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Article

Development and validation of a simple and noninvasive method for salivary uric acid: potential applications for monitoring the salivary uric acid level in healthy volunteers and gout patients

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Serum uric acid (UA) is a traditional biomarker in the clinical diagnosis of gout and hyperuricemia. However, serum treatment and storage are cumbersome, and wounds are susceptible to infection. Therefore, in this study, a simple and noninvasive method was developed to detect the UA in human saliva to monitor the gout. An Inertsil ODS-3 column was used for the analysis under the condition of isocratic elution with the mixed solution phosphate buffer (74 mM, pH=2.2): Methanol=98:2 (v:v) and the UV detection at 284 nm. Using salivary UA data from healthy volunteers (HVs) (n=68) and gout patients (GPs) (n=14), we examined the salivary UA difference in their content. The intra- and inter-day accuracy and precision (RSD %) were less than 2.56%, the limit of detection (LOD) of UA was 5.0 ng/mL, the mean recoveries of the corresponding compounds were 102.48%. Saliva levels of UA in HVs and GPs were 35.26±14.06 μg/mL and 91.96±23.90 μg/mL, respectively. The concentrations of salivary UA in GPs were significantly higher than those in HVs ($p < 0.001$). This method was also expected to monitor the hyperuricemia and other metabolic disorders in the future.

Keywords: Noninvasive. Gout patient. Hyperuricemia. Human saliva. Uric acid.

INTRODUCTION

Uric acid (UA) has both anti- and pro-oxidant effects in tissues, blood and the central nervous system of humans (Riis *et al*., 2018). Moreover, its imbalance is an important clinical index in dysfunctions, such as gout, cardiovascular, and metabolic disorders (Biscaglia *et al*., 2016; Kanbay *et al*., 2016; Li *et al*., 2016; Zhang *et al*., 2015; Abeles, 2015; Soukup *et al*., 2012). Many studies reveal that high level of serum UA is associated with hypertension, stroke, and chronic kidney disease, in addition, increase the risk for Type-2 diabetes and insulin-resistance (Kushiyama *et al*., 2014; Lippi *et al*., 2008; de Oliveira, Burini, 2012). In this study, we present a low-cost, simple and convenient assay for UA detection to monitor the gout.

Gout is one type of arthritis which is caused by the high concentration of UA in the blood. With the increase of the prevalence, more and more patients are suffering from the insufferable joint pain (Zhao, Huang, 2015; Tausche *et al*., 2009). The UA detection in human urine and plasma samples have been extensively investigated for the diagnosis of these diseases (Ross, 1994; Siekmann, 1985; Ellerbe, Cohen, 1990; Zhao, 2013).

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Different methods have been developed to detect UA in the plasma and urine samples, such as high-performance liquid chromatography (HPLC) (Inoue *et al*., 2003; Jen, Hsiao, Liu, 2002; Li, Franke, 2009; Cooper *et al*., 2006; Zhao, 2015), capillary electrophoresis (CE), (Chu *et al*., 2007; Pormsila, Krähenbühl, Hauser, 2009; Xing *et al*., 2008) electrochemical (ED) (Motshakeri *et al*., 2018), mass spectrometry (MS) detection (Dai *et al*., 2007; Luo, Cai, Cheng, 2013). For LC-MS, the advantage of its highly sensitive detection, but the disadvantage of its high cost cannot be accepted. CE and ED methods have good ability to separate and detect the analytes, but the lower reproducibility implied that these methods are not suitable to measure the urine and plasma samples. However, the HPLC-UV method was used to analyze the urine and plasma samples due to its high detection sensitivity good resolution and low-cost (Li *et al*., 2015). The inherent problems of blood and urine, such as the needle jabs, and the hygienic practice during its collection and handling is also another consideration. On the contrary, the human saliva seems to be an ideal biological sample due to being clean and noninvasive compared to blood. Moreover, the content of substances in the saliva is similar to that in blood, thus, the basic health condition of humans can be accessed by the analysis of saliva. The risk of cross infection will then be decreased (Fujii *et al*., 2014). The focus on saliva as a non-invasive sample has occurred because of these characteristics (Higashi *et al*., 2010; Malamud, 2011; Chiappin, *et al*., 2007). More recent studies revealed that UA is existed in saliva and with the capacity to be measured (Shibasaki *et al*., 2012; Nunes, Brenzikofer, Macedo, 2011; Jones, Elwazeer, Taylor, 2018; Vakh *et al*., 2007; Guan, Chu, Ye, 2004). Thus, some researchers found that the amount of UA in plasma is related to that in saliva (Bibi, Green, Nagler, 2008; Deminice, *et al*., 2010). Therefore, the noninvasive human saliva sample could be used as an ideal biological sample to measure UA for the diagnosis of gout.

In this study, we have developed a simple and convenient method to quantify the salivary UA level in healthy volunteers and gout patients. The feasibility of using human salivary UA level as a new noninvasive biological sample for monitoring gout was evaluated by the method.

EXPERIMENTAL

Materials and reagents

Methanol (HPLC, Thermo Fisher Scientific Co., Ltd., USA); Acetonitrile (HPLC, Thermo Fisher Scientific Co., Ltd., USA); ultrapure water was prepared using a Unique-R20 Multi-functional ultra-pure water system (Research Scientific Instruments Co., Ltd., Xiamen, China); Lithium carbonate, disodium hydrogen phosphate, phosphoric acid, UA (purity 99%) (Shanghai Aladdin); Hypoxanthine (internal standard, Beijing Bailingwei). KQ-250DE ultrasonic cleaner (Kunshan Ultrasonic Instrument Co., Ltd., China), HSC-12000 centrifuge (AS ONE Corporation, Japan), BSA124S analytical balances (Sartorius Scientific Instruments Co., Ltd., Beijing), BV101-B Vornado Miniature Vortex Mixer (Benchmark Scientific Inc., USA).

HPLC-UV analysis

In this study, a high-performance liquid chromatograph equipped with a UV detector (Hitachi 1410, Japan) was used to quantify the UA in saliva. The process of analysis was performed using an Inertsil ODS-3 Column (250 mm × 4.6 mm, 4μm) and the mixture of liquid containing phosphate buffer (74 mM, pH=2.2): methanol=98:2 (v:v) as the mobile phase; the column temperature was 40°C, the UV detector wavelength was 284 nm, the flow rate was 1.0 mL/min, the injection volume was 10 μL, and the analysis time was 7 min.

Collection of human saliva

HVs and GPs fasted and beverage 1 h before the saliva collection. And they were also forbidden to brush their teeth 1 h prior to the collection to avoid contamination of saliva with blood due to potential bleeding. Unstimulated saliva (1 mL) samples were collected by direct spitting into a collecting tube from 68 HVs (age: 20-82, 27 males and 41 females, from the College of Pharmacy, Yanbian University) and 14 GPs form Yanbian Universiry Hospital (age: 30-76, 14

males). The samples were stored at -20°C before analysis. HVs and GPs understood the purpose and significance of this experiment and donated saliva after signing an agreement. The experimental procedures were conducted in accordance with the ethical standards of the Helsinki Declaration and approved by the Ethics Committee of Yanbian University.

Pretreatment of UA in the human saliva

A 50 μL thawed human saliva sample was dissolved in 200 mL of acetonitrile and vortexed for 1 min, centrifuged at 12,000 rpm/min for 5 min, and the supernatant liquid was obtained; the remaining samples were then analyzed and the supernatant liquid obtained, combined with the two supernatants, and 50 μL of an internal standard solution (100 μg/mL hypoxanthine) was added.

Method validation

Calibration curves and limit of detection

A 6.0 mg UA standard was added to 10 mL of a 0.01 M lithium carbonate solution to obtain a 600 μg/mL UA standard solution then diluted it into the required concentrations (0.5-600 μg/mL). Each concentration was continuously detected five times. The UA standard solution concentration (μg/mL) was plotted on the abscissa of the calibration curves, and the ratio of the UA peak area to the internal standard peak area was plotted on the ordinate of the calibration curves. The precision (relative standard deviation, RSD %) of each concentration and the limit of detection of UA (S/N = 3) were calculated.

Recovery and precision

Four kinds of saliva samples (2 males and 2 females) were processed as described in section 2.4. One part was used as the blank group. Other groups was added with 50 μL of three groups of UA standard solutions, including low, medium and high (50, 100, 300 μg/mL). They were then centrifuged and dissolved in 200 μL of a lithium carbonate solution. The recovery rate, intra-day and inter-day precision were calculated. The recovery rate is calculated as follows. Firstly, we separately detected concentrations of UA from saliva samples and standard solutions (50, 100, and 300 μg/mL). Secondly, we determined concentrations of UA from saliva spiked three different contents of UA standard solutions. Finally, we subtract concentration of UA before spiked from concentration of UA after spiking. And we use the above data to divide by concentrations of standard solutions of UA and multiply by one hundred percent.

Stability evaluation

Three kinds of saliva samples were processed by using the method which described in section 2.4. 50 μL of the three groups of UA standard solutions containing low, medium and high (50, 100, 300 μg/mL) levels, which were added to the above three samples, then centrifuged and dissolved in 200 μL of lithium carbonate solution. The evaluation about the stability of the saliva samples were finished by detection within 24 hours at room temperature or after the three freeze-thaw cycles. In addition, the samples were stored under -20°C for 15 day to test the storage stability.

Quantitative analysis of UA in saliva of GPs and HVs

Saliva samples were processed using the method described in section 2.4 (68 HVs, 14 GPs). After centrifugation, the supernatant was dissolved in a 200 μL lithium carbonate solution. The concentrations of UA in the saliva from the gout patients and healthy people were recorded. The average content was calculated for quantitative analysis.

Statistical analysis

The statistical analyses were performed using the Mann–Whitney's U-test. A P value of <0.05 (0.001) was considered statistically significant.

RESULTS AND DISCUSSION

LC-UV analysis of UA

In the study, standard solutions of UA as well as pooled human saliva were dissolved by the mobile phase. The optimal separation occurred on an Inertsil ODS-3 column maintained at 40°C with the mixed solution of 74 mmol/L sodium phosphate buffer containing 2.0% methanol (pH 2.2) as the mobile phase and at 284 nm for detection. Figure 1 illustrated the HPLC-UV chromatograms of UA in the saliva from the healthy volunteers and gout patients. The retention times of the internal standard (IS) and UA were 4.6 and 6.0 minute, respectively. The retention time of UA in the saliva of the healthy volunteers and gout patients was consistent with the retention time of standard products. Based on these results, these stated conditions of separation were the best suitable for the detection of UA in human saliva.

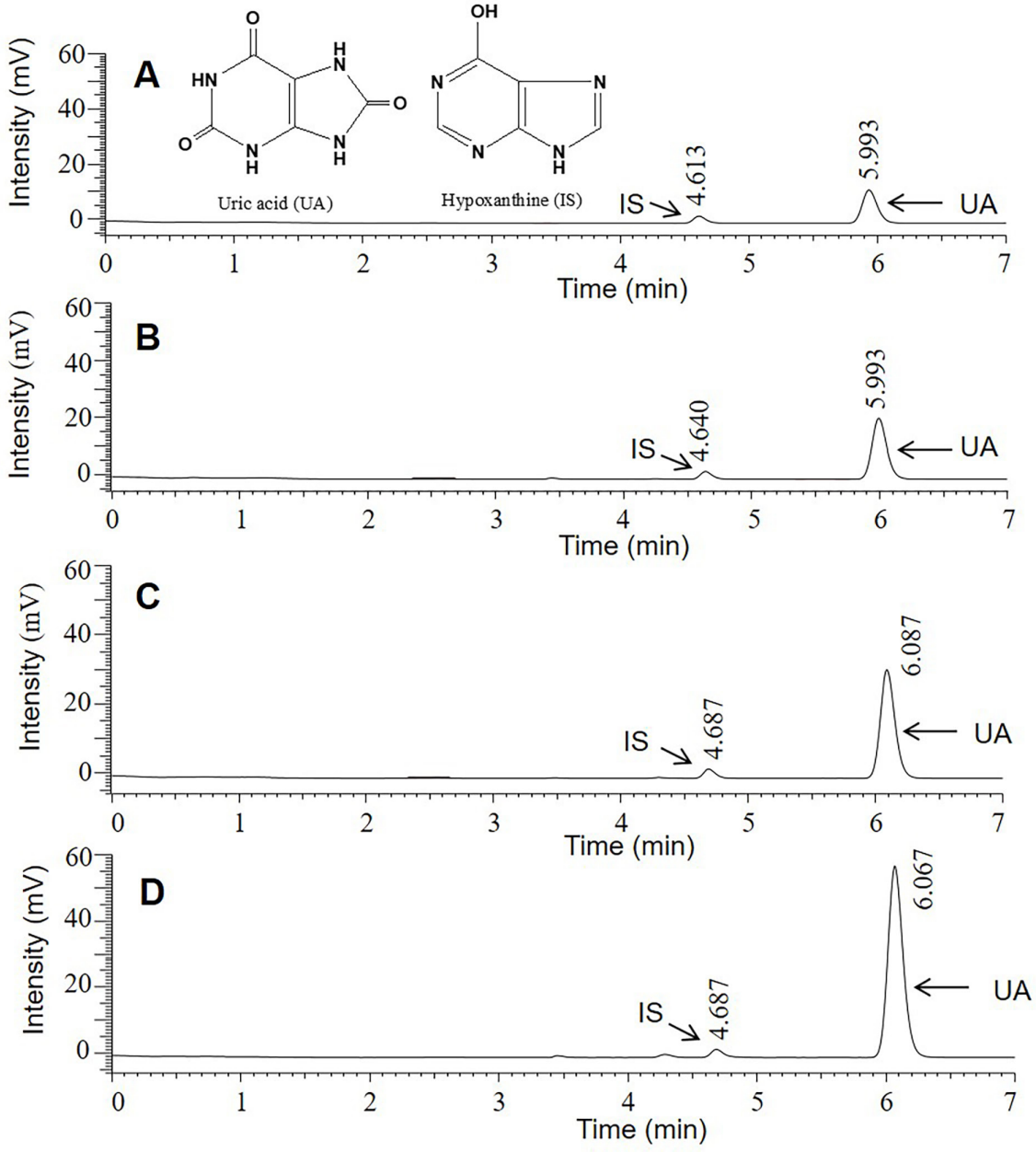

FIGURE 1 - HPLC-UV chromatograms obtained from the analysis of the saliva of healthy volunteers and gout patients.

A: Standard of UA and IS; B: Healthy volunteer female (HV-F); C: Healthy volunteer male (HV-M); D: gout patient male (GP-M).

Validation

In the range of 0.5-600 μg/mL, the calibration curve of UA showed a good linear relation (r^2=0.9999) and its linear equation was y = 0.5333x - 0.4128, the RSD % were 0.54 and 6.42%. Limit of detection (LOD) and limit of quantitation (LOQ) of UA were 5.0 ng/mL and 16.0 ng/mL, respectively. As shown in Table I, the RSD % of the intra-day and inter-day determinations were 0.24-2.56% and 1.62-2.22%, respectively. The accuracies of the determinations were 98.20-105.99%. The mean recoveries of the UA standard in the human saliva were 102.48%. The mean recovery and repeatability showed that the method were suitable for the determination of UA in the human saliva. The stability of UA in the human saliva was shown in Table II. The RSD % of different UA concentrations in the saliva were less than 3.03% within 24 hours at room temperature, three freeze-thaw cycles and 15 days at -20°C. The results indicated that UA was stable in the saliva. The method is simple and precise, and can be used to determine UA in gout patients.

TABLE I - Recovery and precision data of uric acid (UA) from the saliva of healthy volunteers

Compound	UA Amount (mg/mL)	Detection Amount (mg/mL)	Intra-day CV (%) (n=5)	Inter-day CV (%) (n=5)	Recovery (%)	Mean Recovery (%)
UA	0	55.88	1.07	1.72		102.48
	50	107.51 ± 2.42	2.25	2.22	103.26	
	100	161.87 ± 4.14	2.56	2.01	105.99	
	300	350.48 ± 0.83	0.24	1.62	98.20	

TABLE II - Stability of uric acid in human saliva

Concentration (mg/mL)	24h at room temperature		Three freeze-thaw cycles		15 days at -20°C	
	Measured concentration (mg/mL)	CV (%) (n=5)	Measured concentration (mg/mL)	CV (%) (n=5)	Measured concentration (mg/mL)	CV (%) (n=5)
50	50.67 ± 1.96	3.03	51.63 ± 0.05	0.07	54.07 ± 1.68	2.42
100	98.41 ± 2.77	2.47	94.92 ± 0.41	0.36	97.97 ± 1.97	1.73
300	314.7 ± 6.22	1.89	315.50 ± 0.99	0.30	326.14 ± 3.81	1.12

Determination of UA in saliva of HVs and GPs

For clinical research and treatment, the content of UA in blood is used as a medical index for the diagnosis of gout. However, the process of blood collection requires a professional medical staff and the invasive with pain problem are undesirable. On the contrary, some researchers would use human saliva with the advantages of easily storage, relatively clean, painless collection and the optimal collection time of the human saliva was optimized. Figure 2 shows the UA content in saliva collected from men and women of HV at different times over one day. That UA content in the human saliva is relatively stable between 6:30-22:00. At 11:30 a.m., the

UA content in the saliva was very stable, therefore, we chose 11:30 a.m. Thus, some useful information in the diagnosis of gout disorders was provided in order to more accurately determine the human saliva UA level. The saliva of HVs (age 20–82; 27 males and 41 females) and GPs (age 30–76; 14 males) was used as the UA samples to analyze using the recommended procedures. According to the test results of the serum UA level, gout patients were diagnosed at the Affiliated Hospital of Yanbian University. Figure 1 B, C and D shows the HPLC-UV chromatograms of UA in the saliva from healthy volunteer females, healthy volunteer males and the gout patients. The peak related to the UA was achieved with good separation without interference from the endogenous substances in the human saliva. Figure 3 shows the quantitative values of the UA in the saliva of the healthy volunteer males (n=27) and healthy volunteer females (n=41). Comparing the results, the concentration of UA in the men was higher than that in women but without statistical significance. Figure 4 illustrates the statistical analysis of the concentration of UA in the saliva from the HVs (n = 68) and GPs (n = 14). The mean amounts of UA in the saliva from the HVs and GPs were 35.26±14.06 μg/mL and 91.96±23.90 μg/mL, respectively. We found that there is a significantly statistical difference between two groups ($p < 0.001$). These results prove that the detection method of the UA in the human saliva could be used to diagnose and assess the disease activity in GPs.

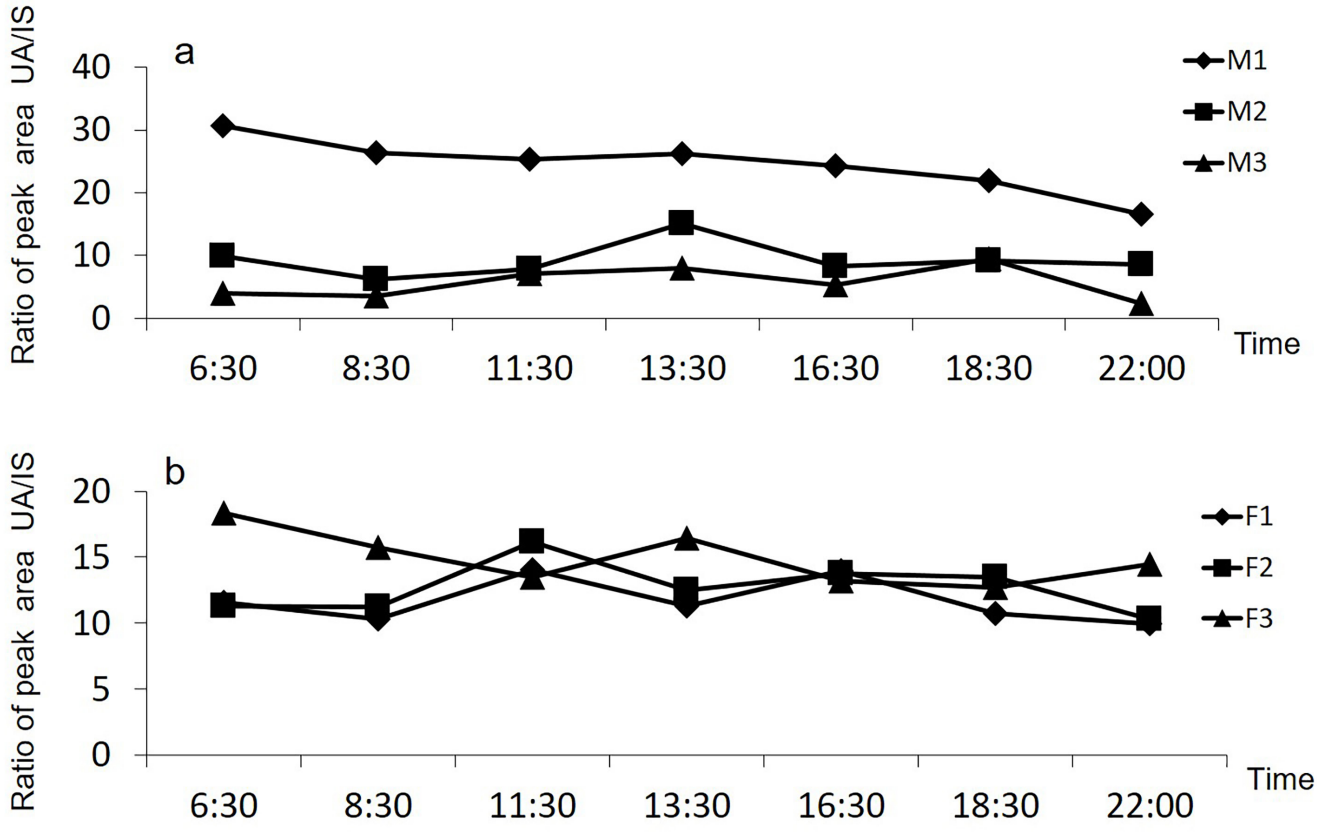

FIGURE 2 - Judgment of the best time to collect saliva from healthy volunteers.

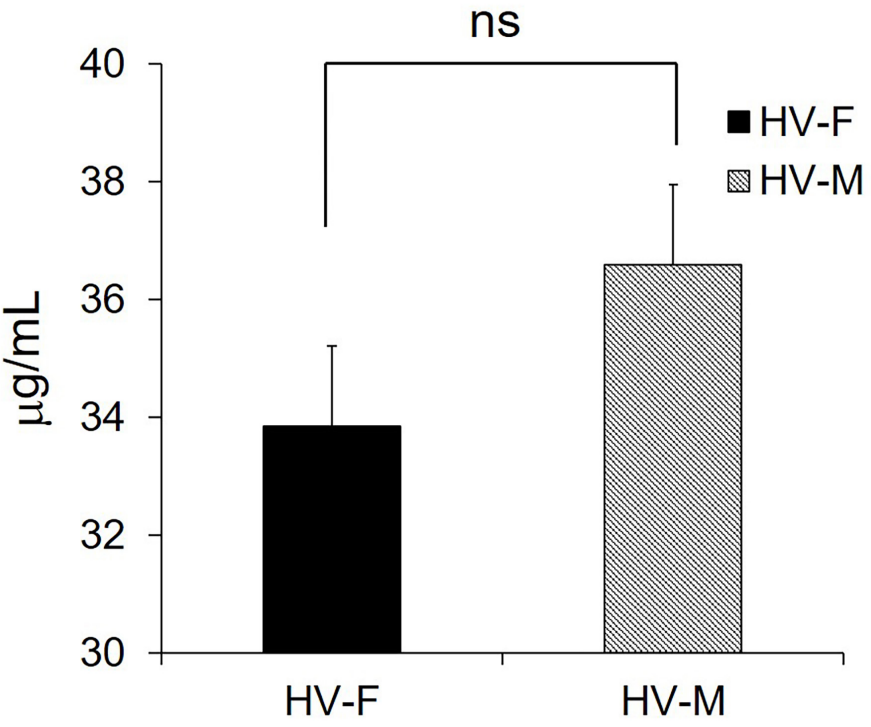

FIGURE 3 - Quantitative values of the uric acid in saliva of healthy volunteer females (HV-F, n=41) and healthy volunteer males (HV-M, n=27).

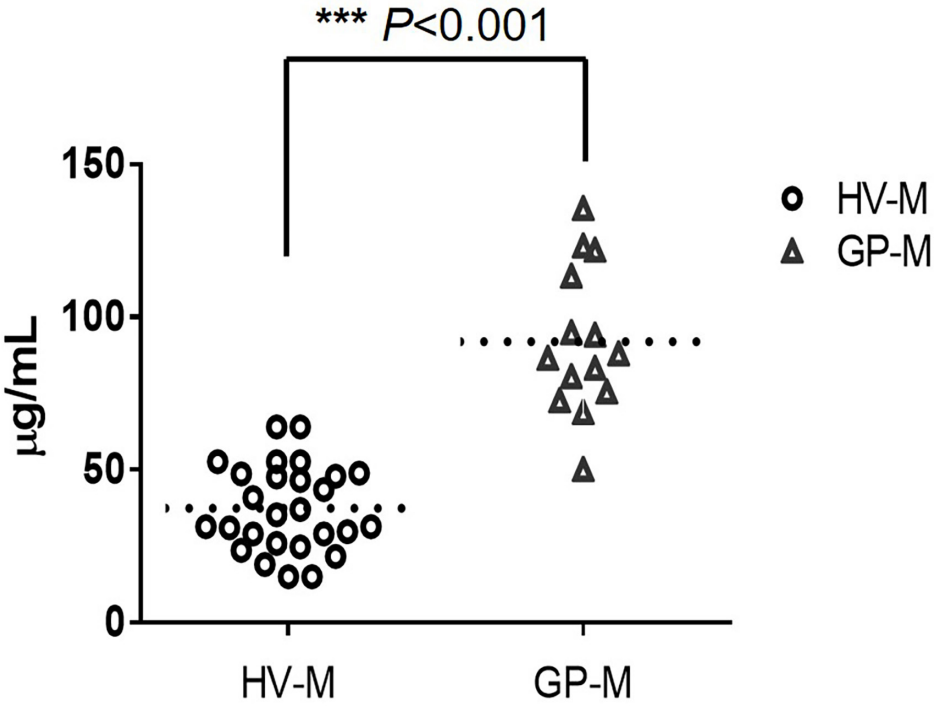

FIGURE 4 - Statistical analysis of UA concentration in the healthy volunteers (male=27) and gout patients (male=14).

HV-M, Healthy volunteer male; GP-M, Gout patient male; (*** $p < 0.001$).

CONCLUSION

A simple and practical quantification procedure for the detection of UA in the saliva from gout patients was developed. Furthermore, rapid detect and separation within 7 min was done by using LC and an ODS-3 Column. The present method has a favorable sensitivity and was proven to be simple and comparable to previous methods such as LC-MS. The UA content of the healthy males was higher than that of the females. Furthermore, comparing the concentrations of UA from the HVs and GPs, we found that there is a significant statistical difference between each other ($p < 0.001$). Consequently, the proposed procedure is useful for the noninvasive salivary monitoring of GPs. The popularity of the method could perfect the health examination setting and monitoring for GPs.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study protocol was approved by the Ethics Committee of Yanbian University, China (YBU 2018-0027).

HUMAN AND ANIMAL RIGHTS

The experimental procedures were conducted in accordance with the ethical standards of the Helsinki Declaration and approved by the Ethics Committee of Yanbian University.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data sets used and/or analysed during this study are available from the corresponding author on request.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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