A novel luminol-based chemiluminescence method for the determination of amikacin sulfate in serum by using trivalent copper-periodate complex

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Abstract A novel chemiluminescence (CL) reaction was based on the oxidizing reaction of luminol by the trivalent copper-periodate complex (K₅[Cu(HIO₆)₂], DPC) in alkaline medium. The CL intensity could be enhanced in the presence of amikacin sulfate (AKS). A new CL method was developed for the determination of AKS by coupling with flow injection (FI) technology. Because of the distinctive oxidative effect of DPC, the luminol-based CL reaction could occur at a low concentration of 10⁻⁷ M. The relative CL intensity was proportional to the concentration of AKS in the range of 4.0 × 10⁻⁹ to 4.0 × 10⁻⁶ g/mL with the detection limit of 1.2 × 10⁻⁹ g/mL. The relative standard deviation was 2.1% for 8.0 × 10⁻⁹ g/mL AKS (n=9). The proposed method was successfully applied to the direct determination of AKS at the level of ng/mL in serum samples. The recovery varied from 97.0% to 106.3%. A possible mechanism of the CL reaction was discussed in detail by relating to the CL kinetic characteristics and electrochemical activities of the oxidant DPC.

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1. Introduction

Amikacin(O-3-amino-3-desoxy-α-d-glucopyranosyl(1-6)-O-[6-amino-6-desoxy-α-d-glucopyrano-syl(1-4)]-N1-(4-amino-2-hydroxy-1-oxobutyl)-2-desoxy-d-streptamine, C₂₂H₄₃N₅O₁₃) sulfate is one of the most broad-spectrum aminoglycoside antibiotics [1,2]. As its structure is shown in Scheme 1, it has the characteristics of being semi-synthetic and water soluble. It can curb drug resistance to gentamicin, kanamycin and tobramycin because of its fewer points susceptible to enzymatic attack than the other
which gave the CL system the characteristic of being selective. The enhanced light emission caused by AKS could be used for the quantitative analysis of AKS concentration in serum samples. A novel, more sensitive and selective chemiluminescence method was developed for trace analysis of AKS in serum by coupling flow injection analysis (FIA) techniques without any pretreatment of samples. Based on the electrochemical reaction and kinetics curve of the CL reaction experiment, the mechanism of the reaction was also discussed.

2. Experimental

2.1. Reagents and chemicals

Luminol (5-aminophthalazinedione) was kindly provided by Shaanxi Normal University (Xi’an, China). The standard medicine of AKS was obtained from Chongqing Institute for Drug Control (Chongqing, China). It was dissolved to prepare the stock solution with the concentration of $1 \times 10^{-4}$ g/mL with distilled water. It was stored at 4°C and diluted to working solution with distilled water. The reagents for DPC preparation were listed as follows: Potassium persulfate (Na2S2O8) was purchased from Shanghai Aijian Chemical Reagent Company (Shanghai, China), cupric sulfate (CuSO4·5H2O) and potassium hydroxide (KOH) were purchased from Chongqing Chemical Reagent Company (Chongqing, China), and potassium periodate (KIO4) was purchased from Shanghai Chemical Reagent Company (Shanghai, China). All of the chemicals were of analytical reagent grade and used without further purification. Doubly distilled water was used throughout the work. The diluted working solutions were prepared and used freshly and daily. A luminol stock solution (0.01 M) was prepared by dissolving 1.772 g luminol in 1 L carbonate buffer (0.1 M), and left to stand for approximately 24 h before use. The luminol solution was stable for at least 1 month when stored in the dark. The DPC stock solution (0.01 M) was prepared by oxidizing Cu (II) in the alkaline medium according to the known method [28]. In briefly, KIO4 (0.23 g), CuSO4·5H2O (0.125 g), Na2S2O8 (0.14 g) and KOH (0.8 g) were added into 30 mL water. The mixture was heated to boil for about 20 min on a hot plate with constant stirring. After the boiling mixture turned intensely red, the boiling continued for another 20 min for the completion of the synthetic reaction. When the mixture was cooled, it was diluted to 50 mL with distilled water. The stock solution obtained was refrigerated at 4°C, which was found fairly stable for several months, and DPC solutions were freshly prepared before use. The complex was confirmed at 415 nm by UV/Visible spectrum with the molar absorptive (ε) of 6230±100 L/mol cm.

2.2. Apparatus

The schematic diagram of the used FIA-CL system in this work is shown in Fig. 1. All the chemicals were delivered to the system with two peristaltic pumps (HL-2, Shanghai Huxi, China) at a flow rate of 2 mL/min. The polytetrafluoroethylene (PTFE) flow tubes (0.8 mm i.d.) were used to connect all the components in the system. A sixteen-port injection valve (Hanzhou, China) was equipped with a loop of 75 μL which was used to process the injection mode. The CL signal was monitored by a BPCL ultra-weak luminescence analyzer (Institute of Biophysics, Chinese Academy of Sciences, Beijing.
China) which consists of a flat coil glass flow cell facing the window of the photomultiplier tube (PMT). Data were acquired and processed with BPCL software running under Windows 98. The UV-Vis-2001 spectrophotometer (Hitachi Ltd., Japan) was used for UV-absorbance detection. The CL spectrum was obtained with the LS45/55 fluorospectrophotometer (PerkinElmer Ltd. America). The electrochemical characteristics of copper metal chelate complex were studied using the CHI832 electrochemical workstation (ChenHua Ltd., Shanghai, China).

2.3. Procedure

As shown in Fig. 1, the flow lines were inserted into the luminol solution, an analyte (a standard AKS solution or a sample containing AKS, respectively) and DPC solutions. The peristaltic pumps were started to wash the whole system at 2.0 mL/min, and then the in-line and real-time determination models were performed by inserting the flow line “b” into the vessel containing the collected samples. When the injection valve was set to the load position, the stream of DPC solution and luminol solution was mixed at “M” point, and then the whole system was run until a stable baseline was recorded. When the injection valve was switched to the inject position, the carrier stream DPC solution bypassed the reagent loop (75 μL analyte solution) and ran directly through the flow cell, producing CL emission. The CL signal was then recorded simultaneously. Under the PMT operated at −800 V, the relative CL intensity ΔI, defined as the difference of CL intensity between in the presence and in the absence of analyte respectively, was proportional to the corresponding concentration of AKS solution.

3. Results and discussion

3.1. Kinetics curve of the CL reaction of luminol-DPC-AKS

In the batch mode, with the experimental parameters kept constant, the typical response curve (intensity versus times) of the CL reaction between luminol solution (1 × 10⁻⁷ M) and DPC (4 × 10⁻⁴ M) in the presence of ASK solution with the concentration of 2 × 10⁻⁷ g/mL was recorded to study the kinetic characteristics of the CL reaction. As shown in Fig. 2, the CL intensity peak appeared within 1 s after AKS was injected into the mixture solution of luminol and DPC. The CL signals would decrease to the baseline within 10 s. Fig. 2 shows that the CL intensity of luminol-DPC could be greatly enhanced by the AKS solution. The CL reaction was obviously quick. The kinetics curve indicated the CL system was rapid and sensitive enough and suitable for the analysis of AKS.

3.2. Optimization of the experiment procedure

As is well-known, luminol is one of the most popular CL reagents for its oxidation in alkaline medium. These luminol-based CL reactions mainly result from the oxidant reaction of luminol with a range of typical oxidants, such as oxygen, hydrogen peroxide, potassium permanganate, ferricyanide, tetravalent cerium ion, lead dioxide and oxygen free radicals. The oxidant reaction of luminol with these typical oxidants can occur when the concentration of luminol is up to 1 × 10⁻⁵ M. The difference of DPC from these typical oxidants enabled the emitted luminal-based CL signal to be clearly observed when luminol was lower than 1.0 × 10⁻⁶ M. So the effect of luminol concentration was examined over the range from 1 × 10⁻⁸ M to 5 × 10⁻⁷ M when the DPC and AKS solutions were fixed at a certain concentration. As shown in Fig. 3, the CL intensity increased with an increase of the luminol concentration in the presented range. For avoidance of PMT saturation and minimal reagent consumption were considered, the optimal concentration of luminol was 1 × 10⁻⁷ M for the subsequent experiment. The use of luminol with a lower concentration also effectively avoided the interference by other common oxidants.

The kinetics test showed that the luminol-based CL was based on the oxidation reaction with DPC and enhanced by AKS. To test the effect of DPC solution, a range of concentrations of DPC (1 × 10⁻³ M–1 × 10⁻³ M) was investigated. As the result in Fig. 4 showed, the CL intensity was increased with the increase of the DPC concentration in a low-concentration range (4 × 10⁻⁴ M), and reached the maximum. Above 4 × 10⁻⁴ M, the CL intensity decreased probably because a higher concentration of DPC caused self-absorption. Therefore, the optimal concentration was 4 × 10⁻⁴ M.

Generally, the CL oxidation of luminol by oxidants often occurs in basic conditions. Additional DPC was obtained in strong alkaline solution (KOH solution). A solution of KOH...
(0.1 M) was added to the luminol solutions to offer different pH of CL system. The effects of pH (8–12) medium were investigated. Fig. 5 shows that the optimal pH of the reaction was 9.0. For obtaining the highest sensitivity and accuracy, a pH 9.0 of luminol solution (KOH solution) was selected as the optimum.

### 3.3. The analytical characteristics of the FI-CL method

Under the optimal experimental conditions, the calibration graph of change of CL intensity, $\Delta I$, against AKS concentration, was measured. There was a biphasic linear relationship between the change in the CL intensity and the AKS concentration over the range of $4.0 \times 10^{-9}$ g/mL–$4.0 \times 10^{-7}$ g/mL and $4.0 \times 10^{-7}$ g/mL–$4.0 \times 10^{-6}$ g/mL. The regression equation and correlation coefficients are listed in Table 1. The detection limit was $1.2 \times 10^{-9}$ g/mL ($3\sigma$) and the relative standard deviation was 2.3% for $8.0 \times 10^{-9}$ g/mL AKS ($n=9$).

### 3.4. Effects of coexisting foreign species

In order to apply the proposed method to determine AKS in a practical sample, the effects of some possibly coexisting foreign inorganic ions and organic compounds were examined.

### 3.5. Analytical applications of the present FIA-CL method

The proposed method was applied to the determination of AKS in human serum. Blank serum sample (2 mL) was collected and transferred to a centrifugal filter unit and then centrifuged at 10,000 rpm for 10 min at 4 °C. Then 1 mL filtrate was transferred to a 100 mL volumetric flask and diluted to the mark with doubly distilled water. The blank serum was injected into the CL system, and the blank signal was recorded. The amount of AKS in human serum and the recovery obtained are shown in Table 2.
concentration (10^{-7} M). In the oxidant reaction of DPC participation, the cuprous complex was regarded as the final product because the electron transfer process was a single electron process.

The CL spectrum of the luminol-DPC system is shown in Fig. 6. The fluorescence emission shows an obvious peak at 430 nm when the luminol was mixed with DPC. The obvious intensive peak appeared at the same wavelength when AKS was joined into the luminol-DPC system. It suggested that a reaction between DPC and luminol occurred and the formation of intermediate states (exited Aminophthalate) was with an emission maximum at 425 nm. The CL intensity can be enhanced in the presence of AKS.

Previous studies on oxidation of L-leucine and threonine by Cu (III)\cite{26,28} suggest that formation of [Cu(H3IO6)2(OH)2]^{5-} is the reactive species of water-soluble Cu (III) periodate complex in alkaline medium. From the oxidant reaction of L-leucine\cite{26} and isoniazid \cite{32} by DPC, it is not difficult to speculate the mechanism of luminol-based CL reaction for the similar characteristics of hydrazide in structure. The mechanism could suffer from a free radical intervention. The CL reaction mechanism of DPC-luminol is proposed in Fig. 7. Fortunately, the CL intensity of DPC-luminol could be enhanced by AKS.

4. Conclusion

In this work, a novel and sensitive CL method for the direct determination of AKS in serum was developed. It was based on the sensitization of AKS on the oxidant reaction of luminol and DPC. Compared with other oxidants such as H_2O_2, K_3Fe(CN)_6, KMnO_4 and KIO_4, DPC has been proved to be a weaker oxidant. However, use of DPC enables the oxidant reaction of luminol to occur at a lower concentration. It makes the CL reaction more selective. Combined with flow injection technique, an attractive FIA-CL method has been developed for its higher sensitivity, lower detection limit and well reproducibility. The detection limit of AKS was at the nanogram level. Recovery tests showed that the DPC-luminol CL method was successfully applied in the determination of AKS in serum without any pretreatment. It indicates that the

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Table 1  The analytical characteristics of the FI-CL method.

| Range of AKS concentration (g/mL) | Regression equation | Correlation coefficient |
|-----------------------------------|---------------------|------------------------|
| 4.0 × 10^{-9}-4.0 × 10^{-7}       | ΔI=14.841c+20.107    | 0.9978                 |
| 4.0 × 10^{-7}-4.0 × 10^{-6}       | ΔI =6.444c^2+246.10  | 0.9970                 |

^a Concentration in ng/mL.

Table 2  Results of recovery tests of AKS in human serum.

| Sample | AKS in sample^a (10^{-9} g/mL) | Added (10^{-9} g/mL) | Total found^a (10^{-9} g/mL) | Recovery (%) | RSD (%, n=3) |
|--------|--------------------------------|----------------------|------------------------------|--------------|--------------|
| No.1   | 4.87                           | 1.00                 | 5.92                         | 105.0        | 0.8          |
|        |                                | 3.00                 | 7.78                         | 97.0         | 0.6          |
|        |                                | 5.00                 | 9.98                         | 102.2        | 1.2          |
| No.2   | 5.23                           | 4.00                 | 9.48                         | 106.3        | 2.5          |
|        |                                | 6.00                 | 11.42                        | 98.8         | 3.1          |
|        |                                | 8.00                 | 13.57                        | 103.2        | 2.6          |
| No.3   | 10.53                          | 6.00                 | 16.62                        | 101.5        | 1.6          |
|        |                                | 10.00                | 20.46                        | 99.3         | 2.1          |
|        |                                | 14.00                | 24.72                        | 101.4        | 3.5          |

^a Average value of the measure (n=3).
Cu (III)-based CL method is a promising reagent. Moreover, Cu (III) can broaden the application of CL method in further studies.

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