Hydrolysis efficiency comparison of two beta-glucuronidases: BG100® and BGTurbo®

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Introduction

During urine drug testing most of the analytes must be de-conjugated from their glucuronide moiety in order to unify the readings and achieve accurate results. To perform this de-conjugation, an efficient chemical or enzymatic hydrolysis is needed.

Unoptimized hydrolysis can lead to incomplete recoveries, complex manual workflows, expensive cost per sample and more important, unreliable results. Understanding the behavior of the β-glucuronidases under typical parameters such as temperature, pH and type of buffer, and other parameters not usually controlled such as urine concentration, is the first step for a successful optimization. Hereby we evaluate **BG100®** and **BGTurbo®** enzymes.

BGTurbo® is a patented high efficiency recombinant β-glucuronidase, developed specifically for urine drug testing applications. It has the highest catalytic efficiency available in the market, for the broadest-spectrum of conjugated analytes, including "hard-to-cleave" glucuronides.

BG100® is a β-glucuronidase, partially purified using a proprietary process, derived from Red abalone (*Haliotis rufescens*).

Methods

In order to find improved catalytic conditions of BGTurbo on human urine, pH was evaluated between 5.4 and 9.0 preparing three buffer solutions: Ammonium acetate (5.4), Sodium phosphate (6.5-7.2) and Ammonium bicarbonate (7.5-9.0). In addition to these buffers, Tris-HCl was also evaluated at different molarities between 5 and 100 mM, to measure buffer molarity impact under the same pH. Hydrolysis temperature was evaluated between 37 and 60°C for BGTurbo. For BG100, pH was assessed between 3.0 and 6.5 preparing sodium acetate and ammonium acetate buffers, varying molarity between 23 and 500 mM (final in hydrolysis mix). BG100 activity was evaluated between 40 and 90°C. For BGTurbo and BG100, activities were measured by the release of phenolphthalein from phenolphthalein glucuronide (PPG) per hour at pH 6.8 and 5.0 respectively, incubating at 37°C. Also, hydrolysis efficiency for both enzymes was evaluated on different urine concentration of normal human urine (UTAK, Valencia, CA).

Results & Discussion

Buffer Effect

Results shown in **Figure 1** indicate that sodium acetate buffer pH 4.8 at 250 mM, results in higher hydrolysis compared to ammonium acetate buffer (pH 4.8) in the whole range of concentrations tested for BG100. As shown in **Figure 2**, for BGTurbo no significant differences were found between ammonium bicarbonate or sodium phosphate buffers, but the latter is more stable and easier to prepare. Tris-HCl acted as an activator on BGTurbo activity, but had a weaker buffering effect than the other buffers evaluated on a wide range urine pH (data not shown). Finally, sodium phosphate was selected for BGTurbo enzyme. And 50 mM (in the mix) was optimum for activity, efficient buffering (authentic urine specimens pH varies from 4.5 to 9.0), as well as compatibility with direct injection in case of a dilute-shoot method.

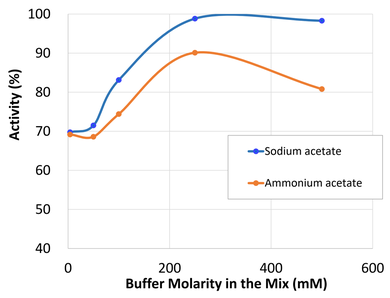

Figure 1: BG100 β-Glucuronidase optimum buffer

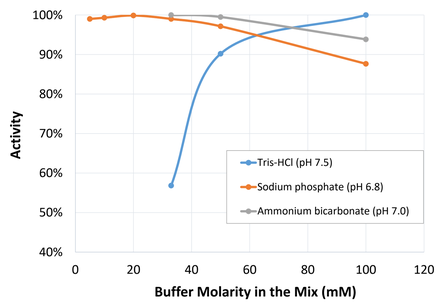

Figure 2: BGTurbo β-Glucuronidase optimum buffer

Effect of pH and Temperature

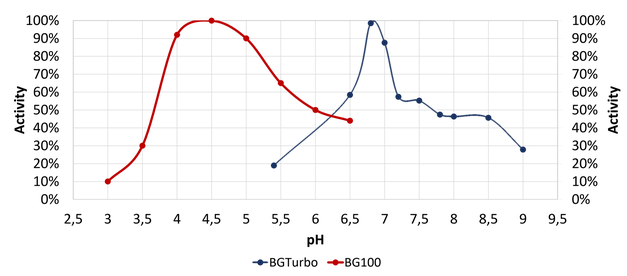

Figure 3: Optimal pH for BGTurbo and BG100 β-glucuronidases. Activity relative to the maximum value reached in 15 and 20 minutes of reaction respectively.

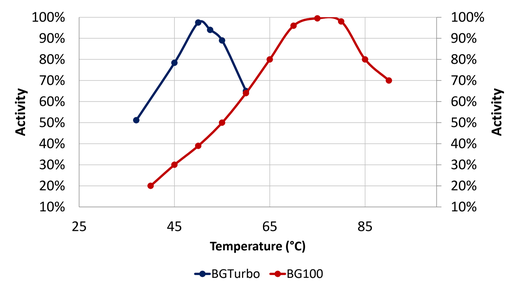

Figure 4: Optimal temperature for BGTurbo and BG100 β-glucuronidases. Activity relative to the maximum value reached in 15 and 20 minutes of reaction respectively.

Urine Effect

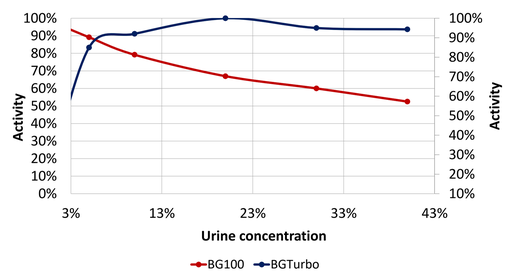

Figure 5: Optimal urine concentration for BGTurbo and BG100 in the hydrolysis mix.

BG100 was more active at acidic pH than BGTurbo enzyme **(Figure 3)**. BG100 performed better at higher temperatures than BGTurbo showing an optimum close to 70°C **(Figure 4).** Interestingly BG100 was affected by the urine concentration (**Figure 5**) while BGTurbo did not show significant activity differences when incubated with urine between 10 and 40 % (v/v) as is shown in **Figure 5.**

ENZYMATIC PERFORMANCE ON DRUGS

Preliminarily, the optimum hydrolysis conditions for BGTurbo and BG100 were identified in our laboratory using PPG as substrate. However, hydrolysis of conjugated drugs can result differently. Each drug-glucuronide and enzyme couple presents a specific affinity and velocity for hydrolysis. **Table 1** shows a comparative study done by Sasaki, T. (2017) from *Northwest Physicians Laboratories.* This study sought to obtain over 80% of recoveries in less than one hour of incubation for codeine-6-glucuronide (C6G) and other conjugates at high concentrations. C6G is known to be one of the "hardest to cleave" conjugates.

Table 1: Incubation times for high drug concentrations

N°	Conjugates	ng/mL	BG100	BGTurbo
1	Morphine-3-β-G	20,000	< 15 min	< 15 min
	Oxymorphone-3-β-G	20,000	< 15 min	< 15 min
	Codeine-6-β-G	**20,000**	**60 min**	**30 min**
	Oxazepam-G	20,000	< 15 min	< 15 min
2	**Dihydrocodeine-6-β-G**	**20,000**	**60% ***	**< 15 min**
	Tapentadol-β-G	10,000	40 min	< 15 min
	Lorazepam-G	10,000	< 15 min	< 15 min
	Temazepam-G	10,000	< 15 min	< 15 min
3	Norbuprenorphine-3-G	2,500	< 15 min	< 15 min
	6-β-Naltrexol-3-β-D-G	5,000	< 15 min	< 15 min
	Naltrexone-3-β-D-G	5,000	< 15 min	< 15 min
	THC-COOH-G	2,500	< 15 min	< 15 min
	Buprenorphine-3-β-D-G	2,500	< 15 min	< 15 min
	Amitriptyline-N-G	**5,000**	**40% ***	**< 15 min**

Time required for >80% hydrolysis recovery or *maximum recovery after 75min

In conclusion, either for opiates confirmation or broad spectrum toxicology panels, we recommend **Table 2** hydrolysis conditions. They depend on the targeted ULOQ for opiates, especially the highest codeine and dihydrocodeine concentrations to be quantitated.

Table 2: Final recommended BGTurbo conditions

ULOQ (ng/ml)	Hydrolysis recovery %	Enzyme:urine v:v	Incubation Time (min)	Protocol objective
2,500	> 90%	2:5	10	< 15 min#
20,000	> 85%	1:1	10	SAHMSA-DOT opiates confirmation ##
20,000	> 85%	2:5	30	

Study done by *ElSohly Laboratories, Inc.* ## *User reports*

Conclusion

Hydrolysis rate depends both on the enzyme and on a large set of incubation conditions. **Temperature** and **pH** are critical and specific for each beta-glucuronidase. Additionally, the **enzyme:urine ratio** and concentration of analytes (and thus **upper limit of quantitation ULOQ**) are critical too. Finally, the **type of buffer**, its **molarity** and **urine dilution** should also be considered. Last but not least, hydrolysis rate depends strongly on the targeted glucuronidated drug. In the case of a broad drug-panel **incubation time** to complete the hydrolysis depends on the slowest cleaving drug. In this case Dihydrocodeine-6-G, codeine-6-G and amitriptyline-N-G are recommended as hydrolysis controls during validation and production.

References

1. Kwan, R. *et al.* (2016) Poster: Streamlining sample preparation with second generation enzymes.
2. Marin, S. *et al.* (2017) Poster: Evaluation of three β-glucuronidase enzymes to determine the best hydrolysis conditions for urine samples in clinical toxicology and pain management.