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QUANTIFICATION OF RIBOFLAVIN, RIBOFLAVIN 5′-PHOSPHATE AND FLAVIN ADENINE DINUCLEOTIDE IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method with fluorimetric detection for the quantification of riboflavin (RB), riboflavin 5′-phosphate (FMN), and flavin adenine dinucleotide (FAD) in plasma, whole blood, and urine is described. Under isocratic conditions with a reversed-phase column, the compounds are completely resolved and eluted within 9 min. Plasma proteins are precipitated with acetonitrile followed by shaking the aqueous phase with chloroform. Urine samples are diluted and injected directly. The reproducibility of this method for the quantification of RB in plasma has a between-day coefficient of variation of 6%. The application of this method is illustrated by analyzing plasma and urine samples from a human subject who received an intravenous dose of FMN equivalent to 25 mg of RB.

INTRODUCTION

Riboflavin (RB), vitamin B_2, participates in biochemical reactions in the form of two coenzymes, riboflavin 5′-phosphate (FMN) and flavin adenine dinucleotide (FAD). A variety of methods is available for analyzing these flavins in biological fluids; however, these methods may lack specificity. Direct fluorimetric methods for the determination of RB [1–3] are potentially non-specific [4]. Microbiological [5] and protozoological methods [6] are relatively sensitive, but not specific. Competitive protein binding assays are only specific for RB [7,8]. Finally, paper [9], thin-layer [10], or high-performance liquid chromatographic (HPLC) assays are specific for RB and may simultaneously quantify FMN and FAD.

Reversed-phase HPLC systems with fluorimetric detection have been used to determine RB in urine [4,11,12] and in hemodialysis fluid [13]. In whole blood, FAD has been determined by HPLC using an amino column [14]. However, these

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methods do not separate the three flavins [4,11–14]. The three flavins have been separated in whole blood with gradient-elution HPLC using an anion-exchange column [15]. Separation of FAD, FMN, RB and some metabolites of RB has been achieved using gradient elution and a C_{18} column [16]. Although isocratic separation of FAD, FMN, and RB using C_{18} columns has been reported with relatively short [15,17] and long [18] retention times, we experienced some difficulties in reproducing the methods that have short retention times when processing a large number of samples.

We report here an isocratic HPLC method which employs direct fluorimetric detection for the quantification of FAD, FMN, RB, and RB-photodegraded products. Separation of the three flavins is achieved in about 9 min. Sample treatment does not result in dilution and, therefore, there is no loss of sensitivity. The application of this HPLC system is illustrated for the quantification of FMN and RB in plasma and RB in urine from a human subject who received an intravenous dose of FMN equivalent to 25 mg of RB. RB plasma concentrations from 22 normal male volunteers with ages ranging between 33 and 86 years are reported. Finally, this method was used to quantify the three flavins in whole blood from five normal male volunteers.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a single-piston pump (Model 110A, Beckman, Fullerton, CA, U.S.A.) with a 50-μl loop. A spectrophotofluorimeter with a xenon lamp (Model 650-10M, Perkin-Elmer, Norwalk, CT, U.S.A.) was set at a wavelength of 470 nm for excitation and 525 nm for emission with a 20-nm bandpath at both wavelengths. The response of the detector was recorded with an integrator (Model 3390, Hewlett-Packard, Avondale, PA, U.S.A.).

Chromatographic conditions

The column was a reversed-phase material of macroporous copolymer, PRP-1, particle size 10 μm, 25 cm $\times$ 4.6 mm I.D. (Hamilton, Reno, NV, U.S.A.) preceded by a guard column of the same material (7 cm $\times$ 2.1 mm). The columns were used at room temperature (approximately 22 °C), and the flow-rate of the mobile phase was set at 1.0 ml/min. The mobile phase was acetonitrile–water–diluted trifluoroacetic acid (10%, v/v)–phosphoric acid (14:84:1.5:0.09). The pH of this mixture was adjusted to 1.8 with 10% sodium hydroxide. The mobile phase was filtered and degassed by filtration through Nylon 66 filters (0.45 μm pore, 47 mm diameter) (Alltech Assoc., Deerfield, IL, U.S.A.).

Reference compounds

RB, FMN, and FAD were obtained from Sigma (St. Louis, MO, U.S.A.). Stock solutions of FMN and FAD were prepared by dissolving 10 mg in 100 ml of 0.1 *M* sodium acetate (pH 6.0). Riboflavin (10 mg) was dissolved in 20 ml of acetonitrile and diluted to 100 ml with water. Stock solutions were stored for two weeks at −20 °C.

Sample preparation

Blood samples were withdrawn from fasting, healthy subjects into vacutainer tubes (Becton-Dickinson, Rutherford, NJ, U.S.A.) containing lithium heparin, immediately placed on ice, and protected from light. Within 15 min, plasma was obtained by centrifugation at 1500 *g*, stored in the dark, and placed in a refrigerator at 4°C and analyzed within 2 h or frozen at −20°C and analyzed within one week.

In a 15-ml test tube, protected from light, 1 ml of acetonitrile was added to 1 ml of plasma to precipitate proteins. The tube was vortex-mixed for 1 min and centrifuged for 5 min at 1500 *g* to separate proteins. The supernatant was transferred to another 15-ml test tube, protected from light, and mixed for 3 min with 6 ml chloroform. The volumes for acetonitrile and chloroform were chosen since they produced the maximum extraction and reproducibility for the three flavins. The aqueous phase was kept at 4°C and injected within 4 h.

Cold water (0.5 ml) was added to 0.5 ml of blood and vortex-mixed for 10 s. The mixture was left to stand for 10 min in an ice bath in order to hemolyze the red blood cells. The hemolyzed blood was treated as above for the plasma samples.

Urine samples were also protected from light and maintained at 4°C during collection times. At the end of the collection periods, total volumes were measured, aliquots frozen at −20°C, and analyzed within one week. Urine samples from subjects without vitamin B_2 supplementation were undiluted and those from subjects after FMN dosing diluted up to 1:40 with water and injected directly.

RB and FMN plasma and urine concentrations were determined by reference to calibration curves in the respective biological fluids. These calibration curves were corrected for endogenous flavins.

Recovery

The recovery of RB was determined in plasma by addition of a trace quantity of [^{14}C]RB (Amersham, Arlington Heights, IL, U.S.A.). The radioactivity was determined in the pellet, in the supernatant after extraction, and in the chloroform phase. Plasma calibration curves were corrected for endogenous flavins. Slopes and intercepts of calibration curves for the three flavins between water and plasma were compared. Finally, a spiked plasma sample containing 500 ng/ml of each flavin was extracted, and the organic phase evaporated under a stream of nitrogen at room temperature. The remaining material was redissolved in 200 μl of acetonitrile and analyzed by HPLC.

Reproducibility

Five known concentrations above the endogenous concentrations of FAD, FMN, and RB were prepared in plasma and urine and analyzed the same day. The within-day reproducibility of the assay was examined by preparing calibration curves ranging from 15 to 575 ng/ml in water, human plasma, and urine. The fluorescence response for each flavin was corrected for endogenous concentrations in plasma and urine. In addition, the within-day reproducibility of this extraction procedure in plasma was compared with three other methods [14,15,18]. Plasma calibration curves were prepared from 15 to 75 ng/ml to determine the concen-

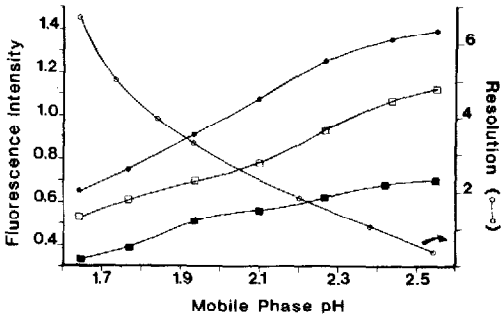

Fig. 1. Fluorescence intensity (y-axis on left) as a function of pH for the three flavins. The resolution between FMN and FAD (y-axis on right) is shown as a function of pH. Resolution (R_s) between these flavins was determined by the following equation: $R_s = 2(t_1 - t_2)/(W_1 + W_2)$, where t_1 is the retention time for FMN, t_2 is the retention time for FAD, W_1 is the width of the FMN peak, and W_2 is the width of the FAD peak. Key: (□) FMN, (●) RB, and (■) FAD.

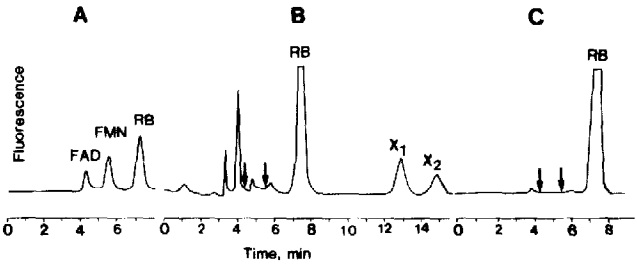

Fig. 2. (A) HPLC profile of authentic flavin standards in water. The peaks correspond to the following concentrations with retention times in parentheses: FAD, 10 ng/ml (4.37 min); FMN, 10 ng/ml (5.70 min); RB, 10 ng/ml (7.47 min). (B) HPLC profile of a human urine sample (subject on a normal diet) exposed to sunlight for 1 h, showing RB and its photodegradation products, indicated as X_1 and X_2. Arrows indicate the absence of FAD and FMN at the retention times of the standards. (C) HPLC profile of a human urine sample diluted 1:20, 2 h after the intravenous administration of FMN. Arrows indicate absence of FAD and FMN. The fluorometer was set at a range of 1.0.

tration of five spiked control plasma samples at concentrations of 25 and 50 ng/ml above the endogenous flavin concentrations. The plasma standards and spiked plasma samples were treated similarly by each extraction method and injected onto the column.

The between-day reproducibility in plasma and urine was examined by analyzing frozen control samples of 50 and 100 ng/ml for each of the three flavins during five consecutive days. Concentrations were determined from their respective daily standard curves after correction for endogenous flavins.

RESULTS AND DISCUSSION

The mobile phase was selected for optimizing the separation of FAD and FMN by lowering the pH and percentage of acetonitrile. The retention times of RB, FMN, and FAD were inversely dependent upon acetonitrile concentration and independent of ionic strength. When pH decreases, the fluorescence of FAD, FMN, and RB decrease as shown in Fig. 1. In addition, retention times for FAD and RB

were practically independent of pH, while the retention time of FMN increased by decreasing the pH. The resolution between FMN and FAD was increased by reduction of pH, as can be seen in Fig. 1. A reasonable retention time of 8 min for RB was observed using 14% acetonitrile. A typical chromatogram of authentic compounds of FAD, FMN, and RB is shown in Fig. 2A. The retention times were 4.37 min for FAD, 5.70 min for FMN, and 7.47 min for RB.

In addition to the separation of the flavins, photodegraded products of RB were separated under these chromatographic conditions. Fig. 2B shows two photodegradation products of RB in a urine sample. These products appeared from a standard RB solution and from a urine sample after they were exposed to sunlight for about 1 h. The urine sample, in Fig. 2B, was from a healthy adult volunteer on a normal diet.

The peak detector response was linearly related to the amounts of flavins injected. The fluorescence response was determined over a wide range of concentrations (30–600 ng/ml). The calibration curves were linear over this concentration range.

The advantage of this extraction procedure is that the plasma sample is practically undiluted before injection onto the column. Similar sample preparation has been reported for the determination of other drugs in plasma with excellent reproducibility [19] and recoveries greater than 84% [20].

After extracting the plasma supernatant with chloroform, the chloroform–acetonitrile phase contained undetectable amounts of FAD and FMN and only small amounts of RB (less than 2% of the initial spiked amount). This observation is in agreement with the low partition coefficient of FMN and FAD between chloroform and phosphate buffer at pH 6.8 [21]. A comparison of the slopes and intercepts obtained from standard curves in plasma and water indicates no statistically significant difference ($p > 0.05$, $n = 5$) for FAD and FMN, but a difference in slope for RB. However, this difference was small; typical slopes of standard curves of RB in water and plasma were 0.432 and 0.435, respectively. Finally, [^{14}C]RB extracted from plasma with this method shows that the radioactivity in the organic phase was 4% (coefficient of variation, C.V. = 4.6%, $n = 5$) of the added radioactivity in the plasma. Therefore, insignificant amounts of FAD and FMN and about 2–4% of RB is lost during the partition procedure with chloroform. On the other hand, 20% (C.V. = 3.8%) of the total radioactivity of RB remains in the protein pellet. This percentage is consistent with the pellet containing 17% FAD and 11% water after protein precipitation with 10% trichloroacetic acid [18].

Table I shows the between-day reproducibility of this extraction procedure for plasma for FAD, FMN, and RB. The between-day reproducibility for RB at different concentrations had a C.V. of less than 8% ($n = 18$). The reproducibility for FMN and FAD had a C.V. ranging from 10 to 20% ($n = 5$).

The within-day reproducibility for this extraction procedure for the three flavins from plasma was compared with three methods previously reported [14,15,18]. The results are presented in Table II. The extraction procedure with 5% ammonium chloride in 10 m*M* sodium dihydrogenphosphate [15] had the best reproducibility, with a dilution of the sample of only 1:1.5. The mixture of 6%

TABLE I

BETWEEN-DAY REPRODUCIBILITY OF THE HPLC ASSAY FOR THE THREE FLAVINS IN PLASMA

Concentrations were determined from standard curves in plasma or in urine having concentrations of 15, 20, 40, 75 and 110 ng/ml above the endogenous concentration.

Flavin	n	Concentration added (ng/ml)	Concentration determined (mean ± S.D.) (ng/ml)
RB	18	50.0	50.7 ± 3.04
		100.0	100.9 ± 7.62
FAD	5	50.0	53.4 ± 7.74
		100.0	100.0 ± 13.18
FMN	5	50.0	48.7 ± 9.45
		100.0	98.6 ± 8.87

trichloroacetic acid and acetonitrile is a very simple procedure that produces a clean supernatant, but loss of sensitivity is observed as a result of a 1:7 dilution of plasma [14]. The extraction procedure with 10% trichloroacetic acid had adequate reproducibility for RB [18] (samples are diluted 1:5), but this method requires maintaining the sample at low temperatures since hydrolysis of FAD in 10% trichloroacetic acid has been observed [21]. In addition to the results presented in Table I, RB analysis in duplicate unknown samples did not differ by more than 3% ($n=10$).

A comparison of slopes and intercepts of calibration curves of RB in water and urine (corrected for endogenous RB) indicated no statistical differences by the Student's t-test ($p>0.05$). Therefore, water calibration curves can be used to determine RB in urine. This conclusion is similar to a previous report that determined RB in urine by preparing calibration curves in a pH 6.0 buffer [6]. The

TABLE II

WITHIN-DAY REPRODUCIBILITY OF THE HPLC ASSAY FOR FLAVINS IN PLASMA, AND COMPARISON WITH OTHER METHODS

Each value represents the mean ± S.D. of five determinations. Concentrations were determined from standard curves in plasma having concentrations of 15, 20, 30, 40, and 75 ng/ml above the endogenous concentration.

Flavin	Concentration added (ng/ml)	Concentration determined (ng/ml)			
		Ref. 14	Ref. 15	Ref. 18	This method
RB	25.0	25.4 ± 9.4	24.7 ± 8.0	24.8 ± 1.6	25.2 ± 6.9
	50.0	50.4 ± 0.8	49.7 ± 3.9	50.1 ± 2.1	50.7 ± 3.0
FAD	25.0	22.4 ± 16.1	24.9 ± 6.0	20.7 ± 6.2	21.5 ± 13.0
	50.0	51.2 ± 4.0	49.6 ± 3.2	45.8 ± 7.0	53.6 ± 15.0
FMN	25.0	26.8 ± 20.0	23.5 ± 4.8	24.7 ± 3.2	25.4 ± 20.7
	50.0	50.8 ± 4.7	50.1 ± 4.0	48.0 ± 5.0	48.2 ± 9.8

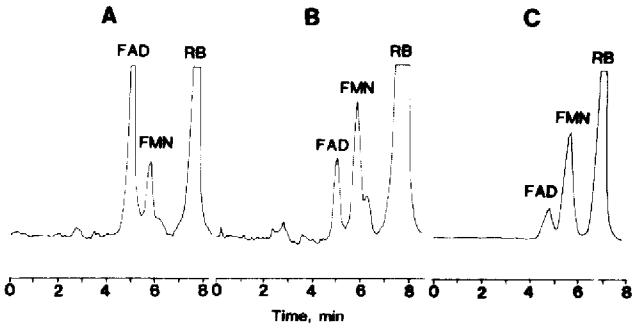

Fig. 3. Typical chromatograms showing the separation of flavins. The fluorometer was set at a range of 0.1 for chromatograms A and B, and at 0.3 for chromatogram C. (A) Whole blood from a healthy volunteer on a normal diet. Concentrations are: FAD, 120 ng/ml; FMN, 10 ng/ml; RB, 50 ng/ml. (B) Plasma sample from the whole blood shown in (A). Concentrations are: FAD, 35 ng/ml; FMN, 38 ng/ml; RB, 94 ng/ml. (C) Plasma from a healthy adult volunteer 0.75 h after the administration of a constant-rate intravenous infusion of FMN equivalent to a total dose of 25 mg of RB. Concentrations are: FAD, 65 ng/ml; FMN, 94 ng/ml; RB, 158 ng/ml.

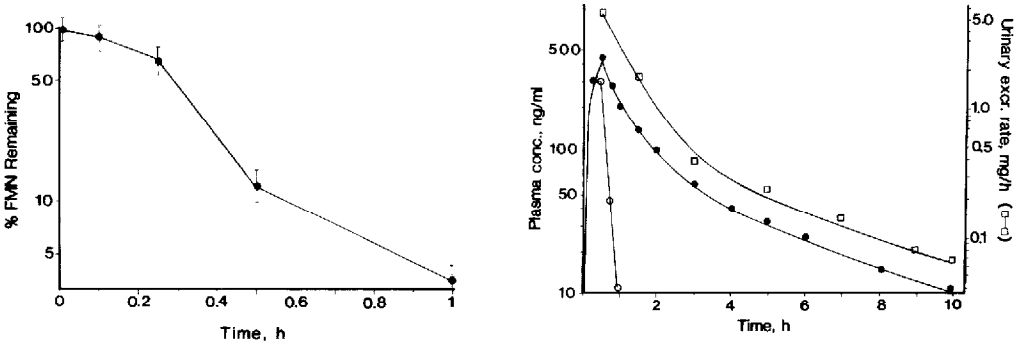

Fig. 4. Percentage FMN remaining in whole blood (on a semi-logarithmic scale) as a function of time. The percentage remaining was determined after correction for endogenous FMN. Lines connect the average experimental observations and the cross-hatched vertical bars represent the standard deviation of three determinations.

Fig. 5. RB (●) and FMN (○) plasma concentrations (semi-logarithmic scale, y-axis on left) as a function of time and RB urinary excretion rate (□) (y-axis on right) as a function of time. Samples were obtained as described in the text from a healthy human volunteer and analyzed by the HPLC method reported here. Endogenous RB and FMN plasma concentrations were subtracted from plasma concentrations after the intravenous dose. Similar corrections were made to the urine data. The solid lines represent the non-linear regression fit of the data.

between-day and within-day reproducibility of RB control samples in urine were less than 8% and less than 4%, respectively.

Fig. 3A and B show typical chromatograms obtained from plasma and whole blood (from a normal subject). The concentration of FAD is higher in blood than in plasma, and RB concentrations are higher in plasma than in blood. These observations agree with previous reports [15,18].

This assay was used to determine the hydrolysis of FMN in vitro in whole blood at 37°C in the dark. A 1-ml volume of 10 μg/ml FMN solution in 0.9% sodium

chloride was added to 10.0 ml of fresh human blood. FMN was analyzed at different times by the method described in sample preparation. Concentrations of FMN were determined from a calibration curve prepared in plasma. Fig. 4 shows a rapid dephosphorylation of FMN with a half-life of about 15 min. By using a manual fluorometric method, a half-life of about 1 h for the hydrolysis of FMN at 37 °C was reported [22]. A rapid degradation of FMN in rabbit blood has been reported based upon a paper chromatographic assay [23]. In addition, the in vivo kinetics of RB and FMN were determined in a healthy male subject after a 30-min constant-rate intravenous infusion of FMN (equivalent to 25 mg of RB). Fig. 5 illustrates the plasma concentrations of RB and FMN, and the urinary excretion rate of RB as a function of time. FMN dephosphorylates in plasma at a rate of about 3% in 1 h [17]. Since FMN is hydrolyzed slowly in plasma [17] in comparison with blood, plasma was separated immediately after obtaining the blood samples. Fig. 5 shows rapid dephosphorylation of FMN, similar to what was observed in vitro (Fig. 4). These results indicate that hydrolysis of FMN occurs mainly in blood and that liver metabolism may play a small role in this hydrolysis. On the other hand, FAD plasma concentrations remained relatively constant after the intravenous administration of FMN.

Undetectable amounts of FMN and FAD were found in urine samples from subjects on a normal diet or after the intravenous infusion. Fig. 2C presents a chromatogram of urine from a healthy volunteer 2.0 h after the intravenous infusion of FMN. The absence of FMN partially disagrees with a previous report that used a manual fluorometric method [22]. FMN was excreted in urine in relatively small amounts compared to RB after an intravenous dose of FMN in one subject [22]. It has been reported that FAD is absent in urine in subjects taking a normal diet [16]. FMN was also absent in urine from subjects that had taken oral multi-vitamins and from subjects ingesting a diet low in RB content [6]. Finally, it has been suggested that FMN is hydrolyzed in the bladder and therefore FMN is not normally present in urine [23].

The sensitivity of this method is adequate to determine normal endogenous concentrations of RB in plasma. Assuming that the signal-to-noise ratio should be at least 3, the lower limit for detection is 1 ng/ml for RB, 2 ng/ml for FMN, and 5 ng/ml for FAD. FAD concentrations in blood ranged from 157.1 to 274.9 ng/ml ($n=5$) in subjects from 27 to 30 years of age. These values agree with previous reports [14,15,18]. FMN plasma concentrations ranged from 5 to 38 ng/ml ($n=5$). RB plasma concentrations ranged from 5 to 30 ng/ml ($n=44$) in subjects after two days of ingesting a diet restricted to RB content (ages ranging from 32 to 86 years). Those values are consistent with the average RB concentration of 11.6 ng/ml in serum in men and women whose ages ranged from 24 to 42 years [15].

In conclusion, the HPLC method reported here provides a simple approach to the determination of RB in plasma and urine and allows the separation of FAD and FMN. This method was used for a three-month period in the analysis of 1500 biological samples. The use of this assay has been illustrated (Fig. 5) for the determination of FMN and RB in plasma samples after an intravenous infusion of FMN to a healthy human volunteer. After intravenous administration, FMN

plasma concentrations decreased rapidly due to its hydrolysis in blood. FMN was absent in urine samples from subjects on a normal diet and after intravenous FMN administration.

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