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Note

Hydrolysis of riboflavin nucleotides in plasma monitored by high-performance liquid chromatography

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Riboflavin (I) carries out its functions in body in the form of one or other of two coenzymes, riboflavin phosphate (FMN, II) and flavin adenine dinucleotide (FAD, III). It was found that both FAD and FMN are hydrolyzed and resynthesized during the incorporation process [1]. Okumura and Yagi [2] reported that FAD and FMN are dephosphorylated very rapidly in whole blood. These authors observed that plasma is responsible for the hydrolysis of both FAD and FMN, the former being hydrolyzed at a faster rate.
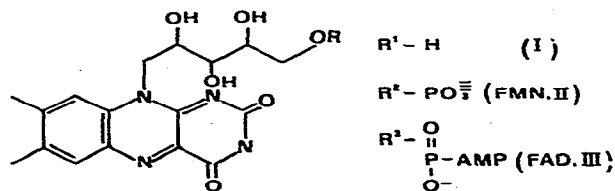
However, these results are not completely consistent with the report of Jusko and Levy [3] that FMN is not decomposed by human blood plasma, and this difference might be ascribed to the analytical methods used for flavins. For this reason, in the present study the hydrolysis of FAD and FMN in blood plasma was monitored by high-performance liquid chromatography (HPLC).

This technique has recently provided a rapid, specific and reproducible method of analyzing nucleotides using normal or reversed-phase columns [4—7].

The method described in this paper is a reversed-phase HPLC procedure for the direct determination of FAD, FMN and riboflavin in blood plasma using a fixed-wavelength detector (254 nm) and ammonium phosphate buffer—acetonitrile as eluent.

EXPERIMENTAL

Reagents and materials

FAD, FMN, riboflavin and nicotinamide were purchased from Sigma (St. Louis, MO, U.S.A.) and diammonium hydrogen phosphate (analytical-reagent grade) from Merck (Darmstadt, G.F.R.). Acetonitrile was of HPLC grade (Chromasolv; Riedel-De Haën, Hannover, G.F.R.). Water was distilled in glass and then passed through a 0.45-μm membrane filter (Type HA, Millipore, Bedford, MA, U.S.A.).

Standard stock solutions were prepared by dissolving riboflavin (10 μg/ml), FAD, FMN (150 μg/ml) and the internal standard, nicotinamide (200 μg/ml), in 0.9% sodium chloride solution. These stock solutions were prepared in amber glass and used within 12 h.

Sample preparation

A 1-ml volume of blood plasma was mixed with 100 μl of FAD or FMN stock solution and fortified with 50 μl of internal standard. Incubation was carried out in the dark at 37°C, and the hydrolysis was stopped by injecting 5-μl aliquots into the liquid chromatograph every 10 min during 1 h. Control blanks were performed under the same conditions using the above solutions containing everything but blood plasma.

Chromatography

A Waters Assoc. (Milford, MA, U.S.A.) analytical liquid chromatograph equipped with a 30 cm × 3.9 mm I.D. μBondapak C_{18} (10 μm) column was fitted with a Waters Model 440 UV detector using low-dead-volume hardware. A pre-column (Waters Assoc., part No. 84550) consisting of a short stainless-steel column (2 cm × 4 mm I.D.) packed with Bondapak—Corasil (37—50 μm) was used to prolong the life of the analytical column. A mobile phase of 10 mM diammonium hydrogen phosphate (adjusted to pH 5.5 with 20% phosphoric acid)—acetonitrile (1000:120) was filtered, degassed and used at a flow-rate of 2.0 ml/min (130 bar). The effluent stream was monitored using a 254-nm filter on the detector. The range setting was fixed at 0.02 a.u.f.s., with the signal monitored by a 10-mV strip-chart recorder (1.0 cm/min) interfaced with a Model 730 Data Module (Waters Assoc.).

RESULTS AND DISCUSSION

Some difficulty was encountered in finding an isocratic eluent to separate rapidly FAD, FMN, riboflavin and the internal standard from each other and from plasma components. The most satisfactory solvent was ammonium

phosphate buffer—acetonitrile; the retention times were strongly dependent upon the acetonitrile concentration and the pH. A mixture of aqueous buffer—acetonitrile (1000:120) and a pH value of 5.5 were selected. Capacity factors in the system as described were: nicotinamide (internal standard), 1.4 min; FAD, 2.3 min; FMN, 3.9 min; riboflavin. 8.1 min.

The chromatograms in Fig. 1 demonstrate the lack of interference and the specificity of the assay procedure for the measurement of FAD, FMN and riboflavin in plasma. Calibration curves were constructed by determining the response from known amounts of FAD, FMN and riboflavin and the internal standard added to 0.9% sodium chloride solution. Assay linearity was demonstrated over the range 0.6—20 μg/ml; the amount of the internal standard was held constant at 10 μg/sample. Typical calibration curves are shown in Fig. 2.
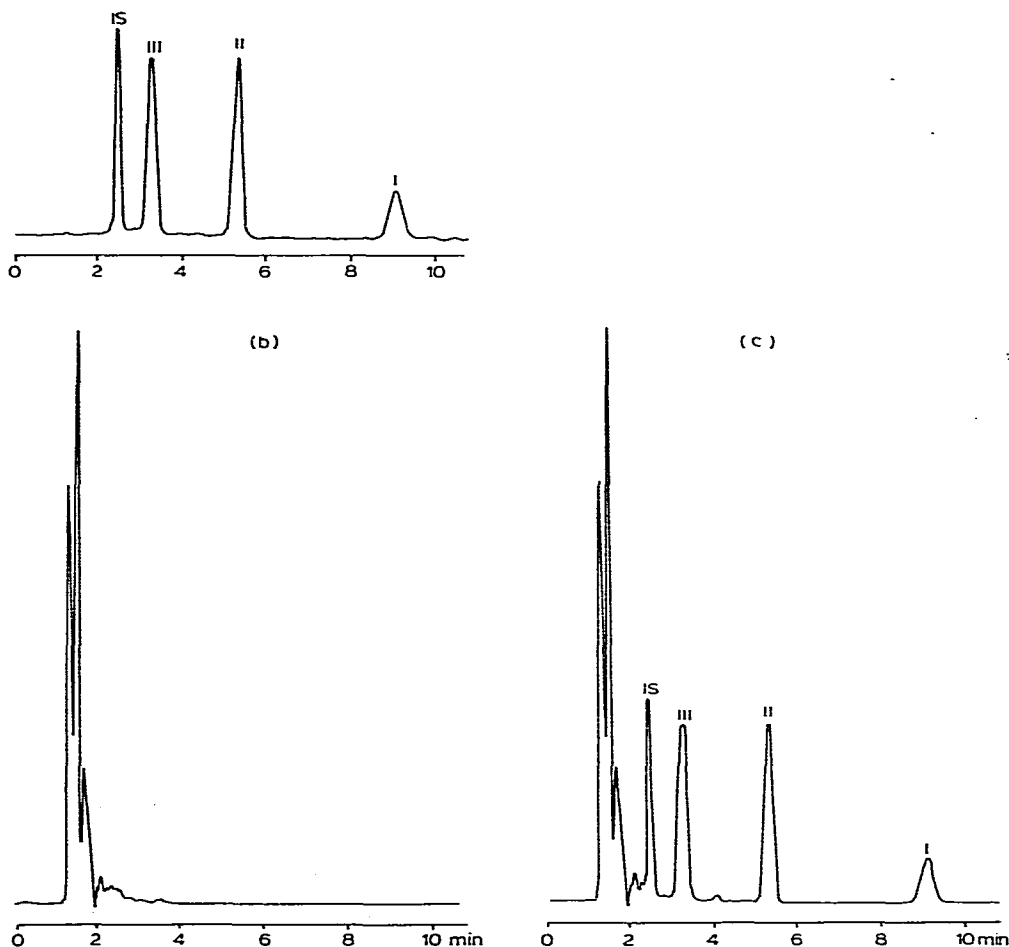

Fig. 1. Chromatograms of: (a) a standard mixture of internal standard (IS, 45 ng), FAD (III, 65 ng), FMN (II, 65 ng) and riboflavin (I, 25 ng); (b) control plasma; (c) plasma fortified with internal standard (IS, 45 ng), FAD (III, 65 ng), FMN (II, 65 ng) and friboflavin (I, 25 ng).
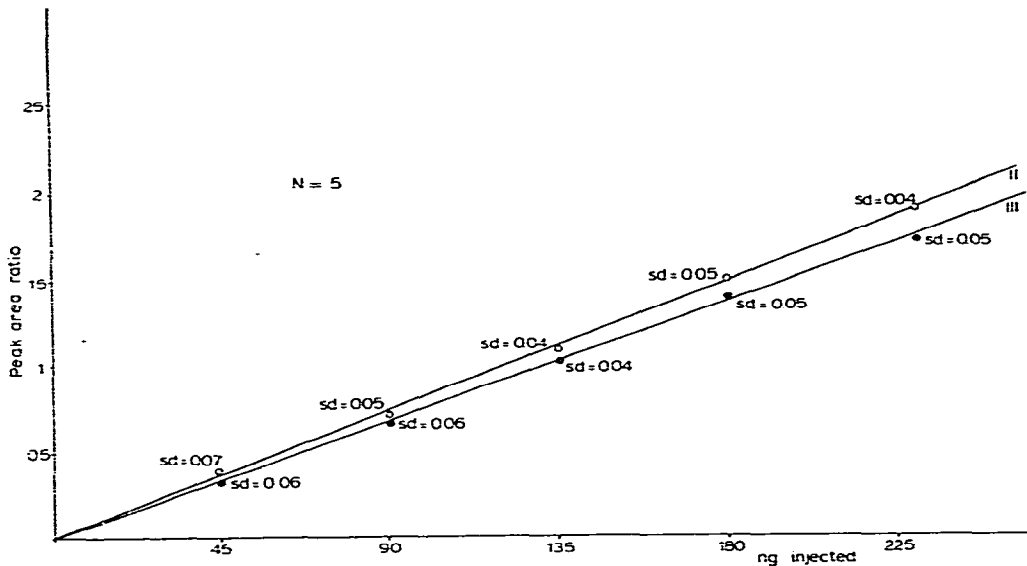
Fig. 2. Calibration graph of the peak area ratio (II or III peak area/internal standard peak area) versus the amount of II and III injected.

TABLE I

HYDROLYSIS OF FAD AND FMN BY BLOOD PLASMA AT 37°C

The reaction mixture consisted of 1 ml of blood plasma, 0.1 ml of FAD or FMN stock solution and 0.05 ml of internal standard. Incubation was at 37°C. Values given express the amount of FAD or FMN remaining as a percentage of the value at time 0.

	Incubation time (min)					
	10	20	30	40	50	60
FAD	68.5	43.4	25.3	15.2	5.1	3.4
FMN	99.4	98.9	98.5	98.3	97.9	97.5

The sensitivity of this method was estimated to be 0.5 μg/ml and was adequate for following the hydrolysis.

The time—course for hydrolysis of FAD and FMN in blood plasma is shown in Table I. FAD added to blood plasma was hydrolyzed to FMN within 60 min; on the other hand, FMN added to blood plasma was hydrolyzed only to a very small extent, comparable to that of the blank. These results indicate that the rapid loss of plasma FAD is due to enzymatic catalysis, while the slow hydrolysis of FMN should not be ascribed to enzymatic activity.

In conclusion, this HPLC method is a simple, reproducible and specific procedure, which allows the monitoring of FAD and FMN hydrolysis in blood plasma. The extension of the procedure to whole blood as well as to red cells has been demonstrated in preliminary experiments.

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