Complete integration of a fully automated flash hydrolysis protocol of glucuronides in urine with LC-MS/MS quantification

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Novel Aspect

Fully automated, online flash hydrolysis, sample preparation and LCMS analysis for glucuronidated drugs of abuse in urine

Introduction

Currently, sample preparation for the detection of glucuronidated drug compounds in urine by liquid chromatography-mass spectrometry (LC-MS) involves multi-stage manual sample preparation methods, which invites human errors. Typical manual sample preparation of glucuronides can take up to two hours. However, a new modified beta-glucuronidase enzyme, BGTurbo®, is fast enough to make serial flash hydrolysis possible for automation. The CLAM-2000 sample preparation module seamlessly integrates sample preparation, LC separation and MS detection of small molecules in an online platform. Using the BGTurbo® enzyme, we developed a serial fully automated and integrated flash hydrolysis, sample preparation, and LCMS analysis method for glucuronides and other drugs of abuse in urine. This method maximizes efficient use of the mass spectrometer using parallel sample processing of four samples simultaneously so that the LCMS system is running constantly. The resulting process defined here for research purposes achieves the same sensitivity and linearity as traditional methods with CV values less than 10%.

Methods

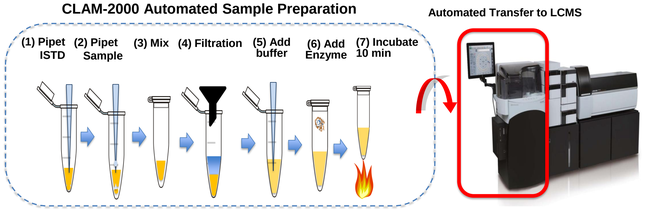
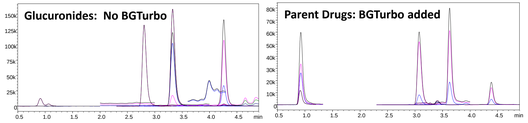


Figure 1. Sample preparation and analysis on CLAM-2000 and LCMS-8050

- Morphine 6 Beta, Codeine 6 Beta, Buprenorphine 6 Beta, Oxazepam 6 Beta, Naltrexone 3 Beta, 11-nor-delta 9-THC (Cerillant, Round Rock, TX)
- Beta-Glucuronidase Enzyme, BGTurbo® (Kura Biotech Inc. Rancho Dominguez, CA)
- Surine (Sigma Aldrich, St. Louis, MO)
- Mobile Phases A/B: 0.1% Formic in Water, 0.1% Formic in Methanol, (Sigma-Aldrich, St. Louis, MO)
- Column: 2.1x50mm Raptor Biphenyl (Restek, PA)
- Gradient: 10% to 95% Methanol over 5.9 minutes
- LC/MS system: Shimadzu Nexera LC system and a Shimadzu 8050 triple quadrupole (Shimadzu, Kyoto, Japan)

Results and Discussion



Figures 2 and 3: Extracted ion chromatograms of glucuronides with no BGTurbo added (Fig 2) and with addition of BGTurbo (Fig 3).

Drug Compound	Conjugated	Free	% Recovery
Morphine	55,350	70,662,834	99.9
Codeine	1,321,361	7,851,914	85.6
Naltrexone	584,424	88,754,377	99.3
Buprenorphine	685	6,189,377	100.0
Oxazepam	649,550	23,941,055	97.4
TCH-COOH	17,696	3,961,203	99.6

Table 1: Peak areas of representative glucuronides subjected to enzymatic hydrolysis with BGTurbo. Free and conjugated peaks areas are shown along with percent recovery of "free" parent.

Optimum Enzymatic Hydrolysis Conditions

- **Use 55 mM Di-Sodium Phosphate Buffer**
- **Prepare a (1:1 Enzyme/Buffer)**
- **Add 30 µL of sample**
- **Add 60 µL of BGTurbo mixture**
- **Incubate at 55 ° C for 10 minutes**

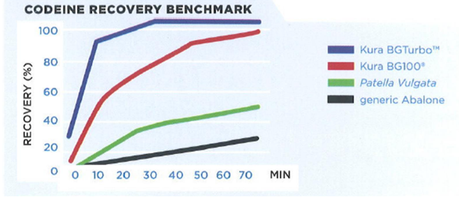


Figure 4: Codeine percent recovery with four different enzymes. BG Turbo exhibits 85% recovery in <10 minutes.

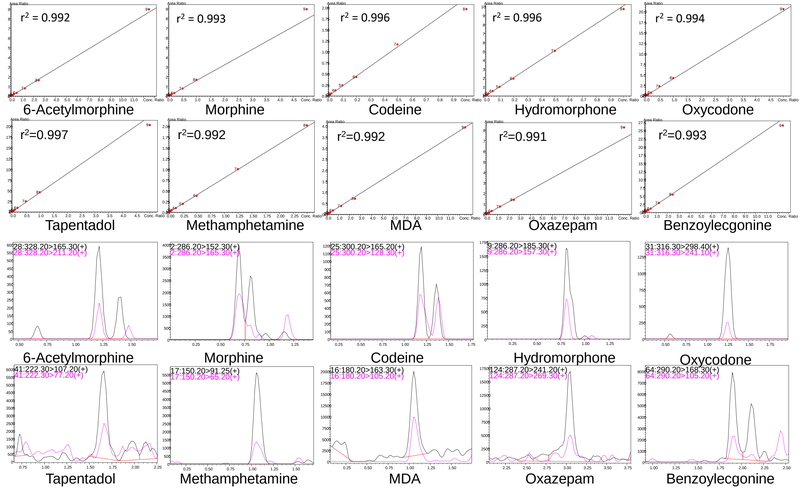
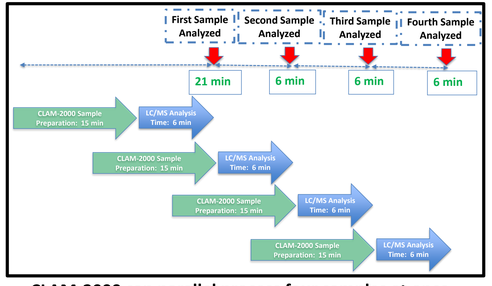


Figure 5: Calibration Curves (L1-L9) and MRM Chromatograms (L1) for ten drug compounds

Comparison of CLAM-2000 and Manual Sample Preparation

> 120 minutes

Centrifuge 10 min → Pipet off supernatant → Add ISTD → Mix → Add Buffer → Add Enzyme → Transfer to oven → **Incubate for 1-2 hr.** → Dilute → Centrifuge 15 min → Transfer to bench → Pipette supernatant → Transfer to LCMS



CLAM-2000 can parallel process four samples at once

Results: Robustness Test

Compound	# Replicates	ISTD	Peak Area %RSD	Peak Area Ratio %RSD
Morphine	60	morphine d3	9.99	4.82
Oxymorphone	60	morphine d3	11.61	6.9
Methamphetamine	60	morphine d3	9.97	4.46
Codeine	60	morphine d3	12.3	6.32
6-MAM	60	morphine d3	12.36	5.52
Hydrocodone	60	morphine d3	11.77	4.89
MDA	60	morphine d3	12.13	5.67
Fentanyl	60	morphine d3	14.80	8.53
Clonazepam	60	morphine d3	13.67	6.67
Tapentadol	60	morphine d3	11.50	5.89
Oxazepam	60	morphine d3	14.02	7.36

Summary

- The CLAM-2000 sample preparation module with a Shimadzu LCMS-8050 was used to serially perform enzymatic hydrolysis of glucuronides in a 35 component drug mixture on an LCMS time scale (5-7 minutes), which allowed the LCMS system to run nonstop after the first sample.
- The use of the Recombinant beta-glucuronidase (BGTurbo®) operating at mild hydrolysis temperatures (55° C) and parallel processing made serial sample preparation and analysis possible
- Representative calibration curves have $R^2 > 0.99$ and have linear dynamic ranges of 1 – 5000 ng/mL (6-Acetylmorphine, Oxycodone, MDA, Tapentadol and Benzoylecgonine) or 1 – 1000 ng/mL (Codeine, Hydromorphone and Methamphetamine) and 10 – 5000 ng/mL (Morphine and Oxazepam).

Future Directions

Development of additional sample preparation procedures for drugs of abuse in various matrices as well development of analysis methods for other small molecule and peptide analytes by CLAM-2000 LC/MS system.

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