Liquid-Chromatographic Determination of Urinary Riboflavin

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We describe a method for determining urinary riboflavin by "high-pressure" liquid chromatography, with fluorometric detection. An aliquot of a 24-h urine specimen is injected directly into the chromatograph and the natural fluorescence of riboflavin is measured as the compound is eluted. Interference from other fluorophores is obviated because of differing retention times. Urinary components other than riboflavin and its analogs or degradation products exhibit no significant fluorescence at the wavelengths used (450 nm excitation, 530 nm emission). Analytical recovery of added riboflavin was 96.5 (SD 1.1)% The CV within-run was 0.7%, between-run it was 4.3%. Concentrations of riboflavin as low as 10 μg/L are readily detected, with a linear relation of response to concentration to at least 2000 μg/L.

Additional Keyphrases: fluorescence detection • vitamin B₂ • nutrition • urine • reference interval

Riboflavin is a water-soluble vitamin (vitamin B₂) long established as an essential nutrient. The daily requirements of the vitamin are usually based on energy and protein requirements (1) and increase during periods of stress (i.e., growth, pregnancy, lactation) or use of oral contraceptives (2). Riboflavin deficiencies have been demonstrated in lower socioeconomic groups in the United States (3, 4), deficiencies that seem to be aggravated by use of oral contraceptives (5, 6). Chlorotetracycline and oxytetracycline cause in vivo increases in urinary concentrations of riboflavin and starvation decreases them (7). Increased urinary concentrations of the vitamin have also been associated with progressive muscular dystrophy (8).

The vitamin is absorbed primarily from the upper gastrointestinal tract, the rate of absorption being regulated by a transport system that determines the upper level of uptake (9). Because the vitamin is water soluble and there is no capacity for storage, the amount in excess of the body's immediate needs is rapidly excreted in the urine. Excretion is greatest during the first hour after ingestion of the vitamin or vitamin-containing foods, and the amount excreted depends on both the amount ingested and the concentration already present in the body. When riboflavin intake is low, excretion is proportional to intake. Free riboflavin is the only flavin excreted by the body in significant amounts (10). Determination of urinary riboflavin concentration appears to be an ideal means for monitoring dietary intake of the vitamin and establishing the vitamin B₂ status of the individual.

Methods of analysis available to date (11–15) involve extractions, oxidation of interfering "pigments," decolorization with peroxide, or column pre-purification—tedious and time-consuming procedures. We use "high-pressure" liquid chromatography (HPLC) with fluorometric detection of the eluted riboflavin peak. An aliquot of urine is injected directly into the chromatograph without pre-processing. Analysis time averages about 10 min per sample.

Materials and Methods

Apparatus. We used a high-performance liquid chromatograph (Model ALC/GPC 204) equipped with a 3.9 mm (i.d.) × 30 cm μ-Bondapak C₁₈ column, a guard column packed with Bondapak C₁₈/Coralis, and a Model 420 fluorescence detector equipped with a F4TSD lamp (all from Waters Associates Inc., Milford, MA 01757).

Reagents. Aconitite and methanol, HPLC grade, were from Burdick and Jackson Laboratories Inc., Muskegon, MI 49442. Alloxazine, riboflavin, lumiflavin, lumichrome, flavin-adenine dinucleotide (FAD), and flavin mononucleotide (FMN) were from Sigma Chemical Co., St. Louis, MO 63178. Hydrochloric acid and oxalic acid dihydrate were from J.T. Baker Chemical Co., Phillipsburg, NJ 08865. HPLC-grade water was from an in-house water-purification system (Continental Water Systems Corp., El Paso, TX 79998).

Stock standard (100 mg/L). Add 10.0 mg of riboflavin to 20 mL of acetonitrile and dilute to 100 mL with HPLC-grade water. Stored at 2–8 °C in a glass bottle that totally excludes light, this standard solution is stable for about 30 days.

Working standard (5 mg/L). Dilute 5.0 mL of the stock standard to 100 mL with HPLC grade water, and store at 2–8 °C in a glass bottle that totally excludes light. Stable for about 30 days.

Patients' samples. From apparently healthy, well-nourished adult volunteers who were not taking any daily vitamin supplements, oral contraceptives, or other medication, we collected 24-h urines in clean, previously unused 2.5-L containers. As a preservative we added 5.0 g of oxalic acid dihydrate before the collection. The containers were taped with masking tape to exclude light, and the samples were refrigerated during and after the collection period. Each specimen was thoroughly mixed and the volume measured; 20-mL aliquots were retained for analyses. The aliquots were centrifuged for 10 min at 2000 x g to remove any sediment. The supernatant solutions were carefully transferred to clean 13 × 100 mm test tubes either for immediate analysis or for storage. During preparation of the urine samples exposure to light must be kept to a minimum. The samples can be stored at −20 °C if the analysis cannot be done within 24 h after the specimen has been collected.

Procedure. Riboflavin standards and urine specimens were chromatographed and detected with a λexcitation of 450 nm (6.5 bandpass) and λemission of 530 nm (long pass, >530 nm). The flow rate of the mobile phase (methanol/water, 34/66 by vol) was 1.0 mL/min. Fluorometer gain was set at 128, and recorder chart speed at 0.5 cm/min. For optimum results the mobile phase should be prepared freshly daily and filtered/degassed through a 0.45-μm type HA filter (Millipore Corp., Bedford, MA 01730). Analysis is at ambient temperature.

To prepare a standard curve, inject various amounts (0.5 to 10.0 μL) of working standard into the instrument. Plot the resulting peak heights vs riboflavin concentration on linear graph paper.

To determine urinary riboflavin concentration, inject 25–100 μL of urine directly into the chromatograph without any pre-treatment of the samples. The volume injected will

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vary with the amount of riboflavin present; however, 25 μL appears to suffice for most samples from "normal" individuals.

The guard column packing should be replaced after about 100 injections, and the analytical column should be flushed with 50 mL of water followed by 50 mL of methanol. The mobile phase may be left in the column when the instrument is shut down; however, for long-term column storage, methanol (700 mL/L) should be pumped through the column first.

Urinary riboflavin concentrations are usually expressed as amount excreted per gram of creatinine. We determined urinary creatinine concentrations (16) with an SMA 6/60® (Technicon Instruments Corp., Tarrytown, NY 10591) within 24 h of receiving the samples.

**Analytical Variables**

Within-day reproducibility was assessed with 11 samples of a frozen pool of urine. Day-to-day reproducibility was assessed by analyses of two different frozen urinary pools, three to four samples per day, for five days. To test analytical recovery, we selected three patients' samples without conscious bias; 0.250 μg of riboflavin was added to the first two samples, 0.126 μg to the third. Six determinations were performed on each of the three samples.

FMN and FAD (the biologically active forms of riboflavin) and of alloxazine, lumichrome, and lumiflavin (degradation products of riboflavin) were investigated for fluorescent and chromatographic interference. Compounds that fluoresce at or close to the wavelengths used for monitoring riboflavin were also examined.

**Reference intervals.** We determined riboflavin concentrations of apparently healthy men (n = 17) and women (n = 21), ages 24 to 65 years.

**Calculations.** Once the chromatograph was obtained, the peak height of the riboflavin peak was measured in cm. Using the standard curve, the concentration, C_r, was obtained. To calculate riboflavin in μg/24 h, use the following expression:

\[
(C_r/V_i) \times V \times 1000 = \text{μg riboflavin/24 h.}
\]

where:

- \(V \) = volume of 24-h urine collection (mL)
- \(V_i \) = volume of urine injected into HPLC (μL)
- \(C_r \) = concentration of riboflavin (μg), from standard curve
- 1000 = to convert from μg/μL to μg/mL

\[
\frac{\text{μg riboflavin/24 h}}{\text{g creatinine/24 h}} = \frac{\text{μg riboflavin}}{\text{g creatinine}}
\]

**Results and Discussion**

In this simple, rapid, and precise quantitative procedure for measuring urinary riboflavin by HPLC, with fluorescence detection, peak height and amount of riboflavin per sample are linearly related to at least 50 μg. A typical chromatogram of a urine sample containing riboflavin is illustrated in Figure 1. The standard curves were used in the calculation of urinary riboflavin concentrations.

This method provides excellent precision with a within-day reproducibility (CV) of 0.7% (mean 284.6, SD 20.7, range 2790–2840 μg/L; n = 11) and day-to-day reproducibility of 2.2% for a low-concentration pool (mean 249.1, SD 5.4 μg/L; n = 15) and 4.3% for a high-concentration pool (mean 622.1, SD 26.1 μg/L; n = 20). Analytical recovery of added riboflavin (n = 18) was 96.5% (SD 1.1%).

We investigated the proposed method for interference from analogs or degradation products of riboflavin. Figure 2 illustrates a chromatogram of a mixture of riboflavin, FAD, FMN, alloxazine, lumichrome, and lumiflavin. FAD and FNM emerged almost coincident with the void volume, followed by a discrete, well-resolved riboflavin peak. Lumiflavin and lumichrome are eluted well after the riboflavin peak, with lumichrome showing a negligible amount of fluorescence. Alloxazine does not interfere, it fluoresces at these wavelengths only at alkaline pH. The fluorescence of riboflavin at 550 nm when excited at 450 nm is both selective and specific. Other biological, pharmaceutical, organic, and inorganic compounds in urine do not exhibit any significant native or natural fluorescence under these analytical conditions. Certain compounds that do fluoresce at wavelengths near those used for monitoring riboflavin (17, 18) were also examined but showed no interference (Table 1).

The separation of riboflavin from other urinary constituents was verified by monitoring at 254 and 280 nm the absorbance of a urine with a high riboflavin content (2000 μg/L). Most urinary components are eluted ahead of riboflavin, giving a distinct peak for the compound of interest when compared with a standard. Retention times for riboflavin were identical when monitored for fluorescence and for ultraviolet absorbance.

Riboflavin in acidic medium decomposes to lumichrome on exposure to light (19, 20), which shows little or no fluorescence at the wavelengths used. Further to prove that the peak designated as riboflavin was due to riboflavin alone, we exposed a urine to fluorescent light for 16 h. A chromatogram was run before and after exposure. After the 16 h the riboflavin peak completely disappeared, as expected. When we then added riboflavin to this sample, a peak was again evident at the expected retention time.
Table 1. Compounds Not Interfering at or near the Wavelengths a Used for Riboflavin Determination

<table>
<thead>
<tr>
<th>Compound</th>
<th>Excitation, nm</th>
<th>Emission, nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylenediamine</td>
<td>460</td>
<td>510</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>470</td>
<td>520</td>
</tr>
<tr>
<td>Dipyridamole</td>
<td>430</td>
<td>540</td>
</tr>
<tr>
<td>Estradiol</td>
<td>490</td>
<td>546</td>
</tr>
<tr>
<td>Estril</td>
<td>490</td>
<td>546</td>
</tr>
<tr>
<td>Estrone</td>
<td>490</td>
<td>546</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>436</td>
<td>525</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>475</td>
<td>530</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>420</td>
<td>570</td>
</tr>
<tr>
<td>Testosterone</td>
<td>450</td>
<td>540</td>
</tr>
<tr>
<td>Tetrahydrocortisone</td>
<td>436</td>
<td>525</td>
</tr>
<tr>
<td>Apresoline HCl</td>
<td>&gt;385</td>
<td>520</td>
</tr>
<tr>
<td>Acriflavin</td>
<td>&gt;385</td>
<td>520</td>
</tr>
<tr>
<td>Urobilin</td>
<td>&gt;385</td>
<td>521</td>
</tr>
</tbody>
</table>

a 450 nm excitation, 530 nm emission. No peak detected under the proposed analytical conditions described.

A preservative being deemed necessary to retard bacterial growth in urine, we evaluated two preservatives (dilute HCl and oxalic acid dihydrate). There was no difference in the riboflavin concentrations over a seven-day period with the preservatives used. We elected to use oxalic acid because it is easy and safe to use. Acidification was preferred over other means of preservation because of riboflavin's stability at acid pH and rapid degradation in alkaline solution (19, 20).

Tables of normal ranges of urinary riboflavin concentrations are available elsewhere (21-23). Compared with these values, our values had wider limits (Table 2), probably because of the small population sampled and our assumption that the individuals studied were healthy, with adequate riboflavin intake and not subject to any stressful conditions. No attempts were made to evaluate the dietary history of each individual, and no conclusions can be made as to whether their diets represent the "typical American diet". Some of the individuals may have been on special diets that were enriched with water-soluble vitamins, which could help explain some of the "higher than expected" values we observed. A suggested guideline for low or deficient vitamin B2 concentrations in individuals is also given in Table 2. By these criteria, only one individual (2.6%) of our subjects was "deficient" and three (7.9%) had "low" concentrations of the vitamin, the rest being "normal," or having an adequate vitamin B2 intake.

With greater emphasis being placed on nutritional profiling of patients, the described procedure should greatly facilitate the ease of quantitating urinary riboflavin concentrations. Although we recognize that urinary riboflavin measurements are not necessarily related to metabolic state of the vitamin, this method can serve as a useful screening procedure for evaluating riboflavin nutriture in humans, particularly in patients on hyperalimentation diets, individuals on oral

Table 2. Reference Intervals for Riboflavin

<table>
<thead>
<tr>
<th>Riboflavin, μg/24 h</th>
<th>This study (n = 38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deficient a</td>
<td>&lt;150</td>
</tr>
<tr>
<td>Low b</td>
<td>150-499</td>
</tr>
<tr>
<td>Acceptable a</td>
<td>85-269</td>
</tr>
<tr>
<td>1-3 yr</td>
<td>70-199</td>
</tr>
<tr>
<td>4-6 yr</td>
<td>27-79</td>
</tr>
<tr>
<td>7-9 h</td>
<td>39-119</td>
</tr>
<tr>
<td>Adult</td>
<td>30-89</td>
</tr>
<tr>
<td>Pregnant, 2nd</td>
<td></td>
</tr>
<tr>
<td>trimester</td>
<td></td>
</tr>
<tr>
<td>Pregnant, 3rd</td>
<td></td>
</tr>
<tr>
<td>trimester</td>
<td></td>
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</table>

a O'Neal et al. (22). b Pearson (21).

Fig. 2. Chromatogram of FMN, FAD, riboflavin, lumiflavin, and lumichrome, showing typical elution pattern.
contraceptives, Crohn's disease patients, and infants and geriatric populations, where riboflavin deficiencies are more prevalent.

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References