A simple and rapid chemiluminescence assay for on-site analysis of paraquat using a portable luminometer

Naoya Kishikawa · Sho Higuchi · Kaname Ohyama · Kenichiro Nakashima · Naotaka Kuroda

Received: 12 September 2012 / Accepted:

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Naoya Kishikawa (✉) · Sho higuchi · Kaname Ohyama · Naotaka Kuroda

Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

e-mail: kishika@nagasaki-u.ac.jp

Kenichiro Nakashima

Faculty of Pharmaceutical Sciences, Nagasaki International University, Sasebo, Japan

Abstract Paraquat (*N,N′*-dimethyl-4,4′-bipyridinium dichloride) is one of the most widely used herbicides owing to its high efficacy and low environmental persistence. However, since paraquat has significant acute toxicity, fatalities are often caused by accidental or voluntary ingestion of paraquat. In consideration of the strong toxicity and fast-acting property of paraquat, on-site analysis in accident scenes should be effective for the immediate medical treatment.

In this study, a simple and rapid chemiluminescence assay for on-site analysis of paraquat was developed using a portable luminometer. The proposed assay is based on luminol chemiluminescence detection of superoxide anion radical resulting from the redox reaction between paraquat and dithiothreitol. An intense chemiluminescence was observed by mixing of paraquat and dithiothreitol in the presence of luminol. Since the chemiluminescence intensity was proportional to the concentration of paraquat, a quantitative measurement of paraquat was possible. The calibration curve for standard paraquat solution was linear from 0.025 to 2.5 μM with the correlation coefficient of 0.992, and the detection limit (blank + 3SD) was 22 nM. The proposed assay could be applied to determine paraquat in beverage samples with a cation exchange clean-up procedure. Since the portable luminometer used in this study is small and lightweight, the proposed assay should be useful to on-site analysis of paraquat.

Keywords Paraquat · On-site analysis · Chemiluminescence · Redox cycle

Introduction

Paraquat (*N,N′*-dimethyl-4,4′-bipyridinium dichloride) is one of the most widely used herbicides owing to its high efficacy and low environmental persistence. However, since paraquat has significant acute toxicity and there is no specific antidote against paraquat [1,2], fatalities are often caused by accidental or voluntary ingestion of paraquat [3-5]. In order to offer sufficient medical treatment for paraquat poisoning, it is important to identify the ingested poison and estimate the dose amount. In consideration of the strong toxicity and fast-acting property of paraquat, on-site analysis before medical transport to hospital should provide effective information for immediate medical treatment. Therefore, it is desirable to develop a simple and rapid assay which allows on-site analysis of paraquat in accident scenes.

Several methods have been reported for the analysis of paraquat using colorimetry [6], spectrophotometry [7], voltammetry [8], capillary electrophoresis (CE) [9], gas chromatography (GC) [10,11], high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [12], post-column colorimetric detection [13] and mass spectrometry (MS) [14-17]. The colorimetry using hydrosulfite as a reagent is very simple and traditional assay for paraquat and has been used for on-site analysis. But, the sensitivity and quantitative capacity of the colorimetry are weak. Spectrophotometric techniques also generally have low sensitivity and selectivity. Voltammetry is not generally used because the method requires a specially designed electrode. Although GC and HPLC methods, especially LC-MS, are sensitive and selective, the chromatographic separation takes a long time. Moreover, the instruments for chromatographic analysis lack portability due to their complexity and heavy weight. Therefore, on-site chromatographic analysis should be virtually impossible.

It is known that chemiluminescence methods have the advantage of high sensitivity, wide linear range and simple instrumentation [18]. Nowadays, simple and portable instruments for chemiluminescence measurement are commercially available from several manufactures. Therefore, such portable instruments should be useful for the development of a simple, rapid and sensitive assay for on-site analysis of paraquat in combination with a specific chemiluminescence reaction for paraquat. For this purpose, we designed a novel chemiluminescence reaction for the

detection of paraquat, based on the toxicity expression mechanism of paraquat. In a living body, paraquat is reduced to paraquat radical via the enzymatic reaction, subsequently paraquat radical is auto-oxidized to paraquat accompanying the generation of superoxide anion radical [19,20]. The generated superoxide anion radical injures plant or living organism. On the other hand, superoxide anion radical can be detected sensitively by luminol chemiluminescence reaction [21]. Therefore, paraquat can be analyzed by the chemiluminescence reaction between luminol and superoxide anion radical that is generated from the redox reaction of paraquat (Fig. 1). In the proposed method, dithiothreitol (DTT) was used for the reduction of paraquat to paraquat radical instead of enzyme. Recently, we successfully developed a simple and sensitive chemiluminescence assay for quinones that have similar redox properties to paraquat by using luminol and DTT as reagents [22,23]. In this study, we developed a simple and rapid chemiluminescence assay for on-site analysis of paraquat based on the measurement of chemiluminescence resulting from the redox reaction of paraquat by portable luminometer.

Materials and methods

Chemicals

Paraquat was purchased from TCI (Tokyo, Japan). Dithiothreitol (DTT) was obtained from Nacalai Tesque (Kyoto, Japan). Luminol and superoxide dismutase (SOD) from bovine erythrocytes (4,470 U/mg) were purchased from Sigma (St. Louis, MO, USA). Sodium hydroxide (NaOH) and acetonitrile (HPLC grade) were from Merck (Tokyo) and Kanto Chemical (Tokyo), respectively. Purified water was prepared by a Simpli Lab UV (Millipore, Bedford, MA, USA) water device. Other chemicals were of extra pure grade. Stock solutions of paraquat were prepared in water and diluted with acetonitrile to prepare the working solutions. Solutions of DTT and luminol were prepared in acetonitrile and 50 mM sodium carbonate buffer (pH 9.7), respectively, just before analysis.

Chemiluminescence measurement of paraquat by portable luminometer

In this study, a portable luminometer Lumitester C-110 (Kikkoman Biochemifa, Tokyo) was employed for the chemiluminescence measurement. Lumitester C-110 is small size (dimensions: width 185 mm; depth 110 mm; height 75 mm), lightweight (700 g) and operated by rechargeable battery. Therefore, Lumitester C-110 is easy to carry to accident scenes. In a small test tube, 100 μL 0.25 mM luminol in 50 mM sodium carbonate buffer (pH 9.7) and 100 μL paraquat acetonitrile solution were added. After the addition of 100 μL 0.1 mM DTT in acetonitrile, the test tube was placed in Lumitester C-110 and the produced chemiluminescence was measured for 60 s. The total intensity was defined as the area under the chemiluminescence delay curve.

Analytical procedure of paraquat in beverage samples

A paraquat in beverage sample was loaded on a preconditioned Omix SCX tip column (Agilent technologies, Santa Clara, CA, USA) by 10-times repeating the aspirate and dispense cycles with 100 μL beverage sample using a manual micropipette. The resin of the pipette tip column was rinsed with 200 μL water, and then paraquat adsorbed on the resin was eluted with 100 μL acetonitrile. The eluted solution was directly used for the chemiluminescence measurement described above.

Results and discussion

Chemiluminescence from paraquat after mixing with luminol and DTT

An intense chemiluminescence was observed by mixing of DTT and paraquat in the presence of luminol (Fig. 2). While, no chemiluminescence was observed in the absence of either DTT or luminol. Since the chemiluminescence was inhibited by the addition of SOD to the reaction mixture, it was thought that superoxide anion radical was involved in the chemiluminescence reaction. In addition, an increase in absorbance at 600 nm, indicating the formation of paraquat

radical [24], was observed by mixing of DTT and paraquat. In consideration of these results, the chemiluminescence reaction mechanism was interpreted as follows. First, paraquat was reduced to paraquat radical by DTT. Successively, paraquat radical converts dissolved oxygen to superoxide anion radical, which reacts with luminol to generate chemiluminescence. By using the chemiluminescence reaction, a quantitative measurement of paraquat was possible because the chemiluminescence intensity was proportional to the concentration of paraquat. Although the chemiluminescence emission maintained more than 180 s, the measurement time was set at 60 s for further studies in consideration of the importance of rapidity for analysis.

Optimization of chemiluminescence conditions

In order to obtain higher sensitivity, chemiluminescence reaction conditions including reagent concentrations and buffer pH were optimized using 1 μM paraquat standard solution. The effects of luminol concentration on the chemiluminescence intensity and signal-to-blank (S/B) ratio were investigated within the range of 0.1 – 0.5 mM. Although the chemiluminescence intensity increased with the increasing of luminol concentration over the tested range, the best S/B ratio was obtained at 0.25 mM because the blank signal also increased with the increasing of luminol concentration (Fig. 3), and thus 0.25 mM was selected as the optimal concentration of luminol. The effects of DTT concentration on the chemiluminescence intensity and S/B ratio were investigated within the range of 0.05 – 0.2 mM. The highest chemiluminescence intensity and the highest S/B ratio were obtained at 0.1 mM (Fig. 4), and thus 0.1 mM was selected as the optimal concentration of DTT. Since the luminol chemiluminescence reaction requires alkaline pH, sodium carbonate buffer was selected as the solvent for luminol. The effects of buffer pH on the chemiluminescence intensity and S/B ratio were investigated within the range of 9.7-10.8. The highest chemiluminescence intensity was obtained at pH 10.8, but the best S/B ratio was obtained at 9.7 owing to the increasing of the blank signal at higher pH (Fig. 5), and thus pH 9.7 was selected as the optimal pH value of the carbonate buffer.

Calibration curve, detection limit and reproducibility

A calibration curve was prepared for standard paraquat solution; a good linear relationship (r = 0.992) between concentration and chemiluminescence intensity was obtained by measuring seven levels in the range 0.025 to 2.5 μM. The regression equation (mean ± SD, n=3) of the calibration curve was I = (170.0 ± 9.1) x 103 C + (22.1 ± 2.0) x 103, where I and C represent the integrated CL intensity and the concentration of paraquat, respectively. The proposed assay can detect the presence of only 1 μL of 5% paraquat product spiked into 500 mL of beverage. The detection limit (blank + 3SD) of paraquat was 22 nM. The sensitivity of the proposed chemiluminescence assay is approximately 380, 17, 10, 8, 8 and 8 times higher than those of CE [8], HPLC-UV [12], spectrophotometry [7], GC-quadrupole-MS [11], HPLC with post-column colorimetric detection [13] and LC-fast atom bombardment-MS [16], respectively. Also, the sensitivity of the proposed assay is almost same as those of LC-time of flight-MS [14], LC-electrospray ionization-MS [15] and LC-tandem MS [17]. Although the proposed assay is less sensitive than voltammetry [9] and GC-ion trap-MS [10], these methods required special and complex equipment. In contrast, the proposed assay enabled sufficiently sensitive determination of paraquat with simple instrument and procedure.

The repeatability of the proposed assay was examined using standard paraquat solutions at three different concentrations (0.25, 1 and 2.5 μM). As shown in Table 1, the relative standard deviations (RSD) for within-day analyses were less than 5.0% and between- day analyses were less than 6.0%; the sufficient repeatability of the assay was proved.

Selectivity of the proposed chemiluminescence assay

In order to evaluate the selectivity of the proposed chemiluminescence assay, the chemiluminescence intensities of typical lethal poisons including potassium cyanide, arsenic trioxide, diethyl chlorophosphate and fenoxycarb (carbamate pesticide) and structural analogues of paraquat such as diquat and 1-methyl-4-phenylpyridinium (MPP^+) were measured after the mixing with luminol and DTT. As shown in Fig. 6, all of the tested poisons did not show significant chemiluminescence except for paraquat and diquat. Diquat is often co-formulated with paraquat

as a herbicide, and it has similar toxicity to paraquat. These results suggest that the proposed chemiluminescence assay can detect paraquat and diquat selectively. Therefore, the proposed assay should be effective for the rapid identification of ingested poison in accident scenes.

Determination of paraquat in beverage samples

The proposed chemiluminescence assay was applied to the determination of paraquat in beverage samples such as tap water, cola, orange and apple juice. The beverage samples spiked with 2.5 μM paraquat were analyzed. However, when paraquat in beverage samples except for tap water was analyzed, a significant reduction of chemiluminescence emission was observed. The reduction might be attributed to the inhibition on the chemiluminescence reaction by components in beverage samples. In order to eliminate the inhibitory components, a cation exchange column purification by Omix SCX tip [25] was carried out before the chemiluminescence measurement. As shown in Table 2, since sufficient recoveries were obtained for all tested beverage samples after the purification procedures. Therefore, it was revealed that the proposed chemiluminescence assay could apply to the determination of paraquat concentration in beverage samples. The proposed assay should provide information to predict the dose amount of paraquat by analyzing residual beverage samples.

Conclusions

A simple chemiluminescecne assay utilizing for on-site analysis of paraquat was developed using a portable luminometer. The proposed assay is based on luminol chemiluminescence detection of superoxide anion radical resulting from the redox reaction between paraquat and DTT. The proposed assay is sensitive, rapid and selective for paraquat. The on-site analysis of paraquat by the proposed assay should be useful for the rapid identification of paraquat and the estimation of the dose amount in accident scenes.

Acknowledgement

This work was supported by a Grant-in-Aid for Adaptable and Seamless Technology transfer Program through target-driven R&D (A-STEP) from Japan Science and Technology Agency (JST).

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Figure Captions

Fig. 1 Mechanism of chemiluminescence detection of paraquat based on the redox cycle

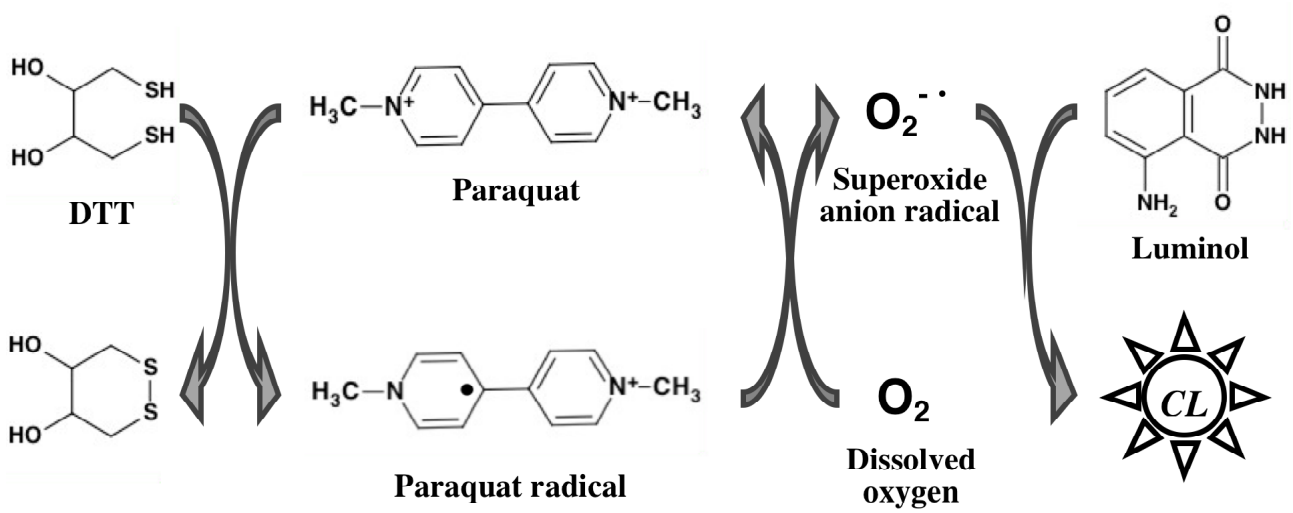
Fig. 2 Time profiles of chemiluminescence emission resulting from the reaction of paraquat with luminol and DTT. The concentrations of paraquat, luminol and DTT are 1 μM, 0.25 mM and 0.1 mM, respectively. The pH of sodium carbonate buffer is 9.7.

Fig. 3 Effects of luminol concentration on chemiluminescence intensity and S/B ratio. The concentrations of paraquat and DTT are 1 μM and 0.1 mM, respectively. The pH of sodium carbonate buffer is 9.7. Chemiluminescence signal expressed as mean ± standard deviation (n=3).

Fig. 4 Effects of DTT concentration on chemiluminescence intensity and S/B ratio. The concentrations of paraquat and luminol are 1 μM and 0.25 mM, respectively. The pH of sodium carbonate buffer is 9.7. Chemiluminescence signal expressed as mean ± standard deviation (n=3).

Fig. 5 Effects of buffer pH on chemiluminescence intensity and S/B ratio. The concentrations of paraquat, luminol and DTT are 1 μM, 0.25 mM and 0.1 mM, respectively. Chemiluminescence signal expressed as mean ± standard deviation (n=3).

Fig. 6 Chemiluminescence responses of several poisons after mixing with luminol and DTT. The concentrations of poisons, luminol and DTT are 1 μM, 0.25 mM and 0.1 mM, respectively. The pH of sodium carbonate buffer is 9.7. Chemiluminescence signal expressed as mean ± standard deviation (n=3).



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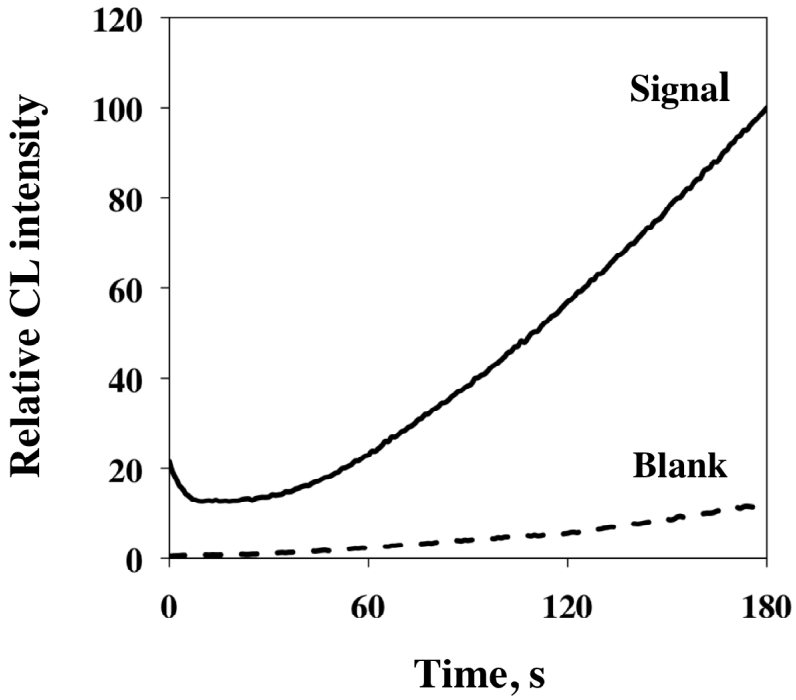

Fig. 2. Time profiles of chemiluminescence emission resulting from the reaction of paraquat with luminol and DTT. The concentrations of paraquat, luminol and DTT are 1 μM, 0.25 mM and 0.1 mM, respectively. The pH of sodium carbonate buffer is 9.7.

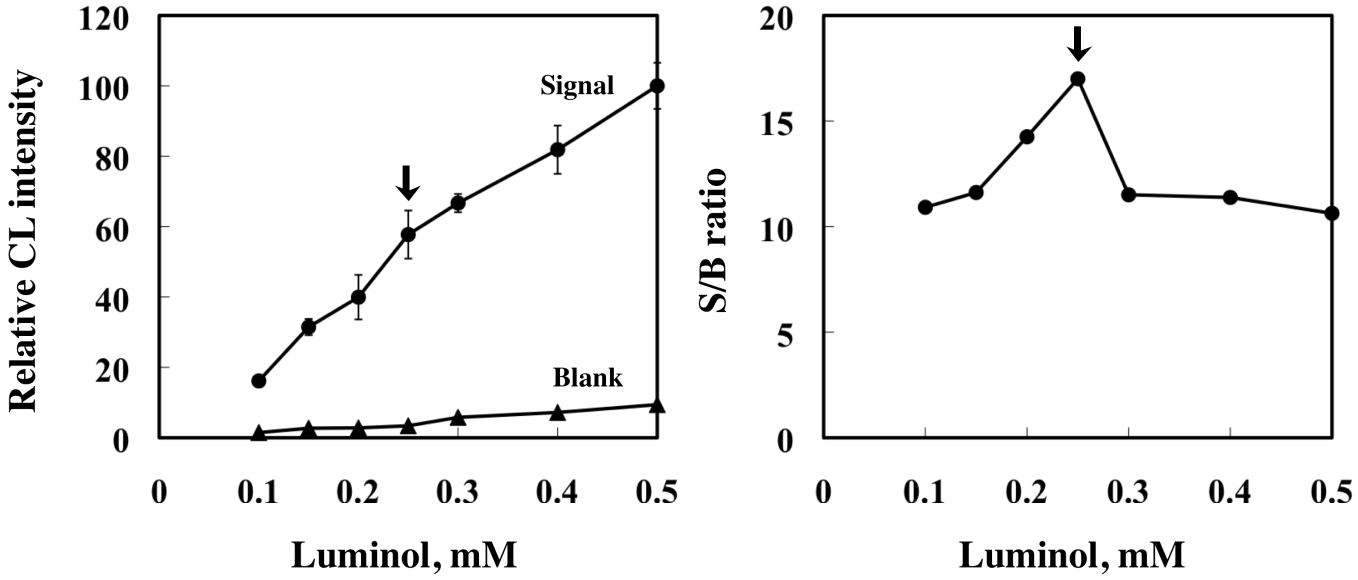

Fig. 3. Effects of luminol concentration on chemiluminescence intensity and S/B ratio. The concentrations of paraquat and DTT are 1 μM and 0.1 mM, respectively. The pH of sodium carbonate buffer is 9.7. Chemiluminescence signal expressed as mean ± standard deviation (n=3).

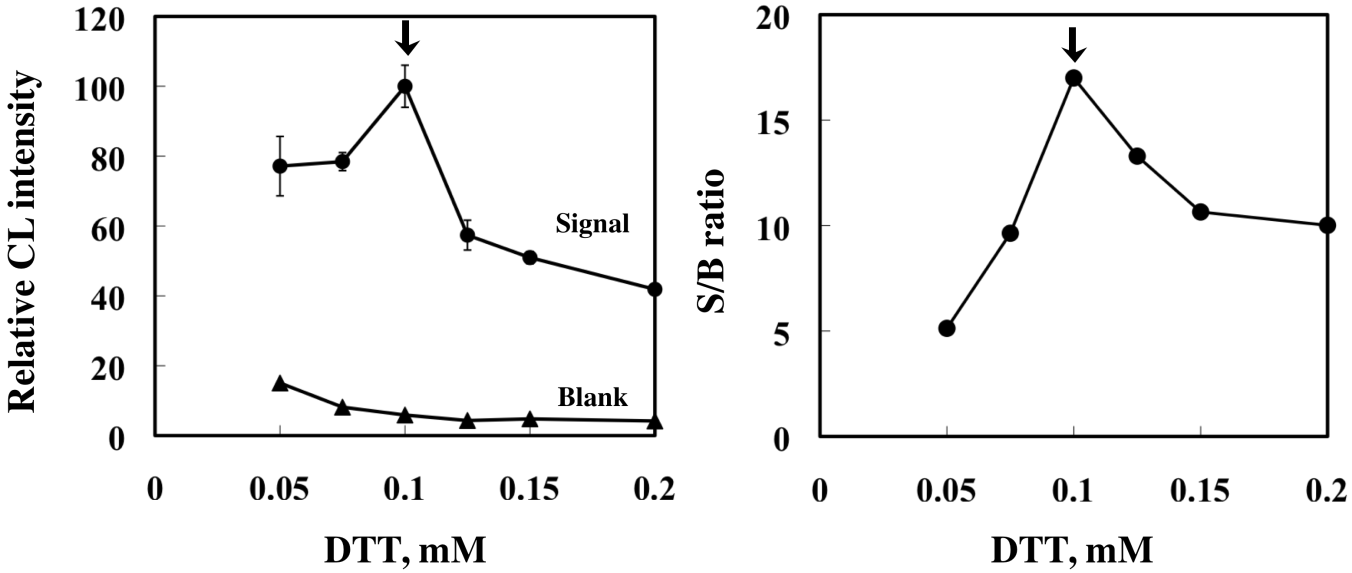

Fig. 4. Effects of DTT concentration on chemiluminescence intensity and S/B ratio. The concentrations of paraquat and luminol are 1 μM and 0.25 mM, respectively. The pH of sodium carbonate buffer is 9.7. Chemiluminescence signal expressed as mean ± standard deviation (n=3).

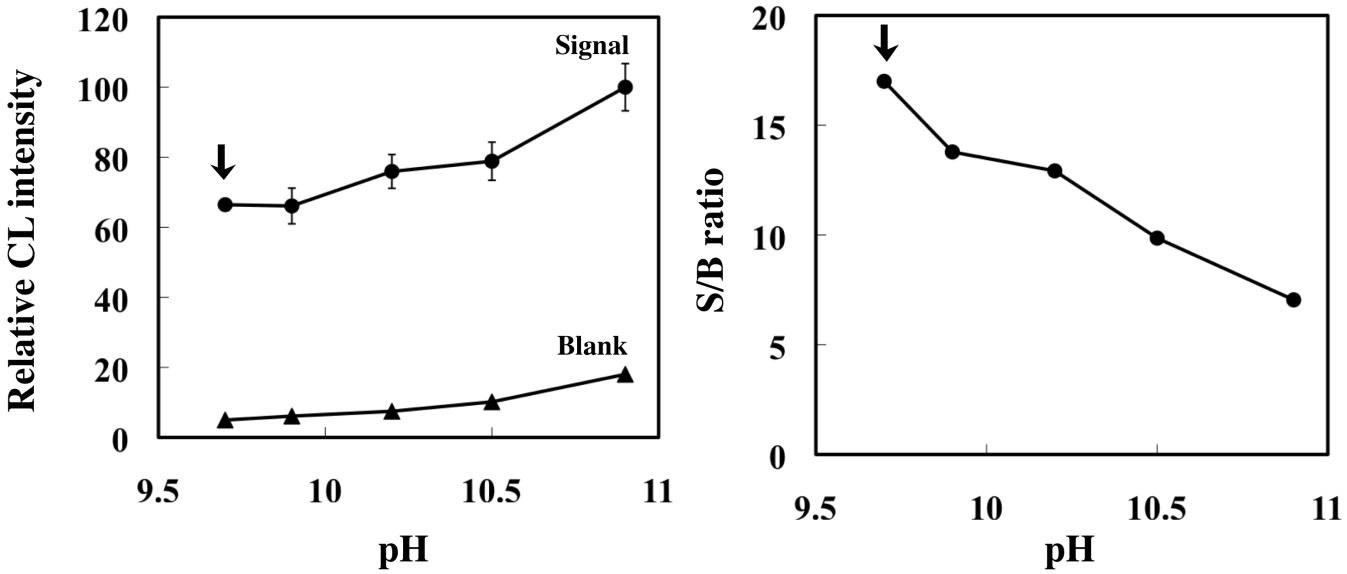

Fig. 5. Effects of buffer pH on chemiluminescence intensity and S/B ratio. The concentrations of paraquat, luminol and DTT are 1 μM, 0.25 mM and 0.1 mM, respectively. Chemiluminescence signal expressed as mean ± standard deviation (n=3).

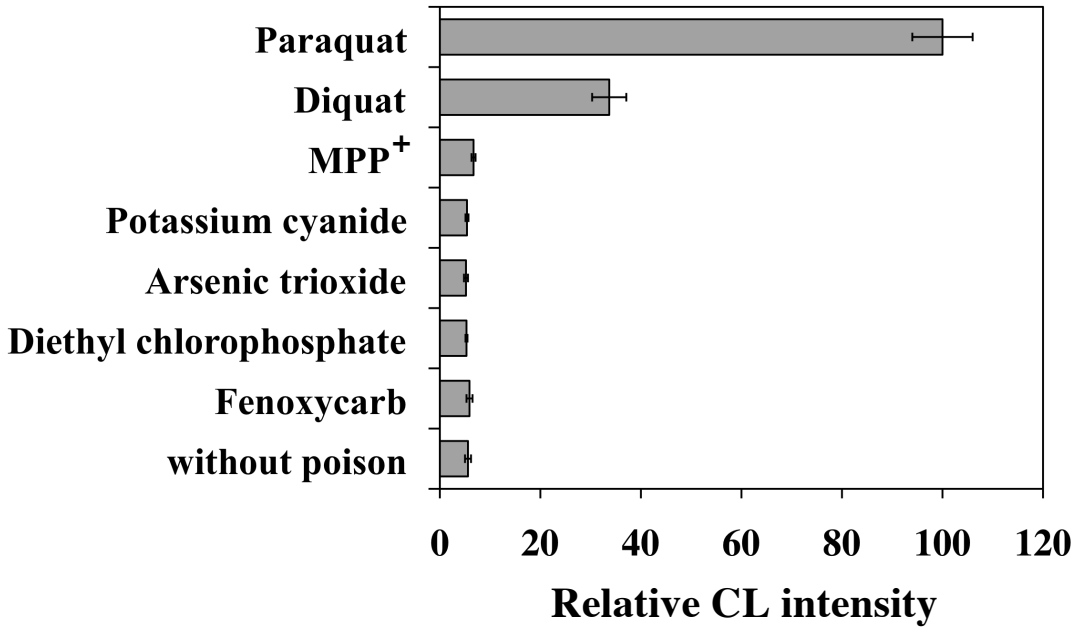

Fig. 6. Chemiluminescence responses of several poisons after mixing with luminol and DTT. The concentrations of poisons, luminol and DTT are 1 μM, 0.25 mM and 0.1 mM, respectively. The pH of sodium carbonate buffer is 9.7. Chemiluminescence signal expressed as mean ± standard deviation (n=3).

Table 1 Method repeatability

Paraquat, μM	Precision, RSD%	
	Within-day (n = 4)	Between-day (n=4)
0.25	5.0	3.3
1	1.4	1.4
2.5	3.3	6.0

Table 2 Determination of paraquat in beverage samples

Sample	Spiked concentration, μM	Found, μM (mean ± SD, n=3)	Recovery%
Tap water	2.50	2.66 ± 0.09	107
Cola	2.50	2.32 ± 0.23	93
Orange juice	2.50	2.62 ± 0.39	105
Apple juice	2.50	2.63 ± 0.05	105