Waters™

Method Development for the Analysis of Endogenous Steroids Using Convergence Chromatography with Mass Spectrometric Detection

Christopher J. Hudalla, Stuart Chadwick, Fiona Liddicoat, Andrew Peck, Kenneth J. Fountain

Waters Corporation

For research use only. Not for use in diagnostic procedures.

Abstract

This method demonstrates the separation power of convergence chromatography utilizing either UV or MS detection. While the limits of detection and quantification presented here are not compatible with the low levels of steroid concentrations typically found in biological samples (e.g. plasma), additional optimization of MS parameters, with the possibility of additional sample derivitization to improve ionization, may help to reach higher sensitivity levels. This could further enable the application of this method to the analysis of steroids for clinical research.

Benefits

- Analysis of endogenous steroids in approximately two minutes

- Minimal sample prep without requiring sample derivitization

- Ability to separate compounds that are structurally very similar

- 50% to 95% reduction in analysis time relative to GC and LC methods enables high-throughput analysis

- Compatible with UV and mass spectrometric detection

Introduction

Steroid biosynthesis is a complex metabolic pathway utilizing simple precursors to synthesize multiple steroidal forms. This biosynthetic pathway is unique to animals and provides a common target for antibiotics and other anti-infective drugs. Precise and accurate steroid analysis is critical for the development of steroid-based therapeutics. For mass spectrometric analysis of steroids, due to their structural similarity, chromatographic separation of the steroids is essential prior to 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.[1] For LC-MS methods, typical analysis times are approximately 12 minutes for HPLC, or four to five minutes with the use of more recent UHPLC methods.[2,3]

This study focuses on the application of convergence chromatography (CC), utilizing liquid CO_2 as the primary mobile phase, for the rapid chromatographic analysis of endogenous steroids (structures shown in Figure 1).
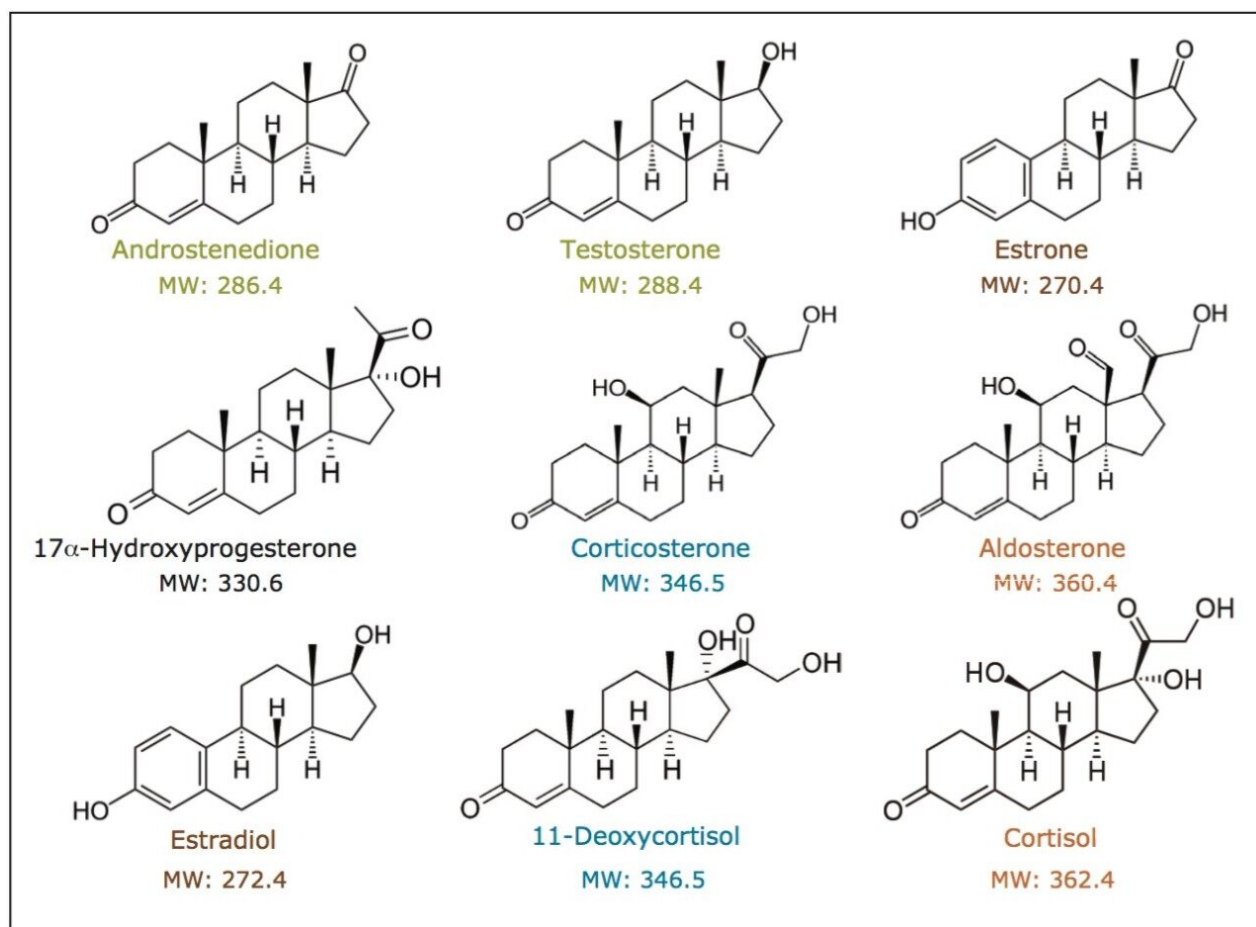
Figure 1. Structures of steroids evaluated. Compounds with the same color font indicate compounds with similar molecular weights that generate similar MS fragments.

Experimental

Sample description

Column screening (UV detection): A mixture of nine steroid standards was prepared at 0.2 mg/mL each, using a diluent of 88:12 methanol/ethanol.

Mass spectrometer evaluations: Often times, matrix interferences can limit the applicability of a technique. For this reason, standards were evaluated in a human plasma matrix. However, to insure these evaluations were indicative of technique sensitivity and not affected by recovery issues during sample preparation, the plasma samples were post-spiked after a 3:1 acetonitrile protein crash of the plasma. After centrifugation, the

supernatant was collected and spiked with a mixture of the nine steroid standards. Spiking of steroids to various levels was achieved by serial dilution of the sample with additional crashed plasma.

Method conditions

Screening columns:

ACQUITY UPC^2 BEH 1.7 μm, 3.0 x 50 mm (p/n 186006562)

ACQUITY UPC^2 BEH 2-Ethylpyridine 1.7 μm, 3.0 x 50 mm (p/n 186006580)

ACQUITY UPC^2 CSH Fluoro-Phenyl 1.7 μm, 3.0 x 50 mm (p/n 186006571)

ACQUITY UPC^2 HSS C$_{18}$ SB 1.8 μm, 3.0 x 50 mm (p/n 186006621)

UPC^2 conditions

System: ACQUITY UPC^2 with PDA detector

Mobile phase A: CO_2 (tank, medical grade)

Modifier B: Methanol (Fisher Optima grade)

Column temp.:   40 °C

ABPR: 1800 psi

Gradient: 2% to 17% modifier B in two minutes, re-equilibration at 2% modifier B for one minute

Flow rate: 3.65 mL/min

UV detection: 220 nm (compensated 380 to 480 nm) [40 pts/s]

Injection volume: 1 μL

UPC^2 conditions

Needle wash:	50:50 methanol/2-propanol
Seal wash:	Methanol

Data management

Empower 3 Software

Make-up flow pump conditions

Solvent:	Methanol with 2.5% water and 0.1% ammonium hydroxide
Flow rate:	0.4 mL/min

MS conditions

Mass spectrometer:	Xevo TQ
Capillary voltage:	1 kV
Desolvation temp.:	500 °C
Desolvation gas flow:	750 L/h
Data management:	MassLynx Software

Here, we present data collected with the ACQUITY UltraPerformance Convergence Chromatography (UPC^2) System. In combination with stationary phases designed specifically for UPC^2, based on the bridged ethylene hybrid, BEH Technology, this technique results in the analysis of steroids in approximately two 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.

Results and Discussion

A generic two-minute screening gradient was used to evaluate the separation of the nine-steroid mixture on four different stationary phases to determine which would provide the best separation. The chromatograms in Figure 2 demonstrate the selectivity differences of the ACQUITY UPC^2 stationary phases, as well as the inherent speed of this chromatographic technique. Based on these results, the ACQUITY UPC^2 BEH stationary phase was chosen for additional application development with mass spectrometric detection.
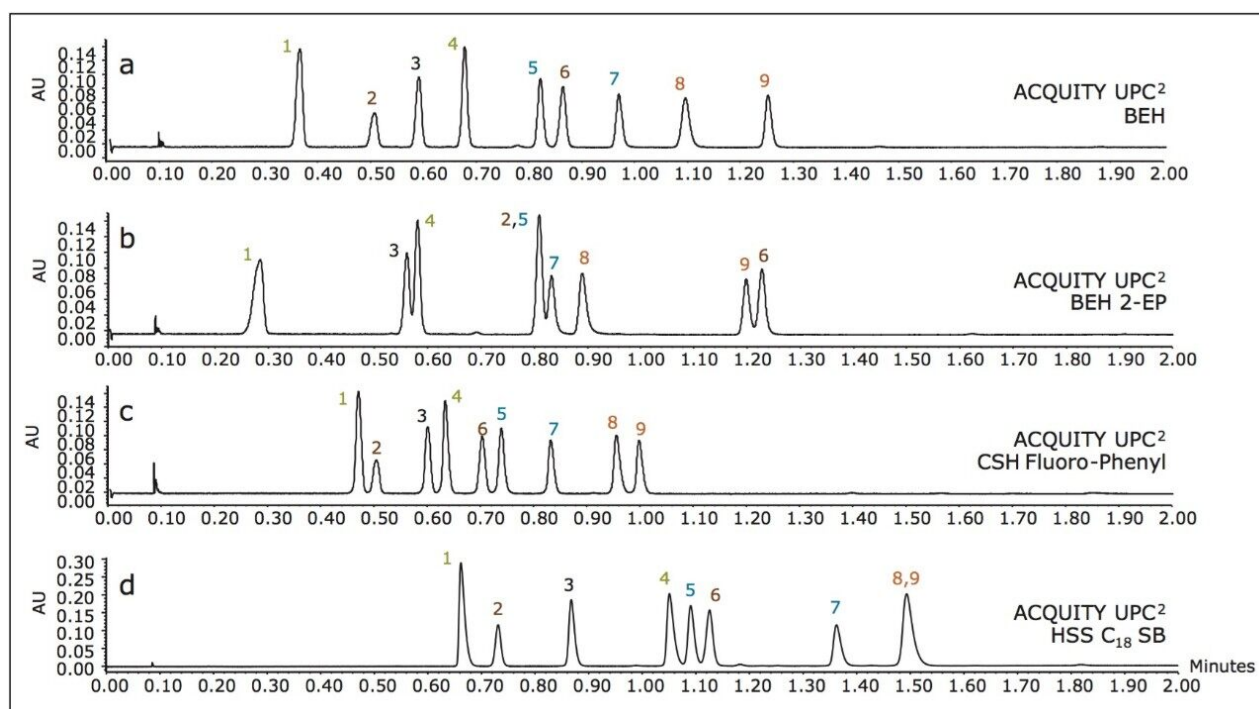
Figure 2. UPC^2 separations (UV) of steroid standards on ACQUITY UPC^2 columns including: (a) BEH, (b) BEH 2-EP, (c) CSH Fluoro-Phenyl, and (d) HSS C$_{18}$ SB. All columns were 3.0 x 50 mm, 1.7-μm configurations except for the HSS C$_{18}$ SB which is a 1.8-μm particle size. Steroid compounds are the following: (1) androstenedione, (2) estrone, (3) 17a-OHP [17a-hydroxyprogesterone, (4) testosterone, (5) 11-deoxycortisol, (6) estradiol, (7) corticosterone, (8) aldosterone, and (9) cortisol. Colored peak assignments denote compounds with similar molecular weights and m/z fragments.

Individual mass spectrometer (MRM) transitions were optimized by direct infusion of standards into the Xevo TQ MS using on-board fluidics, without the connectivity of the ACQUITY UPC^2 System (Table 1). After optimization of transitions, the mass spectrometer was coupled to the UPC^2 system using a mass spectrometer 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	153.1	30	0.005	25	ESI +
		253.2	**15**			
Androstenedione	287.05	97.15	21	0.005	25	ESI +
		109.2	**26**			
Testosterone	289.10	97.15	21	0.005	25	ESI +
		109.15	**26**			
17α-Hydroxy progesterone	331.10	97.15	21	0.005	25	ESI +
		109.1	**26**			
		313.3	16			
11-Deoxycortisol	347.05	**97.11**	**24**	0.005	26	ESI +
		109.14	26			
Corticosterone	347.05	105.1	42	0.005	24	ESI +
		121.1	**28**			
Aldosterone	361.05	97.15	35	0.005	25	ESI +
		315.2	20			
		343.2	**16**			
Cortisol	363.05	**121.2**	**25**	0.005	25	ESI +
		309.2	20			
		327.2	15			
Estradiol	271.00	**145.1**	**38**	0.005	55	ESI -
		183.1	38			

Table 1. Multiple reaction monitoring (MRM) transitions used for the analysis of nine structurally related steroids. MS conditions for the MRM transitions were optimized using IntelliStart in infusion mode only (without the UPC^2 instrument). MRM transitions in bold are transitions chosen for monitoring.
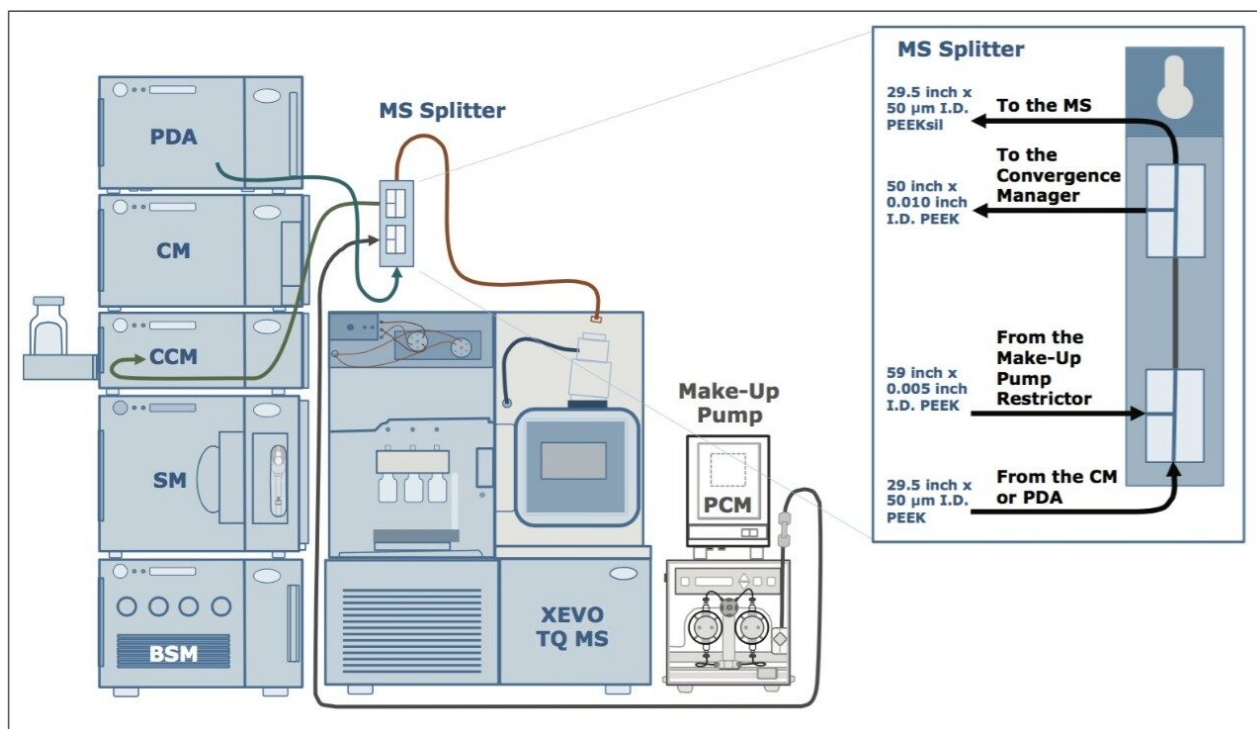
Figure 3. ACQUITY UPC^2 System coupled to a Xevo TQ MS, includes a Binary Solvent Manager (BSM), Sample Manager (SM), Convergence Chromatography Manager (CCM), Column Manager (CM), Photodiode Array (PDA) Detector, Make-up Flow Pump with additional Pump Control Module (PCM). The mass spectrometer splitter is used to connect all components together.

Optimization of make-up flow

The make-up flow introduced through the mass spectrometer splitter has a dual purpose. It facilitates the post-mixer transfer of the sample through the tubing, as the CO_2 in the mobile phase starts to decompress as it reaches the mass spectrometer. This is especially important at low concentrations of the organic modifier in the mobile phase, as seen in the early stages of the current gradient profile. In addition, the use of additives in the make-up flow (such as water, ammonium hydroxide (NH_4OH), or formic acid (FA)) can assist in ionization of the analytes within the mass spectrometer source, thereby improving sensitivity. To optimize the make-up flow and additional MS conditions, a plasma sample spiked with the nine steroids (at 50 ng/mL) was used to evaluate various conditions including: additive used in make-up flow solvent, capillary voltage, desolvation temperature, and gas flow. The results of those evaluations are shown in Figure 4.
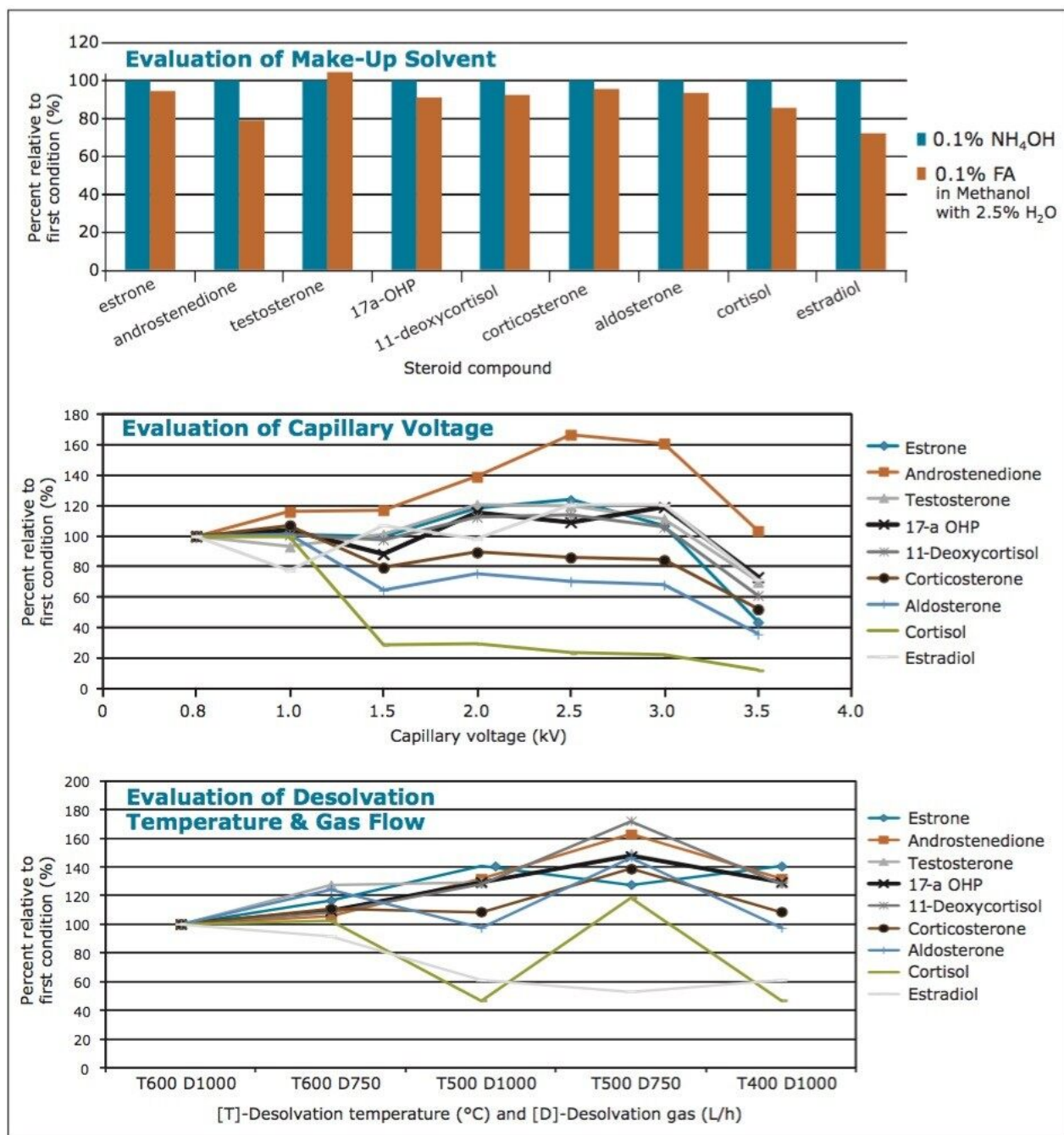
Figure 4. Optimization of make-up flow solvent, capillary voltage (kV), desolvation temperature (°C), and gas flow (L/h). Values reported are in percentages relative to the first conditions evaluated.

The top panel of Figure 4 shows eight of the nine steroids yielded higher MS signals (better ionization) when using ammonium hydroxide as an additive in the make-up flow. In addition, most of the steroid signals were ambivalent to capillary voltages between 0.8 and 3.0 kV, as shown in the middle panel of Figure 4. However, at voltages higher than 1.0 kV, the signal for cortisol diminished dramatically. Based on these evaluations, the

optimum conditions were determined, with the best overall signal obtained for all steroids using a make-up solvent composed of methanol with 2.5% water and 0.1% ammonium hydroxide, and a flow rate of 0.4 mL/min. The optimum results were obtained by MS when using a capillary voltage of 1.0 kV, with a desolvation temperature of 500 °C, and a gas flow of 750 L/h (bottom panel of Figure 4). The resulting chromatography for the nine steroids post-spiked into the human plasma after protein crash is shown in Figure 5.
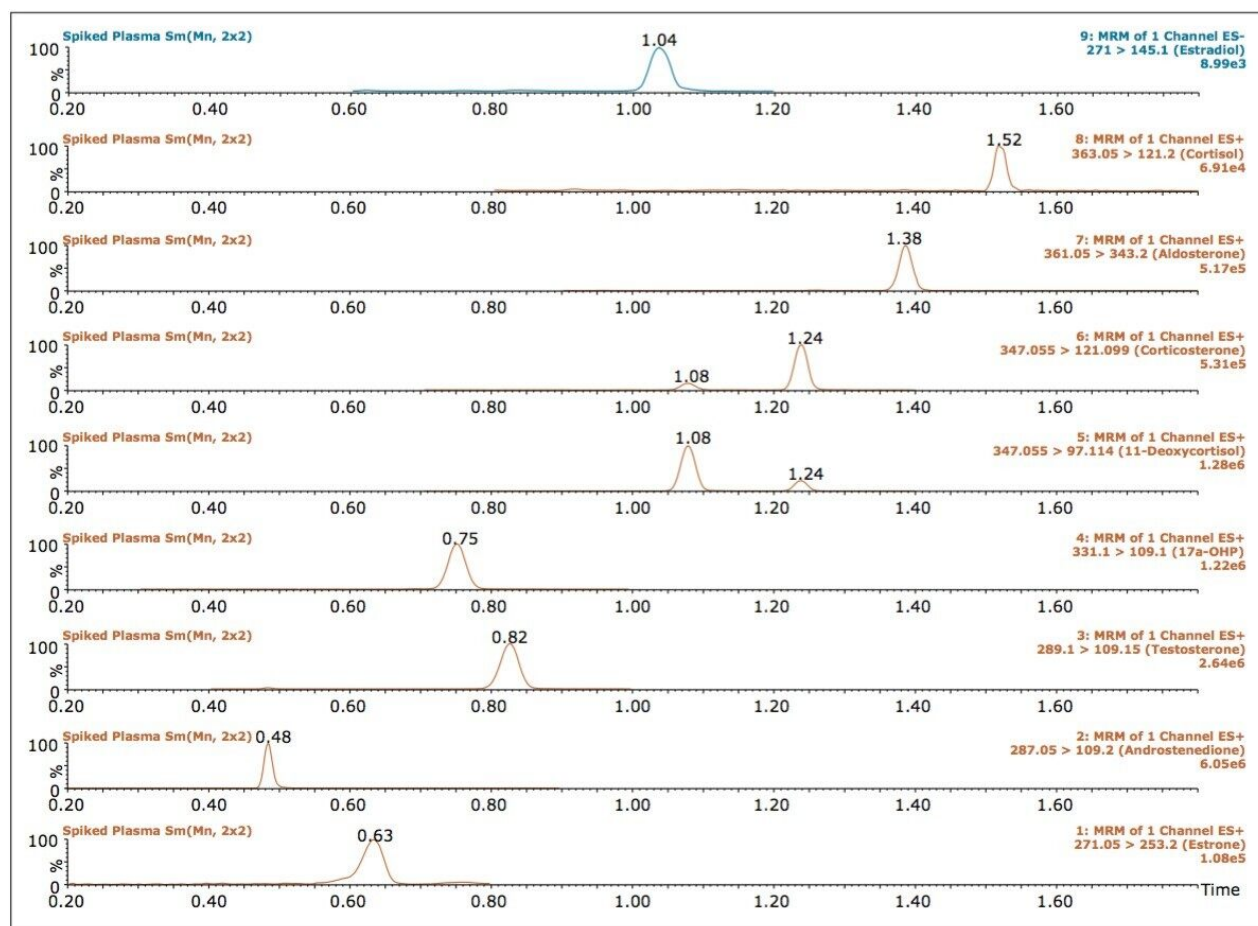

Figure 5. Total ion chromatograms (TIC) of the nine steroids. Standards were post-spiked at a concentration of 50 ng/mL into a 3:1 acetonitrile protein crash of human plasma.

Reproducibility

As in any method development, the accuracy and reproducibility of the method is critical for success. 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 approximately 13.7%. A representative example of the

reproducibility results are shown in Figure 6. Similar results were obtained for the other steroids evaluated.
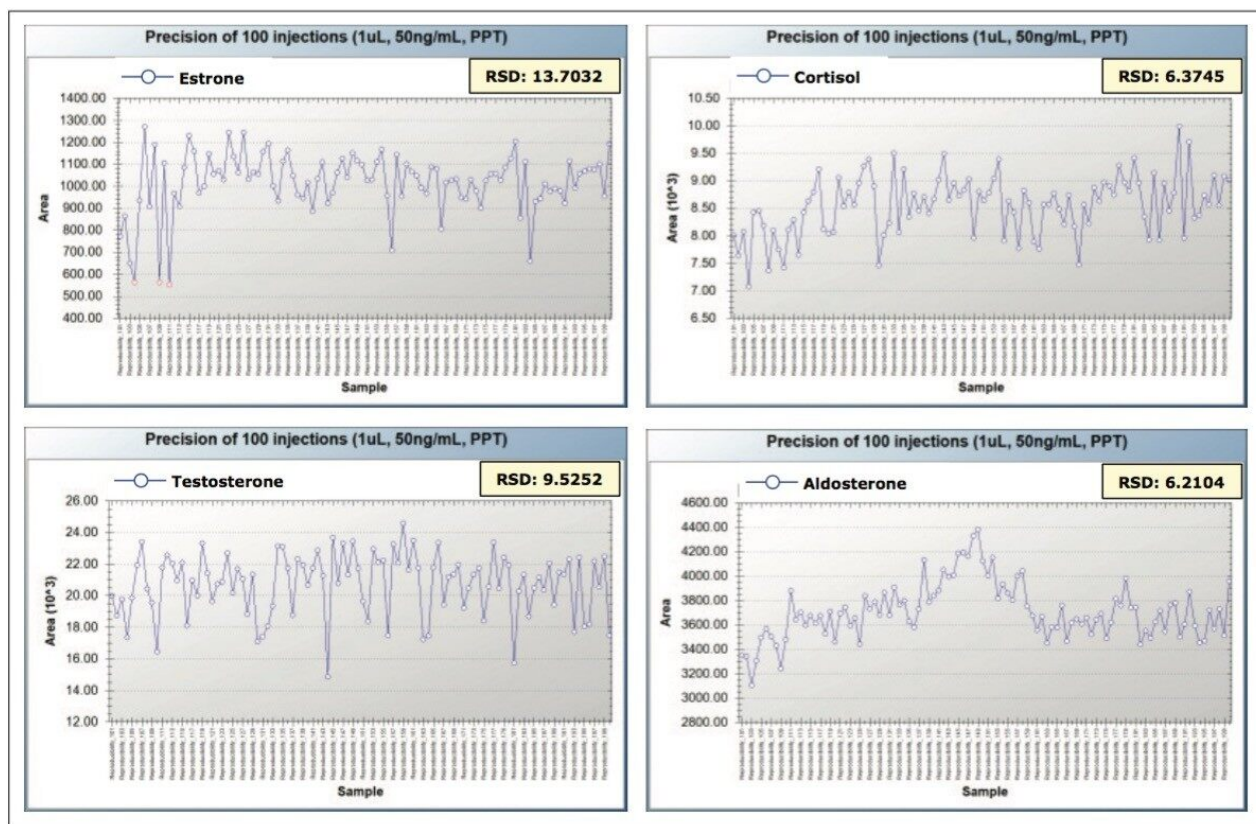

Figure 6. Examples of injection-to-injection reproducibility over 100 injections (1 µL) of steroid-spiked (50 ng/mL) human plasma for estrone (top left), cortisol (top right), testosterone (bottom left), and aldosterone (bottom right). Peak area RSDs range from approximately 5.6% to 13.7%.

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 more complete data for each of the steroids shown in Table 2.
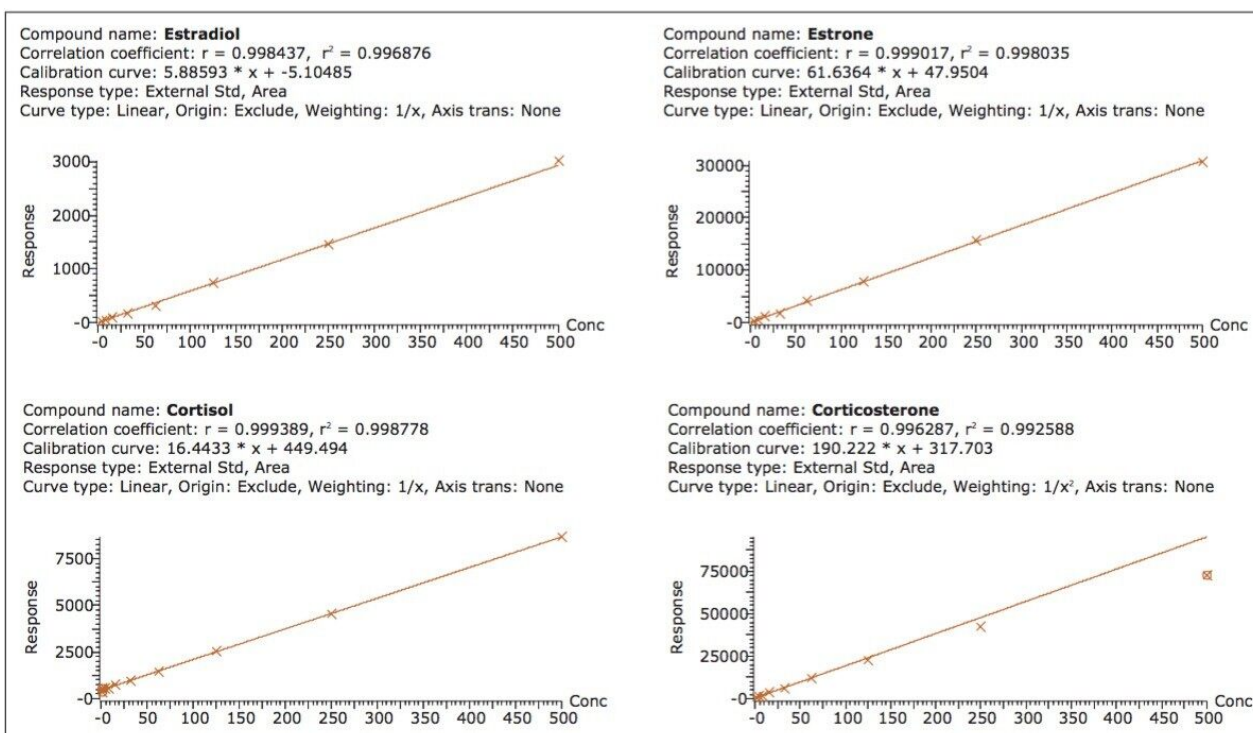
Figure 7. Four representative calibration curves demonstrating the response linearity. All are 5-µL injections of 3:1 acetonitrile crashed human plasma spiked from 0.98 to 500 ng/mL with steroid standards.

Conc (ng/mL)		Estradiol	Cortisol	Aldosterone	Corticosterone	11-deoxy cortisol	17a-OHP	Testosterone	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

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 with reduced analysis times relative to current LC and GC methodologies. UPC^2 offers scientists a unique workflow, application, and environmental impact benefits, compared to LC and GC platforms, with simplified sample preparation. Samples extracted in organic solvents can be injected without additional steps to exchange solvents for RP-compatible diluents. In addition, with CO_2 as the primary mobile phase, the cost of analysis per sample is reduced, using a more environmentally friendly solvent relative to conventional RP methods. This method demonstrates the separation power of convergence chromatography utilizing either UV or MS detection. While the limits of detection and quantification presented here are not compatible with the low levels of steroid concentrations typically found in biological samples (e.g. plasma), additional optimization of MS parameters, with the possibility of additional sample derivitization to improve ionization, may help to reach higher sensitivity levels. This could further enable the application of this method to the analysis of steroids for clinical research.

References

1. Sellers K. Rapid analysis of steroid hormones by GC-MS using the new Rxi-1ms column. Restek Application Note.

2. Licea-Perez H, Wang S, Szapacs ME, Yang E. Development of a highly sensitive and selective UPLC-MS/MS method for the simultaneous determination of testosterone and 5a-dihydrotestosterone in human serum to support testosterone replacement therapy for hypogonadism. *Steroids*. 2008; 73:601-610.

3. Schwarz E, Liu A, Randall H, Haslip C, Keune F, Murray M, Longo N, Pasquali M. Use of steroid profiling by UPLC-MS/MS as a second tier test in newborn screening for congenital adrenal hyperplasia: The Utah experience. *Pediatric Research*. 2009; 66: 230-235.

Featured Products

ACQUITY UPC2 System <https://www.waters.com/134658367>

Empower 3 Chromatography Data Software <https://www.waters.com/513188>

MassLynx MS Software <https://www.waters.com/513662>

ACQUITY UPLC PDA Detector <https://www.waters.com/514225>

720004692, May 2013