Journal of Chromatography B, 921–922 (2013) 87–95

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

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

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ARTICLE INFO

Article history:
Received 10 September 2012
Accepted 9 January 2013
Available online 4 February 2013

Keywords:
Aldehyde oxidase
HPLC–UV
Nicotinamide *N*-methyltransferase
1-Methylnicotinamide
Substrate inhibition

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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http://dx.doi.org/10.1016/j.jchromb.2013.01.030

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

investigated by the addition of 5 μL of 7.05 mM imipramine in DMSO (final concentration 50 μM) [33], or DMSO alone, to the reaction prior to initiation with SAM. Results were calculated and NNMT specific activity was expressed as specific activity ± S.D. NNMT kinetic parameters were calculated as described in Section 2.3.3.

2.4. Data analysis

Correlation coefficient (R^2) was used to evaluate the linearity of all calibration curves. Percentage relative standard deviation (%RSD) was calculated for each standard concentration to assess the accuracy and precision of the method. Imprecision, expressed as %RSD, did not exceed 15% [34]. Statistical comparison of time course experiments was performed using GraphPad Prism (GraphPad, San Diego, USA) using one-way ANOVA with *post hoc* Tukey tests. Statistical analysis of the effect of [1] sample matrix upon MeN and 6-CN retention times and [2] the addition of the AO inhibitor imipramine upon NNMT activity and kinetic parameters was performed using a student's *t*-test with Welch correction. In all instances, $p < 0.05$ was taken as significant.

3. Results

3.1. Linearity range of detection for 1-methylnicotinamide and 6-chloronicotinamide and calculation of limits of detection and quantification

The first stage of the study was to identify retention times for nicotinamide, MeN and 6-CN and to determine the dynamic range of MeN detection for the instrumentation used. Retention times for nicotinamide, MeN and 6-CN were 2.65 ± 0.16, 12.22 ± 0.62 and 21.29 ± 1.09 min (average ± S.D.) respectively (Fig. 1). Previous studies had reported that HPLC–UV detection of MeN was linear within the concentration range of 0.3–30 nmol [26]. In accord with this, linear regression analysis showed that our HPLC–UV detection method was linear within the MeN range of 0.15–30 nmol/100 μL (Fig. 2A, $R^2 = 0.9992$, $p < 0.0001$). Calculations based upon previous studies using rat liver homogenate predicted that MeN concentrations would be in the range of 0.05–1.5 nmol/100 μL injection [26]. In order to measure MeN in such biological samples, we constructed a further 7-point calibration line in the range of 0.05–1.5 nmol which linear regression analysis showed was linear (Fig. 2A, $R^2 = 0.9994$, $p < 0.0001$). Therefore, LOD was determined to be 0.05 nmol/100 μL injection and LOQ to be 0.15 nmol/100 μL injection, with a %RSD of <15% for all calibration points. The detection of 6-CN was linear over the concentration range of 0.15–6 nmol/100 μL injection (Fig. 2B, $R^2 = 0.9998$, $p < 0.0001$).

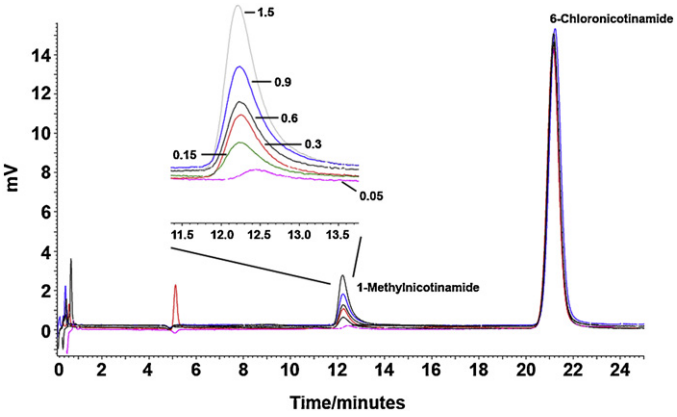

Fig. 1. Retention times of 1-methylnicotinamide and 6-chloronicotinamide. 1-Methylnicotinamide and 6-chloronicotinamide (internal standard) were separated using a Hypersil ODS C18 (250 mm × 4.6 mm, particle size 5 μm, pore diameter 80 Å) column maintained at ambient temperature, using an isocratic mobile phase comprising 7 mM 1-heptanesulphonate, 5 mM potassium dihydrogen phosphate, 0.1 mM ascorbic acid (pH 3.0) and 20 mM trimethylamine at a flow rate of 2 mL/min. Peaks corresponding to 1-methylnicotinamide and 6-chloronicotinamide were detected at a wavelength of 265 nm. The chromatogram is a combination of typical traces produced using various amounts of 1-methylnicotinamide/100 μL as indicated in the expanded inset. Peaks corresponding to 1-methylnicotinamide and the internal standard 6-chloronicotinamide showed excellent baseline resolution with no interference between peaks.

3.2. Chromatographic analysis and quantification of 1-methylnicotinamide in mouse, rabbit and human liver homogenate

Acceptable baseline resolution of peaks corresponding to MeN and 6-CN was observed when using rabbit, mouse and human liver homogenates, and no interference between the analyte of interest and endogenous matrix components, in particular nicotinamide, was observed (Fig. 3). The retention times for nicotinamide, MeN and 6-CN were 4.42 ± 0.14, 11.73 ± 0.18 and 18.16 ± 0.14 min (average ± S.D.) respectively. Although the shift in nicotinamide and 6-CN retention times were significant ($p = 0.0004$ and $p = 0.0009$ respectively), the shift in MeN retention time was not ($p = 0.1136$). Spiking of liver homogenates with 0.6 and 6 nmol MeN/100 μL injection confirmed the retention time of MeN and the lack of interaction with matrix components (data not shown). MeN concentration in mouse, rabbit and human liver was calculated to be 0.0255 ± 0.002, 0.456 ± 0.018 and 0.781 ± 0.043 nmol/mg protein respectively. The intra-assay variation, across all three species, was

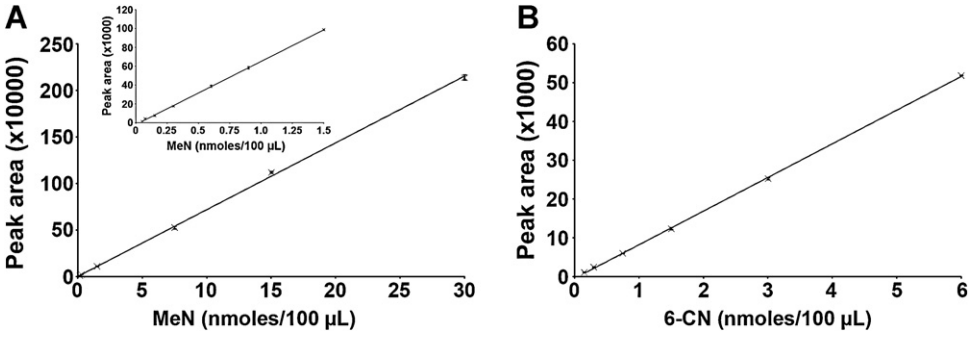

Fig. 2. Calibration curves for 1-methylnicotinamide and 6-chloronicotinamide. (A) 1-Methylnicotinamide. A 0.15–30 nmol/100 μL 6-point calibration curve was produced to demonstrate the linearity of 1-methylnicotinamide detection. The detection of 1-methylnicotimamide was linear over the concentration range of 0.15–30 nmol/100 μL injection. Inset: a 0.05–1.5 nmol/100 μL 7-point calibration curve was produced for the quantification of 1-methylnicotinamide in biological samples. The limit of detection was 0.05 nmol/100 μL and the limit of quantification was 0.15 nmol/100 μL. (B) 6-Chloronicotinamide. A 0.15–6 nmol/100 μL 6-point calibration curve was produced for the quantification of the internal standard 6-chloronicotinamide in biological samples. The detection of 6-chloronicotinamide was linear over the concentration range of 0.15–6 nmol/100 μL. 1 nmol/100 μL 6-chloronicotinamide was used as the internal standard for all biochemical assays.

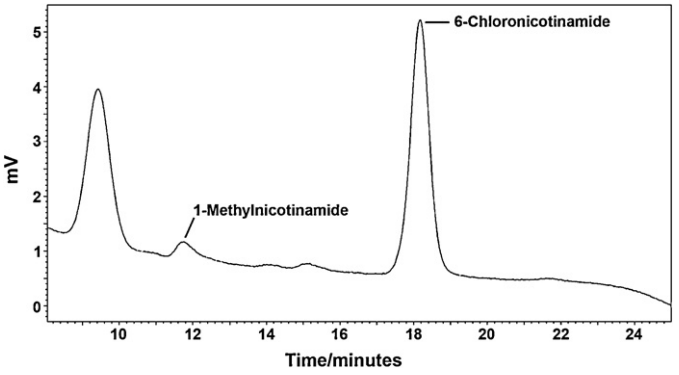

Fig. 3. Detection of endogenous 1-methylnicotinamide and 6-chloronicotinamide internal standard in biological matrix. Liver homogenate was spiked with 1 nmol 6-chloronicotinamide/100 μL (final concentration) followed by detection of endogenous 1-methylnicotinamide as described in legend of Fig. 1. Both 1-methylnicotinamide and 6-chloronicotinamide were resolved with excellent baseline separation and no interference with endogenous components or nicotinamide (not shown).

5.7% (median 5.5%, range 3.9–7.8%, $n = 4$). The inter-assay variation, using mouse liver, was 10.6% ($n = 4$).

3.3. NNMT demonstrated both substrate inhibition kinetics and species differences in specific activity and enzyme kinetic constants

Examination of the Eadie Hofstee plots for NNMT activity in all three species investigated demonstrated a divergence from the linear profile expected (Fig. 4), which is diagnostic for the presence of substrate inhibition [30]. To confirm this, the kinetic data for all three species were modelled using the standard Michaelis–Menten kinetic equation:

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]} \quad \text{(Michaelis-Menten kinetics)} \qquad (1)$$

This resulted in poor kinetic fits for both human and mouse liver NNMT with no fit possible for rabbit liver NNMT (Fig. 4). The poor fit of the curves resulted in the underestimation of both K_m and V_{max} (Table 1). Consequently, the kinetic data were modelled using a number of substrate inhibition kinetic equations in order to obtain a best kinetic fit [29,30]:

$$v = \frac{V_{max} \cdot [S]}{K_m + [S] \cdot (1 + [S]/\mathrm{Ki})} \quad \text{(substrate inhibition kinetics)} \qquad (2)$$

$$v = \frac{V_{max} \cdot (([S]/K_m) + ((\beta[S]^2)/\alpha K_m K_i))}{1 + [S]/K_m + [S]/K_i + [S]^2/\alpha K_m K_i}$$

(two-site model kinetics, random order binding#1) (3)

$$v = \frac{V_{max} \cdot ([S]/K_m + (\beta[S]^2)/(\alpha {K_m}^2))}{1 + 2[S]/K_m + [S]^2/\alpha {K_m}^2}$$

(two site model kinetics, random order binding#2) (4)

Using such a paradigm, good kinetic modelling of the data was obtained in all three species (Fig. 4), from which estimates of V_{max}, K_m and K_i were obtained (Table 1). Kinetic parameters varied significantly amongst the three species used, with human and mouse NNMT demonstrating the highest and lowest V_{max} respectively. In contrast, human and mouse NNMT also demonstrated the lowest and highest affinities for nicotinamide as demonstrated by a significantly higher K_m value for human compared to mouse NNMT.

For comparison, Michaelis–Menten modelling using Eq. (1) was also undertaken using data from which the portions of the inhibited data were truncated (Fig. 4); these data represent kinetics if NNMT were not to undergo substrate inhibition [29]. Predicted kinetics was then plotted and V_{max} and K_m values were obtained (Table 1). These kinetic values were significantly lower than those obtained using Eqs. (2)–(4), yet they also followed the same trend observed for those obtained using substrate modelling kinetics, *i.e.* human NNMT demonstrated the highest V_{max} and K_m.

3.4. Variation of NNMT activity assay conditions

Using mouse liver homogenate, we next decided to determine the effects of longer (>20 min) length of enzyme incubation and the effect of DMSO upon NNMT specific activity.

3.4.1. Length of incubation

The length of incubation had no effect upon the amount of MeN produced per mg protein up to 40 min incubation, suggesting that maximal MeN production occurred within 20 min of reaction initiation (Fig. 5). After 60 min incubation, the amount of MeN produced per mg protein decreased, most likely as a consequence of AO-mediated degradation of MeN. Consequently, specific activity decreased in a time-dependent manner, with highest specific activity observed after 20 min incubation.

3.4.2. Effect of DMSO

The specific activity of rabbit liver NNMT in reactions containing nicotinamide prepared in DMSO was significantly higher than that observed in reactions containing nicotinamide prepared in PBS (0.197 ± 0.014 *vs.* 0.04 ± 0.023 nmol MeN produced/hour/mg protein, $p = 0.0021$, $n = 3$).

3.5. Calculation and comparison of NNMT activity in rabbit, mouse and human liver homogenate

NNMT activity in mouse, rabbit and human liver, using 10 mM nicotinamide as substrate, was calculated to be 0.131 ± 0.013, 0.349 ± 0.056, and 140.6 ± 13.8 nmol MeN produced/hour/mg protein respectively ($n = 3$ for each). The intra-assay variation, assayed using mouse liver at a substrate concentration of 10 mM, was $5.9 \pm 2.23\%$ (median 5%, range 4.2–9.9%, $n = 6$). The inter-assay variation, assayed using mouse liver at a substrate concentration of 10 mM, was 11.6% ($n = 6$).

3.6. Investigating the effect of the inhibition of aldehyde oxidase activity using imipramine

Mouse liver NNMT specific activity significantly increased in the presence of 50 μM imipramine after 20 min incubation by 16% (0.141 ± 0.006 *vs.* 0.164 ± 0.009 nmol MeN produced/hour/mg protein, $p = 0.0347$, $n = 3$). Mouse NNMT still demonstrated substrate inhibition kinetics in the presence of imipramine, although the inhibition of activity was more marked (Fig. 6). Modelling the kinetic parameters V_{max}, K_m and K_i using Eq. (2) showed that V_{max} had increased significantly from 0.18 ± 0.01 to 0.323 ± 0.05 nmol MeN produced/hour/mg protein ($p = 0.0391$, $n = 3$). K_m reduced from 2.6 ± 0.34 to 1.39 ± 3.44 ($p = 0.0315$, $n = 3$), and K_i also reduced from 50.64 ± 8.19 to 12.44 ± 3.44 ($p = 0.0176$, $n = 3$).

4. Discussion

NNMT, which plays a central role in the biotransformation and detoxification of compounds [35], has been linked to multiple tumourigenic processes [15–17] and is therefore a promising candidate target for cancer therapy. One major therapeutic approach will be *via* the design of small-molecule inhibitors of NNMT activity.

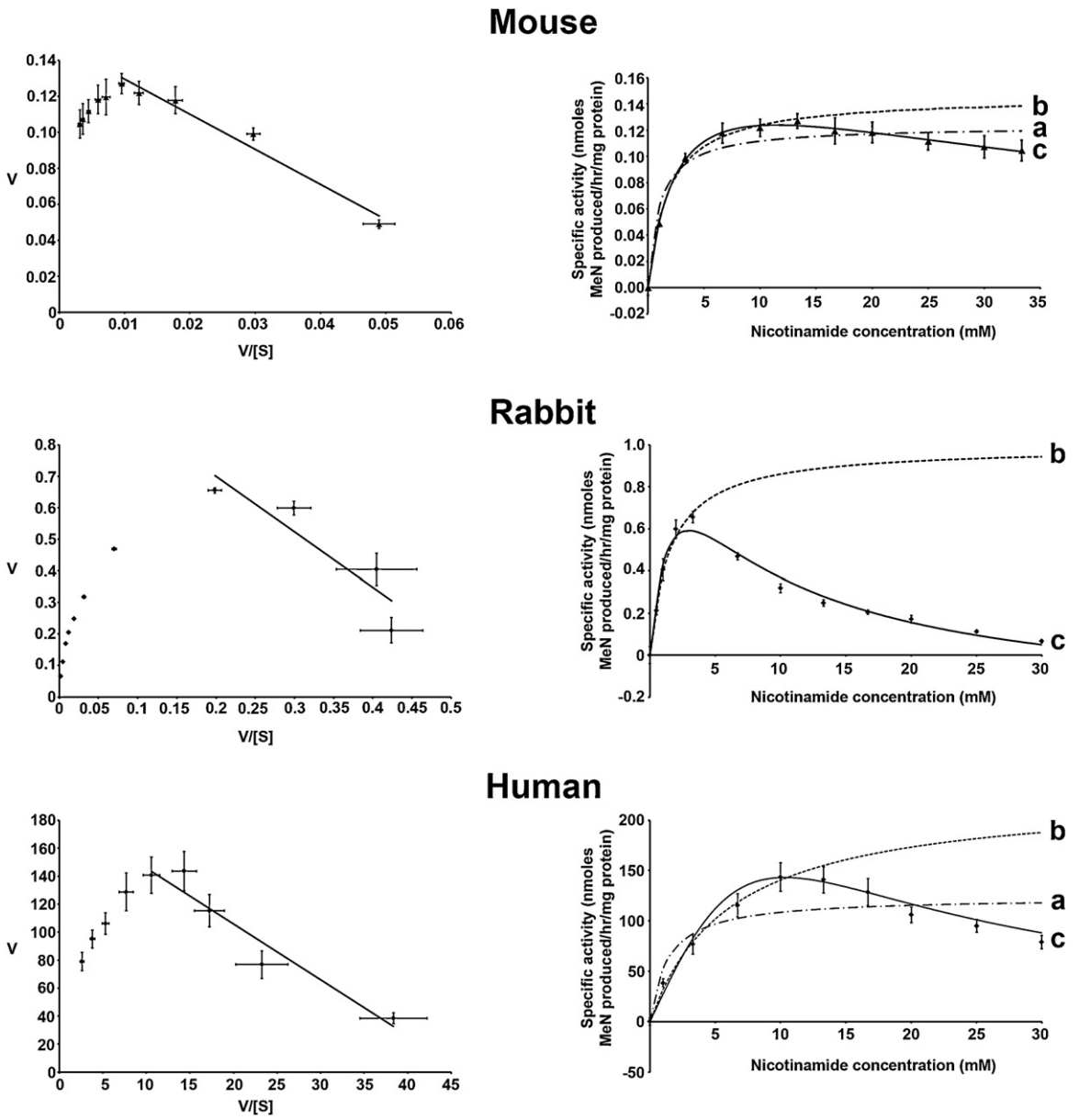

Fig. 4. Effect of substrate concentration upon NNMT specific activity. Liver homogenates were incubated with increasing concentrations of nicotinamide for 20 min after which enzyme activity was terminated by the addition of 1% (final concentration) trichloroacetic acid. 6-Chloronicotinamide internal standard was added as per legend of Fig. 3, centrifuged to precipitate protein and analysed as described in legend of Fig. 1. Peak areas were integrated, normalised for internal standard, and results were calculated and expressed as specific activity (nmoles MeN produced/hour/mg protein). Data were plotted as both Eadie–Hofstee plots of $v/[S]$ *vs.* v (left panels) and kinetic plots of v *vs.* $[S]$ (right panels). Data were modelled using various kinetic models to obtain values for V_{max}, K_m and K_i as appropriate. *a* = Michaelis–Menten kinetics using Eq. (1); *b* = Michaelis–Menten kinetics of truncated data outlined in Section 3.3 using Eq. (1); *c* = substrate inhibition kinetics using the equation from Eqs. (2)–(4) which gave the best fit.

In order to expedite these studies, there is a need for a standardised, quick and relatively high-throughput assay for measuring NNMT activity. Here, we describe an assay which uses ion-pairing reverse-phase HPLC–UV detection of MeN which requires no pre-column derivatisation nor sample manipulation, and is thus amenable for medium- to high-throughput analysis. The assay demonstrated sufficient sensitivity to detect endogenous concentrations of MeN in mouse, rabbit and human liver homogenate, spanning 2.5 orders of magnitude (0.05–30 nmol/100 μL injection) with a LOD (0.05 nmol/100 μL) which is significantly lower than other studies which report detection limits in the range of 0.2–20 nmol/100 μL [24,36]. The assay also demonstrated excellent baseline resolution for MeN and the internal standard 6-CN. Using this assay, we were able to measure endogenous NNMT activity in mouse, rabbit and human liver homogenates and to demonstrate that NNMT demonstrates substrate inhibition kinetics in all three species.

4.1. Comparisons with other described methods

Examination of the literature reveals several reports which have described methods of determining MeN in various different biological matrices [23–25,36,37], with a smaller number applying these for the measurement of NNMT activity [26–28]. A general approach to monitoring the activity of all SAM-dependent methyltransferases is based on the conversion of SAM to *S*-adenosylhomocysteine (SAH) [38]. The lack of enzyme specificity is a major limitation of this assay, and would require purified or

Table 1
Kinetic constants for mouse, rabbit and human NNMT. V_{max}, K_m and K_i (as appropriate) values were calculated using non-linear regression analysis using the equations outlined in the text.

Species	Kinetic constant	Michaelis–Menten	Michaelis–Menten (truncated)[a]	Substrate inhibition
Mouse	V_{max}[b]	0.12 ± 0.003	0.15 ± 0.004	0.18 ± 0.01
	K_m[c]	1.02 ± 0.18	1.77 ± 0.17	2.6 ± 0.34
	K_i[c]	n/a	n/a	50.64 ± 8.19
Rabbit	V_{max}	0.31 ± 0.03	0.99 ± 0.07	2.39 ± 0.35
	K_m	nd[d]	1.52 ± 0.25	3.41 ± 0.13
	K_i	n/a	n/a	3.57 ± 0.16
Human	V_{max}	123.4 ± 6.61	226.0 ± 9.38	404.3 ± 68.49
	K_m	1.36 ± 0.48	6.07 ± 0.53	6.87 ± 2.42
	K_i	n/a	n/a	20.01 ± 2.69

All values are ±SEM and $n = 4$.
[a] Kinetic constants were calculated for data from which the portions of the inhibited data were truncated to allow comparison with observed substrate inhibition kinetic constants.
[b] V_{max} units are nmoles MeN produced/hour/mg protein.
[c] K_m and K_i units are both mM.
[d] nd = K_m value could not be determined using Michaelis–Menten kinetic modelling (Eq. (1)).

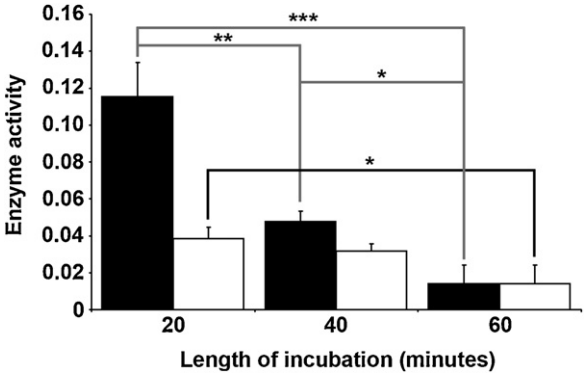

Fig. 5. Effect of variation of length of assay incubation upon NNMT specific activity. Mouse liver homogenate was incubated with 10 mM nicotinamide and 40 μM *S*-adenosylmethionine at 37 °C, after which the reaction was terminated at 20, 40 and 60 min and results analysed as described in legend of Fig. 4. Specific activity (black bars) was calculated and expressed as nmols 1-methylnicotinamide produced/hour/mg protein ± S.D., whereas the amount of 1-methylnicotinamide produced (white bars) was calculated and expressed as nmoles 1-methylnicotinamide produced/mg protein ± S.D. Specific activity decreased with increasing length of incubation, whereas the amount of 1-methylnicotinamide produced peaked after 20 min incubation and decreased after 60 min incubation. *p*-Values over grey bars show statistical comparison of specific activity, whereas *p*-values over black bars show statistical comparison of 1-methylnicotinamide produced; the lack of linking bars indicates that statistical comparison showed no significant difference. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

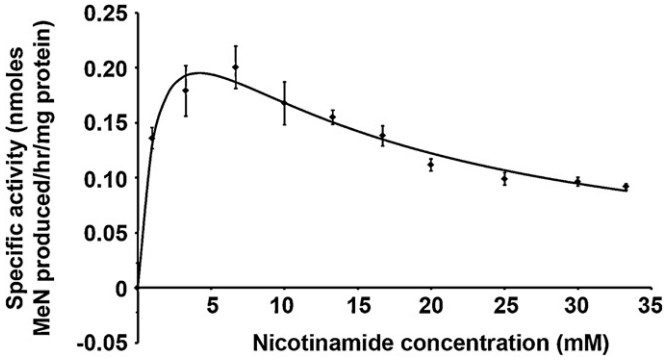

Fig. 6. Effect of imipramine upon the kinetics of mouse liver NNMT. NNMT activity was measured as described in legend of Fig. 4 in the presence of 50 μM imipramine. Results were calculated and expressed as specific activity, plotted as *v vs. S* and modelled for substrate inhibition kinetics using Eq. (2).

recombinant NNMT protein in order to obtain specificity. The fluorescence method of Sano et al. [28] used 4-methylnicotinamide as NNMT substrate, which required post-reaction derivatisation with alkaline 4-methoxybenzaldehyde prior to analysis. Although this method reported a LOD of 100 pmol, which is considerably more sensitive than other published methods, the K_m for 4-methylnicotinamide (0.19 mM) is also significantly lower than that for nicotinamide and thus does not reliably model NNMT catalytic activity.

Previously reported HPLC-based methods require sample manipulation prior to analysis, for example the method of Erb et al. [26] required chloroform/methanol extraction followed by evaporation to dryness and resuspension in aqueous running buffer. Such sample manipulations are time-consuming and can result in loss of sample, and most significantly they are not amenable for use in medium- to high-throughput analysis.

We have previously used the radioisotope method of Rini et al. [27] to measure NNMT activity in both human *post mortem* brain samples [2] and in SH-SY5Y cells stably expressing recombinant NNMT [6]. Although highly sensitive, the method has a number of drawbacks for use in inhibitor screening: (1) potential loss of metabolite yield due to the requirement for organic extraction; (2) inability to definitively identify the radiolabelled compound as MeN; (3) requirement for organic extraction steps reduces the potential for rapid screening; and (4) safety issues surrounding the use of large volumes of radioisotopes. All together, these reduce the attractiveness of the assay in its present form as a screening tool for potential NNMT inhibitors.

In our present study, we have successfully replaced [^{3}H]-MeN detection with HPLC–UV detection of MeN, which is sufficiently sensitive to measure endogenous basal MeN concentration and NNMT activity in the liver of three species – mouse, rabbit and human – which demonstrate species differences in activity. Our modified method only requires deproteination of the homogenate sample, has a LOD (0.05 nmol/100 μL) and LOQ (0.15 nmol/100 μL) which is lower than other published studies, does not require extensive sample manipulation and does not require the use of radioisotopic labelling.

Previous studies have reported a variety of K_m and V_{max} values for mouse liver NNMT, ranging between 0.36 and 0.37 mM for K_m [39,40] and 1.1–16.0 nmol per hour per mg protein [40]. In comparison with these data, our data suggests mouse liver NNMT has both a lower affinity and catalytic turnover than demonstrated in other studies. It is likely that these differences can be ascribed to differences in methodology, for example, length of incubation along with differences in mouse strain and gender [40]. Prior to our study,

the substrate inhibition kinetic behaviour of NNMT (Fig. 4) was unknown, thus it is possible that, in light of our results, inappropriately high concentrations of nicotinamide used in many assays may also have adversely influenced published K_m and V_{max} values. It should also be noted that, in our study, we have used non-linear regression analysis for our calculations of V_{max} and K_m, whereas previous studies have used linear regression analysis such as Lineweaver–Burke [1], although it is unlikely that this alone would be responsible for the large differences observed.

4.2. Extended assay reaction time and nicotinamide diluent influence apparent specific activity

An interesting observation was the decrease in NNMT specific activity after 40 and 60 min incubation compared to that observed after 20 min. It is unlikely that this is due to SAM usage by other methyltransferases as SAM is added in excess in the assay, nor is it likely that it is due to SAM instability as the amount of MeN produced after 60 min incubation is significantly lower than that produced after 20 and 40 min incubation. This decrease in MeN produced after 60 min incubation suggests that it likely arose due to AO-mediated degradation of MeN; this is demonstrated by the increase in NNMT specific activity in the presence of imipramine after 20 min incubation. This decrease in specific activity with increasing period of incubation has significant ramifications for studies which have used incubation periods greater than 20 min [1,4,39], as it is likely that the specific activity values reported are lower than they should be.

DMSO is added to the assay at a final concentration of 6.7% (v/v) as it is used to prepare the nicotinamide stock. This nicotinamide stock solution is prepared at a relatively high concentration (150 mM) to minimise the dilution of the homogenate sample. Preparing nicotinamide using DMSO in this way resulted in a significantly higher specific activity compared to reactions in which nicotinamide was prepared in PBS. This is most likely due to the comparative solubility of nicotinamide in PBS and DMSO at such a high concentration, and it is likely that this results in more nicotinamide being delivered to the assay when prepared in DMSO compared to when it is prepared in PBS. Preparation of nicotinamide in DMSO also increased the reliability of the assay, as evidenced by the significantly smaller standard error (7%) compared to when nicotinamide is prepared using PBS (58%).

4.3. NNMT demonstrated substrate inhibition kinetics

Both Eadie–Hofstee and Michaelis–Menten plots (Fig. 4) showed that NNMT demonstrated substrate inhibition kinetic behaviour when assayed in liver homogenate derived from all three species. This is not without precedent, as a significant number of enzymes demonstrate such behaviour when assayed in similar cell-free assays [41], for example cytochrome P450s [29]. Kinetic modelling using substrate inhibition kinetic equations resulted in good kinetic modelling of our data, providing further confirmation of the presence of substrate inhibition.

Two mechanistic models have been proposed for substrate inhibition. The first is the presence of two binding sites for the substrate, one with a high affinity (catalytically active) and one with a low affinity (catalytically inactive) which is occupied at high concentrations of substrate [29,41]. The localisation of the low affinity site can be either within the active site pocket of the enzyme itself or as a distinct binding site distal to the active site pocket [30]. In the case of both binding sites being within the active site pocket, binding to the lower affinity binding site at high substrate concentrations sterically hinders the binding of the substrate to the catalytically active high affinity binding site [42]. In the case of a distally located low affinity binding site, binding at high substrate concentration produces a conformational change in the enzyme's active site pocket such that substrate binding is reduced [43].

The second mechanism proposed involves the formation of a ternary dead-end complex comprising substrate interacting with another molecule, usually the end-product of cofactor metabolism, within the active site pocket at high substrate concentration [44]. The accumulation of such ternary dead-end complexes results in a significant slowing of the catalytic cycle [30].

Our study is the first to demonstrate that NNMT undergoes substrate inhibition, the conclusion from which is that nicotinamide could, under certain conditions, act as an inhibitor of its own metabolism. It is unclear as to which mechanism NNMT adopts. The recently published crystal structure of NNMT complexed with nicotinamide and SAH showed a single nicotinamide molecule bound in close proximity to SAH, interacting with the active site *via* Tyr 20 and Asp 197 [45]. To produce this crystal structure, NNMT was crystallised in the presence of 10 mM nicotinamide, therefore in light of our data it is unlikely that the putative second allosteric binding site for nicotinamide would have been occupied and as such was not observed, therefore the presence of a low-affinity nicotinamide binding site, either within the active site pocket or a distinct site distal to the active site pocket, cannot be ruled out. Crystallising at much higher nicotinamide concentrations (>35 mM) may reveal the location of this second nicotinamide binding site. In support of the formation of a ternary dead-end complex, the crystal structure described shows such a complex [45].

Substrate inhibition is an important mechanism for the regulation of enzyme activity which plays a number of regulatory roles [41]. For example, tyrosine hydroxylase, the rate-limiting enzyme of dopamine metabolism from tyrosine, undergoes substrate inhibition to ensure steady-state synthesis of dopamine despite large fluctuations in dietary tyrosine levels [46]. It is unclear why NNMT may undergo substrate inhibition, but it may be related to the central importance of nicotinamide to pyridine nucleotide synthesis, the major source of which is *via* the diet [47]. A carnivorous diet, especially one where there are regular intervals between meals, would result in significant fluctuations in hepatic nicotinamide levels. Nicotinamide is a potent inducer of NNMT expression, the consequences of which are potentially deleterious reductions in pyridine nucleotide synthesis and subsequently ATP synthesis. In support of this, we have shown that ectopic expression of high levels of NNMT reduced pyridine nucleotide synthesis [6]. It is thus conceivable that the ability to downregulate NNMT activity using substrate inhibition at times of high dietary influx would allow the cell to maximise pyridine nucleotide synthesis for optimal ATP synthesis *via* oxidative phosphorylation. This would be of particular benefit to those species which have a diet rich in nicotinamide and who feed intermittently as opposed to those species with more herbivorous nicotinamide-deficient diets which tend to graze continuously. NNMT activity in humans, who have a nicotinamide-rich diet as described above, is much more responsive to substrate inhibition compared to that observed in the mouse, who have a diet which is unlikely to result in large variations in nicotinamide intake.

The presence of a second low-affinity nicotinamide binding site, either within or distal to the active site, would provide an excellent target for NNMT inhibitor drug design. Inhibitors which bind to this site may reduce NNMT activity without the need to compete with nicotinamide for the active site. The active site of NNMT is large, as it needs to bind both nicotinamide and SAM. In addition, the crystal structure of NNMT revealed the presence of a "cap" which covers the active site upon binding of nicotinamide and SAM [45]. As such, there are numerous interactions within the active site which need to be taken into account when designing inhibitors. In contrast, it is likely that the interactions within a low-affinity binding site are much simpler, as it is likely to only bind nicotinamide and is unlikely to have a cap. As such, the confirmation of the presence

Fig. 7. Comparison of the primary sequences of mouse (top) and human (bottom) NNMT. Residues involved in the binding of nicotinamide and *S*-adenosylmethionine, and in the catalytic activity of NNMT, are in bold capitals. Amino acid differences are highlighted in grey.

of a low-affinity nicotinamide binding site and the elucidation of the residues involved in the interaction with nicotinamide would be an important step forward for the design of NNMT inhibitors for cancer therapy.

4.4. Imipramine altered mouse liver NNMT kinetic parameters

The addition of imipramine resulted in a small yet significant increase in mouse liver NNMT specific activity. More importantly, it also changed the kinetic parameters V_{max}, K_m and K_i. The increase in V_{max} is unsurprising, as MeN produced by the reaction is no longer metabolised by AO, therefore the measured V_{max} will inevitably increase. The decrease in K_m and K_i, indicating an increase in the affinity of the enzyme for nicotinamide as both a substrate and an inhibitor, is somewhat more surprising, and the reasons for it are as yet unclear. It is possible that these changes may be related to the increased concentration of MeN within the assay, leading to a degree of MeN-mediated inhibition of NNMT activity which is in turn influencing substrate inhibition. With respect to drug screening, this is not critical, as the increase in NNMT specific activity is not large enough to demand the inclusion of imipramine in the assay. However, if an increase in specific activity using imipramine is desired, then this change in kinetic behaviour, in particular the increase in the substrate inhibition properties of the enzyme, should be taken into account.

4.5. NNMT activity demonstrated species differences in various kinetic parameters

It is clear from our results that there are significant species differences in both NNMT specific activity and in kinetic parameters. The reasons for these species differences are unclear, but it may reflect differences in requirement for nicotinamide and/or MeN. There are a number of reasons as to how such differences arise, the most likely being differences in the level of protein expression. It is also possible that they arise from differences in protein sequence. A comparison of human and mouse NNMT (Fig. 7) reveals a significant number of amino acid differences, however those residues identified by Peng et al. [45] as being critical for both the binding nicotinamide and SAM, and the catalytic process, are completely conserved. There are a number of changes in very close proximity to these conserved residues which may induce subtle changes which give rise to altered enzyme activity and the differences in enzyme kinetic constants observed.

5. Conclusion

We have characterised a HPLC–UV method which allows the rapid quantification of MeN and NNMT activity in biological samples which demonstrated a large dynamic range ideal for the detection of MeN concentration and NNMT activity in mouse, rabbit and human liver homogenate. Using this assay, we have provided for the first time evidence that NNMT demonstrates substrate inhibition kinetics, suggesting either the formation of a tertiary non-functional complex or the presence of a low-affinity nicotinamide binding site, allowing nicotinamide to act as an inhibitor of NNMT activity when at high concentrations during *e.g.* dietary influx.

Conflict of interest

The authors have no conflict of interest regarding this work.

Role of funding source

This study was supported by a grant from Parkinson's UK (grant reference G-0505) who had no role in the design, execution, interpretation or the decision to publish this work.

Acknowledgements

The authors would like to thank Dr. David Barlow, Institute of Pharmaceutical Science, King's College London, for his invaluable advice and assistance in the kinetic modelling of the data.

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