〈Original Article〉

Study of *o*-tolidine-reacting protein assay using a novel *o*-tolidine derivative

Hiroaki Ishikawa[1], Koji Ohashi[2], Naomichi Ogitsu[3] and Yoichi Nagamura[4]

Summary We previously reported that *o*-tolidine-reacting protein (OTRP) in serum increased at the early stage of myocardial infarction and should be a specific index for free-radical-induced liver injury. OTRP specifically reacts with *o*-tolidine in 0.2 M citrate buffer (pH5.2); however, we could not obtain sufficient concentration of this reagent because of its poor solubility in buffer, and furthermore this reaction was interfered with by ordinary substances in serum, such as hemoglobin, bilirubin and ascorbic acid. Thus, the OTRP assay was not suitable for daily clinical tests.

Recently, we obtained a novel *o*-tolidine derivative, N,N'-bis (2-hydroxy-3-sulfopropyl) tolidine (SAT-3), from Dojindo Laboratories. This substance is water-soluble and less carcinogenic than *o*-tolidine. In this study, we developed an assay condition for OTRP using SAT-3 and examined the effect of interfering substances. The optimum pH for the assay was pH5.0 at 660 nm and the reaction reached maximum at a concentration of 6.62 mmol/L. Furthermore, the ordinary concentration of interfering substances in serum had no effect on this reaction. The OTRP assay with SAT-3 well correlated with the *o*-tolidine method [$y = 1.829x + 4.260$, $r = 0.980$, $p < 0.0001$]. We developed a novel OTRP assay for routine clinical tests using SAT-3 instead of *o*-tolidine; thus, we have established a superior method for the OTRP assay with SAT-3.

Key words: Free radical, *o*-tolidine, SAT-3

1. Introduction

We previously reported that *o*-tolidine-reacting protein (OTRP) was present in the blood, increased at an early stage of myocardial infarction[1] and might be an independent index for free-radical-induced liver injury[2]. OTRP is a protein belonging to the globulin fraction and specifically reacts with o-tolidine in 0.2 M citrate buffer (pH5.2)[3,4]. We previously developed an automatic analysis method of OTRP for routine clinical examination[4]; however, our devised method was interfered with by bilirubin, ascorbic acid and

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Received for Publication January 7, 2013
Accepted for Publication February 4, 2013

high concentrations of hemoglobin. Recently, we noted that N,N'-bis(2-hydroxy-3-sulfopropyl)tolidine (SAT-3) could be used instead of *o*-toluidine[5], and is water-soluble and less carcinogenic than *o*-tolidine. In this study, we developed assay conditions for OTRP using SAT-3 and investigated the influence of ordinary substances in serum on the new assay method.

2. Materials and methods

1. Sample

Samples of serum without hemolysis, jaundice and chyle were obtained from the Fujita Health University Hospital clinical laboratory. The pooled serum was collected from healthy volunteers. All experiments were performed after receiving written informed consent from hospital staff and volunteers.

2. Materials

SAT-3 was provided by Dojindo Laboratories (Kumamoto, Japan). Interference Check-A Plus Kit was purchased from Sysmex Corporation (Hyogo, Japan). All other chemical reagents were of analytical grade and purchased from Wako Pure Chemical Industries (Osaka, Japan).

3. Reagents

Figure 1 shows the structural formulas of *o*-tolidine and SAT-3, which contains more hydrophilic functional residues, hence being more water soluble and less carcinogenic. The assay reagent for OTRP with *o*-tolidine was prepared as follows: 1 mg *o*-tolidine was suspended in 1 mL of 0.2 M citrate buffer solution (pH5.2) followed by ultrasonic treatment for 3 minutes, then centrifuged at 1,800×g for 10 minutes. The supernatant was used as the reagent for the assay. The assay reagent for OTRP with SAT-3 was prepared as follows: an appropriate concentration of SAT-3 was dissolved in 0.2 M citrate buffer (ranging from pH4.4 to 6.2).

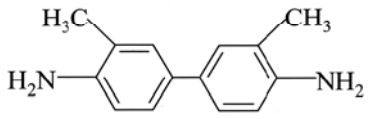
4. Methods

1) Assay conditions for OTRP with automated analyzer

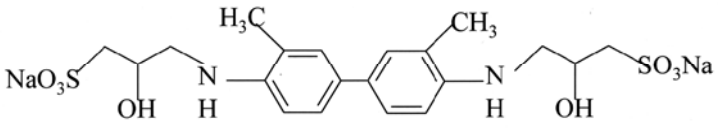
OTRP was measured using an automated analyzer, COBAS-MIRA (Roche Diagnostics, Basel, Switzerland). The OTRP assay with *o*-tolidine or SAT-3 was carried out at 37℃ with the following steps: 10 μL sample was mixed with 400 μL of each reagent, and the mixture was pre-incubated for about 15 minutes to diminish the lag-phase reaction. Thereafter, the increase of absorbance at 405 nm was measured for 5 minutes. We also tried to measure at 660nm wavelength for the specific assay of OTRP with SAT-3, since an absorption maximum of SAT-3 was reported at about 674 nm in 1M sodium acetate buffer (pH5.2)[6,7]. We decided on an increase of 0.001 absorbance per minute as one arbitrary unit of OTRP, since the nature of OTRP was not established.

2) Determination of optimal concentration of SAT-3 for OTRP assay

SAT-3 (16.54mmol/L) was dissolved in 0.2M



***o*-Tolidine**



***N,N'*-Bis(2-hydroxy-3-sulfopropyl)tolidine, disodium salt, tetrahydrate (SAT-3)**

Fig. 1 Structural formulas of o-tolidine and SAT-3

citrate buffer (pH5.0) at 85℃, and the SAT-3 reagent was adjusted to pH 5.0 again. This SAT-3 reagent was diluted with 0.2 citrate buffer (pH5.0) to 0.83 mmol/L, 1.66 mmol/L, 3.3 mmol/L, 6.62 mmol/L and 13.23 mmol/L. We determined the optimal concentration of SAT-3 for OTRP activity by assaying the pool serum at 660 nm.

3) Effects of pH and interfering substances on the SAT-3 method

The optimum pH of OTRP assay with SAT-3 reagent was examined using 0.2M citrate buffer in the pH range from 4.4 to 6.2, and investigated the effect of interfering substances (hemoglobin, free and conjugated forms of bilirubin, chyle and ascorbic acid) on the assay. The interfering substances were used with Interference Check-A Plus and the experiment was carried out according to the manual. The conjugated bilirubin in this kit is a ditaurobilirubin and bovine serum albumin is added to the buffer solution to stabilize this kit.

4) Statistical analyses

Statistical calculations for this study were carried out using software package "Prism 4" purchased from GraphPad Software, Inc. (San Diego CA).

3. Results

1. Effect of SAT-3 concentration on OTRP assay

The effect of SAT-3 concentration on OTRP activity is shown in Fig. 2. The reaction of OTRP

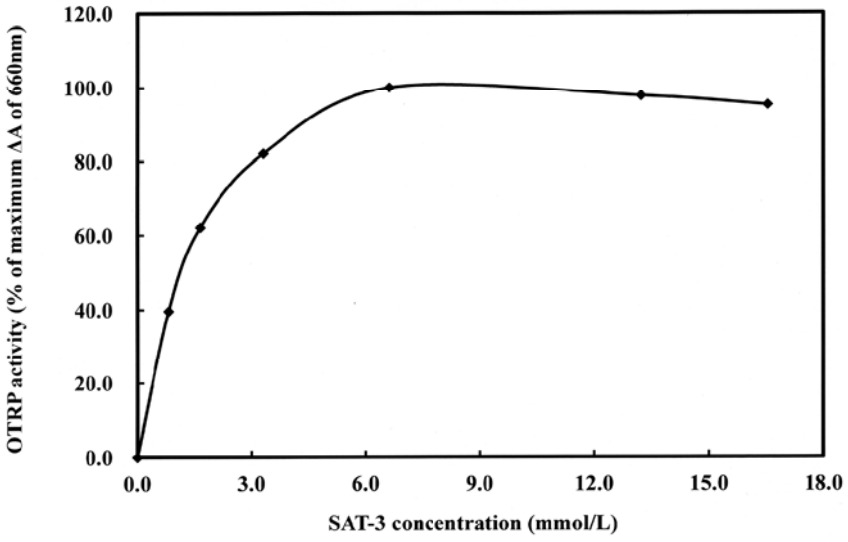


Fig. 2 The ffect of SAT-3 concentrations on OTRP activity

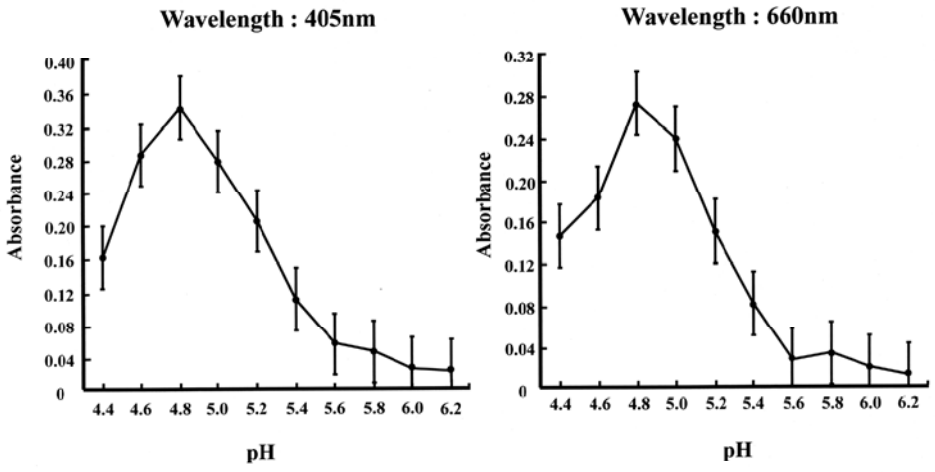


Fig. 3 The optimal pH of OTRP assay with SAT-3

activity with SAT-3 peaked at a concentration of 6.62 mmol/L, and then slightly decreased.

2. Effects of pH on OTRP assay with SAT-3

The optimal pH of the OTRP assay with SAT-3 was examined at wavelengths of both 405 nm and 660 nm. Even though the absorption maximum of oxidized SAT-3 was about 400 nm and 675 nm in a previous report[7], we assayed at both 405 nm and 660nm since the automated analyzer we used could not set the wavelength freely.

As shown in Fig. 3, the maximum absorbance was observed at pH4.8 at both 405 nm and 660 nm. Figure 4 illustrates the time course of OTRP activity at several pH. Of all tested pH, the linearity of the reaction at pH5.0 was the best, though a lag phase was observed for about 60 seconds; thus, the buffer for the OTRP assay with SAT-3 was adjusted to pH5.0.

3. Effects of interfering substances on the OTRP assay

The influences of various interfering substances on the OTRP assay with *o*-tolidine and SAT-3 are shown in Fig. 5. With the exception of chyle, the OTRP assay with *o*-tolidine was affected by hemoglobin, bilirubin and ascorbic acid. The activities of OTRP with o-tolidine gave negative errors by the addition of hemoglobin (>49 mg/dL), free bilirubin (>1.8 mg/dL), conjugated bilirubin (>2.2 mg/dL) and ascorbic acid (>8.0 mg/dL). In contrast, the OTRP assay with SAT-3 at 405 nm was unaffected by hemoglobin, free bilirubin and lipids, but negative errors were observed in the presence of the conjugated bilirubin (>4.4 mg/dL) and ascorbic acid (>8.0 mg/dL). In the assay condition at 660 nm, no influence was observed with the coexistence of hemoglobin, bilirubin and chyle. Even with the addition of 16.0 mg/dL ascorbic acid, the reaction was not affected; therefore, we chose a wavelength of 660 nm for the OTRP assay with SAT-3.

4. Relationship of OTRP assay with between SAT-3 and o-tolidine

Serum OTRP activities in 72 healthy persons

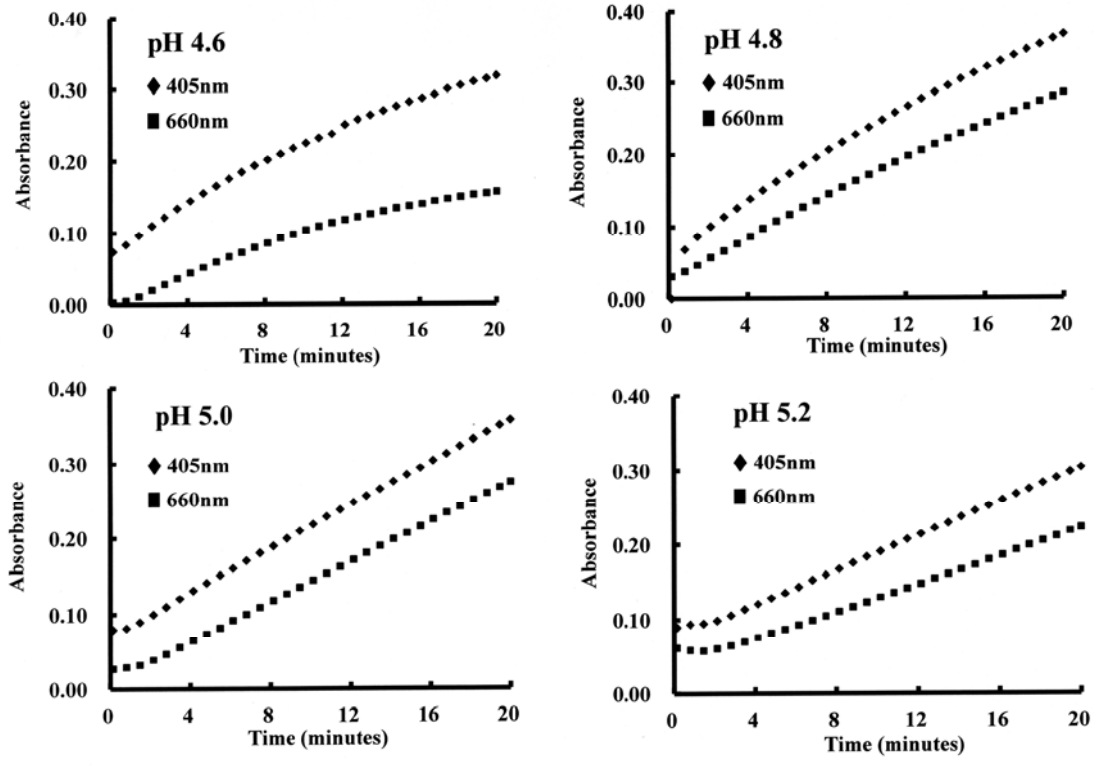


Fig. 4 The time courses of the OTRP assay with SAT-3

were measured with SAT-3 or *o*-tolidine. As shown in Fig. 6, there was a good correlation [y = 1.829x + 4.260, r = 0.980, p <0.0001]. These results indicates that SAT-3 can replace *o*-tolidine in the OTRP assay.

4. Discussion

We previously reported that a protein reacted with *o*-tolidine specifically and had clinical significance in several diseases, although the nature of the OTRP was still unclear[1-3]. We also reported the reaction conditions for the OTRP assay with an automatic analyzing system, COBAS-MIRA. Unfortunately, our assay condition was found to be interfered with by the coexistence of hemoglobin, bilirubin and ascorbic acid in the serum[4]. We suspected that the main reason for the interference was the chemical nature of *o*-tolidine and its poor solubility. This meant that it was very difficult to use o-tolidine for routine clinical OTRP assays, because its chemical nature could not be changed. We searched for an alternative to *o*-tolidine and found SAT-3, which was developed recently as a water-soluble tolidine derivative. We planned to develop a new assay condition for OTRP by substituting SAT-3 for *o*-tolidine. As shown in the previous results, after our various experiments, the optimum pH, substrate concentration and wavelength were set as pH5.0, 6.62 mmol/L and 660 nm, respectively. Furthermore, we investigated the effects of several interfering substances in serum for

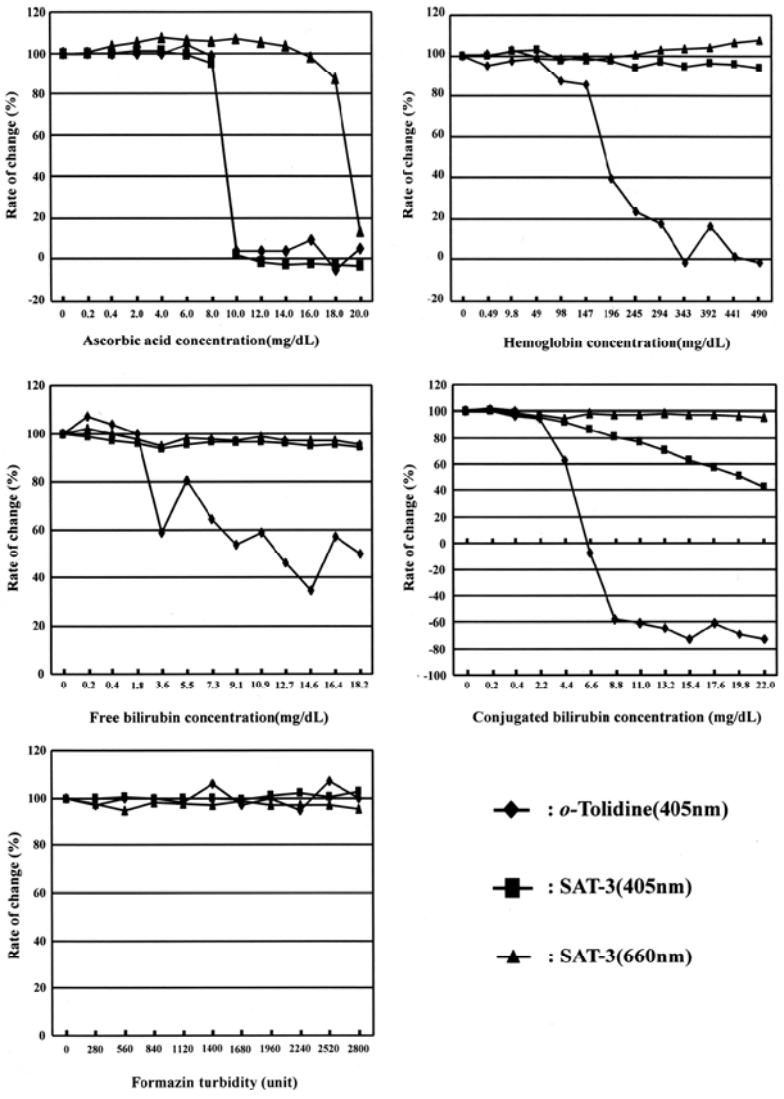


Fig. 5 The effect of interference substances on OTRP assay

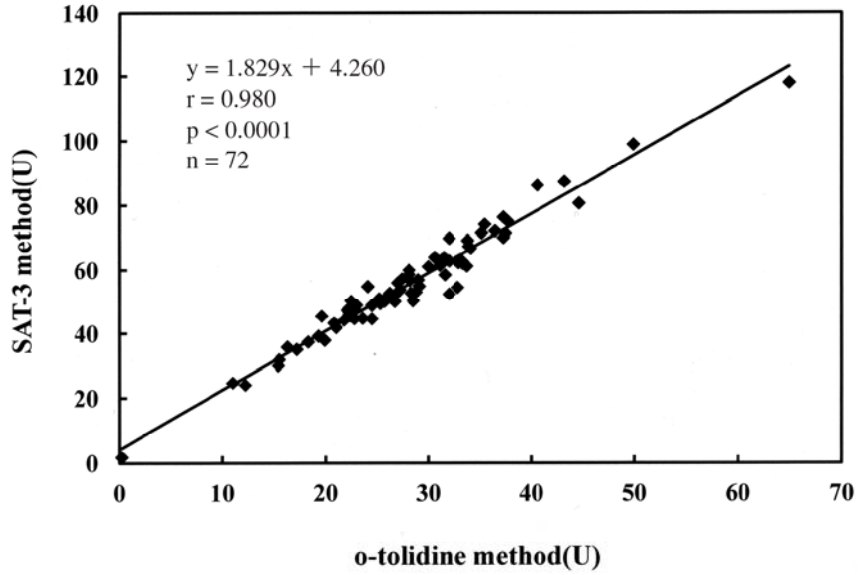


Fig. 6 The relationship of OTRP assay with between SAT-3 and o-tolidine

the new assay system with SAT-3 and confirmed that hemoglobin, bilirubin and lipid did not affect the reaction assayed at 660 nm, except for a high concentration of ascorbic acid. This assay system was affected by ascorbic acid at concentrations above 16 mg/dL; however, we do not think that this problem is serious because the reference interval of ascorbic acid concentration in the blood is 0.70-1.38 mg/dL[8]. Good correlation was observed between the new assay system with SAT-3 and the previous method with o-tolidine. These results showed that replacement of the reagent *o*-tolidine with SAT-3 improved the accuracy of the OTRP assay; thus, we demonstrated that SAT-3 is more useful reagent for OTRP than *o*-tolidine. We hope that our developed method will facilitate the clarification of the nature of OTRP.

Acknowledgements

The authors thank Dojindo Laboratories for supplying SAT-3.

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