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Drug Monitoring and Toxicology

HPLC Determination of Erythrocyte Methotrexate Polyglutamates after Low-Dose Methotrexate Therapy in Patients with Rheumatoid Arthritis

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Background: **Methotrexate (MTX) may produce antiarthritic effects through polyglutamation to methotrexate polyglutamates (MTXPGs), a process that covalently attaches sequential γ-linked glutamic residues to MTX. We sought to develop an innovative HPLC method for the quantification of these metabolites in erythrocytes.**
Methods: **Two alternative approaches were developed. In the first approach, MTXPGs from 50 μL of packed erythrocytes were converted to MTX in the presence of plasma γ-glutamyl hydrolase and mercaptoethanol at 37 °C. In the second approach, MTXPG species (up to the hepta order of glutamation) from 100 μL packed erythrocytes were directly quantified in a single run. In both methods, the MTXPGs were extracted from the biological matrix by a simple perchloric acid deproteinization step with direct injection of the extract into the HPLC. The chromatography used a C_{18} reversed-phase column, an ammonium acetate/acetonitrile buffer, and postcolumn photo-oxidation of MTXPGs to fluorescent analytes.**
Results: **Intra- and interday imprecision (CVs) were <10% at low and high concentrations of analytes for both methods. The limit of quantification was 5 nmol/L. In 70 patients with rheumatoid arthritis receiving weekly low-dose MTX, the mean (SD) total MTXPG concentration measured after conversion of MTXPGs to MTX was similar to the total MTXPG concentration calculated from the sum of individual MTXPG species [117 (56) vs 120 (59) nmol/L; $r = 0.97$; slope = 1.0]. The triglutamate predominated over all other MTXPG species (36% of total), the pentaglutamate was the highest order of glutamation detected, and a stability study revealed no change in the polyglutamation pattern in erythrocytes 48 h after phlebotomy when the specimen was stored at 2–8 °C.**
Conclusion: **The proposed method for quantification of erythrocyte MTXPGs is rapid, sensitive, and accurate and can be applied to the routine monitoring of MTX therapy.**

The folate antagonist methotrexate (MTX)[3] is currently one of the most widely prescribed drugs for the treatment of rheumatoid arthritis *(1, 2)*. Although MTX is among the best tolerated of the disease-modifying antirheumatic drugs, major drawbacks with MTX therapy include the large interpatient variability in the clinical response and an unpredictable appearance of a large spectrum of side effects, including gastrointestinal disturbances, alopecia, increased liver enzymes, bone marrow suppression, and potentially life-threatening pneumonitis and cirrhosis *(3, 4)*. These scenarios complicate the effective dosing of MTX for patients with rheumatoid arthritis.

Because ~95% of a given MTX dose is metabolized within 24 h after administration, there is little value in monitoring low-dose MTX therapy using plasma concentrations *(5)*. However, MTX is intracellularly converted by folylpolyglutamate synthase to methotrexate polyglutamates (MTXPGs) *(6)*. This γ-linked sequential addition of glutamic acid residues enhances the intracellular retention of MTX. Furthermore, this process promotes the sustained inhibition of de novo purine synthesis (5-aminoimidazole carboxamide-ribonucleotide transformylase) *(7)*, thereby promoting the build-up of adenosine, a potent antiinflammatory agent *(8, 9)*.

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[3] Nonstandard abbreviations: MTX, methotrexate; MTXPG, methotrexate polyglutamate; DHFR, dihydrofolate reductase; and RBC, red blood cell.

Although some investigators have advocated the routine monitoring of MTXPGs *(10–17)* and evidence suggests that MTXPGs may be associated with efficacy and toxicity of the drug in the treatment of rheumatoid arthritis *(10, 11)*, psoriasis *(12)*, and cancer *(13, 14)*, very few methods are available for the quantification of MTXPGs in vivo; therefore, clinical monitoring of MTXPGs is not performed routinely *(18–20)*. Investigators have taken advantage of the property of MTX to inhibit the enzyme dihydrofolate reductase (DHFR), an important enzyme that catalyzes the reduction of dihydrofolate to tetrahydrofolate in the presence of NADPH *(18–20)*. Two alternative strategies have previously been developed. In the first strategy, developed by Kamen and Winick *(18)*, MTXPGs were measured with a highly sensitive radiochemical ligand-binding assay. In the second strategy, Shroeder and Heinsvig *(20)* developed an enzymatic assay in which MTXPGs were quantified by measurement of the decrease in absorbance that occurs when NADPH is converted to $NADP^+$.

Because the DHFR enzymatic assay may lack specificity and the radiochemical assay is time-consuming, there is a need for a more rapid and specific method that could be routinely applied to clinical practice. We therefore developed two original HPLC-fluorometry approaches for the quantification of MTXPGs. In the first method, MTXPGs are converted to MTX by plasma γ-glutamyl hydrolase, whereas in the second method, MTXPGs are individually quantified in a single run.

Materials and Methods

CHEMICALS AND PREPARATION OF CALIBRATORS

4-Amino-10-methylpteroylglutamic acid ($MTXPG_1$), 4-amino-10-methylpteroyldiglutamic acid ($MTXPG_2$), 4-amino-10-methylpteroyltriglutamic acid ($MTXPG_3$), 4-amino-10-methylpteroyltetraglutamic acid ($MTXPG_4$), 4-amino-10-methylpteroylpentaglutamic acid ($MTXPG_5$), 4-amino-10-methylpteroylhexaglutamic acid ($MTXPG_6$), and 4-amino-10-methylpteroylheptaglutamic acid ($MTXPG_7$) were purchased from Schircks Laboratories. MTXPGs were ammonium salts. HPLC-grade acetonitrile was purchased from Fisher Chemicals. Lyophilized plasma, hydrogen peroxide (300 mL/L), ammonium hydroxide, glacial acetic acid, potassium phosphate, and mercaptoethanol were purchased from Sigma.

MTX and MTXPGs ($MTXPG_2$, $MTXPG_3$, $MTXPG_4$, $MTXPG_5$, $MTXPG_6$, and $MTXPG_7$) were prepared by dissolution in 0.1 mol/L potassium hydroxide. After dissolution, the final concentration of each calibrator was confirmed using a Hitachi U-2000 spectrophotometer and the molar extinction coefficients ($\epsilon_{256\ nm} = 23\,000$ for all seven MTXPG species). The above calibrators were diluted to a final concentration in water and stored at −70 °C. The 100 μmol/L stock solutions were stable for at least 12 months under these conditions. Calibration curves were prepared by adding known amounts of MTXPGs to a pool of red blood cell (RBC) hemolysates isolated from healthy blood donors (Blood Bank, San Diego, CA). The quantification of total MTXPGs after conversion with plasma γ-glutamyl hydrolase was performed using calibrators consisting of $MTXPG_1$ and an equimolar mixture of $MTXPG_2$–$MTXPG_7$ ($MTXPG_{2–7}$). Alternatively, the quantification of individual MTXPGs was performed using a calibration mixture containing an equimolar amounts of all polyglutamates ($MTXPG_{1–7}$). Calibration curves were fit by linear regression using peak area vs concentrations.

CHROMATOGRAPHIC SYSTEM

The liquid chromatograph was an Agilent 1100 HPLC Chemstation system consisting of a quaternary pump, a system controller, an autoinjector, a sample cooler kept at 4 °C, and a fluorometric detector. The chromatographic separation was performed on a 25 cm × 4.6 mm (i.d.) Terra MS C_{18} column (5-μm particle size; Waters) protected by a guard column. MTX and MTXPGs were detected by use of postcolumn photooxidation with ultraviolet irradiation in the presence of hydrogen peroxide *(21, 22)*. A photochemical reactor unit (Aura Industries) equipped with a 254 nm low-pressure mercury ultraviolet lamp and containing 1 meter of 1/16-inch (o.d.) Teflon tubing (0.25 mm i.d.) assembled as a knitted coil was connected on-line after the analytical column but before the fluorometric detector. Mobile phase A consisted of 10 mmol/L ammonium acetate (pH 6.50) containing 2 mL/L hydrogen peroxide. Mobile phase B consisted of acetonitrile. Chromatograms were acquired and analyzed on a Hewlett-Packard Vector XA computer. The precolumn was changed every 200 injections, and the analytical column demonstrated no deterioration of performance after up to 1000 injections.

DETERMINATION OF TOTAL MTXPG CONCENTRATIONS AFTER CONVERSION TO MTX

Total MTXPGs were measured after conversion to MTX as follows. We added 100 μL of reconstituted plasma to 50 μL of RBC hemolysate in an Eppendorf tube (RBC lysis occurred during the cycle freeze-thaw process). After thoroughly mixing the sample for 30 s, we added 100 μL of buffer containing 100 mmol/L potassium phosphate and 150 mmol/L mercaptoethanol. Samples were incubated for 12–14 h in the dark at 37 °C. After incubation and cooling, we added 25 μL of 700 mL/L perchloric acid to the mixture, vortex-mixed for 10 s, and centrifuged it for 5 min. A total volume of 80 μL was injected into the HPLC system. The chromatographic separation consisted of a 15-min linear gradient from 0% to 25% acetonitrile (mobile phase B) at a flow rate of 1 mL/min. After 15 min, the mobile phase was returned to 100% mobile phase A and reequilibrated for 5 min. One sample was injected every 20 min. The MTX photolytic product was measured at an excitation wavelength set at 400 nm and an emission wavelength at 470 nm. The fluorometric signal was recorded between 10 and 15 min.

DETERMINATION OF INDIVIDUAL MTXPG CONCENTRATIONS

We homogenized 100 μL of packed RBC hemolysate with 150 μL of water, added 25 μL of 700 mL/L perchloric acid to the homogenate, vortex-mixed the mixture for 10 s, and centrifuged it for 5 min. A total volume of 80 μL of supernatant was injected directly into the HPLC system. The HLPC separation of MTXPGs ($MTXPG_{1–7}$) was achieved with a 20-min linear gradient from 0% to 13% acetonitrile (mobile phase B) at a flow rate of 1 mL/min. After 20 min, the mobile phase was returned to 100% mobile phase A and reequilibrated for 10 min. One sample was injected every 30 min. MTXPG photolytic product (hypothetically a pteridine carboxylic acid) was measured at an excitation wavelength set at 274 nm and an emission wavelength set at 470 nm. The fluorometric signal was recorded between 10 and 25 min. Spectral identification was by comparison of the excitation spectrum of the MTX postphotolytic product of RBC extracts with the excitation spectrum of the MTX postphotolytic product in water.

PRECISION, ACCURACY, AND RECOVERIES

Intra- and interday precision and accuracy were determined by analyzing RBC hemolysates to which low and high concentrations of MTXPGs had been added. Intraday analysis was performed with 10 such enriched replicates, whereas interday evaluation was assessed with 3 to 5 replicates on 5 different days. Accuracy was calculated as the percentage difference between the measured concentrations from each enriched sample relative to the target concentration (measured concentration/target concentration × 100%). Imprecision was defined by estimating the CV (%). Recoveries were determined by comparing the peak height of MTXPG-enriched RBC with peak heights for samples prepared at the same concentration in water. The kinetics of conversion of MTXPGs to MTX were determined with an equimolar mixture of the six MTXPGs ($MTXPG_{2–7}$) added to RBC hemolysate at a final concentration of 1000 nmol/L of RBCs. Precision and accuracy were determined by quantification of the MTX concentration after enzymatic treatment, using three to five replicates at each concentration (20, 50, 250, and 500 nmol/L), on 5 different days.

APPLICATION TO RHEUMATOID ARTHRITIS PATIENTS RECEIVING MTX

EDTA-whole blood from rheumatoid arthritis patients who had been receiving weekly low-dose MTX (median, 15 mg; range, 10–25 mg) for more than 3 months (median, 37 months; range, 6–240 months) was drawn immediately before the weekly administration of MTX. Patient consent was obtained, and the study was approved by the local Internal Review Board. After a 5-min centrifugation step to separate plasma and buffy coat from RBCs, the RBCs were washed twice with two volumes of saline. Packed RBCs were stored at −70 °C until analysis. Total MTXPG concentrations measured after conversion with plasma γ-glutamyl hydrolase were compared with the total MTXPGs calculated from the sum of individual MTXPG species by linear regression (coefficient of correlation, slope, and intercept).

In an effort to assess the practical applications of this assay to a clinical setting, we preformed a stability study of RBC MTXPGs post phlebotomy. EDTA-whole blood (10 mL) from patients receiving a weekly stable dose of MTX for at least 3 months was divided into two tubes (within 3 h). RBCs from the first tube were isolated, whereas the second tube of whole blood was transferred to a coolant specimen transportation system containing a frozen cartridge. At 48 h, RBCs were isolated from the blood specimen stored in the transportation system as described above. RBC MTXPG concentrations measured after 48 h under conditions mimicking typical sample transport were compared with the RBC MTXPG concentrations measured just after sample collection.

Results

HPLC DETERMINATION OF TOTAL MTXPG CONCENTRATIONS AFTER CONVERSION TO MTX

HPLC chromatograms for blank RBCs vs RBCs enriched with MTXPGs and converted to $MTXPG_1$ demonstrated the resolution of $MTXPG_1$ from the biological matrix (Fig. 1, A and B, respectively). This conversion of $MTXPG_{2–7}$ (the pool of the six MTXPGs) to MTX was complete after an 8-h incubation at 37 °C in the dark at a concentration of 1000 nmol/L of RBCs (Fig. 1C). In addition, $MTXPG_2$ at a concentration of 1000 nmol/L was completely converted to $MTXPG_1$ in 8 h (not shown). Before enzymatic conversion, no $MTXPG_1$ was detected in RBCs enriched with $MTXPG_{2–7}$, whereas during enzymatic conversion, the $MTXPG_1$ peak appeared (not shown).

Calibration curves demonstrated a linear relationship between peak area and concentration with correlation coefficients >0.995 for $MTXPG_1$ and for $MTXPG_1$ formed after enzymatic conversion of $MTXPG_{2–7}$. The equations describing the calibration curves were as follows: for $MTXPG_1$, $y = 0.401x + 0.281$ LU/s (where LU is luminescence units); $MTXPG_1$ after enzymatic conversion of $MTXPG_{2–7}$, $y = 0.391x - 0.122$ LU/s, where y is the peak area and x is the concentration added. The intra- and interday efficiency of the enzymatic conversion of $MTXPG_{2–7}$ was determined at concentrations ranging from 20 to 500 nmol/L of RBCs. Complete conversion of $MTXPG_{2–7}$ was demonstrated with an accuracy >95% and a CV <6% (Table 1). Furthermore, the slope of $MTXPG_1$ measured after enzymatic conversion compared with the concentration of $MTXPG_{2–7}$ added was 0.95 (intercept, 0.12 nmol/L), thus demonstrating that the method was accurate and converted all MTXPGs to MTX. The mean extraction efficiency for $MTXPG_1$ was 67%. The limit of detection, defined as three times the signal-to-noise ratio,

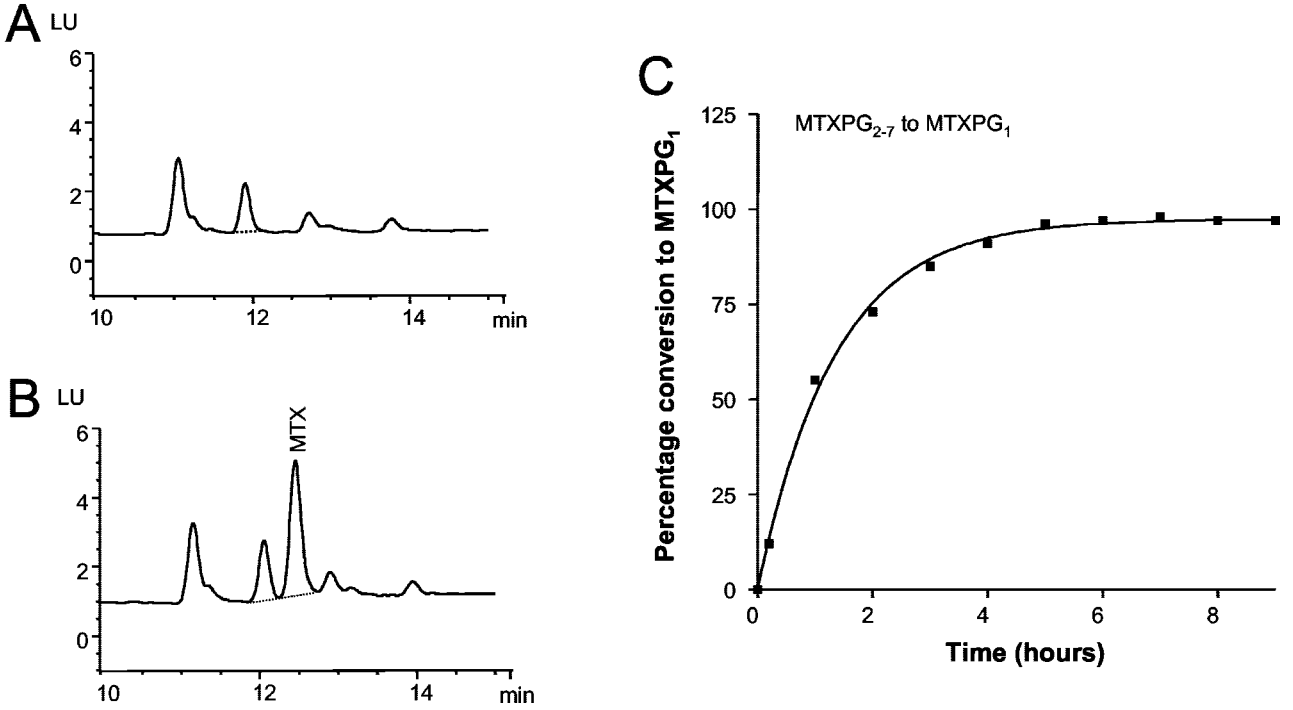

Fig. 1. Chromatograms of a blank RBC sample and RBC sample enriched with $MTXPG_{2-7}$ subjected to enzymatic conversion to MTX.

(*A*), typical chromatogram of a blank RBC sample. The excitation wavelength was set at 400 nm to avoid interferences with MTX photolytic products in the chromatogram. (*B*), typical chromatogram of a RBC sample enriched with $MTXPG_{2-7}$ at a final concentration of 250 nmol/L of RBCs and subjected to enzymatic conversion. The retention time for MTX was 12.5 min. (*C*), conversion of $MTXPG_{2-7}$ to $MTXPG_1$. $MTXPG_{2-7}$ were added at a final concentration of 1000 nmol/L and subjected to enzymatic conversion. The conversion of MTXPGs to MTX was determined by comparing the peak area of $MTXPG_1$ formed during the enzymatic conversion at different times with the peak area of $MTXPG_1$ added to the RBCs at the same concentration. After 8 h of incubation, conversion of $MTXPG_{2-7}$ to $MTXPG_1$ was complete. $MTXPG_2$ added at a concentration of 1000 nmol/L was completely converted to $MTXPG_1$ in 8 h (data not shown). *LU*, luminescence units.

was 5 nmol/L for $MTXPG_1$. The limit of quantification for $MTXPG_1$ or for $MTXPG_1$ formed after enzymatic conversion of $MTXPG_{2-7}$ was 10 nmol/L.

HPLC determination of individual MTXPG concentrations

A chromatogram of a calibration solution containing all seven MTXPGs (25 nmol/L each species) in water (Fig. 2A) illustrates the separation of the MTXPG species. The excitation spectrum of the postcolumn photolytic product was identical for all seven MTXPGs (Fig. 2B). Chromatograms of blank RBCs vs RBCs enriched with MTXPGs show that all seven MTXPG species can be separated from endogenous RBC components (Fig. 2, C and D).

Calibrations curves demonstrated a linear relationship between peak area and concentration with correlation coefficients >0.995 for all seven MTXPGs. Equations describing the calibration curves were as follows:

Table 1. Precision and accuracy for MTX and MTXPGs in RBCs.[a]

		Intraday (n = 10)			Interday (n = 5)		
Calibrator	Target concentration, nmol/L	Mean observed concentration, nmol/L	RSD,[a] %	Mean accuracy of target value, %	Mean observed concentration, nmol/L	RSD, %	Mean accuracy of target value, %
$MTXPG_1$	20.0	19.6	8.9	98.0	19.1	7.6	95.7
	50.0	45.0	4.8	90.0	48.9	8.4	97.9
	250.0	250.2	3.8	100.1	260.8	3.8	104.3
	500.0	504.2	4.1	100.8	517.3	2.3	103.5
$MTXPG_{2-7}$	20.0	19.6	10.1	98.0	19.1	8.4	95.4
	50.0	45.4	4.7	90.7	47.0	4.3	94.1
	250.0	244.5	3.3	97.8	245.6	4.1	98.2
	500.0	473.2	2.3	94.6	492.8	6.1	98.6

[a] The intraday data summarize 10 different replicates at each concentration for each compound ($MTXPG_1$ and $MTXPG_{2-7}$) in one experiment. The interday data summarize five different experiments from 5 consecutive days with three to five replicates each day and at each concentration for each compound.

[b] RSD, relative standard deviation of the mean.

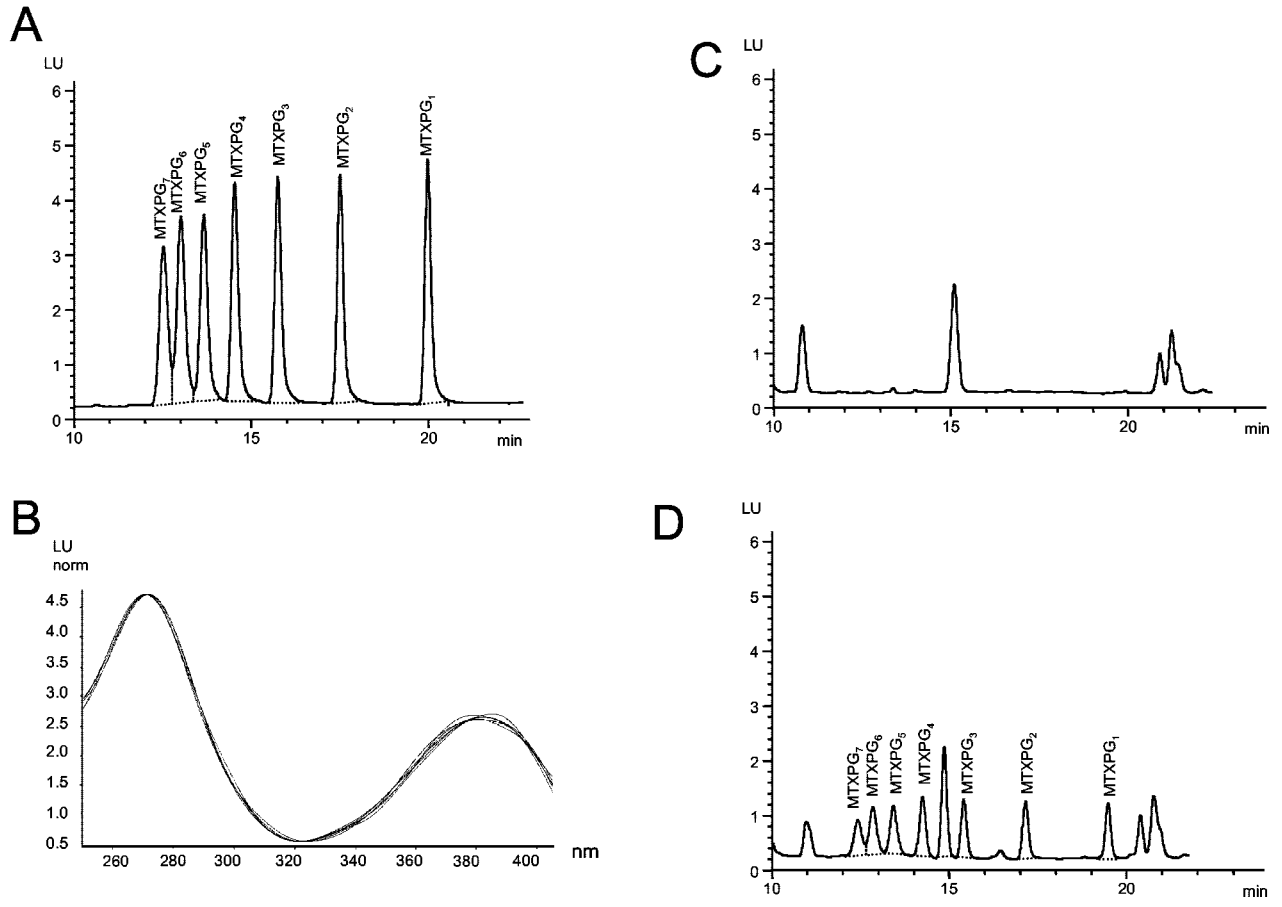

Fig. 2. Chromatograms of calibrators in water, RBC blank, and RBCs with MTXPGs added.

(*A*), chromatogram of $MTXPG_{1-7}$ calibrators in water at a final concentration of 25 nmol/L each. The retention times were as follows: $MTXPG_7$, 12.5 min; $MTXPG_6$, 13.0 min; $MTXPG_5$, 13.7 min; $MTXPG_4$, 14.5 min; $MTXPG_3$, 15.7 min; $MTXPG_2$, 17.5 min; $MTXPG_1$, 19.8 min. (*B*), excitation spectrum of the MTXPG photolytic products. Excitation spectra for $MTXPG_1$–$MTXPG_7$ are overlaid and identical. MTXPGs and MTX photolytic product exhibited a maximum excitation wavelength at 274 nm. (*C*), typical chromatogram of RBC blank. The excitation wavelength was set at 274 nm and the emission wavelength at 464 nm. (*D*), typical chromatogram of a RBC sample enriched with $MTXPG_{1-7}$ at a final concentration of 25 nmol/L each and subjected to the sample treatment procedure. *LU*, luminescence units; *norm*, normalized.

$MTXPG_1$: $y = 0.493x + 0.245$
$MTXPG_2$: $y = 0.540x + 0.130$
$MTXPG_3$: $y = 0.561x + 0.125$
$MTXPG_4$: $y = 0.568x + 0.112$
$MTXPG_5$: $y = 0.668x + 0.01$
$MTXPG_6$: $y = 0.710x + 0.07$
$MTXPG_7$: $y = 0.430x + 0.316$

where y is the peak area, and x is the concentration added. The intra- and interday precision and accuracy of our assay are shown in Table 2. Over a period of 6 months, we performed a total of 22 independent experiments. The interday CV for a control MTXPG mixture added to RBC hemolysate was 4.3–6.4% at a concentration of 25 nmol/L and 3.9–5.8% at a concentration of 50 nmol/L.

The mean extraction recoveries were 66% for $MTXPG_1$, 66% for $MTXPG_2$, 65% for $MTXPG_3$, 66% for $MTXPG_4$, 79% for $MTXPG_5$, 80% for $MTXPG_6$, and 60% for $MTXPG_7$. The limit of detection, defined as three times the signal-to-noise ratio, was 2 nmol/L for each polyglutamate. The limit of quantification for all seven MTXPGs was 5 nmol/L.

Application to patients with rheumatoid arthritis receiving weekly low-dose MTX

Chromatograms for RBCs from rheumatoid arthritis patient receiving MTX therapy are presented in Fig. 3; $MTXPG_6$ and $MTXPG_7$ were not detected in any of the samples. The total long-chain MTXPG concentrations were calculated as the sum of $MTXPG_3$ + $MTXPG_4$ + $MTXPG_5$ ($MTXPG_{3-5}$). The excitation spectra of the MTXPG photolytic products in patient samples compared with the excitation spectrum of MTX photolytic product in water (Fig. 4) highlight the specificity of the analytical method.

In a stability study of MTXPGs in whole blood samples from 20 individuals, MTXPG concentrations and total long-chain $MTXPG_{3-5}$ concentrations were unchanged at 48 h after sample collection and storage in a coolant transportation system. This was evidenced by a slope and coefficient correlation >0.90 between MTXPG concentrations measured within 3 h after phlebotomy and MTXPG concentrations measured after 48 h in conditions that mimicked transportation ($MTXPG_{total}$, slope of 1.0 and

Table 2. Precision and accuracy for MTX and MTXPGs in RBCs.[a]

		Intraday (n = 10)			Interday (n = 5)		
Calibrator	Target concentration, nmol/L	Mean observed concentration, nmol/L	RSD,[b] %	Mean accuracy of target value, %	Mean observed concentration, nmol/L	RSD, %	Mean accuracy of target value, %
	10.0	11.1	4.2	111.1	10.6	7.8	105.5
$MTXPG_1$	25.0	27.4	3.7	109.8	25.8	6.8	103.0
	50.0	49.7	3.7	99.4	50.0	0.5	100.1
	10.0	11.2	4.6	111.9	10.2	7.9	102.2
$MTXPG_2$	25.0	27.2	5.6	108.8	25.0	8.3	100.2
	50.0	49.8	3.6	99.5	50.0	0.4	99.9
	10.0	11.2	6.5	111.7	10.0	12.7	100.0
$MTXPG_3$	25.0	26.8	3.6	107.3	24.7	9.7	98.8
	50.0	49.7	5.4	99.5	50.0	0.6	100.0
	10.0	11.2	6.8	112.3	10.0	9.8	99.7
$MTXPG_4$	25.0	27.3	4.1	109.1	25.0	5.5	100.0
	50.0	49.5	5.7	99.0	49.9	0.6	99.8
	10.0	9.9	5.2	99.0	9.4	5.8	94.1
$MTXPG_5$	25.0	27.1	2.6	108.4	24.9	5.4	99.5
	50.0	49.4	4.3	98.7	49.8	0.7	99.5
	10.0	9.5	6.4	95.1	9.5	8.6	94.5
$MTXPG_6$	25.0	27.1	3.0	108.5	25.2	5.7	100.9
	50.0	48.9	4.4	97.9	49.6	1.4	99.2
	10.0	10.4	8.1	103.8	10.3	2.1	103.0
$MTXPG_7$	25.0	25.2	4.1	100.6	25.2	8.2	100.6
	50.0	44.1	6.5	88.2	48.8	8.3	97.6

[a] The intraday data summarize 10 different replicates at each concentration for each compound in one experiment. The interday data summarize five different experiments from 5 consecutive days with five replicates each day and at each concentration.

[b] RSD, relative standard deviation of the mean.

coefficient of correlation of 0.99; $MTXPG_{3–5}$, slope of 0.95 and coefficient of correlation of 0.93; Fig. 5A). Furthermore, as shown in Fig. 5B, there was no difference in the distribution of RBC MTXPG species after 48 h in our coolant system compared with the distribution of MTXPGs observed just after sample collection.

Finally, in a cohort of 70 rheumatoid arthritis patients receiving weekly MTX therapy (median dose, 15 mg), there was no difference between the mean (SD) total MTXPG concentrations measured after conversion of MTXPGs to MTX [117 (56) nmol/L; n = 70] and the total MTXPG concentrations calculated from the sum of individual MTXPGs [sum of $MTXPG_1$ + $MTXPG_2$ + $MTXPG_3$ + $MTXPG_4$ + $MTXPG_5$ = $MTXPG_{1–5}$: 120 (59) nmol/L; n = 70]. This was evidenced by a slope of 1.0 (intercept, 0.4 nmol/L) and a coefficient of correlation of 0.97 between the total MTXPG concentrations obtained by the two alternative methods (Fig. 6). The triglutamate species predominated over all other MTXPG species (36% of total), and the pentaglutamate form was the most highly glutamated form detected in actual patient samples.

Discussion

Although numerous methods have been developed for the determination of plasma MTX concentrations *(23)*, very few procedures are available for the quantification of MTXPG concentrations in RBCs. In fact, the low concentrations of these analytes (in the nmol/L range in RBCs) and the complexity of the RBC matrix are major obstacles to their accurate quantification. To overcome these limitations, investigators have taken advantage of the property of MTX to inhibit DHFR *(18–20)* and developed two alternative strategies. The method developed by Kamen and coworkers *(18, 19)* associated heat extraction of RBC MTXPGs with HPLC fractionation and quantification by a radiochemical ligand-binding assay. This method has the advantage of being highly sensitive and is capable of quantifying each of the MTXPG species (up to seven glutamic residues). However, it has the disadvantages of being labor-intensive and of requiring the use of radioisotopes. The other alternative method is the DHFR inhibition enzymatic assay developed by Shroeder and Heinsvig *(20)*, in which heat-extracted RBC MTXPGs are quantified by measuring the decrease in absorbance that occurs when NADPH is converted to $NADP^+$. Although this method is suitable for the quantification of MTX in plasma *(24)*, its application to the quantification of MTXPGs in RBCs is questionable because of potential competition between endogenous folate and MTX for DHFR *(25, 26)*

We have developed an innovative and proprietary approach for the quantification of RBC MTXPGs that overcomes the issues associated with these earlier methods. Because MTX can be measured in plasma in the

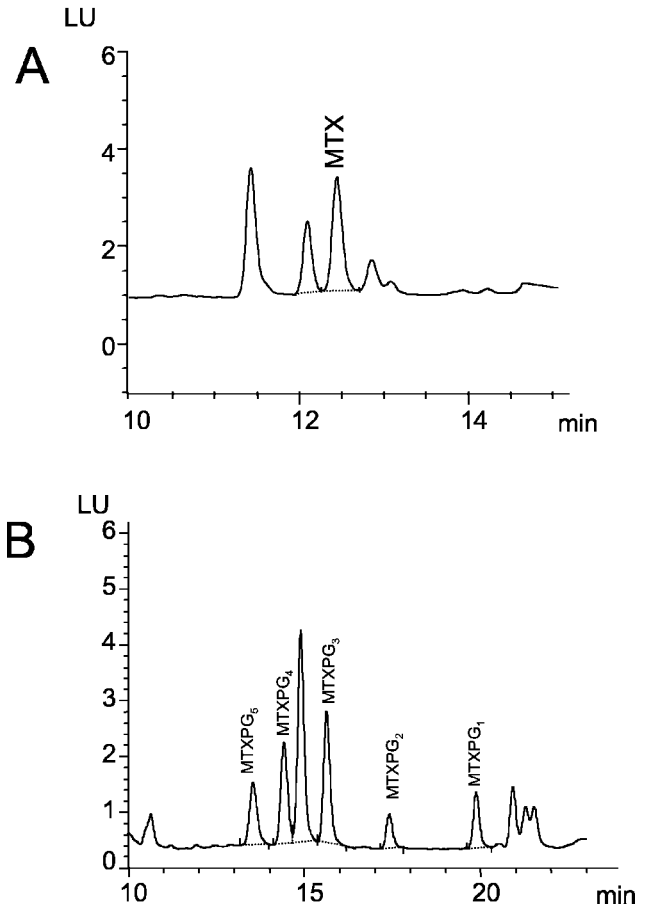

Fig. 3. Chromatograms of a RBC sample from a patient.

The patient had received 17.5 mg of MTX weekly. (*A*), chromatogram after conversion of total MTXPGs to MTX. The total MTX concentration was 185 nmol/L. (*B*), chromatogram with separation of individual MTXPGs. Concentration were as follows: $MTXPG_5$, 39 nmol/L; $MTXPG_4$, 50 nmol/L; $MTXPG_3$, 64 nmol/L; $MTXPG_2$, 10 nmol/L; $MTXPG_1$, 27 nmol/L. $MTXPG_6$ and $MTXPG_7$ were not detected. Total MTXPG concentration was 190 nmol/L. *LU*, luminescence units.

nmol/L range after postcolumn photooxidation to fluorescent products at neutral pH in the presence of hydrogen peroxide *(21, 22)*, we hypothesized that this detection system could be applied to the quantification of MTX and MTXPGs in RBCs. The observation that all MTXPGs exhibited similar excitation spectra after ultraviolet irradiation strongly suggests that the photooxidative process did occur between the heterocyclic and the *p*-aminobenzoyl portion of the MTX molecule.

We applied this detection system to the quantification of RBC MTXPGs. In the first approach, we developed a strategy in which we converted MTXPGs back to MTX with plasma γ-glutamyl hydrolase *(27–29)* during an incubation step at 37 °C in the presence of mercaptoethanol (the sulfhydryl donor for γ-glutamyl hydrolase). However, an incubation time of 8 h was required to efficiently convert all MTXPGs, including MTX diglutamate (up to 1000 nmol/L of packed RBCs; data not shown), a poor substrate for the enzyme *(28, 29)*. This approach has the advantage of being simple and requiring a low volume of RBCs (50 μL). Moreover, because all MTXPGs are converted to MTX, the sensitivity of the method is enhanced and can be applied not only to adult patient samples but also to pediatric patient samples, for which the volume of blood is often limited (i.e., micromethod; data not shown).

One potential drawback of this approach is that it does not separate and quantify long-chain MTXPGs, which are known to have greater effects on the targets of MTX, such as glycinamide ribonucleotide transformylase and 5-aminoimidazole-4-carboxamide-ribonucleotide transformylase *(30)*. We therefore adopted an approach in which we directly measured all MTXPG species. The method is simple and consists of simple deproteinization of the RBC matrix with perchloric acid and direct injection of the

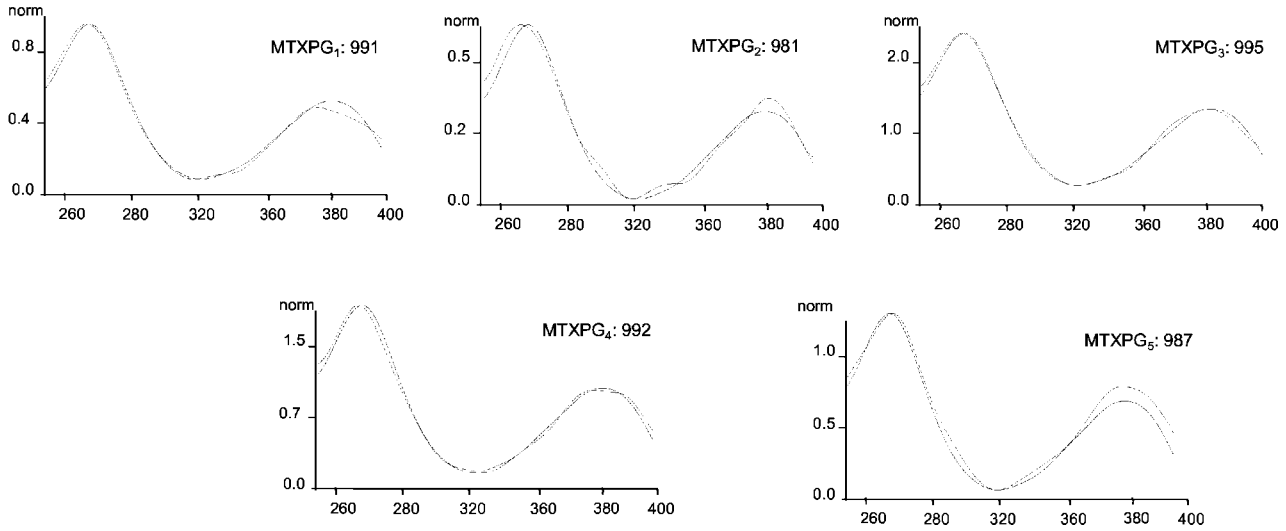

Fig. 4. Excitation spectra of the MTXPG photolytic products in a patient sample compared with the excitation spectrum of the MTX photolytic product in water.

The matching value was >900 for all. *norm*, normalized.

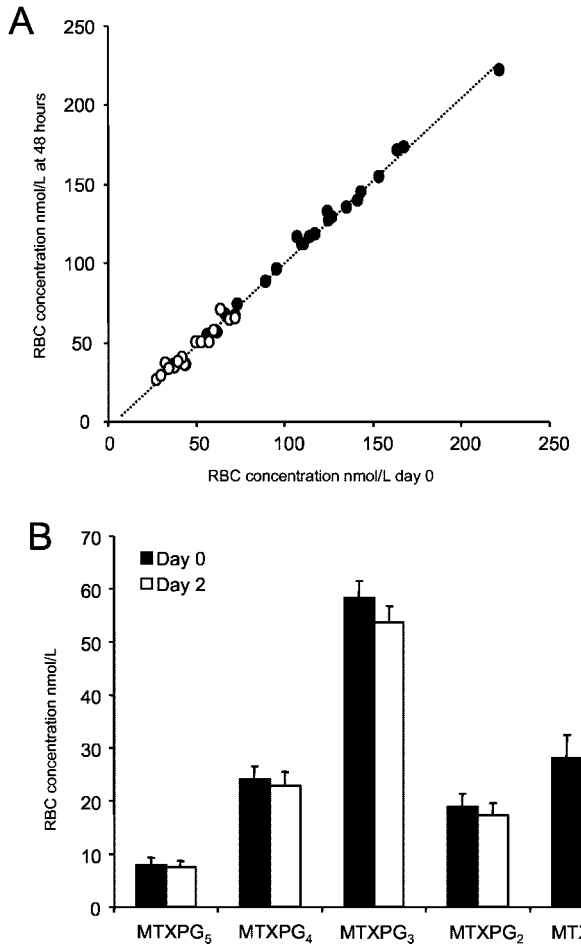

Fig. 5. Stability of MTXPGs in whole blood.

(*A*), stability of MTXPGs in blood from 20 rheumatoid arthritis patients receiving low-dose MTX therapy. The equations for the correlation between MTXPGs concentrations measured just after phlebotomy and MTXPGs concentrations measured after 48 h in conditions that mimicked transportation are as follows: $MTXPG_{total}$, $y = 1.02x + 1.01$ nmol/L ($R^2 = 0.99$); $MTXPG_{3–5}$, $y = 0.95x + 1.46$ nmol/L ($R^2 = 0.93$). (*B*), the distribution pattern of MTXPGs was unaffected 48 h after phlebotomy. *Bars*, SD.

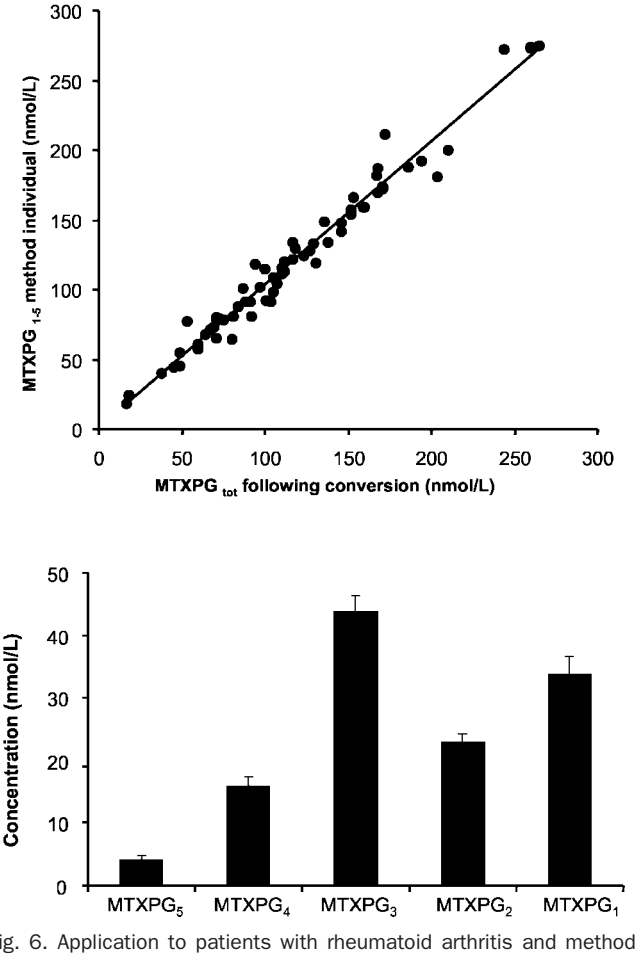

Fig. 6. Application to patients with rheumatoid arthritis and method comparison.

(*Top*), method comparison. The coefficient correlation and slope for the total MTX concentrations measured after enzymatic conversion vs total MTX as the sum of all individual MTXPG species were 0.97 and 1.0, respectively. The equation for the line is: $y = 1.03x + 0.4$ nmol/L ($R^2 = 0.972$). (*Bottom*), individual MTXPG concentrations in the 70 patients. The mean (SD; *bars*) total long-chain MTXPG concentration ($MTXPG_{3–5}$) was 63 (5) nmol/L, which corresponded to 53% of the total MTX.

extract into the HPLC system. The major advantage of this method over the DHFR radioligand assay is the simultaneous quantification of the MTXPGs in a single run without the necessity of collecting multiple fractions *(18, 19)*. In our chromatographic system, the elution of MTXPG species was in order of decreasing number of glutamic residues and was reversed compared with the method developed by Kamen and Winick *(18)*. This difference may be explained by the characteristics of the mobile phase and variations in the ionization state of the carboxylic acid group as hypothesized previously by Pfeiffer and Gregory *(27)*. We also observed slight differences in the extraction recoveries among MTXPG species and speculate that the number of glutamic residues may affect the stability of the MTX moiety during the deproteinization step.

Our method was applied to patients with rheumatoid arthritis receiving weekly low-dose MTX therapy. To assess the application of the method to clinical practice, we conducted a stability study of MTXPGs in conditions that mimicked transportation of specimens from the clinic to the laboratory. The data presented here support the notion that MTXPGs are stable and that there was no alteration in the total RBC MTXPG concentrations or in the distribution pattern of MTXPG species after 48 h storage in a transportation system containing a cold pack. Furthermore, we applied the method to a large population of patients with rheumatoid arthritis receiving weekly low-dose MTX. In this population, the highest order of glutamation observed was the pentaglutamate, with the triglutamate predominating over all other MTXPGs. These data are consistent with previous reports using the radioligand assay *(11, 31)*. It is important to note that circulating RBCs lack folylpolyglutamate synthetase activity and that erythrocyte MTXPGs arise from incorporation of the drug into RBC precursors *(31–33)*. We can

therefore speculate that MTXPG concentrations in RBCs are representative of MTXPG concentrations in less accessible tissues, such as the bone marrow.

There is general agreement that increasing numbers of glutamate residues attached to MTX are associated with increasing in vitro inhibitory activity for target enzymes. For example, longer-chain MTXPGs are more potent than short-chain MTXPGs at inhibiting 5-aminoimidazole carboxamide-ribonucleotide transformylase *(7, 34)* and are therefore presumed to be more effective at promoting the antiinflammatory effects of MTX through adenosine release *(8, 9, 35)*. With such in vitro results in mind, we hypothesize that longer-chain MTXPGs in RBCs would correlate more closely than total MTXPG with the therapeutic response of rheumatoid arthritis patients treated with MTX. In fact, our preliminary data from interim analyses of ongoing prospective clinical studies of rheumatoid arthritis patients on low-dose MTX indicate that the concentrations of $MTXPG_1$ and $MTXPG_2$ are poorly associated with efficacy, whereas the concentrations of RBC MTXPG species with three or more glutamic residues (total long-chain MTXPGs: $MTXPG_{3-5}$) are associated with therapeutic response.

In conclusion, we have developed a rapid, sensitive, and accurate method for the quantification of RBC MTXPGs that can be applied to the routine monitoring of MTX therapy. Because MTX is often the first line of therapy for rheumatoid arthritis patients, this methodology could potentially be useful for practicing physicians to achieve rapid, effective dosing of MTX and to minimize side effects.

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