

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!

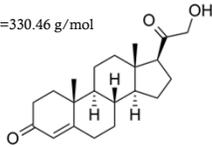
Dynamic Range

A caution on vendors

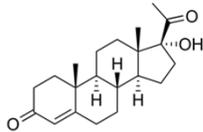
- ◆ Certain vendors will tell you just about anything you want to hear
 - ◆ But they may have developed their method in water solutions
 - ◆ They may have developed their method in analyte free matrix
 - ◆ They may have explored their LoD's and LoQ's in a similar fantasy land.
 - ◆ Do not be deceived – 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

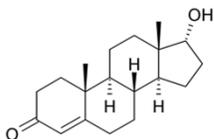


17-OHP, mw=330.46 g/mol

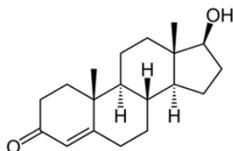


The problem of isobars

Epi-testosterone, mw=288.42 g/mol



Testosterone, mw=288.42 g/mol

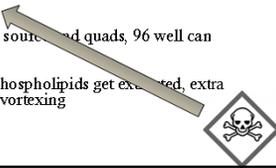


Sample Preps

- ◆ 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, \$\$

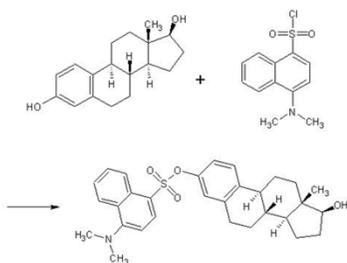
Derivatization

- ◆ 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)

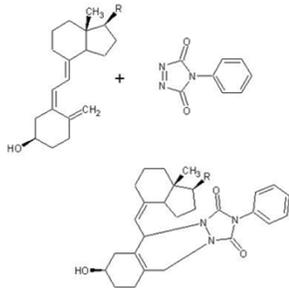
Derivatization

- ◆ 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?
 - ◆ eg 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?

Before you begin

- ◆ 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 vapourize 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.

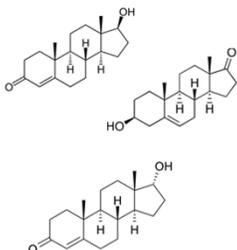
APCI vs ESI

- ◆ 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.

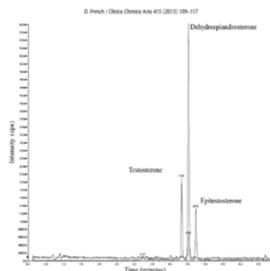
So you've developed a preliminary method...

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?

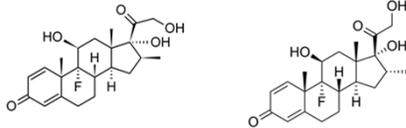
- ◆ Cortisol and prednisolone (the metabolite of prednisone) are very difficult to chromatographically resolve
- ◆ Which means that prednisone and prednisolone administration will cause a problem in a serum cortisol assay
- ◆ It will small in magnitude (5% of the prednisolone concentration in our assay)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

Compound	Molecular Weight (g/mol)	Adult ULN (ng/dL)	Adult ULN (nmol/L)	Testing Concentration (pmol/L)	Result (Aldosterone Quantitative Transition)
11-deoxycorticosterone	336.46	10	0.38	2000.00	No interference observed
11-deoxycortisol	346.46	79	2.3	7.3	No interference observed
18-hydroxy-11-deoxycorticosterone	346.46	15	0.43	288	No interference observed
18-hydroxycorticosterone	382.46	58	1.6	59.2	Interference marginally chromatographically separated
20-deoxycortisol	346.46	5	0.14	1000	No interference observed
corticosterone	346.46	1870	57	182	No interference observed
cortisol	362.46	25000	690	2200	Interference chromatographically separated
dexamethasone	382.46	NA	NA	1000	No interference observed
fludrocortisone	422.48	NA	NA	1000	No interference observed
prednisolone	382.46	NA	NA	1000	No interference observed
prednisone	382.46	NA	NA	1000	No interference observed
Compound in Cocktail	Molecular Weight (g/mol)	Adult ULN (ng/dL)	Adult ULN (nmol/L)	Testing Concentration (pmol/L)	
11-deoxycortisol	346.46	79	2.3	4.3	Any interferences from this mix of steroids is chromatographically separated from aldosterone.
17-hydroxyprogesterone	336.46	265 (Females)	8.6	18.9	
20-hydroxyprogesterone	428.56	3027	75	77.8	
androstenedione	306.39	239 (Females)	7.7	5.4	
cortisol	362.46	25000	690	89	
DHEA	288.42	13	0.39	25.8	
DHEAS	398.49	45700 (Males)	12400	4000	
estradiol	272.38	35.1 (Females)	1.28	22.8	
progesterone	314.46	2708	86	19.9	
testosterone	288.42	60 (Males)	38	25.8	

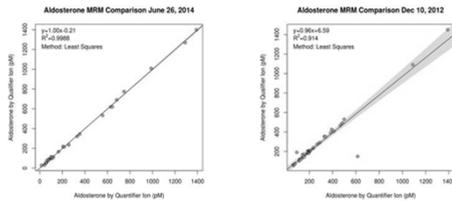
Tube Interferences

- ◆ 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

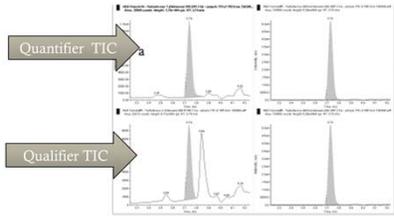
Ion Ratios are Mandatory



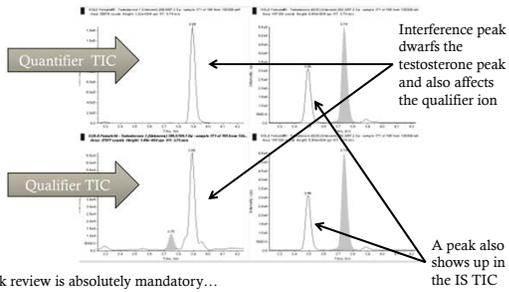
Sometimes it's obvious

- ◆ 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 to 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



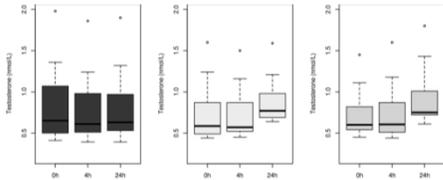
Testosterone F, Gold Top Serum



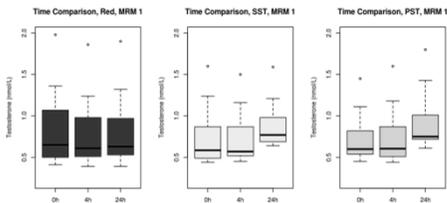
When it is not obvious

- ◆ 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

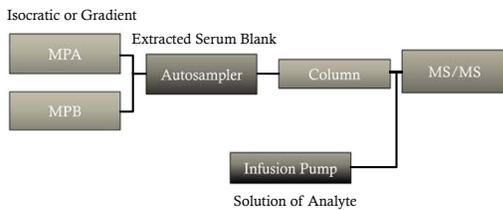
Sitting on the gel...MRM1



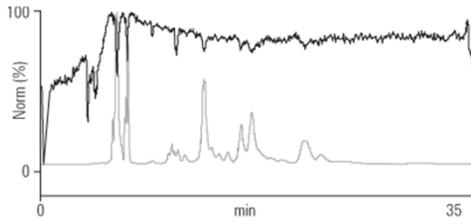
Sitting on the gel...MRM2



Ion Suppression Schematic



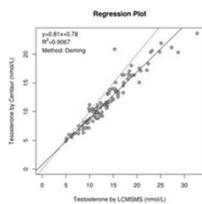
Ion Suppression



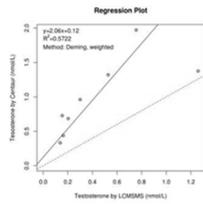
Method Comparison

- 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.

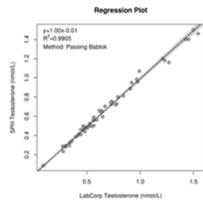
The over-under phenomenon



The over-under phenomenon



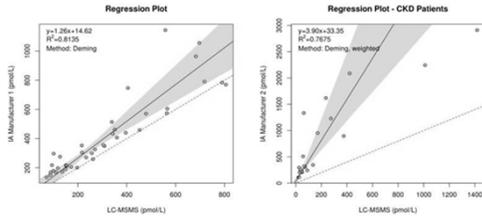
Don't despair



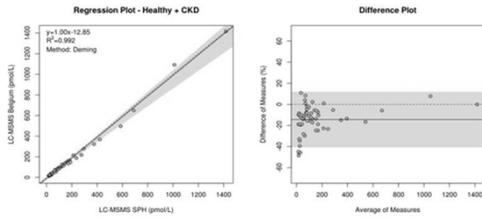
Renal Failure

- ◆ 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
