Method development and validation of an ultra-sensitive LC-MS/MS assay for the quantification of estradiol on the basis of an existing assay for ethinylestradiol

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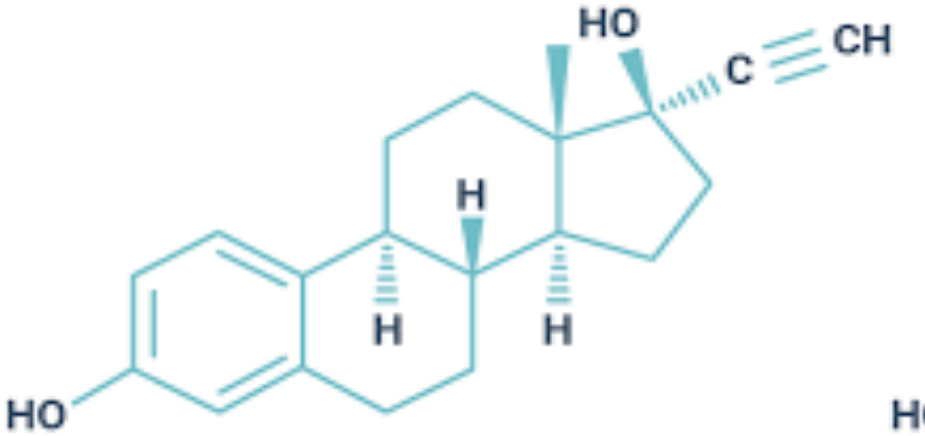
Estradiol

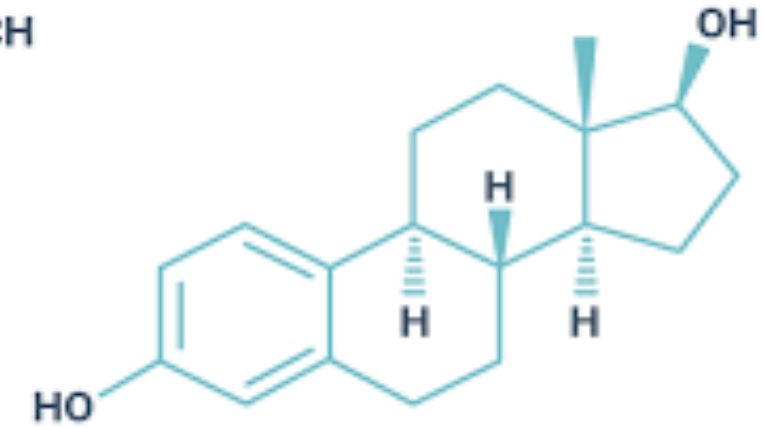
- Estradiol is a naturally occurring hormone circulating endogenously in females.
- It is commercially available in several hormone therapy products for managing conditions associated with reduced estrogen, such as vulvovaginal atrophy and hot flashes.
- Some available forms medicinal products of estradiol are oral tablets, injections, vaginal rings, transdermal patches, sprays, gels, and creams.
- Here we are presenting an ultra-sensitive assay with a LLOQ of 2.00 pg/mL after derivatization with dansyl chloride.
- The assay is simply based on an assay for ethinylestradiol, which is successfully used for a couple of years in our laboratory with the same LLOQ but is running on an API4000.
- The estradiol assay here is running on a QTRAP® 6500+ with an ExionLC™ UHPLC in front.
- Since estradiol is an endogenous compound, calibration standards were prepared in a surrogate matrix whereas QC samples were prepared in low concentration postmenopausal female plasma.
- Special attention was paid to ensure that estradiol was chromatographically separated from estrogen as well from its phase II metabolites.

Ethinylestradiol assay



Ethinylestradiol

Molecular mass: 296.403 g/mol



Estradiol

Molecular mass: 272.38 g/mol

Endogenous 17β-estradiol levels	
Females	25 - 95 pg/mL
Females (ovulation)	75 - 750 pg/mL
Females (postmenopausal)	< 45 pg/mL
Males	12 - 42 pg/mL

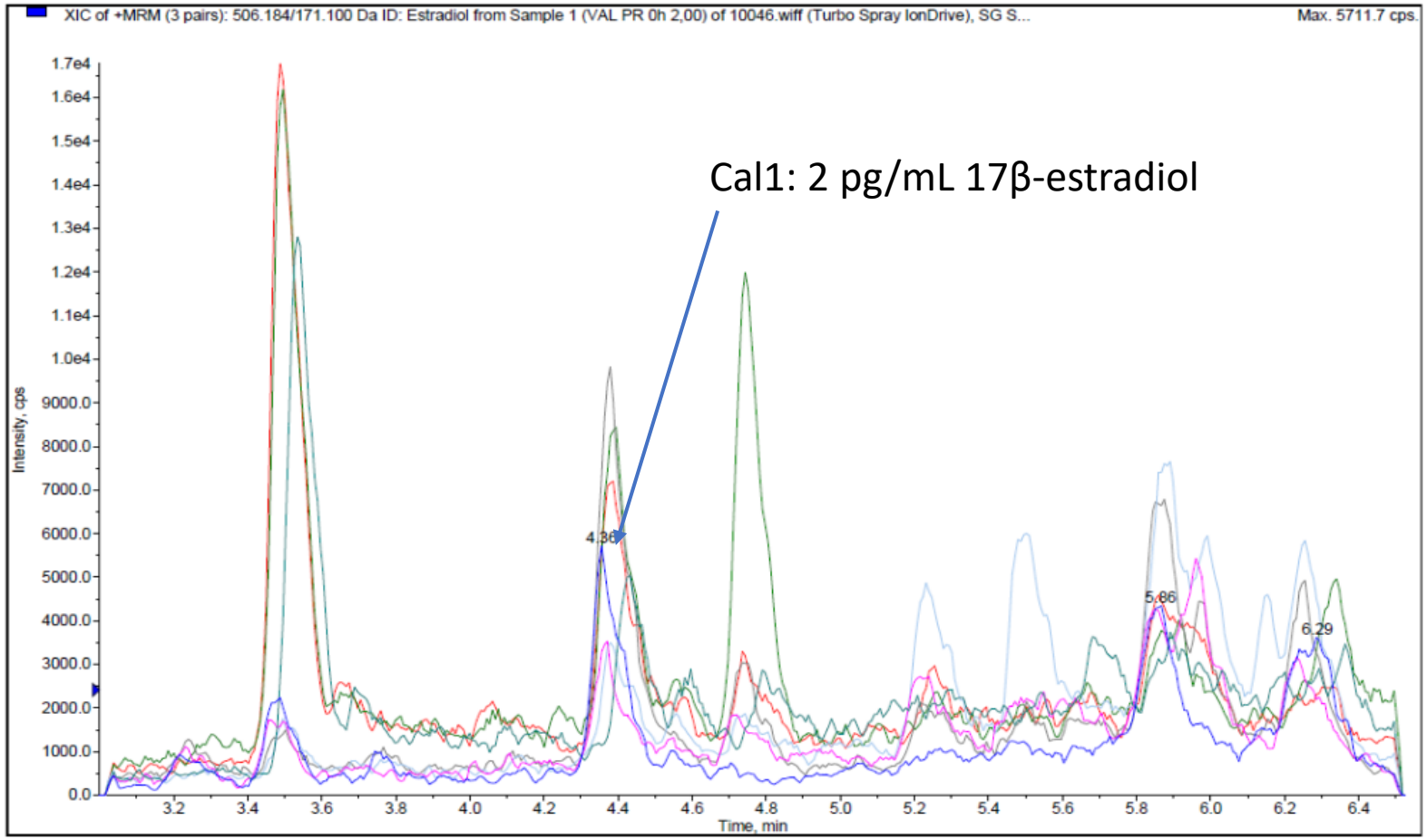
Ethinylestradiol assay

- Plasma samples were processed by liquid-liquid extraction (LLE) and subsequently derivatized.
- Plasma was used as the matrix for the calibration curve.
- The calibration curve was prepared by spiking the blank plasma with known amounts of ethinylestradiol.
- A 500 µL aliquot of plasma was fortified with internal standard (ethinylestradiol-d5) and extracted with 6 mL of hexane/ethyl acetate (9/1).
- The samples were vortexed for 5 min and briefly centrifuged. The organic layer was transferred into a clean test tube and evaporated to dryness.
- The residue was derivatized with dansyl chloride dissolved in acetone and carbonate buffer.
- Sample was extracted again with hexane and evaporated to dryness.
- Sample was reconstructed with 100 µL 0.1 % formic acid/acetonitrile (40/60).
- An 20 µL aliquot was then injected into the HPLC-MS/MS. (API4000®)

17β-estradiol assay

- Plasma samples were processed by liquid-liquid extraction (LLE) and subsequently derivatized.
- Charcoal stripped plasma from menopausal females was used as matrix for the calibration curve.
- The calibration curve was prepared by spiking the charcoal stripped plasma with known amounts of estradiol.
- A 500 µL aliquot of plasma was fortified with internal standard (estradiol-d5) and extracted with 6 mL of hexane/ethyl acetate (9/1).
- The samples were vortexed for 5 min and briefly centrifuged. The organic layer was transferred into a clean test tube and evaporated to dryness.
- The residue was derivatized with dansyl chloride dissolved in acetone and carbonate buffer.
- Sample was extracted again with hexane and evaporated to dryness.
- Sample was reconstructed with 100 µL 0.1 % formic acid/acetonitrile (40/60).
- An 10-µL aliquot was then injected into the HPLC-MS/MS (QTRAP® 6500+).

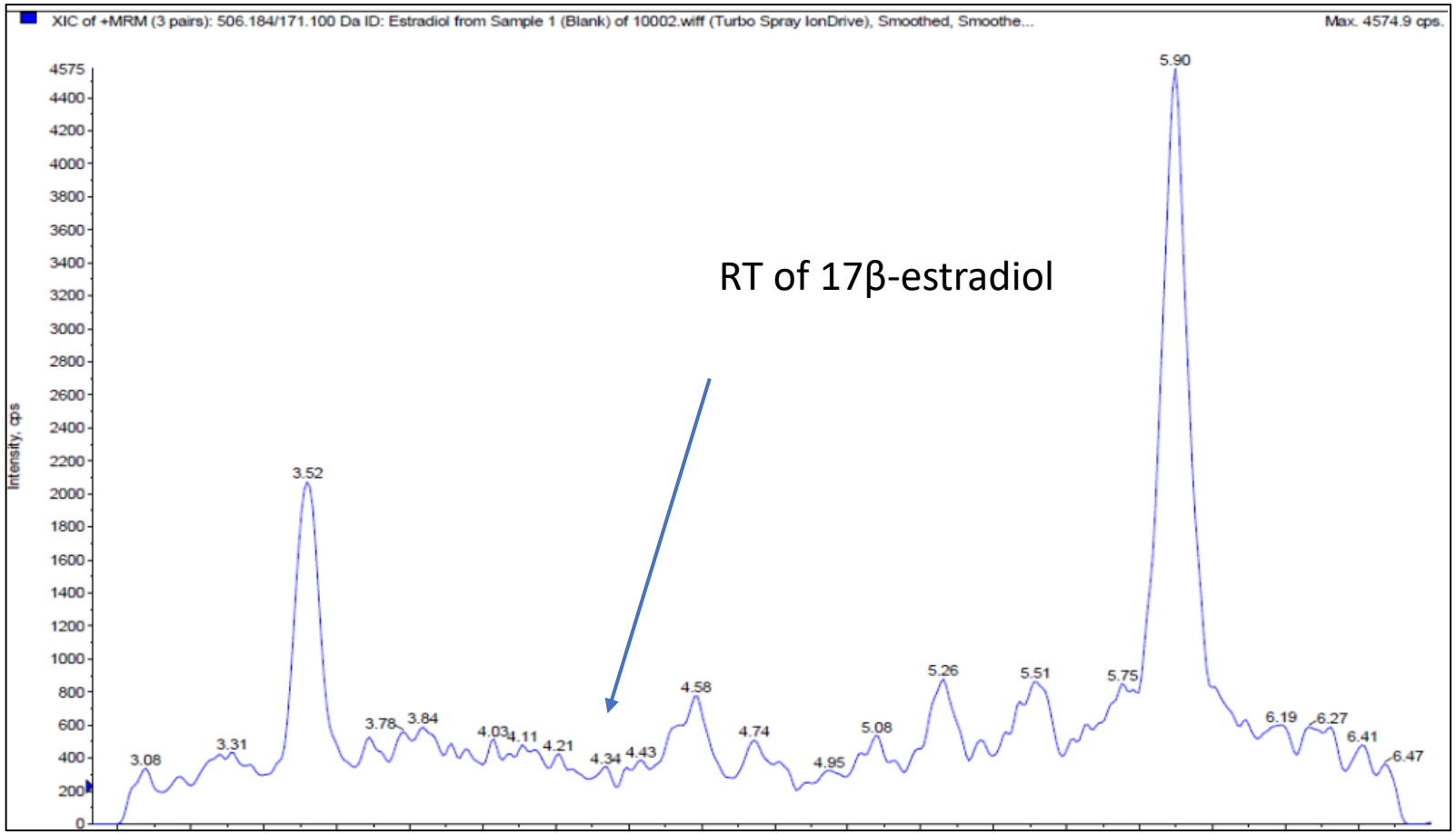
17β-estradiol assay



Overlay EIC of six individual plasma from postmenopausal women + one Ca1 prepared in pooled charcoal stripped plasamm at 2 pg/mL (blue line)

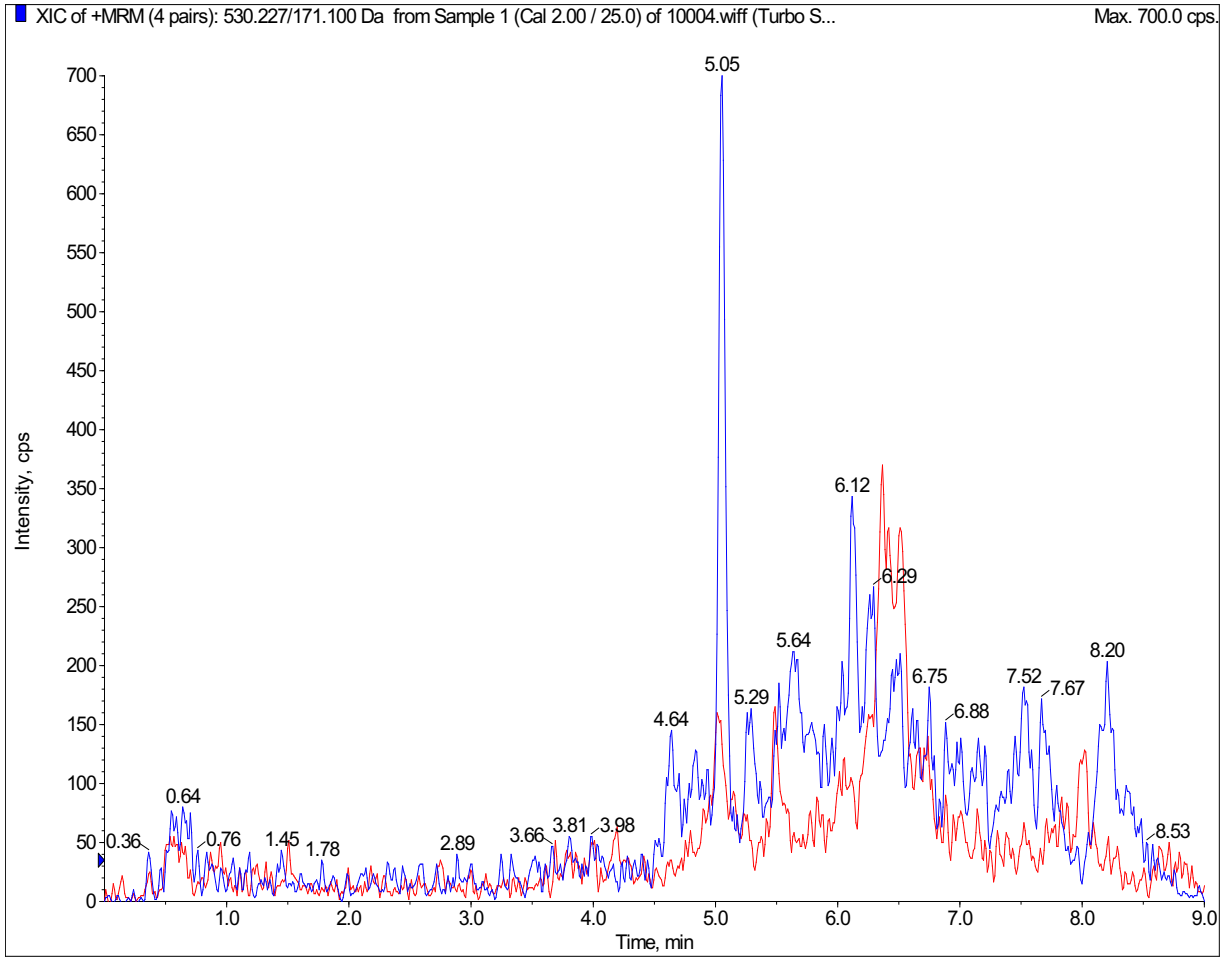
Estradiol levels were between 1 and 5 pg/mL

17β-estradiol assay



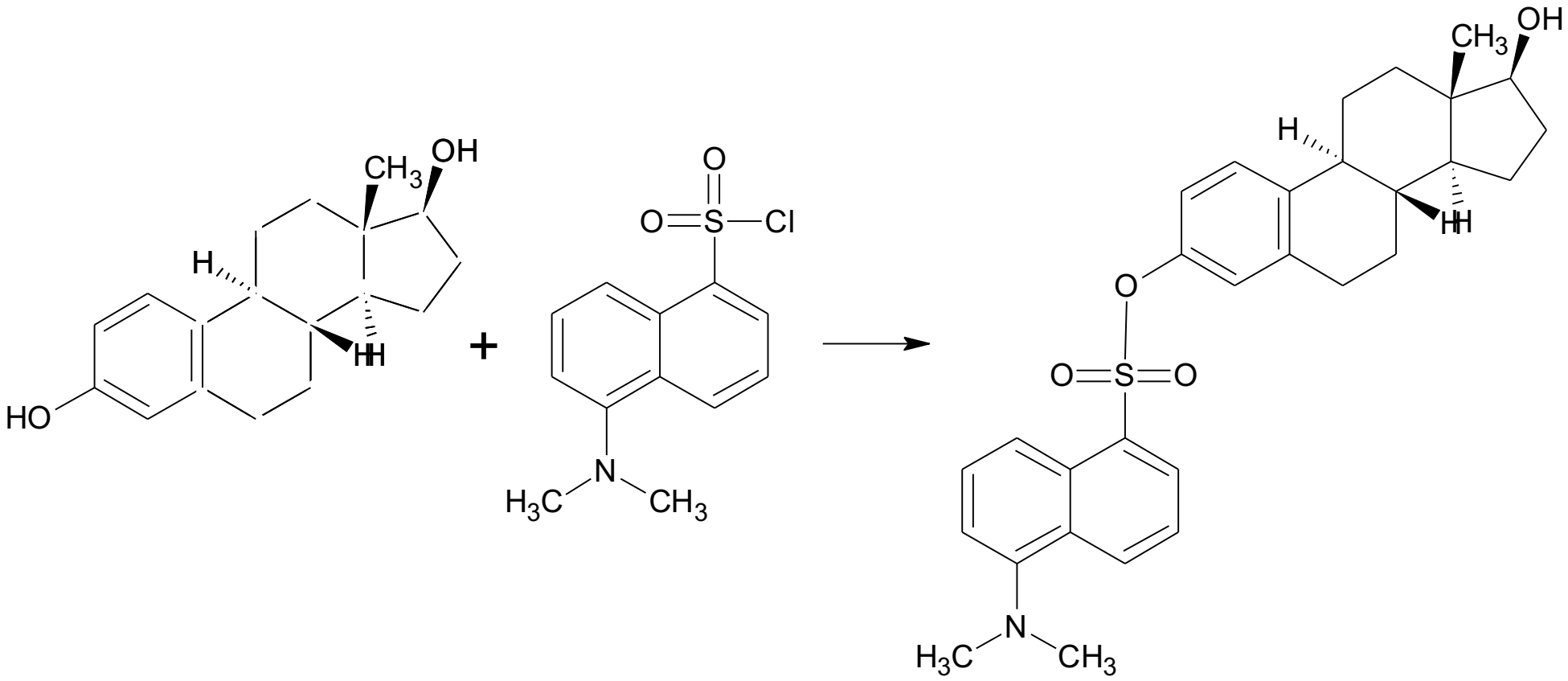
Blank plasma sample from pooled charcoal stripped plasma from menopausal women

Ethinylestradiol assay



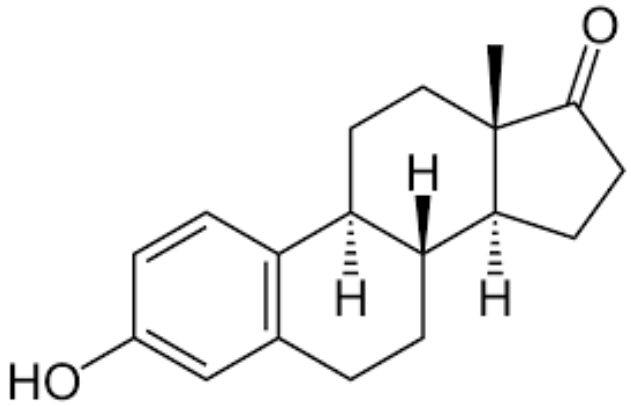
SRM trace of a blank sample (red line) and a LLOQ sample (blue line) at 2 pg/mL (calibration range 2 - 300 pg/mL)
Column: Waters XSelect® HSS T3 3.5 µm; 2.1 x 100 mm
Flow rate: 550 µL/min
Injection volume: 20 µL

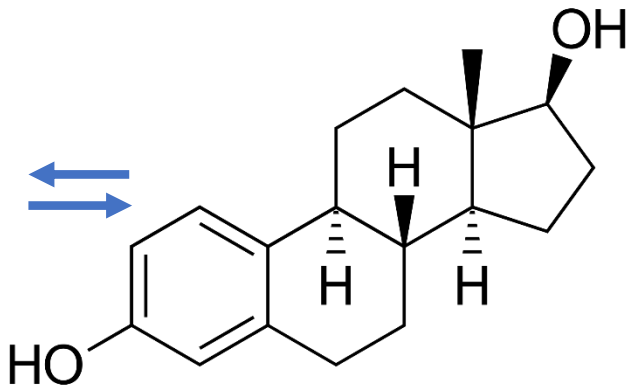
Estradiol derivatization



Mass: 272.4 g/mol Mass: 269.8 g/mol Mass: 505.8 g/mol

17β-estradiol metabolism

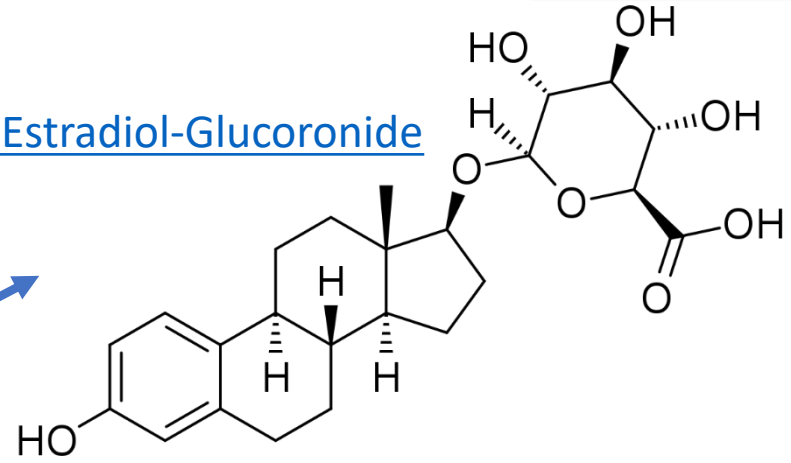




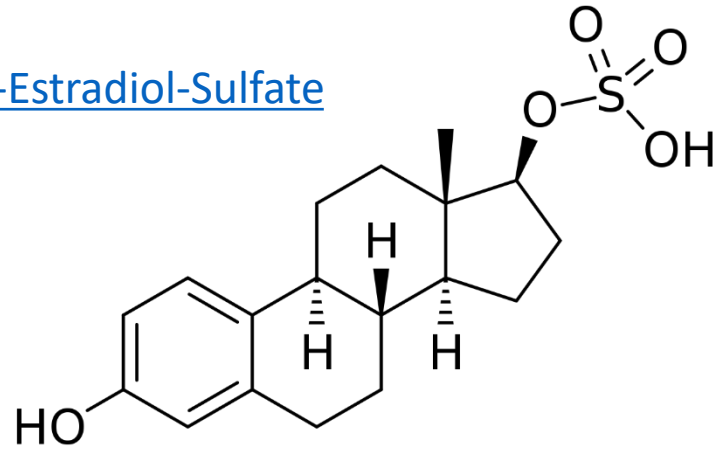
[Estrone](#)
Formula: $C_{18}H_{22}O_2$
Molar mass: 270.366 g/mol

[17β-Estradiol](#)
Formula: $C_{18}H_{24}O_2$
Molar mass: 272.38 g/mol

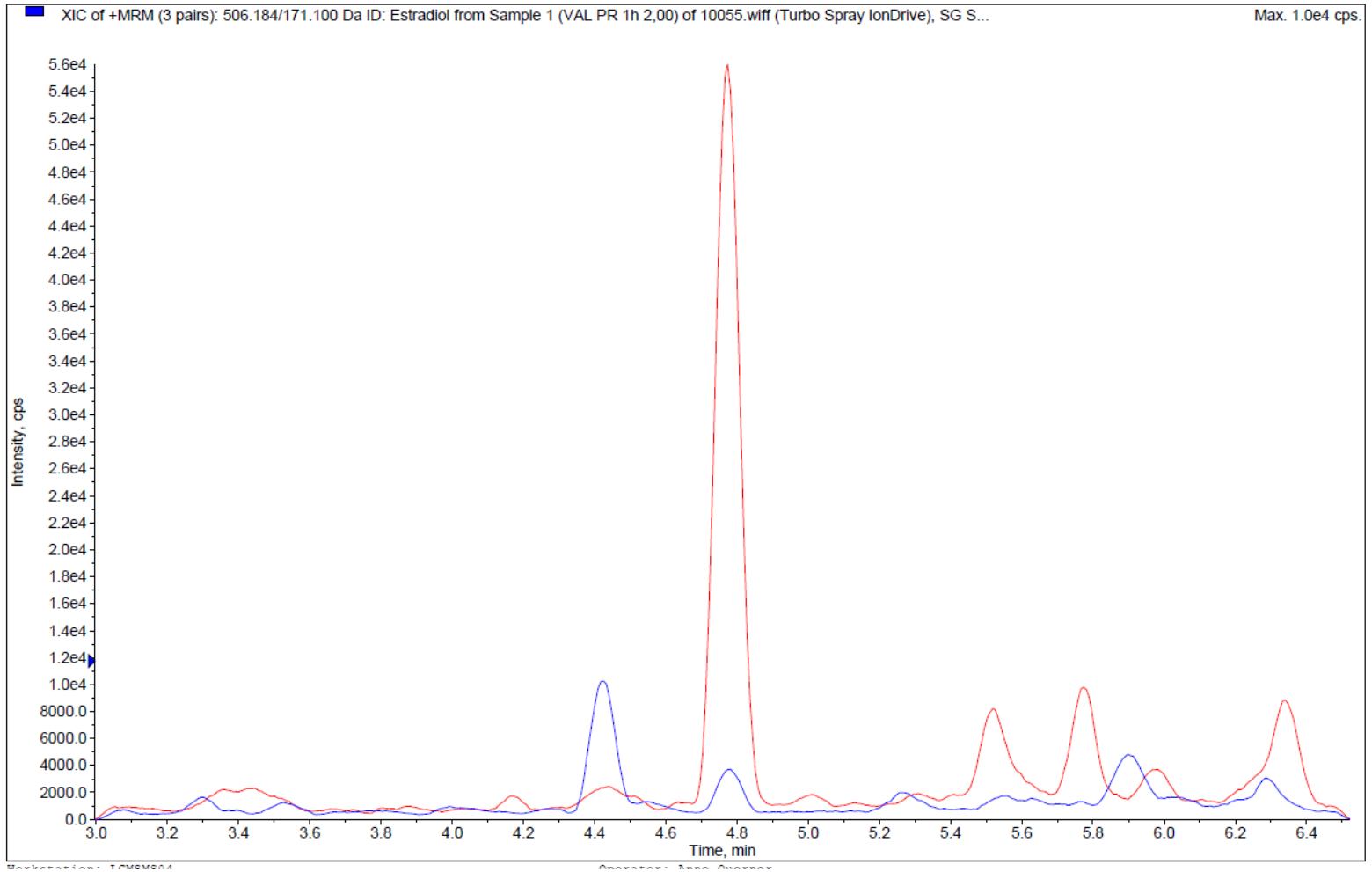
[17β-Estradiol-Glucoronide](#)



[17β-Estradiol-Sulfate](#)



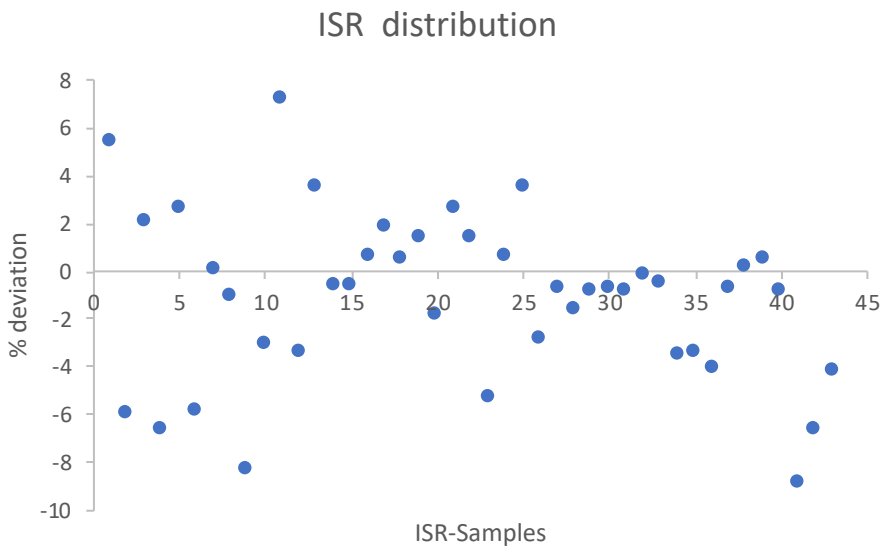
Results

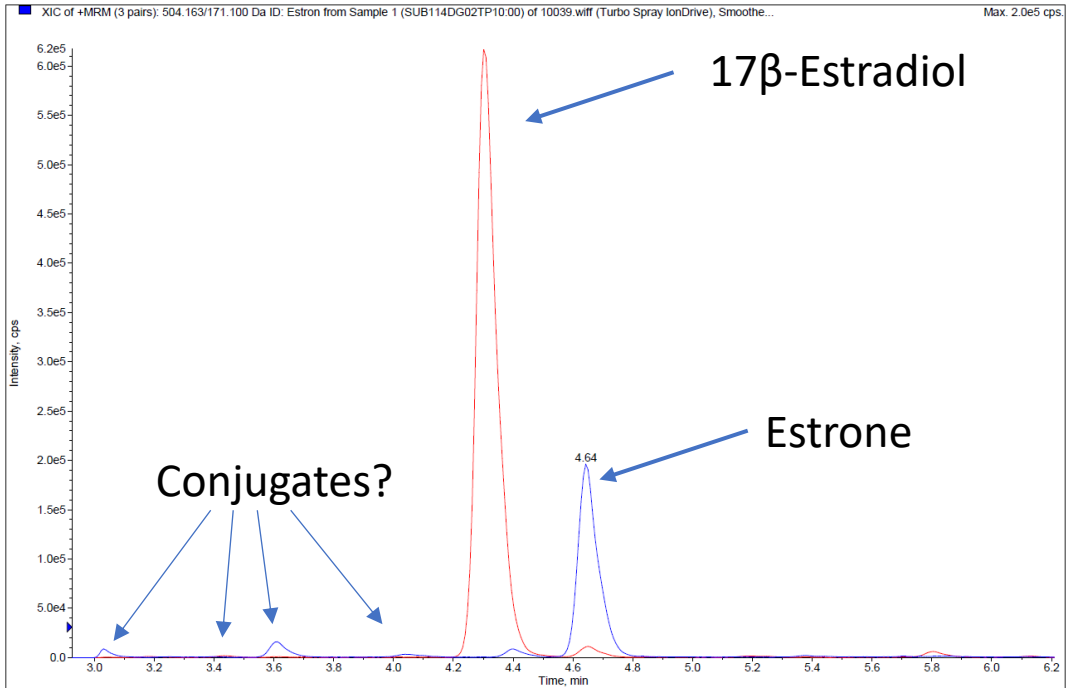


Example of a female plasma sample containing 17β-estradiol (blue trace at 4.43 min) and estrone (red trace at 4.78 min)

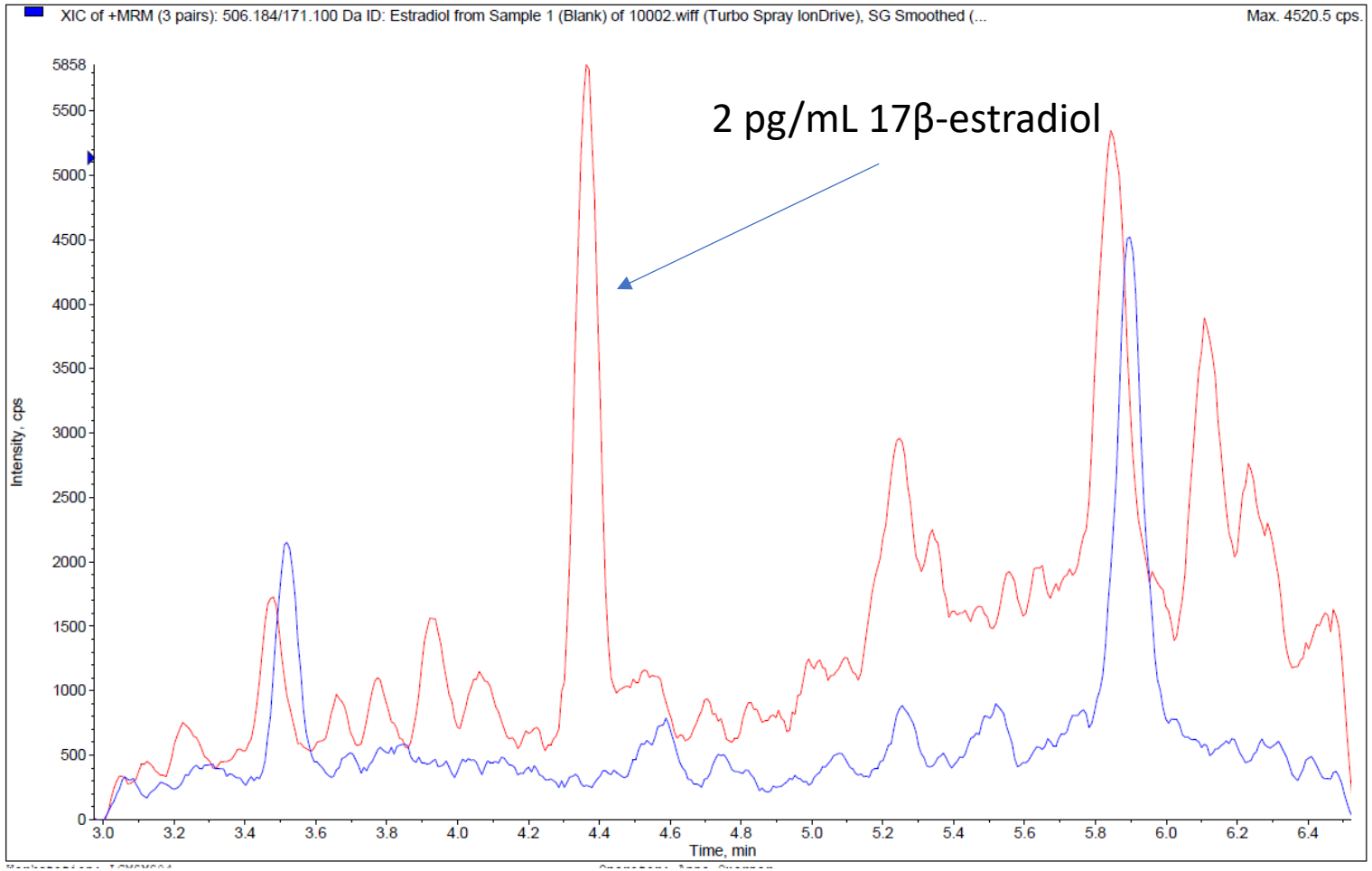
Results

Selectivity against phase II metabolites (conjugates) was achieved by liquid-liquid extraction with hexan and was demonstrated by excellent ISR data (ISR success rate 100%).





Results



Example of a blank surrogate sample (blue trace) and
Cal1 at 2 pg/mL (red trace)

Column: Waters XSelect® HSS T3 3.5 µm; 2.1 x 100 mm
Flow rate: 550 µL/min
Injection volume: 10 µL

MRM transitions

β-Estradiol:	506.2 → 171.1
Estradiol-d5:	511.2 → 156.0
Estron:	504.2 → 171.1

Results

- Validation of the assay was straight, all numbers for precision and inaccuracy including LLOQ were in the single digit range.
- LLOQ A/P: 101.5%/8.0%
- QC levels at 2.00 pg/mL (in surrogate), 3.92 pg/mL, 7.92 pg/mL, 91.92 pg/mL and 226.92 pg/mL
- Inter-assay A/P: 97.9-101.5%/1.7-8.0 %
- Recovery: 84.2-94.1%
- Selectivity: all matrix lots contain estradiol and estrone
- Dilution integrity: concentration diluted 10-fold, A/P 104.8%/1.2 %
- Parallelism: 318.47 pg/mL sample was 5x 1/1 diluted with surrogate matrix, A/P 99.5-100.3%/0.2-3.1%
- Matrix factor: in menopausal plasma (at Low QC level CV of 48.5%)
- Matrix factor: CV of IS for all QCs 2.0-2.8%, normalised MF at MQC 7.5%, HQC 2.7%
- All figures for stability fulfilled the required criteria with no need for extra stabilizers.

Conclusions

- Ultra-sensitve LC-MSMS assay for 17β-Estradiol could be developed and validated.
- Selectivity of the assay against estrone and 17-conjugates could be demonstrated.
- Some experiments like the matrix effect are not conclusive for a an endogenous compound.
- Parallelism experiment demonstrated that the calibration curve in surrogate reflects the concentration-response-relationship of study samples very well.
- Double extraction and the use of a stable isotope internal standard provided the needed robustness of the assay.

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Many thanks for your attention!