Derivatization as a Tool for Reaching Low pg/mL Detection Limits in Salivary Steroidomics

Cato Brede, Stavanger University Hospital, Norway
Steroidomics

The term «steroidomics» was first used by Jan Sjövall [1] and defined as

«detailed characterization and quantification of metabolic profiles of steroids»

Multiplexed quantitation of steroids

- Analytical possibilities
  - GC-MS
  - LC-MS (MRM / SRM, HRMS)
- Snapshot of the steroid metabolism
- Chemometric data analysis
- Cost-efficient
Saliva (advantages)

- Easy accessible
  - Non-invasive
  - Sampling flexibility

- Clinically relevant
  - Biologically active (no SHBG and transcortin)
  - Correlation with blood
  - Diagnostic value (cortisol)
Saliva (challenges)

- Low concentration levels
  - Cortisol and cortisone (ng/mL)
  - Testosterone, DHEA, progesterone, 17-OHP (pg/mL)
  - Estrone, DHT, pregnenolone (low to sub pg/mL)

- Sample preparation
  - LLE
  - SPE
Derivatization

Carbonyl + Hydrazine or hydrazide → Hydrazone + H₂O
Reagents

Girard's reagent T (GRT)

1-benzylpyrrolidine-3-carboxyhydrazide (1-BPH)

2-hydrazinopyridine (2-HP)

4-aminobenzohydrazide (4-ABH)

Isoniazid (INH)
Method development

- Five ketosteroids + five reagents
- LC-MS/MS (MRM)
- Choosing a derivatization reagent
- Optimizing LLE and the derivatization reaction
- Avoiding emulsions
Separation of hydrazones
0.2% formic acid + methanol

1) Cortisol
2) Cortisone
3) Testosterone
4) DHEA
5) Progesterone
Separation of hydrazones
0.1% ammonium hydroxide

1) Cortisol
2) Cortisone
3) Testosterone
4) DHEA
5) Progesterone
Peak height improvements

- Cortisol w/ 4-ABH and ammonia (x 24)
- Cortisone w/ 2-HP and formic acid (x 15)
- Testosterone w/ 4-ABH ammonia (x 7)
- DHEA w/ 4-ABH and ammonia (x 265)
- Progesterone w/ 1-BPH and ammonia (x 2)
Emerging mono- and di-hydrazones with 1 mg/mL of 2-HP

![Graph showing relative response vs. reaction time at 60 °C (min) for various compounds including Progesterone di-hydrazone, Testosterone, Cortisol, Cortisone, DHEA, Melatonin, Aldosterone, Corticosterone di-hydrazone, 21-Hydroxyprogesterone di-hydrazone, 11-Deoxycorticisol, and Estrone.]

- **Progesterone**
- **Dehydroepiandrosterone**
- **Estrone**
Finding the optimum reaction time with 5 mg/mL of 2-HP

Reaction time at 60 ºC (min)

Relative response (%)
Liquid-liquid extraction

![Graph showing extraction efficiency of different compounds with varying butanol concentrations in MTBE](image)

*Relative extraction efficiency (%) vs. Butanol (v/v %) in MTBE*
Dealing with emulsions
Plasma protein precipitation

- Trichloroacetic acid
- Perchloric acid
- Zinc sulfate
- Solvents (acetonitrile, acetone, etc)

Salivary protein precipitation

- 1+1 w/ 20% TCA -> 47%
- 1+3 w/ acetone -> 52%
- 1+1 w/ TCA + acetone + DTT -> 82%

Wikipedia: Astringency

«Astringency is also the dry, puckering mouthfeel caused by tannins found in many fruits such as blackthorn (sloe berries), Aronia chokeberry, chokecherry, bird cherry, quince and persimmon fruits, and banana skins.

The tannins (which are types of polyphenols) bind the salivary proteins, causing them to precipitate or aggregate[2] and lead to a rough "sandpapery" or dry sensation in the mouth. Tannins are found in some red wines and teas.

A small amount of astringency is expected in some wines, especially young red wines made from grapes such as cabernet sauvignon and merlot.»
Tannic acid
Precipitation from 500 μL saliva

- 1000μL acetonitrile
- 200μL 20% TCA
- 200μL 20% PCA
- 100μL tannic acid (100mg/mL)
Breaking the emulsion
Automated sample preparation

- LLE in a 96 well DeepWell plate:
  - 500 μL saliva
  - 50 μL internal standard
  - 100 μL tannic acid (10 mg/mL)
  - 500 μL MTBE w/ 10% butanol

- Evaporation of top layer to dryness in a vacuum centrifuge

- Adding 50 μL 2-HP in methanol (5 mg/mL) and derivatization at 60°C for 30 min
LC-MS/MS (MRM)

- Injection volume: 10 μL
- Column: Acquity BEH C18 2.1 x 50mm (Waters)
- A: 0.2% formic acid  B: Methanol
- Flowrate: 500 μL/min
- Long gradient: 10-95 % B over 10 min
- Short gradient: 10 / 30 / 55 / 99 % B  at 0 / 0.05 / 3 / 3.5 min
- Cycle time: 5.5 min
Insufficient detection limits for saliva

- Aldosterone and 11-deoxycortisol, very high LODs
- Corticosterone, LOD: 81 pg/mL
- 21-hydroxyprogesterone, LOD: 27 pg/mL
- Estrone, LOD: 1 pg/mL
Validated salivary steroids

- Cortisol, LOD: 11 pg/mL
- Cortisone, LOD: 70 pg/mL
- Testosterone, LOD: 1.7 pg/mL
- Progesterone, LOD: 2.5 pg/mL
- DHEA, LOD: 5 pg/mL
- 17-OHP, LOD: 7 pg/mL
Method comparison (cortisol)

\[ y = 0.9025x - 0.0605 \]

\[ R^2 = 0.9911 \]
Reference ranges for cortisol

- Women (7-9): 2.4 – 23 nM (n=90)
- Men (7-9): 1.2 – 23 nM (n=60)
- Women (22-24): 0.21 – 0.26 nM (n=91)
- Men (22-24): 0.19 – 0.25 nM (n=63)
Method comparison (testosterone)

\[ y = 0.862x - 3.6206 \]

\[ R^2 = 0.9845 \]
Reference ranges for testosterone

- Women (7-9): < 6 – 69 pM (n=89)
- Men (7-9): 92 – 344 pM (n=59)
- Women (22-24): < 6 – 17 pM (n=88)
- Men (22-24): 24 – 199 pM (n=60)
New paper in CMS, freely available before September 27
https://authors.elsevier.com/a/1VWTs8STvblSb8

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Multiplexed analysis of steroid hormones in saliva by LC-MS/MS with 2-hydrazinopyridine derivatization

Nirosa Nadarajah a, Øyvind Skadberg b, Joanne Adaway c, Cato Brede b, * 

a Faculty of Health Sciences, Oslo and Akershus University College of Applied Sciences, PO Box 4, NO-0130 Oslo, Norway
b Department of Medical Biochemistry, Stavanger University Hospital, PO Box 8100, NO-4068 Stavanger, Norway
c Department of Clinical Biochemistry, University Hospital of South Manchester NHS Foundation Trust, Southmoor Road, Wythenshawe, Manchester M23 9LT, UK
Adding more analytes

- Pregnenolone
- Androstenedione
- Dihydrotestosterone
Pregnenolone (6 pg/mL)

Internal standard: D8 17-OHP
Androstenedione (21 pg/mL)

Internal standard: D3 Testosterone
Dihydrotestosterone (6 pg/mL)

Internal standard: D3 Testosterone
Conclusion

- 2-HP was favoured because of strong signal and single peaks for the hydrazones

- Tannic acid is a novel protein precipitant and LLE emulsion preventer

- Straightforward sample preparation with LLE and derivatization
People involved

Nirosa Nadarajah, Oslo and Akershus University College of Applied Sciences

Øyvind Skadberg, Stavanger University Hospital

Colleagues at the Department of Medical Biochemistry, Stavanger

Joanne Adaway, University Hospital of South Manchester

Linda Bærheim Ottøy, University of Stavanger

Kåre Jørgensen, University of Stavanger