Simple and Fast Quantitation of Nicotinic Acid and Nicotinamide in Human Plasma by Applying Impact™ Protein Precipitation Plate Technology with Gemini® 3 µm C18 HPLC Columns

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Nicotinic acid and nicotinamide were extracted from human plasma by performing a rapid protein precipitation using Impact Protein Precipitation Plates followed by HPLC analysis using a Gemini 3 µm C18 100 x 4.6 mm HPLC column and positive polarity ESI LC-MS/MS system. Impact technology offers easy, fast protein removal while providing maximized recovery of the target analytes. The Gemini 3 µm C18 HPLC column produced excellent chromatographic resolution, sensitivity, and high peak capacities.

Introduction

Niacin (nicotinic acid) is a water-soluble vitamin that is also referred to as vitamin B3. Nicotinamide (nicotinic acid amide) is the derivative of niacin that is incorporated into the coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). The nicotinamide moiety of NAD and NADP serves as an electron acceptor or donor in biological oxidation-reduction reactions catalyzed by several hundred different enzymes. Nicotinamide is the form of vitamin B3 that is commonly found in nutritional supplements and used to fortify foods.

Materials and Methods

Sample Preparation:
Protein precipitation was performed using an Impact Protein Precipitation Plate.

Step	
1.	Place the Impact plate onto a suitable 96-well sample manifold
2.	Dispense 300 µL acetonitrile into each well of the Impact plate
3.	Add 100 µL of plasma/serum samples to each well of the Impact plate
4.	Mix 3 times by aspirating with a pipette tip
5.	Apply vacuum to filter the sample and collect the purified filtrate

After filtrate is collected, the collection plate containing the purified samples should be covered using a sealing mat. The sample is now ready to be injected onto the LC-MS/MS. If the sample will not be injected onto the LC-MS/MS immediately, transfer the filtrate to amber Verex™ autosampler vials (ambient) to protect from light.

HPLC Conditions:
Column: Gemini 3 µm C18
Dimensions: 100 x 4.6 mm
Part No.: 00D-4439-E0
Mobile Phase: A: 0.1 % Formic acid in water
B: Methanol
Flow Rate: 0.6 mL/min
Gradient:

Time (min)	% B
0	10
2.5	90
2.6	10
4	10

Detection: API 4000™ MS/MS, ESI Positive (ESI+)
Temperature: Ambient
Injection: 2 µL purified plasma

MS/MS Conditions:
SCIEX® API 4000 triple-quadrupole tandem mass spectrometer is used for analysis equipped with an ESI probe operating in positive polarity mode. Under an MRM mode, two channels were monitored for nicotinamide and nicotinic acid (**Table 1**).

Table 1.
MRM Transitions

Peak Name	MRM Channel
Nicotinamide	123.006 ⇒ 80.100
Nicotinic acid	123.981 ⇒ 80.100

Results and Discussion

When developing a method for the analysis of nicotinamide and nicotinic acid, it was important that the method be rapid, sensitive, and accurate in order to accommodate high-throughput labs that analyze 100's to 1000's of samples each week. Traditionally, a protein precipitation step is used for fast cleanup of plasma samples. Protein precipitation is normally performed using a centrifuge tube or a 96-well collection plate, however this process requires that supernatant be collected while being careful not to disrupt pelleted protein in the bottom of the tube or collection plate. This step was greatly simplified by using Impact Protein Precipitation Plates. The Impact plate allows for the analysis of 96 samples at once, eliminates the transfer steps that are com-

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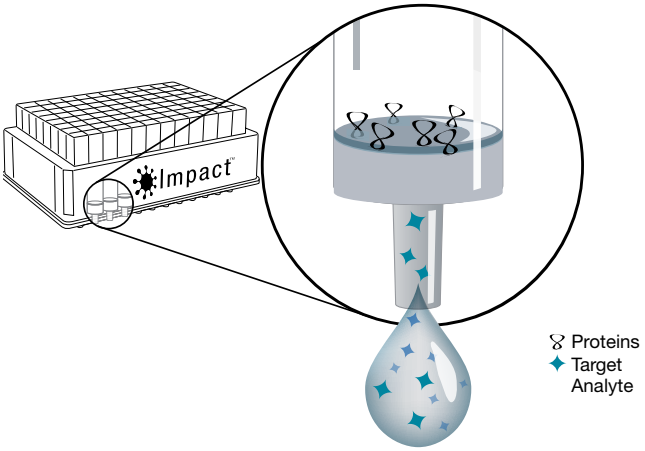
monly associated with protein precipitation, and can also be automated. Protein precipitation was performed within the wells of the Impact™ plate and sample was not allowed to pass through the filter of the plate until vacuum was applied. This ensured that the precipitated protein was left within the wells of the Impact plate while protein free sample was allowed to pass through the filter and into a collection plate (**Figure 1**).

Figure 1.
Protein Precipitation Using Impact Protein Precipitation Plates



After the plasma samples were cleaned up, they were analyzed by LC-MS/MS using a Gemini® 3 µm C18 HPLC column coupled to an API 4000™ triple-quadrapole tandem mass spectrometer. The Gemini 3 µm C18 HPLC column was chosen because it contains a unique silica-organic layer that is grafted onto the base silica which mechanically strengthens the particle while providing excellent efficiencies. Efficiency and resolution were necessary in this analysis because the chemical properties of nicotinamide and nicotinic acid are similar in that they share the same backbone, however nicotinamide contains an amide moiety while nicotinic acid has a carboxylic acid moiety (**Figure 2**). It was also important to minimize tailing and improve peak shape of both target compounds. Using a 100 x 4.6 mm column reduced tailing and provided the desired peak shape of each target compound. Shorter columns, such as a 50 x 4.6 mm, resulted in tailing and undesirable peak shapes indicating that a longer column is necessary to accurately separate nicotinamide and nicotinic acid.

Figure 2.
Chemical Structures of Nicotinamide and Nicotinic Acid

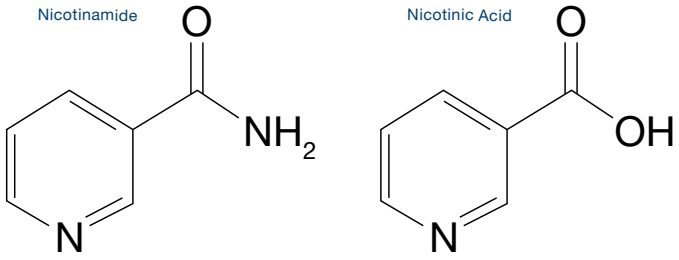


Figure 3.
LC-MS/MS Chromatogram of Nicotinamide and Nicotinic Acid at 1000 ng/mL

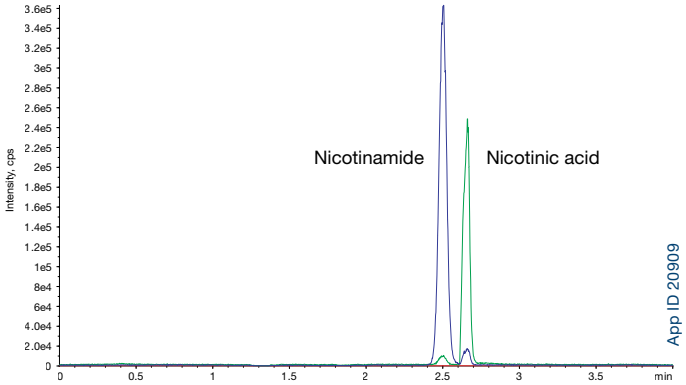


Figure 4.
LC-MS/MS Chromatogram of Nicotinamide and Nicotinic Acid at 10 ng/mL

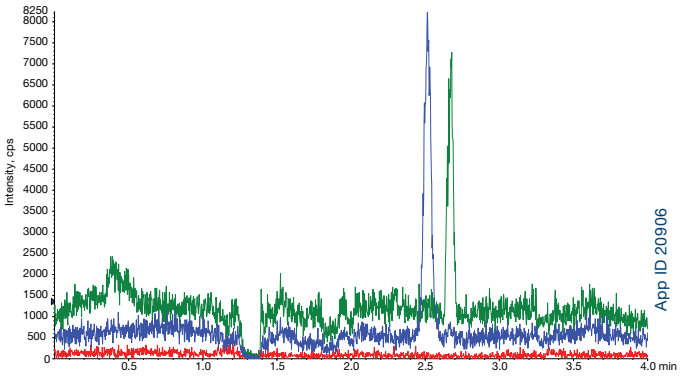
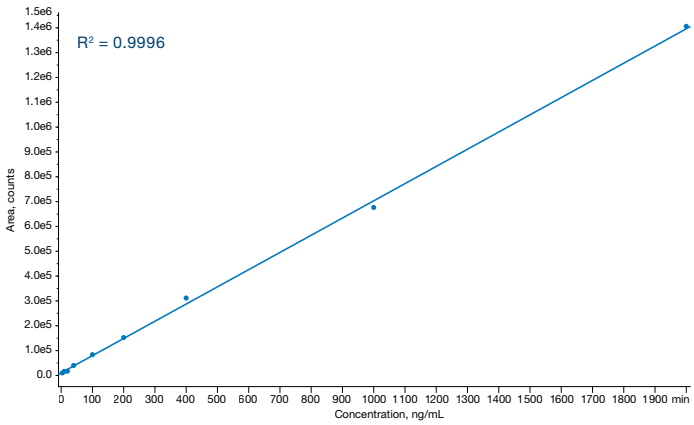


Figure 5.
Representative Standard Curve of Nicotinic Acid at a Concentration Range of 2 to 2000 ng/mL



The reproducibility of our analysis was determined by producing a standard curve of nicotinic acid at a concentration range of 2 to 2000 ng/mL. With R^2 = 0.9996, our method proved to be reproducible even at low levels of detection. The signal-to-noise ratio was also studied at LOD and LOQ. With a signal-to-noise ratio of 4.3 and 3.3, it was determined that we were able to reliably analyze nicotinamide and nicotinic acid from plasma/serum samples at a LOD of 2 ng/mL. The LOQ was subsequently determined to be accurate at 10 ng/mL with signal-to-noise ratios of 12.4 and 10.1.

Table 2.
Signal-to-Noise Ratio of Nicotinamide and Nicotinic Acid at LOD and LOQ

Analyte	LOD		LOQ	
	ng/mL	S/N Ratio	ng/mL	S/N Ratio
Nicotinamide	2	4.3	10	12.4
Nicotinic acid	2	3.3	10	10.1

Conclusion

As the study of vitamins becomes an increasingly important factor in clinical research, high-throughput laboratories must adopt rapid and robust methods to accurately analyze and quantitate vitamins and their derivatives. With these goals in mind, we developed a method that can be easily automated, can be used in high-throughput labs, and provides both sensitivity and speed. The sample preparation step using Impact™ Protein Precipitation Plates is simple, requires no method development, and can process 96 samples at once. The downstream LC-MS/MS analysis using a Gemini® 3 µm C18 HPLC column provides resolution between nicotinic acid and its derivative, nicotinamide, in under 3 minutes. The analysis is also sensitive, with an LOD at 2 ng/mL and a LOQ at 10 ng/mL for both analytes.

APPLICATION

Ordering Information

Gemini® HPLC Columns

3 µm Microbore, Minibore and MidBore™ Columns (mm)

Phases	50 x 1.0	20 x 2.0	30 x 2.0	50 x 2.0	100 x 2.0	150 x 2.0	50 x 3.0	100 x 3.0	150 x 3.0	SecurityGuard™ Cartridges (mm) 4 x 2.0*
										10/pk
C18	00B-4439-A0	00M-4439-B0	00A-4439-B0	00B-4439-B0	00D-4439-B0	00F-4439-B0	00B-4439-Y0	00D-4439-Y0	00F-4439-Y0	AJ0-7596
										for ID: 2.0-3.0 mm

3 µm Analytical Columns (mm)

Phases	20 x 4.0	30 x 4.6	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	SecurityGuard Cartridges (mm) 4 x 3.0*
							10/pk
C18	00M-4439-D0	00A-4439-E0	00B-4439-E0	00D-4439-E0	00F-4439-E0	00G-4439-E0	AJ0-7597
							for ID: 3.2-8.0 mm

5 µm Minibore and MidBore Columns (mm)

Phases	30 x 2.0	50 x 2.0	150 x 2.0	250 x 2.0	50 x 3.0	100 x 3.0	150 x 3.0	250 x 3.0	SecurityGuard Cartridges (mm) 4 x 2.0*
									10/pk
C18	00A-4435-B0	00B-4435-B0	00F-4435-B0	00G-4435-B0	00B-4435-Y0	00D-4435-Y0	00F-4435-Y0	00G-4435-Y0	AJ0-7596
									for ID: 2.0-3.0 mm

5 µm Analytical Columns (mm)

Phases	30 x 4.6	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	SecurityGuard Cartridges (mm) 4 x 3.0*
						10/pk
C18	00A-4435-E0	00B-4435-E0	00D-4435-E0	00F-4435-E0	00G-4435-E0	AJ0-7597
						for ID: 3.2-8.0 mm

* SecurityGuard Analytical cartridges require holder, Part No.: KJ0-4282

Ordering Information

Impact™ Precipitation Products

Part No.	Description	Unit
CE0-7565	Impact Protein Precipitation, Square Well, Filter Plate, 2 mL	2/pk
CE0-7566	Impact Protein Precipitation, Square Well, Long Drip, Filter Plate, 2 mL	2/pk

Impact Starter Kit for Protein Precipitation

Part No.	Description	Unit
CE0-8201	Impact Protein Precipitation Plate (CE0-7565)	2ea
CE0-7566	Collection Plate 2 mL	2ea
AH0-8199	Sealing Mat, Santoprene™	2ea

Accessories

Collection Plates (deep well, polypropylene)		
AH0-7192	96-Well Collection Plate 350 µL/well	50/pk
AH0-7193	96-Well Collection Plate 1 mL/well	50/pk
AH0-7194	96-Well Collection Plate 2 mL/well	50/pk
AH0-8635	96-Well Collection Plate, 2 mL Square/Round-Conical	50/pk

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