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ELECTROCHEMICALLY TREATED GLASSY CARBON ELECTRODE FOR AMPEROMETRIC DETECTION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The application of an electrochemically pre-treated glassy carbon electrode for the amperometric detection of electroactive components, such as tyrosine and oxipurinol, in biological samples was studied in order to demonstrate the usefulness of a pre-anodized electrode in high-performance liquid chromatography. The electrochemical pre-treatment was carried out in 0.2 *M* phosphate buffer (potassium dihydrogenphosphate–potassium hydroxide, pH 6.5) at 1900 mV *vs.* Ag/AgCl for 2 min. The pre-anodized electrode response for the oxidation of lactic acid and pyruvic acid was also studied. The electrochemical treatment enhanced and stabilized the electrode response to the oxidation of tyrosine and both acids.

INTRODUCTION

It has generally been recognized[1,2] that somehow electrochemical treatment of a glassy carbon electrode (GCE) tends to enhance its activity in the oxidation of electroactive components. As pointed out by Ravichandran and Baldwin[2], however, the possibilities of utilizing electrochemical treatment procedures for improving the electrochemical detector response have not been seriously considered. Progress in almost any area of biochemistry depends considerably on reliable methods. Recent advances in electrochemical detectors for high-performance liquid chromatography (HPLC) make them a logical choice for easily oxidizable substances[3]. It would appear certain that the development of procedures for the electrochemical treatment of GCEs will be required for progress in HPLC with electrochemical detection (ED).

In preliminary studies, we found[4–7] that the electrochemical treatment of GCEs in aqueous solutions containing phosphate ions tends to enhance their activity in the oxidation of some electroactive components, such as methionine (Met), glutathione (GSH), uric acid (UA) and xanthine (X), and also that the effectiveness of the electrochemical treatment is strongly dependent on the species undergoing electrolysis and on the electrochemical conditions. Therefore, we attempted to apply an electrochemically treated working GCE in an electrochemical detector for HPLC use. First, we optimized the electrochemical treatment to provide a maximal GC electrode re-

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sponse for Met oxidation[4,5]. The electrochemical treatment tends to enhance the sensitivity to Met oxidation and to stabilize the response with a low background current, even at a high applied potential (*e.g.*, 1700 mV *vs.* Ag/AgCl). GSH[6] and oxypurines, such as UA and X[7], in mammalian tissues were also determined by HPLC with ED using the pre-treated working electrode.

In this paper, we describe a method for the determination of tyrosine (Tyr), oxipurinol (Ox), lactic acid (LA) and pyruvic acid (PA) by HPLC with ED using an electrochemically pre-treated GCE.

EXPERIMENTAL

All experiments were conducted at 25 ± 1°C, unless stated otherwise.

Chemicals

All chemicals were purchased from Wako (Osaka, Japan). Chemicals for electrolyte solutions were of analytical-reagent grade and other chemicals were of reagent grade. The buffers and aqueous solutions were prepared with glass-distilled, deionized water.

A 10-mg amount of Tyr was dissolved in 10 ml of 0.1 *M* hydrochloric acid to give a Tyr stock solution. A 0.5-ml volume of 0.05 *M* sodium hydroxide solution was added to 10 mg of Ox and thoroughly stirred, then 0.1 *M* hydrochloric acid (9.5 ml) was added to the alkaline solution to give an Ox stock solution. Purchased viscous solutions of pure LA and PA were used to prepare stock solutions containing 1.0 mg/ml in 0.1 *M* hydrochloric acid. Each stock solution was diluted appropriately with 0.1 *M* hydrochloric acid just before use to give working solutions.

Chromatography

A Model 655 constant-flow pump (Hitachi, Tokyo, Japan) was employed throughout. Eluates from a 150 mm × 4.6 mm I.D. stainless-steel tube, packed with Chemcosorb 5-ODS-H (5 μm) (Chemco, Osaka, Japan) were amperometrically monitored with the aid of a Model ECP-1 electrochemical deetector (Kotaki, Chiba, Japan) with minor modifications to reduce the background current. It was equipped with a GC-20 working GCE (Tokai Carbon, Tokyo, Japan), an Ag/AgCl reference electrode and a stainless-steel wire as an auxiliary electrode. The mobile phase was 0.2 *M* potassium chloride–0.2 *M* hydrochloric acid–wawter (50:10.6:139.4, v/v) with a pH of 2.0. The flow-rate was 1.0 ml/min. The column temperature was 25 ± 1°C.

Treatment of the GCE

The GCE was polished according to the previously described procedure[7]. The polished GCE (electrode I) surface was in contact with a 0.2 *M* phosphate buffer (potassium dihydrogenphosphate–potassium hydroxide, pH 6.5) and was then anodized with a Model HA-301 potentiostat (Hokuto Denko, Tokyo, Japan) at 1900 mV *vs.* Ag/AgCl for 2 min. The electrode thus treated (electrode II) was used as a working electrode. When the detector response was lowered, the GCE was re-polished and electrochemically re-treated in the same manner as described above.

Serum sample preparation

Serum samples were obtained from healthy volunteers and a patient with renal gout before and after haemodialysis. The patient was dosed orally with 100 mg of allopurinol (1*H*-pyrazolo[3,4-*d*]pyridin-4-ol). Each serum sample (0.5 ml) was mixed with 0.2 *M* perchloric acid (2.0 ml) and the mixture was centrifuged at 3000 *g* for 10 min. The supernatant was passed through a 0.45-μm membrane filter prior to chromatography. A 10-μl volume of the treated serum was injected on to the column.

All electrochemically active components were identified by admixture of authentic internal standards.

RESULTS AND DISCUSSION

Hydrodynamic voltammograms of Tyr

Fig. 1a shows a hydrodynamic voltammogram obtained in the present chromatographic system with electrode II by repeated injections of 100 ng of Tyr at different detector potentials. Fig. 1b illustrates a voltammogram obtained with electrode I under the same chromatographic conditions as used for Fig. 1a. Repeated measurements showed that the voltammograms were reproducible. As can be seen in the figures, the electrochemical treatment reduced the onset potential of Tyr oxidation, but it caused a decrease in the maximum oxidation current generated.

We studied the response of various types of commercially available GCEs for

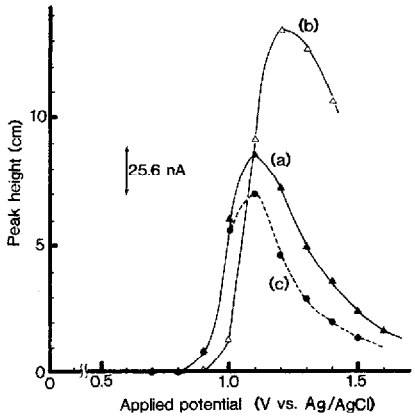

Fig. 1. Hydrodynamic voltammograms obtained by repeated injections of 100 ng of Tyr into the column at different detector potentials when (a) electrode I, (b) electrode II and (c) electrode II′ were used as working electrodes in the electrochemical detector. Mobile phase, 0.2 *M* potassium chloride–0.2 *M* hydrochloric acid–water (50:10.6:139.4, v/v); flow-rate, 1.0 ml/min; column temperature, 25 ± 1°C.

Tyr oxidation and found that the GC-20 employed in this study was the best for HPLC–ED use. As an example, Fig. 1c shows a voltammogram measured with a GC-30S (Tokai Carbon, Tokyo, Japan) working electrode (electrode II′) under the same conditions as in Fig. 1a and b.

The detection limits of Tyr (signal-to-noise ratio = 5) were 1 and 0.8 ng with electrode II (measured at 1150 mV *vs.* Ag/AgCl) and electrode I (measured at 1200 mV *vs.* Ag/AgCl), respectively.

Hydrodynamic voltammogram of Ox

Fig. 2 shows the hydrodynamic voltammograms obtained by repeated injections of 20 ng of Ox at different potentials when (a) electrode I and (b) electrode II were used as the working electrode. Comparison of Fig. 2a and b shows that the electrochemical treatment tends to enhance the electrode response to Ox by lowering the required operating potential and by increasing the maximum oxidation current generated. The detection limit of Ox was 800 pg under the conditions used.

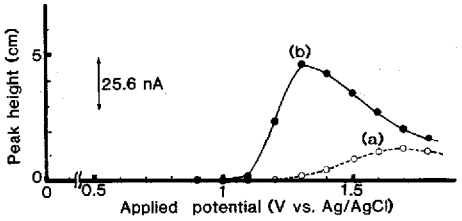

Fig. 2. Hydrodynamic voltammograms obtained by repeated injections of 20 ng of Ox at defferent potentials when (a) electrode I and (b) electrode II were used as working electrodes in the detector. Chromatographic conditions as in Fig. 1.

Hydrodynamic voltammograms of LA and PA

Fig. 3 illustrates the hydrodynamic voltammograms obtained by repeated injections of 10 μg of LA at different detector potentials when (a) electrode I and (b) electrode II were fitted in the detector and all other chromatographic conditions were

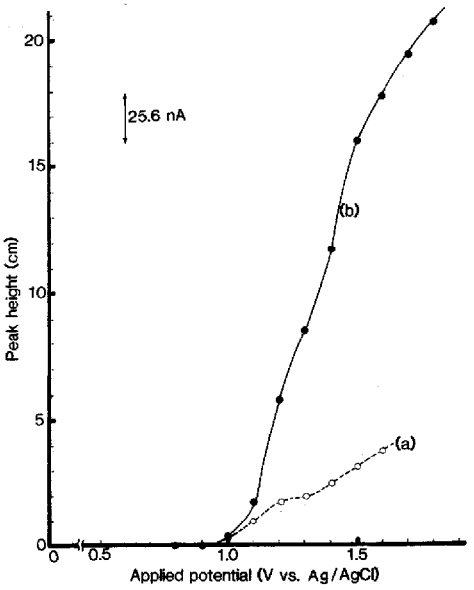

Fig. 3. Hydrodynamic voltammograms obtained by repeated injections of 10 μg of LA at different potentials when (a) electrode I and (b) electrode II were used as working electrodes in the detector. Chromatographic conditions as in Fig. 1.

the same as those in Figs. 1 and 2. The electrochemical modification enhanced the detector response for LA oxidation, whereas the onset potential remained unchanged. The detection limit was estimated to be 30 ng.

Fig. 4 shows the hydrodynamic voltammograms obtained by repeated injections of 10 μg of PA at different detector potentials when (a) electrode I and (b) electrode II were used. The onset potential of electrode I for PA oxidation was 900 mV *vs.* Ag/AgCl and the oxidation current generated gradually increased with increase in the detector potential without showing an optimum current. On the other hand, the response of electrode II to PA oxidation showed a maximum current generated at 1100 mV *vs.* Ag/AgCl. The electrochemical treatment seems to enhance the analytical capability of the detector for the determination of PA by lowering the required operating potential and by increasing the maximum current generated. The detection limit of PA was estimated to be 10 ng.

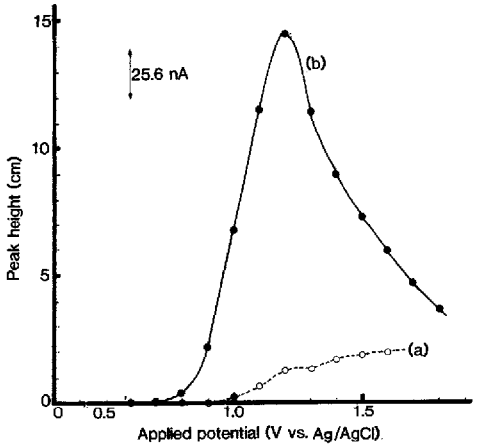

Fig. 4. Hydrodynamic voltammograms obtained by repeated injections of 10 μg of PA at different potentials when (a) electrode I and (b) electrode II were used as working electrodes in the detector. Chromatographic conditions as in Fig. 1.

Fig. 5 shows a typical chromatogram recorded by injecting 10 μl of a test mixture of 10 μg of LA and 10 μg of PA into the HPLC–ED system under the same conditions as in Figs. 3b and 4b. The detector potential was set at 1200 mV *vs.* Ag/AgCl. The peaks of LA and PA were well separated and detected. However, at present we are unable to determine both acids in biological samples. Optimization of the conditions for the determination is now in progress.

Determination of Ox and Tyr in human serum

The applicability of the HPLC–ED method was tested by determining electroactive components in serum. Fig. 6 shows typical chromatograms of deproteinized serum samples obtained from a patient with renal gout (a) before and (b) after haemodialysis. A 10-μl volume of each serum sample was injected and the eluates from the column were monitored amperometrically with electrode II, set at 1150 mV *vs.*

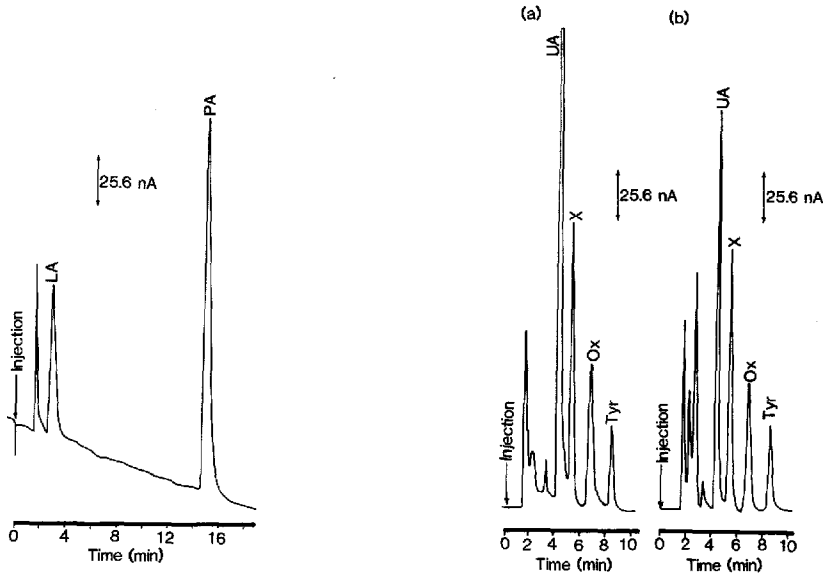

Fig. 5. Typical chromatogram obtained by injecting 10 μl of 10 μg of LA and 10 μg of PA into the column. The eluate was monitored amperometrically with electrode II set at 1200 mV *vs.* Ag/AgCl. Other chromatographic conditions as in Fig. 1.

Fig. 6. Typical chromatograms of serum samples obtained from a patient with renal gout who had been orally dosed 100 mg of allopurinol, (a) before and (b) after haemodialysis. Aliquots (10 μl) of serum samples were injected into the column and the eluate was monitored amperometrically with electrode II set at 1150 mV *vs.* Ag/AgCl. Other chromatographic conditions as in Fig. 1.

Ag/AgCl. The peaks of UA, X, Ox and Tyr were identified; their amounts in the serum before haemodialysis were found to be 45.7, 13.2, 16.2 and 21.7 μg/ml, respectively, and after haemodialysis 23.6, 12.8, 14.7 and 21.0 μg/ml, respectively. This result suggests that haemodialysis was effectively performed with the elimination of oxypurines from the blood of this patient.

Effect of phosphate on the response of electrode

It has been reported[1–6] that phosphate ions modified the surface of glassy carbon under anodic polarization and changed the electrochemical properties of glassy carbon anodes. We experienced an unstable response of electrode I at anodic potentials higher than 1000 mV *vs.* Ag/AgCl in a mobile phase containing phosphate ions. We tested some other compounds whose oxidation onset potential for electrode I was higher than 1000 mV *vs.* Ag/AgCl, such as Met[4], X[7], Tyr, LA and PA, and found that the electrode response was unstable in the phosphate solution, although it was relatively stable for GSH[6], UA[7], ascorbic acid (unpublished work) and catecholamines (unpublished work, whose maximum oxidation potentials are below 1000 mV *vs.* Ag/AgCl. In a mobile phase containing no phosphate ion, such as that used in this study, however, the response of electrode I for Met and Tyr was stable. These results suggested that the surface of the glassy carbon anode was gradually modified

in a phosphate-containing mobile phase at potentials lower than 1900 mV *vs.* Ag/AgCl.

We also studied the effect of electrochemical modification on the electroactive components. The stability of detector response under anodic polarization was improved by this modification for all compounds tested in the mobile phases with or without phosphate. The electrochemical treatment enhanced the sensitivity for detection (for example, about 320-fold for Met), except in the oxidation of Tyr, and decreased the onset potential (for example, about 200 mV for ascorbic acid), except for LA. In most instances the applicability of the electrochemically modified electrode for HPLC–ED was improved.

The mechanism of the enhancement of the GCE response to the oxidation of some electroactive components after the electrochemical modification in phosphate solutions has not yet been elucidated. Takamura *et al.*[8] found that the oxidation peak of the cyclic voltammogram of chloropromazine was enhanced about 30-fold when a GCE, electrochemically pre-treated in 0.5 *M* phosphate buffer (pH 6.7), was used. They postulated[9] that phosphate, adsorbed on the GCE surface, plays a role in enhancing the oxidation of the drug on the basis of an ESCA study of the electrode. We are also planning comparative studies of the electrochemical effects of the modification on the oxidation behaviour of electroactive compounds. They seem to offer some information for establishing the mechanism of oxidation by the modified electrode.

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