

Application Note

## MetaboQuan-R for the UPLC-MS/MS Analysis of Steroids

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diagnostic procedures.*

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## Abstract

A rapid, targeted UPLC-MS/MS methodology has been developed for the semi-quantitative analysis of 14 endogenous steroids in human plasma/serum. This research method has demonstrated to be suitable for the analysis of these analytes at physiologically relevant levels in human plasma/serum. This MetaboQuan-R method is optimized on the same versatile LC-MS platform that has previously been utilized for the analysis of various classes of compounds including bile acids, amino acids, free fatty acids, tryptic peptides, acylcarnitines, tryptophan metabolites, and various lipids. This new steroids assay extends the MetaboQuan-R suite of methodologies, and allows the analysis of an expanding set of metabolites and lipids, when operated sequentially in a targeted multi-omics workflow.

## Benefits

- Simultaneous analysis of 14 steroids in a single analytical run in under ten minutes
- Separation of key isobaric compounds
- High-throughput analysis means larger sample sets can be analyzed rapidly
- A simple analytical workflow without derivatization
- Use of a flexible LC-MS configuration yields versatility for switching from one compound class to another

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## Introduction

Steroid hormones and related compounds are extremely important, biologically active, molecules that are involved in countless essential biological functions (Figure 1). The analysis of these analytes is challenging since so many of them have similar chemical structures giving rise to multiple isobaric interferences. This chemical similarity means that chromatographic separation of these interfering (isobaric) species is essential for selective analysis. This is usually achieved with long chromatographic run times, derivatization or the use of multiple targeted assays.

Here we demonstrate a high-throughput UPLC-MS/MS research method for the semi-quantitative analysis of 14 steroids in human plasma/serum samples, without the need for derivatization, and using a generic, flexible LC-MS platform that has previously been used for multiple other endogenous compound classes (MetaboQuan-R). Thus enabling the high-throughput quantitative analysis of multiple metabolic classes in a single analytical run.

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## Experimental

### Human Plasma Sample Preparation

Human plasma/serum samples were prepared as described previously (Figure 1). Briefly, samples were prepared by liquid/liquid extraction using an ethyl acetate/hexane mixture. After evaporation, the resulting residue was solubilised in a methanol/water mixture prior to analysis by UPLC-MS/MS.

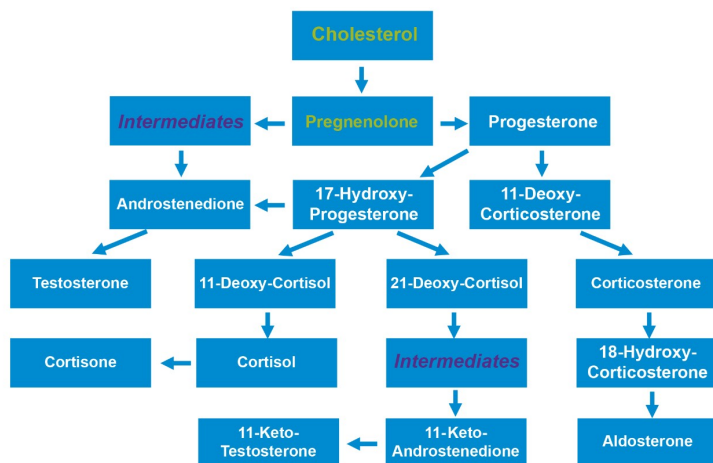


Figure 1. Simplified metabolic pathway for steroids analyzed using MetaboQuan-R (Steroids included in the method are in white).

### UPLC Conditions

UPLC separation was performed with an ACQUITY UPLC I-Class System (Fixed Loop), equipped with a CORTECS T3 2.7  $\mu\text{m}$  (2.1 x 30 mm) Column maintained at 60 °C. A 5  $\mu\text{L}$  aliquot of the sample was injected onto the column and eluted under reversed-phase gradient conditions, where mobile phase A comprised 0.01% formic acid (aq) containing 0.2 mM Ammonium formate and mobile phase B comprised 50% isopropanol in acetonitrile containing 0.01% formic acid and 0.2 mM Ammonium formate. After an initial 2.7 minute hold at 15% mobile phase B, the steroids were eluted from the column and separated with a linear gradient of 15-40% mobile phase B over 4.3 minutes, followed by a 1 minute column wash at 98% mobile phase B at a flow rate of 0.45 mL/min. The column was then re-equilibrated to initial conditions.

### MS Conditions

Steroids were detected using Multiple Reaction Monitoring (MRM) analyses on a Xevo TQ-S micro Mass Spectrometer. All experiments were performed in positive electrospray ionization (ESI+) mode.

The ion source temperature and capillary voltage were kept constant and set to 150 °C and 2.0 kV respectively. The cone gas flow rate was 50 L/hr and desolvation temperature was 650 °C. The MRM transitions employed are detailed in Table 1.

Steroid	MRM transitions	RT (mins)	Cone voltage (V)	Collision energy (eV)
Androstenedione	287.20 > 97.00	4.8	40	22
	287.20 > 109.00		40	22
Testosterone	289.20 > 97.00	5.0	40	22
	289.20 > 109.00		40	26
11-Keto-Androstenedione	301.20 > 109.00	2.0	40	26
	301.20 > 121.00		40	24
11-Keto-Testosterone	303.20 > 121.00	2.5	40	24
	303.20 > 147.10		40	20
Aldosterone	361.20 > 97.00	1.2	40	30
	361.20 > 109.00		40	30
11-Deoxy-Cortisol	347.20 > 97.00	4.0	40	24
	347.20 > 109.00		40	28
21-Deoxy-Cortisol	347.20 > 97.00	3.3	40	24
	347.20 > 109.00		40	28
	347.20 > 121.00		40	24
	347.20 > 175.00		40	18
Corticosterone	347.20 > 97.00	3.6	40	24
	347.20 > 109.00		40	28
	347.20 > 121.00		40	24
	347.20 > 175.00		40	18
11-Deoxy-Corticosterone	331.20 > 97.00	5.0	40	20
	331.20 > 109.00		40	24
17-Hydroxy-Progesterone	331.20 > 97.00	5.4	40	20
	331.20 > 109.00		40	24
Progesterone	315.20 > 97.00	6.5	40	20
	315.20 > 109.00		40	22
Cortisone	361.20 > 121.00	1.6	40	28
	361.20 > 163.10		40	22
18-Hydroxy-Corticosterone	363.20 > 97.00	1.4	40	22
	363.20 > 121.00		40	22
	363.20 > 269.20		40	16
Cortisol	363.20 > 97.00	1.9	40	22
	363.20 > 121.00		40	22
	363.20 > 269.20		40	16

Table 1. List of MS/MS conditions and retention times for Steroids using MetaboQuan-R.

## Informatics

Method information was imported into the LC-MS system using the Quanpedia functionality within MassLynx. This extendable and searchable database produces LC and MS methods as well as processing methods for use in TargetLynx for compound quantification.

## LC Conditions

LC system:	ACQUITY UPLC I-Class FL
Detection:	Xevo TQ-S micro
Column(s):	CORTECS T3 2.7 $\mu\text{m}$ (2.1 x 30 mm)
Column temp.:	60 °C
Sample temp.:	6 °C
Injection volume:	5 $\mu\text{L}$
Flow rate:	0.45 mL/min
Mobile phase A:	0.01% formic acid <sub>(aq)</sub> containing 0.2 mM Ammonium formate
Mobile phase B:	50% isopropanol in acetonitrile containing 0.01% formic acid and 0.2 mM Ammonium formate
Gradient:	After an initial 2.7 minute hold at 15% mobile phase B a linear gradient of 15- 40% mobile phase B was applied over 4.3 minutes, followed by a 1 minute column wash at 98% mobile phase B and re- equilibration back to initial conditions

## MS Conditions

MS system: Xevo TQ-S micro

Ionization mode: ESI positive

Capillary voltage: 2.0 kV

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## Results and Discussion

The 14 steroids detailed in Table 1 were extracted, separated, and detected using the UPLC-MS/MS platform and extraction protocol described. Within this list of 14, there are 5 sets of compounds (see list below) that, due to structural similarity are isobaric, and as a result interfere with each other in the mass spectrometer.

Set A – Testosterone and Androstenedione

Set B – 11-Keto-Testosterone and 11-Keto-Androstenedione

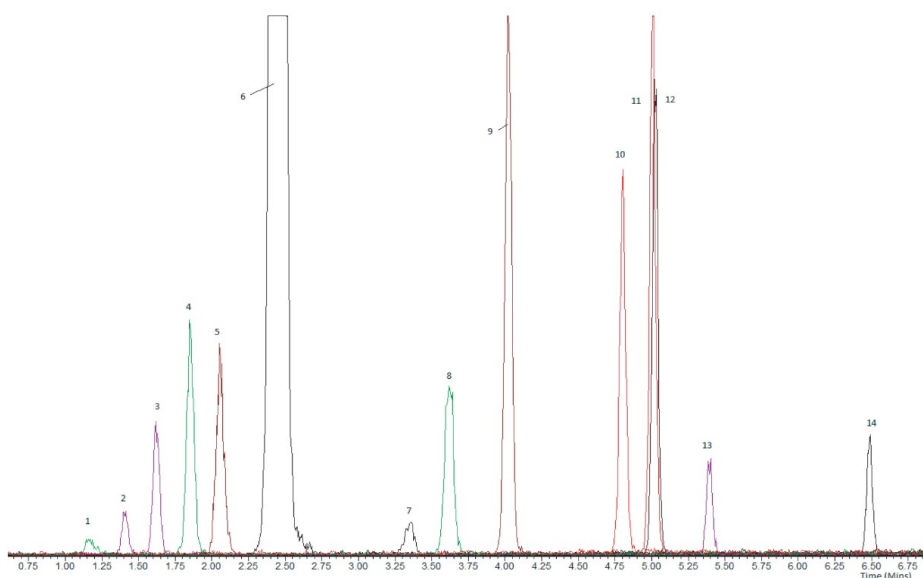
Set C – Aldosterone, Cortisone, 18-Hydroxy-Corticosterone, and Cortisol

Set D – Corticosterone, 11-Deoxy-Cortisol, and 21-Deoxy-Cortisol

Set E – 11-Deoxy-Corticosterone and 17-Hydroxy-Progesterone

In order to selectively analyze all 14 steroids, it is necessary to chromatographically resolve these sets of isobaric steroids. The UPLC separation developed using the MetaboQuan-R platform successfully separated these 5 sets of steroids from each other, such that specific analysis can be achieved for all analytes. Figure 2 shows the UPLC separation for the 14 steroids, including these 5 key isobaric steroid sets.





*Figure 2. UPLC separation of the 14 steroids using the MetaboQuan-R platform, including the 5 sets of isobaric steroids A-E. (1. Aldosterone 2. 18-Hydroxycorticosterone 3. Cortisone 4. Cortisol 5. 11-Ketoandrostenedione 6. 11-Ketotestosterone 7. 21-Deoxycortisol 8. Corticosterone 9. 11-Deoxycortisol 10. Androstenedione 11. Testosterone 12. 11-Deoxycorticosterone 13. 17-Hydroxyprogesterone 14. Progesterone).*

The chromatograms shown in Figure 3 demonstrate the isobaric interference between the two steroids 17-Hydroxy-Progesterone and 11-Deoxy-Corticosterone. Here we can clearly see that both steroids have the same precursor and product ions (MRM transition 331.2 > 97.0). The uppermost chromatogram in Figure 3 is from an extracted pooled human plasma sample, which shows the peak for 17-Hydroxy-Progesterone along with other background peaks. This illustrates the need for a good chromatographic resolution for steroid analysis. This MetaboQuan-R chromatographic method not only successfully separates 17-Hydroxy-Progesterone peak from another interfering steroid of interest, 11-Deoxy-Corticosterone, but also separates it from at least 6 other unknown endogenous isobaric interferences.

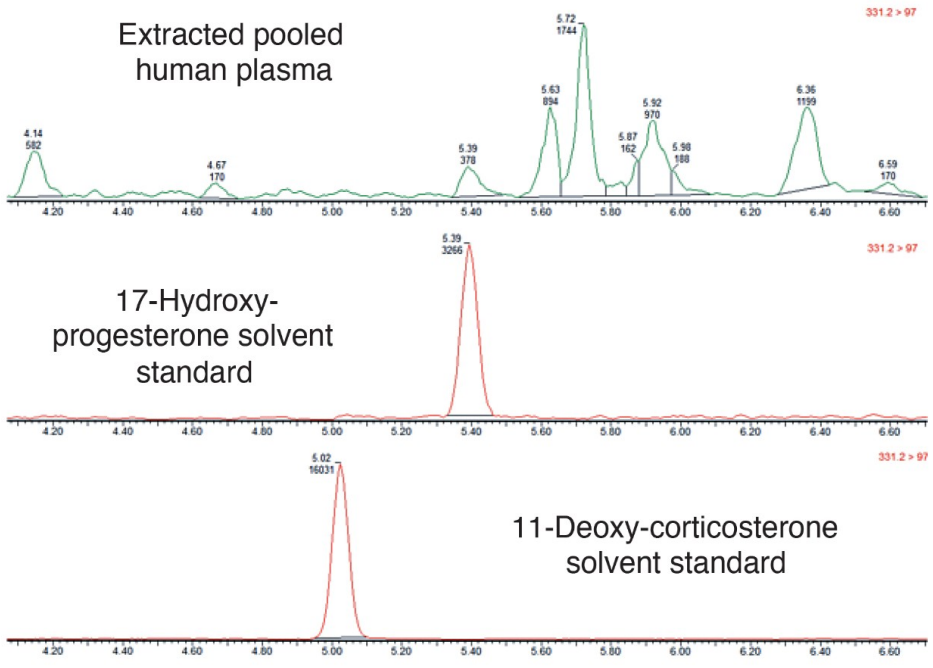


Figure 3. UPLC Chromatograms for MRM transition 331.2 > 97.0 for extracted human plasma and 2 solvent standards of isobaric steroids 17-Hydroxy-Progesterone and 11-Deoxy-Corticosterone.

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## Conclusion

A rapid UPLC-MS/MS methodology has been developed for the research analysis of 14 endogenous steroids in human plasma and serum. This method demonstrated to be suitable for the analysis of these steroids at physiologically relevant levels in human plasma and serum. This method utilizes a flexible reversed-phase UPLC-MS/MS platform that can be used for various compound classes (including bile acids, free fatty acids, amino acids, tryptic peptides, acyl-carnitines, and lipids); thus allowing the assay to be employed as part of a suite of methodologies that can be operated sequentially as part of a targeted multi-omics workflow with broad compound coverage.

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## References

1. Utilizing UPLC Separation to Enable the Analysis of 19 Steroid Hormones in Serum for Clinical Research (Waters Technical Note Number [720006872EN](#), Wardle *et.al*).

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## Featured Products

[ACQUITY UPLC I-Class PLUS System](#)

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