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Quantification of Riboflavin, Flavin Mononucleotide, and Flavin Adenine Dinucleotide in Human Plasma by Capillary Electrophoresis and Laser-induced Fluorescence Detection

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Background: **Riboflavin is the precursor of flavin mononucleotide (FMN) and FAD, which serve as cofactors for several redox enzymes. We have developed a capillary electrophoresis method for the determination of riboflavin and its two coenzyme forms in human plasma.**

Methods: **Trichloroacetic acid-treated plasma was subjected to solid-phase extraction on reversed-phase columns. The analytes were separated by micellar electrokinetic capillary chromatography in uncoated fused-silica capillaries filled with borate buffer containing 50 mmol/L sodium dodecyl sulfate, methanol, and *N*-methylformamide. Native fluorescence was monitored at 530 nm, using an argon laser operating at 488 nm as excitation source.**

Results: **The assay was linear over a concentration range of two orders of magnitude, and the limit of detection was far below physiological concentrations for all vitamers. The within-day and between-day coefficients of variation were 4–9% and 6–12%, respectively. The reference values (median, 5–95 percentiles) obtained by analyzing plasma from 63 healthy subjects were 8.6 nmol/L (2.7–42.5 nmol/L) for riboflavin, 7.0 nmol/L (3.5–13.3 nmol/L) for FMN, and 57.9 nmol/L (44.5–78.1 nmol/L) for FAD.**

Conclusions: **Capillary electrophoresis with laser-induced fluorescence detection allows determination of all riboflavin vitamers far below physiological concentrations. The method may become a useful tool for the assessment of riboflavin status in humans.**

Riboflavin is a water-soluble vitamin that serves as a precursor for flavin mononucleotide (FMN)[1] and FAD *(1, 2)*. These coenzymes are involved in several reduction-oxidation reactions and take part in the metabolism of other vitamins, e.g., folate and vitamin B_6 *(2)*.

Riboflavin deficiency has been claimed to be quite common, and high prevalences have been reported, particularly in developing countries *(3–5)*. The riboflavin status in humans has been assessed by clinical signs, by determination of the urinary excretion of the vitamin *(6)*, and by measurement of the activity ratio of glutathione reductase in erythrocytes *(7, 8)*.

For the determination of vitamin concentrations, several techniques have been developed, including microbiological *(9)*, fluorometric *(9)*, and liquid chromatographic methods *(10)*. Most HPLC methods have been designed for the detection of high concentrations of riboflavin in food, pharmaceutical preparations, and urine *(10)*. Only a few have been used to measure riboflavin in whole blood *(11–14)*, serum *(11, 14, 15)*, or plasma *(16–18)*.

During the last decade, capillary electrophoresis (CE) methods have been developed for several biomedical applications *(19)*. Compared with HPLC, CE generally has the advantage of small sample requirements, short separation times, and high resolution *(20)*. The consumption of organic solvents and other chemicals is usually lower than in HPLC.

We describe here a sensitive and robust CE method for the quantification of low physiological concentrations of riboflavin, FMN, and FAD in human plasma. The method is based on micellar electrokinetic capillary chromatography combined with laser-induced fluorescence (LIF) detection.

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[1] Nonstandard abbreviations: FMN, flavin mononucleotide; CE, capillary electrophoresis; LIF, laser-induced fluorescence; and TCA, trichloroacetic acid.

Materials and Methods

CHEMICALS

Riboflavin, FMN, FAD, and lumiflavin were purchased from Sigma-Aldrich Norway. Isoriboflavin was kindly supplied as a gift by F. Hoffmann-La Roche & Co. (Basel, Switzerland). Sodium dodecyl sulfate, trichloroacetic acid (TCA), and boric acid were obtained from Merck. Methanol (HPLC grade) was purchased from Rathburn Chemicals, and *N*-methylformamide was purchased from Fluka. Doubly distilled water purified on a MilliQ Plus Water Purification System (Millipore) was used for preparation of all aqueous solutions.

INSTRUMENTATION

Solid-phase extraction was performed on a programmable Gilson ASPEC sample processor (Gilson Medical Electronics) equipped with a Rheodyne Model 7010 injector valve and a 3000-μL sample loop.

CE was performed on a Beckman P/ACE System 2210 coupled to a P/ACE LIF Detector (Beckman Instruments). The LIF detector was connected to a 20 mW 488 nm argon laser from Uniphase. The fluorescence was monitored using a 530 nm DF30 band-pass filter from Omega Optical. Beckman System Gold software (Ver. 8.10) was used for system control and data collection and processing.

CAPILLARY ELECTROPHORESIS

Uncoated fused-silica capillaries (Composite Metal Services) with external and internal diameters of 375 μm and 75 μm, respectively, were used. The total capillary length was 67 cm, and the effective separation length was 60 cm. The separation buffer consisted of 100 mmol/L sodium borate, pH 7.9, containing 50 mmol/L sodium dodecyl sulfate, 100 mL/L methanol, and 20 mL/L *N*-methylformamide. All solutions were passed through 0.2 μm membrane filters (Schleicher & Schuell) before use. Capillaries were preconditioned before each electrophoretic run by rinsing in the high-pressure mode (137 kPa) with 0.1 mol/L NaOH for 60 s and the separation buffer for 120 s. Samples were injected for 7 s in the low-pressure mode (3.5 kPa), corresponding to an injection volume of ~25 nL. This was followed by a second injection of separation buffer (for 5 s), to prevent loss of sample material. Electrophoresis was performed at 24 kV (358 V/cm) in the normal polarity mode (positive potential at capillary inlet). The capillary temperature was set at 29 °C. At the end of each electrophoretic run, the capillary was rinsed (137 kPa) with separation buffer for 60 s.

SAMPLE COLLECTION AND STORAGE

EDTA plasma was obtained by collecting blood into Vacutainer Tubes (Becton Dickinson), giving a final EDTA concentration of 4 mmol/L. The samples were immediately placed on ice and centrifuged (2000*g* for 10 min) within 60 min. The plasma was then processed further or stored at −80 °C until use. Sample handling was carried out under dim light to avoid photodegradation of the analytes.

SAMPLE PROCESSING

Plasma samples (500 μL) were mixed with 1500 μL of ice-cold 100 g/L TCA containing 15 nmol/L isoriboflavin, which served as an internal standard. Precipitated protein was removed by centrifugation, and 1500 μL of the supernatant was neutralized by the addition of 480 μL of 2 mol/L K_2HPO_4. Solid-phase extraction using Bond Elut C-18 columns (1 mL/50 mg; Varian) was then carried out automatically on a Gilson ASPEC sample processor. The columns were preconditioned with 500 μL of methanol followed by 1000 μL of 25 mmol/L sodium phosphate buffer, pH 7.0. The columns were then loaded with 1900 μL of neutralized TCA-treated plasma and washed with the same phosphate buffer as used for column conditioning. The analytes were eluted with 200 μL of 600 mL/L methanol in phosphate buffer, and the columns were then loaded with 220 μL of phosphate buffer to reduce the methanol concentration in the pooled eluate, which was used for CE analysis. Between steps, the columns were dried by air pressurization.

CALCULATION OF PLASMA CONCENTRATIONS

Pooled EDTA plasma containing 7.2 nmol/L riboflavin, 6.1 nmol/L FMN, and 56.6 nmol/L FAD was supplemented with 20 nmol/L riboflavin, 20 nmol/L FMN, and 50 nmol/L FAD and used for calibration. The calibrator plasma was processed in the same way as the samples and was run approximately once for every five samples. Calculation of the vitamer concentrations was based on peak area. For each of the vitamers, the sample concentration was calculated using the formula:

$$C_S = C_C \times \frac{A_S}{A_C} \times \frac{A_{IC}}{A_{IS}}$$

where C_S is the vitamer concentration in the sample, C_C is the vitamer concentration in the calibrator plasma, A_S is the peak area of the vitamer in the sample, A_C is the peak area of the vitamer in the calibrator, A_{IC} is the peak area of the internal standard in the calibrator, and A_{IS} is the peak area of the internal standard in the sample.

In addition, we collected plasma in which we obtained the flavin concentration by repeated analysis against the calibrator. This plasma was stored at −80 °C and used for quality control.

STABILITY OF THE INTERNAL STANDARD AND THE RIBOFLAVIN VITAMERS

Because the internal standard was added to the TCA used for plasma protein precipitation, we investigated its stability in 100 g/L TCA. In addition, the stability of this compound was tested at all stages of sample preparation. The stability of riboflavin, FMN, and FAD was investi-

gated in EDTA whole blood, hemolyzed blood, and EDTA plasma and at all stages of sample preparation.

In all stability experiments, samples were placed in the dark at 23 °C, and polypropylene tubes were used for sample processing and storage. To avoid interference from the interconversion of the vitamers, each of the analytes was added separately to aliquots from one donor. Isoriboflavin served as the reference calibrator for riboflavin, FMN, and FAD, and riboflavin was used as the reference calibrator for isoriboflavin. The reference calibrators were added before the elution step to correct for variations in solid-phase extraction and sample injection. Samples were analyzed in duplicate. Plasma concentrations were log-transformed and plotted against incubation time. Data were analyzed by simple linear regression, and the decomposition was given in terms of first-order rate constants.

To study the stability of the internal standard in TCA used for plasma protein precipitation, we studied aliquots of 100 g/L TCA containing 150 nmol/L isoriboflavin incubated for 1, 2, 4, 7, and 14 days. These aliquots were used for precipitating identical plasma samples. Riboflavin was added as a reference calibrator, and the samples were processed according to standard procedures.

We investigated the stability of endogenous riboflavin, FMN, and FAD in whole blood by incubating aliquots from one donor for 1, 2, and 5 h.

To study the stability of the riboflavin vitamers in plasma, we divided a sample containing endogenous vitamin concentrations into four batches, with 500 nmol/L riboflavin, FMN, or FAD added to three of the batches. Plasma aliquots were stored for 1, 4, 8, and 14 days before analysis.

We investigated the stability of the vitamers and the internal standard in TCA-treated plasma before neutralization by adding 500 nmol/L riboflavin, FMN, FAD, or isoriboflavin to the samples. The samples were then incubated for 1, 2, 4, 7, and 12 h before additional processing. The stability of the vitamers and the internal standard was also tested in neutralized TCA-treated plasma. Plasma samples to which 500 nmol/L riboflavin, FMN, FAD, or isoriboflavin had been added were analyzed after incubation for 1, 2, 4, and 7 days. In addition, the stability of the riboflavin vitamers and the internal standard was tested in the methanol-phosphate buffer obtained by solid-phase extraction using the same concentrations of riboflavin, FMN, FAD, or isoriboflavin and incubation for 1, 2, 4, and 7 days.

HEMOLYSIS

Hemolyzed EDTA blood was prepared by sonication (20 kHz for 5 s at 150 W). The hemoglobin concentration of the plasma was measured (Technicon H2^{TM}; Technicon Instruments), and the percentage of hemolysis was calculated by comparing the hemoglobin concentration of the plasma to the total hemoglobin concentration of the blood. Sonicated and nonsonicated blood from the same donor was mixed in different proportions to obtain different degrees of hemolysis. Samples were incubated for 1, 2, and 5 h.

RECOVERY AND ANALYTICAL VARIATION

The recovery was determined by the addition of riboflavin (10 and 30 nmol/L), FMN (10 and 30 nmol/L), and FAD (50 and 100 nmol/L) to plasma containing endogenous analyte. At each of the three concentrations, 15 replicates were analyzed in one analytical run.

To determine within-day precision, we used the data from the recovery experiments. Between-day precision was determined by assaying the same samples on 15 different days over a period of 3 weeks.

LINEARITY AND LIMIT OF DETECTION

The linearity of the assay at vitamin concentrations greater than the endogenous concentrations was determined by the addition of increasing amounts of the vitamers, corresponding to plasma concentrations between 0.3 and 1000 nmol/L greater than the endogenous concentrations, to plasma containing endogenous concentrations of riboflavin, FMN, and FAD.

To determine the limit of detection and assay linearity at concentrations less than the endogenous concentrations, we used photodegradation to prepare plasma devoid of riboflavin. Plasma proteins were precipitated with 100 g/L TCA without internal standard and then exposed

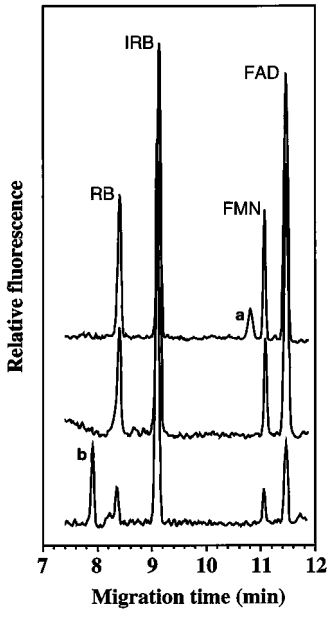

Fig. 1. Electropherograms of human plasma containing different concentrations of riboflavin vitamers.

The *middle electropherogram* shows plasma containing endogenous concentrations of riboflavin (14.0 nmol/L), FMN (7.5 nmol/L), and FAD (85.5 nmol/L). The *top electropherogram* depicts the same plasma with 2 nmol/L of riboflavin, FMN, FAD, and lumiflavin added. The *bottom electropherogram* shows plasma diluted fourfold with photo-degraded plasma. *RB*, riboflavin; *IRB*, isoriboflavin; *FMN*, flavin mononucleotide; *FAD*, flavin adenine dinucleotide; *a*, lumiflavin; *b*, unidentified photodegradation product.

Table 1. Assay recovery.

	Concentration,[a] *nmol/L*							% recovery[b]	
		Added		Expected		Measured			
	Endogenous	Low	High	Low	High	Low	High	Low	High
Riboflavin	6.9	10.0	30.0	16.9	36.9	16.8	36.3	99 (8)	98 (6)
FMN	8.8	10.0	30.0	18.8	38.8	18.7	39.6	99 (4)	103 (9)
FAD	64.0	50.0	100.0	114.0	164.0	109.1	163.2	90 (5)	99 (6)

[a] n = 15 for all concentrations.
[b] Data are given as means with SD in parentheses.

to ultraviolet irradiation at 370 nm for 6 h; neutralizing solution and internal standard were then added. Plasma containing endogenous concentrations of the analytes was not irradiated, but otherwise was prepared in the same way and mixed with irradiated plasma in different proportions ranging from 0% to 100% to obtain different concentrations of the vitamers.

Results

CAPILLARY ELECTROPHORESIS

Electropherograms of a sample containing endogenous concentrations of riboflavin vitamers; a sample with added riboflavin, FMN, lumiflavin, and FAD (2 nmol/L each); and a sample containing 25% of the endogenous concentrations are shown in Fig. 1. The analytes eluted in the order riboflavin, lumiflavin, FMN, and FAD and were baseline-resolved within 12 min and clearly separated from the internal standard. The migration profile was stable over time, and the within- and between-day relative standard deviations (RSD) of the migration times were 1.5% and 2.0%, respectively.

SAMPLE PROCESSING AND STABILITY

Stability of the internal standard and the riboflavin vitamers. The stability of the internal standard was investigated in 100 g/L TCA and at all stages of sample preparation. The stability of riboflavin, FMN, and FAD was tested in EDTA whole blood, hemolyzed blood, and EDTA plasma and at all stages of sample preparation. In all experiments, samples were incubated in the dark at 23 °C.

Table 2. Assay precision.

	Within-day (n = 15)		Between-day (n = 15)	
	Mean ± SD, nmol/L	CV, %	Mean ± SD, nmol/L	CV, %
Riboflavin	6.9 ± 0.6	9	6.6 ± 0.8	12
	16.8 ± 1.3	8	16.6 ± 1.9	11
	36.3 ± 2.1	6	36.7 ± 3.4	9
FMN	8.8 ± 0.4	5	8.3 ± 0.8	9
	18.7 ± 0.7	4	17.9 ± 1.7	9
	39.6 ± 3.6	9	39.3 ± 3.7	9
FAD	64.0 ± 4.4	7	60.6 ± 4.7	8
	108.7 ± 5.1	5	109.1 ± 9.3	8
	163.2 ± 10.5	6	163.4 ± 10.4	6

The internal standard was stable for 14 days when dissolved in the 100 g/L TCA solution used for protein precipitation.

The vitamers were stable for at least 5 h in whole blood and for at least 1 h in blood with 1% hemolysis. This degree of hemolysis did not affect plasma flavin concentrations.

Plasma containing endogenous vitamin concentrations or supplemented with 500 nmol/L riboflavin, FMN, or FAD could be stored for 14 days without changes in the concentrations of riboflavin or FMN. In nonsupplemented plasma, FAD was stable for 14 days, whereas the added FAD degraded at a rate of ~0.03/day.

Riboflavin, FMN, and the internal standard were stable

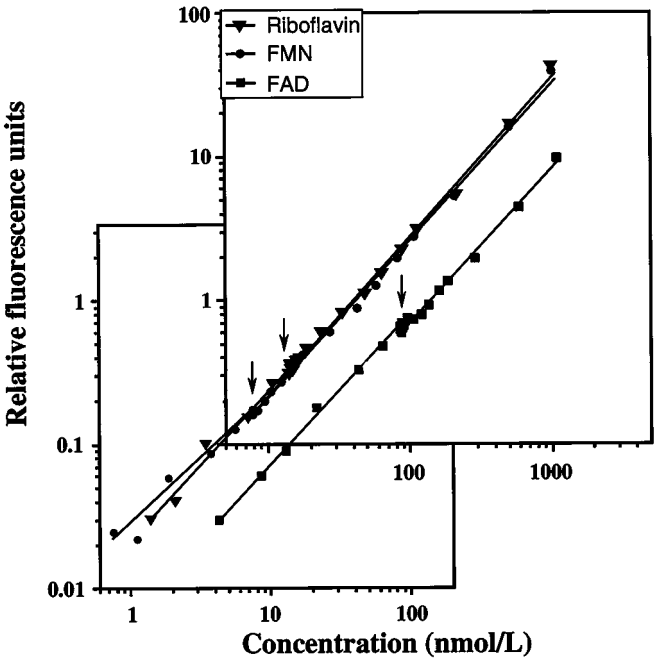

Fig. 2. Linear dynamic range of the CE assay.

The *top panel* shows the linearity in plasma with the vitamers added at concentrations up to 1000 nmol/L greater than the endogenous concentrations. Linear regression equations: $y = -0.043 + 0.025x$ ($r = 0.9987$) for riboflavin; $y = -0.078 + 0.026x$ ($r = 0.9989$) for FMN; and $y = 0.042 + 0.007x$ ($r = 0.9951$) for FAD. The equations are based on data points for endogenous concentrations (indicated by *arrows*) and for concentrations up to 200 nmol/L greater than the endogenous concentrations. The *bottom panel* shows the linearity at concentrations lower than the endogenous concentrations. Data were obtained by diluting native plasma with photo-degraded plasma. Linear regression equations: $y = -0.001 + 0.024x$ ($r = 0.9950$) for riboflavin; $y = 0.006 + 0.022x$ ($r = 0.9935$) for FMN; and $y = -0.008 + 0.008x$ ($r = 0.9980$) for FAD.

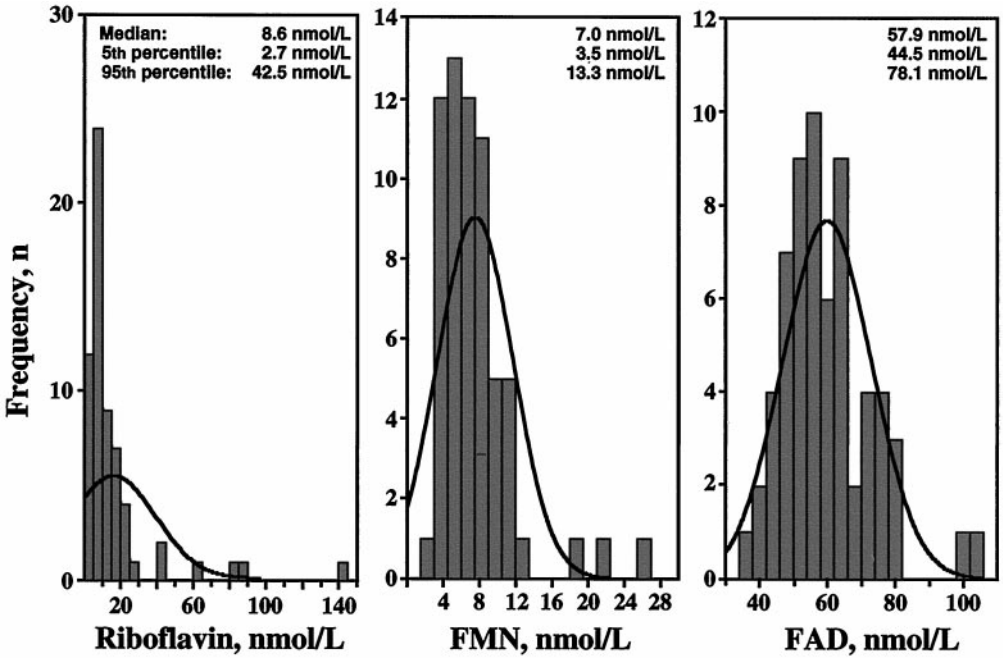

Fig. 3. Vitamin concentrations in healthy subjects.

Frequency distribution of riboflavin, FMN, and FAD in 63 healthy adults (35 men and 28 women), with a mean age of 42 years (range, 23–64 years).

for 12 h in TCA-treated plasma before neutralization, whereas FAD was degraded at a rate of ~0.17/h under these conditions. All vitamers and the internal standard were stable for at least 2 days in neutralized TCA-treated plasma and in the methanol-phosphate buffer obtained by solid-phase extraction.

ASSAY PERFORMANCE

Recovery and analytical variation. The recoveries of riboflavin, FMN, and FAD added to plasma at two concentrations were 90–103% (Table 1). The within-day and between-day CVs were 4–9% and 6–12%, respectively (Table 2).

Linearity and lower limit of detection. The linearity of the assay was tested at concentrations greater than and less than the endogenous vitamer concentrations (Fig. 2), which were 14.0 nmol/L for riboflavin, 7.5 nmol/L for FMN, and 85.5 nmol/L for FAD. The linearity was documented for concentrations up to 200 nmol/L greater than the endogenous concentrations for all vitamers. At concentrations lower than the endogenous concentrations, linearity was observed down to concentrations approaching the limit of detection, which was defined as a signal-to-noise ratio >5:1.

APPLICATION OF THE METHOD

Vitamer concentrations in healthy adults. Riboflavin, FMN, and FAD were measured in plasma from 63 healthy volunteers: 35 men and 28 women. Thirty-six subjects were blood donors, and 27 subjects were recruited from laboratory staff. The mean age was 42 years (range, 23–64 years).

The median concentration (5–95 percentiles) was 8.6 nmol/L (2.7–42.5 nmol/L) for riboflavin, 7.0 nmol/L (3.5–13.3 nmol/L) for FMN, and 57.9 nmol/L (44.5–78.1 nmol/L) for FAD (Fig. 3). Accordingly, the interindividual variation was substantial for riboflavin, whereas it was lower for FMN and, in particular, for FAD. The vitamin concentrations were not related to age ($P > 0.26$, Spearman correlation) or gender ($P > 0.11$, t-test). There was a significant positive correlation between the concentrations of riboflavin and FMN ($r = 0.72$; $P < 0.0001$) and between FMN and FAD ($r = 0.37$; $P < 0.003$), whereas the correlation between riboflavin and FAD ($r = 0.13$; $P = 0.3$) was not significant.

Discussion

To our knowledge, this is the first CE-LIF method for the determination of riboflavin in human plasma. Its main advantages when compared with most HPLC methods *(11–18)* are high sensitivity, validation of analyte stability before and during sample processing, and quantification of all vitamers. Lumiflavin, which is a known photodegradation product of riboflavin *(2)*, is clearly separated from the other analytes.

High sensitivity is obtained by exploiting the native fluorescence of riboflavin and its derivatives, the excitation spectra of which overlap the emission wavelength of 488 nm of the argon laser *(21)*. The lower sensitivity for FAD (Figs. 1 and 2) is related to its lower fluorescence yield compared with riboflavin and FMN *(21)*. In its

present format, the method allows the detection of vitamin concentrations far below the reference values obtained by us (Fig. 2), and therefore should be suitable for the assessment of riboflavin status under deficiency states. Sensitivity enhancement might be obtained by the use of a HeCd laser emitting at 442 nm, which is closer to the excitation maximum of the vitamers. Furthermore, only ~25 nL of the 420-μL eluate from the solid-phase columns is injected into the capillary, and the mass sensitivity could be enhanced by concentrating the eluate before the CE step.

A major portion of the riboflavin vitamers is protein bound *(22, 23)*, and TCA treatment of plasma offers protein precipitation and probably extraction of the protein-bound vitamin fraction. The high ionic strength obtained by this procedure is incompatible with CE, but solid-phase extraction provides a means for sample desalting and for concentration of analytes before CE. We also evaluated organic solvents, e.g., acetonitrile, as protein precipitating agents, but this matrix prevented the retention of analytes on the reversed-phase columns.

Isoriboflavin is an isomeric form of riboflavin (8-demethyl-6-methylriboflavin) and has been used previously as an internal standard in HPLC assays *(15)*. We did not detect endogenous isoriboflavin in human plasma. Isoriboflavin is stable during sample processing and coelutes with the analytes during solid-phase extraction. Inclusion of the internal standard corrects for variable recovery during solid-phase extraction, differences in the amount of sample injected into the capillary, and long-term changes in laser output, and it reduces the within- and between-day CVs from 9–18% (data not shown) to 4–12% (Table 2).

We investigated the stability of the vitamers and the internal standard at different stages of sample processing because analyte degradation would seriously impair assay stability. In neutralized TCA-treated plasma, analytes were stable for at least 2 days at room temperature. This may partly explain the high analytical recovery, which was close to 100% (Table 1), and acceptable within- and between-day CVs (Table 2).

We also demonstrated that the riboflavin vitamers were stable for at least 5 h in whole blood and for several days in plasma at room temperature. This is in agreement with data published by Burch et al. *(24)*, who found that the vitamers were stable in whole blood stored at 4 °C for 48 h. They also reported that endogenous FAD was stable in serum stored at room temperature for several hours, whereas 80% of FAD added to serum was hydrolyzed within 1 h. Others have found that FAD added to whole blood *(25, 26)* or plasma *(16, 26)* is highly unstable and is degraded within minutes. FMN added to whole blood was found to be decomposed within minutes by Nogami et al. *(25)*, but more slowly by other investigators *(26, 27)*; it appears to be relatively stable in plasma *(16, 22, 26)*.

The apparent discrepancies between our stability data and those published by others *(16, 22, 24–27)* can be attributed in part to different incubation temperatures or anticoagulants. Notably, we used EDTA, which has been shown to inhibit the activity of enzymes that hydrolyze FAD and FMN *(28)*. The binding of the vitamers to proteins *(22, 23)* may protect these compounds from degradation, which explains the different stabilities of endogenous vs added vitamers in plasma.

Hemolysis may influence analyte concentrations in plasma, either by the release of flavins from blood cells or by the leakage of cellular enzymes that catalyze the conversion of the vitamers. We found that hemolysis up to 1% does not represent an analytical problem. This percentage of hemolysis is seldom encountered in the clinical setting, and it is higher than the routinely used percentage for interference testing of analytical methods *(29)*.

Our data on analyte stability are the basis for prolonged, unattended sample processing and automated CE injection. In addition, the stability of these analytes allows the measurement of vitamins in clinical samples transported or stored for days as well as epidemiological studies based on frozen samples. We have analyzed plasma samples kept at −80 °C for 32 months. In these samples, the vitamer concentrations, including FAD, were similar to those observed in fresh samples (data not shown).

We found vitamin concentrations similar to those reported by others *(11, 15, 30, 31)*. We also observed a low interindividual variation of FAD compared with variable amounts of riboflavin and FMN. Riboflavin, in particular, varied considerably between subjects *(15, 31)*. This may indicate that the concentration of FAD is tightly regulated *(24, 32–34)*, whereas plasma riboflavin may vary in response to recent vitamin intake *(31)*. Thus, the different flavin species in plasma appear to be under separate regulatory or nutritional influence, and the measurement of all vitamers in plasma may be desirable. The measurement of all vitamers may also be important in the investigation of conditions under which the activities of the enzymes that catalyze the interconversion of the different vitamers are changed, e.g., in riboflavin deficiency *(34, 35)* or thyroid disease *(36, 37)*.

The widely used erythrocyte glutathione reductase activity coefficient is an indirect measure of the FAD concentration in the erythrocytes *(7, 8)*. It does not, however, determine the concentrations of riboflavin and FMN or the relative distribution of different flavin species. It is considered a sensitive and robust index of riboflavin deficiency, but is less suitable for the assessment of riboflavin status at high riboflavin intake *(6)*. Furthermore, the erythrocyte glutathione reductase activity coefficient may give misleading results for certain conditions in which erythrocyte flavin metabolism is altered, e.g., glucose-6-phosphate dehydrogenase deficiency *(6, 7)* and β-thalassemia *(38)*.

In conclusion, we have constructed a robust CE method for the determination of riboflavin species in human

plasma. The monitoring of native fluorescence with LIF detection affords sufficiently high sensitivity to measure vitamin concentrations far lower than the concentrations found in healthy subjects. The stability of the vitamers during sample processing and an almost complete recovery allows precise quantification of all three vitamers. The method thus may become a valuable tool to assess riboflavin status in humans.

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