

## Quantitative Analysis of Dried Bloodspot 17-Hydroxyprogesterone by ACQUITY UPLC-MS/MS for Clinical Research

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Heather A. Brown, Claudia Rossi, Lisa J. Calton

Waters Corporation, Department of Biomedical Science, G. d'Annunzio University Foundation



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## Abstract

In this application note a method for the extraction and UPLC-MS/MS analysis of DBS 17-OHP using the ACQUITY UPLC System with the Xevo TQ MS Detector is described.

### Benefits

- Single non-derivatized liquid-liquid extraction
- Analytical selectivity for 17-hydroxyprogesterone
- Rapid result with 3.5 minute injection-to-injection time

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## Introduction

Measurement of 17-hydroxyprogesterone (17-OHP) by immunoassay is prone to analytical interference arising from cross-reactivity of reagent antibodies with structurally-related steroid metabolites.<sup>1</sup> The dried bloodspot (DBS) has proved popularity as a sample matrix in the pharmaceutical, life sciences and clinical research arena due to simplicity of sample collection and stability of compounds within this matrix. A method for the extraction and UPLC-MS/MS analysis of DBS 17-OHP using the ACQUITY UPLC System with the Xevo TQ MS Detector (Figure 1) is described. The technique features an extended LC gradient to allow qualitative evaluation of the androstenedione (A4) and cortisol chromatographic peaks.



Figure 1. ACQUITY UPLC and Xevo TQ MS Detector.

## Experimental

### Sample description

Two x 3 mm DBS were agitated for 50 minutes in 200  $\mu$ L 50 : 50 acetoneacetonitrile plus 20  $\mu$ L internal standard (95 nmol/L [ $^2\text{H}_8$ ]-17-OHP in 50 : 50 methanol : water). Extract was transferred to a Waters Maximum Recovery vial, evaporated to dryness and reconstituted in 50  $\mu$ L of 55 : 45 mixture of mobile phases A and B.

### Method conditions

## UPLC conditions

|                            |                                                                                                                                                     |
|----------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------|
| System:                    | ACQUITY UPLC System                                                                                                                                 |
| Sample preparation plates: | V bottom polypropylene 96-well microtitre plate for extraction eg Nunc Microwell 96-well plate                                                      |
| Sample preparation vials:  | TruView LCMS Certified Maximum Recovery Vial (p/n 186005662CV)                                                                                      |
| Column:                    | ACQUITY UPLC HSS T3 1.8 $\mu$ m, 2.1 x 50 mm (p/n 186003538) fitted with ACQUITY HSS T3 VanGuard Pre-column 1.8 $\mu$ m, 2.1 x 5 mm (p/n 186003976) |
| Column temp.:              | 60 °C                                                                                                                                               |
| Sample temp.:              | 8 °C                                                                                                                                                |
| Injection volume:          | 20 $\mu$ L (PLNO, load ahead enabled)                                                                                                               |
| Weak wash:                 | 45 % Methanol (aq) 1500 $\mu$ L                                                                                                                     |
| Strong wash:               | Equal parts water, methanol, acetonitrile and isopropanol 500 $\mu$ L                                                                               |
| Flow rate:                 | 0.6 mL/min                                                                                                                                          |
| Mobile phase A:            | 2 mmol/L ammonium acetate, 0.1 % (v/v) formic acid (aq)                                                                                             |
| Mobile phase B:            | 2 mmol/L ammonium acetate, 0.1% (v/v) formic acid in methanol                                                                                       |

## UPLC conditions

|           |                                                                                                                                                                                                    |
|-----------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Gradient: | Binary system: initially 45% mobile phase B increasing linearly to 85% B over 2 min, to 98% B over 0.1 min, holding for 0.4 min before stepping down to 45% B with 1.0 min column re-equilibration |
|-----------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|

## MS conditions

|                      |                                                                        |
|----------------------|------------------------------------------------------------------------|
| System:              | Xevo TQ MS Detector Tuned to unit resolution on MS1 and MS2 (0.7 FWHM) |
| Detection mode:      | Electrospray positive ionization mode                                  |
| Acquisition mode:    | Multiple Reaction Monitoring (see Table 1 for ion transitions)         |
| Capillary voltage:   | 0.7 kV                                                                 |
| Collision energy:    | analyte specific (see Table 1)                                         |
| Cone voltage:        | analyte specific (see Table 1)                                         |
| Source temp.:        | 120 °C                                                                 |
| Desolvation temp.:   | 500 °C                                                                 |
| Inter-channel delay: | 0.01 sec                                                               |
| Inter-scan delay:    | 0.02 sec                                                               |

| Analyte                            | Precursor ion<br>( <i>m/z</i> ) | Product ion<br>( <i>m/z</i> ) | Cone<br>voltage | Collision<br>energy | Dwell time<br>(sec) | MS function<br>(time window min) |
|------------------------------------|---------------------------------|-------------------------------|-----------------|---------------------|---------------------|----------------------------------|
| Cortisol                           | 363                             | 121                           | 26              | 23                  | 0.027               | 2 (0-1.2)                        |
| Androstenedione                    | 287                             | 97                            | 30              | 20                  | 0.018               | 1 (1.4-2.5)                      |
| 17-OHP (quantifier)                | 331                             | 97                            | 28              | 26                  | 0.018               | 1 (1.4-2.5)                      |
| 17-OHP (qualifier)                 | 331                             | 109                           | 28              | 28                  | 0.018               | 1 (1.4-2.5)                      |
| <sup>2</sup> H <sub>8</sub> 17-OHP | 339                             | 100                           | 28              | 26                  | 0.018               | 1 (1.4-2.5)                      |

Table 1. MS Parameters. Optimize precursor and product ions to 1 decimal place.

## Data management

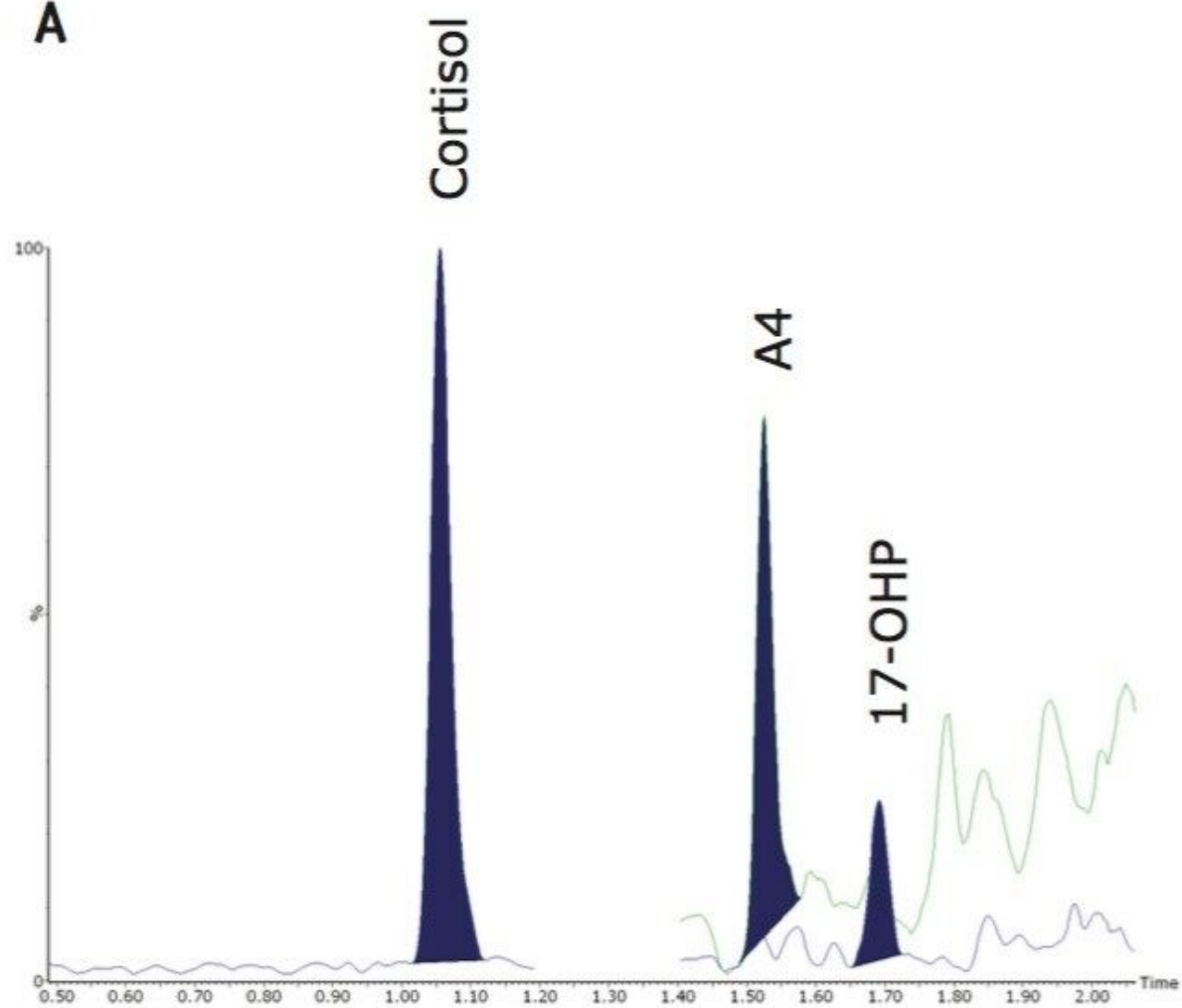
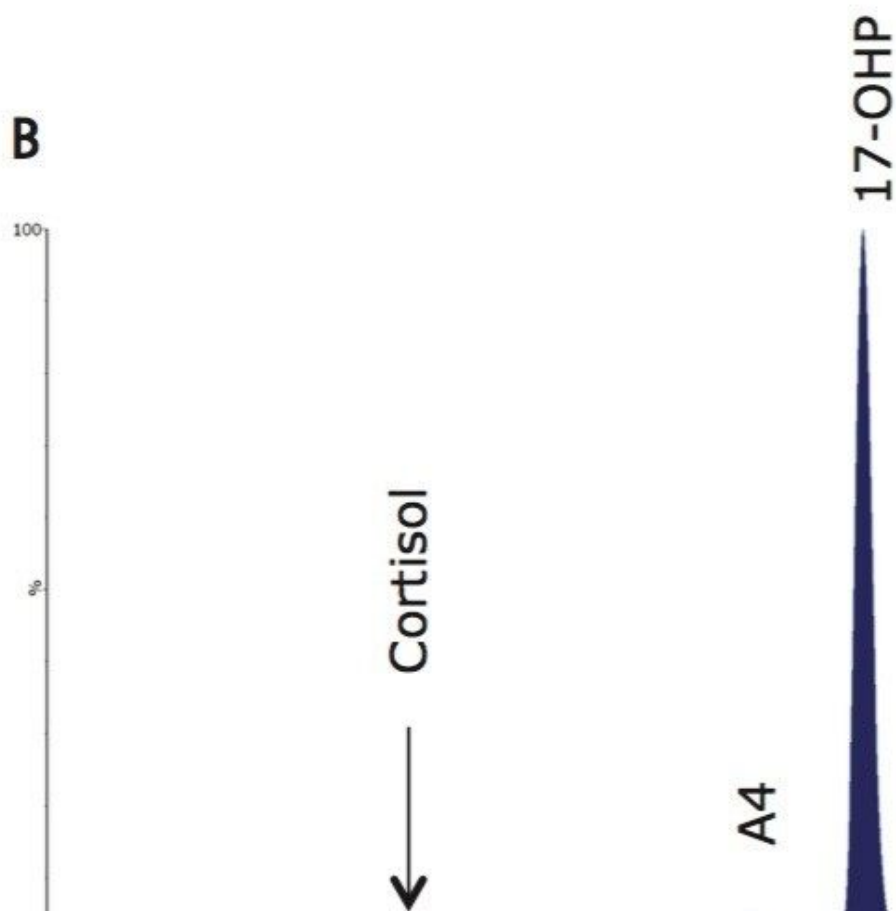
MassLynx v 4.1 incorporating TargetLynx application manager

A function containing one quantifier and internal standard ion transition for both androstenedione and cortisol were added for qualitative evaluation of these compounds, with no adverse effect on 17-OHP detection. MS Function time windows were optimized for instrument duty cycle. Verify chromatogram peak retention time with a single-function MS Method prior to setting time window settings for routine use.

## Results and Discussion

Preparation of 9-point in-house DBS calibration series prepared from saline-washed red blood cells resuspended in spiked stripped-serum enabled linear quantification of 17-OHP between 9.9 – 1270.0 nmol/L with coefficient of determination  $r^2 > 0.997$  and measurements  $\leq 10$  % deviation from nominal calibrator values.

Analytical sensitivity was determined from the peak-to-peak signal to noise ratio (SNR) observed in the 17-OHP chromatogram of 6 reference DBS samples with the lowest SNR (mean 0.5 nmol/L 17-OHP, range 0.5 – 2.9 nmol/L, SNR 1.6 – 4.7). The LLOQ was calculated as the concentration of 17-OHP extrapolated to give SNR  $> 10$  and was determined to be 1.6 nmol/L. The LOD taken as the extrapolated concentration with SNR  $> 3$  was 0.5 nmol/L. Descriptive statistical analysis was conducted using Analyse-it in Microsoft Excel for Windows. The mean (95 % Confidence Interval) was 2.7 (2.2 – 3.1) nmol/L (n=22). Chromatograms from this population are shown in Figure 2A alongside a chromatogram from a separate reference population containing higher concentrations of 17-OHP shown in Figure 2B.

**A****B**

hydroxy-progesterone radioimmunoassays. *Clin Chem* 1992;38:1830 –1837.

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