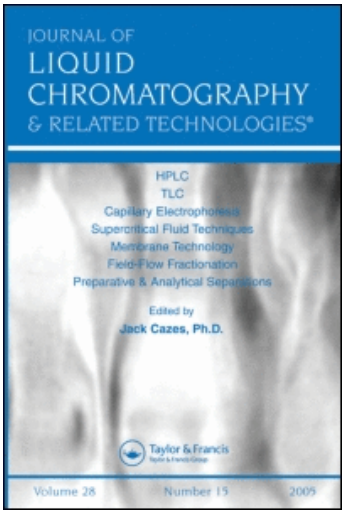

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SIMULTANEOUS DETERMINATION OF ASCORBIC ACID AND URIC ACIDS IN BODY FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTRO-CHEMICAL DETECTION

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ABSTRACT

It has been found that the complete separation of ascorbic acid and uric acid on a column packed with newly developed rigid-type polyvinyl alcohol gels by injecting an aliquot (10 μl) of body fluid without any pretreatment such as deproteinization prior to chromatography can be achieved with good reproducibility. The eluate from the column was amperometrically monitored by aid of an electrochemical detector set at +800 mV vs. Ag/AgCl.

INTRODUCTION

High-peformance liquid chromatography (HPLC) with electrochemical detection (ECD) is coming into widespread

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use for trace determination of easily oxidizable and reducible organic compounds, as described elsewhere (1). Recently, we have developed a method for the determination of ascorbic acid (AA) and uric acid (UA) in body fluids (2-6) and mammalian tissues (7-9) by HPLC-ECD.

AA and UA are the compounds of biological and clinical interest. As described by Fox (10), disorders of purine nucleotide degradation now seem to encompass a range of previously unsuspected disease associations. The adenine nucleotides themselves, ATP, ADP, and AMP remain within the cell so that their estimation requires tissue samples, but metabolic products of nucleotides such as hypoxanthine, xanthine, and UA can escape from the cell to appear in extracellular fluid. On the basis of the above described recognition, UA in body fluids such as serum and urine, not in mammalian tissues, has been studied in relation to diseases such as gout and gouty nephropathy. In 1970, Proctor (11) noted that the loss of the ability to synthesize AA in primates is coincident with the loss of the enzyme uricase. This seems to suggest strongly that UA may replace AA in a defence mechanism against oxygen toxicity. Kelogg and Fridovich (12) found that UA could inhibit lipid peroxidation and lysis of erythrocytes. Ames et al. (13) provided direct evidence that UA provided a general defense mechanism against singlet oxygen and organic hydroperoxides.

As seen in the above description, the development of a method for the one-time determination of AA and UA in biological samples has been required for understanding the biological functions of AA and UA furthermore. In the HPLC-ECD method developed by us (2-9), time for the sample pretreatment has been shortened by injecting an aliquot (10 μl) of the simply deproteinized biological sample onto the reversed-phase column. However, the step for

the deproteinization also seems to be a time-consuming task. Direct injection of body fluid without any pre-treatment onto a column seems to be much more practical for clinical use. Recently, a new rigid-type polymer packing (polyvinylalcohol gels; particle size, 10 μm) has been developed and a column paked with the polymer gels (Asahipak GS-320H) is now commercially available. It has been noted (14) that proteins can be passed through without any interaction with the packing and also that small components such as catecholamines, UA, and ATP can be adsorbed on the column.

In this article, we demonstrate the complete separation of AA and UA in body fluids such as serum, cerebrospinal fluid, and urine on Asahipak GS-320H by injecting an aliquot of each body fluid on it.

MATERIALS AND METHODS

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

All the chemicals were purchased from Wako Pure Chemicals, Co., Ltd., Osaka, Japan. All buffers and aqueous solutions were prepared with glass-distilled deionized water.

The eluate from the column was amperometrically monitored. A liquid chromatograph (Model LC-4A, Shimadzu, Kyoto, Japan) with the column (Asahipak GS-320H, Asahi Chemical Ind. Co., Ltd., Tokyo, Japan; column size, 250 x 7.6 mm i.d.), coupled with an electrochemical detector (Model ECP-1, Kotaki, Funabashi, Chiba, Japan) set at +800 mV *vs.* Ag/AgCl was employwed throughout this study. In the electrochemical detector, a glassy carbon electrode (GC-20, Tokai Carbon Co., Ltd., Tokyo, Japan) was used as a working electrode and electrochemical potential was always positive at the glassy carbon electrode (oxidation mode) relative to the Ag/AgCl

reference electrode. A stainless steel wire was used as a counter electrode. The glassy carbon electrode was hand-polished according to the procedure of Engstrom (15) with minor modification. Finally, the surface of the electrode was polished to mirror-like on 0.3 μm alumina. The electrode thus polished was then throughly sonicated in the deionized, distilled water for 10 min to eliminate such possiblity of alumina catalysis as reported by Zak and Kuwana (16). The polished electrode was inserted in tight-fitting polychlorotrifluoroethylene tube of the electrochemical cell in the electrochemical detector. When the detector sensitivity was lowered, the glassy carbon was re-polished as described above. Before the anodic scans, the electrode was held at a potential of 0.0 V vs. Ag/AgCl. The mobile phase was 0.1 M Na_2HPO_4 containing 0.3 M NaCl (pH 7.0). The flow rate was 1.0 ml/min and the column temperature was 25 ± 1°C.

The stock solutions of AA and UA was carefully prepared as follows. Ten micrograms of AA was dissolved in 10 ml of 2% metaphosphoric acid (MPA) containing 5 mM ethylenediaminetetraacetate (EDTA). The combination of EDTA and MPA has been found to be useful for the stabilization of AA in aqueous solutions (6, 17). The AA stock solution was daily prepared and stored at 4°C. An aliquot (0.05 ml) of 0.05 M sodium hydroxide solution was dropped into 10 mg of UA, and 9.95 ml of 0.1 M hydrochloric acid were then added to the dissolved UA solution. The UA stock solution was also stored at 4°C. Appropriate dilution of each stock solution was done with 2% MPA solution containing 1 mM EDTA just before use.

Human serum, cerebrospinal fluid, and urine samples were obtained from healthy volunteers. An aliquot of each body fluid was directly injected into the

HPLC-ECD system for the analysis of AA and UA. The total time from the sample preparation to the injection was within 30 min in this study, since AA was found to be chemically very labile for forming its degradation product(s) under the present storage conditions (5).

AA and UA were identified and quantified according to the same procedures as employed in our previous report (6). Triplicate injections gave standard deviations of peak height and retention times of 0.5 and 1%, respectively.

RESULTS AND DISCUSSION

We have found (2-6) that the levels of AA and UA in body fluids are extremely high compared to any other naturally occuring electroactive components such as catecholamines (CA's). This finding suggests that the determination of AA and UA in body fluids by HPLC-ECD can scarce be interfered by any other components. Therefore, we have used an artificially prepared solution containing AA and UA as a test solution for optimizing the chromatographic conditions. Fig. 1 shows a typical chromatogram obtained by injecting an aliquot (10 µl) of a test solution containing AA (10 µg/ml) and UA 1 µg/ml) onto the column under the present chromatographic conditions. As seen herein, the complete separation between the peaks of AA and UA has been obtained. The hydrodynamic voltammogram for UA and AA were obtained using the present chromatographic conditions by repeated injection of 1 ng of the respective acids at different electrochemical detector potentials according to the same procedure as used in our previous report (2). The voltammograms revealed that each component gave a maximum oxidation current around +800 mV *vs.* Ag/AgCl. Therefore, the electrochemical detector was

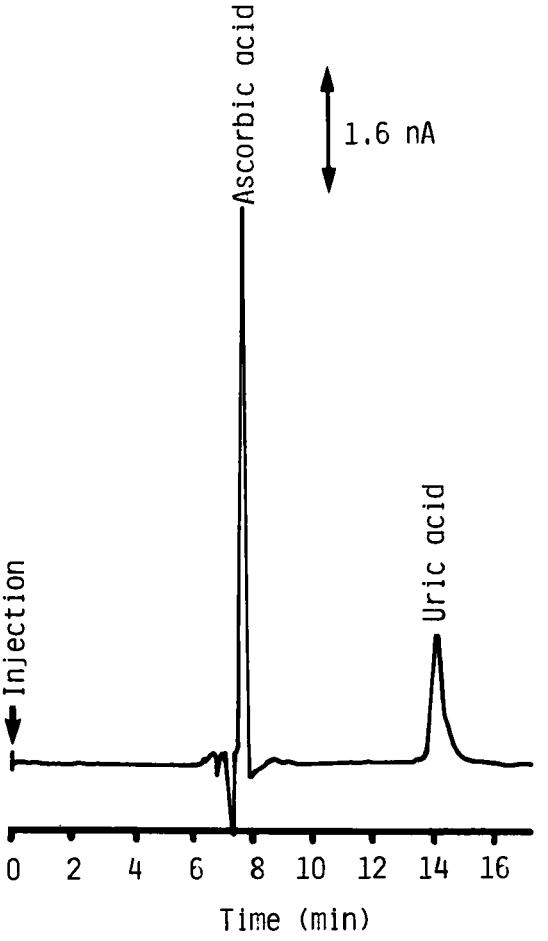

Figure 1. A high-performance liquid chromatogram obtained by injecting 10 µl of a test solution containing ascorbic acid and uric acid into the chromatographic system under the present chromatographic conditions.

set at +800 mV vs. Ag/AgCl. It has been found that the assay limits for quantitation are about 10 pg for AA and UA.

Fig. 2 shows the typical chromatograms obtained by injecting 10 µl of human (a) serum and (b) cerebrospinal fluid into the HPLC-ECD system under the same chromatographic conditions as used in Fig. 1. As seen in Fig. 2, the complete separation of the AA and UA peaks was obtained and any other chromatographic peaks could not be observed. Tentative identification was performed on the basis of retention behavior and co-chromatography with the reference compounds (6). In addition, purity of the UA peak was also checked using uricase, which calalyzes the conversion of UA to allantoin. Since the reaction product, allontoin, cannot be oxidized under the present chromatographic conditions, the disappearance of the substrate (UA) peak indicates the fact that the UA peak does not contain any other electroactive component(s). It was found that the UA peak appeared in the chromatograms (a) and (b) (see Fig. 2) was completely disappeared in the chromatograms obtained by injecting an aliquot of the same human body fluids after the reaction with uricase. The contents of AA and UA in 1 ml of the human serum were estimated to be 9.36 µg and 41.1 µg, respectively. The contents of AA and UA in the human cerebrospinal fluid were estimated to be 39.2 µg/ml and 1.58 µg/ml, respectively. Since the sample preparation did not involve any transfer, the values for AA and UA amounts might be nearly absolute, indicative of the endogenous quantities. Fig. 3 shows a typical chromatogram obtained by injecting 1 µl of human urine onto the column. In Fig. 3, the identification was performed according to the procedure described above. As seen in Fig. 3, the UA peak was identified, whereas the AA peak was not observed. The content of UA in the

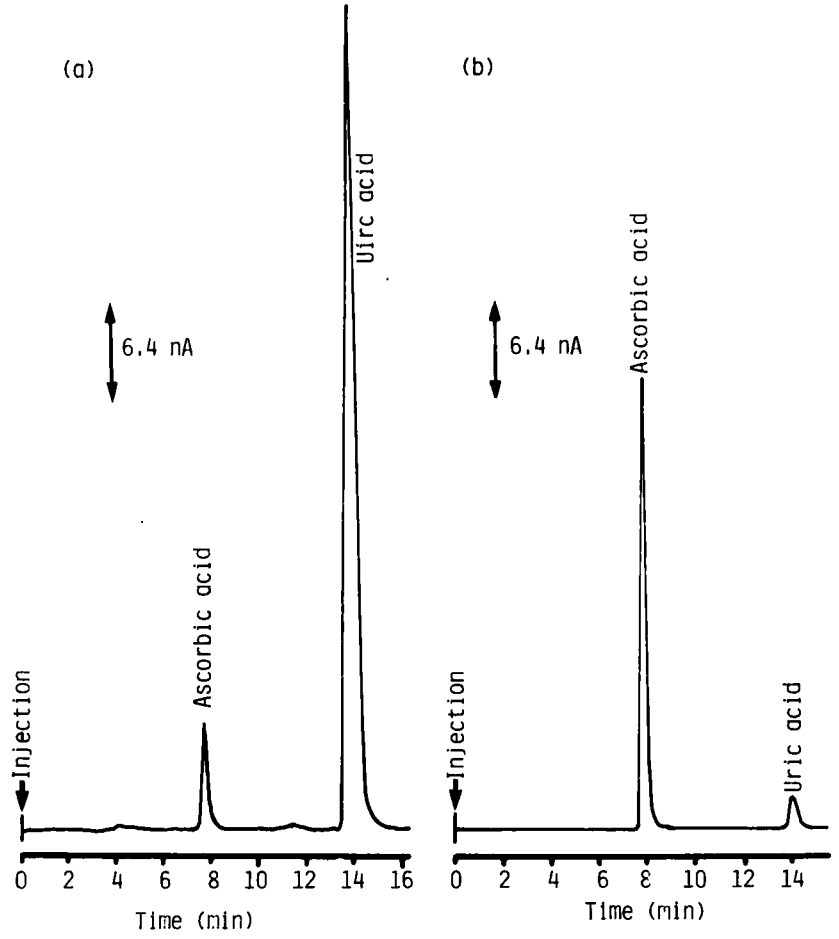

Figure 2. The typical chromatograms of the freshly prepared human (a) serum and (b) cerebrospinal fluid. An aliquot (10 µl) of each sample without any pretreatment such as deproteinization prior to chromatography was injected onto the column.

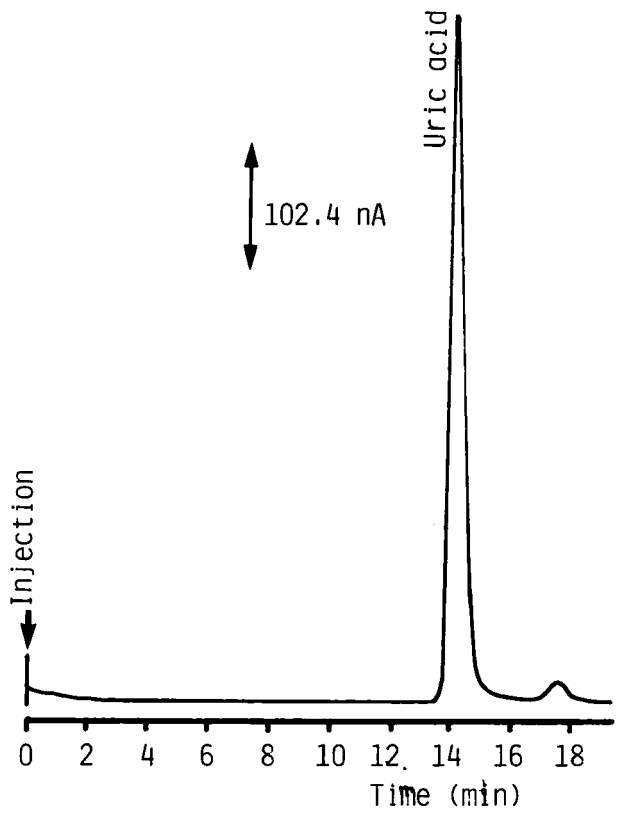

Figure 3. A chromatogram obtained by injecting one microliter of human urine into the same HPLC-ECD system as used in Figs. 1 and 2 under the present chromatographic conditions.

human urine was estimated to be 4.40 mg/ml. Excellent precision of retention times for AA and UA was always obtained in routine analysis over a six-day period for 50 different human urine samples, probably due to the fact that retention time of AA and UA are not affected by the sample matrix.

As demonstrated above, the repeated injections of human body fluids without any treatment procedures

such as deproteinization of body fluids and preconcentration of AA and UA do scarce shorten the column life. Complete separation of AA and UA in body fluids was achieved within 16 min under the present chromatographic conditions (see Figs. 2 and 3). The flow rate can be increased up to 2 ml/min and the separation can be shortened. In our previous report (5), the complete separation of AA and UA was achieved in about 2 min, when the separation was performed on reversed-phase, micro-particulate, chemically-bonded packings. However, it was found (5) that presence of particulate materials such as proteins in the injected samples tended to shorten the column life showing the precipitate on the top of the column. Since we have recognized that the deproteinization procedure seems to be a time-consuming task during the course of AA and UA determination study, use of Asahipak GS-320H seems to be useful in determining UA and AA in body fluids for clinical use due to the fact (14) that proteins can be passed through the column without any interaction under the present chromatographic conditions.

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