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# A New Chemiluminescence Method for Determination of Cytosine Arabinoside in Pharmaceutical Preparations

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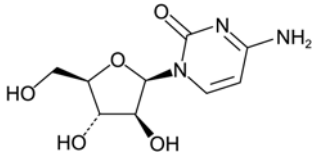
A novel chemiluminescence (CL) system was established for the determination of cytosine arabinoside (Ara-C) in pharmaceutical preparations. It was showed that a clear CL signal was observed when Eosin Y mixed with Fenton reagent. The CL intensity was decreased significantly when Ara-C was added to the reaction system and partially scavenged the hydroxyl radicals in the solution. The extent of decrease in the CL intensity had a good stoichiometrical relationship with the Ara-C concentration. Based on this, we developed a new method for the determination of Ara-C using a flow injection analysis (FIA) technique with CL detection. Under the optimal conditions, the linear range of Ara-C concentration was $6.0 \times 10^{-9} \sim 1.0 \times 10^{-7}$ mol/L (R = 0.9982) with a detection limit of $7.6 \times 10^{-10}$ mol/L (S/N=3) , the RSD was 5.6% for $6.0 \times 10^{-8}$ mol/L Ara-C (n = 11). The method was successfully applied to the determination of Ara-C in injection samples. The possible chemiluminescence reaction mechanism was discussed.

**Key Words :** Flow injection, Chemiluminescence, Eosin Y, Cytosine arabinoside, Hydroxyl radical

## Introduction

Cytosine arabinoside, or cytarabine (1-β-D-arabinofuranosylcytosine; Ara-C, Scheme 1), a pyrimidine nucleoside analogue, is an antitumor chemotherapy drug which is widely used for treatment of leukaemia especially for acute myeloid leukaemias.[1,2] However, cytarabine has a short plasma half-life and a low oral bioavailability (F = 20%), and therefore its efficacy is very dependent on dose and schedule.[3,4] Clinical and experimental studies have revealed that the toxicity of Ara-C is severe, and varies with its concentrations in the dosage,[5] this stimulated more investigations to figure out Ara-C efficacy and toxicandside-effect of different dosage in low,[6,7] intermediate[8,9] and high levels.[10-12] Therefore, it is vital to develop a simple and sensitive method for quantitative analysis of Ara-C to guarantee the quality of its products and ensure the safe use of its associated pharmaceuticals. Several analytical methods such as high performance liquid chromatography (HPLC),[13,14] ultraviolet spectrophotometry (UV)[15] and gas chromatography-mass spectrography (GC-MS),[16,17] have been utilized for this purpose. However, both HPLC and GC-MS methods involve laborious and time-consuming sample pretreatment processes; while the UV method suffers from the lack of high sensitivity.

The chemiluminescence (CL) method was widely employed in the analysis of pharmaceutical compounds due to its simplicity, ease of manipulation, rapidity, and high sensitivity,[18] it therefore could be an effective tool in the analysis of Ara-C. Howerver, to our acknowlege, there is no report on the quantitative determination of Ara-C in its pharmaceutical preparation using a CL method.




**Scheme 1.** The structure of Ara-C.

EosinY (EY, 2-, 4-, 5-, 7-tetrabromofluorescein disodium salt), a xanthene flourescent dye usually adopted in spectroscopic analysis,[19-21] was chosen as the chemiluminescent reagent in our new CL system. Fenton reaction is a typical reaction of generating hydroxyl radical and often used to evaluate the hydroxyl radical-scavenging capacity of plant active constituents.[22,23]

It was observed that a clear CL emission signal occurred when EY reacted with the hydroxyl radicals produced from Fenton reagent in an acidic medium. Ara-C was found to be able to inhibit the CL intensity by partially scavenging the hydroxyl radicals in the reaction system. The degree of CL intensity reduction exhibited a good linear relationship with Ara-C concentration. Based on this, we established a new flow injection chemiluminescence (FI-CL) method for the quantitative assay of Ara-C in its pharmaceutic preparations.

## Experimental

**Reagents and Solutions.** All the chemicals used in this work were analytical grade, and deionized water was used throughout the experiments. Cytosine arabinoside was purchased from Sigma-Aldrich (Shanghai) Trading Co., Ltd. A stock solution of $1.0 \times 10^{-3}$ mol/L cytosine arabinoside was prepared by dissolving 0.0122 g cytarabine in deionized water and then diluting to 50 mL in a brown volumetric flask. A stock solution of $1.0 \times 10^{-3}$ mol/L EY was prepared

by dissolving 0.3459 g EY (Hunan Nanhua Chemicals Co., Ltd.) with 500 mL of deionized water. Hydrogen peroxide (Guang Zhou Xinjian Fine Chemical Plant) was stored in a fridge at −4 °C, and diluted to a suitable concentration just before usage. A stock solution of $5.0 \times 10^{-3}$ mol/L ferrous iron ($Fe^{2+}$) was prepared daily by dissolving 0.1961 g ferrous ammonium sulfate 6-Hydrate (Guangdong Shantou Xilong Chemical Co., Ltd.) in 20 mL of sulfuric acid solution (0.1 mol/L) and diluting to 100 mL with deionized water. The sulfuric acid stock solution (0.1 mol/L) was prepared by diluting 2.72 mL of 98% concentrated sulfuric acid (Shanghai Shiyi Chemicals Reagent Co., Ltd.) to 500 mL with deionized water.

**Apparatus.** The CL measurements were performed using a FI-CL analyzer (IFFL-E, Xi'an Remex Analysis Instrument Co., Ltd. China). This analyzer consisted of two peristaltic pumps and a six-way injection valve equipped with a 75 μL sample loop (as showed in Fig. 1). A PTFE tubing (0.8 mm i.d.) was used to connect all components in the flow system. The flow-cell was a coil of glass tubing (1 mm i.d., total length 100 mm), positioned in front of the detection window of a sensitive photomultiplier tube (PMT) which was operated at a high negative pressure of 850V. The CL signal collected by the PMT was recorded with a computer employing CL analysis software.

The CL and fluorescent spectra were recorded using a spectrofluorometer (RF-5301PC, Shimadzu, Japan). All UV-vis absorption spectra of samples were recorded by a ultraviolet-visible (UV-vis) spectrophotometer (UV-2102 PCS, Shanghai Spectrotech Instruments Co., Ltd.). Any ultrasonic oscillations of reagents and samples were performed using an ultrasonic cleaner (KQ-2200E, Kunshan Ultrasonic Instrument Co., Ltd.).

**Procedure.** As showed in the schematic diagram of experimental manifold (Fig. 1), the solutions of EY and $Fe^{2+}$ were mixed by a 3-way mixing valve and propelled by the peristaltic pump ($P_1$) into the flow-cell as carrier stream at the flow rate of 4.1 mL/min, $H_2O_2$ and sample solutions were merged together by another 3-way mixing valve and propelled by the peristaltic pump ($P_2$) at the flow rate of 2.9 mL/min through a 10 cm tubing into a six-way valve which then injected 75 μL of the mixed solution into the carrier stream. The light output from the flow-cell was detected by a photomultiplier tube (PMT). The Ara-C concentration was calculated based on the decrement of the CL emission intensity ($\Delta I$). Here, $\Delta I = I_0 - I_s$, where $I_0$ and $I_s$ are the CL emission intensities in the absence and presence of Ara-C respectively.

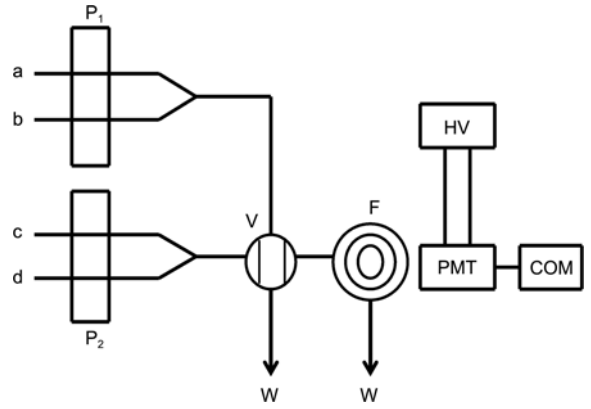



**Figure 1.** Schematic diagram of the flow injection chemiluminescence analysis system. a-$Fe^{2+}$ and $H_2SO_4$ solution; b-(Eosin Y); c-($H_2O_2$ solution); d-sample solution or $H_2O_2$; $P_1$,$P_2$-peristaltic pump; V-6-way injection valve; F-flow cell; W-waste solution; PMT-photomultiplier tube; HV-high negative voltage; COM-computer.

## Results and Discussion

**Kinetic Curve of CL Reaction.** The kinetic characteristic curve of the reactive system is shown in Figure 2. The CL signal generated from the mixed solution of EY and Fenton reagent in an acidic medium reached its maximum intensity at 3.8 s and then extinguished immediately within 4 s thereafter (Fig. 2(a)), indicating that the luminescence reaction was a rapid reaction. In the presence of Ara-C, a relatively weaker CL signal with the same CL emission time and similar peak was obtained (Fig. 2(b)).

**Selection of the Experimental Manifold and Apparatus Parameter.** Various types of the flow injection (FI) manifolds were investigated, the results showed that the maximal CL emission signal was obtained when using the manifold depicted in Figure 1, therefore, this manifold was employed in this study.

The flow rate is important to FI-CL detection. If the flow rate was too high or too low, a suitable CL emission signal could not be obtained. The effects of flow rate on the CL intensity were examined over the range of 2.6 to 4.5 mL/min. It was shown that the CL intensity was greatly enhanced with the increase in the flow rate of the carrier stream delivered by $P_1$, while kept at a constant with the increase in the flow rate of $H_2O_2$ and sample mixed solution propelled by $P_2$. As a result of a compromise between reagent consumption and CL intensity, a flow rate of 4.1 mL/min for the carrier stream and a flow rate of 2.9 mL/min for $H_2O_2$ and sample mixed solution were adopted respectively in our

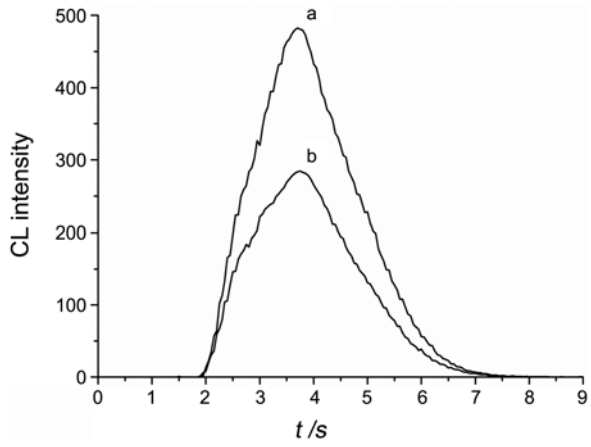



**Figure 2.** The kinetic curve of chemiluminescence. a. 0.005 mol/L $H_2SO_4$ + $4.0 \times 10^{-4}$ mol/L $Fe^{2+}$ + 0.06 mol/L $H_2O_2$ + $3.0 \times 10^{-4}$ mol/L Eosin Y; b. a + $6.0 \times 10^{-8}$ mol/L Ara-C.

experiments.

**Effect of Sulfuric Acid Concentration.** The effect of the concentration of the sulfuric acid used for preparing $Fe^{2+}$ solution on the CL intensity was investigated in the range of 0.001-0.015 mol/L. The concentration of Ara-C used in the optimization experiments was $6.0 \times 10^{-8}$ mol/L. The results showed that the relative CL intensity increased with the increase of sulfuric acid in the solution, and reached a maximal point at 0.005 mol/L. Thus, 0.005 mol/L sulfuric acid was used in the CL reaction system.

**Effect of $Fe^{2+}$ Concentration.** Ferrous iron ($Fe^{2+}$), which functions as the catalyst in the Fenton reagent, is closely associated with the yield of hydroxyl radicals (•OH). The influence of $Fe^{2+}$ concentration on the relative CL intensity was studied in the range of $7.0 \times 10^{-5}$ - $8.0 \times 10^{-4}$ mol/L. The result showed that the relative CL intensity reached its maximal point when the concentration of $Fe^{2+}$ was $4.0 \times 10^{-4}$ mol/L. Thus, $4.0 \times 10^{-4}$ mol/L $Fe^{2+}$ was adopted in our CL system.

**Effect of Hydrogen Peroxide Concentration.** Hydrogen peroxide is the main source of hydroxyl radical, its concentration could exert a comparative strong impact on the CL intensity. The effect of $H_2O_2$ concentration was investigated over the range of 0.0-0.1 mol/L. The results demonstrated that the relative CL intensity increased sharply with the increasing concentration of $H_2O_2$ and then decreased when the $H_2O_2$ concentration was 0.06 mol/L. The probable reason responsible for the decrease of relative CL intensity was that the excessive hydrogen peroxide reacted directly with hydroxyl radicals resulting in the reduction of •OH concentration in the solution, and indirectly inhibited the reaction of •OH with EY.[24] In this study, the $H_2O_2$ concentration of 0.06 mol/L was used for the CL reaction.

**Effect of Eosin Y Concentration.** The effect of EY concentration was investigated over the range of $7.0 \times 10^{-5}$-$6.0 \times 10^{-4}$ mol/L. It was shown that the relative CL intensity increased when the EY concentration ranged from $7.0 \times 10^{-5}$ to $3.0 \times 10^{-4}$ mol/L and decreased after $3.0 \times 10^{-4}$ mol/L. Thus, $3.0 \times 10^{-4}$ mol/L of EY was chosen for the CL reaction in this research.

**Analytical Characteristics.** The calibration curve of relative CL intensity vs. Ara-C concentration obtained under the optimum conditions was linear in the range of $6.0 \times 10^{-9}$ $-1.0 \times 10^{-7}$ mol/L. The regression equation was $\Delta I = 2.0 \times 10^{9}C + 131.14$ with a correlation coefficient of 0.9982. The detection limit was $7.6 \times 10^{-10}$ mol/L (S/N=3) and the relative standard deviation (RSD) for $6.0 \times 10^{-8}$ mol/L Ara-C solution was 5.6% in 11 duplicate measurements.

**Table 1.** Maximum ratio of some interfering species

| Species added | Maximum tolerable concentration ratio |
|---|---|
| $Cl^-$ | 1300 |
| $K^+$, $NO_3^-$, $Ba^{2+}$ | 1000 |
| D-glucose | 800 |
| $Ca^{2+}$ | 500 |
| Lactose, maltose | 300 |
| $Zn^{2+}$, starch | 200 |
| $Mn^{2+}$, $Cu^{2+}$, citric acid | 100 |

**Interference Studies.** To evaluate the selectivity of our proposed method, effects of common inorganic ions, excipients, and additives commonly used in pharmaceutical preparation were investigated by adding these potential interfering species into the $6.0 \times 10^{-8}$ mol/L standard solution of Ara-C. A substance was considered not to interfere when it caused a relative error in Ara-C peak height no more than ± 5%. The results were showed in Table 1. Among the interfering species tested, D-glucose, lactose and maltose are organic reducers, as seen in Table 1, a certain amount of them in the solution did not interfere the determination of Ara-C under the experimental conditions.

**Application.** Two kinds of Ara-C injection were purchased from local hospital, which were manufactured by Zhejiang Hai Zheng Pharmaceutical Co., Ltd. and Pharmacia Italia S.p.A (Italy), respectively. The total freeze-dried powder of Ara-C (100 mg) was dissolved in water, and was diluted to an appropriate concentration which was in the range covered by the calibration curve for the determinations of Ara-C content and recoveries. The results were shown in Table 2. It was demonstrated that the Ara-C contents determined by the proposed CL method were in good agreement with those obtained by the ultraviolet-visible (UV-vis) spectrophotometry method used in China's pharmacopeia.[25]

**Possible Mechanism of the CL Reaction.** The determination of Ara-C proposed in this work was based on its inhibition effect on the CL signal. In order to investigate the reaction mechanism, fluorescence spectra and the UV-vis

**Table 2.** Results of determination of daidzein in tablets (n=5)

| Sample lot number | Labeled amount (mg) | Detectable amount (mg) | | Added (mg) | Found (mg) | Recovery (%) | RSD (%) |
|---|---|---|---|---|---|---|---|
| | | CL method found ± S.D. | UV method found ± S.D. | | | | |
| 94G005/Hai Zheng | 100 | 100.3 ± 0.9 | 98.9 ± 1.0 | 80.0 | 179.3 | 98.8 | 1.35 |
| | | | | 100.0 | 201.2 | 101.2 | 1.27 |
| | | | | 120.0 | 219.9 | 99.7 | 1.02 |
| 090303B/Italia S.p.A | 100 | 100.6 ± 0.7 | 101.1 ± 1.5 | 50.0 | 151.6 | 101.2 | 1.75 |
| | | | | 100.0 | 200.2 | 99.6 | 1.57 |
| | | | | 150.0 | 248.1 | 98.3 | 1.68 |

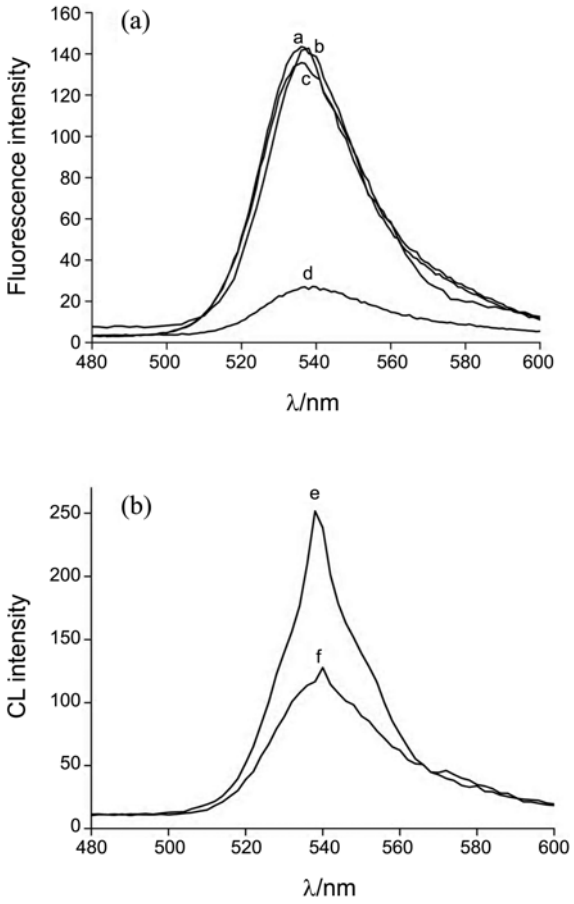



**Figure 3.** Fluorescence spectra (A) and CL spectra (B). a. $3.0 \times 10^{-4}$ mol/L Eosin Y; b. 0.06 mol/L $H_2O_2$ + $3.0 \times 10^{-4}$ mol/L Eosin Y; c. $4.0 \times 10^{-4}$ mol/L $Fe^{2+}$ + $3.0 \times 10^{-4}$ mol/L Eosin Y (in 0.005 mol/L $H_2SO_4$ acidic media); d. $4.0 \times 10^{-4}$ mol/L $Fe^{2+}$ + 0.06 mol/L $H_2O_2$ + $3.0 \times 10^{-4}$ mol/L Eosin Y (in 0.005 mol/L $H_2SO_4$ acidic media); e. (d); f. (d) + $6.0 \times 10^{-8}$ mol/L Ara-C.

absorption spectra of various different CL system components were investigated using a fluorospectrophotometer and a UV-Vis spectrophotometer.

The results showed that fluorescence spectra were observed in any multi-component mixed solution with EY, but not in any of those without EY; moreover, the spectra of EY-$Fe^{2+}$, EY-$H_2O_2$, and EY-$Fe^{2+}$-$H_2O_2$ had almost the same maximum peak position at 539 nm, which was identical with that of the fluorescent spectrum of EY (Fig. 3(a)); this suggested that EY could be the luminophor of the CL system. Figure 3(a) also demonstrated that $Fe^{2+}$ or $H_2O_2$ mixed individually with EY had almost no effects on the spectrum of EY, whereas the mixture of $Fe^{2+}$ and $H_2O_2$ could cause the decrease in fluorescent intensity of EY due to the partial consumption of EY by Fenton reagent.

Figure 3(b) showed the CL emission spectra of EY-$Fe^{2+}$-$H_2O_2$ system in the absence and presence of Ara-C recorded by the spectrofluorometer with the excitation light off. It was seen that both the CL spectra were of identical maximum peak position at 539 nm, which was in agreement with that of the fluorescent spectrum of EY shown in Figure 3(a). This further confirmed that the luminophor of the CL system was EY. The decrease in the CL emission intensity was probably due to the scavenging effect of Ara-C to hydroxyl radicals which were produced from Fenton reagent.

Figure 4 showed the UV absorption spectra of the component mixtures of the CL system and Ara-C. As depicted in Figure 4(a) to 4(c), the characteristics absorption of Ara-C at 280 nm did not decrease in the solutions of Ara-C-$Fe^{2+}$, Ara-C-$H_2O_2$, and Ara-C-EY, which indicated that Ara-C did not react individually with $Fe^{2+}$, $H_2O_2$, and EY. However, the absorption peak of Ara-C disappeared after Fenton reagent was added into the solution (Fig. 4(d)), indicating that Ara-C reacted easily with hydroxyl radicals.

It was shown in Figure 4(e) that the peak height of EY at 520 nm was exactly the same as those of EY-$Fe^{2+}$ and Y-$H_2O_2$, suggesting that there was no reaction between EY and $Fe^{2+}$, as well as EY and $H_2O_2$. Figure 4(f) demonstrated that the maximum absorption peak of EY at 520 nm was decreased significantly after the addition of Fenton reagent, indicating that EY was oxidized by the highly reactive hydroxyl radicals. The decreased absorption peak of EY increased again when Ara-C was added into the solution, suggesting that the hydroxyl radicals in the solution were partially scavenged by Ara-C. Therefore, it could be concluded that the inhibition of the CL emission was resulted from the hydroxyl radical scavenging effect of Ara-C.

Based on the discussion above, the possible mechanism of CL reaction of the proposed CL system could be explained as follow: (1) EY was the luminophor of the CL system, when EY reacted with the hydroxyl radicals from Fenton reagent, a certain amount of energy was released and absorbed by the unreacted EY in the solution to form the excited-state EY, which emitted its characteristic CL at 539 nm when returned to the ground state; (2) the CL emission signal was inhibited when Ara-C was added and partially scavenged the hydroxyl radicals in the CL system. The reaction pathway can be summarized as following:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$$
$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + \cdot OOH + H^+$$
$$\cdot OOH + H_2O_2 \rightarrow \cdot OH + H_2O + O_2$$
$$EY + \cdot OH \rightarrow \text{product} + \text{energy}$$
$$EY + \text{energy} \rightarrow (EY)^*$$
$$(EY)^* \rightarrow EY + h\nu\ (539\ nm)$$
$$[\text{Ara-C-OH}] + \cdot OH \rightarrow [\text{Ara-C-O}] + H_2O$$

## Conclusion

A new flow-injection chemiluminescence method for the determination of Ara-C based on the inhibition effect of Ara-C on CL intensity in EY-$Fe^{2+}$-$H_2O_2$ system was established. Ara-C could be sensitively detected even with a simple setup. It was believed that the reaction of EY with hydroxyl radical was responsible for the CL emission in the CL system, while the degree of the reduction of the CL intensity resulted from the scavenging action of Ara-C on hydroxyl radical had a stoichiometric relationship with Ara-C

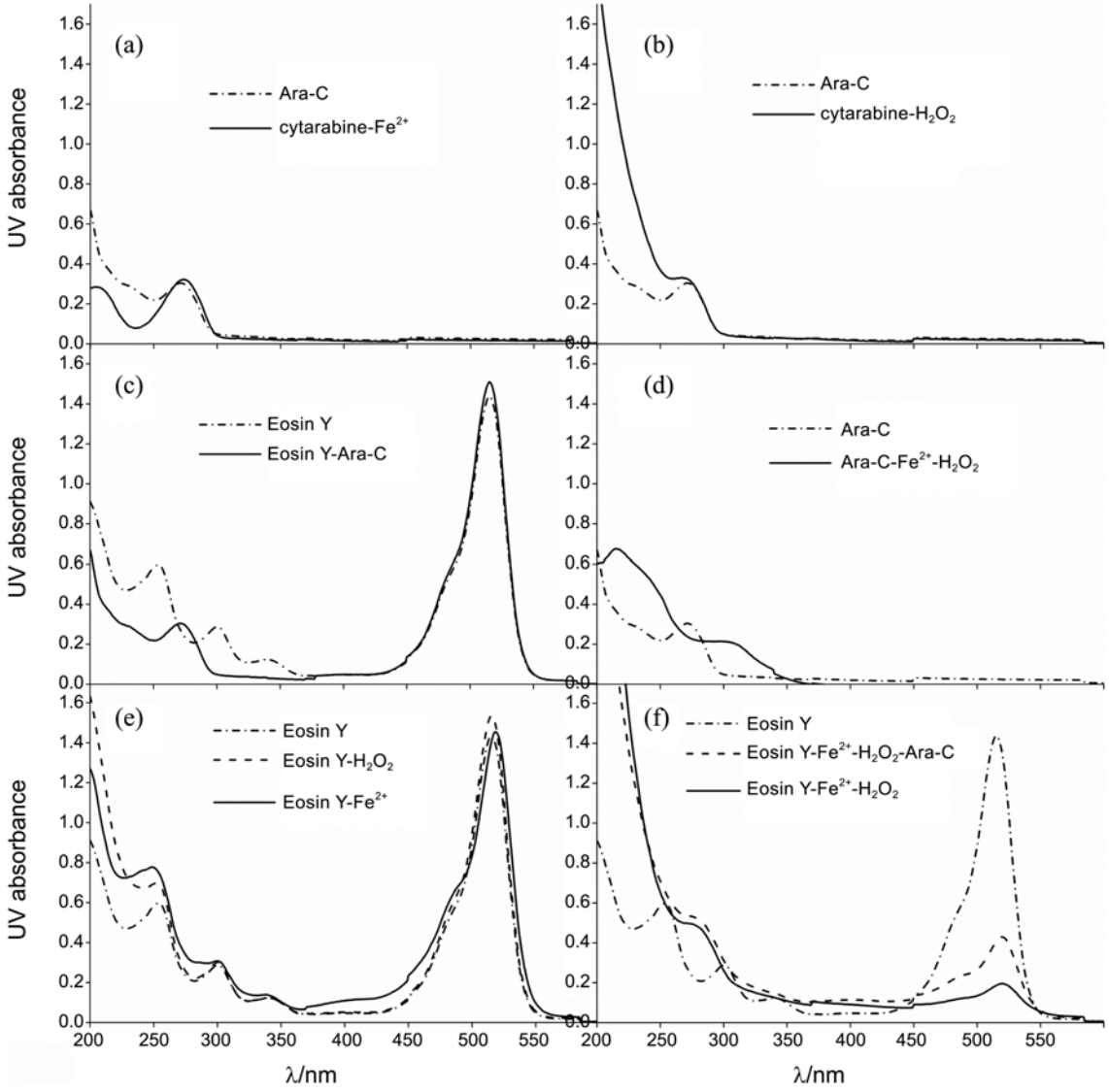



**Figure 4. T**he UV absorption spectra of the system. Ara-C: $6.0 \times 10^{-8}$ mol/L; Eosin Y: $3.0 \times 10^{-4}$ mol/L; $Fe^{2+}$: $4.0 \times 10^{-4}$ mol/L (in 0.005 mol/L $H_2SO_4$ acidic media); $H_2O_2$: 0.06 mol/L.

concentration. The proposed method is not only simple and convenient, but also sensitive and user-friendly. It had been applied to the determination of Ara-C in injection with satisfactory results.

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