APPLICATION INFO

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

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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.

Warfarin an anti clogging agent often prescribed for patients that recently had suffered from a hart attack or have undergone heart surgery. It is sold under the commercial name "Coumadin" or "Miradon". The compound is usually measured in Human Serum.

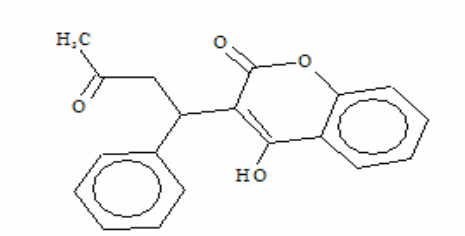

Figure 1 Warfarin

- CAS#000081-81-2
- C19H16O4
- Mw 308.34
- Physical Properties:
  - Water solubility 17 mg/L
  - Log P (Octanol-water) 2.60
  - pKa dissociation constant 5.08

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.

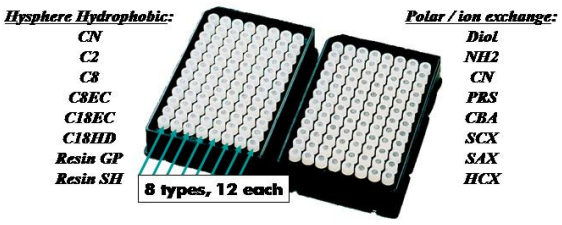

Figure 2 Method Development Cartridge Tray

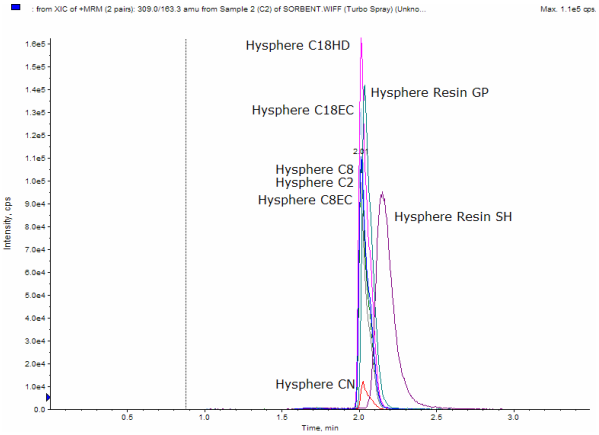

Figure 3: Chromatograms of Warfarin in serum samples during sorbent screening.

From Figure 3 can be derived that C18HD gives the highest signal and best peak shape. Recovery is > 90%.

XLC-MS protocol

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

Figure 4: 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

20 µL of sample is injected using the partial loop fill injection routine.
Washing is performed with two solvents;
Wash solvent1: 50% ACN with 0.1 % Formic Acid.
Wash solvent 2: 90% ACN

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

Table 1: autosampler wash routine.

SPE conditions

Cartridge:	10x2mm HySphere C18HD (Spark Pn:0722.609)	
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 5% ACN with 0.1% Formic Acid	5 mL/min
Elution	2 min with LC gradient	0.75mL/min

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

LC conditions

Column:	Waters Xterra MS C18 4.6 x 50 mm. 3.5µ (Waters Pn:186000432)
Mobile phase A:	0.1 % Formic Acid in Water
Mobile phase B:	0.1 % Formic Acid in Acetonitrile.

Time: (mm:ss)	Flowrate (mL/min)	Perc. A (%)	Perc. B (%)
00:01	0.75	60	40
00:30	0.75	60	40
01:00	0.75	10	90
02:00	0.75	10	90
02:15	0.75	60	40
02:45	0.75	60	40

Table 3: LC gradient.

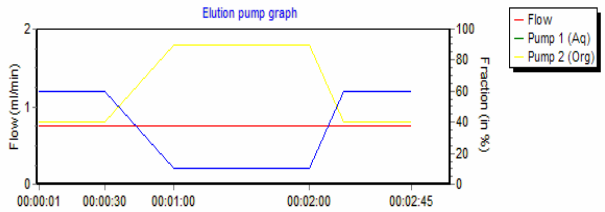

Figure 5: LC gradient.

MS conditions

A Sciex API 3000 MS with a Turbo IonSpray is used. Before entering the MS the LC flow is split to give a 250 µL/min flow into the ESI-Source.

Neb:	15
Cur:	15
IS:	2500
TEM:	450
CAD	7
EP	10

	Warfarin	Propranolol (IS)
Q1 Mass	309.10	260.17
Q3 Mass	163.0	116.2
Dwell (ms)	150	150
DP	36	20
FP	150	200
CE	25	50
CXP	8	30

Table 4: MS parameters

Results

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

- Calibration standards: 0.1; 0.5; 1.0; 5.0; 10; 50; 100; 500 ng/mL.
- QC samples: 0.1; 10; 500 ng/mL.

Chromatograms

Figures 6 and 7 are representative chromatograms of the upper and lower limits of the calibration curve indicating the excellent quantitative suitability of XLC-MS.

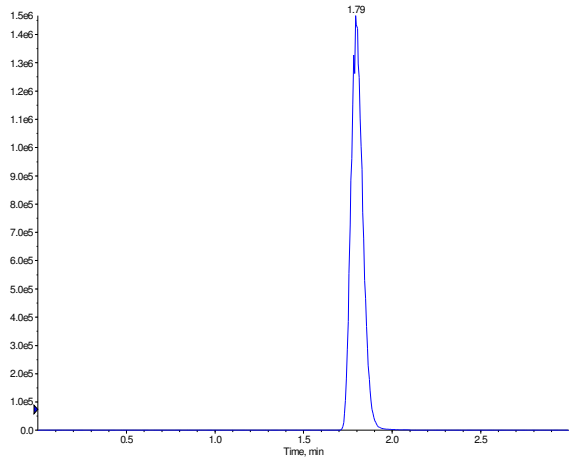

Figure 6: Chromatogram representing 500 ng/mL Warfarin

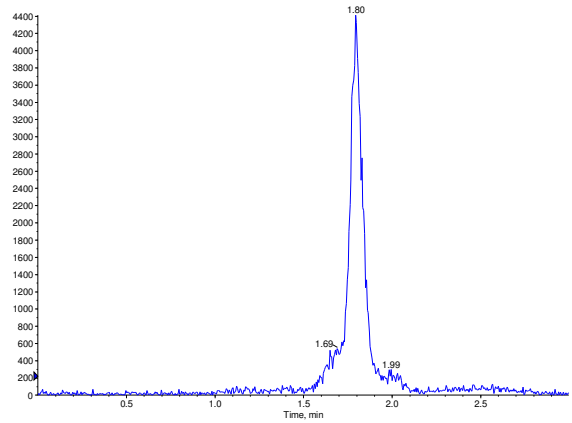

Figure 7: Chromatogram representing 0.1 ng/mL Warfarin

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^2 of **0.999** with a 1/X weighting.

Exp. Conc. (ng/mL)	Sample Name	CV (%)	Accuracy (%)
0.10	0.1	13.54	84.94
0.50	0.5	6.47	92.58
1.00	1	5.10	97.42
5.00	5	3.17	106.33
10.00	10	2.93	108.92
50.00	50	1.26	103.40
100.00	100	0.44	108.72
500.00	500	2.35	97.69

Table 5: Accuracy and Precision calculated from three combined calibration curves. (Number values used: 3 of 3)

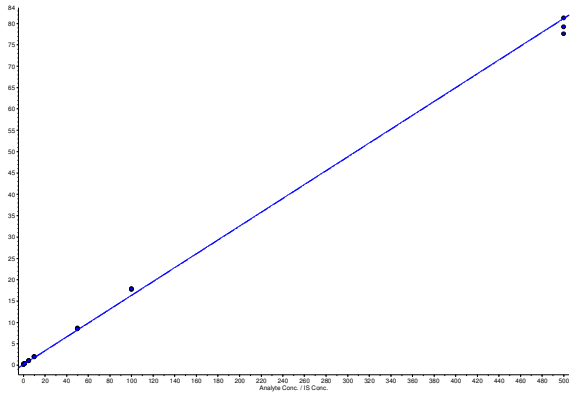

Figure 8: Warfarin Peak area over IS Peak area from three combined calibration curves.

Expected Conc. (ng/mL)	Sample Name	CV (%)	Accuracy (%)
0.100	QC 0.1	6.40	82.58
10.000	QC 10	3.50	108.45
500.000	QC 500	3.41	96.76

Table 6: Accuracy and Precision calculated from three QC series (Number values used: 9 of 9)

Reproducibility

To determine the reproducibility of the XLC-MS method a batch of 170 samples containing 100 ng/mL Warfarin in Serum are processed in an overnight run. Figure 7 displays the peak area uncorrected for internal standard. The calculated RSD is 3.9%.

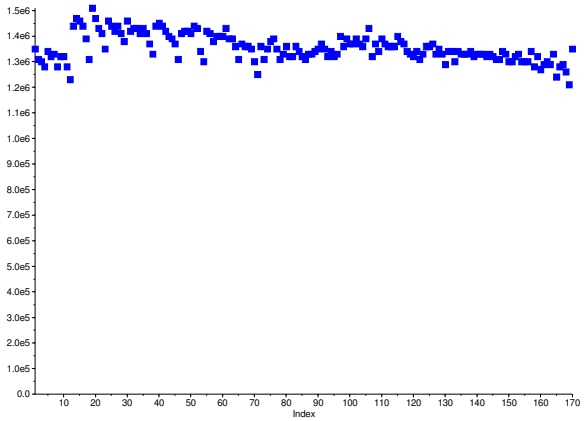

Figure 9: XLC-MS reproducibility, individual peak area of Warfarin prior to IS correction in an overnight run of 170 serum samples.

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 0.1-500 ng/mL (R^2 of 0.999) with an accuracy between 85-110% and a precision of <15% CV; and process a batch of 170 samples with a reproducibility of 3.9% RSD.

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 cycle time is 2.75 minutes per sample. The batch of 170 samples was processed in less than 7 hrs. 50 min. [(170 x 2.75 minutes) + 2.5 minutes].

About Spark

Since 1982 Spark has provided the HPLC and LC/MS markets with state-of-the-art autosamplers, column ovens and sample preparation solutions. Solid Phase Extraction with on-line elution into HPLC and LC/MS systems was pioneered by Spark and introduced in the early 90's. Spark, ISO 9001 certified, does basic research, product development, production, sales and marketing in-house, guaranteeing quality from start to finish. With 25% of the employees working in research and development Spark continues to invest in the future, making sure we can deliver the solutions you need to improve your business results. Innovation and quality are keywords when talking about our development efforts.

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