Enzymatic Hydrolysis of Recalcitrant Steroids with Engineered Arylsulfatases

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INTRODUCTION

- Sulfate is one of the major phase II excretion tags for anabolic-androgenic steroids (AASs).
- The sulfate moiety can be hydrolyzed either by acid or sulfatase enzyme.
  - Acid hydrolysis can lead to analyte degradation or increased matrix interference.
  - Most commercially available sulfatases are either contaminated with impurities or have little to no activity on relevant AASs.
- We have developed novel sulfatase variants that have substantially higher activity on recalcitrant AASs compared to our own sulfatase product, Sulfazyme™ PaS, as well as other sulfatase variants from literature.

Testosterone 17β-Sulfate | **Boldenone 17β-Sulfate** | **Cortisol 21β-Sulfate** | **Dehydroepiandrosterone (DHEA) 3β-Sulfate**

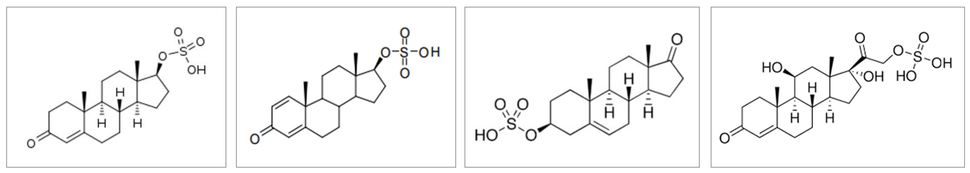


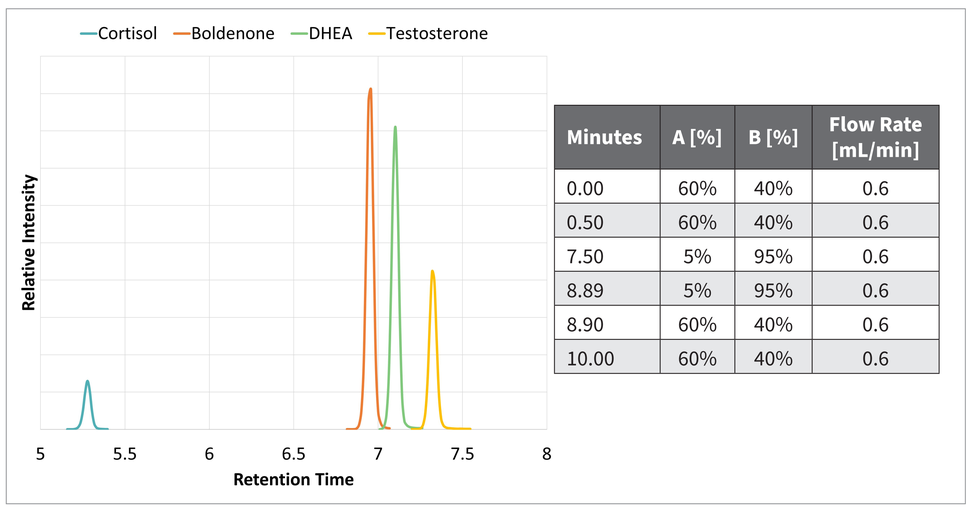
Figure 1. *Chemical structures of 4 sulfated steroids commonly quantified by LC-MS/MS.*

MATERIALS AND METHODS

Sulfazyme™ PaS (from IMCS) is the wild type arylsulfatase from *Pseudomonas aeruginosa*. Sulfazyme™ PaS, Variant 1, and Variant 2, as well as literature variants PaS-PVFV (1) and PaS-LEF (1), were recombinantly expressed in *Escherichia coli*, purified, and compared in *vivo* in a drug free urine control (UTAK). Drug free urine (DFU) was fortified with four different sulfated steroid metabolites at varying concentrations **(Table 1)**. Non-sulfated steroids were separated by a 10-minute LC gradient **(Figure 2)**. Dehydroepiandrosterone (DHEA) 3β-Sulfate and Testosterone 17β-Sulfate were not fully resolved, and therefore, sulfated steroids are not shown. A six-point calibration curve was created using a linear fit for each analyte. Quality control samples were within ± 20% standard deviation of the target values and correlation coefficients were ≥ 0.98.

Table 1. Approximate sulfated steroid concentrations fortified into drug free urine.

Steroid	Concentration (ng/mL)
Boldenone	80 ng/mL
Dehydroepiandrosterone (DHEA)	8000 ng/mL
Cortisol	400 ng/mL
Testosterone	130 ng/mL



Minutes	A [%]	B [%]	Flow Rate [mL/min]
0.00	60%	40%	0.6
0.50	60%	40%	0.6
7.50	5%	95%	0.6
8.89	5%	95%	0.6
8.90	60%	40%	0.6
10.00	60%	40%	0.6

Figure 2. *Extracted ion chromatogram overlays showing separation of 4 steroids and the corresponding gradient.*

200 µL of fortified urine was mixed with 190 µL 100 mM Tris (pH 8.0), 20 µL internal standard and 20 µL enzyme. Samples were incubated at specific time intervals between 15 minutes and 4 hours at 37°C. Hydrolyzed samples were diluted to 50% methanol and filtered. For analysis, 20 µL of sample was injected on a Thermo Scientific™ Vanquish™ UHPLC system coupled with a Thermo Scientific™ Endura™ Triple Quadrupole mass spectrometer using a Phenomenex Kinetex® 2.6 µm Biphenyl 100 Å, 50 x 4.6 mm LC column. Ion source voltage was 4250 in positive mode and 4300 in negative mode. Mobile phase A was 0.1% formic acid in water and mobile phase B was methanol.

RESULTS

Purified Sulfazyme™ PaS, Variant 1, and Variant 2 activities were compared with colorimetric sulfatase substrate 4-nitrocatechol sulfate (pNCS) at 515 nm. Sulfazyme™ PaS, Variant 1 and Variant 2 activities were 3.8, 6.1 and 3.4 U/mg, respectively **(Figure 3)**.

Purified arylsulfatases were incubated with cortisol sulfate (CS), boldenone sulfate (BS), testosterone sulfate (TS), and dehydroepiandrosterone sulfate (DHEAS) for up to 4 hours at 37°C **(Figure 4)**. Hydrolysis was considered complete when samples reached ≥ 80% of the max hydrolyzed amount (~380 ng/mL cortisol, ~7,300 ng/mL DHEA, ~50 ng/mL boldenone, ~105 ng/mL testosterone) for all conditions and performed in DFU buffered with Tris, pH 8.0 at 37°C.

On these steroids, Sulfazyme™ PaS only achieved 39% CS hydrolysis and 5% DHEAS hydrolysis after 4 hours at 37°C and had no discernible activity toward BS or TS. Variant 1 achieved ≥ 80% hydrolysis of BS and TS within 1 hour and ≥ 80% hydrolysis of CS within 3 hours but only achieved 32% DHEAS hydrolysis after 4 hours. Variant 2 achieved ≥ 80% CS, TS, DHEAS hydrolysis within 3 hours but only hydrolyzed 22% of BS in 4 hours.

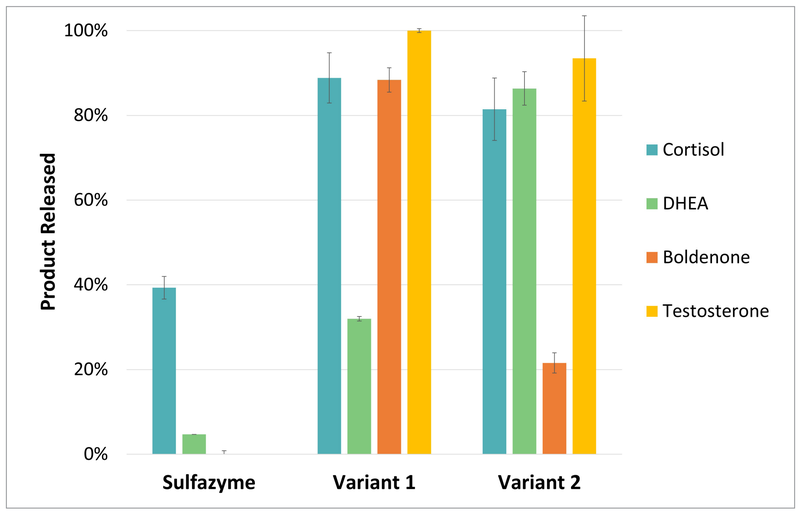


Figure 4. *Sulfazyme™ PaS achieved 39% of CS and 5% of DHEAS hydrolysis after 4 hours and had no discernible activity towards BS or TS. Variant 1 achieved ≥ 80% hydrolysis of BS and TS within 1 hour. Variant 2 achieved ≥ 80% CS, TS, DHEAS hydrolysis within 3 hours.*

The wild type sulfatase from *P. aeruginosa* has been previously engineered by Uduwela *et al.* (1) for increased activity on steroids. We compared the hydrolysis activities on DHEAS and BS of Variant 1, which had the best overall improvement of our variants, to Uduwela *et al.* (1) variants PaS-PVFV and PaS-LEF **(Figure 5)**. For these comparisons, the reactions were performed in Tris buffer, pH 8.0 at 37°C until about 30-40% of the substrate was hydrolyzed to remain in the linear part of the kinetic curve.

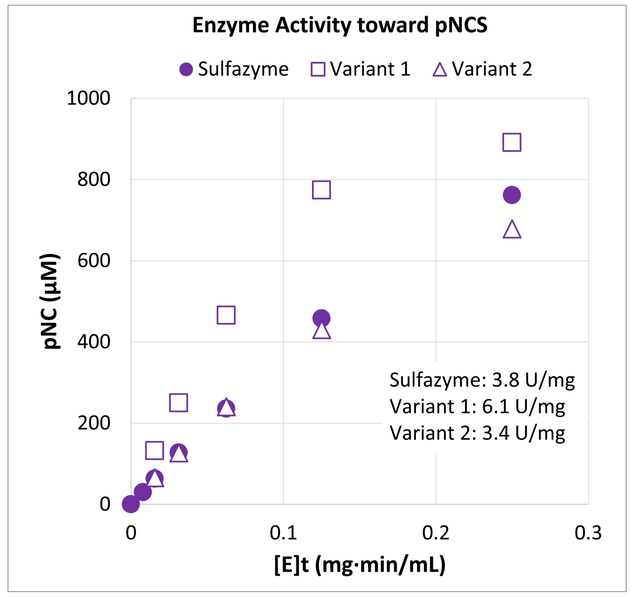


Figure 3. *Enzyme activity comparison between Sulfazyme™ PaS, Variant 1 and Variant 2 toward pNCS.*

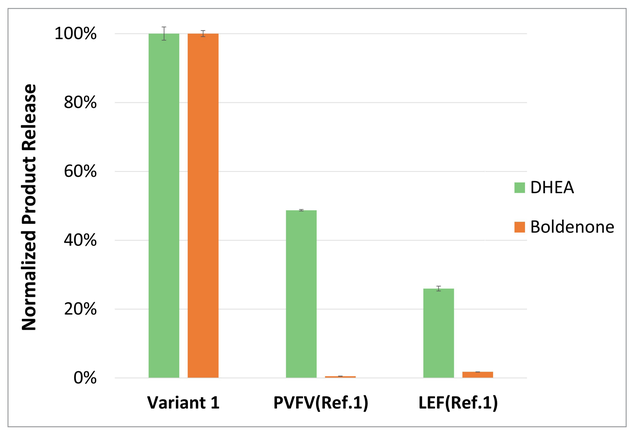


Figure 5. *Relative activities of Variant 1, PaS-PVFV (1), and PaS-LEF (1) on DHEAS and BS. The variants previously characterized in literature, PaS-PVFV and PaS-LEF (1), have about 50% and 26% of the activity on DHEAS relative to Variant 1, respectively, and 1-2% of the activity on boldenone sulfate relative to Variant 1.*

CONCLUSION

- *P. aeruginosa* arylsulfatase has been engineered to dramatically improve hydrolysis on recalcitrant, β-sulfated anabolic-androgenic steroids (AASs) such as DHEAS, CS, BS and TS.
  - Variant 1 hydrolyzed boldenone and testosterone sulfate within 1 hour and cortisol sulfate within 3 hours at 37°C.
  - Variant 2 hydrolyzed dehydroepiandrosterone, testosterone and cortisol sulfate within 3 hours at 37°C.
- Variant 1 showed drastically improved hydrolysis compared to Sulfazyme™ PaS and published variant sulfatases (1).
- Sulfatase enzymes can serve as an alternative to acid hydrolysis for steroid analysis in anti-doping labs.

DISCLOSURE

The authors are employees of Integrated Micro-Chromatography Systems, Inc.

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REFERENCES

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2. Stevenson BJ, Waller CC, Ma P, Li K, Cawley AT, Ollis DL, and MD McLeod (2015). *Pseudomonas aeruginosa* arylsulfatase: a purified enzyme for the mild hydrolysis of steroid sulfates. *Drug Test Anal*, **7**, 903-11.