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Application of a new analytical method using gas chromatography and gas chromatography–mass spectrometry for the azide ion to human blood and urine samples of an actual case

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Abstract

We have established a practical and reliable method to identify and quantify the azide ion in human whole blood and human urine by transforming the ion into pentafluorobenzyl azide ($PFBN_3$). $PFBN_3$ was simply derived from a reaction of the ion with an excess amount of pentafluorobenzyl bromide (PFBBr). The excess amount of PFBBr was removed from the products by its reaction with sodium thiosulfate. $PFBN_3$ in the sample was detected in high sensitivity by gas chromatography with nitrogen–phosphorus detector (GC–NPD) and gas chromatography–mass spectrometry (GC–MS). The lower detection limits of the ion by GC–NPD were 5 ng/ml for human whole blood sample and 0.5 ng/ml for human urine sample at $S/N=3$. On the other hand, they were 100 ng/ml for human whole blood sample and 10 ng/ml for human urine sample by the full-scan mode of GC–MS. The analytical method was applied to identification and quantification of the ion in the actual whole blood and urine samples of the victims in an actual criminal case.

Keywords: Azide; Pentafluorobenzyl azide

1. Introduction

Sodium azide is a useful starting material of heavy metal azide, pure sodium metal, hydrazoic acid, a variety of medicines and a resource of nitrogen gas. Sodium azide is also used for introducing nitrogen functionalities into organic compounds, and often used as an antiseptic and a fungicide for buffer solution, diluting solution and some reagents in biochemistry and clinical examination.

Sodium azide is known as a toxic material [1–13], and must be handled with caution. However, a recent criminal case, which occurred in Japan, of misuse of sodium azide, urged us to establish a practical method of identifying and quantifying a trace amount of azide ion in the actual samples with utmost precision.

The analytical methods reported so far in such cases were volumetric [2,9], spectrophotometric [9], high-performance liquid chromatographic (HPLC) [11–13] and ion chromatographic (IC) methods [14]. The volumetric and spectrophotometric methods are unreliable from qualitative and quantitative points of view, especially in a low concentration of azide ion. The HPLC and IC methods are applicable to quanti-

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fication of a trace amount of the ion with relatively high precision.

On the other hand, more reliable analytical methods have been reported in which anions such as cyanide, thiocyanide or sulfide in biological samples are transformed by a reaction with pentafluorobenzyl bromide (PFBBr) into the corresponding pentafluorobenzyl derivatives. Next, the derivatives were identified and quantified with gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) [15–20]. Thus, we tried to apply the method to azide ion in biological samples. As a result, it was revealed that the azide ion was easily derivatized to pentafluorobenzyl azide ($PFBN_3$) in human whole blood and urine samples, and $PFBN_3$ was unambiguously identified using GC with nitrogen–phosphorus detection (NPD) and GC–MS. The resulting data were highly reproducible and reliable, even with an extremely low concentration of the ion. The method was successfully applied to the whole blood and urine samples of the victims in an actual criminal case, and was demonstrated to be a very useful method for forensic proof.

2. Experimental

2.1. Reagents

Sodium azide and pentafluoropropionic anhydride were purchased from Wako (Osaka, Japan). PFBBr and 2-(trifluoromethyl)benzylamine were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and Aldrich (Milwaukee, WI, USA), respectively. *N*-(2-Trifluoromethyl)benzylpentafluoropropamide was synthesized from (2-trifluoromethyl)benzylamine and pentafluoropropanoic anhydride at the author's laboratory, and the structure was confirmed by MS. Other used reagents and organic solvents were of analytical-reagent grade.

2.2. Preparation of standard solutions and samples

A stock standard solution of azide ion ($1.0 \cdot 10^7$ ng/ml) was prepared by dissolving sodium azide in distilled water. The working standard solutions of the final concentrations of 100 000, 10 000, 1000, 100 and 10 ng/ml were obtained by 10-, 100-, 1000-, 10 000- and 100 000-fold dilution of the standard solution with distilled water, respectively. A solution of internal standard (I.S.) was prepared by dissolving *N*-(2-trifluoromethyl)benzylpentafluoropropamide in *n*-hexane to give a concentration of 96.4 ng/ml (0.3 m*M*). The solutions of PFBBr used for derivatization were prepared by dissolving PFBBr in acetone to give the concentrations of 13.0 and 52.0 mg/ml (50 and 200 m*M*). Saturated solutions of sodium tetraborate and sodium thiosulfate were prepared by dissolving each in distilled water to a concentration of 79.1 mg/ml (0.5 *M*).

Spiked human whole blood and human urine samples (0.5–10 000 ng/ml) were prepared by adding the working standard solutions of azide ion to fresh human whole blood and human urine, which were collected from a healthy volunteer.

2.3. Synthesis of pentafluorobenzyl azide authentic sample

Since no procedure has been reported on the synthesis of $PFBN_3$, we have synthesized and identified it as follows:

A PFBBr (1.01 g, 3.87 mmol) solution in acetone (20 ml) and 3 ml of distilled water saturated with sodium tetraborate were added to a sodium azide (163.2 mg, 2.51 mmol) solution in distilled water (20 ml), and then the mixture was maintained at 60°C for 30 min. After cooling it down to room temperature, 10.0 ml of $7.9 \cdot 10^7$ ng/ml (0.5 *M*) sodium thiosulfate aqueous solution was added, and then the mixture was vigorously shaken for 1 min and maintained at 60°C for 5 min. After cooling it down to room temperature, the derivatives were extracted with *n*-hexane. The combined extracts were washed repeatedly with distilled water and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the crude product was distilled under reduced pressure (61–62°C/7.0 mmHg; 1 mmHg=133.322 Pa) to afford the product as colorless oil (399.0 mg, 71.3%). 400 MHz ^{1}H-nuclear magnetic resonance (NMR) (C^2HCl_3): δ 4.46 (s, 2H, $-CH_2-$). IR (KBr): 2112, 1522, 1507, 1126, 1038 and 933 cm^{-1}. High-resolution (HR) MS found: m/z 223.0115. Calculated for $C_7H_2F_5N_3$: M, 223.0169. MS m/z (relative intensity, %): 223 ($M^{+\cdot}$, 10), 194 (65), 181 (100)

and 117 (55). The ^{1}H-NMR spectra were recorded with an α-400 spectrometer (JEOL, Tokyo, Japan), using tetramethylsilane as an internal standard. The IR spectra and the MS spectra were taken on a Magna 550 series II spectrometer (Nicolet, Madison, WI, USA) and a MS-BU20 spectrometer (JEOL), respectively.

2.4. Derivatizing procedure

To human urine (2.0 ml) in a glass-stoppered test tube (10 ml) was added 0.5 ml of distilled water saturated with sodium tetraborate and 1.0 ml of $1.3 \cdot 10^7$ ng/ml (50 m*M*) PFBBr acetone solution. To human whole blood (0.2 ml) in a glass-stoppered test tube (10 ml) was added 0.2 ml of distilled water, 0.5 ml of distilled water saturated with sodium tetraborate and 0.5 ml of $5.2 \cdot 10^7$ ng/ml (200 m*M*) PFBBr acetone solution. The mixture was maintained at 50°C for 20 min. After cooling it down to room temperature, 1.0 ml of $7.9 \cdot 10^7$ ng/ml (0.5 *M*) sodium thiosulfate aqueous solution was added to the solution, and the mixture was vigorously shaken for 1 min and then maintained at 50°C for 2 min. After cooling it down to room temperature, NaCl (1.0 g) was added, followed by distilled water to give a total amount of 6.0 ml of the mixture, 20 μl of I.S. solution and 1.0 ml of *n*-hexane, and then the mixture was vigorously shaken. After centrifugation (2500 *g*, 3 min), the organic layer was separated and then washed with 1.0 ml of $1.8 \cdot 10^7$ ng/ml (0.5 *M*) hydrochloric acid. A 0.5-μl aliquot of the solution was subjected to GC–NPD determination (qualitative and quantitative analysis), while a 2.0-μl aliquot of the solution was subjected to GC–MS determination (qualitative analysis) in the full-scan mode.

2.5. GC conditions

A HP6890 gas chromatograph (Hewlett-Packard, Wilmington, DE, USA) equipped with a purged packed inlet and a nitrogen–phosphorus detector was used in combination with a HP-5 fused-silica capillary column (15 m×0.53 mm I.D., film thickness 1.5 μm, Hewlett-Packard). Helium was used as the carrier gas at a flow-rate 6.0 ml/min. The column temperature was programmed from 80°C to 230°C at 10°C/min. The temperatures of the injection port and detector were kept at 200 and 280°C, respectively.

2.6. GC–MS conditions

GC–MS analysis was performed on a HP6890 gas chromatograph (Hewlett-Packard) connected to a JMS-AMII15 (JEOL). A HP-5MS fused-silica capillary column (20 m×0.25 mm I.D., film thickness 0.25 μm, Hewlett-Packard) was used. Helium was used as the carrier gas at a flow-rate 1.0 ml/min (constant flow mode). A pulsed splitless injection mode was selected with pulse time of 1.0 min and purge time of 0.7 min. The initial temperature of the column was kept at 40°C for 1 min, and programmed to 90°C at 30°C/min, to 130°C at 10°C/min, and to 300°C at 30°C/min. The temperatures of the injection port, ion source and interface were kept at 250, 210 and 250°C, respectively. The ionization mode was electron impact (current 300 μA, energy 70 eV).

2.7. Preparation of calibration graphs

Spiked human whole blood and human urine samples were derivatized by the procedure described above. Calibration graphs were obtained by plotting the peak-area ratio of $PFBN_3$ to the I.S. versus the concentration of azide by using GC–NPD.

3. Results

3.1. Derivatization of azide ion in human urine and extraction of $PFBN_3$

3.1.1. Optimization of the derivatization

The derivatization of azide ion proceeded smoothly when a spiked human urine sample (1000 ng/ml, 2 ml) was first alkalinized by saturated sodium tetraborate solution, then acetone solution of PFBBr (1.0 ml) was added to the solution, and the reaction mixture was heated at 50°C. Subsequently we examined the reaction to find the optimal reaction time and concentration of PFBBr. The result are shown in Figs. 1 and 2. As can be seen in the figures, the optimal reaction time and concentration are 20 min and $1.3 \cdot 10^7$ ng/ml (50 m*M*), respectively.

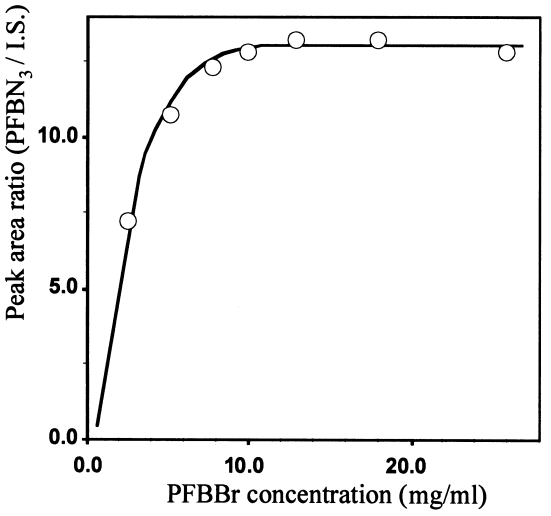

Fig. 1. Effect of PFBBr concentration on the formation of $PFBN_3$ at 50°C for 30 min.

The derivatizing agent PFBBr and the product $PFBN_3$ showed a similar retention time in the widely used nonpolar and slightly polar phase capillary GC columns. Therefore, it was necessary to remove any excess PFBBr from the sample for a precise analysis. We examined a method to remove PFBBr by using ammonia solution, several amines and a variety of anions and found that sodium thiosulfate was the most effective reagent. PFBBr was effectively transformed to the corresponding pentafluorobenzyl thiosulfate when sodium thiosulfate was added to the sample, and the reaction mixture was vigorously shaken and warmed for 1 min. Pentafluorobenzyl thiosulfate is soluble in water and was not extracted with organic solvent. Thus the analysis of $PFBN_3$ was performed with GC and GC–MS with high sensitivity by this treatment (Fig. 3).

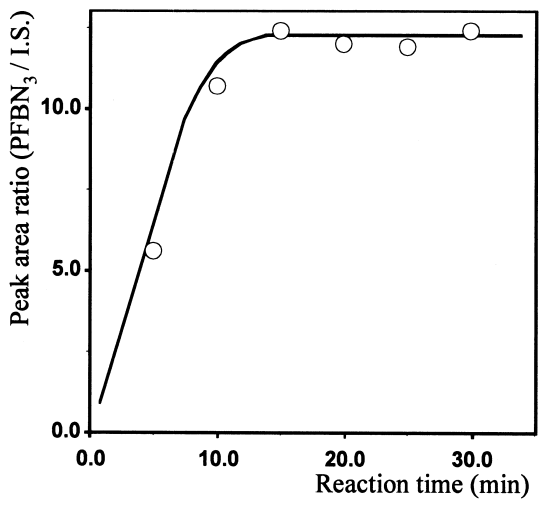

Fig. 2. Effect of reaction time on the formation of $PFBN_3$ at 50°C.

3.1.2. Solvent effect on the extraction of $PFBN_3$ from the reaction mixture

We examined the solvent effect to extract $PFBN_3$ with ethyl acetate, *n*-hexane, toluene and ether. It was found that in each case, more than 99% of $PFBN_3$ was recovered. Therefore, we chose *n*-hexane as the solvent, as it was easily handled and did not extract the by-products.

3.2. Derivatization of the azide ion in human whole blood

Derivatization for human whole blood was carried out in the same way as that for human urine. However, the reaction mixture hardened to gel, and the derivatization was incomplete. We therefore, tried to accelerate the reaction by reducing the amount of the human whole blood sample from 2 ml to 0.2 ml and adding the same amount of water to the sample and then using a PFBBr solution in acetone of higher concentration ($5.2 \cdot 10^7$ ng/ml) in order to reduce the amount of acetone, which resulted in a quantitative yield for derivatization as in the case of the human urine sample.

3.3. GC analysis

GC–NPD and GC with ^{63}Ni electron-capture detection (ECD) analyses were performed for the samples obtained by adding the azide ion to human whole blood and human urine, and then derivatizing the ion by the procedures described above. In the GC–ECD analysis, it was impossible to get any good chromatograms with a high S/N ratio because too many peaks, caused by by-products, were detected. On the contrary, the analysis by GC–NPD was performed at a high S/N ratio and high sensitivity without any significant peaks caused by the by-products, although the sensitivity of GC–NPD analy-

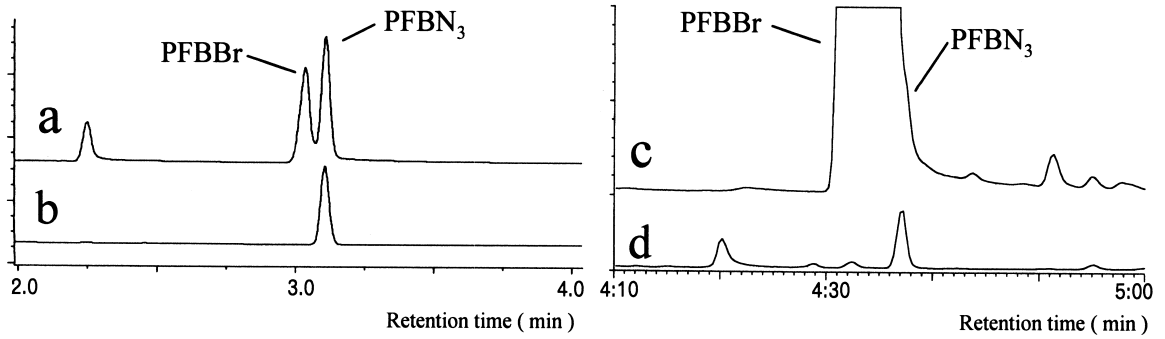

Fig. 3. Gas chromatograms (a and b) and total ion chromatograms (c and d) of the extracted products from the human urine sample spiked with azide ion at 1000 ng/ml before (a and c) and after (b and d) removing excess PFBBr by sodium thiosulfate.

sis was lower than that of ECD. Consequently, GC analysis was performed using NPD.

We tried to make a calibration curve for the samples of human whole blood and urine to which azide ion was added. The data showed a very good linearity with the concentration ranging from 10 to 10 000 ng/ml for human whole blood samples, from 1 to 10 000 ng/ml for human urine samples. The correlation coefficient was 0.999 in both cases. The lower limits of quantification were 10 ng/ml for human whole blood sample and 1 ng/ml for human urine sample. The lower limits of detection were 5 ng/ml for human whole blood sample and 0.5 ng/ml for human urine sample at $S/N=3$. The recoveries of azide ion by derivatization with PFBBr were greater than 90%. The relative standard deviations (RSDs) were 1.2~4.7% as shown in Tables 1 and 2.

3.4. GC–MS analysis

The mass spectrum of the derivatized product $PFBN_3$ is shown in Fig. 4. Molecular ion peak ($M^{+\cdot}$, m/z 223), fragment ion peaks of $[M–N_2H]^+$ at m/z 194 and $[M–N_3]^+$ at m/z 181 were observed in the spectrum, and the chart was the same as that of the standard sample of $PFBN_3$. There was no peak at the position corresponding to $PFBN_3$ in the total ion chromatograms of controls of human whole blood and urine. The lower detection limits of $PFBN_3$ by GC–MS are 100 ng/ml for human whole blood samples and 10 ng/ml for human urine samples. The detection limit was defined as the lowest concentration of the sample for which the same mass spectrum as that of the authentic sample is obtained.

3.5. Application

Recently, a suspect committed a crime at a company in Japan. The 10 victims, who took a small amount of green tea or coffee during a meeting, suffered from nausea, vomiting, headache, palpitations and sweating, which are typical symptoms caused by toxic substances. They were quickly hospitalized. We analyzed the water in the pot used to make the green tea and coffee. The water contained about $7\cdot10^6$ ng/ml (7 mg/ml) of sodium azide, and was deliberately contaminated by the

Table 1
Analytical recovery of azide added to human whole blood

Added concentration (ng/ml)	Recovery±SD ($n=5$) (%)	RSD (%)
10	92.5±4.2	4.5
100	92.8±2.8	3.0
1000	95.5±1.1	1.2
10 000	92.9±3.4	3.7

Table 2
Analytical recovery of azide ion added to human urine

Added concentration (ng/ml)	Recovery±SD ($n=5$) (%)	RSD (%)
1	93.3±4.4	4.7
10	90.6±3.4	3.8
100	92.8±1.3	1.4
1000	94.5±2.2	2.3
10 000	91.1±2.4	2.6

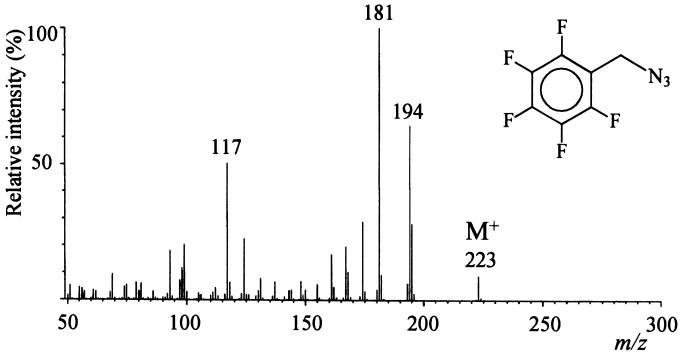

Fig. 4. Mass spectrum of the reaction product ($PFBN_3$).

suspect. We carried out the following qualitative and quantitative analysis: one was for the whole blood samples, which were collected from the victims in the hospital about 1 h after consuming the tainted beverage. The other was for the urine samples from the same victims about 1 h and 2.5 h after the victims drank the green tea or the coffee. The results are shown in Table 3. The gas chromatograms of the whole blood sample (No. 2 in Table 3, 35 ng/ml) and the urine sample (No. 9 in Table 3, 4 ng/ml) are shown together with that of the control in Fig. 5. As seen in Table 3, the analytical method established by us is very useful for identifying and determining the azide ion in the intoxication case.

4. Discussion

Among the analytical methods for azide ion reported so far [12–14,21–41], those applied to the analysis of the ion in the actual biological samples were volumetric [2,9], spectrophotometric [9], HPLC [11–13] and IC methods [14]. Kozlicka-Gajdzinska and Brzyski [2] have identified and quantified the ion in the gastrointestinal contents of a patient who took the ion by mistake by measuring the amount of nitrogen gas generated by oxidation of the ion. Klug and Schneider [9] identified and quantified the ion in the gastric–duodenum contents of the patient by the volumetric method. The ion in the blood was trans-

Table 3
Azide ion concentrations in whole blood and urine samples of victims[a]

Subject No.	Sex	Concentration (ng/ml)		
		Whole blood, about 1 h after drinking	Urine	
			About 1 h after drinking	About 2.5 h after drinking
1	Male	n.q.[b]	n.q.	107
2	Male	35	n.q.	206
3	Female	636	n.q.	52
4	Male	3734	575	265
5	Male	n.q.	n.q.	52
6	Male	n.q.	n.q.	107
7	Male	n.q.	n.q.	81
8	Male	n.q.	n.q.	
9	Male	490	4	
10	Male	1133	3	

[a] All victims are adults.
[b] Not quantified (the lower limit of quantification: whole blood; 10 ng/ml, urine; 1 ng/ml).

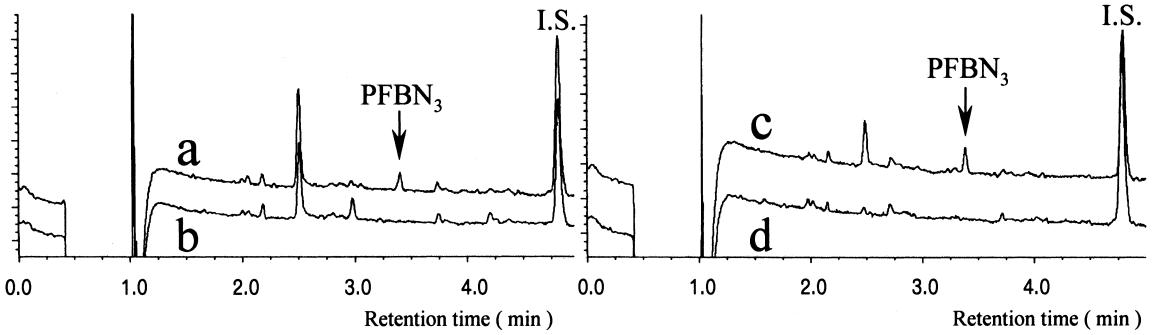

Fig. 5. Gas chromatograms of the extracted products after derivatization with PFBBr from (a) the whole blood sample of victim (subject No. 2 in Table 3, azide ion of 35 ng/ml concentration), (b) the blank human whole blood sample, (c) the human urine sample of victim (subject No. 9 in Table 3, azide ion of 4 ng/ml concentration) and (d) the blank human urine sample.

formed into hydrazoic acid in a Conway microdiffusion cell to absorb with alkaline solution. Ferric chloride solution was added to the solution, and the solution was analyzed by the spectrophotometric method. However, these methods are unreliable and inapplicable to samples that contain an extremely small amount of the ion.

Howard et al. [11] and Lambert et al. [12] identified and quantified the ion by detecting 3,5-dinitrobenzoyl azide which was obtained by the reaction of the ion with 3,5-dinitrobenzoyl chloride in urine [11], blood and internal organs [12] of the patients with the use of HPLC. Marquet et al. [13] also identified and quantified the ion in the blood and internal organs of the patient by a similar method to Howard et al.'s and Lambert et al's with the use of benzoyl chloride, instead of 3,5-dinitrobenzoyl chloride. Kruszyna et al. [14] identified and quantified the ion in spiked blood samples that were transformed into hydrazoic acid to absorb with alkaline solution, and then the solution was analyzed by IC. The lower limits of detection of these methods were 80, 200 and 30 ng/ml of sodium azide concentration, respectively. Therefore, these methods are more sensitive and reliable compared to volumetric and spectrophotometric methods. In contrast to the HPLC and IC methods, our method enabled us to identify the ion unambiguously as $PFBN_3$ by GC–MS with a higher degree of reliability and equal sensitivity and by GC–NPD with more sensitivity and equal reliability.

Analytical methods in which several anions in biological samples were transformed by a reaction with PFBBr into the corresponding pentafluorobenzyl derivatives and then the derivatives was identified and quantified with GC and GC–MS analyses have been reported [15–20].

Among them, the derivatizing method for cyanide, thiocyanide and sulfide ion [15,18] was basically established by Kage and co-workers. In their analytical procedures, the derivatization was carried out in the ethyl acetate–water biphase system using the phase transfer catalyst. The derivatized product, such as pentafluorobenzyl cyanide, was soluble in ethyl acetate, and the separated ethyl acetate layer contained the desired product. However, it was common to use a large excess amount of PFBBr to get a high recovery of the target ion, which resulted in contamination of PFBBr in the product. Since the ratio of the derivatized product ($PFBN_3$) to PFBBr was negligible in our case, it was necessary to remove PFBBr from the products when the $PFBN_3$ was analyzed with a widely used and ordinary column such as the nonpolar and slightly polar phase capillary GC column. We examined the method to remove the excess PFBBr after the derivatization, and found that it was best to react the PFBBr with sodium thiosulfate, followed by an extraction with an organic solvent such as *n*-hexane. Since only one anion site of thiosulfate was benzylated, the monobenzylated thiosulfate was not extracted into the *n*-hexane layer. The derivatization of the excess PFBBr with sodium thiosulfate, however, did not proceed smoothly in the ethyl acetate–water biphase system. Our derivatiza-

tion method in the acetone–water uniphase system solved the problem. The reactions of the azide ion with PFBBr and of PFBBr with sodium thiosulfate proceeded smoothly in the solution. The $PFBN_3$ sample was analyzed without any serious disturbances by contamination of PFBBr by GC–NPD and GC–MS with a nonpolar and slightly polar phase capillary GC column.

The analytical data on the azide ion in the samples of the dead bodies have been reported so far [2,9,11–13]. However, the data pertaining to the surviving patients was the first example, and they are expected to be very useful in considering the movement of the ion within the body, and the excretion of it from the body.

As to the internal standard, we synthesized *N*-(2-trifluoromethyl)benzylpentafluoropropamide for urgent use as it contained the nitrogen atom for GC–NPD and had an adequate retention time for GC. We obtained reliable data using it. Additionally, commercially available 2-nitrotoluene and 3-nitrobenzotrifluoride were also found to give good results.

Several examples, in which up to 9000 ng/ml of the cyanide ion in blood samples presumably generated from the azide ion, have been reported in the sodium azide poisoning cases [10,12,13]. However, cyanide ion was not detected in the blood samples of our case.

Kruszyna et al. [14] reported the interesting phenomenon that it became difficult to detect the azide ion in blood sample in a short time. They considered that this was caused by the oxidative fermentation of the ion. We also had similar findings in both actual and spiked human whole blood and urine samples. We are now examining the cause of these findings, but have not yet obtained any concrete evidence as to whether they were caused by fermentation or by other biological processes, such as interactions with the components of the blood or urine. The phenomenon itself was a very serious problem for the analysis of the ion. We, therefore, examined the method of maintaining the concentration of the azide ion in the samples. The azide ion was added to human whole blood and urine, and the spiked samples were left at room temperature, 4°C and −50°C. The spiked samples which included sodium tetraborate or sodium hydroxide were also examined. In the case of the whole blood samples, after 24 h without the sodium tetraborate or sodium hydroxide, the azide ion was not detected at room temperature. On the other hand, about 60% and a slight decrease in the concentration of the ion were observed at 4°C and −50°C, respectively. The decreasing rate of the ion in the urine samples was very slow compared to that of the whole blood samples. The concentration did not change even after 2 weeks at −50°C, but the decrease of the concentration was about 25% at room temperature after the same period. In contrast to the results, the change in concentration was not observed in both the whole blood and the urine samples, which contained sodium tetraborate or sodium hydroxide even after 1 month at room temperature. Therefore, the preserving method of adding the sodium tetraborate or sodium hydroxide to the samples was demonstrated to be most suitable for our analytical method.

5. Conclusion

We have established a simple, speedy and reliable method of identifying and quantifying the azide ion in the samples of human whole blood and urine by derivatizing the ion to $PFBN_3$. $PFBN_3$ was completely separated by GC and identified by NPD and MS. This method was applied to the human whole blood and urine samples of the actual case. The results demonstrated that the ion in the samples was unambiguously identified and quantified in high precision.

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