On-line SPE LC/MS/MS Configurations for Effective Biological Sample Preparation

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Abstract

During the last ten years, pharmaceutical companies have constantly pushed for shorter analysis time in order to breach the one-thousand-analyses-per-day barrier. With this demand for high speed analysis, new techniques, such as 96-well plates, fast gradients or ultra-high-flow chromatography, are showing promising results.

Recently, we have focused our attention toward on-line extraction techniques for high-throughput analysis. We are injecting a plasma sample, without pretreatment, onto an extraction column at high flow rate (i.e. 4mL/min) (1-3) to remove macromolecular compounds such as proteins, but trap smaller analytes on the head of the column.
Several configurations for direct injection are possible. In the simplest configuration, the extraction column is connected directly to the MS/MS system. Other versions are configured with a single or a dual extraction column coupled to an analytical column. It is often necessary to split the flow. However, in cases where sensitivity is low, this option is not recommended. For efficient high speed analysis, the use of a second pump and a 10 port valve is also a good choice. One line (high flow rate) can be dedicated to the extraction column, while the other (low flow rate) drives the analytical column and the mass spectrometer.
Abstract (continued)

Three different configurations were studied for the analysis of 2 basic drugs in rat plasma. The on-line analysis was performed on an Oasis® HLB extraction column (2.1x20mm, 25 µm) using a Waters Alliance 2790™ in the gradient mode and a 515 stand-alone pump in the isocratic mode. In the dual column configuration, the analytes were backward flushed onto an XTerra column (2.1x30, 3.5 µm) or forward flushed onto a Symmetry column (2.1x30, 3.5 µm). These columns were added to provide additional separation power. The drugs were quantified using a MicroMass Ultima™ triple quadrupole mass spectrometer equipped with an electrospray source and operated in the multiple reaction monitoring mode (MRM).
Common Setup for On-line SPE-LC/MS/MS

This is a common configuration for on-line analysis. Unfortunately, the high loading flow rate is not compatible with the electrospray source. In most applications, the flow is split, resulting in loss of sensitivity.
10 Port Valve Setup for On-line SPE-LC/MS/MS

- **Isocratic HPLC** (4 mL/min)
- **Gradient HPLC** (0.4 mL/min)
- **Oasis® HLB extraction column**
- **10 Port Valve (2 positions)**
- **Mass Spectrometer**
- **C18 Column**
- **Waste**

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On-line Oasis® HLB Extraction Column

- Features
  - Direct plasma injection
  - >100 injections per column
  - Compatible with rapid HPLC gradients
  - Fast cycle time for MS detection

- Description:
  - Sorbent: Oasis® HLB
  - 2.1 mm I.D. x 20 mm 25 µm

Oasis is a trademark of Waters Corporation
# On-line Protocol for Clemastine in Rat Plasma

**Oasis® HLB Extraction Column**

**Goals:** High Loading Flow Rate and Low Elution Flow Rate

**Oasis® HLB Extraction Column**

2.1x20 mm, 25 µm

| Sample preparation: centrifuge rat plasma |
| Sample preparation: Spike 5 mL of rat plasma + 100 µL NH₄OH |
| Sample preparation: 500 µL of spiked rat plasma + 400 µL IS in water |
| Loading: 200 µL at 4 mL/min in 100 % water |
| Elution: 0.4 mL/min gradient 5% ACN to 95 % ACN in 1 minute |
**HPLC Gradient and Wash Conditions**

<table>
<thead>
<tr>
<th>Time</th>
<th>HPLC gradient Flow 4 mL/min</th>
<th>Valve position</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>A 5 B 95</td>
<td>Function</td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td>switch position 2 to 1</td>
</tr>
<tr>
<td>1.0</td>
<td>95 5</td>
<td>switch position 1 to 2</td>
</tr>
<tr>
<td>2.60</td>
<td>95 5</td>
<td></td>
</tr>
<tr>
<td>2.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>5 95</td>
<td></td>
</tr>
</tbody>
</table>

A - Acetonitrile + 0.5 % Formic Acid  
B - Water + 0.5 % Formic Acid
**Oasis® HLB Column Configuration**

**Load position**

- **515 SYSTEM**
- **ALLIANCE HT**
- **MASS SPECTROMETER**
- **WASTE**

**Injection position**

- **515 SYSTEM**
- **ALLIANCE HT**
- **MASS SPECTROMETER**
- **WASTE**

**Specifications**

- **LC₁**: Alliance 2790 - 0.4 mL/min
- **LC₂**: Waters 515 - 4.0 mL/min
- **Loading mobile phase**: 100 % water
- **Eluting mobile phase**: 1 minute gradient 5% ACN to 95% ACN
- **Eluting mobile phase additive**: 0.5 % Formic acid
- **Extraction column temperature**: 40 °C
- **Switching valve**: Rheodyne LabPro 10 ports, 2 position

- **MS**: Quattro Ultima Triple Quadrupole
- **Source**: Electrospray positive
- **Source temperature**: 150 °C
- **Desolvation gas**: 600 L/hr
- **Gas cell**: 1.5e-3 mbar
- **Cone Voltage**: 20 volts
- **Collision energy**: 20
Oasis® HLB LC/MS/MS Analysis of Clemastine at 2.5 ng/mL and 250 ng/mL
# Oasis® HLB Clemastine Calibration Curve

<table>
<thead>
<tr>
<th>Conc. ng/mL N=6</th>
<th>1.0</th>
<th>2.5</th>
<th>5.0</th>
<th>10.0</th>
<th>100</th>
<th>200</th>
<th>250</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>0.98</td>
<td>2.56</td>
<td>5.25</td>
<td>9.5</td>
<td>101.43</td>
<td>201.14</td>
<td>247.1</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.02</td>
<td>0.11</td>
<td>0.12</td>
<td>0.25</td>
<td>2.69</td>
<td>3.2</td>
<td>1.58</td>
</tr>
<tr>
<td>RSD %</td>
<td>2.1</td>
<td>4.4</td>
<td>2.3</td>
<td>2.7</td>
<td>2.6</td>
<td>1.6</td>
<td>0.6</td>
</tr>
</tbody>
</table>

**Response**

- Coefficient of Determination: 0.999397
- Calibration curve: 1.59441e-6 * x^2 + 0.0733900 * x + 0.00620068
- Response type: Internal Std (Ref 1), Area * (IS Conc. / IS Area)
- Curve type: 2nd Order, Origin: Exclude, Weighting: 1/x^2, Axis trans: None
**Oasis® HLB/XTerra® Columns Configuration**

<table>
<thead>
<tr>
<th>Load position</th>
<th>Injection position</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>515 SYSTEM</strong></td>
<td><strong>515 SYSTEM</strong></td>
</tr>
<tr>
<td><strong>OASIS</strong></td>
<td><strong>OASIS</strong></td>
</tr>
<tr>
<td><strong>WASTE</strong></td>
<td><strong>WASTE</strong></td>
</tr>
<tr>
<td><strong>ALLIANCE HT</strong></td>
<td><strong>ALLIANCE HT</strong></td>
</tr>
<tr>
<td><strong>MASS SPECTROMETER</strong></td>
<td><strong>MASS SPECTROMETER</strong></td>
</tr>
<tr>
<td><strong>ANALYTICAL COLUMN</strong></td>
<td><strong>ANALYTICAL COLUMN</strong></td>
</tr>
</tbody>
</table>

**LC$_1$:** Alliance 2790 - 0.4 mL/min  
**LC$_2$:** Waters 515 - 4.0 mL/min  
Loading mobile phase: 100 % water  
Eluting mobile phase: 1 minute gradient 5% ACN to 95% ACN  
Eluting mobile phase additive: 0.5 % Formic acid  
Extraction column temperature: 40 °C  
Switching valve: Rheodyne LabPro 10 ports, 2 position

**MS: Quattro Ultima Triple Quadrupole**  
Source: Electrospray positive  
Source temperature: 150 °C  
Desolvation gas: 600 L/hr  
Gas cell: 1.5e-3 mbar  
Cone Voltage: 20 volts  
Collision energy: 20
Oasis® HLB/XTerra® LC/MS/MS Analysis of Clemastine at 2.5 ng/mL and 250 ng/mL

Clemastine 250 ng/mL

IS

100%
0%

1.33
1.51

MRM of 3 Channels ES+
344.1 > 214.9
2.62e6

Clemastine 2.5 ng/mL

IS

100%
0%

1.33
1.52

MRM of 3 Channels ES+
344.1 > 214.9
1.97e4
Oasis® HLB/XTerra® Clemastine Calibration Curve

<table>
<thead>
<tr>
<th>Conc. ng/mL N=6</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>25</th>
<th>200</th>
<th>250</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>1.01</td>
<td>4.8</td>
<td>9.7</td>
<td>20.3</td>
<td>24.3</td>
<td>206.9</td>
<td>243.1</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.01</td>
<td>0.1</td>
<td>0.2</td>
<td>0.9</td>
<td>0.9</td>
<td>4.2</td>
<td>3.1</td>
</tr>
<tr>
<td>RSD %</td>
<td>1.3</td>
<td>2.0</td>
<td>1.7</td>
<td>4.4</td>
<td>3.5</td>
<td>2.0</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Coefficient of Determination: 0.998500
Calibration curve: $5.01311 \times 10^{-5} \times x^2 + 0.0172052 \times x + 0.0166854$
Response type: Internal Std (Ref 1), Area * (IS Conc. / IS Area)
Curve type: 2nd Order, Origin: Exclude, Weighting: 1/x^2, Axis trans: None

Graph showing the calibration curve with concentration (ng/mL) on the x-axis and response on the y-axis.
Oasis® HLB/Symmetry Columns Configuration

**Load position**

- **515 SYSTEM**
- **ALLIANCE HT**
- **WASTE**
- **MASS SPECTROMETER**
- **ANALYTICAL COLUMN**

**Injection position**

- **515 SYSTEM**
- **ALLIANCE HT**
- **WASTE**
- **MASS SPECTROMETER**
- **ANALYTICAL COLUMN**

**Parameters:**

- **LC$_1$:** Alliance 2790 - 0.4 mL/min
- **LC$_2$:** Waters 515 - 4.0 mL/min
- **Loading mobile phase:** 100 % water
- **Eluting mobile phase:** 1 minute gradient 5% ACN to 95% ACN
- **Eluting mobile phase additive:** 0.5 % Formic acid
- **Extraction column temperature:** 40 °C
- **Switching valve:** Rheodyne LabPro 10 ports, 2 position

- **MS: Quattro Ultima Triple Quadrupole**
- **Source:** Electrospray positive
- **Source temperature:** 150 °C
- **Desolvation gas:** 600 L/hr
- **Gas cell:** 1.5e-3 mbar
- **Cone Voltage:** 20 volts
- **Collision energy:** 20
Oasis® HLB/Symmetry LC/MS/MS Analysis of Clemastine at 2.5 ng/mL and 250 ng/mL
## HLB/Symmetry® Clemastine Calibration Curve

<table>
<thead>
<tr>
<th>Conc. ng/mL N=6</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>25</th>
<th>200</th>
<th>250</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>1.01</td>
<td>4.7</td>
<td>10.4</td>
<td>19.7</td>
<td>25.1</td>
<td>205.7</td>
<td>244.4</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.007</td>
<td>0.1</td>
<td>0.5</td>
<td>0.8</td>
<td>1.1</td>
<td>3.4</td>
<td>2.4</td>
</tr>
<tr>
<td>RSD %</td>
<td>0.7</td>
<td>2.8</td>
<td>4.3</td>
<td>4.0</td>
<td>4.2</td>
<td>1.7</td>
<td>1.0</td>
</tr>
</tbody>
</table>

### Graph

- **Coefficient of Determination:** 0.999149
- **Calibration curve:** $9.46689e-5 \times x^2 + 0.0333011 \times x + 0.00334407$
- **Response type:** Internal Std (Ref 1), Area $\times$ (IS Conc. / IS Area)
- **Curve type:** 2nd Order, Origin: Exclude, Weighting: $1/x^2$, Axis trans: None

![Graph showing the Clemastine Calibration Curve](graph.png)
Benefits of the Oasis® HLB Extraction Column

- No off-line sample preparation
- Rapid isolation of analyte from a complex sample matrix
- Rapid clean up
- Short cycle time (3.0 min)
Dual HLB/XTerra® Columns Configuration

Position 1: Load HLB 1 / Elute HLB 2

- LC1: Alliance 2790 - 0.4 mL/min
- LC2: Waters 515 - 4.0 mL/min
- Loading mobile phase: 100 % water
- Eluting mobile phase: 1 minute gradient 5% ACN to 95% ACN
- Eluting mobile phase additive: 0.5 % Formic acid
- Extraction column temperature: 40 °C
- Switching valve: Rheodyne LabPro 10 ports, 2 position

Position 2: Load HLB 2 / Elute HLB 1

- MS: Quattro Ultima Triple Quadrupole
- Source: Electrospray positive
- Source temperature: 150 °C
- Desolvation gas: 600 L/hr
- Gas cell: 1.5e-3 mbar
- Cone Voltage: 20 volts
- Collision energy: 20