Cetyltrimethylammonium Bromide-Enhanced Chemiluminescence Determination of Uric Acid Using a Luminol-Hexacyanoferrate(III)-Hexacyanoferrate(II) System

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A chemiluminescence (CL) method using flow injection (FI) was developed for the determination of uric acid based on the enhancement chemiluminescence intensity of luminol-hexacyanoferrate(III)-hexacyanoferrate(II) in the presence of cetyltrimethylammonium bromide and the uric acid species. The linear range was $7.0 \times 10^{-10} - 9.0 \times 10^{-7}$ M with a detection limit ($3\sigma$) of $2.58 \times 10^{-10}$ M, which was about two orders of magnitude lower than those reported. The proposed method was used for the determination of uric acid in real samples.

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The determination of uric acid in human urine and serum is very important due to the need for data in the diagnosis and therapy of patients suffering from a range of disorders, such as gout, hyperuricemia, and Lesch-Nyhan syndrome. On the other hand, as a substitute antioxidant, it may play a protection role, because it is involved in many pathological changes and/or damage development. So it is important and necessary to build better detection limits and higher sensitivity methods for the uric acid determination: one need is for the determination of lower physiological uric acid levels based on its higher sensitivity and the other need is for higher physiological uric acid levels based on its higher selectivity by proper diluting the real samples. Different kinds of uric acid detection methods have been developed. These generally suffered from low sensitivity and/or from other problems such as the fouling of electrode in electrochemistry.

The advantages of CL analysis include low limits of detection and a wide linear range, both can generally be achieved with simple, robust, and relatively inexpensive commercial instrumentation. Regarding the determination of uric acid by CL, there are a few references dealing with the issue. The luminol-K$_3$Fe(CN)$_6$-K$_4$Fe(CN)$_6$ system was reported by Kubo et al. and Zhang et al. Details of another CL method based on the enhancement or inhibition CL intensity have been reported. Although these methods could determine trace amounts of uric acid in human urine, they suffered from poor selectivity and unstable and/or expensive reagents. They were also time consuming; a certain amount of uric acid might be oxidized during the time needed.

Due to various unique and advantageous properties of surfactants, which should better facilitate analytical CL measurement, there are a number of reports concerning the application of various kinds of surfactants in CL measurement. Therefore, we investigated the effects of surfactants on emission intensity in luminol-K$_3$Fe(CN)$_6$-K$_4$Fe(CN)$_6$ system and reported significant and useful improvements in signal strength. To the authors’ best knowledge, although the detection of uric acid with luminol-K$_3$Fe(CN)$_6$-K$_4$Fe(CN)$_6$ system had also been described, this was the first method for the determination of uric acid with cetyltrimethylammonium bromide (CTMAB) as sensitizer. On this basis, a simple, sensitive, fast and direct FI-CL method for the determination of uric acid in human fluid was developed. The calibration graphs were linear in the range of $7.0 \times 10^{-10} - 9.0 \times 10^{-7}$ M and a detection limit ($3\sigma$) was $2.58 \times 10^{-10}$ M, which was about two orders of magnitude lower than those reported.

**Experimental**

*Reagents and solutions*

All the chemicals were of analytical reagent grade. Distilled deionized water purified by an Ultra-Pure Water System (Germany) was used throughout. Stock solutions of both 0.01 M luminol (Shannxi Normal University, China) and 1.06 $\times$ 10$^{-3}$ M uric acid (Sigma) were prepared in 0.1 M NaOH, and stored below 4°C. A standard solution for calibration was prepared freshly from the stock solution after adjusting the solution pH to 3.5 before analysis. Other solutions including 1.76 $\times$ 10$^{-3}$ M K$_3$Fe(CN)$_6$, 0.5 M K$_4$Fe(CN)$_6$, and 0.01 M CTMAB were used as received.

*Apparatus and procedures*

A schematic diagram of the flow system was shown in Fig. 1. Two peristaltic pumps were used to deliver all of the flow streams at the flow rate of 2.5 ml/min. The sample solution (50 µl) was injected through the sample injection valve. PTFE tubing (0.8 mm i.d.) was used to connect all components in the flow system. A mixing coil was made by coiling a piece of glass tubing (100 mm × 0.8 mm i.d.) into a spiral disk shape and placing it close to the photomultiplier tube. The CL emission was recorded with a flow injection CL analyzer (IFFM-D,
Results and Discussion

Kinetic profiles

The CL intensity as a function of time was investigated. Figure 2 depicts kinetic curves of the reaction system for uric acid in three different media including neutral (curve 1) and acidic (curve 2) as well as in the presence of CTMAB (curve 3), and clearly demonstrated that all CL reactions were a fast-type luminescence. The time interval between the start of CL and its maximum was 2.0 s for both curves 1 and 2, but 4.0 s for curve 3, and then all decayed quickly. It was also observed that the addition of CTMAB postponed the reaction process, but enhanced CL intensity dramatically.

Optimum conditions for the determination of uric acid

The proper mixing order of reagent solutions is extraordinarily crucial in a CL reaction. In order to obtain the highest CL signal, we checked two mixing orders of reagents by flow injection method. The first was to merge the solution of K₃Fe(CN)₆ with the luminol-K₄Fe(CN)₆-CTMAB-NaOH mixture; and the second was to merge the K₃Fe(CN)₆-NaOH mixture with the luminol-K₄Fe(CN)₆-CTMAB mixture. The results indicated that the first one gave a stronger CL emission and improved the S/N ratio.

Since the CL was observed only in an alkaline medium, the effect of NaOH concentration was tested in the range of 0.1 - 0.7 M. The result showed that the CL signal was the highest and remained stable at 0.6 M. Therefore, 0.6 M NaOH was selected as the reaction medium in the following study.

The CL emission was examined over the range of 1.76 × 10⁻⁵ – 2.11 × 10⁻⁴ M in 0.6 M NaOH solution. Maximum CL intensity was obtained with 1.0 × 10⁻⁴ M K₃Fe(CN)₆; above this value, the intensity was decreased.

The effect of flow rate on intensity was studied in the range of 1.0 – 5.0 ml/min. The CL emission increased with the flow rate, reaching a maximum and constant value at 2.5 ml/min or higher rates. Therefore, this value was selected, since higher rates led to overpressure in the connectors and excessive reagent consumption with little gain in sensitivity.

Effect of sensitizers

In order to investigate which kind of surfactants possessed the best enhancing effect upon the present CL reaction, we studied in detail different types of surfactants, such as two cationic surfactants (CTMAB and CPB), two non-ionic surfactants (Tween 80 and OP), and two anionic surfactants (SDBS and SDS). The experimental results showed that CTMAB enhanced the signals dramatically. Other non-ionic surfactants gave a somewhat enhanced signals whereas anionic surfactants were less effective. Therefore, CTMAB was selected for the following study. The effect of K₃Fe(CN)₆ concentration on the CL intensity was examined in the range of 0.025 – 0.2 M K₃Fe(CN)₆. The maximum intensity was obtained with 0.075 M K₃Fe(CN)₆. Higher concentrations of K₃Fe(CN)₆ lowered the CL intensity.

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Effect of uric acid species

It is well known that the uric acid is a weak acid (pKₐ 5.75). Thus, the uric acid species is strongly dependent on the pH value, as shown in Fig. 4. To identify the effect of uric acid species on the CL signal, we investigated the CL intensity of uric acid in different media, including neutral, acidic and alkaline, and found that acidified uric acid, viz., the molecular species of uric acid, gave the best signal. Furthermore, four different acids (HCl, HNO₃, H₂SO₄ and H₃PO₄) were tested in order to ascertain which was the most suitable. The results showed that HCl gave the highest intensity and that the maximum CL signal occurred at pH = 3.5 (as shown in Fig. 5).

Calibration curve and detection limit

Under the optimum conditions described above, the calibration of emission intensity versus uric acid concentration was linear at the range of 7.0 × 10⁻¹⁰ – 1.0 × 10⁻² M (r = 0.9996) and 1.0 × 10⁻⁷ – 9.0 × 10⁻⁵ M (r = 0.9997); the regression equations were Y = 60099X – 260.25 and Y = 33026X – 625.6, respectively (Y was the relative CL intensity and the X was the concentration of uric acid (µM)). The detection limit of uric acid was 2.58 × 10⁻¹⁰ M (3σ). Figure 6 shows the spectra of five consecutive injections of water (1) and uric acid (2.58 × 10⁻¹⁰ M) (2). The RSD (n = 11) was 0.82% for 1.0 × 10⁻³ M uric acid.

Interference study and application

The effect of common components in human serum and urine on the determination of uric acid was studied. One species was not considered to interfere if it caused a relative error of less than 5% for the determination of 1.0 × 10⁻³ M uric acid. The tolerated ratios of common components in serum and urine to 1.0 × 10⁻³ M uric acid were 300 fold for glucose, 200 fold for urea, 100 fold for sucros, and creatinine, 40 fold for Zn²⁺, 20 fold for creatine and 5 folds for Vc²⁺.

The proposed method was applied to the determination of uric acid in human serum and urine. Without any pretreatment, sample solutions were prepared by directly diluting urine and serum samples with water, so that the final concentration of uric acid was within the working range of determination. The results are listed in Table 1. Recoveries were carried out on samples to which known amounts of uric acid were added.

Possible mechanism of the present CL reaction

The CL emission spectrum was examined by using the fluorescence spectrophotometer. In the luminol-hexacyanoferrate(III)-hexacyanoferrate(II)-CTMAB system, the maximum CL emission wavelength of 425 nm corresponded with results reported in luminol-hexacyanoferrate(III)-hexacyanoferrate(II) system, which suggested that luminol was oxidized to excited 3-aminoththalate by K₃Fe(CN)₆ in alkali medium and hence emitted CL signals. The results showed that CTMAB was not a CL illuminant in the system.

The following experiments were carried out for the further investigating the roles of dissolved oxygen in the reaction; all solutions were purged with nitrogen or oxygen for 5 min. When all solutions were purged with the flow of nitrogen, the relative CL intensity decreased about 40%. These results indicated that the dissolved oxygen played an important role in the reaction, which was in agreement with several reports.

It is well known that the type of species has great effect on its CL intensity. Our experiments indicated that molecular species of uric acid enhanced CL intensity markedly. We think the reason is that uric acid in its molecular species has several pathways that affect the quantum yield and reaction rate, had been discussed by Lin et al. and Townshend et al. In this work, a micelle formed from a cationic surfactant had a hydrophobic center, but allowed ionic interactions at the outside surface. The luminol in alkaline medium was a divalent anion, and the luminol and superoxide radicals and hexacyanoferrate(III)-hexacyanoferrate(II) were attracted onto the micellar surface, which made the interaction of luminol, the

![Fig. 4](image-url) Absorbance of uric acid. Curve 1, neutral uric acid; curve 2, acidified uric acid; curve 3, the mixing of acidified uric acid with CTMAB; luminol, 7.0 × 10⁻⁴ M; K₄Fe(CN)₆, 1.0 × 10⁻⁴ M; K₃Fe(CN)₆, 0.075 M; NaOH, 0.6 M; uric acid, 1.0 × 10⁻³ M; CTMAB, 6.8 × 10⁻⁴ M.

![Fig. 5](image-url) Effect of uric acid species on the CL intensity. Luminol 7.0 × 10⁻⁴ M, K₄Fe(CN)₆ 1.0 × 10⁻⁴ M, K₃Fe(CN)₆ 0.075 M, NaOH 0.6 M, CTMAB 6.8 × 10⁻⁴ M.

![Fig. 6](image-url) The peaks of consecutive injections of water (1) and 2.58 × 10⁻¹⁰ M uric acid (2). Luminol 7.0 × 10⁻⁴ M, K₄Fe(CN)₆ 1.0 × 10⁻⁴ M, K₃Fe(CN)₆ 0.075 M, NaOH 0.6 M, CTMAB 6.8 × 10⁻⁴ M.
superoxide radical and hexacyanoferrate(III)–hexacyanoferrate(II) easy and effective, and hence enhanced the CL of the system. On the other hand, this explained that SDS could not enhance the CL intensity perhaps because SDS could not bring the superoxide radical into better contact with the luminol.

The reaction mechanism can be expressed as follows:

\[
\text{uric acid} + O_2 (\text{aq}) + \text{NaOH} \rightarrow \text{superoxide radical} + \text{luminol} \\
\text{superoxide radical} + \text{luminol} \rightarrow \text{excited aminophthalate} \\
\text{(on the outside surface of surfactant and with hexacyanoferrate(III)-hexacyanoferrate(II) as catalyst)} \\
\text{excited aminophthalate} \rightarrow \text{aminophthalate} + h\nu
\]

**Conclusion**

This paper has described a new FI-CL method for uric acid determination with CTMAB as a sensitizer and molecular species of uric acid as the sample. The proposed method was particularly attractive because of its wide range of four orders of magnitude and its very low detection limit compared with those of other techniques. The procedure was fast and could be accomplished without using any expensive or dangerous reagents. Furthermore, the CL detection system was fairly simple and easy to manipulate. It was believed that it would be useful for the quantitative detection of uric acid for diagnostic and research purposes.

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