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β-Cyclodextrin sensitized chemiluminescence of hemoglobin–hydrogen peroxide–carbonate and its analytical application

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Abstract

The weak chemiluminescence (CL) from the reaction of hemoglobin (Hb) with hydrogen peroxide in an aqueous carbonate solution was significantly enhanced in the presence of β-cyclodextrin (β-CD). The mechanism was discussed by examining CL emission spectrum and the effect of various free radical scavengers on CL emission intensity. The β-CD could form encapsulation complex with heme, which was dissociated from Hb in alkaline medium. The formed complex showed better catalytic activity than free heme thus resulting in an enhanced CL emission intensity. A flow injection CL method for the determination of Hb ranging from 2.0×10^{-9} to 1.0×10^{-5} g ml^{-1} was developed with β-CD as a sensitizer. The detection limit (3σ) for Hb was 1.2×10^{-9} g ml^{-1} (1.9×10^{-11} mol l^{-1}) and the relative standard deviation (R.S.D.) for seven independent detections of 1.0×10^{-7} g ml^{-1} Hb was 2.4%. Since metal ions could not form complex with β-CD, this method showed a good selectivity. The proposed method was used for the determination of Hb in human blood and serum.

Keywords: Hemoglobin; β-Cyclodextrin; Hydrogen peroxide; Chemiluminescence

1. Introduction

The determination of hemoglobin (Hb) in blood is very important due to the need for the diagnosis of acute hemolysis resulted from chemical, immunological, or radiological disruption of red blood cells [1,2]. Many methods including spectrophotometry [3–7], electrochemistry [8–10], fluorometric [11] and CL methods [12–14] have been used for the analysis of Hb. However, the spectrophotometric methods suffer from using toxic and carcinogenic substances such as potassium cyanide, benzidine and *o*-tolidinetime in addition to low sensitivity and long reaction time. The electrochemical methods encounter the problem of low electronic activity of Hb and the fouling of electrode due to the adsorption of protein. Though fluorometric methods offer a relatively higher sensitivity compared to spectrophotometric method, the detection limit cannot be further improved due to the interference of scattering light caused by the use of an excitation light source. This drawback has been overcome in the case of chemiluminescent detection methods since the energy needed for the excitation comes from chemical reaction [12–14]. The chemiluminescence (CL) method with luminol–hydrogen peroxide system has been used for the determination

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of Hb with high sensitivity [12,13]. But the lack of selectivity of this CL system limits their direct application to analysis of complex real samples. Therefore, it is necessary to develop a new method for the determination of Hb with high sensitivity and selectivity.

The ability of cyclodextrins to form inclusion complexes with various molecules is well known [15–17]. The formation of inclusion complex changes the physical and chemical properties of guest compounds; therefore cyclodextrins have been used extensively in optical-based analysis [17,18]. However, their uses in CL method are rather rare compared with those in fluorometric and spectrophotometric methods [19–21]. The CL intensity of the luminol related compound was enhanced seven times by using cyclodextrins as sensitizer [19]. Woolf and Grayeski studied the effect of cyclodextrins on the CL of aqueous peroxyoxalate and pointed out that cyclodextrins could increase the light output by factors up to 300 [20]. The sensitizing effect was attributed to the increase in reaction rate, excitation efficiency, and fluorescence efficiency of the emitting species.

In the present study, a significant increase of the CL emission intensity of Hb–H_2O_2–Na_2CO_3 system is observed upon the addition of β-cyclodextrin (β-CD), while the metal ions show little increase in the place of Hb, thus β-CD displays a selective sensitizing effect. The sensitizing mechanism is discussed by studying the nature of emitter and the characteristic of β-CD. Based on this observation, a flow injection CL method for the determination of Hb with high sensitivity has been developed. The proposed method is free from the interference of most of the transition metal ions and is used for the determination of Hb in blood sample.

2. Experimental

2.1. Reagents

All reagents were of analytical grade and all solutions were prepared with doubly distilled water. Standard Hb (from bovine blood, Sigma) was prepared by dissolving it in distilled water and stored below 4 °C. Hydrogen peroxide solutions were prepared by appropriate dilution 30% solution (Shanghai Chemical Plant, China) with water daily. β-CD (Shanghai Biochemical Reagent Co., China) was recrystallized for three times prior to use. Other reagents were of analytical grade and used as received.

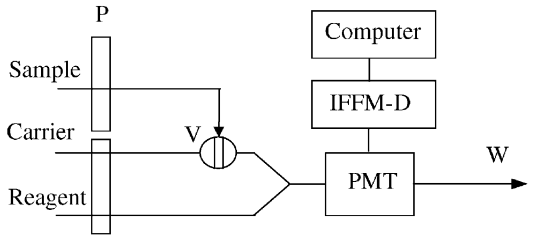

Fig. 1. Schematic diagram of flow injection CL system for the determination of Hb (P: peristaltic pump; V: six-way valve, IFFM-D: ultra weak luminescence analyzer; W: waste).

2.2. Apparatus

Fig. 1 shows the schematic diagram of flow injection system. The two pumps of the ultra weak luminescence analyzer (IFFM-D, Remex Electronic Instrument Limited Co., Xi'an, China) were used to deliver flow streams. Polytetrafluoroethylene (PTFE) tube (0.8 mm i.d.) was used to connect all components in the flow system. The sample was injected into the carrier stream (water) via the six-way injection valve. A Y-shaped mixing element, positioned just before the flow cell inlet, was used for mixing the two streams. The flow cell was a coil of glass tubing (2 mm i.d., total length 100 mm), located in front of the detection window of the photomultiplier tube (PMT). For maximum light collection, the coil was backed with a mirror. The CL emission was converted by PMT to current signals and the output was fed to luminescence analyzer, recorded with a computer via an A/D convert card and special software.

2.3. Procedures

The batch method for recording CL profile was carried out by injecting 5.0 ml Hb solution with a syringe into 10.0 ml mixed solution of Na_2CO_3 and H_2O_2 with or without β-CD in a cuvette.

The CL reaction condition was optimized with the following procedures. By keeping the valve in washing position, water and the mixture of Na_2CO_3 and H_2O_2 solutions were continuously pumped into the manifold

until a stable baseline was established. Volumes of 30 μl of Hb were injected into the carrier stream with a sampling frequency of 60 h^{-1}.

CL spectrum information was obtained with a set of 13 narrow band interference filters (400–745 nm), which were inserted between flow cell and PMT. As the total light transmitted by difference filters was different, they were calibrated by the manufacturer. The recorded data were calibrated according to the manufacturer's instruction.

3. Results and discussion

Preliminary studies were carried out with regard to the effect of medium solution on the CL intensity of Hb–H_2O_2. Na_2CO_3, $NaHCO_3$, NaOH, Na_2CO_3–NaOH, CH_3COONa and NaCl solutions were used as medium solutions, respectively. The strongest CL emission occurred with Na_2CO_3–NaOH solution followed by Na_2CO_3 and NaOH solution, while no CL emission was detected with NaCl solution. It was reported that K_2CO_3 could give out stronger emission than Na_2CO_3 in KIO_4–H_2O_2–CO_3^{2-} system [22], however we did not found significant difference between the two carbonate salts in the present CL reaction system. Therefore, H_2O_2–Na_2CO_3–NaOH system was used for following procedure.

3.1. Effect of various sensitizers

Various sensitizers such as fluorophores and surfactants are often used to increase the photon output of a weak CL system. In the present system, rhodamine B, quinine, fluorescein and dicholrofluorescein do not show the sensitizing effect. Although the CL intensity increases upon the use of some surfactants, the practical application of surfactants is troublesome because they easily adhere to glassware or flow line to cause an unpredictable signal.

Considering the capabilities to produce an organized system and to sensitize light emission, the effect of β-CD on this CL system was investigated. When β-CD was added to H_2O_2–Na_2CO_3 solution, the CL emission intensity increased significantly. A 110-fold increase in CL intensity at the saturated concentration of β-CD was found while the baseline and blank signal showed little increase.

Upon the addition of 1.0 mol l^{-1} formate into H_2O_2–Na_2CO_3 solution containing 1.0×10^{-6} mol l^{-1} Hb, the emission intensity showed a 2.6-fold increase. In comparison with the sensitizing effect of β-CD, this change was very small. Thus, β-CD was used as a sensitizer in this work.

When 1.0 mol l^{-1} formate was added into H_2O_2–Na_2CO_3 solution containing saturated β-CD and 1.0×10^{-6} mol l^{-1} Hb, the emission intensity increased further. However, it also caused the increase of blank emission. The S/N ratio was not improved. Hence, the addition of formate in the running buffer could not further improve the sensitivity.

3.2. Kinetic aspects

The rate of CL reaction plays an important role in the design of a flow CL system. The kinetic curve of CL reaction of Hb–H_2O_2–CO_3^{2-} in the presence of β-CD was investigated with a static method (Fig. 2a). This system showed a fast-type luminescence and the CL intensity reached a maximum value within 1.0 s after the addition of Hb. Compared with that without β-CD (Fig. 2b), the emission intensity increased significantly.

3.3. Optimization of the CL method

The influences of Na_2CO_3 and NaOH concentrations on the CL emission intensity are shown in Fig. 3. CL intensity increased with an increasing Na_2CO_3 concentration in the range from 0.01 to 0.5 mol ml^{-1} and reached a plateau at higher concentrations. The addition of low concentration NaOH was favorable to the emission process. When the concentration of NaOH was higher than 0.01 mol l^{-1}, the emission intensity decreased. Therefore, a 0.5 mol ml^{-1} Na_2CO_3–0.01 mol l^{-1} NaOH solution was selected.

As shown in Fig. 4, the effect of H_2O_2 concentration on CL intensity was significant and higher concentration was beneficial to the CL reaction. But the emission intensity decreased gradually when its concentration was more than 5.0×10^{-3} mol l^{-1}. The most suitable concentration of H_2O_2 was 5.0×10^{-3} mol l^{-1}.

The emission intensity increased and then trended to a maximum value with the increase of β-CD concentration, while the increment of background emission could be ignored (Fig. 5). Higher concentrations

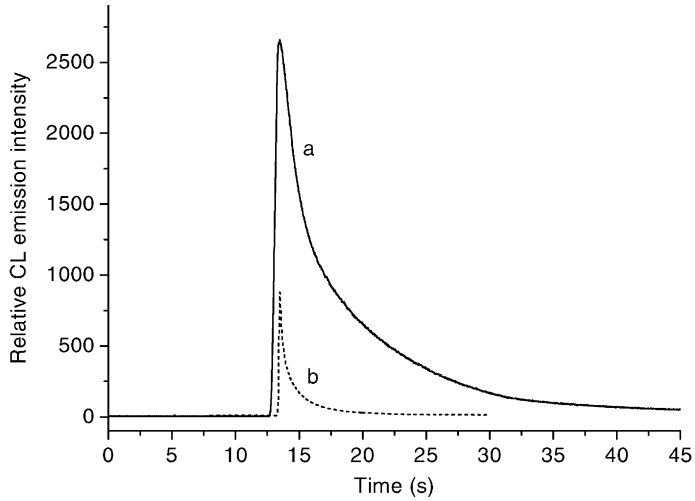

Fig. 2. CL intensity vs. time profile with β-CD (a) and without β-CD (b). Conditions: 1.0×10^{-6} g ml^{-1} Hb, 0.01 mol l^{-1} H_2O_2, 0.1 mol l^{-1} Na_2CO_3, and 1.0×10^{-4} mol l^{-1} β-CD.

than 1.5×10^{-2} mol l^{-1} was not used due to the solubility of β-CD in water. Obviously, a more soluble form of cyclodextrin such as methyl-, hydroxypropyl- or sulfobutyl-ether–β-cyclodextrin would improve the sensitizing efficiency because the sensitizing effect resulted from the encapsulation of heme by cyclodextrin (discussed in Section 3.7.2). However, the improvement is rather limited due to the slow change tendency shown at high β-cyclodextrin concentrations.

The CL intensity was also affected by flow rate of reagents and carrier. The CL intensity increased with increasing flow rate in the range 0.5–2.5 ml min^{-1}.

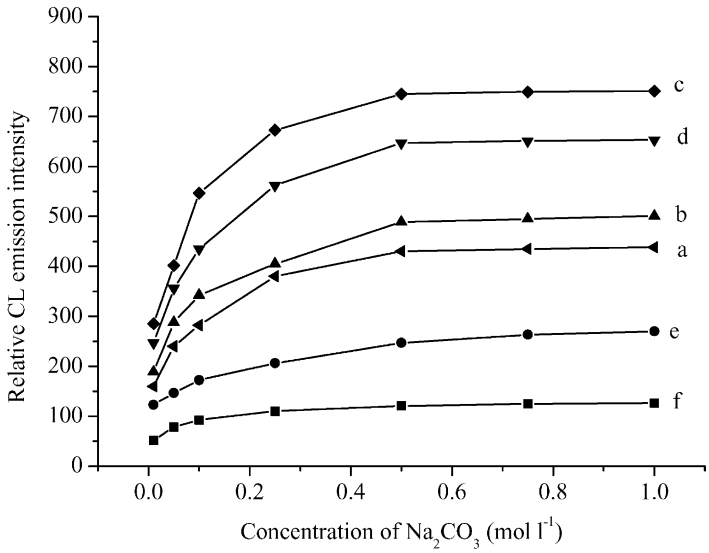

Fig. 3. Effects of concentrations of Na_2CO_3 and NaOH on CL emission intensity. The concentrations of NaOH for a, b, c, d, e and f are 0.0, 0.005, 0.01, 0.05, 0.1 and 0.2 mol l^{-1}, respectively. Conditions: 1.0×10^{-7} g ml^{-1} Hb, 0.01 mol l^{-1} H_2O_2, and 0.01 mol l^{-1} β-CD.

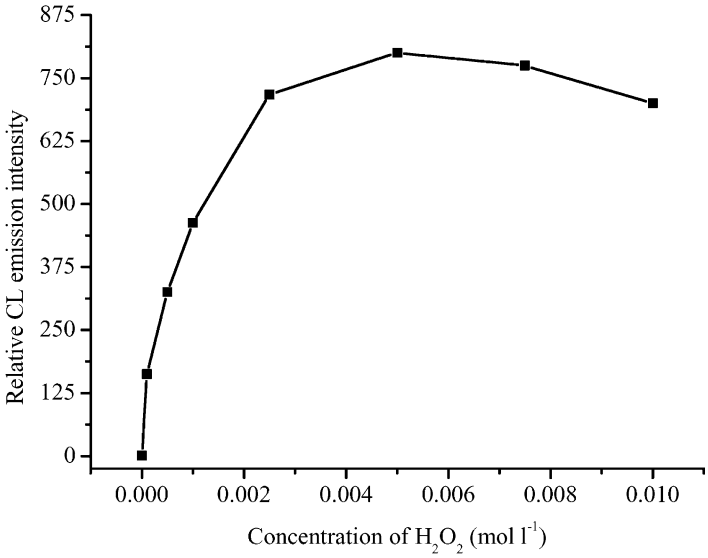

Fig. 4. Effect of H_2O_2 concentration on CL emission intensity. Conditions: 1.0×10^{-7} g ml^{-1} Hb, 0.5 mol l^{-1} Na_2CO_3–0.01 mol l^{-1} NaOH, and 0.01 mol l^{-1} β-CD.

Higher flow rates than 2.5 ml min^{-1} showed a little gain in sensitivity but much consumption of reagents. Therefore, a flow rate of 2.5 ml min^{-1} for each line was used for further studies.

The stability of hydrogen peroxide is not good enough for storage. Therefore, the mixing solution of H_2O_2–β-CD–Na_2CO_3–NaOH should be prepared daily.

3.4. Standard curve and detection limit

Under the selected experimental conditions, a linear calibration graph between 2.0×10^{-9} and 1.0×10^{-5} g ml^{-1} was obtained. The regression equation was $I = 1.24c + 167.4$, where I is the relative emission intensity and c is the concentration of Hb expressed in 10^{-9} g ml^{-1}. The correlation coefficient was 0.9996.

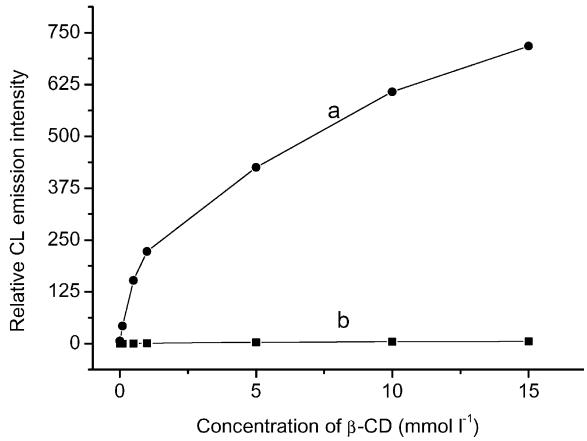

Fig. 5. Influence of β-CD concentration on CL emission intensity (a: sample signal, b: blank). Conditions: 1.0×10^{-7} g ml^{-1} Hb, 0.5 mol l^{-1} Na_2CO_3–0.01 mol l^{-1} NaOH, and 0.005 mol l^{-1} H_2O_2.

The detection limit of Hb was 1.2×10^{-9} g ml^{-1} (three times that of the deviation of blank signal). The relative standard deviation (R.S.D.) for seven determinations of 1.0×10^{-7} g ml^{-1} Hb was 2.4%.

3.5. Interference studies

In order to assess the possible analytical applications of the proposed method, the effects of coexisting compounds were examined by analyzing standard sample solutions containing 1.0×10^{-7} g ml^{-1} of Hb and various excess amount of each compound. The tolerance limit was taken as the amount that caused an error less than 5.0% in emission intensity. Most substances such as anions, sodium citrate and EDTA, did not affect the determination. Transition metal ions at 1.0×10^{-6} mol l^{-1} level did not interference the determination except Co^{2+}, Fe^{2+} and Cr^{3+}. Heparin showed no effect on CL intensity even at 500 times concentration of Hb. Albumin showed a slight inhibitive effect on CL emission when its concentrations was more than 1.0×10^{-5} g ml^{-1}.

The average concentration of albumin in health serum is 0.035–0.055 g ml^{-1}. Thus, the Hb/albumin ratio is very high in blood or hemolyzed serum samples. The interference of albumin can be eliminated by sample dilution. In non-hemolyzed serum the usual concentration of Hb is about 4.0×10^{-5} g ml^{-1}. After a 10000 times dilution, the concentration of albumin is in the range of $3.5–5.5 \times 10^{-6}$ g ml^{-1} and the concentration of Hb is about 4.0×10^{-9} g ml^{-1} which locates in the linear range for Hb determination. The recovery experiment indicated that 6.0×10^{-6} g ml^{-1} albumin did not interfere the determination of sample solution containing 4.0×10^{-9} g ml^{-1} of Hb. Thus, the proposed CL system could be used for the determination of Hb in blood or serum samples.

3.6. Sample analysis

The standard human Hb solution was prepared from fresh human blood. Its concentration was determined by cyanmet-Hb method. The working curve was obtained by properly diluting the above standard solution.

The blood samples were treated with anticoagulant and mixed with the same volume 0.2% acetic acid to break down the red cell. The samples were properly diluted with water for the determination of Hb in whole blood. For the determination of Hb in serum, no anticoagulant was added to the blood samples allowing the blood coagulation naturally. The serum was used directly for the determination after diluting 10000 times with water. The results obtained by proposed method for the determinations of Hb in the blood and serum samples were shown in Table 1.

3.7. Discussion of CL reaction mechanism

3.7.1. The nature of CL emitter

Weber et al. [23] proposed that the catalytic process of Hb to CL reaction of hydrogen peroxide and luminol was similar to the peroxidase-type reaction. However, later studies indicated that the process was due to the formation of free radicals, which were induced by the heme dissociated from Hb in high pH medium [12]. Therefore, the effects of various free radical scavengers on the emission intensity were investigated to illustrate the mechanism of present CL system, and the results were summarized in Table 2. The striking quenching effect was observed upon the addition of ascorbate, a common scavenger of free radicals. The complete quenching of the emission was achieved even at 10^{-4} mol l^{-1} level of ascorbate. The results indicated that free radicals were involved in

Table 1
The results of sample analysis

Sample	Proposed method (mg ml^{-1})	R.S.D.[a] (%)	Reference method[b] (mg ml^{-1})	Added (mg ml^{-1})	Found (mg ml^{-1})	Recovery (%)
Blood 1	112.7	2.3	112.0	10.0	122.5	98
Blood 2	133.2	2.5	134.0	10.0	143.6	104
Serum 1	0.037	3.9		0.010	0.048	103
Serum 2	0.052	3.4		0.010	0.063	102

[a] Five determinations.
[b] This results were supplied by Jiangsu Tumor Hospital.

Table 2
The influence of free radical scavengers on CL emission intensity of Hb–carbonate–hydrogen peroxide[a]

Scavenger	Concentration ($mol\,l^{-1}$)	Relative emission intensity
None	0	282.5
Ascorbate	10^{-6}	104.0
	10^{-5}	45.0
	10^{-4}	0.0
Sodium formate	0.1	337.0
	0.5	514.0
	1.0	722.4
Thiourea	10^{-4}	217.6
	10^{-3}	164.0
	0.01	118.2

[a] Concentration: Na_2CO_3, 0.1 $mol\,l^{-1}$; Hb, 1.0×10^{-6} $mol\,l^{-1}$; H_2O_2, 5.0×10^{-3} $mol\,l^{-1}$.

the present CL process. With increasing concentration of formate, a scavenger for $^{\bullet}OH$, the emission intensity increased up to 2.6-fold at a concentration of 1.0 $mol\,l^{-1}$. Formate ion was known to react with $^{\bullet}OH$ radical and subsequently with O_2 to form $O_2^{\bullet}$ radical [24]. In this way, the efficiency of $O_2^{\bullet}$ generation could be doubled. Considerable quenching of the CL was observed at relatively low concentrations of thiourea, another scavenger for $^{\bullet}OH$, but it did not completely inhibit the light emission even at high concentrations. The effects of two scavengers for $^{\bullet}OH$ on CL emission intensity indicated the formation of $^{\bullet}OH$ radical and another free radical, possibly $O_2^{\bullet}$ radical, during the CL reaction process.

It was reported that $^{\bullet}OH$ or $O_2^{\bullet}$ radicals could react with CO_3^{2-} to form peroxycarbonates, which gave out emission during the decomposition [24–26]. The CL spectrum of Hb and H_2O_2 in carbonate solution was shown in Fig. 6a. There was a broad emission band from 400 to 535 with a maximum value at 440 nm. On the basis of spectrum data, it was concluded partially that the CL emission in the proposed CL reaction system could be attributed to the formation and decomposition of carbon dioxide dimmer [24].

3.7.2. The role of β-CD in the CL process

In the presence of β-CD, the CL spectrum showed similar profile to that without β-CD (Fig. 6b). It indicated that β-CD might not involve in the ultimate emission step but be favorable to one or more intermediate steps due to its powerful inclusive ability. It was reported that the complex of iron-porphyrin with β-CD was proposed as a better substitute for native peroxide proteinase due to its three-dimensional structure [27]. The sensitizing effect resulted from the encapsulation of heme by β-CD [28], leading to the formation of inclusion complex that exhibited high catalytic efficiency.

Metal ions with small size would easily pass in and out the cavity with little or no bonding at all [17].

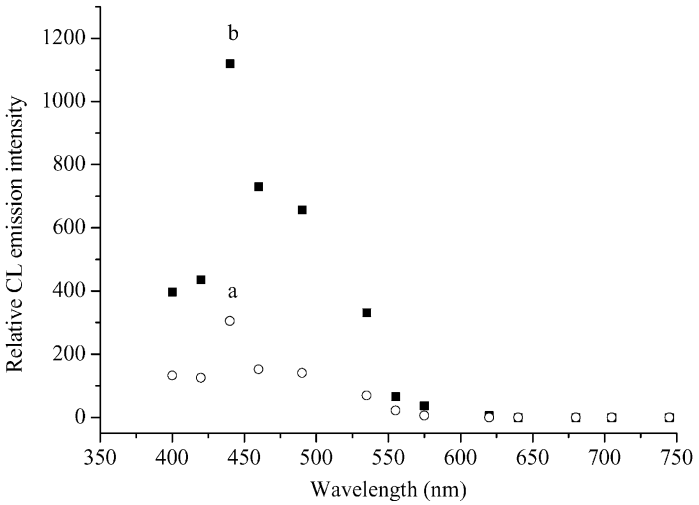

Fig. 6. CL spectra of Hb–H_2O_2–Na_2CO_3 in the absence of β-CD (a) and presence of β-CD (b). Conditions: 1.0×10^{-5} g ml^{-1} Hb, 0.5 $mol\,l^{-1}$ Na_2CO_3–0.01 $mol\,l^{-1}$ NaOH, 0.005 $mol\,l^{-1}$ H_2O_2, and 0.015 $mol\,l^{-1}$ β-CD.

The formation of stable complex between metal ions and β-CD was impossible since geometric rather than the chemical factors were decisive in the formation of complex. Therefore, the sensitizing effect of β-CD on the present CL reaction was highly selective to Hb.

4. Conclusions

A flow injection CL method for the determination of Hb with β-CD as a sensitizer has been proposed. The sensitizing effect of β-CD in the present CL system is due to the formation of complex between β-CD and heme, which shows better catalytic efficiency than heme. Since metal ions cannot form stable complex with β-CD, the enhancement effect shows high selectivity to Hb and has been used for the determination of Hb in human blood and serum.

Acknowledgements

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