1 Simultaneous Determination of Urine Methotrexate, 7-Hydroxy Methotrexate, Deoxyaminopteroic Acid,

2 and 7-Hydroxy Deoxyaminopteroic Acid by UHPLC-MS/MS in Patients Receiving High-dose Methotrexate

3 Therapy

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Objective: Methotrexate (MTX) is widely used for cancer treatment, such as acute lymphoblastic leukemia, osteosarcoma and primary central nervous system lymphoma. The major problem for high-dose MTX therapy is life-threatening toxicities, such as hepatotoxicity, neurotoxicity, mucosal ulcer, and nephrotoxicity, and these toxicities are lethal in some patients. Moreover, there is a big inter- and intra-patient variance of these toxicities, which is not well understood. The most important toxicity in high-dose MTX therapy is nephrotoxicity, which 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), deoxyaminopteroic acid (DAMPA) and 7-hydroxy deoxyaminopteroic 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). This study aims to develop, validate and apply an UHPLC-MS/MS method for the simultaneous determination of MTX, 7-OH MTX, DAMPA and 7-OH DAMPA in human urine.

Method: Method was developed and validated according to the FDA and EMEA guidelines. The method validation including selectivity, carry-over, linearity, accuracy and precision, recovery, matrix effect, dilution integrity, and stability. Bland-Altman plot was used to compare the measurements between urine and blood.

Results: 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).

Conclusion: A fast, simple and stable UHPLC-MS/MS method for simultaneous determination of MTX, 7-OH MTX, DAMPA and 7-OH DAMPA in human urine was developed, validated and applied in clinical practice. Urine might not be able to replace blood for MTX monitoring. 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; deoxyaminopteroic acid; 7-hydroxy deoxyaminopteroic acid; method development and validation

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Introduction

MTX is widely used for cancer treatment, such as acute lymphoblastic leukemia, osteosarcoma and primary central nervous system lymphoma.[1] The major problem for high-dose MTX therapy is life-threatening toxicities, especially nephrotoxicity,[1] which is partly caused by the formation of crystal deposits in the kidney due to the poor water solubility of MTX and its metabolites 7-hydroxy methotrexate (7-OH MTX), deoxyaminopteroic acid (DAMPA) and 7-hydroxy deoxyaminopteroic acid (7-OH DAMPA) under acid conditions.[1-6] Under alkaline conditions, the water solubility of MTX and its metabolites dramatically increases because their carboxylic acid groups are transformed into carboxylate ions. Therefore urine alkalinization, in combination with MTX monitoring-guided glucarpidase detoxification and leucovorin rescue, are routine strategies to overcome MTX-induced toxicities.[1, 7]

In clinical studies, urine alkalinization increases MTX clearance and decreases high-dose MTX-induced toxicities.[8, 9] However, over-alkalinization increases the risk of acid-base disturbance. A study found that a reduction of the urine pH threshold from 8 to 7 did not affect the clearance of MTX, the rates of nephrotoxicity and the length of hospital stay.[10] Therefore, urine pH is routinely monitored for alkalinization adjustment. Glucarpidase, an efficient drug for MTX detoxification, can rapidly and completely transform MTX into its inactive form, DAMPA, which can form crystal deposits in the urine due to its poor water solubility.[4, 7] Therefore the urine level of DAMPA is recommended to monitor in patients receiving glucarpidase therapy.[4]

Predicting MTX-induced toxicities is a challenge for clinicians due to huge intra- and inter-individual variances of the MTX pharmacokinetics and pharmacodynamics.[1, 2, 5, 11] Therefore, 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,

73 DAMPA, and 7-OH DAMPA), which have similar chemical structures with MTX.[2, 5, 12, 13] Various

74 chromatographic based assays have been developed for MTX, 7-OH MTX and DAMPA analysis in human

75 blood plasma,[2, 3, 5, 13-26] but these methods have some disadvantages, such as the time-consuming procedure

76 for sample pretreatment[15, 16] and a long turnaround time (5 to 60 min).[2, 3, 15, 17, 21] MTX is mainly excreted in

77 the urine, which is more convenient to obtain compared to blood, especially for children; therefore, urine is

78 potential to replace blood for MTX monitoring. However, only two methods were developed for MTX

79 analysis in human urine.[23, 24] One method analyzed MTX by using a high sample volume (500 μL) and a low

80 upper limit of detection (0.11 μmol/L),[23] the other analyzed MTX and 7-OH MTX with a long turnaround

81 time (6.6 min).[24]

82 This study was aimed to develop and validate a fast, accurate, and robust ultra high-performance liquid-

83 chromatography tandem mass/mass spectrometry (UHPLC-MS/MS) method for the simultaneous

84 determination of MTX, 7-OH MTX, DAMPA, and 7-OH DAMPA in human urine, and to apply it in

85 patients with primary central nervous system lymphoma receiving high-dose MTX therapy. The influence of

86 urine pH on plasma and urine MTX and 7-OH MTX levels was established and the correlation between the

87 plasma and urine levels of both MTX and 7-OH MTX was evaluated to find out whether urine could replace

88 blood for monitoring.

89 **Experimental**

90 *Reagents and chemicals*

91 MTX (Lot: 100138-201606, 99.8% purity) was obtained from the National Institutes for Food and Drug

92 Control (Beijing, China). 7-OH MTX (Lot: 11-NSR-30-2, 95.23% purity), DAMPA (Lot: 1-JMS-61-4, 96%

93 purity), 7-OH DAMPA (Lot: 10-JHY-49-2, 95% purity), MTX-D_3 (Lot: 12-ZCA-5-1, 95% purity, 99.0%

94 isotopic purity, internal standard, IS), and DAMPA-D_3 (Lot: 1-TEK-173-1, 95% purity, 98.5% isotopic

95 purity, IS) were purchased from the Toronto Research Chemicals INC (Toronto, Canada). Methanol and

96 formic acid were purchased from Fisher Scientific (Waltham, USA), while ultrapure water was generated
97 from a Millipore Ultra pure water system (Bedford, USA). Analytes- and IS-free urine were obtained from
98 healthy volunteers and checked to ensure they did not contain any of the analytes and IS.

99 *Instrumentations*

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

103 *LC and MS conditions*

104 A BEH C18 column (Waters, 2.1 × 50 mm, 1.7 μm particles) was used for separation by using methanol
105 (A, 0.1% formic acid) and water (B, contain 5% methanol, 0.1% formic acid) as mobile phase with a flow
106 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
107 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
108 autosampler and column oven were set at 10 °C and 37 °C.

109 Positive electrospray ionization was performed at 550 °C with an ion spray voltage of 5500 V. Curtain
110 gas, ion source gas 1, and ion source gas 2 were set at 35, 55, and 55 psi, respectively. Medium collision gas
111 was used. The quantitative and qualitative ion pairs, ion collision energy, declustering potential, entrance
112 potential, and collision cell exit potential are given in Appendix 1. The chemical structure and mass
113 spectrometry of analytes and IS are shown in Fig. 1.

114 *Preparation of stock and working solutions*

115 MTX (4000 μmol/L), 7-OH MTX (2000 μmol/L), DAMPA (4000 μmol/L), 7-OH DAMPA (2000
116 μmol/L), MTX-D_3 (220 μmol/L), and DAMPA-D_3 (300 μmol/L) were dissolved in ultrapure water
117 containing 16 mmol/L NaOH (for dissolution). The four analytes were mixed together to obtain a series of
118 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,

119 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
120 μmol/L for MTX-D_3 and 0.2 μmol/L for DAMPA-D_3. The working solutions of QC samples were 0.02, 0.06,
121 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-
122 OH DAMPA. All stock and working solutions were stored at –80 °C before use.

123 *Preparation of calibration and quality control (QC) samples*

124 Ten μL of analytes- and IS-free urine were mixed with 10 μL of a working solution and 10 μL of IS
125 (contain 0.1 μmol/L MTX-D_3 and 0.2 μmol/L DAMPA-D_3); then, 300 μL of methanol (with 15% water and
126 0.1% formic acid) was added for protein precipitation and extraction. After 5-min vortex mixing, 30-min
127 storage at 4 °C, and 2-min centrifugation at 12000 × *g*, 2 μL of the supernatant was injected for analysis. A
128 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,
129 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
130 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
131 prepared.

132 *Sample collection and preparation*

133 Patients with primary central nervous system lymphoma receiving high-dose MTX therapy were enrolled.
134 Then, 1 mL of venous blood was collected at about 13, 37, and 61 h after infusion, and 1 mL of urine was
135 obtained from the patients' natural urine at similar time points. For urine, after 5-min centrifugation at 3000
136 × *g*, 10 μL of urine was spiked with 10 μL of IS (contain 0.1 μmol/L MTX-D_3 and 0.2 μmol/L DAMPA-D_3)
137 and 10 μL of water containing 16 mmol/L NaOH (for dissolution); then, 300 μL of methanol (containing 15%
138 water and 0.1% formic acid) was added for protein precipitation. After 5-min vortex mixing, 30-min storing
139 at 4 °C, and 2-min centrifugation at 12000 × *g*, 2 μL of the supernatant was injected for analysis. The final
140 concentration of IS was 0.003 μmol/L for MTX-D_3 and 0.006 μmol/L for DAMPA-D_3. The plasma MTX
141 and 7-OH MTX levels were determined by our previously validated LC-MS/MS method.[22]

142 *Method validation*

143 Method validation was performed according to the guidelines including the selectivity, carry-over, lower
144 limit of quantitation (LLOQ), calibration curve, accuracy, precision, dilution integrity, recovery, matrix
145 effect, and stability.[27, 28]

146 *Selectivity and LLOQ*

147 To evaluate the selectivity, analytes- and IS-free urine from 10 individuals was used. LLOQ was regarded
148 as the lowest concentration of the calibration curve (0.02 μmol/L for MTX and DAMPA, and 0.1 μmol/L for
149 7-OH MTX and 7-OH DAMPA). The selectivity was acceptable when the interfering peak areas in the
150 analytes- and IS-free urine were less than 20% of the analytes peak areas in the LLOQ sample. For LLOQ
151 samples, the mean bias should be within ± 20%, and the within-run and between-run coefficient of variation
152 (CV) should be less than 20%.[27, 28]

153 *Carry-over and linearity*

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

160 *Accuracy and precision*

161 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,
162 0.6, 9, and 15 μmol/L for 7-OH MTX and 7-OH DAMPA were analyzed to evaluate the intra-day and inter-
163 day accuracy and precision (20 days). The bias and imprecision of QC samples should be within ± 15% (±
164 20% for LLOQ) and less than 15% (20% for LLOQ), respectively.

165 *Recovery and matrix effect*

166 To evaluate the recovery and matrix effect, three batches of QC samples at 0.06, 0.12, 1.8, and 3 μmol/L

167 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,

168 28]: (A) analytes and IS in blank urine from 10 different individuals with protein precipitation and extraction,

169 (B) analytes and IS in post-protein precipitated urine matrix from 10 different individuals, (C) analytes and

170 IS in methanol (with 15% water and 0.1% formic acid). The ratios of (A/B) × 100% and (B/C) × 100% were

171 defined as the recovery and matrix factor. The ratios of $(A_{analyte}/B_{analyte})/(A_{IS}/B_{IS})$ × 100% and

172 $(B_{analyte}/C_{analyte})/(B_{IS}/C_{IS})$ × 100% were defined as the IS normalized recovery and matrix factor. At all QC

173 levels, the IS normalized recovery should be consistent, and the IS normalized matrix factor should be

174 precise (CV < 15%).[27, 28]

175 *Dilution integrity and stability*

176 To evaluate the dilution integrity, 10-fold and 100-fold dilution of samples by blank urine at 10 and 100

177 times of the highest QC levels were used for 7-OH MTX and 7-OH DAMPA; 10-fold, 100-fold and 1000-

178 fold dilution of samples at 10, 100 and 1000 times of the highest QC levels were used for DAMPA, and 10-

179 fold, 100-fold, 1000-fold, and 10000-fold dilution of samples at 10, 100, 1000, and 10000 times of the

180 highest QC levels were used for MTX. The bias and precision of diluted samples should be within ± 15%

181 and less than 15%, respectively.

182 To evaluate the stability of analytes during sample preparation, analysis, and storage, QC samples at 0.06,

183 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

184 DAMPA were measured under various conditions (in urine: 24 °C for 15 h, 4 °C for 22 h, three freeze-thaw

185 cycles from –80 °C to 24 °C, and –80 °C for 2 weeks and 4 weeks; post extraction: 24 °C for 2 h, 6 h, 10 h,

186 24 h, and 113 h, 4 °C for 8 h, 12 h, 24 h, and 111 h, 10 °C for 10 h, 24 h, 48 h, 72 h, 96 h, and 120 h, two

187 freeze-thaw cycles from –80 °C to 24 °C, and –80 °C for 15 days). Analytes were considered to be stable

188 under a certain condition when the bias of QC samples was within ± 15%.

189 *Application*

190 Patients with primary central nervous system lymphoma receiving high-dose MTX therapy (about 3.5

191 g/m^2) were enrolled. Urine and blood samples were collected at similar time points every morning (about 13,

192 37 and 61 h after infusion). Urine concentrations of MTX and its three metabolites were measured by this

193 method, while plasma concentrations of MTX and 7-OH MTX were determined by our previously validated

194 LC-MS/MS method.[22] The clinical characteristics of enrolled patients including age, sex, height, body

195 weight, body surface area, MTX dose, sampling time, alanine transaminase, serum creatinine, urine volume

196 and urine pH were recorded.

197 *Statistical analysis*

198 In this study, urine drug levels were supposed to predict plasma drug levels, which should be normally

199 distributed for multiple linear regression. However, when all plasma drug levels at three sampling time

200 points were analyzed as a whole, its distribution was non-normal even after logarithmic transformation

201 because the plasma drug levels decreased significantly with time. Therefore, the plasma drug levels were

202 separated into three groups according to their sampling time points which was further restricted within ± 2 h

203 to reduce the variance, but each subgroup was still not normally distributed. After a logarithmic

204 transformation, they were normally distributed, except for 7-OH MTX at 13 h after dosing ($P = 0.049$). The

205 difference of sampling time between urine and blood varied greatly between individuals, and it was

206 restricted (within ± 0.5 h, ± 1 h, and ± 2 h) before regression to reduce the bias. The Durbin-Watson test

207 (1.24 to 2.13) indicated that the residuals were independent, and it was another prerequisite for multiple

208 linear regression, which was performed to find out the relationship between the logarithmic transformed

209 plasma drug levels and the covariates (including logarithmic transformed urine drug levels, age, gender,

210 body weight, body surface area, dose, alanine transaminase, and serum creatinine). The influence of urine

211 pH (< 7 or ≥ 7) on dose-normalized plasma and urine drug levels (μmol/L per g/m^2) was evaluated by a *t*-

212 test or a nonparametric test after restriction of the sampling time (13.5 ± 0.5 h, 38 ± 1 h and 62 ± 1 h). SPSS

213 software (version 17.0, SPSS Inc., Chicago, USA) was used for statistical analysis including the student's *t*-

214 test, nonparametric test, normality test (Kolmogorov-Smirnov and Shapiro-Wilk test), and multiple linear

215 regression. The statistical significance was defined as P value < 0.05.

216 **Results**

217 *LLOQ and selectivity*

218 Typical chromatograms of the UHPLC-MS/MS method are shown in Fig. 2. Some peaks were observed

219 at the elution time of analytes and IS; however, their responses were far less than 20% of the responses of

220 the four analytes at the LLOQ level and 5% of that of the IS. The two IS did not affect the measurement of

221 all analytes. The bias and imprecision of LLOQ samples were –11.40% to 10.10% and < 13.66% for MTX,

222 –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%

223 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

224 7-OH MTX, 68.2 for DAMPA, and 40.6 for 7-OH DAMPA.

225 *Carry-over and linearity*

226 There was no carry-over effect for all analytes. The typical linear regression equation is $y = 3.32\,x +$

227 0.0121, $r = 0.9998$ for MTX, $y = 0.651\,x + 0.00393$, $r = 0.9980$ for 7-OH MTX, $y = 4.24\,x + 0.00218$, $r =$

228 0.9994 for DAMPA, and $y = 1.06\,x + 0.017$, $r = 0.9991$ for 7-OH DAMPA (x, analytes concentration; y,

229 peak area ratio of the analytes to IS).

230 *Accuracy and precision*

231 Table 1 shows the intra-day and inter-day accuracy and precision of the method. At five QC levels, the

232 intra-day and inter-day bias and imprecision were –1.30% to 6.81% and < 4.73% for MTX, –4.02% to 4.47%

233 and < 6.98% for 7-OH MTX, –8.00% to 7.61% and < 9.04% for DAMPA, and –5.10% to 3.76% and < 8.92%

234 for 7-OH DAMPA, respectively.

235 *Recovery and matrix effect*

236 At four QC levels, the IS normalized recovery and matrix factor were 102.59% to 108.72% and 97.61% to

237 99.73% (CV < 4.98%) for MTX, 96.03% to 104.97% and 117.65% to 124.70% (CV < 10.55%) for 7-OH

238 MTX, 102.05% to 109.49% and 98.60% to 104.84% (CV < 4.43%) for DAMPA, and 92.34% to 104.85%

239 and 156.57% to 172.93% (CV < 20.68%) for 7-OH DAMPA (detail in Table 2).

240 *Dilution integrity and stability*

241 The bias and imprecision of diluted samples indicated that 10-fold and 100-fold dilution for 7-OH MTX

242 and 7-OH DAMPA, 10-fold, 100-fold and 1000-fold dilution for DAMPA, and 10-fold, 100-fold, 1000-fold

243 and 10000-fold dilution for MTX did not affect the analysis (data not shown).[27, 28] At four QC levels, MTX,

244 7-OH MTX, DAMPA, and 7-OH DAMPA were stable under all tested conditions with the bias ranging from

245 –10.53% to 16.00% (Appendix 2).

246 *Method application*

247 A total of 171 urine and blood samples from 38 patients were enrolled and analyzed. DAMPA was

248 observed in 100 urine samples, while 7-OH DAMPA was only observed in 43 urine samples. The clinical

249 characteristics of our patients are summarized in Appendix 3. In multiple regression, plasma MTX and 7-

250 OH MTX levels increased with the urine drug levels, age, serum creatinine and alanine transaminase. The

251 correlation was poor between urine and blood for MTX and 7-OH MTX at three sampling time points (R^2:

252 0.16 to 0.51, detail in Table 3). Therefore we concluded that urine might not replace blood for MTX

253 monitoring. Dose-normalized urine and plasma MTX and 7-OH MTX levels were similar in patients with

254 different urine pH values (pH < 7 or ≥ 7). Unexpectedly, at 62 h after dosing, 7-OH MTX plasma level was

255 higher in patients with urine pH ≥ 7 compared to those with urine pH < 7 (n = 19). (Appendix 4)

256 **Discussion**

257 *Method development and validation*

258 One-step protein precipitation was efficient and simple,[25] and it was used in our previous methods[13, 22] and 259 many other studies for MTX analysis.[14, 17, 24, 26] However, a pretreatment by pure methanol resulted in an 260 asymmetric peak for 7-OH DAMPA. To solve this problem, various proportions of water (10%, 15%, 20% 261 and 25%) were added in methanol for protein precipitation, and symmetric peaks were obtained when the 262 water proportion was equal to or higher than 15%. Therefore methanol containing 15% water was used for 263 protein precipitation, but some precipitates were observed at the bottom of the supernatants of the post-264 extracted samples after storing at 10 °C for 10 min or longer. To solve this problem, the post-extracted 265 samples were stored at 4 °C for a period of time (10, 20, 30 and 40 min) for complete formation of the 266 precipitates, and following a 2-min centrifugation at 12000 × *g* to remove it. The results indicated that 267 storing at 4 °C for 30 min was efficient for complete formation of the precipitates, and it was used in the 268 present study. Leading and tailing peaks were observed when the injection volume was higher than 2.5 μL. 269 Therefore, a 2 μL injection volume was used for analysis, which was comparable to those in published 270 studies (0.5 to 2 μL).[13, 17, 18, 22, 23] The HPLC conditions were similar to our previously published studies with 271 minor modifications, including an extension of the gradient elution time (from 0.5 min to 1 min) for 272 complete separation of the four analytes and a reduction of the column washing time (from 0.8 min to 0.6 273 min).[13, 22] The 3-min run time was much shorter than those in many published methods (5.52 to 6.6 min) for 274 the analysis of MTX and its metabolites,[2, 15, 24] and close to two studies (3.0 and 3.6 min) for MTX 275 analysis.[18, 20]

276 The recovery and matrix factor of analytes were comparable to the observations in published studies in 277 various biological fluids including human urine, plasma, serum, and cerebrospinal fluid (recovery: 72% to 278 126% for MTX, 67% to 122% for 7-OH MTX, and 54.4% to 105.1% for DAMPA; matrix factor: 70.5% to 279 118% for MTX, 90% to 105% for 7-OH MTX, and 101% to107% for DAMPA).[2, 3, 15-18, 23, 24, 26] The possible

280 reasons for the matrix induced response enhancement for both 7-OH MTX (117.65% to 124.70%) and 7-OH
281 DAMPA (156.57% to 172.93%), and the big inter-individual variance of 7-OH DAMPA matrix factor (CV
282 < 20.68%) were summarized as follows: (1) the one-step protein precipitation for sample extraction retained
283 many matrix in the post-extracted samples;[29, 30] (2) the fast separation procedure of the HPLC method could
284 not efficiently separate all of the matrix from the analytes;[30, 31] (3) MTX-D_3 and DAMPA-D_3 were used for
285 the quantitation of 7-OH MTX and 7-OH DAMPA, respectively. However, the chemical structures and
286 retention times were different between the two internal standards and the two analytes, therefore their matrix
287 effects could not be well compensated.[30] The matrix effects of both 7-OH MTX and 7-OH DAMPA might
288 be well compensated by using an efficient sample purification technology, such as solid-phase extraction, an
289 efficient separation procedure, and/or the isotope internal standards.[29-31] All analytes were stable in urine and
290 post-extracted urine matrix under tested conditions, which was consistent with the results in previous
291 studies.[2, 3, 15-17, 21, 23, 24]

292 *Method application*

293 Before multiple regression, plasma drug levels were separated into three groups according to their
294 sampling time points, which was restricted within ± 2 h. Moreover, the difference of the sampling time
295 between urine and blood was also restricted (within ± 0.5 h, ± 1 h and ± 2 h), and within ± 1 h was the best
296 to reduce the variance and to ensure the normal distribution of the data. The multiple-regression results
297 indicated that the plasma drug levels increased with the urine drug levels, age, serum creatinine and alanine
298 transaminase. MTX was mainly excreted in the urine,[1] which could explain the correlation between
299 increased plasma MTX levels and elevated urine MTX levels. Interestingly, the urine 7-OH MTX levels
300 increased with the plasma 7-OH MTX levels at 37 hours after dosing, although urine was the minor route for
301 7-OH MTX excretion.[32, 33] Plasma MTX levels increased with serum creatinine at 13 hours after dosing,
302 which was caused by the reduced urine drug excretion due to impaired renal function.[11] Moreover, the renal

303 function decreased with age, which could explain that the plasma MTX levels increased with age at 13 hours
304 after dosing. MTX and 7-OH MTX plasma levels increased with alanine transaminase, which could be
305 elucidated by the following reasons: both MTX and 7-OH MTX were transformed into their polyglutamates
306 mainly via folypolyglutamate synthase in the liver;[34, 35] these polyglutamates could not be transported out of
307 the cells when the number of their glutamate residues was greater than three, which resulted in significant
308 accumulation of these polyglutamates in the liver;[35, 36] in the case of liver injury, these polyglutamates were
309 released into the blood with the death of hepatocyte and transformed back into MTX and 7-OH MTX via
310 blood gamma-glutamyl hydrolase.[22, 37] In the present study, due to a poor correlation between the urine and
311 blood drug levels, urine could not replace blood for MTX monitoring in patients receiving high-dose MTX
312 therapy.

313 Alkalinization was routinely performed for patients receiving high-dose MTX therapy to enhance the
314 renal excretion of MTX and to reduce toxicity risk.[8, 9] In the present study, dose-normalized plasma and
315 urine levels of both MTX and 7-OH MTX varied greatly between individuals (Appendix 4), which could be
316 explained by the following reasons: the small sample size; the different MTX dose, sampling time, urine
317 volume, patients' physiological status and pharmacokinetic parameters of analytes between individuals.[11]
318 The dose-normalized plasma and urine MTX and 7-OH MTX levels were similar between patients with
319 different urine pH (< 7 or ≥ 7), which might be explained by the similar alkalinization treatment of enrolled
320 patients. Dose-normalized 7-OH MTX plasma level was higher in patients with the urine pH ≥ 7 compared
321 to those with the urine pH < 7 at 62 hours after dosing. This unexpected result might be also explained by
322 the reasons given for the big variance of the drug levels between individuals.

323 *Deficiencies of the study*

324 (1) The results in the present study should be treated with caution due to the great inter-individual
325 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.

Conclusion

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 (recovery 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 (R^2, 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.

Acknowledgments

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References

1. Hospira. Label for Methotrexate injection. Available at: http://www.accessdata.fda.gov/drugsatfda_docs/label/2011/011719s117lbl.pdf2011.

350 2. R. C. Schofield, L. V. Ramanathan, K. Murata, M. Grace, M. Fleisher, M. S. Pessin, and D. C. Carlow,
351 *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **2015**, *1002*, 169.
352 3. M. Uchiyama, T. Matsumoto, T. Matsumoto, S. Jimi, Y. Takamatsu, K. Tamura, and S. Hara, *Biomed.*
353 *Chromatogr.*, **2012**, *26*, 76.
354 4. F. Berga, P. Luna, C. Martorell, J. Rey, I. Gomila, S. Gimenez, A. Costa-Bauza, M. A. Elorza, I.
355 Sanchez, F. Grases, and B. Barcelo, *Clin. Chim. Acta*, **2018**, *487*, 1.
356 5. E. Klapkova, J. Kukacka, K. Kotaska, I. Suchanska, R. Urinovska, and R. Prusa, *Clin. Lab.*, **2011**, *57*,
357 599.
358 6. S. A. Jacobs, R. G. Stoller, B. A. Chabner, and D. G. Johns, *J. Clin. Invest.*, **1976**, *57*, 534.
359 7. L. B. Ramsey, F. M. Balis, M. M. O'Brien, K. Schmiegelow, J. L. Pauley, A. Bleyer, B. C. Widemann,
360 D. Askenazi, S. Bergeron, A. Shirali, S. Schwartz, A. A. Vinks, and J. Heldrup, *Oncologist*, **2018**, *23*, 52.
361 8. T. E. Sand, and S. Jacobsen, *Eur. J. Clin. Pharmacol.*, **1981**, *19*, 453.
362 9. O. Mir, S. Ropert, A. Babinet, J. Alexandre, F. Larousserie, J. P. Durand, E. Enkaoua, P. Anract, and F.
363 Goldwasser, *Cancer Chemother. Pharmacol.*, **2010**, *66*, 1059.
364 10. S. A. Drost, J. R. Wentzell, P. Giguere, D. L. McLurg, M. Sabloff, S. Kanji, and T. T. Nguyen,
365 *Pharmacotherapy*, **2017**, *37*, 684.
366 11. S. Mei, X. Li, X. Jiang, K. Yu, S. Lin, and Z. Zhao, *J. Pharm. Sci.*, **2018**, *107*, 1454.
367 12. J. B. Oudart, B. Marquet, C. Feliu, C. Gozalo, Z. Djerada, and H. Millart, *Ann. Biol. Clin. (Paris)*, **2016**,
368 *74*, 333.
369 13. S. Mei, L. Zhu, X. Li, J. Wang, X. Jiang, H. Chen, J. Huo, L. Yang, S. Lin, and Z. Zhao, *Anal. Sci.*,
370 **2017**, *33*, 665.
371 14. R. C. Schofield, L. V. Ramanathan, K. Murata, M. Fleisher, M. S. Pessin, and D. C. Carlow, *Methods*
372 *Mol. Biol.*, **2016**, *1383*, 213.
373 15. M. S. Roberts, N. S. Selvo, J. K. Roberts, V. M. Daryani, T. S. Owens, K. E. Harstead, A. Gajjar, and C.
374 F. Stewart, *J Liq Chromatogr Relat Technol*, **2016**, *39*, 745.
375 16. M. A. Al-Ghobashy, S. A. Hassan, D. H. Abdelaziz, N. M. Elhosseiny, N. A. Sabry, A. S. Attia, and M.
376 H. El-Sayed, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **2016**, *1038*, 88.
377 17. D. Wu, Y. Wang, Y. Sun, N. Ouyang, and J. Qian, *Biomed. Chromatogr.*, **2015**, *29*, 1197.
378 18. C. C. Christianson, C. J. Johnson, and S. R. Needham, *Bioanalysis*, **2013**, *5*, 1387.
379 19. R. Bouquié, G. Deslandes, B. N. Bernáldez, C. Renaud, E. Dailly, and P. Jolliet, *Analytical Methods*,
380 **2013**, *6*, 178.
381 20. E. den Boer, S. G. Heil, B. D. van Zelst, R. J. Meesters, B. C. Koch, M. L. Te Winkel, M. M. van den
382 Heuvel-Eibrink, T. M. Luider, and R. de Jonge, *Ther. Drug Monit.*, **2012**, *34*, 432.
383 21. H. L. Cheng, S. S. Chiou, Y. M. Liao, C. Y. Lu, Y. L. Chen, and S. M. Wu, *Anal. Bioanal. Chem.*, **2010**,
384 *398*, 2183.
385 22. S. Mei, X. Shi, Y. Du, Y. Cui, C. Zeng, X. Ren, K. Yu, Z. Zhao, and S. Lin, *J. Pharm. Biomed. Anal.*,
386 **2018**, *158*, 300.
387 23. A. Barbieri, L. Sabatini, P. Indiveri, R. Bonfiglioli, V. Lodi, and F. S. Violante, *Rapid Commun. Mass*
388 *Spectrom.*, **2006**, *20*, 1889.
389 24. J. Bluett, I. Riba-Garcia, K. Hollywood, S. M. Verstappen, A. Barton, and R. D. Unwin, *Analyst*, **2015**,
390 *140*, 1981.
391 25. G. Fabrizi, M. Fioretti, and L. Mainero Rocca, *Biomed. Chromatogr.*, **2016**, *30*, 1297.
392 26. P. Koufopantelis, S. Georgakakou, M. Kazanis, C. Giaginis, A. Margeli, S. Papargiri, and I. Panderi, *J.*
393 *Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **2009**, *877*, 3850.
394 27. EMEA. Committee for Medicinal Products for Human Use, Guideline on Bioanalytical Method
395 Validation. EMEA/CHMP/EWP/192217/2009 Rev. 1 Corr. 2** (21July2011). Available at:

396 http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf2
397 011.
398 28. FDA. Guidance for industry: bioanalytical method validation. Available at:
399 https://www.fda.gov/media/70858/download2018.
400 29. H. Jiang, H. Cao, Y. Zhang, and D. M. Fast, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **2012**,
401 *891-892*, 71.
402 30. N. R. Srinivas, *Biomed. Chromatogr.*, **2011**, *25*, 740.
403 31. L. Hu, J. E. Agbokponto, L. Ding, B. Liu, F. Shi, and C. Gong, *Biomed. Chromatogr.*, **2015**, *29*, 53.
404 32. B. Winograd, R. J. Lippens, M. J. Oosterbaan, M. J. Dirks, T. B. Vree, and E. van der Kleijn, *Eur. J.*
405 *Clin. Pharmacol.*, **1986**, *30*, 231.
406 33. P. Bore, A. Iliadis, J. Catalin, S. Just, and J. P. Cano, *Cancer Drug Deliv.*, **1987**, *4*, 177.
407 34. T. S. Mikkelsen, C. F. Thorn, J. J. Yang, C. M. Ulrich, D. French, G. Zaza, H. M. Dunnenberger, S.
408 Marsh, H. L. McLeod, K. Giacomini, M. L. Becker, R. Gaedigk, J. S. Leeder, L. Kager, M. V. Relling, W.
409 Evans, T. E. Klein, and R. B. Altman, *Pharmacogenet. Genomics*, **2011**, *21*, 679.
410 35. A. K. Fotoohi, and F. Albertioni, *Leuk. Lymphoma*, **2008**, *49*, 410.
411 36. D. French, W. Yang, C. Cheng, S. C. Raimondi, C. G. Mullighan, J. R. Downing, W. E. Evans, C. H.
412 Pui, and M. V. Relling, *Blood*, **2009**, *113*, 4512.
413 37. S. Inoue, M. Hashiguchi, S. Kawai, and M. Mochizuki, *Yakugaku Zasshi*, **2009**, *129*, 1001.
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416 **List of Supplemental Digital Content**

417 Supplemental Digital Content 1: Appendix 1-4.docx.

418 Appendix 1. Mass parameters for analytes.

419 Appendix 2. The stability of MTX, 7-OH MTX, DAMPA, and 7-OH DAMPA in human urine.

420 Appendix 3. Clinical characteristics, plasma and urine concentrations of MTX and 7-OH MTX in patients

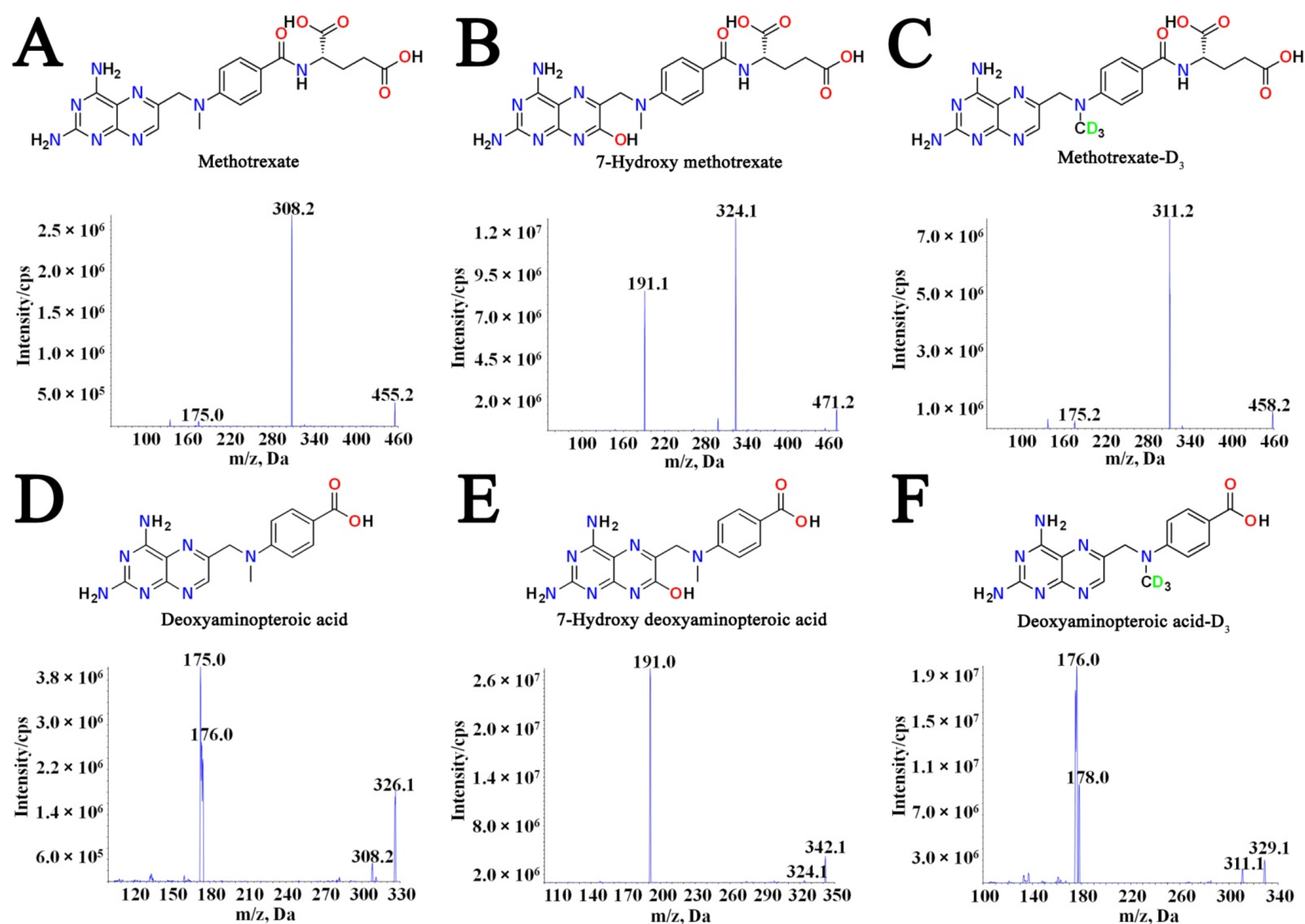
421 with primary central nervous system lymphoma receiving high-dose MTX therapy (average ± standard

422 deviation, n = 171).

423 Appendix 4. The influence of urine pH on dose-normalized plasma and urine concentrations of MTX and 7-

424 OH MTX in patients with primary central nervous system lymphoma receiving high-dose MTX therapy.

425 **Figures**



426

427 Fig. 1 Chemical structure and mass spectrometry of analytes and internal standards. (A) methotrexate
428 (about 10 ng/mL); (B) 7-hydroxy methotrexate (about 50 ng/mL); (C) methotrexate-D_3 (about 10 ng/mL);
429 (D) deoxyaminopteroic acid (about 10 ng/mL); (E) 7-hydroxy deoxyaminopteroic acid (about 50 ng/mL); (F)
430 deoxyaminopteroic acid-D_3 (about 10 ng/mL).

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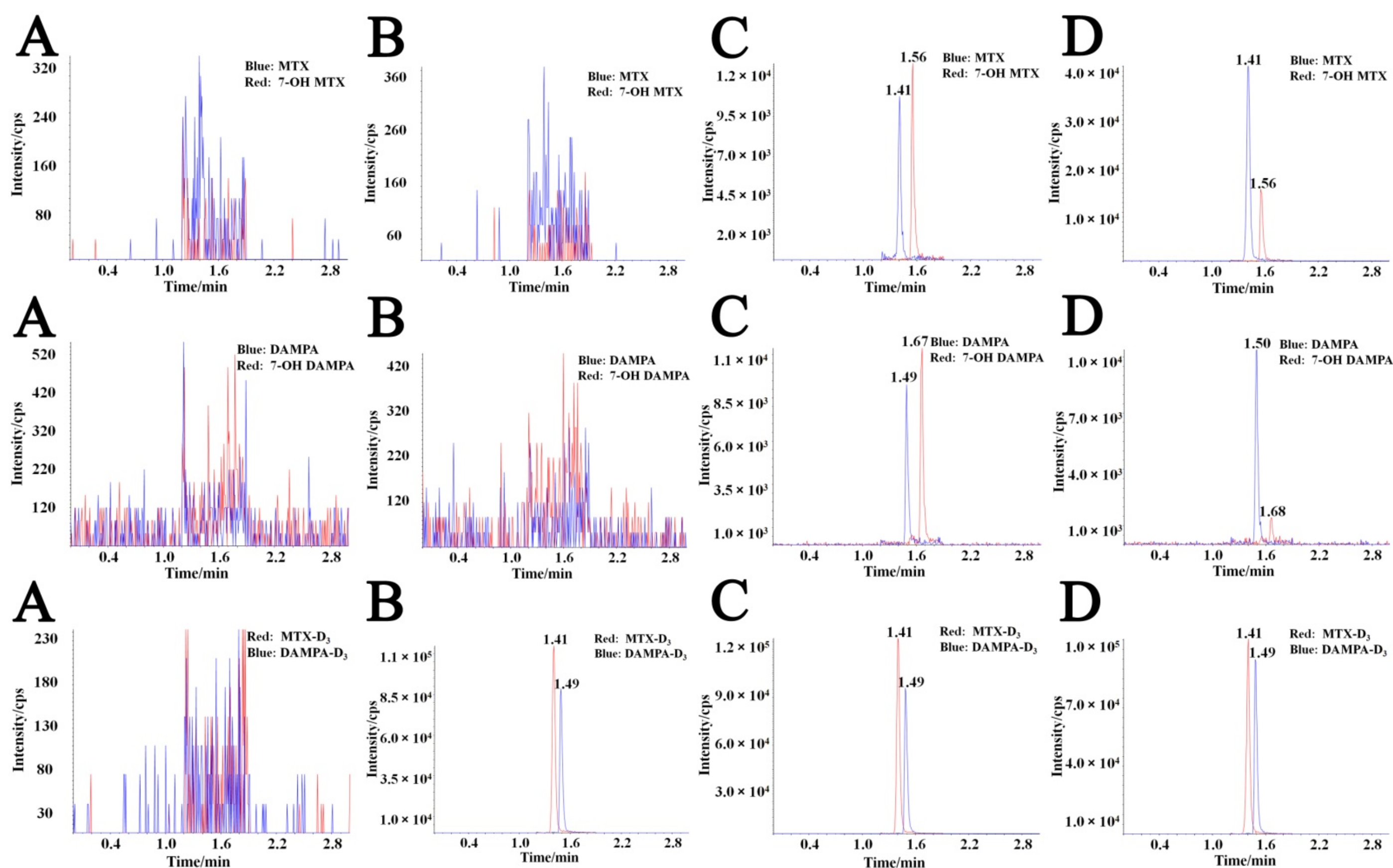

Fig. 2 Typical spectrum of methotrexate (MTX), 7-hydroxy methotrexate (7-OH MTX), deoxyaminopteroic acid (DAMPA) 7-hydroxy deoxyaminopteroic acid (7-OH DAMPA), and internal standard methotrexate-D_3 (MTX-D_3) and deoxyaminopteroic acid-D_3 (DAMPA-D_3) obtained from: (A) blank plasma; (B) blank plasma only spiked with internal standard; (C) lower limit of quantitation (0.02 μmol/L for MTX and DAMPA, and 0.1 μmol/L for 7-OH MTX and 7-OH DAMPA); (D) sample from a patient.

439 Tables

440 Table 1 Precision and accuracy for the determination of MTX, 7-OH MTX, DAMPA, and 7-OH DAMPA in human urine

Measurement	Analyte	Nominal concentration/µM	Bias, %	Coefficient of variation, %
Intra-day, n = 5	MTX	0.02	–1.30	3.16
		0.06	6.67	1.65
		0.12	1.67	2.96
		1.8	0.74	1.77
		3	–0.22	1.07
	7-OH MTX	0.1	–4.02	3.22
		0.3	–1.33	6.98
		0.6	3.83	3.29
		9	3.33	5.17
		15	0.89	2.75
	DAMPA	0.02	–8.00	9.04
		0.06	7.61	1.85
		0.12	0.28	0.48
		1.8	5.37	0.81
		3	1.67	4.19
	7-OH DAMPA	0.1	–5.10	8.92
		0.3	0.78	7.65
		0.6	–1.28	2.58
		9	2.15	1.21
		15	–0.89	3.70
Inter-day, n = 20	MTX	0.02	–0.46	4.32
		0.06	6.81	4.73
		0.12	5.19	3.36
		1.8	2.13	2.33

	3	–0.99	1.97
7-OH MTX	0.1	0.20	6.21
	0.3	2.59	5.14
	0.6	4.47	5.03
	9	0.95	4.31
	15	–1.67	3.67
DAMPA	0.02	–0.06	4.91
	0.06	4.38	3.35
	0.12	4.24	2.96
	1.8	2.58	3.02
	3	–0.34	2.06
7-OH DAMPA	0.1	–1.86	5.31
	0.3	0.98	4.63
	0.6	3.76	4.17
	9	2.07	3.77
	15	–1.20	3.42

441 Abbreviations: MTX, methotrexate; 7-OH MTX, 7-hydroxy methotrexate; DAMPA, deoxyaminopteroic acid; 7-OH DAMPA, 7-hydroxy deoxyaminopteroic
442 acid.

443 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/ μM	Recoveries of analytes, %	Recoveries of IS, %	IS normalized 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	0.06	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
	0.12	103.1 ± 6.2	100.6 ± 3.9	102.6 ± 6.8	6.68	101.4 ± 5.8	103.5 ± 7.0	98.1 ± 2.9	2.99
	1.8	101.3 ± 4.1	97.2 ± 2.4	104.3 ± 5.1	4.93	94.6 ± 7.6	97.0 ± 7.4	97.6 ± 3.2	3.32
	3	101.6 ± 5.8	97.1 ± 5.2	104.7 ± 4.5	4.28	105.3 ± 6.0	105.6 ± 6.1	99.7 ± 1.8	1.76
7-OH MTX	0.3	99.2 ± 7.6	94.5 ± 2.5	105.0 ± 7.3	6.94	119.5 ± 6.1	97.7 ± 6.8	123.0 ± 13.0	10.55
	0.6	96.5 ± 9.1	100.6 ± 3.9	96.0 ± 10.3	10.70	128.3 ± 5.8	103.5 ± 7.0	124.7 ± 11.9	9.53
	9	101.4 ± 9.8	97.2 ± 2.4	104.3 ± 10.3	9.87	113.5 ± 4.7	97.0 ± 7.4	117.8 ± 12.1	10.24
	15	100.5 ± 5.2	97.1 ± 5.2	103.7 ± 5.0	4.79	123.8 ± 6.2	105.6 ± 6.1	117.6 ± 9.7	8.28
DAMPA	0.06	102.4 ± 4.2	93.5 ± 2.8	109.5 ± 4.7	4.29	71.7 ± 10.5	68.7 ± 11.6	104.8 ± 4.6	4.43
	0.12	103.0 ± 4.5	99.3 ± 3.1	103.8 ± 5.2	5.04	71.9 ± 10.6	73.3 ± 12.7	98.6 ± 4.3	4.37
	1.8	100.6 ± 5.2	98.7 ± 3.0	102.0 ± 6.1	5.96	69.1 ± 11.9	68.6 ± 12.1	101.0 ± 3.6	3.59
	3	99.6 ± 6.9	95.0 ± 7.2	105.0 ± 4.7	4.51	77.4 ± 10.6	76.3 ± 10.4	101.5 ± 1.6	1.56
7-OH DAMPA	0.3	98.1 ± 7.4	93.5 ± 2.8	104.8 ± 7.5	7.19	114.5 ± 5.5	68.7 ± 11.6	171.4 ± 33.0	19.25
	0.6	91.5 ± 9.2	99.3 ± 3.1	92.3 ± 10.4	11.28	123.0 ± 11.3	73.3 ± 12.7	172.9 ± 35.8	20.68
	9	101.1 ± 5.2	98.7 ± 3.0	102.5 ± 5.6	5.46	104.4 ± 3.1	68.6 ± 12.1	156.6 ± 28.3	18.07
	15	97.9 ± 5.6	95.0 ± 7.2	103.3 ± 4.2	4.04	117.7 ± 4.2	76.3 ± 10.4	157.5 ± 26.6	16.86

444 Abbreviations: MTX, methotrexate; 7-OH MTX, 7-hydroxy methotrexate; DAMPA, deoxyaminopteroic acid; 7-OH DAMPA, 7-hydroxy deoxyaminopteroic
445 acid; IS, internal standard (MTX-D_3 for MTX and 7-OH MTX, DAMPA-D_3 for DAMPA and 7-OH DAMPA); CV, coefficient of variation.
446

447 Table 3 Multiple linear regression results between logarithmic transformed plasma drug levels and covariates (the blood sampling time was restricted within ± 448 2 h, and the difference of sampling time between blood and urine was restricted within ± 1 h)

Time after dose/h	logarithmic transformed plasma drug levels	Normality of logarithmic transformed plasma drug levels	Goodness of fit, R^2	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	< 0.001	Age (0.47)	< 0.001	1.55	0.61
					SCR (0.38)	0.001		
					ALT (0.30)	0.009		
	7-OH MTX	0.049	0.16	0.005	ALT (0.40)	0.005	1.52	0.0080
37	MTX	0.55	0.22	0.017	MTXU (0.35)	0.031	2.02	0.99
					ALT (0.32)	0.047		
	7-OH MTX	0.79	0.34	0.001	7-OH MTXU (0.46)	0.003	1.24	0.66
					ALT (0.32)	0.031		
61	MTX	0.11	0.34	0.004	MTXU (0.59)	0.004	1.38	0.25
	7-OH MTX	0.18	0.21	0.031	ALT (0.46)	0.031	2.13	0.40

449 Abbreviations: MTX, methotrexate; 7-OH MTX, 7-hydroxy methotrexate; SCR, serum creatinine; ALT, alanine transaminase; MTXU, logarithmic transformed 450 urine methotrexate levels; 7-OH MTXU, logarithmic transformed urine 7-hydroxy methotrexate levels.

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