Simultaneous Determination of Urine Methotrexate, 7-Hydroxy Methotrexate, Deoxyaminoptericoic Acid, and 7-Hydroxy Deoxyaminoptericoic Acid by UHPLC-MS/MS in Patients Receiving High-dose Methotrexate Therapy

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Nephrotoxicity, the most important toxicity in high-dose methotrexate (MTX) therapy, is partly caused by the formation of crystal deposits in the kidney due to poor water solubility of MTX and its metabolites 7-hydroxy methotrexate (7-OH MTX), deoxyaminoptericoic acid (DAMPA) and 7-hydroxy deoxyaminoptericoic acid (7-OH DAMPA). Plasma MTX level-guided urine alkalinization, leucovorin rescue and glucarpidase detoxification are common strategies to overcome MTX-related nephrotoxicity. However, overestimation is a problem for MTX analysis by immunoassays due to the cross-reactivity of MTX metabolites (7-OH MTX and DAMPA). An UHPLC-MS/MS method for the simultaneous determination of MTX, 7-OH MTX, DAMPA and 7-OH DAMPA in human urine was developed, validated and applied in clinical practice. Samples were treated by one-step protein precipitation and analyzed within 3 min. The calibration range was 0.02 to 4 μmol/L for MTX and DAMPA, and 0.1 to 20 μmol/L for 7-OH MTX and 7-OH DAMPA. For all analytes, the intra-day and inter-day bias and imprecision were –8.0 to 7.6 and <9.0%, the internal standard normalized recovery and matrix factor were 92.34 to 109.49 and <20.68%. The plasma MTX and 7-OH MTX levels increased with the urine drug levels, age, serum creatinine and alanine transaminase, but urine could not replace blood for MTX monitoring due to their poor correlation (R², 0.16 to 0.51). Dose-normalized urine and plasma MTX and 7-OH MTX levels were similar between different patient groups (urine pH <7 or ≥7). Due to the large inter-individual variance of the analytes levels in both plasma and urine, these findings should be treated with caution.

Keywords UHPLC-MS/MS, urine, methotrexate, 7-hydroxy methotrexate, deoxyaminoptericoic acid, 7-hydroxy deoxyaminoptericoic acid, method development and validation

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MTX plasma level is routinely monitored in clinical practice for leucovorin dose adjustments, especially in patients receiving high-dose MTX therapy. Immunoassays are widely used methods for MTX monitoring with significant overestimations, especially at low levels due to cross-reactivity caused by MTX metabolites (7-OH MTX, DAMPA, and 7-OH DAMPA), which have similar chemical structures with MTX. Various chromatographic based assays have been developed for MTX, 7-OH MTX and DAMPA analysis in human blood plasma, but these methods have some disadvantages, such as the time-consuming procedure for sample pretreatment and a long turnaround time (5 to 60 min). MTX is mainly excreted in the urine, which is more convenient to obtain compared to blood, especially for children; therefore, urine is potential to replace blood for MTX monitoring. However, only two methods were developed for MTX analysis in human urine. One method analyzed MTX by using a high sample volume (500 µL) and a low upper limit of detection (0.11 µmol/L), the other analyzed MTX and 7-OH MTX with a long turnaround time (6.6 min). This study was aimed to develop and validate a fast, accurate, and robust ultra high-performance liquid-chromatography tandem mass/mass spectrometry (UHPLC-MS/MS) method for the simultaneous determination of MTX, 7-OH MTX, DAMPA, and 7-OH DAMPA in human urine, and to apply it in patients with primary central nervous system lymphoma receiving high-dose MTX therapy. The influence of urine pH on plasma and urine MTX and 7-OH MTX levels was established and the correlation between the plasma and urine levels of both MTX and 7-OH MTX was evaluated to find out whether urine could replace blood for monitoring.

Experimental

Reagents and chemicals
MTX (Lot: 100138-201606, 99.8% purity) was obtained from the National Institutes for Food and Drug Control (Beijing, China). 7-OH MTX (Lot: 11-NSR-30-2, 95.23% purity), DAMPA (Lot: 1-JMS-61-4, 96% purity), 7-OH DAMPA (Lot: 10-JHY-49-2, 95% purity), MTX-D₃ (Lot: 12-ZCA-5-1, 95% purity, 99.0% isotopic purity, internal standard, IS), and DAMPA-D₃ (Lot: 1-TEK-173-1, 95% purity, 98.5% isotopic purity, IS) were purchased from the Toronto Research Chemicals (Canada). 7-OH MTX (Lot: 11-NSR-30-2, 95.23% purity), MTX-D₃ (Lot: 100138-201606, 99.8% purity) was obtained from the National Institutes for Food and Drug Control (Beijing, China), and ultrapure water was generated from a Millipore Ultrapure water system (Bedford, USA). Methanol and formic acid were purchased from Waltham, USA, while Millipore Ultra pure water system (Bedford, USA). Analytes- and IS-free urine were obtained from healthy volunteers and checked to ensure they did not contain any of the analytes and IS.

Instrumentations
An Acquity UHPLC H-Class (Waters, MA, USA) tandem 5500 QTRAP mass system (AB SCIEX, CA, USA) was used for analysis. Data was acquired and processed by using Analyst software (AB SCIEX, CA, USA, Ver. 1.6).

LC and MS conditions
A BEH C18 column (Waters, 2.1 × 50 mm, 1.7 µm particles) was used for separation by using methanol (A, 0.1% formic acid) and water (B, contain 5% methanol, 0.1% formic acid) as mobile phase with a flow rate of 0.4 mL/min under gradient elution as follows: initial, 5.5% A; 0 - 1.0 min, 5.5% A-90% A; 1.0 - 1.6 min, 90% A; 1.6 - 1.7 min, 90% A-5.5% A; 1.7 - 3.0 min, 5.5% A (1.3 min for equilibration). The autosampler and column oven were set at 10 and 37°C. Positive electrospray ionization was performed at 550°C with an ion spray voltage of 5500 V. Curtain gas, ion source gas 1, and ion source gas 2 were set at 35, 55, and 55 psi, respectively. Medium collision gas was used. The quantitative and qualitative ion pairs, ion collision energy, declustering potential, entrance potential, and collision cell exit potential are given in Table S1 (Supporting Information). The chemical structure and mass spectrometry of analytes and IS are shown in Fig. 1.

Preparation of stock and working solutions
MTX (4000 µmol/L), 7-OH MTX (2000 µmol/L), DAMPA (4000 µmol/L), 7-OH DAMPA (2000 µmol/L), MTX-D₃ (220 µmol/L), and DAMPA-D₃ (300 µmol/L) were dissolved in ultrapure water containing 16 mmol/L NaOH (for dissolution). The four analytes were mixed together to obtain a series of working solutions of calibrators at 0.02, 0.04, 0.2, 0.4, 2, and 4 µmol/L for MTX and DAMPA, and 0.1, 0.2, 1, 2, 10, and 20 µmol/L for 7-OH MTX and 7-OH DAMPA. The two IS were also mixed together at 0.1 µmol/L for MTX-D₃ and 0.2 µmol/L for DAMPA-D₃. The working solutions of QC samples were 0.02, 0.06, 0.12, 1.8, and 3 µmol/L for MTX and DAMPA, and 0.1, 0.3, 0.6, 9, and 15 µmol/L for 7-OH MTX and 7-OH DAMPA. All stock and working solutions were stored at −80°C before use.

Preparation of calibration and quality control (QC) samples
Ten µL of analyte- and IS-free urine were mixed with 10 µL of a working solution and 10 µL of IS (contain 0.1 µmol/L MTX-D₃ and 0.2 µmol/L DAMPA-D₃); then, 300 µL of methanol (with 15% water and 0.1% formic acid) was added for protein precipitation and extraction. After 5-min vortex mixing, 30-min storage at 4°C, and 2-min centrifugation at 10000 × g, 2 µL of the supernatant was injected for analysis. A series of calibration samples at 0.02, 0.04, 0.2, 0.4, 2, and 4 µmol/L for MTX and DAMPA, and 0.1, 0.2, 1, 2, 10, and 20 µmol/L for 7-OH MTX and 7-OH DAMPA, and QC samples at 0.02, 0.06, 0.12, 1.8, and 3 µmol/L for MTX and DAMPA, and 0.1, 0.3, 0.6, 9, and 15 µmol/L for 7-OH MTX and 7-OH DAMPA were prepared.

Sample collection and preparation
Patients with primary central nervous system lymphoma receiving high-dose MTX therapy were enrolled. Then, 1 mL of venous blood was collected at about 13, 37, and 61 h after infusion, and 1 mL of urine was obtained from the patients' natural urine at similar time points. For urine, after 5-min centrifugation at 3000 × g, 10 µL of urine was spiked with 10 µL of IS (contain 0.1 µmol/L MTX-D₃ and 0.2 µmol/L DAMPA-D₃) and 10 µL of water containing 16 mmol/L NaOH (for dissolution); then, 300 µL of methanol (containing 15% water and 0.1% formic acid) was added for protein precipitation. After 5-min vortex mixing, 30-min storing at 4°C, and 2-min centrifugation at 12000 × g, 2 µL of the supernatant was injected for analysis. The final concentration of IS was 0.003 µmol/L for MTX-D₃ and 0.006 µmol/L for DAMPA-D₃. The plasma MTX and 7-OH MTX levels were determined by our previously validated LC-MS/MS method.

Method validation
Method validation was performed according to the guidelines including the selectivity, carry-over, lower limit of quantitation (LLOQ), calibration curve, accuracy, precision, dilution integrity, recovery, matrix effect, and stability.
Selectivity and LLOQ

To evaluate the selectivity, analyte- and IS-free urine from 10 individuals was used. LLOQ was regarded as the lowest concentration of the calibration curve (0.02 μmol/L for MTX and DAMPA, and 0.1 μmol/L for 7-OH MTX and 7-OH DAMPA). The selectivity was acceptable when the interfering peak areas in the analyte- and IS-free urine were less than 20% of the analyte peak areas in the LLOQ sample. For LLOQ samples, the mean bias should be within ±20%, and the within-run and between-run coefficient of variation (CV) should be less than 20%.27,28

Carry-over and linearity

To validate the carry-over of the analytes and IS, a blank sample was analyzed immediately following the highest concentration of the calibration sample. The carry-over was acceptable when the peak area of the blank sample was less than 20% of the peak area of the LLOQ sample for the analytes, 28 and 5% for the IS (laboratory standard). The method of weighted least-squares (weighting factor = 1/x^2) was used for linear regression. The bias of each level of the calibrator should be within ±15% and the correlative coefficient of linear regression function should be higher than 0.995.

Accuracy and precision

Five replicates of QC samples at 0.02, 0.06, 0.12, 1.8, and 3 μmol/L for MTX and DAMPA, and 0.1, 0.3, 0.6, 9, and 15 μmol/L for 7-OH MTX and 7-OH DAMPA were analyzed to evaluate the intra-day and inter-day accuracy and precision (20 days). The bias and imprecision of QC samples should be within ±15% (±20% for LLOQ) and less than 15% (20% for LLOQ), respectively.

Recovery and matrix effect

To evaluate the recovery and matrix effect, three batches of QC samples at 0.06, 0.12, 1.8, and 3 μmol/L for MTX and DAMPA, and 0.3, 0.6, 9, and 15 μmol/L for 7-OH MTX and 7-OH DAMPA were prepared:27,28 (A) analytes and IS in blank urine from 10 different individuals with protein precipitation and extraction, (B) analytes and IS in post-protein precipitated urine matrix from 10 different individuals, (C) analytes and IS in methanol (with 15% water and 0.1% formic acid). The ratios of (A/B) × 100% and (B/C) × 100% were defined as the recovery and matrix factor. The ratios of (A_analyte/B_analyte)/(A_IS/B_IS) × 100% and (B_analyte/C_analyte)/(B_IS/C_IS) × 100% were defined as the IS normalized recovery and matrix factor. At all QC levels, the IS normalized recovery should be consistent, and the IS normalized matrix factor should be precise (CV <15%).27,28

Dilution integrity and stability

To evaluate the dilution integrity, 10-fold and 100-fold dilution of samples by blank urine at 10 and 100 times of the highest QC levels were used for 7-OH MTX and 7-OH DAMPA; 10-, 100-, and 1000-fold dilution of samples at 10, 100, and 1000 times of the highest QC levels were used for DAMPA, and 10-, 100-, 1000-, and 10000-fold dilution of samples at 10, 100, 1000, and 10000 times of the highest QC levels were used for MTX. The bias and precision of diluted samples should be within ±15% and less than 15%, respectively.

To evaluate the stability of analytes during sample preparation, analysis, and storage, QC samples at 0.06, 0.12, 1.8, and 3 μmol/L for MTX and DAMPA, and 0.3, 0.6, 9, and 15 μmol/L for 7-OH MTX and 7-OH DAMPA were measured under various conditions (in urine: 24°C for 15 h, 4°C for 22 h, three freeze-thaw cycles from –80 to 24, and –80°C for 2 and 4 weeks; post extraction: 24°C for 2, 6, 10, 24, and 113 h, 4°C for

Fig. 1 Chemical structure and mass spectrometry of analytes and internal standards. (A) Methotrexate (about 10 ng/mL); (B) 7-hydroxy methotrexate (about 50 ng/mL); (C) methotrexate-D3 (about 10 ng/mL); (D) deoxyaminopteroyl acid (about 10 ng/mL); (E) 7-hydroxy deoxyaminopteroyl acid (about 50 ng/mL); (F) deoxyaminopteroyl acid-D3 (about 10 ng/mL).
8, 12, 24, and 111 h, 10°C for 10, 24, 48, 72, 96, and 120 h, two freeze-thaw cycles from −80 to 24, and −80°C for 15 days). Analytes were considered to be stable under a certain condition when the bias of QC samples was within ±15%.

Application

Patients with primary central nervous system lymphoma receiving high-dose MTX therapy (about 3.5 g/m²) were enrolled. Urine and blood samples were collected at similar time points every morning (about 13, 37, and 61 h after infusion). Urine concentrations of MTX and its three metabolites were measured by this method, while plasma concentrations of MTX and 7-OH MTX were determined by our previously validated LC-MS/MS method.22 The clinical characteristics of enrolled patients including age, sex, height, body weight, body surface area, MTX dose, sampling time, alanine transaminase, serum creatinine, urine volume and urine pH were recorded.

Statistical analysis

In this study, urine drug levels were supposed to predict plasma drug levels, which should be normally distributed for multiple linear regression. However, when all plasma drug levels at three sampling time points were analyzed as a whole (about 13, 37, and 61 h after infusion). Urine concentrations of MTX and its three metabolites were measured by this method, while plasma concentrations of MTX and 7-OH MTX were determined by our previously validated LC-MS/MS method.22 The clinical characteristics of enrolled patients including age, sex, height, body weight, body surface area, MTX dose, sampling time, alanine transaminase, serum creatinine, urine volume and urine pH were recorded.

Results

LLOQ and selectivity

Typical chromatograms of the UHPLC-MS/MS method are shown in Fig. 2. Some peaks were observed at the elution time of analytes and IS; however, their responses were far less than 20% of the responses of the four analytes at the LLOQ level and 5% of that of the IS. The two IS did not affect the measurement
of all analytes. The bias and imprecision of LLOQ samples were –11.40 to 10.10 and <13.66% for MTX, –7.00 to 19.50 and <20.89% for 7-OH MTX, –8.00 to 9.40 and <16.59% for DAMPA, and –12.02 to 6.98 and <14.20% for 7-OH DAMPA. The signal-to-noise ratio of LLOQ was 57.9 for MTX, 84.8 for 7-OH MTX, 68.2 for DAMPA, and 40.6 for 7-OH DAMPA.

**Carry-over and linearity**

There was no carry-over effect for all analytes. The typical linear regression equation is \( y = 3.32x + 0.0121, \) \( r = 0.9998 \) for MTX, \( y = 0.651x + 0.00393, \) \( r = 0.9980 \) for 7-OH MTX, \( y = 4.24x + 0.00218, \) \( r = 0.9994 \) for DAMPA, and \( y = 1.06x + 0.017, \) \( r = 0.9991 \) for 7-OH DAMPA (x, analytes concentration; \( y, \) peak area ratio of the analytes to IS).

**Accuracy and precision**

Table 1 shows the intra- and inter-day accuracy and precision of the method. At five QC levels, the intra- and inter-day bias and imprecision were –1.30 to 6.81 and <4.73% for MTX, –4.02 to 4.47 and <6.98% for 7-OH MTX, –8.00 to 7.61 and <4.98% for DAMPA, and –10.53 to 16.00% for 7-OH DAMPA, respectively.

**Recovery and matrix effect**

At four QC levels, the IS normalized recovery and matrix factor were 102.59 to 108.72% and 97.61 to 99.73% (CV <20.68%) for 7-OH DAMPA (detail in Table S2).

**Dilution integrity and stability**

The bias and imprecision of diluted samples indicated that 10- and 100-fold dilution for 7-OH MTX and 7-OH DAMPA, 10-, 100-, and 1000-fold dilution for DAMPA, and 10-, 100-, 1000-, and 10000-fold dilution for MTX did not affect the analysis (data not shown).27,28 At four QC levels, MTX, 7-OH MTX, DAMPA, and 7-OH DAMPA were stable under all tested conditions with the bias ranging from –10.53 to 16.00% (Table S2).

**Method application**

A total of 171 urine and blood samples from 38 patients were enrolled and analyzed. DAMPA was observed in 100 urine samples, while 7-OH DAMPA was only observed in 43 urine samples. The clinical characteristics of our patients are summarized in Table S3. In multiple regression, plasma MTX and 7-OH MTX levels increased with the urine drug levels, age, serum creatinine and alanine transaminase. The correlation was poor between urine and blood for MTX and 7-OH MTX at three sampling time points (\( R^2: 0.16 \) to 0.51, detail in Table 3). Therefore we concluded that urine might not replace blood for MTX monitoring. Dose-normalized urine and plasma MTX and 7-OH MTX levels were similar in patients with different urine pH values (pH <7 or ≥7). Unexpectedly, at 62 h after dosing, 7-OH MTX plasma level was higher in patients with urine pH ≥7 compared to those with urine pH <7 (\( n = 19 \)) (Table S4).

**Discussion**

**Method development and validation**

One-step protein precipitation was efficient and simple,25 and it was used in our previous methods13,22 and many other studies for MTX analysis.14,17,24,26 However, a pretreatment by pure methanol resulted in an asymmetric peak for 7-OH DAMPA. To solve this problem, various proportions of water (10, 15, 20, and 25%) were added in methanol for protein precipitation, and symmetric peaks were obtained when the water proportion was equal to or higher than 15%. Therefore methanol containing 15% water was used for protein precipitation, but some precipitation were observed at the bottom of the supernatants of the post-extracted samples after storing at 10 \( \text{C} \) for 10 min or longer. To solve this problem, the post-extracted samples were stored at 4 \( \text{C} \) for a period of time (10, 20, 30, and 40 min) for complete formation of the precipitates, and following a 2-min centrifugation at 12000 \( \text{g} \) to remove it. The results indicated that storing at 4 \( \text{C} \) for 30 min was efficient for complete formation of the precipitates, and it was used in the present study. Leading and tailing peaks were observed when the injection volume was higher than 2.5 \( \mu \text{L} \). Therefore, a 2-\( \mu \text{L} \)}
The possible reasons for the matrix effect of analytes, such as matrix factor, matrix effect, and recoveries, were similar to our previously published studies with minor modifications, including an extension of the gradient and sample purification technology, such as solid-phase extraction, for the quantitation of 7-OH MTX and 7-OH DAMPA, 7-hydroxy deoxyaminopteroic acid; IS, internal standard (MTX-D₃ for MTX and 7-OH MTX, DAMPA-D₃ for DAMPA and 7-OH DAMPA); CV, coefficient of variation.

### Table 2
The recovery and matrix effect of MTX, 7-OH MTX, DAMPA, and 7-OH DAMPA in human urine (mean ± standard deviation, n = 10)

| Drug       | Nominal concentration/ analytes recoveries, % | CV of IS normalized recoveries, % | Matrix factor of analytes, % | Matrix factor of IS, % | IS normalized matrix factor, % | CV of IS normalized matrix factor, % |
|------------|---------------------------------------------|----------------------------------|-----------------------------|------------------------|------------------------------|------------------------------------|
| MTX        | 102.7 ± 6.7, 94.5 ± 2.5, 108.7 ± 7.4        | 6.81                             | 95.6 ± 6.2                  | 97.7 ± 6.8             | 97.9 ± 4.9                  | 4.98                               |
| 7-OH MTX   | 0.3                                           | 6.94                             | 119.5 ± 6.1                 | 97.7 ± 6.8             | 123.0 ± 13.0                | 10.55                              |
| 7-OH DAMPA | 0.6                                           | 10.70                            | 123.8 ± 5.8                 | 103.5 ± 7.0            | 124.7 ± 11.9                | 9.53                               |
| DAMPA      | 4.28                                          | 9.87                             | 113.5 ± 4.7                 | 97.0 ± 7.4             | 117.8 ± 12.1                | 10.24                              |
|            | 15.4                                          | 4.79                             | 123.8 ± 6.2                 | 105.6 ± 6.1            | 116.7 ± 9.7                 | 8.28                               |

### Table 3
Multiple linear regression results between logarithmic transformed plasma drug levels and covariates (the blood sampling time was restricted within ±1 h)

| Time after dose/h | Logarithmic transformed plasma drug levels | Normality of logarithmic transformed plasma drug levels | Goodness of fit, R² | Equation significance (F test) | Covariates and its standardized coefficient | Significance of standardized coefficient (t-test) | Residual independence (Durbin-Watson test) | Normality of residual |
|-------------------|------------------------------------------|------------------------------------------------------|----------------------|--------------------------------|-----------------------------------------------|----------------------------------------------|------------------------------------------|----------------------|
| 13                | MTX                                      | 0.94                                                 | 0.51 Corton                  | <0.001                        | Age (0.47)                                     | <0.001                                        | 1.55                                     | 0.61                 |
|                   |                                           | SCR (0.38)                                           | 0.001                                 |                                |                                               |                                              |                                          |                      |
|                   |                                           | ALT (0.30)                                           | 0.009                                 |                                |                                               |                                              |                                          |                      |
| 37                | 7-OH MTX                                 | 0.049                                                | 0.16                        | 0.005                          | MTXU (0.35)                                    | 0.031                                         | 2.02                                     | 0.99                 |
|                   |                                           | ALT (0.40)                                           | 0.045                        |                                |                                               |                                              |                                          |                      |
|                   | 7-OH MTX                                 | 0.79                                                 | 0.34                        | 0.001                          | MTXU (0.35)                                    | 0.031                                         | 2.02                                     | 0.99                 |
|                   |                                           | ALT (0.40)                                           | 0.047                        |                                |                                               |                                              |                                          |                      |
| 61                | MTX                                      | 0.11                                                 | 0.34                        | 0.004                          | MTXU (0.59)                                    | 0.031                                         | 1.38                                     | 0.25                 |
|                   |                                           | ALT (0.46)                                           | 0.031                        |                                |                                               |                                              |                                          |                      |

### Abbreviations:
- MTX, methotrexate; 7-OH MTX, 7-hydroxy methotrexate; DAMPA, deoxyaminopteroic acid; 7-OH DAMPA, 7-hydroxy deoxyaminopteroic acid; IS, internal standard (MTX-D₃ for MTX and 7-OH MTX, DAMPA-D₃ for DAMPA and 7-OH DAMPA); CV, coefficient of variation.

injection volume was used for analysis, which was comparable to those in published studies (0.5 to 2 μL). The HPLC conditions were similar to our previously published studies with minor modifications, including an extension of the gradient elution time (from 0.5 to 1 min) for complete separation of the four analytes and a reduction of the column washing time (from 0.8 to 0.6 min). The 3 min run time was much shorter than those in many published methods (5.52 to 6.6 min) for the analysis of MTX and its metabolites, and close to two studies (3.0 and 3.6 min) for MTX analysis.

The recovery and matrix factor of analytes were comparable to the observations in published studies in various biological fluids including human urine, plasma, serum, and cerebrospinal fluid (recovery: 72 to 126% for MTX, 67 to 122% for 7-OH MTX, and 54.4 to 105.1% for DAMPA; matrix factor: 70.5 to 118% for MTX, 90 to 105% for 7-OH MTX, and 101 to 107% for DAMPA). The possible reasons for the matrix induced response enhancement for both 7-OH MTX (117.65 to 124.70%) and 7-OH DAMPA (156.57 to 172.93%), and the big inter-individual variance of 7-OH DAMPA matrix factor (CV < 20.68%) were summarized as follows: (1) the one-step protein precipitation for sample extraction retained many matrix in the post-extracted samples; (2) the fast separation procedure of the HPLC method could not efficiently separate all of the matrix from the analytes; (3) the fast separation procedure, and/or the isotope internal standards. All analytes were stable in urine and
post-extracted urine matrix under tested conditions, which was consistent with the results in previous studies.\textsuperscript{2,3,15,17,21,23,24} Method application Before multiple regression, plasma drug levels were separated into three groups according to their sampling time points, which was restricted within ±2 h. Moreover, the difference of the sampling time between urine and blood was also restricted (within ±0.5, ±1, and ±2 h), and within ±1 h was the best to reduce the variance and to ensure the normal distribution of the data. The multiple-regression results indicated that the plasma drug levels increased with the urine drug levels, age, serum creatinine and alanine transaminase. MTX was mainly excreted in the urine,\textsuperscript{1} which could explain the correlation between increased plasma MTX levels and elevated urine MTX levels. Interestingly, the urine 7-OH MTX levels increased with the plasma 7-OH MTX levels at 37 h after dosing, although urine was the minor route for 7-OH MTX excretion.\textsuperscript{3,23} Plasma MTX levels increased with serum creatinine at 13 h after dosing, which was caused by the reduced urine drug excretion due to impaired renal function.\textsuperscript{11} Moreover, the renal function decreased with age, which could explain that the plasma MTX levels increased with age at 13 h after dosing. MTX and 7-OH MTX plasma levels increased with alanine transaminase, which could be elucidated by the following reasons: both MTX and 7-OH MTX were transformed into their polyglutamates mainly via folypolyglutamate synthase in the liver;\textsuperscript{34,35} these polyglutamates could not be transported out of the cells when the number of their glutamate residues was greater than three, which resulted in significant accumulation of these polyglutamates in the liver;\textsuperscript{36} in the case of liver injury, these polyglutamates were released into the blood with the death of hepatocyte and transformed back into MTX and 7-OH MTX via blood gamma-glutamyl hydrolase.\textsuperscript{2,27} In the present study, due to a poor correlation between the urine and blood drug levels, urine could not replace blood for MTX monitoring in patients receiving high-dose MTX therapy. 

Alkalinization was routinely performed for patients receiving high-dose MTX therapy to enhance the renal excretion of MTX and to reduce toxicity risk.\textsuperscript{8,9} In the present study, dose-normalized plasma and urine levels of both MTX and 7-OH MTX varied greatly between individuals (Table S4), which could be explained by the following reasons: the small sample size; the different MTX dose, sampling time, urine volume, patients’ physiological status and pharmacokinetic parameters of analytes between individuals.\textsuperscript{11} The dose-normalized plasma and urine MTX and 7-OH MTX levels were similar between patients with different urine pH (<7 or ≥7), which might be explained by the similar alkalinization treatment of enrolled patients. Dose-normalized 7-OH MTX plasma level was higher in patients with the urine pH ≥7 compared to those with the urine pH <7 at 62 h after dosing. This unexpected result might be also explained by the reasons given for the big variance of the drug levels between individuals. Deficiencies of the study 

(1) The results in the present study should be treated with caution due to the great inter-individual variance of the drug levels in both blood and urine. (2) The sample size was small. (3) The sampling time between urine and blood was different. (4) The influence of the renal and liver function on the urine drug levels was not evaluated due to lack of cases (9 samples with slight renal impairment; 10 samples with slight liver impairment). (5) The influence of co-medications on the urine drug levels was not evaluated.

Conclusions An accurate and robust UHPLC-MS/MS method for simultaneous determination of MTX, 7-OH MTX, DAMPA, and 7-OH DAMPA in urine was developed, validated, and applied in clinical practice. The simple and efficient (recognition 92.34 to 109.49\%) one-step protein precipitation for sample pretreatment and the short analysis time (3 min) were suitable for clinical application. The calibration range (expanded by dilution factors) could cover most of the clinical samples. The inter-individual variance of matrix factor for all analytes (<20.68\%) could ensure the accuracy of analysis. All processes during sample collection, pretreatment, and storage did not affect the analysis. Plasma MTX and 7-OH MTX levels increased with urine drug levels, age, serum creatinine and alanine transaminase, all of which should be considered in clinical practice. Urine might not replace blood for MTX monitoring due to their poor correlation (R², 0.16 to 0.51). Urine pH (<7 or ≥7) did not affect dose-normalized urine and plasma MTX and 7-OH MTX levels, but these results did not mean that alkalinization is not important for patients receiving high-dose MTX therapy. In fact, alkalinization is a key strategy for toxicity prevention in patients receiving high-dose MTX therapy. Due to the deficiencies of the study, these findings should be treated with caution and further and larger studies are warranted to confirm these results.

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Supporting Information This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/

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