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Journal of Chromatography A, 1051 (2004) 193–197
JOURNAL OF CHROMATOGRAPHY A
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Simultaneous determination of nitrite, nitrate, thiocyanate and uric acid in human saliva by capillary zone electrophoresis and its application to the study of daily variations

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Available online 20 July 2004

Abstract

Simultaneous determination of nitrite (NO_2^-), nitrate (NO_3^-), thiocyanate (SCN^-) and uric acid in human saliva was performed by capillary zone electrophoresis using a coated capillary with reversed electroosmotic flow (EOF), using a 100 mM sodium phosphate buffer at pH 6.5 as a running buffer. Saliva samples were deproteinized with acetonitrile and filtered through a membrane filter. The important advantages of the reported method are: simple operation, short analysis time, minimal sample pre-treatment and sample dilution. In order to evaluate the daily variations of the anionic components, the concentrations were determined in the human saliva of four healthy volunteers upon waking and at 2 h intervals during a day.

Keywords: Nitrite; Nitrate; Thiocyanate; Inorganic anions; Uric acid

1. Introduction

Saliva is a complex liquid consisting of secretions from several salivary glands. It contains a number of compounds, namely proteins, peptides, amino acids, hormones, electrolytes and lipids [1]. The salivary composition is controlled by the autonomic nervous system and influenced by a number of physiological factors [2]. For example, levels of nitric oxide metabolites in human body fluids, nitrite (NO_2^-) and nitrate (NO_3^-), can be used as indicators of oxidative and nitrosative stress [3–6]. Since thiocyanate (SCN^-) is an end product of detoxification of hydrogen cyanide included in cigarette smoke, its excretion in urine and saliva can provide a useful marker of exposure in smokers and nonsmokers [7]. An elevated blood level of uric acid (hyperuricemia) may indicate an increased risk of gout, and it has been reported that the detectable levels of uric acid in human saliva were related to the levels in the blood of gout patients [8]. Many methods have been reported for the determination of the above anionic components in saliva, including UV–vis spectrophotometry [6,8–10], flow injection analysis [9,11], ion chromatography [12,13] and high performance liquid chromatography [10,14,15]. Capillary electrophoresis (CE) is widely used for inorganic ion analysis as a simple alternative to ion chromatography, particularly in environmental fields. Additionally, determinations of NO_2^-, NO_3^- and/or SCN^- in human saliva have been also performed by micellar electrokinetic chromatography using zwitterionic micelles [16], capillary isotachophoresis [17] and capillary zone electrophoresis (CZE) using a high ionic strength electrolyte buffer [18]. The aim of this work was to develop an accurate, fast and simple CZE method for the simultaneous determination of NO_2^-, NO_3^-, SCN^- and uric acid in saliva. A preliminary study of the daily variations of the salivary anionic components has been performed.

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doi:10.1016/j.chroma.2004.06.053

2. Experimental

2.1. Chemicals

Sodium nitrite ($NaNO_2$), potassium nitrate (KNO_3), potassium thiocyanate (KSCN), and fumaric acid were of analytical grade, and purchased from Kanto Kagaku (Tokyo, Japan), Kishida (Osaka, Japan), Wako (Osaka, Japan) and Nakalai Tesque (Kyoto, Japan), respectively. Sodium urate was of extra pure grade and purchased from Wako. Hexadimethrine bromide (polybrene, PB) was purchased from Sigma–Aldrich Japan (Tokyo, Japan), and dextran sulfate sodium salt (DS) was from ICN Biomedicals (Aurora, Oh, USA). Other reagents were of analytical grade.

2.2. Saliva sampling procedure

Saliva samples of four healthy volunteers (three males and one female, nonsmokers) were collected in Salivette tubes (Sarstedt, Nümbrecht, Germany) upon waking, at 2 h intervals during a day, and at the points until 11:00 after waking the following morning. The study was approved by the Institutional Ethics Committee in AIST and informed consent was obtained from all volunteers prior to their participation. The collected samples were kept in a refrigerator or a cool box before receipt from the volunteers at our laboratory. Immediately after receipt, the Salivette tubes were centrifuged at 2500 rpm for 10 min. The saliva samples were divided into several portions of an appropriate volume for analysis and stored at −20 °C.

2.3. Standard and sample preparation

An artificial saliva medium, namely SAGF medium (Gal–Fovet artificial saliva) [19] without potassium thiocyanate as given in Table 1, was used in preparing the following standard solutions to propose a simple and versatile analytical method. Stock standard solutions of 10 mM $NaNO_2$, 10 mM KNO_3, 10 mM KSCN, and 1 mM sodium urate were prepared by dissolving in the artificial saliva medium. A standard solution was prepared by mixing the stock standard solutions and diluting with the artificial saliva medium at the concentrations of 13.8 μg/mL (as NO_2^-), 18.6 μg/mL (as NO_3^-), 43.56 μg/mL (as SCN^-) and 33.42 μg/mL (as uric acid). A frozen saliva sample was thawed to room temperature prior to use. A mixture of 90 μL of the saliva sample (or the standard solution), 10 μL of internal standard solution (2 mM fumaric acid dissolved in the artificial saliva medium) and 100 μL of acetonitrile was filtered through a 0.45 μm membrane filter prior to sample injection.

Table 1
Composition of the SAGF medium without potassium thiocyanate [19]

Reagent	Concentration (mg/L)	Reagent	Concentration (mg/L)
NaCl	125.6	$Na_2SO_4{\cdot}10H_2O$	763.2
KCl	963.9	NH_4Cl	178.0
KH_2PO_4	654.5	$CaCl_2{\cdot}2H_2O$	227.8
Urea	200.0	$NaHCO_3$	630.8

2.4. Apparatus and analytical methods

CE separations were performed on a G1600A Agilent capillary electrophoresis system (Yokogawa Analytical Systems, Tokyo, Japan). Fused-silica capillaries from Polymicro Technologies (Phoenix, AZ, USA) with dimension of 33 cm (24.5 cm to the detector) × 50 μm i.d. × 365 μm o.d. were coated with successive multiple ionic polymer layers, namely SMIL(3)-coating [20]. Separations were performed in 100 mM sodium phosphate buffer at pH 6.5 as a running buffer. Prior to each analysis, the capillary was rinsed with 5% (w/v) PB solution dissolved in the phosphate buffer for 5 min, then the running buffer for 3 min and finally conditioned by applying the voltage of −10 kV for 2.5 min. A sample was injected hydrodynamically for 6 s at 5 kPa (50 mbar). The applied voltage was −10 kV and UV detection at 214 nm was performed. The ratio of the peak area of the analyte to that of the internal standard was used for quantitative purposes.

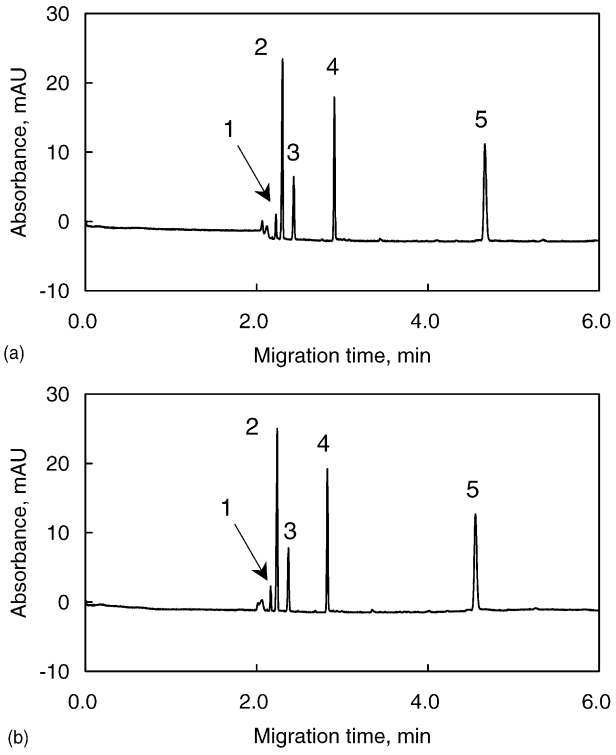

Fig. 1. Effect of the capillary preconditioning step, applying voltage prior to the sample injection. Capillary preconditioning prior to the injection of a standard solution: (a) 5 min flush with 5% (w/v) PB solution buffer and 3 min flush with buffer; (b) addition of voltage preconditioning for 2.5 min at 10 kV. Capillary: SMIL(3)-coated, 33 cm (24.5 cm to the detector) × 50 μm i.d.; running buffer: 100 mM phosphate buffer (pH 6.5). Sample: human saliva from volunteer B. Peak identification – 1: nitrite, 2: nitrate, 3: thiocyanate, 4: fumarate (internal standard), 5: urate.

3. Result and discussion

3.1. Method development

The direction of electroosmotic flow (EOF) can be reversed (i.e. from cathode to anode) by changing the charge on the capillary surface from negative to positive. EOF reversion is particularly useful for the analysis of anions by increasing the operating speed, since the anionic ions migrate through the capillary due to the combination of their electrophoretic mobility and EOF. A SMIL(3)-coated capillary with a stable wall modification of PB/DS/PB [20] was used in this study, nevertheless the EOF gradually decreased during successive analysis. Since the deterioration of capillary coating layer may relate to the fact that the salivary matrix of high ionic strength includes an equivalent volume of acetonitrile, a rinse step with a 5% (w/v) PB solution for 5 min was adopted for the process of the capillary preconditioning before each analysis. As shown in Fig. 1a, however, a drop in the electropherogram baseline is always observed after 2 min, in spite of an adequate rinse with the run buffer. This corresponds to the elution of residual bromide ions of PB on the capillary surface. Thus, the equilibrium process of the applied voltage was added to the preconditioning process. Under the optimum capillary preconditioning, the electropherogram provided an adequate baseline as shown in Fig. 1b. Concerning the robustness of the analytical method, the running buffer in the range of pH 6.0–7.5 was shown to have no effect on the selectivity.

3.2. Daily variations of salivary anionic components

Fig. 1b shows a representative electropherogram of salivary sample solutions. The analytes of interest are not interfered with by any components in the artificial saliva medium such as chloride and sulfate. Several neutral species (corresponding to the EOF marker) were detected at ca. 9 min (data not shown). Daily variations of NO_2^-, NO_3^-, SCN^- and uric acid were determined using saliva samples from four volunteers A–D, and the results are summarized in Fig. 2. Since salivary NO_2^- and NO_3^- concentrations increased after a high NO_3^- meal and these levels were sustained for at least 5 h post NO_3^- ingestion [21], the higher concentrations of NO_2^- and NO_3^- in saliva samples from volunteer C seemed to be related to the effects of diet. Concerning SCN^-, elevated levels were present in some data points such as volunteer A at 11:00 (day 1) and volunteer D at 17:00. However, there was no effect of passive smoke exposure, because the volunteers stayed in the nonsmoking room of our laboratory.

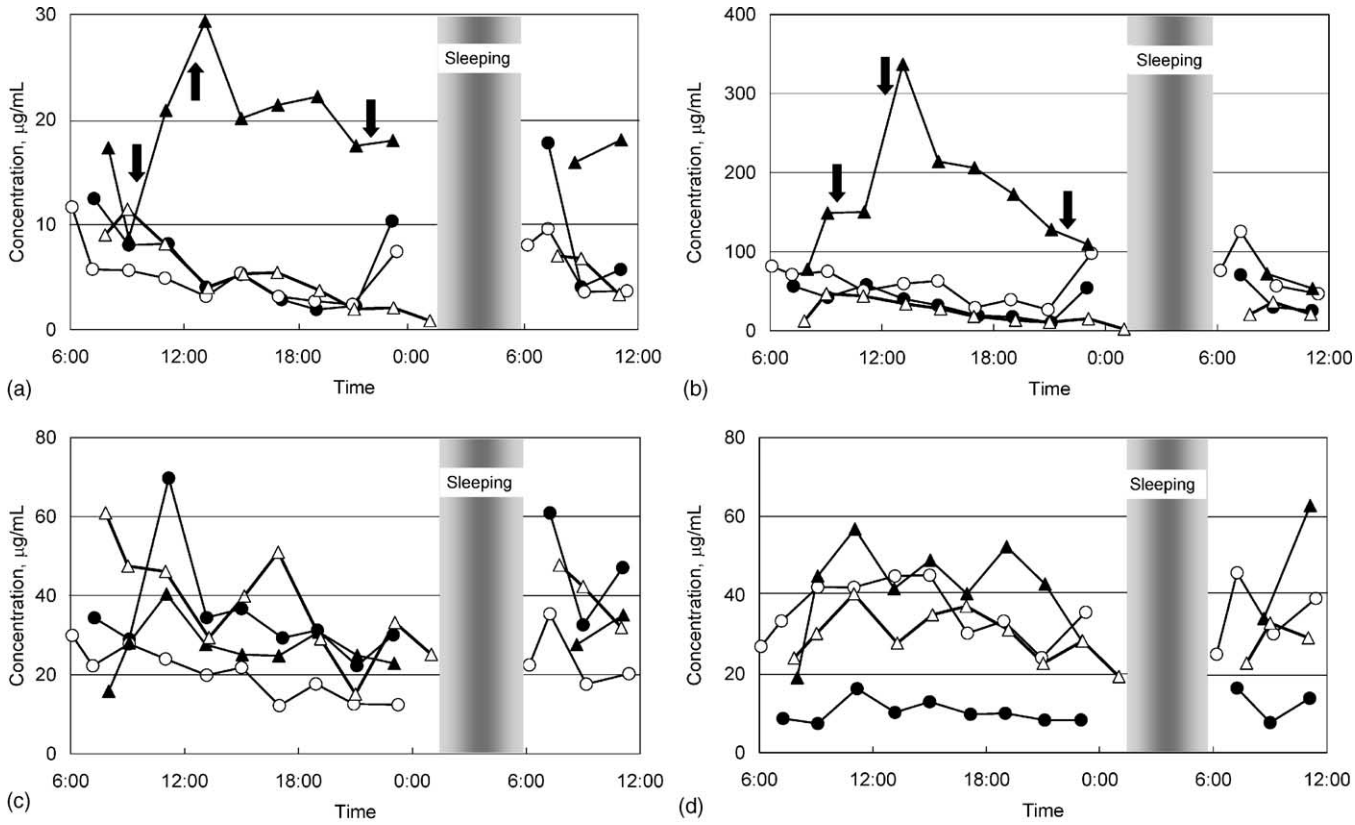

Fig. 2. Daily variations of (a) nitrite, (b) nitrate, (c) thiocyanate and (d) urate in human saliva. Volunteers – A: closed circle, B: open circle, C: closed triangle, D: open triangle. The arrows in the figure show the mealtimes of volunteer C.

Table 2
Linearity data and limit of detection (LOD) obtained from the standard solutions

Component	Concentrations[a] (μg/mL)	Regression line	r	LOD[b] (μg/mL)
NO_2^-	1, 2.5, 5, 10, 20, 50	$y = 0.030\,x + 0.006$	1.0000	0.7
NO_3^-	10, 25, 50, 100, 200, 500	$y = 0.031\,x - 0.116$	0.9998	1.1
SCN^-	4, 10, 20, 40, 80, 200	$y = 0.017\,x - 0.028$	1.0000	5.4
Uric acid	4, 10, 20, 40, 80, 200	$y = 0.026\,x + 0.038$	0.9995	2.3

[a] Two determinations at each concentration.
[b] S/N = 3.

3.3. Analytical validation

3.3.1. Linearity and limit of detection

The linearity was examined using a standard solution with the addition of different volumes of stock standard solutions. Each concentration was prepared in duplicate. The obtained correlation coefficient between the concentration of analytes and the ratio of all peak areas to the internal standard for all calibration curves is adequate as given in Table 2. Based on these linearity data, standard solutions prepared at a single concentration are considered to be sufficient for accurate quantification. The limits of detection (LODs, S/N = 3) were calculated from the baseline noise for a time period from 0.5 to 1.5 min using Agilent Technologies ChemStation software and are given in Table 2.

3.3.2. Accuracy and repeatability

The accuracy was examined using a standard addition method. Each concentration was prepared in triplicate, with the results shown in Table 3. In order to evaluate the repeatability of this method, multiple sample preparations from the same saliva and the same standard solution were analyzed. The results, expressed as the R.S.D., are given in Table 4.

Table 3
Accuracy of the analytical procedure using the standard addition method

Component	Concentration (μg/mL)[a]		Recovery (%)
	Spiked	Found[b]	
NO_2^-	2.189	2.287	104.5
	4.379	4.930	112.6
	6.568	7.326	111.5
	8.757	9.507	108.6
	10.946	11.871	108.4
NO_3^-	19.869	21.192	106.7
	39.738	43.681	109.9
	59.607	63.707	106.9
	79.476	86.369	108.7
	99.345	108.090	108.8
SCN^-	8.206	8.729	106.4
	16.413	17.890	109.0
	24.619	25.987	105.6
	32.825	36.489	111.2
	41.032	45.326	110.5
Uric acid	7.615	7.543	99.1
	15.229	15.973	104.9
	22.844	21.678	94.9
	30.459	31.360	103.0
	38.074	38.929	102.2

[a] Concentration of the components in saliva used in this experiment: NO_2^-, 3.977 μg/mL; NO_3^-, 21.875 μg/mL; SCN^-, 20.246 μg/mL; uric acid, 20.246 μg/mL.
[b] Average results based on three replications at each concentration.

3.3.3. Stability of saliva samples

The stability was determined of both the collected saliva sample and the salivary sample solution for injection. As given in Table 5, the concentration of the anionic components remained stable over a period of 48 h in the refrigerator and for 4 h at room temperature. The salivary NO_2^- concentration increased during storage at room temperature since some bacterial contaminant from the tongue reduced NO_3^- to NO_2^- [21]. By contrast, the prepared salivary sample solution after deproteinizing was stable over a period of 8 h at room temperature.

Table 4
Repeatability of the analytical procedure

Component	Standard solution		Saliva sample	
	Concentration (μg/mL)	R.S.D. (%) (n = 6)	Concentration (μg/mL)	R.S.D. (%) (n = 6)
NO_2^-	21.1	1.5	4.7	4.7
NO_3^-	25.0	3.1	20.6	2.3
SCN^-	23.0	1.3	15.2	3.4
Uric acid	36.9	5.0	11.0	3.3

Table 5
Change in the components of salivary samples stored at room temperature (RT) and at 3 °C

Storage period	Component	Collected saliva		Sample solution after deproteinizing, RT
		3 °C	RT	
4 h	NO_2^-	97.4	97.6	96.4
	NO_3^-	99.6	97.1	97.4
	SCN^-	102.9	100.1	97.4
	Uric acid	104.2	103.2	105.3
8 h	NO_2^-	91.6	108.9	97.4
	NO_3^-	92.1	91.6	104.6
	SCN^-	93.0	92.8	104.8
	Uric acid	97.9	99.8	99.4
24 h	NO_2^-	99.6	133.5	104.8
	NO_3^-	97.8	81.4	105.1
	SCN^-	100.2	80.8	117.5
	Uric acid	98.6	82.6	96.9
48 h	NO_2^-	97.3	101.1	104.4
	NO_3^-	100.4	51.2	101.7
	SCN^-	101.4	78.7	107.0
	Uric acid	99.4	60.3	97.4

4. Conclusion

A fast and simple CE method was implemented for the analysis of salivary NO_2^-, NO_3^-, SCN^- and uric acid, and the acceptable analytical validation data was obtained. In this work, preliminary determinations of the daily variations of the salivary NO_2^-, NO_3^-, SCN^- and uric acid were performed without explicit consideration of either endogenous or exogenous factors. This analytical method would be of practical use for the study of oxidative and/or nitrosative stress, such as the determination of nitric oxide metabolites in human body fluids.

Acknowledgement

The authors would like to thank Dr. Y. Ishihama (Eisai Co. Ltd.) for his helpful advice regarding the SMIL(3)-coated capillary. We also acknowledge Dr. E. Niki of AIST for helpful support.

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