Reviews

Methotrexate Polyglutamates Analysis by Chromatography Methods in Biological Matrices: A Review

Yiming ZHANG,*, Liyu SUN,*,** Libo ZHAO,**,*** Xiaoling WANG,**,*** Zhigang ZHAO,*,**† and Shenghui MEI*,**†**

**Department of Pharmacy, Beijing Tiantan Hospital, Capital Medical University, 119 Nansihuan West Road, Fengtai District, Beijing 100070, P. R. China*
***Department of Clinical Pharmacology, College of Pharmaceutical Sciences, Capital Medical University, Beijing 100069, P. R. China*
****Department of Pharmacy, Beijing Children's Hospital, Capital Medical University, National Center for Children's Health, Beijing 100045, P. R. China*

Methotrexate (MTX) is used as an immunosuppressant and antineoplastic drug in clinical practice. MTX is a parent drug and converts to MTX polyglutamates (MTXPGs) to exhibit its biological activity. Clinical studies found that MTXPG levels were associated with MTX response and toxicities, especially at low doses. Due to huge variance of MTX response and toxicities between individuals, therapeutic drug monitoring is necessary for its use in individualized therapy. Various chromatography methods coupled with ultraviolet-visible detector, fluorescence detector and mass spectrometry have been reported for MTXPG analysis in various biological matrices. The aim of this paper is to review the chromatographic based methods for the measurement of total and/or individual MTXPGs. We searched Embase, Science Direct and PubMed databases using "methotrexate polyglutamate" and "chromatography" as search terms, and found 745 articles. Of those, 14 articles were extracted for this study. The key steps for method development (sample pretreatment, parameter optimization of liquid chromatography and mass spectrometry, selection of internal standard) and validation (lower limit of quantitation, accuracy, precision, recovery, matrix effect and stability) were analyzed and summarized, which might be helpful for researchers to develop their own methods.

Keywords Methotrexate polyglutamates, analysis, biological matrices, chromatography, mass spectrometry, method development, method validation, review

(Received February 21, 2021; Accepted May 12, 2021; Advance Publication Released Online by J-STAGE May 21, 2021)

Yiming ZHANG received a bachelor's degree in clinical pharmacy in 2021 at the Capital Medical University. During her internships at Beijing Tiantan Hospital, she developed a strong interest in clinical pharmacology and analytical chemistry. Currently, she is studying for a master's degree at Beijing Children's Hospital and is involved in pediatric pharmaceutical care.

Liyu SUN received her bachelor's degree from Capital Medical University in China in 2022. Starting in 2022, she will be a master's student at Peking University. His scientific interests are related to the clinical pharmacokinetics and the application of blood drug concentration monitoring.

Libo ZHAO received his PhD at Tongji Medical College of Huazhong University of Science and Technology in 2008. Since 2016, he has been director of the Clinical Research Center, Beijing Children's Hospital. He is doctoral supervisor and his scientific interests includes clinical pharmacology and population pharmacokinetic models.

† To whom correspondence should be addressed.
E-mail: ttyyzzg1022@126.com (Z. Z.); meishenghui1983@126.com (S. M.)

1 Introduction

Methotrexate (MTX, $MTXPG_1$) is structurally similar to folic acid (Fig. 1), which results in anti-proliferative and anti-inflammatory properties, through inhibition of purine biosynthesis and accumulation of adenosine.[1] High doses of MTX is widely used as chemotherapy for various neoplastic diseases, such as acute lymphoblastic leukemia, malignant lymphoma, and osteosarcoma.[2] This drug can also be administered at low doses to treat autoimmune diseases such as psoriatic arthritis, rheumatoid arthritis, and juvenile idiopathic arthritis.[3]

After 24 h intravenous administrations of MTX, 80 – 90% of the total dose is excreted through the kidneys.[4] In the liver, MTX is metabolized to its major active metabolite, 7-hydroxymethotrexate.[5] MTX and 7-hydroxymethotrexate could crystallize in the kidneys due to their poor water solubility under acidic conditions, which is responsible for MTX renal toxicity.[5,6] In the intestine, MTX could convert to 2,4-diamino-N10-methylpteroic acid, which is much less toxic than MTX.[7] Methotrexate polyglutamates (MTXPGs) are formed in cells by folylpolyglutamate synthetase, while gamma-glutamyl hydrolase can remove glutamate residues from MTXPGs.[8,9] The composition of these MTXPGs is determined by the equilibrium between the two enzymes.[10] Compared to MTX, the glutamylated MTX ($MTXPGs_{2-7}$) have increased polarity and a higher anionic feature, and poor affinity for multidrug resistance-associated proteins, resulting in prolonged intracellular retention.[11,12] Evidence suggests that MTXPGs display not only longer half-lives but also higher efficacy than MTX.[13,14] Additionally, long-chain MTXPGs ($MTXPGs_{3-7}$) have higher inhibition effects on key enzymes (dihydrofolic acid reductase, thymidylate synthase, and 5-aminoimidazole 4-carboxamide ribonucleotide transformylase) over short-chain MTXPGs (MTX, $MTXPG_2$).[15,16] Therefore, MTX has also been known as a prodrug.[17]

MTX has therapeutic effects for patients with rheumatoid arthritis or juvenile idiopathic arthritis in about 60% of patients,[18] however, its efficacy[7] and toxicity (gastrointestinal disturbances,[19] alopecia, liver damage, and bone marrow suppression) varies greatly among individuals.[20] Several categories of factors could influence MTXPG concentration in patients treated with MTX:[21,22] 1. Genetic background, enzymes in the folate and adenosine pathways;[23,24] 2. patient-related factors, such as age, renal function (glomerular filtration rate) and smoking status;[25] and 3. treatment-related factors, such as MTX dose, treatment duration and co-medications.[26,27]

The therapeutic window of MTX is narrow. Therefore, therapeutic drug monitoring is important for individualized therapy of MTX to enhance efficacy and decrease toxicity.[28,29] MTX plasma levels are associated with its toxicities at high doses, and MTX plasma levels are routinely monitored in clinical practice.[30] In recent years, MTXPG levels were found to be related to efficacy and side effects at low doses,[31] and MTXPGs were used as alternative biomarkers in clinical practice.[14,15]

MTXPGs in white blood cell lines have a short steady-state time, and they might be highly correlated with MTX response.[32] However, measuring MTXPG levels in mature red blood cells (RBCs) has been widely applied in clinical practice due to its feasibility.[30,33] Folylpolyglutamate synthetase is only present in red cell precursors, and mature RBCs are unable to accumulate or excrete MTXPGs.[8,32] RBCs lifetime is about 90 days, so erythrocyte MTXPG concentration is reflective of the MTXPG status in bone marrow at the time of erythrocyte formation.[8,10]

This paper aims to summarize and analyze methodological literature for MTXPG detection based on chromatography in different biological matrices, which might be helpful for analysts in developing their own methods.

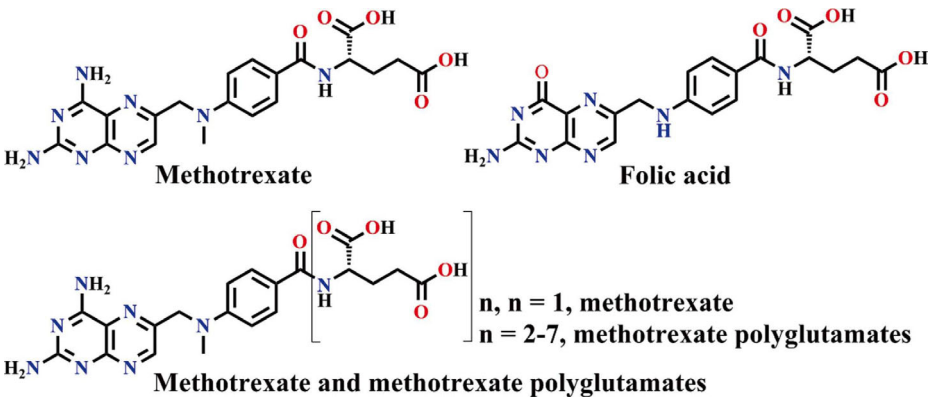
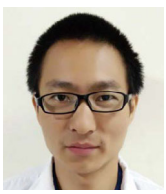


Fig. 1 Chemical structures of folic acid, methotrexate and methotrexate polyglutamates.

Xiaoling Wang did her master's in pharmacy at Beijing University of Chinese Medicine in 2006. From 2012, she has worked as an office director of child drug use expert committee of the National Health Commission of the People's Republic of China. She is master tutor and her scientific interests are pediatric clinical pharmacy and pharmacology, pediatric pharmaceutical care, pharmacist education and training.

Zhigang Zhao graduated from the medical department of Peking University in 1990. In 2016, he received a doctorate in clinical pharmacy from the Capital Medical University. He is the director of the department of clinical pharmacy, school of pharmacy, Capital Medical University. He is doctoral supervisor in clinical pharmacology and his scientific interest including clinical pharmacology, clinical pharmacy, and pharmacogenomics.



Shenghui Mei got his doctorate in Capital Medical University in 2011. After graduation, he worked in the department of pharmacy, Beijing Tiantan Hospital. His major work was therapeutic drug monitoring and individualized therapy. His study field including biopharmaceutical analysis, population pharmacokinetic modeling and pharmacogenomics.

2 Literature Search and Data Extraction

Articles on "methotrexate polyglutamate" and "chromatography" in all fields published between 1981 to 2019 were searched in Embase, Science Direct and PubMed network databases, as well as reference lists of relevant articles. Then after filtering and analyzing the results, 14 articles were selected. Sample preparation procedures, separation and detection parameters, and method validation results were extracted from these papers and summarized. The advantages and limitations of current analytical methods, including high performance liquid chromatography (HPLC) in combination with ultraviolet (UV)-visible (three articles), fluorescence (FD) (four articles) or mass spectrometry (MS) detection (seven articles), were discussed.

3 Analysis of Total and Individual MTXPGs

To determine the concentration of MTXPGs in biological matrices, researchers have developed two strategies: one entails the conversion of MTXPGs into MTX (determining total MTXPGs); the other involves determining each component of MTXPGs (individual MTXPGs). However, previous research works have established that long-chain MTXPGs have a stronger correlation with MTX exposure and effectiveness compared to short-chain MTXPGs.[34] Thus, there may be considerable clinical interest in the quantitation of individual MTXPGs. $MTXPG_3$, the most abundant metabolite (48.9%) over all other MTXPGs is highly associated with adverse effects, in connection with the administration of weekly low doses of MTX for the treatment of juvenile idiopathic arthritis.[18,35] Therefore, to simplify the detection method, some researcher have selected choose $MTXPG_3$ to represent intracellular MTXPG levels.[36]

3·1 Release of MTXPGs from blood cells

For MTXPGs analysis, sample preparation is the limiting and labor-intensive step of the entire analytical procedure.[6] Choosing an optimal technique for sample preparation is vital for the measurement of MTXPGs in different matrices, especially in erythrocyte. The results are shown in Table 1.

RBCs lysis was obtained *via* freeze-thaw cycle (–80,[18,37-39] –70,[40-42] and –60°C[43]), chemical cracking[18,38-40,42,44] and blood drying process.[36,38] To determine total MTXPGs, RBC pellets[40] or drying blood spots[38] should be re-dissolved in lysis agent (water). For individual MTXPG analysis, twice washing of fresh human blood samples with sterile normal saline are sufficient for removing polyglutamate hydrolase in the plasma prior to RBC lysis to avoid conversion of MTXPGs to MTX.[18,41,42] However, washing just once has also been used in other matrices (HT29 and MCF-7 cells).[44-46]

3·2 Conversion of MTXPGs to MTX

Human plasma, containing polyglutamate hydrolase, is used to convert MTXPGs to MTX.[38,40,47] Phosphate buffer was used for pH control. Mercaptoethanol[38,47] and ascorbic acid[37,43] were added to avoid oxidation.[42] Under the moderate condition of phosphate buffer with mercaptoethanol, incubation time for conversion of MTXPGs to MTX by polyglutamate hydrolase is 6 to 14 h at 37°C.[38,47] Moreover, ascorbic acid creates acidity that accelerates the conversion reaction,[43] resulting in a short incubation time (2 to 3 h at 37°C).[37,43] Reduction by sodium dithionite was also used for the conversion of MTXPGs to MTX.

3·3 Protein precipitation and analyte extraction

After being released from erythrocytes, parts of MTXPGs were bounded to proteins. Therefore, protein precipitation (PP) was an essential step to release MTXPGs from its bounded proteins before detection. Perchloric acid,[36,38,40,42,48] trichloroacetic acid (TCA)[37,43,44,46,47] and boiling water[18,41] followed by fast centrifugation have been used to remove proteins from biological samples, and 70% perchloric acid is the most widely applied agent.[36,38,40] However, perchloric acid resulted in a relatively high ionic strength and low pH, thus a number of subsequent purification steps (solid phase extraction, SPE) were required.[18] Hroch *et al.*[47] used 0.8 M TCA in 40% (v/v) acetic acid for PP. PP by heating was also developed.[39] Thermal extraction (pH 7.85) was first applied for the analysis of folate polyglutamates, which is structurally similar to MTXPGs.[49] The extraction of folate needs a "Wilson and Horne" buffer, consisting of ascorbic acid (antioxidant), mercaptoethanol (to scavenge formaldehyde released by ascorbic acid) and 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid/*N*-cyclohexyl-2-aminoethanesulfonic acid[18] buffer system (to control pH at 7.85). Van Haandel *et al.* applied thermal extraction for MTXPG deproteinization using a modified buffer (ammonium bicarbonate buffer adjusted to pH 7.5 with formic acid).[42]

Three kinds of SPE cartridges were used for sample purification with different elution solvents and steps. Recovery rates obtained from the Sep-Pak Cl8 cartridge ranged from 77 – 102,[45] 61 – 81,[44] and 69 – 91%.[46] Oasis MAX cartridge was reported by Mo *et al.*,[37] with recovery rates of 70% (total MTXPGs) and 27 – 71% (individual MTXPGs). The recovery rates for these SPE methods were acceptable. Only one method using SPE for sample purification reported matrix effect (85 – 115%) for total and individual $MTXPGs_{1-5}$ analysis in dry blood spots.[38] The drawbacks of SPE included high cost, low reproducibility, and time consuming process, all of which limited its clinical application.[37] The recovery rate of MTXPGs in human blood pretreated by SPE combined with PP was 27 – 71%[38] (Hawwa *et al.*, 2014) and 31.2 – 50.6%[42] (Van Haandel *et al.*, 2009).

Liquid–liquid extraction (LLE) was also performed alone or in combination with PP for sample pretreatment,[37,39] but large amounts of organic solvents were required for better recovery. Therefore, evaporation was required to remove the solvents followed by re-dissolving the residues before sampling. Ethyl acetoacetate[37] and acetonitrile[39] were used for LLE, and recovery ranged from 26.2 – 30.7[37] and 60 – 108%,[39] respectively.

3·4 Selection of IS

E. den Boer *et al.* found that recovery and matrix effects differed greatly between different MTXPGs.[48] A stable isotopic internal standard (IS) was vital for reliable quantitation of MTXPGs, especially for methods with multistep sample preparation procedures.[42] Various IS were used in MS detection-based methods, including methotrexate-d_3,[18,36] ($^{13}C_5$,^{15}N)-MTX,[48] doxofylline[37] and aminopterin.[39] E. den Boer *et al.* used ($^{13}C_5$,^{15}N)-MTX as IS and obtained acceptable matrix effect results (95 – 99%, coefficient of variation (CV) < 20%). The chemical structure of MTXPGs is similar to that of folic acid polyglutamtes, therefore Van Haandel *et al.* used folate polyglutamates as IS for MTXPG analysis.[18] Since folate supplementation is very common, they screened 12 samples (2 patients receiving folate supplementation and 10 individual donors) to confirm the potential influence of folate polyglutamates. Theophylline was originally employed as IS by Mo *et al.*,[37] but interference was observed. Doxofylline was demonstrated to be an ideal IS with high specificity, proper

Table 1 Sample preparation and chromatographic conditions for methotrexate polyglutamates analysis by liquid chromatography based methods

Author	Matrix	Analyte	Sample preparation	Column; temperature/°C	Mobile phase		Flow rate; run time; elution
					A	B	
K. Brady[36]	Dried capillary blood	$MTXPG_3$	Lysis by drying process; PP by 70% perchloric acid	Accucore pentafluorophenyl analytical column (50 × 2.1 mm, 2.6 µm); NA	0.1% formic acid with 0.01% triethylamine and CH_3CN		1 mL/min; 6 min; NA
A. F. Hawwa[38]	Dried blood spots	Total MTXPGs	Lysis by drying process and freeze-thaw cycle; convert MTXPGs to MTX by polyglutamate hydrolase; PP by 70% perchloric acid; SPE, Oasis MAX cartridges (1 mL/30 mg) on a Waters Extraction Manifold (Waters, USA)	Atlantis T3-C18 column (150 × 2.1 mm, 3 µm; Waters); 30°C	10 mM ammonium bicarbonate buffer adjusted to pH 7.5 using formic acid	CH_3CN	0.15 mL/min; 20 min; 0 – 10 min, 8 – 20% B; 10 – 20 min, 98% A
		$MTXPG_{1-5}$	Lysis by drying process and freeze-thaw cycle; PP by 70% perchloric acid; SPE, Oasis MAX cartridges (1 mL/30 mg) on a Waters Extraction Manifold (Waters, USA)	Atlantis T3-C18 column (150 × 2.1 mm, 3 µm; Waters); 30°C	10 mM ammonium bicarbonate buffer adjusted to pH 7.5 using formic acid	CH_3CN	0.15 mL/min; 30 min; 0 – 20 min, 0 – 20% B; 20 – 30 min, 100% A
E. den Boer[48]	Human blood	$MTXPG_{1-5}$	Lysis, NA; PP by 16% perchloric acid	Waters Acquity ethylene bridged hybrid C18 column (100 × 2.1 mm, 1.7 µm); 35°C	10 mM ammonium bicarbonate adjusted to pH 10 with 25% ammonium hydroxide	Methanol	0.3 mL/min; 6 min; 0 – 0.5 min, isocratic hold 5% B; 0.5 – 4 min, 5 – 40% B; 4 – 4.25 min, 40 – 100% B; 4.25 – 4.75 min, isocratic 95% B; 4.75 – 5 min, 100 – 5% B; 5 – 6 min, isocratic 5% B
X. Mo[37]	Human blood	Total MTXPGs	Lysis by freeze-thaw cycle; convert MTXPGs to MTX by polyglutamate hydrolase; PP by trifluoroacetic acid; LLE by ethyl acetoacetate	XB-C18 column (100 × 2.1 mm, 3 µm; Welch Materials Inc., USA); room temperature	CH_3CN (1% formic acid)–20 mM ammonium formate solution (30:70, v/v)		0.2 mL/min; 3 min; 0 – 3 min, 100% mobile phase
L. Van Haandel[18]	Human blood	$MTXPG_{1-7}$	Lysis by freeze-thaw cycle; PP by boiling	Phenomenex Synergy Hydro-RP LC column (50 × 1 mm, 4 µm); NA	10 mM ammonium bicarbonate buffer with 5 mM of the *N*,*N*-dimethylheptyl-amine adjusted to pH 7.5 with formic acid	CH_3CN with 5 mM of *N*,*N*-dimethyl-heptylamine	200 µL/min; 12 min; 0 – 1 min, 90% A: 10% B; 1 – 10 min, 90% A: 10% B–70% A: 30% B; 10 – 12 min, 90% A:10% B
L. Van Haandel[41]	Human blood	Total MTXPGs	Lysis by freeze-thaw cycle; convert MTXPGs to MTX by reduction; PP by boiling	Phenomenex Inertsil ODS-3 analytical column (150 × 4.6 mm, 5 µm, 100 Å); NA	10 mM ammonium acetate in water	Methanol	1 mL/min; 7 min; 0 – 7 min, 30% B
L. Van Haandel[42]	Human blood	$MTXPG_{1-7}$	Lysis by freeze-thaw cycle; PP by 70% perchloric acid; SPE, Oasis HLB cartridges (30 mg)	Phenomenex Synergy Hydro-RP LC column (50 × 1 mm, 4 µm, 80 Å); NA	10 mM ammonium bicarbonate buffer with 5 mM *N*,*N*-dimethylhexylamine adjusted to pH 7.5 with formic acid	CH_3CN with 5 mM *N*,*N*-dimethyl-hexylamine	200 µL/min; 20 min; 0 – 1 min, 90% A; 1 – 10 min, 90 – 70% A; 10 – 12 min, 70% A; 12 – 20 min, 90% A
G. Chen[39]	Caco-2 cells	$MTXPG_{1-5}$	Lysis by freeze-thaw cycle; LLE by acetonitrile	C8 column (150 × 3.9 mm, 5 µm; Symmetry Shield RP8, Waters); 40°C	0.1% formic acid	CH_3CN	0.5 mL/min; 15 min; 0 – 7 min, 10 – 90% B; 7 – 9 min, 90% B; 9 – 10 min, 90 – 10% B; 10 – 15 min, 10% B
M. Hroch[47]	Human blood	Total MTXPGs	Lysis, NA; convert MTXPGs to MTX by polyglutamate hydrolase; PP by 0.8 M trichloracetic acid in 40% (v/v) acetic acid	Gemini C18 110A RP column (150 × 4.6 mm, 5 µm); 30°C	50 mM ammonium acetate buffer (pH 5.5)-CH_3CN–hydrogen peroxide (890:110:0.25, v/v)		0.6 mL/min; 13 min; 0 – 13 min, 100% mobile phase
H. Li[43]	Human blood	Total MTXPGs	Lysis by freeze-thaw cycle; convert MTXPGs to MTX by polyglutamate hydrolase; LLE by sodium hydroxide solution and methanol	HP Zorbax StableBondSB-C18 column (150 × 4.6 mm, 5 µm); 40°C	CH_3CN–50 mM ammonium acetate in water (7:93, v/v)		1 mL/min; 8.5 min; 0 – 8.5 min, 100% mobile phase
T. Dervieux[40]	Red blood cells	$MTXPG_{1-7}$	Lysis by water; PP by 70% perchloric acid	Terra MS C18 column (250 × 4.6 mm, 5 µm; Waters); 4°C	10 mM ammonium acetate (pH 6.50) containing 2 mL/L hydrogen peroxide	CH_3CN	1 mL/min; 30 min; 0 – 20 min, 0 – 13% B; 20 – 30 min, 100% A
R. Durand[45]	HT29 cell line	$MTXPG_{1-4}$	Lysis by water; PP by boiling; SPE, Sep-Pak C18 column (Waters Associates Milford, MS)	C18 µBondapak column (30 × 0.39 cm, 10 µm; Waters Associates, Milford, MS, USA); NA	5 mM potassium phosphate buffer (pH 7.4) containing 2.5 mM tetrabutyl ammonium nitrate	Methanol	2 mL/min; 40 min; 0 – 10 min, 80% A:20% B; 10 – 40 min, 20 – 30% B

Table 1 (Continued)

Author	Matrix	Analyte	Sample preparation	Column; temperature/°C	Mobile phase A	Mobile phase B	Flow rate; run time; elution
J. Jolivet[44]	MCF-7 cells	$MTXPG_{1-5}$	Lysis by water; PP by trichloroacetic acid; SPE, Sep-Pak C18 cartridge (Water Associates)	Radial-Pak C8 cartridge (Waters Associates); NA	10 mM monopotassium phosphate with 5 mM tetrabutyl ammonium phosphate (pH 5)	CH_3CN	2 mL/min; 25 min; 0 – 15 min, 21 – 27% B and 3.95 to 3.65 mM tetrabutyl ammonium phosphate; 15 – 25 min, 27% B and 3.65 mM tetrabutyl ammonium phosphate
J. Jolivet[46]	MCF-7 cells	$MTXPG_{1-3,6}$	Lysis by water; PP by trichloroacetic acid; SPE, Sep-Pak C18 cartridge (Water Associates)	C18 µBondapak column (30 × 0.39 cm; Waters Associates); NA	5 mM tetrabutyl ammonium phosphate	CH_3CN	1 mL/min; 35 min; 0 – 15 min, 30 – 40% B; 15 – 35 min, 40% B

[Abbreviations] MTXPGs: methotrexate polyglutamates, $MTXPG_1$ (MTX): methotrexate, $MTXPG_2$: 4-amino-10-methylpteroyldiglutamic acid, $MTXPG_3$: 4-amino-10-methylptero-yltriglutamic acid, $MTXPG_4$: 4-amino-10-methylpteroyltetraglutamic acid, $MTXPG_5$: 4-amino-10-methylpteroylpentaglutamic acid, $MTXPG_6$: 4-amino-10-methylpteroylhexa-glutamic acid, $MTXPG_7$: 4-amino-10-methylpteroyl-heptaglutamic acid, PP: protein precipitation, LLE: liquid-liquid extraction, SPE: solid phase extraction, RP: reverse phase, NA: not available, CH_3CN: acetonitrile.

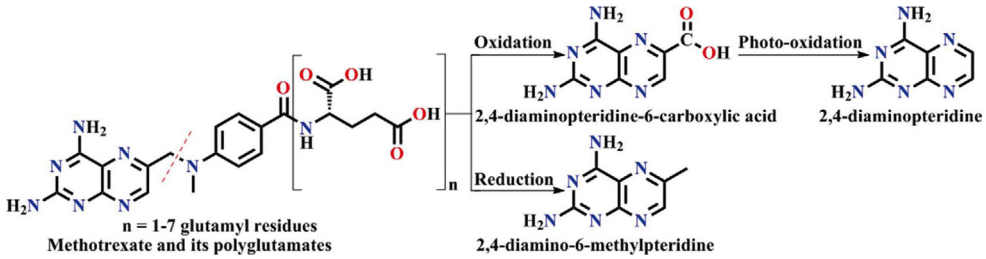


Fig. 2 Formation of fluorescent derivatives under oxidative or reductive conditions. The most commonly used excitation and emission wavelength was 367 and 463 nm for both reduction and oxidation products.

retention time, and acceptable stability during analysis, and the matrix effect was 167.2 to 198.3% with acceptable CV (intra- and inter-day, 5.3 – 8.3% and 6.3 – 9.4%).[37] The anti-folate aminopterin was successfully used as IS by Chen *et al.*[39]

3·5 Chromatographic conditions

LC coupled with FD and MS have been widely applied for MTXPGs analysis. Most of the HPLC methods were performed on C18 and C8 columns. Other kinds of columns such as Phenomenex Synergy Hydro-reverse phase LC column,[18,42] phenomenex Inertsil ODS-3 analytical column[41] and Accucore pentafluorophenyl analytical column,[36] also have been reported.

Isocratic elution is performed for single analyte determination (MTX or $MTXPG_3$).[36,37,41,43,47] Gradient elution was widely used for the determination of individual MTXPGs with acceptable separation efficiency and peak shape.

Mixtures of water and acetonitrile were mainly used for separation. Formic acid was added to achieve higher signal intensity for mass detection and better peak shape. Methanol has also been used for separation.[41,48] In order to form the oxidation product, hydrogen peroxide was added in FD methods[40,47] (Fig. 2). Additionally, ionization suppression was observed under high aqueous elution conditions, especially for long-chain MTXPGs.[41-43]

For ion-pair ultra performance liquid chromatography-MS/MS method, ion-pair agents affected the separation efficacy of analytes. Previous studies have described the hydrophobicity for ion-pair agents: *N*,*N*-dimethylheptylamine > *N*,*N*-dimethylhexylamine > *N*,*N*-dimethylpentylamine.[18] But a low boiling point of the ion-pair agent was generally considered to be beneficial for MS detection (*N*,*N*-dimethylpentylamine > *N*,*N*-dimethylhexylamine > *N*,*N*-dimethylheptylamine). Van Haandel *et al.*[50] found that *N*,*N*-dimethylhexylamine could interfere with the analysis under the multiple reaction monitoring model, because *N*,*N*-dimethylhexylamine had the same molecular weight as glutamyl residue. So they replaced *N*,*N*-dimethyl-hexylamine with *N*,*N*-dimethylheptylamine.

The pH of the mobile phase was an important influencing factor for column retention of MTXPGs (MTX, pK_a 4.8, 5.5). Under reductive conditions, the largest volume of fluorescent product was formed between pH 5.5 – 6.[47] The ionization efficacy of MTXPGs improved with the increase of buffer alkalinity.[18] Under an oxidative environment, Dervieux *et al.* found that a higher fluorescence response and a shorter retention time were obtained at pH 6.5 than at pH 5.5.[40,47] To enhance selectivity, pH 7.5 was most commonly used in LC-MS methods.[18,38,42] Details are described in Table 1.

3·6 Mass detection

A triple quadrupole mass spectrometry system was used in most of the methods under multiple reaction monitoring model with positive electrospray ionization. The most commonly used quantitative ion pair was *m/z* 455.2 > 308.2 for $MTXPG_1$ (MTX), *m/z* 584.4 > 308.2 for $MTXPG_2$, *m/z* 713.3 > 308.2 for $MTXPG_3$, *m/z* 842.3 > 308.2 for $MTXPG_4$, *m/z* 971.6 > 308.2 for $MTXPG_5$, *m/z* 1100.4 > 308.1 for $MTXPG_6$, and *m/z*

Table 2 Method validation results for methotrexate polyglutamates analysis by liquid chromatography based methods

Author	Matrix	Analyte	Linear range/nM; LOQ/nM	Bias, %		Coefficient of variation, %	
				Intra-day	Inter-day	Intra-day	Inter-day
K. Brady[36]	Dried capillary blood	$MTXPG_3$	5 to 100; 5	–11.1 to 8	–7.2 to 5.1	2.0 to 10.9	3.1 to 10.8
A. F. Hawwa[38]	Dried blood spots	$MTXPG_{1\text{-}5}$; Total MTXPGs	10 to 400; 5 10 to 400; 5	–8.6 to 14.4; –1.6 to 8.8	–9.6 to 13.0; 4.4 to 19.6	2.0 to 19.4; 7.7 to 14.3	1.6 to 11.3; 3.3 to 7.0
E. den Boer[48]	Human blood	$MTXPG_{1\text{-}5}$	0.97 to 250; 1	–10.0 to 15.0	–3.6 to 12.8	1.0 to 4.3	5.9 to 14.7
X. Mo[37]	Human blood	Total MTXPGs	2.2 to 220; 1	6.5 to 8.5	1.5 to 3.9	5.3 to 8.3	6.3 to 9.4
L. Van Haandel[18]	Red blood cells	$MTXPG_{1\text{-}7}$	1 to 100; 1	–9.7 to 8	–8.1 to 7	1.1 to 30.3	4.9 to 60.1
L. Van Haandel[41]	Human blood	Total MTXPGs	10 to 500; 10	–2.9 to 6	–1.3 to 1.3	1.2 to 8.8	1.6 to 9.6
L. Van Haandel[42]	Human blood	$MTXPG_{1\text{-}7}$	2.5 to 100; 2.5	–23.2 to 9.8	–11.4 to 7.9	3.2 to 20.6	7.2 to 23.7
G. Chen[39]	Caco-2 cells	$MTXPG_{1\text{-}5}$	2 to 250; 2	–3.4 to 6.6	–5.45 to 12.1	3.4 to 15.1	4.29 to 18.4
M. Hroch[47]	Human blood	Total MTXPGs	25 to 400; 32.9	–17.3 to 3.1	–12.0 to –4.7	3.8 to 18.8	5.1 to 18.3
H. Li[43]	Human blood	Total MTXPGs	4.4 to 440; 2.6	NA	NA	1.8 to 3.1	2.8 to 7.8
T. Dervieux[40]	Red blood cells	$MTXPG_{1\text{-}7}$	10 to 50; 5	–11.8 to 12.3	–5.9 to 5.5	2.6 to 8.1	0.4 to 12.7
R. Durand[45]	HT29 cell line	$MTXPG_{1\text{-}4}$	NA	NA	NA	NA	NA
J. Jolivet[44]	MCF-7 cells	$MTXPG_{1\text{-}5}$	NA	NA	NA	NA	NA
J. Jolivet[46]	MCF-7 cells	$MTXPG_{1\text{-}3,6}$	NA	NA	NA	NA	NA

[Abbreviations] MTXPGs: methotrexate polyglutamates, $MTXPG_1$ (MTX): methotrexate, $MTXPG_2$: 4-amino-10-methylpteroyldiglutamic acid, $MTXPG_3$: 4-amino-10-methylptero-yltriglutamic acid, $MTXPG_4$: 4-amino-10-methylpteroyltetraglutamic acid, $MTXPG_5$: 4-amino-10-methylpteroylpentaglutamic acid, $MTXPG_6$: 4-amino-10-methylpteroylhexa-glutamic acid, $MTXPG_7$: 4-amino-10-methylpteroyl-heptaglutamic acid, LOQ: limit of quantitation, NA: not available.

1229.4 > 308.1 for $MTXPG_7$. However, an unidentified endogenous compound that exhibits the same *m/z* as MTX (455.2) and forms a 308.10 fragment was reported by Chen *et al.* and Van Haandel *et al.*[39,42] The authors declared that it did not interfere in the analysis. However, when Hawwa *et al.* optimized their MS parameters, they found that this unidentified endogenous compound could influence MTX analysis. So they selected the latter product ion (175.05) for the analysis of all MTXPGs.[38]

3·7 Fluorescence detection

Fluorescence detection of MTXPGs requires pre-column or post-column derivatization of MTXPGs under different conditions to generate oxidized or reduced fluorescent products (Fig. 2).[41,43,47] The most commonly used excitation and emission wavelength was 367 and 463 nm for both reduction and oxidation products.

For pre-column derivatization, derivatization reagent(s) were added to the sample prior to chromatographic separation.[6,41] Van Haandel *et al.* chose sodium dithionite to reduce MTXPGs to 2,4-diamino-6-methylpteridine within 45 min. The reduction product (2,4-diamino-6-methylpteridine) has higher fluorescence absorptivity and better chromatographic behavior than the oxidation product (2,4-diaminopteridine-6-carboxylic acid).[18,41] Total MTXPG concentrations determined by fluorescence detector were on average 30% higher than those observed by LC-MS/MS methods, possibly due to its poor selectivity.[41]

Post-column derivatization was performed after column separation and before detection by adding reagents to the eluent.[51] Compared to pre-column derivatization, post-column derivatization provides several disadvantages, including additional post column dead volume, complex equipment and baseline fluctuations.[51] Dervieux *et al.* successfully applied post-column photochemical derivatization reaction for total MTXPGs analysis,[40] while other researchers found interferences.[52] Van Haandel *et al.* improved chromatographic performance but failed to obtain specific and sensitive data, mainly due to the false identification and over-estimation.[42]

The fluorescent compound (2,4-diaminopterin-6-carboxy-aldehyde) formed by photo-oxidation will be further degraded to 2,4-diaminopterin.[47] Therefore, Li *et al.* chose a coulometric electrochemical cell, which displays stable and reproducible fluorescence signal.[43] Also, the addition of an online electrochemical cell between the HPLC column and the fluorescence detector did not affect the quality of the chromatogram.[43] However, studies have shown that when post-column derivatization was combined with HPLC/ultra performance liquid chromatography, the enlarged dead volume could increase peak width, which might affect analysis accuracy.[51]

3·8 UV-visible detection

MTXPGs have UV absorption at 254 and 313 nm, therefore, both 254[45,46] and 313 nm[44] have been reported for MTXPGs analysis. Samples were pretreated by boiling or TCA to remove proteins, and further purified by SPE. However, there has been no methodological literature using UV detection for 30 years, perhaps due to its low selectivity and sensitivity.

3·9 Method validation

3·9·1 Lower limit of quantitation and calibration curve

Due to the low concentration of MTXPGs, a low limit of quantitation (LOQ) is crucial for MTXPG analysis. The LOQ in LC-MS methods was 1 ng/mL, both for total and individual MTXPGs.[18,37,48] The LOQs of LC-FD methods for total and individual MTXPGs were 2.6 and 5 ng/mL, respectively.[40,43] Method validation details are illustrated in Table 2. For total MTXPG analysis, the linear range was 2.2 – 400 ng/mL for LC-MS/MS methods,[37,38] while it was 4.4 – 500 ng/mL for fluorescence methods.[41,43] For individual MTXPG analysis, the linear range was 0.97 – 400 ng/mL for LC-MS/MS methods,[38,48] and 10 – 50 ng/mL for fluorescence methods.[40] In general, the linear range was comparable between LC-MS/MS and fluorescence methods for both total and individual MTXPG analysis.

3·9·2 Matrix effect and recovery

Matrix effect is an significant drawback of LC-MS based methods. Positive electrospray ionization was applied for the

Table 3 Matrix effect and recovery for methotrexate polyglutamates analysis by liquid chromatography based methods

Author	Matrix; pretreatment	Analyte; detector	Internal standard	Matrix effect, %; recovery, %						
				$MTXPG_1$	$MTXPG_2$	$MTXPG_3$	$MTXPG_4$	$MTXPG_5$	$MTXPG_6$	$MTXPG_7$
K. Brady[36]	Dried capillary blood; PP	$MTXPG_3$; MS	d_3-MTX	—	—	NA; 87.6	—	—	—	—
A. F. Hawwa[38]	Dried blood spots; PP, SPE	Total MTXPGs; MS	No	85 to 115; 70	—	—	—	—	—	—
		$MTXPG_{1-5}$; MS	No	85 to 115; 71	85 to 115; 70	85 to 115; 27	85 to 115; 44	85 to 115; 49	—	—
E. den Boer[48]	Human blood; PP	$MTXPG_{1-5}$; MS	($^{13}C5$,^{15}N)-MTX	98; 98 to 100	99; 98 to 100	97; 98 to 100	95; 98 to 100	99; 98 to 100	—	—
X. Mo[37]	Human blood; PP, LLE	Total MTXPGs; MS	Doxofylline	167.2 to 198.3; 26.2 to 30.7	—	—	—	—	—	—
L. Van Haandel[18]	Red blood cells; PP	$MTXPG_{1-7}$; MS	d_3-MTX	NA; 48 to 54.2	NA; 60.4 to 65	NA; 67.7 to 72.7	NA; 64 to 72.8	NA; 63.9 to 76.3	NA; 62.2 to 78	NA; 53.7 to 85.9
L. Van Haandel[41]	Human blood; PP	Total MTXPGs; FD	No	107.7 to 111.7; 60.1 to 63.7	—	—	—	—	—	—
L. Van Haandel[42]	Human blood; PP, SPE	$MTXPG_{1-7}$; MS	No	NA; 31.2	NA; 33.4	NA; 39.6	NA; 44.2	NA; 43.0	NA; 50.6	NA; 47.8
G. Chen[39]	Caco-2 cells; LLE	$MTXPG_{1-5}$; MS	Aminopterin	93 to 102; 60 to 79	95 to 99; 73 to 83	100 to 102; 73 to 98	88 to 98; 71 to 78	100 to 101; 95 to 108	—	—
M. Hroch[47]	Human blood; PP	Total MTXPGs; FD	No	NA; 70.3 to 72.8	—	—	—	—	—	—
H. Li[43]	Human blood; LLE	Total MTXPGs; FD	No	NA; 87.9 to 118	—	—	—	—	—	—
T. Dervieux[40]	Red blood cells; PP	$MTXPG_{1-7}$; FD	No	NA; 66	NA; 65	NA; 65	NA; 66	NA; 79	NA; 80	NA; 60
R. Durand[45]	HT29 cell line; PP, SPE	$MTXPG_{1-4}$; UV	No	NA; 100	NA; 77 to 85	NA; 80 to 84	NA; 80 to 84	NA; 90 to 102	—	—
J. Jolivet[44]	MCF-7 cells; PP, SPE	$MTXPG_{1-5}$; UV	No	NA; 71	NA; 71	NA; 71	NA; 71	NA; 71	—	—
J. Jolivet[46]	MCF-7 cells; PP, SPE	$MTXPG_{1-3,6}$; UV	No	NA; 69 to 91	NA; 69 to 91	NA; 69 to 91	—	—	NA; 69 to 91	—

[Abbreviations] PP: protein precipitation, LLE: liquid–liquid extraction, SPE: solid phase extraction, d_3-MTX: methotrexate-d_3, $MTXPG_1$ (MTX): methotrexate, $MTXPG_2$: 4-amino-10-methylpteroyldiglutamic acid, $MTXPG_3$: 4-amino-10-methylptero-yltriglutamic acid, $MTXPG_4$: 4-amino-10-methylpteroyltetraglutamic acid, $MTXPG_5$: 4-amino-10-methylpteroylpentaglutamic acid, $MTXPG_6$: 4-amino-10-methylpteroylhexa-glutamic acid, $MTXPG_7$: 4-amino-10-methylpteroylheptaglutamic acid, NA: not available.

analysis of MTXPGs. To minimize quantitation errors caused by matrix, sample extraction, purification and separation are required. Only five articles reported their matrix effect results (Table 3). For total MTXPG analysis, Mo *et al.*[37] used PP for sample treatment and isocratic elution for separation, and they found stable matrix induced response enhancement (matrix effect: 167.2 to 198.3%, CV: 1.8 – 7.7%). Hawwa *et al.*[38] used PP combined with SPE for total MTXPG extraction and purification, and they obtained acceptable matrix effect without using IS. For individual MTXPG analysis, E. den Boer *et al.*[48] used 16% perchloric acid for PP, and their matrix effect was acceptable. Hawwa *et al.*[38] used PP combined with SPE for individual MTXPG extraction and purification, and acceptable matrix effect was obtained without using IS. Van Haandel *et al.*[18] remove proteins by boiling, and ion suppression was observed at the elution time of $MTXPG_1$ and $MTXPG_2$, therefore methotrexate-d_3 was used as IS to compensate the ion suppression effect for $MTXPG_1$ and $MTXPG_2$. In addition, the inter-day coefficient of variation of this method varied from 4.9 to 60.1%. Chen *et al.*[39] used acetonitrile for extraction of MTXPGs from Caco-2 cells, and no significant matrix effect was observed for $MTXPGs_{1-5}$.

Recovery is important for analysis especially when analyte level is low. Matrix type and pretreatment process are two major influential factors for recovery. Human erythrocyte hemolysate was the most commonly used matrix for MTXPG analysis. For total MTXPG analysis, Hawwa *et al.*[38] reported a recovery rate of 70% by using 70% perchloric acid combined with SPE for sample pretreatment. Horch *et al.*[47] obtained 70.3 – 72.8% recovery by using 0.8 M TCA in 40% (v/v) acetic acid for sample pretreatment. Using a combination of PP (methanol) and extraction (methylene chloride), Li *et al.*

Table 4 Stability of methotrexate polyglutamates in various biological matrices

Author	Matrix	Analyte	Analytes in matrix			Post-treatment	
			Room temperature/ bias, %	Long term/ bias, %	Freeze thaw/ bias, % (cycles)	Room temperature/ bias, %	Autosampler/ bias, %
K. Brady[36]	Dried capillary blood	$MTXPG_3$	30 days; NA	NA	NA	NA	NA
A. F. Hawwa[38]	Dried blood spots	$MTXPG_{1-5}$	2 months; 0.79 to 1.07	–80°C for 2 months; 0.89 to 1.14	NA	NA	NA
		Total MTXPGs	2 months; 0.85 to 1.01	–80°C for 2 months; 0.92 to 1.04	NA	NA	NA
E. den Boer[48]	Human blood	$MTXPG_{1-5}$	NA	–80°C for 3 month; –19 to 6	3; –7 to 7	NA	4°C for 14 days; –7 to 12
X. Mo[37]	Human blood	Total MTXPGs	4 h; –12.92 to 12	–80°C for 1 month; –11.88 to 10	2; –14.8 to 10.5	6 h; –6.5 to 11.76	
L.Van Haandel[18]	Human blood	$MTXPG_{1-7}$	NA	4°C for at least 1 month; –20 to 20	3; –20 to 20	NA	4°C for 24 h; –20 to 20
L. Van Haandel[41]	Human blood	Total MTXPGs	NA	NA	3; 5.9 to 24.4	NA	7°C for 24 h; 5.8 to 13.4
L. Van Haandel[42]	Human blood	$MTXPG_{1-7}$	NA	NA	NA	NA	NA
G. Chen[39]	Caco-2 cells	$MTXPG_{1-5}$	4 h; –13 to 16	–20°C for 30 days; –10 to 16	3; –13 to 10	NA	4°C for 24 h; –14 to 9
M. Hroch[47]	Human blood	$MTXPG_3$	NA	NA	2; –14.3 to –8	NA	25°C for 24 h; –15 to 15
H. Li[43]	Human blood	Total MTXPGs	NA	NA	NA	NA	NA
T. Dervieux[40]	Red blood cells	$MTXPG_{1-7}$	NA	2 to 8°C for 48 h; NA	NA	NA	NA
R. Durand[45]	HT29 cell line	$MTXPG_{1-4}$	NA	NA	NA	NA	NA
J. Jolivet[44]	MCF-7 cells	$MTXPG_{1-5}$	NA	NA	NA	NA	NA
J. Jolivet[46]	MCF-7 cells	$MTXPG_{1-3,6}$	NA	NA	NA	NA	NA

[Abbreviations] MTXPGs: methotrexate polyglutamates, $MTXPG_1$ (MTX): methotrexate, $MTXPG_2$: 4-amino-10-methylpteroyldiglutamic acid, $MTXPG_3$: 4-amino-10-methylptero-yltriglutamic acid, $MTXPG_4$: 4-amino-10-methylpteroyltetraglutamic acid, $MTXPG_5$: 4-amino-10-methylpteroylpentaglutamic acid, $MTXPG_6$: 4-amino-10-methylpteroylhexa-glutamic acid, $MTXPG_7$: 4-amino-10-methylpteroyl-heptaglutamic acid, NA: not available.

reported a high recovery rate (87.9 – 118%).[43] A low recovery (24.11 – 32.57%) was observed using TCA for PP.[37]

For individual MTXPGs, the recovery of $MTXPGs_{1-7}$ in erythrocyte pretreated by PP was 48 – 85.9[18] and 65 – 80%.[40] The recovery of $MTXPGs_{1-7}$ in human blood pretreated by PP combined with SPE was 31.2 – 50.6% (without IS), and the recovery increased with the increase of the glutamate residues.[42] The recovery of $MTXPGs_{1-5}$ in human blood pretreated by PP alone was 98 – 100%.[48] The recovery of $MTXPG_3$ in dry capillary blood pretreated by PP was 87.6%,[36] while the recovery of $MTXPGs_{1-5}$ in dry blood spots pretreated by PP combined with SPE was 27 – 71% (without IS).[38] Therefore, using SPE for sample purification could dramatically decrease recovery. The recovery of $MTXPGs_{1-6}$ in cells pretreated by PP combined with SPE or LLE alone was 60 – 108%.[39,44-46] Meesters[11] used matrix-assisted laser desorption/ionization-MS for individual MTXPG analysis using aminopterin as IS. The sample pretreatment combined PP (TCA) and SPE (Oasis HLB 96-well SPE plate), and the recovery was 71.3 – 97.7%.

3·9·3 Stability

$MTXPGs_{1-5}$ in dried blood were stable at room temperature and –80°C for 2 months.[38] In human blood, total MTXPGs were stable at room temperature for 4 h.[37,39] $MTXPGs_{1-5}$ were stable in human blood at –80°C for 1 month, while $MTXPGs_{1-7}$ were stable at 4°C for 1 month[18] and at 2 – 8°C for 48 h.[48] In Caco-2 cells, $MTXPGs_{1-5}$ were stable at room temperature for 4 h and at –20°C for 1 month.[39] After 3 freeze-thaw cycles, MTXPGs were stable in human blood and Caco-2 cells.[18,37,39,41,47,48] For post extracted samples, total MTXPGs was stable at room temperature for 6 h[37] and at 7°C for 24 h,[41] while $MTXPGs_{1-5}$ were stable at 25°C for 24 h[47] and at 4°C for 14 days.[48] After sample treatment, $MTXPGs_{1-7}$ released from human blood and $MTXPGs_{1-5}$ released from Caco-2 cells were stable at 4°C for 24 h.[18,39]

4 Conclusion

This paper reviewed several published chromatographic-based methods for MTXPG analysis. These methods can be divided into two categories according to analytes, total MTXPGs and individual MTXPGs. Gamma-glutamyl hydrolase in human plasma could convert MTXPGs into MTX, and low speed centrifugation and twice washing with saline were used to remove this enzyme for individual MTXPG analysis. Enzyme hydration was commonly used to convert MTXPGs to MTX for total MTXPG analysis. To release the analytes from cells, dry process, freeze-thaw cycle, and hypotonic solution like water were used, and freeze-thaw cycle was suggested for its simplicity and efficacy. Mercaptoethanol phosphate and ascorbic acid were used to avoid the decomposition of MTXPGs during the analyte release process. Acid precipitation, LLE and SPE alone or in combination with other pretreatment strategies were used for analyte extraction and purification. Perchloric acid precipitation coupled with SPE purification was suggested for its efficacy. Gradient elution by acetonitrile on reversed phase

column was commonly used for separation in the literature and these strategies were suggested for method development. Fluorescence, UV or MS have been used for the detection of MTXPGs, and MS was suggested due to the following reasons: reduction or oxidation of analytes were required before fluorescence detection; MS was much more selective and sensitive than fluorescence. MTXPGs were stable under routine analysis process.

Various methods have been successfully developed for total and/or individual MTXPG analysis, but many questions still remained to be solved. They include the conversion rate of MTXPGs to MTX by various methods for total MTXPG analysis; the lack of isotope IS for MTXPG analysis by MS; the relatively long separation time; lack of commercial kit for MTXPG analysis by chromatographic-based methods for routine clinical practices; whether total MTXPG analysis could replace individual MTXPG analysis for MTX dose adjustment. It is hoped that this review will be helpful for researchers in the development of their own methods.

5 Acknowledgements

Thanks are given to our patients and nurses. This work was supported by the Beijing Municipal Administration of Hospitals (ZYLX201827), National Key R&D Program of China (2020YFC2008306), and Beijing Municipal Health Bureau (2018000021469G238).

6 References

1. J. M. Kremer, *Arthritis Rheum.*, **2004**, *50*, 1370.
2. P. Koźmiński, P. K. Halik, R. Chesori, and E. Gniazdowska, *Int. J. Mol. Sci.*, **2020**, 21.
3. E. S. Chan and B. N. Cronstein, *Nat. Rev. Rheumatol.*, **2010**, *6*, 175.
4. S. Mei, X. Shi, Y. Du, Y. Cui, C. Zeng, X. Ren, K. Yu, Z. Zhao, and S. Lin, *J. Pharm. Biomed. Anal.*, **2018**, *158*, 300.
5. S. Mei, Y. Cui, D. Zhang, C. Zeng, X. Ren, K. Yu, S. Lin, and Z. Zhao, *Anal. Sci.*, **2020**, *36*, 1479.
6. F. Karami, S. Ranjbar, Y. Ghasemi, and M. Negahdaripour, *J. Pharm. Anal.*, **2019**, *9*, 373.
7. M. F. Silva, C. Ribeiro, V. M. F. Goncalves, M. E. Tiritan, and A. Lima, *Biomed. Chromatogr.*, **2018**, *32*, e4159.
8. B. C. Bostrom, G. R. Erdmann, and B. A. Kamen, *J. Pediatr. Hematol. Oncol.*, **2003**, *25*, 114.
9. T. Yamamoto, K. Shikano, T. Nanki, and S. Kawai, *Sci. Rep.*, **2016**, *6*, 35615.
10. J. M. Dalrymple, L. K. Stamp, J. L. O'Donnell, P. T. Chapman, M. Zhang, and M. L. Barclay, *Arthritis Rheum.*, **2008**, *58*, 3299.
11. R. J. Meesters, E. den Boer, R. de Jonge, J. Lindemans, and T. M. Luider, *Rapid Commun. Mass Spectrom.*, **2011**, *25*, 3063.
12. S. Pan, J. Korell, L. K. Stamp, and S. B. Duffull, *Eur. J. Clin. Pharmacol.*, **2015**, *71*, 1509.
13. S. I. Rahman, E. Siegfried, K. H. Flanagan, and E. S. Armbrecht, *J. Am. Acad. Dermatol.*, **2014**, *70*, 252.
14. A. F. Hawwa, A. AlBawab, M. Rooney, L. R. Wedderburn, M. W. Beresford, and J. C. McElnay, *Arthritis Res. Ther.*, **2015**, *17*, 295.
15. S. Pan, L. K. Stamp, S. B. Duffull, M. L. Barclay, J. M. Dalrymple, J. Drake, M. Zhang, and J. Korell, *Clin. Pharmacokinet.*, **2014**, *53*, 1161.
16. M. L. Becker, L. van Haandel, R. Gaedigk, A. Lasky, M. Hoeltzel, J. Stobaugh, and J. S. Leeder, *Arthritis Rheum.*, **2010**, *62*, 1803.
17. M. B. Ćalasan, E. den Boer, M. C. de Rotte, S. J. Vastert, S. Kamphuis, R. de Jonge, and N. M. Wulffraat, *Ann. Rheum. Dis.*, **2015**, *74*, 402.
18. L. van Haandel, M. L. Becker, T. D. Williams, J. S. Leeder, and J. F. Stobaugh, *Bioanalysis*, **2011**, *3*, 2783.
19. C. Roubille, V. Richer, T. Starnino, C. McCourt, A. McFarlane, P. Fleming, S. Siu, J. Kraft, C. Lynde, J. Pope, W. Gulliver, S. Keeling, J. Dutz, L. Bessette, R. Bissonnette, and B. Haraoui, *Ann. Rheum. Dis.*, **2015**, *74*, 480.
20. J. Li and P. Gwilt, *Cancer Chemother. Pharmacol.*, **2002**, *50*, 373.
21. K. I. Halilova, E. E. Brown, S. L. Morgan, S. L. Bridges, Jr., M. H. Hwang, D. K. Arnett, and M. I. Danila, *Int. J. Rheumatol.*, **2012**, *2012*, 978396.
22. A. Lima, M. Bernardes, R. Azevedo, J. Monteiro, H. Sousa, R. Medeiros, and V. Seabra, *Toxicol. Sci.*, **2014**, *142*, 196.
23. G. J. Leclerc, T. A. York, T. Hsieh-Kinse, and J. C. Barredo, *Leuk. Res.*, **2007**, *31*, 293.
24. G. J. Leclerc, C. Mou, G. M. Leclerc, A. M. Mian, and J. C. Barredo, *Leukemia*, **2010**, *24*, 552.
25. E. den Boer, M. C. de Rotte, S. M. Pluijm, S. G. Heil, J. M. Hazes, and R. de Jonge, *J. Rheumatol.*, **2014**, *41*, 2167.
26. T. Dervieux, M. E. Weinblatt, A. Kivitz, and J. M. Kremer, *Ann. Rheum. Dis.*, **2013**, *72*, 908.
27. S. L. Goss, C. E. Klein, Z. Jin, C. S. Locke, R. C. Rodila, H. Kupper, G. R. Burmester, and W. M. Awni, *Clin. Ther.*, **2018**, *40*, 309.
28. A. K. Pannu, *Curr. Drug. Metab.*, **2019**, *20*, 714.
29. M. F. Silva, C. Ribeiro, V. M. F. Gonçalves, M. E. Tiritan, and Á. Lima, *Biomed. Chromatogr.*, **2018**, *32*, e4159.
30. T. Dervieux, D. Furst, D. O. Lein, R. Capps, K. Smith, M. Walsh, and J. Kremer, *Arthritis Rheum.*, **2004**, *50*, 2766.
31. C. Takahashi, Y. Kaneko, Y. Okano, H. Taguchi, H. Oshima, K. Izumi, K. Yamaoka, and T. Takeuchi, *RMD Open*, **2017**, *3*, e000363.
32. J. Korell, S. B. Duffull, J. M. Dalrymple, J. Drake, M. Zhang, M. L. Barclay, and L. K. Stamp, *Br. J. Clin. Pharmacol.*, **2014**, *77*, 493.
33. H. J. Mohamed, M. J. Sorich, S. M. Kowalski, R. McKinnon, S. M. Proudman, L. Cleland, and M. D. Wiese, *Eur. J. Clin. Pharmacol.*, **2015**, *71*, 411.
34. M. C. Montesinos, M. Takedachi, L. F. Thompson, T. F. Wilder, P. Fernández, and B. N. Cronstein, *Arthritis Rheum.*, **2007**, *56*, 1440.
35. A. Sandhu, V. Dhir, A. Bhatnagar, V. Dhawan, J. Kaur, A. Sood, S. Naidu, S. Ahmad, N. Varma, A. Sharma, and S. Sharma, *Ther. Drug Monit.*, **2017**, *39*, 157.
36. K. Brady, Y. Qu, D. Stimson, R. Apilado, R. Vezza Alexander, S. Reddy, P. Chitkara, J. Conklin, T. O'Malley, C. Ibarra, and T. Dervieux, *J. Appl. Lab. Med.*, **2019**, *4*, 40.
37. X. Mo, Y. Wen, B. Ren, J. Chen, C. Xu, X. Wang, M. Huang, and X. Chen, *J. Chromatogr. B*, **2012**, *907*, 41.
38. A. F. Hawwa, A. Albawab, M. Rooney, L. R. Wedderburn, M. W. Beresford, and J. C. McElnay, *PLoS One*, **2014**, *9*, e89908.
39. G. Chen, J. P. Fawcett, M. Mikov, and I. G. Tucker, *J. Pharm. Biomed. Anal.*, **2009**, *50*, 262.
40. T. Dervieux, D. Orentas Lein, J. Marcelletti, K. Pischel, K. Smith, M. Walsh, and R. Richerson, *Clin. Chem.*, **2003**, *49*, 1632.
41. L. v. Haandel, A. A. M. Heemskerk, M. L. Becker, J. S. Leeder, and J. F. Stobaugh, *Anal. Methods*, **2010**, *2*, 831.

42. L. van Haandel, M. L. Becker, J. S. Leeder, T. D. Williams, and J. F. Stobaugh, *Rapid Commun. Mass Spectrom.*, **2009**, *23*, 3693.
43. H. Li, W. Luo, Q. Zeng, Z. Lin, H. Luo, and Y. Zhang, *J. Chromatogr. B*, **2007**, *845*, 164.
44. J. Jolivet, R. L. Schilsky, B. D. Bailey, and B. A. Chabner, *Ann. N. Y. Acad. Sci.*, **1982**, *397*, 184.
45. R. Durand, G. Fabre, J. P. Cano, J. Catalin, O. A. Ahmed, and S. Just, *J. Appl. Toxicol.*, **1983**, *3*, 189.
46. J. Jolivet and R. L. Schilsky, *Biochem. Pharmacol.*, **1981**, *30*, 1387.
47. M. Hroch, J. Tuková, P. Dolezalová, and J. Chládek, *Biopharm. Drug Dispos.*, **2009**, *30*, 138.
48. E. den Boer, R. J. Meesters, B. D. van Zelst, T. M. Luider, J. M. Hazes, S. G. Heil, and R. de Jonge, *Anal. Bioanal. Chem.*, **2013**, *405*, 1673.
49. M. W. Dong, J. Lepore, and T. Tarumoto, *J. Chromatogr.*, **1988**, *442*, 81.
50. L. C. Garratt, C. A. Ortori, G. A. Tucker, F. Sablitzky, M. J. Bennett, and D. A. Barrett, *Rapid Commun. Mass Spectrom.*, **2005**, *19*, 2390.
51. A. Jones, S. Pravadali-Cekic, G. R. Dennis, and R. A. Shalliker, *Anal. Chim. Acta*, **2015**, *889*, 58.
52. E. P. Quinlivan, A. D. Hanson, and J. F. Gregory, *Anal. Biochem.*, **2006**, *348*, 163.