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Validation of an Automated Method to Remove β-Glucuronidase from Hydrolyzed Pain Management Urine Sample

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Abstract

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.

Intrduction

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 column1. 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® 2.6µm Phenyl-Hexyl 50 x 4.6mm core-shell HPLC column. The chromatographic conditions were adequately efficient to accommodate fifty one (51) pain panel compounds in less then six (6) minutes.

Experimental Conditions

HPLC

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 water 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, AB 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, 10^6 units (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

Premature Column Death After Only 15 Injections

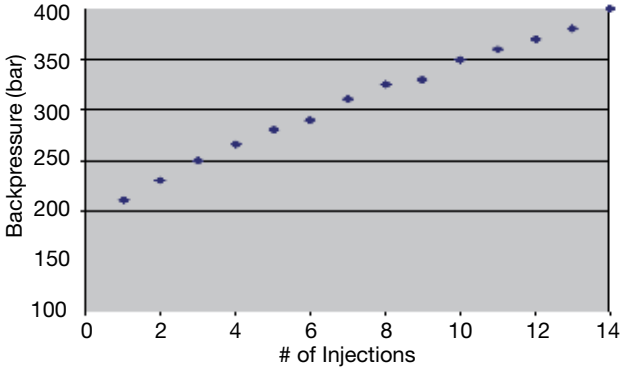

Figure 2. Number of injections vs. backpressure for protein precipitated samples

Stable Backpressure After Cleanup with Impact 96-Well Plate

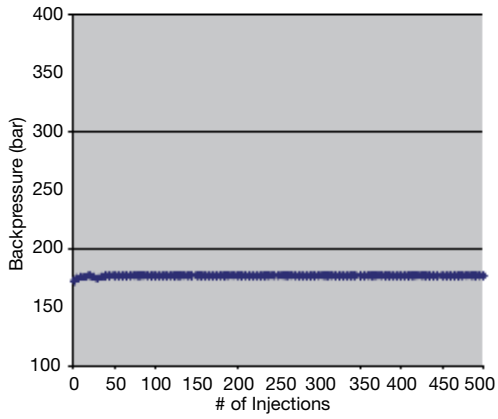

Figure 3. LC/MS/MS analysis of 51 compounds in less than 6 minutes

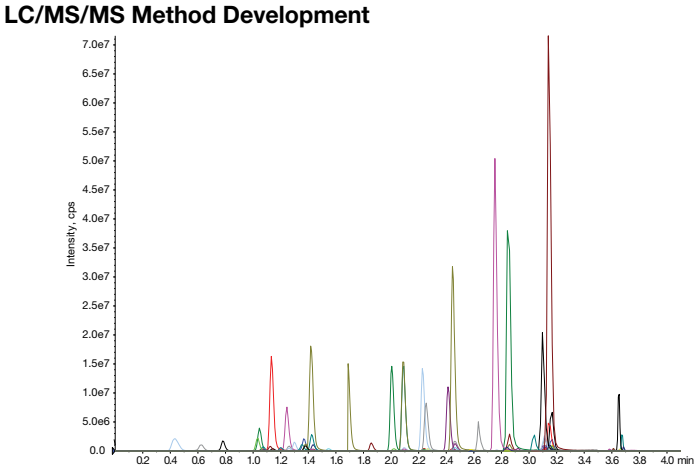

Figure 4. Calibration curve for benzodiazepines

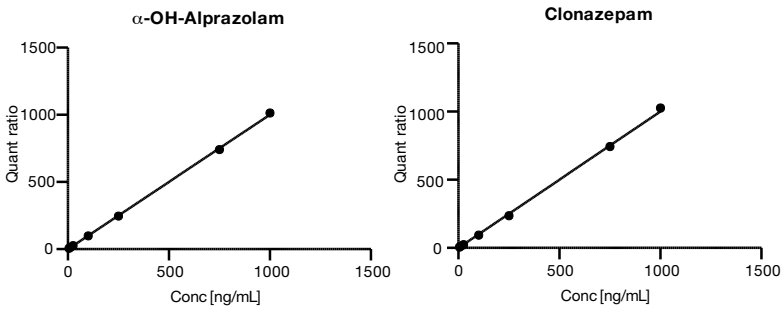

Figure 5. Calibration curve for opiates

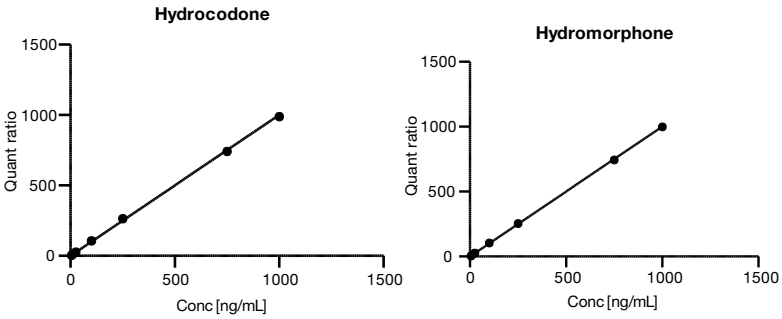

Figure 6. Calibration curve for amphetamines

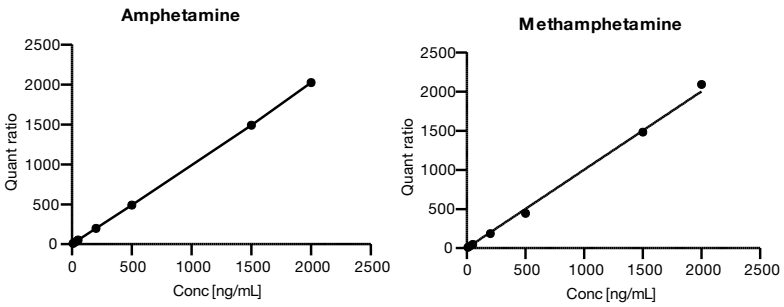

Figure 7. Calibration curve for analgesics

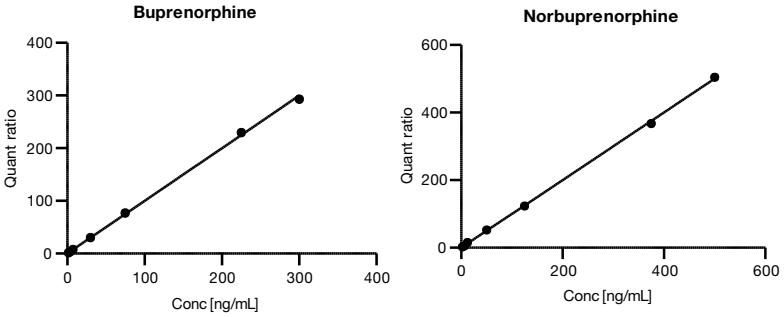

Figure 8. Lower and upper limits of quantitation (ng/mL)

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
Norfentanyl	0.5	100
Norhydrocodone	10	2000
Noroxycodone	5	1000
Nortriptyline	10	2000
Oxazepam	5	1000
Oxycodone	5	1000
Oxymorphone	5	1000
Phencyclidine	2.5	500
Propoxyphene	10	2000

Figure 8. Lower and upper limits of quantitation (ng/mL)

Drug Name	LLOQ	ULOQ
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
Gaba	80	16000
Sovaldi	10	2000
THCA	12.5	500

Figure 9. Method Precision for High Conc.

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
Gaba	8000	2.84	3.07	2.23	1.9
THCA	250	1.37	5.56	5.19	1.46

Figure 10. Method Precision for Low Conc.

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
Gaba	800	1.98	5.19	4.97	1.29
THCA	25	2.68	7.67	6.8	2.37

Figure 11. 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	Gaba	98.58
Hydromorphone	103.56	Oxycodone	100.4	THCA	90.26

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. In addition, great method precision was achieved at both low and high quantitation levels (**Figures 9-10**).
4. The method produced minimal matrix effect despite a simple sample preparation procedure from an inherently dirty sample matrix (**Figure 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