Steroid hormones are derived from cholesterol and perform a number of important physiological functions. The steroids are synthesized mainly by endocrine glands – such as the gonads, adrenals and placental – and are then circulated through the blood stream. The main role of steroid hormones is to coordinate physiological and behavioral responses.

Liquid chromatography triple quadrupole mass spectrometry (LC-MS/MS) has become an essential clinical research tool for analysis of endogenous steroids because of its ability to simultaneously analyze multiple analytes with high sensitivity, and excellent specificity and reproducibility.

In this study, thirteen major steroids were quantified. A robust, sensitive, reliable and fast method is presented for the quantification of the major endogenous steroids in human serum using LC-MS/MS in both positive and negative ionization modes in a single run. This quantitative method demonstrates a wide dynamic range, excellent linearity, accuracy and reproducibility.

**Experimental**

**Sample Preparation:**
- Thirteen steroid standards and four isotopic labeled internal standards are listed in Table 1.
- Serum sample preparation: 250 mg human serum was obtained from UKF Laboratories, Inc. It was spiked with 500 ng/mL acetonitrile, vortexed for 1 minute and centrifuged for 4 min at 10,000 rpm. 500 µL supernatant was transferred and diluted with 500 µL of water. 2 µL is injected onto LC-MS/MS.

**LC Method:**
- Agilent 1200 Infinity UHPLC series binary pump, well plate sampler, thermostatted column compartment
- Column: Extend C18, 1.8µm 1.8 mm, 60 bar
- Column temperature: 50 ºC
- Injection volume: 2 µL
- Autosampler temp: 4 ºC

**MS Method:**
- Agilent 6460 triple quadrupole mass spectrometer ion mode: Agilent Jet Stream pos/neg
- Gas temperature: 350 ºC
- Drying gas (nitrogen): 35 psi
- Sheath gas (nitrogen): 350ºC
- Sheath flow: 11 L/min
- Capillary voltage: +3000V/3000V
- Nozzle voltage: +200/-200V
- Q1/Q2 Resolution: 1.2/0.7 unit
- Switching dwell time: 48 msec
- Delta EMK: +200/-200V

**Results and Discussion**

**Quantitative Analysis of Steroids in Blood by Positive and Negative Ionization using QQQ LC-MS/MS**

Yinan Yang and Andrej Szczesiakiewski, Agilent Technologies Inc.

Thirteen steroid standards and four isotopic labeled internal standards are listed in Table 1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ion Mode</th>
<th>RT (min)</th>
<th>MRM</th>
<th>Dwell (usec)</th>
<th>Frag (V)</th>
<th>CE (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androstenedione</td>
<td>B+</td>
<td>3.17</td>
<td>249.0/171.0</td>
<td>185</td>
<td>100</td>
<td>47.40</td>
</tr>
<tr>
<td>Estradiol</td>
<td>B+</td>
<td>3.33</td>
<td>263.0/151.0</td>
<td>192</td>
<td>100</td>
<td>62.78</td>
</tr>
<tr>
<td>17α-Oestradiol</td>
<td>B+</td>
<td>3.68</td>
<td>263.0/151.0</td>
<td>192</td>
<td>100</td>
<td>62.12</td>
</tr>
<tr>
<td>Androsterone</td>
<td>B+</td>
<td>3.68</td>
<td>263.0/151.0</td>
<td>192</td>
<td>100</td>
<td>62.12</td>
</tr>
<tr>
<td>Progesterone</td>
<td>B+</td>
<td>4.95</td>
<td>247.0/171.0</td>
<td>185</td>
<td>100</td>
<td>52.27</td>
</tr>
<tr>
<td>Testosterone</td>
<td>B+</td>
<td>5.60</td>
<td>305.0/105.0</td>
<td>192</td>
<td>100</td>
<td>72.95</td>
</tr>
<tr>
<td>Dehydroepiandrosterone (DHEA)</td>
<td>B+</td>
<td>6.97</td>
<td>277.0/141.0</td>
<td>185</td>
<td>100</td>
<td>57.95</td>
</tr>
<tr>
<td>Dehydroepiandrosterone sulfonate (DHEAS)</td>
<td>B+</td>
<td>6.97</td>
<td>277.0/141.0</td>
<td>185</td>
<td>100</td>
<td>57.95</td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>B+</td>
<td>6.97</td>
<td>277.0/141.0</td>
<td>185</td>
<td>100</td>
<td>57.95</td>
</tr>
<tr>
<td>Testosterone-d3</td>
<td>B+</td>
<td>8.42</td>
<td>271.2/253.2</td>
<td>192</td>
<td>100</td>
<td>75.10</td>
</tr>
<tr>
<td>Estradiol-d5</td>
<td>B+</td>
<td>10.87</td>
<td>276.2/171.0</td>
<td>185</td>
<td>100</td>
<td>57.95</td>
</tr>
<tr>
<td>DHEA</td>
<td>B+</td>
<td>14.31</td>
<td>412.5/141.0</td>
<td>185</td>
<td>100</td>
<td>57.95</td>
</tr>
<tr>
<td>DHEAS</td>
<td>B+</td>
<td>14.31</td>
<td>412.5/141.0</td>
<td>185</td>
<td>100</td>
<td>57.95</td>
</tr>
</tbody>
</table>

**Method C**: The method was validated using the following parameters:
- **Sensitivity**: Excellent signal levels were observed for most analytes.
- **Reproducibility**: Intra-day and inter-day reproducibility were excellent with RSD values ranging from 0.8 to 1.2% for most analytes.
- **Linearity**: Excellent linearity was observed with correlation coefficients (R²) ranging from 0.999 to 1.000 for all analytes.
- **Accuracy**: The accuracy was determined from the calibration curves and was found to be within ±10% for all analytes.
- **Precision**: The precision was determined from replicate injections of the same sample and was found to be within ±5% for all analytes.

**Conclusion**

- **Baseline separation of thirteen steroids with the exception of estradiol is achieved under 8.5 minutes. However, estradiol is not isobaric to androsterone or estrone, so the quantitation calculation is not possible.**
- **The calibration curves show excellent linearity (> 0.995) with greater than three orders of dynamic range.**
- **Great accuracy, precision, reproducibility, and signal stability of LC-MS/MS (QQQ) analyses were observed.**
- **Eight steroids were detected in pooled human serum after being crashed with acetonitrile and diluted with water (Figure 4). The detected hormone levels are listed in Table 2. The simple sample preparation procedure used is a 6 factor dilution from the original sample. Estradiol, estron and DHEA, which have lower normal levels, are not detected at very low levels. Increasing injection volume and using an enrichment column or drying down the extracts are alternatives that would improve detection limits. In a future study, a double channel stripped serum will be used.**