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Chemiluminescence Investigation of Detection of Rutin in Medicine and Human Urine Using Controlled-Reagent-Release Technology

Zhenghua Song* and Lin Wang

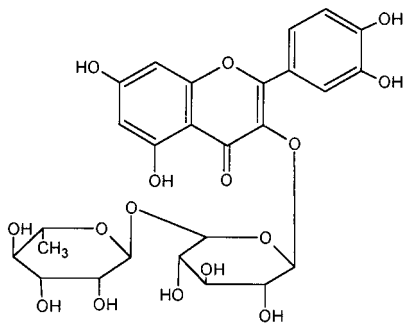
Department of Chemistry, Northwest University, Xi'an 710069, People's Republic of China

A novel continuous-flow sensor based on chemiluminescence (CL) detection was developed for the determination of rutin in pharmaceutical preparations and human urine by controlled-reagent-release technology. The analytical reagents involved in the CL reaction, including luminol and hexacyanoferrate(III), were both immobilized on an anion-exchange column in a flow-injection system. The CL signal produced by the reaction between luminol and hexacyanoferrate(III), which were eluted from the column through sodium phosphate injection, was decreased in the presence of rutin. CL intensity was inhibited by rutin; the decrement of CL intensity was linear over the logarithm of the rutin concentration range of 1.0−400 ng·mL^{-1}, and the detection limit was 0.35 ng·mL^{-1} (3σ). The whole process, including sampling and washing, could be completed in 1.5 min with a relative standard deviation of <3.5%. The flow sensor showed remarkable stability and could be easily reused >450 time; the sensor proposed was applied successfully to the determination of rutin in pharmaceutical preparations and human urine.

Keywords: *Rutin; flow injection; chemiluminescence; urine*

INTRODUCTION

Among all flavonoid derivatives that possess high pharmacological and physiological activities, rutin has



the most potent therapeutic action and is widely known as vitamin P. It was thought to be an activating factor for vitamin C, and its action is to preserve and protect capillaries, promote circulation, and lower cholesterol levels. It acts as an antioxidant and helps prevent vitamin C and adrenaline from being oxidized by copper-containing enzymes (*1*, *2*). Further studies indicated that orally administered rutin is absorbed into the bloodstream in the upper part of the small intestine. Excessive amounts are excreted in the urine (*3*).

All flavonoids are electroactive, easily subjected to either oxidation or reduction electrode reactions; hence, rutin can be determined by electrochemical means (*4*−*6*). Depending on its maximal absorption at 260 or 360 nm obtained by coloring reaction with some reagents, some spectrophotometric methods (*7*−*10*) had been carried out for the determination of rutin. Some solid extraction and chromatography methods (*11*−*14*) have been introduced due to rutin's low solubility in water. Also reported were some methods based on its fluorescent effect (*15*). However, these methods have relatively

* Author to whom correspondence should be addressed (e-mail songzhenghua@hotmail.com).

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complex devices, time-consuming procedures, or poor sensitivity.

Progress in flow-injection (FI) chemiluminescence (CL) analysis has received much attention in various fields for its high sensitivity, rapidity, and simplicity. He et al. reported an FI-CL method (*16*) for the detection of rutin, in which the CL reactants were directly introduced into CL cell and the limit of detection was 6.7 ng·mL^{-1}. However, a limitation of the application of this method is the need to prepare large quantities of analytical reagents and continuously deliver them into the reaction zones. This is undesirable not only for operational convenience and for the simplicity of the detection device but also for the cost, environment, and resource considerations (*17*).

An effective approach to solve this problem is to immobilize CL reagents on a solid phase. Such controlled-reagent-release technology has not been applied to the determination of rutin so far. In this work, a new CL method for the determination of rutin based on the inhibition of rutin in the CL reaction is presented. The CL reagents, luminol and $K_3Fe(CN)_6$, used in this sensor were both immobilized on Amberlyst A-27 (Rohm and Haas Co.) anion-exchange resins. Through injection of 200 μL of sodium phosphate, the reagents on the anion-exchange column were eluted, and in the presence of rutin, the CL intensity was decreased, by which rutin could be detected. The decreased CL intensity is linear over the logarithm of the rutin concentration range from 1.0 to 400 ng·mL^{-1} with a relative standard deviation (RSD) of <3.5%. The proposed method has been applied successfully for the determination of rutin in pharmaceutical preparations and human urine.

MATERIALS AND METHODS

Materials. All reagents used were of analytical reagent grade. Doubly distilled water was used throughout. NaOH, Na_3PO_4, and $K_3Fe(CN)_6$ were obtained from the Xi'an Chemical Agent plant. The stock standard solution of rutin (obtained from Shaanxi Institute for Drug Control) was dissolved to 0.2 mg·mL^{-1} with 80% methanol aqueous solution (v/v) and was stored at 4 °C in a brown flask of 50 mL. Working standards were prepared daily from the stock solution by appropriate dilution. Stock standard luminol solution, 2.5×10^{-2} mol·L^{-1}, was prepared by dissolving 4.40 g of luminol (Fluka, Biochemika) in 1 L of 0.5 mol·L^{-1} NaOH solution. Amberlyst A-27 anion-exchange resin (20+ to 50− mesh) purchased from Rohm and Haas Co. was used for immobilization of luminol and hexacyanoferrate(III).

Two preparations containing rutin with different batch numbers (990868 and 000327, respectively) were purchased on the local market. The labeled content of rutin for each is 25 mg per tablet. They were analyzed directly or administered to volunteers in the subsequent experiments.

Preparation of Immobilization Reagent Column. A 0.5 g of Amberlyst A-27 anion-exchange resin was stirred with 100 mL of 2.5×10^{-2} mol·L^{-1} luminol or 1.0×10^{-2} mol·L^{-1} $K_3Fe(CN)_6$ for 12 h, after which time the resin was filtered, washed with doubly distilled water, and dried before being stored. The most convenient method to determine the amounts of luminol and hexacyanoferrate(III) is to measure the concentration change of immobilization solutions. The concentration was detected at 360 nm for luminol and at 420 nm for hexacyanoferrate(III) by UV−vis spectrophotometry. In the proposed method, the amounts of luminol and hexacyanoferrate(III) immobilized were 1.9 and 1.0 mmol·g^{-1} resin, respectively.

To prepare a column with immobilized reagents, resins (0.18 g) containing immobilized luminol and hexacyanoferrate(III) were mixed and packed into a glass column with an i.d. of 5 mm a total volume of ~0.6 mL and furnished with glass wool at both ends to prevent loss of the resins.

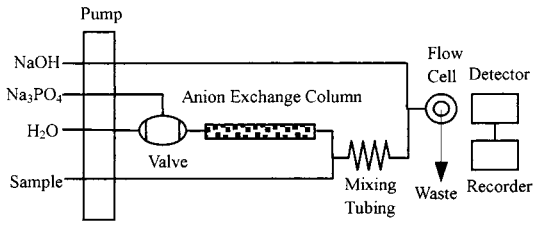

Figure 1. FI manifold for the determination of rutin.

Apparatus. The FI system used in this work is shown in Figure 1. A peristaltic pump (Shanghai Meter Electromotor plant, model ND-15, 15 rpm) was used to generate the flows. PTFE tubing (1 mm i.d.) was used in the flow system. Eluant solution of sodium phosphate (200 μL) was injected into the carrier stream by a six-way valve. The CL emission cell is a twisty glass tube (1 mm i.d., 15 cm length), which provides a large surface area exposed to the adjacent photomultiplier tube (PMT) (Hamamatsu, model IP28). Extreme precautions were taken to ensure that the sample compartment and PMT were light-tight. The CL detected without wavelength discrimination and the PMT output were amplified and quantified by a luminometer (Northwest Non-ferrous Geology Institute of China, model GD-1) connected to a recorder (Shanghai Dahua Instrument and Meter Plant, model XWT-206).

General Procedures. The carrier water and the solutions (NaOH, sample, and Na_3PO_4) were propelled at a constant flow rate on each flow line. The pump was started to wash the whole flow system until a stable baseline was recorded. Then 200 μL of eluant solution of sodium phosphate was injected into the carrier stream, and luminol and hexacyanoferrate(III) were eluted quantitatively, which were then mixed with the sample stream; the mixed solution was delivered to the CL cell, and the peak height of the CL signal was detected with the PMT and the luminometer. The PMT voltage used ranged from 700 to 750 V (to maximize the signal-to-noise ratio). The concentration of sample was quantified by decreased CL intensity, $\Delta I = I_0 - I_s$, where I_0 and I_s are CL signals in the absence and in the presence of rutin, respectively.

Determination of Rutin in Pharmaceutical Preparations. No fewer than 30 tablets were weighed, ground to a fine powder, and mixed. A sample equivalent to ~100 mg of powder was weighed accurately, transferred into a 250 mL calibrated flask, and made up to volume with water. According to the proposed method, the sample was then diluted to the concentration with the calibration range without pretreatment. As reference method (*18*), a 10 mL volume of previously filtered pharmaceutical preparations was diluted to 100 mL for the UV spectrophotometry.

Determination of Rutin in Human Urine. Three apparently healthy male volunteers were administered rutin tablets orally in the morning on an empty stomach. According to the marked content, the net dosage of rutin they took was 100 mg each. From then on, first-voided urine samples were collected in dark glass bottles at different times. In the whole procedure, all volunteers were prevented from eating. Urine was analyzed directly after dilution to 10^5 with water.

RESULTS AND DISCUSSION

Selection of Eluant. The immobilized luminol and hexacyanoferrate(III) can be eluted from the resin by various anions. In this system, the characteristics of several different salts including NaCl, NaAc, $NaNO_3$, $NaSO_4$, and Na_3PO_4 were evaluated. The results obtained clearly demonstrated that CL intensity was enhanced with the increase of the ion strength of the eluant injected as Table 1 shows. It was found that sodium phosphate was the best eluant with the highest

Table 1. Character of Eluants for Rutin Determinatins[a]

type of CL intensity[b]	relative CL intensity				
	NaCl	NaAc	$NaNO_3$	Na_2SO_4	Na_3PO_4
I	94	78	89	142	166
II	67	61	59	104	116
III	27	17	30	38	50

[a] The concentration of eluant was 1.0×10^{-4} mol·L^{-1}. [b] I, CL intensity in the absence of rutin; II, CL intensity in the presence of 50 ng·mL^{-1} rutin; III, decreased CL intensity.

Table 2. Effect of Molar Ratio between Immobilized Luminol and Hexacyanoferrate(III)[a]

molar ratio luminol/ hexacyanoferrate(III)	relative CL intensities		decreased CL intensity
	blank[b]	signal[c]	
3:1	161	141	20
2:1	143	105	38
1:1	129	101	28
1:2	101	74	27
1:3	95	70	25

[a] The concentration of each eluant was 1.0×10^{-4} mol·L^{-1}. [b] CL intensity in the absence of rutin. [c] CL intensity in the presence of 50 ng·mL^{-1} rutin.

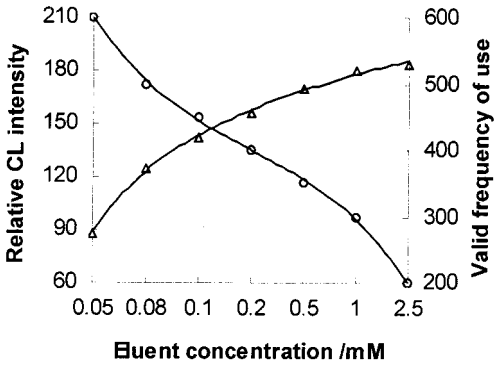

Figure 2. Effect of eluant concentration: (○) on valid frequency of use for the column; (△) on CL intensity.

CL intensity. Therefore, sodium phosphate was chosen for subsequent work.

Selection of Molar Ratio of the Immobilized Luminol and Hexacyanoferrate(III). To test the influence of the mixing ratio, resins (0.18 g) with different mixing ratios were packed into a glass column with an internal diameter of 5 mm and total volume of ~0.6 mL. With the injection of 200 μL of sodium phosphate (1.0×10^{-4} mol·L^{-1}), different amounts of luminol and hexacyanoferrate(III) were eluted from the resins and CL intensity proceeded as shown in Table 2. The best signal-to-noise ratio was found with a molar ratio of 2:1 of luminol to hexacyanoferrate(III).

Selection of Eluant Concentration. Various concentrations of sodium phosphate were injected through the anion-exchange column with immobilized luminol and hexacyanoferrate(III). The results obtained are shown in Figure 2. Valid frequency of use was defined as the actual injection times for a column using a certain concentration of eluant while the relative deviation of CL intensity is <5%. The figure clearly demonstrates that with the increase of eluant concentration the CL intensity increased but the frequency of use of the sensor decreased considerably. To obtain long lifetime and high CL intensity, 1.0×10^{-4} mol·L^{-1} sodium phosphate was chosen as a compromise in the following work.

Effect of NaOH Concentration. The CL reaction of luminol and hexacyanoferrate(III) proceeds under an alkaline condition. The effect of the NaOH concentration was examined, and the results are illustrated in Figure 3. The CL intensity and the decreased CL intensity varied significantly with NaOH concentration. To maximize the sensitivity, an NaOH concentration of 0.5 M was chosen as optimum.

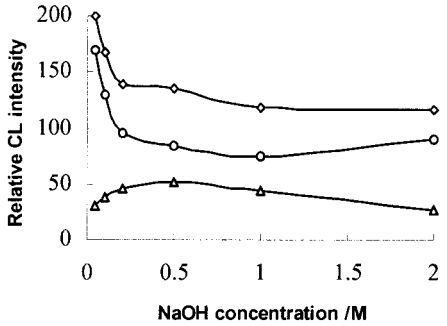

Figure 3. Effect of NaOH concentration with 50 ng·mL^{-1} rutin: (◇) luminol−hexacyanoferrate(III) system (I_0); (○) luminol−hexacyanoferrate(III)−rutin system (I_s); (△) decreased CL intensity ($\Delta I = I_0 - I_s$).

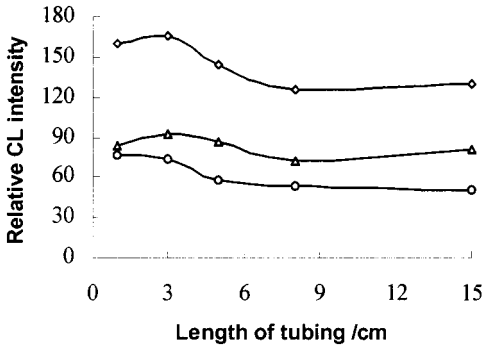

Figure 4. Effect of the length of the mixing tubing with 50 ng·mL^{-1} rutin: (◇) luminol−hexacyanoferrate(III) system (I_0); (○) luminol−hexacyanoferrate(III)−rutin system (I_s); (△) decreased CL intensity ($\Delta I = I_0 - I_s$).

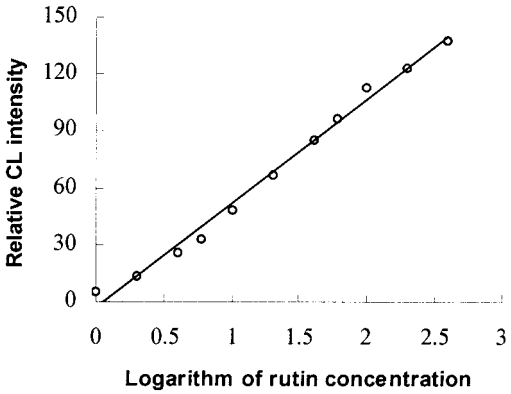

Figure 5. Calibration curve for the determination of rutin. The concentration of rutin was 1.0−400 ng·mL^{-1}.

Effect of Length of Mixing Tubing. The length of the mixing tubing was also adjusted to yield maximum light emission around the cell. It was found that 3 cm of mixing tubing afforded the best results with regard to sensitivity and reproducibility (Figure 4).

Effect of Flow Rate. The CL intensity increases with the increase of total flow rate, and the rate of 2.0 mL·min^{-1} was chosen as a compromise between good precision and lower reagent consumption in the subsequent work.

Practical Lifetime of the Column. Two hundred microliters of sodium phosphate at a constant concen-

Table 3. Tolerable Concentration with Respect to Rutin for Some Interfering Species (<5% Error)

species	tolerable concn ($\mu g \cdot mL^{-1}$)	species	tolerable concn ($\mu g \cdot mL^{-1}$)	species	tolerable concn ($\mu g \cdot mL^{-1}$)
Cr^{3+}	5.2	potassium oxalate	17	cholesterol	3.8
Zn^{2+}	6.5	citric acid	9.6	phenobarbital	46
Mg^{2+}, Ca^{2+}, S^{2-}, SO_3^{2-}	30	malic acid	10	methanol	800
Co^{2+}, Ni^{2+}	12	urea	60	ethanol	920
Fe^{3+}, Fe^{2+}	0.056	uric acid	0.15	globulin	1.5
Cu^{2+}	0.064	sucrose	170	myoglobin	1.2
I^-, NO_3^-, Ac^-, CO_3^{2-}, HCO_3^-	60	glucose	18	lysozyme	0.5

Table 4. Results of Rutin in Different Pharmaceutical Preparations[a]

sample batch	results by proposed method					results by UV
	found ($ng \cdot mL^{-1}$)	added ($ng \cdot mL^{-1}$)	total ($ng \cdot mL^{-1}$)	recovery (%)	content (mg/tablet)	content (mg/tablet)
990868	26.0	10	36.4	104	26.0	25.7
000327	24.9	10	34.7	98.0	24.9	24.5

[a] Average of five determinations.

tration of 1.0×10^{-4} $mol \cdot L^{-1}$ was injected through the column with immobilized luminol and hexacyanoferrate-(III), and the CL intensity (I_0) was recorded. During >500 injections, the I_0 became decreased and fluctuated considerably. Therefore, the column with immobilized CL reagents could be used stably up to 450 times.

Performance of the System for Rutin Measurements. The calibration curves for rutin were obtained under the optimized conditions. It was found that the decreased CL intensity was linear with the logarithm of rutin concentration. As Figure 5 shows, the linear range is from 1.0 to 400 $ng \cdot mL^{-1}$, and the regression equation is $\Delta I = 73.4 \log C_{rutin} - 45.0$, $\gamma = 0.9911$. The RSD of nine determinations were 3.3, 2.6, and 0.6% with rutin solutions of 2.0, 40, and 200 $ng \cdot mL^{-1}$, respectively, and the limit of detection was 0.35 $ng \cdot mL^{-1}$ (3σ).

Interference Study. The effect of foreign species was tested by analyzing a standard solution of rutin (50 $ng \cdot mL^{-1}$) to which increasing amounts of interfering species were added. The tolerable limit of a foreign species was taken if it caused a relative error of <5%. The interference study for the proposed method for rutin was focused on some common metal ions and organic species easily found in medicine tablets and human urine. The tolerable concentrations of some interfering inorganic ions and organic compounds are listed in Table 3. One of the main interferences was caused by some metal ions, which can be masked by EDTA. From the table it can be seen that most of the compounds in urine such as urea and uric acid do not interfere with the detection under the optimized conditions. It should be noted that the interference of some proteins easily found in urine was studied preliminarily. The total urinary protein in a healthy adult is ~100 $\mu g \cdot mL^{-1}$; therefore, these proteins do not interfere with the determination of rutin after urine is diluted at 10^5 with water.

Operational Stability of the Sensor. The major requirements of a sensor are sensitivity and stability. Sensitivity of the system has been achieved in the above studies by selecting the most efficient conditions. It was found that the CL-based sensor exhibited a very good operational stability when >450 successive measurements were performed over a 3 day period.

APPLICATIONS

Determination of Rutin in Pharmaceutical Preparations. Following the procedure described under Materials and Methods, the proposed method was applied to the determination of rutin in pharmaceutical preparations. Two different preparations were purchased on the local market. The measured rutin contents are listed in Table 4. The results obtained by using the proposed method were 24.9 and 26.0 mg per tablet, which were in good agreement with results obtained by using the method described in the *Pharmacopoeia* (*18*), and the recovery was from 98 to 104%.

Table 5. Results of Rutin in Human Urine Samples[a]

sample	found ($ng \cdot mL^{-1}$)	added ($ng \cdot mL^{-1}$)	total ($ng \cdot mL^{-1}$)	recovery (%)	RSD% ($n = 7$)
1	5.1	10.0	15.6	105	1.3
2	5.3	10.0	15.6	103	1.5
3	5.5	10.0	15.4	98.2	0.8
4	4.7	10.0	14.3	95.8	1.2
5	4.6	10.0	14.4	97.6	2.2
6	4.4	10.0	15.1	107	2.2
7	4.6	10.0	14.9	103	1.8
8	5.1	10.0	14.8	96.5	2.6
9	5.1	10.0	16.1	110	1.6
10	4.9	10.0	16.1	112	2.2
11	4.5	10.0	13.8	92.3	2.4
12	4.2	10.0	15.1	109	2.5
13	3.9	10.0	12.9	90.0	1.6
14	3.2	10.0	14.8	116	2.8
15	2.1	10.0	12.0	87.5	2.9

[a] Average of 5 determinations in 15 samples from 3 volunteers.

Determination of Rutin in Human Urine. Results of a preliminary applicability test of the proposed method are summarized in Table 5. Urine samples were collected from volunteers in our group, diluted with distilled water directly, and sometimes supplemented with rutin to test the recovery of the method. Thus, urinary rutin could be determined relatively simply by FI-CL without any pretreatment procedures. Although noted for relatively few samples, the results of trial determinations were characterized by rather high standard deviations, and they suggest that this FI-CL needs further improvement to eliminate the possible matrix effect of samples of some unique composition. A more sensitive system would help in this respect. Nevertheless, this preliminary version of FI-CL has shown simplicity and expediency for the determination of rutin in urine from human subjects.

CONCLUSIONS

The proposed method offers advantages of simplicity, rapidity, high sensitivity, low reagent consumption, and wide linear range for the determination of rutin. It has a potential application in pharmaceutical analysis. In

the determination of rutin in human urine, sample dilution is necessary to preclude interference and achieve satisfactory reproducibility. The method makes possible rapid and direct determination of rutin in body fluids without tedious pretreatment.

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