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Quantification of steroids in in-vitro and in-vivo assays with on-line SPE-LC-MS/MS

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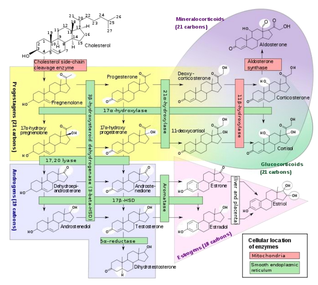
As part of testing drugs for their endocrine disrupting effect the OECD approved in-vitro test, H295R steroidogenesis assay, is used to assess test compounds potential to affect steroid hormone synthesis. For measuring the steroids in the final cell extracts a LC-MS/MS is developed using on-line solid phase extraction for detection of androgens, corticosteroids, estrogens and gestagens in cell extracts.
The cell extract is centrifugated at high speed (12000 g) and 100 µL is injected on the LC-MS/MS with a dual-gradient pump. The steroids are retained on a short HLB enrichment column and eluted on an analytical column with a gradient with ammoniumfluoride (0,2 mM) and methanol (estrogens) or formic acid (0,1%) and acetonitrile (androgens, gestagens and corticosteroids). The method is also developed for detection of the steroids in plasma and gonad tissue. The method is validated for 15 steroids and the limit of detection is below 100 ppt for most steroids and 10 ppt for the estrogens.



Materials and method

Extraction

100 µL of Sample
300 µL ACN/2% FAc
IS (0.3 ng)

-20 °C, 20 min.

10000G, 7 min, 4 °C

Clean-up

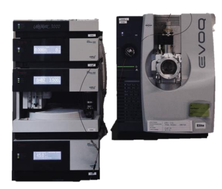
50 mg Zsep

5000G, 10 min, 4 °C

50 °C, N_2

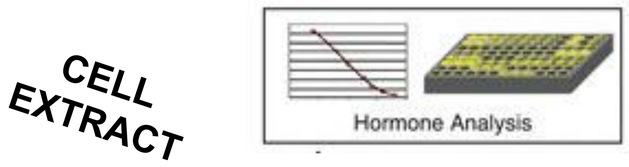
Analysis

300 µL 10% ACN



LC-MS/MS

Bruker EVOQ Elite MS/MS
Thermo Ulltimate 3000 HPLC with DGP-3600RS dual-gradient pump



CELL EXTRACT

700 µL of Sample
+ IS (2 ng)

12000G, 10 min, 4 °C

On-line SPE

On-line SPE-LC

LC with dual gradient pump.

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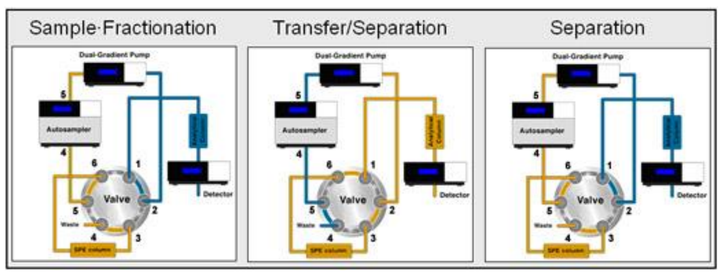


0-1 min.: Sample on SPE column

1-3 min.: Analytes from SPE column to analytical column

3-11 min.: Separation of analytes on analytical column

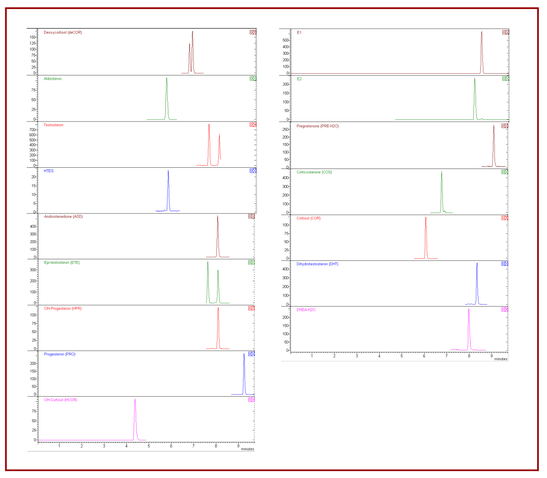
Pump A:
A: Acetonitril
B: 0.1% Formic acid
Flowrate: 0.25 ml/min

Time	%A
0	10
0.5	25
8	90
11	90
11.5	10
16	10

Pump B:
A: Methanol
B: 0,1% Formic acid
C: Methanol/Acetonitril (1/1)
Flowrate: 0.1-1.0 ml/min

Time	%A	%C	Flow
0	10	0	1
1	10	0	1
1.1	10	0	0.1
3.5	10	0	0.1
4	0	100	1
6	0	100	1
7	10	0	1
16	10	0	1

Enrichment column: HLB (100 mm*1,8; 15 µm)
Analytical column: Column: C8 (100 mm*1.8; 2.7 µm)



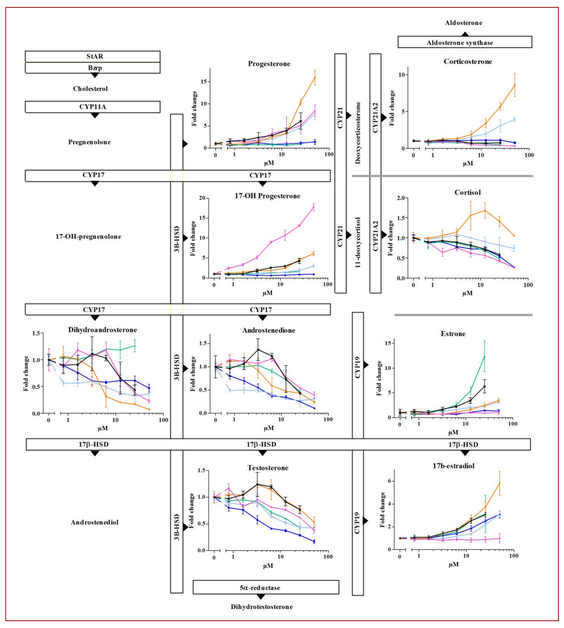
Results

Comparison with ELISA

Comparable results for Testosteron and β-Estradiol using ELISA and on-line SPE-LC-MS/MS with the Perchlorat (inhibitor) and Forskolin (inducer) assay.

Hormone profiles for Bisphenol

BPA (dark blue), BPB (green), BPE (light blue), BPF (orange), 658 BPS (pink), HPP (black) from the H295R steroidogenesis assay. The six test compounds generally led to increased progestagen and estrogen levels, and decreased androgen levels in the H295R assay



Conclusion

- On-line SPE for large volume injection (100 µL) and purification established
- Used for quantification of steroids in the H295R in vitro assay and in vivo samples (plasma and testes)
- Limit of detection, for in-vitro samples, below 100 ppt for most steroids and below 10 ppt for estrogens
- Comparable results with ELISA by analysing QC-samples and steroidprofiles demonstrated after adding EDC's to the cell assay