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Optimisation of Benzodiazepine Immunoassay Using β-Glucuronidase Enzymatic Hydrolysis: A Comparison of Five Different β-Glucuronidase Enzymes

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Abstract

Background: Hydrolysis improves the sensitivity of drug detection for drug classes such as opiates/opioids and benzodiazepines, which are highly metabolized by glucuronidation and sulfation and should be implemented in analytical procedures to convert conjugated metabolites into the free or unbound form. This study was aimed to compare different enzymes to make an informed decision. **Methods:** In this study, the CEDIA Benzodiazepine assay was compared with the LC-MS-MS method using 150 positive urine samples and 50 negative urine samples. The samples were analysed without adding any enzyme and then by adding different enzymes to compare their performance. **Results:** The Kura *Escherichia coli* enzyme performed better than the Roche *Escherichia coli* enzyme which had 20% false-positive results. Kura BG-100 enzyme performed well but Kura B-One enzyme performed better The Kura B-One enzyme had only 11.5% false-positive results. When double the volume of Kura B-One enzyme was used to test to see if it will have any impact on reducing the number of false negatives, it performed worse. Kura Turbo enzyme behaved similarly to Kura BG-100. **Conclusions:** The β-glucuronidase enzymes comparison allowed us to identify the Kura B-One enzyme as the enzyme of choice for our operation because it reduces the false positives from 20% to 11.5% when compared with the Roche enzyme. It also improved the detection of oxazepam. The Kura B-One enzyme has a short incubation time for hydrolysis when used with the LC-MS-MS method. As a result, we improved the overall turn-around time and reduced the number of false positives that needed confirmation.

Keywords

β-Glucuronidase Enzyme, CEDIA, Benzodiazepine, 7-Amino Clonazepam, 7-Amino Clonazepam

1. Introduction

Using *β*-glucuronidase is a preferred method of hydrolysis over acid-catalysed hydrolysis, which is known to induce benzodiazepine degradation and transformation to increase cross-reactivity [1] [2] [3]. Metabolised forms of benzodiazepines undergo a process called glucuronidation during metabolism that attaches a glucuronic acid for increased solubility [4] [5]. *β*-glucuronidase is an enzyme that is used to de-conjugate *β*-glucuronides during urinary drug testing for benzodiazepines. Hydrolysis improves the sensitivity of drug detection for drug classes such as opiates/opioids and benzodiazepines, which are highly metabolized by glucuronidation and sulfation and should be implemented in analytical procedures to convert conjugated metabolites into the free or unbound form. Enzyme hydrolysis of urine using the *β*-glucuronidase to liberate conjugated drugs improves detectability [6] [7]. Only trace amounts of parent benzodiazepines are present in urine following extensive metabolism and conjugation [8]. It is also important to understand the difference between different immunoassays methods and what they can detect and if there are any limitations [9]. The Thermo Fisher CEDIA high sensitivity assay performed better when compared to other immunoassays [10] [11]. This study compared five different enzymes with the CEDIA immunoassay. The enzymes tested are *β*-glucuronidase from *Escherichia coli* from Roche and four different enzymes are obtained from Finden Kura which are B-One *β*-Glucuronidase, BG-100 *β*-Glucuronidase, BG Turbo *β*-Glucuronidase, and *β*-Glucuronidase from *Escherichia coli*.

2. Materials and Methods

A total of 200 urine samples were tested for Benzodiazepine without adding enzyme and then tested separately after adding different enzymes. The 200 urine samples had 150 positive samples and 50 negative samples. The method used in this study was CEDIA® Benzodiazepine assay from Thermo Fisher (Catalogue number 1775561). The samples were tested using the Beckman-Coulter 5810 chemistry analyser. All results were confirmed using Liquid Chromatography with tandem mass spectrometry (LC-MS/MS). *β*-glucuronidase from *Escherichia coli* was obtained from Roche (Catalogue number 127680), B-One *β*-Glucuronidase was obtained from Finden Kura (Catalogue number B-One-10 mL), BG-100 *β*-Glucuronidase was obtained from Finden Kura (Catalogue number BG100-10 mL), *β*-glucuronidase from *Escherichia coli* was obtained from Finden Kura (Catalogue number EBG), BG Turbo *β*-Glucuronidase was obtained from Finden Kura (Catalogue number BG Turbo-25 mL). Phosphate Buffer Saline was

obtained from Thermo Fisher (Catalogue number TM4121).

Roche enzyme *Escherichia coli* which has the β-glucuronidase activity of ≥200 U/mL is validated and recommended by Thermo Fisher to be used with the CEDIA Benzodiazepine assay. Because the concentrations of β-glucuronidase enzymes and their origin are different, an equivalent concentration of glucuronidase activity of 200 U/l is used to test and compare all other enzymes using phosphate buffer saline to prepare the required concentration to maintain the optimum pH of the assay and the enzymes which are pH 7. The ratio of the enzyme added is about 5 µL glucuronidase activity of 200 U/L to each 1 mL of the CEDIA reagent 1. If a β-glucuronidase enzyme is added at a higher concentration that will lead to a lot of false-positive results and changes in the dynamics of the reactions. For some enzymes, the concentration is expressed as PS-U/mL. PS-U is a product-specific unit and identifies as "*One Product Specific Unit will liberate 1.0 µg of phenolphthalein from phenolphthalein glucuronide in 5 minutes at pH 6.8 and 20˚C*". On chemistry analysers, it is not always possible to change the incubation time of a reaction as it is linked to the other mechanics that make the analyser fast and robust. In the case of the Beckman-Coulter chemistry analyser, the incubation time is fixed at 3.3 minutes when the tested sample is added to reagent number 1 of the assay.

In this study, the CEDIA Benzodiazepine assay was compared with LC-MS-MS using 150 positive urine samples and 50 negative urine samples. The samples were analysed without adding any enzyme and also after making different preparation with 5 different enzymes to compare how they perform.

3. Results

The Kura *Escherichia coli* enzyme performed better than the Roche *Escherichia coli* enzyme which had 20% false-positive results. The false negatives were less when the Roche enzyme was used only because the false positives were much higher because the Roche enzyme elevated all the baseline of results in general. Kura BG-100 enzyme performed well but Kura B-One enzyme performed better. The Kura B-One enzyme had only 11.5% false-positive results. Consequently, the true negatives were better when the Kura B-One enzyme was used. When double the volume of Kura B-One enzyme was added to test if it will have any impact on reducing further the number of false negatives, it performed worse. Kura Turbo enzyme behaved similarly to Kura BG-100. The results are summarised in Table 1.

The false negatives were mainly 7-Amino Clonazepam, 7-Aminonitrazepam and oxazepam. They were detected, but not up to the cut-off level to be reported as positive. Using the Kura B-One enzyme improved the detectability and the reporting of oxazepam only. According to the manufacturer, CEIA immunoassay cross-reactivity for 7-Amino Clonazepam is 39% at a concentration of 515 ng/mL and the cross-reactivity for 7-Aminonitrazepam is 44% at a concentration of 450 ng/mL as shown in Table 2.

Serial dilutions of 7-Amino Clonazepam and 7-Aminonitrazepam standard materials were tested using the Kura BG100 and Kura B-One enzymes. The cross-reactivity and consequently detectability was improved at a concentration of 62.5 ng/mL for 7-Amino Clonazepam and 7-Aminonitrazepam as shown in Table 3.

Table 1. Comparing CEDIA Benzodiazepine assay using five different enzymes with LC-MS-MS.

Comparison Between CEDIA Benzodiazepine Immunoassay and LC-MS/MS Using Different Enzymes	True Positives	True Negatives	False Positives	False Negatives
Without using any enzyme	123	49	21	7
Escherichia coli enzyme (from Roche)	127	31	40	2
Escherichia coli enzyme (from Kura)	122	49	22	7
BG-100 enzyme (from Kura)	123	49	22	6
B-One enzyme (from Kura)	124	48	23	5
Double the volume of B-One enzyme (from Kura)	122	45	26	7
Turbo enzyme (from Kura)	123	49	22	6

Table 2. Cross-reactivity of Benzodiazepine and metabolites—High Sensitivity at 200 ng/mL Cut-off level.

Benzodiazepines and metabolites	Tested Concentration (ng/mL)	Positive/Negative	Cross-reactivity (%)
α-Hydroxyalprazolam	110	Positive	182
α-Hydroxytriazolam	140	Positive	143
Alprazolam	100	Positive	200
7-Aminoclonazepam	800	Positive	25
7-Aminoflunitrazepam	225	Positive	89
7-Aminonitrazepam	500	Positive	40
Bromazepam	300	Positive	67
Chlordiazepoxide	2000	Positive	10
Clobazam	450	Positive	44
Clonazepam	350	Positive	57
Clorazepate	100	Positive	200
Delorazepam	100	Positive	200
Demoxepam	1500	Positive	13
Desalkylflurazepam (Norfludiazepam)	110	Positive	182
Diazepam	80	Positive	250
Estazolam	115	Positive	174
Flunitrazepam	125	Positive	160
Flurazepam	70	Positive	286
Lorazepam	250	Positive	80
Lorazepam glucuronide	400	Positive	50
Lormetazepam	175	Positive	114

Continued

Medazepam	200	Positive	100
Nitrazepam	290	Positive	69
Nordiazepam (Desmethyldiazepam)	70	Positive	286
Oxazepam	200	Positive	100
Oxazepam glucuronide	350	Positive	57
Prazepam	140	Positive	143
Temazepam	130	Positive	154
Temazepam glucuronide	250	Positive	80
Triazolam	90	Positive	222

Table 3. Cross-reactivity of 7-Amino Clonazepam and 7-Aminonitrazepam using Kura BG-100 enzyme and Kura B-One enzyme (Cut-off—200 ng/mL).

Kura BG-100 enzyme				Kura B-One enzyme			
Serial of Standard Dilution	**Drug**	**LC-MS-MS ng/mL**	**CEDIA ng/mL**	**Serial of Standard Dilution**	**Drug**	**LC-MS-MS ng/mL**	**CEDIA ng/mL**
1	7-Aminonitrazepam	2500	649	1	7-Aminonitrazepam	2500	624
2		1250	618	2		1250	576
3		500	500	3		500	458
4		250	399	4		250	379
5		125	331	5		125	307
6		62.5	218	6		62.5	216
7		31.25	168	7		31.25	158
8	7-Amino Clonazepam	2500	646	8	7-Amino Clonazepam	2500	623
9		1250	578	9		1250	531
10		500	456	10		500	419
11		250	375	11		250	352
12		125	302	12		125	273
13		62.5	215	13		62.5	218
14		31.25	149	14		31.25	159

4. Discussion

Studies comparing CEDIA and EMIT immunoassay methods with LC-MS/MS method disputed specificity with no data regarding the false-positive and without specifying the metabolites [9] [12] [13]. Another study stated that the rates of enzyme hydrolysis depend on the configuration of the substrate as well as on the enzyme preparation used. The rate of cleavage was highest with the (S)-(+)-glucuronide and β-glucuronidase from *Escherichia coli*. This enzyme possesses the highest degree of stereoselectivity; it hydrolyses the (S)-(+)-isomer more than 400 times faster than the (R)-(−)-form. Bovine liver glucuronidase is less

stereoselective, whereas glucuronidase preparations of molluscan origin exhibit little stereoselectivity. The ready hydrolysis of one of the glucuronides by an enzyme from an intestinal microorganism may play a role in the enterohepatic circulation of oxazepam [14]. Enzyme preparations from *Escherichia coli*, *Helix pomatia*, and *Patella vulgate* were examined and found capable of reducing oxazepam or oxazepam glucuronide into nordiazepam (desmethyldiazepam). Nordiazepam formation was positively correlated with incubation temperature, incubation time, oxazepam concentration, and enzyme concentration. A study found that enzymatic hydrolysis using *β*-glucuronidase enzymes (*Escherichia coli*, *Helix pomatia*, *and Patella vulgate*) caused < 2.5% nordiazepam formation that was relative to the amount of oxazepam present in the system [15]. This unusual reductive transformation also occurs in other benzodiazepines with a hydroxyl group at the C3 position and converting temazepam into diazepam and lorazepam into delorazepam by about 1% [16]. These findings are suggesting the detection of nordiazepam, diazepam or delorazepam in biological samples subjected to testing involving enzyme-catalyzed hydrolysis should be interpreted with care. Another study found that after enzymatic hydrolysis of the urine samples, a 2 - 19-fold increase in the concentration of the designer benzodiazepines flubromazolam was found, highlighting the value of hydrolysis for this analyte [17]. It was shown in another study that the amount of 7-amino-flunitrazepam metabolite quantitated by GC-MS, however, accounted for only 15% - 20% of the total OnLine immunoassay crossreactive flunitrazepam metabolites [18]. Another study evaluated EMIT, EPIA, and Online immunoassays with the GC-MS method and although differences in the performances of the investigated assay systems were observed, they all seem appropriate for clinical use in detecting benzodiazepine intake in drug abusers when enzymatic hydrolysis is included [19].

In this study, the Roche enzyme has 20% false-positive results, while the Kura B-One enzyme has 11.5% false positive. Consequently, the true negative was improved when using Kura B-One. In our laboratory, all positive results are retested by LC-MS-MS for confirmation. Using an enzyme that doesn't produce lots of false positives, reduce the unnecessary testing for confirmation which also improves the turn-around time for reporting. Because all negative results are reported without confirmation, false negatives constitute a problem in failing to report drug use. Analysing the results from LC-MS/MS for false-negative samples showed that the Kura B-One enzyme improves the detectability of oxazepam. Kura BG-100 and Kura B-One enzymes performed better than the other enzymes in detecting 7-Amino Clonazepam and 7-Aminonitrazepam at a cut-off level of 200 ng/mL. Both of these drugs are detected through their cross-reactivity with the assay. There are methods described to measure 7-aminonitrazepam using HPLC also [20]. The prospects of this study should help other laboratories to choose an enzyme that suits their needs and workflow. Also to realise the differences between these enzymes.

5. Conclusion

The β-glucuronidase enzyme comparison allowed us to identify the Kura B-One enzyme as the enzyme of choice for our operation because it reduces the false positives from 20% to 11.5% when compared with the Roche enzyme. As a result, the number of samples that need to go for confirmation on LC-MS-MS was reduced. It also improved the detection of oxazepam, 7-Amino Clonazepam and 7-Aminonitrazepam. Additionally, it has the least incubation time for hydrolysis when also used for confirmation using LC-MS/MS method which improved the overall turn-around time.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Abbreviations

CEDIA: Cloned Enzyme Donor Immunoassay.

EMIT: Enzyme-Multiplied Immunoassay Technique.