Spectrophotometric Determination of Hydrogen Peroxide, Glucose, Uric Acid, and Cholesterol Using Peroxidase-like Activity of an Fe(III) Complex of Thiocalix[4]arenetetrasulfonate Attached to an Anion-exchanger

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An iron(III) complex of thiocalix[4]arenetetrasulfonate attached to an anion-exchanger (Fe^{3+} -TCAS_{A-500}) showed high peroxidase-like catalytic activity at pH 5–8 for the formation of quinoid dye, following the color reaction between 3-methyl-2-benzothiazolinone hydrazone (MBTH) and *N*-ethyl-*N*-(3-sulfopropyl)aniline (ALPS) in the presence of H_2O_2 . This catalytic activity of Fe^{3+} -TCAS_{A-500} for the MBTH-ALPS system was applied for the spectrophotometric determination of H_2O_2 , glucose, uric acid, and cholesterol. The calibration curves were linear in the concentration range from 1.0 to 15 μg of H_2O_2 in a 1.0-mL sample solution, and from 5.0 to 60 μg of glucose, 2.0 to 30 μg of uric acid, and 11.6 to 116 μg of cholesterol in a 0.5-mL sample solution. The apparent molar absorptivity of H_2O_2 was determined as $2.31 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$, which was about 70% of that by peroxidase under the same conditions. The determination method using Fe^{3+} -TCAS_{A-500} was applied for the determination of glucose and uric acid in both control sera I and II.

Keywords Peroxidase-like activity, thiocalix[*n*]arene, iron(III) complex, photometric determination, hydrogen peroxide, glucose, uric acid, cholesterol, anion-exchanger

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Introduction

Calix[*n*]arenes are a class of macrocycles that have been investigated extensively in various fields because of their high potential for forming host-guest complexes.^{1,2} A large number of calix[*n*]arene derivatives have been developed by modifying either the upper- or lower-rim of their structure for use as analytical reagents for separations or sensors of various ions and molecules, among other applications.^{3,4} However, derivatives like thiocalix[*n*]arenes are interestingly able to form very stable metal complexes without any modification of their rims.^{5–8} They have a unique structure where the methylene units of the parent calix[*n*]arenes are replaced by sulfur (–S–) atom linkages, as shown in Fig. 1.^{9–11} Motivated by the complexation properties of thiocalix[*n*]arenes, we prepared an anion-exchanger modified with Fe^{3+} -thiocalix[4]arenetetrasulfonate (Fe^{3+} -TCAS_{A-500}) as shown in Fig. 1, and demonstrated high peroxidase-like catalytic activity.^{12,13}

In clinical analyses, various enzymes are frequently utilized for determining trace amounts of vital compounds in numerous components of vital fluids with high selectivity and specificity.¹⁴ However, these enzymes generally have some disadvantages regarding stability, purity, handling and storage. To improve these disadvantages, many artificial mimesis have been

developed for use instead of these enzymes.^{15–17} However, most of them have not always been developed for the purpose of analytical reagents for clinical analyses, although they have exhibited high activities as effective catalysts. So far, only a few artificial mimesis have been developed, which could be easily prepared and showed high enzyme-like activities.^{12,18} Moreover, they were actually usable instead of acting as enzymes in clinical analyses. As an example, we developed Fe^{3+} -TCAS_{A-500} with high peroxidase-like activity, which was easy to prepare and handle, very stable, and could be used repeatedly. In addition, we found new methods in which the activity of Fe^{3+} -TCAS_{A-500} could be used for the photometric and fluorometric determinations of H_2O_2 , glucose and uric acid in clinical analyses.^{12,13,19,20}

For the spectrophotometric determination of H_2O_2 in clinical analyses, 4-aminoantipyrine (4-AAP) and phenol or *N,N*-dialkylaniline derivatives were frequently employed in enzymatic methods using peroxidase as a coupler and a hydrogen donor, respectively.¹⁴ Based on the concept that the amount of the vital samples should be minimized in clinical analyses, new chromogenic reagents with higher sensitivity and selectivity have been developed. So far, in order to determine as small an amount of H_2O_2 as possible, MBTH and Trinder's reagents have been developed as a new coupler and hydrogen donors, respectively.^{21–24} Mizoguchi *et al.* demonstrated that Trinder's reagents were characteristic of producing quinoid dyes with larger molar absorptivity and longer wavelength of their λ_{max} by coupling with 4-AAP or MBTH, and the characteristics

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Fig. 1 Structure of sodium thiacalix[4]arenetetrasulfonate, and the proposed structure of Fe^{3+} -TCAS_{A-500}.

were dependent on substituent groups (especially, R^1 and R^2) in the structure shown in Fig. 2.²³ Further, as a coupler with Trinder's reagents, MBTH was superior to 4-AAP especially in sensitivity.

In previous reports, we have shown that color reactions between 4-aminoantipyrine (4-AAP) and phenol or between 3-methyl-2-benzothiazolinone hydrazone (MBTH) and *N*-(3-sulfopropyl)aniline (HALPS) could be used for the spectrophotometric determination of H_2O_2 or glucose using Fe^{3+} -TCAS_{A-500}.^{12,13} However, those methods have still some disadvantages regarding reaction conditions, such as narrow optimum pH range, relatively long reaction time, *etc.* In this study we have attempted to alleviate this problem by following the color reactions between MBTH and Trinder's reagents (Fig. 2) using Fe^{3+} -TCAS_{A-500}. In addition, we have also investigated new methods for the spectrophotometric determination of H_2O_2 , glucose, uric acid, and cholesterol.

Experimental

Reagents and apparatus

Sodium thiacalix[4]arenetetrasulfonate (TCAS, Fig. 1), prepared according to methods described in literature,²⁵ was donated by the Cosmo Research Institute. DEAE cellulofine A-500 (an anion-exchanger of the cellulose-type with diethylaminoethyl groups) was purchased from Seikagaku Kogyo Co. (Tokyo, Japan). It was washed with water several times and subsequently dried over phosphorous pentoxide (P_2O_5) under reduced pressure before use. Horseradish peroxidase (POD, EC 1.11.1.7; Type VI; activity, 100 units mg^{-1}), cholesterol esterase (COE, EC 3.1.1.13; *Pseudomonas sp.*; 10 – 30 units mg^{-1}), MBTH, and control sera I and II were purchased from Wako Pure Chemical Industries Co. (Osaka, Japan). Glucose oxidase (GOD, *Aspergillus niger*; activity, 100 – 250 units mg^{-1}), uricase (*Candida sp.*, recombinant, expressed in *E. coli*; activity, ≥ 2 units mg^{-1}), and cholesterol

HALPS ($R^1=H; R^2=H; R^3=H; R^4=H$)

ALPS ($R^1=H; R^2=H; R^3=C_2H_5; R^4=H$)

ADPS ($R^1=OCH_3; R^2=H; R^3=C_2H_5; R^4=H$)

TOOS ($R^1=CH_3; R^2=H; R^3=C_2H_5; R^4=OH$)

ADOS ($R^1=OCH_3; R^2=H; R^3=C_2H_5; R^4=OH$)

DAOS ($R^1=OCH_3; R^2=OCH_3; R^3=C_2H_5; R^4=OH$)

HDAOS ($R^1=OCH_3; R^2=OCH_3; R^3=H; R^4=OH$)

Fig. 2 Structure of Trinder's reagent.

oxidase (COD, EC 1.1.3.6; *Rhodococcus sp.*; activity, ≥ 30 units mg^{-1}) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Sodium *N*-ethyl-*N*-(3-sulfopropyl)aniline (ALPS), sodium *N*-ethyl-*N*-(3-sulfopropyl)-3-methoxyaniline (ADPS), sodium *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline (TOOS), sodium *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methoxyaniline (ADOS), sodium *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (DAOS), and sodium *N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (HDAOS) were supplied by Dojindo Laboratories (Kumamoto, Japan). All other chemicals were of either analytical or reagent grade and were used without further purification.

The sample solutions of cholesterol and cholesteryl palmitate were prepared as follows. Cholesterol (11.6 mg) was dissolved in ethanol (100 mL), and then this stock solution was diluted to the desired concentration in distilled water before use. According to methods described in literature,²⁶ cholesteryl palmitate (20.0 mg) was dissolved in a 10% solution of Triton X-100 in isopropyl alcohol (50 mL), and then this stock solution was diluted to the desired concentration with a 10% solution of Triton X-100 in isopropyl alcohol before use. The anion-exchanger modified with Fe^{3+} -TCAS (Fe^{3+} -TCAS_{A-500}) was prepared according to method A, described in the literature.¹² It contained 100 μmol of Fe^{3+} -TCAS per 1 g of dry anion-exchanger.

The absorption spectra and absorbances were recorded on a Shimadzu UV-1600 PC double-beam spectrophotometer using a 10-mm quartz cell.

Method for the spectrophotometric determination of H_2O_2 using the MBTH-ALPS system

The spectrophotometric determination of H_2O_2 was carried out by measuring the absorbance at 597 nm of the quinoid dye produced on the color reaction between MBTH and ALPS. Fe^{3+} -TCAS_{A-500} (20 mg, 100 $\mu mol g^{-1}$) was added to a mixture containing the sample solution (1.0 mL, 1.0 – 15 μg of H_2O_2) and the reagent solution A (5.0 mL). The mixture was incubated at room temperature for 10 min. Then, the Fe^{3+} -TCAS_{A-500} was filtered off, and the absorbance at 597 nm of the supernatant was measured against a reagent blank solution.

POD was used instead of Fe^{3+} -TCAS_{A-500} as a reference. A

mixture of the sample solution (1.0 mL), POD solution (0.5 mL, 25 units mL⁻¹) and reagent solution B (4.5 mL) was incubated at room temperature for 10 min. The absorbance at 597 nm of this reaction mixture was also measured against a reagent blank solution.

The reagent solutions A and B mentioned above consisted of a 1:1:3 and 1:1:2.5 (v/v) mixture of 0.1 mg mL⁻¹ MBTH, 1.0 mg mL⁻¹ ALPS, and buffer solutions, respectively. The buffer solutions used were 0.1 M CH₃COONa-0.1 M CH₃COOH for pH 4–7, 1/15 M Na₂HPO₄-1/15 M KH₂PO₄ for pH 6–8, 0.1 M NH₄OH-0.1 M NH₄Cl for pH 8–9, 0.05 M Na₂B₄O₇ (0.2 M H₃BO₃ + 0.05 M NaCl) for pH 8–9, and 0.2 M Na₂CO₃-0.2 M NaHCO₃ for pH 10.

GOD/Fe³⁺-TCAS_{A-500}, uricase/Fe³⁺-TCAS_{A-500}, COD/Fe³⁺-TCAS_{A-500} based methods for the determination of glucose, uric acid, and cholesterol, respectively

Glucose, uric acid, and cholesterol were indirectly determined by measuring the amount of H₂O₂ produced in the following reactions (1), (2), and (3) catalyzed by the corresponding oxidase, respectively:

Fe³⁺-TCAS_{A-500} (20 mg) was added to a mixture containing the sample solution (0.5 mL), the corresponding oxidase solution (0.5 mL) and the reagent solution A (5.0 mL); the mixture was incubated at 40°C for 30 min. Then, the Fe³⁺-TCAS_{A-500} was filtered off, and the absorbance at 597 nm of the supernatant was measured against a reagent blank solution. In the case of the reference POD, a mixture of the sample solution (0.5 mL), the corresponding oxidase solution (0.5 mL), the POD solution (0.5 mL, 25 units mL⁻¹) and the reagent solution B (4.5 mL) were incubated at 40°C for 30 min. The absorbance at 597 nm of this reaction solution was measured against a reagent blank solution.

The corresponding oxidase solutions, GOD (32 units mL⁻¹), uricase (2.5 units mL⁻¹), and COD (1.6 units mL⁻¹) were used for the determination of glucose, uric acid, and cholesterol, respectively.

Results and Discussion

Activity of Fe³⁺-TCAS_{A-500} for color reactions

Tamaoka *et al.* demonstrated that Trinder's reagents, such as ALPS, ADOS, ADPS, and TOOS, were very useful for the spectrophotometric determination of H₂O₂ in the color reactions where 4-AAP was used as a coupler.^{21,22} In this study, MBTH was used as a coupler in color reactions in place of 4-AAP, because MBTH was coupled with hydrogen donors, such as Trinder's reagents, to produce quinoid dyes with larger molar absorptivity and longer wavelength of their λ_{max} than those by 4-AAP, as pointed out by Mizoguchi *et al.*²³ The peroxidase-like activity of Fe³⁺-TCAS_{A-500} was evaluated using the color reactions between MBTH and Trinder's reagents for 7 μg of H₂O₂ in buffer solutions of various pH levels. Figure 3 shows the absorbance at λ_{max} for the corresponding quinoid dye produced through color reactions by Fe³⁺-TCAS_{A-500} at each pH. The data observed for DAOS and HDAOS are not shown because of the relatively low absorbance in all buffer solutions.

The λ_{max} of the corresponding quinoid dye was observed at 597, 564, 554, and 588 nm in the relatively longer wavelength region than that for 4-AAP-Trinder's reagent system for ALPS, ADOS, ADPS, and TOOS, respectively.^{21,22} As shown in Fig. 3, Fe³⁺-TCAS_{A-500} exhibited high activity in acetate buffer of pH 5–7 and ammonia buffer of pH 8 for all Trinder's reagents, whereas it exhibited relatively weak activity in phosphate, borate, and carbonate buffer solutions. As pointed out by Tamaoka *et al.*,^{21,22} each optimum pH for color reactions between 4-AAP and Trinder's reagents in the presence of H₂O₂ by POD ranged generally from 5.5 to 9.5. Also in the MBTH-Trinder's reagent system, it will not be suitable in a carbonate buffer of pH 10. In phosphate and borate buffer solutions, a coordination of phosphate and borate ions to Fe³⁺ ion of Fe³⁺-TCAS_{A-500} will result in decreasing the activity of Fe³⁺-TCAS_{A-500}.

In the previous methods,^{12,13} Fe³⁺-TCAS_{A-500} exhibited high activity at pH 10–11 and at pH 5–6 for the color reaction between 4-AAP and phenol and that between MBTH and HALPS, respectively. However, for each color reaction between MBTH and Trinder's reagents in this study, Fe³⁺-TCAS_{A-500} exhibited high and constant activity over a relatively wide range of pH 5–8. Hence, the MBTH-Trinder's reagent system used in this study was more effective than the 4-AAP-phenol or MBTH-HALPS system for the activity of Fe³⁺-TCAS_{A-500}. The MBTH-ALPS system was selected for the determination of H₂O₂ using Fe³⁺-TCAS_{A-500} in place of POD, because the quinoid dye produced in this system was the most stable in the reaction solution and its color reaction proceeded most quantitatively for various concentrations of H₂O₂ (data not shown). When using vital fluids, such as blood and urine as samples, it is advantageous to perform measurements over a relatively longer wavelength region so that interference by foreign substances in the samples can be prevented. Accordingly, measurement at 597 nm in the MBTH-ALPS system is more suitable for the determination of vital compounds, such as glucose and uric acid, in blood and urine samples.

POD is well known to catalyze an oxidation of substrates (AH₂) by H₂O₂. This reaction is a three-step cyclic process, in which POD is first oxidized by H₂O₂ and then reduced back to the native form in two sequential steps involving the formation of two high-valent oxoiron(IV or V) intermediates, Compounds I and II.²⁷ During the reaction, AH₂ was oxidized with Compounds I and II to the AH[•] radical, and then the oxidation product (A) was produced through the dismutation of the AH[•] radical. If Fe³⁺-TCAS_{A-500} will catalyze the color reaction in this study *via* any redox system similarly to POD, Fe³⁺-TCAS_{A-500} should be able to exhibit its catalytic activity through the redox cycle, such as Fe³⁺ ⇌ Feⁿ⁺ (n = 4 or 5). As shown in Fig. S1 (Supporting Information), the MBTH(-NH[•]) radical produced during the reaction will be coupled with ALPS to produce the quinoid dye. However, it was very difficult to investigate oxidation states of Fe³⁺-TCAS in the course of the catalytic cycle, because Fe³⁺-TCAS was on the anion-exchangers. Unfortunately, there is no evidence to prove the reaction pathway shown in Fig. S1.

Determination of H₂O₂ using Fe³⁺-TCAS_{A-500}

In order to establish the optimum conditions for determining

Fig. 3 Effect of the pH on the activity of Fe³⁺-TCAS_{A-500} in the MBTH-Trinder's reagent system for 7 µg H₂O₂. The mixture (20 mg of Fe³⁺-TCAS_{A-500}, 1.0 mL of H₂O₂ solution, and 5.0 mL of the reagent solution A) was incubated at room temperature for 30 min. After Fe³⁺-TCAS_{A-500} was filtered off, the absorbance at $\lambda_{\max}$ of the quinoid dye in the supernatant was measured against the reagent blank solution. The constituents of the reagent and buffer solutions were the same as in the text. (A) ALPS, (B) ADOS, (C) ADPS, (D) TOOS. ●, Acetate buffer; ○, phosphate buffer; ■, ammonia buffer; □, borate buffer; △, carbonate buffer.

Fig. 4 Effect of the concentration of the MBTH solution on the activity of Fe³⁺-TCAS_{A-500} in the MBTH-ALPS system in a pH 5 acetate buffer solution for 7 µg H₂O₂. The conditions were the same as in Fig. 3, except for the concentration of the MBTH solution.

H₂O₂ using Fe³⁺-TCAS_{A-500}, experiments for some factors that may influence the activity of Fe³⁺-TCAS_{A-500} for the color reaction between MBTH and ALPS were carried out using the MBTH-ALPS system for 7 µg of H₂O₂ in an acetate buffer solution of pH 5.

As shown in Figs. 4 and 5, the absorbance at 597 nm was dependent on the concentrations of MBTH and ALPS. However, contrary to our expectation, the absorbance decreased gradually with an increase in the concentration of MBTH. As shown in Fig. S1, we proposed that the MBTH(-NH[•]) radical as an intermediate participated in the formation of the quinoid dye. In the presence of a high concentration of MBTH, it will be understandable that a part of the MBTH(-NH[•]) radical react mutually to produce a MBTH dimer without producing the quinoid dye. This reactivity of the MBTH(-NH[•]) radical could result in the inhibition of the activity of Fe³⁺-TCAS_{A-500} by increasing the concentration of MBTH. Nevertheless, because of the constant, near-maximum absorbance at 0.1 mg mL⁻¹ of MBTH and over 0.5 mg mL⁻¹ of ALPS, optimum MBTH and ALPS concentrations of 0.1 mg mL⁻¹ and 1.0 mg mL⁻¹, respectively, were selected for the chromogen. Further, as the absorbance was almost constant for the incubation temperature

Fig. 5 Effect of the concentration of the ALPS solution on the activity of Fe^{3+} -TCAS_{A-500} in the MBTH-ALPS system in a pH 5 acetate buffer solution for 7 μg H_2O_2 . The conditions were the same as in Fig. 3, except for the concentration of the ALPS solution.

Fig. 6 Effect of the incubation time on the activity of Fe^{3+} -TCAS_{A-500} in the MBTH-ALPS system in a pH 5 acetate buffer solution for 7 μg H_2O_2 . The conditions were the same as in Fig. 3, except for the incubation time.

range from 20 to 50°C, room temperature was selected as the incubation temperature. In the previous methods,^{12,13} the reaction solution was incubated for 60 and 30 min for the maximum and constant absorbance of the quinoid dye in the color reaction between 4-AAP and phenol and that between MBTH and HALPS, respectively. However, in the method using the MBTH-ALPS system, the absorbance at 597 nm approached the maximum value and remained constant after just 5 min of incubation, as shown in Fig. 6. Thus, 10 min of incubation was considered sufficient.

Under the optimum conditions described above, a linear calibration curve shown in Fig. 7 was obtained over the range between 1.0 and 15 μg of H_2O_2 in the sample solution (1.0 mL), when using the MBTH-ALPS system. The correlation coefficient and relative standard deviation ($n = 5$) were greater than 0.996 and 2.5%, respectively, for 7 μg of H_2O_2 . The apparent molar absorptivity for H_2O_2 was $2.31 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$, which was about 70% of that for the corresponding reaction by POD. In the previous methods,^{12,13} we demonstrated

that the apparent molar absorptivity ($\text{L mol}^{-1} \text{ cm}^{-1}$) for H_2O_2 using the 4-AAP-phenol and the MBTH-HALPS systems were 3.0×10^3 and 2.4×10^4 , respectively. Accordingly, the method using the MBTH-ALPS system for the determination of H_2O_2 was superior to that using the 4-AAP-phenol or the MBTH-HALPS system with regard to its sensitivity, wide range of optimum pH, and shorter incubation time.

Interference by substances that intrinsically exist in blood, or may be added to blood as an anticoagulant was investigated using the MBTH-ALPS system. As shown in Table 1, the ascorbate ion and human serum albumin (HSA) showed severe interference. When using the present method for the sample solution containing ascorbic acid, the addition of ascorbate oxidase to sample solutions, in analogy with the methods using POD in clinical analyses, would be effective for minimizing such interference.²⁸ HSA is known to serve as a transport protein carrying various compounds, such as metal ions, fatty acids, and drugs.²⁹ As HSA has good binding capacity for such compounds, MBTH and/or ALPS could also be adsorbed by HSA, which would lead to severe interference by HSA for the activity of Fe^{3+} -TCAS_{A-500}. However, the interference by HSA may be suppressed by diluting the sample solution to the desired concentration of HSA.

Effect of repetitive use of Fe^{3+} -TCAS_{A-500} on the activity

In order to elucidate whether Fe^{3+} -TCAS_{A-500} (100 $\mu\text{mol/g}$) can be used repeatedly and exhibit catalytic activity for color reaction, the effect of repeated use of Fe^{3+} -TCAS_{A-500} on such activity was investigated for 15 μg of H_2O_2 (0.44 μmol). The molar ratio of Fe^{3+} -TCAS_{A-500} on Fe^{3+} -TCAS_{A-500} to H_2O_2 in the sample solution was 2.0:0.44. Fe^{3+} -TCAS_{A-500} was used repeatedly after being separated from the reaction mixture, washed with water and dried. In terms of the repetitive use of Fe^{3+} -TCAS_{A-500} separated from the reaction solution, the activity of Fe^{3+} -TCAS_{A-500} for 15 μg of H_2O_2 after use 10 times was over 90% of the activity obtained during the first run. As such, we found that Fe^{3+} -TCAS_{A-500} can be used repeatedly and still show catalytic activity for color reaction. Moreover, the catalytic activity of Fe^{3+} -TCAS_{A-500} remained constant even after being allowed to stand for over six months at room temperature in a desiccator. One of the characteristics of Fe^{3+} -TCAS_{A-500} is that Fe^{3+} -TCAS_{A-500} can be used repeatedly after being separated from the reaction mixture, while POD is difficult to be separated.

Previously, Iki *et al.* demonstrated that thiocalix[4]arenes were oxidized to sulfinyl- or sulfonylthiocalix[4]arenes with oxidizing agents such as H_2O_2 or $\text{NaBO}_3 \cdot 4\text{H}_2\text{O}$; that is, the epithio groups (-S-) of thiocalix[4]arenes were oxidized to >SO or >SO₂, respectively.^{10,30} If the epithio groups of Fe^{3+} -TCAS_{A-500} could be oxidized with H_2O_2 , in terms of the repetitive use of Fe^{3+} -TCAS_{A-500}, the Fe^{3+} complex of oxidized Fe^{3+} -TCAS_{A-500} would become unstable and so the activity of Fe^{3+} -TCAS_{A-500} would decrease. However, as described before, the activity level of Fe^{3+} -TCAS_{A-500} was maintained even after 10 uses. No consideration toward oxidation of the epithio groups of Fe^{3+} -TCAS_{A-500} was needed in this study because only a small amount of H_2O_2 existed in the reaction system.

Application for the determination of glucose

As shown in reaction (1), glucose was oxidized with GOD to produce H_2O_2 corresponding to the amount of glucose. Glucose was indirectly determined by measuring the quinoid dye produced through the color reaction catalyzed by Fe^{3+} -TCAS_{A-500}. As each optimum pH for the activities of GOD and Fe^{3+} -TCAS_{A-500} in the color reaction was in the same region, GOD and Fe^{3+} -TCAS_{A-500} were added simultaneously to the

Fig. 7 Calibration curves for the determination of H_2O_2 , glucose, uric acid, cholesterol, and cholesteryl palmitate using the activity of Fe^{3+} -TCAS_{A-500} in the MBTH-ALPS system. The determination methods were the same as in the text. (A) H_2O_2 , (B) glucose, (C) uric acid, (D) cholesterol, (E) cholesteryl palmitate. ●, The method using Fe^{3+} -TCAS_{A-500}; ○, the method using POD.

sample solution. For the determination of glucose using GOD and Fe^{3+} -TCAS_{A-500}, the optimum conditions for GOD concentration ($1.0 - 48 \text{ units mL}^{-1}$), incubation time (10 - 60 min), and incubation temperature ($25 - 50^\circ\text{C}$) were investigated. Almost maximum and constant absorbance at 597 nm was observed over 16 units mL^{-1} of GOD, in 30 min or more of incubation time, and in the range of 40 to 50°C of incubation temperature. Hence, 32 units mL^{-1} of GOD, 30 min of incubation time, and 40°C of incubation temperature were

selected as the optimum conditions. Although only 5 min was needed for the color reaction in the MBTH-ALPS system by Fe^{3+} -TCAS_{A-500}, 30 min of incubation was required in the GOD- Fe^{3+} -TCAS_{A-500} method. As shown in Fig. 7, for a sample solution containing 5.0 - 60 μg of glucose in 0.5 mL, a straight calibration curve was obtained under the GOD- Fe^{3+} -TCAS_{A-500} method. The correlation coefficient and relative standard deviation ($n = 5$) were greater than 0.992 and 4.0%, respectively, for 30 μg of glucose. The apparent molar absorptivity for

Table 1 Effect of foreign substances on the activity of Fe^{3+} -TCAS_{A-500} by the method using the MBTH-ALPS system for 7.0 μg H_2O_2 in a pH 5 acetate buffer solution

Substance	Added/ μg	Error ^a , %	Substance	Added/ μg	Error ^a , %
Heparin	70	+1.9	Glycine	70	+1.9
NaF	70	-2.0	Citrate	70	+1.2
EDTA	70	-5.2	Fe^{3+}	70	+1.8
Ascorbate	70	-95	K^+	70	-0.5
	30	-39	Ca^{2+}	70	-2.0
	7	-7.8	PO_4^{3-}	70	-2.5
Albumin	10×10^3	-22	CO_3^{2-}	70	+3.0
(HSA)	5×10^3	-6.5	NH_4^+	70	+2.1
	1×10^3	+2.8	Br^-	70	+2.1
			I^-	70	+4.3

a. Error, % = $100 \times (\text{H}_2\text{O}_2(\text{found}) - \text{H}_2\text{O}_2(\text{added})) / \text{H}_2\text{O}_2(\text{added})$.

Table 2 Glucose amount in diluted control sera I and II

Method	Glucose amount (mean $\pm$ SD, $n = 4$ each)	
	Control serum I ^a ($\mu\text{g}/0.5 \text{ mL}$)	Control serum II ^a ($\mu\text{g}/0.5 \text{ mL}$)
GOD-POD	28.9 ± 0.03	32.9 ± 0.91
GOD- Fe^{3+} -TCAS _{A-500}	26.0 ± 0.06	30.4 ± 1.02

a. Control sera I and II were diluted 20 and 50 times with distilled water, respectively.

glucose was $2.21 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$, which was about 40% of that by the GOD-POD method. Moreover, the GOD- Fe^{3+} -TCAS_{A-500} method was applied for the determination of glucose in control sera I and II; the sample solutions were applied after dilution to 20 and 50 times with distilled water, respectively. Table 2 shows that the observed amounts of glucose by the GOD- Fe^{3+} -TCAS_{A-500} method were satisfactory for both control sera I and II. The interference by HSA was completely diminished by diluting each serum with distilled water, indicating that the removal of HSA from each serum was unnecessary.

Application for the determination of uric acid

Uric acid was determined using the uricase- Fe^{3+} -TCAS_{A-500} method by measuring the quinoid dye produced through reaction (2) and the color reaction, catalyzed by uricase and Fe^{3+} -TCAS_{A-500}, respectively. Similar to the GOD- Fe^{3+} -TCAS_{A-500} method, uricase and Fe^{3+} -TCAS_{A-500} were added simultaneously to the sample solution. For the determination of uric acid using uricase and Fe^{3+} -TCAS_{A-500}, the optimum conditions for uricase concentration (0.1 – 5.0 units mL^{-1}), incubation time (5 – 60 min), and incubation temperature (25 – 50°C) were investigated. As almost maximum and constant absorbance at 597 nm was obtained, 2.5 units mL^{-1} of uricase, an incubation time of 30 min, and an incubation temperature of 40°C were selected as the optimum conditions as shown for the uricase- Fe^{3+} -TCAS_{A-500} method. As shown in Fig. 7, a straight calibration curve was obtained for a sample solution containing 2.0 – 30 μg of uric acid in 0.5 mL under the uricase- Fe^{3+} -TCAS_{A-500} method. The correlation coefficient and relative standard deviation ($n = 5$) were greater than 0.990 and 4.5%, respectively, for 20 μg of uric acid. The apparent molar absorptivity for glucose was $2.43 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$, which was about 55% of that by the uricase-POD method. Moreover,

Table 3 Uric acid amount in diluted control sera I and II

Method	Uric acid amount (mean $\pm$ SD, $n = 4$ each)	
	Control serum I ^a ($\mu\text{g}/0.5 \text{ mL}$)	Control serum II ^a ($\mu\text{g}/0.5 \text{ mL}$)
Uricase-POD	2.84 ± 0.43	5.29 ± 0.09
Uricase- Fe^{3+} -TCAS _{A-500}	2.58 ± 0.53	4.85 ± 0.23

a. Control sera I and II were diluted 12 times with distilled water.

the uricase- Fe^{3+} -TCAS_{A-500} method was applied for the determination of uric acid in control sera I and II diluted 12 times with distilled water. Table 3 shows that the observed amounts of uric acid by the uricase- Fe^{3+} -TCAS_{A-500} method were satisfactory for both control sera I and II. Moreover, uric acid was determined without the removal of HSA from each serum.

Application for the determination of cholesterol

Cholesterol in blood is known to exist in two types: free cholesterol and cholesteryl esters. The cholesterol level in blood is thus evaluated as the sum of these two types.³¹ Generally, cholesteryl esters are first hydrolyzed with cholesterol esterase (COE) to produce free cholesterol and long-chain fatty acids, and then, the free cholesterol produced is determined. Hence, in this study, the method for the determination of free cholesterol was investigated. Free cholesterol was indirectly determined by measuring H_2O_2 produced through reaction (3) catalyzed by COD. For the determination of free cholesterol using COD and Fe^{3+} -TCAS_{A-500}, the optimum conditions for a COD concentration (0.1 – 2.0 units mL^{-1}), incubation time (20 – 60 min), and incubation temperature (25 – 45°C) were investigated. Almost maximum and constant absorbance at 597 nm was obtained over 0.8 units mL^{-1} of COD, in 30 min or more of incubation time, and in the range of 35 to 45°C of incubation temperature. Based on these results, the method for the determination of free cholesterol as previously described was established. For a sample solution containing 11.6 – 116 μg of free cholesterol in 0.5 mL, a straight calibration curve was obtained when using the COD- Fe^{3+} -TCAS_{A-500} method, as shown in Fig. 7. The correlation coefficient and relative standard deviation ($n = 5$) were greater than 0.994 and 5.5%, respectively, for 34.8 μg of free cholesterol. The apparent molar absorptivity for free cholesterol was $2.36 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$, which was about 88% of that by the COD-POD method.

The COD- Fe^{3+} -TCAS_{A-500} method was used for the determination of cholesteryl palmitate to confirm its use for cholesteryl ester determination. After a mixture containing COE solution (17 units mL^{-1} , 0.5 mL), cholesteryl palmitate solution (0.5 mL), and HEPES buffer (pH 7.0, 2.0 mL) was incubated for 10 min at 40°C, the free cholesterol produced in the reaction solution was determined using the COD- Fe^{3+} -TCAS_{A-500} method. As described before, a 10% solution of Triton X-100 in isopropyl alcohol was used for the preparation of cholesteryl palmitate solution. No interference by Triton X-100 for each activity of COE, COD and Fe^{3+} -TCAS_{A-500} was observed even if its amount in the sample solution was over 10%. Also no interference by isopropyl alcohol was observed under the conditions of this study. As shown in Fig. 7, the calibration curve was straight for a sample solution containing 18.8 – 62.5 μg of cholesteryl palmitate in 0.5 mL, and the apparent molar absorptivity for cholesteryl palmitate was about 43% of that determined by the COD-POD method. Using the COD- Fe^{3+} -TCAS_{A-500} method, the determination of both free

cholesterol and cholesteryl esters in blood was investigated. Unfortunately, the results were unsatisfactory for the determination of both free cholesterol and cholesteryl esters in control sera I and II, possibly because of severe interference by HSA.

In conclusion, Fe^{3+} -TCAS_{A-500} showed high peroxidase-like activity for catalyzing the color reaction between MBTH and ALPS in buffer solutions of pH 5–8. The use of the MBTH-ALPS system is advantageous because of the high sensitivity and more suitable reaction conditions (especially, optimum pH and incubation time), as compared to the previous methods using the 4-AAP-phenol or the MBTH-HALPS system. The method using the MBTH-ALPS system and Fe^{3+} -TCAS_{A-500} was useful not only for the spectrophotometric determination of H_2O_2 but also for the determination of glucose, uric acid, and cholesterol. Moreover, the method using Fe^{3+} -TCAS_{A-500} was applicable to the determination of glucose and uric acid in control sera without any interference by HSA.

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Supporting Information

Figure for proposed pathway of the quinoid dye formation. This material is available free of charge on the Web at <http://www.jsac.or.jp/analsci/>.

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