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DETERMINATION OF CYANIDE AND THIOCYANATE IN BLOOD PLASMA AND RED CELLS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUOROMETRIC DETECTION

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SUMMARY

A method for the determination of cyanide and thiocyanate in blood plasma and red cells of humans was established. It involved high-performance liquid chromatography and fluorometric detection by the Kŏnig reaction. Calibration curves for cyanide and thiocyanate were linear in the range 1—200 pmol and 2—300 pmol, respectively. Clean-up methods for the determination of cyanide and thiocyanate in red cells were also developed. These methods were applied for the determination of cyanide and thiocyanate in the blood of smokers and non-smokers.

INTRODUCTION

Much is now known about the metabolism of cyanide in normal humans. Cyanide is converted to thiocyanate by an irreversible reaction catalyzed by rhodanese (EC 2.8.1.1), which is a mitochondrial enzyme in liver and kidney [1, 2]. On the other hand, several workers have demonstrated in vivo conversion of thiocyanate to cyanide by an erythrocytic enzyme, "thiocyanate oxidase", and suggested the physiological effects of its conversion [3—7]. Recently, Vesey and Wilson [8] have reported that thiocyanate oxidase activity was not observed in erythrocytes, and have pointed out that the earlier reported cyanide assays in erythrocytes were not suitable for the determination of cyanide because haemoglobin in erythrocytes oxidized thiocyanate chemically to give cyanide. However, the presence of thiocyanate oxidase in body fluids cannot be ruled out clinically, since a large dose of thiocyanate as a hypotensive agent caused cyanide poisoning [9]. Therefore, an accurate and reliable method for the determination of cyanide and thiocyanate in biological samples is desired.

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A large number of reports have been published for the determination of cyanide and thiocyanate in biological fluids. A usual method for the determination of cyanide in blood plasma and erythrocytes is the pyridine–pyrazolone method developed by Epstein [10] coupled with a microdiffusion method using a Conway cell [11] or Cavett flask [12]. For the determination of thiocyanate in blood plasma, the method of Boxer and Rickards [13] has been widely used. In the assay, thiocyanate is first oxidized to cyanide under mild acidic conditions and the hydrogen cyanide liberated is then absorbed to alkali by aeration; the cyanide is determined colorimetrically using the pyridine–pyrazolone method.

We have already reported a method for the simultaneous determination of cyanide and thiocyanate using high-performance liquid chromatography (HPLC) and colorimetric detection by the König reaction [14]. Recently, we found that the reaction products formed from cyanide and thiocyanate by the König reaction have an intense fluorescence [15]. A few picomoles of both anions can be determined by this reaction.

In the present work, we devised a highly sensitive method for the determination of cyanide and thiocyanate by HPLC employing this fluorescence reaction, as well as the development of a pretreatment method for the determination of both anions in human plasma and red cells.

EXPERIMENTAL

Reagents

A standard solution of cyanide (0.1 *M*) was prepared by dissolving potassium cyanide (Wako Pure Chemicals, Osaka, Japan) in 0.1 *M* sodium hydroxide; the concentration of cyanide was calibrated by titration with silver nitrate according to the Liebig-Dénigés method [16]. A standard solution of thiocyanate (Wako) was prepared with redistilled water. Other chemicals were of analytical reagent grade.

Chromatographic conditions

Fig. 1 is a flow diagram of the HPLC system, which consists of reciprocating pumps (PSU-2.5, Seishin Seiyaku Co., Tokyo, Japan), a variable-wavelength fluoromonitor (RF-530, Shimadzu Seisakusho Co., Kyoto, Japan) with a xenon lamp and 12-μl flow cell, a variable-input recorder (SS-250F, Sekonic Co., Tokyo, Japan) and a sample injector (VMW-6, Seishin Seiyaku Co., Tokyo, Japan). HPLC conditions were as follows: column, a strong base anion-exchange resin, TSK Gel LS-222 (6 μm, 100 mm × 3 mm I.D., Toyo Soda Co., Tokyo, Japan); eluent, 0.1 *M* sodium acetate buffer (pH 5.0) containing 0.2 *M* sodium perchlorate (flow-rate, 0.5 ml/min); chlorination reagent, 0.1% chloramine T aqueous solution (flow-rate, 0.1 ml/min); pyridine–barbituric acid reagent, a mixture of barbituric acid (1.5 g), pyridine (15 ml), concentrated hydrochloric acid (3 ml) and redistilled water (82 ml) (flow-rate, 0.1 ml/min); excitation and emission wavelengths of the detector were set at 583 and 607 nm, respectively.

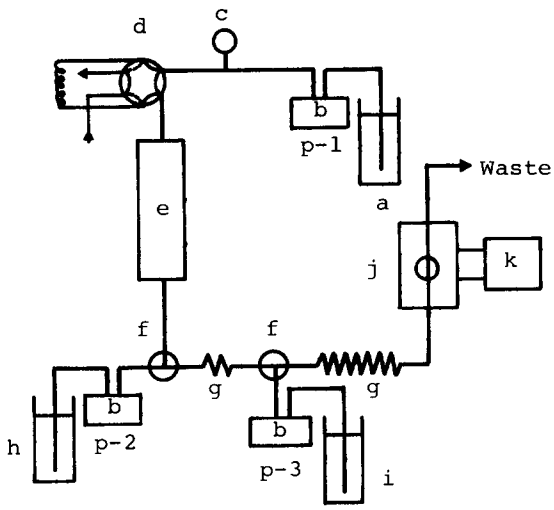

Fig. 1. Schematic diagram of HPLC: a = eluent (p-1), b = pump, c = pressure gauge, d = sample injector, e = analytical column, f = mixing joint, g = mixing coil, h = chloramine T reagent (p-2), i = pyridine—barbituric acid reagent (p-3), j = fluorescence spectromonitor, k = recorder.

Determination of cyanide in red cells

Isolation of red cells. Fresh heparinized blood, obtained by the standard techniques, was used. For the determination of total cyanide in red cells, the heparinized blood was centrifuged at 1500 *g* for 20 min and the plasma and buffy coat were removed with a micropipette. The packed red cell volume (haematocrit) was determined using a haemocytometer. Red cells were washed four times with 0.9% sodium chloride and centrifuged at 1500 *g* for 20 min.

Determination of cyanide. Portions of red cells (0.2 or 0.4 ml) were placed in the outer well of the Conway cell and 1.0 ml of 0.1 *M* sodium hydroxide was placed in the center chamber. The Conway cell and ground-glass cover were coated with silicone grease, and a glass cover was placed on top of the microdiffusion cell, leaving a small space for the addition of a hemolyzing agent. To the samples described above for the determination of total cyanide 1.0 ml of 20% ascorbic acid was added, and to those for the determination of stable cyanide 1.0 ml of redistilled water was added. After about 1 h, 1.5 ml of 10% sulphuric acid were added to the outer chamber. Subsequently, the ground-glass cover was moved to seal the microdiffusion cell. These cells were rotated carefully to mix the solutions in the outer chamber. The cells were rotated every 30 min. Cyanide in the sample was allowed to diffuse for 4 h at room temperature and the hydrogen cyanide liberated was absorbed into the sodium hydroxide solution in the center chamber. An aliquot in the center chamber solution was applied to HPLC.

Determination of thiocyanate in red cells

An appropriate volume of red cells prepared for total cyanide assay was added to 0.3 *M* phosphate buffer (pH 8.0) containing 0.2 *M* sodium perchlorate. The mixture was agitated once and maintained for 30 min at room temperature. Then the red cell suspension was filtered with an Amicon

Centriflo ultrafiltration membrane cone (Amicon, Lexington, MA, U.S.A.) by centrifuging at 1000 *g* for 20 min. The filtrate was submitted to HPLC.

Determination of thiocyanate in blood plasma

A 100 μl volume of blood plasma was mixed with 500 μl of 10% perchloric acid in a microtube. After 10 min, the suspension was centrifuged at 8000 *g* for 5 min. The supernatant was injected for HPLC.

RESULTS AND DISCUSSION

Detection and HPLC separation of cyanide and thiocyanate

In order to find the optimum detection conditions, we examined the reaction time (reaction coil length) and the concentration of chloramine T and pyridine–barbituric acid reagents by using a flow injection system consisting of the HPLC apparatus (Fig. 1) with the separation column removed. In this series of studies, 2.5 μ*M* cyanide and thiocyanate aqueous solutions were submitted to the test sample and the pH of the eluent was adjusted to 5.0 with acetate buffer as reported previously [14, 15].

The effect of the concentration of chloramine T reagent on response for cyanide and thiocyanate is shown in Fig. 2. The peak heights remained constant between the concentrations of 0.08% and 0.3%. In this range, the time required for chlorination was within about 1 min. The concentration of chloramine T reagent, therefore, was fixed at 0.1%.

Various concentrations of pyridine—barbituric acid reagent containing 3 ml of concentrated hydrochloric acid in 100 ml of reagent solution were prepared and their effects on response for cyanide were examined (Fig. 3). The optimum

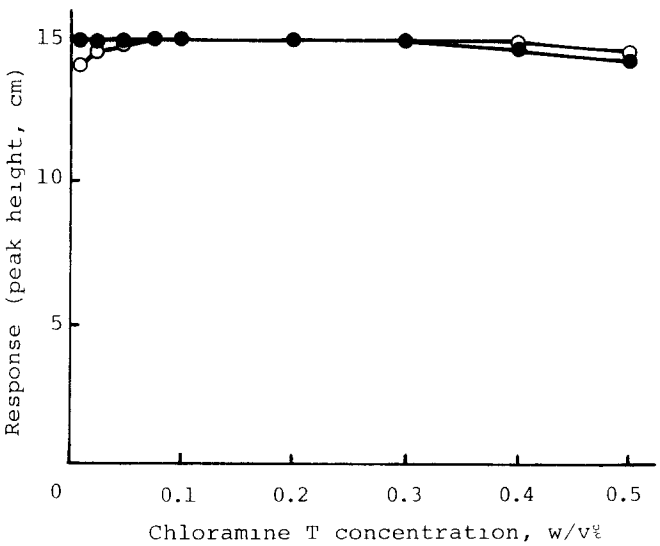

Fig. 2. Effect of chloramine T concentrations on fluorescence intensity. Conditions of flow injection system (FIA): p-1, 0.1 *M* acetate buffer (pH 5.0); p-2, chloramine T; p-3, 15% (v/v) pyridine—1.5% (w/v) barbituric acid containing 3 ml of concentrated hydrochloric acid in 100 ml, sample volume, 20 μl. Flow-rates and detection wavelength are given under Experimental. (○), Thiocyanate (50 pmol); (●), cyanide (50 pmol).

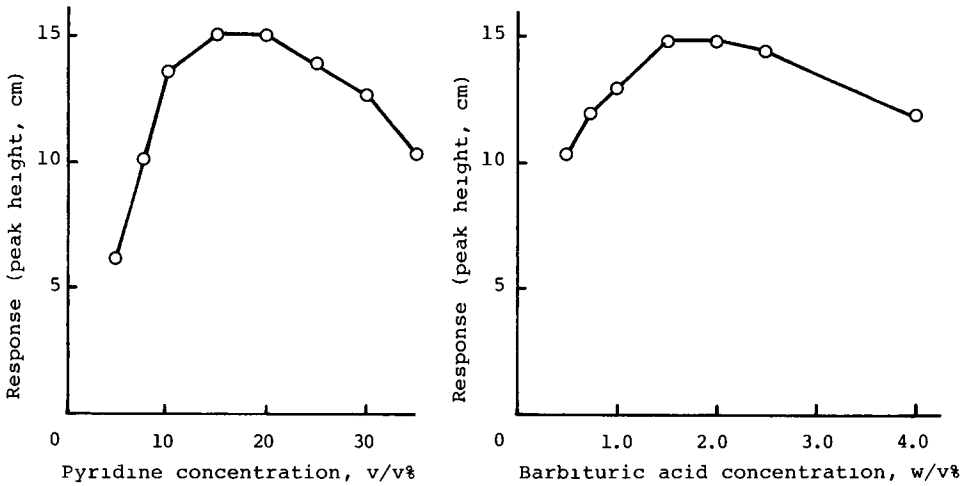

Fig. 3. Effect of pyridine and barbituric acid concentrations on fluorescence intensity. FIA conditions: p-2, 0.1% (w/v) chloramine T; p-3, pyridine—1.5% (w/v) barbituric acid (left) and 15% (v/v) pyridine—barbituric acid (right) containing 3 ml of concentrated hydrochloric acid in 100 ml. Other conditions as in Fig. 2. (○), Cyanide (50 pmol).

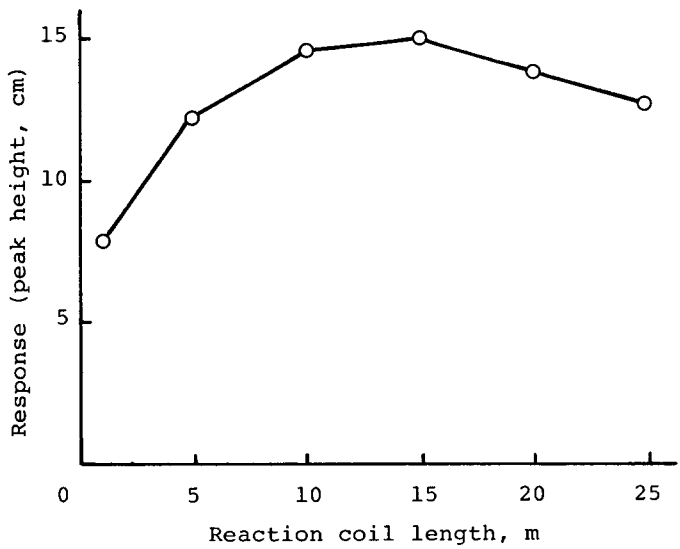

Fig. 4. Effect of reaction coil length on fluorescence intensity. FIA conditions: p-3, 15% (v/v) pyridine—1.5% (w/v) barbituric acid containing 3 ml of concentrated hydrochloric acid in 100 ml. Other conditions as in Fig. 2. (○), Cyanide (50 pmol).

concentrations of the components of pyridine—barbituric acid reagent were estimated at 15% (v/v) and 1.5% (w/v), respectively, as described in Experimental.

The effect of the reaction time on response for cyanide is shown in Fig. 4. Reaction time means the period for the reaction with barbituric acid and glutaconic aldehyde formed from cyanogen chloride and pyridine in the reaction coil. It is well known that the reaction product decomposes rapidly

[17]. In the flow system, however, reproducible peak heights proportional to the concentrations of cyanide and thiocyanate were obtained with a reaction coil of 15 m × 0.5 mm I.D.

The above data were almost the same as those obtained using HPLC with colorimetric detection [14].

For the separation of cyanide and thiocyanate, we used the same conditions as those described in the earlier report [14], except that the separation column length was changed from 15 to 10 cm. Under these chromatographic conditions, cyanide and thiocyanate were separated at the respective retention times of 6 and 9 min (Fig. 5), and were determined in the range 1—200 pmol and 2—300 pmol, respectively, in a sample size of 20 μl. The limit of detection for each anion was 0.4 pmol.

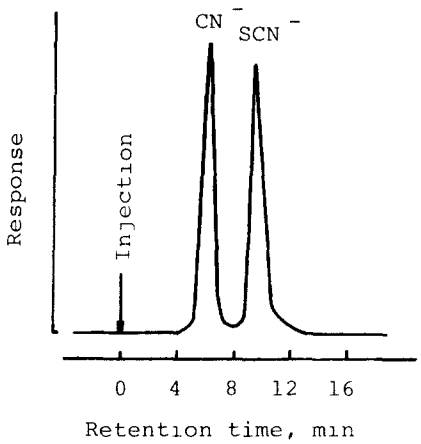

Fig. 5. Typical chromatogram of cyanide and thiocyanate (10 pmol of each). HPLC conditions are given under Experimental.

Regeneration of the separation column

When a sample containing heavy metal ions was submitted to the column, the chromatograms of cyanide and thiocyanate were not reproducible because the heavy metal ions were adsorbed on the column. In this case, the column was regenerated with 40 ml of 0.1 *M* sodium hydroxide solution containing 1% EDTA, followed by about 40 ml of eluent.

Cyanide in blood plasma and red cells

The normal molar ratio of thiocyanate to cyanide in blood plasma is more than 50:1. Furthermore, when cyanide is added to blood plasma in vitro, and left at room temperature, it survives only briefly (Fig. 6). Since a concentration step was involved for the determination of cyanide in blood plasma reported earlier [3—7], this step must be treated with some caution as suggested by Pettigrew and Fell [18]. On the other hand, Vesey and Wilson [8] suggested that the amount of cyanide released on acidifying the whole blood was much greater than the total amount from the blood plasma and erythrocytes assayed separately. The value also varied with blood plasma thiocyanate concentrations. This suggests that this artifactual release of cyanide from the whole blood was

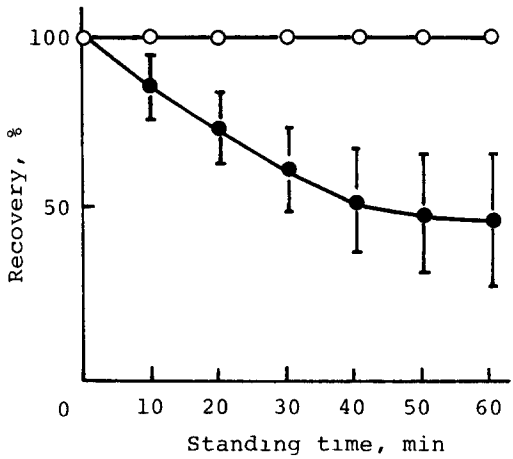

Fig. 6. Disappearance of cyanide added to plasma. Conditions: 1.0 μM cyanide is added to plasma (•) and saline (○). The mixtures are left to stand at room temperature in the outer wells of the Conway cells; then sulphuric acid is added to each sample. Other conditions are given under Experimental.

due to the oxidation of thiocyanate by methaemoglobin under acidic conditions.

Table I indicates the effect of ascorbic acid as the reducing reagent for the determination of cyanide in red cells containing thiocyanate by the microdiffusion method. Ascorbic acid effectively prevented artifactual formation of cyanide. These data were obtained from the determination of the total cyanide in red cells, where thiocyanate was not washed out by saline. The same result was obtained for the determination of stable cyanide in red cells, where thiocyanate and the free cyanide were washed out by saline as suggested by Vesey and Wilson [8]. The procedure for the determination of the total cyanide was as described in Experimental.

TABLE I

EFFECT OF ASCORBIC ACID ON CYANIDE* FORMATION IN THE PRESENCE OF RED CELLS AND THIOCYANATE

Agent	Thiocyanate added (μM)	Cyanide added (μM)	Cyanide recovery (%)
None	0	1.0	98.9
	200	1.0	398.0
Ascorbic acid**	0	1.0	100.6
	200	1.0	102.8

*Measured as total cyanide (see Experimental).
**20% Aqueous solution.

Thiocyanate in blood plasma and red cells

Methods for the determination of thiocyanate in red cells have not been reported, except Lang's [1] method. He has obtained approximately

quantitative recoveries of thiocyanate from whole blood using trichloroacetic acid as the deproteinizing reagent. But it was difficult to obtain quantitative and reproducible recoveries for thiocyanate by the method of Lang. Consequently, the new pretreatment method of red cells for the determination of thiocyanate was developed.

Blood plasma (100 μl) was deproteinized with 10% perchloric acid (400 μl) and centrifuged. Portions (20 μl) of the supernatant were then analyzed for thiocyanate by HPLC. The specificity of this deproteinization procedure was checked by a series of recovery tests, by adding various concentrations of potassium thiocyanate standard solution to blood plasma.

It was noted that thiocyanate in red cells was completely washed out by saline. So, the effective extraction of thiocyanate from red cells was accomplished by the use of phosphate buffer, pH 8.0, containing 0.3 *M* sodium perchlorate. Table II shows the results of the recovery tests for the determination of thiocyanate in blood plasma and red cells.

TABLE II

RECOVERY TESTS OF THIOCYANATE FROM BLOOD PLASMA AND RED CELLS

	Amount added (μM)	Recovery (%) (mean ± S.D.)*
Plasma	5 0	98.3 ± 2.1
	50.0	97.6 ± 3.3
Red cells	2 5	101.5 ± 1.4
	10.0	100.2 ± 0.7

*Mean of six analyses.

TABLE III

DETERMINATION OF CYANIDE AND THIOCYANATE IN HUMAN BLOOD SAMPLES

Subjects	Red cells			Plasma
	Total cyanide (μM)	Stable cyanide (μM)	Thiocyanate (μM)	Thiocyanate (μM)
1	0 15	0.15	10.6	13.3
2*	0 42	0.40	80 8	82.8
3*	0 44	0.42	84.7	90.0
4*	0 27	0.27	8.0	12.0
5	0 15	0 15	12.4	17 5
6	0 13	0 13	19.3	28.6
7*	0.27	0.25	34 7	44 4

*Cigarette smoker

Application

The results for the determination of cyanide and thiocyanate in blood plasma and red cells are shown in Table III. Blood plasma thiocyanate concentrations in smokers were much higher than in non-smokers, and cyanide

concentrations in red cells of smokers tended to be higher but the difference was not significant. A marked difference between the total cyanide and the stable cyanide in red cells was neither observed. From these data, it is suggested that most cyanide in red cells exists as a "stable cyanide" and may have no toxic effects in vivo.

CONCLUSION

A new fluorometric method for the specific and sensitive determination of cyanide and thiocyanate based on the König reaction was developed using HPLC. The sensitivity of this sytem was more than one order of magnitude better than that obtained by the earlier colorimetric method using HPLC [15]. Anions present in environmental and biological materials mostly did not affect the chromatogram of cyanide and thiocyanate. It seems that our pretreatment methods are effective for the accurate determination of cyanide in red cells in the presence of large amounts of thiocyanate.

REFERENCES

1 K. Lang, Biochem. Z., 259 (1933) 243.
2 R.G. Baumeister, H. Schievelvein and G. Zickgraf-Rüdel, Arzneim.-Forsch., 25 (1975) 4056.
3 F. Goldstein and F. Reiders, Amer. J. Physiol., 167 (1951) 47.
4 J. Chung and J.L. Wood, J. Biol. Chem., 246 (1971) 555.
5 J.D. Oram and B. Reister, Biochem. J., 100 (1966) 373.
6 B. Sörbo and J.G. Llungren, Acta Chem. Scand., 12 (1958) 470.
7 F. Maloof and M.J. Soodak, J. Biol. Chem., 239 (1964) 1955.
8 C.J. Vesey and J. Wilson, J. Pharm. Pharmacol., 20 (1978) 20.
9 K.L. Pines and M.M. Crymble, Proc. Soc. Exp. Biol. Med., 81 (1952) 160.
10 J. Epstein, Anal. Chem., 19 (1947) 272.
11 R.L. Moran, G.E. Isom and J.L. Way, Toxicol. Appl. Pharmacol., 50 (1979) 323.
12 S. Baar, Analyst, 91 (1966) 268.
13 G.E. Boxer and J.C. Rickards, Arch. Biochem. Biophys., 39 (1952) 292.
14 T. Imanari, S. Tanabe and T. Toida, Chem. Pharm. Bull., 30 (1982) 3800.
15 T. Toida, S. Tanabe and T. Imanari, Chem. Pharm. Bull., 29 (1981) 3763.
16 W.J. Williams, Handbook of Anion Determination, Butterworths, London, 1979, p. 73.
17 G.V.L.N. Murty and T.S. Viswanathan, Anal. Chim. Acta, 25 (1961) 293.
18 A.R Pettigrew and G.S. Fell, Clin. Chem., 18 (1972) 996.