A Quantitative and Selective Analysis of Aldosterone and Cortisol in Plasma by LC-MS/MS for Clinical Research

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Introduction

Liquid chromatography triple quadrupole mass spectrometry (LC-MS/MS) is ideally suited for the rapid, simultaneous analysis of multiple analytes. The ability of an LC/MS system to identify compounds based on chromatographic retention and molecular mass is especially critical when dealing with an application such as steroid analysis. As demonstrated in Figure 1, all steroids share a common structure, making them difficult to differentiate by other methodologies. Through the use of LC-MS/MS, it is possible to confidently perform accurate quantitation.

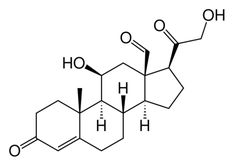

Figure 1. Chemical structure for Aldosterone

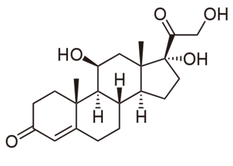

Figure 2. Chemical structure for Cortisol

A highly sensitive and specific method has been developed for the quantitation of two mineralocorticoids for clinical research – aldosterone and cortisol. The described method achieves the required functional sensitivity and is capable of quantitating analytes over a sufficiently wide dynamic range. The analysis is achieved through the use of ultra high performance liquid chromatography (UHPLC) to quickly separate compounds of interest from any interference. Two different sample preparation procedures are evaluated and compared.

Experimental

LC Instrumentation and Methodology
Chromatographic separation was achieved using an Agilent 1290 LC system consisting of one 1290 UHPLC binary pump, one well plate sampler with a thermostat and one temperature-controlled column compartment.

Parameter	Value
Analytical Column	Agilent Poroshell 120 EC-C18, 2.1 x 100 mm, 2.7
Column Temp	55°C
Injection Volume	40 µl
Autosampler Temp	4°C
Needle Wash	Flush port for 10 seconds
Mobile Phase A	2 mM NH4 Acetate in H_2O
Mobile Phase B	2 mM NH4 Acetate in Methanol

Table 1. LC Parameters

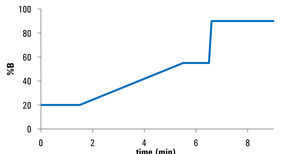

Figure 3. LC gradient with a flow rate of 0.35 ml/min.

MS Instrumentation and Methodology
MS analysis was performed with an Agilent 6460 Triple Quadrupole Mass Spectrometer coupled with an Agilent JetStream Ionization Source.

Ion mode:	AJS negative
Gas temperature:	350 °C
Drying gas (nitrogen):	8 L/min
Nebulizer gas (nitrogen):	35 psi
Sheath gas (nitrogen):	350 °C
Sheath flow:	10 L/min
Capillary voltage:	3000V
Nozzle voltage:	1500V
Q1/Q3 Resolution:	0.7 unit
Delta EMV:	500V

Compound	Prec Ion	Prod Ion	Dwell	Frag (V)	CE (V)
Aldosterone (qual)	359.2	331.2	50	110	12
Aldosterone	359.2	189.1	50	110	16
Aldosterone-d7	366.2	338.3	50	110	12
Cortisol (qual)	361.2	297.2	50	130	32
Cortisol	361.2	282.1	50	130	40
Cortisol-d4	365.2	286.2	50	130	40

Table 2. MRM Parameters

Sample Preparation

In additions to developing an accurate quantitative QQQ method, two sample preparation techniques were explored to ensure the optimal robustness and sensitivity were achieved. A liquid-liquid-extraction (LLE) procedure was developed and then adapted for use with Agilent's Versatube supported-liquid extraction (SLE) tubes. Half the amount of sample was used for the SLE procedure so a smaller SLE bed mass could be used, allowing the procedure to be done in the 96-well plate format. This simplified the sample handling, which in turn made the sample preparation faster.

Liquid-Liquid Extraction (LLE) Procedure
1. Pipet 500 µL of each calibrator, QC, unknown and blank into a 10 mL glass tube
2. Add 60 µL of ISTDs solution
3. Add 2.5 mL of MTBE
4. Vortex for 5 min
5. Centrifuge for 5 min at 3000 RPM at 5°C to separate aqueous and organic layer
6. Pipet 2 mL of the organic layer (top) into another tube, taking care to not disturb the aqueous/organic interface
7. Evaporate under nitrogen at room temperature
8. Reconstitute with 125 µL of 2 mM NH4 Acetate in MeOH:H2O 20:80
9. Vortex for 1 min
10. Transfer into autosampler vial or plate

Supported-Liquid Extraction (SLE) Procedure
1. Pipet 250 µL of each calibrator, QC, unknown and blank onto the SLE material
2. Add 50 µL of ISTDs solution
3. Apply a short burst of vacuum and wait for 5 min to absorb the aqueous solution into the material
4. Add 800 µL of MTBE and wait for 5 min to allow the MTBE to extract the analytes of interest
5. Apply vacuum for 1 min. to elute MTBE
6. Repeat the extraction with another 800 µL of MTBE
7. Evaporate under nitrogen at room temperature
8. Reconstitute with 125 µL of 2 mM NH4 Acetate in MeOH:H2O 20:80
9. Vortex for 1 min
10. Transfer into autosampler vial or plate

Results

LLE vs SLE: Aldosterone
When comparing the described LLE and SLE procedures, both achieved excellent linearity ($r^2 \geq 0.995$) and lower limits of quantitation (LLOQ = 50pmol/L with S/N ≥ 6 and %RSD <10%). It is important to notice, however, these results are achieved with half the amount of sample with the SLE protocol.

	Range	Equation	R^2
LLE	50-7000 pmol/L	y=9.99e-4*x-7.00e-4	0.998
SLE	50-7000 pmol/L	y=5.77e-4*x-9.78e-4	0.997

Table 3. LLE vs SLE Aldosterone calibration curves

Aldosterone	Extraction	Intraday	Interday
QC 1	LLE	7.26	9.19
	SLE	7.64	10.13
QC 2	LLE	7.15	7.93
	SLE	6.34	10.42
QC 3	LLE	0.70	4.02
	SLE	3.55	8.69

Table 4. Aldosterone intra- and interday reproducibility

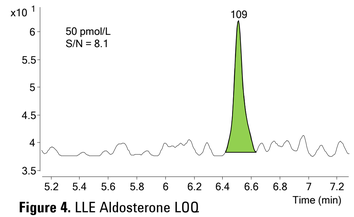

Figure 4. LLE Aldosterone LOQ

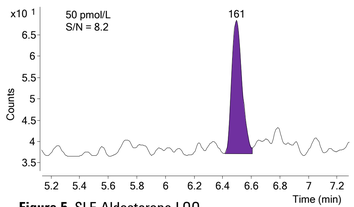

Figure 5. SLE Aldosterone LOQ

Results and Discussion

LLE vs SLE: Cortisol
When comparing the described LLE and SLE procedures, both achieved excellent linearity ($r^2 \geq 0.995$). For the purposes of this experiment, a LLOQ was not determined. However, at the lowest concentration calibrator the, SLE procedure resulted in a better signal-to-noise ratio despite a lower response. Further optimization of the SLE procedure could improve the recovery of cortisol.

	Range	Equation	R^2
LLE	10-1500 nmol/L	y=0.007*x-0.035	0.998
SLE	10-1500 nmol/L	y=0.004*x-0.019	0.996

Table 5. LLE vs SLE Cortisol calibration curves

Cortisol	Extraction	Intraday	Interday
QC 1	LLE	4.08	2.96
	SLE	2.93	4.15
QC 2	LLE	2.61	2.00
	SLE	1.49	2.02
QC 3	LLE	1.55	1.68
	SLE	1.20	4.91

Table 6. Cortisol intra- and interday reproducibility

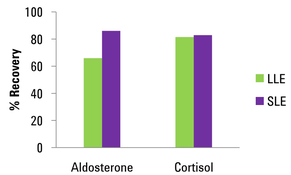

Figure 8. LLE vs SLE recovery

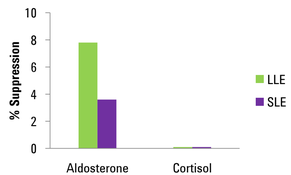

Figure 9. LLE vs SLE suppression

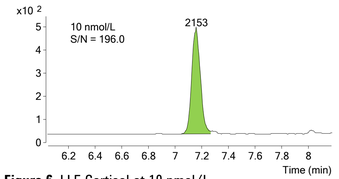

Figure 6. LLE Cortisol at 10 nmol/L

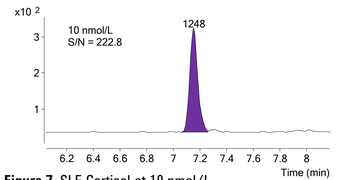

Figure 7. SLE Cortisol at 10 nmol/L

Recovery, Suppression
SLE greatly improved the recovery and suppression for aldosterone over LLE. Equivalent results have been demonstrated for cortisol (Figures 8 and 9)

Interferences
11-deoxycortisol, corticosterone, 17-hydroxyprogesterone, androstenedione, DHEA ,DHEAS, progesterone and testosterone were injected at appropriate concentrations to investigate chromatographic interferences. No interfering peaks were observed at the retention times for aldosterone or cortisol.

Conclusions

A robust and accurate quantitative LC/MS/MS method has been developed for the simultaneous determination of aldosterone and cortisol in plasma. A comparison of LLE and SLE procedures showed significant improvements for aldosterone and equivalent results for cortisol when using SLE while only using half as much sample material. The easy to handle 96-well plate format of the SLE also improved the efficiency of sample preparation.

Reference
J Clin Pathol, 457-462, Vol 65, No 5, 2012, Van Der Gugten JG, Dubland J, Liu H-F, et al. J Clin Pathol