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Short communication

Quantification of riboflavin in human urine using high performance liquid chromatography–tandem mass spectrometry☆

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ABSTRACT

We developed a selective method to measure riboflavin in human urine. Sample preparation involved solid phase extraction and concentration of the target analyte in urine. The urine concentrate was analyzed using high performance liquid chromatography–tandem mass spectrometry. Riboflavin concentrations were quantified using an isotopically labeled internal standard. The limit of detection was 11 ng/mL, and the linear range was 4.4–20,000 ng/mL. The relative standard deviation at 100, 1000, and 5000 ng/mL was 17%, 17%, and 12%, respectively. The accuracy was 90%. On average, 100 samples, including calibration standards and quality control samples, were prepared per day. Using our method, we measured concentrations of riboflavin in human urine samples that were collected from participants in a study where riboflavin was used as a surrogate chemical to simulate exposure to an environmental toxicant.

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1. Introduction

Riboflavin, also known as vitamin B-2, is a water-soluble micronutrient and is primarily eliminated in the urine. This micronutrient, which plays an important role in human health, has also been used successfully as a marker of medication consumption to monitor participants' compliance in epidemiologic studies [1,2]. In a novel application, riboflavin was used as a surrogate chemical to simulate exposure to an environmental toxicant (Lynn Wilder, personal communication).

Analytical methods utilizing high performance liquid chromatography (HPLC)–fluorescence spectroscopy [3–7], HPLC–photo diode array detection [8], HPLC–matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (HPLC–MALDI-TOF) [9], capillary electrophoresis (CE) light emitting diode (LED) fluorescence spectroscopy [10], and CE laser induced fluorescence (LIF) spectroscopy [11,12], have traditionally been used for the determination of riboflavin.

Because of its selectivity, tandem mass spectrometry (MS/MS) is the definitive analytical approach for the determination of environmental toxicants [13] or biological markers of disease in human matrices [14] for biomonitoring purposes. To our knowledge, no methods to date have been published for the determination of riboflavin in human urine using MS/MS. While the traditional methods used for the determination of riboflavin in human matrices offer adequate sensitivity, none provide selectivity comparable to that of MS/MS. We present here a method that makes use of the selectivity of MS/MS, coupled to a liquid chromatography system, to quantify riboflavin in human urine.

2. Experimental

2.1. Chemicals

Riboflavin ($C_{17}H_{20}N_4O_6$) (98%) was purchased from Acros Organics (Morris Plains, NJ, USA). The d_8-riboflavin ($C_{17}H_{12}D_8N_4O_6$) was purchased from Beta Chem (Leawood, KS, USA). OASIS® HLB solid phase extraction (SPE) cartridges (30-μm particle size, 3-cc volume, 60-mg sorbent bed) were purchased from Waters (Milford, MA, USA). Analytical grade methanol and acetonitrile were purchased from Tedia Company Inc. (Fairfield, OH, USA), formic acid (98%) was purchased from Acros Organics, and deionized water was obtained from a DI Reagent Grade Water Purification System (Aqua Solutions®, Jasper, GA, USA).

☆ "The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention".

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Table 1
Multiple reaction monitoring MRM transitions for quantification of riboflavin.

Analyte	Precursor → product	Entrance potential (V)	Collision exit potential (V)	Collision energy (V)	Declustering potential (V)	Dwell time (msec)
Riboflavin-Q[a]	377 → 243	10	18	35	70	200
Riboflavin-C_1[b]	377 → 172	10	13	57	70	200
Riboflavin-C_2[c]	377 → 198	10	13	58	70	200
d_8-Riboflavin[d]	385 → 251	10	20	39	70	200

[a] Q – quantification ion.
[b] C_1 – confirmation ion.
[c] C_2 – confirmation ion.
[d] Used as internal standard.

2.2. Instrumentation

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) analyses were performed using an Agilent 1200 series high performance liquid chromatograph coupled to a SCIEX API 4000 triple quadrupole mass spectrometer equipped with a turbo-ionspray probe (Applied Biosystems, Foster City, CA, USA).

Chromatographic separation was performed using an ATLANTIS® HILIC Silica column (4.6 × 100 mm, 3 μm, Waters) with acetonitrile:water:formic acid (80:20:0.2) as the mobile phase at an isocratic flow rate of 700 μL/min for 4 min. Analyst Ver. 1.4.1 (Applied Biosystems) software was used for data acquisition and analysis.

Optimized instrument settings specific for riboflavin and d_8-riboflavin are as follows: curtain gas setting 20, ion source gas 1 setting 40, ion source gas 2 setting 0, ion spray voltage setting 5500 V, Turbo Heater temperature setting 650 °C, and collision gas setting 7. The protonated ions $(M+H)^+$ for riboflavin and d_8-riboflavin were selected in the first quadrupole, and the collision energy was adjusted to achieve maximum sensitivity for each ion transition. Quantification and confirmation ion transitions and their optimized values for entrance potential (EP), declustering potential (DP), collision energy (CE), collision exit potential (CXP), and dwell time are listed in Table 1.

2.3. Method to eliminate endogenous riboflavin from urine

The method used to eliminate endogenous riboflavin from human urine reported by Chen et al. [7] was modified by the addition of an ice/water bath and exposure to direct sunlight. Urine was collected from volunteers and pooled according to established protocols for the collection of biological specimens for use in the development and validation of laboratory methods and the preparation of quality assurance/quality control materials. The urine collection protocol was approved by CDC's Institutional Review Board. Five hundred milliliters of urine was aliquoted into a 1-L clear glass bottle and placed in an ice/water bath. After exposure to 32 h of sunlight over four days, the urine was analyzed to determine its endogenous concentration of riboflavin. This "blank" urine pool was used as a control to ensure that no contamination in the analytical system occurred and for preparing standard calibration curves and QC pools.

2.4. Preparation of standards and quality control samples

A stock solution (100 μg/mL) of native riboflavin was prepared by dissolving 5.0 mg of compound into 50 mL of water. The stock solution was aliquoted into 4-mL portions and stored in 10-mL amber vials at or below −20 °C. Eight spiking solutions were prepared each week by diluting the stock solution with water to create a calibration curve where a 25-μL aliquot added to a 50-μL urine sample resulted in the following concentrations: 4.4, 14.6, 48.6, 162, 540, 1800, 6000, 20,000 ng/mL. One urine-based calibration curve, prepared as indicated above, was used for the quantification of riboflavin in each analytical run.

A stock solution of d_8-riboflavin was prepared by dissolving 3.7 mg of compound in 10 mL of acetonitrile and was stored in amber vials at or below −20 °C. The internal standard (ISTD) spiking solution of d_8-riboflavin was prepared weekly by diluting the stock solution with acetonitrile so that a 20 μL aliquot added to a 50 μL urine sample resulted in a d_8-riboflavin concentration of 16 μg/mL. The ISTD solution prepared as described was used to spike all blanks, standards, quality control and unknown samples.

QC sample pools were prepared by spiking pooled urine with riboflavin at 100 ng/mL (low-concentration pool), 1000 ng/mL (medium-concentration pool), and 5000 ng/mL (high-concentration pool). QC samples were aliquoted into 1.3-mL portions in amber vials, and stored at or below −20 °C. Characterization of QC materials was performed by running duplicate QCs for all three levels over a period of 20 days. After statistical analysis of the resulting data, one QC sample from each of the three levels was added to each analytical run.

2.5. Sample preparation

Sample urine and blank urine for the standard calibration curve, 50 μL, was pipetted into a 15-mL screw-capped glass tube, and 20 μL of ISTD and 25 μL of appropriate calibrator solution were added. After adding 1.5 mL of deionized water, the urine was vortex-mixed before being extracted on a vacuum manifold using OASIS® HLB 3-cc solid phase extraction (SPE) cartridges. The SPE cartridges were first conditioned with 1 mL of methanol followed by 1 mL of deionized water. After the samples were loaded onto the SPE cartridges, the cartridges were washed with 2 × 1 mL of 20% methanol in water. Cartridges were briefly dried under vacuum. Riboflavin was eluted from the cartridge with 2 × 1 mL of 100% methanol into 15-mL conical tubes. Sample extracts were concentrated to dryness at 40 °C using 12 psi of nitrogen. After concentration, 300 μL of 100% methanol was added to each tube and the tubes were vortex-mixed for about 15 s. After concentrating for 10 additional minutes, samples were reconstituted with 50 μL of 100% methanol, vortex-mixed, and transferred to amber autosampler vials for analysis. A 1-μL volume of sample was injected onto the liquid chromatography (LC) column.

2.6. Validation procedure

The method was validated by evaluating the linearity, limit of detection (LOD), accuracy, precision, and extraction efficiency. Twenty runs were performed over 20 days; each validation run consisted of an eight-point urine-based calibration curve (concentrations 4.4, 14.6, 48.6, 162, 540, 1800, 6000, 20,000 ng/mL), four spiked urine samples (concentrations 4.4, 14.6, 48.6, 162 ng/mL), to be evaluated as unknowns for calculating the LOD, a urine blank and low-, medium-, and high-QC samples ($n = 2$) for calculating the within-day (WD) and between-day (BD) precision. Linearity was evaluated by calculating the average R^2 values from the

20 calibration curves. The LOD was calculated as $3s_0$ where s_0 is the estimated standard deviation at zero concentration [15]. Precision was calculated for each of the three QC levels (100, 1000, 5000 ng/mL) as the relative standard deviation (RSD) using 20 runs over 20 days. Accuracy was calculated as the slope of a linear regression analysis of a plot of the expected concentration of the spiked QC materials versus the calculated concentration of the materials where a slope of one represents 100% accuracy.

The extraction efficiency was evaluated at 432 ng/mL and 10,000 ng/mL. For each concentration evaluated, six blank urine samples were spiked prior to the SPE cleanup step, and six additional blank urine samples were prepared concurrently but were spiked after the SPE cleanup step. The latter represented 100% recovery. After the SPE step, all samples were spiked with internal standard solution and analyzed according to the method. Extraction efficiency was calculated with the following equation: extraction efficiency (%) = $A_1/A_2 \times 100$ where A_1 is the area ratio (native area divided by the ISTD area) of samples spiked prior to SPE and A_2 is the area ratio of samples spiked after SPE.

2.7. Effects of exposure to standard lighting

Long-term stability of riboflavin has been reported for up to six months when solutions were stored at either 4 or −20 °C [7]. Because riboflavin is a known photosensitive compound, degradation caused by exposure to standard laboratory lighting was determined by evaluating the effectiveness of storing riboflavin solutions in amber glass compared to clear glass. A 16-μg/mL solution of native riboflavin was prepared in deionized water and divided into a reference pool and a test pool. The reference pool was protected from exposure to light by storing it in an amber vial at or below −20 °C. The test pool was divided into clear and amber vials and placed on a laboratory bench where both vials were exposed continually to standard laboratory lighting. At times t = 0, 3, 6, 9, 11.5, 27, and 50 h, 50-μL aliquots from the amber (n = 3) and clear (n = 3) test vials were dispensed into separate vials, labeled with the time interval, and stored at or below −20 °C. After 50 h, three aliquots of the reference vial and all the time-interval test vials were transferred to individual conical tubes, spiked with internal standard, evaporated to dryness, reconstituted with 100% methanol, and analyzed. The riboflavin relative concentration change was determined by plotting the average response (area of native analyte/area of ISTD) of the three test aliquots divided by the average response (area of native analyte/area of ISTD) of the three reference aliquots versus the time interval.

3. Results and discussion

3.1. Chromatography

Riboflavin eluted at 2.63 min and the LC–MS/MS run time for each sample was 4 min. Fig. 1 shows a chromatogram of quantitation and confirmation ions in a spiked urine sample at a concentration of 4.4 ng/mL, which is equal to the concentration of the lowest calibration standard. This concentration is lower than the calculated LOD and has a signal-to-noise (S/N) ratio greater than 3:1. This is not uncommon because the LOD value we report is based on the precision of repeat measurements, a statistically determined value [15] and, therefore, does not necessarily reflect a concentration level for any specific injection that produces a S/N ratio of 3:1.

3.2. Performance characteristics

The isotopically labeled compound, d_8-riboflavin, was used specifically to minimize any potential matrix effects [16]. The

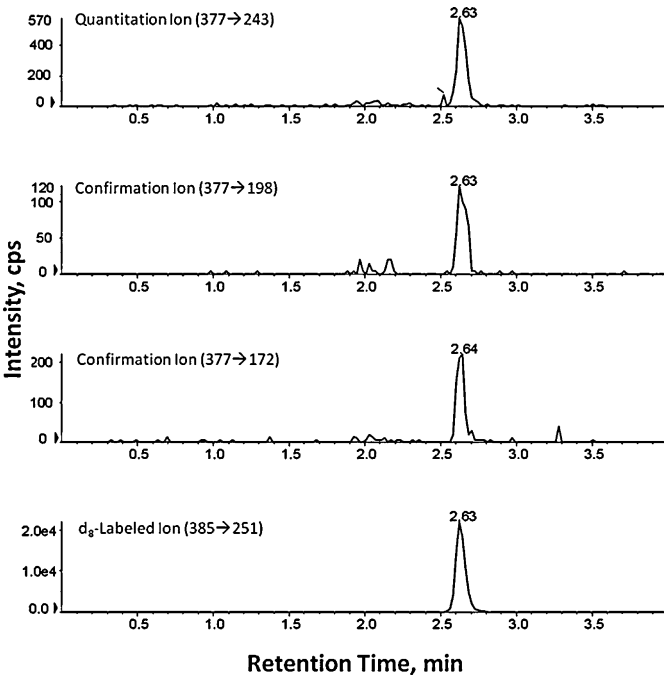

Fig. 1. HPLC–MS/MS chromatogram of spiked urine sample (prepared using cleanup method) at a concentration of 4.4 ng/mL.

endogenous concentration of riboflavin in the pooled urine used as the matrix for constructing calibration curves and preparing QC pools was calculated to be 1.3 ng/mL. This is slightly higher than the value reported by Chen et al. [7]. The following performance characteristics were determined using a urine-based standard curve. The linear range of the method was 4.4–20,000 ng/mL (Fig. 2). The average R^2 value was calculated with n = 20 and was 0.9997. RSDs were 17%, 17%, and 12% at 100, 1000, and 5000 ng/mL, respectively. The accuracy was 90%. The LOD was 11 ng/mL. The extraction efficiency was 96% at 432 ng/mL and 87% at 10,000 ng/mL.

3.3. Exposure to standard laboratory lighting

Because riboflavin is a photosensitive compound, precaution must be taken when handling specimens and making standards. To avoid exposure to laboratory lighting, amber vials were used when available, specifically to store urine samples, stock standards, and QC materials; when amber vials were not available, samples were processed with the laboratory lights off to reduce sample exposure

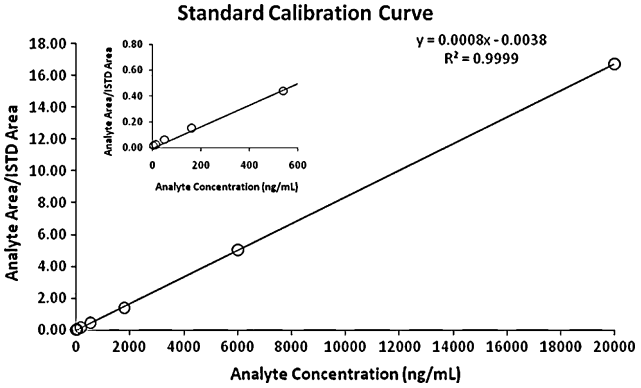

Fig. 2. A typical standard calibration curve for riboflavin using human urine as the matrix. Curve was produced using standard solutions at concentrations of 4.4, 14.6, 48.6, 162, 540, 1800, 6000, 20,000 ng/mL using a 1/x regression fit. Insert shows the linearity fit of the five lowest standards.

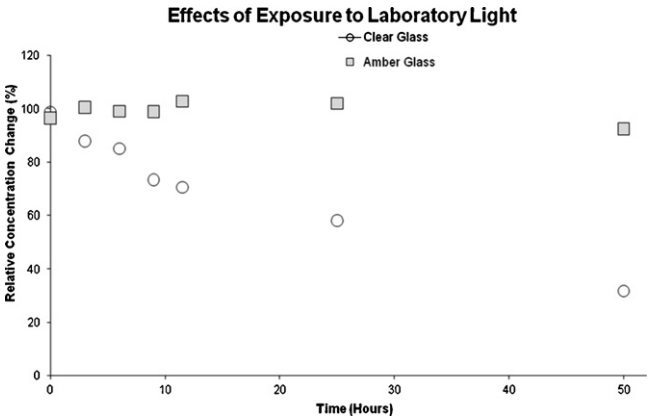

Fig. 3. The effect of exposure to laboratory light on riboflavin was shown by following with time the concentration of a solution of riboflavin stored in clear and amber glass. At each time interval, three aliquots were taken from the vials exposed to light in amber or clear glass (test) and the vial kept in the dark (reference). The RSD for these measurements was <9%. The riboflavin relative concentration change was determined by calculating the average response (area of native analyte/area of ISTD) of the three test aliquots divided by the average response (area of native analyte/area of ISTD) of the three reference aliquots.

to light. When exposed to light for 12 and 50 h, riboflavin concentration stored in amber vials decreased by 2% and 8%, respectively; riboflavin concentration stored in clear glass vials decreased by 30% and 69%, respectively. These results are consistent with those presented by Chen et al. [7]. A plot comparing the relative concentration change of riboflavin stored in clear vials versus amber vials is shown in Fig. 3.

3.4. Method application

The method was used to analyze human urine samples for a study where riboflavin was orally administered to participants to serve as a surrogate to simulate exposure to an environmental toxicant (Lynn Wilder, personal communication). In the study, participants were asked to ingest a known dose of riboflavin and then collect timed, discrete, total-void urine samples for a period of 24 h. Study investigators obtained all relevant human subjects approvals for the study. Calculated concentrations ranged from <LOD to 65,000 ng/mL. Fig. 4 shows a graph of calculated concentrations from a subset of unknown samples.

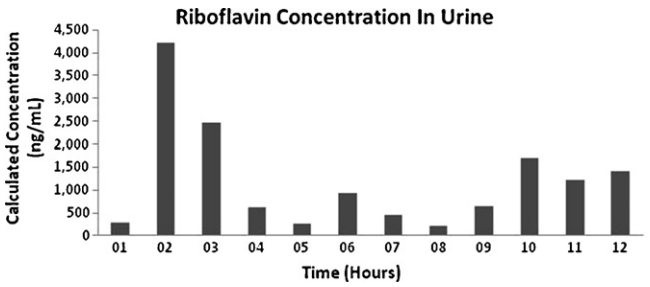

Fig. 4. Calculated concentrations of urinary riboflavin from a subset of human samples.

4. Conclusions

The results presented here suggest that the proposed method, which uses MS/MS, is a highly selective way to quantify riboflavin in human urine. This method, which makes use of the selectivity of MS/MS, has the advantage of low-sample volume, a wide linear range, and an average daily throughput of 100 samples, and is suitable for epidemiological studies.

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