APPLICATIONS

# Validation of an Automated Solution for Post Hydrolysis Enzyme Removal and LC/MS/MS Analysis of Illicit and Pain Related Compounds from Urine

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*The escalating abuse of pain medicines has mandated that health care providers and practitioners routinely perform urine drug testing. Most laboratories opt for a dilute-and-shoot approach, along with enzymatic hydrolysis that requires no sample cleanup. The addition of extra protein as a result of hydrolysis using β-glucuronidase to the urine can result in fouling of the LC column and loss of productivity. In this work, we present an automated method that uses an Impact™ Protein Precipitation 96-well plate to remove a majority of the proteins from hydrolyzed urine.*

## Introduction

The gradual dependence and addiction of pain medication has made pain management and monitoring one of the fastest growing clinical market segments today. The escalating abuse of pain medicines has mandated that health care providers and practitioners routinely perform urine drug testing using a reliable approach that is fast and cost effective. To meet the demand for a large accrual of samples, most laboratories adopt a dilute-and-shoot approach that requires virtually no sample cleanup. However, the samples must undergo some form of hydrolysis procedure to de-conjugate the metabolized compounds back to their native form. An enzymatic procedure using β-glucuronidase is the most readily accepted form of hydrolysis. However, the addition of extra protein to the urine can result in plugging or otherwise fouling (**Figure 1**) of the LC column[1]. The continuous increase in system pressure resulting from denatured enzyme within the column will reduce the column and assay performance. In this work, we present a validated procedure that uses an Impact Protein Precipitation 96-well plate to remove the enzyme (and other proteins) from urine (**Figure 2**). To increase productivity and accuracy, we utilized a Tecan Freedom EVO® 100 Liquid Handling System (or similar) in conjunction with a Kinetex® 2.6 µm Phenyl-Hexyl 50 x 4.6 mm core-shell HPLC column. The chromatographic conditions were adequately efficient to accommodate fifty one (51) pain panel compounds in less than six (6) minutes.

## Experimental Conditions

### HPLC Conditions

**Column:** Kinetex 2.6 µm Phenyl-Hexyl
**Dimensions:** 50 x 4.6 mm
**Part No.:** 00B-4495-E0
**Mobile Phase:** A: 0.1 % Formic acid in Water
B: 0.1 % Formic acid in Methanol
**Gradient:**

| Time (min) | % B |
|---|---|
| 0.0 | 5 |
| 5.5 | 95 |
| 6.0 | 95 |
| 6.01 | 5 |
| 6.5 | 5 |

**Flow Rate:** 1.0 mL/min
**Injection Volume:** 5 µL
**Instrument:** Shimadzu® XR
**Detection:** MS/MS, SCIEX QTRAP® 6500 (Positive Ionization)
**Sample Preparation Automation:** Tecan Freedom EVO® 100 with MultiChannel Arm™ 96 (MCA96)

**Note:** Additional equilibration time is included in the acquisition method

### *Sample Preparation*

Sample Hydrolysis Procedure

A 500 µL sample of urine was diluted with 100 µL acetate buffer (pH 4.5-4.8) and 20 µL β-glucuronidase, 106 units per mL (DR2100, www.campbellscience.com) in a 96-well collection plate. The samples were vortexed for 10-15 seconds and then incubated in a water bath at 63 °C for 30 minutes.

Dilute-and-Shoot Protocol

The hydrolyzed samples were sealed and centrifuged for 10 minutes at 2000 rpm (or the maximum possible speed by the centrifuge). The supernatant was then transferred to a LC/MS/MS for analysis.

Protein Precipitation

A 100 µL volume of the hydrolyzed sample was loaded directly to an Impact (2 mL Square Well Filter Plate, Part No. CE0-7565) Protein Precipitation 96-well plate that had been pre-loaded with 400 µL methanol. The plate was sealed and then vortexed for 2 minutes at the maximum possible speed. A vacuum of 2-7" of Hg was applied for 2-3 minutes until filtrate was collected. The resulting extract was then evaporated to dryness and reconstituted in starting mobile phase before being transferred for LC/MS/MS analysis.

**Figure 1.**
Number of injections vs. backpressure for dilute-and-shoot samples

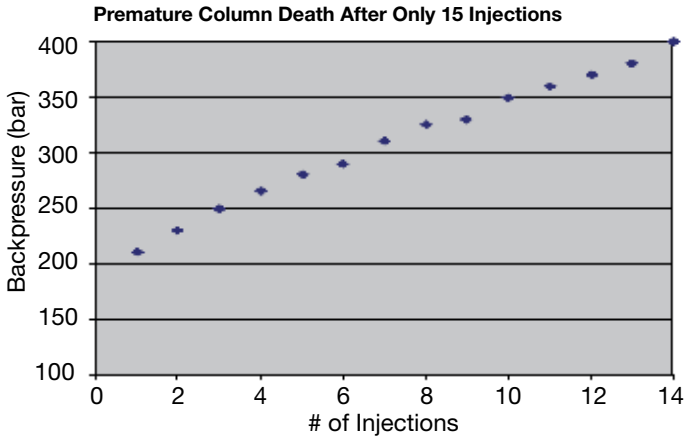

**Figure 2.**
Number of injections vs. backpressure for protein precipitated samples

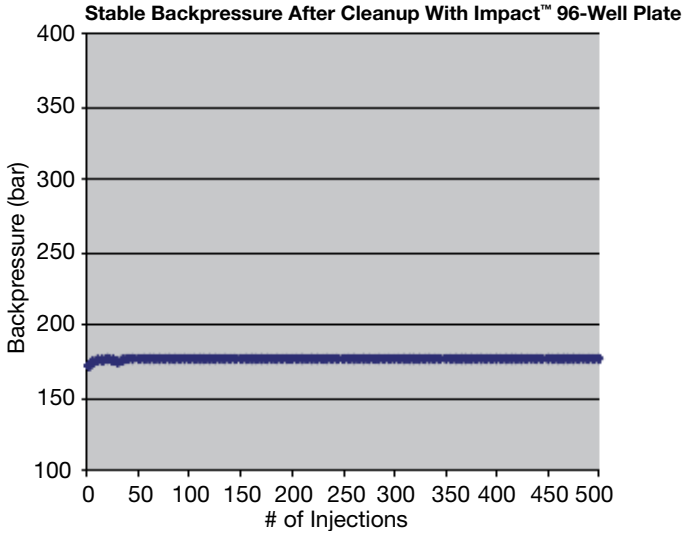


**Figure 3.**
LC/MS/MS analysis of 51 compounds in less than 6 minutes

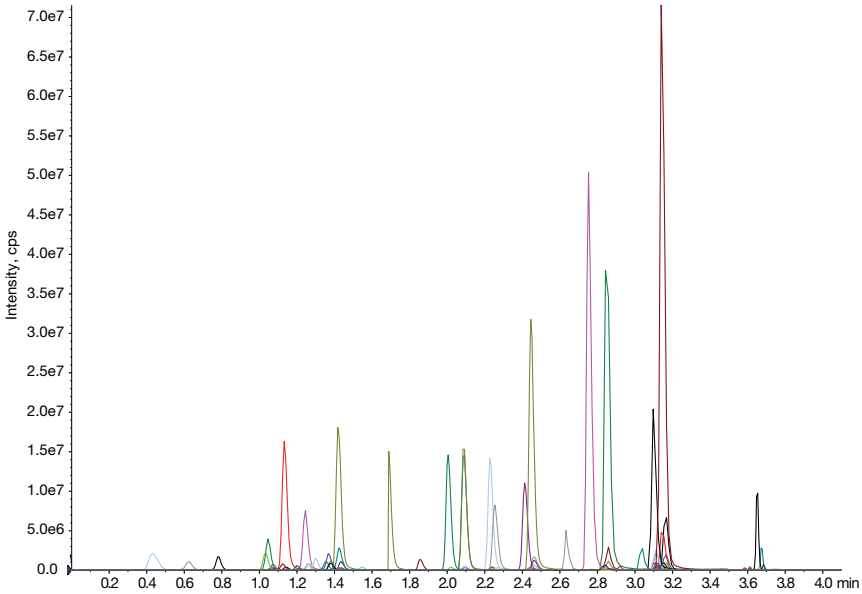


**Figure 4.**
Calibration curve for benzodiazepines

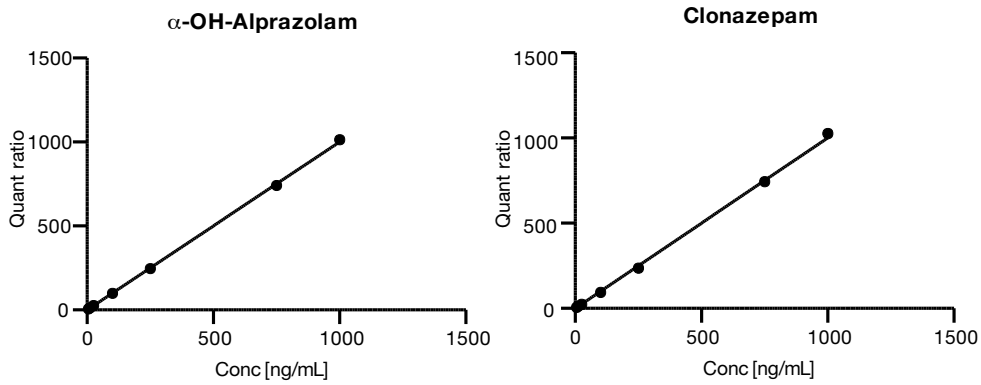

**Figure 5.**
Calibration curve for opiates

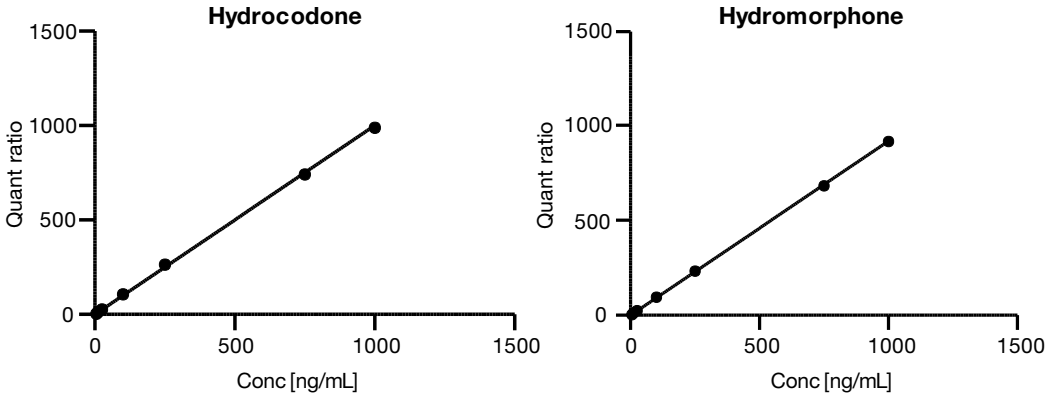


**Figure 6.**
Calibration curve for amphetamines

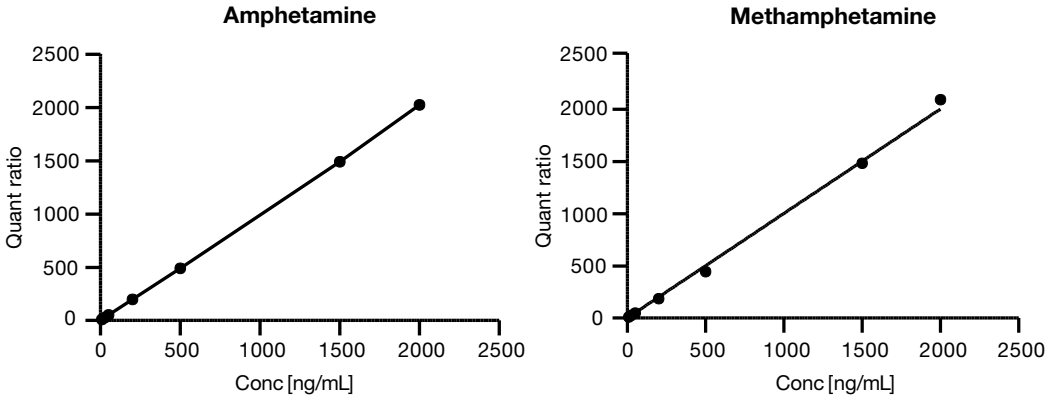


**Figure 7.**
Calibration curve for analgesics

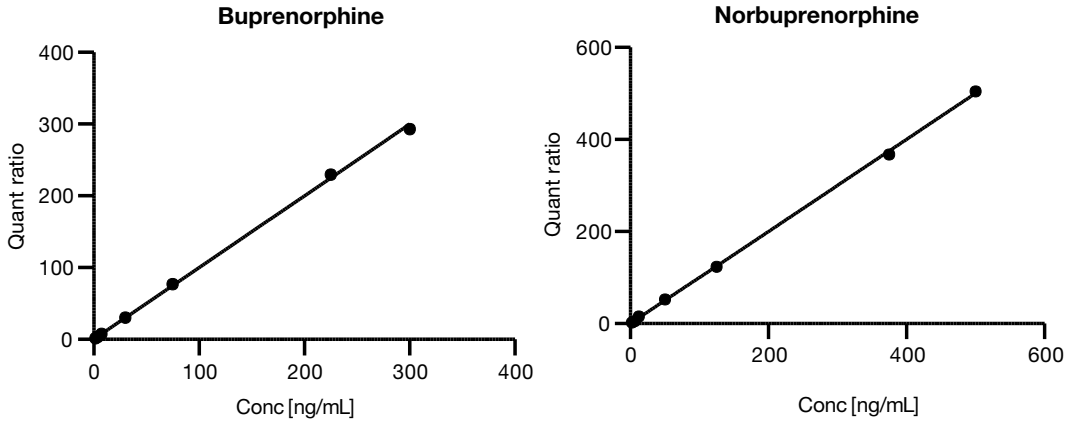

**Figure 8.**
Matrix effect summary

| Compound | % Matrix Effect | Compound | % Matrix Effect | Compound | % Matrix Effect |
|---|---|---|---|---|---|
| 6-MAM | 97.39 | MDMA | 105.72 | Oxymorphone | 106.48 |
| α–OH-Alprazolam | 105.88 | Meperidine | 100.88 | PCP | 96.62 |
| Alprazolam | 103.71 | Meprobamate | 109.15 | Propoxyphene | 94.88 |
| Amitriptyline | 101.26 | Methadone | 104.77 | Temazepam | 102.66 |
| Amphetamine | 103.66 | Methamphetamine | 96.82 | Tramadol | 102.9 |
| Benzoylecgonine | 107.37 | Morphine | 98.97 | Butalbital | 95.53 |
| Buprenorphine | 118.52 | Naloxone | 106.81 | Phenobarbital | 97.9 |
| Carisoprodol | 113.79 | Norbuprenorphine | 102.84 | Methylphenidate | 101.19 |
| Clonazepam | 88.85 | Nordiazepam | 101.99 | Desipramine | 100.38 |
| Codeine | 110.88 | Norfentanyl | 104.61 | Imipramine | 99.75 |
| EDDP | 98.41 | Norhydrocodone | 105.19 | 7-Aminoclonazepam | 120.89 |
| Fentanyl | 102.21 | Nortriptyline | 102.04 | Zolpidem | 109.61 |
| Hydrocodone | 98.5 | Oxazepam | 103.37 | Gabapentin | 98.58 |
| Hydromorphone | 103.56 | Oxycodone | 100.4 | THCA | 90.26 |

**Figure 9.**
Lower and upper limits of quantitation (ng/mL)

| Drug Name | LLOQ | ULOQ |
|---|---|---|
| 6-MAM | 2.5 | 500 |
| α-OH-Alprazolam | 5 | 1000 |
| Alprazolam | 5 | 1000 |
| Amitriptyline | 10 | 2000 |
| Amphetamine | 10 | 2000 |
| Benzoylecgonine | 5 | 1000 |
| Buprenorphine | 1.5 | 300 |
| Carisoprodol | 10 | 2000 |
| Clonazepam | 5 | 1000 |
| Codeine | 10 | 2000 |
| Cyclobenzaprine | 10 | 2000 |
| EDDP | 10 | 2000 |
| Fentanyl | 0.5 | 100 |
| Hydrocodone | 5 | 1000 |
| Hydromorphone | 5 | 1000 |
| Lorazepam | 5 | 1000 |
| MDMA | 10 | 2000 |
| Meperidine | 5 | 1000 |
| Meprobamate | 10 | 2000 |
| Methadone | 10 | 2000 |
| Methamphetamine | 10 | 2000 |
| Morphine | 5 | 1000 |
| Naloxone | 10 | 2000 |
| Norbuprenorphine | 2.5 | 500 |
| Nordiazepam | 5 | 1000 |

**Figure 9. *Continued***
Lower and upper limits of quantitation (ng/mL)

| Drug Name | LLOQ | ULOQ |
|---|---|---|
| Norfentanyl | 0.5 | 100 |
| Norhydrocodone | 10 | 2000 |
| Noroxycodone | 5 | 1000 |
| Nortriptyline | 10 | 2000 |
| Oxazepam | 5 | 1000 |
| Oxycodone | 5 | 1000 |
| Oxymorphone | 5 | 1000 |
| PCP | 2.5 | 500 |
| Propoxyphene | 10 | 2000 |
| Temazepam | 5 | 1000 |
| Tramadol | 5 | 1000 |
| Butalbital | 12.5 | 2500 |
| Phenobarbital | 12.5 | 2500 |
| Tapentadol | 5 | 1000 |
| Methylphenidate | 5 | 1000 |
| Desipramine | 5 | 1000 |
| Imipramine | 5 | 1000 |
| 7-Aminoclonazepam | 5 | 1000 |
| Duloxetine | 25 | 1000 |
| Zolpidem | 5 | 1000 |
| MDPV | 5 | 1000 |
| Mephedrone | 5 | 1000 |
| Methylone | 5 | 1000 |
| Gabapentin | 80 | 16000 |
| Sovaldi | 10 | 2000 |
| THCA | 12.5 | 500 |

**Figure 10.**
Method precision for high conc. (replicates = 12)

| Analyte | Conc. (ng/mL) | Within-Run Precision (%) | Within-Laboratory Precision (%) | Interday Precision (%) | Intraday Precision (%) |
|---|---|---|---|---|---|
| 6-MAM | 250 | 1.67 | 2.15 | 1.15 | 0.73 |
| α-OH-Alprazolam | 500 | 2.68 | 3.53 | 2.33 | 0.4 |
| Alprazolam | 500 | 2.62 | 2.41 | 1.2 | 1.57 |
| Amitriptyline | 1000 | 7.29 | 8.23 | 2.64 | 2.75 |
| Amphetamine | 1000 | 3.2 | 2.79 | 1.49 | 2.16 |
| Benzoylecgonine | 500 | 2.17 | 3.47 | 2.95 | 1.17 |
| Buprenorphine | 150 | 12.23 | 12.23 | 6.12 | 6.12 |
| Carisoprodol | 1000 | 6.19 | 15.09 | 12.3 | 6.17 |
| Clonazepam | 500 | 3.37 | 3.95 | 2.55 | 1.49 |
| Codeine | 1000 | 3.19 | 4.84 | 3.78 | 1.05 |
| EDDP | 1000 | 5.79 | 9.73 | 5.34 | 5.72 |
| Fentanyl | 50 | 3.16 | 4.46 | 2.88 | 1.26 |
| Hydrocodone | 500 | 2.36 | 2.31 | 0.82 | 0.96 |
| Hydromorphone | 500 | 1.99 | 3.52 | 3.18 | 1.3 |
| Lorazepam | 500 | 3.4 | 3.59 | 1.22 | 0.46 |
| MDMA | 1000 | 1.91 | 2.99 | 2.27 | 0.39 |
| Meperidine | 500 | 3.04 | 3.52 | 2.52 | 1.79 |
| Meprobamate | 1000 | 6.25 | 6.23 | 3.38 | 3.34 |
| Methadone | 1000 | 5.29 | 9.87 | 4.8 | 9.61 |
| Methamphetamine | 1000 | 2.84 | 6.29 | 4.94 | 2.66 |
| Morphine | 500 | 1.63 | 2.78 | 2.28 | 0.38 |
| Naloxone | 1000 | 2.92 | 2.61 | 0.6 | 1.45 |
| Norbuprenorphine | 250 | 5.89 | 5.48 | 3.14 | 3.81 |
| Nordiazepam | 500 | 2.63 | 2.41 | 0.99 | 0.41 |
| Norfentanyl | 50 | 2.49 | 2.67 | 1.93 | 1.67 |
| Norhydrocodone | 1000 | 1.75 | 1.58 | 0.68 | 1.02 |
| Nortriptyline | 1000 | 3.54 | 3.38 | 2.09 | 2.35 |
| Oxazepam | 500 | 2.92 | 2.76 | 0.38 | 0.88 |
| Oxycodone | 500 | 3.29 | 3.47 | 2.4 | 2.11 |
| Oxymorphone | 500 | 1.73 | 2.4 | 1.92 | 0.96 |
| PCP | 250 | 5.04 | 8.56 | 1.27 | 6.8 |
| Propoxyphene | 1000 | 8.76 | 14.67 | 10.16 | 5.95 |
| Temazepam | 500 | 2.91 | 3.87 | 1.8 | 1.81 |
| Tramadol | 500 | 1.74 | 2.77 | 2.39 | 1.03 |
| Butalbital | 1250 | 2.12 | 4.31 | 1.36 | 3.99 |
| Phenobarbital | 1250 | 4.57 | 6.79 | 4.88 | 1.18 |
| Desipramine | 500 | 2.95 | 2.98 | 1.71 | 1.65 |
| Imipramine | 500 | 2.64 | 4.68 | 3.79 | 0.78 |
| 7-Aminoclonazepam | 500 | 2.92 | 3.58 | 2.38 | 1.16 |
| Zolpidem | 500 | 3.06 | 3.73 | 2.61 | 1.5 |
| MDPV | 500 | 3.01 | 3.48 | 2.43 | 1.69 |
| Gabapentin | 8000 | 2.84 | 3.07 | 2.23 | 1.9 |
| THCA | 250 | 1.37 | 5.56 | 5.19 | 1.46 |

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**Figure 11.**
Method precision for low conc. (replicates = 12)

| Analyte | Conc. (ng/mL) | Within-Run Precision (%) | Within-Laboratory Precision (%) | Interday Precision (%) | Intraday Precision (%) |
|---|---|---|---|---|---|
| 6-MAM | 25 | 4.31 | 6.15 | 4.21 | 1.24 |
| α-OH-Alprazolam | 50 | 2.32 | 5.58 | 4.95 | 1.12 |
| Alprazolam | 50 | 2.92 | 5.62 | 4.96 | 1.23 |
| Amitriptyline | 100 | 5.8 | 8.08 | 3.62 | 4.3 |
| Amphetamine | 100 | 3.27 | 13.93 | 13.38 | 2.08 |
| Benzoylecgonine | 50 | 3.26 | 6.27 | 5.37 | 0.45 |
| Buprenorphine | 15 | 16.06 | 15.38 | 4.33 | 6.33 |
| Carisoprodol | 100 | 10.31 | 17.58 | 13.72 | 3.8 |
| Clonazepam | 50 | 4.92 | 5.15 | 3.6 | 3.26 |
| Codeine | 100 | 3.14 | 4.28 | 3.29 | 1.53 |
| EDDP | 100 | 5.64 | 5.94 | 4.01 | 3.56 |
| Fentanyl | 5 | 4.47 | 4.47 | 0 | 0 |
| Hydrocodone | 50 | 2.57 | 11.19 | 10.85 | 1 |
| Hydromorphone | 50 | 2.93 | 5.04 | 4.38 | 1.55 |
| Lorazepam | 50 | 3 | 4.27 | 2.66 | 1.48 |
| MDMA | 100 | 1.8 | 2.95 | 2.6 | 1.14 |
| Meperidine | 50 | 2.61 | 5.02 | 4.49 | 1.34 |
| Meprobamate | 100 | 8.75 | 15.31 | 3.13 | 12.16 |
| Methadone | 100 | 5.76 | 6.93 | 3.38 | 5.13 |
| Methamphetamine | 100 | 2.66 | 3.99 | 3.27 | 1.34 |
| Morphine | 50 | 3.29 | 5.01 | 4.21 | 1.84 |
| Naloxone | 100 | 3.57 | 3.51 | 1.85 | 1.97 |
| Norbuprenorphine | 25 | 14.14 | 14.38 | 7.62 | 7.16 |
| Nordiazepam | 50 | 3.49 | 5.03 | 3.63 | 0 |
| Norfentanyl | 5 | 2.16 | 3.28 | 2.68 | 1.02 |
| Norhydrocodone | 100 | 3.75 | 3.56 | 2.22 | 2.5 |
| Nortriptyline | 100 | 4.92 | 5.7 | 4.46 | 3.41 |
| Oxazepam | 50 | 2.79 | 9.64 | 8.95 | 2.28 |
| Oxycodone | 50 | 3.1 | 4.54 | 3.55 | 1.26 |
| Oxymorphone | 50 | 3.55 | 4.95 | 4.14 | 2.28 |
| PCP | 25 | 9.8 | 8.29 | 3.35 | 4 |
| Propoxyphene | 100 | 12.18 | 13.32 | 4.36 | 6.94 |
| Temazepam | 50 | 2.32 | 4.63 | 3.53 | 1.9 |
| Tramadol | 50 | 2.97 | 4.75 | 4.14 | 1.84 |
| Butalbital | 125 | 5.08 | 6.28 | 3.68 | 5.22 |
| Phenobarbital | 125 | 0 | 6.79 | 4.88 | 1.18 |
| Methylphenidate | 50 | 4.77 | 5.01 | 3.42 | 3.07 |
| Desipramine | 50 | 3.66 | 4.96 | 3.77 | 1.73 |
| Imipramine | 50 | 3 | 7.05 | 6.46 | 1 |
| 7-Aminoclonazepam | 50 | 2.72 | 11.62 | 11.31 | 0.45 |
| Zolpidem | 50 | 3.16 | 5.2 | 4.5 | 1.79 |
| MDPV | 50 | 2.97 | 4.33 | 3.54 | 1.61 |
| Gabapentin | 800 | 1.98 | 5.19 | 4.97 | 1.29 |
| THCA | 25 | 2.68 | 7.67 | 6.8 | 2.37 |

## Results and Discussion

1. The presence of additional protein (β-glucuronidase) in a urine sample can denature in the column during a normal chromatographic run. The resulting fouling will quickly plug the HPLC column and render it useless (**Figure 1**). However, removal of the protein from the samples by a simple precipitation procedure can enhance the longevity of the column (**Figure 2**).

2. The protein precipitation method evaluated here produced good chromatographic separation of the compounds (**Figure 3**) with acceptable calibration parameters (**Figures 4-7**).

3. The method produced minimal matrix effect despite a simple sample preparation procedure from an inherently dirty sample matrix (**Figure 8**).

4. In addition, great method precision was achieved at both low and high quantitation levels (**Figures 10-11**).

## Conclusion

- Performing protein precipitation after enzymatic hydrolysis provides cleaner extracts and extends the HPLC column lifetime.
- Fifty one (51) compounds and fourteen (14) deuterated analogs were efficiently and quickly resolved in 6 minutes using a Kinetex® Phenyl-Hexyl core-shell HPLC column.
- The sample preparation proved to be robust requiring no method development.
- This method produced excellent calibration range.
- The calculated matrix effect results for all the evaluated analytes fell within acceptable limits.
- This bioanalytical method is proven to be suitable for a wide range of acidic, basic, and neutral compounds.

## References

1. S Huq, S Sadjadi, and S Countryman, "Removal of Beta-Glucuronidase Enzyme from Urine Post-Hydrolysis to Improve Assay Performance and Column Lifetime." Mass Spec Application for Clinical Laboratory Conference, 2013

# APPLICATIONS

## Ordering Information

### Impact™ Precipitation Products

| Part No. | Description | Unit |
|---|---|---|
| **Impact Precipitation Products** | | |
| **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** | | |
| **CE0-8201** | Impact Protein Precipitation Plate (CE0-7565) (2 ea)<br>Collection Plate 2 mL (2 ea)<br>Sealing Mat, Santoprene™ (AH0-8199) (2 ea) | ea |

### Kinetex® HPLC Columns

**2.6 µm Minibore Columns (mm)**

| Phases | 30 x 2.1 | 50 x 2.1 | 75 x 2.1 | 100 x 2.1 | 150 x 2.1 | SecurityGuard™ ULTRA Cartridges‡ 3/pk |
|---|---|---|---|---|---|---|
| **EVO C18** | 00A-4725-AN | 00B-4725-AN | — | 00D-4725-AN | 00F-4725-AN | AJ0-9298 |
| **F5** | 00A-4723-AN | 00B-4723-AN | — | 00D-4723-AN | 00F-4723-AN | AJ0-9322 |
| **Biphenyl** | 00A-4622-AN | 00B-4622-AN | — | 00D-4622-AN | 00F-4622-AN | AJ0-9209 |
| **XB-C18** | 00A-4496-AN | 00B-4496-AN | 00C-4496-AN | 00D-4496-AN | 00F-4496-AN | AJ0-8782 |
| **C18** | 00A-4462-AN | 00B-4462-AN | 00C-4462-AN | 00D-4462-AN | 00F-4462-AN | AJ0-8782 |
| **C8** | 00A-4497-AN | 00B-4497-AN | 00C-4497-AN | 00D-4497-AN | 00F-4497-AN | AJ0-8784 |
| **HILIC** | 00A-4461-AN | 00B-4461-AN | 00C-4461-AN | 00D-4461-AN | 00F-4461-AN | AJ0-8786 |
| **Phenyl-Hexyl** | 00A-4495-AN | 00B-4495-AN | 00C-4495-AN | 00D-4495-AN | 00F-4495-AN | AJ0-8788 |
| | | | | | | for 2.1 mm ID |

**2.6 µm MidBore™ Columns (mm)**

| Phases | 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 |
|---|---|---|---|---|---|---|
| **EVO C18** | — | 00B-4725-Y0 | — | 00D-4725-Y0 | 00F-4725-Y0 | AJ0-9298 |
| **F5** | — | 00B-4723-Y0 | — | 00D-4723-Y0 | 00F-4723-Y0 | AJ0-9321 |
| **Biphenyl** | — | 00B-4622-Y0 | — | 00D-4622-Y0 | 00F-4622-Y0 | AJ0-9208 |
| **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 |
| **HILIC** | 00A-4461-Y0 | — | — | — | 00F-4461-Y0 | AJ0-8779 |
| **Phenyl-Hexyl** | — | 00B-4495-Y0 | — | 00D-4495-Y0 | 00F-4495-Y0 | AJ0-8781 |
| | | | | | | for 3.0 mm ID |

**2.6 µm Analytical Columns (mm)**

| Phases | 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 |
|---|---|---|---|---|---|---|
| **EVO C18** | — | 00B-4725-E0 | — | 00D-4725-E0 | 00F-4725-E0 | AJ0-9296 |
| **F5** | — | 00B-4723-E0 | — | 00D-4723-E0 | 00F-4723-E0 | AJ0-9320 |
| **Biphenyl** | — | 00B-4622-E0 | — | 00D-4622-E0 | 00F-4622-E0 | AJ0-9207 |
| **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 |
| **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 |

‡ 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 comparative data within 45 days for a FULL REFUND.

# APPLICATIONS

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