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Evaluation of β-glucuronidase Enzymes and Sample Cleanup Methods

A summary of the article: Development and Validation of a Novel LC-MS/MS Opioid Confirmation Assay: Evaluation of β-glucuronidase Enzymes and Sample Cleanup Methods

He, S. Yang, A., Wu, H.B., Lynch, L. K. (2016) Journal of Analytical Toxicology 40:5, 323-329.

Overview:

With the rise in use and prescription of opioids for pain management over the past few years, an increase in the need to monitor opioid usage has emerged. Typically, testing for opioids and their metabolites in urine is done through immunoassay screening and GC-MS analysis, which involves laborious sample preparation and cleanup. This study sought to develop and validate a more efficient and accurate method for detecting opioids in urine via β-glucuronidase hydrolysis, protein precipitation sample cleanup, and LC-MS/MS assay. Four different β-glucuronidases and three different protein precipitation plates were compared to determine the best enzyme and plate to use for opioid confirmation.

Materials and Methods:

The following β-glucuronidases were compared: IMCSzyme®, a genetically modified β-glucuronidase (IMCS), BG100™ (Kura Biotech) from red abalone *Haliotis rufescens* entrails, EBG (Kura Biotech), a recombinant β-glucuronidase from *E. coli*, and β-glucuronidase from *P. vulgata* (Sigma-Aldrich). The four enzymes were tested using the buffers, incubation temperatures and concentrations listed in Table 1.

Table 1. Hydrolysis Conditions

β-glucuronidase	Buffer/pH	Temp. °C	Enzyme Conc. (kU/mL)
IMCSzyme®	Proprietary buffer / pH 6.8	55 °C	10
BG100™	0.2 M sodium citrate / pH 4.8	68 °C	20
EBG	0.2 M potassium phosphate buffer / pH 6.8	46 °C	10.5
P. vulgata	0.2 M acetate buffer / pH 5.0	65 °C	10

β-glucuronidases were then tested at these conditions with 200 µL of hydrolysis controls containing 1 µg/mL morphine-3-β-D-glucuronide (M3G), morphine-6-β-D-glucuronide (M6G), codeine-6-β-D-glucuronide (C6G), hydromorphone-3-β-D-glucuronide (H3G), oxymorphone-3-β-D-glucuronide (O3G), buprenorphine-glucuronide (BG), norbuprenorphine-glucuronide (NBG) as well as internal standards and incubated for 1 hour.

The plates compared for sample preparation were the Supelco 96-well protein precipitation plate (Sigma-Aldrich), Impact™ protein precipitation plate (Phenomenex), and Isolute PLD+ protein precipitation plate (Biotage AB). For sample extraction, 100 µL of the urine was transferred to a protein precipitation plate after hydrolysis. Then 300 µL of acetonitrile was added before applying a vacuum and collecting the filtrate, which was diluted 5-fold with mobile phase A (10mM ammonium formate).

The samples were analyzed using an Agilent HPLC with a Kinetex 2.6 µm Phenyl-Hexyl 100 Å, 50 × 4.6 mm LC column at 30°C and a flow rate of 0.7 mL/min. The LC method used is found in Table 2.

Table 2. Liquid Chromatography Method

% Mobile Phase A (10 mM ammonium formate)	% Mobile Phase B (0.1% formic acid in methanol)	Time (minutes)
80	20	0
60	40	0.7
0	100	4.5
0	100	6.0
80	20	8.0

reference LC-MS/MS method were analyzed by this experimental method.

The LC was coupled with a 5500 QTrap (ABSciex) with an electrospray ionization source operated in positive mode with a curtain gas at 20 psi, ion spray voltage of 5000 V, ion source temperature of 700, and medium collision gas. The signal of each compound was normalized against the internal standard fentanyl-d5 and concentration was calculated from prepared calibration curves.

Sixty-two patient urine samples previously tested positive for opioids using the standard GC-MS assay as well as 22 patient samples that tested positive using a

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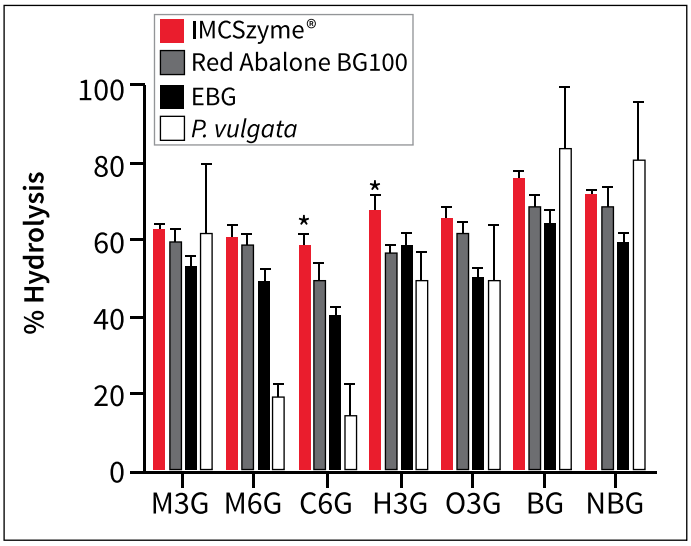
Results:

The hydrolysis efficiency test of the four β-glucuronidases showed the IMCSzyme® had a significantly higher percent hydrolysis of C6G and H3G than the other enzymes.

Figure 1. Hydrolysis Efficiency



*Evaluation of the hydrolysis efficiency of four β-glucuronidase enzymes for seven opioid glucuronide conjugates after 1-h incubation. Substrate concentrations were 1 μg/mL for each enzyme reaction. Results are reported as mean ± SD. *p < 0.05 compared with the other three groups.*

The protein precipitation plate comparison revealed that the Supelco plate had the best recovery (88-103%) and the least significant impact of matrix effects when compared to the other two plates.

The 62 patient urine samples tested with the new LC-MS/MS method showed 96.6% of the peaks identified previously by the GC-MS method. Also, some additional peaks were detected which indicated that samples previously thought to be negative for certain opioids were actually positive. Analysis of the 22 patient urine samples previously tested with a reference LC-MS/MS method revealed 93.3% of the peaks were confirmed with the new method.

Conclusions:

This study revealed that out of four β-glucuronidases tested, IMCSzyme® is the most effective in opioid hydrolysis with half the enzyme concentration as the red abalone β-glucuronidase.The new LC-MS/MS method coupled with IMCSzyme® hydrolysis and the Supelco precipitation plate yielded excellent results for opioid detection in urine. The new method significantly reduces the sample preparation time that is necessary for GC-MS analysis while increasing sensitivity.

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