Reliable Measurement of Endogenous Drug Using True Plasma Matrix – A Case Study for Determination of Methylnicotinamide in Human Plasma Using LC-MS/MS

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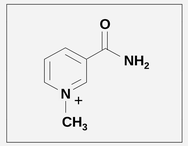
INTRODUCTION

Methylnicotinamide (MNA) is a primary metabolite of Vitamin B3. A reliable quantitation method using unaltered matrix was desired for determining endogenous concentrations of MNA in healthy volunteers and for supporting safety of proposed MNA dosing.



Methylnicotinamide (MNA)

Using unaltered matrix for standard (STD) and QC preparations normally requires an LLOQ being set at much higher concentration than endogenous levels. As a result, the samples with drug concentrations at or close to endogenous levels can not be analyzed. Standard addition method is an alternative approach. However, it is not practical for clinical studies due to less robustness of the method and higher cost. The standard addition method is often complicated by the dynamic responses through the quantitation range and selection of the amount of drug spiked into each pre-dosed or dosed sample.

METHOD

In our methodology, all standard, QC and clinical samples are analyzed using calibration curve method while standard addition method is used only for determining endogenous level of drug in the matrix used for standard and QC preparation. The screened human plasma lots consisting of the lowest levels of endogenous MNA were pooled and used for preparation of standard and QC samples. The concentration of endogenous MNA in the pooled plasma was calculated as Equation 1 and then was added to the spiked concentrations (10 to 480 ng/mL) for the nominal value of each standard or QC sample.

$$C_{end} = \frac{R_b \times S}{R_{cal} - R_b}$$

C_{end} = Endogenous concentration of MNA in plasma blank
R_b = Average peak area ratio for blank
R_{cal} = Average peak area ratio for spiked plasma sample
S = Spiked concentration (ng/mL)

Equation 1. Standard Addition Method

Mass Spectrometry

AB Sciex API 3000 / 4000
Polarity/Interface : Positive/TurboIon Spray
Scan type : Multiple Reaction Monitoring
Methylnicotinamide: 137 → 94
Methylnicotinamdie-d3: 140 → 97 (I.S.)

Liquid Chromatography

Pump : Shimadzu 10ADvp, Autosampler : Perkin Elmer 200
Column: Aquasil C18 (50 x 2.1 mm)
MP A : MeOH/10 mM acetate (pH 4.0)/HFBA (5/95/0.2)
MP B : MeOH

Sample Clean-up : Protein precipitation using methanol

RESULTS AND DISCUSSION

The concentration of MNA in pooled plasma matrix was determined to be 7.6 ng/mL using standard addition method. Figures 1 to 4 show typical data observed during validation.

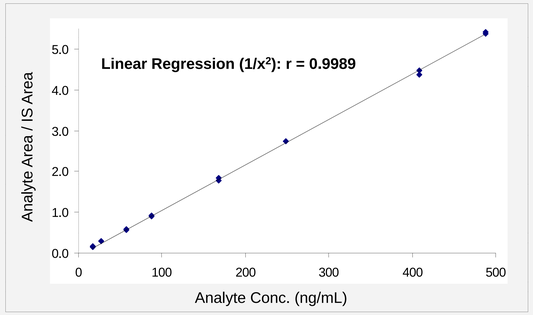


Figure 1. Calibration Curve for MNA

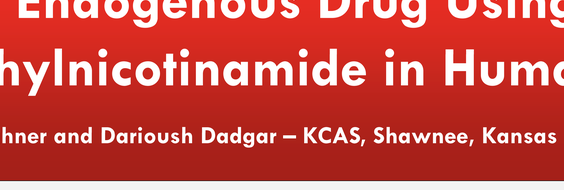


Figure 2. Chromatogram of a Solvent Blank Sample (No I.S.) Injected after ULOQ

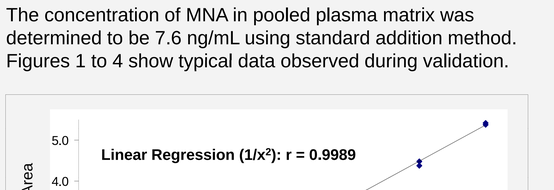


Figure 3. Chromatogram of an Extracted LLOQ sample (17.6 ng/mL)

Figure 4. Chromatogram for the Pooled Plasma Blank Consisting of 7.6 ng/mL MNA

The full validation were completed with reliable LLOQ being set at 17.6 ng/mL (10 ng/mL spiked into pooled plasma). The back calculated values based on calibration curve method were against the nominal values for evaluating the accuracy. The LLOQ was extended to lower concentration at endogenous concentration at 7.6 ng/mL.

The specificity and accuracy for analysis of endogenous compounds can not be directly evaluated. We have used solvent blank to control the potential contamination and carryover effect during analytical process and tested the recoveries of drug spiked into the plasma to evaluate the selectivity and accuracy. The recoveries for varied amounts of drug spiked into different lots of matrix were between 90.0% to 92.7% during validation as the data shown in **Table 1**, where three replicates are measured for each sample with CV% from 0.8 to 2.7. The matrix effect with the same amount (30 ng/mL) of drug spiked into six different lots of plasma is shown in **Table 2**. No significant matrix effect was observed.

Endogenous Level	10 ng/mL Spiked	20 ng/mL Spiked	40 ng/mL Spiked
10.9	19.0	N/A	N/A
21.6	29.3	38.1	N/A
32.5	38.2	48.3	N/A
43.9	49.5	58.2	83.9

Table 1. The Mean Concentration Determined for Selected Plasma Matrices with Different Amounts of Drug Spiked.

	Total Conc.[1] (ng/mL)	n	Avg. Analyte Rf [2]	Avg. I.S. Area	Avg. Rf/I.S. Ratio
Lot 1	39.5	6	942	95,047	0.00991
Lot 2	36.5	6	928	92,886	0.00999
Lot 3	36.4	6	1,006	103,389	0.00973
Lot 4	38.7	6	921	91,243	0.01009
Lot 5	38.4	6	952	96,169	0.00990
Lot 6	39.2	6	887	91,380	0.00971
	Mean		939	95,016	0.00989
Overall	**SD**		39.53	4,550.1	0.000147
	% CV		4.2	4.8	1.5

[1] Total conc. = Spiked conc. (30 ng/mL) + Plasma conc.
[2] Average analyte response factor (Rf = Analyte area/total concentration).

Table 2. Statistics for Spiked Samples at Low QC levels (30 ng/mL) over six different lots of Plasma.

The inter- and intra-batch results for QC samples are shown in **Table 3**. Different amounts of MNA above endogenous level could be quantitatively recovered with good precision and accuracy.

Batch		17.6 ng/mL (LLOQ)	37.6 ng/mL (Low)	208 ng/mL (Mid)	328 ng/mL (High)
1	**Mean**	16.6	36.4	200	335
	% CV	4.4	1.9	1.2	1.7
	% Bias	-5.7	-3.2	0.4	2.0
2	**Mean**	16.7	36.9	210	335
	% CV	2.6	3.0	1.3	1.2
	% Bias	-5.1	-1.9	1.1	2.0
3	**Mean**	16.2	36.7	213	335
	% CV	2.7	2.3	2.6	1.6
	% Bias	-7.9	-2.4	2.4	2.0
Overall	**Mean**	16.5	36.7	211	335
	n	18	18	18	18
	% CV	3.4	2.4	1.9	1.4
	% Bias	-6.2	-2.5	1.3	2.0

Table 3. Statistics for spiked QC Samples above endogenous level.

One batch was conducted during validation with pooled blank plasma, endogenous level at 7.6 ng/mL, being used as LLOQ standard and sensitivity QC samples. The accuracy of the mean for all standard levels including LLOQ were between 94.3 and 102.8%. The result for QC at endogenous level is shown in **Table 4**. The method with extended LLOQ to endogenous level was not fully validated. The robustness of the method was not a problem, but the specificity of the method for endogenous concentrations could not be fully evaluated with available technology.

n	mean	CV%	Bias (%)
6	7.89	7.5	3.8

Table 4. Intra-run Statistics for Extended LLOQ at Endogenous Level.

In a clinical study, 211 pre-dosed samples were measured for endogenous concentrations using calibration curve method. Only one sample was found below 7.6 ng/mL and the concentration for all other predosed samples were determined to be between 8.35 and 95.8 ng/mL with 72% of the samples being above 17.6 ng/mL.

CONCLUSION

A robust LC-MS/MS method for determination of endogenous drug MNA was developed and validated using unaltered plasma as matrix for standard and QC preparation. A concentration range from 10 to 480 ng/mL above the endogenous level was fully validated. The clinical samples at low endogenous levels can also be directly analyzed for providing additional information. The methodology employed in this work is reliable and practical and has shown many advantages over other methodologies. In our current knowledge the methodology in this work has been used only by **KCAS** for supporting preclinical and clinical studies .