Determination of Endogenous Cortisol in Human Plasma Using LC-MS/MS Techniques with Combined Calibration Curve and Standard Addition Methods

Yansheng Liu, Yu-Hui Fu, David Winburn, Rodney Boughner, Gene Ray–KCAS, Shawnee, Kansas;
Stephen Wanaski–Marathon Pharmaceuticals, Northbrook, IL; Daniel Selness–Spaulding Clinical Research, West Bend, WI

INTRODUCTION

Cortisol is a glucocorticoid, released by the adrenal gland in the response to stress and low blood sugar. It is challenging to analyze endogenous compounds such as cortisol using true matrix in preparation of QC samples as required by regulation guidance. Techniques using pre-treatment of plasma or stable isotope labeled drug as the surrogate analyte have been reported. In this work we report a validated bioanalytical method for determination of cortisol in human plasma using spiked cortisol in untreated plasma for preparation of QCs and in diluted plasma for preparation of standards with combined calibration curve and standard addition methods. This work represents a continuous effort in our lab for analysis of endogenous compounds using true reference material and true matrix.

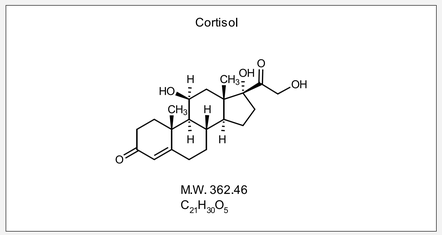

Figure 1. Chemical Structure of Cortisol

METHOD

In our methodology, the screened human plasma lots consisting of the low endogenous levels of cortisol were pooled and used for preparation of QC samples. Other low levels of endogenous cortisol were pooled and diluted with buffer (1:1 ratio) for preparation of standards. The endogenous concentration of cortisol in the pooled plasma was measured using standard addition method and then was added to the spiked concentration for determining the nominal value of each standard and each QC sample. The spiked levels were 0 to 500 ng/mL. Following a sample cleanup procedure, the sample was analyzed with LC-MS/MS techniques. The concentrations of cortisol in QC and clinical samples are determined only by calibration curve method.

Liquid Chromatography

HPLC : Shimadzu 10 ADvp
Autosampler : Perkin Elmer 200
Column : Pursuit C18 (50 x 2.0 mm) 5µ
MP A : 0.1% Formic Acid in DI H_2O
MP B : 0.1% Formic Acid in Methanol

Mass Spectrometry

AB Sciex API 3000 with Turbo-ion Spray interface
Polarity : Positive
Scan type : Multiple Reaction Monitoring
Cortisol: m/z 363 → 121
Cortisol-d_4: m/z 367 → 121 (IS)

RESULTS

Figures 2 and 3 show typical chromatograms for pooled plasma blank consisting of endogenous cortisol at 53.3 ng/mL and the blank that was spiked with100 ng/mL of cortisol. The endogenous concentration of cortisol was initially determined using standard addition method based on equation 1. The potential contamination and carryover effect were controlled during analytical process by using buffer blank.

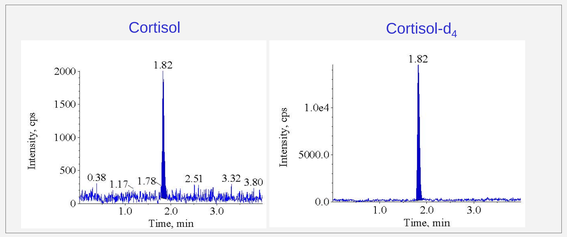

Figure 2. Chromatogram Observed for Plasma Blank (Endogenous Level at 53.3 ng/mL)

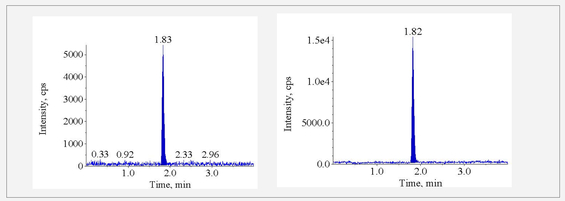

Figure 3. Chromatogram Observed for QCs Spiked with 100 ng/mL of Cortisol (Nominal Value at 153 ng/mL).

$$C_{end} = \frac{R_{end} \times C_s}{R_{tot} - R_{end}}$$

C_{end} = Average concentration of endogenous cortisol
R_{end} = Average peak area ratio for endogenous cortisol
R_{tot} = Average peak area ratio for total cortisol (C_s + C_{end})
C_s = Spiked concentration (ng/mL)

Equation 1. Standard Addition Method

The method was fully validated over a range of **40.4 to 540 ng/mL** using a weighted linear regression ($1/x^2$). A typical calibration curve in validation is shown in **Figure 4**.

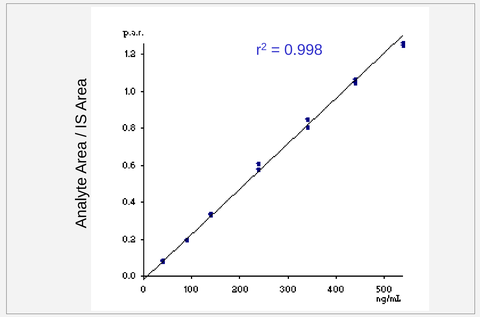

Figure 4. A Typical Calibration Curve for Cortisol

The inter- and intra-batch results for QC samples are shown in **Table 1**. All statistical data are based on the data obtained using calibration curve method. The matrix effect was evaluated for Cortisol in six different lots of blank plasma at varied endogenous concentrations and spiked concentration of 100 ng/mL (**Table 2**).

Batch		40.4* (LLOQ)	53.3* (End)	103 (Low)	153 (Mid)	428 (High)
1	**Mean**	43.3	52.4	105	151	403
	% CV	7.7	6.2	2.5	7.1	2.8
	% Bias	7.1	-1.8	2.3	-1.3	-5.9
2	**Mean**	44.8	53.4	97.9	146	397
	% CV	6.6	5.7	4.3	4.0	1.8
	% Bias	10.9	0.2	-5.0	-4.8	-7.3
3	**Mean**	42.2	51.8	99.8	149	410
	% CV	7.9	10.1	7.7	4.9	2.1
	% Bias	4.4	-2.9	-3.1	-2.8	-4.2
Overall	**Mean**	43.4	52.5	101	148	403
	% CV	7.4	7.2	5.9	5.4	2.5
	% Bias	7.5	-1.5	-1.9	-3.0	-5.8

Table 1. Precision and Accuracy Results for Cortisol Quality Control Samples (n = 6 replicates for each QC per batch).
* Endogenous concentrations measured by standard addition method.

Plasma lot	**C_{end}** (ng/mL)	**C_s** (ng/mL)	Avg IS Normalized MF
Lot 1	73.6	100	0.9093
Lot 2	34.2	100	1.0465
Lot 3	47.7	100	0.9553
Lot 4	65.7	100	0.9548
Lot 5	23.5	100	1.0141
Lot 6	53.9	100	0.9823
mean			**0.9770**
n (Lots)			**6**
SD			**0.04854**
% CV			**5.0**

Table 2. Matrix Effect for Cortisol in Human Plasma

The effect of hemolytic and lipemic plasma matrices was evaluated at the presence of 100 ng/mL of cortisol spiked above the endogenous levels and no significant effect was observed for the assay (data not shown). Cortisol stabilities were also evaluated and passed the criteria for validation including stabilities in whole blood and plasma matrices.

This method was subsequently used for an open-label, single dose, randomized cross-over pharmacodynamic study to evaluate the cortisol response in healthy volunteers after administration of two different doses of cosyntropin injectable suspension. Incurred sample reanalysis (ISR) from this clinical study indicated that 139 over a total of 140 ISR samples passed the criteria well. The data from a single subject are presented in **Figure 5** with 12 pharmacokinetic sampling time points over 24 hours for each period.

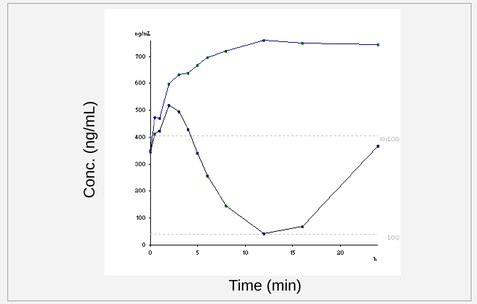

Figure 5. Cortisol Concentrations measured for plasma samples obtained from single subject, but two treatments.

CONCLUSION

The standards were prepared in diluted plasma (1 to 1 ratio) that could be used to mimic and track the behavior of authentic plasma matrix successfully. This bioanalytical method showed acceptable accuracy, precision, selectivity, reproducibility, and sufficient stabilities. The validated bioanalytical method and the established methodology by KCAS have been demonstrated to be simple and reliable in quantitation of endogenous compound in clinical samples using authentic analyte and matrix in preparation of QC samples.

ACKNOWLEDGEMENTS

The authors would like to thank each of organizations that made this research possible. We would also like to acknowledge many other KCAS scientists who have contributed to work.