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# Erythrocyte Methotrexate-polyglutamate Assay Using Fluorescence Polarization Immunoassay Technique: Application to the Monitoring of Patients with Rheumatoid Arthritis

Sachie INOUE,[a] Masayuki HASHIGUCHI,[a] Shinichi KAWAI,[b] and Mayumi MOCHIZUKI*,[a]

[a]Division for Evaluation and Analysis of Drug Information, Keio University Faculty of Pharmacy,
1−5−30 Shibakoen, Minato-ku, Tokyo 105−8512, Japan, and [b]Division of Rheumatology,
Department of Internal Medicine (Omori), Toho University School of Medicine,
6−11−1 Omori-Nishi, Ota-ku, Tokyo 143−8541, Japan

Methotrexate (MTX), i.e., MTX-polygluatmate 1 (MTX-PG$_1$), exerts its antirheumatic effects mainly by ≤6 (MTX-PG$_{2-7}$) via folypolyglutamyl synthase in cells. The authors developed a new method using fluorescence polarization immunoassay to determine MTX-PG$_{1-7}$ concentrations in erythrocytes (RBC). MTX-PG$_{2-7}$ in RBC of rheumatoid arthritis (RA) patients receiving MTX was converted to MTX in the presence of plasma γ-glutamyl hydrolase and mercaptoethanol at 37°C. The MTX in RBC was extracted in a perchloric acid deproteinization step then on a solid-phase extraction column. The concentration of MTX was measured by TDX analyzer. The mean MTX recovery rate was 76.1% (n=8). The intraday and interday coefficients of variation were <11.3% (n=8) and <12.4% (n=3), respectively, at low and high concentrations (30−300 nmol/l). The calibration curve was linear over the range 30−300 nmol/l. The total concentration of MTX-PGs (mean±S.D.) in RBC obtained from 95 Japanese RA patient blood samples was 97.3±8.1 nmol/l for the MTX dose of 0.13±0.05 mg/week/kg. This newly developed method for the quantification of MTX-PGs in RBC is sensitive and accurate and can be applied for routine monitoring of MTX therapy in RA patients.

Key words——methotrexate; methotrexate-polyglutamate; rheumatoid arthritis; fluorescence polarization immunoassay

## INTRODUCTION

Methotrexate (MTX), i.e., MTX-polyglutamate 1 (MTX-PG$_1$), is the most widely used disease-modifying antirheumatic drug in the treatment of rheumatoid arthritis (RA).[1,2] However, it is known that there are large interindividual differences in the response to MTX therapy in patients with RA.[3,4] Those differences are mainly thought due to variations in individual pharmacokinetics of MTX.[5] Once MTX is transported into the cell, it progressively adds ≤6 MTX polyglutamates (MTX-PG$_{2-7}$) via folypolyglutamyl synthetase (FPGS) and is retained within cells for a long time. On the other hand, MTX-PG$_{2-7}$ is converted back into MTX by γ-glutamyl hydrolase (GGH), and MTX is transported to the extracellular space.[5-8] Therefore intracellular MTX-PG$_{1-7}$ (MTX-PGs) may be the main compounds exerting the antirheumatic effects of MTX; thus monitoring MTX-PGs in erythrocytes (RBC) as a substitute[7,9] for the MTX-PG concentration in target cells such as mononuclear cells, lymphocytes, or synovial cells could be helpful in adjusting the optimal MTX dose in RA patients. Although several methods to determine the concentration of MTX-PGs in RBC[10-12] have been developed, the concentrations of MTX-PGs in RBC are extremely low, and a method to determine MTX-PGs in RBC has not been completely established.

The present report describes a simple and accurate method for determination of MTX-PGs in RBC using the fluorescence polarization immunoassay (FPIA) method and its usefulness in monitoring RA patients receiving MTX.

## MATERIALS AND METHODS

**Chemicals**　　MTX and lyophilized plasma were purchased from Sigma-Aldrich (Tokyo, Japan). Mercaptoethanol, perchloric acid, potassium hydroxide, dipotassium hydrogenphosphate, potassium dihydrogenphosphate, and methanol were obtained from Wako Pure Chemical Industries (Tokyo, Japan). An Oasis HLB 30 mg/ml solid extraction column was purchased from Waters (Tokyo, Japan).

**Drug Solutions**　　MTX was dissolved in 0.1 M potassium hydroxide. After dissolution, stock solu-

tions were prepared by diluting to $100\,\mu$mol/l in water. Working standard solutions of MTX in water $(6, 4, 2, 1, 0.6\,\mu$mol/l) were prepared from stock solutions by serial dilution. The $100\,\mu$mol/l stock solutions were stable at $-80°$C for $\geq 12$ months. Drug-free RBC used for validation of the method were obtained from 5 healthy volunteers. Calibrators were prepared by adding known amounts of working standard solutions to $190\,\mu$l of blank RBC to obtain the final concentrations of 300, 200, 100, 50, and 30 nmol/l for MTX.

**Extraction Procedure**    Total MTX-PGs in RBC were measured after conversion from MTX-PG$_{2-7}$ to MTX as follows. Four hundred microliters of reconstituted plasma (source of GGH) was added to $200\,\mu$l of RBC hemolysates. The RBC were disrupted in a freeze-thaw cycle. After mixing the sample for 30 s, $400\,\mu$l of buffer containing 100 mmol/l of potassium phosphate (pH 7.4) and 150 mmol/l of mercaptoethanol was added to the sample. After incubating the sample at $37°$C for 12–14 h, MTX-PG$_{2-7}$ was converted to MTX. After incubation and cooling, $100\,\mu$l of 70% perchloric acid was added to the mixture. After 10 s of vortex-mixing, the mixture was centrifuged at 10000 rpm for 5 min. Seven hundred fifty microliters of the supernatant solution was injected into the solid-phase extraction sorbent. After washing with 1 ml of 5% methanol and $500\,\mu$l of 20% methanol, MTX was eluted with 2 ml of methanol. The eluate was evaporated under nitrogen at $60°$C, and the residue was dissolved in $130\,\mu$l of drug-free plasma. Next, a $65\,\mu$l aliquot was injected into a TDX analyzer (Abbott, Tokyo, Japan).

**Patient Samples**    Ninety-five samples of heparinized peripheral whole blood (5 ml) were obtained from 36 Japanese RA patients comprising 31 women and 5 men whose mean ($\pm$S.D.) age and body weight were $58.8 \pm 2.5$ years and $53.3 \pm 1.7$ kg, respectively, and who had been receiving the same dose of MTX for $>1$ month. After a 10-min centrifugation step to separate plasma and RBC, the RBC were washed twice with two volumes of saline. All collected blood samples were stored at $4°$C, and RBC and plasma were separated within 48 h.[12] RBC were stored at $-8$ $°$C until analysis. This study protocol was approved by the Institutional Review Board of the Kitasato Institute Hospital and written informed consent for participation was given by all patients.

## RESULTS

**Recovery and Linearity**    The recovery rate was determined by assaying blank RBC spiked with 5 different known concentrations (30–300 nmol/l) and blank plasma spiked with same amounts of MTX in 8 replicates. The results are shown in Table 1. The recovery rate ranged from 74.6% to 78.1% ($76.1 \pm$ 7.5; mean $\pm$ S.D.; $n=8$). The analysis of calibrators gave a linear curve over the range tested (Fig. 1). The linearity curves were defined by the following equation: $y=0.7658x+2.5629$ ($r^2 = > 0.999$), where $y$ is the measured concentration of RBC in samples indicated by the TDX analyzer and $x$ is the spiked MTX concentration.

**Precision**    Intraday and interday precision determined by assaying blank RBC spiked with 5 different known concentrations of MTX is shown in Table 2. Intraday precision was assessed by assaying 8 samples at 5 drug concentrations (30, 50, 100, 200, and 300 nmol/l). Interday precision was evaluated by assaying 3 samples at 5 concentrations (30, 50, 100,

Table 1.  Methotrexate Recovery Rates

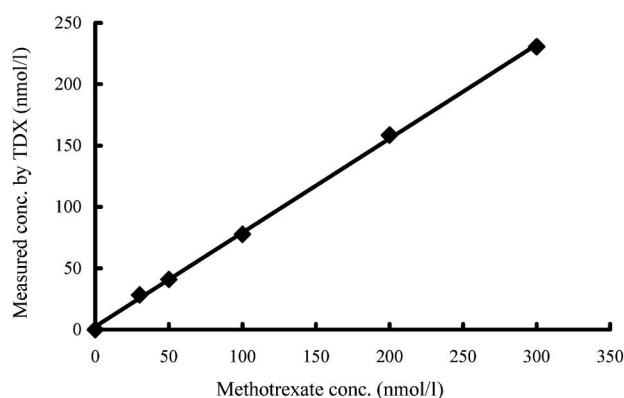| MTX concentration (nmol/l) | Recovery rate (%) (mean $\pm$ S.D., $n=8$) |
|---|---|
| 30 | $78.1 \pm 8.8$ |
| 50 | $77.1 \pm 8.6$ |
| 100 | $75.0 \pm 8.1$ |
| 200 | $75.6 \pm 6.8$ |
| 300 | $74.6 \pm 6.2$ |



Fig. 1.  Linear Regression Curves of the Concentration of Methotrexate Determined by TDX Analyzer and Spiked Methotrexate in the Calibrators

Table 2. Precision of Determination of Methotrexate in Spiked Erythrocytes

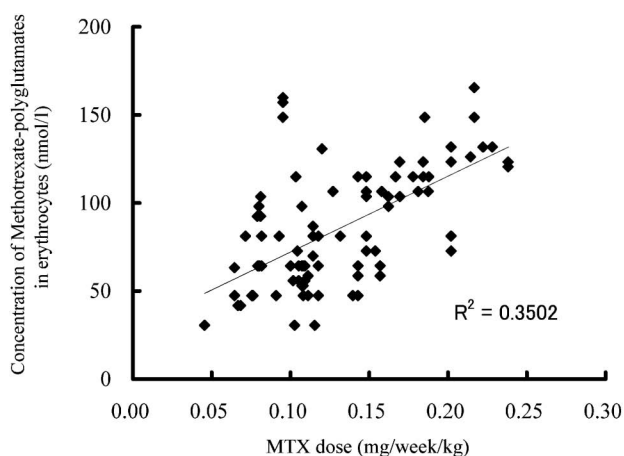| Spiked MTX concentration (nmol/l) | Intraday variation ($n=8$) | | Interday variation ($n=3$) | |
|---|---|---|---|---|
| | Measured concentration (nmol/l) (mean $\pm$ S.D.) | CV (%) | Measured concentration (nmol/l) (mean $\pm$ S.D.) | CV (%) |
| 30 | 23.44 $\pm$ 2.65 | 11.30 | 28.33 $\pm$ 3.49 | 12.33 |
| 50 | 38.54 $\pm$ 4.31 | 11.19 | 40.89 $\pm$ 4.95 | 12.12 |
| 100 | 81.48 $\pm$ 8.33 | 10.23 | 77.86 $\pm$ 6.26 | 8.04 |
| 200 | 151.25 $\pm$ 13.56 | 8.97 | 158.49 $\pm$ 9.09 | 5.74 |
| 300 | 223.66 $\pm$ 18.50 | 8.27 | 230.64 $\pm$ 12.36 | 5.35 |



Fig. 2. Methotrexate Polyglutamate Concentration in Erythrocytes *versus* Methotrexate Dose in 95 Blood Samples from Japanese Rheumatoid Arthritis (RA) Patients

200, and 300 nmol/l) on 5 days. Precision was quantitated by calculating intraday and interday coefficients of variation (CVs) according to the formula: $[(\text{SD/mean measured concentration}) \times 100]$. In the determined range, intraday and interday CVs were < 11.3% and < 12.3%, respectively. The limit of quantification for MTX was 30 nmol/l.

**Patient Samples** The concentrations of MTX formed from MTX-PG$_{2-7}$ by enzymatic conversion could be measured in all 95 blood samples. The mean MTX-PG concentration in RBC was 97.3 $\pm$ 8.1 nmol/l for the MTX dose of 0.13 $\pm$ 0.05 mg/week/kg (Fig. 2).

## DISCUSSION

Although numerous methods have been developed for the determination of plasma MTX concentrations,[13–15] there are few methods for the quantifica-tion of MTX-PG concentrations in RBC. Because the RBC matrix is very complex, accurate quantification of MTX-PGs in RBC is difficult. Moreover, in RA patients the MTX dose is markedly lower than that administered to patients with leukemia and other cancers. To overcome these limitations, several investigators attempted to develop methods to determine MTX-PG concentrations in RBC. However, those methods had several disadvantages.[12] The radio-chemical ligand-binding assay developed by Kamen *et al*.[10] and Kamen and Winick[16] is time-consuming and requires the use of radioisotopes. An alternative method, the dihydrofolate reductase inhibition en-zymatic assay developed by Shroeder and Heinsvig[11] and Imbert *et al*.[17] lacks specificity. The recently de-veloped HPLC-fluorometry approach[12,18] is sensitive and accurate but uses the specific technique of postcolumn photooxidation with ultraviolet irradia-tion. Therefore we developed an easier-to-use, more accurate method for the quantification of MTX-PGs in RBC. It was confirmed that MTX-PG$_{2-7}$ (the pool of 6 MTX-PGs) was converted completely to MTX in the presence of plasma GGH and mercaptoethanol (the sulfhydryl donor for GGH) with an 8-h incuba-tion step at 37°C in the dark at an RBC concentration of 1000 nmol/l.[19–21] Therefore according to previous reports we judged that MTX−PG$_{2-7}$ in RBC of RA patients receiving MTX was converted completely to MTX under our incubation conditions. In a prelimi-nary study, we examined the experimental conditions for the conversion from MTX-PG$_{2-7}$ to MTX and confirmed that the concentration of MTX reached plateau after incubating RBC samples ($n=5$/patient) obtained from 3 RA patients receiving MTX at 37°C for 8–14 h in the dark (data not shown). Using a

solid column sorbent, the present method allowed determination of the concentration of MTX-PGs in RBC using the TDX analyzer. It has been confirmed that the cross-reactivity of MTX with a folic acid compound, MTX metabolites, and other chemical analogues was $<1\%$ in the measurement range of the TDX analyzer. Recently, Hayashi et al.[24] reported a method for determination of MTX-PGs in RBC using TDX analyzer. This method differs from our developed method in the following respect that they measured all MTX-PG$_{2-7}$ directly without hydrolyzing them to MTX, basing on their observation that anti-MTX monoclonal antibody shows reactivity to MTX-PG$_7$ as equal to MTX.

Our newly developed method was applied to the quantification of MTX-PGs in RBC of 95 blood samples from 36 Japanese RA patients receiving MTX. The MTX dose ranged from 2.0 to 13.0 mg/week, and the MTX-PGs in all RBC samples could be quantitated.

Our method has certain limitations. This method cannot measure each MTX-PG compound concentration separately. Dervieux et al.[22] postulated that longer-chain MTX-PGs (MTX-PG$_{4-7}$) in RBC might correlate more closely than total MTX-PGs with the response to MTX therapy in RA patients. Another report commented on the relation of the total MTX-PG concentration and therapeutic response to MTX.[23]

In conclusion, we developed an accurate and sensitive method for quantification of MTX-PGs in RBC. This method can be applied to routine monitoring of MTX therapy in RA patients to adjust the individual optimal MTX dose. The TDX analyzer is a commonly used measuring instrument; therefore monitoring using our newly developed method can be adopted to various clinical practices.

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