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

R.E. Shoup, Xiaoli Ren, Angela P. Johnson, Donald A. Gray, Simone Evarts, Lynn Gill, and Brian D. Beato

BAS Analytics
A Division of Bioanalytical Systems, Inc.
2701 Kent Avenue
West Lafayette, IN
47906 USA

With the success of "combination therapies" using reverse transcriptase inhibitors, antiinfectives, and protease inhibitors in the treatment of HIV infection, BAS Analytics developed a single method for profiling six protease/reverse transcriptase inhibitors in human plasma. The method utilizes robotic solid phase extraction at neutral pH and is generally applicable to all the analytes and their internal standards.

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, antiinfectives, 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 delavirdine (Pharmacia & Upjohn) in human plasma samples (see ***F1***).

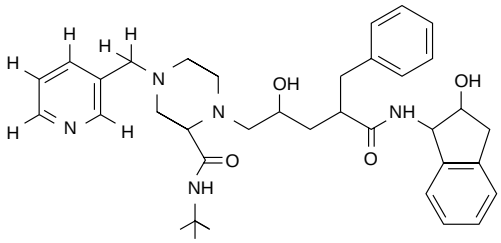
The method utilizes robotic solid phase extraction at neutral pH on a Zymark RapidTrace system. The procedure was purposely designed to be generally applicable toward all of the analytes as well as their five respective internal standards. Plasma samples (100 mL) were diluted with an internal standard 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.

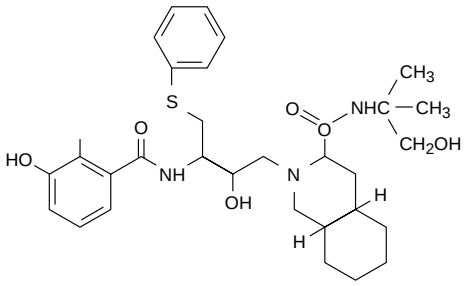
F1

Structures of six protease inhibitors.

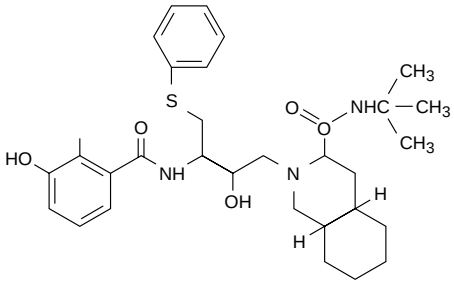
Indinavir



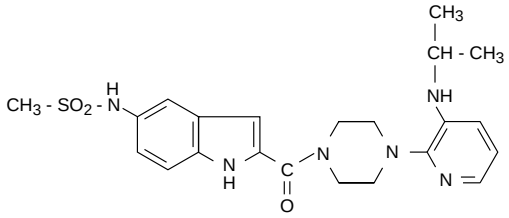
Nelfinavir M8



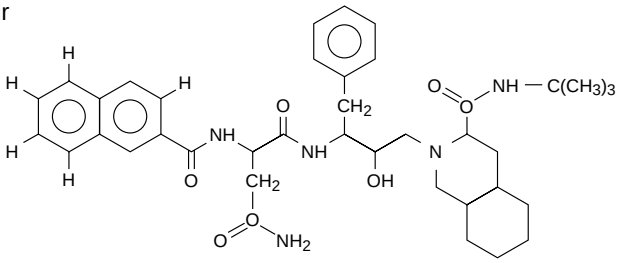
Nelfinavir



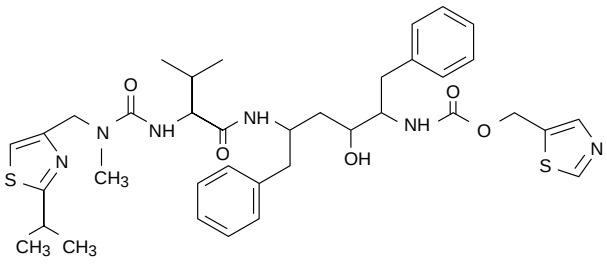
Delavirdine



Saquinavir



Ritonavir



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 MultiProbe 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

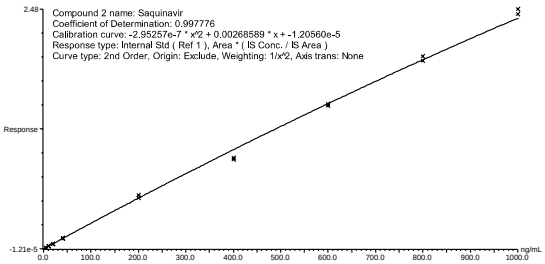
Sample dilution: Dilution factor of 5 was verified

Freeze/thaw stability: At least 4 cycles

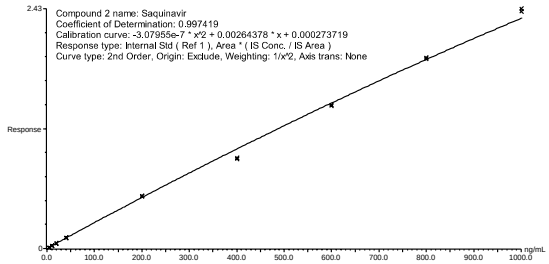
F2

Dynamic ranges. Response plotted versus concentration (ng/mL).

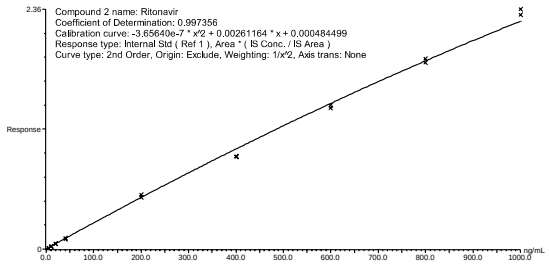
Saquinavir
5.0-1,000 ng/mL in mobile phase A



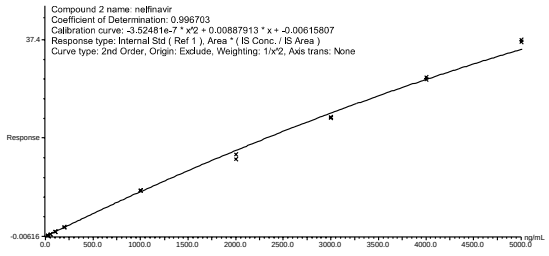
Saquinavir
5.0-1,000 ng/mL in mobile phase B



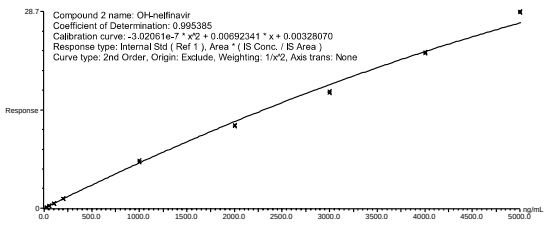
Ritonavir
5.0-1,000 ng/mL in mobile phase A



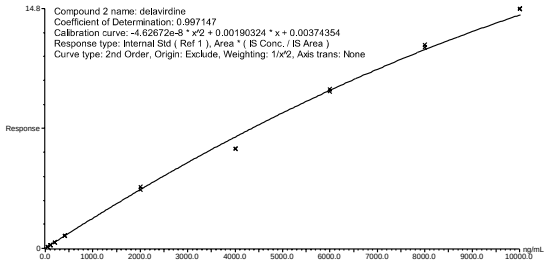
Nelfinavir
25.0-5,000 ng/mL in mobile phase B



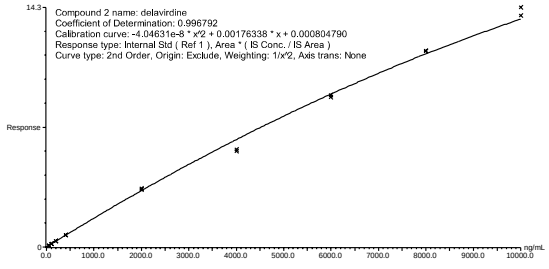
Nelfinavir M8 Metabolite
25.0-5,000 ng/mL in mobile phase B



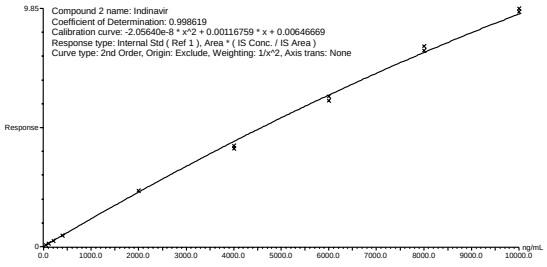
Delavirdine
50-10,000 ng/mL in mobile phase A



Delavirdine
50-10,000 ng/mL in mobile phase B



Indinavir
50-10,000 ng/mL in mobile phase A



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

T1

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

Saquinavir MP A									
Nominal conc.	1000	800	600	400	200	40.0	20.0	10.0	5.00
Precision (%)	1.6	1.8	1.4	2.2	2.1	3.5	3.7	2.0	4.9
Accuracy (%)	101.7	101.5	100.1	91.4	103.6	102.4	99.5	100.3	99.7
Saquinavir MP B									
Nominal	1000	800	600	400	200	40.0	20.0	10.0	5.00
Precision (%)	1.5	1.1	1.0	1.3	0.9	1.8	2.8	1.8	2.5
Accuracy (%)	102.4	101.7	99.1	91.2	103.2	101.6	100.2	102.0	98.8
Ritonavir MP A									
Nominal	1000	800	600	400	200	40.0	20.0	10.0	5.00
Precision (%)	2.6	1.8	2.1	1.0	2.1	3.6	2.9	1.2	6.2
Accuracy (%)	103.9	100.2	99.1	91.2	102.8	100.8	100.1	104.4	97.7
Nelfinavir MP B									
Nominal	5000	4000	3000	2000	1000	200	100	50.0	25.0
Precision (%)	3.7	4.2	3.7	3.5	5.1	2.3	2.3	2.6	5.4
Accuracy (%)	101.7	104.2	98.3	91.3	102.1	100.9	100.7	103.4	98.1
Nelfinavir M8 MP B									
Nominal	5000	4000	3000	2000	1000	200	100	50.0	25.0
Precision (%)	2.4	3.2	2.6	1.2	4.7	0.6	2.1	2.2	6.0
Accuracy (%)	105.4	101.8	94.2	94.0	102.2	103.5	98.3	102.5	98.7
Delavirdine MP A									
Nominal	10000	8000	6000	4000	2000	400	200	100	50.0
Precision (%)	1.3	2.1	0.9	1.2	2.2	0.9	2.1	3.1	5.9
Accuracy (%)	103.4	102.4	99.7	89.3	102.4	102.2	99.6	103.9	97.9
Delavirdine MP B									
Nominal	10000	8000	6000	4000	2000	400	200	100	50.0
Precision (%)	2.7	1.8	1.9	2.3	1.7	1.8	1.2	1.4	5.2
Accuracy (%)	103.0	102.7	98.2	91.0	102.8	101.8	99.6	103.2	98.3
Indinavir MP A									
Nominal	10000	8000	6000	4000	2000	400	200	100	50.0
Precision (%)	1.1	2.5	2.6	1.2	2.9	3.0	5.4	5.0	4.2
Accuracy (%)	101.5	103.4	96.9	94.7	103.2	96.9	102.5	103.2	98.2

T2

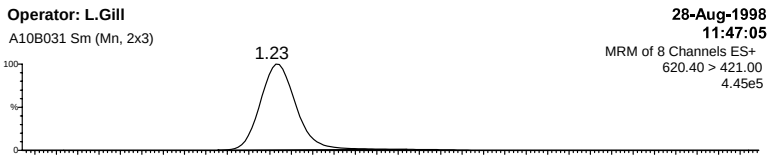
Precision and accuracy for quantitation limits and quality controls.

		HIGH QC	MID QC	LOW QC	ULOQ	LLOQ
	Conc. (ng/mL):	**7500**	**3750**	**150**	**10000**	**50.0**
DELAVIRDINE A	Intra-day precision	3.6	1.5	2.6	1.8	2.4
	Intra-day accuracy	89.6	96.1	96.2	101.5	99.5
	Inter-day precision	1.8	1.9	0.3	-	-
	Inter-day accuracy	91.0	97.2	96.4	-	-
		7500	**3750**	**150**	**10000**	**50.0**
INDINAVIR A	Intra-day precision	2.4	1.9	16.6	1.8	6.8
	Intra-day accuracy	93.7	100.6	96.1	101.5	98.6
	Inter-day precision	2.8	2.6	2.0	-	-
	Inter-day accuracy	92.3	98.0	94.9	-	-
		750	**375**	**15.0**	**1000**	**5.00**
SAQUINAVIR A	Intra-day precision	3.1	2.2	4.4	2.2	3.2
	Intra-day accuracy	98.0	95.0	102.9	98.8	106.5
	Inter-day precision	1.6	2.6	1.9	-	-
	Inter-day accuracy	99.3	97.6	103.1	-	-
		750	**375**	**15.0**	**1000**	**5.00**
RITONAVIR A	Intra-day precision	2.5	2.4	3.4	2	6.8
	Intra-day accuracy	100.6	95.1	105.1	100.2	110.8
	Inter-day precision	2.2	2.7	2.4	-	-
	Inter-day accuracy	102.8	98.1	108.1	-	-
		3750	**1875**	**50.0**	**5000**	**25.0**
NELFINAVIR B	Intra-day precision	3.5	3.7	3.3	4	2.4
	Intra-day accuracy	106.2	98.1	105.6	97.5	103.4
	Inter-day precision	0.5	2.3	2.5	-	-
	Inter-day accuracy	106.5	100.3	108.7	-	-
		3750	**1875**	**50.0**	**5000**	**25.0**
NELFINAVIR M8 B	Intra-day precision	4.6	2.8	3.4	3.7	1.5
	Intra-day accuracy	99.4	92.6	95.7	99.8	100
	Inter-day precision	1.4	2.3	4.4	-	-
	Inter-day accuracy	98.0	95.1	106.1	-	-

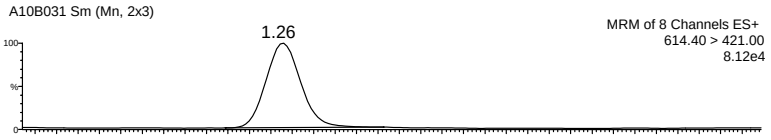
F3

Typical chromatograms of low concentration quality control sample extract prepared according to the validated procedure (mobile phase A). Retention times shown in units of minutes.

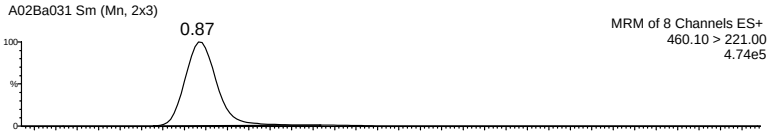
D6-indinavir



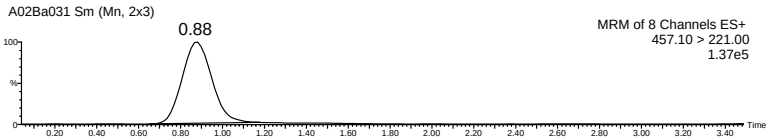
Indinavir



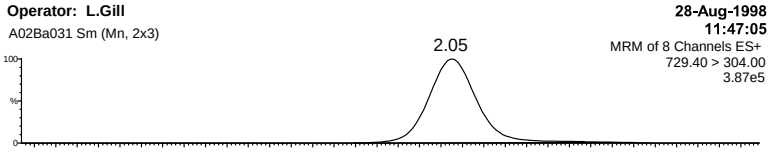
Delavirdine Internal Standard



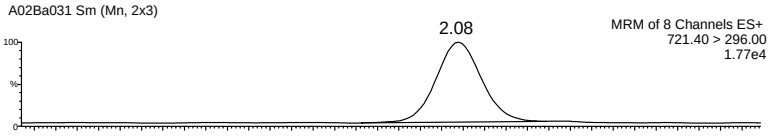
Delavirdine



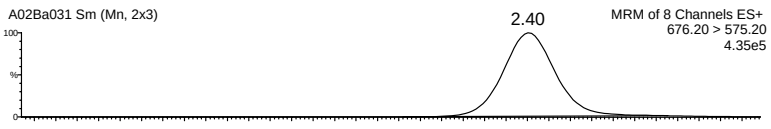
Ritonavir Internal Standard



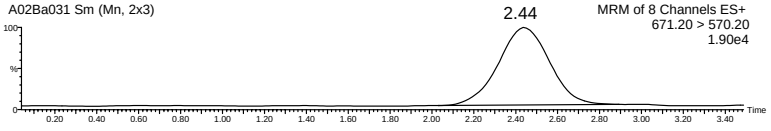
Ritonavir



Saquinavir Internal Standard



Saquinavir



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 B

Nelfinavir metabolit

Internal Standard:
reserpine

LC Conditions:
Mobile phase B

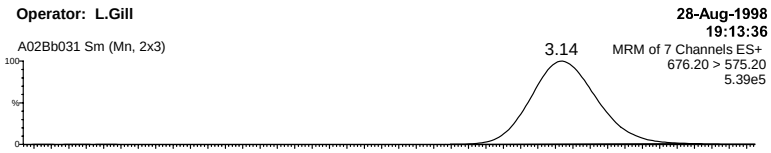
Conclusions

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

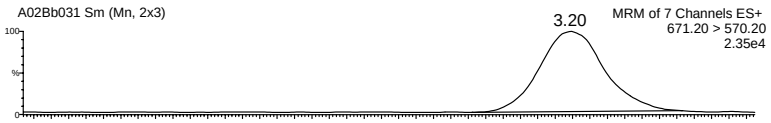
F4

Typical chromatograms of low concentration quality control sample extract prepared according to the validated procedure (mobile phase B).

Saquinavir Internal Standard

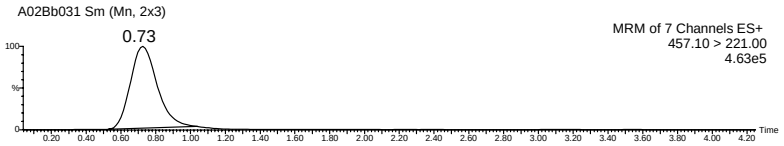


Saquinavir

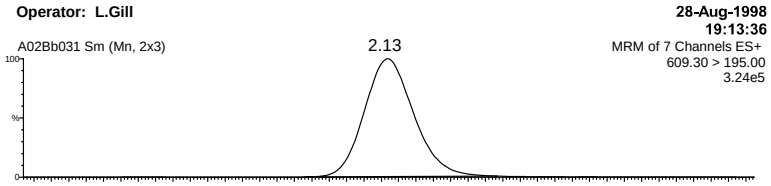


Delavirdine Internal Standard

Delavirdine

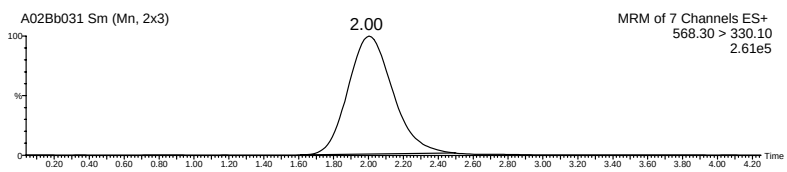


Reserpine



Nelfinavir M8 Metabolite

Nelfinavir



and accuracy, and limit of quantitation tests.

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.