**Steroid Profile v.140528 SCBrown**

**Service Code: Steroid-D4**

**Summary:** Profile up to 15 -4 steroids by LLE of blood plasma, serum, or tissue samples. Target steroid analytes are chromatographically separated on a 2.1mm x50mm Biphenyl column in a 20 min cycle. All analytes and Internal Standards are measured by ESI ionization--pos and/or neg polarity (analyte dependent) on a UHPLC-QQQ mass spectrometer using MRM methods and reported as total pG/mL (biofluids) or pM (tissues--normalized to wet tissue weight). CV's are generally 10%.

Container: cryovial

Normal Volume: 500 uL

Minimal Volume: 50uL

Special Handling: Dry ice; store @ -800 C

Sample Collection:

Reference:

**Table I: Analytes reported. Others on special request:**

|  |  |  |  |
| --- | --- | --- | --- |
| **Analyte** | **Abbr.** | **MRM** | **LOQ**  **pG/ml; pG (OC)** |
| Aldosterone | Aldo | 359>189 | 40; 0.8 |
| Androsterone | AN | 291.2>255 | 50; 1.0 |
| Androstenedione | A4 | 287.2>97.0 | 5; 0.1 |
| Corticosterone | B | 347.2>121.0 | 50; 1.0 |
| Cortisol | F | 363.2>121.0 | 100; 2.0 |
| Cortisone | E | 361.2>163.0 | 125; 2.5 |
| 11-deoxycortisol | S | 347.2>97.0 | 50; 1.0 |
| 11-deoxycorticosterone | 11-DOC | 331.2>97.0 | 18; 0.35 |
| Estradiol | E2 | 271.2>145.1 | 10; 0.2 |
| Estrone | E1 | 269.2>145.1 | 10; 0.2 |
| 17-Hydroxyprogesterone | 17-OHP4 | 331.2>97.0 | 13; 0.25 |
| Progesterone | P4 | 315.2>97.0 | 5; 0.1 |
| Testosterone | T | 289.2>97.0 | 5; 0.1 |
| Dehydroepiandrosterone sulfate | DHEAS | 367.2>97.0 | 500; 10 |

**Table II: Internal standards and corresponding analytes**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Internal Standards** | **Source** | **Cat#** | **Analytes quantified** |  |
| Cortisol-d4 | C/D/N Isotopes | D-5280 | Cortisol; |  |
| DHEAS-d6 | Sigma-Aldrich | 723266-1mg | DHEAS |  |
| Cortisone-d7 | Sigma-Aldrich | 705586-5mg | Cortisone; Aldosterone |  |
| Corticosterone-d8 | C/D/N Isotopes | D-5822 | Corticosterone |  |
| Estradiol-d4 | C/D/N Isotopes | D-4318 | Estradiol |  |
| 11-Deoxycortisol-d5 | Sigma-Aldrich | 710784-5mg | 11-Deoxycortisol |  |
| 11-DOC-d8 | C/D/N Isotope | D-5732 | 11-DOC |  |
| 17-OH-progesterone-d8 | C/D/N Isotopes | D-5650 | 17-OH Progesterone |  |
| Progesterone-d9 | C/D/N Isotopes | D-5385 | Progesterone |  |
| Testosterone-d3 | C/D/N Isotopes | D-3793 | Testosterone; Androstenedione |  |
| Estrone-d4 | C/D/N Isotopes | D-3650 | Estrone |  |

**Materials**

1. Steroid authentic standards and stable-isotope labeled internal standards (see Table II)
2. LC/MS grade methanol, water
3. ACS grade MTBE, acetonitrile, chloroform, 2-butanol, ammonium fluoride, ammonium sulfate
4. N2 drying/heating block
5. Benchtop Centrifuge
6. Vortex mixer
7. Agilent 6490 triple quad mass spectrometer
8. Agilent 1290 LC System

**PROCEDURES:**

**Plasma/Serum Sample Preparation (w/o DHEAS)**

1. Aliquot 100 ul DI H2O & 100 ul serum/plasma sample to labeled 1.5 mL eppendorf centrifuge tubes;
2. Add 150 ul methanol (MeOH) to each centrifuge tube, then 20 ul of internal standard mixture and 225 ul acetonitrile (ACN), in that order;
3. Mix for 5 min on shaker, incubate for 5 min, Mix again for 5 min;
4. Centrifuge @ 15,000 rpm and 4 0C for 5 min., transfer supernatant to a labeled 2 mL autosampler vial;
5. Add 1.0 mL MTBE and 300 ul DI H2O, Mix for 10 min., incubate for 5 min., vortex again for 5 min.;
6. Allow to stand to facilitate phase separation, transfer MTBE to a new, labeled, 2 mL autosampler vial, dry under UHP N2 @ 37 0C;
7. Reconstitute sample in 200 ul 50:50 (v/v) MeOH/DI H2O, vortex mix for 15s and transfer to 350 ul autosampler vial insert.

**LIPID EXTRACTION PROTOCOL - Tissues**

1. Start with 5-20 mg of tissue samples or cell pellets harvested from 1x106 – 3x106 cells at around confluence (2 such dishes) (modify adding proportional amounts of reagents for smaller or larger sample size of the starting material).
2. Make up volume up to 0.6 ml with water for plasma and disperse in 0.6 ml of water for tissues or Cell pellets
3. Add 2.25 ml of methanol – chloroform mixture (2:1, v/v) containing 0.01 % of BHT
4. Add 10 l of 4mM heptadecanoic (C17:0) acid (internal standard)
5. Vortex well for plasma samples and homogenize with polytron homogenizer for cell pellets and tissues. Follow the same protocol for all the samples for rest of the procedure
6. Add 0.75 ml more of chloroform and 0.75 ml of 0.9 % NaCl
7. Vortex well
8. Centrifuge in table top centrifuge (at ~ 3000 rpm) for 5-6 min
9. Remove the upper layer
10. Transfer the lower layer into a separate tube
11. Dry under nitrogen and go for the next step of methyl ester derivative preparation or, if not ready, then
12. Re-dissolve in about 200 l of chloroform
13. Save under nitrogen until ready for the next step

**Additional Extraction for DHEAS**

After performing MTBE extraction of serum/plasma sample perform the following extraction on the aqueous phase:

1. Add 300 ul of 1 M Ammonium sulfate;
2. Add 1.0 ml of a 50:50 (v/v) Chloroform/2-Butanol mixture to the vial containing the aqueous phase, Mix for 10 min, incubate for 5 min, Mix again for 5 min.;
3. Allow vial to stand for phase separation, then transfer the organic layer to a 2 mL autosampler vial
4. Dry under UHP N2.
5. Reconstitute sample in 50:50 (v/v) MeOH/DI H2O as in MTBE procedure;
6. Vortex mix for 15s then transfer to 350 ul vial inserts.

**LCMS Calibration**

Working calibration standards are prepared from a mixed stock standard solution prepared in 50:50 (v/v) MeOH/DI H2O. Stock standards are prepared fresh on a monthly basis. Twelve calibration standard concentration levels are prepared—To either cover the working range of the mass spectrometer (i.e. cortisol, DHEAS), or to cover the expected concentration range of a particular analyte in actual samples (i.e. estradiol, 17-OH-Progesterone). An internal standard stock solution is prepared with labeled standards and included in all calibration standards, QC standards, blanks and analytical samples at the following concentrations:

Internal Standard Stock Standard Conc. (pG/mL)

Cortisol-D4 5000

DHEAS-D6 10000

Cortisone-D7 1500

Corticosterone-D5 2500

Estradiol-D4 200

Testosterone-d3 2500

All other D-steroids 500

**LC-MS procedure**

1. LC column: Restek 2.1mm x 50mm 1.9um Pinnacle DB Biphenyl; at 50 °C.
2. Mobile phase A: 0.25 mM ammonium fluoride in water
3. Mobile phase B: 0.25 mM ammonium fluoride in methanol
4. Gradient: 0min, 30%B, 1.5min, 20%B, 3.5min, 60%B, 9.0min, 80%B, 12.0min, 98%B, 15.5min, 98%B, 15.6min, 15%B, 16.25min, 30%B, 17.0min, 30%B; flow rate: 200ul/min
5. Autosampler: 4°C, 10 uL injection
6. Agilent 6490 QQQ: ESI+/-, Method: **XXX** or equivalent
7. Collect standard curve data first, then sample data if system is suitable. Analytes are quantitated by isotope ratio per Table II.

**Table III: MRM transitions monitored:**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Analyte** | **MRM 1** | **MRM 2** | **Polarity** | **Quant or Qual?** |
| Cortisol | 363.2>121 | 363.2>327.2 | + | Quant; Qual |
| Cortisol-d4 | 367.2>121.1 | 367.2>121.1 | + | Quant |
| Cortisone | 361>163 | 361>163 | + | Quant |
| Cortisone-d7 | 368>163 | 368>163 | + | Quant |
| Corticosterone | 347.2>121 | 347.2>311.2 | + | Quant; Qual |
| Corticosterone-d5 | 352.2>121 | 352.2>121 | + | Quant |
| 11-Deoxycortisol | 347.2>97 | 347.2>109.1 | + | Quant; Qual |
| 11-Deoxycortisol-d5 | 352.2>113 | 352.2>100.1 | + | Quant |
| 11-DOC | 331.2>97 | 331.2>109.1 | + | Quant; Qual |
| 11-DOC-d8 | 339.1>100.1 | 339.1>112.9 | + | Quant |
| 17-OH Progesterone | 331.2>97 | 331.2>109.1 | + | Quant; Qual |
| 17-OH-Progesterone-d8 | 339.2>113 | 339.2>113 | + | Quant |
| Progesterone | 315.2>97 | 315.2>109.1 | + | Quant; Qual |
| Progesterone-d9 | 324.2>100.1 | 324.2>113.1 | + | Quant |
| Dihydrotestosterone | 291>255 | 291>255 | + | Quant |
| Testosterone | 289.2>97 | 289.2>109.1 | + | Quant; Qual |
| Testosterone-d3 | 292>97 | 292>97 | + | Quant |
| Androstenedione | 287>97 | 287>109.1 | + | Quant; Qual |
| Aldosterone | 361.2>343.2 | 361.2>343.2 | + | Qual |
| Aldosterone | 359>189 | 359>189 | - | Quant |
| Estradiol | 271.2>145.1 | 271.2>183.1 | - | Quant; Qual |
| Estradiol-d4 | 275.2>147 | 275.2>187.1 | - | Quant |
| Estrone | 269>145.1 | 269>183.1 | - | Quant; Qual |
| DHEAS | 367.2>97 | 367.2>97 | - | Quant |
| DHEAS-d6 | 373.1>97.9 | 373.1>97.9 | - | Quant |
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