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Simultaneous determination of cyanide and thiocyanate in blood by ion chromatography with fluorescence and ultraviolet detection

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Abstract

An ion chromatographic method for the simultaneous determination of cyanide and thiocyanate in blood has been developed. After extraction by adding water and methanol to blood, cyanide was derivatized with 2,3-naphthalenedialdehyde and taurine to give a fluorescent product of 1-cyanobenz[*f*]isoindole. This compound was detected with high sensitivity by fluorometry and the underivatized thiocyanate was detected by ultraviolet absorption. The detection limits were 3.8 pmol ml^{-1} for cyanide and 86 pmol ml^{-1} for thiocyanate, and the recoveries from blood were ca. 83% and ca. 100%, respectively. The proposed method was successfully applied to the analysis of both anions in blood from smokers, non-smokers and fire victims.

Keywords: Cyanide; Thiocyanate

1. Introduction

The analysis of cyanide in biological fluids is of interest because cyanide acts not only as an acute toxicant, which binds to and inhibits the activity of cytochrome oxidase, but also as a chronic toxicant [1]. Cyanide is usually metabolized in vivo to thiocyanate by rhodanese, which is a mitochondrial enzyme in liver and kidney [2]. Therefore, an accurate and reliable method for simultaneous determination of cyanide and thiocyanate has been often desired for studies in forensic science and clinical medicine. However, no simple method for the simultaneous determination of these compounds has been developed yet because of the presence of many interfering substances in the blood matrix.

Numerous methods for determination of trace cyanide have been developed. In forensic science, the microdiffusion–spectrophotometric method has been adopted by using a Conway cell [3] and in recent years, this has become increasingly replaced by headspace gas chromatography (HS-GC) [4,5]. However, thiocyanate causes the artifactual formation of cyanide when blood is treated with phosphoric acid to release hydrogen cyanide. Sano et al. [6] developed a selective reversed-phase high-performance liquid chromatography (HPLC) method employing fluorescence derivatization with 2,3-naphthalenedialdehyde (NDA) and the primary amine, taurine, and applied it to the determination of cyanide in red cells from healthy persons [7]. We

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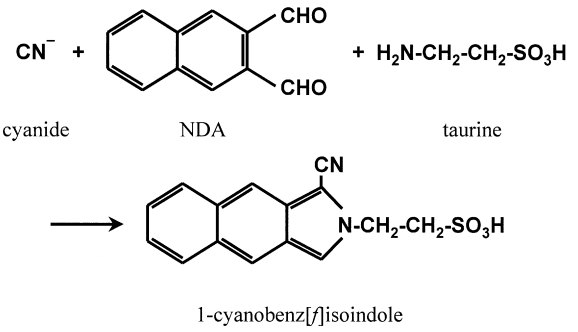

Fig. 1. Reaction of cyanide with NDA and taurine.

recently reported an improved method for the determination of cyanide and applied it to whole blood from healthy persons and fire victims [8]. In that study, we found that thiocyanate was never derivatized and could not be detected with fluorescence detection.

Several other HPLC methods have been developed for the determination of thiocyanate in biological fluids. These methods involve derivatizations based on the formation of a red complex with iron(III) [9], the König reaction [10] and the cerium(IV) redox reaction [11]. We have previously reported a simple ion chromatographic (IC) method with ultraviolet detection and have determined trace thiocyanate in human serum without the use of these derivatization operations [12].

Imanari and co-workers developed a simultaneous HPLC method using the König reaction for determination of cyanide and thiocyanate in human urine [10], and human plasma and red cells [13]. However, this method is rather complicated, because the sample must be pretreated differently to determine each anion and because of the need for two post-column reagents. Thus, a rapid method for the simultaneous determination of both anions that uses a single treatment would be preferable.

The goal of this work was to develop a simple, sensitive and precise IC method for the simultaneous determination of cyanide and thiocyanate in blood, employing both fluorescence and ultraviolet detection, which allows blood from healthy persons, smokers and fire victims to be analyzed directly after extraction followed by the fluorescence derivatization with NDA and taurine, as shown in Fig. 1.

2. Experimental

2.1. Reagents

NDA was obtained from Tokyo Kasei Kogyo (Tokyo, Japan) and methanol (Wako, Tokyo, Japan) was of HPLC grade. Taurine, potassium cyanide and potassium thiocyanate were of analytical reagent grade (Wako). All other reagents used were of analytical reagent grade. Deionized, distilled water was used for all procedures. NDA was dissolved in a small amount of methanol, and then diluted by 50 μmol ml^{-1} borate–100 μmol ml^{-1} phosphate buffer to give 2 μmol ml^{-1} solution. A 50 μmol ml^{-1} taurine solution was prepared by dissolving taurine in 50 μmol ml^{-1} borate–100 μmol ml^{-1} phosphate buffer. The stock standard solutions of cyanide and thiocyanate at 1 mg ml^{-1} were prepared with potassium cyanide in 1% sodium hydroxide and potassium thiocyanate in water, respectively. Working standard solutions were prepared by dilution of stock solutions with water.

2.2. IC apparatus and conditions

The IC system (Shimadzu, Kyoto, Japan) consisted of an LC-10AD pump, a DGU-3A on-line degasser, an SIL-10A autoinjector, an RF-10A fluorescence detector and an SPD-10AV ultraviolet detector, where the detectors were connected in tandem. The system was controlled by an FMV-5120D5 computer (Fujitsu, Tokyo, Japan), a CBM-10A communications bus module and CLASS-LC10 software (Shimadzu). Cyanide derivative was detected by fluorescent detection (418 nm excitation, 460 nm emission), and thiocyanate was detected by ultraviolet detection (210 nm). A TSK-gel IC-Anion-SW anion-exchange column (50×4.6 mm I.D., Tosoh, Tokyo, Japan) was used as a separation column, and the eluent was 10 μmol ml^{-1} phosphate buffer (pH 6.1)–methanol (1:1, v/v). The column temperature was ambient and the flow-rate was 1.0 ml min^{-1}. Sample injection volume was 20 μl.

2.3. Blood samples

Blood samples of 13 healthy persons (six smokers and seven non-smokers) were taken by venipuncture into heparinized tubes on the day of analysis or the day before. The samples were stored in a refrigerator at 4°C. Blood samples of 17 deceased fire victims and one suicide who ingested potassium cyanide were taken from the heart into heparinized tubes at the autopsy, and these were stored at −80°C. Carboxyhemoglobin in the blood of fire victims was measured by a CO oximeter at the autopsy.

2.4. Pretreatment of blood samples and derivatization procedure

To a 0.1-ml aliquot of blood, 0.5 ml of water was added followed by addition of 2 ml of methanol. After vortex-mixing, the mixture was centrifuged at 1600 *g* for 10 min. The derivatization procedure was as follows: a 0.5-ml aliquot of the supernatant or standard solution was placed in a 1.5-ml sample tube, and 0.1 ml each of 2 μmol ml^{-1} NDA and 50 μmol ml^{-1} taurine solution were added. After standing for 30 min at room temperature, a 20-μl aliquot of the mixture was injected into the IC system.

2.5. HS-GC for the determination of cyanide in blood

The HS-GC instrument consisted of a HP5890 SERIES II gas chromatograph with nitrogen–phosphorus detector (Hewlett-Packard, USA), GS-Q capillary column (30 m×0.53 mm I.D., J&W Scientific, USA) and HP ChemStation analysis system (Hewlett-Packard). Temperatures of the injection and the detector were 200°C and 250°C, respectively. The initial temperature of the column oven was 110°C (4 min constant) and the final was 160°C (10°C min^{-1}). HS equilibrium was performed in a screw cap septum vial (8 ml), to which was added 0.5 ml of blood sample and 0.3 ml of water, and then the vial was sealed. A 0.2-ml volume of 50% phosphoric acid was added through the septum using a syringe. The mixture was allowed to stand at 50°C for 30 min, and 0.5 ml of the gas phase in the sealed vial was injected into the GC system using a syringe [4,5].

3. Results and discussion

3.1. Separation and detection of cyanide derivative and thiocyanate

It was previously shown that the derivatization reaction with NDA and taurine is specific for cyanide, and that thiocyanate is not derivatized [8]. This indicates that both the cyanide derivative and thiocyanate exist as anions in the reaction mixtures and could therefore be simultaneously determined by anion chromatography. A previous paper reported the determination of thiocyanate in serum by IC using phosphate eluent (4 μmol ml^{-1} potassium dihydrogenphosphate–2 μmol ml^{-1} dipotassium hydrogenphosphate) and ultraviolet detection [12]. But the NDA–taurine–cyanide derivative (1-cyanobenz[*f*]isoindole) does not dissolve in the above phosphate eluent and has little ultraviolet absorption. Use of a phosphate buffer–methanol solution as the eluent could achieve good resolution for both anions.

Phosphate and methanol concentrations, pH and detection wavelength were varied to find the optimum conditions for the simultaneous determination of both anions in blood. The selected IC conditions were 10 μmol ml^{-1} phosphate buffer (pH 6.1)–50% methanol as eluent, 418 nm excitation and 460 nm emission for fluorescence detection, and 210 nm for ultraviolet detection. The fluorescence and ultraviolet chromatograms of a standard mixture of 38 pmol ml^{-1} cyanide and 1722 pmol ml^{-1} of thiocyanate are shown in Fig. 2.

The calibration graphs were linear in the range 3.8–7690 pmol ml^{-1} of cyanide and 0.17–172 nmol ml^{-1} of thiocyanate. In the analysis of cyanide standard solutions, the reagent blank showed a minor peak of cyanide corresponding to ca. 1.5 pmol ml^{-1}, which was the same as reported in our previous paper [8]. Thus, the lower limit for cyanide standard solution was estimated to be 3.8 pmol ml^{-1}, which was the concentration that gave a peak height 2.5-times that of the reagent blank peak. The detection limit of thiocyanate was 86 pmol ml^{-1}, which was

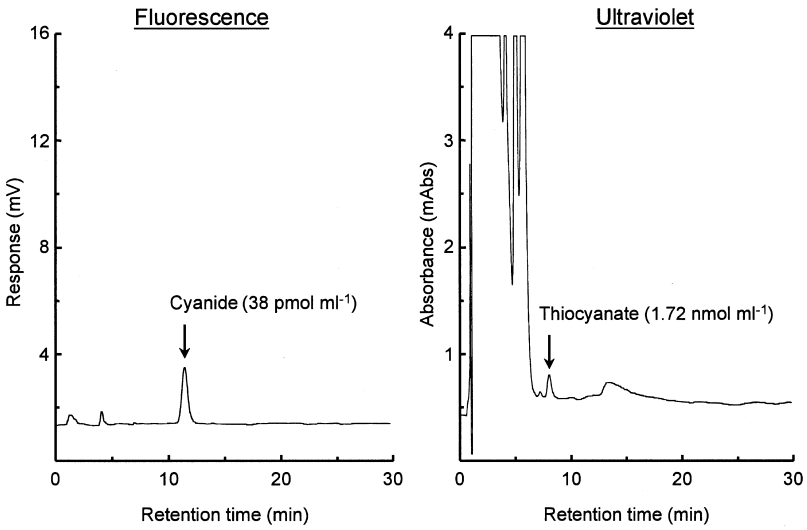

Fig. 2. Chromatograms of the standard solution. Eluent: 10 μmol ml^{-1} phosphate buffer (pH 6.1)–50% methanol. Flow-rate: 1.0 ml min^{-1}. Detection wavelength: 418 nm (excitation) 460 nm (emission) for fluorescent detection, 210 nm for UV detection. Sample injection volume: 20 μl.

defined as the concentration that produced a signal equal to 3-times the background noise level.

3.2. Recovery in blood

The recoveries of cyanide and thiocyanate were determined by spiking blood samples from a healthy subject with known concentrations of these compounds. As shown in Table 1, the recovery of cyanide was ca. 83% with standard deviations (S.D.s) in the range 0.9–1.2%. The recovery of thiocyanate was ca. 100% with S.D.s in the range 3.9–5.1%. Other methods that use acidification to liberate cyanide from methemoglobin obtained more than 90% recoveries of cyanide from whole blood [13–15]. However, when the acidification is used to liberate cyanide, the cyanide is liberated as hydrogen cyanide, which is a gas, and thus must be collected in an alkaline liquid phase for IC method. Therefore, the determination of both anions needs at least two measurements. The proposed method needs only a single pretreatment of blood, and shows constant recovery and a good S.D. Therefore, it is sufficient for practical analysis.

Table 1
Recovery of cyanide and thiocyanate from blood

	Spiked (nmol ml^{-1})	Found, mean±S.D.[a] (nmol ml^{-1})	Recovery, mean±S.D.[a] (%)
Cyanide	–	0.46±0.01	–
	3.84	3.69±0.05	84.1±1.2
	38.43	32.75±0.34	84.0±0.9
	115.30	94.93±1.29	81.9±1.1
Thiocyanate	–	14.8±1.1	–
	17.2	31.6±0.9	97.6±5.1
	172.2	189.0±6.7	101.2±3.9

[a] S.D.=Standard deviation (n=5).

3.3. Determination of cyanide and thiocyanate in blood from smokers and non-smokers

Blood samples from 13 healthy persons, consisting of six smokers and seven non-smokers, were analyzed. Typical chromatograms are shown in Fig. 3A and Fig. 3B. The chromatogram using fluorescence detection (Fig. 3A) shows that the cyanide peak (retention time 11.2 min) is free from interference. The chromatogram using ultraviolet detection (Fig. 3B) shows that the thiocyanate peak (7.8 min) suffers a little interference from the other anions in the blood. The results are summarized in Table 2. The concentrations of cyanide in blood were not very different between the smokers and the non-smokers. However, thiocyanate concentrations in the blood of smokers (mean, 55.8 nmol ml^{-1}) were clearly higher than they were in the blood of non-smokers (mean, 10.6 nmol ml^{-1}). The blood samples from smokers were taken 30–60 min after smoking, and the results indicate that the cyanide from tobacco smoke is immediately metabolized to thiocyanate in vivo [16].

3.4. Determination of cyanide and thiocyanate in blood from fire victims and a suicide

The subjects included 17 fire victims and one suicide who ingested potassium cyanide. Typical fluorescence and ultraviolet chromatograms of the blood of fire victims are shown in Fig. 3C and Fig. 3D. The results are summarized in Table 3. For fire victims, the concentration of carboxyhemoglobin (CO-Hb) in blood, which is one of the indicators of death by fire, is also shown. The cyanide con-

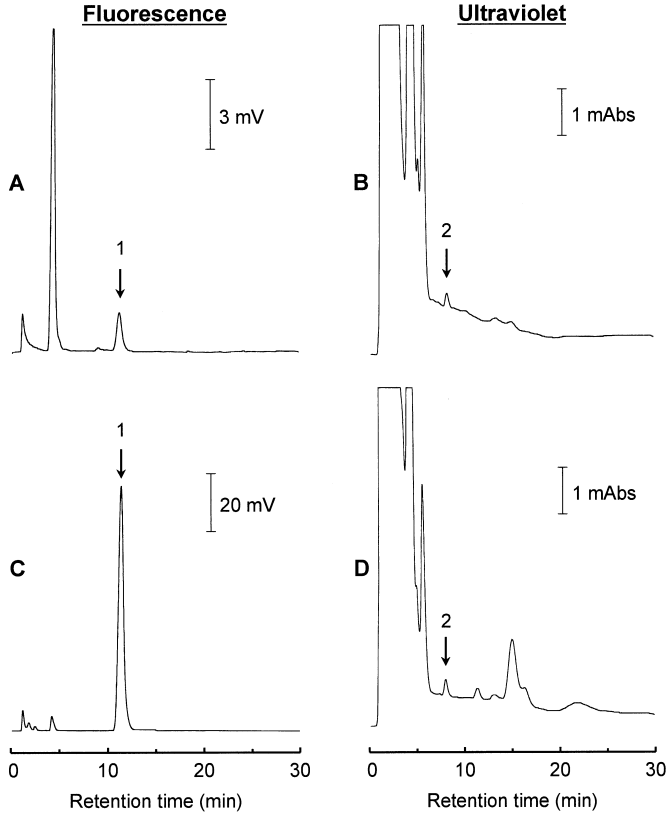

Fig. 3. Chromatograms of blood from (A, B) a healthy person (containing 638 pmol ml^{-1} cyanide and 62.6 nmol ml^{-1} thiocyanate) and (C, D) a fire victim (containing 42.5 nmol ml^{-1} cyanide and 74.6 nmol ml^{-1} thiocyanate). (1) Peak from the cyanide derivative; (2) peak from thiocyanate. The chromatograms in (A and B), and in (C and D), were obtained simultaneously. Chromatographic conditions as in Fig. 2.

Table 2
Concentrations of cyanide and thiocyanate in blood from healthy smokers and non-smokers

Sample	Smoker (S) or non-smoker (N)	Cyanide (pmol ml^{-1})	Thiocyanate (nmol ml^{-1})
1	S	618	62.5
2	S	686	55.3
3	S	509	60.1
4	S	591	96.1
5	S	530	28.6
6	S	650	32.2
7	N	434	6.2
8	N	537	5.2
9	N	525	11.9
10	N	510	3.6
11	N	550	15.9
12	N	547	15.0
13	N	525	16.0
Mean±S.D.[a]	S	597±62	55.8±22.3
	N	518±37	10.6±5.0

[a] S.D.=Standard deviation.

centrations in blood samples were also determined by the HS-GC method, and were found to be strongly correlated with the values obtained with the present method (r^2=0.978).

There was no clear correlation between cyanide and thiocyanate in blood from the fire victims, which is in agreement with the results of Anderson and Harland [17] and Toida et al. [18]. This is probably due to the fact that most persons who die in fires die in, or soon after, the fire, leaving little or no time for the cyanide to be metabolized to thiocyanate before death. Thus, the concentrations of these compounds in the blood of fire victims were mainly affected by their smoking and eating habits and by other environmental factors before death. Concentrations were also strongly affected by the particular conditions of a fire.

Table 3
Concentrations of cyanide and thiocyanate in blood from fire victims and a suicide

Sample	Cyanide (nmol ml^{-1})	Thiocyanate (nmol ml^{-1})	CO-Hb (%)
1	121.75	132.6	75
2	3.49	55.4	67
3	1.73	27.6	75
4	3.92	64.1	42
5	15.91	142.6	63
6	39.39	52.2	60
7	40.97	44.2	76
8	49.69	126.7	50
9	49.96	237.5	88
10	28.75	225.8	94
11	25.71	64.8	2
12	0.78	8.8	1
13	3.87	22.8	10
14	81.35	27.2	80
15	40.38	66.5	12
16	42.51	74.6	74
17	26.74	60.1	7
18[a]	852.30	112.1	–

[a] Suicide who ingested potassium cyanide.

Recently, an alternative metabolic pathway of cyanide based on its condensation with cystine or mercaptoalbumin has been reported [19,20]. According to these studies, when the exposure rate exceeds the cyanide-to-thiocyanate conversion rate, higher amounts of other cyanide metabolites, such as 2-aminothiazoline-4-carboxylic acid, are formed. Therefore, these other pathways might be important factors in the cause of death from fire.

The proposed method thus provides a rapid and sensitive method for the simultaneous determination of cyanide and thiocyanate in blood by a simple pretreatment. The method eliminates the acidification step and depresses the artifactual formation of cyanide from large amounts of thiocyanate. The method should be useful for forensic science and clinical medicine.

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