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

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Abstract

Acid hydrolysis provides the most efficient conversion of 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 they 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 complain about 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 rotors available for 96-well plates do not allow for sufficient speed to bring down the enzyme. The resulting samples have a high level of solubilized enzyme that can precipitate out on the head of the column during the gradient and negatively impacts 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 clean up 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 enzyne can be removed using Impact™ 96-well plates prior to analysis.

Experimental Conditions

Sample Hydrolysis Procedure:

A 500 µL sample of urine was diluted with 100 µL buffer and 20 µL 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 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 by the centrifuge). The supernatant was then transferred to an HPLC autosampler vial for analysis.

Protein Precipitation:

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

HPLC Conditions:

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
	3.0	15
Flow Rate:	0.5 mL/min	
Detection:	UV @ 220 nm	
Injection Volume:	50 µL	
Temperature:	Ambient	
Instrument:	Agilent® 1100	

Figure 1. **Premature Column Death After Only 15 Injections**

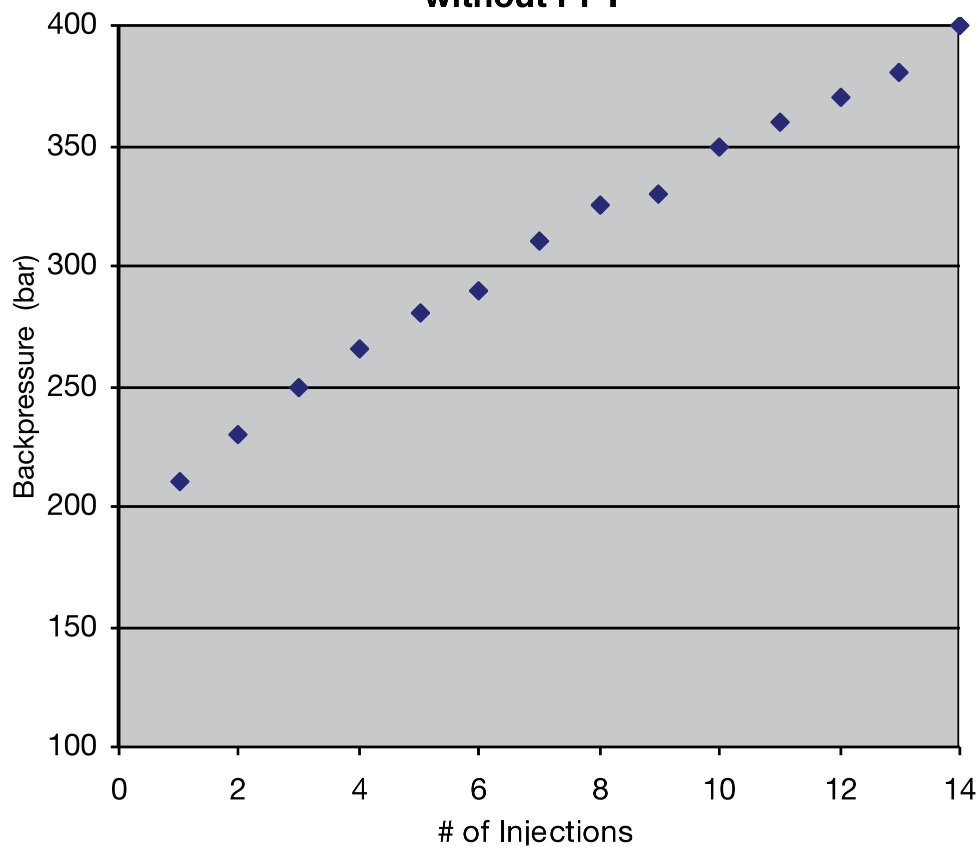


Figure 2. Stable Pressure After Cleanup using Impact

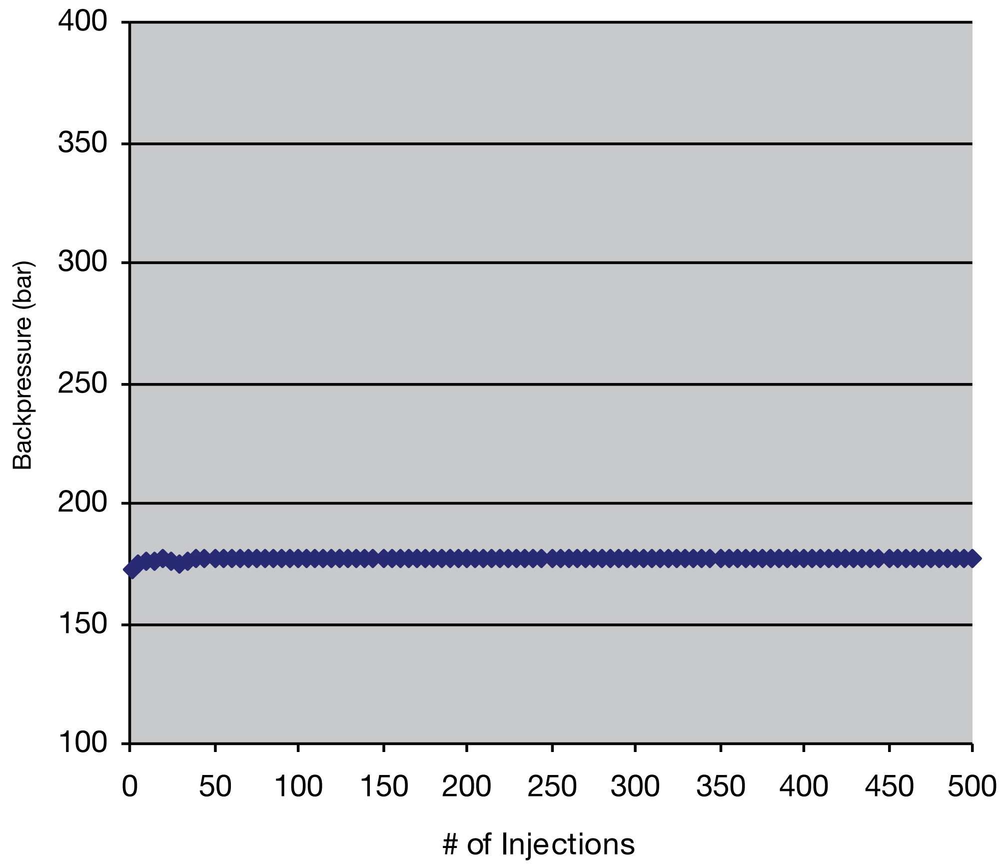
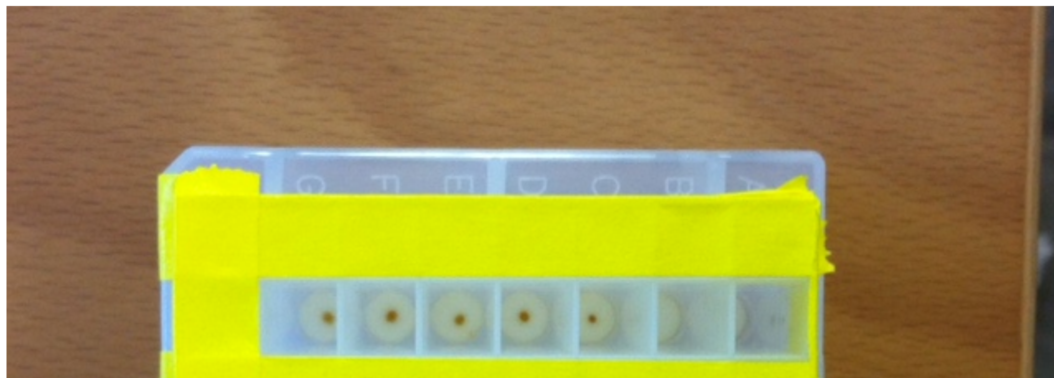


Figure 3. Precipitated Enzyme on Impact Plate



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

Results and Discussions

When the hydrolyzed samples were directly injected onto the HPLC column, we observed death after only 15-20 injections, which was consistent with results we had heard from customers (**Figure 1**). Column death was defined as a backpressure exceeding the HP 1100 limit of 400 bar. The results were repeated using three separate columns to ensure that the results were valid.

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. We transferred this protocol to a customer site and observed more than 1,000 injections with no increase in backpressure.

Looking in the wells of the Impact 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).

Conclusion

Hydrolysis using beta-glucuronidase results in a solution that contains 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 removes the enzyme and is suitable for high-throughput environments.

Once the enzyme is removed, acceptable column lifetime is observed.

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