TWO-DIMENSIONAL UPLC-MS/MS METHODS FOR ANALYSIS OF SERUM CORTISOL, ANDROSTENEDIONE, AND 17-HYDROPROGESTERONE FOR CLINICAL RESEARCH

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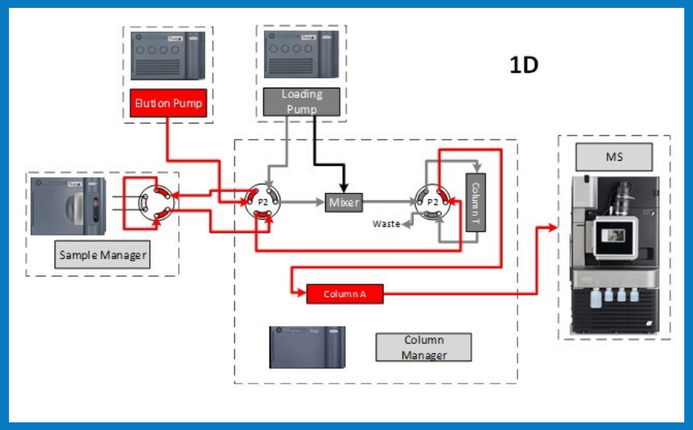
INTRODUCTION

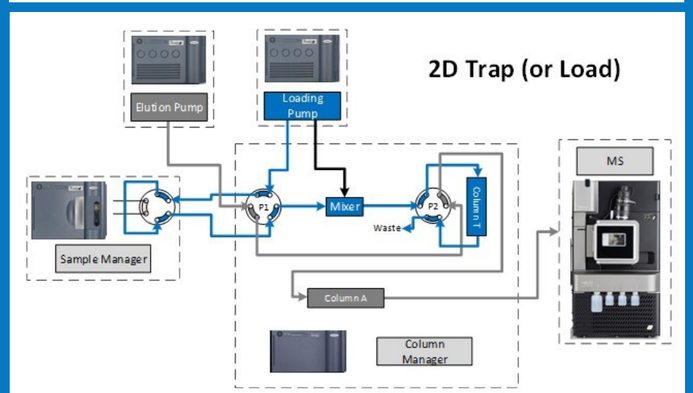
Extensive sample preparation is frequently employed in clinical research applications to reduce interferences from biological matrices, to reach the desired analytical sensitivity requirements and to ensure solvent compatibility with the LC-MS/MS system. Using LC chromatographic methodology to perform sample clean-up can simplify sample preparation, reducing preparation time and removing potential human sources of error. Online sample clean-up can also provide other benefits, such as allowing larger injection volumes to improve analytical sensitivity. Here we describe the use of a single, flexible UPLC®-MS/MS system configured to provide two flow paths for analytical chromatography; a two-dimensional (2D) trapping/ back-elution path and a conventional single-dimensional path. Using the 2D flow path, the analytes of interest are focused onto the first column using a loading gradient. This allows for the use of large injections volumes to improve analytical sensitivity before being eluted onto the second column for analytical separation. Additionally, the trap column can be washed and re-equilibrated during the chromatographic column's separation time to prepare it for the next injection. The system is applied in the online sample clean-up of steroid hormones (serum cortisol, androstenedione and 17-hydroxyprogesterone) following a simple protein precipitation extraction to demonstrate the utility of 2D chromatography in clinical research applications.

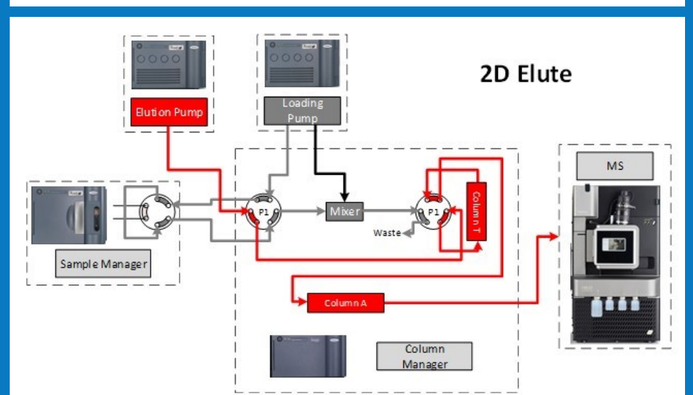
METHODS

- Charcoal stripped serum-based steroid hormone calibrator and control samples were prepared over the ranges of 1.7–1,380 nmol/L (Cortisol), 0.17–349 nmol/L (Androstenedione) and 0.38–757 nmol/L (17-hydroxyprogesterone) with methanol and zinc sulfate.
- All samples were diluted with internal standard and zinc sulfate(aq) solution and precipitated with methanol, followed by centrifugation at ~25,000 g for five minutes. 100 µL of each extracted sample was injected onto the 2D UPLC system.
- The 2D UPLC system was configured with two BSM binary pumps, a fixed loop Sample Manager, a two-position heated Column Manager with 2-position 6-port fluidic valves, and a Xevo™ TQ-S micro tandem quadrupole mass spectrometer. The valves in the column manager were configured to allow operation of the system either as a direct UPLC system or a trap and elute two-dimensional UPLC system. Waters ACQUITY™ UPLC HSS C18 SB VanGuard™ pre-column was used in the first dimension and an ACQUITY UPLC HSS T3 Column was used as the analytical column in the second dimension.
- The extracted samples were delivered to the trap column with conditions allowing the analytes to adsorb to the column while matrix interferences were flushed to waste. Samples were loaded onto the trap column using an isocratic water/methanol/formic acid gradient. The method automatically toggled the required valves and allowed the same analytical gradient used in 1D chromatography to be delivered in the reverse direction eluting the analytes from the trap column to the analytical column. Multiple reaction monitoring (MRM) tandem mass spectrometry with positive ion mode electrospray ionization was used for mass analysis. MassLynx™ instrument control software was used to control the system, with data analysis using TargetLynx™ software.

1D/2D Rapid Switch configuration enables flexibility between standard 1D methods and reproducible online sample clean-up with high method sensitivity

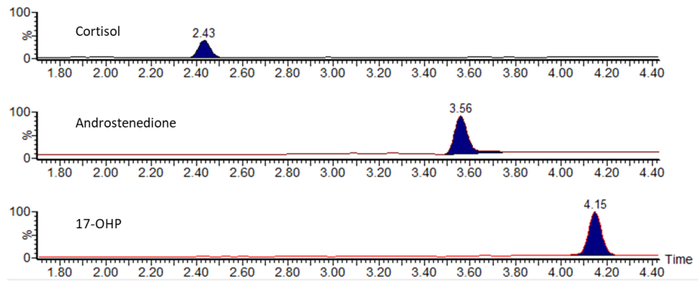






RESULTS

Below is a typical chromatogram of a Low QC injection.



Linearity

Calibration curve correlation coefficients were calculated for cortisol, androstenedione, and 17- hydroxyprogesterone and were shown to be >0.99 across the analyzed concentration ranges.

Precision

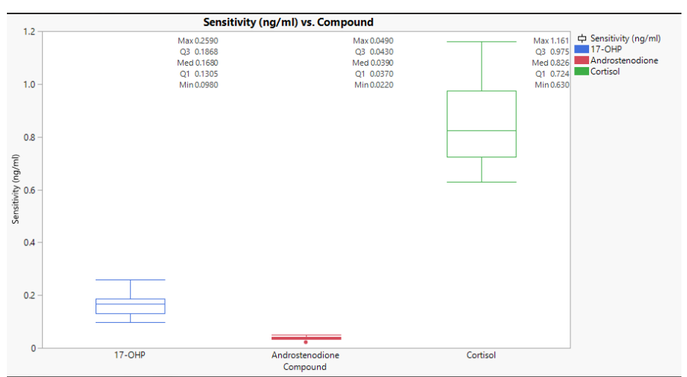
Total precision and repeatability of the method was assessed by quantification of serum samples in five replicates at low, mid, and high concentrations across five days (n=25). All results were <10% CV.

	Low QC Level			Medium QC Level			High QC Level		
Compound	Conc (nmol/L)	Total Precision (% CV)	Repeatability (≤ % CV)	Conc (nmol/L)	Total Precision (% CV)	Repeatability (≤ % CV)	Conc (nmol/L)	Total Precision (% CV)	Repeatability (≤ % CV)
Cortisol	6.9	9.1%	6.2%	69.1	5.0%	1.4%	691	4.6%	3.2%
Androstenedione	1.7	6.6%	8.6%	17.5	4.1%	3.0%	175	3.1%	2.8%
17 -hydroxyprogesterone	5.3	3.5%	5.4%	174	3.2%	3.2%	530	2.4%	2.5%

Analytical Sensitivity

The analytical sensitivity was assessed by extracting and quantifying serum samples using 10 replicates from low to high concentrations across three days (n=30). Results from an example data set of 24 replicates is shown below:

Compound	Conc (nmol/L)	Conc (nmol/L)	S/N	Precision (%CV)
Cortisol	1.9	0.7	≥ 58	19.2%
Androstenedione	0.16	0.05	≥ 19	13.7%
17 -hydroxyprogesterone	0.37	0.12	≥ 16	22.3%



Previous results have shown Signal-to-noise (ptp) of >10:1 and % CV's of <20% at 0.7 ng/ml for cortisol, 0.05 ng/ml for androstenedione and 0.12 ng/ml for 17-hydroxyprogesterone.

CONCLUSION

- Trap and back-elute UPLC-MS/MS is demonstrated for application in the analysis of three steroid hormones (cortisol, androstenedione and 17-hydroxyprogesterone) in serum for clinical research.
- The flexible configuration allows switching between direct UPLC analysis and trap and back-elute two-dimensional UPLC for online sample clean-up with no hardware changes.
- Sample preparation can be simplified, requiring only a simple protein precipitation extraction reducing the cost of required consumables.
- Trap and back-elute UPLC can be used to allow injection of larger sample volumes, providing high analytical sensitivity from 100 µL of serum, as demonstrated for cortisol (1.9 nmol/L), androstenedione (0.16 nmol/L), and 17-hydroxyprogesterone (0.37 nmol/L).

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