To hydrolyze, or not to hydrolyze, that is the question for HRMS Pain Management Testing

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Background: The demand for urine drug testing to monitor patients receiving treatment for chronic pain is continuing to increase. Specific identification of prescribed medications and detection of unprescribed or illicit drugs is required to insure treatment compliance and to prevent diversion. There are many things to consider when setting up pain management testing methods, including should screening be done by immunoassay or mass spectrometry? Is one mass spectrometry method sufficient for screening or is it better to develop individual methods for each drug class? What is the optimal sample preparation to insure identification of all drugs and metabolites of interest? Should sample preparation include hydrolysis or should the analytical method monitor the conjugated drugs? The objective of this study was to develop and validate a liquid chromatography high resolution mass spectrometry (LC-HRMS) screening panel (50 drugs and metabolites) for pain management testing while taking these questions into consideration.

Methods:

Two sample preparation approaches were evaluated: 1) enzyme hydrolysis following by protein precipitation / sample filtration (1:30 overall dilution) and 2) dilute and shoot (1:30 overall dilution). For enzyme hydrolysis, urine samples were hydrolyzed with β-glucuronidase (IMCSzme™, DPX Labs) at 55°C for 1 hour. The reaction was mixed with acetonitrile (3x) and applied to a protein precipitation filter plate (Supleco). The filtrate was then diluted 1:5 with mobile phase A. Chromatographic separation was performed using a Phenomenex Kinetex Phenyl-Hexyl column (50x4.6mm, 2.6µm) with a 5 minute gradient (7 min run time) from 5-95% organic (MPA - 10mM ammonium formate and MPB – 0.1% formic acid in methanol). An ABSciex TripleTOF®5600 operating in positive-ion HRMS full scan mode with IDA triggered acquisition of HRMS product ion spectra was used for mass detection. PeakView® and MasterView™ (AB Sciex) were used for targeted data analysis and optimized for the identification of 50 drugs and phase I metabolites and an additional 15 phase II conjugated

metabolites (opioids-19, sedative/hypnotics-20, illicits-9, other-2, conjugated metabolites-15). The method validation of this qualitative screening method included, determination of the lower limit of detection, matrix effect, hydrolysis efficiency and precision at the cut-off. Twenty-four pain managment urine samples were prepared with and without hydrolysis and the results were compared. The same samples were also tested using a traditional immunoassay screen (Microgenics immunoassay on an Advia 1800) and equivalent GC-MS or LC-MS/MS methods that monitor classes of drugs for comparison.

Results: The lower limit of detection for all 50 analytes ranged from 1-25 ng/mL. The assay was linear for all analytes from 25-2000 ng/mL. No significant ion suppression was observed. Hydrolysis efficiency ranged from 65-88% for all possible conjugated metabolites. Precision at the cut-off (10 or 100 ng/mL) for each analyte ranged from 2-15%. There was good overall concordance between the new HRMS method and the traditional immunoassay, GC-MS and LC-MS/MS panel testing, with no apparent decrease in sensitivity or specificity due to merging the analysis of all analytes into one method. When evaluating the need for hydrolysis, there were a total of 124 unconjugated drugs/metabolites and 14 conjugated metabolites detected in the hydrolyzed urine samples. The detection of the 14 conjugated metabolites reflects the sometimes incomplete hydrolysis, but the parent drug was identified as well in all instances. In contrast, there were 113 unconjugated drugs/metabolites and 32 conjugated metabolites detected in the same urine samples that were unhydrolyzed. There were 7 examples in which the conjugated metabolite was identified but the parent drug was missed and 6 examples in which the parent and the conjugated metabolite were both not identified. These included 4 benzodiazepines and 2 opioids. For the unhydrolyzed samples there were 25 examples in which both the parent and conjugated metabolite were identified.

Conclusions: HRMS offers a great platform for testing large panels of compounds in one analytical run as shown here by this method. Since conjugated metabolites are highly polar, they are sometimes difficult to analyze within the same analytic run as parent drugs and phase I metabolites. Sample preparation and chromatographic conditions that are optimal for parent drugs and phase I metabolites are often poor for conjugated metabolites. For this reason, sample preparation for drug panels often includes hydrolysis. However, hydrolysis adds sample

preparation and labor time to the overall process. These issues are of particular concern when analyzing pain management drug such as opioids and benzodiazepines. It is appealing to try and analyze the conjugated metabolites directly; however, the results from this study show that by doing this some compounds may still be missed. In addition, directly analyzing the conjugated metabolites adds to the data analysis time as there are additional peaks/analytes that need to be reviewed for each sample.