

Microplate analytical method for quinones by pulse photo-irradiation and chemiluminescence detection

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Abstract

Quinones are widely distributed in the nature and have various bioactivities. Besides, quinones also considered as toxicological intermediates which cause a severe dangerous effects. Hereby, sensitive, simple, and rapid method is reported for quinones determination. The proposed method employed time resolved fluorescence (TRF) microplate reader based chemiluminescent (CL) detection for the first time as a novel approach for measurement. Under pulse photo-irradiation, the unique photochemical characteristic of quinones is exploited to liberate reactive oxygen species (ROS) which reacted with photosensitized CL reagent. L-012, luminol analogue, was selected for its high sensitivity. Under our investigation, para-quinones showed high CL response when compared to ortho-quinones. A linear response was obtained for studied quinone concentrations in the range of 0.05-50 μM for 1,4-naphthquinone and of 0.05-150 μM for 2-methyl-1,4-naphthoquinone and 9,10-anthraquinone with detection limit (blank + 3SD) of 0.01 μM . The proposed method allowed the rapid determination of large number of samples in very short time (96 sample/ 125 sec). The proposed method was successfully applied for determination of menadione in spiked human serum.

Introduction

Quinones are a widely distributed class of compounds which occur naturally in many biological systems (e.g. pyrroloquinoline quinone, lapachone, and emodin). Quinones (e.g. ubiquinone, plastoquinone, and menaquinone) have important roles in the biochemistry of energy production and serve as vital links in electron transport chains involved in cellular respiration and photosynthesis¹. Nowadays, quinones (e.g. doxorubicin, mitomycin, and mitoxantrone) form the second largest class of cytotoxins approved as anticancer agents for clinical use by FDA. Besides, they represent a class of molecules preventing and treating

several illnesses such as coagulopathy, osteoporosis, Alzheimer, cardiovascular diseases, and microbial, protozoal, and malarial infections (e.g. vitamin K, idebenone, phaeosphenone, and atovaquone)². Additionally, quinones are practically used for long time either industrially or agriculturally.³ However, with these beneficial effects, quinones represent a class of toxicological intermediates which can create a variety of hazardous effects *in vivo*, including acute cytotoxicity, immunotoxicity, and carcinogenesis. Quinones are also regarded as harmful environmental pollutants that have been detected in environmental samples (e.g. anthraquinones, naphthoquinones, and phenanthraquinone). Owing to the inherent reactivity of quinones and their involvement in a number of essential biological and chemical processes, a substantial amount of research has focused on quinones chemistry and biochemical toxicology⁴.

The quantification of quinones in different matrices considers an interesting research point in order to know their physiological and toxicological processes. Several analytical methods have been reported for determination of quinones in pharmaceutical formulation, plants, as well as biological samples including titrimetric method^{5,6}, potentiometric methods⁷, differential pulse polarography^{8,9}, and spectrophotometric methods¹⁰⁻¹². The most of these methods lacked sensitivity and simplicity. Although the high sensitivity achieved by the fluorescence detection, quinones lack native fluorescence and have to be reduced to their hydroquinone form prior to fluorescence detection. Different procedures are used for quinone reduction, either chemically,¹³⁻¹⁵ electrochemically¹⁶ or photochemically^{17,18}. Although the fluorescence detection may accomplish the required sensitivity, the methods lacked simplicity and rapidity because the reduction processes required many additional systems component such as reducing column, electric reactor or photoreactor.

In last decade the using of chemiluminescence (CL) for determination of trace and ultra-trace concentration of organic and inorganic species is interestingly increased.^{19,20}

However, direct determination of quinones by CL has been restricted because quinones cannot produce CL signal directly. On the other hand, several quinones can be detected by CL due to their ability to generate ROS when exposed to UV irradiation, a property that allows their determination by mixing with CL reagent.^{21,22} Recently, our laboratory reported a novel photo-induced (PI)-CL method combined with high performance liquid chromatography (HPLC) for quinones determination in both biological and environmental samples.²³⁻²⁵ These methods showed high sensitivity but also lacked simplicity and rapidity because they required both online photoreactor and additional pumping system for post-column reagent delivery. For that reasons, it is indispensable to develop a simple and high through-put method for quinone monitoring.

In the way to developing more streamlined alternatives, several researchers have advanced microplate-based techniques. These techniques are based on the standard 96 or 384 well microplate format that can greatly reduce sample volume, save time and low cost alternative to conventional technique.²⁶ However, to date there is no simple technique that utilizes merits of microplate reader to measure widely abundant quinones.

The application of microplate reader for quinones quantification by PI-CL techniques seemed to be quite difficult. Since, CL mode of microplate reader lacked the light source for photo-irradiation. Furthermore, fluorescence mode showed interference from scattering and stray light originated from the excitation light source. The only mode accomplishes the criteria; photo-irradiation and measurement while the excitation lamp turned off is time resolved fluorescence (TRF) mode of microtiter plate reader. Actually, TRF mode succeeds to detect CL from the well in presence of quinones and CL reagent, L-012 after pulse photo-irradiation. Another merit of TRF mode is longer measuring time (100 reading/well) achieved to obtain sufficient statistical precision. In this piece of work, we have exploited PI-CL assay for quinones quantification. Quinones under pulse photo-irradiation of xenon flash lamp of

TRF mode of microtiter plate reader can liberate ROS that react with photosensitized L-012, as a single reagent without oxidant or catalyst addition, to generate intense CL.

Herein, a sensitive, simple and rapid microplate reader CL assay has been developed for quantitative measurement of quinones. The proposed method precludes the drawbacks of previously published methods.

1. **Experimental**

2.1 Chemicals and reagents

All of the chemicals used throughout this study were of extra pure grade. 9,10-Anthraquinone, 1,4-naphthoquinone, luminol, isoluminol, and n-hexane (HPLC grade) were from Nacalai Tesque (Kyoto, Japan); 1,2-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, 1-aminoanthraquinone, lucigenin, 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one (MCLA), plumpagin, and 9,10-phenanthraquinone were from TCI (Tokyo, Japan); 2-methyl-1,4-Napthoquinone (menadione), acetonitrile, and methanol (HPLC grade) were from Kanto chemical company (Tokyo, Japan); 2-amino-3-chloro-1,4-napthoquinone, Alizarine, 8-Amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione (L-012), NaOH were from Wako Pure Chemical Industry (Osaka, Japan), beef liver catalase (20 mg/ml) and bovine erythrocytes superoxide dismutase (SOD) (4,470 U/mg) were purchased from Boehringer (Mannheim GmbH, Germany) and Sigma Aldrich (St. Louis, MO, USA), respectively. 2-methyl-3-(glutathion-S-yl)-1,4-napthoquinone (thiodione) and benzo[a]pyrene-7,8-dione were synthesized in our laboratory based on previous published papers and confirmed by MS.^{27,28} Purified water was obtained using Simpli lab-UV (Millipore, Bedford, MA, USA) water device. Stock solution of quinones were prepared in acetonitrile and kept at 4 °C until used. Working solutions were prepared from stock solution by appropriate dilution with acetonitrile. Stock solution of L-012 was prepared in 0.2M

carbonate buffer pH 9.4 and kept at 4 °C until use. Working solution of L-012 (10 µM) was prepared by appropriate dilution of the stock solution by the same buffer solution. All other chemicals were prepared in purified water unless otherwise indicated.

2.2 Instrumentation and measurement procedure

All luminescence measurements were made using a multimode microplate reader (SpectrMax M5, California, USA). Data acquisition was performed on Softmax[®] Pro 5 software. Microplate reader was operated at laboratory temperature (~ 25 °C). To optimize data collection for a particular assay, the instrument was performed by flashing excitation lamp at 250 nm, delaying time at 500 µs, integration time at 1500 µs, and data acquisition at 450 nm. The microplate was automatically shaken for 3 s. Each well was read 100 times from top in order to obtain highly precise and accurate results. Disposable transparent polystyrene, flat bottom 96-well microplates (Greiner Bio One, CLLSTAR #655180, Germany) were used for all CL measurements. HORIBA F22 pH meter was used for adjusting carbonate buffer pH.

2.3 Assay procedure for typical quinones

The proposed method was highly simple that proceeded as follow: 100 µl of 0.2M carbonate buffer (pH 9.4) plus 50 µl of quinones were added into each well of the microplate. Then 50 µl of 10 µM of L-012 was added. The microplate was transferred to SpectraMax M5 whose adjusted as previously mentioned. Data were expressed as the mean of triplicate measurement. Standard curves were obtained by plotting CL intensity against analyte concentration. In order to study the effect of various scavengers, 50 µl of different concentration (Table 1) was added to each well before addition of quinones and L-012.

2.4 Determination of menadione in human serum

To 1 ml serum containing menadione, 3 ml of methanol was added. After vortex-mixing for 1 min, the solution was kept at 4 °C for 5 min to promote the precipitation of proteins and centrifuged at 1585 x g to separate the suspended matter. The liquid phase was extracted three times with n-hexane (3 ml) and the organic phases were pooled to another tube with 200 µl of 70% methanol to prevent loss of menadione and evaporated to dryness under an atmosphere of nitrogen. The extract was reconstituted with acetonitrile and then analyzed by microplate reader as described above. The analytical recovery was calculated by comparing CL intensity from spiked sample and standard sample prepared post-extracted blank plasma.

2. Results and Discussion

CL methods in pharmaceutical and biomedical analysis seemed to have become quite important in recent years because such methods are often fairly sensitive and require relatively simple instrumentation if carried out either in batch mode or in flow systems.²⁹ However, a major obstacle of conventional CL method is a limited number of target analytes can be detected. Photo-induced CL was suggested as a new approach to expand the detectability of interested compounds.³⁰ Our laboratory investigated novel methods for quinones determination either in biological or environmental samples by using HPLC.²³⁻²⁵ In this work, we continued to exploit unique photochemical properties of quinones to improve both the simplicity and sensitivity of their determination.

One of the most representative examples of CL reactions is the oxidation of luminol and luminol-type derivatives in alkaline medium. Oxidant such as hydrogen peroxide (H₂O₂) in addition to the catalyst such as metal ions or metallocomplex can be used to enhance CL response. The requirement of strong oxidant in assay system is frequently inconvenient and causes intense light emission background. Recently, the development of PI-CL technique can

solve the problem of strong oxidant addition through generation of an oxidative species during reaction itself to react with reductive CL reagents. In the reported luminol-based PI-CL system for quinones quantification,²²⁻²⁵ mercury lamp has been used as a light source. Owing to self-oxidation of luminol or its derivatives and accordingly high background can be produced; the luminol was added after the light irradiation. The drawback of that is the loss of ROS during transport process decreased the sensitivity. In this work, microplate reader built in xenon flash lamp was used as light source. This lamp shown a convenient source of ultraviolet and visible light and generate a minimal amount of heat made it a suitable source for our study.

Indeed, the proposed method possessed significant merit comparing to other PI-CL methods²²⁻²⁵ because strong CL can be observed from sample without oxidant and catalyst addition under simple and rapid measurement of microplate reader.

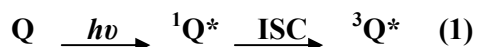
3.1 Confirmation of ROS from photochemical reaction

First and foremost, in preliminary test, both quinone and L-012 alone hadn't CL response. Also, their combination in absence of pulse photo-irradiation showed no CL. But a high response was achieved when they were combined in one well under pulse photo-irradiation. This means that a powerful oxidant like ROS has been generated to react with L-012 to liberate CL. Second evidence was our previous experience in dealing with quinones under UV irradiation to generate ROS and CL enhancer.²³⁻²⁵ Although these evidences might be enough to confirm ROS generation under our condition; we also investigated the quenching effect of various ROS scavenger. SOD, catalase, sodium azide, and uric acid were selected as selective scavenger of superoxide anion radical, H₂O₂, singlet oxygen, and hydroxyl radical, respectively. The quenching effect of these scavengers on menadione as an

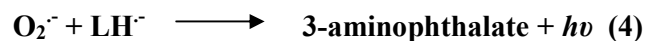
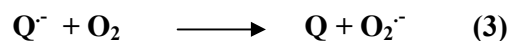
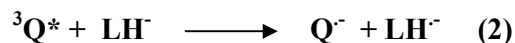
example of studied quinones shown in Table 1 supposed that hydroxyl radical, superoxide anion, and H₂O₂ play an important role in CL release by the proposed method.

3.2 Suggested mechanism of CL reaction

The principal of the proposed method based mainly on the unique photochemical properties of quinones. Quinones like many carbonyl compounds can absorb the light or energy to transfer to excited singlet state (S₁) which transferred to triplet state (T₁) via intersystem crossing mechanism (Reaction 1).



T₁ of the quinone readily reduced by electron donor to liberate semiquinone with subsequent electron transfer to molecular oxygen to generate superoxide anions radical and quinone again (oxygen-dependent type I reactions). In our case, luminol or its derivatives acted as electron donor to generate luminol radical which reacted subsequently with superoxide anion to generate strong CL (Reaction 2-4)^{31,32}. The role of oxygen was confirmed by the quenching of CL intensity (Table 1) with the addition of sodium sulphite that used in electroanalytical experiment to reduce the solution of O₂ concentration to level lower than achieved by bubbling nitrogen³³.



Under our condition, superoxide anion was probably more susceptible to spontaneous dismutation to H₂O₂ (Reaction 5). This H₂O₂ in presence of trace metals elicit hydroxyl radical via fenton reaction (Reaction 6). The liberated H₂O₂ and hydroxyl radical could be reacted with luminol radical to reinforce CL (Reaction 7)





The liberation of superoxide anion radical, H_2O_2 , and hydroxyl radical was confirmed by selective scavenger as illustrated previously. The role of trace metal found as impurities was also confirmed by chelator addition; EDTA quenched the CL reaction (Table 1). The difference between the proposed method and previously reported method is the source of UV irradiation.²³⁻²⁵ In the previous method mercury lamp was used to decompose quinones and generate ROS and CL enhancer (3,6-dihydroxyphthalic acid) which initiated the first step in luminol CL reaction pathway (conversion of luminol monoanion to luminol radical). In our case, Xenon flash lamp (1 joule/flash) was used as a source of UV irradiation; the energy of lamp is weak and also gave pulse not continuous radiation like mercury lamp. This pulse photo-irradiation was able to excite the quinones to triplet state but not decompose them as previous methods. Another evidence confirmed our suggested mechanism was the employment of luminol derivative alone without addition of oxidant and catalyst. Superoxide anion radical and H_2O_2 alone are not sufficient to act as luminol primary oxidant to generate luminol radical which entered the CL pathway terminated by aminophthalate.³⁴

3.3 Quinones Structure

The proposed method based mainly on the photochemical specificity of quinones. There are some substituents can affect triplet state photoreactivity of these compounds. Electron donating group such as hydroxyl and amino group exhibit rapid internal conversion instead of intersystem crossing enhanced by both intra- and inter-molecular hydrogen bonds. Intermolecular interaction involving charge transfer (CT) has been proved to be another mechanism for efficient radiationless deactivation of the excited state of quinones.³⁵ This is obviously appeared in the proposed method: 1-amino-anthraquinone, 2-amino-3-chloro-1,4-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, plumbagin, and alizarin showed no CL

reaction. It is well-known that solvent polarity affects the electronic structure of the T_1 of carbonyl compounds. It was found that the T_1 state of ortho-quinones like 1,2-naphthoquinone, 9,10-phenanthraquinone, benzo[a]pyrene-7,8-dione has dominantly $\pi\pi^*$ character in polar solvent;³⁶ therefore, these compounds also showed very weak CL under our method. Figure 1 showed the CL response of the studied quinones to the proposed method.

3.4 Optimisation of experimental parameters

Many investigations were carried out to establish the most favourable condition to produce intense CL and low background effect. Three of simple and widely distributed quinones; 1,4-naphthoquinone, menadione, and 9,10-anthraquinone were selected in this study.

3.4.1 Media pH and type of buffer

The influence of buffer pH on CL intensity of quinones was tested. A wide pH range from 3 to 11 was checked. The intense CL was achieved in carbonate buffer pH 9 to 10 (Fig.2). In order to select the optimum pH a tight range of pH between 8 and 10.2 also tested. Although the high CL intensity obtained at pH 10.8, pH 9.4 was selected for further experiment in concern to method sensitivity, signal /blank ratio (S/B). At pH 9.4, both high CL intensity and S/B ratio achieved in the carbonate buffer when compared with other buffers.

3.4.2 CL reagent and its concentration

Luminol, isoluminol, L-012, lucigenin, and MCLA are extensively used for ROS detection biologically; therefore, these reagents were tested to the proposed method. Among CL reagent, L-012 gave the maximum CL intensity and high S/B ratio. Moreover, L-012 showed high CL sensitivity for superoxide anion radical in addition to considerable CL intensity to other ROS when compared to other reagent³⁷; therefore L-012 was selected for ongoing work. The influence of the L-012 concentration on CL intensity and S/B ratio was

also examined. It was found that CL intensity increased linearly as the concentration of L-012 increased, however, the background noise was also increased (Fig. 3). The best S/B ratio was found at 10 μ M of L-012.

3.4.3 Irradiation and measurement wavelength

The influence of both irradiation and measurement wavelengths on CL intensity and S/B ratio was examined. A considerable range of wavelength was tested ranged from 250 nm to 400 nm. It was found that CL intensity and S/B ratio decreased with increasing excitation wavelength. Nearly all examined quinones gave optimum results at 250 nm. As most of quinones compound showed absorbance maximum at nearly 250 nm, this point was selected as irradiation wavelength. The influence of measurement wavelength ranging from 400 nm to 480 nm was studied. It was found that both CL intensity and S/B ratio increased with increasing wavelength till 450 nm then decreasing again; therefore 450 nm was selected as optimum measurement wavelength for further work. Measurement at 450 nm confirmed the reaction of liberated ROS with luminol anion radical to produce amino phthalate³⁸, the final product of multistep reaction path of CL reagent.

3.4.4 Delaying and Integration time

Important setting for obtaining the best results in TRF assays are delaying and integration time. Firstly, the integration delay is the amount of time elapses between the flash of the lamp and beginning of data acquisition from well. To examine the influence of delaying time on CL intensity and S/B ratio, a wide range of time (from 0 μ s to 600 μ s) was selected. It was found that both CL intensity and S/B ratio increased as delaying time increased, achieving optimum results for 500 μ s. Secondary, the integration time is the amount of time required for each well to be read. The optimum results related to both intensity and sensitivity achieved for 1500 μ s (Fig. 4).

3.4.5 Organic solvent

Owing to the low solubility of most typical quinones in water, organic solvent should be used to dissolve these compounds. Various water miscible organic solvents were examined as quinones dissolving agent such as acetonitrile, methanol, ethanol, acetone, dimethyl sulfoxide, and dimethyl formamide. It was known that most of organic solvent acted as ROS scavenger.³⁹ The high intensity and sensitivity were achieved when acetonitrile used as dissolving agent, therefore it was used for further experiment.

3. Method Validity and applicability

Under the optimum experimental conditions, linear relationship was observed by plotting relative CL intensity versus quinone concentrations. The calibration curve prepared for standard studied quinones concentration; was linear in the range 0.05–150 μM with good correlation coefficients (>0.998) as shown in Table 2. Also, the detection limits (blank + 3SD) for studied quinones obtained by the proposed method was 0.01 μM . The proposed method was found to be more or less equal sensitive to our previous method for determination of 1,4-naphthoquinone and 9,10-phenanthraquinone.²⁴ Our method was 30 and 45 fold more sensitive to spectrophotometric method for determination of 1,4-naphthoquinone and menadione⁴⁰. It was 560 times more sensitive when compared with the reported HPLC⁴¹ for determination of 9,10-phenanthraquinone in technical and commercial formulation. Another worth of the proposed method is the rapidity; 16 sec required for each sample in triplicate compared to long time under other methods.

The order of CL intensity and detection sensitivity for all the studied quinones after pulse photo-irradiation were as follows: 9,10-anthraquinone > 2-methy-1,4- naphthoquinone > 1,4-naphthoquinone. This order is agreed with order of quantum yield of intersystem crossing (Φ_{ISC}), e.g. $\Phi_{\text{ISC}} = 1, 0.86,$ and 0.74 for 9,10-anthraquinone, menadione, 1,4-

naphthoquinone, respectively.⁴² The method repeatability expressed as within and between days precision was examined. Three concentrations of studied quinones were selected for this study as shown in Table. 3. It was found that the relative standard deviations (RSD) within-day and between-day (n=5) were 3.5–9.3% and 5.5–9.4%, respectively, so acceptable reproducibility was obtained. The applicability of the method was demonstrated by the determination of menadione in spiked serum sample. The serum was subjected to protein precipitation, liquid–liquid extraction to an organic phase, evaporation and reconstitution with acetonitrile. Under this condition the recovery of menadione from serum was higher than 88% as shown in Table 4. The proposed method was successfully applied for menadione without any interference of co-existing biological substances. Our method shown reasonable sensitivity, simplicity, and rapidity when compared to previously published paper.⁴³ So, the proposed method can be applied for clinical studies of menadione whose shown a wide anticancer applicability (the complete data under investigation in our laboratory to be published later.

Conclusion

A novel, simple, and sensitive analytical method was established for determination of quinones. The method exploited the photochemical specificity of quinones to enhance their quantification. The proposed method utilized for first time TRF microplate reader based CL detection with pulse photo-irradiation as a novel technique for the measurement. Pulse photo-irradiation of quinones and CL reagent succeed to induce CL without addition of trigger reagent. The proposed method offers merits to the quinones measurement methods due to it based on microplate reader which significantly saves time (96 sample/125 sec) and cost while maintains high accuracy and precision. The proposed method may be applied for routine analysis and in quality control laboratories for quantitative measurement of studied quinones either in row material or commercial formulation.

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Figures

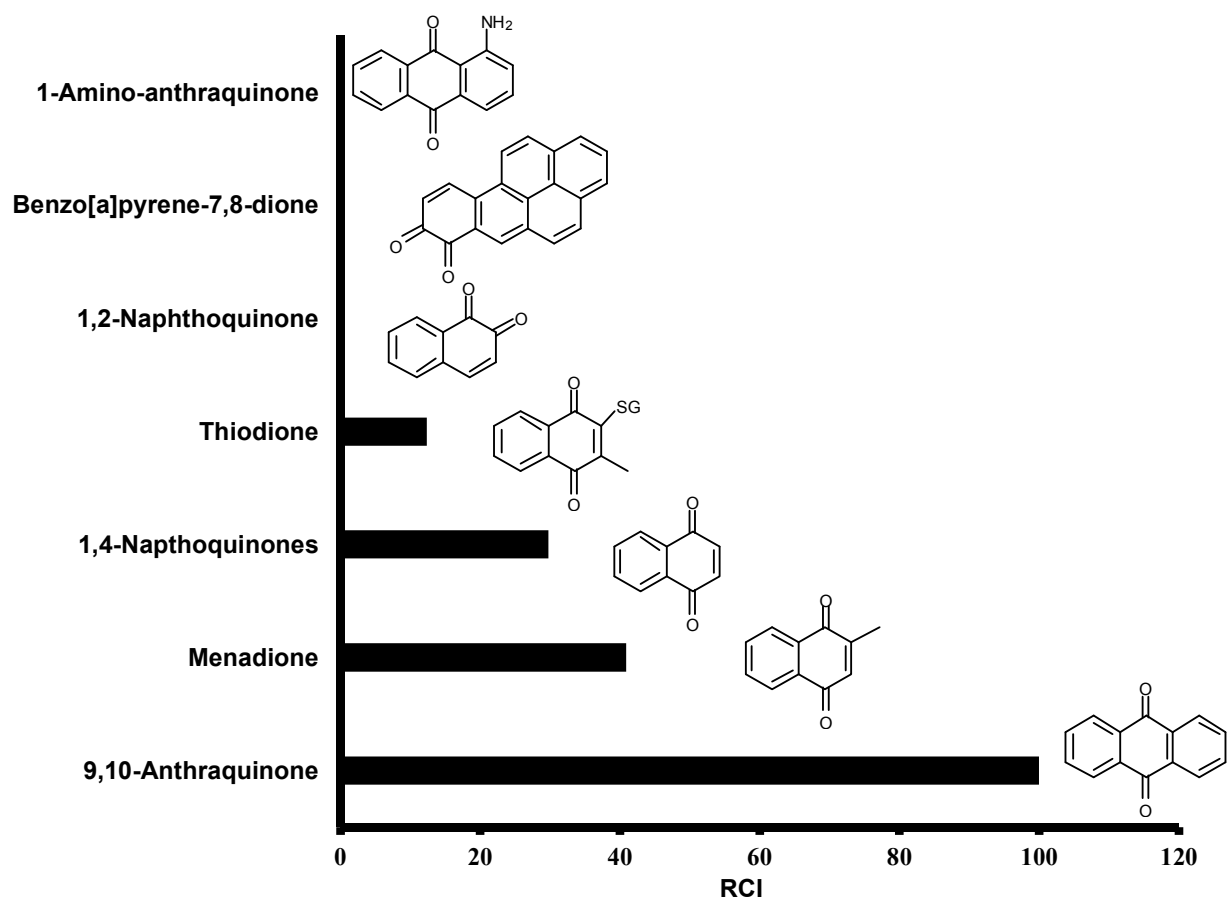


Fig.1. CL response of studied quinones to the proposed method

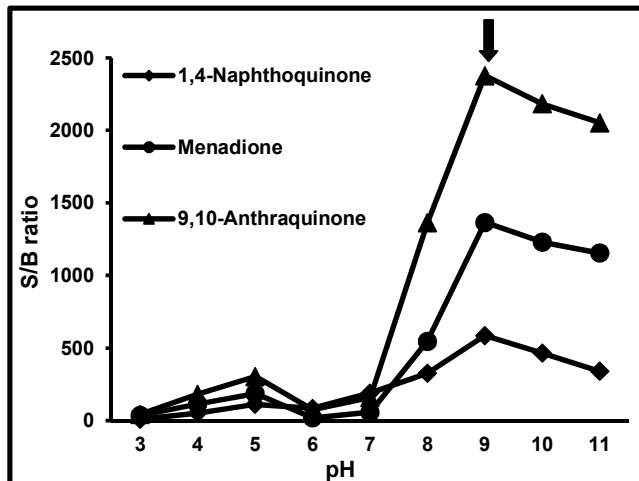


Fig.2. Effect of pH on S/B ratio of studied quinones, obtained at 10 μ M L-012, 500 μ s of delaying time, and 1500 μ s of integration time.

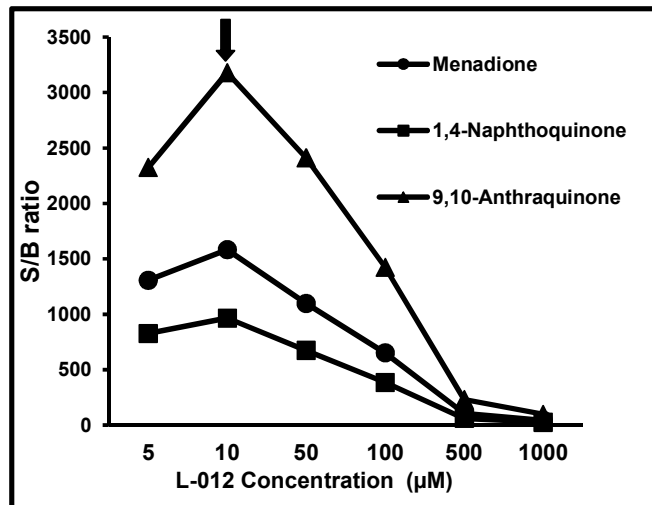


Fig.3. Effect of CL reagent concentration on S/B ratio of studied quinones, obtained at pH 9.4, 500 μs of delaying time, and 1500 μs of integration time.

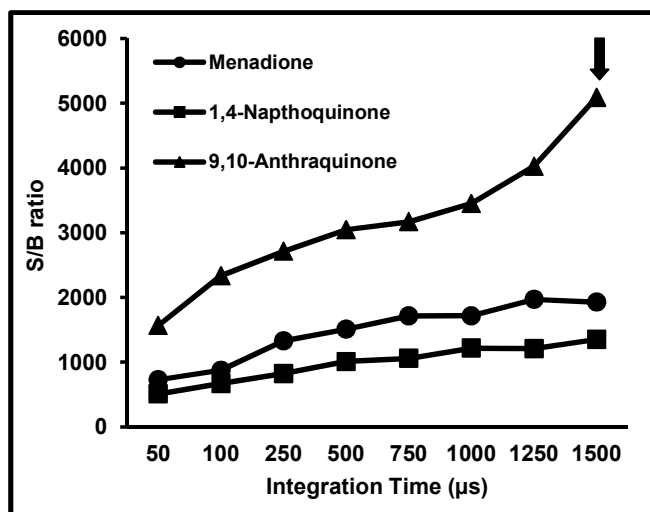


Fig.4. Effect of integration time on S/B ratio of studied quinones, obtained at pH 9.4, 10 µM L-012, 500 µs of delaying time.

Tables

Table 1. The effect of scavenger on CL intensity of menadione

Scavenger	Concentration (mg/ml)	RCI*
without Scavenger		100
SOD	0.02	14.9
Catalase	1	7.89
Sodium azide	0.065	82
	0.65	50
Uric acid	0.00168	68
	0.0168	8
EDTA	3.72	29
	18.6	4.5
Sodium sulphite	2.5	19.9

* CL intensity of 100 μ M of menadione without scavenger was considered as 100

Table 2. Analytical parameter and regression characteristic of studied quinones by the proposed method

Quinone	Range (μM)	Calibration equation^a	Correlation coefficient (<i>r</i>)	Detection limit (μM)^b (pmol/well)
1,4-Naphthoquinone	0.05-50	$Y = 740.75X + 237.74$	0.999	0.01(10.4)
Menadione	0.05-150	$Y = 936.89X + 1151.6$	0.998	0.01(8.6)
9,10-Anthraquinone	0.05-150	$Y = 1397.6X + 1667.6$	0.999	0.01(7.8)

^aChemiluminescence intensity versus concentration (μM)

^bDetectio limit (blank+ 3SD)

Table3. Method repeatability of studied quinones

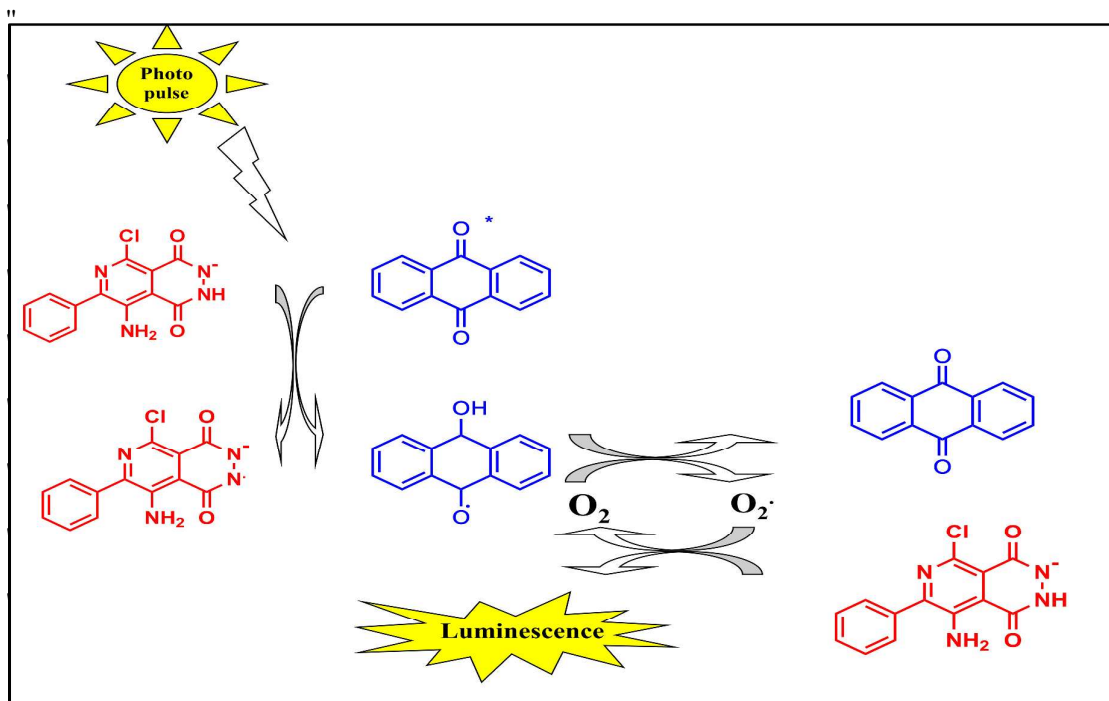
Quinone	Concentration (μM)	Precision (RSD, %)	
		within day (n=5)	Between day (n=5)
1,4-Naphthoquinone	0.05	4.6	6.8
	1	5.3	6.3
	50	5.0	5.6
Menadione	0.05	5.8	6.1
	1	4.3	7.3
	100	5.3	6.7
9,10-Anthraquinone	0.05	9.3	9.4
	1	6.6	5.6
	100	3.5	5.5

Table 4. Recovery of menadione from human serum determined by proposed method

Concentration ($\mu\text{g/ml}$)		Recovery % (mean \pm RSD)
Add	Found \pm SD	
4.3	4.17 \pm 0.06	96.98 \pm 5.9
8.6	8.08 \pm 0.06	94.06 \pm 5.8
17.2	15.23 \pm 0.02	88.54 \pm 1.7

Table of contents entry

A novel and rapid chemiluminescence assay for quinones was developed. The method employed pulse photo-irradiation of time resolved fluorescence microplate reader to generate reactive oxygen species which can be detected by the reaction with luminol derivatives.



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