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Short communication

Determination of riboflavin by high-performance liquid chromatography with riboflavin-depleted urine as calibration and control matrix

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Abstract

A simple method, exposure to natural-light, was developed to remove riboflavin from urine to enhance its use as the biological matrix for the preparation of calibration and control samples. Riboflavin-depleted urine containing less than 1 ng/ml of riboflavin was used to validate a high-performance liquid chromatography with fluorescence detection method for the determination of urinary riboflavin. The linearity of the assay ($r^2 = 0.999$) was acceptable over the range of 10–5000 ng/ml. The intra-assay and inter-assay CVs were 3.3% and 9%, respectively. Subsequent stability studies found that urine riboflavin was stable for up to 6 months at 4 or −20 °C.

Keywords: Riboflavin; Urine; HPLC; Stability

1. Introduction

Many methods have been reported in the determination of urinary riboflavin [1–6], a biomarker used to assess the status of Vitamin B_2 and monitor the compliance of certain medications [7,8]. A problem that came to our attention was the availability of appropriate matrices that can be used to prepare calibration standard and quality control samples. As a common practice in analytical laboratories, matrices used to prepare calibration and quality control samples should closely resemble the matrix to be analyzed. The endogenous nature of riboflavin complicates the implementation of this practice. The concentration of riboflavin in urine is greatly influenced by many factors such as dietary intake, nutritional supplement use, health and physical condition, etc., so urine has not been an appropriate matrix. Many researchers have used non-biological solutions such as water [2], or diluted acetic acid [3] as the matrices while others used a urine and buffer combination [1].

Based on the fact that riboflavin is light sensitive, we developed a simple method to degrade riboflavin from urine. Using this riboflavin-depleted urine as the matrix, the method reported by Gatautis and Naito [2] was slightly modified and validated. The changes of the method included increasing the methanol content in the mobile phase from 34 to 50%, reducing the injection volume from 25–100 μl to 5 μl, reducing flow rate from 1 ml/min to 0.7 ml/min, and shortening the injection time from over 10 min to 6 min.

Although stability of riboflavin has been studied in mixed parenteral nutrition solution [9], milk powders [10], antibiotic solutions [11] and mixtures of liposomes and riboflavin [12], limited data have been published on the short term stability of urinary riboflavin [1,2] and no data have been published on long term stability of urinary riboflavin. We now present our studies on the stability of urinary riboflavin during light exposure and long-term storage at 4 or −20 °C.

2. Materials and methods

2.1. Chemicals and reagents

Riboflavin was purchased from Sigma (Saint Louis, MO, USA). HPLC-grade methanol was purchased from Burdick & Jackson (Muskegon, MI, USA) or Fisher Scientific (Fairlawn,

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NJ, USA). A MilliQ filter apparatus (Millipore, Boston, MA, USA) was used for purification of water.

2.2. Method to remove riboflavin from urine

Urines collected from healthy adult voluteers were pooled and fortified with 1% sodium fluoride. The urinary pH was 7.5. Four hundred milliliters of urine were aliquoted into a 500-ml clear glass bottle and exposed to natural-light for 1 week. Urine was analyzed by HPLC to confirm the concentration of riboflavin to be less than 1 ng/ml. After this light-exposure process, the urine was considered "blank" and used to prepare calibration and quality control samples.

2.3. Standards and controls

Calibrator and quality control aqueous stock solutions were prepared at 100 and 1000 μg/ml and stored in the dark at 4 °C. Calibrator samples used in analysis were prepared at 10, 100, 1000 and 5000 ng/ml by adding calibrator stock solution to the "blank" urine. The quality control samples were prepared at 10, 40, 400 and 4000 ng/ml in a similar fashion but from separate stocks.

2.4. Sample preparation

Approximately 1 ml of urine was transferred to the amber glass autosampler vials (Waters, Milford, MA, USA). Samples were first centrifuged if turbidity was observed.

2.5. Instrumentation and HPLC analysis

Five microliters of urine specimen was directly injected onto a YMC-Pack Pro C_{18} column (150 × 4.6 mm i.d., particle size 5 μm, pore size 12 nm) (Waters, Milford, MA, USA). The instrument system consisted of a Waters 600 LC, a Waters 717 *plus* autosampler and a Waters 474 scanning fluorescence detector that used Waters's Millennium software (Version 3.05). The column was maintained at room temperature. The mobile phase was MilliQ water and methanol (50:50 by volume). The run-time was 6 min with a flow rate at 0.7 ml/min. Riboflavin was detected with the excitation and emission wavelengths set at 450 and 530 nm, respectively. The concentration of riboflavin was determined from the peak area and comparison of this peak area with the calibration curve (peak area versus concentration of calibrators, linear with $1/x^2$ weighting).

2.6. Effects of light exposure on urinary riboflavin

To study the effects of light exposure on urinary riboflavin, samples containing riboflavin at 200 and 2000 ng/ml were prepared and 1-ml volume was aliquoted into 1-ml amber glass vials and clear glass vials. All samples were stored in a refrigerator and protected from light exposure. Two replicates of each concentration in each type of vial were removed from the refrigerator and placed on the laboratory bench to be exposed to fluorescent light for specified times (3, 6, 12, 24, 48 and 72 h). At hour-0, all samples were analyzed. The peak areas of riboflavin in samples exposed to light were compared to the peak areas of riboflavin in samples not exposed to light (hour-0).

2.7. Stability of riboflavin stored at 4 or −20 °C for 6 months

Stability of urinary riboflavin stored at 4 °C and −20 °C was studied for a period of 6 months. At a specified time (0, 2, 3, 4, 5 and 6 months), riboflavin control samples in urine were made at 300 and 3000 ng/ml. Approximately 1 ml was aliquoted into six amber glass autosampler vials for each concentration. The six vials were divided into two sets. One set ($N = 3$) was placed at 4 °C and another was placed at −20 °C. At month 6, all samples were collected and analyzed with a freshly-made calibration curve and quality control samples.

3. Results and discussion

Despite the fact that only 5 μl of the urine sample was injected, riboflavin displayed a strong signal even at the concentration of 10 ng/ml, the lower limit of quantification (LLOQ) (Fig. 1). Riboflavin appeared to separate well from other fluorescent compounds, which were eluted ahead of riboflavin in the solvent front. Potential interference from other fluorescent compounds in urine or lumichrome decomposed from riboflavin had been studied by Gatautis and Naito [2]. They

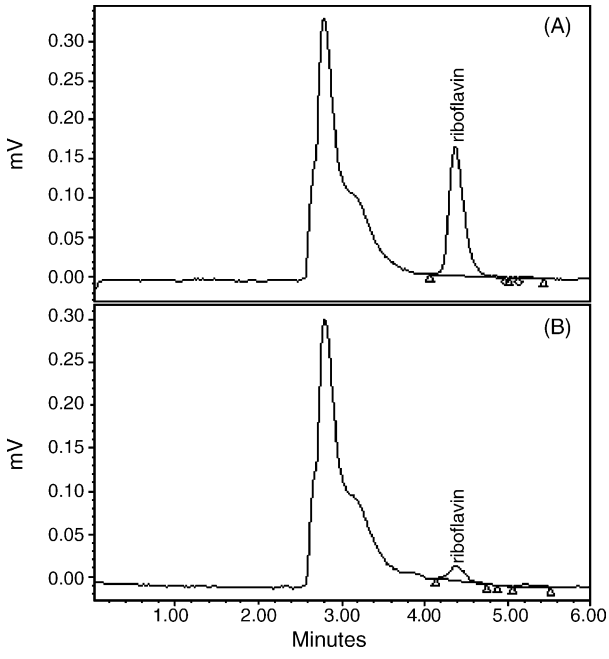

Fig. 1. HPLC chromatograms of urinary riboflavin: at the lower limit of quantitation (10 ng/ml) (A) and in a riboflavin-depleted "blank" urine (B).

indicated that the detection of riboflavin was selective and optimized when the fluorescence excitation and emission wavelengths were set at 450 and 530 nm, respectively. Although a riboflavin peak was observed in the "blank" urine (Fig. 1), the signal was only 11% of the LLOQ intensity. It indicated that riboflavin was degraded to a level which would not be able to interfere with the accuracy of the analysis. This also indirectly indicates that the light-induced riboflavin-degradation products were separated from the riboflavin.

The concentrations of urinary riboflavin vary greatly depending on dietary intake, nutritional supplement use, health and physical condition. In an analysis of urine from six healthy adults in our laboratory, riboflavin varied from 126 to 7833 ng/ml. In order for urine to be used as "blank" matrix, riboflavin should be removed. As shown in this study, exposing the urine to natural-light for 1 week effectively creates riboflavin-free urine. Compared to other published studies that used non-biological matrices in preparation of calibration curve and quality control samples [2,3], the "blank" urine used in our method certainly was more appropriate. In the method reported by Smith [1]), urine used in preparation of calibration standards contained 0.3 μg/ml of riboflavin. Calibrators prepared with such urine would not be able to detect lower concentrations of riboflavin that may be required in the clinical laboratory.

The precision and accuracy of our method were evaluated at 10, 40, 400 and 4000 ng/ml. The coefficients of variation for intra- and inter-assay did not exceed 3.3% and 9%, respectively, and the mean concentrations from the target did not deviate more than 10% and 11%, respectively (Table 1). For eight analytical runs through a period of 2 months, the mean correlation coefficient was $r^2 = 0.999$ over the range of 10–5000 ng/ml. This calibration range is suitable for both clinical and compliance measurements. In a clinical laboratory, analysis of urinary riboflavin ranges from tens to thousands ng/ml [2,13,14]. In drug compliance studies, riboflavin concentrations often exceed 1000 ng/ml [7,8,15].

When handling specimens for determination of riboflavin, precaution must be taken. To avoid exposure of light, light-resistant containers were used and samples were processed in an area with reduced light. When exposed to light for 24 and 72 h, riboflavin stored in amber glass vials decreased by 4% and 9%, respectively (Fig. 2), while riboflavin stored in clear glass vials decreased by 52% and 81%, respectively. Gatautis and Naito [2] reported that riboflavin completely disappeared from urine after exposure to fluorescent light for 16 h. Since details of the experiment were not presented, it is not possible to determine what caused the more rapid depletion of riboflavin in the previous study. The "blank" urine used in our study was prepared by exposure to light at room temperature for 7 days. Bacteria could degrade riboflavin [2,3], but its effects on our "blank" urine was not studied. Effects of urine pH on riboflavin had been studied by Gatautis and Naito [2] and Lambert et al. [3]. They both reported that riboflavin was more stable in acidic than in alkaline or neutral conditions. As normal urine pH ranges from 4.6 to 8.6, our stability findings, conducted with urine at pH 7.5, would certainly apply to more acidic urine (most cases), but stability may be more limited in those few urines with higher pH.

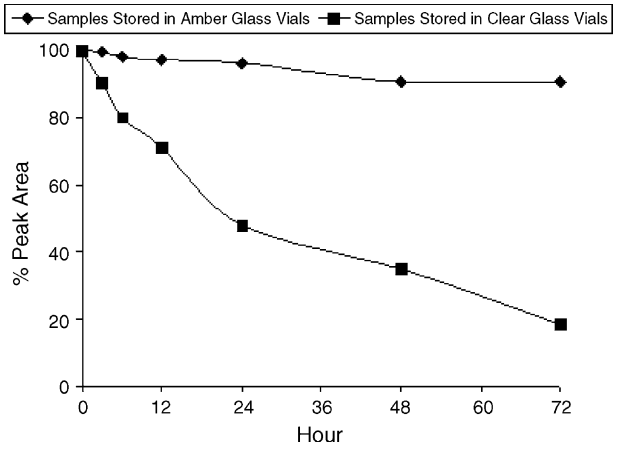

Fig. 2. Effects of exposure to light on urinary riboflavin was depicted by comparison of urinary riboflavin stored in amber glass vials to those stored in clear glass vials over a period of 72 h. Data were the means of two concentrations (200 and 2000 ng/ml) (CVs not >5.9%). %Peak area was the percentage differences of samples exposed to light and samples not exposed to light (hour-0). %Peak area at hour-0 was normalized to 100%.

Our study indicates that riboflavin in urine is stable for up to 6 months when stored at 4 or −20 °C. The CV for each concentration (300 or 3000 ng/ml) at either temperature did not exceed 8% and the mean concentration from the target did not deviate more than 6% over the entire time-course of the experiment. Long term stability of riboflavin is important in drug compliance studies. In some complex studies, thousands of samples may be collected from multiple locations through a period of time and analysis may not be performed until all samples are accumulated.

It is worthwhile to emphasize the simplicity of the analytical method described here. Isoriboflavin had been reported to be used as internal standard to compensate any loss of sample during preparation [3]. Because sample was directly injected onto the column without any processing in our method, loss of sample during extraction was not a

Table 1
Precision and accuracy for analysis of urinary riboflavin

Target concentration (ng/ml)	Intra-assay[a]		Inter-assay[b]	
	%Target	%CV	%Target	%CV
10	90	3.3	89	9.0
40	94	2.5	95	3.1
400	94	0.7	96	2.9
4000	96	1.0	96	3.4

[a] %Target and %CV of intra-assay were calculated based on the mean and standard deviation of five replicates in a single run.

[b] %Target and %CV of inter-assay were calculated based on the mean and standard deviation of 22 replicates from five different runs.

concern. Since external standard calibration is sufficient, the use of internal standard was not necessary. With no pre-analytical processing, the time and steps to handle the sample are reduced so that any riboflavin loss is minimized.

4. Conclusions

"Blank" urine with depleted riboflavin (less than 1 ng/ml) provides an appropriate matrix for the preparation of calibration standards and quality control samples. When protected from exposure to light, urinary riboflavin is stable at 4 or −20 °C for at least 6 months.

Acknowledgments

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