Removal of Beta-Glucuronidase Enzyme from Urine Post-Hydrolysis to Improve Assay Performance and Column Lifetime

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Abstract

In order to facilitate their elimination via urinary excretion, many drugs are converted into more hydrophilic forms via glucuronidation or hydroxylation. Prior to analysis by HPLC, glucuronidated drugs must undergo a hydrolysis step. Acid hydrolysis provides the most efficient hydrolysis of glucuronidated drug metabolites, but the resulting samples corrode metal components in the analytical instrumentation, requiring increased maintenance frequency and cost. Acid hydrolysis can also destroy important opiate metabolites such as 6-monoacetylmorphine (6-MAM), the unique metabolite of heroin.

To overcome these challenges, many labs use an enzymatic process, but these have their own associated problems. If the enzymes are not removed from the sample prior to analysis, they will continue to react with drugs like codeine-6-glucuronide in the autosampler vial indefinitely, resulting in different measured values depending on the time point of analysis.

Many labs using enzymatic hydrolysis also suffer from reduced column lifetime that is often associated with an increase in column backpressure. Beta-glucuronidase is a 332 kDa enzyme that remains solubilized after the incubation. Centrifuging samples post-hydrolysis is a common step to help remove the enzyme before analysis. This process works well in tubes, but the current centrifuges available for 96-well plates do not allow for sufficient speed to precipitate the enzyme. The resulting samples have a high level of solubilized enzyme that can precipitate out on the head of the column during the analysis and negatively impact column lifetime.

The increase in popularity of the 96-well plate format for high-throughput analysis has created a need for a simple technique to remove the enzyme that does not add significant cost to the assay. In this work we demonstrate a simple post-hydrolysis step that removes the enzyme and significantly improves column lifetime.

Introduction

As the average age of healthcare patients increases, pain management medicine has a greater need for less expensive, quicker, more accurate, and more reproducible methods for analyzing the presence and concentration of the pain management medication in vivo. Chronic pain affects approximately 86 million people in the United States. Pain management centers have been established all over the country to help treat these chronic conditions. During treatment, routine testing is critical to prevent abuse. To decrease the cost per sample most laboratories try to limit the amount of sample cleanup and favor “dilute-and shoot” strategies. In such approaches, the hydrolyzed sample is centrifuged and the supernatant diluted with the appropriate volume of mobile phase for injection on the LC/MS/MS. The centrifugation step requires high speeds to fully pelletize the solubilized beta-glucuronidase enzyme.

This approach works very well in individual tubes where the centrifuge spin rate is sufficient. However, in some centrifuge models, the rotor arms that are used with 96-well plates reduce the maximum spin rate that can be achieved. The resulting extract has a significant portion of solubilized enzyme that, when injected, precipitates on the head of the HPLC column resulting in a rapid increase in backpressure and short column life. In this work, we demonstrate that this enzyme can be effectively removed using Impact™ Protein Precipitation 96-well plates prior to analysis.

Materials and Methods

All reagents and solvents were HPLC or analytical grade. Beta-glucuronidase was purchased from Campbell Science (Rockford, IL, USA). Analyses were performed using an Agilent® HP1100 LC system (Agilent Technologies, Palo Alto, CA USA) equipped with UV/VIS detector.

Sample Preparation and Protein Precipitation

Sample Hydrolysis Procedure

A 500 µL sample of urine was diluted with 100 µL buffer and 20 µL of 1,000,000 units/mL beta-glucuronidase in a 96-well collection plate. The samples were vortexed for 5-6 seconds and then incubated in a water bath at 63 °C for 30 minutes. The buffer was prepared by adding 800 mL of DI Water (Millipore or equivalent) and 111 mL of Glacial Acetic Acid to a 1 L volumetric flask. The final volume was adjusted to the line with a 50 % KOH solution and mixed by inversion several times before being transferred to a clean 1 L amber bottle. The final buffer solution had a pH reading between 4.7-4.8.

“Dilute-and-Shoot” Protocol

The hydrolyzed samples were sealed and centrifuged for 10 minutes at 2000 rpm (or the maximum possible centrifuge speed). The supernatant was then transferred to HPLC autosampler vials for analysis.

Column:	Kinetex 2.6 µm C18	
Dimensions:	50 x 3.0 mm	
Part No.:	00B-4462-Y0	
Mobile Phase:	A: 10 mM Ammonium formate	
	B: Methanol	
Gradient:	**Time (min)**	**B (%)**
	0	15
	0.08	15
	1.25	100
	1.33	100
	1.34	15
	3.00	15
Flow Rate:	0.5 mL/min	
Detection:	UV @ 220 nm	
Injection Volume:	50 µL	
Temperature:	Ambient	
Instrument:	Agilent® 1100	

Protein Precipitation
A 100 µL volume of the hydrolyzed sample was loaded directly onto an Impact Protein Precipitation 96-well plate (Phenomenex, Torrance, CA) that had been pre-loaded with 300 µL acetonitrile (ACN) or methanol (MeOH). The membrane used in the Impact product holds the ACN until vacuum is applied. The plate was sealed and then vortexed for 2 min at the maximum possible speed. A vacuum of 2-7 inches Hg was applied for 2-3 min until filtrate was collected. The resulting extracts were then evaporated to dryness and reconstituted in starting mobile phase before being transferred to HPLC autosampler vials for analysis.

HPLC Conditions

HPLC analysis was performed with a Kinetex® 2.6 µm C18, 50 x 3.0 mm column packed with core-shell particle media, providing high resolving power and fast analysis time.

Results and Discussion

When beta-glucuronidase hydrolyzed samples were directly injected onto the HPLC column, we observed column lifetime failure after only 15-20 injections, consistent with (**Figure 1**). Column lifetime was defined as a backpressure exceeding the Agilent® HP 1100 limit of 400 bar. The results were repeated using three separate columns to ensure that the results were reproducible.

The same hydrolyzed urine sample that was filtered using the Impact™ Protein Precipitation Plate showed stable pressure readings in excess of 500 injections (**Figure 2**). Further injections were not done as we did not see any reason to expect pressure to suddenly increase. When we transferred this protocol to a customer site, more than 1,000 injections with no increase in backpressure were observed. Looking in the wells of the Impact Protein Precipitation 96-well plate after the precipitation procedure, the precipitated enzyme was clearly visible (**Figure 3**). We have also observed, through data not presented here, that the enzyme can be removed using more selective techniques such as solid phase extraction (SPE).

Figure 1.
Premature Column Death After Only 15 Injections

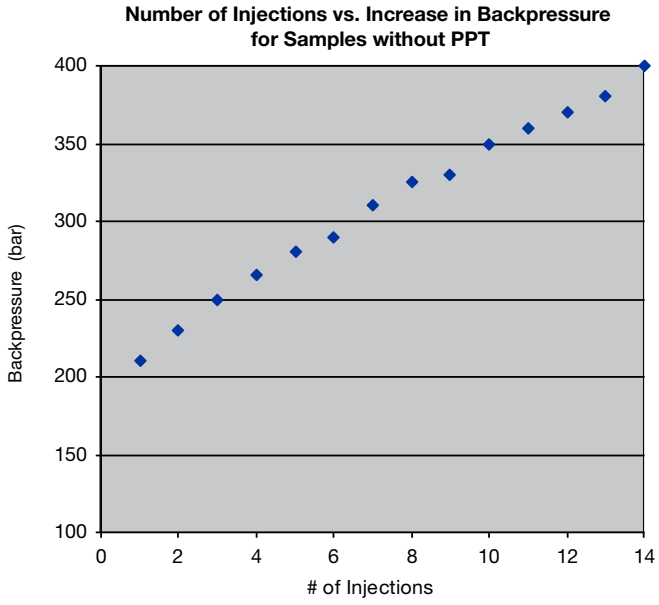

Figure 2.
Stable Pressure After Cleanup using Impact

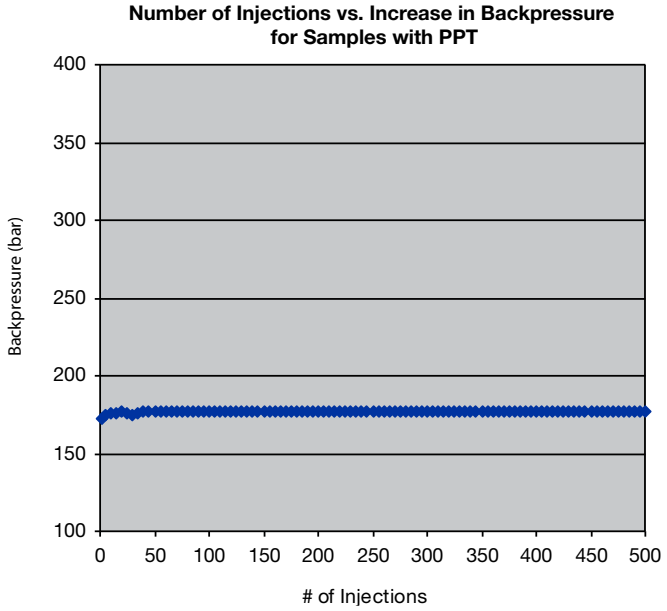

Figure 3.
Precipitated Enzyme on Impact Plate

Trapped-precipitated-enzyme on Impact following enzymatic hydrolysis using acetonitrile

Conclusion

In this study we presented a simple and effective method for removing beta-glucuronidase from enzymatically hydrolyzed urine samples. Hydrolysis using beta-glucuronidase results in a sample solution containing a significant amount of solubilized enzyme that must be removed before HPLC analysis. Centrifugation in 96-well plates is not effective in removing the solublized enzyme. Performing a protein precipitation step using Impact Protein Precipitation 96-well plates effectively removes the enzyme and was suitable for high-throughput testing. Once the enzyme was removed, an acceptable column lifetime was observed.

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

Kinetex® HPLC Columns

2.6 µm Analytical Columns (mm)

	30 x 4.6	50 x 4.6	75 x 4.6	100 x 4.6	150 x 4.6	SecurityGuard™ ULTRA Cartridges* 3/pk
XB-C18	—	00B-4496-E0	00C-4496-E0	00D-4496-E0	00F-4496-E0	AJ0-8768
C18	00A-4462-E0	00B-4462-E0	00C-4462-E0	00D-4462-E0	00F-4462-E0	AJ0-8768
C8	—	00B-4497-E0	00C-4497-E0	00D-4497-E0	00F-4497-E0	AJ0-8770
PFP	00A-4477-E0	00B-4477-E0	00C-4477-E0	00D-4477-E0	00F-4477-E0	AJ0-8773
HILIC	—	00B-4461-E0	00C-4461-E0	00D-4461-E0	00F-4461-E0	AJ0-8772
Phenyl-Hexyl	—	00B-4495-E0	00C-4495-E0	00D-4495-E0	00F-4495-E0	AJ0-8774

for 4.6 mm ID

2.6 µm MidBore™ Columns (mm)

	30 x 3.0	50 x 3.0	75 x 3.0	100 x 3.0	150 x 3.0	SecurityGuard ULTRA Cartridges* 3/pk
XB-C18	00A-4496-Y0	00B-4496-Y0	00C-4496-Y0	00D-4496-Y0	00F-4496-Y0	AJ0-8775
C18	00A-4462-Y0	00B-4462-Y0	00C-4462-Y0	00D-4462-Y0	00F-4462-Y0	AJ0-8775
C8	00A-4497-Y0	00B-4497-Y0	00C-4497-Y0	00D-4497-Y0	00F-4497-Y0	AJ0-8777
PFP	00A-4477-Y0	00B-4477-Y0	00C-4477-Y0	00D-4477-Y0	00F-4477-Y0	AJ0-8780
HILIC	00A-4461-Y0	—	—	—	00F-4461-Y0	AJ0-8779
Phenyl-Hexyl	—	—	—	00D-4495-Y0	00F-4495-Y0	AJ0-8781

for 3.0 mm ID

2.6 µm Minibore Columns (mm)

	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	SecurityGuard ULTRA Cartridges* 3/pk
XB-C18	00A-4496-AN	00B-4496-AN	00D-4496-AN	00F-4496-AN	AJ0-8782
C18	00A-4462-AN	00B-4462-AN	00D-4462-AN	00F-4462-AN	AJ0-8782
C8	00A-4497-AN	00B-4497-AN	00D-4497-AN	00F-4497-AN	AJ0-8784
PFP	00A-4477-AN	00B-4477-AN	00D-4477-AN	00F-4477-AN	AJ0-8787
HILIC	00A-4461-AN	00B-4461-AN	00D-4461-AN	00F-4461-AN	AJ0-8786
Phenyl-Hexyl	00A-4495-AN	00B-4495-AN	00D-4495-AN	00F-4495-AN	AJ0-8788

for 2.1 mm ID

*SecurityGuard ULTRA cartridges require holder, Part No.: AJ0-9000

guarantee

If Phenomenex products in this technical note do not provide at least an equivalent separation as compared to other products of the same phase and dimensions, return the product with your comparative data within 45 days for a FULL REFUND.

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