APPLICATIONS

Quantitation of Human Plasma and Breast Milk Thiamine Monophosphate (TMP) and Thiamine by Applying Impact™ Protein Precipitation Plate Technology with Gemini® 3 µm NX-C18 HPLC Columns

Shuguang Li, Seyed Sadjadi, Sky Countryman, and Erica Pike
Phenomenex, Inc., 411 Madrid Ave., Torrance, CA 90501 USA

Thiamine monophosphate (TMP) and thiamine were extracted from human plasma and human breast milk by performing a rapid protein precipitation using Impact Protein Precipitation Plates followed by HPLC analysis using a Gemini 3 µm NX-C18 100 x 3.0 mm HPLC column with fluorescence detection. Impact technology offers easy, fast protein removal while providing maximized recovery of the target analytes. The Gemini 3 µm NX-C18 HPLC column produced excellent chromatographic resolution, sensitivity, and high peak capacities.

Introduction

Vitamin B1, also known as thiamine, is mainly present in human body fluids and tissue as thiamine diphosphate (TDP), thiamine monophosphate (TMP) and free thiamine. TDP, the most abundant thiamine derivative, is well described as a cofactor of several important enzymes, whereas TMP and thiamine are thought to be simple intermediates for which no specific role has currently been defined. TDP is commonly monitored from whole blood while TMP has reportedly shown higher concentrations in cerebrospinal fluids as compared to TDP. Recently, TMP and thiamine were studied in human breast milk from 636 lactating women in a Maela refugee camp[1]. The studies illustrated that vitamin B6 in breast milk consisted of TMP and thiamine. A ratio of TMP to total thiamine of > 63% was associated with a 7.5-fold higher risk of low whole blood TDP and a 4-fold higher risk of deficient total breast thiamine, respectively[1]. Monitoring the body's concentration of TMP and thiamine is an aid in researching diseases related to their deficiencies. For this reason, it is important that analysis techniques be both sensitive and accurate.

Materials and Methods

Protein Precipitation

1. Place the Impact plate onto a suitable 96-well sample manifold
2. Dispense 100 µL of human plasma or breast milk into each well of the Impact plate
3. Add 300 µL of methanol 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 in a collection plate

Transfer 50 µL to an autosampler vial (or allow it to remain in the collection plate). Add 50 µL of water and 50 µL of derivatizing reagent (15 % sodium hydroxide solution plus 200 µL of 30 mM $K_3Fe(CN)_6$). Cover the vial or collection plate with a lid or sealing mat, respectively. Vortex for 15 seconds. Put the vial or collection plate into an autosampler. The sample is now ready to be injected onto the HPLC-FLD.

HPLC Conditions

An Agilent® 1100 HPLC system (Agilent Technologies, Inc., Santa Clara, CA, USA) was used with a Shimadzu® RF-20A Prominence® Fluorescence Detector (Shimadzu, Japan) for LC/FLD analysis.

Column: Gemini NX-C18 3 µm
Dimensions: 100 x 3.0 mm
Part No. : 00D-4453-Y0
Mobile Phase: A: 25 mM Na_2HPO_4, 10 % Methanol (pH 7.0)
B: 25 mM Na_2HPO_4, 70 % Methanol (pH 7.0)

Gradient:

Time (min)	% A
0.0	3
0.25	25
0.75	25
3.00	35
4.00	100
5.00	100
5.10	3
8.00	3

Flow Rate: 750 µL
Detection: Fluorescence (Excitation: 375 nm, Emission: 435 nm)
Temperature: 25 °C
Injection: 20 µL

Figure 1.
Protein Precipitation Using Impact Protein Precipitation Plates

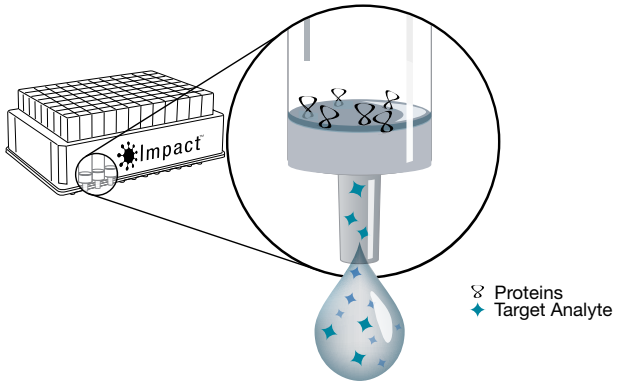

Figure 2.
200 nmol/L of TMP and thiamine in water filtered by an Impact Protein Precipitation Plate

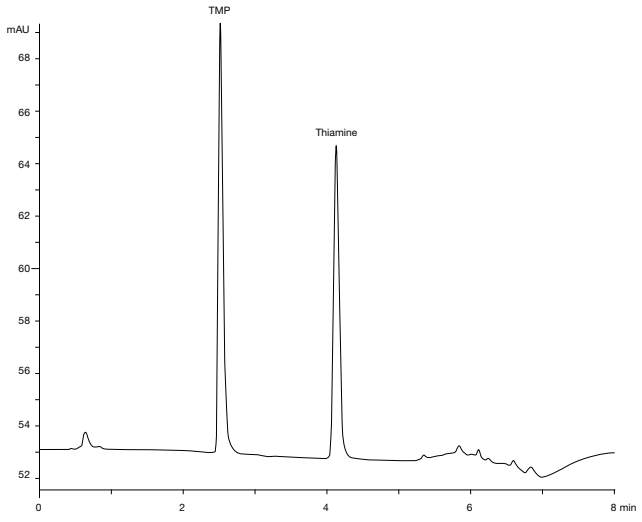

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Figure 3a.
2.5 nmol/L of TMP and thiamine in water filtered by an Impact Protein Precipitation Plate

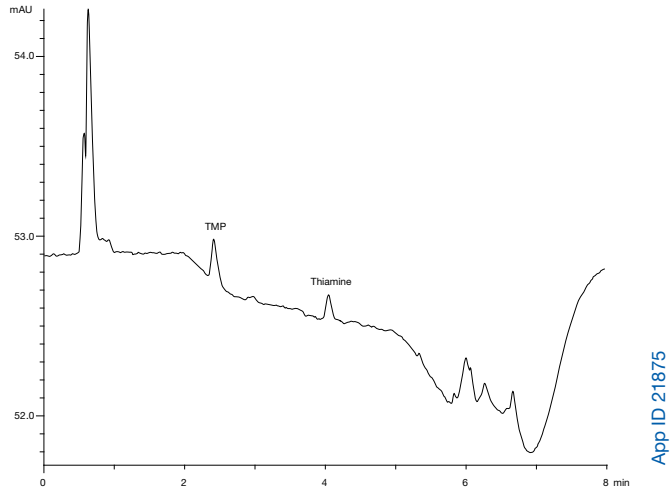


Figure 3b.
Comparison of TMP and thiamine at 25 nmol/L vs. blank control (overlay and expended chromatograms for human plasma filtered by an Impact Protein Precipitation Plate)

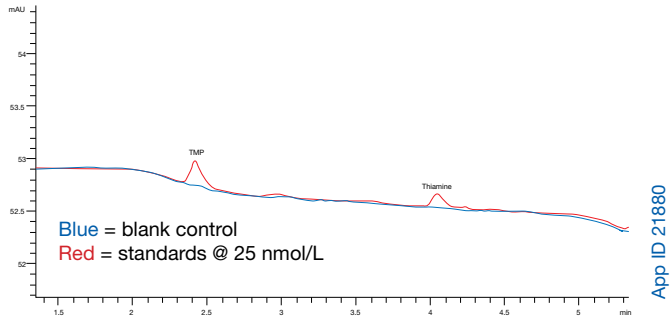


Figure 4.
Higher level TMP (93.6 nmol/L) and thiamine (240.8 nmol/L) in human breast milk

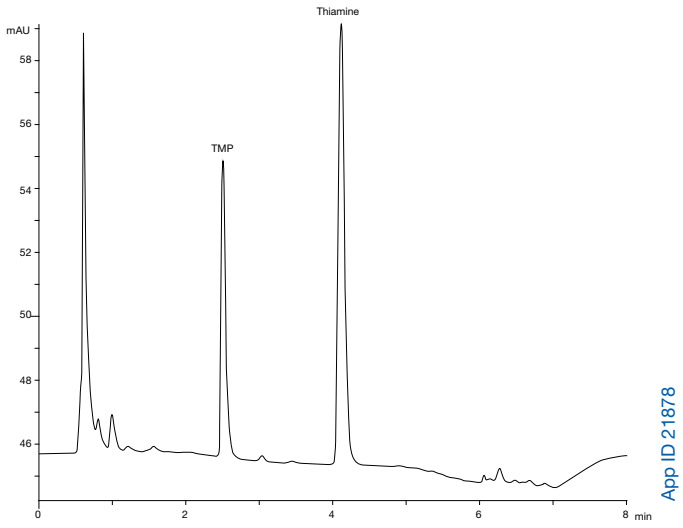


Figure 5.
Lower level TMP (37.2 nmol/L) and thiamine (66.4 nmol/L) in human breast milk

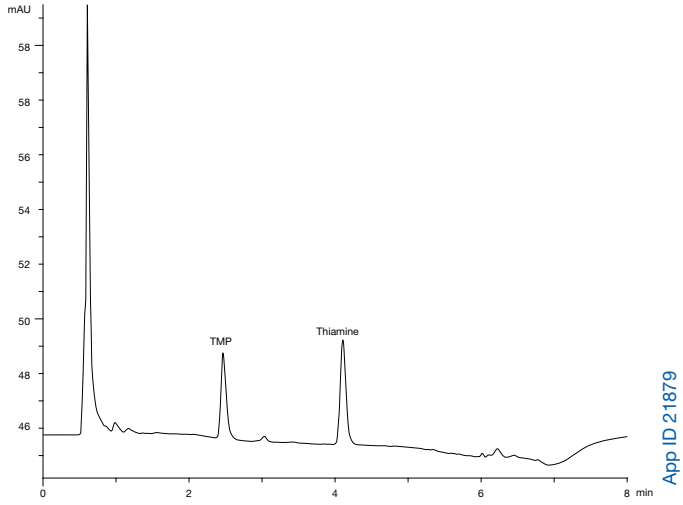


Figure 6.
Standard curve of TMP filtered by an Impact Protein Precipitation Plate at a concentration range of 0 to 200 nmol/L

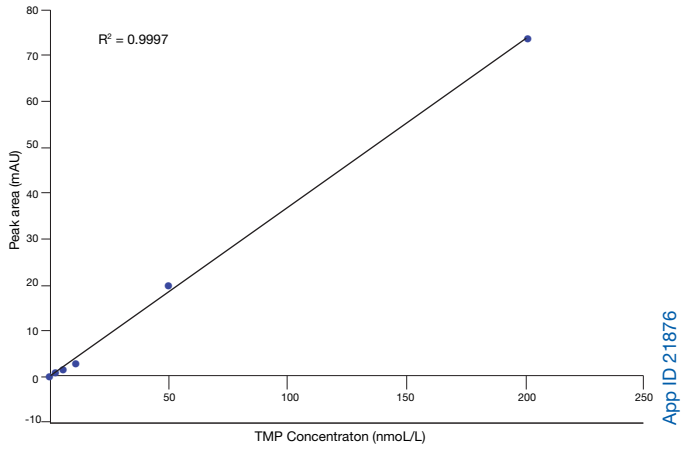


Table 1.
Standard curve (6-point) of TMP

TMP Standard Curve (6-point)	
Concentration (nmol/L)	Peak Area (mAU)
0	0
2.5	0.8
5	1.5
10	3.1
50	19.4
200	73.6

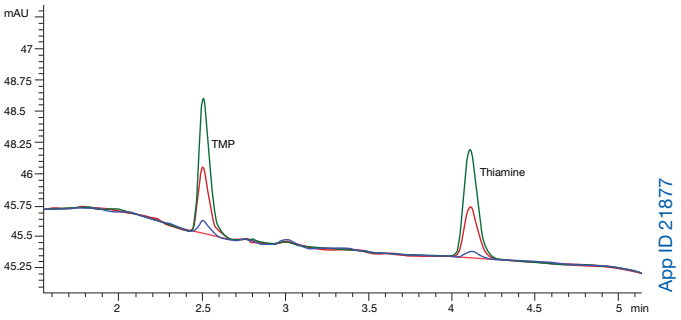
Table 2.
Recovery of TMP and thiamine from human plasma after cleanup with an Impact Protein Precipitation Plate

Recovery for Human Plasma TMP			Recovery for Human Plasma Thiamine		
Added TMP (nmol/L	Observed (nmol/L)	Recovery (%)	Added Thiamine (nmol/L)	Observed (nmol/L)	Recovery (%)
0	3.0		0	5.0	
5	7.0	87.5	5	10.8	108.0
10	12.8	98.5	10	18.4	122.7
	Mean	**93.0**		**Mean**	**115.4**

Table 3.
Accuracy studies for TMP and thiamine from human plasma after cleanup with an Impact Protein Precipitation Plate

	TMP Spiked Plasma Controls			Thiamine Spiked Plasma Controls		
Control No.	Expected (nmol/L)	Results (nmol/L)	Accuracy (%)	Expected (nmol/L)	Results (nmol/L)	Accuracy (%)
Control 1	40	38.7	96.8	40	34.2	85.5
Control 2	20	20.0	99.8	20	19.9	99.4
Control 3	10	10.7	107.0	10	8.2	81.8
		Mean	**101.2**		**Mean**	**88.9**

Figure 7.
Overlay of endogenous and spiked TMP and thiamine in plasma filtered by Impact



Blue = endogenous TMP (3 nmol/L) and thiamine (5 nmol/L)
Red = spiked TMP (5 nmol/L) and thiamine (5 nmol/L)
Green = spiked TMP (10 nmol/L) and thiamine (10 nmol/L)

Table 4.
Retention time reproducibility studies

	Retention Time (RT)	
Inj. No.	TMP (min)	Thiamine (min)
100	2.487	4.032
150	2.534	4.133
180	2.528	4.143
240	2.491	4.122
400	2.411	4.045
420	2.452	4.061
490	2.44	4.04
Mean	2.4818	4.0822
STEDV	0.0393	0.0483
%CV	**1.58**	**1.18**

Results and Discussion

When developing a method for the analysis of TMP and thiamine, 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 or breast milk 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 commonly 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**).

After the protein precipitation step, the plasma and breast milk samples were derivatized and analyzed by HPLC-FLD using a Gemini® 3 µm NX-C18 HPLC column. The Gemini 3 µm NX-C18 HPLC column 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 separation of TMP and thiamine (**Figures 2**, **3a**, and **3b**) was crucial in order to accurately quantify each compound in plasma (**Figure 7**) and breast milk (**Figures 4** and **5**).

The reproducibility of our analysis was determined by producing a standard curve of TMP at a concentration range of 0 to 200 nmol/L, resulting in a correlation coefficient of R^2 = 0.9997 (**Figure 6**). Thiamine was also subjected to a linearity curve at a concentration range of 0 to 200 nmol/L, resulting in a correlation coefficient of R^2 = 0.9993 (not shown). Even at low levels of detection, our method proved to be reproducible for both TMP and thiamine.

Compared with a water blank, the lower level TMP and thiamine standards (2.5 nmol/L) can be clearly distinguished (**Figure 3b**), showing that our extraction and HPLC method are extremely sensitive. Endogenous levels of TMP and thiamine were also studied in plasma (**Figure 7**), which showed that 3 nmol/L and 5 nmol/L were present, respectively.

Resulting recoveries of both target compounds averaged 93 % for human plasma TMP and 115 % for thiamine (**Table 2**). Accuracy studies resulted in an average accuracy of 101.2 for TMP and 88.9 for thiamine (**Table 3**), suggesting that our method not only provided acceptable recoveries but was also accurate and reproducible.

Retention times (RT) were stable by comparing several retention times between injection number 100 and 490, resulting in % CV's of 1.58 % for TMP and 1.18 % for thiamine (**Table 4**).

Conclusion

Vitamins are becoming a popular means to diagnose and treat disease states. With vitamin testing on the rise, laboratories must develop rapid, reliable, and robust analytical methods that can be easily incorporated into a high-throughput setting. Protein precipitation has always been a popular sample preparation method however the process can be improved upon. Using Impact Protein Precipitation Plates, TMP and thiamine were cleaned up from plasma and breast milk providing benefits such as minimal method development and processing time as well as ease of use. The resulting cleanup method can also be automated, allowing laboratories to save time by increasing productivity while improving reproducibility and reducing the risk of human error. Preparation by automated protein precipitation is also a versatile sample preparation technique as the resulting extract can be analyzed by several different methods including HPLC/UV, LC/MS/MS, and HPLC-FLD. Using HPLC-FLD, our separation method on the Gemini 3 µm NX-C18 HPLC column provided excellent resolution of TMP and thiamine and was sensitive enough to detect down to low levels, making it applicable to clinical analysis.

References

1. Wolfgan Stuetz, Verena Ilona Carrara, Rose McGready, Sue Jean Lee, Hans Konrad Biesalski and Francois Henry Noste. PLOS ONE, 2012.

APPLICATIONS

Ordering Information

Gemini® HPLC Columns

3 µm Microbore, Minibore and Narrow Bore Columns (mm)

Phases	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*
									/10pk
NX-C18	00M-4453-B0	00A-4453-B0	00B-4453-B0	00D-4453-B0	00F-4453-B0	00B-4453-Y0	00D-4453-Y0	00F-4453-Y0	AJ0-8367

for ID: 2.0-3.0 mm

3 µm Analytical Columns (mm)

Phases	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	SecurityGuard™ Cartridges (mm) 4 x 3.0*
					/10pk
NX-C18	00B-4453-E0	00D-4453-E0	00F-4453-E0	00G-4453-E0	AJ0-8368

for ID: 3.2-8.0 mm

5 µm Minibore and Narrow Bore Columns (mm)

Phases	30 x 2.0	50 x 2.0	150 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*
								/10pk
NX-C18	00A-4454-B0	00B-4454-B0	00F-4454-B0	00B-4454-Y0	00D-4454-Y0	00F-4454-Y0	00G-4454-Y0	AJ0-8367

for ID: 2.0-3.0 mm

5 µm Analytical Columns (mm)

Phases	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	SecurityGuard™ Cartridges (mm) 4 x 3.0*
					/10pk
NX-C18	00B-4454-E0	00D-4454-E0	00F-4454-E0	00G-4454-E0	AJ0-8368

for ID: 3.2-8.0 mm

Australia
t: 02-9428-6444
f: 02-9428-6445
auinfo@phenomenex.com

Austria
t: 01-319-1301
f: 01-319-1300
anfrage@phenomenex.com

Belgium
t: 02 503 4015 (French)
t: 02 511 8666 (Dutch)
f: +31 (0)30-2383749
beinfo@phenomenex.com

Canada
t: (800) 543-3681
f: (310) 328-7768
info@phenomenex.com

Denmark
t: 4824 8048
f: +45 4810 6265
nordicinfo@phenomenex.com

Finland
t: 09 4789 0063
f: +45 4810 6265
nordicinfo@phenomenex.com

France
t: 01 30 09 21 10
f: 01 30 09 21 11
franceinfo@phenomenex.com

Germany
t: 06021-58830-0
f: 06021-58830-11
anfrage@phenomenex.com

India
t: 040-3012 2400
f: 040-3012 2411
indiainfo@phenomenex.com

Ireland
t: 01 247 5405
f: +44 1625-501796
eireinfo@phenomenex.com

Italy
t: 051 6327511
f: 051 6327555
italiainfo@phenomenex.com

Luxembourg
t: +31 (0)30-2418700
f: +31 (0)30-2383749
nlinfo@phenomenex.com

Mexico
t: 001-800-844-5226
f: 001-310-328-7768
tecnicomx@phenomenex.com

The Netherlands
t: 030-2418700
f: 030-2383749
nlinfo@phenomenex.com

New Zealand
t: 09-4780951
f: 09-4780952
nzinfo@phenomenex.com

Norway
t: 810 02 005
f: +45 4810 6265
nordicinfo@phenomenex.com

Puerto Rico
t: (800) 541-HPLC
f: (310) 328-7768
info@phenomenex.com

Sweden
t: 08 611 6950
f: +45 4810 6265
nordicinfo@phenomenex.com

United Kingdom
t: 01625-501367
f: 01625-501796
ukinfo@phenomenex.com

United States
t: (310) 212-0555
f: (310) 328-7768
info@phenomenex.com

All other countries: Corporate Office USA
t: (310) 212-0555
f: (310) 328-7768
info@phenomenex.com

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Ordering Information

Impact™ Protein Precipitation Plates

Part No.	Description	Unit
Impact Precipitation Plates		
CE0-7565	Impact Protein Precipitation, Square Well, Filter Plate, 2 mL	2/box
Impact Starter Kit for Protein Precipitation		
CE0-8201	Impact Protein Precipitation Plate (2 ea) Collection Plate 2 mL (2 ea) Sealing Mat, Santoprene™ (AH0-8199) (2 ea)	ea

Accessories

Collection Plates (deep well, polypropylene)		
AH0-7192	Strata® 96-Well Collection Plate 350 µL/well	50/pk
AH0-7193	Strata 96-Well Collection Plate 1 mL/well	50/pk
AH0-7194	Strata 96-Well Collection Plate 2 mL/well	50/pk
AH0-8635	Strata 96-Well Collection Plate, 2 mL Square/Round-Conical	50/pk
AH0-8636	Strata 96-Well Collection Plate, 2 mL Round/Round, 8 mm	50/pk
AH0-7279	Strata 96-Well Collection Plate, 1 mL/well Round, 7 mm	50/pk
Sealing Mats		
AH0-8597	Sealing Mats, Pierceable, 96-Square Well, Silicone	50/pk
AH0-8598	Sealing Mats, Pre-Slit, 96-Square Well, Silicone	50/pk
AH0-8631	Sealing Mats, Pierceable, 96-Round Well 7 mm, Silicone	50/pk
AH0-8632	Sealing Mats, Pre-Slit, 96-Round Well 7 mm, Silicone	50/pk
AH0-8633	Sealing Mats, Pierceable, 96-Round Well 8 mm, Silicone	50/pk
AH0-8634	Sealing Mats, Pre-Slit, 96-Round Well 8 mm, Silicone	50/pk
AH0-7362	Sealing Tape Pad	10/pk
Vacuum Manifolds		
AH0-8950	Strata 96-Well Plate Manifold, Universal with Vacuum Gauge	ea

guarantee

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