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Flow-injection chemiluminescence determination of dihydralazine sulfate based on hexacyanoferrate(III) oxidation sensitized by eosin Y

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Abstract

A novel flow-injection chemiluminescence (CL) method for the determination of dihydralazine sulfate (DHZS) is described. The method is based on the reaction between DHZS and hexacyanoferrate(III) in alkaline solution to give weak CL signal, which is dramatically enhanced by eosin Y. The CL emission allows quantitation of DHZS concentration in the range 0.02–2.8 $\mu g\,ml^{-1}$ with a detection limit (3σ) of 0.012 $\mu g\,ml^{-1}$. The experimental conditions for the CL reaction are optimized and the possible reaction mechanism is discussed. The method has been applied to the determination of DHZS in pharmaceutical preparations and compared well with the high performance liquid chromatography (HPLC) method.
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Keywords: Chemiluminescence; Flow-injection analysis; Dihydralazine sulfate; Hexacyanoferrate(III); Pharmaceutical analysis

1. Introduction

Dihydralazine sulfate (DHZS), which contains two hydrazine groups, is a well-known antihypertensive compound. It can induce peripheral vasodilation, thereby reducing peripheral vascular resistance and thus lowering elevated blood pressure. Several methods have been reported for the determination of DHZS in pharmaceutical preparations, including colorimetry [1,2], spectrophotometry [3–9], liquid chromatography [10–12] and also chemiluminescence (CL) method [13,14].

Though CL methods have been reported, they suffered from the disadvantages of low sensitivity or time-consuming pretreatment procedure. Halvatzis et al. [13] reported the CL determination of DHZS using *N*-bromosuccinimide (NBS) as the oxidant. The method involves cumbersome degradation of DHZS to 2,3-dihydrophthalazine-1,4-dione in alkaline medium before CL measurement. An and co-workers [14] determined DHZS using electrochemiluminescence method, which was based on the oxidation of DHZS using rapid alternate positive rectangular electrical pulse in the presence of H_2O_2, and the linear range was 2.0×10^{-4} to 7.5×10^{-3}%. This method causes much complexity in operation process and is not sensitive enough for DHZS analysis in some real samples.

Hexacyanoferrate(III), a commonly used oxidant, has been used in CL analysis of isoniazid [15], rutin [16], thiamine [17], tetracycline and its major degradation products [18,19] by direct oxidation in basic solution. In recent studies, we found that oxidation of DHZS by $Fe(CN)_6^{3-}$ in NaOH solution to give weak CL signal, which could be significantly enhanced by eosin Y. Based on these observations, a novel flow-injection CL method for the determination of DHZS is developed. The CL emission is linearly related to the concentration of DHZS in the range 0.02–2.8 $\mu g\,ml^{-1}$. Compared with the reported CL method for the assay of DHZS, the proposed method merits some advantages: (1) needs no pretreatment. Unlike the CL method reported by Halvatzis et al. [13], which can be carried out only after a 4 h delay to degrade DHZS to chemiluminogenic product in alkaline medium, the proposed method can be used for DHDS analysis without any pretreatment, and hence sim-

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plifying the procedure and shortening the analysis time; (2) higher sensitivity. The detection limit of the present method is 0.012 μg ml^{-1}, which is not only much lower than those reported by Halvatzis et al. [13] and by An and co-workers [14] but also lower than the traditional UV method. Therefore, the present assay offers the advantages of simplicity, rapidity and high sensitivity for the determination of DHZS. The proposed method has been applied to the analysis of DHZS in compound antihypensive tablets and agrees well with the HPLC method.

2. Experimental

2.1. Reagents

A stock solution of 0.5 mg ml^{-1} DHZS (Institute of Shaanxi Pharmaceutical Industry) was prepared by dissolving appropriate amount of DHZS in warm water and diluting to the mark with water. A stock solution of 0.02 M $Fe(CN)_6^{3-}$ was prepared by dissolving 0.658 g of potassium hexacyanoferrate(III) (Xi'an Chemical Reagent Co.) in 0.1 M NaOH and diluting with the same alkaline solution to 100 ml. An aqueous eosin Y (Guangzhou Chemical Reagent Factory) solution of 0.01 M was used. All the reagents were of analytical reagent grade, and doubly distilled water was used throughout.

2.2. Apparatus

The flow stream employed in this work is shown in Fig. 1. An automatic sampler (IFIS-C, Xi'an Ruike Electronic Science-Tech. Co. Ltd.), equipped two peristaltic pumps and a six-way injection valve, was used to deliver all flow streams. PTFE tubing (0.8 mm i.d.) was used to connect all components in the flow systems. A mixing coil (glass tubing, 100 mm × 2 mm i.d.) was used as a flow cell and was positioned in front of the detection window of the

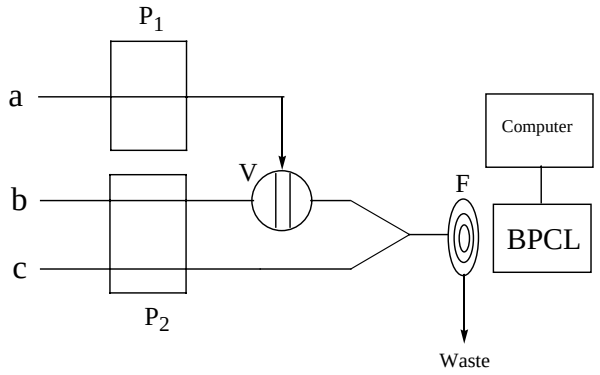


Fig. 1. Schematic diagram of the flow-injection CL system used for the determination of DHZS. (a) Sample solution + eosin Y; (b) water carrier; (c) $Fe(CN)_6^{3-}$ + NaOH; P_1, P_2, peristaltic pump; V, injection valve; F, CL flow cell; BPCL, BPCL ultra-weak luminescence analyzer.

CR-150 photomutiplier tube (operated at −950 V, Hamamatsu, Tokyo, Japan). The sample solution was injected into water stream by a six-way valve and merged with the oxidant stream, and then reached the mixing coil, producing the CL signal. The CL emission was recorded with a computerized BPCL ultra-weak luminescence analyzer (Institute of Biophysics, Academia Sinica, Beijing, China). Data acquisition and treatment were performed with BPCL software running under Windows 95.

CL spectrum was achieved with a set of interference filters, which were set between the mixing coil and PMT. Flow-injection method described as in Section 2.3.1 was used to obtain the CL emission at different wavelength bands. Absorption spectra were obtained with a S2000 miniature fiber optic spectrometer (OceanOptics, Inc., USA).

2.3. Procedures

2.3.1. Procedure for flow-injection analysis

Using the flow system schematically shown in Fig. 1, flow lines were inserted into the mixture of eosin Y and sample solution, carrier (water) and $Fe(CN)_6^{3-}$ alkaline solution, respectively. The pumping was continued till a stable baseline was recorded, and then a 120 μl sample solution (DHZS in 0.1 mM eosin Y) was injected into a carrier stream (water) by a six-way injection valve. This carrier stream was then merged with the oxidant stream ($Fe(CN)_6^{3-}$ in 0.3 M NaOH) at the mixing tee, producing the CL signal. The concentration of the sample was quantified by the CL increase (ΔI_{CL}) of the system, which was obtained by subtracting the CL blank (produced by the oxidation of eosin Y) from the total CL emission recorded. The flow rate of P_1, P_2 were set at 2.0 and 3.0 ml min^{-1}, respectively.

2.3.2. Procedure for dosage form

The average tablet weight was calculated from the weight of 20 tablets. They were then finely powdered, homogenized and a portion of the powder, equivalent to 4.2 mg of DHZS was accurately weighed and dissolved with 25 ml of double-distilled water. The resulting mixture was filtered and the filtrate was diluted with water in a calibrated 50 ml flask for further sample analysis. This solution was further diluted with water appropriately so that the final DHZS concentration was within the working range, and then analyzed according to the procedure described in Section 2.3.1.

3. Results and discussion

Preliminary studies showed that the mixture of DHZS with NaOH solution could give out weak CL emission. The kinetic profile of this process is shown in Fig. 2. With a 20 μg ml^{-1} DHZS, the maximum peak was obtained at 22 s after adding DHZS to NaOH solution and then the signal slowly decreased. At the same time, the absorbance

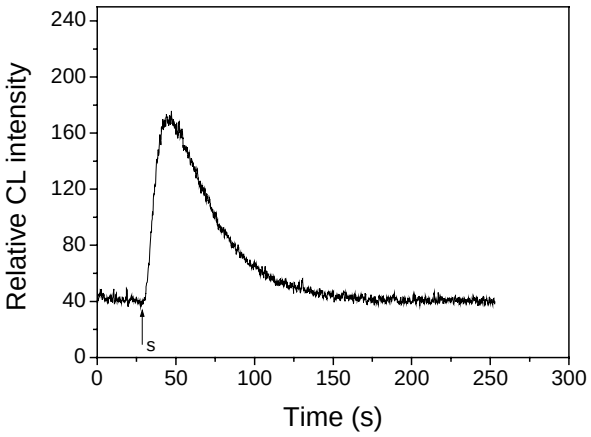


Fig. 2. CL profile of DHZS oxidation by dissolved oxygen in alkaline solution. The CL signal was recorded by adding 1.0 ml of 20 μg ml^{-1} DHZS to 8.0 ml of 0.3 M NaOH at point S.

spectra of DHZS before and after the reaction were also recorded (Fig. 3). It can be seen that the absorbance of DHZS at 305 nm was almost lost after the reaction, indicating that the structure of DHZS had changed in this process.

3.1. Kinetic aspect

Before carrying out the flow-injection method, the kinetic characteristics of the proposed CL reaction were studied by using the batch method. Upon adding DHZS or eosin Y to $Fe(CN)_6^{3-}$ basic solution gave out a weak CL signal, respectively. On injection of the mixture of DHZS and eosin Y, a strong CL emission was recorded. The CL signal from the reaction of eosin Y with $Fe(CN)_6^{3-}$ will be the background of the flow-injection analysis. Experiments showed that the proposed CL reaction was a flash type luminescence and the time interval between the start of CL and its maximum is only about 0.5 s.

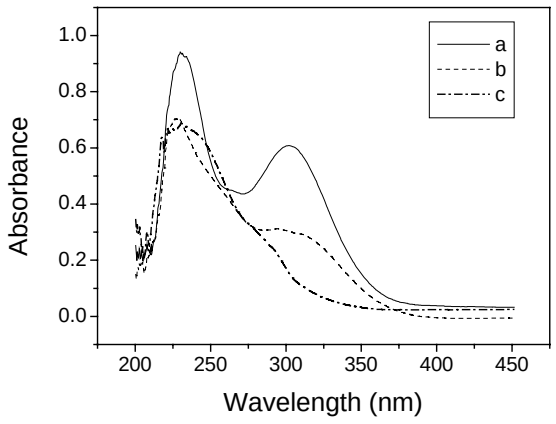


Fig. 3. Absorption spectra of DHZS added to alkaline solution at different times: (a) 0 min; (b) 10 min; (c) 40 min. The reaction was carried out by adding 0.1 M NaOH to 30 μg ml^{-1} DHZS.

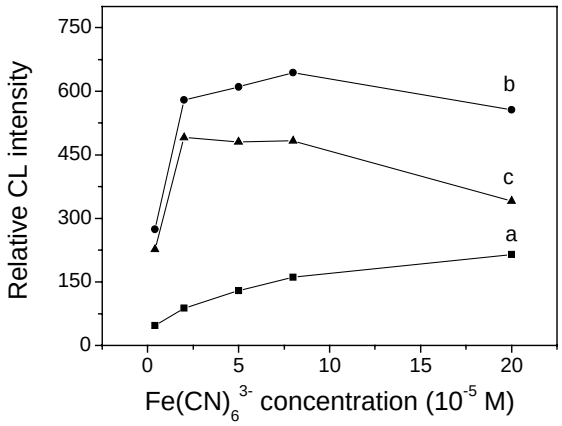


Fig. 4. Effect of $Fe(CN)_6^{3-}$ concentration on the CL intensity: (a) $Fe(CN)_6^{3-}$ + eosin Y; (b) $Fe(CN)_6^{3-}$ + eosin Y + DHZS; (c) ΔI_{CL}, which was obtained by subtracting the background signal (curve a) from the total CL emission recorded (curve b). DHZS, 0.2 μg ml^{-1}; eosin Y, 0.1 mM; NaOH, 0.3 M.

3.2. Select of oxidant

Preliminary studies showed that periodate, hydrogen peroxide, $Fe(CN)_6^{3-}$ and even dissolved oxygen in basic solution could oxidize DHZS to produce CL emission. Experiments showed that $Fe(CN)_6^{3-}$ gave out the strongest CL signal in the presence of eosin Y. Hence, $Fe(CN)_6^{3-}$ was selected.

The effect of $Fe(CN)_6^{3-}$ concentration on the CL intensity was examined over the range 4.0 μM–0.2 mM in 0.3 M NaOH solution and the results are shown in Fig. 4. Maximum ΔI_{CL} was obtained in the range 20–80 μM of $Fe(CN)_6^{3-}$. Therefore, the concentration of $Fe(CN)_6^{3-}$ was selected at 50 μM for the further experiment.

3.3. Effect of NaOH concentration

Preliminary studies showed that $Fe(CN)_6^{3-}$ could react with DHZS to produce CL emission in basic solution. In the present study, NaOH was selected as the basic medium, and its concentration was tested in the range 0.04–0.6 M. As can be seen form Fig. 5, ΔI_{CL} was the highest and remained stable over the range 0.2–0.3 M. Therefore, a 0.3 M NaOH was selected as the reaction medium in the following study.

3.4. Effect of sensitizers

CL reaction generates the product in an excited state. If this excited species is fluorescent, the radiative relaxation is possible and we observe light generation—direct CL. If there is another molecule present in the system, which is capable to "take" (somehow) excitation energy and become excited itself, the energy transfer occurs. The originally excited molecule is called "primary emitter" or "donor (of excitation energy)", the other one is called "acceptor (of excitation energy)". If acceptor is more fluorescent than donor

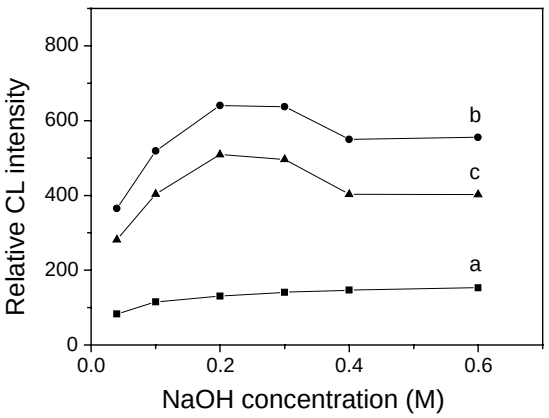


Fig. 5. Effect of NaOH concentration on the CL intensity: (a) $Fe(CN)_6^{3-}$ + eosin Y; (b) $Fe(CN)_6^{3-}$ + eosin Y + DHZS; (c) ΔI_{CL}, which was obtained by subtracting the background signal (curve a) from the total CL emission recorded (curve b). DHZS, 0.2 μg ml^{-1}; $Fe(CN)_6^{3-}$, 50 μM; eosin Y, 0.1 mM.

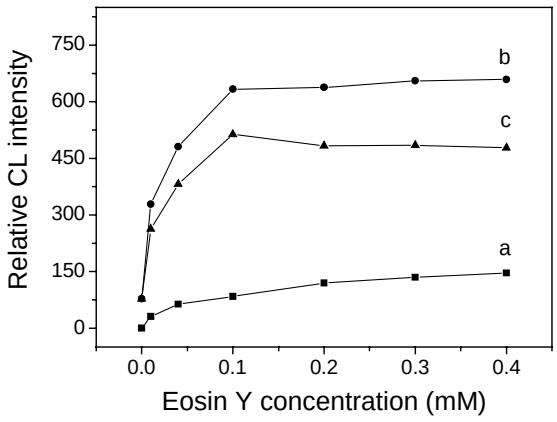


Fig. 6. Effect of eosin Y concentration on the CL intensity: (a) $Fe(CN)_6^{3-}$ + eosin Y; (b) $Fe(CN)_6^{3-}$ + eosin Y + DHZS; (c) ΔI_{CL}, which was obtained by subtracting the background signal (curve a) from the total CL emission recorded (curve b). DHZS, 0.2 μg ml^{-1}; $Fe(CN)_6^{3-}$, 50 μM; NaOH, 0.3 M.

(at suitable wavelength), the sensitization process occurs and often leads to a large increase in CL emission. The above process, called sensitization, has been extensively used in the CL analysis of a variety of compounds such as sulfite [20], thiol-containing substances [21,22] or sensitizers (pipemidic acid [23], quinine [24], etc.).

In order to obtain a high sensitivity, some fluorophores were selected and their sensitizing effects on the present CL reaction were examined. As shown in Table 1, eosin Y gave the highest CL increase for this system. Thus, eosin Y was selected as sensitizer in the present CL system. The effect of eosin Y concentration on the CL signal was studied in the range 0–0.4 mM. As can be seen from Fig. 6, ΔI_{CL} was increased with increasing the concentration of eosin Y up to 0.1 mM, thereafter remaining almost constant up to 0.4 mM. As a compromise of high sensitivity and low CL background, the concentration of eosin Y was chosen at 0.1 mM.

3.5. Effect of flow rate

The flow rate is an important parameter in the CL reaction because the time taken to transfer the excited product into flow cell is critical for maximum collection of the emitted light. The effect of flow rates on the CL emission was investigated and experiments shows that CL intensity increased with increasing the flow rate over 0.5–5.0 ml min^{-1}. Because of a high flow rate led to a great consumption of reagents with little gain in sensitivity, hence, a flow rate of 3.0 ml min^{-1} for carrier solution and oxidant solutions was selected for the further studies.

Table 1
Effect of different sensitizers on CL emission of $Fe(CN)_6^{3-}$—DHZS system[a]

Sensitizer[b]	ΔI_{CL}
None	43
Eosin Y	506
Rhodamine B	53
2,7-dichlorofluorescein	168
Riboflavine	117
Salicylic acid	61

[a] Conditions: $Fe(CN)_6^{3-}$, 50 μM; NaOH, 0.3 M; DHZS, 0.2 μg ml^{-1}.
[b] The concentration of each sensitizer was 0.1 mM.

3.6. Stability of DHZS

DHZS is stable in acidic conditions and the CL signal of the proposed system remains almost unchanged for a month. However, DHZS is unstable in basic medium and can degrade to 2,3-dihydrophthalazine-1,4-dione [13]. The effect of alkaline medium on the CL emission was investigated by adding 0.01 M NaOH to DHZS solution. As shown in Fig. 7, with the addition of NaOH, the CL signal decreased with reaction time went on and showed no difference with the

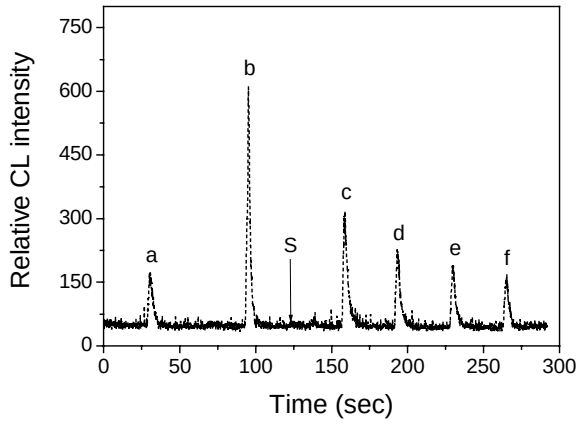


Fig. 7. Dependence of CL signal on time upon addition of 0.01 M NaOH to DHZS: (a) $Fe(CN)_6^{3-}$ + eosin Y; (b) $Fe(CN)_6^{3-}$ + eosin Y + DHZS; (c)–(f) were the CL emission upon addition of 0.01 M NaOH (at point S) to the sample solution (DHZS in 0.1 mM eosin Y) at different time. DHZS, 0.2 μg ml^{-1}; $Fe(CN)_6^{3-}$, 50 μM; NaOH, 0.3 M; eosin Y, 0.1 mM.

Table 2
Tolerance limit of some foreign substances on the determination of DHZS (0.7 μM)

Substance	Mole ratio to DHZS
Zn^{2+}, Mg^{2+}, K^{+}, Al^{3+}, Pb^{2+}, NH_4^{+}, Cl^{-}, SO_4^{2-}, glucose, sucrose, EDTA	≥500
Br^{-}, Fe^{2+}, hydrazine, phthalate, pyridoxine hydrochloride, dibazol	100
Riboflavin, hydrochlorothiazide, reserpine	50
Promethazine, citric acid, Co^{2+}	10
Cu^{2+}	1
Thiamine	0.5

background signal after about 150 s. Therefore, to maintain a stable CL signal, DHZS should be prepared in neutral or acidic solutions.

3.7. Limit of detection, linear calibration range and precision

Under the selected conditions given above, the ΔI_{CL} showed a linear relationship with the concentration of DHZS in the range 0.02–2.8 μg ml^{-1} ($r = 0.9983$, $n = 10$). According to IUPAC, the detection limit was determined from three times the standard deviation of the blank signal (3σ) as 0.012 μg ml^{-1}, where the blank signal was the CL signal produced by the oxidation of eosin Y. The relative standard deviation ($n = 7$) was 2.2% for 0.2 μg ml^{-1} of DHZS.

3.8. Interference study

In order to assess the possible analytical applications of the described CL method, the effect of foreign substances was tested by analyzing a standard solution of DHZS (0.7 μM) to which increasing amounts of interfering species were added. The tolerable concentration ratios with respect to 0.7 μM of DHZS for interference at 5% level were shown in Table 2. It can be seen that most of co-existing substance in compound antihypertensive tablets have almost no effect on the determination of high concentration of DHZS.

Table 3
Results of determination of DHZS in Tabellae Reserpini Compositae

Sample	Amount labeled (mg)	Amount found (mg)[a]	
		Proposed method	HPLC method
Tablet 1	4.2	4.10 ± 1.3	4.05 ± 2.2
Tablet 2	4.2	4.41 ± 2.8	4.22 ± 1.8

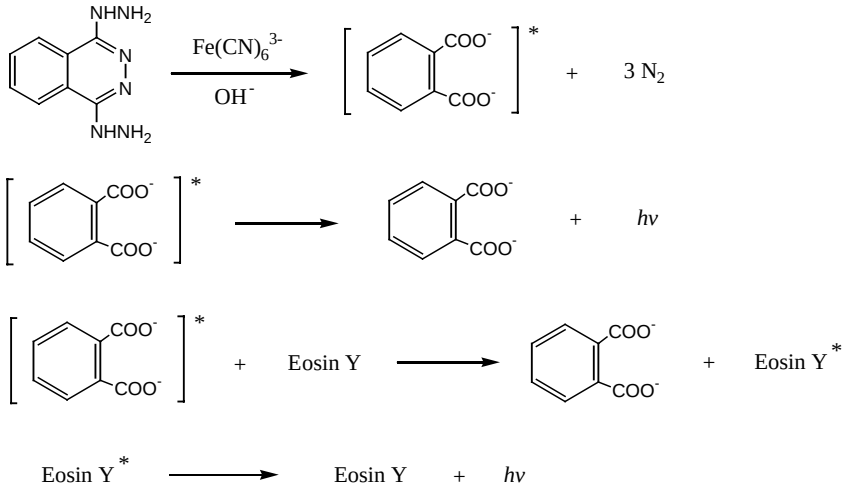
[a] Average of four measurements (±RSD).

3.9. Sample analysis

Following the procedure detailed in Section 2.3.2, the proposed method was applied to the determination of DHZS in commercially available compound antihypertensive tablets, Tabellae Reserpini Compositae (4.2 mg DHZS, 3.1 mg hydrochlorothiazide and 0.032 mg reserpine per tablet). The results are listed in Table 3, which agrees well with those obtained by the HPLC method [12].

3.10. Possible mechanism of the present CL reaction

The proposed CL reaction mechanism is studied in detail. First of all, no CL was recorded when DHZS was replaced by the same concentration of hydrazine, indicating that the proposed CL emission cannot produce by hydrazino group alone. Secondly, no CL signal was observed when DHZS was degraded in basic solution, showing that CL emission was relevant to the hydrazino groups of DHZS. The above results indicate that the proposed CL emission can only be



Scheme 1.

generated by DHZS itself, while not by its alkaline degradation products (2,3-dihydrophthalazine-1,4-dione and phthalate) or its hydrazino groups alone. Thirdly, in order to identify the emitter of the proposed CL reaction, the CL spectrum was recorded by a series of interference filters (400–745 nm) and the experimental results showed that the maximum CL emission is about at 535 nm, which is similar to the fluorescence emission maximum of eosin Y (545 nm) reported in the literature [25]. Based on the above experimental results, we suggest the CL reaction occurred as follows: In basic solution, DHZS is oxidized by $Fe(CN)_6^{3-}$ to generate the electronically excited phthalate. This reaction is similar to the CL reaction of luminol, which generates 3-aminophthalate. However, the lack of electron-donating group (3-amino group) in its structure makes it give only weak CL emission. In the presence of eosin Y, the excited phthalate can transfer its excitation energy to eosin Y, producing the excited eosin Y, and a strong light emission occurs when the excited eosin Y goes to its ground state level. The possible mechanism stated above can be expressed as in Scheme 1.

4. Conclusion

In summary, a novel CL method has been developed for the determination of DHZS. The method is based on the reaction of DHZS with $Fe(CN)_6^{3-}$ in alkaline medium to give weak CL emission, which is enhanced by eosin Y and thus leading to a dramatic increase in CL emission. Compared with the other reported methods for measuring DHZS, the proposed method for quantitative DHZS analysis is simple, fast and needs only inexpensive reagents and instrumentation.

Though the energy-transferred CL analysis has been widely used for the determination of a variety of compounds, to the best of our knowledge, this is the first report using DHZS as an energizer, and its CL characteristics are still under investigation.

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