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Determination of erythrocyte methotrexate polyglutamates by liquid chromatography/tandem mass spectrometry after low-dose methotrexate therapy in Chinese patients with rheumatoid arthritis

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ABSTRACT

Methotrexate (MTX) is currently one of the most widely used drugs for treatment of rheumatoid arthritis (RA) through polyglutamation of methotrexate polyglutamates (MTXPGs), a process attaching sequential γ-linked glutamic residues to MTX. A new and sensitive LC/MS/MS method was developed and validated for determination of whole-blood MTX and total MTX (MTX + MTXPGs), and then concentration of MTXPGs was calculated. To determine whole-blood MTX, whole blood was precipitated with 50% trifluoroacetic acid, and extraction was performed using ethyl acetoacetate. Analytes were subjected to LC/MS/MS analysis using positive electrospray ionization. To determine whole-blood total MTX, whole blood was incubated with ascorbic acid (200 mM) at 37 °C for 3 h to enzymatically convert the MTXPGs to MTX, and then processed with the same method mentioned above. Recoveries of spiked MTX at ppb (ng/mL) level were between 26.2% and 37.8% with intra- and inter-day precision less than 15.8% and 11.8%, respectively. The lower limit of detection (LLOD) and lower limit of quantitation (LLOQ) were 0.5 ng/mL and 1 ng/mL, respectively. The sensitive LC/MS/MS method was fully validated with high selectivity and acceptable accuracy and precision, which was successfully applied to determine the erythrocyte methotrexate polyglutamates in patients with RA.

1. Introduction

Methotrexate (MTX), a folate antagonist, is the most commonly used antirheumatic drug for the treatment of rheumatoid arthritis (RA). It is characterized as a disease-modifying agent which relieves joint inflammation and pain, slows disease progression, and prevents disability by delaying joint destruction [1–3]. However, its clinical response varied among patients with only 50–60% of the effectiveness in managing RA [4]. Furthermore, a large portion of patients experienced MTX related side effects including gastrointestinal disturbances, increased liver enzymes, alopecia, bone marrow suppression, and potentially life-threatening pneumonitis and cirrhosis [5,6]. Low-dose MTX is now the first-line therapy for the treatment of RA, which is generally given orally to RA patients at a single weekly dose (American College of Rheumatology Subcommittee on Rheumatoid Arthritis Guidelines 2002). Since 95% of a given MTX dose is metabolized within 24 h after administration and there is a lack of correlation between dose of MTX and disease activity, determination of MTX plasma concentration following low dose oral administration is of little significance. It has been reported that MTX is intracellularly converted to methotrexate polyglutamates (MTXPGs) by folylpolyglutamate synthase after its entry into cells [7,8]. The γ-linked sequential addition of glutamic acid residues selectively modifies the properties of MTX and enhances the intracellular retention of MTX. The intracellular level of MTXPGs is suggested to be associated with the efficacy and toxicity of MTX in the treatment of RA, psoriasis, ankylosing spondylitis and cancer [9–11]. Therefore, monitoring MTXPGs level is more significant in evaluating the pharmacokinetic–pharmacodynamic profile of MTX therapy [12,13]. Diverse analytical methods have been reported for determination of MTXPGs in erythrocyte. A radiochemical ligand-binding assay was first reported by Kamen and Winick [14], which determined MTXPGs level by measuring the reduction in absorbance when NADPH was converted to $NADP^+$ [15]. HPLC

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fluorescence methods with post-column photo or electrochemical oxidation were also developed for individual quantification of MTXPGs [16,17]. Recently, an LC/MS/MS method has been reported to determine the MTXPGs fingerprint in human erythrocyte with protein precipitation followed by solid-phase extraction on-line [18]. However, these assay methods have some defaults including high cost, time-consuming, low specificity and exhibiting interferences in some patients, which limit their widespread use in clinical application. The aim of this study is to develop a sensitive and simple LC/MS/MS method to quantify the total MTXPGs in erythrocyte using liquid–liquid extraction from a small size of whole-blood sample.

2. Experimental

2.1. Materials

4-Amino-10-methylpteroylglutamic acid ($MTXPG_1$), 4-amino-10-methylpteroyldiglutamic acid ($MTXPG_2$), 4-amino-10-methylpteroyltriglutamic acid ($MTXPG_3$), 4-amino-10-methylpteroyltetraglutamic acid ($MTXPG_4$), 4-amino-10-methylpteroylpentaglutamic acid ($MTXPG_5$), 4-amino-10-methylpteroylhexaglutamic acid ($MTXPG_6$), and 4-amino-10-methylpteroylheptaglutamic acid ($MTXPG_7$) were purchased from Schircks Laboratories (Switzerland). MTXPGs were ammonium salts. Doxofylline injection (internal standard, IS) was purchased from Hansheng pharmaceutical limited company of ZheJiang province of China. HPLC-grade acetonitrile was purchased from Fisher Chemicals and ultra-pure water was obtained from a Milli Q-plus system (Billerica, MA, USA). Formic acid, ammonium formate and trifluoroacetic acid, HPLC-grade, were purchased from Sigma (USA). Ethyl acetoacetate and other reagents were of analytical grade. Blank human whole blood was obtained from the Guangzhou Blood Donor Service (Guangzhou, China).

2.2. Standard solution and calibration curve

(1) The stock solutions of MTX and equimolar mixed-$MTXPG_{2-7}$ were prepared by dissolving the accurately weighed reference compounds in acetonitrile/water (50:50, v/v) to give the final concentrations of 84 μg/mL and 1 μg/mL. The concentration of stock solution of doxofylline injection was 10 mg/mL which was also diluted with acetonitrile/water (50:50, v/v) to 4 μg/mL and 1 μg/mL according to different methods. All the solutions were stored at −80 °C and brought to room temperature before use. (2) The stock solution of MTX (84 μg/mL) was then serially diluted with acetonitrile/water (50:50, v/v) to obtain the calibration working standard solutions at concentrations of 20, 40, 100, 200, 500, 1000, 2000 ng/mL. The working standards were analyzed with LC/MS/MS and a linear regression calibration equation was generated. (3) Matrix-matched calibration curve was prepared by spiking blank human whole blood with MTX standard solutions to give concentrations of 1, 2, 5, 10, 25, 50, 100 ng/mL. (4) Quality control (QC) samples were prepared by spiking control human whole blood with independently prepared MTX standard solutions to give concentrations of 2, 10, and 50 ng/mL for MTX.

2.3. Sample preparation procedure for whole-blood MTX

20 μL of the internal standard working solution (1 μg/mL) was added to 400 μL human whole blood (after a freeze–thaw cycle) and vortexed for 30 s. Then, 400 μL trifluoroacetic acid was added and the mixture was vortexed for 30 s and centrifuged at 20,000 × *g* for 3 min. 700 μL of the supernatant was transferred to another clean tube, and 3 mL ethyl acetoacetate was then added. The mixture was vortexed for 1 min and centrifuged at 20,000 × *g* for 5 min. And then 3 mL of the organic phase was pipetted into another 5-mL tube and evaporated to dryness. The residues were dissolved in 100 μL mobile phase and an aliquot (8 μL) of the reconstituent was injected into the LC/MS/MS for analysis.

2.4. Sample preparation procedure for whole-blood total MTX (MTX + MTXPGs)

100 μL human whole blood (after a freeze–thaw cycle) was mixed with 100 μL ascorbic acid (200 mM), vortexed, and incubated at 37 °C for 3 h to enzymatically convert the MTXPGs to MTX. After the incubation, 5 μL of the internal standard working solution (4 μg/mL) was added followed by vortex for 30 s. Then the sample was added with 100 μL trifluoroacetic acid for precipitation. After vortex and centrifugation, 260 μL of the supernatant was transferred to another clean eppendorf tube, and 1.5 mL ethyl acetoacetate was added. The mixture was vortexed for 1 min and centrifuged at 20,000 × *g* for 5 min. 1460 μL of the organic phase was pipetted into another 1.5-mL tube and evaporated to dryness. The residues were dissolved in 50 μL mobile phase and an aliquot (8 μL) of the reconstituent was injected into the LC/MS/MS for analysis.

2.5. Liquid chromatographic and mass spectrometric conditions

An Agilent 1200 HPLC system (Agilent Technologies, USA) was used for solvent and sample delivery. Chromatographic separation was achieved by using a XB-C18 column (XB-C_{18}, 3 μm, 2.1 mm × 100 mm, Welch Materials Inc., USA) with a filter (SS, 0.5 μm) at room temperature. The mobile phase consisted of acetonitrile (1% formic acid)–20 mM ammonium formate solution (30:70, v/v), and pumped at a flow rate of 0.2 mL/min. The injection volume was 8 μL. The total run time was 3 min for each sample.

An Agilent 6410 Triple Quad mass spectrometer (Agilent Technologies, USA) equipped with an ESI source was used for mass analysis and detection. Mass spectrometric analysis was performed in the positive ion mode (ESI+) and set up in the multiple reaction monitoring (MRM) mode. The column effluent was monitored at the following transitions: 455.2 → 308.2 (*m*/*z*) for MTX and 267.2 → 181.2 (*m*/*z*) for doxofylline with a dwell time of 200 ms and 220 ms for each channel, respectively. Nitrogen was used as the nebulizer gas, collision gas and drying gas. The drying gas was set at 310.3 kPa with gas temperature, 350 °C and gas flow rate, 8 L/min. The Capillary voltage was set at 4000 V. The collision energies were 16 and 20 V for MTX and doxofylline, respectively. The fragmentor voltages were 160 and 120 V for MTX and doxofylline, respectively. The electron multiplier voltages for MTX and doxofylline were 400 V. The instrument was controlled by the Masshunter workstation (Agilent Technologies, USA).

2.6. Method validation

The method was validated for selectivity, accuracy, precision, recovery, calibration curve range, stability and matrix effect according to FDA guidelines. The $MTXPG_{2-7}$ mixed equimolarly were also analyzed to validate the selectivity of MTX which might not be interfered by MTXPGs. The absolute and relative matrix effects (MEs) on the spectral response of MTX and IS were assessed as described by Matuszewski et al. [19] with slight modifications. Each analytical run of routine analysis included a blank whole blood, a blank whole blood spiked with IS, a set of calibration samples, a set of QC samples and unknowns.

In order to verify the efficiency of the enzymatic conversion of MTXPGs to MTX, blank whole-blood samples were spiked with mixtures of MTXPGs or MTX and analyzed following the sample

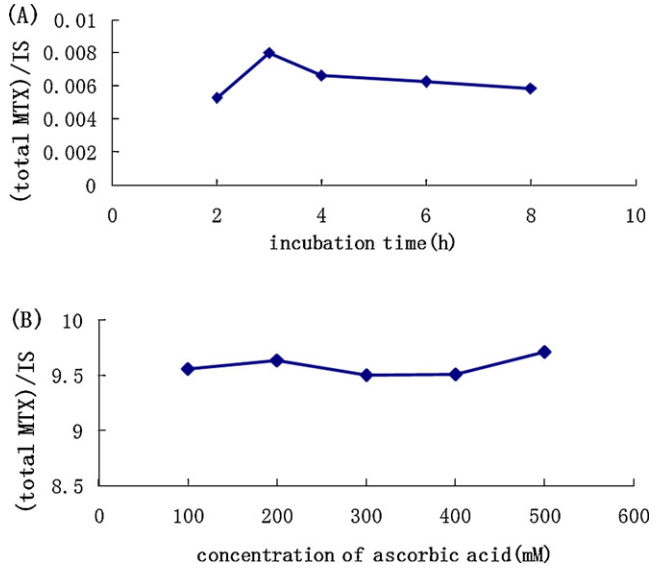

Fig. 1. The effect of incubation time (A) and concentration of ascorbic acid (B) on the whole-blood total MTX (MTX + MTXPGs) content determined by the LC/MS/MS method described.

preparation procedure for whole-blood total MTX (MTX + MTXPGs) described above.

3. Results and discussion

3.1. Sample preparation procedure

There are many methods for extracting MTX from plasma, whole blood, urine and other matrixes, most of which employ solid phase extraction (SPE) to increase efficiency. However, high cost and low reproducibility are the main drawbacks for SPE method. Liquid–liquid extraction is also performed but usually requires a big sample size for better sensitivity. In the current study, we used trifluoroacetic acid and ethyl acetoacetate to increase efficiency for extracting MTX, which only required a small sample volume. It is possible that trifluoroacetic acid provides a proper pH condition in which MTX can be easily extracted by ethyl acetoacetate. Previous study showed that amphoteric substance containing heterocyclic nitrogen was extracted by trifluoroacetic acid more easily.

In whole blood, a portion of the MTX enters erythrocyte and converts to MTXPGs by folylpolyglutamate synthase. In order to determine whole-blood total MTX (MTX + MTXPGs), erythrocyte were destroyed by a freeze–thaw cycle, and MTXPGs were released into the plasma. The endogenous polyglutamate hydrolase in the plasma catalyzed the conversion of MTXPGs to MTX [20,21]. The acidic condition created by adding ascorbic acid accelerated this conversion. The similar procedure was also used to determine the whole-blood total 5-MTHF and MTX by converting 5-methyltetrahydrofolate (5-MTHF) polyglutamates to 5-MTHF [22] and MTXPGs to MTX [17], respectively. We also replaced ascorbic acid with mercaptoethanol, which was mentioned in the method developed by Dervieux [16], but with lower recovery and longer incubation time. In order to identify the appropriate length of time for enzymatic incubation, blood samples from patients with treatment for more than three months were collected, and incubated for different length of time at 37 °C with diverse concentrations of ascorbic acid (Fig. 1). The last data point in Fig. 1(B) was abnormally higher than others possibly due to degradation of the internal standard (IS). The incubation time of 3 h and ascorbic acid of 200 mM were selected based on the optimal results.

The application of an internal standard is considered to be advantageous for analytical methods especially with liquid–liquid extraction, off-line solid-phase extraction or derivatization. However, most published methods for quantification of MTX and its metabolites did not employ the usage of internal standard. Among the methods with an internal standard, theophylline, 8-chlorotheophylline, aminopterin or OHM, the metabolite of MTX was used [23–26]. Ideally, the isotopically labeled internal standard is preferred in multistep sample preparation procedure. However, d3-MTX is extremely expensive. Theophylline was originally employed as IS in our study, but had interference when eluted. Arilin had a proper retention time, high specificity, and no interference, but was unstable as the concentration of ascorbic acid increased. Finally, doxofylline was demonstrated to be an ideal IS with high specificity, a proper retention time close to the one for MTX, and without degradation at around 200 mM of ascorbic acid. It was still stable when ascorbic acid was increased to 400 mM.

3.2. The LC/MS/MS analysis for MTX

ESI was chosen as the ionization source for the analysis of the target compound; the signal intensity for MTX in whole blood was high when using the ESI source, and the regression curves were linear. MTX and doxofylline formed predominant protonated molecules $[M+H]^+$ at m/z 455.2 and 267.2 in the mass spectra, respectively. Fragmentation of these ions using collision induced dissociation resulted in strong product ions at m/z 308.2 for MTX and 181.2 for doxofylline with collision energies ramped from 8 to 30 eV. These product ions were selected to obtain the maximum sensitivity under multiple reaction monitoring (MRM) spectra (Fig. 2).

The mobile phase was optimized using different proportions of acetonitrile and water to achieve good sensitivity, efficiency and peak shape. An acidic modifier (formic acid) was added in the mobile phase to improve peak response, while ammonium formate was added in water to obtain better peak shape. Thus, a mobile phase consisted of acetonitrile (1% formic acid)/20 mM ammonium formate was chosen with the ratio of 30:70 (v/v). Each chromatographic run was completed within 3 min.

3.3. Method validation

3.3.1. Selectivity

Selectivity was assessed by comparing the chromatograms of five different batches of blank human whole blood and incubated whole blood with samples at LLOQ levels. No endogenous peak were observed at the retention time of the analytes from any of the batches evaluated, indicating no significant interference in the MRM channel for the analytes at the expected retention time. Representative chromatograms of blank human whole blood, the whole blood with MTX at LLOQ levels, whole blood sample from patient, and the whole blood with $MTXPG_{2-7}$ mixed equimolarly were shown in Fig. 3(A2–D2). The selectivity was also validated for whole blood after incubation, with the chromatograms shown in Fig. 3(A1–D1). The retention times for MTX and IS were 1.69 and 2.10 min, respectively.

3.3.2. Matrix effects (ME)

The ME occurred when a biological sample contained a component that did not yield a signal in the MRM channel used for the target analytes but co-eluted with the analytes and affected the spectral response of the analytes [27]. The presence of the ME could decrease or increase the response of the analytes and thus affect the sensitivity of a developed method. The data for ME at QC concentrations of MTX and working concentration of IS from five different batches of human whole blood and incubated whole blood were

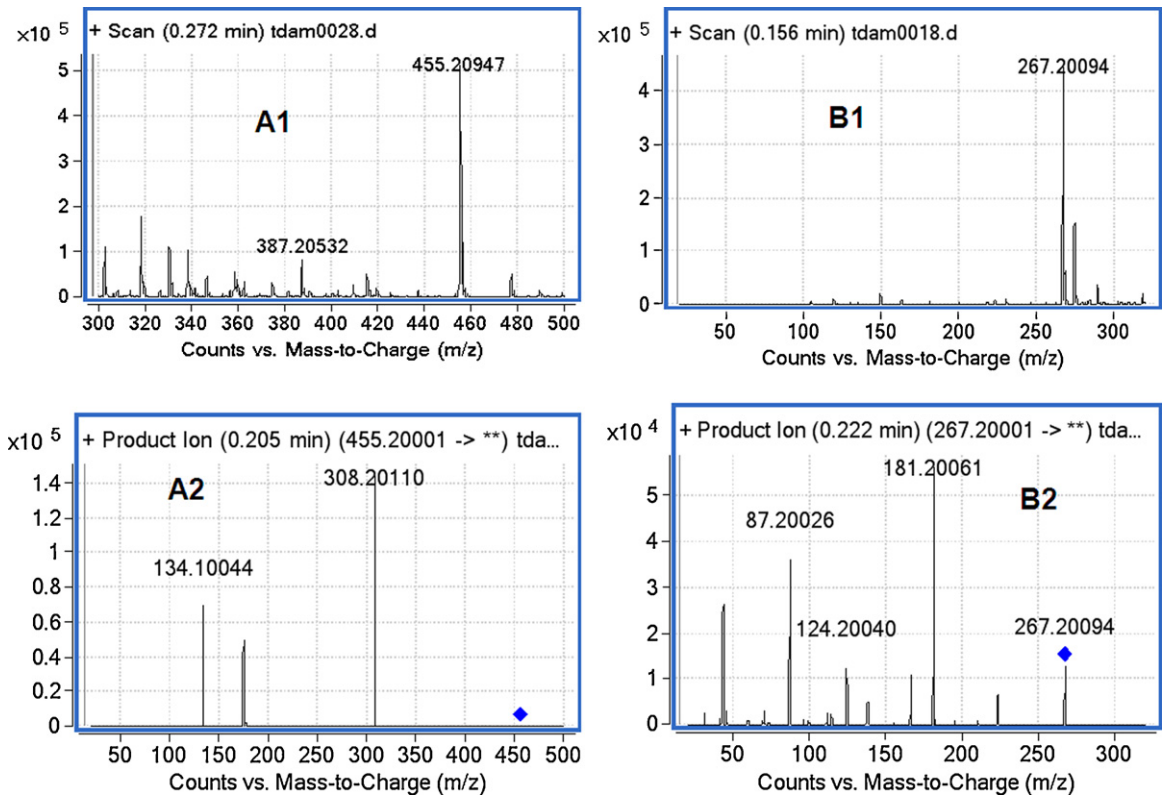

Fig. 2. Full-scan mass spectrum of methotrexate (A1) and doxofylline (B1), and its MS/MS product ion spectrum (A2) and (B2) in the positive ion mode using electrospray ionization (ESI, $[M+H]^+$).

shown in Table 1. The results demonstrated an ionization enhancement effect for MTX and IS under the present LC/MS/MS conditions. Notably, the ionization enhancement for the analytes was consistent over the QC concentration ranges without showing any analyte concentration-dependence. The RSD (%) of ME at different concentrations of MTX and IS from five different batches of human whole blood and incubated whole blood was considered to be the assessment of the relative ME effect. The variability was acceptable,

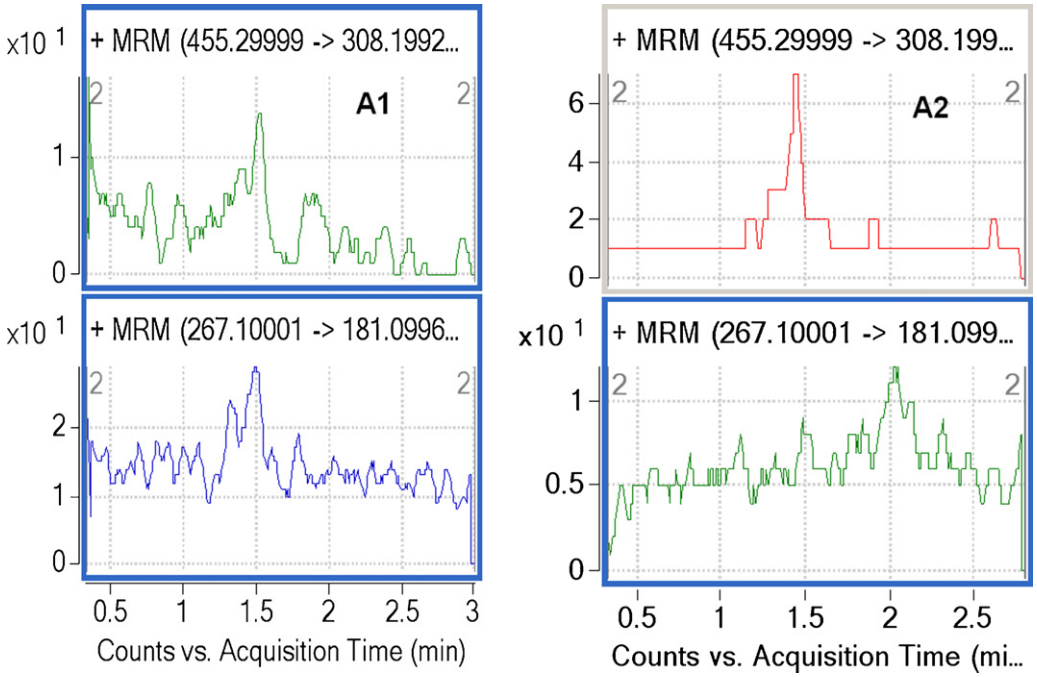

Fig. 3. Representative multiple reaction monitoring (MRM) chromatograms of MTX and doxofylline (IS) in human incubated whole blood (A1–D1) and whole blood (A2–D2). (A) Blank whole blood sample; (B) whole blood sample with MTX at an LLOQ level (1 ng/mL) and (C) blood sample from a patient dosing for more than two months, and (D) blood sample with equimolarly mixed-MTXPG2–7 (total concentration was equivalent to MTX at 45 ng/mL).

Table 1
Matrix effect data for MTX at 2, 10, and 50 ng/mL and IS in five different lots of human whole blood and incubated whole blood ($n = 5$).

Nominal conc. (ng/mL)	Whole blood		Incubated whole blood	
	ME[a] (mean ± SD, %)	RSD[b] (%)	ME[a] (mean ± SD, %)	RSD[b] (%)
2	123.6 ± 7.5	6.1	191.9 ± 3.4	1.8
10	113.4 ± 9.6	8.5	198.3 ± 5.2	2.6
50	114.2 ± 15.4	13.5	191.2 ± 5.0	2.6
IS	103.9 ± 0.70	0.7	167.2 ± 12.9	7.7

[a] The ME (matrix effect) expressed as the ratio of the peak area of MTX in spiked human whole blood and incubated whole blood after extraction over that of the same analyte in mobile phase multiplied by 100.
[b] RSD = relative standard deviation (%RSD = SD/mean × 100).

indicating that the relative ME for the analytes was minimal in this study (Table 1).

Moreover, the ionization enhancement did not affect the slopes or linearity of the established calibration curves, the ratio of analyte over IS, and the precision and accuracy throughout the process. Thus, the present analytical method was considered reliable with high sensitivity for determination of MTX in human whole blood and incubated whole blood, in spite of the matrix effects existed.

In addition, the "cross-talk" between the two MS/MS channels was assessed by separately injecting MTX and IS. The response was monitored in the other channel. No "cross-talk" was observed between channels (Fig. 4).

3.3.3. Linearity and lower limit of quantification

A seven-point calibration curve was prepared as described in Section 2.2, and the slope, intercept and correlation coefficient (r^2) for each standard curve from each analytical run were determined automatically by the Agilent Masshunter software program. The regression equation of these curves and their correlation coefficients (r^2) were calculated as follows: $Y = 6.5366X - 0.0144$ ($r^2 = 0.9980$) for whole blood; $Y = 3.3948X - 0.0067$ ($r^2 = 0.9993$) for incubated whole blood. Overall, the linearity was satisfactory over the concentration ranges studied.

The lowest concentration on both of the calibration curves was 1 ng/mL. Both of the responses of MTX at 1 ng/mL were >8 times of the baseline noise. The precision and accuracy at this concentration were acceptable, with <7.03% of the CVs and <8.32% of the relative errors for whole blood, and <6.93% of the CVs and <4.56% of the relative errors for incubated whole blood. Thus, the lowest concentration on the calibration curve was accepted as the LLOQ. The LOD value was the same (0.5 ng/mL) for whole blood and incubated whole blood.

3.3.4. Accuracy and precision

Intra- and inter-day precision was assessed from the results of QC samples. The intra- and inter-day precision and accuracy data for whole blood and incubated whole blood were summarized in Table 2. The intra-day precision ranged over 9.5–15.8% and 5.3–8.3%, while the inter-day precision ranged over 9.5–11.8% and 6.3–9.4% for whole blood and incubated blood, respectively. The accuracy ranged between 99.1% and 108.5% for whole blood and incubated whole blood. The results above demonstrated that the precision and accuracy of the method were acceptable.

Table 3
The recovery (extraction efficiency) for MTX in human whole blood and incubated whole blood ($n = 5$).

Blood sample	Nominal concentration (ng/mL)	Recovery[a]	
		Mean ± SD	%RSD
Whole blood	2	29.3 ± 0.68	2.3
	10	37.8 ± 1.21	3.2
	50	36.2 ± 4.79	13.2
Incubated whole blood	2	30.7 ± 1.87	6.1
	10	26.9 ± 1.17	4.4
	50	26.2 ± 2.09	7.9

RSD, relative standard deviation.
[a] The recovery (extraction efficiency) of analytes from human whole blood and incubated whole blood after the extraction procedure was determined by comparing the areas of extracted analytes with that of the non-extracted pure standards that represent 100% recovery.

3.3.5. Recovery and stability

Table 3 showed the recovery (extraction efficiency) of MTX from human whole blood and incubated whole blood following ethyl acetoacetate extraction. The recovery of MTX ranged between 29.3–37.8% and 26.2–30.7% for whole blood and incubated whole blood, respectively. The results were similar at all concentrations. These data indicated that the extraction efficiency for MTX was satisfactory without concentration dependence. van Haandel et al. [18] reported that after protein precipitation and extraction by SPE, mean recovery of MTX in whole blood was 31.2%, which was similar to our results. The reason for the low recovery might be mainly attributed to the protein precipitation step.

The results for stability of MTX in whole blood and incubated whole blood under different storage conditions were presented in Table 4. MTX was stable without significant degradation under various conditions. No degradation product was detected under the LC/MS condition after two freeze/thaw cycles. MTX in whole blood could therefore be stored at room temperature for 4 h and −80 °C for 30 days. Analysis of the QC samples following ethyl

Table 2
Intra- and inter-day precision and accuracy data for assays of MTX in human whole blood and incubated whole blood ($n = 5$).

Group	Quality control conc. (ng/mL)	Intra-assay ($n = 5$)			Inter-assay ($n = 3$)		
		Mean ± SD	Accuracy (%)	%RSD	Mean ± SD	Accuracy (%)	%RSD
Whole blood	2	2.09 ± 0.33	104.5	15.8	2.03 ± 0.24	101.5	11.8
	10	9.91 ± 1.17	99.1	11.8	10.01 ± 1.16	100.1	11.6
	50	52.6 ± 5.02	105.2	9.5	50.9 ± 4.82	101.8	9.5
Incubated whole blood	2	2.17 ± 0.16	108.5	7.4	2.23 ± 0.14	111.5	6.3
	10	10.71 ± 0.57	107.1	5.3	10.16 ± 0.88	101.6	8.7
	50	53.23 ± 4.41	106.5	8.3	51.93 ± 4.90	103.9	9.4

RSD = relative standard deviation (%RSD = SD/mean × 100); accuracy% = (mean calculated concentration/quality control concentration) × 100%.

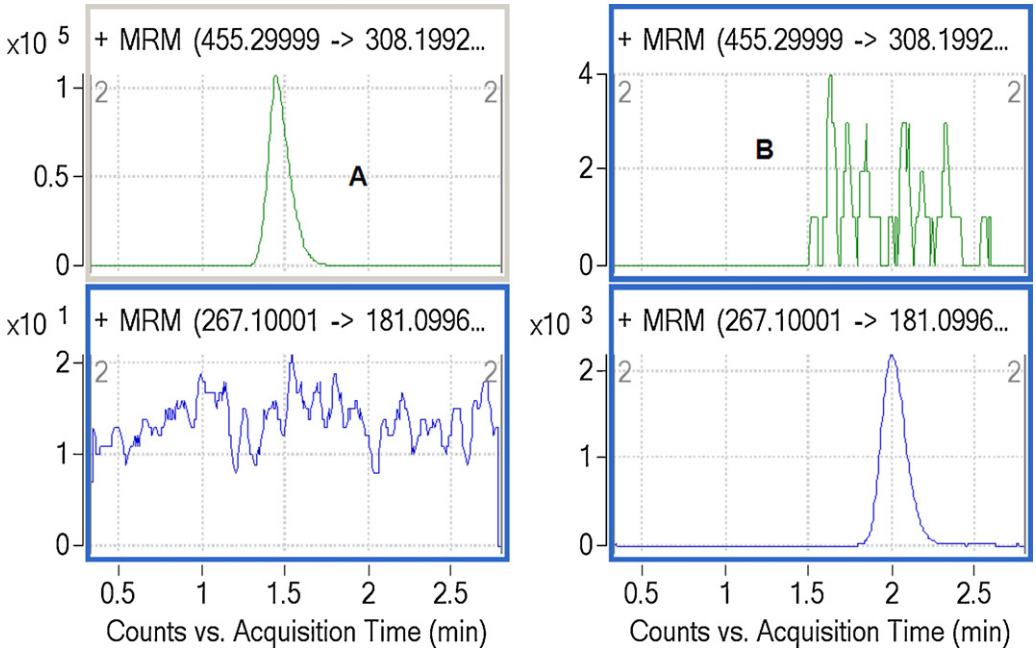

Fig. 4. Representative multiple reaction monitoring (MRM) chromatograms of methotrexate and doxofylline for cross-talk assessment. (A) a blank mobile phase sample spiked with methotrexate; (B) a blank mobile phase sample spiked with doxofylline.

acetoacetate extraction showed no significant degradation after 6 h at room temperature. These results indicated that MTX was stable under routine laboratory conditions and no specific procedure (e.g., acidification or adding organic solvents) was needed to stabilize the compound for drug monitoring and pharmacokinetic study. MTXPGs were also stable, the stability of which was estimated indirectly through the experiment of enzymatic conversion (see Section 3.3.6).

Table 4
Stability of MTX in human whole blood and incubated whole blood under various storage conditions ($n = 5$).

Storage condition	Blood sample	Nominal concentration (ng/mL)	Calculated concentration	
			Mean ± SD	Er[a] (%)
−80 °C/1 month				
	Whole blood	2	1.98 ± 0.10	−1.00
		10	9.54 ± 0.69	−4.60
		50	53.05 ± 4.57	6.10
	Incubated whole blood	2	2.10 ± 0.10	5.00
		10	9.83 ± 0.67	−1.70
		50	46.48 ± 2.42	−7.04
−80 °C/2 freeze–thaw cycles				
	Whole blood	2	1.99 ± 0.18	−0.50
		10	9.62 ± 0.64	−0.38
		50	53.03 ± 3.58	6.06
	Incubated whole blood	2	1.98 ± 0.23	−1.00
		10	9.89 ± 1.01	−1.10
		50	44.00 ± 1.40	−12.00
4 h at room temperature				
	Whole blood	2	1.92 ± 0.08	−4.00
		10	9.61 ± 0.46	−3.90
		50	53.15 ± 1.54	6.30
	Incubated whole blood	2	2.15 ± 0.09	7.50
		10	9.91 ± 0.50	−0.9
		50	47.41 ± 3.87	−5.18
6 h at room temperature (extracted samples)				
	Whole blood	2	2.02 ± 0.17	1.00
		10	9.93 ± 0.95	−0.70
		50	49.69 ± 4.12	−0.62
	Incubated whole blood	2	1.97 ± 0.10	−1.50
		10	10.78 ± 0.35	7.80
		50	54.41 ± 1.47	8.82

[a] Mean relative error = 100 × (overall mean assayed concentration-added concentration)/added concentration.

3.3.6. Confirmation of the completeness of MTX produced by enzymatic conversion from MTXPGs

In order to investigate the completeness of the enzymatic conversion of MTXPGs to MTX, blank whole-blood samples from three volunteers were spiked with mixtures of MTXPG2-MTXPG6 at a final concentration of 100 ng/mL (220.05 nmol/L) with mixture of equimolar or at a proportion of 2:5:3:3:1. The ratio was estimated according to previous studies [16,18]. Blood samples spiked with MTXPGs or MTX were analyzed following the above described procedure for whole-blood total MTX (MTX + MTXPGs). The enzymatic conversion rates were 92.1% (RSD = 13.1%, $n = 3$) and 94.3% (RSD = 14.8%, $n = 3$), respectively. Similar result of enzymatic conversion was also reported in other studies [16,17], in which enzymatic conversion rates were more than 90%.

Stability of the enzyme was observed in the repeated conversion experiment using the same concentration of mixed MTXPGs spiked in the same blank whole-blood, which were stored at −80 °C for one month. The enzymatic conversion rates were almost the same as those of one month ago, which were 90.5% (RSD = 14.2%, $n = 3$) and 94.6% (RSD = 13.5%, $n = 3$), respectively. These results indicated that the enzyme and MTXPGs spiked in blank whole-blood were stable at −80 °C for one month. Blood samples spiked with MTXPGs were also stable without interconversion at −80 °C for 6 months at concentrations up to 90.8 ng/8 × 10^{12} RBCs [28]. Dervieux et al. previously reported that MTXPGs concentrations were stable without interconversion after storage in a transportation system containing a cold pack for 48 h [16].

3.3.7. Application of the MTX analytical method in patients with rheumatoid arthritis

The developed and validated method was applied to determinate the concentrations of whole-blood MTX and whole-blood total MTX (MTX + MTXPGs) in rheumatoid arthritis patients with low dose treatment of MTX (10–15 mg, intravenous injection). Blood samples were collected from patients receiving treatment for more than three months to reach the constant concentration. The mean constant concentration of MTXPGs was 10.1 ng/mL (0.4–59.4 ng/mL, $n = 130$), which was generated by subtracting the whole blood MTX from the whole-blood total MTX (MTX + MTXPGs) following blood volume conversion. The MTXPGs concentrations in the current study were lower than the ones published previously. Dervieux et al. [29] reported that the $MTXPG_3$ concentration was 31.2 ng/mL (1.6–103.1 ng/mL, $n = 226$) in erythrocyte in patients receiving low-dose treatment of MTX (10–17.5 mg for more than 3 months) weekly. In another study reported by Brooks [28], the mean $MTXPG_{1-5}$ concentration was 21.8 ng/8 × 10^{12}RBC (6.5–36.0 ng/mL, $n = 18$) in patients with inflammatory bowel disease who received similar dose of MTX as patients with RA. The discrepancy may account for sampling from distinct ethnic groups (Caucasian vs Chinese) and detecting using different methods (HPLC with direct protein precipitation vs LC/MS/MS with liquid–liquid extraction). Some studies revealed that the gene polymorphisms of MDR1 3435C>T, GGH −401C>T or RFC1 80G>A might affect MTXPGs levels in erythrocyte and drug efficacy in RA patients [30,31]. The mutant frequencies of the genes mentioned above are different between Caucasian and Chinese. For example, the mutant frequencies of MDR1 3435T are 54% and 40% in Caucasian and Chinese, respectively; while the ones of GGH −401T are 31% and 21% in Caucasian and Chinese, respectively (Allele frequencies compiled from hapmap database, www.hapmap.org).

Our result was similar to the one reported by Li et al. [17], in which the total MTXPGs concentrations were between 1.1 ng/mL and 8.7 ng/mL in patients ($n = 6$) with MTX (10 mg) treatment for 48 h through oral administration. However, it is difficult to compare the MTXPGs levels between these two studies, because the sample size from Li's study was too small ($n = 6$). The individual difference would affect the pharmacokinetics of MTXPGs.

MTX is given weekly to patients with rheumatoid arthritis. Therefore, it is applicable to monitor MTXPGs levels indirectly by this method.

4. Conclusion

A rapid and sensitive LC/MS/MS method with liquid–liquid extraction from a small size of whole-blood sample has been developed for determination of MTXPGs concentration in erythrocyte, which is more convenient for monitoring the MTX therapy in rheumatoid arthritis patients. This method has been successfully applied to determine the concentrations of erythrocyte MTXPGs in a pharmacokinetic–pharmacodynamic study in patients with rheumatoid arthritis.

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