RAPID UPLC-MS/MS DRIED BLOOD SPOT ANALYSIS OF STEROID HORMONES USING THE XEVO TQ-S MICRO FOR CLINICAL RESEARCH

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INTRODUCTION

Dried Blood Spots (DBS) are an established microsampling technique providing a low-cost approach of collecting, shipping and analysing samples for clinical research. Ligand-binding assays (LBAs) are the established frontline testing methodologies for DBS samples in steroid hormone analysis. Although rapid, the relatively low analytical specificity of the LBAs may necessitate follow-up, using liquid chromatography – tandem mass spectrometry (LC-MS/MS). Additionally, analysis of steroid hormones panels provides greater detail about the underlying enzyme activity, compared with single-analyte LBAs, which is important for the assessment of biomarkers in clinical research. The challenge is to create a LC-MS/MS methodology which separates interferences, whilst maximizing throughput. Ultra Performance Liquid Chromatography (UPLC™) on the Waters™ ACQUITY UPLC™ combined with CORTECS™ 2.7µm particle columns provides UPLC separations at high linear velocities with minimal loss in column performance.

METHODS

Materials

- Certified androstenedione, 17-hydroxyprogesterone (17-OHP), cortisol, 11-deoxycortisol and 21-deoxycortisol reference material purchased from Merck (Poole, UK) was used to prepare calibrators and QC material in stripped serum from Golden West Biologicals (CA, USA) and then mixed 50/50 (v/v) with red blood cells from BioIVT (West Sussex, UK).
- Dried Blood Spots (DBS) were prepared by aliquoting 75µL of sample onto Whatman 903 Protein Saver cards from Merck (Poole, UK).
- Total precision was determined by extracting and quantifying five replicates of tri-level QC material on one occasion per day over five consecutive days (n=25). Repeatability was determined by analysing five replicates at each QC level.

Methods

- 2 x 3mm DBS samples were added to a 96 well plate, followed by addition of internal standard (90% methanol) and shaken for 5 minutes. Water was added and the plate was shaken for 1 minute prior to SPE.
- Sample supernatant was loaded onto a Waters Oasis™ MAX µElution plate and washed with 1% ammonia in 10% acetonitrile, Analytes were eluted using 70%$_{(aq)}$ acetonitrile. Water as added prior to injection.
- Sample preparation was automated using the Tecan Freedom Evo 100 Liquid Handler.
- Using a Waters ACQUITY UPLC I-Class System, samples were injected onto a 2.1 x 50mm Waters CORTECS C18 2.7µm column with a pre-column CORTECS C18 VanGuard, using a methanol and 0.05mM ammonium fluoride gradient and analyzed with a Waters Xevo™ TQ-S micro detector (Figure 1) in positive ESI, using Multiple Reaction Monitoring (Table 1).
- The analysis time per sample was approximately 2.3 minute injection to injection.

Figure 1. The ACQUITY UPLC I-Class with Xevo TQ-S micro

Analyte	MRM Transition (m/z)	Cone Voltage (kv)	Collision Energy (eV)
17-OHP	331.3 > 97.1 (109.1)	50	20
17-OHP-$^{13}C_3$	334.3 > 100.1	50	20
Androstenedione	287.3 > 97.1 (109.1)	50	20
Androstenedione-$^{13}C_3$	290.3 > 100.1	50	20
Cortisol	363.3 > 121.1 (91.1)	50	22 (50)
Cortisol-$^{13}C_3$	366.3 > 124.1	50	22
11-Deoxycortisol	347.3 > 97.1 (109.1)	50	22
11-Deoxycortisol-$^{13}C_3$	350.3 > 100.1	50	22
21-Deoxycortisol	347.3 > 121.1 (311.2)	50	22 (14)
21-Deoxycortisol-$^{2}H_4$	351.3 > 100.1	50	22

Table 1. MRM parameters for the analysis of androstenedione, 17-OHP, cortisol, 11-deoxycortisol, 21-deoxycortisol and their internal standards (Qualifier ions in parentheses)

RESULTS

Chromatography

- The CORTECS C_{18} 2.7µm column provides baseline resolution between androstenedione, 17-OHP, cortisol, 11-deoxycortisol and 21-deoxycortisol (Figure 2).
- Figure 2 also demonstrates the baseline resolution of structurally similar steroid hormones.

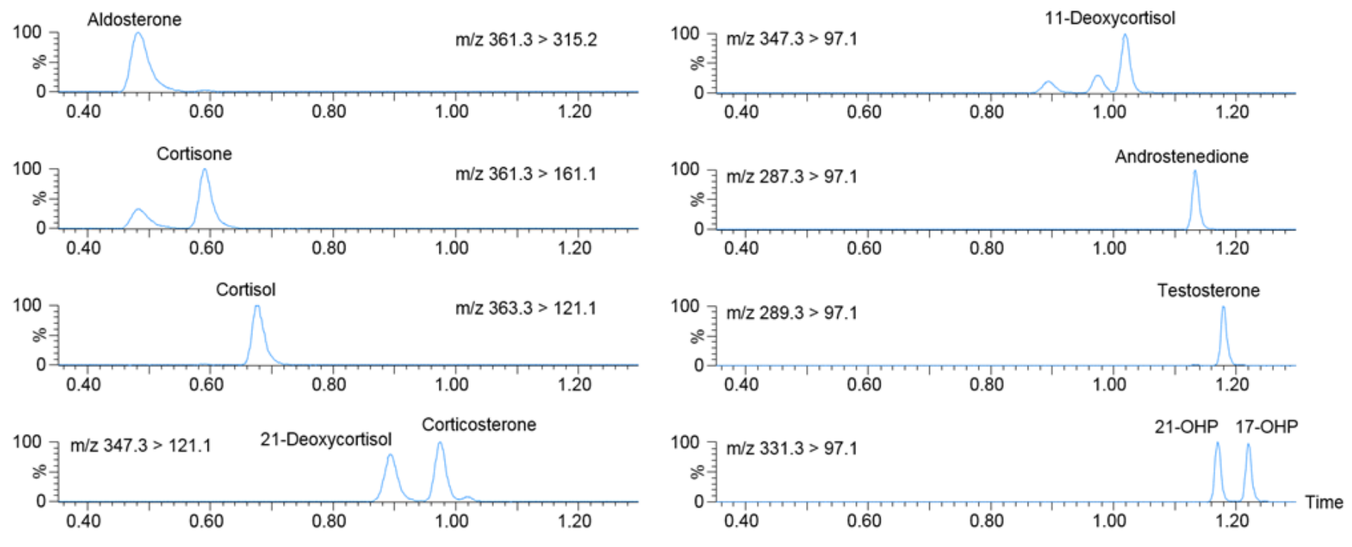

Figure 2. Separation using the CORTECS C_{18} 2.7µm column demonstrating baseline resolution of the steroid hormone isobars

Workflow

- A rapid run time (2.3 minutes injection to injection) and automated SPE provides a high throughput of samples.
- Figure 3 illustrates the workflow for the analysis of 4 plates (384 samples) of DBS samples from preparation to result, which can be performed within 18 hours.

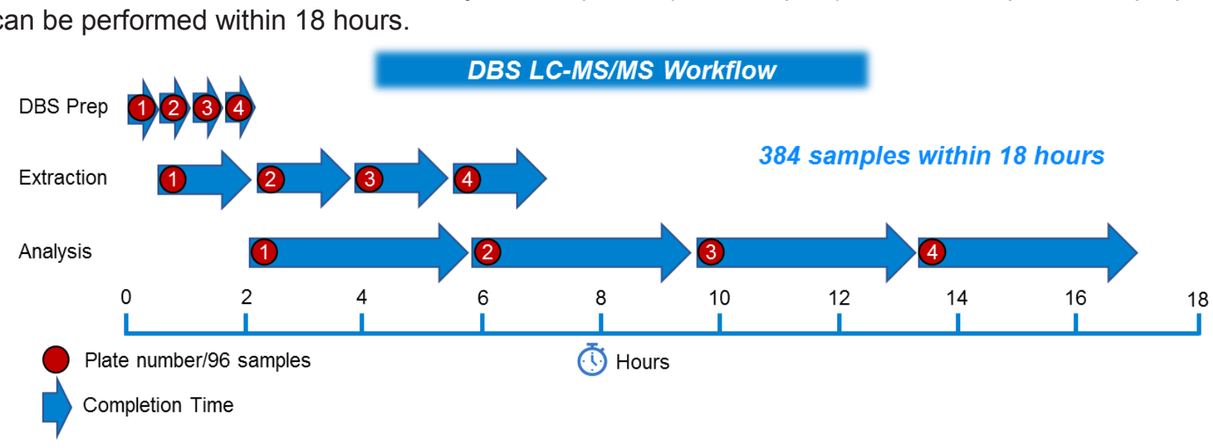

Figure 3. The DBS LC-MS/MS workflow for the analysis of steroid hormones in 384 samples

'RAPID SEPARATION OF STEROID HORMONES, COUPLED TO OFFLINE AUTOMATED SPE, ENABLES ANALYTICALLY SENSITIVE AND SELECTIVE ANALYSIS OF 4 PLATES OF DBS SAMPLES WITHIN 18 HOURS'

Calibration and Analytical Sensitivity

- The calibration lines were linear across the calibration range of 0.5 – 500 ng/mL for androstenedione and 11-deoxycortisol, and 1.0 – 500 ng/mL for 17-OHP, cortisol and 21-deoxycortisol, with correlation coefficients (r^2) >0.995.
- The analytical sensitivity of the method for the method was calculated using S/N (PtP), where S/N was >10. The LLOQ for this method was 0.5 ng/mL for androstenedione and 11-deoxycortisol, and 1.0 ng/mL for 17-OHP, cortisol and 21-deoxycortisol.

Precision

- In-house QC concentrations were 2, 5, 50 and 400 ng/mL for androstenedione, 17-OHP, cortisol, 11-deoxycortisol and 21-deoxycortisol.
- Total precision and repeatability was ≤9.3% for the steroid hormones (Figure 5).
- Accuracy of the QCs to the nominal steroid hormone concentrations ranged from 94.2% - 110.1%.

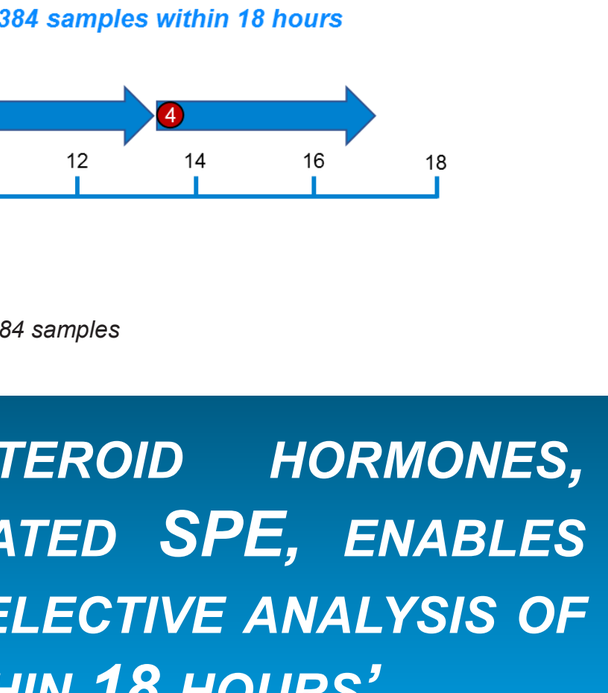

Figure 4. Chromatograms demonstrating the analytical sensitivity for the analysis of androstenedione, 17-OHP, cortisol, 11-deoxycortisol and 21-deoxycortisol

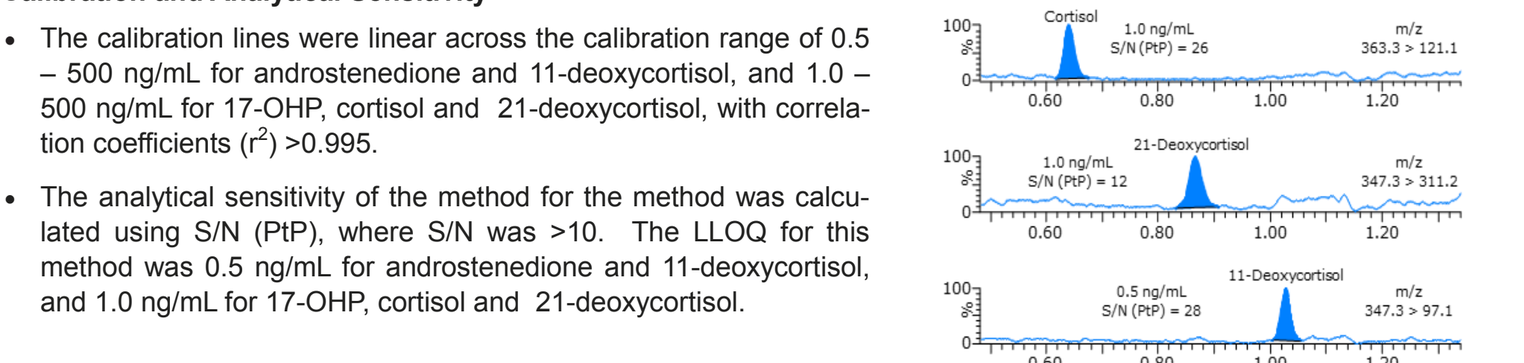

Figure 5. Total precision and repeatability for the analysis of in-house QC samples over five occasions (n=25) for androstenedione, 17-OHP, cortisol, 11-deoxycortisol and 21-deoxycortisol

CONCLUSION

- **A clinical research method to quantify androstenedione, 17-OHP, cortisol, 11-deoxycortisol and 21-deoxycortisol in DBS samples has been developed.**
- **The use of CORTECS 2.7µm columns coupled to offline automated Oasis MAX µElution sample preparation, provides a high throughput, analytically sensitive and selective method for analysing steroid hormones in DBS.**
- **The method demonstrates excellent linearity, precision and accuracy for all the steroid hormones**

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