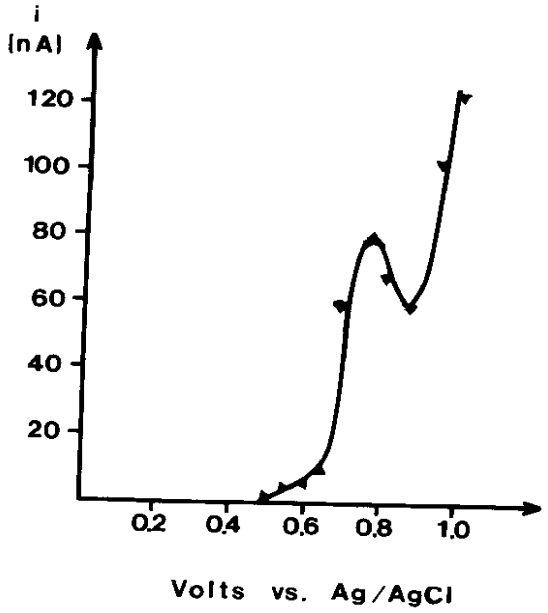

Figure 4. HDV of 2 ThU at polyCoPC-CME. Mobile phase was 5% CH_3CN/95% 0.050 M phosphate buffer (pH, 3.0).

The utility of the polyCoPC-CME for LCED of thiol compounds such as L-cysteine, glutathione, and 2 ThU is illustrated in (**Figure 5**), in which is presented a chromatogram recorded at an applied potential of +0.75 V for a laboratory prepared mixture of the compounds mentioned above. As can be seen from this figure, all three compounds present well-formed peaks; and, of the three, only 2 ThU presents both UV and electrochemical response.

For the polyCoPC-CME, peak currents at +0.75 V were linear for sample solutions containing 2 ThU concentrations ranging from 0.3-16 μmol/L (or 55-3000 μg/L). This was evidenced by least-squares analysis of data taken for five concentrations over this range (slope of the calibration curve, 1.1 nA/μM; correlation coefficient, 0.999). The limit of detection under the above conditions was 55μg/L or 7 pmol injected (signal/noise = 3).

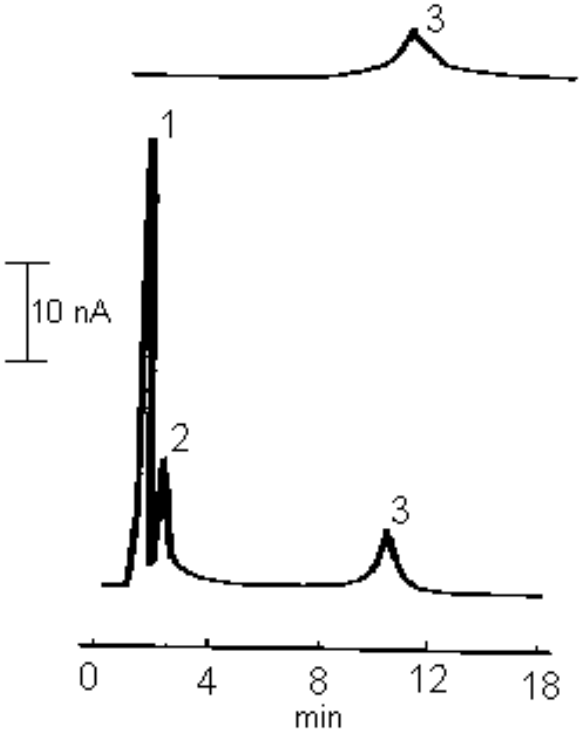

Figure 5. Chromatogram of a mixture of: (1) 2.0 mM L-cysteine, (2) 2.0 mM glutathione, (3) 0.50 mM 2ThU (1:1:1v/v/v): (A) electrochemical detection at a polyCoPC-CME at+0.75 V vs. Ag/AgCl and (B) u.v. detection at 280 nm.

The stability of the polyCoPC-CME response was checked by repeated injection of samples containing added 2 ThU. The results showed that, as long as the electrode was maintained at +0.75 V vs. Ag/AgCl, only a gradual decrease in peak current was observed. For example, for samples doped with 2 ThU, the CME retained 90-95% of its original activity after 5 hours of continuous chromatography and more than 20 separate injections. Operation at a higher potential caused a much more rapid decrease in electrode response and thus should be avoided in practice. However, the surface of the polyCoPC-CME could be rapidly and reproducibly renewed (±5% relative standard deviation) and , at +0.75 V, required an equilibration time of only 12-15 minutes in the flow stream. As a result, even though a single CME surface could in principle be used for work extending over several days, our usual practice consisted of creating and conditioning a new electrode surface at the start of each day's work.

Conclusion

The LCED assay described here should be highly attractive for the determination of 2 ThU in relevant physiological samples. The method is extremely sensitive, offering a detection limit below those reported for much more complex LCUV-based assays. Most important, the selectivity of the detection at the polyCoPC-CME is such that no derivatization or sample treatment (other than routine particulate filtration) is required for 2 ThU determination. In case of fouling of the electrode surface by sample constituents, the polymeric cobalt phthalocyanine electrode surface could be renewed with a variability of less than 10% and an equilibration time of less than 15 minutes. Furthermore, the response of the electrodes is extremely stable, with more than 95% of the initial activity retained after 10 hours of continuous use.

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