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Automating the Extraction and Analysis of Urinary Free Cortisol Using Instrument Top Sample Prep (ITSP), GERSTEL MultiPurpose Sampler and Agilent LC-MS/MS

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Introduction

The measurement of urinary free cortisol (UFC) is used in the investigation of possible Cushing's syndrome. Cortisol is a steroid-hormone synthesized from cholesterol by a multienzyme cascade in the adrenal glands. It is the main glucocorticoid in humans and acts as a gene-transcription factor influencing a multitude of cellular responses in virtually all tissues. Only a small percentage of circulating cortisol is biologically active (free), with the majority of cortisol inactive (protein bound). As plasma cortisol values increase, free cortisol (i.e., unconjugated cortisol and hydrocortisone) increases and is filtered through the glomerulus. UFC in the urine correlates well with the concentration of plasma free cortisol. UFC represents excretion of the circulating, biologically active, free cortisol.

Cushing's syndrome is a hormonal disorder caused by prolonged exposure of the body's tissues to high levels of the hormone cortisol. Cushing's syndrome is relatively rare and most commonly affects adults aged 20 to 50. Signs and symptoms of Cushing's syndrome vary, but most people with the disorder have upper body obesity, a rounded face, increased fat around the neck, and relatively slender arms and legs. Children tend to be obese with slowed growth rates.

Presented in this application note is a fully automated miniaturised solid phase extraction (SPE) method for the extraction and clean-up of UFC, followed by tandem mass spectrometry detection. Performance data is derived from spiked urine and commercially available QC materials. Currently the automated extraction takes four and a half minutes, which fits well within the LC cycle time of seven minutes, this allows for highest possible throughput for this clinically important assay.

Instrumentation

GERSTEL MPS 2, fitted with 500 µl syringe and LC injection valve Instrument Top Sample Preparation (ITSP) Hardware Kit
Maestro Version 1.3.7.69
Agilent 6410 Triple Quadrupole Mass Spectrometer with HotBox and electrospray source
Agilent 1200 Series HPLC
- G1312B Binary Pump SL
- G1316B Thermostatted Column Compartment SL
- G1379B Degasser

Figure 1 – GERSTEL MPS System for cortisol extraction and clean-up

Method

A set of calibrators, eight points, were prepared spanning the range 0.1 to 50 µg/dL in synthetic urine[1]. See table 1. The calibrators were prepared in synthetic urine after it was found that commercially available urine contained moderate (~ 5 µg/dL) endogenous levels of cortisol.

For sample preparation, 1 ml of standard, sample urine or QC urine is pipetted into a standard 2 ml glass screw top autosampler vial and the vial capped. The sample is then placed on the tray of the MultiPurpose Sampler (MPS). See Figure 1.

The following aspects of sample preparation are fully automated, conducted via the MPS and ITSP Kit.

Cal Level	Analyte µg/dL	
	Cortisol	Cortisone
Std_01	0.099	0.101
Std_02	0.248	0.254
Std_03	0.496	0.509
Std_04	0.992	1.018
Std_05	4.96	5.09
Std_06	9.92	10.18
Std_07	24.8	25.4
Std_08	49.6	50.9

Table 1 – Levels of calibrators in synthetic urine

25 µl of a solution of cortisol-d2 in synthetic urine (200 µg/dL) is automatically added to the vial to act as an internal standard. This gives an in vial concentration of 5 µg/dL, which is a good mid-point on the calibration curve.

A 10 mg C8 ITSP SPE cartridge is solvated with 100 µl of methanol and then equilibrated with 100 µl of HPLC grade water. 500 µl of the standard, sample or QC urine is then loaded onto the SPE cartridge, before the cartridge is washed with 100 µl of 40% methanol in water. Analytes are eluted with one 100 µl aliquot of methanol into a 300 µl high recovery vial.

Sample analysis is fully automated by means of an external injection valve and loop fitted onto the MPS, 2 µl of sample is injected. Separation is achieved by means of an Agilent Eclipse Plus 2.1 x 50 mm; 3.5 µm particle size column. The chromatographic mobile phases consisted of 2 mM ammonium acetate plus 0.1% formic acid in water (eluent A) and 2 mM ammonium acetate plus 0.1% formic acid in methanol (eluent B). Gradient conditions are listed in the table below. See table 2.

Column flow rate was 0.6 ml/min throughout the chromatographic run whilst the column temperature was maintained at 60 °C.

An Agilent 6410B tandem mass spectrometer with electrospray source was used in positive ionization mode. Instrument analysis time was 7 minutes per sample using the conditions listed in table 3. Column effluent was directed to waste for the first 2 minutes of the chromatographic run and for the final 3 minutes to ensure the mass spectrometer source remained clean.

Time	Flow	% Eluent A	% Eluent B
0.0	0.6	75	25
0.5	0.6	75	25
4.5	0.6	20	80
4.7	0.6	0	100
4.8	0.6	0	100
5.0	0.6	75	25
7.0	0.6	75	25

Table 2 – Gradient conditions for separation

Parameter	Cortisol	Cortisone	Cortisol-d2
Precursor ion	363.2	361.2	365.2
Product ion (Q)	121.1	163.1	122.1
Dwell	50	50	50
Fragmentor (V)	100	140	100
Collision Energy (Q)	30	20	25

Gas Temp (°C):350 Gas Flow (1/min):10
Nebulizer (psi):60 Capillary (v):4000

Table 3 – Selected MS conditions for analysis

Results

Calibration curves were constructed for both cortisol and cortisone. Linear calibrations were achieved from the eight point calibration standards. Correlation coefficients of 0.9986 and 0.9992 were obtained for cortisone and cortisol respectively. Calibration standards were run at the beginning and end of the analytical run and plotted on the same graph. See Figure 2.

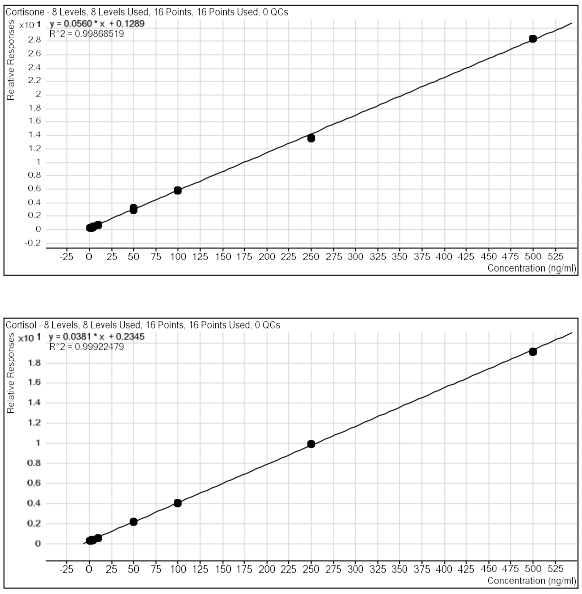

Figure 2 – Calibration curves for cortisone and cortisol

In order to fully validate the method two commercially available quality control materials (Seronorm Urine L1 & L2, Alere, UK) were extracted and analysed for cortisol only, using the procedure described above. See Table 4.

In addition to these quality control samples mixed gender human urine (Seralabs, UK) was spiked with 1, 5 and 10 µg/dL of cortisone and cortisol and the concentrations determined against the synthetic urine calibration standards minus the endogenous concentrations already present in the urine. See Table 5.

Sample	Urine L1	Urine L2
Replicate_1	5.71	24.58
Replicate_2	5.63	24.86
Replicate_3	5.94	24.11
Replicate_4	6.01	24.62
Replicate_5	5.54	24.16
Replicate_6	5.87	23.96
Replicate_7	5.83	24.73
Replicate_8	5.87	24.5
Target Value	6.3	23.7
Range ± 10%	5.7 – 6.9	21.3 – 26.1
Average	5.80	24.4
Pass/Fail	Pass	Pass
S.D	0.16	0.32
% RSD	2.76	1.32

Table 4 – Showing the results of the quality control urine materials

Table 4 shows excellent precision and accuracy for the QC urine samples. The QC materials were within 10% of the published concentration values.

Figures 3 & 4 show representative chromatograms from the 0.1 µg/dL and 50 µg/dL standards. Figure 5 shows a typical chromatogram from an extracted urine quality control sample. Shown in all these figures are clean chromatograms where the analytes of interest are the only major contributing peaks.

Discussion

Presented is a fully automated method for UFC featuring automated internal standard addition and automated SPE clean-up and extraction. Sample preparation is coupled directly to LC-MS/MS and is fully integrated within the Masshunter software. Alternatively, it is possible to configure a standalone workstation. The system is capable of handling 98 samples in 12 hours and 21 minutes. Sample processing time is under 5 minutes fitting within the analytical runtime of the LC analysis ensuring highest possible throughput. Indicative limits of detection of the proposed method are < 0.1 µg/dL.

References

1. McCurdy D, Lin Z, Inn KGW, Bell R, Wagner S, Efurd, DW, Steiner R, et al. Second interlaboratory comparison study for the analysis of 239PU in synthetic urine at the µBq (~100 aCi) level by mass spectrometry. Journal of Radioanalytical and Nuclear Chemistry. 2005;263/2:447-4.

	Cortisol					Cortisone			
Sample	Unspiked Urine	1 µg/dL Spike	5 µg/dL Spike	10 µg/dL Spike		Unspiked Urine	1 µg/dL Spike	5 µg/dL Spike	10 µg/dL Spike
Replicate_1	5.08	6.05	10.22	15.74		7.93	8.95	13.84	21.17
Replicate_2	5.06	6.31	10.23	15.63		7.60	9.15	12.99	20.06
Replicate_3	5.18	6.10	10.13	15.55		7.71	8.77	12.93	19.82
Replicate_4	5.15	6.40	10.68	15.47		7.98	9.58	13.86	20.53
Replicate_5	4.91	6.28	10.97	15.43		7.63	9.13	13.41	20.50
Replicate_6	5.09	6.17	10.43	15.48		7.81	10.20	13.82	20.63
Replicate_7	4.99	6.35	10.98	15.47		8.18	10.13	13.87	19.80
Replicate_8	5.02	6.29	10.28	16.03		7.60	9.42	13.53	20.28
Average	5.06	6.24	10.49	15.60		8.38	9.22	13.53	20.35
S.D	0.09	0.12	0.34	0.20		0.27	0.34	0.39	0.46
% RSD	1.72	1.98	3.27	1.29		3.19	3.70	2.89	2.26
Spike	0.00	0.99	4.96	9.92		0.00	1.02	5.09	10.18
Recovered Spike	0.00	1.18	5.43	10.54		0.00	0.83	5.15	11.97
Recovery %	0.0	119.3	109.5	106.3		0.0	81.8	101.1	117.5

Table 5 – Recovery data for the spiked urine samples at 1, 5 and 10 µg/dL
NB: It is work noting that the urine used in these experiments contained moderate levels of cortisol and cortisone and was not blank.

Figure 3 – Showing representative chromatogram from the 0.1 µg/dL standard.

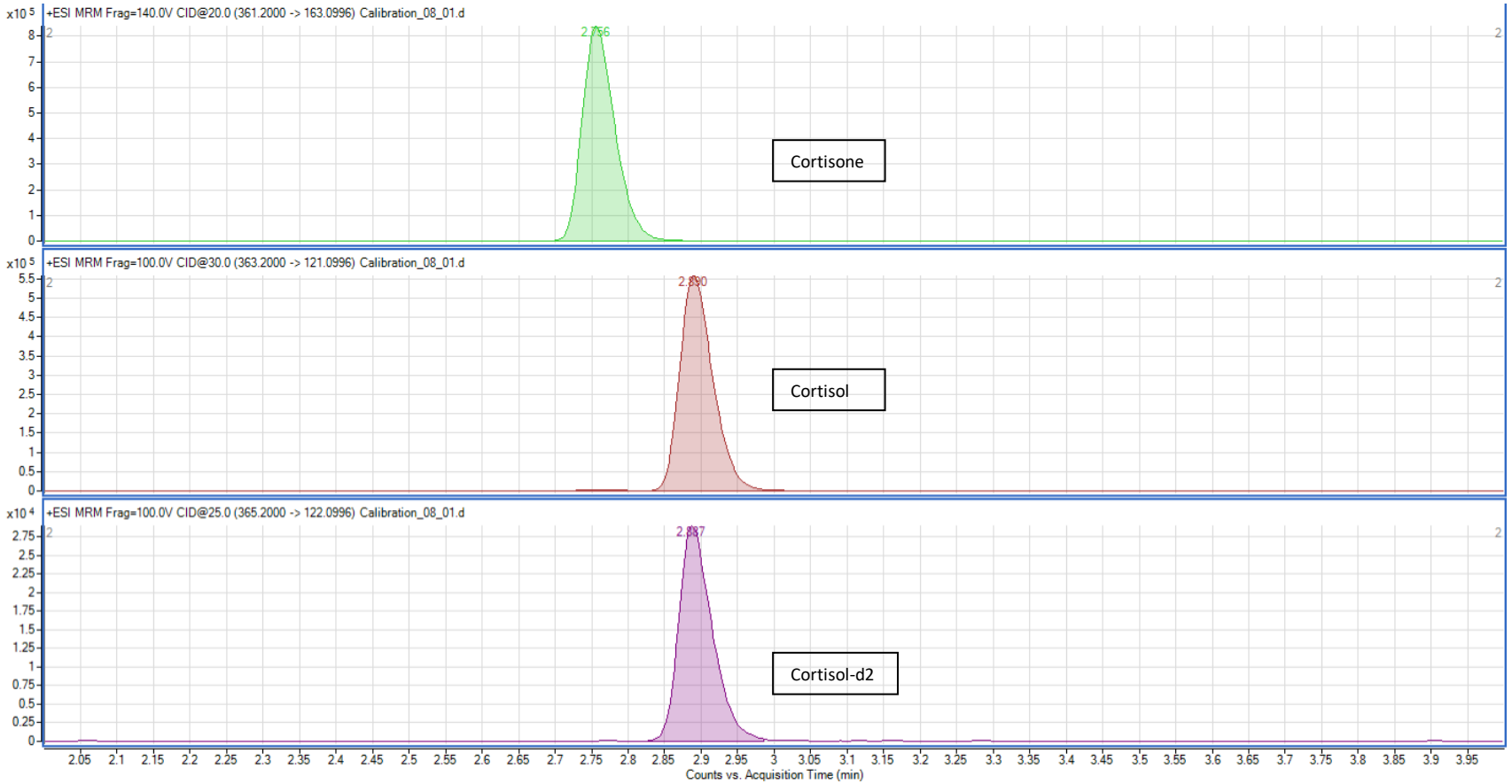

Figure 4 – Showing respresentative Chromatogram from the 50 µg/dL standard

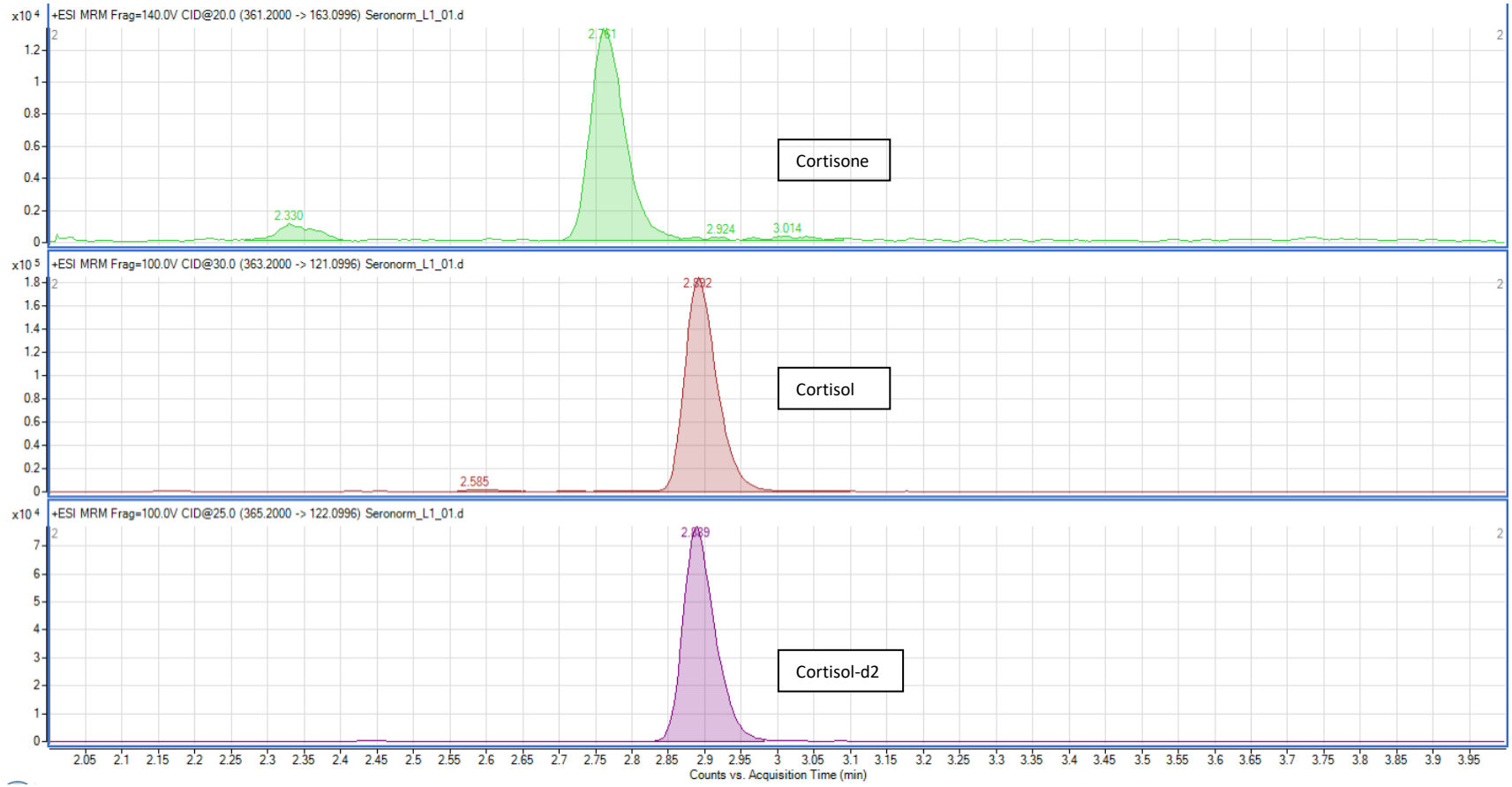

Figure 5 – Typical chromatogram from an extracted urine quality control material