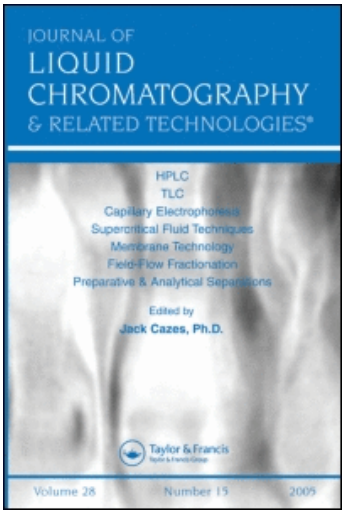

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URINARY URIC ACID DETERMINATION BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

Determination of urinary uric acid has been attempted by reversed-phase high-performance liquid chromatography with electrochemical detection. We have found that the electrochemical detection method is suitable for monitoring eluate from reversed-phase column and also that the minimum detectable quantity of uric acid using an electrochemical detector is about 10 pg. Complete separation of uric acid was achieved in about 8 min under the present chromatographic conditions.

INTRODUCTION

Since Kissinger and his coworkers' (1) pioneering work on normal-phase high-performance liquid chromatography (NPHPLC) with electrochemical detection (ECD), the most widely used methods of analysis for catecholamines have been varied in the electrochemical approach. It has been found (2,3) that uric acid (UA) in biological fluids can be determined by NPHPLC-ECD. Clinical laboratory estimations of serum concentrations of UA are currently based on one of two chemical principles: 1) an oxidation-reduction reaction

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in which UA reduces colorless phosphotungstic acid to tungsten blue and measurement at 700 nm (4) and 2) an enzymatic conversion of UA and oxygen by uricase to allantoin and hydrogen peroxide (5). In the latter, there is more heterogeneity of methods due to ancillary linked indicator systems (6). Although the first method is satisfactory for routine analysis, its usefulness is limited because of the presence of other reducing substances such as ascorbic acid (AA) in biological samples. The enzymatic methods proposed until now are sensitive to uricase inhibitors and have poor reproducibility, although the specificity of these procedures are high compared with the phosphotungstic acid method. It has generally been recognized that normal UA levels in human biological fluids (e.g., serum) of different generation must be determined at diagnosis for gout and several other diseases. HPLC may be an obvious candidate as reference method for the determination of UA levels in biological samples. Different separation principles and detectors have been used for the determining UA by aid of HPLC. Ion-exchange (normal-phase) columns have been used, coupled with ultraviolet detection (UVD) (7) or ECD (2,3). Reversed-phase high-performance liquid chromatography (RPHPLC) has been also used with UVD (8) or ECD (9-11). Recently, various types of electrochemical detector of good quality are now commercially available. The inherent higher sensitivity of ECD compared with UVD has been the subject of several publications (9,12). However, there has been no report for the determination of urinary UA by RPHPLC-ECD.

In this article, we describe a simple, rapid, easy, sensitive, accurate and selective method for the determination of UA in human urine samples by RPHPLC-ECD.

MATERIALS AND METHODS

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

A constant flow pump (Model TWINCLE, Jasco, Tokyo, Japan) allowed a mobile phase to pass through a 25 cm x 4.6 mm I.D. stainless steel column packed with "Fine SIL C_{18}" (Jasco, Tokyo, Japan; particle size, 10 μm) as a reversed-phase adsorbent. Eluate from the column was electrochemically monitored by aid of an electrochemical detector (Model ECP-1, Kotaki, Funabashi, Chiba, Japan) under the potentiostatic condition (+800 mV vs. Ag/AgCl). In addition, eluate from the column was spectrophotometrically monitored by aid of an ultraviolet absorption detector (Model UVIDEC-100-III, Jasco, Tokyo, Japan) set at 254 nm, which was situated between the reversed-phase column and the electrochemical detector, unless otherwise stated. The column temperature was 25 ± 1°C. The mobile phase was 0.2 M phosphase buffer (KH_2PO_4-H_3PO_4, pH 3.0). The flow rate was 2.0 ml/min. For the spectrophotometrical and electrochemical detectors, the limits of detection of UA were in the nanogram and picogram range, respectively, under the present chromatographic conditions.

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

UA was quantified by comparing the peak height in the respective chromatogram with value from a standard curve. Triplicate injections gave standard deviation of peak heights and retention times of 0.5% and 1%, respectively.

All the chemicals used in this study were the same one as used in our previous reports (13-17). All buffers and aqueous solutions were prepared with glass-distilled deionized water.

An aliquot (0.1 ml) of each human urine sample was diluted with 9.9 ml of the phosphate buffer used as the mobile phase. The diluted urine was passed through a 0.45 μm membrane filter (EKICRODISC 13, Gelman Sci. Jpn. Ltd., Tokyo Japan) to remove the particulate matter (e.g., proteins). The stock solution containing UA(1 mg/ml) was prepared according to the procedure previously

described (16) and was stored at -80°C. Appropriate dilution of the stock solution with the mobile phase was done just before use. 10 μl of each solution containing UA was injected into the chromatographic system for the separation and determination of UA.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram of the simply deproteinized human urine monitored by the electrochemical detector under the potentiostatic condition (+800 mV vs. Ag/AgCl). As seen here, the UA peak was completely separated as a distinguished one under the present chromatographic conditions. It has generally been recognized that the pretreatment of urine samples previous HPLC analysis is necessary to avoid interferences and extend the column life. Presence of particle materials tends to shorten the column life. One of the most simple pretreatment procedures is the filtration of the liquid sample through a membrane filter to remove the particulate matter. In this way, particles such as proteins are separated from urine sample. Repeated injections of the simply deproteinized human urine samples did not seem to shorten the column life. In addition, interference materials in determining UA were also removed from the human urine samples by the simple filtration procedures, as shown in Fig. 1. The repeated chromatographic examinations revealed that any trace amount of UA was not resultant on the membrane filter, through which the urine sample was passed. Since the sample preparation did not involve any transfer, the value for UA might be nearly absolute, indicative of an endogenous quantity. Content of UA in the human urine, whose chromatogram is shown in Fig. 1, was estimated to be 971 μg/ml.

The peak X component was always appeared in the human urine samples. The component seemed to be chemically very labile in human urine samples, probably forming its degradation product(s) under the present experimental conditions. The Peak X near the so-called

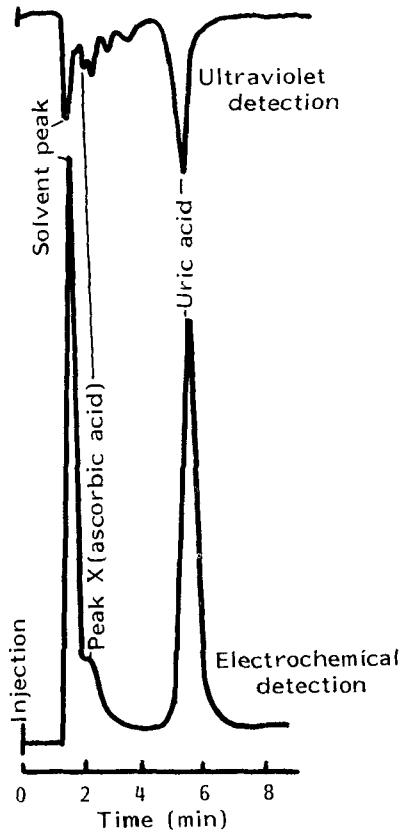

Figure 1. The typical reversed-phase high-performance liquid chromatography, obtained by injecting 10 µl of the simply deproteinized human urine into the RPHPLC-ECD system. Ten microliters of the urine was injected onto the reversed-phase column by using a microsyringe. The eluent was 0.2 M phosphate buffer (KH_2PO_4-H_3PO_4, pH 3.0). The column temperature was 25 ± 1°C. The flow rate was 2.0 ml/min. Eluate from RPHPLC was electrochemically monitored by aid of the electrochemical detector under the potentiostatic condition (+800 mV vs. Ag/AgCl). The sensitivity of the detector was set at 256 nA full scale. At the same time, the eluate was spectrophotometrically monitored by aid of the ultraviolet absorption detector set at 254 nm. The chromatogram is also shown as a reference.

solvent peak was finally identified as AA. Generally, AA is found to be chemically very labile for forming its degradation products, some of which have been found to be electrochemically active (17). Thus, before starting to develop a method for the determination of AA by RPHPLC-ECD, storage conditions for urine samples should be optimized for the determination of AA in biological samples. In the chromatograms of freshly prepared human urine or serum samples,the AA peak was always observed, whereas the AA peak was not observed in the chromatograms of the long-storaged human urine or serum samples. On the basis of the above findings, we are now aiming to develop a method for the determination of AA in biological samples by RPHPLC-ECD. Under the present experimental conditions, the analytical recoveries of AA in biological samples were found to be less than 60%.

As described above, the RPHPLC-ECD method developed in this studyis free from any interferences of the urinary UA determination by urinary reducing substances such as AA. UA standard solutions added to human urine were analyzed with good precision at concentrations comparable to those in physiological samples. The recoveries of UA in different standard solutions added to one hundred different human urine samples prepared on five different days were found to be 99.2 ± 0.5% under the present experimental conditions.

Excellent precision of retention time for UA was always obtained in routine analysis over a six-day period for three hundred different human urine samples, probably due to the fact that the retention time of UA is not affected by the sample matrix. In our laboratory, however, the calibration graphs were daily obtained before and after the UA determination study. Repeated injection gave an average precision (R.S.D) of less than 2%.

CONCLUSION

We have developed a method for the determination of urinary UA by RPHPLC-ECD. The RPHPLC-ECD method has been proven to be

powerful method for the separation and quantitation of urinary UA. It has generally been recognized that the so-called ECD method is more sensitive than the UVD method. However, the higher sensitivity is not crucial in the analysis of urinary UA because of the fact the UVD is adequately sensitive for the determination of urinary UA concentrations (see Fig. 1). Juge and Del-Razo (18) have concluded that the RPHPLC-ECD method developed by them is a powerful method for the determination of urinary UA. At this present stage, no single method is considered to be a standard comparison (reference) method for the estimation of UA in biological samples such as serum and urine. At least, we believe that the RPHPLC-ECD method is also an obvious candidate as a reference method for the determination of UA in urine as well as in any other biological samples.

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