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Internal Hydrolysis Indicator for Sample Specific Monitoring of β-Glucuronidase Activity

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Abstract

Metabolized forms of benzodiazepines (benzos) can cause issues with mass spectrometry identification. Benzodiazepines undergo a process called glucuronidation during metabolism that attaches a glucuronic acid for increased solubility. Often in clinical testing an enzymatic hydrolysis step is implemented to increase the sensitivity of benzodiazepines by hydrolyzing β-D-glucuronic acid from benzodiazepine-glucuronide conjugates in urine samples using the β-Glucuronidase enzyme. In this study resorufin β-D-glucuronide, a substrate of the β-Glucuronidase enzyme, was added to patient samples to determine if proper hydrolysis had occurred. The presence of resorufin as an Internal Hydrolysis Indicator (IHI) shows the activity and efficiency of the enzyme in each patient sample. Synthetic/patient urine samples were obtained and mixed with hydrolysis buffer containing resorufin β-D-glucuronide. The β-Glucuronidase enzyme was used to hydrolyze the benzodiazepine analytes as well as resorufin β-D-glucuronide. The enzymatic hydrolysis addition increased the positivity rate of benzodiazepines by 42.5%. The β-Glucuronidase substrate resorufin (IHI) displayed variability in area counts between patient samples. Comparative studies with internal standards and resorufin (IHI) showed no correlation between recovery and analyte variability. Hydrolysis reactions greatly improved the sensitivity of benzodiazepines by liquid chromatography time-of-flight mass spectrometry analysis. The large variation in resorufin (IHI) area counts amongst patient samples indicates possible variability in enzymatic hydrolysis activity. The enzymatic hydrolysis step is a part of the extraction procedure and should be controlled for in each patient sample.

Introduction

Drug testing is an effective means for clinical, diagnostic or therapeutic monitoring. However, drug detection is complicated by the fact that parent drug forms are not always present in urine samples. Consumed opioids, opiates, benzodiazepines (benzos), as well as other drugs and hormones can undergo a process called glucuronidation, in which a UDP-glucuronosyl transferase enzyme utilizes UDP to attach a sugar moiety (glucuronic acid) forming a glycosidic bond (1). Glucuronidation occurs during metabolism in order to solubilize compounds for urinary excretion.

Benzos are versatile drugs commonly used in the treatment of correcting pain, delirium, depression and sleep apnea (2). They have varied half-lives, ranging from several hours to days (3). An overdose can result in symptoms such as drowsiness, dizziness and occasionally coma (3). For many benzos, the parent drug is typically short lived. Glucuronidation occurs in over 75% of metabolized benzos (4) and can result in difficulties for detection due to the relative polarity when compared to non-glucuronidated compounds (5). The metabolized benzos are consequently more commonly identified; however, a single metabolite may have numerous parent drug origins. Glucuronidated compounds are notoriously difficult to include in routine detection methods due to large differences in extraction efficiencies, chromatographic performance and costs associated with dedicated isotopically labeled internal standards

(ISs) giving rise to complex strategies to ensure accuracy (5). Alternatively, an enzymatic hydrolysis step using β-Glucuronidase may be implemented to increase the sensitivity of benzos by hydrolyzing β-D-glucuronic acid from benzo-glucuronide conjugates in urine samples.

In routine clinical mass spectrometry, ISs are added to each sample to determine if an assay is performing correctly from patient to patient. ISs provide information on extraction inefficiencies and ion suppression/enhancement on a sample-by-sample basis. In contrast to monitoring the enzymatic hydrolysis step it is common for labs to test a single glucuronidated control at the beginning of their batch. This strategy will monitor generic enzymatic activity in the control, but it does not provide any information for whether each individual patient sample has successfully undergone enzymatic hydrolysis. To date, glucuronidated Internal Hydrolysis Indicators (IHIs) are not commonly used in the clinical lab to determine if the enzymatic hydrolysis step has worked as expected on a sample-by-sample basis. Additionally, it has been shown that some natural glucuronides commonly found in Japanese herbal medicines act as potent inhibitors of the β-Glucuronidase enzyme (6). Rather than using a single control to provide an indication of enzyme addition as well as enzyme function for an entire batch of samples, we investigated the use of a known glucuronidated substrate as an IHI (resorufin β-D-glucuronide) added to each sample as a mechanism for monitoring enzymatic activity from patient to patient. By monitoring the formation of the non-glucuronidated product in each sample, we were able to verify enzyme addition as well as a mechanism to establish minimum enzyme activity for use on a sample-by-sample basis.

Methods

Laboratory assays

Samples of synthetic urine (7) or patient urine were obtained and 200 µLs of sample was transferred into a 96 deep well plate. A premixed hydrolysis buffer (IMCS, Columbia, SC) containing 7 µg/mL resorufin β-D-glucuronide (Millipore-Sigma, Billerica, MA) was then added to the urine sample. 1,600 units of the β-Glucuronidase enzyme (IMCS) was added to the reaction mixture and incubated at room temperature for 15 min. After incubation, 180 µLs of sodium bicarbonate (0.1 M, pH 9.0) extraction buffer was mixed into the reaction. The reaction mixture was then transferred to an SLE + fixed well plate column, which was attached to a 96 deep well plate (Biotage, Charlotte, NC). The SLE + fixed well plate column and attached deep well plate was transferred to an Automated Liquid Dispenser instrument (SPEware Inc., Baldwin Park, CA) that loaded the sample onto the column (using positive gas pressure) and eluted the analytes off with 800 µLs of ethyl acetate. Once complete, the SLE + plate was disposed of and the collection plate was covered with an Anti-Cross Talk plate adapter (Biotage) and dried using a CEREX 96-well plate nitrogen dryer (SPEware Inc.). Dried samples were then reconstituted in 200 µLs of reconstitution buffer (9:1 of clinical laboratory reagent water: Methanol Optima) in preparation for liquid chromatography time-of-flight mass spectrometry (LC-TOF-MS) analysis. The assay was calibrated with a single point calibration at the cutoff concentration for all analytes. This was verified with two quality controls, one placed at 50% of the cutoff concentrations and one placed at 150% of the cutoff concentrations. Each reportable analyte was represented in all quality controls and the calibrator.

LC-TOF-MS parameters

After extraction and enzymatic hydrolysis, urine samples were analyzed by a previously validated qualitative LC-TOF-MS method (8). The HPLC system consisted of a series 1260 binary pump (Agilent Technologies) with G1379B vacuum solvent degassers, a well plate autosampler (1260 series G1379B) and a thermostatted column compartment (G1316B) with a 10-port two-position switching valve. The TOF mass spectrometer used was an Agilent 6230 equipped with dual-spray jet stream electrospray ionization (ESI) source. A Phenomenex Kinetex F5 column (2.1 × 50 mm, 2.7 µM) was used for liquid chromatography analyte separation. Mobile phase solvents consisted of 5 mM ammonium formate in water, pH 3.5 (A) and Fisher Optima grade Methanol (B).

B-Glucuronidase activity

Enzyme activity was monitored by LC-TOF-MS detection of the non-glucuronidated product. Opioids and benzodiazepine glucuronidated compounds (morphine-glucuronide, oxazepam-glucuronide and lorazepam-glucuronide) were used to determine enzymatic activity of the β-Glucuronidase enzyme in addition to the IHI resorufin-glucuronide. Enzyme concentrations were tested at 3,200 units, 1,600 units and 800 units with incubation times of 5, 10, 15, 30 and 60 min. Additionally, sulfatase activity (which was not expected) was monitored using tapentadol-o-sulfate as a substrate and monitoring for tapentadol-o-sulfate recovery.

Results

Glucuronidase optimization

Through the addition of resorufin-glucuronide and subsequent monitoring of resorufin, the IHI, we were able to identify sufficient glucuronidase activity by the β-Glucuronidase enzyme on a sample-by-sample basis. Glucuronidated benzodiazepines and opioids were initially tested with the enzymatic hydrolysis method to determine an appropriate incubation time. Oxazepam-glucuronide, lorazepam-glucuronide and morphine-glucuronide were treated with the β-glucuronidase enzyme and incubated for various time intervals. An optimal hydrolysis time was achieved around 15 min with 1,600 units of enzyme used, as shown in Figure 1A. In Figure 1B, no observable sulfatase activity was detected using tapentadol-o-sulfate, while glucuronidase activity was observed on morphine-glucuronide and resorufin-glucuronide.

Method comparison

Extending the extraction method to include an enzymatic hydrolysis step had an acceptable impact on test cost and completion time. There was a minor addition in completion time of ~20–30 min due to aliquoting and incubating buffer and enzyme in each sample using a liquid handler. The cost per billable test increased approximately 2.1% for the test specimens. Of the 668 patient samples tested, 284 samples showed a benzodiazepine to be present using an enzymatic hydrolysis step that was not previously observed when tested without a hydrolysis step (a positivity rate increase of 42.5%). The increased sensitivity in benzodiazepine detection due to the enzymatic hydrolysis addition more than justified the minor cost and time increase observed.

Qualitative agreement studies were conducted to ensure that the results generated with the new enzymatic hydrolysis step agreed with the results generated without the enzymatic hydrolysis step. A total of 668 patient samples were analyzed, using both methods, over 8 separate runs/extractions in order to test agreement. Of the 37

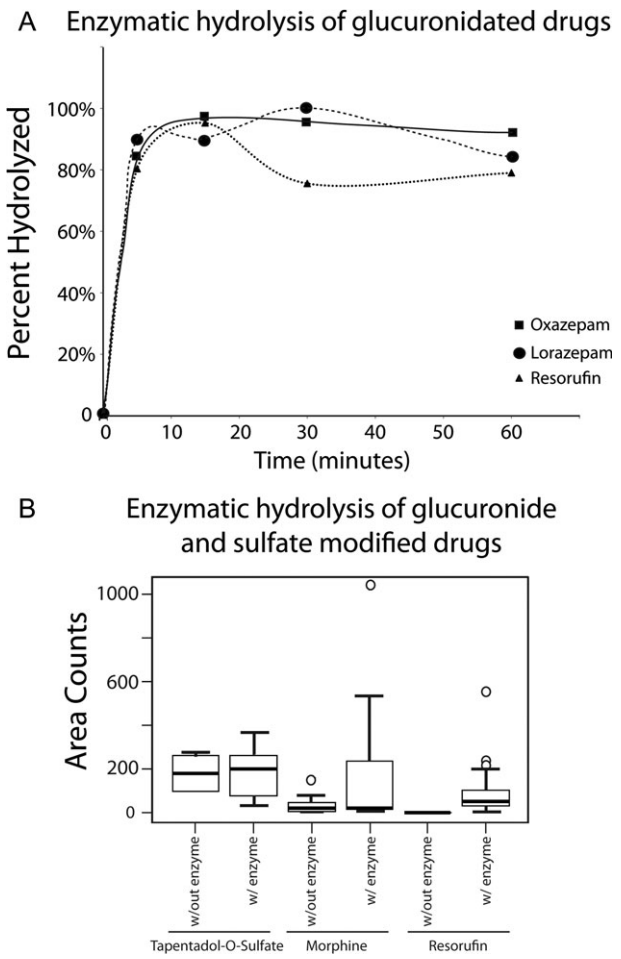

Figure 1. Monitoring enzymatic hydrolysis efficiency on glucuronidated drugs. Graph displaying enzymatic hydrolysis, at 1,600 units of enzyme, of oxazepam-glucuronide, lorazepam-glucuronide and resorufin-glucuronide throughout an hour time interval (A). A box plot is displayed to represent the enzymatic hydrolysis of tapentadol-o-sulfate, morphine-glucuronide and resorufin-glucuronide within a 15 min time interval in the presence or absence of the β-Glucuronidase enzyme at 1,600 units (B). The area counts of the non-glucuronidated forms of each compound were monitored as a result of the enzymatic hydrolysis.

drugs/drug metabolites analyzed, the largest discrepancies were observed with the benzodiazepines between the old (non-enzymatic) and new (enzymatic hydrolysis) method. This was to be expected since the enzymatic hydrolysis step is targeted to hydrolyze the β-D-glucuronic acid from glucuronidated compounds. Therefore this increase in positive samples for benzos would correlate to an improvement in the method for detection purposes. Patient samples that had different results between the two methods were sent for confirmatory testing and agreed with the enzymatic hydrolysis method results.

Determination of IHI utility

The detection of resorufin, the non-glucuronidated enzyme product, by LC-TOF-MS in each patient sample indicated enzymatic activity had occurred, and therefore analytes that were glucuronidated would have presumably been hydrolyzed as well. Resorufin area counts (an indication of successful enzymatic activity) were observed to vary widely from patient to patient indicating a potential, previously unaddressed need to monitor enzymatic activity in each sample.

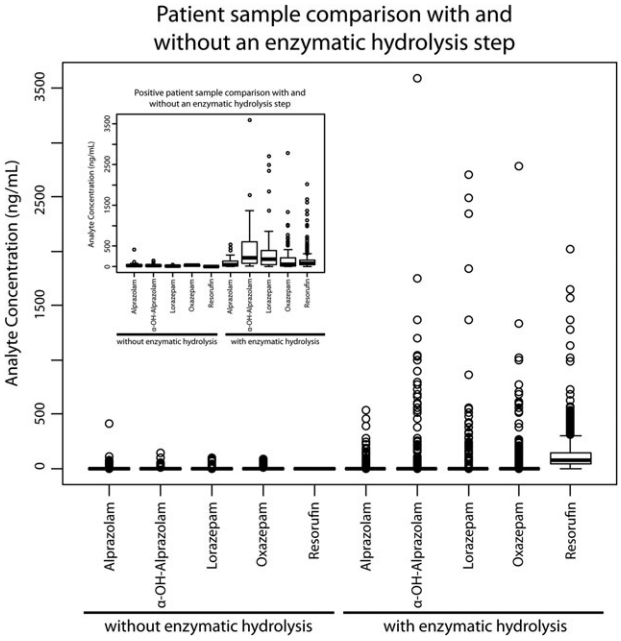

Figure 2. Increase in benzodiazepine concentration with the use of an enzymatic hydrolysis step. Box plot of benzodiazepines and resorufin with/without the enzymatic hydrolysis step. Main graph provides distributions of all results (present/not detected) while the inset provides distributions for present results only. The box represents 50% of data with a central median line (above/below median 75% and 25% of data). The "whiskers" are 1.5 times box height, representing remaining data (non-outliers).

Figure 2 shows a comparison of patient samples that were treated with and without an enzymatic hydrolysis step. Benzodiazepine concentrations increased approximately 3-fold with the addition of the hydrolysis step. Resorufin was not detected in the patient samples without an enzymatic hydrolysis step; however, samples that included an enzymatic hydrolysis step showed the presence of the resorufin analyte. Of note, resorufin area counts varied greatly in patient samples indicating potential variability of extraction/enzymatic activity from patient to patient.

In order to determine if the variability of the resorufin area counts might be attributed to variation in recovery for a given sample, we compared variability of the ISs. The ISs present in the extraction procedure (6-acetylmorphine-d6 (6-AM-d6), α-OH-alprazolam-d5, diazepam-d5, meperidine-d4, methamphetamine-d5, morphine-d3 and norfentanyl-d5) were plotted to see if they correlated with variability in area counts to resorufin (Figure 3). The greatest variability observed was with methamphetamine-d5, and the least variability was with α-OH-alprazolam-d5. IS area counts were investigated further to test the possibility that the observed resorufin area count variations correlated with recovery issues. In Figure 4 resorufin and the IS area counts were compared directly over several batches of different patient samples. The R^2 values ranged from 0.01 to 0.0973, demonstrating a lack of strong correlation and indicating the resorufin variability was likely independent of extraction inefficiency as monitored by the included ISs.

Discussion

A large majority of metabolized drugs undergo glucuronidation before urinary excretion, making presumptive and/or definitive methods

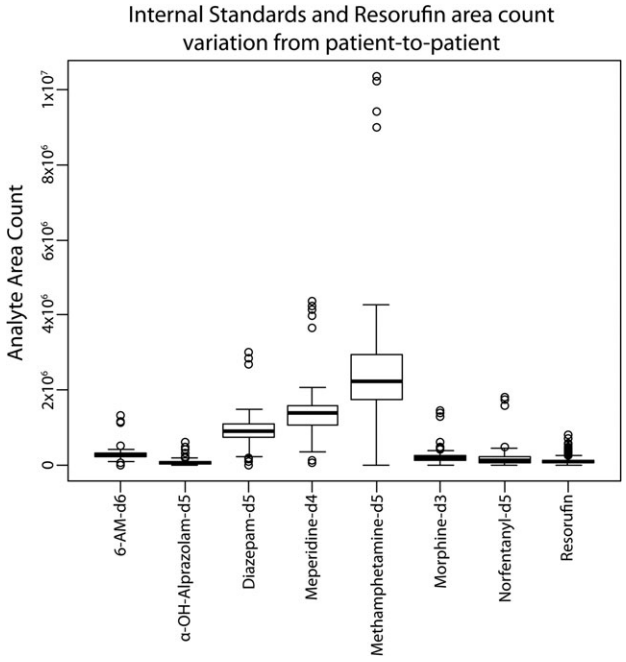

Figure 3. Distribution of ISs area counts and resorufin (IHI). The box represents 50% of the data with a central line that represents the median (above median 75% of data, below 25%). The "whiskers" extend 1.5 times the height of the box representing the remaining data. All points outside the plot represent outliers.

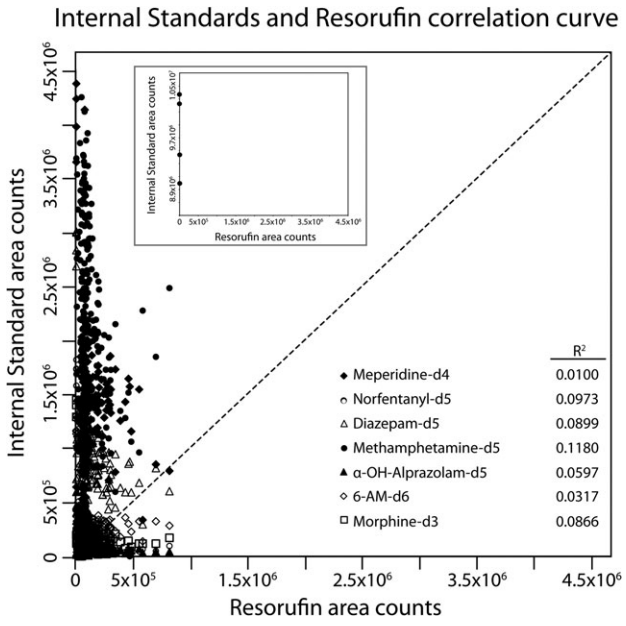

Figure 4. Correlation between the IHI resorufin area counts and IS area counts. Graphical representation investigating the correlation between ISs and resorufin (internal hydrolysis indicator) area counts.

more complex and expensive. However, the issue of glucuronidation of drug analytes in urine samples can be addressed through the addition of a hydrolysis step (enzymatic or non-enzymatic). In this study an enzymatic hydrolysis step was implemented to cleave glucuronic acid from the glucuronidated metabolites, allowing for improved detection of the included drugs. An observed positivity rate increase of 42.5% for glucuronidated analytes was obtained and confirmed as detailed in the results section. The hydrolysis reaction expectedly improved the sensitivity, especially of benzodiazepines, by mass spectrometry.

Criteria for qualitative analysis by LC-TOF-MS in a production setting makes use of retention time matching, accurate mass matching, quality scores, area counts and chromatography. Retention times must be within 0.05 min of the expected retention time to be considered present for each analyte. The mass must be within 10 ppm and the compound score must be ≥70 for an analyte to be considered positive. Additionally a minimum area count threshold must be met for each analyte (varies amongst analytes) and the peaks must be near Gaussian shape with no co-eluting compounds or tailing. Furthermore, the deuterated markers from the IS added during extraction must be present with good qualitative criteria.

The use of a single well control for monitoring glucuronidase activity to indicate adequate performance for all patients could lead to false negative results due to enzyme inhibition and/or lack of addition. Enzymatic hydrolysis monitoring with an IHI revealed that enzyme activity varied greatly from patient to patient. In some cases no hydrolysis was observed, even though the rest of the patient samples in the same batch displayed observable hydrolysis. Use of an IHI, such as the conversion of resorufin-glucuronide to resorufin demonstrated here, is a cost effective method to ensure each patient sample undergoes hydrolysis.

Conclusion

Inclusion of an enzymatic hydrolysis step is a variable that should be controlled on a sample-by-sample basis. Expecting each sample has undergone hydrolysis to the same extent based on a limited set of initial controls is not enough and is analogous to having a limited number of samples with ISs to act as a surrogate for the rest of the associated batch. Although recovery factors play a minor role in the resorufin variability observed, it is possible that compounds present in the patient specimen (such as Japanese herbal medicines) can alter the enzyme activity for each individual sample. If left unassessed, an increased potential for false negatives or falsely lowered concentrations may be observed. The method presented here provides a simple and cost effective approach for the integration of an IHI with existing assays, providing a mechanism to further enhance the quality of urine drug detection by mass spectrometry.

Acknowledgments

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