TECHNICAL NOTE 73517

Quantification of six steroids in human serum by TurboFlow chromatography coupled to tandem mass spectrometry for use in clinical research

Authors: Claudio De Nardi,[1] Paolo Brambilla,[2] Chiara Fania,[3] Maura Brambilla[2]

[1]Thermo Fisher Scientific GmbH,

Dreieich, Germany

[2]Ospedale di Desio, Desio, Italy
[3]Università degli Studi Milano-Bicocca,

Milano, Italy

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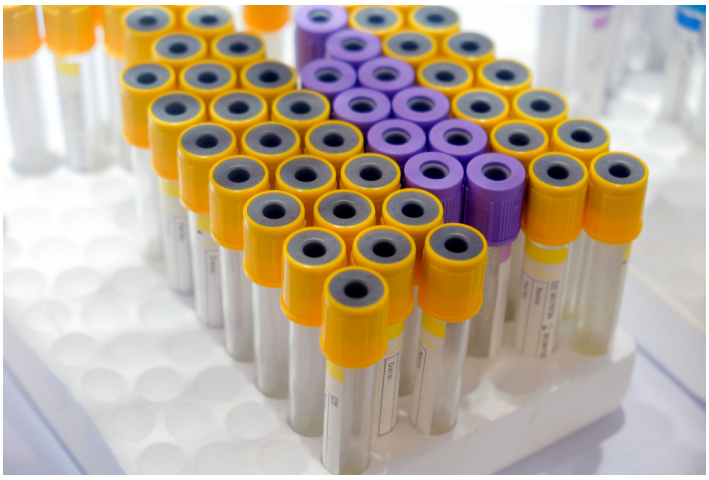


Application benefits

- Simple pre-injection sample preparation
- Quantification of six different steroids in serum in 7 minutes

Goal

Development and implementation of an analytical method for the quantification of six steroids in human serum on a Thermo Scientific™ TSQ Quantis™ triple-stage quadrupole mass spectrometer using Thermo Scientific™ TurboFlow™ online sample preparation for clinical research

Introduction

Liquid chromatography (LC) coupled to triple quadrupole mass spectrometry (MS/MS) has gained widespread popularity and emerged as the technology of choice for determination of steroid hormones in biological matrices.

There are several advantages that LC-MS offers for these specific assays: superior specificity compared to immunoassays, as well as the possibility to achieve high-throughput capabilities and required sensitivity in low sample volumes. While the ability to analyze and quantify several steroids simultaneously in one LC-MS/MS run offers some significant advantages, there is a stronger demand for LC-MS/MS assays to be more sensitive and robust. Here we report a robust, reliable LC-MS quantification assay of six steroids in human serum. The list of analytes includes 17-hydroxyprogesterone, androstenedione, cortisol, dehydroepiandrosterone sulphate (DHEAS), progesterone, and testosterone. Serum samples were extracted by offline protein precipitation with concomitant addition of the internal standards.

Extracted samples were injected onto a Thermo Scientific™ Transcend™ TLX system using TurboFlow technology for online sample preparation and chromatographic separation followed by detecteion on a TSQ Quantis triple-stage quadrupole mass spectrometer with heated electrospray ionization (H-ESI II) operated in polarity switching mode. Detection was performed by selected reaction monitoring (SRM) using six isotopically labelled internal standards for quantification. Method performance was evaluated using calibrators and controls from Chromsystems Instruments & Chemicals (Munich, Germany) in terms of limits of quantification, linearity ranges, carryover, accuracy, and intra- and inter-assay precision.

Experimental

Target analytes

A list of analytes, corresponding internal standards, and concentration ranges covered by the calibrators are reported in Table 1.

Sample preparation

Homemade calibrators (eleven levels including blank) were prepared by mixing 1:1 (v/v) at each level the two sets of calibrators (six levels including blank) from Chromsystems and further diluting the lowest calibrator using the blank matrix. Controls samples (three levels) were obtained by mixing 1:1 (v/v) the two sets of controls from the same supplier to get to lower concentration levels and to have more volume of each calibrator and control. Nominal concentrations for calibrators and controls are reported in Tables 2 and 3, respectively. Samples were processed by adding 100 µL of methanol containing the internal standards to 100 µL of serum sample followed by vortex-mixing. Precipitated samples were left at room temperature for 5 min, vortex-mixed again, left at 4 °C for 15 min, and centrifuged for 10 min at maximum speed. The supernatant was transferred to a clean plate or vial.

Table 1. Analytes, internal standards, and concentration ranges covered by the calibrators

Analyte	Internal standard	Concentration range (ng/mL)
17-hydroxyprogesterone	d_8-17-hydroxyprogesterone	0.049–10.8
Androstenedione	$^{13}C_3$-androstenedione	0.096–7.00
Cortisol	d_4-cortisol	4.89–141
DHEAS	d_5-DHEAS	57.5–2947
Progesterone	d_9-progesterone	0.081–12.3
Testosterone	d_3-testosterone	0.027–5.75

Table 2. Nominal concentrations for calibrators

Analyte	Units	CAL 1	CAL 2	CAL 3	CAL 4	CAL 5	CAL 6	CAL 7	CAL 8	CAL 9	CAL 10
17-hydroxyprogesterone	pg/mL	0.400	1.80	10.0	16.2	49.0	241	481	975	1931	10800
Androstenedione	pg/mL	0.711	3.56	10.7	32.0	96.0	191	383	730	2380	7000
Cortisol	ng/mL	0.036	0.181	0.540	1.63	4.89	10.0	20.2	40.6	73.0	141
DHEAS	ng/mL	0.426	2.13	6.39	19.2	57.5	298	513	1023	2011	2947
Progesterone	pg/mL	0.600	3.00	9.00	27.0	81.0	383	1020	2540	5000	12300
Testosterone	pg/mL	0.200	1.00	3.00	9.00	27.0	128	493	1480	2930	5750

Table 3. Nominal concentrations for controls

Analyte	Units	CAL 1	CAL 2	CAL 3
17-hydroxyprogesterone	pg/mL	146	725	2863
Androstenedione	pg/mL	148	580	4725
Cortisol	ng/mL	12.7	29.9	87.0
DHEAS	ng/mL	137	768	2475
Progesterone	pg/mL	159	1525	7400
Testosterone	pg/mL	103	750	3880

Liquid chromatography

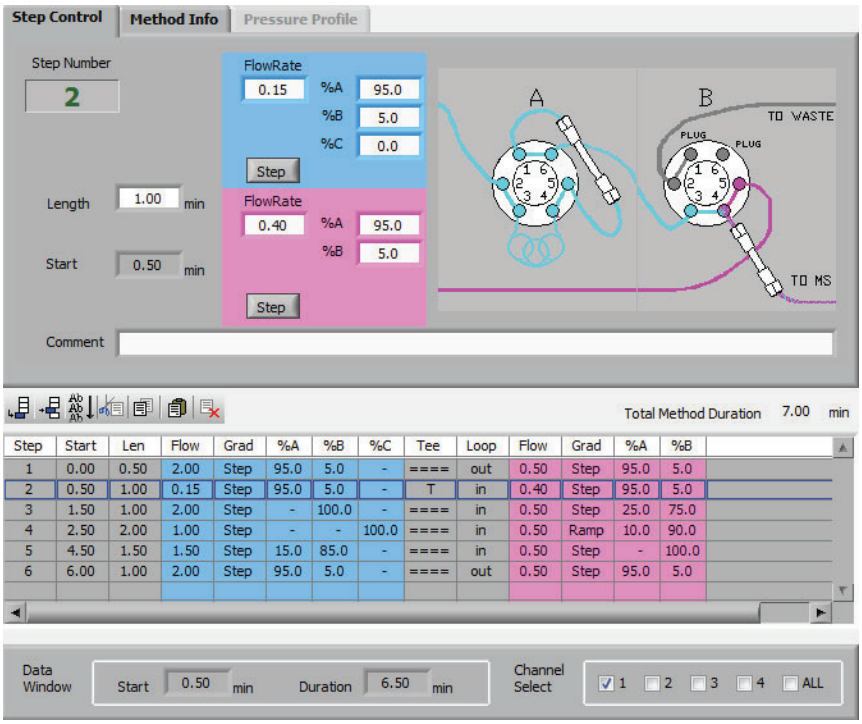
The supernatant was injected onto a Transcend TLX-2 system; only one TLX channel was used for the scope. Online sample cleanup was performed using a 0.5 × 50 mm Thermo Scientific™ TurboFlow™ Cyclone-P™ column. Chromatographic separation was achieved on a 50 × 2.1 mm (2.6 µm) Thermo Scientific™ Accucore™ biphenyl analytical column (P/N 17826-052130) kept at 40 °C. Details of the method are reported in Figure 1. Injection volume was 80 µL. Total runtime was 7.0 min.

Mass spectrometry

Analytes and internal standards were detected by SRM on a TSQ Quantis triple-stage quadrupole mass spectrometer with heated electrospray ionization operated in positive and negative (DHEAS only) ionization mode. Two SRM transitions for each analyte were included in the acquisition method for quantification and confirmation, respectively. Mass spectrometric conditions are reported in Table 4.

Table 4. MS settings

Source type	Heated electrospray ionization (H-ESI II)
Vaporizer temperature	350 °C
Capillary temperature	325 °C
Spray voltage (positive/negative mode)	3500/3000 V
Sheath gas	45 AU
Sweep gas	1 AU
Auxiliary gas	10 AU
Data acquisition mode	Selected-reaction monitoring (SRM)
Collision gas pressure	2.0 mTorr
Cycle time	0.300 s
Q1 mass resolution (FWMH)	0.7
Q3 mass resolution (FWMH)	0.7



Mobile Phases

A	Water
B	Methanol
C	Acetonitrile Methanol 2-propanol 40/40/20 (v/v/v)
A	0.2 mM Ammonium Fluoride in Water
B	Methanol / Water 95/5 (v/v) + 0.2 mM Ammonium Fluoride

Figure 1. LC method description including both TurboFlow samples cleanup (blue) and analytical separation (pink)

Method evaluation

The method performance was evaluated in terms of limit of quantification (LOQ), linearity of response within the calibration range, carryover, accuracy, and intra- and inter-assay precision. LOQ evaluation was based on the lowest calibrator with a percentage bias between nominal and back-calculated concentration within ±20% (±15% for the others). The acceptance criterion for all the controls was the same bias to be within ±20%. Carryover was calculated in terms of percentage ratio between peak area of the highest calibrator and a blank sample injected just after it. Analytical accuracy was evaluated in terms of percentage bias between nominal and average back-calculated concentrations on the quality control samples at three levels prepared and analyzed in replicates of five on three different days. Intra-assay precision for each day was evaluated in terms of percentage coefficient of variation (%CV) using the same controls in replicates of five (n=5). Inter-assay precision was evaluated as the %CV on the full set of samples (control samples at three levels in replicates of five prepared and analyzed on three different days).

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 4.1 software.

Results and discussion

LOQ values for each analyte are reported in Table 5. The method showed an excellent linearity within the calibration range for each analyte with a correlation factor (R^2) always above 0.999. A representative chromatogram of cortisol and 17-hydroxyprogesterone at the lowest calibration level together with their internal standards is reported in Figure 2. Representative calibration curves for the same analytes are reported in Figure 3.

No significant carryover was observed, with no signal detected in the blank injected immediately after the highest calibrator.

Table 5. Analytes and corresponding LOQ

Analyte	LOQ
17-hydroxyprogesterone	49.0 pg/mL
Androstenedione	96.0 pg/mL
Cortisol	0.540 ng/mL
DHEAS	6.39 ng/mL
Progesterone	81.0 pg/mL
Testosterone	27.0 pg/mL

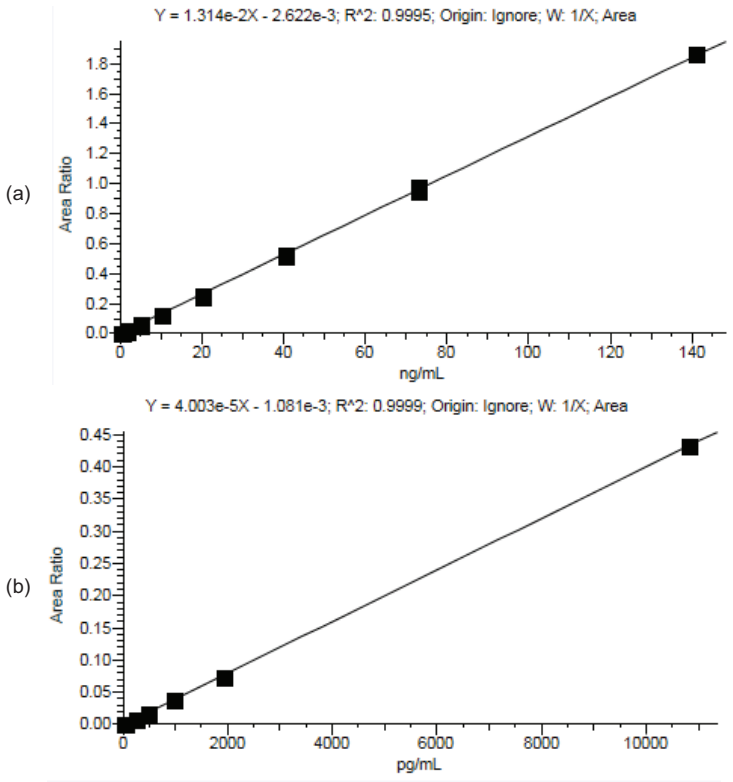


Figure 3. Representative calibration curves for (a) cortisol and (b) 17-hydroxyprogesterone – day 3

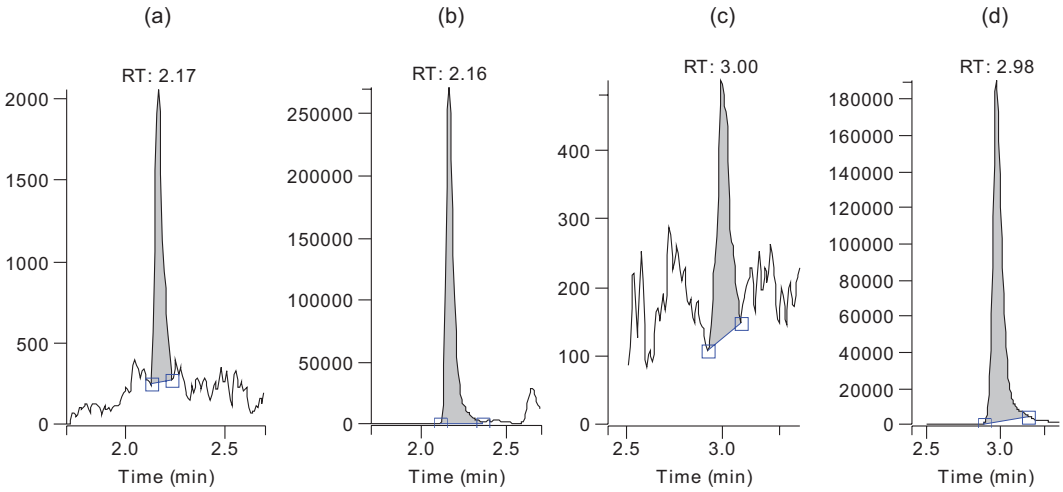


Figure 2. Representative chromatograms of the LOQ for (a) cortisol, (b) d_4-cortisol, (c) 17-hydroxyprogesterone and (d) d_8-17-hydroxyprogesterone

The data presented in this report demonstrate excellent accuracy of the method with a percentage bias between nominal and average back-calculated concentration for the used control samples ranging between -5.4% and 5.1% (Table 6). The %CV for intra-assay precision was always below 6.9%. The maximum %CV for inter-assay precision was 4.4%. Results for intra- and inter-assay precision are reported in Table 7 and Table 8, respectively.

Table 6. Analytical accuracy results

Analyte	Level	Units	Nominal concentration	Average calculated concentration	Bias (%)
17-hydroxyprogesterone	CTRL1	pg/mL	146	150	2.6
	CTRL2		725	715	-1.4
	CTRL3		2863	3009	5.1
Androstenedione	CTRL1	pg/mL	148	142	-4.1
	CTRL2		580	566	-2.4
	CTRL3		4725	4816	1.9
Cortisol	CTRL1	ng/mL	12.6	12.3	-2.4
	CTRL2		29.9	29.0	-3.0
	CTRL3		87.0	87.8	0.9
DHEAS	CTRL1	ng/mL	137	129	-5.4
	CTRL2		768	775	0.9
	CTRL3		2475	2596	4.9
Progesterone	CTRL1	pg/mL	159	163	2.6
	CTRL2		1525	1507	-1.1
	CTRL3		7400	7560	2.2
Testosterone	CTRL1	pg/mL	103	105	2.3
	CTRL2		750	729	-2.8
	CTRL3		3880	3874	-0.2

Table 7. Intra-assay precision results

Analyte	Level	Units	Nominal concentration	Day 1		Day 2		Day 3	
				Average calculated concentration	CV (%)	Average calculated concentration	CV (%)	Average calculated concentration	CV (%)
17-hydroxyprogesterone	CTRL1	pg/mL	146	155	2.1	150	5.1	144	1.8
	CTRL2		725	726	4.3	720	5.8	699	1.5
	CTRL3		2863	3024	0.8	3015	1.6	2987	0.8
Androstenedione	CTRL1	pg/mL	148	143	2.9	140	2.0	143	1.6
	CTRL2		580	567	1.5	567	2.3	564	0.8
	CTRL3		4725	4730	3.8	4925	1.3	4793	1.2
Cortisol	CTRL1	ng/mL	12.6	12.2	4.7	12.5	5.4	12.3	0.9
	CTRL2		29.9	28.3	1.8	29.9	2.5	28.9	1.9
	CTRL3		87.0	86.9	3.4	89.1	1.0	87.3	1.2
DHEAS	CTRL1	ng/mL	137	129	6.9	128	1.1	131	1.8
	CTRL2		768	786	3.2	763	2.1	776	1.7
	CTRL3		2475	2642	1.2	2571	0.9	2574	1.0
Progesterone	CTRL1	pg/mL	159	161	2.1	161	1.5	167	1.9
	CTRL2		1525	1486	2.3	1540	3.3	1497	1.4
	CTRL3		7400	7304	3.0	7726	1.0	7650	1.3
Testosterone	CTRL1	pg/mL	103	102	5.7	107	0.3	107	1.5
	CTRL2		750	723	2.4	754	2.4	710	1.5
	CTRL3		3880	3836	5.2	3931	1.1	3854	2.4

Table 8. Inter-assay precision results

Analyte	Level	Units	Nominal concentration	Average calculated concentration	CV (%)
17-hydroxyprogesterone	CTRL1	pg/mL	146	150	4.4
	CTRL2		725	715	4.3
	CTRL3		2863	3009	1.2
Androstenedione	CTRL1	pg/mL	148	142	2.3
	CTRL2		580	566	1.6
	CTRL3		4725	4816	2.8
Cortisol	CTRL1	ng/mL	12.6	12.3	4.0
	CTRL2		29.9	29.0	3.1
	CTRL3		87.0	87.8	2.3
DHEAS	CTRL1	ng/mL	137	129	4.0
	CTRL2		768	775	2.6
	CTRL3		2475	2596	1.6
Progesterone	CTRL1	pg/mL	159	163	2.3
	CTRL2		1525	1507	2.8
	CTRL3		7400	7560	3.1
Testosterone	CTRL1	pg/mL	103	105	3.8
	CTRL2		750	729	3.3
	CTRL3		3880	3874	3.3

Conclusion

A robust, reliable, and reproducible liquid chromatography-tandem mass spectrometry method for clinical research focused on quantification of six steroids in human serum was implemented on a Transcend TLX system coupled to a TSQ Quantis triple-stage mass spectrometer. The use of TurboFlow online sample cleanup minimizes offline sample preparation. Used with mass spectrometry, TurboFlow cleanup provides higher sensitivity compared to the traditional LC-MS approach. The outstanding quality of data also highlights the performance of the Accucore biphenyl analytical column, which can enable every clinical research laboratory to meet the critical challenges of developing, optimizing and implanting a reliable, accurate and robust LC-MS method.

Find out more at **thermofisher.com/clinicalresearch**

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