Simplifying Urine Drug Testing with "Flash Hydrolysis" Using New Recombinant β-Glucuronidase Enzymes

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ABSTRACT

The prevalence of urine drug testing (UDT) has increased significantly over the last decade with LC-MS/MS being the gold standard for detecting and quantifying drugs. As testing volumes rise, laboratories are continually looking for improvements to workflow and efficiency. One (historically) time consuming step in sample preparation is enzyme hydrolysis, required to cleave conjugated metabolites. Over the past decade, "second generation" β-glucuronidase enzymes have reduced the required incubation time from 2+ hours to as little as 15 minutes. Although the incubation time is reduced, the number of steps required in sample preparation remains the same. This research explores the possibility of adding enzyme to a sample as a "reagent" and using "flash hydrolysis" to cleave glucuronides. Flash hydrolysis results in a simple one-vial, "dilute-and-shoot" approach.

Flash hydrolysis was evaluated using commercial blank urine fortified with several glucuronidated reference standards, as well as positive patient samples. Compounds were chosen to represent commonly tested drugs and spanned several different drug classes. Opiates, opioids, benzodiazepines, and TCAs were all studied. The efficiency of the enzyme was measured immediately after the enzyme was added and across several time points.

Though a full method validation has yet to be performed, preliminary data indicate that flash hydrolysis sample preparation does provide sufficient cleavage of the main glucuronide conjugates relevant to UDT. Furthermore, the non-quenching of the hydrolysis reaction did not have any adverse effects on 6-MAM or morphine concentrations. Simplifying the sample preparation into a single vial process without any incubation time reduces both consumable and labor costs, as well as potential for error or contamination. Additionally, this workflow is more conducive to analyzing rush or stat samples due to the ease of preparing a single sample.

INTRODUCTION

The prevalence of urine drug testing (UDT) has significantly increased over the past decade. One ongoing debate in UDT is whether to hydrolyze the sample and measure the combined total concentrations (glucuronide plus free) or measure glucuronides directly in the analytical method. Each of these protocols has advantages and disadvantages.

Direct detection of glucuronide conjugates is useful due to its quick and easy sample preparation, typically a simple addition of internal standard and dilution of the sample. This simple prep reduces consumable and labor costs, as well as total preparation time, versus hydrolysis protocols. Furthermore, elimination of the hydrolysis incubation step is more conducive to preparation and analysis as the sample is received instead of batch preparation and analysis, which is very useful, especially in any rush or "stat" situation. However, glucuronide standards can add significant cost, not only due to the price of reference standards, but also the overhead of maintaining appropriate QC and documentation for the additional compounds in the test method.

Historically, hydrolysis incubation times required up to three hours. Over the past 10 years, manufacturers have introduced "second-generation" recombinant β-glucuronidase enzymes that reduce the incubation time to <60 minutes, and even as little as 15 minutes. Although 30 to 60 minutes may be required for complete hydrolysis of all conjugated metabolites, previous work(1) shows that the majority of the glucuronides are nearly completely hydrolyzed during the first few minutes after the enzyme is added to the sample. As a result, these new enzymes can simply be added as a "reagent" and the sample analyzed by LC-MS/MS using this "flash hydrolysis" preparation, i.e. without (semi-)lengthy incubation. By omitting the incubation, protein precipitation, centrifugation and possibly a sample transfer step, the advantages of "direct detection" can be achieved without the additional costs/overhead associated with direct detection. To determine the utility and effectiveness of flash hydrolysis, common glucuronides across drug classes were studied.

EXPERIMENTAL

Standards: Glucuronidated reference standards were either donated by Lipomed (Cambridge, MA) or purchased from Sigma Aldrich (Round Rock, TX). **Table 1** lists the glucuronides studied.

Samples: Drug free urine (UTAK, Valencia, CA) was fortified with the glucuronide standards. Conjugated metabolites were chosen to represent several drug classes of interest, including benzodiazepines, opiates, opioids, and tricyclic antidepressants. The target compounds were divided into six different mixes and grouped similar to what would be observed in biological samples. The mixtures and concentrations of the fortified samples are shown in **Table 1**. Fortified concentrations were chosen based on concentrations observed in patient urine specimens. Patient samples confirmed positive for the target drugs were also analyzed to evaluate the enzyme performance on authentic specimens.

Enzymes: Two enzymes from two different manufacturers were evaluated in this study: IMCSzyme RT (IMCS, Irmo, SC) and B-One Mastermix (Kura Biotech, Rancho Dominguez, CA).
The IMCSzyme RT kit included a vial of enzyme and a vial of buffer. According to manufacturer information, the activity of IMCSzyme RT is 200,000 modified Fishman units (mFU) per mL. The industry standard mFU is defined as: 1 unit will liberate 1 µg of phenolphthalein from phenolphthalein glucuronide in **60 minutes** at 25°C at pH 6.8.
B-One Mastermix is an "all-in-one" solution that contains the enzyme and buffer in a single bottle. According to manufacturer information, the activity of this solution is >12,000 Product Specific Unit (PSU) per mL. PSU is defined on the package insert as: 1 µg of phenolphthalein from phenolphthalein glucuronide in **5 minutes** at pH 6.8 and 20°C.

Sample Preparation: Samples were hydrolyzed based upon manufacturers recommended protocol, which is outlined in **Table 2**. Slight modifications were made to accommodate the appropriate dilution factor for our instrument. It should be noted that 5 µL of IMCSzyme RT and 100 µL of B-One enzyme solution (buffer plus enzyme) was used for this study. It is possible to increase the amount of enzyme to achieve faster hydrolysis.

Sample Analysis: All samples were analyzed by LC-MS/MS on a SCIEX 4500 with a Shimadzu Prominence LC stack. The method used in this research was an R&D (non-validated) method used to explore the utility of flash hydrolysis. Enzyme manufacturers both recommend that after enzyme addition, the sample be left at "room temperature" for approximately 15 minutes without dilution with water before analysis. However, to fully evaluate how quick the hydrolysis reaction achieved completion (defined as ≥80% for this research), the sample was placed in the autosampler immediately after enzyme addition. Sample injection occurred within 1 to 2 minutes after the enzyme was added (Time=0, or T0). Using "true T0" is not typical of how samples are prepared in a laboratory environment, as it usually takes 20 to 60 minutes to prepare a batch of samples. Additional time is also required from when the sample rack is loaded into the autosampler and when the actual sample is injected for analysis. As a result, a few hours may pass from enzyme addition to sample injection and analysis. However, for informative purposes, this "true T0" workflow was used. To more closely mimic room temperature and maintain hydrolysis "incubation" temperature to study reaction progress, the autosampler was kept at 20°C and replicate injections were acquired.

The LC-MS/MS method was 8.2 minutes. For calculation purposes, 8.35 minutes was used as the time between injections to include some autosampler overhead. A baseline sample (no hydrolysis) was analyzed both at the beginning and end of the replicate injections. An average of the peak areas from the baseline sample was used to determine the extent of hydrolysis over time by comparing glucuronide peak areas from the hydrolyzed samples to the baseline peak area.

	Analyte	Conc (ng/mL)
Mix 1	Codeine-6β-glucuronide	20,000
	Morphine-3β-glucuronide (M3)	20,000
	Oxymorphone-3β-glucuronide	20,000
Mix 2	Morphine-6β-glucuronide (M6)	20,000
	Hydromorphone glucuronide	20,000
	Lorazepam glucuronide	10,000
Mix 3	Tapentadol glucuronide	10,000
	Naltrexone glucuronide	5,000
	6β-Naltrexol glucuronide	5,000
Mix 4	Naloxone glucuronide	10,000
	Buprenorphine glucuronide	2,500
	Norbuprenorphine glucuronide	2,500
Mix 5	Oxazepam glucuronide	10,000
	Temazepam glucuronide	10,000
Mix 6	Amitriptyline glucuronide	10,000

***Table 1**. A list of the compounds and spiked concentrations used in this study. Target metabolites were chosen based upon frequency of detection. Spiked concentrations are in the higher range of what is typically observed in positive samples.*

Sample Preparation	
IMCSzyme RT	**B-One (Kura)**
50 µL urine 150 µL buffer 5 µL IMCSzyme RT 10 µL int stand	50 µL urine 100 µL B-One 10 µL int stand
295 µL water	350 µL water

***Table 2:** Sample preparation protocol for each of the enzymes used in this study is outlined in the table. Manufacturer recommendations were utilized, with small modifications included to maintain the appropriate dilution factor of the urine. The B-One mix included the buffer so no additional buffer was necessary. The activity of enzyme used per hydrolysis reaction were 83 PSU equivalent of IMCSzyme RT or 1,200 PSU of B-One (See "Experimental" section for definition of PSU and information on enzyme activity).*

1.

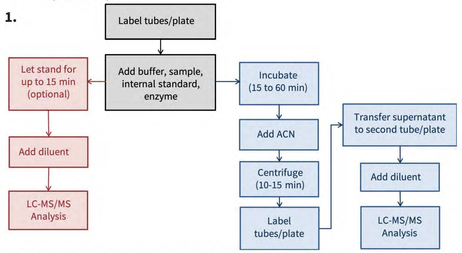


***Figure 1.** Flow diagram comparing flash hydrolysis preparation to traditional hydrolysis preparation. Flash hydrolysis not only saves incubation time, there are also fewer steps, which reduces the potential for error or contamination.*

RESULTS & DISCUSSION

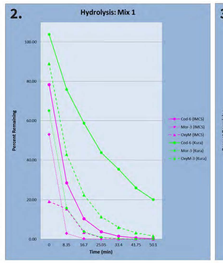
	Analyte	Enzyme	Percent Remaining 0 min	8.35 min	16.7 min	25.1 min	33.4 min	41.75 min	50.1 min
Mix 1	Codeine-6β-glucuronide (C6)	IMCSzyme RT	78	28	10	3.7	1.5	0.6	0.20
		B-One	100	76	59	44	35	26	20
	Morphine-3β-glucuronide (M3)	IMCSzyme RT	53	2.9	ND	ND	ND	ND	ND
		B-One	65	16	3.8	0.84	0.24	ND	ND
	Oxymorphone-3β-glucuronide	IMCSzyme RT	19	15	3.4	0.77	0.17	0.05	0.02
		B-One	89	43	22	11	6.0	3.2	1.6
Mix 2	Morphine-6β-glucuronide (M6)	IMCSzyme RT	96	84	71	61	55	51	44
		B-One	91	72	59	52	42	35	29
	Hydromorphone glucuronide	IMCSzyme RT	81	24	6.8	2.0	0.62	0.25	0.14
		B-One	61	31	15	7.8	4.0	2.0	1.0
	Lorazepam glucuronide	IMCSzyme RT	5.2	2.4	2.4	2.5	2.5	2.6	2.4
		B-One	2.4	2.4	2.4	2.3	2.3	2.3	2.3
Mix 3	Tapentadol glucuronide	IMCSzyme RT	52	1.5	1.2	1.1	1.1		
		B-One	6.8	0.82	0.74	0.75	0.69		
	Naltrexone glucuronide	IMCSzyme RT	76	17	4.1	1.1	0.32		
		B-One	24	1.9	ND	ND	ND		
	6β-Naltrexol glucuronide	IMCSzyme RT	78	15	2.7	0.63	0.12		
		B-One	26	3.7	0.59	ND	ND		
Mix 4	Naloxone glucuronide	IMCSzyme RT	78	21	5.3	1.6	0.37		
		B-One	3.6	ND	ND	ND	ND		
	Buprenorphine glucuronide	IMCSzyme RT	19	ND	ND	ND	ND		
		B-One	ND	ND	ND	ND	ND		
	Norbuprenorphine glucuronide	IMCSzyme RT	8.8	ND	ND	ND	ND		
		B-One	7.3	ND	ND	ND	ND		
Mix 5	Oxazepam glucuronide	IMCSzyme RT	20	2.4	2.4	2.3	2.4		
		B-One	2.8	2.8	2.8	3.0	2.9		
	Temazepam glucuronide	IMCSzyme RT	36	2	1.8	1.9	1.8		
		B-One	2.3	2.3	2.3	2.2	2.3		
Mix 6	Amitriptyline glucuronide	IMCSzyme RT	62	39	24	15	9.0		
		B-One	1.5	ND	ND	ND	ND		

*ND = not detected

***Table 3:** A summary of all compounds tested and the effective hydrolysis over time is presented. Hydrolysis is considered complete when ≤80% of the conjugate remained and is highlighted in green. Two points are highlighted in yellow because it was close to 80% complete, which could be considered complete within the estimated margin of error in the experiments. Sufficient hydrolysis of most compounds was achieved in less than 15 minutes. The exceptions were: 1) amitriptyline (TCA), which took approximately 20 minutes for >80% hydrolysis using IMCSzyme; 2) C6, which took approximately 50 minutes using B-One, and M6, which was a challenge for both enzymes (see **Figures 2** and **3** for more detail.)*

Sample	Drug Class	Compound	ng/mL
12439	Benzodiazepines	Oxazepam	116
		Temazepam	103
		Lorazepam	76
	Opiates	Codeine	>25000
		Morphine	1316
13052	Opioids	Naltrexone	932
		6-β-Naltrexol	3371
13152	Opioids	Tapentadol	>30000
		Desmethyltapentadol	>30000

***Table 4.** Patient samples positive for the drugs studied were selected and analyzed to evaluate the performance of the enzymes on "real world" samples. A list of samples and their positive analytes are shown in this table. The concentrations are total concentrations from a validated, production method using hydrolysis and a 45 to 60 minute incubation. Note that the concentrations of codeine, 6-β-naltrexol, tapentadol, and desmethyltapentadol are well above the ULOQ for these compounds. The ULOQ for codeine is 10000 ng/mL and the ULOQ for the other three compounds is 2500 ng/mL. Hydrolysis trends are shown in Figures 4 and 5.*



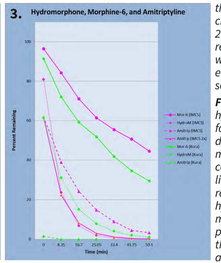
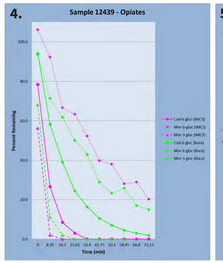


***Figure 2.** Not surprisingly, the opiate glucuronides, especially at the high concentrations used in Mix 1, presented some hydrolysis challenges. IMCSzyme RT achieved >80% hydrolysis within 15-20 minutes, while it took approximately 50 minutes for B-One to reach 80%. Codeine-6-glucuronide was one of the two compounds where a significant difference in performance between the two enzymes was observed. (Morphine-6-glucuronide data can be seen in Figure 3.)*

***Figure 3.** Morphine-6-glucuronide (M6) presented the biggest hydrolysis challenge, as neither enzyme achieved >80% efficiency for M6 in the 50 minutes used in the initial study. (Figure 4 presents data over an extended time window.) However, M6 is such a minor metabolite, and M3 would be present at significantly higher concentrations. Therefore, incomplete hydrolysis of M6 would have little, if any, impact on interpretation of overall urine drug testing results. IMCSzyme RT performed slightly better on hydrolysis of hydromorphone-glucuronide, but amitriptyline hydrolysis was much better using the B-one enzyme. Amitriptyline hydrolysis was performed with 10 µL of IMCSzyme RT (vs originally 5 µL) Increasing the amount of enzyme resulted achieving >80% hydrolysis approximately 5 minutes faster. Even though some performance differences were observed between the enzymes, all compounds except M6 achieved >80% hydrolysis in under 30 minutes.*



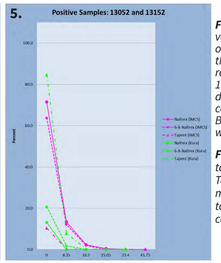
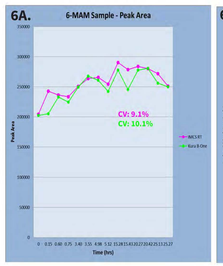


***Figure 4.** Overall, hydrolysis trends for the compounds behaved very similarly to the fortified samples. One exception was observed: codeine-6 glucuronide using the Kura enzyme. In the fortified sample (Mix 1), approximately 50 minutes were required to reach 80% completion, whereas data for Sample 12439 show completion in approximately 30 minutes. This difference requires further investigation. Morphine-6 was the only conjugate requiring at least 60 minutes to reach 80% completion. Benzodiazepine glucuronides were immediately cleaved by T0 so were omitted from the graph for clarity.*

***Figure 5.** One positive naltrexone sample and one positive tapentadol sample were analyzed using flash hydrolysis. (See Table 4 for detailed information.) Trend lines for the drugs/metabolites are shown in Figure 5. Performance was similar to the fortified samples and both enzymes cleaved >80% of the conjugate in under 10 minutes.*



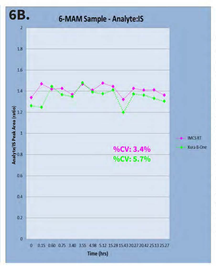


***Figure 6.** The hydrolysis reaction is never quenched when using flash hydrolysis. To evaluate any potential effects this continual hydrolysis reaction may have on 6-MAM or morphine, a sample that tested positive for 6-MAM (100 ng/mL) was prepared using the flash hydrolysis protocol and analyzed over a period of 25+ hours. During this time, the sample remained in the 20°C autosampler. Peak areas are shown above in **Figure 6a**. Although it appears that 6-MAM is increasing over time, when the ratio of the areas of analyte: internal standard are plotted, there is no significant difference – either increasing or decreasing – over time, as can be seen in **Figure 6b**. The percent CV for each experiment are listed in their respective figures. Data is not shown, but a sample that was positive for morphine (>20,000 mg/mL) was prepared and analyzed over approximately 25 hours to test if 6-MAM would be formed. 6-MAM was not detectable in the final LC-MS/MS injection when either enzyme was used.*

CONCLUSION

- Flash hydrolysis is a simple, quick sample preparation for analysis of urine samples in urine drug testing.
- Reducing the number of steps in the preparation protocol and omitting the incubation time not only significantly save labor and consumable costs, it also reduces potential for error or contamination.
- For the glucuronide conjugates studied in this research, most were sufficiently hydrolyzed in less than 15 minutes. All were completely hydrolyzed within approximately an hour. Considering that the time between adding enzyme to a sample and actual injection of the sample for analysis often exceeds 1 hour (and can be several hours), incomplete hydrolysis for any of the compounds studied is not an issue. An autosampler temperature of 20°C mimics room temperature and assists in complete hydrolysis.
- Fast hydrolysis results for real samples were comparable to results obtained on blank urine spiked with standards.
- Although the hydrolysis reaction is never quenched, no degradation of 6-MAM was observed over the course of 25+ hours. No formation of 6-MAM from a high concentration morphine sample was observed when the hydrolysis reaction continued for 25+ hours.
- The lack of enzyme clean-up from the sample did not affect assay performance or have any detrimental effects on the LC column or MS interface.
- Although there were some differences between the two enzymes studied, the performance within the urine drug testing workflow were very comparable.
- Though a full method validation is necessary (e.g. matrix effects), flash hydrolysis is promising to reduce costs, simplify sample preparation, and make the workflow conducive to prepping "one-off"/rush/stat samples with minimal overhead.

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REFERENCES

1. Sasaki, T. A., *Comparison and Evaluation of Recombinant β-glucuronidase Enzymes for Urine Drug Testing*, Presentation S81, SOFT-TIAFT Conference, Boca Raton, 2017

DISCLOSURE

Lipomed donated reference standards for this work. IMCS provided a travel stipend to the author.