APPLICATION NOTE

Improving Benzodiazepine Immunoassay Sensitivity by Rapid Glucuronide Hydrolysis Technology

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Overview

Benzodiazepines (BZDs) are widely prescribed for general anesthesia and the treatment of sleep disorders, anxiety-induced depression, stress, muscle spasms, seizures, and alcohol withdrawal. Clinical and forensic toxicology laboratories routinely utilize immunoassays to rapidly screen BZDs in urine samples. The challenges to this detection method are low sensitivity to glucuronidated metabolites in urine and meeting lower cut-offs for more potent generation of BZDs. Studies suggest that hydrolyzing the glucuronides with a catalytic enzyme improves the sensitivity and specificity of the assays, reducing the number of false negatives. The novel recombinant β-glucuronidase enzyme, IMCSzyme®, has been reported for a rapid hydrolysis of glucuronidated BZDs at room temperature for analysis on LC-MS/MS. This application note focuses on implementing this rapid hydrolysis technique to enhance the sensitivity of immunoassay and to reduce false negatives for a detection of BZDs in urine.

Introduction

Benzodiazepines (BZDs) are prescribed for general anesthesia and the treatment of sleep disorders, anxiety-induced depression, stress, muscle spasms, seizures, and alcohol withdrawal [1]. It is important to monitor metabolites in urine to reassure the patient's adherence to the treatment plan. A fast, qualitative method to screen the presence of BZDs in urine is immunoassays. Although several immunoassay methods are commercially available [2], one of the limitations of BZD immunoassays for monitoring compliance of BZD therapy is the relative low cross-reactivity of antibody used in the immunoassays against certain glucuronidated BZD. The lower cross-reactivities towards the glucuronide metabolites would increase the number of false negatives.

In order to improve sensitivities of the immunoassays urine samples are often pre-treated with β-glucuronidase to deconjugate the glucuronic acid from BZD. The enzyme pre-treatment has been suggested to improve the sensitivity of BZD immunoassays, especially in the case of lorazepam [3-7]. This process requires added steps for the laboratories prior to screening with the immunoassays. In the recent work, EMIT™ (enzyme multiplied immunoassay technique) for benzodiazepines was reported to have a false negative rate of 35.5% [3]. In another study, three different immunoassay kits (KIMS™, CEDIA™ and HS-CEDIA™) were screened on nearly 300 real patient samples that were previously confirmed for benzodiazepines with LC-MS/MS. Despite the incorporate of the hydrolysis step, the higher sensitivity kit (HS-CEDIA) missed 22% of BZD-positive urine samples [4]. The results from these studies strongly suggest that the current hydrolysis method utilized in these immunoassay kits is suboptimal.

Recently, a pain medication monitoring laboratory has reported that the novel genetically modified β-glucuronidase enzyme, IMCSzyme, can rapidly deconjugate glucuronides from parental BZDs in urine at ambient temperature to rapidly increase the urinalysis process for the LC-MS/MS method [8]. This study focuses on the implementation of IMCSzyme to reduce pre-treatment time without requiring a heating step prior to the immunoassay detection of BZDs in urine. The transfer of this rapid hydrolysis technique using the novel β-glucuronidase from the LC-MS/MS technique to the immunoassay screening process will likely alleviate sample processing bottle necks and reduce the number of false negatives from the first screening process.

Materials and Method

7-Aminoclonazepam, α-hydroxyalprazolam, alprazolam, clonazepam, diazepam, lorazepam, midazolam, nordiazepam, oxazepam, temazepam, lorazepam glucuronide, oxazepam glucuronide, temazepam glucuronide, 7-aminoclonazepam-d4, clonazepam-d4, diazepam-d5, midazolam-d4, oxazepam-d5, and temazepam-d5 were purchased from Cerilliant Corporation. IMCSzyme, a genetically modified β-glucuronidase enzyme, was from Integrated Micro-Chromatography Systems, Inc. BZD immunoassay kit was purchased from Neogen Corporation. The kit was used according to the vendor's specifications including the standard β-glucuronidase recommended by Neogen which is referred to as Enzyme S.

The immunoassay detection was performed according to the kit manufacturer's recommended protocol with a slight modification in the enzyme pre-treatment step. In brief, Enzyme S and IMCSzyme were diluted in corresponding hydrolysis buffers at 100 and 1000 units/mL, respectively. 20 µL of each of the twelve authentic patient urine samples was mixed with 20 µL of diluted enzyme/buffer solution. The urine samples with the buffer/enzyme mixture were then incubated at ambient temperature (20°C) for 10 minutes to allow for hydrolysis, instead of at 37°C for 15 minutes as recommended by the manufacturer's protocol.

10 µL of the kit urine calibration containing 0 ng/mL (negative control) or 200 ng/mL BZDs (reference cut-off) was added to each well in duplicates. Drug free urine sample spiked with oxazepam at 200 ng/mL and unhydrolyzed patient urine samples were diluted 10 fold. The hydrolyzed samples were diluted an additional 5 fold to achieve a final 10 fold dilution. 10 µL of the diluted

samples was added to each well in duplicates on immunoassay plate. 100 µL of drug-conjugate was added to each well and incubated at ambient temperature for 45 minutes. After washing the wells, signals were developed by incubating with 100 µL of substrate for 30 minutes and adding 100 µL of stop solution. The absorbance was measured at wavelength of 450 nm on a plate reader (SpectroMax M2). The BZD concentrations in urine were confirmed using LC-MS/MS. Parent BZDs were spiked in drug-free urine as calibrators at 0, 50, 100, 200, 500, and 1,000 ng/mL. The master mix containing enzyme, hydrolysis buffer, and internal standards (200 ppb in 50% methanol) was prepared at a ratio of 8: 2: 5, respectively. 50 µL urine samples were mixed with 75 µL of master mix and incubated at 55°C for 30 minutes for glucuronide deconjugation. Hydrolyzed samples were extracted using DPX WAX tips according to the manufacturer's protocol. In brief, the tips were pre-conditioned with 30% methanol and washed with MilliQ water. Next, the hydrolyzed urine samples were aspirated and dispensed three times to allow analytes to bind to the resins. The tips were then washed with MilliQ water and analytes were eluted in acetonitrile with 1% formic acid. The eluent was evaporated and reconstituted in 200 µL of 5% methanol.

Samples were analyzed on Thermo TSQ Vantage triple quadrupole instrument coupled with an Agilent 1260 HPLC using an Agilent Poroshell EC-C18 column (3.0 x 50 mm, 2.7 µm) heated to 50°C. The mobile phase solvents were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Injection volume was 5 µL. Samples were separated chromatographically for 6 minutes. Mass spectrometer parameters were as follow: electrospray voltage, 4000 V; gas pressure, 60 psi.

Results

The signal intensity is inversely proportional to the amount of BZDs in urine samples due to the competitive binding of urine metabolite and drug/HRP-conjugate. Drug-free urine spiked with 200 ng/mL oxazepam resulted in a higher absorbance reading than the cut-off calibrator provided by the manufacturer (Figure 1). This difference in absorbance between the calibrator and urine sample may be due to the urine matrix interfering with the binding affinity of the antibody towards the drug analytes. Therefore, the cut-off reading was based on the value of oxazepam-spiked drug-free urine. Samples with absorbance level above 0.994 were regarded as negative, while those samples with absorbance levels below 0.994 were regarded as positive for BZDs.

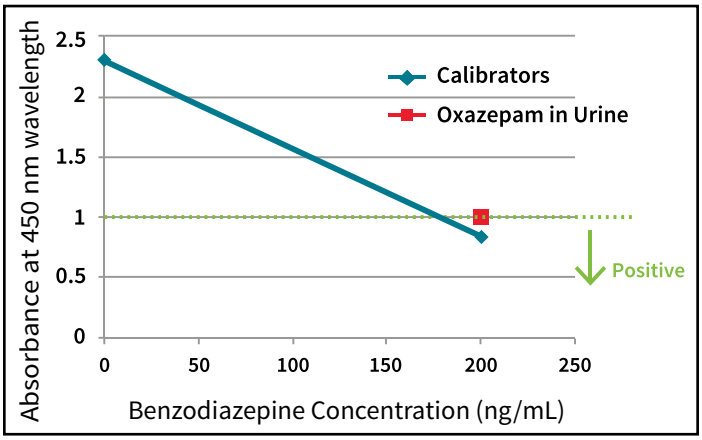

Figure 1. An inverse correlation plot between the concentration of BZDs and absorbance at 450 nm wavelength from ELISA screening kit. Calibrators (◆) were provided by the ELISA kit. Oxazepam in Urine (■) was prepared by spiking drug-free urine with 200 ng/mL of oxazepam. The cut-off value (• • • •) was based on the value of oxazepam-spiked drug-free urine.

Twelve authentic patient urine samples were screened using the ELISA kit, with and without enzyme pre-treatment (Figure 2). Seven out of 12 patient samples were classified as negatives without enzyme treatment (Table 1). There was no improvement in immunoassay sensitivity despite treating with Enzyme S. In comparison, pre-treating with IMCSzyme yielded four true negatives, and eight true positives. The overall absorbance signal improvement was achieved with the pre-treatment of IMCSzyme, whereas pre-treatment with Enzyme S did not significantly improve the sensitivity of the kit.

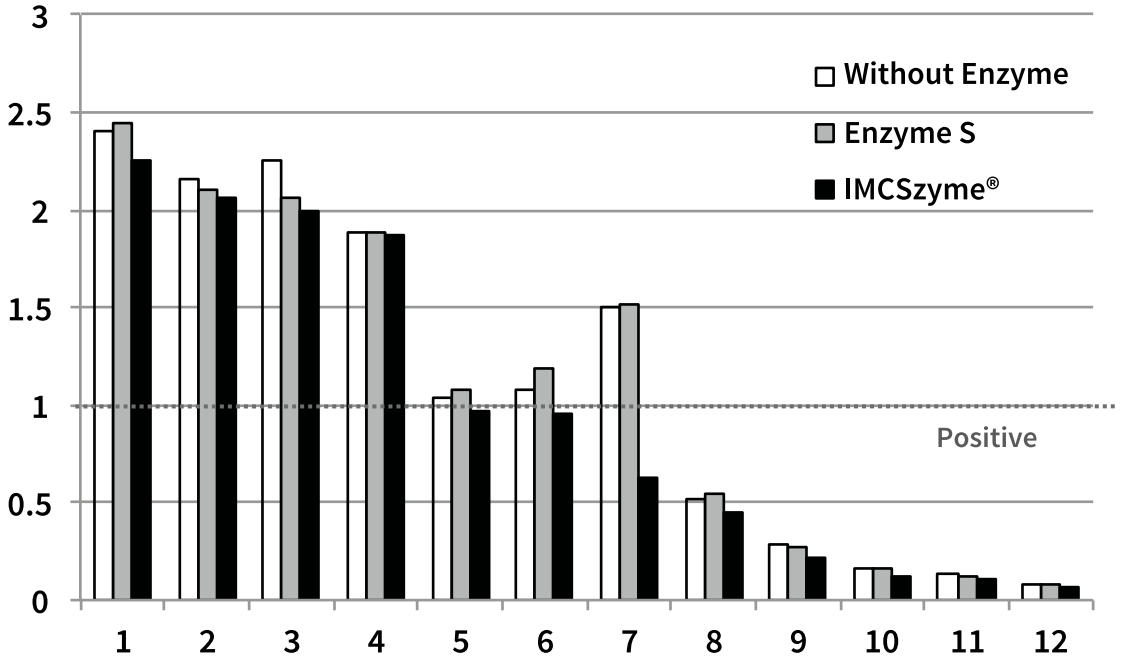

Figure 2. Absorbance measured at a wavelength of 450 nm from the ELISA screening of twelve patient urine samples. The readings below a cut-off (0.994) were regarded as positive for BZDs.

Table 1. Screening results from ELISA without enzyme, with Enzyme S, or IMCSzyme pre-treatment. The false negatives are highlighted.

Sample Number	No Enzyme	Enzyme S	IMCSzyme®
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	+
6	-	-	+
7	-	-	+
8	+	+	+
9	+	+	+
10	+	+	+
11	+	+	+
12	+	+	+

The screening results were analyzed using LC-MS/MS to confirm the concentrations of ten BZDs in the twelve patient samples. There were eight samples that were confirmed positive. Three samples were found to be free of any BZDs and the fourth negative sample containing less than 200 ng/mL of BZD. This finding aligns well with the ELISA results for samples treated with IMCSzyme. Without IMCSzyme pre-treatment, ELISA screening produced false negative on sample# 5, 6, and 7 (Table 2). Sample# 5 and 6 contains a near cut-off level of 7-aminoclonazepam at 209 and 378 ng/mL, respectively (Table 2). Especially for sample # 5, the ELISA readings were only slightly below the cut-off level of 0.994. Sample# 7 contains 1,179 ng/mL of lorazepam which has been previously reported to be associated with the majority of the false negative ELISA screenings due to the poor sensitivity of the immunoassay to its glucuronidated form. [4, 9]

Table 2. Positive and negative screening results compared with the corresponding LC-MS/MS concentrations of BZDs in twelve authentic patient urine samples.

Sample Number	ELISA Result with IMCSzyme®	LC-MS/MS Confirmation	
		Compound Name	Concentration (ng/mL)
1	-	-	-
2	-	-	-
3	-	-	-
4	-	Midazolam	20
5	+ ¥	7-Aminoclonazepam	209
6	+ ¥	7-Aminoclonazepam	378
7	+ ¥	Lorazepam	1,179
8	+	α-Hydroxyalprazolam	145
9	+	Alprazolam	42
10	+	Diazepam	14
		Nordiazepam	107
		Oxazepam	1,105
		Temazepam	296
11	+	Diazepam	25
		Nodiazepam	132
		Oxazepam	1,525
		Temazepam	1,097
12	+	Diazepam	27
		Nodiazepam	772
		Oxazepam	66,769
		Temazepam	3,664

¥ False negative reported by immunoassay screening without enzyme pre-treatment or with Enzyme S treatment

Conclusions

The study demonstrated the importance of a proper pre-treatment step with β-glucuronidase enzyme prior to immunoassay detection of BZDs in urine. The pre-treatment step increases sensitivity of the assay and reduces a percentage of false negatives. The ELISA kit manufacturer recommended this step to be performed at 37 ˚C for 15 minutes using Enzyme S. We were able to reduce the incubation time and completely eliminate the heating step. Using IMCSzyme β-glucuronidase enzyme, this step can be performed at ambient temperature for only 10 minutes. There were no false negative with ELISA screening of twelve patient urine samples using IMCSzyme. To further validate a larger sample pool of real patient samples will be tested along with other commercial kits.

References

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