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# Flow injection-chemiluminescence determination of puerarin in pharmaceutical preparations

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**Abstract**

Strong chemiluminescence was observed when cerium(IV) reacted with rhodamine 6G in sulfuric acid medium in the presence of puerarin. This phenomenon has been utilized to design a sensitive and selective flow injection-chemiluminescence method for the determination of puerarin. Under the optimum conditions, the proposed procedure has a linear range between $1.3 \times 10^{-9}$ and $8.0 \times 10^{-7}$ g/mL, with a detection limit of $8.4 \times 10^{-10}$ g/mL puerarin and a relative standard deviation of 1.86% ($n$ = 11) at $5.0 \times 10^{-8}$ g/mL puerarin. The method was successfully applied to the determination of puerarin in pharmaceutical preparations. The mechanism of this chemiluminescence reaction has been proposed.


*Keywords:* Flow injection; Chemiluminescence (CL); Cerium(IV); Rhodamine 6G; Puerarin

## 1. Introduction

Puerarin (4′,7-dihydroxy-8-β-D-glucosylisoflavone), whose chemical structure is shown in Fig. 1, is a C-glycoside compound. It is present in the large amount of the active components of *Puerariae radix*, a commonly used Chinese herb, which exerts sedative and antipyretic actions and is often used to treat influenza, wrist stiffness and headache [1]. A number of investigations were carried out internationally to identify the physiological activities of puerarin such as antiproliferative effects on human cancer cell lines [2], inhibiting alcohol dehydrogenase [3], improvement of blood circulation, prevention of cardiovascular diseases [4,5], treatment for arrhythmia [6], and inhibiting xanthine oxidase [7]. Recent researches demonstrate that puerarin is an effective antioxidant and shows effects against glutamate excitotoxicity on cultured mouse cerebral cortical neurons [8,9]. Moreover, the latest studies indicate that puerarin has numerous biological roles, including antihyperglycemic effect [10], hepatoprotective activity relating to the inhibition of beta-glucuronidase [11], and estrogenic effect [12]. Because puerarin has been prepared in the form of commercially available pharmaceutical preparations that are consumed by the public, it is very important to develop a sensitive and rapid method for the determination of puerarin. Most reported methods for the analysis of puerarin were based on high-performance liquid chromatography (HPLC) [13–20], high-speed counter-current chromatography (HSCCC) [21], capillary electrophoresis (CE) [22,23] or micellar electrokinetic chromatography (MEKC) [24,25] separation with UV, MS, and electrochemical detection, which required a preconcentration process and consume much more analytical time. In addition, ultraviolet (UV) spectrophotometry [26–28], near infrared (NIR) spectral technology [29] and thin layer chromatographic (TLC) internal standard method [30] were reported to use for the determination of puerarin. However, these methods suffered from relatively lower sensitivity.

Because of low detection limit, wide linear range, high analytical frequency, simple and inexpensive instrumentation, analytical procedure applying chemiluminescence (CL)

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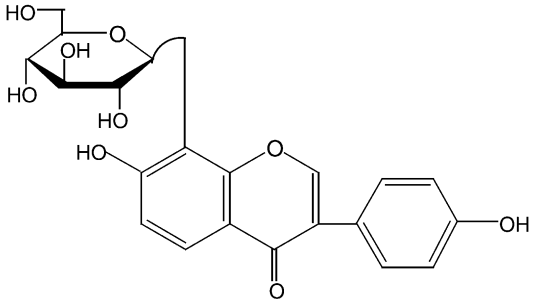


Fig. 1. Structural formula of puerarin.

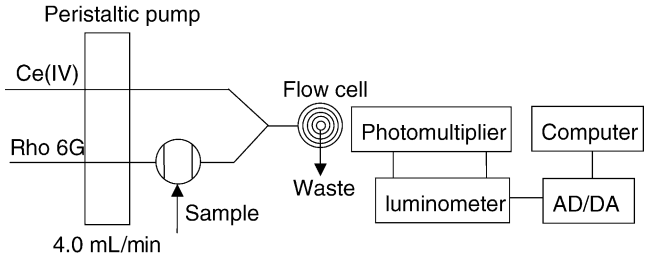


Fig. 2. Schematic diagram of the FI-CL system used for the determination of puerarin.

coupled with flow injection analysis (FIA) has extensively been applied in the different fields of analytical chemistry, including the determination of many pharmaceutical compounds [31–39]. Our preliminary experiments showed that weak CL emission could be produced when cerium(IV) reacted with rhodamine 6G in sulfuric acid medium, and the CL intensity was strongly enhanced by puerarin. This phenomenon allowed us to develop a sensitive method for the determination of puerarin in pharmaceutical preparations by using a simple flow injection system. To the best of our knowledge, this is the first time that a CL method has been applied to the determination of puerarin.

## 2. Experimental

### 2.1. Reagents

All chemicals were of analytical grade and were used without further purification. Redistilled water was used throughout. $Ce(SO_4)_2{\cdot}4H_2O$ was obtained from Shanghai Yaolong Metal Company (Shanghai, China) and prepared in sulfuric acid solution daily. Rhodamine 6G was obtained from Merck (Darmstadt, Germany). Puerarin was obtained from Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). A stock solution of 0.02 mol/L rhodamine 6G was prepared by dissolving 0.479 g rhodamine 6G in 50 mL redistilled water. A stock solution of $1.0 \times 10^{-4}$ g/mL puerarin was prepared with 50% ethanol and standard solutions by diluting with redistilled water. The solutions of puerarin were stored in brown bottles and kept at 0–4 °C.

### 2.2. Instruments

CL measurements were made using a lab-built flow injection CL analyzer, including a model FIA-2400 flow injection (FI) system (Xintong Scientific Instrument Company, China) and a model 1P-21 photomultiplier tube (Binsong Electronic Company, China), which was amplified and quantified by a model GD-1 luminometer (Ruike Electronic Co. Ltd., China) biased at −850 V. The flow cell was a flat glass coil placed in front of photomultiplier tube. An IBM compatible personal computer was used for data acquisition. The CL and fluorescent spectra were measured by using a model RF-5301 fluorimeter (Shimadzu, Japan). The UV–vis spectra were conducted on a model UV-2401PC spectrophotometer (Shimadzu, Japan).

### 2.3. General procedure

A schematic diagram of the FI-CL system is shown in Fig. 2. The solutions of $3.0 \times 10^{-3}$ mol/L cerium(IV) containing 0.8 mol/L $H_2SO_4$ and $5.0 \times 10^{-6}$ mol/L rhodamine 6G were pumped continuously at a rate of 4.0 mL/min into the flow cell. The sample solution was introduced using a 100 μL loop valve injector. The light output from the flow cell was detected by the photomultiplier tube. The full CL intensity versus time curve was recorded. The concentration of puerarin was determined by measuring the enhanced CL intensity according to:

$$\Delta I = I_S - I_0$$

where $I_S$ and $I_0$ are the CL signals in the presence and absence of puerarin, respectively.

For the UV spectrophotometry determination, working standard solutions of puerarin were prepared by diluting stocking solution at the concentration of $1.0 \times 10^{-6}$, $3.0 \times 10^{-6}$, $5.0 \times 10^{-6}$, $7.0 \times 10^{-6}$, $9.0 \times 10^{-6}$ g/mL with redistilled water. The absorbance at the wavelength of 250 nm of standard and appropriate sample solutions was measured on the UV-2401PC spectrophotometer.

### 2.4. Sample preparation

The injection of puerarin was purchased locally, which was manufactured by Beijing Union Pharmaceutical Factory. The injection is the solution of puerarin dissolved in 0.9% NaCl with a concentration of puerarin 0.1 g: 2 mL. A total of 2 mL of the puerarin injection solution was diluted appropriately with redistilled water and the final sample concentration was in the working range.

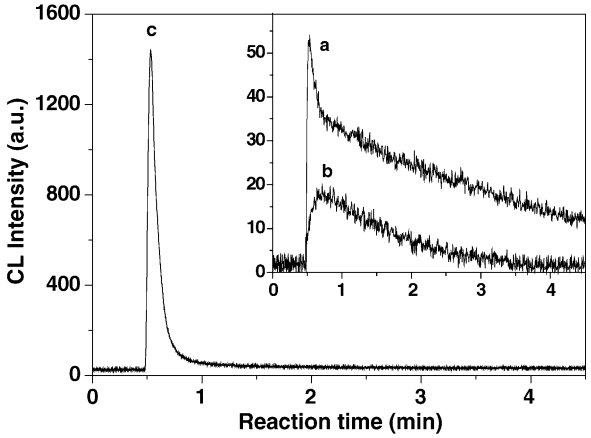


Fig. 3. Kinetic profile of the CL reaction. Conditions: (a) Ce(IV) + Rho 6G; (b) Ce(IV) + puerarin; (c) Ce(IV) + Rho 6G + puerarin. Ce(IV): $1.7 \times 10^{-3}$ mol/L, Rho 6G: $1.4 \times 10^{-5}$ mol/L, puerarin: $3.3 \times 10^{-5}$ g/mL.

## 3. Results and discussion

### 3.1. Kinetic profile of the CL reaction

The CL intensity with respect to time, as shown in Fig. 3, was investigated with a static system. For the oxidation reaction between cerium(IV) and rhodamine 6G, weak CL was observed, and then the signal decreased slowly (Fig. 3a). When cerium(IV) and puerarin were mixed, a weak light emission was also obtained, and the maximal peak was obtained at 10 s after mixing two reagents (Fig. 3b). However, a strong enhancement of the CL emission of cerium(IV)–rhodamine 6G reaction was observed in the presence of puerarin (Fig. 3c).

### 3.2. Optimization of the CL reaction

The effects of various oxidants, including $H_2O_2$, $K_3Fe(CN)_6$ in sodium hydroxide medium and $KMnO_4$, cerium(IV) in sulfuric acid medium on CL intensity were studied. The CL was obtained when $KMnO_4$ or cerium(IV) was used as an oxidant in sulfuric acid medium. However, the CL emission produced by $KMnO_4$ oxidation was very weak. Therefore, cerium(IV) was selected as an oxidant in the coming work.

A series of experiments were conducted to establish the optimum reaction conditions for the cerium(IV)–rhodamine 6G-puerarin CL system.

#### 3.2.1. Effect of cerium(IV) concentration

The effect of cerium(IV) concentration upon the CL intensity was examined in the range $8.0 \times 10^{-4}$ to $1.0 \times 10^{-2}$ mol/L. It was found that the CL emission increased until $3.0 \times 10^{-3}$ mol/L cerium(IV) and then sharply decreased, as shown in Fig. 4a. Lower concentrations of oxidant produced lower CL emission, whereas the CL emission decreased with higher cerium(IV) concentrations. Because of the increased collisional energy transfer between molecules

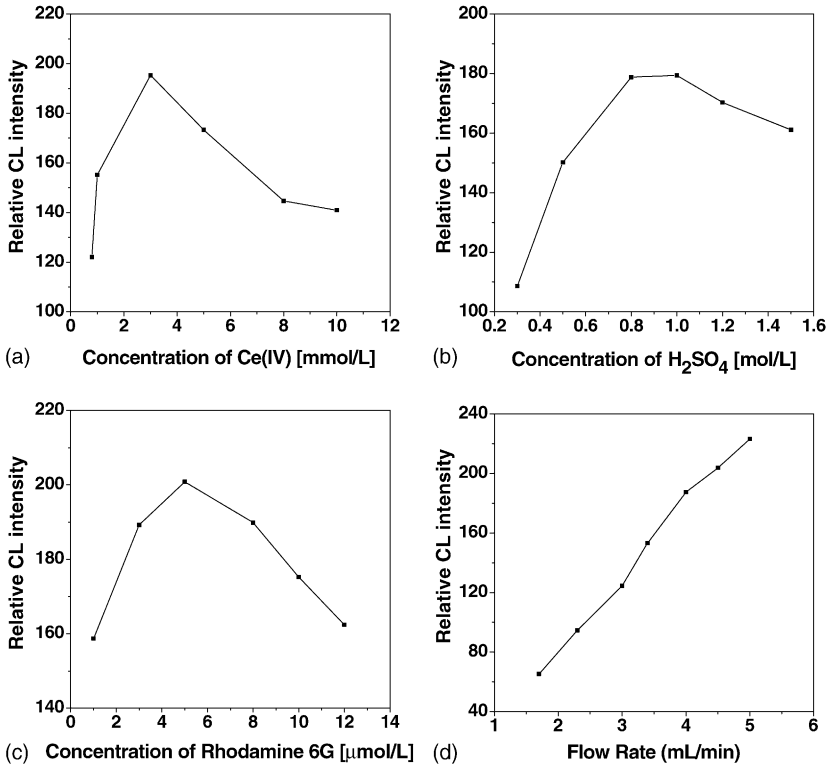


Fig. 4. Effects of the concentration of (a) cerium(IV); (b) $H_2SO_4$; (c) rhodamine 6G and (d) flow rate on the CL intensity.

Table 1
Maximum ratio of some interfering species (≤5.0% error)

| Species added | Maximum tolerable concentration ratio |
|---|---|
| Glucose, sorbose | 2500 |
| $Na^+$, $K^+$, $Cl^-$, ethanol, methanol | 2000 |
| EDTA | 1500 |
| $H_2PO_4^-$ | 1000 |
| $Mg^{2+}$ | 200 |
| $Zn^{2+}$ | 150 |
| $CH_3COO^-$ | 80 |

caused by higher cerium(IV) concentration, some excited-state rhodamine 6G molecules return to the ground state by a non-radiative internal transfer process, which would decrease the chemiluminescent quantum yield. Therefore, $3.0 \times 10^{-3}$ mol/L cerium(IV) was used for the further work.

### 3.2.2. Effect of $H_2SO_4$ concentration

The effect of the concentration of sulfuric acid used for preparing cerium(IV) solution was studied in the range 0.3–1.5 mol/L, as shown in Fig. 4b. It was found that the plateau of CL intensity was reached in the range 0.8–1.0 mol/L and then CL intensity decreased with increasing sulfuric acid concentration. Thus, 0.8 mol/L was then chosen to prepare and dilute the cerium(IV) solution.

### 3.2.3. Effect of rhodamine concentration

The effect of the concentration of rhodamine 6G on the CL intensity was examined over the range $1.0 \times 10^{-6}$ to $1.2 \times 10^{-5}$ mol/L. The results in Fig. 4c show that $5.0 \times 10^{-6}$ mol/L rhodamine 6G provides maximum CL intensity. Lower concentrations of rhodamine 6G gave the lower CL emission, and higher concentrations caused the decreased CL emission, which may be due to that some excited-state rhodamine 6G molecules return to the ground state by a non-radiative internal transfer process, which would decrease the chemiluminescent quantum yield because of the increased collisional energy transfer between molecules caused by higher rhodamine 6G concentration.

### 3.2.4. Effect of flow rate

The flow rates of solutions are very important to the CL reaction and should be regulated. At the flow rates that are too slow or too high, CL is not emitted in the flow cell and hence the emitter cannot be detected. Under the above selected conditions, the effect of flow rate on the CL intensity of puerarin was studied over the range 1.7–5.0 mL/min in each stream, as shown in Fig. 4d. It was found that CL intensity increased with increasing flow rate, indicating a fast dynamic process of this reaction. As a compromise between reagent consumption and CL intensity, 4.0 mL/min of flow rate was recommended.

## 3.3. Calibration

Under the optimum conditions described previously, a log–log calibration graph was linear over the range of $1.3 \times 10^{-9}$ to $8.0 \times 10^{-7}$ g/mL of puerarin solutions with the equation $y = 0.9207x + 8.7457$ ($r = 0.9992$, $n = 11$), where $y$ is the log (peak height), and $x$ the log (concentration). The relative standard deviation (R.S.D.) for $5.0 \times 10^{-8}$ g/mL puerarin measurement was 1.86% ($n = 11$). The detection limit, defined as three times the S.D. for the reagent blank signal, was $8.4 \times 10^{-10}$ g/mL puerarin. The sample solutions can be analyzed at a rate of 120 samples $h^{-1}$.

## 3.4. Interference studies

In order to assess the possible analytical applications of the described CL method, the effect of concomitant species on the determination of puerarin in real samples was studied by analyzing synthetic sample solutions containing $5 \times 10^{-8}$ g/mL puerarin and various excess amounts of some common additives used in the preparation of pharmaceutical formulations. A substance was considered not to interfere when the variation in puerarin peak height was less than ≤5.0%. The results are shown in Table 1.

## 3.5. Application

In order to evaluate the validity of the proposed method for the determination of puerarin in pharmaceutical preparations, the recovery test was carried out by adding known amounts of puerarin standard to puerarin injection solutions. As shown in Table 2, the recoveries were 102.5 and 106.8%, indicating that the method is reliable for the quantitation of puerarin in pharmaceutical preparations. The reliability of the proposed method was also evaluated by comparing the results with those obtained from the UV spectrophotometry method [26]. No significant differences were observed between the both methods.

Table 2
Results of the determination of puerarin in injection samples by FI-CL and UV method[a]

| Sample | Claimed | Amount (mg) | | Added (mg) | Recovered (mg) | Recovery (%) |
|---|---|---|---|---|---|---|
| | | CL method, found ± S.D. | UV method, found ± S.D. | | | |
| 030406[b] | 100.0 | 100.6 ± 0.53 | 99.2 ± 0.38 | 100.0 | 203.1 ± 0.87 | 102.5 ± 0.41 |
| 030713[b] | 100.0 | 101.1 ± 0.46 | 99.5 ± 0.44 | 100.0 | 207.9 ± 0.92 | 106.8 ± 0.35 |

[a] Mean values ($n = 5$).
[b] Lot number.

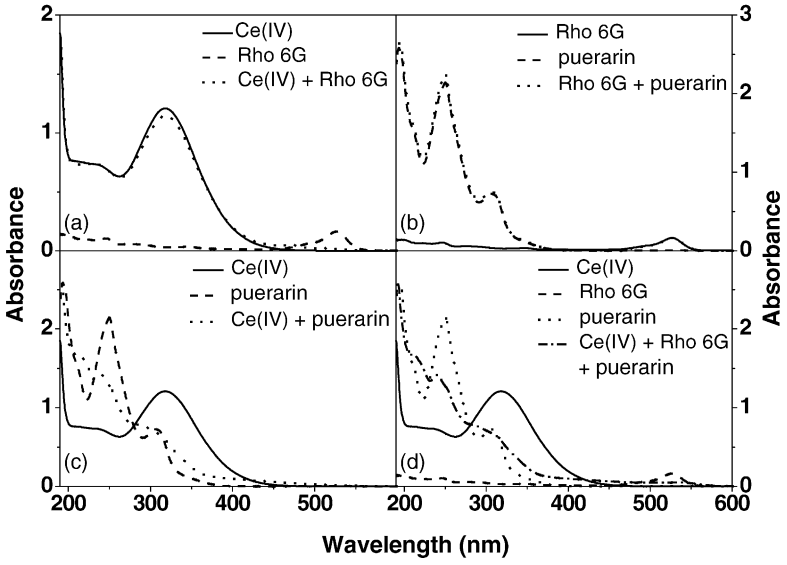


Fig. 5. Absorption spectra of the cerium(IV)–rhodamine 6G-puerarin reaction. Reference solution, water. Conditions: (a) Ce(IV) + Rho 6G; (b) Rho 6G + puerarin; (c) Ce(IV) + puerarin; (d) Ce(IV) + Rho 6G + puerarin. Ce(IV): $3.0 \times 10^{-4}$ mol/L, Rho 6G: $2.0 \times 10^{-6}$ mol/L, puerarin: $3.0 \times 10^{-5}$ g/mL.

### 3.6. CL mechanism

It was found that the reaction of cerium(IV) with rhodamine 6G in sulfuric acid medium could produce weak CL, which was greatly enhanced by puerarin. The CL spectra of the cerium(IV)–rhodamine 6G reaction in the absence and presence of puerarin were measured. Both CL spectra were almost identical with a maximum wavelength at about 556 nm, which was in agreement with that of the fluorescent spectrum of rhodamine 6G. Thus, the luminophor could be ascribed to rhodamine 6G.

The UV–vis absorption spectra of the cerium(IV)–rhodamine 6G-puerarin CL reaction, as shown in Fig. 5, were recorded. Fig. 5a indicates that the absorption of rhodamine 6G at 525 nm decreases after the addition of cerium(IV), and a new peak at 471 nm emerges, which suggests that rhodamine 6G was oxidized by cerium(IV) to form a new compound. Similarly, the 250 nm peak of puerarin decreases in the presence of cerium(IV) (Fig. 5c). Fig. 5b shows that the absorption spectrum of the mixing solution of rhodamine 6G and puerarin is exactly the addition of the spectrum of rhodamine 6G and puerarin, thus there is no reaction between rhodamine 6G and puerarin. Fig. 5d demonstrates that rhodamine 6G and puerarin are oxidized by cerium(IV) in the cerium(IV)–rhodamine 6G-puerarin system. Moreover, the oxidized product of puerarin was found to be non-fluorescent. The 351 nm peak was observed in the fluorescent spectrum taken from the mixture of the cerium(IV), rhodamine 6G, and puerarin solution, which was affirmed to be the maximum wavelength of the fluorescent spectrum of cerium(III) [40]. It was demonstrated that cerium(IV) was reduced to cerium(III). Based on the above discussion, the mechanism of the cerium(IV)–rhodamine 6G-puerarin CL reaction can be explained as shown in Scheme 1. Rhodamine 6G and puerarin are oxidized by cerium(IV) in sulfuric acid medium to form the excited-state cerium(III). The reaction rate between cerium(IV) and puerarin is faster than that of cerium(IV) with rhodamine 6G. Thus, the presence of puerarin can accelerate the generation of the excited-state cerium(III), and then energy is transferred from cerium(III)* to rhodamine 6G to form the excited-state rhodamine 6G, which emits its characteristic radiation at 556 nm.


Scheme 1.

$$\mathrm{Ce(IV) + Rho\,6G \xrightarrow[H_2SO_4]{slow} Ce(III)^* + Rho\,6G_{OX}}$$

$$\mathrm{Ce(IV) + PU \xrightarrow[H_2SO_4]{fast} Ce(III)^* + PU_{OX}}$$

$$\mathrm{Ce(III)^* + Rho\,6G \rightarrow Ce(III) + Rho\,6G^*}$$

$$\mathrm{Rho\,6G^* \rightarrow Rho\,6G + h\nu}$$

where Rho 6G, Rho $6G_{OX}$, PU and $PU_{OX}$ are rhodamine 6G, the oxidized form of Rho 6G, puerarin and the oxidized form of PU; $h\nu$ is the CL emission.

## 4. Conclusion

A novel CL method has been established for the determination of puerarin, based on the enhancement by puerarin of the CL of cerium(IV)–rhodamine 6G reaction. It offers the advantages of simplicity, rapidity, high sensitivity and wide linear range and has a potential application in pharmaceutical preparations.

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