Chapter 23

Development of an Assay for Methotrexate and Its Metabolites 7-Hydroxy Methotrexate and DAMPA in Serum by LC-MS/MS

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Abstract

Methotrexate (MTX) is a folic acid antagonist that is widely used as an immunosuppressant and chemotherapeutic agent. After high-dose administration of MTX serum levels must be monitored to determine when to administer leucovorin, a folic acid analog that bypasses the enzyme inhibition caused by MTX and reverses its toxicity. We describe a rapid and simple turbulent flow liquid chromatography (TFLC) method implementing positive heated electrospray ionization (HESI) for the accurate and precise determination of MTX, 7-hydroxymethotrexate (7-OH MTX), and 4-amino-4-deoxy-N^{10}-methylpteroic acid (DAMPA) concentrations in serum. MTX is isolated from serum samples (100 μL) after protein precipitation with a methanolic solution containing internal standard (MTX-D_3) followed by centrifugation. The supernatant is injected into the turbulent flow liquid chromatography which is followed by electrospray positive ionization tandem mass spectrometry (TFLC-ESI-MS/MS) and quantified using a six-point calibration curve. For MTX, 7-OH MTX, and DAMPA the assays were linear from 20 to 1000 nmol/L. Dilutions of 10-, 100-, and 1000-fold were validated giving a clinically reportable range of 20 to 1.0×10^6 nmol/L. Within-day and between-day precisions at concentrations spanning the analytical measurement ranges were less than 10 % for all three analytes.

Key words Methotrexate, Carboxypeptidase-G2, Therapeutic drug monitoring, Mass spectrometry, Turbulent flow liquid chromatography

1 Introduction

Methotrexate (MTX) is a folic acid antagonist that is widely used as an immunosuppressant and chemotherapeutic agent. MTX exerts its cytotoxic effects by competitively inhibiting dihydrofolate reductase (DHFR), the enzyme responsible for converting folates to tetrahydrofolates; the folate carrier that functions in the transfer of carbon units. A normal dividing cell uses large amounts of reduced folates to maintain ongoing purine and thymidine synthesis and the demand is even greater for rapidly dividing malignant cells [1].

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High-dose MTX is mainly used for the treatment of leukemia and osteosarcoma. Intermediate and lower dose MTX regimens are used to treat malignant gestational trophoblastic disease, breast and bladder cancer, ALL, and acute promyelocytic leukemia [1, 2]. In addition to its antiproliferative activity, MTX also has anti-inflammatory and immunomodulating properties and is a first-line treatment for a growing number of autoimmune rheumatologic, dermatologic, and gasteroenterologic conditions [1, 2]. After high-dose administration of MTX serum levels must be monitored to determine when to administer leucovorin, a folic acid analog that bypasses the enzyme inhibition caused by MTX and reverses its toxicity [3]. Patients in renal failure who are given high-dose MTX are sometimes given carboxypeptidase-G2 ($CPDG_2$) ($CPDG_2$) to reverse the effects of MTX [3–5]. $CPDG_2$ is an enzyme that converts MTX into glutamate and 4-amino-4-deoxy-N10-methylpteroic acid (DAMPA) that are much less toxic and readily excreted. DAMPA cross-reacts considerably in immunoassays rendering them unsuitable for monitoring patients who have been given $CPDG_2$ therapy [6].

MTX assays using mass spectrometry have been described previously [7–9]. However the objective of this study was to develop an MTX assay performed by LC-MS with the following characteristics: analytically sensitive with a clinically useful dynamic range; good specificity with no interference from metabolites or other compounds; suitable analytical transferability for a high volume clinical laboratory, and the accurate measurement of 7-OH MTX and DAMPA to support clinical trials utilizing $CPDG_2$ and related compounds. The following chapter describes a rapid and simple turbulent flow method implementing positive heated electrospray ionization for the accurate and precise determination of MTX, 7-OH MTX, and DAMPA concentrations in serum.

2 Materials

2.1 Samples

Serum samples are required. All samples should be processed and analyzed within 4 h of collection or refrigerated for analysis up to 24 h after collection or frozen for analysis up to 6 months.

2.2 Solvents and Reagents

1. Human drug-free pooled normal serum (UTAK Laboratories).
2. Mobile Phase A (10 mM ammonium formate/0.1 % formic acid in water): Remove 8.4 mL of water from a 4 L bottle. Add 2.8 mL of ammonium hydroxide, cap, and invert ten times. Add 5.6 mL of formic acid and degas for 5 min by sonication. The mobile phase is stable at room temperature, 18–24 °C, up to 1 month.

3. Mobile phase B (10 mM ammonium formate/0.1 % formic acid in methanol): Remove 8.4 mL of methanol from a 4 L bottle. Add 2.8 mL of ammonium hydroxide, cap, and invert ten times. Add 5.6 mL of formic acid and degas for 5 min by sonication. The mobile phase is stable at room temperature, 18–24 °C, up to 1 month.
4. Mobile phase C (acetonitrile/2-propanol/acetone, 6:3:1): In a 1000 mL graduated cylinder, add 600 mL acetonitrile, 300 mL of 2-propanol, and 100 mL of acetone into a 2 L HPLC solvent bottle. Degas the solution for 5 min by sonication. The mobile phase is stable at room temperature, 18–24 °C, up to 1 month.
5. Autosampler aqueous wash (water/acetic acid/acetonitrile, 8.8:1:0.2): In a 500 mL graduated cylinder, add 440 mL of water, 50 mL of acetic acid, and 10 mL of acetonitrile and transfer into an HPLC wash bottle. Degas the solution for 5 min by sonication. The wash solution is stable at room temperature, 18–24 °C, up to 1 month.
6. Autosampler organic wash (acetonitrile/2-propanol/acetone, 6:3:1): In a 1000 mL graduated cylinder, add 600 mL acetonitrile, 300 mL of 2-propanol, and 100 mL of acetone and transfer into a 2 L HPLC solvent bottle. Degas the solution for 5 min by sonication. The mobile phase is stable at room temperature, 18–24 °C, up to 1 month.
7. Extraction solution (30 ng/mL MTX-D_3 in methanol with 0.1 % formic acid): Add approximately 150 mL of methanol and 200 μL formic acid into a 200 mL volumetric flask. Add 60 μL of MTX-D_3 stock (100 μg/mL) to the same volumetric flask. Bring to volume with methanol and mix well. The extraction solution is stable for up to 6 months when stored at −20 °C.

2.3 Internal Standards and Standards

1. Primary standards: MTX (Sigma-Aldrich), 7-OH MTX (Santa Cruz Biotechnology Inc.), and DAMPA (Schircks Laboratories).
2. Primary internal standard (I.S.): MTX-D_3 (Cerilliant) 100 μg/mL in 0.1 N sodium hydroxide.
3. Standard stock solutions:
   (a) Methotrexate MTX (1 mg/mL): Using an analytical balance weigh 25 mg and place in a 25 mL volumetric flask. Bring to volume with methanol containing 0.1 N sodium hydroxide and mix well. Then prepare a 100 μg/mL stock solution from the previous 1 mg/mL stock solution: Add 900 μL of methanol to a 2 mL amber vial and add 100 μL of MTX stock (1 mg/mL) and mix well. Both stock solutions are stable up to 6 months when stored at −20 °C.

(b) 7-Hydroxymethotrexate, 7-OH MTX (1 mg/mL): Using an analytical balance weigh 25 mg and place in a 25 mL volumetric flask. Bring to volume with methanol containing 0.1 N sodium hydroxide and mix well. Then prepare a 100 μg/mL stock solution from the previous: Add 900 μL of methanol to a 2 mL amber vial and add 100 μL of 7-OH MTX stock (1 mg/mL) and mix well. Both stock solutions are stable for up to 6 months when stored at −20 °C.

(c) DAMPA (1 mg/mL): Using an analytical balance weigh 25 mg and place in a 25 mL volumetric flask. Bring to volume with methanol containing 0.1 N sodium hydroxide and mix well. Then prepare a 100 μg/mL stock solution from the previous: Add 900 μL of methanol to a 2 mL amber vial and add 100 μL of DAMPA stock (1 mg/mL) and mix well. Both stock solutions are stable for up to 6 months when stored at −20 °C.

(d) Combined standard (MTX, 7-OH MTX, and DAMPA): To obtain a combined standard containing, 4.54 μg/mL, 9.40 μg/mL, and 3.25 μg/mL respectively, add 1656 μL of drug-free serum to a 2 mL amber glass vial. Then add 91, 188, and 65 μL of MTX, 7-OH MTX, and DAMPA from each respective 100 μg/mL stock solution and mix well. Stock solution is stable for up to 6 months when stored at −20 °C.

2.4 Calibrators and Controls

1. Calibrators: Prepare calibrators 1–8 by making serial dilutions of the combined standard according to Table 1. For each dilution step add the appropriate amount of previous solution as shown in the table to a 10.0 mL volumetric flask and fill with drug-free human serum. Vortex mix the volumetric flask after each dilution step (*see* **Note 1**).
2. Controls: MTX controls were purchased from UTAK laboratories at the following concentrations: 0.023, 0.034, 0.227, and 0.341 μg/mL. Currently there are no commercially available controls for 7-OH MTX and DAMPA. To prepare 7-OH MTX and DAMPA controls follow Table 2 for the procedure. For each control add the appropriate amount of 100 μg/mL stock solution into a 10 mL volumetric flask then bring to volume with drug-free serum. These two sets of controls are made on separate days and from separate lots of material than the calibrators (*see* **Note 1**).
3. Check the new lot of standards by verifying five unknown patient samples concentrations with the current lot of calibrators. The agreement between the two calculated concentrations must be within 10 %.
4. Establish a range for the new lot of controls by collecting data points over 20 consecutive runs and establish the mean and standard deviation.

Table 1
Calibrator preparation

Calibrator	Volume of previous standard (mL)	Drug-free serum (mL)	Final concentrations (μg/mL) MTX	7-OH MTX	DAMPA
1	1.0 (combined std.)	9.0	0.454	0.940	0.325
2	5.0	5.0	0.227	0.470	0.163
3	5.0	5.0	0.114	0.235	0.081
4	5.0	5.0	0.057	0.118	0.041
5	5.0	5.0	0.028	0.059	0.020
6	5.0	5.0	0.014	0.029	0.010
7	5.0	5.0	0.007	0.015	0.005
8	5.0	5.0	0.004	0.007	0.003

MTX: 1 μg/mL = 2201.8 nmol/L
7-OH MTX: 1 μg/mL = 2127.1 nmol/L
DAMPA: 1 μg/mL = 3075.7 nmol/L

Table 2
7-OH MTX and DAMPA control preparation

Control	Volume of 7-OH MTX 100 μg/mL stock	Volume of DAMPA 100 μg/mL stock	Final concentration (μg/mL) 7-OH MTX	DAMPA
Low	24 μL	8 μL	0.235	0.081
Mid	47 μL	16 μL	0.470	0.161
High	71 μL	24 μL	0.705	0.244

2.5 Analytical Equipment and Supplies

1. Thermo Scientific Transcend TLX-2 with Agilent 1200 pumps coupled to a TSQ Quantum Ultra triple quadrupole mass spectrometer running Aria software 1.6.3 (Thermo Scientific).
2. TurboFlow column (TFC): Cyclone-P 50 × 0.5 mm, 60 μm particle size, 60 Å pore size (Thermo Scientific). Analytical column: Hypersil Gold C8 2.1 × 50 mm, 5 μm particle size (Thermo Scientific).
3. Column heater (Thermo Scientific).
4. Eppendorf 1.5 mL microcentrifuge tubes and National Scientific 2 mL amber glass vials with inserts and pre-slit caps or equivalent.

2.6 Instrument Operating Conditions

1. Turbulent flow liquid chromatography (TFLC): Chromatographic separations were performed using a Thermo Scientific Transcend TLX-2 which was comprised of a PAL autosampler (CTC Analytics), a low-pressure mixing quaternary pump (loading pump), a high-pressure binary pump

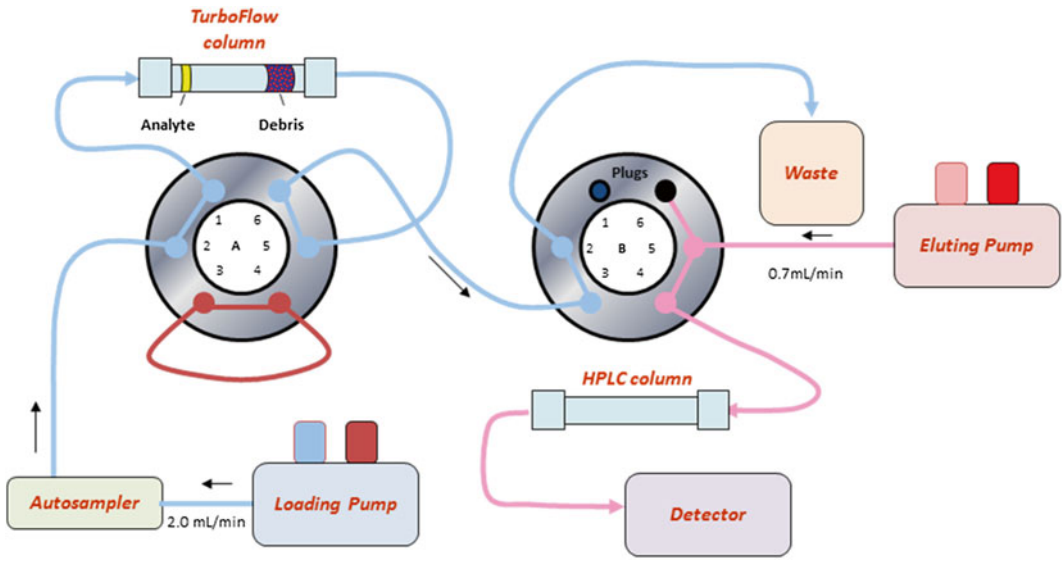

Fig. 1 Transcend TLX-2 valve configuration

(eluting pump), and a six-valve switching module with six-port valves (Fig. 1). The system was controlled via Aria software, version 1.6.2. The TurboFlow column used was a Cyclone-P 50×0.5 mm, 60 µm particle size, and a 60 Å pore size. The analytical HPLC column was a Hypersil Gold C8 2.1×50 mm, 5 µm particle size. The temperature of the analytical column was maintained at 70 °C using a column heater. The analytes were loaded on the TurboFlow column in 100 % mobile phase A and transferred to the HPLC column with 80 % mobile phase B using a 200 µL transfer loop. The loading and eluting mobile phase composition for the HPLC column was identical to that of the TurboFlow column. The integration parameters for all four analytes were similar with a baseline window of 20, area noise factor of 5, peak noise factor of 10, and an integration window of 15 s. The retention times of the TFLC-ESI-MS/MS ion chromatograms of the analytes can be seen in Fig. 2.

2. Tandem mass spectrometry: Mass spectrometric detection was performed using a Thermo Scientific TSQ Quantum Ultra triple-quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source operating in a positive ion mode. MS/MS conditions are depicted in Table 4. Nitrogen (99.995 % purity) was used as the desolvation gas, and ultrapure argon (99.999 % purity) was used as the collision gas. The mass transitions from the protonated molecular ion $[M+H]^+$ to the most abundant product ions were used as the quantifying ions for each analyte (Table 5). The SRM acquisition method was run in unit resolution (0.7) in both Q1 and Q3 with a scan width and scan rate of 0.050 m/z and 0.100 s, respectively.

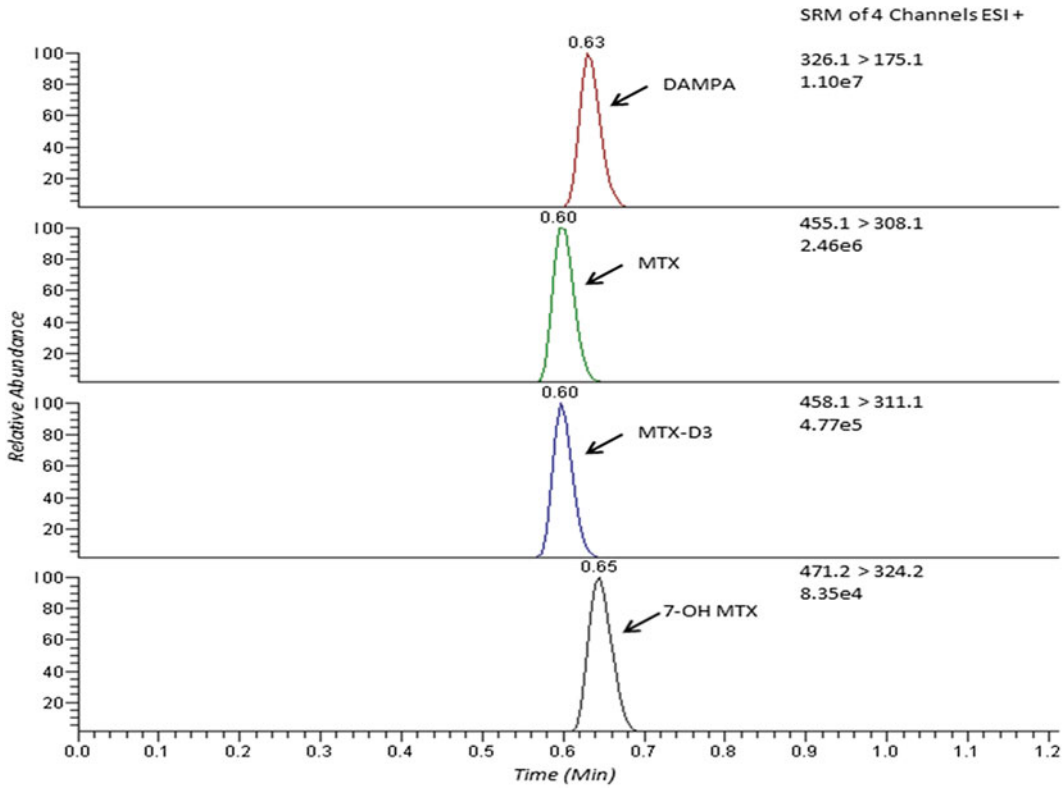

Fig. 2 TFLC-ESI-MS/MS ion chromatograms of MTX, 7-OH MTX, DAMPA, and MTX-D_3 (I.S.) product ions

3 Methods

3.1 Stepwise Procedure

1. Run a system suitability to confirm the system performance (*see* **Note 2**).
2. To a 1.5 mL microcentrifuge tubes pipette 100 µL serum (calibrators, controls, or patient specimen) (*see* **Note 3**).
3. Add 200 µL extraction solution.
4. Cap and vortex each sample vigorously.
5. Centrifuge for 10 min at 13,000 × *g*.
6. Transfer 200 µL of the supernatant into glass inserts and place in 2 mL amber glass vials.
7. Place all samples in the autosampler and inject 50 µL of the sample into the TFLC-ESI-MS/MS. Ion chromatograms for all analytes are shown in Fig. 2.

Table 3
TurboFlow and HPLC operating parameters

A. HPLC method. TurboFlow parameters HPLC parameters													
Step	*Start*	*s*	*Flow*	*Grad*	*%A*	*%B*	*%C*	*Tee*	*Loop*	*Flow*	*Grad*	*%A*	*%B*
1	0:00	30	2.00	Step	100			====	Out	0.7	Step	100	
2	0:30	45	0.15	Step	100			T	In	0.7	Step	100	
3	1:15	15	2.00	Step			100	====	In	0.7	Ramp	20	80
4	1:30	15	2.00	Step			100	====	Out	0.7	Step	20	80
5	1:45	30	2.00	Step			100	====	In	0.7	Step	20	80
6	2:15	45	2.00	Step	20	80		====	Out	0.7	Step	20	80
7	3:00	45	2.00	Step	20	80		====	In	0.7	Step		100
8	3:45	75	2.00	Step	100			====	Out	0.7	Step	100	
B. Mobile phase composition													
Mobile phase													
Loading pump A:	10 mM ammonium formate/0.1 % formic acid in water (v/v)												
Loading pump B:	10 mM ammonium formate/0.1 % formic acid in methanol (v/v)												
Loading pump C:	Acetonitrile/2-propanol/acetone (6:3:1 v/v)												
Eluting pump A:	10 mM ammonium formate/0.1 % formic acid in water (v/v)												
Eluting pump B:	10 mM ammonium formate/0.1 % formic acid in methanol (v/v)												
Aqueous wash 1:	Water/acetic acid/acetonitrile (8.8:1:0.2 v/v)												
Organic wash 2:	Acetonitrile/2-propanol/acetone (6:3:1 v/v)												

3.2 Analysis

1. Instrumental operating parameters are shown in Tables 3 and 4.
2. The data are analyzed using LCquan 2.6 software (Thermo Scientific).
3. Standard curves are based on linear regression analysis for MTX, 7-OH MTX, and DAMPA. Weighted linear regression models with weights inversely proportional to the X values were used. The analysis compared I.S. peak area to sample peak area (*y*-axis) versus analyte concentration (*x*-axis) using the quantifying ions indicated in Table 5.

Table 4
MS/MS tune settings

Spray voltage (V):	4500
Vaporizer temperature (°C):	380
Sheath gas pressure (arbitrary units):	60
Ion sweep gas pressure (arbitrary units):	2.0
Aux gas pressure (arbitrary units):	15
Capillary temperature (°C):	235
Collision pressure (mTorr):	1.5
Data window (min):	1:45–3:15

Table 5
Analyte precursor and product ions

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	CE (V)	Tube lens (V)	Skimmer (V)	CFPW (s)
MTX	455.1	308.1	18	105	10	5
7-OH MTX	471.2	324.2	11	105	10	5
DAMPA	326.1	175.1	18	110	10	5
MTX-D3	458.1	311.1	18	105	10	5

Optimized m/z may change based on instrument and tuning parameters

4. Acceptability of each run is confirmed if the calculated control concentrations fall within two standard deviations of the target mean values. Target values are established as the mean of 20 separate runs. If any control is greater than three standard deviations from the mean the run cannot proceed and trouble-shooting procedure must commence.
5. Typical coefficients of correlation of the standard curve are >0.995 (*see* **Note 4**).

4 Notes

1. Individual sets of calibrators and controls can be aliquoted and stored at −80 °C for 1 year. For each calibrator or control, aliquot 125 μL into a glass insert and place in a 2 mL amber glass vial and cap. Thaw completely before use.

2. A system suitability should be performed each day the method is run. The suitability includes running a test mix with all analytes to ensure proper retention time, integration, and sensitivity.
3. A new standard curve should be generated with each analytical run to ensure method performance.
4. The MTX, 7-OH MTX, and DAMPA assays were linear from 0 to 0.454 μg/mL, 0.007 to 0.940 μg/mL, and 0.003 to 0.325 μg/mL, respectively. Dilutions of 10-, 100-, and 1000-fold were validated for all analytes. Within-day and between-day precisions at concentrations spanning the analytical measurement ranges were less than 10 % for all three analytes.

References

1. LaCasce AS (2014) Therapeutic use and toxicity of high-dose methotrexate. In: Post TW (ed) UpToDate. Waltham, MA. Accessed 1 Oct 2014
2. Lennard L (1999) Therapeutic drug monitoring of antimetabolic cytotoxic drugs. Br J Clin Pharmacol 47:131–143
3. Widemann BC, Adamson PC (2006) Understanding and managing methotrexate nephrotoxicity. Oncologist 11:694–703
4. DeAngellis LM, Tong WP, Lin S, Fleisher M et al (1996) Carboxypeptidase G2 rescue after high-dose methotrexate. J Clin Oncol 14:2145–2149
5. Widemann BC, Sung E, Anderson L et al (2000) Pharmacokinetics and metabolism of the methotrexate metabolite 2,4-diamino-N-methylpteroic acid. J Pharmacol Exp Ther 294:894–901
6. Albertioni F, Rask C, Eksborg S et al (1996) Evaluation of clinical assays for measuring high-dose methotrexate in plasma. Clin Chem 42:39–44
7. Kumar VS, Law T, Kellogg M (2010) Liquid chromatography-tandem mass spectrometry (LC-MS-MS) method for monitoring methotrexate in the setting of carboxypeptidase-G2 therapy. In: Garg U, Hammett-Stabler CA (eds) Clinical applications of mass spectrometry: methods in molecular biology 603. Humana Press, New York
8. Guo P, Wang X, Liu L et al (2007) Determination of methotrexate and its major metabolite 7-hydroxymethotrexate in mouse plasma and brain tissue by liquid chromatography-tandem mass spectrometry. J Pharm Biomed Anal 43:1789–1795
9. Nair H, Lawrence L, Hoofnagle AN (2012) Liquid chromatography-tandem mass spectrometry work flow for parallel quantification of methotrexate and other immunosuppressants. Clin Chem 58:943–945