Determination of Monovalent Inorganic Anions in Human Saliva by Ion Chromatography Using Microcolumn Coated with Micellar Zwitterionic Bile Acid Derivative.

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Anions such as nitrite, nitrate, iodide, and thiocyanate ions contained in human saliva have been determined by ion chromatography using a microcolumn coated with micellar bile acid derivative, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, where only a phosphate buffer solution was used as the mobile phase. The detection limits obtained by the present system were 1.1 μM for nitrite and nitrate, 2.3 μM for thiocyanate, and 0.48 μM for iodide when the direct UV absorption detection of the anions was performed at 230 nm. The present system was applied to the determination of inorganic anions in human saliva, especially in relation with smoking habit, age and sex. Thiocyanate ion was detected at the significantly higher level in saliva for the smokers, compared to the non-smokers. Furthermore, the thiocyanate levels in saliva depended on the age and sex, while the concentrations of nitrite, nitrate, and iodide ions were rather independent of these parameters.

The determination of inorganic anions contained in biological fluids is of important medical interest, because some inorganic anions play a physiologically important role in human body. For example, thiocyanate ion prohibits iodine uptake in the thyroid gland[1] and also affects improvement of the hypertensive situations. So far, many analytical methods have been developed for such a purpose. Some of them are the spectrophotometric method based on the color-forming reactions,[1] and linear sweep polarography.[2] Another method is flow injection analysis using a modified electrode detector.[3] Gas chromatography with derivatization reaction has been also reported,[4,5] although the derivatization reaction procedure is fairly time-consuming and the derivatization conditions are rather difficult to be regulated. In addition, these methods provide quantitative information about only a single analyte, which is not enough for the survey of the biological sources or medical diagnosis. Ion chromatography has been recognized as one of the most effective methods for the determination of inorganic anions, in which ion exchange mode is most commonly used. Even so, in convenient ion exchange chromatography, iodide and thiocyanate ions are generally associated with the long retention time, thus requiring an increasing ionic strength in the mobile phase[6] and its selectivity is rather poor.

Recently the present authors have investigated the enantiometric separation by high performance liquid chromatography (HPLC) technique using micellar bile salt and their derivatives as the mobile phase additives.[7—9] In these experiments, it has been noted that the micellar bile salts and their derivatives existed in the stationary phase as the adsorbents as well as in the mobile phase as the additives. Furthermore, we have examined a possibility to use a microcolumn coated with micellar bile salt for the separation of the enantiomers in the HPLC mode[10] and also for the separation of cations in the ion chromatography mode,[11,12] where the bile salts or their derivatives were not contained in the mobile phase. In this separation microcolumn system, bile acid derivatives in the micelle forms are adsorbed on the ODS (octadecylsilica) stationary phase by the hydrophobic interaction.[9] The characteristic property of bile acid derivatives is that they possess both positive and negative charges in a molecule. Then it is expected that they act as a zwitterionic separator. In the present work, hence, we have examined the potential separation ability of the zwitterionic function of micellar bile acid derivative coated on the stationary phase for the separation of inorganic anions such as nitrite, nitrate, iodide, and thiocyanate ions. Consequently it has been found that these anions in aqueous solution as well as in human saliva could be separated by using ODS coated with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) as the stationary phase, where only a phosphate buffer was used as the mobile phase.

Experimental

Apparatus. An ion chromatography system employed in the present work was almost the same as in the previous work.[12] It consisted of a microfeeder (model MF-Z; Azumadenki Kogyo, Tokyo) equipped with a 0.5-ml gastight syringe (model ML-522; JASCO, Tokyo) as the pump, a microvalve injector with 0.02 μl injection volume, a 150×0.35 mm i.d. microcolumn packed with Develosil ODS-5 (5 μm; Nomura Chemical, Seto) and a UVIDEC-100V UV detector (JASCO). A data processor (Chromatopac C-R4AX; Shimadzu Instrument Co., Kyoto) was utilized for the peak area measurements in the chromatograms.

Reagents. The reagents used were of analytical reagent grade. As zwitterionic bile acid derivative, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was obtained from Dojindo Laboratories (Kumamoto). The anions examined were obtained as their sodium salts from Wako Pure Chemical Industries (Osaka). These reagents were used without further purifucation. Purified water was prepared in the laboratory by using a Milli-Q System (Nihon Millipore Kogyo, Tokyo).

Preparation of Separation Column. First, aqueous solution of 30 mM (M=mol dm^{-3}) CHAPs was passed through a microcolumn for 20 min at a flow rate of 2.8 μl min^{-1} to adsorb CHAPS on the ODS. Then the column was conditioned with a phosphate buffer (10 mM each of NaH_2PO_4 and Na_2HPO_4, pH 5.6), which was used as the mobile phase for separation. The stability of the CHAPS-coated ODS column was examined by flowing the phosphate buffer through the column for 24 h with monitoring the eluent by the UV absorption detection at 230 nm. During this procedure, no elution of CHAPS was observed. In addition, the separation column prepared above maintained almost the same separation characteristics for more than 6 months, even when the column was used for other experiments or preserved with filling pure water inside during nonuse.

Results and Discussion

Separation Characteristics of Diverse Anions. CHAPS has the zwitterionic structure with both positive ($-\overset{|}{\underset{|}{N}}^{+}-$) and negative ($-[SO_3]^-$) charges in the molecule. In addition, it is further considered that CHAPS is adsorbed on the ODS in the micelle form by the hydrophobic interaction.[10] Thus CHAPS is a potential ion separator along with some size exclusion capability, even when it is immobilized on the stationary phase. Therefore, in the present experiment, the separation characteristics of the micellar CHAPS-coated ODS as the stationary phase was examined for the separation of diverse anions, especially monovalent anions.

A typical chromatogram for anion separation obtained by the present system is shown in Fig. 1, where 10 mM phosphate buffer (pH 5.6) was used as the mobile phase and the wavelength of the UV detector was set at 230 nm. Five kinds of anions were quite well baseline-separated by the microcolumn coated with micellar CHAPS. The retention times of these monovalent anions obtained by the present system are summarized in Table 1. Under the conditions shown in Fig. 1, bromide ion could not be chromatographically separated from nitrate ion. This problem was fortunately overcome by choosing the detection wavelength at 230 nm, because the UV absorption intensity of bromide ion at 230 nm was about 1/100 of that of nitrate. Thus only nitrate ion could be detected at 230 nm, unless the concentration of bromide ion was not so high. The divalent anions were hardly retained on the present column, and then they did not interfere with the determination of the monovalent anions.

Analytical Figures of Merit. The calibration graphs for the determination of nitrate, nitrite, iodide, and thiocyanate ions were obtained by measuring the peak areas of the analyte anions, and each of these analytes was linear up to 20 mM. The detection limits of the analytes, which were estimated as the concentration corresponding to signal-to-noise ratio of 3 in the peak height measurement, were 1.1 μM for nitrate and nitrite ions, 0.48 μM for iodide ion and 2.3 μM for thiocyanate ion.

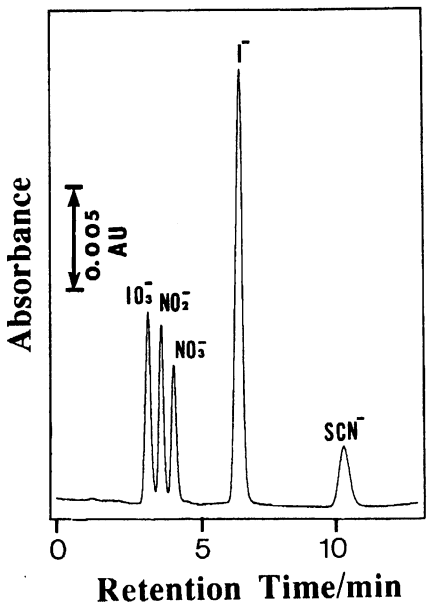

Fig. 1. Chromatogram of monovalent anions in artificial sample. Column: Develosil ODS-5 coated with CHAPS micelles (150×0.35 mm i.d.), mobile phase: 10 mM NaH_2PO_4–10 mM Na_2HPO_4, flow rate: 2.8 μl min^{-1}, wavelength of UV detection: 230 nm, analyte ions: 1 mM each of iodate, nitrite, nitrate, iodide, and thiocyanate ions.

Table 1. Retention Times of Monovalent Anions in Ion Chromatography Using the Stationary Phase Coated with Micellar CHAPS and the Mobile Phase of the Phosphate Buffer

Anions	IO_3^-	NO_2^-	NO_3^-	I^-	SCN^-
Retention time/min	3.40	3.93	4.40	6.98	11.05

The recovery test was performed by adding the 0.1—0.5 mM standard solutions of the anions into the saliva samples. Consequently the recovery values obtained were in the range of 95—103% for all anions.

Determination of Anions in Human Saliva. The present separation system was applied to the determination of inorganic anions contained in human saliva. In the determination of thiocyanate ion in saliva samples, usually the proteins in the samples should be excluded in some proper methods prior to analysis.[5] However, in the present experiment the proteins or large molecules were eluted as the non-retained components in the chromatograms for saliva samples,[13] and thus only filtration was required for removal of solid particles in the samples. The saliva sample was taken in a syringe by sucking up manually. In this procedure, the head of the syringe needle was covered with cotton cloths, which was removed before the saliva sample was injected through the microvalve injector. Figure 2 shows a chromatogram of inorganic anions contained

in saliva, in which nitrite, nitrate, iodide, and thiocyanate ions were identified from the retention times. The quantitative results obtained are summarized in Table 2. The reproducibilities (relative standard deviations) of analytical values in Table 2 were ca. 1% in the measurements of 10 replicate samples.

The stabilities of the analytes in human saliva samples were also examined. Then no significant differences in the retention times and concentrations of the analytes were observed for the samples preserved over two weeks.

Comparison of Thiocyanate Levels between Smokers and Non-Smokers. It has been reported that the thiocyanate levels differ between smokers and non-smokers.[1,2] Bendtsen and Hansen[1] determined the thiocyanate levels in saliva by the spectrophotometric method to be 1.04 mM for non-smokers and 3.62 mM for the smokers, while Cai and Zhao[2] found to be 0.3 mM for the former and 4.0 mM for the latter, respectively, by polarography.

The present experimental method was also applied to examine the difference in the thiocyanate levels between the smokers and non-smokers. The chromatograms of inorganic anions for smoker's and non-smoker's saliva are shown Fig. 3, where other monovalent anions were also observed. It is seen in the figure that the thiocyanate ion level is higher for the smoker, compared to the non-smoker. The average levels of thiocyanate for the non-smokers and smokers (each ten samples were analyzed) were 0.325 and 2.67 mM, respectively. As has been reported, the thiocyanate level was higher for the smoker, although the average value determined in the present experiment was somewhat lower than those reported earlier.[1,2]

Dependence of Thiocyanate Levels on Age and Sex. Thiocyanate ion is also contained in vegetables such as cabbage family, although tobacco smoke is the larger source of thiocyanate ion.[1] In order to examine the dependence of the thiocyanate levels on the age, saliva samples were collected from the non-smokers and their family who were also non-smokers. In these experiments, since other anions could be observed simultaneously, the concentrations of nitrite, nitrate, and iodide ions were also determined at the same time, using the present method. Figures 4 and 5 show the relationships between the concentrations of the inorganic anions and

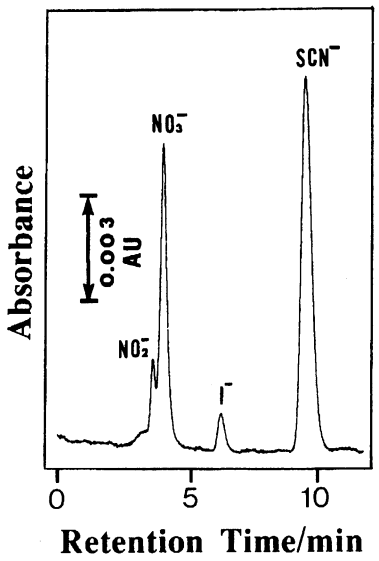

Fig. 2. Chromatogram of monovalent anions contained in human saliva. Experimental conditions are the same as in Fig. 1.

Table 2. Determination of NO_2^-, NO_3^-, I^-, and SCN^- Contained in Human Saliva

Samples	Found/mM			
	NO_2^-	NO_3^-	I^-	SCN^-
No.1	0.62	ND[a]	0.01	0.42
No.2	0.50	0.48	ND[a]	0.59
No.3	0.29	0.26	ND[a]	0.97
No.4	0.29	1.05	0.05	2.62
No.5	0.08	0.25	ND[a]	0.29
No.6	0.34	0.74	0.17	0.56
No.7	0.40	0.46	0.01	0.27
No.8	0.16	0.96	ND[a]	0.44

a) Not detected.

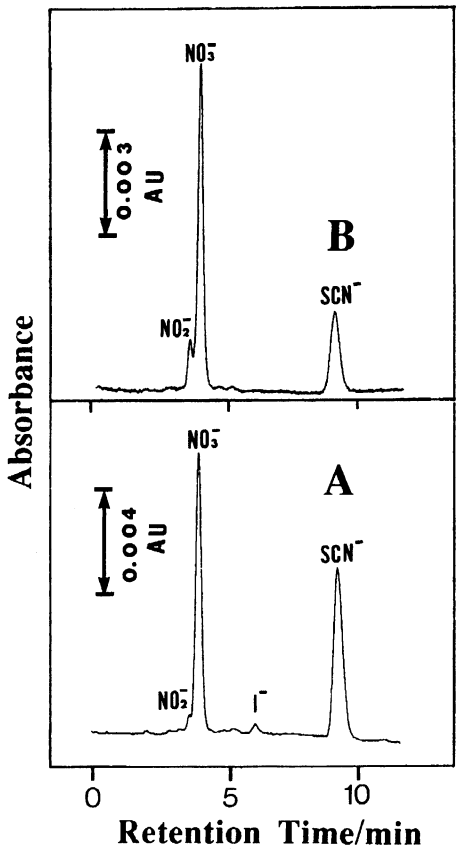

Fig. 3. Chromatograms of monovalent anions in human saliva from a smoker (A) and a non-smoker (B). Experimental conditions are the same as in Fig. 1.

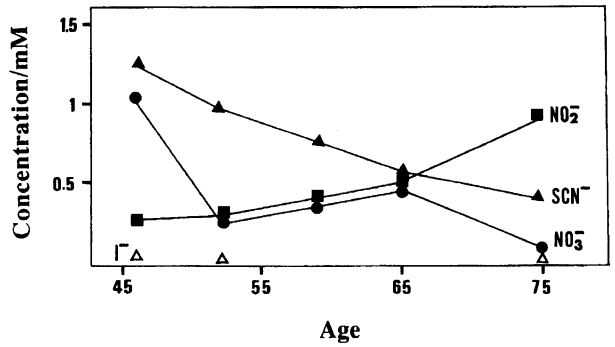

Fig. 4. Relationships between the concentrations of inorganic anions and age for women.

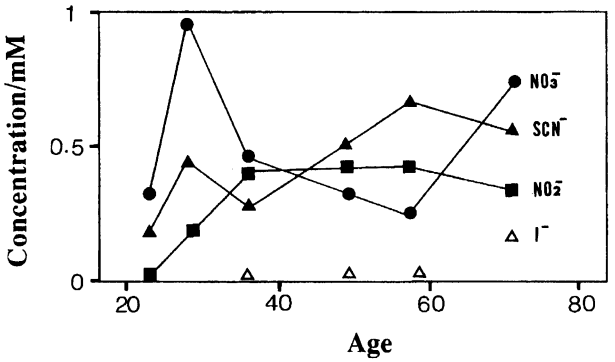

Fig. 5. Relationships between the concentrations of inorganic anions and age for men.

the age for women and men, respectively. In the case of women, the thiocyanate level decreased at the older age, while other anion levels were independent of the age. On the other hand, in the case of men, the thiocyanate level was rather independent of the age.

It should be added here that the determination of thiocyanate ion in human saliva and urine by ion chromatography using an ODS column coated with cetyldimethylamine was reported by Michigami et al.,[14] after the present paper was submitted.

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