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Analysis of riboflavin and riboflavin cofactor levels in plasma by high-performance liquid chromatography

Callinice D. Capo-chichi[a], Jean-Louis Guéant[a,*], François Feillet[a,b], Farès Namour[a], Michel Vidailhet[a,b]

[a]*Laboratoire de Pathologie Cellulaire et Moléculaire en Nutrition, EP CNRS 616, Faculté de Médecine de Nancy, B.P. 184, 54505 Vandœuvre-Lès-Nancy Cédex, France*

[b]*Service de Pédiatrie, Hôpital d'Enfants, Allée du Morvan, 54500 Vandœuvre-Lès-Nancy Cédex, France*

Abstract

We describe an assay which determines simultaneously riboflavin (RF), flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) in plasma, using galactoflavin (GF) as an internal standard. The flavins were extracted on a C_{18} Sep-Pack cartridge after protein precipitation with 10% trichloroacetic acid, and were analyzed on a C_{18} RP-HPLC with 85% phosphate–magnesium acetate buffer (pH 3.4) and 15% acetonitrile. FAD, FMN, GF and RF extraction recoveries were 101.0±5.6, 97.0±6.5, 97.0±2.0 and 95.0±4.1%, and reproducibilities were 5.9, 6.8, 2.1 and 4.3%, respectively. FAD, FMN and RF values in infant and adolescent plasma were in the range 53.5–108.2, 9.0–25.1 and 12.7–53.4 n*M*, and 36.5–157.20, 7.1–24.6 and 8.2–57.8 n*M*, respectively. Using GF as an internal standard improved the quantification of these B2 vitamers.

Keywords: Riboflavin; Flavin nucleotides; Vitamers

1. Introduction

Riboflavin (RF) is a micronutrient which is metabolized into two coenzymes, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). These coenzymes are needed for the activity of flavoenzymes implicated in redox reactions [1]. Several authors have used the determination of the erythrocyte glutathione reductase activity coefficient (EGRAC) to evaluate the riboflavin status in humans [2–5], but this method is indirect and lacks sensitivity and specificity in some cases such as inherited glucose-6-phosphate dehydrogenase deficiency [1]. The analysis of flavin coenzymes can be achieved by high-performance liquid chromatography (HPLC), coupled either to a spectrophotometer [6,7] or to a spectrofluorometer [8–12]. The quantification needs to be evaluated by comparing B2 vitamers with an internal standard. The aim of the present study was to improve the HPLC method described by Bötticher [10] for routine analysis in order to have FAD, FMN and RF in the same run by modifying the extraction procedure and using galactoflavin (GF) as an internal standard. Galactoflavin is a flavin derivative with an isoalloxazine ring in common with FAD, FMN and

*Corresponding author. Tel.: +33-83-153-484; fax: +33-83-153-591.

E-mail address: jean-louis.gueant@facmed.u-nancy.fr (J.-L. Guéant)

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RF, which is responsible for their fluorogenic properties. Our procedure allows the simultaneous quantification of FAD, FMN and RF (B2 vitamers) in plasma. The recovery of GF in the analysis guarantees the validity of the extraction procedure.

2. Experimental

FAD and FMN were obtained from Sigma (St. Louis, MO, USA), galactoflavin from Merck Sharp & Dohme (Whitehouse Station, NJ, USA) and riboflavin from Merck (Darmstadt, Germany). FAD, FMN, GF and RF standard solutions were prepared separately in pure distilled water (120, 220, 206 and 265 μ*M*, respectively) under low-intensity light. The standard solutions were placed in separate tubes covered with aluminium foil, and stored at 4°C until analysis. Under these conditions the standard solutions could be stored for 3 months without attenuation of fluorescence intensity. The purity of the flavin standards was 94, 95, 99 and 99%, respectively, for FAD, FMN, GF and RF. The molecular weights of FAD, FMN, GF and RF are 829.5, 456.3, 484.4 and 376.4, respectively.

Methanol (99.8% purity), magnesium acetate (98% purity) and orthophosphoric acid (80% purity) were obtained from Prolabo (Fontenay sous-Bois, France). Potassium dihydrogen phosphate (99.5% purity) was from Merck (Darmstadt, Germany), and acetonitrile (99.7% purity) was from SDS (Peypin, France).

2.1. Standard solutions

The working flavin standards were prepared daily by diluting the stock solutions in a mixture containing (v/v) methanol solution A (10 m*M* potassium dihydrogen phosphate and 15 m*M* magnesium acetate, adjusted to pH 3.4 with orthophosphoric acid).

Standards were prepared individually or mixed together for the determination of the retention times. Each standard solution was analyzed in four repeat runs. Calibration curves were obtained by plotting FAD (2–240 n*M*), FMN (2–220 n*M*), GF (2–206 n*M*) and RF (2–265 n*M*) peak areas as a function of the concentration of each compound.

2.2. Blood collection and handling

Fasting venous blood from 10 healthy infants (6–37 months), 10 healthy adolescents (10–15 years) and 10 malnourished infants (6–37 months) was collected in 5 ml heparinized tubes (wrapped in aluminium foil) and centrifuged at 2200 *g* (CR 4.11 Jouan, Winchester, VA, USA) for 5 min at 4°C. Plasma was aliquoted into plastic tubes and immediately frozen at −20°C until analysis.

Fifteen aliquots of the same plasma sample, each spiked with FAD, FMN, GF and RF (240, 220, 206 and 265 n*M*, respectively), were used to estimate the flavin recoveries and reproducibilities.

2.3. Sample preparation and vitamin extraction

All assays were carried out under low-intensity light. A 1 ml aliquot of plasma was used for the vitamin extraction procedure. The internal standard solution was added at a final concentration of 206 n*M* galactoflavin. A 1 ml aliquot of 15 m*M* magnesium acetate solution was then added and the mixture incubated at 65°C for 15 min. Proteins were precipitated by adding 0.5 ml of 10% trichloroacetic acid (TCA) followed by centrifugation at 3200 *g* (MR 14.11 Jouan) for 10 min at 4°C. The supernate was kept and the pellet then rinsed once with 1 ml of the 15 m*M* magnesium acetate solution and centrifuged. The two supernates were combined together. The optimal concentration of TCA was evaluated in preliminary experiments performed with either 5, 10 or 20% TCA.

2.4. Supernate desalting

The mixture of supernates was pooled onto a C_{18} Sep-Pack cartridge (Waters, Milford, MA, USA) conditioned previously with 2 ml of methanol and 2 ml of solution A. The Sep-Pack column was rinsed with 2 ml of solution A prior to the elution of flavin compounds in 2 ml (v/v) of a methanol–solution A mixture. A 100 μl sample of the extract was used for the HPLC run.

2.5. HPLC run: separation and identification of B2 vitamers

Mobile-phase optimization was achieved by using potassium dihydrogen phosphate (10 m*M*) and varying the concentration of magnesium acetate (10, 15 and 20 m*M*), acetonitrile (10, 15 and 20%) and the pH (3.4 and 5.0).

Routine analysis of the plasma B2 vitamers was carried out on a C_{18} reversed-phase column (250 mm×4 mm, 5 μm) (Interchim, Montluçon, France) with an isocratic eluent (15% acetonitrile in solution A) at a flow-rate of 1 ml/min. The eluent was filtered through a 0.45 μm membrane (Gelman-Sciences, Ann Arbor, MI, USA) and degassed under vacuum prior to use in HPLC. The HPLC system was composed of two 501 pumps (Waters, Milford, MA, USA) connected to an RF-535 fluorescence HPLC monitor and a CR-6A chromatopac integrator (both from Shidmadzu, Kyoto, Japan). The spectrofluorometer was set at 445 nm for the excitation wavelength and 530 nm for the emission wavelength.

2.6. Statistical analyses

The statistical analyses were performed with STATVIEW 4.02 software (Abacus Concepts, Berkeley, CA, USA). The Mann–Whitney U-test was used to compare the concentration of the B2 vitamers between control infants and adolescents, and between control and malnourished infants. Differences were considered significant for $P < 0.05$.

2.7. Extraction recovery and reproducibility

Standard solutions of FAD, FMN, GF and RF (240, 220, 206 and 265 n*M*, respectively) were prepared either with or without preincubation with B2-deficient plasma.

The extraction recovery (ER) was estimated according to the formula

$$\mathrm{ER} = 100 \cdot (C_f - C_i / C_s)$$

where C_f is the concentration of FAD, FMN, GF or RF obtained from the enriched plasma, C_i is the initial concentration, and C_s the concentration of the corresponding standard solution.

The reproducibility (coefficient of variation, C.V.) was estimated according to the formula

$$\text{C.V.} = 100 \cdot (\text{standard deviation} / \text{mean of the recovered concentration})$$

The detection limits were calculated by considering the mean values + 2 SD ($n = 4$) of the concentrations corresponding to the dilution point at which the fluorescence intensity was not significantly different from that of the dilution point just before.

3. Results and discussion

Optimal results were obtained as follows: flavin compounds were extracted from plasma after incubation in magnesium acetate (15 m*M*) for 15 min, this was followed by precipitation of proteins with 10% TCA, the supernatant then extracted on a C_{18} Sep-Pack cartridge before the C_{18} RP-HPLC was run with 85% solution A–15% acetonitrile.

Protein precipitation was partially achieved with 5% TCA, while 20% TCA hydrolyzed FAD into FMN. Flavin compounds were not eluted when HPLC elution was performed with a mobile phase containing 10 m*M* magnesium acetate; with 20 m*M* magnesium acetate, FAD was not clear of the FMN peak. Flavin compounds were not eluted with 10% acetonitrile, while elution in 20% acetonitrile reduced the retention times and did not allow the separation of FAD from the FMN peak. The retention times extended to 13 min when the eluent was at pH 5.0.

A significant correlation was observed between the fluorometric signal of flavins and their concentrations. The regression line correlations were: $y = 169.6x - 570.2$ ($r = 0.99$), $y = 161.4x + 4.0$ ($r = 1$), $y = 172.4x + 5.4$ ($r = 1$) and $y = 225.8x + 307.5$ ($r = 1$) for FAD, FMN, GF and RF, respectively, where y is the fluorescence intensity and x the flavin concentration. These calibration equations allowed the quantification of FAD, FMN, GF and RF up to 240, 220, 206 and 265 n*M*, respectively.

The detection limits were estimated at 4.0 n*M* for

FAD, 3.4 n*M* for FMN, and 2.9 n*M* for both GF and RF. The RF detection limit was of the same order of magnitude as that estimated by Bötticher [10]. By comparison, Zempleni estimated the detection limits of FMN and RF to be 3.0 and 9.0 n*M*, respectively.

FAD, FMN, GF and RF extraction recoveries were 101.0±5.9, 97.0±6.5, 97.0±2.0 and 95.0±4.1%, respectively, when using the regression line obtained by plotting 'standard peak area' against the corresponding 'standard concentration'. The flavin extraction recovery and reproducibility obtained using galactoflavin as internal standard and by plotting the 'vitamer peak area/GF peak ratio' versus 'standard concentration' are shown in Table 1. They are compatible with the use of our method for routine analysis of blood samples.

The recovery of the B2 vitamers obtained using our procedure was higher than that found by Lambert et al. [9] in serum (72.6% for RF) using isoriboflavin as an internal standard, and that of Zempleni [12] in plasma (82.4% for FMN and 94.4% for RF). Recovery was of the same order of magnitude as that found by Bötticher et al. [10] in serum and whole blood (98±4% for RF).

The retention times of FAD, FMN, GF and RF before extraction were 3.04±0.42, 4.04±0.05, 5.82±0.03 and 7.21±0.04 min, respectively. The retention times were not significantly modified by extraction (2.99±0.1, 3.97±0.03, 5.80±0.03 and 7.18±0.03 min, respectively). The coefficients of variation of retention times were <0.7%, demonstrating that the flavin components were not affected by the extraction procedure.

Table 1
Flavin recovery and reproducibility obtained from plasma enriched with FAD, FMN, GF and RF, when using galactoflavin as an internal standard ($n = 15$)[a]

	Theoretical conc. (n*M*)	Measured conc. (mean±SD) (n*M*)	Recovery[b] (mean±SD)	C.V. (%)
FAD	240	237.7±9.2	99.0±3.8	3.8
FMN	220	218.3±12.9	99.0±5.8	5.8
RF	265	258.9±7.6	97.0±2.8	2.9

[a] FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; RF, riboflavin; SD, standard deviation; C.V., coefficient of variation.

[b] The calibration was performed by estimating the ratio 'vitamer peak area/internal standard peak area'.

The elution followed the polarity order (FAD> FMN>GF>RF) with the GF position being intermediate between those of the RF cofactors (FAD and FMN) and RF (Fig. 1). This feature makes GF an excellent internal standard for the detection method described here.

The Sep-Pack step was indispensable in desalting the supernate, and in avoiding interference in the fluorescence intensity of all three B2 vitamers (FAD, FMN and RF) due to quenching by anions, particularly chloride [13]. In addition, this extraction step improved the separation of the FAD from the FMN peak in the chromatogram. Without the Sep-Pack cartridge extraction, the retention time of FAD shifted from 2.99 to 3.27 min and, therefore, was not well clear of the FMN peak. No artifactual peaks were observed in the elution profile of B2 vitamers extracted from the spiked plasma (Fig. 1A). By comparison, Floridi et al. [8] obtained a chromatographic profile of FAD with artifactual peaks at retention times between 0 and 6 min.

Our method was applied to evaluate FAD, FMN and RF concentrations in plasma from 10 control infants (6 to 37 months) and 10 control adolescents (12 to 15 years). The results are presented in Table 2. We did not have the opportunity to collect blood from healthy children aged from 4 to 12 years. The results show that the median concentration of plasma FAD was six-fold and 3.5-fold higher than that of FMN and RF, respectively (Fig. 1B). The FAD concentrations represented approximately 73.7±16.7% of the total plasma B2 vitamers, as described by others [14]. No significant differences were observed for FAD, FMN and RF concentrations between the infant and adolescent groups (Table 2).

The concentration of plasma flavocoenzymes reported by Zempleni [12] in control plasma (55.4 n*M*) is lower than the median of plasma flavocoenzymes (sum of FAD and FMN) quantified by our procedure. This shows that the presence of chloride anions from TCA might attenuate the flavin fluorescence intensity, as described by Ellinger [13], when the sample extract is not desalted prior to the HPLC run.

Some malnourished infants ($n = 10$) exhibited low plasma FAD concentrations (Fig. 1C). The plasma concentrations of FAD, FMN and RF in those malnourished infants were [median (range)] 10.7 (7.5–26.8), 10.5 (2.5–15.3) and 33.5 (14.6–68.7)

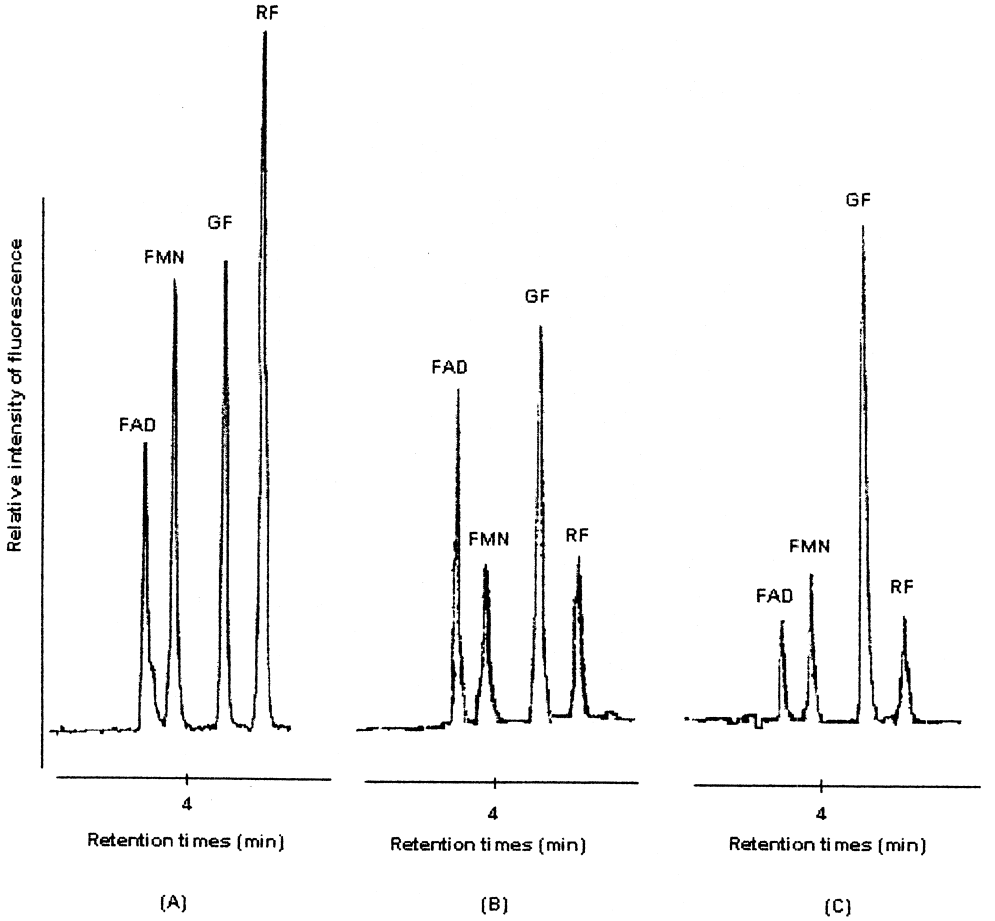

Fig. 1. HPLC chromatogram of flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), galactoflavin (GF) and riboflavin (RF), from plasma enriched with FAD, FMN, GF and RF (240, 220, 206 and 265 n*M*, respectively) (A), from plasma with a normal riboflavin concentration (B), and from plasma deficient in riboflavin (C). The sample was injected onto a C_{18} reversed-phase column (250 mm×4 mm; 5 μm) and eluted at a flow-rate of 1 ml/min with an isocratic eluent (15% acetonitrile and an 85% mixture of 10 m*M* potassium dihydrogen phosphate containing 15 m*M* magnesium acetate, adjusted to pH 3.4 with orthophosphoric acid). FAD, FMN, GF and RF were detected by spectrofluorometry with an excitation wavelength of 445 nm and an emission wavelength of 530 nm. FAD, FMN, GF and RF were cleared with respective retention times of 2.99±0.01, 3.97±0.03, 5.80±0.02 and 7.18±0.03 min.

Table 2
Plasma B2 vitamer concentrations in 10 infants and 10 adolescents [median (range)][a]

	Infants (6–37 months, $n = 10$)	Adolescents (10–15 years, $n = 10$)
FAD (n*M*)	73.0 (53.5–108.3)	96.3 (36.5–157.2)
FMN (n*M*)	10.8 (9.0–25.1)	12.5 (7.1–17.5)
RF (n*M*)	20.9 (12.7–53.4)	18.5 (8.2–57.8)

[a] FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; RF, riboflavin.

n*M*, respectively. The median plasma FAD concentration was three-fold lower than that of RF ($P < 0.001$). In these cases, the FAD concentrations in the plasma represented only 25.1±12.1% of the total plasma B2 vitamers, instead of the 73.7±16.7% observed in healthy children. Therefore, our results show that the simultaneous determination of the three plasma B2 vitamers is more reliable than measuring only FAD [8] or RF [10] or FMN and RF [12].

In conclusion, the simultaneous detection of ribo-

flavin and its derived cofactors using our HPLC method allows the estimation of the relative distribution of each vitamer, and can therefore be used to detect cases of riboflavin deficiency due to either malnutrition or inherited disorders.

References

[1] J.C. Bates, Eur. J. Clin. Nutr. 51 (1997) S38.
[2] R.A. Bayoumi, B. Rosalki, Clin. Chem. 22 (1970) 327.
[3] D. Glatzle, W. Körner, O. Christeller, O. Wiss, Int. J. Vit. Res. 40 (1970) 166.
[4] O.A. Ajayi, Hum. Nutr. Clin. Nutr. 40 (1984) 383.
[5] T.R.C. Sisson, Fed. Proc. 46 (1987) 1883.
[6] P. Pietta, A. Calatroni, J. Chromatogr. 229 (1982) 445.
[7] D. Blanco, L.A. Sanchez, M.D. Gutiérrez, J. Liq. Chromatogr. 17 (1994) 1525.
[8] A. Floridi, C.A. Palmerini, C. Fini, M. Pupita, F. Fidanza, Int. J. Vit. Nutr. Res. 5 (1985) 5187.
[9] W.E. Lambert, P.M. Cammaert, A.P. De Leenheer, Clin. Chem. 31 (1985) 1371.
[10] B. Bötticher, D. Bötticher, Int. J. Vit. Nutr. Res. 57 (1987) 273.
[11] D.W. Batey, C.D. Eckert, Anal. Biochem. 188 (1990) 164.
[12] J. Zempleni, Ann. Nutr. Metab. 39 (1995) 224.
[13] P. Ellinger, M. Holden, Biochem. J. 38 (1944) 147.
[14] H. Ohkawa, N. Ohishi, K. Yagi, Biochem. Int. 4 (1982) 187.