



DETERMINATION OF MIDAZOLAM IN SERUM BY XLC-MS USING SYMBIOSIS™ PHARMA

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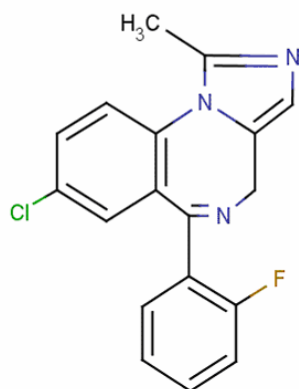
APPLICATION INFO

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).

Midazolam, is a benzodiazepine and is insoluble in water (pH=7), but is soluble in aqueous solutions under acidic conditions.

Chemically, midazolam HCl is 8-chloro-6-(2-fluorophenyl)-1-methyl-4 H -imidazo[1,5-a][1,4]benzodiazepine hydrochloride.



Midazolam, Mw = 325.77 with LogP = 3.13, C₁₈H₁₃ClFN₃, CAS#59467-70-8, Water solubility 10.0 g/L at pH 3.4

Midazolam is used to produce sleepiness or drowsiness and to relieve anxiety before surgery or certain procedures. It is also used to produce loss of consciousness before and during surgery.

Method Development



Figure 1: Symbiosis™ Pharma

The XLC mode of Symbiosis™ Pharma in conjunction with the HySphere™ method development cartridge tray (Spark p.n. 0722.650) enables "quick sorbent screening" for the most suitable SPE cartridge and optimal clean-up conditions.

The following data was obtained in less than 1 hour using generic pre-defined SPE conditions of Symbiosis™ Pharma.

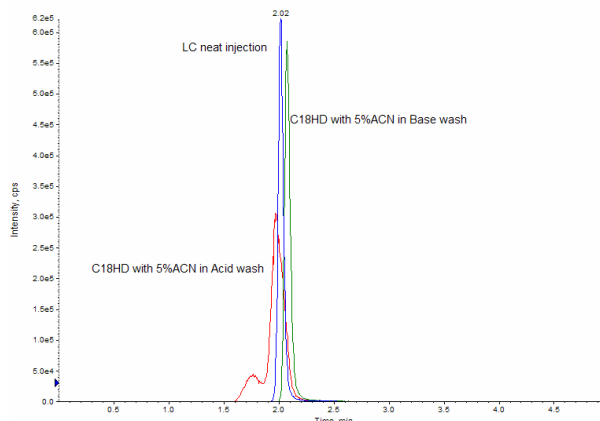


Figure 2: Chromatograms of Midazolam in serum after sorbent screening using the HySphere™ hydrophobic MD tray. Here only the C18HD results are shown.

From figure 2 can be derived that the HySphere™ C18HD cartridge with base wash gives the highest signal and also the best peak shape.

With the HySphere™ C18HD cartridge and SPE optimization the recovery compared to an LC injection area is higher than 90%.

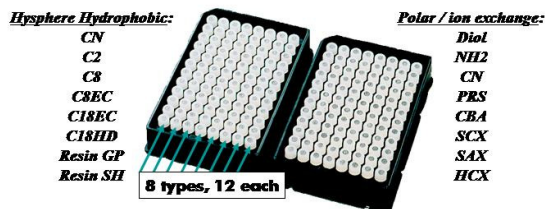


Figure 3: Method development cartridge tray

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



XLC-MS Protocol

Autosampler conditions

20 μL of sample is injected using a standard autosampler configuration. Washing is performed with two wash solvents; Wash solvent 1: 40% ACN - 60% Water with 0.1% formic acid. Wash solvent 2: 90% ACN - 10% Water.

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 10% ACN in 1% NH_4OH	5 mL/min
Sample Loading:	0.5 mL 10% ACN in 1% NH_4OH	1 mL/min
Washing:	1 mL 10% ACN in 1% NH_4OH	5 mL/min
Elution	1 min with LC mobile phase	
Matrix:	Serum	

LC conditions

Column:	Waters Xterra C18 ms 3 mm x 50 mm 3 μ
Mobile phase A:	2 mM Ammonium acetate in water
Mobile phase B:	ACN
Flow	1.0 mL/min
Mobile Phase	30 % Mobile Phase A/ 70% Mobile Phase B

MS conditions

A Sciex API 3000 with a Turbo IonSpray is used. The LC flow is split 1 to 4 before entering the MS.

Table 3: MS parameters

Neb	9
Cur	10
IS	5000
TEM	450
CAD	4
Gas	7000

Table 4: Compound dependable MS settings

	Midazolam	Midazolam D5
Q1 mass	326.3	331.1
Q3 mass	291.1	296.2
Dwell time	150	150
DP	39	39
FP	300	300
EP	10	10
CE	39	39
CXP	16	16

Result

The following samples are prepared in human plasma.

- Calibration standards: 1.0; 2.0; 5.0; 10; 50; 100; 150; 200; 250 ng/mL
- QC samples: 1; 2; 125; 180; 250 ng/mL

Chromatograms

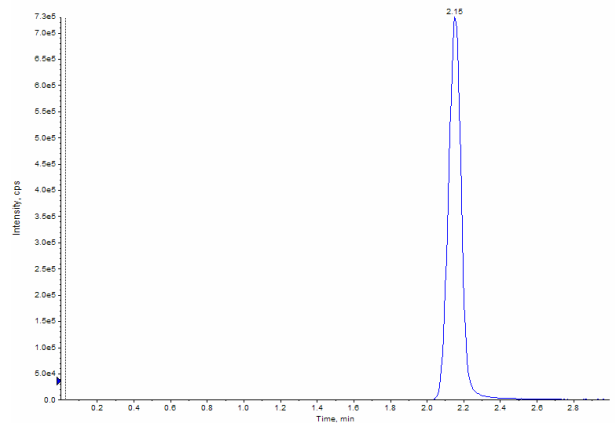


Figure 4: Chromatogram of real sample representing 250 ng/mL Midazolam.

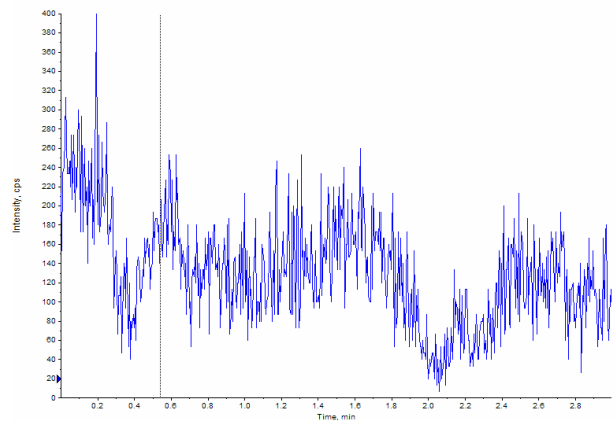


Figure 5: Chromatogram representing blank plasma after a 250 ng/mL sample.

Linearity, Accuracy and Precision

A calibration standard was determined by injecting five sets of calibration standards and QCs. All points are used to determine the calibration curve with $r = 0.9979$ using a $1/X$ weighting.

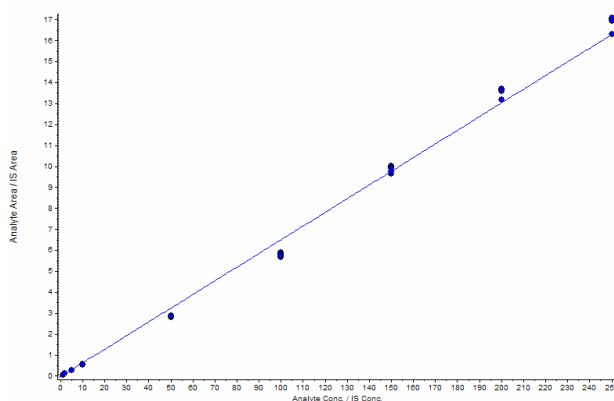


Figure 6: Calibration curve of Midazolam

Table 5: Accuracy and precision of 5 combined calibration curves

Sample (ng/mL)	CV (%)	Accuracy (%)
1	6.34	115
2	1.96	111
5	0.35	89.7
10	1.56	89.6
50	1.05	88.5
100	1.56	89.0
150	1.59	100.9
200	1.53	103.8
250	1.93	103.5

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

Sample (ng/mL)	CV (%)	Accuracy (%)
QC 1	1.66	83.5
QC 2	2.11	98.8
QC 125	1.64	100
QC 180	2.61	101
QC 250	1.40	105

Conclusions

The development of this assay on the **Symbiosis™ Pharma**, demonstrated the speed of transfer from an off-line, to an on-line XLC-MS assay, (~2 days, with optimization).

This study shows how to develop a XLC-MS method with an absolute recovery >90% and a set of calibration standards with a linear range from 1 to 250 ng/mL ($r = 0.9979$) and an accuracy between 86-115%. The carry-over is minimized by using the Valve Wash option of the Reliance™ autosampler and not detectable with the used detection method.

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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