Supplementary material

Chemicals and Reagents

Testosterone, progesterone, 3α-hydroxy-5α-pregnan-20-one (allopregnanolone, AP), allopregnanolone-2,2,3,4,4-D_5 (AP-D_5, deuterated internal standard), Amplifex^{TM}-Keto Reagent Kit and human albumin (≥ 96%) as blank matrix were supplied from Sigma-Fluka (St. Louis, MO, USA). Acetonitrile, methanol, formic acid and ammonium formate were of LC-MS purity grade (Sigma-Fluka, St. Louis, MO, USA); the ultra-pure water was purified by a Milli-Q Plus185 system from Millipore (Milford, MA, USA). Phree Phospholipid Removal Tubes (1.0 mL) were supplied by Phenomenex (Torrance, CA, USA).

Sample processing

Aliquots (200 µL) of the obtained serum samples were spiked with 50 µL of the internal standard solution, vortexed (90 sec) and added with 1.0 mL of acetonitrile/methanol (70/30; + 1.0% formic acid) for protein precipitation. The samples were then sonicated (10 min, 4°C), centrifuged (15.000 rpm, 10 min, 4°C) and the supernatants were purified by Phree-SPE cartridges to remove endogenous phospholipids. The obtained eluates were evaporated to dryness by a Mod. 5305 Concentrator Plus (Eppendorf AG, Hamburg, Germany) and subsequently derivatized with 50 µL of Amplifex Keto Reagent. After one hour at room temperature and in the dark, the samples were added with 50 µL methanol/water (70/30), centrifuged (15.000 rpm, 10 min, 10°C) and transferred in autosampler vials for LC-MS/MS analysis.

Working solutions

A stock solution was serially diluted with methanol to obtain 8 working solutions in the range 16 ± 2000 pg/mL for the target analytes. The internal standard solution (IS) was prepared by diluting a stock solution of AP-D_5 with methanol up to the concentration of 1000 pg/mL. All solutions were stored at –20°C until use.

LC-MS/MS conditions

The HPLC system consisted of an Agilent 1200 Series Binary Pump equipped with a vacuum degasser, an autosampler and a thermostatted column compartment (Agilent, Waldbronn, Germany). The analytical column was a Kinetex XB-C18 column (100 x 2.1 mm; 2.6 µm particle sizex) preceded by a UHPLC C18 SecurityGuard cartridge (2.1 mm) (Phenomenex, Torrance, CA, USA), set at 40°C.

The mobile phase was (A) water/acetonitrile 90/10 (v/v; + 3 mM ammonium formate, + 0.1% formic acid) and (B) acetonitrile/water 90/10 (v/v; + 3 mM ammonium formate, 0.1% formic acid) under the following gradient: 0 to 1.5 min, isocratic at 20% (B); 1.5 to 2 min, linear gradient from 20 to 25% (B); 2 to 11 min, linear gradient from 25 to 45% (B); 11 to 14 min, isocratic at 45% (B), 14 to 15 min, linear gradient from 45 to 90% (B), 15 to 25 min, isocratic at 90% (B), 25 to 26 min, linear gradient from 90 to 20% (B). The flow-rate was 0.4 mL/min and a pre-equilibration period of 10 min was used between each run. The injection volume was 10 µL and the autosampler was maintained at 4 °C.

Mass spectrometric detection was performed in ESI positive mode using an Agilent QQQ-MS/MS (6410B) triple quadrupole mass analyzer (Agilent, Waldbronn, Germany). The ESI ion source parameters were as follows: capillary voltage, 3000 V; nebulizer (N_2) pressure, 30 psi; drying gas (N_2) temperature, 350°C; drying gas flow, 10 L/min; electron multiplier voltage, 700. Data were acquired in multiple reaction monitoring (MRM) mode using high purity nitrogen (15 psi) as collision gas (15 psi) and a dwell time of 30 msec. Instrument control, data acquisition, qualitative and quantitative data analysis were performed by MassHunter workstation software, version B.05.00 (Agilent, Waldbronn, Germany).

Neat solutions (50,000 pg/mL) of each analyte and AP-D_5 were derivatized with Amplifex Keto Reagent and infused directly into the mass spectrometer at a flow of 10 µL/min to optimize MS/MS acquisition parameters. For each compound, three MRM transitions (one parent and three product

ions) were monitored; the effectiveness of the selected MRM transitions was validated by LC-MS/MS analyses of blank matrix samples processed as described and spiked with the derivatized analytes and the deuterated IS (50,000 pg/mL).

Calibration samples

Aliquots (200 μL) of blank human albumin were spiked with 50 μL of the IS solution and 50 μL of the working solutions and then subjected to the sample processing described above. The concentration range for the target analytes in the obtained calibration samples (n=8) was 8 ± 1,000 pg/mL and the concentration of AP-D_5 was 500 pg/mL.

Each calibration sample was analyzed in triplicate on three separate days. The most abundant MRM transition (quantifier) was selected for quantification and the less abundant as qualifier transitions for confirmation.

Calibration curves were calculated from the peak-area ratio of each analyte quantifier transition to the deuterated IS; the ratio was then plotted on the y-axis against the nominal analyte concentration to generate the standard curves by the method of least squares using a weighed (1/x) linear regression model.

For progesterone, which presents two keto groups in the moiety at both C3 and C20, the derivatisation procedure led to two derivatives (cis/trans isomers) and the sum of these peak areas was used to for quantification.

The sensitivity of the procedure was evaluated by calculating the limit of quantitation (LOQ) for the target analytes from their corresponding calibration plots as $10\sigma/S$, where σ and S are the standard deviation and the slope of the regression line. The found LOQ values (6 pg/mL for allopregnanolone and 4 pg/mL for progesterone and testosterone) were then validated by analyzing blank serum samples fortified with analytes at the calculated LOQ levels.