RAPID ANALYSIS OF ENDOGENOUS STEROIDS USING CONVERGENCE CHROMATOGRAPHY COUPLED WITH MASS SPECTROMETRIC DETECTION

コンバージェンスクロマトグラフィー、UPC²/MS による内因性ステロイドの高速分析

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INTRODUCTION

Steroid regulation plays a central role in the health and development of adults and children. Many disease states will differ based on subtle variations in a complex series of interactions among the many different steroids. Clinical research into disease mechanisms related to steroids often depends on the simultaneous quantitative analysis of multiple steroid levels. For mass spectrometric analysis of steroids, because of their structural similarity, chromatographic separation of the steroids is essential prior to their analysis. Typical research analyses utilize either gas (GC/MS) or liquid (LC/MS) chromatographic methods. GC/MS methods require sample derivitization prior to analysis resulting in analysis times of approximately 40 minutes. For LC/MS methods, typical analysis times are about 12 minutes for HPLC or 4 to 5 minutes with the use of more recent UHPLC methods.

This study focuses on the application of Convergence Chromatography for clinical research, utilizing liquid CO_2 as the primary mobile phase, for the rapid chromatographic analysis of endogenous steroids (structures shown in Figure 1). Here we present data collected with the ACQUITY® UltraPerformance Convergence Chromatography (UPC²®) system. In combination with stationary phases designed specifically for UPC², based on the high strength silica (HSS) and bridged ethylene hybrid (BEH™) technology, this technique results in the analysis of steroids in approximately 2 minutes. After initial method development using UV detection, the system was coupled to a tandem quadrupole mass spectrometer for analysis of steroid spiked plasma samples. In addition to the significant reduction in analysis time relative to other techniques, Convergence Chromatography minimizes the consumption of mobile phase solvents (e.g. methanol) thereby generating less waste for disposal and significantly reducing the cost of analysis per sample.

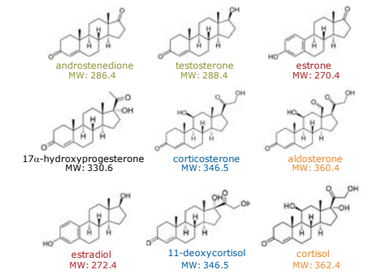

Figure 1. *Steroid structures for the current investigation. Colors denote similar structures resulting in similar m/z MS fragments.*

METHODS

INITIAL METHOD DEVELOPMENT

Column Screening:

A mixture of the 9 steroid standards was prepared at 0.2 mg/mL each, using a diluent of 88:12 methanol/ethanol. A generic 2-minute screening gradient was used to evaluate the separation of this mixture on four different stationary phases to determine which would provide the best separation.

Columns:

ACQUITY UPC² BEH, 1.7 µm, 3.0 x 50 mm
ACQUITY UPC² BEH 2-Ethylpyridine, 1.7 µm, 3.0 x 50 mm
ACQUITY UPC² CSH Fluoro-Phenyl, 1.7 µm, 3.0 x 50 mm
ACQUITY UPC² HSS C_{18} SB, 1.8 µm, 3.0 x 50 mm

Screening Conditions:

Instrument:	ACQUITY UPC² with PDA detection
Mobile Phase A:	CO_2 (tank, medical grade)
Modifier B:	Methanol (Fisher Optima® grade)
Needle Wash:	50:50 Methanol/Isopropanol
Seal Wash:	Methanol
Flow Rate:	3.65 mL/min
Gradient:	2% to 17% Modifier B in 2 minutes Re-equilibration at 2% Modifier B for 1 min.
CCM Pressure:	1800 psi
Column Temp.:	40 °C
UV Detection:	220 nm (compensated for 380-480 nm)
Injection Vol.:	1 µL

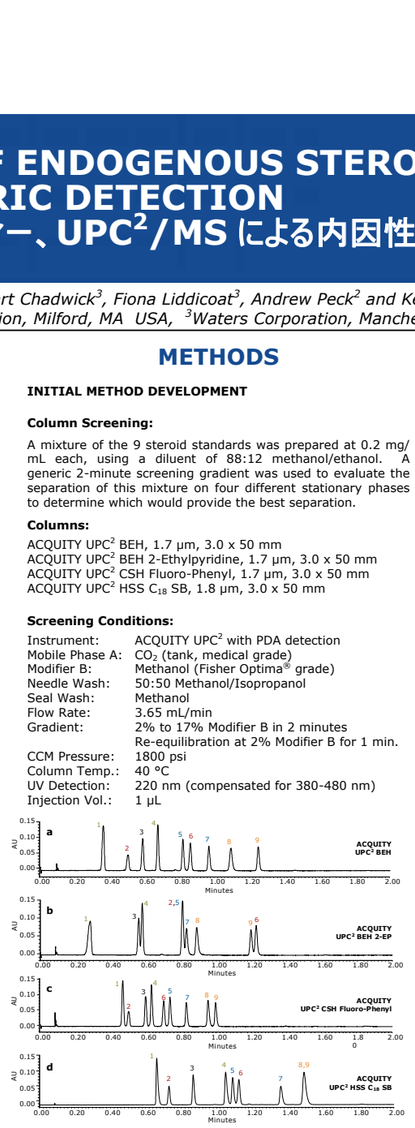

Figure 2. *UPC² separations (UV) of steroid standards on ACQUITY UPC² columns: BEH (a), BEH 2-EP (b), CSH Fluoro-Phenyl (c), and HSS C_{18} SB (d). All columns were 1.7 µm, 3.0 x 50 mm configurations except the HSS which is a 1.8 µm particle size. Steroid compounds are androstenedione (1), estrone (2), 17a-OHP [17 α-hydroxyprogesterone] (3), testosterone (4), 11-deoxycortisol (5), estradiol (6), corticosterone (7), aldosterone (8), and cortisol (9). Colored peak assignments denote compounds with similar molecular weights and m/z fragments.*

Column Selection:

The chromatograms in Figure 2 demonstrate the selectivity differences of the ACQUITY UPC² stationary phases, as well as the inherent speed of this chromatographic technique. Based on these results, the ACQUITY BEH stationary phase was chosen for additional application development with mass spectrometric detection.

MASS SPEC EVALUATIONS

Sample Prep:

Plasma samples were prepared from a 3:1 acetonitrile protein crash of human plasma. After centrifugation, the supernatant was collected and spiked with a mixture of the 9 steroid standards. Spiking of steroids to various levels was achieved by serial dilution of the sample with additional crashed plasma.

Xevo® TQ MS:

Transitions were optimized by direct infusion of standards into the Xevo TQ MS using the on-board fluidics, without the connectivity of the UPC² system (Table 1). After optimization of transitions, the Mass Spec system was coupled to the UPC² system using a Mass Spec splitter, incorporating the addition of a make-up flow pump, to facilitate sample flow into the MS and subsequent ionization (Figure 3).

Compound	Precursor	Product	Collision Energy	Dwell	Cone Voltage	Mode
estrone	**271.05**	133.1 **253.2**	30 **15**	0.005	25	ESI +
androstenedione	**287.05**	97.15 **109.2**	21 **26**	0.005	25	ESI +
testosterone	**289.10**	97.15 **109.15**	21 **26**	0.005	25	ESI +
17α-hydroxy progesterone	**331.10**	97.15 313.3	21 16	0.005	25	ESI +
11-deoxycortisol	**347.05**	**97.11** 109.14	**24** 26	0.005	26	ESI +
corticosterone	**347.05**	105.1 **121.1**	42 **28**	0.005	24	ESI +
aldosterone	**361.05**	97.15 315.2 **343.2**	35 20 **16**	0.005	25	ESI +
cortisol	**363.05**	**121.2** 309.2 327.2	**25** 20 15	0.005	25	ESI +
estradiol	**271.00**	**145.1** 183.1	**38** 38	0.005	55	ESI -

Table 1. *Multiple Reaction Monitoring (MRM) transitions used for the analysis of 9 structurally related steroids. Mass Spec conditions for the MRM transitions were optimized using IntelliStart™ in **infusion** mode only (without the UPC² instrument). MRM transitions in **bold** are transitions chosen for monitoring.*

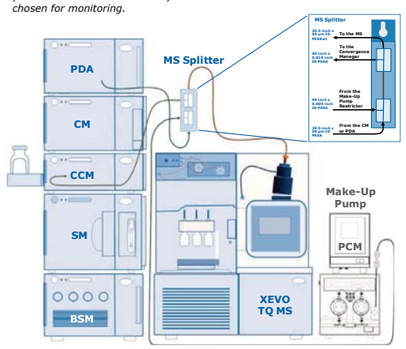

Figure 3. *UPC² System coupled to a XEVO TQ MS, includes a Binary Solvent Manager (BSM), Sample Manager (SM), Convergence Chromatography Manager (CCM), Column Manager (CM), Photo-Diode Array detector (PDA), Make-Up flow pump with additional Pump Control Module (PCM). The Mass Spec Splitter is used to tie all components together.*

RESULTS

Additional Parameter Optimization

The make-up flow introduced through the Mass Spec Splitter has a dual purpose:

- To keep the sample moving through the tubing as the CO_2 in the mobile phase starts to decompress as it reaches the mass spec. This is especially important at low concentrations of the organic modifier in the mobile phase, as in the early stages of the current gradient profile.
- The use of additives in the make-up flow (e.g. water, ammonium hydroxide [NH_4OH], or formic acid [FA]) can assist in ionization of the analytes within the mass spec source, improving sensitivity.

To optimize the make-up flow and additional mass spec conditions, a plasma sample spiked with the 9 steroids (at 50 ng/mL) was used to evaluate various conditions:

- Additive used in make-up flow solvent
- Capillary Voltage
- Desolvation Temperature and Gas Flow

The results of those evaluations are shown below in Figure 4.

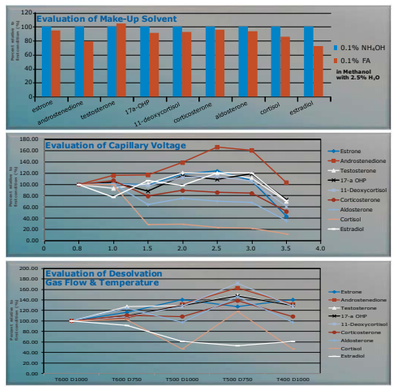

Figure 4. *Optimization of Make-up Flow Solvent, Capillary Voltage (kV), and Desolvation Temperature (°C) and Gas Flow (L/hr). Values reported are in percent relative to the first conditions evaluated.*

Based on these evaluations, the following settings were chosen:

- Make-Up Solvent: MeOH + 2.5% Water + 0.1% NH_4OH
- Make-Up Flow Rate: 0.4 mL/min
- Capillary Voltage: 1.0 kV
- Desolvation Temperature: 500°C
- Desolvation Gas Flow: 750 L/hr
- Data System: MassLynx® 4.1

resulting in the chromatography shown in Figure 5 for the 9 steroids spiked into human plasma after protein crash.

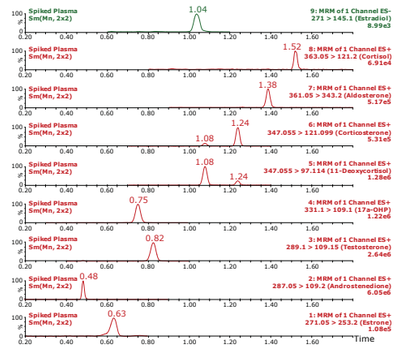

Figure 5. *Total Ion Chromatograms (TIC) of the 9 steroids. Standards were post spiked at a concentration of 50 ng/mL into a 3:1 acetonitrile protein crash of human plasma.*

Reproducibility

To evaluate reproducibility of the method, the peak areas for the individual steroids were monitored over the course of 100 injections (using 1 µL injection volumes of 50 ng/mL steroid spiked in plasma). The RSD values for the peak areas ranged from 5.6 to about 13.7%. A representative example of the reproducibility is shown below in Figure 6.

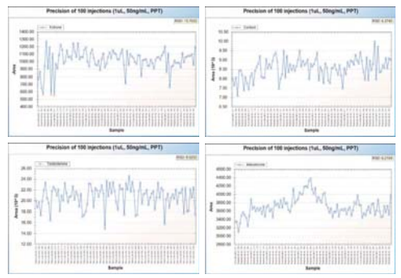

Figure 6. *Examples of injection to injection reproducibility over 100 injections (1 µL) of steroid spiked (50 ng/mL) human plasma for estrone (top left), cortisol (top right), testosterone (bottom left), and aldosterone (bottom right). Peak area RSD values range from approximately 5.6 to 13.7%.*

Linearity of Response

To evaluate the linearity of response, calibration curves were generated using 5 µL injections of the spiked steroid plasma samples (after 3:1 acetonitrile protein crash). Concentrations of the steroids ranged from 0.98 to 500 ng/mL. Representative calibration curves are shown in Figure 7, with the more complete data presented in Table 2 for each of the steroids.

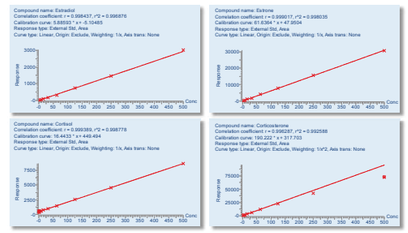

Figure 7. *Representative calibration curves demonstrating the response linearity for the steroids: estradiol (top left), estrone (top right), cortisol (bottom left), and corticosterone (bottom right). All are 5 µL injections of 3:1 ACN crashed human plasma from 0.98 to 500 ng/mL.*

Conc (ng/mL)		Compound (% Dev)								
		Estradiol	Cortisol	Aldo-sterone	Cortico-sterone	11-Deoxy cortisol	17a-OHP	Testo-sterone	Androstene-dione	Estrone
Std 0.5	1.0	BLQ	E	10.8	-4.2	1.7	18.7	E	E	BLQ
Std 1	2.0	BLQ	E	(44.5)	0.8	-8.8	-10.2	E	-15.2	BLQ
Std 2	3.9	BLQ	16.6	-15.3	11.4	3.4	8.5	E	-20.9	-14.9
Std 3	7.8	23.1	-26.2	-9.2	4.7	12.6	-2.6	-5.3	28.3	4.4
Std 4	15.6	-2.6	9.5	-6.0	9.4	6.7	-3.5	-12.1	11.0	18.8
Std 5	32.3	-8.5	1.3	0.9	-1.3	-4.4	-12.2	6.1	(26.4)	-14.9
Std 6	62.5	-16.0	-3.7	12.7	-2.7	-0.2	-5.6	-1.8	-1.9	6.4
Std 7	125.0	-0.3	3.6	4.0	-6.0	8.1	5.9	15.6	-1.3	0.0
Std 8	250.0	-1.2	-1.0	8.8	-12.1	-9.0	2.0	2.4	(-40.1)	1.2
Std 9	500.0	2.9	0.0	-6.6	(-24.1)	-10.0	-1.0	-4.8	(-20.6)	-1.0

□% Dev <20%
▨% Dev 20-25%
▩% Dev >25%

BLQ – Below limit of quantification
E – Rejected standard due to endogenous levels in the blanks
( ) – Rejected standard (large deviation)

Table 2. *Percent deviation from the calibration curve for each steroid at each level of spiking.*

CONCLUSION

- **Convergence Chromatography enables fast, accurate analysis of steroids for clinical research with reduced analysis times relative to current LC and GC methodologies.**
- **UPC² offers scientists a unique workflow, application and environmental impact benefits compared to LC and GC platforms:**
  - **Sample preparation is simplified**
    - Does not require sample derivatization.
    - Samples extracted in organic solvents can be injected without the additional steps to exchange solvents for RP compatible diluents.
  - **More environmentally friendly mobile phase.**
- **With CO_2 as the primary mobile phase, the cost of analysis per sample is reduced.**
- **While the limits of detection and quantitation for several of the steroids presented here do not meet the current levels required for clinical research, it is important to note that this is a first attempt with a new technology as proof of concept. Additional efforts to increase the sensitivity for these applications is on-going.**

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