APPLICATION INFO

DETERMINATION OF RITONAVIR IN PLASMA BY ON-LINE 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 info shows the possibility to perform "quick sorbent screening" for the most suitable SPE cartridge and the optimal clean-up conditions for the determination of Ritonavir, with the use of the XLC-mode of Symbiosis™ Pharma together with the HySphere™ method development cartridge tray (Spark p.n. 0722.650).

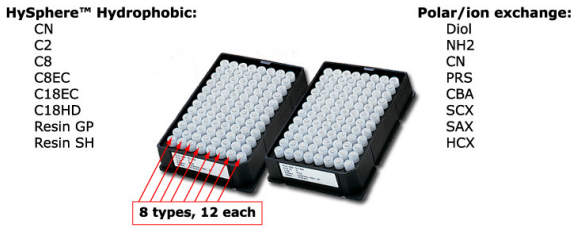
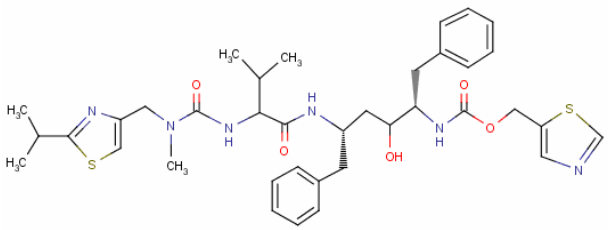


Figure 1: Method development cartridge tray

Ritonavir is used, alone or in combination with other medicines, in the treatment of the infection caused by the human immunodeficiency virus (HIV). HIV is the virus that causes acquired immune deficiency syndrome (AIDS). It is sold as Norvir ™ by Abbott Laboratories



Ritonavir, Mw 720.3 with LogP = 4.9,
$C_{37}H_{48}N_6O_5S_2$,CAS#155213-67-5,
water solubility: <<0.001 g/L

Method Development

Figure 2: Symbiosis™ Pharma

The following "quick sorbent screening" data was obtained in less than 1 hour using generic pre-defined SPE conditions of the Symbiosis™ Pharma.
From the range of cartridges available in the HySphere™ hydrophobic method development tray, the HySphere™ C18HD cartridge gives the highest signal and also the best peak shape (see figure 3).

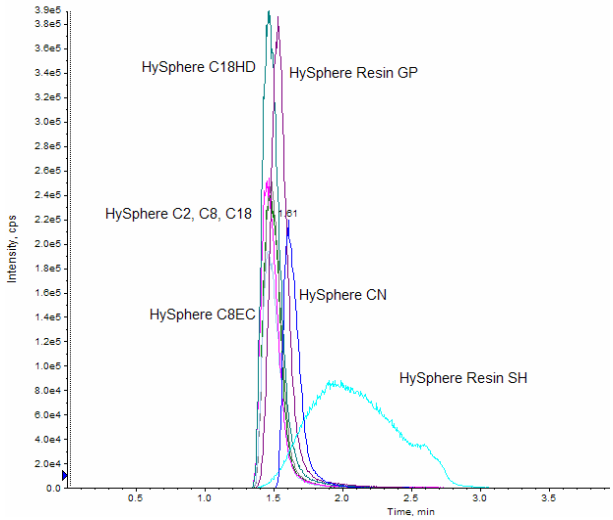


Figure 3: Chromatograms of Ritonavir in plasma after sorbent screening using the HySphere™ hydrophobic method development tray.

In this "sorbent screening" approach each run was closely monitored and the results were used for determining the next step in the process.
This process can also be performed unattended, but then the whole set of data must be evaluated afterwards to find the best conditions.

Recovery compared to a LC injection area is higher then 170%.

XLC–MS Protocol

Autosampler Conditions

50 µL of sample is injected using a standard Reliance™ autosampler configuration.
Washing is performed with two wash solvents;
Wash solvent 1: 50% ACN - 50% Water with 0.1% Formic Acid.
Wash solvent 2: 50 % ACN 1% HCl pH 1.5.

Table 1: Autosampler wash routine.

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

SPE conditions

Cartridge:	10 x 2 mm HySphere C18HD (Spark PN:0722.609)	
Solvation:	1 mL, ACN	5 mL/min
Equilibration:	1 mL, 30% ACN in 0.1 % FA	5 mL/min
Sample Loading:	1 mL, 30% ACN in 0.1 % FA	2 mL/min
Washing:	1 mL, 30% ACN in 0.1 % FA	5 mL/min
Elution	2 min. with LC Gradient	
Matrix:	Plasma	

LC conditions

Column:	Waters Sunfire C18 2.1X50 mm
Mobile phase A:	0.1% Formic Acid in Water
Mobile phase B:	0.1% Formic acid in ACN

Table 2: LC gradient

Time (mm:ss)	Flow (mL/min.)	A (%)	B (%)
00:01	0.25	35	65
00:05	0.25	35	65
02:00	0.25	5	95
02:30	0.25	5	95
03:00	0.25	35	65
04:00	0.25	35	65

MS Conditions

A Sciex API3000 with a Turbo IonSpray (ESI pos) is used.

Table 3: MS parameters

IS	5500
TEM	400
CAD	6
GS2	7
Neb	15

Table 4: Compound dependable MS settings

	Ritonavir	Ritonavir D6
Q1 mass	721.26	727.26
Q3 mass	296.1	302.1
Dwell time	150	150
ESI	Pos	Pos
DP	56	56
FP	270	270
EP	10	10
CE	29	29
CXP	26	26

Result

After the optimization of the SPE protocol the method was tested for reproducibility, linearity, precision and recovery.

The following samples are spiked in pure plasma. The samples contain 90% plasma.

- Calibration standards:0.25; 0.5; 1.0; 2.5; 5; 10; 25; 50; 100 ng/mL
- QC samples: 0.5; 10; 100 ng/mL

In the LC mode, the compound has hydrogen and sodium adducts. This sodium adduct is not available in the cleaned XLC extract.

Chromatograms

Two example chromatograms of the assay: one representing a plasma sample and one a blank.

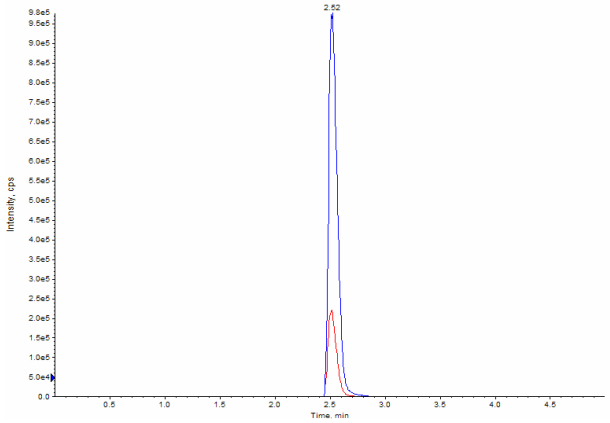


Figure 7: Chromatogram representing 100 ng/mL Ritonavir.

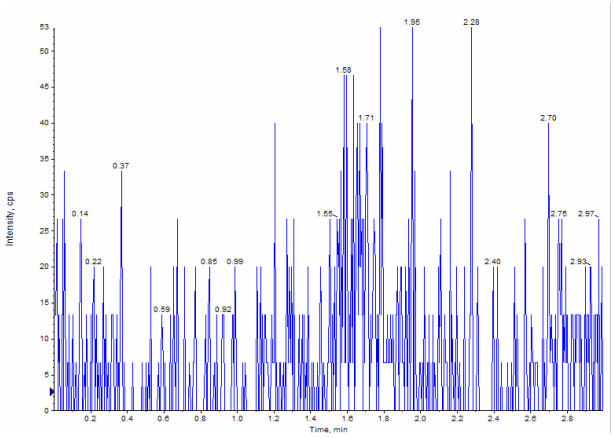


Figure 8: Chromatogram representing blank

Linearity, Accuracy and Precision

Linearity was evaluated by injecting a full set of calibration standards. Regression analysis of the calibration data was determined, with a correlation coefficient (r) of 0.9996 and a 1/X Weighting.

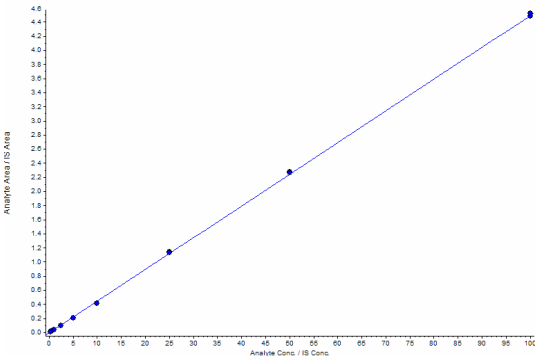


Figure 9: Calibration curve of Ritonavir R=0.9996

Table 5: Accuracy calculated from three combined sets of Calibration standards

Sample (ng/mL)	CV (%)	Accuracy (%)
0.25	2.19	115
0.5	3.97	104
1.0	0.52	94.1
2.5	2.63	89.7
5.0	1.20	92.6
10	0.21	92.6
25	0.45	101
50	0.56	101
100	0.63	100

Table 6: Accuracy and precision calculated from eight combined sets of QC standards.

Sample (ng/mL)	CV (%)	Accuracy (%)
0.50	2.44	107
10.0	2.36	92.6
100	2.21	94.3

Recovery

In the XLC mode, the HySphere C18HD peak area of the neat solution is 170 % compared to a standard LC injection. This is caused by the shifted equilibrium between hydrogen- and sodium adducts, since sodium is in the cleaned XLC extract.

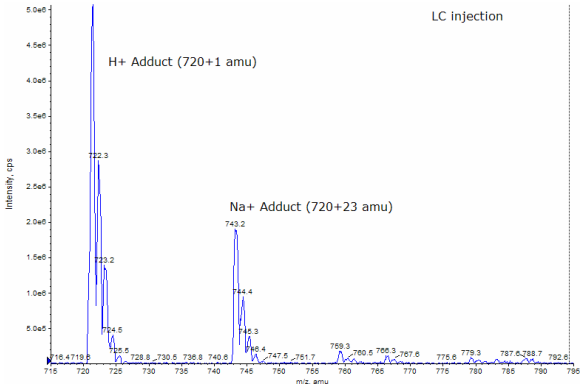


Figure 10: Hydrogen and Sodium adduct of Ritonavir in an LC injection.

Conclusions

The development of this assay on the **Symbiosis™ Pharma** was done in ~2 days, with optimization.

This study shows how to develop a XLC-MS method with a good recovery and a set of calibration standards with a linear range from 0.25 to 100 ng/mL (r = 0.9996) and an accuracy between 89-115%.

The carry-over is minimized by using the Valve Wash option of the Reliance™ autosampler, the carryover was reduced to levels below the detection limits.

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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