APPLICATION NOTE

Steroid profiling from dried blood spot samples using QSight 220/QSight 225 MD mass spectrometer.

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Introduction

Inborn errors of metabolism include a wide variety of conditions with abnormal steroidogenesis caused by mutations affecting the enzymatic activity in the steroid biosynthesis pathways. 21-hydroxylase deficiency and 11- β hydroxylase deficiency impact the normal synthesis of glucocorticoids and mineralocorticoids.

The 21-hydroxylase enzyme converts 17-hydroxyprogesterone (17-OHP) to 11-deoxycortisol (11-DECOL) and progesterone to 11-deoxycorticosterone. Impaired activity of this enzyme results in increased 17-OHP, defective synthesis of cortisol and mineralocorticoids, increased synthesis of androstenedione (ADIONE) and decreased central feedback (increased production of adrenocorticotropic hormone) (Figure1).

The 11- β hydroxylase enzyme (11β-H) converts 11-DECOL to cortisol (CORT) and 11-deoxycorticosterone to corticosterone. Its deficiency increases production of 11-DECOL and 11-deoxycorticosterone.[1]

Measurement of increased 17-OHP concentration in dried blood spot (DBS) by immunoassays (IA) is associated to analytical interference arising from cross-reactivity of the reagent antibodies with other structurally-related steroid metabolites, particularly 17-hydroxypregnenolone [2, 3, 4]. To allow more specific marker measurement we report here an ultra-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method for steroid profiling from dried blood spot (DBS) samples.[2,3] It allows the simultaneous specific determination of 17-OHP, ADIONE, CORT [2, 3] and additional steroids such as 21-deoxycortisol (21-DECOL) and 11-DECOL to enable distinction between 21-H and 11β-H deficiency.

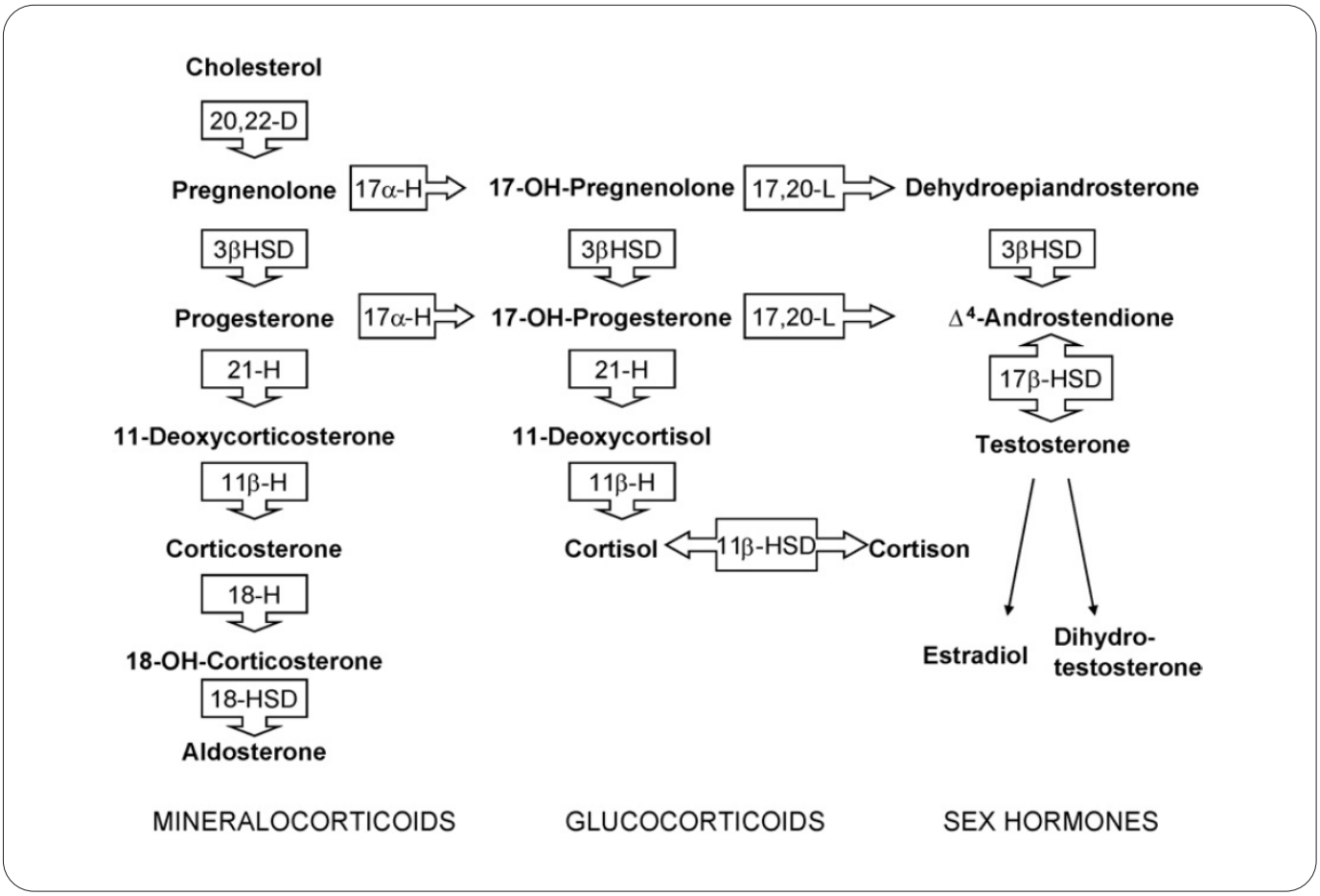

Figure 1: Pathways of steroid biosynthesis: (3β-HSD: 3β-hydroxysteroid dehydrogenase, 11βHSD: 11β-hydroxysteroid dehydrogenase, 18-HSD: 18-hydroxysteroid dehydrogenase, 17α-H: 17α-hydroxylase, **21-H: 21-hydroxylase, 11β-H: 11β-hydroxylase**, 18-H: 18-hydroxylase, 20,22-D: 20,22 desmolase, 17,20-L: 17,20-lyase).[1]

Method

Two 3.2 mm DBS disks were punched (Panthera-Puncher™ 9; Revvity) into a microplate well, and incubated with 210 µL of extraction solution, at 30 °C for one hour under stirring at 650 rpm. During the incubation an adhesive microplate cover was used to minimize evaporation. We obtained a higher extraction yield by adding the extraction solution in two steps as follows:

first 30 µL of water and after five minutes we added 180 µL of methanol containing the internal standards 2H5-ADIONE (2.5 nMol), $2H_4$-CORT (46 nMol), $2H_5$-11-DECOL (7.52 nMol) and 2H8-17OHP (4.8 nMol), included in CHS™ MSMS Steroids Kit (p/n 3070-0020).

After incubation the supernatant was transferred to a new microplate, evaporated under nitrogen stream and reconstituted in 60 µL of 55:45 mobile phase A: mobile phase B (the composition of mobile phases is reported in section "LC conditions").

Finally, 10 µL of the obtained solution was injected in the LC-MS/MS system.

The quantitative determination of 17OHP, CORT. ADIONE, 11-DECOL and 21-DECOL was performed using calibration curves for each steroid to final concentration of 1.10, 2.30, 4.68, 9.39, 18.70, 37.50, 75.00, 150.00, 300.00 nMol (for CORT 4.68, 9.39, 18.70, 37.50, 75.00, 150.00, 300.00 nMol).

Mass spectrometry conditions

The LC-MS/MS analysis was performed using a QSight ® 220 MD mass spectrometer coupled with LX-50 UHPLC and equipped with ESI source operating in positive ion mode. Table 1 outlines the MS instrumental source parameter settings. The optimized MRM transition parameters for analytes involved in this assay are shown in Table 3.

Table 1: MS conditions.

ESI voltage (V)	5000
HSID temp (°C)	275
Nebulizer gas setting	325
Drying gas setting	110
Source temp. (°C)	375
Dwell time (msec)	30

LC Conditions

The UHPLC separation was performed using an Acquity UPLC HSS T3 column (2.1 x 50 mm, 1.8 µm) and Acquity HSS T3 VanGuard Pre-column (2.1 x 5 mm, 1.8 µm), from Waters. The LC was run with gradient conditions reported below:

- **Mobile phases:**
  - A. 1.2 mMol ammonium acetate, 0.1 % (v/v) formic acid in 98% water/2% methanol
  - B. 1.2 mMol ammonium acetate, 0.1 % (v/v) formic acid in methanol
- **Injection volume:** 10 µl
- **Column temperature:** 60 °C
- **Gradient**

Table 2: UHPLC gradient.

Time	Flow Rate (µL/min)	%B
0	600	45
2	600	57
2.5	600	98
3	600	98
3.1	600	45
4.1	600	45

Table 3: Optimized MRM parameters (*$^{2}H_{5}$-11-DECOL has been used as internal standard for both 11-DECOL and 21-DECOL).

Compound	Precursor (m/z)	Fragment (m/z)	CCL2	CC	EV
17OHP quant	331.1	97.0	-64	-34	30
17OHP qual	331.1	109.0	-60	-40	30
CORT quant	363.1	121.0	-75	-36	30
CORT qual	363.1	97.0	-80	-60	30
ADIONE quant	287.1	97.0	-60	-34	28
ADIONE qual	287.1	109.0	-50	-34	28
11-DECOL quant	347.1	109.0	-60	-42	25
11-DECOL qual	347.1	97.0	-64	-36	25
21-DECOL quant	347.1	311.2	-60	-23	28
21-DECOL qual	347.1	121.0	-70	-46	28
$^{2}H_{8}$-17OHP quant	339.1	100.0	-64	-36	30
$^{2}H_{8}$-17OHP qual	339.1	113.0	-60	-40	30
$^{2}H_{4}$-CORT quant	367.1	121.0	-75	-36	30
$^{2}H_{4}$-CORT qual	367.1	97.0	-80	-60	30
$^{2}H_{5}$-ADIONE quant	292.1	100.0	-60	-34	28
$^{2}H_{5}$-ADIONE qual	292.1	113.0	-50	-34	28
$^{2}H_{5}$-11-DECOL quant *	352.1	113.0	-60	-42	25
$^{2}H_{5}$-11-DECOL qual *	352.1	100.0	-64	-36	25

Results

The LLOQ results were achieved using stable isotope-labeled analogues of steroid analytes. Measured amounts of these were added to real blood from normal subjects. Stable isotope- labeled analogues were used in place of steroid analytes, which are usually present in unstripped blood at physiological levels, as they have the same basic structures, provide an equivalent instrumental response, and can be easily distinguished from corresponding steroid analytes by mass spectrometry due to their different m/z value.[5]

LLOQ was calculated from S/N obtained by RMS mode, based on the lowest point of the calibration curves for each steroid.

Table 4.: Results obtained during steroid panel analysis.

Compound	LLOQ (nMol)	Accuracy (%)	Linearity (R^2)	Calibration Range (nMol)	Retention Time (RT)
17OHP	0.053	90-110	0.996	1.1 - 300 nMol	2.5
CORT	0.30	90-105	0.999	4.7 - 300 nMol	1.0
ADIONE	0.0064	90-108	0.996	1.1 - 300 nMol	2.0
11-DECOL	0.025	91-109	0.996	1.1 - 300 nMol	1.6
21-DECOL	0.29	94-105	0.999	1.1 - 300 nMol	1.4

Extracted ion chromatograms (EICs)

Figures 2-6 illustrate the EICs of 17OHP, ADIONE, 11-DECOL, CORT, and 21-DECOL respectively. All the compounds are shown at a concentration of 1.1 nMol (4.68 nMol for CORT) in DBS matrix. These chromatograms are representative of the panel as a whole.

Total ion chromatograms (TICs)

Figure 7 shows steroid profiling obtained using the shallow gradient detailed in the experimental section.

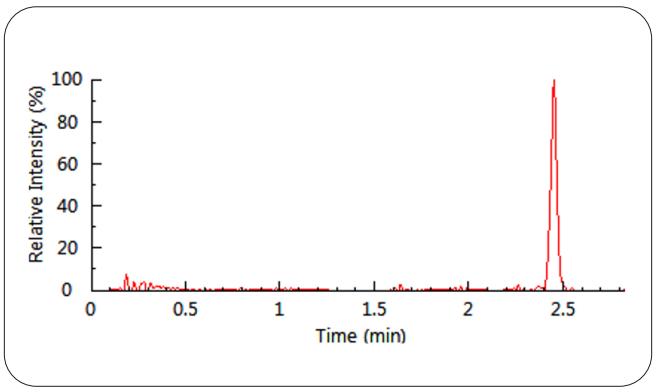

Figure 2: EIC of 17OHP in DBS Matrix.

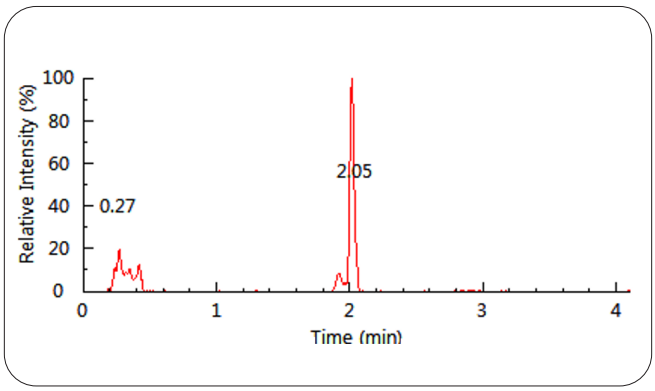

Figure 3: EIC of ADIONE in DBS Matrix.

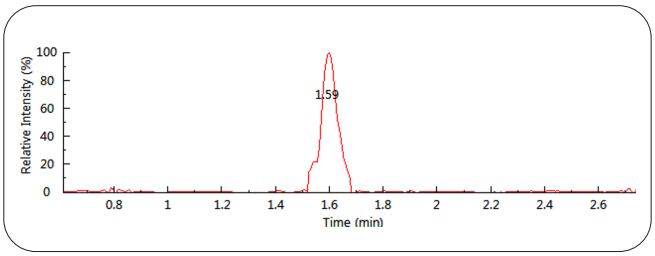

Figure 4: EIC of 11-DECOL in DBS Matrix.

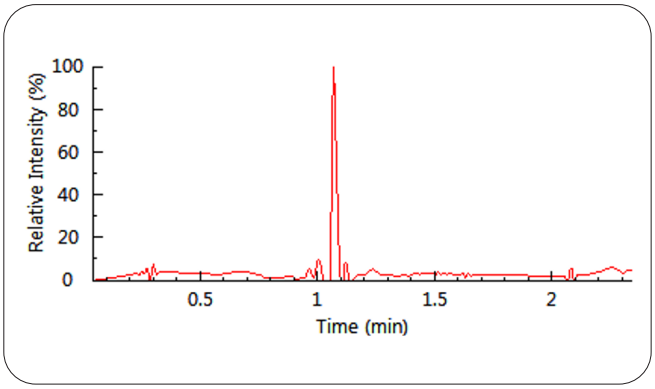

Figure 5: EIC of CORTISOL in DBS Matrix.

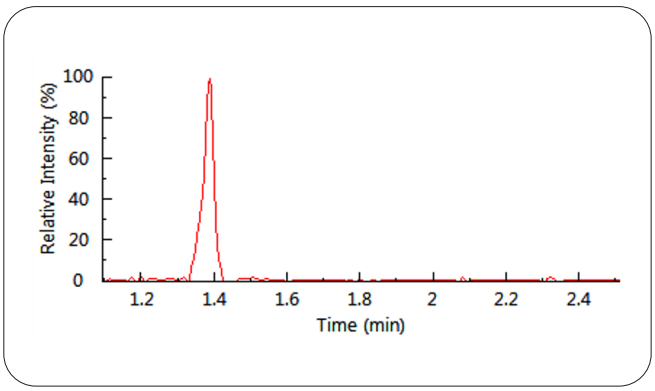

Figure 6: EIC of 21-DECOL in DBS Matrix.

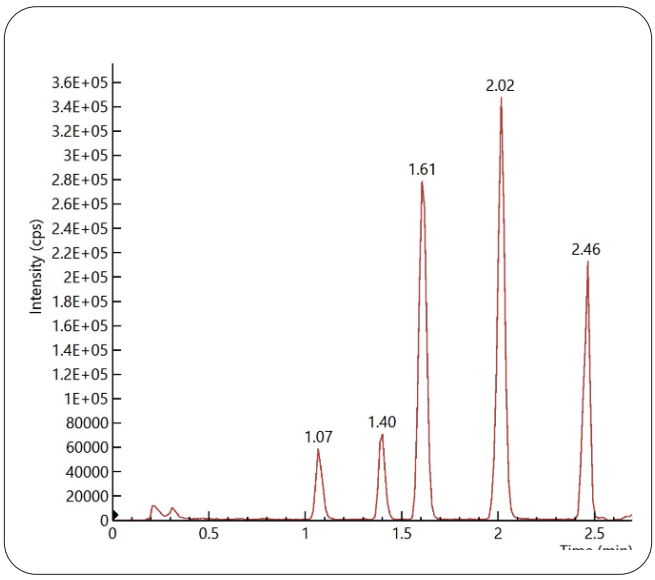

Figure 7. Steroid profiling by MS/MS in DBS matrix.

Figure 8 shows a representative DBS profiling for a confirmed 21-H deficiency (red TIC trace) and a DBS profiling for a healthy newborn (blue TIC trace). Traditionally, the confirmation of both 21-H and 11β-H deficiencies using LC-MS/MS was based on findings of elevated levels of ADIONE and 17OHP, a decreased level of CORT and an elevation in the ratio (ADIONE + 17OHP)/CORT. However, ADIONE is an indirect marker, which is not a potential substrate for 21-H[6]. Furthermore, ADIONE and 17OHP elevations are present in both 21-H and 11β-H deficiencies. In contrast, 21-DECOL and 11-DECOL are specific markers, in fact their concurrent determination allows differentiation between 21-H deficiency and 11β-H deficiency, improving the specificity of the assay.[4] In the example shown here, an increase in 21-DECOL highlights a case of 21-H deficiency.

The steroid panel evaluated contains a pair of structural isomers: 21-DECOL and 11-DECOL, which have identical precursor ions and similar mass spectrometric fragmentation patterns. Despite similarities in fragmentation, two dominant product ions for 21-DECOL were observed at m/z 121,0 and 311,2 whilst dominant product ions at m/z 97,0 and 109,0 were found for 11-DECOL. In biological samples, another endogenous steroid with the same nominal molecular mass, corticosterone, is also present in addition to these two isobaric species mentioned. The reported method chromatographically resolves the three isobaric compounds: 21-DECOL, 11-DECOL and corticosterone (Figure 9). Thus, this method allows prompt confirmation and differentiation between the two main forms of enzymatic deficiencies (21-H and 11β-H).

Linearity

All analytes produced a linear response over the measured concentration range (R^2>0.99). The accuracy was acceptable for bioanalysis (Table 3). Figures 10 and 11 illustrate the linearity of 17-OHP and ADIONE and are representative of results achieved for all the analytes.

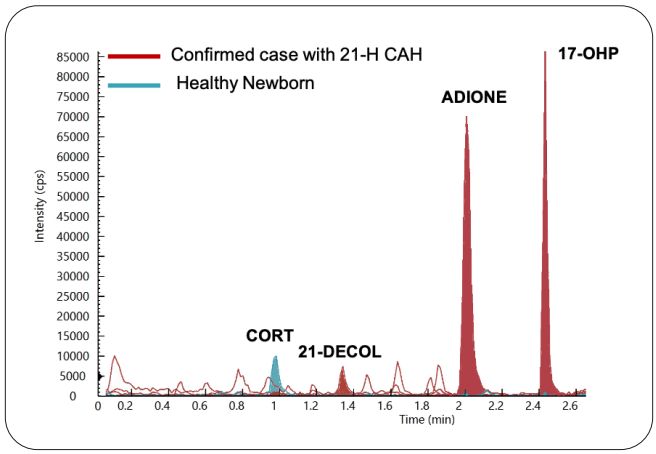

Figure 8: Steroid profiling by MS/MS in blood spots. Blue TIC trace: healthy newborn; red TIC trace: confirmed case with 21-H. (The healthy and positive newborn are both full-term and weigh more than 3200 g).

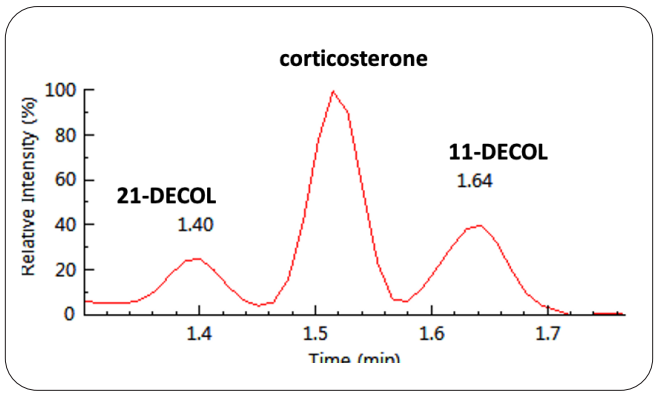

Figure 9: The chromatogram obtained through the final UHPLC-MS/MS shows a good resolution among the three isobaric compounds: 21-DECOL, corticosterone and 11-DECOL.

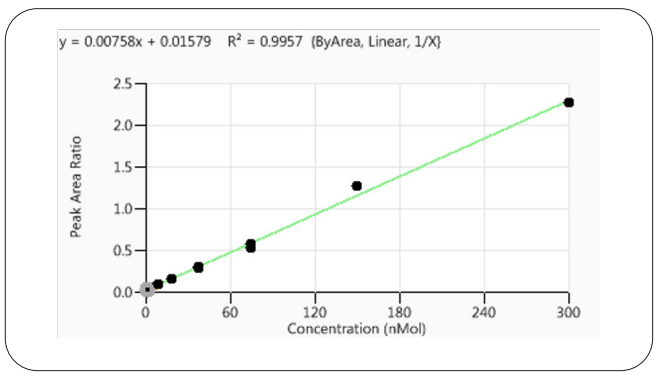

Figure 10: Calibration Curve for 17OHP (331.1/97.0).

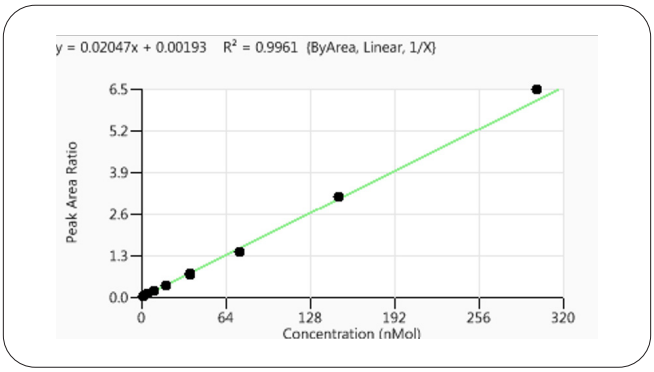

Figure 11: Calibration Curve for ADIONE (287.1/97.0).

Conclusions

The results obtained confirm the applicability of LC-MS/MS method for profiling steroids in DBS samples with analytical sensitivity and specificity on QSight 200 series mass spectrometers. This reported method can be potentially used as a 2nd Tier test to improve the analytical interference arising from cross-reactivity associated with immunoassay measurement. In addition, it allows prompt differentiation between 21-H and 11β-H deficiencies.

References

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