Utilizing Purified β-Glucuronidase and Arylsulfatase to Accurately Quantitate Metabolites in Human Urine

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ABSTRACT

Several metabolites are known to be glucuronidated and sulfated in the human body. Monitoring the aglycones with the gluruconidated and sulfated metabolites creates six additional masses to monitor on tandem mass spectrometry, which can limit the total number of metabolites being monitored. In addition, the conjugated standards are very expensive and often not available. Herein, we report the use of purified β-glucuronidase (IMCSzyme®) and recombinant arylsulfatase that effectively cleave glucuronides and sulfates at neutral pH with no detectable CYP activity or esterase activity. The initial studies explored urinary corticosteroid metabolites on tandem mass spectrometry before and after cleaving glucuronides and sulfates with the purified enzymes. Among corticosteroids, glucuronidated allo-tetrahydrocortisol (**aTHF**) has been reported to be recalcitrant substrate for enzymatic deconjugation. We demonstrate rapid hydrolysis within 10 minutes, while the reported 4 hour incubation using crude snail enzyme is inadequate for recovering the higher levels of **aTHF** glucuronide (1, 2).

INTRODUCTION

The study and quantitation of metabolites can give insights to health state and human diseases. This poster focuses on cortisol metabolism as an example. Cortisol is quantitatively the major glucocorticoid product of the adrenal cortex. The deficiency of adrenal steroid excretion is found in Addison's disease leading to hypotension, and on the other hand the overproduction is found in Cushing's syndrome leading to hypertension (3). Quantitative measurement of cortisol in both serum and urine are widely done by immunoassays, however the limitations are cross reactivity and low sensitivity. Recently, gas chromatography or liquid chromatography coupled to tandem mass spectrometry, abbreviated GC-MS/MS and LC-MS/MS respectively, have proven to improve specificity and sensitivity (3). This facilitates reliable measurement of cortisol and enables the detection of individual cortisol metabolites.

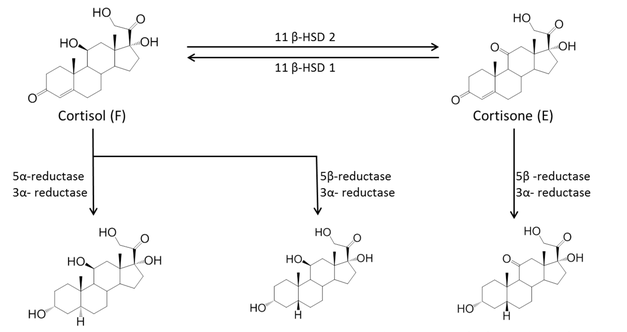

Figure 1. *Structures of cortisol and its metabolites. The enzymes involved in the conversion of cortisol into cortisone*

Metabolism of cortisol is illustrated in ***Figure 1***. In 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) (4). Moreover, A-ring reductases (5α, 5β and 3α reductase) convert cortisol and cortisone to their tetrahydro-metabolites, allo-tetrahydrocortisol (**aTHF**), tetrahydrocortisol (**THF**) and tetrahydrocortisone (**THE**). Medical diagnostic is generally based on the metabolite ratios. However, most of these compounds are excreted in urine as conjugates and reports mention the use of β-glucuronidase and arylsulfatase to deconjugate prior to MS analysis (2, 5-7).

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 (8-11). Therefore, the genetically modified β-gluruconidase and recombinant arylsulfatase were selected to map the metabolites susceptible to glucuronidation and sulfation. The hydrolysis was tracked using the plateau level of **aTHF** in urine since the conjugated analytes were not commercially available.

MATERIALS AND METHOD

Helix pomatia β-glucuronidase type HP-2 (with arylsulfatase activity) was purchased from Millipore Sigma. Purified genetically modified β-glucuronidase (IMCSzyme®) and recombinant arylsulfatase were provided by Integrated Micro-Chromatography Systems, LLC. Steroid-free synthetic urine, cortisol, cortisol-D4 were purchased from Cerilliant. Cortisone, allo-tetrahydrocortisol, tetrahydrocortisol, and tetrahydrocortisone were purchased from Toronto Research Chemicals. RP tips were purchased from DPX. Formic acid and methanol were LC grade from Fisher Scientific.

Five overnight urine samples were obtained from volunteers with written informed consent. The urine sample containing the highest **aTHF** was selected to determine the optimal incubation time. The hydrolysis was performed using 100 µL of urine, 50 µL of buffer (Rapid Hydrolysis Buffer for IMCS enzymes, and 1 M sodium acetate buffer pH 4.5 for *H. pomatia*), 10 µL of internal standard (1 µg/mL of cortisol-D4 in methanol), and one of the following amount of enzymes: 20 µL of crude extract from *H. pomatia*, 15 µL of IMCSzyme, 20 µL of IMCS arylsulfatase, mixture of the latter two. Incubation was at 55 °C and time was varied from 0 to 4 hours. Incubation temperature and enzyme amounts were based on prior internal studies on other substrates. The enzyme amounts were fixed at 15 µL of IMCSzyme, 20 µL of IMCS arylsulfatase and incubation time was fixed at 10 minutes for five different urine samples. All hydrolyzed urine samples were extracted with DPX RP tips and eluted with methanol. The eluent was dried down and reconstituted in 20% methanol. Analyses were performed using a Thermo TSQ Endura triple quadrupole system with a Thermo Vanquish UPLC equipped with a Phenomenex Kinetex 2.6 µm Phenyl-Hexyl 100 Å, LC Column (50 x 4.6 mm). Mobile phase A was water with 0.1% formic acid and mobile phase B was methanol. Flow rate was 0.6 mL/min starting at 20% B for 0.5 min, 45% B at 1.5 min, 55% B at 7.5 min, 95% B at 8 min for 0.8 min, then back to 20% B at 9 min and re-equilibrated for 2 minutes. MRM transitions are shown in ***Table 1***.

Table 1. *Multiple Reaction Monitoring Transitions*

Compound	Retention Time (min)	RT Window (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)
Cortisol-D4	6.47	0.8	Negative	411.183	335.151	16	87
Cortisol-D4	6.47	0.8	Negative	411.183	286.097	36	87
Cortisol-D4	6.47	0.8	Negative	411.183	301.183	33	87
Cortisol	6.49	0.8	Negative	407.183	331.151	16	87
Cortisol	6.49	0.8	Negative	407.183	282.111	38	87
Cortisol	6.49	0.8	Negative	407.183	297.111	31	87
Cortisone	6.51	0.8	Negative	405.183	329.169	13	77
Cortisone	6.51	0.8	Negative	405.183	359.169	10	77
Cortisone	6.51	0.8	Negative	405.183	301.169	20	77
Allo-Tetrahydro Cortisol	7.32	0.8	Negative	411.183	301.151	37	88
Allo-Tetrahydro Cortisol	7.32	0.8	Negative	411.183	335.222	17	88
Allo-Tetrahydro Cortisol	7.32	0.8	Negative	411.183	365.222	11	88
Tetrahydro Cortisol	7.71	0.8	Negative	411.213	301.151	38	90
Tetrahydro Cortisol	7.71	0.8	Negative	411.213	335.222	18	90
Tetrahydro Cortisol	7.71	0.8	Negative	411.213	365.222	11	90
Tetrahydro Cortisone	8.64	0.8	Negative	409.183	333.151	18	89
Tetrahydro Cortisone	8.64	0.8	Negative	409.183	305.151	24	89
Tetrahydro Cortisone	8.64	0.8	Negative	409.183	363.151	11	89

RESULTS

Since cortisol-D4, tetrahydrocortisol, and allo-tetrahydrocortisol have the same major transition, it is important to separate them on LC gradient. ***Figure 2*** shows extracted chromatogram of each analytes and their corresponding retention time.

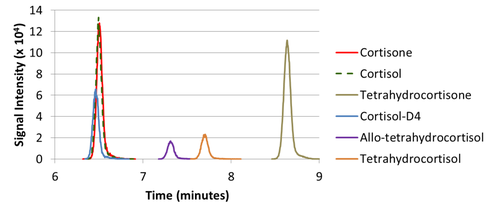

Figure 2. *Extracted ion chromatograms showing the separation of cortisol, cortisone, allo-tetrahydrocortisol, tetrahydrocortisol, and tetrahydrocortisone at 100 ng/mL.*

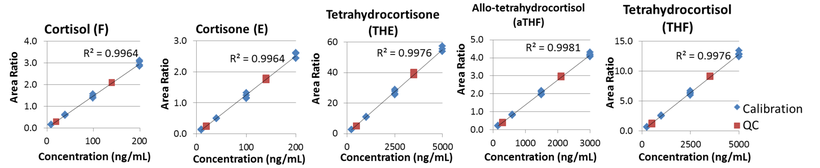

Figure 3. *Calibration and quality control curves of cortisol and the metabolites in steroid-free urine using IMCSzyme and IMCS arylsulfatase. The square of the Pearson correlation coefficient was greater than 0.99 for all five analytes. Quality control samples were within 20% relative standard deviation.*

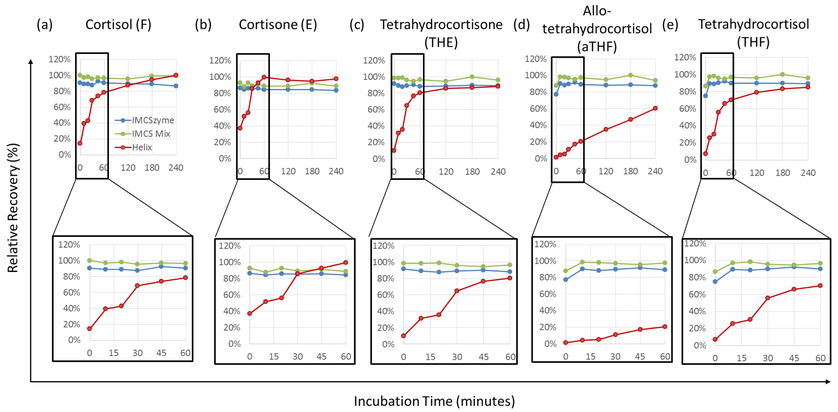

Figure 4. *Hydrolysis of a human urine sample containing the highest amount of **aTHF** for determining hydrolysis efficiency versus incubation time. The hydrolysis percentage is calculated to the maximum amount as 100%; (a) cortisol, (b) cortisone, (c) tetrahydrocortisone, (d) allo-tetrahydrocortisol, and (e) tetrahydrocortisol. A complete hydrolysis indicating by a plateau recovery was reached after 10 minutes of incubation using both enzymes, IMCSzyme and IMCS arylsulfatase (IMCS Mix).*

Hydrolysis time was determined by incubating the urine samples using a fixed amount of different enzymes and their combinations over 4 hour incubation time period at 55 °C. Five urine samples were screened to identify the one containing the highest concentration of **aTHF**, which was reported to be the hardest to enzymatically hydrolyze (2). IMCSzyme-containing samples showed maximal recovery of **F**, **E**, **THE** without incubating at elevated temperature (***Figures 4a, b, c***), while **aTHF** and **THF** required 10 minute incubation at elevated temperature (***Figure 4d, e***). In comparison, the same urine sample hydrolyzed with crude extract from *H. pomatia* indicated **aTHF** levels that were still significantly below the levels measured when urine was processed with IMCSzyme (***Figure 4d***). The difference % between IMCSzyme-hydrolyzed samples with and without IMCS arylsulfatase is around 5-10% indicating that the majority of cortisol metabolites in urine are glucuronidated while 5-10% are sulfated (***Figure 4a-e***).

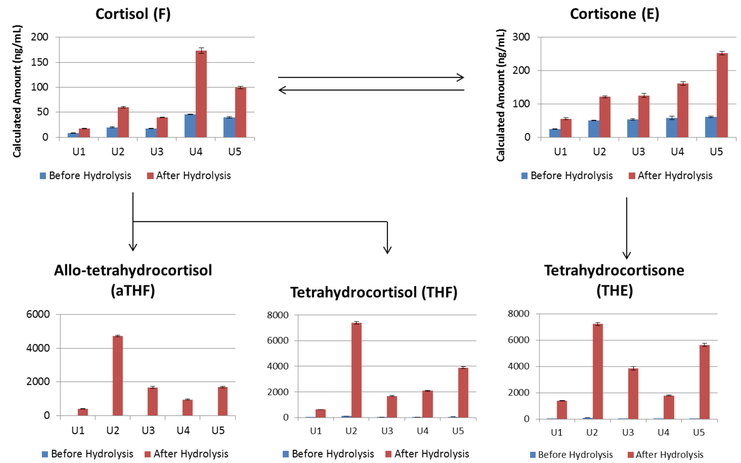

Figure 5. *Average calculated amount of cortisol, cortisone, allo-tetrahydrocortisol, tetrahydrocortisol, and tetrahydrocortisone in five different urine samples with or without enzymatic hydrolysis using IMCSzyme and IMCS arylsulfatase. Error bars indicates ± 1 standard deviation.*

CONCLUSION

The sample hydrolysis step allows the detection of total cortisol metabolites in urine, which include both free and conjugated forms. Several reports pointed to the ratio between **aTHF** and **THF** to **THE** amounts ((aTHF+THF)/THE) as an indication to monitor 11β-HSD activity and to further study its biological relevance in pathologenesis of human diseases (1, 2). As 11β-HSD mentioned earlier, 11β-HSD isozymes involve in the inter-conversion between the biological active **F** and the inactive variant **E**. It is important to completely hydrolyze conjugated metabolites to accurately determine this ratio. ***Figure 4*** shows that the ratio is 1.71 using IMCSzyme or IMCS mix, while it is only 0.98 using crude extract from *H. pomatia*. The ratio calculation could vastly affect the interpretation of 11β-HSD activity, leading to incorrect correlation to disease pathogenicity. In ***Figure 5***, the measurements of **F** and **E** were improved 2-4 fold. **THF** and **THE** were improved by 60-400 fold. Finally, **aTHF** was undetectable before hydrolysis but the level went up as high as 4,700 ng/mL after hydrolysis. The total metabolite detection resulted from sample hydrolysis eliminates the need of costly conjugated standards for tandem mass spectrometry.

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