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Note

Determination of the B_2 vitamer flavin—adenine dinucleotide in whole blood by high-performance liquid chromatography with fluorometric detection

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Vitamin B_2 is a generic term for the three naturally occurring B_2 vitamers flavin—adenine dinucleotide (FAD), flavin mononucleotide (FMN) and riboflavin (Rb) [1]. The first two are phosphorylated while the latter is not. Vitamin B_2 occurs in various foodstuffs mainly as FAD and is widely distributed in all leafy vegetables, in meat, fish, eggs and in milk and milk products [2]. In the gut Rb taken up with food is phosphorylated to FMN by the intestinal mucosa during absorption. In tissue cells FMN can be converted to FAD. This vitamer serves as the coenzyme in most of the oxidation—reduction reactions catalysed by the flavin enzymes.

The determination of vitamin B_2 in blood is often used to establish the vitamin B_2 status of man [1]. Various microbiological [3] and manual fluorometric [1, 3—6] methods have been described for the determination of vitamin B_2 in blood. However, all these methods have one or more disadvantages. Microbiological methods [3] are rather time-consuming and do not allow the analysis of the specific B_2 vitamers. Furthermore, they cannot be easily automated and their results are poorly reproducible. Also most of the fluorometric methods described do not allow a separate analysis of the three B_2 vitamers [3—6], they are laborious and, like the microbiological methods, not easy to automate. On account of these drawbacks we set out to develop a reliable high-performance liquid chromatographic (HPLC) method for the analysis of FAD in whole blood of humans.

EXPERIMENTAL

Apparatus

Chromatography was performed using a HPLC system incorporating a Knauer FR30 constant-flow pump (Salm and Kipp, Breukelen, The Nether-

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lands), a Micromeritics 725 Autoinjector (CLI, Schijndel, The Netherlands), and a Perkin-Elmer 204A fluorescence spectrophotometer with scanning options (Perkin-Elmer, Delft, The Netherlands) equipped with a 100-μl Hellma fluorescence flow-cell, type QS 176.51, having a centre height of 15 mm (Hellma, Müllheim, G.F.R.). A Knauer stainless-steel column (25 cm × 4.6 mm I.D.) was home-packed with Hypersil ODS 5 μm (Shandon Southern Products, Astmoor, Great Britain; Cat. No. 580x9) by the balanced-density slurry technique using a Haskel pump type MCP 110 (Ammann Technik, Stuttgart, G.F.R.). The elution profiles were displayed on a Kipp BD-8 recorder (Kipp Analytica, Delft, The Netherlands).

Reagents

10% (w/v) trichloroacetic acid (TCA) and 4.5 *M* sodium acetate buffer (pH 6.2). The HPLC mobile phase was an aqueous phase containing 0.3 *M* KH_2PO_4 and 16.7% (v/v) methanol. The pH of this liquid was adjusted to 2.9 using H_3PO_4. FAD (Boehringer, Mannheim, G.F.R.; Cat. No. 104736) was dissolved in water to a concentration of 29 μmol/l. FMN was from Fluka (Buchs, Switzerland), Cat. No. 83810. Rb (Hoffmann-La Roche) was freshly dissolved in water at 50°C. Vitamin B_2 working standard solution with final concentrations of FAD, FMN and Rb of 60, 20 and 10 nmol/l, respectively, were made up in a solution of 30 ml of water, 90 ml of the TCA solution and 80 ml of the sodium acetate buffer. The working standard solution was stored in the dark at −20°C and was stable for at least two months.

Chromatographic conditions

Analysis of FAD was carried out by injecting 240-μl aliquots of a sample extract or the working standard solution onto the Hypersil ODS column. The column was eluted isocratically with a flow-rate of 2.0 ml/min using the mobile phase specified under Reagents, whose composition was the result of an optimization procedure for the best HPLC separation. The effluent was monitored with the fluorescence detector set at optimum wavelengths for excitation and emission (see below). The recorder was set at 10 mV full-scale. Duration of the chromatographic analysis was about 22 min per sample. The concentration of FAD in the original sample was calculated from peak heights with the working standard solution as the reference. In routine analysis this solution was run before each series of five samples.

Selection of fluorometric parameters

As shown in Fig. 1, under the chromatographic conditions employed the B_2 vitamers have their excitation maximum at a wavelength of 470 nm and their emission maximum at a wavelength of 525 nm. Accordingly this wavelength pair was chosen for the fluorometric detection. The fluorescence intensities proved to be pH-dependent. The fluorescence intensities of FMN and Rb showed a maximum value between pH 3.5 and pH 7.5, whereas for FAD the fluorescence intensity was maximal between pH 2.7 and pH 3.1. Thus for optimum sensitivity, the pH of the mobile phase was fixed at 2.9 in the optimization procedure for the best HPLC separation.

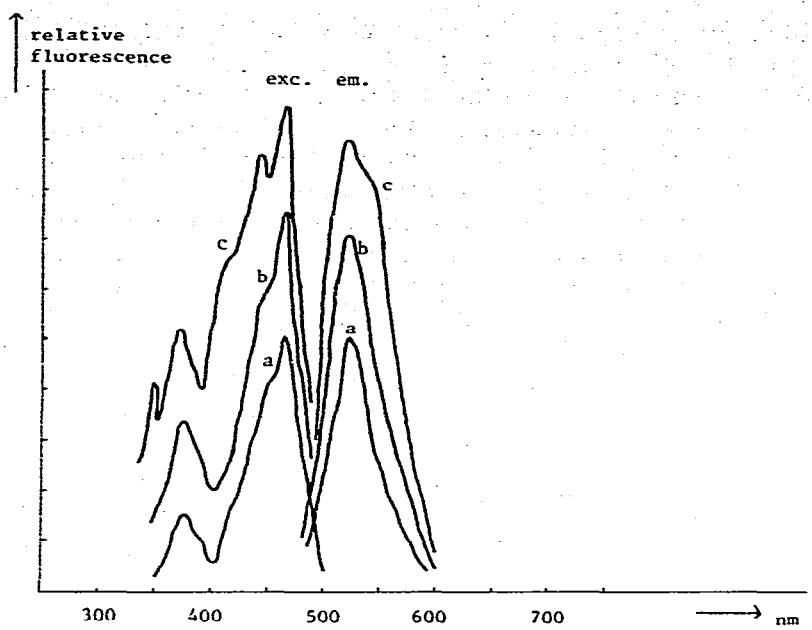

Fig. 1. Excitation (exc.) and emission (em.) spectra of equimolar concentrations of the B_2 vitamers FAD (a), FMN (b) and Rb (c) in the HPLC mobile phase.

Preparation of sample extracts

As vitamin B_2 is light-sensitive, the preparation of sample extracts was carried out in the dark as much as possible. Blood samples were collected from veins of normal healthy Dutch volunteers, directly into Venoject evacuated tubes (Terumo Co., Tokyo, Japan) containing sodium heparinate as the anticoagulant. One millilitre of such a blood sample was slowly transferred, with thorough mixing using a Vortex mixer, to a tube containing 3.0 ml of 10% (w/v) TCA solution. The tube was allowed to stand for about 30 min in the dark at 4°C. Thereafter, 2.0 ml of the sodium acetate buffer were slowly added with thorough mixing using a Vortex mixer, and the tube was then centrifuged at 2000 *g* and 4°C for 15 min. The supernatant, being the sample extract, was either used for direct analysis of FAD by HPLC or stored in the dark at 4°C for analysis within one week.

Recovery tests

The recovery of FAD added to whole blood was determined by analysing portions of 1 ml of whole blood to which 10 μl of an aqueous solution containing 29 μmol FAD per litre had been added.

RESULTS

Range of applicability of HPLC procedure

As far as has been investigated, the fluorescence response of FAD in the HPLC procedure described was linear up to a concentration corresponding to about 900 nmol/l in the blood sample. As mentioned below, reference values

of FAD in human blood were found in the range 260—390 nmol/l. In blank experiments only a solvent peak was recorded. As can be seen in Fig. 2, the HPLC procedure affords excellent separation between FAD, FMN and Rb. Typical elution profiles of the B_2 vitamers from the working standard solution, from a normal blood sample, and from a whole blood sample obtained from a healthy adult volunteer 12 h after supplementation with 60 mg of FMN are shown in this figure. Although our method was not specifically developed for FMN and Rb and no recovery checks have been made for these vitamers, it is worth noting that the concentrations of FMN and Rb in whole blood appear to be very low compared to the concentration of FAD. A significant increase in the concentrations of FMN and Rb in whole blood was observed after supplementation of a healthy adult volunteer with 60 mg of FMN (Fig. 2c).

Assuming the signal-to-noise ratio should be at least 3, the detection limits of the HPLC method described in this paper were found to correspond to blood concentrations of 20 nmol/l for FAD, 15 nmol/l for FMN and 10 nmol/l for Rb. The sensitivity for FAD is high in relation to concentrations in blood even in conditions of vitamin B_2 deficiency. Values below 150 nmol/l have been observed in various disease states [7].

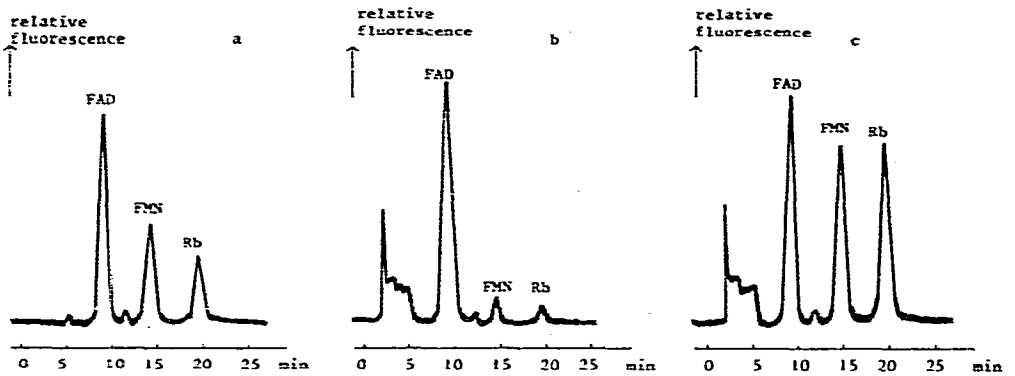

Fig. 2. Typical elution profiles of the B_2 vitamers from (a) the working standard solution, (b) a whole blood sample, and (c) a whole blood sample obtained from a healthy adult volunteer 12 h after supplementation with 60 mg of FMN.

Efficiency of the extraction procedure

To investigate the efficiency of the extraction of FAD by the procedure described under Experimental (preparation of sample extracts) 1.0-ml aliquots of a series of ten different whole blood samples were extracted in the presence of radioactive ^3H_2O ($15 \cdot 10^4$ dpm). After centrifugation and removal of the supernatant the pellet was extracted twice more with 3 ml of 10% (w/v) TCA and 2.0 ml of sodium acetate buffer. The three separate supernatants of each sample were analysed for FAD and ^3H_2O. It was found that 17.0 ± 2.1% (mean ± S.D.) of the total amount of FAD and 11.0 ± 0.9% (mean ± S.D.) of the total amount of ^3H_2O were still present in the pellet after the first extraction. From these results it was concluded that the efficiency of the extraction of FAD by the method described amounted to 94.0% for blood samples of 1 ml. Assuming

that whole blood contains 85.0% of water [8] and taking into account the volumes of sample and added liquors, the concentration of FAD in the original whole blood sample was finally calculated by multiplying the concentration of FAD found in its supernatant by a factor of [(5 + 0.85)/94.0] · 100 = 6.22.

Precision and recovery

In order to test the within-assay and between-assay precisions of the method, several specimens of one and the same whole blood sample with and without the addition of 290 nmol FAD per litre were stored at −20° C and analysed for FAD on a series of consecutive days. The results are given in Table I. The coefficient of variation (C.V.) of the between-assay for FAD in whole blood samples is somewhat greater than the C.V. of the within-assay, although both are sufficiently small. Table I also shows that the recovery of FAD added to whole blood samples is good.

TABLE I

PRECISION OF THE METHOD FOR DETERMINATION OF FAD

	Within-assay precision		Between-assay precision	
	Whole blood	Recovery test*	Whole blood	Recovery test*
n	19	6	10	9
Mean	310 nmol/l	93.1%	300 nmol/l	97.6%
S.D.	9 nmol/l	3.8%	13 nmol/l	3.8%
C.V.	2.9%		4.4%	

*Recovery tests were performed as described under Experimental (recovery tests).

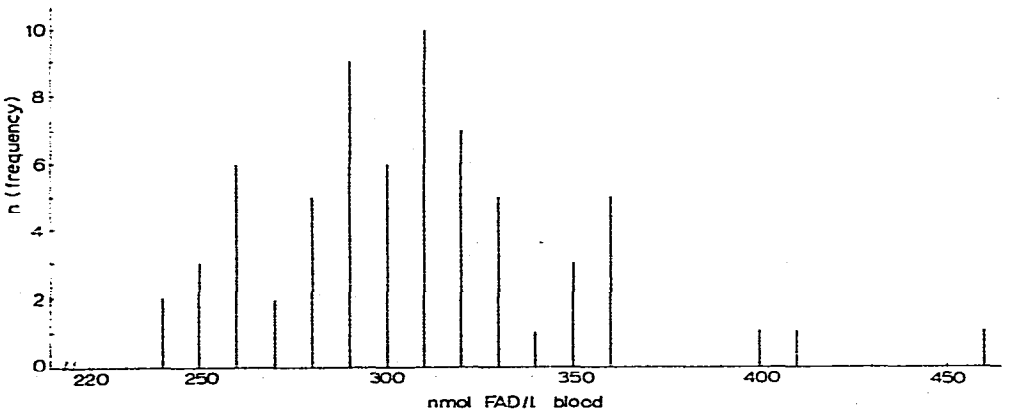

Fig. 3. Frequency distribution of the concentration of FAD in whole blood samples of normal healthy Dutch volunteers (n = 70).

Reference values

From the analysis of FAD in whole blood samples of a group of 70 normal healthy Dutch volunteers (41 males and 27 females), a total range for FAD of

240—460 nmol/l was found with a mean value of 310 nmol/l. The frequency distribution of FAD in these samples seems to be non-gaussian (Fig. 3). For the assessment of a "normal" range, the distribution-free method described by Rümke and Bezemer [9] was used. By setting the limits of percentiles at 2.5 and 97.5% with a reliability of 95%, a reference range was obtained of 260—390 nmol FAD/l whole blood. Accordingly, a concentration of FAD lower than 260 nmol/l would be regarded as below normal.

DISCUSSION

The HPLC method specifically developed for the analysis of FAD in whole blood described in this paper is, in fact, able to separate FAD, FMN and Rb from each other and from interfering compounds. A reliable and sensitive detection of FAD has been obtained by selecting the optimal pH of the mobile phase and optimal adjustment of the fluorescence detector. At these settings, the other two vitamers are also individually detected with sensitivities even slightly greater than for FAD but the development of over-all determination procedures for these two vitamers was not attempted in the present paper. As part of several research projects, about 800 determinations were carried out within three weeks. The precision and recovery figures confirmed the reliability of the method. Finally, we conclude that a reliable HPLC method for the fluorometric analysis of FAD in whole blood is now available which is suitable for large-scale routine analysis.

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