Sensitized Chemiluminescence Determination of Fluorouracil in Pharmaceutical and Biological Fluids Based on Potassium Permanganate Oxidation in the Presence of Formaldehyde

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A novel chemiluminescence (CL) reaction system was established for the anticancer drug fluorouracil (5-FU). We developed a formaldehyde-sensitized CL emission mechanism for 5-FU-KMnO₄-HCl systems by comparing the fluorescence emission with CL spectra. The illuminant was the singlet-state bimolecular oxygen ¹O₂(¹Δ_gΔ_g), which is from ¹O₂(¹Δ_g) produced in the reaction system, and emits CL spectra at 639 nm. The presence of formaldehyde can sensitize CL emission by accelerating the generation of ¹O₂(¹Δ_g).

Key words: fluorouracil; chemiluminescence; flow injection; pharmaceutical analysis; biological fluids

The optimum conditions for CL emission were investigated. The relationship between the relative CL intensity and the concentration of the analyte showed good linearity. The determination limit was $3 \times 10^{-8} \text{ g mL}^{-1}$. The relative standard deviation (SD) was 1.8%. The proposed method was applied to the determination of fluorouracil in pharmaceutical preparations and biological fluids, and satisfactory results were obtained. J. Clin. Lab. Anal. 21:213–219, 2007. © 2007 Wiley-Liss, Inc.

INTRODUCTION

Fluorouracil (5-FU, 5-fluoropyrimidine-2,4(1H,3H)-dione) is an anticancer drug that has been used for many years to treat a wide variety of solid tumors in various organs, including the breast, pancreas, stomach, colon, and rectum. 5-FU is a fluorinated pyrimidine and acts as a prodrug. The antineoplastic effects of 5-FU are caused by the active metabolites (1). In colorectal cancer, continuous infusion of 5-FU is known to be superior to bolus administration. Furthermore, in breast cancer the tissue concentration of 5-FU metabolites is reported to predict tumor response (2). In pancreatic carcinoma and many other solid tumors, the most effective mode of action, the tissue kinetics of 5-FU, and the tissue concentrations required for the inhibition of tumor growth are unknown. Therefore, it is of great importance to determine 5-FU and its metabolites in tissue cultures, body fluids, and tissues because such information may ultimately permit the design of more effective schedules of 5-FU administration in these cancers. Recent clinical research has shown that some metabolites and derivatives of anticancer drugs have stronger bioactivity than

the anticancer drugs themselves. Quality control of drug dosage, monitoring in biological fluids, and research into the metabolism and action of anticancer drugs are important analytical tasks. Therefore, it is necessary to establish a sensitive analytical technique.

Several methods can be used to quantify anticancer drugs. The Chinese Pharmacopoeia described a UV-spectrophotometry detection method for 5-FU determination in its injection form (3). The United States Pharmacopoeia Convention described an HPLC method for 5-FU determination (4). Other methods include potentiometry (5), capillary electrophoresis (6,7), and high-performance liquid chromatography (HPLC) (8,9).

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However, these methods are complicated, have a low sensitivity, and involve harsh analytical conditions or expensive instruments.

Chemiluminescence (CL) is becoming a powerful analytical tool with widespread application in various fields owing to its high sensitivity, wide dynamic range, and simple instrumentation (10,11). A critical review described the use of acidic potassium permanganate to generate CL during the oxidation of both organic compounds and inorganic species (12). Several studies have reported that very sensitive CL emission can be generated by oxidation of hydroxyl-containing drugs with KMnO_4 in acidic medium (13,14). Formaldehyde was found to enhance the CL emission intensity of such a system, which laid the foundation for using HCHO as a sensitized agent for CL analysis, and stimulated researchers to explore its application as a sensitized agent for analysis of melatonin and some of its derivatives (15), hydralazine (16), naftopidil (17), and estrogens (18). Although the CL method has been used for pharmaceutical analysis, only a few studies have used the CL method to detect 5-FU.

The main purpose of this study was to develop a new, sensitized CL system based on the use of acidified KMnO_4 as the oxidant in the presence of formaldehyde for determination of the anticancer drug 5-FU. The proposed sensitized CL system was successfully applied for the determination of 5-FU in injection and biological fluids, and satisfactory results were obtained. A CL mechanism was also developed.

METHODS AND MATERIALS

Chemicals

All chemicals used in this study were of analytical-reagent grade. Deionized water was used throughout. The 5-FU was obtained from Fluka (Buchs, Switzerland). We prepared a stock standard solution ($5.0 \times 10^{-4} \text{ g mL}^{-1}$) by dissolving 25.0 mg of 5-FU in 2 mL of 0.2 mol L^{-1} NaOH and diluting it with deionized water to 50 mL. We freshly prepared more dilute solutions by diluting the stock solution with deionized water. The 5-FU injection was provided by Jinyao Aminophenol Inc. (Tianjin, China). We prepared a stock solution of KMnO_4 ($5 \times 10^{-2} \text{ mol L}^{-1}$) by dissolving a weighed amount of KMnO_4 in water and diluting it to volume. Stock solutions of HCOH (2.5%) and HCl (3 mol L^{-1}) were prepared daily.

Apparatus

The FI system, as shown in Fig. 1, is a MPI-B-FI-CL analysis system (Xi'an Remex Electronic Science-Tech Co., Xi'an, China) that consists of two peristaltic pumps

Fig. 1. Schematic diagram of the flow injection CL analysis system. P, peristaltic pump; V, sampling inlet valve; C, flowing cell; PMT, photomultiplier tube; AMP, amplifier; HV, high voltage; R, recorder; W, waste. **a:** Sample solution. **b:** HCOH solution. **c:** KMnO_4 solution. **d:** HCl solution.

working at a constant flow rate (30 rpm) and a six-way injection valve with a sample loop ($120 \mu\text{L}$), which is automatically operated by a computer equipped with software for MPI-B flow-injection analysis. The flow cell is a glass tube that is twisted in order to produce a large surface area exposed to the adjacent photomultiplier tube (PMT) (Hamamatsu, Japan).

PTFE tubing (0.8 mm i.d.) was used to connect all components in the flow system. Because there is no reaction between the KMnO_4 and HNO_3 solutions, a mixture of the two solutions was used as carrying solution, and flowed into a flow cell through a three-way pipe. A mixture of the sample and HCOH solution was then injected from a sample valve, and the CL signal was produced immediately.

The flow cell was a glass tube that was twisted in order to produce a large surface area exposed to the adjacent PMT. The signal from the CL reaction was recorded. Fluorescence spectra were recorded with a RF-5301PC spectrofluorometer (Shimadzu, Japan) to study the fluorescence characteristics.

Sample Preparation

No less than five bottle injections selected from the same group randomly were weighed to obtain the mean mass per bottle. Working solutions were prepared by appropriate dilution of this sample solution so that the final analytic concentration was within the working range.

A 1-mL volume of serum sample was deproteinized by the addition of 4.0 mL of 10% trichloroacetic acid (CCl_3COOH) in a centrifuge tube, which was then centrifuged for 15 min at 4000 rpm. The supernatant liquid was used for determination of 5-FU concentration. Serum samples from patients were kindly provided by the Taian Cancer Hospital.

Procedure

As shown in Fig. 1, all solutions were continuously pumped into the manifold. A $120\text{-}\mu\text{L}$ mixture of 5-FU

and HCOH solutions was injected into a mixed stream of KMnO_4 and HCl solutions. The mixed solution was transferred into the CL flow cell and immediately gave rise to an intensive CL signal. The signal from the CL reaction was recorded by an analyzer. We constructed calibration graphs by plotting the intensity (peak height) of the CL signal vs. the concentration of analyte.

RESULTS AND DISCUSSION

Optimization of Experimental Conditions

Effect of sample volume and flow rate

The role of the sample volume and flow rate is critical, for instance, when the sample volume was too small or too large, the maximum CL could not be obtained. The highest emission was obtained when the injected sample volume was $120\ \mu\text{L}$. The CL intensity increased with increasing flow rate. However, a flow rate of $3.0\ \text{mL min}^{-1}$ for all solutions is recommended because it allows greater precision and economy in the use of reagents.

Effects of acidic media

The kind and concentration of the acid used in the reaction has a very significant influence on the CL emission intensity. Several acids, including HCl, H_2SO_4 , HNO_3 , H_3PO_4 , and $\text{H}_6\text{P}_4\text{O}_{13}$, were used to test the effects of an acidic medium on the CL signal. The experimental results are shown in Fig. 2. The highest and most stable emission was observed for the 5-FU- KMnO_4 -HCOH system in HCl medium. The optimum concentration of the acidic medium was $2.0\ \text{mol L}^{-1}$ HCl.

Effect of KMnO_4 concentration

In this CL system, KMnO_4 was used as the oxidant. The KMnO_4 concentration not only affected the sensitivity, it also influenced the linear range for the assay. The dependence of the KMnO_4 concentration on the CL intensity was investigated for $1.0 \times 10^{-6}\ \text{g mL}^{-1}$ analytes. As shown in Fig. 3, the maximum CL intensity was obtained with a KMnO_4 concentration of $5 \times 10^{-4}\ \text{mol L}^{-1}$.

Effect of sensitizers

The KMnO_4 -5-FU system produced only a weak CL emission. Various compounds, such as rhodamine B, HCOH, H_2O_2 , Na_2SO_3 , $\text{Na}_2\text{S}_2\text{O}_3$, and $\text{Na}_2\text{S}_2\text{O}_4$, were tested as sensitizers for the KMnO_4 -5-FU system. It was found that only HCOH enhanced the CL signal. The effect of HCOH concentration on the CL intensity was investigated in the range of 0.5–3%, as shown in Fig. 4.

Fig. 2. Effect of HCl concentration on the relative CL intensity. KMnO_4 : $5 \times 10^{-4}\ \text{mol L}^{-1}$; HCOH: 2%; 5-FU: $1 \times 10^{-5}\ \text{g mL}^{-1}$.

Fig. 3. Effect of potassium permanganate concentration on the relative CL intensity. 5-FU: $1 \times 10^{-5}\ \text{g mL}^{-1}$; HCl: $2.0\ \text{mol L}^{-1}$; HCOH: 2%.

The CL intensity was enhanced with increasing HCOH concentrations. The maximum CL intensity was obtained when the HCOH concentration was 2.5%.

Interference Studies

We investigated the influence of some common excipients used in drugs, metal ions in the human body, and several organic compounds on the CL intensity for the determination of $4 \times 10^{-6}\ \text{g mL}^{-1}$ 5-FU by comparing the CL emissions obtained using 5-FU solution alone and those obtained using 5-FU with a foreign

Fig. 4. Effect of formaldehyde concentration on the relative CL intensity. 5-FU: $1 \times 10^{-5} \text{ g mL}^{-1}$; KMnO_4 : $5 \times 10^{-4} \text{ mol L}^{-1}$; HCl : 2.0 mol L^{-1} .

species added. Tolerance was defined as the amount of foreign species that produced an error no greater than $\pm 5\%$ in the determination of 5-FU. The tolerated ratios of foreign substances to $4 \times 10^{-6} \text{ g mL}^{-1}$ 5-FU were as follows: 100-fold for magnesium stearate, glucose, fructose, sucrose, galactose, and sodium citrate; 50-fold for lactose; 25-fold for starch and dextrin; 10-fold for sodium benzoate; 100-fold for Ni^{2+} , Ca^{2+} , Co^{2+} , Mg^{2+} , Zn^{2+} , Ba^{2+} , Al^{3+} , and Cu^{2+} ; 10-fold for Fe^{3+} ; and twofold for Br^- . There was feeble interference from equal amounts of hemoglobin, myoglobin, bilirubin, vitamin B_1 , I^- , and Fe^{2+} .

Analytical Performance of the CL System

The CL kinetic characteristics of the 5-FU-HCOH- KMnO_4 -HCl system were studied in detail. We found that the reaction rate in solution was very fast: from the reagent mixing to the peak maximum, only 2.8 sec elapsed, and it took 8.5 sec for the signal to return to zero again.

Under the optimum conditions described above, the linearity of the calibration graph of 5-FU was examined in the range from $1.0 \times 10^{-7} \text{ g mL}^{-1}$ to $8.0 \times 10^{-5} \text{ g mL}^{-1}$. The calibration graph consisted of three parts in order to improve the accuracy. The basic parameters of the calibration graph are listed in Table 1. The detection limit (3σ) for 5-FU was $3 \times 10^{-8} \text{ g mL}^{-1}$. The relative standard deviation (RSD) was 1.8% for 11 determinations of $2.0 \times 10^{-6} \text{ g mL}^{-1}$ 5-FU. The RSDs were 2.0% and 1.6% for intraday variations and 2.2% and 1.7% for interday variations for five determinations at concentrations of $2.0 \times 10^{-7} \text{ g mL}^{-1}$

TABLE 1. Linear range and regressive equations of the calibration curves

5-FU concentration (g mL ⁻¹)	Regressive equation (I = aC+b)	Correlation coefficient (r)
1.0×10^{-7} – 1.0×10^{-6}	$I = 1.2C + 41.2$	0.9977
1.0×10^{-6} – 1.0×10^{-5}	$I = 5.2C + 49.1$	0.9987
1.0×10^{-5} – 8.0×10^{-5}	$I = 15.6C + 89.7$	0.9968

TABLE 2. Recovery experiments

Content (10 ⁻⁶ g– mL ⁻¹)	Added (10 ⁻⁶ g mL ⁻¹)	Found (10 ⁻⁶ g mL ⁻¹)	Recovery n = 7 (%)
0.21	0.2	0.40	95.0
	0.4	0.62	102.5
	0.6	0.85	106.7
2.35	2.0	4.36	100.5
	4.0	6.32	99.5
	6.0	8.50	102.5

TABLE 3. Determination results of 5-Fu in pharmaceutical samples

Batch number	Nominal (mg/bottle)	Proposed method (mg/bottle)	RSD n = 7 (%)	Official method (mg/bottle)
0406111	250	255.3	2.1	253.6
0502121	250	248.9	1.9	250.1

and $6.0 \times 10^{-5} \text{ g mL}^{-1}$ 5-FU, respectively. The results indicate that the proposed CL system has good linearity and relatively high sensitivity and precision.

Pharmaceutical Analysis

To evaluate the validity of the proposed method for the determination of 5-FU in pharmaceutical preparations, we conducted recovery studies on samples to which known amounts of 5-FU were added. The results are given in Table 2. The recoveries were in the range of 95.0–106.7%.

The proposed method was applied to the determination of 5-FU in injections. The obtained results are summarized in Table 3 along with the labeled contents. The results agree well with those obtained by the official method (3). There was no significant difference between the labeled contents and the results obtained by the proposed method.

Determination of Spiked Serum

It was previously reported that the concentration of 5-FU in the serum was 0.28–1.25 $\mu\text{g mL}^{-1}$ after injection

TABLE 4. Determination result in different patient serum samples

Sample No.	Content (10^{-6} g mL $^{-1}$)	Added (10^{-6} g mL $^{-1}$)	Found (10^{-6} g mL $^{-1}$)	Recovery (%)	RSD n = 5 (%)
1	6.1	5.0	11.4	106.0	1.9
2	3.2	5.0	8.1	98.0	2.0
3	4.8	5.0	9.9	102.0	1.8
4	2.2	5.0	7.0	96.0	2.1
5	0.8	5.0	6.0	104.0	2.2

of 500 mg 5-FU (19). To make the sample concentration of the drug within the linear range of determination, we diluted the serum sample appropriately. The standard addition method was used to avoid matrix effects. Different cancer patients were injected with 750 mg 5-FU, and their serum samples were tested within 1 hr. The results for the contents and recoveries are summarized in Table 4.

CL Mechanism

Fluorescence characteristics

We examined the fluorescence spectra to obtain more information about the enhanced CL mechanism. The fluorescence emission spectra of different reaction systems were observed, as shown in Fig. 5.

The native fluorescence emission of 5-FU-HCl system in the absence and the presence of HCOH showed a broad peak center at 366 nm, and the peak intensity increased in the presence of HCOH. The presence of KMnO_4 can lead to a decrease in fluorescence emissions from the 5-FU-HCl system. This is because 5-FU in HCl solution can be oxidized by KMnO_4 to its oxide form without fluorescence characteristics.

Mechanism of the CL reaction

Spectroscopic studies have demonstrated that the C2-C3 double bond of the indole ring in melatonin and some of its derivatives are oxidized by KMnO_4 in the presence of formaldehyde in H_2SO_4 medium. The maximum CL peak was at 640 nm (15). The CL derived from manganese(IV) oxidations was relatively weak, and the presence of formaldehyde in phosphoric acid medium showed a wavelength of maximum emission at approximately 710 nm (20). The CL spectrum showed a peak hand at 640 nm for the KMnO_4 -HCOH system with uric acid or methotrexate as the reductant (21,22). The maximum wavelength of CL emission is (635 nm) (23).

As shown in Fig. 4, the presence of formaldehyde increased the amount of Mn^{2+} and therefore the concentration of Mn^{3+} , which substantially facilitated the direct CL reaction. Excess formaldehyde, however,

Fig. 5. Fluorescence emission spectra of 5-FU (B), 5-FU- KMnO_4 (C), 5-FU- KMnO_4 -HCOH (D), and 5-FU-HCOH (E). $\lambda_{\text{ex}} = 318$ nm; KMnO_4 : 5×10^{-4} mol L $^{-1}$; HCOH: 2.0%; 5-FU: 5×10^{-4} g mL $^{-1}$.

stabilized Mn as Mn^{2+} , and thus the amount of Mn^{3+} in the solution decreased. This resulted in diminution of the CL emission. The current experimental results are very similar to previous findings (24). It may be that HCOH did not change the emitter of the CL reaction, but increased the luminescence quantum number. This suggests that the proper HCOH concentration increases the amount of singlet oxygen in the CL reaction system.

To investigate the reaction product that generates the CL, we examined the CL spectrum of the reaction using a series of interference filters. The CL spectra of KMnO_4 -HCOH-HCl solution in the absence and presence of 5-FU were recorded in the range of 360–730 nm. When 5-FU was absent, no obvious CL spectrum was observed. Although there was weak fluorescence for the 5-FU- KMnO_4 -HCOH-HCl system, the CL emission was stronger.

As shown in Fig. 6, the CL spectrum showed a peak hand at 639 nm for the 5-FU- KMnO_4 -HCOH-HCl system. The CL peak wavelengths are very different from those of fluorescence peaks for the reaction systems. Singlet-state bimolecular oxygen $^1\text{O}_2(^1\Delta_g)$ was the illuminant, and its theoretically maximum

Fig. 6. CL spectra of 5-FU-KMnO₄-HCOH-HCl. KMnO₄: 5×10^{-4} mol L⁻¹; HCl: 2.0 mol L⁻¹; HCOH: 2.0%; 5-FU: 5×10^{-4} g mL⁻¹.

wavelength of CL emission is 645 nm (23). The CL spectrum for the studied systems showed a peak hand at 639 nm, which basically coincides with the theoretically maximum wavelength. The deviation may have been caused by the use of different media.

Therefore, it is possible that there was $^1\text{O}_2$ ($^1\Delta_g$) (singlet-state oxygen) in the reaction of potassium permanganate and 5-FU in the acidic medium. The presence of HCOH may serve to accelerate the generation of $^1\text{O}_2$ ($^1\Delta_g$), which is further transferred to be illuminant $^1\text{O}_2^1\text{O}_2$ ($^1\Delta_g^1\Delta_g$). When it was transferred to triplet-state oxygen $^3\text{O}_2$ ($^3\Sigma_g$), the CL peak was produced at 639 nm. Therefore, the mechanism may be summarized by the following scheme:

CONCLUSIONS

A novel CL reaction system was established for 5-FU. The CL spectrum for the 5-FU-KMnO₄-HCOH-HCl system showed a peak hand at 639 nm, which basically coincides with the theoretically maximum wavelength. The illuminant was a singlet-state bimolecular oxygen $^1\text{O}_2^1\text{O}_2$ ($^1\Delta_g^1\Delta_g$). The proposed CL system has good linearity, relatively high sensitivity and precision, and is

potentially useful for analyses of fluorouracil in biological samples and investigations of anticancer activity.

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