

Flow injection analysis of ascorbic acid using carriers modified with metal–porphine as oxidative solid catalyses

Akimasa Iwado^a, Masaki Mifune^a, Hiromichi Akizawa^a, Noriko Motohashi^b,
Yutaka Saito^{a,*}

^a Faculty of Pharmaceutical Sciences, Okayama University, Tsushima-Naka, Okayama 700-8530, Japan

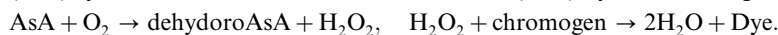
^b Kobe Pharmaceutical University, Motoyamakita-machi, Higashinada-ku, Kobe 658-8558, Japan

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Dedicated to Professor Terumichi Nakagawa on the occasion of his retirement and 63rd birthday.

Abstract

To investigate one of practical applications of the supports modified with metal–porphines as artificial solid-catalysts, columns into which the supports were packed were supplied to catalytic columns for a flow injection analysis (FIA) system for determination of ascorbic acid (AsA) by the following reactions:



Among the columns tested, the column containing silica gels modified with Co–tetrakis(carboxyphenyl)porphine catalyzed most rapidly the oxidation reaction of AsA that is accompanied by the formation of hydrogen peroxide. The resulting hydrogen peroxide was determined by FIA system equipped with the column containing glass beads modified with Mn–tetrakis(carboxyphenyl)porphine, which gave a linear calibration curve and large peak-areas of the range corresponding to AsA concentration between 0.2 and 10 $\mu\text{mol/ml}$. The results indicated that some supports modified with metal–porphine would be applicable to the FIA for AsA as the solid catalyses which function as if the immobilized enzymes.

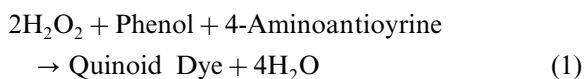
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1. Introduction

We have recently revealed that aminopropyl-glass beads immobilized with Mn^{3+} –porphine ($\text{Mn-P}_{\text{Glass}}$) exhibit the peroxidase (POD)-like

activity in the following colored reaction Eq. (1) catalyzed by native POD [1]:



This POD-like activity of $\text{Mn-P}_{\text{Glass}}$ is useful for determination of hydrogen peroxide by using reaction (1) in place of POD as well as the anion-exchange resins modified with Mn–por-

* Corresponding author

E-mail address: saito@pharm.okayama-u.ac.jp (Y. Saito).

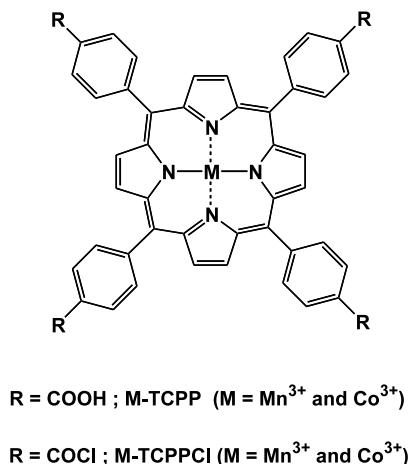


Fig. 1. Structures of metal-porphines.

phine (Mn-P_{Resin}) reported previously [2,3]. In particular, the glass beads as the mother support are superior in sensitivity to the supports of Mn-P_{Resin}, because the anion-exchange resins have electrostatic interactions with phenol and the resulting anionic dye in the neutral pH range. To avoid this demerit and gain the sensitivity, *N,N*-diethylaniline (DEA), which gives a cationic dye, should be used as a chromogen, while DEA reacts more slowly than phenol in the similar reaction of (1). In other words, the method using Mn-P_{Glass}, seems to be more suitable to flow injection analysis (FIA) than that using Mn-P_{Resin}, since a short time is required for determination of hydrogen peroxide. On the Other hand, we also reported that supports modified with Co³⁺-porphine catalyzed an oxidative reaction of ascorbic acid (AsA) [4] by the following scheme (2):



We then suggested that AsA could be spectrophotometrically determined by measuring hydrogen peroxide by using reaction (1). In the present study, to investigate one of the practical applications of supports modified with Co- and Mn-porphines as practical solid-catalyses, we would try to determine AsA by an FIA system using the supports as reactive columns. As a result, we could elucidate two columns packed with supports each serving as a reactor for AsA and solid mimesis of POD in the FIA system.

2. Experimental

2.1. Reagents and chemicals

Metal-tetrakis(4-carboxyphenyl)porphines (M-TCPP, M = Mn³⁺ and Co³⁺, see Fig. 1) were prepared from reagent grade tetrakis(4-carboxyphenyl)porphines (H₂-TCPP, Wako Junyaku, Osaka, Japan) by the methods described in the literatures [5,6]. Metal-tetrakis-(4-chloroformylphenyl)porphine (M-TCPPCl, refer to Fig. 1), acid chloride of M-TCPP, was prepared from M-TCPP and thionyl chloride as reported in the previous papers [4].

Muromac MSA-1 (100–200 mesh, Muromachi Kagakukogyo, Inc. Tokyo, Japan) and Amberlite IRA 900 (24–42 mesh, Organo Co., Tokyo, Japan) were used as mother anion-exchange resins. They were washed with 1 mol/l HCl_{aq}, 1 mol/l NaOH_{aq}, water, successively, and finally were washed with acetone and dried up. Aminopropyl controlled-pore glass beads (particle size; 120–200 mesh) and aminopropyl silica gels (Develosil-NH₂-5, particle size; 5 μm and -NH₂-10, particle size; 10 μm) were purchased from CPG Inc. (Lincoln Park, NJ, USA) and Nomura Chemicals (Kyoto, Japan), respectively, and used as mother inorganic supports.

The anion-exchange resins modified with Co- and Mn-TCPP (25 μmol M-TCPP per g dry resin, M-TCPP_{Resin}), on which M-TCPP was firmly bonded with electrostatic and physical interactions, were prepared by using the anion-exchange resins in chloride form as described previously [2,3]. For preparation of silica gels or glass beads firmly linked by M-TCPP (M-TCPP_{Silica} and -TCPP_{Glass}, M-TCPP 10–30 μmol/g dry support) through peptide bonds [4], a mixture of M-TCPPCl (50–150 μmol) and silica gels or glass beads (5 g) in dioxane were refluxed until the mixture became clear, when all the M-TCPPCl was linked to the supports. All of the modified supports were stable to temperature, in moisture and storage, and M-TCPP on the supports was not eluted at all with water.

Aqueous solutions of AsA were prepared in requirement from AsA (reduced form, Aldrich, WI, Japan).

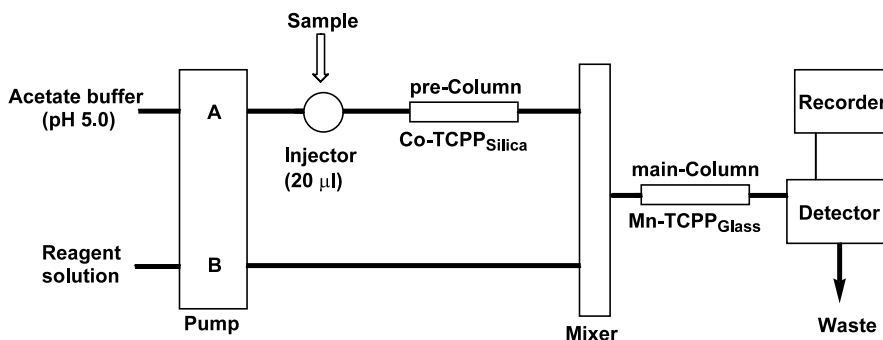


Fig. 2. System of the flow analysis of AsA.

2.2. Apparatus

As shown in Fig. 2, the apparatus was constructed with two dual cylinder pumps (Jasco 880-PU, Japan), a sample injector equipped with 20 μ l sample loop (Rheodyne model 7125, USA), a mixer (Jasco 880-30) and a UV/VIS detector (Jasco 875-UV) for HPLC with a Chromatocorder 21 (System Instruments, Japan). The catalytic pre-column (4×150 mm) and main-column (4×30 mm) packed with Co-TCPP_{Silica} and Mn-TCPP_{Glass}, respectively, were stainless columns in thermostat (35 °C).

2.3. Procedure

A chromogen solution containing 3.5 mg/ml phenol, 0.5 mg/ml 4-aminoantipyrine (AAP) and Palitzch borate buffer (pH 8.5) solutions (1:1:3), was made to flow with the pump B and 0.1 mol/l acetate-buffer (pH 5.0) with the other pump A. The flow rate of the pumps A and B were adjusted to 0.9 and 2.0 ml/min, respectively. The samples containing 0.2–10.0 μ mol of AsA per 1.0 ml were injected into the acetate-buffer-flow tube. The

quinoid dye resulted from hydrogen peroxide was detected at 505 nm, and the absorbances were recorded. The calibration curve for AsA was obtained on the basis of peak-areas.

3. Result and discussions

3.1. Pretest conducted by a batch system

Before the construction of the FIA system, we examined optimal conditions the supports modified with Co- and Mn-porphines for the oxidation of AsA and the formation of the quinoid dye, respectively, by using batch methods. As results, 12 μ mol of AsA in a pH 5.0 acetate-buffer (5.3 ml) was completely oxidized by the incubation for 30 min at 35 °C. Particularly, it should be noted that the 20 μ mol/g Co-TCPP_{Silica} catalyzed most rapidly the oxidation reaction of AsA among the tested Co-TCPP_{Silica} (5, 10, 20 and 30 μ mol/g). For the formation of the quinoid dye resulted from hydrogen peroxide, the similar conditions reported previously [1] were suitable.

3.2. Conditions for oxidative reaction (1) of AsA in the pre-column

To examine the reaction conditions of the FIA system, 20 μ l of 8.0 μ mol/ml AsA solution was injected as a sample solution, and the resulting peak was initially evaluated regarding peak-height, -width, -area and -form. We then decided to use the peak-area and -form mainly, as a criterion in the selection of the conditions.

Table 1
Effect of the length of column-I ($\phi = 4$ mm) on the peak

Column (mm)	Retention time (min)	Peak-area	Peak-width (min)
4 \times 50	2.21	3.87×10^4	4.6
4 \times 100	2.83	3.93×10^4	4.7
4 \times 150	3.69	7.82×10^4	4.3

Flow rate: 0.9 ml/min at 35 °C.

As a carrier, we selected Co-TCPP_{Silica} (20 $\mu\text{mol/g}$) making reference to the pretest conducted by a batch method. The pretest also indicated that the optimal pH and temperature for the oxidative reaction (2) were pH 5.0 and 35 °C, respectively.

Table 1 represents the effect of the length of pre-column on the peak. As shown in Table 1, the peak-area exhibited a tendency to increase as the column becomes longer. Furthermore, in the cases of the 50 and 100 mm-columns, the peak-areas obtained were variable in each injections. It is probably that the longer column, i.e. longer reaction time, is preferred to make the complete progress of the oxidative reaction Eq. (2), because the reaction-rate of the reaction (2) is rather slow.

Since the flow rate also affects the reaction-rate, i.e. the peak-area we examined the flow rate around 1.0 ml/min. As the result, it was revealed that the rate of 0.9 ml/min gave enough peak-area and -form for the determination. In these conditions, AsA was almost completely converted into DhAsA by oxidative activity of the pre-column.

3.3. Conditions for color-reaction (2) of H_2O_2 in the main-column

Mn-TCPP was used as a metal-porphine, which we previously reported to show a strong POD-like activity for H_2O_2 when fixed on an anion-exchange resin [2,3,8]. We have also reported that Mn-TCPP_{Glass} shows a strong POD-like activity and Mn-TCPP_{Glass} can be used as a reaction catalysis in place of immobilized POD for the determination of hydrogen peroxide [1]. As chromogens, among a lot of reagents reported [9–13], phenol and AAP were selected because they can give a quinoid dye more rapidly than chlorophenol [14,15] and *N,N*-diethylaniline with AAP [2,9].

As a mother carrier, two anion-exchange resins, glass beads and silica gels were examined by using five columns, 4 \times 15, 4 \times 20, 4 \times 30, 4 \times 50 and 2 \times 50 mm as a mother carrier. As the result, the column-length was revealed not to affect the sensitivity but the linearity of the calibration curve. Among the 15 columns tested, only three columns shown in Fig. 3 gave a linear calibration curve. As seen from Fig. 3, the Mn-TCPP_{Glass}

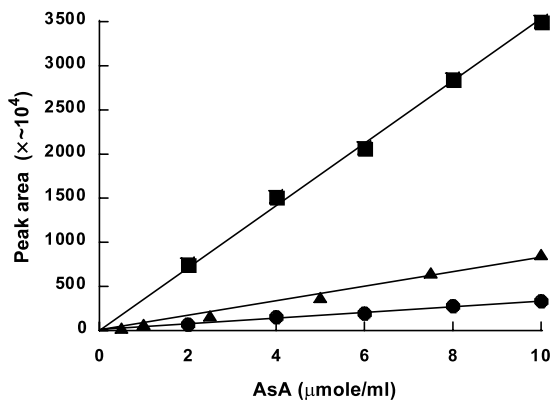


Fig. 3. Effect of mother carriers on the calibration curves. (●) Mn-TCPP_{Glass} column (4 \times 30 mm); (▲) Mn-TCPP_{Silica} column (4 \times 15 mm); (■) Mn-TCPP_{Resin} column (4 \times 20 mm).

column gave much larger peak-area than Mn-TCPP_{Silica} and -TCPP_{Resin} columns did. Thus, we selected Mn-TCPP_{Glass} packed in a 4 \times 30 mm column as a solid mimetic of the immobilized POD in this study. Probably, since the resulting quinoid dye exhibits an anionic property, the more dye may be adsorbed on the mother resin and silica gels than the glass beads.

The optimal pH for the Mn-TCPP_{Glass} column is around pH 7.5. Accordingly, the pH of the flow containing hydrogen peroxide from the pre-column should be adjusted to about pH 7.5 before it runs into the main-column. Both the flow rate and the pH would affect the POD-like activity of the Mn-TCPP_{Glass} column. We then examined the effect of pH on peak-area by using various buffer solutions as well as flow rates. As the result, the maximum peak-area was obtained, when borate buffer (pH 8.5) containing chromogens was mixed into the acetate-buffer (pH 5.0) at a flow rate of 2.0 ml/min. Thus, borate buffer (pH 8.5) was chosen as a component of the carrier solution. In these conditions, the absorbance of quinoid dye by Mn-TCPP_{Glass} reached up over 90% to that by POD [1].

3.4. Calibration curve and reproducibility

A part of the charts used for calibration is shown in Fig. 4. The peaks were sharp enough for the determination. The calibration curve was

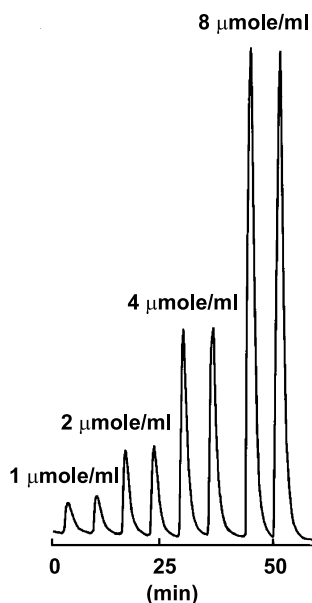


Fig. 4. A part of the chart for calibration curves.

linear within the AsA concentration between 0.20 and 10.0 $\mu\text{mol/ml}$. The coefficient of variation ($n = 10$) was 4.1% for 8.0 $\mu\text{mol/ml}$ AsA solution. As well known, numerous FIA methods for determination of AsA were reported to date as summarized in a recent review [16]. However, most of such method used compliment method, expensive reagent or special devices. As compared with these FIA methods for AsA, the present simple FIA method is not good in sensitivity, but simplicity and convenience. For an example, an assay on average took less than 6 min, which was shorter than that in the case of the electrochemical fiber/biosensor FIA system [7]. The durability of this system was examined using 4.0 $\mu\text{g/ml}$ ASA solution. The peak-areas are almost constant from first injection to 100th injection.

Table 2
Effect of foreign equimolar molecules to AsA

Molecule	KCl	CaCl ₂	KBr	NH ₄ Ac ^b	NaHCO ₃	NaH ₂ PO ₄	FeCl ₃
Error(%) ^a	-3.9	7.0	0.4	-7.8	-9.5	-15.6	-68.4

Foreign molecules (8.0 μmol) were added into 8.0 $\mu\text{mol/ml}$ AsA solution.

^a $\{(\text{Found AsA} - \text{added AsA}) / \text{added AsA}\} \times 100$.

^b NH₄Ac=CH₃COONH₄.

Table 3
AsA content (%) in vitamin compounds and soft drinks

Supplier	This FIA (%) ^a	InPh (%) ^a
<i>Vitamin compound</i>		
A granules (Shionogi)	112 ^b	81
B granules (Kowa)	104	112
C granules (Esuesu)	112	125
D tablet (Morishita-Jintan)	107	113
<i>Soft drink</i>		
A _s (Otsuka)	125	136
B _s (Suntory)	161	157
C _s (Takeda)	133	93
D _s (Suntory)	120	112

^a Content (%) = (observed AsA/indicated AsA) \times 100 (%).

^b Coefficient of variation ($n = 10$) was 3.4%.

The above results indicate that the Mn-TCPP_{Glass} and the Co-TCPP_{Silica} columns play roles just like the columns packed with the immobilized enzymes such as POD and ascorbate-oxidase.

3.5. Effect of foreign substances

Under the conditions of the procedure, some ions were examined for interference as summarized in Table 2. The largest decrease in peak-area was observed in the presence of equimolar FeCl₃ to AsA among the molecules examined. This interference may be caused from the reaction between AsA and Fe³⁺ ion.

3.6. Application

The present FIA method was applied to the determination of AsA in vitamin compounds and soft drinks. The results are summarized in Table 3 together with those obtained by an official indo-

phenol method (InPh) [17]. The AsA contents obtained by both methods were roughly agreeable with each other. The InPh method gave illegitimately lower AsA-values (81 and 93%) than the indicated values. The recoveries of AsA were 95 and 98% for the soft drinks B_s and C_s spiked with 50% AsA of the indicated value, respectively.

4. Conclusion

In conclusion, some supports modified with metal–porphine would be applicable to the FIA method for ASA as the solid catalyses which act as if were immobilized enzymes.

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