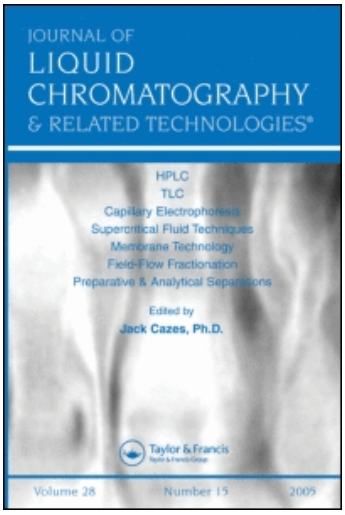

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Electrochemical Detection of Ascorbic and Uric Acids in the Eluate from Reversed-Phase High-Performance Liquid Chromatography with Newly Developed Rigid-Type Porous Polymer Packing (Polymetacrylate Gels)

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ELECTROCHEMICAL DETECTION OF ASCORBIC AND URIC ACIDS IN THE ELUATE FROM REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH NEWLY DEVELOPED RIGID-TYPE POROUS POLYMER PACKING (POLYMETACRYLATE GELS)

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ABSTRACT

It has been found that the combination of ethylenediaminetetraacetate and metaphosphoric acid is useful for the stabilization of ascorbic acid (AA) and precipitation of proteins in body fluids and also that the complete separation of catecholamines, AA, and uric acid (UA) on a column packed with a newly developed rigid-type porous polymer packing (polymetacrtlate gels) can be achieved. On the basis of the above findings, we have developed a method for the determination of endogenous AA and UA in body fluids such as human serum, urine, and cerebrospinal fluid by reversed-phase high-performance liquid chromatography with electrochemical detection. Determination of AA and UA in each body fluid has been carried out by injecting 10 µl of each simply deproteinized body fluid into a chromatographic system.

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INTRODUCTION

Uric acid (UA) and ascorbic acid (AA) are the compounds of biological and clinical interest. Therefore, numerous techniques have been developed to estimate the concentrations of AA and UA in biological samples. High-performance liquid chromatography (HPLC) with electrochemical detection (ECD) is coming into widespread use for trace determination of easily oxidizable and reducible organic compounds such as catecholamines (CA's) (1), AA (2, 3), and UA (2-6). The widespread use of the HPLC-ECD techniques seems to be catalized by recent development of various packing materials of high quality. The application of porous polymers as a group of chromatographic packings shows much promise because of the fact (7) that the range of reversed-phase chromatography has been expanded dramatically to allow operation over pH limits of 1 to 13 by using the chemically inert macroporous poly(styrenedivinylbenzene) phase. Recently, a new rigid-type porous polymer packing (polymetacrylate gels; particle size, 6 μm) has been developed and a column packed with the porous polymer gels (RSpak DE-613) is now commercially available (8). Recently, we have found (9) that the complete separation of AA, UA, norepinephrine (NE), epinephrine (E), and dopamine (DM) on RSpak DE-613 can be achieved and also that AA tends to decompose for forming its degradation products during the course of the chromatographic separation. Most recently, we have found that the combination of ethylenediaminetetraacetate (EDTA) and metaphosphoric acid (MPA) is useful for the stabilization of AA. On the basis of the above informations, we aimed to developed a method for the quantitative determination of CA's, AA, and UA in biological samples by one-time HPLC-ECD. As a preliminary study, we have developed a method for the determination of endogenous UA in body fluids (9).

In this article, a rapid, easy, sensitive, selective and accurate method for the quantitative determination of AA and UA in human body fluids by one-time HPLC-ECD.

MATERIALS AND METHODS

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

All the chemicals were the same one as used in our previous reports (3, 5, 6, 9). All buffers and aqueous solutions were prepared with glass-distilled deionized water.

A chromatographic system employed in this study was completely the same one as employed in our previous report (9). Briefly, a liquid chromatograph (Model LC-3A, Shimadzu, Kyoto, Japan) with a reversed-phase porous polymer column (RSpak DE-613, Showadenko K.K., Tokyo, Japan; column size, 150 mm x 6.5 mm I.D.; particle size, 6 μm), coupled to an electrochemical detector (Model ECP-1, Kotaki, Funabashi, Chiba, Japan) set at +800 mV vs. Ag/AgCl was employed in this study. The mobile phase was a 0.1 M K_2HPO_4-citric acid buffer, whose pH was adjusted to 4.6, containing 1 mM EDTA. Addition of EDTA in the buffer did not introduced any significant pH change. As will be described later, EDTA was added into the buffer as a stabilizer of AA during the course of the chromatographic separation. The flow rate was 1.0/ml. The column temperature was 25 ± 1°C.

The stock solutions of AA and UA were prepared according to the procedures described previously (3). The AA stock solution was daily prepared and stored at 4°C. When the chromatographic examinations revealed that AA in the stock solution was decomposed, the AA solution was discarded. Appropriate dilution of each stock solution was done with 0.2 M MPA solution containing 1 mM EDTA just before use.

An aliquot (0.5 ml) of human serum or cerebrospinal fluid was mixed with 1.0 ml of 0.2 M MPA solution containing 5 mM EDTA (pH 2.0) on a thermomixer at 0°C and the mixture was passed through a 0.45 μm membrane filter (Ekicrodisc 13, Gelman Sci. Jpn. Ltd., Tokyo, Japan). 10 μl of the filtrate was injected into the MPA solution into the chromatographic system. 5 μl of human urine was diluted with 1.0 ml of the MPA solution with EDTA and the diluted urine was also passed through the membrane filter prior to chromatography. The total times from the sample collection to the filtration were less than 10 min. It has been found that AA in each filtrate is chemically stable for at least 6 hrs and 2 days at 4 and -20°C, respectively. MPA and EDTA was added into body fluids as the stabilizing agents of AA.

AA and UA were identified on the basis of retention times by comparison with standards and also co-chromatography with standard solutions of AA and UA with various concentrations in different solvent conditions.

AA and UA were quantified by comparing the peak heights in the respective chromatograms with values from the respective standard curves. Triplicate injections gave standard deviations of peak height and retention times of 0.5 and 1%, respectively.

RESULTS AND DISCUSSION

In our previous reports (3, 5, 6, 9, 10), it has been found that the levels of AA and UA in body fluids are extremely high compared to any other naturally occuring electroactive components such as CA's. During the course of the CA determination study, we have recognized that the AA and UA peaks tend to interfere with the quantitative determination of CA's in biological

samples, probably due to the so-called column-to-column, lot-to-lot, and manufacture-to manufacture variations. Therefore, we have used an artificially prepared solution containing AA, UA, NE, E, and DM as a test solution for optimizing the chromatographic conditions. Fig. 1 shows a typical reversed-phase high-performance liquid chromatogram obtained by injecting 10 μl of a test solution containing AA, UA, NE, E, and DM onto the resersed-phase column system under the present chromatographic conditions. The concentration of each component was 1 μg/ml. As seen here, the complete separation of NE, E, DM, AA, and UA has been achieved.

Since it has been described (8) that chromatographic data, obtained by use of column packed with octadecyl-silica gels, are helpful in determining the composition of a mobile phase for use with RSpak DE-613, a phosphate buffer (KH_2PO_4-H_3PO_4, pH 2.0), which had been used as a mobile phase for separating CA's, AA, and UA on a column packed with octadecyl-silica gels (3, 5, 6), was used for the preliminary separation study. After the above study, we have found that the phosphate buffer is not useful for the complete separation of CA's, AA, and UA on RSpak DE-613. In addition, we have found that when a 0.2M K_2HPO_4-citric acid buffer (pH 4.6) is used as a mobile phase, the complete separation of CA's, AA, and UA on the porous polymer column can be carried out. However, AA has been found to form its degradation products during the course of the chromatographic separation, probably due to the its instability in the mobile phase of high pH value. It has generally been recognized (11) that at high pH, AA decomposes at a substantially higher rate than at low pH. Fortunately, a 0.2M K_2HPO_4-citric acid buffer (pH 4.6) containing 1 mM EDTA has been found to be useful as a mobile phase

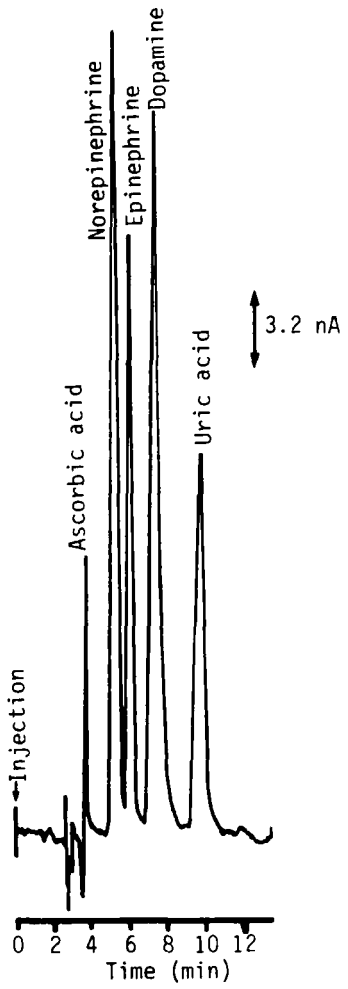

Figure 1. A reversed-phase high-performance liquid chromatogram obtained by injecting 10 μl of an artificially prepared test solution containing ascorbic aicd, uric acid, norepinephrine, epinephrine, and dopamine into the chromatographic system. For explanation of the chromatographic conditions, see the text.

for the separation and determination of CA's, AA, and UA by one-time HPLC-ECD. EDTA seems to stabilize AA at least within the chromatographic separation. In addition, as shown in Fig. 1, the complete separation of CA's, AA, and UA has been carried out under the present chromatographic conditions.

The hydrodynamic voltammograms for NE, E, DM, AA, and UA have been recorded under the present electrochemical conditions. The onset potentials of NE, E, DM, AA, and UA at the glassy carbon-mobile phase have been found to be +400, +400, +300, -100, and +400 mV vs. Ag/AgCl. In addition, it has been found that the oxidation currents of NE, E, DM, AA, and UA reach a plateau around +700 mV vs. Ag/AgCl. On the basis of the above informations, the electrochemical detector was set at +800 mV vs. Ag/AgCl. The minimum detectable quantities of NE, E, DM, AA, and UA were found to be about 10 pg under the present chromatographic conditions.

The chromatographic system and conditions optimized above have been applied for the separation and determination of AA and UA in body fluids. Fig. 2 shows the liquid chromatograms of the simply deproteinized human (a) serum, (b) cerebrospinal fluid, and (c) urine. As seen there, the AA and UA peaks were appeared as the respective distinguished ones. Any other chromatographic peaks of biological components were not obaserved due to the fact that concentrations of any other electrochemically active components such as biogenic amines in each simply deproteinized body fluid were too low to be detected by the electrochemical detector. The AA and UA contents in the human serum, cerebrospinal fluid, and urine, whose chromatograms are shown in Fig. 2, are tabulated in Table I. Since the sample preparation did not involve any transfer, the values for AA and UA

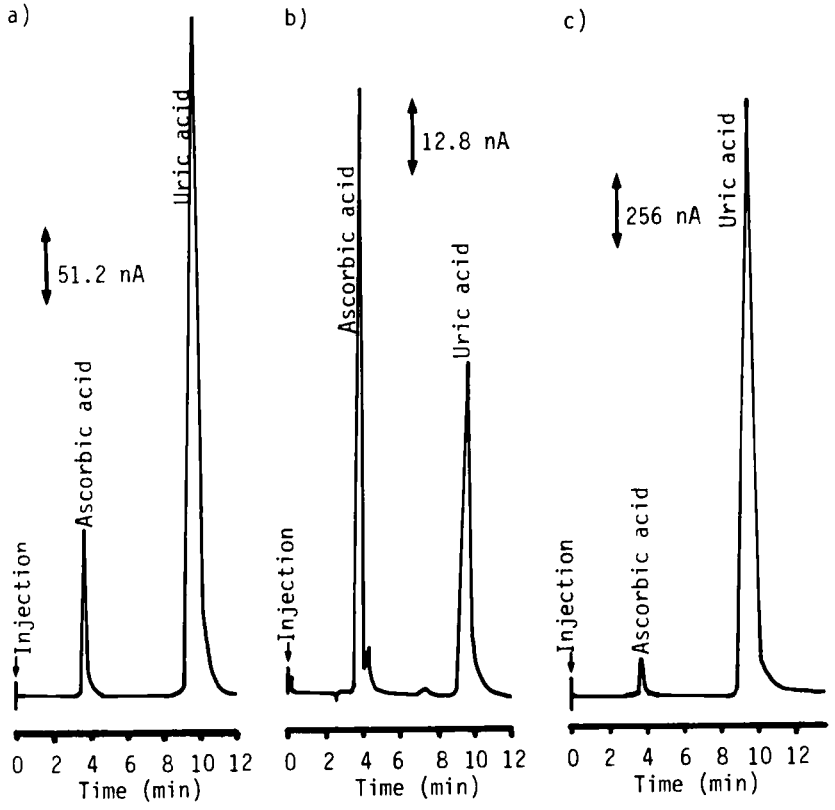

Figure 2. The typical chromatograms of the simply deproteinized human (a) serum, (b) cerebrospinal fluid, and (c) urine prepared according to the procedures described in the text. An aliquot (10 μl) of each simply deproteinized body fluid was injected onto the porous polymer column. For further explanation of the chromatographic conditions, see the text.

Table I. Ascorbic acid and uric acid concentration in human serum, urine, and cerebrospinal fluid.

	Ascorbic acid (μg/ml)	uric acid (μg/ml)
Human serum*	12.8	40.5
Human urine*	36.1	490.0
Human cerebrospinal fluid*	9.01	4.05

* The Chromatogram of each body fluid is shown in Fig. 2.

amounts might be nearly absolute, indicative of an endogenous quantity. AA and UA standard solutions added to human serum were analyzed with good precision at concentrations comparable to those in biological samples. The recoveries of AA and UA in different standard solutions added to twenty different human serum samples prepared on four different days were found to be 99.6 ± 0.5% and 99.8 ± 0.4%, respectively, under the present experimental conditions. Excellent precision of retention times for AA and UA was always obtained in routine analysis over a six-day period for 100 different human serum samples, probably due to the fact that the retention times of AA and UA is not affected by the sample matrix. However, the calibration graphs were daily obtained before and after before and after AA and UA determination study in our laboratory. Both calibration graphs always coincided well with each other.

Most recently, the HPLC-ECD method developed in this study has been applied for the quantitative determination of CA's, UA, and AA in a simply deproteinized tissue-extract and allowed to determine UA, AA, NE, E,

and DM by one-time HPLC-ECD (Yoshiura, Iwamoto, and Iriyama, to be published elsewhere).

The first steps in the estimation of CA's from body fluids aim at the removal of interfering matter and the preconcentration of the substances of interest. The adsorprion of CA's on alumina is considerably specific and achieves a high degree of concentration. Anton and Sayre (12) optimized the alumina treatment procedure for the determination of CA's in biological samples, not by HPLC-ECD, but by HPLC with fluorescence detection. Therefore, we aimed to optimize the alumina treatment procedure and have then optimized the procedure (13). In addition, we have found (14) that the adsorption by alumina is not completely specific to catechol compounds, but shared by any other elecroactive biological components such as UA and AA and (15) that the recovery of UA after the alumina treatment of the brain-extract is around 20%. However, the recovery of AA has been found to be not constant under the alumina treatment conditions. Thus, we have not been able to develope a method for the determination of AA, UA, NE, E, and DM in body fluids by one-time HPLC-ECD. We are now aiming to optimize the alumina treatment procedure for obtaining the constant recoveries of CA's, AA, and UA.

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