Development and Characterization of an Enzyme Formulation for Sulfatase and Glucuronidase Hydrolysis in a Single Step

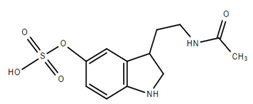
J. L. Callejas, M. Vargas and M. Rozas
Kura Biotec, Puerto Varas, Chile
Presented at ASMS Conference in June 2019

ThP 155

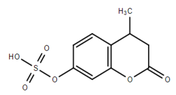
Introduction

A common challenge in steroid determination during MS-coupled drug testing is the heterogeneity of the analyte due to conjugation with a glucuronic or sulfate moiety. Aryl-sulfatases and β-glucuronidases allow homogenization of the sample by hydrolyzing the conjugated sulfate or glucuronide, respectively. Fine-tuning the ideal conditions for enzyme storage and substrate hydrolysis is critical for achieving maximum hydrolysis, and the subsequent accurate and reproducible quantification of the desired analyte.
Kura Biotec has developed two recombinant enzyme preparations carrying aryl-sulfatase activity: **ASPC™**, with only aryl-sulfatase activity and **BGS™**, with both aryl-sulfatase and β-glucuronidase activities for hydrolysis of both conjugates in a single reaction.

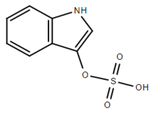
In this study, we determined the optimal pH and temperature conditions for activity of both enzyme preparations, **BGS™** and **ASPC™,** using p-nitrocatechol sulfate (PNCS) and phenolphthalein glucuronide as substrates. Secondly, the stability of the aryl-sulfatase stored at 4°C was calculated in a 24 month period. Finally the performance of **BGS™** and **ASPC™** was evaluated in the hydrolysis of 2 steroids (sulfate and glucuronide estrone) analytes and 4 sulfated metabolites from human gut microbiota.



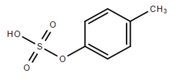
n-Acetylserotonin-O Sulfate



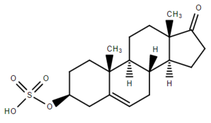
4-Methylumbelliferyl Sulfate



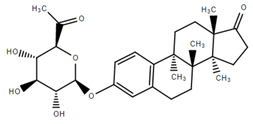
Indoxyl Sulfate



p-Cresyl Sulfate



Estrone Sulfate



Estrone Glucuronide

Methods

Aryl-sulfatase activity assay: We determined aryl-sulfatase activity monitoring hydrolysis of p-nitrocatechol-sulfate (PNCS) for 30 min at pH 7 and 45°C, unless otherwise indicated.

β-glucuronidase activity assay: We determined β-glucuronidase activity using a 30-min assay, with phenolphthalein glucuronide (PPG) as the substrate, at 37°C and pH 6.8, unless otherwise indicated.

Enzymatic Hydrolysis evaluation: 2 groups of analytes were used to evaluate the hydrolytic performance of **BGS** and **ASPC**: steroids and gut microbiota-derived metabolites. For the first group, steroids estrone-sulfate and estrone-glucuronide were spiked into human urine at a concentration of 100 ng/mL and incubated at 45 °C for hydrolysis (1). Gut microbiota-derived metabolites were at a concentration of 12.810 ng/mL for 4-methylumberlliferyl sulfate, 14.920 ng/mL for N-Acetylserotonin-o-Sulfate, 9.410 ng/mL for p-cresyl sulfate and 10.660 ng/mL forindoxyl sulfate and incubated at 37°C (2).

Stability assay: The stability of **ASPC** and **BGS** upon storage at 4°C was monitored using the above mentioned assays. Projections over extended periods of time were simulated in OriginPro using kinetic constants of destabilization obtained from accelerated stability studies.

Effect of pH and Temperature

We monitored the activities of **BGS** (mix of β-glucuronidase and aryl-sulfatase) and **ASPC** (aryl-sulfatase) over a wide range of pHs and temperatures to find the optimal pH and temperature conditions for the hydrolysis reaction. The results in figure 1 show a similarity in the temperature required to achieve maximal efficiency for β-glucuronidase and aryl-sulfatase activity, which ranges between 50 °C - 55 °C for **BGS** and **ASPC.** On the other hand, the optimal pH is slightly different for the aryl-sulfatase (pH 7.2) and the beta-glucuronidase (pH 6.8). However, **ASPC** has >80% of activity in a pH range of 6.8 – 7.5 while **BGS** can be used in a pH range of 6.8 - 7.0 depending on which enzyme is required to be more active.

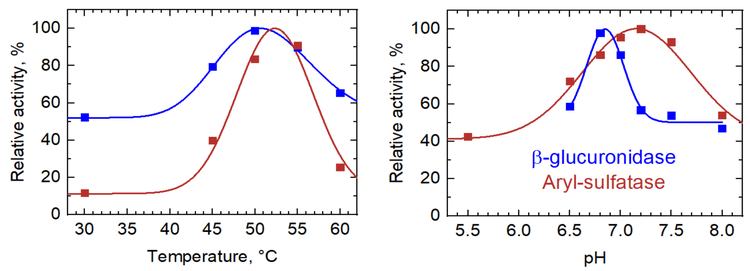


Figure 1: Evaluation of activity at different temperatures (left) and pH (right) for two enzymes; β-glucuronidase (blue) and aryl-sulfatase (red) to determine the optimal temperature and pH range for BGS (mix of β-glucuronidase and aryl-sulfatase) and ASPC (aryl-sulfatase).

Aryl-sulfatase Stability

The stabilities of **ASPC** and **BGS** were calculated from an aryl-sulfatase batch with activity >200,000 U/mL, stored at 4 °C and analyzed periodically for one month. The data obtained was analyzed with the software OriginPro. Figure 3 shows the projection calculated by the software which indicates that after 24 months of storage, the enzyme´s activity decreases by 1%.

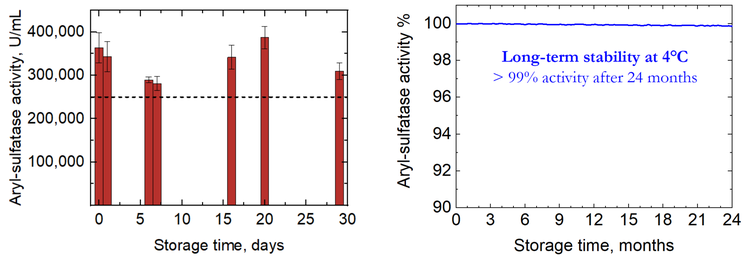


Figure 3: Evaluation of the stability of aryl-sulfatase in ASPC and BGS stored at 4 °C. The 24-month projection was simulated with the software OriginPro following accelerated stability studies with the Arrhenius method.

Enzymatic Hydrolysis Evaluation

The efficiency of **BGS** was evaluated with 6 analytes belonging to two families. The steroids were >80% deconjugated after 15 minutes of incubation for estrone-sulfate and after 10 min of incubation for estrone-glucuronide (1). The sulfates from gut microbiota-derived metabolites were evaluated using **ASPC**. The incubation time to reach >80% of deconjugation varied widely between these analytes: 4-methylumberlliferyl sulfate was the fastest to be deconjugated, in 5 minutes of incubation. N-Acetylserotonin-o-Sulfate and p-cresyl sulfate required 3 and 4 hours, respectively. After 4 hours of incubation Indoxyl sulfate reached 50% of deconjugation, being the hardest analyte to cleave in this study (2). Importantly, all tested metabolites were successfully deconjugated.

Table 1: Hydrolysis efficiency of BGS in the deconjugation of sulfate and glucuronide estrones and ASPC in the deconjugation of four sulfated gut-microbiota co-metabolism.

Product	Analyte´s Family	Analyte	Incubation Time*
BGS	Steroids	Estrone-S	15 min
		Estrone-G	10 min
ASPC	Human microbiota metabolites	4-Methylumbelliferyl Sulfate	5 min
		N-Acetylserotonin-o-Sulfate	3 h
		p-Cresyl Sulfate	4 h
		Indoxyl Sulfate	4 h (50%)

*Incubation time required to reach >80% of deconjugation.

Conclusions

- The optimal temperature range for **BGS** and **ASPC** is 50°C - 55°C.
- The optimal pH range for **BGS** is 6.8 - 7.0
- The optimal pH for **ASPC** is 6.8 – 7.5.
- **BGS** and **ASPC** can be stored at 4°C for 24 months without significant loss in activity..
- **ASPC** and **BGS** can successfully deconjugate sulfates and glucuronides from analytes in chemically complex samples.

References

1. Determine the optimal conditions to hydrolyze conjugated steroids with different enzymes (BGTurbo®, ASPC and BGS). Internal report developed by SiliCycle 2019
2. M. S. P. Correia, C. Ballet, H. Meistermann, L. P. Conway, D. Globisch Bioorg. Med. Chem. 2019, 27, 955-962.

Contact Information: www.kurabiotec.com - info@kurabiotec.com
Sales & Customer Support - (800) 3321448