HOMOGENEOUS ENZYME IMMUNOASSAY FOR QUANTITATIVE DETERMINATION OF METHOTREXATE

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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/m^2) 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 0.050 µmol/L enables clinical determination of non-toxic status.

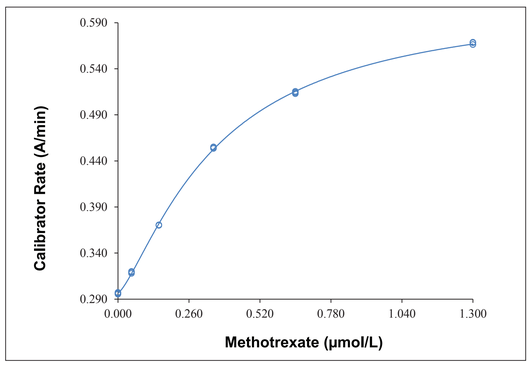
METHOD

The ARK™ Methotrexate II Assay is a homogeneous enzyme immunoassay for the quantitative determination of methotrexate in human serum or plasma on automated clinical chemistry analyzers. The assay was evaluated on the Beckman AU680 analyzer. The assay consists of two reagents, six-level calibrators, six-level (0.070, 0.400, 0.800, 5.0, 50.0, 500.0 µmol/L) quality controls, and dilution buffer. Performance of the assay was determined by assessing precision, limit of quantitation, linearity, endogenous substances interference, high sample dilution (1:10 serial dilutions), on-board auto dilution (at 1:10 and 1:50), cross-reactivity, and method comparison.

RESULTS

CALIBRATION CURVE

The assay system uses 6 calibrators which contain methotrexate at concentrations of 0.000, 0.050, 0.150, 0.350, 0.650, and 1.300 µmol/L.



PRECISION

Precision was determined as described in CLSI Protocol EP5-A3. Six-level controls and negative pooled human serum with supplemented methotrexate were assayed in quadruplicate twice a day for 20 days. Mean determinations of methotrexate, standard deviation (SD) for within-run, between-day, and total (within-lab) coefficients of variation (% CVs) were calculated.

Calibration Range Control Levels

Sample	N	Mean (µmol/L)	Within Run		Between Day		Total (Within Lab)	
			SD	CV (%)	SD	CV (%)	SD	CV (%)
Control Low	160	0.069	0.002	2.84	0.001	1.23	0.002	3.00
Control Mid	160	0.411	0.006	1.40	0.002	0.43	0.006	1.40
Control High	160	0.811	0.014	1.79	0.008	0.97	0.017	2.05
Serum Low	160	0.070	0.002	2.50	0.001	1.49	0.002	2.88
Serum Mid	160	0.404	0.008	1.86	0.003	0.65	0.008	1.92
Serum High	160	0.846	0.016	1.93	0.008	0.95	0.017	2.06

High Range Control Levels

Sample	N	Mean (µmol/L)	Within Run		Between Day		Total (Within Lab)	
			SD	CV (%)	SD	CV (%)	SD	CV (%)
Control 5	160	4.868	0.007	1.44	0.036	0.74	0.077	1.58
Control 50	160	49.660	1.108	2.23	0.397	0.80	1.141	2.30
Control 500	160	493.769	8.012	1.62	2.483	0.50	8.012	1.62
Serum 5	160	5.247	0.076	1.45	0.028	0.54	0.078	1.49
Serum 50	160	51.614	0.723	1.40	0.285	0.55	0.777	1.51
Serum 500	160	507.988	7.632	1.50	4.240	0.83	8.538	1.68

LIMIT OF BLANK, LIMIT OF DETECTION, AND LIMIT OF QUANTITATION

Limit of Blank (LoB), Limit of Detection (LoD), and Lower Limit of Quantitation (LLoQ) were evaluated according to CLSI EP17-A2. The LLoQ of the ARK Methotrexate II Assay is defined as the lowest concentration for which acceptable inter-assay precision (≤0.010 SD where RMS SD is used for SD) with recovery (Grand Mean – Nominal ±0.010 µmol/L) is observed. The criteria of LLoQ were met at all levels tested. Criteria for LLoQ were met at 0.030 µmol/L with 4.87% CV and 113.6% recovery.

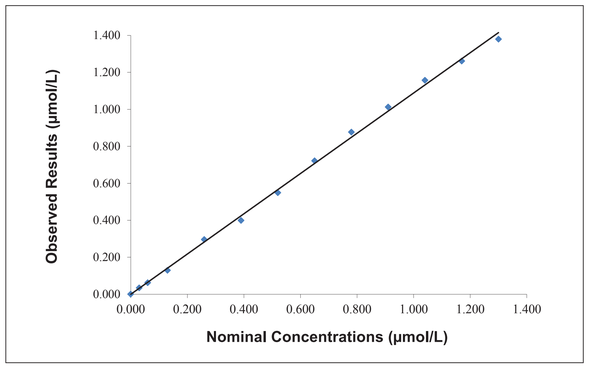
Criterion	N	Methotrexate (µmol/L)
Limit of Blank (LoB); 57th value = 0.000 µmol/L, 58th value = 0.000 µmol/L	60	0.000
Limit of Detection (LoD); LoB + 1.652SD, where SD = 0.002	60	0.004

Limit of Quantitation (LoQ)

Conc. Tested (µmol/L)	Mean Recovery (µmol/L)	Recovery (%)	RMS SD	CV (%)	N
0.030	0.034	113.6	0.002	4.87	40
0.040	0.043	107.1	0.002	4.01	40
0.050	0.052	104.2	0.002	4.00	40

LINEARITY

Linearity studies were performed according to CLSI Protocol EP6-Ed2 by testing concentrations of methotrexate within the assay calibration range (0.000 to 1.300 µmol/L). Negative pooled human serum was supplemented with methotrexate (1.600 µmol/L) and then diluted proportionally in serum. Weighted linear regression analysis (intercept set to zero) was performed in which the varying observed standard deviations were factored into calculation to generate a fitted slope. The slope was then multiplied against nominal concentrations to determine predicted results. Less than 10% deviation from linearity was observed over the range 0.030 to 1.300 µmol/L.



MANUAL HIGH SAMPLE DILUTION

Manual High Sample Dilutions were tested with spiked human serum samples and ARK Methotrexate II High Range Controls (5, 50, and 500 µmol/L). Serial 1:10 dilutions were performed using the ARK Methotrexate II Dilution Buffer.

Spiked Level	Dilution Factor	Recovery (%)
Serum 2 µmol/L	10 (1:10)	102.3
Serum 20 µmol/L	100 (1:10 × 2)	102.3
Serum 200 µmol/L	1000 (1:10 × 3)	100.0
Serum 1200 µmol/L	1000 (1:10 × 3)	99.9
Serum 1200 µmol/L	10000 (1:10 × 4)	94.0
Control 5 µmol/L	10 (1:10)	94.3
Control 50 µmol/L	100 (1:10 × 2)	93.2
Control 500 µmol/L	1000 (1:10 × 3)	91.9

ON-BOARD AUTO DILUTION

On-Board Auto Dilutions on the Beckman Coulter AU680® Analyzer were tested with spiked human serum samples and ARK Methotrexate II High Range Controls (5, 50, and 500 µmol/L). Analyzer automated 1:10 and 1:50 dilutions were both tested using the ARK Methotrexate II Dilution Buffer.

1:10 Auto Dilution	
Spiked Level	Recovery (%)
Serum 2 µmol/L	108.9
Control 5 µmol/L	96.3

1:50 Auto Dilution	
Spiked Level	Recovery (%)
Serum 2 µmol/L	108.9
Serum 20 µmol/L	100.5
Control 5 µmol/L	96.3
Control 50 µmol/L	106.7

ENDOGENOUS INTERFERING SUBSTANCES

Clinically high concentrations of potentially interfering endogenous substances were spiked in serum with known levels of methotrexate (0.050 and 0.500 µmol/L) and evaluated along with a serum control of methotrexate. Measurements of methotrexate were not affected by the presence of interfering substances at the levels tested.

Endogenous Substance	Conc. Tested	± µmol/L from Control (0.050 µmol/L Methotrexate)	% Interference (0.500 µmol/L Methotrexate)
Human Albumin	12 g/dL	0.002	-1.04
Conj. - Bilirubin	72 mg/dL	0.001	1.96
Unconj. - Bilirubin	72 mg/dL	0.003	0.23
Cholesterol	500 mg/dL	0.005	3.49
Human IgG	12 g/dL	0.003	2.42
Hemoglobin	1000 mg/dL	-0.006	-2.72
Rheumatoid Factor	1080 IU/mL	0.001	3.52
Triglycerides	1000 mg/dL	-0.007	7.48
Uric Acid	30 mg/dL	0.000	1.60

SPECIFICITY

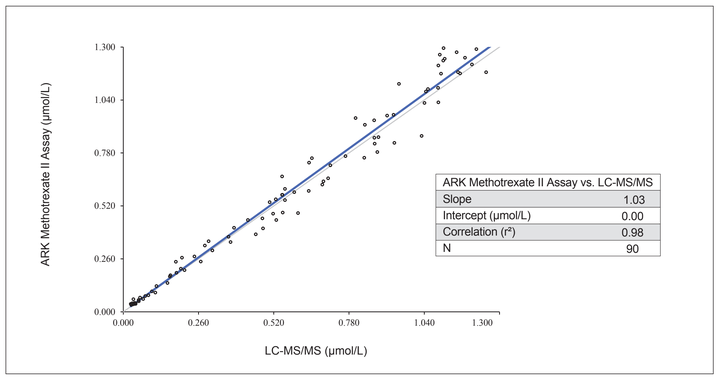
A high concentration of each compound (potentially co-administered drugs, folate derivatives, and compounds of similar structure) was spiked into serum with known levels of methotrexate (0.050 and 0.500 µmol/L) and assayed alongside methotrexate serum control. The major metabolite, 7-hydroxymethotrexate (7-OH-MTX), was also tested. The following compounds at the levels tested shown below did not interfere with the measurement of methotrexate (<10% interference).

Metabolite	Interference (%)	
	0.050 µmol/L Methotrexate	0.500 µmol/L Methotrexate
7-Hydroxymethotrexate (50 µmol/L)	8.72%	0.58%

Compound Tested	Conc. Tested (µmol/L)
Adriamycin	1000
Cyclophosphamide	2200
Cytosine	1000
Dihydrofolic Acid	1000
Tetrahydrofolic Acid	1000
DL-6-Methyl-5,6,7,8-Tetrahydropterine	1000
Folic Acid	1000
Folinic Acid	1000
5-Fluorouracil	3000
6-Mercaptopurine	1000
5-Methyltetrahydrofolic Acid	1000
Prednisolone	1000
Pyrimethamine	1000
Sulfamethoxazole	1600
Vinblastine	1000
Vincristine	1000
Trimethoprim	150
Triamterene	25

METHOD COMPARISON

Correlation studies were performed using CLSI Protocol EP9-A3. All samples were in serum. Results from the ARK Methotrexate II Assay were compared to results from liquid chromatography with tandem mass spectrometry (LC-MS/MS). Passing-Bablok regression analysis results are shown below.



CONCLUSIONS

ARK Methotrexate II Assay demonstrates accurate and precise quantitation of methotrexate. ARK Methotrexate II reagents, calibrators, controls, and dilution buffer are provided in liquid form ready-to-use. Ability to measure methotrexate accurately and with fast turn-around time will enable clinically useful, routine monitoring of methotrexate.

PROPOSED INTENDED USE

The ARK Methotrexate II 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 help ensure appropriate therapy.

REGULATORY STATUS

The performance characteristics of the ARK Methotrexate II Assay have not been established. The assay has not been cleared by the U.S. FDA for in vitro diagnostic use