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Determination of azide in biological fluids by use of electron paramagnetic resonance

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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.

Keywords: Azide; Electron paramagnetic resonance; Copper; Blood; Urine

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 h-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.

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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 $6000 \times g$ 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, 50–200 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, 500–2000 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, 500–2000 μM N_3^- by analyzing six 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 °C 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 °C 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

Fig. 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 four 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

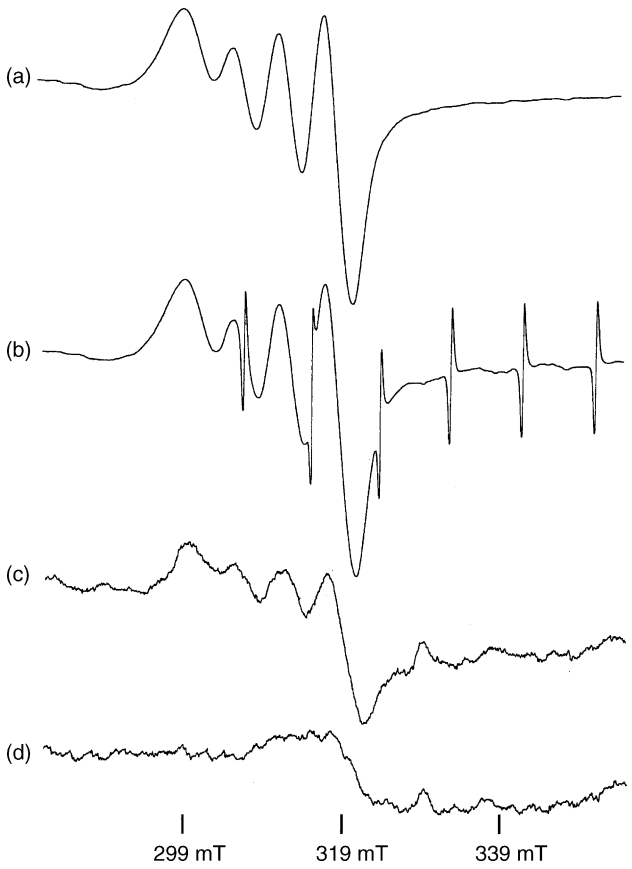

Fig. 1. EPR spectra of $Cu(N_3)_2(Mp)_x$ complex in 10 μl of benzene as a function of N_3^- concentrations. (a) Extract from 200 μM N_3^- aqueous solution measured at gain setting of 160. (b) Mn^{2+} in MgO was measured together with $Cu(N_3)_2(Mp)_x$ to calibrate the magnetic field. (c) Extract from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