

# Multiplexed Analysis of Steroid Hormones Using ionKey/MS

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## Abstract

In this application, we report the use of the newly developed 150- $\mu$ m ionKey/MS System for the multiplexed quantitation of five steroid compounds in human serum. The use of the ionKey Source and the 150- $\mu$ m iKey Separation Device yielded a 100-400 fold increase in on-column sensitivity while at the same time decreasing solvent usage by 150 fold as compared to standard flow methods. The increased on-column sensitivity allowed for simplification of the steroid extraction procedure which in turn streamlined the sample preparation and reduced per/sample assay cost.

## Benefits

A highly analytically sensitive multiplexed assay was developed for targeted quantitation of five steroids in human serum. The use of the ionKey source and the 150  $\mu$ m iKey Separation Device yielded a 100–400 fold increase in on-column sensitivity while at the same time decreasing solvent usage by 150 fold as compared to standard flow methods. The increased on-column analytical sensitivity allowed for simplification of the steroid extraction procedure which in turn streamlined the sample preparation and reduced per/sample assay cost.

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## Introduction

The measurement of steroids in human serum is an important clinical research tool. Traditionally, these assays are performed using a variety of biochemical techniques including radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and chemiluminescent immunoassay (CLIA). However, immunoassays suffer from antibody cross-reactivity with structural isomers which has been shown to result in an overestimation of steroid levels. Recently, LC-MS/MS has emerged as a viable alternative for this important assay in the clinical research setting. While providing improved accuracy as compared to antibody based techniques, standard flow LC-MS/MS assays also consume high levels of solvent and often require time-consuming sample extraction procedures such as liquid-liquid or solid phase extraction to adequate analytical sensitivity.

In this application, we report the use of the newly developed 150  $\mu$ m ionKey/MS System for the multiplexed quantitation of five important steroid compounds in human serum: testosterone, dihydrotestosterone, progesterone, cortisone, and cortisol. The reduced flow method results in a 150 fold decrease in solvent consumption and a 100–400 fold increase in on-column analytical sensitivity.<sup>1</sup>

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## Experimental

## Method conditions

### LC conditions

LC system:	nanoACQUITY UPLC
Sample loop:	5 µL
Column:	iKey BEH C <sub>18</sub> 130, 1.7 µm, 150 µm x 50 mm
Column temp.:	45 °C
Flow rate:	3.06 µL/min
Mobile phase A:	Water + 0.1%formic acid
Mobile phase B:	Methanol + 0.1% formic acid
Volume injected:	0.5 µL using partial loop mode

### Gradient:

Time (min)	%A	%B	Curve
Initial	90.0	10.0	Initial
0.25	90.0	10.0	6
1.00	45.0	55.0	6
7.50	5.0	95.0	6
8.00	55.0	10.0	6
12.00	55.0	10.0	6

### MS conditions

Mass spectrometer:	Xevo TQ-S
Acquisition mode:	MRM
Ionization mode:	ESI positive
Capillary voltage:	3.2 kV
Source temp.:	100 °C
Source offset:	50 V
Collision gas:	argon
Dwell times for all compounds:	0.011 s

### Sample preparation

Serum samples were precipitated with 3.7 volumes of ice cold methanol containing stable isotope-labeled internal standards for each steroid at a level of 10 ng/mL. Samples were incubated at -80 °C for 30 minutes, centrifuged at 3270 x g for 10 minutes and supernatant was collected. All sample preparation and injections were conducted in 96-well plates. 0.5 µL of extracted serum was injected and separation was performed using a nanoACQUITY UPLC connected to an ionKey source using a 150 µm iKey packed with BEH C<sub>18</sub> (1.7 µm particles). The column effluent was monitored using a Xevo TQ-S Mass Spectrometer operated in multiple reaction monitoring (MRM) positive ion electrospray mode.

Compound	MRM time acquisition window (min)	MRM transition	Cone (V)	Collision energy (V)
testosterone	4.1–5.3	289.24>97.03	50	20
dihydrotestosterone	4.5–5.5	291>255	46	14
d3 testosterone	4.1–5.3	292.2>97.03	50	20
d3 dihydrotestosterone	4.5–5.5	294.1>258.2	46	14
progesterone	4.9–6	315>109	20	26
<sup>13</sup> C <sub>3</sub> progesterone	4.9–6	318.2>112.2	20	26
cortisone	3.5–4.6	361>163.05	25	30
cortisol	3.5–5	363>327.14	25	16
d4 cortisol	3.5–5	367.2>331	25	22
d7 cortisone	3.5–4.6	368.2>169	25	22

Table 1. MRM transitions and instrument settings for each compound. (Broccardo 2013)

## Data analysis

Quantification was performed using linear regression against a standard curve in MassLynx Software. Peak areas for each compound were normalized to the corresponding internal standard in each sample.

## Results and Discussion

Chromatographic separation of the five compounds is illustrated in Figure 1. An average peak width of 6s was achieved.

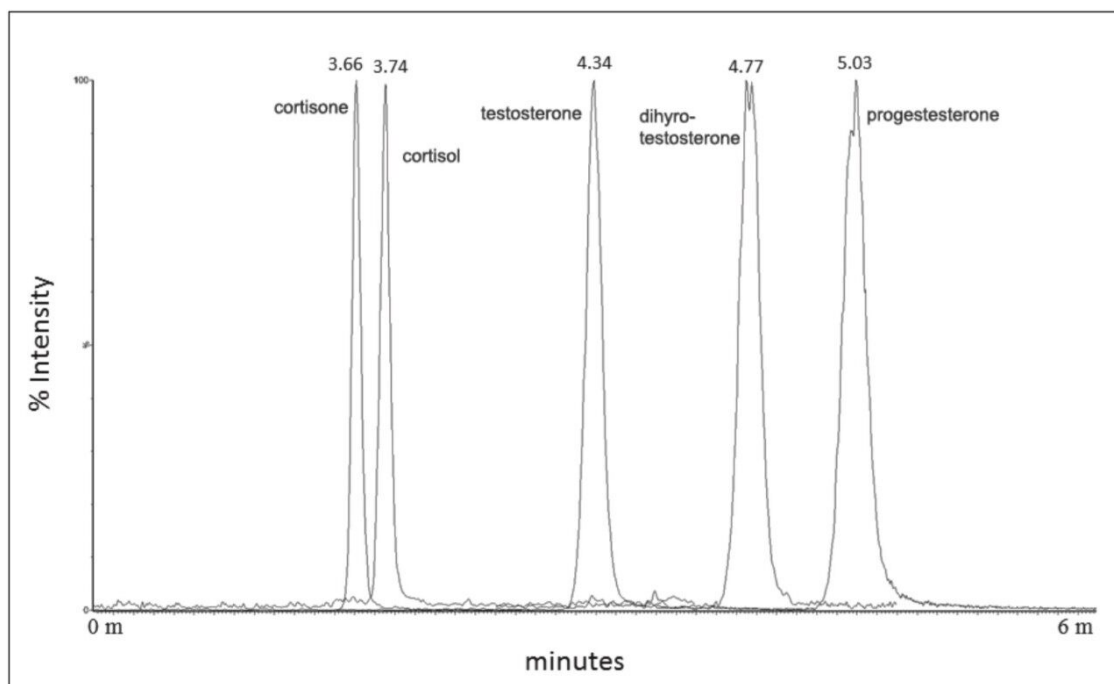


Figure 1. Chromatographic separation of the five steroid compounds in human serum. (Broccardo 2013)

A typical calibration curve is shown in Figure 2. The correlation coefficients for all compounds were 0.99 or greater.

The lower limit of quantification (LOQ) and lower limit of detection (LOD) for all five compounds is listed in Table 2. These values represent a 100–400 fold increase in on-column analytical sensitivity as compared to published standard flow assays which typically require injection volumes of 50–200  $\mu\text{L}$  of extracted sample; only 0.5  $\mu\text{L}$  of extracted sample is used in the assay presented here.

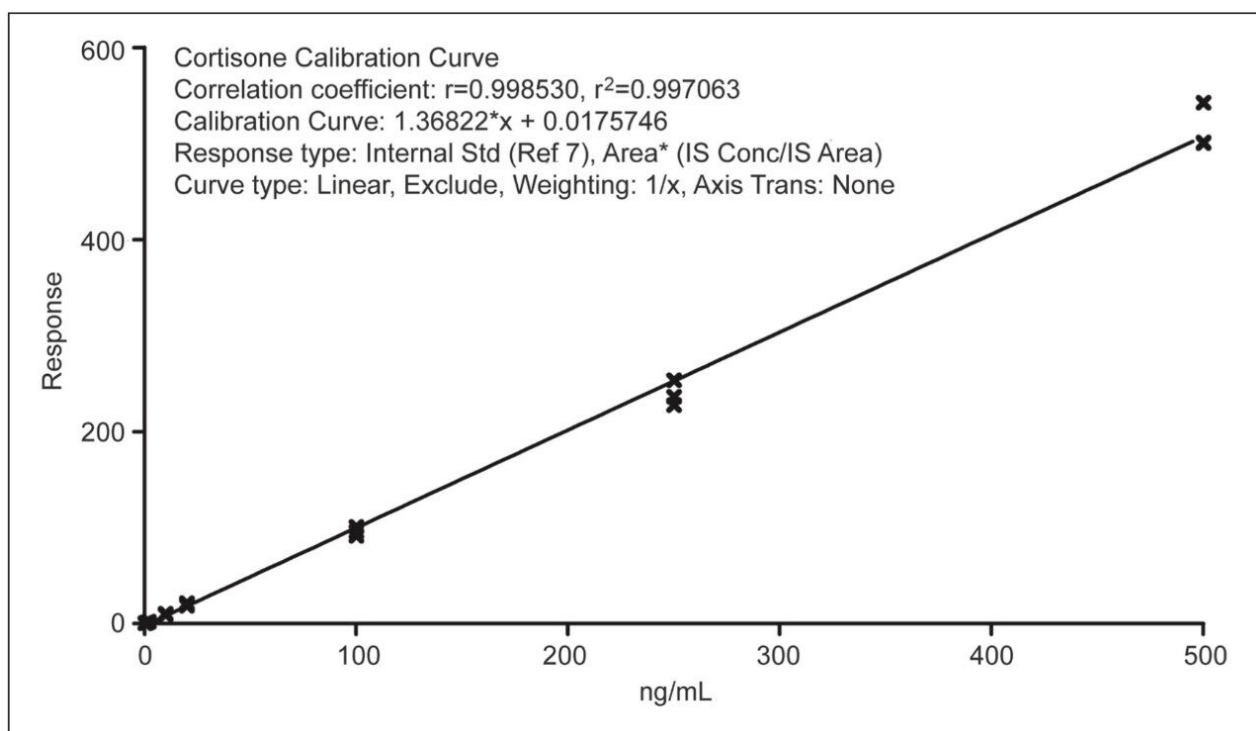


Figure 2. Calibration curve for cortisone measured in human serum. (Broccardo 2013)

Analyte	LOD (ng/mL)	LOQ (ng/mL)
testosterone	0.12	0.41
dihydrotestosterone	0.42	1.40
progesterone	0.03	0.40
cortisone	0.09	0.29
cortisol	0.57	1.90

Table 2. LOD and LOQ values for the five steroids in human serum.  
 (Broccardo 2013)

## Conclusion

- The ionKey/MS System with the Xevo TQ-S and 150  $\mu$ m iKey enabled the development of a low flow MRM assay of five steroids in human serum for clinical research.
- The low flow regime resulted in an increase in on-column analytical sensitivity and a 150 fold decrease

in solvent consumption, as compared to standard flow multi-use methods in the literature.

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## References

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