Robust Extraction, Separation and Quantitation of Structural Isomer Steroids From Human Plasma by SPE with LC-MS/MS Detection

Jon Bardsley[1], Kean Woodmansey* and Stacy Tremintin[2]; [1]Thermo Fisher Scientific, Tudor Road, Runcorn, UK, WA7 1TA; [2]Thermo Fisher Scientific, 1228 Titan Way, Sunnyvale, California, 94085, USA

ABSTRACT

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 which cannot be differentiated by MS/MS alone. This may lead to inaccurate analysis by over estimation of concentration levels. Separation prior to MS/MS 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 interferences, separate isomers and detect 12 steroids, with an assessment of method performance is reported here.

INTRODUCTION

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 a rugged 2.6 µm solid-core particles 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 are presented to achieve separation of common groups of steroids required for routine analysis. 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.

MATERIALS AND METHODS

Sample Preparation

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 50000 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 method. Blank plasma was also extracted as a baseline concentration as some of the compounds used are endogenous in high concentrations.

Method 1 (ACN)	Method 2 (MeOH)
Cortisol	Hydrocortisone
Corticosterone	Cortisone
Estradiol	21-deoxycortisol
Trembolone	Aldosterone
Nandrolone	21-Deoxycortisone
Testosterone	11-Deoxycortisol
Methyltestosterone	Corticosterone
17, Hydroprogesterone	Testosterone
Androstenedione	17a Hydroxyprogesterone
Progesterone	11-Deoxycorticosterone
25-Hydroxy Vitamin D3	Androstenadione
	Progesterone

Table 1. List of Compounds Determined

400 µL of each sample was mixed 1:1 with zinc sulfate (2%), centrifuged, and the supernatant 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.

HPLC Conditions

Instrumentation:

Thermo Scientific 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 Preheater (P/N 6732.0110)
MS Connection Kit Vanquish (P/N 6720.0405)

Thermo Scientific™ TSQ Quantiva™ Triple-Stage Quadrupole Mass Spectrometer (IQLAAEGAAXFAOUMZZZ)

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

Compound	Polarity	Precursor (m/z)	Product (m/z)
Androstenedione	+ve	287.2	97.1
Testosterone	+ve	289.3	109.1
Testosterone-d3	+ve	292.2	97.1
Progesterone	+ve	315.2	97.1
d9-Progesterone	+ve	324.4	100.2
11-Deoxycorticonsterone	+ve	331.2	97.1
17a OH progesterone	+ve	331.2	313.2
21-Deoxycortisone	+ve	345.1	163.2
11-Deoxycortisol	+ve	347.2	97.1
21-Deoxycortisol	+ve	347.2	311.2
Corticosterone	+ve	347.2	329.2
Cortisone	+ve	361.2	163.2
Aldosterone	+ve	361.2	343.2
Hydrocortisone	+ve	363.2	121.1

Table 2. Compound Transition Details

Data Processing

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

RESULTS

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

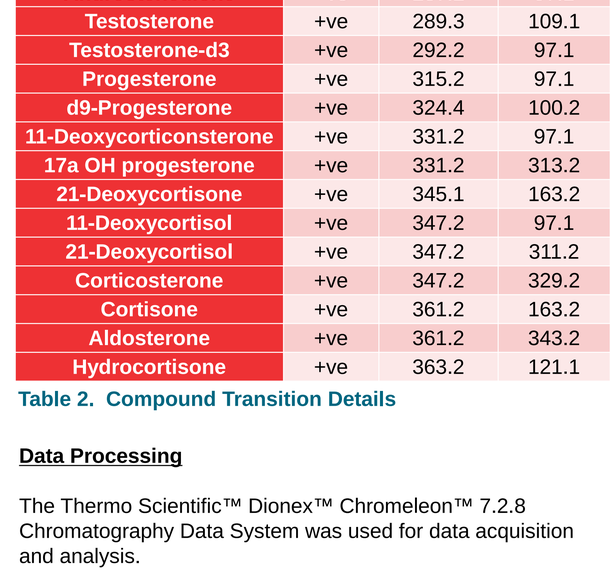

Figure 1. Separation of common steroids of interest with an acetonitrile gradient

Separation of this closely related set of compounds was achieved with a 12 minute gradient demonstrated in Figure 2. Table 3 shows superior peak widths and increased peak capacity of the biphenyl column compared to a C18 using the same gradient.

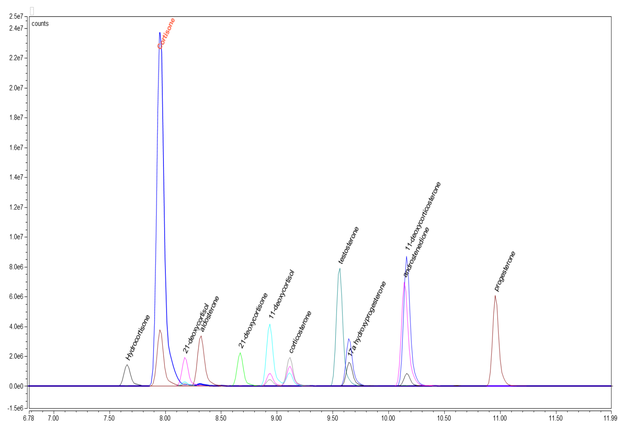

Figure 2. Example chromatogram showing separation of the components

	Biphenyl	C18
Average peak width (min)	0.058	0.066
Gradient time	9.5	9.5
Peak Capacity	165	146

Table 3. Average Peak Widths and Peak Capacity Comparison

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), 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.

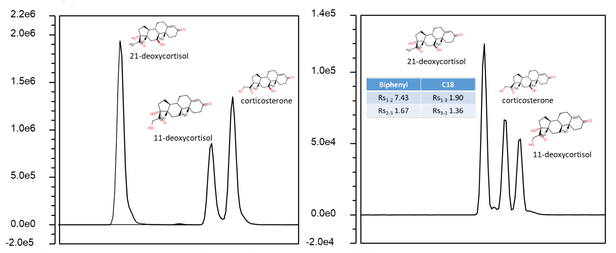

Figure 3. Separation of structural isomers 21-deoxycortisol, 11-deoxycortisol, and corticosterone on biphenyl (left) and C18 (right), all with m/z 347.2

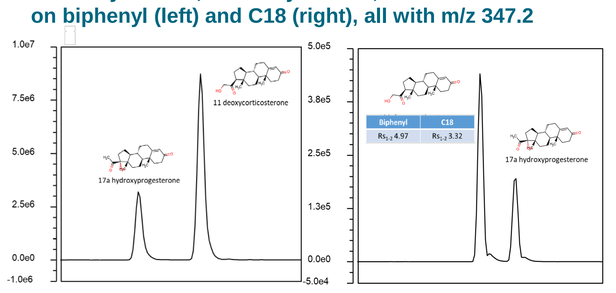

Figure 4. Separation of structural isomers 17α-hydroxyprogesterone and 11-deoxycorticosterone on biphenyl (left) and C18 (right), both with m/z 331.2

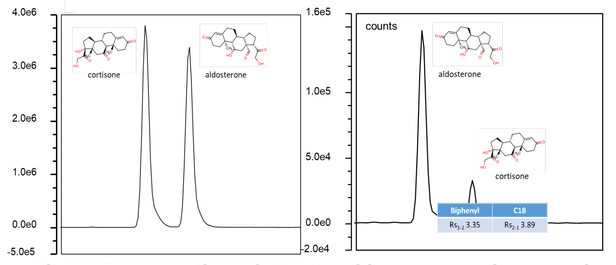

Figure 5. Separation of structural isomers cortisone and aldosterone on biphenyl (left) and C18 (right), both with m/z 361.2

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.

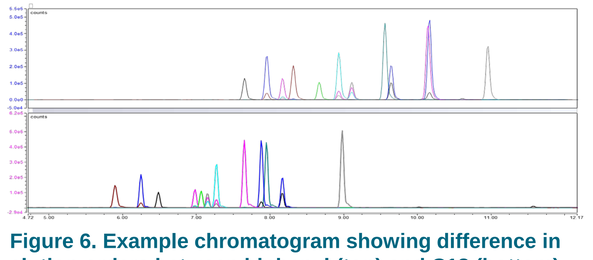

Figure 6. Example chromatogram showing difference in elution orders between biphenyl (top) and C18 (bottom) chemistries

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.

	MeanRT	%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
17a-Hydroxyprogesterone	9.65	0.064%	25
11-Deoxycorticosterone	10.17	0.034%	25
11-Deoxycorticosterone	10.17	0.034%	25
Progesterone	10.96	0.054%	25

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

Excellent accuracy and precision values were observed from QC samples (n=6). Both accuracy and precision displayed single digit values showing the assays ability to record accurate concentration information over the calibration range of 50 – 50000 pg/mL. In addition the coefficient of determination values are displayed showing excellent correlation over the 3 orders of magnitude range.

Compound	Mean Precision Low PBS QC (%)	Mean Accuracy Low PBS QC (%)	Mean Precision High PBS QC (%)	Mean Accuracy High PBS QC (%)
Hydrocortisone	4.21	102.3	7.55	98.3
Cortisone	5.83	100.6	2.96	99.5
21-Deoxycortisol	5.89	100.3	5.89	105.5
Aldosterone	4.62	102.5	4.72	104.5
21-Deoxycortisone	2.74	95.0	6.07	105.9
11-Deoxycortisol	1.30	107.2	3.66	103.0
Corticosterone	4.08	102.4	2.49	105.2
Testosterone	5.77	97.9	3.00	104.9
17a-Hydroxyprogesterone	4.38	96.5	4.69	95.8
11-Deoxycorticosterone	2.73	98.2	3.10	105.5
Androstenadione	4.74	98.8	3.92	100.0

Table 5. A&P values for each compound at low and high QC levels.

CONCLUSIONS

The Thermo Scientific Accucore Biphenyl Column offers alternative selectivity to the 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 with an increase in overall resolution of structural isomers
- Stable retention time from extracted plasma
- Accurate and precise methodology across 1000 fold concentration range

REFERENCES

Find out more at www.thermofisher.com/appslab

Find out more at www.thermofisher.com/biphenyl

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