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Luminol chemiluminescent determination of hydrogen peroxide at picomole levels using high-performance liquid chromatography with a cation-exchange resin gel column

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Abstract

The optimum conditions of high-performance liquid chromatography combined with luminol chemiluminescence detection were established for the determination of hydrogen peroxide at picomole levels using a cation-exchange gel column. The gel column with distilled water as the mobile phase allowed a good separation of H_2O_2 without causing any irreversible binding of H_2O_2 to the column surface. The detection limit and the quantification limit of H_2O_2 were 4 and 6–600 pmol, respectively. The suitability of the present method was verified by the determination of H_2O_2 present in coffee drinks.

1. Introduction

Hydrogen peroxide (H_2O_2) is an oxidizing agent and has been used in sterilization and bleaching of food products. Previous studies have reported the formation of H_2O_2 in the oxidation of oily foods [1], in the oxidation of ascorbic acid in beverages [2] and in several food systems, and this has still not been well explained [3]. H_2O_2 has been found to cause duodenal cancer in mice following oral intake [4].

H_2O_2 is generally determined by means of electrochemical [5,6], spectrophotometric [7,8], fluorimetric [9,10] and chemiluminescence [11–16] techniques. Among these methods, luminol chemiluminescence assay is the most popular because of its excellent sensitivity. In both food and biological systems, it is known that ascorbic acid, tocopherols and lipid peroxides interfere with the chemiluminescent reaction in H_2O_2 determination [17]. Therefore, separation of H_2O_2 from these compounds is needed.

We have previously reported the presence of phospholipid hydroperoxides in human blood plasma, rat liver and brain using high-performance liquid chromatography with chemiluminescence detection (HPLC–CL) with a mixture of luminol and cytochrome *c* as a postcolumn chemiluminescent reagent with a silica-based aminopropyl column [17,18]. In the determination of lipid hydroperoxides, cytochrome *c* is favoured as a catalyst because of its higher hydrophobicity than that of microperoxidase, a haem peptide prepared from cytochrome *c* [19].

In this work, the optimum conditions of HPLC–CL, consisting of a cation-exchange resin gel column and a mixture of luminol and mi-

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croperoxidase as chemiluminescent reagent, were studied in order to determine H_2O_2 present in aqueous systems. The method was based on separation of H_2O_2 with the gel column and postcolumn detection of H_2O_2-dependent chemiluminescence catalysed by microperoxidase under alkaline conditions. Under the recommended conditions, the determination of 6–600 pmol of H_2O_2 was quantitative without irreversible binding of H_2O_2 to the column surface. The detection limit of the total system was 4 pmol. The suitability was verified by the determination of H_2O_2 present in coffee drinks.

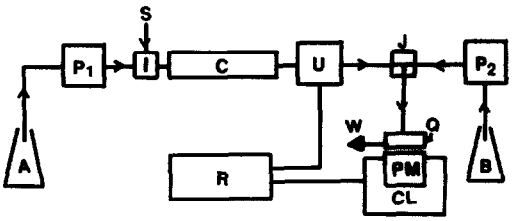

Fig. 1. Schematic diagram of HPLC–CL system for the assay of hydrogen peroxide. A = mobile phase (distilled water); P_1 and P_2 = pumps (Jasco 880PU), flow-rate 1 ml/min; I = sample injection valve (Rheodyne Model 7125, 20 μl); S = sample (20 μl); C = HPLC column (Ionpak KS-801, 300 mm × 8 mm I.D., in a Jasco 860CO column oven at 24°C); U = UV detector (Jasco 875UV); J = mixing joint (Kyowa Seimitsu Y type); B = chemiluminescent reagent consisting of 1.0 μg/ml luminol and 1.0 μg/ml microperoxidase in borate buffer (200 m*M* $H_3BO_3 \cdot KCl$–Na_2CO_3, pH 10.4); Q = flow cell; PM = photomultiplier; CL = chemiluminescence detector (Jasco 825CL); R = multiple recorder and integrator; W = waste.

2. Experimental

2.1. Reagents

Distilled water (HPLC grade) and hydrogen peroxide (30% aqueous solution) were obtained from Kanto Chemical (Tokyo, Japan) and Santoku Chemical (Tokyo, Japan), respectively. The hydrogen peroxide concentration was standardized by iodimetry [20]. Luminol (3-aminophthaloylhydrazine) was purchased from Wako (Osaka, Japan). Peroxidase (from *Arthromyces ramosus*, EC 1.11.1.7) was a gift from Suntory (Osaka, Japan) [21]. Cytochrome *c* (Type IV from equine heart), microperoxidase (MP-11 from equine heart cytochrome *c*) and catalase (C-10 from bovine liver, EC 1.11.1.6) were obtained from Sigma (St. Louis, MO, USA). Other chemicals were of analytical-reagent grade.

2.2. HPLC–CL

A schematic diagram of the HPLC–CL system for H_2O_2 determination is shown in Fig. 1. The system consisted of an HPLC column packed with a cation-exchange resin gel of sulphonated styrene–divinylbenzene copolymer (Shodex Ionpak KS-801, 300 mm × 8 mm I.D.; Showa Denko, Tokyo, Japan) placed in a column oven (Jasco 860CO; Japan Spectroscopic, Tokyo, Japan) at 24°C, two HPLC pumps (Jasco 880PU) with a Rheodyne Model 7125 sample loop injection (20 μl), a UV detector (Jasco 875UV) and chemiluminescence detector (Jasco 825CL) equipped with a spiral flow cell (200 μl).

The chemiluminescent reagent was prepared by dissolving luminol (1.0 μg/ml) and microperoxidase (1.0 μg/ml) in 200 m*M* $H_3BO_3 \cdot KCl$–Na_2CO_3 buffer at pH 10.4. The mixture was filtered through a cellulose nitrate filter (pore size 0.45 μm; Advantec Toyo, Tokyo, Japan) before use. The pH of the chemiluminescent reagent was stable at least for 48 h after preparation. The flow-rate of distilled water used as the mobile phase and that of the chemiluminescent reagent were 1 ml/min.

The chromatograms with chemiluminescence and UV detection (at 210 nm) were simultaneously recorded with a multiple pen recorder (SS-250F; Sekonic, Tokyo, Japan). The chemiluminescence peak area of H_2O_2 was calculated with a Chromatocorder 12 (System Instruments, Tokyo, Japan). The assays were performed in a temperature-controlled room at 24°C. The concentration of H_2O_2 present in a sample solution was determined from a calibration graph prepared with standard H_2O_2 solutions.

3. Results and discussion

3.1. Optimum conditions of HPLC–CL for H_2O_2 determination

We employed Ionpak KS-801, an HPLC column packed with a cation-exchange resin gel, in order to avoid the irreversible binding of H_2O_2 to the column surface. Nahum *et al.* [22] reported that silica-based columns are not suitable for H_2O_2 detection owing to the binding of H_2O_2 to the column surface.

First, a standard solution of H_2O_2 (18 nmol) was assayed by HPLC–CL using a chemiluminescent reagent consisting of luminol and cytochrome *c* [18] that has been employed in the determination of lipid hydroperoxides. A sharp chemiluminescence peak ascribed to H_2O_2 appeared at 11.0 min (Fig. 2). H_2O_2 at nanomole levels was also detectable by UV spectrophotometry (210 nm).

To increase the sensitivity, peroxidases other than cytochrome *c* were examined as catalysts,

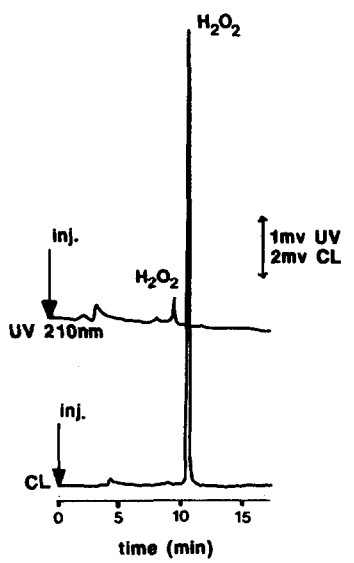

Fig. 2. Typical chromatogram of hydrogen peroxide (18 nmol) recorded by HPLC–CL with an Ionpak KS-801 column. Chemiluminescent reagent, mixture of 1.0 μg/ml luminol and 10.0 μg/ml cytochrome *c* in 50 m*M* borate buffer (pH 10.4) [19]. Other analytical conditions as in Fig. 1.

i.e., microperoxidase, *A. ramosus* peroxidase and horseradish peroxidase (HRP). Each peroxidase (1.0 μg/ml of microperoxidase, 1.0 μg/ml of *A. ramosus* peroxidase and 10.0 μg/ml of HRP) was added to a 200 m*M* $H_3BO_3 \cdot KCl$–Na_2CO_3 buffer that contained luminol (1.0 μg/ml). The chemiluminescent reagent consisting of microperoxidase and luminol was adjusted to pH 10.4 [18], that of *A. ramosus* peroxidase and luminol to pH 9.0 [21] and that of HRP and luminol to pH 10.4 [18]; 6, 20 and 60 pmol of H_2O_2, respectively, were introduced into the HPLC–CL system. Microperoxidase yielded H_2O_2-dependent chemiluminescence, *i.e.*, $7.4 \cdot 10^5$ counts at 6 pmol, $2.2 \cdot 10^6$ counts at 20 pmol and $7.2 \cdot 10^6$ counts at 60 pmol of H_2O_2. *A. ramosus* peroxidase yielded slightly higher chemiluminescence than that of microperoxidase. Both peroxidases gave linear relationships between integrated chemiluminescence counts and amount of H_2O_2 ($r = 0.99$; data not shown). In the absence of the catalyst, the integrated chemiluminescence count for 600 pmol of H_2O_2 was only 7200, indicating the importance of peroxidase as the catalyst in producing H_2O_2-dependent chemiluminescence. The signal-to-noise ratios for microperoxidase and *A. ramosus* peroxidase were 100 times higher than those for cytochrome *c* and HRP. This might be due to the difference in the hydrophilicity of the two peroxidases compared with cytochrome *c* and HRP. The high hydrophilicity of microperoxidase and *A. ramosus* peroxidase would result in dissolution of the chemiluminescent reagent by the column mobile phase (distilled water). This may relate to the high chemiluminescence production and baseline low noise (high signal-to-noise ratio) when using these peroxidases. As a result, both microperoxidase and *A. ramosus* peroxidase were suitable for H_2O_2 detection with this HPLC–CL system. Fig. 3 shows the HPLC–CL trace of H_2O_2 (60 pmol per 20-μl injection) for the total system, employing microperoxidase as a catalyst. Considering the commercial availability of the catalysts, we selected microperoxidase as the catalytic constituent of the luminol-containing chemiluminescent reagent.

The optimum concentrations of luminol and

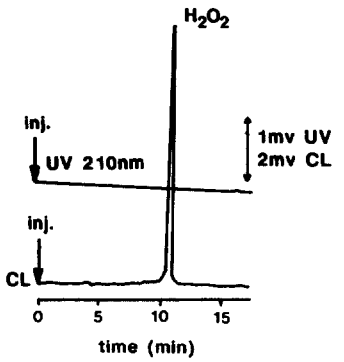

Fig. 3. Chromatogram of hydrogen peroxide (60 pmol) for the total system. Microperoxidase (1.0 μg/ml) was employed as a catalyst instead of cytochrome *c*. Other analytical conditions as in Fig. 1.

microperoxidase in the chemiluminescent reagent were examined. When the luminol concentration was fixed at 1.0 μg/ml in the chemiluminescent reagent, the chemiluminescence yield increased with increase in microperoxidase concentration from 0.1 to 5.0 μg/ml (Fig. 4a). The best signal-to-noise ratios at H_2O_2 amounts of 60 pmol (24) and 600 pmol (235) were observed with 1.0 μg/ml of microperoxidase. Much higher concentrations of microperoxidase (*i.e.*, over 2.0 μg/ml) caused significant increases in the noise levels and resulted in instability of the chemiluminescence baseline. This might result from the impurities in the microperoxidase. In the same way, 1.0 μg/ml of luminol was sufficient to yield the maximum H_2O_2-dependent chemiluminescence in HPLC–CL (Fig. 4b).

Considering the HPLC conditions, the column temperature and the mixing joint temperature were set at 24°C, taking into account the column pressure and retention time of H_2O_2. Above 24°C, the baseline (background counts) in chemiluminescence detection became unstable.

From these results, the optimum HPLC–CL conditions for the determination of H_2O_2 as given in Fig. 1 were confirmed. With the optimized HPLC–CL conditions, the calibration line from 6 to 600 pmol (6, 20, 60, 200 and 600 pmol) of H_2O_2 showed good linearity (correlation coefficient $r = 0.9992$). In four determinations of each H_2O_2 concentration, the standard errors

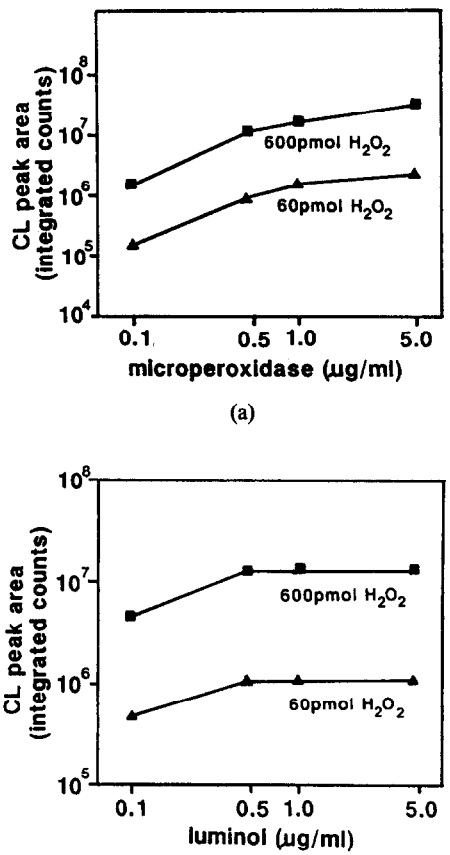

Fig. 4. Effect of microperoxidase and luminol concentrations on H_2O_2-dependent chemiluminescence. (a) In the chemiluminescent reagent, graded amounts (0.1, 0.5, 1.0, 5.0 μg/ml) of microperoxidase were included, in which the luminol concentration was fixed at 1.0 μg/ml in 200 m*M* borate buffer (pH 10.4). (b) In the chemiluminescent reagent, graded amounts (0.1, 0.5, 1.0, 5.0 μg/ml) of luminol were included, in which the microperoxidase concentration was fixed at 1.0 μg/ml in 200 m*M* borate buffer (pH 10.4). Other analytical conditions in Fig. 1; 60 and 600 pmol of H_2O_2 were determined.

were within 5% of the mean values. The detection limit of the total system was 4 pmol of H_2O_2 (signal to noise ratio = 3).

3.2. Interferences

Under the established optimum HPLC–CL conditions, ascorbic acid was found to give a

quenching peak (negative chemiluminescence peak) with a retention time of 5.5 min and was clearly separated from H_2O_2 (data not shown). In order to prevent the deterioration of the column, particles and proteins in the sample solution should be eliminated prior to the HPLC–CL analysis.

3.3. Application

Three types of powdered instant coffees (normal, caffeine-free and espresso types) and coffee beans for making drip coffee were purchased commercially. Coffee drinks were prepared by dissolving 2.0 g of powdered coffee in 140 ml of boiling water. The drip coffee was prepared by pouring 150 ml of boiling water on to 15.0 g of freshly milled roast coffee beans on a filter-paper. To each coffee drink (9.9 ml) 0.1 ml of 20% (w/v) sulphosalicylic acid solution was added to precipitate proteins. An aliquot of this sample suspension was then diluted 100-fold with distilled water. Prior to application to the HPLC–CL system, the diluted sample was filtered through a cellulose nitrate filter disc (pore size 0.45 μm) to remove proteins precipitated and particles. A typical chromatogram of H_2O_2 found in the drip coffee is shown in Fig. 5. The H_2O_2 content in coffee drinks was 67.1–165.0 μM (Table 1). The value determined here was identical with that in a previous report [3] in which an electrochemical method was used for the determination. The negative (quenching) peak in Fig. 5 might be ascribed to antioxidants, such as chlorogenic acid and ascorbic acid, present in the coffee drink. The chemiluminescence peak of H_2O_2 disappeared completely after treating the sample solution with catalase before the filtration procedure, indicating that H_2O_2 detected in the coffee drinks was not an artifact.

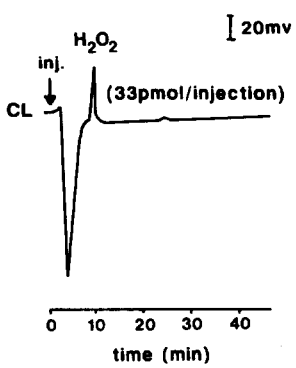

Fig. 5. Chromatogram of H_2O_2 in coffee drinks. Analytical conditions as in Fig. 1.

Table 1
Determination of hydrogen peroxide found in coffee drinks by HPLC–CL.

Coffee drink	H_2O_2 found (μM)
Normal	67.1 ± 3.8
Caffeine-free	64.7 ± 2.1
Espresso	99.7 ± 9.7
Drip	165.0 ± 5.3

Analytical conditions as in Fig. 1. Data are means ± standard errors ($n = 4$). Normal, caffeine-free and espresso coffee drinks were prepared by dissolving 2.0 g of powdered instant coffee in 140 ml of boiling water. Drip coffee was prepared by pouring 150 ml of boiling water on to 15.0 g of freshly milled coffee beans on a filter-paper.

4. Conclusions

The proposed HPLC–CL method is suitable for the determination of H_2O_2 with good selectivity, sensitivity and reproducibility. A cation-exchange resin gel column (Ionpak KS-801) is used that does not cause irreversible binding of H_2O_2 to the column surface. The sensitivity was equivalent to that of the most sensitive fluorimetric method. The small sample volume required as compared with those in other methods with the same sensitivity is another advantage of this HPLC–CL method. The applicability was verified by the determination of H_2O_2 present in coffee drinks. An environmental advantage is that no organic solvent is used in the column mobile phase.

Measurement of the oxygen metabolite H_2O_2 in biological fluids is of interest because it might indicate participation of toxic oxygen species in tissue injury [23–27]. The present HPLC–CL method should be useful also for application to biological systems.

5. References

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