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Short communication

Reversed-phase high-performance liquid chromatography of nicotinic acid mononucleotide for measurement of quinolinate phosphoribosyltransferase

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Abstract

A system has been developed for the determination of quinolinate phosphoribosyltransferase (QPRT) activity in liver and kidney homogenates using HPLC. A product, nicotinic acid mononucleotide (NaMN), is separated by reversed-phase chromatography (a Tosoh ODS 80TS was used as an analytical column) using a mixture of 10 m*M* KH_2PO_4–K_2HPO_4 buffer (pH 7.0) containing 1.48 g/l tetra-*n*-butylammonium bromide–acetonitrile (9:1, v/v) as a mobile phase. The flow-rate was 1.0 ml/min, the detection wavelength was 265 nm. The column temperature was maintained at 40°C. Under these conditions, NaMN was eluted at about 8.1 min. Sample preparation was very straightforward. The reaction mixture of QPRT assay was stopped by immersing the tube into a boiling water bath, the resulting supernatant was filtered, and the filtrate was directly injected into a HPLC system. The total HPLC analysis time was approximately 20 min.

Keywords: Nicotinic acid mononucleotide; quinolinate phosphoribosyltransferase; Enzymes

1. Introduction

Quinolinic acid (QA) is not only a key intermediate in the de novo biosynthesis of NAD from tryptophan [1], but also an endogenous neurodegenerative compound [2]. QA is metabolized to form nicotinic acid mononucleotide (NaMN) in the presence of 5-phopshoribosylpyrophosphate (PRPP) by quinolinate phosphoribosyltransferase (QPRT; EC 2.4.2.19) [1]. The biosynthesis of QA is concerned with two enzymes, 3-hydroxyanthranilic acid oxygenase (3-HAO; EC 1.13.11.6) and α-amino-β-carboxumuconate-ϵ-semialdehyde decarboxylase (ACMSD; EC 4.1.1.45) [3]. 3-HAO catalyses the reaction of 3-hydroxyanthranilic acid (3-HA) to α-amino-β-carboxumuconate-ϵ-semialdehyde (ACMS), and then ACMS is decarboxylated to form α-amino-muconate-ϵ-semialdehyde (AMS) by ACMSD. On the other hand, ACMS spontaneously cyclizes to form QA. Therefore, the fate of QA is subject to the enzyme activities of 3-HAO, ACMSD and QPRT.

Nutritional factors that affect the activity of ACMSD are well known [4–8] and the activity of 3-HAO is known to increase in Huntington's disease [9] and epilepsy in mice [10], however, those that

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affect the activity of QPRT are little known. This is because there are no methods for measuring non-radiochemical assay of QPRT. To measure the activity of QPRT, [2,3,7,8-^{14}C]QA has been used as the substrate [11], however, it is now impossible to get the radiochemical from commercial sources because its production was discontinued. Although Kalikin and Calvo [12] reported high-performance liquid chromatographic analysis of the products of the reaction catalyzed by QPRT, this could be only applied to the purified enzyme reaction. Therefore, we have investigated the method of non-radiochemical assay of QPRT and succeeded in a rapid non-radiochemical high-performance liquid chromatography (HPLC) method.

2. Experimental

2.1. Chemicals

QA and tetra-*n*-butylammonium bromide were purchased from Wako (Osaka, Japan). NaMN and PRPP (tetra sodium salt) were from Sigma. All other chemicals used were of the purest grade available from commercial sources.

2.2. HPLC system

The HPLC system consisted of an LC-9A liquid chromatograph (Shimadzu, Kyoto, Japan), a Model 7125 syringe-loading sample injector (Rheodyne, Cotati, CA, USA), a Tosoh 80TS column (250×4.6 mm I.D., average particle size 7 μm) (Tosoh, Tokyo, Japan), and an SPD-6A spectrophotometer (Shimadzu) and Chromatopac C-R6A (Shimadzu) for data processing. The mobile phase was a mixture of 10 m*M* KH_2PO_4–K_2HPO_4 buffer (pH 7.0) containing 1.48 g/l tetra-*n*-butylammonium bromide–acetonitrile (9:1, v/v), the flow-rate was 1.0 ml/min, the detection wavelength was 265 nm. The column temperature was maintained at 40°C with a CTO-6A column oven (Shimadzu). Under these conditions, NaMN, the main product of the QPRT reacted mixture was eluted at about 8.1 min. Peak identity was confirmed by co-elution with standard.

2.3. Animal experiment

Male Wistar rats (6 weeks old) were obtained from CLEA Japan (Tokyo, Japan). The rats were housed in an ordinary wire-bottomed cage and fed ad libitum an ordinary commercial diet MF (the Oriental Yeast, Tokyo, Japan) during 14 days. The enzyme preparation was done as follows. The rats were killed by decapitation, the liver and kidneys of each animal were removed, and a portion (approximately 0.25 g) was immediately homogenized with a PTFE-glass homogenizer in five volumes of cold 50 m*M* KH_2PO_4–K_2HPO_4 buffer (pH 7.0). These homogenates were centrifuged at around 55 000 *g* for 20 min to measure the activity of QPRT.

2.4. NaMN-degrading enzyme activity

The incubation medium (a final volume of 500 μl) contained 50 μl of 500 m*M* KH_2PO_4–K_2HPO_4 buffer (pH 7.0), 50 μl of 1 m*M* NaMN, 350 μl of water, and 50 μl of enzyme source. The reaction was started by addition of the enzyme source and the incubation was carried out at 37°C for 1 h. Heat treatment (the reaction tube was placed in a water boiling bath for 5 min) was used to stop the reaction, because acid treatment to stop the reaction was not suitable for HPLC analyses. After the reaction was stopped, the tube was cooled on ice for 5 min, and then centrifuged at 10 000 *g* for 5 min. The resulting supernatant was filtered through a 0.45-μm microfilter and the filtrate (20 μl) was injected into the HPLC system.

2.5. QPRT assay

The incubation medium (a final volume of 500 μl) contained 50 μl of 500 m*M* KH_2PO_4–K_2HPO_4 buffer (pH 7.0), 50 μl of 10 m*M* QA, 50 μl of 10 m*M* PRPP, 10 μl of 100 m*M* $MgCl_2$, 260 μl of water, and 50 μl of enzyme source. The reaction was started by addition of the enzyme source and the incubation was carried out at 37°C for 1 h. Heat treatment (the reaction tube was placed in a water boiling bath for 5 min) was used to stop the reaction. After the reaction was stopped, the tube was cooled on ice for 5 min, and then centrifuged at 10 000 *g* for

5 min. The resulting supernatant was filtered through a 0.45-μm microfilter and the filtrate (20 μl) was injected into the HPLC system.

3. Results and discussion

3.1. Selection of analytical columns

QPRT catalyzes the reaction of QA+PRPP→NaMN+CO_2+pyrophosphate. Of the substrates and products, UV-active compounds are QA and NaMN. Therefore, we firstly developed the separation method of standard QA and NaMN using the Chemcosorb ODS-H column, which is a standard reversed-phase column used in our laboratory. As a result, the separation was sufficient when a mixture of 10 m*M* KH_2PO_4–K_2HPO_4 buffer (pH 7.0) containing 1.48 g/l tetra-*n*-butylammonium bromide–acetonitrile (9:1, v/v) was used as a mobile phase. The retention times for NaMN and QA were ca. 6 min and ca. 10 min, respectively, on this column. Then, the reacted mixture of QPRT and the standard NaMN solution were co-applied on this column, however, the peak of the standard NaMN partly overlapped the compound(s) including enzyme sources. In order to survey a suitable column for the separation of NaMN and the interfering compound(s), the reacted mixture of QPRT and the standard NaMN solution were applied on several types of reversed-phase columns [Tosoh ODS 80TM and Tosoh ODS 80TS (Tosoh), Develosil ODS-MG-5 and ODS-P-5 (Nomura, Seto, Japan), Capcell Pak C_{18} (Shiseido, Tokyo, Japan), STR ODS and ODS-II (Shimadzu Techno-Research, Kyoto, Japan), Shim-Pack CLC-ODS (Shimadzu), Puresil C_{18} (Nihon Millipore, Tokyo, Japan), and Mightysil RP-18 GP (Kanto, Tokyo, Japan)] under the fixed mobile phase condition. Among these, the Tosoh ODS 80TS column (250×4.6 mm I.D., average particle size 5 μm) was selected because it gave the best separation of NaMN and the interfering peaks and the results were very reproducible. This stationary phase has no silanol groups, is fully end-capped and has a low metal content. Fig. 1A shows the separation on this column of standard NaMN and QA dissolved in water.

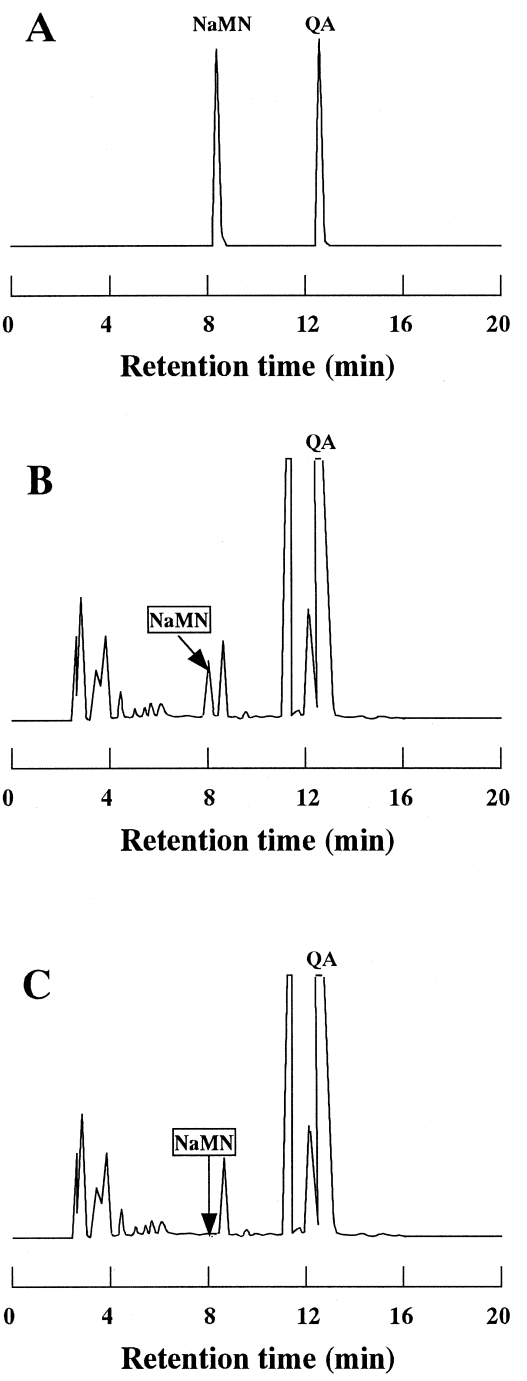

Fig. 1. Chromatograms of the reaction mixture of QPRT. (A) Standard NaMN (1.2 nmol) and QA (1.8 nmol). (B) Supernatant of the reaction mixture of QPRT assay (1 h incubation); sample size, 20 μl. (C) Supernatant of the reaction mixture of QPRT assay (0 h incubation); sample size, 20 μl.

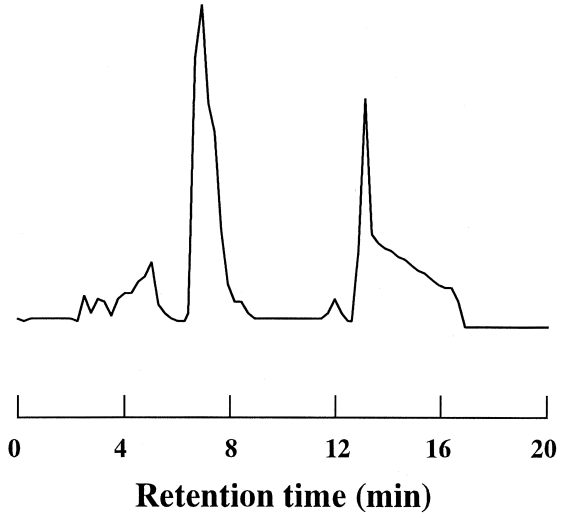

Fig. 2. Chromatogram of acidified NaMN. Chromatogram of the standard NaMN (1.6 nmol) dissolved in 7% perchloric acid; sample size, 20 μl. Analytical conditions were described in Section 2.2.

3.2. Calibration curve and limit of detection

The concentration of standard NaMN was determined by using the molar absorptivity value of 3300 M^{-1} cm^{-1} at 265 nm in water. The peak area of the NaMN (molecular mass 335.2) was linear in the range of 20 pmol (6.7 ng) to 2000 pmol per injection (20 μl), with a correlation coefficient of 0.99. The amount of NaMN formed in the QPRT reaction was calculated from the following equation because the integrated peak area of 1 nmol of NaMN under the HPLC conditions was 74 000 μV s: NaMN (nmol)=(integrated peak area eluted at around 8.1 min)/74 000 μV s.

The detection limit was 10 pmol at a signal-to-noise ratio of 5:1. The relative standard deviations (RSDs) in the analyses of NaMN (500 pmol injection, $n=10$) were within 1% within-series. We periodically measured the standard NaMN stored at −20°C (freezing and thawing were done each time when measured) and it was found that the standard solution sample was stable for at least 5 months.

The reaction mixture of liver QPRT incubated for 1 h was immediately injected five times. The mean peak area of NaMN±SD was 26520±344 μV s. The RSD within-series was 1.3%. After the same reaction mixture was kept for 4 days and 8 days at 4°C, it was reanalyzed five times. The resulting mean peak areas±SDs were 26282±433 μV s (RSD=1.65%) and 26409±445 (RSD=1.69%), respectively. Therefore, the mean±SD of all values ($n=15$) was 26405±435 μV s and the RSD between-series was 1.65%. Accordingly, it was found that this method was highly reproducible.

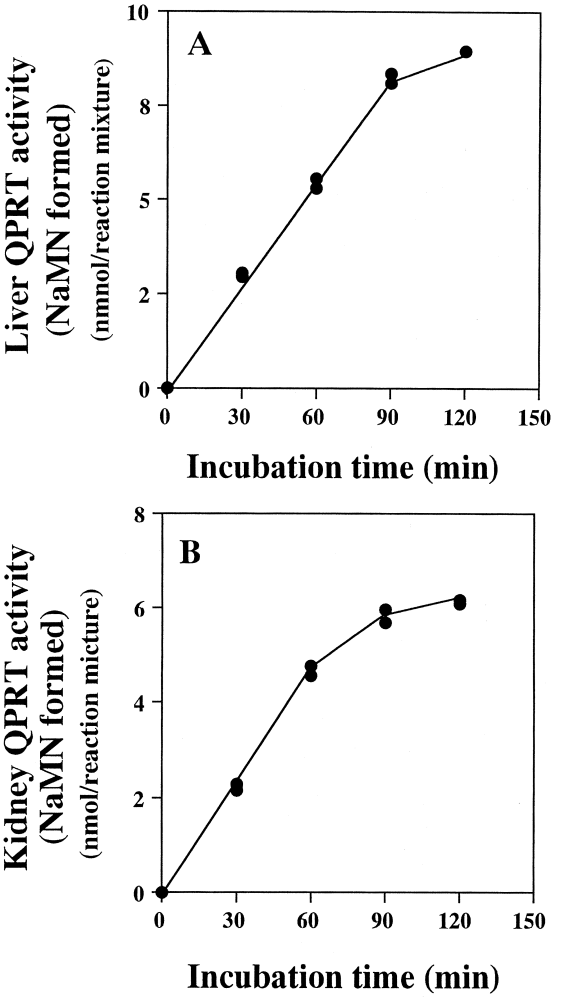

Fig. 3. Time course of QPRT reaction performed with the standard assay condition described in Section 2.5 except for incubation time. (A) Rat liver homogenate was used as an enzyme source. (B) Rat kidney homogenate was used as an enzyme source. Two separate experiments were done in each incubation time.

Table 1
QPRT activity in liver and kidneys of rat[a]

	Activity (μmol/h/g of tissue)
Liver	0.46±0.04
Kidney	0.26±0.02

[a] Rats were housed in a normal wire-bottomed cage and fed ad libitum a commercial diet. Other details are described in the Experimantal section. Values are means±SD for five animals.

3.3. NaMN-degrading enzyme activity

The enzyme reaction was stopped by heating rather than by treatment with acid because the latter resulted in poor peak shapes (see Fig. 2).

Even when NaMN was incubated with a homogenate of rat liver and kidney (the conditions are descried in the Experimental section), the peak areas of the added standard NaMN between the reacted mixtures of 0 h and 1 h incubation were almost the same. Therefore, it was found that NaMN was not metabolized under these conditions. Nishizuka and Hayaishi [1] reported that a rat liver homogenate prepared and incubated with QA-2,3,7,8-^{14}C under the conditions described by them formed only NaMN. This report [1] means that there is no NaMN-degrading activity in rat liver homogenate.

3.4. QPRT activity in liver and kidney

Fig. 1B and C show the chromatograms of the reacted mixture of rat liver QPRT assay incubated for 1 h and 0 h, respectively. When PRPP, another co-substrate, was omitted in the standard reaction mixture (see Section 2.5), the peak of NaMN was not observed (data not shown). The chromatogram of the reacted mixture of rat kidney was almost the same as shown in Fig. 1B.

The reaction was linear up to 1.5 h under the conditions shown in Fig. 3A, when a rat liver homogenate was used as a enzyme source, and it was linear up to 1.0 h, when a rat kidney homogenate was used (Fig. 3B).

The activities of QPRT in rat liver and kidneys are shown in Table 1. These values of rat liver and kidney QPRT were almost the same as those reported previously which were measured by a radiochemical assay [13].

Information on the changes in the activity of QPRT by nutritional factors and chemicals in vivo are little known because non-radiochemical assay method of QPRT had not been reported. We developed the accurate and easily reproducible HPLC method to measure QPRT activity. The pretreatment of sample prior to injection into HPLC was not needed. This method is suitable for the measurement of liver and kidney QPRT activities.

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