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Fast, Simple Urine Drug Extraction with In-well Room Temperature Hydrolysis and Enzyme Removal to Extend LC Column Life

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Introduction

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) is an essential tool for detecting the presence of drugs and their metabolites in urine samples. However, highly water-soluble glucuronide conjugates can affect the sensitivity and specificity of these tests. Many laboratories use hydrolysis to cleave Glucuronic Acid from the parent compound to overcome this challenge, thereby improving analyte detection by LC-MS/MS. One widely used hydrolysis technique in urine drug testing is enzymatic hydrolysis using β-glucuronidase enzymes.

Next-generation β-glucuronidase enzymes, such as IMCSzyme® RT, are highly active at room temperature and require less enzyme for complete hydrolysis. This reduction in enzyme quantity puts less protein on column and prolongs column life. However, due to endogenous metabolites in human urine samples, not all next-generation β-glucuronidase enzymes can hydrolyze glucuronidated metabolites equally. Previously, IMCS has reported that naturally occurring components of urine, such as urea, as well as metabolites from diet, such as vitamins and flavonoids, can have a negative impact on β-glucuronidase hydrolysis efficiency and not all β-glucuronidases are equally affected.

In this technical note, we evaluate the hydrolysis efficiency of IMCSzyme RT by 1) hydrolyzing 4 human urine samples at a range of enzyme amounts, and 2) hydrolyzing 96 human urine samples at a fixed amount of enzyme. We also evaluated how sample treatment using β-Gone™ Plus β-glucuronidase removal plates can improve column life and peak quality of analytes compared to Dilute-and-Shoot treatment methods, using a Kinetex™ 2.6 µm Biphenyl LC column.

Sample Preparation

Urine samples were a subset of those submitted for drug screening. Selected samples were screened and confirmed positive for Morphine, Oxymorphone, Hydromorphone, Codeine, and Temazepam. Certified Drug-Free Urine (DFU) was used as a control sample and fortified with glucoronidated drug standards. IMCSzyme RT was used for hydrolysis of the samples and protein concentration was based on A280 resulting in approximately 2 µg/µL. Hydrolyzed samples were diluted to 40 % Methanol with 5 % Formic Acid in Methanol elution solvent and filtered through a β-Gone Plus plate (Part No.: 8E-S323-TGA) to remove residual hydrolysis enzyme. Samples were diluted to 10 % Methanol with Water prior to injection on LC-MS/MS.

Hydrolysis with a range of enzyme amounts: 12 human urine samples were pooled into 4 subsets to make samples positive for specific analytes: (1) Morphine, Hydromorphone, Buprenorphine, Norbuprenorphine, Temazepam, Oxazepam; (2) Oxymorphone and Hydromorphone; (3) Hydromorphone; and (4) Codeine. Control samples were fortified with the same glucuronidated metabolites at 100 ng/mL of free base. Control and human samples were hydrolyzed at room temperature (~20 °C) for 15 minutes with IMCSzyme RT at multiple enzyme concentrations ranging from 0 – 200 µg (**Table 1**).

Hydrolysis with 20 µg of enzyme: 96 human urine samples positive for at least one of the analytes (Morphine, Oxymorphone, Hydromorphone, Codeine, O-Desmethyl Tramadol, Tapentadol, Norbuprenorphine, Buprenorphine, Oxazepam, and/or Temazepam) were hydrolyzed with IMCSzyme RT using 100 µL of urine, 20 µg of enzyme and 300 µL of room temperature buffer. 20 µL of internal standard were also added to each sample.

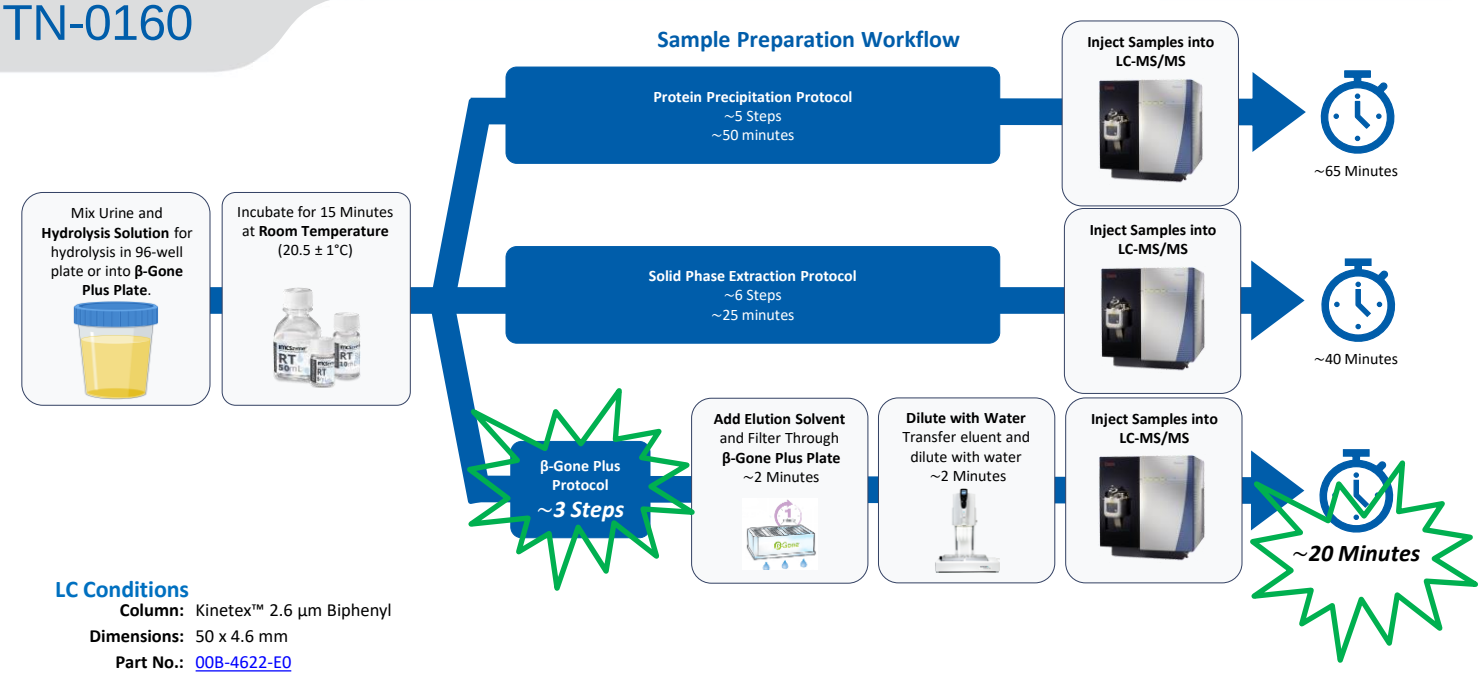
IMCSzyme RT Dilute-and-Shoot Samples: Based on ≥ 80 % analyte recovery hydrolysis results 20 µg of IMCSzyme RT were used per reaction, mixed with 100 µL of urine, 300 µL of buffer and 20 µL of Methanol to represent internal standard. Samples were diluted to 500 µL final volume with 10% Methanol and 0.5% Formic Acid.

Table 1. IMCSzyme RT Master Mix Preparations.

Urine Sample (µL)	IMCSzyme RT at 2 µg/µL (µL)	IMCSzyme RT in Reaction (µg)	Water (µL)*	Room Temperature Hydrolysis Buffer (µL)	Internal Standard (µL)
100	0	0	50	300	20
	2	4	48		
	4	8	46		
	6	12	44		
	8	16	42		
	10	20	40		
	30	60	20		
	50	100	0		

*Water was added after hydrolysis, so every sample had equivalent volume.

Sample Preparation Workflow



LC Conditions

Column: Kinetex™ 2.6 µm Biphenyl
Dimensions: 50 x 4.6 mm
Part No.: 00B-4622-E0
Mobile Phase: A: 0.1 % Formic Acid in Water
B: 0.1 % Formic Acid in Acetonitrile

Gradient:

Time (min)	%B
0	5
0.5	5
1	95
1.5	95
1.55	5
2.35	5

Flow Rate: 0.5 mL/min
Injection Volume: 10 µL
Temperature: 40 °C
Instrument: Thermo Scientific™ Vanquish™ HPLC
Detection: MS/MS
Detector: Thermo Scientific TSQ Endura™ MS

MS/MS Conditions

Electrospray: 1000 V
Sheath Gas: 55 arb
Auxiliary Gas: 11 arb
Sweep Gas: 1 arb
Ion Transfer Tube Temperature: 300 °C
Vaporizer Temperature: 300 °C

Table 2. MRM Transitions

Analyte	Retention Time (min)	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)
Oxymorphone-Glucuronide	1.10	478.183	284.040	28	142
			460.097	22	
Morphine 3β-Glucuronide	1.12	462.183	211.012	42	144
			286.040	29	
Hydromorphone-3-Glucuronide	1.23	462.183	184.986	47	168
			286.054	28	
Morphine	2.09	286.4	157.100	39	136
			165.100	40	
			183.100	32	
Morphine-D_3	2.09	289.1	165.054	41	119
Oxymorphone	2.29	302.152	227.058	29	108
			284.058	19	
Oxymorphone-D_3	2.29	305.152	287.040	20	106
Naloxone Glucuronide	2.34	504.17	310.169	28	96
			328.222	21	
			486.222	20	
Hydromorphone	2.54	286.122	157.000	42	127
			184.986	30	
Hydromorphone-D_3	2.54	289.183	184.929	30	130
Naltrexol Glucuronide	2.77	520.27	326.468	21	229
			501.994	38	
Dihydrocodeine-Glucuronide	2.83	478.183	199.040	47	151
			302.111	31	
Codeine-6-Glucuronide	2.85	476.213	282.169	26	160
			300.111	30	
Naloxone	2.91	328.22	267.879	24	126
			310.022	17	
Naloxone-D_5	2.91	333.2	315.151	21	106
Dihydrocodeine	2.93	302.183	199.058	33	122
			201.058	29	
Dihydrocodeine-D_6	2.93	308.239	202.000	34	139
Codeine	2.99	300.19	165.111	42	129
			215.111	25	
Codeine-D_6	2.99	306.183	218.111	26	128
Naltrexol	3.17	343.865	254.240	31	107
			326.169	21	
Naltrexol-D_3	3.17	347.24	329.240	21	128
Tapentadol Glucuronide	3.24	398.183	107.169	45	129
			222.183	22	

Analyte	Retention Time (min)	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)
Norbuprenorphine-Glucuronide	3.34	590.304	396.208	37	156
			414.222	35	
Tapentadol	3.79	222.183	107.111	26	105
			121.111	21	
Tapentadol-D_3	3.79	225.183	107.111	26	102
Buprenorphine-Glucuronide	4.07	644.243	396.222	55	281
			414.222	49	
			468.262	40	
Norbuprenorphine	4.15	414.27	340.110	30	162
			396.208	27	
Norbuprenorphine-D_3	4.15	417.274	343.165	29	164
Oxazepam-Glucuronide	4.54	463	241.000	38	111
			287.000	13	
Lorazepam Glucuronide	4.63	498.7	276.875	37	109
			322.889	13	
Amitriptyline-Glucuronide	4.81	454.183	233.040	19	130
			278.111	22	
Temazepam-Glucuronide	4.83	477.152	255.000	36	115
			301.058	13	
Buprenorphine	4.9	468.365	396.151	40	194
			414.222	35	
Buprenorphine-D_4	4.9	472.274	400.222	40	186
Oxazepam	5.34	287	241.000	23	104
			269.000	15	
Oxazepam-D_5	5.34	292	245.986	23	106
Amitriptyline	5.39	278.243	117.111	23	96
			233.111	17	
Amitriptyline-D_3	5.39	281.183	233.040	18	103
Lorazepam	5.42	322.091	275.960	22	109
			303.875	15	
Lorazepam-D_4	5.42	326.7	280.946	23	106
Temazepam	5.9	301.091	255.058	22	99
			282.986	14	
Temazepam-D_5	5.9	306.091	260.040	23	98
cTHC Glucuronide	6.29	521.335	327.111	22	128
			345.111	12	
cTHC	7.14	345.274	299.111	19	132
			327.040	15	
cTHC-D_3	7.14	348.312	330.111	15	121

Have questions or want more details on implementing this method? We would love to help!
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Results and Discussion

Ninety-six urine samples were hydrolyzed with IMCSzyme RT using 20 µg of enzyme in the reaction for Dilute-and-Shoot and β-Gone workflows. Samples were considered positive (+) with ≥ 25 ng/mL of drug and negative (-) with < 25 ng/mL of drug (**Table 3**).

Control and pooled urine samples were analyzed. Hydrolysis results were converted into % hydrolysis due to the differing concentration of drug in control and patient samples. Hydrolysis was considered complete at ≥ 80% hydrolysis. 20 µg of enzyme was found to be sufficient for adequate hydrolysis of targeted analytes.

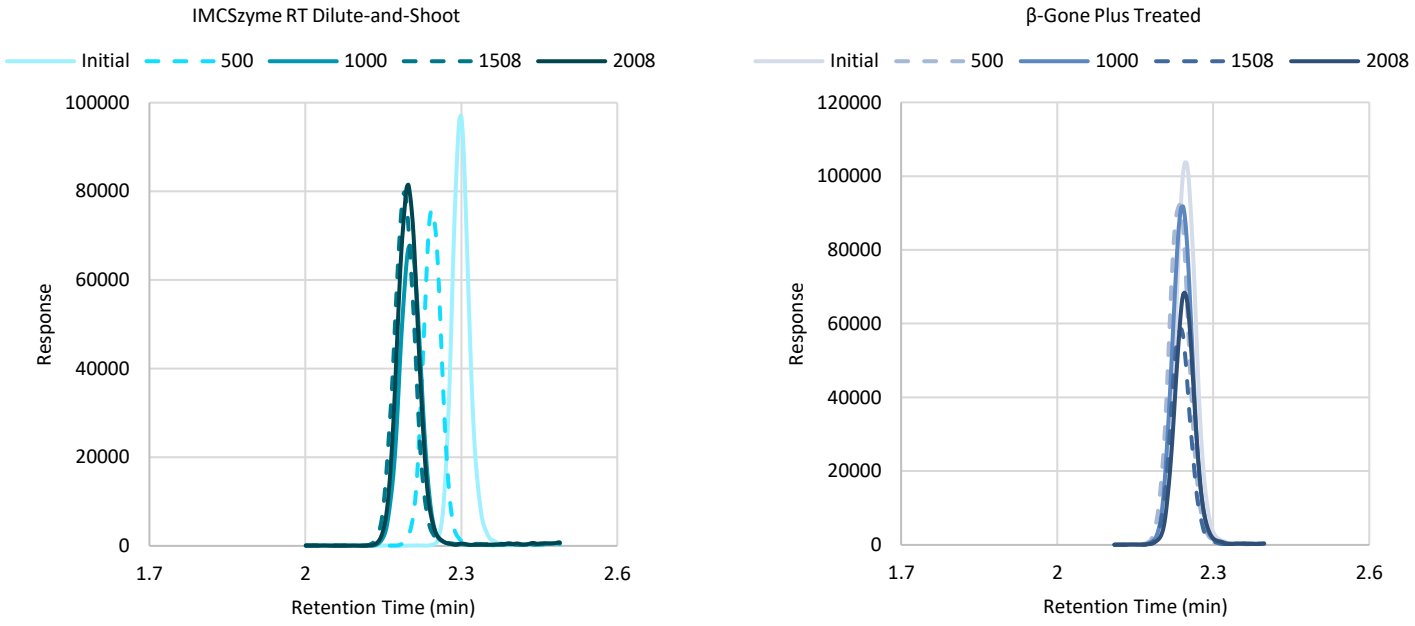
IMCSzyme RT with Dilute-and-Shoot and IMCSzyme RT processed with β-Gone Plus were compared. The injection sequence involved injecting one blank and three neat standard injections (10-minute run time). This was followed by injecting 96 Dilute-and-Shoot or β-Gone Plus treated samples. The sample injections were accelerated to 2.35 minutes to reduce run time and mobile phase waste, but column wash and column equilibration times remained equivalent between methods. This sequence was repeated 20 times for IMCSzyme RT Dilute-and-Shoot samples and the IMCSzyme RT processed with β-Gone Plus. Each sequence finished with a blank and three neat standard injections for a total of 2008 injections for IMCSzyme RT Dilute-and-Shoot and IMCSzyme RT processed with β-Gone Plus.

All sample treatment methods started with a brand new Kinetex 2.6 µm Biphenyl, 50 x 4.6 mm column from the same batch to reduce variability. Total Ion chromatograms (TICs) of Morphine were plotted. The full width at half max and asymmetry of Morphine peaks were calculated. Full width at half max should be as low as possible. Low values indicate a nice tall sharp peak while high values would indicate a short wide peak. Peak symmetry should be around 1, values higher than 1 indicate asymmetrical fronting and values less than 1 indicate asymmetrical tailing. The TICs of Morphine show that IMCSzyme RT Dilute-and-Shoot samples caused significant retention time shifting (**Figure 1**). Full width at half max started out at 0.03, increased to 0.04 by 200 injections and stayed at 0.04 up to 2000 injections. Peak symmetry started out at 1.2 and fluctuated between 0.8 and 1.2 through 2000 injections.

Table 3. Summary of Drug Screening Results on 96 Urine Samples. Samples are Considered Positive at >25 ng/mL and Negative <25 ng/mL.

Drug	Positives	Negatives
Temazepam	7	89
Morphine	17	79
Oxymorphone	36	60
Hydromorphone	51	45
Codeine	5	91

Figure 1. Example Total Ion Chromatogram for Morphine.



Conclusions

The combination of IMCSzyme® RT and the β-Gone™ Plus plate provided fast, in-well room temperature hydrolysis and efficient removal of residual β-glucuronidase enzyme in urine samples. The total sample preparation time using β-Gone Plus and IMCSzyme RT was 20 minutes. This is significantly shorter than using traditional SPE or manual protein precipitation. Enzyme removal using β-Gone Plus resulted in cleaner samples and extended column life of the Kinetex™ 2.6 µm Biphenyl LC column compared to Dilute-and-Shoot analysis. This workflow hydrolyzes even the most challenging urine samples and reduces the risk of LC column clogging and costly instrument downtime.

Ordering Information

Kinetex Analytical Columns

2.6 µm Analytical Columns (mm)							SecurityGuard™ ULTRA Cartridges‡
Phases	30 x 4.6	50 x 4.6	75 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	3/pk
EVO C18	00A-4725-E0	00B-4725-E0	—	00D-4725-E0	00F-4725-E0	00G-4725-E0	AJ0-9296
PS C18	00A-4780-E0	00B-4780-E0	—	00D-4780-E0	00F-4780-E0	00G-4780-E0	AJ0-8949
Polar C18	00A-4759-E0	00B-4759-E0	—	00D-4759-E0	00F-4759-E0	—	AJ0-9530
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
F5	00A-4723-E0	00B-4723-E0	—	00D-4723-E0	00F-4723-E0	—	AJ0-9320

for 4.6 mm ID

‡SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000

β-Gone β-Glucuronidase Removal Products

Part No.	Description	Unit
8B-S139-TAK	1 mL Tubes, Recombinant Enzyme	100/Box
8B-S322-DAK	1 mL Tubes, Non-Recombinant Enzyme	100/Box
8E-S139-TGA	96-Well Plate, Recombinant Enzyme	1/Box
8E-S322-DGA	96-Well Plate, Non-Recombinant Enzyme	1/Box
8E-S323-TGA	96-Well Plate Plus 30 mg/well, Recombinant/Non-Recombinant Enzyme	1/Box
8E-S323-UGA	96-Well Plate Plus 60 mg/well, Recombinant/Non-Recombinant Enzyme	1/Box
8N-S323-TUK	2 mL Centrifuge Tubes, Recombinant and Non-Recombinant Enzyme	100/Box

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