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DETERMINATION OF OXIDIZABLE INORGANIC ANIONS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION AND APPLICATION TO THE DETERMINATION OF SALIVARY NITRITE AND THIOCYANATE AND SERUM THIOCYANATE

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SUMMARY

A sensitive method for the simultaneous determination of oxidizable inorganic anions (sulphide, thiocyanate, thiosulphate and nitrite) was developed by use of high-performance liquid chromatography and fluorimetric detection based on the formation of fluorescent cerium(III) by a redox reaction with cerium(IV). The detection limits are 0.1 nmol for both thiocyanate and nitrite, 0.3 nmol for thiosulphate and 0.8 nmol for sulphide per 10-μl injection volume. This system can be utilized for the determination of salivary thiocyanate and nitrite and serum thiocyanate.

INTRODUCTION

Small amounts of thiocyanate and thiosulphate ions, which are metabolic products of sulphur-containing compounds, are present in the human body as normal constituents. The concentration of thiocyanate, which is also a detoxication product of cyanide, increases in the urine and plasma of smokers [1,2] and patients receiving nitroprusside as an antihypertensive agent [3,4]. The urine of patients with sulphite oxidase deficiency contains large amounts of thiosulphate [5–7], formed by the enzyme-catalysed reaction of sulphite with β-mercaptopyruvate.

Nitrite ion, which in excessive amounts may induce methaemoglobinaemia in infants and form carcinogenic nitrosamines when present with secondary or tertiary amines and amides [8,9], is also present in human plasma and saliva at lower levels [10,11].

Electrochemical detection (ED) is a powerful method for the measurement of these oxidizable anions. Kägedal et al. [12] and Kawanishi et al. [13] have

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achieved the determination of micromole per litre levels of thiosulphate in human urine with mercury-based ED and in human urine and plasma with glassy carbon-based dual ED, using high-performance liquid chromatography (HPLC). On the other hand, the nitrite concentration in the human body has been measured by gas chromatography [11] and by HPLC with UV detection [14]. For the determination of thiocyanate in biological samples, two different spectrophotometric methods based on the formation of a red complex with iron(III) ion [2,15] and the König reaction [1,16] have been reported. Toida et al. [17] have reported a sensitive, specific method for the determination of thiocyanate and cyanide in blood plasma and red blood cells by HPLC with fluorescence detection using a modified König reaction.

Cerium(IV) ion, which is converted into fluorescent cerium(III) by a redox reaction, is also available as a highly sensitive oxidizing reagent for oxidizable compounds, and Wolkoff and co-workers and Katz and co-workers have applied this reagent to the monitoring of phenols [18], comparative serum and urine analyses [19], carbohydrates [20], aromatic acids in urine [21] and thiosulphate and polythionate in mining wastewater and environmental samples [22] after eluting from chromatographic columns. Lee and Field [23] have described the simultaneous determination of nitrite, nitrate, thiosulphate and iodide ions using an anion-exchange column fitted with a copperized cadmium reductor to reduce nitrate to nitrite and cerium(IV) fluorescence detection, and applied the method to the determination of nitrate and nitrite in drinking and sea water.

We describe here a sensitive HPLC system with cerium(IV) fluorescence detection for the simultaneous separation and determination of sulphide, thiocyanate, thiosulphate and nitrite and the application of this system to measurements of thiocyanate and nitrite in human saliva and bound–free thiocyanate in human serum.

EXPERIMENTAL

Materials

All chemicals were of analytical-reagent grade. Doubly distilled water, after passage through an anion-exchange resin, was used to prepare all standards and eluents. Sulphide ion was prepared by dissolving $Na_2S \cdot 9H_2O$ in 0.1 *M* ammonium chloride solution. All other inorganic anions were prepared by dissolving their sodium salts in water.

Post-column reagent

A 0.75 *M* sulphuric acid solution of $2.5 \cdot 10^{-4}$ *M* cerium(IV) sulphate, to which 0.003% sodium bismuthate was added to prevent the selfreduction of cerium(IV) [20], was used. The reagent solution was cooled to room temperature after warming for 30 min at 50°C.

Eluent

The mobile phase was 0.05 *M* sodium nitrate solution. The solution was filtered through a 0.45-μm membrane filter (Type TM-2P) (Toyo Roshi, Tokyo, Japan) and deaerated prior to use.

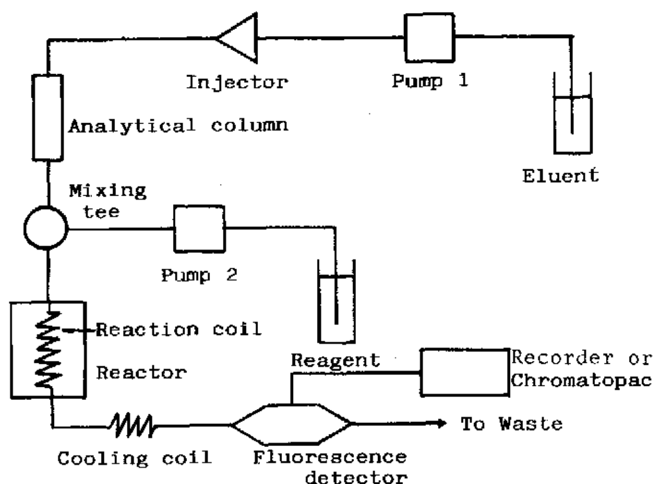

Fig. 1. Flow diagram of the HPLC system.

Column preparation

A stainless-steel column (150 mm×4 mm I.D.) was slurry-packed with the strongly basic ion-exchange resin TSK gel QAE-2SW (silica-type anion exchanger, mean particle diameter 5 μm) (Toyo Soda, Tokyo, Japan) suspended in the mobile phase.

HPLC system

Fig. 1 is a flow diagram of the chromatographic system employed.

The HPLC system consisted of two pumps, a Model BIP-1 (pump 1) (Jasco, Tokyo, Japan) to provide the mobile phase at a flow-rate of 0.8 ml/min and a Hitachi 633 chemical pump (pump 2) (Hitachi, Tokyo, Japan) which introduced the cerium(IV) post-column reagent at a flow-rate of 0.3 ml/min, a Rheodyne Model 7125 injection valve equipped with a 100-μl loop (Rheodyne, CA, U.S.A.), an RF-500 LC fluorescence spectromonitor equipped with a 5-μl flow cell (Shimadzu, Kyoto, Japan), a Hitachi 638-0250 temperature-controlled reactor and an RC-150 recorder (Jasco) or a Chromatopac C-R1B integrator (Shimadzu). A 5 m×0.25 mm I.D. of coiled Teflon tubing was immersed in the reactor as the reaction section. The column was maintained at room temperature. The reagent solution from pump 2 was combined with the effluent and the resulting solution was delivered to the reaction coil immersed in the reactor maintained at 80°C to provide a residence time of about 15 s and then passed through the fluorescence detector. The fluorescence signals were recorded by the recorder or integrator at 360 nm with excitation at 260 nm.

Determination of nitrite and thiocyanate in human saliva

After thoroughly rinsing the mouth with distilled water and discarding the first few millilitres of saliva, about 5 ml of saliva were collected in a beaker and were centrifuged at 2000 *g* for 10 min. The supernatant solution was filtered with an

Amicon MPS-1 micropartition system (Amicon, Lexington, MA, U.S.A.) by centrifuging at 1000 *g* for 20 min and then 10 μl of filtrate were submitted to HPLC.

Determination of bound-free thiocyanate in human serum

Human serum diluted two-fold with distilled water was filtered with an Amicon Centriflo ultrafiltration membrane cone by centrifuging at 1000 *g* for 30 min and then 50 μl of the filtrate were submitted to HPLC.

RESULTS AND DISCUSSION

Separation of sulphide, thiocyanate, thiosulphate and nitrite

The HPLC separation for four oxidizable inorganic anions was examined under the above chromatographic conditions. The excitation maximum of cerium(III) produced from the redox reaction of the ions with cerium(IV) in the presence of sulphuric acid was at 260 nm and the emission maximum was at 360 nm.

Imanari and co-workers have already reported two mobile phase systems for the separation of inorganic anions involving the oxidizable inorganic anions by use of a TSK gel QAE-2SW column: 0.05 *M* phosphate (pH 7.5) [24] and sodium acetate (pH 5.48) [25] buffers containing 0.05 *M* sodium nitrate. For the separation of sulphide, thiocyanate, thiosulphate and nitrite, the acetate buffer gave good resolution, but also gave a high fluorescence background caused by the reaction of acetate ion with cerium(IV). The phosphate buffer gave poor resolution between the sulphide and nitrite peaks. Only when sodium nitrate was used in the eluent were a minimal fluorescence background and a favourable separation for the four anions was observed.

The effect of sodium nitrate concentration on the retention time for the separation of the anions on a column packed with QAE-2SW resin is shown in Fig. 2. Although the four anions were separated within the concentration range 0.07–0.2

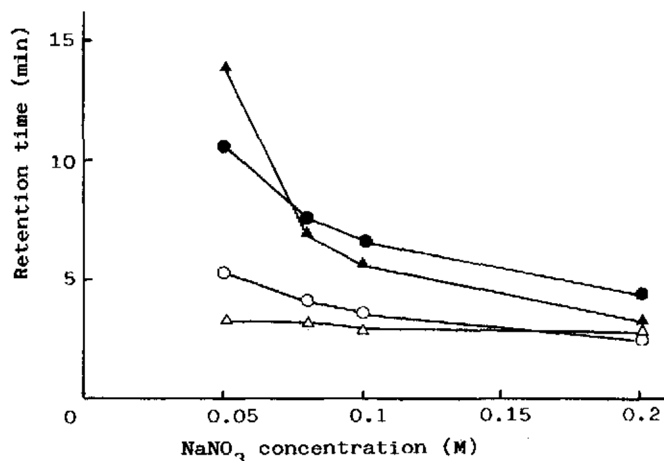

Fig. 2. Effect of the concentration of sodium nitrate on the retention time for the separation of sulphide (△), thiocyanate (●), thiosulphate (▲) and nitrite (○).

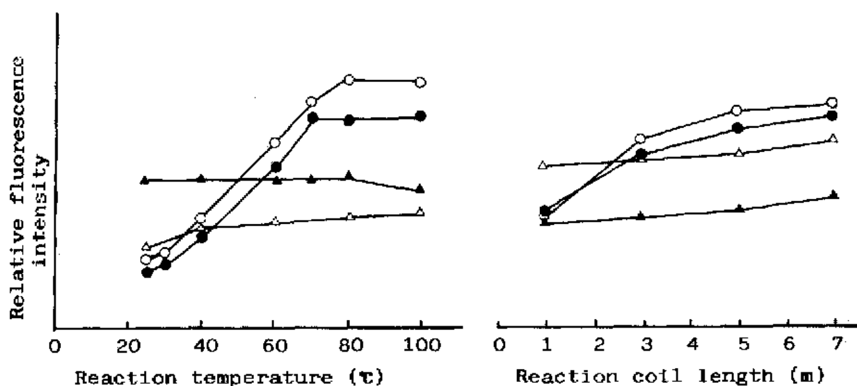

Fig. 3. Effects of the reaction temperature and coil length on the fluorescence response developed with the reactions of sulphide (△), thiocyanate (●), thiosulphate (▲) and nitrite (○) with cerium(IV).

M sodium nitrate, the thiocyanate and thiosulphate peaks and also the nitrite and sulphide peaks were poorly resolved. However, all four anions were completely separated when 0.05 *M* sodium nitrate was used. Under these conditions, sulphide ion was eluted first at a retention time of 3.3 min, and nitrite, thiocyanate and thiosulphate were eluted within 20 min in this order.

Post-column conditions

As the fluorescence responses for the four anions were held constant in each concentration range from $1\cdot10^{-4}$ to $5\cdot10^{-4}$ *M* for cerium(IV) sulphate and from 0.25 to 1.25 *M* for sulphuric acid containing 30 mg/l sodium bismuthate, $2.5\cdot10^{-4}$ *M* cerium(IV) sulphate and 0.75 *M* sulphuric acid were therefore chosen as the optimum concentrations of the post-column reagents. On the other hand, the reaction rates of both nitrite and thiocyanate with cerium(IV) depended on the temperature and reached a maximum temperature higher than 80°C, whereas those of thiosulphate and sulphide were almost independent of temperature, as shown in Fig. 3. Taking into consideration the increase in the background fluorescence with increase in temperature, the reaction temperature was set at 80°C.

The fluorescence intensity gradually increased with increasing coil length (Fig. 3), but the peaks for the four anions were broadened considerably owing to diffusion and the background fluorescence also increased. Consequently, a 5 m × 0.25 mm I.D. coiled tubing was chosen as optimum. We were able to reduce the reaction time to less than 15 s, compared with the method of Lee and Field [23], which required a residence time of 1.5 min.

A typical chromatogram obtained with a standard mixture of the ions under the established conditions is illustrated in Fig. 4.

The limits of detection were 0.1 nmol for both nitrite and thiocyanate, 0.3 nmol for thiosulphate and 0.8 nmol for sulphide (three times the baseline noise level with an injection volume of 10 μl). A linear relationship was observed for the four anions up to at least 10 nmol per 10-μl injection volume. The relative standard deviation for the method was less than 8%.

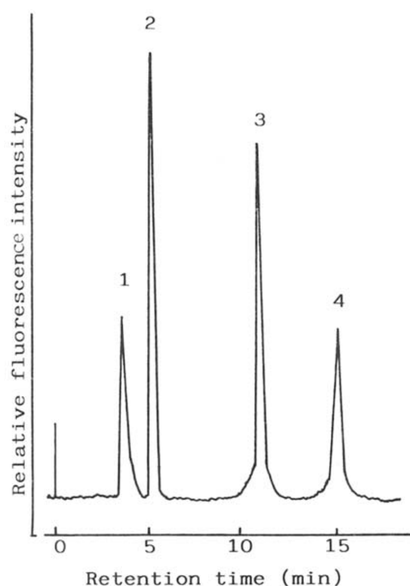

Fig. 4. Typical chromatogram of standard. Peaks: 1 = sulphide; 2 = nitrite; 3 = thiocyanate; 4 = thiosulphate. Concentration of anions: 10 μM.

Determination of thiocyanate and nitrite in human saliva

The method was applied to the simultaneous determination of thiocyanate, thiosulphate, sulphide and nitrite in human saliva. Chromatograms for a 10-μl sample of ultrafiltered saliva from two smokers and one non-smoker exhibited four peaks, as shown in Fig. 5.

A prominent peak at a retention time of 10.8 min and a small peak at 5.0 min, both of which disappeared on adding the iron (III) nitrate and hydrogen peroxide reagents, had retention times corresponding to the peaks of thiocyanate and nitrite ions. The chromatograms also showed that there was not detectable amount of thiosulphate, the concentration of which in human saliva has not yet been reported. The sulphide peak was not confirmed owing to overlapping with the peaks of other components in saliva. The recoveries of nitrite and thiocyanate ions added to saliva at a concentration of 0.5 mM were 99.3 and 99.1%, respectively.

The within-run coefficients of variation (C.V.s) were 6.03% for nitrite at 1.5 nmol per 10 μl and 5.88% for thiocyanate at 10 nmol per 10 μl ($n=5$ at each concentration). It was difficult to evaluate exactly the between-run precision for both ions as the concentration of nitrite in saliva tended to increase from day to day.

The determination of nitrite in saliva may be affected by contamination from the atmosphere and by microorganisms which reduce nitrate to nitrite. In any event, it is preferable for the determination of nitrite in body fluids to be carried out immediately after preparation.

The thiocyanate level in saliva was clearly higher in smokers (0.89 and 1.30 mM) than in non-smokers (0.40 mM), whereas for the nitrite concentration in saliva no significant difference between smokers (0.03 and 0.19 mM) and non-

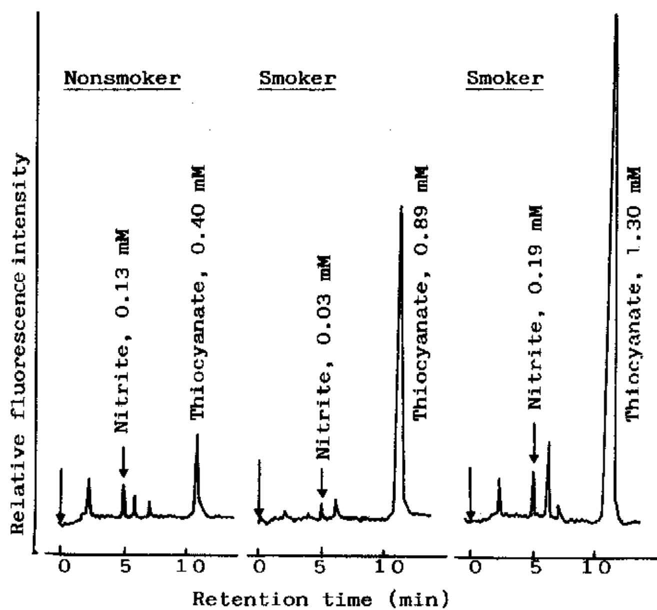

Fig. 5. Chromatograms of human saliva. Sample: 10 μl of ultrafiltered saliva.

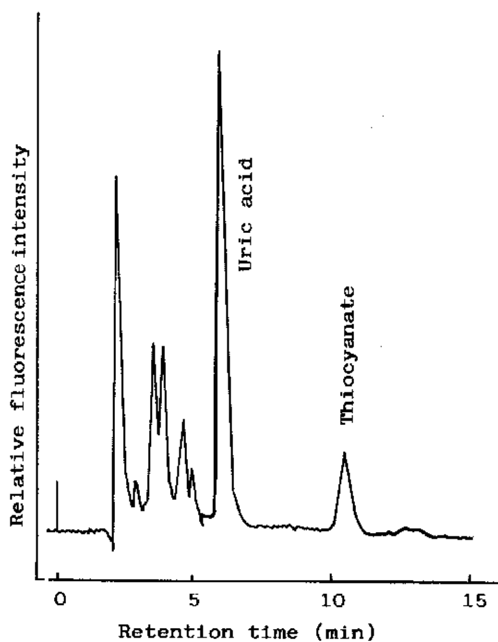

Fig. 6. Chromatogram of human serum. Sample: 50 μl of ultrafiltered serum.

TABLE I

DETERMINATION OF BOUND-FREE THIOCYANATE IN HUMAN SERUM

Subject No.	Concentration (μM)	
	Smoker	Non-smoker
1	50.4	19.2
2	46.4	12.4
3	65.6	12.0
4	38.4	10.4
5		8.4
6		13.2
Mean ± S.D.	50.2 ± 11.4	12.6 ± 3.3

smokers (0.13 mM) was observed, although the amount of the ion varied with the diet [26], as shown in Fig. 5. These results agreed with data previously reported [27,28].

Determination of bound–free thiocyanate in human serum

Fig. 6 shows the chromatogram for a 50-μl sample of ultrafiltered human serum. Only the thiocyanate peak was completely separated from the other peaks. According to Tanaka et al. [11] and Kawanishi et al. [13], the concentrations of nitrite and thiosulphate ion in normal human plasma are very low, in the ranges of 0.17–17.2 and 2.41–3.04 μM, respectively. It was difficult to detect clearly these levels of both ions using the present method.

Under physiological conditions serum thiocyanate is 40% protein-bound, exclusively to albumin [29]. The thiocyanate ion present in the filtrate of ultrafiltered serum, therefore, is of the free type. The recovery of thiocyanate was about 63% when 0.1 mM was added to serum. The within- and between-run C.V.s were 2.9 and 3.7% at the 0.48 nmol per 50μl level ($n=5$). Table I shows the concentrations of bound–free thiocyanate in the serum of smokers and non-smokers. The concentration of bound–free thiocyanate in the serum of smokers was higher than that in the serum of non-smokers, as also was the total thiocyanate concentration [1,2].

CONCLUSION

An HPLC method was developed with post-column cerium(IV) fluorescence detection for the determination of oxidizable inorganic anions (sulphide, thiocyanate, thiosulphate and nitrite). The method is applicable to the determination of nitrite and thiocyanate in saliva and thiocyanate in serum, although the detection system is inferior to ED in terms of sensitivity. As thiocyanate accelerates the endogenous synthesis of carcinogenic N-nitroso compounds [28,30], the measurement of the concentrations of salivary thiocyanate and nitrite is of importance in epidemiological studies. In addition, the method may serve for the

investigation of the pharmacokinetics of thiosulphate as a neutralizing agent for several antineoplastic drugs [31,32].

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