Application Note

Clinical Research

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Analysis of Multiclass Steroids in Serum Using Agilent Ultivo Triple Quadrupole LC/MS

Automation-ready SLE sample preparation using Agilent Chem Elut S, 96-well plates

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Abstract

This application note presents a robust workflow for quantitative screening of a wide panel of steroids from serum matrix using the Agilent Ultivo triple quadrupole LC/MS (Ultivo LC/TQ) system. The workflow includes an automation-ready 96-well plate-based supported liquid extraction sample preparation protocol, a 14-minute chromatographic separation method, and TQ multiple reaction monitoring (MRM) parameters for confident analysis of 15 steroids. The workflow method performance was evaluated using ChromSystems steroid panels 1 and 2 certified reference samples, and the results were satisfactory. Observed target peak area response %RSD <10% and retention time %RSD was <0.5%. All 15 targets displayed excellent linearity, with R^2 >0.995, and method accuracy was within 85 to 120% across the linearity range (%RSD <10%, n = 3). The average recovery of targets for three QC levels was within 82 to 112%, with an intraday repeatability %RSD of ≤12% (n = 3) and interday reproducibility %RSD of ≤10% (n = 3).

Introduction

Steroids are endogenous cholesterol metabolites that hold specific roles in various biological processes.[1] The biological availability of many steroids is only at trace concentrations, still, their quantitative determination is relevant for clinical research interpretation. Analytical specificity with conventional immunoassays is often challenged by interferences due to the cross-reactivity of reagent antibodies with structurally similar steroids. However, analytical methods based on LC/MS/MS offer reduced interferences, better specificity, and improved sensitivity for accurate measurement of target steroids.[2,3]

This application note discusses an LC/MS/MS workflow for clinical research using automation-ready supported liquid extraction (SLE) sample preparation followed by reverse phase chromatography, which enables sensitive measurement of 15 steroids from serum with specificity. The method performance was characterized using certified steroids reference samples from ChromSystems.

Experimental

Chemicals and reagents

All 15 steroid standards, respectively labeled standards (as internal standards), and DC Mass Spect Gold serum were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Sample preparation, chromatography, and Ultivo TQ method parameters are optimized using these individual standards. When not in use, all standards were stored as per the manufacturer's recommendation either at –20 °C or –80 °C. LC/MS/MS MassCheck steroids in serum samples (panels 1 and 2) and internal standard mix (part number 72044) were purchased from ChromSystems (Graefelfing, Germany) and were used as reference samples to evaluate the method performance. LC/MS grade acetonitrile and methanol were purchased from Agilent. All other reagents including ammonium fluoride, ethyl acetate (EA), and *tert*-butyl methyl ether (MTBE) were purchased from Sigma-Aldrich. Ultrapure Milli Q water was produced using an in-house water purification system (Merck Millipore, MA, USA).

Equipment and consumables

Laboratory equipment and consumables used for sample preparation were as follows:

- Chem Elut S, 2 mL 96WP 400 µL (part number 5610-2004)
- Positive pressure manifold, PPM-96 (part number 5191-4116)
- Sealing gasket, for PPM-96 (part number 5191-4117)
- Plate Holder, for PPM-96 (part number 5191-4120)
- 96-well plate mats (part number 5043-9317)
- Well plate for collection, 2 mL (part number 5043-9302)
- Screw vial, fixed insert, amber, 100/pk (part number 5188-6592) (optional)
- Vial screw cap 100/pk (part number 5190-7024) (optional)
- Vacuum concentrator, 96-well plate format
- Centrifuge, 96-well plate format
- Ultrasonic bath
- Vortex mixer >500 rpm
- Multichannel pipettes and matching tips
- 2 mL disposable self-lock vials

Instrumentation

Chromatographic separation was performed on an Agilent 1260 Infinity II liquid chromatography system using a Poroshell 120 EC-C18 column. The 1260 Infinity II LC system consisted of:

- 1260 Infinity II binary pump (G7112B)
- 1260 Infinity II multisampler (G7167A)
- 1260 Infinity II multicolumn compartment (G7116A)

A 0.3 µm inline filter (part number 5067-6189) was installed between the autosampler injector valve port 6 and the multicolumn compartment. The LC conditions are listed in Table 1, and Ultivo LC/TQ source parameters, optimized using Agilent MassHunter Optimizer software (version 1.2), are included in Table 2.

Table 1. Agilent 1260 Infinity II LC parameters.

Parameter	Value
Needle Wash	Standard wash, flush port, 20 s; 60/40 acetonitrile/water with 0.1% formic acid
Autosampler Temperature	6 °C
Injection Volume	15 µL
Analytical Separation	Agilent Poroshell 120 EC-C18, 3.0 × 50 mm, 2.7 µm analytical column (p/n 699975-302) with Agilent InfinityLab Poroshell EC C18, 3.0 × 5 mm, 2.7 µm UHPLC guard column (p/n 823750-911)
Column Temperature	45 °C
Mobile Phase A	Water with 0.5 mM ammonium fluoride
Mobile Phase B	Methanol with 0.5 mM ammonium fluoride
Gradient	Time (min) %B Flow rate (mL/min) 0.00 35 0.50 2.00 50 0.50 3.50 50 0.50 4.00 60 0.50 5.50 60 0.50 6.00 70 0.50 7.50 70 0.50 9.00 95 0.50 9.50 95 0.50 11.00 95 0.75 11.25 35 0.75 14.00 35 0.50

Table 2. Agilent Ultivo LC/TQ mass spectrometer configuration and parameters.

Parameter	Value
Configuration	Agilent Ultivo LC/TQ (G6465B) equipped with an Agilent Jet Stream (AJS) Electrospray ion source
Ionization Polarity	Positive/Negative
MS/MS Mode	MRM
Resolution MS1/MS2	Unit/Unit
Drying Gas Temperature	300 °C
Drying Gas Flow	10 L/min
Nebulizer Pressure	50 psi
Sheath Gas Temperature	250 °C
Sheath Gas Flow	9 L/min
Capillary Voltage (Pos/Neg)	2,500 V
Nozzle Voltage (Pos/Neg)	1,000 V
Diverter Valve to MS	From 3.6 to 10.0 min

MRM optimization

Target-specific MRM transitions, fragmentor voltage, and collision energies were optimized using MassHunter Optimizer software (version 1.2). The MS/MS optimization was performed without chromatographic separation, using 2 µL injections of neat solutions of individual analytes at about 1,000 µg/L. The LC parameters along with optimized TQ fragmentor and collision energy values were added to compose the final MRM method for data acquisition. The optimized MRM settings used for the analysis are included in Table 3.

Table 3. MRM parameters of standards and internal standards: dwell: 20 (ms).

Target Number	Target Name	Precursor (*m/z*)	Fragmentor (V)	Product 1 Quant (*m/z*)	CE (V)	Product 2 (*m/z*)	CE (V)
1	Aldosterone	361.2	125	343	16	315	20
ISTD (1)	Aldosterone-d4	365.2	125	347	15	346	15
2	Corticosterone	347.2	121	329	12	121	24
ISTD (2)	Corticosterone-d8	355.3	121	100	30	NA	NA
3	Cortisol	363.2	121	121	24	327	12
ISTD (3)	Cortisol-d4	367.2	121	121	24	331	13
4	Cortisone	361.2	138	163	24	121	36
ISTD (4)	Cortisone-d8	369.2	138	168	24	124	36
5	11-Deoxycortisol	347.2	116	97	28	109	32
ISTD (5)	11-Deoxycortisol-d5	352.2	141	100	28	113	32
6	21-Deoxycortisol	347.2	129	311	12	121	24
ISTD (6)	21-Deoxycortisol-d8	355.3	129	319	12	125	28
7	Androstenedione	287.2	111	97	24	109	24
ISTD (7)	Androstene-3,17-dione-$^{13}C_3$	290.2	111	100	24	112	24
8	11-Deoxycorticosterone	331.2	131	97	28	109	30
ISTD (8)	11-Deoxycorticosterone-d8	339.2	131	100	28	113	28
9	DHEA (Dehydroepiandrosterone)	306.3	70	289	0	271	4
ISTD (9)	DHEA-d5	311.3	70	294	0	276	8
10	DHEAS (Dehydroepiandrosterone sulfate)	288.2 (271.2)	75 (115)	271 (253)	4 (8)	NA	NA
10	DHEAS	367.4 (Neg)	150	97	40	NA	NA
ISTD (10)	DHEAS--d6	294.2 (277.2)	75 (115)	277 (259)	4 (8)	NA	NA
ISTD (10)	DHEAS-d6	375 (Neg)	150	100	40	NA	NA
11	DHT (Dihydrotestosterone)	291.2	136	255	13	105	41
ISTD (11)	DHT-d3	294.3 (311.3)	136 (80)	258 (294)	13 (4)	NA	NA
12	17β-Estradiol	273.2	90	107	30	135	16
ISTD (12)	17β-Estradiol-d5	278.2	90	109	30	138	16
13	17α-Hydroxyprogesterone	331.2	131	109	30	97	28
ISTD (13)	17α-Hydroxyprogesterone-$^{13}C_3$	334.2	131	100	28	112	30
14	Progesterone	315.2	131	97	24	109	28
ISTD (14)	Progesterone-$^{13}C_3$	318.2	131	100	28	112	24
15	Testosterone	289.2	126	97	24	109	28
ISTD (15)	Testosterone-d3	292.2	126	97	24	109	28

Sample preparation

Serum sample cleanup was performed using Chem Elut S supported liquid extraction (Chem Elut S) 2 mL 96-well plates. A positive pressure manifold 96 processor (PPM-96) was used to perform SLE. A 350 µL aliquot of the serum sample spiked with internal standard was loaded onto the SLE sorbent first. After a 5-minute equilibration, the elution was performed with a water-immiscible solvent using gravity or gentle pressure. A 50/50 mix of ethyl acetate and MTBE was used as SLE elution solvent. Figure 1 describes the optimal sample preparation procedure in detail.

Reference samples for performance verification

Reference samples of all 15 steroid targets are available as two separate panels from ChromSystems. Aldosterone, corticosterone, cortisol, cortisone, 11-deoxycortisol, and 21-deoxycortisol constitute panel 1 and the remaining nine targets constitute panel 2. Lyophilized reference samples were reconstituted as per the manufacturer's protocol and used to verify the workflow performance. The reference sample set includes blank control, six-level calibrators, and three QC levels: low-range QC (LQC), mid-range QC (MQC), and high-range QC (HQC), respectively.

Data acquisition and analysis

ChromSystems reference blanks, calibrators, and three technical preparations of each QC level from both panels were spiked with 30 µL internal standard mix and subjected to the in-house developed SLE sample preparation protocol. LC/TQ data of each processed sample were acquired in three replicates using the Ultivo equipped with the AJS source. Data were acquired and processed by MassHunter LC/MS Data Acquisition software (version 1.2) and MassHunter Quantitative Analysis software (version 10.0), respectively. Sensitivity assessment was performed using the level 1 serum calibrator reference sample. Method characteristics like precision, accuracy, linearity, and recovery were evaluated using reference samples. Recovery repeatability (%RSD) values were calculated from three intraday technical preparations of each QC level. The sample extraction and analysis were repeated over 3 days to assess interday QC recovery deviation (%RSD) and workflow reproducibility. Target responses, from a diluent injection performed immediately after the highest calibrator, were used to compute the carryover.

350 µL serum (spike 30 µL ISTD and mix well).

SLE cleanup using Agilent Chem Elute S 2 mL 96WP 400 µL with ppm 96

- Load the sample and apply 2 to 3 psi of pressure.
- Equilibrate for 5 minutes.
- Elute using 500 µL of 50/50 EA/MTBE; elute by gravity.
- When there is no visible liquid left in the wells, repeat the elution two more times (2x 500 µL 50/50 EA/MTBE) gravity elution.
- When there is no visible liquid left in all of the wells, apply ~6 psi to dry the cartridge.

Remove the collection plate, evaporate to dryness, and reconstitute using 60 µL of diluent (70/30 mobile phase A/B).

Analyze

Figure 1. Agilent PPM-96 based SLE workflow for sensitive measurement of 15 steroids in serum using the Agilent Ultivo LC/TQ.

Results and discussion

SLE sample cleanup and elution profile

The synthetic media of Chem Elut S provided easy extraction of all 15 target analytes and allowed efficient removal of the matrix, including salts and phospholipids. Controlled particle and pore size enabled consistent elution flow and offered batch-to-batch reproducibility compared to conventional LLE. The 96-well plate-based SLE sample cleanup procedure is amenable to automation, which increases sample preparation throughput. The Agilent Poroshell 120 EC-C18 column offered sufficient separation of all 15 analytes in under 10 minutes. The targets corticosterone, 11-deoxycortisol, and 21-deoxycortisol with similar MRM transitions are well separated using the described chromatographic method. The LC methodology also separates another critical pair with similar molecular fragmentation patterns; 11-deoxycorticosterone and 17α-hydroxyprogesterone, thus enabling confident MRM-based quantitation. A typical MRM trace overlay of 15 analyzed steroids in serum is depicted in Figure 2.

Method detection limit

Method detection limit (MDL) represents the minimum concentration of a target in a given matrix that can be measured with 99% confidence that the analyte concentration is greater than zero. The workflow MDL was assessed with MassHunter Quantitative Analysis software using seven replicate injections of calibrator 1 reference samples and the observed results are included in Table 4.

The calculated MDL values for 12 targets were much lower than level 1 reference calibrator concentrations, whereas for three targets 11-Deoxycorticosterone, DHT, and 17β-Estradiol, the MDL values were close to the L1 calibrator concentration. The MRM overlay of blank and calibrator 1 reference samples for all 15 targets are included in Figure 3.

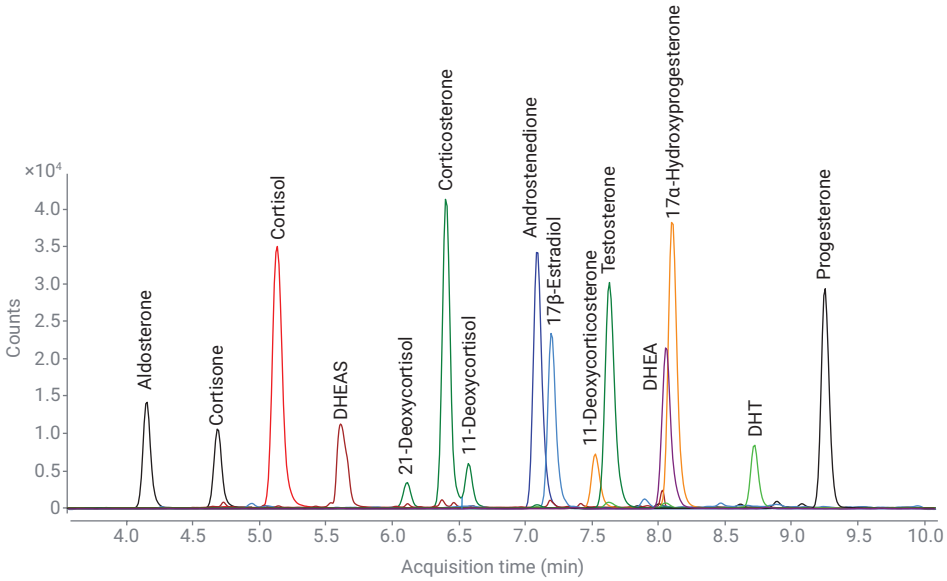


Figure 2. MRM trace overlay of 15 steroids detailing the elution profile.

Table 4. Elution time, MDL, and calibration range details of all 15 targets.

Number	Target	Retention Time (min)	Calculated MDL (ng/L)	Reference Calibrator Range L1 to L6 (ng/L)
1	Aldosterone	4.2	8	22 to 2,860
2	Corticosterone	6.4	90	593 to 55,200
3	Cortisol	5.1	377	10,200 to 284,000
4	Cortisone	4.7	72	1,020 to 39,800
5	11-Deoxycortisol	6.6	12	99 to 14,500
6	21-Deoxycortisol	6.1	20	64 to 5,000
7	Androstenedione	7.1	40	204 to 13,400
8	11-Deoxycorticosterone	7.5	35	51 to 2,830
9	DHEA	8.1	381	1,010 to 54,600
10	DHEAS	5.9	43,528	119,000 to 5,675,000
11	DHT	8.7	40	53 to 1,350
12	17β-Estradiol	7.2	35	40 to 4,990
13	17α-Hydroxyprogesterone	8.2	17	106 to 22,100
14	Progesterone	9.3	23	137 to 24,000
15	Testosterone	7.6	14	47 to 11,500

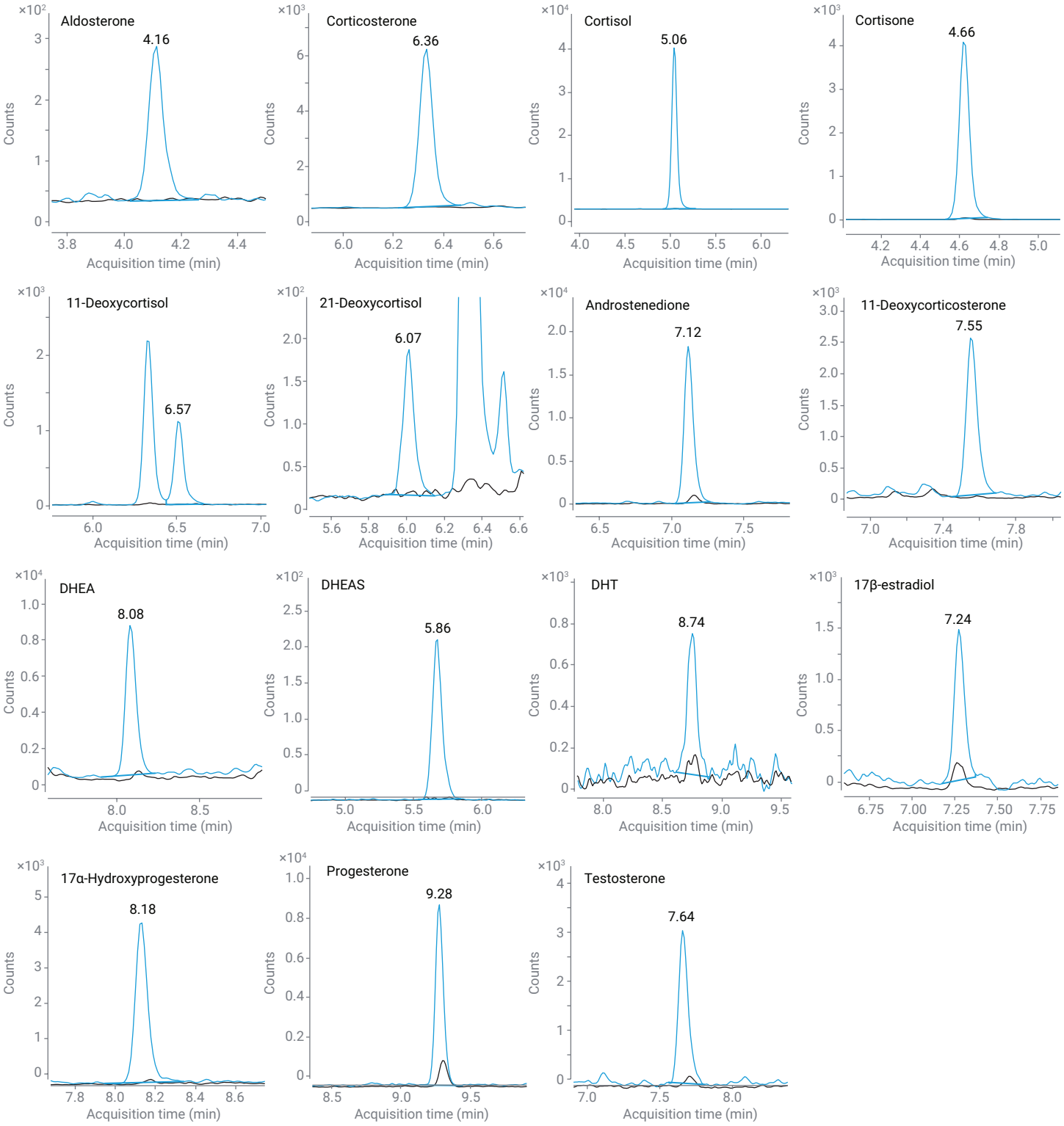


Figure 3. MRM trace overlay of blank serum (black trace) and calibrator 1 reference sample (blue trace) for all fifteen targets.

Linearity study using reference calibrators

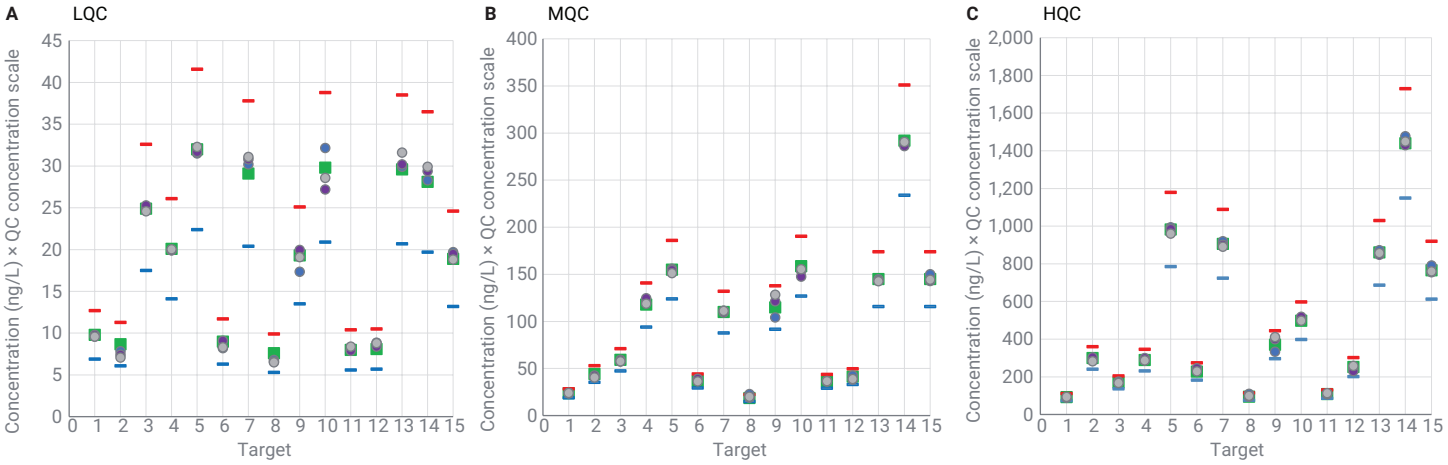
When compared with the control matrix, the analyte peak response for the lowest calibrator level was significantly exceeding the acceptable signal-to-noise values for quantitation, thus ensuring easy target identification and sensitive detection. Signal linearity curves for each analyte were constructed using six calibrator reference levels with internal standard correction. All 15 targets displayed a linear response with R^2 values >0.999 (calibration model type: linear; origin: ignore; weight: 1/x). The linear regression coefficient and slopes from all three batches were consistent, and thus confirmed the workflow reproducibility in the given analytical range.

Precision and accuracy

Method precision was determined by calculating the %RSD of the target RT and response ratio using three replicate injections of each calibration level. Satisfactory RT and response ratio precision values for all analytes were obtained for both panels, with %RSD <0.4% and <10%, respectively. The average accuracy value for each serum calibration level was calculated from three replicate injections. Accuracy for all three analytes across the calibration range was within 85 to 115%, with %RSD <10%.

Recovery and repeatability

The efficiency of the workflow for target recovery was assessed using three levels of certified reference QC samples (LQC, MQC, and HQC). Three technical preparations of each QC level were processed using the SLE 96-well plate-based protocol, and each preparation was injected into the LC/TQ in three replicates. Using respective calibration curve equations, the recovery (%) was calculated for each QC preparation and average recovery values were calculated (Figure 4). The intraday recovery repeatability was measured as %RSD of average recovery values, calculated using three technical preparations. Recoveries for overall analytes were within 82 to 112% which is well within the allowed deviation, with intraday %RSD ≤12%.



No:	Target	QC Concentration Scale
1	Aldosterone	×10¹
2	Corticosterone	×10²
3	Cortisol	×10³
4	Cortisone	×10²
5	11-Deoxycortisol	×10¹
6	21-Deoxycortisol	×10¹
7	Androstenedione	×10¹
8	11-Deoxycorticosterone	×10¹

No:	Target	QC Concentration Scale
9	DHEA	×10²
10	DHEAS	×10⁴
11	DHT	×10¹
12	17β-Estradiol	×10¹
13	17α-Hydroxyprogesterone	×10¹
14	Progesterone	×10¹
15	Testosterone	×10¹

Figure 4. Summarized recovery results of three QC levels from three batch preparations. (A) LQC, (B) MQC, and (C) HQC. The target list and concentration scale are listed in the table.

Interday reproducibility

Day-to-day workflow method reproducibility was assessed by computing %RSD of average recovery results from three consecutive days. The observed interday reproducibility was within 10 %RSD for all three QC levels (Figure 5). These results confirm the consistency of SLE extraction and LC/TQ methodology for routine quantitative analysis of steroids.

Carryover analysis

Target response from a solvent blank injection immediately after the highest calibration level was compared against that from calibrators to assess percent carryover. The observed carryover in the solvent injection was <0.05% when compared with the level 6 calibrator, and <3% when compared with the level 1 calibrator response. No significant carryover was observed from high concentration calibrator samples to subsequent blank injections.

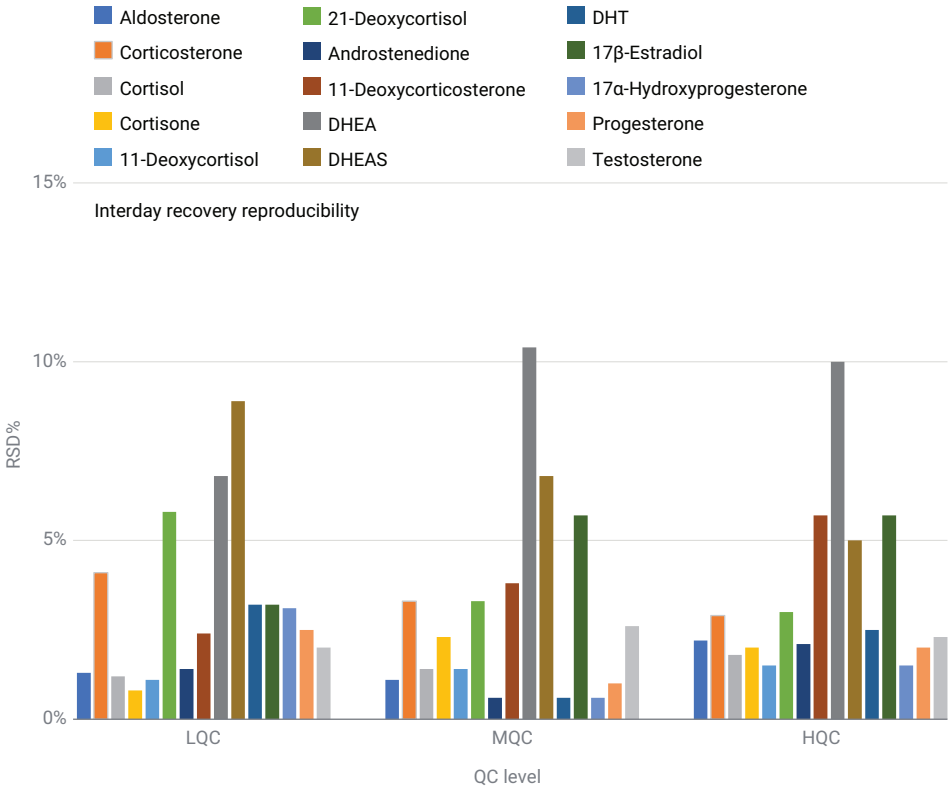


Figure 5. Recovery reproducibility %RSD of all 15 steroids across 3 days for all QC levels.

Conclusion

The newly developed LC/MS/MS workflow with SLE cleanup and Ultivo TQ MRM detection allows simultaneous quantitation of 15 steroids in serum that fulfills clinical research demand for sensitivity and selectivity. The automation-ready SLE sample cleanup offers significant time and labor savings compared to traditional liquid-liquid extraction (LLE). The use of the Agilent Poroshell 120 EC-C18 column helped to reduce gradient elution time with sufficient chromatographic resolution of all critical pairs for unambiguous TQ detection. The workflow sensitivity offers analytical confidence for detecting trace levels of steroids like 17β-estradiol and testosterone by using only a 350 µL serum sample size. The method was found to be linear for all targets with acceptable precision and accuracy results across the reference sample calibration range. The interday multi-level QC statistics illustrated the workflow suitability for confident day-to-day operation. The workflow offers a single convenient and fast alternative method for comprehensive measurement of multi-class steroids from serum to the standard practice of analyzing these targets using multiple methods.

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