Spectrophotometric Determination for 10^{-8} M Levels of L-Ascorbic Acid Using the Diazotization of 5,10,15,20-Tetrakis(4-aminophenyl)porphine

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A water-soluble porphyrin having four aminophenyl groups 5,10,15,20-tetrakis(4-aminophenyl)porphine (TAPP) diazotizes with nitrite ion and produces a large spectral change (ε: molar absorptivity, $\Delta\varepsilon = \varepsilon_{sample} - \varepsilon_{blank} = 3.0 \times 10^5\ M^{-1}\ cm^{-1}$, 434 nm). The coexistence of L-ascorbic acid (AsA) strongly interferes with this reaction. Therefore, a highly sensitive spectrophotometric determination of AsA was developed using this effect. For the determination of trace amounts of AsA, the calibration curve was linear in the concentration range of 8.0×10^{-8} M—5.0×10^{-7} M (14—88 ppb). The detection limit (3σ) was 1.0×10^{-8} M (1.76 ppb), and the relative standard deviation was 1.86% (2.0×10^{-7} M (35 ppb) AsA, 6 determinations). This method is more than 20 times highly-sensitive than the indophenol method as standard spectrophotometry for AsA. This method has been applied to the determination of AsA in soft drinks and drugs.

Key words water-soluble porphyrin; L-ascorbic acid; diazotization; soft drink; drug; spectrophotometric determination

L-Ascorbic acid (AsA) plays important roles in life support in living bodies, *e.g.*, substance metabolism or biosynthesis of collagen. AsA is commonly added to juices, soft drinks, canned foods, cooked meats and drugs for the purpose of preventing degradation, retaining flavor and improving nutrition. Thus, the determination of AsA has become an important subject in the fields of biochemistry and food chemistry.[1,2]

There are two major methods for the spectrophotometric determination of AsA. The indophenol method[3,4] is limited by its low selectivity and the poor stability of the chromogenic reagent. Although the dinitrophenylhydrazine method[5,6] has high selectivity, its procedure is complicated. Both these techniques have low sensitivity, although the molar absorptivity (ε) of the reagents can be as much as several thousand. Many means of determining AsA have been reported besides spectrophotometry. For example, the electrochemical method[7] involves interference due to coexisting impurities with oxidation potentials close to that of AsA. Though its sensitivity is high, the chemiluminescence technique[8] is often altered by background influence. Hence, a new highly-sensitive determination method for AsA has been desired.

Porphyrin compounds are known as analytical reagents with highly sensitive spectrophotometric properties. They have a Soret band with molar absorptivity on the order of several hundred thousandth; therefore, many methods using porphyrin have been reported such as those for determining metal ions and anion species.[9-14] We have recently reported a phenomenon in which a water-soluble porphyrin having four aminophenyl groups 5,10,15,20-tetrakis(4-aminophenyl)porphine (TAPP) is diazotized with nitrite ion and produces a large spectral change ($\Delta\varepsilon = \varepsilon_{sample} - \varepsilon_{blank} = 3.0 \times 10^5\ M^{-1}\ cm^{-1}$, 434 nm).[15] This reaction strongly interferes with the coexistence of AsA. This interference effect was applied to the determination of AsA because of its selectivity. A highly sensitive spectrophotometric determination method for 10^{-8} M levels of AsA has been developed and has been used to evaluate AsA in soft drinks and drugs.

Experimental

Reagents All reagents used were of analytical reagent grade and were used as received. Deionized and distilled water was used for all solution preparations. TAPP was purchased from Tokyo Chemical Co., Ltd. A stock solution of TAPP was prepared by dissolving it in water with a small amount of hydrochloric acid. Fresh stock solutions of AsA and sodium nitrite were prepared daily. All the solutions described above were stored in polystyrene bottles in a refrigerator. All working solutions were prepared just before use and temperature-controlled using a water bath with a thermostat.

Apparatus A Hitachi model 200-10 double beam spectrophotometer (with a 1-cm cell) and a Hitachi model 057 X-Y recorder were used for the spectral measurements. A Horiba F-8AT pH meter was used for the pH measurements. A Yamato-Komatsu CTE42A thermostat circulator with a water bath was used to control the determination temperature.

Recommended Procedure One milliliter of 2.5×10^{-5} M TAPP aqueous solution, 1.0 ml of 1.0 M hydrochloric acid solution and 1.0 ml of 1.0×10^{-2} M EDTA solution are mixed. The sample solution containing AsA is added to this mixture. One milliliter of 1.8×10^{-5} M sodium nitrite solution is added and diluted to 10 ml with water and the resulting solution is allowed to react for 20 min at 35 °C. The change in absorbance is then measured after 30 min as ΔA, which is given by $\Delta A = A_{blank} - A_{sample}$ at 434 nm.

Results and Discussion

Mechanism of Spectral Change Figure 1 shows the spectral change of TAPP. When the aminophenyl group diazotized, the absorbance of the Soret band decreased significantly (approximately $\Delta\varepsilon = 3.0 \times 10^5\ M^{-1}\ cm^{-1}$, 434 nm). The mechanism of this chromogenic reaction can be explained as follows: When the amino-phenyl group in TAPP was diazotized with nitrite ion, the diazo-phenyl group produced was easily transformed into a kind of quinoid form by the electron-attractive property of the porphine ring at the para position. In general, when a chromogenic reagent is converted to a quinoid form, its absorbance is enhanced by the extension of the π-electronic resonance plane.[9] In this reagent, however, the 18π-electronic resonance structure at the porphine ring was disturbed due to the $+I$ effect of the outer quinoid form; hence, its absorbance was decreased.[15,16] Such decrease of absorbance at the Soret band has been exemplified by the use of π–π^* electron transition.[17] In this system, the change in the absorbance is quantitative in a certain range where the decreased width of the absorbance by the

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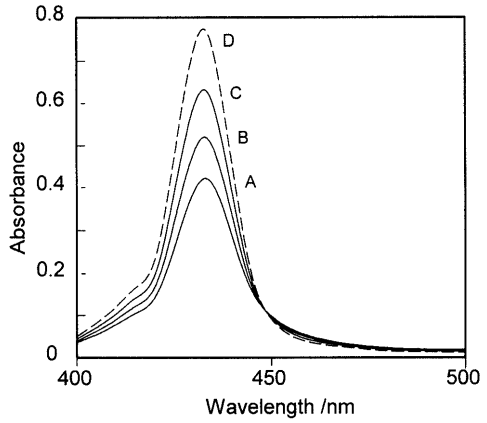

Fig. 1. Spectral Change of TAPP Aqueous Solution

$[TAPP]_T = 2.50 \times 10^{-6}$ M, $[NaNO_2]_T$ = A, B, C: 1.75×10^{-6} M, D: 0 M, [AsA] = A, D: 0 M, B: 5.0×10^{-7} M, C: 5.0×10^{-6} M.

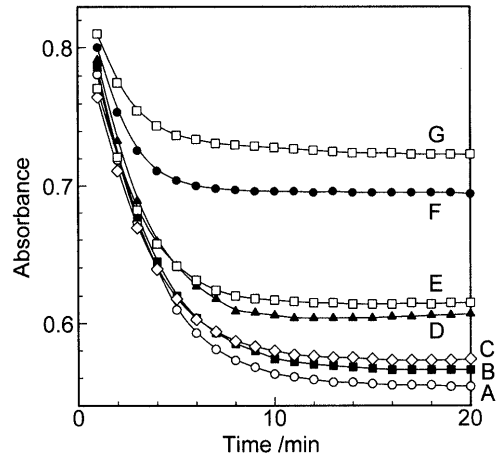

Fig. 2. The Time *versus* Absorbance Plots in TAPP Aqueous Solution

$[TAPP]_T = 2.5 \times 10^{-6}$ M, $[NaNO_2]_T = 1.75 \times 10^{-6}$ M, $[AsA]_T$ = A: 0 M, B: 1.0×10^{-7} M, C: 1.0×10^{-6} M, D: 5.0×10^{-6} M, E: 1.0×10^{-5} M, F: 5.0×10^{-5} M, G: 1.0×10^{-4} M.

diazotization becomes less with a certain amount of AsA coexisting in the solution. The decrease in absorbance is not restored even if AsA is added to the system after the diazotization, nor does the spectrum change when only AsA is added to the TAPP solution. From these findings, it is assumed that the absorbance change is interfered with so long as AsA is reduced and consumes nitrite ion. The diazotization reaches an equilibrium within 5—20 min in this system (Fig. 2); therefore, the reaction time was determined to be 20 min. When the nitrite solution is added to the system before the sample is spiked with AsA, the diazotization occurs first, and the reproducibility in the determination becomes improper, because the diazotization is much faster than the reduction. Nitrite solution was therefore added at the end of the procedure.

Concentration of Nitrite Ion The concentration of nitrite ion added should always be $[AsA] < [NO_2^-] < [TAPP]$ because nitrite ion and TAPP react in a molar ratio of 1 : 1 when the spectrum change occurs.[15] The linear range of the calibration graph changed in proportion to the concentration of added nitrite ion. Therefore, the added quantity of nitrite was determined to be 1.8×10^{-6} M which should provide good linearity in the calibration graph.

Table 1. Tolerance Limits of Foreign Ions for Determination of AsA[a]

Foreign ion	Tolerance limit[b] (M)
K^+, Na^+, Ca^{2+}, Mn^{2+}, Mg^{2+}, NH_4^+, Cl^-, NO_3^-, SO_4^{2-}, CO_3^{2-}, PO_4^{3-}, glucose, lactose, sucrose, galactose, fructose, glycine, citric acid, EDTA	2.0×10^{-4}
Cu^{2+}, Zn^{2+},[c] Br^-, CH_3COO^-, D-xylose	1.0×10^{-4}
Al^{3+}[c]	2.0×10^{-5}
I^-	1.0×10^{-5}
VO_3^-, Fe^{2+},[c] SO_3^{2-}	2.0×10^{-6}
Fe^{3+},[c] Ti^{4+}[c]	2.0×10^{-7}
H_2O_2, L-cysteine	1.4×10^{-7}
NO_2^-	2.0×10^{-8}

a) AsA taken: 2.0×10^{-7} M. *b*) Deviation of ±3.0% was allowed for absorbance of AsA. *c*) The interfering metal ions Fe^{2+}, Fe^{3+}, Ti^{4+}, Al^{3+} and Zn^{2+} were masked completely by adding 10^{-3} M of ethylenediaminetetraacetic acid.

Effect of pH The reagent TAPP is soluble in water at pH < 3, because it is soluble by virtue of the protonation of amino groups. The acid dication form of TAPP (H_4P^{2+} at 434 nm) is stable at pH < 1.5. In general, AsA is stable in the very low pH range. In this study, AsA reacted quantitatively in the pH range of 0.1—1.5. All subsequent determinations were carried out at pH 1.0.

Reaction Temperature The effect of temperature on the reaction was examined (0 °C < T < 45 °C). The diazotization rate was so slow that the reaction was not completed within 20 min at low temperature (T < 25 °C), while the sensitivity became lower based on the degradation of the diazonium salt at higher temperatures (T > 40 °C). All subsequent determinations were carried out at 35 °C from the point of view of stability and sensitivity.

Calibration Graphs A linear calibration graph was obtained in the range of 8.0×10^{-8} M—5.0×10^{-7} M (14—88 ppb) AsA. The lowest detection limit (3σ) was 1.0×10^{-8} M (1.76 ppb). The relative standard deviation was 1.86% (2.0×10^{-7} M (35 ppb), 6 determinations).

Interference of Foreign Ions Table 1 shows that many foreign ions were tolerated in rather large amounts. Reducing sugars and Cu^{2+} are known as interfering substances in the determination of AsA by the indophenol method, however, they only slightly interfered using this method. Most of the interfering metal ions, such as Fe^{2+}, Fe^{3+}, Ti^{4+}, Al^{3+} and Zn^{2+}, were completely masked by adding EDTA. Excess EDTA had no effect on the reaction system. All of the following determinations with real samples were carried out with the addition of 1.0 ml of 1.0×10^{-2} M EDTA. For hydrogen peroxide and cysteine, the tolerance limit was less than twice the nitrite ion concentration. However, this does not become a problem in actual determination, because there are few samples in which cysteine and AsA are included at concentrations of the same order except for certain drugs. The tolerance limit was less than 0.1 the AsA concentration in the case of nitrite ion. A separation method using ion chromatography is necessary to apply this method to biological samples such as urine and serum which have higher concentrations of nitrite.

Determination of AsA in Soft Drinks and Pills This method was used to determine of AsA in diluted soft drinks

Table 2. Determination of AsA in Soft Drinks[a]

	[AsA] M (R.S.D./%)[b]			
	Sample A (Mix vegetable juice)	Sample B (Soft drink)	Sample C (Soft drink)	Sample D (Green tea)
This method	3.17×10^{-4} (0.664)	0.956×10^{-3} (0.588)	2.29×10^{-3} (1.85)	2.10×10^{-3} (2.41)
AsA content	2.99×10^{-4}	1.00×10^{-3}	2.39×10^{-3}	2.20×10^{-3}

a) All drinks were diluted with water to the determination range (× 1000—10000). *b*) 5 determinations.

Table 3. Determination of AsA in Pills[a]

	[AsA] mg/l tablet (R.S.D./%)[b]		
	Sample A (Cold pill)	Sample B (Vitamin tablets)	Sample C (Vitamin C tablets)
This method	88.9 (1.18)	93.2 (3.27)	169 (2.64)
AsA content	83.3	100	167

a) All pills were dissolved in water to the determination range. *b*) 5 determinations.

and drugs. Canned soft drinks were purchased and diluted with water to 10^6 in volume just before use (Table 2). All drug samples were prepared by dissolving the pills with water (Table 3). The value that was obtained agreed with each generally recognized value.

This is the most sensitive of all spectrophotometric determinations of AsA, and has comparatively good selectivity, although the separation of nitrite ion is a problem. However, in preliminary experiments we have succeeded in separating nitrite ion using ion chromatography with an anion exchange column. This technique will be applied to the simultaneous detection of AsA and nitrite using ion chromatography.

Acknowledgment This work was supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists 3237.

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