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TECHNICAL NOTE 21882

Robust extraction, separation, and quantitation of structural isomer steroids from human plasma by SPE-UHPLC-MS/MS

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Keywords

Steroid hormone, Vanquish Horizon UHPLC, TSQ Quantiva, Accucore Biphenyl column, LC-MS/MS, SPE

Application benefits

- Separation of structural isomers for accurate detection
- Alternative selectivity to C18 phase with an increase in overall resolution of structural isomers
- Stable retention time from extracted plasma
- Accurate and precise analytical method across 1000-fold concentration range

Goal

Achieve separation of 12 steroid hormones including structural isomers. Comparison to more popular C18 phase is assessed, as well as extraction from human plasma using polymeric solid phase extraction.

Introduction

Accurate measurement of steroids in plasma is an important requirement in clinical research laboratories. Triple quadrupole mass spectrometry (MS/MS) is now a standard platform in this area for detection due to speed and sensitivity, however this group of compounds contains many structural isomers that cannot be differentiated by MS/MS alone. This may lead to inaccurate analysis by over estimation of concentration levels. Separation prior to MS/MS detection must be achieved, typically by liquid chromatography (LC). An analytical method utilizing LC-MS/MS combined with solid phase extraction of plasma samples is used to remove many matrix

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interferences, separate isomers, and detect 12 steroids, with an assessment of method performance is reported here.

Effective liquid chromatography gradient conditions were used to provide excellent retention time precision by combining the Thermo Scientific™ Vanquish™ Horizon UHPLC system with a Thermo Scientific™ Accucore™ Biphenyl analytical column. These columns feature rugged 2.6 µm solid-core particles that ensure high efficiencies and enable compatibility with both HPLC and UHPLC platforms. Biphenyl bonded phases offer unique selectivity for aromatic and moderately polar analytes providing an increase in resolution of structural isomers, particularly when methanol is used in the mobile phase.

Two LC-MS/MS methods to achieve separation of common groups of steroids required for routine analysis are presented. In the first method, excellent separation is achieved using acetonitrile gradient for rapid separation of common steroids of interest. The second method presents quantitative data at low concentration levels (50 pg/mL) from plasma extracts using methanol as mobile phase B. This provided extra retention and selectivity to fully resolve structurally similar compounds that proved to be difficult to separate on C18 phases.

Experimental

Consumables

- Fisher Scientific™ Optima™ UHPLC-MS grade water (P/N 10154604)
- Fisher Scientific™ Optima™ UHPLC-MS grade methanol (P/N A458-1)
- Fisher Scientific™ Optima™ UHPLC-MS grade acetonitrile (P/N A956-1)
- Fisher Scientific™ Ammonium fluoride, extra pure (P/N A/4920/53)
- Thermo Scientific™ SOLAµ™ HRP plate (P/N 60209-001)
- Thermo Scientific™ Accucore™ Biphenyl column, 100 × 2.1 mm, 2.6 µm (P/N 17826-102130)
- Zinc sulfate (P/N Z/1550/53)
- Thermo Scientific™ WebSeal™ 96 well square well plates (P/N 60180-P135)
- Thermo Scientific™ WebSeal™ mats (P/N 60180-M102)

Sample preparation

Method 1 (ACN)	Method 2 (MeOH)
Cortisol	Hydrocortisone
Corticosterone	Cortisone
Estradiol	21-Deoxycortisol
Trenbolone	Aldosterone
Nandrolone	21-Deoxycortisone
Testosterone	11-Deoxycortisol
Methyltestosterone	Corticosterone
17α-Hydroxyprogesterone	Testosterone
Androstenedione	17α-Hydroxyprogesterone
Progesterone	11-Deoxycorticosterone
25-Hydroxy vitamin D3	Androstenadione
	Progesterone

Matrix: Pure solutions, human plasma (lithium heparin), and phosphate buffer solution (PBS)

Method 1

Compounds were prepared at 100 ng/mL in mobile phase starting conditions.

Method 2

PBS was spiked with various concentrations of compounds to produce a calibration curve from 50 to 50,000 pg/mL. PBS and human plasma from a pooled source including male and female donors were spiked as QC samples at 500 and 5000 pg/mL to assess the analytical method. Blank plasma was also extracted as a baseline concentration as some of the compounds used are endogenous in high concentrations.

Four hundred microliters of each sample were mixed 1:1 with zinc sulfate (2%) and centrifuged. The supernatant was added to a SOLAµ HRP plate, preconditioned with methanol, then equilibrated with water. After sample loading the SPE device was washed with 20% methanol. All the compounds eluted with 40 µL of elution solvent (80% acetonitrile, 20% methanol). The extract was diluted to 100 µL with mobile phase A. The samples were loaded into the autosampler set to 10 °C ready for injection onto the liquid chromatography system.

LC conditions

Instrumentation

Designed with innovative technology, the Vanquish Horizon UHPLC system delivers a new standard in high-end UHPLC. This fully integrated and biocompatible system features high sample capacity for high-throughput workflows, industry-leading pumping

performance, amazingly high S/N and linearity, two-mode thermostatting, and active preheating.

- Vanquish Horizon UHPLC system consisting of the following:
  - System Base Vanquish Horizon (P/N VH-S01-A)
  - Binary Pump H (P/N VH-P10-A)
  - Split Sampler HT (P/N VH-A10-A)
  - Column Compartment H (P/N VH-C10-A)
  - Active Pre-heater (P/N 6732.0110)
  - MS Connection Kit Vanquish (P/N 6720.0405)

Separation conditions

Method 1

Mobile phase A:	Water
Mobile phase B:	Acetonitrile
Gradient:	40 to 45% mobile phase B over 2.5 minutes 45 to 100% mobile phase B over 2.5 minutes 2 minute equilibration time
Flow rate:	0.6 mL/min
Column temperature:	30 °C, still air, active pre-heating
Injection volume:	1 µL
Injection wash solvent:	1:1 mix of mobile phase A and B

Method 2 - Extended method for separation of closely related isobaric compounds from plasma extract

Mobile phase A:	0.2 mM ammonium fluoride in water
Mobile phase B:	0.2 mM ammonium fluoride in methanol
Gradient:	5 to 100% mobile phase B over 12 minutes, 3 minute equilibration time
Flow rate:	0.6 mL/min
Column temperature:	40 °C, still air, active pre-heating
Injection volume:	20 µL
Injection wash solvent:	1:1 mix of mobile phase A and B

MS conditions

Instrumentation

Detection was performed with the Thermo Scientific™ TSQ Quantiva™ triple quadrupole mass spectrometer, which uses active ion management to exceed even the most stringent analytical requirements with superb sensitivity, speed, and dynamic range.

MS settings (Method 2 settings shown only)

Table 1. MS source parameters

MS Source Parameters	Setting
Source	Thermo Scientific™ Ion Max™ source with HESI-II probe
Polarity	Positive ionization
Spray voltage (V)	3500
Vaporizer temperature (°C)	400
Sheath gas pressure (psi)	50
Aux gas pressure (Arb)	15
Ion transfer tube temperature (°C)	350
CID gas pressure (mTorr)	1.5

Data processing

The Thermo Scientific™ Chromeleon™ 7.2.8 Chromatography Data System was used for data acquisition and analysis.

Table 2. Compound transition details

Compound	Polarity	Precursor (*m/z*)	Product (*m/z*)	Collision Energy (V)	RF Lens (V)
Androstenedione	Positive	287.2	97.1	22	91
Testosterone	Positive	289.3	109.1	26	83
Testosterone-d3	Positive	292.2	97.1	24	79
Progesterone	Positive	315.2	97.1	23	87
d9-Progesterone	Positive	324.4	100.2	25	83
11-Deoxycorticosterone	Positive	331.2	97.1	24	79
17 -Hydroxyprogesterone	Positive	331.2	313.2	15	80
21-Deoxycortisone	Positive	345.1	163.2	26	89
11-Deoxycortisol	Positive	347.2	97.1	26	77
21-Deoxycortisol	Positive	347.2	311.2	16	85
Corticosterone	Positive	347.2	329.2	16	78
Cortisone	Positive	361.2	163.2	23	90
Aldosterone	Positive	361.2	343.2	17	94
Hydrocortisone	Positive	363.2	121.1	29	86

Results and discussion

Separation of 11 steroids was achieved with an acetonitrile gradient within 4.2 minutes. Direct comparison to an equivalent biphenyl chemistry was conducted with an Accucore Biphenyl column showing greater resolution of closely eluting trenbolone, nandrolone, and testosterone (peaks 4-6, Figure 1).

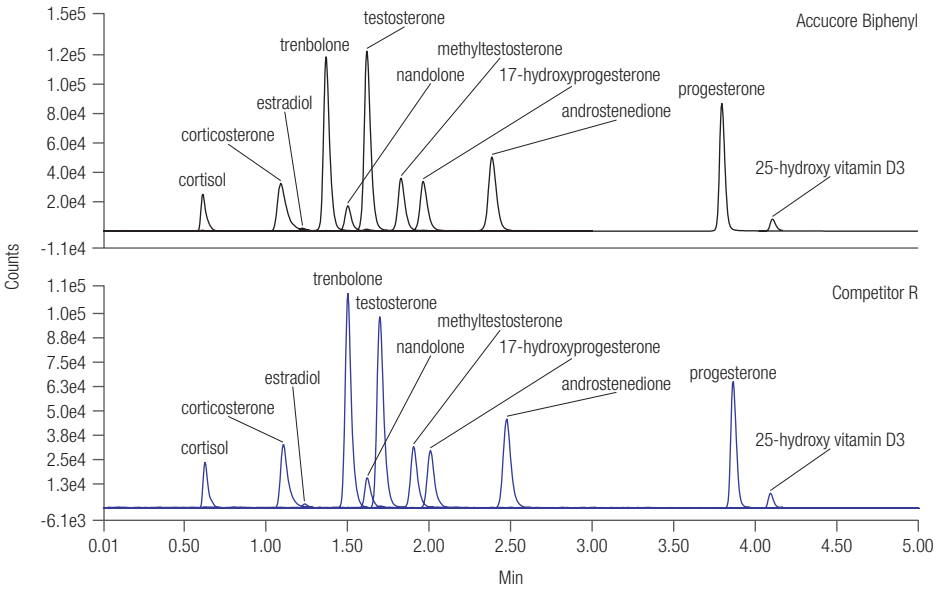

Figure 1. Separation of common steroids of interest with an acetonitrile gradient

Separation of this closely related set of compounds was achieved with a 12-minute gradient demonstrated in Figure 2.

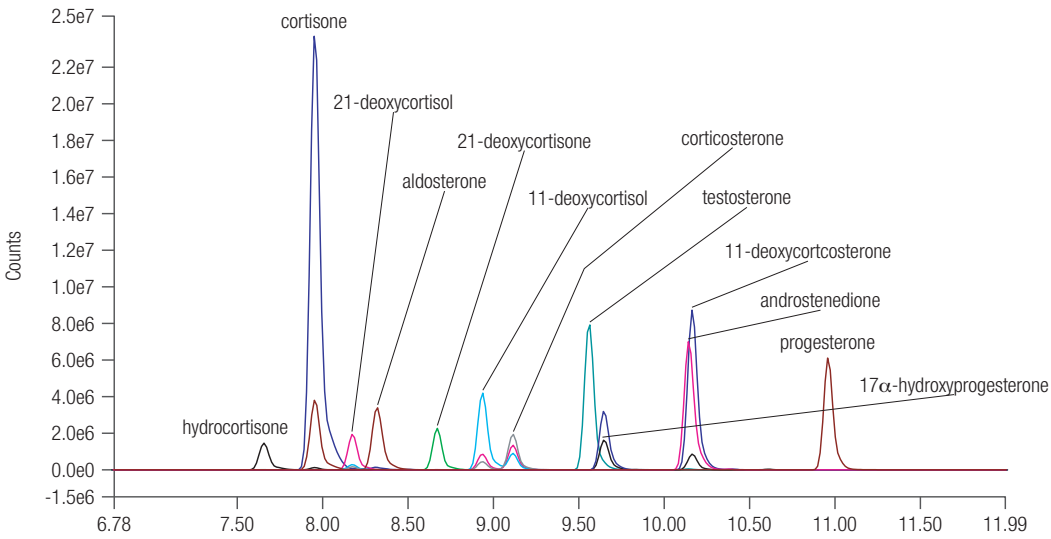

Figure 2. Example chromatogram showing separation of the components

Increased resolution was observed between two of three groups of isomers, along with some changes in elution orders. Figure 3 shows the separation of structural isomers 21-deoxycortisol, 11-deoxycortisol, and corticosterone on biphenyl (left) and C18 (right) phases, all with molar mass 346.467 g/mol. Resolution between each peak is greater on the biphenyl chemistry with the greatest increase between 21-deoxycortisol and 11-deoxycortisol from 1.9 on the C18 to 7.93 on the biphenyl. Figures 4 and 5 show two other critical pairs of isomers and the change in resolution between the two phases.

Biphenyl	C18
Rs_{1-2} 7.43	Rs_{1-3} 1.90
Rs_{2-3} 1.67	Rs_{3-2} 1.36

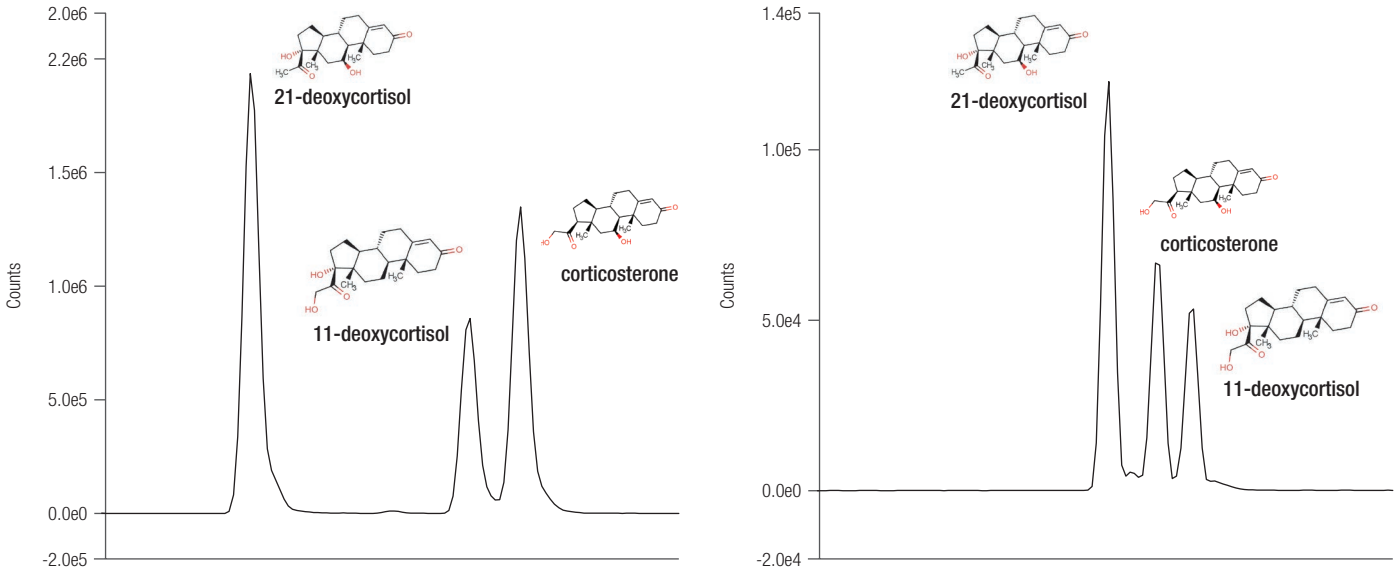

Figure 3. Separation of structural isomers 21-deoxycortisol, 11-deoxycortisol, and corticosterone on biphenyl (left) and C18 (right), all with *m/z* 347.2

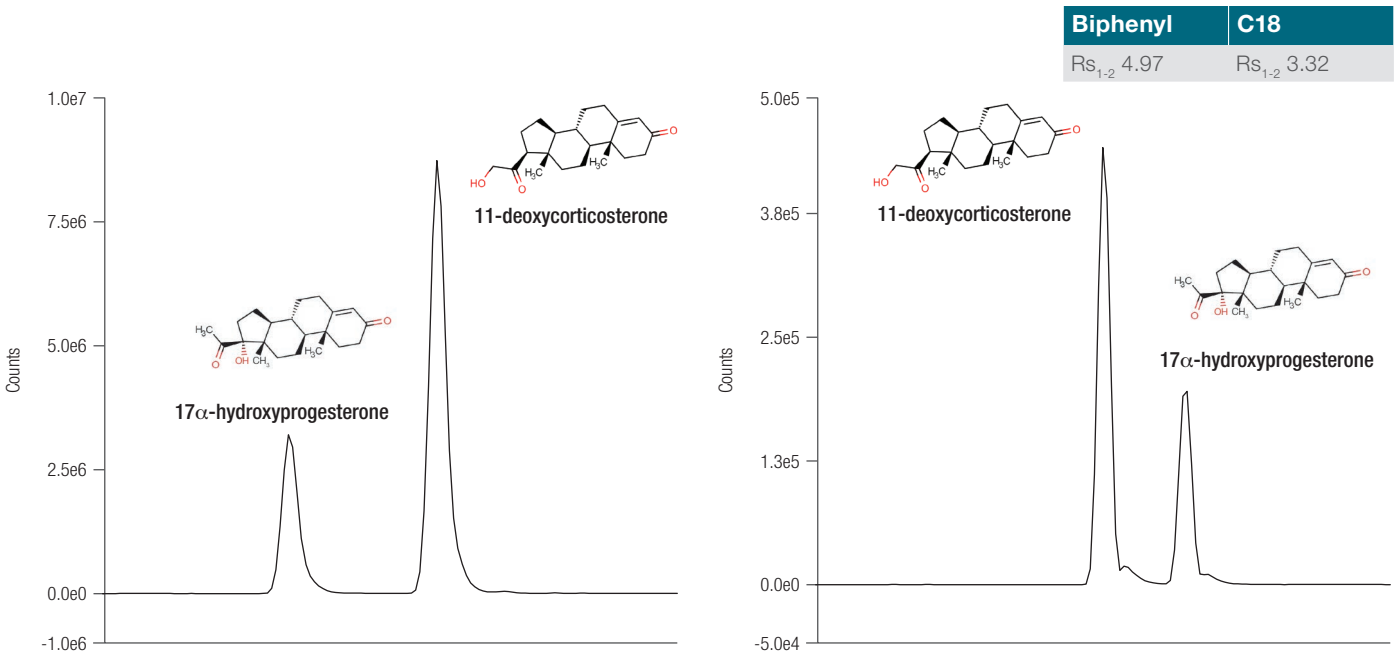

Figure 4. Separation of structural isomers 17 -hydroxyprogesterone and 11-deoxycorticosterone on biphenyl (left) and C18 (right), both with *m/z* 331.2

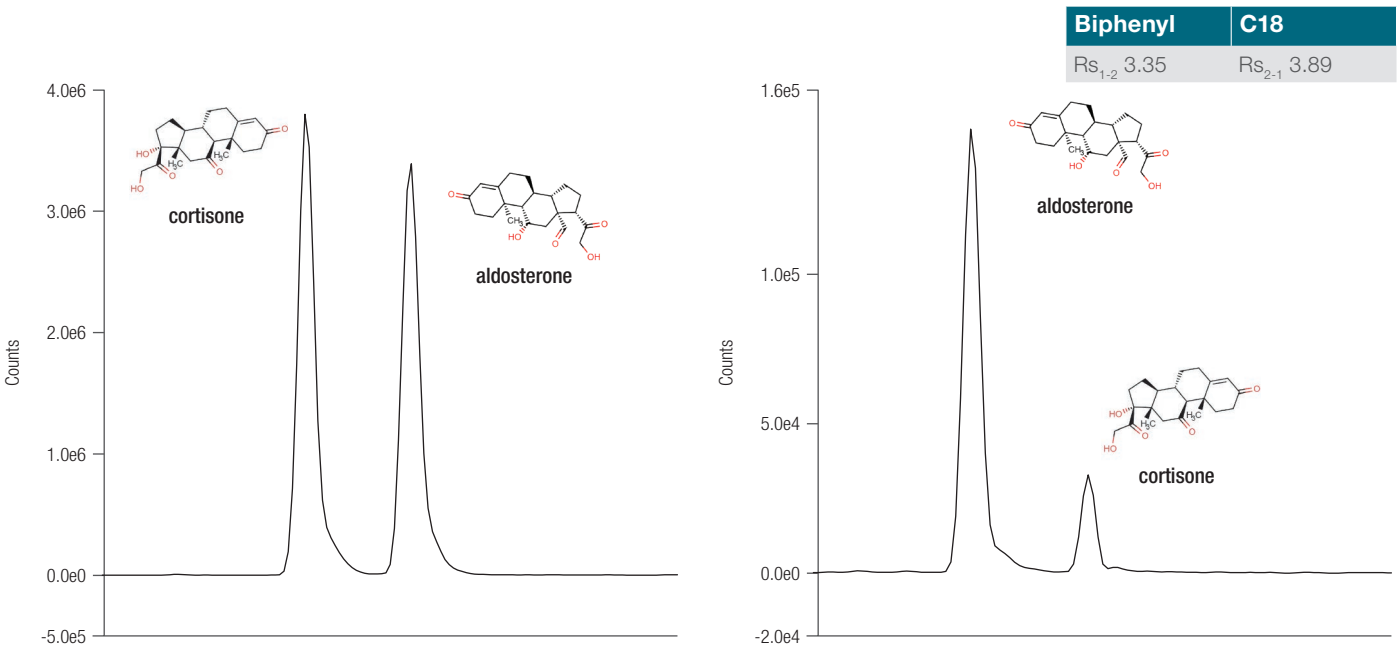

Figure 5. Separation of structural isomers cortisone and aldosterone on biphenyl (left) and C18 (right), both with *m/z* 361.2

Table 3 shows superior peak widths and increased peak capacity of the biphenyl column compared to a C18 column run on the same gradient. The elution order across the compound range also differs between biphenyl and C18 phase. This is highlighted in Figure 6 and can be a useful tool for difficult separations of structurally similar compounds, such as this steroid panel.

Table 3. Average peak widths and peak capacity comparison (refer to Figure 6)

	Biphenyl	C18
Average peak width	0.058	0.066
Gradient time	9.5	9.5
Peak capacity	165	146

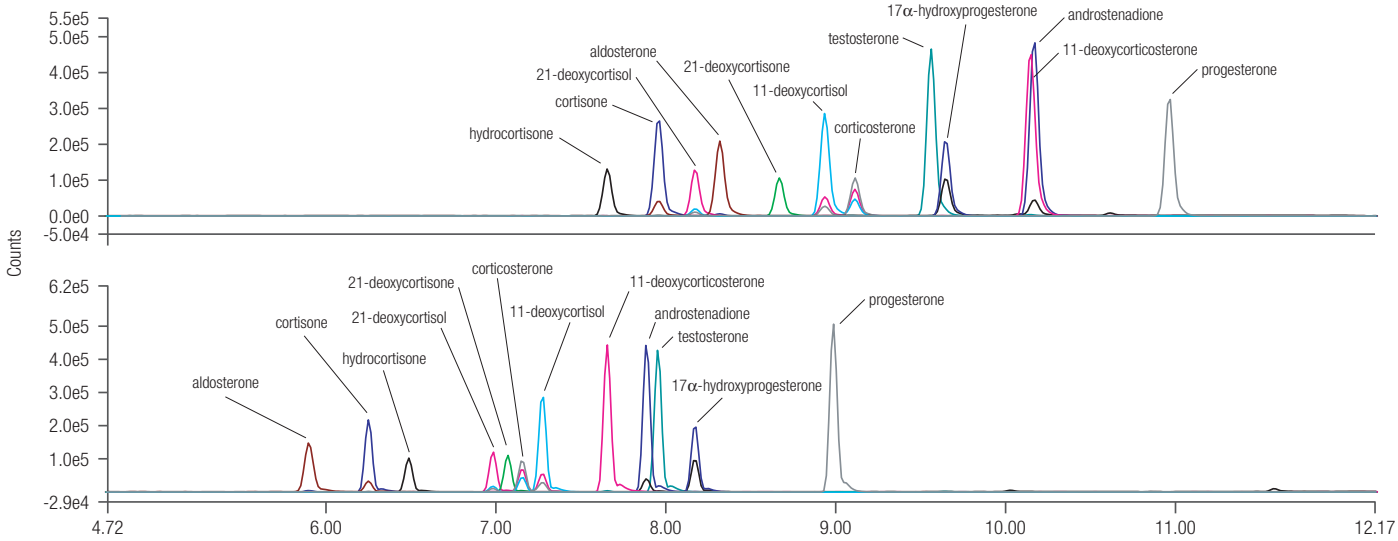

Figure 6. Example chromatogram showing difference in elution orders between biphenyl (top) and C18 (bottom) chemistries

Table 4 shows retention time stability of the QC samples combining PBS and plasma extracts combined. Each compound demonstrated less than 0.099% relative standard deviation (RSD). Stable retention times from both extracted PBS and plasma is critical for confidence in the analytical results, particularly when measuring structural isomers where co-elution can cause inaccurate measurements.

Table 4. Retention time (RT) stability over 25 injections including extracted and non-extracted samples

Compound	Mean RT	%RSD	N
Hydrocortisone	7.65	0.099%	25
Cortisone	7.96	0.034%	25
21-Deoxycortisol	8.18	0.079%	25
Aldosterone	8.32	0.080%	25
21-Deoxycortisone	8.67	0.082%	25
11-Deoxycortisol	8.94	0.095%	25
Corticosterone	9.11	0.053%	25
Testosterone	9.56	0.042%	25
17α-Hydroxyprogesterone	9.65	0.064%	25
11-Deoxycorticosterone	10.17	0.034%	25
Androstenadione	10.17	0.034%	25
Progesterone	10.96	0.054%	25

Excellent accuracy and precision values were observed from QC samples (n=6) as shown in Table 5. Both accuracy and precision displayed single digit values showing the method's ability to record accurate concentration information over the calibration range of 50–50,000 pg/mL. In addition, the coefficient of determination values are displayed showing excellent correlation over the three orders of magnitude range.

Table 5. Accuracy and precision values for each compound showing coefficient of determination of each calibration curve and mean values of the low and high QC levels

Compound	Coefficient of Determination	Mean Precision from Nominal at Low PBS QC (n=6)	Mean Accuracy from Nominal at Low PBS QC (n=6)	Mean Precision from Nominal at High PBS QC (n=6)	Mean Accuracy from Nominal at High PBS QC (n=6)
Hydrocortisone	0.99276	4.21%	102.3%	7.55%	98.3%
Cortisone	0.99487	5.83%	100.6%	2.96%	99.5%
21-Deoxycortisol	0.99820	5.89%	100.3%	5.89%	105.5%
Aldosterone	0.99851	4.62%	102.5%	4.72%	104.5%
21-Deoxycortisone	0.99704	2.74%	95.0%	6.07%	105.9%
11-Deoxycortisol	0.99516	1.30%	107.2%	3.66%	103.0%
Corticosterone	0.99170	4.08%	102.4%	2.49%	105.2%
Testosterone	0.99711	5.77%	97.9%	3.00%	104.9%
17α-Hydroxyprogesterone	0.98220	4.38%	96.5%	4.69%	95.8%
11-Deoxycorticosterone	0.99929	2.73%	98.2%	3.10%	105.5%
Androstenadione	0.99697	4.74%	98.8%	3.92%	100.0%

Plasma QCs were also prepared by standard addition and extracted alongside unspiked plasma for comparison (Table 6). By comparing the spiked to unspiked plasma, the accuracy of the method for extraction of the endogenous compounds can be measured. Good accuracy values were observed across the compound set.

Table 6. Extracted plasma and extracted spiked plasma at 500 pg/mL

Compound	Extracted Plasma Levels (pg/mL)	Spiked Plasma Extracts (pg/mL)	Difference between Spiked and Unspiked Plasma (pg/mL)	Accuracy from Nominal (%)
Hydrocortisone	412	880	469	94%
Cortisone	ND	470	470	94%
21-Deoxycortisol	83	501	418	84%
Aldosterone	ND	529	529	106%
21-Deoxycortisone	ND	498	498	100%
11-Deoxycortisol	240	825	585	117%
Corticosterone	40*	559	519	104%
Testosterone	6*	520	515	103%
17α-Hydroxyprogesterone	80	501	421	84%
11-Deoxycorticosterone	5*	490	486	97%
Androstenedione	5*	516	511	102%

**Extrapolated ND – Not detected*

Conclusions

The Thermo Scientific Accucore Biphenyl column offers alternative selectivity to a C18 column for clinical research. This high-resolution column separates isomers and steroids utilizing superior combination of hydrophobic, aromatic, and polar selectivity.

The analytical method described herein demonstrates:

- Separation of structural isomers for accurate detection
- Alternative selectivity to C18 phase with an increase in overall resolution of structural isomers
- Stable retention time from extracted plasma
- Accurate and precise analytical method across 1000-fold concentration range

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Find out more at **thermofisher.com/biphenyl**
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