

## Confidence in Your Calibrators: MassTrak Endocrine Steroid Calibrators and Quality Control Sets for the LC-MS/MS Analysis of Steroid Hormones

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### Abstract

The routine analysis of steroid hormones is critical in understanding the function of metabolic pathways that impact sexual characteristics, inflammation and blood pressure. Liquid Chromatography-Mass Spectrometry (LC-MS) is fast becoming a sought-after technique in steroid analysis due to the advantages it provides over traditional ligand-binding techniques. These benefits include improvements in analytical sensitivity and selectivity, and the capability of multi-analyte quantitative detection in a single run. However, many LC-MS methods lack harmonization or standardization. The Waters MassTrak Endocrine Steroid Calibrator and Quality Control Sets (IVD) contain metrologically traceable calibrators, aiding laboratories in their compliance to ISO 15189, and provide confidence in the accuracy of results when using validated LC-MS methods.

The MassTrak Endocrine Steroid Calibrator and Quality Control Sets performance was demonstrated using the ACQUITY UPLC I-Class PLUS and Xevo TQ-S micro Triple Quadrupole Mass Spectrometer and an in-house developed LC-MS/MS methodology.

### Benefits

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- Metrologically traceable calibrators and QCs that aid laboratories in their compliance to ISO 15189
- Confidence in the accuracy steroid hormones and provides a path to laboratory method harmonization
- Lyophilized calibrators and QCs that reduce sample preparation time

## Introduction

The analysis of steroid hormones is critical in our understanding of the dysfunction of steroid biosynthetic pathways that impact sexual characteristics, inflammation, and blood pressure.

Analysis of steroid hormones have traditionally been performed using ligand-binding techniques. These techniques can be analytically sensitive and highly automatable, providing high throughput of samples. However, the technique is held back by the inability to detect panels of analytes and is impacted by problems with selectivity of the reagents being used, therefore affecting the reliability of the result, particularly at lower steroid hormone concentrations. More recently, LC-MS/MS has become a sought-after technique in steroid analysis, as it has been established that it can overcome the limitations observed in ligand-binding methods, while providing similar levels of analytical sensitivity.

LC-MS/MS methods in clinical laboratories are often based on laboratory developed tests (LDTs), validated to local regulatory guidelines. These guidelines are constantly evolving and there is increasing demand for all aspects of clinical methods to comply with these changing regulations. This includes the calibrator and QC materials used to generate and independently check the accuracy of the calibration within the method. There is a growing need for metrological traceable calibration materials to replace the in-house prepared materials to aid in compliance with ISO 15189.

The Waters MassTrak Endocrine Steroid Calibration and Quality Control Sets (IVD) (Figure 1) contains a range of steroid hormones in lyophilized serum that have been sourced to obtain the highest level of metrological traceability available. In order to demonstrate the quality of materials found in this product, we have shown the proof of concept performance of the materials using solid phase extraction (SPE) and separation and detection of the samples using the ACQUITY UPLC I-Class PLUS with Xevo TQ-S micro Triple Quadrupole Mass Spectrometry.



Figure 1. The Waters MassTrak Endocrine Steroid Calibration and Quality Control Sets.

## Experimental

The MassTrak Endocrine Steroid Calibrator and Quality Control Sets contain the following steroid hormones in lyophilized serum: dehydroepiandrosterone sulfate (DHEA-S), cortisol, 21-deoxycortisol, corticosterone, 11-deoxycortisol, androstenedione, 11-deoxycorticosterone, testosterone, dehydroepiandrosterone (DHEA), 17-hydroxyprogesterone (17-OHP), dihydrotestosterone (DHT), and progesterone. Assigned concentrations for the calibration range and QCs are found in Table 1.

Steroid hormone	Calibrator range (nmol/L)	QCs (nmol/L)
DHEA-S	61–29392	527, 4770, 19061
Cortisol	3–1388	30, 279, 931
21-Deoxycortisol	0.14–141	0.64, 6.2, 94
Corticosterone	0.14–141	0.65, 6.4, 93
11-Deoxycortisol	0.15–144	0.64, 6.3, 94
Androstenedione	0.17–169	0.74, 7.4, 114
11-Deoxycorticosterone	0.03–59	0.11, 1.1, 40
Testosterone	0.05–74	0.18, 1.7, 52
DHEA	0.90–224	2.9, 28, 110
17-OHP	0.15–293	0.66, 6.2, 207
DHT	0.10–8.2	0.35, 3.4, 5.8
Progesterone	0.08–164	0.40, 3.6, 114

Table 1. Concentration ranges of the MassTrak Endocrine Steroid Calibrator and Quality Control Sets.

The calibrators and QCs are reconstituted following the instructions for use (IFU), prior to sample preparation and analysis.

## Sample Description

Sample preparation was performed using protein precipitation, followed by Solid Phase Extraction (SPE).

### Protein Precipitation

To 125 µL of serum sample, 25 µL of internal standard (SIL) in 50/50 (v/v) methanol/water was added and mixed for 1 minute. 250 µL of methanol was added, followed by mixing for 5 minutes. The sample was diluted with 550 µL water prior to mixing for 1 minute and centrifugation for 10 minutes at 5000 g.

### Solid Phase Extraction

An Oasis MAX µElution Plate was conditioned and equilibrated with 150 µL methanol and water, respectively. 625 µL of supernatant was loaded on to the SPE plate and 10 µL of supernatant (for DHEA-S) was directly transferred to the 1 mL 96-well collection plate. Washes were performed using 150 µL 1% formic acid in 10%

acetonitrile, then 150  $\mu\text{L}$  1% ammonia in 10% acetonitrile. Samples were eluted with 35  $\mu\text{L}$  60% acetonitrile into the 1 mL 96-well collection plate, already containing 10  $\mu\text{L}$  of protein precipitation supernatant. 35  $\mu\text{L}$  of 50 mM ammonium bicarbonate pH 7.4 (0.03% acetic acid) was added and the mixed for 1 minute and sealed prior to injection onto the LC-MS/MS system.

## LC Conditions

LC system:	ACQUITY UPLC I-Class PLUS FTN
Sample needle:	30 $\mu\text{L}$
Column:	CORTECS C <sub>8</sub> , 90 Å, 2.1 mm x 100 mm, 2.7 $\mu\text{m}$
Precolumn:	CORTECS C <sub>8</sub> VanGuard Cartridge, 2.1 mm x 5 mm, 2.7 $\mu\text{m}$
Column temp.:	50 °C
Sample temp.:	8 °C
Injection volume:	25 $\mu\text{L}$
Flow rate:	0.3 mL/min
Mobile phase A:	0.1 mM Ammonium fluoride in water
Mobile phase B:	0.1 mM Ammonium fluoride in methanol
Run time:	7.8 minutes

## Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.300	60	40	Initial
1.25	0.300	60	40	6
3.00	0.300	47.5	52.5	6
4.00	0.300	47.5	52.5	6
5.00	0.300	35	65	6
6.25	0.300	10	90	11
7.00	0.600	60	40	11
7.60	0.300	60	40	11

## MS Conditions

MS system: Xevo TQ-S micro Triple Quadrupole Mass Spectrometry

Ionization mode: Positive/Negative ESI

Capillary voltage: 2.5 kV

## MRM Parameters

Analyte	MRM		Cone (V)	Collison (V)	Scan window (min)
DHEA-S	367.2>97.0	Quantifier	40	30	1.00–2.00
	367.2>80.0	Qualifier	40	80	
	373.2>98.0	SIL	40	30	
Cortisol	363.3>121.1	Quantifier	40	24	2.50–4.70
	363.3>91.1	Qualifier	40	24	
	366.3>124.1	SIL	40	24	
21-Deoxycortisol	347.3>121.1	Quantifier	40	24	2.50–4.70
	347.3>311.2	Qualifier	40	14	
	351.3>121.1	SIL	40	24	
Corticosterone	347.3>121.1	Quantifier	40	24	2.50–4.70
	347.3>97.1	Qualifier	40	24	
	351.3>121.1	SIL	40	24	
11-Deoxycortisol	347.3>97.1	Quantifier	40	24	2.50–4.70
	347.3>109.1	Qualifier	40	24	
	350.3>100.1	SIL	40	24	
Androstenedione	287.3>97.1	Quantifier	70	24	4.71–5.80
	287.3>109.1	Qualifier	70	24	
	290.3>100.1	SIL	70	24	
11-Deoxycorticosterone	331.3>97.1	Quantifier	70	22	4.71–5.80
	331.3>109.1	Qualifier	70	22	
	334.3>100.1	SIL	70	22	
Testosterone	289.3>97.1	Quantifier	70	22	4.71–5.80
	289.3>109.1	Qualifier	70	22	
	292.3>100.1	SIL	70	22	
DHEA	271.3>213.3	Quantifier	40	14	4.71–5.80
	271.3>197.3	Qualifier	40	16	
	274.3>216.3	SIL	40	14	
17-OHP	331.3>97.1	Quantifier	70	22	4.71–5.80
	331.3>109.1	Qualifier	70	22	
	334.3>100.1	SIL	70	22	
DHT	291.3>255.3	Quantifier	40	14	5.80–6.80
	291.3>159.2	Qualifier	40	18	
	294.3>258.3	SIL	40	14	
Progesterone	315.3>97.1	Quantifier	70	24	5.80–6.80
	315.3>109.1	Qualifier	70	24	
	318.3>100.1	SIL	70	24	

## Method Events

Time	Event	Action
0.01	Flow state	Waste
1.01	Flow state	LC
6.75	Flow state	Waste

## Data Management

MS software:

MassLynx v4.2 with TargetLynx XS

## Results and Discussion

Chromatographic separation of the twelve steroid hormones was achieved using the CORTECS C<sub>8</sub>, 2.7 µm, 2.1 mm x 100 mm Column, with baseline resolution of steroid hormone isomers which cannot be differentiated with MRM alone (Figure 2). This includes separation of 21-deoxycortisol, corticosterone and 11-deoxycortisol, in addition to the isomeric pair of 11-deoxycorticosterone and 17-OHP.



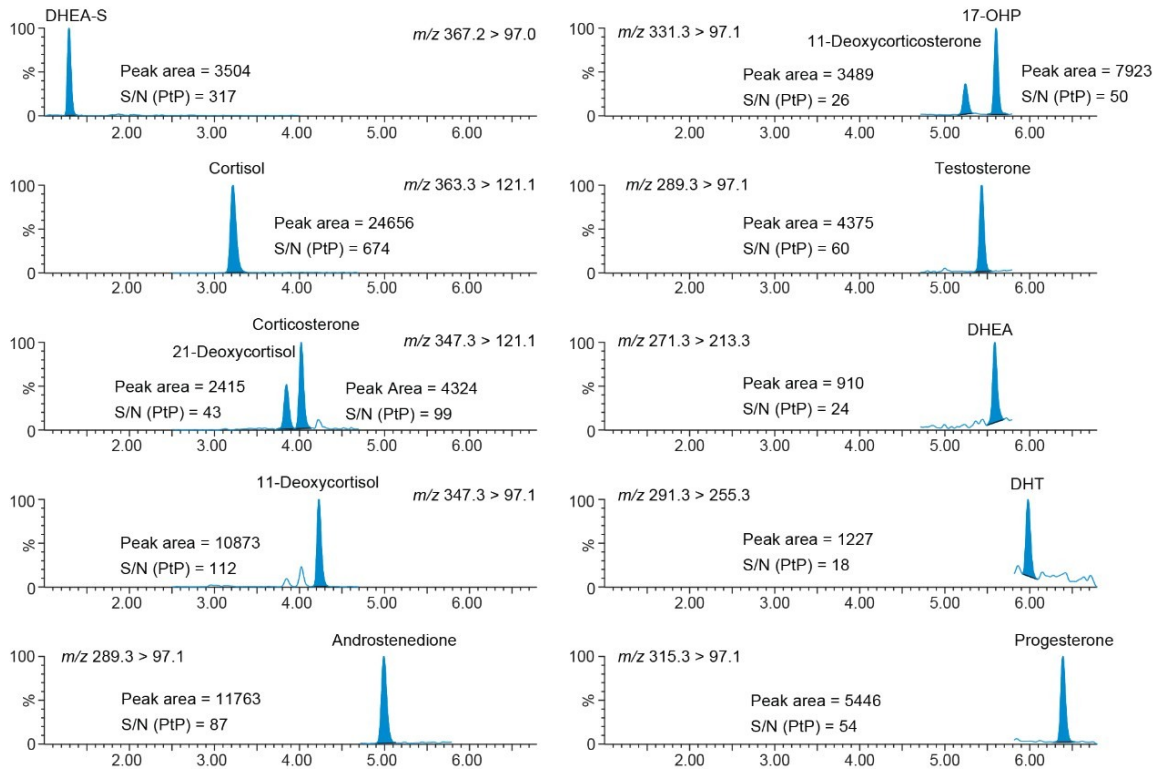


Figure 2. Performance characteristics of the extracted C1 calibrator from the MassTrak Endocrine Steroid Calibrator Set analyzed using the ACQUITY UPLC I-Class/Xevo TQ-S micro IVD System. Linearity of the calibration ranges was demonstrated with mean  $r^2$  values for the calibration lines  $>0.994$  across the 12 steroid hormones. Analytical sensitivity of the method was determined through the signal:noise (S/N) evaluation of the low calibration (C1) standard For the steroid hormones. The S/N (PtP) was  $>10$  at each of calibrator 1 concentrations across five analytical runs. This is summarized in Table 2 and an example of the S/N at the low calibrator can also be seen in Figure 2.

Compound	Calibrator range (nmol/L)	Mean $r^2$	Mean S/N (PtP) at Cal 1
DHEA-S	61-29392	0.994	186
Cortisol	3-1388	0.997	277
21-Deoxycortisol	0.14-141	0.996	26
Corticosterone	0.14-141	0.995	52
11-Deoxycortisol	0.15-144	0.999	86
Androstenedione	0.17-169	0.999	79
11-Deoxycorticosterone	0.03-59	0.996	20
Testosterone	0.05-74	0.995	59
DHEA	0.90-224	0.994	27
17-OHP	0.15-293	0.995	55
DHT	0.10-8.2	0.998	16
Progesterone	0.08-164	0.998	42

Table 2. Summary of calibration linearity and analytical sensitivity performance of the steroid hormones in the MassTrak Endocrine Steroid Calibrator Set.

Total precision and repeatability were determined by extracting and quantifying five replicates of three level QC material per day over five separate days (n=25). Low, mid and high concentrations were 527, 4770, and 19061 nmol/L for DHEA-S; 30, 279, and 931 nmol/L for cortisol; 0.64, 6.2, and 94 nmol/L for 21-deoxycortisol; 0.65, 6.4, and 93 nmol/L for corticosterone; 0.64, 6.3, and 95 nmol/L for and 11-deoxycortisol; 0.74, 7.4, and 114 nmol/L for androstenedione; 0.11, 1.1, 40 nmol/L for 11-deoxycorticosterone; 0.66, 6.2, and 207 nmol/L for 17-OHP; 2.9, 28, and 110 nmol/L for DHEA; 0.35, 3.4, and 5.8 nmol/L for DHT, and 0.40, 3.6, and 114 nmol/L for progesterone. Total precision and repeatability were determined to be  $\leq 7.7\%$  CV across all steroid hormones at the three QC concentrations (Figure 3).

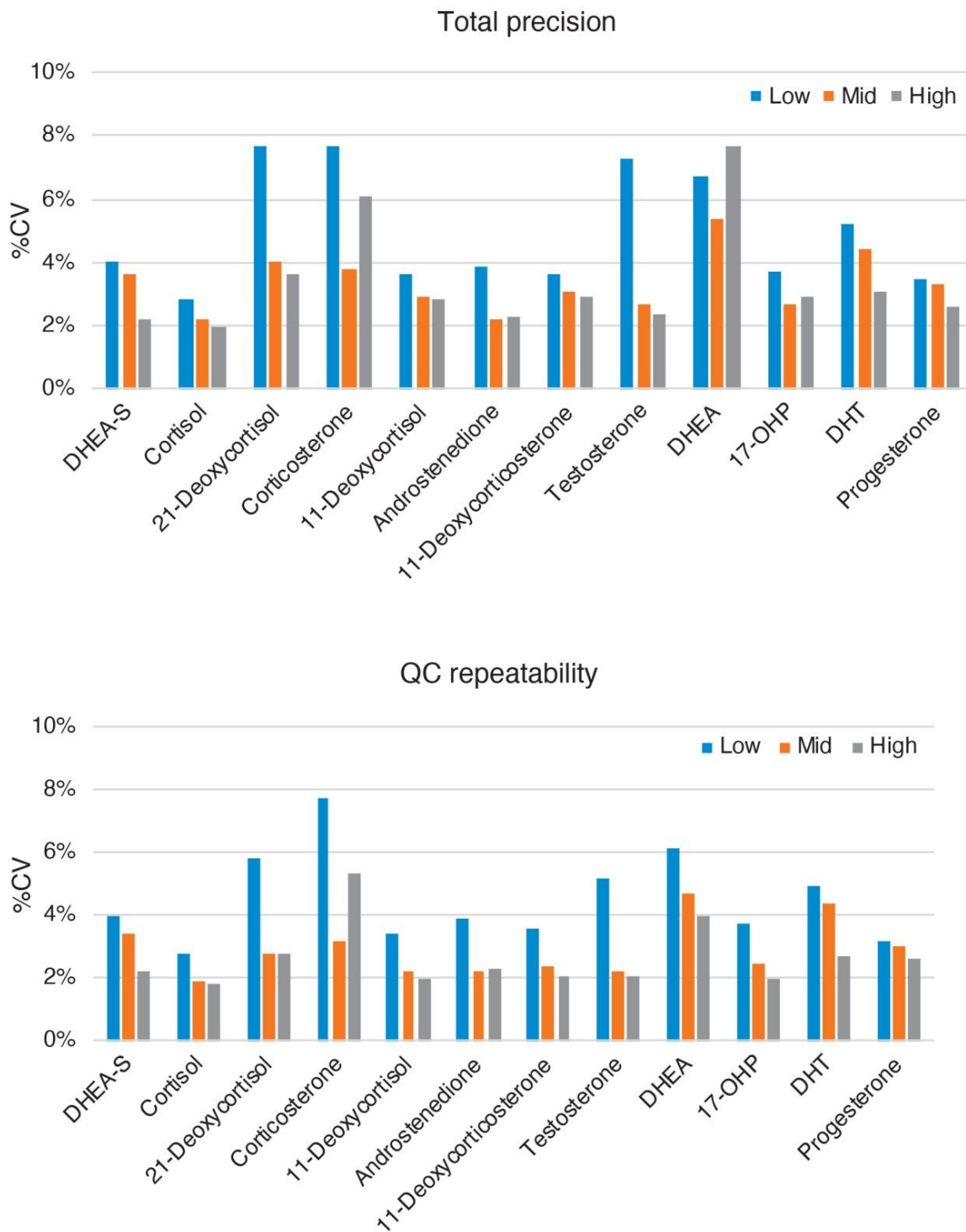


Figure 3.

Total precision and repeatability for the analysis of the twelve steroid hormones in the MassTrak Endocrine Steroid Quality Control Set. In addition, the accuracy of the QCs was evaluated in comparison to the calibrators over the five analytical runs. The mean accuracy for the QCs across the 12 steroid hormones at the three concentrations ranged from 91.0%–112.4% (Table 3).

Analyte	QC Accuracy		
	Q1	Q2	Q3
DHEAS	91%	102%	112%
Cortisol	102%	106%	98%
21-Deoxycortisol	99%	100%	109%
Corticosterone	102%	96%	108%
11-Deoxycortisol	103%	100%	103%
Androstenedione	100%	97%	103%
11-Deoxycorticosterone	104%	98%	103%
Testosterone	106%	107%	99%
DHEA	102%	100%	100%
17-OHP	105%	101%	99%
DHT	109%	108%	111%
Progesterone	102%	105%	97%

Table 3. Accuracy of the MassTrak Endocrine Steroid Quality Control Set analyzed in replicates of five at three concentrations over five analytical runs.

Accuracy was assessed for DHEA-S, cortisol, androstenedione, testosterone, 17-OHP, and progesterone through the analysis of EQA samples from UK NEQAS. The data obtained was compared to the mass spectrometry method mean for the samples (ALTM for progesterone, as LC-MS values were unavailable) and Altman-Bland agreement was performed on the data set. Altman-Bland agreement for DHEA-S, cortisol, androstenedione, testosterone, 17-OHP, and progesterone provided a mean method bias within  $\pm 7.8\%$ , demonstrating excellent agreement with the EQA method values for the steroid hormones (Figures 4a-f).

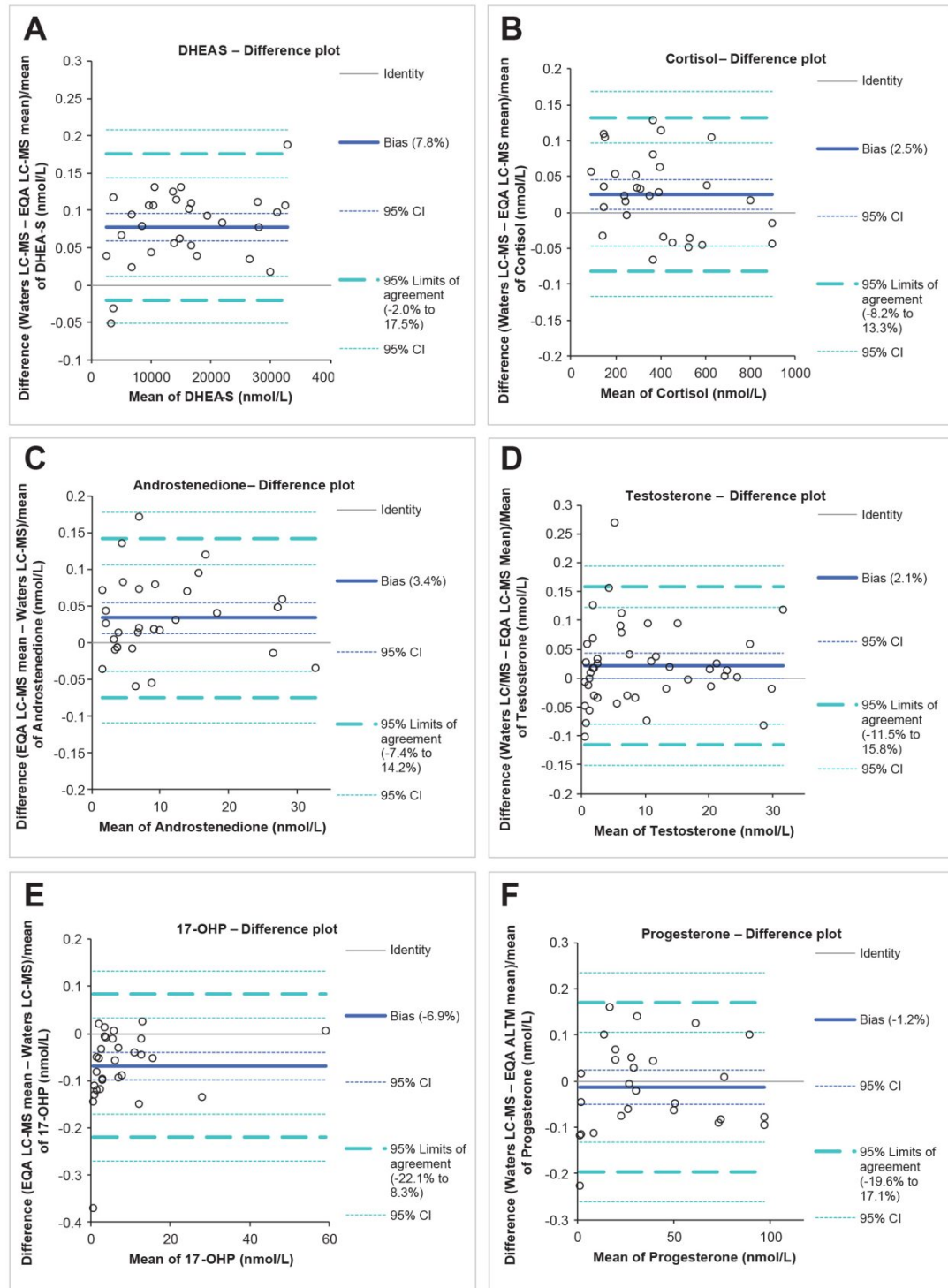


Figure 4.

Altman-Bland agreement comparing the Waters LC-MS/MS method to the EQA scheme MS method mean for: (a) DHEA-S, (b) cortisol, (c) androstenedione, (d) testosterone, (e) 17-OHP, and (f) progesterone (ALTM mean). Accuracy for DHT was assessed through the analysis of Royal College of Pathologists of Australasia Quality Assurance Program (RCPA QAP) samples (n=4). Altman-Bland agreement demonstrated a mean bias of 6.6% (range -1% to 13%) compared to the all laboratory mean (n≥8).

## Conclusion

Through this proof of performance evaluation, it has been demonstrated the MassTrak Endocrine Steroid Calibrator and Quality Control Sets (IVD) can provide precise and accurate quantification of the 12 steroid hormones in serum.

The ACQUITY UPLC I-Class PLUS with Xevo TQ-S micro Triple Quadrupole Mass Spectrometer is able to provide sufficient analytical sensitivity to analyze low levels of the steroid hormones in the set by using only 125  $\mu$ L sample volume. Excellent levels of precision across the calibration range have been demonstrated with total precision and repeatability of  $\leq 7.7\%$  CV. In addition, the accuracy of the QC set was established with accuracies ranging from 91.0%–112.4%. An indication of metrological traceability through agreement to EQA samples was also shown, with the method providing excellent agreement to samples from the EQA (DHEA-S, cortisol, androstenedione, testosterone, 17-OHP, and progesterone) and RCPA (DHT) with mean method bias within  $\pm 7.8\%$  compared to method mean values from the schemes.

## Disclaimer

*This method is an example of an application using the instrumentation, software and consumables described in this document. This method has not been cleared by any regulatory entity for diagnostic purposes. The end user is responsible for completion of the method development and validation. MassTrak Endocrine Steroid Calibrator and Quality Control Sets are not available for sale in all countries. For information on availability, please contact your local sales representative.*

## Featured Products

- [ACQUITY UPLC I-Class PLUS System <https://www.waters.com/134613317>](https://www.waters.com/134613317)
- [Xevo TQ-S micro Triple Quadrupole Mass Spectrometry <https://www.waters.com/134798856>](https://www.waters.com/134798856)
- [MassLynx MS Software <https://www.waters.com/513662>](https://www.waters.com/513662)
- [TargetLynx <https://www.waters.com/513791>](https://www.waters.com/513791)

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