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# RAPID DETERMINATION OF THIOCYANATE IN BIOLOGICAL FLUIDS BY USE OF ELECTRON SPIN RESONANCE

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# 電子スピン共鳴法を用いたチオシアン酸イオンの迅速定量

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## Summary

A simple and sensitive method has been developed for determination of thiocyanate ion ($SCN^-$), the main metabolite of cyanide, in biological fluids and beverages. The procedure was based on the formation of a paramagnetic complex $Cu(SCN)_2$(4-methylpyridine)$_2$ in benzene, followed by its detection by electron spin resonance (ESR). The complex showed a characteristic four-peak hyperfine structure with a *g*-value of 2.132 at room temperature. By the present method, $SCN^-$ at concentrations from 5 μM to 2 mM in 500 μl solution could be determined. The recoveries were more than 95% for $SCN^-$ added to blood, plasma, urine, milk and beverages at 200 μM. Several anions and cations in biological fluids did not interfere with the formation of the complex in the presence of large amounts of $Cu^{2+}$. Our method is recommendable, because it takes only less than 10 min to determine $SCN^-$ and the produced complex is quite stable.

*Key words :* Thiocyanate; Cyanide metabolite; Electron spin resonance; Copper; Blood; Urine

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## Introduction

Sensitive colorimetric determinations of thiocyanate ($SCN^-$), the main metabolite of cyanide, are being performed using chloramine T and pyridine-pyrazolone reagent with the molar absorption $\varepsilon_{630}$ of 100,000 [1,2]. In these methods, however, it takes about 30 min for the complete coloration; cyanide ion ($CN^-$) should be completely evaporated before the determination of $SCN^-$, because $CN^-$ similarly reacts with these reagents. Although $SCN^-$ reacts with $Fe^{3+}$ to produce selective absorption around 470 nm, the molar ratios of $SCN^-$ to $Fe^{3+}$ as well as the wavelengths of the absorption maxima vary according to the concentrations of $SCN^-$ and $Fe^{3+}$ [3]. Gas chromatography can be also used for sensitive determination of $SCN^-$, but it requires the derivatization of $SCN^-$ to be lasted for 30 min [4]. $SCN^-$ is known to immediately form ternary complexes with transition metals in the presence of pyridine (Py), and the complexes can be extracted with chloroform [5]. The molar ratio of the constituents in the ternary complex with $Cu^{2+}$ was reported to be constantly 1 : 2 : 2 for $Cu^{2+}$, $SCN^-$ and Py, respectively. Such an $SCN^-$ complex can be used for the colorimetric determination of either $SCN^-$ or $Cu^{2+}$ [5]; however, the molar absorption of the ternary complex was not large with its $\varepsilon_{405}$ of as low as 850 [5]. The extraction of the complex with an organic solvent like chloroform reduced the interference by impurities to some extent.

Most transition metals show their electron spin resonance (ESR) signals only at low temperatures. $Cu^{2+}$, however, sometimes shows its ESR signals even at room temperature.

Furthermore, $Cu^{2+}$ was found to react with $SCN^-$ 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 $SCN^-$ with $Cu^{2+}$ to the determination of $SCN^-$ has been studied, since only paramagnetic species could be sensitively detected by the ESR method even for crude materials [6]. In addition, to extract the $Cu(SCN)_2$ complex from aqueous crude samples, 4-methylpyridine, *i.e.*, γ-picoline (Pi) and benzene have been used to obtain $Cu(SCN)_2(Pi)_2$, in place of pyridine (Py) and chloroform, which had been used in the previous work [5] to decrease nonspecific reactions of $Cu^{2+}$ with anions being contained in biological fluids and beverages.

## Experimental

*Materials*

A standard solution of KSCN (100 mM) and chemicals of atomic absorption grade or of analytical grade were obtained from Wako Pure Chemical Ltd. (Osaka); polypropylene tubes with caps (200 – 1000 μl) from Eppendorf AG (Hamburg, Germany); quartz flat cells for X band ESR measurements (80 μl) from JEOL (Tokyo). Blood and urine were obtained from healthy volunteers. Beverages were obtained from local stores.

*ESR spectroscopy*

ESR measurements were performed on a JEOL JES-FE2XG ESR spectrometer (Tokyo). For determination of the hyperfine splitting and *g*-values, the calibration of magnetic field was performed by using signals of $Mn^{2+}$ [6]. The modulation width and microwave power were found most suitable at 2 mT and 65 mW, respectively. Gain setting was varied from 1 to $10^4$ according to signal heights. Optical absorption spectra were recorded with a Shimadzu UV 2200 spectrophotometer (Kyoto).

*Assay procedure for thiocyanate*

In case of a blood sample, it was centrifuged to obtain plasma as sample solution, since $SCN^-$ is distributed mainly in plasma [2]. A 500-μl volume of diluted KSCN standard solution or each sample solution was placed in a tube and mixed with 125 μl of solution containing both 2 M $Na_2SO_4$ and either 0.125 or 0.5 M $CuSO_4$. The pH of the solution was adjusted to 4 – 8 with either NaOH or $H_2SO_4$ solution using pH test paper, if necessary. To the solution, 100 μl of benzene containing 10% (v/v) Pi was added, mixed well for 10 s and centrifuged for 30 s. The benzene layer (about 90 μl) was transferred to another tube containing 20 μl of 0.5 M $Na_2SO_4$ solution and mixed well for 5 s to shift hydrophilic compounds to the aqueous layer. It was centrifuged for 15 s and 80 ml of benzene layer was placed in an ESR cell to measure an ESR peak height difference between 296 and 317 mT at room temperature (Fig. 1a). For the colorimetric measurements, it required 2 – 3 ml of the final solution; for this purpose, the volumes of the sample and reagents were proportionally (about 30-fold) increased at every step.

## Results and discussion

*Optimization of conditions*

In the previous report [5], Py and chloroform had been used to extract $Cu(SCN)_2$ from water to an organic layer. In the present work, however, Pi and benzene were used due to the following two reasons. Firstly, the interfering ESR signals in chloroform resulting from anions listed in group C of Table 1 were about ten times as large as those 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 each interfering anion. 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 at the bottom of the aqueous solution in a tube. When benzene was used as a solvent, Pi had to be used, because of higher solubility of Pi than that of Py in benzene. Benzene containing 10% (v/v) Pi was used in the present method, because the complete extraction of the complex was achieved with benzene containing Pi at the concentrations of 5 – 20%. The $Cu(SCN)_2(Pi)_2$ 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.

$CuSO_4$ was chosen as a source of $Cu^{2+}$, since the final benzene layer extracted from $CuSO_4$ solution at even higher than 1 M did not show any ESR signal. $Na_2SO_4$ was added at the initial and final steps for extracting $Cu(SCN)_2(Pi)_2$ with high recovery rates using its salting-out effect.

$Cu(Cl)_2(Py)_2$ produced from 100 mM $Cl^-$ showed an ESR signal 1.9 times higher than that of $Cu(SCN)_2(Py)_2$ produced from 200 μM $SCN^-$ in chloroform. On the other hand, $Cu(Cl)_2(Pi)_2$ produced from 100 mM $Cl^-$ showed an ESR signal about 11 times lower than that of $Cu(SCN)_2(Pi)_2$ produced from 200 μM $SCN^-$ in benzene. These results also indicate that not chloroform but benzene should be used for extraction of the complex from biological fluids like blood or urine containing as

**Table 1.** Effects of 100 mM anions on the determination of 200 μM $SCN^-$

| Group | Anions(100 mM) | Reaction with $Cu^{2+}$ | Interfering ESR 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 | Strong | No |
| C | $NO_3^-$(0.19)[b], $Br^-$(0.18) $Cl^-$(0.09), $CH_3COO^-$(0.02) $HCOO^-$(0.02), $S_2O_4^{2-}$(0.02) | Weak | Small |
| D | $I^-$, $NO_2^-$ | 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 ESR peak height ratio of an anion at 100 mM to $SCN^-$ at 200 μM.

high as100 mM $Cl^-$.

The ESR signals of the complexes were compared for Pi, 3-methylpyridine and 2-methylpyridine. The signal height decreased in the order of Pi, 3-methylpyridine and 2-methylpyridine, indicating that the methyl group in position 2 interfered with the complex formation.

*Reliability of the method*

Figure 1 shows typical ESR spectra of the $Cu(SCN)_2(Pi)_2$ complex extracted into benzene from aqueous solutions at various concentrations of $SCN^-$. The ESR spectrum of $Cu(SCN)_2(Pi)_2$ was characterized by four peaks with the hyperfine splitting of $5.8 \pm 0.1$ mT. The peak height difference between 296 and 317 mT corresponded to the $SCN^-$ concentration. The *g*-value of the center of the signal was $2.132 \pm 0.001$. Although the data shown in Fig. 1 dealt only with clean $SCN^-$ solutions, crude biological samples such as blood and urine gave no impurity signals in their spectra. Endogenous $SCN^-$ is present in human blood; the pattern was similar to that shown in Fig. 1c.

Figure 2 shows an optical absorption spectrum of the $Cu(SCN)_2(Pi)_2$ complex. It showed its maximum at 405 nm. However, unfortunately, the ε value at 405 nm for this complex was as low as 850, which is the same as that of the $Cu(SCN)_2(Py)_2$ complex [5].

Figure 3 shows the calibration curve for the ESR response as a function of $SCN^-$ concentration in aqueous solution. Good linearity was found in the range of 5 to 500 μM. The detection limit was about 5 μM.

The recoveries of 200 μM $SCN^-$ added to biological fluids and beverages are listed in Table 2. They were more than 95% for blood, plasma, urine and milk, even though large amounts of precipitates were formed. 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; but the recoveries were more than 95% in all beverages at 100 mM $Cu^{2+}$. Substances in any of matrices were confirmed not to exhibit ESR signals.

Using 500 μl of sample solution, the relative standard deviation for six determinations was about 5%, when the concentrations were greater than 5 μM.

The effects of various anions were examined using 100 mM of each anion, 200 μM $SCN^-$ and either 25 or 100 mM $Cu^{2+}$, because such anions are expected to be included at relatively high concentrations in biological samples. The results are listed in Table 1. Anions in group A did not cause any error at the

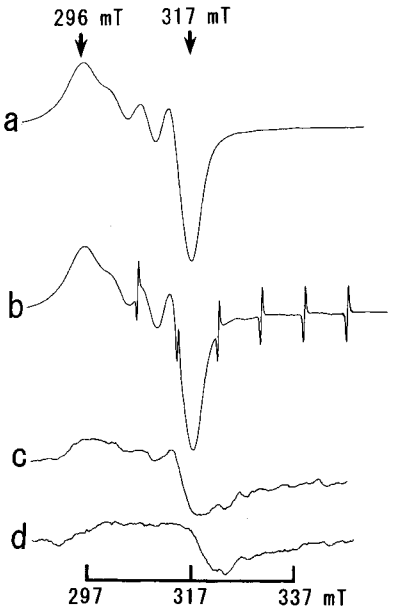


**Fig. 1.** ESR spectra of the $Cu(SCN)_2(Pi)_2$ complex in benzene as a function of $SCN^-$ concentrations. (a): extract from 200 μM $SCN^-$ solution being measured at gain setting of 75. (b): $Mn^{2+}$ in MgO measured together with the $Cu(SCN)_2(Pi)_2$ complex to calibrate the magnetic field. (c): extract from 5 μM $SCN^-$ solution being measured at gain setting of 1,000. (d): extract from $SCN^-$-free water being measured at gain setting of 1,000; the observed small signals are derived from impurities being contained in the quartz ESR cell used.

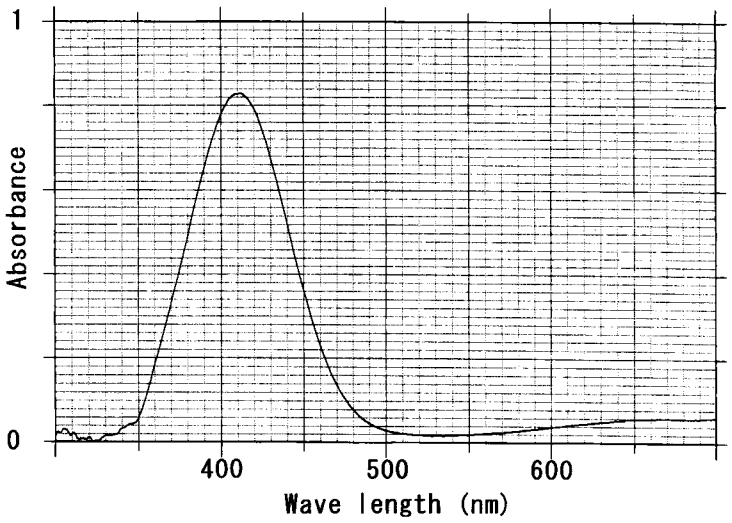


**Fig. 2.** Absorption spectrum of the $Cu(SCN)_2(Pi)_2$ complex at 1 mM $SCN^-$ in benzene.

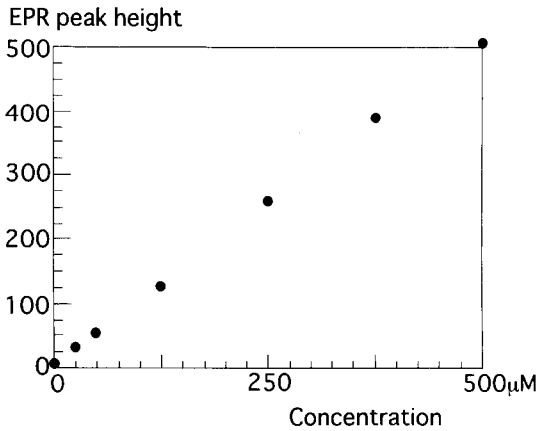


**Fig. 3.** Correlation between the ESR peak height difference between 296 and 317 mT and $SCN^-$ concentration. Gain setting for the ESR was 1,000. Each point represents the mean of six determinations.

**Table 2.** Recoveries of 200 μM $SCN^-$ added to biological fluids and beverages

| Sample | 25 mM $Cu^{2+}$ (%) | 100 mM $Cu^{2+}$ (%) |
|---|---|---|
| Blood | 95> | 95> |
| Plasma | 95> | 95> |
| Urine | 95> | 95> |
| Milk | 95> | 95> |
| Orange juice | 80> | 95> |
| Coffee | 70> | 95> |
| Green tea | 60> | 95> |

concentrations of both 25 and 100 mM $Cu^{2+}$. Anions in group B did not show any ESR signals and reduced the ESR signals of $Cu(SCN)_2(Pi)_2$, 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 the anions in group B. Anions in group C showed small ESR signals of their complexes with $Cu^{2+}$ in benzene having different hyperfine structures and *g*-values from those of $SCN^-$, but interfered with the determination of $SCN^-$, when the concentrations of $SCN^-$ were low and the concentrations of the anions were high. Especially, $Cl^-$ concentrations 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 concentrations of $SCN^-$ are dealt with. Anions in group D, $I^-$ and $NO_2^-$ showed large ESR signals of their complexes with $Cu^{2+}$ in benzene having different hyperfine structures and *g*-values from those of $SCN^-$, but they did interfere with the signals of $SCN^-$. However, this problem could be overcome by adding a 2-fold molar amount of $K_2CrO_4$ to the test solution at nearly neutral pH to oxidize $I^-$ or $NO_2^-$; $SCN^-$ could be measured without any oxidation.

The effects of various metal ions on the present assay were also examined at the concentrations of 1 mM each metal ion, 1 mM $Cu^{2+}$ and 5 mM $SCN^-$. The production rate of $Cu(SCN)_2(Pi)_2$ from solution containing both $Cu^{2+}$ and each metal ion was compared with that of the solution without a test metal ion. As results, there was no interference by $Ti^{4+}$, $V^{5+}$, $Cr^{3+}$, $Mn^{2+}$, $Fe^{3+}$, $Zn^{2+}$, $As^{3+}$, $Se^{4+}$, $Zr^{4+}$, $Mo^{6+}$, $Ru^{3+}$, $Pd^{2+}$, $Ag^+$, $W^{6+}$, $Pb^{2+}$ and $La^{3+}$. The production rates were reduced in the solutions containing following three metal ions: 70% reduction by $Co^{2+}$, 60% by $Ni^{2+}$ and 20% by $Cd^{2+}$. However, the production rates of $Cu(SCN)_2(Pi)_2$ could be recovered to more than 95%, when the concentrations of these three metal ions were decreased to one tenth of $Cu^{2+}$ concentration. All metal ions mentioned above did not show any ESR signals derived from complexes between each metal ion and $SCN^-$.

*Conclusion*

To our knowledge, this is the first report to analyze thiocyanate by ESR. Our method does not require deproteinization; the time needed for whole procedure of analysis is only less than 10 min. It seems useful for analysis of thiocyanate in biological samples including those in cyanide poisoning cases.

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