Cortisol measurement in urine: LC-MS/MS method validation and preliminary clinical application

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Measurements of free cortisol amount excreted in the urine are useful for assessing suspected iatrogenic hypothalamic-pituitary-adrenal axis suppression and Cushing syndrome (1, 2). In this study, we aimed to develop a rapid, sensitive and selective liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for the determination of free cortisol levels in urine samples.

Cortisol, methanol (LC-MS grade) and ammonium formate were purchased from Sigma, and d4-cortisol was purchased from Cambridge Isotope Laboratories. A 250µl aliquot of centrifuged urine sample and 50 µL of the working internal standard (IS; d4-cortisol, 0.5 mg/L) were added to vials, and the vials were vortex-mixed. Urine with IS was injected with mobile phases as detailed in Table 1. The cartridge was washed for 9.6 min to remove hydrophilic constituents. After 9.6 min, the mobile phase composition was changed by step gradient to 5:95; 5 mmol/L ammonium formate-methanol and the column effluent was directed to the LC column connected to a MS interface. Details about the analysis conditions are shown in Table 1.

Quantitative analysis was performed in the multiple-reaction monitoring (MRM) mode, and the transitions monitored were *m/z* 363.4 > 121 and 363.4 > 97 for cortisol and *m/z* 367.3 > 121 and 367.3> 97 for d4-cortisol. Quantification was performed based on the *m/z* 121 product ion, whereas the *m/z* 97 product ion was used for confirmation of cortisol and d4-cortisol identity. Chromatograms of cortisol standards in MRM mode are shown in Figure 1.

The calibrators were prepared at 2, 5, 10, 50 and 100 µg/L using charcoal stripped urine. Assay precision, accuracy, and linearity were determined by using enriched samples. Precision experiments to determine intra-day precisions and inter-day precisions were performed using three replicates of each of four levels of quality control materials across three independent analytical runs. The intra-day and inter-day precisions at four different concentrations (5, 25, 50, 80 µg/L) for quality control samples were about 1.9–4.5% and 2-

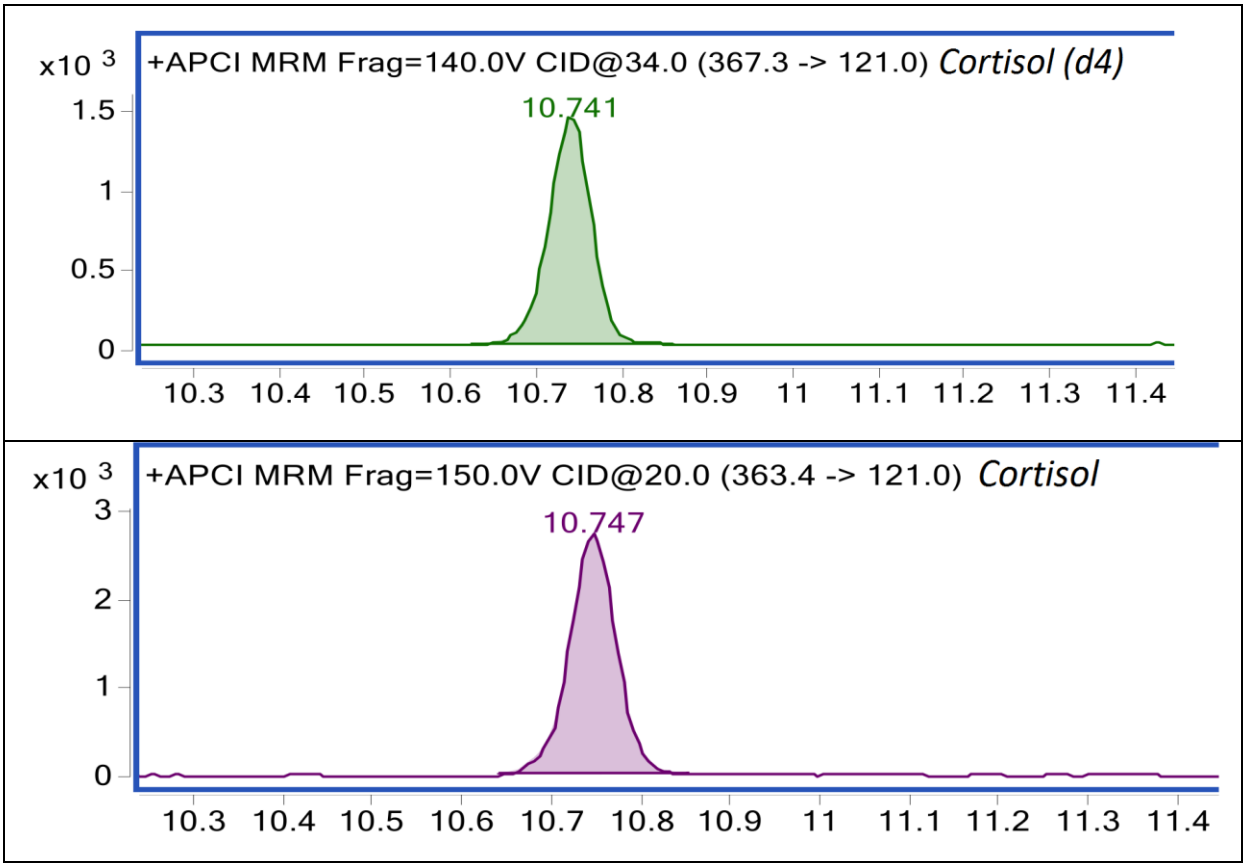
5.4%, respectively. Each run was controlled using a 5-point calibration curve. Calibration curve of cortisol is shown in Figure 2. In addition, the accuracy of the method was about 103.4% for cortisol. The method was linear from 2.0 to 500.0 ng/mL (r^2) > 0.999). The limits of detection and quantification were 1 μg/L and 2 μg/L.

We highly recommend the use of this sensitive, simple and reliable LC-MS/MS method for the diagnosis of some adrenal diseases in clinical laboratories.

Table 1. Analysis conditions

Instrumentation	LC	: Agilent 1200 Series HPLC System	
	MS/MS	: Agilent 6420 triple quadrupole	
LC Conditions	Solvent A	: 5 mM Ammonium formate	
	Solvent B	: 100% Methanol	
	Gradient	: Time (min)	% A
		0.20	90 %
		8.0	60%
		9.0	40%
		9.6	5%
		11.0	5%
		11.1	90%
	Column	: Poroshell 120 EC-C18 column [50 x 3.0 mm (i.d.); 2.7µm particles; Agilent	
	Column Temperature	: 50°C	
	Injection volume	: 5 µL	
	Flow rate	: 0.6 mL/min	
Autosampler syringe wash solvent	Methanol-water (75:25 by volume)		
MS/MS Conditions	Ion source	: APCI (positive ion)	
	Drying gas flow	: 7 L/min	
	Nebulizer	: 35 psig	
	Drying gas temp.	: 325°C	
	Capillary	: 3000 V	
	Detection	: Multiple Reaction Monitoring	
	Transitions	: Cortisol (d_4)	: 367.3 > 121
		: Cortisol	: 363.4 > 121

Figure 1. Chromatograms of Internal Standard (Cortisol d4) and Cortisol



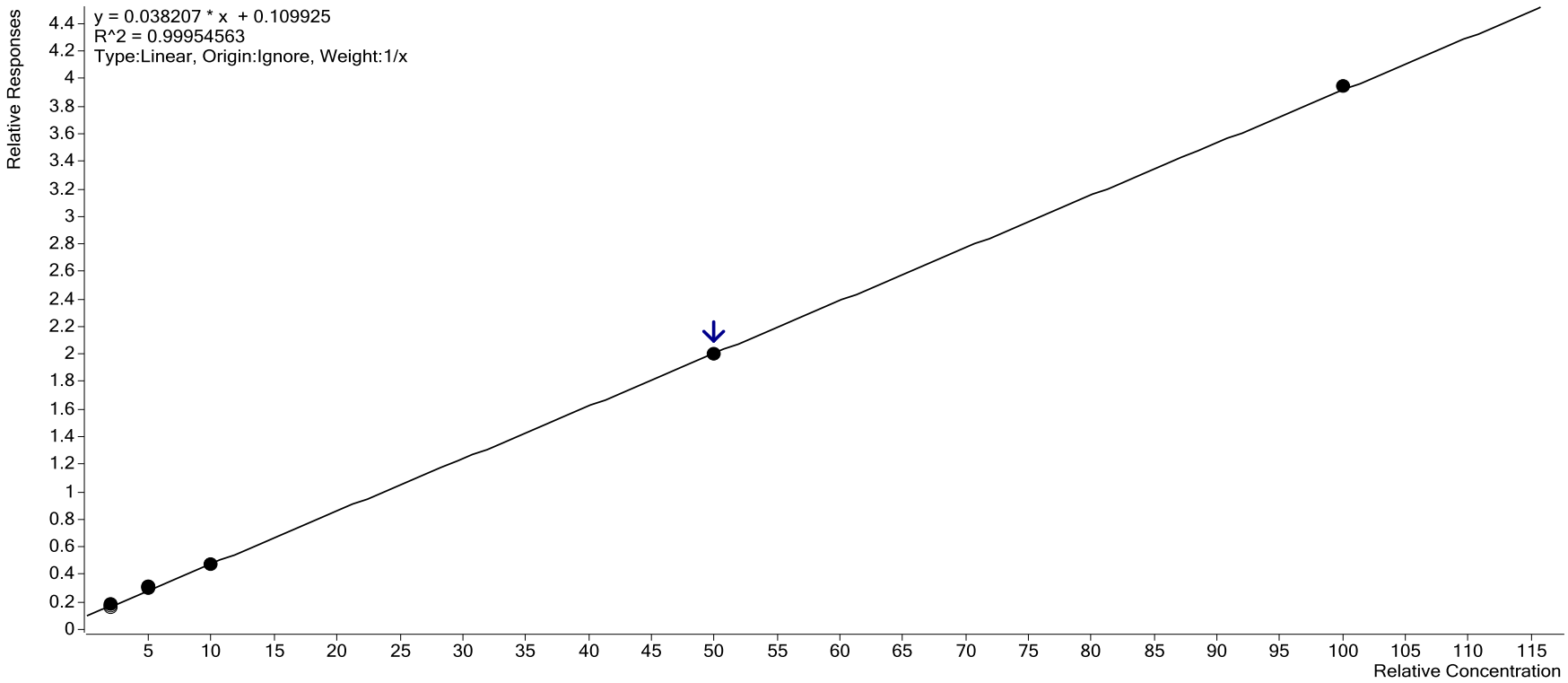


Figure 2. Calibration Curve of Cortisol

Level 1: 2 µg/L, Level 2: 5 µg/L, Level 3: 10 µg/L, Level 4: 50 µg/L, Level 5: 100 µg/L.

References

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2- Persichilli S, Gervasoni J, Iavarone F, Zuppi C. A simple liquid chromatography-tandem mass spectrometry method for urinary free cortisol analysis: suitable for routine purpose. Clin Chem Lab Med. 2010;48(10):1433-7.