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Development of an Evaluation Method for Hydroxyl Radical Scavenging Activities Using Sequential Injection Analysis with Chemiluminescence Detection

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A method for evaluating hydroxyl radical (·OH) scavenging activities using sequential injection analysis (SIA) with chemiluminescence (CL) detection was developed. In this system, CL was produced by the reaction of luminol with ·OH generated from the Fenton reaction. The scavenging activity was expressed as a diminution rate of the CL due to the scavenging of ·OH by a sample. The SIA system allows the automation of a series of experimental procedures including Fenton's reaction, scavenging of ·OH, and luminol CL reaction. The evaluation of scavenging activities in one sample (n = 3) was completed within 3.0 min. Relative standard deviations (n = 3) of scavenging activity with 700 μM L-ascorbic acid were 2.6% (intraday) and 3.7% (interday). The SIA-CL system was applied to measure ·OH scavenging activities of several antioxidants and pharmaceuticals.

Keywords Hydroxyl radical, sequential injection analysis, chemiluminescence, luminol

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Introduction

Oxygen is indispensable for many living organisms, including human beings. However, oxygen also generates reactive oxygen species (ROS) through various mechanisms such as biological metabolism, smoking, and consumption of alcohol. Even though ROS are essential for killing bacteria that is engulfed by macrophages in order to protect the body from infection, excessive ROS is an underlying cause of oxidative damage. Oxidative stress is implicated in causing the progress of several diseases, such as cancer, cerebral infarction, and diabetes, as well as aging.[1–3] As a result, much attention has recently been given to antioxidants, such as vitamins, catechins, and other plant-based compounds.[4,5]

ROS include hydroxyl radical (·OH), superoxide anion (O_2^-), singlet oxygen ($^1O_2$), hypochlorite (OCl^-), and hydrogen peroxide (H_2O_2). Hydroxyl radicals mainly form through a Fenton reaction *in vivo*, as shown in reaction (1).[6]

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + \cdot OH \quad (1)$$

·OH is believed to be the most harmful ROS to health, because they have the highest reactivity among various ROS. In particular, there is considerable evidence that ·OH can damage DNA bases.[4] Therefore, a simple and rapid screening method to find ·OH scavengers from various sources is needed.

Various methods to measure the reaction product of ·OH have been developed: fluorometry,[5,7] absorptiometry,[8,9] electron paramagnetic resonance (EPR),[10,11] and chemiluminescence (CL) batch method.[12] However, these methods require relatively large amounts of sample and reagent consumption, and also measurement procedures are complicated and time-consuming. Additionally, the sensitivity of EPR methods are generally low because these methods are based on the measurement of the electron paramagnetic resonance spectrum of unstable ·OH adducts. We used the SIA-CL method this time to overcome these weak points. The SIA system is able to automate the manipulation of sample and reagent solution mixing and transportation to the measurement point. Furthermore, this method requires less sample material than other methods and does not require as much time for analysis.

In our laboratory, we have reported sequential injection analysis (SIA) with CL methods for the evaluation of antioxidative activity against OCl^-, $^1O_2$, O_2^-, and nitric oxide.[13–15] As mentioned above, ·OH is regarded as the most reactive species of ROS, and as such, ·OH scavenger should be effective to protect from serious oxidative stress caused by ·OH. Therefore, in this research, we developed the SIA-CL detection method to detect ·OH for the evaluation of ·OH scavenging activity. In the proposed method, CL was produced by the reaction of luminol with ·OH generated from the Fenton reaction. The luminol emits light by reacting with ·OH.[12] ·OH which has short length of life (about two nanoseconds[5]) can be detected easily by monitoring the emission of the light. The scavenging activity was expressed as a diminution rate of the CL due to the scavenging of ·OH by a sample. The SIA system

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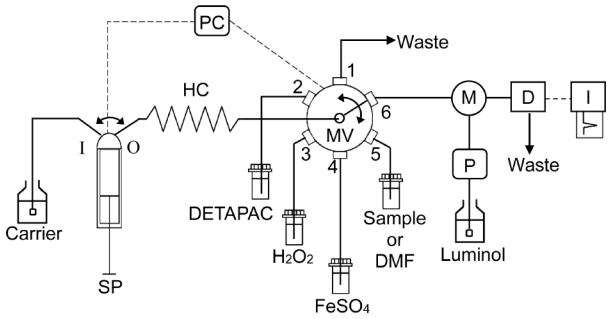

Fig. 1 SIA system for antioxidant assay of hydroxyl radical. SP, syringe pump, Cavro XL 3000 (syringe volume: 1 mL); HC, holding coil (length: 1 m, 0.5 mm, i.d.); MV, multi-port valve, Cavro Smart Valve; M, mixing tee; P, pump, Shimadzu LC-10ADvp; D, chemiluminescence detector, Shimadzu CLD-10A; I, integrator, Shimadzu Chromatopac C-R8A; PC, personal computer, NEC Alie NX; CL reagent solution, 200 μM luminol solution.

allows the automation of a series of experimental procedures including Fenton's reaction, scavenging of ·OH and luminol CL reaction. Specifically, ·OH scavenging activities of well-known pharmaceuticals were measured. The results obtained using this method are highly reproducible with sufficient accuracy.

Experimental

Reagents and solutions

Luminol, *N,N*-dimethylformamide (DMF), and α-tocopherol, and edaravone were purchased from Nacalai Tesque (Kyoto, Japan); L-ascorbic acid and H_2O_2 (30%, v/v) were purchased from Wako Pure Chemical Ind. (Osaka, Japan). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a hydrophilic α-tocopherol derivative) was purchased from Aldrich (Milwaukee, WI) and *N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid (Hepes), diethylenetriaminepentaacetic acid (DETAPAC), acetylsalicylic acid, and chlorpheniramine were obtained from Sigma Chemical Co. (St. Louis, MO). Ferrous sulfate ($FeSO_4$) was purchased from Ishizu (Osaka, Japan). Water was deionized using an Autostill WG 220 distiller from Yamato Scientific Co., Ltd. (Tokyo, Japan) and was passed through a Puric-Z from Japan Organo Co., Ltd. (Tokyo, Japan). All other solvents and reagents were of analytical grade.

Apparatus

The SIA-CL system used for determining the scavenging activity of ·OH is shown in Fig. 1. The SIA system consisted of a Cavro XL 3000 syringe pump (volume: 1 mL; Cavro Scientific Instruments Inc., Sunnyvale, CA), a six-port multiport valve (Cavro Smart Valve), holding coil (1 m × 0.5 mm i.d., polytetrafluoroethylene tube), a CL detector from Shimadzu Co. (Kyoto, Japan), and an integrator (Chromatopac C-R8A, Shimadzu). The CL reagent (200 μmol L^{-1} luminol solution) was pumped by an LC-10AD pump (Shimadzu). The syringe pump and multiport valve were computer-controlled with Pump: Link Evaluation Software (Cavro). Table 1 shows the sequence of operations for the SIA program. The operations performed for each measurement are as follows. The tubing of the SIA system and the syringe pump were initially filled with carrier solution. Then, the zones for the sample (with or without antioxidants) and for the reagents for generated ·OH (H_2O_2, $FeSO_4$, and DETAPAC) were sequentially aspirated into the holding coil by the syringe pump. Subsequently, the multiport valve was switched to the port connected with a CL detector, the flow direction was reversed, and the composite zone was propelled to the mixing tee and introduced to the CL detector. In these steps, the generation of ·OH by Fenton's reaction and the scavenging of ·OH by the sample occurred successively. The composite zone was then introduced to the stream of the luminol solution (flow rate: 1.0 mL min^{-1}) by an HPLC pump. The resulting CL signal derived from unscavenged ·OH was recorded at the peak height. Each measurement was repeated in triplicate and the mean and standard deviation (SD) of peak-height values were used in this experimental analysis. All measurements were carried out at ambient temperature (24 ± 4°C).

Table 1 The sequence of operations for the SIA system

Step	Event	Valve position of SP	Valve position of MV	Volume/ μL	Flow rate/ mL min^{-1}
1	Aspiration of carrier solution	I	—	600	1.5
2	Aspiration of sample or blank	O	6	5	0.4
3	Aspiration of H_2O_2	O	3	10	0.4
4	Aspiration of DETAPAC	O	2	10	0.4
5	Aspiration of $FeSO_4$	O	5	10	0.4
6	Propulsion to the CL detector	O	4	635	1.5

Measurement of vitamins and pharmaceutical ·OH scavenging activities

The scavenging activities of L-ascorbic acid, α-tocopherol, Trolox, edaravone, acetylsalicylic acid, and chlorpheniramine were measured by the SIA-CL system. Trolox, L-ascorbic acid, and α-tocopherol are well-known antioxidants. Edaravone, a free radical scavenger; acetylsalicylic acid, an anti-inflammatory drug; and chlorpheniramine, which is a histamine antagonist are all approved pharmaceuticals by the Ministry of Health, Labour and Welfare.

Scavenging activity was calculated *via* the following equation:

$$\text{Scavenging activity (\%)} = (CLI_B - CLI_S)/CLI_B \times 100 \quad (2)$$

Here, CLI_B and CLI_S represent the CL intensities obtained from the blank (DMF) and the sample, respectively. The IC_{50} value, defined as the sample concentration that produces 50% scavenging activity against ·OH, was used to compare the sample compounds in terms of effectiveness. The IC_{50} value was calculated from a regression of the scavenging activity against the logarithmic concentration of each sample compound. Smaller IC_{50} values of the sample indicate the ·OH scavenging activity is more active.

Results and Discussion

Optimization of the SIA-CL system

The ·OH generating reagents were prepared according to our previous study[12] except for $FeCl_2$. In the present study, we used $FeSO_4$ because $FeCl_2$ exhibited low solubility. A 100 mM Hepes buffer (pH 7.4) was employed as a carrier solution. In this study, the effects of aspiration volume and flow-rate of the carrier solution, the concentrations of reagents, and the aspiration order on the CL intensities were investigated. The CL intensity obtained from the DMF (blank) was used as the index for the

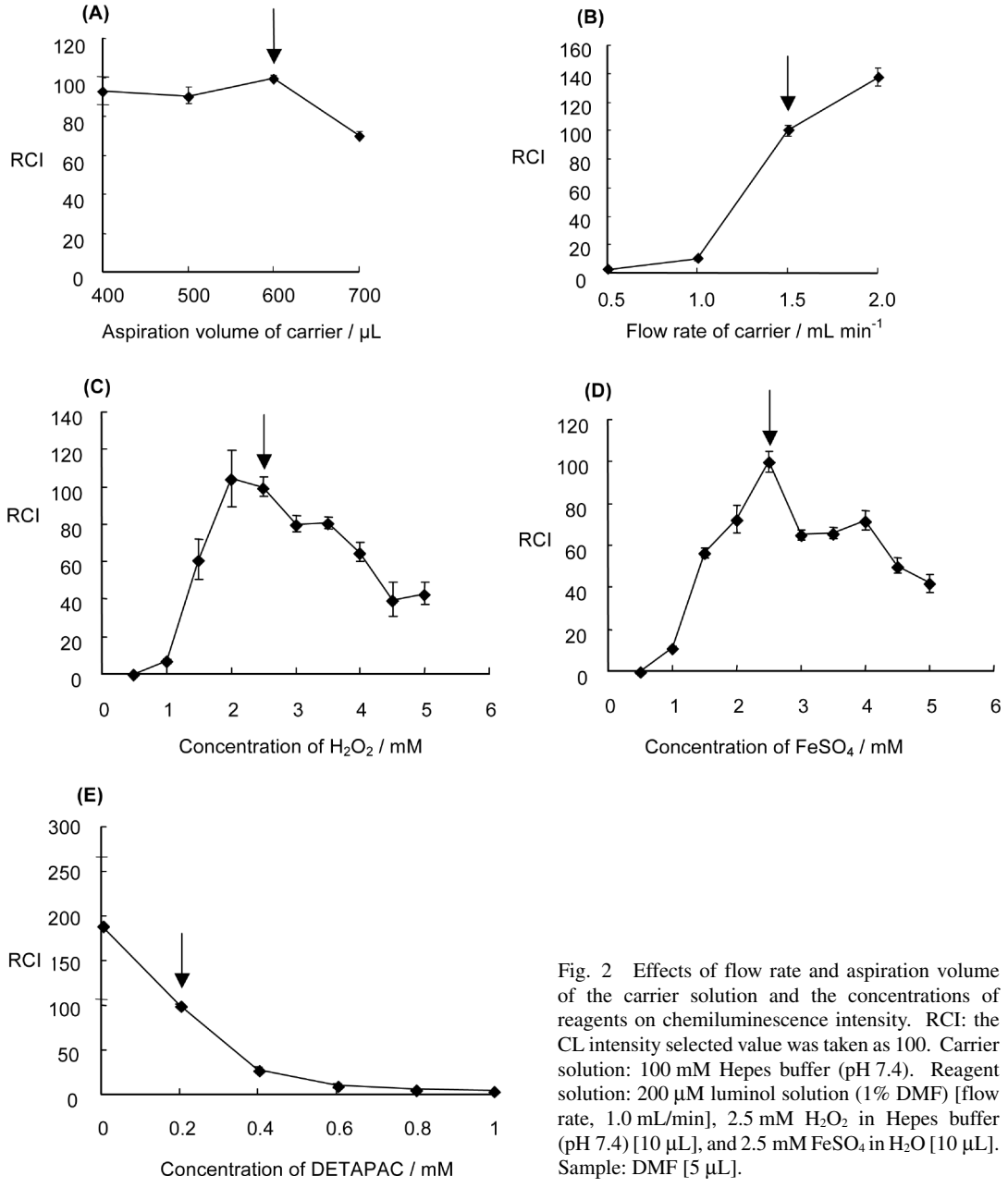

Fig. 2 Effects of flow rate and aspiration volume of the carrier solution and the concentrations of reagents on chemiluminescence intensity. RCI: the CL intensity selected value was taken as 100. Carrier solution: 100 mM Hepes buffer (pH 7.4). Reagent solution: 200 μM luminol solution (1% DMF) [flow rate, 1.0 mL/min], 2.5 mM H_2O_2 in Hepes buffer (pH 7.4) [10 μL], and 2.5 mM $FeSO_4$ in H_2O [10 μL]. Sample: DMF [5 μL].

optimization of SIA-CL conditions. These results of the study are shown in Fig. 2. The largest CL intensity and minimum SD (RSD ≤ 5%) were selected as the optimum conditions.

The effect of the aspiration volume of the carrier solution was examined over the range of 400 – 700 μL (Fig. 2(A)). Because of the minimum SD, an aspiration carrier volume of 600 μL was employed. A higher flow-rate of the carrier solution generally provides shorter analysis time but also possibly lowers the sensitivity due to the shorter time of passing through the CL detector. The effect of the flow-rate of the carrier solution was examined over the range of 0.5 – 2.0 mL/min (Fig. 2(B)), because the flow-rate could control the time to reach the mixture containing sample, ·OH generating reagents and luminol to the detector. The maximum CL intensities were attained at 2.0 mL/min.; however, minimum SD was obtained from 1.5 mL/min. On the basis of this result, 1.5 mL/min was selected as the measuring condition. The concentration effects of H_2O_2, $FeSO_4$, and DETAPAC were investigated (Figs. 2(C) – 2(E)). We selected the concentrations to be 2.5 mM H_2O_2, 2.5 mM $FeSO_4$, and 0.2 mM DETAPAC. Since the aspiration order of the reagents and sample influenced the CL intensity, the effect of the order of reagent aspiration for the generation of ·OH was examined (Table 2). The maximum CL intensity was obtained at the following order: sample, H_2O_2, DETAPAC, and $FeSO_4$. This system was divided into 1) ·OH generation, 2) ·OH and mixture of the luminol, and 3) emission of light detection. Because all these processes were automated with a PC, all those processes can be controlled precisely. Therefore, the detection of unstable OH, and the evaluation of scavenging activities of a compound can be carried out with good reproducibility.

Signal responses and reproducibility

The typical signal responses obtained from L-ascorbic acid (0 – 2.0 mM, $n = 3$) are shown in Fig. 3. The CL intensities were inhibited by L-ascorbic acid in a dose-dependent manner. The evaluation of scavenging activities in one sample ($n = 3$) was completed within 3.0 min. The relative standard deviations (RSD) of the scavenging activity were examined using 700 μM

Table 2 Optimization for the aspiration order of scavenging activity

Aspiration order of reagent				CL intensity (RCI ± SD, n = 3)
1	2	3	4	
Sample	H_2O_2	DETAPAC	$FeSO_4$	100[a] ± 5.07
Sample	DETAPAC	H_2O_2	$FeSO_4$	59.60 ± 5.46
Sample	DETAPAC	$FeSO_4$	H_2O_2	21.88 ± 3.70
Sample	$FeSO_4$	DETAPAC	H_2O_2	53.45 ± 6.57
DETAPAC	Sample	H_2O_2	$FeSO_4$	77.71 ± 6.49
DETAPAC	Sample	$FeSO_4$	H_2O_2	46.77 ± 8.22
DETAPAC	H_2O_2	Sample	$FeSO_4$	87.25 ± 7.34
H_2O_2	Sample	DETAPAC	$FeSO_4$	44.56 ± 5.97
H_2O_2	DETAPAC	Sample	$FeSO_4$	26.98 ± 10.59

a. RCI: the CL intensity selected value was taken as 100.
Carrier solution: 100 mM Hepes buffer (pH 7.4). Reagent solution: 200 μM luminol solution (1% DMF) [flow rate, 1.0 mL/min], 2.5 mM H_2O_2 in Hepes buffer (pH 7.4) [10 μL], 2.5 mM $FeSO_4$ in H_2O [10 μL]. Sample: DMF [5 μL].

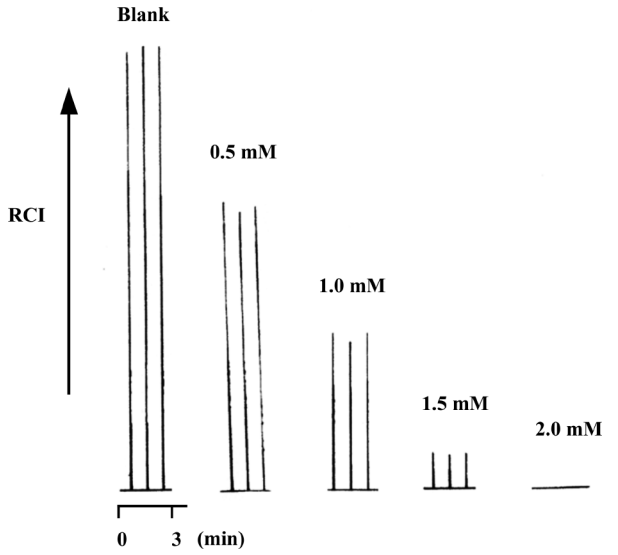

Fig. 3 Signal responses obtained for blank and L-ascorbic acid solutions. RCI: the CL intensity selected value was taken as 100. Carrier solution: 100 mM Hepes buffer (pH 7.4). Reagent solution: 200 μM luminol solution (1% DMF) [flow rate, 1.0 mL/min], 2.5 mM H_2O_2 in Hepes buffer (pH 7.4) [10 μL], and 2.5 mM $FeSO_4$ in H_2O [10 μL]. Sample: DMF or L-ascorbic acid [5 μL].

L-ascorbic acid. The acceptance criterion for reproducibility was defined as an RSD at each concentration not exceeding 5%. The intraday (n = 3) and interday (n = 3) reproducibility of the scavenging activities were 2.6 and 3.7%, respectively.

Evaluation of ·OH scavenging activity of vitamins and pharmaceuticals

Various concentration of L-ascorbic acid, α-tocopherol, Trolox, edaravone, acetylsalicylic acid, and chlorpheniramine were measured by this SIA-CL system. Table 3 shows the relationship between the scavenging activity and the sample concentration, as well as the IC_{50} value for each test compound. Linear relationships between the scavenging activities and the logarithmic concentrations were obtained ($r \geq 0.931$), except for chlorpheniramine. L-ascorbic acid, which is a representative antioxidant, showed strong scavenging activity. α-Tocopherol showed weaker scavenging activity than Trolox (hydrophilic α-tocopherol derivative). Therefore, ·OH scavenging activity might be influenced by the polarity of the compounds. Also, the ·OH scavenging of Trolox was found to be approximately two times more than that of L-ascorbic acid. This result was consistent with other reports.[16] Among the tested pharmaceuticals, edaravone showed most effective ·OH scavenging activity. This result was consistent with its role as a free radical scavenger.[17] On the other hand, acetylsalicylic acid showed extremely weak ·OH scavenging activity, and chlorpheniramine did not show any ·OH scavenging activity. Since the proposed SIA-CL method allowed simple and rapid measurement of ·OH scavenging activity, the method should be useful to discover new beneficial effects of existing pharmaceuticals.

Table 3 IC_{50} values of vitamins and pharmaceuticals

Compound	Equation	r	IC_{50}/mM
L-Ascorbic acid	$y = 88.8 \log(x) + 330$	0.933	0.7
α-tocopherol	$y = 49.0 \log(x) + 101$	0.931	91
Trolox	$y = 79.0 \log(x) + 338$	0.969	0.23
Edaravone	$y = 467.0 \log(x) + 225$	0.958	0.2
Acetylsalicylic acid	$y = 35.7 \log(x) - 78$	0.997	168.3
Chlorpheniramine	—[a]	—	—

y = scavenging activity (%), x = concentration (M).
a. Calculation was not possible.
Blank: DMF.

Conclusions

A method for evaluating ·OH scavenging activities using SIA was developed. A Fenton reaction was employed in order to generate ·OH. The aspiration volume and flow-rate of the carrier solution, the aspiration order, and the concentrations of H_2O_2, DETAPAC, and $FeSO_4$ were optimized for the SIA system. Scavenging activities against L-ascorbic acid, α-tocopherol, and Trolox were measured under these optimum conditions.

The SIA-CL system was able to determine rapid and reproducible detection with minimum consumption of the sample and reagents. This method is useful for the screening of compounds possessing scavenging activities against ·OH. The scavenging activity of common antioxidants and pharmaceuticals against ·OH was successfully determined using this method.

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