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RAPID METHOD FOR DETERMINATION OF RIBOFLAVIN IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple, specific, and sensitive high-performance liquid chromatographic (HPLC) method for the determination of riboflavin directly in urine samples using a fixed-wavelength spectrofluorometer is described. Centrifuged raw urine samples (50 μl) are injected onto a reversed-phase microparticulate C_{18} column. The eluent is 0.01 *M* KH_2PO_4 (pH 5.0)—methanol (65:35). This method is capable of differentiating riboflavin from riboflavin-5-phosphate, non-riboflavin fluorescing components in urine, and photo-degraded riboflavin. The method shows good reproducibility and is linear to at least 12 μg/ml. The sensitivity of this procedure, at the 95% confidence limit, determined by linear regression analysis, is estimated to be 0.05 μg/ml using peak height and 0.07 μg/ml using peak area. This HPLC method is compared to an automated fluorometric method for riboflavin. The coefficient of linear regression of this comparison is $Y = 0.858 + 0.893X$, where X is the HPLC method and Y is the fluorometric method.

INTRODUCTION

Riboflavin, vitamin B_2, is an enzyme co-factor vitamin found in most multiple vitamin or B-complex preparations. In man, riboflavin is excreted in urine apparently only as free riboflavin [1, 2]. Riboflavin excreted into the urine is often used as a measure of the relative bioavailability of vitamin formulations. The U.S.P. riboflavin assay [3] is a fluorometric method using an excitation wavelength of 440 nm and emission wavelength at 565 nm. Riboflavin is quantitated by comparing the fluorescence of the sample in the oxidized state (fluorescing form) with the reduced state (leuco or non-fluorescing form). Mellor and Maass [4] developed an automated fluorometric method for the determination of riboflavin in human urine. Their method, a modification of the U.S.P. procedure, used an excitation wavelength at 440 nm and emission wavelength at 560 nm.

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Several other assay methods have been described to determine riboflavin in biological fluids: a microbiological method [5], a protozoological assay [6] and a thin-layer chromatographic method [7].

Several high-performance liquid chromatographic (HPLC) methods to determine riboflavin in non-biological fluids using either normal-phase columns with a fluorometer [8] or reversed-phase columns with UV detection [9, 10] have been described. Williams and Slavin [11] described a HPLC method for the direct determination of riboflavin in urine using a variable-wavelength fluorometer and a 10-μl sample volume. The reproducibility of their method was not reported. They also noted the presence of detectable amounts of riboflavin phosphate in the urine from a subject on a riboflavin supplement.

The method described in this paper is a reversed-phase HPLC procedure for the direct determination of riboflavin in urine using a fixed-wavelength fluorometer, 50 μl sample volume, and phosphate buffer—methanol eluent. The concentration of riboflavin and the presence of riboflavin phosphate were determined in urine specimens from subjects who had received a multiple vitamin formulation or from control subjects on a low riboflavin diet. The reproducibility and sensitivity of this method as well as a comparison of the assay results of this HPLC method with the results from an automated fluorometric method [4] are reported.

EXPERIMENTAL

Apparatus

A Spectra-Physics Chromatronix Model 3500 high-performance liquid chromatograph (Santa Clara, CA, U.S.A.) equipped with a Valco (Houston, TX, U.S.A.) sample injection valve with a 50-μl sampling loop and a Hewlett-Packard Model 7120A strip chart recorder (10 mV input and 0.5 cm/min chart speed) was used. The detector was a fixed-wavelength spectrofluorometer (range: 4) (LDC Model 1209 FluoroMonitor, Laboratory Data Control, Riviera Beach, FL, U.S.A.). The excitation lamp was a low-pressure hot cathode mercury lamp with a phosphor coating which emitted near UV light with a range of 320—400 nm. The wavelength range for the emission filter was 400—700 nm. Peak areas were determined with a Spectra-Physics Autolab minigrator. A refrigerated centrifuge (Sorvall RC-3, Norwalk, CN, U.S.A.) was used to prepare the urine samples.

Chromatographic conditions

The column was a reversed-phase micro-particulate C_{18} (μBondapak C_{18}, particle size 10 μm, 30 cm × 4 mm, Waters Assoc., Milford, MA, U.S.A.) preceded by a C_{18} precolumn (Co:Pell ODS, 7 cm × 2.1 mm, Whatman, Trenton, NJ, U.S.A.). The eluent was 0.01 *M* KH_2PO_4 (pH 5.0)—methanol (65:35) at a flow-rate of 2.0 ml/min. The mobile phase was prepared by mixing exact volumes of methanol (distilled-in-glass, spectroscopic grade, Burdick and Jackson, Muskegon, MI, U.S.A.) and 0.01 *M* potassium monobasic phosphate solution adjusted to pH 5.0 with 1 *N* sodium hydroxide and then filtering through a 0.5-μm filter.

Standards

Riboflavin standard stock solutions were prepared to contain 100 μg/ml of U.S.P. reference standard by addition of 100 mg of riboflavin, previously dried at 105°C for 2 h, 750 ml of water and 1.2 ml of glacial acetic acid to a 1-liter flask, dissolving with heat, and diluting to volume with water. This stock solution was diluted with either urine or 0.1 *M* sodium acetate buffer (adjusted to pH 6.0 with acetic acid) to contain 1, 2, 6 and 10 μg/ml of riboflavin. All solutions were protected from light. These standards were injected onto the column via the sampling loop. The chromatogram was recorded and the peak areas or peak heights were determined.

Riboflavin-5-phosphate standard stock solution was prepared to contain 100 μg/ml in distilled water and then diluted with urine to a concentration of 10 μg/ml.

Sample analysis

Approximately 10 ml of urine were centrifuged at 1400 *g* for 10 min. A portion of the supernatant liquid was injected onto the column via the sampling loop. The chromatogram was recorded and the peak areas or peak heights were determined.

System suitability test

The resolution factor (10) for riboflavin relative to riboflavin-5-phosphate should be greater than 3. After three or more injections of a single standard, the relative standard deviation of response should be less than 2%.

RESULTS AND DISCUSSION

The retention time of riboflavin is dependent upon the methanol concentration. A ratio of 65:35 (aqueous buffer—methanol) was selected to maximize sample throughput. Total sample time was about 5 min. A typical chromatogram of riboflavin and riboflavin-5-phosphate in urine is shown in Fig. 1.

Although the excitation wavelength maximum for riboflavin is 440 nm, the excitation energy emitted by this lamp was sufficient to cause fluorescence. The output of this detector was limited, but adequate for this assay. UV absorbance could not be used in the direct determination of riboflavin in urine since large UV absorbance occurred at the solvent front and tailed into the riboflavin peak as was observed by Williams and Slavin [9].

Pooled centrifuged urine containing about 0.3 μg/ml of endogenous riboflavin, based on this HPLC assay, was spiked with riboflavin from 0.2 to 10 μg/ml. Similarly, riboflavin standards in 0.1 *M* sodium acetate (pH 6.0) from 0.4 to 10 μg/ml were prepared. The spiked urine and buffer samples were injected onto the column. The responses (peak area and peak height) of the spiked urine samples were then corrected for endogenous levels of riboflavin.

Least-square linear regression analysis was used to determine the slope, *y*-intercept, and correlation coefficient for the spiked urine samples and the standards in buffer. The results of these two analyses, given in Table I, indicate that all responses were linear with concentration to at least 10 μg/ml of riboflavin. The slopes and *y*-intercepts for corrected standards in urine and stan-

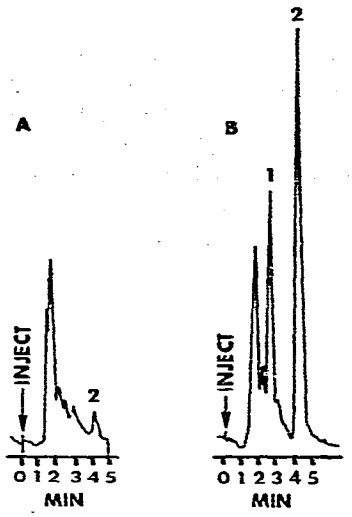

Fig. 1. Chromatograms of riboflavin and riboflavin-5-phosphate in urine. (A) Urine blank with approximately 0.1 μg/ml of endogenous riboflavin (2); (B) same urine spiked with 2.0 μg/ml each of riboflavin-5-phosphate (1) and riboflavin (2).

TABLE I

LINEARITY OF RESPONSE IN HUMAN URINE AND BUFFER SOLUTIONS

Least-square regression analysis*	Urine		Buffer (pH 6.0)	
	Peak height	Peak area	Peak height	Peak area
n	28	29	6	6
Slope	53.3	1.55×10^4	53.9	1.58×10^4
y	0.03	150	0.15	−490
r	0.999	0.999	0.999	0.999

*n = number of samples assayed; y = y-intercept; r = correlation coefficient.

dards in buffer were practically equivalent. These results indicate that standards in buffer can be used in the determination of riboflavin in urine.

The sensitivity of this method was estimated by linear regression analysis to be, at the 95% confidence limit, 0.05 μg/ml using peak height and 0.07 μg/ml using peak area. Only those concentrations less than 1.3 μg/ml were used in the calculation of sensitivity.

The precision of this method was determined by assaying samples of known concentrations of riboflavin in urine. The responses (both peak height and peak area) were corrected for endogenous riboflavin which was calculated to be 0.34 μg/ml. The results are given in Table II. The mean calculated concentration ± standard deviation and differences between actual and calculated concentration are reported. As is shown in Table II, this method showed good accuracy and reproducibility. The pooled coefficient of variation was <1% at concentrations greater than 1 μg/ml and 6.3% at concentrations less than 1 μg/ml using peak height as the response and 1.8% at concentrations greater than 1 μg/ml and

TABLE II

PRECISION AND ACCURACY IN THE DETERMINATION OF RIBOFLAVIN IN URINE

Theoretical concentration (μg/ml)	Calculated concentration (μg/ml)					
	Using peak height			Using peak area		
	Concentration (mean ± standard deviation)	*n*	% Difference	Concentration (mean ± standard deviation)	*n*	% Difference
10.0	9.95 ± 0.005	4	0.5	9.95 ± 0.03	4	0.5
6.0	6.10 ± 0.006	3	1.7	6.07 ± 0.04	3	1.2
3.0	3.00 ± 0.007	2	0	3.10 ± 0.02	2	3.3
2.0	2.06 ± 0.007	2	3	2.03 ± 0.06	2	1.5
1.0	1.00 ± 0.05	3	0	1.03 ± 0.02	3	3
0.4	0.37 ± 0.005	4	7.5	0.35 ± 0.02	4	12.5
0.2	0.16 ± 0.06	3	20.0	0.15 ± 0.03	3	25.0

3.3% at concentrations less than 1 μg/ml using peak area. The average percent difference in calculated concentration relative to theoretical concentration was 1% using peak height as the response and 1.9% using peak area at concentrations greater than 0.5 μg/ml.

In bioavailability studies of multiple vitamin formulations, the subject must collect his/her urine over a 24-h period. This collected urine is usually stored at room temperature. These urine specimens are then stored at 5°C until all legs of a cross-over design are complete. Therefore, the stability of riboflavin in urine at room temperature and at 5°C was determined. Pooled urine, spiked with riboflavin (10 μg/ml), protected from light and stored at room temperature, was periodically sampled up to 24 h. Similarly, riboflavin-spiked urine was stored under refrigeration (5°C) and periodically sampled up to two weeks. Pooled urine spiked with riboflavin (10 μg/ml) was also exposed to direct natural sunlight at room temperature. These samples were then assayed according to this procedure.

For those samples protected from light and stored at room temperature or 5°C, no change in riboflavin peak response was observed. The spiked urine samples exposed to natural light showed a decrease in the peak response of riboflavin and the appearance of a second peak (retention time was about 30 sec longer than riboflavin) which also disappeared upon prolonged exposure to light as is shown in Fig. 2. These results indicate that this HPLC procedure can differentiate between photodegraded and undegraded riboflavin. Riboflavin in urine, when protected from light, appeared to be stable in urine for at least 24 h at room temperature and at least 2 weeks at 5°C.

This HPLC method was compared to an automated fluorometric method for the determination of riboflavin in urine. Urine samples from human subjects receiving a 10-mg riboflavin tablet or a multivitamin tablet containing 10 mg of riboflavin as well as urine samples from the same subjects on a low riboflavin diet and no riboflavin supplement were assayed by this HPLC—fluorometric method and by an automated fluorometric method [2]. It should be noted that no detectable levels of riboflavin phosphate were observed in these urine specimens by the HPLC method. In the fluorometric method, the apparent riboflavin content of urine was calculated from the difference in fluorescent intensity before and after reduction with sodium hydrosulfite, by comparison

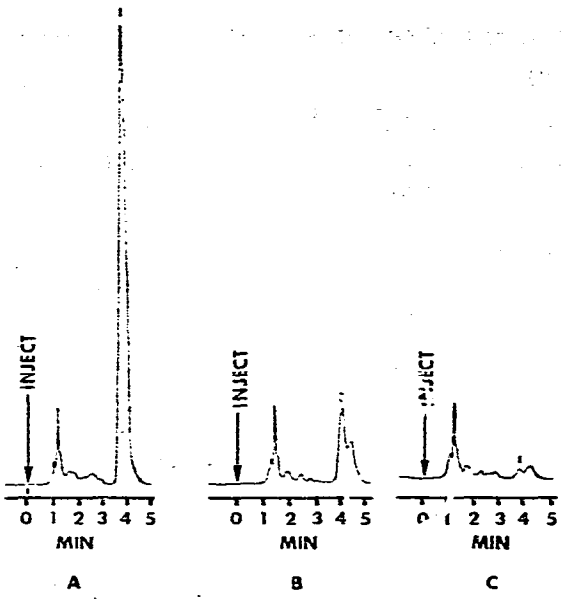

Fig. 2. Stability of riboflavin in urine in natural light. Riboflavin (10 μg/ml) (1) under following experimental conditions: A = initial, B = 1 h in natural sunlight, C = 4 h in natural sunlight.

with the intensity of known concentrations of riboflavin assayed simultaneously.

The data from these two determinations were compared using linear regression analysis and paired *t*-test. A graphical representation of this comparison is given in Fig. 3. The coefficients of the linear regression analysis are as follows: $Y = 0.865 + (0.888 \pm 0.050)\ X$ ($p = 0.05$) where X = HPLC data and Y = fluorescence data; $r = 0.991$. Both coefficients were significantly greater than

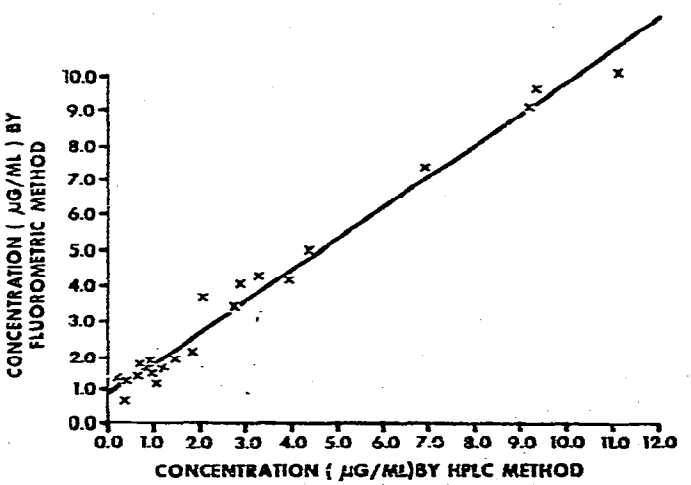

Fig. 3. Comparison of the HPLC method with a fluorometric method for the determination of riboflavin concentration in human urine.

zero. The paired t-test indicated that the fluorometric and HPLC results were significantly different [$p = 0.01$ ($t_{value} = 5.303$, $df = 23$)].

In the comparison of the HPLC and fluorometric method a few points at higher concentrations heavily weighed the estimation of the overall slope. Linear regression analysis of those riboflavin concentrations less than 5 μg/ml were determined. The regression equation is as follows: $Y = 7.3 + (0.98 \pm 0.13) X$ ($p = 0.05$, $n = 20$, $r = 0.97$) where X = determinations by HPLC, Y = determinations by fluorometric method. For concentrations less than 5 μg/ml, the slope was not significantly different from one ($p = 0.05$, $n = 20$). The y-intercept was still significantly greater than zero.

These analyses indicated that the assay results from this HPLC method and an automated fluorometric differed, but were highly correlated. The HPLC method has been demonstrated to be specific. As was shown in Figs. 1 and 2, this method differentiated riboflavin, riboflavin phosphate, and photodegradation products of riboflavin. The fluorometric method may not have the same degree of specificity, and may be quantitating a residual fluorescing component of urine which is also reduced to a non-fluorescing component.

In conclusion, this HPLC method, which uses a fixed-wavelength spectrofluorometer for the determination of riboflavin directly in urine, is a simple, reproducible and sensitive procedure. Assay results of human urine specimens by this HPLC method and by an automated fluorometric method were different, but highly correlated.

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