# Waters™

Application Note

# Quantification of Corticosteroids and Androgens in Serum, utilizing Waters™ MassTrak™ Steroid Serum Sets 2 & Sets 3 (RUO)

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*Apenas para utilização em pesquisas. Não se destina à utilização em procedimentos de diagnóstico.*

## Abstract

A clinical research method for the quantification of seven endocrine steroid hormones in human serum by UPLC-MS/MS.

## Benefits

· Analytical selectivity of the chromatographic method provides separation of isobaric species

· LC-MS/MS enables high sample- throughput using multi- well plate automation

· Excellent agreement (within ±6%) to the EQA Mass spectrometer mean and in-house panel mean for testosterone, androstenedione, 17-OHP, DHEAS, cortisol, 11-deoxycortisol, and 21-deoxycortisol

· Lyophilized calibrators and QCs that reduce sample preparation time, aid in method harmonization and assist with metrological traceability in accordance with ISO 15189

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## Introduction

Steroid hormones encompass a large class of small molecules that play a central role in metabolic processes, such as the regulation of sexual characteristics, blood pressure, and inflammation. Enzymes that form part of the steroid biosynthetic pathway are pivotal in these metabolic processes and their dysfunction can be examined through the correct measurement of steroid hormones in a clinical research setting.

Quantification of steroids is more beneficial using LC-MS/MS, as opposed to traditional ligand binding techniques such as immunoassay, as it can provide higher analytical sensitivity and selectivity and allows for simultaneous quantification of multiple analytes. However, many LC-MS methods lack harmonization or standardization. The Waters MassTrak™ Steroid Serum Sets (RUO) contains a range of steroid hormones in lyophilized serum that are metrologically traceable to the highest level of metrological traceability available, aiding laboratories in their compliance to ISO 15189, and provide confidence in the accuracy of results when using validated LC-MS clinical research methods.

The MassTrak Steroid Serum Cal Set 2 (RUO) is intended for the quantitative determination of androgens and progestogens in human serum to aid with monitoring of physiological markers. Whilst the MassTrak Steroid Serum Cal Set 3 (RUO) is intended to be used for the quantitative determination of androgens and glucocorticoids in human serum to aid with monitoring of physiological markers.

Here we describe a clinical research method utilizing Waters MassTrak Steroid Serum Sets 2 and 3 (RUO), Cals and QCs and Oasis™ PRiME HLB µElution plate technology for the extraction of testosterone, androstenedione, 17-hydroxyprogesterone (17-OHP), dehydroepiandrosterone sulfate (DHEAS), Cortisol, 11-deoxycortisol, and 21-deoxycortisol from serum samples, which has been automated using the Hamilton Microlab STAR Liquid Handler. Chromatographic separation was performed on a ACQUITY™ UPLC™- I Class Plus (FL-I) System, using a ACQUITY UPLC HSS T3 Vanguard™ Pre-Column and ACQUITY UPLC HSS T3 Column, accompanied by a Xevo™ TQ-S micro Mass Spectrometer (Figure 1).

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*Figure 1. Waters ACQUITY UPLC I–Class Plus System and Xevo TQ-S micro.*

# Experimental

## Sample Preparation

For this study Waters MassTrak Steroid Serum Cal Set 2 (RUO)(p/n: 186010563 < https://www.waters.com/nextgen/us/en/shop/standards--reagents/186010563-masstrak-steroid-serum-cal-set-2-ruo.html> ), MassTrak Steroid Serum QC Set 2 (RUO)(p/n: 186010564 < https://www.waters.com/nextgen/us/en/shop/standards--reagents/186010564-masstrak-steroid-serum-qc-set-2-ruo.html> ), MassTrak Steroid Serum Cal Set 3 (RUO) (p/n: 186010565 < https://www.waters.com/nextgen/us/en/shop/standards--reagents/186010565-masstrak-steroid-serum-cal-set-3-ruo.html> ) and MassTrak Steroid Serum QC Set 3 (RUO)(p/n: 186010566 < https://www.waters.com/nextgen/us/en/shop/standards--reagents/186010566-masstrak-steroid-serum-qc-set-

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MassTrak Steroid Serum Cal Set 2 and Set 3 (RUO), QC Set 2 and Set 3 (RUO) contains the following analytes in a range of concentrations.

| | Compound | Calibrator range (ng/mL) | QC range (ng/mL) |
|---|---|---|---|
| Set 2 | Testosterone | 0.01–20 | 0.03–9 |
| | Androstenedione | 0.05–20 | 0.15–9 |
| | 17-OHP | 0.1–100 | 0.3–80 |
| | DHEAS | 50–8000 | 150–5000 |
| Set 3 | Androstenedione | 0.15–50 | 0.15–50 |
| | 17-OHP | 0.3–300 | 0.5–150 |
| | Cortisol | 3–500 | 9–400 |
| | 11-deoxycortisol | 0.1–100 | 0.3–70 |
| | 21-deoxycortisol | 0.1–100 | 0.3–70 |

Table 1. MassTrak Steroid Serum Cal Set 2 and Set 3, QC Set 2 and Set 3 concentration range for each analyte.

(To convert SI units to nmol/L multiply by 3.470 for testosterone (ng/mL to nmol/L), 3.494 androstenedione (ng/mL to nmol/L), 3.028 for 17-OHP (ng/mL to nmol/L), 2.716 for DHEAS (ng/mL to nmol/L), 2.761 for Cortisol (ng/mL to nmol/L), and 2.889 for 11-deoxycortisol and 21-deoxycortisol (ng/mL to nmol/L)).

## Sample Extraction

Prior to extraction it is suggested to centrifuge all samples for five minutes at 3000 x g. Extraction was performed using the Hamilton Microlab Star Liquid Handler. To 100 µl of sample, 25 µl of MassTrak Steroid Internal Standard Mix RUO was added to each well of sample. (2 ng/mL of testosterone $^{13}C_3$, 4 ng/mL of androstenedione $^{13}C_3$, 10 ng/mL of 17OHP $^{13}C_3$, 3000 ng/mL of DHEAS $^2H_6$, 100 ng/mL of cortisol $^{13}C_3$, 4 ng/mL of 11-deoxycortisol $^{13}C_3$ and 10 ng/mL of 21-deoxycortisol $^2H_4$). 200 µl of LC-MS Grade Methanol and 550 µl of LC-MS grade Water is added to each well. The samples are mixed after each reagent addition. Samples then

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centrifuged for five minutes at 4000 x g.

An aliquot of each of the pre-treated samples (600 μl) was loaded into individual wells of the Oasis PRiME HLB μElution Plate (p/n: 186008052 <https://www.waters.com/nextgen/us/en/shop/sample-preparation--filtration/186008052-oasis-prime-hlb-96-well--elution-plate-3-mg-sorbent-per-well-1-p.html> ) and slowly pulled through at low vacuum (100 mbar). Consecutive washes with 150 μl of 0.1% (v/v) ammonia in 35% (v/v) methanol$_{(aq)}$ and 150 μL 0.1% (v/v) formic acid in 35% (v/v) methanol$_{(aq)}$ were preformed to reduce potential ionic interference. Analytes were eluted using 30 μL of 85/15 (v/v) acetonitrile/methanol, followed by addition of 70 μL water.

## LC Conditions

| | |
|---|---|
| LC system: | ACQUITY UPLC I Class Plus (FL-I) |
| Column(s): | ACQUITY UPLC HSS T3 (2.1 x 50 mm, 1.8 μm) (p/n: 186003538) |
| Pre Column: | ACQUITY UPLC HSS T3 VanGuard (p/n: 186003976) |
| Column temperature: | 50 °C |
| Sample temperature: | 10 °C |
| Loopsize: | 50 μL |
| Needle: | 20 μL |
| Injection volume: | 20 μL |
| Flow rate: | 0.6 mL/min |
| Mobile phase A: | Water with 2 mM ammonium acetate +0.1% formic acid |

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| | |
|---|---|
| Mobile phase B: | Methanol with 2 mM ammonium acetate +0.1% formic acid |
| Needle wash solvent: | 100% Methanol |
| Purge solvent: | 40% Methanol(aq) |
| Run time: | 4.7 min |
| Gradient: | See Table 1 |

## MS Conditions

| | |
|---|---|
| MS system: | Xevo TQ-S micro |
| Resolution: | MS1 (0.75 FWHM), MS2 (0.50 FWHM) |
| Acquisition mode: | Multiple Reaction Monitoring (MRM) |
| Capillary voltage: | 1.0 kV |
| Polarity: | ESI +/- |
| Source temperature: | 150 °C |
| Desolvation temperature: | 600 °C |
| MS inter-scan delay: | 0.01 s |
| Inter-channel delay: | 0.02 s |
| Polarity/Mode switch interscan: | 0.015 s |

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## Data Management

Chromatography/ MS software:                MassLynx™ v4.1 Software with TargetLynx™

## Gradient

| Time (min) | Flow (mL/min) | %A | %B | Curve |
|---|---|---|---|---|
| Initial | 0.600 | 55 | 45 | Initial |
| 1.0 | 0.600 | 55 | 45 | 6 |
| 3.5 | 0.600 | 35 | 65 | 6 |
| 3.51 | 0.600 | 2 | 98 | 11 |
| 4.0 | 0.600 | 55 | 45 | 11 |

*Table 2. Gradient table for the separation of the steroid hormones. Operating back pressure at the initial conditions was approximately 9000–10000psi.*

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## MRM Settings

| Compound | Parent ion (*m/z*) | Product ion (*m/z*) | Dwell time (s) | Cone (eV) | Collision (V) | ESI mode |
|---|---|---|---|---|---|---|
| Androstenedione (Quan) | 287.1 | 96.95 | 0.043 | 45 | 20 | + |
| Androstenedione (Qual) | 287.1 | 108.9 | 0.043 | 45 | 20 | + |
| Androstenedione $^{13}C_3$ | 290.3 | 100.03 | 0.043 | 45 | 20 | + |
| Testosterone (Quan) | 289.2 | 97 | 0.043 | 45 | 20 | + |
| Testosterone (Qual) | 289.2 | 108.9 | 0.043 | 45 | 20 | + |
| Testosterone $^{13}C_3$ | 292.2 | 100.03 | 0.043 | 45 | 20 | + |
| 17-OHP(Quan) | 331.5 | 96.98 | 0.043 | 55 | 24 | + |
| 17-OHP (Qual) | 331.2 | 108.97 | 0.043 | 55 | 24 | + |
| 17-OHP $^{13}C_3$ | 334.3 | 100.04 | 0.043 | 55 | 24 | + |
| DHEAS (Quan) | 367.1 | 96.85 | 0.03 | 45 | 30 | - |
| DHEAS (Qual) | 367.1 | 79.9 | 0.03 | 45 | 75 | - |
| DHEAS $^{2}H_6$ | 373.2 | 97.9 | 0.03 | 45 | 30 | - |
| Cortisol (Quan) | 363.1 | 120.9 | 0.03 | 45 | 24 | + |
| Cortisol (Qual) | 363.1 | 97 | 0.03 | 45 | 24 | + |
| Cortisol $^{13}C_3$ | 366.3 | 124.05 | 0.03 | 45 | 24 | + |
| 11-Deoxycortisol (Quan) | 347.2 | 97 | 0.03 | 45 | 24 | + |
| 11-Deoxycortisol (Qual) | 347.2 | 120.9 | 0.03 | 45 | 18 | + |
| 11-Deoxycortisol $^{13}C_3$ | 350.3 | 100.04 | 0.03 | 45 | 24 | + |
| 21-Deoxycortisol (Quan) | 347.2 | 175.1 | 0.03 | 45 | 30 | + |
| 21-Deoxycortisol (Qual) | 347.2 | 120.9 | 0.03 | 45 | 18 | + |
| 21-Deoxycortisol $^{2}H_4$* | 351.3 | 120.9 | 0.03 | 45 | 18 | + |
| 21-Deoxycortisol $^{2}H_4$ | 351.3 | 177.15 | 0.03 | 45 | 18 | + |

* Alternative product ion.

Table 3. MRM parameters for testosterone, androstenedione, 17-OHP, DHEAS, cortisol, 11-deoxycortisol, and 21-deoxycortisol, and their stable isotope labelled internal standards. The scan window for the analytes were 1.40–2.75 mins for DHEAS, cortisol, 11-deoxycortisol, and 21-deoxycortisol (+/- switching), and 2.76–3.80 mins for testosterone, androstenedione, and 17-OHP. Mobile phase was directed to waste at all other times. Dwell times were set to automatic for all analytes, bar DHEAS, which was set to 0.03 s, with 15 points across the peak.

# Results and Discussion

The chromatographic selectivity of the column is demonstrated through the baseline resolution of isobaric

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steroid species; 11-deoxycortisol and 21-deoxycortisol (please see Analysis of Corticosteroids and Androgens in Serum for Clinical Research 720005999 (2017), which demonstrated the separation of 17-OHP and 21-OHP and testosterone and epitestosterone). No significant interferences were observed at the retention time of testosterone, androstenedione, 17-OHP, DHEAS, cortisol, 11-deoxycortisol, and 21-deoxycortisol, when each of these compounds were individually examined. No system carryover was observed from high concentration samples into subsequent blank injections. Analytical sensitivity using signal:noise (S/N) of the low calibrator of each set (C1) was >10:1 at each of calibrator one concentrations across several analytical runs.
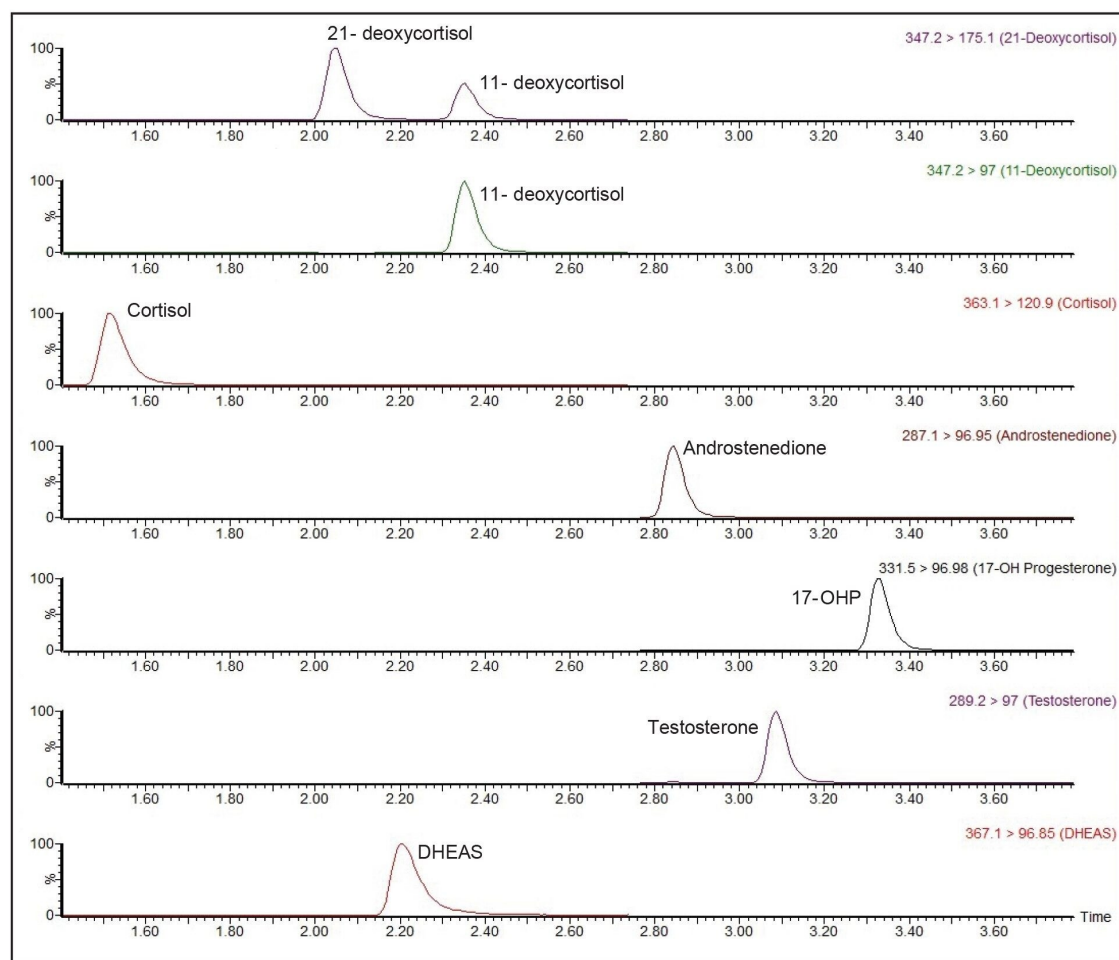


*Figure 2. Chromatographic selectivity on the ACQUITY UPLC HSS T3 Column for the selection of steroid hormones.*

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The clinical reasearch method allows for precise quantification of all analytes at low concentrations. Total precision was determined by extracting and quantifying two replicates per QC level over five days. Repeatability was assessed by analysing four replicates at each QC level in a single analytical run. All results for all analytes were <10%CV, with the majority of results <5%CV.

| Set | Compound | Total precision (%CV) | | | | Repeatability (%CV) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | QC 1 | QC 2 | QC 3 | QC 4 | QC 1 | QC 2 | QC 3 | QC 4 |
| Set 2 | Testosterone | 6.3 | 2.8 | 2.6 | 2.1 | 3.1 | 1.8 | 1.3 | 0.4 |
| | Androstenedione | 2.5 | 2.3 | 3.2 | 3.9 | 1.5 | 1.8 | 1.00 | 0.8 |
| | 17-OHP | 2.1 | 6.9 | 3.1 | 5.1 | 2.2 | 1.1 | 1.3 | 1.0 |
| | DHEAS | 3.3 | 2.7 | 2.7 | 3.3 | 4.8 | 4.4 | 2.5 | 2.0 |
| Set 3 | Androstenedione | 1.6 | 1.9 | 1.7 | 1.2 | 0.8 | 0.8 | 0.4 | 1.2 |
| | 17-OHP | 3.2 | 1.9 | 3.4 | 1.6 | 1.1 | 1.5 | 1.1 | 0.8 |
| | Cortisol | 1.6 | 1.1 | 2.2 | 1.3 | 0.3 | 0.5 | 1.7 | 0.9 |
| | 11-Deoxycortisol | 2.2 | 2.2 | 2.1 | 2.1 | 1.7 | 1.2 | 1.4 | 1.1 |
| | 21-Deoxycortisol | 9.9 | 9.0 | 3.8 | 3.8 | 5.7 | 2.2 | 3.1 | 1.7 |

*Table 4. Total precision and repeatability for the analysis of MassTrak Steroid Serum Cals Set 2 and 3 (RUO), measuring MassTrak Steroid Serum Set 2 and 3 (RUO) QC material.*

The clinical research method was shown to be linear for all analytes over the calibration ranges specified in Table 1 when different ratios of low and high concentration pools of the analytes were combined and analysed. Furthermore, calibration lines created using Set 2 and Set 3 were analysed over a five-day period and were linear with a co-efficient of determination of ($r^2$) >0.999 for all analytes.

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| Linear coefficient of determination ($r^2$) | | | | | | |
|---|---|---|---|---|---|---|
| Set | Compound | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
| Set 2 | Testosterone | 0.999963 | 0.99978 | 0.999527 | 0.999651 | 0.99987 |
| | Androstenedione | 0.999926 | 0.99993 | 0.99985 | 0.999733 | 0.998004 |
| | 17-OHP | 0.999992 | 0.999688 | 0.999288 | 0.999419 | 0.999498 |
| | DHEAS | 0.997901 | 0.99956 | 0.997784 | 0.998498 | 0.999319 |
| Set 3 | Androstenedione | 0.999817 | 0.999907 | 0.999601 | 0.999867 | 0.999873 |
| | 17-OHP | 0.999148 | 0.999657 | 0.998784 | 0.99904 | 0.999412 |
| | Cortisol | 0.999303 | 0.999883 | 0.999188 | 0.999805 | 0.999777 |
| | 11-Deoxycortisol | 0.998635 | 0.999251 | 0.99968 | 0.999164 | 0.999893 |
| | 21-Deoxycortisol | 0.999906 | 0.999936 | 0.999317 | 0.999568 | 0.999849 |

Table 5. Linear co-efficient of determination for each analyte, in each set, over a five-day period.

Accuracy was assessed for testosterone, androstenedione, 17-OHP, and cortisol through the analysis of EQA material. The data obtained over a four-day period, was compared to the mass spectrometry clinical research method mean for each EQA sample and the mean % difference from target was tabulated (Table 6). All analytes were assessed at the low, medium, and high end of the calibration line for each MassTrak Steroid Serum Set, which yielded excellent results across the range (±10%). Due to the unavailability of EQA material for 11-deoxycortisol and 21-deoxycortisol, in house panels were used to assess the accuracy of these compounds and the low and medium range. Deming regression and Linear regression was preformed (Table 7). No Statistically significant bias was observed for each compound, with a mean method bias of ±1.3% for Set 2 and ±1.0% for Set 3.

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| | Compound | Low | Med | High | N= | Mean % diff from target | |
|---|---|---|---|---|---|---|---|
| Set 2 | Testosterone | 106% | 93% | 93% | 52 | 96% | EQA material |
| | Androstenedione | 98% | 95% | 98% | 56 | 95% | |
| | 17-OHP | 93% | 107% | 110% | 56 | 102% | |
| | DHEAS | 103% | 94% | 107% | 54 | 101% | |
| Set 3 | Androstenedione | 94% | 97% | 101% | 56 | 98% | |
| | 17-OHP | 97% | 107% | 110% | 48 | 102% | |
| | Cortisol | 107% | 104% | 101% | 42 | 104% | |
| | 11-Deoxycortisol | 98% | 106% | n/a | 54 | 102% | In house panels |
| | 21-Deoxycortisol | 101% | 99% | n/a | 51 | 100% | |

Table 6. Accuracy summary table, EQA Material - % Difference of EQA Mean Target for LCMS to Waters measured concentration. Due to the unavailability of EQA material for 11-deoxycortisol and 21-deoxycortisol, in house panels were used to assess the accuracy of these compounds and the low and medium range.

| | Compound | Samples | Deming equation | Mean bias | Linear fit (r) |
|---|---|---|---|---|---|
| Set 2 | Testosterone | 54 | y=0.06072 + 0.9217x | -3.93% | 1.000 |
| | Androstenedione | 58 | y=0.06383 + 0.9835x | -3.52% | 0.999 |
| | 17-OHP | 56 | y=-0.2981 + 1.109x | 1.50% | 0.999 |
| | DHEAS | 54 | y=-116.9 + 1.08x | 0.63% | 0.999 |
| Set 3 | Androstenedione | 56 | y=-0.1134 + 1.019x | -2.38% | 0.999 |
| | 17-OHP | 48 | y=-0.2102 + 1.102x | 2.13% | 1.000 |
| | Cortisol | 42 | y=4.028 + 0.9946x | 3.49% | 0.999 |
| | 11-Deoxycortisol | 54 | y=-0.02675 + 1.063x | 1.73% | 1.000 |
| | 21-Deoxycortisol | 51 | y=-0.003172 + 0.9937x | 0.05% | 0.999 |

Table 7. Deming regression comparing Waters LC-MS/MS method to the EQA scheme MS method for testosterone, androstenedione, 17-OHP, DHEAS, and Cortisol analysis. 11-Deoxycortisol and 21-Deoxycortisol comparison to in house panels and medium range.

# Conclusion

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Through this proof of performance evaluation, it has been demonstrated the MassTrak Steroid Serum Calibrator and Quality Control Sets 2 and 3 (RUO) can provide precise and accurate quantification of steroid hormones in serum. An analytically sensitive and selective clinical research method has been developed for the analysis of testosterone, androstenedione, 17-OHP, DHEAS, cortisol, 11-deoxycortisol, and 21-deoxycortisol in serum using Waters Xevo TQ-S micro (FL).

The Xevo TQ-S micro Mass Spectrometer enables this clinical research method to provide sufficient analytical sensitivity to analyse low levels of testosterone, androstenedione, 17-OHP, DHEAS, cortisol, 11-deoxycortisol, and 21-deoxycortisol by using only 100 μL of sample volume.

The assay's accuracy (±6%) and precision (±10%) have been confirmed through comparison to EQA LC-MS/MS means, In House Panels and QC material for testosterone, androstenedione, 17-OHP, DHEAS, cortisol, 11-deoxycortisol, and 21-deoxycortisol. The addition of automation or liquid handlers, in particular this study utilized the Hamilton Microlab STAR improves laboratory workflow and helps remove operator errors.

## References

1. Foley D, Calton L. Analysis of Corticosteroids and Androgens in Serum for Clinical Research. Waters Application Note. 720005999. May 2017.

## Disclaimer

*This clinical research method is an example of an application using the instrumentation, software and consumables described in this document. This clinical research method has not been cleared by any regulatory entity for diagnostic purposes. MassTrak Endocrine Steroid Calibrator and Quality Control Sets (RUO) 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>

Xevo TQ-S micro Triple Quadrupole Mass Spectrometry <https://www.waters.com/134798856>

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MassLynx MS Software <https://www.waters.com/513662>

TargetLynx <https://www.waters.com/513791>

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