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Determination of uric acid in human saliva by high-performance liquid chromatography with amperometric electrochemical detection

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Abstract

The aim of the present study is to establish a highly sensitive method for the determination of uric acid (UA) in human saliva. The monitoring of UA levels in less invasive biological samples such as saliva is suggested for the diagnosis and therapy of gout, hyperuricemia, and the Lesch–Nyhan syndrome, and for detecting such conditions as alcohol dependence, obesity, diabetes, high cholesterol, high blood pressure, kidney disease, and heart disease. Reversed-phase high-performance liquid chromatography with electrochemical detection (HPLC–ED) was employed for the determination of UA obtained by solid-phase extraction from saliva. To quantify UA, we compared the ED efficiencies of an amperometric ED (Ampero-ED) with a single electrode and a coulometric ED (Coulo-ED) with a multiple electrode array. The results showed that the detection limits ($S/N = 3$) were 3 nM for Ampero-ED and 6 nM for Coulo-ED, and the linearity of the calibration curves of 60–6000 nM had correlation coefficients exceeding 0.999. In addition, the total analytical time was 10 min. In the sample preparation of UA in saliva, an Oasis MAX solid-phase cartridge was used. The recoveries of UA spiked at 0.6 and 3 μM in saliva were above 95% with a relative standard deviation (RSD) of less than 15%. Therefore, the present method may be used in the routine and diagnostic determination of UA in human saliva.

Keywords: Uric acid

1. Introduction

Uric acid (2,6,8-trihydroxypurine, UA) (Fig. 1), the major nitrogenous compound in urine, is the product of purine metabolism in the human body [1,2].

The presence of elevated UA levels is a sign of gout, hyperuricemia, or Lesch–Nyhan syndrome [3]. Similarly, elevated UA levels are related to other conditions including increased alcohol consumption, obesity, diabetes, high cholesterol, high blood pressure, kidney disease, and heart disease. Many epidemiological studies have suggested that serum UA is also a risk factor for cardiovascular disease [4]. Thus, for the diagnosis of patients suffering from a range of disorders associated with altered purine metabolism, the screening of UA in human physiological fluids is indispensable.

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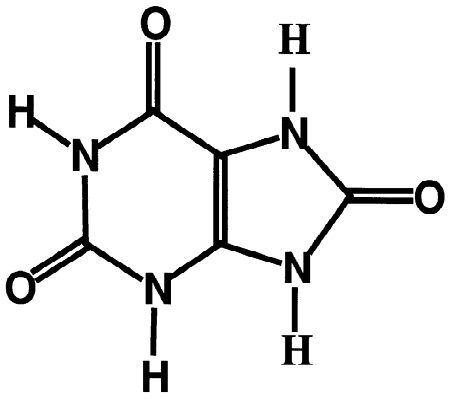

Fig. 1. Structure of UA: M_w 168.1.

The method that is currently used for the quantification of UA in biological samples is a direct method. A method for detecting serum UA based on its strong reducing activity in solvent has been proposed, but the detection limit is low [5]. The separation and detection of UA in human biological samples was achieved by high-performance liquid chromatography (HPLC) [1,3,6–31]. Naturally, most of the target biological samples were human blood and urine. Recently, it was reported that the detectable levels of UA in human saliva were similar to those in the blood of gout patients [32]. Therefore, the use of less invasive biological samples such as saliva for monitoring UA was investigated for the diagnosis and therapy of gout, hyperuricemia, and Lesch–Nyhan syndrome, etc. However, highly sensitive methods for the determination of trace amounts of UA in a small volume of human saliva are not available.

In the present study, reversed-phase HPLC with electrochemical detection (HPLC–ED) was employed for the determination of UA obtained by solid-phase extraction (SPE) from human saliva. To quantify UA, we compared the detection efficiencies of an ultraviolet detector (UV), and the electrochemical detectors for amperometric ED (Ampero-ED) with a single electrode and coulometric ED (Coulo-ED) with a multiple electrode array. We describe herein a simple, rapid, sensitive, accurate, and selective method for the determination of UA in human saliva by HPLC.

2. Experimental

2.1. Materials and standard solution

UA, methanol, sodium phosphate, phosphoric acid, and hydrochloric acid were purchased from Wako Pure Chemicals (Osaka, Japan). Distilled water was purified by the Milli-Q gradient A10 Elix system (Millipore, Bedford, MA, USA).

A stock solution of UA was prepared by dissolving and mixing 10.0 mg of the compound in 100 ml of distilled water, and potassium hydroxide was added to a final concentration of 10 m*M*, to give a final UA concentration of 600 μ*M*. Standards for HPLC analysis were prepared by appropriate dilutions of this solution with 10 m*M* potassium hydroxide. The working standards were 60, 30, 6, 3, 0.6, 0.3, and 0.6 μ*M*.

2.2. Human saliva collection and storage

The saliva samples for this study were originally collected from six healthy volunteers (four males and two females). None of them suffered from systemic or salivary gland disease that could affect the saliva. They were requested to fast 2 h before saliva withdrawal. The saliva pH ranged from 7.0 to 8.0 in all the subjects. The saliva collected from the whole mouth while chewing a polyester sponge was centrifuged at 3000 rev./min for 30 min to remove cellular elements. The clear supernatant fluid was stored at −20 °C until analysis.

2.3. Apparatus and instrument conditions for HPLC with ultraviolet (UV) detector

The HPLC system consisting of a pump, a column oven, an auto injector, and an ultraviolet (UV) detector was from the Shiseido NANOSPACE SI-1 Series. The column oven was controlled at 40 °C. Separation of compounds was achieved using a CAPCELL PAK C_{18} UG 120 (150×2.0 mm, 5 μm) column (Shiseido, Tokyo, Japan). Isocratic potassium phosphate buffer (74 m*M*, pH 3.0) was used as the mobile phase. The flow-rate was 0.2 ml/min and the injection volume was 20 μl. The UV detection wavelength was 284 nm with a total run time of 10 min. After the injection of each sample, water

containing 10 m*M* potassium hydroxide was injected for blank monitoring. Comparing the HPLC retention times with those of the authentic standards enabled the identification of UA.

2.4. Apparatus and instrument conditions for HPLC with amperometric detector (Ampero-ED)

The HPLC system consisting of a pump, a column oven, an auto injector, and an amperometric electrochemical detector (Ampero-ED) was from the Shiseido NANOSPACE SI-1 Series. Separation of compounds was achieved using a CAPCELL PAK C_{18} UG 120 (150×2.0 mm, 5 μm) column (Shiseido). The column oven was controlled at 40 °C. Isocratic potassium phosphate buffer (74 m*M*, pH 3.0) was used as the mobile phase. The flow-rate was 0.2 ml/min and the injection volume was 20 μl. The analytical cell potential $E = +600$ mV vs. Ag/AgCl with a total run time of 10 min was used for detection. Working and reference electrodes used in the electrochemical detector were glassy carbon and Ag/AgCl, respectively. After the injection of each sample, water containing 10 m*M* potassium hydroxide was injected for blank monitoring. Comparing the HPLC retention times with those of the authentic standards enabled the identification of UA.

2.5. Apparatus and instrument conditions for HPLC with coulometric detector (Coulo-ED)

The HPLC system consisting of a pump, a column oven, an auto injector, and a multiple electrode array electrochemical detector containing cells from Coul Array model 6210 and database from Coul Array System Win 32 vol.1.0 (ESA, Chelmsford, MA, USA) was used. The cell potentials of Coulo-ED consisted of an increasing array (Ch_1–Ch_4; +100, 130, 160, and 190 mV vs. palladium). Working and reference electrodes used in the electrochemical detector were porous graphite and palladium, respectively. Separation of the compounds was achieved using a CAPCELL PAK C_{18} UG 120 (150×2.0 mm, 5 μm) column (Shiseido). The column oven was controlled at 40 °C. Isocratic potassium phosphate buffer (74 m*M*, pH 3.0) was used as the mobile phase. The flow-rate was 0.2 ml/min, and the injection volume was 20 μl by an auto-sampler. The total run time was 10 min. After the injection of each sample, water containing 10 m*M* potassium hydroxide was injected for blank monitoring. Comparing the HPLC retention times with those of the authentic standards enabled the identification of UA.

2.6. Sample preparation of UA by solid-phase extraction (SPE)

Sample preparation and clean-up of human saliva were carried out using an SPE cartridge. An Oasis MAX SPE cartridge was used, which consisted of *n*-vinylpyrrolidone and divinylbenzene polymer that provided the mixed-phase mode (reversed-phase and strong anion-exchange). The SPE cartridge was conditioned with 1.0 ml of methanol followed by 1.0 ml of water. After 50 μl of 1 M potassium hydroxide were added to 450 μl of the saliva samples (pH adjusted to 10.0), the samples were vortex-mixed for 10 min and then applied onto the conditioned SPE cartridge under vacuum. The cartridge was washed with 0.5 ml of water (pH adjusted to 10.0 with 1 M potassium hydroxide), and then eluted with 2.0 ml of water (pH adjusted to 2.0 with 1 M hydrochloric acid). The SPE eluate was evaporated to dryness and the dried residues were reconstituted in 500 μl of the mobile phase. The obtained samples (injection volume, 20 μl) were measured by HPLC.

3. Results and discussion

3.1. Optimization of HPLC–UV and ED conditions

Firstly, we examined the HPLC–UV detection of UA standard solutions. The UV absorption spectrum of UA showed a maximum at 284 nm. This absorption maximum at 284 nm can be used to determine UA. The limit of detection (LOD) of HPLC–UV was calculated as 3× the response of the analyzed concentration in the blank. The calculated LOD was 180 nM (3.6 μmol) for HPLC–UV detection. In addition, the limit of quantification (LOQ) was 600 nM in saliva samples according to the signal-to-noise (S/N) ratio = 10. A UA standard was used to investigate the linearity of this method. The UA concentration versus the peak area was plotted. The response was found to be linear in the investigated

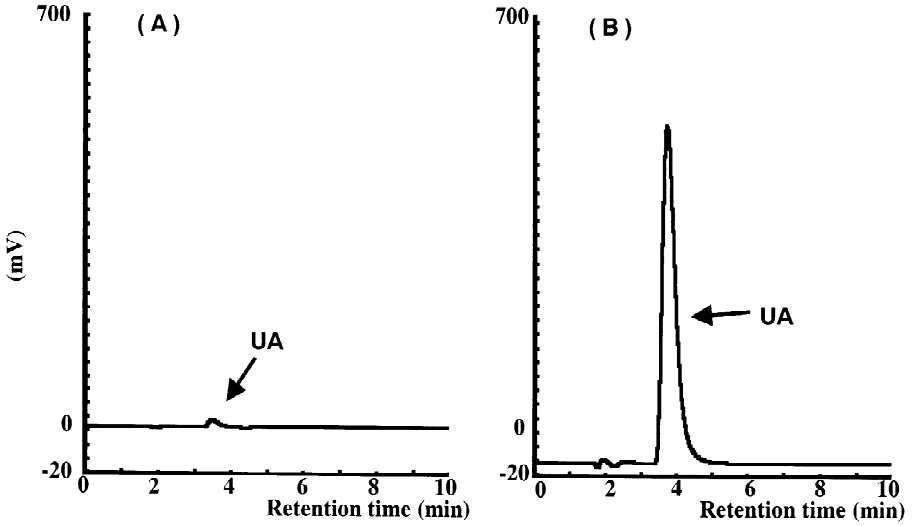

Fig. 2. Chromatograms of UA standard (6 μM) obtained by (A) HPLC–UV detection at 284 nm and (B) Ampero-ED at +600 mV.

range of 0.6–60 μM with correlation coefficients (*r*) higher than 0.999. When the UA standard was consecutively measured (0.6 and 60 μ*M*), the averages of the retention times were 3.59 min (RSD = 0.4%, $n = 10$) and 3.63 min (RSD = 1.8%, $n = 10$). Moreover, the accuracy of the RSDs of peak area were 2.2 and 2.1%, respectively. The HPLC–UV chromatogram and the summarized conditions are shown, respectively, in Fig. 2 and Table 1. We see from Fig. 2 and Table 1 that the HPLC–ED method proved to be more sensitive and useful than the peak response of the UV method. Thus, it is possible that this method is useful for sensitive determination of UA in biological samples.

In the determination of UA by HPLC–Ampero-ED, it is generally known that UA oxidation potentials shift with increasing pH. The pH of the supporting electrolyte influences the electron transfer rate constants. For this reason, we chose phosphoric acid as the mobile phase. Hydrodynamic voltammograms of UA measured at various pH values are shown in Fig. 3. Based on the results, we decided to use phosphoric acid in water at pH 3.0 as the mobile phase. We also decided to use the above mobile phase for the HPLC–Ampero-ED of UA standard. As a result, LOD and LOQ were, respectively, 3 nM, (60 pmol) with $S/N = 3$ and 10 nM with $S/N = 10$. The Ampero-ED calibration curve of UA, which was

Table 1
Analytical parameters of HPLC for UA

Parameter	UV	Ampero-ED	Coulo-ED
Detection limit (pmol)	3600	60	120
RSD of retention time (%)[a]	0.4	0.8	0.6
Limit of detection (nM)	180	3	6
Limit of quantification (nM)	600	10	20
RSD of peak area (%)[a]	2.2	0.8	1.4
Linear range (μM)	0.6–60	0.06–6	0.06–6
Correlation coefficient (*r*)	0.9996	0.9997	0.9999

[a] $n = 10$.

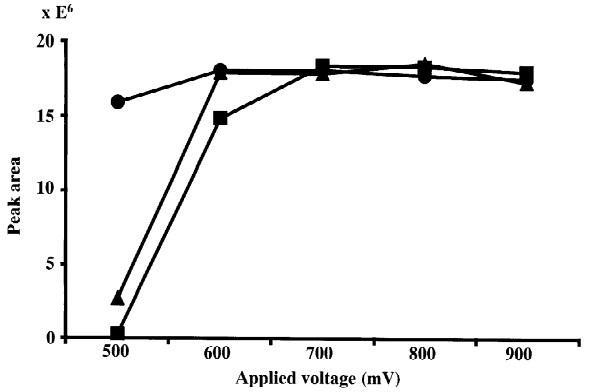

Fig. 3. Hydrodynamic voltammograms of UA standard (6 μM) obtained by HPLC–Ampero-ED. ●, pH 2.0; ▲, pH 3.0; ■, pH 4.0.

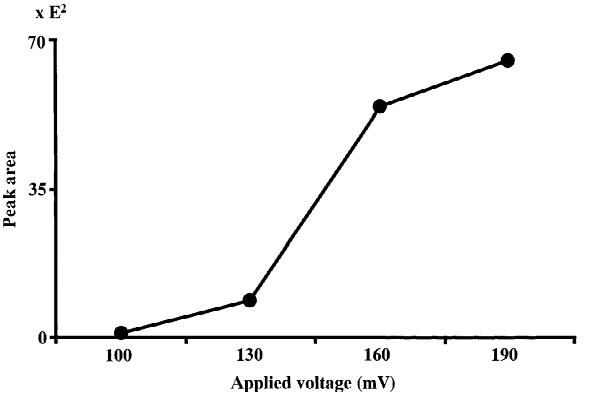

Fig. 4. Hydrodynamic voltammogram of UA standard (6 μM) obtained by HPLC–Coulo-ED.

constructed by plotting the concentration versus the peak area, showed good linearity in the 0.6 to 6 μM range (r higher than 0.999). When the UA standard was consecutively measured at 0.06 and 6 μM, the average retention times were 3.85 min (RSD = 0.8%, $n = 10$) and 3.88 min (RSD = 1.0%) with peak response accuracies of 0.8% (RSD, $n = 10$) and 1.6% (RSD, $n = 10$), respectively. The HPLC–Ampero-ED chromatogram and the summarized conditions are shown, respectively, in Fig. 2 and Table 1.

In the same way, the efficiency of HPLC–Coulo-ED was demonstrated. We optimized the potentials for this system by using the same mobile phase as that of HPLC–Ampero-ED. Fig. 4 depicts the voltammogram of UA. When UA passes through the multiple electrode array, it is normally detected by three contiguous electrodes. The first electrode (Ch_1) will oxidize a small amount of the UA, whereas the second (Ch_2) or the dominant (Ch_3) electrode will oxidize a large amount. The UA standard solution having a known retention time will give a predictable response at all three electrodes and the ratio across these three electrodes is constant and independent of concentration. In these conditions, LOD and LOQ were, respectively, 6 nM (120 pmol) with $S/N = 3$, and 2 μmol/l with $S/N = 10$. The calibration curve for the UA standard, which was constructed by plotting the concentration versus the Ch_3 peak area, showed good linearity in the 0.06 to 6 μM range (r higher than 0.999). The RSDs of the retention times (average 3.16 min) and peak area were 0.6 and 1.4% for the 0.06 μM standard, and those of the retention times (average 3.20 min) and peak areas were 0.5 and 0.9% for 6 μM, respectively. In addition, confirmation of actual samples was examined to compare with matching ratio (R) between UA standard and the actual sample. The HPLC–Coulo-ED chromatogram and the summarized conditions are shown, respectively, in Fig. 5 and Table 1.

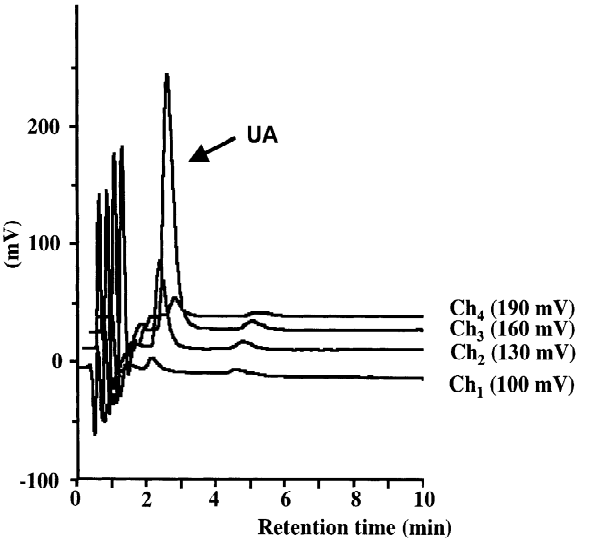

Fig. 5. Chromatograms of UA standard (6 μM) obtained by HPLC–Coulo-ED.

Table 2
Recovery test of UA in human saliva

Spiked conc. (μM)	Recovery±SD % ($n=5$)
0.6	98.5±10.0
3.0	103.2±10.4

This HPLC method enables the precise determination of standards and may be used in detecting trace amounts of UA in small volumes of saliva.

3.2. Pretreatment of UA in human saliva using SPE cartridge

Samples were extracted using an SPE cartridge (Oasis MAX, Waters). The SPE cartridge with the mixed-phase mode (reversed-phase and anion-exchange) was examined in terms of recovery, relative standard deviation (RSD) and cleanness. The extractions using SPE cartridges were performed according to the above-described method. The recovery rate of UA was higher than 95% with the Oasis MAX (Table 2). Therefore, the SPE method is suitable for use with UA.

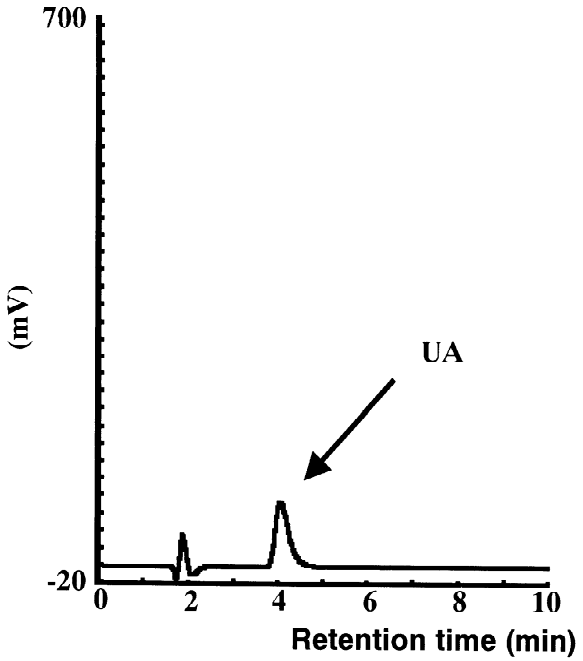

Fig. 6. Typical chromatogram of UA in human saliva obtained by HPLC–Ampero-ED.

3.3. Application

We examined six saliva samples for the presence of UA using HPLC–ED. Measurements made by HPLC–UV failed to detect UA in a small number of samples. Using HPLC–ED detection, these results from the saliva samples ranged from 7.3 to 265.0 μ*M*, with the average being 137.5 μ*M*. In addition, the chromatogram of the sample is shown in Fig. 6.

These data suggest that the LC–ED method can detect trace amounts of UA in human saliva. In addition, we have demonstrated that the LC–UV method may give erroneous values, which may be due to the non-specific and non-selective detection of UA in coexisting biological compounds in saliva.

4. Conclusions

Uric acid is a final metabolite product of purine nucleotide catabolism in humans. Therefore, the method for determination of this compound is useful for clinical diagnosis of gout. In this study, the interferences from endogenous compounds were removed by using the SPE technique. In addition, the development of an analytical technique for the accurate quantification of UA in human saliva is desired for the diagnosis and therapy of gout, hyperuricemia, and Lesch–Nyhan syndrome, etc. We have demonstrated here that HPLC–ED is the method of choice for the accurate determination of UA levels in human saliva, and that this may be extended to include other human biological samples. Further studies are necessary to examine the correlation between trace amounts of UA in saliva and the diagnosis of gout, hyperuricemia, and Lesch–Nyhan syndrome, etc.

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