Integration of steroids analysis in serum using LC-MS/MS with full-automated sample preparation

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Introduction
Currently sample preparation for the detection of steroids in serum by liquid chromatography-mass spectrometry (LC-MS/MS) involves complex offline extraction methods such as solid phase extraction or liquid/liquid extraction, all of which require additional sample concentration and reconstitution in an appropriate solvent. These sample preparation methods are time-consuming, often taking 1 hour or more per sample, and are more vulnerable to variability due to errors in manual preparation. Our approach to offering a high sensitivity steroid detection method and timely, automated analysis of multiple samples is to use the automated sample preparation system coupled to the detection capabilities of a high-sensitivity triple stage quadrupole mass spectrometer.

Materials and Methods
10 steroid hormones (cortisol, aldosterone, 11-deoxycortisol, corticosterone, 17-OHP, androstenedione, DHEA, DHEAS, progesterone and testosterone) in serum were verified using CHS™ MSMS Steroids Kit (PerkinElmer, USA). Serum sample was loaded directly into the automated sample preparation system (CLAM-2000 Shimadzu, Japan). The CLAM-2000 was programmed to perform protein precipitation using acetonitrile followed by filtration and sample collection. The sample is then transported using an arm from the CLAM-2000 to the HPLC without human intervention for LC-MS/MS analysis. The treated samples were trapped using a MAYI-ODS column and then separated by Core-Shell Biphenyl HPLC column at 40 °C with a binary gradient system at a flow rate of 0.3 ml/min in 12 min.
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**HPLC**

- Mobile Phase A: 1mM ammonium fluoride – water
- Mobile Phase B: Methanol
- Mobile Phase C: 10mM ammonium formate – water
- Column temperature: 40 °C
- Analytical Column: Kinetex Biphenyl (50mm L x 2mm I.D., 2.6µm)
- Guard Column: MAYI-ODS column (5mm L x 2mm I.D.)
- Gradient Program:

**Injection Volume**: 30 µL

**Mass (LCMS-8060 triple quadrupole mass spectrometry)**

- Ionization: heated ESI
- Nebulizing Gas Flow: 3 L / min
- Drying Gas Pressure: 7 L / min
- Heating gas flow: 13 L/min
- DL Temperature: 120 °C
- BH Temperature: 450 °C
- Interface Temperature: 370 °C
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Results

We evaluated this system using calibrator and control serum spiked with 10 steroids contained in the kit and carried out concurrent analysis over a range of concentrations for each steroid: cortisol (1.51-320 ng/mL), aldosterone (0.03-1.14 ng/mL), 11-deoxycortisol (0.08-18 ng/mL), corticosterone (0.29-62 ng/mL), 17-OHP (0.12-26 ng/mL), androstenedione (0.08-18 ng/mL), DHEA (0.31-65 ng/mL), DHEAS (12.9-2750 ng/mL), progesterone (0.12-26.5 ng/mL) and testosterone (0.03-7.2 ng/mL). The calibration curves that were generated had linear regression values of $r^2 > 0.997$ for each curve. The reproducibility (N=3) at seven concentrations, including LLOQ of each compounds was excellent (CV<10%). We found that the sample preparation time was reduced from 60 minutes to 6 minutes by the automated system. Thus sample preparation and LC/MS/MS analysis can be performed in parallel to accelerate throughput.
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![Calibration Curves](image)

Fig. 3  Calibration Curves (L1-L7) and MRM Chromatograms (L1) of 10 Steroids
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* Traditional sample preparation (protein precipitation)

60min

Add ACN with IS → Shake for 15 min → Centrifuge for 30 min → Transfer the supernatants → Dry for >30 min → Reconstitute

* Automated sample preparation process by CLAM-2000

6min

Add ACN with IS → Shake for 3 min → Filtrate for 2 min

Fig.4 Comparison with a time required for sample preparation

Fig.5 Analytical Flow with Parallel Processing
Conclusions

We completed steroid analysis using the automated sample preparation system coupled to LC-MS/MS. The results show the capability of the system for large sample set analyses with improved accuracy and precision by eliminating human error associated with manual sample handling.


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