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Automated analysis of the serum antioxidative activities against five different reactive oxygen species by sequential injection system with a chemiluminescence detector

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ABSTRACT

Background: There is a growing evidence that reactive oxygen species (ROS) may cause many pathologic conditions including chronic diseases, neurodegenerative disorders, cancer and aging. There are a number of methods to measure the total antioxidative activity of the serum. However, since the lifetime and oxidative activity of various types of ROS are all different, to measure simply the total antioxidative activity of the serum is not enough. Therefore, to aid the diagnosis and to improve the therapeutic strategy, it is important to develop a simple and reliable method of assaying antioxidative activity of the serum.
Methods: A method that combines sequential injection analysis (SIA) and luminol chemiluminescence (CL) detection was employed for the measurement of antioxidative activities of human serum. We collected sera from healthy subjects ($n=42$) and patients with diabetes ($n=39$) and rheumatoid arthritis ($n=25$) and tested the sensitivity, reproducibility and reliability of our method.
Results: Since the operation is automatically controlled by a personal computer, we obtained a satisfactory repeatability without the need of much manpower. The time required for obtaining the antioxidative activity against one ROS for one individual is less than 3 min. Although the value of antioxidative activity varies from subject to subject, there were a certain relationship between the disease and the antioxidative values of each type of ROS. The results suggest that the measurement of antioxidative activity against different ROS may provide us with valuable information regarding the disease state.
Conclusions: The evaluation of antioxidative activities against each ROS by the proposed method should be more informative to understand the antioxidative status of biological fluid.

1. Introduction

Reactive oxygen species (ROS) generated in biological systems are involved in signal regulation, production of energy, phagocytosis and defense mechanism against infection. However, the excess generation of ROS induces harmful biological oxidation. Indeed, ROS have been implicated in the pathophysiology of several conditions including diabetes, infertility, rheumatoid arthritis and cardiovascular diseases [1–4]. Much evidence indicates that exposure to ROS cause deleterious changes in cell function by a number of oxidative modification such as lipid peroxidation, enzyme inactivation and oxidative DNA damage, ultimately results in cell death [5–8]. In these aspects, the evaluation of the degrees of oxidative damage caused by ROS is essential in order to clarify the contribution of ROS to several diseases.

On the other hand, it is known that there are different kinds of ROS including superoxide anion (O_2^-), nitric oxide (NO), hydrogen peroxide (H_2O_2), hypochlorite anion (ClO^-) and singlet oxygen ($^1O_2$). Since each ROS has different chemical features including lifetime and oxidative activity, the oxidative damage on the living body and resulting disease should be different. Therefore, it is important to evaluate the degrees of oxidative damage due to each

Abbreviations: CL, chemiluminescence; DMF, *N*, *N*-dimethylformamide; HEPES, *N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid; HX, Hypoxanthine; LPO, lactoperoxidase; NOR1, (±)-(*E*)-4-methyl-2-[(*E*)-hydroxyimino]-5-nitro-6-methoxy-3-hexenamide, ROS, reactive oxygen species; SIA, sequential injection analysis; XOD, xanthine oxidase.

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ROS in order to understand the relationship between ROS generation and pathogenesis.

In this study, we focused on the endogenous antioxidative activity of human serum as an indicator of oxidative damage due to each ROS. It is known that human serum possess antioxidants and antioxidative enzymes to protect the body against oxidation by ROS. The concentrations and/or activities of them should decrease by the scavenging of excessively generated ROS in the body of patients. As a result, the antioxidative activity of serum of patients might be lower than that of healthy human.

The task of quantifying antioxidative activity of biological samples can be approached in three different ways. Firstly, the concentrations of all of the individual molecules that are currently recognized as antioxidants are measured. However, it does not account for the influence of undiscovered antioxidants or the substances that are technically difficult to be assayed. Secondly, the antioxidative activity can be measured by the decreasing ratio of 1,1-diphenyl-2-picrylhydrazyl radical or peroxy radical scavenging activities [9,10]. However, these methods can be applied only for specific radicals. Thirdly, ferric reducing ability of plasma method [11] and total radical-trapping potential method [12] are famous for assay of antioxidative activity of biological sample; however, they are based on the total reduction ability of samples. Since ROS differ from each other with respect to chemical features including reactivity and life-time, antioxidative activities of biological samples against each ROS should be different. However, as mentioned above, all the assays for the total antioxidative activity of biological samples cannot meet the requirement. Therefore, the method for determination of antioxidative activities against each ROS will be required.

In our laboratory, antioxidative activity measurement method for plant extracts has been developed, based on batch method with luminol chemiluminescence (CL) detection [13]. Luminol emits light when it reacts with ROS. In this method, the attenuation of luminol CL due to scavenging of ROS by sample was measured as the antioxidative activity. Successively, luminol CL detection was hyphenated with sequential injection analysis (SIA) for determination of antioxidative activities against ClO^-, $^1O_2$, O_2^- and NO [14–16]. Employing SIA makes it possible to reduce sample volume, reagent consumption and human error and obtain repeatable results, comparing with flow injection analysis [17].

In this study, we exploit the SIA-CL method to automated analysis of the serum antioxidative activity against five different ROS. The method has an advantage for determination of antioxidative activities against each ROS because different ROS can be generated by altering the reagents in the SIA system. The developed method was applied to the serum samples collected from healthy subjects, diabetic and rheumatoid patients and the measured antioxidative activities were compared.

Table 1
Carrier solution and ROS generation reagent for the SIA-CL measurement.

ROS	Carrier solution	ROS reagent
ClO^-	50 mmol/l borate buffer (pH 9.5)	38.3 mmol/l NaClO/carrier solution
NO	100 mmol/l Hepes buffer (pH 8.2)	2 mmol/l NOR1/DMSO:100 mmol/l HCl = 1:1
$^1O_2$	100 mmol/l sodium acetate buffer (pH 4.5)	150 mmol/l H_2O_2/carrier solution 80 mg/ml LPO/carrier solution 250 mmol/l NaBr/carrier solution
O_2^-	100 mmol/l Hepes buffer (pH 8.2)	1.6 U/ml XOD/carrier solution 1.0 mmol/l HX/carrier solution
H_2O_2	50 mmol/l borate buffer (pH 9.5)	100 mmol/l H_2O_2/carrier solution

2. Material and methods

2.1. Reagents and solution

Hypoxanthine (HX), L-ascorbic acid, boric acid, sodium hydroxide, H_2O_2 and sodium acetate were purchased from Wako Pure Chemical (Osaka, Japan); xanthine oxidase (XOD), *N*, *N*-dimethylformamide (DMF), luminol and sodium hypochlorite were from Nacalai Tesque (Kyoto, Japan); (±)-(*E*)-4-methyl-2-[(*E*)-hydroxyimino]-5-nitro-6-methoxy-3-hexenamide (NOR1) was from Dojindo (Kumamoto, Japan); *N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid (HEPES), lactoperoxidase (LPO) and sodium bromide were from Sigma (St. Louis, MO, USA). Water was deionized using an Autostill WG 220 (Yamato Kagaku, Tokyo, Japan) and passed through a Puric-Z (Organo, Tokyo, Japan). All other solvents and reagents were of analytical grade. Luminol was dissolved in DMF and then diluted with water (DMF content was 1%). The reagents used for generation of each ROS are summarized in Table 1.

2.2. Apparatus and manifold design

The SIA-CL system (Fig. 1) consisted of a Cavro XL 3000 syringe pump (volume 1 mL, Cavro Scientific Instruments Inc., CA, USA), a

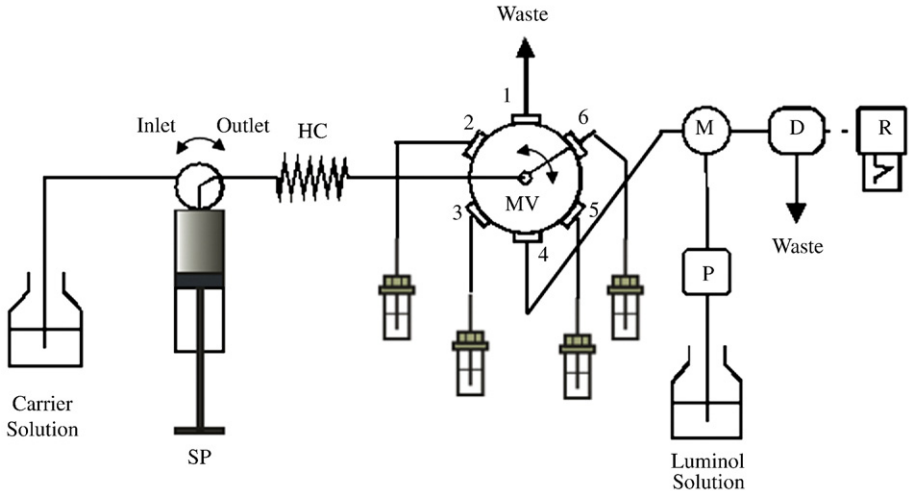

Fig. 1. Scheme of the SIA-CL system for the measurement of antioxidative activity of human serum against each ROS. SP, syringe pump; HC, holding coil; MV, multiport valve; M, mixing tee; P, pump: D, detector; R, recorder.