

The Determination of Uric Acid in Human Saliva by Liquid Chromatography with Electrochemical Detection

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Abstract

A method employing high-performance liquid chromatography with electrochemical detection (LC-ED) has been successfully developed for the determination of uric acid in human saliva. The optimal chromatographic conditions were found to comprise a mobile phase containing 5 % methanol, 95 % 50 mM trichloroacetic acid, adjusted to pH 2.7, in conjunction with a Hypersil C₁₈ 250 mm x 4.6 mm column at a flow rate of 0.6 mL min⁻¹. Hydrodynamic voltammetric studies were undertaken to optimize the operating potentials required for electrochemical detection. It was found that an applied potential of +1.2 V was optimum for the determination of uric acid. The proposed method was evaluated by carrying out replicate uric acid determinations on spiked and unspiked human saliva samples (%CV = 8.8 %). The data suggest that the method holds promise for clinical applications.

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Introduction

The aim of the current study was to investigate the possibility of determining uric acid (2,6,8-trihydroxypurine) (I) in human saliva using high performance liquid chromatography and electrochemical detection (LC-ECD).

The study of uric acid is important as it represents one of the most important antioxidants in humans contributing to more than 70 % of the total antioxidants present in human saliva^[1]. In fact, humans and other higher apes exhibit relatively high levels of uric acid and in blood it is near its solubility point. Relatively small increases can lead to illness such as gout, the prevalence and incidence of which has been reported to be increasing in the UK^[2]. Nevertheless, it has been suggested that these high levels of uric acid are one of the reasons for our lengthen life spans compared to many other species and there is debate on the relative health effects of uric acid^[3,4]. However, uric acid has also been shown to be associated with conditions such as, type 2 diabetes^[5], congestive heart failure, hyperurice-

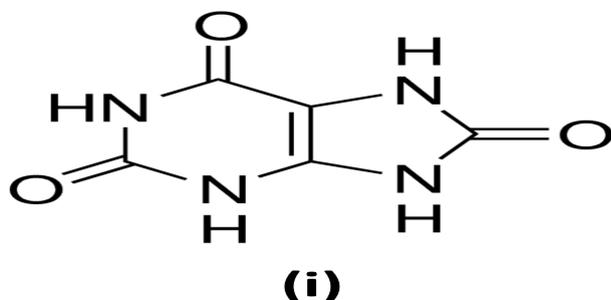
mia obesity, hypertension^[6], depression^[7], cognitive ability^[8], Lesch-Nyhan syndrome^[9] and kidney stone formation^[10].

Levels of uric acid in saliva for healthy individuals would appear to vary, but generally are in the mid to high μM range. Owen-Smith *et al.*^[11] have shown uric acid levels in the saliva of healthy individuals to be in the range of 120 μM to 400 μM with levels as high as over 1 mM were found in the saliva of gout sufferers.

As a reflection of the importance of monitoring uric acid, various analytical techniques have been utilised for its determination. Table 1 summaries a number of recent chromatographic approaches utilised for the determination of uric acid. HPLC coupled with electrochemical detection is relatively straightforward and highly sensitive requiring no derivatisation step or expensive sophisticated equipment. In this study we have chosen to investigate the levels of uric acid in human saliva as this has a number of practical advantages over that of blood and plasma both in terms of non-invasive nature of sample collection and the greater willingness of individuals to donate such samples.



Our present proof-of-concept study was divided into three parts. Initially, we optimized the HPLC conditions needed for the separation of uric acid on a C₁₈ reversed-phase column and optimized the electrochemical conditions required via hydrodynamic voltammetry. Finally, we investigated the possibility of using the optimized system for the determination of uric acid in human saliva samples.



Experimental

Chemicals and Reagents

All standard reagents were purchased from Fisher Scientific or BDH and used as received. Deionised water was obtained from a Purite RO200-Stillplus HP System, (Purite, Oxon, UK). Uric acid was obtained from Sigma Aldrich (Poole, Dorset, UK). Stock solutions were made by dissolving the required mass in 20 mM NaOH. Human saliva was obtained from a healthy volunteer after fasting for one hour.

Apparatus

Cyclic voltammetry (CV) was performed with a Sycopel Scientific AEW2 potentiostat interfaced to a PC for data

acquisition and processing using the Electrochemistry Program version 3.00 (both Sycopel Scientific Ltd, Tyne and Wear, UK).

High-Performance Liquid Chromatography

HPLC studies were undertaken using a system consisting of an IsoChrom pump (Spectra Physics), with a 250 mm x 4.6 mm Hypersil Hypurity C₁₈, 5 µm column connected to a 7125 valve manual injector fitted with a 50 µL sample loop (Rheodyne, Cotati, CA). Sample extracts were analyzed using a mobile phase consisting of 50 mM trichloroacetic acid, adjusted to pH 2.7 with sodium hydroxide modified with 5 % methanol at a flow rate of 0.6 mL min⁻¹.

Electrochemical Detection

The detector cell was obtained from BAS (Congleton, Cheshire, U.K.) The cell consisted of a two-piece thin-layer cell, formed from an upper Teflon block containing a glassy carbon electrode (3 mm diameter), and a bottom steel block serving as the pseudo reference/counter electrode. Teflon gaskets were purchased from BAS. An EG&G Princeton Applied Research (Princeton, NJ) model 362 scanning potentiostat was used to control the potential at +1.2 V versus the pseudo reference/counter steel electrode. Chromatograms were recorded using a Siemens Kompenosograph X-T C1012 chart recorder.

Hydrodynamic Voltammetry (HDV)

HDV was performed by injecting fixed volumes of a standard solution of uric acid and varying the applied potential between + 0.5 and + 1.5 V. HDVs were constructed by plotting the recorded peak current for a fixed concentration against the applied potential. The optimum potential was determined from the position of the plateau of the hydrodynamic wave.

Table 1: HPLC and related approaches to the determination of uric acid in human saliva and human body fluids.

	Sample	Detection Limit	Linear Range	Analytical Technique	Reference
Uric acid	Human urine and saliva	0.21 ng	0.7–100 ng of UA (0.07–10 mg/L, taking into account the sample volume injected).	LC/MS. Anion exchange chromatography.	[14]
Uric acid	Human saliva	3 nM for amperometric and 6 nM for coulometric detection	60 to 6000 nM	Reversed-phase HPLC with electrochemical detection.	[15]
Uric acid, salivary peptides histatins 1, 3, 5 and statherin				HPLC with diode-array detection.	[16]
Uric acid	Serum	0.032 ng	6.0 to 200 mg/kg	Isotope dilution coupled with liquid chromatography/mass spectrometry electrospray ionization.	[17]
Uric acid	Human urine	0.02 mg/L	0.25 to 100 mg/L	Ion-exclusion column using water as mobile phase. UV detection at 254 nm.	[18]
Uric acid	Gout patients' plasma and urine	0.5 µg/mL	5 to 200 µg/mL	Microemulsion electrokinetic chromatography.	[19]
Uric acid	Human urine	0.5 µg/L	0.10 to 20 mg/L	Ion chromatography with conductivity detection.	[20]
Uric acid	Dental calculi	--	--	LC/MS	[21]
Uric acid	Human urine	0.3 µM	1 to 100 µM	Flow injection analysis with UV detection at 735 nm based on the reduction of Prussian Blue.	[22]

Sample Preparation

Approximately 1 to 2 mL of human saliva was collected from a healthy human volunteer in a 2 mL Eppendorf tube. This was sonicated for 15 minutes to destroy any cells that could be present^[12,13]. A suitable aliquot of this was diluted ten times in mobile phase and then syringe filtered (PTFE, 0.2 μm). The resulting solution was then introduced to the LC-ECD system. Quantification was achieved by external calibration.

Results and Discussion

Liquid Chromatographic separation of Uric Acid

Initial studies were made using a mobile phase consisting of 50 mM phosphate pH 3.0 buffers with 0.1 % EDTA at a flow rate of 1.0 mL/min. Under these conditions uric acid was found to co-elute with reduced glutathione (GSH). Milton and Trevithick^[23] utilised a mobile phase of chloroacetic acid and trichloroacetic acid (TCA) adjusted to pH 2.7, modified with 2 % methanol, for the determination of GSH in vertebrate lens fluid. It was believed that a similar approach could be used to separate GSH and uric acid. The pKa values of uric acid and GSH are known to be 3.89 and 2.12 respectively, hence at pH 2.7, GSH would be expected to be in its ionic form, and hence form an ion pair with trichloroacetic acid. However, under the same conditions, uric acid would not be ionised, and so not form an ion-pair with the trichloroacetic acid, allowing for a mechanism to separate the two analytes. Investigations showed that an organic modifier concentration of 2 % methanol was insufficient to stop collapse of the stationary phase and so was increased to 5 % and consequently a mobile phase of 5 % methanol, 95 % 50 mM TCA adjusted to pH 2.7 was found to optimal, and was used in further studies.

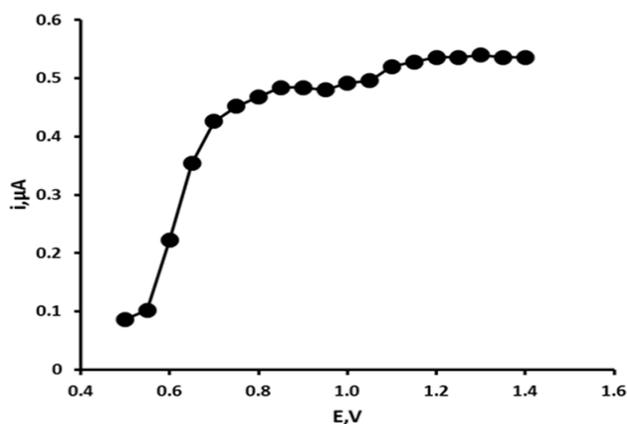


Figure 1: Hydrodynamic voltammogram of uric acid.

Hydrodynamic Voltammetric Studies

The electrochemical behaviour of uric acid has been understood for some time^[24]. It is known to undergo an irreversible $2e^-$, $2H^+$ oxidation to form the corresponding unstable 4,5-diol, which then hydrolyses to the electrochemically inactive allantoin. To identify the optimum applied potential required for the determination of uric acid hydrodynamic voltammograms were constructed over the potential range + 0.5 V to + 1.4 V. Figure 1 shows the resulting HDV for uric acid. Uric acid was found to give a potential dependant response between + 0.5 V and + 0.75 V, with current response above this potential forming

two plateaus between + 0.80 V and + 1.0 V and + 1.0 V and + 1.4 V. Consequently, an applied potential of + 1.2 V was used in further investigations.

Effect of Flow Rate

Studies have demonstrated that the sensitivity of an amperometric detector is dependent on flow rate^[25], and the effects of this parameter were studied over the range 0.1 mL min⁻¹ to 1.0 mL min⁻¹ (Figure 2). Peak height was seen to increase with flow rate from 0.1 mL min⁻¹ to 0.6 mL min⁻¹; beyond on this value peak height became independent of the flow rate. Below flow rates of 0.4 mL min⁻¹, the current response moved from amperometric to approaching more coulometric values. However, using such low flow rates resulted in excessively long retention times, and hence further studies were undertaken using a flow rate of 0.6 mL min⁻¹ as this gave the best balance between sensitivity and chromatographic performance.

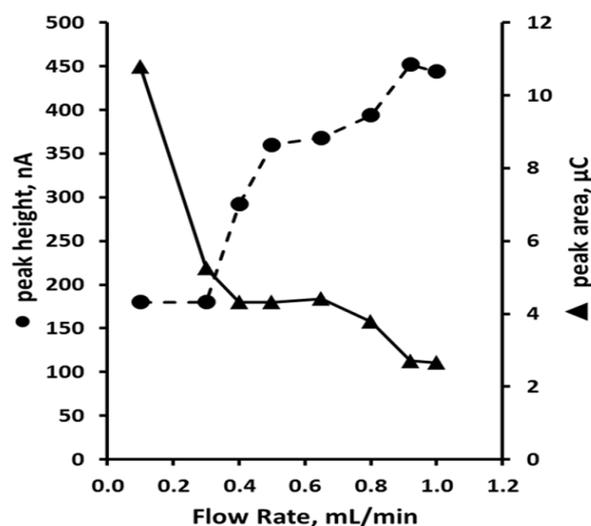


Figure 2: Effect of flow rate on peak height \bullet and peak area \blacktriangle for uric acid. Chromatographic conditions: 95 % TCA pH 2.7, 5 % methanol, +1.2 V.

Studies of Possible Interferences

Paracetamol, aspirin, salicylic acid, caffeine, xanthine, hypoxanthine, L-ascorbic acid, L-cysteine, urea, phenyl red, tyrosine, indole and reduced glutathione (GSH) were investigated as possible interferences under the optimized LC-ECD parameters. Paracetamol, indole, L-cysteine, xanthine, hypoxanthine and L-ascorbic acid were found to give oxidative responses, at concentrations of 0.1 mM. However, these were removed from the retention times of uric acid, and hence did not interfere.

Calibration Plot, Limit of Detection, and Precision

Standard solutions containing uric acid in the concentration range 0.0 - 1.0 mM were prepared in mobile phase and determined by the optimized LC-ECD procedure. The calibration plot (figure 3) was found to be linear from 3.4 ng to 2.8 μg , injected on column with a slope of 2.761 nA/ng, with an R^2 value of 0.9987 for uric acid. The limit of detection was calculated by making replicate current measurements at the appropriate retention times uric acid ($n = 5$) for a blank solution; the detection limit based on three times the mean of these measurements gave a value of 1.0 ng on column.

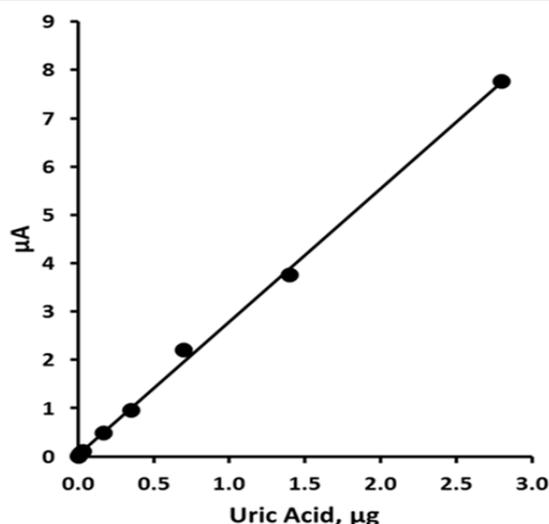


Figure 3: Calibration curve for uric acid. Chromatographic conditions: 95 % TCA pH 2.7, 5 % methanol, 0.6 mL min⁻¹, +1.2 V.

Analytical Application

To assess the performance of the LC-ECD, seven replicate determinations of uric acid in spiked and unspiked human saliva samples were undertaken. Aliquots of the saliva were prepared using the procedure described in the sample preparation section. Quantification was achieved by external calibration. Figure 4 shows representative chromatogram of an unadulterated human saliva extract. Uric acid (RT = 7.9 min) were well resolved from other endogenous saliva components and a mean uric acid concentration of 234.5 µM (% CV = 4.1 %) was found. Recoveries and precision gained for a 120 µM uric acid fortification of the original sample are summarized in Table 2. The method can be seen to give reliable data at the concentrations investigated here.

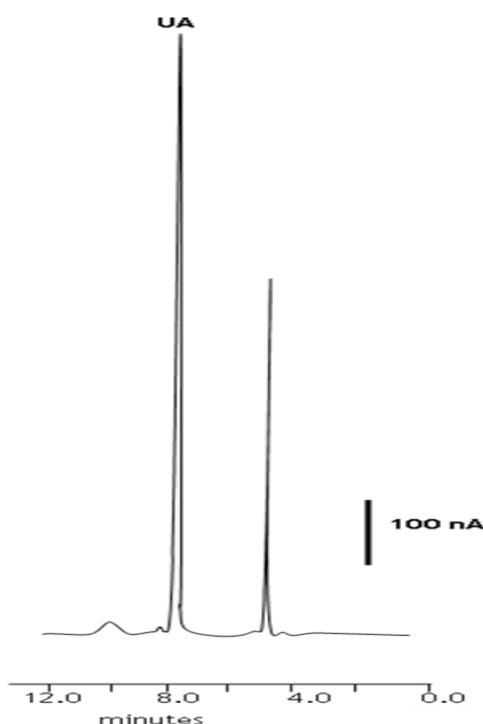


Figure 4: Representative chromatogram of an unadulterated human saliva sample obtained by LC-ECD. Chromatographic conditions: 95

% TCA pH 2.7, 5 % methanol, 0.6 mL min⁻¹, +1.2 V. UA = uric acid.

Table 2: Precision and recovery data for uric acid in human saliva.

Uric Acid	Native, µM	Added, µM	Found, µM	% Recovery
1	234.5	120.0	357.3	102.3
2	234.5	120.0	347.3	94.0
3	234.5	120.0	347.3	94.0
4	234.5	120.0	347.3	94.0
5	234.5	120.0	347.3	94.0
6	234.5	120.0	327.5	77.5
			mean	92.6
			sd	8.1
			%CV	8.8

Conclusions

An assay involving LC-ECD has been successfully developed for the determination of endogenous levels of uric acid in human saliva. The chromatographic separation is achieved using an octadecyl reversed-phase column in conjunction with methanolic trichloroacetic acid as the mobile phase. Little sample pre-treatment was required other than simple dilution of the sample in mobile phase and its subsequent filtration, before introduction to the HPLC system. Therefore, this assay should be readily applicable to the monitoring of these compounds in relation to oxidative stress and other diseases in humans. This demonstrates several advantages over previously reported methods, as these require more extensive sample preparation, such as solid phase extraction and centrifugation^[15]. It should be noted that further studies have been made on human urine and we have found it possible to successively determine endogenous levels of uric acid using the same sample preparation and LC-ECD conditions described here. In further studies the levels of uric acid present in the saliva of gout sufferers or haemodialysis patients will be investigated using the developed method. Alternative stationary phase technologies will also be investigated to overcome possible issues with the low pH of the mobile phase employed.

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