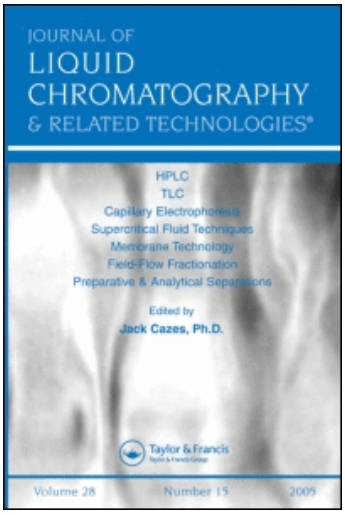

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A Method for the Determination of Methionine in Human Serum by High-Performance Liquid Chromatography with Electrochemical Detection

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A METHOD FOR THE DETERMINATION OF METHIONINE IN HUMAN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

We have found remarkable enhancement of electrochemical response for the analysis of methionine by use of a glassy carbon electrode preanodized in a 0.2 M phosphate buffer (KH_2PO_4-KOH, pH 6.5) at +1.9 V vs. Ag/AgCl for 2 min. On the basis of this finding, we have developed a method for the determination of methionine in human serum by reversed-phase high-performance liquid chromatography with electrochemical detection using the electrochemically pretreated glassy carbon electrode. The minimum detectable quantity of methionine has been found to be about 1 ng.

INTRODUCTION

Methionine (Met) is the compound of biological and clinical interest (1-4). Therefore, many methods for

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the analysis of Met as well as any other amino acids have been proposed. Quantitative determination of amino acids has commonly been carried out by high-performance liquid chromatography (HPLC) with fluoresecence detection (5) or visible absorption detection (6). However, it has generally been recognized that derivatization of amino acids for enhancing detectability introduces additional steps in their analysis. Therefore, we aimed to develop a sensitive and selective method for the determination of amino acids without derivatization.

HPLC with electrochemical detection (ED) is coming into widespread use for trace determination of easily oxidizable organic compounds, as described elsewhere (7). Met has been also recognized as an electroactive compound (8, 9). Therefore, Met may be determined by HPLC-ED. To our knowledge, there has been no report on the determination of Met by HPLC-ED. As a preliminary study, we found that the applied potential required for the maximum oxidation current of Met was +1.7 V vs. Ag/AgCl under the same chromatographic conditions as used in our previous study (10). However, we were unable to measure Met in human serum under the existing chromatographic conditions because of the observed excessive background current generated at the glassy carbon (GC) electrode. The GC electrode used as a working electrode in the electrochemical detector as well as the low sensitivity of the detector limited analysis of Met. Recently, we have found that when GC electrodes are preanodized in electrolyte solutions containing phosphate ions, the electrochemical pretreatment enhances significantly the analytical capability of the GC electrodes for the determination of Met. From an analytical standpoint, pretreatment procedures has been sought that can provide a reproducibly active surface after electrode polishing (11). Engstrom (11) has found the effectiveness of electrochemical

treatment of GC electrodes depends on the electrochemical species undergoing electrolysis. As pointed out by Ravichandran and Baldwin (12), however, the potential of electrochemical pretreatment procedures to improve analytical capability of electrochemical detectors has not been seriously considered. Therefore, our goal was to optimize an electrochemical pretreatment procedure to provide a maximal GC electrode response for Met oxidation with low background current. A sensitive method for determination of Met in biological samples by HPLC-ED may have clinical applications.

In this paper, we have developed a method for the determination of Met in human serum by HPLC-ED with a GC electrode preanodized in a phosphate buffer.

MATERIALS AND METHODS

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

All the chemicals were purchased from Wako Pure Chemicals, Osaka, Japan. Chemicals used in preparing buffer and electrolyte solutions were analytical reagent grade and any other chemicals were reagent grade. They were used without further purification. The buffers and aqueous solutions were prepared with glass-distilled deionized water. A portion (10 mg) of Met was dissolved in 10 ml of 0.1 N HCl and the Met solution was used as a stock solution. A portion (10 mg) of tyrosine (Tyr) was also dissolved in 10 ml of 0.1 N HCl for the preparation of its stock solution. The stock solution of uric acid (UA) was prepared according to the procedure previosuly described (13). Each stock solution was placed in a dark cold room (5 ± 1°C). Appropriate dilution of each stock solution was done with 0.1 N HCl just before use.

The HPLC-ECD system was similar to that of previous study (13). Briefly, a liquid chromatographic pump (TRIROTAR III, Jasco, Tokyo, Japan) with a 250- x 4.6-mm i.d. stainless-steel tube packed with Fine SIL C_{18} (particle size, 5 μm; Jasco, Tokyo, Japan), coupled with an electrochemical detector (Model ECP-1, Kotaki, Funabashi, Chiba, Japan). Eluate from the reversed-phase column was amperometrically monitored by aid of the electrochemical detector, in which electrochemical potentials were always positive at a GC electrode (oxidation mode) relative to the Ag/AgCl reference electrode. The GC electrode was short length of 5-mm diameter rod (GC-20, Tokai Carbon Co., Ltd., Tokyo, Japan) inserting in tight-fitting polychlorotrifluoro-ethylene (diflon) tube. A stainless-steel wire was used as an auxiliary electrode. To diminish the background current, the electrochemical detector purchased was slightly reformed in our laboratory to perform background current substraction. The range of zero adjustment was exchanged from 1 μA to 10 μA by changing the circuit around the knob of zero adjustment from Fig. 1 (a) to Fig. 1 (b). After the minor reformation, the background current was drastically diminished and thus almost zero drift of a baseline can be recorded in a chromatogram. In addition, the electrochemical detector response for Met analysis was enhanced by using the electrochemically pretreated GC electrode according to the procedure recently optimized and used in our most recent study (14). At first, the GC electrode was polished according to the procedure of Engstrom (11) with some minor modification. Finally, the surface of the GC electrode was polished to mirror-like on 0.3 μm alumina. The electrode was then thoroughly sonicated in highly purified water for 10 min to eliminate the possibility to alumina catalysis as reported by Zak and Kuwana (15). The surface

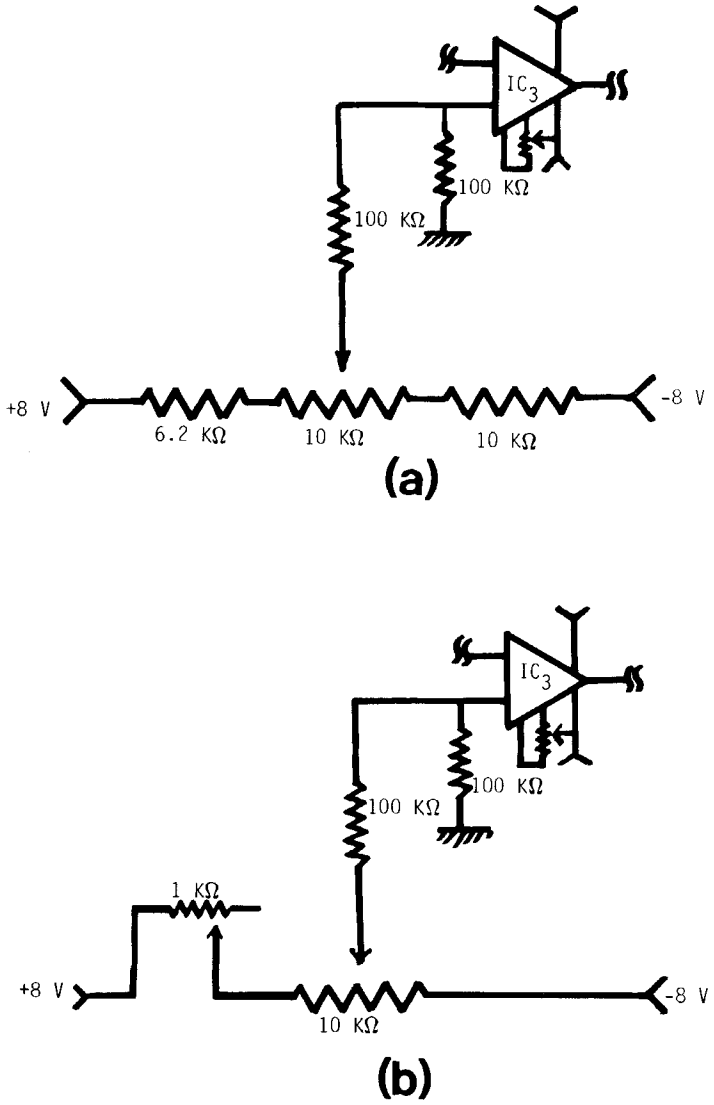

Figure 1. Circuits of the electrochemical detector around the knob of zero adjustment (a) before and (b) after the reformation.

of the polished GC electrode inserted in the diflon tube was electrochemically further treated by using a system shown in our previous report (14). Electrochemical pretreatment of the GC electrode was performed with a potentiostat (Model HA-301, Hokuto Denko Ltd., Tokyo, Japan). The polished GC electrode was anodized in a 0.2 M phosphate buffer (KH_2PO_4-KOH, pH 6.5) at +1.9 V for 2 min. As will be demonstrated later, the electrochemical pretreatment of the polished GC electrode enhanced the electrochemical detector response for Met analysis. When the detector sensitivity was lowered, the GC electrode was re-polished and electrochemically re-treated as described above and the GC electrode could be used repeatedly as a sensitive working electrode. The electrochemical pretreatment of the GC electrodes employed in this study has been found to enhance their response for some other electroactive components such as Tyr, tryptophane, glutathione (GSH), UA, xanthine, lactic acid, and pyruvic acid. Most recently, we have developed a method for the determination of GSH by HPLC-ED with the GC electrode preanodized in the phosphate buffer (14).

The mobile phase was a 0.02 M phosphate buffer (KH_2PO_4-H_3PO_4, pH 3.0) and the flow rate was 1.0 ml/min. It has been found that the effects of the electrochemical pretreatment of the GC electrode are unaltered in the mobile phase. The column temperature was 25 ± 1°C. The detector was set at +1.7 V in monitoring Met eluated from the column, unless otherwise stated.

The blood samples were obtained from healthy volunteers for preparing serum samples. An aliquot (0.5 ml) of each freshly prepared human sample was vigorously mixed with 1.0 ml of a 0.2 M perchloric acid on a thermomixer and the mixed solution was centrifuged at 3,000 *g* for 10 min. The supernatant thus obtained was

filtrated through a 0.45 μm membrane filter and an aliquot (10 μl) of the filtrate was injected into the HPLC-ED system.

Tentative identification of peak components was performed on the basis of retention behavior and co-chromatography with the reference compounds.

RESULTS AND DISCUSSION

It has been found that the onset potential and the potential for yielding the maximum oxidation current of Met are +1.0 V and +1.7 V, respectively, under the present chromatographic conditions. Therefore, the electrochemical detector was set at +1.7 V throughout this study. Fig. 2 shows a typical chromatogram obtained by injecting 200 ng of Met onto the column. The chromatogram monitored by the electrochemical detector using the polished but electrochemically untreated GC electrode is shown as a dashed line in Fig. 2. Comparison of the chromatograms in Fig. 2 reveals the effectiveness of the electrochemical treatment of the GC electrode for enhancing the detector response.

Fig. 3 shows a calibration graph of Met obtained under the present chromatographic conditions. The usual detector response has been found to be linear over a range (1-1,000 ng) for Met. A non-linear response tends to occur when more than 1,000 ng was injected.

Fig. 4 shows a chromatogram obtained by injecting an aliquot (10 μl) of the deproteinized human serum preparation onto the reversed-phase column using the electrochemical treatment of the GC electrode and conventional chromatographic conditions. As seen herein, a Met peak is observed in the chromatogram. The amount of Met in the human serum sample was estimated to be 7.16 μg/ml serum. The Met concentration obtained by our procedure

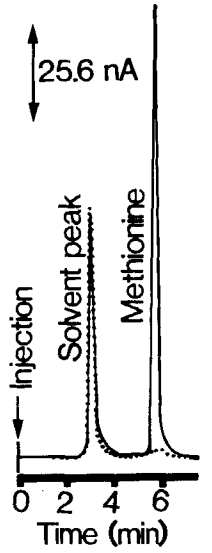

Figure 2. A typical chromatogram obtained by injecting 200 ng of methionine onto the column under the present chromatographic conditions. A chromatogram recorded by use of the polished but electrochemically untreated glassy carbon electrode is also shown as a dashed line.

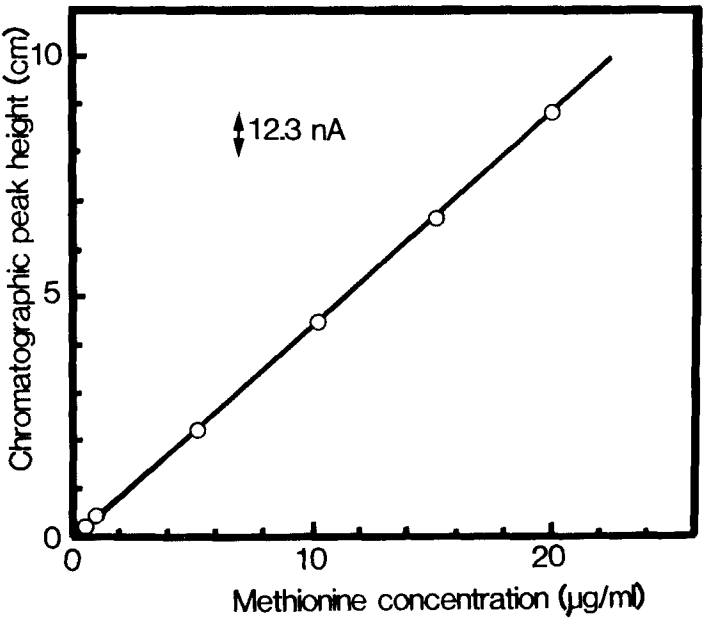

Figure 3. A calibration curve for methionine under the present chromatographic conditions.

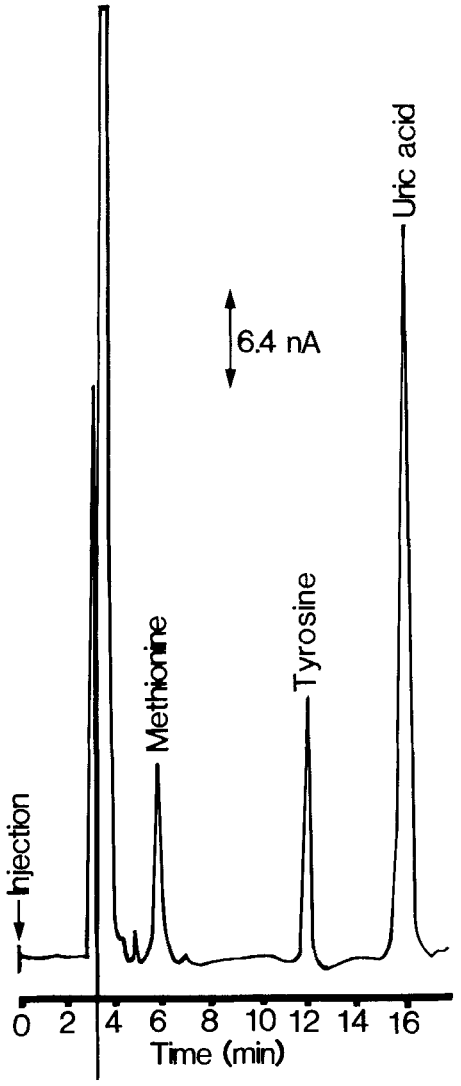

Figure 4. A chromatogram obtained by injecting an aliquot (10 µl) of the deproteinized human serum preparation onto the reversed-phase column under the present chromatographic conditions.

corresponds to the report (16) that serum samples prepared from healthy men contain 3.88-7.31 μg/ml serum.

Activity of the electrochemically pretreated GC electrodes for HPLC-ED is stable for at least 100 injections of the deproteinized serum samples. Met standard solutions added to human serum were analyzed with good precision at concentrations comparable to those in biological samples. The recoveries of Met in different human serum samples prepared on four different days were found to be 98.7 ± 1% under the present chromatographic conditions. Excellent precision of the retention time observed for Met was obtained in routine analysis over a six-day period for fifty different human serum samples, because the retention time is not affected by the sample matrix. The response of the electrochemical detector with the electrochemically treated polished GC electrode, whose switch was off for three days, was found to show the same activity as obtained three days before. When the detector sensitivity becomes too low to detect Met from the column, the GC electrode must be re-polished and electrochemically re-treated, and the treated electrode can be used again. In our laboratory, calibration curves for Met have been obtained before and after the experiments and even in the course of the analysis, if necessary.

As indicated in Fig. 2, UA and Tyr serve as markers in the chromatogram. In a preliminary study, we found that the minimum detectable quantities of UA and Tyr are also about 1 ng under the present chromatographic conditions. This finding suggests that the HPLC-ED method developed in this study may allow quantitative determination of Met, UA, and Tyr by a single chromatographic analysis. We have already developed a method for the determination of UA in body fluids such as serum (17), urine (18) and cerebrospinal fluid (19) and mammalian

tissues (20) by HPLC-ED with the polished but electrochemically untreated GC electrode. According to these methods (17-20), the minimum detectable quantities are about 10 pg. Therefore, we recommend use of the HPLC-ED methods when it is necessary to determine only UA in biological samples.

As described above, the HPLC-ED system, in which the polished and then electrochemically pretreated GC electrode is used as a working electrode, gives excellent separation and quantitation of Met in human serum in spite of the injection of deproteinized body fluid onto the column. Detector response is linear over a range (1-1,000 ng of Met). We now hope to improve procedures for electrochemical pretreatment of the GC electrodes for enhancing the Met oxidation rate at much lower potentials than +1.7 V. Recently, Henderson and Griffin (21) have proposed a method for the determination of adenosine and other purine metabolites by HPLC-ED. However, they were unable to exceed +1.65 V under their chromatographic conditions because of excessive background current generation (greater than 1 μA). We also observed a similar problem in the first step of this study. We have eliminated the problem by reforming the commercial electrochemical detector as described in the experimental section, as well as by using the electrochemically pretreated GC electrode as a working electrode. Ravichandran and Baldwin (12) have found enhancement of the electrochemical detector response for NADH, ascorbic acid, and hydrazine by using electrochemically pretreated GC electrodes. It has generally been recognized that many biological compounds of clinical and biological interest undergo electrolysis at activation-controlled rates and, as a result, at potentials drastically exceeding their thermodynamic potentials. For these compounds, detection by an electrochemical detector

cannot provide optimum levels of sensitivity and selectivity and in extreme cases provide no useful quantitation at all. As demonstrated in this study, the electrochemical treatment procedure seems to be simple and rapid. Therefore, the treatment procedure proposed in this study as well as in our previous study (14) appears useful for the analysis of serum electroactive components such as Met and GSH.

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