

Introduction

ABSTRACT

Background

Methotrexate (MTX), a classical antifolate, can be safely administered over a wide dose range as maintenance chemotherapy for acute lymphoblastic leukemia and treatment of nononcologic diseases including rheumatoid arthritis or psoriasis. When combined with leucovorin (LV) rescue, high-dose MTX (HDMTX; doses of 1,000–33,000 mg/m²) is usually administered as a prolonged i.v. infusion for a variety of cancers, including acute lymphoblastic leukemia, lymphoma, osteosarcoma, breast cancer, and head and neck cancer. HDMTX can be safely administered to patients with normal renal function by vigorously hydrating and alkalinizing the patient to enhance the solubility of MTX in urine. Serum levels may reach 1000 µmol/L or more. Pharmacokinetically guided LV rescue by monitoring MTX serum levels is required to prevent potentially lethal MTX toxicity. Ability to measure MTX accurately at 0.05 µmol/L enables clinical determination of non-toxic status.

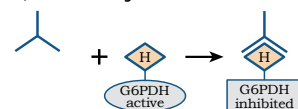
Objective

Evaluate the analytical performance of a new ARK Methotrexate Assay.

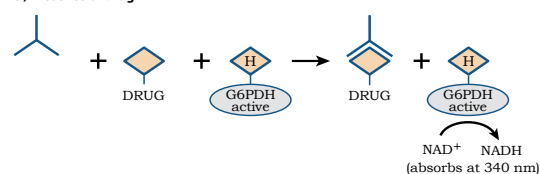
Method

The ARK™ Methotrexate Assay is a homogenous enzyme immunoassay for quantifying MTX in human serum or plasma. The assay was evaluated on the Roche/Hitachi 917 system. Increasing reaction rate correlates to increasing MTX concentration for a six point calibration curve (0 to 1.20 µmol/L). Six-level (0.07, 0.40, 0.80, 5.0, 50.0, and 500.0 µmol/L) quality controls were run. Performance of the assay was determined by assessing precision, limit of quantitation, linearity, endogenous interference, specificity, proficiency samples from the Heath Control scheme and method comparison to Abbott TDX® MTX II Assay.

A) Absence of drug



B) Presence of drug



RESULTS

Total Precision (%CV) for controls was 7.2% (0.07 µmol/L), 3.7% (0.40 µmol/L), 5.6% (0.80 µmol/L), 4.9% (1:10 of 5.0 µmol/L), 5.2% (1:100 of 50.0 µmol/L), and 6.4% (1:1000 of 500.0 µmol/L) respectively. Limit of Detection and Quantitation were comparable to that of the Tdx method: LOD ≤ 0.02 µmol/L and LOQ was 0.04 µmol/L (12%CV, 98.8% analytical recovery). Analytical recovery was within 10% for nominal values 0.15 to 1.00 µmol/L. The ARK assay was linear from 0.035 to 1.26 µmol/L. Endogenous substances did not interfere with measurement of MTX at the levels tested. Crossreactivity to the major metabolite, 7-hydroxy MTX, was equivalent to that of Tdx; 20 µmol/L of 7-hydroxy MTX in the presence of its parent molecule MTX (0.2 µmol/L in serum) resulted as 0.12% crossreactivity in both assays. Recoveries of MTX in proficiency samples from the Heath Control scheme were within 10% of spiked and consensus values. For method comparison (35 samples): ARK = 0.99 Tdx - 0.00 (r² = 0.99) using Passing Bablok regression analysis.

Precision

Precision was determined as described in CLSI/NCCLS Protocol EP5-A2. The six-level ARK Methotrexate Control and pooled human specimens containing methotrexate were used in the study. Each level was assayed in quadruplicate twice a day for 20 days. Each of the runs per day was separated by at least two hours. The within run, between day, total SD, and percent CVs were calculated.

Sample	N	Within Run			Between Day		Total	
		Mean (µg/mL)	SD	CV (%)	SD	CV (%)	SD	CV (%)
ARK Methotrexate Control								
LOW	160	0.06	0.005	8.2	0.005	7.3	0.007	10.7
MID	160	0.37	0.011	3.0	0.008	2.1	0.014	3.8
HIGH	160	0.76	0.032	4.3	0.030	4.0	0.045	5.9
5*	160	4.8	0.15	3.1	0.13	2.8	0.20	4.2
50*	160	49	1.36	2.8	2.32	4.8	2.72	5.6
500*	160	476	15.17	3.2	30.75	6.5	34.66	7.3
Patient Pool								
LOW	160	0.07	0.006	9.1	0.005	7.5	0.008	11.7
MID	160	0.41	0.013	3.3	0.026	6.4	0.029	7.2
HIGH	160	0.82	0.037	4.5	0.043	5.2	0.057	7.0
5*	160	4.6	0.14	3.1	0.183	4.0	0.24	5.3
50*	160	45	1.33	3.0	2.63	5.9	2.93	6.6
500*	160	461	11.84	2.6	27.04	5.9	29.60	6.4

*Samples were diluted in ARK Methotrexate Dilution Buffer. Mean result and SD were multiplied by the dilution factor.

Limit of Detection, Limit of Blank, and Lower Limit of Quantitation

Limit of quantitation was evaluated according to CLSI/NCCLS EP17-A. Pooled human serum was supplemented with methotrexate to give concentrations of 0.02, 0.03, 0.04, and 0.05 µmol/L.

Limit of Quantitation			
Nominal (µmol/L)	N	Grand Mean (µmol/L)	RMS SD
0.00	60	0.002	0.0039
0.02	60	0.020	0.0048
0.03	40	0.036	0.0048
0.04	40	0.045	0.0049
0.05	40	0.054	0.0051

Criterion	N	MTX Concentration (µmol/L)
Limit of Blank (LoB); • µB + 1.645 SD, where SD = 0.005	60	0.01
Limit of Detection (LoD); • LoB + 1.652 SD, where SD = 0.005	60	0.02
Limit of Quantitation (LoQ); • LoQ - 2 SD > LoD	40	0.04

Analytical Recovery

Samples were prepared by volumetric addition of methotrexate (Cerilliant Certified Stock solution 99.8% purity) to human serum negative for methotrexate. Drug concentrations across the assay range (0.06, 0.10, 0.30, 0.60, and 1.00 µmol/L) were tested, six replicates. The results were averaged and compared to the theoretical target concentration and percentage recovery calculated.

For methotrexate concentrations >0.1 µmol/L, the percentage recovery ranged from 98.3% to 102.2%. At 0.06 µmol/L, recovery was within 0.01 µmol/L (0.007 µmol/L), 111.1%. An overall mean percentage recovery was 102.1%.

Target (µmol/L)	Mean (µmol/L)	SD	CV (%)	Recovery (%)
0.06	0.067	0.008	12.2	111.1
0.10	0.100	0.006	6.3	100.0
0.30	0.295	0.010	3.6	98.3
0.60	0.613	0.029	4.7	102.2
1.00	0.988	0.090	9.1	98.8

Mean percent recovery: 102.1

Specificity

Specificity studies were conducted using Clinical and Laboratory Standards Institute (CLSI) Guideline EP7-A2: Interference Testing in Clinical Chemistry. Two lots were tested (D2 and D3).

CROSSREACTIVITY TO 7-HYDROXYMETHOTREXATE, THE MAJOR METABOLITE

The ARK Methotrexate Assay did not crossreact with the major metabolite 7-OH-MTX.

Reagent Lot	7-OH MTX (µmol/L)	Serum Control (µmol/L)	MTX (µmol/L)	Cross Reactivity (%)
Cross Reactivity 7-OH MTX in the presence of 0.05 µmol/L MTX				
D2	5.0	0.06	0.06	0.01
D3	5.0	0.04	0.05	0.02
Cross Reactivity 7-OH MTX in the presence of 0.50 µmol/L MTX				
D2	50.0	0.44	0.47	0.07
D3	50.0	0.46	0.48	0.05

CROSSREACTIVITY TO THE MINOR, INACTIVE METABOLITE 2,4-DIAMINO-N¹⁰-METHYLPYTEROIC ACID (DAMPA)

The ARK Methotrexate Assay crossreacts substantially with the minor metabolite DAMPA. Tests were performed in the absence of the parent drug methotrexate. Crossreactivity to DAMPA ranged 64.3 to 100%. The assay should not be used during possible compassionate therapy with glucarpidase (carboxypeptidase G2) that rapidly converts circulating methotrexate to DAMPA.

DRUGS THAT CROSSREACT

The ARK Methotrexate Assay crossreacts slightly with triamterene and trimethoprim, however these drugs may be contraindicated for MTX cancer treatment due to additional adverse effects if co-administered. The structures of these compounds closely match the pteridine ring moiety of methotrexate.

Compound	Tested (µmol/L)	Apparent MTX (µmol/L)	Cross Reactivity (%)
MTX Absent			
Triamterene	25	0.46	1.85
Trimethoprim	100	0.17	0.17
MTX Present 0.05 µmol/L			
Triamterene	25	0.89	3.32
Trimethoprim	100	0.16	0.12
MTX Present 0.50 µmol/L			
Triamterene	25	1.04	2.31
Trimethoprim	100	0.99	0.54

CROSSREACTIVITY TO FOLATE ANALOGS AND OTHER COMPOUNDS

The ARK Methotrexate Assay did not crossreact (≤ 0.01%) with folate analogs or other compounds at ≥ 1000 µmol/L as tested.

Compound	Tested (µmol/L)
Adriamycin	1000
Cyclophosphamide	1500
Cytosine	1000
Dihydrofolic Acid	1000
DL-6-Methyl-5,6,7,8-Tetrahydropterine	1000
Folic Acid	1000
Folic Acid (leucovorin)	1000
5-Fluorouracil	3000
6-Mercaptopurine	1000
5-Methyltetrahydrofolic acid	1000
Prednisolone	1000
Pyrimethamine	1000
Sulfamethoxazole	1600
Tetrahydrofolic Acid	1000
Vinblastine	1000
Vincristine	1000

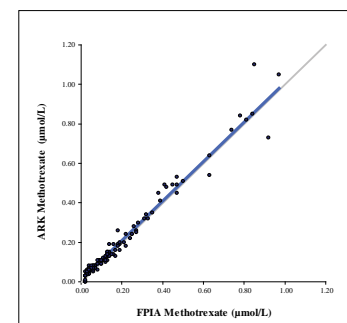
Endogenous Interference

Interference studies were conducted using CLSI/NCCLS Protocol EP7-A2 as a guideline. Clinically high concentrations of potentially interfering endogenous substances in serum with known levels of methotrexate (approximately 0.05 and 0.50 µmol/L) were evaluated. Measurement of methotrexate by the ARK Methotrexate Assay was not affected by the presence of interfering substances at the levels tested.

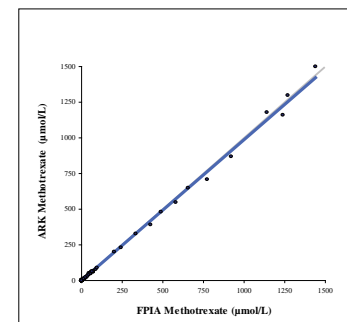
Interfering Substance	Interferent Concentration	Serum Control Concentration (µmol/L)	Test Sample Concentration (µmol/L)	Recovery (%)
Methotrexate (~ 0.05 µmol/L)				
Albumin	12 g/dL	0.05	0.06	-
Bilirubin (conjugated)	70 mg/dL	0.05	0.06	-
Bilirubin (unconjugated)	70 mg/dL	0.05	0.06	-
Cholesterol	400 mg/dL	0.05	0.06	-
Gamma-Globulin	12 g/dL	0.05	0.06	-
Hemoglobin	1000 mg/dL	0.04	0.05	-
Intralipid®	500 mg/dL	0.05	0.05	-
Rheumatoid Factor	1100 IU/mL	0.05	0.06	-
Triglycerides	749 mg/dL	0.04	0.04	-
Uric Acid	30 mg/dL	0.05	0.04	-
Methotrexate (~ 0.5 µmol/L)				
Albumin	12 g/dL	0.48	0.45	92.8
Bilirubin - conjugated	70 mg/dL	0.48	0.51	105.5
Bilirubin - unconjugated	70 mg/dL	0.48	0.52	106.9
Cholesterol	400 mg/dL	0.47	0.49	105.4
Gamma-Globulin	12 g/dL	0.48	0.51	105.5
Hemoglobin	1000 mg/dL	0.49	0.45	93.2
Intralipid®	500 mg/dL	0.43	0.45	105.1
Rheumatoid Factor	1100 IU/mL	0.43	0.41	96.1
Triglycerides	749 mg/dL	0.49	0.45	91.4
Uric Acid	30 mg/dL	0.48	0.50	102.8

Method Comparison

Correlation studies were performed using CLSI/NCCLS Protocol EP9-A2. Results from the ARK Methotrexate Assay were compared with results from Fluorescence Polarized Immunoassay method (monoclonal FPIA). Methotrexate concentrations by FPIA ranged 0.02 to 1440 µmol/L (µM). ARK Methotrexate values ranged 0.00 to 1500 µmol/L.



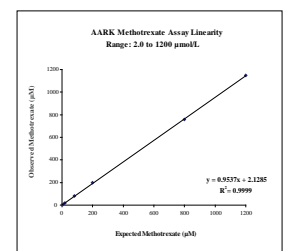
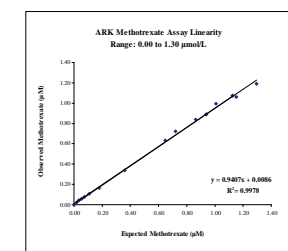
MTX sample conc. Range 0.02 to 0.97 µmol/L	
95% Confidence Interval	
Slope	1.00 (1.00 to 1.02)
y-intercept	0.01 (0.01 to 0.01)
Correlation Coefficient (r ²)	0.971 (0.958 to 0.980)
Number of Samples	111



MTX sample conc. Range 0.02 to 1440 µmol/L	
95% Confidence Interval	
Slope	0.99 (0.96 to 1.00)
y-intercept	0.01 (0.01 to 0.01)
Correlation Coefficient (r ²)	0.998 (0.997 to 0.998)
Number of Samples	157

Linearity

Linearity studies were performed as suggested in CLSI/NCCLS Protocol EP6-A. Samples containing methotrexate were prepared proportionally in pooled human serum. The ARK Methotrexate Assay was linear throughout the calibration range from the limit of detection (LoD = 0.02 µmol/L) and for samples between 2 and 1200 µmol/L (diluted into the calibration range with ARK Methotrexate Dilution Buffer). Regression of assayed methotrexate concentrations was linear throughout the range.



Proficiency Samples (Heath Control (UK NEQAS) Sample Evaluation)

Heath Controls (UK NEQAS: United Kingdom National External Quality Assessment Scheme, LGC Standards, Queens Road, Teddington, Middlesex TW11 0LY U.K.) and CAP TDM Survey Samples (College of American Pathologists; Northfield, IL) were evaluated. The mean and standard deviation (SD) of the consensus reported predicate methotrexate concentrations are shown. Mean measurements of six replicates are shown. Tests by the ARK assay were considered within the consensus range for the predicate FPIA device.

Sample ID	Heath QC - FPIA Consensus		ARK Methotrexate Assay		
	Mean (µmol/L)	SD	Mean (µmol/L)	SD	%CV
MTX 0610	10.79	1.39	11.40	1.08	9.5
MTX 0710	0.27	0.02	0.26	0.004	1.6
MTX 0810	0.41	0.03	0.46	0.010	2.3
MTX 1010	0.03	0.02	0.02	0.000	0.0
MTX 1110	3.75	0.30	3.50	0.09	2.6
MTX 1210	0.10	0.02	0.09	0.005	6.4

Sample ID	CAP Survey - FPIA Consensus		ARK Methotrexate Assay		
	Mean (µmol/L)	SD	Mean (µmol/L)	SD	%CV
CHM01	35.174	2.558	32.17	0.983	3.1
CHM02	8.123	0.457	7.67	0.427	5.6

Conclusions

The ARK Methotrexate Assay provided quantitative measurement MTX in serum and plasma on the Roche/Hitachi 917 and correlated with Tdx Methotrexate II Assay. Its homogeneous enzyme immunoassay technology is well-suited for routine TDM of MTX on automated clinical laboratory systems.

INTENDED USE

The ARK Methotrexate Assay is a homogeneous enzyme immunoassay intended for the quantitative determination of methotrexate in human serum or plasma on automated clinical chemistry analyzers. The measurements obtained are used in monitoring levels of methotrexate to ensure appropriate therapy.

MEASUREMENT RANGE

The range of the ARK Methotrexate Assay calibration curve is 0.00 - 1.20 µmol/L. Specimens containing methotrexate in higher concentrations are assayed by dilution of the specimen into the range of the calibration curve. Report assayed values exceeding the LoD according to the information provided for LoQ. Multiply the assayed result by the dilution factor for specimens containing methotrexate above the calibration range.

Regulatory Status - USA

Pending FDA 510(k) clearance