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# HPLC–UV method for measuring nicotinamide *N*-methyltransferase activity in biological samples: Evidence for substrate inhibition kinetics

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ABSTRACT

Nicotinamide *N*-methyltransferase (NNMT, E.C. 2.1.1.1) *N*-methylates nicotinamide to produce 1-methylnicotinamide. Enhanced NNMT activity is a feature of many types of cancer, and has been linked to processes such as tumour metastasis, resistance to radiotherapy and tumour drug resistance. As such, inhibition of NNMT activity is a promising therapeutic target for cancer therapy. To screen for NNMT inhibitors, there is a need for a standardised, rapid and cost-effective NNMT assay. Here, we describe a cell-free assay coupled with ion-pairing reverse-phase HPLC–UV detection of 1-methylnicotinamide which requires minimal sample manipulation, is linear over 2.5 orders of magnitude with limits of detection and quantification of 0.05 and 0.15 nmol 1-methylnicotinamide/100 μL injection respectively. The assay was sufficiently sensitive to measure basal hepatic 1-methylnicotinamide concentration and NNMT activity in mouse, rabbit and human liver. 1-Methylnicotinamide concentration and the NNMT kinetic parameters specific activity, $V_{max}$ and $K_m$ all demonstrated species differences. NNMT also demonstrated substrate inhibition kinetics in all three species, which again was species-specific in term of calculated $K_i$. This assay demonstrates improved sensitivity over other previously published methods whilst lacking many of their drawbacks such as extensive sample preparation, use of non-physiological substrates and radioisotopic labelling.



## 1. Introduction

Nicotinamide *N*-methyltransferase (NNMT, E.C. 2.1.1.1) *N*-methylates nicotinamide to 1-methylnicotinamide (MeN) using *S*-adenosylmethionine (SAM) as cofactor [1]. Enhanced expression of NNMT has been linked with a variety of diseases such as Parkinson's disease [2,3], hepatic cirrhosis [4] and chronic obstructive pulmonary disease [5]. Such enhanced expression has been proposed to serve as a protective response to the underlying pathogeneses in these diseases [4–6]. NNMT expression is also significantly enhanced in many cancers [7–14], which in contrast is reported to be associated with several fundamental processes of tumour progression such as metastasis and proliferation [15–17]. As such, NNMT has been suggested as both a possible diagnostic biomarker [18] and prognostic marker [19] for cancer therapy.

Enhanced NNMT expression may play a role in tumour resistance to therapy by reducing intracellular concentrations of the radiation-sensitiser nicotinamide [9,20]. We have recently shown that the expression of NNMT in SH-SY5Y human neuroblastoma cells, which have no endogenous NNMT expression, increased their resistance to cytotoxic challenge [6], thus suggesting that NNMT may also play a role in acquired drug resistance.

In light of this accumulated evidence, NNMT is a strong candidate as a therapeutic target for the treatment of cancer. Small molecule NNMT inhibitors have the potential for increasing the efficacy of cancer treatment. The vast majority of methyltransferase inhibitors such as *S*-adenosylethionine are analogues of SAM, the consequence of which, due to the ubiquitous use of SAM as the methyl donor in all methyltransferase reactions, is that current NNMT inhibitors demonstrate an inherent non-selectivity [21,22]. To obtain the desired selectivity, inhibitors based upon the structure of nicotinamide are required. In order to screen potential NNMT inhibitors, there is a need for a reliable, low-cost assay for NNMT activity. A variety of NNMT activity assays have been reported, however the majority of these methods rely on pre-column derivatisation [23–25], sample drying [4,26], the use of

*Abbreviations:* 6-CN, 6-chloronicotinamide; AO, aldehyde oxidase; DMSO, dimethylsulphoxide; HPLC–UV, high-performance liquid chromatography-ultraviolet detection; LOD, limit of detection; LOQ, limit of quantification; MeN, 1-methylnicotinamide; NNMT, nicotinamide *N*-methyltransferase; PBS, phosphate-buffered saline; RSD, relative standard deviation; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine.

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radioisotopes [27] or substrate mimics [28]. Here, we describe a simple assay for NNMT activity which uses HPLC–UV detection of MeN which requires no sample manipulation, and apply it to the detection of NNMT activity in mouse, rabbit and human liver. We also provide evidence that NNMT demonstrates substrate inhibition kinetics in all three species.

## 2. Materials and methods

Unless otherwise stated, all chemicals were obtained of the highest purity from Sigma, Poole, UK.

### 2.1. UV spectral scans of 1-methylnicotinamide and 6-chloronicotinamide

Absorbance scans for 0.1 mM MeN, nicotinamide and 6-chloronicotinamide (6-CN, internal standard) in the wavelength range of 190–800 nm were produced using a Jasco UV/Visible v4.55 spectrophotometer (Great Dunmow, Essex, UK).

### 2.2. HPLC–UV detection of 1-methylnicotinamide, 6-chloronicotinamide and nicotinamide

#### 2.2.1. Instrumentation and chromatographic conditions

The HPLC–UV method for the separation and detection of nicotinamide and related metabolites as described by Erb et al. [26] was used as the basis for the detection of MeN. HPLC–UV was performed using a Thermo Separation Products SpectraSYSTEM incorporating an AS3000 autosampler, SCM1000 degasser and P4000 quaternary gradient pump running an isocratic gradient comprising 7 mM 1-heptane sulphonate, 5 mM potassium dihydrogen orthophosphate (BDH Chemicals Ltd., Poole, UK), 0.1 mM ascorbic acid (pH 3.0) and 20 mM trimethylamine. Peaks corresponding to MeN, nicotinamide and 6-CN were detected using a wavelength of 265 nm, identified *via* UV spectral scan described above, using a LabChrom L-7400 UV detector. A Hypersil ODS C18 (250 mm × 4.6 mm, particle size 5 μm, pore diameter 80 Å) column maintained at ambient temperature was used for chromatographic separation. Columns were protected with a C18 (4.0 mm × 2.0 mm) guard cartridge (Phenomenex Ltd., Cheshire, UK). Injection volume was 100 μL with a flow rate of 2.0 mL/min and a 40 min cycle time per sample.

#### 2.2.2. Calibration curves

MeN and 6-CN calibration curves were prepared in mobile phase as 100 nmol/μL stocks. To demonstrate the linearity of the HPLC–UV detection of MeN and 6-CN, a 0.15–30 nmol/100 μL 6-point standard curve for MeN and a 0.15–6 nmol/100 μL 6-point standard curve for 6-CN were produced. Using the concentration of MeN in mouse liver reported by Erb et al. [26], we calculated that the expected rabbit liver MeN concentration, at a homogenate protein concentration of 10–20 mg/mL, to be approximately 0.2–0.8 nmol/100 μL injection. Thus a further 0.05–1.5 nmol/100 μL 7-point calibration curve was produced for MeN which was subsequently used to calculate the concentration of MeN for all biochemical analyses. Calibration curves were generated by plotting integrated peak area against amount of analyte per 100 μL injection volume. The limit of detection (LOD) for MeN was determined as the lowest concentration at which a peak area could be integrated using a signal:noise ratio of 12. From this value, the limit of quantification (LOQ) was calculated as 3 × LOD.

### 2.3. NNMT assay

#### 2.3.1. Liver homogenate preparation

Male New Zealand White rabbits and male C57BL/6 mice were obtained from the Biomedical Services Unit, King's College, London, UK. After acquisition from suppliers, animals were allowed to rest and acclimatise before use. Human liver cytosol, comprising pooled cytosol from 50 mixed-gender donors, was obtained from Invitrogen Life Technologies (Paisley, UK) at a concentration of 20 mg/mL.

Liver homogenate samples were prepared by homogenisation of tissue in phosphate-buffered saline (PBS) (Invitrogen Ltd., Paisley, UK) pH 7.2 at 4 °C, followed by centrifugation at 600 × *g* for 10 min to precipitate particulate material. Protein concentration was measured using the BioRad Dc protein assay (BioRad, Hemel Hempstead, UK) as per manufacturer's instructions. Homogenate (500 μL aliquots) were either used fresh or stored at −80 °C prior to analysis.

#### 2.3.2. NNMT activity assay

Determination of NNMT activity was based upon the method of Rini et al. [27]. Dimethylsulphoxide (DMSO) or 150 mM nicotinamide in DMSO (50 μL) was added to 500 μL liver homogenate (7.2 mg/500 μL average, range 6.55–11.5 mg/500 μL) in triplicate, followed by 200 μL of 150 μM SAM in PBS to initiate the reaction. The final concentration of nicotinamide and SAM in the reaction mixtures was 10 mM and 40 μM, respectively. The reaction was incubated in a shaking water bath at 37 °C for 20 min, after which the reaction was terminated by the addition of 75 μL of 10% trichloroacetic acid followed by vortexing for 5 s. At this point, 7.5 μL of a 1.11 mM 6-CN solution (corresponding to 1 nmol 6-CN/100 μL injection) was added prior to centrifugation at 16,000 × *g* for 15 min to precipitate protein. Chromatographic analysis of the extracted supernatant was performed as described in Section 2.2.1, with each triplicate injected in triplicate. Peak areas corresponding to MeN and 6-CN were integrated and the peak area for MeN was normalised using 6-CN. NNMT initial velocity was calculated, from which NNMT specific activity was calculated and expressed as nmoles MeN produced/hour/mg of protein ± S.D.

#### 2.3.3. Comparison of NNMT kinetic parameters in mouse, rabbit and human liver

Liver cytosol was incubated with 1, 3.3, 6.7, 10, 13.3, 16.7, 20, 25, 30 and 33.3 mM nicotinamide (final concentration), each in triplicate. NNMT initial velocities were calculated and expressed as specific activity and plotted using Eadie Hofstee plots ($V_i$/[*S*] *vs.* $V_i$). The kinetic constants $K_m$ and $V_{max}$ were calculated using non-linear regression analysis of $V_i$ *vs.* [*S*] [29,30] using GraphPad Prism (GraphPad, San Diego, USA), and expressed as mM and nmol MeN produced/hour/mg protein ± SEM respectively.

#### 2.3.4. Effect of incubation duration and dimethylsulphoxide upon NNMT activity

*2.3.4.1. Incubation duration.* The effect of increasing the length of reaction incubation upon MeN production and NNMT specific activity was investigated by terminating the reaction after 20, 40 and 60 min, each in triplicate. Results were calculated and NNMT activity expressed as both nmoles MeN produced/mg protein ± S.D. and specific activity.

*2.3.4.2. Effect of DMSO.* To investigate the effects of DMSO (final concentration 6.7%) upon the specific activity of NNMT, homogenate was incubated as described in Section 2.3.2, with nicotinamide prepared either in DMSO or in PBS. Specific activity was calculated and expressed as nmol MeN produced/hour/mg protein ± S.D.

#### 2.3.5. Effect of the inhibition of aldehyde oxidase-mediated degradation of 1-methylnicotinamide upon mouse liver kinetic parameters

The effect of inhibiting aldehyde oxidase (AO), responsible for the catabolism of MeN [31,32], upon measured NNMT activity was