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Simple and rapid method for determining nicotinamide adenine dinucleotide synthetase activity by high-performance liquid chromatography

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Abstract

A method is described for the determination of nicotinamide adenine dinucleotide synthetase (NADS) activity in human blood. Using high-performance liquid chromatography (HPLC), the formed NAD is separated from the substrates and the other blood components in less than 13 min. The activity of NADS determined by HPLC is closely correlated with that determined by the conventional spectrophotometric method, which requires two steps of enzyme reaction. The present method is simple and reliable and facilitates the routine analysis of NADS activity. © 1997 Elsevier Science B.V.

Keywords: Nicotinamide adenine dinucleotide synthetase

1. Introduction

In the final step of the Preiss–Handler pathway for nicotinamide adenine dinucleotide (NAD) biosynthesis [1], NAD synthetase (NADS) transfers an amino group from glutamine (Gln) to nicotinic acid adenine dinucleotide (NAAD) to form NAD. NAD is a coenzyme for oxidoreduction, and considered to play an important role in preventing oxidative stress in tissues. The ability of tissues to supply reducing equivalents and to maintain normal levels of glutathione appears to be NADH dependent [2].

Impaired rate of NAD synthesis in erythrocytes was reported in some disorders (e.g., pyruvate kinase deficiency [3], enolase deficiency [4] and thalassemia [5]). The NADS activity was reduced in erythrocyte from lead exposed subjects [6–8]. Zerez et al. [6] were the first to report that NADS activity was markedly decreased in three workers exposed to lead. The World Health Organization (WHO) referred to the decrease in NADS activity as one of the important effects of lead on humans [7]. Recently, Morita et al. [8] demonstrated the dose–effect relationship of NADS activity versus blood lead concentration (Pb-B), indicating that the activity could be useful as an biological effect index. However, the analysis can not be used for routine tests because of the complicated and time-consuming procedures.

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The purpose of this study was to develop a simple and reliable method for determining NADS activity in erythrocytes, using high-performance liquid chromatography (HPLC).

2. Experimental

2.1. Chemicals

NAAD, adenosine 5′-triphosphate (ATP) and tris-[hydroxy-methyl]aminomethane (Tris) were obtained from Sigma (St. Louis, MO, USA). NAD, magnesium chloride, potassium chloride, Gln, potassium dihydrogenphosphate and methanol were purchased from Wako (Osaka, Japan). The water used in the present study was deionized and distilled with a Model WF-12 distiller (Yamato, Tokyo, Japan).

2.2. Biological materials

The investigations were carried out on heparinized venous blood obtained from 28 male lead workers employed in a glass factory, and from some control subjects with no history of occupational exposure to lead. Blood samples were stored at room temperature and the activity was determined one day after sampling.

2.3. Sample preparation

Fifty μl of whole blood were hemolyzed by 350 μl of water and 40 μl of the hemolysate were used for the NADS reaction. The total volume of the reaction mixture was 400 μl placed in a microtube (1.5 ml) with cap, and consisted of 30 m*M* Tris–HCl buffer (pH 7.4), 60 m*M* KCl, 2 m*M* $MgCl_2$, 0.5 m*M* ATP, 15 m*M* Gln, 0.5 m*M* NAAD and 40 μl of the hemolysate. The reaction was carried out at 37°C for 60 min and stopped by boiling the microtube for 2 min before cooling the tubes in ice cold water. After centrifugation at 12 000 *g* for 10 min at 4°C, 40 μl of the supernatant was used for NAD determination by HPLC.

2.4. HPLC determination

A liquid chromatograph (Shimadzu, Kyoto, Japan) consisting of a pump (LC-10A), an automatic sample injector (SIL-10A), a column oven (CTO-10A), a detector (SPD-10A) and a data processor (C-R4A) was used. The column (150×4.6 mm) packed with reversed-phase silica (Senshu Pak, Senshu Science, Tokyo, Japan) was used for the separation. The mobile phase was 3% methanol containing 10 m*M* KH_2PO_4. The flow-rate, oven temperature and detector wavelength were set at 1 ml/min, 40°C and 254 nm, respectively. Samples were cooled to 4°C during a series of analyses, and 40 μl of the supernatant was injected at 16-min intervals. An aqueous solution of NAD (100 μ*M*) was injected into the HPLC system as the standard.

2.5. Calibration of NADS activity

NADS activity was calculated by the amounts of formed NAD. The following equation was used for the calibration of the NADS activity.

$$\text{NADS activity } (\mu\text{mol/h/g Hb}) = S_A/S_T \cdot C_{St} \cdot V \cdot f/C_{Hb}$$

where S_A and S_T are peak area of sample NAD and that of standard NAD, C_{St} the concentration of standard NAD used (100 μ*M*), V is the volume of reaction mixture (0.0004 l), f (20 000) is the factor for volume of blood used (5 μl) to convert the blood volume of 100 ml and C_{Hb} is hemoglobin (Hb) concentration of blood used (g/100 ml). The peak area is automatically calculated by the software which is originally provided with the data processor (C-R4A).

2.6. Other analysis

NADS activity was also determined by the spectrophotometric method by Morita et al. [8]. Pb-B was determined by flameless atomic absorption spectrometry (Z-8000, Hitachi, Tokyo, Japan). Hemoglobin concentration was determined by a hemoglobin

counter (COBAS-ARGOS, Roche Japan, Tokyo, Japan), which directly measured hemoglobin spectrophotometrically as cyanmethemoglobin.

3. Results and discussion

3.1. Chromatographic separation and enzyme reaction

The formed NAD is completely separated from the substrates (NAAD and ATP) and blood components in the present HPLC system (Fig. 1A). Very low levels of NAD are formed in the absence of NAAD (Fig. 1B). In the absence of ATP, Gln, or enzyme solution, the blank level is also the same as the low levels. The blank levels were less than 1% of the control activity and did not vary among the subjects. Hence the blank for each sample can be omitted in routine analysis. There was no effect of boiling on NAD levels in the reaction mixture. NAD added to a reaction mixture was also stable during the incubation for 60 min at 37°C and recovery was almost 100%. The reactions of both enzymes from controls and lead workers were linear over a broad range with respect to the amounts of enzyme solution added (less than 80 μl). As the reaction time is prolonged, the amount of NAD formed was linearly increased up to 120 min (Fig. 2).

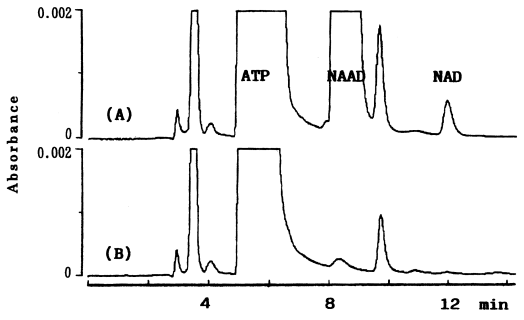

Fig. 1. Chromatogram of NADS reaction mixture. (A) and (B) show the reaction mixture in the presence or absence of substrate, NAAD, respectively.

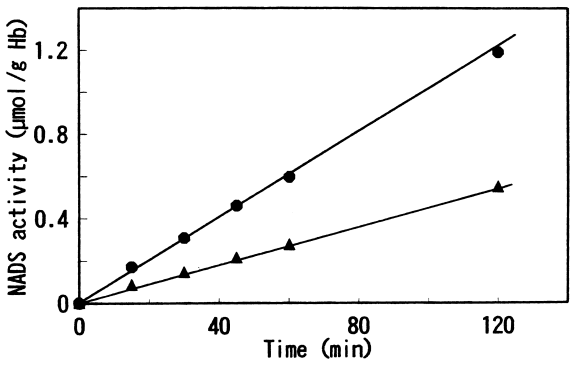

Fig. 2. Dependence of NADS activity (μmol/g Hb) on the duration of incubation time. (●) Control subject, (▲) lead worker.

3.2. Dependence of the activity on substrate concentrations

Fig. 3 shows the effects of substrate concentrations on the NADS activity. The effects were investigated in both the control subject and lead worker. It is appeared that NADS activity is remarkably decreased in a lead worker even at the higher concentrations of ATP or Gln (Fig. 3A,B). However, the depressed activity of NADS in a lead worker tends to be partly recovered with increasing NAAD concentration (Fig. 3C).

Concentrations of 0.5 m*M* ATP, 15 m*M* Gln and 0.5 m*M* NAAD are a good compromise for the NADS determination. The chosen conditions are the most convenient and economical, without an inconveniently high concentration of NAAD. The concentrations are also sufficient to discriminate the decreased activity in lead exposed subjects. With the highest NADS activity among the present samples, the NAD formed in the reaction mixture is about 3 μ*M* which is 0.6% of the substrate NAAD concentration (0.5 m*M*).

From the double reciprocal plot of the activity versus each of the substrate concentrations in Fig. 3, apparent K_m value and V_{max} were determined for the enzymes from a normal and a lead worker as shown in Table 1. In the present study, we measured only one subject as a control, therefore, mean and standard deviation (S.D.) values of the K_m and V_{max} could not be determined.

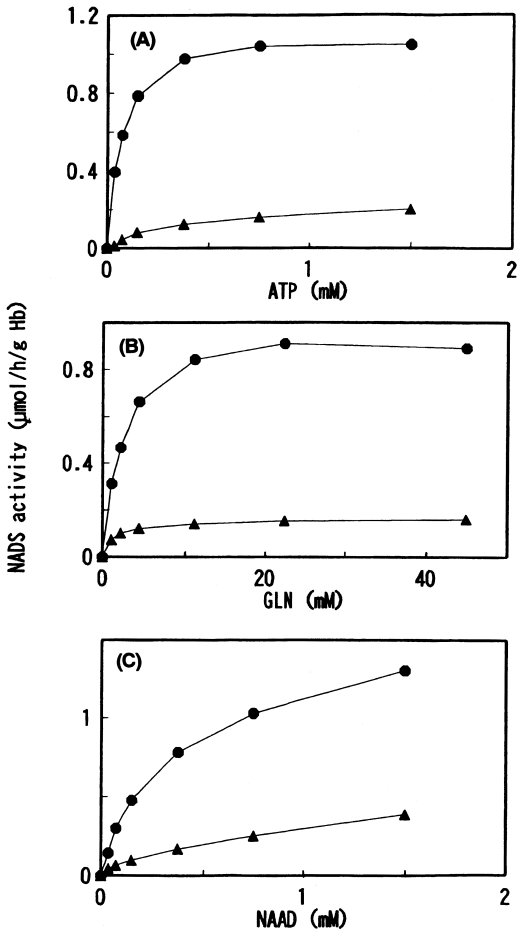

Fig. 3. Effect of substrate concentrations on the NADS activity. (●) Control subject, (▲) lead worker.

In control subjects, Zerez et al. [6] reported that K_m values of ATP, Gln and NAAD were 0.154±0.027, 2.17±0.42 and 0.108±0.028 (mean±S.D.) m*M*, respectively. The K_m of Gln was nearly the same as in our study. The K_m of ATP was slightly higher than ours and NAAD slightly lower. The small differences in the values found by Zerez et al. and those of the present study might be due to the differences in NADS reaction conditions and in the method of NAD determination. For the enzyme solution, Zerez et al. [6] used partially purified enzyme, while we used crude hemolysate which was more convenient for routine analyses. The mean and S.D. values of K_m and V_{max} for the control subjects remain to be investigated by the present method. In the present data V_{max} was extremely low in a lead worker. However, V_{max} is not only influenced by lead but also by the amount of enzyme present in the reaction mixture.

Table 1
K_m and V_{max} of NADS in the both of control and lead exposed subjects shown in Fig. 3

Substrates	Control subject		Lead exposed subject	
	K_m (m*M*)	V_{max} (μmol/h/g Hb)	K_m (m*M*)	V_{max} (μmol/h/g Hb)
ATP	0.08	1.33	0.33	0.24
Gln	2.38	1.18	1.54	0.15
NAAD	0.31	1.43	1.14	0.67

3.3. Analytical precision

The detection limit of this method is dependent on the HPLC determination and is about 0.01 μmol/h/g Hb in the entire procedure. On the other hand, the detection limit of NADS activity in the spectrophotometric procedure is about 0.05 μmol/h/g Hb. To evaluate the precision of the method, we calculated within-run coefficient of variation (C.V.) for samples from a control and a lead worker. Ten replicates were assayed for both samples in a single analytical run. The C.V.s were 2.8% and 5.0% for the NADS activities of 0.8 (control subject) and 0.3 (lead worker) μmol/h/g Hb, respectively. The results indicate that the present method has good precision to detect normal and abnormal activity. The C.V. described here is the variation of the entire procedure including the assay and analysis by HPLC. In the conventional method, the C.V. might be high, although the precision of the method has not been reported so far [6,8]. The higher C.V. in the conventional method might be due to the fact that the method required two enzyme reactions including a complicated cycling assay, and high blank levels in the spectrophotometrical determination [8].

3.4. Comparison with the conventional method

Fig. 4 demonstrates the comparison of NADS activities between the present HPLC method and the conventional spectrophotometric method [8]. The activities of both methods are well correlated (r = 0.908), indicating that the activity obtained with the

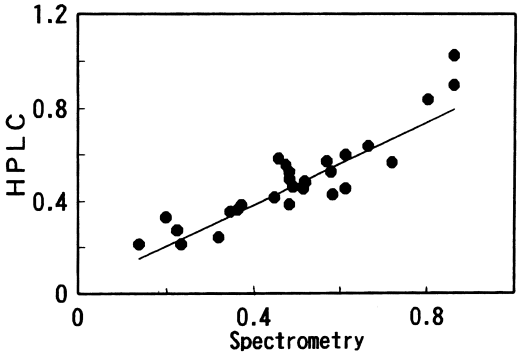

Fig. 4. Relationship between NADS activity (μmol/h/g Hb) determined by the spectrophotometric (x) and HPLC (y) methods. $y=0.933x+0.0295$ ($r=0.908$, $n=28$).

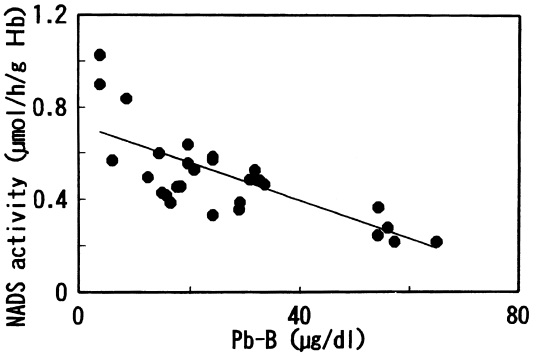

Fig. 5. Relationship between Pb-B and NADS activity in 28 lead workers. NADS = 0.00824Pb-B + 0.724 ($r=0.749$).

present method may be compared with the data from the method previously reported.

NADS activity has been determined by a spectrophotometric method [6–8]. Zerez et al. [6] used partially purified enzyme for the NADS enzyme reaction, while Morita et al. [8] reported a simpler method using whole blood. Both methods required two steps of enzyme reaction, because of the low concentration of NAD formed in the reaction mixture. The procedure was very complicated and time-consuming and not appropriate for routine analyses. In the present study, we developed a simple method for the determination of NADS activity using HPLC, which separated NAD from substrates and blood components.

3.5. Biological monitoring in 28 lead workers

Fig. 5 shows the relationship between Pb-B and NADS activity obtained with the present HPLC method in lead workers. NADS activity is well correlated with Pb-B. The correlation coefficient is of the same order as that obtained by the previous method (data not shown). These results indicate that NADS activity by the present method is useful for the biological monitoring of lead exposure.

4. Conclusions

A simple and reliable method has been developed for the analysis of NADS activity in human blood, using HPLC. The NADS activity found using HPLC is well correlated with that found using the conventional spectrophotometric method [8]. The HPLC method dose not require the two steps of enzyme reaction as in the spectrophotometric method [6,8], and therefore, simplifies the procedure. NADS activity determined with the HPLC method is well correlated with Pb-B. Hence the present HPLC method is more useful for routine analyses in clinical laboratories and for biochemical research on the enzyme.

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