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Summer studentship report

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Project title: *Development of a comprehensive salivary steroid profile utilising liquid chromatography-tandem mass spectrometry (LC-MS/MS) and gas chromatography-mass spectrometry (GC-MS).*

Introduction:

Steroids are crucial to the proper functioning of human body by regulating growth, blood pressure, glucose metabolism, stress and reproduction. They are primarily produced within the adrenal gland by enzymatic pathways. Altered urinary and serum steroid profiles have shown to be associated with several adrenal related conditions such as, Cushing's syndrome (2) Addisons disease (3), Congenital Adrenal Hyperplasia (2) and Primary aldosteronism (2). Recently, saliva has been utilised by several laboratories as an alternative matrix, saliva sampling is a non-invasive, stress free, convenient and a real time monitoring method for analysis of these steroids. While salivary cortisol/cortisone is already used clinically (e.g., for diagnosing Cushing's syndrome), the broader salivary steroid profile remains poorly understood. In this project we aim to use multiple different coupled mass spectrometry steroid profiling techniques, on both gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS) platforms, to assess the salivary steroid profile within both male and female pooled saliva samples (6 healthy individuals, 3 males and 3 females). Using these semi quantifiable techniques, approximate concentrations of both free and conjugated steroids were investigated within both male and female samples. The result of this project will facilitate the development and validation of a LC-MS method for the quantification of salivary steroids, therefore enabling investigations into steroid profiles within healthy and diseased populations.

Materials and Methods:

Healthy Controls:

Saliva was collected by passive drool from 3 males and 3 females. To maximise steroid recovery and reduce interference salvia was collected in the morning for 20 minutes, 30 minutes after eating or drinking and an hour after brushing teeth. Saliva samples were pooled based on sex in equal portions from each participant.

Sample Preparation:

Liquid chromatography mass spectrometry

To compare conjugated and free steroid concentrations both a hydrolysed and non-hydrolysed sample set was prepared each using 1.5 mL of saliva. For the hydrolysed samples, enzymatic hydrolysis was performed for 3 hours at 55°C using sulfatase from Helix Pomatia to deconjugate steroids to study the total steroids. All samples were then extracted using via liquid-liquid extraction with MTBE and reconstituted in 50% vol/vol methanol solution before being injected into the LC-MS/MS for analysis.

Gas chromatography mass spectrometry

Both a hydrolysed and non-hydrolysed sample set was prepared each using 3 mL of saliva. For the hydrolysed samples, C18 solid phase extraction with methanol and water was performed prior to enzymatic hydrolysis for 3 hours at 55°C using sulfatase from Helix Pomatia. All samples were then extracted using C18 solid phase extraction with methanol and water and derivatised with both methoxamine hydrochloride and trimethylsilyl imidazole. Samples were then cleaned before injection on GC-MS via liquid-liquid extraction with cyclohexane.

Sample analysis:

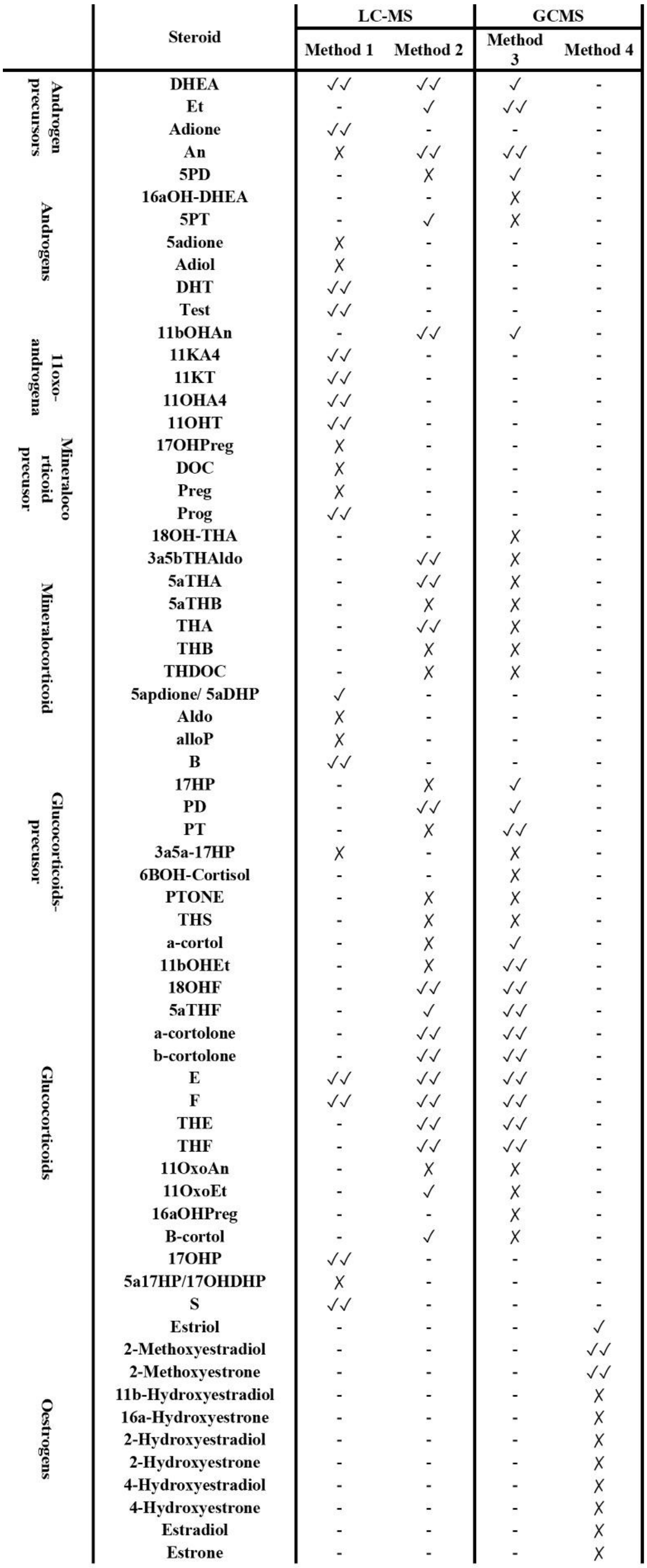
Samples were analysed using previously published methods (2,4). Steroids commonly present in serum were investigated using LC-MS/MS analysis (Method 1). Steroids commonly present in urine were investigated using both LC-MS/MS (Method 2) and GC-MS analysis (Method 3). Oestrogens were investigated using GC-MS analysis (Method 4). Steroids were evaluated for whether they were quantifiable S/N of greater than 10:1 or detectable S/N greater than 3:1 or if they were not present. The LC-MS/MS used in the analysis of these samples was a Waters Acquity UPLC system coupled with a Waters Xevo TQXS triple quadrupole mass spectrometer. The GC-MS used in the analysis of these samples was a Shimadzu GC2030 GC system coupled to a Shimadzu GCMS-QP2020 NX mass spectrometer

Results:

The quantifiability and detectability of each steroid assessed and the method which it was investigated in is shown in table 1. A total of 66 different steroids were investigated, of which 31 were quantifiable with an additional 8 being detectable.

For method 1(steroid commonly present in serum) a total of 25 steroids were assessed, results showed 14 quantifiable, 1 detectable and 10 undetectable steroids. For method 2 (steroid commonly present in urine) a total of 29 steroids were assessed: 13 quantifiable, 5 detectable and 11 undetectable steroids. Steroids analysed within both methods show conformality. All the 11-oxygenated androgens were quantifiable, with 11KA4 only quantifiable in females. In method 1, glucocorticoids, E and F showed greater concentrations than any other steroids analysed, and progesterone was quantified only in females. Hydrolysis significantly increased the quantifiable concentrations for 5apdione, DHEA, Adione, DHT and 11OHA4 (see Fig 1). In method 2, hydrolysed and non-hydrolysed samples showed negligible difference in concentration. E showed a significant increase in concentration in the hydrolysed sample for female. Glucocorticoids were the most quantifiable steroid group with 10 steroids being either quantifiable or detectable while glucocorticoid precursors were the least with only one steroid being quantifiable. Males and females showed almost similar variation in both, hydrolysed and non-hydrolysed sets except for PD (see Fig 2).

Table 1: *Table showing detectability of all steroids analysed across all 4 methods used. Steroids were categorised into 3 categories quantifiable with a signal to noise of greater than 10:1 (✓✓), detectable with a signal to noise of greater than 3:1 (✓) and not detectable with a signal to noise of less than 3:1 (✗). Steroids that were not analysed within the specific method are defined by (-)*



	Steroid	LC-MS		GCMS	
		Method 1	Method 2	Method 3	Method 4
Androgen precursors	DHEA	✓✓	✓✓	✓	-
	Et	-	✓	✓✓	-
	Adione	✓✓	-	-	-
	An	✗	✓✓	✓✓	-
Androgens	5PD	-	✗	✓	-
	16aOH-DHEA	-	-	✗	-
	5PT	-	✓	✗	-
	5adione	✗	-	-	-
	Adiol	✗	-	-	-
	DHT	✓✓	-	-	-
	Test	✓✓	-	-	-
11oxo-androgena	11bOHAn	-	✓✓	✓	-
	11KA4	✓✓	-	-	-
	11KT	✓✓	-	-	-
	11OHA4	✓✓	-	-	-
	11OHT	✓✓	-	-	-
Mineralocorticoid precusor	17OHPreg	✗	-	-	-
	DOC	✗	-	-	-
	Preg	✗	-	-	-
	Prog	✓✓	-	-	-
Mineralocorticoid	18OH-THA	-	-	✗	-
	3a5bTHAldo	-	✓✓	✗	-
	5aTHA	-	✓✓	✗	-
	5aTHB	-	✗	✗	-
	THA	-	✓✓	✗	-
	THB	-	✗	✗	-
	THDOC	-	✗	✗	-
	5apdione/ 5aDHP	✓	-	-	-
	Aldo	✗	-	-	-
	alloP	✗	-	-	-
	B	✓✓	-	-	-
Glucocorticoids-precusor	17HP	-	✗	✓	-
	PD	-	✓✓	✓	-
	PT	-	✗	✓✓	-
	3a5a-17HP	✗	-	✗	-
	6BOH-Cortisol	-	-	✗	-
	PTONE	-	✗	✗	-
	THS	-	✗	✗	-
Glucocorticoids	a-cortol	-	✗	✓	-
	11bOHEt	-	✗	✓✓	-
	18OHF	-	✓✓	✓✓	-
	5aTHF	-	✓	✓✓	-
	a-cortolone	-	✓✓	✓✓	-
	b-cortolone	-	✓✓	✓✓	-
	E	✓✓	✓✓	✓✓	-
	F	✓✓	✓✓	✓✓	-
	THE	-	✓✓	✓✓	-
	THF	-	✓✓	✓✓	-
	11OxoAn	-	✗	✗	-
	11OxoEt	-	✓	✗	-
	16aOHPreg	-	-	✗	-
	B-cortol	-	✓	✗	-
	17OHP	✓✓	-	-	-
	5a17HP/17OHDHP	✗	-	-	-
	S	✓✓	-	-	-
Oestrogens	Estriol	-	-	-	✓
	2-Methoxyestradiol	-	-	-	✓✓
	2-Methoxyestrone	-	-	-	✓✓
	11b-Hydroxyestradiol	-	-	-	✗
	16a-Hydroxyestrone	-	-	-	✗
	2-Hydroxyestradiol	-	-	-	✗
	2-Hydroxyestrone	-	-	-	✗
	4-Hydroxyestradiol	-	-	-	✗
	4-Hydroxyestrone	-	-	-	✗
	Estradiol	-	-	-	✗
	Estrone	-	-	-	✗

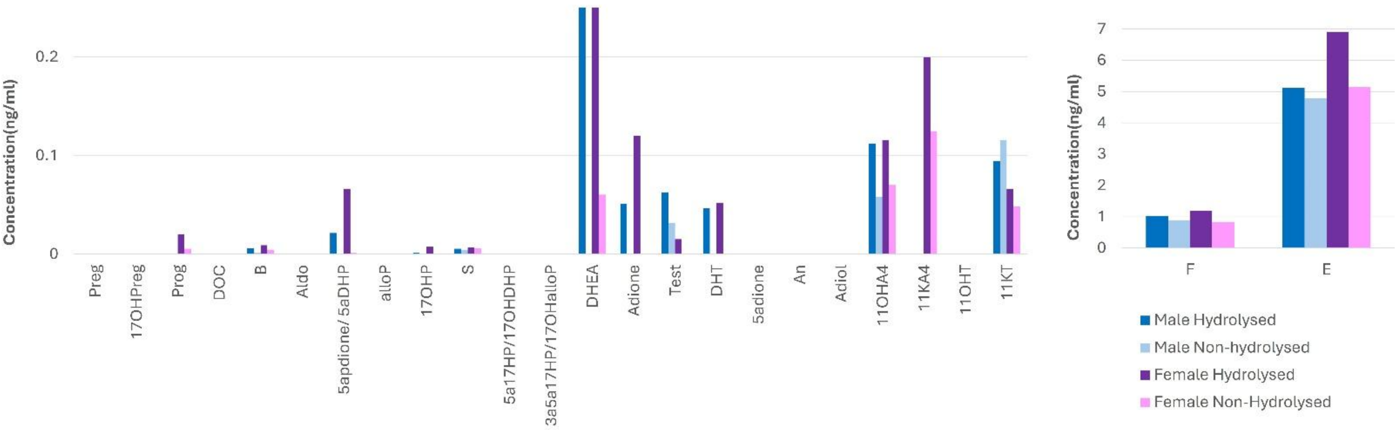


Figure 1: *Bar graph depicting all quantifiable and detectable steroids assessed within LC-MS method 1 separated into hydrolysed (free + conjugated steroid concentration) and non-hydrolysed (free steroid concentration) for males and females.*

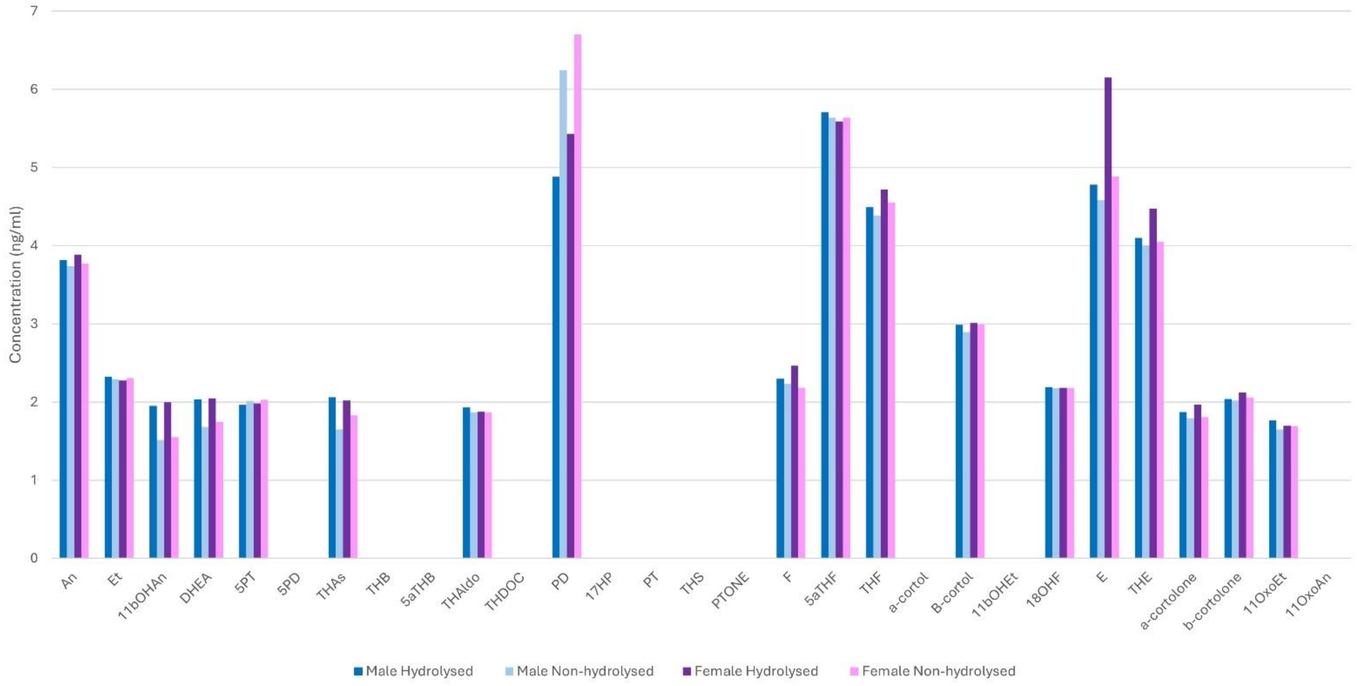


Figure 2: *Bar graph depicting all quantifiable and detectable steroids assessed within LC-MS method 2 separated into hydrolysed (free + conjugated steroid concentration) and non-hydrolysed (free steroid concentration) for males and females.*

Method 3 and 4 were both preformed on the GC-MS. For method 3 (steroid commonly present in urine) a total of 32 steroids were assessed, results showed 13 quantifiable, 6 detectable and 17 undetectable steroids. For method 4 (oestrogens) a total of 11 steroids were assessed results showed 2 quantifiable, 1 detectable and 8 undetectable steroids. Despite GC-MS having lower sensitivity compared to LC-MS/MS, the GC-MS results were similar to those of LC-MS/MS, with most glucocorticoids analysed falling within the quantifiable range and most mineralocorticoids remaining undetectable.

Discussion:

In this project we have assessed the salivary steroid profile within healthy controls through the screening of 66 steroids of multiple classes including mineralocorticoids, glucocorticoids, androgen precursors, androgens, oestrogens and their metabolites. We have achieved this using 4 separate mass spectrometry couple assays 2 using LC-MS and 2 using GC-MS, these methods showed conformity between each method aiding in our confidence with this profile. Using these methods we have demonstrated that 31 steroids were highly excreted (quantifiable), 8 were excreted in lower concentrations (detectable) and 27 were undetectable. We have gained an understanding of the classes of steroids excreted with most of these quantifiable steroids being within the glucocorticoid and androgen class. Precursor steroids, mineralocorticoids and oestrogens are minorly excreted evidencing a higher level of metabolism within saliva. Future methods for salivary steroid profiling should focus on these steroids and investigate a greater variety of downstream glucocorticoid metabolites so a full profile can be investigated. Future work should also focus on improving the sensitivity of the assay although improving sensitivity by increasing sample load can prove difficult due to variability of collection volumes and can introduce matrix effects.

The presence of conjugated steroids within saliva was also investigated. In this research we found through the comparison of non-hydrolysed and hydrolysed samples an increase in concentration of steroids post hydrolysis providing evidence of the presence of these conjugated steroids. For most of the steroids analysed, concentrations of conjugated steroids were minimal and within the standard deviation of our data set although for steroids: 5apdione, DHEA, Adione, DHT and 11OHA4 we saw a larger increase, therefore it is not yet known whether hydrolysis is required or whether it provides a greater level of information within saliva.

Conclusion:

Here we have successfully investigated whether 66 steroids are present within saliva, whilst not an exhaustive list of steroids this salivary steroidome profile will aid in the development of a single LC-MS quantitative mass-spectrometry method for the analysis of steroids within saliva and will fuel further investigations into more extensive profiles. It is hoped that this single LC-MS quantitative mass-spectrometry method for the analysis of steroids within saliva will fuel further investigations into salivary steroid concentrations within healthy and disease populations investigating adrenal related conditions as well as stress etc.

In this project I have gained hands-on experience in a range of analytical and laboratory techniques. I developed proficiency in performing varied techniques of steroid extraction which include carrying out liquid-liquid extraction, solid phase extraction on saliva samples and using LCMS and GCMS to analyse the quantifiable, detectable and undetectable salivary steroids by interpreting the chromatograms generated. I honed my skills in using Excel and R software to generate tables, graphs for large datasets and perform statistical analysis. Furthermore, I enhanced my scientific communication abilities through writing report, crafting a presentation and conveying the results of my work to both, scientific and general audience. It was a wholesome experience strengthening my practical knowledge, as I look forward to pursuing a career in research.

Acknowledgements

I would like to thank BMSS and ChromSoc for allowing me the opportunity to do this summer studentship, the experience I have gained has been invaluable.

I would also like to thank those who have supported and supervised me through these 6 weeks, Dr Angela Taylor, Dr Joshua Bain and Dr Fozia Shaheen.

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