

Utilization of a Novel Ag(III)-Luminol Chemiluminescence System for Determination of *d*-Penicillamine in Human Urine Samples

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Um novo sistema de quimioluminescência (CL), sensível, (reação $[\text{Ag}(\text{HIO}_6)_2]^{5-}$ -luminol), acoplado com injeção em fluxo, é introduzido para determinação de *d*-penicilamina. O sistema é baseado no efeito do aumento da reação de quimioluminescência por penicilamina em meio alcalino. Sob condições ótimas, a intensidade de CL é proporcional às concentrações de *d*-penicilamina com uma boa linearidade. O intervalo linear é de 1,5-150 ng mL⁻¹ e o limite de detecção do método, 0,8 ng mL⁻¹. O desvio padrão relativo é 0,23% para 11 repetições de 0,06 µg mL⁻¹ de *d*-penicilamina. O método tem sido aplicado com sucesso às determinações de *d*-penicilamina em preparações farmacêuticas e amostras de urina, proporcionando resultados satisfatórios. Com base nos espectros de quimioluminescência e no experimento de aprisionamento de radicais livres realizado neste trabalho, um possível mecanismo de reação para esse sistema reacional é sugerido.

A novel sensitive chemiluminescence (CL) system ($[\text{Ag}(\text{HIO}_6)_2]^{5-}$ -luminol reaction system), coupled with a flow injection, is introduced for determination of *d*-penicillamine. The system is based on the enhancement effect of chemiluminescence reaction by penicillamine in alkaline medium. Under the optimum conditions, the CL intensity is proportional to the concentrations of *d*-penicillamine with a good linearity. The linear range covers 1.5-150 ng mL⁻¹ and the limit of detection of the method is 0.8 ng mL⁻¹. The relative standard deviation is 0.23% for 11 repeated measurements of 0.06 µg mL⁻¹ *d*-penicillamine. The method has been successfully applied to the determinations of *d*-penicillamine in pharmaceutical preparations and urine samples, giving rise to satisfactory results. Based on the chemiluminescent spectra and free radical trapping experiment performed in this work, a possible reaction mechanism for this reaction system is suggested.

Keywords: Ag(III)-luminol, chemiluminescence, *d*-penicillamine, kinetic characteristic

Introduction

Penicillamine (2-amino-3-mercapto-3-methylbutanoic acid) (PA) is an unphysiological sulfur-containing amino acid that belongs to the aminothiols family. It is a product of hydrolytic degradation of penicillin, and has no antibiotic properties.¹ Of the two enantiomeric forms, *d*-penicillamine (*d*-PA) has found many therapeutic applications, such as in treatments of scleroderma (a genetic disease), Wilson's disease, and as well as rheumatoid arthritis. It is also helpful in treating another rare inherited disease, cystinuria. Severe adverse reactions to the oral uses of this drug have been

described in subjects in which abrupt incremental dosing of the drugs is started. This suggests that monitoring the concentrations of this drug in biological fluids over the course of therapy is in high demand.²

Various methods have been developed for the determination of *d*-PA in biological specimens and pharmaceutical formulations, including capillary electrophoresis with laser-induced fluorescence,³⁻⁵ online vapor-phase generation combined with Fourier transform infrared spectrometry,⁶ HPLC (with fluorescence,^{7,8} chemiluminescence,⁹ and UV detections,^{2,10,11} respectively), spectrophotometry,¹²⁻¹⁴ and electrochemical methods.^{15,16} The methods of capillary electrophoresis,³⁻⁵ and online vapor-phase generation,⁶ are high cost, time-consuming and need special training. The HPLC method with either UV or

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fluorescent detection can improve detecting selectivity; pre or post-column derivatizations and expensive derivatizing reagents are inevitably needed.

Chemiluminescence (CL) method, when compared with the methods mentioned above, has the advantages of better sensitivities, lower limits of detection and costs. So far, there seems only two reports on application of CL for determination of *d*-PA, which utilized tris(bipyridyl) ruthenium(II)-peroxydisulfate,¹⁷ and copper-luminol-hydrogen peroxide systems.¹⁸

Recently, we reported a new CL system based on the reaction of $[\text{Ag}(\text{HIO}_6)_2]^{5-}$ with luminol and the determinations of cortisol by using this CL system.¹⁹ Subsequently, we also found that *d*-PA could remarkably enhance the CL intensities of $[\text{Ag}(\text{HIO}_6)_2]^{5-}$ -luminol reaction in alkaline medium. In this work, we report the determinations of *d*-PA by this CL system coupled with a flow injection analysis (FIA), in pharmaceutical preparations and urine samples. The limit of detection of this measuring system is as low as 0.8 ng mL^{-1} for *d*-PA with a linear concentration range of $1.5 - 150 \text{ ng mL}^{-1}$. On the basis of the chemiluminescent spectra recorded in this work, we also propose a reaction mechanism.

Experimental

Reagents and solutions

Luminol and *d*-PA were purchased from Sigma-Aldrich. Tablets of *d*-PA were purchased from commercial sources. AgNO_3 , KIO_4 , $\text{K}_2\text{S}_2\text{O}_8$, KOH , and acrylonitrile were obtained either from Beijing Chemical Reagent Company (Beijing, China) or from Tianjin Chemical Reagent Company (Tianjin, China). All the above reagents are of analytical grade and used as received without further purification. A 0.020 mol L^{-1} luminol solution was prepared by dissolving 0.8860 g luminol in 7.0 mL 1.0 mol L^{-1} NaOH and then diluted with water to 250 mL . Bis(hydrogenperiodato)argentate(III) ($[\text{Ag}(\text{HIO}_6)_2]^{5-}$) was synthesized according to the procedure described previously.²⁰ Stock solutions of the Ag(III) complex, prepared from the solid state compound obtained, was used freshly and daily; their concentrations were determined spectrophotometrically at 362 nm by use of the molar absorptivity of $\epsilon = 1.26 \times 10^4 (\text{mol L}^{-1})^{-1}\text{cm}^{-1}$.²¹ All the stock solutions and standards were stored in a refrigerator at $4 \text{ }^\circ\text{C}$ and protected from light. Double-distilled water (referred to pure water thereafter) was used as carrier flow and for the preparation of solutions. The diluted working solutions were prepared and used freshly and daily.

Instruments

The flow-injection system (shown in Figure 1) consisted of two peristaltic pumps and a six-way injection valve. CL emissions were recorded with a flow-injection CL analyzer (IFFL-D) (Xi'an Remax Electronic Science-Tech Co. Ltd.) controlled by a personal computer. A 970CRT spectrofluorophotometer (Sanco Co., Shanghai, China) was used to record fluorescence spectra, and 300 nm was selected as excitation wavelength. The CL spectra of this system were also recorded with a 970CRT spectrofluorophotometer, whose excitation light output slit being set to zero.

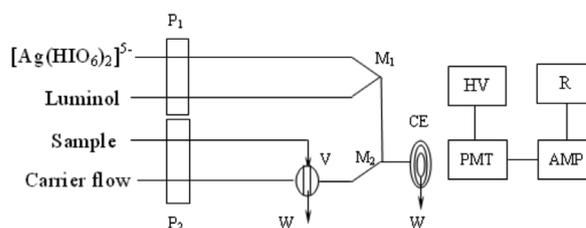


Figure 1. Schematic diagram of the FIA-CL system for the determination of penicillamine: P₁ and P₂, peristaltic pump; V, six-way injection valve; M₁ and M₂, manifold I and manifold; CE, CL reaction cell; W, waste; HV, high voltage source; PMT, photomultiplier tube; AMP, amplitude; R, recorder.

CL signal measurements

As shown in Figure 1, the four flow pathways were for carrier flow, Ag(III), luminol and sample solutions, respectively. Pure water was used as a carrier flow. The Ag(III) and luminol solutions, merged previously through a three-way pipe, passed into the flow cell and a base CL signal was created subsequently. Sample solutions were injected from a sample valve, and were then brought into the flow pathway by the carrier flow; subsequently, they were combined with the mixed solution of Ag(III) and luminol in manifold M₂ before reaching the detector. The enhanced CL signals, produced immediately, were recorded.

The flow-rates of the Ag(III) and luminol solutions were all set at 2.0 mL min^{-1} , while those of the sample and carrier were all set at 2.5 mL min^{-1} . A calibration graph was prepared by plotting the emission intensities *versus* the *d*-PA concentrations.

Results and Discussion

Effect of luminol concentration

CL intensities influenced by the luminol concentrations in $0.0050 \text{ mol L}^{-1}$ KOH medium had been collected and are

shown in Figure 2. Obviously, the CL intensities continue to increase with increasing the luminol concentrations. But the noises increased slightly as well. A graph was made by plotting relative CL intensity (S/N ratio) versus the luminol concentration. The best S/N ratio was at luminol concentration of $8.0 \times 10^{-8} \text{ mol L}^{-1}$ and was thus employed in our measurements.

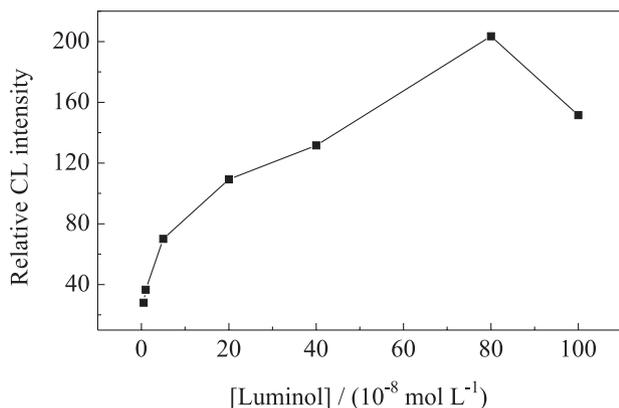


Figure 2. Effect of luminol concentration on the CL intensity. Conditions: $[d\text{-PA}] = 8.0 \times 10^{-8} \text{ g mL}^{-1}$; $[\text{Ag(III)}] = 1.0 \times 10^{-5} \text{ mol L}^{-1}$ (in 0.040 mol L^{-1} KOH solution). Luminol: in $0.0050 \text{ mol L}^{-1}$ KOH solution. Carrier flow: pure water.

Effect of the Ag(III) concentration

Preliminary experiment showed that the change of Ag(III) concentrations in 0.040 mol L^{-1} KOH medium could influence the CL emission intensities remarkably. The effect of the Ag(III) concentration in the range of 1.0×10^{-8} - $5.0 \times 10^{-5} \text{ mol L}^{-1}$ on the intensities are shown in Figure 3. From Figure 3, a concentration of $5.0 \times 10^{-6} \text{ mol L}^{-1}$ for the Ag(III) complex was chosen as the most suitable one for further studies. The CL intensities became lower at higher concentrations of Ag(III) solution, due probably to the absorption by the Ag(III) complex itself.

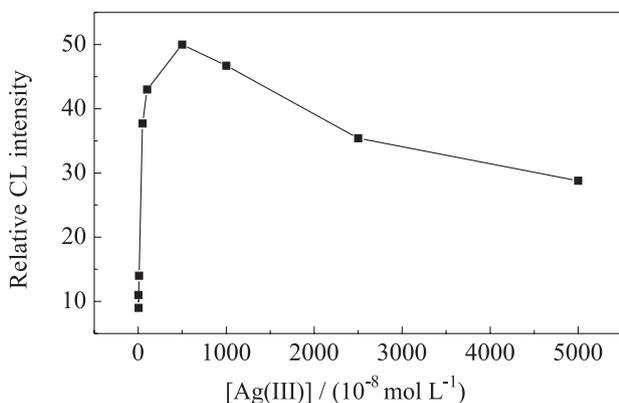


Figure 3. Effect of $[\text{Ag(III)}]$ concentration on the CL intensity. Conditions: $[d\text{-PA}] = 8.0 \times 10^{-8} \text{ g mL}^{-1}$; $[\text{Luminol}] = 8.0 \times 10^{-7} \text{ mol L}^{-1}$ (in $0.0050 \text{ mol L}^{-1}$ KOH solution); Ag(III): in 0.040 mol L^{-1} KOH solution.

Changing $[\text{OH}^-]$ in both Ag(III) and luminol solutions affects CL intensities

The hydroxide concentrations in both Ag(III) and luminol solutions could affect the CL intensities significantly. Plots of CL intensities versus $[\text{OH}^-]$ in $[\text{Ag}(\text{HIO}_6)_2]^{5-}$ and luminol solutions, expressed by Line a and b, respectively, are shown in Figure 4. After analysis of the influences, together with S/N ratios, $[\text{OH}^-] = 0.0050 \text{ mol L}^{-1}$ in luminol solution, and $[\text{OH}^-] = 0.080 \text{ mol L}^{-1}$ in Ag(III) solution were chosen for further studies.

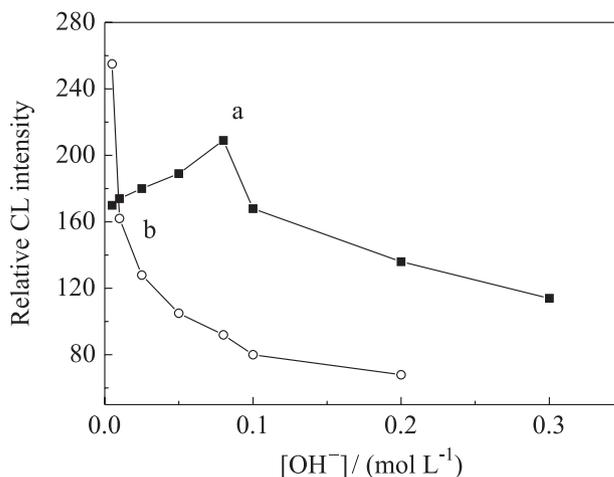


Figure 4. Effect of $[\text{OH}^-]$ in $[\text{Ag(III)}]$ and luminol solutions on the CL intensity. Line a: $[\text{OH}^-]$ in Ag(III) solution on CL intensity. Conditions: $[d\text{-PA}] = 8.0 \times 10^{-8} \text{ g mL}^{-1}$; $[\text{Ag(III)}] = 1.0 \times 10^{-5} \text{ mol L}^{-1}$; $[\text{Luminol}] = 2.0 \times 10^{-7} \text{ mol L}^{-1}$ (in $0.0050 \text{ mol L}^{-1}$ KOH solution). Line b: $[\text{OH}^-]$ in luminol solution on CL intensity. Conditions: $[d\text{-PA}] = 8.0 \times 10^{-8} \text{ g mL}^{-1}$; $[\text{Luminol}] = 2.0 \times 10^{-7} \text{ mol L}^{-1}$; $[\text{Ag(III)}] = 1.0 \times 10^{-5} \text{ mol L}^{-1}$ (in 0.040 mol L^{-1} KOH solution).

Interference study

The influence of various foreign species on the determination of $0.1 \mu\text{g mL}^{-1}$ *d*-PA was investigated. It was considered that a foreign substance would not interfere if the substance caused a relative error of $\leq \pm 5\%$. Results showed that the following substances had no inference: 100-fold starch, glucose, urea, Na^+ , K^+ , Cl^- , SO_4^{2-} , and NO_3^- , 50-fold lactose, and fructose, 2-fold EDTA, glycine, and glutamic acid. KI interfered seriously.

Analytical characteristics

Under the optimum conditions mentioned above, the CL analysis was performed by a standard addition method. The calibration plot of CL signal versus *d*-PA concentration was linear over the range of 1.5×10^{-9} - $1.5 \times 10^{-7} \text{ g mL}^{-1}$. The linear relationship can be expressed by: $I = -78.3 + 1.77 \times 10^{10}C$ with a correlation

Table 1. Determination results of *d*-PA in tablet and urine samples

Sample	Original concentration / (g mL ⁻¹)	Added / (g mL ⁻¹)	Found / (g mL ⁻¹)	Recovery / (%)	RSD / (%) n = 5
Penicillamine tablet	1.0 × 10 ⁻¹⁰	1.0 × 10 ⁻¹⁰	2.064 × 10 ⁻¹⁰	103.2	2.1
	1.0 × 10 ⁻¹⁰	1.0 × 10 ⁻⁹	1.119 × 10 ⁻⁹	101.7	1.9
	1.0 × 10 ⁻¹⁰	2.0 × 10 ⁻⁹	2.110 × 10 ⁻⁹	100.5	1.7
Urine	0	1.5 × 10 ⁻⁹	1.638 × 10 ⁻⁹	109.2	2.0
	0	5.0 × 10 ⁻⁹	5.275 × 10 ⁻⁹	105.5	1.9
	0	1.0 × 10 ⁻⁸	1.043 × 10 ⁻⁸	104.3	1.8

coefficient of 0.998, where I stands for CL intensities and C denote the *d*-PA concentrations (in g mL⁻¹). The limit of detection was 0.8 × 10⁻⁹ g mL⁻¹. The limit of detection was calculated as the concentration of *d*-PA required to yield a net peak which is three times the standard deviation of the background signal (3σ). The relative standard deviation was 0.23% for 11 repeated measurements of 0.06 μg mL⁻¹ *d*-PA.

Sample analysis

d-PA was determined in PA containing tablets and in human urine samples by the proposed method. No fewer than 20 tablets were weighed to obtain the mean mass *per* tablet. An accurately weighed portion of powder from each homogenized tablet, containing 125 mg *d*-PA, was dissolved in water in a small beaker. The solution was filtered, and the residue was washed several times with water. The filtrates were combined together, and were then transferred to a 100 mL volumetric flask and diluted to the mark with water. Working solutions were prepared by appropriate dilutions of this sample solution so that the final concentrations of *d*-PA were within the linear range.

To evaluate the validity of the proposed method for the determination of *d*-PA in human urine samples, the recovery was investigated by adding *d*-PA in human urines. No further pretreatment was required for urine samples when the urine samples were diluted 10⁵ multiples by water. The results are summarized in Table 1.

Kinetic characteristics of the CL reaction

The CL kinetic characteristic for the reaction of *d*-PA with luminol and Ag(III) solutions in a basic medium were investigated (Figure 5). It was showed that *d*-PA could enhance CL signals evidently when it was mixed with luminol and Ag(III) basic solutions. The rate of the reaction was very fast, only 3.8 s being needed from the Ag(III) droplet dropping into the mixture of luminol and sample solutions to the peak maximum.

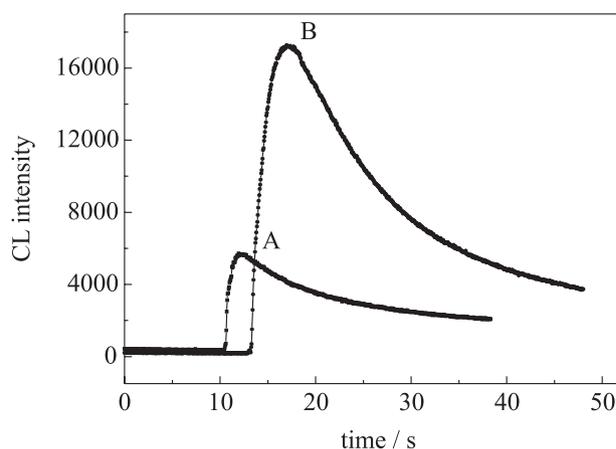


Figure 5. Characteristic kinetics of the CL reaction: (A) Luminol-[Ag(HIO₆)₂]⁵⁻ system; (B) *d*-PA-Luminol-[Ag(HIO₆)₂]⁵⁻ system. Conditions: [*d*-PA] = 1.0 × 10⁻⁶ g mL⁻¹. [Luminol] = 2.0 × 10⁻⁶ mol L⁻¹ (in 0.0050 mol L⁻¹ KOH solution); [Ag(III)] = 5.0 × 10⁻⁷ mol L⁻¹ (in 0.10 mol L⁻¹ KOH solution).

Fluorescent spectrum

In this system, [Ag(HIO₆)₂]⁵⁻ *viz.* Ag(III) can be reduced by penicillamine to Ag(I). Fluorescence spectra of (a) luminol, (b) *d*-PA, (c) luminol-Ag(III), (d) luminol-Ag(III)-*d*-PA, were obtained (λ_{ex} = 300 nm), and are displayed in Figure 6. Spectra for (a) and (b) showed no fluorescence while (c) and (d) showed that *d*-PA can enhance fluorescence intensity of luminol-Ag(III) reaction. The maximum fluorescence peak of (d) is near 435 nm which is the maximum emission wavelength of aminophthalate (a CL species from luminol). It appears that aminophthalate is perhaps the only fluorescent species in Ag(III)-luminol-*d*-PA system.

Chemiluminescence spectra

To investigate the mechanism of these CL reactions, the CL spectra of the Ag(III)-luminol reaction in the absence and the presence of *d*-PA were obtained using a Senco 970CRT spectrofluorophotometer with a flow-injection system whose excitation light output slit

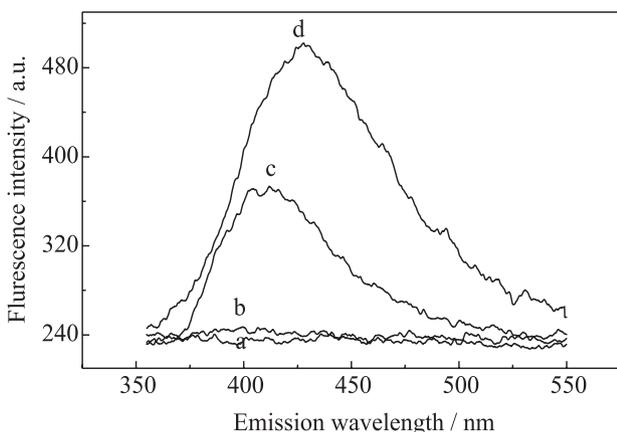


Figure 6. Fluorescence emission spectra of Luminol (a); *d*-PA (b); Luminol-[Ag(HIO₆)₂]⁵⁻ (c); *d*-PA-luminol-[Ag(HIO₆)₂]⁵⁻ (d); $\lambda_{ex} = 300$ nm. Conditions: [*d*-PA] = 1.0×10^{-3} g mL⁻¹; [Luminol] = 8.0×10^{-5} mol L⁻¹ (in 0.0050 mol L⁻¹ KOH solution); [Ag(III)] = 5.0×10^{-4} mol L⁻¹ (in 0.040 mol L⁻¹ KOH solution).

being set at zero. The CL spectra showed the maximum wavelength of CL emission enhanced by *d*-PA was the same as the one unenhanced, and the relative CL intensity was higher when the *d*-PA was presented (Figure 7). The CL spectrum appears to be independent of *d*-PA, suggesting that the luminophor of luminol-Ag(III)-*d*-PA is still 3-aminophthalate.

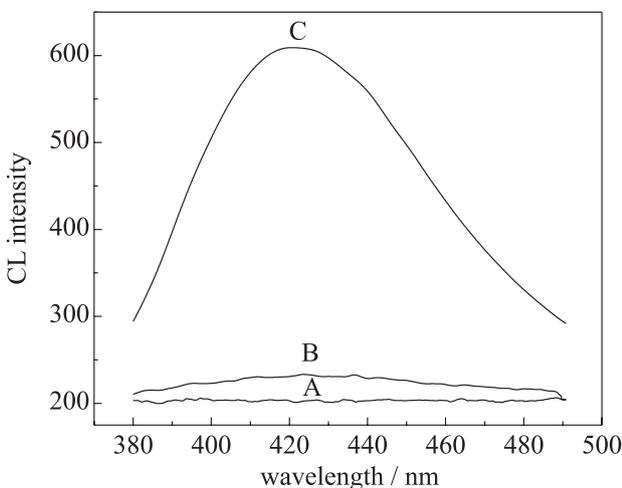


Figure 7. CL spectra for the luminol-[Ag(HIO₆)₂]⁵⁻ CL system: (A) *d*-PA; (B) luminol-[Ag(HIO₆)₂]⁵⁻; (C) *d*-PA-luminol-[Ag(HIO₆)₂]⁵⁻. Conditions: [*d*-PA] = 1.0×10^{-3} g mL⁻¹; [Luminol] = 8.0×10^{-5} mol L⁻¹ (in 0.0050 mol L⁻¹ KOH solution); [Ag(III)] = 5.0×10^{-4} mol L⁻¹ (in 0.040 mol L⁻¹ KOH solution).

Free radical trapping experiment

Subsequently, a free radical trapping experiment was undertaken. Under the same optimal conditions used for the CL measurements, a 50 mL Ag(III) (2.5×10^{-5} mol L⁻¹)

alkaline solution was added drop-wisely to a 50 mL solution of luminol and *d*-PA (5.0×10^{-7} mol L⁻¹, respectively) that both contained 8% acrylonitrile. The solution of luminol/PA was flushed for 30 min with nitrogen gas before Ag(III) solution was added. The reaction mixture was stirred at 40 °C for about 3 h under nitrogen gas. Both the luminol and *d*-PA solutions produced precipitates of polyacrylonitrile. This observation implied that free radicals were most likely involved in the reactions of luminol and *d*-PA with Ag(III).

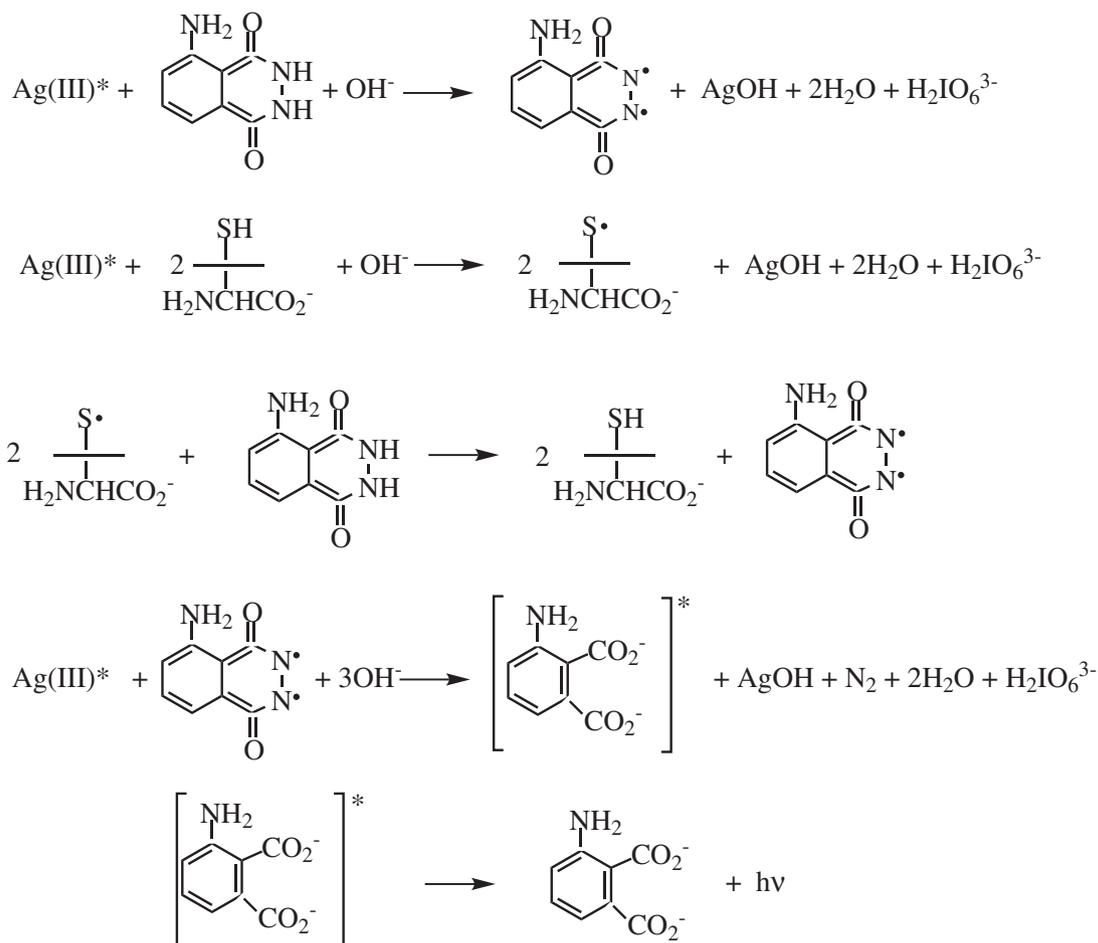
Possible CL reaction mechanism

The free radical trapping experiment shows that free radicals are likely present during the reactions of luminol and *d*-PA with Ag(III). Perhaps thiol group in *d*-PA is very easy to be oxidized, the reaction rate of *d*-PA with Ag(III) is faster than luminol with Ag(III), implied that free radicals of *d*-PA are transferred to luminol. Based on the experiments mentioned above, a CL mechanism is suggested as described in Scheme 1.

Luminol can be oxidized by some strong oxidants such as H₂O₂, K₃Fe(CN)₆, HIO₄, KMnO₄, and BrO⁻ to produce CL. So the CL systems based on the reactions of luminol have been widely used in the quantitative determinations of many inorganic and organic compounds.²²⁻²⁵ Commonly, the mechanism of luminol-H₂O₂ CL system is suggested due to the reaction of luminol radical with superoxide anion (O₂^{•-}).^{26,27} Metal / metal ion or some strong oxidizing species catalysts such as Ag, Au could efficiently catalyze the H₂O₂ decomposition to form superoxide anion.²⁸ During the H₂O₂ decomposition and superoxide anion reaction with luminol, excited aminophthalate are generated which emits photons upon relaxation to the ground state, that is essential for luminol CL system.^{29,30}

As for our Ag(III)-luminol-PA CL system, free radical trapping of this system implied that free radicals in the reaction of both luminol and PA with Ag(III) course are formation. The CL spectra, fluorescent spectra of these two reactions gave rise to a common absorption peak at about 435 nm, suggesting that all these CL reactions shared a common emitting species, presumably from the spectrum of aminophthalate (a CL species from luminol). This is also a free radical reaction.

The mechanisms of the reactions leading to enhancement or inhibition of luminol CL are still not fully understood.³¹ Thus, there is necessary to develop new CL reactions, enlarging the application scope and investigating the mechanisms of CL reactions.



Scheme 1. A reaction mechanism suggested for the CL reaction.

Conclusions

d-PA enhances the CL signals of luminol by reaction with $[\text{Ag}(\text{HIO}_6)_2]^{5-}$ in basic media. Based on this fact, a flow-injection chemiluminescence method for the determination of *d*-PA in pharmaceutical preparations and human urine samples has been developed. The proposed method is sensitive, fast, simple, and not require sophisticated reagents and equipments. This method has also been used for the determinations of *d*-PA in real pharmaceutical tablets and human urine samples with satisfactory results. And the limit of detection of method is lower than other methods reported previously.

Acknowledgments

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