Rapid Automated Immunoassay for Therapeutic Drug Monitoring of Nevirapine Using ARK NVP-Test®: Method Validation, Application and Comparison with HPLC Method

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Abstract

Background: Therapeutic drug monitoring (TDM) in HIV disease may increase antiretroviral (AVR) efficacy by reducing toxicity, preventing drug resistance and managing drug-drug interactions. Measuring AVRs with current techniques (e.g. HPLC) is costly, time consuming and requires specialized equipment and skilled technicians. A new rapid automated enzyme immunoassay has been developed for determining plasma nevirapine (NVP) concentrations. Results using the new method were compared to those from a standard HPLC method. **Methods:** The ARK NVP-Test is based on competitive binding to antibody between drug in the sample and drug-labeled enzyme. Drug concentration is measured spectrophotometrically (Roche MIRA® bench top analyzer) in terms of enzyme activity. Each test uses 4μl of sample. The calibration standards ranged from 1 - 12 μg/ml. Assay sensitivity was 0.5μg/ml. Patient samples and proficiency testing (PT) samples were run and compared to HPLC results. **Results:** Validation data for controls (0.5 to 8 μg/ml) show inter-assay precision <8.5 CV% (n = 36). Accuracy was -16.4% deviation at 0.5μg/ml and within 6.5% for remaining controls (n=36) (Table 3). No interference was noted from other AVR drugs or blank plasma samples. Patient samples analyzed by HPLC and the ARK NVP-Test yielded the following results: $y = 0.9x + 0.36$, $R^2 = 0.97$, n = 34 (Figure 1). PT samples showed excellent agreement with target values (Table 7). **Conclusions:** ARK NVP-Test for measuring nevirapine in plasma was validated. The test is an automated EIA that requires minimum expertise, small sample volume, no sample pre-treatment and provides the first result within 30 minutes. All reagents are supplied ready-to-use. The test showed good correlation with HPLC and is well suited for routine TDM use. It may also provide a cost-effective way to determine NVP concentrations in areas with high HIV prevalence and limited testing resources.

COBAS MIRA Parameters

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

Sample Volume (µL)	4
Reagent 1 Volume (µL)	150
Reagent 2 Volume (µL)	75
Assay Temperature (°C)	37
Wavelength (nm)	340
Throughput (tests/hour)	72

HPLC Procedure

Plasma nevirapine (NVP) levels were determined by a validated reverse-phase high-performance liquid chromatography (RF-HPLC) using UV detection. Briefly: plasma was mixed with perchloric acid, mixed, and centrifuged to precipitate plasma proteins. The supernatant was injected directly onto a C-18 RF column and NVP was separated using a buffer of pH 6.8 which included 15% acetonitrile. UV detection was at 280nm. The preferred sample size for HPLC is 300-500µl.

Inter-Assay Precision Study 1

Table 2. Nine QC samples were tested using the ARK Nevirapine 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. (µg/mL)	**Assayed (Mean ± SD)**	**Precision (CV%)**	**Accuracy (Bias %)**
0.4 µg/mL	0.38 ± 0.03	8.59	-4.75
0.5 µg/mL	0.47 ± 0.03	6.21	-6.15
0.75 µg/mL	0.70 ± 0.03	4.50	-6.60
1.5 µg/mL	1.53 ± 0.06	3.96	1.83
2.5 µg/mL	2.57 ± 0.09	3.46	2.83
3.0 µg/mL	3.10 ± 0.16	5.13	3.23
5.0 µg/mL	4.88 ± 0.21	4.28	- 2.46
6.0 µg/mL	5.66 ± 0.29	5.09	-5.73
10.0 µg/mL	10.15 ± 0.67	6.62	1.54

Inter-Assay Precision Study 2

Table 3. Four QC samples were tested using the ARK Nevirapine Assay on the COBAS MIRA analyzer. The data are derived from 3 days: 2 runs per day, 6 replicates per run with a total of 36 replicates of each control level.

Conc. (µg/mL)	**Assayed (Mean ± SD)**	**Precision (CV%)**	**Accuracy (Bias %)**
0.5 µg/mL	0.42 ± 0.03	8.2	-16.4
2.5 µg/mL	2.66 ± 0.07	2.8	6.2
5.0 µg/mL	5.05 ± 0.15	3.0	1.1
8.0 µg/mL	7.97 ± 0.28	3.5	-0.3

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 nevirapine concentration.

PIs That Do Not Cross-react	Level Tested (µg/mL)
Amprenavir	10
Atazanavir	10
Indinavir	10
Nelfinavir	10
Ritonavir	6
Saquinavir	12
Lopinavir	12
Tipranavir	10

NNRTIs That Do Not Cross-react	Level Tested (µg/mL)
Efavirenz	12

Lower Limit of Quantitation

Table 5. Pooled human serum samples were supplemented with known amounts of nevirapine 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	0.20 ± 0.02	9.92	0

Analytical Recovery

Table 6. Pooled human serum samples were supplemented with known amounts of nevirapine. Each sample was then assayed 10 times. The amount of nevirapine recovered from nominal ranged from 89.5% to 105.2%.

Conc. Tested (µg/mL)	Recovery (Mean ± SD)	(%)
0.3	0.30 ± 0.02	98.7
0.4	0.39 ± 0.01	97.0
0.5	0.47 ± 0.03	94.0
0.75	0.67 ± 0.02	89.5
1.5	1.53 ± 0.03	101.7
2.5	2.63 ± 0.07	105.2
3.0	3.07 ± 0.07	102.2
5.0	5.04 ± 0.15	100.8
6.0	5.60 ± 0.23	93.3
10.0	10.46 ± 0.39	104.6

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 nevirapine in duplicate by the ARK Nevirapine Assay and the mean compared to target values.

I.D.	Target (µg/mL)	ARK Mean (µg/mL)
UC-13-D	2.65	2.82
UC-13-E	0.38	0.31
UC-13-F	6.71	6.64
J-13-D	2.45	2.70
J-13-E	5.92	6.06
J-13-F	0.37	0.30

I.D.	Target (µg/mL)	ARK Mean (µg/mL)
UC-14-D	7.13	6.40
UC-14-E	2.85	2.74
UC-14-F	0.43	0.38
J-14-D	0.32	0.23
J-14-E	1.68	1.73
J-14-F	5.11	4.90

Endogenous Interference

Table 8. Seven (7) hyperlipidemic, 5 hyperbilirubinemic and 1 hypergammaglobulinemic plasma samples obtained from individual patients were supplemented with 5.0 µg/mL of nevirapine and tested. The endogenous substances tested did not interfere significantly with the ARK Nevirapine Assay.

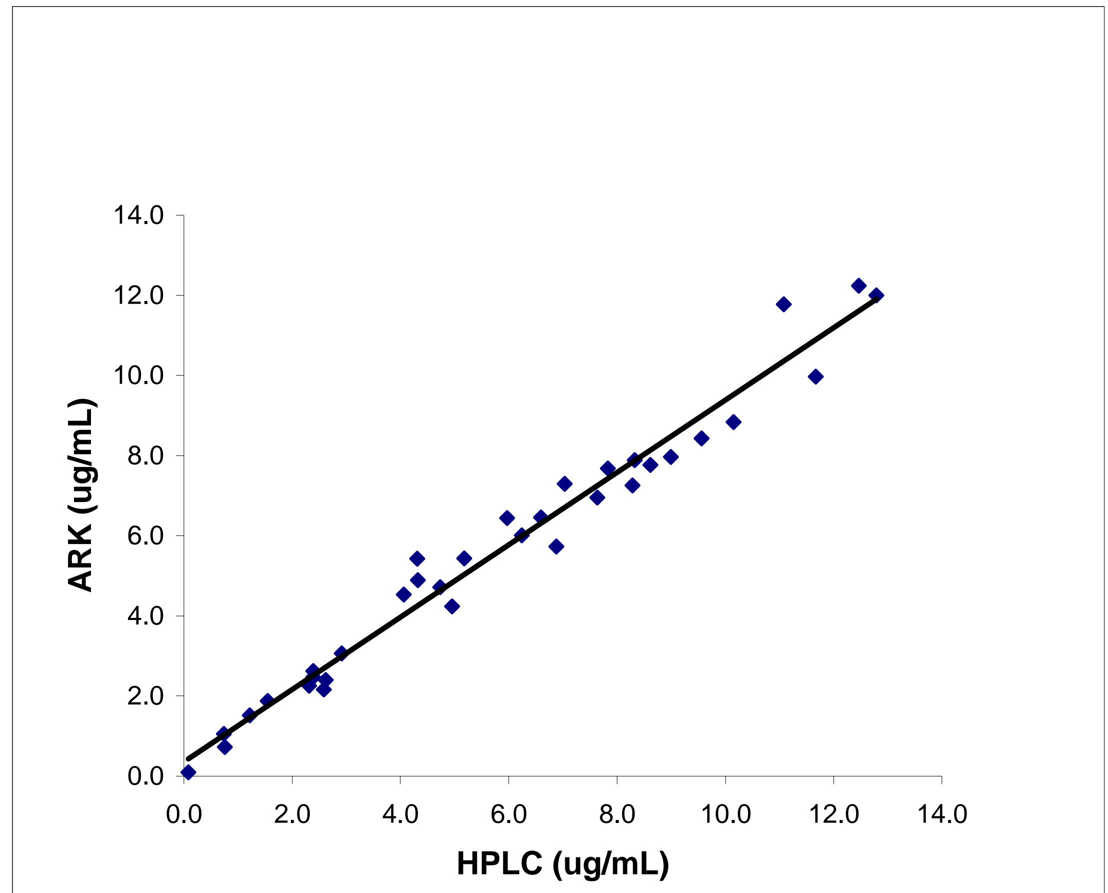
Endogenous Substance	Endogenous Substance Concentration Range	Nevirapine Recovery (%)
Cholesterol	304 - 346 mg/dL	97.4–102.4
Triglyceride	255 - 350 mg/dL	101.2 – 102.0
Total Bilirubin	26.7 - 31.4 mg/dL	102.9 – 105.6
Gamma Globulin	10,000 mg/dL	105.0

Comparative Analysis

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

ARK Nevirapine Assay vs HPLC

Slope	0.90
Intercept (µg/mL)	0.36
Correlation	0.97
N	34



INTER-ASSAY AVERAGE BACK CALCULATED CALIBRATION STANDARDS

Table 9. Calibration standards were run on three days. A total six calibration curves were generated along with QC samples on the COBAS MIRA for validation. Precision of calibrators was <1.3% CV and accuracy was within 3.4% deviation.

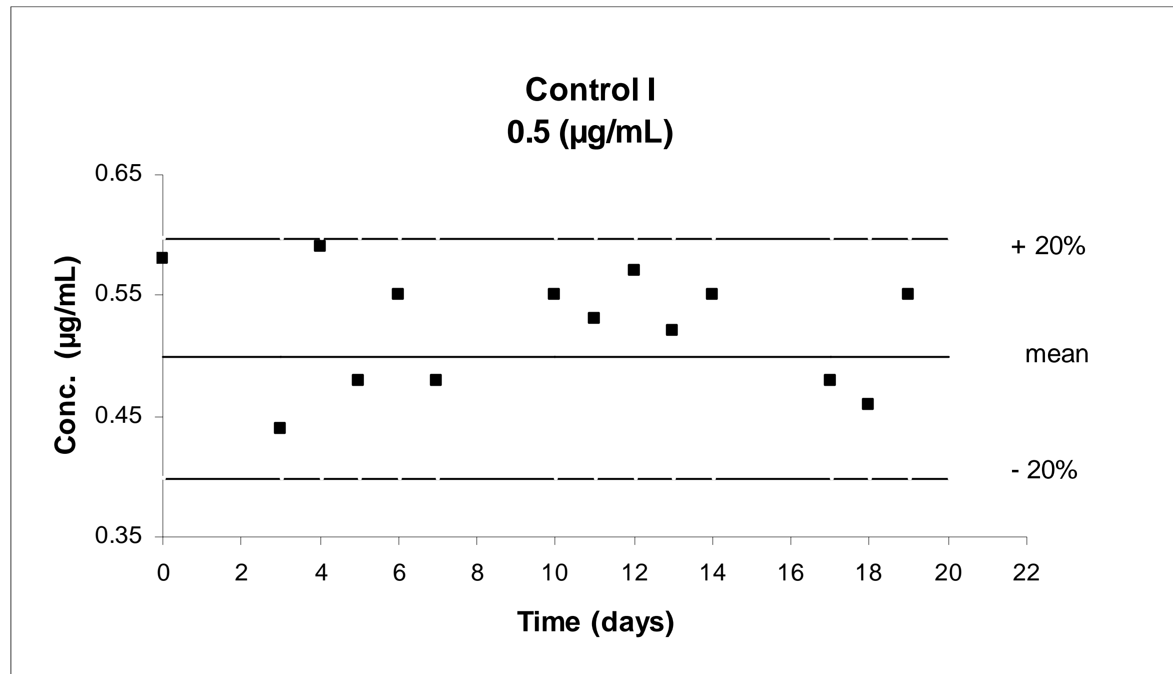
	Standards					Logit/Log4 Math Function * Calibration Parameters			
Run ID	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	A	B	Ro	Kc
022805 Run 1	0.980	2.020	4.061	7.679	12.000	-1.41064	1.11200	499.809	625.226
022805 Run 2	0.966	2.063	3.988	7.807	12.000	-1.39981	1.03569	499.307	652.307
030105 Run 1	0.982	2.031	4.002	7.865	12.000	-1.41031	1.10384	504.317	624.430
030105 Run 2	0.961	2.073	3.987	7.759	12.000	-1.38201	1.06198	496.614	642.749
030205 Run 1	0.953	2.077	4.027	7.618	11.999	-1.43014	1.06687	496.991	645.461
030205 Run 2	0.964	2.063	4.007	7.702	12.000	-1.35211	1.07599	497.990	631.486
Theoretical (µg/mL)	1	2	4	8	12				
Mean (µg/mL)	0.968	2.055	4.012	7.738	12.000				
SD	0.011	0.023	0.028	0.090	0.000				
% CV	1.2	1.1	0.7	1.2	0.0				
% dev	-3.2	2.7	0.3	-3.3	0.0				
N	6	6	6	6	6				

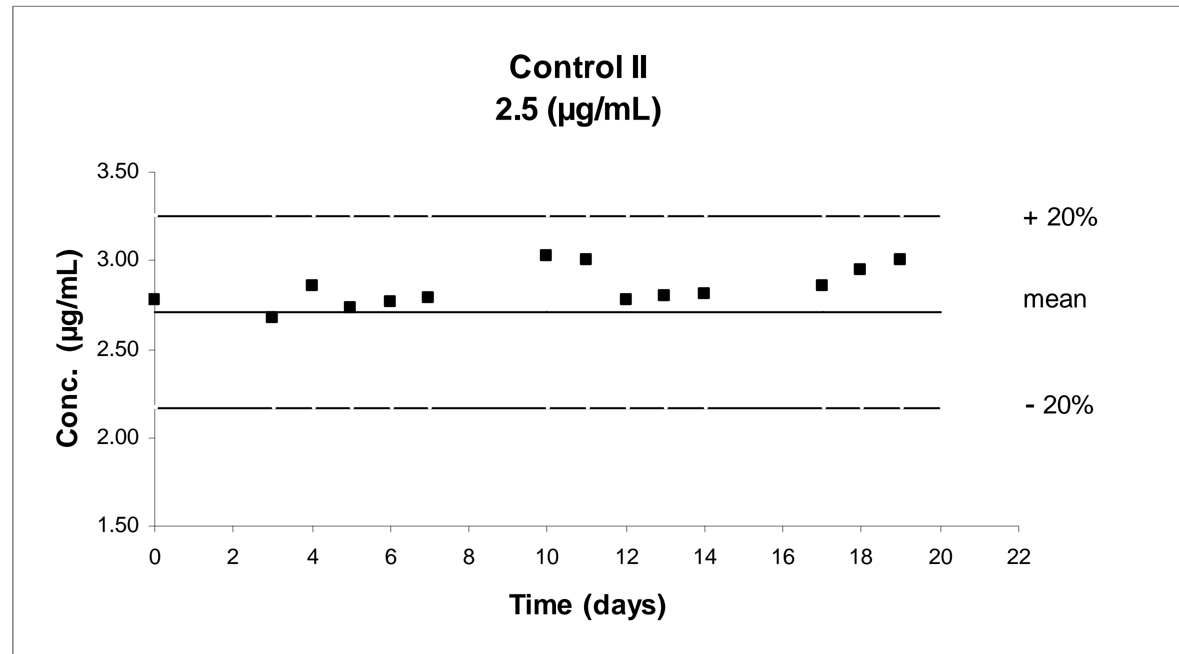
*The parameters of the standard curve mathematical function are derived from the reaction rates and concentrations of the standards. The concentration of the unknown is derived by placing the rate of an unknown into the mathematical function. These calculations are automatically performed by the COBAS MIRA chemistry analyzer.

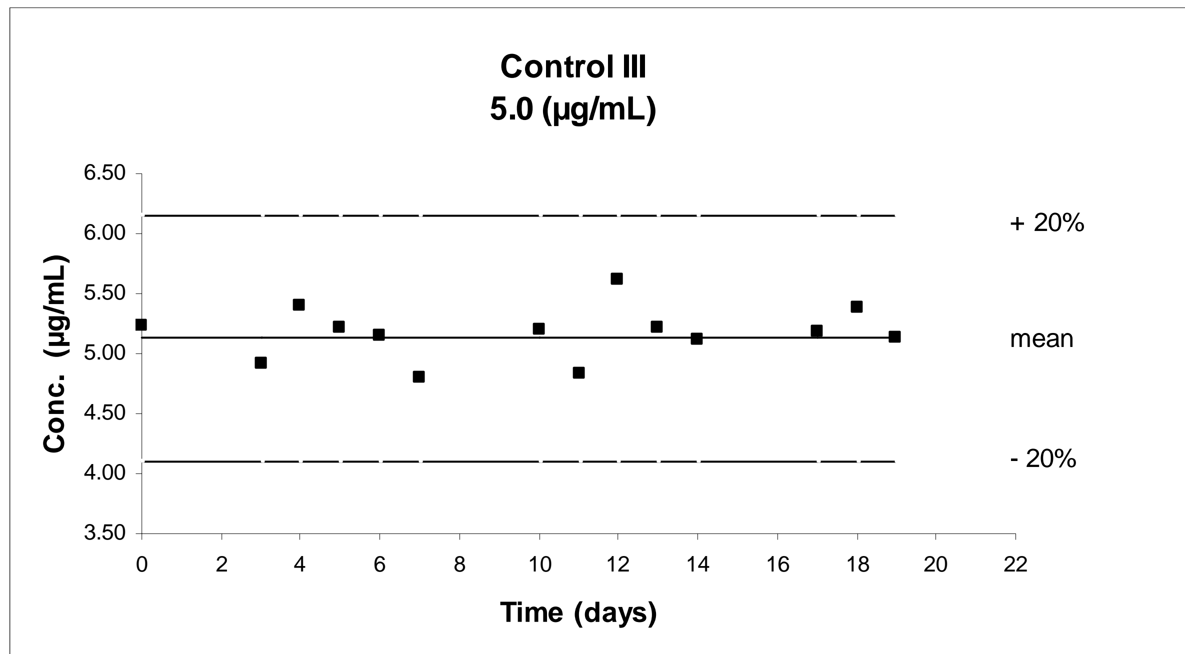
Calibration Stability

Figure 2. Control limits were calculated from fixed percentages (±20%) of control means (µg/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 (±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 calibration curve was stable for 19 days. The assay performance is shown in the following Levey-Jennings graph.


Control I
0.5 (µg/mL)
0.65
0.55
0.45
0.35
Conc. (µg/mL)
+ 20%
mean
- 20%
0
2
4
6
8
10
12
14
16
18
20
22
Time (days)


Control II
2.5 (µg/mL)
3.50
3.00
2.50
2.00
1.50
Conc. (µg/mL)
+ 20%
mean
- 20%
0
2
4
6
8
10
12
14
16
18
20
22
Time (days)


Control III
5.0 (µg/mL)
Conc. (µg/mL)
6.50
6.00
5.50
5.00
4.50
4.00
3.50
0
2
4
6
8
10
12
14
16
18
20
22
Time (days)
+ 20%
mean
- 20%

Conclusions

The ARK Nevirapine Assay is an accurate and precise method to conveniently measure nevirapine 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 NVP
- Ready-to-use liquid reagents and calibrators
- Calibration stability
- Rapid turn-around time