APPLICATION INFO

DETERMINATION OF ATORVASTATIN AND ITS ORTHO-, PARA-HYDROXY METABOLITES IN SERUM BY XLC-MS USING 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.

Atorvastatin a HMG-CoA reductase inhibitor (statins) is used with diet changes to reduce the amount of cholesterol and certain fatty substances in the blood. Lowering cholesterol and fats levels in blood may help to prevent heart disease, angina (chest pain), strokes, and heart attacks. Atorvastatin is usually measured together with the two metabolites Para- and Ortho-Hydroxy Atorvastatin in human serum. Deuterated isotopes are used as Internal Standard [IS].

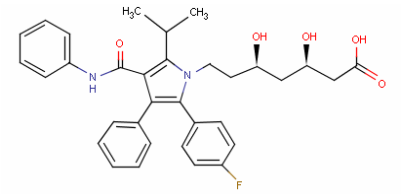

Figure 1 Atorvastatin

- CAS# 134523-00-5
- C33H35F1N2O5
- Mw 558.66
- Physical Properties:
  - Water solubility 0.01 mg/L
  - Log P (Octanol-water) 5.6
  - pKa dissociation constant 4.6

Method Development

The AMD mode of Symbiosis™ Pharma in conjunction with the HySphere method development cartridge tray (Spark PN 0722.650) 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 pre-defined SPE conditions of the Symbiosis™ Pharma.

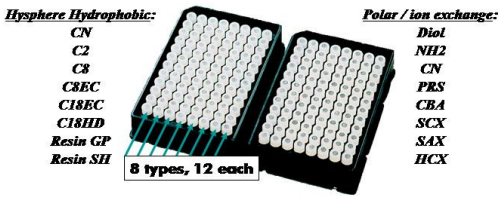

Figure 2: Method Development Cartridge Tray

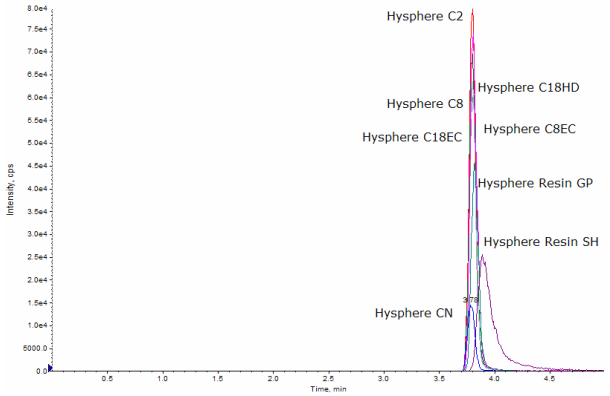

Figure 3: Chromatograms of Atorvastatin in serum after sorbent screening using the HySphere hydrophobic MD tray.

From Figure 3 can be derived that C2 gives the highest signal and also the best peak shape. The overall recovery >95%, is calculated from the peak area of a LC run with a neat solution and a XLC run with a serum sample (figure 4).

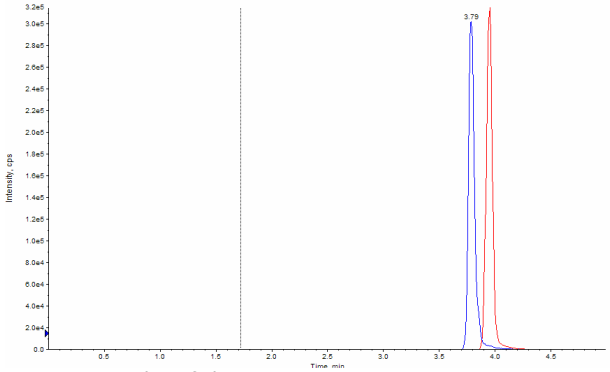

Figure 4: Overlay of chromatograms LC-run Atorvastatin in neat solution and a XLC-run of Atorvastatin in serum using the HySphere C2.

XLC-MS protocol

The serum samples are processed with the developed XLC-MS method (as described below) using Symbiosis™ Pharma and an 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 sample is injected using the partial loop fill injection routine.
Washing is performed with two wash solvents;
Wash solvent1: 50% ACN with 0.1 % Formic Acid.
Wash solvent 2: 90% ACN.

Wash solvent	Wash volume	Valve wash
1	700 µL	No
2	700 µL	No
1	700 µL	Yes
2	700 µL	Yes
1	1500 µL	Yes

Table 1: Autosampler wash routine.

SPE conditions

Cartridge:	10x2mm HySphere C2 (Spark Pn:0722.605)	
Solvation:	1 mL ACN	5 mL/min
Equilibration:	1 mL 5% ACN with 2% NH4OH	5 mL/min
Sample Loading:	1 mL 5% ACN with 2% NH4OH	2 mL/min
Washing:	1 mL 5% ACN with 2% NH4OH	5 mL/min
Elution	4 min. with Gradient LC	

Table 2: SPE settings.

LC conditions

Column:	Waters Nova Pak C18 3.9 x 150 mm. 4µ (Waters Pn: WAT086344)
Mobile phase A:	0.1% Formic Acid in Water.
Mobile phase B:	0.1% Formic Acid in ACN.
Run time:	5 minutes @ 0.5 ml/min

Time (mm:ss)	Flow (mL/min.)	A (%)	B (%)
00:01	0.5	60	40
00:05	0.5	60	40
02:25	0.5	0	100
03:00	0.5	0	100
03:30	0.5	60	40
05:00	0.5	60	40

Table 3: LC conditions

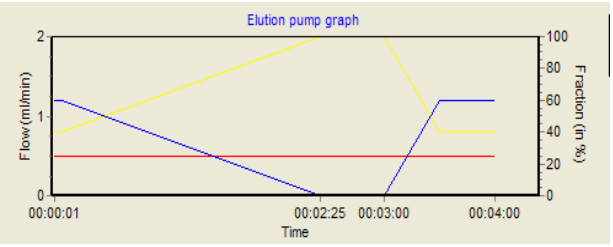

Figure 6: LC Conditions

MS Conditions

A Sciex API 3000 with a Turbo IonSpray is used. Before entering the MS the LC flow is spitted 1:1.

Neb:	
Cur:	20
IS:	4000
TEM:	450
CAD:	4.00
GS1	80
GS2	80

	Ator-vastatin	Ortho-Hydroxy	Para-Hydroxy
Q1	559.27	575.26	575.26
Q3	249.90	440.10	440.00
Dwell	150	150	150
DP	41	36	41
FP	220	370	370
EP	11	7	8
CE	65	29	27
CEP	26	24	24
CXP	8	10	10

Table 4: MS parameters

Results

The following samples are prepared in new born calf serum using Atorvastin-D5; Ortho- Atorvastin-D5 and Para- Atorvastin-D5 (100 ng/mL) as internal standard.

- Calibration standards: 0.5; 1.0; 5.0; 10; 50; 100; 500 and 1000 ng/mL.
- QC samples: 1.0; 50; and 500 ng/mL.

Chromatograms

Figures 6 and 8 are representative chromatograms of the upper and lower limits of the calibration standards indicate excellent quantitative suitability of the XLC-MS method. The carry over shown in the Blanc injection (figure 8) immediately after the HLOQ (figure 7) is calculated as <0.02%.

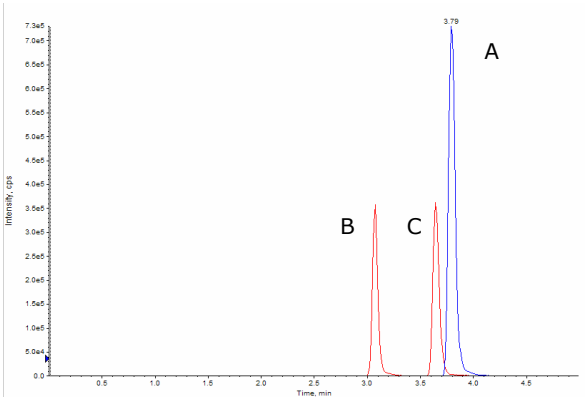

Figure 7: Chromatogram representing 1000 ng/mL Atorvastatin (A); Ortho- Hydroxy-Atorvastatin (B) and Para- Hydroxy-Atorvastatin(C).

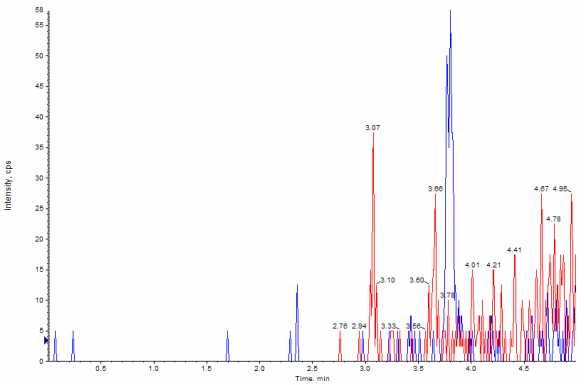

Figure 8: Chromatogram representing a blank

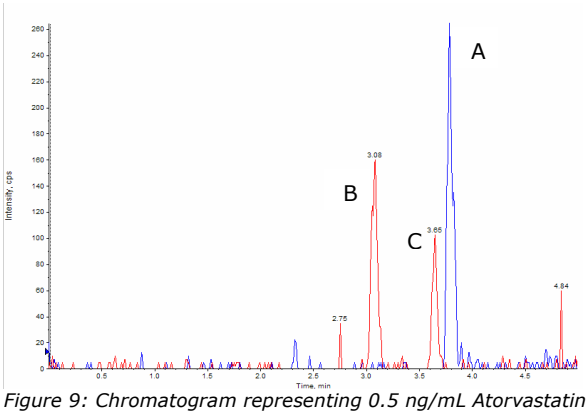

Figure 9: Chromatogram representing 0.5 ng/mL Atorvastatin (A); Ortho-Hydroxy-Atorvastatin(B) and Para- Hydroxy-Atorvastatin (C).

Linearity, Accuracy and Precision

A calibration curve was determined by combining the results of 3 repeated injections of the full set of calibration standards, using deuterated isotopes as internal standards. This resulted in a R^2 of **0.999** with a 1/X weighting for Atorvastatin and the metabolites Orto-Hydroxy-Atorvastatin and Para-Hydroxy-Atorvastatin.

Atorvastatin:

Exp. Conc. (ng/mL)	CV (%)	Accuracy (%)
0.50	18.8	108
1.00	13.2	100
5.00	5.04	95.8
10.00	3.77	99.1
50.00	4.32	98.7
100.0	2.37	96.7
500.0	2.97	97.4
1000.0	0.23	101

Table 5a: Accuracy and Precision calculated from three combined sets of calibration standards Atorvastatin.

	CV (%)	Accuracy (%)
QC-1	9.44	88.5
QC-2	3.54	97.0
QC-3	4.29	99.2

Table 5b: Accuracy and precision calculated from three combined sets of QC standards Atorvastatin.

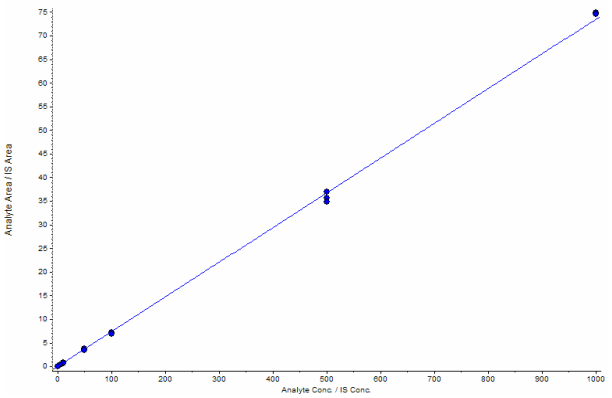

Figure 10: Atorvastatin Peak area vs. concentration from three sets of calibration standards.

Ortho-Hydroxy-Atorvastatin:

Exp. Conc. (ng/mL)	CV (%)	Accuracy (%)
0.50	19.2	100.
1.00	15.5	85.9
5.00	5.08	93.3
10.00	4.59	94.2
50.00	11.0	96.3
100.0	1.62	100.
500.0	1.81	97.0
1000.0	4.29	101.

Table 6a: Accuracy and precision calculated from three combined sets of calibration standards Ortho-Hydroxy-Atorvastatin.

Sample name	CV (%)	Accuracy (%)
QC-1	13.9	85.2
QC-2	3.05	93.4
QC-3	7.76	95.2

Table 6b: Accuracy and precision calculated from three combined sets of QC standards Ortho-Hydroxy-Atorvastatin.

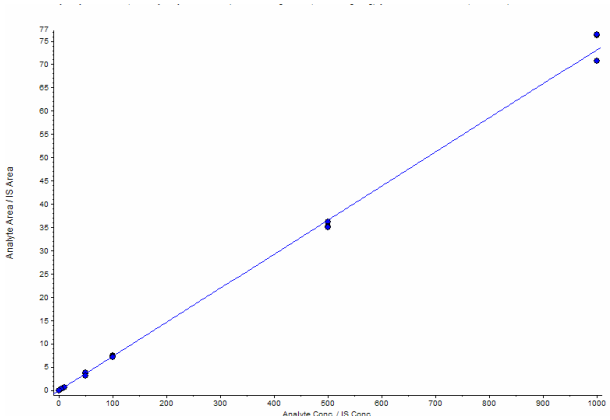

Figure 11: Ortho-Hydroxy-Atorvastatin Peak area vs. concentration from three sets of calibration standards.

Para-Hydroxy-Atorvastin:

Exp. Conc. (ng/mL)	CV (%)	Accuracy (%)
0.50	9.59	118
1.00	9.25	102
5.00	3.16	94.8
10.00	3.23	97.4
50.00	2.69	98.9
100.0	1.57	96.3
500.0	5.73	94.8
1000.0	3.01	103

Table 7a: Accuracy and precision calculated from three combined sets of calibration standards Para-Hydroxy-Atorvastatin.

Sample name	CV (%)	Accuracy (%)
QC-1	8.59	85.4
QC-2	2.60	98.9
QC-3	3.40	97.2

Table 7b: Accuracy and Precision calculated from three combined sets of QC standards Para-Hydroxy-Atorvastatin.

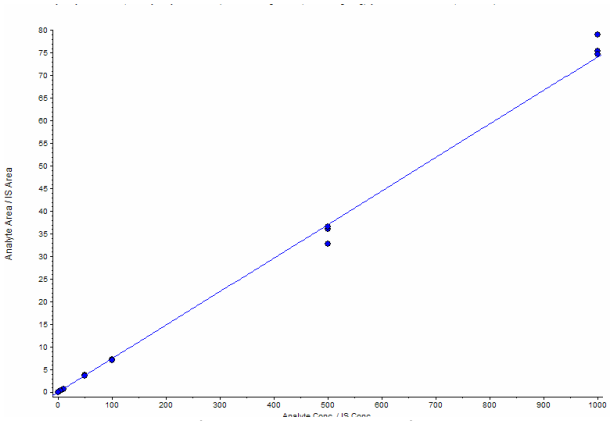

Figure 12: Para-Hydroxy-Atorvastatin Peak area vs. concentration from three sets of calibration standards

Reproducibility

To determine the reproducibility of the XLC-MS method a batch of 110 samples containing 100 ng/mL Atorvastatin in Serum are processed in an overnight run. Figure 13 displays the peak area corrected for internal standard (RSD is 3.1%).

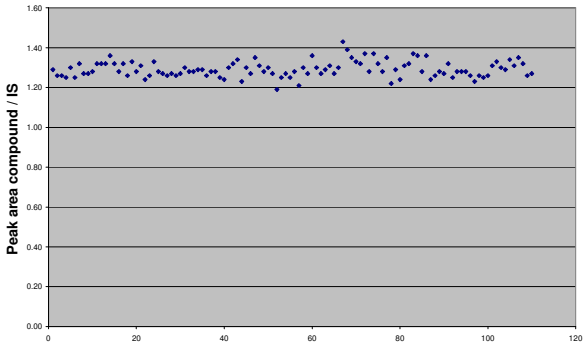

Figure 13: XLC-MS reproducibility, individual peak area of Atorvastatin over peak area IS in an overnight run of 110 serum samples.

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 >95% for Atorvastin and its metabolites; run the various sets of calibration standards with a linear range from 0.5-1000 ng/mL (R^2 of 0.999) with an accuracy between 85-115% and a precision of <15% CV; and process a batch of 110 samples with a reproducibility of 3.1%

The total XLC-MS cycle time consists of the sample preparation time + LC-MS time. Since the sample prep is executed in parallel with the LC, the total XLC-MS time is 5.0 minutes per sample. The batch of 110 samples was processed in 9 hrs.

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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