Fluorometric Assay of Rat Tissue *N*-Methyltransferases with Nicotinamide and Four Isomeric Methylnicotinamides

Akira SANO,* Naoto ENDO and Shoji TAKITANI

Faculty of Pharmaceutical Sciences, Science University of Tokyo, 12, Ichigaya-funagawara-machi, Shinjuku-ku, Tokyo 162, Japan. *Received July 29, 1991*

Nicotinamide (NA) and its four isomeric methyl analogs [2-, 4-, 5- and 6-methylnicotinamides (MNs)] were tested as substrates for nicotinamide *N*-methyltransferase (NNMT) and amine *N*-methyltransferase (ANMT) using rat liver, kidney, spleen and brain 9000 × *g* supernatant fluids as model enzyme preparations. The *N*-methylated products were determined fluorometrically by their reaction with acetophenone or 4-methoxybenzaldehyde to form fluorescent 2,7-naphthyridine derivatives, and the lower limits of the determination were 8—30 pmol/100 μl. *N*-Methyltransferase activities were detected in the liver with NA, 4-MN and 5-MN, and in the brain and spleen with 4-MN. On this basis, 5-MN is considered to be a selective substrate for NNMT in addition to NA, which is a known methyl acceptor for this enzyme. Although 4-MN appears to serve as a methyl acceptor for both ANMT and NNMT, it seems to be essentially a selective substrate for brain ANMT because of the absence of NNMT in brain. The fluorometric methods used here are also very useful because of their simplicity, sensitivity and selectivity.

Keywords amine *N*-methyltransferase; nicotinamide *N*-methyltransferase; 4-methylnicotinamide; 5-methylnicotinamide; nicotinamide; fluorometry; rat tissue; brain; liver

In mammalian tissues, several *N*-methyltransferases have been discovered and play an important role in the biotransformation of various compounds of both endogenous and exogenous origin.[1] Among them, nicotinamide *N*-methyltransferase (NNMT, EC 2.1.1.1) and amine *N*-methyltransferase (ANMT) catalyze the *N*-methylation of a wide variety of azaheterocycles in the presence of cofactor *S*-adenosylmethionine (AdoMet).[2,3] However, there appear to be some interesting differences in their tissue distribution and substrate specificity. NNMT is known to present in the liver and kidney of human,[4] rat[5-9] and pig,[2,9] whereas ANMT has been found in the brain[10,11] of human, monkey, rat and rabbit and in the liver[3] of rabbit. Although some pyridine derivatives such as pyridine, quinoline and 1,2,3,4-tetrahydroisoquinoline are a substrate common to the two enzymes,[2,3] nicotinamide (NA), which is the best substrate for NNMT,[2] is not a substrate for ANMT.[3] In contrast to NA, 4-phenylpyridine is a good substrate for ANMT[3] but not for NNMT.[2]

Recently, we reported the applicability of 4-methylnicotinamide (4-MN) as a new substrate for the assay of rat liver NNMT activity.[12] However, the assay of *N*-methyltransferases in various rat tissues using other MN isomers as substrates has not been examined so far. In this study, simple, sensitive and selective assay systems for each of the two enzymes, especially ANMT, were sought by evaluating the methyl accepting ability of NA and all isomeric MNs using rat liver, kidney, spleen and brain 9000 × ***g*** supernatant fluids as model enzyme preparations. The use of such NA derivatives also offers the advantage of allowing convenient determination of *N*-methylated products by the two fluorometric methods, A and B shown in Fig. 1. Method A, which is applicable to 1,2-, 1,5- and 1,6-dimethylnicotinamides (DMNs) as well as 1-methylnicotinamide (MNA), is a modified fluorometric method for determining MNA[13] utilized in NNMT assay with NA.[2,5,6,8] It is

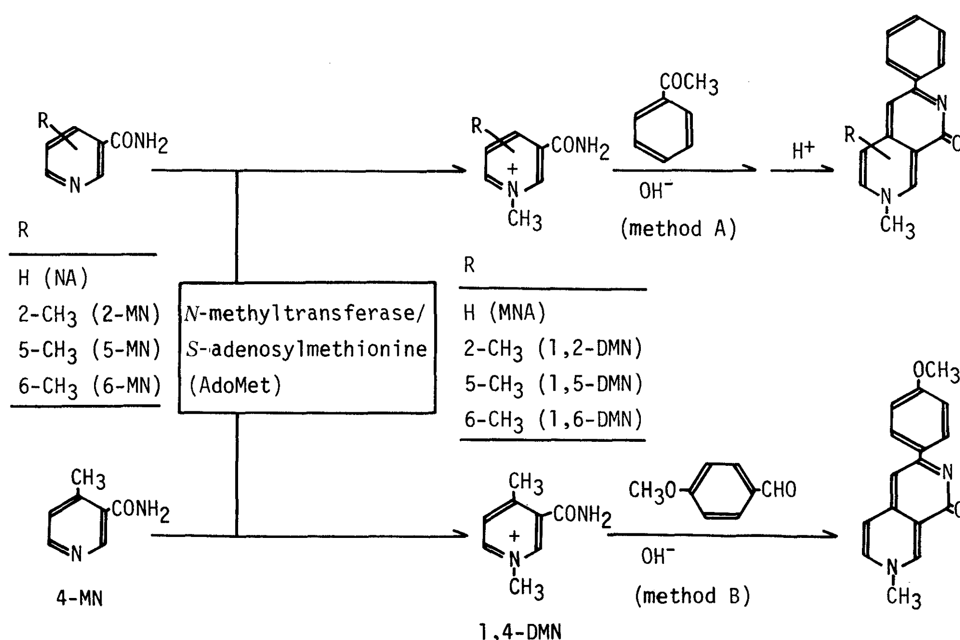

Fig. 1. Principle of *N*-Methyltransferase Assay Systems

© 1992 Pharmaceutical Society of Japan

NII-Electronic Library Service

based on the formation of fluorescent 2,7-naphthyridine derivatives by their reaction with acetophenone.[14] Method B[12] has recently been developed for the determination of 1,4-DMN, which is also converted to a similar naphthyridine derivative by the reaction with 4-methoxybenzaldehyde.

Experimental

Apparatus A Shimadzu RF-510 spectrofluorometer equipped with a xenon lamp was used with a 0.4 × 1 cm quartz cell at room temperature; spectral bandwidths of 5 and 10 nm were used in excitation and emission, respectively. All fluorescence excitation and emission spectra were uncorrected. A high-performance liquid chromatography (HPLC) system consisted of a Shimadzu LC-6A pump, a Rheodyne 7125 injector with 20-μl sample loop and a 10-μm SSC-ODS-262 column (100 × 6 mm i.d., Senshu Scientific Co.) with a guard column (LiChroCART RP-18, E. Merck). The mobile phase was acetonitrile–water–formic acid (20 : 80 : 2, v/v/v). The column temperature was ambient and the flow rate was 1.0 ml/min. Detection was carried out with a Hitachi F-1000 fluorescence spectrophotometer equipped with a flow-cell (12 μl) and a xenon lamp. All melting points were determined with a Mitamura Riken micro melting point apparatus and are uncorrected. Mass spectra (MS) were obtained on a Hitachi M-80 spectrometer in the electron impact mode.

Chemicals AdoMet *p*-toluenesulfonate and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. Formic acid (98—100%) was obtained from Nacalai Tesque. NA and MNA were purchased from Tokyo Kasei Co. and 6-MN from Aldrich Chemical Co. Water used was purified on a Milli RO-Milli Q system (Millipore Ltd.). 2-[15] and 4-[16] MNs were synthesized according to the published methods. 5-MN was obtained through the reaction of ethyl 5-methylnicotinate[17] with ammonia water.[15] mp 162—163 °C. MS *m/z*: 136 (M^+). Authentic 1,4-DMN was synthesized as a chloride salt from 4-MN *via* its methiodide according to the published method.[18] 1,2-, 1,5- and 1,6-DMNs were also synthesized as chlorides in the same manner. Analytical data were as follows: 1,2-DMN: mp 268—269 °C (dec.). MS *m/z*: 150 ($M^+ - HCl$). *Anal.* Calcd for $C_8H_{11}ClN_2O$: C, 51.48; H, 5.94; Cl, 19.00; N, 15.01. Found: C, 51.30; H, 5.88; Cl, 18.94; N, 15.15. 1,5-DMN: mp 233—235 °C (dec.). MS *m/z*: 151 ($M^+ - Cl$). *Anal.* Calcd for $C_8H_{11}ClN_2O$: C, 51.48; H, 5.94; Cl, 19.00; N, 15.01. Found: C, 51.23; H, 5.89; Cl, 18.93; N, 15.11. 1,6-DMN: mp 233—234 °C (dec.). MS *m/z*: 150 ($M^+ - HCl$). *Anal.* Calcd for $C_8H_{11}ClN_2O$: C, 51.48; H, 5.94; Cl, 19.00; N, 15.01. Found: C, 50.60; H, 5.86; Cl, 18.88; N, 14.94. All other chemicals used were of analytical reagent grade.

Tissue Preparation Male Sprague-Dawley rats (230—260 g) were killed by cervical dislocation. The livers, kidneys, spleens and brains were removed, washed with cold 0.9% (w/v) sodium chloride and homogenized 1 : 4 (w/v) in cold 5 mM potassium phosphate buffer (pH 7.5) with a Teflon–glass homogenizer. The homogenates were centrifuged at 9000 × ***g*** for 20 min at 4 °C. The supernatant was kept in small aliquots at −20 °C until assayed. Protein concentration was determined by the method of Lowry *et al.*[19] using BSA as the standard and adjusted to 0.5 mg/ml (liver), 5 mg/ml (brain) or 10 mg/ml (kidney and spleen).

Assay Conditions for Measurement of *N*-Methyltransferase Activity The reaction mixture consisted of 25 μl of 2 mM dithiothreitol, 25 μl of 0.8 M Tris–HCl buffer (pH 8.6), 50 μl of 8 mM substrate (NA or 2-, 4-, 5- or 6-MN), 100 μl of 0.4 mM AdoMet in 0.1 mM sulfuric acid and 200 μl of enzyme preparation. The mixture was incubated at 37 °C for 60 min. When 4-MN was used as the substrate, the reaction was terminated by heating in a boiling water bath for 2 min. In the case of NA, and 2-, 5- and 6-MNs, the reaction was terminated by addition of 1.6 ml of ethanol. To prepare the blanks, the same procedures were used except that the reaction was stopped immediately without incubation. The mixtures were centrifuged at 1000 × ***g*** for 10 min. A 100-μl aliquot of the supernatant was submitted to fluorometric determination.

Fluorometric Determination of MNA and 1,2-, 1,5- and 1,6-DMNs (Method A) Sample solution (100 μl) was taken in a 1.5-ml glass-stoppered test tube which was placed in an ice-water bath, and 50 μl of 20% (v/v) acetophenone in ethanol and 50 μl of 5 M potassium hydroxide in 50% (v/v) aqueous ethanol were added. After 30 min at 0 °C, the mixture was acidified with 1 ml of formic acid, heated in a boiling water bath for 5 min and then cooled to room temperature. The fluorescence intensity was measured with excitation at 375 nm and emission at 430 nm.

Fluorometric Determination of 1,4-DMN (Method B) The procedure used was the same as that reported previously.[12]

Identification of Enzyme Reaction Products by HPLC When NA or 5-MN was used as the substrate, the final reaction mixture obtained by method A was diluted with an equal volume of acetonitrile, and a 20-μl aliquot of the mixture was injected into the HPLC system in which fluorescence was monitored with excitation at 375 nm and emission at 430 nm. When 4-MN was tested, the final reaction mixture obtained by method B was acidified by addition of 500 μl each of formic acid and acetonitrile, and then a 20-μl aliquot of the mixture was injected into the HPLC system. In this case, fluorescence was monitored with excitation at 395 nm and emission at 459 nm which were the optimal wavelengths for the fluorophore in acidic medium.

Results

Fluorometric Determination of MNA and DMNs As described above, the fluorescence reaction of MNA with acetophenone was considered to proceed as shown in Fig. 1 (method A),[14] and consequently this reaction was expected to be applicable to 1,2-, 1,5- and 1,6-DMNs because of the lack of substituent at position 4 on the pyridine ring. However, when the previous method[13,14] was applied directly, 1,2-DMN gave extremely low fluorescence intensity as compared with those of MNA, 1,5-DMN and 1,6-DMN. Thus various reaction conditions were re-examined and some modifications were necessary as follows: a higher concentration of potassium hydroxide (5 M) and prolonged reaction time (0 °C for 30 min) in the condensation reaction of the analytes with acetophenone; a higher concentration of formic acid (100%) in the acid treatment. Although the corresponding conditions used in the previous method,[14] 2 M potassium hydroxide, reaction at 0 °C for 20 min and 60% formic acid, sufficed for determining MNA, 1,5-DMN and 1,6-DMN, those were also treated by the modified procedure.

The fluorescence characteristics and relative fluorescence intensities of MNA, and 1,2-, 1,5- and 1,6-DMNs obtained by method A are summarized in Table I. They showed blue fluorescence having similar spectra for the maximal wavelengths for fluorescence excitation and emission, and the formation of analogous 2,7-naphthyridine derivatives was suggested. Their fluorescence intensities were of almost the same magnitude except that 1,2-DMN gave relatively low fluorescence intensity. The fluorescences were stable for at least 3 h at room temperature. Linear calibration curves were obtained over the concentration ranges given in Table I. As little as 8—30 pmol of these could be determined and relative standard deviations were satisfactory. A 10-fold molar ratio of the following substances did not affect the determination of 1 nmol/100 μl of MNA or the DMNs: alanine, aspartic acid, cysteine, histidine, methionine, phenylalanine, proline, tryptophan, tyrosine, glutathione, α-ketoglutaric acid, pyruvic acid, glucose, fructose, sac-

Table I. Analytical Data for MNA, and 1,2-, 1,5- and 1,6-DMNs Obtained by Method A

Compound	λ_{max} (nm) Ex	λ_{max} (nm) Em	RFI[a]	Determination range (nmol/100 μl)	Relative standard deviation (%)[b]
MNA	374	430	100	0.01 —1.0	3.0 (0.05), 1.7 (1.0)
1,2-DMN	372	423	32	0.03 —3.0	2.1 (0.2), 2.5 (2.0)
1,5-DMN	376	427	133	0.008—1.0	2.9 (0.05), 1.1 (1.0)
1,6-DMN	380	435	127	0.008—1.0	1.5 (0.05), 1.0 (1.0)

a) Relative fluorescence intensity at optimal wavelengths; MNA is arbitrarily taken as 100. *b*) $n = 10$; numbers in parentheses are the relevant concentrations in nmol/100 μl.

NII-Electronic Library Service

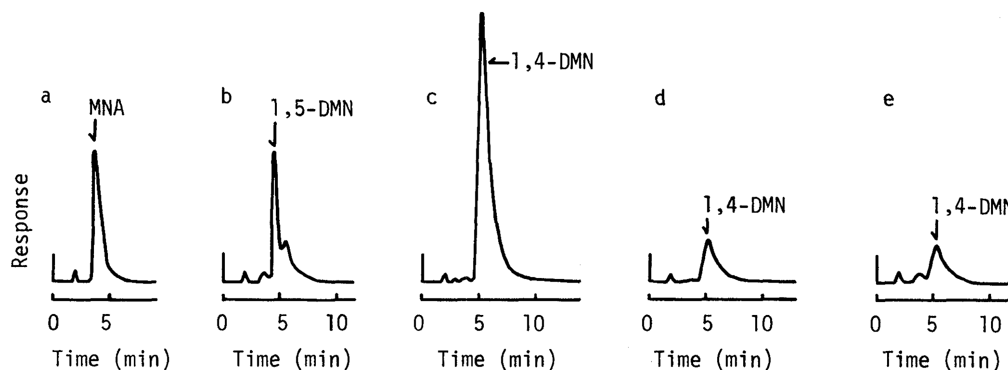

Fig. 2. HPLC Chromatograms of Enzyme Reaction Mixtures after Fluorescence Derivatization

a, MNA formed from NA by liver homogenate; b, 1,5-DMN formed from 5-MN by liver homogenate; c, d and e, 1,4-DMN formed from 4-MN by liver (c), brain (d) and spleen (e) homogenates.

TABLE II. Rat Tissue *N*-Methyltransferase Activity

Tissue	Activity (pmol/mg protein/min)[a]				
	NA	2-MN	4-MN	5-MN	6-MN
Liver	86.5±4.9	ND	211±14	90.3±20.8	ND
Kidney	ND	ND	ND	ND	ND
Spleen	ND	ND	3.6±0.1	ND	ND
Brain	ND	ND	9.8±0.9	ND	ND

a) Mean±standard deviation ($n=3$). ND: not detected.

charose, uric acid, nicotinic acid, dithiothreitol, AdoMet, NA, 2-MN, 5-MN and 6-MN.

1,4-DMN did not show any fluorescence by method A, but by method B, it could be converted to a similar naphthyridine derivative (Fig. 1) and determined over the concentration range of 20—2000 pmol/100 μl with relative standard deviations of below 2%.[12]

These 2,7-naphthyridine derivatives formed by methods A and B could be detected after separation by reversed-phase HPLC with a mobile phase of aqueous organic solvents containing formic acid[20] (see Experimental section). This approach was employed for the confirmation of the enzyme reaction products as described below.

Rat Tissue *N*-Methyltransferase Activity The enzyme assay conditions used were essentially the same as those reported by Clark *et al.*,[6] except that various NA derivatives were tested as methyl acceptor substrates. When 2—5 nmol of the authentic *N*-methylated products were added to the incubation mixtures of the blanks, more than 80% recoveries were obtained. As shown in Table II, *N*-methylating activities were detected in the liver with NA, 4-MN and 5-MN, and in the brain and spleen with 4-MN. In the other cases, *N*-methylated products were not detected even if higher concentrations of the substrates, AdoMet or protein were used. The identification of the *N*-methylation products was verified by HPLC after the fluorescence reaction of the enzyme reaction mixture (Fig. 2). The methylated products from NA and 5-MN, shown for the liver preparation, yielded fluorescence peaks at 3.8 min (Fig. 2a) and 4.5 min (Fig. 2b), respectively, which were identical to those of the reaction products resulting from MNA and 1,5-DMN, respectively. The reaction product obtained from 4-MN with the liver, brain and spleen preparations gave a fluorescence peak at 5.3 min, which was also identical to that of the reaction product resulting from authentic 1,4-DMN (Fig. 2c, d, e). Other minor peaks shown in Fig. 2 were negligible because these also appeared in chromatograms resulting from the blanks of enzyme assay.

Discussion

The proposed fluorometric methods permitted the determination of pmol amounts of the *N*-methylated derivatives and the recoveries of those added to the enzyme reaction mixtures were satisfactory. Therefore, the fact that 2- and 6-MNs did not act as methyl acceptors in all of the tissues seemed to be due to the steric hindrance caused by the methyl group at position 2 or 6 on their pyridine ring.

Liver *N*-methyltransferase was assayed using NA and 5-MN, whose actions as a methyl acceptor were very similar. NNMT activity has been demonstrated in the liver and kidney of rat and NA is known to be the best substrate for NNMT[5,6,8,9] but not ANMT.[3,5] Therefore, 5-MN is also considered to be a selective substrate for NNMT. MNA is a common biological component[13] and is sometimes used as an inhibitor of NNMT.[4,6,9] In such instances, the combined use of the assay system with 5-MN and a suitable separation technique, preferably HPLC, is probably capable of determining the *N*-methylated product without interference from the MNA.

In this study, however, kidney NNMT activity was not detected toward any of the substrates tested and was also not observed in the microsomal or cytosolic fractions. The reason is presumably lower enzyme activity in the kidney, because the activities of NNMT in rat kidney are reported to vary from about 2%[9] to 27%[8] of that in the liver, and consequently detectable *N*-methylation products may not be formed in the enzyme reaction.

4-MN is considered to serve as a methyl acceptor for both NNMT and ANMT because *N*-methylating activities were detected not only in the liver but also in the brain and spleen. The applicability of 4-MN as the substrate for rat liver NNMT has been described.[12] On the other hand, the enzyme activity detected in the brain with 4-MN is higher than those reported for the brains[10] of human, monkey, rat and rabbit toward other substrates such as 4-phenylpyridine and is comparable to that reported for human brain with 1,2,3,4-tetrahydroisoquinoline.[11] Based on the above findings together with the fact that NNMT

NII-Electronic Library Service

is not present in brain,[5,8] it may be concluded that 4-MN acts as a selective substrate for brain ANMT and is a useful substrate for the assay of the ANMT, though the reason 4-MN alone exhibits a methyl accepting ability among the NA derivatives tested is unknown.

The spleen homogenate appears to catalyze the *N*-methylation of 4-MN similarly to the brain and therefore it is believed that there is some ANMT-like enzyme, though not NNMT-like enzyme. Details are not clear at present, however.

In the present study, 4- and 5-MNs were discovered as new substrates for brain ANMT and liver NNMT, respectively. Although methyl conjugation is usually considered to be a detoxication process,[1] *N*-methylation of azaheterocyclic compounds sometimes increases their chemical reactivity and toxicity. For example, potent parkinsonogenic species such as 1-methyl-4-phenylpyridinium[10] and 1-methyl-1,2,3,4-tetrahydroisoquinoline[2,11] are known to be produced from their precursor amines by the action of ANMT and/or NNMT. Therefore, in order to elucidate the biotransformation of such amines, the measurements of *N*-methyltransferase activities, particularly brain ANMT, are of great interest. Further studies on the application of the proposed assay systems and the optimization of enzyme reaction conditions for the assay of brain ANMT with 4-MN are in progress.

Acknowledgement This work was supported in part by the Research Aid of Inoue Foundation for Science.

References

1) R. Weinshilboum, *Pharmac. Ther.*, **43**, 77 (1989).
2) T. A. Alston and R. H. Abeles, *Arch. Biochem. Biophys.*, **260**, 601 (1988).
3) S. S. Ansher and W. B. Jakoby, *J. Biol. Chem.*, **261**, 3996 (1986).
4) J. Rini, C. Szumlanski, R. Guerciolini and R. M. Weinshilboum, *Clin. Chim. Acta*, **186**, 359 (1989).
5) G. L. Cantoni, *J. Biol. Chem.*, **189**, 203 (1951).
6) B. R. Clark, J. T. Murai, A. Pomeranz, P. A. Mills, R. M. Halpern and R. A. Smith, *Cancer Res.*, **35**, 1727 (1975).
7) J. Hoshino, U. Kühne and H. Kröger, *Biochim. Biophys. Acta*, **719**, 518 (1982).
8) K. Shibata, *Agric. Biol. Chem.*, **50**, 1489 (1986).
9) H. Taguchi, H. Muto, K. Inamori, K. Okumura and Y. Shimabayashi, *Bitamin*, **62**, 559 (1988).
10) S. S. Ansher, J. L. Cadet, W. B. Jakoby and J. K. Baker, *Biochem. Pharmacol.*, **35**, 3359 (1986).
11) M. Naoi, S. Matsuura, T. Takahashi and T. Nagatsu, *Biochem. Biophys. Res. Commun.*, **161**, 1213 (1989).
12) A. Sano, N. Takimoto and S. Takitani, *Chem. Pharm. Bull.*, **37**, 3330 (1989).
13) B. R. Clark, R. M. Halpern and R. A. Smith, *Anal. Biochem.*, **68**, 54 (1975).
14) A. Sano, Y. Asabe, M. Suzuki and S. Takitani, *Bunseki Kagaku*, **32**, E93 (1983).
15) A. Dornow, *Chem. Ber.*, **73**, 78 (1940).
16) J. M. Bobbitt and D. A. Scola, *J. Org. Chem.*, **25**, 560 (1960).
17) D. S. Noyce and J. A. Virgilio, *J. Org. Chem.*, **38**, 2660 (1973).
18) A. Sano, M. Ogawa and S. Takitani, *Anal. Sci.*, **3**, 359 (1987).
19) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
20) Presented at the 102nd Annual Meeting of the Pharmaceutical Society of Japan, Osaka, April 1982, Abstract of Papers, p. 315.

NII-Electronic Library Service