Spectrofluorimetric Determination of 10^{-7} M Levels of Thiol Compounds Using Silver(I)–5,10,15,20-Tetrakis(4-sulfophenyl)porphine Complex

Keiji TAKAHATA and Shukuro IGARASHI*

Department of Materials Science, Faculty of Engineering, Ibaraki University, 4–12–1 Nakanarusawa, Hitachi, 316 Japan. Received July 7, 1995; accepted September 11, 1995

5,10,15,20-Tetrakis(4-sulfophenyl)porphine (TPPS) has a strong fluorescence, while the silver(I) complex [$Ag(I)_2$–TPPS] has very little fluorescence. Also, the $Ag(I)_2$–TPPS complex easily releases the silver ion from the porphine ring in the presence of thiol compounds such as L-cysteine, glutathione and 2-mercaptoethanol. Based on these results, a highly sensitive spectrofluorimetric determination of 10^{-7} M levels of thiol compounds was developed. Calibration curves were linear in the concentration ranges of 0—5×10^{-7} M for L-cysteine, 0—5×10^{-7} M for glutathione and 0—2×10^{-6} M for 2-mercaptoethanol. Detection limits (3σ) were 1×10^{-10} M for L-cysteine, 5×10^{-11} M for glutathione and 2×10^{-10} M for 2-mercaptoethanol. Precisions were 1.89% for L-cysteine (2.5×10^{-7} M), 3.33% for glutathione (2.5×10^{-7} M) and 2.33% for 2-mercaptoethanol (1.0×10^{-6} M).

Key words thiol compound; water-soluble porphyrin; spectrofluorimetric determination; dissociation reaction of silver ion

Thiol compounds (thiols) such as L-cysteine and glutathione widely exist in animals, plants and microorganisms. In living tissues, naturally occurring thiols are well known to play important roles, *e.g.*, acting as a detoxication agent, a protection agent and so on. Hence, several medicines contain naturally occurring thiols for the above described purpose. For their determination, two different types of fluorigenic reagents, *e.g.*, halogenated benzofurazans such as 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole[1] and *N*-substituted maleimides such as *N*-(9-acridinyl)maleimide (NAM),[2] have been reported. However, with the diversity of medicine, the development of a more highly sensitive fluorigenic reagent for thiols has been strongly desired.

Porphyrin compounds not only have a very high molar absorptivity, reaching several hundred thousand $M^{-1}cm^{-1}$ in the range 400—500 nm (the so-called Soret band), but also a strong red fluorescence. For these reasons, porphyrins are attractive from an analytical point of view, and many highly sensitive spectrophotometric or fluorimetric determinations of metal ions using porphyrins have been developed.[3–6] The determination of inorganic anions such as sulfide ions and iodide ions, based on the dissociation reaction of silver ion with silver(I)–anionic water-soluble porphyrin 5,10,15,20-tetrakis(4-sulfophenyl)porphine (TPPS) [$Ag(I)_2$–TPPS], has been reported.[7] Also, the spectrophotometric determination of cyanide ions has been reported, based on the fact that the complexation of silver ions with cationic water-soluble porphyrins, such as 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphine (TMPyP), in alkaline media is inhibited by cyanide ions.[8]

In this paper, using the respective properties of the fluorescence spectra of TPPS and $Ag(I)_2$–TPPS, and the reaction in which silver ion dissociates from $Ag(I)_2$–TPPS in the presence of an organic thiol compound, a spectrofluorimetric determination for 10^{-7} M levels of thiols is demonstrated.

Materials and Methods

Apparatus A Hitachi Model F-4500 spectrofluorimeter with a 10-mm square quartz cell was used for the fluorescence measurement. A Hitachi Model 200-10 double-beam spectrophotometer with a 10-mm cell and a Hitachi Model 057 X-Y recorder were used for the measurement of the absorption spectra. A Horiba Model F-8AT pH meter was used for the pH measurements.

Reagents All of the chemicals used were of analytical reagent grade and were used without further purification. Doubly distilled water was used in all of the experiments. TPPS was prepared as described by Fleisher *et al.*[9] Solutions (10^{-4} M) of Ag(I) were prepared from a 1000 mg l^{-1} atomic absorption stock solution (Wako Co.). A 2.5 M sodium nitrate solution was prepared by dissolving 106.24 g of $NaNO_3$ (Wako Co.) in 500 ml of doubly distilled water. Standard solutions (10^{-4} M) of L-cysteine (Kanto Co.), glutathione (Wako Co.), 2-mercaptoethanol (Kanto Co.), glycine (Wako Co.), L-glutamic acid (Wako Co.) and L-methionine (Kanto Co.) were prepared by dissolving and diluting them with doubly distilled water, and they were adjusted to approximately pH 5. Under these conditions, the oxidation of thiols was negligible in the solution.[10] Also, the concentration of thiols was determined with NAM.[2] Each solution was prepared before use.

Procedure A 0.5 ml aliquot of 10^{-4} M TPPS solution, 10 ml of 2.5 M sodium nitrate solution, 1.0 ml of 10^{-4} M Ag(I) solution, and 2 ml of 1 M sodium hydroxide solution were placed in a 50 ml amber volumetric flask. After standing for 5 min, a 30 ml sample solution containing the thiols was added and then the mixture was diluted to 50 ml with water. After 10 min, the fluorescence intensity of the solution was measured at $\lambda_{em}=640$ nm with an excitation wavelength at $\lambda_{ex}=413$ nm.

Results and Discussion

Spectrophotometric and Fluorimetric Properties The absorption maxima for TPPS and $Ag(I)_2$–TPPS in 0.5 M $NaNO_3$ solution were 414 nm ($\varepsilon=41.4\times10^4$ $M^{-1}cm^{-1}$) and 443 nm ($\varepsilon=7.9\times10^4$ $M^{-1}cm^{-1}$), respectively.[7] The excitation and emission maxima for TPPS were 413 and 640 nm, respectively. In addition, TPPS showed approximately a 20 times higher fluorescence intensity than that of TMPyP.[11] The complexation of silver ion with TPPS is shown in reaction [A]. The $Ag(I)_2$–TPPS complex was produced with increasing concentrations of silver ion and the fluorescence of TPPS was quenched.

$$TPPS+2Ag(I)\rightarrow Ag(I)_2\text{–}TPPS+2H^+ \quad [A]$$

On the other hand, the dissociation of silver ion with $Ag(I)_2$–TPPS is shown in reaction [B]. Namely, the $Ag(I)_2$–TPPS dissociated the silver ion with an increasing concentration of coexistent thiol and the fluorescence of TPPS was revived.

* To whom correspondence should be addressed.

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$Ag(I)_2$–TPPS + thiols → TPPS + silver-thiols complex [B]

The fluorescence spectra changes in reactions [A] and [B] are shown in Fig. 1.

Optimum Conditions of Silver Complex Formation Influence of pH: The $Ag(I)_2$–TPPS complex was quantitatively formed and gave a constant fluorescence intensity over the pH range of 12.0—12.9. Due to hydrolysis of the silver ion, the complex formations were carried out at pH = 12.5, which is the center of the complexation pH range.

Influence of Salt Concentration: The $Ag(I)_2$–TPPS became more stable with increasing $NaNO_3$ concentration. The oxidation of the silver(I) complex [$Ag(I)_2$–TPPS, λ_{max} = 443 nm] to the silver(II) complex [Ag(II)-TPPS, λ_{max} = 423 nm] was inhibited by the addition of salt. Potassium nitrate (KNO_3) and potassium sulfate (K_2SO_4), as well as $NaNO_3$, showed a similar effect. The 0.5 M $NaNO_3$ was chosen for the complexation.

Influence of Light: When light was irradiated into the reaction system, the oxidation of the silver(I) complex to the silver(II) complex was promoted. The result was identified by the absorption spectra.[7] Therefore, the complexation was carried out in an amber volumetric flask.

Composition of Silver Complex: The composition of the silver(I) complexes of TPPS was confirmed by the molar ratio method.[7] The results revealed the formation of a 2 : 1 (metal:ligand) complex. At the equivalence point (2 : 1) of silver ion and TPPS, the spectra of free TPPS remained due to incomplete complexation; however, those of its reagent blank were completely quenched by adding excess silver ion.

Also, the $Ag(I)_2$–TPPS complex was stable for at least 4 h under the optimum conditions.[7]

Reactivity of $Ag(I)_2$–TPPS The $Ag(I)_2$–TPPS complex rapidly released the silver ion in the presence of 10^{-7} M levels of thiols having a mercapto group. L-Cysteine and glutathione reacted with $Ag(I)_2$–TPPS at a 1 to 1 molar ratio. On the other hand, 2-mercaptoethanol reacted with the porphyrin complex at a 2 to 1 ratio. Each molar ratio result is shown in Fig. 2. Because of the different molecular structures of thiol compounds, the molar ratio of thiol to $Ag(I)_2$–TPPS was changed. Namely, L-cysteine and glutathione have functional groups such as amino and/or carboxyl groups, besides the mercapto group. But 2-mercaptoethanol does not have such a functional group.

Also, the $Ag(I)_2$–TPPS complex did not release the silver ion in the presence of a 10^{-6} M level of glycine, L-glutamic acid or L-methionine.

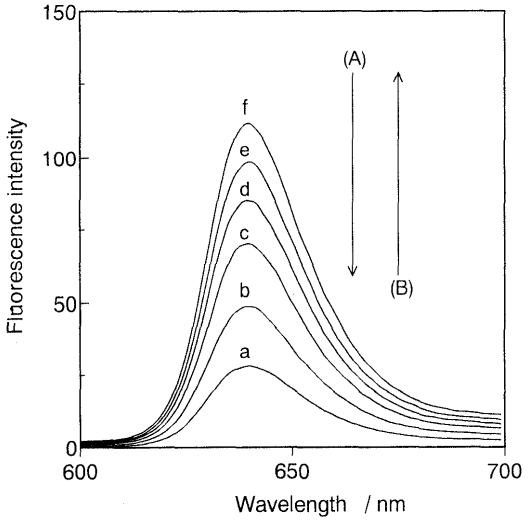

Fig. 1. Emission Spectra of Ag(I)–TPPS System

(A) Various concentrations of Ag(I) ion, (B) various concentrations of glutathione. Glutathione concentration ($\times 10^{-6}$ M): a = 0, b = 0.25, c = 0.5, d = 0.75, e = 1.0, f = 1.25. [TPPS] = 1.0×10^{-6} M, excitation wavelength (λ_{ex}) = 413 nm.

Determination of Thiols Calibration curves, detection limits and precision were obtained for L-cysteine, glutathione and 2-mercaptoethanol under the conditions stated above.

For L-cysteine, the calibration curve was linear in the range of 0—5×10^{-7} M (r = 0.996). The limit of detection (3σ) was 1×10^{-10} M and the relative standard deviation (n = 5) was 1.89% at 2.5×10^{-7} M.

For glutathione, the calibration curve was linear in the range of 0—5×10^{-7} M (r = 0.997). This determination range of glutathione was lower than the NAM method,[2] which was at the 10^{-6} M level (0.4×10^{-6}—16×10^{-6} M). The limit of detection (3σ) was 5×10^{-11} M, and the relative standard deviation (n = 5) was 3.33% at 2.5×10^{-7} M.

For 2-mercaptoethanol, the calibration curve was linear in the range of 0—2×10^{-6} M (r = 0.991). The limit of detection (3σ) was 2×10^{-10} M and the relative standard deviation (n = 5) was 2.33% at 1.0×10^{-6} M.

The presence of 1000 times Na^+, K^+, NO_3^-, and SO_4^{2-}, the presence of 100 times Mg^{2+} and Ca^{2+}, the presence of 10 times glycine, L-glutamic acid and L-methionine and the presence of an equivalent of Fe^{2+}, Cu^{2+}, Zn^{2+}, sodium sulfite(SO_3^{2-}) and L-ascorbic acid did not interfere with

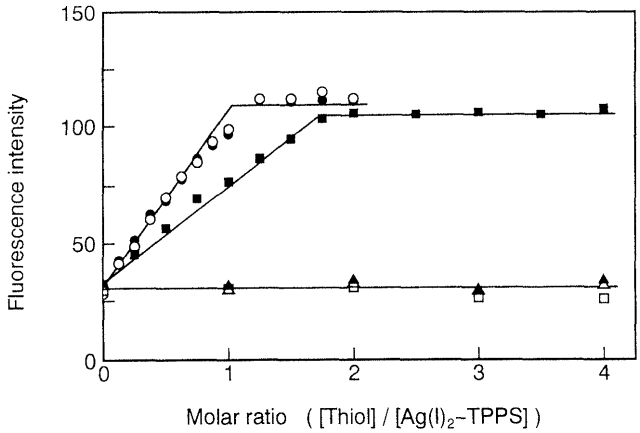

Fig. 2. The Molar Ratio Method in the Thiol–$Ag(I)_2$–TPPS System

[$Ag(I)_2$–TPPS] = 1.0×10^{-6} M; ●, L-cysteine; ○, glutathione; ■, 2-mercaptoethanol; □, glycine; ▲, L-glutamic acid; △, L-methionine.

Table 1. Determination of Glutathione in Medicines

Sample	Glutathione content (mg)	Proposed method		NAM[a] method	
		Glutathione (mg)	(R.S.D. (%))[b]	Glutathione (mg)	(R.S.D. (%))[b]
A[c]	100	104	(0.99)	115	(2.14)
B[d]	100	108	(0.80)	103	(1.81)

a) *N*-(9-Acridinyl)maleimide. *b*) Relative standard deviation, 5 determinations. *c*) An antidote for medical poisoning. *d*) A collyrium for cataracts.

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the determination of 2.5×10^{-7} M glutathione.

Application to Practical Samples The proposed method was applied to the determination of glutathione in actual medicines. The results obtained for the determination of this species in two medicine samples (Sample A: an antidote for medical poisoning, and Sample B: a collyrium for cataracts) are summarized in Table 1 and compared with those obtained by the NAM method.[2] The glutathione content of the medicine is also shown in Table 1. In the proposed method, the recommended procedure was carried out at pH 12.5. At this high pH, thiols such as L-cysteine and glutathione may be air oxidized to disulfide, and this reaction may interfere with the accuracy of the determinations. However, the results of this method agreed with that by the NAM method, carried out at pH 3.5, because the complexation and the dissociation of silver ion in this method are very rapid compared to the oxidation of thiols.

Moreover, in order to apply the proposed method to a more extensive practical sample, a proper pretreatment method for the complicated matrix should be used, and therefore increased selectivity for individual thiols should be achieved. With respect to the latter point, combination with a high performance liquid chromatography(HPLC)-post column system[12] or an enzyme reaction[13] such as the NAM method reported previously, will provide an illuminating method.

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