The Development of a Method for the Determination of Cortisol and 6-Beta-Hydroxycortisol in Human Urine Using 2-D Chromatography

J. Jeppson, E. Dibbern, C. Kafonek, R. Nachi, R. Islam, and C. Sheldon
Celerion, Lincoln, NE USA

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Overview

- A 2D-UPLC–MS/MS method was developed and qualified for the quantification of COR and 6BH in one method, eliminating the need for two separate methods.
- 2D system with trap and analytical column.
- Less sample preparation time provides faster turn-around time.

Introduction

Cortisol (COR), a glucocorticoid hormone produced in the cortex of the adrenal gland, stimulates glucose formation and copper enzymes and suppresses the production of inflammation-promoting cytokines and collagen. COR is known as the "fight or flight" hormone because it is typically released in response to stress and it curbs anabolic activities while preparing the body for action.

COR is metabolized to 6-β-hydroxycortisol (6BH) by cytochrome P450 3A4 (CYP3A4), the most abundant and versatile member of the cytochrome P450 family of oxidative enzymes. CYP3A4 is located in both the liver and the intestines and is involved in the metabolism of approximately half of all drugs. It is also a key player in the endogenous metabolic pathways for cholesterol, steroid hormones, and eicosanoid synthesis. Although there are multiple P450 enzymes with monooxygenase activity, CYP3A4 also has epoxygenase activity critical to its role in oxidizing unsaturated carbon-carbon bonds.

The ratio of 6BH to COR in urine is sometimes used in drug-drug interaction studies to confirm inhibition (e.g. with itraconazole) and induction (e.g. with rifampin) effects on CYP3A4. The 6BH/COR ratio exhibits minimal inter-day variation compared to the concentrations of COR and 6BH.

A method for the quantitation of COR and 6BH in human urine using 2D UPLC-MS/MS has been developed for pharmacokinetic assessment of samples. Previously, two chromatography methods were used to analyze the two compounds and the extraction was completed manually. The goal of this project was to automate the extraction and decrease the chromatography time.

Methods

Sample preparation

1. 0.100 mL of urine
2. Add 0.0500 mL of internal standard (6BH-d4 and COR-d4)
3. Add 0.1 mL of 20:80 MeOH:H_2O
4. Filter through a 1.2 µm, Millipore 96-well filter plate

Chromatography

1. Columns
   a. Waters, ACQUITY UPLC® HSS T3 Van Guard, 5 x 2.1 mm, 1.8 µm, 100Å (trap column)
   b. Waters, CORTECS® UPLC® Phenyl, 150 x 2.1 mm, 1.6 µm (analytical column)
2. Mobile Phase
   a. Quaternary Pump (trap column)
      10:90 MeOH:H_2O
      100% ACN
   b. Binary Pump (analytical column)
      95:5 ACN:25 mM $HCOONH_4$, pH 4.5 w/ HCOOH
      5:95 ACN:25 mM $HCOONH_4$, pH 4.5 w/ HCOOH

LC-MS/MS

Mass spectrometer: AB Sciex Triple Quad 6500
Source: ESI, Negative mode

Ions monitored:			
COR formate adduct:	407.2	→	331.2
COR-d_2 formate adduct (IS):	411.2	→	335.2
6BH formate adduct:	423.2	→	347.2
6BH-d_4 formate adduct (IS):	427.2	→	206.1

The total injection cycle time was approximately 7 minutes. The 2D aspect of this method did not increase the injection cycle time required to analyze COR and 6BH within the same injection while maintaining resolution from cortisone, aldosterone, and other endogenous steroids. Initial trap time of 1.0 minute was followed by elution on Phenyl column for additional 6.0 minutes. Selectivity from prednisolone, a synthetic therapeutic steroid that is isobaric with cortisol, was also demonstrated.

Results

Table 1. Inter-batch Precision and Accuracy for COR

	LLOQ	QC A	QC B	QC C
	2.00 ng/mL	5.38 ng/mL	30.1 ng/mL	375 ng/mL
Inter-batch Mean	1.94	5.72	29.3	388
Inter-batch % CV	8.5	6.7	7.1	5.9
Inter-batch % Bias	-3.0	6.5	-2.7	3.5
n	18	18	18	18

Table 2. Inter-batch Precision and Accuracy for 6BH

	LLOQ	QC A	QC B	QC C
	30.0 ng/mL	92.0 ng/mL	459 ng/mL	5640 ng/mL
Inter-batch Mean	30.6	86.0	448	6030
Inter-batch % CV	6.2	5.0	4.2	5.2
Inter-batch % Bias	2.0	-6.4	-2.4	6.9
n	18	18	18	18

Table 3. Matrix Effect for COR in Human Urine

		Low Spike				High Spike			
Lot	Mean Basal Level (ng/mL)	Nominal Spike (ng/mL)	Expected Concentration (ng/mL)	Measured Concentration (ng/mL)	% Bias	Nominal Spike (ng/mL)	Expected Concentration (ng/mL)	Measured Concentration (ng/mL)	% Bias
1	18.3	2.00	20.3	20.8	+2.5	375	393	361	-8.2
2	32.4	2.00	34.4	33.1	-3.8	375	407	396	-2.8
3	19.0	2.00	21.0	21.0	+0.0	375	394	398	+1.0
4	5.45	2.00	7.45	7.87	+5.6	375	380	381	+0.1
5	3.94	2.00	5.94	5.22	-12.1	375	379	395	+4.2
6	6.45	2.00	8.45	7.66	-9.3	375	381	393	+3.0
7	14.2	2.00	16.2	16.0	-1.2	375	389	400	+2.8
8	47.0	2.00	49.0	44.9	-8.4	375	422	423	+0.2
9	3.98	2.00	5.98	5.74	-4.0	375	379	383	+1.1
10	6.33	2.00	8.33	8.94	+7.3	375	381	379	-0.6

No significant matrix effect was observed in the lots that were fortified with COR.

Table 4. Matrix Effect for 6BH in Human Urine

		Low Spike				High Spike			
Lot	Mean Basal Level (ng/mL)	Nominal Spike (ng/mL)	Expected Concentration (ng/mL)	Measured Concentration (ng/mL)	% Bias	Nominal Spike (ng/mL)	Expected Concentration (ng/mL)	Measured Concentration (ng/mL)	% Bias
1	300	30.0	330	333	+0.9	5630	5930	6420	+8.3
2	281	30.0	311	289	-7.1	5630	5911	6590	+11.5
3	43.4	30.0	73.4	68.1	-7.2	5630	5673	6400	+12.8
4	67.1	30.0	97.1	94.7	-2.5	5630	5697	5950	+4.4
5	62.9	30.0	92.9	85.8	-7.6	5630	5693	5990	+5.2
6	55.7	30.0	85.7	86.7	+1.2	5630	5686	5770	+1.5
7	162	30.0	192	179	-6.8	5630	5792	5930	+2.4
8	243	30.0	273	268	-1.8	5630	5873	6220	+5.9
9	29.7	30.0	59.7	58.6	-1.8	5630	5660	6330	+11.8
10	92.5	30.0	123	127	+3.7	5630	5723	6570	+14.8

No significant matrix effect was observed in the lots that were fortified with 6BH.

Figure 1. Representative Chromatogram of 2-Period Method for 6BH (1.9 min) and COR (4.9 min) LLOQ

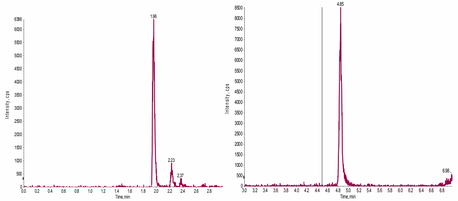


Figure 2. Representative Chromatogram of 2-Period Method for 6BH and COR ULOQ

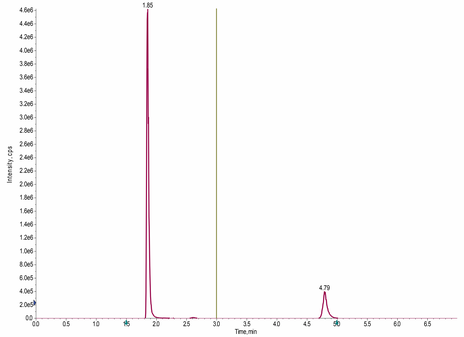


Figure 3. Representative Chromatogram of Separation of Cortisol (5.0 min) and Prednisolone (4.8 min)

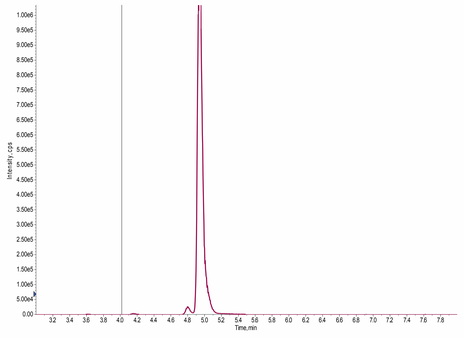
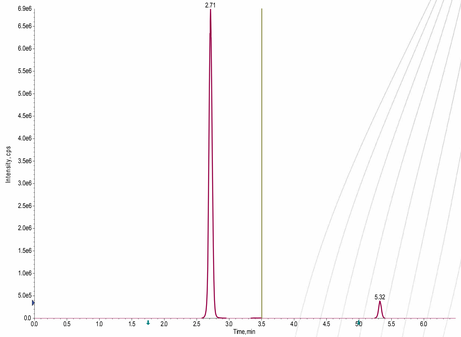


Figure 4. Representative Chromatogram after 96 samples



One trap column used for entire 96-well plate.

Some peak broadening was noted for the cortisol peak in some batches which limited batch length.

Filtration of the diluted samples improved the peak shape, but continued work on cortisol peak broadening will be needed.

Conclusions

A method has been developed that allows for the rapid, accurate, and reproducible quantitation of cortisol and 6-beta-hydroxycortisol in human urine samples. The 2D chromatography greatly reduced sample preparation time and allowed two methods with different standard ranges to quantitate in one method.

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