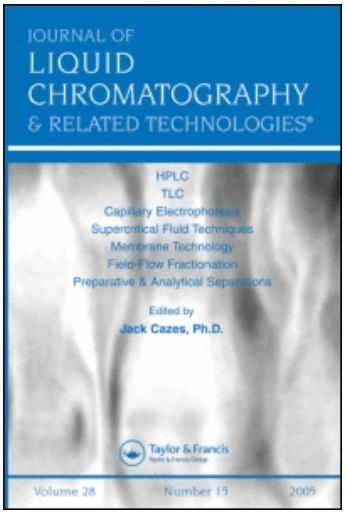

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A Uricase Method for the Peak Identification of Uric Acid Appeared in a Liquid Chromatogram Amperometrically Monitored

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A URICASE METHOD FOR THE PEAK IDENTIFICATION OF URIC ACID APPEARED IN A LIQUID CHROMATOGRAM AMPEROMETRICALLY MONITORED

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ABSTRACT

A uricase method for the peak identification of uric acid appeared in a liquid chromatogram monitored by aid of an electrochemical detector has been developed. Uricase (EC 1.7.3.3, from Candida utilis) catalyzes the conversion of uric acid to allantoin. We have found that uric acid can be oxidized under the chromatographic conditions employed in this study, whereas allantoin cannot be oxidized. The complete disappearance of a uric acid peak in a chromatogram of a biological sample after

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the uricase treatment indicates that the uric acid peak does not contain any other electroactive components. We observed the complete disappearance of the uric acid peaks in the chromatograms of human serum and gastric body.

Uric acid (UA) is the end-product of purine catabolism in man and the compound of biological and clinical interest. As described in our previous report (1), for a better understanding of the relationship between some complications such as gouty nephropathy and serum urate levels, studies of UA in mammalian tissues seem to be essential. Recently, we have developed a method for the determination of UA in body fluids such as serum (2, 3), urine (4, 5), and cerebrospinal fluid (5, 6) and in mammalian tissues (1, 7) by high-performance liquid chromatography (HPLC) with electrochemical detection (ED). Krstulovic et al. (8) also have developed a method for the determination of UA in amniotic fluid by HPLC-ED. In our previous reports (1-7), tentative identification of a UA peak was performed on the basis of retention behavior and co-chromatography with the reference compound. Krstulovic et al. (8) tested the identity of a UA peak using an enzyme peak-shift reaction with uricase, which catalizes the conversion of UA to allantoin. Since the reaction product (allantoin) does not absorb at 280 nm, the disappearance of the substrate peak was taken as an indication of the peak identity (8). When Krstulovic et al. (8) performed the peak identification, eluate from a reversed-phase column was spectrophotometrically monitored by aid of an ultraviolet absorption detector. Most recently, we have found that allantoin cannot be oxidized under the same chromatographic conditions as used in our previous study (1-7). The complete disappearance of a uric acid peak in a chromatogram, which was amperometrically monitored by aid of an electrochemical detector, after the uricase treatment indicates that the

uric acid peak did not contain any other electroactive components.

In this paper, we demonstrate the enzymatic treatment procedure for checking the UA peak purity.

MATERIALS AND METHODS

Uricase (EC 1.7.3.3, from Candida utilis, 2.5 IU/mg powder) was obtained from Oriental Yeast Co., Ltd., Tokyo, Japan. All other reagents were obtained in analytical reagent grade from Wako Pure Chemicals, Osaka, Japan. All buffers and aqueous solutions were prepared with glass-distilled deionized water. The stock solution of UA was carefully prepared according to the procedure previously described (2, 3), since it has been found that the solubility of UA and its salt in aqueous solutions depends markedly on the pH value. These substances are only sparingly soluble under acid conditions. An aliquot (0.05 ml) of a 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 into the dissolved UA solution. The UA stock solution was stored at 4°C. Appropriate dilution of the stock solution was done with a 0.1 M borate buffer (H_3BO_4-NaOH, pH 8.0) containing 10 mM ethylenediaminetetraacetate (EDTA) just before use. A portion (1 mg) of uricase was dissolved in an aliquot (10 ml) of the borate buffer (pH 8.0) containing 10 mM EDTA. The presence of EDTA seems to stabilize uricase in the buffer solution. The enzyme solution thus prepared was added into biological samples, whose pH was adjusted to pH 8.0, for converting UA to allantoin for the UA peak identification.

A chromatographic system employed in this study was completely the same as used in our previous report (1). Briefly, a liquid chromatographic constant flow pump

(Model 655, Hitachi, Tokyo, Japan) with a 50- 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, Funabshi, Chiba, Japan) set at +800 mV vs. Ag/AgCl was used in this separation and determination study. In this study, eluate from the column was also spectrophotometrically monitored by aid of an ultraviolet absorption detector (Model UVIDEC-100-III, Jasco, Tokyo, Japan) set at 284 nm, which was situated between the column and the electrochemical detector. The mobile phase was a 0.2 M phosphate buffer (KH_2PO_4-H_3PO_4, pH 2.0). The flow rate was 1.0 ml/min. The column temperature was 25 ± 1°C. An aliquot (10 μl) of each sample solution was injected onto the column. For the spectrophotometrical and electrochemical detectors, the limits of UA detection were in the nanogram and picogram range, respectively, under the present chromatographic conditions.

Absorption spectra were recorded on a Hitachi 340 recording spectrophotometer (Hitachi, Tokyo, Japan).

The pH optimum of the reaction step catalyzed by uricase has been found to be around pH 8.0. Therefore, each biological sample was first buffered to pH 8.0 and then incubated with uricase for an appropriate period. An aliquot (1 ml) of freshly prepared human serum was mixed with 9 ml of a 0.1 M borate buffer (H_3BO_4-NaOH, pH 8.0) containing 10 mM EDTA and the value for pH of the diluted serum (Sup I) was found to be 8.0. A portion (123.2 mg) of gastric body excised from a patient with gastric ulcer provided by Dr. Kunio Nitta (Teikyo University School of Medicine) was homogenized in 10 ml of the borate buffer (pH 8.0) containing 10 mM EDTA and the homogenate was centrifuged at 3,000 g for 5 min in order to obtain the supernatant (Sup I). An aliquot (1 ml) of each Sup I was mixed with an aliquot (0.5 ml)

of the uricase solution and the mixture (Sup II) was incubated at 37°C. The conversion of UA to allantoin by uricase was studied as a function of the incubated time. The enzymatic reaction was stopped by the addition of a 0.4 M perchloric acid solution (1 ml) and each reaction mixture was centrifuged at 3,000 g for 5 min. An aliquot (10 µl) of the supernatant (Sup III) was injected onto the column. An aliquot (1 ml) of each SupI was also mixed with an aliquot (0.5 ml) of the highly purified water in place of the uricase solution and the mixture (Sup IV) was incubated under the same conditions as used in the above described incubation study. The conversion of UA to allantoin in artificially prepared aqueous solutions was also studied as a function of the incubated time. An aliquot (1 ml) of the UA stock solution was mixed with an aliquot (99 ml) of the borate buffer with 10 mM EDTA (pH 8.0) and an aliquot (1 ml) of the thus diluted UA solution (Sup V) was mixed with an aliquot (0.5 ml) of the uricase solution. The reaction mixture was also incubated at 37°C to study the conversion of UA to allantoin as a function of the incubated time. After the incubation for an appropriate period, the enzymatic reaction was stopped by the addition of an aliquot (1 ml) of a 0.4 M perchloric acid solution. The reaction mixture was centrifuged at 3,000 g for 5 min and an aliquot (10 µl) of the supernatant (Sup VI) was injected onto the column.

RESULTS AND DISCUSSION

Fig. 1 (a) shows an absorption spectrum of a 0.2 M phosphate buffer solution (KH_2PO_4-H_3PO_4, pH 2.0) containing UA (5 µg/ml). As seen in Fig. 1 (a), the absorption maximum of UA in the phosphate buffer used as the mobile phase in this study was located at 284 nm. Therefore, the ultraviolet absorption detector was set

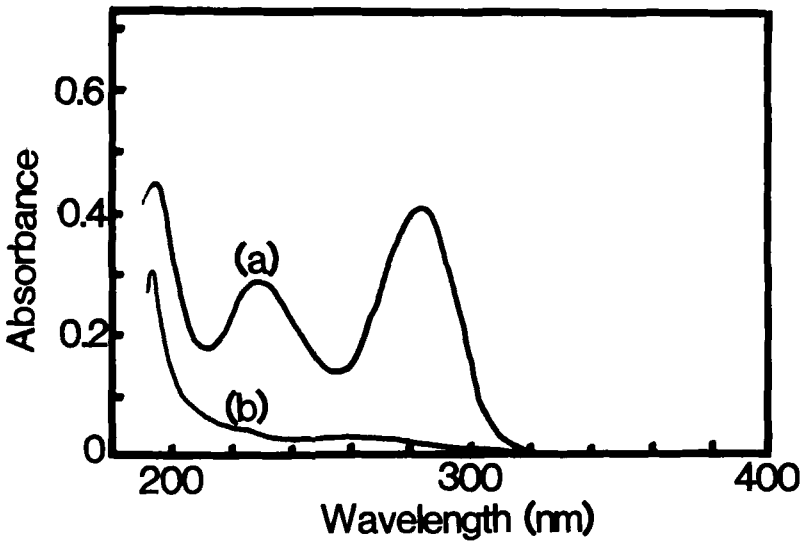

Fig. 1. Ultraviolet absorption spectra of (a) uric acid and (b) allantoin in 0.2 M phosphate buffer (KH_2PO_4-H_3PO_4, pH 2.0).

at 284 nm. Fig. 1 (b) shows an absorption spectrum of the phosphate buffer (pH 2.0) containing allantoin (100 μg/ml). Comparison of the absorption spectra (a) and (b) in Fig. 1 revealed that the absorption coefficient of allantoin is extremely small. Therefore, it was impossible to detect allantoin eluted from the column by aid of the ultraviolet absorption detector, when an aliquot of each Sup III was injected.

Fig. 2 (a) shows a chromatogram obtained by injecting an aliquot (20 μl) of the phosphate buffer solution containing allantoin (100 μg/ml) onto the column, when eluate from the column was monitored by aid of the ultraviolet absorption detector set at 210 nm. Fig. 2 (b) shows a chromatogram obtained by injecting an aliquot (20 μl) of the same allantoin solution used in

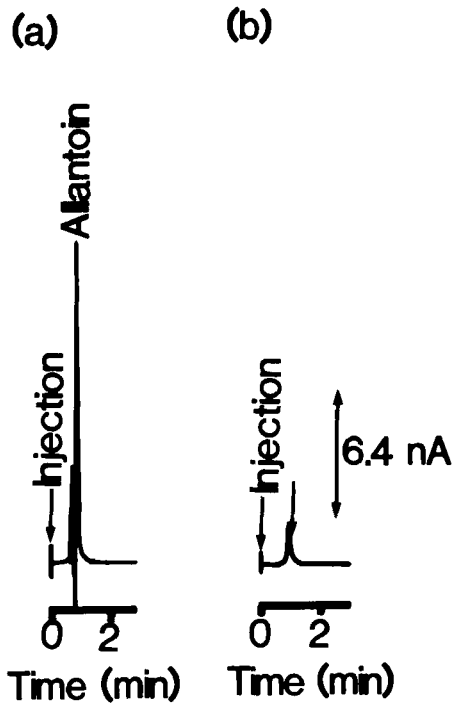

Fig. 2. The typical liquid chromatograms obtained by injecting an aliquot (20 µl) of 0.2 M phosphate buffer (KH_2PO_4-H_3PO_4, pH 2.0) containing allantoin (100 µg/ml) onto the column under the present chromatographic conditions. The chromatograms (a) and (b) were recorded by monitoring eluate from the column by aid of the ultraviolet absorption and electrochemical detectors, respectively.

Fig. 2 (a), when eluate from the column was amperometrically monitored. As seen in Fig. 2 (b), any peak except for the so-called solvent peak could not be appeared. This observation suggests that allantoin can scarecely be oxidized under the present electrochemical conditions employed in this liquid chromatographic study. Therefore, the compelte disappearance of a UA peak in a liquid

chromatogram of a biological sample after the uricase treatment indicates that the peak identified as the UA one on the basis of retention behavior and co-chromatography with the reference compound contains only UA as the electroactive component.

Fig. 3 shows a chromatogram obtained by injecting an aliquot (10 μl) of the Sup V preparation onto the column eluate from the column was amperometrically monitored in Fig. 3. Fig. 4 shows the change of UA as a function of the incubation time of the Sup V. In Fig. 4, eluate from the column was amperometrically monitored and each peak height was plotted as a function of the incubated time. As seen in Fig. 4, the conversion of UA to allantoin by uricase was achieved by 15 min incubation.

Fig. 5 (a) shows a chromatogram obtained by injecting an aliquot (10 μl) of the Sup IV from human serum after 15 min incubation onto the column, when eluate from the column was monitored by aid of the electrochemical detector. As seen in Fig. 5 (a), the UA peak was obtained as a distinguished single one and any other peaks were not appeared in the chromatogram. Fig. 5 (b) represents a chromatogram of the Sup II obtained from human serum after 15 min incubation. As seen in Fig. 5 (b), the complete disappearance of the UA peak was performed by the enzymatic treatment of the human serum for 15 min at 37°C. This observation indicates that the UA peak appeared in Fig. 5 (a) contained only UA as the electroactive components.

Fig. 6 (a) shows a chromatogram of the same sample as used in Fig. 5 (a), when an aliquot (10 μl) of the sample was injected and eluate from the column was monitored by aid of the ultraviolet absorption detector set at 284 nm. Fig. 6 (b) represents a chromatogram of

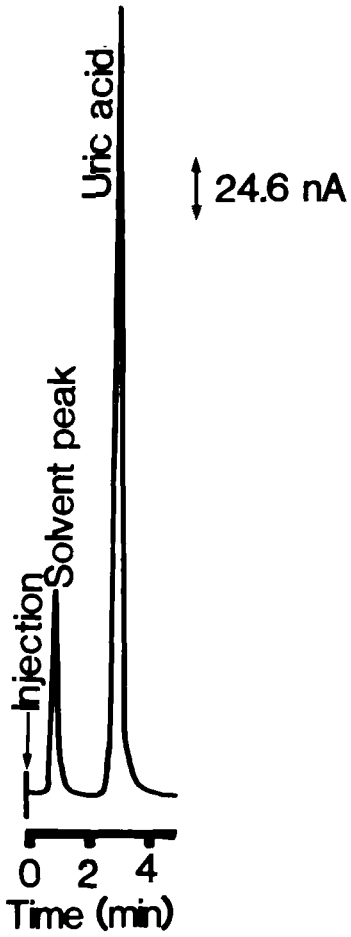

Fig. 3. A liquid chromatogram obtained by injecting an aliquot (10 μl) of the Sup V (see the text) onto the column under the present chromatographic conditions. Eluate from the column was amperometrically monitored by aid of the electrochemical detector.

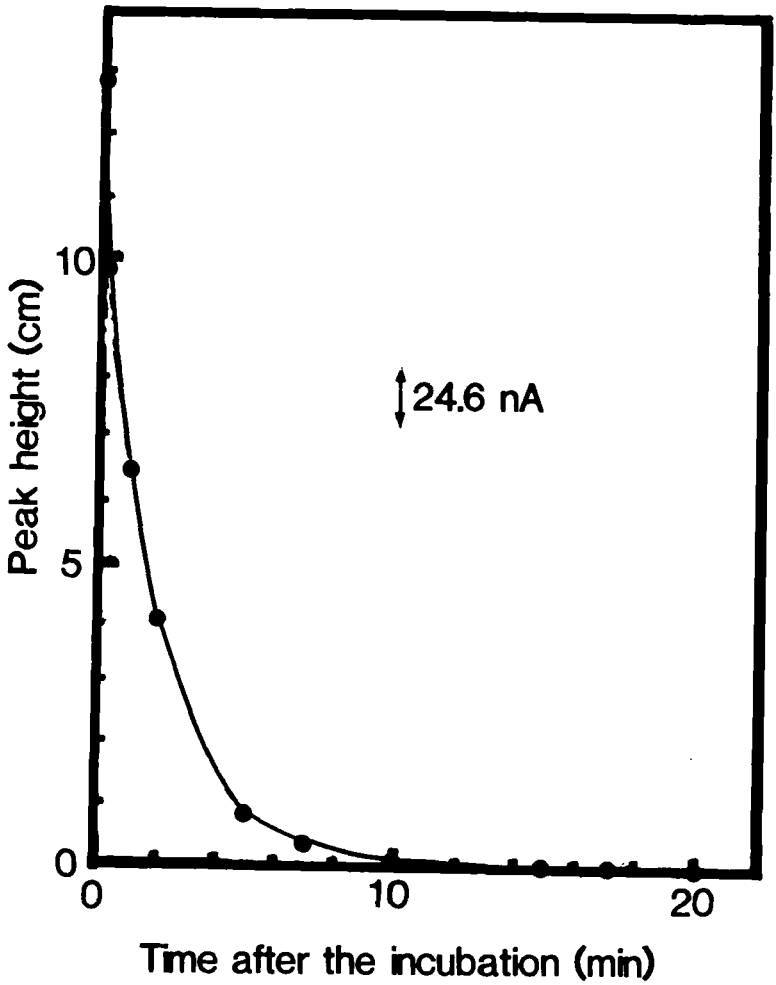

Fig. 4. Change of uric acid as a function of the incubation time of the same sample as used in Fig. 3.

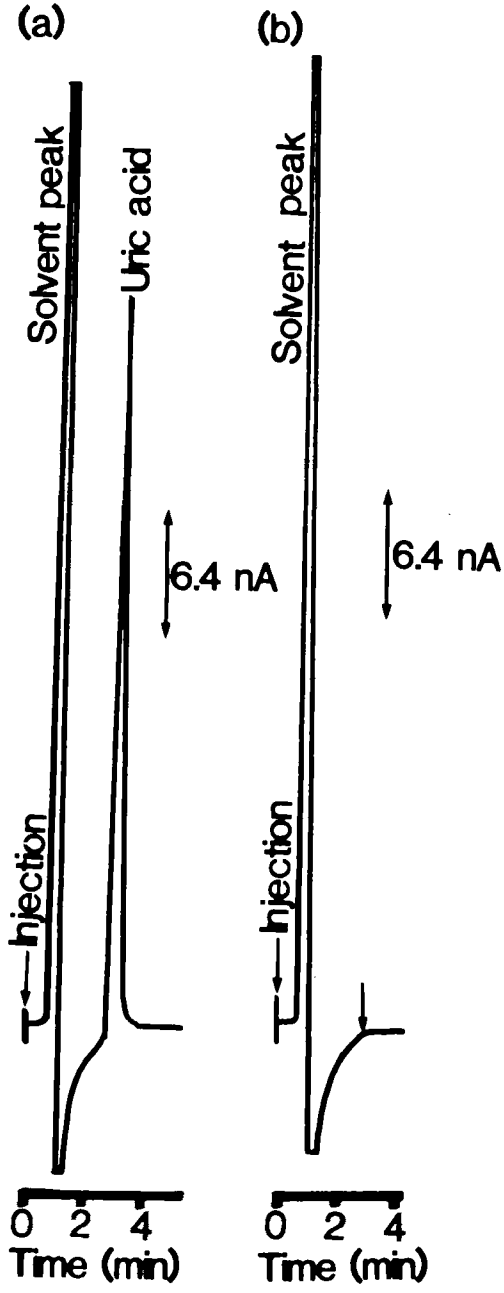

Fig. 5. The typical liquid chromatograms of (a) the Sup IV and (b) the Sup II obtained from human serum after 15 min incubation. Eluate from the column was amperometrically monitored.

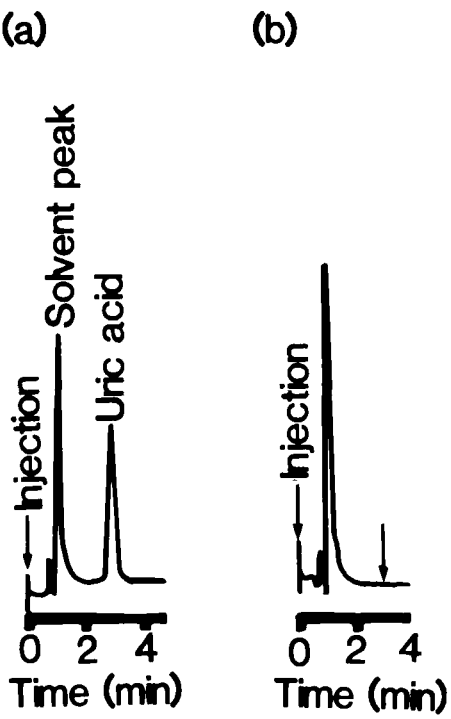

Fig. 6. The chromatogram of the same (a) Sup IV and (b) Sup II as used in Fig. 5 after 15 min incubation. Eluate from the column was spectrophotometrically monitored.

the same sample as used in Fig. 5 (b) under the same chromatographic conditions as used in Fig. 6 (a). As seen in Fig. 6 (b), the UA peak was completely disappeared indicating that the UA peak did not contain any other ultraviolet-absorbing components, which disturb the quantitative determination of UA in human serum samples.

Fig. 7 (a) shows a chromatogram obtained by injecting an aliquot (10 μl) of the freshly prepared Sup II from the human gastric ulcer tissue onto the column, when eluate from the column was amperometrically

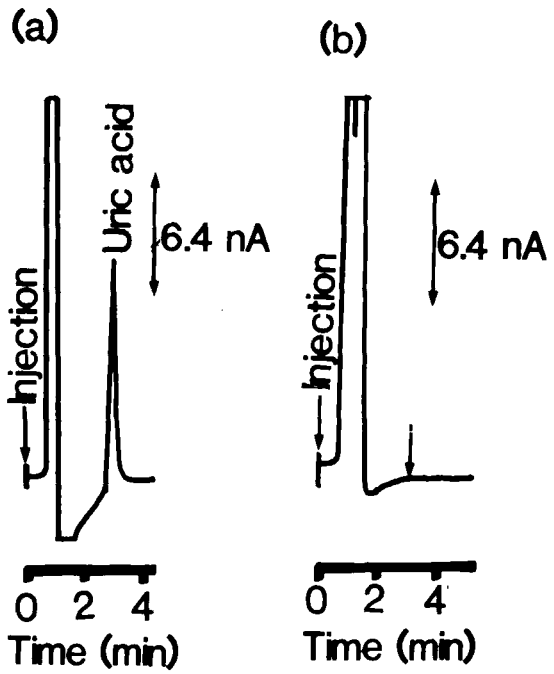

Fig. 7. The chromatograms obtained by injecting an aliquot (10 µl) of the Sup II, which was obtained from the human gastric ulcer tissue, (a) before and (b) after one hour incubation onto the column. Eluate from the column was monitoerd by aid of the electrochemical detector.

monitored. As seen in Fig. 7 (a), the UA peak was appeared as a distinguished one. Fig. 7 (b) shows a liquid chromatogram of the same Sup II preparation as used in Fig. 7 (a) after one hour incubation. It has been found that conversion of UA to allantoin by uricase is generally slower in tissue-extracts than in body fluids such as serum, urine, amniotic fluid, and cerebrospinal fluid. Therefore, the incubation was conducted for one hour in Fig. 7. Fig. 8 (a) represents a chromatogram of the same sample as used in Fig. 7 (a), when eluate from

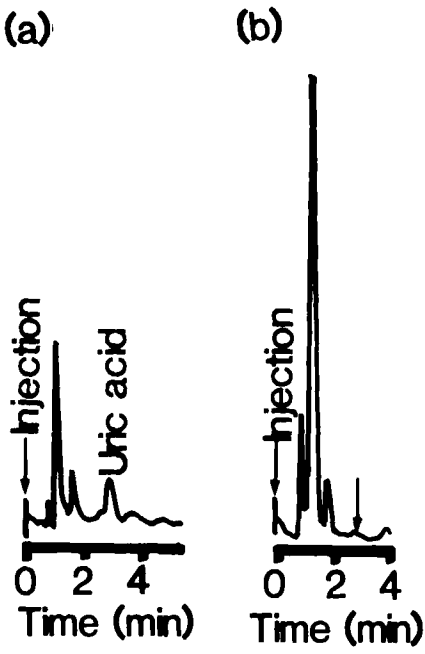

Fig. 8. The chromatograms of the same Sup II as used in Fig. 7 (a) before and (b) after one hour incubation. Eluate from the column was monitored by aid of the ultraviolet absorption detector.

the column was monitored by aid of the ultraviolet absorption detector. As indicated by an arrow in Fig. 8 (a), the UA peak was identified by the tentative identification procedures on the basis of retention behavior and co-chromatography with the reference compound. Fig. 8 (b) shows a chromatogram of the same sample as used in Fig. 7 (b) under the same chromatographic conditions as used in Fig. 8 (a). As seen in Fig. 8 (b), the disappearance of the UA peak was attained.

As described above, we have demonstrated the effectiveness of the uricase peak-disappearance reaction

method for testing the purity of a UA peak in a liquid chromatogram of a biological sample, when eluate from the column is spectrophotometrically and/or electrochemically monitored. The HPLC-ED and -ultraviolet absorption detection (UAD) methods seem to be useful for the determination of UA in biological samples within the experimental results shown in this paper. However, we have found also that the UA peak in a chromatogram of the rat gastric body tissue-extract does not contain any other electroactive components, but contains any other ultraviolet-absorbing components. We have found (2) that the retention time and peak height of UA is scarecely affected by the sample matrix as well as by the presence of some overlapping electroactive components, when eluate from a column was amperometrically monitored by aid of the electrochemical detector. Therefore, we recommend use of the HPLC-ED method in determining UA in biological samples because of its high selectivity and sensitivity.

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