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Determination of Cytochrome *c* in Human Serum and Pharmaceutical Injections Using Flow Injection Chemiluminescence

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Abstract It was found that the complex of cytochrome *c* (Cyt *c*) and hydrogen peroxide could significantly catalyze the chemiluminescence (CL) reaction from luminol–hydrogen peroxide, and a sensitive, rapid, and simple CL procedure was proposed for the determination of Cyt *c* in a flow injection system for the first time. The increment of CL intensity was linear over the concentration of Cyt *c* ranging from 5 to 700 ng ml^{-1}, with a detection limit of 2 ng ml^{-1} (3σ). At a flow rate of 2.0 ml min^{-1}, a complete analytical process could be performed in 30 s with a relative standard deviation of less than 4.0%. The proposed method was applied successfully for the assay of Cyt *c* in pharmaceutical injections and human serum, and the recoveries were from 98.0% to 108.8% and 92.5% to 109.0%. The possible mechanism of Cyt *c* enhanced CL reaction was also discussed.

Keywords Cytochrome *c* · Luminol · Hydrogen peroxide · Chemiluminescence · Flow injection

Introduction

Cytochrome *c* (Cyt *c*) is a small heme protein (molecular weight about 12 kDa) and consists of a single polypeptide chain of 104 amino acid residue and covalently attached to one heme group, which was found loosely associated to the inner membrane of mitochondria [1–3]. Cyt *c* plays a key role in the biological respiratory chain, whose function is to receive electrons from Cyt *c* reductase and deliver them to Cyt *c* oxidase [4]. In addition, Cyt *c* released from mitochondria translocates to the cytosol in apoptotic cells, which is considered a critical, early event in the induction of the caspase cascade that ultimately leads to programmed cell death [5–7].

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High-performance liquid chromatography [8–10] and capillary electrophoresis (CE) [11–13] with electrochemical or ultraviolet detection were reported for the determination of Cyt *c*. Since it was first reported by Albrecht in 1928 [14], the chemiluminescence (CL) resulting from the reaction of luminol and oxidants has been extensively studied and applied to the determination of inorganic and organic species [15–17]. Compared with other methods for the assay of Cyt *c*, CL method offers the advantages of simplicity of apparatus, low reagent consumption, higher sensitivities, and higher sample throughput [18, 19]. Robert Feissner reported a CL procedure for the determination of Cyt *c* using a variety of luminol-based substrates, giving a linear ranging from 4 to 400 ng [20].

In this work, it was first found that the complex of Cyt *c* and hydrogen peroxide could significantly catalyze the CL reaction from luminol–hydrogen peroxide, and a sensitive and simple procedure was proposed for the determination of Cyt *c* in a flow injection (FI) system. The increment of CL intensity was correlated with Cyt *c* concentration in the range of 5 to 700 ng ml^{-1} with a relative standard deviation of less than 4.0% and a limit of detection of 2 ng ml^{-1} (3σ). At a flow rate of 2.0 ml min^{-1}, the determination could be performed in 30 s, including sampling and washing. The flow injection CL method exhibited both high sensitivity and good selectivity giving a throughput of 120 samples per hour.

Experimental

Reagents

All reagents used were of analytical grade. Water purified in a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout. Luminol (Fluka, biochemika) was obtained from Xi'an Medicine Purchasing and Supply Station, China. Hydrogen peroxide was purchased from Xi'an Chemical Reagent Plant. Cyt *c* was obtained from Sigma (St. Louis, MO, USA), and stock solution (1 mg ml^{-1}) was prepared by dissolving 10 mg Cyt *c* to 10 ml in a calibrated flask and stored below 4 °C. Cyt *c* pharmaceutical injections were purchased from the local market. Working strength solutions were prepared daily from the above stock solution as required. Luminol (2.5×10^{-2} mol l^{-1}) was prepared by dissolving 4.4 g luminol at 1 l of 0.1 mol l^{-1} NaOH solution.

Apparatus

The FI system used in this work is shown schematically in Fig. 1. A peristaltic pump was utilized to deliver all flow streams. PTFE tubing (1.0 mm i.d.) was used as connection

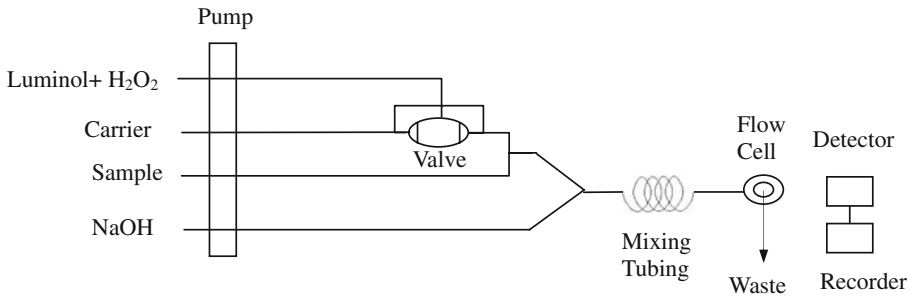


Fig. 1 Schematic diagram of the flow injection system for determination Cyt *c*

material in the flow system, and the whole system was pumped. A six-way valve with a loop of 100 μl was employed for sampling. The flow cell was made by coiling 15 cm of colorless glass tube (2 mm i.d.) into a spiral disk shape with a diameter of 2 cm and placed close to the photomultiplier tube (PMT). The apparatus (Model IFFL-DD, Xi'an Remax Electronic Science-Tech.) integrate the sampling system, the PMT, the luminosity meter, and the recorder.

General Procedures

All solutions were propelled at a constant flow rate of 2.0 ml min^{-1}. Luminol mixing with hydrogen peroxide was injected into the carrier system by a six-way valve quantitatively until a stable baseline was recorded, which was merged with Cyt *c* in the mixing tube. Then, the solution mixed was delivered into the CL flow cell and generated CL in an alkaline medium. The peak height of the CL was therefore detected with the PMT and the luminometer. By measuring the increment of the CL intensity, the concentration of Cyt *c* could be quantitatively determined, viz. $\Delta I = I_s - I_o$, where I_s and I_o are the CL intensity in the presence and absence of Cyt *c*, respectively.

Results and Discussion

CL Intensity–Time Profile

Prior to the flow system being carried out, the kinetic curve was examined by dynamic method. The kinetic profile for CL intensity of luminol–hydrogen peroxide was tested using 5.0×10^{-6} mol l^{-1} luminol and 5.0×10^{-5} mol l^{-1} hydrogen peroxide in 0.025 mol l^{-1} sodium hydroxide solution. As Fig. 2 shows, the Cyt *c* greatly catalyzed the CL reaction between luminol and hydrogen peroxide. A CL signal in the presence of Cyt *c* was observed with an evident increment compared with that in the absence of Cyt *c*. The CL intensity approached maximum at 5.0 s, which was 0.8 s faster than that in the absence of Cyt *c*, and then declined in the following 30 s.

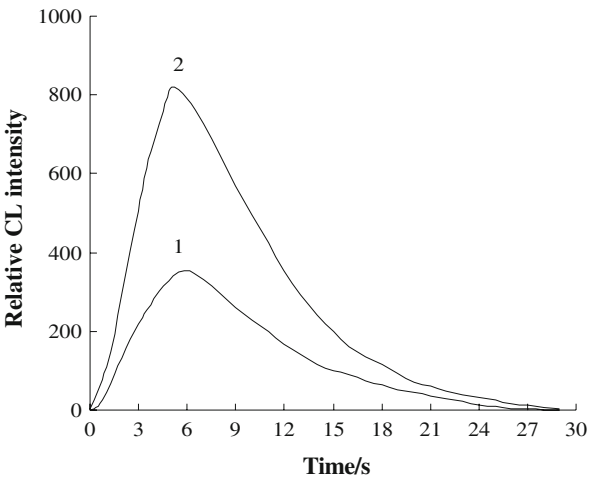


Fig. 2 Kinetic CL intensity–time profile in dynamic system. *1* CL intensity in the absence of Cyt *c*. *2* CL intensity in the presence of Cyt *c* (100 ng ml^{-1})

Effect of Luminol and Hydrogen Peroxide Concentrations

The effect of luminol on the reaction studied was investigated over the concentration range from 1.0×10^{-6} to 2.5×10^{-5} mol l^{-1}. It was found that the CL intensity increased steeply with an increase in luminol concentration up to 5.0×10^{-6} mol l^{-1}, above which it decreased slightly. Therefore, the luminol concentration of 5.0×10^{-6} mol l^{-1} was selected for the analysis. Similarly, the influence of hydrogen peroxide on the reaction rate was monitored. When increasing hydrogen peroxide concentration, the signal intensity rose rapidly up to 5.0×10^{-5} mol l^{-1} and then went down slowly. Thus, 5.0×10^{-5} mol l^{-1} was assumed the optimum hydrogen peroxide concentration for the following experiments.

Effect of NaOH Concentration

Owing to the nature of the luminol reaction, which is more favored under alkaline conditions, NaOH was introduced into the luminol solution to increase the sensitivity of the system. A series of NaOH solutions with different concentrations (0.01, 0.025, 0.05, 0.1, and 0.25 mol l^{-1}, respectively) were tested. The CL intensity versus concentration of NaOH plot reached maximum at 0.025 mol l^{-1}, and this concentration was employed in subsequent experiments.

Effect of Flow Rate and the Length of Mixing Tubing

The CL intensity was related to the flow rate. A lower flow rate caused broadening of the peak and slowed sampling rates. The ratio of signal-to-noise increased at higher flow rates. Moreover, higher flow rates could lead to an unstable baseline. So, a flow rate of 2.0 ml min^{-1} was selected as an appropriate condition considering both the good precision and lower solution consumption. The effect of the length of mixing tube on CL intensity was also tested in pursuit of producing maximum CL intensity in flow cell. It was observed that 5.0 cm of mixing tube afforded the best results with good sensitivity and reproducibility. Accordingly, 5.0 cm was considered as an optimum length.

Performance of Proposed Method for Cyt *c* Determination

Under optimum conditions, the linearity of the results was examined by measuring a series of standard solutions. The enhanced CL intensity was found to be proportional to Cyt *c* concentration, and the response to the concentration was linear over the range from 5 to 700 ng ml^{-1} with the detection limit of 2 ng ml^{-1} (3σ, $n=5$). The regression equation was, $\Delta I = 3.38C_{\mathrm{Cyt}\,c} + 76.12$, $r^2=0.9984$, and the relative standard deviation was less than 4.0%. At a flow rate of 2.0 ml min^{-1}, the determination could be performed in 30 s, including sampling and washing.

Interference Study

The influence of foreign species was examined by analyzing standard solution of Cyt *c* (25 ng ml^{-1}) to which increasing amounts of interfering species was added. The tolerable limit of foreign substances was taken as the amount that caused an error of ±5% in peak height. The tolerated concentrations were over 100 μg ml^{-1} for fructose, methanol, and ethanol; 2.0 μg ml^{-1} for L-hydrochloric acid lysine; 1.0 μg ml^{-1} for NO_3^-, I^-, SO_4^{2-}, PO_4^{3-}, $Cr_2O_7^{2-}$, borate, oxalate, tartrate, citrate, sucrose, glucose, and malic acid; 0.5 μg ml^{-1} for Mg^{2+},

Ca^{2+}, Ba^{2+}, Zn^{2+}, L-threonine; 0.3 μg ml^{-1} for uric acid and L-leucine; 0.1 μg ml^{-1} for L-cysteine, 10 ng ml^{-1} for L-histidine, and formaldehyde; 0.5 ng ml^{-1} for Cu^{2+}, Fe^{3+}, Fe^{2+}, and Mn^{2+}. It should be noticed that the interference of some biospecies often found in serum was tested. The total serum proteins in a healthy adult are about 67–83 mg ml^{-1} [21], which showed no interference in the determination of Cyt *c* after diluting 5.0×10^5 fold with water.

Selectivity of the Luminol–H_2O_2–Cyt *c* CL System

The selectivity of the luminol–H_2O_2–Cyt *c* CL system was studied. Upon addition of 10 ng ml^{-1} of species such as Mg^{2+}, Ca^{2+}, Ba^{2+}, Zn^{2+}, NO_3^-, I^-, SO_4^{2-}, PO_4^{3-}, $Cr_2O_7^{2-}$, L-hydrochloric acid lysine, L-threonine, L-leucine, L-cysteine, L-histidine to the system of luminol–H_2O_2 and luminol–H_2O_2–Cyt *c*, the results are listed in Table 1. It was found that these species did not cause obvious CL intensity changes at the same condition. Otherwise, 1 ng ml^{-1} of Fe^{2+}/Fe^{3+} could result in more than 15% CL intensity changes. It is clear that the selectivity of the luminol–H_2O_2–Cyt *c* CL system for Cyt *c* over other species is good in the absence of Fe^{2+}/Fe^{3+}.

Application

Determination of Cyt *c* in Pharmaceutical Injections

The proposed method was applied to the determination of Cyt *c* in pharmaceutical injections. The injection was diluted by an appropriate factor and then determined directly by the proposed method followed by the procedure introduced in the "Experimental" section without any pretreatment. In order to evaluate the validity of the proposed method for the determination of Cyt *c* in pharmaceutical injections, standard addition methodology was implemented to test the recovery. The results are listed in Table 2. The recoveries for the different concentration levels varied from 98.0% to 108.8% with a relative standard deviation of less than 3.0%.

Determination of Cyt *c* in Human Serum

The serum samples were supplied by the Hospital of Northwest University. To prepare the spiked samples, known quantities of Cyt *c* were spiked into 1.0 ml of serum. After homogenization, the spiked samples were diluted 5.0×10^5 fold, and the influence of foreign species existing in serum could be eliminated. In order to evaluate the validity of the

Table 1 The comparison of CL intensity with different reaction systems.

Systems	I_{CL}	RSD% (n=5)
Luminol–H_2O_2–Cyt *c*	476±10	1.8
Luminol–H_2O_2–Cyt *c*– species[a]	476±15	2.1
Luminol–H_2O_2	354±5	1.3
Luminol–H_2O_2–species[a]	354±10	1.6

The concentration of Cyt *c* is 10 ng ml^{-1}

[a] Mg^{2+}, Ca^{2+}, Ba^{2+}, Zn^{2+}, NO_3^-, I^-, SO_4^{2-}, PO_4^{3-}, $Cr_2O_7^{2-}$, L-hydrochloric acid lysine, L-threonine, L-leucine, L-cysteine, L-histidine

Table 2 Determination of Cyt *c* in pharmaceutical injections.

Sample no.	Added ng ml^{-1}	Found ng ml^{-1}	RSD%	Recovery%	Content of Cyt *c* mg ml^{-1}	
					By CL	Labeled
1	0	17.9	1.26	98.0	6.7	7.5
	30	47.3	1.45			
2	0	131.1	2.08	98.0	8.2	7.5
	60	189.9	2.23			
3	0	92.3	0.98	98.1	7.7	7.5
	60	151.1	0.51			
4	0	67.7	1.38	105.1	8.4	7.5
	90	162.3	1.26			
5	0	25.2	1.03	108.8	6.3	7.5
	90	114.4	2.65			

The average of five determinations

proposed method, recovery studies were carried out on samples to which known amounts of Cyt *c* were added. The results are given in Table 3, indicating recoveries from 92.5% to 109.0%. The concentrations of Cyt *c* determined by the proposed method were compared with the concentration spiked, and good agreement was obtained.

The FI–CL method demonstrated its usefulness for the determination of Cyt *c*. The relative enhancement effect of Cyt *c* on the original CL derived from the luminol–hydrogen peroxide system and the increased CL intensity were detected quantitatively. Furthermore, the measurement of Cyt *c* in spiked human serum by this method showed good precision and recovery, confirming the suitability of the method. The proposed method is rapid, sensitive, and suitable for the determination of Cyt *c*.

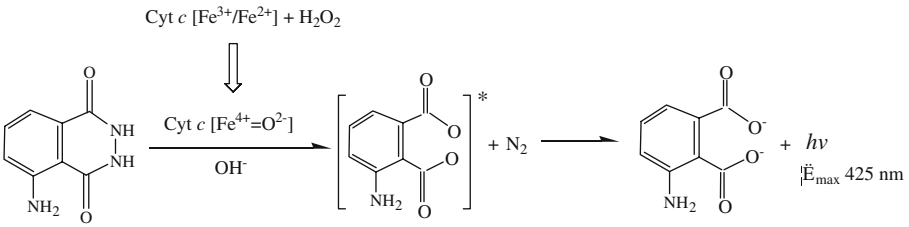
Table 3 Determination of Cyt *c* in spiked human serum.

No.	Added ng ml^{-1}	Found ng ml^{-1}	RSD%	Recovery%
1	0	15.3	0.80	109.0
	10	26.2	2.56	
2	0	9.2	1.26	94.0
	20	28.0	0.72	
3	0	9.2	1.12	107.9
	30	48.0	1.60	
4	0	86.0	2.16	92.5
	40	123.0	3.87	
5	0	66.8	1.74	95.8
	50	114.7	0.26	
6	0	54.3	1.03	102.4
	60	115.7	0.87	
7	0	38.2	2.19	92.7
	70	103.1	1.79	
8	0	70.2	2.00	103.8
	100	174.0	2.09	

The average of five determinations

Possible Mechanism

The possible mechanism of Cyt *c* enhanced CL reaction was discussed using UV absorbance and CL, and the results were listed in Table 3. As Table 4 shows, CL intensity in the presence of Cyt *c* was more than two times of that in the absence of Cyt *c* in flow system. The UV absorption of Cyt *c* measured at 408 nm decreased from 0.6610 to zero in the presence of hydrogen peroxide suggesting that Cyt *c* reacts with hydrogen peroxide, which accorded with the literature [22]. The active heme center of Cyt *c* consists of a porphyrin ring where the four pyrrole nitrogens are coordinated to the Fe atom forming a square planar complex. [23]. It was also reported that the active heme center of Cyt *c* consists of a porphyrin ring, which could be oxidized to a ferryl (Fe^{4+}=O^{2-}) state by hydrogen peroxide [24]. So, it was suggested that the ferryl [Fe^{4+}=O^{2-}] state in Cyt *c* could accelerate the oxidation between luminol and hydrogen peroxide. The mechanism of the enhanced effect of Cyt *c* on luminol–hydrogen peroxide CL reaction could be presented as followings.



Conclusion

The proposed FI–CL method for the assay of Cyt *c* offers the advantages of simplicity of apparatus, less time consumption, and higher sample throughput compared with the CL procedure reported by Robert Feissner [20]. The satisfactory performance in an assay of Cyt *c* in pharmaceutical preparations and human serum demonstrated that the proposed CL method is practical and suitable not only for quality control analysis but also for biological samples, confirming the promise for pharmacological and clinical research.

Table 4 The results of UV absorbance and dynamic CL.

Reaction mixture	I_{CL} [a]	$A_{408\ nm}$ [b]
Cyt *c*	0	0.6610
Cyt *c* + H_2O_2	–	0
Cyt *c* + luminol	–	0.6621
H_2O_2 + luminol	354	–
Cyt *c* + H_2O_2 + luminol	820	–

[a] $C_{\mathbf{Cyt\ c}}$, 100 ng ml^{-1}

[b] $C_{\mathbf{Cyt\ c}}$, 10 μg ml^{-1}; NaOH, 0.025 mol l^{-1}; H_2O_2, 5.0×10^{-5} mol l^{-1}; and luminol, 5.0×10^{-6} mol l^{-1}

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