Determination of Hormones in Serum by LC/MS/MS Using Agilent Bond Elut Plexa SPE

Application Note

Bio-analysis, Pharmaceutical, Clinical Research

Authors

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Abstract

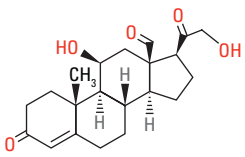
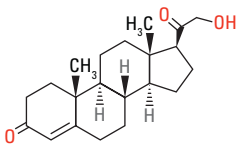
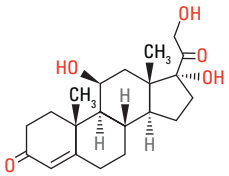
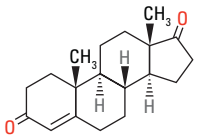
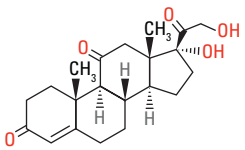
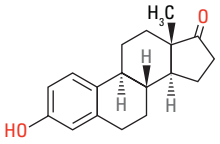
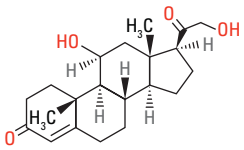
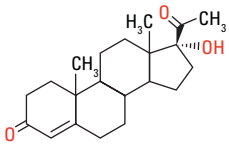
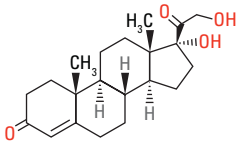
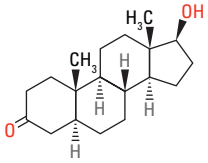
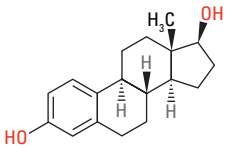
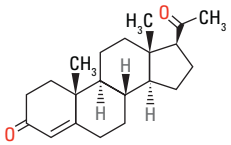
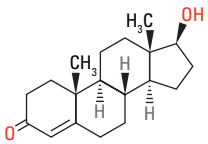
A method for the determination of 13 hormones and their respective internal standards in serum was developed. Hormones were extracted from serum using Agilent Bond Elut Plexa Solid Phase Extraction (SPE) (30 mg, 1 mL) cartridges. They were separated on an Agilent InfinityLab Poroshell HPH-C8 column (2.1 × 50 mm, 2.7 µm), and analyzed by liquid chromatography tandem mass spectrometry (LC/MS/MS). Hormones were analyzed using both positive and negative electrospray ionization (ESI) using a 1 mM ammonium fluoride mobile phase to improve hormone responses in both modes. Overall recoveries ranged from 80 to 105%, with relative standard deviations (RSDs) between 2.8 and 5.8%. This application note demonstrates the ease-of-use and effectiveness of Bond Elut Plexa SPE cartridges for hormone determination in serum.

Introduction

A method using polymeric solid phase extraction (SPE) was developed for the determination of steroid hormones in serum. Table 1 shows the structures and chemical formulas for the 13 analyzed hormones. Agilent Bond Elut Plexa has a unique polymeric architecture with a hydroxylated, nonretentive, amide-free surface. Proteins and lipids bind minimally to the polymeric surface, resulting in cleaner samples and reduced matrix interferences. The nonpolar PS-DVB core is ideal for retaining small molecules such as hormones. This application note demonstrates the effectiveness and ease-of-use of Bond Elut Plexa SPE cartridges.

Agilent Technologies

Table 1. Molecular formulas and structures for 13 analyzed hormones. Isobaric pair information is also included.

Hormone	Molecular formula	Structure	Hormone	Molecular formula	Structure
Aldosterone	$C_{21}H_{28}O_5$		11-Deoxycorticosterone	$C_{21}H_{30}O_3$	
Cortisol	$C_{21}H_{30}O_5$		Androstenedione	$C_{19}H_{26}O_2$	
Cortisone	$C_{21}H_{28}O_5$		Estrone	$C_{18}H_{22}O_2$	
Corticosterone	$C_{21}H_{30}O_4$		17αOH Progesterone	$C_{21}H_{30}O_3$	
11-Deoxycortisol	$C_{21}H_{30}O_4$		Dihydrotestosterone (DHT)	$C_{19}H_{30}O_2$	
β-Estradiol	$C_{18}H_{24}O_2$		Progesterone	$C_{21}H_{30}O_2$	
Testosterone	$C_{19}H_{28}O_2$		**Isobaric pairs:** Aldosterone and cortisone 11-Deoxycorticosterone and 17αOH progesterone 11-Deoxycortisol and corticosterone		

Experimental

Reagents and chemicals

All reagents were HPLC grade or higher. Methanol was bought from Honeywell (Muskegon, MI, USA.) Water was purified using an EMD Millipore Milli-Q Integral System (Darmstadt, Germany.) Reagent-grade formic acid (FA, p/n G2453-85060) was from Agilent Technologies. Ammonium fluoride was from Sigma-Aldrich (St. Louis, MO, USA). 11-Deoxycorticosterone d7 was from Toronto Research Chemicals (Toronto, Ontario, Canada). All other hormones and internal standards were from Sigma-Aldrich. Serum (DC Mass Spect Gold, MSG4000) was bought from Golden West Biologicals, Inc. (Temecula, CA, USA). Serum was stored at –70 °C until day of use.

Equipment and materials

- Eppendorf pipettes and repeater
- Concentration evaporation system
- Vortexer and multitube vortexers (VWR, Radnor, PA, USA)
- Agilent Vac Elut SPS manifold 24 with collection rack for 13 × 100 mm test tubes (p/n 12234022)
- Agilent Bond Elut Plexa, 30 mg, 1 mL straight barrel cartridges (p/n 12109301)
- Agilent MS analyzed amber screw-top glass vials with write-on spot (p/n 5182-0716)
- Agilent bonded screw cap, PTFE/red silicone septa (p/n 5190–7024)
- Agilent vial insert, 250 µL, deactivated glass with polymer feet (p/n 5181–8872)

Instrumentation

Analysis was performed on an Agilent 1290 Infinity LC system consisting of:

- Agilent 1290 Infinity binary pump (G4220A)
- Agilent 1290 Infinity high performance autosampler (G4226A) equipped with an Agilent 1290 FC/ALS thermostat (G1330B)
- Agilent 1290 Infinity thermostatted column compartment (G1316C)

The LC system was coupled to an Agilent 6460A triple quadrupole LC/MS/MS system equipped with Agilent Jet Stream Electrospray ionization technology. Agilent MassHunter workstation software was used for all data acquisition and analysis.

Sample preparation

A hormone standard mix and hormone internal standard mix were each prepared in methanol. Table 2 lists the concentrations for each analyte and its respective internal standard. Each internal standard was kept at the same concentration as its respective analyte. For example, aldosterone was at a concentration of 50 ng/mL in the hormone standard mix, and aldosterone-d4 was 50 ng/mL in the hormone internal standard mix.

Table 2. Hormone and internal standard concentrations are provided. Internal standards were spiked at the same concentration as parent analytes.

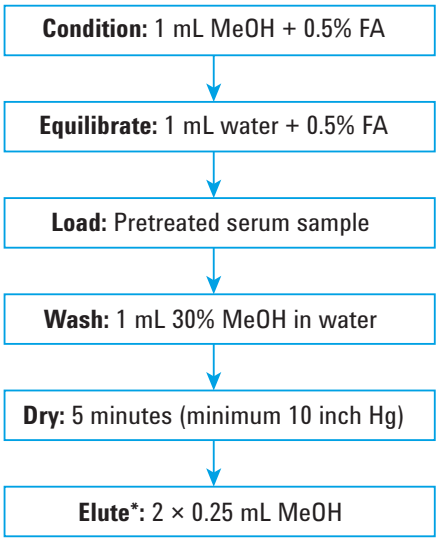
Analyte/internal standard	Standard concentration, each (ng/mL)
Aldosterone/aldosterone d4	50
Cortisol/cortisol d4	50
Cortisone/cortisone d8	50
Corticosterone/corticosterone d4	50
11-Deoxycortisol/11-deoxycortisol d5	50
β-Estradiol/β-estradiol d5	500
Testosterone/testosterone d3	50
11-Deoxycorticosterone/11-deoxycorticosterone d7	50
Androstenedione/androstenedione $^{13}C3$	50
Estrone/estrone d3	500
17αOH Progesterone/17αOH progesterone d8	50
DHT/DHT d3	500
Progesterone/progesterone d9	50

Sample pretreatment

Prior to the SPE protocol, 400 µL of 0.5% formic acid (FA) in water was added to 100 µL of serum. Samples were spiked with 20 µL of hormone standard mix and 20 µL of hormone internal standard mix. The final concentration was 100 ng/mL for β-estradiol, β-estradiol d5, estrone, estrone d3, DHT, and DHT d3. All other hormones and internal standards were at a final concentration of 10 ng/mL in serum. Samples were vortexed and centrifuged to remove any serum particulates.

After SPE, the collected eluent was evaporated to dryness under nitrogen at 40 °C, then reconstituted with 100 µL of 50/50 MeOH/water. Sample was vortexed, then transferred to a vial with a glass insert for LC/MS/MS analysis.

SPE Protocol



*Gravity elution was performed. After the eluent flow stopped, a low vacuum (<5 inch Hg, 3-5 seconds) was applied to fully collect all eluent.

Figure 1. SPE protocol for hormone determination using an Agilent Bond Elut Plexa 30 mg, 1 mL cartridge.

Instrument conditions

Parameter	Value
HPLC	
Column:	Agilent InfinityLab Poroshell HPH-C8, 2.1 mm × 50 mm, 2.7 µm (p/n 699775–706)
Guard:	Agilent InfinityLab Poroshell HPH-C8, UHPLC guard column, 2.1 mm × 5 mm, 2.7 µm (p/n 821725–922)
Mobile phase:	A) 1 mM ammonium fluoride in water B) acetonitrile (ACN)
Flow rate:	0.4 mL/min
Column temperature:	40 °C
Autosampler temperature:	4 °C
Injection volume:	10 µL
Needle wash:	1:1:1:1 ACN/MeOH/isopropanol/water with 0.2 % FA
Gradient:	Time (min) % B 0 20 8 50 9 95
Stop time:	10 minutes
Post-time:	1 minute
MS	
Electrospray Ionization (ESI)	
Gas temperature:	250 °C
Gas flow:	11 L/min
Nebulizer:	35 psi
Sheath gas heater:	350 °C
Sheath gas flow:	11 L/min
Capillary:	Positive 3,000 V; Negative 3,500 V
Nozzle voltage:	Positive 0 V; Negative 1,800 V
Delta EMV (+):	300 V
Delta EMV (–):	0 V

Table 3. LC/MS/MS dMRM parameters and retention times for target analytes.

				Product ion				
Analyte	**RT (min)**	**Precursor ion (*m/z*)**	**Frag (V)**	**Quant ion (*m/z*)**	**CE (V)**	**Qual ion (*m/z*)**	**CE (V)**	**Ionization mode**
Aldosterone	1.55	361.2	100	343.2	15	315.2	16	Positive
Cortisol	1.98	363.2	105	121.1	24	91.1	72	Positive
Cortisone	2.05	361.2	133	163.1	24	121	32	Positive
Corticosterone	3.01	347.2	110	329.2	12	121	24	Positive
11-Deoxycortisol	3.22	347.2	133	109.1	28	97.1	24	Positive
β-Estradiol	4.02	271.17	158	145.1	44	143	64	Negative
Testosterone	4.17	289.2	104	109.1	24	97.1	24	Positive
11-Deoxycorticosterone	4.51	331.2	133	109.1	28	97.1	24	Positive
Androstenedione	4.70	287.2	84	109.1	24	97.1	20	Positive
Estrone	4.77	269.15	158	145.1	44	143	64	Negative
17αOH Progesterone	5.01	331.2	133	109.1	28	97.1	24	Positive
DHT	5.34	291.2	158	255.1	8	77.1	80	Positive
Progesterone	6.82	315.2	104	109.1	24	97.1	24	Positive

Table 4. LC/MS/MS dMRM parameters and retention times for internal standards.

				Product ion		
Analyte	**RT (min)**	**Precursor ion (*m/z*)**	**Frag (V)**	**Quant ion (*m/z*)**	**CE (V)**	**Ionization mode**
Aldosterone d4	1.54	365.4	100	347.2	15	Positive
Cortisol d4	1.97	367.24	104	121	24	Positive
Cortisone d8	2.02	369.25	143	169.1	24	Positive
Corticosterone d4	3.01	363.2	105	121	24	Positive
11-Deoxycortisol d5	3.20	352.26	133	100.0	24	Positive
β-Estradiol d5	4.02	276.2	150	147.1	35	Negative
Testosterone d3	4.13	292.2	104	97.1	24	Positive
11-Deoxycorticosterone d7	4.51	338.27	84	100.1	20	Positive
Androstenedione $^{13}C3$	4.70	290.4	104	100.1	20	Positive
Estrone d3	4.77	272.19	158	148.1	40	Negative
17αOH Progesterone d8	4.96	339.28	138	100.1	28	Positive
DHT d3	5.31	294.25	120	258.2	12	Positive
Progesterone d9	6.75	324.29	104	100.1	24	Positive

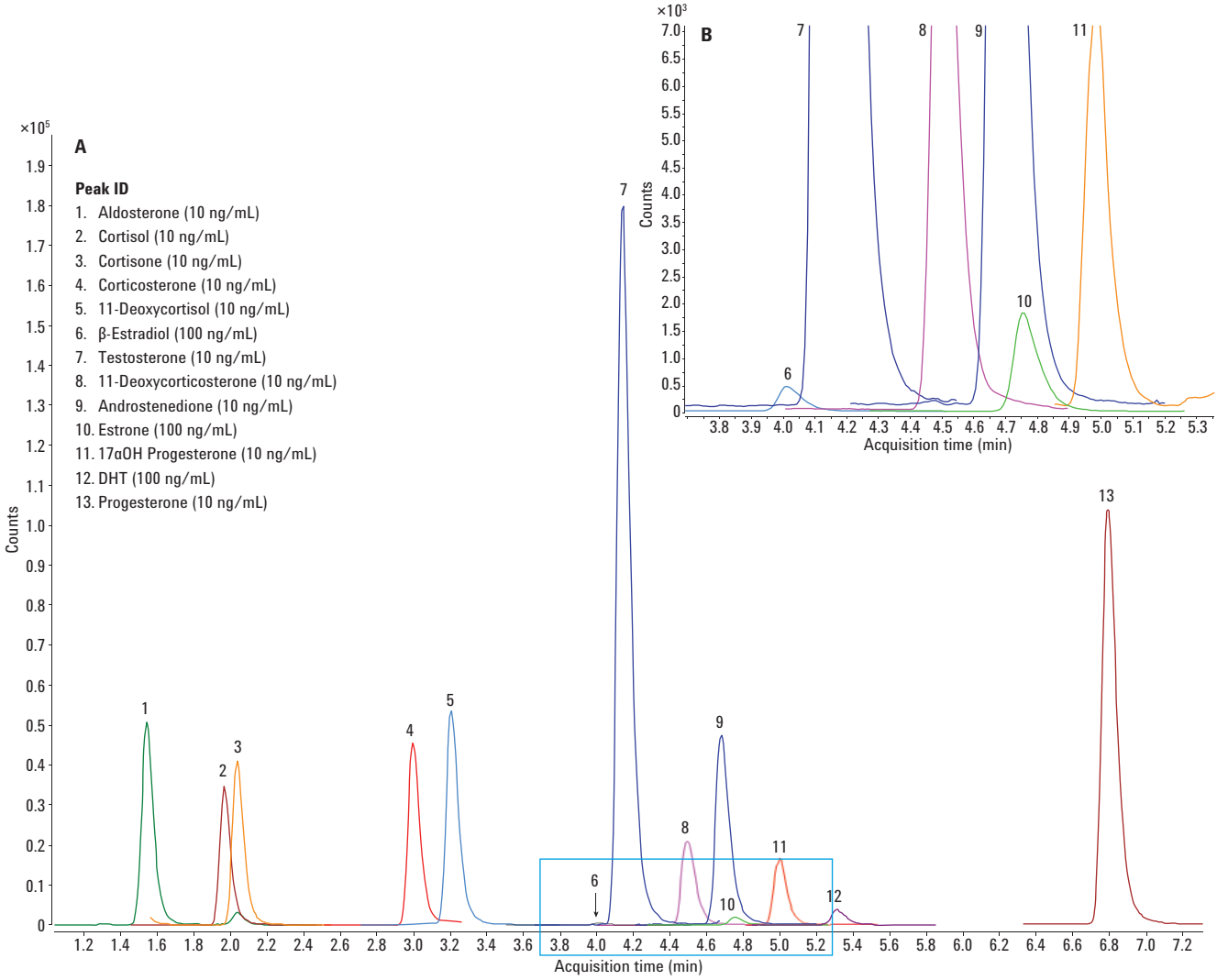


Figure 2. Final dMRM chromatogram of 13 analyzed hormones in serum (A), with a zoomed in chromatogram (B) showing β-estradiol and estrone. Concentrations are provided in the legend.

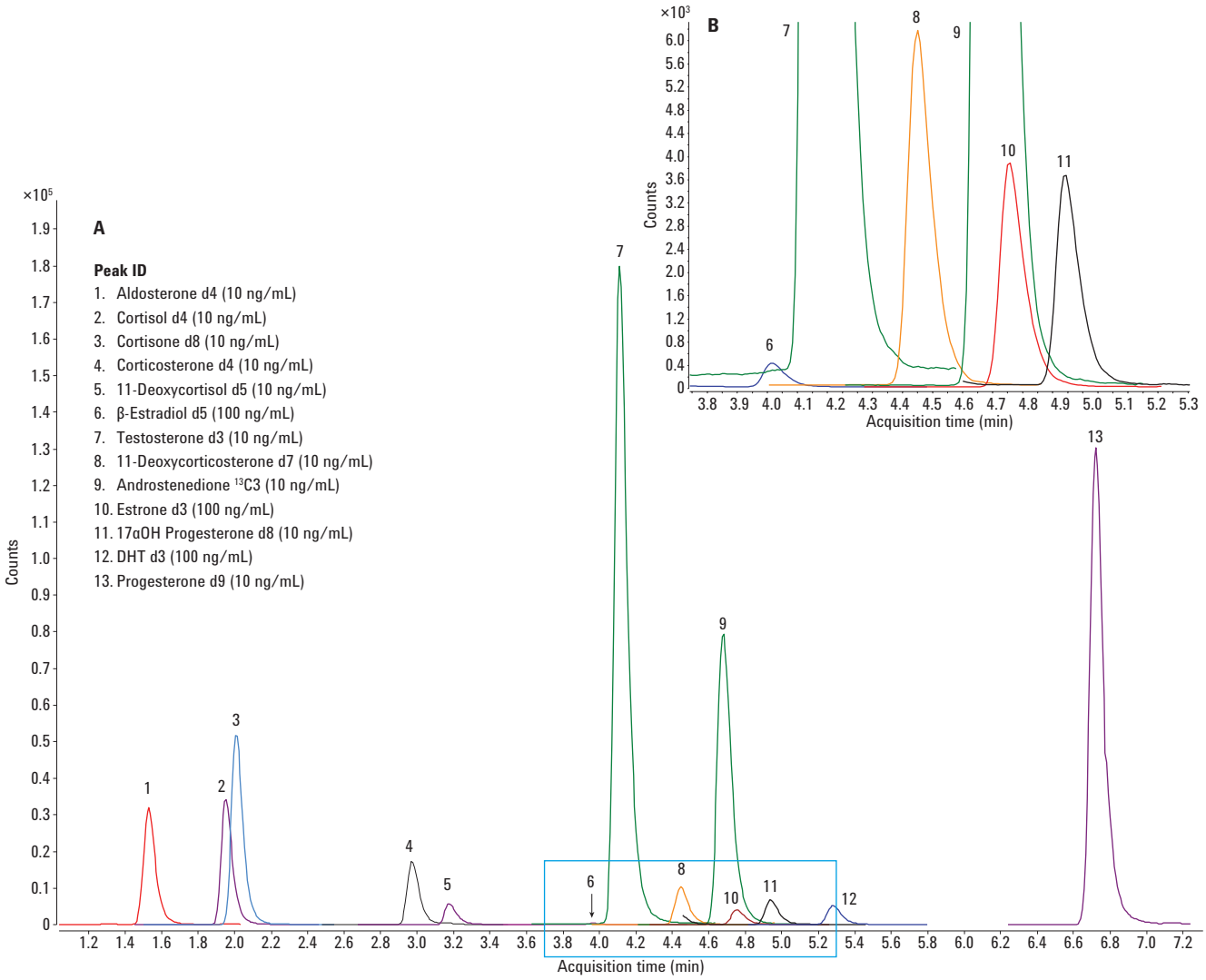


Figure 3. Final dMRM chromatogram of 13 analyzed hormone internal standards in serum (A) with a zoomed in chromatogram (B) showing β-estradiol d5 and estrone d3. Concentrations are provided in the legend.

Results and Discussion

SPE Optimization

When developing an SPE method, it is important to use a strong enough wash solvent to remove matrix interferences, while ensuring your analytes of interest remain retained on the cartridge. By collecting and analyzing the wash eluent from each of the candidate wash solvents (100% water, 10% MeOH in water, 20% MeOH in water, and so forth), hormone loss can be determined. After evaluation, a 30% MeOH in water wash was chosen as the optimal wash for this protocol.

Similar to the wash step, the elution solvent can be optimized as well. The goal of the elution step is to choose a solvent that is strong enough to elute all the target analytes, without eluting matrix interferences that may be retained on the sorbent during the SPE procedure. For this application note, 80% MeOH in water, 90% MeOH in water, 100% MeOH, and 50/50 MeOH/ACN elution solvents were evaluated. The 100% MeOH elution solvent provided the highest hormone recoveries, and was therefore chosen for this application.

LC/MS/MS Optimization

During initial LC/MS/MS method development, 11 hormones were analyzed: aldosterone, cortisol, cortisone, corticosterone, 11-deoxycortisol, androstenedione, 11-deoxycorticosterone, testosterone, 17αOH progesterone, DHT (negative mode), and progesterone. Two negative mode analytes (β-estradiol and estrone) were later added to the method, and therefore were not included in method development chromatograms.

An Agilent InfinityLab Poroshell 120 EC-C8 column (2.1 × 50 mm, 2.7 µm) was compared to an Agilent InfinityLab Poroshell 120 EC-C18 column (2.1 × 50 mm, 2.7 µm) (Figure 4). The shorter alkyl chain of the EC-C8 delivered slightly shorter retention times for the hormones, while still separating all isobaric pairs.

Conditions

Mobile phase A:	Water + 0.1% formic acid
Mobile phase B:	Methanol + 0.1% formic acid
Gradient program:	0–8 minutes: 40% mobile phase B to 55% mobile phase B, 8.1 minutes: 95% mobile phase B, hold
Total run time:	10 minutes + 1 minute post-time
Flow rate:	0.4 mL/min
Injection volume:	10 µL

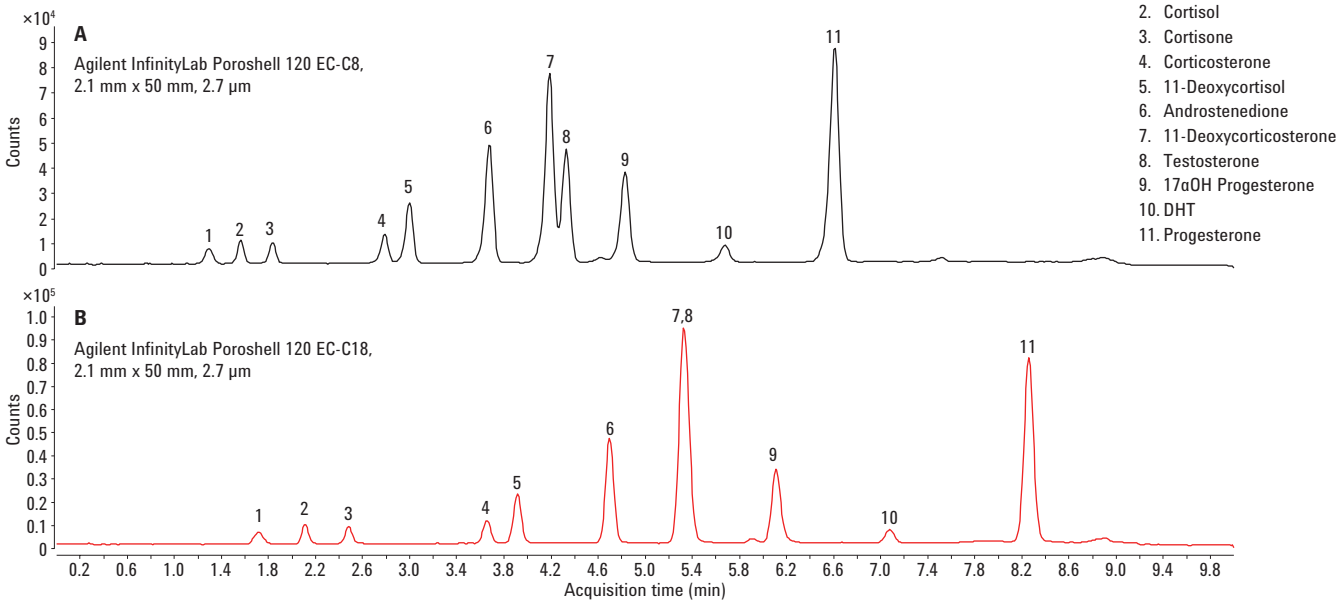


Figure 4. Comparison of separation and retention for 11 hormones (100 ng/mL) using Agilent InfinityLab Poroshell 120 EC-C8 and EC-C18 columns. β-Estradiol and estrone were added to the final method after this chromatogram was generated, and are not included in this chromatogram.

Formic acid (0.1%), ammonium hydroxide (0.02%, pH 10.5), and ammonium fluoride (pH 6.2) mobile phases were also evaluated (Figures 5-7). High pH mobile phase with ammonium hydroxide (0.02%) has been shown to improve ionization of hormones analyzed in negative mode [1]. Ammonium fluoride has been shown to improve responses in both positive and negative modes [2,3].

Since most silica-based LC columns are not recommended for mobile phases with a pH higher than 8 or 9, an Agilent InfinityLab Poroshell HPH-C8 column was used for evaluation. This column is stable up to a pH of 11, and provides very similar selectivity to an InfinityLab Poroshell 120 EC-C8 column. Therefore, the InfinityLab Poroshell HPH-C8 column was used for method optimization with both ammonium hydroxide and ammonium fluoride mobile phases, and chosen for the final analytical method. Ammonium fluoride (1 mM) mobile phase yielded the highest analyte responses, and was used for the final chromatographic method.

Conditions

Mobile phase A:	0.1% Formic acid in water or 0.02% ammonium hydroxide in water
Mobile phase B:	Methanol
Gradient program:	0–9 minutes: 40% mobile phase B to 60% mobile phase B 9–11 minutes: 60% mobile phase B to 95% mobile phase B
Total run time:	12 minutes + 1 minute post-time
Flow rate:	0.4 mL/min
Injection volume:	10 µL

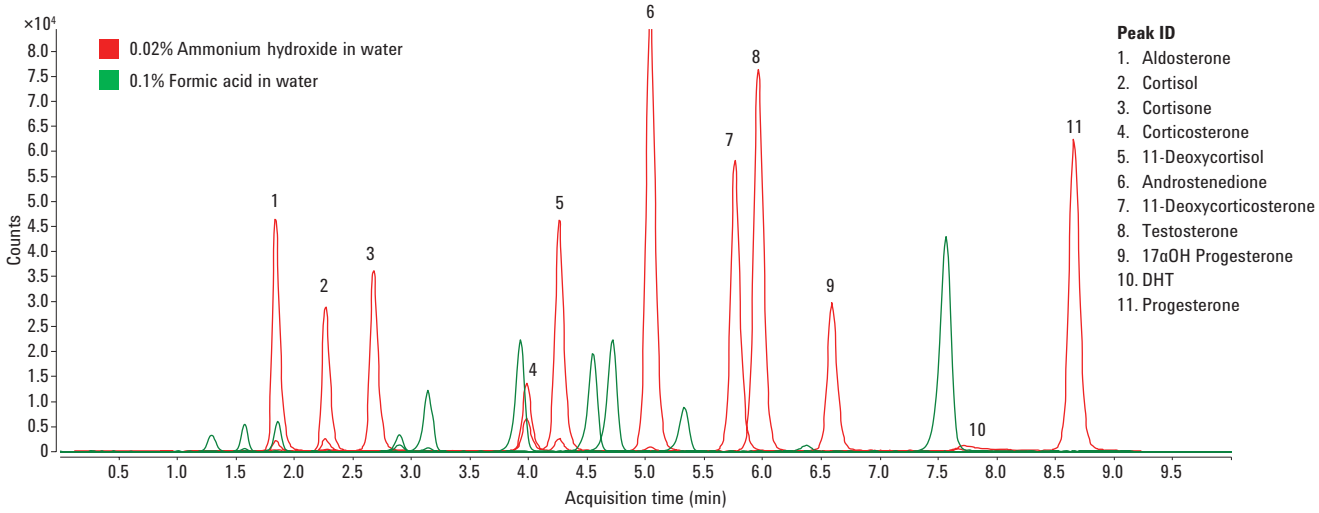


Figure 5. Chromatogram for a 100 ng/mL hormone sample with 0.1% formic acid in water (green trace) and 0.02% ammonium hydroxide in water (red trace) using an Agilent InfinityLab Poroshell HPH-C8, 2.1 mm × 50 mm, 2.7 µm column. The LC/MS/MS gradient was kept the same for comparison purposes. β-Estradiol and estrone were added to the method after these chromatograms were generated, and are not included.

Conditions

Mobile phase A:	1 mM ammonium fluoride in water or 0.02 % ammonium hydroxide in water
Mobile phase B:	Methanol
Gradient program:	0–9 minutes: 40% mobile phase B to 60% mobile phase B 9–11 minutes: 60% mobile phase B to 95% mobile phase B
Total run time:	12 minutes + 1 minute post-time
Flow rate:	0.4 mL/min
Injection volume:	10 µL

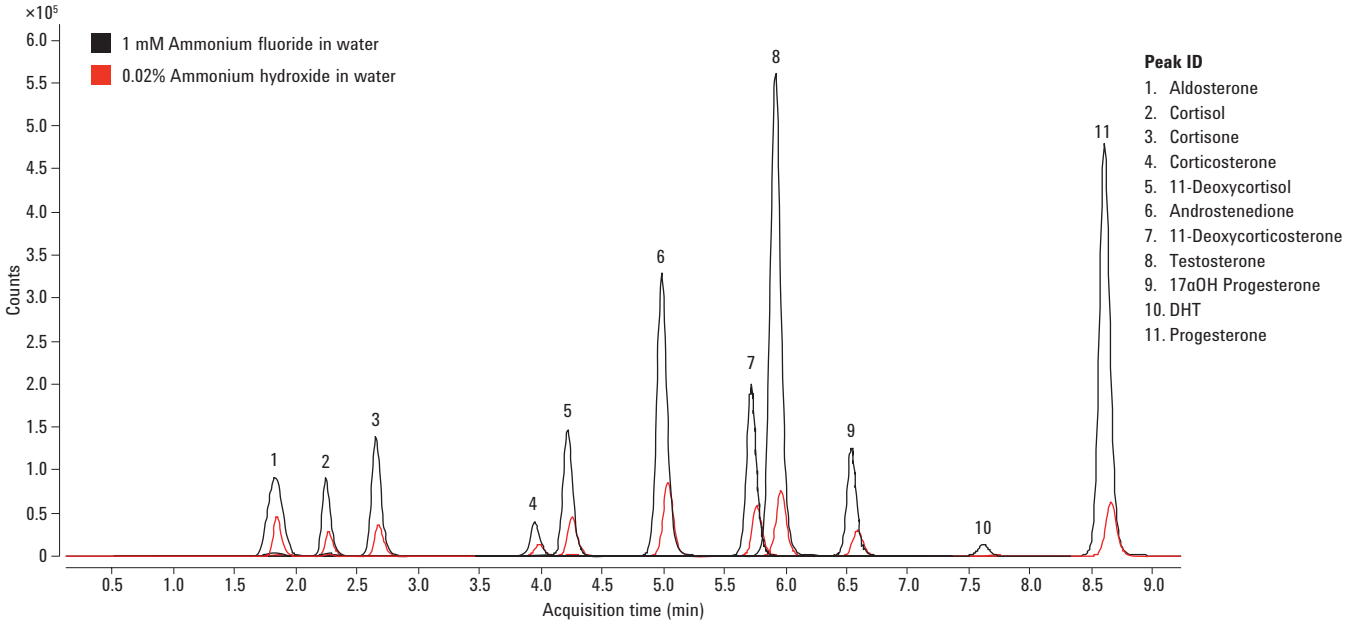


Figure 6. Total ion chromatogram for a 100 ng/mL hormone sample with 1 mM ammonium fluoride in water (black trace), and 0.02% ammonium hydroxide in water (red trace). An Agilent InfinityLab Poroshell HPH-C8, 2.1 mm × 50 mm, 2.7 µm was used. The LC/MS/MS gradient was kept the same for comparison purposes. β-Estradiol and estrone were added to the method after these chromatograms were generated, and are not included.

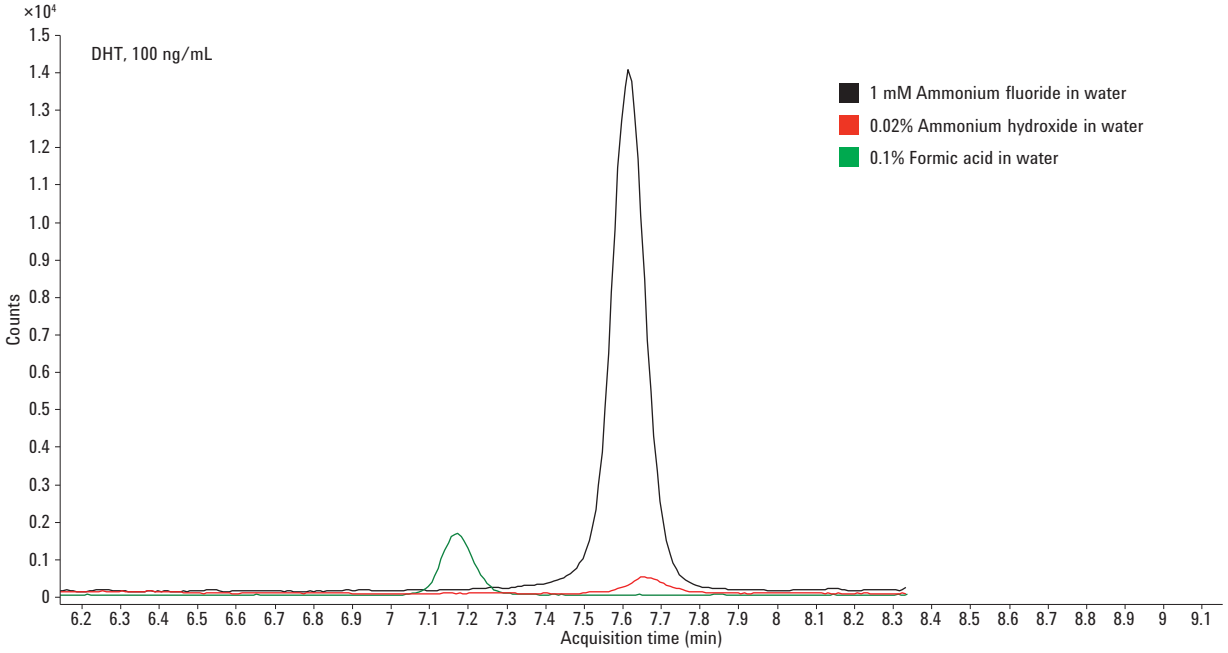


Figure 7. MRM chromatogram overlay for DHT (100 ng/mL; negative mode) with three mobile phases: 1 mM ammonium fluoride in water (black), 0.02% ammonium hydroxide in water (red), and 0.1% formic acid in water (green). An Agilent InfinityLab Poroshell HPH-C8, 2.1 mm × 50 mm, 2.7 µm was used. Gradient conditions are provided in the descriptions of Figures 5 and 6.

Recovery and reproducibility

Recovery and reproducibility were evaluated by extracting prespiked and blank serum samples using the SPE protocol previously described. Serum samples were prespiked with a concentration of 100 ng/mL for β-estradiol, estrone, and DHT. The remaining 10 hormones were prespiked at a concentration of 10 ng/mL. Blank and spiked serum samples were extracted using the SPE protocol. Blank samples were postspiked after final dry down at a concentration of 10 and 100 ng/mL, as described earlier. Percent recovery and RSD values were determined by comparing average peak areas for prespiked and postspiked samples (n = 5).

Table 5. Percent recovery and RSD values (shown in parentheses) are provided (n = 5).

Analyte	Fortified concentration (ng/mL)	% Recovery (RSD)
Aldosterone	10	94 (5.8)
Cortisol	10	102 (5.4)
Cortisone	10	99 (5.7)
Corticosterone	10	97 (5.1)
11-Deoxycortisol	10	97 (3.9)
β-Estradiol	100	81 (5.3)
Testosterone	10	86 (3.3)
11-Deoxycorticosterone	10	91 (4.6)
Androstenedione	10	92 (4.7)
Estrone	100	80 (3.9)
17αOH Progesterone	10	105 (4.2)
DHT	100	88 (2.8)
Progesterone	10	90 (4.0)

Conclusions

A method was developed for the determination of 13 hormones, and their respective internal standards in serum using Agilent Bond Elut Plexa SPE cartridges. Analyte response with an Agilent 6460 triple quadrupole LC/MS/MS operating in both positive and negative mode was greatly enhanced with the addition of 1 mM ammonium fluoride. An Agilent InfinityLab Poroshell HPH-C8 (2.1 mm × 50 mm, 2.7 µm) column yielded fast run times and baseline separation of three isobaric pairs, allowing for more reproducible integration and quantification. The wide pH range makes it an ideal column choice for method development. The method provided excellent recoveries (80-105%) and low RSD values (2.8-5.8%). Agilent Bond Elut Plexa SPE products require little method development and are simple to use, making them the ideal choice for biological analysis.

References

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