Determination of folic acid by chemiluminescence based on peroxomonosulfate-cobalt(II) system

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Abstract

Based on the chemiluminescence (CL) phenomena of folic acid in peroxomonosulfate-cobalt(II) system, a rapid and sensitive CL method was developed for determination of folic acid in pharmaceutical preparations and its urinary metabolism processes. Under the optimum conditions, the relative CL intensity was linear over the concentration ranging from 10^{-9} to 8×10^{-7} mol L⁻¹ ($R^2=0.9991$) with a detection limit as low as 6×10^{-10} mol L⁻¹ (S/N = 3) and relative standard deviation was 2.63% for 2×10^{-8} mol L⁻¹ folic acid ($n=11$). This method has been successfully applied to the determination of folic acid in tablets and human urine. The blank CL emission was yielded owing to the formation of singlet oxygen molecular pair from the quenching experiment of 1,4-diazabicyclo[2.2.2]octane, and pterine-6-carboxylic acid might be the degradation intermediate in this system and it also acts an energy acceptor and sensitizes the chemiluminescence based on the studies of the CL and fluorescence spectra.

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1. Introduction

Folic acid, otherwise known as vitamin M or pteroylglutamic acid (PGA), is made up of a bicyclic pterine linked by a methylene bridge to *para*-aminobenzoic acid, which is joined by peptide linkage to a single molecule of L-glutamic acid. Folic acid is susceptible to cleavage under acidic conditions, light, and high temperature [1]. Folic acid can promote the formation of red blood cells and is identified as an anti-anemia and growth factor. It prevents neural tube defects like spina bifida, while its ability to lower homocysteine suggests it might have a positive influence on cardiovascular disease. The role of this vitamin in maintaining good health may extend beyond these clinical conditions to encompass other birth defects, several types of cancer, dementia, affective disorders, Down's syndrome, and serious conditions affecting pregnancy outcome [2,3].

Therefore, it is important to develop simple, sensitive and accurate methods for being able to detect folic acid. But analysis of folic acid is not easy due to its lower stability, presence in lower concentration in biological systems, and complex extraction and detection techniques. Methods of analysis for folic acid are grouped into microbiological, bio-specific procedures, chromatographic, and chemical methods [1]. Although microbiological assay is the most commonly used method, it is time consuming, needs great care and skill [4]. Chromatographic methods have the advantage of separating and quantifying different forms of folic acid and its derivations and minimum interference from enzymes but involve set up cost, a complex extraction, and purification procedure [5,6]. Enzyme protein binding assay is much cheaper, rapid, and easier but there exists considerable variation between different kits and self-life of kits is very short [7].

Chemiluminescence (CL) is a powerful analytical technique that has excellent sensitivity, wide linear dynamic range and requires relatively simple and inexpensive instrumentation [8,9]. To our knowledge, there were a few reports for the determination of folic acid based on CL analysis system [10–12]. But the proposed methods had their own shortages in the respectively

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CL system such as poor linearity, lack of selectivity or high limit of detection. Furthermore, they almost had been developed for the measurement of folic acid only in tablets.

In our research, we found that a strong CL signal was given out when a trace amount of folic acid was added to peroxomonosulfate(PMS)/Co(II) mixed solution and the CL intensity was strongly dependent on folic acid concentration. Based on this phenomenon, a new, rapid, simple, sensitive and inexpensive method is proposed to determine folic acid. Comparing with the other CL methods, it has more widely linear range and much lower detection limit. Furthermore, the method has been used for the determination of folic acid not only in real pharmaceutical preparations but also in human urine with satisfactory results.

2. Experimental

2.1. Reagents

All the reagents used in these experiments were of analytical grade or higher without further purification. Water was purified using a compact ultra pure water system ($18.3 \text{ M}\Omega \text{ cm}^{-1}$, Barnstead, Iowa, USA). A $10^{-4} \text{ mol L}^{-1}$ folic acid stock solution was prepared by dissolving 4.41 mg of folic acid (Sigma–Aldrich, St. Louis, USA) in 1 mL of 0.1 mol L^{-1} NaOH solution and diluting to 100 mL with water, then stored at 4°C and protected from the light. Solutions of KHSO_5 available in the form of a triple salt ($2\text{KHSO}_5 \cdot \text{KHSO}_4 \cdot \text{K}_2\text{SO}_4$) as Oxone (Alfa Aesar–A Johnson Matthey Company, Ward Hill, USA) and cobalt(II) sulfate heptahydrate (Beijing Chemical Reagent Company, Beijing, China) were prepared daily. 1,4-Diazabicyclo[2.2.2]octane (DABCO) is the products of Acros Organics (New Jersey, USA). Cobalt(II) chloride hexahydrate, cobalt(II) acetate tetrahydrate and cobalt(II) nitrate hexahydrate were purchased from Beijing Chemical Reagent Company (Beijing, China).

2.2. Apparatus and procedure

Batch chemiluminescence experiments were carried in a BPCL luminescence analyzer (Institute of Biophysics, Chinese Academy of Sciences, Beijing, China). A flow CL analyzer (Lumiflow LF-800, Microtec NITI-ON, Funabashi, Japan) was used for flow-injection chemiluminescence experiments. UV–vis absorbance spectra were collected by a UV-2401 Spectrophotometer (Shimadzu, Japan) and the fluorescence spectra were measured by an F-2500 fluorescence spectrometer (Hitachi, Japan).

The flow-injection analysis (FIA) system is illustrated in Fig. 1. PTFE tubing (1.0 mm i.d.) was used as connection material in the flow system. Reagent solutions (R1–R3) were delivered by two peristaltic pumps (SJ-1211, Atto, Tokyo, Japan) through three flow lines. Folic acid standard solution or sample solution was injected into the carrier stream (water) with a $150 \mu\text{L}$ loop injector. The flow cell was a flat spiral-coiled colorless glass tube (1.0 mm i.d.; total diameter of the flow cell, 3 cm) and placed closed to the window of the photomultiplier tube (PMT, operated at -800 V). Before each measurement, the instruments were allowed to carry solutions for 10 min to achieve

Fig. 1. Schematic diagram of the flow-injection system. R1, H_2O as carrier stream; R2, $0.01 \text{ mol L}^{-1} \text{HSO}_5^-$; R3, $0.01 \text{ mol L}^{-1} \text{Co}^{2+}$; P1 (4.0 mL min^{-1}), P2, P3 (0.5 mL min^{-1}), peristaltic pump; S, $150 \mu\text{L}$ sample injector; M, mixing tube; F, flow cell; W, waste; PMT, photomultiplier tube.

good mechanical and thermal stability. The concentration of folic acid was quantified via the peak height of the relative CL intensity obtained by subtracting the blank CL intensity which emitted when mixing PMS and Co(II) solutions.

2.3. Sample preparation

The tablets were purchased from Changzhou Pharmaceutical Factory (sample 1, Changzhou, China) and Tianjin Feiying Pharmaceutical Co. Ltd. (sample 2, Tianjin, China). Not less than 10 tablets were ground into fine power and mixed. A sample equivalent to one tablet were weighed accurately and dissolved in 1 mL of 0.1 mol L^{-1} NaOH solution and diluting to 100 mL with water. Then the solution was filtered and appropriately diluted when determined.

Urine samples were collected in dark glass bottle and diluted at 10^4 with distilled water before determination. Sometimes urine samples were supplemented with folic acid to test the recovery of the method. In view of the photo lability of folic acid, all the sample solutions should be protected from blazing light during the experiment.

3. Results and discussion

3.1. Batch chemiluminescence

The catalytic decomposition of peroxymonosulfate by cobaltous ion was first reported in 1956 [13], and cobalt was proved to be the best catalyst activator of this peroxide [14]. The dynamic profile of chemiluminescence of folic acid in PMS/Co(II) in batch experimental is shown in Fig. 2. From the chemiluminescence (CL) kinetic curve, a weak luminescence was yielded when the CoSO_4 , $\text{Co(NO}_3)_2$ and CoCl_2 solution was added to PMS solution, which would be the blank of determination of PGA in this system. There was a strong CL when the cobalt acetate (CoAc_2) solution was added to PMS solution, whose blank would be too high to suit for CL analysis, because aliphatic monocarboxylic acids, such as acetic and butyric, were found to enhance the CL emission generation from the PMS/ CoSO_4 reaction [15]. A very strong luminescence was given after the adding of the solution of $10^{-6} \text{ mol L}^{-1}$ PGA to the mixing solution of PMS and Co(II) except PMS/ CoCl_2 solution whose intensity as low as its blank. The latter phenomenon might due to the fact

Fig. 2. Effect of counter ion of Co(II) on the chemiluminescence. The concentrations of folic acid, HSO_5^- , and Co^{2+} are 10^{-6} , 0.01 and 0.01 mol L $^{-1}$, respectively. The volume of each reagent is 50 μL . Different Co^{2+} solution is injected into system firstly (at time of 25–45 s in the graph), and then folic acid is injected into system after 30 s.

that the peroxymonosulfate ion can be consumed by oxidizing chloride ion through reactions (1) and (2) [16].

So the counter ion of cobalt greatly affected the CL signal greatly. For further experiments, CoSO_4 was employed since PMS itself contained sulfate and hydrogen sulfate anions. Based on the results of batch chemiluminescence, the flow-injection analysis (FIA) system was developed for determination of folic acid.

3.2. Determination of PGA by FIA-CL of PMS/Co(II) system

3.2.1. Optimization of flow-injection CL system

In order to determinate the optimum operating condition of the flow system, the CL intensity for 2×10^{-8} mol L $^{-1}$ folic acid was measured with respect to the reaction variables.

The CL intensity decreased with the length of mixing tube according to Fig. 3A because of the catalytic decomposition of peroxymonosulfate, so it was no necessary to set up mixing tube and PMS and Co(II) was mixed at the input of the flow CL analyzer, which was a little different from our previous researches [17,18]. The CL intensity decreased slightly with the flow rates of the reagent solutions in the range of 0.5–2.5 mL min $^{-1}$ from Fig. 3B, so the flow of 0.5 mL min $^{-1}$ was select as an appropriate condition in the view of analytical precision and lower solution consumption. But the CL intensity increased almost linearly with the flow rates of carrier steam in the range of 0.5–5.0 mL min $^{-1}$ because the higher flow rate of the carrier solution was, the more enough the reagent solutions mixed in the flow cell. A flow rate of 4.0 mL min $^{-1}$ was selected considering the stability of peristaltic pump.

The concentration of PMS and Co(II) had a very important effect on the relative CL intensity for the determination of folic

Fig. 3. Effect of mixing tube length (A), flow rate (B) and PMS or Co concentration (C) on relative chemiluminescence intensity in FIA system ($n=5$).

acid, as is shown in Fig. 3C. The effect of PMS concentration on the CL signal was investigated in the 0.002–0.06 mol L $^{-1}$ range. The relative CL intensity reached its peak at the concentration of 0.01 mol L $^{-1}$ PMS, so it was operated as one of the FIA optimum condition. Although the CL signal increase with the concentration of CoSO_4 in the range of 0.001–0.05 mol L $^{-1}$, the relative CL intensity grew nearly stable when concentration was over 0.01 mol L $^{-1}$. So the concentration of 0.01 mol L $^{-1}$ CoSO_4 was select in further experiments for lower cobalt consumption.

Fig. 4. Typical recorder response for the determination of standard folic acid solution ($10^{-8} \text{ mol L}^{-1}$).

3.2.2. Analytical performance

Under the optimum experimental conditions as above, the calibration curve of relative CL intensity versus folic acid concentration over range 10^{-9} to $8 \times 10^{-7} \text{ mol L}^{-1}$ was obtained. Fig. 4 shows typical recorder response for the determination of standard folic acid solution. The relative CL intensity which has subtracted the blank from the maximum peak increased linearly with the increasing PGA concentration, as expressed by the equations $\Delta I = 3.095 C_{\text{FA}} (10^{-9} \text{ mol L}^{-1}) + 17.53$ ($R^2 = 0.9991$).

The determination limit was $6 \times 10^{-10} \text{ mol L}^{-1}$ ($S/N=3$) of folic acid in the PMS/Co(II) system. The present method has a wider linear range and much lower detection limit than previously reported [10–12]. The relative standard deviation was found to be 2.63% by 11 replicate determination of $2 \times 10^{-8} \text{ mol L}^{-1}$ folic acid.

3.2.3. Interference studies

The effect of various interferences for determination folic acid was investigated. The tolerable concentration ratios with respect to $2.0 \times 10^{-8} \text{ mol L}^{-1}$ folic acid for interference at 5% level were examined. Thousand-fold excess lactose, glucose, fructose, dextrin, starch, magnesium stearate, albumin, Na^+ , Al^{3+} , Mg^{2+} , Zn^{2+} , Ca^{2+} , NO_3^- , CO_3^{2-} , HPO_4^{2-} , NH_4^+ , 500-fold urea, uric acid, Fe^{2+} , 100-fold ascorbic acid, Cl^- , 10-fold I^- , had almost no effect on the determination of PGA.

3.2.4. Determination of folic acid in pharmaceutical tablets

The excipients of starch, lactose and magnesium stearate in PGA tablets do almost not interfere in the determination, so the flow method proposed has adequate selectivity for the analysis of folic acid in pharmaceutical preparation analysis. The measured folic acid contents are listed in Table 1. The recoveries for PGA in spiked samples were found to be acceptable, between 98 and 104%. The results obtained agreed well with the PGA content that labeled on the bottle of pharmaceutical preparations.

3.2.5. Determination of folic acid in human urine

The proposed method was also applied to the determination of folic acid in human urine samples. Folic acid tablets (10 mg) were taken orally in morning with empty stomach. From then on, urine samples were collected in dark glass bottle periodically. After urinary PGA was diluted, it could be determined by this FIA-CL method without any pre-treatment. The results of the

Table 1

Determination of folic acid in tablet samples of different companies

Sample	Sample 1		Sample 2	
	1	2	1	2
Measured ($10^{-8} \text{ mol L}^{-1}$)	1.14	2.41	1.13	2.42
R.S.D ($n=5$, %)	3.62	0.77	4.14	5.64
Added ($10^{-8} \text{ mol L}^{-1}$)	1.00	2.00	1.00	2.00
Total ($10^{-8} \text{ mol L}^{-1}$)	2.18	4.43	2.11	4.46
Recovery (%)	1.04	1.01	0.98	1.02
Content (mg tablet^{-1})	5.04	5.32	4.99	5.35
Label (mg tablet^{-1})	5.00	5.00	5.00	5.00

Sample 1: Changzhou Pharmaceutical Factory, Sample 2: Tianjin Feiying Pharmaceutical Co. Ltd.

Fig. 5. Metabolism of folic acid in human urine ($n=3$).

metabolic profile of PGA are shown in Fig. 5. The excretive folic acid reached a maximum in 5 h after taking the tablets, and the total PGA excreted through urine was 7.61 mg in total volume 1551 ml in 10 h.

3.3. The chemiluminescence mechanism of PGA in the PMS/Co(II) system

The transition metal ion-catalyzed decomposition of peroxymonosulfate has been reported by several studies. And cobalt, the best catalyst, activates the PMS through reactions (3)–(5) with the formation of several radical, such as $\text{SO}_5^{\bullet-}$, $\text{SO}_4^{\bullet-}$, $\bullet\text{OH}$, which have been identified by optical pulse radiolysis [14,19–22].

The evident blank CL emission was yielded from Fig. 2 owing to the formation of singlet oxygen molecular pair, $(^1\text{O}_2)_2^*$, which has higher energy than the ground state triplet oxygen. CL phenomena occur when the molecular pair lose excess energy and turn to the latter according to reactions (6)–(9) [19,20,23,24].

Table 2
Effect of DABCO on the CL signal

Concentration (mol L ⁻¹)	Blank (Count)	Signal (Count)
0	64	2351
10 ⁻⁶	52	1895
10 ⁻⁵	45	1592
10 ⁻⁴	32	1383
10 ⁻³	11	1244
10 ⁻²	Not detected	Not detected

The concentrations of folic acid, HSO₅⁻, and Co²⁺ are 10⁻⁶, 0.01 and 0.01 mol L⁻¹, respectively. The volume of each reagent is 50 μL.

And the mechanism can be confirmed by the effect of the singlet oxygen quencher 1,4-diazabicyclo[2.2.2]octane (DABCO) [25], as shown in Table 2. The blank and the signal CL emission all decreased with the increasing of the DABCO concentration, and the CL cannot be detected when the DABCO concentration is above 0.01 mol L⁻¹. And it can also infer from the phenomena that the CL signal of the system was given out by energy transfer from higher energy state singlet oxygen.

Which compound acts the energy acceptor and sensitizes the CL emission is a little difficult to interpret. Folic acid might not be the acceptor, because its fluorescence intensity is very weak from Fig. 6. The maximum fluorescence wavelength of folic acid is 446 nm which is different from the maximum CL wavelength located at ca. 460 nm according to Fig. 7. The fluorescence intensity and the adsorption of PGA were decreased to blank very quickly under experimental concentration of PMS and Co(II) solutions, so it is difficult for us to find degradation intermediates that might be CL acceptor.

Interestingly, the fluorescence intensity increased dramatically and the maximum fluorescence wavelength shifted from 446 to 465 nm after folic acid solution was treated with ultraviolet (UV) irradiation. High performance liquid chromatography methods have been used for the assay of folic acid and its

Fig. 6. Fluorescence spectra of folic acid and after irradiated with high pressure mercury lamp for 1 h. The concentration of folic acid was 10⁻⁵ mol L⁻¹.

Fig. 7. CL spectra of the Co/PMS/PGA reaction. The concentrations of folic acid, HSO₅⁻, and Co²⁺, are 10⁻⁶, 0.01 and 0.01 mol L⁻¹ respectively. The volume of each reagent is 50 μL. Batch CL of each spectrum was repeated three times.

photodegradation products [26]. The photolysis of folic acid showed that it degraded to pterine-6-carboxylic acid (PCA) and *p*-aminobenzoyl-glutamic acid, as are concluded in Scheme 1. The fluorescence spectrum of UV-irradiated folic acid was quite similar to that of pterine-6-carboxylic acid, which is a strong fluorescence molecule. But the pteridine moiety of folic acid shows very weak fluorescence, which is due to an intramolecular electron transfer from the *p*-aminobenzoyl-glutamic acid moiety to the pteridine moiety [27,28].

The maximum emission fluorescence wavelength of pterine-6-carboxylic acid is 465 nm, which is accordance with the CL spectrum of folic acid in PMS/Co(II) system. It can be inferred that PCA might be the degradation intermediate in PMS/Co(II) system and it also acts an energy acceptor and sensitizes the CL emission. PCA and other intermediates were oxidized to inorganic compound by the radicals, such as SO₅^{•-}, SO₄^{•-}, •OH, from the fact that the fluorescence intensity and the adsorption

Scheme 1. The photodegradation of folic acid.

of PGA were decreased to blank [29]. The chemiluminescence mechanism of PGA in the PMS/Co(II) system can be summarized as the following reactions:

4. Conclusion

A flow-injection chemiluminescence method based on peroxomonosulfate-cobalt(II) system was proposed for determination of folic acid. The presented method had a good sensitivity, selectivity, precision and wider linear range, which allowed application in determination of PGA in pharmaceutical preparations and biological sample analysis. Additionally, the enhancing of the chemiluminescence emission by folic acid due to the energy transfer from $^1\text{O}_2$ to its degradation intermediate pterine-6-carboxylic acid was confirmed.

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