WHITE PAPER

LC-MS/MS Steroid Analysis Solutions for Clinical Research

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Introduction

Steroid hormones are synthesized in the adrenal cortex, the gonads, and the placenta; are all derived from cholesterol, and many are of clinical importance. Because all steroid hormones are derived from cholesterol, they are not soluble in plasma and other body fluids. As a result, steroids are bound to transport proteins that increase their half-life and ensure ubiquitous distribution. In the past, steroids usually were analyzed individually, using gas chromatography-mass spectrometry (GC-MS) or immunoassay. Immunoassays (IAs) are among the most sensitive and precise analytical methods; however, recent studies showed that many immunoassays lack specificity as a result of cross-reactivity. GC-MS is sensitive and specific, but requires tedious and time-consuming sample preparation. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is more specific and offers simpler approaches to sample preparation without sample derivatization steps. Here, LC column recommendations and chromatographic conditions for a 19-analyte steroid panel and additional sub-groups of steroids are presented along with sample preparation methods using supported liquid extraction (SLE) and solid phase extraction (SPE). Specific recommendations for male and female hormones in addition to cortisol are included.

LC-MS/MS Selectivity Screening

Steroids are a class of structurally related endogenous compounds. Several have the same chemical formula, and therefore the same m/z, and must be chromatographically separated for accurate identification and quantitation by LC-MS/MS. Labs desire high-throughput methods which consolidate analytes into one panel with fast chromatographic run times. Meeting these criteria can be challenging in a single LC-MS/MS method. Here, six bonded silica solid core or "core-shell" LC columns (**Table 1**) were evaluated to determine selectivity and optimum separation conditions for a 19-analyte steroid panel (**Table 2**) for clinical research. The optimum column for the separation of Estrone and Estradiol was also established. **Figure 1** demonstrates the different interactions that functional groups on the steroid molecules have with the different LC stationary phases.

Table 1. Core-shell Columns Screened for Selectivity of Steroid Analytes.

Phase	Phase Chemistry	Property
C18	C18	Traditional bonded silica C18
XB-C18	C18 modified with di-isobutyl side chains	Inactive surface, selectivity for acidic compounds
EVO C18	C18 with organo-silica and stabilizing ethane crosslinking	1-12 pH stable bonded C18
Polar C18	C18 modified with polar groups	100% aqueous stable, improved polar analyte retention
Biphenyl	Silica modified with biphenyl groups	Improved aromatic and polar analyte selectivity
Phenyl-Hexyl	Silica modified with phenyl-hexyl groups	Balanced hydrophobic and aromatic selectivity

Table 2. 19-Analyte Steroid Panel.

Label	Steroid Analytes – Positive Mode	Molecular Weight
1	Androstenedione (A4)	286.41
2	Dehydroepiandrosterone (DHEA)	288.42
3	Testosterone	288.42
4	Dihydrotestosterone (DHT)	290.44
5	Progesterone	314.46
6	Pregnenolone	316.48
7	11-Deoxycorticosterone	330.46
8	17-OH-Progesterone	330.46
9	11-Deoxycortisol	346.46
10	21-Deoxycortisol	346.46
11	Corticosterone	346.46
12	Cortisone	360.44
13	18-OH-Corticosterone	362.46
14	Cortisol	362.46

Label	Steroid Analytes – Negative Mode	Molecular Weight
1	Estrone (E1)	270.37
2	Estradiol (E2)	272.38
3	17-OH-Pregnenolone	332.48
4	Aldosterone	360.44
5	Dehydroepiandrosterone Sulfate (DHEAS)	391.48

Revision: 0
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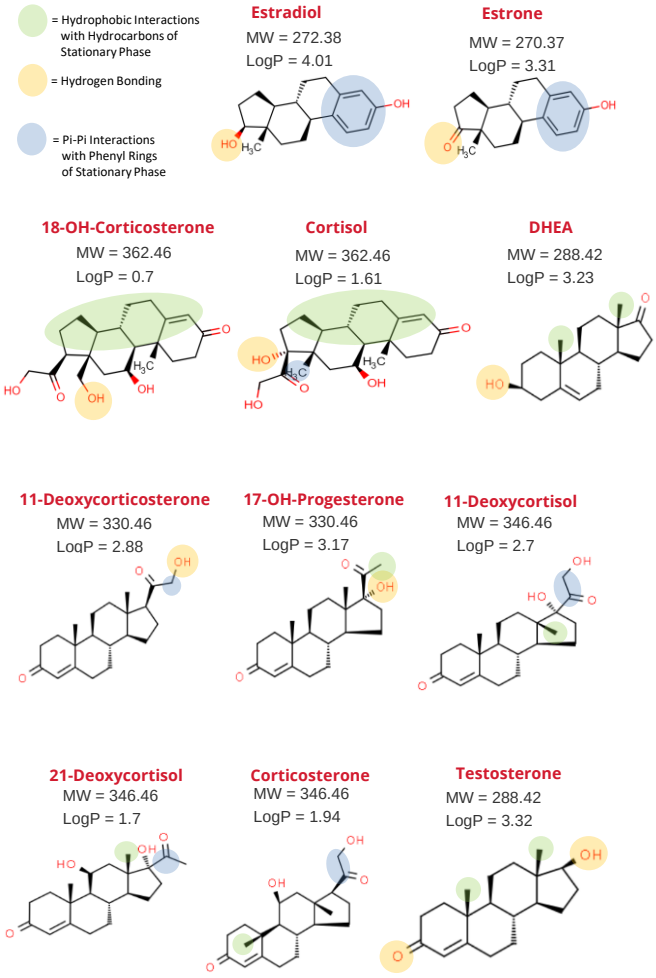
Figure 1. Selected Steroid Structures and Interaction with Screened Stationary Phases.



LC Conditions

Columns: Kinetex 2.6 µm C18 (00B-4462-AN)
Kinetex 2.6 µm XB-C18 (00B-4496-AN)
Kinetex 2.6 µm EVO C18 (00B-4725-AN)
Kinetex 2.6 µm Polar C18 (00B-4759-AN)
Kinetex 2.6 µm Biphenyl (00B-4622-AN)
Kinetex 2.6 µm Phenyl-Hexyl (00B-4495-AN)

Dimensions: 50 x 2.1 mm

Mobile Phase: A: 0.5 mM Ammonium Fluoride in Water
B: Methanol

Gradient:

Time (min)	%B
0	40
6.5	95
7	95
7.5	40
8	40

Flow Rate: 0.6 mL/min
Injection Volume: 5 µL
Temperature: 40 °C
LC System: Agilent® 1290 Infinity
Detection: MS/MS
Detector: SCIEX® 7500 Triple Quad™

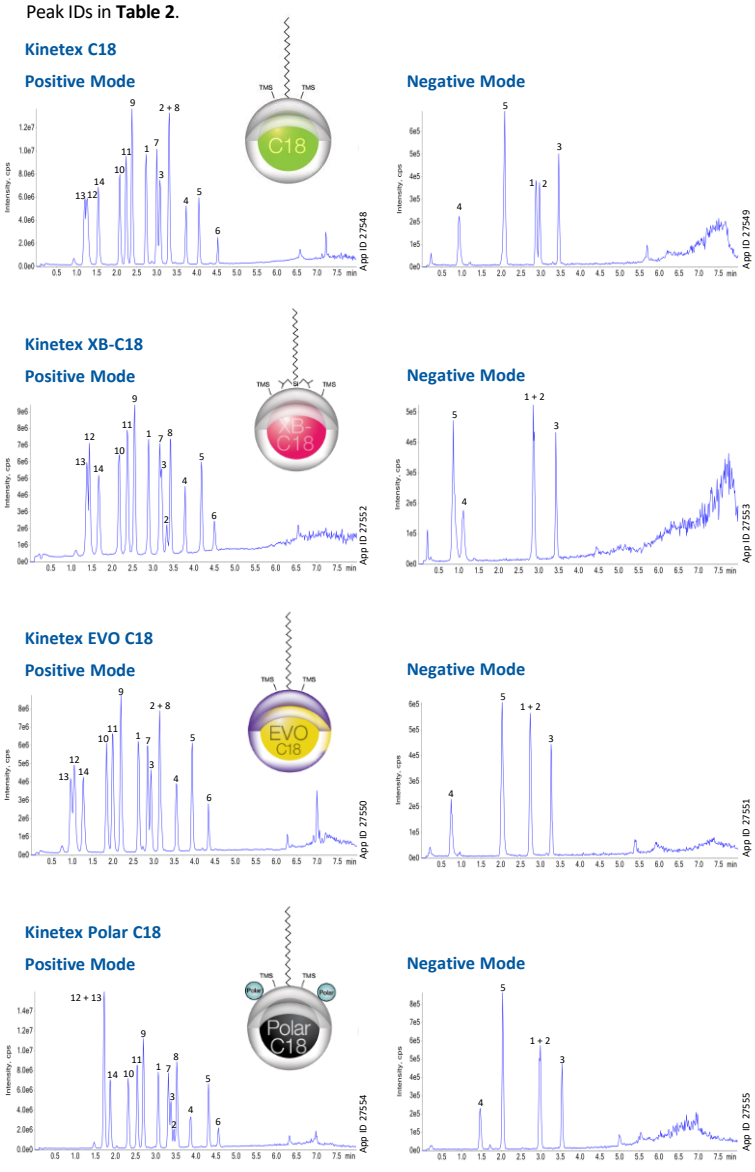
MS/MS Conditions

Ion Source: ESI
Polarity: Positive or Negative
Source Temperature: 700 °C
GS1: 60 psi
GS2: 60 psi
CUR: 40 psi
CAD: 10
IS: 3000 V or -3000 V
EP: 10 V or -10 V

The traditional Kinetex™ C18 column worked best to chromatographically separate the complete steroid clinical research panel. Estrogens had the best separation on the Biphenyl phase, which demonstrated the highest level of selectivity for Estrone and Estradiol. For a panel of androgens including Testosterone, Androstenedione, DHT, DHEA, and DHEAS, the traditional C18 or EVO C18 would be the best option. For a panel of adrenal steroids including 11-Deoxycortisol, 17-Hydroxyprogesterone, 17-Hydroxypregnenolone, and Pregnenolone, any of the above Kinetex core-shell columns have the correct selectivity to separate the analytes.

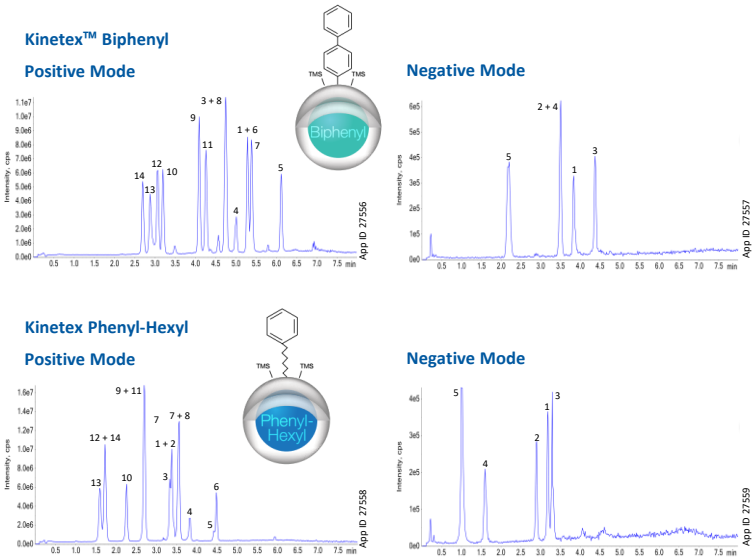
The Phenyl-Hexyl column did not sufficiently separate all steroid isomers in the clinical research panel. However, it was sufficient to separate Estrone and Estradiol. For the isomers Cortisol and 18-Hydroxycorticosterone, the traditional C18 and the specialized EVO C18 and Polar C18 phases showed the greatest selectivity and best separation. The Phenyl-Hexyl phase did not provide full baseline separation of the isomers Testosterone and DHEA, nor did it resolve Corticosterone and 11-Deoxycortisol. 11-Deoxycorticosterone was not separated from 17-OH-Progesterone with the Phenyl-Hexyl column. The chromatograms for all six columns screened for the 19-analyte panel in positive and negative ionization mode can be seen in **Figure 2**. The HPLC conditions presented for the Kinetex core-shell 2.6 µm C18, 50 x 2.1 mm column provided the best separation for all 19 steroids with a fast 8-minute run time.

Figure 2. Chromatographic Profiles of Steroid Analytes Using Core-shell columns. Peak IDs in **Table 2**.



Have questions or want more details on implementing this method? We would love to help!
Visit www.phenomenex.com/Chat to get in touch with one of our Technical Specialists

Figure 2 Cont'd. Chromatographic Profiles of Steroid Analytes Using Core-shell columns.



After extensive screening, the following LC conditions and column were determined to be the most suitable for steroid panel analysis and were used for determining the optimal sample preparation protocol:

LC Conditions

Column: Kinetex 2.6 µm C18
Dimensions: 50 x 3.0 mm
Part No.: 00B-4462-Y0
Mobile Phase: A: 0.5 mM Ammonium Fluoride in Water
B: Methanol

Gradient:

Time (min)	%B
0	40
2	50
4.5	75
5	95
6	95
6.5	40
8	40

Flow Rate: 0.8 mL/min
Injection Volume: 5 µL
Temperature: 30 °C
LC System: Agilent® 1290 Infinity
Detection: MS/MS
Detector: SCIEX® 7500 Triple Quad™

MS/MS Conditions

Ion Source: ESI
Polarity: Positive or Negative
Source Temperature: 700 °C
GS1: 60 psi
GS2: 60 psi
CUR: 40 psi
CAD: 10
IS: 3000V or -3000V
EP: 10 V or -10 V

Steroid Panel Solid Phase Extraction (SPE)

Steroid analysis for clinical research can require very low limits of detection which can be accomplished using solid phase extraction (SPE) to yield high recoveries and very clean extracted samples, free from matrix interferences.

Sample Preparation

Six calibrators and all spiked samples were prepared in stripped serum. The 19 analytes were separated into two sets to correspond to the standard concentration ranges for clinical research methods for each given steroid; one set (low level analytes) at pg/mL concentrations and another set (high level analytes) at ng/mL concentrations. All samples were pre-treated with 1 % Formic Acid prior to analysis. No other pretreatments were assessed.

Several washes combinations were considered. First, Methanol in Water starting at 10 % Methanol, increasing to 90 % Methanol in increments of 10 %, were evaluated. The best Water / Methanol wash that yields high recoveries for the entire panel was 30 % Methanol in Water. Two additional two-step wash protocols, 1 % Formic Acid in Water followed by 30 % Methanol in Water, and 30 % Methanol in Water followed by Hexane, were also studied. The best overall wash solvent combination that cleaned up the sample and still yielded high recoveries was 1 % Formic Acid in Water followed by 30 % Methanol in Water. Once the best wash combinations were determined, five elution solvent combinations were explored: 1:1, 1:4, and 1:9 Methanol/Acetonitrile, 2 % Formic Acid in Methanol, and Ethyl Acetate. The best elution solvent for the entire 19-analyte panel was Methanol / Acetonitrile (1:4, v/v). The optimized protocol is listed below:

Step	Description
Sample Pre-treatment:	Combine 500 µL of serum sample and 500 µL of 1 % Formic Acid in Water.
Condition:	Strata™-X 33 µm Polymeric Reversed Phase, 30 mg 96-well plate (Part No.: 8E-S100-TGB) with 1 mL of Methanol.
Equilibrate:	1 mL Water.
Load:	1 mL of pre-teated sample spiked with 10 µL of internal standard.
Wash 1:	1 mL 1 % Formic Acid in Water.
Wash 2:	1 mL 30 % Methanol in Water, apply vacuum for 3-4 min at 15-20 in Hg.
Elute:	2x 500 µL of Methanol / Acetonitrile (1:4, v/v).
Dry:	Under a gentle stream of Nitrogen at 45 °C.
Reconstitute:	100 µL of 0.5 mM Ammonium Fluoride in [Water / Methanol (60:40, v/v)].

Recovery, Matrix Effect, and Process Efficiency were determined by preparing spiked samples at three concentrations (Low, Mid, and High; **Tables 4** and **5**) for the pg/mL analytes and the ng/mL analytes. A set of spiked samples at each concentration level for each group of analytes were prepared in triplicate: samples spiked with analytes before extraction (pre-spiked samples, *PS*) and samples spiked after extraction (post-spiked samples, *PoS*). Unextracted samples containing the analytes from each group prepared in reconstitution solvent were also prepared at each of the three concentration levels in duplicate. All samples were injected in duplicate (n=4 for unextracted samples and n=6 for pre-spiked and post-spiked samples).

The Lower Limit of Quantitation (LLOQ, the lowest calibrator) for the pg/mL analytes were initially chosen based on typical limits of quantitation required for clinical research. Final LLOQ concentrations were determined by choosing the lowest concentration that produced a linear calibration curve with a R^2 of >0.99. All peaks at the LLOQ had a signal to noise (S/N) ratio of at least 3:1 and met all other LC-MS/MS criteria (correct retention time, ion ratios within 20 %, etc.). LLOQs for the ng/mL analytes were determined by extracting duplicate samples at progressively lower concentrations. All analyte peaks for these steroids at the LLOQ had at least a 10:1 S/N ratio and met all other LC-MS/MS criteria.

Recovery, Matrix Effect, and Process Efficiency of each analyte, at each concentration, were calculated based on work by Matuszewski, *et al.*[1] All values are reported as a percent.

$$Recovery = \left[\frac{Average\ Area\ Counts\ of\ PS\ Samples}{Average\ Area\ Counts\ of\ PoS\ Samples}\right] x\ 100$$

Recovery measures the percent of analyte recovered from the SPE extraction.

$$Matrix\ Effect = \left[\frac{Average\ Area\ Counts\ of\ PoS\ Samples}{Average\ Area\ Counts\ of\ Unextracted\ Samples}\right] x\ 100$$

Matrix Effect measures any changes in response related to ion suppression or ion enhancement from the mass spectrometer because of the matrix.

$$Process\ Efficiency = \left[\frac{Average\ Area\ Counts\ of\ PS\ Samples}{Average\ Area\ Counts\ of\ Unextracted\ Samples}\right] x\ 100$$

Process efficiency is a measurement of differences in response from recovery and matrix effect combined.

Results

The Kinetex™ 2.6 µm C18 column provided a fast 8-minute chromatographic separation and good selectivity for the entire steroid panel in both positive ion mode and negative ion mode (**Figure 3**). All isomers with the same m/z were chromatographically resolved. Calibration curves (**Figure 4**), with a linear fit and 1/x weighting, showed good linearity with R^2 values of >0.99 for all analytes (**Table 3**). Accuracy of spiked, stripped serum samples in triplicate at three concentrations were within 80-120 %. Recovery of the lowest concentration spiked samples were 81-116 % for all analytes except DHEAS which was 54 %. Recovery of mid-concentration spiked samples were 80-96 % for all analytes except DHEAS which was 61 %. Recovery of high-concentration spiked samples were 82-102 % for all analytes except DHEAS which was 59 % (**Table 4**). To improve recovery of DHEAS, a different elution solvent should be considered. Precision calculated as %RSD of spiked samples were <15 % for all analytes (**Table 5**). For select groups of steroid analytes, a stronger organic wash may reduce matrix effect without sacrificing recovery.

Figure 3. Total Ion Chromatograms (TICs) of Steroid Isomers Fully Separated in an 8-minute Method Using a Kinetex C18 Column.

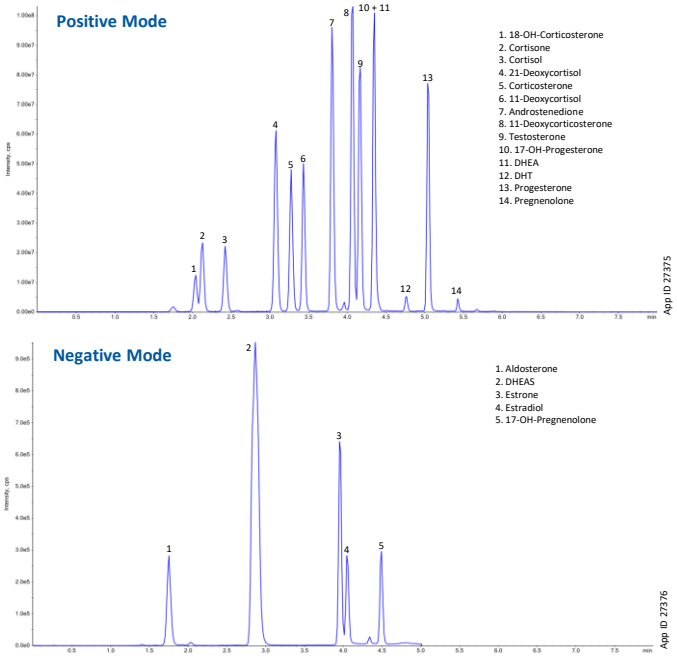


Figure 4. Example Calibration Curves.

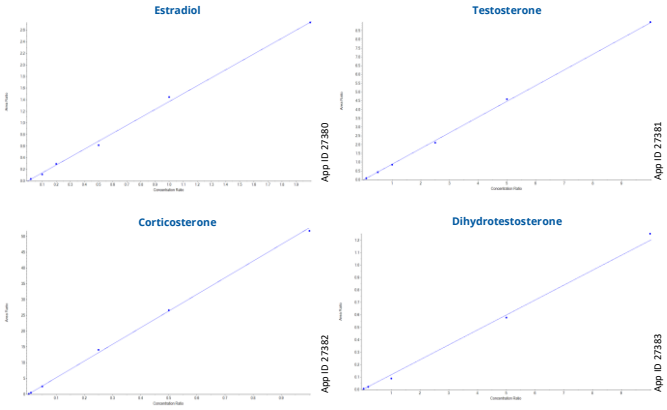


Table 3. SPE Analyte Calibration Curve, LLOQ, and S/N Data.

Analyte	Calibration Equation	R^2	LLOQ (pg/mL)	S/N
11-Deoxycorticosterone	y=0.91019x - 0.01371	>0.999	10	15.8
11-Deoxycortisol	y=0.92022x + 0.01119	>0.999	10	22.4
17-OH-Progesterone	y=1.09404x - 0.04160	>0.996	50	6.9
18-OH-Corticosterone	y=0.24256x + 0.00335	>0.997	50	8.3
21-Deoxycortisol	y=0.60591x + 0.00786	>0.996	10	8.2
Aldosterone	y=0.20181x + 0.00135	>0.998	10	3.2
Androstenedione	y=0.64551x + 0.01859	>0.999	10	20.5
Estradiol	y=1.36625x + 0.00205	>0.995	10	13.4
Estrone	y=0.98883x + 0.00741	>0.993	10	37.9
Progesterone	y=2.40103x + 0.09331	>0.995	10	14.6
Testosterone	y=0.89557x - 0.01050	>0.999	10	29.0
Analyte	**Calibration Equation**	**R^2**	**LLOQ (ng/mL)**	**S/N**
Corticosterone	Y=52.77043x – 0.02846	>0.999	0.05	34.4
Cortisol	Y=13.29607x + 0.18652	>0.999	N/A*	N/A*
Cortisone	Y=0.35557x – 0.00735	>0.997	0.2	13.1
DHEA	Y=13.50761x - 0.07797	>0.996	1	27.1
DHEAS	Y=16.81832x – 0.08771	>0.995	N/A*	N/A*
Dihydrotestosterone	Y=0.12000x - 0.00106	>0.993	1	10.3
Pregnenolone	Y=0.21205x + 0.03652	>0.992	5	6.0

*Note: LLOQ data is not available for DHEAS as it was not possible to obtain DHEAS-free serum. LLOQ data is not available for Cortisol because appropriate testing levels are much higher tan the detection capabilities of the instrument.

Figure 5. Peak Examples at LLOQ.

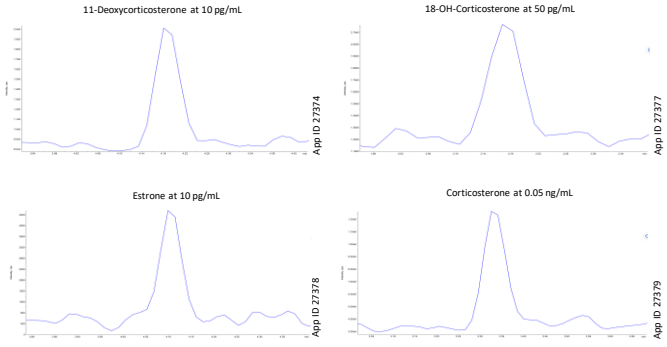


Table 4. SPE Recovery and Accuracy Data.

	Low Concentration			Mid Concentration			High Concentration		
Analyte	Expected (pg/mL)	Recovery (%)	Accuracy (%)	Expected (pg/mL)	Recovery (%)	Accuracy (%)	Expected (pg/mL)	Recovery (%)	Accuracy (%)
11-Deoxycorticosterone	50	115 ± 10.3	91.3	100	91 ± 3.5	90.8	200	95 ± 3.7	80.2
11-Deoxycortisol	250	107 ± 13.7	95.9	500	89 ± 4.5	99.8	1000	97 ± 5.4	91.1
17-OH-Progesterone	250	110 ± 12.3	88.5	500	95 ± 4.1	94.6	1000	96 ± 3.3	86.5
18-OH-Corticosterone	250	116 ± 13.2	103.1	500	89 ± 6.3	97.5	1000	96 ± 5.9	90.1
21-Deoxycortisol	250	115 ± 14.2	98.8	500	81 ± 8.0	88.0	1000	95 ± 7.3	85.6
Aldosterone	250	113 ± 12.1	94.5	500	93 ± 7.7	103.0	1000	98 ± 4.5	91.9
Androstenedione	50	114 ± 10.1	87.6	100	92 ± 4.5	92.0	200	97 ± 5.0	83.1
Estradiol	50	106 ± 13.1	81.0	100	90 ± 8.7	83.4	200	102 ± 7.1	81.0
Estrone	50	114 ± 10.5	87.7	100	91 ± 5.5	96.1	200	95 ± 4.3	86.4
Progesterone	250	103 ± 11.2	88.2	500	88 ± 5.1	89.5	1000	92 ± 1.9	80.2
Testosterone	50	107 ± 8.3	92.8	100	96 ± 2.2	96.5	200	100 ± 3.8	85.0
Analyte	Expected (ng/mL)	Recovery (%)	Accuracy (%)	Expected (ng/mL)	Recovery (%)	Accuracy (%)	Expected (ng/mL)	Recovery (%)	Accuracy (%)
Corticosterone	0.25	107 ± 6.7	101.1	0.5	92 ± 4.5	101.1	1	91 ± 6.6	96.7
Cortisol	1	108 ± 7.8	101.4	2	90 ± 4.5	113.4	4	92 ± 5.9	114.3
Cortisone	1	100 ± 3.2	116.5	2	91 ± 4.4	90.5	4	95 ± 4.5	80.5
DHEA	1	81 ± 13.6	117.9	2	80 ± 13.2	118.1	4	82 ± 12.5	117.4
DHEAS	1	54 ± 4.5	98.2	2	61 ± 2.7	91.3	4	59 ± 0.7	87.0
DHT	5	87 ± 7.6	92.8	10	85 ± 4.1	86.0	20	86 ± 6.4	85.1
Pregnenolone	5	88 ± 4.4	114.1	10	84 ± 13.7	103.5	20	87 ± 6.6	105.7

Table 5. SPE Precision, Matrix Effect, and Process Efficiency Data.

	Low Concentration			Mid Concentration			High Concentration		
Analyte	Precision (%RSD)	Matrix Effect	Process Efficiency	Precision (%RSD)	Matrix Effect	Process Efficiency	Precision (%RSD)	Matrix Effect	Process Efficiency
11-Deoxycorticosterone	10.28	0.369	0.426	3.49	0.507	0.460	3.71	0.500	0.476
11-Deoxycortisol	13.75	0.377	0.404	4.46	0.497	0.445	5.40	0.462	0.446
17-OH-Progesterone	12.26	0.157	0.173	4.10	0.218	0.207	3.32	0.233	0.224
18-OH-Corticosterone	13.23	0.365	0.424	6.35	0.473	0.420	5.92	0.397	0.381
21-Deoxycortisol	14.17	0.383	0.439	7.96	0.500	0.407	7.31	0.480	0.454
Aldosterone	12.07	0.376	0.425	7.69	0.509	0.473	4.48	0.455	0.446
Androstenedione	10.12	0.360	0.409	4.54	0.496	0.453	5.00	0.492	0.478
Estradiol	13.07	0.461	0.486	8.73	0.524	0.473	7.09	0.442	0.449
Estrone	10.47	0.434	0.496	5.52	0.520	0.474	4.32	0.461	0.440
Progesterone	11.20	0.372	0.384	5.09	0.449	0.394	1.89	0.413	0.380
Testosterone	8.31	0.417	0.445	2.17	0.507	0.485	3.79	0.499	0.497
Analyte	Precision (%RSD)	Matrix Effect	Process Efficiency	Precision (%RSD)	Matrix Effect	Process Efficiency	Precision (%RSD)	Matrix Effect	Process Efficiency
Corticosterone	6.70	0.703	0.752	4.54	0.776	0.715	6.62	0.798	0.724
Cortisol	7.75	0.696	0.754	4.47	0.781	0.705	5.89	0.838	0.774
Cortisone	3.24	0.739	0.739	4.36	0.837	0.766	4.51	0.862	0.816
DHEA	13.57	0.212	0.171	13.23	0.207	0.166	12.48	0.234	0.193
DHEAS	4.46	0.773	0.421	2.66	0.835	0.511	0.74	0.753	0.446
DHT	7.56	0.761	0.663	4.12	0.840	0.712	6.38	0.794	0.686
Pregnenolone	4.35	0.995	0.878	13.73	1.176	0.985	6.58	1.033	0.900

The developed SPE method is effective and reproducible for cleanup of blood serum samples. Clean samples are needed for low level detection of steroids and to provide accurate LC results. Strata™-X, 30 mg 96-well plate allows for high-throughput and a fast method. The Kinetex™ 2.6 µm C18, 50 x 3.0 mm LC column provides full chromatographic separation of all steroid analytes, with resolution of all isomers, in just 8 minutes allowing for accurate quantitation using LC-MS/MS. For labs interested in quantitation of 17-OH-Pregnenolone, further SPE method development is needed to eliminate an interference. An additional wash or a different elution solvent may achieve this.

Steroid Panel Supported Liquid Extraction (SLE)

SLE of serum using can provide very clean extracted samples for steroid analysis with less method development and a more streamlined workflow than SPE.

Sample Preparation

Two sets of calibrators and spiked samples were prepared in stripped serum to include low level (pg/mL) analytes and high level (ng/mL) analytes. Five or six calibrators were prepared dependent on appropriate concentration ranges for each analyte. A spiked standard, negative control, matrix blank, and extraction blank were prepared. Two pre-treatments were examined for the Novum PRO SLE method: 2 % Formic Acid in Water and pH 6 Ammonium Acetate buffer. Six elution solvents were evaluated: Dichloromethane, Ethyl Acetate, 1:3 Hexane/ Ethyl Acetate (1:3, v/v), Hexane /Ethyl Acetate (1:4, v/v), Dichloromethane / Ethyl Acetate (90:10, v/v), and Dichloromethane / Ethyl Acetate (50:50, v/v). All 6 elution solvents were tested with both pre-treatments, resulting in 12 combinations total. The best pre-treatment and elution solvent combination for the 19-analyte steroid panel was the pH 6 Ammonium Acetate buffer and either Dichloromethane or Ethyl Acetate for elution. The optimized protocol is listed below:

Step	Description
Sample Pre-treatment:	Combine 200 µL of serum sample and 200 µL of pH 6 Ammonium Acetate in Water.
Load:	400 µL of preteated sample onto a Novum™ PRO SLE, MAX 96-well plate (Part No.: 8E-S539-5GA).
Apply:	Vacuum briefly. Wait 5 minutes before eluting.
Elute 1:	900 µL of Dichloromethane or Ethyl Acetate. Briefly apply vacuum.
Elute 2:	900 µL of Dichloromethane or Ethyl Acetate (same solvent used for **Elute 1**). Briefly apply vacuum.
Dry:	Under a gentle stream of Nitrogen at 45 °C
Reconstitute:	100 µL of 0.5 mM Ammonium Fluoride (aq) / Methanol (60:40, v/v)

For low level steroid analytes (pg/mL): Concentration ranged from 10-3000 pg/mL, dependent on appropriate level for each analyte. Concentrations for spiked standards were 100, 200, 40, or 1500 pg/mL, depending on the analyte. Internal standard was spiked in all calibrators, standards, and negative controls at 200 pg/mL.

For high level steroid analytes (ng/mL): Concentration ranges from 0.1 - 1000 ng/mL dependent on appropriate level for each analyte. Concentrations for spiked standards were 2.5, 5, 15, 25, or 50 ng/mL, dependent on analyte. Internal standard was spiked in all calibrators, standards, and negative controls at 10 or 20 ng/mL, dependent on analyte.

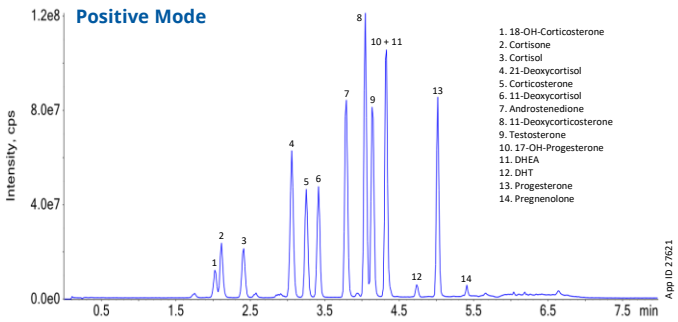
The Lower Level of Quantitation (LLOQ, the lowest calibrator) for the pg/mL analytes were initially chosen based on typical limits of quantitation required for clinical research. Final LLOQ concentrations were determined by choosing the lowest concentration that produced a linear calibration curve with a R^2 of >0.99. All peaks at the LLOQ had a S/N ratio of at least 3:1 and met all other LC-MS/MS criteria (correct retention time, ion ratios within 20 %, etc.). LLOQs for the ng/mL analytes were determined by extracting duplicate samples at progressively lower concentrations. All analyte peaks for these steroids at the LLOQ had at least a 10:1 S/N ratio and met all other LC-MS/MS criteria.

Results

The same Kinetex™ 2.6 µm C18 column method used for SPE method development provided a fast 8-minute chromatographic separation and good selectivity for steroid analytes in both positive ion mode and negative ion mode (**Figure 6**). Calibration curves (**Figure 7**), with a linear fit and 1/x weighting, showed good linearity with R^2 of >0.99 for all analytes except 17-OH-Pregnenolone and DHEAS when using the Dichloromethane as the elution solvent, and R^2 of >0.980 for all analytes when using Ethyl Acetate as the elution the solvent (**Table 6**). Accuracy of samples using Dichloromethane as the elution solvent were 80-112 %, except for 17-OH-Pregnenolone and DHEAS (**Table 7**). Accuracy of all samples using the Ethyl Acetate elution solvent were 91-118 %.

Percent recovery ranged from 13-87 % using Dichloromethane as the elution solvent (**Table 7**). Some analytes had notably lower recoveries using Dichloromethane: 18-OH Corticosterone (13 %), Aldosterone (21 %), Progesterone (28 %), Cortisol (24 %), and Cortisone (52 %). Ethyl Acetate yielded recoveries were 84-110 % for all analytes except DHEAS. DHEAS was not recovered using SLE. SPE would need to be used for samples where DHEAS quantitation is desired.

Figure 6. Total Ion Chromatograms (TICs) of Steroid Isomers Fully Separated in an 8-minute Method Using a Kinetex C18 Column.



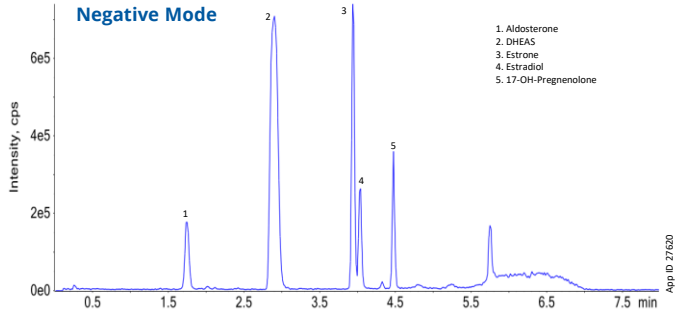


Figure 7. Example Calibration Curves.

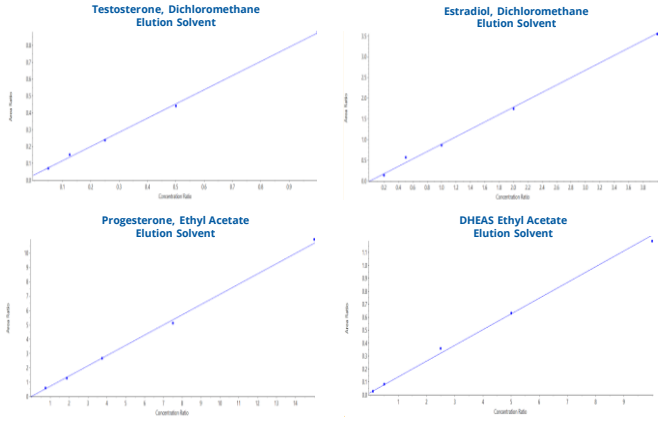
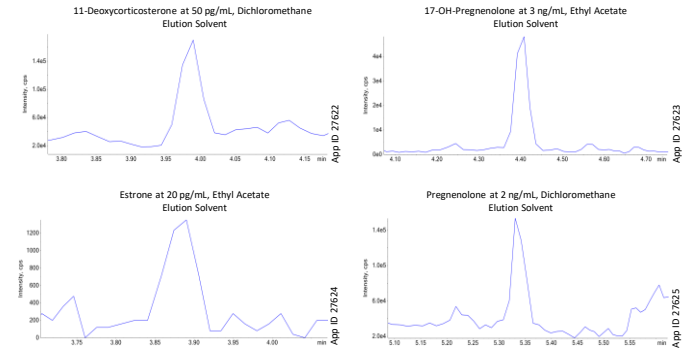


Table 6. SLE Analyte Calibration Curve, LLOQ, and S/N Data.

	Dichloromethane				Ethyl Acetate			
Analyte	Calibration Equation	R^2	LLOQ (pg/mL)	S/N	Calibration Equation	R^2	LLOQ (pg/mL)	S/N
11-Deoxycorticosterone	y = 0.312x - 0.008	0.987	50	5.8	y = 0.241x - 0.055	0.999	100	4.4
11-Deoxycortisol	y = 0.761x + 0.017	0.999	20	21.4	y = 0.768x + 0.026	0.999	20	12.3
17-OH-Progesterone	y = 0.998x - 0.104	0.993	150	18.7	y = 0.872x + 0.263	0.991	150	8.2
18-OH-Corticosterone	y = 0.018x - 0.052	0.869	750	8.1	y = 0.060x + 0.005	0.995	150	7.7
21-Deoxycortisol	y = 2.034x + 0.123	0.993	40	27.2	y = 2.627x + 0.355	0.992	40	16.2
Aldosterone	y = 0.104x - 0.015	0.933	50	9.7	y = 0.393x + 0.004	0.999	10	11.0
Androstenedione	y = 0.676x - 0.006	0.994	10	8.0	y = 0.665x - 0.010	0.994	10	4.7
Estradiol	y = 0.892x - 0.001	0.990	40	8.2	y = 1.056x - 0.094	0.980	40	6.2
Estrone	y = 0.564x - 0.014	0.999	50	10.0	y = 0.556x + 0.034	0.999	20	6.3
Progesterone	y = 0.329x + 0.013	0.988	150	15.7	y = 0.712x + 0.005	0.998	150	13.5
Testosterone	y = 0.841x + 0.032	0.997	10	10.6	y = 0.953x + 0.048	0.992	10	5.8
Analyte	Calibration Equation	R^2	LLOQ (ng/mL)	S/N	Calibration Equation	R^2	LLOQ (ng/mL)	S/N
17-OH-Pregnenolone	N/A	N/A	5	12.6	y = 3.934x + 1.278	0.992	3	18.5
Corticosterone	y = 2.050x + 0.002	0.999	0.01	12.2	y = 1.037x - 0.003	0.999	0.04	13.8
Cortisol	y = 0.403x + 0.007	0.993	0.02	12.6	y = 0.440x - 2.450	0.995	0.02	10.8
Cortisone	y = 1.127x + 0.013	0.999	0.2	33.9	y = 0.612x - 0.001	0.998	0.15	16
DHEA	y = 1.597x + 1.439	0.991	0.6	15.3	y = 1.770x + 3.853	0.995	0.2	12.5
DHEAS	N/A	N/A	N/A	N/A	y = 0.121x + 0.019	0.997	1	16.7
Dihydrotestosterone	y = 0.916x - 0.030	0.999	5	11.9	y = 0.795x + 0.044	0.991	5	11.8
Pregnenolone	y = 0.597x - 0.016	0.998	2	19	y = 0.895x - 0.218	0.997	10	36

Figure 8. Peak Examples at LLOQ.



Have questions or want more details on implementing this method? We would love to help!
Visit www.phenomenex.com/Chat to get in touch with one of our Technical Specialists

Table 7. SLE Recovery and Accuracy Data.

	Dichloromethane			Ethyl Acetate		
Analyte	Expected (pg/mL)	Recovery (%)	Accuracy (%)	Expected (pg/mL)	Recovery (%)	Accuracy (%)
11-Deoxycorticosterone	200	92.59	114.32	200	156.33	111.15
11-Deoxycortisol	200	78.25	105.97	200	90.22	104.78
17-OH-Progesterone	1500	66.94	102.80	1500	110.75	111.73
18-OH-Corticosterone	1500	13.39	107.50	1500	77.63	104.02
21-Deoxycortisol	400	77.28	106.95	400	95.44	104.13
Aldosterone	100	20.61	131.65	100	90.61	92.57
Androstenedione	100	78.83	100.25	100	109.07	101.13
Estradiol	400	74.74	101.08	400	91.97	91.67
Estrone	200	71.61	107.32	200	93.30	101.42
Progesterone	1500	28.37	80.13	1500	85.37	102.90
Testosterone	100	86.94	111.32	100	97.39	109.77
Analyte	Expected (ng/mL)	Recovery (%)	Accuracy (%)	Expected (ng/mL)	Recovery (%)	Accuracy (%)
17-OH-Pregnenolone	15	76.67	N/A	15	87.03	135.00
Corticosterone	2.5	76.18	105.81	2.5	88.95	93.60
Cortisol	5	24.34	107.72	5	84.25	100.29
Cortisone	5	52.43	101.13	5	91.21	94.20
DHEA	5	69.14	104.95	5	88.01	152.18
DHEAS	5	0.50	N/A	5	1.15	117.43
Dihydrotestosterone	25	95.33	92.27	25	98.61	99.72
Pregnenolone	50	66.41	91.10	50	84.45	75.94

Table 8. SLE Precision, Matrix Effect, and Process Efficiency Data.

	Dichloromethane			Ethyl Acetate		
Analyte	Precision (%RSD)	Matrix Effect	Process Efficiency	Precision (%RSD)	Matrix Effect	Process Efficiency
11-Deoxycorticosterone	6.27	0.68	0.63	9.00	0.26	0.41
11-Deoxycortisol	2.34	0.85	0.67	5.62	0.78	0.71
17-OH-Progesterone	7.94	0.44	0.30	11.57	0.43	0.48
18-OH-Corticosterone	12.98	0.99	0.13	6.85	0.84	0.66
21-Deoxycortisol	4.39	0.98	0.76	9.07	0.97	0.93
Aldosterone	21.27	0.96	0.20	7.64	0.96	0.87
Androstenedione	0.86	0.84	0.66	5.22	0.60	0.66
Estradiol	10.34	0.95	0.71	9.52	1.06	0.97
Estrone	3.84	0.91	0.65	5.31	0.86	0.80
Progesterone	9.11	0.75	0.21	5.08	0.51	0.44
Testosterone	5.45	0.82	0.72	5.26	0.64	0.62
Analyte	Precision (%RSD)	Matrix Effect	Process Efficiency	Precision (%RSD)	Matrix Effect	Process Efficiency
17-OH-Pregnenolone	N/A	1.02	0.78	13.13	0.94	0.82
Corticosterone	14.40	0.99	0.76	7.27	0.90	0.80
Cortisol	3.45	0.99	0.24	5.96	0.96	0.81
Cortisone	7.93	1.01	0.53	6.97	0.94	0.86
DHEA	9.72	0.65	0.45	29.62	1.25	1.10
DHEAS	N/A	0.96	0.01	20.30	1.08	0.01
Dihydrotestosterone	2.52	0.50	0.48	5.13	0.26	0.26
Pregnenolone	7.55	0.658	0.437	8.59	0.55	0.26

The developed SLE method is effective and reproducible for cleanup of serum samples. Extraction using Novum™ PRO SLE, MAX 96-well plates provides a fast, high-throughput method. The Kinetex™ 2.6 µm C18, 50 x 3.0 mm column provides full chromatographic separation of steroid analytes including structural isomers in just 8 minutes, allowing for accurate quantitation using LC-MS/MS.

Testosterone

Testosterone is an androgenic steroid responsible for the development of male reproductive organs, maintaining (or increasing) muscle mass, and bone density. As anabolic steroids, testosterone has been used (or abused) to increase muscle mass and enhance athletic performance. The concentration of testosterone is lower in the female population than men and in general diminishes with advancing age. Monitoring body concentration of testosterone can aid in understanding disease states related to the hormonal imbalance. Strata™-X-A gave cleaner extracted serum samples for testosterone than Strata-X. The strong mixed mode, anion exchange moiety may retain some charged interfering compounds.

Sample Preparation

Step	Description
Condition:	Strata-X-A 30 mg/3 mL tube (Part No.: 8B-S123-TBJ) with 2 mL Methanol followed by 2 mL Water.
Load:	Into individually labeled test tubes, combine 0.25 mL serum sample, calibrator, or CQ sample, 1 mL DI Water, and 0.1 mL working internal standard solution (2 ng/mL).
Wash:	With 0.6 mL 50 % Methanol in Water.
Dry:	5 minutes under high vacuum.
Elute:	With 2 washes of 0.3 mL Methanol.

Derivatization with Hydroxylamine can increase sensitivity for testosterone by LC-MS/MS. To derivatize: following evaporation of elution solvent, dry at 50-55 °C under a gentle Nitrogen stream. Add 50 µL 25 % Hydroxylamine solution and heat at 60-65 °C for 5-10 min, then add 200 µL 5 % aqueous Formic Acid and vortex the tubes. Transfer the solution to autosampler vials and inject 25 µL on column. Inject 20 µL onto LC-MS/MS system.

LC Conditions

Column: Kinetex 1.7 µm C18
Dimensions: 30 x 2.1 mm
Part No.: 00A-4475-AN
Mobile Phase: A: 0.1 % Formic Acid + 1 mM Ammonium Formate in Water
B: 0.1 % Formic Acid + 1 mM Ammonium Formate in Acetonitrile
Gradient:

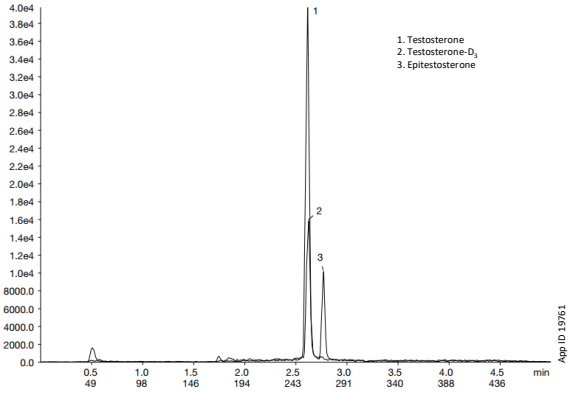
Time (min)	%B
0	10
2.5	90
3.5	90
3.6	10
5	10

Flow Rate: 0.4 mL/min
Injection Volume: 2 µL
Temperature: 55 °C
Detection: MS/MS (ESI+)
Detector: SCIEX® API 5000™

Results

As demonstrated in **Figure 9**, the Kinetex™ 1.7 µm C18, 30 x 2.1 mm LC column efficiently separated Testosterone from its isomeric form Epitestosterone. This column provides a very high degree of selectivity, even in a short dimension, resulting in superior chromatographic separation in a short run time. By using the Strata™-X-A cartridge, cleaner samples from serum were obtained.

Figure 9. Separation of Testosterone and Epitestosterone by LC-MS/MS.



LC Conditions

Column:	Kinetex 1.7 µm Biphenyl
Dimensions:	50 x 2.1 mm
Part No.:	00B-4628-AN
Mobile Phase:	A: 0.1 % Formic Acid in Water
	B: 0.1 % Formic Acid in Methanol

Gradient:	**Time (min)**	**%B**
	0	80
	1	80
	2	90
	3	95
	6	95
	6.1	80
	8	80

Flow Rate:	0.4 mL/min
Injection Volume:	10 µL
Temperature:	Ambient
Detection:	MS/MS
Detector:	SCIEX® 6500

MS/MS Conditions

Ion Source:	ESI
Polarity:	Positive
Source Temperature:	450 °C
GS1:	30 psi
GS2:	10 psi
CUR:	20 psi
CAD:	High
IS:	4000 V

Estrogen

Estrogens are common hormones responsible for many diverse biological processes and are important for a variety of testing purposes. They can be found in a wide range of concentrations in females but are typically analyzed at low levels that can present analytical challenges such as matrix interferences and sensitivity limitations in men, post-menopausal women and children. Immunoassays are commonly used to perform analysis of estrogens in biologic samples, though mass spectrometry methods are generally preferred due to their greater specificity and sensitivity. Estrogens have poor ionizations due to a lack of ionizable functional groups which cause mass spectrometric approaches to rely on derivatization methods to achieve a sufficient lower level of quantitation. Introducing derivatization by binding charged moieties to the analytes, estrogen ionization is enhanced thus increasing sensitivity for quantitation at lower endogenous concentrations. By coupling this technique with a supported liquid extraction technique, matrix contaminants are reduced, and samples can be concentrated further increasing analyte sensitivity.

Sample Preparation

Step	Description
Sample Pre-treatment:	200 µL spiked plasma + 200 µL 50 mM Sodium phosphate buffer, pH unadjusted.
Load:	Novum™ PRO SLE, MAX 96-Well Plate (Part No.: 8E-S539-5GA), wait 5 minutes.
Elute:	With Hexane / Ethyl Acetate (1:3, v/v).
Dry:	Under Nitrogen.
Add:	50 µL Dansyl Chloride / Sodium Bicarbonate (50:50, v/v).
Incubate:	60 °C for 10 minutes.

Results

Estradiol and Estriol were linear in the range of 10 pg/mL to 500 pg/mL with a $R^2 \geq 0.995$. Estrone was linear in the range of 5 pg/mL to 500 pg/mL with a $R^2 \geq 0.995$. CVs ranged from 1 % to 7 % and recovery was 92 % or higher for all analytes. The LOQs were able to reach 10 pg/mL for β-Estradiol and Estrone and 5 pg/mL for Estriol. Dansyl Chloride derivatization in conjunction with LC-MS/MS produced a sensitive method consisting of the simultaneous determination of 3 estrogens in plasma using SLE and an 8-minute analysis, offering advantages in sensitivity over existing underivatized techniques. This method was able to reach an LLOQ of of 10 pg/mL in plasma. The method shows good precision, accuracy, and recovery over the entire calibration range of 10 to 1000 pg/mL (**Table 9**).

Figure 10. Chromatogram of Estrogens.

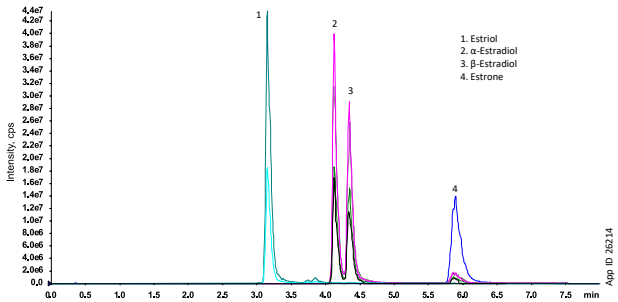


Figure 11. LLOQ of Estrogens.

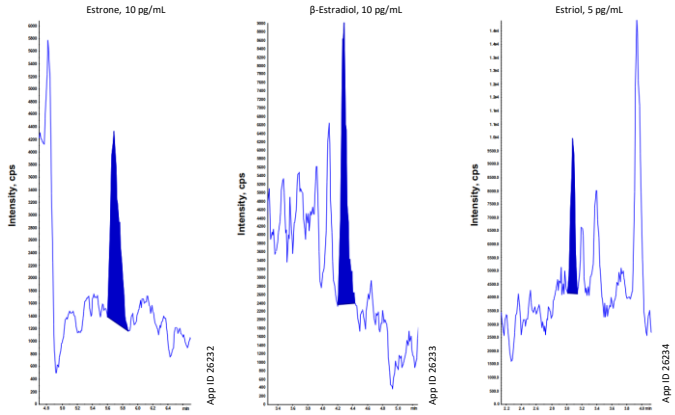


Table 9. Recovery, % CV and LLOQ Data for Estrogens.

Analyte	Retention Time (min)	% Recovery	% CV	LLOQ
Estrone	5.90	111	7	10 pg/ml
β-Estradiol	4.35	92	7	10 pg/ml
Estriol	3.15	99	1	5 pg/ml

Cortisols, Androgens, and Pregnancy Hormones

The glucocorticoids affect metabolism in several ways by stimulating gluconeogenesis and decreasing the glucose use by cells. Cortisol, the most clinically important glucocorticoid, accounts for about 95% of all glucocorticoid activity. Androgens are responsible for the development of secondary sexual characteristics in men. Testosterone is the major androgen. Estrogens promote proliferation and growth of specific cells in the body that are responsible for the development of most of the secondary sexual characteristics in women. The progestins are responsible for the preparation of the uterus for pregnancy and the breasts for lactation. The best pretreatment and elution solvent combination for these groups of steroids would most likely be the pH 6 Ammonium Acetate buffer and either Dichloromethane or Ethyl Acetate.

LC Conditions

Column: Kinetex 5 µm EVO C18
Dimensions: 100 x 2.1 mm
Part No.: 00D-4633-AN
Mobile Phase: A: 5 mM Ammonium Formate in Water
B: Methanol
Gradient:

Time (min)	%B
0	80
8	80
8.5	90
8.51	95
10	95

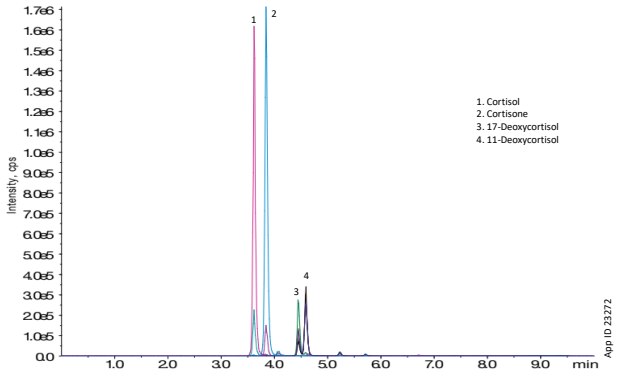
Flow Rate: 0.6 mL/min
Injection Volume: 10 µL
Temperature: 22 °C
Detection: MRM
Detector: SCIEX® API 5000™

Results

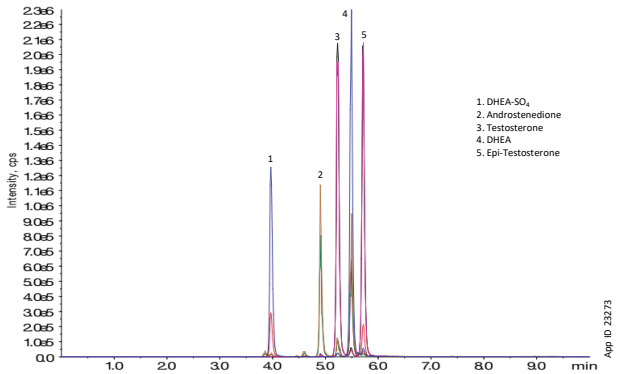
As seen in **Figure 12**, a single Kinetex™ 5 µm EVO C18 column was able to successfully separate individual steroids within the cortisol, androgen, and pregnancy hormone groups. This would help labs eliminate the need for multiple columns for different groups of steroid hormones.

Figure 12. Representative Chromatograms of Steroids.

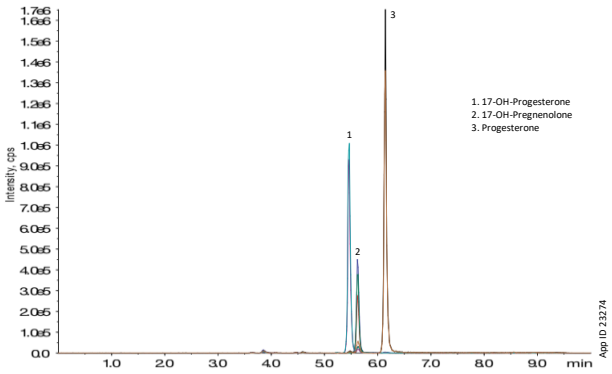
Cortisols

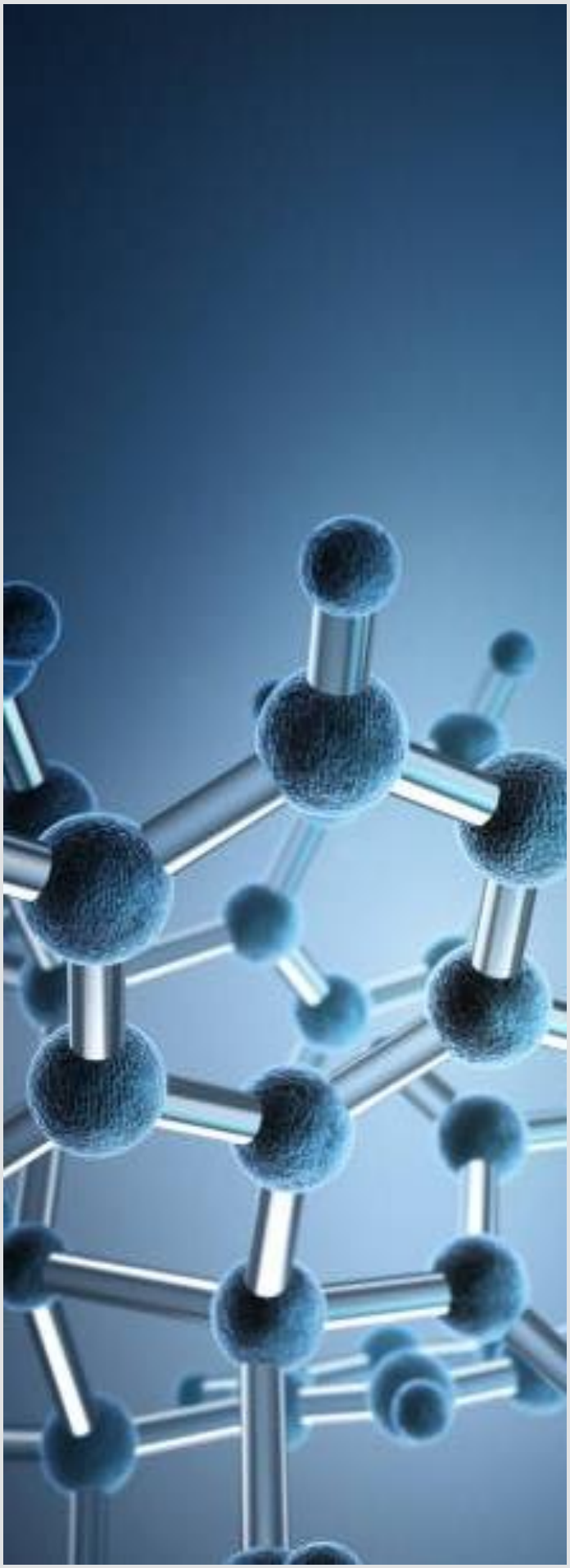


Androgens



Pregnancy Hormones





Summary

This white paper outlines multiple sample preparation and LC-MS/MS conditions for the analysis of steroids in serum or plasma for clinical research. Kinetex™ core-shell columns, combined with SLE or SPE for sample clean up, can provide fast, sensitive, specific quantitation of steroids by LC-MS/MS for a large panel or selected groups of steroid analytes.

References

1. Matuszweski, B.K., Constanzer, M.L., and Chavez-Eng, C.M. 2003. Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC-MS/MS. *Anal. Chem.* 75, 3019-3030.

Need a different column size or sample preparation format?

No problem! We have a majority of our available dimensions up on www.phenomenex.com, but if you can't find what you need right away, our super helpful Technical Specialists can guide you to the solution via our online chat portal www.phenomenex.com/Chat.

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