Asian Journal of Chemistry; Vol. 27, No. 3 (2015), 813-817

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2015.17120

# Determination of 5-Fluorouracil in its Injection and Biological Fluid by Enhanced Chemiluminescence Based on Luminol-Ag(III) Complex Reaction in Alkaline Solution

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*Received*: 25 January 2014; *Accepted*: 19 June 2014; *Published online*: 19 January 2015; AJC-16677

A novel chemiluminescence method was developed for the determination of 5-fluorouracil based on the chemiluminescence reaction between Ag(III) complex $[Ag(HIO_6)_2]^{5-}$ and luminol in alkaline solution. Chemiluminescence emission of $[Ag(HIO_6)_2]^{5-}$-luminol in alkaline medium was different from that in acidic medium. A possible mechanism of enhanced chemiluminescence emission was suggested. The enhanced effect of 5-fluorouracil on chemiluminescence emission of the $[Ag(HIO_6)_2]^{5-}$-luminol system was found. The effect of the reaction conditions on chemiluminescence emission was examined and optimized. The chemiluminescence intensity was proportional to the logarithm of 5-fluorouracil concentration with correlation coefficient (r) of 0.9954 in the range of 10-5000 ng/mL. Under the optimized conditions, the limit of detection (LOD) was 3 ng/mL. The chemiluminescence system was applied for the determination of 5-fluorouracil in its injection and biological fluid with the recoveries of 94.6-104 % and with the RSD of 1.2-2.8 %. The proposed flow-injection chemiluminescence method provides high sensitivity necessary for analysis of 5-fluorouracil in pharmaceutical and biological fluids at clinically relevant concentrations.

**Keywords: Enhanced chemiluminescence, Flow injection, 5-Fluorouracil, Pharmaceutical analysis.**

## INTRODUCTION

5-Fluorouracil (5-FU, 5-fluoropyrimidine-2,4(1*H*,3*H*)-dione) is a cytostatic agent that has been widely used in the treatment of various solid tumours for more than 20 years and is still considered to be among the most active antineoplastic agents in advanced colourectal cancer and malignancies of the head and neck. In pancreatic carcinoma and many other solid tumors, the most effective mode of action, the tissue kinetics of 5-fluorouracil and the tissue concentrations required for the inhibition of tumor growth are unknown. Recently 5-fluorouracil in association with irinotecan and radiotherapy has been used for the treatment of rectal cancer[1]. Quality control of drug dosage, monitoring in biological fluids and research into the metabolism and action of anticancer drugs are important analysis tasks. Therefore, it is necessary to establish a sensitive analytical technique.

Breda and Barattè, recently, reviewed numerous chromatographic and non-chromatographic methods of analysis reported in the literature for 5-fluorouracil in biological matrices[2]. Various chromatographic methods for the analysis of 5-fluorouracil in biological matrices include high performance liquid chromatography (HPLC), gas chromatography (GC), GC-mass spectrometry (MS) and HPLC-MS. The United States Pharmacopoeia Convention described an HPLC method for 5-fluorouracil determination[3]. The Chinese Pharmacopoeia described an UV-spectrophotometry detection method for 5-fluorouracil determination in its injection form[4]. Other methods include potentiometry[5] and capillary electrophoresis[6,7]. 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. Chemiluminescence has been used as diagnostic tool[8]. Its clinical applications in routine testing and the diverse applications in clinical research were reviewed[9]. A series of flow injection-chemiluminescence systems were reported for the analysis of many drugs using different chemiluminescence reagents, such as *tris*(2,2'-bipyridine) ruthenium(II), potassium permanganate, cerium(IV) and luminol. To our best of knowledge, there was only a chemiluminescence method for the determination of 5-fluorouracil. This method was based on potassium permanganate oxidation in the presence of formaldehyde and was used for the determination of 5-fluorouracil

in pharmaceutical and biological fluid with the detection limit of 30 ng/mL[10].

In our previous work, a new chemiluminescence reaction system with Ag(III) complex in acidic medium without luminol was developed for the determination of fluoroquinolones synthetic antibiotics[11-13]. A chemiluminescence reaction of Ag(III) complex with luminol in alkaline medium was used for the determination of cortisol and 10-hydroxycamptothecin[14,15]. Recently, we found that chemiluminescence emission of Ag(III) complex-luminol in alkaline medium could be enhanced by 5-fluorouracil.

The main purpose of this study was to discuss the enhanced mechanism of 5-fluorouracil to chemiluminescence and to develop an enhanced chemiluminescence method for determination of anticancer drug 5-fluorouracil in its injection and biological fluid.

## EXPERIMENTAL

The flow-injection system used for chemiluminescence was an IFFM-E analysis system (Remex Electronic Sci-Tech. Co. Ltd, Xi'an, China) consisting of two peristaltic pumps working at a constant flow rate (60 rpm) and a six-way injection valve with a sample loop (120 mL) automatically operated by a computer-equipped operation system of IFFM-E flow injection analysis. A F-7000 Fluorescence spectrophotometer (Hitachi, Japan) was used for studying chemiluminescence mechanism. A TGL-16M centrifuge (Xiangyi centrifuge Co., Hunan, China) was used in sample treatment.

All chemicals used in this study were of analytical reagent grade. Deionized water was used throughout. The 5-fluorouracil (= 99 %) was obtained from Fluka (Buchs, Switzerland). Stock standard solution ($5 \times 10^{-4}$ g/mL) was prepared by dissolving 25 mg of 5-fluorouracil in 2 mL of 0.2 M NaOH and diluting it with deionized water to 50 mL and stored in a refrigerator at 4 °C to keep dark. More dilute solutions were prepared freshly by diluting the stock solution with deionized water. The 5-fluorouracil injection was provided by Jinyao Aminophenol Inc. (Tianjin, China).

Sodium periodate ($NaIO_4$, 99.5 %) was purchased from Tianjin Kermel Chemical Reagent Company (Tianjin, China). Potassium peroxydisulfate ($K_2S_2O_8$, 99.5 %) was purchased from Beijing Chemical Reagent Company (Beijing, China). Silver nitrate ($AgNO_3$, 99.8 %) and potassium hydroxide (KOH, 82 %) were purchased from Tianjin Damao Chemical Reagent Company (Tianjin, China). The Ag(III) complex {*bis*-(hydrogenperiodato) argentate(III) complex anion $[Ag(HIO_6)_2]^{5-}$} stock solution was prepared by oxidizing Ag(I) in the alkaline medium according the known method[16]. The concentration of Ag(III) complex solutions prepared was determined accord to the literature[17].

All chemicals were of analytical reagent grade and used without further purification and deionized water was used throughout.

**Treatment of injection sample:** Sample solutions for analysis were prepared as follows. The average bottle weight was calculated from the weight of 5 bottles that were randomly selected from the same group. An accurately weighed portion of each homogenized sample containing 5 mg of 5-fluorouracil was dissolved with 2 mL of 0.1 M NaOH and water in a small beaker. The solution was filtered and the residue was washed with water several times, then transferred into a 25 mL calibrated flask and diluted to the volume with water. Working solutions were prepared by appropriate dilutions, so that the final concentration was in the linear range.

**Treatment of urine and serum samples:** Urine and serum samples were provided by Hebei University Hospital. 1 g aliquot of $PbO_2$ powder was added to 5 mL of blank uric and followed by stirring for 10 min to eliminate uric acid, thiourea and ascorbic acid. After centrifugation for 15 min at 10,000 rpm, the supernatant was filtrated and then the filtrate was applied to a cation exchange column (4 × 1.2 cm) for cleanup. The clear liquid was diluted with deionized water to make different concentrations of 5-fluorouracil in the linear range.

The protein of a 1 mL volume of serum sample was removed by adding 4 mL 10 % (v/v) trichloroacetic acid ($CCl_3COOH$) in a centrifuge tube, which was shaken for 5 min, then centrifuged at 4,000 rpm for 15 min. The supernatant was diluted with deionized water to make different concentrations of 5-fluorouracil in the linear range.

**Procedures:** The procedure for FIA is shown in Fig. 1. The flow lines a and b were inserted into luminol alkaline solution and 5-fluorouracil solution, respectively and then mixed with $[Ag(HIO_6)_2]^{5-}$ from the flow lines (c) to produce chemiluminescence when the injection valve was switched to the position of injection. The concentration of 5-fluorouracil was quantified by the peak height of the chemiluminescence signals.

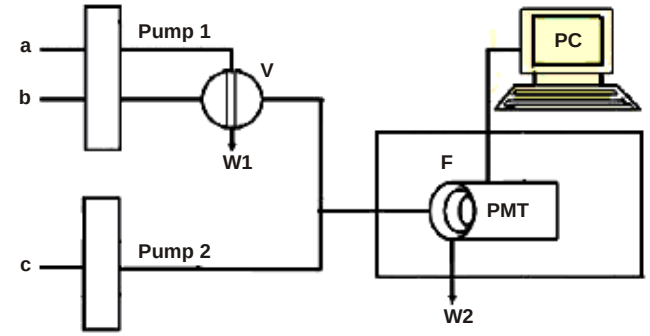



Fig. 1. Schematic diagram of flow-injection-chemiluminescence (CL) system. (a) 5-Fluorouracil solution or samples; (b) luminol and KOH solution; (c) $[Ag(HIO_6)_2]^{5-}$ solution. V, injection valve; F, spiral glass flow cell; PMT, photomultiplier tube; Pump1 and Pump 2, peristaltic pumps

## RESULTS AND DISCUSSION

**Kinetic characteristic of the chemiluminescence reaction:** An attempt was made to research and develop a new and sensitive chemiluminescence system that could be applied for the chemiluminescence determination. The chemiluminescence kinetic characteristics of the reactions system were investigated. The investigation of the chemiluminescence intensity-time profiles was performed with the static chemiluminescence analysis. The result is shown in Fig. 2. It was shown that the reaction rate in solution was very fast; from reagent mixing to peak maximum only 0.3 s was needed for

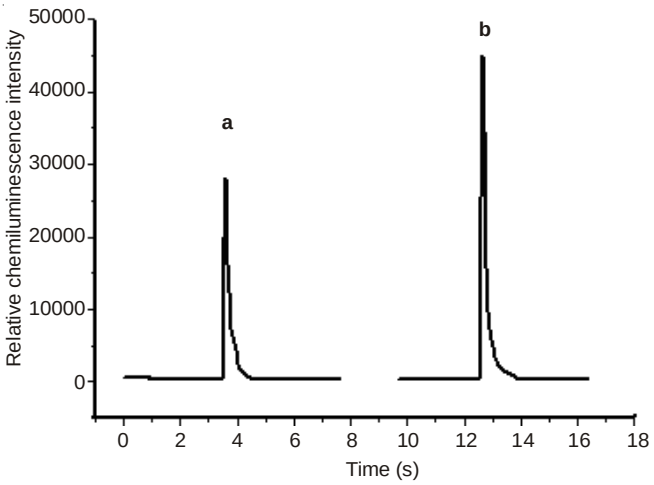



Fig. 2. Kinetic curve of the chemiluminescence reaction system in alkaline medium without 5-fluorouracil (a) and with 5-fluorouracil (b)

$[Ag(HIO_6)_2]^{5-}$-luminol-KOH system and it took 2 s for the signal to return to zero again. The kinetic curve also indicated that the chemiluminescence could be enhanced by 5-fluorouracil.

**Possible mechanism of chemiluminescence emission:** In order to obtain more information about the chemiluminescence emission mechanism and the enhanced mechanism of 5-fluorouracil to chemiluminescence, the fluorescence spectra of different systems in alkaline medium were recorded and chemiluminescence spectra were observed by an F-7000 fluorescence spectrophotometer (taken off lamp-house), as shown in Figs. 3 and 4.

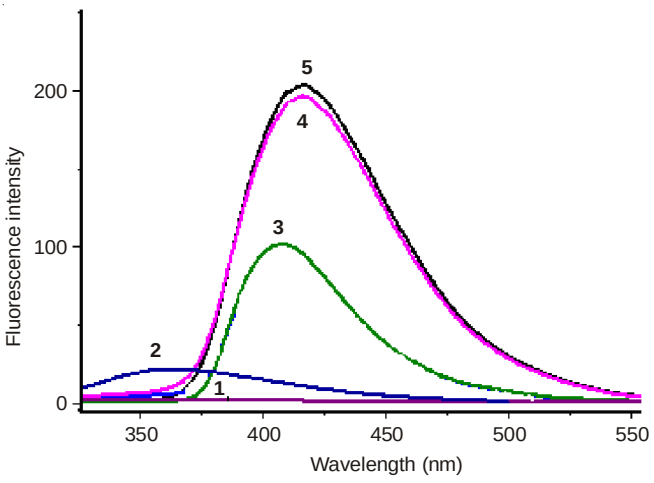



Fig. 3. Fluorescence emission spectra of different systems in alkaline medium at $\lambda_{ex}$ = 285 nm. (1) $[Ag(HIO_6)_2]^{5-}$, 0.02 mM; (2) 5-fluorouracil, 16 ng/mL; (3) luminol, 50 ng/mL; (4)-(1) + (3); (5)-(1) + (2) + (3)

No fluorescence emission was observed for $[Ag(HIO_6)_2]^{5-}$; a weak and broader fluorescence at about 365 nm was observed for 5-fluorouracil; a broader fluorescence peak at 407 nm was observed for luminol; and a stronger fluorescence peak at 420 nm was observed for $[Ag(HIO_6)_2]^{5-}$-luminol with and without 5-fluorouracil. Our previous work had shown that Ag(III) complex has two forms, $[Ag(HIO_6)_2]^{5-}$ and $[Ag(HIO_6)(OH)(H_2O)]^{2-}$ [$Ag(III)^*$] and the last could be active

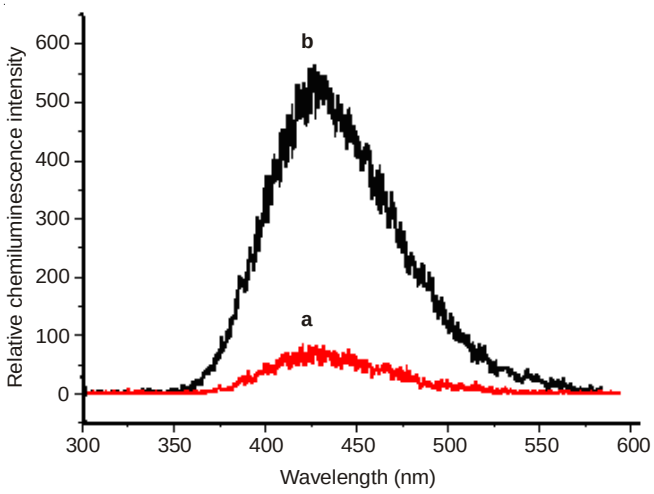



Fig. 4. Chemiluminescence (CL) spectra of the reaction systems in alkaline medium. (a) 0.02 mM $[Ag(HIO_6)_2]^{5-}$ + 50 ng/mL luminol + 0.16 M KOH; (b)-(a) + 16 ng/mL 5-FU

center[18]. $Ag(III)^*$ could oxidize luminol to diazonaphthoquinone, resulting in conjugation effect to be increased due to the formation of -N=N- in diazonaphthoquinone. Therefore the fluorescence wavelength increased from 407 to 420 nm and the intensity increased.

Our previous work also showed that $[Ag(HIO_6)_2]^{5-}$ in alkaline medium in the absence of luminol could not produce chemiluminescence emission, but $[Ag(HIO_6)_2]^{5-}$ can enhance the chemiluminescence emission of luminol in alkaline medium[11,15]. Fig. 3 shows that chemiluminescence spectrum at 425 nm was observed for $[Ag(HIO_6)_2]^{5-}$-luminol with and without 5-fluorouracil in alkaline medium, which was similar to the chemiluminescence spectra of $H_2O_2$-luminol in alkaline medium. Both chemiluminescence spectra in Fig. 3 had similar emission wavelength range and profile, which suggested that they provided with the same emitter. Therefore, like $H_2O_2$, $Ag(III)^*$ could oxidize luminol to produce luminol free radicals, which could be excited by $Ag(III)^*$ further.

Otherwise, our test showed that, if the dissolved oxygen in luminol and 5-fluorouracil solution was removed by passing $N_2$ gas for 5 min, the chemiluminescence intensity decreased by 45 %. The result showed that a reaction of $Ag(III)^*$ with dissolved oxygen in the solution had taken place, which could produce superoxide radical ($O_2^{\bullet-}$) and $O_2^{\bullet-}$ forms singlet oxygen ($1O_2^{\bullet-}$), which could oxidize the luminol free radicals[12,15,18].

Based on the chemiluminescence mechanism of the luminol-$H_2O_2$ system and the above discussion, a possible chemiluminescence mechanism of this reaction system could be suggested as shown in **Scheme-I**.

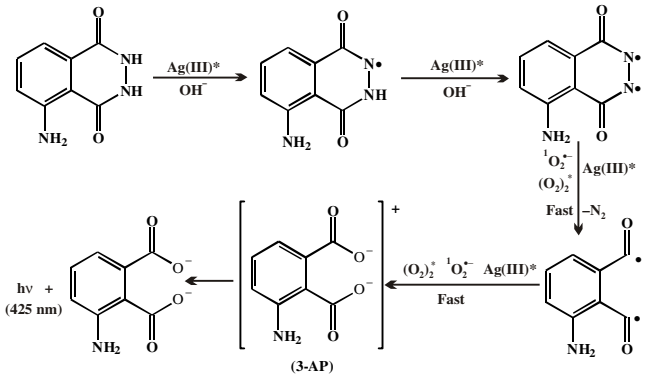
It is shown in Fig. 3 that 5-fluorouracil has a strongly enhancing effect on the luminol-$[Ag(HIO_6)_2]^{5-}$ chemiluminescence system and the luminol-$[Ag(HIO_6)_2]^{5-}$ chemiluminescence system with and without 5-fluorouracil showed a broad chemiluminescence emission at 425 nm. All these chemiluminescence reactions shared a common emitting species (luminol free radical). Any factors that facilitate the formation of the key intermediates luminol radical and luminol endoperoxide as well as increasing the excitation efficiency and quantum yield of the emitter will enhance the chemiluminescence

$$Ag[(HIO_6)_2]^{5-} + H_2O \longrightarrow Ag[(HIO_6)(OH)(H_2O)]^{2-}\{Ag(III)^*\} + H_2IO_6^{3-}$$

$$Ag(III)^* + H_3O^+ \longrightarrow Ag^+ + H_5IO_6 + O_2^{\bullet-}$$

$$2O_2^{\bullet-} \longrightarrow (O_2)_2^*$$

$$Ag(III)^* + O_2 \text{ (dissolved)} \longrightarrow O_2^{\bullet-} \dashrightarrow {}^1O_2^{\bullet-}$$




**Scheme-I**

emission. 5-Fluorouracil could produce $O_2^{\bullet-}$ and $OH^{\bullet}$, as well as $^1O_2^{\bullet-}$ in alkaline solution[15,19], which could cause luminol free radical to be excited, enhancing chemiluminescence emission.

**Effect of sample volume and flow rate on chemiluminescence:** The role of sample volume and flow rate is critical. For instance, if the sample volume was too small or too large, the maximum chemiluminescence could not be obtained. The highest emission was produced when the injected sample volume was 120 mL. The chemiluminescence intensity increased with increasing flow rate. A flow rate of 3 mL/min for all solutions was recommended because of the greater precision and economy in the use of reagents.

**Effect of alkaline medium on chemiluminescence:** The type and concentration of the alkaline used in the reaction had a significant influence on the chemiluminescence emission intensity. Therefore, $Na_2CO_3$, $NaHCO_3$, $Na_2CO_3$-$NaHCO_3$, KOH or NaOH with the same concentration was added to the luminol solution to test the effect of alkaline medium on the chemiluminescence signal. The highest and most stable chemiluminescence intensity was observed in KOH medium. The effect of KOH concentration of 0.10-0.18 M on the chemiluminescence intensity of 0.5 μg $mL^{-1}$ 5-fluorouracil-0.2 μg/mL luminol-0.02 mM $[Ag(HIO_6)_2]^{5-}$ system was investigated. The result showed that the chemiluminescence intensity was increased markedly with increasing KOH concentration up to 0.16 M and decreased when over 0.16 M. Hence, 0.16 M KOH was used as the optimum concentration for further test.

**Effect of luminol concentration on chemiluminescence**: The concentration of luminol had a very important effect on the chemiluminescence intensity for the determination of 5-fluorouracil. The dependence of luminol concentration in the range of 0.1-1.0 μg/mL on the chemiluminescence intensity was investigated for 0.5 μg/mL 5-fluorouracil. The result showed that chemiluminescence intensity increased obviously with increasing luminol concentration from 0.1 to 0.25 μg/mL and chemiluminescence intensity decreased obviously when over 0.25 μg/mL. After the analysis of S/N ratio and the sensitivity of the system, the best concentration of luminal was selected to be 0.25 μg/mL for next test.

**Effect of $[Ag(HIO_6)_2]^{5-}$ concentration on chemiluminescence:** In the chemiluminescence system, $[Ag(HIO_6)_2]^{5-}$ was used as an oxidant. The $[Ag(HIO_6)_2]^{5-}$ concentration not only influenced the sensitivity, but also influenced the linear range for the assay. The influence of $[Ag(HIO_6)_2]^{5-}$ concentration on the chemiluminescence signal was tested in the range of 0.01-0.05 mM on the chemiluminescence emission of 0.5 μg/mL 5-fluorouracil. The result showed that chemiluminescence intensity increased with increasing $[Ag(HIO_6)_2]^{5-}$ concentration from 0.01 to 0.02 mM, but decreased when over 0.02 mM. Therefore 0.02 mM $[Ag(HIO_6)_2]^{5-}$ was selected as an optimum concentration.

**Analytical performance of chemiluminescence systems:** The influence of some common excipients on the chemiluminescence intensity was investigated for determining 5-fluorouracil by comparing with the chemiluminescence emissions. The tolerance content was defined as the amount of coexisting species that produced an error not exceeding ± 5 % in the determination of 5-fluorouracil. The tolerated ratios of foreign substances to 50 ng/mL analyte were 100-fold for lactose, D-galactose, glucose, sucrose, sodium benzoate dextrin, $Ba^{2+}$, $Mg^{2+}$, $Ca^{2+}$, $Co^{2+}$ and $Ni^{2+}$; 50-fold for starch; 25-fold for polyethyleneglycol, ascorbic acid and $Cu^{2+}$; 10-fold for $Fe^{3+}$, $Cu^{2+}$, $Zn^{2+}$, $I^-$ and $Br^-$. The data show that there was little interference. Since urine samples had been pretreated by deposition and cation exchange and serum samples had been pretreated with trichloroacetic acid, there was no interference in the analysis of real samples.

Under the optimized conditions, the linearity between peak height (y) and logarithm of 5-fluorouracil concentration (log C) or 5-fluorouracil concentration (C) was evaluated. The regression equations are given in Table-1. The calibration graph for peak height toward concentration consisted of two parts in order to improve the veracity. It is shown that the proposed chemiluminescence system has wide dynamic range of 10-5000 ng/mL.

The limit of detection (LOD) was determined as the sample concentration that produces a peak with a height three times of the level of baseline noise. The LOD was 3 ng/mL. The relative standard deviation (RSD) was 1.7 % for eleven determinations of the analyte at 50 ng/mL. The LOD of this method was one order of magnitude lower than that of the reported chemiluminescence method[10] and lower than those of bulk HPLC methods[2]. The high assay sensitivity allowed a high fold dilution of the samples before analysis to avoid sample matrix effects. The proposed chemiluminescence system has satisfactory linearity, sensitivity and precision.

**Sample analysis:** The proposed method and UV-method[4] were applied for the determination of 5-fluorouracil in its injections with labeled content of 0.25 g/bottle. The results obtained by the two methods were 0.260 and 0.253 g/bottle, respectively and there was no significant difference between the results and the labeled contents based on Student *t*-test ($p = 0.05$).

In order to evaluate the validity of the proposed method, recovery test was carried out on the injection samples to which known amounts of 5-fluorouracil were added at three concen-

TABLE-1
PERFORMANCE OF THE METHOD FOR DETERMINATION OF 5-FLUOROURACIL

| Regressive equation | Linear range (ng/mL) | Correlation coefficient (r) | LOD (ng/mL) | RSD n = 11 |
|---|---|---|---|---|
| y = 833.7 Log C-323.33 | 10 -5000 | 0.9954 | | |
| y = 26.79 C + 129.2 | 10-500 | 0.9992 | 3.5 | 1.7 |
| y = 0.4469 C + 1569 | 500-5000 | 0.9948 | | |

TABLE-2
DETERMINATION OF 5-FLUOROURACIL IN SERUM AND URINE SAMPLES

| Sample | Content (ng/mL) | Added (ng/mL) | Found (ng/mL) | Recovery (%) | RSD, n = 5 |
|---|---|---|---|---|---|
| Injection | 50 | 40.0 | 89.3 | 98.3 | 2.4 |
| | | 50.0 | 100.7 | 101 | 1.9 |
| | | 60.0 | 109.3 | 98.8 | 1.5 |
| Serum | nd | 5.0 | 4.83 | 96.6 | 3.7 |
| | | 20.0 | 20.86 | 104 | 2.8 |
| | | 50.0 | 49.89 | 99.8 | 1.2 |
| Urine | nd | 5.0 | 4.73 | 94.6 | 1.5 |
| | | 35.0 | 35.40 | 101 | 2.4 |
| | | 50.0 | 49.33 | 98.7 | 1.4 |

nd: not detected

tration levels. The result is given in Table-2. The recovery was 98.3-101 % with the RSD of 1.5-2.4 %.

It was previously reported that the concentration of 5-fluorouracil in the serum was 0.28-1.25 μg/mL after injection of 500 mg 5-fluorouracil[20]. The urine and serum samples of patient after the administration of 5-fluorouracil could not be obtained, so urine and serum samples were taken from healthy postulant. The proposed method was applied for the determination of 5-fluorouracil in the samples spiked with 5-50 ng/mL.

Table-2 showed that for spiked serum samples the recovery of 5-fluorouracil was in the range of 96.6-104 % with an RSD of 1.2-2.8 % and for spiked urine samples the recovery was in the range of 94.6-101 % with an RSD of 1.4-2.4 %.

## Conclusion

Based on the strong enhancing effect of 5-fluorouracil on luminol-$[Ag(HIO_6)_2]^{5-}$ chemiluminescence reaction, a new flow-injection chemiluminescence method was proposed for the determination of 5-fluorouracil. The new method offers several advantages over other methods, such as being faster, using simple instrumentation and the reagents being stable and inexpensive. It is indicated that the proposed method provides high sensitivity necessary for analysis of 5-fluorouracil in pharmaceutical and biological fluids at clinically relevant concentrations.

## ACKNOWLEDGEMENTS

This work was supported by the Natural Science Foundation of Hebei Province (B2014201171).

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