IMPROVED HOMOGENEOUS ENZYME IMMUNOASSAY FOR THE QUANTITATIVE DETERMINATION OF METHOTREXATE

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BACKGROUND

Methotrexate (MTX) is used at high doses (HDMTX, 1,000-33,000 mg/m2 by prolonged i.v. infusion) with leucovorin (LV) rescue for a variety of cancers. 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. HDMTX safety is improved by monitoring serum creatinine and MTX levels during renal clearance of MTX, which can initially exceed 1000 µmol/L. Delayed clearance can cause acute kidney injury, further delaying clearance and leading to severe toxicity. MTX is monitored at 12-24h intervals until it drops to a safe level for discharge, <0.05 µmol/L. MTX levels during clearance also inform LV dosing. Accurate measurement of MTX, especially at 0.050 µmol/L is an important clinical tool.

METHOD

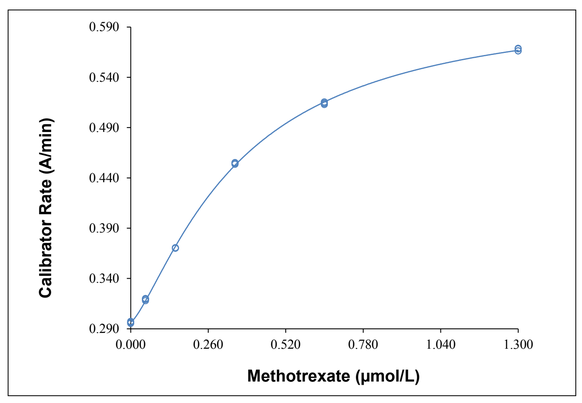
The ARK Methotrexate II Assay is a homogeneous enzyme immunoassay for the quantitative determination of methotrexate in human serum or plasma on automated clinical chemistry analyzers. The assay uses recombinant rabbit monoclonal antibodies and recombinant enzyme-drug conjugate; these were selected to maintain or improve specificity and for analytical performance characteristics. It consists of two reagents, a six-level calibrator set, and controls.

The assay was developed and characterized using the Beckman AU680 analyzer by optimizing reagents for repeatability precision profile performance using techniques published by T. M. Houts[1] and W. A. Sadler[2]. The low and flat precision profile allowed the analytical measurement interval to be improved at both the low (from 0.040 to 0.030 µmol/L) and high (from 1.20 to 1.30 µmol/L) ends while simultaneously improving precision and linearity.

RESULTS

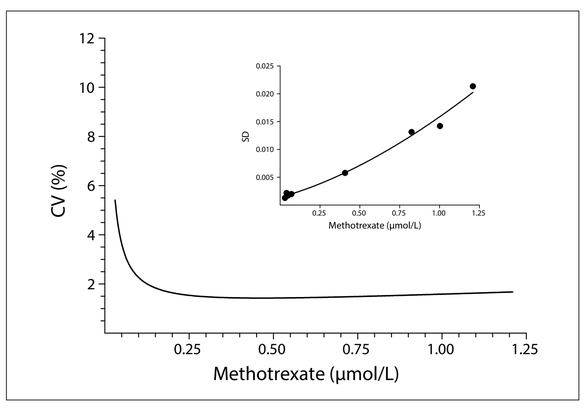
CALIBRATION CURVE

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.



REPEATABILITY PRECISION PROFILE

8 levels of serum supplemented with methotrexate were evaluated for repeatability in a 20-day, 2 runs per day, 4 replicates per run precision study. The graph shows the precision profile generated by the Variance Function Program[2] for the ARK Methotrexate II Assay on the Beckman AU680 Analyzer. The valid range for the precision profile is from 0.030 to 1.21 µmol/L.



PRECISION

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

20 Day Precision Results								
Serum	N	Mean (µmol/L)	Within Run		Between Day		Total (Within Device)	
			SD	CV (%)	SD	CV (%)	SD	CV (%)
Low	160	0.070	0.002	2.50	0.001	1.49	0.002	2.88
Mid	160	0.404	0.008	1.86	0.003	0.65	0.008	1.92
High	160	0.846	0.016	1.93	0.008	0.95	0.017	2.06

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

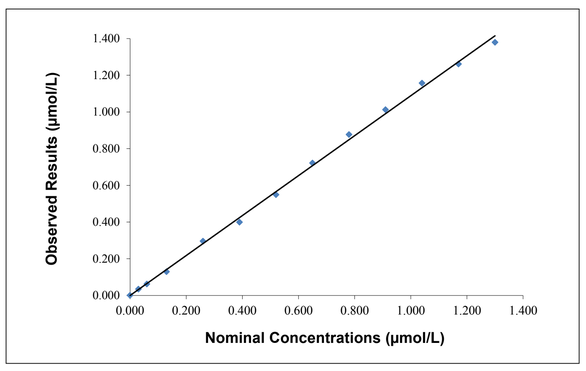
Limit of Blank (LoB), Limit of Detection (LoD), and Lower Limit of Quantitation (LLoQ) were evaluated according to CLSI EP17-A2. The LLoQ of the ARK Methotrexate II Assay is defined as the lowest concentration for which acceptable precision (<20% CV using RMS SD) recovery (± 15%) are observed. The criteria 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. Variance-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/.



EXTENDED MEASUREMENT RANGE: 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

EXTENDED MEASUREMENT RANGE: 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

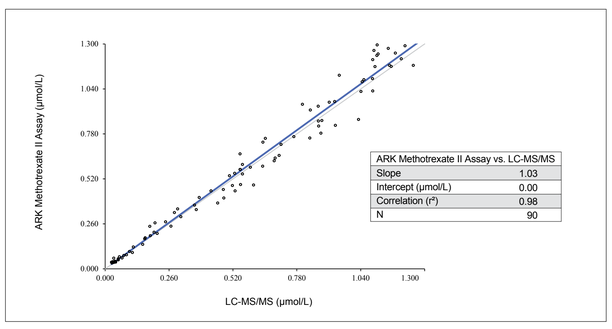
A high concentration of each compound (potentially co-administered drugs, folate derivatives, and compounds of similar structure) was spiked into serum with known levels of methotrexate (0.050 and 0.500 µmol/L) and assayed alongside methotrexate serum control. The major metabolite, 7-hydroxymethotrexate (7-OH-MTX), produced less than 0.005 µmol/L or 10% interference. The additional compounds shown below did not interfere (<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. 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.

The ARK Methotrexate II Assay has been successfully applied to additional analyzers from Abbott, Beckman, Siemens, QuidelOrtho, and Roche.

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.

Specimens obtained from patients who have received glucarpidase (carboxypeptidase G2) as a high dose methotrexate rescue therapy should not be tested with the ARK Methotrexate II Assay.

REGULATORY STATUS

The ARK Methotrexate II Assay is FDA cleared and CE marked under IVDR.

REFERENCES

[1] Houts, T.M. Useful estimates of assay performance from small data sets. Poster presented at AACC's 36th Annual Oak Ridge Conference (2004). DOI: 10.1373/clinchem.2004.036996

[2] Sadler, W.A. Variance Function Program available from Australasian Association for Clinical Biochemistry. https://aacb.asn.au/AACB/AACB/Resources/Variance-Function-Program.aspx