TN-0162

LC-MS/MS Analysis of a Comprehensive Steroid Panel from Serum for Using Supported Liquid Extraction

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Introduction

Steroid analysis for clinical research can require very low limits of detection which demand high recoveries and very clean extracted samples. Supported Liquid Extraction (SLE) can provide very clean extracted samples with less method development and a streamlined workflow compared to solid phase extraction (SPE). Laboratories desire high-throughput methods which consolidate analytes into one panel with fast chromatographic run times. Chromatographic separation of steroid isomers with the same m/z is necessary for accurate quantitation by LC-MS/MS. 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 Novum™ PRO SLE 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

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 also 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 (DCM), Ethyl Acetate, 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.

Step	Description
Sample Pre-treatment:	Combine 200 µL of serum sample, 10 µL of internal standard (IS), and 200 µL of pH 6 Ammonium Acetate in Water.
Load:	410 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): Concentrations ranged from 10-3000 pg/mL, dependent on the appropriate level for each analyte. Concentrations for spiked standards were 100, 200, 400, 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): Concentrations ranged from 0.1 -1000 ng/mL dependent on the 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.

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 Novum™ PRO SLE 96-well Plate and SCIEX® 7500 Triple Quad™.

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, Dichloromethane (pg/mL)	Calibration Range, Ethyl Acetate (pg/mL)	Internal Standard (IS)	Internal Standard Concentration (pg/mL)
11-Deoxycorticosterone	50-400	100-400	Androstenedione-$^{13}C_3$	200
11-Deoxycortisol	20-400	20-400	11-Deoxycortisol-D_5	200
17-OH-Progesterone	150-3000	150-3000	17-OH-Progesterone-$^{13}C_3$	200
18-OH-Corticosterone	750-3000	150-3000	11-Deoxycortisol-D_5	200
21-Deoxycortisol	40-800	40-800	11-Deoxycortisol-D_5	200
Aldosterone	50-200	10-200	Estrone-$^{13}C_3$	200
Androstenedione	10-200	10-200	Androstenedione-$^{13}C_3$	200
Estradiol	40-800	40-800	Estradiol-D_5	200
Estrone	50-400	20-400	Estrone-$^{13}C_3$	200
Progesterone	150-3000	150-3000	17-OH-Progesterone-$^{13}C_3$	200
Testosterone	10-200	10-200	Testosterone-D_3	200
Analyte	**Calibration Range, Dichloromethane (pg/mL)**	**Calibration Range, Ethyl Acetate (pg/mL)**	**Internal Standard (IS)**	**Internal Standard Concentration (ng/mL)**
17-OH-Pregnenolone	N/A	3.0-75	DHEAS-D_5	10
Corticosterone	0.1-12.5	0.1-50	Cortisol-D_4	10
Cortisol	0.2-100	0.2-100	Cortisol-D_4	10
Cortisone	0.2-50	0.2-100	Cortisol-D_4	10
Dehydroepiandrosterone (DHEA)	1-100	0.2-100	DHEA-D_5	10
Dehydroepiandrosterone Sulfate (DHEAS)	N/A	1-100	DHEAS-D_5	10
Dihydrotestosterone (DHT)	5-125	5-500	Cortisol-D_4	20
Pregnenolone	2-500	10-1000	Pregnenolone-$^{13}C_2$,D_2	20

Results and Discussion

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 Kinetex™ 2.6 µm C18 column provided a fast 8-minute chromatographic separation and good selectivity for steroid analytes in both positive ion mode and negative ion mode (**Figures 1** and **2**). Calibration curves (**Figure 3**), 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 Dichloromethane as the elution solvent, and R^2 of >0.980 for all analytes when using Ethyl Acetate as the elution the solvent (**Table 3**). Accuracy of samples using Dichloromethane as the elution solvent were 80-112 %, except for 17-OH-Pregnenolone and DHEAS (**Table 4**). Accuracy of all samples using the Ethyl Acetate elution solvent were 91-118 %. Representative chromatograms for select analytes at the LLOQ are shown in **Figure 4**.

Percent recovery ranged from 13-87 % using Dichloromethane as the elution solvent (**Table 4**). 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 recovery was 1 % using SLE. SPE would need to be used for samples where DHEAS quantitation is desired (TN-0156).

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.

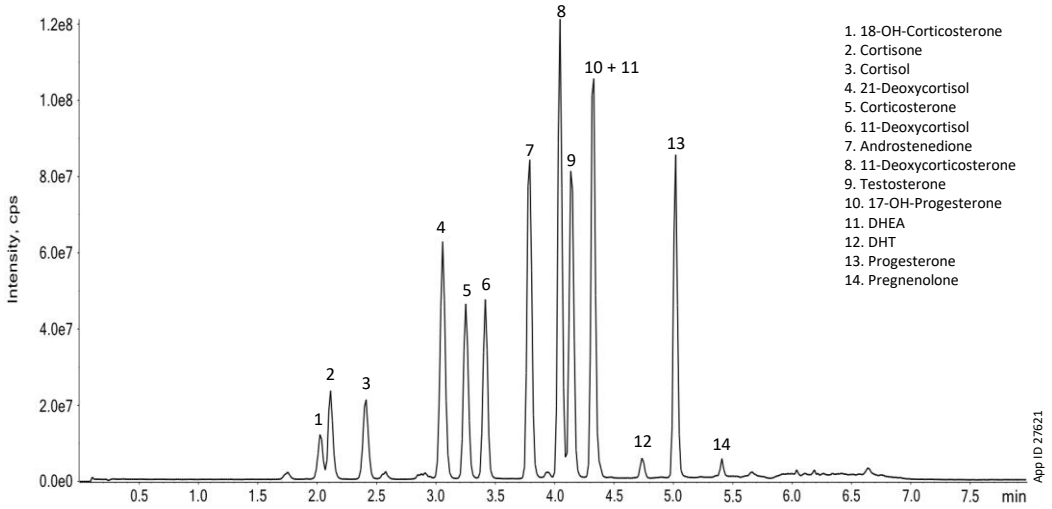

Figure 2. TIC in Negative Ion Mode of Steroid Isomers Fully Separated in an 8-minute Method Using a Kinetex C18 Column.

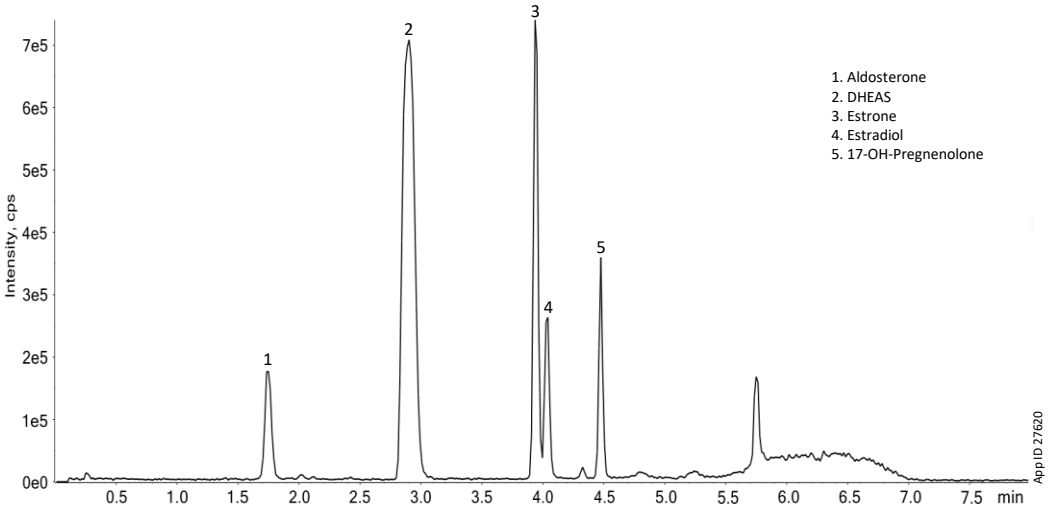

Figure 3 . Example Calibration Curves.

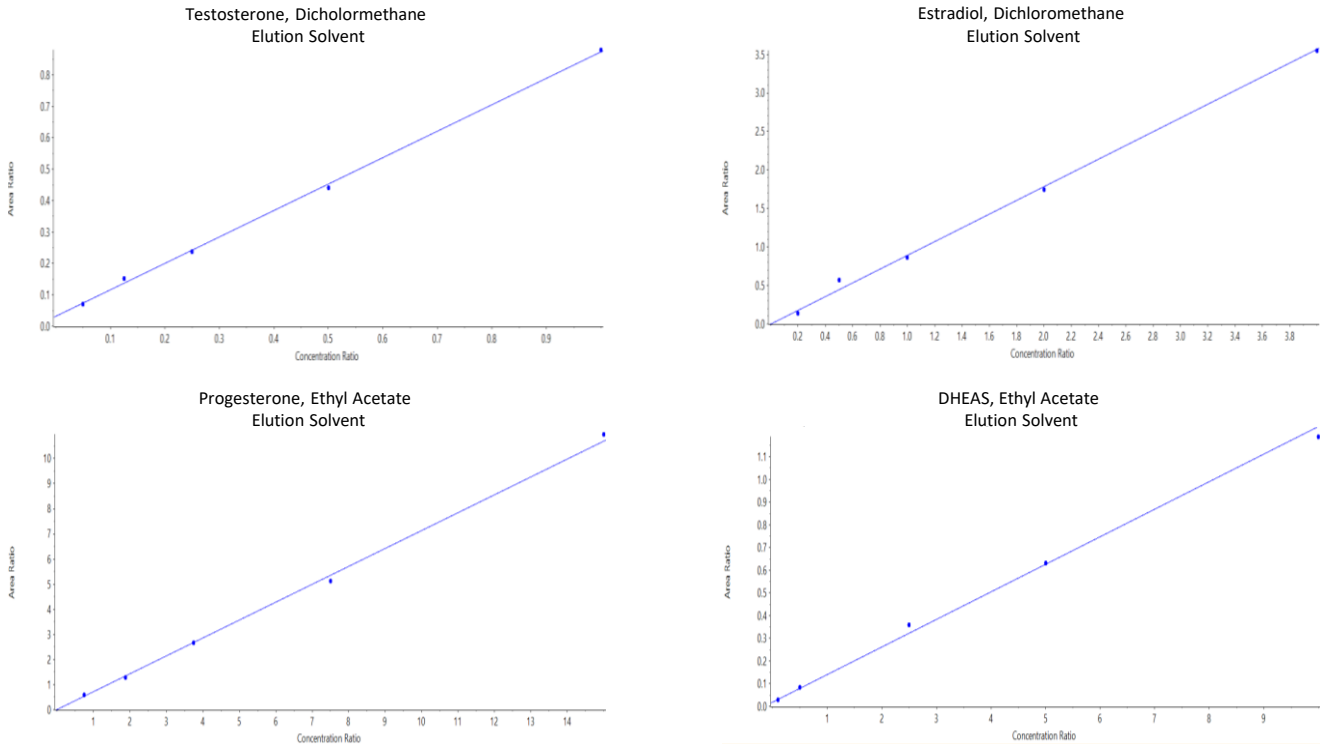

Figure 4 . Peak Examples at LLOQ.

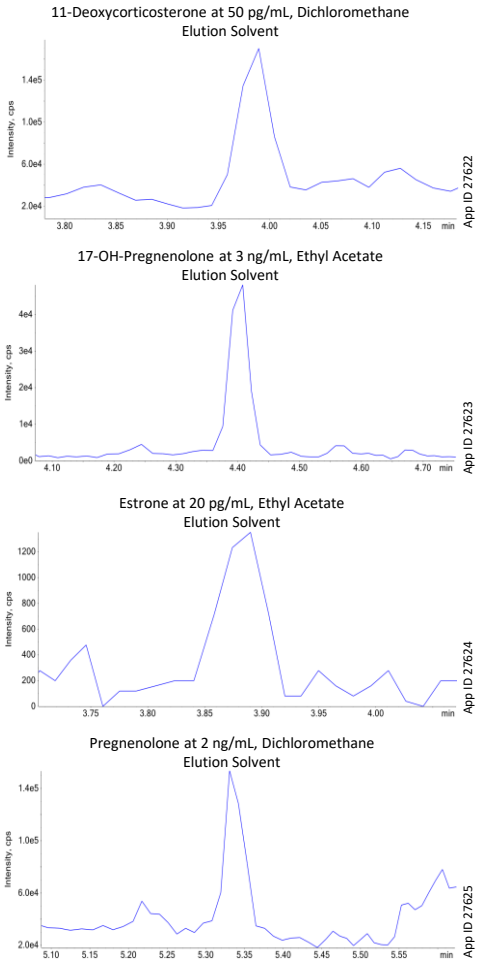

Table 3 . Analyte Calibration Curve, LLOQ an S/N Data.

	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

Table 4. 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 5. 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

Conclusions

This 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 extraction method. The Kinetex™ 2.6 µm C18, 50 x 3.0 mm column provides full chromatographic separation of 19 steroid analytes including structural isomers in just 8 minutes for fast, accurate quantitation using LC-MS/MS.

Ordering Information

Kinetex 2.6 µm Analytical Columns (mm)						SecurityGuard™ ULTRA Cartridges*
Phases	**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

Novum Supported Liquid Extraction (SLE) 96-Well Plates				
Novum SLE 96-Well Plates	**MINI**	**MAX**	**PRO MINI**	**PRO MAX**
Maximum Sample Volume (after dilution)	300 µL	400 µL	300 µL	400 µL
Recommended Elution Volume	1 x 1 mL	2 x 900 µL	1 x 1 mL	2 x 900 µL
Part No.	8E-S138-FGA	8E-S138-5GA	8E-S539-FGA	8E-S539-5GA
Unit	1/pk	1/pk	1/pk	1/pk

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