



# DETERMINATION OF CORTISOL IN SERUM BY XLC-MS/MS USING THE SYMBIOSIS™ PHARMA

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APPLICATION INFO

## Introduction

**Symbiosis™ Pharma** is Spark Holland's unique solution for integrated online SPE-LC-MS automation (XLC-MS). The system offers large flexibility in processing different types of samples selecting one of the three fully automated operational modes LC-MS; XLC-MS; AMD (Advanced Method Development).

This Application note will present a study that demonstrates the capabilities of the AMD-mode to speed-up method development. The presented results were obtained within 2 days and show a XLC-MS protocol that generates acceptable accuracy, precision and linearity over the calibration curve.

**Cortisol** the most potent glucocorticoid produced by the human adrenal, is synthesized from cholesterol. Its production is stimulated by pituitary adrenocorticotropic hormone (ACTH) which is regulated by cortisol releasing factor (CRF).

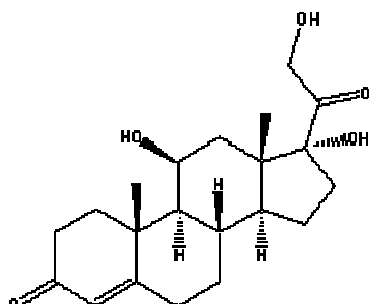


Figure 1: Cortisol

- CAS#000050-23-7
- C<sub>21</sub>H<sub>30</sub>O<sub>5</sub>
- Mw 362.46
- Physical Properties:
  - Water solubility 320 mg/L
  - Log P (Octanol-water) 1.61
  - pKa dissociation constant 12.7

## Method Development

The AMD mode of Symbiosis™ Pharma in conjunction with the HySphere Method Development Cartridge tray enables "quick sorbent screening" for most suitable SPE cartridge and optimal wash conditions for clean-up. The following data was obtained in less than 1 hour using generic SPE conditions pre-defined in the Symbiosis™ Pharma.

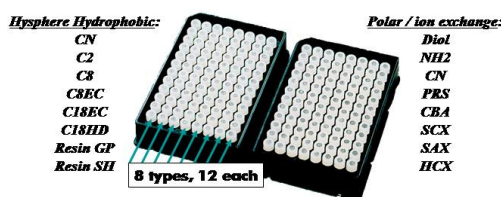


Figure 2: Method Development Cartridge Tray

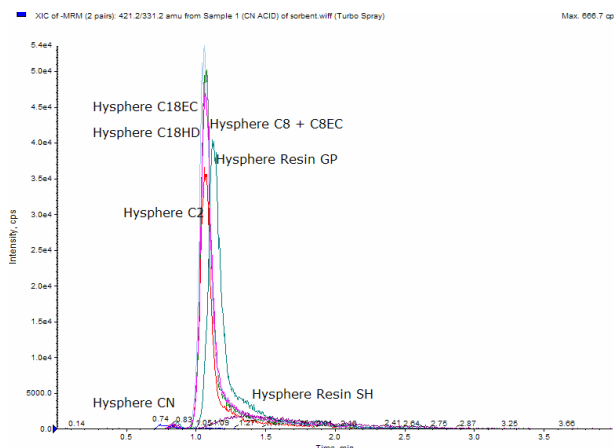


Figure 3: Chromatograms of Cortisol in serum samples during sorbent screening.

From Figure 3 can be derived that C18EC gives the highest signal and also the best peak shape. The recovery calculated from figure 4 is >90%.

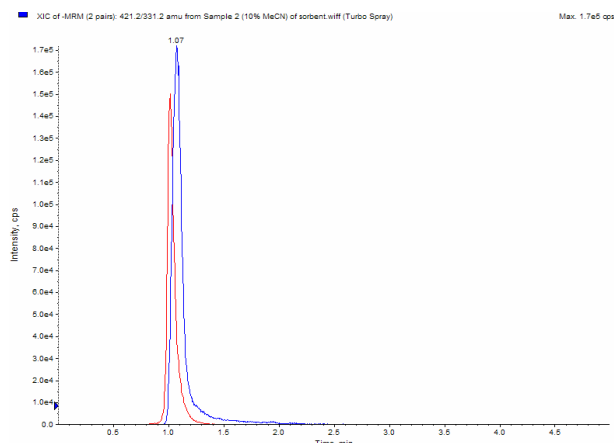


Figure 4: LC and XLC Chromatograms representing recovery of Cortisol using HySphere C18-EC.

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[HTTP://WWW.SPARKHOLLAND.COM](http://www.sparkholland.com)



## XLC-MS protocol

The plasma samples are processed with the developed XLC-MS method (as described below) using the Symbiosis™ Pharma and API-3000 System.



Figure 5: Symbiosis™ Pharma System

- The XLC-MS method contains a protocol for:
- the autosampler (injection and wash routine)
  - the Online SPE (extraction and clean-up)
  - the LC gradient
  - MS settings\*

\* The MS settings are stored in a separate MS acquisition file except when using the Analyst 1.4.1. software the XLC-MS method is then incorporated in the original Analyst acquisition method (.dam file).

### Autosampler Conditions

50 µL of sample is injected using the partial loop fill injection routine. Washing is performed with two solvents: Wash solvent 1: 50% ACN with 0.1 % Formic Acid. Wash solvent 2: 90% ACN.

Wash solvent	Wash volume
1	700 µL
2	700 µL
1	1500 µL

Table 1: wash routine autosampler.

### SPE conditions

Cartridge:	10x2mm HySphere C18EC (Spark Pn:0722.602)	
Solvation:	1 mL ACN	5 mL/min
Equilibration:	1 mL 5% ACN with 0.1 % Formic Acid	5 mL/min
Sample Loading:	1 mL 5% ACN with 0.1 % Formic Acid	1 mL/min
Washing:	1 mL 10% ACN with 0.1 % Formic Acid	5 mL/min
Elution	2 min with Isocratic LC	

Table 2: SPE settings; Total SPE time is 2 min 30 sec.

### LC conditions

Column:	Waters Xterra MS C18 4.6 x 50 mm. 3.5 µ (Waters Pn: 186000432)
Mobile phase:	40% ACN in 10 mM ammonium acetate.
Run time:	5 minutes @ 0.25 ml/min

Table 3: LC conditions

## MS conditions

A Sciex API 3000 with a Turbo IonSpray is used.

	Cortisol	Cortisol-D3
Q1 Mass	421.17	424.17
Q3 Mass	331.20	334.20
Dwell (ms)	150	150
DP	-31	-31
FP	-130	-130
CE	-24	-24
CXP	-17	-17
Neb:	15	
Cur:	15	
IS:	-4000	
TEM:	350	
CAD:	10	
EP:	-10	

Table 4: MS parameters

## Results

The following samples are prepared in new born calf serum using Cortisol-D3 (100 ng/mL) as internal standard.

- Calibration standards: 1.0; 2.5; 5.0; 10; 25; 50; 100; 250; 500 ng/mL.
- QC samples: 50; 100; 300 ng/mL.

### Chromatograms

Figures 6 and 8 are representative chromatograms of the upper and lower limits of the calibration curve indicating the excellent quantitative suitability of XLC-MS. The carry over shown in (figure 7) Blanc injection is calculated as <0.01%.

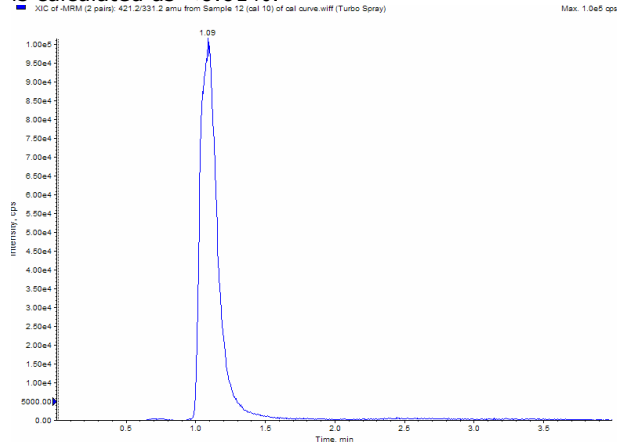


Figure 6: Chromatogram representing 500 ng/mL Cortisol

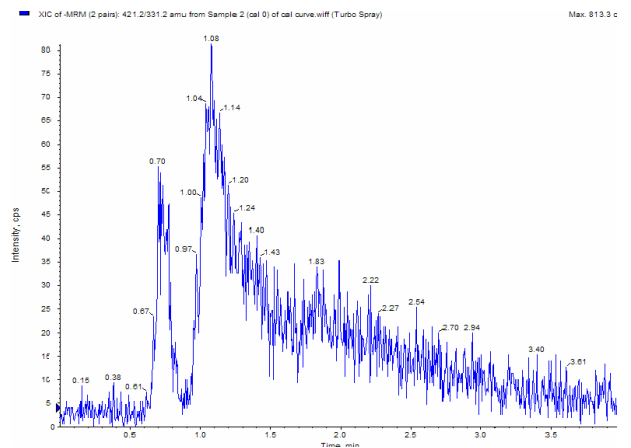


Figure 7: Chromatogram representing Blanc

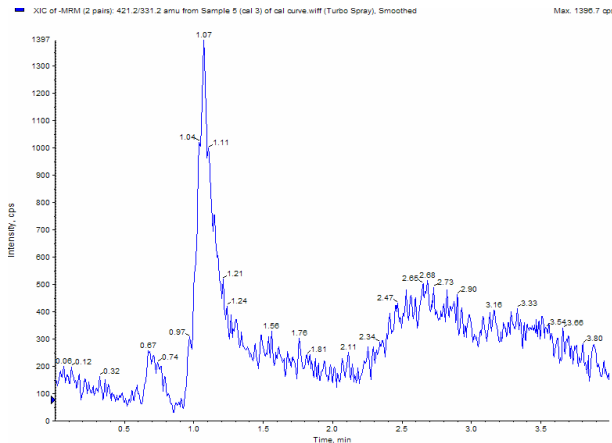


Figure 8: Chromatogram representing 2.5 ng/mL Cortisol.

### Linearity, Accuracy and Precision

A calibration curve was determined by combining the results of 3 repeated injections of the full set of calibration standards. This resulted in a R<sup>2</sup> of **0.999** with a 1/X weighting.

Exp. Conc. (ng/mL)	CV (%)	Accuracy (%)
1.00	14.1	115
2.50	9.31	84.9
5.00	6.51	103
10.00	4.04	102
25.00	3.13	95.1
50.00	2.17	98.2
100.0	0.81	98.0
250.0	1.29	99.6
500.0	1.52	101

Table 5: Accuracy and precision calculated from three combined sets of calibration standards.

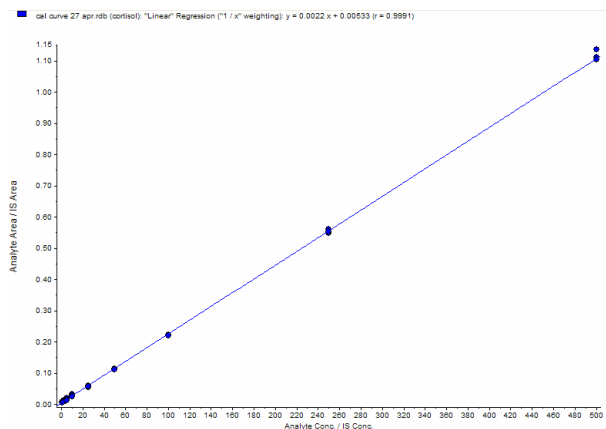


Figure 9: three combined calibration curves.

Expected Conc. (ng/mL)	Sample Name	CV (%)	Accuracy (%)
51.0	QC 1	2.04	103
53.5	QC 2	4.17	99.3
61.0	QC 3	3.45	98.0
101.0	QC 4	2.79	96.7
301.0	QC 5	1.53	101

Table 6: QC series

### Reuse of cartridges

Cartridge reuse is an effective option to reduce cost. The consumable cost per sample can be reduced to 50 cents by reusing the cartridges 6 times. Cartridge reuse will always increase the risk of carry over and can even result to incorrect data.

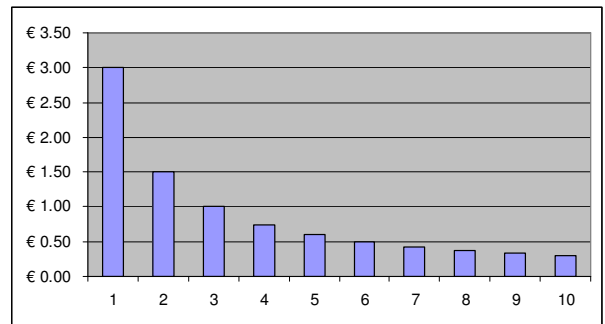
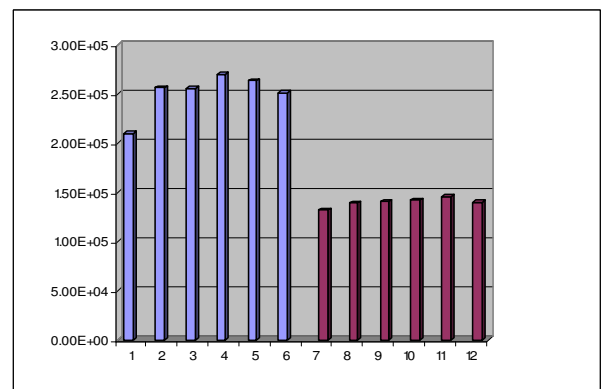


Figure 10: Cartridge cost when reused.

To determine the effect on performance of cartridge reuse, 2 series of 6 injections were performed using a single cartridge. There is no significant decrease in accuracy and precision after 6x cartridge reuse.



Sample name	%CV	Accuracy
QC2	2.75	100
QC4	1.57	101

Figure 11: Performance reused cartridges, peak area of 6 QC-4 and 6 QC-2 samples

## Conclusions

From this study it is concluded that within a time frame of two days it is possible to develop a XLC-MS method with an absolute recovery >90%; run three sets of calibration standards with a linear range from 1-500 ng/mL (R<sup>2</sup> of 0.999) with an accuracy between 85-115% and a precision of <15% CV.

Furthermore it is concluded that reusing the cartridge up to six times will not have a significant impact on the analytical performance for this particular application. It is advised though to check the effect at all times when cartridge reuse is considered.

The total XLC-MS cycle time consists of the sample preparation time + LC-MS runtime. Since the sample prep is executed in parallel with the LC, the total XLC-MS time is 2.5 minutes per sample.

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