THE ANALYSIS OF URINARY FREE CORTISOL BY ON-LINE SOLID PHASE EXTRACTION LC/MS/MS

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ABSTRACT

Introduction: The measurement of urinary free cortisol (UFC) is of clinical importance in the diagnosis of Cushing's syndrome. Cushing's is a disease caused by the autonomous and excessive production of cortisol. The majority of routine methods for determining 24 hr-UFC concentrations involve RIA or HPLC with UV detection. In general, the performance of immunoassays is compromised due to the cross-reactivity of cortisol metabolites leading to the amounts of UFC being over estimated[1,2]. In the clinical laboratory LC/MS/MS is now becoming more common due to the gains in specificity and accuracy compared to immunoassays[3].
The objective of this study was to asses the feasibility of using an automated on-line solid phase extraction (SPE) tandem mass spectrometry technique for the analysis of UFC. A Symbiosis™ automated SPE instrument (Spark Holland, Netherlands) coupled to a Waters® Quattro micro™ tandem quadrupole mass spectrometer (Waters, Manchester, UK) was used for the analysis.
Methodology: For the initial study, twenty UFC patient samples were analysed at South Manchester University Hospital by their validated LC/MS/MS method for cortisol[4]. After analysis the samples were stored at -20°C prior to analysis by XLC/MS/MS. Calibrators were prepared in urine-like electrolyte solution (ULE solution Fluka) over the concentration range 0.5-500ng/mL and QC samples were prepared independently by the hospital laboratory. Sample preparation consisted of adding 10µL of the internal standard (d_2-cortisol) to 240µL of each calibrator, QC, and patient sample to give a final concentration of approximately 20ng/mL. Using an injection volume of 20µL, automated SPE using Oasis® HLB cartridges was carried out employing a two stage wash protocol. The analytes were eluted from the cartridge directly onto an analytical column using a linear gradient from 20-45% acetonitrile and detected by MRM tandem mass spectrometry.
Results: The assay was shown to be linear over the range 0.5-500ng/mL with a correlation co-efficient (R^2) of > 0.999. The calculated concentrations for the QC samples were all within ±11% of the expected concentration. The limit of quantification (LOQ) of the assay was determined to be 0.5ng/mL (S/N of 15:1) and the cortisol determination of 10 replicate injections of a spiked urine sample at a concentration of 17ng/mL gave a precision of 2.8%CV. The calculated XLC/MS/MS cortisol concentrations of the twenty patient samples were in good agreement with the LC/MS/MS measurements from South Manchester Hospital, using linear regression analysis (y=1.0165x-0.7887). Recovery of the on-line extraction was determined to be 98.3% using the Automated Method Development functionality of the Symbiosis system.
Conclusion: A method for the direct analysis of cortisol in urine using XLC/MS/MS technology has been investigated and has been shown to have good linearity (R^2 >0.999), sensitivity (LOQ 0.5ng/mL) and precision (2.8%CV).

INTRODUCTION

The measurement of urinary free cortisol (UFC) is of clinical importance in the diagnosis of Cushing's syndrome. Cushing's is a disease caused by the autonomous and excessive production of cortisol. The majority of routine methods for determining 24hr-UFC concentrations involve RIA or HPLC with UV detection. In general the performance of immunoassays is not good due to the cross-reactivity of cortisol metabolites leading to the amounts of UFC being over estimated.[1,2] In the clinical laboratory LC/MS/MS is now becoming more common due to the gains in specificity and accuracy compared to immunoassays.[3]
Here we describe the analysis of UFC by an automated on-line solid phase extraction (XLC) MS/MS technique. The method uses a Spark Holland Symbiosis™ automated SPE instrument coupled to a Waters® Quattro micro™ tandem quadrupole mass spectrometer (Figure 1).
The entire system is controlled through MassLynx using the new Symbiosis™ Pharma software which is available in SCN 587 for use with MassLynx 4.1.

Figure 1. Symbiosis™ Pharma / Quattro micro™ system.

METHODS

Sample preparation

Calibrators were prepared in urine like electrolyte solution (ULE solution Fluka) over the concentration range 0.5-500ng/mL and six QC samples were prepared independently.

Forty 24hr urine samples collected into plain containers were received for the investigations into possible Cushing's syndrome. After determination of the urine volume, an aliquot was taken and kept at 8°C for a maximum of 1 week and analysed at South Manchester University Hospital by their 'in house' routine LC/MS/MS method. Aliquots were stored at -20°C prior to analysis by XLC/MS/MS at Waters Manchester.

10µL of the internal standard (d_2-cortisol) was added to 240µL of each calibrator, QC, and patient sample to give a final concentration of 20ng/mL.

Experimental

This experiment details the analysis of UFC by an automated XLC/MS/MS method.
20µL of the prepared sample was loaded on to the Oasis® HLB SPE cartridge (new cartridge per sample) and washed with aqueous high pH and low pH containing 5% ACN. The analytes were eluted from the cartridge using a gradient described in the XLC Conditions section and detected by MRM tandem mass spectrometry

XLC Conditions

XLC System:	Spark Holland Symbiosis™ Pharma
SPE Cartridge:	Waters® Oasis® HLB 1x10mm 30µm
Conditioning:	Acetonitrile 1mL
Equilibration:	Water 1mL
Load:	Water 1mL
Wash:	Water 2% Ammonium Hydroxide 1mL
Wash:	5% Acetonitrile 2% Formic Acid 1mL
Elution mode:	Standard (LC Pump)
Elution time:	3.5 mins
Injection Volume:	20µL

LC Conditions

HPLC Column:	Waters® Sunfire™ C18 2.1x50mm, 3.5µm
Eluents:	A: 2mM Ammonium acetate+0.1% formic acid in water B: Acetonitrile
Column Temp:	30°C
Sample Temp:	4°C
Flow Rate:	0.2 mL/min
Gradient:	Time %A %B 0.0 80 20 2.0 55 45 2.5 55 45 3.0 5 95 3.5 5 95 3.6 80 20
Total Run Time:	5.5 minutes

MS Conditions

MS System:	Waters® Quattro micro™
Ion Mode:	Electrospray positive ionisation
Capillary Voltage:	1.00kV
Source Temp:	120°C
Desolvation Temp:	400°C
Desolvation Gas Flow:	1150L/hour
Cone Gas Flow:	50L/hour
Detection Mode:	MRM (Table 1)
Dwell Time:	0.20 seconds
Inter-scan Delay:	0.05 seconds
Collision Gas:	Argon (3.30x10-3mbar)

Compound	Transition	Cone (V)	Collision (eV)
Cortisol	363.20>121.10	22	24
d_2-Cortisol	365.20>122.10	22	24

Table 1. MRM Transitions and for cortisol and the internal standard.

RESULTS

Linearity

The data were processed using QuanLynx™ quantification software, using the ApexTrack™ integration algorithm. A linear fit was applied with a 1/x weighting. The correlation coefficient (R^2) for cortisol was 0.9999 (Figure 2) and the calculated concentrations for the calibrators were all within ±4% of the assigned values. The assay was shown to be linear up to 1000ng/mL (R^2 0.9993), using calibrators over the concentration range 0.5-1000ng/mL.

Accuracy

The calculated concentrations and the % deviations for the six QC samples were all acceptable (mean %RSD -4.4%).

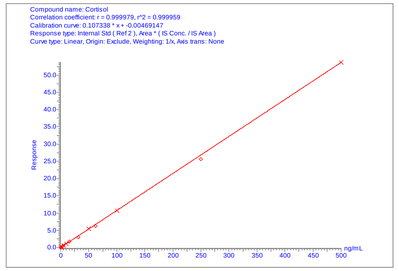

Figure 2. Calibration line for cortisol. X= Calibrator ◇= QC

Precision

Cortisol determination of 10 replicate injections of a spiked urine sample at a concentration of 17ng/mL gave a precision of 2.8%CV.

The intra and inter-day precision of the assay was calculated using three commercially available materials (Bio-Rad). The intra and inter-assay variations for each level (n=5) are shown in Tables 2 an 3 respectively.

	QC 1	QC 2	QC 3
mean	12.38ng/mL	37.68ng/mL	109.46ng/mL
std dev	0.36	0.60	3.72
%CV	2.88	1.60	3.39

Table 2. Intra– assay variation of three commercial quality control samples (n=5).

	QC 1	QC 2	QC 3
mean	11.42ng/mL	34.98ng/mL	98.08ng/mL
std dev	0.31	2.48	4.24
%CV	2.73	7.08	4.32

Table 3. Inter– assay variation of three commercial quality control samples analysed over five consecutive days.

Limit of Quantification

The limit of quantification (LOQ) was determined to be 0.5ng/mL with a S/N of 15:1.

Recovery

Using the Automated Method Development (AMD) functionality of the Symbiosis™ system an extraction recovery experiment was performed and the recovery was calculated to be 98.3%.

Carry Over

Carry over was determined by analysing a high cortisol standard (1000ng/mL) followed by a water blank. The area for the water blank was divided by the area for the cortisol standard and expressed as a percentage to give a value of 0.03%.

Patient Analysis

Forty anonymised patient samples were analysed using the Symbiosis™/Quattro micro™ . Two samples were excluded from the method comparison because the measured cortisol concentration was out of the calibration range of the South Manchester University Hospital method.

The calculated cortisol concentrations obtained were in good agreement with the LC/MS/MS measurements, as shown in Figure 3.

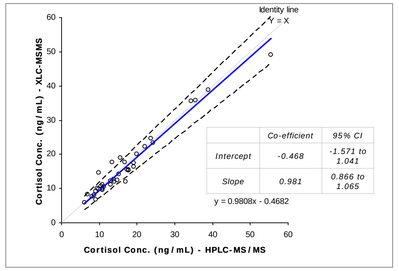

Figure 3. Passing-Bablok method comparison.

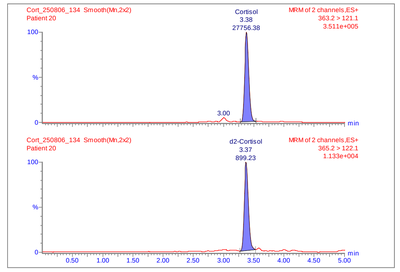

Figure 4. Chromatogram to show a patient with an elevated cortisol level (480.5ng/mL).

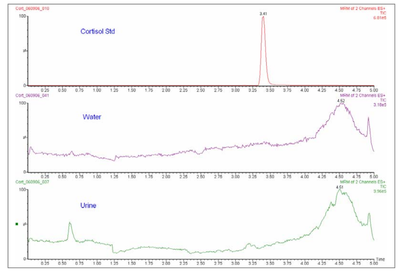

Figure 5. Retention time of cortisol and the ion suppression chromatograms for the analyses of water and urine.

The Passing-Bablok method comparison shows four samples that clearly fall outside the 95% confidence limits, further investigation is required to determine the cause of this.

One of the forty patient samples analysed showed elevated cortisol levels of clinical significance 480.5ng/mL (Figure 4).

Ion Suppression

The ion suppression was assessed by infusing a solution of cortisol (1000ng/mL) at 10µL/min post-column and monitoring the MRM transition for cortisol when injecting a water blank and a urine sample.

Minimal ion suppression was observed (Figure 5) and the use of a deuterated internal standard will compensate for these minor ion suppression effects.

DISCUSSION

The dedicated on-line SPE functionality of the Symbiosis™ Pharma instrument allows for high-throughput analysis. The system has two SPE cartridge clamps enabling parallel SPE and analytical operation. Sample preparation is no longer the rate limiting step in high through put LC/MS/MS assays when using the Symbiosis Pharma system.

A five minute method for the direct analysis of UFC has been investigated using the Symbiosis™ Pharma / Quattro micro™ system. The method demonstrates excellent linearity (R^2 <0.999), sensitivity and precision when injecting only 20µL of urine.

The method developed was compared with a routine LC/MS/MS method. The calculated cortisol concentrations for forty patients were in good agreement with the measurements from South Manchester University Hospital.

CONCLUSION

- The integrated Symbiosis™ Pharma / Quattro micro™ system is easy to use with the new MassLynx driver, enabling complete control of the system.
- A method for the direct analysis of cortisol in urine has been investigated and shown to have good linearity, sensitivity and precision.

References
1. C Lee. Beta hydroxy cortisol interferes with immunoassay of UFC. ClinChem 1996;42:1290-1
2. BEP Murphy. How much 'UFC' is really cortisol ? Clin Chem 2000;46:793-4
3. RL Taylor, D Machacek, RJ Singh. Validation of a high-throughput liquid chromatography–tandem mass spectrometry method for urinary cortisol and cortisone. Clin Chem 2002;48:1511-1519
4. SJ McCann, S Gillingwater and BG Keevil 'Measurement of urinary free cortisol using liquid chromatography-tandem mass spectrometry: comparison with the urine adapted ACS:180 serum cortisol chemiluminescent immunoassay and development of a new reference range' Ann Clin Biochem 2005; 42: 112–118

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