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Note

High-performance liquid chromatographic determination of nicotinamide in rat tissue samples and blood after extraction with diethyl ether

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Nicotinamide and nicotinic acid play an important role in biological functions. The combined amounts of nicotinamide and nicotinic acid in biological specimens are usually measured as niacin using *Lactobacillus planturum* [1]. However, the pharmacological effects of nicotinamide and nicotinic acid are different, e.g.: (1) nicotinic acid exhibits a vasodilating effect [2] and a hypolipemic action [3], but nicotinamide does not; (2) unlike nicotinic acid, nicotinamide prevents streptozotocin-induced diabetes [4]; (3) the feeding of large amounts of nicotinamide to young rats inhibits the gain in body weight, but nicotinic acid has no effect [5]; (4) the intraperitoneal injection of large amounts of nicotinamide into mice induces an abnormal increase in the content of liver NAD, but nicotinic acid has no effect [6]; (5) urethane-induced mutagenesis and urethane-induced tumorigenesis in the lungs of mice are significantly inhibited by the oral or intraperitoneal injection of nicotinamide, but not nicotinic acid [7]. Furthermore, NAD biosynthesis from nicotinic acid occurs only in the liver, kidney and heart, whereas NAD biosynthesis from nicotinamide takes place in all organs of rats [8].

Thus, the individual determination of nicotinamide and nicotinic acid is important. Kitada et al. [9] reported the simultaneous determination of nicotinamide and nicotinic acid in fish and meats by high-performance liquid chromatography (HPLC). Hengen et al. [10], Trugo et al. [11] and Tyler and

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Shrago [12] reported the HPLC determination of nicotinic acid from plasma and urine, instant coffee and cereals, respectively. However, owing to interfering peaks, the identification of nicotinic acid or nicotinamide is difficult and requires special skill. De Vries et al. [13] and McKee et al. [14] reported the HPLC determination of nicotinamide. These methods are adequate for pharmacokinetic work. However, they have inadequate sensitivity and are impractical for the determination of endogenous nicotinamide as required in biochemical and nutritional studies. Furthermore, the pretreatment of the samples is cumbersome since ion-exchange column chromatography is needed.

During experiments to measure liver N^1-methyl-2-pyridone-5-carboxamide (2-py), a major metabolite of nicotinamide and nicotinic acid, we found that nicotinamide and 2-py were extracted from tissue with diethyl ether with a modified method for 2-py reported previously [15]. In this paper, we report a practical micro-determination method for nicotinamide in rat organs and blood.

EXPERIMENTAL

Chemicals

Nicotinamide was purchased from Wako Pure Chemical Industries (Osaka, Japan). N′-Methylnicotinamide was from Sigma (St. Louis, MO, U.S.A.). All other chemicals used were of the highest purity and were obtained from commercial sources.

Liquid chromatography

HPLC was performed on a Shimadzu HPLC system, an LC-4A using a liquid chromatography spectrophotometric detector, SPD-2AS (Shimadzu, Kyoto, Japan), a syringe-loading sample injector, Model 7125 (Rheodyne, Cotati, CA, U.S.A.), and a 5-ODS-H column (150×4.6 mm I.D., particle size 5 μm, Chemco Scientific, Osaka, Japan). A degassed solution of 10 m*M* potassium dihydrogenphosphate and acetonitrile (96:4, v/v, pH 4.5) was used as the mobile phase at a flow-rate of 1.0 ml/min. The detection wavelength was 260 nm. The column temperature was maintained at 25°C. The HPLC system was interfaced with a Shimadzu Chromatopac C-R3A for data processing.

Extraction of nicotinamide from organ and blood

Tissue samples. Organs (liver, kidney and brain) removed from rats (1 g) were added to 4.5 ml of water and 500 μl of 1 mg/ml N′-methylnicotinamide, and homogenized with a Teflon-glass homogenizer (volume of homogenate, 5.3 ml). A screw-capped vial containing 1 ml of this homogenate and 4 ml of water was placed in a boiling water-bath for 10 min. In this procedure, NAD and NADP were converted stoichiometrically into nicotinamide. After cooling on ice, the sample was centrifuged at 10 000 *g* for 10 min. The supernatant was retained. The precipitated materials were again extracted with 5 ml of water as described above. The nicotinamide in the pooled supernatant (9.5 ml) was analysed by HPLC.

Blood. Blood (0.1 ml) was mixed with 1.88 ml of water and 20 μl of 1 mg/ml

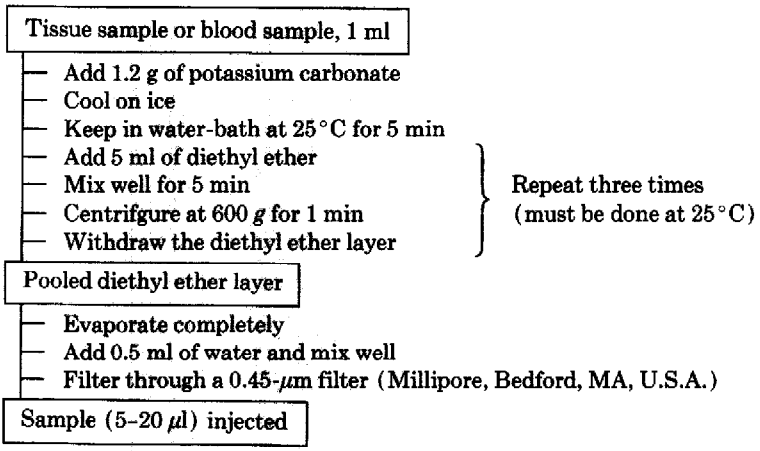

Fig. 1. Procedure for extraction of nicotinamide from tissue samples and blood.

N′-methylnicotinamide, and the mixture was put in a boiling water-bath for 10 min. After cooling on ice, the sample was centrifuged at 10 000 *g* for 10 min. The supernatant (1.9 ml) was used for analysis.

Nicotinamide was extracted from tissue samples or blood samples with diethyl ether as shown in Fig. 1. This nicotinamide content represents the sum of free nicotinamide and nicotinamide converted from NAD and NADP.

RESULTS

Calibration curve and limit of detection

The calibration curve for nicotinamide was linear in the range from 10 pmol to 30 nmol per injection, with a correlation coefficient of 0.99999; linear regression equation: nicotinamide (pmol) = (0.0277991 ± 0.000145) × (integrated peak area) + (1.9407 ± 1.95378). The amount of nicotinamide was calculated using the following equation: nicotinamide (pmol) = integrated peak area/36. The detection limit was 10 pmol (1.22 ng) at a signal-to-noise ratio of 5:1. This sensitivity was about the same as that of the microbiological method [1].

Recovery of nicotinamide and N′-methylnicotinamide

A known amount of nicotinamide (1000 nmol) was added to the rat liver prior to the homogenization and the liver sample was prepared as described in Experimental. Nicotinamide was extracted from the liver sample according to the procedure shown in Fig. 1. The recovery was calculated from the following equation: recovery (%) = (integrated peak area of endogenous nicotinamide + added nicotinamide/10 μl) − (integrated peak area of endogenous nicotinamide/10 μl) × (500 μl/10 μl) × (9.5 ml/1 ml) × (5.3 ml/1 ml) × (1/integrated peak area of added nicotinamide prior to the homogenization) × 100. The recovery of nicotinamide from rat liver was 98.3 ± 2.0% (mean ± S.D., $n = 5$), and those from the other tissues were also satisfactory (Table I).

N′-Methylnicotinamide (pyridine-3-carboxymethylamide, which is not N^1-

TABLE I

RECOVERIES OF NICOTINAMIDE AND THE INTERNAL STANDARD, N′-METHYLNICOTINAMIDE, IN LIVER, KIDNEY, BRAIN AND BLOOD

Sample	Recovery (mean ± S.D., $n=6$) (%)	
	Nicotinamide	N′-Methylnicotinamide
Liver	98.3 ± 2.0	99.7 ± 5.1
Kidney	98.0 ± 2.4	99.5 ± 3.4
Brain	98.4 ± 2.7	97.6 ± 5.5
Blood	99.2 ± 2.9	97.2 ± 1.0

methylnicotinamide, an endogenous metabolite of nicotinamide and nicotinic acid) was available as the internal standard. As described in Experimental, N′-methylnicotinamide was added to the organ or blood prior to homogenization. The recoveries of N′-methylnicotinamide from each organ and blood were satisfactory (Table I).

A typical chromatogram of the reference nicotinamide and N′-methylnicotinamide is shown in Fig. 2A. Nicotinamide is eluted at ca. 5.8 min and N′-methylnicotinamide at 10.8 min. The total HPLC analysis time is ca. 15 min.

Total nicotinamide content in rat tissue samples and blood

The chromatogram of the extract of rat liver sample is shown in Fig. 2B. When the tissue sample was directly injected, the nicotinamide peak was observed by other peaks. However, all the interfering peaks were removed following extraction with diethyl ether. Nicotinamide in tissue samples and blood samples was

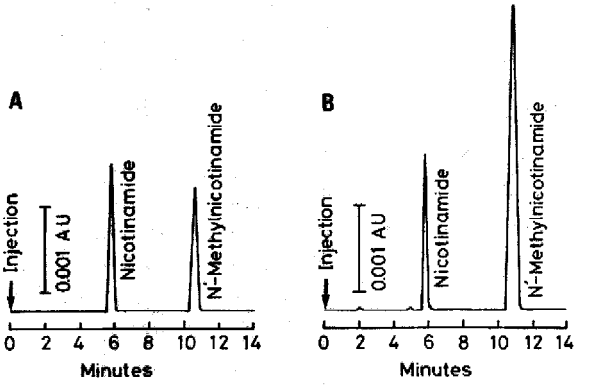

Fig. 2. Chromatogram of nicotinamide and N′-methylnicotinamide. Conditions: column, 5-ODS-H (150 × 4.6 mm I.D.); mobile phase, 10 m*M* potassium dihydrogenphosphate–acetonitrile (96:4, v/v, pH 4.5); flow-rate, 1 ml/min; wavelength, 260 nm; oven temperature, 25°C. (A) Chromatogram of standard nicotinamide (426 pmol) and N′-methylnicotinamide (570 pmol). (B) Chromatogram of the extract of rat liver sample: sample size, 10 μl combining 447 pmol nicotinamide and 1460 pmol N′-methylnicotinamide.

TABLE II

NICOTINAMIDE IN RAT LIVER, KIDNEY, BRAIN AND BLOOD

Male Wistar rats (six weeks old) obtained from Clea Japan were fed ad libitum a stock diet CE-2 for seventeen days. The rats were kept individually in a wire-bottomed cage. The room temperature was 22±2°C and the humidity ca. 60%. The light-darkness schedule was 06:00-18:00 (light) and 18:00-06:00 (dark). The liver, kidney and brain were removed at ca. 09:00 under pentobarbital anaesthesia, and blood was taken from an artery. These organs and blood were used for determining nicotinamide.

Rat No.	Nicotinamide content			
	Liver (nmol/g)	Kidney (nmol/g)	Brain (nmol/g)	Blood (nmol/ml)
1	1326	919	396	171
2	1641	962	455	191
3	1647	932	427	181
4	1767	964	444	163
5	1414	944	368	160
6	1603	1005	463	138
Mean±S.D.	1566±164	954±30	426±38	167±18

characterized on the basis of its retention time and entire UV spectrum between 200 and 350 nm. The chromatograms of the extract of rat kidney, brain and blood are essentially the same. The mean ($n=6$) nicotinamide contents in liver, kidney, brain and blood of rat fed a stock diet CE-2 (obtained from Clea Japan, Tokyo, Japan) were 1566±164, 954±30, 426±38 nmol/g wet mass and 167±18 nmol/ml, respectively, as shown in Table II.

DISCUSSION

Under the HPLC conditions described, the elution times of the major compounds of nicotinamide metabolism, NAD^+, NADH, $NADP^+$, NADPH, nicotinic acid, quinolinic acid, N^1-methylnicotinamide and 2-py were 2.14, 2.29, 1.47, 1.50, 2.26, 1.48, 3.07 and 4.68 min, respectively. Therefore, these compounds do not interfere with the determination of nicotinamide, which has a retention time of 5.80 min. Furthermore, these compounds, except for 2-py, cannot be extracted with diethyl ether.

Since the ion-exchange clean-up steps usually result in severe dilution of the sample, samples from nutritionally deficient subjects cannot be measured. However, this clean-up step does not result in dilution, but rather in enrichment of the sample by a factor of two. The evaporation of diethyl ether is not time-consuming. Furthermore, the extraction with diethyl ether eliminates all the interfering peaks (Fig. 2B). So, it is considered that there are no interfering compounds in biological materials, including foods, for the determination of nicotinamide. The sensitivity of the method is comparable with that of the microbiological method (1.22 ng) [1]. Therefore, the method is adequate for determining endog-

enous nicotinamide in tissue samples and blood. A major metabolite of nicotinamide, 2-py, is also separated under the HPLC conditions described. However, the levels of 2-py in liver, kidney, brain and blood were below the detection limit. It is probable that the small peak just before the peak of nicotinamide is 2-py (Fig. 2B). As 2-py is known to be formed mainly in the liver [16], it is excreted very rapidly into urine. Indeed, urinary excretion in the rats fed a CE-2 diet was very high, ca. 1 μmol per day (unpublished data). Conversely, urinary excretion of nicotinamide in rats was below the detection limit. The level of nicotinamide excreted in unchanged form is low, the predominant metabolites being N^1-methylnicotinamide and 2-py.

We believe that this method will be useful in the field of niacin studies.

REFERENCES

1 E.E. Snell and L.D. Wright, J. Biol. Chem., 139 (1941) 675.
2 M. Weiner and J.V. Eys, Clinical Pharmacology, Vol. 1, Nicotinic Acid: Nutrient-Cofactor-Drug, Marcel Dekker, New York, 1983, p. 273.
3 M. Weiner and J.V. Eys, Clinical Pharmacology, Vol. 1, Nicotinic Acid: Nutrient-Cofactor-Drug, Marcel Dekker, New York, 1983, p. 253.
4 P.S. Schein, D.A. Cooney and M.L. Vernon, Cancer Res., 27 (1967) 2324.
5 K. Shibata and K. Tanaka, Bull. Teikoku-Gakuen, 12 (1986) 1.
6 N.O. Kaplan, A. Goldin, S.R. Humphrey, M.M. Ciotti and F.E. Stolzenbach, J. Biol. Chem., 219 (1956) 287.
7 H. Goto, D. Nomura, C. Hasegawa and Y. Sakamoto, Abstracts of the 10th Meeting of the Japanese Study Group of Tryptophan, Kyoto, Dec. 13, 1986, p. 20 (in Japanese).
8 K. Shibata, T. Hayakawa and K. Iwai, Agric. Biol. Chem., 50 (1986) 3037.
9 Y. Kitada, M. Inoue, K. Tamae, M. Imou, A. Hasuike, M. Sakai and K. Tanigawa, Eiyo to Shokuryo (in Japanese), 35 (1978) 121.
10 N. Hengen, V. Seiberth and M. Hengen, Clin. Chem., 24 (1978) 1740.
11 L.S. Trugo, R. Macrae and N.M.F. Trugo, J. Micronutr. Anal., 1 (1985) 55.
12 T.A. Tyler and R.R. Shrago, J. Liq. Chromatogr., 3 (1980) 269.
13 J.X. De Vries, W. Günthert and R. Ding, J. Chromatogr., 221 (1980) 161.
14 R.W. McKee, Y.A. Kang-Lee, M. Panaqua and M.E. Swendseid, J. Chromatogr., 230 (1982) 309.
15 K. Shibata, T. Kawada and K. Iwai, J. Chromatogr., 417 (1987) 173.
16 K. Shibaba, Vitamins (Japan), 61 (1987) 39.