
Abstract

Rapid automated immunoassay for Therapeutic Drug Monitoring of Lopinavir using ARK LPV-Test: Method validation, application and comparison with HPLC method

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Objective: The objective of the present study was to validate and test a new automated immunoassay method for Lopinavir [LPV] and compare results to our current validated HPLC method. This test, which uses a bench-top analyzer (Roche MIRA®), can be run with minimum expertise in contrast to current HPLC and LC/MS/MS techniques. It has the advantage of reducing turnaround time from days to hours providing a rapid result of benefit to the clinician and the patient. Therapeutic drug monitoring (TDM) of HIV protease inhibitors may help to increase efficacy (1, 2), reduce toxicity (1, 2) and reduce the development of resistance mutations (1, 2).

Methods: The ARK LPV-Test™ is a two-reagent homogeneous enzyme immunoassay technique used for the specific analysis of LPV in human serum or plasma (Table 1). The test is based on competitive binding to antibody between drug in the sample and drug-labeled enzyme in the reagents. Known enzyme activity in the reagent decreases upon binding of the antibody to drug in the patient sample and this change can be observed spectrophotometrically as in standard immunoassays. The ARK LPV-Test was validated by the company and independently at the UCSD laboratory. Validation was based on FDA and other guidelines (3, 4). Samples from treated patients, proficiency testing samples and blank plasma samples were run using the ARK LPV-Test and results compared to the UCSD HPLC method results. For the immunoassay 50µl of centrifuged sample was added to a sample tube. The assay uses 4µl of sample and each sample is run in duplicate. The calibration standards ranged from 1-12 µg/ml and used a Logit/Log

curve fit. Assay sensitivity was tested between 0.2 and 0.5 µg/ml. Other antiretroviral drugs were tested for interference.

Results: Validation data show inter-assay precision CVs ranging from 4.3% to 13.8% (± 0.1 SD) and accuracy within 8% deviation from expected (n=42) (Table 3). Precision at 0.2µg/ml was 16.02%CV (Table 5) and meets the LOD acceptance criteria for immunoassays (4). Precision at 0.4µg/ml was 13.1%CV (Table 2). There was no interference from other antiretroviral drugs (Table 4) and blank plasma samples showed no measurable levels. Patient samples analyzed by HPLC and the ARK LPV-Test yielded the following results: $y=1.01x + 0.23$ ug/ml, $R = 0.98$ (n = 31) (Figure 1). Proficiency samples generally gave good agreement between the two methods (Table 7).

Conclusions: A fast, automated test for measuring lopinavir in plasma was validated and evaluated. The test takes about 1.5 hour to test 25 specimens including setup time and requires no advanced operator expertise. No sample pre-treatment is required and sample volume is 50µl. The reagents and calibrators are supplied ready to use by ARK Diagnostics Inc., Fremont, California. The ARK LPV-Test showed good correlation with the validated UCSF Pediatric Pharmacology HPLC method. **The test is suited equally to rapid monitoring of LPV for TDM or for doing pharmacokinetic assessments, providing useful and beneficial information to the clinician and the patient. It is also suitable for point-of-contact clinical sites, providing these observations for drug therapy and for monitoring adherence to drug regimes.**

COBAS MIRA Parameters

Table 1. Parameters have been established for the ARK Lopinavir Assay on the COBAS MIRA System:

Sample Volume (μL)	4
Reagent 1 Volume (μL)	150
Reagent 2 Volume (μL)	75
Assay Temperature ($^{\circ}\text{C}$)	37
Wavelength (nm)	340
Throughput (tests/hour)	72

HPLC Procedure

Plasma lopinavir levels were determined by a validated reverse-phase high-performance liquid chromatography (RF-HPLC) using UV detection. Briefly: plasma was mixed with acetonitrile (ACN) at a 1:1.2 dilution, mixed, and centrifuged to precipitate plasma proteins. The supernatant was injected directly onto a C-18 RF column and LPV was separated using a buffer of pH 3.1 and 50% ACN. UV detection was at 215nm. The preferred sample size for HPLC is 300-500 μl .

Inter-Assay Precision Study 1

Table 2. Five QC samples were tested using the ARK Lopinavir Assay on the Cobas Mira analyzer. The data are derived from 5 days: 2 runs per day, 4 replicates per run with a total of 40 replicates of each control level.

Conc. ($\mu\text{g}/\text{mL}$)	Assayed (Mean \pm SD)	Precision (CV%)	Accuracy (Bias %)
0.4 $\mu\text{g}/\text{mL}$	0.40 \pm 0.05	13.13	0
0.5 $\mu\text{g}/\text{mL}$	0.51 \pm 0.07	12.92	2.0
2.5 $\mu\text{g}/\text{mL}$	2.62 \pm 0.11	4.28	4.8
5.0 $\mu\text{g}/\text{mL}$	4.99 \pm 0.24	4.89	- 0.2
8.0 $\mu\text{g}/\text{mL}$	8.09 \pm 0.57	7.09	1.1

Inter-Assay Precision Study 2

Table 3. Four QC samples were tested using the ARK Lopinavir Assay on the Cobas Mira analyzer. The data are derived from 4 days: 1 or 2 runs per day, 6 replicates per run with a total of 42 replicates of each control level.

Conc. ($\mu\text{g}/\text{mL}$)	Assayed (Mean \pm SD)	Precision (CV%)	Accuracy (Bias %)
0.5 $\mu\text{g}/\text{mL}$	0.47 \pm 0.07	13.80	-5.40
2.5 $\mu\text{g}/\text{mL}$	2.70 \pm 0.12	4.39	7.98
5.0 $\mu\text{g}/\text{mL}$	4.99 \pm 0.22	4.32	-0.22
8.0 $\mu\text{g}/\text{mL}$	7.89 \pm 0.59	7.89	-6.05

Specificity

Table 4. Antiretrovirals whose chemical structure or concurrent therapeutic use would suggest possible cross-reactivity were tested at the levels indicated. None of the compounds tested gave an apparent lopinavir concentration.

Protease Inhibitors That Do Not Cross-react	Level Tested (µg/mL)
Amprenavir	10
Atazanavir	10
Indinavir	10
Nelfinavir	10
Ritonavir	10
Saquinavir	12

Nonnucleoside Reverse Transcriptase Inhibitors That Do Not Cross-react	Level Tested (µg/mL)
Efavirenz	12
Nevirapine	12

Lower Limit of Quantitation

Table 5. Pooled human serum samples were supplemented with known amounts of lopinavir at the concentrations shown below. Each sample was then assayed 20 times. The lowest concentration measured with acceptable accuracy and precision is 0.20 µg/mL.

Conc. (µg/mL)	Assayed (Mean ± SD)	Precision (CV%)	Accuracy (Bias %)
0.2 µg/mL	0.22 ± 0.04	16.02	10.0

Analytical Recovery

Table 6. Pooled human serum samples were supplemented with known amounts of lopinavir. The amount of lopinavir recovered from nominal ranged from 90.6% to 112.4%.

Concentration Tested ($\mu\text{g/mL}$)	Recovery (%)
0.5	90.6
0.75	102.5
1.5	105.3
2.5	101.0
3.0	112.4

Concentration Tested ($\mu\text{g/mL}$)	Recovery (%)
5.0	106.2
6.0	100.9
8.0	101.6
9.0	103.8

AIDS Clinical Trials Group Proficiency Testing

Table 7. Proficiency testing samples were prepared by the AIDS Clinical Trials Group (5). High, medium, and low concentrations of protease inhibitors and nonnucleoside reverse transcriptase inhibitors were added to drug-free EDTA plasma. The samples were assayed for lopinavir in duplicate by the ARK Lopinavir Assay and the mean compared to RF-HPLC result.

I.D.	HPLC ($\mu\text{g/mL}$)	ARK Mean ($\mu\text{g/mL}$)	I.D.	HPLC ($\mu\text{g/mL}$)	ARK Mean ($\mu\text{g/mL}$)
UC-13-B	4.32	4.25	I/NAT6 - 98	7.45	7.47
J-13-B	6.86	7.58	J-11-A	1.82	1.89
J- 13-C	2.54	2.47	J-13-A	0.41	0.64
UC-11-B	3.30	3.50	J-13-B*	6.86	6.66
J-11-B	0.28	0.29	I/NAT6 - 98*	7.45	6.98
I/NAT6- 97	2.43	2.48	UC 11- A*	10.52	11.10

* Manually diluted w/neg serum 1:4 prior to loading onto Cobas Mira; result multiplied by a factor of 4.

Endogenous Interference

Table 8. Seven (7) hyperlipidemic, 5 hyperbilirubinemic and 1 hypergammaglobulinemic plasma samples obtained from individual patients were supplemented with 6.0 µg/mL of lopinavir and tested. The endogenous substances tested did not interfere significantly with the ARK Lopinavir Assay.

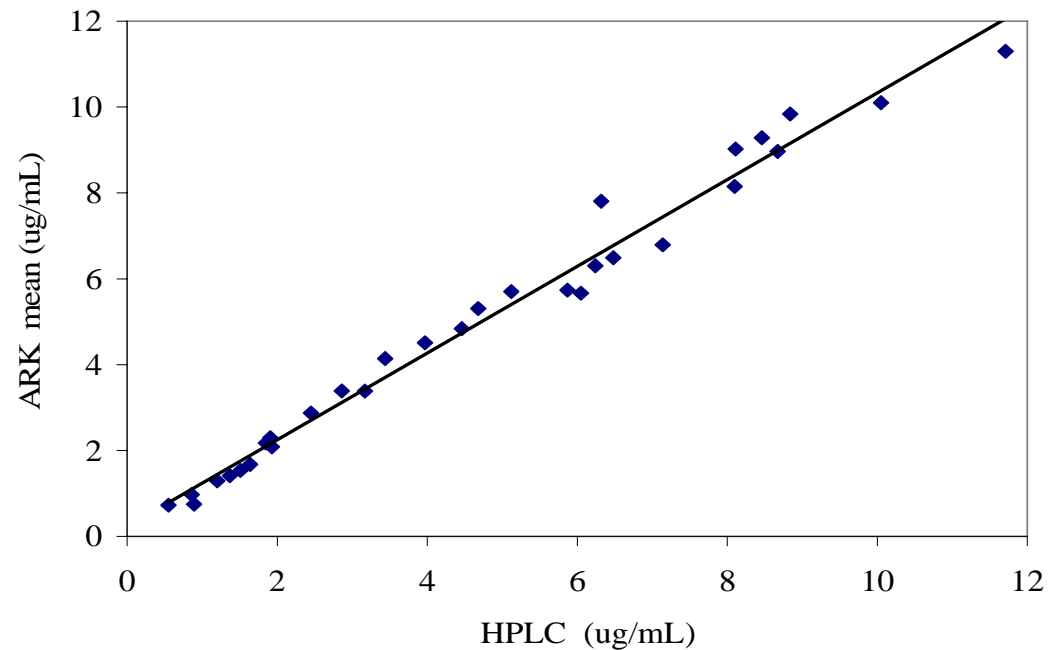
Endogenous Substance	Endogenous Substance Concentration Range	Lopinavir Recovery (%)
Triglyceride	304 - 346 mg/dL	97-107
Cholesterol	255 - 350 mg/dL	97 - 99
Total Bilirubin	26.7 - 31.4 mg/dL	106 - 111
Gamma Globulin	10,000 mg/dL	109

Comparative Analysis

Figure 1. Patient samples dosed with Kaletra (lopinavir and ritonavir) were analyzed using the ARK Lopinavir Assay on the COBAS MIRA chemistry analyzer and HPLC, and results compared. Results are shown in the figure below.

ARK Lopinavir Assay vs HPLC

Slope	1.01
Intercept ($\mu\text{g/mL}$)	0.23
Correlation	0.98
N	31



Inter-Day Calibration Reproducibility

Table 9. Calibration standards were run on four days. A total seven calibration curves were generated along with QC samples on the Cobas Mira for validation. Precision of calibrators was <2.3% CV and accuracy was within 5% deviation.

Run	Cal 1 ($\mu\text{g/mL}$)	Cal 2 ($\mu\text{g/mL}$)	Cal 3 ($\mu\text{g/mL}$)	Cal 4 ($\mu\text{g/mL}$)	Cal 5 ($\mu\text{g/mL}$)
1	0.97	2.04	4.03	7.73	11.89
2	0.96	2.07	4.00	7.64	12.00
3	0.94	2.12	3.95	7.70	12.00
4	0.97	2.01	4.05	7.62	11.93
5	1.00	1.99	4.06	7.77	11.98
6	0.99	2.00	4.12	7.43	12.00
7	0.96	2.05	4.06	7.52	12.00

Theoretical					
Conc. ($\mu\text{g/mL}$)	1.0	2.0	4.0	8.0	12.0
Mean ($\mu\text{g/mL}$)	1.0	2.0	4.0	7.6	12.0
SD	0.02	0.04	0.06	0.12	0.05
%CV	2.2	2.2	1.4	1.6	0.4
%dev	-3.1	2.1	0.9	-4.6	-0.3
N	7	7	7	7	7

Table 10. Logit/Log 4 curve fitting parameters.

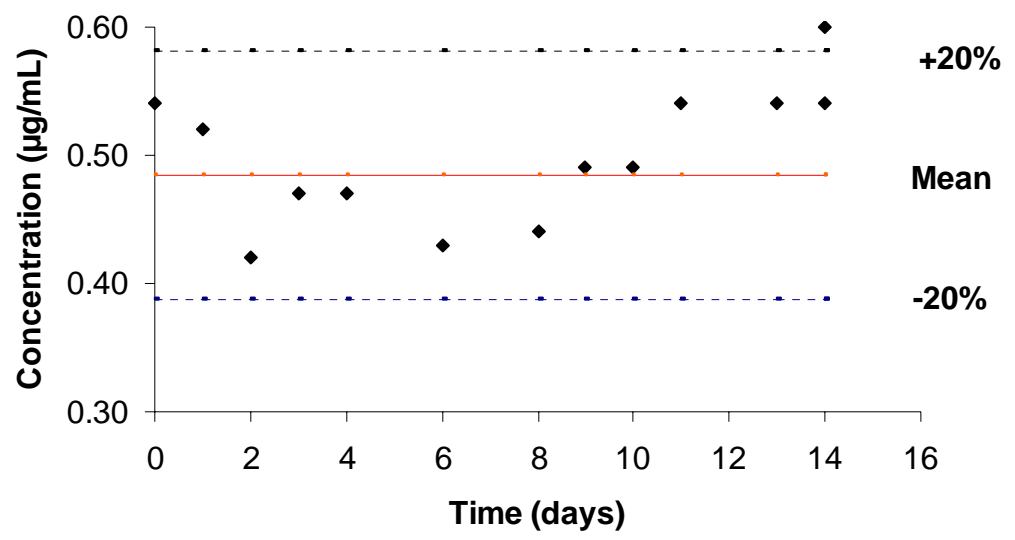
Run	A	B	Ro	Kc
1	-1.4446	1.168	559.111	540.135
2	-1.40203	1.30697	558.495	506.836
3	-1.4446	1.16800	559.111	540.135
4	-1.40203	1.30697	558.495	506.836
5	-1.44975	1.29989	560.032	511.951
6	-1.4278	1.30183	557.991	508.78
7	-1.4952	1.237736	553.11	533.017

Calibration Stability

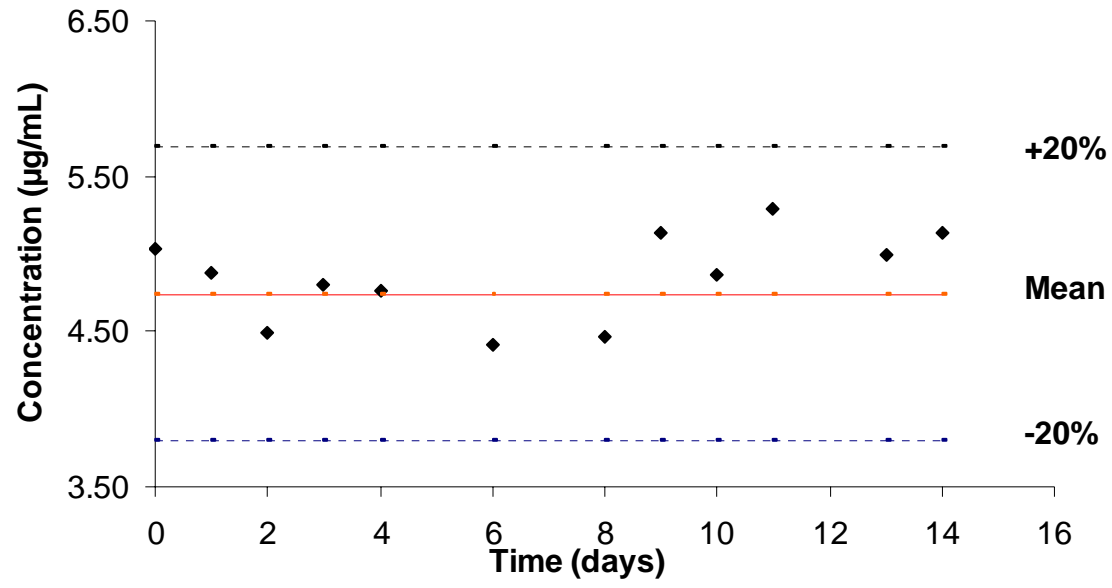
Figure 2. Control limits were calculated from fixed percentages ($\pm 20\%$) of control means ($\mu\text{g/mL}$) established from a within-run precision study ($n=20$). Thereafter, a single determination of QC levels was assayed daily. Values were calculated using a stored calibration curve. If any value was not within its control limits ($\pm 20\%$), the control was retested. If, after retesting, the value was within its control limit, calibration was verified. If, however, the value was still not within its control limits, calibration was repeated.

In this study, the curve was stable for 14 days. The assay performance is shown in the following Levey-Jennings graph.

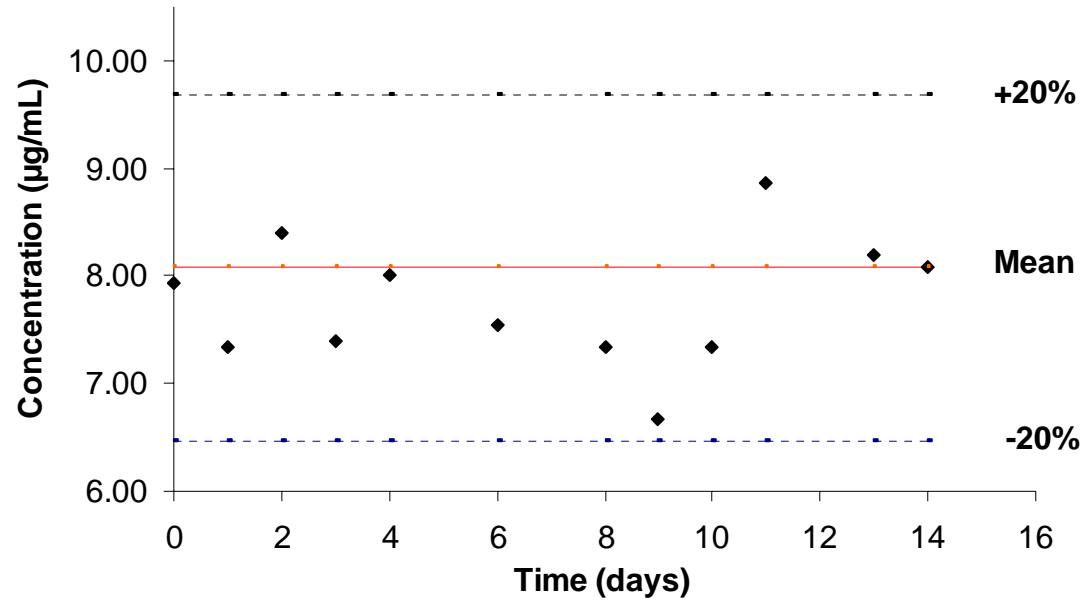
Low Control



Mid Control



High Control



Conclusions

The ARK Lopinavir Assay is an accurate and precise method to conveniently measure lopinavir in serum or plasma. The assay offers the following advantages to laboratories:

- No sample extraction or pretreatment
- High specificity and good sensitivity
- Small sample size
- Excellent correlation to HPLC method for LPV
- Ready-to-use liquid reagents and calibrators
- Calibration stability
- Rapid results with 1 hour turn-around time

References:

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