APPLICATION NOTE

QSight® 200 Series Mass Spectrometer

Measurement of Cortisol & Cortisone in Urine

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Introduction

Steroid hormones are a fat-soluble compound that are derived from cholesterol. These compounds help to control the metabolism of inflammatory response, immune function, salt and water balance and development of sexual characteristics among others[1].

Particularly, glucocorticoids are a class of steroid hormones that bind to the glucocorticoid receptor and contribute to the hypothalamic-adrenal-pituitary feedback system. They are part of the feedback mechanism in the immune system that down regulate immune response[1].

Cortisol is a steroid hormone synthesized in the adrenal glands, it is the main glucocorticoid in humans and acts as a gene transcription factor influencing a variety of cellular responses in many tissues. Cortisol plays a key role in the metabolism of carbohydrates, in the maintenance of vascular tone, regulates the immune response and the body's response to stress. Its production is under the control of the pituitary gland hypothalamus. Cortisol exists in the urine in free (unconjugated) and conjugated (e.g., glucuronide-conjugated and sulfate-conjugated) forms. Approximately 1–2% of protein-unbound circulating cortisol is excreted in the urine; therefore, the level of cortisol in the urine reflects the level of the protein-unbound (biologically active or free) form of cortisol in the plasma[2].

Whereas Cortisone, cortisol metabolite, is the inactive form of cortisol. The enzyme 11β-Hydroxysteroid dehydrogenase catalyzes the interconversion of physiologically active 11β-hydroxyl glucocorticoids as cortisol and inactivate 11-keto glucocorticoids such as Cortisone[3].

These steroids are normally measured with immunoassay techniques. However, these assays suffer from serious disadvantages such as sample matrix effects and lack of specificity resulting from cross-reactivity with structurally related endogenous steroids[2,4]. Therefore, the use of liquid chromatography-tandem mass spectrometry (LC-MS/MS) represents a better alternative for the determination and quantification of these compounds due to the high sensitivity and specificity of the technique[2,4].

Here, an LC-MS/MS method is described that allows the simultaneous detection and quantification of cortisol and cortisone in urine using the QSight® 200 series mass spectrometer coupled with a commercially available kit.

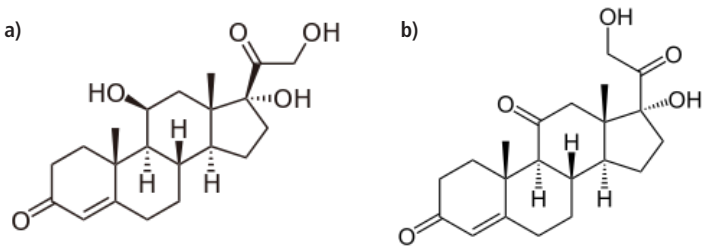

Figure 1- Chemical structures of a) Cortisol and b) Cortisone

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Experimental

Chemicals and Materials

The LC91010 Eureka kit for determination of cortisol and cortisone in urine by LC-MS/MS (Eureka Lab division, Ancona, Italy) consisted of quality control (QC) samples, calibrators, extraction solution, stable isotope labeled internal standard, mobile phase A and B, glass vials and caps, as well as an analytical column.

Sample Preparation

The samples were prepared according to the instructions provided by the kit manufacturer. 50 µL of the urine sample (calibrators or QCs) was mixed with 450 µL of reagent solvent A (Eureka Lab division, Ancona, Italy). The solution was then vortexed for 10 seconds and 10 µL were directly injected into the mass spectrometer. The reagent A solution was previously prepared, and it contains both the extraction solution, and the internal standard (Cortisol-d4).

Mass Spectrometry Conditions

LC-MS/MS analysis was performed using the QSight® 200 series mass spectrometer coupled with the LX-50 UHPLC (both manufactured by PerkinElmer, Waltham, MA, USA) and equipped with an ESI source operating in positive ion mode, using 50 ms dwell times. Table 1 outlines the Mass Spec (MS) ion source parameter settings. The optimized compound dependent parameters for MRMs monitored in this assay are shown in Table 2.

Table 1. *MS ion source settings .*

ESI Voltage (V)	5000
HSID Temp (°C)	275
Nebulizer Gas Setting	250
Drying Gas Setting	110
Source Temp. (°C)	250

Table 2. *Compound dependent parameters for MRMs monitored (EV = entrance voltage, CC = collision energy, CCL2 = Collision cell lens 2).*

Compound	Type	Q1	Q2	CCL2	CC	EV
Cortisol	Quan	363.1	121.0	-75	-36	30
	Qual	363.1	97.0	-80	-60	30
Cortisone	Quan	361.1	163.1	-71	-35	14
	Qual	361.1	91.0	-113	-90	14
Cortisol-d4(IS)	Quan	367.1	121.0	-75	-36	30
	Qual	367.1	97.0	-80	-60	30

LC Conditions

LC separation was performed using a reverse phase C18 (50 x 2.1mm, 1.8 um) column, while maintaining the column oven at a temperature of 60°C. The chromatographic separation was achieved with a 5 minute gradient (Table 3) using the autosampler settings reported below (Table 4). Mobile phase A and B, in addition to the column were included in the kit mentioned above.

Table 3. *LC gradient used to separate Cortisol and Cortisone*

Time	Flow Rate (µL/min)	%A
0	400	60
1	400	60
2.5	400	5
3.5	400	5
3.51	400	60
5.0	400	60

Table 4. *Autosampler settings*

Parameter	Value
Injection Volume	10 uL
Aspirate flow	5 uL/sec
Inject sample rate	10 uL/sec
Clean valve flow rate	20 uL/sec
Valve clean solvent 1	100uL
Valve clean solvent 2	100 uL

Results and Discussion

The results showed good separation of cortisol and cortisone at base line using the above-mentioned gradient as can be seen in figure 2.

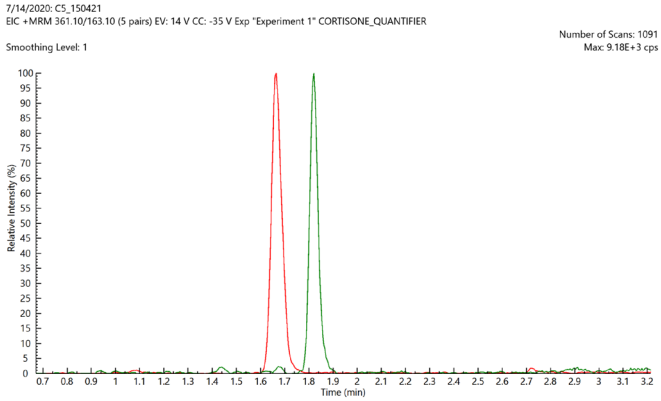

Figure 2. *Cortisol and cortisone detection at LC-MS/MS detection at 60.8 and 122.9 ng/mL respectively*

The linearity of the LC-MS/MS method was assessed using 6 different calibration concentrations of urine spiked with cortisol and cortisone as showed in Table 5. With a weighting of 1/X, both analytes demonstrate an R2 > 0.99 over the measurement range (1.9 – 115.1 ng/mL for cortisol and 2.6-290.8 ng/mL for cortisone). The linearity results are reported in Table 5 and Figure 3.

The LLOQs reported in the table 5 , were calculated from S/N obtained by RMS model, based on the lowest point of the calibration curves for each analytes.

The average accuracy of the kit calibrators, obtained by analyzing 3 different extractions of the calibration curves, is displayed in Table 5. Accuracy falls well within +/- 10% of the expected values provided in the kit insert (Table 5), for both analytes. In the same table, precision of the method is also reported, which was lower than 6%.

Table 5. *Analytical performance for cortisol and cortisone*

Compound	Linearity range (ng/mL)	Linearity (R^2)	Retention Time (RT)	LLOQ (ng/ml)	Level	Concentration (ng/mL)	Accuracy % (n=3)	Precision % (n=3/level)
Cortisol	0.25-300	0.998	1.8	0.9	C1	1.9	103	4.1
					C2	6.6	98	5.3
					C3	10.0	94	1.2
					C4	52.0	101	1.6
					C5	60.8	102	1.8
					C6	115.1	100	0.3
Cortisone	0.25-600	0.997	1.6	0.26	C1	2.6	103	6.0
					C2	29.4	102	2.0
					C3	80.6	93	1.8
					C4	122.9	98	0.7
					C5	155.5	102	2.3
					C6	290.8	96	3.4

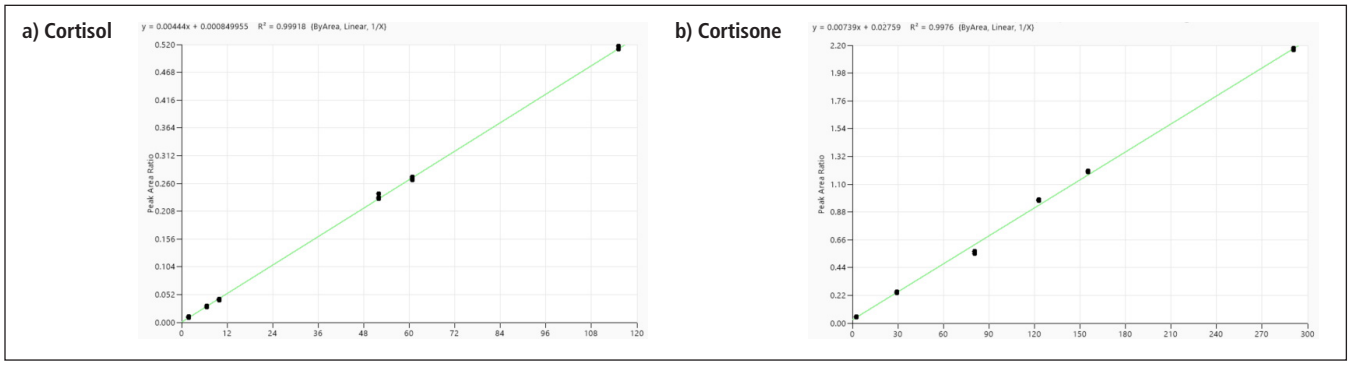

Figure 3. *Regression curves for Cortisol and Cortisone*

Finally, the matrix effect was evaluated by analyzing different urine samples spiked with 2 ng/mL of cortisol and cortisone, and the signal was compared with a purified standard at 2 ng/mL. A matrix effect of less than 30% was observed for both cortisol and cortisone. (Table 6).

Table 6. *Matrix effect*

Analyte	Spiked Amount (ng mL)	Matrix Effect
Cortisol	2 ng/mL	28%
Cortisone	2 ng/mL	27%

Conclusion

The QSight® 200 series UHPLC-MS/MS system exhibited excellent sensitivity for the determination of cortisol and cortisone in urine samples. Linearity, accuracy and precision were also excellent, demonstrating that this method in combination with the Eureka kit provide a superb solution for routine measurement of these two analytes in urine.

Bibliography

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