APPLICATION INFO

Determination of Vinorelbine in Rat Plasma by XLC-MS using the Symbiosis™ Pharma System

March 2006

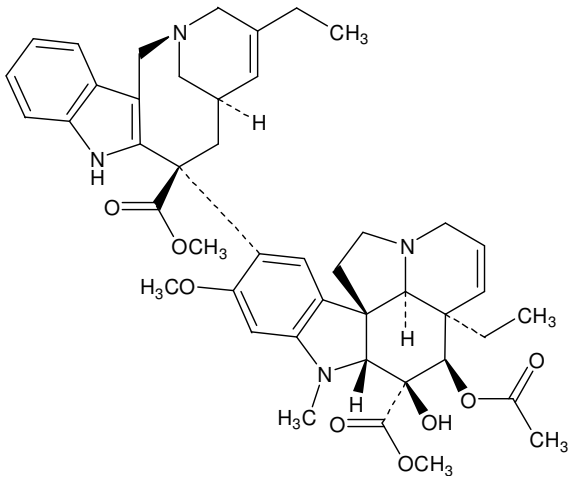
Charles River Laboratories
Preclinical Services Montreal

Introduction

The **Symbiosis™ Pharma** is Spark Holland's unique solution for integrated on-line 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, or AMD (Advanced Method Development).

A sensitive, specific, accurate and precise assay was developed for the on-line extraction and determination of vinorelbine in rat plasma by XLC-MS, using the **Symbiosis™ Pharma**. This method was developed as part of an evaluation, to introduce this new technology into our bioanalytical laboratory. This application note demonstrates the ease of transfer of an off-line sample pretreatment to an on-line protocol, with a concomitant increase in productivity.

Vinorelbine is a semi-synthetic derivative of vinblastine, a vinca alkaloid, found in the Madagascar periwinkle. This compound has demonstrated a wider range of antitumor activity than the other vinca alkaloids, and is commonly used in the treatment of breast cancer and non-small-cell lung cancer.



Vinorelbine, $C_{45}H_{54}N_4O_8$, MW 778.95

Vinorelbine acts at the cellular level by binding to tubulin, inhibiting the cells ability to make mitotic spindles in metaphase, thus preventing cell division. The compound also interferes with the cells ability to synthesize DNA and RNA.

Previous Assay

Our previous assay involved off-line protein precipitation, with acetonitrile:methanol:trifluoroacetic acid (50:50:0.1, v/v), which was diluted with mobile phase prior to analysis. A reverse phase LC-MS cycle-time of 25 min was employed to allow for a column wash, in addition to keeping system carry-over to within FDA bioanalytical guidelines (≤ 20% of the LLOQ response), due to the "sticky" nature of this compound.

Typical preparation time for 80 samples was two thirds of an average working day (7.5 hours), followed by a day and a half run (~33 hours). Therefore, a batch of samples would take approximately 38 hours to analyse from start to finish.

XLC-MS Protocol

During the evaluation, our previously developed assay was transferred onto the **Symbiosis™ Pharma.** An SPE elution profile was developed overnight using XLC mode with a spiked plasma sample at the desired ULOQ. This profile was later optimized for breakthrough, recovery and carry-over using AMD mode.

Autosampler Conditions

Control rat plasma (K_2 EDTA, 50 µL) was spiked with a vinorelbine standard solution (5 µL), and then diluted with internal standard working solution (200 µL; methanol:water:control rat plasma 5:85:10, v/v/v). This sample cocktail was gently mixed, centrifuged and then injected using the **Reliance™** autosampler with µL-Pickup mode, to ensure the preservation of sample volume.

Autosampler Wash Solvents

Wash Solvent 1	Methanol:Water (5:95, v/v)
Wash Solvent 2	Methanol:Trifluoroacetic Acid (100:0.1, v/v)
Wash Solvent 3	Methanol:Ammonium Hydroxide (100:0.2, v/v)

Autosampler Wash Routine

Wash Solvent	Wash Volume	Valve Wash
1	500 µL	No
2	1500 µL	Yes
3	1500 µL	Yes
1	500 µL	Yes

SPE Conditions

Cartridge	10 x 2 mm HySphere-C18 HD, 7 µm (Spark PN:0722.609)	
Solvation 1	Methanol:Ammonium Hydroxide - 1 mL (100:0.2, v/v)	5 mL/min
Solvation 2	Methanol:Trifluoroacetic Acid - 1 mL (100:0.1, v/v)	5 mL/min
Equilibration	Methanol:Water:Trifluoroacetic Acid - 1 mL (10:90:0.1, v/v/v)	2.5 mL/min
Sample Loading	Methanol:Water:Trifluoroacetic Acid - 1 mL (10:90:0.1, v/v/v)	2 mL/min
Washing	Methanol:Water:Trifluoroacetic Acid - 1 mL (10:90:0.1, v/v/v)	2.5 mL/min
LC Elution	4:00 min, with LC Pump	1.25 mL/min
Clamp Wash 1	Methanol:Ammonium Hydroxide - 0.5 mL (100:0.2, v/v)	2.5 mL/min
Clamp Wash 2	Methanol:Trifluoroacetic Acid - 0.5 mL (100:0.1, v/v)	5 mL/min
Clamp Wash 3	Methanol:Water:Trifluoroacetic Acid - 0.5 mL (10:90:0.1, v/v/v)	2.5 mL/min
Valve Wash	Methanol:Trifluoroacetic Acid (100:0.1, v/v)	-
Sample Matrix	Control Rat Plasma (K_2 EDTA) - 50 µL, diluted with Methanol:Water:Control Rat Plasma (5:85:10, v/v/v) - 500 µL	

LC Conditions

Column	Phenomenex® Onyx™ C_{18}, 4.6 x 50 mm
Mobile Phase A	5 mM Ammonium Acetate in Methanol:Water:Trifluoroacetic Acid (10:90:0.025, v/v/v)
Mobile Phase B	5 mM Ammonium Acetate in Methanol:Water:Trifluoroacetic Acid (90:10:0.025, v/v/v)

LC Gradient

Time (mm:ss)	Flow (mL/min)	A (%)	B (%)
00:01	1.25	50	50
01:45	1.25	0	100
03:00	1.25	0	100
03:01	2.50	0	100
04:00	2.50	0	100
04:01	2.00	50	50
04:30	2.00	50	50

MS Conditions

MDS Sciex API 3000™ LC-MS/MS System - Ionics™ HSID Interface Flow Dependent Parameters

Parameter	Setting
Ionisation Mode	(+) ESI
Curtain Gas (N_2)	12 dac
Ion Spray Voltage	5500 V
Source Temperature	550 °C
HSID Temperature	300 °C
Nebulizer Gas (Zero Air)	12 dac
Auxiliary Gas (Zero Air)	8 L/min
Interface Heater	On
CAD Gas (N_2)	6 dac

MDS Sciex API 3000™ LC-MS/MS System - Ionics™ HSID Interface Mass Dependent Parameters

Parameter	Vinorelbine	Internal Standard (Vinblastine)
Scan Mode	Multiple Reaction Monitoring	
Q1/Q3 Resolution	Unit/Unit	
Q1 mass (amu)	779.4	811.5
Q3 mass (amu)	122.1	224.2
Dwell Time (ms)	200	100
Declustering Potential (V)	30	30
Deflector Potential (V)	50	50
Entrance Potential (V)	-10	-10
Collision Energy (eV)	80	60
Collision Exit Potential (V)	8	7

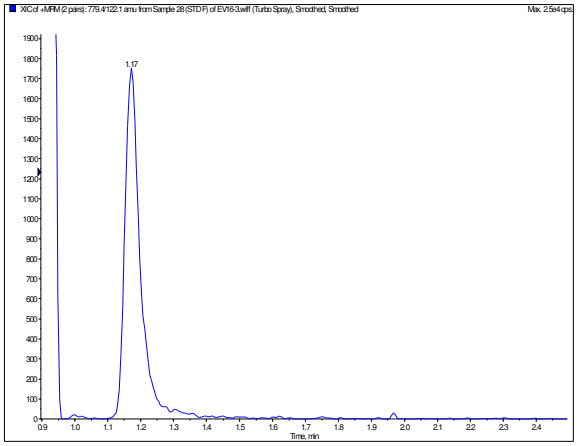
Results

The following samples are prepared in control rat plasma:

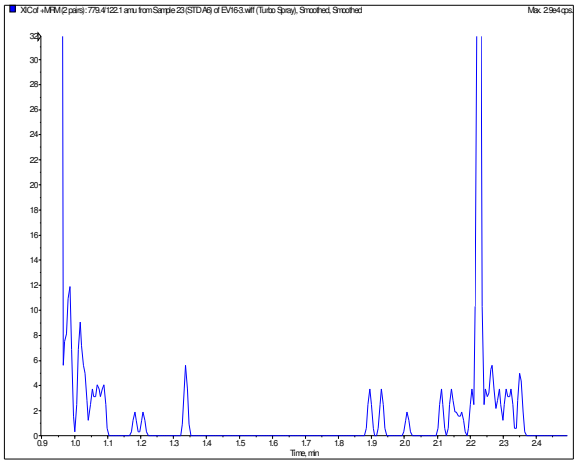
- Calibration Standards: 20, 40, 80, 100, 200, 400, 800, 1000, 2000, 4000, 8000, 10000 and 20000 ng/mL
- QC Samples: 20, 60, 750 and 17000 ng/mL, as 6 replicates for each concentration, from 6 individual plasma lots
- Double Blanks, from 6 individual plasma lots
- Single Blanks, from 6 individual plasma lots

Typical Chromatography

200 ng/mL Vinorelbine in Rat Plasma (RT 1.17 min)



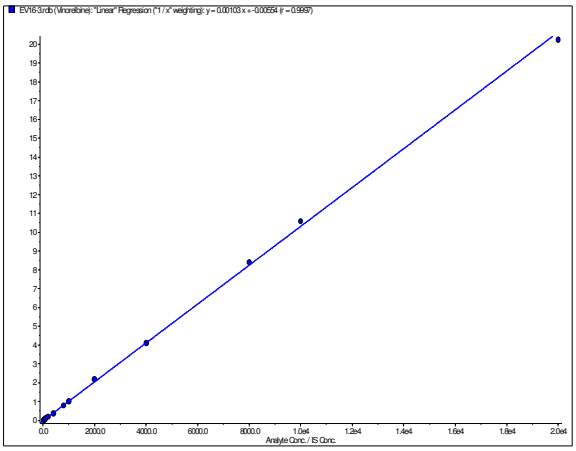
Rat Plasma Double Blank



Linearity, Accuracy, Precision And Carry-Over

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 0.9997 and a 1/concentration weighting.

Vinorelbine Calibration Curve - 20 to 20000 ng/mL



Calibration Standard Back-Calculated Accuracy

Calibration Standard (ng/mL)	Accuracy (%)
20 (LLOQ)	117
40	97.8
80	87.5
100	105
200	99.1
400	91.8
800	96.5
1000	97.7
2000	106
4000	99.7
8000	102
10000	103
20000 (ULOQ)	98.0

(Acceptance Criteria: ≤ ±15% of the Theoretical Value; ≤ ±20% for the LLOQ)

QC Sample Mean Accuracy And Precision Over 6 Individual Plasma Lots

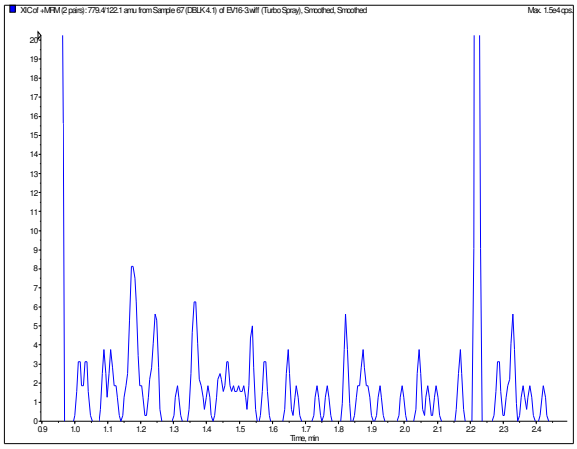
QC Sample (ng/mL)	Acceptable Replicates	Accuracy (%)	CV (%)
20	4/6	113	4.3
60	6/6	99.6	6.7
750	6/6	95.6	3.7
17000	6/6	100	4.6

(Acceptance Criteria: ≤ ±15% of the Theoretical Value; ≤ ±20% for the LLOQ)

Immediate And Accumulative Carry-Over

Double blank samples injected immediately after the ULOQ, and after 6 replicate injections of the high QC sample (17000 ng/mL), showed the absence of any obvious peak.

Rat Plasma Double Blank After 6 High QC Sample (17000 ng/mL) Injections



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, (~1.5 days, with optimization).

Off-line sample preparation time was reduced from two thirds to one third of a typical working day. In addition, the automated overlap between the extraction of the "next" sample, during the chromatographic run of the "current" sample, the production of a "cleaner" final extract (versus protein precipitation), and the reduced carry-over from the **Reliance™** autosampler, allowed for an XLC-MS cycle-time of 6.0 min. Therefore, in comparison with our previous assay, a typical batch size of 80 samples would take approximately 10.5 hours to analyse from start to finish, versus 38 hours. Faster analysis times could be achieved with further optimization of the **Symbiosis™ Pharma.**

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.

Spark System Solutions BV
Bendienplein 5
7815 SM Emmen, the Netherlands

P +31 591631700
F +31 491645900
E Solutions@Sparkholland.com

About Charles River Laboratories Montreal

Charles River Laboratories Montreal, one of the world's largest toxicology CROs, provides a comprehensive range of services dedicated to *in vitro* and *in vivo* drug metabolism / toxicokinetics / pharmacokinetics, and extensive analytical chemistry and bioanalytical capabilities to support both preclinical and clinical trials. Specialties include LC-MS/MS, immunology, immunoassay, CYP450, and QWBA assessments.

Charles River Laboratories Montreal Inc.
22022 Transcanadienne
Senneville, Quebec, H9X 3R3, Canada

P +1 (514) 6308200
F +1 (514) 6308230
E bioanalysis@ca.crl.com