Application Note

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Analysis of Adrenal Steroids in Dried Blood Spots and Serum Using Agilent Ultivo LC/TQ

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Abstract

This application note describes a LC/MS/MS based method for the analysis of adrenal steroids extracted from dried blood spots and blood serum using an Agilent Ultivo LC/TQ system. The steroid profile consists of 11 adrenal steroids analyzed in both matrices. The analytical LC/MS/MS method was performed within 7 minutes per injection. The determined limits of detection and quantification were nearly the same in both matrices with less than 15% interday variation and 10% intraday variation. In summary, the Ultivo system showed good overall performance.

Introduction

The human metabolism depends essentially on the endocrinologic function of hormones. The adrenal steroids, derived from the adrenal glands, are comprised of a group of different biomolecules affecting and regulating the electrolyte and water homeostasis in the kidneys. Moreover, a malfunction caused by genetic mutations of the corresponding steroids or their biosynthesis might result in adrenal insufficiency causing different disorders like Congenital Adrenal Hyperplasia comprising 21-hydroxylase- and 11-hydroxylase deficiency as well as hyperandrogenemia or cushing's syndrome.[1-3]

In the context of clinical research and in order for a better understanding of the interaction and synergy of the steroids, it is important to be able to analyze the steroid profile in different matrices. Therefore, biochemical and mass spectrometry based approaches were established to investigate steroid hormones and their concentrations.[4-7]

The measurement of the overall steroid profile including 21-deoxycortisol (21-F), 11-deoxycortisol (S), 4-Androstendione (4-A), 17-hydroxyprogesterone (17-OHP), cortisol (F), testosterone (T), progesterone (Prog), deoxycorticosterone (DOC), dihydrotestosterone (DHT), corticosterone (B) and dehydroandrosteronesulfate (DHEAS) enables the determination and discrimination of different disorders that are caused by altered steroid biosynthesis pathways in less than 7 minutes of analysis time. This study transfers the existing method to an Agilent system combining the Infinity II 1290 with an Ultivo TQ mass spectrometer. Beside the aim of the method transfer onto another LC/MS/MS system the overall performance should be determined.

Experimental

LC configuration and parameters

Configuration	
Agilent Pump	Infinity II 1290 High Speed Pump, G7120A
Agilent Autosampler	Infinity II 1290 Multisampler, G7167B
Agilent Column Compartment	Infinity II 1290 MCT, G7116B
Needle Wash	Standard Wash, Flush Port, 2 sec; 50/50 MeOH/H_2O
Autosampler Temperature	8 °C
Injection Volume	10 µL
Analytical Column	2.1 × 50 mm sub-2 µm C18 column
Column Temperature	23 °C
Mobile Phase A	H_2O + 0.1% FA
Mobile Phase B	MeOH + 0.1% FA
Flow rate	350 µL/minute
Gradient	**Time (min) %B** 0.00 47 0.47 51 1.74 51 3.70 70 4.50 70 4.51 95 5.00 95 5.01 47 6.00 47
Stop Time	6.0 minutes

LC/TQ mass spectrometer configuration and parameters

Configuration	
Agilent Ultivo Triple Quadrupole Mass Spectrometer Equipped with JetStream Ion Source (G6465A)	
Ionization Mode	Positive and Negative
Drying Gas Temperature	260 °C
Drying Gas Flow	11 L/min
Nebulizer Pressure	38 psi
Sheath Gas Temperature	400 °C
Sheath Gas Flow	12 L/min
Nozzle Voltage	400 V
Capillary Voltage, Positive	4,500 V
Capillary Voltage, Negative	4,500 V

MRM parameters: dynamic MRM, 500 MS cycle time, MS1 resolution = wide, MS2 resolution = unit								
Analyte	ISTD	Precursor (*m/z*)	Product (*m/z*)	RT (min)	RT win. (min)	Frag (V)	CE (V)	+/–
4-A	D7-4A	287.2	97.1	3.7	0.8	124	21	Pos.
21-F	D8-21-F	347.2	311.1	2.9	0.8	124	5	Pos.
B	D8-21-F	347.2	120.9	3.1	0.8	124	21	Pos.
DHEAS	D6-DHEAS	367.2	96.8	3.5	1.0	182	40	Neg.
DHT	D3-DHT	291.2	255.2	4.7	0.8	119	5	Pos.
DOC	D8-DOC	331.2	96.9	4.2	0.8	129	21	Pos.
F	D2-F	363.2	120.9	2.2	0.8	129	25	Pos.
OHP	D8-OHP	331.2	97.0	4.3	0.8	119	21	Pos.
Prog	D9-Prog	315.2	97.0	5.1	0.8	124	21	Pos.
S	D2-S	347.2	97.0	3.3	0.8	119	21	Pos.
T	D8-OHP	289.2	97.0	4.1	0.8	119	21	Pos.
D2-F	–	365.2	121.9	2.1	0.8	124	17	Pos.
D2-S	–	349.2	96.9	3.3	0.8	124	20	Pos.
D3-DHT	–	294.3	258.2	4.7	0.8	124	5	Pos.
D6-DHEAS	–	373.2	97.8	3.5	1.0	182	41	Neg.
D7-4A	–	294.3	100.0	3.6	0.8	129	17	Pos.
D8-21-F	–	355.3	319.2	2.8	0.8	124	5	Pos.
D8-DOC	–	339.3	99.9	4.2	0.8	134	20	Pos.
D8-OHP	–	339.3	113.0	4.2	0.8	134	20	Pos.
D9-Prog	–	324.3	100.0	5.0	0.8	134	17	Pos.

Chemicals and Reagents

Analytical Standards:

- 21-F (21-Deoxycortisol), Sigma, Darmstadt, Germany, Order-No. P-9521
- S (11-Deoxycortisol), Sigma, Darmstadt, Germany, Order-No. R-0500
- 4-A (4-Androstendione), Sigma, Darmstadt, Germany, Order-No. A-9630
- 17-OHP (17-Hydroxyprogesterone), Sigma, Darmstadt, Germany, Order-No. H-5752
- F (Cortisol), Sigma, Darmstadt, Germany, Order-No. H-4001
- T (Testosterone), Sigma, Darmstadt, Germany, Order-No. T-1500
- DOC (Deoxycorticosterone), Sigma, Darmstadt, Germany, Order-No. R-0500
- Prog (Progesterone), Sigma, Darmstadt, Germany, Order-No. P-0130
- DHT (Dihydrotestosterone), Sigma, Darmstadt, Germany, Order-No. 10300
- B (Corticosterone), Sigma, Darmstadt, Germany, Order-No. 27840
- DHEAS (Dehydroandrosteronesulfate), Sigma, Darmstadt, Germany, Order-No. D-5297
- D2-F (Cortisol-D_2), CDN Isotopes, Augsburg, Germany, Order-No. D-2878
- D8-21-F (21-Deoxycortisol-D_9), CDN Isotopes, Augsburg, Germany, Order-No. D-5821
- D8-17-OHP (17-Hydroxyprogesterone-D_8), CDN Isotopes, Augsburg, Germany, Order-No. D-5650
- D7-4-A (4-Androstendione-D_7), CDN Isotopes, Augsburg, Germany, Order-No. D-5305
- D3-DHT (Dihydrotestosterone-D_3), CDN Isotopes, Augsburg, Germany, Order-No. D-5079
- D2-S (11-Deoxycortisol-D_2), CDN Isotopes, Augsburg, Germany, Order-No. D-5901
- D8-DOC (Deoxycorticosterone-D_8), CDN Isotopes, Augsburg, Germany, Order-No. D-5732
- D6-DHEAS (Dehydroandrosteronsulfate-D_6), Sigma, Darmstadt, Germany, Order-No. 723266
- D9-Prog (Progesterone-D_9), Sigma, Darmstadt, Germany, Order-No. P-070

Standards and curve preparation

Calibrator preparation

Erythrocytes were washed once and mixed with steroid free serum (ratio 55:45) obtained from MP Biomedicals (Order No. 07-166804) serving as the matrix. The solid analytical standard compounds were dissolved with methanol. Standard stock solutions were mixed and diluted prior to addition to the matrix. The steroid containing blood matrix was spotted and dried on filter cards. Serum calibrators were made by adding standard stock solution into steroid free serum. The final analytical concentrations (nM) for each calibrators were as follows:

Calibrator Abbrev.	Cortisol (nM)	DHEAS (nM)	All Other Steroids (nM)
C1	20	200	5
C2	100	1,000	25
C3	300	3,000	75
C4	500	5,000	125
C5	800	8,000	200

Internal Standard preparation

The internal standard compounds were diluted in methanol and the mixture containing different ISTD concentrations was added to each punched blood spot or serum sample prior sample extraction.

Quality control preparation

Quality control samples were prepared accordingly to the calibrators except for the final concentrations, which were as follows:

Quality Control	Cortisol (nM)	DHEAS (nM)	All Other Steroids (nM)
QC Blank	0	0	0
QC1	37.5	400	10
QC2	375	800	20
QC3	750	4,000	100

Sample preparation

The sample extraction was performed as follows. Briefly, 2 × 4.7 mm punchings or 15.2 µL serum were placed into a 96-well microtiter plate. After addition of 20 µL internal standard mix solution samples were further incubated for one minute at 600 rpm. Next, 200 µL 50/50 (v/v) acetone:acetonitrile was added with further incubation for 50 min (blood spot) or 20 minutes (serum) at 300 U/min. The serum sample was centrifuged for 10 minutes at 2,800 U/min. Supernatants were transferred into a new 96-well microtiter plate and dried under constant nitrogen flow at 35 °C. Samples were reconstituted with 80 µL 50% methanol and 50 mM formic acid for 30 minutes at 400 U/min. After a final centrifugation step for 10 minutes at 2,800 U/min 10 µL of sample was used for mass spectrometric analysis.

Data analysis

The acquired raw data were analyzed using the MassHunter Workstation Quantitative Analysis software, version B.09.00 Build 9.0.647.0 for QQQ. Quantification was performed using an external 5 point calibration curve and linear regression with 1/x weighting. Intensities were normalized to the internal standards and concentrations were calculated based on the external calibration samples. The overall quantification was performed using the Agile2 software algorithm and quantification by height. In addition peaks were smoothed using Quartic/Quintic Savitzky-Golay algorithm (Smoothing Function Width = 15 and 5 for Smoothing Gaussian Width). For noise determination the noise Algorithm RMS and a noise SD multiplier of 5 was applied.

Results

The existing LC/MS/MS method for analysis of 11 adrenal steroids was transferred from a non-Agilent system onto an Agilent Ultivo system. Herein, the overall performance regarding detection and quantification limits as well as precision were analyzed. An example of the total ion chromatograph (TIC) for a calibrator sample is shown in Figure 1.

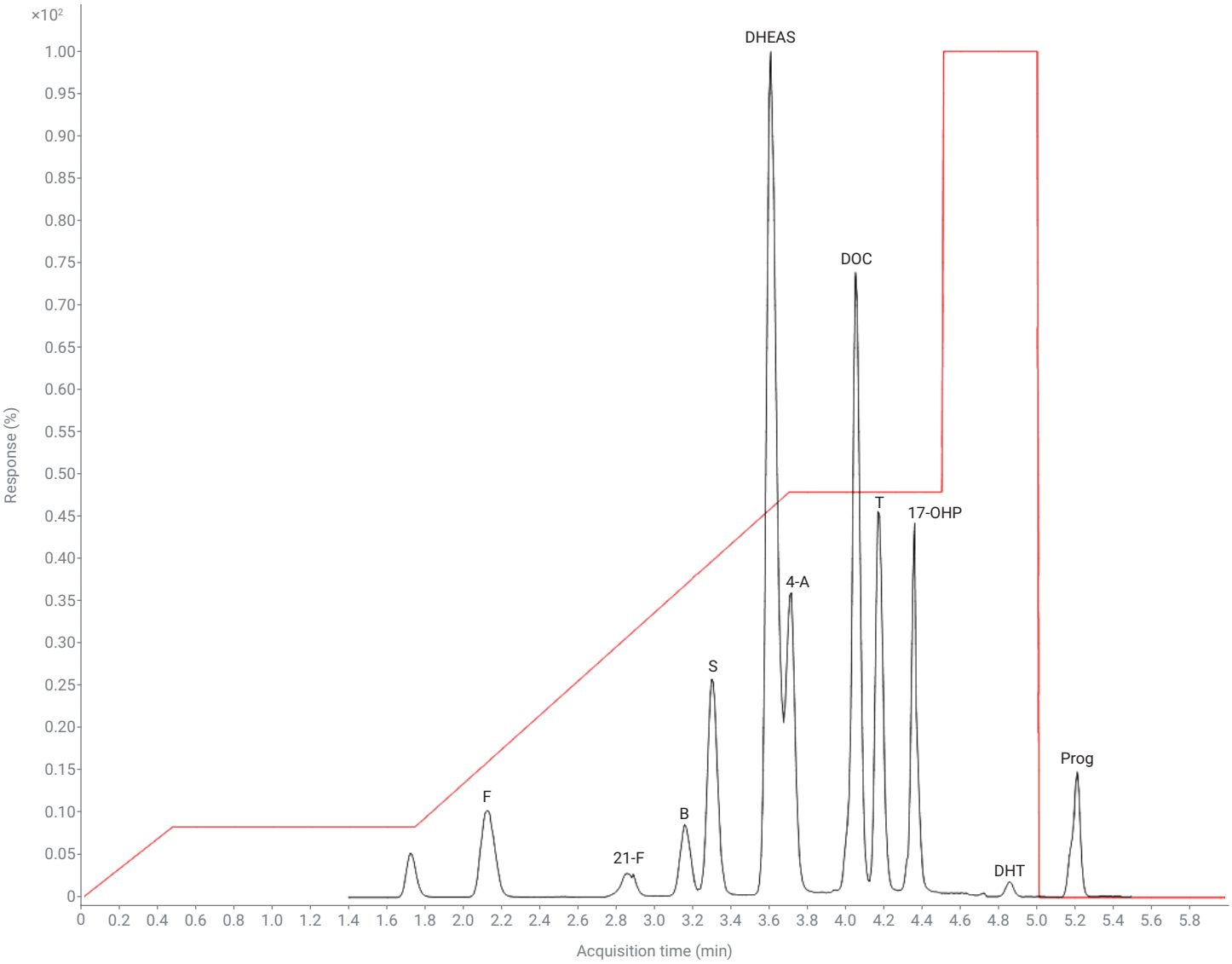

Figure 1. Gradient profile (red) and typical total ion chromatogram for the applied dynamic MRM method and calibrator sample extracted from dried blood.

All experimental results are shown in Table 1 (dried blood) and Table 2 (serum). In detail, varying analyte recovery was observed between the different concentration levels of the controls (10, 20, and 100 nM) for dried blood and serum with reference to the target concentrations. Overall, experiments for the determination of the limit of detection (LOD) as well as the lower limit of quantification (LOQ) for the different analytes were performed on six independent days in triplicate with a predefined concentration of 5 nM. Herein, the mean LOD was defined as the extrapolated concentration that give a signal-to-noise ratio of 3:1 (smoothed chromatogram, Noise PeakToPeak). For the lower LOQ a signal-to-noise threshold of 10:1 was defined, respectively.

In summary, all adrenal steroids of interest, extracted from the different matrices, showed acceptable limits of detection and quantification.

The intraday variances were measured for three different concentration levels (K1, K2, K3) with up to eight replicates for each level. Furthermore, interday variation was determined on six independent days for the same concentration levels used for the intraday measurements and almost three replicates per level and day. Results are shown in Table 1 (dried blood) and Table 2 (serum). The resulting values represent the mean range for the different concentrations.

Conclusion

The Agilent Ultivo MS/MS system is suitable to analyze a broad spectrum of adrenal steroids extracted from dried blood spots and serum in less than 7 minutes. The overall performance is comparable to other LC/MS/MS systems. The method transfer process in this example was done without any problems.

Table 1. General LC/MS/MS method performance for the Agilent Ultivo for adrenal steroids extracted from dried blood spots.

Anaylte	F	S	21-F	Prog	17-OHP	4-A	T	DHT	B	DOC	DHEAS
LOD (nM)	0.57	0.24	0.70	0.12	0.41	0.11	0.05	0.95	0.55	0.10	0.23
LOQ (nM)	1.89	0.80	2.32	0.40	1.35	0.36	0.18	3.16	1.83	0.33	0.77
Recovery (%)	97 to 101	90 to 94	100 to 111	87 to 97	98 to 113	92 to 105	92 to 101	83 to 99	103 to 106	93 to 101	92 to 97
Intraday CV (%)	3 to 5	3 to 5	3 to 6	8 to 13	2 to 6	3 to 5	2 to 8	4 to 8	3	3 to 6	3 to 4
Interday CV (%)	5 to 7	6 to 8	6 to 11	6 to 12	7 to 9	11 to 17	5 to 12	7 to 12	7 to 10	6 to 12	5 to 6

Table 2. General LC/MS/MS method performance for the Agilent Ultivo for adrenal steroids extracted from serum.

Anaylte	F	S	21-F	Prog	17-OHP	4-A	T	DHT	B	DOC	DHEAS
LOD (nM)	0.35	0.14	0.70	0.11	0.35	0.11	0.09	1.25	0.34	0.15	0.18
LOQ (nM)	1.15	0.46	2.34	0.37	1.16	0.36	0.28	4.15	1.13	0.48	0.59
Recovery (%)	61 to 75	58 to 69	73 to 74	57 to 63	74 to 78	67 to 75	65 to 75	59 to 61	57 to 64	64 to 66	59 to 63
Intraday CV (%)	3 to 6	5 to 8	5 to 8	5 to 11	5 to 8	5 to 8	5 to 7	6 to 7	6 to 9	6 to 9	4 to 7
Interday CV (%)	6 to 8	9 to 13	8 to 14	7 to 11	8 to 12	12 to 19	10 to 13	7 to 9	8 to 13	10 to 12	7 to 8

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