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Determination of Methotrexate, 7-Hydroxymethotrexate, and 2,4-Diamino-N¹⁰-methylpteroic Acid by LC-MS/MS in Plasma and Cerebrospinal Fluid and Application in a Pharmacokinetic Analysis of High-Dose Methotrexate

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Abstract

A rapid and robust method for measuring methotrexate (MTX) and its two primary metabolites, 7-hydroxymethotrexate (7-OHMTX) and 2,4-diamino-N¹⁰-methylpteroic acid (DAMPA), was developed for use in pharmacokinetic studies of plasma and cerebrospinal fluid samples collected from infants with malignant brain tumors. Sample aliquots (100 μ L) were prepared for bioanalysis of MTX and metabolites using a Waters Oasis HLB microelution SPE plate. Chromatography was performed using a Phenomenex Synergi Polar-RP 4 μ 75 $\times$ 2.0mm ID column heated to 40°C. A rapid gradient elution on a Shimadzu HPLC system was used, with mobile phase A consisting of water/formic acid (100/0.1 v/v) and mobile phase B consisting of acetonitrile/formic acid (100/0.1 v/v). Column eluent was analyzed using AB Sciex QTRAP 5500 instrumentation in electrospray ionization mode. The ion transitions (m/z) monitored were 455.2 $\rightarrow$ 308.1, 471.1 $\rightarrow$ 324.1, and 326.2 $\rightarrow$ 175.1 for MTX, 7-OHMTX, and DAMPA respectively. The method was linear over a range of 0.0022 – 5.5 μ M for MTX, 0.0085 – 21 μ M for 7-OHMTX, and 0.0031 – 7.7 μ M for DAMPA. The method was applied to the analysis of serial plasma samples obtained from infants diagnosed with malignant brain tumors receiving high-dose MTX and results were compared to MTX concentrations from a TDx-FLx FPIA.

Keywords

Methotrexate; 7-OHMTX; LC-MS/MS; DAMP

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

INTRODUCTION

High-dose methotrexate (HDMTX) is used to treat many adult and pediatric malignancies including acute lymphoblastic leukemia (ALL), non-Hodgkin lymphoma, and osteosarcoma [1–5]. Recently the use of HDMTX in combination with other anticancer drugs in infants and young children with medulloblastoma has increased to provide an alternative to craniospinal irradiation due to the incidence of long-term neurocognitive and neuroendocrine effects [6, 7]. Children treated on these regimens have shown improvement in progression free and overall survival with less morbidity compared to patients receiving radiation and chemotherapy [6, 8–10].

Upon systemic administration MTX is primarily excreted by the kidneys but also undergoes hepatic metabolism to an active metabolite, 7-hydroxymethotrexate (7-OHMTX). 7-OHMTX exhibits cytotoxic activity and, due to its low water solubility, can precipitate in the renal tubules, contributing to the potential for acute kidney injury [11]. In the rare instances where patients have severe renal dysfunction, a MTX rescue agent, glucarpidase (carboxypeptidase G2 or CPG2), can be administered as an adjunct treatment, triggering rapid systemic conversion of MTX to 2,4-diamino-N¹⁰-methylpteroic acid (DAMPA), an inactive and nontoxic metabolite of MTX [12].

The physiological and pathological changes that take place in infants and young children with primary CNS tumors make it important to thoroughly study the disposition of MTX and its metabolites (7-OHMTX and DAMPA) in this patient population. For example, renal tubular function in infants is comparable to adults by 3–7 months, glomerular filtration rate (normalized for body surface area) by 5–12 months, and renal blood flow by about 5 months [13, 14]. Furthermore, infants with brain tumors can develop fluid collections after surgical resection, which may not circulate freely with normal cerebrospinal fluid (CSF) spaces. We have recently shown that the presence of a post resection fluid collection can alter MTX disposition leading to delayed MTX excretion [15–17].

Although our study suggested that MTX can be administered safely to infants with post resection fluid collections, it was only with intensive and protracted MTX monitoring requiring a sensitive MTX method. Additionally, we did not assess the role of the 7-OHMTX metabolite with the incidence of grade 3 or 4 toxicity. Lastly, since we were monitoring MTX therapy in very young infants receiving MTX in the context of multiagent chemotherapy, including nephrotoxic drugs (e.g., cisplatin), we viewed the ability to measure the DAMPA metabolite as important in the event of compromised renal function. Thus, the objective of this study was to develop and validate a sensitive and robust LC-MS/MS assay for measuring MTX and metabolites 7-OHMTX and DAMPA in plasma and CSF samples collected from infants and young children diagnosed with malignant brain tumors.

MATERIALS AND METHODS

Chemicals and Reagents

MTX was obtained from Sigma Aldrich (St. Louis, USA). Methanol and phosphoric acid (>85% purity) were purchased from Fisher Scientific (Fairlawn, NJ, USA). MTX-d3 was obtained from Toronto Research Chemical, Inc. (Toronto, Canada). 7-OHMTX and $^{13}\text{C}^2\text{H}_3$ -7-OHMTX were purchased from Alsachim (Strasbourg, France). Acetonitrile was purchased from Honeywell (VWR International, Pittsburgh, PA) and formic acid from Fluka Biochemika (Buchs, Switzerland). All water was prepared using Millipore Milli-Q UV Plus and Ultra-Pure Water System (Tokyo, Japan). Human CSF was purchased from Bioreclamation LLC (Liverpool, USA). Blank human plasma was obtained from Life Blood (Memphis, USA). All other chemicals were from standard sources and of the highest purity available.

Chromatographic Conditions

The HPLC system consisted of a Shimadzu (Kyoto, Japan) system controller (CBM-20A), pumps (LC-30AD), autoinjector (SIL-30AC), online degasser, and column heater set to 40°C. Chromatographic separation was accomplished using a Synergi Polar-RP C18 column (4µm, 75 mm × 2.0 mm ID; Phenomenex, USA). The analytes and ISTD were rapidly separated using a gradient elution. Initial conditions consisted of 85% mobile phase A (water/formic acid, 100/0.1, v/v) and 15% mobile phase B (acetonitrile/formic acid, 100/0.1, v/v). These conditions were held for 1.5 minutes at which time mobile phase B was increased to 20% for 2 minutes followed by a flush of 90% mobile phase B for an additional 2 minutes. Total sample acquisition time was 7.0 minutes.

Instrument Conditions

Detection was achieved using an AB Sciex QTRAP 5500 with an ESI source (Applied Biosystems, Singapore). Instrument settings and sample acquisition were controlled using Analyst software (Version 1.5, Applied Biosystems, Foster City, CA). Multiple Reaction Monitoring (MRM) and positive ion mode with unit resolution for both Q1 and Q3 were used in the detection of the analytes and ISTDs. The optimized MS/MS conditions were as follows: ion spray source temperature at 500°C, curtain gas (cur) pressure at 25 psi, gas 1 (G1) set at 40 psi, gas 2 (G2) set at 20 psi, ion spray voltage (IS) was set at 5500 V, collision-activated dissociation (CAD) was set at medium, declustering potential (DP) was 46 V, and the collision energy (CE) was set at 40 V for MTX and 20 V for the remaining analytes. The ion transitions monitored (m/z) were 455.2→308.1, 471.1→324.1, and 326.2→175.1 for MTX, 7-OHMTX, and DAMPA respectively; 458.2→311.2 and 475.2→328.3 were monitored for MTX-d3 and $^{13}\text{C}^2\text{H}_3$ -7-OHMTX, respectively.

Preparation of Stocks and Calibrators

MTX, 7-OHMTX, DAMPA, and $^{13}\text{C}^2\text{H}_3$ -7-OHMTX were prepared to a concentration of 1 mg/mL in 5% NH_4OH in water. MTX-d3 was prepared to a concentration of 0.2 mg/mL in 0.05% NH_4OH in water. Working solutions were prepared from stocks by combining the appropriate volumes of each analyte and then diluting using water/methanol 70/30 v/v.

Calibrators were prepared such that when 10 μL was spiked into 100 μL of matrix, the final calibrator concentration would be 0.0022, 0.011, 0.017, 0.11, 0.549, 1.1, 2.2, 4.4, and 5.5 μM for MTX; 0.0085, 0.042, 0.064, 0.43, 2.1, 4.2, 8.5, 17, and 21 μM for 7-OHMTX; and 0.0031, 0.015, 0.023, 0.15, 0.77, 1.5, 3.1, 6.1, and 7.7 μM for DAMPA. The same procedure was used to prepare a combined working ISTD solution with a final concentration of 0.22 μM for MTX-d3 and 0.42 μM for $^{13}\text{C}_2\text{H}_3$ -7-OHMTX. Master stock and working stock solutions were stored at -80°C .

Sample Preparation

All samples were prepared for analysis with solid phase extraction using an Oasis 30 μm HLB 96-well microelution plate (Waters, Wexford, Ireland). Blank plasma samples were included in every extraction and analysis to verify there were no interfering peaks with the analytes of interest. To 100 μL aliquots of plasma or CSF, 10 μL of combined ISTD working solution was added followed by 125 μL of water/phosphoric acid (96/4, v/v). Prior to addition of samples, the 96-well plate was pre-conditioned under gentle vacuum with methanol followed by water. After the samples had been transferred to the appropriate wells of the plate, they were washed twice with 200 μL water and eluted with 100 μL of methanol. Sample eluents were then dried down completely using house nitrogen gas on a plate warmer set to 45°C for approximately 15 minutes prior to being reconstituted with 75 μL of a solution of mobile phase A/mobile phase B (90/10, v/v).

Linearity and Lower Limit of Quantitation

Calibration curves were evaluated using least square linear regression weighted with $1/x^2$. The coefficient of determination (r^2) was used to evaluate the linearity of each calibration curve. The LLOQ was defined as the lowest concentration in the calibration curve that had accuracy within 20% of the accepted true value and a signal/noise (S/N) ratio greater than 5 [18,19]. The lower limit of detection (LOD) was defined as the lowest concentration resulting in S/N of 3.

Accuracy and Precision

Intra- and inter-day precision and accuracy were performed in plasma and CSF using five quality control samples each at the LLOQ (0.0022, 0.0085, 0.0031 μM), low (0.0055, 0.021, 0.0077 μM), medium (0.82, 3.18, 1.15 μM), and high (3.85, 14.9, 5.37 μM) quality control concentrations for MTX, 7-OHMTX, and DAMPA, respectively. The acceptance criteria for precision were $\pm 15\%$ at the low, medium, and high concentrations for MTX and 7-OHMTX and $\pm 20\%$ at the assay LLOQ for all analytes and the low, medium, and high concentrations for DAMPA.

Selectivity, Carryover, Matrix Effects, Recovery

Selectivity was assessed by extraction of blank samples and samples spiked at the LLOQ in six different sources of human plasma. In consideration of the feasibility/costs of obtaining multiple lots of human CSF, only one source of pooled CSF was evaluated. Sample carryover was assessed by monitoring wash samples after injections of the high concentration calibrator to ensure any peak area did not exceed 20% of the LLOQ. Matrix

effects were evaluated by comparing post extraction spiked samples to mobile phase spiked samples. The matrix factor (MF) was calculated by dividing the instrument response of the post extracted samples by the instrument response of mobile phase spiked samples. Both low (0.0055 μ M MTX, 0.021 μ M 7-OHMTX, 0.0077 μ M DAMPA) and high (3.85 μ M MTX, 14.9 μ M 7-OHMTX, 5.37 μ M DAMPA) quality controls were evaluated in six different lots of human plasma and a single lot of CSF (n=6). Recovery was assessed with the low and high quality controls for each respective analyte by calculating the ratio of the instrument response of extracted samples to that of samples prepared by spiking blank unextracted matrix at the same concentrations.

Stability Studies

Stability was assessed for all three analytes in both human plasma and CSF. Pooled samples were prepared at the same low and high quality controls as in the accuracy and precision experiments. Three samples at each concentration were analyzed immediately to establish an average time zero baseline value. All other aliquots were subjected to the appropriate stress, analyzed, and compared to baseline value. Freeze thaw stability, short-term stability, and extract stability experiments were performed for both matrices. Long-term stability studies were completed for human plasma. Samples were considered stable with $\pm$ 20% change in concentration.

Cross-Validation to TDx-FLx FPIA

To compare the method reported here to the TDx-FLx FPIA, 30 plasma samples from pediatric patients receiving high-dose MTX were analyzed with both methods [20,21]. Pediatric patients diagnosed with a malignant brain tumor received 2.5 or 5 g/m² HDMTX as an intravenous infusion over 24 hours during induction therapy for treatment of a malignant brain tumor (SJYC07; NCT00602667). Samples were first analyzed via TDx-FLx FPIA and then by LC-MS/MS. The TDx-FLx FPIA assay was carried out by the St. Jude Pharmaceutical Sciences Clinical Lab according to instructions recommended by Abbott Laboratories as part of routine clinical monitoring. The Passing and Bablok (PB) regression test was implemented in R (version 3.1.3, R Foundation for Statistical Computing, Vienna, Austria) to analyze the results from the cross-validation studies.

Application of the LC-MS/MS Method to a Plasma Pharmacokinetic Study

To demonstrate the applicability of the LC-MS/MS method, concentration measurements were analyzed from a single patient who received HDMTX (5 g/m²) on our institutional protocol (SJYC07), which was approved by our institutional review board. Pre-hydration began the night before and the total MTX dosage was diluted in 5% dextrose in water with 40 mEq/L sodium bicarbonate to a concentration of 10 mg/mL. A 10% loading dose was administered over the first hour and the remainder over 23 hours. Urine pH was monitored throughout drug administration and bolus doses of sodium bicarbonate were administered if urinary pH $\leq$ 6. Leucovorin rescue was administered starting at hour 42 at 15 mg/m² every 6 hours for five doses. Samples for pharmacokinetic analysis were collected prior to the start of HDMTX and at 6, 23, 42, 66, and 90 hours after the start of infusion. Samples were centrifuged at room temperature and plasma supernatant was stored at -80° C until analysis. After thawing, plasma was assayed for MTX, 7-OHMTX, and DAMPA according to the

procedure described in this report. A three compartment model was simultaneously fit to MTX and 7-OHMTX concentration-time data (two compartments for MTX and one compartment for 7-OHMTX) using the maximum likelihood algorithm, as implemented in ADAPT5 [22]. A non-compartmental analysis was implemented in R 3.1.3 using the PK package.

RESULTS AND DISCUSSION

Chromatography

Due to its hydrophilic nature, MTX was not sufficiently retained with traditional C18 columns. For this reason, a Phenomenex Synergi Polar-RP column was selected for its ability to better retain polar compounds. During method development, DAMPA exhibited a significantly longer retention time compared to the other two analytes so a gradient was selected to efficiently separate the compounds. Due to the low organic content required to elute all three analytes, a mobile phase flush was added after elution to prevent organic buildup on the column. The column heater was added to improve consistency in peak shape. Both MTX-d3 and $^{13}\text{C}^2\text{H}_3$ -7-OHMTX were evaluated as internal standards for DAMPA. $^{13}\text{C}^2\text{H}_3$ -7-OHMTX appeared to correlate better with DAMPA, so it was selected to serve as the IS for the validation. Chromatograms of the three analytes at the LLOQ are shown in Figure 1.

Mass Spectrometry

MTX and its two metabolites were measured using an AB Sciex QTRAP 5500 triple quad with an ESI source at unit resolution for both Q1 and Q3. Positive ionization was selected since all three analytes ionized well under these conditions. All instrument parameters were optimized by direct infusion of the compound using a syringe pump. Q1 scans were used to select the parent ion followed by product ion scans to select the daughter ions monitored. The source of the daughter ions was also confirmed with precursor ion scans. The LOD was identified as 0.0004 μM for MTX and 7-OHMTX and 0.0023 μM for DAMPA in both matrices (plasma and CSF).

Accuracy and Precision

The intra-day and inter-day precision and accuracy results for plasma and CSF are listed in Tables 1 and 2, respectively. Accuracy is presented as percentage error and precision as percent CV. For plasma, the intra-day accuracy and precision were within 20% and 15% for the LLOQ and quality controls, respectively, for all analytes. Likewise, the inter-day precision and accuracy in plasma were within acceptable limits for all three analytes with the exception of DAMPA at the highest quality control of 5.37 μM , which had a percent coefficient of variation of 19.0%. This was due to a single sample that had about half of the internal standard expected. If this specific sample is removed, the percent coefficient of variation is then 12.4%. However, this concentration was left in for completeness of the results.

The CSF intra-day and inter-day precision and accuracy studies were all within 20% and 15% for LLOQ and the quality controls, respectively, for all three analytes. Our method

precisely and accurately quantitates CSF MTX, 7-OHMTX, and DAMPA concentrations making this method very useful for research applications to assess the relevance of CSF MTX, 7-OHMTX, and DAMPA exposure.

Selectivity, Carryover, Matrix Effects, Recovery

Selectivity was assessed in both human plasma and CSF, and no interfering peaks were observed for either matrix. Carryover was assessed by injecting wash samples after injection of the high concentration calibrator, and no significant carryover was observed for any of the analytes. Matrix effects and recovery experiment data presented in Table 3 demonstrate a slight ion enhancement in human plasma though matrix factor never exceeded 1.08. In CSF, the matrix factor ranged from 0.940 to 1.01 (Table 3), demonstrating acceptable matrix effects.

The recovery was consistent and ranged from 80.8% to 91.0% for all analytes in human plasma. In CSF, the recovery was also consistent and ranged from 76.7% to 103.8%. For both plasma and CSF, the ISTD peak height values trended with analytes in regards to magnitude and direction of both matrix effect and recovery, indicating the degree of matrix effect did not affect analyte quantitation.

Stability

Stability was determined for MTX, 7-OHMTX, and DAMPA and reported as the percent change of the average of three stressed samples compared to the original time zero samples. Samples were considered stable if $\pm 20\%$ change in concentration was observed, and no differences were observed between low and high concentrations for any of the stability conditions. Short-term stability studies in plasma at room temperature demonstrated an average percent change for MTX, 7-OHMTX, and DAMPA of $\pm 16.7\%$ from baseline for 120 hours. In CSF, all three components were stable at room temperature for 48 hours. At 4°C, MTX and 7-OHMTX in plasma were stable for 120 hours, though because of methodological problems the results for DAMPA were not interpretable. In CSF at 4°C, MTX, 7-OHMTX, and DAMPA were stable for 48 hours. Plasma extracts for MTX, 7-OHMTX, and DAMPA were stable at 4°C for 120 hours, and CSF extracts at 4°C were stable for 48 hours. Finally, through 4 FTS cycles at -80°C , the average percent change for MTX and 7-OHMTX in human plasma was $\pm 10.2\%$ from baseline. However, DAMPA was only stable for 2 FTS cycles. In CSF, MTX, 7-OHMTX, and DAMPA were all stable for 2 FTS cycles at -80°C . MTX, 7-OHMTX, and DAMPA were all stable at -80°C for at least 270 days with the exception of the high 7-OHMTX quality control, which had a -21% loss at 270 days.

Cross-Validation to TDx-FLx FPIA

As a component of this LC-MS/MS validation, a cross-validation was performed for MTX between the TDx-FLx FPIA (analyzed for clinical purposes) and LC-MS/MS method. The cross-validation involved samples collected from pediatric patients enrolled on SJYC07 who received HDMTX as either a 5 g/m² or 2.5 g/m² 24 hr infusion for treatment of malignant brain tumors. A comparison of the 30 plasma samples showed good agreement, with a coefficient of determination (R^2) of 0.997. Shown in Figure 2 is the PB regression for the

LC-MS/MS and the FPIA in our analysis indicating a proportional bias of ~ 8% and a constant bias of ~ 0.03 μM , with 95% CI for the slope and y-intercept of 0.88 to 0.93 and -0.047 to 0.0011, respectively. Shown in Figure 3 is a Bland-Altman plot describing the mean difference between the 2 methods and the 95% CI. The average bias was 3.47 μM (95% CI -5.45 to 12.4 μM) and only three samples (10%) were outside the 95% CI.

Application to a Pharmacokinetic Study

We analyzed MTX, 7-OHMTX, and DAMPA in samples collected from an 18-month-old patient enrolled on our institutional brain tumor protocol (SJYC07). All values for DAMPA were BLQ and thus were not included in the model. The representative plasma concentration-time profile for MTX and 7-OHMTX for this patient is depicted in Figure 4, including the pharmacokinetic model fits of the observed MTX and 7-OHMTX data. In this patient, the estimated area under the curve ($\text{AUC}_{0-\infty}$) for MTX and 7-OHMTX was 1,398 $\mu\text{M}\times\text{hr}$ and 432 $\mu\text{M}\times\text{hr}$, respectively. The quantitation of MTX and 7-OHMTX is valuable for future investigations on the relationship between systemic exposure (AUC) and toxicity or response in our patient population.

As might be expected for a compound that has been used clinically for over 60 years, many assay methods have been published for methotrexate. These assay methods vary widely, but could be broadly divided into those that are immunoassay-based versus those that are chromatography-based. Of the methods that are chromatography-based, they can be divided based upon the detection methodology into those that are non-mass spectrometry (i.e., primarily UV detection) and mass spectrometry. Each method has a minor advance over previously published methods (e.g., measures different metabolites 7-OH, DAMPA, or MTX polyglutamates; measures components in different matrices including plasma, serum, urine, and CSF; uses different extraction approaches including protein precipitation, solid phase extraction; has different LLOQ or LOD; reports different aspects of the method (stability results, comparison with immunoassay)), and rather than try to compare our method with all previously published methods, only the most recently published methotrexate mass spectrometry method will be compared with the current method (Schofield, J Chrom B, 2015). The method by Schofield and colleagues measured methotrexate, 7-OH MTX, and DAMPA and used 100 μL sample volume. However, they used serum and we used plasma, they used protein precipitation and we used solid phase extraction to extract the sample, we measured MTX and metabolites in plasma and CSF and they did not measure in CSF, and we had much more sensitive method with a MTX and 7-OH MTX LLOQ of 0.0022 μM and 0.0085 μM compared to 0.01 μM and 0.02 μM for Schofield. Thus, our method is a more sensitive method that could be used to measure MTX and metabolites in more clinical matrices (e.g., CSF), and represents an advance over previously published methods.

CONCLUSION

The method described in this report represents a LC-MS/MS assay designed to measure MTX, 7-OHMTX, and DAMPA in human plasma and CSF using a 96-well solid phase extraction plate. This presents a single method that has the capability to analyze every component needed for a pharmacokinetic study in very young pediatric patients, including

MTX and its primary metabolite concentrations, validated in human plasma and CSF, and very small sample volumes. Additionally, the use of an Oasis 96-well elution plate for sample preparation allows the method to be adapted to liquid handling systems for automation, enabling high throughput sample analysis and also minimizing solvent usage while reducing sample analysis costs and time. Furthermore, a follow-up cross-validation analysis was performed to compare the validated LC-MS/MS method to the clinical TDx-FLx FPIA, with excellent agreement. Overall, the technique described herein proved to be a fast and reliable method for quantitating MTX and metabolite concentrations in human plasma and CSF, permitting application to a pharmacokinetic study in a very young child treated with HDMTX.

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Figure 1.

Human plasma chromatograms of (A) LLOQ of MTX (B) LLOQ of 7-OHMTX (C) LLOQ of DAMPA (D) ISTD MTX-d3 (E) MTX scan of human plasma (F) 7-OHMTX scan of blank human plasma (G) DAMPA scan of blank human plasma (H) ISTD $^{13}\text{C}_2\text{H}_3$ -7-OHMTX

Figure 2. Scatter-plot of all samples plotted by reference TDx-FLx FPIA (Method A) values on the x-axis and the comparator LC-MS/MS (Method B) values on the y-axis ($n = 30$). The solid line represents the slope of the Passing-Bablok regression, the shaded region represents the 95% confidence interval of the regression, and the dashed line is the line of unity.

Figure 3.

Bland and Altman plot for MTX measured by TDx-FLx FPIA and LC-MS/MS. The difference between the TDx-FLx FPIA and LC-MS/MS is plotted against the average of the two methods. The dotted black line is the mean and the dotted gray lines are the upper and lower 95% confidence intervals.

Figure 4. MTX (open square; solid line model fit) and 7-OHMTX (open circle; dashed line model fit) plasma concentration–time profile in a 18-month-old child after HDMTX infusion (5 g/m^2 over 24 hours).

Table 1

Intra-day and inter-day (3 days) precision and accuracy of MTX, 7-OHMTX, and DAMPA in human plasma, n=6.

	Target conc. (µM)	Mean conc. (µM)	Precision (%CV)	Accuracy (%)
Intra-day n=6				
MTX	0.0022	0.0018	19.0	82.8
	0.0055	0.0054	8.3	98.2
	0.82	0.81	1.6	98.7
	3.85	3.73	5.1	97.0
7-OHMTX	0.0085	0.0091	6.5	106.9
	0.021	0.022	3.3	103.3
	3.18	3.19	3.5	101.5
	14.90	14.86	3.2	99.7
DAMPA	0.0031	0.0031	7.5	102.3
	0.0077	0.0075	9.4	97.2
	1.15	1.12	11.9	97.7
	5.37	5.26	12.7	97.9
Inter-day n=6 for 3 days				
MTX	0.0022	0.0019	16.8	85.6
	0.0055	0.0053	10.1	96.7
	0.82	0.82	4.1	99.2
	3.85	3.76	4.3	97.7
7-OHMTX	0.0085	0.0093	5.4	109.1
	0.021	0.023	6.4	108.1
	3.18	3.29	4.4	103.5
	14.90	15.09	4.3	101.3
DAMPA	0.0031	0.0032	8.8	103.5
	0.0077	0.0077	6.8	100.2
	1.15	1.17	9.7	101.4
	5.37	6.02	19.0	112.2

MTX – methotrexate; 7-OHMTX – 7-hydroxymethotrexate; DAMPA – 2,4-diamino-N¹⁰-methylptericoic acid; %CV – percent coefficient of variation

Table 2

Intra-day and inter-day (3 days) precision and accuracy of MTX, 7-OHMTX, and DAMPA in human CSF, n=6.

	Target conc. (μM)	Mean conc. (μM)	Precision (%CV)	Accuracy (%)
Intra-day n=6				
MTX	0.0022	0.0023	12.4	103.0
	0.0055	0.0053	6.1	95.8
	0.824	0.800	1.6	97.1
	3.85	3.71	3.4	96.5
7-OHMTX	0.0085	0.0088	6.0	103.4
	0.021	0.020	2.0	95.3
	3.18	3.12	2.3	98.3
	14.9	14.3	3.1	95.9
DAMPA	0.0031	0.0032	7.9	102.7
	0.0077	0.0075	5.7	97.8
	1.15	1.11	2.3	96.5
	5.37	5.62	12.0	104.6
Inter-day n=6 for 3 days				
MTX	0.0022	0.0021	15.8	97.2
	0.0055	0.0054	8.5	98.9
	0.824	0.812	3.7	98.6
	3.85	3.78	3.3	98.1
7-OHMTX	0.0085	0.0081	9.6	95.0
	0.021	0.0207	5.1	97.4
	3.18	3.16	2.8	99.4
	14.9	14.5	4.6	97.0
DAMPA	0.0031	0.0030	11.6	97.6
	0.0077	0.0076	9.2	98.8
	1.15	1.14	2.9	99.4
	5.37	5.27	9.9	98.1

MTX – methotrexate; 7-OHMTX – 7-hydroxymethotrexate; DAMPA – 2,4-diamino-N¹⁰-methylptericoic acid; %CV – percent coefficient of variation

Table 3

Matrix effects and recovery in human plasma (n=6) and CSF (n=3) of MTX, 7-OHMTX, and DAMPA.

	Human Plasma			CSF		
	Recovery		Matrix Effects	Recovery		Matrix Effects
	Avg (%)	%CV	Avg	Avg (%)	%CV	Avg
MTX						
Low QC	86.5	1.82	1.08	103.8	7.1	1.01
High QC	90.4	1.45	1.03	98.7	7.6	0.950
7-OHMTX						
Low QC	80.8	6.68	0.90	95.6	11.6	0.9932
High QC	84.2	12.28	1.05	76.7	11.9	0.940
DAMPA						
Low QC	91.0	3.89	1.07	98.0	2.8	0.993
High QC	90.2	0.80	1.01	100.6	5.6	0.963

MTX – methotrexate; 7-OHMTX – 7-hydroxymethotrexate; DAMPA – 2,4-diamino-N¹⁰-methylpterioic acid; Avg – average; %CV – percent coefficient of variation