New Sensitivity and Selectivity Gains with the QTRAP® 6500 System and SelexION™ Technology for Steroid Analysis

For Research Use Only. Not for use in diagnostic procedures.

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Introduction

- The two major challenges facing steroid researchers (in fact, all researchers!) using LC/MS/MS are:
  - Sensitivity
  - Selectivity

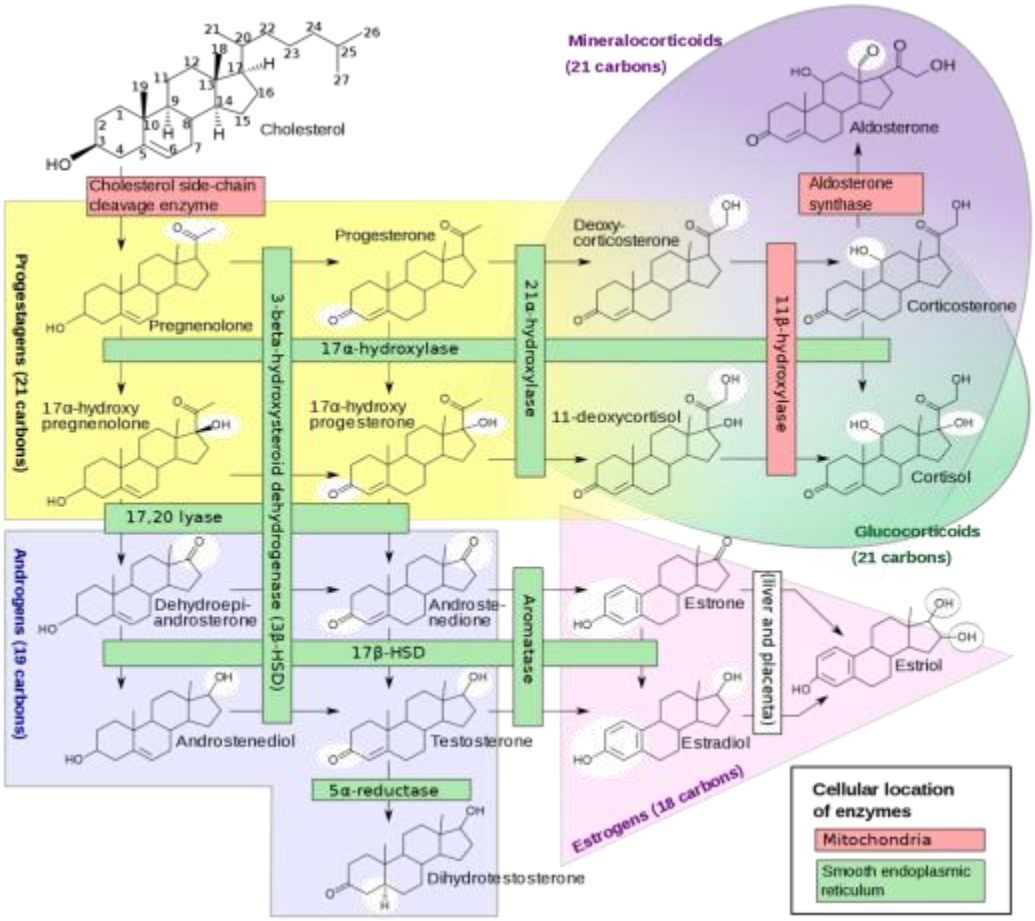
Challenges for LC-MS/MS Analysis of Steroids

Sensitivity:

- Low ionization efficiencies

Selectivity:

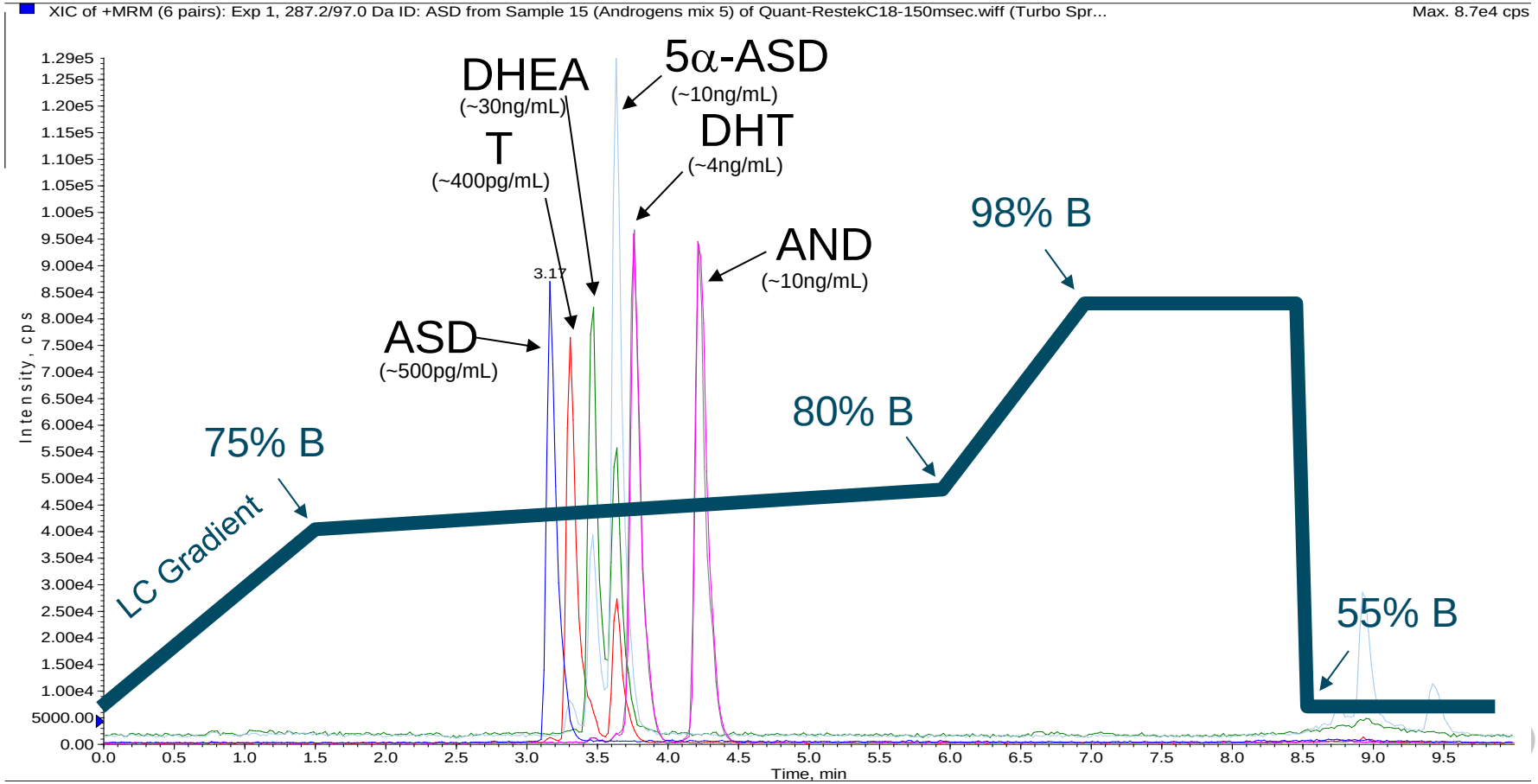
- Common precursor ion masses
- Similar fragmentation patterns
- Similar chromatographic retention properties
- Lots of endogenous interferences



http://en.wikipedia.org/wiki/Steroidogenesis#Steroidogenesis

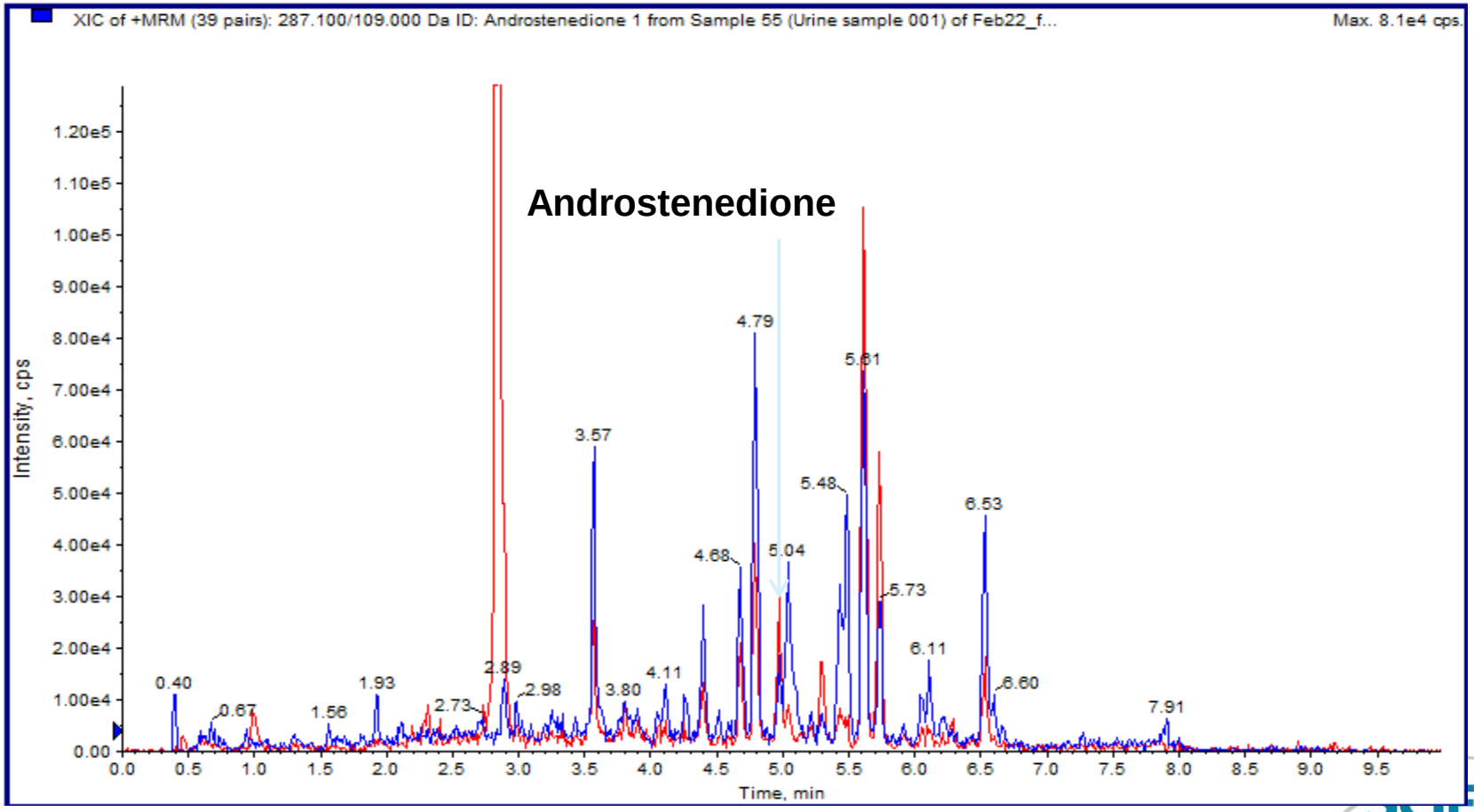
Analysis of 6 androgens in 60:40 methanol:water

- Similar retention times
- Common fragment ions complicate the analysis



Analysis of androstenedione in urine sample

- Enzymatic hydrolysis performed, to remove glucuronides
- Numerous interferences observed



Agenda

- Pushing the limits of sensitivity with the **QTRAP® 6500** LC/MS/MS system
- Enhanced selectivity using **SelexION™ ion mobility** technology and LC/MS/MS

SCIEX Triple Quad™ and QTRAP® 6500 LC/MS/MS System

SCIEX Triple Quad™ and QTRAP® 6500 System

- IonDrive™ technology provides a new level of performance
  - Up to 10x greater sensitivity
  - Up to 6 orders dynamic range
  - Improved robustness
- Advanced dual RF electronics allows 2 modes of operation:
  - High Sensitivity (5-1250 Da)
  - Extended mass range (5-2000 Da)
- Compatible with SelexION™ ion mobility technology
- 2x faster scanning MRM^3
- Same compact footprint as 4500 and 5500 system (32” x 32” x 24”)

IonDrive™ Technology
Create more ions. Capture more ions. Detect more ions.

IonDrive™
Turbo V
Source

IonDrive™
QJet Ion
Guide

IonDrive™
High Energy
Detector

Actual MRM Sensitivity Gains +ESI mode

Verapamil

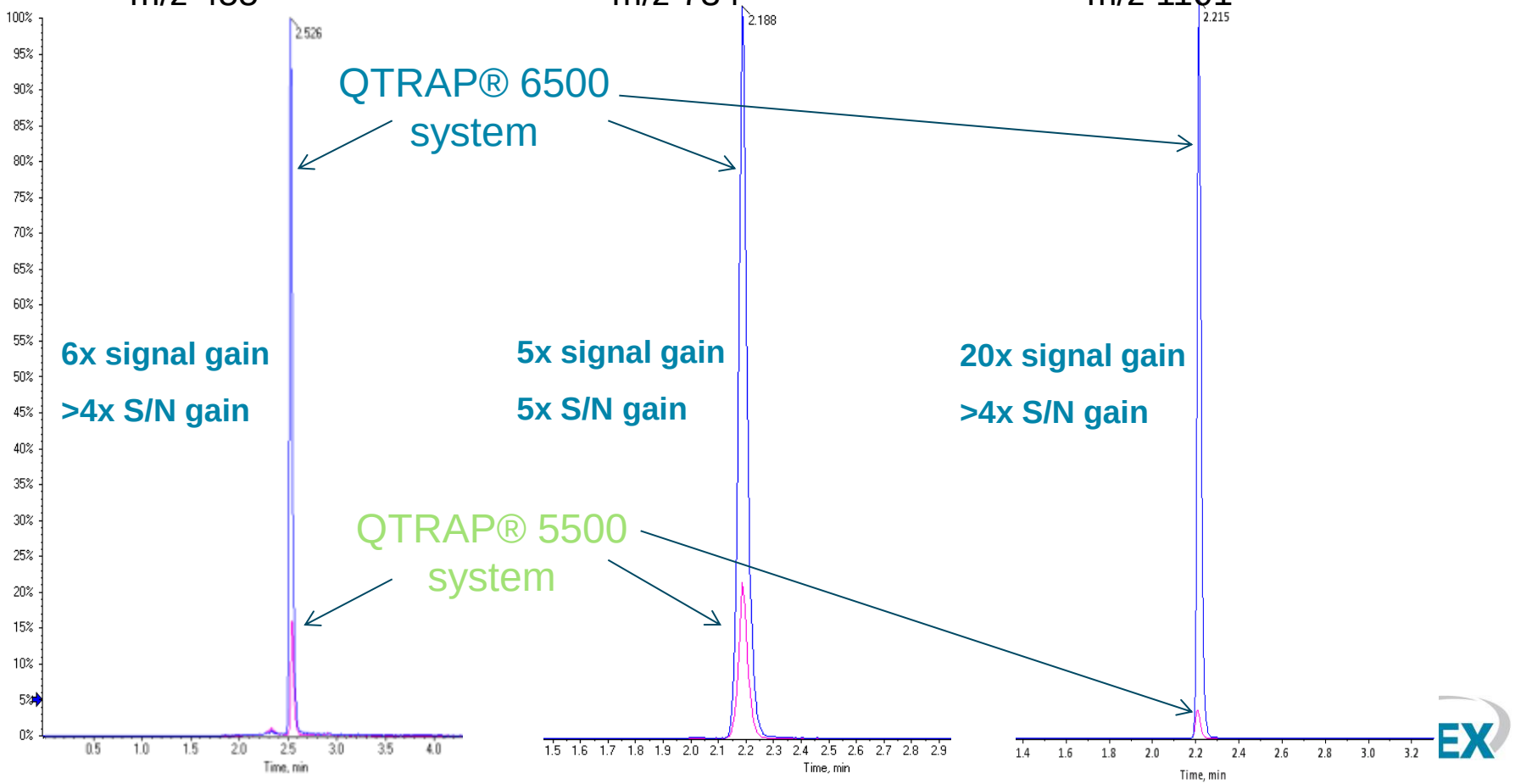
m/z 455

Erythromycin

m/z 734

Ginsenoside Rb2

m/z 1101

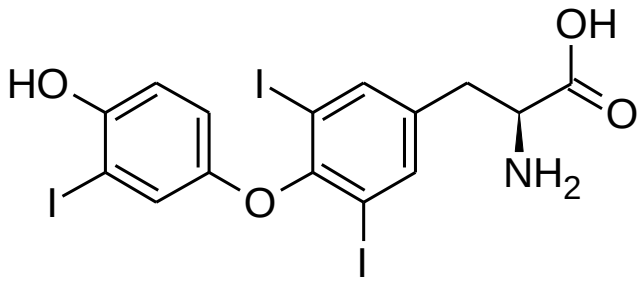


Thyroid Research:

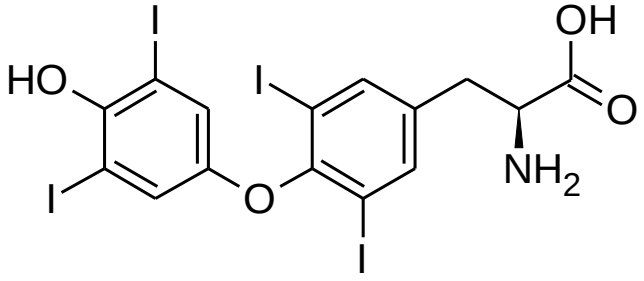
Analysis of Free T3 and Free T4

Thyroid hormones, T3 and T4

- Thyroid hormones circulate in the blood as mostly protein bound hormone (about 99.9% bound)
- The major binding protein is Thyroxine Binding Globulin (TBG) and transthyretin. Only the unbound form of the hormones are biologically active.
- Free T3 (2-4 pg/mL) and free T4 (8-15 pg/mL) are present in very low concentrations in serum.



3,3,5-triiodothyronine (T3)



Thyroxine (T4)

Sample Preparation

- Free (unbound) fraction of T3 and T4 was isolated from the protein-bound fraction by ultrafiltration, using a filter having 10kDa molecular-weight cut-off.
- 500uL of serum was subjected to ultrafiltration at 30C, for 60 minutes.
- 25uL of internal standard was added to 100uL of the ultrafiltrate
- 50uL was directly injected onto LC/MS/MS system

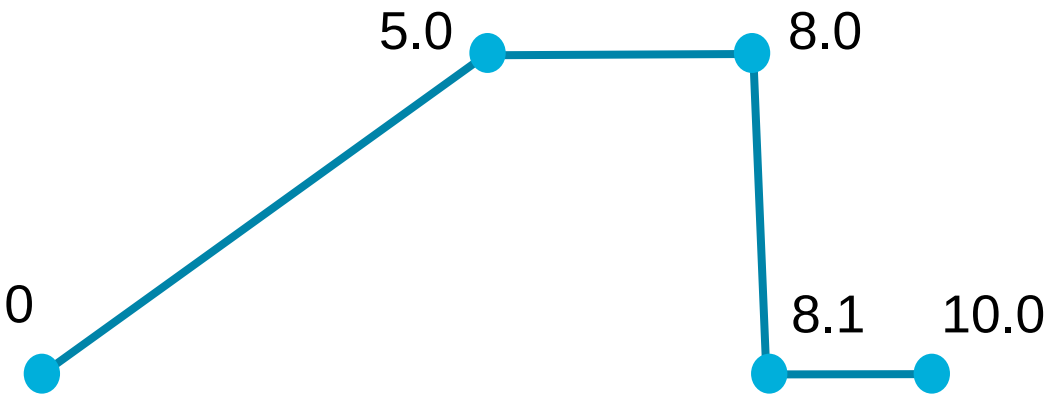
MS Method

- Triple Quad™ 6500 system
- Positive ESI mode

Name	Q1	Q3
T3 (1)	651.7	605.8
T3 (2)	651.7	479.0
T4 (1)	777.7	731.6
T4 (2)	777.7	604.8

LC Method

- Phenomenex Kinetex C18, 50x4.6mm, 2.6um @ 0.6 mL/min
- A = Water + 0.1% acetic acid
- B = Methanol + 0.1% acetic acid

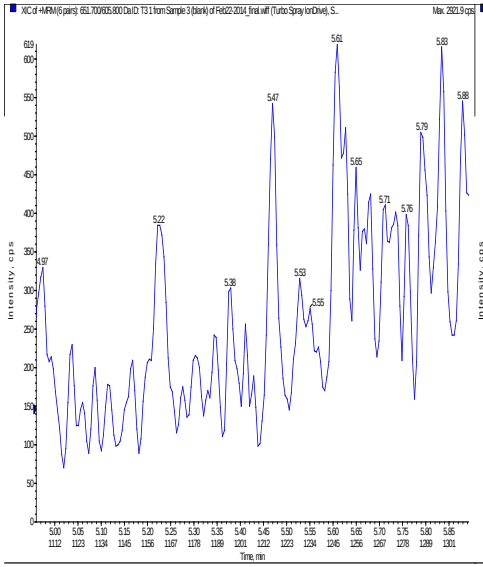


Time	%B
0	20
5	95
8	95
8.1	20
10	20

LOQ < 0.5 pg/mL

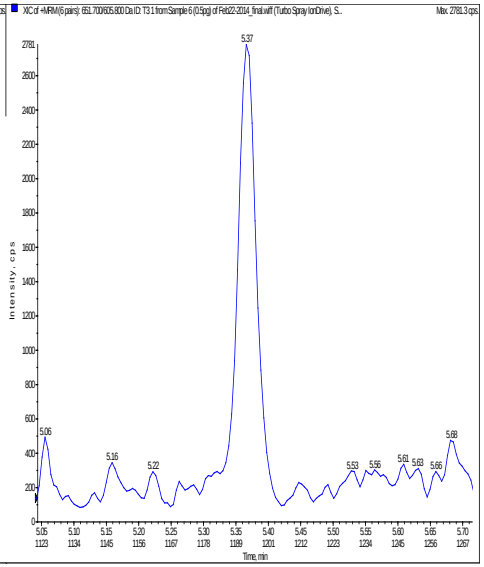
T3

Blank injection



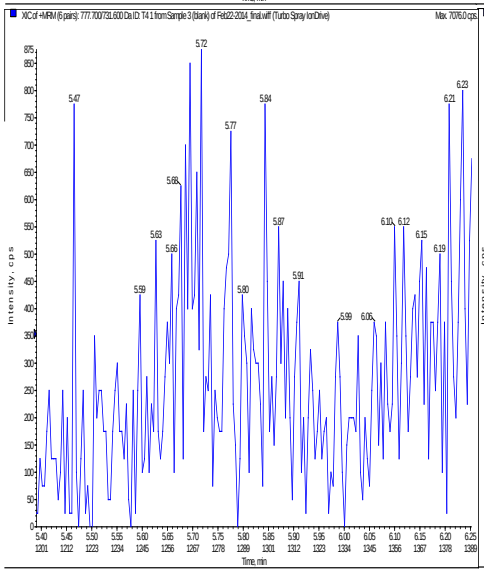
T3

0.5 pg/mL
(S/N = 48)



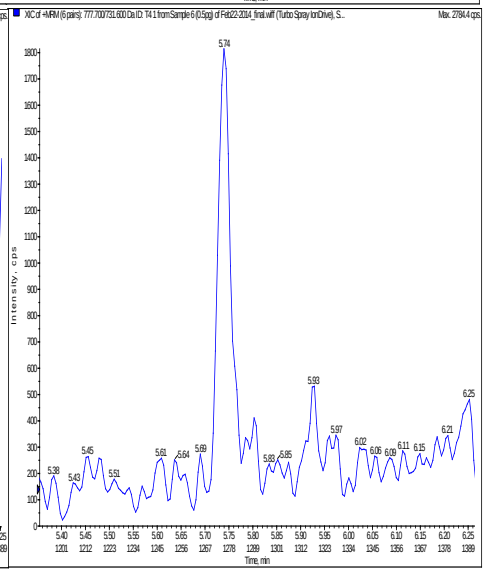
T4

Blank injection

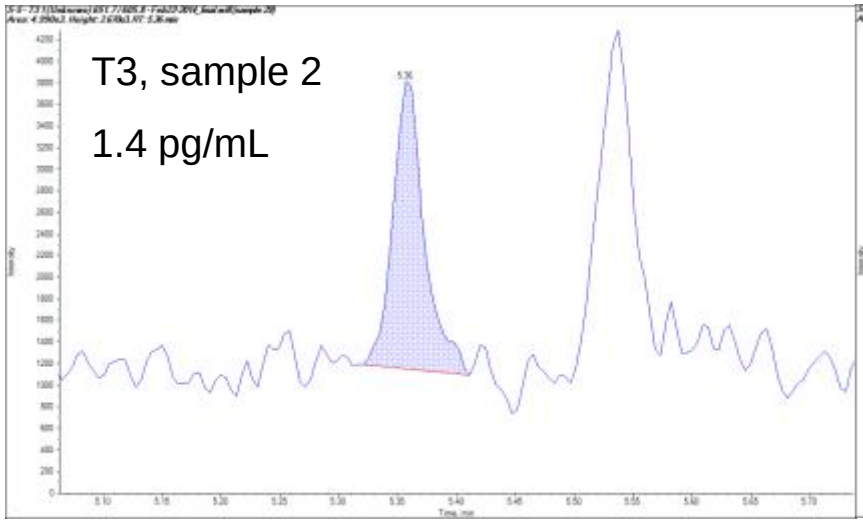


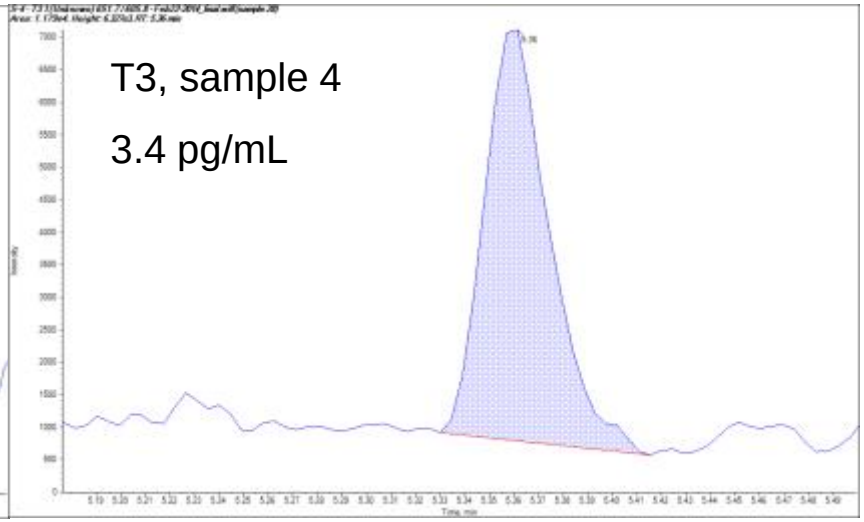
T4

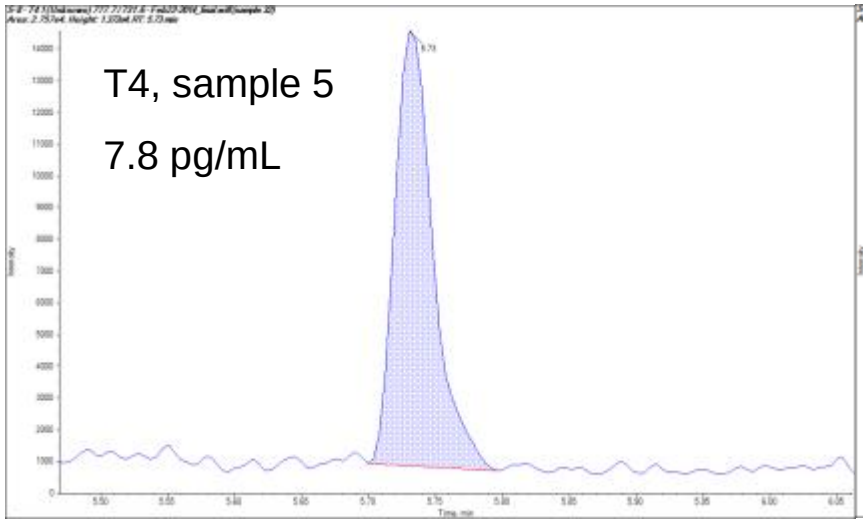
0.5 pg/mL
(S/N = 60)

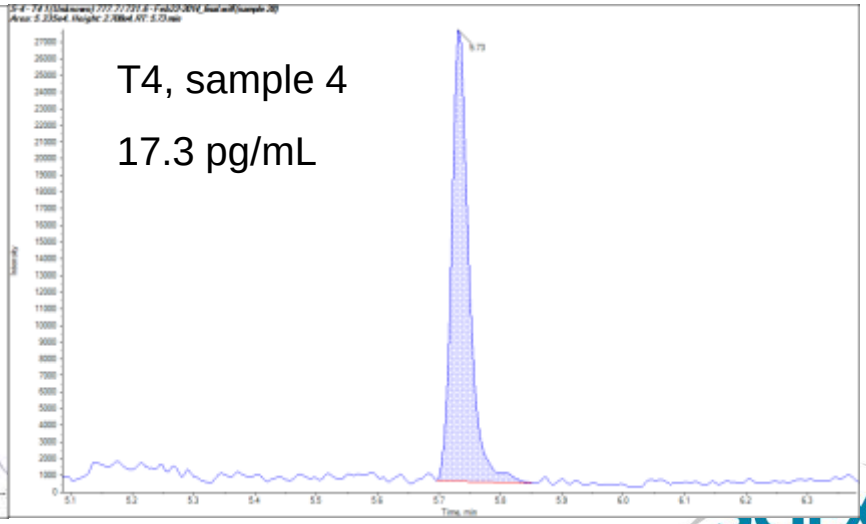


Measurements in serum









Steroid Research:

Analysis of Aldosterone

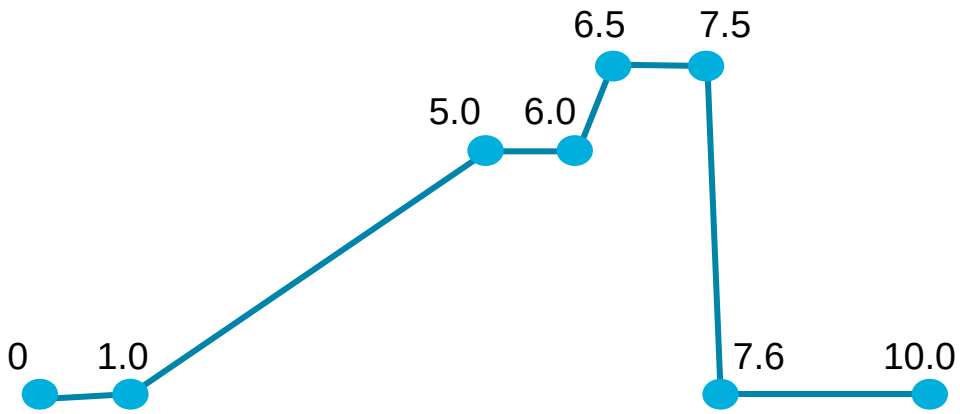
MS Method

- Triple Quad™ 6500 system
- Negative ESI mode

Name	Q1	Q3
Aldosterone (1)	359.2	189.0
Aldosterone (2)	359.2	331.1
Aldosterone-d7	366.2	338.2

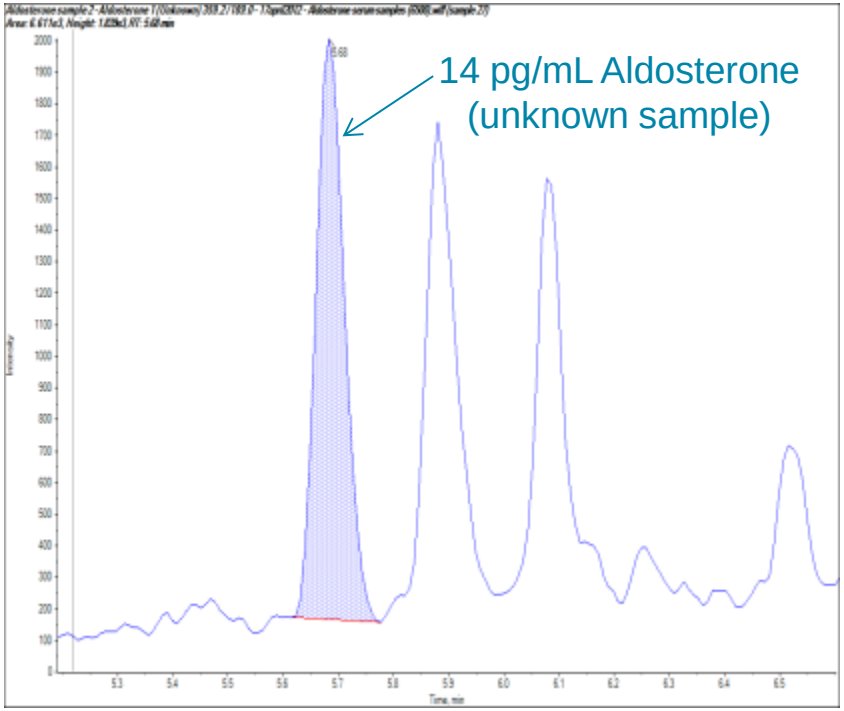
LC Method

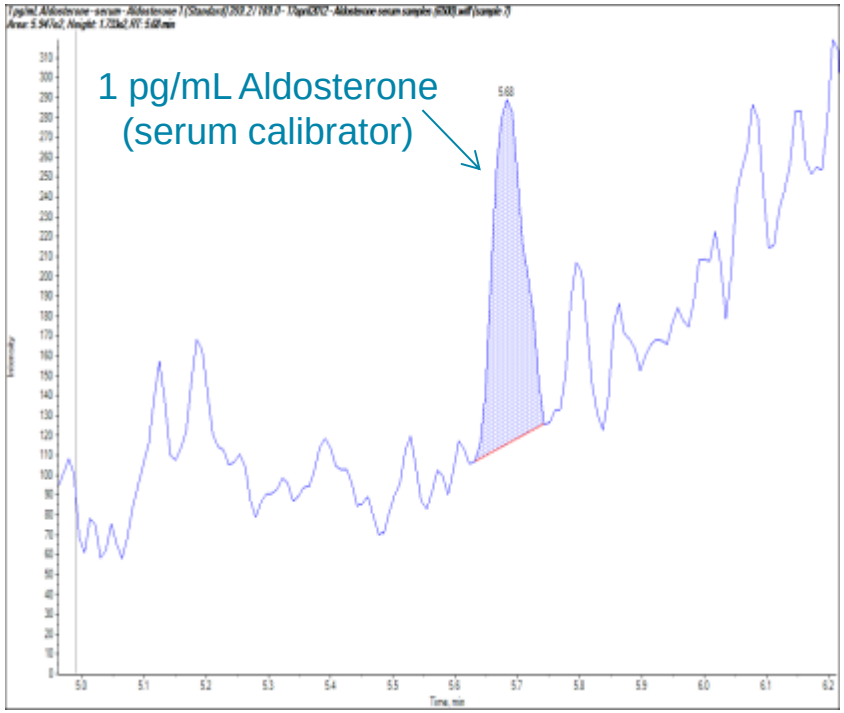
- Phenomenex Gemini-NX C18, 150x3.0mm, 5um @ 0.5 mL/min
- A = Water + 2mM ammonium acetate
- B = Methanol + 2mM ammonium acetate



Time	%B
0	20
1	20
5	70
6	70
6.5	90
7.5	90
7.6	20
10	20

LOQ = 1 pg/mL

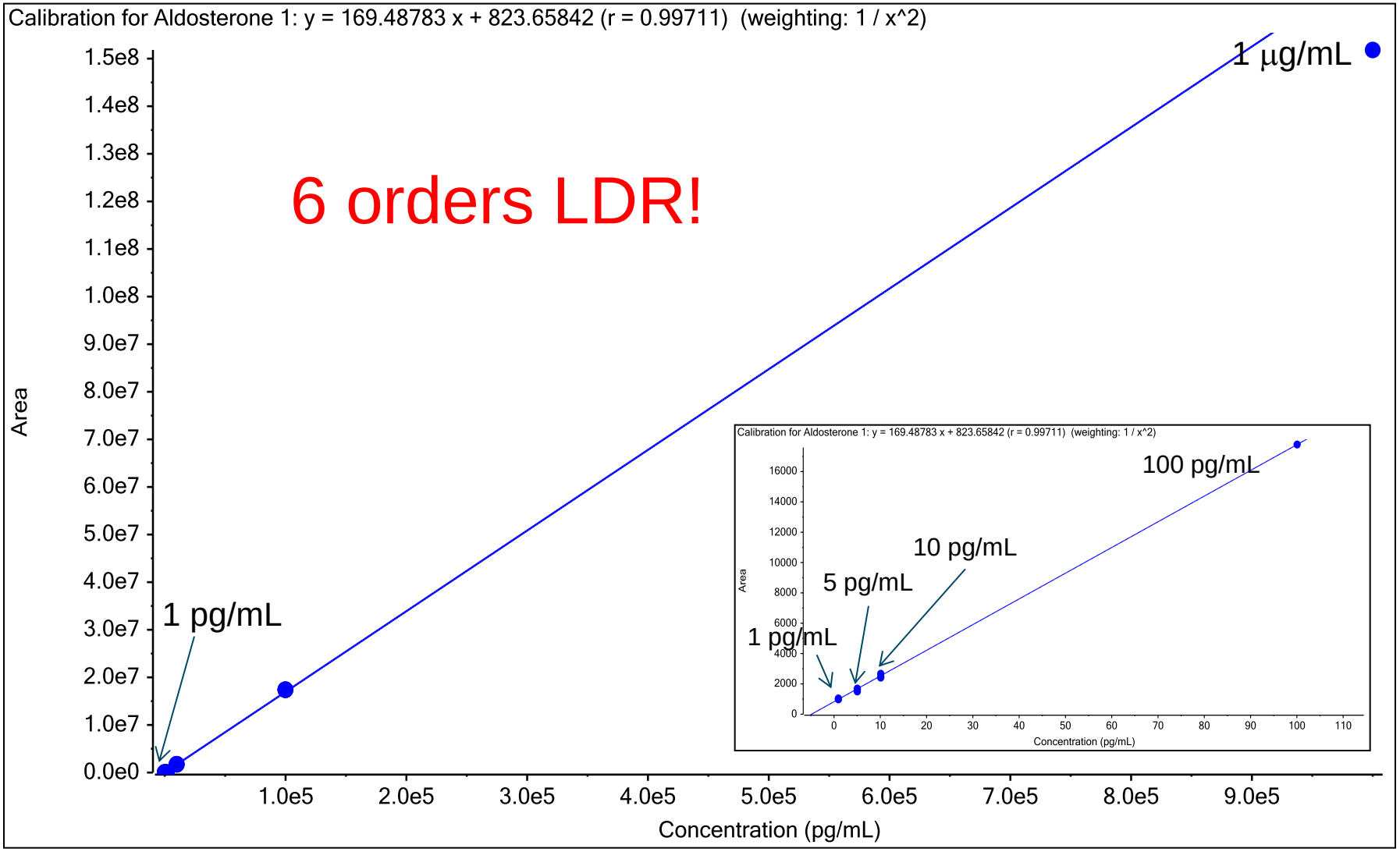




14 pg/mL (39.6 pmol/L) aldosterone in human serum sample

LOQ = 1 pg/mL (2.8 pmol/L) aldosterone in human serum

Linear Dynamic Range: 1pg/mL – 1μg/mL Aldosterone



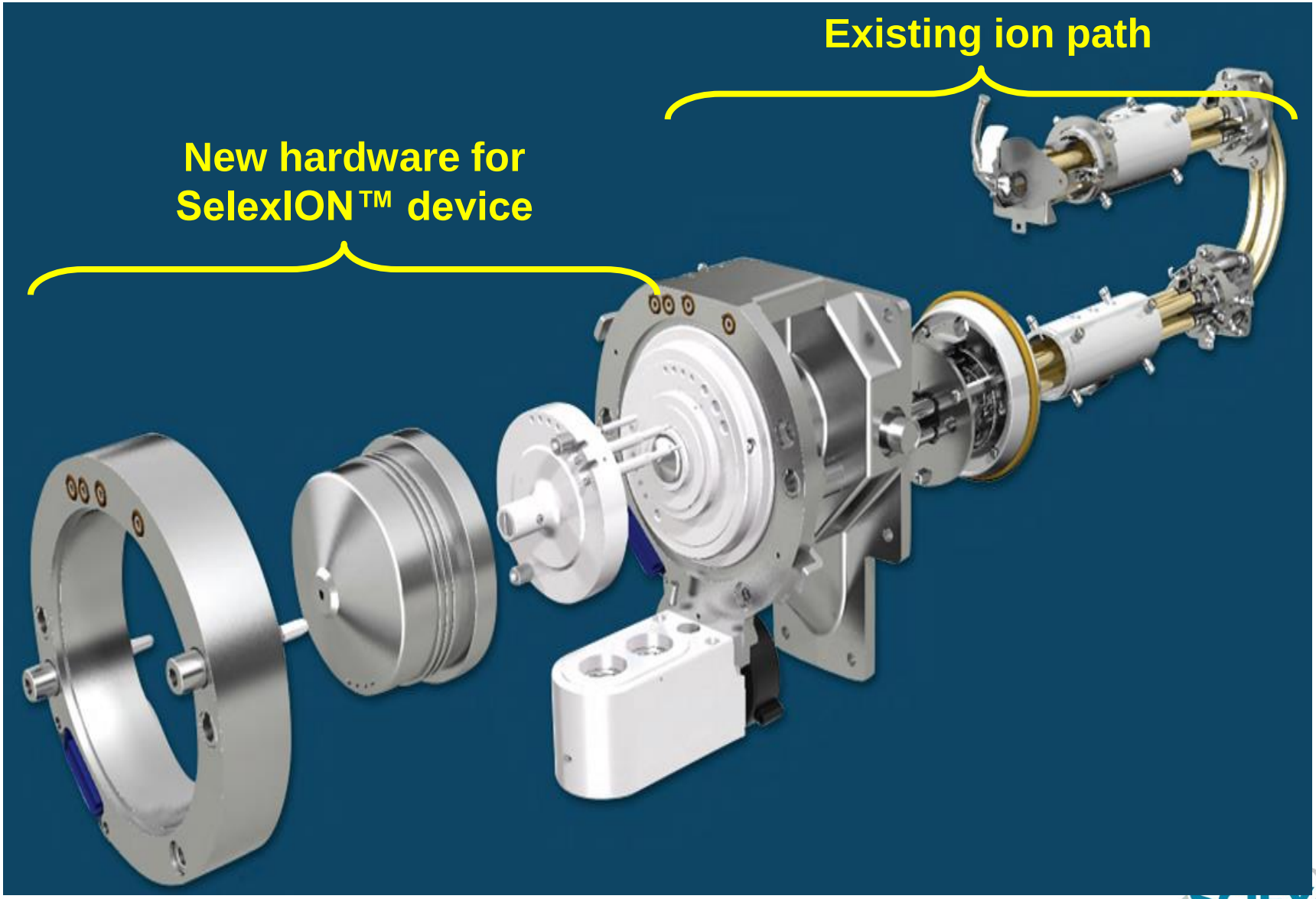
SelexION™ Ion Mobility Technology

Revolutionizing the way we do LC-MS/MS

- Benefits of ion mobility separations
  - Separation of isobaric interferences
  - Removal of chemical background noise
  - Enables simplified sample preparation
  - Enables faster LC run-times
  - Improves LLOQ
  - Improves data quality

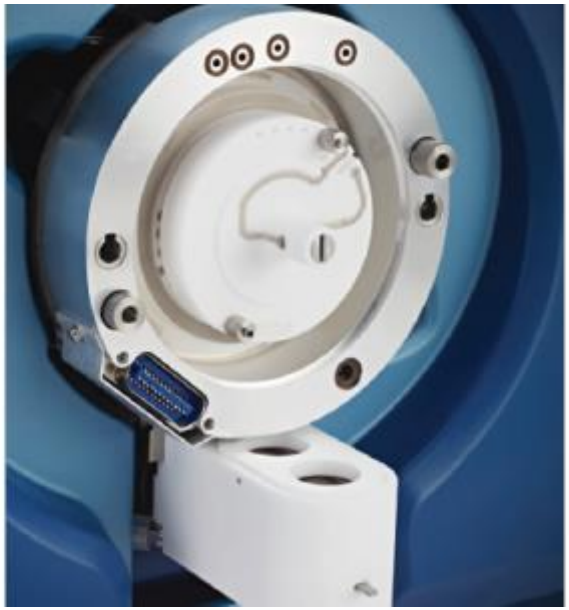
- Features of the SelexION™ device
  - Installation without breaking vacuum
  - Ability to operate in “transparent” mode
  - User-defined resolution setting
  - Rapid MRM scanning
  - Enhanced separations using chemical modifiers

SCIEX Triple Quad™ 6500 system, equipped with SelexION™ technology

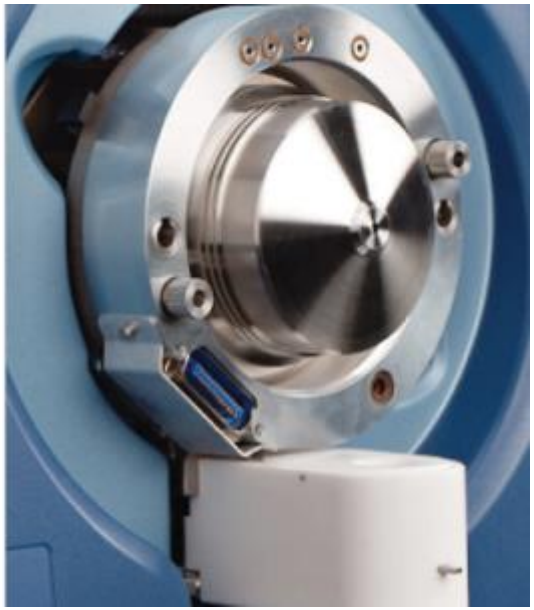


1. Orifice Plate

2. Ion Mobility Cell

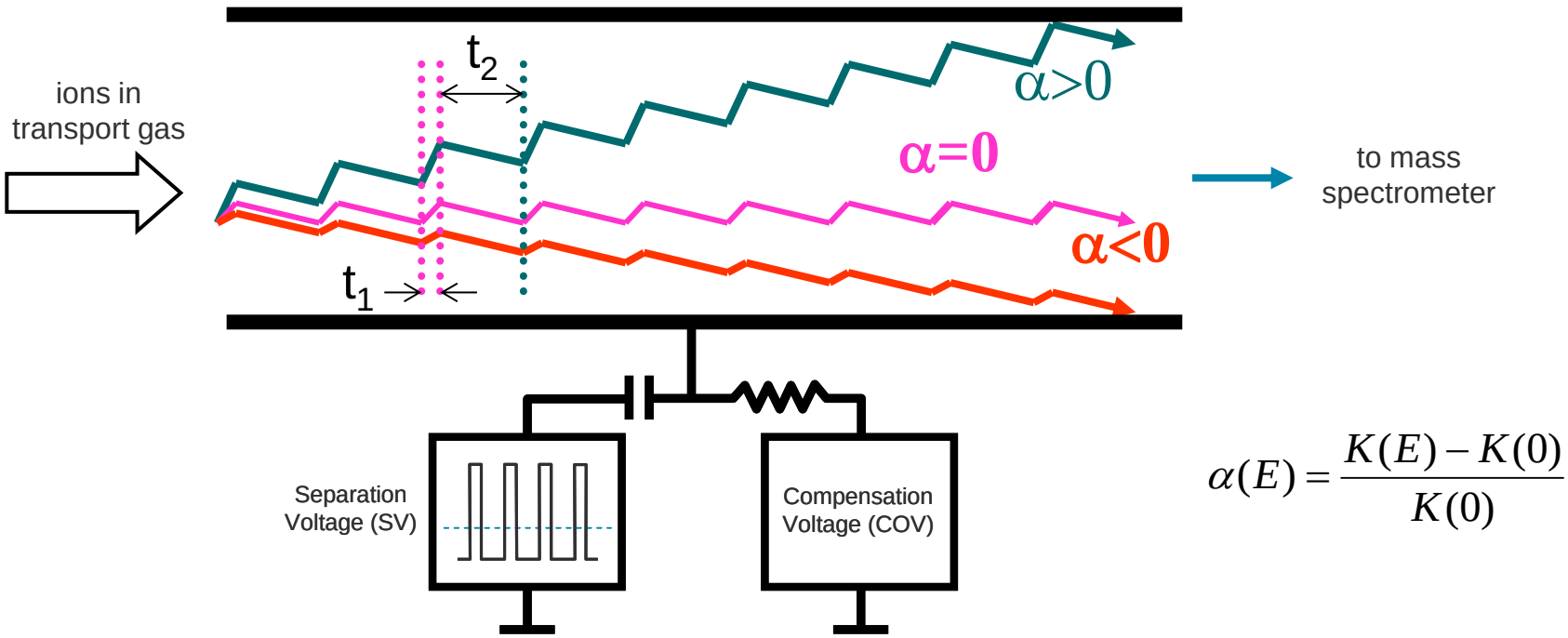


3. Curtain Plate



Robust, easy-to-install, hardware components:

- No tools required
- No cables
- No need to break vacuum
- Installation in about 2 minutes

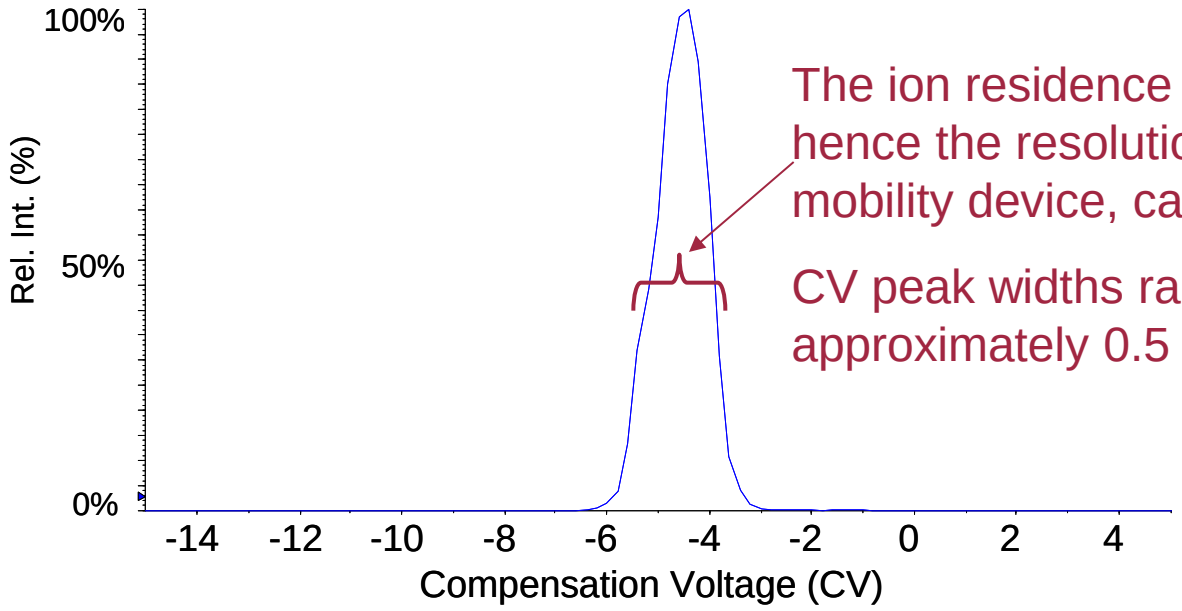


$$\alpha(E) = \frac{K(E) - K(0)}{K(0)}$$

- Ions migrate towards one of the planar electrodes
  - If an ion's high-field mobility K(E) is larger than its low-field mobility K(0), $\alpha>0$
  - If an ion's low-field mobility K(0) is larger than it high-field mobility K(E), $\alpha<0$

- Any ion can be steered back onto the center-line, by application of a compound-specific DC compensation voltage (CV).

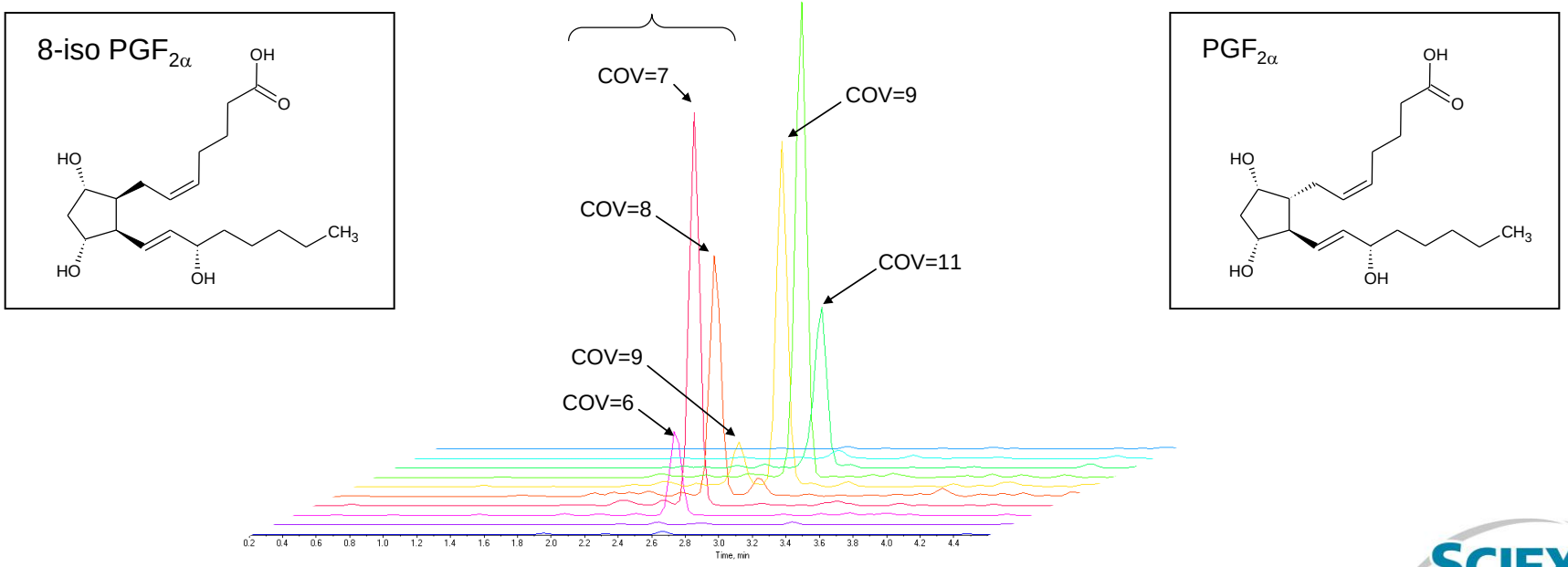
Tuning the SelexION™ Ion Mobility Device



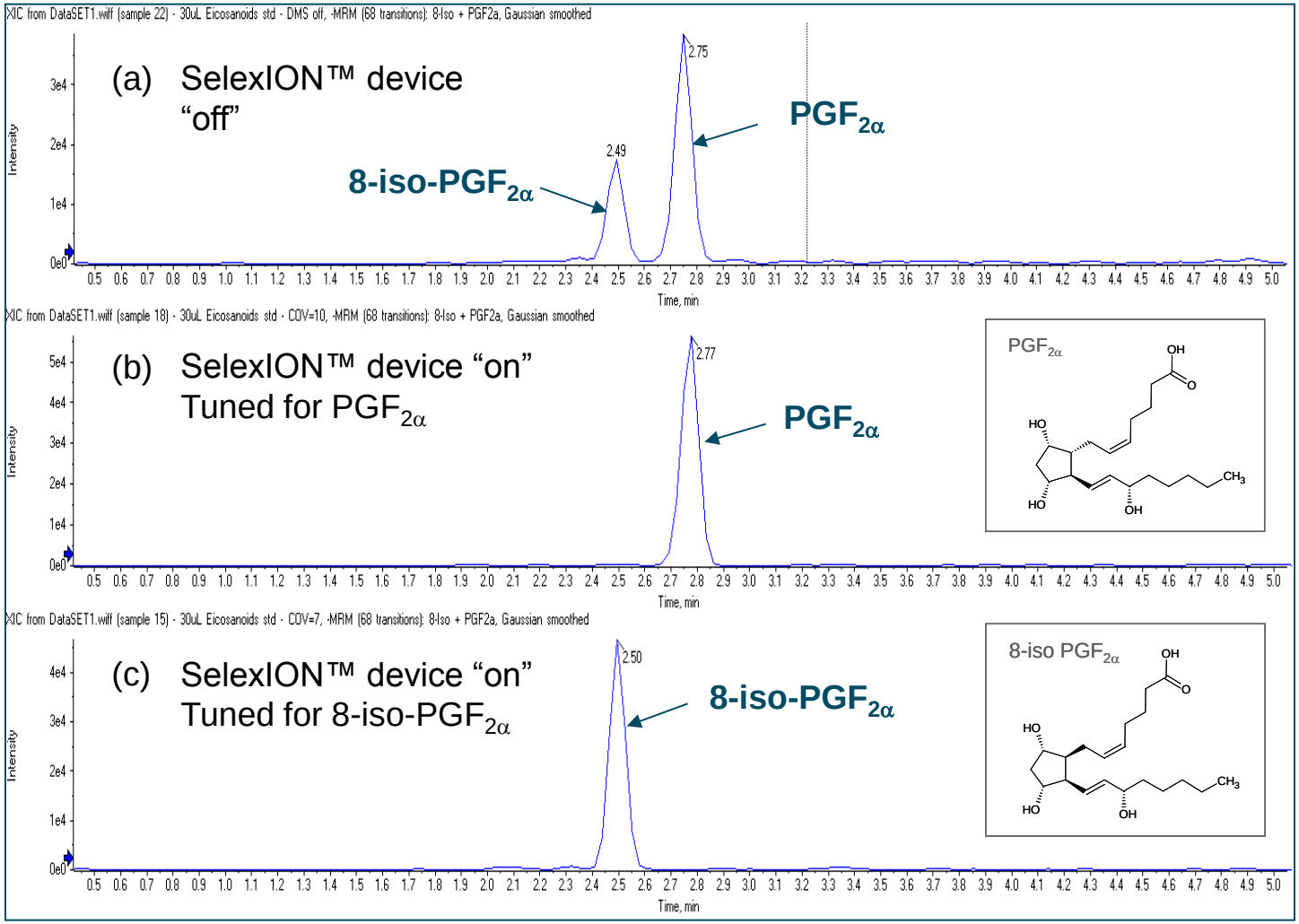
- In infusion mode, the compensation voltage (CV) parameter is ramped in order to determine the optimized value.
- Note that this is analogous to tuning any other compound-specific parameter, e.g. the collision energy (CE)

Resolving isobaric prostaglandins with SelexION™ technology

- Optimization of the SelexION™ device for isomers is accomplished by ramping the COV parameter.
- At COV=10, only $PGF_{2\alpha}$ is transmitted. At COV=7, only 8-iso-$PGF_{2\alpha}$ is transmitted.



Resolving isobaric prostaglandins with SelexION™ technology

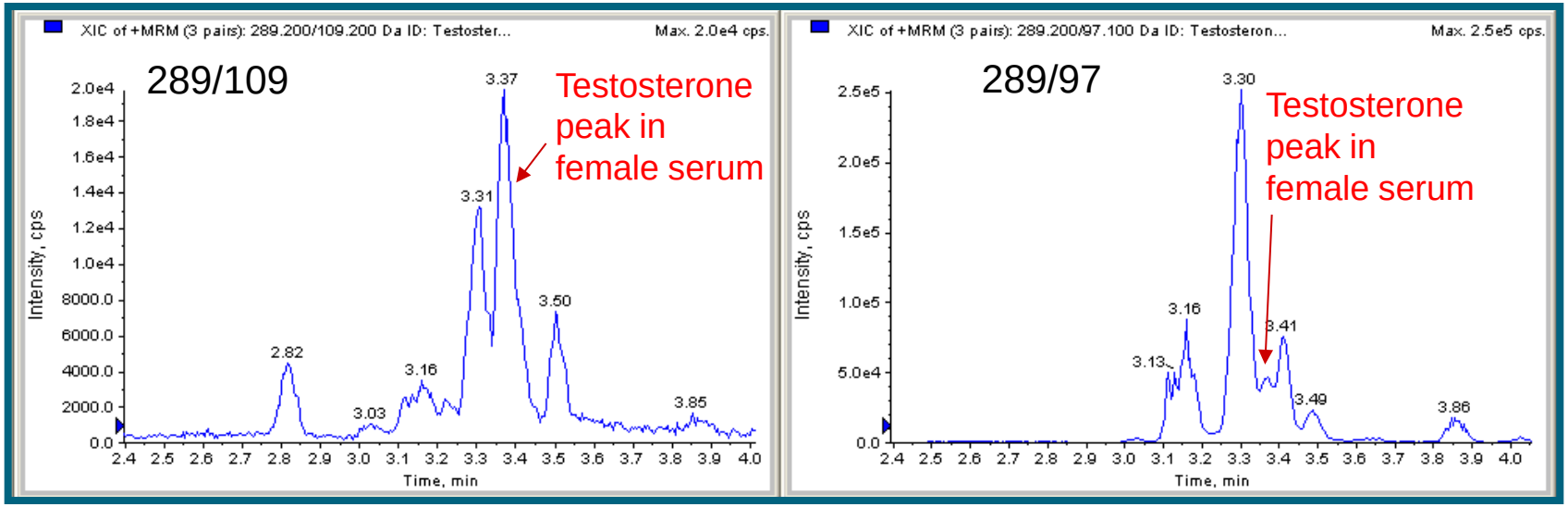


Q1	Q3	CE
353.2	309.2	-28

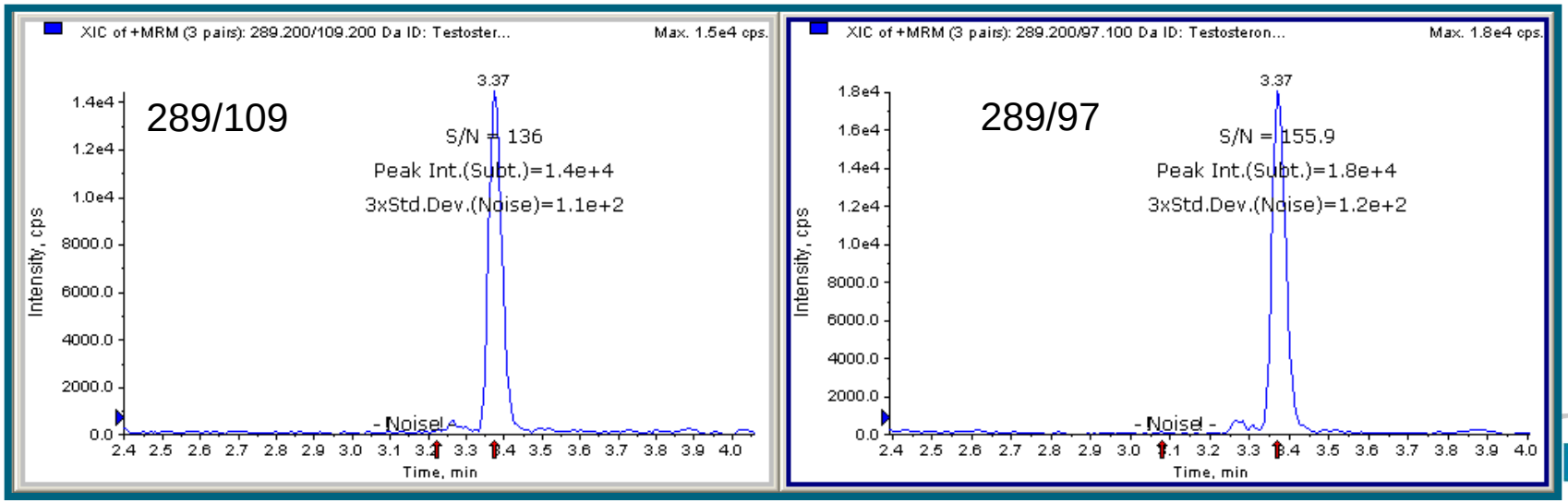
Q1	Q3	CE	CV
353.2	309.2	-28	10.0

Q1	Q3	CE	CV
353.2	309.2	-28	7.0

Protein Precipitation (PPT) – LC-MS/MS



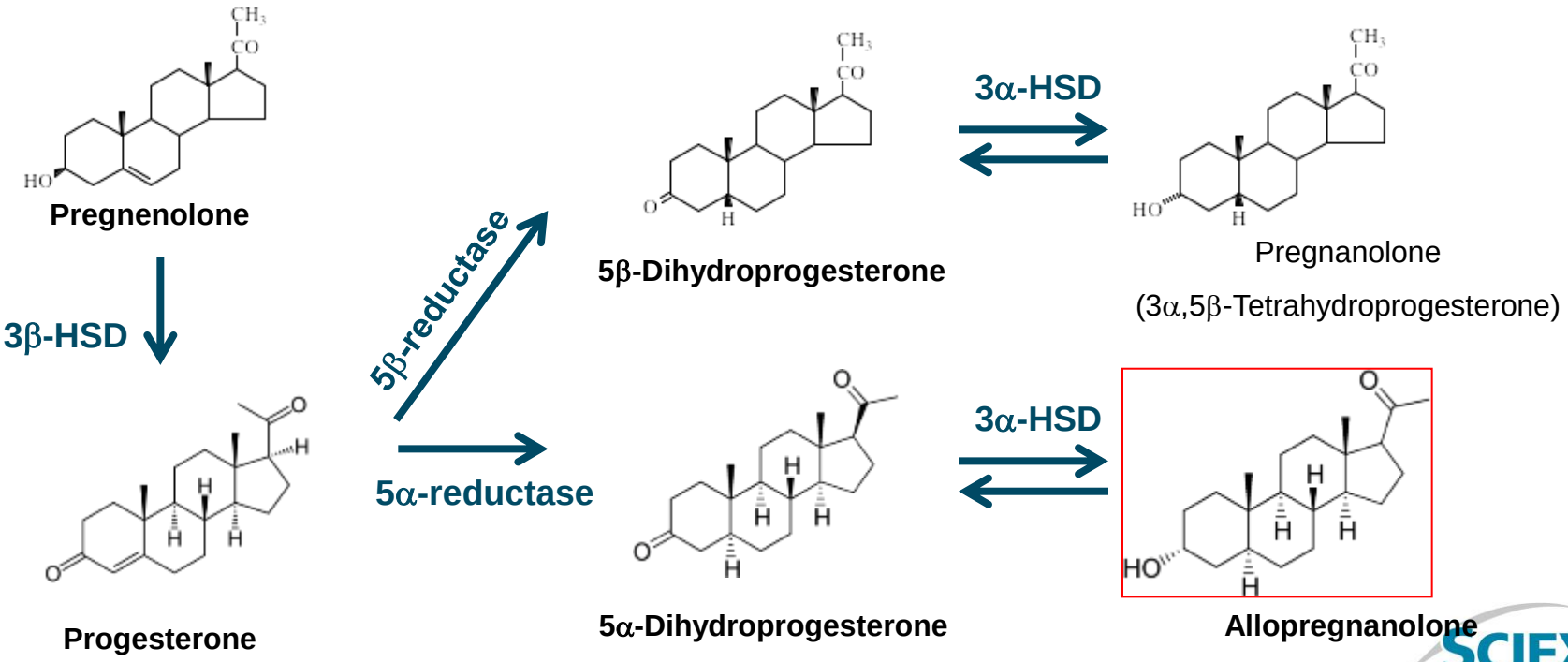
Protein Precipitation (PPT) – LC-MS/MS with SelexION™ Technology



Analysis of the Neuro-active Steroid Allopregnanolone

Metabolism of Neurosteroids

- The most familiar steroid hormones, commonly measured in blood for research purposes, are the mineralocorticoids, glucocorticoids and the androgens and estrogens.
- One alternative pathway that has been of great interest to researchers is the production of compounds by 5α-reductase , followed by 3α-hydroxysteroid oxido reductase.



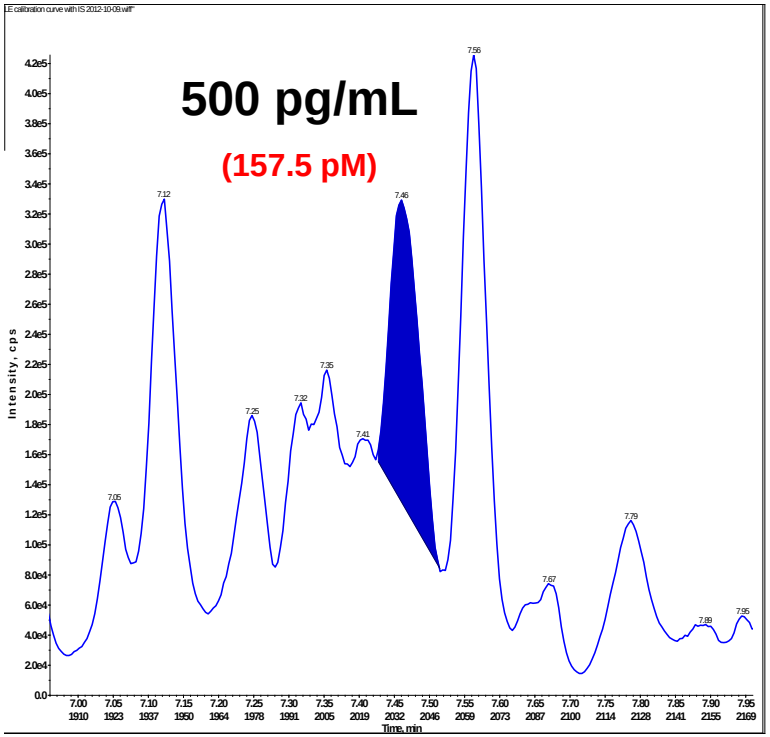
Allopregnanolone: Why LC-MS/MS?

Currently, the assays in use are an RIA (but supplies of the antibody are very low) and GC/MS (which is only performed by a few academic labs).

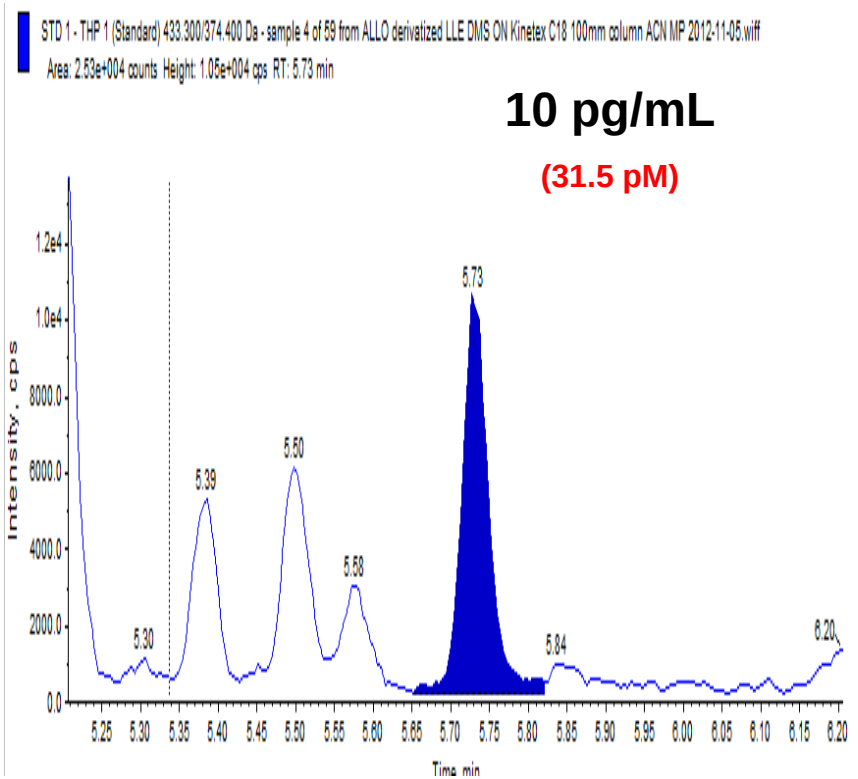
Availability of the assay is a real backlog in trying to perform research studies.

- Sensitivity
  - Potential for increased sensitivity by LC/MS/MS
- Sample prep
  - 5-day process with GC/MS
  - Very labor intensive for GC/MS
  - Many opportunities for errors
  - Limited number of samples/run
  - Long sample run time for GC/MS

Early days for LC-MS/MS…



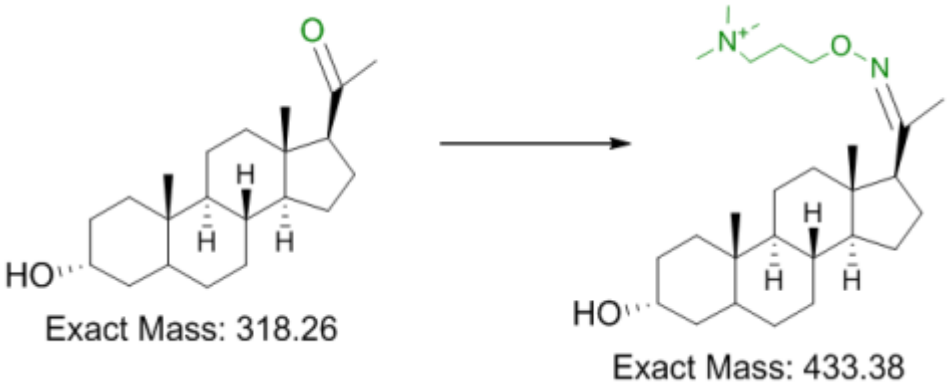
Current Method



- **Keys to success**
  1. Derivatization with the SCIEX Amplifex™ Keto reagent
     - Add reagent at room temp; 30 minutes; no need for fume hood
  2. Removal of interferences with SelexION™ ion mobility technology

Sample preparation

- Plasma samples
  - Collected from pregnant, postpartum and normal females
- Liquid-liquid extraction
  - Extract 100uL of plasma, using 1:1 (v:v) ethyl acetate:hexane mixture
- Derivatization
  - Add 50uL of SCIEX Amplifex™ Keto reagent to the dried, extracted sample
  - Allow to react for 1 hour.

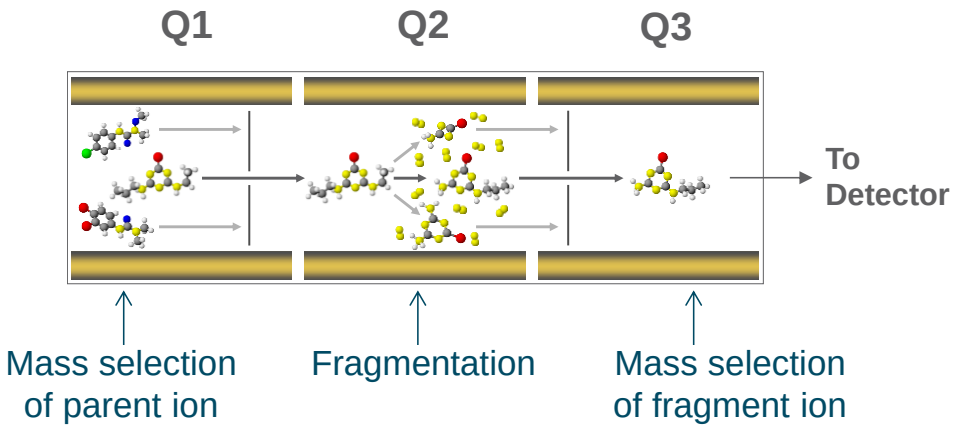


Allopregnanolone Derivatized Allopregnanolone

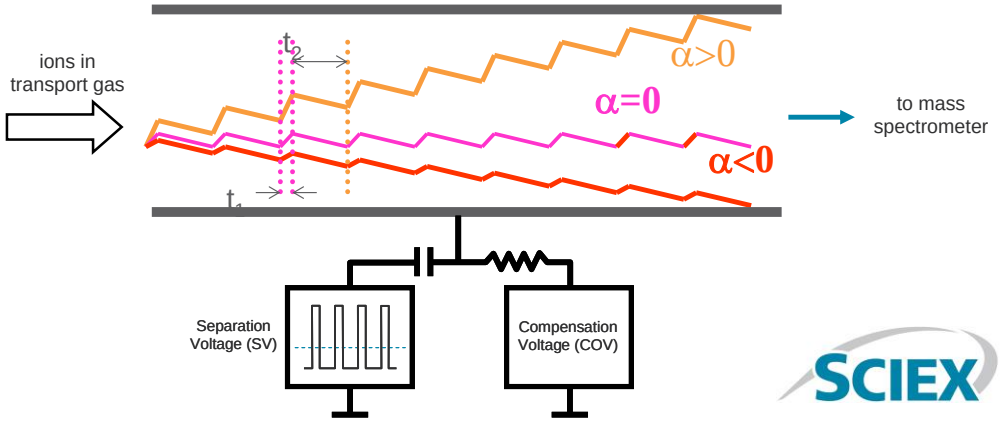
- Steroids are non-polar, and are difficult to ionize
- Derivatization enhanced the sensitivity, by improving the ionization efficiency

MS/MS and Ion Mobility

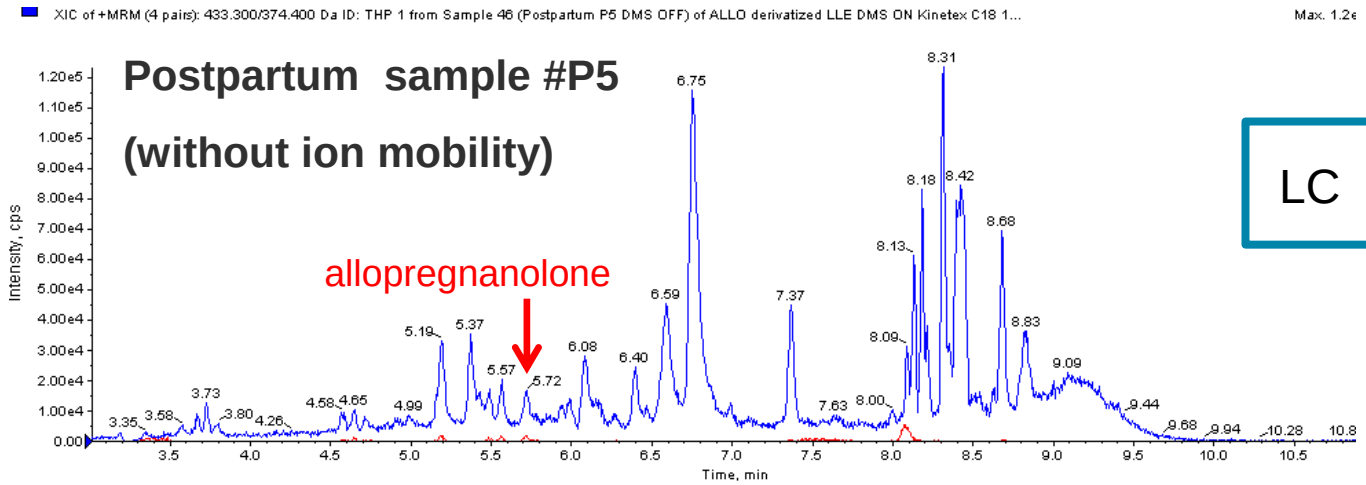
- MS/MS separates compounds based on differences in the masses of the ions (both precursor ions and fragment ions).

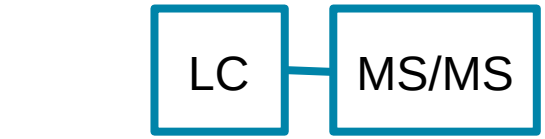


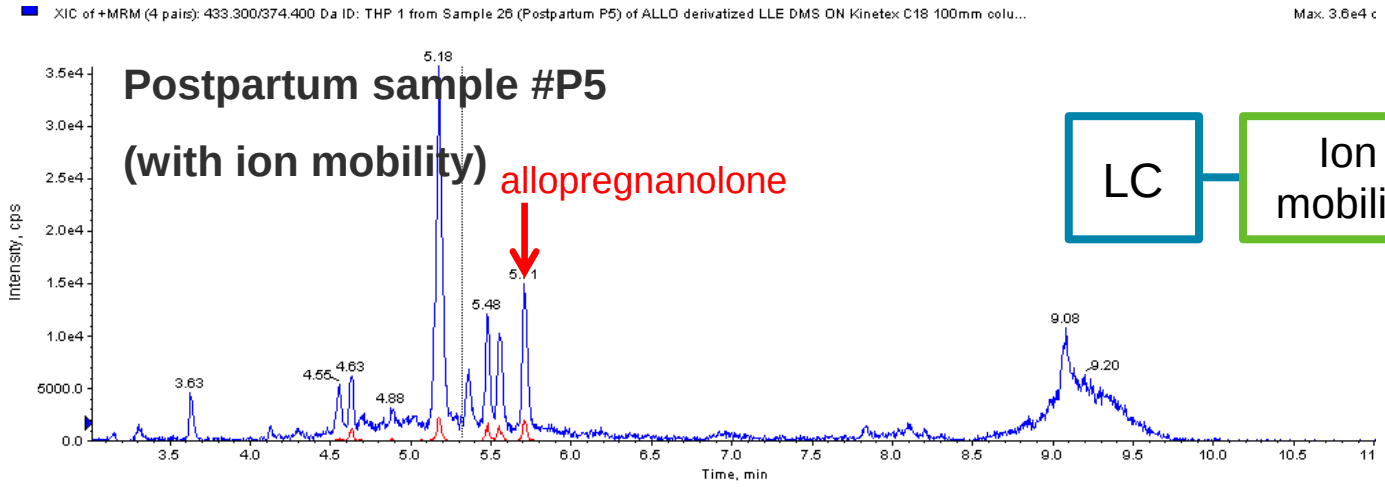
- Ion mobility separates compounds based on differences in the size/shape of the ionized compounds.



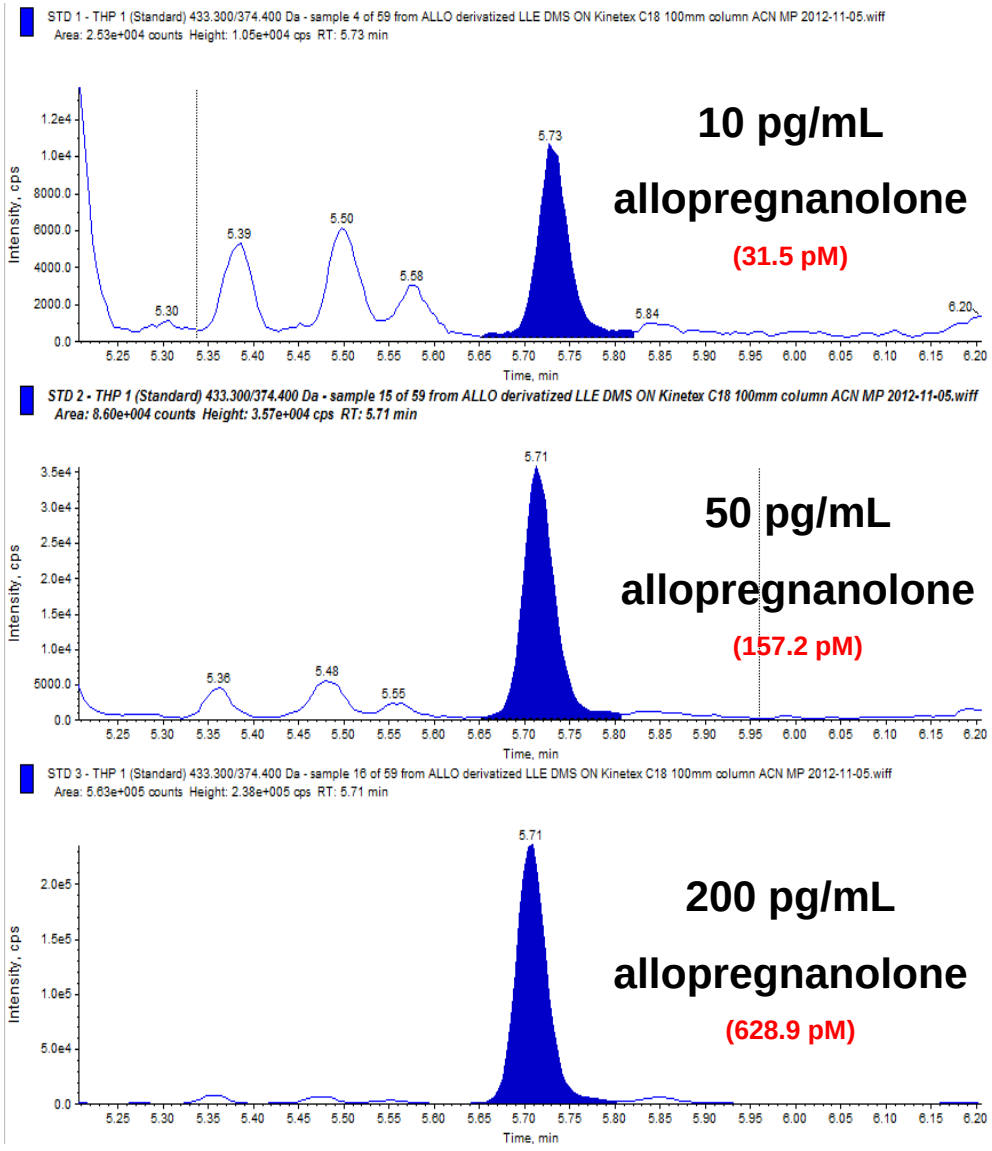
Assessing the effect of the SelexION™ ion mobility device



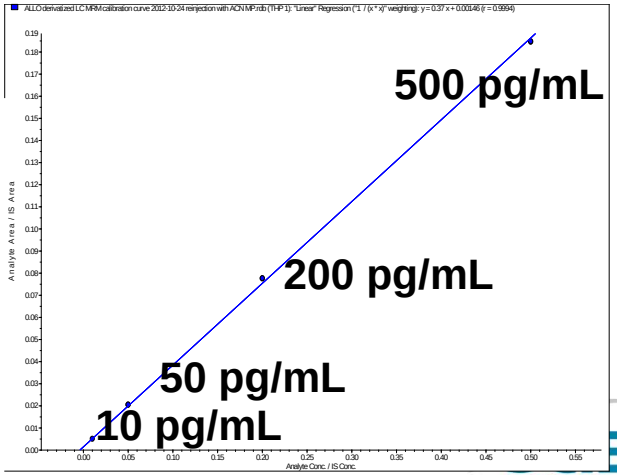




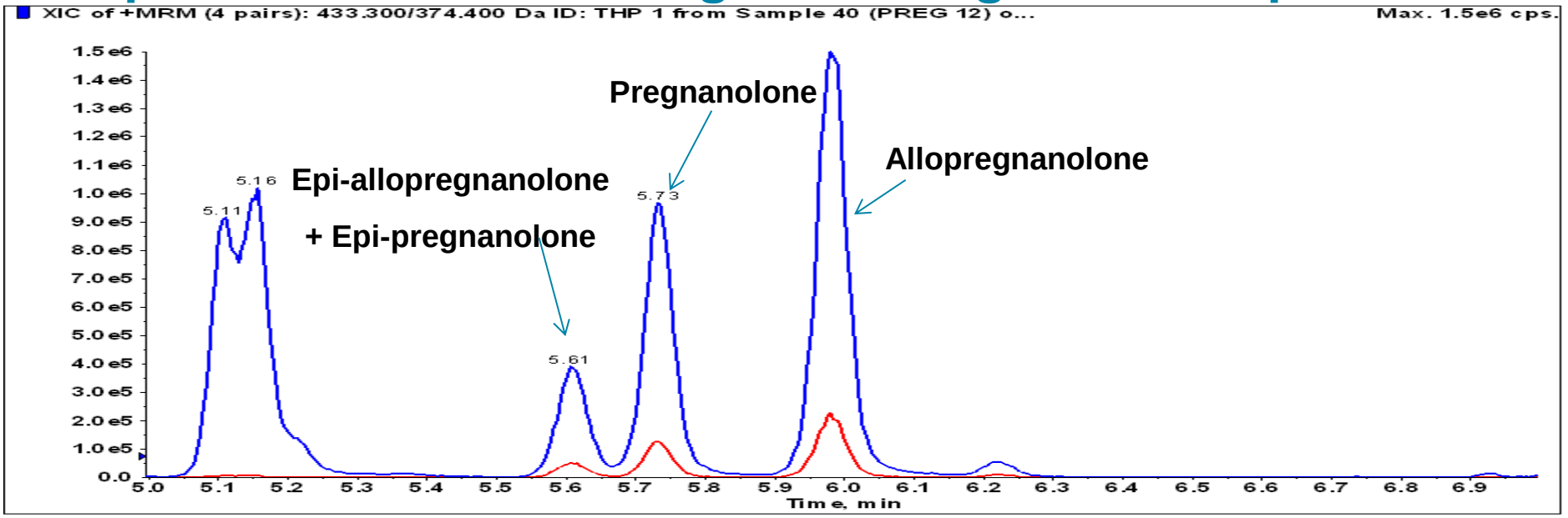
Sensitivity of the LC-ion mobility-MS/MS method

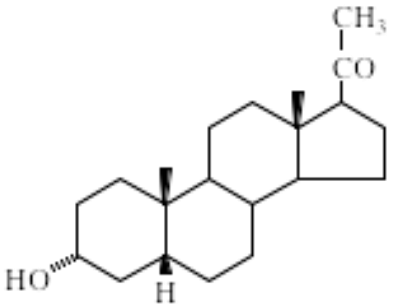


- Using this very sensitive method, it was possible to measure <10pg/mL
- Only 10uL were injected
- The instrument response was linear over the calibration range covering from 10 - 25,000 pg/mL

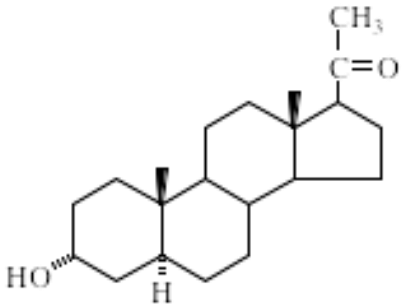


Representative chromatogram: “Pregnant” sample

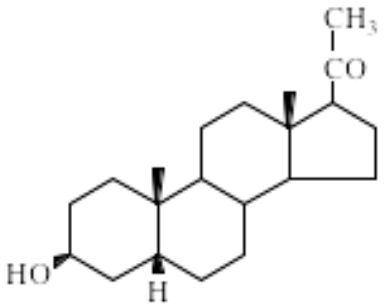




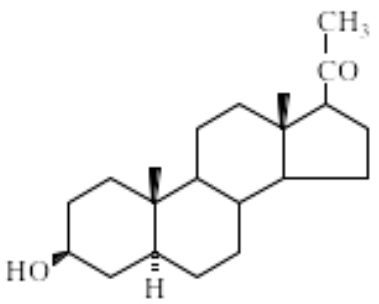
Pregnanolone
(3α,5β-THP)



Allopregnanolone
(3α,5α-THP)

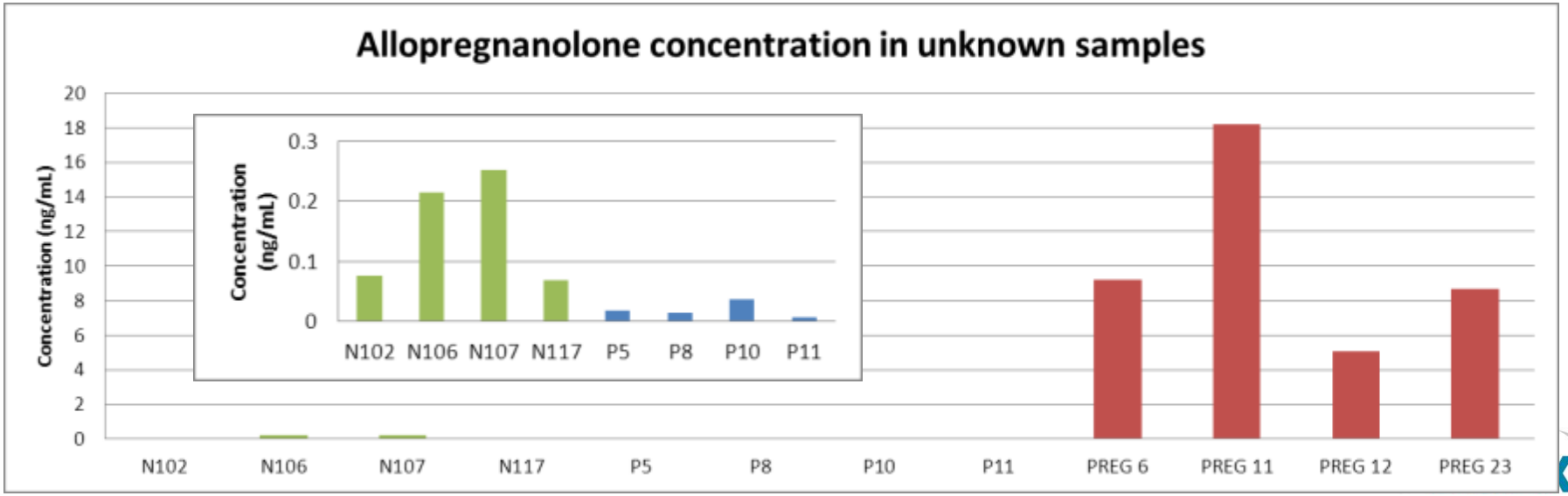


Epipregnanolone
(3β,5β-THP)



Epiallopregnanolone
(3β,5α-THP)

Sample Name	File Name	Analyte Peak Area (counts)	Analyte Peak Height (cps)	Analyte Concentration (ng/mL)	Analyte Retention Time (min)	IS Peak Area (counts)	IS Peak Height (cps)	IS Retention Time (min)	Calculated Concentration (ng/mL)
N102	ALLO derivatized LLE DMS ON I	7.65E+04	2.42E+04	N/A	5.83	2.81E+06	8.82E+05	5.81	0.0756
N106	ALLO derivatized LLE DMS ON I	2.35E+05	7.33E+04	N/A	5.85	3.13E+06	9.85E+05	5.82	0.215
N107	ALLO derivatized LLE DMS ON I	2.30E+05	7.14E+04	N/A	5.85	2.62E+06	8.11E+05	5.82	0.252
N117	ALLO derivatized LLE DMS ON I	7.08E+04	2.31E+04	N/A	5.83	2.82E+06	8.88E+05	5.8	0.0693
P5	ALLO derivatized LLE DMS ON I	2.04E+04	6.22E+03	N/A	5.84	2.81E+06	8.75E+05	5.82	0.0174
P8	ALLO derivatized LLE DMS ON I	1.67E+04	5.47E+03	N/A	5.84	2.81E+06	8.75E+05	5.82	0.0137
P10	ALLO derivatized LLE DMS ON I	3.69E+04	1.17E+04	N/A	5.83	2.68E+06	8.25E+05	5.81	0.0364
P11	ALLO derivatized LLE DMS ON I	1.09E+04	3.16E+03	N/A	5.82	2.82E+06	8.68E+05	5.8	0.00763
PREG 6	ALLO derivatized LLE DMS ON I	9.78E+06	3.04E+06	N/A	5.83	3.08E+06	1.00E+06	5.8	9.24
PREG 11	ALLO derivatized LLE DMS ON I	1.78E+07	5.63E+06	N/A	5.83	2.86E+06	9.16E+05	5.81	18.2
PREG 12	ALLO derivatized LLE DMS ON I	4.87E+06	1.52E+06	N/A	5.83	2.79E+06	8.67E+05	5.8	5.07
PREG 23	ALLO derivatized LLE DMS ON I	8.53E+06	2.63E+06	N/A	5.83	2.85E+06	8.98E+05	5.81	8.71



Conclusions

- Keys to success, for LC-MS/MS method:
  - Ion mobility separation using the SelexION™ device
  - Derivatization using the SCIEX Amplifex™ Keto reagent
  - Highly sensitive SCIEX QTRAP® 6500 LC/MS/MS system
- Great potential for research into:
  - Detecting individual differences in metabolism of reproductive and neurosteroid hormones
  - Predicting individual differences in responses to SSRIs and other medications.
- The QTRAP® 6500 system, combined with SelexION™ ion mobility technology, provides researchers with the sensitivity and selectivity required to measure low-level endogenous steroids in complex biological matrices.

Answers for Science.
Knowledge for Life.™

SCIEX

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