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Short communication

Determination of uric acid and *p*-aminohippuric acid in human saliva and urine using capillary electrophoresis with electrochemical detection Potential application in fast diagnosis of renal disease

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Abstract

The monitoring of uric acid (UA) and *p*-aminohippuric acid (PAH) levels in biological samples is routinely carried out in clinical laboratories as an indication of renal disease. With the aim of investigation of the correlation between the trace amounts of UA and PAH in human saliva or urine and renal diseases, we carried out the determination of UA and PAH in human saliva and urine by using capillary electrophoresis with electrochemical detection (CE-ED) in this work. Under the optimum conditions, UA, PAH and three coexisting analytes could be well separated within 21 min at the separation voltage of 14 kV in 80 mmol/L borax running buffer (pH 9.2). Good linear relationship was established between peak current and concentration of analytes over two orders of magnitude with detection limits ($S/N = 3$) ranged from 5.01×10^{-7} to 2.00×10^{-6} mol/L for all analytes. The result shows that this proposed method could be successfully applied for the study on the correlation between the levels of UA and PAH in human saliva and urine and renal diseases, and provide an alternative and convenient method for the fast diagnosis of renal disease.

Keywords: Capillary electrophoresis; Electrochemical detection; Renal disease; Saliva; Urine; Uric acid; *p*-Aminohippuric acid

1. Introduction

The kidneys play a vital role in maintaining the body's stable internal chemical environment. They are responsible for filtering impurities from the body, draining waste products of the body and maintaining the necessary balance of chemicals in the liquid form [1]. Once kidneys are disorder, people will suffer from loss of role within the family and workplace, loss of renal function and mobility, loss of physical skills and cognitive abilities, loss of sexual function [2,3] and medical disorders of diabetes and hypertension [4,5]. Recent years, renal disease has been the focus of many diseases. In the USA, a survey has shown that more than 372 000 people suffer from renal failure at the end of 2000 (about 0.13% of the population) and the number of patients is growing at a rate of 6% per year. In 2010, the number of patients will get to 650 000 [6,7]. In Europe and Asia, there are less detailed analyses for trends, but the data available indicate a similar pattern to that in the USA [8].

Several compounds in biological fluids can serve as marker for diagnose of renal function such as UA and PHA [9]. The presence of elevated UA levels [10] and an imbalance in PAH levels [11–13] have been acknowledged as a sign of renal diseases. Thus, monitoring of UA and PAH in human physiological fluids is indispensable for the diagnosis of renal disease.

So far, a limited number of methods including colorimetry [13,14], HPLC [15–19] and CE with UV [20–22] have been only developed for the quantitative analysis of these renal markers in human urine or serum, most of them suffer from long analysis time, low resolution, short column lifetime and etc. Capillary electrophoresis is becoming increasingly recognized as an important analytical separation technique due

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to its speed, efficiency, reproducibility, ultra-small sample volume, and ease of clearing up the contaminants. In combination with electrochemical detection, CE-ED offers high sensitivity and good selectivity for electroactive species. The advantages of capillary electrophoresis include fast analysis time, high resolution, and ultra-small sample volume. Besides, diluted sample solutions can often be directly injected into the capillary without complicated sample pretreatment. Garcia et al. has described the separation of creatine, creatinine, and UA in urine by microchip CE with pulsed ED [23]. Fanguy et al. have separated UA by microchip CE-ED [24]. In addition, CE with polarity reversal and ED have been applied for the determination of UA in human serum [25]. However, none of them have examined the saliva samples as well as uncovered the relativity between the levels of UA and PAH in human saliva and urine samples.

In this work, we reported a sensitive and reliable method for the simultaneous determination of UA, PAH and their exogenous and endogenous co-existing potential interferences namely xanthine (Xan), hypoxanthine (HX) and ascorbic acid (Vc) in human saliva and urine by CE-ED, and furthermore, confirmed the close relativity between the levels of UA and PAH in human saliva and urine and the renal disease, which suggests a potential application in fast primary diagnosis of renal disease. The molecular structures of above ingredients were shown in Fig. 1.

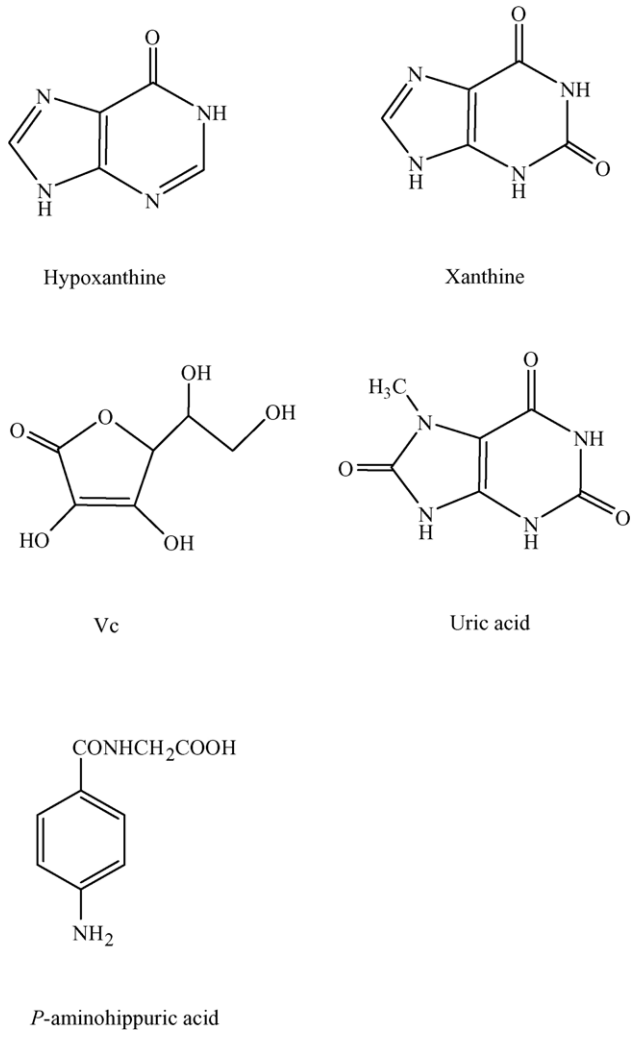

Fig. 1. Molecular structures of HX, PAH, Vc, UA and Xan.

2. Experimental

2.1. Reagent and solutions

HX, PAH, Vc, UA and Xan were all purchased from Sigma (St. Louis, MO, USA), and were all used as received. All chemicals were of analytical grade.

Stock solution of Vc and PAH (1.0×10^{-2} mol/L) was prepared in doubly distilled water, and those of HX, UA and Xan were dissolved in 0.05 mol/L NaOH aqueous solution to reach the final concentration of 0.01 mol/L. All standard solutions were stored in a 4 °C refrigerator and were stable for 4 days. All solutions were diluted to the desired concentration with the running buffer (80 mmol/L H_3BO_3–$Na_2B_4O_7$ buffer with pH value from 8.2 to 9.2). Before use, all solutions were filtered through 0.22 μm nylon filters. All experiments were performed in a air-conditioned room at 25 °C.

2.2. Apparatus

A CE-ED system has been described previously [26,27]. A ±30 kV high-voltage power supply (Shanghai Institute of Nuclear Research, China) provided a separation voltage between the ends of the capillary. The inlet end of the capillary was held at a positive potential and the outlet end of capillary was maintained at ground. The separations were proceeded in a 75 cm length of 25 μm i.d. and 360 μm o.d. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA). Samples were all injected electrokinetically, applying 14 kV for 6 s.

A three-electrode electrochemical cell consisting of a laboratory-made 300 μm diameter carbon disc working electrode, a platinum auxiliary electrode and a saturated calomel electrode (SCE) as the reference electrode, was used in combination with a BAS LC-4C amperometric detector (Bioanalytical Systems Inc., West Lafayette, IN, USA). The carbon disc electrode was made of a piece of 300 μm diameter graphite rod from polishing technique as descried in a previous report [28]. Before use, the surface of the carbon-disk electrode was successively polished with emery paper and alumina power, sonicated in doubly distilled water, and finally was positioned carefully opposite the outlet of the capillary with the aid of a micromanipulator (CORRECT, Tokyo, Japan) and arranged in a wall-jet configuration. The electropherograms were recorded using a chart record (Shanghai Dahua Instrumental Factory, China). A YS 38-1000 220 V alternate constant-voltage power supply (Shanghai Instrumental Transformer Factory, Shanghai,

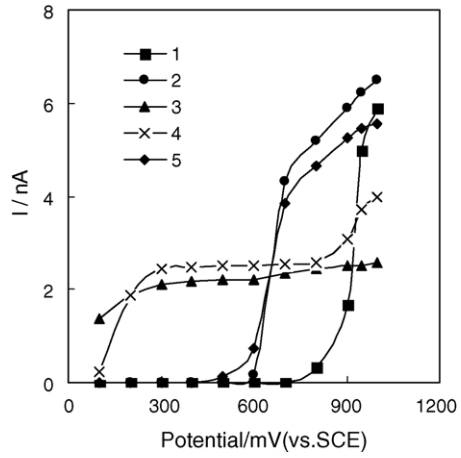

Fig. 2. Hydrodynamic voltammograms (HDVs) of HX (1), PAH (2), Vc (3), UA (4) and Xan (5) in CE-ED. Fused-silica capillary: 25 μm i.d. × 75 cm; working electrode: 300 μm diameter carbon disk electrode; running buffer: 80 mmol/L(BB. pH 9.2); separation voltage: 14 kV; injection time: 14 kV/6 s; concentrations of five analytes: HX (5.0×10^{-5} mol/L), PAH (1.0×10^{-4} mol/L), Vc (1.0×10^{-4} mol/L), UA (1.0×10^{-4} mol/L) and Xan (1.0×10^{-4} mol/L).

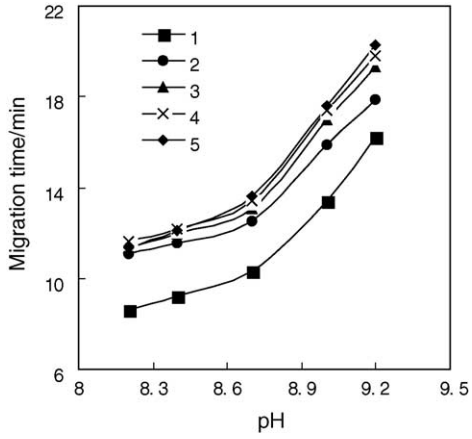

Fig. 3. Effect of the running buffer pH on the migration time of the analytes. Working potential was +950 mV (vs. SCE); concentrations of five analytes: HX (1.0×10^{-4} mol/L), PAH (1.0×10^{-4} mol/L), Vc (2.0×10^{-4} mol/L), UA (1.0×10^{-4} mol/L) and Xan (1.0×10^{-4} mol/L); other experimental conditions and labels are the same as in Fig. 2.

China) was employed to suppress the voltage fluctuation of the power line. The whole system was assembled in a air-conditioned room at 25 °C in order to minimize the variation of running buffer viscosity, which is important to the reproducibility of the experiment results.

2.3. Sample preparation

Urine and saliva samples from apparently healthy volunteers and patients with renal disease were collected from Shanghai Changzheng Hospital. None of them suffered from systemic or salivary gland disease that could affect the saliva. They were requested to fast 30 min before saliva withdrawal. Urine and saliva samples were diluted with running buffer, then filtered through 0.22 μm nylon filters, and the filtrate were injected directly to the CE-ED system for analysis. Before use, all solutions were stored in a 4 °C refrigerator.

3. Results and discussion

3.1. Effect of the potentials applied to the working electrode

In amperometric detection, the potential applied to the working electrode directly affects the sensitivity, detection limit and stability of this method. Therefore, hydrodynamic voltammetry experiment was investigated to obtain optimum detection. As shown in Fig. 2, the peak current of Vc does not have obvious changes, while, the peak current of other three analytes increases rapidly when the applied potential exceed +100 mV for UA, +500 mV for Xan, +600 mV for PAH, and +700 mV for HX, respectively. However, when applied potential is greater than +950 mV (versus SCE), although the peak current of the analytes still have certain increase, both the baseline noise and the background current increase substantially, which is a big disadvantage for sensitive and stable detection. Therefore, the potential applied to the working electrode is maintained at +950 mV (versus SCE), where the background current is not too high and the signal-to-noise ($S/N = 3$) ratio is the highest.

3.2. Effects of the pH and concentration of the running buffer

The acidity of the running buffer affects the zeta potential (ξ), the electro osmotic flow (EOF) as well as the migration time and the separation of the analytes. The effect of the running buffer pH on the migration time of the analytes was investigated in the pH range of 8.2–9.2. As shown in Fig. 3, the migration time of all analytes increase with the increasing pH value. When pH is below 8.4, Xan and Vc cannot be separated. When pH value is 8.7–9.0, Xan cannot be separated from UA. At pH 9.2, the five analytes can be well separated, however, higher pH value results in longer analysis times, and the analytes are more susceptible to oxidation. Therefore, pH 9.2 was selected as the optimum pH value for this work.

In addition to the pH value, the concentration of the running buffer is another important factor affecting the resolution, migration time and the peak current of the analytes. So the effect of the running buffer concentration on migration time was also studied, and the optimum running buffer concentration is 80 mmol/L (pH 9.2).

3.3. Effect of separation voltage and injection time

For a given capillary length, the separation voltage determines the electric field strength, which affects both the velocity of electroosmotic flow (EOF) and the migration velocity of the analytes, which in turn determines the migration

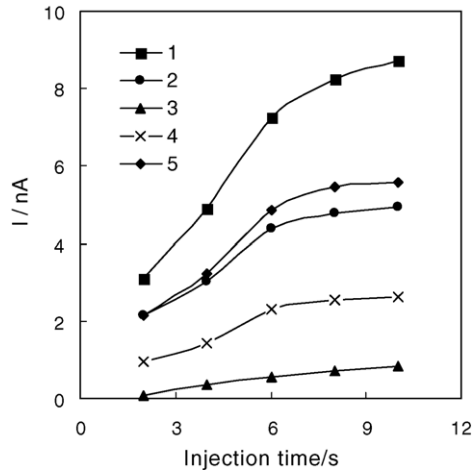

Fig. 4. Effect of injection time on the peak current of the analytes. Experimental conditions and labels are the same as in Fig. 3.

time of the analytes. As expected, higher separation voltage gives shorter migration time for all analytes. However, when the separation voltage exceeds 16 kV, Xan, Vc, and UA cannot be well separated, and baseline noise becomes larger. Therefore the optimum separation voltage selected is 14 kV, at which good separation can be obtained for all analytes within 21 min.

The injection time determining the amount of sampling affects both peak current and peak shape. The effect of injection time on peak current is studied by varying injection time from 2 to 10 s at 14 kV. As shown in Fig. 4, the peak current increases with increasing sampling time. When the injection time is longer than 8 s, peak current nearly levels off and peak broadening becomes more severe. In this experiment, 6 s (14 kV) is selected as the optimum injection time.

Through the experiments above, the optimum conditions for HX, PAH, Vc, UA and Xan have been decided. The typical electropherogram for a standard mixture solution of the five analytes is shown in Fig. 5A, from which we can see good separation can be achieved within 21 min.

3.4. Reproducibility, linearity, detection limits and recovery

The reproducibility of the peak current is estimated by making repetitive injections of a standard mixture solution (2.0×10^{-4} mol/L for Vc, 1.0×10^{-4} mol/L for HX, PAH, UA and Xan, respectively) under the selected optimum conditions ($n = 7$). The relative standard deviations (R.S.D.s) for the peak current are 0.39, 0.66, 2.39, 0.63 and 0.55% for HX, Vc, PAH, UA and Xan, respectively. The high reproducibility indicates that this method is accurate and sensitive.

A series of the standard mixture solutions of HX, PAH, Vc, UA and Xan with concentration ranging from 2.0×10^{-6} to 5.0×10^{-4} mol/L are tested to determine the linearity for all analytes at the carbon disc electrode in this method. The results of regression analysis on calibration curves and detection limits are presented in Table 1. Determination limits are evaluated on the basis of a signal-to-noise ratio of 3. The calibration curves exhibit satisfactory linear behavior over two orders of magnitude with the detection limit ranging from

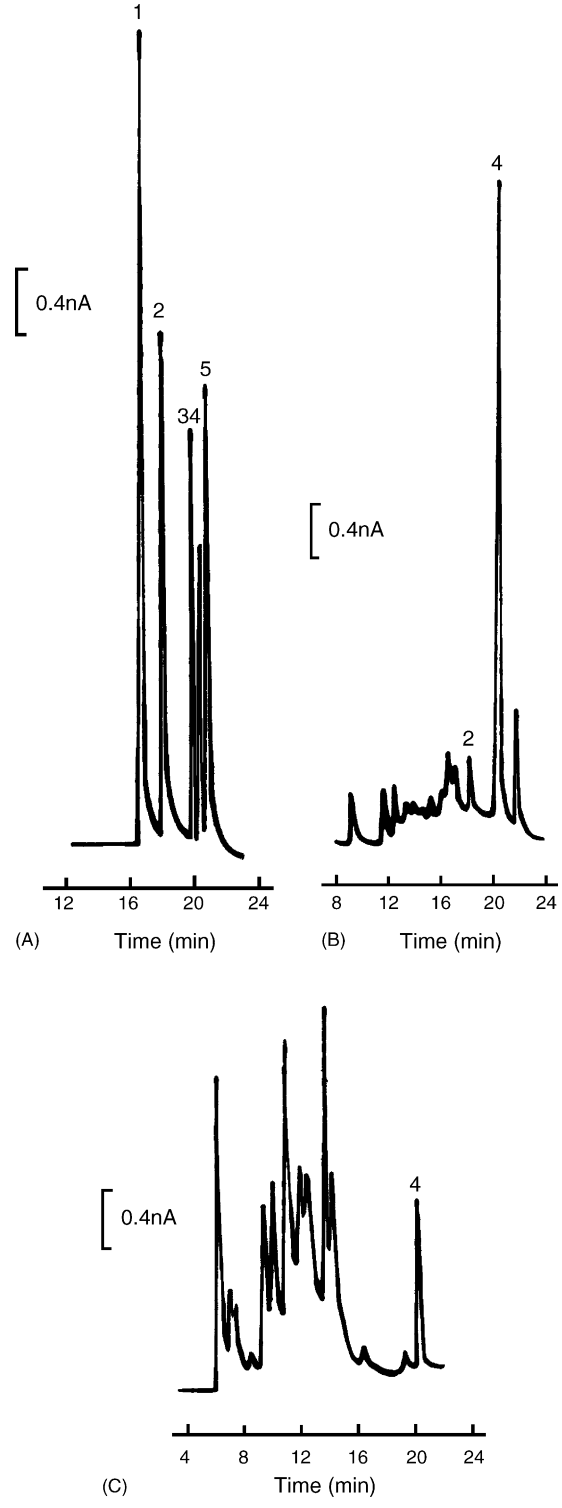

Fig. 5. Electropherograms of the standard mixture solution (A), and samples of patient urine sample (B), and patient saliva (C). Experimental conditions and labels are the same as in Fig. 3. Peak identification: (1) HX; (2) PAH; (3) Vc; (4) UA; (5) Xan.

Table 1
The results of regression analysis on calibration curves and the detection limits[a]

Compound	Regression equation $Y = aX + b$[b]	Correlation coefficient	Linear range ($\times 10^{-4}$ mol/L)	Detection limit ($\times 10^{-6}$ mol/L)
HY	$Y = 1.04 \times 10^5\ X + 0.0962$	0.9996	0.01−2	0.50
PAH	$Y = 3.13 \times 10^4\ X - 0.0177$	0.9999	0.01–2	0.80
Vc	$Y = 1.26 \times 10^4\ X - 0.0010$	0.9999	0.02–2	2.00
UA	$Y = 3.86 \times 10^4\ X + 0.0655$	0.9997	0.01–2	0.66
Xan	$Y = 2.72 \times 10^4\ X - 0.0267$	0.9998	0.01–2	0.92

[a] CE-ED conditions are the same as in Fig. 3.
[b] In the regression equation, the X value is the concentration of analytes (mol/L) and the Y value is the peak current (nA).

Table 2
Assay results of UA in urine and saliva of healthy volunteers and patients with renal disease[a]

	Healthy volunteer (mol/L)		Patient (mol/L)	
	Urine	Saliva	Urine	Saliva
Sample 1	1.99×10^{-3} (96.9%)[b]	9.60×10^{-5} (94.2%)	6.39×10^{-3} (97.2%)	3.55×10^{-4} (96.3%)
Sample 2	1.93×10^{-3} (97.5%)	9.25×10^{-5} (90.2%)	5.10×10^{-3} (97.6%)	2.42×10^{-4} (95.2%)
Sample 3	0.76×10^{-3} (98.3%)	8.80×10^{-5} (95.3%)	4.59×10^{-3} (99.1%)	2.19×10^{-4} (93.2%)

[a] CE-ED conditions are the same as in Fig. 3.
[b] The data in parentheses refer to the recovery.

5.01×10^{-7} to 2.00×10^{-6} mol/L for all five analytes, and the correlation coefficients are in the range of 0.9996–0.9999.

To further evaluate the precision and accuracy of the method, the recovery experiments under the optimum conditions are also conducted with the urine and saliva samples ($n = 3$). Accurate amounts of standard were added to the actual samples, and the recovery values can be obtained by comparing the increase of the peak height before and after the addition of standards. The average recoveries are listed in Table 2.

3.5. Application and discussion

HX and Xan are the metabolites of purine bases in nucleic acids through a series of biochemical pathway, and Vc is also a common compound in human fluids. Furthermore, HX, Xan and Vc are electroactive compounds which might interfere the determination of PHA and UA, therefore PAH and UA were determined at the presence of these three co-existing potential interferences. Under the optimum conditions, CE-ED is employed for the determination of UA and PAH in human saliva and urine, where the exogenous and endogenous co-existing compounds produce no interference for the quantitation. Typical electropherograms for real-life samples are shown in Fig. 5B and C. By comparing the migration times of analytes with those of the standard mixture solution (Fig. 5A), UA content in human saliva and urine can be determined, and PAH content can be determined in human urine, this lack of PAH in saliva may be the result of levels of PAH below the limit of detection after dilution. The experimental results of healthy volunteers and patients with renal disease are presented in Tables 2 and 3, from which we can readily find the close relativity of UA and PAH levels in human saliva and urine for both healthy volunteers and renal disease patients. The UA and PAH levels in urine samples collected from renal disease patients is about several times higher than that collected from healthy volunteers, similarly the UA levels in saliva samples collected from renal disease patients is also about several times higher than that collected from healthy volunteers.

Table 3
Assay results of PAH in urine of healthy volunteers and patients with renal disease[a]

	Healthy volunteer (mol/L)	Patient (mol/L)
Sample 1	0.44×10^{-4} (97.9%)[b]	2.82×10^{-4} (97.3%)
Sample 2	0.37×10^{-4} (97.9%)	1.02×10^{-4} (98.6%)
Sample 3	0.22×10^{-4} (98.8%)	0.97×10^{-4} (98.1%)

[a] CE-ED conditions are the same as in Fig. 3.
[b] The data in parentheses refer to the recovery.

4. Conclusions

We developed a sensitive and reliable method for the simultaneous determination of UA, PAH and their exogenous and endogenous co-existing ingredients namely HX, Vc and Xan in human saliva and urine by CE-ED, and especially we confirmed the close relativity between the levels of UA and PAH and the renal disease, and developed a fast method for primary diagnosis of renal disease in clinic.

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