Simultaneous Determination of Six Protease/Reverse Transcriptase Inhibitors in Human Plasma Utilizing LC/MS/MS

Several newly introduced inhibitors of HIV-encoded protease have garnered widespread excitement and acceptance for their ability to increase CD4+ counts and reduce viral load. Combination therapies involving reverse transcriptase inhibitors, antifungals, and protease inhibitors have been very successful and are likely to continue. Also, there is interest in screening potential clinical trial subjects in order to confirm the absence of other experimental medications. For these reasons, we have developed a single method which can profile the protease inhibitors indinavir (Merck), saquinavir (Roche), ritonavir (Abbott Laboratories), nelfinavir and nelfinavir M8 metabolite (Agouron Pharmaceutical), and the reverse transcriptase inhibitor de-}

lavirdine (PharMacia & Upjohn) in human plasma samples (see F1).

The method utilizes robotic solid phase extraction at neutral pH on a Zymark RapidTrace system. The procedure was purposely designed to be generally applicable toward all of the analytes as well as their five respective internal standards. Plasma samples (100 mL) were diluted with an internal standard mixture and water, and the analytes were trapped on short C18 extraction columns. Extracts were subjected to chromatography on a short cyano bonded phase column and passed into the electrospray (ESI) source of a MicroMass QuattroLC mass spectrometer operated in the positive ion mode. The instrument was instructed to measure the daughter ions of each analyte over the respective retention time windows for the six analytes and five internal standards. Although the initial intention was to measure all of the compounds in a single injection, better quality results were obtained for nelfinavir and nelfinavir M8 metabolite by using the same column and ionization mode with a lower pH mobile phase. Due to concomitant retention time changes, the group of six analytes was best measured using two mobile phases.

The analytical method presented in this article was fully validated; however, due to the scope of this project, not all of the validation data will be presented here.
Sample Preparation

Zymark RapidTrace System

Heparinized human plasma samples were thawed and vortexed. A 100 µL aliquot of plasma was manually transferred to a 13 x 100 test tube, to which were added 100 µL of an internal standard mixture and 500 µL water. The internal standard mixture consisted of 1000 ng/mL $^{13}$C$_3$-delavirdine, 1000 ng/mL D$_6$-indinavir, and 500 ng/mL reserpine, D$_5$-saquinavir, and D$_8$-ritonavir.

The mixture was then aspirated by the RapidTrace system according to a program file which pre-conditions the C18 columns, applies the sample, follows with 2 mL pH 6 phosphate buffer and water rinses, air-dries, and elutes with 3 mL ethyl acetate. The solvent is collected by the RapidTrace system in a new tube, and the extracts are evaporated to dryness and reconstituted in 250 µL of 0.01% formic acid solution in 35% aqueous acetonitrile.

Packard Multi Probe 204 System

The same sample mixture could be created robotically on the Packard system, straight from sample vials and reagent reservoirs, and applied to 25 mg Varian 96 well C18 SPE plates which were similarly pre-conditioned. A pulsed vacuum source drew down the samples at a controlled rate, via the MultiProbe software. The phosphate buffer and water rinses proceeded as above, and the SPE array was eluted in a deep well plate. The solvent layer was evaporated and reconstituted as before.

Sample Requirements and Validated Stability Data

Volume: 100 µL
Matrix: Human heparinized plasma
Sample dilution: Dilution factor of 5 was verified
Freeze/thaw stability: At least 4 cycles
Stability in matrix: At least 46 hours at room temperature; at least 46 hours refrigerated
Processed extract stability: At least 2 days
Long term stability in frozen matrix: At least 5 months at either -20 °C or -80 °C
Heat treatment stability: At least 1 hour at 56 °C

Instrumental Analysis

Autosampler: BAS Sample Sentinel SS-4000 with 20 µL loop
Pump: BAS PM-80 isocratic pump with LC-26 on-line degasser
Column: Zorbax SB-CN column, 2.1 x 50 mm, 5 µm
Source: MicroMass Z-spray, positive ion, electrospray

Detector: MicroMass QuattroLC triple quadrupole system
Flow rate: 0.5 mL/min
Mobile phase A: 41% 20 mM ammonium acetate buffer (pH 5.0), 59% methanol
Mobile phase B: 0.3% formic acid, 46% methanol, 54% water
### Saquinavir MP A

<table>
<thead>
<tr>
<th>Nominal conc. (ng/mL)</th>
<th>7500</th>
<th>3750</th>
<th>150</th>
<th>10000</th>
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<tbody>
<tr>
<td>Precision (%)</td>
<td>1.6</td>
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<td>1.4</td>
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<td>Accuracy (%)</td>
<td>101.7</td>
<td>101.5</td>
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### Saquinavir MP B

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<tr>
<td>Accuracy (%)</td>
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<td>101.7</td>
<td>99.1</td>
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<td>2.1</td>
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<tr>
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<td>100.2</td>
<td>99.1</td>
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<td>102.8</td>
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### Nelfinavir MP B

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### Delavirdine MP B

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<tr>
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<td>102.7</td>
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<td>102.8</td>
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### Nelfinavir B

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<th>Nominal conc. (ng/mL)</th>
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<th>5000</th>
<th>25.0</th>
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<td>Accuracy (%)</td>
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<td>97.6</td>
<td>103.1</td>
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<td>92.6</td>
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### Nelfinavir M8 B

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<td>98.0</td>
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T1

Inter-day calibration standard precision and accuracy (N=6). Concentrations expressed in ng/mL.

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T2

Precision and accuracy for quantitation limits and quality controls.
Injections

Delavirdine

*Internal Standard:* $[^{13}C_3]$ delavirdine

*LC Conditions:* Mobile phase A or B

Saquinavir

*Internal Standard:* D5-saquinavir

*LC Conditions:* Mobile phase A or B

Indinavir

*Internal Standard:* D6-indinavir

*LC Conditions:* Mobile phase A

Ritonavir

*Internal Standard:* D8-ritonavir

*LC Conditions:* Mobile phase A

Nelfinavir

*Internal Standard:* reserpine

*LC Conditions:* Mobile phase A

Nelfinavir metabolite

*Internal Standard:* reserpine

*LC Conditions:* Mobile phase B

Conclusions

It was possible to automate the overnight extraction process and provide quantitative data on all six analytes the following morning. Recoveries were greater than 80%, and each run was completed in less than 5 minutes. Using a 100 µL sample volume, a lower limit of quantitation of 5-50 ng/mL was achieved. This can be lowered to 1 ng/mL if necessary. Coefficients of variation and biases of the means were less than ±15% for all of the validation tests performed, including stability protocols, inter- and intra-day precision.
Typical chromatograms of low concentration quality control sample extract prepared according to the validated procedure (mobile phase B).

Saquinavir Internal Standard

Operator: L. Gill

A02Bb031 Sm (Mn, 2x3)  
MRM of 7 Channels ES+  
1.4  
5.3x10^5

Saquinavir

A02Bb031 Sm (Mn, 2x3)  
MRM of 7 Channels ES+  
3.20  
2.35x10^4

Delavirdine Internal Standard

A02Bb031 Sm (Mn, 2x3)  
MRM of 7 Channels ES+  
0.73  
1.77x10^6

Delavirdine

A02Bb031 Sm (Mn, 2x3)  
MRM of 7 Channels ES+  
0.73  
4.63x10^4

Reserpine

Operator: L. Gill

A02Bb031 Sm (Mn, 2x3)  
MRM of 7 Channels ES+  
21.3  
3.24x10^5

Nelfinavir M8 Metabolite

A02Bb031 Sm (Mn, 2x3)  
MRM of 7 Channels ES+  
1.08  
2.78x10^5

Nelfinavir

A02Bb031 Sm (Mn, 2x3)  
MRM of 7 Channels ES+  
2.00  
2.61x10^4

The method can also be implemented on a 96 well SPE system (Packard MultiProbe 204) to create a validation curve, to pipette QC and study samples automatically, and to perform the sample extractions.

The method is currently in use for a number of protocols involving combination therapies with two or three of these drugs. In a few cases, the limited sample volume requirements have proven ideal for pediatric pharmacokinetic studies.

Acknowledgements

Special thanks go to David Morris of GlaxoWellcome, Andy Brown of Roche Products, Ltd., Rita Chiou of Merck, Larry Sennello of Abbott Laboratories, Richard Daniels of Agouron Pharmaceuticals, and Dean Knuth and Barbara Carel of Pharmacia & Upjohn for providing the reference standards necessary for this validation.