Hydrolysis of sulfated steroids, toxic endobiotics and xenobiotics using purified sulfatase for quantitation of sulfated and unconjugated compounds

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ABSTRACT

In the human body, sulfation and glucuronidation are major detoxification processes to facilitate the clearance of xeno- and endogenous chemicals and byproducts. Due to the limited number of high purity sulfated reference materials and the high cost of existing sulfated reference materials, sulfated metabolite monitoring is challenging. In addition, steroid monitoring using gas chromatography mass spectrometry requires the cleavage of sulfate esters prior to the derivatization step. However, currently available sulfatases show limited effectiveness in part due to the dearth of high-quality reagents and poor characterizations. Herein, we report a methodical characterization of a purified sulfatase that cleave various sulfated analytes. Shotgun metabolomics was performed by comparing mass spectra from plasma and urine samples with or without sulfatase treatment to determine feasibility of monitoring sulfo-conjugates on a larger scale.

INTRODUCTION

Sulfatases derived from different organisms have been documented with different hydrolysis efficiencies on a range of sulfated compounds (1), which include a chromogenic substrate (*para*-nitrophenyl sulfate: PNPS) and nine different sulfated steroids **(Table 1)**. The five sulfatases summarizedin the table showed no activity against alpha linkages and poor activity towards sulfated steroids. Sulfatase derived from *Pseudomonas aeruginosa* showed the highest enzymatic activity relative to the other four sulfatases, though all tested sulfatases showed little or no detectable catalytic conversion of androsterone sulfate (AS), etiocholanolone sulfate (ECS) and epitestosterone sulfate ETS **(Table 1)**.

Integrated Micro-Chromatography Systems, Inc (IMCS) provides purified enzymes, such as IMCSzyme®, a genetically modified β-glucuronidase, for clinical and forensic toxicology applications. While there are extensive studies on glucuronidases in drug use monitoring, there are limited reports on the effective conversion of sulfonated analytes. Tackling the sulfo-conjugated metabolites requires well-characterized and high-quality sulfatase to ensure that analytes are not falsely attributed to the wrong metabolic pathways. Here, we present IMCS-PSF, a purified sulfatase, as a part of the expanded portfolio of high-quality enzyme products for monitoring sulfated metabolites, another product of phase II metabolism.

A recent study on the hydrolysis profile of terbutaline metabolites compared the use of purified β-glucuronidase and purified sulfatase to crude snail enzyme mixture (2). Terbutaline could be glucuronidated or sulfated during phase II metabolism **(Figure 1a)**. The use of individual purified enzymes allows the degree of sulfation or glucuronidation to be quantitated independently. Based on the initial study, the results from a single patient's urine sample suggest that the majority of terbutaline metabolites in urine was sulfated and IMCS-PSF achieved maximum conversion within 15 minutes of incubation **(Figure 1b)**. However, crude enzyme sourced from snail entrails showed no significant release of terbutaline from its conjugated forms (Figure 1B). Using the purified sulfatase and glucuronidase, excretion profile of sulfo-conjugated terbutaline, glucuronidated terbutaline and free terbutaline in urine was reported **(Figure 1c)**. By monitoring the sulfo and glucuronidated metabolites, terbutaline ingestion was detectable up to 3 days post-ingestion. For early characterization studies of IMCS-PSF, a purified sulfatase, hydrolysis profiles of various commercially available sulfated metabolites were generated in both urine and plasma matrices.

Table 1

Substrate	Sulfate position	*Pseudomonas aeruginosa*	*Helix pomatia*	*Haliotis rufescens*	*Patella vulgata*	*Klebsiella pneumoniae*
Cortisol sulfate	21	no data	no data	no data	no data	no data
Tapentadol sulfate	aromatic	no data	no data	no data	no data	no data
para-nitrophenyl sulfate (PNPS)	aromatic	7	6	5	3	2
Estadiol sulfate (ES)	aromatic	7	5	3	2	0
Dehydroepiandrosterone sulfate (DHEAS)	3β	3	3	3	1	0
Epiandrosterone sulfate (EAS)	3β	3	3	2	2	0
Andresterone sulfate (AS)	3α	0	0	0	0	0
Etiocholanolone sulfate (ECS)	3α	0	2	2	0	0
Testosterone sulfate (TS)	17β	2	1	1	0	0
Boldenon sulfate (BS)	17β	2	2	0	0	0
Nandrolone sulfate (NS)	17β	3	1	0	0	0
Epitestosterone sulfate (ETS)	17α	0	0	0	0	0

100 mM substrate at 37°C. The activity (μmol·min^{-1}[g protein]$^{-1}$) is represented on a logarithmic scale with:

0	no detectable activity
1	0.005-0.05
2	0.05-0.5
3	0.5-5
4	5-50
5	50-500
6	500-5000
7	5000-50000

Substrate range for five sulfatase sources with 100 μM substrate at 37°C (modified from (1))

1a.

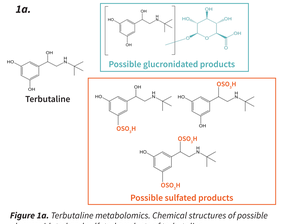

Figure 1a. *Terbutaline metabolomics. Chemical structures of possible glucuronidated and sulfated products of terbutaline.*

1b.

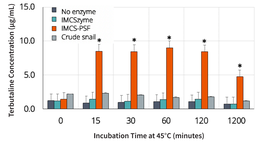

Figure 1b. *Terbutaline metabolomics. Comparison of the temporal hydrolysis profile of terbutaline metabolites upon hydrolysis with no enzyme, IMCSzyme®, IMCS-PSF and crude snail enzyme. The error bars indicate ± 1 S.D. (n=3). *p ≤ 0.001 is based on analysis of variance (ANOVA).*

1c.

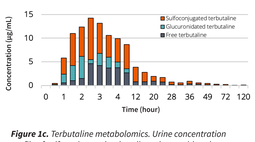

Figure 1c. *Terbutaline metabolomics. Urine concentration profile of sulfoconjugated terbutaline, glucuronidated terbutaline and free terbutaline. 100 μl of urine collected at each time point was added with 50 μl water, 25 μl 1 M Tris HCl buffer pH 8 and 25 μl water, IMCS-PSF or IMCSzyme®. The mixture was incubated at 45°C for 1 h. Figures are modified from (2).*

MATERIALS AND METHODS

Chromogenic assays to measure glucuronidase, sulfatase, and esterase were performed using phenolphthalein glucuronide (25°C), para-nitrocatechol sulfate (37°C), and Calcein-AM (37°C), respectively. The incubation time was 30 minutes.

Human urine and plasma were separated into three sets. The first set was tested without enzymatic hydrolysis, the second was tested with β-glucuronidase incubation and the third aliquot was tested with sulfatase incubation. All incubated samples were performed in triplicates, and analytes were isolated using reverse phase dispersive pipette extraction technique. Samples were first eluted in 100% methanol and dried down using CentriVap, then reconstituted with 20% methanol in water and injected into either UPLC-MS/MS or UPLC-qTOF with a reverse phase column. For MS/MS, TSQ-Endura with binary flex Vanquish was used to optimize hydrolysis conditions of sulfated metabolites.

RESULTS

The majority of the commercial enzymes are sourced from organisms such as snail, limpets or abalone which are known to contain several different interfering metabolites in the crude solution. Therefore, a highly pure, recombinant sulfatase was developed. According to the manufacturer, crude enzyme sourced from snail entrails contains both glucuronidase and sulfatase activities. The chromogenic assays not only confirmed the manufacturer's statement but also revealed that the crude enzyme also contained another enzyme like esterase **(Figure 2)**. This mixture of enzymes can skew the profiling of sulfated and glucuronidated metabolites which may be further altered by the presence of esterase. Identifying pure sulfatase with no esterase and glucuronidase activity is important aspect in ensuring that sulfated metabolites are accurately monitored.

As part of the early characterization two other substrates, cortisol and tapentadol metabolites, were studied. Synthetic urine samples fortified with known concentrations of glucuronide or sulfate conjugates were incubated with IMCSzyme® or IMCS-PSF and complete hydrolysis of conjugates was achieved within the first hour of incubation at 40°C. **(Figure 3a, 3b)**. Since plasma contains endogenous cortisol metabolites, only the recovery of tapentadol in plasma was monitored. Hydrolysis of these conjugates plateaued after 1 hour of incubation with quantitative recoveries (> 85%) within the first hour of incubation at 40°C **(Figure 3c)**.

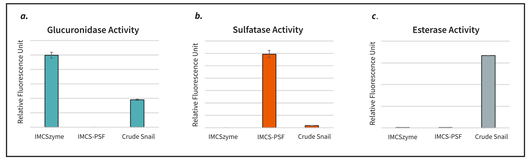

Figure 2. *Chromogenic assays to detect relative activities of IMCSzyme, IMCS-PSF and crude snail enzyme. (a) Glucuronidase activity using phenolphthalein glucuronide at 25°C. (b) Sulfatase activity using para-nitrocatechol sulfate at 37°C. (c) Esterase activity using Calcein-AM at 37°C.*

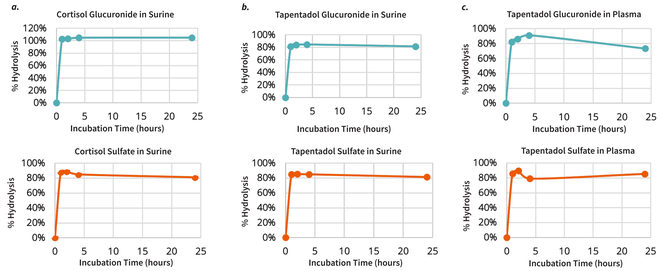

Figure 3. *Hydrolysis profiles up to 24 hours of glucuronidated or sulfated metabolites at known concentrations. (a) Cortisol glucuronide and sulfate in synthetic urine (Surine™) incubated with IMCSzyme® or IMCS-PSF, respectively. (b) Tapentadol glucuronide and sulfate in synthetic urine (Surine™) incubated with IMCSzyme® and IMCS-PSF, respectively. (c) Tapentadol glucuronide and sulfate in human plasma incubated with IMCSzyme® and IMCS-PSF, respectively.*

Cortisol is the major glucocorticoid product of the adrenal cortex. The metabolism of cortisol is illustrated in **Figure 4a** and clinical diagnostic is generally based on the ratio of these metabolites. In the kidney and colon, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) inactivates cortisol **(F)** to cortisone **(E)**, while in the liver and adipose tissue, F is regenerated from E by 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) (3). Moreover, A-ring reductases (5α, 5β and 3α reductase) convert cortisol and cortisone to tetrahydro-metabolites called allo-tetrahydrocortisol **(aTHF)**, tetrahydrocortisol **(THF)** and tetrahydrocortisone **(THE)**. Levels of free and total **aTHF**/**THF** ratio can be used to support clinical diagnosis of pathologies related to cortisol metabolism such as polycystic ovary syndrome (4).

In this study, cortisol metabolite distributions in plasma and urine of the same human volunteer were compared. Samples were treated with no enzyme, IMCSzyme® or IMCS-PSF. The distributions are vastly different in urine compared to plasma. Level of **F** level was higher in plasma compared to urine, on the other hand the inactive **E** level was higher in urine compared to plasma **(Figure 4b)**. Enzyme hydrolysis did not significantly increase levels of **F** or **E**, indicating that neither metabolites are glucuronidated or sulfated in plasma and urine matrices. The analytes **F** and **E** are detected in urine and plasma, whereas the metabolites **(aTHF, THF, THE)** are only present in urine. Assuming IMCS-PSF is effective against the metabolites of cortisol, the recoveries from treating the samples with each enzyme indicate that **aTHF**, **THF** and **THE** were mostly secreted as glucuronidated form in urine **(Figure 4b)**.

4a.

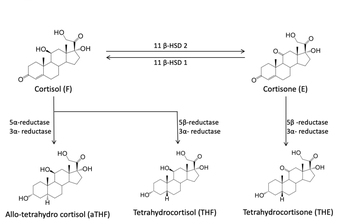

Figure 4a. *Cortisol metabolomics. Structures of cortisol and its metabolites.*

4b.

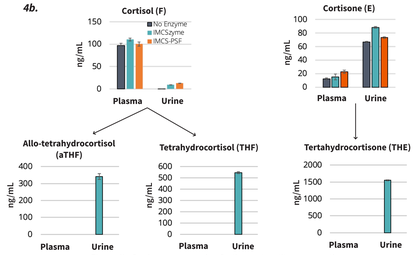

Figure 4b. *Cortisol metabolomics. Cortisol metabolite concentrations in plasma and urine of the same human volunteer.*

In our previous work presented in 2017 at the American Society for Mass Spectrometry conference, the concentrations of cortisol and its metabolites in urine were monitored primarily using glucuronidase (IMCSzyme®) and a mixture of IMCSzyme® and IMCS-PSF. The study showed that aTHF conjugates were the slowest to be cleaved when incubated with crude enzyme from snail **(Figure 5)**. IMCSzyme processed aTHF glucuronide within 15 minutes and showed higher recoveries than 4-hour incubation with crude enzyme **(Figure 5d)**. The slower hydrolysis of aTHF glucuronide had been previously reported by Cuzzola *et al.* but this may not be a concern using the new generation of beta-glucuronidase (4).

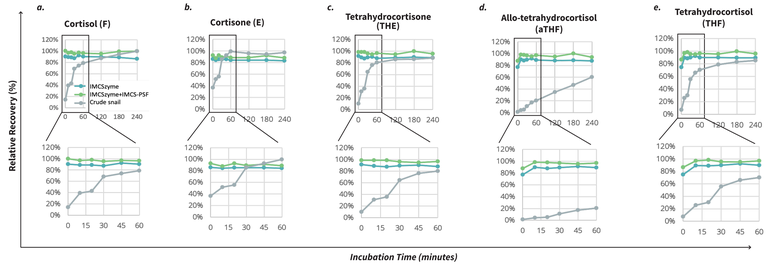

Figure 5. *Hydrolysis of a human urine sample containing the highest amount of **aTHF** for determining hydrolysis efficiency versus incubation time. The hydrolysis percentage is calculated to the maximum amount as 100%; (a) cortisol, (b) cortisone, (c) tetrahydrocortisone, (d) allo-tetrahydrocortisol, and (e) tetrahydrocortisol. A complete hydrolysis indicating by a plateau recovery was reached after 10 minutes of incubation using both enzymes, IMCSzyme and IMCS-PSF.*

Next, this study explored the general application of shotgun metabolomic profiling for finding various glucuronidated and sulfated compounds in plasma and urine using two purified enzymes. Mass spectra obtained from UPLC-qTOF between untreated and enzyme-treated samples were compared to screen for any increase in compound concentration as a result from deconjugation event. When treated with purified β-glucuronidase, a mass shift of 176.13 would be related to O-linked glucuronide while a mass shift of 139.67 would be related to N-linked glucuronide. When treated with purified sulfatase, a mass shift of 79.89 would be related to the loss of sulfate conjugation. Preliminary data gathered from UPLC-qTOF and analyzed using Global Metabolomics Cloud Plots (5) showed multiple features that changed when samples are treated with IMCSzyme or IMCS-PSF **(Figure 6)** which indicates the presence of unconjugated species of multiple metabolites. Overall, the glucuronidated and sulfated patterns are different in plasma compared to urine. By identifying glucuronidated and sulfated metabolites, we envision a characterization table of various commercially available β-glucuronidase and sulfatase products with hydrolysis efficiencies towards different metabolites.

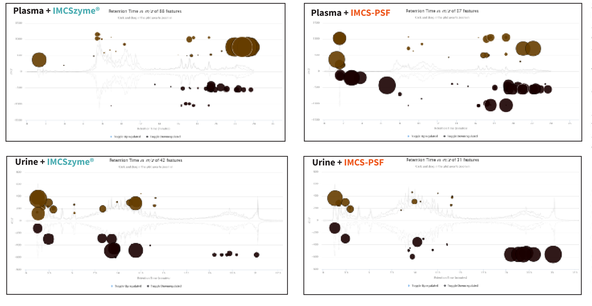

Figure 6. *Cloud plots of plasma and urine treated with IMCSzyme® or IMCS-PSF, compared to their corresponding biological matrices without enzyme treatment. Features with fold change ≥ 3 are displayed with directional fold change colors (yellow: increased intensity, red: decreased intensity), the retention time, and the mass-to-charge ratio. The size of each bubble corresponds to the log fold change of the feature: the larger the bubble, the larger the fold change. The y coordinate of each feature corresponds to the mass-to-charge ratio of the compound as determined by mass spectrometry.*

CONCLUSION

Purified β-glucuronidase and sulfatase could be used to treat biological samples in mild incubation conditions, facilitating the discovery of conjugated metabolites which are difficult to detect by MS if they remain conjugated. The use of crude enzymes can impact accuracy because they contain unwanted esterase activity that could produce side reactions when converting metabolites. The use of individual purified enzymes enables both discovery and quantification of conjugated xenobiotic or endogenous metabolites by mass spectrometry.

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