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Three-minute high-performance liquid chromatographic assay for NMN adenylyltransferase using a 20-mm-long reversed-phase column

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Abstract

NMN adenylyltransferase (NAD pyrophosphorylase; NMNAT) reversibly catalyzes the synthesis of NAD from ATP and NMN. In this paper, we describe a rapid and sensitive high-performance liquid chromatographic assay for NMNAT, which uses a 20-mm-long C_{18} reversed-phase (RP) column. The activity was measured by separating in less than 3 min the substrates (NMN and ATP) from the product (NAD) with 0.1 *M* potassium phosphate, pH 6.0, at a 2 ml/min flow-rate and 22°C. NAD was directly quantitated from its ultraviolet absorbance. Amounts of NAD as small as 25 pmol could be measured. The activity value closely agreed with that determined by the spectrophotometric assay. This method was successfully applied to the determination of NMNAT activity in human placental and bull testis extracts, as well as in rat pheochromocytoma (PC12) cells.

Keywords: Enzymes; NMN adenylyltransferase; NAD pyrophosphorylase; NAD; ATP; NMN

1. Introduction

Nicotinamide adenine dinucleotide (NAD) is ubiquitous in living cells, where it participates in cellular oxidation–reduction reactions. In addition, NAD can be degraded at either of the two high-energy bonds to produce the energy to drive DNA repair and recombination reactions and to ADP-ribosylate proteins [1].

In mammalian cells the main biosynthetic pathway leading to the formation of NAD involves the enzyme NMNAT (EC 2.7.7.1), which catalyzes the conversion of NMN and ATP into NAD and inorganic pyrophosphate (PP_i). The relevance of this enzymatic activity is shown by its correlation with crucial cellular events, like mitosis [2] and DNA synthesis [3]. Furthermore, NMNAT appears to be involved in the cellular response to the potent oncolytic agent, tiazofurin [4]. In view of its possible role in the regulation of cellular levels of NAD, over recent years we have been studying NMNAT from several sources. In our laboratory the enzyme has been for the first time purified to homogeneity from yeast [5], human placenta [6], and bull testis [7].

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The need for a rapid and reliable assay for measuring NMNAT activity in different organisms prompted us to develop a continuous spectrophotometric coupled assay based on the reduction by alcohol dehydrogenase (ADH) of the NAD formed [8]. This assay can be conveniently used as a rapid routine test in place of the tedious spectrophotometric two-step, coupled assay [9]. However, the application of the spectrophotometric methods has some drawbacks, mainly due to the necessity of a coupled enzymatic reaction, which may be subject to interferences. For these reasons a sensitive and reproducible assay based on the direct visualization and quantitation of the reaction components was developed. In the present report we describe a new and rapid high-performance liquid chromatographic technique for the determination of NMNAT activity in biological samples. With respect to previously reported HPLC-based procedures for the determination of NAD [8,10–12], this method is unexpensive, and it does not involve gradient elution, labeled substrates, or time-consuming chromatographic runs.

2. Experimental

2.1. Materials

NAD, nucleotides, nucleosides, and bases were purchased from Sigma (St. Louis, MO, USA). Potassium phosphate, dithiotreitol, EDTA, and HPLC grade methanol were from Merck (Darmstadt, Germany). Water was purified by a Milli-Q system from Millipore. Other reagents were of analytical grade.

2.2. Biological samples preparation

2.2.1. Human placental extract

Term human placenta was obtained immediately after delivery and immersed in a solution containing 0.129 *M* sodium citrate, pH 7.5, to reduce blood coagulation. The tissue was trimmed free of adherent membranes, washed with 0.9% NaCl, and cut into small pieces. These pieces were then blotted on paper towels to remove as much blood as possible. The tissue was suspended in 4 volumes of buffer A (100 m*M* potassium phosphate buffer, pH 7.4, containing 1 m*M* DTT, 1 m*M* $MgCl_2$, 0.5 m*M* EDTA and 1 m*M* phenylmethylsulfonyl fluoride) and homogenized five times for 10 s each with 10-s intervals in a Waring Blendor. The homogenate was centrifuged at 13 000 *g* for 20 min and to the supernatant 1 *M* acetic acid up to pH 5.0 was added dropwise. After 10 min stirring, the pellet was collected by centrifugation at 25 000 *g* for 10 min and dissolved in a small volume of buffer A, containing 3 *M* KCl. The suspension was adjusted to pH 7.4 by adding 1 *M* KOH, stirred 30 min and centrifuged at 25 000 *g* for 20 min. The pellet was resuspended in 100 m*M* buffer A containing 3 *M* KCl. The resulting supernatant was referred as the human placental extract.

2.2.2. Bull testis extract

The extract was prepared following the same procedure used for human placental extract, with minor modifications.

2.2.3. PC12 cell extract

PC12 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 10% horse serum. Cells in the logarithmic phase were harvested by scraping and washing with phosphate-buffered saline and centrifuged at 800 *g* for 10 min. The pellet ($14 \cdot 10^6$ cells) was suspended in buffer A (1 ml). The cell suspension was sonicated on ice for 5×5 s and centrifuged at 16 000 *g* for 5 min at 4°C. An aliquot (5–50 ml) of the supernatant was used as the source of enzyme for the assay of NMNAT activity.

2.3. HPLC system

The analysis was performed with a Beckman System Gold apparatus (Berkeley, CA, USA), endowed with two HPLC pumps, a mixing chamber, an injector valve, an UV absorbance detector, and an integrating software. A 20×4.6 mm I.D. LC-18 guard column, 5-mm particle size (Supelco, Bellefonte, PA, USA) was used as analytical column.

2.4. Enzyme assay

The standard assay mixture contained 43 m*M* Tris, pH 7.5, 17 m*M* $MgCl_2$, 10 m*M* NaF, 2.3 m*M* ATP, 1 m*M* NMN, and the appropriate amount of sample, in a final volume of 540 μl for both human placental and bull testis extracts. Incubations were done at 37°C for different times, ranging from 5 to 60 min. The reaction was stopped by adding 100 μl of assay mixture to 50 μl of ice-cold 1.2 *M* $HClO_4$ in an Eppendorf tube. After 10 min at 0°C proteins were removed by 1 min centrifugation on microfuge; 130 μl of supernatant was then neutralized by the addition of 35 μl of 1 *M* K_2CO_3 and the $KClO_4$ formed was removed by centrifugation. NMNAT activity was calculated after HPLC identification and quantitation of NAD produced, as described under 'Chromatographic conditions', Section 2.5. One unit of activity is defined as the amount of enzyme that catalyzes the synthesis of 1 μmol of NAD per minute at 37°C.

2.5. Chromatographic conditions

The analytical column, 20×4.6 mm I.D. LC-18, was thermostatted, equilibrated and eluted with 0.1 *M* potassium phosphate buffer, pH 6.0 (buffer 1), containing different percentages of methanol, ranging from 0 to 0.8, at a flow-rate of 2.0 ml/min. For each assay mixture, an aliquot of the neutralized perchloric acid supernatant was injected and the eluate absorbance was recorded at 254 nm. Every twenty sample injections the column was washed for 5 min with buffer 1 containing 20% methanol and then it was equilibrated with buffer 1 before the following run. The concentrations of stock nucleotides NMN and ATP used in enzyme assay were determined from their molar absorbances.

2.6. Other analyses.

Spectrophotometric determination of NMNAT activity was performed according to [8]. Protein content was measured by the method of Bradford [13], using crystalline bovine serum albumin fraction V as the standard.

3. Results

The aim of our work was to develop a rapid isocratic HPLC method that allowed the determination of NMNAT activity in biological samples, through the separation and quantitation of NAD produced. In order to select the optimum chromatographic conditions for the separation of NAD from the substrates, NMN and ATP, and other related compounds, four different percentages of methanol in buffer 1 were tested as the mobile phase, at different column temperature values. The results obtained show that the retention time decreases with increasing temperature (20–28°C) and increasing methanol (0–0.8%). Maintenance of constant temperature of the solvents and column was found to be critical for reproducible retention times. With variations in temperature, retention times for components having a stronger affinity for the column matrix were altered to a greater degree than those for the compounds eluted earlier. By lowering the temperature a significantly better resolution was achieved.

For the determination of NMNAT activity in the biological samples described under Experimental, Section 2 the most satisfactory conditions were buffer 1 without methanol at 22°C; however if the sample to be analyzed does not contain inosine the mobile phase can be changed to 0.8% methanol to shorten the retention time of NAD to 1.47 min.

Fig. 1a shows the separation of a standard compounds mixture (NMN, ATP, ADP-ribose, nicotinamide, inosine, and NAD), where NAD is completely separated in less than 3 min. The method allows the determination of NAD concentration as low as 0.5 μM in the reaction mixtures, thus rendering the determination comparable to the spectrophotometric one. In Fig. 1b and c the proposed HPLC method was applied to the determination of NMNAT activity in human placental extract, using NMN and ATP as the substrates and including in the assay mixture NaF in order to inhibit ATP degrading activities (see Experimental, Section 2). As shown, the low amount of inosine observed at 30 min (Fig. 1c) does not interfere with NAD determination.

Fig. 2 describes the proportionality of the assay as a function of the amount of human placental extract present in the incubation mixture; the amount of

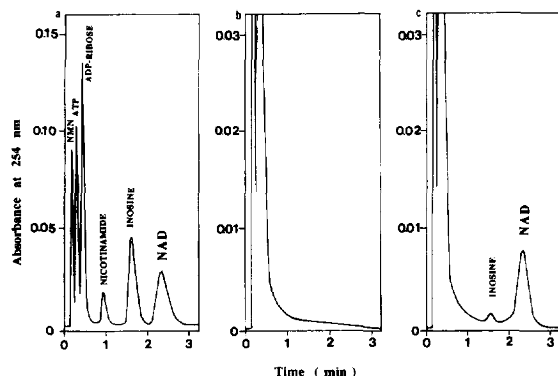

Fig. 1. HPLC-based assay of NMNAT activity in human placental extract. (a) Representative separation of 2 nmol each of NMN, ATP, ADP-ribose, nicotinamide, inosine and NAD, on LC-18 guard column, equilibrated with buffer 1 (0.1 *M* potassium phosphate buffer, pH 6.0) at a flow-rate of 2.0 ml/min. The column was thermostatted at 22°C. (b, c) Separation of a representative assay mixture, containing 43 m*M* Tris, pH 7.5, 17 m*M* $MgCl_2$, 10 m*M* NaF, 2.3 m*M* ATP, 1 m*M* NMN, and 50 μl of human placental extract in 0.54 ml total volume, at time 0 (b) and after 30 min incubation (c). After deproteinization, 20 μl of neutralized perchloric acid supernatant was injected into HPLC. See Experimental, Section 2, for details.

NAD produced increased linearly with the amounts of human placental extract used. The rate of production of NAD was also proportional to the incubation time over the range explored (Fig. 3). The concentration of substrates was always in large excess, about 25–100 times, over the K_m values. Multiple determinations of activity for each extract sample gave reproducible values, the coefficient of variation being approximately 3.7%.

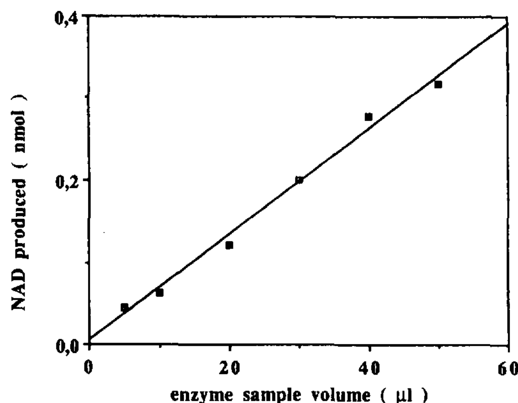

Fig. 2. NMNAT activity of samples containing increasing volumes of human placental extract after 30-min incubation at 37°C. The 0.54-ml mixture contained 43 m*M* Tris, pH 7.5, 17 m*M* $MgCl_2$, 10 m*M* NaF, 2.3 m*M* ATP, 1 m*M* NMN. After deproteinization, 20 μl of neutralized perchloric acid supernatant was injected into HPLC. See Experimental, Section 2, for details. Each data point represents the mean of three independent experiments, made in duplicate.

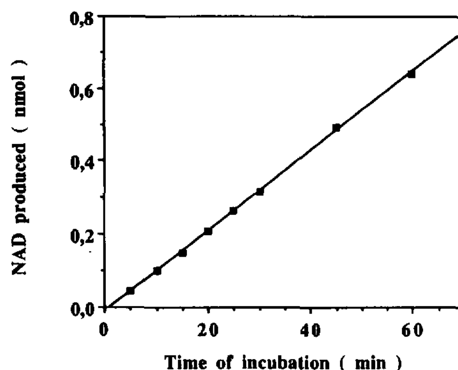

Fig. 3. Time course of NMNAT activity. The 0.54-ml mixture contained 43 m*M* Tris, pH 7.5, 17 m*M* $MgCl_2$, 10 m*M* NaF, 2.3 m*M* ATP, 1 m*M* NMN, and 50 μl of human placental extract. After deproteinization, 20 μl of neutralized perchloric acid supernatant was injected into HPLC. See Experimental, Section 2, for details. Results are given as means from three independent experiments.

In order to evaluate the accuracy of this HPLC method the enzyme activity in human placental extract and in bull testis extract was measured by both the spectrophotometric method [8] and the present assay. Table 1 shows the excellent agreement existing between the proposed method and the spectrophotometric assay.

In order to test the applicability of this assay to a cell extract, NMNAT activity was also measured in cultured PC12 cells, as described under Experimental, Section 2. Enzyme activity of 0.55 mU/mg (mean from five independent determinations) was obtained; this value is very similar to that observed in the ovarian carcinoma cells, but lower than that found in human myelogenous leukemic K562 cells [10]. The addition of nicotinamide up to 5 m*M* to the reaction mixture, to inhibit contaminating NADase activity, did not result in any increase of NMNAT activity. In order to evaluate the sensitivity of the assay, different amounts of PC12 cell supernatant were included in the reaction mixture and the activity was determined as described earlier. The results

Table 1
Comparison of the HPLC method with the spectrophotometric coupled assay

Sample	Activity[a] (mean±S.D.) (mU/ml)	
	HPLC method	Spectrophotometric coupled assay
Human placenta[b]	10.8±0.4	12.8±2.0
Bull testis[c]	5.5±0.2	5.9±0.8

[a]Determined as described under Experimental (Section 2) and by the method reported by Balducci et al. [8].
[b]Human placental extract, preparation is described under Experimental (Section 2).
[c]Bull testis extract, preparation is described under Experimental(Section 2).

indicated that the lowest number of cells which can be used is $7 \cdot 10^4$ cells (4.5 μg protein).

4. Discussion

NMNAT catalyzes the reversible transfer of the adenylyl moiety of ATP to the phosphoryl group of NMN to form NAD, thus playing a central role both in de novo and salvage synthesis of pyridine dinucleotide.

Interest in this enzyme has also increased with the discovery that, in patients undergoing tiazofurin therapy, low level of NMNAT activity is associated with the development of drug resistance [4]. Therefore the measurement of NMNAT activity during drug therapy would enable those individuals who may become unduly resistant to tiazofurin to be quickly identified.

NMNAT activity can be determined in different ways [8–10]. As it has been mentioned in the introduction, we have previously described a continuous spectrophotometric coupled assay, based on the reduction by ADH of the NAD formed, which can be conveniently used as a rapid routine test [8]. However, the biological matrix in which the enzyme is present, e.g. when dealing with crude extracts, may disturb the accurate spectrophotometric detection of NADH. For these reasons we have developed a new, non radioactive and sensitive RP-HPLC technique for the determination of NMNAT activity in biological samples. This method represents a noteworthy improvement over the previously reported HPLC procedures [8,10–12] for the following reasons: (a) it does not require a gradient, (b) owing to the use of a guard column the analysis is very fast and not expensive, (c) the components of the reaction mixture are monitored, allowing the reaction course to be controlled and the possible presence of side-reaction products, like inosine and nicotinamide, to be detected, (d) the direct measurement of NAD by UV absorbance eliminates drawbacks associated with radioactive methods, such as the availability of labeled substrates and their cost.

In addition, the high sensitivity in NAD detection (25 pmol) renders the proposed method suitable for the measurement of low levels of NMNAT activity. Therefore the HPLC method described herein represents a useful alternative to the radioactive assay [10], when dealing with tumor cells of patients undergoing tiazofurin therapy, who usually are leukopenic with very low cell numbers.

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