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Quantitation of Clinical Research Steroid Analytes from Serum Utilizing Solid Phase Extraction with LC-MS/MS

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Introduction

Steroid analysis for clinical research can require very low limits of detection which demand high recoveries from solid phase extraction (SPE) and very clean extracted samples. Laboratories desire high-throughput methods which consolidate analytes into one panel with fast chromatographic run times. Accurate quantitation makes it necessary to chromatographically separate steroids with the same m/z. Meeting these criteria can be challenging in a single LC-MS/MS method. In this technical note, we present an effective sample cleanup method for steroid analysis that targets 19 steroid analytes, utilizing a Polymeric Reversed Phase Strata™-X 96-well plate. A Kinetex™ core-shell 2.6 μm C18, 50 x 3.0 mm column was employed for fast chromatographic separation.

Sample Preparation

Six calibrators and all spiked samples, with analytes and internal standards, were prepared in stripped serum. Two sets of spiked samples were prepared and analyzed, one at pg/mL concentration and the other at ng/mL concentration. These ranges correspond to the standard testing range for each given steroid compound.

Step	Description
Sample Pretreatment:	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-treated 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:	2 aliquots of 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) 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 in duplicate (n=4 for unextracted samples and n=6 for pre-spiked and post-spiked samples).

Recovery, Matrix Effect, and Process Efficiency of each analyte, at each concentration, were calculated as below. All values are reported as a percent.

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

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

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

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

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

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

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



Table 1. MRM Transitions for Steroid Analytes Extracted from Serum Using the Strata™-X 96-well Plate.

Analyte (Positive Ion)	Q1 m/z	Q3 m/z	CE (V)	CXP (V)	Q0D (V)
18-OH-Corticosterone	363.21	121.059	34	20	20
18-OH-Corticosterone	363.21	269.185	22	18	40
Cortisone	361.2	163.062	32	32	30
Cortisone	361.2	121.2	38	30	30
Cortisol	363.21	146.936	37	18	40
Cortisol	363.21	115	132	22	20
21-Deoxycortisol	347.22	311.222	24	18	10
21-Deoxycortisol	347.22	121.068	32	35	20
Corticosterone	347.22	90.99	78	15	30
Corticosterone	347.22	121.024	30	18	10
11-Deoxycortisol	347.22	97.019	31	10	10
11-Deoxycortisol	347.22	109.021	45	18	30
Androstenedione	287.2	109.026	33	18	40
Androstenedione	287.2	97.021	27	12	50
11-Deoxycorticosterone	331.22	97	26	15	40
11-Deoxycorticosterone	331.22	109.052	32	18	40
Testosterone	289.21	97.042	31	12	40
Testosterone	289.21	109.047	39	18	40
17-OH-Progesterone	331.22	109.023	42	18	30
17-OH-Progesterone	331.22	97.006	29	18	30
Dehydroepiandrosterone (DHEA)	271.2	196.9	28	32	0
Dehydroepiandrosterone (DHEA)	271.2	213.1	24	28	30
Dihydrotestosterone	291.23	105	44	20	40
Dihydrotestosterone	291.23	159.095	28	18	50
Progesterone	315.23	109.063	35	18	40
Progesterone	315.23	97.019	28	18	40
Pregnenolone	299.23	159.09	36	30	30
Pregnenolone	299.23	105	58	30	0
Cortisol-D ₄	367.24	121.03	31	18	20
Cortisol-D ₄	367.24	97	35	12	40
11-Deoxycortisol-D ₅	352.25	100.07	31	12	30
11-Deoxycortisol-D ₅	352.25	113.097	70	18	30
Androstenedione- ¹³ C ₃	290.21	100.047	27	12	30
Androstenedione- ¹³ C ₃	290.21	112.071	33	18	30
Testosterone-D ₃	292.24	97.043	29	5	30
Testosterone-D ₃	292.24	109.061	34	5	30
17-OH-Progesterone- ¹³ C ₃	334.24	112.088	46	18	40
17-OH-Progesterone- ¹³ C ₃	334.24	100.051	41	12	40
Dehydroepiandrosterone-D ₅	276.2	121.04	36	20	20
Pregnenolone- ¹³ C ₂ D ₂	303.3	160.95	30	25	0

Analyte (Negative Ion)	Q1 m/z	Q3 m/z	CE (V)	CXP (V)	Q0D (V)
Aldosterone	359.18	189.1	-24	-9	-50
Aldosterone	359.18	331.021	-26	-13	-30
Dehydroepiandrosterone Sulfate (DHEAS)	367.15	96.95	-42	-9	-60
Dehydroepiandrosterone Sulfate (DHEAS)	367.15	79.97	-121	-10	-30
Estrone	269.15	145.073	-50	-3	-60
Estrone	269.15	159.23	-50	-8	-100
Estradiol	271.17	143.105	-66	-11	-40
Estradiol	271.17	145	-49	-4	-50
17-OH-Pregnenolone	331.22	303.3	-29	-12	-20
17-OH-Pregnenolone	331.22	287.166	-28	-10	-40
Dehydroepiandrosterone Sulfate-D ₅	372.19	97.955	-40	-12	-70
Estrone- ¹³ C ₃	272.16	148.05	-51	-10	-30
Estrone- ¹³ C ₃	272.16	162	-49	-6	-70
Estradiol-D ₅	276.2	145.052	-63	-9	-70
Estradiol-D ₅	276.2	187.151	-51	-5	-100

Table 2. Analyte Calibration Ranges and Internal Standard Concentrations.

Analyte	Calibration Range (pg/mL)	Internal Standard (IS)	Internal Standard Concentration (pg/mL)
11-Deoxycorticosterone	10-1000	Androstenedione- ¹³ C ₃	100
11-Deoxycortisol	10-1000	11-Deoxycortisol-D ₅	500
17-OH-Progesterone	50-1000	17-OH-Progesterone- ¹³ C ₃	500
18-OH-Corticosterone	50-1000	11-Deoxycortisol-D ₅	500
21-Deoxycortisol	10-1000	11-Deoxycortisol-D ₅	500
Aldosterone	10-1000	Estrone- ¹³ C ₃	500
Androstenedione	10-1000	Androstenedione- ¹³ C ₃	100
Estradiol	10-1000	Estradiol-D ₅	500
Estrone	10-1000	Estrone- ¹³ C ₃	500
Progesterone	10-1000	17-OH-Progesterone- ¹³ C ₃	500
Testosterone	10-1000	Testosterone-D ₃	100

Analyte	Calibration Range (ng/mL)	Internal Standard (IS)	Internal Standard Concentration (ng/mL)
Corticosterone	0.05-25	Cortisol-D ₄	25
Cortisol	0.2-100	Cortisol-D ₄	25
Cortisone	0.2-25	Cortisol-D ₄	25
Dehydroepiandrosterone (DHEA)	1-100	DHEA-D ₅	25
Dehydroepiandrosterone Sulfate (DHEAS)	0.2-100	DHEAS-D ₅	25
Dihydrotestosterone (DHT)	1-250	Cortisol-D ₄	25
Pregnenolone	5-500	Pregnenolone- ¹³ C ₂ D ₂	100



Results and Discussion

The Kinetex™ 2.6 µm C18 column provided a fast 8-minute chromatographic separation and good selectivity for steroid isomers in both positive ion mode (Figure 1) and negative ion mode (Figure 2). Calibration curves (Figure 3), with a linear fit and 1/x weighting, showed good linearity with R^2 values >0.992 for all analytes (Table 3). Accuracy of spiked, stripped serum samples in triplicate at three concentrations were within 80-120%. Precision calculated as %RSD of spiked samples were <15% for all analytes. Recovery of the lowest concentration spiked samples were 81-116% for all analytes except DHEAS which is 54%. Recovery of mid-concentration spiked samples were 80-96% for all analytes except DHEAS which is 61%. Recovery of high-concentration spiked samples were 82-102% for all analytes except DHEAS which is 59%. To improve recovery of DHEAS, a different elution solvent should be considered. A Methanol / Water (30:70, v/v) wash was chosen for maximum recovery for the entire panel of 19 analytes. For select groups of steroid analytes, a stronger organic wash may reduce matrix effect without sacrificing recovery. For labs interested in quantitation of 17-OH-Pregnenolone, further SPE method development is needed to eliminate interference. An additional wash or a different elution solvent may achieve this.

Figure 1. Total Ion Chromatogram (TIC) in Positive Ion Mode of Steroid Isomers Fully Separated in an 8-minute Method Using a Kinetex C18 Column.

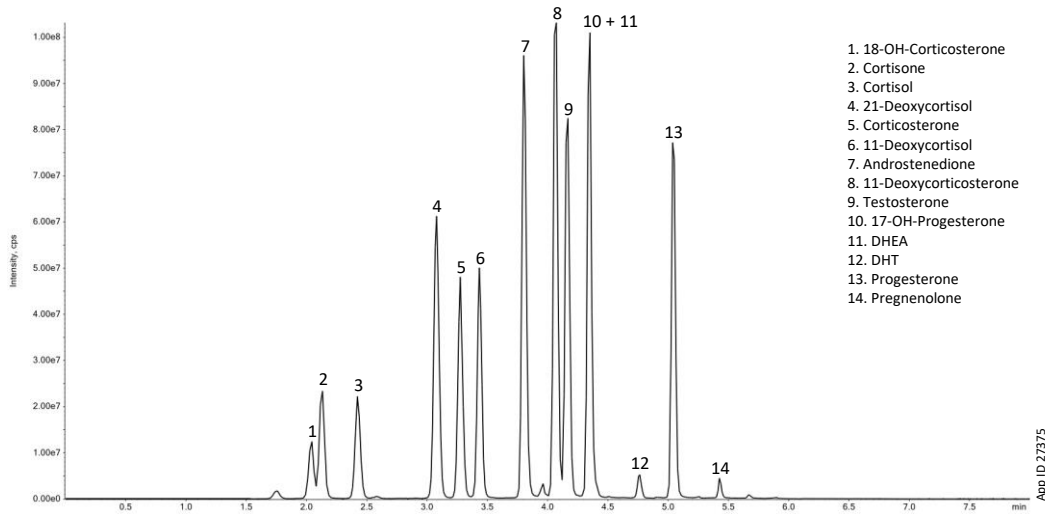
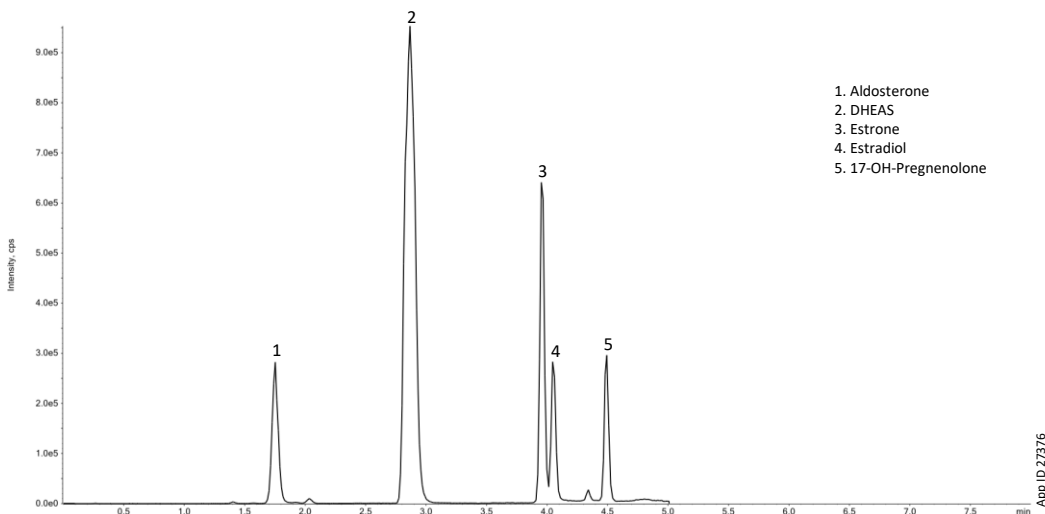


Figure 2. TIC in Negative Ion Mode of Steroid Isomers Fully Separated in an 8-minute Method Using a Kinetex C18 Column.



Note: Chromatographic Separation of 17-OH-Pregnenolone is Shown Here, but the Analyte is Not Included in Calibrator and QC Data. Further Development is Required for 17-OH-Pregnenolone.



Figure 3 . Example Calibration Curves.

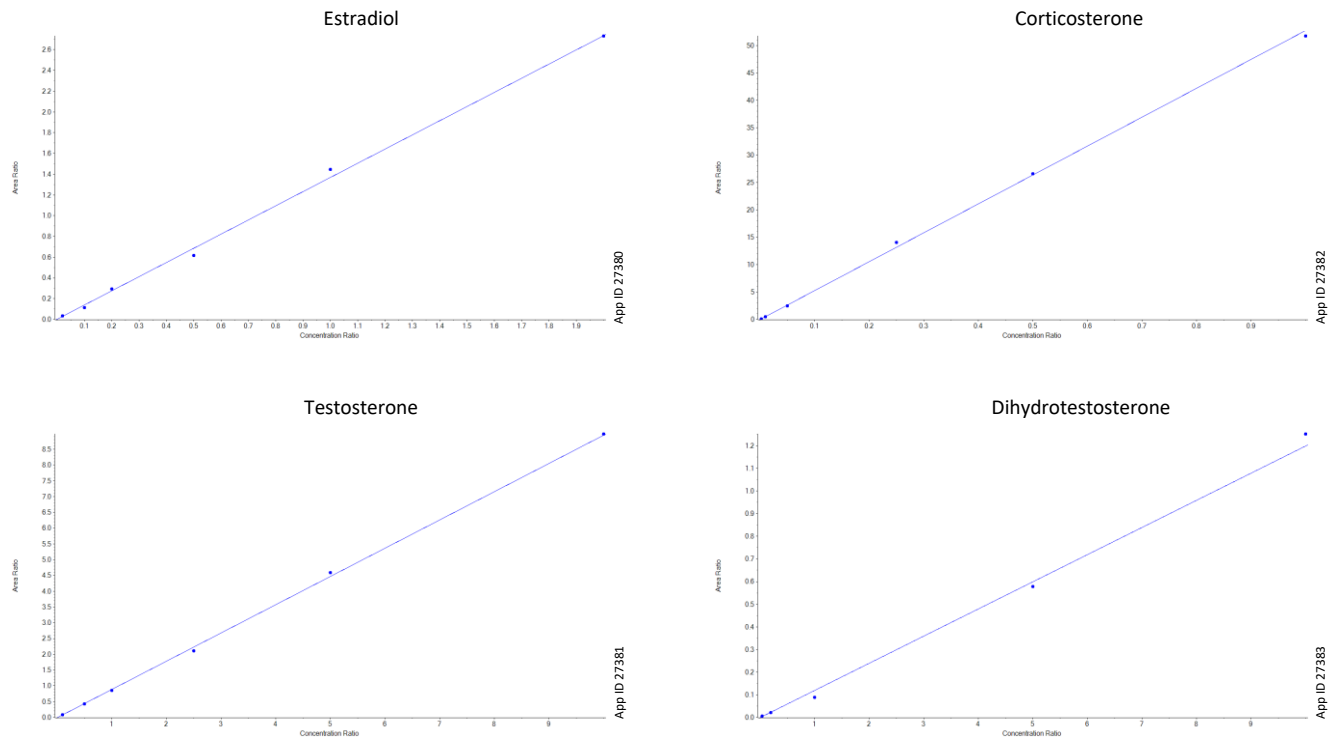


Figure 4 . Peak Examples at LLOQ.

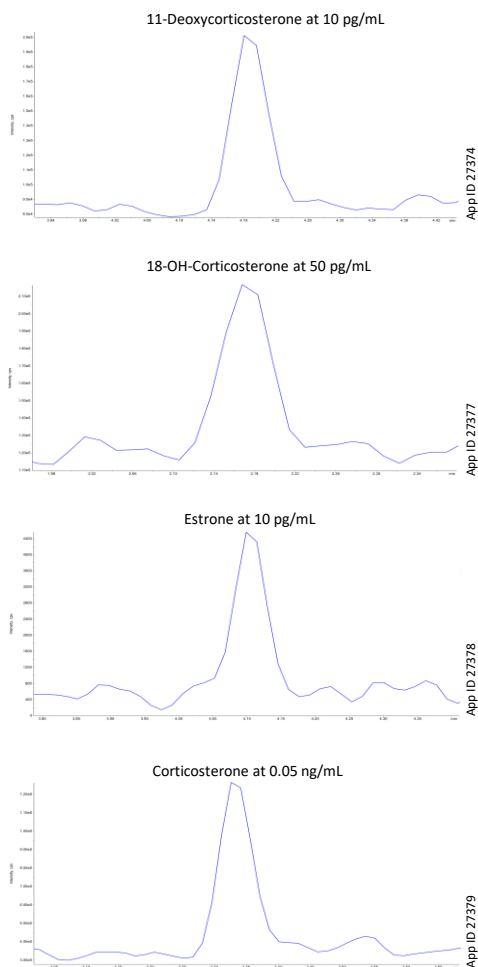


Table 3 . Analyte Calibration Curve, LLOQ an S/N Data.

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 than the detection capabilities of the instrument.



Table 4. Recovery and Accuracy Data.

Analyte	Low Concentration			Mid Concentration			High Concentration		
	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. Precision, Matrix Effect, and Process Efficiency Data.

Analyte	Low Concentration			Mid Concentration			High Concentration		
	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



Conclusions

The SPE method described here provides high recovery and reproducibility for a panel of 19 steroid analytes in serum. The Kinetex™ 2.6 µm C18 column gave good separation of the steroid isomers in 8 minutes. The SPE method and LC separation combined with a SCIEX® 7500 Triple Quad™ provided sensitive, accurate quantitation of 19 steroid analytes.

Ordering Information

Phases	Kinetex 2.6 µm Analytical Columns (mm)					SecurityGuard™ ULTRA Cartridges*
	30 x 3.0	50 x 3.0	75 x 3.0	100 x 3.0	150 x 3.0	3/pk
EVO C18	00A-4725-Y0	00B-4725-Y0	—	00D-4725-Y0	00F-4725-Y0	AJ0-9297
PS C18	00A-4780-Y0	00B-4780-Y0	—	00D-4780-Y0	00F-4780-Y0	AJ0-8950
Polar C18	—	00B-4759-Y0	—	00D-4759-Y0	00F-4759-Y0	AJ0-9531
Biphenyl	—	00B-4622-Y0	—	00D-4622-Y0	00F-4622-Y0	AJ0-9208
XB-C18	00A-4496-Y0	00B-4496-Y0	00C-4496-Y0	00D-4496-Y0	00F-4496-Y0	AJ0-8775
C18	00A-4462-Y0	00B-4462-Y0	00C-4462-Y0	00D-4462-Y0	00F-4462-Y0	AJ0-8775
C8	00A-4497-Y0	00B-4497-Y0	00C-4497-Y0	00D-4497-Y0	00F-4497-Y0	AJ0-8777
HILIC	00A-4461-Y0	—	—	00D-4461-Y0	00F-4461-Y0	AJ0-8779
Phenyl-Hexyl	—	00B-4495-Y0	—	00D-4495-Y0	00F-4495-Y0	AJ0-8781
F5	—	00B-4723-Y0	—	00D-4723-Y0	00F-4723-Y0	AJ0-9321

*SecurityGuard ULTRA Cartridges require holder, Part No.: [AJ0-9000](#)

for 3.0 mm ID

Strata™-X Format	Sorbent Mass	Part Number	Unit
Tube			
	30 mg	8B-S100-TAK**	1 mL (100/box)
	30 mg	8B-S100-TBJ	3 mL (50/box)
	60 mg	8B-S100-UBJ**	3 mL (50/box)
	100 mg	8B-S100-EBJ	3 mL (50/box)
	100 mg	8B-S100-ECH	6 mL (30/box)
	200 mg	8B-S100-FBJ	3 mL (50/box)
	200 mg	8B-S100-FCH	6 mL (30/box)
	500 mg	8B-S100-HBJ	3 mL (50/box)
	500 mg	8B-S100-HCH	6 mL (30/box)
Giga™ Tube			
	500 mg	8B-S100-HDG	12 mL (20/box)
	1 g	8B-S100-JDG	12 mL (20/box)
	1 g	8B-S100-JEG	20 mL (20/box)
	2 g	8B-S100-KEG	20 mL (20/box)
	5 g	8B-S100-LFF	60 mL (16/box)
Teflon® Tube			
	200 mg	8B-S100-FBJ-T	3 mL (50/box)
	200 mg	8B-S100-FDG-T	12 mL (20/box)
96-Well Plate			
	10 mg	8E-S100-AGB	2 Plates/Box
	30 mg	8E-S100-TGB	2 Plates/Box
	60 mg	8E-S100-UGB	2 Plates/Box
96-Well Microelution Plate			
	2 mg	8M-S100-4GA	ea

**Tab-less tubes available. Contact Phenomenex for details.



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