Determination of doxorubicin in pharmaceutical preparation and rat plasma with luminol-K₃Fe(CN)₆ chemiluminescence system

Han-Chun Yao*, Er-Jun Xu, Wen-Yuan Zeng, Xiao-Ying Zeng, Min Zhang, Juan Chen

School of Pharmaceutical Science, Department of Pharmaceutical Analysis, Zhengzhou University, Zhengzhou, Henan 450001, PR China

Abstract

A novel and rapid flow injection chemiluminescence (FI-CL) method was established for the determination of doxorubicin, which was based on the phenomenon that CL intensity of the reaction between K₃Fe(CN)₆ and luminol in alkaline solution could be strongly enhanced by doxorubicin. Under optimum conditions, the relative CL intensity of the system was linear with the concentration of doxorubicin in the range from 2.0 × 10⁻⁹ g/mL to 5.0 × 10⁻⁷ g/mL with a correlation coefficient of 0.9993 (n = 9). The detection limit (3s) was 4.25 × 10⁻¹⁰ g/mL, and the relative standard deviation (RSD) for 1.0 × 10⁻⁷ g/mL doxorubicin (n = 11) was 0.33%. The method was applied to the determination of doxorubicin in pharmaceutical preparation and rat plasma, and the percentage recovery of doxorubicin in rat plasma was between 89.8% and 102.2%. The possible CL mechanism was also discussed briefly.

1. Introduction

Doxorubicin is a cytotoxic anthracycline antibiotic widely used in acute lymphocytic leukemia and various carcinomas [1]. However, the clinical use of doxorubicin is limited by a cumulative dose-dependent irreversible chronic cardiomyopathy, which can subsequently lead to congestive heart failure, with an ultimate mortality rate of 20–40% [2,3]. Therefore, for its potential pharmaceutical effects on health, the development of a simple, rapid, and sensitive method for the determination of doxorubicin in pharmaceutical preparations and biological fluids would be highly desirable.

Several analytical methods have been reported for the determination of doxorubicin in real samples, including high-performance liquid chromatography (HPLC) [4–7], ultraviolet-visible (UV-vis) spectrophotometry [8,9], and fluorescence detection [10–12]. However, these methods suffered from the disadvantages of low sensitivity and narrow linear range, or were time consuming. In recent years, the sensitive analytical technique based on chemiluminescence (CL) systems has received considerable attention with the characteristics of low detection limit, wide calibration range, and short analysis time. Although the CL method has been successfully applied to the analysis of pharmaceutical compounds and biological samples, the reports concerning the use of the CL...
The reported methods and the proposed method for the determination of doxorubicin were relatively few [13–15]. Ahmed et al. [15] reported a selective HPLC method for the determination of doxorubicin and its metabolite doxorubicinol in rat plasma. The method was based on the sensitization reaction followed by peroxyoxalate CL detection. A comparison of analytical figures of merit of the proposed method with previously reported methods is shown in Table 1[4,8,9,15].

In the current study, a new and sensitive CL method for the rapid determination of doxorubicin was developed, which was based on the enhanced effect exerted by doxorubicin on the luminol-K₃Fe(CN)₆ CL reaction. The proposed approach offered good accuracy and precision, which was successfully used for the determination of doxorubicin in the commercial preparation and biological fluid. The possible mechanism was also discussed briefly.

2. Methods

2.1. Apparatus and reagents

CL measurements were carried out by means of a flow injection CL analyzer, including a model IFMM-E flow injection system and a model IFFS-A luminometer (Xi’an, Ruimai Electronic Company, Xi’an, China). The CL spectra were acquired on a model RF-5301 spectrofluorometer (SHIMADZU Enterprise Management Co., Ltd., Shimadzu, Japan). UV-vis absorption spectra were acquired on a UV-2550 spectrometer (SHIMADZU Enterprise Management Co., Ltd., Shimadzu, Japan).

All the reagents were of analytical grade and all solutions were prepared in Milli-Q (The Millipore ultrapure water Co., Ltd., Millipore, U.S.A) ultrapure water. Doxorubicin was purchased from Dalian Meilun Biology Technology Company (Dalian, China). A 1.0 × 10⁻⁴ g/mL doxorubicin stock solution was prepared by dissolving 0.01000 g doxorubicin in water and diluting to 100 mL, and the working solution was diluted from the stock solution as required. A 1.0 × 10⁻³ M luminol solution was prepared by dissolving 0.17716 g luminol (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) in 100 mL 1.0M NaOH solution. The working solution of luminol was prepared by dissolving K₄Fe(CN)₆ in NaOH solution containing K₃Fe(CN)₆ solution. Stock solutions of 5.0 × 10⁻³M K₃Fe(CN)₆ and 0.2 M K₄Fe(CN)₆ were also prepared and diluted with water.

2.2. Procedures

As shown in Fig. 1, one peristaltic pump was used to carry K₃Fe(CN)₆ solution, and another to carry luminol solution, sample solution, and carrier stream. The pumps started to wash the whole flow system until a stable baseline was achieved. The flow rate was set at 1.5 mL/min for all lines. Luminol solution [with K₃Fe(CN)₆ in NaOH solution] and sample solution were pumped into the mixing element. The K₃Fe(CN)₆ solution was simultaneously injected into the carrier stream (water) through a six-way injection valve, and then merged into the mixed stream of luminol solution and sample solution. Finally, the mixtures reacted in the flow cell to generate the CL signal. The concentration of doxorubicin was quantified based upon the net CL intensity changes (ΔI_{CL}), ΔI_{CL} = I_{CL} - I_{BL}, where I_{CL} and I_{BL} are the CL intensities of the doxorubicin and blank solution, respectively.

2.3. Plasma sample preparation

Whole blood was collected from healthy Sprague-Dawley rats weighing 200–250 g (Experimental Animal Center of Henan Province) after injection via a tail vein. After the centrifugation at 3,500 rpm for 5 minutes, the supernatant was collected as plasma samples. In addition, 0.1 mL ethanol and 0.3 mL

---

**Table 1 – The reported methods and the proposed method for the determination of doxorubicin.**

<table>
<thead>
<tr>
<th>Methods</th>
<th>Linear range (g/mL)</th>
<th>Limit of detection (g/mL)</th>
<th>Sample matrix</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC</td>
<td>1.0 × 10⁻⁸–2.0 × 10⁻⁶</td>
<td>6.25 × 10⁻⁷</td>
<td>Human plasma sample</td>
<td>[4]</td>
</tr>
<tr>
<td>Visible spectrophotometry</td>
<td>4.0 × 10⁻⁷–4.0 × 10⁻⁶</td>
<td>3.4 × 10⁻⁸</td>
<td>Pharmaceutical preparations</td>
<td>[8]</td>
</tr>
<tr>
<td>CE</td>
<td>1.16 × 10⁻⁸–2.9 × 10⁻⁷</td>
<td>5.8 × 10⁻¹⁰</td>
<td>Human plasma sample</td>
<td>[9]</td>
</tr>
<tr>
<td>HPLC-PO-CL</td>
<td>1.16 × 10⁻⁹–5.8 × 10⁻⁷</td>
<td>2.61 × 10⁻¹⁵</td>
<td>Rat plasma sample</td>
<td>[15]</td>
</tr>
<tr>
<td>FI-CL</td>
<td>2.0 × 10⁻⁸–5.0 × 10⁻⁷</td>
<td>4.25 × 10⁻¹⁰</td>
<td>Pharmaceutical preparations and rat plasma sample</td>
<td>Proposed method</td>
</tr>
</tbody>
</table>

CE = capillary electrophoresis; FI-CL = flow injection chemiluminescence; HPLC = high-performance liquid chromatography; PO-CL = peroxyoxalate chemiluminescence.
dichloromethane are added to the plasma sample, and then vortexed for 1 minute. The mixture was centrifuged at 10,000 rpm for 10 minutes and the organic layer was transferred into the tube. The organic layer was left to evaporate to dryness under nitrogen. The residue was dissolved in water and diluted appropriately within the linear range of the determination.

3. Results and discussion

3.1. Kinetic characteristics of the reaction

The kinetic behavior of the CL reaction of luminol-K$_3$Fe(CN)$_6$-doxorubicin was studied by a static method. As shown in Fig. 2, when $3.0 \times 10^{-5}$M K$_3$Fe(CN)$_6$ solution was injected into luminol solution, a CL reaction was initiated immediately. Subsequently, a stronger CL signal was observed when K$_3$Fe(CN)$_6$ solution was injected into the mixed solution of luminol and doxorubicin. These findings indicated that the doxorubicin enhanced the CL intensity dramatically, and the rate of the reaction was so fast that the CL intensity reached the maximum value within 2.5 seconds.

3.2. Optimization of CL reactions

3.2.1. Single factor experiment

In order to optimize conditions for the determination of doxorubicin, various parameters, including the concentrations of luminol, NaOH, K$_3$Fe(CN)$_6$, and K$_4$Fe(CN)$_6$, were investigated first using a univariate approach. The studied range was selected based on the influence of variable on the CL signal, and the optimizing criterion was based on the maximum signal and good repeatability. All of these experiments were performed with a $1.0 \times 10^{-7}$ g/mL doxorubicin standard solution and a photomultiplier tube (PMT) voltage of 800 V.

The experimental results showed that the relative CL intensity reached the maximum value when the K$_3$Fe(CN)$_6$ concentration was $2.0 \times 10^{-5}$ M. However, the CL signal increased rapidly with the increasing the concentration of NaOH and luminol under the selected range. Moreover, K$_4$Fe(CN)$_6$ can effectively decrease the blank signal and increase the stability of the CL reaction. Therefore, it is not possible to obtain the optimal concentrations of luminol, NaOH and K$_3$Fe(CN)$_6$ by the single factor test, and with the consideration of reproducibility and linearity, an orthogonal experiment was thus designed to obtain the optimum conditions.

3.2.2. Orthogonal experiment

According to the results of the single factor test, an orthogonal experiment (four factors and three levels) was designed and the studied factors were the concentrations of NaOH, luminol, K$_3$Fe(CN)$_6$, and K$_4$Fe(CN)$_6$. The influences of variables were studied by the range analysis. As shown in Table 2, K$_3$Fe(CN)$_6$ was the most important factor, following by NaOH, luminol, and K$_4$Fe(CN)$_6$.
and K₄Fe(CN)₆. Therefore, 3.0 × 10⁻⁵M K₃Fe(CN)₆, 0.3M of NaOH, 5.0 × 10⁻³M of luminal, and 0.004M K₄Fe(CN)₆ were the optimal combination of this study. Moreover, another single factor experiment was performed to verify the optimal conditions, which were obtained by the above orthogonal experiment. As shown in Fig. 3, the optimized conditions coincided with the results of the orthogonal experiment, and the reproducibility and linear relationship of the system were also satisfactory.

### 3.3. Analytical characteristics

Under the above optimum conditions, calibration graph over the range of 2.0 × 10⁻⁹ g/mL to 5.0 × 10⁻⁷ g/mL doxorubicin with a correlation coefficient of 0.9993 was obtained. The regression equation was

\[ I = 580.24 + 688.25C, \quad (C, \text{g/mL}) \]

The detection limit, as defined by the International Union of Pure and Applied Chemistry (IUPAC), was determined to be 4.25 × 10⁻¹⁰ g/mL, and the relative standard deviation (RSD) was 0.33%, which was obtained from a series of 11 standards each containing 1.0 × 10⁻⁷ g/mL of doxorubicin. The sample measurement frequency was calculated about 120 samples h⁻¹.

### 3.4. Interference

The influences of different metal ions and some excipients used in pharmaceutical preparations on the CL intensity were investigated by determining the CL emission of the solutions containing 1.0 × 10⁻⁷ g/mL doxorubicin and foreign species. The tolerance limit of the foreign species was taken as a relative error ±5% in the peak height. The results indicated that 1,000-fold CaCl₂, KCl, (NH₄)₂SO₄, lactose and glucose, 500-fold sodium citrate and sodium carbonate, 200-fold Mg²⁺ and...

### Table 3 – Results of determination of doxorubicin in injections.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Added (×10⁻⁷ g/mL)</th>
<th>Found¹ (×10⁻⁷ g/mL)</th>
<th>RSD (%)</th>
<th>Recovery (%)</th>
<th>Content (mg/injection)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00</td>
<td>1.08</td>
<td>0.3</td>
<td>103.8</td>
<td>10.8</td>
</tr>
<tr>
<td></td>
<td>0.80</td>
<td>1.91</td>
<td>1.4</td>
<td>104.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>2.12</td>
<td>0.3</td>
<td>95.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.20</td>
<td>2.23</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.00</td>
<td>1.08</td>
<td>0.7</td>
<td>101.3</td>
<td>10.8</td>
</tr>
<tr>
<td></td>
<td>0.80</td>
<td>1.89</td>
<td>0.6</td>
<td>96.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>2.04</td>
<td>0.8</td>
<td>99.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.20</td>
<td>2.27</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹ Average of five measurements [± relative standard deviation (RSD)].
3.5. Sample analysis

3.5.1. Determination of doxorubicin in injections

The proposed method for the determination of doxorubicin was applied to the commercial pharmaceutical formulation. Two different batch numbers of doxorubicin injections (Zhejiang Hisun Pharmaceutical Co. Ltd, Taizhou, China) were diluted appropriately with water prior to measurement, so that the concentration of doxorubicin was in its linear response range. To validate the developed method, the recovery of doxorubicin was examined by spiking known amounts of doxorubicin into the sample solutions. The results were listed in Table 3 with an acceptable recovery range of 95.8–104.0%.

3.5.2. Determination of doxorubicin in rat plasma

First, the extraction recovery was examined on plasma samples spiked with known amounts of standard doxorubicin. The results were listed in Table 4, with recoveries of 96.0–102.5%, indicating a suitable extraction method for doxorubicin in plasma.

The proposed method was then used for the determination of doxorubicin in rat plasma after injection at a dose of 5 mg/kg via a tail vein. Blood samples were obtained from the orbital vein right after injection of the drug. According to the procedure for the plasma samples treatment, the final samples were obtained and analyzed by the proposed method with the standard addition. The recoveries were obtained ranging from 89.8% to 102.2%, as shown in Table 5. It can be seen that the developed method can be easily performed and affords good precision and accuracy when applied to the plasma samples.

3.6. Possible reaction mechanism

The CL reaction system of luminol and K₃Fe(CN)₆ has been extensively studied. It was confirmed that the excited state of 3-aminophthalate, which was the oxidation product of luminol, was the emitter in this system and the maximum wavelength of CL emission spectra of the system was 425 nm [16]. In order to explore the possible mechanism of luminol-K₃Fe(CN)₆-doxorubicin system, the following experiments were performed. First, the CL emission spectra were examined on an RF-5301 model spectrofluorometer whose excitation lamp was shut off. As shown in Fig. 4, the maximum emission wavelength of the luminol-K₃Fe(CN)₆-doxorubicin was located at 425 nm, and doxorubicin just increased the relative CL intensity. Therefore, we deduced that doxorubicin did not change the mechanism of the CL reaction, the luminophor was still the excited state of 3-aminophthalate, and the addition of doxorubicin did not lead to the generation of a new luminophor for this system. Next, UV-vis absorption spectra of luminol, K₃Fe(CN)₆, and doxorubicin were measured, as depicted in Fig. 5. It can be seen that doxorubicin had two notable absorption peaks at about 233 nm and 253 nm (Fig. 5C), and luminol had two distinct absorption peaks at 296 nm and 346 nm (Fig. 5B). The addition of doxorubicin to luminol revealed that no complex formed between the species and the absorption spectrum of the mixture system was approximately the sum of these two individual spectra (Fig. 5A). However, the absorption of K₃Fe(CN)₆ decreased after doxorubicin was added into it, which implied that the

<table>
<thead>
<tr>
<th>Sample</th>
<th>Added (×10⁻⁶ g/mL)</th>
<th>Found (×10⁻⁶ g/mL)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.50</td>
<td>5.00</td>
<td>7.99</td>
<td>89.8</td>
</tr>
<tr>
<td>2</td>
<td>3.22</td>
<td>5.00</td>
<td>8.33</td>
<td>102.2</td>
</tr>
<tr>
<td>3</td>
<td>3.78</td>
<td>5.00</td>
<td>8.57</td>
<td>95.8</td>
</tr>
</tbody>
</table>

* Average of five measurements [± relative standard deviation (RSD)].
reaction occurred between doxorubicin and K₃Fe(CN)₆ (Fig. 5E).

Several papers have reported that polyphenol can be oxidized to phenoxy radicals and produce energy that induces the transition of phenoxy radicals from its ground state to an excited electronic state [17–20]. Doxorubicin, an anthracycline glycoside antibiotic that contains phenolic hydroxyl, can be easily oxidized to its excited state by strong oxidant. Therefore, according to the aforementioned experiments and discussion, the possible enhancement mechanism of doxorubicin in the system may be concluded as follows (Scheme 1): doxorubicin can be oxidized and produce energy that induces the transition of oxidized doxorubicin from its ground state to an excited electronic state. When the excited oxidized doxorubicin molecule returns to the ground state, it could transfer energy to the ground state of 3-aminophthalate ions, and form more excited 3-aminophthalate ions, which return to the ground state with enhanced CL phenomena.

4. Conclusion

A rapid and simple flow injection chemiluminescence technique for the determination of doxorubicin with luminol and K₃Fe(CN)₆ system was established for the first time. Compared with other methods, the current work had a high sensitivity, good precision, convenience, and wide linear range. It has been successfully applied to the determination of doxorubicin in pharmaceutical preparation and rat plasma, and was potentially useful for analysis of doxorubicin in more complicated biological samples.

REFERENCES

[16] Yang D, Li HY, Li ZY, et al. Determination of rutin by flow injection chemiluminescence method using the reaction of
luminol and potassium hexacyanoferrate (III) with the aid of response surface methodology. Luminescence 2010;25:436–44.


