Supplemental data: HPLC analysis of total MTX in plasma and RBC

Total MTX was determined by HPLC using the method of Deen et al., as modified by Lobo and Bathasar [1, 2]. The method provides a high sensitivity HPLC method for measuring total (bound and unbound MTX) in plasma, which was further modified as below for RBC concentrations. Briefly, MTX (figure 1), a folic acid (figure 2) analogue, is rapidly metabolized by the addition of up to 7 glutamate conjugates. Although MTX and MTX polyglutamates can be determined individually, we chose to convert the different MTX forms to a fluorescent derivative, 2,4-diamino-6-methylpteridine (figure 3), which has a reported detection sensitivity of 1.25 ng in rat and mouse plasma.

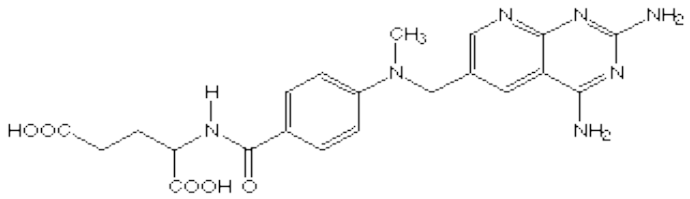

Figure 1. Chemical structure of MTX

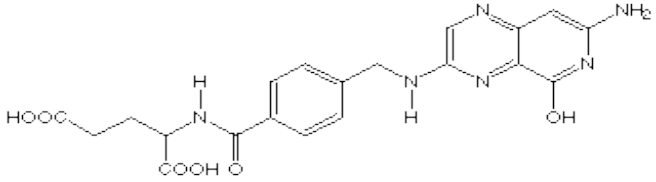

Figure 2. Chemical structure of folic acid

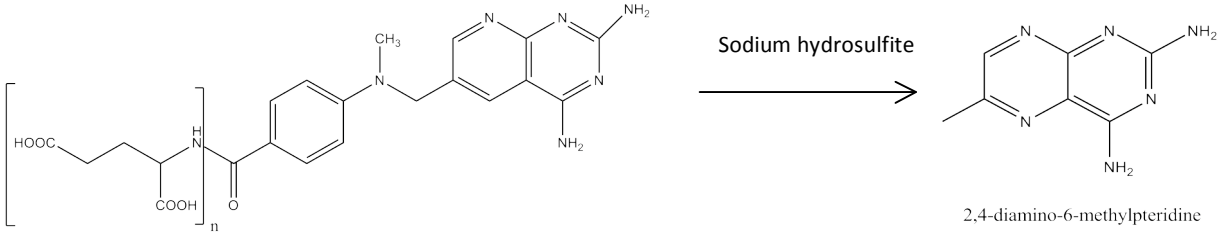

Figure 3. Chemical structure of the reduced product of $MTXGlu_n$ with sodium hydrosulfite

Methods:

Chemicals and reagents

MTX and folic acid were acquired from Sigma (St. Louis, MO, USA). 4-amino-10-methylpteroyl-tri-γ-L-glutamic acid was purchased from Schircks Laboratories (Jona, Switzerland).

Chromatographic instrumentation and system

The HPLC system consists of Waters components, including a C18 column (150 × 3.9 mm), a Model 626 solvent delivery system, a Model 474 fluorescence detector and a 717 autosampler. Waters Empower software was used for instrument control, data acquisition and processing.

0.1 M Tris (trishydromethylaminomethane) buffer (pH 7.0)-methanol (9:1) was used as mobile phase and was filtered (0.45 μm pore size) and degassed with built in Waters degasser. The isocratic condition at a flow rate of 0.7 ml/min was used for separation. The fluorescence was detected at excitation and emission wavelengths of 367 nm and 463 nm, respectively.

Sample preparation

Mice were injected with K/BxN serum and treated with nanoparticles (NPS, n = 4), MTX (n = 4), or a combination of NPS + MTX (n = 4) as outlined in the Materials and Methods section. On day 9 after the K/BxN serum transfer, mice were sacrificed and the blood collected through the inferior vena cava. The plasma was separated and the RBCs were washed in phosphate buffered saline (PBS) x 3 prior to MTX measurements.

Standard and quality control samples

Stock solutions of MTX (50 μg/ml) and folic acid (50 μg/ml) were prepared from MTX powder in HPLC grade water and stored at -20 ^{0}C. Working standards were diluted to concentration range of 10-500 ng/ml with PBS pH 7.4. Folic acid (5.0 μg/ml) was used as an internal standard as it forms a different derivative and its retention time in chromatogram is shorter than that of MTX. Quality control samples (QCs) for MTX were prepared by spiking mouse plasma with appropriate amounts of MTX.

Derivatization of MTX in plasma

The reduction of total MTX in mouse plasma was carried out in plastic vials. A reaction solution was prepared containing 50 μl plasma (blank mouse plasma in case of standards), 25 μl of folic acid (5.0 μg/ml), 100 μl of PBS (containing MTX in case of standards), 100 μl of 2 M sodium acetate-5 M acetic

acid buffer (pH 6.0), 50 µl of freshly prepared sodium hydrosulfite (10 mg/ml) solution. The solution was heated at 92^{0}C for 45 min and covered with aluminum foil. The solution was then centrifuged at 16,000 g 4 ^{0}C for 15 min. A 175-µl aliquot of the supernatant was injected on the column. (1 ng to 972 ng of MTX was injected). The detection limit was 1 ng per injection.

Derivatization of MTXGlu3 in RBC

For total MTXGul3 in mouse red blood cell (RBC), the adopted procedure, as above, was used with slight modification. Three volumes of water were mixed with 1 volume RBC for cell lysis. The solution was heated at 99 ^{0}C for 7 min, centrifuged at 16 000 g for 5 min, and the supernatant recovered for analysis. The reduction of MTXGlu3 was performed in plastic vials. A reaction solution was prepared containing 450 µl RBC supernatant (blank mouse RBC supernatant in case of standards spiked with MTXGlu3), 75 µl of folic acid (5.0 µg/ml), 100 µl of 2 M sodium acetate-5 M acetic acid buffer (pH 6.0), 50 µl of freshly prepared sodium hydrosulfite (10 mg/ml) solution. The solution was heated at 92 ^{0}C for 45 min. The mixture was centrifuged at 16,000 g 4 ^{0}C for 15 min. A 175-µl aliquot of the supernatant was injected on the column (8 ng to 73 ng MTXGlu3 per injection). But no peak from sample RBC was observed.

MTX recovery in plasma

Plasma spiked with standard MTX, MTX concentration was ranged from 5ng/ml to 5000 ng/ml. The linear standard curve (HPLC peak area versus the concentration of MTX in reaction mixture) was showed in Figure 4 and R-square is above 0.99. Three different concentration of MTX was spiked in plasma, reduced, centrifuged and then injected. With obtained peak area and the standard curve, the accuracy of measurement was calculated based on the experimental concentration and the theoretic value (see table one). The sample MTX in plasma was processed as the QCs and no MTX was detected.

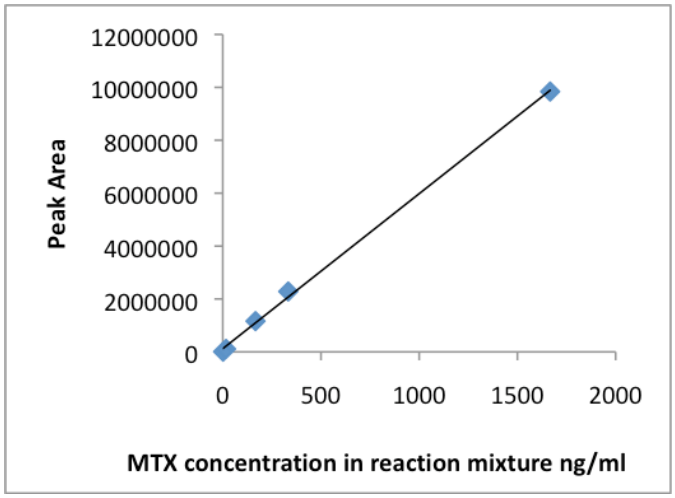

Figure 4. MTX in buffer standard solution (1.67-1667 ng/ml)

	Rxn vol 0.3 ml		
MTX HPLC area	Spiked conc. ng/ml	RXn conc. ng/ml	accuracy
1168243	166.6	163.8	98.3
2312920	333.3	361.8	108.5
11120579	1666.6	1885.4	113.1

Table 1: The known amount of MTX (spiked) in plasma

MTXGlu3 in RBC

In order to obtain standard curve, RBC was spiked with 0.875 µg of MTXGlu3 in 0.9 ml reaction mixture, then after incubation and reduction, supernatant with a volume of 5, 50, 100, 175 µL was injected, respectively. The standard curve for MTXGlu3 in ng per injection versus HPLC peak area was showed in Figure 5. R-square is over 0.99. The sample in the RBC was handled the same way as the standard sample. No peak was observed.

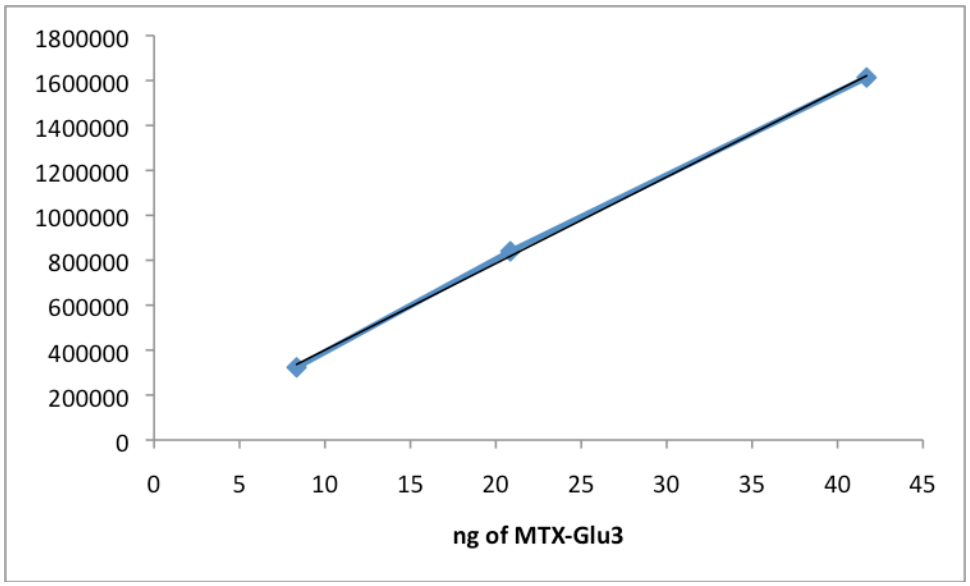

Figure 5. Known amount of MTXGlu3 in RBC supernatant

This method can be used to measure the total MTX in both plasma and RBC since the MTX and MTXGlu3 can be reduced into the same form and with the same elution retention time.

Results:

Plasma and RBC lysates were analyzed. There was no detectable MTX in any of the treatment groups.

NPS1	MTX1	NPS+MTX1
NPS2	MTX2	NPS+MTX2
NPS3	MTX3	NPS+MTX3
NPS4	MTX4	NPS+MTX4

1. Lobo ED, Balthasar JP: Highly sensitive high-performance liquid chromatographic assay for methotrexate in the presence and absence of anti-methotrexate antibody fragments in rat and mouse plasma. *J. Chromatogr. B. Biomed. Sc.i Appl.* 736, 191-199 *(*1999).

2. Deen WM, Levy PF, Wei J, Partridge RD: Assay for methotrexate in nanomolar concentrations with simultaneous detection of citrovorum factor and vincristine. *Anal. Biochem.* 114, 355-361 (1981).