Reagentless chemiluminescence flow sensor for the determination of riboflavin in pharmaceutical preparations and human urine

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A novel continuous-flow sensor based on chemiluminescence (CL) detection was developed for the determination of riboflavin at pg ml\(^{-1}\) levels by the immobilization of the reagents. It was found that the CL intensity from the oxidation between luminol and periodate could be enhanced in the presence of riboflavin. The increase of CL emission was correlated with the riboflavin concentration in the range from 0.04 to 200 ng ml\(^{-1}\), and the detection limit was 0.02 ng ml\(^{-1}\) (3\(\sigma\)). Considering the effective reaction ions, luminol and IO\(_4^-\) was immobilized on anion-exchange resin. The system could produce an evident CL signal by water as eluant and it was also shown that the flow sensor could greatly improve the selectivity and sensitivity for determination of riboflavin with a high signal-to-noise ratio. A complete analysis, including sampling and washing, could be performed in 0.5 min with a relative standard deviation of less than 3.0%. The flow sensor was applied successfully to the determination of riboflavin in pharmaceutical preparations and human urine samples.

Introduction

Riboflavin is one of the water-soluble B-group vitamins essential to general health. The deficiency of riboflavin is associated with numerous diseases. Orally administered riboflavin appears mainly in a free state in biological tissues and is excreted through urine for the most part (more than 60% of single oral dosage).\(^1\) It is generally agreed that the classical microbiological procedures for measuring haematic and urinary riboflavin are tedious, may be subject to interference by unknown factors, and require up to 72 h before the results can be obtained.\(^2\) The protein-binding assay is a sensitive and specific assay but requires time-consuming preparation of extracts of the riboflavin and the evaporation of the extraction prior to running the assays.\(^3\)–\(^5\) Some sensitive methods depend upon the fluorescence properties\(^6\)–\(^12\) and electrical activity\(^13\)–\(^15\) of the riboflavin. When dealing with biological samples, these methods also require considerable preparation and/or treatment of the sample before and during the assay. Other analytical methods for the determination of riboflavin have been reported, including spectrophotometry,\(^16\)–\(^18\) IR\(^19\) and chemiluminescence (CL).\(^20\)–\(^23\) To achieve a selective detection, the techniques of HPLC\(^22\)–\(^26\) and capillary electrophoresis\(^27\) were applied to detect riboflavin in complex samples, such as multivitamin tablets and food.

With the movement toward automation in sample assays, more and more research is being done with continuous-flow systems. This format is inherently easier to lead to rapid results and there is great flexibility as to the type of detection system. Gong and Zhang developed a flow-injection (FI) optosensor\(^10\)–\(^11\) using silica gel or immobilized \(\beta\)-cyclodextrin as a fluorescence-enhancing reagent. A drawback to these approaches is that the solid phase needs to be regenerated after each injection. Some attempts to use CL detection in FI systems were also reported. In a previous paper,\(^28\) we have proposed an FI method based on the K\(_2\)Fe(CN)\(_6\)-luminol CL system for the determination of riboflavin, and the limit of detection was 0.01 µg ml\(^{-1}\). Perez-Ruiz \textit{et al.}\(^29\) also described an FI-CL method for determination of riboflavin and riboflavin 5’-phosphate based on their reduced forms, RFH\(_2\) and FMNH\(_2\), decreasing the H\(_2\)O\(_2\)-luminol CL emission intensity. However, this method has a complex device to produce RFH\(_2\) and FMNH\(_2\).

It is well known that luminol reacts with periodate\(^30\),\(^31\) in alkaline medium, producing strong CL. We found that the CL intensity from the oxidation between luminol and periodate could be enhanced in the presence of riboflavin. The CL reagents, luminol and periodate, used in this sensor, were both immobilized on anion-exchange resin. Through the injection of 200 µl eluant, the reagents on the anion-exchange resin column were eluted and in the presence of riboflavin, the CL intensity was enhanced, by which riboflavin could be sensed. The increase of the CL intensity is linear with the concentration of riboflavin in certain ranges. The method has a rather low limit of detection down to 20 pg ml\(^{-1}\), thus it can be applied directly in the assay of the human urine and some pharmaceutical preparations without any pre-treatment.

Experimental

Reagents

All solutions were prepared from analytical-reagent grade materials in deionized distilled water throughout. Riboflavin and potassium periodate were purchased from Xi’an Chemical Reagent Plant. Luminol (Fluka, Biochemika) was obtained from Xi’an Medicine Purchasing and Supply Station, China. A standard solution of riboflavin (50.0 µg ml\(^{-1}\)) was stored at 4 °C and protected from light. Working strength solutions were prepared daily from the above stock solution as required. Luminol was used as supplied to prepare a 0.25 mol l\(^{-1}\) stock standard solution in 0.5 mol l\(^{-1}\) NaOH in a 1000 ml calibrated flask. A 3.0 \(\times\) 10\(^{-2}\) mol l\(^{-1}\) stock standard solution of KIO\(_4\) was made by dissolving the solid in distilled water and diluting to 250 ml in a calibrated flask.

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Preparation of immobilized reagents column

Amberlyst A-27 (1.0 g) was shaken with 50 ml 0.25 mol l⁻¹ luminol or 0.04 mol l⁻¹ potassium periodate for 96 h, then the resin was filtered, washed with doubly distilled water and dried. The most convenient method to determine the amounts of luminol and potassium periodate immobilized was to measure the losses of these reagents from the immobilization solutions. The concentration was detected at 360 nm for luminol and at 225 nm for potassium periodate by UV-Vis. The amounts of luminol and periodate immobilized were 1.99 mmol g⁻¹ and 1.01 mmol g⁻¹ resin, respectively. To prepare a column with immobilized reagents, resins containing immobilized luminol (0.05 g) and potassium periodate (0.10 g) were mixed together and packed into a glass column with an internal diameter of 3 mm and total volume of about 0.5 ml, and plugged with glass wool at both ends to prevent the resin from leaking.

Apparatus

The flow injection system used in this work is shown in Fig. 1. A peristaltic pump (Shanghai Meter Electromotor plant, Model ND-15, 15 rpm) was used to generate the flows. PTFE tubing (1 mm id) was used in the flow system. Eluant (200 µl) was injected into the carrier stream and luminol and potassium periodate were released quantitatively. Before reaching the flow cell, the streams of luminol, periodate, sodium hydroxide and analyte were combined in a mixing tubing (50 mm in length). The CL emission cell was a coiled glass tubing (1 mm id, 15 cm length) in order to produce 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 signal produced in the flow was detected without wavelength discrimination, and the PMT output was amplified and quantified by a luminosity meter (Northwest Non-Ferrous Geology Institute of China, Model GD-1) connected to a recorder (Shanghai Dahua Instrument and Meter Plant, Model XWT-206).

Optimization condition for the immobilization of CL reagents

Several immobilization methods were studied. First, the resin was treated with the mixture 0.05 M KIO₄–0.06 M luminol (in 0.5 M NaOH). Second, periodate and luminol anions were separately immobilized on resins, and then the different weights of these resins were mixed and packed into the column. The sensor prepared with the first method had no CL signal observed. This consequence may be due to the interaction between KIO₄ and luminol in an alkaline media. The treatment of anion-exchange resin with different concentrations of KIO₄ and luminol solution was also examined. KIO₄ in the concentration range higher than 0.01 M gave out a stable signal when the resin was treated for 96 h and a higher concentration of KIO₄ can cause a longer lifetime of the flow sensor. In this work, a 0.05 M KIO₄ solution was used for the sensor preparation.

Optimal manifold design for the FI-CL system

The assay was carried out by a continuous-flow mode. Two different manifolds (Fig. 1 and Fig. 2) were designed. Through injection of 200 µl eluant (1.0 × 10⁻⁴ mol l⁻¹ of Na₃PO₄), the reagents on the anion-exchange resin column were eluted and in the presence of riboflavin, the CL intensity was enhanced, and the increase of CL intensity was recorded. While the column

Determination of riboflavin in human urine samples

Two apparently healthy male volunteers took riboflavin tablets orally in the morning on an empty stomach. According to the marked content, the net dosage of riboflavin they took was 30 mg per person. From then on, first-voided urine samples were collected in dark glass bottles after 2, 3, 4, 6.5, 12, 18 and 25 h, respectively. Urine was analyzed directly after dilution with water.

Results and discussion

Time profile of the CL reaction

Before carrying out the flow injection method, the batch method for the CL profiles was used. Without any special eluant, the mixture of luminol and periodate rinsed by pure water gave out an evident CL signal. The signal reached maximum intensity in 5 s and then became extinguished within 15 s after eluant was injected. On joining of the sample into the above mixing solution, an enhanced CL signal was recorded. The peak heights of the CL emission were proportional to the concentration of riboflavin.

Determination of riboflavin in pharmaceutical preparations

Not less than 20 tablets were weighed then ground to a fine powder and mixed. A sample equivalent to approximately 100 mg preparations was weighed accurately and made up to 100 ml with water. As the proposed method, the sample was then diluted to the concentration with the calibration range (0.8 to 200 ng ml⁻¹) without pre-treatment. As the reference method, the sample was filtered and the clear filtrate was used for the spectrophotometry. Some pharmaceutical preparations of riboflavin (Batch No. 990521, produced by Pharmaceutical Plant of Kanghua, Xi’an; Batch No.960113, produced by Pharmaceutical Plant of Chengde, Hebei Province) were tested following the same procedures, respectively.
with immobilized reagents was put in front of the injection valve (Fig. 1) or behind the injection valve (Fig. 2), two significantly different results were observed. The whole analysis process, including sampling and washing, could be accomplished in 0.5 min while the column with immobilized reagents was put in front of the injection valve, whereas it took more than 2.5 min with a higher background using the other method. Therefore, the flow system, as shown in Fig. 1, was employed for subsequent work.

Selection of eluant

Different eluants (200 µl) were injected through the column with immobilized reagents and releasing different amounts of luminol and periodate, thus producing the CL emission. In this flow system, the characteristics of several eluants including NaCl, Na₂CO₃, Na₂SO₄, Na₃PO₄ and H₂O were evaluated and the results are shown in Table 1. It can be seen that sodium sulfate gives a maximum CL emission while sodium carbonate shows some inhibitive effects on the CL reaction. Nevertheless, it was observed that a continuous flow of eluant through the column results in a rather short lifetime of sensor down to only a few hours. It was shown that the immobilized luminol and periodate anions on the anion exchange resin undergo dissociation with water, thus releasing trace amounts of luminol and periodate from the column, and the increase of riboflavin CL signal could be easily observed. In this case, the column could be used over 80 h. As a compromise between higher CL intensity and longer lifetime of the column (discussed in the Application section), water was used as eluant in subsequent work.

Effect of pH on CL and sensor lifetime

The best pH of the eluant (water) on the performance of the system was evaluated. It was found that, along with the increase of pH in the eluant, the CL intensity increased while the lifetime of the sensor decreased considerably (Fig. 3). This phenomenon is probably due to the quantities of hydroxide ions in the eluant increasing. pH 6.0 was then chosen as a compromise between higher CL intensity and longer lifetime of the column (discussed in the Application section), water was used as eluant in subsequent work.

<table>
<thead>
<tr>
<th>Type of CL intensity</th>
<th>H₂O</th>
<th>NaCl</th>
<th>Na₂CO₃</th>
<th>Na₂SO₄</th>
<th>Na₃PO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>108</td>
<td>186</td>
<td>62</td>
<td>257</td>
<td>213</td>
</tr>
<tr>
<td>II</td>
<td>154</td>
<td>243</td>
<td>77</td>
<td>343</td>
<td>272</td>
</tr>
<tr>
<td>III</td>
<td>46</td>
<td>57</td>
<td>15</td>
<td>86</td>
<td>59</td>
</tr>
</tbody>
</table>

a The concentration of each eluant was 1.0 \times 10^{-4} \text{mol l}^{-1} (except H₂O).

Effective molar ratio of immobilized luminol and periodate

To examine the influence of the mixing ratio, resins (0.15 g) with different mixing ratios were packed into columns with the same internal diameter and volume. By injection of distilled water at a fixed volume of 200 µl, different amounts of luminol and periodate were eluted from the resins and emitted CL signals with different intensity. As Fig. 4 shows, the CL intensity dropped drastically from the beginning to the next day, then it went down gradually. The most stable CL signal was found with a molar ratio of 1:2 (luminol to periodate) and a middling CL intensity is in favor of measuring a catalytic effect of riboflavin on CL reaction.

Effect of NaOH concentration

Owing to the nature of the luminol reaction, which is more favored under basic conditions, potassium hydroxide was introduced into the manifold through a flow line to improve the sensitivity of the system. A NaOH concentration less than 0.05 M leads to an apparent decrease in ΔI. The maximum intensity was found with 0.1 M NaOH. While the concentration of NaOH is higher than 0.2 M, there is a scattering effect in the flow cell due to the discrepancy between the refractive index of various components. Thus 0.1 M NaOH was selected as an optimal condition.

Effect of flow rate and the length of mixing tubing

The CL signal was also dependent on the flow rate of carrier and eluant. The signal-to-noise ratio increased at a higher flow rate because the higher flow rate would have an impact on the rate of contact of sample molecules with the ion-exchange resin. The lower flow rate caused broadening of the peak and slowing down of the sampling rates. Nevertheless, the high flow rate...
could lead to an unstable baseline and shortening of the sensor lifetime. A rate of 2.0 ml min\(^{-1}\) was then chosen as a compromise between good precision and lower reagent consumption.

The length of the mixing tubing was also adjusted to yield maximum light emission in the cell. It was found that 50 mm of mixing tubing afforded the best results as regards sensitivity and reproducibility.

**Performance of the sensor for riboflavin measurements**

A series of standard solutions were injected into the manifold depicted in Fig. 1 under the optimized conditions to test the linearity of riboflavin. The linear range, the correlation coefficients, relative standard deviations and the detection limit are summarized in Table 2. At a flow rate of 2.0 ml min\(^{-1}\), the determination of analyte could be performed in 0.5 min, including sampling and washing, giving a throughput of about 100 times per hour with a relative standard deviation of less than 2.0%.

**Selectivity studies**

The selectivity study for the proposed sensor for riboflavin was focused on some common metal ions, pharmaceuticals and ingredients. The following maximum tolerable concentration excesses of other species caused no interferences (relative error < 5% in the presence of 1.0 ng ml\(^{-1}\) riboflavin): 500 for Cl\(^-\), NO\(_3\)\(^-\), Ac\(^-\), I\(^-\), SO\(_4\)\(^2-\), PO\(_4\)\(^3-\), Cr\(_2\)O\(_7\)\(^2-\), NH\(_4\)\(^+\), Mg\(^{2+}\), Ca\(^{2+}\), Ba\(^{2+}\), Zn\(^{2+}\), Ni\(^{2+}\), Mn\(^{2+}\), Cr\(^{3+}\), borate, oxalate, tartrate, citrate, salicylic acid, malic acid, methanol, ethanol, urea, Tween-80, polyvinyl alcohol, CTMAB (cetyltrimethylammonium bromide), glucose, sucrose, sodium dodecylbenzenesulfonate, gelatine, tris-hydroxymethylaminomethane, starch, dextrin, 100 mg of urea, salt and mannitol, 50 for EDTA and 8-hydroxyquinoline, 200 for Cl\(^-\), P\(_2\)O\(_5\)\(^3-\), Cr\(_2\)O\(_7\)\(^2-\), N\(_2\)H\(^4+\), M\(_{2}\)g, C\(_{2}\)a, B\(_{2}\)a, Zn\(^{2+}\), N\(_{2}\)i\(^{2+}\), Mn\(^{2+}\), Cr\(^{3+}\), borate, oxalate, tartrate, citrate, salicylic acid, malic acid, methanol, ethanol, urea, Tween-80, polyvinyl alcohol, CTMAB (cetyltrimethylammonium bromide), glucose, sucrose, sodium dodecylbenzenesulfonate, gelatine, tris-hydroxymethylaminomethane, starch, dextrin, 100 for CO\(_3\)\(^2-\) and mannitol, 50 for EDTA and 8-hydroxyquinoline, 10 for uric acid and Cu\(^{2+}\). Common excipients such as starch and sugar in tablets do not interfere in the determination.

Compounds abundant in human urine such as urea, salt and glucose have no effect on the determination of riboflavin at ng ml\(^{-1}\) levels except for uric acid, therefore urine samples to be analyzed should be diluted with adequate water to avoid the inhibitive effect of uric acid. The flow-sensor proposed has a high selectivity for riboflavin.

**Operational stability of the sensor**

Eluant (200 µl water, pH 6.0) was flow-injected through the system and the CL intensity (I\(_0\)) was recorded to test the lifetime of the immobilized column. The total experiment lasted for eight days and the system was tested for over six hours per day. The results are listed in Table 3. The average CL intensity was calculated in ten spot check determinations. As Table 3 shows, the CL intensity in the first day fluctuated and was unstable. This is possibly due to the swelling procedure of the resin in water. With the increase of soaking time, the interaction between water and resin tended to form a kind of equilibrium and the CL signal (I\(_0\), I\(_1\) and ΔI\(_1\)) came to be steady. Therefore, the sensor could be used stably for over five days with a relative deviation less than 3.0%.

**Applications**

**Determination of riboflavin in pharmaceutical preparations**

Following the procedure described in the Experimental section, the proposed method was applied to the determination of riboflavin in pharmaceutical preparations. Two different preparations were purchased from the local market. The measured riboflavin contents (an average of five determinations) are listed in Table 4. The results obtained by the proposed method were 3.95 mg per tablet and 4.72 mg per tablet, which were well in...
agreement with results obtained by the method described in the Pharmacopoeia,18 and the recovery was from 101 to 107%.

Determination of riboflavin in human urine

The proposed method was also applied with preliminary success to the determination of riboflavin in human urine. Urine samples were collected from volunteers, diluted with distilled water directly and sometimes supplemented with riboflavin to test the recovery of the method. Thus, urinary riboflavin could be determined relatively simply by FI-CL without any pretreatment procedures. The results of trial determinations are summarized in Table 5. To eliminate the interference of uric acid, the urinary riboflavin concentration has to be diluted to pg ml\(^{-1}\) levels with a uric acid concentration lower than 5.0 ng ml\(^{-1}\). However, due to the strong photolysis of riboflavin,15 all the work solutions should be protected from intense light during the course of the experiment.

Metabolic curve of riboflavin in human urine

Two healthy men took riboflavin tablets on an empty stomach in the morning. From then on, the urine samples were collected and riboflavin was determined. The metabolic profile of riboflavin in urine is shown in Fig. 5. From the curve, it can be seen that riboflavin was metabolized rapidly after taking the riboflavin tablets. The total riboflavin excreted through urine was 20.56 mg in a total volume of 1.42 l in 25 h. The riboflavin concentration reached its maximum after four hours and dropped sharply within a few hours, and the riboflavin metabolism ratio in 6.5 h was 57.9% in the body of the volunteers. The result corresponds to the one given in the document.1

**Table 4** Results of riboflavin in different pharmaceutical preparations\(^a\)

<table>
<thead>
<tr>
<th>Pharmaceutical tablet</th>
<th>Riboflavin supplement/ ng ml(^{-1})</th>
<th>Found/ ng ml(^{-1})</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
<th>Content/ mg tablet(^{-1})</th>
<th>Pharmacopoeia method/ mg table(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch 1</td>
<td>0</td>
<td>39.5</td>
<td>101.6</td>
<td>1.85 ((n = 7))</td>
<td>3.95</td>
<td>3.91</td>
</tr>
<tr>
<td></td>
<td>64</td>
<td>104.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Batch 2</td>
<td>0</td>
<td>66.1</td>
<td>106.3</td>
<td>2.17 ((n = 7))</td>
<td>4.72</td>
<td>4.85</td>
</tr>
<tr>
<td></td>
<td>64</td>
<td>134.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) The average of five determinations in sample

**Table 5** Results of riboflavin in human urine samples\(^a\)

<table>
<thead>
<tr>
<th>Individual urine</th>
<th>Time/h</th>
<th>Riboflavin supplement/ pg ml(^{-1})</th>
<th>Mean/ pg ml(^{-1})</th>
<th>Recovery (%)</th>
<th>RSD ((n = 5))</th>
<th>Riboflavin in urine (M_{\text{urine}}/V_{\text{urine}})</th>
<th>Riboflavin metabolism ratio in urine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>0</td>
<td>40.4</td>
<td>107</td>
<td>2.31</td>
<td>2.12/210</td>
<td>7.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80</td>
<td>126.2</td>
<td>95.9</td>
<td>4.42</td>
<td>4.20/140</td>
<td>14.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80</td>
<td>136.7</td>
<td>110</td>
<td>3.17</td>
<td>8.72/160</td>
<td>29.07</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4</td>
<td>0</td>
<td>109.0</td>
<td>1.52</td>
<td>2.32/160</td>
<td>7.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80</td>
<td>197.0</td>
<td>110</td>
<td>4.55</td>
<td>1.28/250</td>
<td>4.27</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6.5</td>
<td>0</td>
<td>28.3</td>
<td>102</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>80</td>
<td>109.5</td>
<td>109</td>
<td>4.86</td>
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<td></td>
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<tr>
<td></td>
<td>5</td>
<td>12</td>
<td>0</td>
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<td>94.6</td>
<td>113.5</td>
<td></td>
</tr>
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<td></td>
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<td>90.3</td>
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<td></td>
<td>6</td>
<td>18</td>
<td>0</td>
<td>41.3</td>
<td>90.3</td>
<td>3.46</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>80</td>
<td>113.5</td>
<td>92.5</td>
<td>2.70</td>
<td>0.84/260</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>25</td>
<td>0</td>
<td>32.4</td>
<td>92.5</td>
<td>0.84/260</td>
<td>2.78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80</td>
<td>110.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total: 68.19%

\(^a\) The average of five determinations in two volunteers’ urine.

Determinations of riboflavin in human urine

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Metabolic curve of riboflavin in human urine

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**Fig. 5** Time–concentration profile after single oral dose of riboflavin.

Conclusions

The proposed sensor is more rapid, simple and sensitive than the existing manual and automated methods, and offers significant advantages in ease of use. Preliminary evidence is presented that the analytical performance of the proposed sensor is sufficient for clinical research and routine control determination of riboflavin in human urine and pharmaceutical preparations.

**References**
