Peroxyoxalate Chemiluminescence Assay of Hydrogen Peroxide and Glucose Using 2,4,6,8-Tetrathiomorpholinopyrimido[5,4-*d*]-pyrimidine as a Fluorescent Component

Kenichiro NAKASHIMA*, Kouichi MAKI*, Shinki KAWAGUCHI*, Shuzo AKIYAMA*, Yukie TSUKAMOTO and Kazuhiro IMAI****

**School of Pharmaceutical Sciences, Nagasaki University, Bunkyo-machi, Nagasaki 852, Japan*
***Branch Hospital Pharmacy, University of Tokyo, Mejirodai, Bunkyo, Tokyo 112, Japan*

Peroxyoxalate chemiluminescence (CL) assay of hydrogen peroxide (H_2O_2) or glucose was developed by using 2,4,6,8-tetrathiomorpholinopyrimido[5,4-*d*]pyrimidine as a fluorescent component and bis(2,4,6-trichlorophenyl)oxalate (TCPO) as an oxalate. Linear relationships between CL intensity and final concentration of H_2O_2 from 10^{-8} to 10^{-4} M were obtained. The detection limit at the ratio of CL intensities for sample and blank (S/B) of 3 was 10 nM. The precision for five replicate measurements at 10^{-5} and 10^{-6} M of H_2O_2 were 17.6 and 15.7% of relative standard deviations, respectively. α-D-Glucose was transformed to β-D-glucose with mutarotase and converted to H_2O_2 and D-gluconic acid with glucose oxidase, which was detected by using peroxyoxalate CL reaction. A linear calibration graph was obtained up to 1.5×10^{-4} M of glucose solution. The method was applied to the assay of glucose in human serum. The recovery was 98.2% (n=4). The method correlated well with the conventional colorimetric method (r=0.968).

Keywords Peroxyoxalate chemiluminescence, 2,4,6,8-tetrathiomorpholinopyrimido[5,4-*d*]pyrimidine, hydrogen peroxide, glucose, glucose oxidase, mutarotase

Peroxyoxalate chemiluminescence (CL) has been applied to the quantitative analysis of fluorescent compounds[1-10], hydrogen peroxide[11-14] and substrates from which hydrogen peroxide is enzymatically produced.[14-19] Recently, we have synthesized some pyrimido[5,4-*d*]pyrimidine derivatives as fluorescent components in peroxyoxalate CL reaction system[20,21] and we have applied these derivatives to the photographic detection of hydrogen peroxide and glucose.[22]

This paper describes peroxyoxalate CL assay of hydrogen peroxide or glucose using 2,4,6,8-tetrathiomorpholinopyrimido[5,4-*d*]pyrimidine (TMP) as a fluorescent component and bis(2,4,6-trichlorophenyl)-oxalate (TCPO) as an oxalate. The transformation of α-D-glucose to β-D-glucose with mutarotase (EC 5.1.3.3) and enzymatic conversion of D-glucose to hydrogen peroxide and D-gluconic acid with glucose oxidase (GOD, EC 1.1.3.4) are also demonstrated.

Experimental

Apparatus

A lumiphotometer TD-4000 (Laboscience, Tokyo, Japan) was used for all measurements of CL. A small glass test tube (50×5 mm, i.d.) was used for CL reaction.

Chemicals

TCPO was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Glucose, hydrogen peroxide (30%, v/v), imidazole, trishydroxymethylaminomethane (Tris), control serum, and a kit for *o*-toluidine method (Glucose-Test Wako) were obtained from Wako Pure Chemical Co. (Osaka, Japan). 3-(*N*-Morpholino)-2-hydroxypropanesulfonic acid (MOPSO), *N*-2-hydroxyethylpiperazine-*N'*-2-hydroxypropane-3-sulfonic acid (HEPPSO) and 2-(cyclohexylamino)ethanesulfonic acid (CHES) were purchased from Dojindo Lab. (Kumamoto, Japan). TMP was synthesized using our previous method.[20] Dipyridamole (DP) and GOD as type X with a specific activity of 128000 units/g were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Mutarotase derived from porcine kidney with a specific activity of 10000 units/ml was obtained from Wako. Water was deionized and distilled once. The other chemicals used were of analytical reagent grade.

Imidazole buffer

Imidazole (6.80 g) was dissolved in *ca.* 800 ml water. After the pH was adjusted to 6.75 with 10% HNO_3, the solution was diluted to 1 l with water (100 mM imidazole buffer, pH 6.75). Similarly, 10 mM imidazole buffer (pH 8.0) was prepared with 0.68 g imidazole.

Serum sample

Normal human serum specimens were obtained from healthy volunteers in our department.

Assay procedure for hydrogen peroxide

One hundred microliters of H_2O_2 in acetonitrile (5×10^{-8} - 5×10^{-4} M), imidazole buffer (10 mM, pH 8.0), TMP in acetone (10 nM), and acetonitrile were successively pipetted into a reaction tube and mixed. When a 100 µl portion of TCPO in CH_3CN (1 mM) was injected into the tube, CL produced was instantaneously measured with a lumiphotometer. The total CL intensity was defined as the area under the CL decay curve.

Assay procedure for glucose

To a test tube were successively added 200 µl of imidazole buffer (100 mM, pH 6.75), 100 µl of glucose solution, 100 µl GOD (30 U/ml), and 20 µl mutarotase solution (100 U/ml). After mixing, the mixture was incubated at 35 °C for 10 min; then the tube was immediately immersed in an ice bath to stop the enzymatic reaction. A 100 µl portion of the mixture was transferred to a reaction tube. One hundred microliters of TMP in acetone (10 nM) and 200 µl of CH_3CN were successively added. After mixing for 10 s, a 100 µl portion of TCPO in CH_3CN (1 mM) was added and the CL produced was instantaneously measured.

For the assay of glucose in serum, normal human serum or control serum was diluted 100-fold with water. By using a 100 µl aliquot of diluted serum, glucose was assayed by the method described above.

o-Toluidine colorimetric method

To 0.05 ml of sample solution was added 5.0 ml of reagent solution (*o*-toluidine-boric acid solution). The mixture was heated for exactly 8 min on boiling water bath. After it was chilled with tap water for 3 min, absorbance at 635 nm of the resulting solution was measured.

Results and Discussion

Hydrogen peroxide assay

In a preliminary examination, effects of pH and concentration of buffer on CL intensity were tested. Imidazole buffer, Tris buffer, and Good buffers (HEPPSO, MOPSO, CHES) with pHs from 3 to 10 were examined according to the procedure for H_2O_2 assay described in the text using DP as a fluorescent component. The chemiluminescence was produced at pH above 5 of all buffers used. Among these, when the imidazole buffer of 10 mM (pH 8.0) was used the largest CL was obtained (Fig. 1). On the other hand, Tris and Good buffers gave considerably smaller CL intensities than imidazole buffer. Then, effect of water content in the reaction system was examined by using imidazole buffer (10 mM, pH 8.0). The water content also affected CL intensity. The CL intensity decreased with increasing content of water. The hydrolysis of TCPO would precede a formation of an active intermediate such as 1,2-dioxetane-3,4-dione.

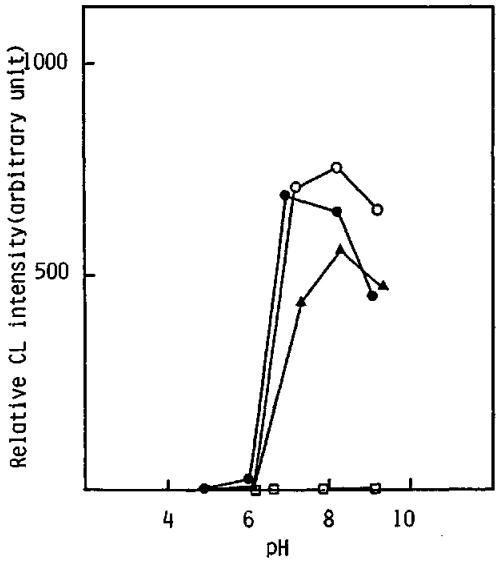

Fig. 1 Effect of pH of imidazole buffer on CL intensity. Final concentration: TCPO, 0.2 mM; H_2O_2, 0.5 µM; DP, 0.1 µM. Imidazole buffer: (□) 0.1 mM; (▲) 1 mM; (○) 2 mM; (●) 10 mM.

In the peroxyoxalate CL reaction, selection of fluorescent components is very important to obtain strong CL. Thus many fluorescent compounds have been studied on their CL properties.[6,8,10,23-25] Among these, DP, one of the pyrimido[5,4-*d*]pyrimidine derivatives, was found to luminesce strongly in peroxyoxalate CL reaction.[10] Therefore, several kinds of derivatives were synthesized and their properties as fluorescent components were evaluated together with some other fluorescent compounds by flow injection method.[20,21] As a result, pyrimido[5,4-*d*]pyrimidine derivatives were found to be available as fluorescent components. Therefore, some derivatives were tested again as luminescent enhancers in this study. The CL intensity was given as the area under the CL decay curve. By using a series of known concentrations of fluorescent compounds (10^{-9} - 10^{-6} M), calibration curves were prepared (Table 1). As described in a previous paper[21], the relative CL intensity (RCI) for each fluorescent compound was estimated from the slope of the calibration curve for each compound. The slope (or RCI) of DP was arbitrarily taken as 1.0. As shown in Table 1, TMP showed the largest relative CL intensity. Consequently, TMP was selected as a fluorescent component for the following experiments. Figure 2 shows the effect of TMP concentration on the linearity of the correlation between CL intensity and H_2O_2 concentration. The CL intensity increased with an increase in TMP concentration. However, the blank CL (*B*) also increased. The *S*/*B* ratios for 1.2 nM, 2

Table 1 Comparison of relative chemiluminescence intensities for fluorescent compounds as luminescent enhancers

Fluorescent compound	Equation of calibration curve[a]	r[b]	RCI[c]
2,4,6,8-Tetrathiomorpholinopyrimido[5,4-*d*]pyrimidine (TMP)	$Y=22.7X-13.6$	0.983	3.90
2,4,6,8-Tetramorpholinopyrimido[5,4-*d*]pyrimidine	$Y=10.3X-20.6$	0.985	1.78
Dipyridamole (DP)	$Y=5.8X-26.7$	0.971	1.00
Perylene	$Y=2.2X-14.2$	0.984	0.38
Rhodamine B	$Y=0.36X-7.8$	0.997	0.06
9,10-Bis(phenylethynyl)anthracene	$Y=0.91X-28.9$	0.991	0.16
9,10-Diphenylanthracene	$Y=0.39X-34.0$	0.988	0.07

a. Y, relative CL; X, concentration of fluorescent compound (nM). b. r, coefficient of correlation. c. The RCI of dipyridamole was arbitrarily taken as 1.0.

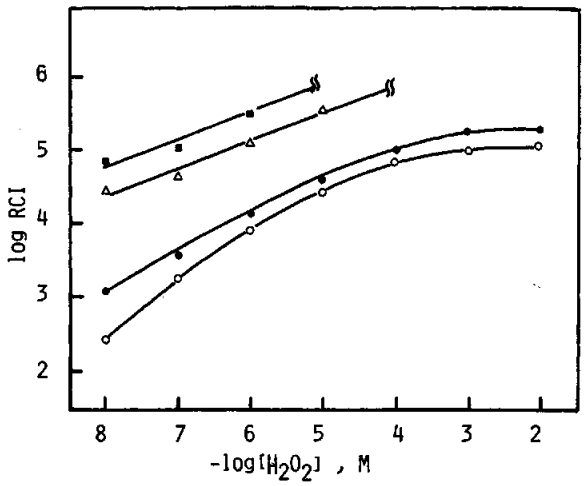

Fig. 2 Effect of TMP concentration on the linearity of the correlation between CL intensity and H_2O_2 concentration. RCI: relative chemiluminescence intensity. Final concentration: TCPO, 0.2 mM; imidazole buffer, 2 mM (pH 8). TMP: (○) 1.2 nM; (●) 2 nM; (△) 0.2 μM; (■) 20 μM.

Table 2 Precision of the method for the determination of H_2O_2

H_2O_2 conc./M	Mean CL intensity (arbitrary units)	RSD (n=5), %
10^{-7}	21.2	16.2
10^{-6}	233	15.7
10^{-5}	1940	17.6

nM, 200 nM and 20 μM for TMP were 2.82, 3.08, 1.47 and 1.42, respectively. Consequently, TMP of the final concentration of 2 nM was used hereafter.

Under the experimental conditions, a linear calibration curve for H_2O_2 was obtained from 10^{-8} to 10^{-4} M. The detection limit was 10 nM at the CL intensity ratio of sample to blank (S/B) was 3. Precision for five replicates measurements was 15.7–17.6% of relative srandard deviations (RSDs, Table 2).

Glucose assay

It is known that D-glucose consists of 36.5% of α-form and 63.5% of β-form in water, and that GOD specifically acts on β-D-glucose to generate H_2O_2. On the other hand, mutarotase easily transforms α-D-glucose to β-D-glucose.[26,27] Thus, in this experiment, glucose was completely converted to hydrogen peroxide and D-gluconic acid with both GOD and mutarotase. Generally, 50 mM (pH 5.6) of phosphate buffer or acetate buffer has been used as a buffer solution for the GOD reaction.[28-31] On the other hand, the imidazole buffer (pH 7–8) has been commonly used for peroxyoxalate CL reaction.[32] Thus we examined the effect of a combination of these buffers for enzymic reaction and/or CL reaction as a function of CL intensity with 10^{-6} M H_2O_2. The water content of the system was fixed at 40%(v/v). Under the condition, the CL produced by using phosphate–imidazole buffer or acetate–imidazole buffer was 1.5 to 2 times smaller than that by imidazole buffer only at pHs from 6 to 9. Thus we used the imidazole buffer for both the enzymatic and CL reactions.

The time course for the GOD reaction is illustrated in Fig. 3. When 5.0 U of mutarotase was used, the enzymic reaction completed in about 20 min at 35° C with more than 30 U of GOD. On the other hand, as shown in Fig. 4, 20 U of mutarotase was found to be necessary for transformation of D-glucose in a short time. The calibration curve for glucose was linear up to 1.5×10^{-4} M (r=0.996). The RSD for the ten replicate measurements for 2.8×10^{-5} M of glucose solution was 11.0%.

The method was applied to the determination of glucose in human serum. The recovery rate was 98.2% (n=4), which was calculated from the slopes of calibration curves using standard glucose solution and spiked serum. The proposed method (y) was compared with the conventional *o*-toluidine colorimetric method (x) for the determination of glucose in normal human serum or control serum. As shown in Fig. 5, a good correlation was obtained ($y=0.94x+10.6$; r=0.968; n=13).

In conclusion, a simple and sensitive peroxyoxalate CL method for the detection of H_2O_2 was developed by

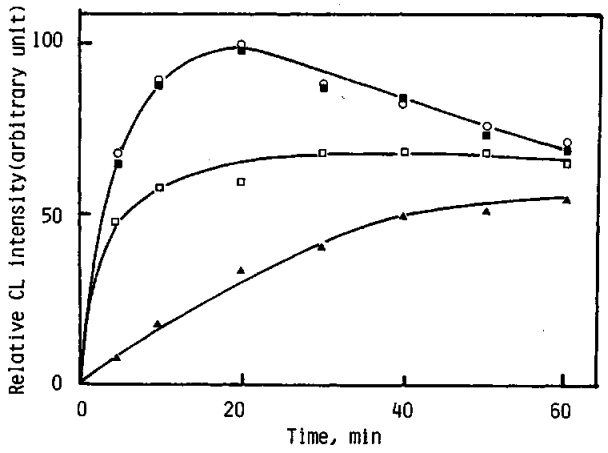

Fig. 3 The time course for GOD reaction. GOD amount (units in the reaction mixture): (▲) 5; (□) 20; (○) 30; (■) 40. Mutarotase, 5 U. To 2 ml of 100 ml of 100 mM imidazole buffer (pH 6.75) were added 1 ml of glucose (4 mg/dl), 0.2 ml of mutarotase (25 U/ml), and 1 ml of GOD (5, 20, 30, 40 U/ml), mixed and incubated at 35°C. A 100 µl aliquot of the reaction mixture was periodically removed and the relative CL intensity was measured.

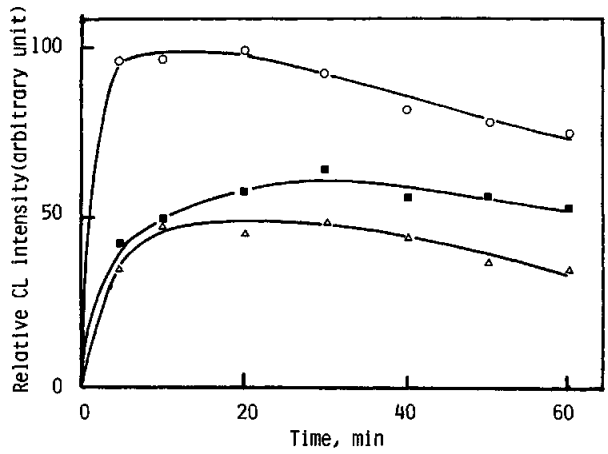

Fig. 4 The time course for mutarotase reaction. Mutarotase amount (units in the reaction mixture): (△) 0; (■) 5; (○) 20. GOD, 30 U. The other experimental conditions were shown in Fig. 3.

using TMP as a fluorescent component, which could be used in the determination method of glucose. As the glucose assay method developed is sensitive and comparable to known CL methods[14,19,28,31], it should be useful for the micro-assay of glucose in a biomedical study. On the other hand, TMP concentration used as a fluorescent component was only 10 nM. This might be advantageous for the use of fluorescent components as postcolumn reagents in a high-performance liquid chromatography or in a flow injection analysis. The proposed method will be able to apply the flow injection method for the assay of glucose by using immobilized enzyme.

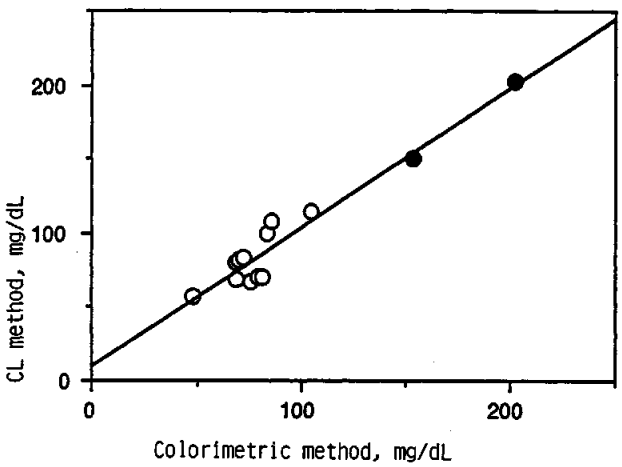

Fig. 5 Correlation between amounts of glucose in serum for the CL method and the colorimetric method. Serum sample: (○) normal human serum; (●) control serum (adjusted to contain 150 or 200 mg/dl of glucose).

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