ARK™ HOMOGENEOUS ENZYME IMMUNOASSAYS FOR VORICONAZOLE AND POSACONAZOLE

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Introduction

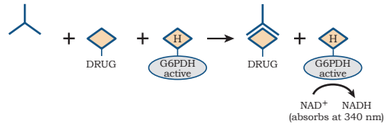
ABSTRACT

Background
Despite the availability of newer antifungal agents, invasive fungal diseases remain a leading cause of morbidity and mortality in immunocompromised patients. Liquid-stable, homogeneous enzyme immunoassays are in development for voriconazole and posaconazole. Individualization of drug therapy is important for effective treatment of difficult infections, but also to maximize the outcomes of vulnerable immunocompromised patients on prophylactic therapy.

Methods
Each assay system consists of two reagents, six calibrators and tri-level controls. Calibration of the posaconazole assay ranged 0.00 to 8.00 µg/mL and that for the voriconazole assay ranged 0.00 to 16.00 µg/mL. The assays were evaluated on the Roche/Hitachi 917 analyzer. Precision, limit of quantitation, analytical recovery, specificity, linearity and accuracy were studied.

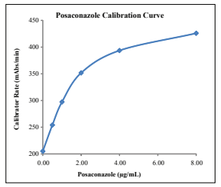
A) Absence of drug

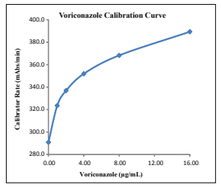
B) Presence of drug



Calibration Curve

The assay systems use 6 calibrators.
Posaconazole Assay: 0.00, 0.50, 1.00, 2.00, 4.00 and 8.00 µg/mL
Voriconazole Assay: 0.00, 1.00, 2.00, 4.00, 8.00 and 16.00 µg/mL





Endogenous Interference

Interference studies were conducted using CLSI/NCCLS Protocol EP7-A2 as a guideline. Clinically high concentrations of the following potentially interfering substances in serum with known levels of posaconazole (approximately 1.0 and 5.0 µg/mL) were evaluated. Each sample was assayed using the ARK Posaconazole Assay, along with a serum control of posaconazole. Measurement of posaconazole resulted in ≤10% error in the presence of interfering substances at the concentrations tested.

Substance	Concentration	% Control (PSZ 1.00 µg/mL)	% Control (PSZ 5.00 µg/mL)
Cholesterol	300 mg/dL	100.8	91.6
Hemoglobin	1000 mg/dL	108.1	103.2
Rheumatoid Factor	1069 IU/mL	100.2	101.5
Triglycerides	1000 mg/dL	94.3	91.1

Endogenous interference for the voriconazole assay is pending.

Precision

Tri-level controls containing posaconazole were assayed in quadruplicate twice a day for 5 days. The study was performed at an early stage of development using calibration 0.00, 0.50, 1.50, 3.00, 6.00 and 12.00 µg/mL.

5-Day Precision Verification: Posaconazole Assay

			Within Run		Between Day		Total	
Sample	N	Mean (µg/mL)	SD	CV (%)	SD	CV (%)	SD	CV (%)
QC LOW (1.00 µg/mL)	40	1.07	0.042	4.0	0.041	3.8	0.049	4.5
QC MID (5.00 µg/mL)	40	4.82	0.129	2.7	0.101	2.1	0.182	3.8
QC HIGH (10.00 µg/mL)	40	9.97	0.493	4.9	0.095	1.0	0.749	7.5

Tri-level controls containing voriconazole were assayed in quadruplicate twice a day for 5 days. Precision was tested at 1.50, 5.00 and 10.00 µg/mL.

5-Day Precision Verification: Voriconazole Assay

			Within Run		Between Day		Total	
Sample	N	Mean (µg/mL)	SD	CV (%)	SD	CV (%)	SD	CV (%)
QC LOW (1.50 µg/mL)	40	1.52	0.048	3.2	0.051	3.4	0.070	4.5
QC MID (5.00 µg/mL)	40	4.82	0.198	4.1	0.089	1.9	0.246	5.1
QC HIGH (10.00 µg/mL)	40	9.80	0.528	5.4	0.213	2.2	0.523	5.3

Analytical Recovery

Accuracy (analytical recovery) was performed by adding concentrated posaconazole or voriconazole drug into human serum. Two analytical runs of three replicates of each sample were assayed. The results of the six replicates were averaged and compared to the theoretical target concentration and the percentage recovery was calculated.

Analytical Recovery: Posaconazole Assay

Target (µg/mL)	Mean (µg/mL)	SD	CV (%)	Recovery (%)
0.40	0.40	0.03	7.4	100.0
0.80	0.78	0.01	1.4	96.9
1.20	1.19	0.02	1.7	98.8
1.60	1.53	0.03	2.1	95.6
3.00	2.80	0.04	1.5	93.3
6.00	5.34	0.14	2.6	89.0
7.00	6.49	0.22	3.4	92.7

Analytical Recovery: Voriconazole Assay

Target (µg/mL)	Mean (µg/mL)	SD	CV (%)	Recovery (%)
1.20	1.17	0.063	5.4	97.1
3.00	3.19	0.112	3.5	106.2
6.00	6.25	0.134	2.1	104.1
9.00	9.33	0.346	3.7	103.6
12.00	12.97	0.495	3.8	108.1

Lower Limit of Quantitation

Limit of quantitation was evaluated. Pooled human serum was supplemented with known amounts of posaconazole or voriconazole and assayed 40 times. The LLOQ was estimated as the lowest concentration for which acceptable inter-assay precision (≤20% CV) and recovery (±15%) is observed.

LLOQ: Posaconazole Assay

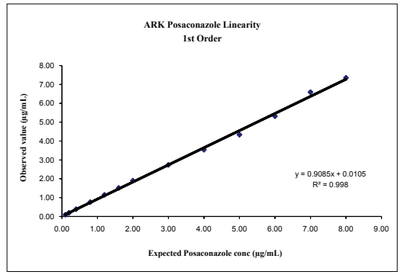
Conc. Tested (µg/mL)	Mean (µg/mL)	RMS SD	CV (%)	Recovery (%)	N
0.08	0.075	0.012	16.4	93.4	40
0.10	0.095	0.015	15.7	94.8	40
0.20	0.194	0.006	3.2	97.0	40

LLOQ: Voriconazole Assay

Conc. Tested (µg/mL)	Mean (µg/mL)	RMS SD	CV (%)	Recovery (%)	N
0.50	0.38	0.049	13.0	75.4	40
0.60	0.56	0.035	6.4	92.8	40
0.70	0.67	0.034	5.0	96.0	40

Linearity

Linearity studies were performed as suggested in CLSI/NCCLS Protocol EP6-A. Negative pooled human serum was supplemented with posaconazole to give 9.00 µg/mL and then diluted proportionally. The assay was considered linear when the percentage difference between the predicted 1st order and 2nd order polynomial was within 10% above 0.50 µg/mL and ±0.10 µg/mL ≤ 0.50 µg/mL. Regression plots of observed versus expected concentrations are shown for the entire linear range determined on the Roche/Hitachi 917, 0.10 to 8.00 µg/mL.

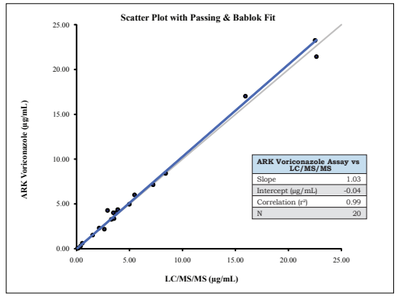


Nominal (µg/mL)	Result (µg/mL)	Predicted Line 1st Order	Predicted 2nd Order	Difference (2nd from 1st order)	
0.10	0.09	0.10	0.16	0.06	µg/mL
0.20	0.19	0.19	0.24	0.05	µg/mL
0.40	0.38	0.37	0.41	0.04	µg/mL
0.80	0.76	0.74	0.75	1.83	%
1.20	1.15	1.10	1.09	-0.65	%
1.60	1.51	1.46	1.44	-1.69	%
2.00	1.90	1.83	1.79	-2.16	%
3.00	2.74	2.74	2.67	-2.32	%
4.00	3.54	3.64	3.58	-1.89	%
5.00	4.33	4.55	4.50	-1.23	%
6.00	5.31	5.46	5.44	-0.45	%
7.00	6.59	6.37	6.40	0.39	%
8.00	7.35	7.28	7.37	1.28	%

Linearity for the voriconazole assay is pending

Method Comparison: Voriconazole Assay

Clinical specimens from patients treated with voriconazole were analyzed. Passing-Bablock regression of the comparison is shown in the figure below.



Specificity in the Presence of Other Triazole Antifungals

POSACONAZOLE ASSAY
Other triazole Antifungals; fluconazole, itraconazole and voriconazole were tested in the presence of 3.00 µg/mL of posaconazole. The ARK Posaconazole Assay did not crossreact with fluconazole, and voriconazole, and slight crossreactivity (up to 1.33%) with itraconazole was observed.

% Crossreactivity = 100 × (mean value Test – mean value Control)÷(Conc. Compound Tested)

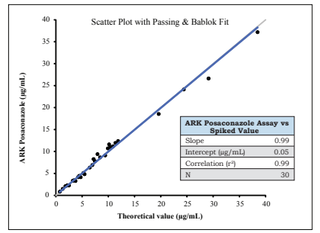
Sample	Crossreactivity (%)
Posaconazole (3.00 µg/ml) + Itraconazole (20 µg/ml)	1.33
Posaconazole (3.00 µg/ml) + Fluconazole (20 µg/ml)	0.12
Posaconazole (3.00 µg/ml) + Voriconazole (20 µg/ml)	0.17

VORICONAZOLE ASSAY
Other triazole Antifungals; fluconazole, itraconazole and posaconazole and N-oxide voriconazole metabolite were tested in the presence of 1.50 or 5.00 µg/mL of voriconazole. The ARK Voriconazole Assay did not crossreact with fluconazole, itraconazole and posaconazole; and slight crossreactivity (up to 1.35%) with N-oxide voriconazole was observed.

Sample	Crossreactivity (%)
Voriconazole (1.50 µg/ml) + Itraconazole (20 µg/ml)	-0.58
Voriconazole (1.50 µg/ml) + Fluconazole (20 µg/ml)	-0.47
Voriconazole (1.50 µg/ml) + Posaconazole (20 µg/ml)	-0.43
Voriconazole (1.50 µg/ml) + Vor-N-Oxide (20 µg/ml)	1.35
Voriconazole (5.00 µg/ml) + Itraconazole (20 µg/ml)	-0.71
Voriconazole (5.00 µg/ml) + Fluconazole (20 µg/ml)	-0.88
Voriconazole (5.00 µg/ml) + Posaconazole (20 µg/ml)	-1.23
Voriconazole (5.00 µg/ml) + Vor-N-Oxide (20 µg/ml)	0.83

Accuracy - Recovery: Posaconazole Assay

Simulated specimens were made throughout the proposed calibration range; samples from thirty individuals (21 serum & 9 plasma) were spiked with known concentrations of posaconazole. Passing-Bablok regression analysis was performed using Analyze-It software to assess relative accuracy between the observed and expected concentrations. The analysis gave a slope of 0.99, intercept of 0.05 and a correlation of 0.99 calculated using Pearson's Correlation (r^2). The strong correlation suggests that serum and plasma are equivalent matrices.



KKGT CONTROL: Proficiency Testing for Antifungal Drugs

Association for Quality Assessment in Therapeutic Drug Monitoring and Clinical Toxicology (The Hague, The Netherlands; and Department of Medical Microbiology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands). KKGT Antifungal Control Serum 2013 was evaluated. The sample was tested with a total of 10 replicates. The mean ARK result, standard deviation, %CV, and percentage recovery versus the spiked value were calculated.

KKGT Control	Spiked Value (µg/mL)	ARK Result (µg/mL)	SD	CV (%)	N	Recovery (%)
Posaconazole	1.99	2.00	0.02	1.0	10	100.7
Voriconazole	2.98	2.94	0.09	3.2	10	98.8

The UK NEQAS Antibiotic Assay-Antifungal Panel Sample

Fifteen antifungal panel samples (UK NEQAS for Antibiotic Assays, Department of Microbiology, North Bristol NHS Trust, Southmead Hospital, Bristol BS10 5NB, UK) were evaluated. ARK test results are singlicate determinations. Relative recoveries (%) versus the weighed-in value and the All Laboratory Trimmed Mean (ALTM) were calculated. All the ARK Voriconazole results fell within 2SD of the ALTM and all the posaconazole result except two samples (#3209 and #3411) fell within 2SD of the ALTM.

Posaconazole Assay

Sample ID	Weigh-in Value (µg/mL)	ALTM Value ± 2SD (µg/mL)	ARK Result (µg/mL)	RecoveryWeigh-in (%)	Recovery ALTM (%)
3198	1.63	1.49 ± 0.68	1.43	87.7	96.0
3199	3.54	3.56 ± 0.94	3.20	90.4	89.9
3200	0.68	0.83 ± 0.20	0.71	104.4	85.5
3201	4.27	4.51 ± 1.02	4.22	98.8	93.6
3202	0.92	0.87 ± 0.28	0.83	90.2	95.4
3203	6.00	5.58 ± 1.40	4.76	79.3	85.3
3204	0	0.10 ± 0.00	0.01	< LLOQ	< LLOQ
3205	0.49	0.42 ± 0.20	0.36	73.5	85.7
3206	2.10	2.29 ± 0.40	1.79	85.2	78.2
3207	4.99	5.58 ± 1.30	4.81	96.4	86.2
3208	0.19	0.20 ± 0.04	0.19	100	95.0
3209	2.90	3.07 ± 0.60	2.38	82.1	77.5
3410	0	0.29 ± 0.80	0	< LLOQ	< LLOQ
3411	3.34	3.06 ± 0.48	2.43	72.8	79.4
3412	0.64	0.61 ± 0.14	0.58	90.6	95.1

Voriconazole Assay

Sample ID	Weigh-in Value (µg/mL)	ALTM Value ± 2SD (µg/mL)	ARK Result (µg/mL)	RecoveryWeigh-in (%)	Recovery ALTM (%)
3198	0.52	0.52 ± 0.22	0.40	< LLOQ	< LLOQ
3199	2.10	1.99 ± 0.50	2.40	114.3	120.6
3200	5.01	4.88 ± 1.00	5.65	112.8	115.8
3201	2.91	3.14 ± 1.30	3.13	107.6	99.7
3202	3.79	4.31 ± 0.98	4.21	111.1	97.7
3203	0.35	0.37 ± 0.20	0.19	< LLOQ	< LLOQ
3204	7.12	5.99 ± 1.16	6.35	89.2	106.0
3205	1.60	1.51 ± 0.40	1.77	110.6	117.2
3206	5.87	6.59 ± 1.20	6.48	110.4	98.3
3207	0	0 ± 0.16	0.02	< LLOQ	< LLOQ
3208	1.00	0.90 ± 0.22	0.82	82	91.1
3209	8.80	9.03 ± 1.80	8.37	95.1	92.7
3410	1.10	1.18 ± 0.20	1.02	92.7	86.4
3411	0.35	0.44 ± 0.12	0.16	< LLOQ	< LLOQ
3412	2.01	2.29 ± 0.50	2.40	119.4	104.8

Conclusions

The ARK Posaconazole Assay and the ARK Voriconazole Assay enable quantitative measurements of the respective drugs in human serum with excellent precision and specificity at low concentrations. Ability to measure trough levels accurately and with fast turn-around time will enable clinically useful, routine therapeutic drug monitoring of these antifungal drugs.

PROPOSED INTENDED USE

The ARK Posaconazole and Voriconazole Assays are homogeneous enzyme immunoassays intended for the quantitative determination of posaconazole or voriconazole, respectively, in human serum or plasma on automated clinical chemistry analyzers. The measurements obtained are used in monitoring levels of posaconazole or voriconazole, respectively, to help ensure appropriate therapy.

REGULATORY STATUS

The performance characteristics of the ARK Posaconazole Assay and the ARK Voriconazole Assay have not been established. The assays have not been cleared or approved by the U.S. FDA for in vitro diagnostic use.

Assays for the next generation