*J Pharm Anal* Vol 1, No 2, 104 - 107 (2011)

ORIGINAL ARTICLES

# Chemiluminescence determination of melamine with Luminol-$K_3Fe(CN)_6$ system

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**Abstract:** A sensitive chemiluminescence (CL) method was developed for determining melamine in urine and plasma samples based on the fact that melamine can remarkably enhance the chemiluminescence of Luminol-$K_3Fe(CN)_6$ system in alkaline medium. The determination conditions were optimized. Under optimum conditions, the chemiluminescence intensity had a good linear relationship with melamine in the range of $9.0\times10^{-9}-7.0\times10^{-6}$ g/mL with a correlation coefficient of 0.9992. The detection limits ($3\sigma$) were 3.54 ng/mL for urine sample and 6.58 ng/mL for plasma sample. The average recoveries of melamine were 102.6% for urine sample and 95.1% for plasma sample. Melamine in samples was extracted with liquid-liquid extraction procedures and the assay results coincided very well with that determined with flow injection chemiluminescence method. The method provides a reproducible and stable approach for sensitive detection and quantification of melamine in urine and plasma samples.

**Keywords:** chemiluminescence; flow injection; melamine; urine; plasma

## 1 Introduction

Melamine, an important trimer of cyanamide, is a common chemical intermediate. It is usually used to produce melamine formaldehyde resin in commercially. Melamine (Figure 1), containing 66.6% nitrogen, was deliberately added to milk and fodders to elevate the protein content in 2008, which has aroused concern all over the world. Previous toxicological study has demonstrated that melamine is of low toxicity whereas excessive exposure in animals may cause renal stones [1,2]. Melamine is also responsible for the development of urinary tract stones and acute kidney failure in infants in China as a result of ingestion of melamine-adulterated powdered infant formulas [3]. Therefore, it has been regulated by the Ministry of Health that the upper limits of melamine content in dried infant formulas, liquid milk and dairy products that contain milk above 15% should be 1 mg/kg, 2.5 mg/kg and 2.5 mg/kg, respectively.

To protect the development of dairy products and the people's safety, it is extremely important and necessary to monitor the amount of melamine in the food and fodders. Many methods for detecting melamine have been established, including gas chromatography-mass spectrometry (GC-MS) [4-6], liquid chromatography [7-9], liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) [3, 10], reversed phase liquid chromatography-mass spectrometry (RPLC-MS) [11], liquid chromatography-tandem mass spectrometry (LC-MS) [12,13], capillary zone electrophoresis [14] and Raman spectroscopy [15-17]. The studies are more focused on the melamine detection in milk and pork. Wang [18] adopted Luminol-Myoglobin system determine melamine in milk products. However, few researches have reported melamine analysis in urine or plasma samples [19]. Furthermore, few studies on the chemiluminescence (CL) method for the determination of melamine have been reported so far. It has been found that melamine could enhance the CL intensity of Luminol-$K_3Fe(CN)_6$ system remarkably in alkaline solution. This method has advantages such as high sensitivity, low cost and simple analysis apparatus. A great deal of success has been achieved when it is applied to the assay of melamine. In this work, a chemiluminescence method was developed and validated for the analysis of melamine in urine and plasma.

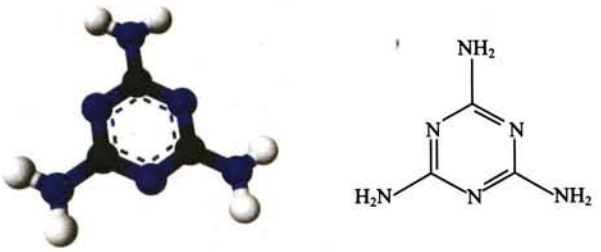


**Figure 1** The structure of melamine

Received 28 October 2010; Accepted 30 December 2010
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## 2 Experimental designs

### 2.1 Apparatus and reagents

An IFFM-E chemiluminescence analyzer (Xi'an Remex Analysis Instrument Co., Ltd., China) was used for flow-injection analysis and CL intensity recording.

Luminol standard solution (0.01 M) was prepared by dissolving 0.2715 g Luminol (Aldrich, Sigma-Aldrich Quimica) in a small amount of concentrated NaOH followed by diluting to 150 mL with distilled water. $K_3Fe(CN)_6$ standard solution (0.01 M) was prepared by dissolving 0.3294 g $K_3Fe(CN)_6$ (Xi'an Chemical Reagent Factory, Xi'an, China) in distilled water and diluting to 100 mL. Melamine standard solution (0.001 g/mL) was prepared by dissolving 0.050 g melamine (Chengdu Kelong Chemical Reagent Factory, Chengdu, China) in 2 mL $H_2SO_4$ and diluting to 50 mL. All the standard solutions were stored at 4 ℃ in a refrigerator before use. Trichloroacetic acid (Xi'an Chemical Reagent Factory, Xi'an, China) and plumbi acetas (Xi'an Chemical Reagent Factory, Xi'an, China) were of analytical grade.

### 2.2 Sample preparation

Urine samples were obtained from the children who had suffered from melamine in a hospital (the Second Affiliated Hospital, Lanzhou University, China) and liquid-liquid extraction was adopted. 1 mL of urine sample was vigorously mixed with 6 mL of 1% trichloroacetic acid solution for 1min followed by adding 2 mL plumbi acetas (2%). The mixture was centrifuged for 5 min at 4000 rpm and the supernatant was collected. The extract procedure was repeated and the top layers were combined and centrifuged at 4000 rpm for 5 min one more time. The entire upper aqueous layer was collected in a separate tube and dried via evaporating the solvent under a gentle flow of nitrogen gas. Finally, the prepared sample was dissolved with distilled water and diluted to test the recovery of the method [20, 21].

Another sample was supplied with sheep plasma. The plasma was first centrifuged for 10 min at 4000 rpm and the top aqueous layer (serum) was collected in a tube. 1 mL of obtained serum spiked with melamine solution in tube A was vortexed with 6 mL of 1% trichloroacetic acid solution for 1 min. The mixture was sonicated for 20 min followed by adding 1 mL plumbi acetas (2%) for protein precipitation. Then the mixture was centrifuged for 5 min at 4000 rpm and the supernatant solution was transferred into tube B. 5 mL water was added to tube A and the same extraction procedure was repeated. The supernatant was also transferred into tube B. Then the solution in tube B was centrifuged at 4000 rpm for 5 min. The supernatant was removed into tube C and evaporated to dryness under a stream of nitrogen. The residue was dissolved with distilled water and diluted to test the recovery of the method.

### 2.3 Procedure

Flow-injection system (Xi'an Remex Analysis Instrument Co., Ltd.) used for the determination of melamine is shown in Figure 2. All the streams were driven by peristaltic pump at the fixed flow rate. Luminol solution was mixed with $K_3Fe(CN)_6$ solution and then emerged with melamine solution in photomultiplier tube. The voltage for photomultiplier tube was 650 V and the pump running speed was 1.2 mL/min. The CL intensity was fixed and the CL signal was recorded by computer.

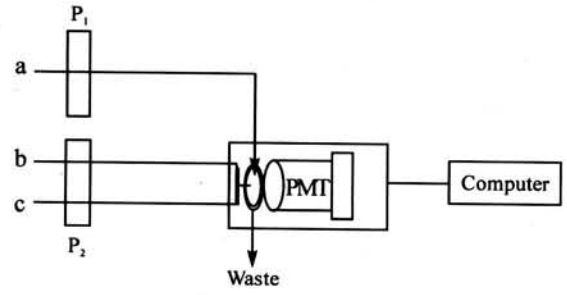


**Figure 2** Schematic diagram of flow injection chemiluminescence determination. a, melamine; b, Luminol $5.0\times10^{-6}$ M; c, $K_3Fe(CN)_6$ $1.0\times10^{-5}$ M; PMT, photomultiplier tube; $P_1$, $P_2$, peristaltic pump.

## 3 Results and discussion

### 3.1 Optimization of CL system

#### 3.1.1 Selection of oxidant

The characteristics of several oxidants, including Luminol-$KMnO_4$, Luminol-$H_2O_2$, Luminol-$K_3Fe(CN)_6$, Luminol-$KIO_4$, $KMnO_4$-$Na_2SO_3$, $Ce(SO_4)_2$-$Na_2SO_3$, $H_2O_2$-fluorescein sodium, and $KMnO_4$-HCHO systems in the presence of melamine, were evaluated. It was found that melamine had more effective enhancement for Luminol-$K_3Fe(CN)_6$ CL system than the other systems.

#### 3.1.2 Effect of $K_3Fe(CN)_6$ concentration

The concentration of $K_3Fe(CN)_6$ affected the emission of relative CL intensity and the influence of $K_3Fe(CN)_6$ concentration was examined in the range of $1.0\times10^{-7}-7.0\times10^{-5}$ M. The relative CL intensity increased sharply with the increase of $K_3Fe(CN)_6$ concentration at the initial stage. When the concentration was over $1.0\times10^{-5}$ M, the relative CL intensity did not increase significantly. Therefore, the optimum concentration of $K_3Fe(CN)_6$ was $1.0\times10^{-5}$ M.

#### 3.1.3 Effect of Luminol concentration

The CL emission intensity depends on the concentration of Luminol as well. The Luminol concentration varied from $1.0\times10^{-8}$ to $9.0\times10^{-6}$ M to study its influence on the relative CL intensity. When the concentration was over

$5.0\times10^{-6}$ M, only slight increment of relative CL intensity was observed. Therefore, $5.0\times10^{-6}$ M Luminol concentration was employed.

#### 3.1.4 Effect of NaOH concentration

The effect of NaOH concentration on the relative CL intensity was also examined under the optimized Luminol and $K_3Fe(CN)_6$ concentrations as discussed above. According to melamine being dissolved by $H_2SO_4$ (0.1 M) at first, the concentration of $H_2SO_4$ was considered as well. It was confirmed that melamine was diluted before determination and the effect of $H_2SO_4$ could be omitted. The relative CL intensity was improved from 150 to over 1700 as NaOH concentration increased from 0.001 to 0.1 M. When NaOH concentration was 0.03 M, the relative CL intensity displayed the maximal value. While over 0.03 M the relative CL intensity signal did not increase remarkably any more. Therefore, 0.03 M of NaOH was regarded as the optimized concentration for the subsequent studies.

### 3.2 Calibration curve and the detection limit

Under the optimum concentrations of Luminol ($5.0\times10^{-6}$ M), $K_3Fe(CN)_6$ ($1.0\times10^{-5}$ M) and NaOH (0.03 M) as mentioned above, the relative CL intensity ($\triangle I$) was proportional to the concentration of melamine($c$) in the range of $9.0\times10^{-9}$ – $7.0\times10^{-6}$ g/mL. The linear regression equation was $\triangle I = 12.425c + 171.6$, and the correlative coefficient was 0.9992. The detection limits ($3\sigma$) were 3.54 ng/mL for urine sample and 6.58 ng/mL for plasma sample, respectively.

### 3.3 Precision and recovery

The melamine stock solution was diluted to $1.0\times10^{-9}$ g/mL, $1.0\times10^{-8}$ g/mL and $1.0\times10^{-7}$ g/mL, and 9 samples were prepared and examined at each concentration. Intra-day and inter-day precisions were evaluated as well as listed in Table 1. The relative standard deviation (RSD) ranged from 1.73% to 3.20%.

The recovery was obtained by measuring melamine concentration of a series of solutions, which were prepared by mixing the initial melamine solution of $5.0\times10^{-6}$ g/mL with solutions containing melamine $2.5\times10^{-6}$, $5.0\times10^{-6}$ and $7.5\times10^{-6}$ g/mL, respectively. As shown in Table 2, the average recovery was 104.6%, which was within the acceptable range.

**Table 1** Intra-day and inter-day precisions of melamine ($n=9$)

| Sample | Concentration (ng/mL) | RSD (%) | |
|---|---|---|---|
| | | Intra-day | Inter-day |
| Melamine | 10 | 2.37 | 2.95 |
| | 100 | 2.01 | 3.20 |
| | 1000 | 1.73 | 2.67 |

**Table 2** Determination results of melamine recovery ($n=9$)

| Sample | Initial ($\times10^{-6}$ g/mL) | Added ($\times10^{-6}$ g/mL) | Measured ($\times10^{-6}$ g/mL) | Recovery (%) | Average recovery (%) |
|---|---|---|---|---|---|
| Melamine | 5.0 | 2.5 | 7.65 | 106.0 | 104.6 |
| | | 5.0 | 10.22 | 104.4 | |
| | | 7.5 | 12.76 | 103.5 | |

### 3.4 Interference

Under the selected experimental conditions, the interference tests of some foreign species were performed. 1000-fold of $Zn^{2+}$, $Ca^{2+}$, $Mg^{2+}$, $NO_3^-$, $CO_3^{2-}$, $PO_4^{3-}$ and amylaceum, 100-fold of $Al^{3+}$ and $Ni^{2+}$, 10-fold of $Cu^{2+}$, $Ba^{2+}$ and $NH_4^+$ were tested. No obvious interference was observed in the determination of $1.0\times10^{-9}$ g/mL of melamine at the confidence level of 95%.

## 4 Applications

### 4.1 Determination of melamine in urine samples

The urine samples of five children who were three to ten months old and had ingested melamine were collected, and diluted to the desired concentration. The samples were injected into the flow-injection system, and the melamine content in each urine sample was obtained as listed in Table 3.

The recovery of melamine in urine samples was estimated by mixing urine samples with three solutions containing different melamine content respectively. The results obtained are shown in Table 4. As can be seen, an acceptable recovery range (from 101.4% to 104.0%) was found by the proposed method, and the method was suitable for melamine detection.

**Table 3** Determination of melamine in five different urine samples

| Urine samples | Measured ($\times10^{-6}$ g/mL) | RSD (%, $n=3$) |
|---|---|---|
| ZY001 | 1.29 | 2.12 |
| WW1L043 | 0.48 | 3.29 |
| JM017 | 4.51 | 1.76 |
| WWN039 | 3.30 | 2.64 |
| LZ025 | 9.34 | 2.35 |

**Table 4** Determination of melamine recovery in urine samples ($n=9$)

| Sample | Initial ($\times10^{-6}$ g/mL) | Added ($\times10^{-6}$ g/mL) | Measured ($\times10^{-6}$ g/mL) | Recovery (%) | Average recovery (%) |
|---|---|---|---|---|---|
| Urine | 9.4 | 5.0 | 14.60 | 104.0 | 102.6 |
| | | 10.0 | 19.54 | 101.4 | |
| | | 15.0 | 24.77 | 102.5 | |

### 4.2 Determination of melamine in sheep plasma samples

As discussed in experimental section, serum was firstly separated from plasma sample by centrifuging followed by

adding melamine to prepared serum samples. Then the obtained samples were extracted and the melamine content was determined. The recovery was evaluated by adding melamine solutions with different concentrations into the samples, and the measured results are listed in Table 5. The recovery was within the desired range (from 94.0% to 96.0%). It was proved that extracting solvents were collected correctly and melamine could be extracted effectively. According to the recovery, the method could be applied to determine melamine in the plasma sample.

**Table 5** Determination of melamine recovery in sheep plasma samples ($n=9$)

| Sample | Added ($\times10^{-6}$g/mL) | Measured ($\times10^{-6}$g/mL) | Recovery (%) | Average recovery (%) |
|---|---|---|---|---|
| Plasma | 0.5 | 0.47 | 94.0 | 95.1 |
| | 1.0 | 0.96 | 96.0 | |
| | 1.5 | 1.43 | 95.3 | |

## 5 Conclusion

FI-CL method has been successfully developed to determine the content of melamine both in urine and plasma. The determination conditions were optimized as Luminol in $5.0\times10^{-6}$M, $K_3Fe(CN)_6$ in $1.0\times10^{-5}$M and NaOH in 0.03 M. The chemiluminescence intensity had a good linear relationship with melamine in a wide concentration range (from $9.0\times10^{-9}$ g/mL to $7.0\times10^{-6}$ g/mL) and the average recovery was in an acceptable range (102.6% in urine and 95.1% in plasma). It has been proved that this method has such advantages as convenience, high sensitivity and selectivity to determine melamine in urine and plasma. The detection limits of melamine determined by the proposed method (3.54 ng/mL for urine sample and 6.58 ng/mL for plasma sample) were lower than those of official methods (100 ng/mL of melamine for HPLC method [22]). This method may be applied in detecting melamine of other samples. Moreover, FI-CL system also has high potential applications in other areas.

### Acknowledgments

In this work, Xi'an Jiaotong University Analysis Chemistry Laboratory provided lots of technical support for melamine determination, and we gratefully acknowledge all the cooperators.

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