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Abstract

A simple and sensitive method has been developed for the determination of azide ion (N_3^-) in biological fluids and beverages. The procedure was based on the formation of a ternary complex $Cu(N_3)_2$(4-methylpyridine)$_x$ in benzene, followed by its detection by electron paramagnetic resonance. The complex in benzene showed a characteristic four-peak hyperfine structure with a *g*-value of 2.115 at room temperature. Cu^{2+} reacted with N_3^- most strongly among common metals found in biological fluids. Several anions and metal ions in biological fluids did not interfere with the determination of N_3^- in the presence of large amounts of Cu^{2+} and oxidants. In the present method, N_3^- at the concentration from 5 μM to 2 mM in 100 μl solution could be determined with the detection limit of 20 ng. The recoveries were more than 95 % for N_3^- added to 100 μl of blood, urine, milk and beverages at 200 μM. Our method is recommendable because it takes less than 10 min to determine N_3^- and the produced complex is quite stable.

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1. Introduction

Sodium azide is used in industry as an explosive in automobile safety bags and an antifungal for diagnostic reagents. A few years ago, however, several poisoning cases of sodium azide occurred in Japan [1]. When poisoning occurred, quick and decisive determination of toxic substances is required using the sample as small as possible. Demonstration of the presence of azide ion (N_3^-) was mostly based on a color reaction [1-3]. A colorimetric determination of N_3^- in blood, beverages and waste-water, however, required large amounts of N_3^-, e.g., 4 – 20 μg. Also the measuring processes such as vaporization of N_3^- as HN_3 gas and condensation of HN_3 in alkaline solution to avoid interferences from the other substances resulted in 1 hr-measurement time. To avoid the distillation, a liquid chromatographic determination [4,5] was proposed using 80 – 200 ng N_3^-. Those methods, however, took time for the derivatization of N_3^- after the deproteinization of samples.

N_3^- is known to form immediately ternary complexes with transition metals in the presence of pyridine (Py), and the complexes can be extracted with chloroform [3]. These azide complexes were used for the colorimetric determination of either N_3^- or transition metals [1-3]; however, the molar absorption of azide complex with Cu in chloroform, $\varepsilon_{435} = 2500$, was not large and less than twice that in aqueous solution, $\varepsilon_{385} = 1600$ [2,3]. The extraction of the complex with organic solvents reduced the

interference by impurities to some extent.

Cu^{2+} having an EPR signal at room temperature was found to react with N_3^- most strongly among several transition metals commonly contained in biological fluids. In the present study, the applicability of the paramagnetic character of the complex of N_3^- with Cu^{2+} to the determination of N_3^- has been studied since only paramagnetic species could be sensitively detected by EPR method even for crude materials [6]. In benzene solution, the quantitation of the complex of N_3^- with Cu^{2+} means the quantitation of N_3^-, since the present study showed that one Cu^{2+} ion combined with two N_3^- ions always in benzene, whereas one Cu^{2+} ion combined with either one, two or four N_3^- ions in water [2,3]. In addition, to extract $Cu(N_3)_2$ complex from aqueous crude samples, 4-methylpyridine (Mp) and benzene have been used in place of Py and chloroform used previously [3], to decrease nonspecific reactions of Cu^{2+} with anions contained in biological fluids and beverages.

2. Experimental

2.1. Materials

The chemicals of atomic absorption grade or of analytical grade were obtained from Wako Pure Chemical Ltd., Japan. A 200-μl polypropylene tube with cap was obtained from Eppendorf AG, Germany, a 20-μl quartz hematocrit capillary for EPR measurement, from Drummond Scientific Co., USA, and putty for sealing the capillary, from Modulohm A/S, Denmark. Blood and urine were obtained from healthy volunteers with their informed consent. Beverages were obtained from local stores. A vortex mixer and a centrifuge with 6000× g were used to transfer $Cu(N_3)_2(Mp)_x$ from

water to benzene.

2.2. Preparation of calibration and quality control samples

Sodium azide dissolved in water at 1 M was used as the stock solution. Standard solutions at 0.5, 1.5, 20, 50 and 200 mM N_3^- in 1 M Na_2SO_4 were prepared daily by using the stock solution,. To obtain calibration standards at 5, 15, 50, 200, 500 and 2000 μM N_3^-, 1 μl of appropriate standard solution was added to 99 μl of sample, respectively. Intra-day variations were assessed on quality control samples at 5, 15, 50, 200, 500 and 2000 μM N_3^- by analyzing 6 times for each concentration on the same day. Inter-day variations were assessed on the quality control samples prepared and analyzed daily for 5 days. Recovery was calculated as the percentage of the concentration found in the sample to the nominal concentration spiked.

2.3. EPR spectroscopy

EPR measurements were performed on a JEOL JES-FE2XG ESR spectrometer, Japan. For determination of the hyperfine splitting and *g*-value, the calibration of magnetic field was performed by using signals of Mn^{2+} [6]. Modulation width of 2 mT was found to be the most suitable setting. Microwave power of 65 mW was applied, since power saturation appeared at higher power than 70 mW. Gain setting could be varied from 1 to 10^4 according to signal heights. The accuracy of the EPR method was compared with a colorimetric method measured by a Shimadzu UV 2200 spectrophotometer, Japan.

2.4. Assay procedure for azide

A 100-μl volume of sodium azide standard solution or each sample solution (except blood) is placed in a tube and mixed with 25 μl of solution containing either 0.125 or 0.5 M $CuSO_4$. The pH of the solution is adjusted to 4 – 8 with either NaOH or H_2SO_4 solution, if necessary. In case of a blood sample, 100 μl of blood is centrifuged to obtain plasma. To the precipitate, 25 μl of 0.1 M Na_2SO_4 solution is added and mixed, followed by centrifugation to obtain the supernatant. This extraction is repeated once. The mixture of plasma and two supernatants is added with 25 μl of solution containing both 2 M Na_2SO_4 and 0.125 M $CuSO_4$. If the mixture of plasma and two supernatants contains hemoglobin, the mixture is heated for 2 min at 100 ℃ in a heat block.

To the solution prepared above, 20 μl of benzene containing 10 % (v/v) Mp is added and mixed for 10 s, and centrifuged for 30 s. The benzene layer is shifted to another tube containing 10 μl of 0.5 M Na_2SO_4 and mixed for 10 s to remove hydrophilic compounds from benzene layer. After centrifugation for 30 s, 10 μl of benzene layer is placed in a quartz capillary, and put in EPR cavity. EPR spectrum is measured at 20 ℃ and, the difference in the peak at 319 mT (i.e. the vertical length between the signal top at 317 mT and the signal bottom at 321 mT) is considered to be the amount of N_3^- . For the colorimetric measurements, the volumes of sample and reagents are proportionally increased at every step.

3. Results and discussion

3.1. EPR spectrum

Figure 1 shows EPR spectra of $Cu(N_3)_2(Mp)_x$ extracted into benzene from aqueous solutions at various concentrations of N_3^-. The EPR spectrum of $Cu(N_3)_2(Mp)_x$ was characterized by 4 peaks with the hyperfine splitting of 5.8±0.1 mT and the *g*-value of the center of the signal, 2.115±0.001 as listed in Table 1. The line shapes of $Cu(N_3)_2(Mp)_x$ complex in benzene extracted from several samples were the same as that extracted from aqueous standard solution, respectively. Therefore, the difference in the peak at 319 mT could be used for quantitation of N_3^- in various samples.

3.2. Suitable solvent and ternary component

In the previous report [3] chloroform and Py had been used to extract $Cu(N_3)_2$ from water to an organic layer. In the present work, however, benzene and Mp were used due to following two reasons. Firstly, the interfering EPR signals in chloroform resulting from anions listed in group C of Table 2 were larger than those in benzene. For example, under the condition of 100 mM Cu^{2+}, 100 mM Cl^- and 200 μM N_3^-, the signal of $Cu(Cl)_2(Py)_x$ was 1.9 times higher than that of $Cu(N_3)_2(Py)_x$ in chloroform, whereas that of $Cu(Cl)_2(Mp)_x$ was 11 times lower than that of $Cu(N_3)_2(Mp)_x$ in benzene. This may be due to higher polarity of chloroform than that of benzene, resulting in dissolution of weaker complexes between Cu^{2+} and interfering anions. Secondly, benzene layer is easy to take out, since it is lighter than the aqueous layer containing precipitates, whereas the chloroform layer sinks together with precipitates.

The ternary components such as Mp, 3-methylpyridine, 2-methylpyridine and Py were compared. The *g*-value, hyperfine splitting and relative signal height are listed

in Table 1. The signal height decreased in the order of Mp, 3-methylpyridine and 2-methylpyridine, indicating that the methyl group at position 2 interfered with the complex formation of N atom in pyridine ring. The color of the complex of 2-methylpyridine was also the weakest among these complexes. When benzene was used as a solvent, Mp had to be used because of higher solubility of Mp than that of Py in benzene. Benzene containing 10 % Mp was used for the following study since the complete extraction of the complex was achieved with benzene containing 5 – 20 % Mp.

3.3. Molar ratio of N_3^- to Cu^{2+}

To determine the molar ratio of N_3^- to Cu^{2+} in the complex, the titration was performed as follows. Each 0 to 100 μl of 2 mM N_3^- in 0.5 M Na_2SO_4 solution was mixed with 100 to 0 μl of 1 mM Cu^{2+} in 0.5 M Na_2SO_4 solution, respectively, and they were extracted with 20 μl of benzene containing 10 % Mp, respectively. The maximum EPR signal was obtained when the molar ratio N_3^- / Cu^{2+} was 2, where N_3^- was converted to $Cu(N_3)_2(Mp)_x$ in a 93 % yield. When the molar ratio was smaller than 0.86, i.e., 3 mM N_3^- and 3.5 mM Cu^{2+} in benzene, N_3^- was converted to $Cu(N_3)_2(Mp)_x$ in a 100 % yield. Species like CuN_3^+ and $Cu(N_3)_4^{2-}$ were observed in aqueous solution [2,3]. Therefore, Na_2SO_4 was added in the assay to extract $Cu(N_3)_2(Mp)_x$ completely with benzene.

3.4. Effect of anions

$CuSO_4$ was chosen as a source of Cu^{2+} since the final benzene layer extracted from

even 1 M $CuSO_4$ solution did not show any EPR signals. The effects of various anions were examined using 100 mM each anion, 200 μM N_3^- and either 25 or 100 mM Cu^{2+}, because such anions are expected to be included at relatively high concentration in biological samples. The results are listed in Table 2. Anions in group A did not cause any errors at the concentrations of both 25 and 100 mM Cu^{2+}. Anions in group B did not show any EPR signals and reduced the EPR signals of $Cu(N_3)_2(Mp)_x$ only when their concentrations were higher than those of Cu^{2+}; they caused negative errors at 25 mM Cu^{2+} but not at 100 mM Cu^{2+}, indicating that some extent of Cu^{2+} was consumed for formation of complexes with these anions in group B. Anions in group C showed small EPR signals of their complexes with Cu^{2+} in benzene having different hyperfine structures and *g*-values from those of N_3^-, but interfered with the determination of N_3^- when the concentrations of N_3^- were low and the concentrations of the anions were high. Especially, concentrations of Cl^- are close to 100 mM in human blood and urine. Although the binding of Cl^- with Cu^{2+} is weak, the possibility of interference by Cl^- should be kept in mind when samples containing low concentration of N_3^- are dealt with. Therefore, the steps of washing out hydrophilic compounds into 0.5 M Na_2SO_4 solution were added in the assay. When benzene containing weak Cu^{2+} complexes with anions, such as $Cu(Cl)_2(Mp)_x$ and $Cu(NO_3)_2(Mp)_x$, was mixed with 0.5 M Na_2SO_4 solution, these complexes were dissolved in aqueous layer showing no EPR signals in benzene layer. In contrast, $Cu(N_3)_2(Mp)_x$ remained in benzene layer completely, making the determination of 5 μM N_3^- possible as shown in Fig. 1 (c). Anions in group D, I^-, NO_2^- and SCN^- showed large EPR signals of their complexes with Cu^{2+} in benzene having different hyperfine

structures and *g*-values from those of N_3^-, but they did interfere with the determination of N_3^-. However, this problem could be overcome by adding a 4-fold molar amount of $KMnO_4$ to the test solution to oxidize I^-, NO_2^- or SCN^-. After 5 min, any EPR signals originated from either I^-, NO_2^-, SCN^- or $KMnO_4$ were not observed. In $KMnO_4$ solution, however, N_3^- could be measured without its oxidation when the pH of the solution was higher than 7.

3.5. Effect of metal ions

The effects of various metal ions on the present assay were examined at the concentrations of 1 mM each ion, 1 mM Cu^{2+} and 5 mM N_3^-. The production rate of $Cu(N_3)_2(Mp)_x$ from solution containing both test ion and Cu^{2+} was compared with that of the solution without the test ion. As results, there was no interference by V^{5+}, Cr^{6+}, Cr^{3+}, Mn^{7+}, Mn^{2+}, Fe^{3+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, As^{3+}, Se^{4+}, Mo^{6+}, Ag^{+}, Cd^{2+}, W^{6+}, Pb^{2+} and La^{3+}. The production rates were reduced in the solutions containing following four metals: 50 % reduction by Pd^{2+}, 40 % by $Ti^{4+}$and Zr^{4+}, and 20 % by Ru^{3+}, respectively. However, the production rates of $Cu(N_3)_2(Mp)_x$ could be recovered to more than 95 % when the concentrations of these four metals were decreased to one tenth of Cu^{2+} concentration. All metal ions mentioned above did not show any EPR signals derived from complexes between each metal ion and N_3^-.

3.6. Optical absorption and concentration linearity

The optical absorption spectrum of $Cu(N_3)_2(Mp)_x$ in benzene showed its maximum at 435 nm. The ε value at 435 nm was 2500, the same as that of $Cu(N_3)_2(Py)_x$ in

chloroform [3]. The concentrations of N_3^- from 0 to 500 μM measured by EPR method (y in μM) and those measured by colorimetric method (x in μM) were well satisfied with the equation y = 1.00 x + 3.5 with the correlation coefficient of 0.99.

The calibration equations calculated on 36 points (six concentrations such as 5, 15, 50, 200, 500 and 2000 μM, six determinations per each concentration) were excellent for all six samples as listed in Table 3.

3.7. Stability and recovery

The $Cu(N_3)_2(Mp)_x$ complex in benzene was quite stable; more than 95 % (*n*=6) remained unchanged after 7 days, when it was kept in a tube with a tight cap under room light at room temperature. In the assay of N_3^- in beverages such as coffee, green tea and orange juice, 100 mM Cu^{2+} was used instead of 25 mM Cu^{2+} used for aqueous solution, blood, urine and milk, respectively. The lower recoveries from beverages at 25 mM Cu^{2+} indicated that Cu^{2+} was consumed to form water-soluble complexes with various substances, such as citric acid and ascorbic acid.

Recovery and coefficient of variation of the determination for N_3^- spiked into 100 μl of biological fluids at 5 - 200 μM are listed in Table 4. The corresponding values for the solutions spiked at 5, 500 and 2000 μM were not listed in the table, since the recoveries were lower than 37 % at 5 μM in all six samples, whereas the deviation from the nominal value as well as the coefficient of variation of determination were lower than 5 % at 500 and 2000 μM in all six samples, respectively. When blood was hemolyzed, however, the recovery was reduced according to the degree of hemolysis, irrespective to the concentrations of Cu^{2+}. The recoveries of N_3^- at 200 μM were 0 %

and 80 % in hemolyzed blood and in the heat-denatured hemolyzed blood, respectively. This suggests that intact hemoglobin combines with N_3^- but denatured one does not. Large amounts of substances in any of matrices were confirmed not to exhibit EPR signals in benzene layer, shown in Fig. 1(d) as an example of blood.

3.8. Accuracy, precision, limit of quantitation and limit of detection

Fig. 1 (c) shows the spectrum of 20 ng of N_3^- extracted from100 μl aqueous solution at 5 μM. Here, 5 μM is considered as the limit of detection in aqueous solution, since characteristic 4-peak structure can be observed and S/N ratio is better than 3 at the concentration higher than 5 μM. Limit of quantitation in aqueous solution was 15 μM, since the deviation from the nominal value as well as the coefficient of variations of six determinations was less than 5 % when the concentration was higher than 15 μM. Intra-day and inter-day variation of recovery and coefficient of variation of the determination for N_3^-, spiked into 100 μl of biological fluids at 15 - 200 μM are listed in Table 4. The limit of quantitation was 50 μM in these samples, since all the recoveries were in the range of 80 – 100 %, and all the coefficients of variation were lower than 20 %, respectively. The limit of detection in these samples was 15 μM, since 4-peak structure was observed and S/N ratio was better than 3 at this concentration. The absolute amount of detection limit was 60 ng of N_3^- using 100 μl of sample solution. The detection limits of the colorimetric method reported were 20 μg [1] and 4 μg [2], and those of HPLC method, 80 ng [4] and 200 ng [5], respectively, using 1 ml of sample solution in each method.

4. Conclusion

In the present method N_3^- in plasma and urine was sensitively determined within 10 min without tedious distillation used in the colorimetric method [1,2] and without derivatization of N_3^- used in the HPLC method [4,5]. This method can be used for the concentration of N_3^-. That is, N_3^- in aqueous solution was extracted with 0.2 volume of benzene as $Cu(N_3)_2(Mp)_x$ and from that benzene, N_3^- was extracted again with 0.2 volume of 0.01 M NaOH solution, resulting in 25-fold concentration with 90 % recovery of N_3^-.

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References

[1 K. Tsuge, M. Kataoka, Y. Seto. J. Anal. Toxicol. 25 (2001) 228.

[2] E.A. Neves, E. De Oliveira, L. Sant' Agostino. Anal. Chim. Acta 87 (1976) 243.

[3] R.G. Clem, E.H. Huffman. Anal. Chem. 38 (1966) 926.

[4] W.E. Lambert, M. Piette, C. Van Peteghem, A.P. De Leenheer. J. Anal. Toxicol. 19 (1995) 261.

[5] P. Marquet, S. Clement, H. Lotfi, M.F. Dreyfuss, J. Debord, D. Dumont, G. Lachatre. J. Anal. Toxicol. 20 (1996) 134.

[6] K. Minakata, O. Suzuki. Anal. Chim. Acta 512 (2004) 355.

Figure captions

Fig. 1. EPR spectra of $Cu(N_3)_2(Mp)_x$ complex in 10 μl of benzene as a function of N_3^- concentrations. (a): extract from 200 μM N_3^- aqueous solution measured at gain setting of 160. (b): Mn^{2+} in MgO was measured together with $Cu(N_3)_2(Mp)_x$ to calibrate the magnetic field. (c): extract from 5 μM N_3^- aqueous solution measured at gain setting of 2000. (d): extract from N_3^--free blood measured at gain setting of 2000.

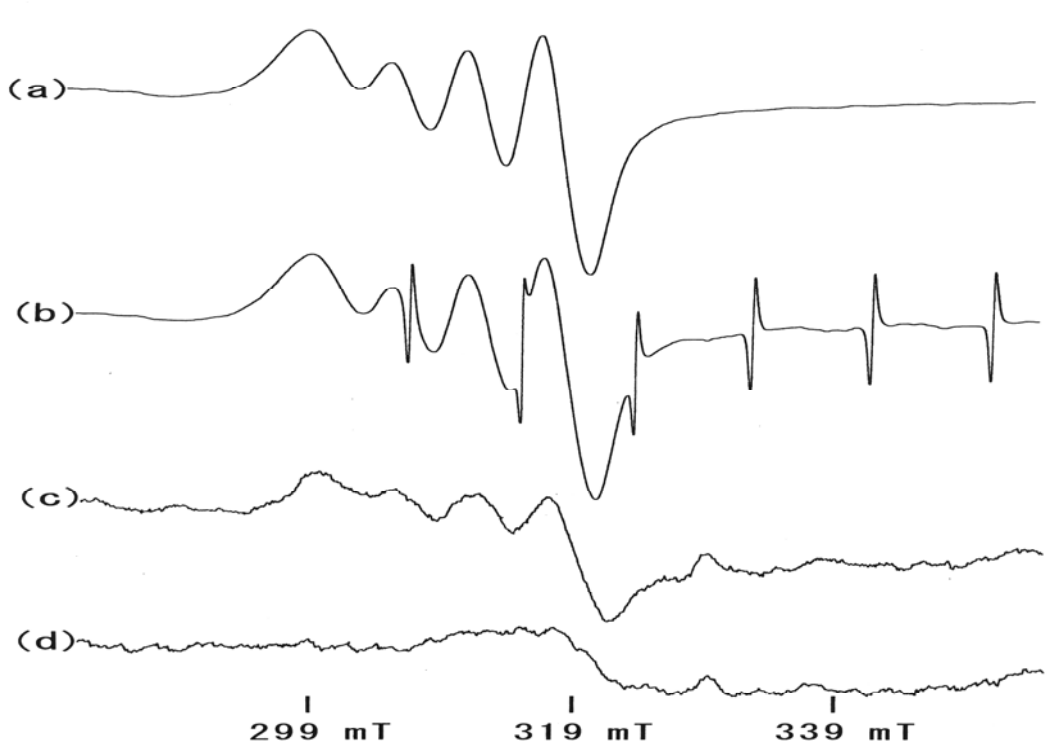

Table 1

EPR parameters of Cu-azide complexes

Components	*g*-Value ± 0.001	Hyperfine splitting in mT ± 0.1	Peak height in arbitrary unit ± 0.01
4-Methylpyridine in benzene	2.115	5.8	1.0
3-Methylpyridine in benzene	2.113	5.3	0.69
2-Methylpyridine in benzene	2.096	6.4	0.21
Pyridine in benzene	2.115	5.8	0.66
Pyridine in chloroform	2.115	5.8	0.68

Each value represents mean for six determinations.

Table 2

Effects of 100 mM anions on the determination of 200 μM N_3^-

Group	Anions(100 mM)	Reaction with Cu^{2+}	Interfering EPR signals
A	SO_4^{2-}, CN^-, F^-, HCO_3^- HPO_4^{2-}, HSO_3^-, $S_2O_5^{2-}$	Weak	No
B[a]	EDTA, oxalate, citrate tartrate, ascorbate, $S_2O_3^{2-}$	Strong	No
C	Cl^-(0.09)[b], Br^-(0.18), NO_3^-(0.19) CH_3COO^-(0.02), $HCOO_4^-$(0.02)	Weak	Small
D	I^-, NO_2^-, SCN^-	Strong	Large

[a] Anions of group B gave negative errors, only when the concentrations of anions were greater than that of Cu^{2+}.

[b] Each value shown in parenthesis is an EPR peak height ratio of an anion at 100 mM to N_3^- at 200 μ M.

Table 3

Calibration curve, quantitation range and detection limit for azide ion spiked at 5 – 2000 μM in biological fluids.

Sample	Calibration equation μM	Correlation coefficient (*r*)	Qantitation range μM	Detection limit μM
Blood	y = 0.965 x – 1.8	0.998	15 – 2000	15
Urine	y = 1.002 x + 4.7	0.999	15 – 2000	15
Milk	y = 0.973 x + 3.4	0.998	50 – 2000	15
Coffee	y = 0.961 x – 2.2	0.998	50 – 2000	15
Green tea	y = 0.963 x + 0.8	0.998	50 – 2000	15
Orange juice	y = 0.955 x – 1.8	0.998	50 – 2000	15

Table 4

Recovery and coefficient of variation of determination for azide ion spiked at 15 – 200 μM in biological fluids. Values for azide ion at 5, 500 and 2000 μM, see the text.

Sample	N_3^- added	Intra-day (6 times)		Inter-day (5 days)	
		Recovery	Coefficient of	Recovery	Coefficient of
	μM	%	variation %	%	variation %
Blood	15	63	15	59	20
	50	91	16	89	17
	200	98	5	97	6
Urine	15	80	16	72	18
	50	100	10	96	12
	200	101	5	99	5
Milk	15	59	43	59	32
	50	84	14	82	16
	200	97	5	97	5
Coffee	15	63	33	60	34
	50	85	17	83	18
	200	96	5	96	5
Green tea	15	59	30	58	35
	50	83	16	83	18
	200	97	3	96	5
Orange juice	15	57	34	57	32
	50	82	15	80	16
	200	95	4	95	5