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Note

Determination of flavin adenine dinucleotide in biological tissues by high-performance liquid chromatography with electrochemical detection

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Flavin adenine dinucleotide (FAD) is one of the essential electron carriers in the oxidation of energetic molecules. It may be interesting to quantify this cofactor in order to explore the cellular energetic metabolism. At present time, high-performance liquid chromatography (HPLC) with fluorimetric detection, based on the fluorescence characteristics of FAD, is the usual method for its determination[1,2]. In this paper, we report the determination of FAD by HPLC with electrochemical detection[3], based on the reduction of FAD. This method is rapid, selective and sensitive, and has been applied to tissue extracts of fish (liver, brain and muscle).

EXPERIMENTAL

Reagents and chemicals

Sodium dihydrogenphosphate and acetonitrile (Merck) were of analytical-reagent grade. FAD (purity 94–99%) was supplied by Sigma and stored at −20°C. Standard solutions (10^{-3} *M*) were prepared just before their chromatographic analysis.

Sample preparation

The extraction procedure used was the same as that for nucleotides[4]. The frozen tissues (200–300 mg) were mixed at −20°C with 2 ml of trichloroacetic acid–diethyl ether solution (0.6 *M*) 30 min before their homogenization.

The tubes were placed in crushed ice–salt, a Polytron was used for tissue homogenization and 2 ml of trichloroacetic acid–diethyl ether were added. Then FAD and nucleotides were extracted with 4 ml of trichloroacetic acid–water (10:90, w/v). After centrifugation at 30 000 *g* (2–4°C) for 20 min, the supernatant solution was extracted three times with three times its volume of cooled diethyl ether to eliminate the trichloroacetic acid. Between each extraction, the ether was removed with a vacuum pump. Finally, after bubbling nitrogen through to remove traces of diethyl ether, the extract was filtered through a 0.45-μm filter (Millex-HV 25 mm, not sterile) and injected into the chromatograph.

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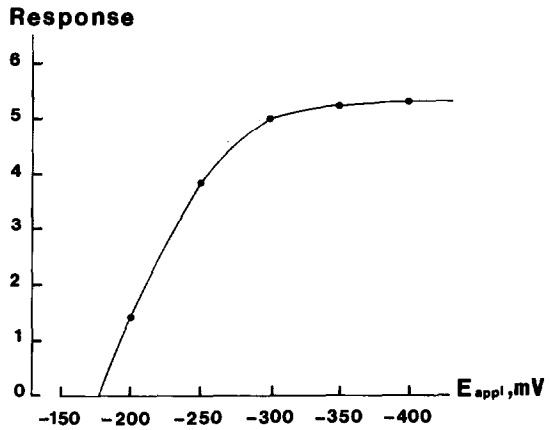

Fig. 1. Voltammogram of FAD (500 pmol injected). Effect of applied potential (E_{appl}, mV) on the response intensity.

High-performance liquid chromatography

The analysis was performed using a Kontron 414 pump with a 7125 Rheodyne injection valve fitted with a 50-μl loop, a Hypersil APS, NH_2 (particle size 5 μm) column (150 mm × 4.6 mm I.D.) (Interchim) protected by a pre-column (50 mm × 2 mm I.D.) packed with the same stationary phase (Hypersil APS, 5 μm; Shandon); these guard columns were packed in our laboratory.

The mobile phase was acetonitrile–0.1 *M* NaH_2PO_4 (30:70, v/v) of pH 3.05, filtered through a 0.22-μm filter (GVWP, Durapore). The flow-rate was 0.8 ml/min.

FAD was detected with a Bioanalytical Systems electrochemical detector consisting of an LC4B amperometric detector with a TL5 glassy carbon electrode and a silver–silver chloride reference electrode at a potential of −280 mV.

Data analysis was carried out on an Anacomp 220 microcomputer (Kontron Analytical).

RESULTS AND DISCUSSION

The voltammogram (Fig. 1) indicated that the optimal potential (E) was −280 mV. This potential was chosen taking into account that the FAD reduction potential is E_0 = −250 mV at pH 7.0.

The calibration graph for FAD (Fig. 2) was linear over the range 2–500 pmol injected. The linearity of the detector was verified by injecting a standard containing FAD at different concentrations and the reproducibility was tested by injecting the same standard solutions at different periods, the coefficient of variation being about 2–3%. The limit of detection was 1.25 pmol of FAD.

Fig. 3 shows a typical chromatogram obtained for fish liver; FAD has a retention time of 13 min. The recovery of FAD added (2 nmol) to the extract of liver was higher than 95%. The FAD concentration in this tissue was 16.9 nmol/g of fresh tissue. This quantitative measurement was carried out by injection of FAD standard solution containing a known concentration (external standard method). It is impor-

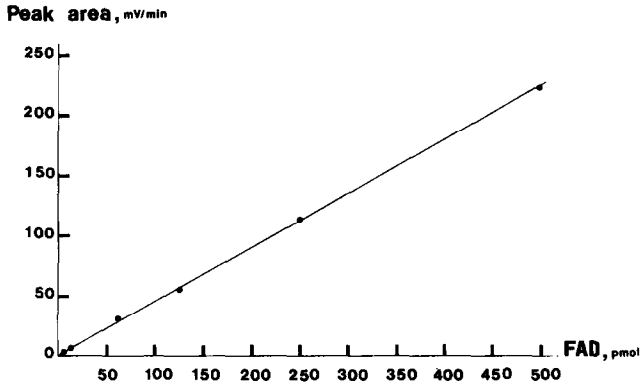

Fig. 2. Calibration graph for FAD. Standard solutions prepared in mobile phase.

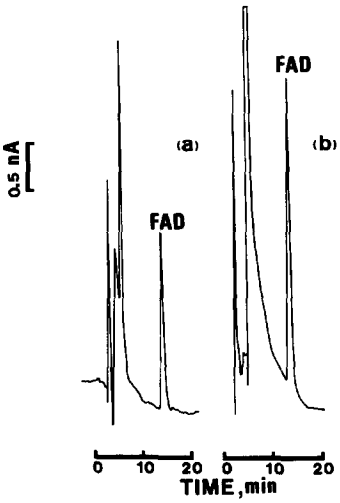

Fig. 3. Chromatograms of (a) standard FAD (25 pmol injected) and (b) extract from fish liver. Range, 5 nA full-scale; chart speed, 0.2 cm/min.

tant always to integrate the chromatograms in the same way for high reproducibility of the results; in this study, the precision of FAD determination was 3%.

This method of quantification of FAD with electrochemical detection is rapid and as sensitive as fluorimetric detection, and can be applied to the determination of FAD in animal tissues.

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