Delivering accurate steroid results with LC-MS/MS:
How owning an expensive bicycle does not make you a good cyclist

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Learning Objectives

- Understand the technical challenges of associated with endogenous steroid analysis in human serum.
- Why is it challenging?
- Approaches to the sample preparation – advantages and disadvantages
- Consider automation
- Choosing ionization mode
- Not all instruments can achieve sensitivity
- Identifying interferences
- Managing interferences: “Terror on every side!”
- Anecdotes in unexpectedness
Steroid Structure

- It is steroid structure that makes the technical problem of steroid analysis challenging.
- All steroids share the same characteristic arrangement of four cycloalkane rings.
- All are derivatives of cholesterol shown on the right.

Steroid Metabolic Diversity

- Lipids (steroid precursor, structural)
- Bile Acids (cholesterol excretion/dietary fat emulsifiers)
- Counter-regulatory (glucocorticoids: cortisol)
- Electrolyte (mineralocorticoids: aldosterone, DOC)
- Ca Homeostasis (25(OH)D, 1,25(OH)2D, 24,25(OH)2D)
- Sexual Development (T, DHEA, AD, E2, Prog)
- And all the pharmaceutical analogues!

Dynamic Range
A caution on vendors

- Certain vendors will tell you just about anything you want to hear
- But they may have developed their method in water solutions
- They may have developed their method in analyte free matrix
- They may have explored their LoD's and LoQ's in a similar fantasy land.
- Do not be deceived - speak to a lab that is using that instrument for the analyte of interest in production
- Email one of us
- As a vendor sows, so shall they reap…

The problem of isobars

- DOC, mw=330.46 g/mol
- 17-OHP, mw=330.46 g/mol

The problem of isobars

- Epi-testosterone, mw=288.42 g/mol
- Testosterone, mw=288.42 g/mol
Sample Preps

- **Dilute and Shoot**
  - Purpose is to precipitate and dilute the protein/lipid content of the specimen while solubilizing the steroid.
  - Will effectively release steroid from binding globulins – which almost all steroids have (why?).
  - Internal Standard is often added to the crash reagent.
- Advantages – easy, fast, all-in-one, highly scalable, 96-well compatible
- Disadvantages – manner of IS addition matters, dirty source/quads, subject to clogging (inline filter needed)

Sample Preps

- **Liquid liquid extraction**
  - Organic solvent is mixed with sample and vortexing causes the steroid to enter the organic layer.
  - Organic layer is sampled, dried down, reconstituted and injected.
  - Hexane, Ethyl Acetate, Methyl tert-butyl Ether, Diethyl Ether, Dichloromethane, Chloroform
- Advantages – clean sample, clean source and quads, 96 well can work.
- Disadvantages – odor, volatiles, phospholipids get extracted, extra pipetting steps, relies on uniform vortexing

Sample Prep

- **Solid Phase Extraction (SPE)**
  - Works like an HPLC column with a shorter lifespan.
  - Sample is loaded into cartridge or 96 well format, washed and then eluted with organic.
- Advantages – very clean sample, sample has been significantly concentrated, plates can be re-used if this is validated, amenable to online formats
- Disadvantages – Extra steps, $$
Derivatization

- Certain steroids are often measured with derivatization because they do not ionize well.
- Examples include
  - 1,25(OH)2 VitD
  - Estradiol
  - Aldosterone (though not really necessary anymore)

Derivatization

- Derivatization reagents are something that we would generally prefer to avoid.
- Example
  - dansylation of E2
  - PTAD and 1,25VitD

E2 Dansylation
1,25(OH)2D PTAD

Before you begin

- Needs questions:
  - What steroid do I want to analyze?
  - Is there any other steroid that I can concomitantly measure that will be useful?
  - How often am I going to have to run this?
  - What is my current volume for this test?
  - Has anyone published a method?
    - Collect all the papers and read them!

Before you begin

- Throughput Questions:
  - If this test becomes popular, am I going to be able to handle the throughput?
  - Will the sample prep need automation or can I get away with pipetting by hand?
  - Should I be working in Eppendorf or 96 well format?
  - Do I have the onsite expertise to make this work?
    - Or will I get adequate support from the company?
Before you begin

- Analytical questions tied to clinical need:
  - What precision do I need to accomplish to accommodate the clinical needs?
  - What are the clinical decision limits?
    - e.g. Testosterone: 8 nmol/L and 2 nmol/L
    - Cortisol: 50 nmol/L, 500 nmol/L
    - VitD 20 nmol/L
  - What analytical range and LoQ do I need to accomplish?

- Analytical questions tied to technical need:
  - Can I buy standard material? In what form?
  - How will I prepare the standards?
  - Do I have a good IS – is it free of analyte?
  - Do I have analyte-free matrix? (Golden West Double Stripped)

- Method validation questions:
  - Do I have a reliable comparator lab?
  - How am I going to get the comparator specimens?
  - Can I get gel-free comparator specimens?
  - What is a comparison going to cost me?

APCI vs ESI

- Practically speaking ESI is usually attempted first.
- ESI supports a wider range of molecular weights than APCI and is better for thermally labile compounds.
  - Recall that APCI requires introduction of carrier gas and rapid heating in order to vaporize the HPLC outflow in preparation for the chemical ionization process.
  - ESI can produce multiply charged ions and therefore has a functional m/z range which is much higher.
  - ESI does not tend to have good signals for uncharged, non-basic, low polarity compounds.
  - ESI is very sensitive to the presence of contaminants – alkali metals, phospholipids.
APCI vs ESI

- APCI generally ionizes low polarity compounds better than ESI and may be a good choice (e.g., certain steroids)
- But APCI requires heated gas flow to vaporize solvents and so generally operates at higher temperatures (350-550 °C)
- Will not work as well on thermally labile compounds
- APCI tolerates higher flow rates
- APCI is less affected by ion suppression effects.

So you’ve developed a preliminary method…

Targeted Interference Investigation

- On the right we have Testosterone, DHEA and Epi-testosterone
- All have molecular weight 288.42 g/mol
- In positive ion mode, they will all form an precursor ion of 289 g/mol
- Their fragments will be very similar
Targeted Interference Investigation

What about the interference we cannot resolve chromatographically?

- Cortisol and prednisolone (the metabolite of prednisone) are very difficult to chromatographically resolve
- Which means that prednisone and prednisolone administration will cause a problem in a serum cortisol assay
- It will small in magnitude (5% of the prednisolone concentration in our assay) but it will be significant

Isobaric Compounds: Planning

- You are going to need to obtain solutions of all the potentially interfering compounds.
- The list is going to have to be fairly comprehensive and you should go to great lengths to make sure that you have captured the isobaric compounds.
- You should either see no effect from the potential interfering compound or you should see it chromatographically resolved.
- This may represent a financial barrier
- The companies may help you
Isobaric Compounds: Planning

- The good news:
  - Xenobiotic compounds have fewer potential interferences
  - Unlikely for both MRMs to be equally affected – ion ratio will catch a problem specimen.
  - Not all xenobiotics are interference free – of course.
- Betamethasone vs Dexamethasone

Example - Aldosterone

<table>
<thead>
<tr>
<th>Compound</th>
<th>Release Weight (g)</th>
<th>Anti (kg)</th>
<th>Anti (mg)</th>
<th>Tube Concentration (IU)</th>
<th>Result (Reference Value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldosterone</td>
<td>0.15</td>
<td>0.01</td>
<td>0.15</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.15</td>
<td>0.01</td>
<td>0.15</td>
<td>0.01</td>
<td>0.00</td>
</tr>
</tbody>
</table>

There can be no a priori confidence that collection tubes will not contain unexpected interferences.

- This is particularly true of gel-containing tubes.
- This is a well-known phenomenon with testosterone but not all is understood by all about this.
- Relate story about aldosterone and tube types.
Ion Ratios are Mandatory

- If there is an interference due to a gel-containing tube, it is very likely that it will affect one MRM over another.
- For this reason, we always look at quantifier and qualifier ions and only release samples that fall within acceptable expected limits for the ion-ratios.
- These percentage limits are too sensitive at low concentrations and so for aldosterone, my practice is to plot MRM1 vs MRM2 and decide whether it matters.

Sometimes it’s obvious

- There is a non-coeluting interference well known to affect the quantitation of testosterone using ESI affecting the 289 → 97 transition and the 289 → 109 to a lesser extent.
- This is very easy to see and so chromatographically separate but it is so large that it may contribute to the integration of the analyte peak.
- Some think it adequate to have chromatographic separation … but is this enough?
Testosterone F, Red Top Serum

Testosterone F, Gold Top Serum

When it is not obvious

- In pediatric and female specimens, there is a co-eluting interference that is time-dependent and cannot be dealt with chromatographically.
- This can only be seen by letting the samples sit on the gel and analyzing T after a period of exposure.
- 10 samples collected from females in red top, SST and PST tubes.
- Exposed to gel for 0, 4 and 24 hours respectively.
Sitting on the gel…MRM1

Sitting on the gel…MRM2

Ion Suppression Schematic
Ion Suppression

Method Comparison

- What to expect when you are doing a comparison with IA
  - Biases extremely common
  - Correlation coefficients can be quite poor (~0.8) and sometimes shockingly poor.
  - Bias may go a different direction between the low end and the high end.

The over-under phenomenon
The over-under phenomenon

Don’t despair

Renal Failure

- Chronic kidney disease (CKD) introduces very significant positive bias in certain analyses – especially steroid analyses.
- This leads to (massive) overestimation of steroids by IA methods in CKD.
- For this reason, it is very much worthwhile to know the creatinines of your subjects so that you can produce comparisons with and without CKD.
Aldosterone: no CKD and CKD

Same Subjects: LC-MS/MS vs. LC-MS/MS

Conclusions

- In order to accurately quantify steroids you need to be very aware of the much higher likelihood of interferences
- Must do systematic spiking experiments
- Predictable Isobaric
- Mystery in-source reactions
- Mystery Isobaric
- Mystery tube/matrix interferences
- Ion ratios are absolutely mandatory
- Peak review is mandatory
Conclusions

- Expect poor comparison with IA
- Expect good comparison with LC-MSMS
- Examine the CKD phenomenon