

Validation of a Sensitive Assay to Quantify the Biomarker Cortisol in Human Saliva Utilizing HPLC with Tandem Mass Spectrometric Detection

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ABSTRACT

Purpose

To develop and validate an LC-MS/MS method for the determination of cortisol in human saliva containing 1.5 mM citric acid. Among other functions, cortisol serves to help the body manage stress and has been termed “the stress hormone” because it is also secreted in higher levels during the body’s response to stress. Therefore, the levels of cortisol can be used as a biomarker and pharmacodynamic indicator to support pharmacokinetic findings. Previous reported methodologies have only focused on the determination of cortisol in urine samples.

Methods

Saliva QC and study samples were back-calculated against cortisol calibration curves prepared in water, and the concentration of the QC samples were corrected for endogenous cortisol levels in saliva. The sample preparation employed a simple dilution methodology prior to sample analysis. HPLC separation was achieved using an aquasil C18 reverse phase column (50 x 3 mm) with a mobile phase mixture of ammonium acetate, water, methanol, and acetonitrile. Cortisol eluted at approximately 4 minutes with a total run time of 6.5 minutes. Analysis was performed on an Applied Biosystems API 4000 tandem mass spectrometer. Negative ESI mass spectra were acquired with MRM mode.

Results

This assay was validated over a linear range of 0.500 to 100 ng/mL using 0.050 mL of saliva. Inter-batch accuracy ranged from 88 to 105%. Inter-batch precision ranged from 5.9 to 16.5%. The intra-batch accuracy and precision ranged from 86 to 114% and 1.3 to 20%, respectively. Cortisol was found to be stable in human saliva containing 1.5 mM citric acid at -20 °C for at least 4 months, on the benchtop for at least 40 hours and after 3 freeze thaw cycles at approximately -20 °C. Dilution integrity, endogenous cortisol levels, and matrix selectivity were also demonstrated.

Conclusions

This analytical method was demonstrated to be accurate, precise, sensitive and rugged for the measurement of cortisol in human saliva containing 1.5 mM citric acid. The method is being used to quantify cortisol in human saliva as a biomarker in ongoing clinical studies.

METHODOLOGY

Instrumentation:

- CTC Analytics HTS PAL Leap Technologies autosampler
- Agilent 1100 series pumping system
- Applied Biosystems API 4000 LC-MS/MS

Experimentation:

Analyte:	Cortisol
Matrix:	Human Saliva Containing 1.5 mM Citric Acid
Method:	LC-MS/MS in negative ESI/MRM mode
Column:	Aquasil C18 Reverse Phase (50 x 3 mm)
Injection Volume:	35 µL
Run Time:	6.5 minutes
Retention Time:	~4 minutes (cortisol and cortisol-d4 IS)
Calibration Range:	0.500 – 100 ng/mL

RESULTS

Figure 1: Representative Calibration Curve for Cortisol in Water

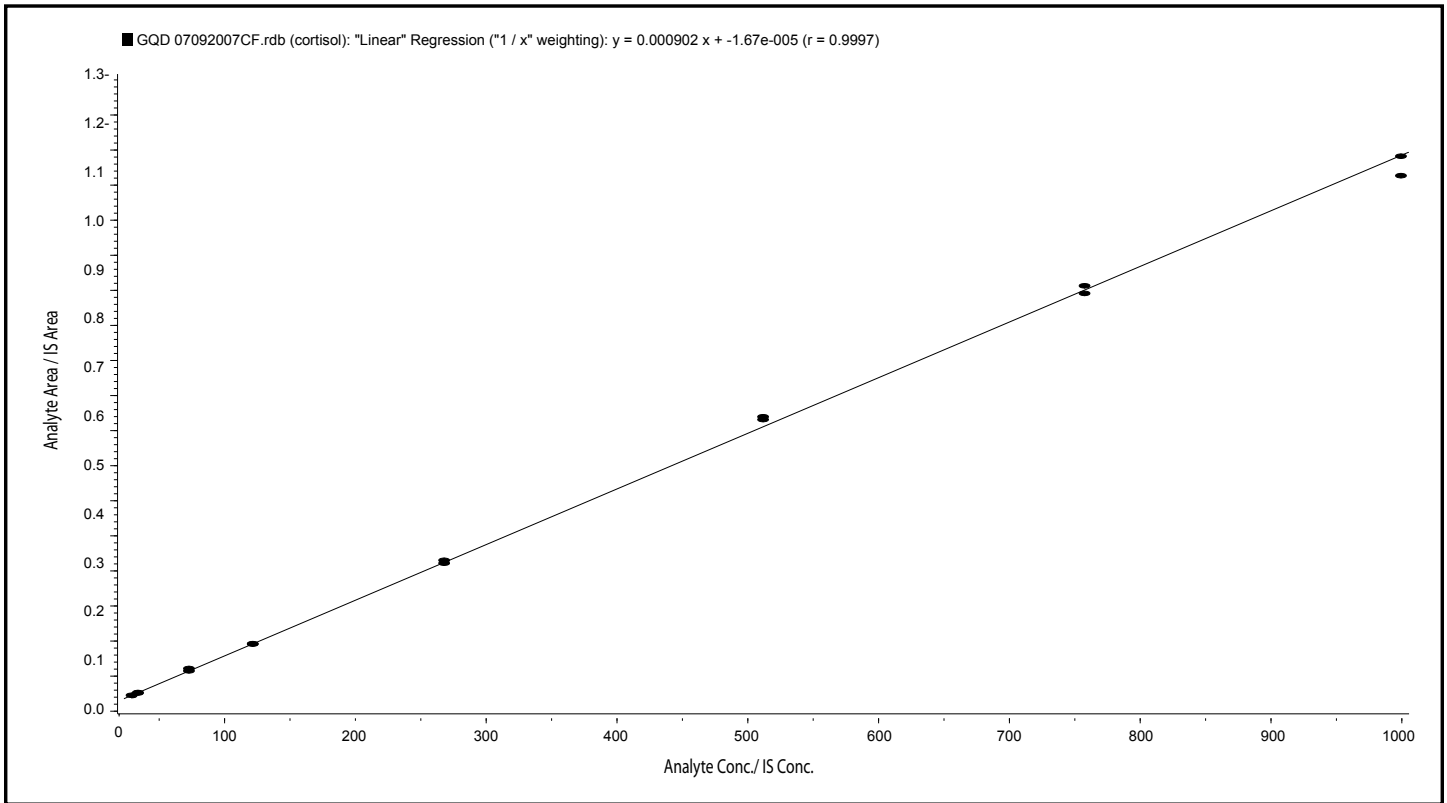


Fig. 1

Figure 2: Representative ESI Chromatograms for a 0.500 ng/mL Cortisol Standard in Human Saliva Containing 1.5 mM Citric Acid

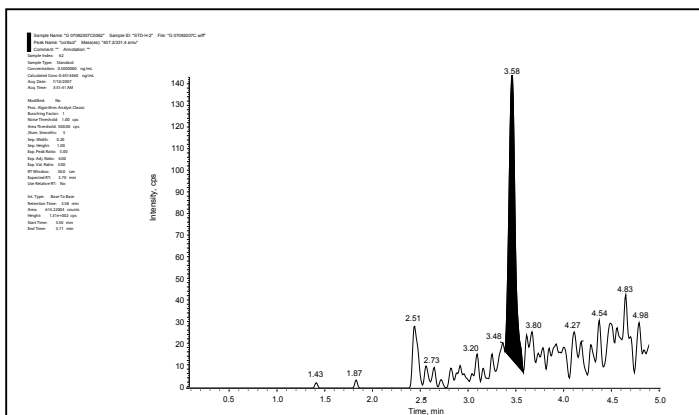


Fig. 2a

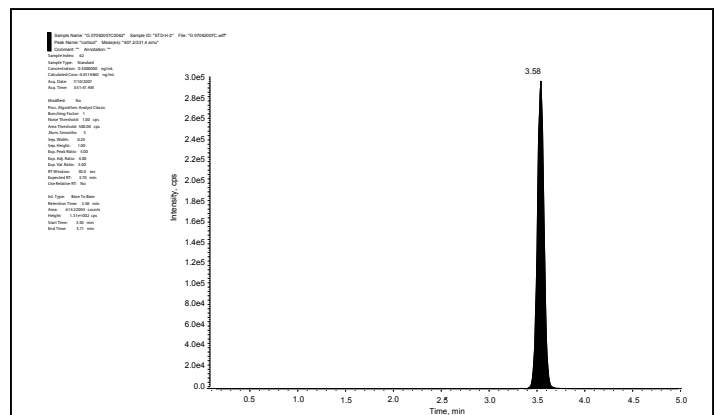


Fig. 2b

Figure 3: Representative ESI Chromatograms for a 100 ng/mL Cortisol Standard in Human Saliva Containing 1.5 mM Citric Acid

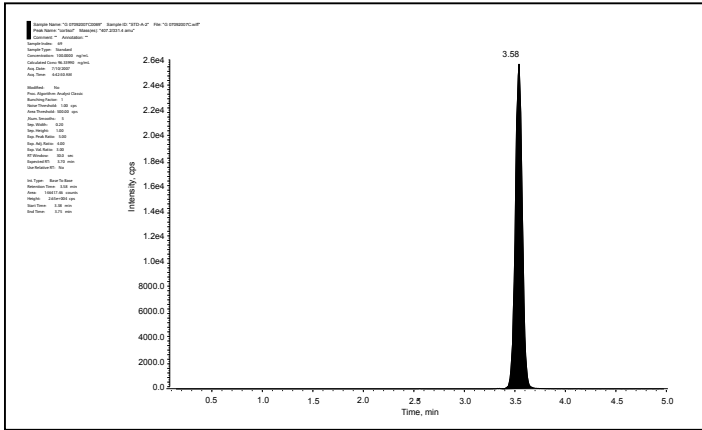


Fig. 3a

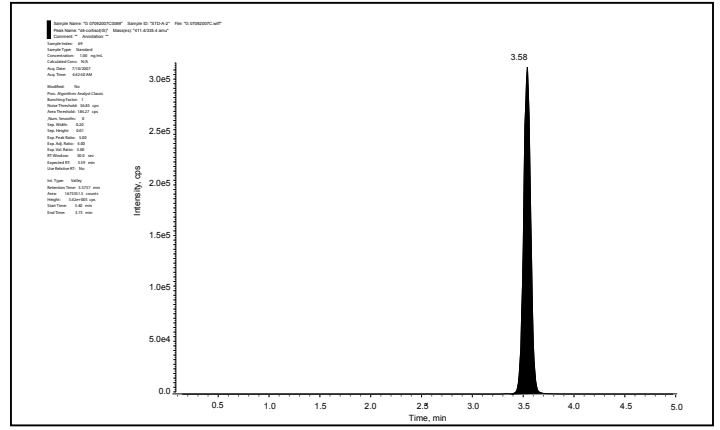


Fig. 3b

Figure 4: Representative ESI Chromatograms for a Zero-Level Cortisol Standard in Human Saliva Containing 1.5 mM Citric Acid

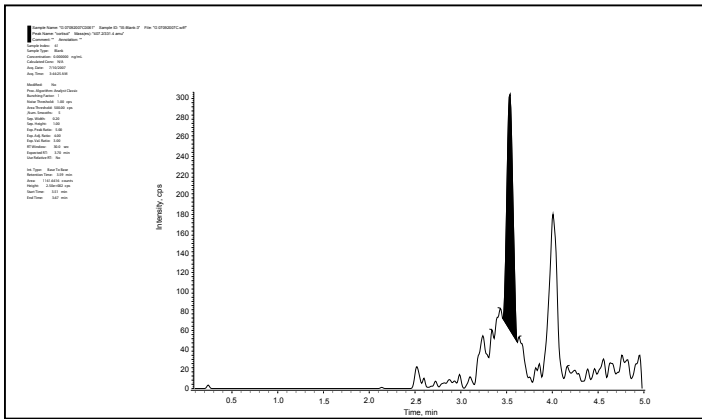


Fig. 4a

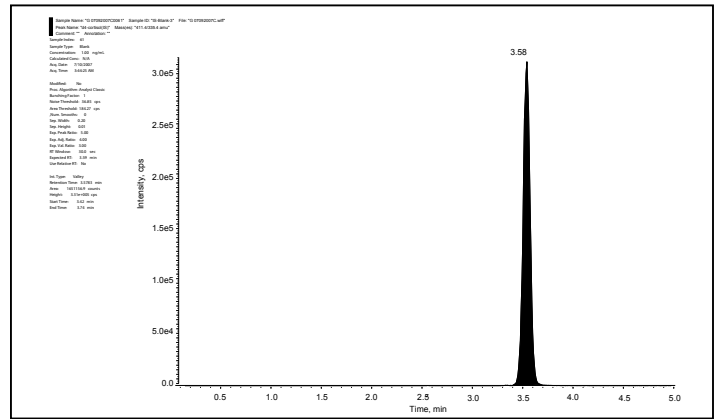


Fig. 4b

Table 1: Back Calculated Concentrations of Cortisol Calibration Standards in Water

Additional Runs		Nominal Concentration of Cortisol														Acceptance Ratio ^a	
LCMS File Name	Replicate	0.500 ng/mL		1.00 ng/mL		5.00 ng/mL		10.0 ng/mL		25.0 ng/mL		50.0 ng/mL		75.2 ng/mL			100 ng/mL
		Calculated Conc.	Acc.	Calculated Conc.	Acc.	Calculated Conc.	Acc.	Calculated Conc.	Acc.	Calculated Conc.	Acc.	Calculated Conc.	Acc.	Calculated Conc.	Acc.	Calculated Conc.	Acc.
GQD 06282007 DF	1	0.497	99%	0.665 ^b	67% ^b	5.35	107%	9.25	93%	24.1	96%	49.2	98%	74.9	100%	101	101%
	2	0.710 ^b	142% ^b	0.918	92%	5.33	107%	10.2	102%	27.9	112%	45.9	92%	74.9	100%	102	102%
	MEAN	0.497	99%	0.918	92%	5.34	107%	9.71	97%	26.0	104%	47.6	95%	74.9	100%	102	102%
n		1		1		2		2		2		2		2		2	
Linear Regression Analysis:		Intercept = -5.5e-005 Slope = 0.000926 Correlation Coeff. = 0.9991 Weighting = 1/x															
KQD 07062007 DR1	1	0.584	117%	1.11	111%	5.19	104%	9.77	98%	24.7	99%	50.1	100%	75.9	101%	100	100%
	2	0.409	82%	0.884	88%	5.14	103%	10.3	103%	24.0	96%	48.8	98%	75.2	100%	101	101%
	MEAN	0.497	99%	0.997	100%	5.16	103%	10.0	100%	24.4	98%	49.5	99%	75.6	101%	101	101%
n		2		2		2		2		2		2		2		2	
Linear Regression Analysis:		Intercept = -3.85e-005 Slope = 0.000891 Correlation Coeff. = 0.9998 Weighting = 1/x															
Additional Runs		Nominal Concentration of Cortisol														Acceptance Ratio ^a	
LCMS File Name	Replicate	0.503 ng/mL		1.01 ng/mL		5.03 ng/mL		10.1 ng/mL		25.2 ng/mL		50.3 ng/mL		75.5 ng/mL			101 ng/mL
		Calculated Conc.	Acc.	Calculated Conc.	Acc.	Calculated Conc.	Acc.	Calculated Conc.	Acc.	Calculated Conc.	Acc.	Calculated Conc.	Acc.	Calculated Conc.	Acc.	Calculated Conc.	Acc.
KQD 10252007 CF	1	0.482	96%	1.02	101%	5.01	100%	10.2	101%	25.6	102%	51.3	102%	73.6	97%	98.5	98%
	2	0.480	95%	1.32 ^b	131% ^b	5.20	103%	9.93	98%	26.3	104%	51.6	103%	74.4	98%	103	102%
	MEAN	0.481	96%	1.02	101%	5.10	101%	10.1	100%	25.9	103%	51.4	102%	74.0	98%	101	100%
n		2		1		2		2		2		2		2		2	
Linear Regression Analysis:		Intercept = 0.000138 Slope = 0.00109 Correlation Coeff. = 0.9997 Weighting = 1/x															

^a Acceptance ratio = Acceptable Calibrators / Total Calibrators.
^b Standard did not meet acceptance criteria and was excluded from regression analysis.

Primary Runs		Nominal Concentration of Cortisol										Acceptance Ratio ^a
LCMS File Name	Replicate	END Blank		QC-L		QC-M		QC-H		Acceptance Ratio ^a		
		Measured Conc. ng/mL	%Acc.	Measured Conc. ng/mL	%Acc.	Measured Conc. ng/mL	%Acc.	Measured Conc. ng/mL	%Acc.			
GQD 07092007 CR1	1	1.05	NA	2.29	92%	41.0	100%	72.7	102%	17/18		
	2	0.901	NA	2.26	91%	43.2	105%	71.1	100%			
	3	1.00	NA	2.15	87%	41.2	101%	71.8	101%			
	4	0.931	NA	2.28	92%	41.0	100%	72.0	101%			
	5	0.984	NA	2.09 ^b	84% ^b	41.8	102%	70.0	99%			
	6	1.03	NA	2.40	97%	38.6	94%	72.0	101%			
	MEAN	0.983	NA	2.24	90%	41.1	100%	71.6	101%			
SD	0.056		0.11		1.5		0.9					
% CV	5.7		4.8		3.6		1.3					
n	6		6		6		6					
Acceptance Ratio ^a	NA		5/6		6/6		6/6					
Primary Runs		Nominal Concentration of Cortisol										Acceptance Ratio ^a
LCMS File Name	Replicate	END Blank		QC-L		QC-M		QC-H		Acceptance Ratio ^a		
		Measured Conc. ng/mL	%Acc.	Measured Conc. ng/mL	%Acc.	Measured Conc. ng/mL	%Acc.	Measured Conc. ng/mL	%Acc.			
KQD 07172007 FR1	1	1.35	NA	2.44	86%	40.4	98%	74.2	104%	16/18		
	2	1.51	NA	2.57	90%	41.2	100%	69.9	98%			
	3	1.41	NA	2.53	89%	41.3	100%	74.4	104%			
	4	2.39 ^c	NA	2.47	86%	41.9	101%	72.2	101%			
	5	1.27	NA	2.37 ^b	83% ^b	41.7	101%	73.3	103%			
	6	1.23	NA	2.32 ^b	81% ^b	40.8	99%	70.2	98%			
	MEAN	1.35	NA	2.45	86%	41.2	100%	72.4	101%			
SD	0.11		0.09		0.5		2.0					
% CV	8.3		3.9		1.3		2.7					
n	5		6		6		6					
Acceptance Ratio ^a	NA		4/6		6/6		6/6					
Primary Runs		Nominal Concentration of Cortisol										Acceptance Ratio ^a
LCMS File Name	Replicate	END Blank		QC-L		QC-M		QC-H		Acceptance Ratio ^a		
		Measured Conc. ng/mL	%Acc.	Measured Conc. ng/mL	%Acc.	Measured Conc. ng/mL	%Acc.	Measured Conc. ng/mL	%Acc.			
KQD 07252007 DR1	1	0.646	NA	2.24	89%	46.6	113%	78.1	110%	12/18		
	2	0.840	NA	1.85 ^b	73% ^b	48.3 ^b	118% ^b	75.6	106%			
	3	1.19	NA	2.00 ^b	79% ^b	46.1	112%	82.6 ^b	116% ^b			
	4	0.915	NA	2.44	97%	45.6	111%	80.6	113%			
	5	1.23	NA	2.80	111%	47.1	115%	84.0 ^b	118% ^b			
	6	1.36	NA	1.91 ^b	75% ^b	46.5	113%	79.6	112%			
	MEAN	1.03	NA	2.21	87%	46.7	114%	80.1	113%			
SD	0.27		0.36		0.9		3.0					
% CV	26.5		16.5		2.0		3.8					
n	6		6		6		6					
Acceptance Ratio ^a	NA		3/6		5/6		4/6					
Overall MEAN	1.11	NA	2.30	88%	43.0	105%	74.7	105%	45/54			
Overall SD	0.23		0.24		2.9		4.4					
Overall % CV	21.0		10.4		6.6		5.9					
n	17		18		18		18					
Acceptance Ratio ^a	NA		12/18		17/18		16/18					

Table 2: Inter- and Intra-Batch Precision and Accuracy of Cortisol QC Samples in Human Saliva Containing 1.5 mM Citric Acid

Specificity

Six lots of blank human saliva containing 1.5 mM citric acid were screened. The endogenous levels of cortisol were not found to affect quantitation of cortisol.

Matrix Effect

Matrix effect tests were performed for the methodology by assaying at least three replicates at low and high cortisol concentrations in six lots of blank human saliva containing 1.5 mM citric acid.

A matrix enhancement was noted at low concentrations. The internal standard response was not affected by the presence of matrix.

Recovery

Recovery was evaluated at three concentrations across the range of the method with an average recovery of 147% upon comparison of saliva extracts versus spiked reagent solutions. Recovery at the low concentrations was impacted by the observed matrix enhancement.

Carryover

No carryover was observed when blank reagent solutions were injected immediately following injections of high standards and/or QC's.

Stability

The following cortisol stability assessments were made:

Freeze/Thaw Cycles:

Stable in human saliva containing 1.5 mM citric acid for at least 3 freeze/thaw cycles when stored at -20 °C in polypropylene tubes.

Autosampler:

Processed human saliva containing 1.5 mM citric acid samples are stable at approximately 4-8 °C for at least 7 days.

Long-term Freezer Storage Stability:

Stable at approximately -20 °C in human saliva containing 1.5 mM citric acid for at least 120 days (4 months).

CONCLUSIONS

- The validated work herein provides a specific and sensitive LC-MS/MS method for the determination of cortisol in human saliva containing 1.5 mM citric acid for pharmacokinetic studies.
- Due to the presence of endogenous levels of cortisol in saliva, prior to conducting study sample analysis, the actual concentration of cortisol in prepared human saliva quality control samples is determined by analysis in three separate batches. The nominal concentration of the quality control samples is a result of the average concentration across these three batches.
- The stability of cortisol was established during freezer storage for at least 120 days (-20 °C), during storage of processed samples (4-8 °C) and for three freeze/thaw cycles (-20 °C).