Developing and Investigating Response Markers to Methotrexate in Rheumatoid Arthritis

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Contents

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List of Abbreviations

2,4-diamino-N10-methylpteroic acid	DAMPA
Acetonitrile	ACN
Adenosine monophosphate	AMP
Alanine transaminase	ALT
American College of Rheumatology	ACR
Aminoimidazolecarboxamidotibonucleotide	AICAR
Anti-citrullinated protein antibodies	ACPA
Anti-cyclic peptide	Anti-CCP
Area under the curve	AUC
Aspartate transaminase	AST
Below the lower limit of quantification	BQL
Bioavailability	F
Case report forms	CRF
Central Manchester University Hospitals NHS Foundation Trust	CMFT
Centre for Advanced Discovery and Experimental Therapeutics	CADET
Chromatin Immunoprecipitation-sequencing	CHiP-Seq
Chromosome capture conformation	3C
Clearance	Cl
Coefficient of variation	CV
Compliance-Questionnaire-Rheumatology	CQ-R
Computational Shared Facility	CSF
Confidence interval	CI
Connexin	Cx
C-reactive protein	CRP
Deuterium-labelled MTX	MTX-d_3
Dihydrofolate reductase	DHFR
Disability Adjusted Life Year	DALY
Disease activity score	DAS
Disease modifying anti-rheumatic drugs	DMARD
ENCyclopedia of DNA Elements	ENCODE
Erythrocyte sedimentation rate	ESR
Estimated glomerular filtration rate	eGFR
European League Against Rheumatism Collaborative	EULAR
European Medicines Agency	EMA
Expression quantitative trait loci	EQTL
Fibroblast-like synoviocytes	FLS
Fluorescent polarisation immunoassay	FPIA
Folylpolyglutamate	FPGS
Food and Drug Administration	FDA
Forced expiratory volume in one second	FEV_1
Forced vital capacity	FVC
Genome wide association study	GWAS

Glomerular filtration rate	GFR
Haemoglobin	Hb
Han Chinese individuals	CHB
High performance liquid chromatography triple-quadrupole mass spectrometry	HPLC-QQQ-MS
Highly active anti-retroviral treatment	HAART
High-performance liquid chromatography	HPLC
Human immunodeficiency virus	HIV
Human leucocyte antigen	HLA
Ibadan, Nigeria	YRI
Identity by descent	IBD
Intercompartmental clearance	Q
Interquartile range	IQR
Leflunomide-induced pneumonitis	LFN-P
Linkage disequilibrium	LD
Lower limit of detection	LLOD
Lower limit of quantification	LLOQ
Lymphoblastoid cell lines	LCL
Macrophage-like synoviocytes	MLS
Major histocompatibility complex	MHC
Matrix metalloproteinases	MMP
Medical Research Council	MRC
Medication Adherence Revised Scale	MARS
Medication Possession Ratio	MPR
Medicines and Healthcare products Regulatory Agency	MHRA
Methotrexate	MTX
Methotrexate-induced interstitial lung disease	MI-ILD
Methotrexate-pneumonitis	MTX-P
Minor allele frequency	MAF
MTX-polyglutamate	MTX-PG
National Centre for Biotechnology Information	NCBI
National Institute for Health and Care Excellence	NICE
National Institute for Health Research	NIHR
National Patient Safety Agency	NPSA
Non-steroidal anti-inflammatory drugs	NSAIDS
Odds ratio	OR
Polymerase chain reaction	PCR
Prediction interval	PI
Principle component analysis	PCA
Randomised controlled trial	RCT
Research Ethics Committee	REC
Rheumatoid Arthritis	RA
Rheumatoid Arthritis Medication Study	RAMS
Rheumatoid Factor	RF
Ritchie Articular Index	RAI

Selected reaction monitoring	SRM
Sequence specific primers	SSP
Signal to noise ratio	SNR
Single nucleotide polymorphism	SNP
Solid phase extraction	SPE
Solute carrier family 19 member 1	SCL19A1
Solute carrier organic anion transporter 1B1	SLCO1B1
Standard deviation	SD
Summaries of Product Characteristics	SPC
Tokyo, Japan	JPT
Transcription factor	TF
United Kingdom	UK
Untranslated region	UTR
Upper limit of normal	ULN
Visual analogue score	VAS
Volume of distribution	V
Wellcome Trust Manchester Clinical Research Facility	WTCRF
Western European ancestry	CEU
White cell count	WCC

Abstract

The University of Manchester
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Developing and Investigating Response Markers to Methotrexate in Rheumatoid Arthritis
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Introduction

Rheumatoid arthritis (RA) is a multisystem disease associated with early mortality. Methotrexate (MTX) is the first-line therapy in RA but is associated with significant adverse events and response is not universal. There is, therefore, a need to identify early those patients with RA unlikely to respond or develop toxicity to MTX. One of the major influences on drug response is adherence and MTX can cause a range of side effects known to impact on adherence such as pneumonitis (MTX-P). The gold standard measurement of adherence would be direct detection of MTX or its metabolites in a biochemical assay. Currently, there are no reliable markers that predict response to MTX but some studies have suggested measurement of MTX levels may predict response. Previous studies have suggested that MTX-P may occur in individuals genetically predisposed to the disease. The aims of this research are to i) develop an assay to measure MTX levels; ii) test the ability of the assay to measure adherence; iii) investigate if MTX levels are associated with response; and iv) conduct a genome wide association study (GWAS) investigating MTX-P.

Methods

An assay to measure MTX and 7-OH-MTX in urine and plasma was developed using HPLC-SRM-MS and the assay was used to measure levels in a cohort of RA patients to develop a pharmacokinetic model. Simulations of the model were used to determine the ability of the assay to monitor adherence and the model was validated in a separate cohort of patients with RA. An observational study of RA patients was used to measure MTX and 7-OH-MTX levels to investigate if levels are associated with response. Finally, a GWAS investigating MTX-P was conducted.

Results

Results of the pharmacokinetic model demonstrated that MTX is the preferred analyte to monitor adherence. The model was validated in a separate cohort of patients with RA demonstrating the ability of the assay to measure adherence. MTX levels were not associated with disease response in this cohort. A GWAS of MTX-P demonstrated three SNPs associated with disease ($p < 5 \times 10^{-5}$) with subsequent bioinformatics analysis showing a potential functional role for rs7514182.

Conclusion

Adherence to MTX may be a significant barrier to patients achieving full response to therapy. The development of a direct test to detect adherence based on measuring MTX levels using HPLC-SRM-MS has been developed in urine and blood. The assay was shown to be accurate in several domains from EMA guidelines and was validated in a separate cohort of patients. Finally, this program of work has investigated genetic markers associated with MTX-P. The results demonstrated a potential SNP associated with disease which demonstrates a functional role in the development of pulmonary fibrosis.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Finally, I thank my family for their endless support from medical school, life as a junior doctor and as a clinical research fellow.

Preface

I graduated from the University of Newcastle upon Tyne with MBBS (with merit) in 2005. Whilst an undergraduate, I undertook an intercalated degree and was awarded an MRes in Biomolecular Sciences with distinction with funding from the Arthritis Research Campaign. During this time, I investigated the role of matrix metalloproteinase 3 promoter polymorphism in the susceptibility, severity and progression of rheumatoid arthritis using a candidate gene approach. Following graduation, I completed my foundation and core medical training within the North West Deanery. I was successful in applying to a specialty training post in Rheumatology and General Internal Medicine. I secured the MRCP(UK) postgraduate examination in 2010. During specialty training I embarked on the part-time MSc Clinical Rheumatology degree at the University of Manchester and was awarded distinction in 2012. During this time I analysed a genome wide association study of psoriatic arthritis. In 2013, I was awarded a North West England MRC Clinical Pharmacology and Therapeutics Clinical Research Training Fellowship and in 2014 I was awarded a National Institute of Health Research Manchester Musculoskeletal Biomedical Research Unit grant for the development of a methotrexate assay.

Publications Arising from this Thesis

Bluett J, Riba-Garcia I, Hollywood K, et al. A HPLC-SRM-MS based method for the detection and quantification of methotrexate in urine at doses used in clinical practice for patients with rheumatological disease: a potential measure of adherence. Analyst. 2015;140:1981-7.

Publications Arising During the Course of the Thesis

Bowes J, Budu-Aggrey A, Huffmeier U, et al. Dense genotyping of immune-related susceptibility loci reveals new insights into the genetics of psoriatic arthritis. Nat Commun. 2015;6:6046.

Bluett J, Morgan C, Thurston L, et al. Impact of inadequate adherence on response to subcutaneously administered anti-tumour necrosis factor drugs: results from the Biologics in Rheumatoid Arthritis Genetics and Genomics Study Syndicate cohort. Rheumatology (Oxford, England). 2015;54:494-9.

Hope H, Bluett J*, Hyrich K, Cordingley L, Verstappen SM. Psychological factors predict adherence to methotrexate (MTX) in rheumatoid arthritis (RA); findings from a systematic review of rates, predictors and associations with patient outcomes. Arthritis and Rheumatology. 2014;66:S892-S93.

Bluett J, Ibrahim I, Plant D, et al. Association of a complement receptor 1 gene variant with baseline erythrocyte sedimentation rate levels in patients starting anti-TNF therapy in a UK rheumatoid arthritis cohort: results from the Biologics in Rheumatoid Arthritis Genetics and Genomics Study Syndicate cohort. Pharmacogenomics J. 2014;14:171-5.

Bluett J, Davies C, Harris J, Herrick A. Cervical spine calcinosis in systemic sclerosis. J Rheumatol. 2013;40:1617-18.

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Chapter 1: Introduction

1 Introduction

1.1 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a common multisystem autoimmune disease that affects up to 1% of the adult population and has a 3:1 female:male preponderance [1]. The hallmark of RA is symmetrical synovitis with joint destruction; however, significant extra-articular features can also be present [2].

1.1.1 The Clinical Features of RA

The range of RA presentations is broad, as is the clinical course; ranging from mild, self-limiting arthritis to rapidly progressive joint inflammation with destruction. Most patients experience joint pain, stiffness and swelling due to the synovitis and early morning stiffness, which typically lasts more than an hour. The smaller joints of the hands are often affected but all synovial joints may be involved, including the cervical spine. The joints become swollen, erythematous and warm due to inflammation as shown in Figure 1-1 [2].

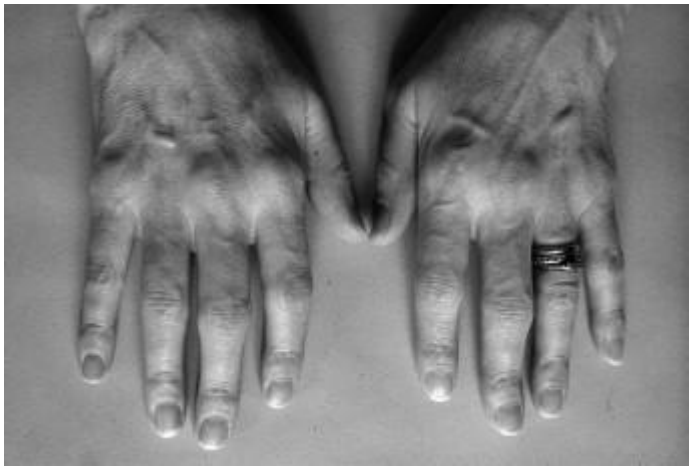


Figure 1-1. Early rheumatoid arthritis with active synovitis demonstrated by swollen joints. [a]

With ongoing inflammation within the joint, irreversible joint destruction occurs causing joint deformities as shown in Figure 1-2 [3].

[a] This article was published in Rheumatology, volume 1, Hochberg A. J. Silman J. S. Smolen M. E. Weinblatt and M. H. Weisman, Clinical features of Rheumatoid arthritis, p 770, Copyright Elsevier (2003).

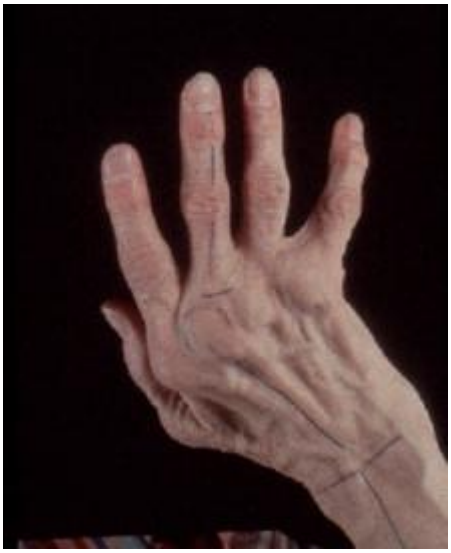


Figure 1-2. Rheumatoid hand demonstrating destructive erosive disease with ulnar deviation and Z-thumb deformities. [b]

However, not all patients will progress over time to develop severe joint destruction and the time until patients develop joint destruction varies [4]. RA not only affects the musculoskeletal system; extra-articular features may include anaemia, liver function abnormalities, interstitial lung disease, keratoconjunctivitis sicca, peripheral neuropathy and membranous nephropathy [2]. RA is associated with reduced life expectancy of three to ten years compared to the general population and is also associated with significant morbidity [5, 6]. Co-morbidities such as respiratory disease and cardiovascular disease are more common in RA [7]. An important co-morbidity is interstitial lung disease, a chronic fibrotic lung disease present in up to 47% of patients with RA causing significant shortness of breath [8]. Prognosis of rheumatoid interstitial lung disease is poor with a median survival of three years following diagnosis [9]. Physical disability is an important outcome in RA and increases as joint damage increases; 32% of adults with RA are unable to work after only five years of disease [10-12]. This represents significant financial costs to both the patient and the state. A disability adjusted life year (DALY) is a measure of disability and mortality developed by the World Health Organisation and the World Bank. One DALY represents one lost year of healthy life. Within the United Kingdom (UK), it is estimated that RA causes 103 DALYs lost per 100,000 population and that the national annual direct and indirect cost of RA is €6,577 million per annum [13].

The disease is associated with rheumatoid factor (RF) and/or anti-citrullinated protein antibodies (ACPA) in the blood in approximately 88% and 45%, respectively, of early inflammatory arthritis patients [14, 15]. RF is an antibody targeting the Fc portion of IgG. Whilst its role in the

[b] Reprinted from International Congress Series, 1295, Sollerman, C, How I do it—MP joint arthroplasty, 144-153., Copyright (2006), with permission from Elsevier.

pathogenesis of RA is yet to be elucidated, it is a useful non-specific marker for classification purposes of RA. The presence of RF is, however, non-diagnostic as false-positives occur; for example, in infectious endocarditis [16]. ACPA are antibodies directed against citrulline containing proteins and commonly detected using the anti-cyclic peptide (anti-CCP) antibody test. Citrulline is an amino acid developed by post-translational modification of arginine [17]. It is hypothesised that smoking is an environmental trigger that leads to the development of an immune response against citrullinated proteins in patients with a genetic predisposition which leads to the development of RA [18]. The presence of anti-CCP antibodies, which detect many ACPAs, has a specificity and sensitivity of 95% and 68% for RA respectively [19]. Anti-CCP is a more specific test compared to RF for RA. Patients with ACPA and/or RF are termed seropositive RA and have a worse prognosis compared to seronegative with a higher burden of joint erosion [20, 21].

1.1.2 The Classification Criteria of RA

There is currently no biological test to confirm if someone has RA but a set of clinical characteristics are used to aid diagnosis. This has the inherent problem of misclassification as no set of criteria will be 100% accurate; there will therefore be false negatives and false positives. Criteria for RA are hence termed classification criteria and not diagnostic criteria. In the development of classification criteria, a balance must be found between the stringency of exclusion criteria to reduce the false positive rate (increased specificity) whilst allowing for a high enough sensitivity (ability to detect true positives) to correctly classify patients with RA. For example, polyarticular gout is a form of polyarthritis, characterised by high levels of serum uric acid that may mimic RA. If the criteria excluded patients with raised uric acid levels, this would reduce the chance of falsely classifying patients with gout as RA but it would not allow for a dual diagnosis of gout and RA. Classification criteria are required for case definition of patients to be used in clinical research. The aim is to create a set of criteria so that case cohorts are more homogenous, thus reducing heterogeneity. Case definition heterogeneity reduces the power of studies because, when the study includes patients with different diseases, bias may be introduced and power to detect associations with specific disease subsets is also reduced.

In 2010, the American College of Rheumatology (ACR) and European League Against Rheumatism Collaborative (EULAR) formed a joint working group in order to revise the previous 1987 classification criteria for RA (Table 1-1) [22]. The 2010 classification criteria for RA have a reported sensitivity and specificity of 73.5 and 71.4%, respectively [23].

Target population (Who should be tested?): Patients who	
1) have at least 1 joint with definite clinical synovitis (swelling)	
2) with the synovitis not better explained by another disease	
Classification criteria for RA (score-based algorithm: add score of categories A–D; a score of ≥6/10 is needed for classification of a patient as having definite RA)	
A. Joint involvement	
1 large joint	0
2 – 10 large joints	1
1 – 3 small joints (with or without involvement of large joints)	2
4 – 10 small joints (with or without involvement of large joints)	3
> 10 joints (at least 1 small joint)	5
B. Serology‡	
Negative rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA)	0
Low-positive RF or low-positive ACPA	2
High-positive RF or high-positive ACPA	3
C. Acute phase reactants (at least 1 test results is needed for classification)	
Normal CRP and normal ESR	0
Abnormal CRP or abnormal ESR	1
D. Duration of symptoms	
< 6 weeks	0
≥ 6 weeks	1

Table 1-1. Revised 2010 ACR/EULAR Criteria for the Classification of Rheumatoid Arthritis. ‡ Negative refers to IU values that are less than or equal to the upper limit of normal (ULN) for the laboratory and assay; low-positive refers to IU values that are higher than the ULN but ≤3 times the ULN for the laboratory and assay; high-positive refers to IU values that are >3 times the ULN for the laboratory and assay. Adapted from Aletaha et al. [22].

1.1.3 Pathogenesis of RA

RA is an autoimmune disease, characterised by immune destruction of the joints. However, the molecular mechanisms underlying RA are yet to be fully uncovered.

A normal joint, as shown in Figure 1-3, is surrounded by a joint capsule that protects and supports it [24]. Hyaline cartilage covers and cushions the ends of adjacent bones during joint movement and compression. Hyaline cartilage is a connective tissue containing cells (chondrocytes), fibrous macromolecules and a ground substance (matrix). The matrix contains water, collagen types II, V, VI, IX and XI, proteoglycans, enzymes, growth factors and lipids [25]. Type II collagen is vital in cartilage; the fibrils form a network with negatively charged proteoglycans trapping large amounts of water and salts, allowing cartilage to be compressed like a sponge. Molecules such as fibronectin and laminins bind to many cartilage molecules and chondrocytes through interaction with integrins (transmembrane proteins that engage cells with their environment) maintaining cartilage structure. The joint capsule is lined with the synovium which produces synovial fluid, a

clear fluid that lubricates and nourishes the cartilage inside the joint capsule. Within the normal synovium are macrophage-like synoviocytes (MLS) and fibroblast-like synoviocytes (FLS). MLS cells express receptors for the Fc domains of IgG, suggesting an immunological role in the development of RA. FLS cells produce hyaluronic acid, a lubricating fluid. Associated with many joints is the enthesis which anchors a muscle or tendon to the bone allowing the muscle to contract and the joint to flex (Figure 1-3).

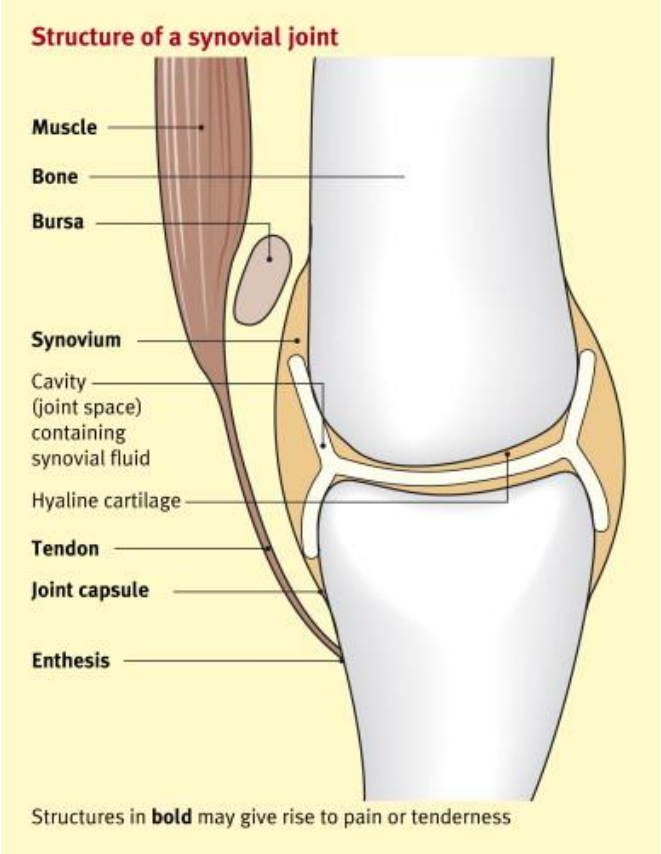


Figure 1-3. Diagram of a joint.[c]

In RA, the synovium transforms becoming hypercellular with an inflammatory cell infiltrate including macrophages, T and B lymphocytes, dendritic cells and natural killer cells as shown in Figure 1-4.

[c] Reprinted from Medicine, 38, Dacre, J., Worrall, J., Rheumatological Examination, 133-138., Copyright (2010), with permission from Elsevier.

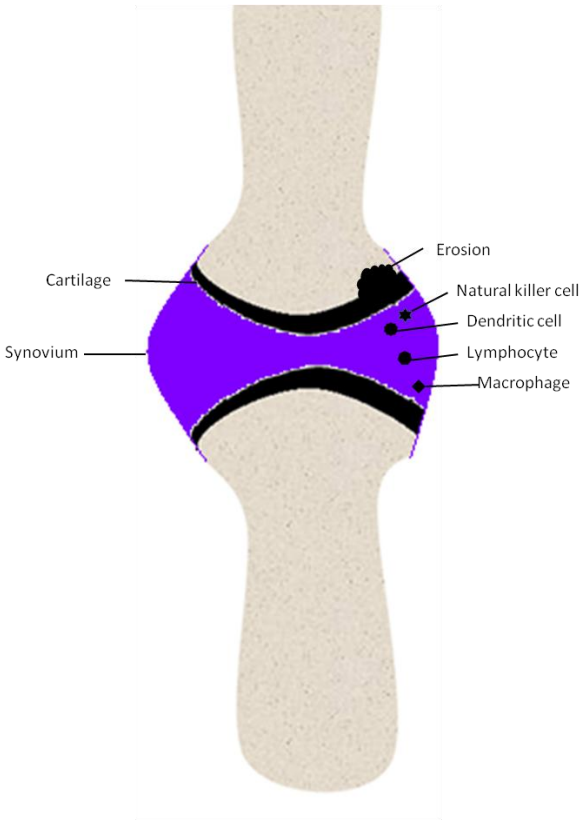


Figure 1-4. Diagram illustrating the inflamed joint in RA.

At the interface between the synovium and cartilage is a transformed synovium, termed pannus, which covers the cartilage with fibroblast cells that degrade the extracellular matrix of cartilage. Once joint destruction has occurred, re-modelling of the bone will not return the joint to its pre-diseased state. It is therefore essential that treatment of RA aims to reduce bone and joint destruction to prevent early disability and that effective treatment is started early in the disease process.

1.1.4 Measuring Disease Activity and Response in RA – The Disease Activity Score-28 and EULAR Response Criteria

In order to assess whether a treatment is effective or not, validated measures of efficacy are required. Physician's clinical judgement does not adequately predict which patients are in remission and which patients will develop joint damage [26]. In RA, the aim of treatment is to reduce the level of joint inflammation, reduce disease activity and ultimately prevent joint damage. One of the most commonly used measures of disease activity is based on change in the disease activity score (DAS-28). DAS -28 is a composite score designed to assess disease activity in RA with a continuous scale. DAS-28 was initially developed using data from a prospective study of patients with early RA (n=227), where decisions to start or stop therapy classified patients as high or low disease activity [27]. Discriminant analysis was used to determine factors that discriminate

between high and low disease activity. The DAS-28 comprises of a 28 swollen and tender joint count (Figure 1-5), a visual analogue score (VAS: 0-100mm, Figure 1-6) and an objective biochemical marker of inflammation (ESR (erythrocyte sedimentation rate) or CRP (C-reactive protein)). CRP is an acute phase protein, produced by the liver, with pro-inflammatory effects [28]. ESR is the rate at which erythrocytes sediment over an hour, ESR is increased by many disease states including inflammation and infection [29]. The variables are combined in a composite score as shown in Box 1-1.

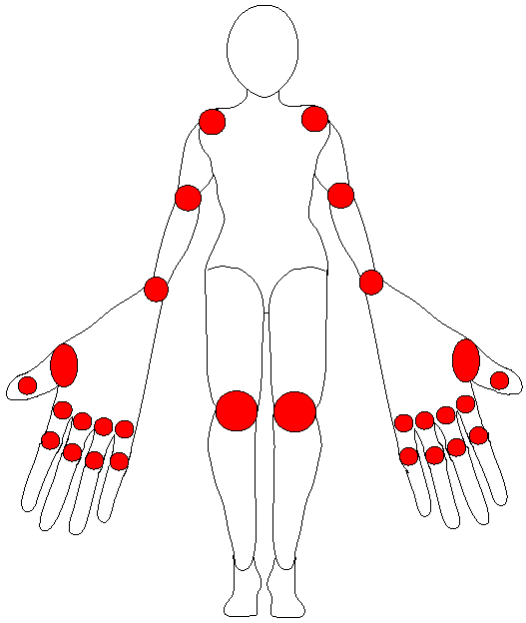


Figure 1-5. Diagram of joints used for the 28 swollen and tender joint counts.

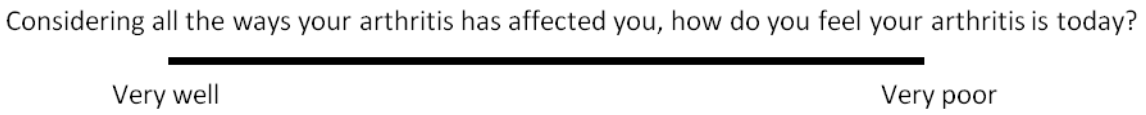


Figure 1-6. Visual analogue score used to assess patients global assessment of disease activity.

$$DAS(CRP) - 28 = 0.56 \times \sqrt{28TJC} + 0.28 \times \sqrt{28SJC} + 0.36 \times \ln(CRP + 1) + 0.014 \times GH + 0.96$$

$$DAS(ESR) - 28 = 0.56 \times \sqrt{28TJC} + 0.28 \times \sqrt{28SJC} + 0.70 \times \ln ESR + 0.014 \times GH$$

Box 1-1. Calculation for the DAS-28(CRP) and DAS-28(ESR). TJC = tender joint count. SJC = swollen joint count. GH = patient global assessment of disease activity.

The DAS-28 has been validated in several studies and has been shown to correlate with functional disease activity (Health Assessment Questionnaire) [27, 30, 31], radiographic progression [27, 32] and increases in DAS-28 have been associated with worsening function [33]. A DAS-28 change of

1.2 is considered clinically significant [34]. The EULAR response criteria have been developed to categorise response to treatment over a period of time and have been validated in previous studies [34, 35]. The EULAR response criteria categorise treatment response as good, moderate or no response according to change in DAS-28 and the level of DAS-28 reached (Table 1-2).

DAS-28 at endpoint	**Improvement in DAS-28 from baseline**		
	> 1.2	> 0.6 and ≤ 1.2	≤ 0.6
≤ 3.2	Good Response	Moderate Response	No Response
> 3.2 and ≤ 5.1	Moderate Response	Moderate Response	No Response
> 5.1	Moderate Response	No Response	No Response

Table 1-2. The EULAR response criteria.

However, the DAS-28 is not a panacea and there are disadvantages with its use. The DAS-28 formulae are weighted such that the tender joint count is valued more than swollen joint count. Global health and tender joint count can be elevated due to illnesses other than active RA, including fibromyalgia and depression, which can lead to a false elevation of the DAS-28 [36, 37]. There is significant inter-observer variability in performing the tender and swollen joint counts, which can affect the measurement of response over time if joint counts are completed by different clinicians and/or research nurses [38]. Recently, objective biochemical tests of disease activity have been developed that show better correlation with radiographic joint progression than previous biomarkers, such as CRP, but are not currently available in Europe [39].The VECTRA® DA is a blood test that measures 12 biomarkers associated with disease activity developed through screening candidate biomarkers and identifying biomarkers associated with measures of disease activity. In a validation study, a high VECTRA® DA activity level was associated with radiographic progression over one year compared to moderate activity level (p = 0.021) [40]. Correlation with change in VECTRA® DA and DAS-28 is, however, modest (ρ = 0.51) suggestive of poor discriminant validity [41]. Imaging such as ultrasound may be a more sensitive and specific modality for joint synovitis, but its use and interpretation is operator dependent and further studies are required to determine the prognostic benefit of treatment of subclinical synovitis [42, 43].

1.1.5 Treatment of RA

Treatment of RA is multidisciplinary, involving both pharmacological and non-pharmacological therapies. The aims of treatment are to reduce joint inflammation, damage and disability. Disease modifying anti-rheumatic drugs (DMARDs) are a class of drugs that have been shown to reduce disease progression in RA. EULAR guidelines suggest conventional oral synthetic DMARDs are first line (e.g.methotrexate, sulphasalazine and leflunomide) treatment for RA, with the aim to achieve

disease remission [44]. Should response be poor, combination of DMARDs can be introduced. Biologic DMARDs are drugs that have been designed to block specific molecules to reduce joint inflammation (e.g. etanercept and rituximab). Biologics are more effective at treating joint inflammation compared to combinations of conventional DMARDs, but due to their expense they are often withheld until MTX and another synthetic DMARD fail to control disease activity [45]. The biologics (with the exception of tocilizumab) are often given with MTX as it has been shown that dual therapy is superior to biologic therapy alone [46, 47].

The central role of MTX is emphasised by the National Institute for Health and Care Excellence (NICE) in their RA clinical guidelines, which state that patients should be offered "*a combination of disease-modifying anti-rheumatic drugs (including MTX and at least one other DMARD, plus short-term glucocorticoids) as first-line treatment*" [48]. A good response to MTX is not universal and early more aggressive treatment is essential in reducing progression of joint damage [49]. Time on ineffective therapy therefore leads to ongoing joint inflammation, damage and resultant disability, given the central role of MTX it is essential that the predictors of response and adverse events to MTX are better understood [50].

1.2 Methotrexate

MTX is classed as a conventional synthetic DMARD and is the treatment of first choice for most patients with RA. Prognosis and outcome are improved when DMARD therapy is started within a few weeks of RA onset, the period before joint and bone destruction occur, demonstrating the need for early effective treatment to prevent joint destruction [51].

1.2.1 MTX – Mechanism of Action

MTX was first introduced in 1948 as an antiproliferative drug to treat leukaemia (1 g/m^2) but is used in much lower oral doses in RA (5-25 mg per week) [52]. The first study of MTX in RA demonstrating efficacy was published in 1985 and the drug was approved by the Food and Drug Administration (FDA) as a therapy for RA in 1988 [53]. Despite almost 30 years' experience with low dose MTX in RA, the exact mechanism of action of the drug remains unclear. MTX is thought to act as an anti-metabolite through folate antagonism but it also has anti-inflammatory actions [54]. Its major metabolite, 7-OH-MTX, is metabolised by hepatic aldehyde oxidase and is less potent compared to the parent drug [55]. MTX and 7-OH-MTX have a similar molecular structure to folic acid as shown in Figure 1-7 [56]. A proposed mechanism of action of MTX is summarised in Figure 1-8.

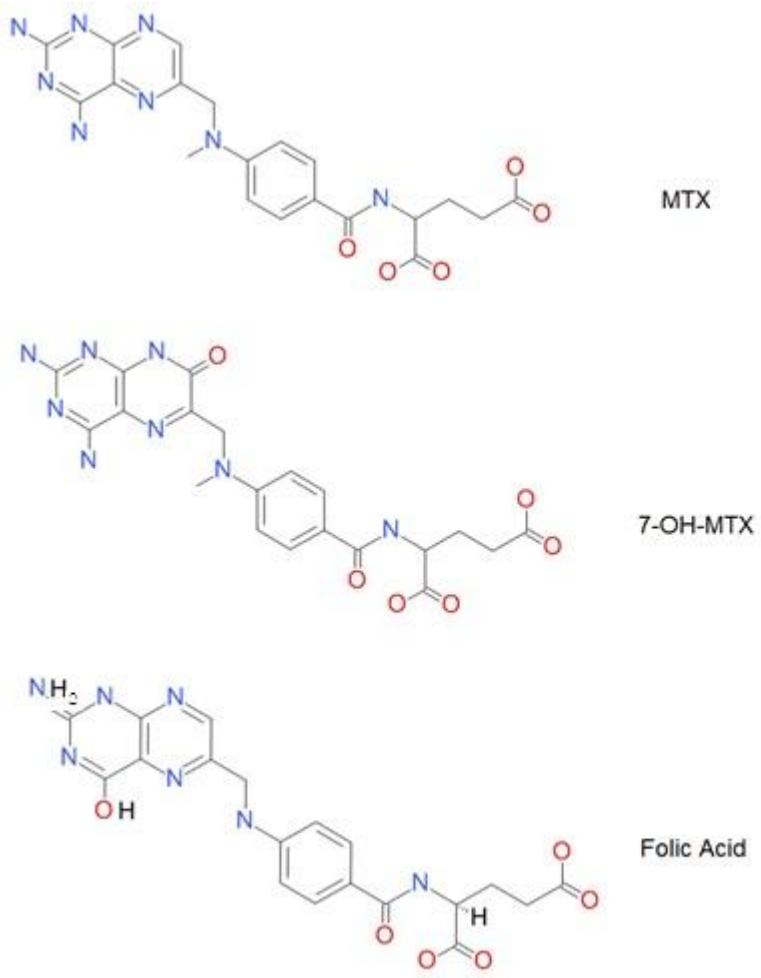


Figure 1-7. Molecular structure of MTX, 7-OH-MTX and folic acid. The molecules closely resemble each other but their pharmacodynamics are distinctive.

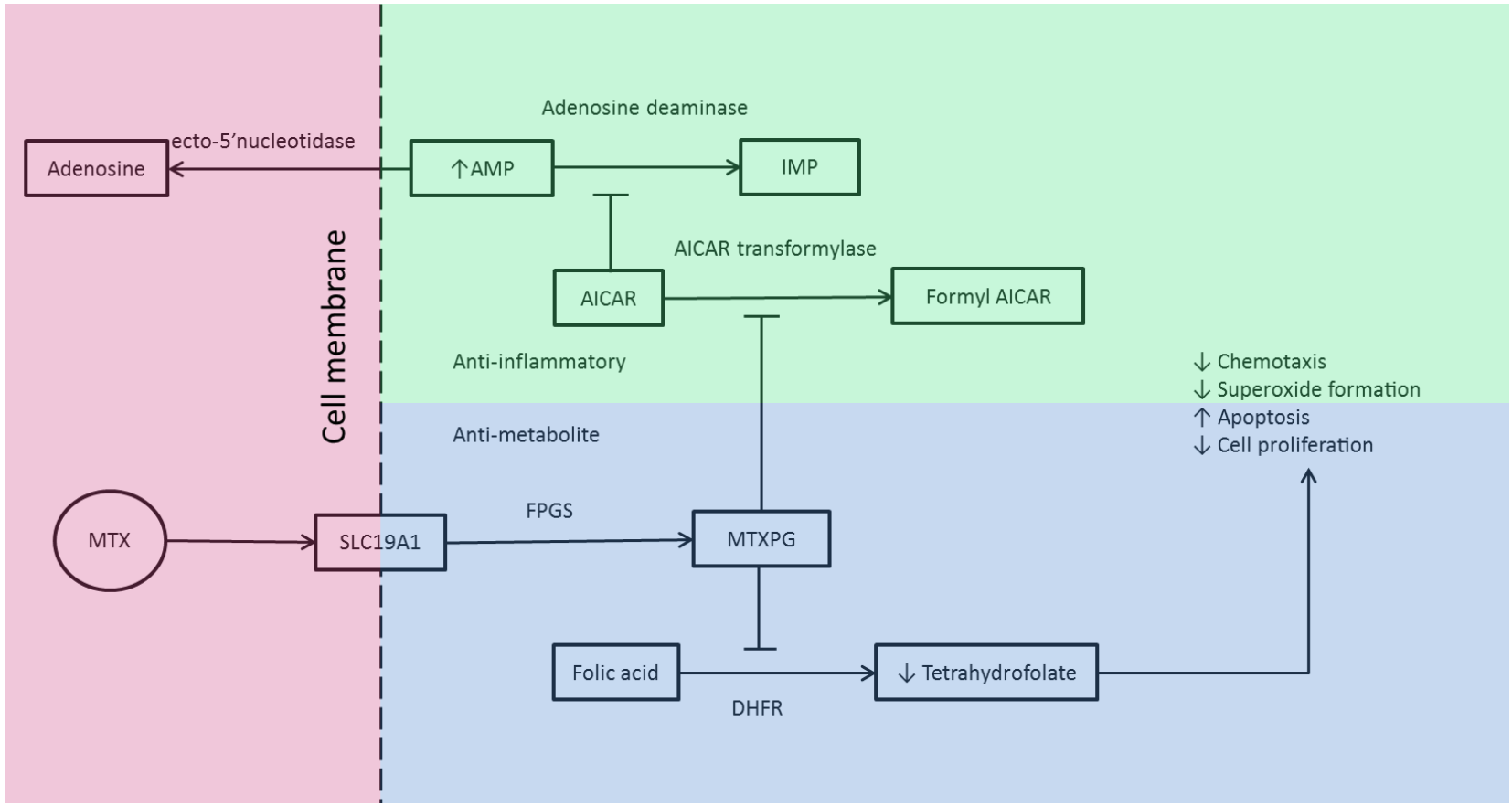


Figure 1-8. Proposed mechanism of action of MTX. Research has demonstrated that MTX has both anti-inflammatory and anti-metabolic properties. A simplified pathway by which MTX may exert these effects is shown.

1.2.2 Anti-metabolite

MTX is used in the treatment of haematological malignancies, albeit at much higher doses than is used in RA, where its action as an anti-metabolite inducing apoptosis in cancer cells is vital. MTX enters cells including hepatocytes via several transport carriers including reduced folate carrier (solute carrier family 19 member 1, SLC19A1) and solute carrier organic anion transporter 1B1 (SLCO1B1) [57, 58]. MTX is metabolised to an active form MTX-polyglutamate (MTXPG) by the enzyme folylpolyglutamate (FPGS) [59], which sequentially polyglutamates MTX through the addition of glutamic acid residues. Acting as a folate antagonist, MTXPG competitively inhibits dihydrofolate reductase (DHFR) which would otherwise convert folic acid to tetrahydrofolate. Tetrahydrofolate is a coenzyme responsible for the generation of folate cofactors, required for purine and pyrimidine synthesis. Blocking the pathway halts cellular proliferation and promotes apoptosis. MTX may, therefore, inhibit the proliferation of stimulated T-lymphocytes and induce clonal apoptosis of activated T-lymphocytes, thereby reducing inflammation [60].

There is a paucity of clinical research demonstrating that MTX acts as an anti-metabolite in RA. In one study, a randomised double-blind multicentre trial lasting 48 weeks, patients (n=434) received MTX with either placebo, folic acid (1 mg/day) or folinic acid (2.5 mg/week). The group receiving concomitant folic acid supplementation with MTX required a higher MTX dose to obtain the same clinical response compared to the placebo group ($p < 0.001$) supporting the role of MTX as an anti-metabolite drug in RA through antagonism of the folate pathway, but these results have not been consistently replicated [61, 62].

1.2.3 Anti-inflammatory Actions

1.2.3.1 Production of Superoxides

Monocytes are cells of the immune system that migrate to the synovium and can differentiate into macrophages in RA where they are responsible for joint inflammation and destruction [63, 64]. Low dose MTX has been found to act as an inhibitor of monocyte chemotaxis and monocytic production of ammonia and hydrogen peroxide responsible for cytotoxicity *in vitro*; this may be secondary to DHFR inhibition reducing tetrahydrofolate levels which act as a methyl donor to the superoxides [54].

1.2.3.2 Adenosine

Adenosine is an anti-inflammatory agent which is increased by MTX therapy [65, 66]. MTXPG inhibits aminoimidazolecarboxamidotibonucleotide (AICAR) transformlyase leading to an increase in AICAR levels [67]. It is currently unclear how and if AICAR causes an increase in extra-cellular adenosine required to reduce joint inflammation. Research suggests that it may inhibit adenosine

deaminase, resulting in increased adenosine monophosphate (AMP) which is directly released by cells [68, 69]. AMP is then converted into adenosine by ecto-5'nucleotidase in the extracellular matrix [67, 70]. Adenosine, in turn, is thought to act via the A2a extracellular adenosine receptor to inhibit T-cell activation and expansion [71]. This model is supported in patients with adenosine deaminase deficiency: the enzyme irreversibly deaminates adenosine to inosine and deficiency, therefore, results in increased levels of extracellular adenosine resulting in lymphopenia, immunodeficiency and premature death [72].

1.3 Response to MTX

Not all patients respond to MTX. One study reported that after two years, the probability of patients remaining on MTX and other DMARDs was only 55%, with drug survival being used as a marker of response [73]. There are a number of reasons that patients may not respond to a drug, including genetic, environmental and patient centred-factors or the development of adverse events that limit the continuation of the drug. One of the most important factors in chronic long-term conditions that require the long-term taking of medication is adherence [74].

1.3.1 Adherence

Human non-adherence may have first occurred when Eve ate the apple in the garden of Eden and Hippocrates noted that Physicians should "*keep aware of the fact that patients often lie when they state that they have taken certain medicines*" [75]. Much has progressed since the work of Hippocrates and non-adherence is no longer viewed as patients deliberately concealing or lying to their Doctor but a health behaviour which requires positive intervention to promote healthy behaviour [74]. On a societal level, non-adherence significantly increases healthcare costs with one study reporting a cost to the NHS of £500 million every year [76]. Given the importance of non-adherence, the World Health Organisation has published evidence-based guidance for clinicians to improve medication adherence [74].

Medication compliance is defined as the '*the extent to which a patient acts according to the prescribed interval and dose of a dosing regimen*' [77]. For the purpose of this report, compliance and adherence are considered interchangeable terms. Medication persistence, however, is defined as '*the duration of time from initiation to discontinuation of therapy*' [77]. There is, therefore, a wide spectrum of deviations that could constitute non-adherence. Non-adherence can include patients who do not take their medication at all (complete non-adherence), drug-holidays (a period of time taking no medication), catch-up dosing (following a drug holiday, an increased dosing frequency to catch-up on missed doses) or patients who do not take the dose recommended by the prescribing health care provider. Patients who do not take their medication

may still be adherent however, particularly if they have been medically advised to withhold their medication due to an adverse event. Currently, there is no gold-standard for the measurement of adherence; therefore, estimates of medication adherence vary widely due to differences in study design, study populations, definitions of adherence and methods of measurement of adherence and/or persistence [78].

There is no doubt, however, that non-adherence is a significant problem, both through wasted health care resources and because of the effect of non-adherence on disease control [74]. It is estimated that, of the 3.2 billion annual prescriptions dispensed in the United States, approximately half are not taken as prescribed, leading to significant disability and premature mortality [79, 80]. Adherence is higher for acute illnesses and persistence to medication reduces substantially after 6 months of treatment for a chronic illness, such as RA [81-83]. Often the prescriber is blinded to adherence and is unable to determine if a patient is taking their medication as prescribed. One study evaluating clinician's ability to determine patient adherence to highly active anti-retroviral treatment (HAART) for human immunodeficiency virus (HIV) determined that clinicians did not correctly establish patient-reported adherence in one third of cases [84]. Non-adherence due to drug holidays can increase the risk of adverse events due to the rebound effect of cessation of therapy; for example, during beta-blocker therapy there is an increase in sensitivity of the β-receptors to catecholamines. Thus, on cessation of therapy there is a rebound effect due to an exaggerated response to catecholamines and with recurrent drug holidays or lack of compliance to the dosing schedule, there is a risk of recurrent first-dose effects [85]. For medications such as MTX, the dose is often increased slowly over months; should there be a drug holiday, there is the danger of an increased risk of toxicity as the MTX is taken suddenly at full-dose with no titration.

1.3.2 Measurement of Adherence

Adherence is an important variable that can alter the outcome of a medical intervention. It is therefore vital that clinical trials measure adherence to ensure that the intervention cohort is adherent to medication which affects the outcome measurement, allowing the investigation of the dose-response relationship and analysis of treatment efficacy. Despite this, one systemic literature review showed that, of 192 randomised controlled trials, only 33.3% of publications reported the results of adherence analysis [86]. In clinical practice measurement of adherence is important as clinicians have been shown to be poor at judging adherence. Non-adherence is a significant cause of non-response to treatment leading to disease progression and, in RA, joint damage and functional disability [87]. In any study that evaluates adherence there is the possibility of white-coat compliance – where subjects are aware that their adherence is being

monitored and therefore the adherence is increased during the study period (the Hawthorn effect). Despite this, even in clinical trials where participants are required to strictly adhere to the medication to explore response, adherence has a wide reported range of 43 to 78% [82, 88, 89].

There are two categories of measurement of medication adherence – indirect and direct:

Indirect	**Direct**
Patient self-reporting	Directly observed therapy
Pill counts	Detection of drug/metabolite
Prescription claims	Detection of marker given with the drug
Electronic monitoring devices	

A number of methods of detecting or measuring adherence have been developed and are discussed below.

1.3.2.1 Patient Self-Reporting

Patient self-reporting includes patient diaries, patient interviews and adherence questionnaires to establish adherence. These methods are simple, easy to use and often quick to administer; questionnaires have the added advantage of standardised measurements of adherence to reduce clinical trial heterogeneity. However, those who report compliance may not be correct. For example, one meta-analysis that included 11 studies (n = 1,684 patients) comparing electronic monitoring devices with patient self-reporting found a pooled correlation coefficient of 0.45, demonstrating poor correlation between the two assessment methods [90].

1.3.2.2 Pill Counts

Pill counts are based upon counting the number of returned tablets to determine the number of tablets a patient has taken. However, analysis of timing compliance, drug holidays (and catch-up doses) and not taking the drug as medically advised is not possible. It is also possible that the subjects could discard the remaining tablets prior to their return or that adherence is increased through the Hawthorn effect [91].

1.3.2.3 Prescription Claims

Prescription claims measure the date and frequency of the exchange of prescriptions for medication and compare these with the expected frequency of prescriptions over a follow-up period using pharmacy databases. Adherence can be calculated in a number of ways, the most common of which is the Medication Possession Ratio (MPR). The MPR is calculated as the number of prescribed days of medication available during a course of treatment divided by the total duration of days of a course [92]. A cut-off value (typically ≥ 80%) may be used to dichotomise the adherent and non-adherent cohort. Prescription claims, therefore, capture medication taking

behaviours such as drug holidays and forgetting to collect a repeat prescription. However, this only determines the collection of the medication and is therefore an indirect measurement of drug adherence. Furthermore, in situations where a patient temporarily withholds their medication based on healthcare advice, use of a prescription claims database may yield a falsely reduced rate of adherence. The cut-off values used are often arbitrary, with no evidence that patients need to adhere ≥ 80% in order to obtain optimal effect of their medication over time.

1.3.2.4 Electronic Monitoring Devices

Electronic monitoring devices monitor the opening of a container and can record the time and date of opening. The disadvantage of this method of measurement of adherence is that it only records opening of the container and there may be instances of accidental activation of the container (e.g. for refilling the container). Furthermore, patients using the devices report increased anxiety as a result of known surveillance; this may lead to the refusal to use the device in the future in a subgroup of patients and there is a risk of falsely increased adherence (Hawthorn effect) [93].

1.3.2.5 Directly Observed Therapy

Directly observed therapy is currently used clinically for patients with poor compliance to anti-tuberculosis medication and with parenteral antipsychotic medication [94]. However, it is resource demanding and, for oral medications, patients can avoid the taking of medication through not swallowing.

1.3.2.6 Detection of Drug/Metabolite

The direct measurement of a drug/metabolite has known advantages and disadvantages, but therapeutic drug monitoring is currently employed for a number of drugs such as the anti-epileptic, phenytoin. The sensitivity of the test to detect adherence depends on the sensitivity of the assay utilised and the pharmacokinetics of the drug/metabolite. If a drug has a short half-life and is eliminated from the body rapidly, such as intravenous adenosine, measurement of the metabolite would be negative after 24 hours, producing a false-negative result. Biochemical tests are often expensive and require sample collection which can be inconvenient to patients. Testing for adherence also requires consent for testing, which non-adherent patients may not consent to. Detection of drug/metabolite may also be prohibitively expensive compared to other methods of adherence detection and costs should be weighed against costs of non-adherence. As there is often a wide-range of inter-individual pharmacokinetics of medication, detection of drug/metabolite is only able to provide information about adherence on a dichotomous scale (taken or not-taken). Relating the level of drug/metabolite to determine if the drug was taken on

time (timing compliance) has difficulties for medication with wide inter-individual variation in drug levels over time. Measurement in the clinic has further disadvantages as it is known that there is improved compliance five days before and after a healthcare appointment [95, 96]. Despite all the disadvantages, the major advantage of measurement of drug/metabolite is that it provides an objective measurement of adherence which cannot be obtained from indirect measurements.

1.3.2.7 Detection of a Biochemical Marker Given with Drug

The use of drug marker within a drug (such as phenobarbitone) and measurement of phenobarbitone has been trialled previously [97]. However, as with measurement of drug/metabolite, whilst the measurement is objective, it can only provide a dichotomous result.

1.3.3 Adherence to MTX in RA

Adherence to MTX in RA has been assessed using a number of different indirect measures of adherence. This section will describe results from a literature review (manuscript under review) investigating the adherence to oral MTX in RA and if MTX adherence affects response.

Nine studies have assessed adherence to MTX in RA as summarised in Table 1-3 [73, 87, 98-103]. Adherence rates vary between 59 to 107% (the latter figure representing overdosing of MTX).

Study	Adherence method	Definition of MTX adherence	n	MTX adherence	95% CI/SD
Self reported					
Contreras-Yanez et al.[73]	7 day DRR	≥ 80% of doses taken correctly over the past week	10	78%	NP
de Thurah et al.[101]	CQ-R	CQ-R score >25th percentile	85	BL 77%	NP
			65	9 mo. 77%	NP
Salt et al.[102]	MARS	MARS score >39	77	92%	NP
Prescription Claims					
Harley et al.[99]	MPR	≥ 80% prescriptions filled	1,668	64%	24-102
Grijalva et al.[100]	MPR	% of prescription filled	NP	59%	31-82
Grijalva et al.[104]	MPR	% of prescriptions filled excluding the last prescription	2,933	80%	NP
Cannon et al.[87]	MPR	≥80% prescriptions filled (first time user)	85	80%	NP
	MPR	≥ 80% prescriptions filled (established user)	370	85%	NP
Electronic monitoring					
de Klerk et al.[103]	MEMS	% of correctly taken doses	23	107%	98-117
Waimann et al.[98]	MEMS	% of correctly taken doses	76	63%	20%

Table 1-3. Comparison of oral MTX rates of adherence across nine studies utilising different methods of adherence measurement. DRR = Drug Record Registry; CQ-R = Compliance Questionnaire-Rheumatology; MARS = Medication Adherence Revised Scale; MPR = Medication Possession Ratio; CMG = Continuous Medication Gap; MEMS=Medication Electronic Monitoring System; NP= information not presented; BL = Baseline; 9 mo = 9 months.

1.3.3.1 Patient Self-Reporting

Three studies have reported adherence to MTX in RA using patient self-reporting, the results of which are shown in Table 1-3 [73, 101, 102].

A study by Contreras-Yanez et al. used a seven day diary to record the patient reported day, timing and dose of MTX ingestion before a clinic appointment [73]. Adherence was calculated as a percentage of expected MTX use over a six month period. Patients were defined as adherent if they took 80% or more of MTX as prescribed. The study sample was small (n=10), adherence may be falsely raised due to the Hawthorn effect and as it is known that there is improved compliance five days before and after a healthcare appointment, these factors may falsely inflate the measurement of adherence [95, 96].

de Thurah et al. [101] investigated MTX adherence with the use of a questionnaire, the Compliance-Questionnaire-Rheumatology (CQ-R), a validated measure that consists of 19 items on a Likert scale with a weighted score from 0 to 100 [105, 106]. Adherence was defined as the percentage of participants who were in the upper quartile of the CQ-R; however, using this cut-off for adherence has not been clinically validated as being meaningful and influencing the clinical outcome of patients with RA, nor does it allow for calculating timing compliance.

Salt et al. [102] used the validated Medication Adherence Revised Scale (MARS) questionnaire in which participants rate the frequency they engaged in non-adherence behaviours on a 5-point Likert scale with 5=never, 4=rarely, 3=sometimes, 2=often, and 1=very often [107, 108]. Questions include forgetting to take their medication, missing a dose and avoid using it. A score > 39 was arbitrarily chosen to define adherence, but it is not known whether this affects clinical outcome in RA.

1.3.3.2 Prescription Claims

Four studies have evaluated adherence using prescription claims databases. Two studies defined adherence as a dichotomy and reported that the proportion of patients who claimed ≥ 80% of their MTX course over follow-up ranged from 64 to 84% [87, 99]. Adherence did not significantly vary between first-time users of MTX and established users in one study [87]. Two studies reported the MPR values, which ranged from 59 to 80% [100, 104]. Three studies recruited patients from the United States where pharmacy databases are used for billing insurance programs. These pharmacy databases are not designed to collect data relating to adherence and may be inaccurate in data collection which could lead to variability in the results; for example, data on physician-advised cessation of therapy due to infection cannot be captured in these records which would falsely reduce the MPR. Cannon et al. recruited former members of the

armed forces from the Veterans Affairs Rheumatoid Arthritis registry [109]. Consequently, the majority of the patients were male (92%), which may affect the generalisability of the results to female RA patients [103].

1.3.3.3 Electronic Monitoring

de Klerk et al. analysed patient compliance in RA, polymyalgia rheumatica and gout using electronic medication monitors [103]. The study enrolled 127 patients of which 23 were prescribed MTX for RA over a six month period. Compliance was defined as the percentage of prescribed doses taken over the study period. Correct dosing was defined as the percentage of days within which the correct number of doses were taken. Timing compliance was defined as the percentage of doses taken within a set period of time. A total of 26,685 days were monitored. The results demonstrated that MTX compliance was high (107%) indicating overdoses. Correct dosing and timing compliance was reported as 88 and 82% respectively and there was a decline in compliance over time. There are a number of limitations of the study. First, no analysis of those who declined to participate was included; it might be reasonable to speculate that non-participants would be less adherent compared with more motivated patients who participated in the study. Second, adherence may be falsely raised due to the Hawthorn effect, but this was not explored. Third, the small number of participants included may limit the generalisability of the results to the wider population.

Waimann et al. investigated adherence using the electronic monitoring system within publicly funded outpatient rheumatology clinics in the United States [98]. As participants were accessing healthcare through publicly funded means they were more likely to be from economically disadvantaged backgrounds. A total of 76 participants with RA were recruited and followed up for up to two years. MTX adherence was found to be 63%; however, there was significant overdosing in 14% of participants and underdosing in 22%. The study revealed that those patients who declined to participate were more likely to be female and/or of Hispanic ethnicity.

1.3.3.4 Measurement of drug/metabolite

No study was identified from the literature review that investigated adherence by utilising a validated test of measurement of drug/metabolite. This is perhaps surprising given the wide measures of adherence obtained from indirect methods of adherence detection thus far. However, one study by Woolf et al. suggested that MTXPG levels could be used as a measure of adherence [110]. The study recruited 35 patients prescribed oral MTX and determined that there is minimal inter-individual variability in MTXPG levels between 24 and 52 weeks and suggested that measurement of MTXPG levels may be a marker of drug adherence. However, the study had

a low number of patients to base normal levels of MTXPG and requires validating in patients on a wide-range of MTX drug doses. Furthermore, there are inherent difficulties in measuring MTXPG levels. Erythrocytes need to be either separated at source or the whole blood sample frozen at source and centrifuged within two days otherwise the MTXPG levels are not stable [111]. Requiring samples to be tested within two days increases the cost of the assay as samples cannot be processed as a large batch thus reducing throughput.

1.3.4 Adherence and Response to MTX in RA

It may seem logical to assume that reduced adherence causes reduced response to MTX in RA, but that is not necessarily true. MTX has a long mechanism of action so reduced adherence over a short time period may not necessarily affect overall response. One study has assessed the association between adherence and response to oral MTX in RA [87]. The study by Cannon et al. used prescription claims data to calculate MPR and adherence was defined as MPR ≥ 80%. Response was measured by the mean DAS-28 score calculated from 90 days after the first MTX prescription claim until the end of the MTX course. Multivariate linear regression was used to identify an association between MPR ≥ 80% and DAS-28 with covariates of age, disease duration, gender, African American race, past history of smoking, RF status, anti-CCP status and concurrent therapy. There was no statistically significant difference between the mean MTX dose in the adherent cohort compared to the non-adherent. In the univariate model, MPR ≥ 80% was significantly ($p < 0.05$) associated with lower mean ESR, CRP, tender joint and swollen joint counts but not VAS. The results demonstrated that adherence was significantly associated with lower mean DAS-28 over the MTX course ($p < 0.05$, $\beta = -0.37$ 95% CI: -0.67, -0.07). The model suggests that mean DAS-28 is 0.37 lower in the adherent cohort compared to the non-adherent; however, given that a DAS-28 change of 1.2 is considered clinically meaningful, these results may not be clinically relevant [34].

1.3.5 Summary of Adherence to Oral Low Dose MTX in RA

There is a wide variation in the measurement of adherence reported in the literature. This variation may reflect the differences in definition and measurement of adherence, sample characteristics and size, study design and statistical models.

All of the studies in the literature used indirect measurements of adherence which have inherent limitations. There is an assumption that the self-reporting, pill bottle opening or collection of a prescription is equivalent to taking the medication and there is the risk of estimating falsely high adherence due to the Hawthorn effect. The wide variation in adherence recorded indicates that more direct measures of adherence are required. In order to develop direct measurements of

adherence to MTX it is vital to understand the pharmacokinetics. This is particularly important for MTX use in RA as the dosing schedule is weekly; hence, a test that only detects MTX for four hours after ingestion is unlikely to be as useful as one that can detect adherence up to six days following ingestion, which is important since MTX is taken once weekly.

1.4 Pharmacokinetics

The medieval physician/alchemist Paracelsus stated: "*Only the dose makes a thing not a poison*" and this remains true today [112]. Pharmacokinetic studies in their simplest form examine the body's metabolism of a drug. Factors influencing pharmacokinetics include drug absorption, distribution, metabolism and eventual excretion.

Pharmacokinetic studies are an essential investigation in drug development to establish drug bioavailability, effect of different conditions on drug levels (such as impaired renal function), evaluation of drug-drug interactions, to individualise drug dose or to adjust timing to improve efficacy.

There are four stages that represent the pharmacokinetics of any drug taken orally:

1. Absorption
2. Distribution
3. Metabolism
4. Elimination

Absorption relates to the absorption of the drug from the site of administration into the measured bodily fluid. Lack of absorption of the drug into the blood stream or a delay of absorption may result in inefficacy of that drug. Prior to absorption, oral drugs must be dissolved to enable absorption into the blood stream and this process begins in the stomach. For most oral preparations, it may take between 0.3 to 3.3 hours for drug transit to occur from beyond the stomach into the duodenum, depending on the fasting state and the preparation of the drug (e.g. coated vs. non-coated) [113]. Drug absorption into the blood stream occurs across the gastrointestinal membrane. Absorption can be a passive process by diffusion across a concentration gradient or an active process whereby drug is actively transported across the membrane by a drug carrier. Active transportation can, therefore, occur against a concentration gradient. As active transportation requires the use of a carrier protein, unlike passive diffusion, the rate of transport can be limited due to saturation kinetics. At saturation point, any increase in dose/concentration will not increase the rate of absorption from the gastrointestinal tract. This theoretical maximum rate of absorption is termed V_{max}. During oral absorption a maximum

concentration is reached (C_{max}) at a particular time (T_{max}), this represents the time at which the rate of absorption equals the rate of drug elimination.

The peak time and maximum concentration relate to the bioavailability of the drug and may correlate with the pharmacological effect of a drug. Bioavailability is defined by the European Agency for the Evaluation of Medicinal Products as "*the rate and extent to which the active substance or active moiety is absorbed from a pharmaceutical form and becomes available at the site of action*" [114]. There are many factors that may influence a drugs bioavailability including, drug excipients, gastrointestinal blood flow, gut motility and co-administered medication. Drug excipients are chemicals which are not active at the site of drug action, but affect the pharmacokinetics of a drug, for example coatings that affect the rate that the drug is dissolved in gastric juices.

Following absorption, the drug is distributed within the body. Distribution is a reversible process where the drug is distributed between different sites of the body which reaches equilibrium. In relation to MTX, distribution occurs between the plasma, erythrocytes, liver and other organs such as the bone marrow [115]. There are therefore many compartments to which MTX is distributed.

Metabolism is the process of conversion of the drug administered to another chemical. The metabolite may be an inactive chemical or may be active and affect physiology.

Elimination of the drug is the irreversible loss of the parent drug from the site of measurement, usually due to irreversible metabolism or excretion. Excretion is the elimination of the drug in its parent form. In the case of MTX, 93.7 % is eliminated via the renal tract [116].

For a drug taken orally a typical plot of concentration and time is shown in Figure 1-9:

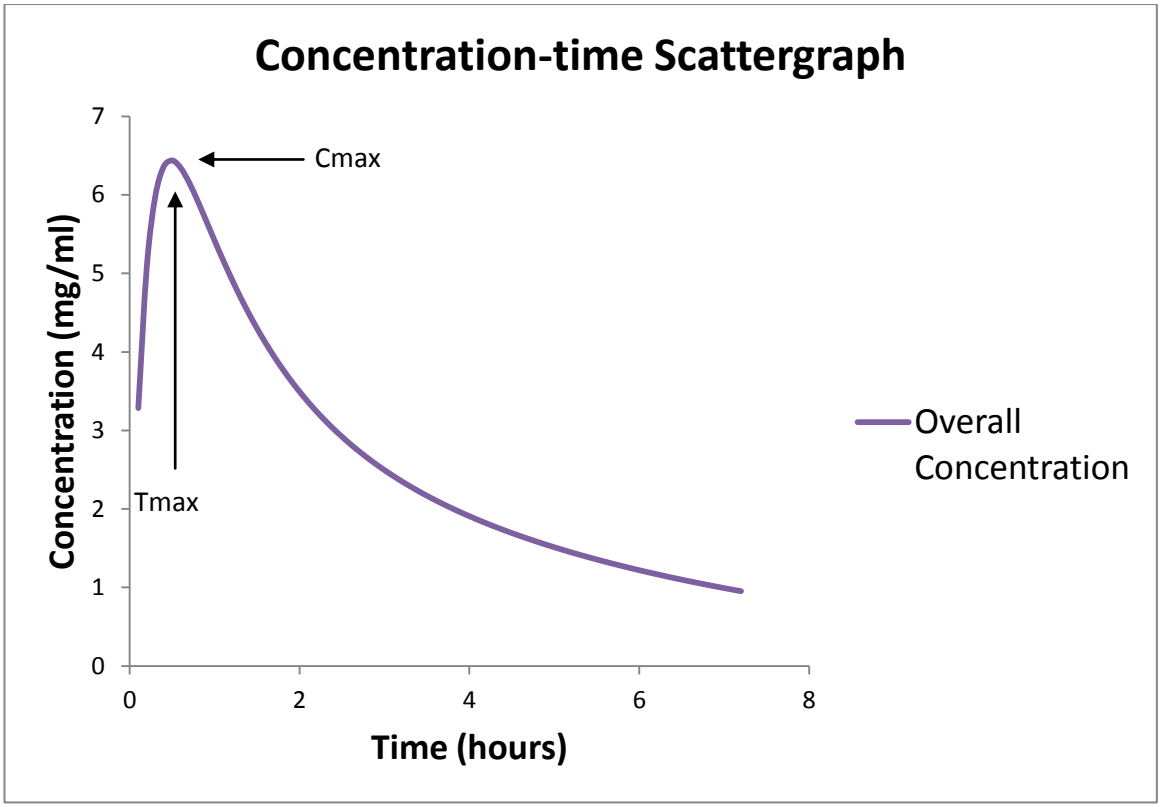


Figure 1-9. Example of a concentration-time scatter graph for an orally absorbed drug. Arrows indicate the C_{max} and T_{max} of the drug that occurs due to the rate of absorption equalling the rate of elimination.

The amount of drug available in the circulation is represented by the following equation:

$$F\ X\ dose = \int_0^{\infty} AUC\ X\ KVd$$

Where F is the bioavailable fraction (the proportion of dose administered that is absorbed into the circulation); AUC is area under the curve and represents the absolute bioavailability of the drug from time point 0 to infinity. K is an elimination rate constant. V_d is the drug's volume of distribution, a theoretical concept that relates to the theoretical volume required for a given mass of drug to be diluted in to give the observed concentration. V_d is a theoretical volume and relates to the property of the drug rather than the volume of plasma in which it is diluted. For example, consider a given mass of drug administered via the intravenous route. If the drug is highly absorbable into tissues, it will have a low concentration in plasma after administration; measurement of the plasma concentration of the drug will yield a high theoretical volume of distribution since:

$$concentration = mass/volume$$

V_d is therefore related to the degree of drug tissue binding.

Due to the complex nature of drug pharmacokinetics, a simplified model is derived from experimental data in order to create formulae that best describe the dataset for the majority of results. The most common model used is the compartmental model [117]. The compartmental model separates the body into a number of theoretical compartments. The model describes the concentration-time profile which allows derivation of other pharmacokinetic parameters such as V_d and the rate of elimination. The number of compartments depends on the time it takes for the drug to reach equilibrium in distribution i.e. the rate of transfer of drug from blood into a compartment such as the target organ equals the rate of transfer back from this compartment. A slower rate of distribution suggests that the drug is treated differently by the different organs/tissues it is distributed into, so the overall kinetics observed are a result of a number of different kinetic interactions; in this case, a two compartment model is required. Drugs that reach equilibrium rapidly generally have fewer mathematical compartments and have simpler equations. This is the case where a drug is distributed in liver/kidney (termed the central compartment); as these organs are highly perfused, drug distribution will be much faster compared with the bone marrow. Therefore, the pharmacokinetics of a drug that has a two compartment model will show early peaking during the distribution phase, a rapid decline as the drug distributes within the central compartment, then a slower decline during which poorly perfused organs (termed peripheral compartment) uptake the drug. At a certain time point, an equilibrium between the central and peripheral compartment will exist (the post-distribution phase) where the concentration on a semilogarithmic plot reduces linearly with time as shown in Figure 1-10.

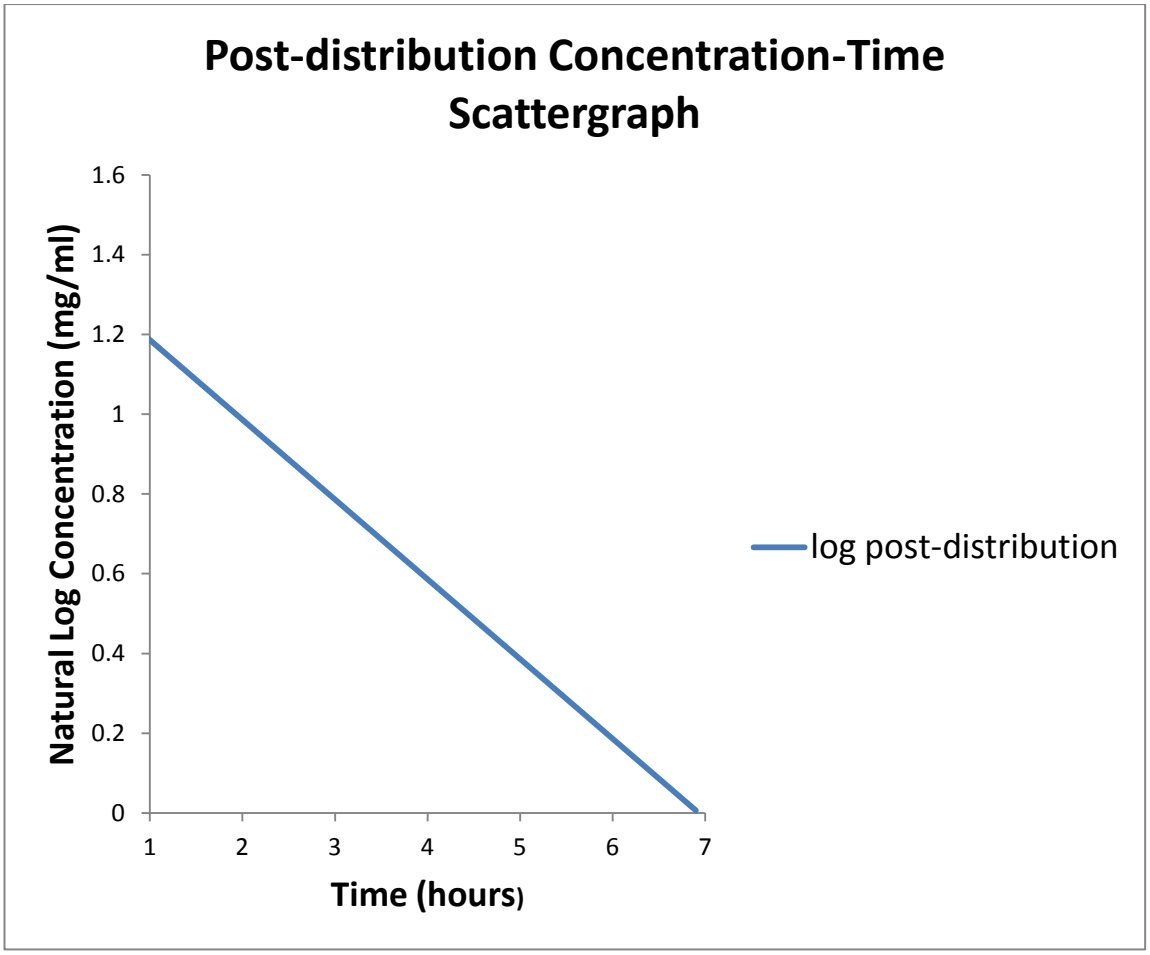


Figure 1-10. Example of post-distribution concentration-time semilogarithmic scatter graph demonstrating equilibrium reached between the different compartments causing the concentration to reduce proportionally over time.

A population pharmacokinetic model is based upon drug plasma-concentration versus time data, and is, therefore, only as accurate as the results from experimental data. That, in turn, is dependent on the frequency of blood sampling, the amount of time over which blood sampling occurs and the sensitivity/specificity of the drug assay. Results from pharmacokinetic studies may therefore vary substantially due to variation in study design.

Concentration-time models that are best fitted to a two compartment model require two exponential terms to describe the data. For example, in a two compartment model for a drug administered intravenously, there is no absorption phase as absorption is instantaneous. A representative equation for a two compartment model without an absorption phase is:

$$concentration = Ae^{-\propto t} + Be^{-\beta t}$$

Where A and α relate to drug distribution and B and β relate to the post-distribution phase.

A drug administered orally has an additional absorption phase requiring a further exponential term as shown below:

$$Concentration = Ae^{-\propto t} + Be^{-\beta t} + Ce^{-\kappa t}$$

Where:

$$C = -(A + B)$$

Where κ relates to the drug absorption.

Using this formula, as time increases, absorption exponentially reaches 0 from negative, and distribution exponentially reaches 0 from positive, but is slower to reach 0 compared to absorption, giving the overall concentration curve as shown in Figure 1-11.

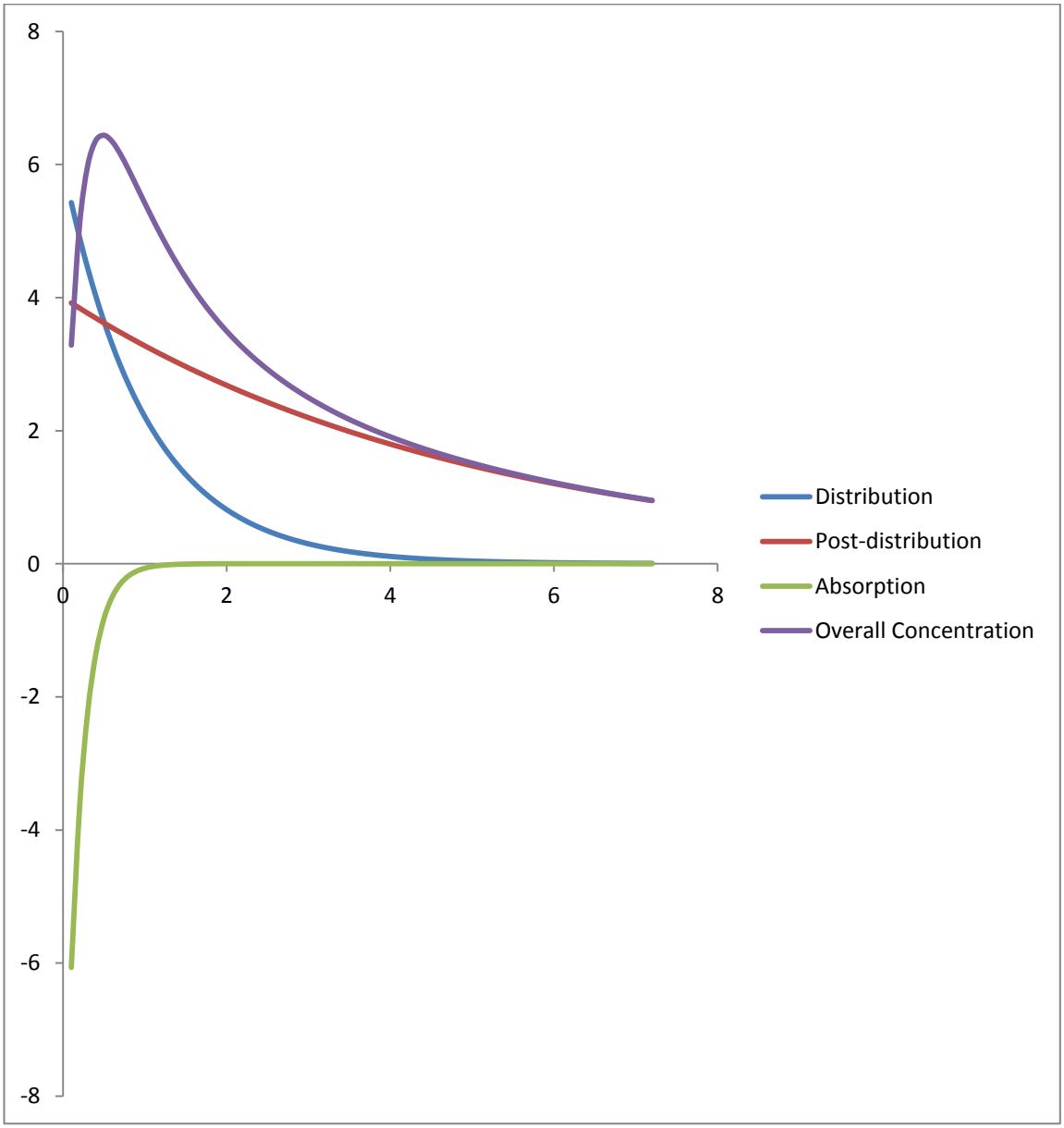


Figure 1-11. Concentration-time scatter graph for an orally absorbed drug with two compartments demonstrating the overall concentration and the theoretical formulae that explain the drugs absorption, distribution and post-distribution phases.

The elimination of a drug can be characterised by the elimination half-life ($T_{1/2}$), which is the amount of time after administration of a drug at which the mass of unchanged drug in the sampling fluid becomes half of the initial mass of the drug in the sampling fluid. Drug elimination from the body is termed clearance and is defined as "*the hypothetical volume of blood (plasma or serum) or other biological fluids from which the drug is totally and irreversibly removed per unit time*" [118]. Measured clearance may be:

- Total body clearance
- Organ specific clearance e.g. renal, hepato-biliary
- Metabolic clearance – the clearance of drug removed by metabolism

Many drugs, including MTX, have significant renal clearance. Renal excretion can occur via a combination of the following processes:

- Passive diffusion through the glomeruli
- Active tubular secretion
- Passive resorption back into the blood stream

1.4.1 Establishing a Pharmacokinetic Model

There can be wide inter-individual differences in the pharmacokinetics of a drug. These differences can be due to covariates that are measurable, such as age, weight, creatinine clearance and random non-predictable effects, such as inter-individual variability and intra-individual variability. The study of pharmacokinetic variability within a population is termed population pharmacokinetics. In order to establish a pharmacokinetic model, multiple blood samples are taken from a cohort of subjects who have taken the drug at specified times. The blood samples are analysed to determine the concentration of the drug and a drug concentration-time graph is plotted. A model is a simplified version of the pharmacokinetics of the drug which explains the majority of the data. Population pharmacokinetic models can be formulated in a number of ways but the most common is using nonlinear mixed effects modelling [119]. Mixed effects models include fixed (e.g. drug doses, gender or time points that blood samples are taken) and random effects (e.g. unexplained inter-individual variability in plasma MTX levels) on the observations, allowing the non-measurable influences to be estimated. Covariates can be incorporated into the model if they significantly alter the pharmacokinetics of the drug and improve the variability explained by the model. Development of a pharmacokinetic model is required to determine how sensitive a particular assay is at measuring drug adherence over time, simulations can be performed to predict the proportion of patients with detectable

concentrations of drug over time after a given dose of drug to determine how sensitive the assay will be at detecting non-adherence at different time points following drug ingestion.

1.5 Pharmacodynamics

Pharmacodynamics describes what the drug does to the body in order to exert the pharmacological effect [120]. The drug at the action site during steady state will be in equilibrium with the drug level in blood. Drugs typically produce effects through interaction with a protein receptor, which causes a cascade of second messenger signalling. Drugs can activate the cascade (full agonist), block the cascade (antagonist) or partially activate the cascade (partial agonist). For example, the drug propanolol is a beta-adrenergic blocking agent and antagonises the effect of noradrenaline at the sino-atrial node in the heart. Noradrenaline normally causes tachycardia; propanolol will, therefore, only have an effect after exercise as that activates the sympathetic nervous system causing the release of noradrenaline [121]. The majority of drugs display a dose-response-toxicity relationship whereby increases in drug dose lead to an increase in response to treatment and toxic side effects. However, this relationship is not linear; it is sigmoidal for dose-response, due to saturation of the target enzyme, and exponential for dose-toxicity (Figure 1-12).

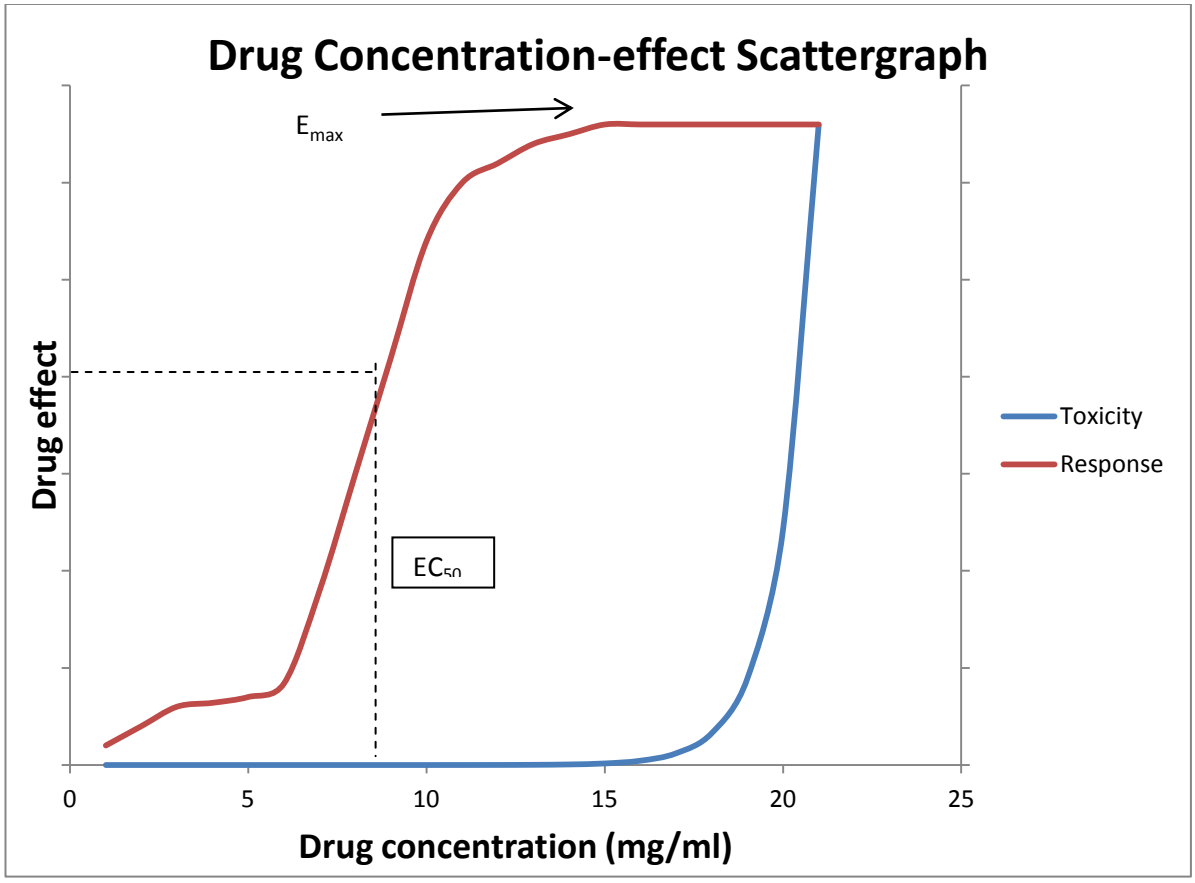


Figure 1-12. Scatter graph demonstrating the balance between drug concentration and toxicity or response. EC_{50} represents the drug concentration that causes half of the maximal effect.

E_{max} is defined as the maximal effect of the drug where all receptors are occupied by the drug and increases in drug concentration produce no increase in drug effect, whilst the EC_{50} is the drug concentration that causes half of the maximal effect [117]. Drugs can be highly selective or non-selective in their receptor binding. The difference seen with drug therapeutic and adverse effects may be due to the drug binding to different receptors with different EC_{50}. The ratio of adverse effect EC_{50}/therapeutic effect EC_{50} is used to characterise the safety of the drug, termed the therapeutic index.

The example above demonstrates that drug concentration is related to effect. There are, however, situations when drug concentration is not a good indicator of effect, including:

- Drugs administered at concentrations which give a maximal effect i.e. EC_{max}.
- Drugs that act irreversibly: for drugs that are complete antagonists and block the receptor effect, termination of these effects will not occur until there is synthesis of new receptors; there is, therefore, no direct relationship between drug concentration and effect as effect relates to an individual's ability to synthesise more receptor.
- Delayed distribution: for drugs that bind to receptors at a site to which the drug is slowly distributed (such as the bone marrow), the effect will increase as the drug concentration in blood falls due to redistribution. This means that a lower concentration of drug in the blood equates to an increased effect.
- The drug response measured is not an adequate measurement of drug-receptor binding.
- Prodrugs: if a drug is a prodrug, its metabolite is the active drug producing the desired effect. If the prodrug is measured by the drug assay and not its metabolite, there may be dissociation between concentration and effect.

1.6 Inter-individual Variability

Not all patients will respond the same way to the same dose of medication due to inter-individual variability. In practice, clinicians tailor the dose to the individual based on response, increasing the dose in patients who have not responded.

Inter-individual variability may occur for many reasons including:

- Genetic polymorphism
- Renal impairment
- Hepatic impairment
- Time of day the drug is administered – chronopharmacology
- Age
- Body-weight
- Drug-drug interactions

1.7 Pharmacokinetics of Methotrexate

1.7.1 Absorption

MTX is actively absorbed in the proximal jejunum by enzymes including SLC19A1, but demonstrates inter-individual variability of up to five fold in the fraction of the MTX dose absorbed over time [115, 122, 123]. Individuals may vary greatly, therefore, in their absorption of MTX and may reach saturation of absorption of MTX whereby further increases in the dose will not increase C_{max} [124]. Once absorbed, MTX is transported through the portal vein to the liver.

The pattern of absorption was explored by Seideman et al. [125]. In that study, the authors recruited nine patients (two male, seven female) with RA who were receiving low-dose MTX. Following an overnight fast, MTX levels were measured in plasma before administration, at hourly intervals for 8 hours and thereafter at 24, 48, 72, 96, 144 and 168 hours after dosage using high-performance liquid chromatography (HPLC) with fluorometric detection. It was shown that the absorption phase tends to follow zero or first-order kinetics (i.e. plasma-concentration-time profile is linear or exponential during absorption of drug). From a separate study, the mean absorption time has been estimated to be 1.2 hours [122].

The effect of food on absorption of MTX has also been evaluated [126]. Oguey et al. recruited 10 patients with RA and randomised patients to receive intravenous or oral MTX, with or without breakfast following an overnight fast. After oral administration, MTX was detectable from 30 minutes. Fasting patients achieved higher C_{max} than non-fasting patients (0.71 +/- 0.34 vs. 0.49 +/- 0.22 μM, $p < 0.02$), but bioavailability was not affected by food. This may be explained by prolonged gastric retention causing slower release of MTX from the stomach and a flattened absorption profile without affecting overall absorption as measured by bioavailability.

1.7.2 Distribution

Following absorption MTX is distributed within the body in compartments including erythrocytes, T-lymphocytes, fibroblasts, synovial fluid and the liver [122, 127-129]. In plasma, up to 60% of MTX is bound to albumin [122, 130], whilst intracellularly, MTX is polyglutamated (Figure 1-8). Erythrocyte MTX increases with plasma MTX after administration. Plasma MTX AUC but not dose of MTX administered correlates with steady-state erythrocyte MTX, indicating that measuring MTX levels at steady-state may be used to estimate erythrocyte MTX levels [131]. The mean V_d of MTX has been estimated to be 0.42 – 1.01 L/Kg [130, 132].

1.7.3 Metabolism

Following transport via the portal vein to the liver, a proportion of MTX is converted to the less active 7-OH-MTX by hepatic aldehyde oxidase [133]. 7-OH-MTX is a 10-fold less potent antagonist of DHFR and is 8 times less effective as an inhibitor of AICAR transformlyase compared to the parent drug [55]. Cellular uptake of MTX occurs by passive diffusion and active transport by SLC19A1 (Figure 1-8) [134]. Following absorption, MTX is polyglutamated into its active form with up to six glutamate particals, by the enzyme folyl-polyglutamate synthetase [128]. Deglutamation of MTX is required prior to cellular elimination. Following deglutamation, further oxidation via hepatic aldehyde oxidase can occur.

A study by Baggott et al. demonstrated that there appears to be two phenotypes controlling the metabolism of MTX to 7-OH-MTX in patients with RA [116]. In 29 patients with RA who received an oral dose of MTX (7.5 – 22.5mg), the total MTX excreted as 7-OH-MTX in urine over 72 hours was tested. The results show a non-normal distribution with significant variation in metabolism ($p < 0.025$), suggesting that there are fast metabolisers and slow metabolisers (Figure 1-13).

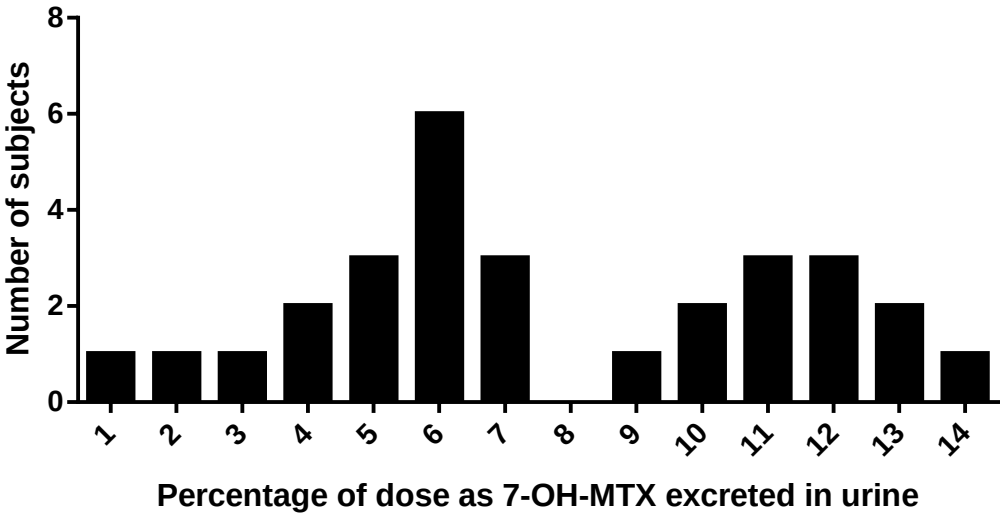


Figure 1-13. Percentage of oral methotrexate renally excreted as 7-OH-MTX over 72 hours in 29 patients with RA. Adapted from Baggott et al. [116].

The percentage of MTX excreted as 7-OH-MTX did not significantly correlate with the MTX dose; however, the number of subjects tested was low and the study may not have been sufficiently powered to detect a difference. The findings could be due to inter-individual variability in absorption reducing the amount of MTX available to metabolise to 7-OH-MTX; genetic variation altering the effectiveness of hepatic aldehyde oxidase; drug-drug interactions affecting the metabolism of MTX; enzymes other than hepatic aldehyde oxidase also being responsible for

metabolism such as bacterial gut enzymes or that MTX in a proportion of subjects is sequestered into cells and polyglutamated more efficiently so that less 7-OH-MTX is produced.

MTX is metabolised to MTXPG intracellularly (Figure 1-8, Section 1.2.1). MTXPG is retained within the cell and are more efficient inhibitors of purine and pyrimadine synthesis than MTX [135].

1.7.4 Excretion

Renal excretion is the main route of elimination of MTX and between 86-100% is excreted as MTX [116]; for example, Seideman et al. demonstrated that within 24 hours of MTX ingestion, 80.5% of MTX is excreted [125]. MTX is freely filtered through the renal glomeruli and is additionally actively excreted across the renal tubules, which demonstrate saturation kinetics [136]. Renal clearance of MTX may, therefore, exceed creatinine clearance due to active excretion and show non-linear elimination over time. Patients with renal impairment, therefore, have reduced ability to excrete MTX, increasing the risk of toxicity [137]. The effect of renal insufficiency on the pharmacokinetics of intramuscular MTX has been evaluated by Bressolle et al. [138]. The authors recruited 77 patients (16 men, 61 women) who fulfilled the 1987 ACR RA classification criteria. Following an overnight fast, each patient received their usual dose of MTX (7.5 to 15mg) intramuscularly. MTX levels in plasma were measured at baseline, two and eight hours after administration. The subjects were categorised according to their creatinine clearance (< 45, 45-60, 61-80 and > 80 ml/min). Baseline demographics revealed that patients with creatinine clearance > 80 ml/min were of higher weight, whilst those with creatinine clearance < 45 ml/min had higher ESR values and older age (Table 1-4), which may be confounding factors that affect the MTX pharmacokinetics.

Parameters	**Creatinine clearance ml/min**			
	< 45	**45-60**	**61-80**	**> 80**
Weight (Kg)	53.3 (85)	54.6 (6.5)	65.6 (11.8)	71.1 (14.9)
ESR (mm/hr)	49.1 (23.5)	34.1 (21.1)	23.2 (17.7)	22.6 (17.1)
Age (years)	74.6 (6.0)	67.4 (5.8)	65.5 (8.4)	52.8 (10.9)

Table 1-4. Mean (SD) demographic and clinical characteristics at baseline for patients adapted from Bresolle et al. [138]. The results demonstrate that there is an association between creatinine clearance and weight, ESR and age which may be potential covariates to consider in a population pharmacokinetic model.

The results demonstrated that MTX levels were significantly higher at two and eight hours in patients with impaired renal function; $T_{1/2}$ therefore increased with worsening renal function as shown in Table 1-5.

Parameters	Creatinine clearance ml/min			
	< 45	**45-60**	**61-80**	**> 80**
MTX concentration (t=2 hours) (μM) **	0.66 (0.18)	0.51 (0.13)	0.49 (0.11)	0.45 (0.12)
MTX concentration (t=8 hours) (μM) ***	0.21 (0.12)	0.13 (0.052)	0.12 (0.039)	0.11 (0.04)
T ½ elimination (hours) ***	22.7 (9.8)	13.1 (2.9)	11.9 (2.6)	10.8 (3.3)

Table 1-5. Mean (SD) pharmacokinetic characteristics for patients adapted from Bresolle et al. [138]. The results demonstrate that as creatinine clearance declines MTX clearance declines. ** $p < 0.01$, *** $p < 0.001$.

7-OH-MTX is also eliminated via the kidneys, but at a slower rate than MTX and may therefore be a more suitable biomarker for adherence to MTX [125].

1.7.5 Inter-individual Variability of MTX Pharmacokinetics

In the studies published thus far, the pharmacokinetics of MTX demonstrate a triexponential function or 2-compartment model; however, there is wide inter-individual variability in the pharmacokinetics of MTX as shown in Table 1-6, Table 1-7, Table 1-8 and Table 1-9. In one study C_{max} showed considerable variability ranging from 0.3 to 1.6 μM [126]. Furthermore, the bioavailability has a wide range from 28 to 88% in one study and a five-fold range in another [126, 139]. In contrast, intra-individual differences of bioavailability range from 3 to 30% [139]. It is likely that some of the variation seen between studies is due to study design, dose given and sensitivity of the MTX/7-OH-MTX assay. For example, Lebbe et al. [139] conducted a pharmacokinetic study after 15mg oral MTX that lasted 8 hours and determined $T_{1/2}$ to be 3.3 hours whilst Seideman et al. [125] conducted a pharmacokinetic study after 15mg oral MTX that lasted 168 hours and determined $T_{1/2}$ to be 49 hours.

Paper	n	MTX Dose	C_{max} (μM)	T_{max} (minutes)	$T_{1/2}$ (hr)	AUC (nM hr)
Seideman et al. [125]	9	15mg			49 (95% CI 36-86)	3533 (95% CI: 2962-4073)
Hermen et al. [122]	41	10mg/m^2			6.1	
Oguey et al. [126]	10	15mg	0.71 (0.34)	78		
Lebbe et al. [139]	10	15mg		83 (26)	3.3 (1.1)	
Bologna et al. [140]	22	7.5-10mg	0.6 (0.17)	35 (19)	10.3 (4.86)	
Kremer et al. [141][a]	15	7.5mg	0.547 (0.16)	79 (29)		

Table 1-6. MTX pharmacokinetics measured in plasma following an oral dose of MTX. [a] samples measured in serum. Values are mean. Numbers in brackets represent the standard deviations unless otherwise stated. Note that there is wide variation in the elimination $T_{1/2}$ and T_{max}.

Paper	n	MTX Dose	C_{max} (μM)	T_{max} (minutes)	$T_{1/2}$ (hr)	AUC (nM hr)
Seideman et al. [125]	9	15mg			49 hours (20 -309)	2473 (1147-3854)

Table 1-7. 7-OH-MTX pharmacokinetics measured in plasma following an oral dose of MTX. Values are mean. Numbers in brackets represent the 95% confidence interval.

Paper	n	Urine collection period (hours)	% of dose excreted in urine
Seideman et al. [125]	9	24	Median 80.5% (95% CI: 63.1-94.9)
Hermen et al. [122]	41	48	Mean 48.1 (SD: 18.3)

Table 1-8. Summary of papers investigating the percentage of MTX excreted in urine in RA patients following oral ingestion.

Paper	n	Urine collection period (hours)	Median % of dose excreted in urine
Seideman P et al. [125]	9	24	3.53% (95% CI: 2.81-6.15)
Baggott et al. [116]	29	72	7.19 (range 0.94-13.2)

Table 1-9. Summary of papers investigating the percentage of 7-OH-MTX excreted in urine in RA patients following oral ingestion.

One study by Godfrey et al. attempted to explain the inter-individual variability by incorporating patient specific data into regression equations in order to predict pharmacokinetic parameters [142]. The authors recruited 62 patients with RA; 12 were receiving long-term MTX whilst 50 were new starters. Patients received either oral or intramuscular MTX with all patients contributing to both an intramuscular and oral pharmacokinetic study. Patients were fasted and allowed to take their usual medications. The pharmacokinetic studies were conducted every 6 months. Blood was collected at baseline and 0.5, 1, 2, 3, 4, 6, 8, 24 hours after MTX administration. Urine was collected also for 24 hours. Using an extended least squares approach to nonlinear mixed effects modelling the authors estimated variability in terms of patient specific information such as age or body weight. The final regression model determined that MTX pharmacokinetics was a function of weight, creatinine clearance and gender as shown below:

$$Cl\ (l\ h^{-1}) = (\theta 2 * Cl_{CR}\ * 0.06 + \ \theta 3 * WT) * (1 + \theta 4 * GEN)$$

$$v_c\ (l) = 0.5 * WT$$

$$v_p\ (l) = 0.6 * WT + \ \theta 7 * AGE$$

$$ka_{po}\ (h^{-1}) = (\theta 11 + \ \theta 12 * DOSE) * (1 + \ \theta 13 * FED)$$

$$LAG_{po}\ (h) = \theta 14$$

Where:

Cl = MTX clearance

Θ2 = 0.081 (SE = 0.00862)

Θ4 = 0.312 (SE = 0.0135)

Θ11 = -0.0416 (SE = 0.014)

Θ12 = -0.496 (0.068)

Θ13 = 0.225 (SE = 0.0173)

Θ14 = 0.934 (SE = 0.0181)

AGE = age (years)

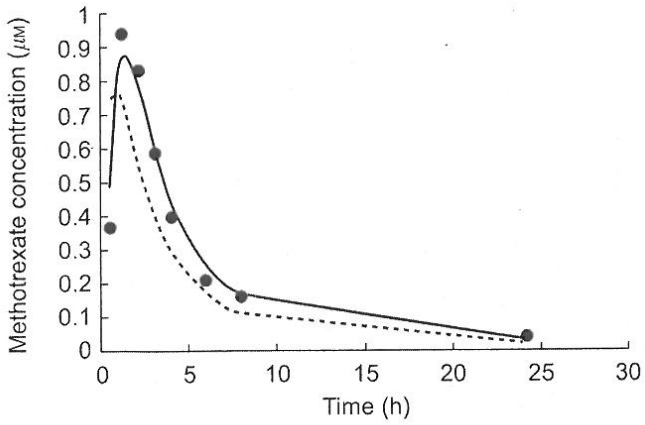
Ka_{po} = absorption rate constant

Cl_{CR} = creatinine clearance

WT = weight (kg)

LAG_{PO} = oral absorption lag time

Figure 1-14 displays the observed and model predicted MTX concentrations versus time for a subject (69 year old female weighing 69 kg with a creatinine clearance of 69 ml/min) following oral administration of 27.5 micromoles MTX in the non-fasted state.



Representative model predicted (---) and observed (●) methotrexate concentrations *vs* time profile. Solid line (———) is prediction using individualized pharmacokinetic parameter estimates.

Figure 1-14. Observed and model predicted MTX concentrations verses time for a representative patient[d].

Despite the wide inter-individual difference in pharmacokinetics of MTX, currently clinicians do not carry out therapeutic drug monitoring of MTX as there is conflicting evidence that this predicts response (Section 1.8).

1.8 MTX Pharmacokinetic-Pharmacodynamic Studies

To date, there have been a limited number of studies published investigating the relationship between pharmacokinetic-pharmacodynamics of MTX and treatment response, either in terms of correlating with adverse events or efficacy.

1.8.1 Adverse Events

In one study conducted by Shoda et al. sixty-nine Japanese patients fulfilling the 1987 ACR classification criteria for RA were recruited [143]. MTX was divided into one, two or three portions at intervals of 12 hours. Serum MTX levels were measured at 1, 2 and 3 hours after intake of the first portion of MTX. Serum MTX was measured by a fluorescence polarisation immunoassay. Adverse reactions to MTX were recorded from the patients' records and included nausea, gastrointestinal distress, skin or mucosal lesions, liver dysfunction (both AST and ALT levels two-fold that of the upper limit of normal or higher), leucopenia (WCC < 3000/μl), anaemia (Hb < 8 mg/dl with a decrease by more than 2 mg/dl in within three months), thrombocytopenia (platelets < 100000 /μL), interstitial pneumonia and fever. Concomitant therapy was also recorded.

[d] Reprinted from British Journal of Clinical Pharmacology, 46, Godfrey C, Sweeney K, Miller K, Hamilton R, Kremer J. The population pharmacokinetics of long-term methotrexate in rheumatoid arthritis, 369-76., Copyright (1998) Blackwell Science Ltd, with permission from John Wiley and Sons.

Patients were followed up for a mean of 39.7 +/- 12.0 months. No patient took more than 16 mg of MTX per week and none took folic acid. The average C_{max} was 0.15 +/- 0.07 µM and T_{max} 2.0 +/- 0.8 hours. Interestingly, there was no correlation between the serum creatinine and C_{max} or $T_{max,}$ which conflicts with data from other studies [138]. A total of 12 patients developed adverse reactions; C_{max} was significantly associated with the development of adverse reactions ($p < 0.001$) as was the maintenance dosage of MTX per week ($p < 0.03$). At a threshold of 0.16 µM, a sensitivity of 81% and 67% to predict adverse events was observed.

The number of patients in this cohort was modest and only 12 patients developed an adverse event, raising the possibility of a false-positive result (a type 1 statistical error). The C_{max} is also much lower in the cohort compared to Oguey et al. [126] who determined the C_{max} in patients taking 15mg of MTX to be 0.71 µM. However, that may be explained because the total dose was split into portions taken 12 hours apart, and sampling occurred after the first portion, therefore it is expected that the C_{max} in this study would be lower than in previous studies [126]. It is interesting that creatinine levels did not correlate with MTX levels; this may be due to the lower dose of MTX used and the multi-dosing regimen. A disadvantage of this retrospective study is that not all of the adverse events will have been recorded, particularly minor common adverse events such as nausea, which clinicians may not record. Further work is needed in other populations to determine whether the C_{max} can be used to predict adverse events consistently.

1.8.2 Drug Efficacy

Despite the variability in the pharmacokinetic studies of MTX, levels of MTX and 7-OH-MTX or other metabolites may be useful as biomarkers of efficacy and this has been investigated in a number of studies. For example, it is known that 7-OH-MTX is less efficacious in animal models of arthritis compared to MTX [55] and one study by Baggott et al. investigated the percentage of total MTX dose excreted as 7-OH-MTX [144]. The authors recruited 39 patients with RA starting MTX. Patients were admitted to a research centre for three 24-hour visits over a seven week period. A 24 hour urine collection started after the sixth weekly dose of MTX. Joint swelling, pain and tenderness were assessed clinically at each visit by a trained registered nurse. MTX and 7-OH-MTX were measured using high performance liquid chromatography triple-quadrupole mass spectrometry (HPLC-QQQ-MS). The results demonstrated that those patients with marked improvement in joint assessments had a significantly lower amount of MTX excreted as 7-OH-MTX ($p = 0.01$), suggesting that the greater the amount of MTX retained in the parent form rather than converting to the less active 7-OH-MTX form, the better the outcome.

There are however, a number of potential confounders in that study which make the results difficult to interpret with certainty. The lack of classification criteria used for RA may have resulted in case heterogeneity. The study failed to use recognised measures of disease activity such as DAS-28, biomarkers, imaging or inflammatory markers. When low numbers of patients are recruited, an outcome with imprecise subjective measurements may increase the possibility of a type I error (falsely rejecting the null hypothesis). Measurement of MTX and 7-OH-MTX may also have been unreliable; the samples were stored for up to six years and may have undergone degradation during that time [145].

A study by Hornung et al. measured erythrocyte intracellular MTX levels in a cohort of 76 patients with RA [131]. Forty patients were initiating MTX for the first time at a starting dose of 5 to 10mg per week, the remaining 36 patients were prescribed long term MTX at a stable dose for three months prior to MTX level measurement. No patient received concomitant DMARDS. Patients were clinically evaluated by the same physician at weeks 0, 12-16, 28 and 52. Patients were classified as responders if they fulfilled the core criteria of the ACR response criteria on at least one occasion [146]. Steady-state erythrocyte MTX levels were measured at 0, 6, 8, 12, 16, 20, 24, 28 and 52 weeks by a radio chemical-ligand binding assay. Furthermore, for each patient, MTX plasma concentration was measured before intake of MTX and at 1, 2, 4, 6, 12 and 24 hours to calculate the bioavailability or AUC for each patient.

The results demonstrated that the weekly dose of MTX and steady-state erythrocyte-MTX levels were poorly correlated (R^2 = 0.16). In the group starting MTX for the first time higher MTX dose, mean erythrocyte-MTX and AUC plasma MTX were significantly associated with responder status as shown in Table 1-10, but not in those patients established on MTX.

	Responders	**Non responders**	***p*-value**
Erythrocyte-MTX, nM mean (SD)	25.74 (12.99)	17.44 (7.51)	0.013
AUC mg/l h	2.70 (0.86)	1.99 (0.75)	0.002
MTX dose, mg/wk	11.1 (4.2)	7.9 (1.9)	< 0.001

Table 1-10. Pharmacokinetic results in responders and non-responders from the Hornung et al. study demonstrating that responder status was significantly associated with higher erythrocyte levels, AUC and MTX dose in patients starting MTX. Numbers are mean (SD). Adapted from [131].

The results suggest that higher dose MTX correlates to higher bioavailability and erythrocyte-MTX levels and that correlates with response to treatment. However, the classification of response and non-response was imprecise, without the requirement for a prolonged response to MTX. As the study is an open prospective study, participants could have taken concomitant therapy such as corticosteroids during the course of follow-up, which would cause them to become temporary responders and there is therefore the potential for bias within the study.

A study conducted in 1995 by Bolonga et al. controlled for the use of concomitant therapy during the follow-up period [140]. The study was a prospective study of RA patients (n=22) investigating if MTX pharmacokinetic parameters correlate with clinical response. Each participant was prescribed 10 mg of intramuscular MTX weekly except one patient who was prescribed 7.5 mg due to renal impairment. No non-steroidal anti-inflammatory drugs were allowed during the study. Three patients were recruited who were on a stable dose of prednisolone for at least six weeks. Disease activity was measured using the Ritchie Articular Index (RAI), a predecessor of the DAS-28 [147]. RAI was recorded by the same physician in the morning prior to administering MTX. Blood sampling occurred before, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12 and 24 hours after MTX. Urine was collected for 24 hours. MTX levels were measured using a fluorescence polarisation assay.

The results of this experiment showed a mean C_{max} of 0.6 μM and T_{max} of 0.58 hours. MTX AUC was significantly associated with lower RAI (p = 0.01) and early morning stiffness (p = 0.03). Whilst a small study, the authors did control for concomitant medication and utilised a composite score for assessing disease activity. However, the doses of MTX tested were low in comparison to those widely prescribed in practice now and it is not clear whether the same relationships between MTX AUC and outcome will remain.

Furthermore, a study by Lafforgue et al. [148] was not consistent with the results of Bologna et al [140]. The authors recruited 46 patients with RA starting MTX for the first time. MTX was administered intramuscularly with a mean dose of 11.3 +/- 2.78 mg (range 5 to 17.5 mg per week). During the follow-up period a stable dose was maintained; however, if the patient was a non-responder, MTX was stopped and replaced with another drug. Non-steroidal anti-inflammatory drugs (NSAIDs) and steroids were continued at the same dose at recruitment. The pharmacokinetics of MTX were studied at the beginning of treatment after the first dose. Serum MTX levels were measured before and at 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12 and 24 after the first MTX injection. Twenty-four hour urine was also collected in 24 patients. Levels were measured using fluorescence polarisation immunoassay. Response to treatment was measured monthly with the number of swollen and tender joints, physician global assessment and patient global assessment. Responders were classified according to a more than 50% decrease in at least two of the variables compared with the baseline results.

The results showed that there was no difference in-between the pharmacokinetics of MTX between responders and non-responders. There was wide inter-individual variability with $T_{1/2}$ range of 1.9 to 30.9 hours. C_{max}, 24 hour clearance and $T_{1/2}$ did not correlate with MTX dose, age of patients or creatinine clearance. The lack of correlation between MTX levels and response may be due to the study design. Pharmacokinetics were measured following the first dose of MTX,

before the steady state of MTX was reached, therefore the levels would not have reached equilibrium between the sampling site and effector site. The wide inter-individual variability may be due to the lack of steady state of MTX at the time of sampling.

Two studies by Dervieux et al. have examined the relationship between MTXPG levels and response to MTX in RA [149, 150]. In a prospective study, the authors recruited 48 MTX-naïve RA patients [149]. MTX dose was increased every 4-6 weeks aiming for disease control. Response to therapy was measured using DAS-28. After six months of therapy MTXPG levels were measured. The authors showed that a lower change in DAS-28 from baseline (poor response) was associated with higher MTX dose (p = 0.02) administered and lower MTXPG levels (p = 0.01). In a second much larger cross sectional study (n=226) Dervieux et al. recruited RA patients on MTX therapy for a median of 51 months (IQR: 19-97) [150]. The participants had a long median disease duration (8.6 years, IQR: 4.2-17.9). Disease status was measured according to the number of tender and swollen joints (0-22) and physician's global assessment of disease activity (0-10 cm on VAS). Using multivariate linear regression, the authors demonstrated that higher MTXPG levels were independently associated with lower number of tender and swollen joints and physician global assessment of disease (p < 0.01, = 0.01 and = 0.01 respectively). Higher MTX dose was again associated with poorer response with a higher number of tender and swollen joints and physician global assessment of disease (p < 0.01, = 0.03 and = 0.01, respectively). However, MTXPG levels are not stable over time and samples are required to be tested within two days; increasing the cost of the assay as samples cannot be processed as a large batch thus reducing throughput [111].

Overall, these studies suggest that individuals may vary in their ability to polyglutamate MTX despite higher MTX doses which leads to poorer response. The association between MTXPG and disease activity or response has not been consistently replicated [151]. There are a number of possible reasons for this. The studies often include small sample sizes and may be underpowered [149]. Use of cross-sectional studies allows for increased recruitment of patients, but makes direct comparison of MTXPG levels according to disease activity status difficult as subjects will have been taking MTX for varying amounts of time [150, 151]. Furthermore, these studies have measured MTXPG levels in patients on MTX for a long period of time. Six months is a prolonged period for patients to be on ineffective medication leading to ongoing joint damage. An earlier biomarker of poor response is needed so that clinicians can add in more effective therapy.

1.9 Measurement of MTX and 7-OH-MTX

There is a wide variability between studies in the pharmacokinetics of MTX (section 1.7.5). This may be due to the different techniques employed in measurement of MTX and/or 7-OH-MTX, the low number of subjects recruited and the wide inter-individual metabolism of MTX.

As shown in Table 1-11, there is a wide variability in the performance of assays published within the literature. In clinical practice MTX is currently used at high doses in acute lymphoblastic leukaemia where it is routine practice to measure MTX levels in plasma to guide folinic acid rescue therapy [52]. The commonly used method of MTX measurement for high dose MTX therapy is the fluorescent polarisation immunoassay (FPIA) and enzyme-multiplied immunoassay technique [152] from Abbott and Siemens. The immunoassays have the advantage that they are easy to perform, have a rapid turn-around time and samples require little sample preparation prior to testing. However, they cross react with other substances such as 2,4-diamino-N10-methylpteroic acid (DAMPA), reducing their specificity and have a lower limit of quantification of 10nM [139, 142, 153-155]. Furthermore, it has been suggested that the major metabolite of MTX, 7-OH-MTX may be a more suitable measurement of adherence due to its longer half-life in plasma [125]. In order to detect adherence and investigate whether MTX/7-OH-MTX levels correlate to change in disease activity or are associated with response, an assay which detects MTX/7-OH-MTX over a prolonged period of time and therefore has a low lower limit of quantification (LLOQ) is required. Assays with dual detection of MTX/7-OH-MTX in urine may have the added advantage of being more acceptable to patients than the need for a blood sample.

HPLC (High performance liquid chromatography) methods have been developed for MTX detection which use fluorometric detection [156-159] and/or utilise solid phase extraction (SPE) which increases the cost of the assay and time for measurement [158]. HPLC separates solutes according to their physiochemical properties; following separation a method to detect the analyte of interest is required. Fluorometric detection of MTX is subject to interference by folates, thus reducing specificity and sensitivity [160]. The development of a method for the detection of these compounds using HPLC-SRM-MS (Selected Reaction Monitoring-Mass Spectrometry), specifically has the advantage of improved specificity with minimal cross-reaction and improved LLOQ [161]. Using SRM-MS as a method of detection improves specificity through using the fragmentation and mass/charge properties of the analyte; it is therefore a powerful technique for trace quantitative analysis. Methods utilising HPLC-SRM-MS to measure MTX have been developed but they are designed for use in plasma alone [162], to measure MTX alone [163, 164] or have an LLOQ too high for the measurement of adherence [144, 165, 166]. There is therefore a need for a HPLC-

SRM-MS assay without solid phase extraction to be developed that can measure MTX and 7-OH-MTX in urine and blood for the detection of adherence to low dose MTX in RA.

Study	Assay	Biofluid	Lower limit of quantification	
			MTX	7-OH-MTX
Herman et al. [122]	Radiochemical ligand binding assay.	Plasma and urine	4nM	Not tested
Oguey et al. [126] and Lebbe et al. [139]	Fluorescence polarization immunoassay	Plasma	10nM	Not tested
Kremer et al. [141]	Fluorescence polarization immunoassay	Plasma	10nM	Not tested
Mendu et al. [152]	Fluorescence polarization immunoassay	Plasma	60nM	Not tested
Godfrey et al. [142]	Fluorescence polarization immunoassay	Serum and urine	10nM	10nM
Bologna et al. [140]	Fluorescence polarisation immunoassay	Plasma	10nM	Not tested.
Seideman et al. [125] and Beck et al. [167]	HPLC with fluorometric detection	Urine	0.2nM	1nM
Baggott et al. [116] and Fox et al. [159]	HPLC with fluorometric detection	Plasma and urine	10nM	10nM
Hartmann et al. [158]	HPLC with fluorometric detection and solid phase extraction	Plasma and urine	220nM	44nM
Schwartz et al. [157]	HPLC with fluorometric detection	Plasma and urine	110nM	110nM
Hirai et al. [156]	HPLC with fluorometric detection and solid phase extraction	Urine	10nM	10nM
Rule et al. [166]	HPLC-QQQ-MS with solid phase extraction	Plasma and urine	2000nM	110nM
Turci et al. [164]	HPLC-QQQ-MS with solid phase extraction	Urine	1nM	Not tested
Barbieri et al. [163]	HPLC-QQQ-MS with solid phase extraction	Urine	1nM	Not tested
Schwartz et al. [168]	HPLC-QQQ-MS with protein precipitation and liquid-liquid extraction	Plasma and urine	110nM	110nM
den Boer et al. [162]	HPLC-QQQ-MS with protein precipitation	Plasma	5nM	Not tested

Table 1-11. Published methods of MTX or 7-OH-MTX measurement in urine and plasma with lower limit of quantification. Note that there is no assay developed that can measure MTX and 7-OH-MTX in urine and plasma.

1.10 Summary of Adherence and Response to MTX in RA

In summary, MTX is the first line therapy in RA but response is not universal. Adherence to MTX has been shown to affect response but adherence estimates vary widely due to the indirect methods of measurement employed thus far. A more direct method of measurement is therefore required. MTX and 7-OH-MTX have been shown to be detectable up to six days after MTX ingestion and measurement of these analytes may, therefore, allow for a more accurate measurement of adherence. MTX AUC and MTXPG levels have been associated with disease activity and response in a number of studies but the results are conflicting. The sample sizes tested are generally modest and the doses of MTX assessed are not always representative of doses used in current clinical practice. MTXPG levels are resource intensive to measure, requiring freezing at source and testing within two days. MTX levels in plasma correlate with erythrocyte MTX and may, therefore, provide an improved biomarker of response and adherence to MTX, due to the ease of measurement.

1.11 Adverse Events: Methotrexate-Induced Pneumonitis

There are a number of reasons why a patient may not continue on a drug, which include both lack of efficacy (which may be mediated by adherence) but also because of the development of adverse events to that drug. Indeed, fear of adverse events may reduce adherence and thereby reduce efficacy. Reassuring patients that they are not at high risk of an adverse event may, therefore, improve adherence and efficacy whilst avoiding harmful effects in patients at risk. An adverse drug reaction is defined as "*a response to a drug which is noxious and unintended, and which occurs at doses normally used in man*" [169]. Adverse drug reactions are responsible for up to one fifth of hospital re-admissions [170]. Within the context of MTX, toxicity has been reported to be a concern for patients starting therapy and is associated with reduced adherence to therapy [171]. The ability to stratify patients into risk for the development of side effects may therefore reduce the incidence of adverse events, improve adherence, clinical outcome and be cost beneficial to the NHS.

Adverse drug reactions are classified into six different subtypes according to the mechanism of action as shown in Table 1-12.

Reaction subtype	Features	Examples	Management
Dose related	• Related to pharmacological action of the drug • Low mortality	• Toxic effects: Bradycardia due to beta-blockers • Side effects: Anticholinergic effects of tricyclic antidepressants	• Withhold or reduce dose
Non-dose related	• Rare • Unpredictable • Not related to pharmacological action of drug	• Immunological reactions: MTX-pneumonitis • Idiosyncratic reactions: Acute porphyria	• Withhold and avoid in future
Dose and time related	• Related to the cumulative dose	• Adrenal suppression by corticosteroids	• Withhold or reduce dose
Time related	• Usually dose-related	• Carcinogenesis	• Withhold and avoid in future
Withdrawal	• Occurs after withdrawal of drug	• Opiate withdrawal syndrome	• Reintroduce and withdraw slowly
Failure of therapy	• Common • Dose-related • May be caused by drug-drug interactions	• Oral contraceptive failure after introduction of rifampicin	• Increase dose • Consider alternative drug to avoid interaction

Table 1-12. Classification of adverse drug reactions adapted from [172].

MTX is associated with a potentially life-threatening lung disease, MTX-pneumonitis (MTX-P), which is listed as an adverse event in patient-related reading material provided when patients start therapy. Indeed, the National Patient Safety Agency (NPSA) recommends that patients taking MTX are monitored for the development of pulmonary symptoms [173].

1.11.1 Pathophysiology

The pathophysiology of MTX-P has not yet been fully established. Several theories exist including that it occurs as a result of a direct toxic drug reaction [174] or an idiosyncratic hypersensitivity reaction [175]. MTX-P can occur at both low and high-dose therapy, suggesting that MTX-P is more likely to represent a hypersensitivity reaction rather than be due to dose-dependent folate antagonism or a direct toxic drug reaction (Table 1-12) [176-178]. Bronchoalveolar lavage and histological findings of MTX-P have also supported the theory of a hypersensitivity reaction as there is an immune response to MTX localised within the lungs with corresponding inflammation, cytokine release and activation of T-cells with CD4+ predominance [179-182]. Chemokines are

secreted by lung fibroblasts and epithelial cells, causing the chemotaxis of eosinophils into the lung parenchyma [182]. MTX has also been shown to stimulate lung epithelial cells to release the proinflammatory cytokine IL-8 [183].

1.11.2 Clinical Features of MTX-P

The main clinical features of MTX-P are dyspnoea with hypoxia. However, these symptoms are non-specific and are the reason why, in part, diagnosis and classification of MTX-P is challenging. A number of classification criteria have been proposed to facilitate further research (Section 1.11.3) but all have been criticised for failing to capture important aspects of the condition or for being too stringent. Due to the immunological nature of the disease, fever and malaise can occur; indeed, fever is one of the classification criterion [184]. Most cases of MTX-P are thought to occur within the first year of starting MTX [177, 185, 186]. In a literature review of all the available cases of MTX-P in the English-language literature, Kremer et al. reported that the median duration of MTX therapy to time to development of MTX-P was 36 weeks but with a wide range interval (1-480 weeks) (n=51 MTX-P cases) [177]. The duration of treatment prior to symptom-onset does not form part of any current classification criteria for MTX-P.

The signs and symptoms of MTX-P can manifest in other diseases, making the diagnosis challenging; these include infection, interstitial lung disease, adult respiratory distress syndrome, malignancy, extrinsic allergic alveolitis and pulmonary oedema. Indeed, interstitial lung disease occurs in 20-40% of patients with RA (so-called rheumatoid lung) [187, 188]. Diseases such as malignancy, interstitial lung disease and extrinsic allergic alveolitis would not, however, be expected to resolve following withdrawal of MTX; this feature is only captured in one of the current classification criteria (Carson et al [189].).

1.11.3 Classification Criteria

There are currently three classification criteria for MTX-P as shown below (Table 1-13). None are clinically validated and MTX-P is therefore a diagnosis of exclusion [177, 184, 189].

Carson et al. 1987	**Searles et al. 1987**	**Kremer et al. 1997**
Clinical 1. Clinical course consistent with hypersensitivity *Radiology* 2. Resolving infiltrates on chest x-ray after discontinuing MTX *Infection exclusion* 3. Exclusion of infection or other pulmonary disease *Histology* 4. Pathology consistent with drug induced injury (i.e. hypersensitivity pneumonitis or other toxic drug reaction) 3 or 4 out of the above 4 – probable MTX-P 2 of above – possible MTX-P 1 of above – unlikely MTX-P	*Clinical* 1. Acute onset of dyspnoea 2. Fever > 38°C 3. Tachypnoea ≥ 28/min and non-productive cough *Laboratory* 4. WBC ≤ 15 X 10^9/L (+/- eosinophilia) 5. PO_2 on room air < 55mg/Hg at admission *Infection* 6. Negative blood and sputum cultures (obligatory) *Radiological* 7. Pulmonary interstitial or alveolar infiltrates *Pulmonary function tests* 8. Restrictive pattern, decreased diffusion *Histopathology* 9. Bronchioloitis/interstitial pneumonitis with giant cells without evidence of pathogenic microorganisms 6 out of 9 criteria – Definite MTX-P 5 out of 9 criteria – Probable MTX-P 4 out of 9 criteria – Possible MTX-P	*Major criteria* *Histology* 1. Hypersensitivity pneumonitis by histopathological examination *Radiology* 2. Radiologic evidence of pulmonary interstitial or alveolar infiltration *Infection* 3. Blood (if febrile) cultures and initial sputum (if produced) cultures negative for pathogenic organisms* *Minor criteria* *Clinical* 1. Shortness of breath < 8 weeks 2. Dry cough *Laboratory* 3. O_2 saturations ≤ 90% 4. WBC < 15,000/mm^3 *Pulmonary function tests* 5. DLCO ≤ 70% predicted * microbiological criteria met if afebrile and no sputum production Definite case of MTX-P: Major criteria 1 Major criteria 2 and 3 and at least 3 minor criteria Probable case of MTX-P Major criteria 2 and 3 and 2 minor criteria

Table 1-13. Summary of MTX-P classification criteria.

The Carson criteria were developed based on expert opinion. The authors reviewed the medical records of patients with RA receiving MTX. 168 records were reviewed of which seven fulfilled criteria for probable MTX-P and two fulfilled the criteria for possible MTX-P (prevalence 5.4%). There were no statistically significant demographic or treatment related differences that predicted patients who would develop MTX-P. A weakness of the Carson criteria is the broad and

vague definitions. For example, there is no obligation that infection is excluded; therefore, a lower respiratory tract infection would result in an acute illness resembling a hypersensitivity reaction with pyrexia, dyspnoea and hypoxia. MTX would be withdrawn due to an acute illness and the chest x-ray would show resolution which would coincide with MTX cessation. Conversely, the ambiguous criteria allow for flexibility, which is required for case recruitment in the real world. For example, in clinical practice not all cases of suspected MTX-P will have documented evidence of negative blood and sputum cultures, but the clinical suspicion and disease course may be in keeping with MTX-P.

The Searles criteria were also based on expert opinion. The authors conducted a retrospective case note review of RA patients prescribed MTX within their local hospital and defined 4/73 patients as having developed MTX-P (prevalence 5.5%). The criteria require that infection is excluded by negative sputum and blood cultures and are more specific than the Carson criteria.

Criteria defined by Kremer et al. were modified from the Searles criteria. The criteria have excluded the need for negative cultures if the patient is apyrexial and there is no sputum production. The authors conducted a retrospective cohort review of MTX-P which occurred between 1981 and 1993. Cases were required to be either definite or probable. Twenty-nine patients fulfilled the criteria of MTX-P; of these, the most predominant symptoms were cough and shortness of breath with pyrexia noted in 69%.

1.11.4 Investigations

The full blood count may be normal but MTX-P has been associated with a mild peripheral eosinophilia which can develop in type 1 hypersensitivity reactions [177].

Due to the inflammatory infiltrate within the lung parenchyma, the lung loses its elasticity and pulmonary function tests reveal a restrictive pattern, represented by a reduced forced expiratory volume in one second (FEV_1) to forced vital capacity (FVC) ratio. Gas transfer testing demonstrates a reduced carbon monoxide transfer factor due to reduced diffusion across alveoli. The spirometry and blood results are not, however, specific to MTX-P and other diseases may demonstrate these features such as allergic bronchopulmonary aspergillosis [190].

Imaging demonstrates the inflammation within the lung parenchyma. Chest x-ray may show localised or diffuse interstitial shadowing [177]. High resolution CT scanning is a more sensitive imaging modality to detect MTX-P and demonstrates patchy ground glass shadowing as shown in Figure 1-15 [191]. Bronchoalveolar lavage demonstrates an increase in the CD4+/CD8+ ratio and the presence of inflammatory cells; however, that finding is not specific to MTX-P and is a supportive but non-diagnostic finding [192].

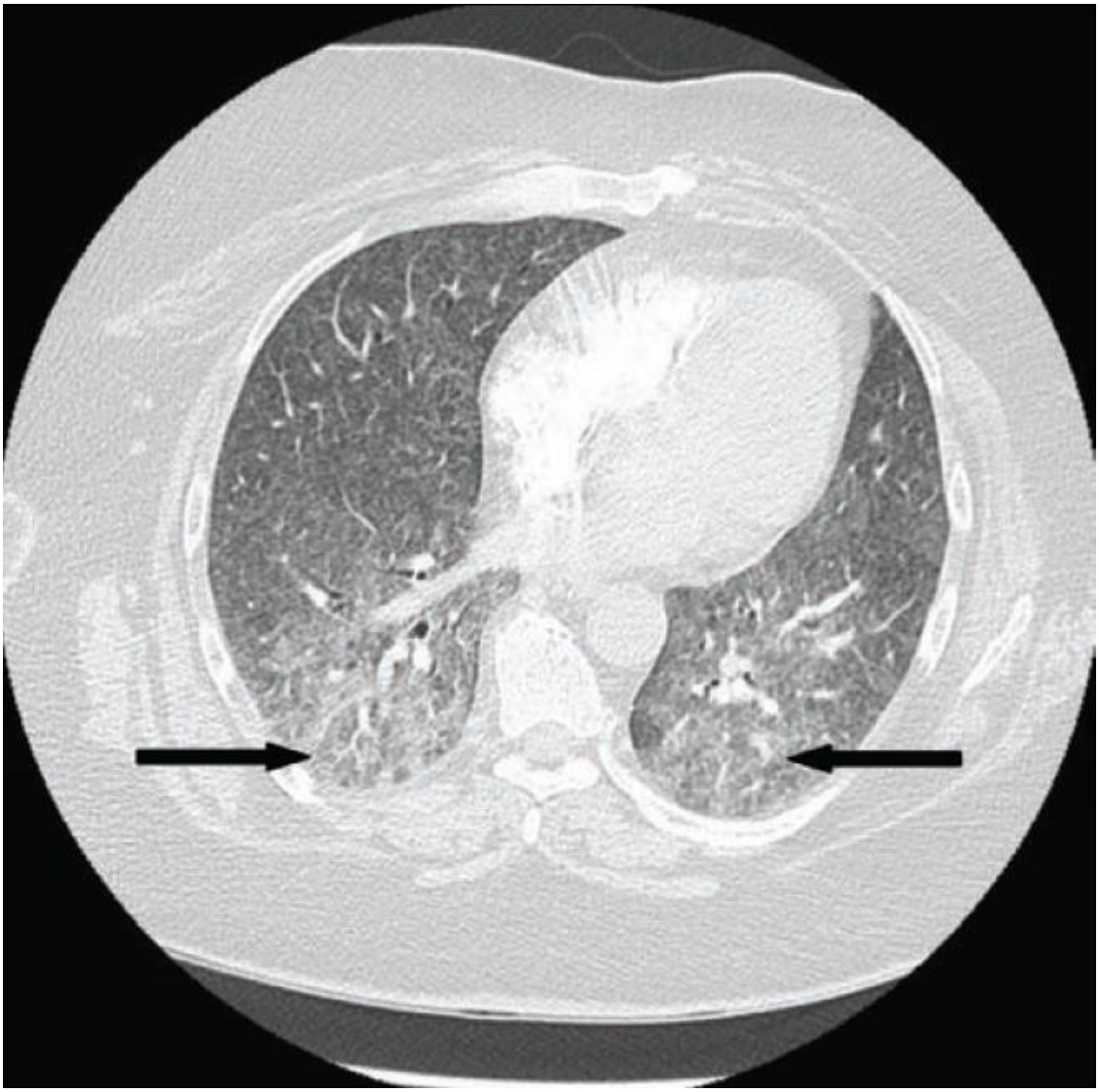


Figure 1-15. High-resolution CT scan of MTX-P. Note the ground-glass shadowing present bilaterally (arrows)[e].

1.11.5 Epidemiology

There is a broad range of estimates of prevalence of MTX-P in the literature, ranging from 0.7 to 7.3% [193, 194]. This is in part due to the rarity of the disease requiring large case recruitment, variation in case definition and study design heterogeneity.

The most recent study was a prospective study by Sathi et al. published in 2011 and was the largest prospective study to date, recruiting 223 patients starting low-dose MTX for all indications [195]. The diagnosis of MTX-P was made using the Carson et al. criteria. Baseline measurements included chest x-ray to exclude pre-existing interstitial lung disease. The study demonstrated a prevalence of 1.06% and an incidence of one case every 192 patient-years. A recent meta-analysis

[e] This figure was originally published in Can Respir J 2013;20(3):153-155. Reproduced with permission.

of randomised controlled trials (RCT) investigating MTX and lung disease in RA concluded that compared to all comparator agents, MTX is associated with a relative risk of MTX-P of 7.81 (95% CI 1.76-34.72, I^2=0%); however interestingly, there were no cases of MTX-P in the 13 RCTs published after 2002 [196]. There may be several explanations for this observation. In 2002, there may have been a change in the surveillance for pulmonary disease (for example, the improved access to HRCT with improved diagnostic accuracy) that may have led to a reduced likelihood of pulmonary symptoms being attributed to MTX-P, reducing the estimated prevalence. Later trials may have recruited fewer patients with risk factors for MTX-P such as advanced age and prior DMARD use, though this is unlikely to be a significant factor given that these risk factors are poorly predictive of MTX-P.

1.11.6 Non-Genetic Risk Factors

Risk factors for the development of MTX-P may be divided into either genetic or non-genetic risk factors. Non-genetic risk factors have been associated with the development of MTX-P in RA (Box 1-2); they are however, poorly predictive. In 1997 Alarcón et al. [197] conducted a multicentre case-control study to identify predictive factors for MTX-P. The study defined MTX-P according to the Kremer et al. criteria [177]. Controls were matched by the time to development of MTX-P in the index case. The authors classified 27 cases as definite and two as probable MTX-P and matched these to 82 controls. Multivariate logistic regression was utilised to investigate predictive baseline factors. The study found that age 60 years or older (OR_{adj} 5.1 95% CI 1.2 – 21.1), diabetes (OR_{adj} 35.6 95% CI 1.3 – ∞), hypoalbuminaemia (OR_{adj} 19.5 95% CI 3.5 – 109.7) and previous use of DMARDs (OR_{adj} 5.6 95% CI 1.2 – 27.0) were associated with an increased risk of MTX-P. The advantage of the use of a case-control study is that they are particularly useful for the study of rare diseases such as MTX-P as case recruitment is not as difficult as it would be for a prospective cohort study; it is also cost-effective as there is no long follow up period. However, despite the robust study design, the confidence intervals are wide, reflecting the small sample size.

A similar study by Ohosone et al. [198] recruited six patients with RA who developed MTX-P according to the Searles criteria. The cases were compared with controls with RA who did not develop MTX-P (n=278). MTX-P was significantly associated with older age (p < 0.005), pre-existing interstitial pulmonary abnormalities (p < 0.001) and adverse events to previous DMARD therapy (p < 0.01). Similar to the study by Alarcón et al. [197] the sample size tested was small, increasing the possibility of a type 2 statistical error.

- Age > 60 years
- Diabetes mellitus
- Hypoalbuminaemia
- Previous DMARD use
- Pre-existing lung disease
- Adverse events to previous DMARD therapy

Box 1-2. Risk factors for MTX-P.

1.11.7 Prognosis and Treatment

Mortality estimates for MTX-P in RA are high, between 18-25% [177, 199]. The wide variation in mortality may be due to the rarity of disease and difficulty in case ascertainment. Development of MTX-P earlier than six months of MTX therapy is associated with an increased mortality compared to later onset MTX-P [200]. Survivors of MTX-P have a good prognosis overall and there is no evidence that survivors are at increased risk of chronic interstitial lung disease. This is captured by the Carson et al. criterion, which requires that there are resolving lung infiltrates after MTX discontinuation.

The first-line therapy for any drug reaction is the immediate withdrawal of the offending drug. In view of the autoinflammatory nature of MTX-P, corticosteroids are usually prescribed to reduce inflammation and antibiotics administered until infection has been excluded [177]. However, there are no published guidelines on treatment of MTX-P and no evidence for the correct dose or route of corticosteroid therapy.

1.12 The Genetic Study of Disease

Although any two humans are >99% identical at their gene sequence, important differences do occur and are responsible for phenotypic features such as hair colour, height and, in some cases, the development of adverse events to drugs. A polymorphism is a variation of a gene occurring with fairly high frequency (>1%) in the population. A polymorphism associated with disease suggests that the polymorphism is involved in the aetiology of the disorder or is physically close (linked) to the causal gene (linkage disequilibrium). The most common type of polymorphism is a single nucleotide polymorphism (SNP). Since SNPs occur with a high density within the genome they provide useful markers in genetic studies of disease. SNPs are labelled with rs tags as universal nomenclature.

SNPs have different effects depending on their position and type of SNP (substitution, deletion or insertion). SNPs that lie within the exon can be non-synonymous SNPs altering the amino acid sequence of the protein they encode or synonymous SNPs which do not change the amino acid

sequence due to the redundant nature of the genetic code [201]. Intronic SNPs may be spliced from the transcribed mRNA, lie within the promoter region and regulate gene expression or lie in the untranslated regions (UTR) at the flanking region of the gene, termed the 5' and 3' termini of the gene. SNPs may impact on protein folding, active sites, protein-protein interactions, protein solubility or stability, and promoter, enhancer or operator SNPs may alter gene expression through transcription factor binding [202]. Genetic polymorphisms may affect drug response through a number of different mechanisms that include altering enzymes responsible for drug metabolism [203], affecting the sensitivity of the target to the drug [204] or altering drug transporters [205].

There are a number of different types of genetic studies that may be undertaken to investigate genetic risk factors of MTX-P which may be family based such as linkage studies and twin studies or non-family based such as association studies [206]. Due to the rarity of MTX-P the recruitment of families with MTX-P for family based studies would not be possible; the most relevant type of genetic study is, therefore, the association study.

1.12.1 Association Studies

Association studies compare the frequency of polymorphisms between patients and controls [207]. If a particular allele or genotype is significantly more frequent in a disease cohort compared to healthy controls it suggests that the polymorphism is causal for disease or in close proximity (linkage disequilibrium) to the causal polymorphism. Initially, association studies were conducted by investigating variants within candidate genes; however, associations were frequently erroneous and not replicated in subsequent studies. The current approach is the genome wide association study (GWAS). GWAS allow genome scanning to investigate polymorphisms across the whole genome that may be associated with disease in a hypothesis-free approach [208]. However, there are some disadvantages: Due to the large number of SNPs tested, there may be a high rate of false-positive findings, if no correction is made for multiple testing [209, 210]. For that reason, stringent significance thresholds have been agreed for claims of confirmed ($p < 5 \times 10^{-8}$) or suggestive ($p < 10^{-4}$) association.

1.13 The Genetic Basis of MTX-P

Prior to investigating genetic risk factors for the development of MTX-P it is necessary to establish that there is a genetic basis. Previous theories have suggested that the prevalence of MTX-P varies geographically due to genetic differences that increase the risk of developing MTX-P. Leflunomide-induced pneumonitis (LFN-P) is a disease that occurs in RA patients taking leflunomide and has similar clinical characteristics to MTX-P with a higher prevalence in the

Japanese population [211-213]. One theory exists that the Japanese population is at increased risk of LFN-P and MTX-P due to genetic differences compared to Europeans; however, this theory has not been consistently supported [214]. For example, a study by Shidara et al. [214] identified, from a prospective observational cohort of patients with RA, a subset of 18 patients who later developed MTX-P according to the Carson criteria. The age-adjusted incidence of MTX-P among the total patient population was 3.775 per 1,000 cases, lower than the suggested overall incidence in the wider literature.

MTX is widely used as a treatment for the chronic skin condition psoriasis. There is however a dearth of epidemiological studies within the literature examining the incidence of MTX-P in psoriasis and publications are limited to case reports. That suggests that MTX-P may occur less frequently in diseases other than RA. The observation suggests that the risk of MTX-P may be increased by factors that increase the risk of RA and not psoriasis, such as the strong genetic association of RA and HLA-DR4 alleles [215].

The ability to stratify patients according to risk of development of MTX-P would be of great benefit. One theory exists that there may be genetic risk factors that increase the risk of MTX-P although evidence for this is weak. The major histocompatibility complex (MHC) is a region located on chromosome 6 which encodes the human leucocyte antigen (HLA) proteins and transcription regulators. It encodes HLA-A, B and C molecules, which present both self and non-self peptides to CD8+ T-cells as well as DR DP and DQ molecules that present self peptides to CD4+ T cells thereby modulating the immune response. For many rare adverse events to synthetic drugs, genes or alleles within the MHC region have been found to be responsible. For example, HLA-A 31:01 is associated with carbamazepine-induced hypersensitivity skin reactions [216].

A recent study by Furukawa et al. recruited 55 Japanese patients with RA who developed MTX-induced interstitial lung disease (MI-ILD) and 709 Japanese RA patients without [217]. In a case-control study, the HLA loci were genotyped using a commercially available kit. The results suggested that HLA-A 31:01 was significantly associated with the development of MTX-P (p_{corr} = 1.93 X 10^{-3}, OR = 2.97). HLA-A 31:01 is associated with carbamazepine-induced hypersensitivity syndrome in the European population, suggesting an association between the locus and the development of immune-mediated drug hypersensitivity [216]. However, the paper does not describe the criteria for MI-ILD used to define the cases, the matching of cases and controls or the quality control steps used to ensure there was no error in genotyping.

The association between HLA-A and MTX-P would be unexpected biologically. HLA-A molecules present processed peptide fragments to CD8+ T_c lymphocytes stimulating T_c lymphocyte

proliferation and not CD4+ T_H lymphocytes. Therefore functionally it would be expected that the ratio of CD4:CD8 would be reduced, in contrast to results from bronchoalveolar lavage, which show that the opposite is true in MTX-P. There is therefore a need to validate the association independently in a European cohort as the association may be due to population stratification, errors in genotyping or misclassification of cases and controls.

1.13.1 Bioinformatic Analysis

Whilst GWA studies investigate SNPs associated with disease, the SNPs identified may be in linkage disequilibrium with the true disease causing variants (Section 3.11.10.1). A GWAS may therefore lead to a number of SNPs within a particular region that are associated with disease. Further research post-GWAS is required to identify the true disease causing SNP including investigating the potential function of the SNP. There is a wealth of information available in publicly available databases of experimental functional investigations which can be interrogated to provide evidence for the function of a SNP. The advantage of bioinformatic analysis is the reduced need to replicate previous experiments, thereby reducing cost and analysis time.

1.14 Pharmacogenetics and Pharmacogenomics

Pharmacogenetics is the study of inherited variations in drug response and describes the influence that a single gene has on drug response. The aim of a pharmacogenetic study is to identify genetic variation (polymorphism) which affects the response to therapy; this would allow the development of stratified medicine, determining which patients are more likely to respond or develop an adverse event to a drug.

Genotyping technology has evolved over the past decade and there is now access to high throughput genotyping platforms which can quickly genotype a proportion of the genome paving the way to pharmacogenomics. Pharmacogenomics is the investigation of the effect of multiple genes on drug response [218]. Pharmacogenomic research may also identify key biochemical pathways which are responsible for the response to medication, allowing for the development of drugs to target these pathways. It is hoped that by studying the pharmacogenomics of MTX-P it will:

- Provide clues to the pathogenesis of disease, which may lead to novel therapies.
- Discover genetic markers of disease susceptibility to aid diagnosis of a clinically heterogeneous disease.
- Identify genetic markers of disease severity, allowing individual-tailored approaches to clinical management.

The U.S FDA has recognised the important role that pharmacogenomics plays and has published a list of 166 FDA-approved drugs with pharmacogenomic information in their labelling [219]. The majority of these genetic associations that have been discovered affect the risk of adverse reactions through three mechanisms:

1. Affecting drug transport.
2. Affecting drug metabolism.
3. Affecting the immune response.

Five adverse reactions that are listed are associated with variation at the MHC locus suggesting an interaction between the drug and the immune system which would be expected to be dose-independent.

Typically, the effect size (odds ratio) for polymorphisms that affect drug response are much higher than the effect sizes for polymorphisms that increase the risk of genetically complex diseases (such as RA) [220]. This has the advantage of smaller sample sizes being required in pharmacogenomic studies in order to power a study that can detect a large effect size; for rare phenotypes such as MTX-P this is of particular advantage. The major challenge of pharmacogenomic research is recruitment of an adequate number of subjects due to the low number of patients who experience significant adverse events. Pharmacogenomic studies can therefore be time consuming and are required to be multi-centred studies.

1.15 Summary of MTX-P

In summary, MTX-P is a life-threatening adverse reaction to MTX that occurs in patients with RA. Risk of toxicity is a concern for patients starting MTX therapy and is associated with reduced adherence to therapy which may therefore affect response. Research to date has suggested that the risk of developing MTX-P may be increased in genetically pre-disposed subjects, with one previous study in a Japanese population implicating variants at the HLA locus with susceptibility to MTX-ILD. That finding requires independent replication and the search for variants predisposing to MTX-P ideally requires a hypothesis-free GWAS. Bioinformatics support should be sought for variants associated with disease to hypothesise a functional role and biological plausibility.

1.16 Summary

MTX is a first-line therapy in the treatment of RA that is economically advantageous. However, response is not universal and drug persistence is limited beyond two years; this may be, in part, due to waning efficacy over time and the development of adverse events. Efficacy is associated with adherence but methods to measure adherence are limited to indirect methods only which have inherent limitations; the development of a biochemical assay for direct measurement of

adherence would therefore be a major breakthrough. Adverse events affect response to MTX in two ways; directly due to cessation of MTX due to the adverse event and indirectly as fear of adverse events reduces adherence. One of the most important adverse events to MTX due to its high fatality rate is the development of MTX-P. This is a rare disease but rare adverse events to other drugs have been associated with genetic variation. The detection of genetic variants that are associated with MTX-P would be a major clinical advance, allowing for the stratification of individuals at risk of MTX-P.

Chapter 2: Hypothesis and Aims

2 Hypothesis

RA patients with reduced adherence to MTX experience reduced response. Whilst, MTX pharmacokinetics vary widely between individuals, there is some evidence to suggest that measuring MTX levels may predict response in patients with RA. Not all response to therapy is beneficial however; a proportion of patients with RA develop life-threatening MTX-P as a consequence of MTX therapy. Risk of developing MTX-P may be increased due to genetic variation. This thesis will investigate whether MTX and 7-OH-MTX levels can be measured to detect adherence using HPLC-SRM-MS, are associated with MTX treatment response and if there are genetic risk factors for developing MTX-P. The hypotheses for this work are:

1. Measuring MTX and 7-OH-MTX in plasma and urine using HPLC-SRM-MS can accurately predict adherence to MTX in RA.
2. MTX and 7-OH-MTX AUC at steady state in plasma are correlated with change in DAS-28 over three months and/or are associated with EULAR response criteria.
3. MTX-P is an autoimmune hypersensitivity reaction that occurs in a genetically susceptible individual with rheumatoid arthritis who is prescribed MTX.

2.1 Aims

The aims of the study with respect to the first hypothesis are to:

1. Develop a HPLC-SRM-MS assay to measure MTX and 7-OH-MTX levels in plasma and urine.
2. Perform a pharmacokinetic study of MTX and 7-OH-MTX in plasma and urine of patients with RA.
3. Develop a pharmacokinetic model of MTX and 7-OH-MTX in plasma to determine the ability of the HPLC-SRM-MS assay to measure adherence over time.
4. Validate the pharmacokinetic model by measuring MTX and 7-OH-MTX levels in plasma of patients with RA recruited to a prospective observational study.

The aims of the study with respect to the second hypothesis are to:

1. Measure MTX and 7-OH-MTX levels in plasma of patients with RA prescribed MTX recruited in a prospective observational study.
2. Investigate the correlation between three month MTX AUC and 7-OH-MTX AUC and change in DAS-28 over three months.
3. Investigate the association between three month MTX AUC and 7-OH-MTX AUC and EULAR response at three months.

The aims of the study with respect to the third hypothesis are to:

1. Recruit cases with physician diagnosed MTX-P and age-sex matched RA controls prescribed MTX without the development of MTX-P.
2. Conduct a genome wide association study (GWAS) to identify genetic markers that increase the risk of MTX-P in patients with RA.
3. Perform a bioinformatics search of available databases to investigate SNPs with a potential functional role in the development of MTX-P

Chapter 3: Methods

3 Methods: Investigation of MTX/7-OH-MTX Levels in Urine and Plasma of Patients with RA

3.1 Measurement of MTX and 7-OH-MTX in Urine and Plasma

3.1.1 Study Setting and Funding

This research was undertaken in the Arthritis Research UK Centre for Genetics and Genomics in the Institute of Inflammation and Repair, The Centre for Advanced Discovery and Experimental Therapeutics (CADET) and The National Institute for Health Research (NIHR)/Wellcome Trust Clinical Research Facility in Manchester. The sponsor for this study was The University of Manchester. The research was funded by a three year North West England Medical Research Council (MRC) Clinical Pharmacology and Therapeutics Research Training Fellowship to the author; a grant award to the author from the NIHR Manchester Musculoskeletal Biomedical Research Unit and genotype consumable funding from the NIHR Manchester Musculoskeletal Biomedical Research Unit.

The Rheumatoid Arthritis Medication Study (RAMS) study (REC ref: 08/H1008/25) is a one year prospective multi-centre observational study designed to identify predictors of response to MTX in patients with RA.

3.1.2 RAMS Recruitment

RA patients who are starting MTX were informed about the study and invited to participate. Inclusion and exclusion criteria for the study are as follows:

Inclusion Criteria

- Age 18 and above.
- Physician diagnosis of RA or early undifferentiated polyarthritis.
- About to start MTX as monotherapy or in combination with other DMARDs, including oral steroids, for the first time. This can also include patients in whom MTX is being added in to another DMARD such as sulfasalazine or hydroxychloroquine; or patients who are taking oral steroids.
- Informed consent.

Exclusion Criteria

- Patients with known contraindications to MTX such as women of childbearing age not taking adequate contraception, excess alcohol consumption or abnormal liver function tests.

- Participating in a blinded clinical trial such that not all the medication being taken by the patient is known.

3.1.3 Baseline Clinical Assessments

Baseline clinical and demographic data were collected via a Case Report Form (CRF) during an interview with the patient and supplemented by data from the patient's case notes prior to MTX commencement. In addition, blood and urine samples (a subset of the overall RAMS cohort) were taken, aliquoted and stored in -80°C freezers. The following data is collected:

- Demographic data: age, gender, height, smoking history.
- Clinical data: Date of onset of RA symptoms, ACR 1987 RA classification criteria, current medication and the DAS-28 components (swollen and tender joint count and patient VAS of global disease activity).
- Laboratory data: CRP, ESR, creatinine, RF and anti-CCP are recorded from the case notes.
- Blood samples: A 10ml sample for plasma, external CRP testing and RF testing was collected prior to MTX commencement. Samples are sent to the unit via post in specially prepared packaging.

3.1.4 Follow-up Clinical Assessments

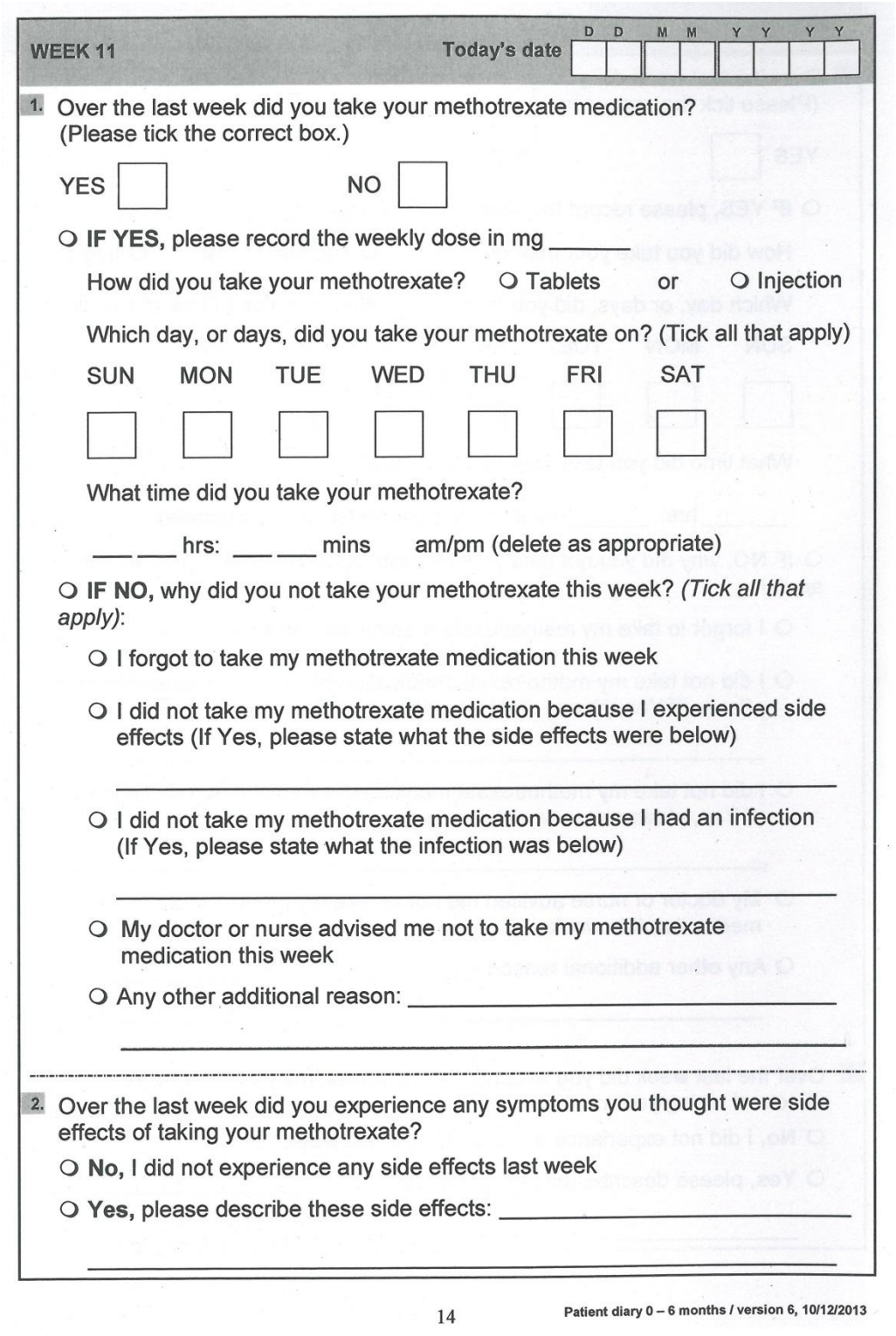
At three months the DAS-28 components and MTX dose are recorded and a 10 ml blood sample for plasma is collected.

At six and 12 months, the following data were collected:

- Clinical data: The DAS-28 components.
- Medication History: Details of the current dose, day of week and time the patient takes MTX and whether any additional DMARDs have been added and the date when added.
- Laboratory data: CRP, ESR, creatinine, RF and anti-CCP are recorded from the case notes.
- Blood sample: A 10ml sample for plasma, external CRP and RF testing. Samples are sent to the unit via post in specially prepared packaging.

3.1.5 Patient Diary

At baseline and six months patients were given a diary to record MTX adherence each week. They were asked if they took their MTX, the dose of MTX, the day of week that MTX was taken and time that MTX was taken as shown in Figure 3-1 (version 6).



WEEK 11 Today's date D D M M Y Y Y Y

1. Over the last week did you take your methotrexate medication? (Please tick the correct box.)

YES ☐ NO ☐

❍ **IF YES,** please record the weekly dose in mg _____

How did you take your methotrexate? ❍ Tablets or ❍ Injection

Which day, or days, did you take your methotrexate on? (Tick all that apply)

SUN MON TUE WED THU FRI SAT

☐ ☐ ☐ ☐ ☐ ☐ ☐

What time did you take your methotrexate?

___ ___ hrs: ___ ___ mins am/pm (delete as appropriate)

❍ **IF NO,** why did you not take your methotrexate this week? *(Tick all that apply)*:

❍ I forgot to take my methotrexate medication this week

❍ I did not take my methotrexate medication because I experienced side effects (If Yes, please state what the side effects were below)

_____

❍ I did not take my methotrexate medication because I had an infection (If Yes, please state what the infection was below)

_____

❍ My doctor or nurse advised me not to take my methotrexate medication this week

❍ Any other additional reason: _____

_____

2. Over the last week did you experience any symptoms you thought were side effects of taking your methotrexate?

❍ **No,** I did not experience any side effects last week

❍ **Yes,** please describe these side effects: _____

_____

14 Patient diary 0 – 6 months / version 6, 10/12/2013

Figure 3-1. Patient diary card for the RAMS study.

3.2 Development of a HPLC-SRM-MS Assay in Urine and plasma

3.2.1 Study Design

This was an experimental study to develop an assay to measure MTX and 7-OH-MTX in urine and plasma using HPLC-SRM-MS using the guidelines of assay bioanalytical method validation by the

European Medicines Agency (EMA) as a benchmark [221]. MTX-free urine was obtained from patients recruited to RAMS. Whole blood was obtained from healthy volunteers recruited to the National Repository Study, a study designed to recruit healthy volunteers as a comparison cohort and for protocol, technique and method development. Ethical approval for the National Repository Study was granted by the North West Research Ethics Committee (REC reference 99/8/084).

3.2.2 Method Development and Validation

An assay is defined as "*the process of estimating an unknown concentration or potency. The unknown is compared with a standard of known concentration or potency*" [222]. The method development stage often runs alongside method validation. The method development stage is designed to select the ideal analysis technique, sample preparation method and chromatographic conditions. A well-developed assay designed to measure adherence requires high performance in order to be reliable and show validity. Performance is tested in several domains based on guidelines from the EMA [221]:

- Accuracy – the assay is required to measure the true value of the analyte. This is calculated by spiking a known concentration of analyte into water or the biological matrix of interest (e.g. urine or whole blood) and comparing with the assay measurement. Mean concentrations should be within 15% of the expected value.
- Linearity – the assay signal increases proportionally to the concentration of the analyte.
- Precision – relates to the closeness of repeated measurements of an analyte to each other. An assay with high precision will return the same concentration for repeated measurements of the same sample. Precision is often calculated statistically as the coefficient of variation (CV). CV should be ≤ 15%.
- Lower limit of quantification (LLOQ) – the lowest concentration of the analyte in a sample that can be quantified with a certain amount of accuracy and precision. The LLOQ should be five times greater than the signal observed for a blank matrix injection.
- Lower limit of detection (LLOD) – the lowest concentration of the analyte that produces an assay signal which can be separated from the background noise.
- Stability – the analyte is required to be stable over time prior to testing. Mean concentration at each time point should be ± 15% of the expected concentration.
- Carryover – carryover is the detection of analyte in a blank sample following injection of a high concentration sample. Carryover occurs due to contamination of the blank sample. Carryover in the blank sample following a high concentration standard should not be greater than 20% of the LLOQ.

HPLC-SRM-MS was chosen as the preferred analysis technique as published studies have shown that this technique has a lower LLOQ and improved specificity in MTX/7-OH-MTX measurement compared to other techniques (Section 1.9).

3.2.3 HPLC-QQQ-MS

A standard system for HPLC-SRM-MS is shown in Figure 3-2.

Figure 3-2. Standard system setup for HPLC-SRM-MS. Samples first undergo sample preparation to remove molecules that affect the specificity of the assay.

3.2.4 Sample Preparation

Sample preparation is required prior to HPLC-SRM-MS in order to remove molecules that may affect the specificity of the assay to detect the analyte of interest whilst not affecting the recovery of the analyte of interest. There are numerous methods available for sample preparation. Protein precipitation with organic solutions is a simple method for removing large proteins from samples. An organic solution such as methanol precipitates the protein out of solution. The protein free supernatant is then dried under vacuum, resuspended and centrifuged. Protein precipitation has the advantage that it is economical and requires little in the way of specialist expertise. Solid phase extraction (SPE) is another method of sample preparation which separates analytes through a solid phase extraction cartridge based on their polarity or other physiochemical properties [223]. A solid stationary phase sorbent is used to isolate and concentrate the analyte, with a solvent to interrupt the retention of the target analyte and sorbent. SPE is highly selective and therefore may increase the assay sensitivity by reducing noise but adding SPE to a method requires further validation steps, increases the time for sample preparation and the overall cost of the assay.

3.2.5 HPLC

A biofluid such as urine will contain multiple molecules. In order to accurately and reproducibly detect the analyte/s of interest (MTX and 7-OH-MTX in this case), they must be separated from other solutes. HPLC is a process that separates solutes according to their physiochemical properties (e.g. charge, hydrophobicity). A layer of coated beads is present within a column which is referred to as the stationary phase. The stationary phase has a selected polarity which will attract or repel molecules within the biofluid depending on the molecule's polarity. A mobile

phase flows through the column which will compete with analyte molecules for binding to the stationary phase causing displacement and elution of the analyte. The spherical beads within the column have diameters up to 10 µm; this small diameter increases separation, but slows the flow of the mobile phase, so high pressure is required to drive the biofluid through the column. Consequently, it is necessary to remove solutes which may contaminate the column causing it to block and preventing elution of the analyte of interest. High pressure forces the biofluid through the column, flushing the column with the mobile phase. Depending on polarity, the solutes will elute at different times as they will spend a different amount of time in the stationary phase bound to the beads of the column [224]. Separation will therefore occur due to different solutes eluting at different times (the retention time) in the mobile phase. Formic acid is often added to the mobile phase as a mobile phase modifier in order to improve the separation of solutes by altering the pH of the mobile phase and improving the ionisation of the eluent, which is vital for the QQQ-mass spectrometer [225]. Different properties of columns will affect the retention time. For example, as column length increases, separation between solutes increases due to more time for the solutes to separate, improving resolution as shown in Figure 3-3 but at the cost of longer analysis time, reduced throughput and consequently cost.

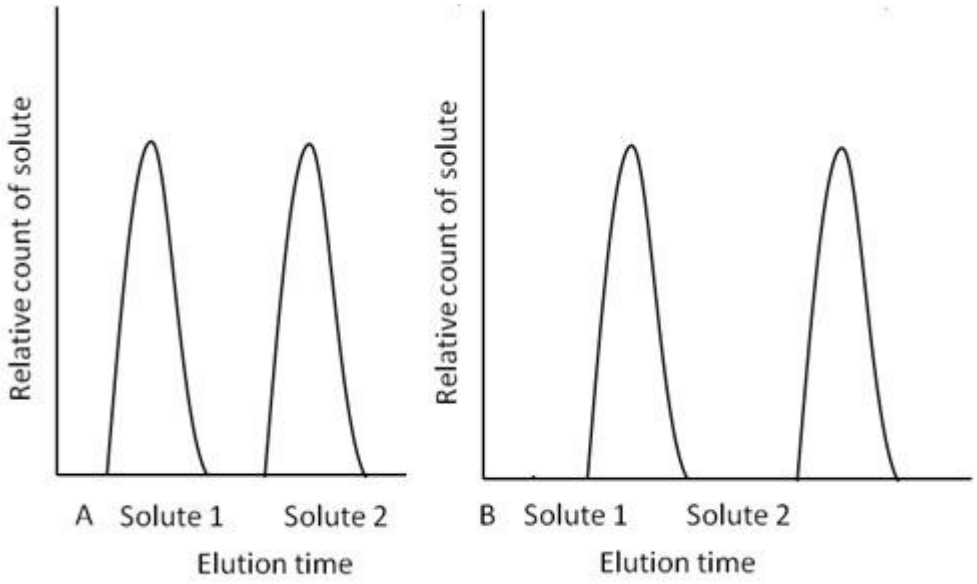


Figure 3-3. The effect of increasing column length on assay specificity. Figure A presents results expected from a short column, figure B presents results expected from a longer column showing improved resolution between solute 1 and 2 but at the expense of a longer run time.

The ideal column and chromatography conditions will allow adequate separation of solutes to allow detection of analyte, but without broadening the peak so that the detection waves of different solutes do not cross-over as that reduces specificity. Reverse phase chromatography is a method whereby the column contains a layer of non-polar compounds maintaining the solute in

the stationary phase. Polar solutions such as acetonitrile (ACN) are used as the mobile phase. Polar solutes will, therefore, spend more time within the mobile phase and will elute at a lower retention time. A hydrophobic analyte such as MTX will initially be bound in the stationary phase if the mobile phase is aqueous; gradually the mobile phase is changed to organic in a gradient of increasing concentration so that the analyte becomes mobile and elutes at a particular retention time [9]. There are numerous mobile phases available, but the majority of methods use methanol or ACN. The advantage of ACN is that it is highly competitive for interaction with sites on the stationary phase, causing the analyte to move faster through the column with a shorter run time.

3.2.6 SRM-MS

Following separation with HPLC there is a need to improve specificity of the assay with further separation of the eluent, to allow detection of solutes which may be similar in their physiochemical properties. The first stage of QQQ-MS requires the eluent to be nebulised and ionised. Droplets are formed by the nebulisation of the eluent into a spray. Ionisation occurs through electrospray ionisation resulting in charged droplets of the solute [226]. Ionisation of the analyte can be either favoured (ion enhancement) or not (ion suppression) by the presence of other compounds within the biofluid (termed matrix) which will increase or reduce the detection of the analyte (matrix effects) [227]. For this reason, an internal standard that behaves similarly to the analyte of interest is spiked into samples to correct for differences in ion suppression/enhancement and the effect of sample preparation on the analytes. An internal standard is chosen that is similar in structure to the analyte of interest and therefore will elute at the same time and be subject to the same matrix effects. The effect of the matrix is the difference between the mass spectrometric response for an analyte in aqueous solution compared to the response at the same concentration in a biological matrix (such as urine) [228]. The effect of sample preparation on the spectrometric response is calculated by spiking the biofluid with a known concentration of analyte, performing sample preparation and measuring the output and comparing this with sample preparation first and then spiking the biofluid; this is termed recovery and is calculated by:

Extraction recovery = response extracted sample with analyte ÷ response post extracted spiked sample

Following ionisation, the droplets are heated causing them to reduce in size, increasing the surface charge of the droplet causing it to transform into the gas phase. The QQQ-MS consists of three quadrupoles (Q1, 2 and 3), which are used to select the analyte of interest for detection (selected reaction monitoring) [225]. Each quadrupole consists of four cylindrical rods; a

radiofrequency voltage is applied between pairs of rods and a direct current voltage across the rods to create an oscillating electric field. The oscillating electric field causes the ions to spiral down the quadrupole. In Q1, analytes with a specified mass/charge (*m/z*) ratio are selected by successfully travelling through Q1 whilst other ions will collide with the rods and be destroyed. In Q2, the collision cell, the precursor ions collide with gas molecules to fragment the ions. The fragments have a specific *m/z* ratio depending on the fragmentation properties of the precursor ion. A fragment of the analyte of interest is selected in Q3. The time allowed for selection of the analyte in Q3 is the dwell time and the analysis can be multiplexed so that different analytes can be detected in the same sample. The detector consists of a conversion dynode. Ions of the analyte of interest collide into the conversion dynode which produces electrons in response, producing an electrical signal proportional to the number of ions that strike the conversion dynode and this is used to produce the spectrometric response.

Tuning of the mass spectrometer settings is required in order to create optimum conditions for ionisation and fragmentation. A constant stream of analyte and mobile phase is introduced to the ion source to provide a steady signal from the analyte ions. Parameters that affect the nebulisation improving the production of ions are altered (e.g. nebuliser gas flow, eluent flow rate). In addition parameters affecting the mass analyser for the SRM are adjusted (e.g. collision cell energy, gas flow, quadruple voltage and dwell time).

HPLC-SRM-MS methods are not, however, a panacea. The methods require a high level of technical skill and involve sensitive equipment that is more liable to breaking down compared to immunoassays requiring extended down-time which can take a prolonged period to repair [229, 230].

3.2.7 Reagents and Chemicals

MTX, 7-OH-MTX and deuterium-labelled MTX (MTX-d_3) were purchased from Toronto Research Chemicals (catalog number: M260675, H945925 ,M260677). Their chemical structures can be seen in Figure 3-4. MTX-d_3 is similar to MTX in its molecular structure but it contains a stable isotope of hydrogen, termed deuterium, which can be separated from MTX due to its different mass/charge ratio and therefore act as an internal standard. LC-MS grade acetonitrile (ACN), water, methanol and formic acid were purchased from Sigma-Aldrich®. Human urine, used for both spiking of samples and to prepare calibration curves was obtained from pooled baseline (pre-MTX) samples (n=3) from the RAMS cohort. Use of these samples had the advantage that the biological matrix used for method validation is from a cohort of patients in whom the assay will be

used. Human whole blood was obtained from the National Repository Study. Human pooled plasma was purchased from the NHS Blood and Transplant service.

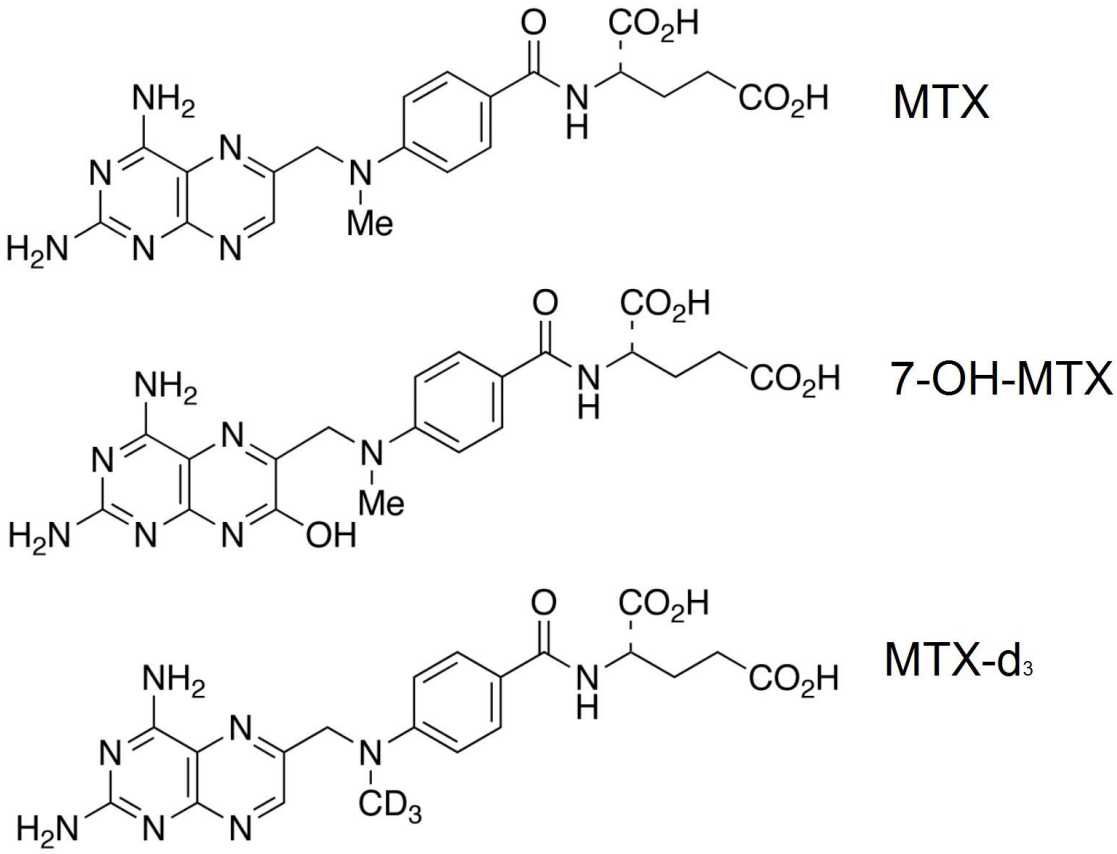


Figure 3-4. Chemical structures of MTX, 7-OH-MTX and MTX-d_3.

3.2.8 Internal Standards in Assay Development

MTX-d_3 was used as the internal standard. The internal standard is added in a constant concentration to samples (50 nM) and used for calibration by comparing the signal for MTX-d_3 and standards of MTX/7-OH-MTX. The internal standard corrects for loss of analyte during sample preparation and matrix effects. MTX-d_3 was chosen as it is similar in structure to MTX and 7-OH-MTX so that the effects of sample preparation and matrix effects should be similar to the internal standard and the analytes of interest but the mass/charge of MTX-d_3 in Q3 is different owing to the deuterium so that the signal can be resolved from MTX and 7-OH-MTX. Use of an internal standard controls for variations in recovery and ionisation.

3.2.9 Preparation of Standards and Samples

Stock solutions were prepared in water at concentrations of 0.1 nM, 1 nM, 10 nM, 100 nM and 1000 nM for MTX/7-OH-MTX and 500 nM for MTX-d_3 and stored at -80°C. A working solution was prepared freshly for each batch of samples. Samples were spiked with the required amount of MTX and 7-OH-MTX. Protein precipitation was performed by the addition of 200 µl organic solvent (ACN or methanol) with 12.5nM MTX-d_3 to 50 µl of sample, and subsequently vortex mixed at room temperature for ten minutes. Samples were then centrifuged at 10,000g for ten minutes. The supernatant was removed and dried in a concentrator (Eppendorf concentrator plus) for at least three hours at room temperature and reconstituted in 50 µl water prior to LC-MS/MS analysis (Figure 3-5). Standard concentrations up to 1000 nM for MTX and 7-OH-MTX were based upon previous pharmacokinetic studies in plasma suggesting that mean C_{max} is lower than 1000 nM [126, 140, 141].

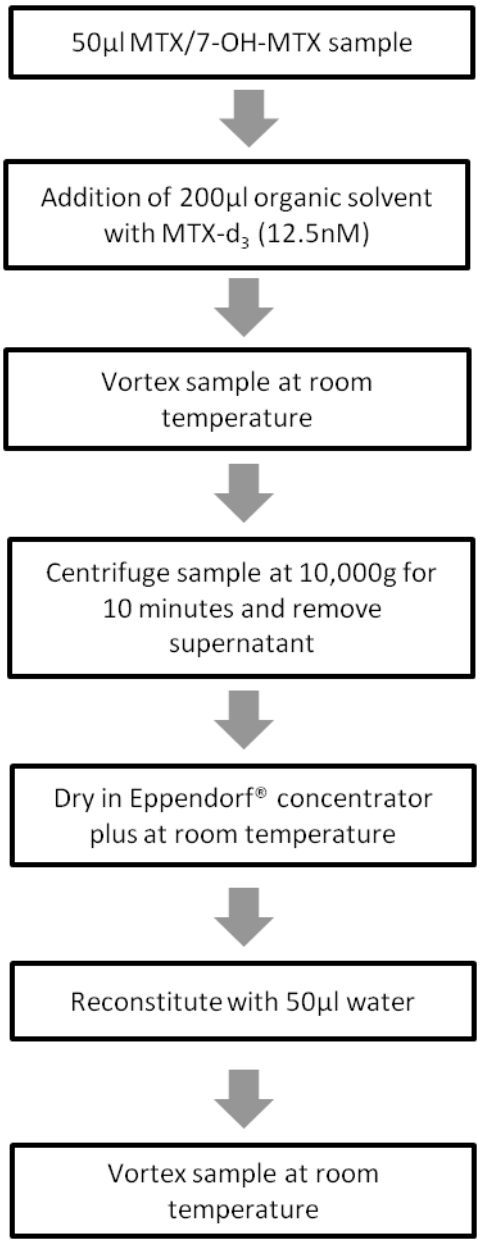


Figure 3-5. Sample preparation protocol for protein precipitation.

3.2.10 Instruments

A manual front loading injector was used to inject 5 µl of sample. Analyses were performed on an Agilent® 6460 triple quadrupole mass spectrometer with an electrospray ionisation source operated in the positive ion mode.

3.2.11 Chromatographic Conditions

Chromatographic separation was performed using a Thermo Scientific™ Hypersil GOLD HPLC column with a particle size 1.9 µm, 100 mm length and 2.1 mm diameter maintained at 21 °C. The mobile phase consisted of acetonitrile (ACN) with 0.1% formic acid as the organic solvent (B) and water with 0.1% formic acid as the aqueous phase (A). The system was equilibrated in 5% buffer B

at a flow rate of 0.3 mL/min. Separation of the analytes was achieved by increasing the percentage of buffer B until elution occurred at the required time. The elution gradient was subsequently produced (Table 3-1); the total chromatographic run time was 11 minutes to achieve full equilibration of the system.

Time after injection (min)	Buffer A (%)	Buffer B (%)
0.00	95	5
2.00	95	5
2.50	75	25
7.50	75	25
7.51	95	5
10.00	95	5

Table 3-1. Gradient elution timetable.

The final 95% A concentration is for re-equilibration of the column to ensure that for the next sample, the column is equilibrated and the retention time will be the same as for the previous sample.

3.2.12 Mass-Spectrometry Detection

Analyses were performed on an Agilent® 6460 triple quadrupole mass spectrometer equipped with an electrospray ionisation (JetStream source) operated in positive ion mode and MTX/7-OH-MTX were detected using the following SRM *m/z* transitions: 455.1>308.1 for MTX, 471.1>324.1 for 7-OH-MTX, and 458.1>311.1 for MTX-d_3. The mass spectrometer settings were optimised manually as follows: cone voltage 1500 V, capillary voltage 4000 V, collision energy 25 eV, source temperature 350 °C, desolvation temperature 350 °C with a nitrogen gas flow of 10 L/min and cone gas flow 11 L/min. Nitrogen was used as collision gas at a pressure of 45 psi. Quantification of the calibration curve was calculated using the peak-area ratio of the analyte to internal standard using Agilent MassHunter Workstation Software Quantitative analysis B.04.00. Signal to noise ratio was calculated by dividing the greatest height of the signal range above the mean noise value divided by the variance.

3.2.13 Assay Validation

Assay optimisation was first completed in water to negate the effect of the biological matrix and then moved to testing of urine and plasma (Figure 3-6).

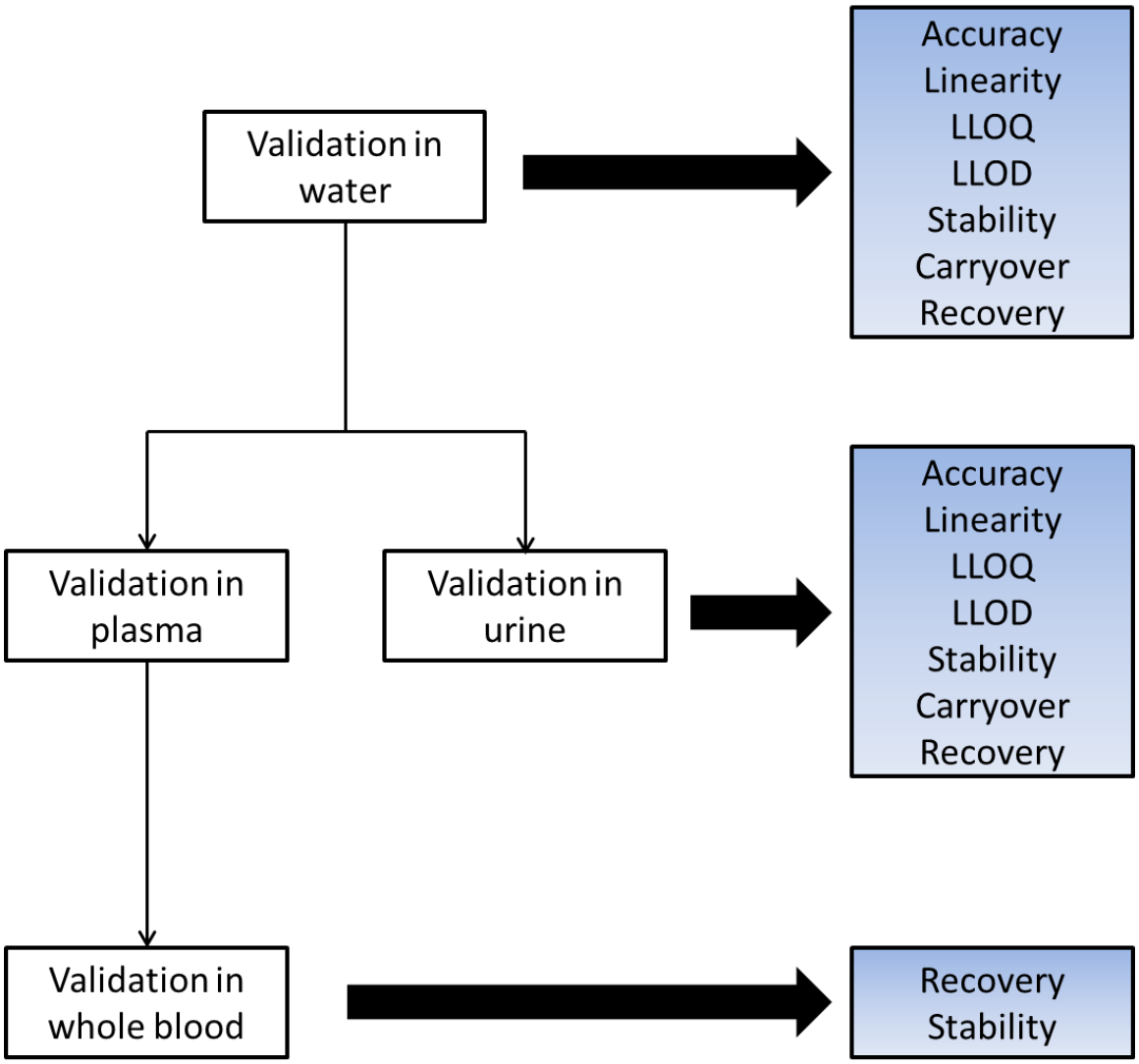


Figure 3-6. Assay validation workflow.

3.3 Assay Validation in Water

3.3.1 Lower Limit of Quantification and Lower Limit of Detection

The LLOQ and LLOD were measured from the linearity experiment. A signal five times greater than the noise signal observed was considered to be the LLOQ. A signal discernible from the background noise by visual check of the spectrograph was considered to be the LLOD.

3.3.2 Carryover

Carryover was assessed by the signal following injection of 1000 nM MTX/7-OH-MTX and then a blank sample (Figure 3-7). EMA requirements are that carryover is less than 20% of the LLOQ.

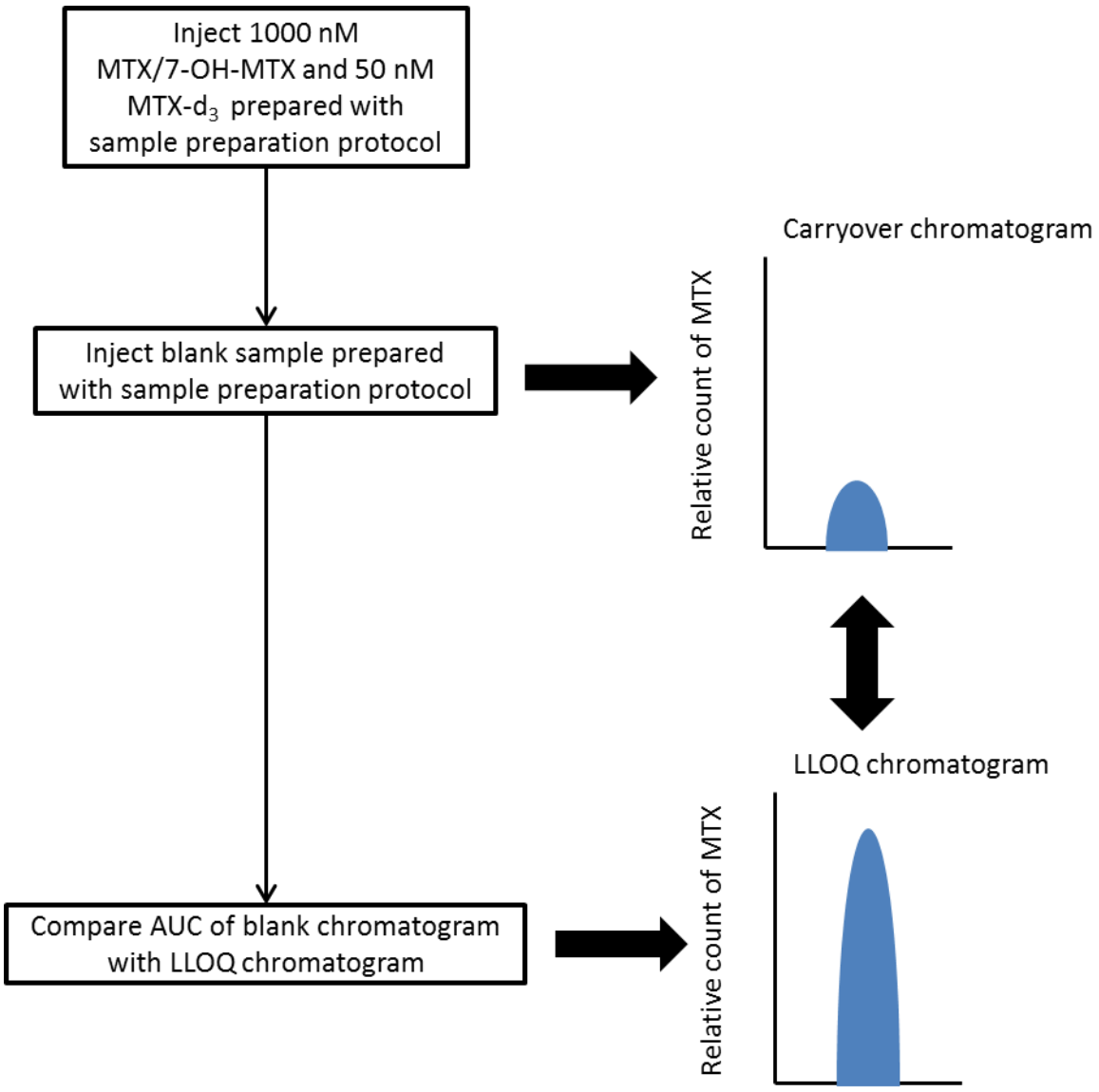


Figure 3-7. Flowchart demonstrating the workflow of the carryover experiments.

3.3.3 Accuracy and Linearity

Stock solutions were defrosted at room temperature and vortexed. MTX and 7-OH-MTX were prepared in 50µl samples according to Table 3-2 in LC-MS grade water.

A1 (µl)	A2 (µl)	A3 (µl)	A4 (µl)	A5 (µl)	H_20 (µl)	Final MTX/7-OH-MTX Concentration (nM)
50.0						1000
25.0					25.0	500.0
12.5					37.5	250.0
	50.0					100.0
	25.0				25.0	50.00
		50.0				10.00
		25.0			25.0	5.000
			37.5		12.5	0.750
			25.0		25.0	0.500
				50.0		0.100
				37.5	12.5	0.075
				25.0	25.0	0.050

Table 3-2. Concentrations used for accuracy and linearity method optimisation of MTX and 7-OH-MTX in water. Each sample was spiked to a final concentration of 50 nM MTX-d_3. Concentrations of stock solutions for MTX/7-OH-MTX were A1: 1000 nM, A2: 100 nM, A3 10 nM, A4 1 nM and A5 0.1 nM.

Samples were spiked and then vortexed at room temperature. Samples were prepared in triplicate. The peak-area ratio of the analyte to internal standard was used to calculate measured concentration. The CV was calculated to provide a measure of variability across the 3 triplicates with a CV ≤ 15% considered acceptable. The mean measured concentration for each concentration level was used to measure accuracy according to the following formula:

Accuracy = measured concentration ÷ expected concentration X 100

Linearity was assessed using MS-Excel (Microsoft), charting the mean measured concentration against expected with standard deviations. Linear least-squared regression was used to calculate the correlation coefficient R^2, a measure of the reliability of the linear relationship between the measured and calculated concentrations.

3.3.4 Precision

Precision (intraday) relates to how close repeated measurements of the same sample are to each other. Precision was calculated by repeated measurements (n=5) of 0.5 nM, 0.75 nM, 5 nM, 25 nM, 50 nM and 250 nM MTX/7-OH-MTX. At the LLOQ samples were tested in triplicate. CV was calculated to provide a measure of variability across the repeated measurements. A CV < 15% is considered satisfactory.

3.3.5 Recovery

Biofluid samples require some sample preparation to remove large proteins which reduce the specificity of the assay due to increasing noise and can block the column if they are insoluble in the mobile phase causing a system shutdown. Sample preparation techniques can however reduce the amount of analyte that remains in the biofluid (recovery). Use of an internal standard is designed to reduce the effect that sample preparation has on recovery. The internal standard is molecularly similar to the analytes of interest and therefore should be affected in the same way the analytes of interest are by sample preparation thereby correcting for any loss of analyte. In order to ensure that the assay required little sample preparation, is rapid and economical it was decided to utilise protein precipitation initially. The workflow of protein precipitation is presented in Figure 3-5. To investigate the recovery of MTX and 7-OH-MTX 11 different concentrations were freshly prepared in water (0.5, 1, 5, 10, 25, 50, 100, 250, 500, 750 and 1000 nM) and underwent the protein precipitation protocol with either ACN or methanol with 12.5 nM MTX-d_3 producing a final concentration of 50 nM MTX-d_3 following rehydration. A further experiment using ACN as the organic solution for the protein precipitation protocol with samples prepared in triplicate was conducted. Nine concentrations (0.5, 0.75, 5, 10, 50, 100, 250, 500 and 1000 nM) of MTX/7-OH-MTX were tested for recovery comparing samples that underwent the protein precipitation protocol and samples that did not. Samples were measured with and without sample preparation using ACN as the organic solvent. The mean measured concentration for each concentration level was compared to the concentration of the equivalent sample which went through the protein precipitation process (Figure 3-5).

3.3.6 Stability

The assessment of stability is vital in assay validation. If the analytes are not stable, it is important that the limits of stability are known so that the sample collection protocol can be modified to ensure that there is no significant loss of analyte in the biofluid. MTX/7-OH-MTX samples were prepared from stock solutions and vortexed at room temperature at eight different concentrations for MTX (0.5, 0.75, 5, 10, 50, 100, 250 and 1000 nM) and seven for 7-OH-MTX (0.75, 5, 10, 50, 100, 250 and 1000 nM) and stored at either room temperature or -80°C. To avoid the effect of freeze-thaw-freezing of samples, samples were aliquoted according to their intended time of testing. At the allocated time, samples underwent the protein preparation protocol (Figure 3-8). Stability was tested at five time points (0, 3, 24, 72 and 168 hours).

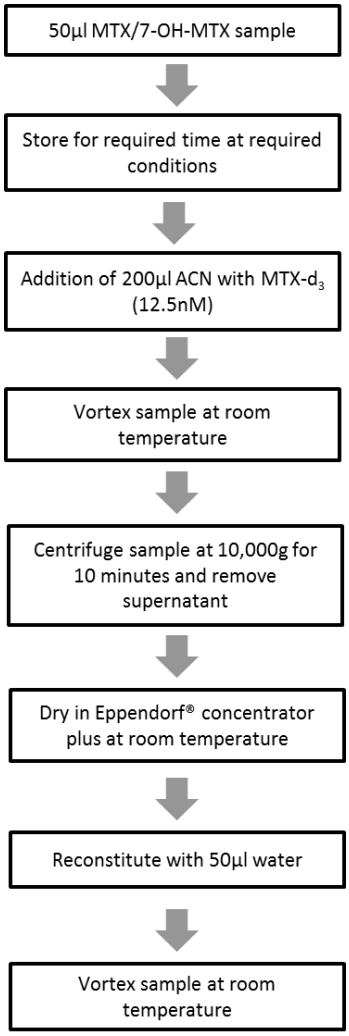


Figure 3-8. Sample preparation protocol for testing stability of MTX/7-OH-MTX over time at room temperature and -80°C.

3.4 Assay Validation in Urine

Following passing assay development and validation requirements in water, the assay was moved to testing MTX/7-OH-MTX and MTX-d_3 concentrations in urine. Assays in biofluids often have higher LLOQ, lower recovery, accuracy and precision due to matrix effects. Assay optimisation was therefore completed in water first as there is no matrix and allows optimisation for the detection of the analytes of interest to improve sensitivity, moving to biofluids allows for optimisation to improve assay specificity.

3.4.1 Preparation of Standards and Samples

Stock standards were prepared as per Section 3.2.9. Human MTX-free urine, used for both spiking of samples and to prepare calibration curves, was obtained from pooled samples (n=3) donated from patients with RA participating in RAMS. Pooled samples are used to account for inter-individual differences in matrix effects (Section 3.2.7). Urine was stored at -80°C. Frozen urine was

thawed at room temperature, vortex mixed and spiked to the required concentration. Samples were prepared in 1.5 ml safe-lock tubes (Eppendorf®).

3.4.2 LLOD and LLOQ

The LLOD and LLOQ were determined by an experiment of lower concentrations. A signal five times greater than the noise signal observed was considered to be the LLOQ. A signal discernible from the background noise by visual check of the spectrograph was considered to be the LLOD.

3.4.3 Carryover

Carryover was assessed by the chromatogram signal following injection of 1000 nM MTX/7-OH-MTX in urine that underwent sample preparation and then a blank sample.

3.4.4 Accuracy and Linearity

Accuracy and linearity were tested in six concentrations of MTX and 7-OH-MTX in urine following the sample preparation protocol. Linearity and accuracy were tested in triplicate in the following concentrations for MTX/7-OH-MTX: 5 nM, 10 nM, 50 nM, 100 nM, 500 nM and 1000 nM. The concentration of MTX-d_3 remained constant at 50 nM.

3.4.5 Precision

Precision (intraday) was calculated by repeated measurements (n=5) of the same urine sample spiked with MTX/7-OH-MTX/MTX-d_3 and protein precipitated. The concentrations of MTX/7-OH-MTX were 5 nM, 10 nM, 50 nM, 500 nM and 1000 nM. MTX-d_3 remained constant at 50 nM. CV was calculated to provide a measure of variability across the repeated measurements. A CV ≤ 15% is considered satisfactory.

3.4.6 Recovery

Recovery was performed by comparing the measured concentration of MTX and 7-OH-MTX in samples that were spiked with MTX/7-OH-MTX/MTX-d_3 and subjected to the sample preparation protocol to analyte-free urine samples that went through the sample preparation protocol with no spiked analyte and were subsequently spiked as shown in Figure 3-9. The following concentrations were prepared in triplicate of MTX/7-OH-MTX: 5 nM, 10 nM, 25 nM, 50 nM, 100 nM, 250 nM, 500 nM, 750 nM and 1000 nM as described in Table 3-2. The internal standard MTX-d_3 remained at 50 nM in all samples. Fresh calibration curves were prepared daily in water. Calibration curves were prepared in water as it was known at the time of the experiment that the calibration in water was linear. The extraction recovery percentage was calculated with the following equation:

Mean measured concentration on spiking sample followed by the sample preparation protocol ÷ Mean measured concentration on the sample preparation protocol followed by spiking sample X 100

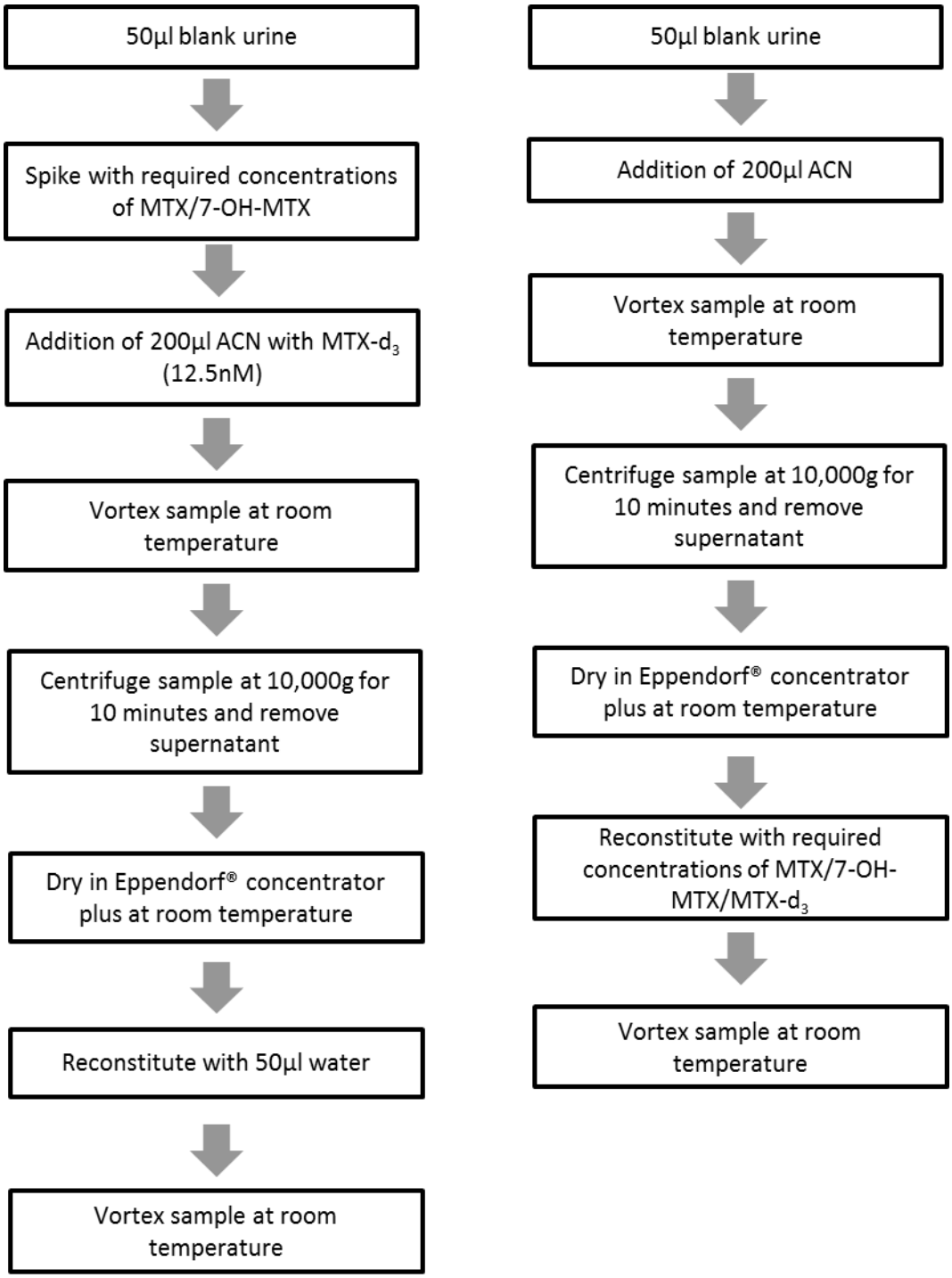


Figure 3-9. Sample preparation for recovery experiment showing process for spike then protein precipitation (left) and protein precipitation then spike (right).

3.4.7 Stability

Stability of the analyte in urine was assessed by spiking urine samples with MTX/7-OH-MTX at 5 nM (MTX only), 10 nM, 50 nM, 500 nM and 1000 nM from stock solutions in triplicate. To avoid the effect of freeze-thaw-freezing, samples were aliquoted according to their intended time of testing. Samples were subsequently stored at -80°C or room temperature in dark conditions. Samples were spiked with the internal standard MTX-d_3 and precipitated with ACN at the following time points: 3, 72 and 168 hours. Fresh calibration curves were prepared daily in urine. Analyte stability was calculated as a percentage of loss of analyte compared to the baseline sample.

3.5 Assay Validation in Plasma

3.5.1 Preparation of Standards and Samples

Stock standards were prepared as per section 3.2.9. Human MTX-free plasma, used for both spiking of samples and to prepare calibration curves was purchased from the NHS Blood and Transplant service. Pooled samples are used to account for inter-individual differences in matrix effects (Section 3.2.7). Plasma was stored at 3 °C as per the NHS Blood and Transplant service instructions. Plasma was warmed to room temperature, vortex mixed and spiked to the required concentration. Samples were prepared in 1.5 ml safe-lock tubes (Eppendorf®).

3.5.2 Recovery

Recovery was assessed in plasma similar to in urine at the following concentrations of MTX/7-OH-MTX: 2.5 nM, 5 nM, 10 nM, 50 nM, 100 nM, 500 nM, 750 nM and 1000 nM. MTX-d_3 remained constant at 50 nM. Samples were prepared as triplicates. Recovery was first assessed comparing ACN to methanol as the organic solvent to investigate the organic solution which led to a recovery closest to 100% for MTX/7-OH-MTX and then investigated as per Figure 3-9 using the ideal organic solvent for protein precipitation at the following concentrations of MTX/7-OH-MTX: 1 nM, 2.5 nM, 5 nM, 100 nM, 250 nM, 750 nM and 1000 nM.

3.5.3 Precision

Precision (intraday) was calculated by repeated measurements (n=5) of the same plasma sample spiked with MTX/7-OH-MTX/MTX-d_3 and protein precipitated. The concentrations of MTX/7-OH-MTX were 0.5 nM, 0.75 nM, 25 nM, 500 nM and 750 nM. MTX-d_3 remained constant at 50 nM. CV was calculated to provide a measure of variability across the repeated measurements. A CV ≤ 15% was considered satisfactory.

3.5.4 Accuracy and Linearity

Linearity and accuracy were tested in triplicate samples in the following concentrations for MTX/7-OH-MTX: 0.5 nM, 0.75 nM, 1, 2.5 nM, 5 nM, 10 nM, 25 nM, 50 nM, 100 nM, 250 nM, 500 nM, 750 nM and 1000 nM following sample preparation as shown in Figure 3-5. The concentration of MTX-d_3 remained constant at 50 nM.

3.5.5 LLOQ

The LLOQ was determined from measurement of plasma spiked with MTX/7-OH-MTX/MTX-d_3 and then protein precipitated. The concentrations of MTX/7-OH-MTX were 0.05 nM, 0.075 nM, 0.1 nM, 0.25 nM, 0.5 nM, 0.75 nM and 1 nM. The LLOQ was defined as a signal five times greater than the noise signal observed.

3.5.6 Carryover

Carryover was assessed by the chromatogram signal following injection of 1000 nM MTX/7-OH-MTX in urine that underwent sample preparation and then a blank sample.

3.5.7 Stability

Plasma samples for future testing will not be stored at room temperature for a prolonged period of time (less than one hour); therefore the stability of samples stored at -80 °C was tested. Stock solutions and plasma were warmed to room temperature and vortexed. Plasma samples were spiked with the required MTX/7-OH-MTX concentration at day 0. Six concentrations were tested (1 nM, 2.5 nM, 5 nM, 100 nM, 250 nM and 750 nM). To avoid the effect of freeze-thaw-freezing of samples, samples were aliquoted according to their intended time of testing. Samples were subsequently stored at -80°C. At the allocated time, samples were thawed, vortexed at room temperature and underwent protein precipitation with organic solvent and MTX-d_3 as per Figure 3-8 at three and 28 days. To minimise the effect of interday variation, a fresh calibration curve was prepared daily in plasma. Analyte stability was calculated as a percentage of loss of analyte compared to the baseline sample.

3.6 Assay Validation in Whole Blood

Samples obtained from participants in the future would be whole blood samples that are transported for up to three days at room temperature as whole blood and then centrifuged to obtain the plasma. Therefore it was necessary to investigate the stability and recovery from whole blood.

3.6.1 Preparation of Standards and Samples

Human MTX-free whole blood was obtained on the morning of spiking and testing from a volunteer recruited to the National Repository Study. 80 ml of whole blood was collected into K_2EDTA collection tubes from a participant following informed consent. Whole blood was immediately aliquoted into 5 ml aliquots and spiked with MTX/7-OH-MTX to create the following final concentrations: 5 nM, 100 nM, 250 nM, 500 nM and 750 nM as per Table 3-3.

A1 (µl)	A2 (µl)	A3 (µl)	Final MTX/7-OH-MTX Concentration (nM)
37.5			750
25			500
12.5			250
	50		100
		25	5

Table 3-3. Concentrations used for assay validation of MTX and 7-OH-MTX in whole blood. Concentrations of stock solutions for MTX/7-OH-MTX were A1: 100 µM, A2: 10 µM and A3: 1000 nM.

3.6.2 Recovery

Following preparation of whole blood the samples were immediately centrifuged at 1500 g for 10 minutes at 4 °C. The plasma was aliquoted into 50 µl aliquots and protein precipitated with 200 µl methanol with 12.5 nM MTX-d_3 and underwent the sample preparation protocol. Samples were prepared as triplicates. A standard calibration curve was performed in plasma obtained from the NHS Blood and Transplant service. Measured MTX and 7-OH-MTX were expressed as a percentage of the initial spiked concentration of MTX/7-OH-MTX. Sample preparation is shown in Figure 3-10.

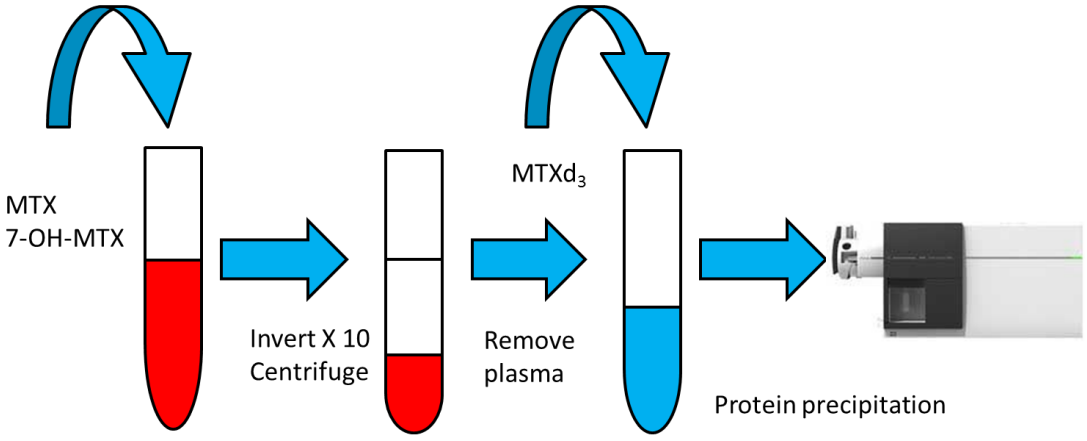


Figure 3-10. Diagram illustrating whole blood preparation for investigating recovery of MTX/7-OH-MTX.

3.6.3 Stability

Whole blood samples from RAMS may take up to 72 hours until they are received in the laboratory when the samples are centrifuged and the plasma stored at -80 °C. An experiment was

therefore conducted examining the stability of whole blood stored at room temperature. Due to ethical constraints in the amount of blood that could be collected, blood was collected so that whole blood could be tested at baseline, 24 and 48 hours after preparation. Whole blood was spiked with MTX/7-OH-MTX at baseline and at the required time samples were centrifuged at 1500 g for 10 minutes at 4°C. The plasma was aliquoted into 50 µl aliquots and protein precipitated with 200 µl methanol with 12.5 nM MTX-d_3 and underwent the sample preparation protocol. Samples were prepared as triplicates. Each day a fresh standard calibration curve was performed in pooled plasma from the NHS Blood and Transplant service. Measured MTX and 7-OH-MTX were expressed as a percentage of the measured MTX and 7-OH-MTX concentrations on day 0.

3.7 Measurement of MTX and 7-OH-MTX Metabolites in Urine and Blood of Patients with Rheumatoid Arthritis: The MEMO Study

3.7.1 Ethical Approval

Ethical approval for the MEMO study was approved by the North West Research Ethics Committee 28th October 2013 (REC reference: 13/NW/0653). A substantial amendment was approved 28th October 2014. Further approvals were obtained from the Scientific Advisory Board at the NIHR/Wellcome Trust Manchester Clinical Research Facility (WTCRF) and the Research and Development Department at Central Manchester University Hospitals NHS Foundation Trust (CMFT). The protocol was reviewed by the Medicines and Healthcare products Regulatory Agency (MHRA) who commented that the study was not a clinical trial of a medicinal product under the scope of Directive 2001/20/EC as it is not designed to ascertain or verify/compare the efficacy or safety of MTX. The sponsor for this study was The University of Manchester. The author prepared the protocol, patient information sheet, consent form and obtained all the regulatory approvals (Appendix 1). The author was the Chief Investigator responsible for the conduct of the study.

3.7.2 Study Design

This was an experimental study to investigate the performance of the HPLC-SRM-MS assay to measure MTX adherence in patients with RA treated with MTX. An observational cohort of RA patients who were treated with MTX as part of their routine care were recruited. Patients were admitted to the WTCRF and undertook directly observed therapy of oral MTX. Blood and urine samples were subsequently collected over a seven day period and stored for testing using the HPLC-SRM-MS assay. The results were used to develop a pharmacokinetic model to establish the sensitivity of the assay to correctly identify adherence over time (section 3.8).

3.7.3 Recruitment

Twenty patients who were receiving oral MTX therapy as part of their routine care for treatment of RA were recruited. This sample size was thought to be sufficient based on previous studies investigating adherence assays [231, 232].

Patients suitable for this study were identified from the RAMS cohort by the RAMS investigators and in clinic by the rheumatology team at the Kellgren Centre for Rheumatology, CMFT. Prospective suitable patients participating in RAMS were contacted initially in writing with a reply slip to indicate an expression of interest/non-interest with a copy of the patient information sheet from the Arthritis Research UK Epidemiology Unit, Manchester (Appendix 1). As well as contacting RAMS participants, the consultant rheumatology team at the Kellgren Centre for Rheumatology, CMFT informed eligible patients that there was a study they would be eligible to participate in and asked if they would be happy to be sent some information about it. Those who were happy to be sent information had their hospital number passed onto the MEMO study team, who conducted the same mailing as for potential participants identified from the RAMS study. Those who did not reply were contacted within one month with a further letter (Appendix 1). Patients eligible and expressing interest were telephoned to explore any questions they may have and if they wished to take part. Patients expressing an interest in taking part in the study were invited to attend an initial screening visit at the WTCRF to discuss the project in further detail and ensure they fulfilled the inclusion criteria with a member of the study team. Informed written consent was taken by a qualified person from the research team (Appendix 1).

3.7.4 Inclusion and Exclusion Criteria

The following inclusion and exclusion criteria applied:

Inclusion criteria

- Physician diagnosis of RA or early undifferentiated polyarthritis.
- Patients who are currently being treated with MTX as part of their routine care for treatment of RA/undifferentiated polyarthritis.
- Patients who have been on MTX for at least three months.
- Patients on a stable dose of MTX (no alteration in the past four weeks) of 5mg to 25mg per week orally.
- Patients must be willing to participate in the study after providing informed consent.
- Patients on a stable dose of concomitant folic acid supplementation.

Participants were required to have been taking MTX for at least three months with no alteration in their dose in the past four weeks to ensure that the MTX levels were at steady state.

Exclusion criteria

- Age < 18.
- Liver enzymes twice the upper limit of normal.
- Significantly impaired renal function (eGFR < 30 ml/min).
- Patients who are pregnant or taking inadequate contraception.
- Patients who are breast-feeding.
- Patients with a known allergic hypersensitivity to methotrexate.
- Conception should be avoided during the period of methotrexate administration and for at least six months thereafter.
- Pre-existing blood dyscrasias, such as significant marrow hypoplasia, leukopenia, thrombocytopenia or anaemia (Hb < 9.0 g/dL).
- Patients unable to stay at the WTCRF for 24 hours due to a significant comorbidity that may affect their safety when left unmonitored e.g. suicidal intent.
- Patients unable to give informed consent.
- Subjects must refrain from blood donation for one month prior to screening visit, during the study and 2 months after completion of the study.
- Non-adherence to MTX.

The exclusion criteria were based on the contraindications from the Summaries of Product Characteristics (SPC) for MTX [233]. Refraining from blood donation was required so that the volume of distribution was physiological and not affected by blood donation. Adherence to MTX was required so that the MTX levels were at steady state.

3.7.5 Screening Procedures

The first clinic visit was the pre-study (screening) visit to ensure participants fulfilled the inclusion and exclusion criteria, could discuss details of the study and provide informed consent. Consenting individuals were assigned a unique linked-anonymised identification number which was used to identify biological samples. This visit took place at the WTCRF and proceeded accordingly:

1. Consent	Written and verbal consent for inclusion into study
2. History taking	(a) Past medical history (b) Concomitant medications including side effects to medication (c) Social history – smoking, alcohol, diet (d) Contact details – GP (e) Last menstrual period and contraception history if applicable
3. Physical examination	(a) Height and weight (b) Blood pressure (c) Temperature (d) Musculoskeletal examination
4. Blood sampling	(a) 1 X 4ml EDTA monovette tube (full blood count, FBC) (b) 1 X 5ml Serum gel monovette tube (urea and electrolytes, U&E and liver function tests, LFTs)

3.7.6 Visit 1

Participants were admitted to the WTCRF to coincide with the day they normally take their MTX and underwent routine observations (temperature, blood pressure and pulse rate). Participants ingested a single dose of oral MTX at their usual prescribed dose and time after the first blood sample (time point = 0). Subsequent blood sampling was according to the timeline below. A standard operating procedure for blood sampling including aseptic technique was utilised by all practitioners involved in the study. The following timeline was followed for all subjects:

1. Consent	Ensure patient still happy to continue with study
2. Case note review	Review laboratory results
3. Interview	(a) Ensure patient well (b) New medications since screening visit (c) Dietary history
4. Physical examination	(a) Temperature (b) Blood pressure (c) Pulse
5. Inclusion/Exclusion criteria	Review and update inclusion/exclusion criteria
If all inclusion and exclusion criteria are satisfied then continue	
6. Blood sampling	1 X 9ml tube
7. Methotrexate	Single dose of methotrexate taken as the patient usually takes their medication and at the dose normally prescribed for treatment of their rheumatoid arthritis
8. Blood sampling	Blood sampling at the following times: 0, 1, 2, 4, 8, 16, 24 hours
9. Urine collection	Subject empties bladder at time point = 0 Commence 24 hour urine collection
10. Discharge	Discharge from Clinical Research Facility

3.7.7 Visit 2

Visit 2 took place at the WTCRF within 7 days of visit 1 at a date/time mutually convenient to the participant and WTCRF. Subjects underwent the following consultation:

1. Interview	Ensure patient still happy to continue with study
2. Physical examination	(a) Temperature (b) Blood pressure (c) Pulse
3. Blood sampling	1 X 9ml tube
4. Urine collection	Patient will empty bladder at time point 0 and urine collection will occur over the next 4 hours

3.7.8 Visit 3

Visit 3 took place at the WTCRF within 7 days of visit 1. Subjects underwent the following consultation:

1. Interview	Ensure patient still happy to continue with study
2. Physical examination	(a) Temperature (b) Blood pressure (c) Pulse
3. Blood sampling	1 X 9ml tube
4. Urine collection	Patient will empty bladder at time point 0 and urine collection will occur over the next 4 hours

3.7.9 Sample Collection

A standard operating procedure was prepared for staff at the WTCRF describing how samples were to be collected and processed (Figure 3-11).

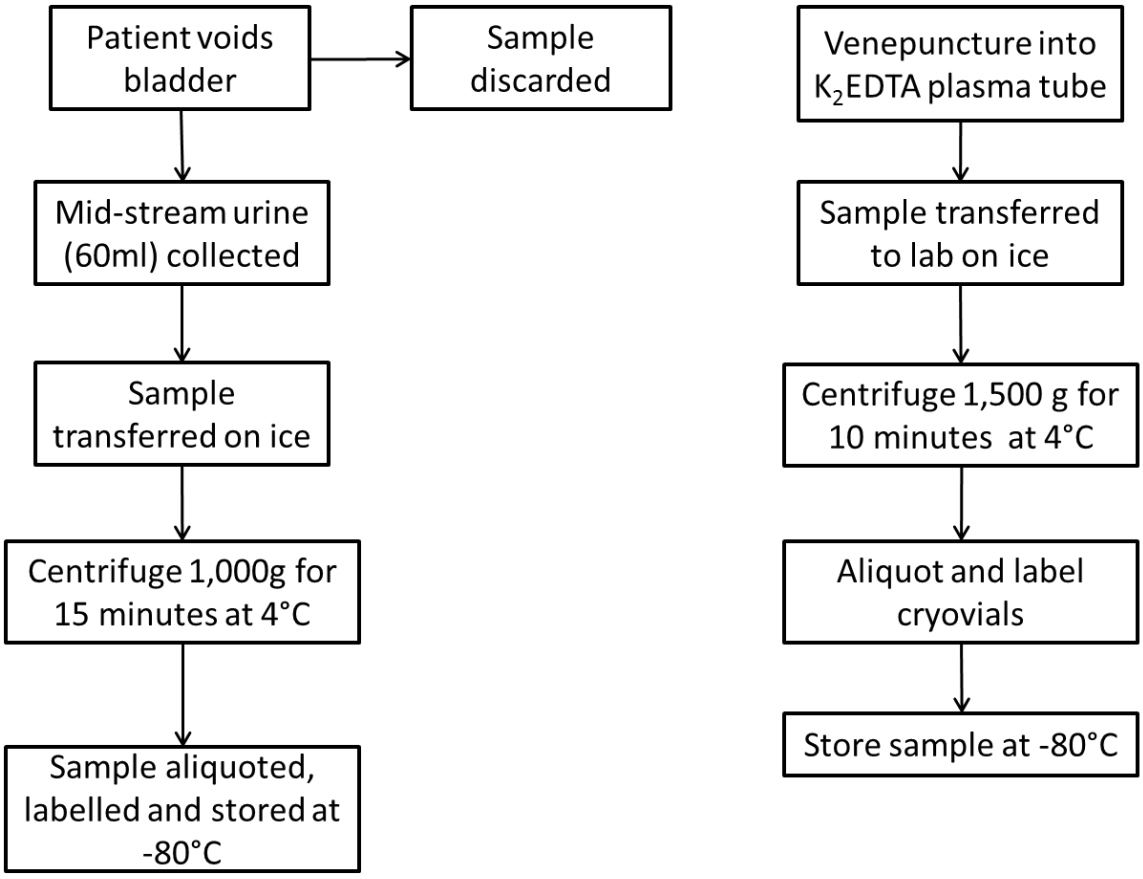


Figure 3-11. Sample collection procedure for urine (left) and plasma (right) used in the MEMO study.

3.7.9.1 Urine Collection

When the patient was admitted (time=0), they completely voided their bladder and the sample discarded.

3.7.9.2 Visit 1

For every subsequent urination, a mid-stream urine sample (60ml) was collected in chemical free and sterile collection pots. Samples were placed on ice and centrifuged for 15 minutes at 1,000 g at 4°C. Samples were immediately aliquoted to remove the sediment into 5ml containers and labelled with the date, time, volume of urination and participant ID. Samples were stored at -80°C.

3.7.9.3 Visit 2 and 3

When the patient was admitted, they completely voided their bladder, the sample was not collected.

For the subsequent urination, a mid-stream urine sample (60ml) was collected in chemical free and sterile collection pots. Samples were placed on ice and centrifuged for 15 minutes at 1,000 g at 4°C. Samples were immediately aliquoted to remove the sediment into 5ml containers and labelled with the date, time, volume of urination and participant ID. Samples were stored at -80°C.

3.7.10 Plasma Collection

Blood was preferably to be taken using a solid needle and vacutainer system to reduce the risk of haemolysis; should the veins be unfavourable, a butterfly vacutainer system could be used. Blood was drawn into K_2EDTA (lavender top) plasma collection tubes. Samples were placed on ice for a maximum of 30 minutes prior to sample preparation. The plasma fraction was prepared as soon as possible by centrifugation at 1500 g for 10 minutes at 4°C. Samples were divided immediately into aliquots (0.5ml) in cryovials (Greiner, cat. no. 122261/122263) and frozen by placing in a -80°C freezer. Samples were labelled with the patient ID, date and time of collection.

3.7.11 Case Report Forms

Case report forms were used (Appendix 1). The following data was collected:

- Inclusion/Exclusion criteria
- Demographic data: Age, gender, height, weight and smoking history.
- Clinical data: Co-morbidities, drug history, MTX dose, adverse events to MTX, blood pressure, components of DAS-28 and timing of MTX ingestion.
- Blood samples: Urea and electrolytes, liver function profile, full blood count, timing of urine and blood sampling.

3.7.12 Measurement of MTX and 7-OH-MTX in Plasma and Urine

Plasma and urine samples in aliquots of 50 µl were spiked with MTX-d_3 in triplicate and subjected to the sample preparation protocol prior to measurement of MTX and 7-OH-MTX using HPLC-SRM-MS. In addition, urine samples were corrected for creatinine concentration. Creatinine was measured using the creatinine Jaffé reaction and analysed on a Roche® Cobas analyser by colleagues at CMFT [234]. Samples were diluted with blank urine/plasma as required if the measurement of MTX/7-OH-MTX was above the calibration curve. Quality control samples of known MTX/7-OH-MTX samples were used in every run to ensure that the HPLC-SRM-MS assay

was measuring accurately. Samples were processed in triplicates and those with CV ≤ 15% and within the calibration curve were accepted as accurate.

3.8 Pharmacokinetic Model Development

3.8.1 Plasma Pharmacokinetic Model

A population pharmacokinetic model was developed by a colleague (Thierry Wendling) in the School of Pharmacy, the University of Manchester. Estimation of the population median and variance parameters was performed using a Bayesian approach and uninformative priors for all parameters. Based on visual inspection of the concentration-time profiles of MTX and 7-OH-MTX and previously published data, a two-compartment model for MTX and one compartment model for 7-OH-MTX was fitted to the data. For the metabolite 7-OH-MTX, as there is no direct ingestion of 7-OH-MTX it is not possible to calculate the parameters of this compartment directly, therefore apparent formation and clearance of 7-OH-MTX are estimated as is common in pharmacokinetic studies investigating metabolites [235]. Covariates (body weight and serum creatinine levels) for the model parameters were tested to try to explain part of the variability in the parameters. Visual predictive checks were undertaken to evaluate the ability of the model to describe the data. Pharmacokinetic parameters were reported with their relative standard error to provide an estimate of uncertainty in the parameters. The amount of variability in the model that is explained by each parameter was expressed as coefficient of variation. Finally, simulations were performed to predict the proportion of patients with detectable concentrations of both MTX and 7-OH-MTX over time to estimate how long after ingestion of MTX the assay is able to detect adherence for a given dose and if MTX or 7-OH-MTX is the most sensitive analyte in plasma samples. The analysis was performed using NONMEM version 7.3.0 (ICON Development Solutions, Hanover, Maryland, USA) [236].

3.8.2 Urine Data

Urine concentrations of MTX and 7-OH-MTX are an expression of MTX/7-OH-MTX clearance and depend on numerous random unpredictable factors such as the creatinine clearance, how frequently a person passes urine and how much water they drink. It is not possible to produce a pharmacokinetic model of the urine data as it is a function of clearance and non-measurable effects. In order to correct for urine concentration, MTX/7-OH-MTX concentrations were corrected for urine creatinine and were described for each participant categorised by MTX dose over time as observational data. This data is indicative of how long the urine assay can detect adherence but not predictive.

3.9 Validation of the Pharmacokinetic Model and Investigation of MTX/7-OH-MTX levels and Response

Following development of the pharmacokinetic model of MTX and 7-OH-MTX, it was validated using plasma samples from the RAMS study.

3.9.1 Selection of Patients for Measurement of MTX/7-OH-MTX Levels in Plasma

A subset of patients from RAMS were selected to measure MTX/7-OH-MTX levels in plasma at three months to validate the pharmacokinetic model of adherence and investigate whether MTX/7-OH-MTX levels correlate with change in disease activity following MTX therapy.

3.9.1.1 Patient Eligibility

In addition to the inclusion/exclusion criteria of RAMS, patients were required to be on MTX as monotherapy with no second DMARD added until the three month DAS-28 or plasma sample was collected, whichever was the later. This was required to ensure that any response to therapy was due to MTX and not a second DMARD. Patients were excluded if they were prescribed oral steroids for more than 30 days. Oral steroids are often used as induction therapy to reduce the burden of disease, as there is often a lag period before the effects of MTX modify disease activity of RA, steroids may therefore confound the analysis of MTX response. Patients were excluded if the date/time of MTX ingestion for the week that the three month blood sample was taken was not diarised; this was so the time interval between MTX ingestion and blood sampling could be calculated.

3.9.1.2 Sample Eligibility

In order to be eligible, the three month blood samples must have been sent for external CRP measurement. This was chosen so that the three month CRP measurements were taken on the day that the swollen and tender joint count was completed to ensure that the DAS-28 was as accurate as possible. Samples were also required to have been received within three days of venepuncture and marked with the date and time of venepuncture.

3.9.2 Pharmacokinetic Model Validation

External validation of the population model was conducted by plotting, over time, the dose-normalised observed concentrations of the sparse RAMS samples along with the median of the predicted concentrations and a 90% prediction interval. Where there were more than ten patients taking the same dose individual MTX concentrations were plotted against the median predicted concentration. In order to check that the model predicts reasonably well, the proportion of

subject with detectable concentrations were binned according to time and compared to the simulation results from MEMO to externally validate the simulations.

3.10 Investigating the Correlation between MTX/7-OH-MTX Levels and Change in Disease Activity

Measurements of MTX levels from the RAMS cohort (Section 3.9) were used to investigate their correlation with change in disease activity and association with EULAR response. Time from MTX ingestion, dose of MTX and MTX plasma concentration were used to calculate individual estimates of clearance and bioavailability from the pharmacokinetic model by a colleague (Dr Thierry Wendling). MTX AUC was calculated according to Box 3-1 [237].

$$AUC = F \times dose / CL$$

Box 3-1. Formula for calculating AUC.

3.10.1 Measurement of Response

Response to MTX was measured using the change in DAS(CRP)-28 over a three month period. DAS(CRP)-28 was calculated as previously described (Section 1.1.4). Change in DAS-28 over three months and the three month DAS-28 were used to categorise response according to the EULAR response criteria (Section 1.1.4) [34].

3.10.2 Statistical Analysis

Moderate and good EULAR responders were grouped together. Logistic regression was used to determine if MTX AUC was significantly associated with EULAR response. In addition, linear regression was used to determine if AUC correlated with change in DAS-28 over three months. Analysis was undertaken using Stata Version 13.1 [238].

3.11 Methods: Genome Wide Association Study Investigating Genetic Predictors of MTX-P

3.11.1 Study setting and funding

This research was undertaken in the Arthritis Research UK Centre for Genetics and Genomics in the Institute of Inflammation and Repair. The research was funded by the National Institute for Health Research (NIHR) Manchester Musculoskeletal Biomedical Research Unit and a three-year North West England Medical Research Council (MRC) Clinical Pharmacology and Therapeutics Clinical Research Training Fellowship. The study was designed and ethical approval gained by a colleague (Dr Sally Owen) prior to the commencement of my PhD.

3.11.2 Ethical Approval

Ethical approval for this research study was granted by the National Research Ethics Service Committee North West 31st August 2011 (REC ref: 11/NW/0492).

3.11.3 Study Design

This is a case control genome wide association study.

3.11.4 Subject Recruitment

3.11.4.1 MTX-P Cases

Rheumatologists working within the NHS in the United Kingdom identified patients with RA who developed MTX-P. Participants interested in taking part in the study were sent a consent form and blood kit (EDTA vial). Blood was taken and the sample was sent to the University of Manchester via Royal Mail for DNA extraction.

3.11.4.2 Controls

Controls were identified from RAMS. In order to be eligible controls were required to have one year of continuous MTX therapy without the development of MTX-P. One year was selected as the median duration of MTX therapy to time to development of MTX-P was previously reported as 36 weeks [177]. Controls were age:sex matched to cases using Stata [238].

3.11.5 Sample Size Requirement

Adequate power in case-control GWAS studies is vital. Due to multiple testing of genetic variants the *p*-value in GWA studies is required to be set low (typically 5×10^{-8}) and an adequate number of samples are required to avoid false positives. A study of 43 cases and 129 controls would have 80% power to detect an odds ratio of 15 at genome wide association levels ($< 5 \times 10^{-8}$) assuming a minor allele frequency (MAF) of 30% under a recessive model. Power calculations were performed in Quanto [239].

3.11.6 Inclusion and Exclusion Criteria

Table 3-4 describes the inclusion and exclusion criteria relevant to this study.

Inclusion Criteria	Exclusion Criteria
• Treated with methotrexate • Developed physician diagnosed drug induced pneumonitis requiring hospitalisation • Provide informed consent • Self-declared European Caucasian descent	• Participants unwilling or unable to donate a blood sample or provide informed consent

Table 3-4. Inclusion and exclusion study for the MTX-P study.

3.11.7 Data Collection

A proforma was sent to the participating Rheumatologists for case note review and data collection (Appendix 2). Data collection included the Searles and Carson criteria for MTX-P, ACR (American College of Rheumatology) 2010 criteria for RA, family history of RA, smoking status, start/stop dates of DMARDs, dose of MTX, concomitant medication and outcome of the pneumonitis episode.

3.11.8 DNA Extraction

DNA extraction was performed using a standard phenol-chloroform extraction method on whole blood as described previously by technicians within the Centre for Musculoskeletal Research [240].

3.11.9 Genotyping Platform

Genotyping was performed using the Illumina Infinium HumanCoreExome 12 BeadChip kit (Illumina, San Diego, USA) using 200 ng of DNA according to the manufacturer's guidelines [241]. The Illumina HumanCoreExome BeadChip contains 264,909 SNPs of which the majority are located within the exome. This genotyping platform is reported to genotype 45% of variation across the European genome with MAF > 1% with an average of one marker every 5.5 kb. Genotype calling was performed using GenomeStudio software (Illumina). The platform was used as it is economically viable and because of its high coverage of the exome. As MTX-P is an idiosyncratic drug reaction, targeting known metabolic pathways of MTX are unlikely to be of benefit as the dose of MTX does not affect the probability of developing MTX-P, therefore a genome wide approach was required. Given that HLA-A 31:01 has been associated with MTX-P it is logical that the genotyping platform chosen preferentially genotypes the exome and provides good coverage across the MHC region.

3.11.10 Data Quality Control

Quality control of the genotyped data is vital for analysis and reliable interpretation of results. Any change in the frequency of a genotype in comparing cases and controls can lead to an erroneous result. Data quality control was undertaken using Plink [242], SMARTPCA.pl software [243] for running principle component analysis (PCA) and R statistical software (http://cran.r-project.org/) on a Unix emulator (http://mobaxterm.mobatek.net/) to connect to the University of Manchester Computational Shared Facility (CSF). The CSF is a high performance computing cluster enabling fast analysis of data. A flowchart summarising the steps taken in quality control is shown in Figure 3-12.

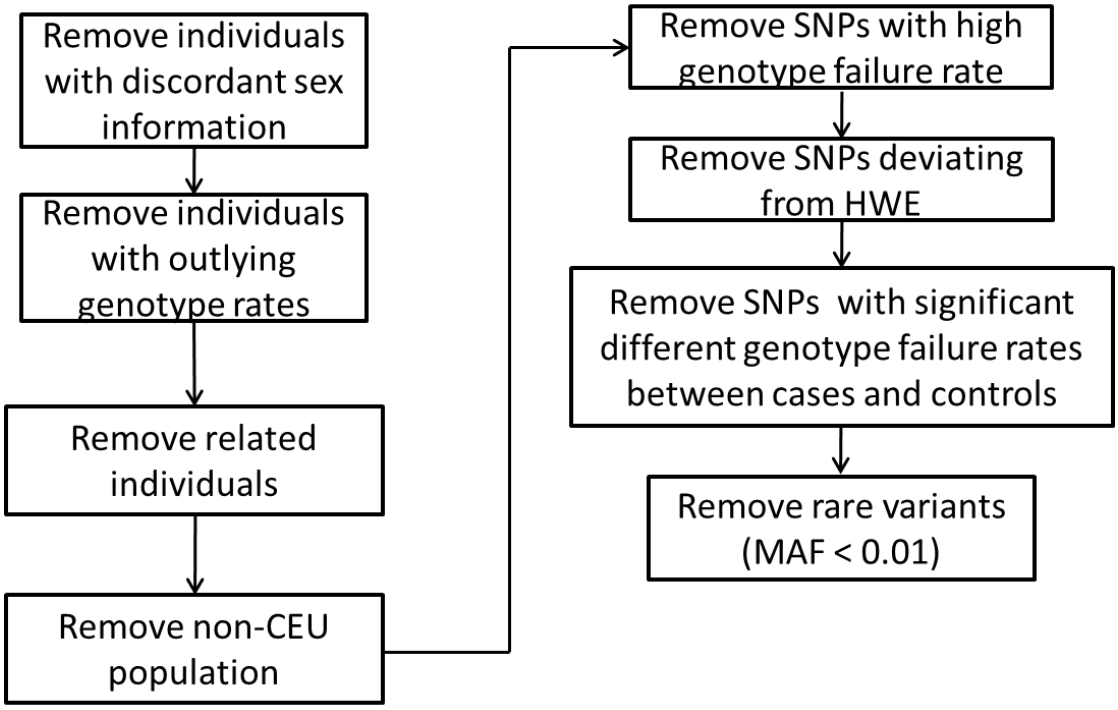


Figure 3-12. Flowchart summarising the steps taken in quality control of the genotyped data.

3.11.10.1 Linkage Disequilibrium

Linkage disequilibrium (LD) is of great importance in the study of population genetics. Due to the close proximity of loci, alleles are often inherited from a parent en-bloc. For example, because of the close linkage between loci in the MHC region, loci are often inherited together more than would be expected by chance alone. The distance between loci is not the only factor that affects the degree of LD; other factors include migration, non-random mating and the rate of recombination [244]. The specific sequence of alleles that are inherited together is termed the haplotype and SNPs that are used to identify haplotype sequences are termed tag SNPs. LD is measured by D' or the R^2 correlation coefficient as shown in Box 3-2.

Consider loci A and B. At each loci there is the possibility of genotypes AA, Aa, aa and BB, Bb, bb.

$D = p(AB) - p(A)*p(B)$

Where p(A) is the observed probability of allele A for locus 1.

$R^2=D^2/p(A)*p(a)*p(B)*p(b)$

Where R^2 is the correlation coefficient between 2 markers.

When $R^2 = 1$ there is perfect agreement i.e. allele A is always associated with allele B.

Box 3-2. The calculation of D' and R^2 correlation coefficient.

3.11.10.2 The Hardy-Weinberg Principle

The Hardy-Weinberg principle theorises that in a large randomly mating population, the relative proportions of the different genotypes remains constant from one generation to another [201]. Therefore, for any population the frequency of each allele is expected to conform to expected genotype frequencies as shown in Box 3-3.

Calculate the frequency of the alleles observed, and let:

p = frequency of allele α
q = frequency of allele β

According to the Hardy-Weinberg equation (HWE):

$$p^2 + 2pq + q^2 = 1$$

therefore:

p^2= Expected frequency of αα genotype
$2pq$ = Expected frequency of αβ genotype
q^2 = Expected frequency of ββ genotype

Box 3-3. The calculation the Hardy-Weinberg Principle. The proportion of expected genotypes can be compared with the observed genotypes to determine genotype error has occurred.

3.11.10.3 International HapMap Project

The international HapMap project aims to produce a map of haplotypes across the human genome [245]. The project is the result of collaboration among scientists in Japan, the U.K., Canada, China, Nigeria, and the U.S. As the frequencies of haplotypes vary according to the population under study, four populations have been genotyped. The four populations are U.S.

residents of northern and western European ancestry (CEU), Ibadan, Nigeria (YRI), Tokyo, Japan (JPT) and Han Chinese individuals from Beijing, China (CHB). To create the map the HapMap consortium re-sequenced a large region of the human genome to discover novel SNPs. The results of this study can be can be used to exclude individuals of divergent ancestry from a case-control study using principle component analysis (PCA) (section 3.11.10.5).

3.11.10.4 1000 Genomes Project

The 1000 genomes project (http://www.1000genomes.org/) aims to detect all polymorphisms with a frequency > 1% by re-sequencing the genomes of 1000 individuals [246].

The information contained within the 1000 genomes project can be used to infer or impute genotypes that are likely to exist in an individual by haplotype matching between the study sample and the reference panel [247-249]. This reduces the costs of direct genotyping in a study whilst increasing the number of genotypes available for analysis.

3.11.10.5 Population Stratification

Population stratification is the presence of a subpopulation within the cases or controls, for example a proportion of cases being of Japanese descent in a study of participants of mostly European descent. Population stratification is a potential confounder as, using the example above, the comparison between cases and controls may detect a significant difference between cases and controls which is associated with a phenotype not being investigated (such as height as Japanese subjects tend to be shorter than Europeans).

Within the UK population there is only modest population stratification and statistical methods now allow for adjustment by principle component analysis (section 3.11.10.5) [250].

3.11.11 Individual Sample Quality Control

Quality control was initially individual sample based rather than SNP marker quality control; prioritising retention of SNP data over individual samples improves the overall coverage of the genome. To exclude individuals with poor quality genotyping the following steps were employed:

1. Identification of individuals with discordant sex information.
2. Identification of individuals with outlying genotype rates.
3. Identification of individuals related to each other or duplicate samples.
4. Identification of individuals of divergent ancestry.

Individuals whose labelled gender does not match the gender that their genetics indicate are first excluded. Individuals who are male would not be heterozygous for markers not present in the Y-chromosome. During the genetic analysis, when there is a sample labelled as male with

unexpected heterozygosity at the X-chromosome only polymorphism the genotype-calling algorithm will label the genotype as missing. Samples labelled as male which are genetically female will therefore have a large amount of missing data and are identified as discordant. Female samples are expected to have a homozygosity of < 0.2. Samples labelled as female which are genetically male will have a higher than expected homozygosity rate and are labelled as discordant. Sex discordant samples can occur due to incorrect labelling from the recruiting site or mislabelling of samples. Individuals that are identified as sex discordant are therefore removed from further analysis.

Individuals with outlying genotype rates are excluded. Due to technical issues such as pipetting small volumes, human error, poor quality or quantity of extracted DNA and the possibility of contamination, the genotype can be called incorrectly or the assay may fail (genotype failure) [251]. Individuals with outlying genotype failure rate are excluded by a visual check of a graph plotting each individual's genotype failure rate. In order to exclude individuals where samples have been cross-contaminated with other samples, individuals with a high heterozygosity rate are excluded through examination of a graph plotting each individual's heterozygosity rate.

The third step is to exclude individuals who are related to each other. This is a required quality control step as if the cases included individuals that are related to each other there would be a higher number of shared SNPs in the case population compared to the controls causing a type 1 statistical error. The software excludes (prunes) regions of high LD (section 3.11.10.1) which are shared in any normal population so that the SNPs remaining are independent markers to reduce computational requirements. Every sample is then compared with every other sample in a matrix to investigate if samples bear a close resemblance to each other and share a higher than would be expected proportion of independent markers. The threshold for duplicate samples was >0.185, halfway between the expected identity by descent (IBD) for second- and third-degree relatives [252]. If IBD is > 0.185, one of the samples is excluded from further analysis.

Principal component analysis (PCA) is a method to exclude individuals of divergent ancestry [253]. The genotype distribution of samples is compared to those of known ancestry from the HapMap database [245]. The genotypes of three known ancestry populations – Europeans, Asia and Africa are compared to each other; the genotyped samples are compared with those populations to determine with which population they share more genetic markers. The result is displayed as a plot where each dot represents an individual in the study.

3.11.12 Marker Quality Control

The second phase of quality control excludes SNPs that are of poor quality where the genotype cannot be reliably determined. The following steps are employed:

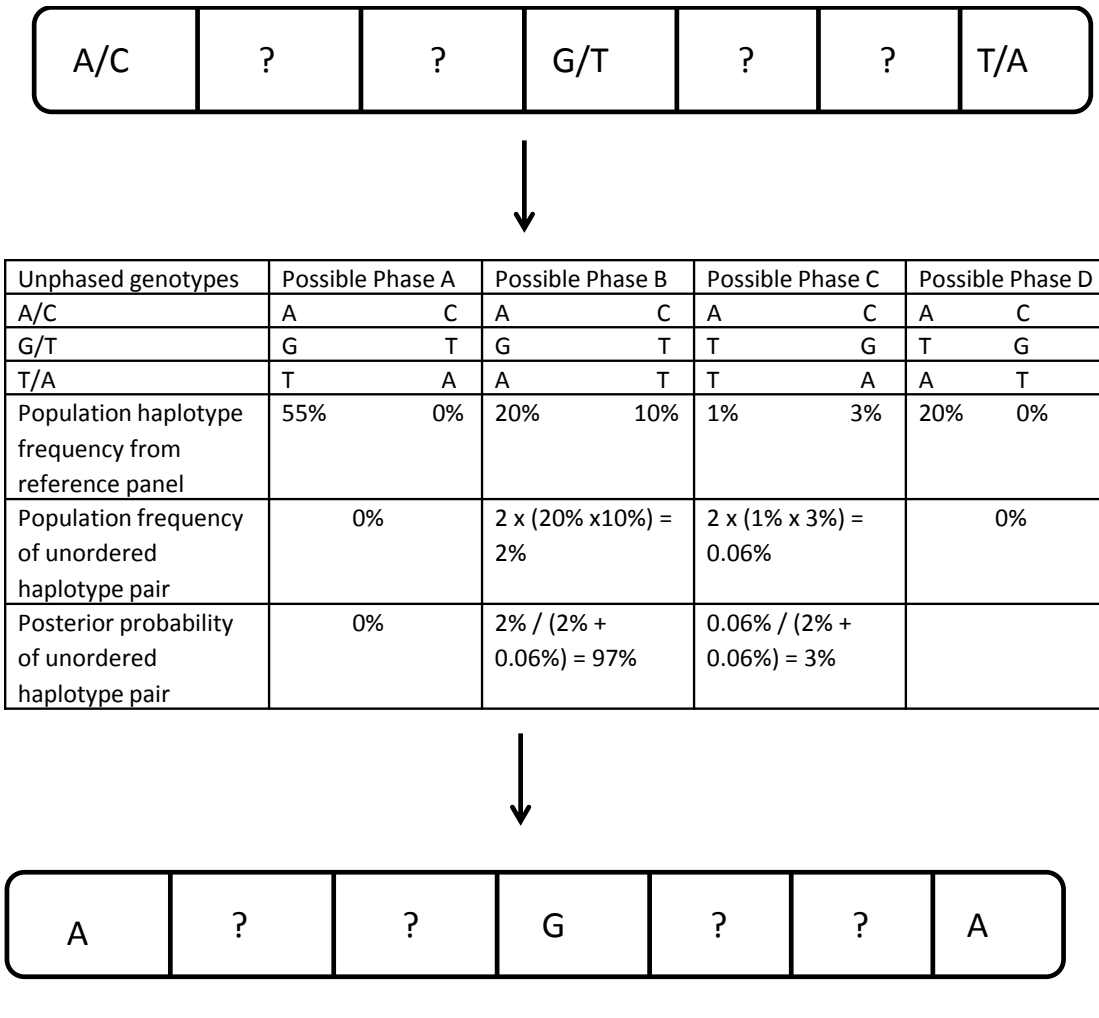
1. Remove SNPs with high genotype failure rate.
2. Remove SNPs showing significant deviation from HWE.
3. Remove SNPs with different genotype failure rates between cases and controls.
4. Remove markers with a very low minor allele frequency (MAF).

SNPs with a high genotype failure rate (> 1%) were removed due to the inaccuracy of the genotyping of these SNPs. SNPs which showed significant deviation from HWE ($p < 10 \times 10^{-7}$) (section 3.11.10.2) were removed. Deviation from HWE suggests a genotype calling error. SNPs with significant differences ($p < 10 \times 10^{-7}$) in successful genotyping frequency between cases and controls were excluded to reduce the risk of confounding [254].

SNPs which have a low MAF (< 1%) are regarded as rare variants. Rare variants are removed as they can cause type 1 statistical errors as the association is based on a low number of cases. For example, if 1% of the case cohort had a rare variant compared with 0.05% of the control cohort, the relative risk would be 20, rejecting the null hypothesis.

3.11.13 Imputation

A disadvantage of GWAS is that the power to detect association is dependent on the coverage of the genotype chip. Imputation is an *in silico* method that predicts genotypes that are not directly genotyped, thereby reducing costs, increasing genotype coverage and increasing the power of a study to detect association [249]. Imputation uses a known reference panel of haplotypes to infer the genotype of the study sample as shown in Figure 3-13. The statistic R^2 is calculated as an estimate of the accuracy of imputation at a particular locus.



Unphased genotypes	Possible Phase A		Possible Phase B		Possible Phase C		Possible Phase D	
A/C	A	C	A	C	A	C	A	C
G/T	G	T	G	T	T	G	T	G
T/A	T	A	A	T	T	A	A	T
Population haplotype frequency from reference panel	55%	0%	20%	10%	1%	3%	20%	0%
Population frequency of unordered haplotype pair	0%		2 x (20% x10%) = 2%		2 x (1% x 3%) = 0.06%		0%	
Posterior probability of unordered haplotype pair	0%		2% / (2% + 0.06%) = 97%		0.06% / (2% + 0.06%) = 3%			

Figure 3-13. Genotyping results for a fictitious sample (a) showing heterozygosity at three genotyped loci. Haplotype phasing predicts the most likely sequence of alleles on each chromosome by comparing with a reference panel (b) producing the final phased sequence (c). The haplotypes are then modelled by comparing to the haplotype panel to genotype the missing loci.

3.11.14 HLA-31:01 Imputation

SNP2HLA is a method of imputation that can infer classical HLA alleles from SNPs that have been directly genotyped [255]. A $R^2 \leq 0.75$ was used as a quality control cut-off for determination of imputed genotypes.

3.11.15 HLA-31:01 Validation of Imputed Genotype

Prior to imputation, a subset of pneumonitis samples (n=24) were directly genotyped at The University of Liverpool. Genotyping was undertaken using polymerase chain reaction (PCR) to amplify DNA with detection using gel electrophoresis. The genotyping method is an established wet lab technique amplifying HLA-A 31:01 with sequence specific primers (SSP) in a PCR. The genotyping method has been described previously [216]. The following primers were used for the PCR reaction (Metabion, Germany):

Forward Primer 5’-gatagagcaggagaggcct-3’

Reverse Primer 5’-agcgcaggtcctcgttcaa-3’

Sample DNA was plated out at 20ng/µl concentration and 23 µl of mastermix was added to each sample. The mastermix and PCR temperature cycle was undertaken as shown in Table 3-5 and Table 3-6.

Reagent	Concentration	Volume per 23 µl reaction (µl)
Gotaq buffer	1X	2.5
$MgCl_2$	23 mM	1.5
dNTPs	10 mM per dNTP	0.2
Forward primer	20 µM	1.25
Reverse primer	20 µM	1.25
GoTaq® Hot Start Polymerase	1 U	0.125
DNA	20 ng/ µl	2

Table 3-5. Mastermix used for PCR of HLA-A 31:01.

Temperature (ºC)	Time	Cycles
95	3 min	
95	15 s	
70	15 s	5 cycles
72	30 s	
95	15 s	
65	15 s	21 cycles
72	30 s	
95	15 s	
55	1 min	9 cycles
72	2 min	
72	7 min	
4	Hold	

Table 3-6. PCR temperature cycle used to PCR HLA-A 31:01.

After PCR, the product was loaded onto a 1.5% agarose gel over an electric current. Known positive and negative controls were used for quality control checks. Cohen’s kappa was used to assess the degree of concordance between imputed data and the genotyped results.

3.11.16 Bioinformatic Analysis

A number of publicly available databases of experimental functional investigations were interrogated to provide evidence of the function of SNPs associated with MTX-P.

3.11.16.1 The ENCODE (ENCyclopedia of DNA Elements) Project

The ENCODE project is a consortium with the aim to catalogue all regulatory elements in the human genome sequence [256]. A regulatory element may be identified through its ability to bind transcription factor binding sites, open chromatin or DNase footprint analysis. The experiments are wet-lab experiments that have been conducted and are not based on predictive models. However, the limitation of ENCODE is that a SNP may be a regulatory element in one cell type such as the lung fibroblast and not others. Unless the experiment has been conducted in the lung fibroblast, ENCODE will not demonstrate evidence of the SNP being a regulatory element.

3.11.16.2 Chromatin Immunoprecipitation-Sequencing

Chromatin Immunoprecipitation-sequencing (ChIP-Seq) is an experiment designed to detect specific transcription factor (TF) binding across the genome in a particular cell-line [257]. The process involves cross-linking of DNA strands to fix the TF and chromatin complex. A TF-antibody which targets a specific TF binds to regulatory regions of the genome that are associated with a TF causing immunoprecipitation. The antibody-TF-regulatory region complexes that have been precipitated undergo high-throughput sequencing to detect the regulatory region that has been immunoprecipitated. The regulatory region is then mapped to the genome. The process is repeated for different TFs in different cell lines to create a map of specific TF-binding sites across the genome.

3.11.16.3 DNase Hypersensitivity Sites and Footprint Analysis

Over two metres of DNA is wrapped into tightly packed units within the cell nucleus. The DNA is wrapped around four histone proteins to form a nucleosome much like beads on a string [258]. Units of nucleosomes form chromatin. The accessibility of regulatory regions within DNA depends on how tightly the chromatin is wrapped. Histones can be modified regulating DNA transcription, particular histone modifications are associated with up or down-regulation of transcription [259].

Open chromatin is accessible to TFs which can bind to the regulatory regions and control gene expression. Whether chromatin is open or not depends on many factors including TF binding, histone modification and DNA methylation [201]. Open chromatin and hence regulatory regions can be detected through their sensitivity to DNase I, an endonuclease that cleaves DNA. As the chromatin is open, it is susceptible to DNase I binding and cleaving and is, therefore, a DNase hypersensitivity site. The cleaved DNA can then be sequenced and mapped to the human genome to determine DNase hypersensitivity sites and hence open chromatin as performed by the ENCODE consortium [256].

DNase hypersensitivity is, however, not consistent throughout the site [260]. Active TF binding to the regulatory region reduces the ability of DNase to cleave the DNA at the site, reducing the nucleotide count detected through sequencing producing a footprint as shown in Figure 3-14. Analysis of DNase I footprints with TF binding sites obtained from ChIP-Seq can provide specific nucleotide sequences that are associated with specific TF binding sites.

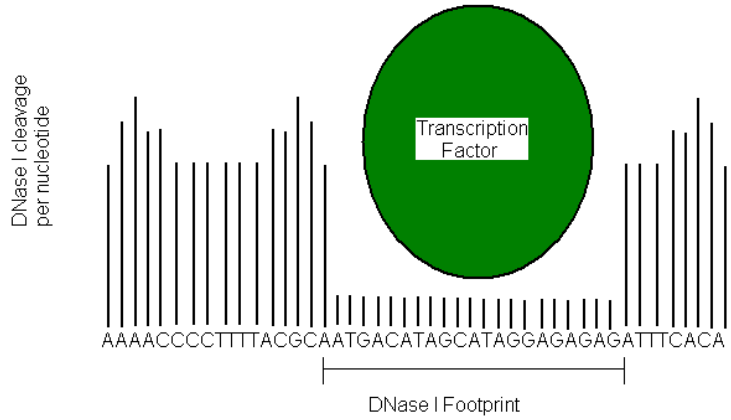


Figure 3-14. TF binding in a DNase hypersensitivity site producing a footprint.

3.11.16.4 Expression Quantitative Trait Loci (eQTL)

Allelic variation at regulatory regions may affect TF binding and gene expression. It is now possible to perform whole-genome gene expression assays which measure RNA expression in a cell [261]. SNPs are correlated to quantitative gene expression levels, termed expression quantitative trait locus (eQTL). Two effects are possible, *cis* and *trans. Cis* effects are seen when the SNP is in close proximity to the gene. *Trans* effects are seen when the SNP is distant to the gene being expressed. *Trans* effects may be seen due to microRNA effects which bind to mRNA resulting in repression [262]. RNA is extracted from a specific cell type in genotyped individuals of a specified ancestry. Transcript levels in the cells are quantified using microarrays or next generation sequencing [263]. A disadvantage of relying on eQTL data alone is that it is based on the disease-causing variant being genotyped or a SNP in LD with the disease causing variant being genotyped, which is not always the case. Whole genome eQTL results are available in publicly searchable databases as summarised in Table 3-7.

Paper	Population	Sample type	Number of samples	Database application
Yang, TP et al. [264]	8 HapMap 3 populations [265]	Lymphoblastoid cell lines (LCL)	726 individuals	Genevar
	Gencord population - collection of cell lines from umbilical cords of individuals of Western European origin [266]	Primary fibroblasts, EBV-immortalized B-cells (LCLs) and T-cells	85 individuals	Genevar
	Twin population from TwinsUK adult registry [267, 268]	Adipose, LCLs and skin	856 twins (856 female twins (154 monozygotic twin pairs, 232 dizygotic twin pairs and 84 singletons)	Genevar
	UK adult twin registry [269]	Adipose, LCLs and skin	156 LCL, 160 skin, 166 fat	Genevar
Montgomery, ST et al. [270]	HapMap 3 population (CEU)	LCLs	60	GTEX
Schadt, EE et al. [271]	Caucasian	Liver tissue	427	GTEX
Gibbs, JR et al. [272]	Neurologically normal subjects	Brain tissue	600 samples from 150 individuals	GTEX
Stranger, BE et al. [273]	HapMap 2 population (CEU, CHB, JPT, YRI)	LCLs	270 individuals	GTEX
Stranger, BE et al. [274]	Gutenberg Heart Study population	Monocytes	730 women 760 men	GHS express
Gamazon, ER et al. [275]	HapMap CEU and YRI	LCLs	87 CEU and 89 YRI	SCAN

Table 3-7. Summary of eQTL databases and their sources publicly available. EBV = Epstein Barr virus.

3.11.16.5 RegulomeDb

The amount of functional data derived from experimental studies from large consortia is increasing at an exponential rate. The majority of this data is scattered across numerous databases and manual integration of the data is time-consuming. RegulomeDb is a web-based tool which integrates information from a variety of databases including ENCODE, ChIP-seq assay results from the National Centre for Biotechnology Information (NCBI) sequence read archive (http://www.ncbi.nlm.nih.gov/sra), eQTL databases, predicted DNAse footprints and binding

motif alterations [276]. The database scores the likelihood of a SNP being functional as shown in Table 3-8.

Category	Description
	Likely to affect binding and linked to expression of a gene target
1a	eQTL + TF binding + matched TF motif + matched DNase footprint + DNase peak
1b	eQTL + TF binding + any motif + DNase footprint + DNase peak
1c	eQTL + TF binding + matched TF motif + DNase peak
1d	eQTL + TF binding + any motif + DNase peak
1e	eQTL + TF binding + matched TF motif
1f	eQTL + TF binding/DNase peak
	Likely to affect binding
2a	TF binding + matched TF motif + matched DNase footprint + DNase peak
2b	TF binding + any motif + DNase footprint + DNase peak
2c	TF binding + matched TF motif + DNase peak
	Less likely to affect binding
3a	TF binding + any motif + DNase peak
3b	TF binding + matched TF motif
	Minimal binding evidence
4	TF binding + DNase peak
5	TF binding or DNase peak
6	Motif hit

Table 3-8. RegulomeDb scoring system.

A disadvantage of the RegulomeDb database is that it is not updated with newer versions of data and it does not contain all the databases available such as GHS-express.

3.11.16.6 ASSIMILATOR

ASSIMILATOR is a program developed to summarise data from a variety of sources to identify evidence that the SNP has functional relevance [277]. Assimilator includes data from ENCODE tracks and eQTL data not available in previously described databases [278].

3.11.16.7 Bioinformatic Investigation

SNPs showing association with MTX-P were selected for a bioinformatics search. 1000 genomes (release 20110521) was used to calculate and filter SNPs in high LD (r^2>0.8) with the lead SNP. These SNPs were taken forward for bioinformatic interrogation using Genevar, GTX, GHS express, SCAN, RegulomeDb and ASSIMILATOR.

3.11.17 Statistical Analysis

3.11.17.1 The Null Hypothesis and Statistical Significance

The null hypothesis (H_0) used for statistical analysis is that the genotype of cases and controls does not significantly differ. Statistical significance was set as $p \leq 5 \times 10^{-8}$ as reaching genome wide significance and $p \leq 5 \times 10^{-5}$ as a variant of interest worthy of further investigation [279]. Association was tested using the Cochran-Armitage Test for trend except for HLA-A*31:01 where the allelic test of association was used as MHC expression is co-dominant [280].

3.11.18 Interpretation of Results

Visual inspection of the results is required to ensure quality control of the data. A quantile-quantile *p*-value plot is a scatter graph of the expected log *p*-values under the null hypothesis against the observed log *p*-values [281]. A spuriously high number of SNPs showing association in the quantile-quantile plot can indicate significant population stratification. A plot with few genotypes deviating from the null hypothesis suggests that there is no unaccounted population structure in the dataset. The expected *p*-value depends on the degrees of freedom and number of tests being conducted in the GWAS. It is expected that only a minority of variants will deviate from the expected *p*-value if population structure has been controlled by PCA or has an insignificant effect. The genomic inflation factor is a statistical test that is used to investigate unexpected deviation from the null distribution [282]. The genomic inflation factor is a ratio of the median observed chi^2 distribution and the median expected chi^2 distribution. A genomic inflation factor > 1 suggests a degree of systematic bias which may be due to population stratification, undetected sample duplications or related individuals within the case or control population.

Manhattan plots are scatterplots that provide an overall summary of the results, describing the position of the SNP on the x-axis, separated by chromosomes and –log(*p*-value). LocusZoom graphs are scatterplots of a region within the chromosome [283]. LocusZoom graphs plot the genomic location on the x-axis, the gene at the particular locus and –log(*p*-value) of the genotype association. Each SNP is colour coded representing the strength of LD between SNPs. A locus with strong evidence for association to disease should have a cluster of loci in proximity that also show evidence for association to disease due to LD with the lead SNP.

3.12 Contribution of the Author

The candidate conceived the project idea and design of the MEMO study in conjunction with his supervisory team. MTX/7-OH-MTX assay development and validation was conducted by the candidate under supervision from Dr Isabel Garcia. The candidate was the chief investigator for

the MEMO study. The candidate designed and wrote the protocol and application to the ethics committee. In addition, the candidate consented all participants into the MEMO study and performed all clinical assessments. Measurement of MTX/7-OH-MTX levels in plasma and urine from samples obtained from the RAMS and MEMO studies were undertaken by the candidate. Measurement of clinical biochemical, haematological and urine creatinine were performed by the clinical pathology labs at CMFT. Development of the pharmacokinetic model, validation of the model and calculation of AUC using samples from RAMS was performed by Dr Thierry Wendling at the School of Pharmacy, University of Manchester. Study design and ethical approval for the MTX-P GWAS was obtained by Dr Sally Owen. DNA extraction was performed by technicians within the Centre for Musculoskeletal Research, University of Manchester. Genotyping of MTX-P cases and controls was undertaken by the candidate. The candidate undertook the quality control and analysis of the MTX-P genotyping results. The candidate is also responsible for all other data analysis, data presentation and interpretation of the results.

Chapter 4: Results

4 Developing a HPLC-SRM-MS Assay to Measure MTX/7-OH-MTX Levels in Urine and Plasma

4.1 Introduction

MTX is the first-line therapy in RA but response is not universal. Up to 41% of RA patients are non-adherent to MTX as assessed by indirect measures but no published study has described adherence to MTX using a validated direct measure of adherence detection [100]. In order to develop a direct test of adherence to MTX it is vital to develop an assay that has a low lower limit of detection/quantification so that adherence can be measured for an adequate time after ingestion of MTX. A published pharmacokinetic study of MTX and 7-OH-MTX in RA suggests that 7-OH-MTX may be a more sensitive direct measure of adherence in plasma due to its longer $T_{1/2}$ [125]. Assays to detect MTX/7-OH-MTX in urine and/or plasma have been developed but they have cross-reaction with other substances [139-142, 152], are validated for use in urine alone [156, 163, 164, 167], plasma alone [162], have a high LLOQ [157-159, 166, 168] or detect MTX alone [140, 162-164].

This section describes the results of experiments conducted to develop an assay to measure MTX/7-OH-MTX levels in urine and plasma of patients taking low-dose (5-25mg) oral MTX. Given the association between low adherence and MTX AUC to response [87, 140], measuring MTX/7-OH-MTX levels may predict response to MTX in RA. Therefore the aim was to develop an assay to measure MTX and 7-OH-MTX levels in urine and plasma of patients with RA taking low-dose oral MTX. The results of the urine assay contained within this section have been published in the Analyst Journal [284].

4.2 Assay Optimisation in Water

Targeted liquid chromatrography mass spectrometry data is represented as a mass chromatogram, where the X-axis represents time after injection of the sample (the retention time), the y-axis represents signal intensity and the area under the curve represents the total amount of analyte detected. A typical mass chromatogram is shown in Figure 4-1 following injection of 250nM MTX/7-OH-MTX with 50nM MTX-d_3 in water.

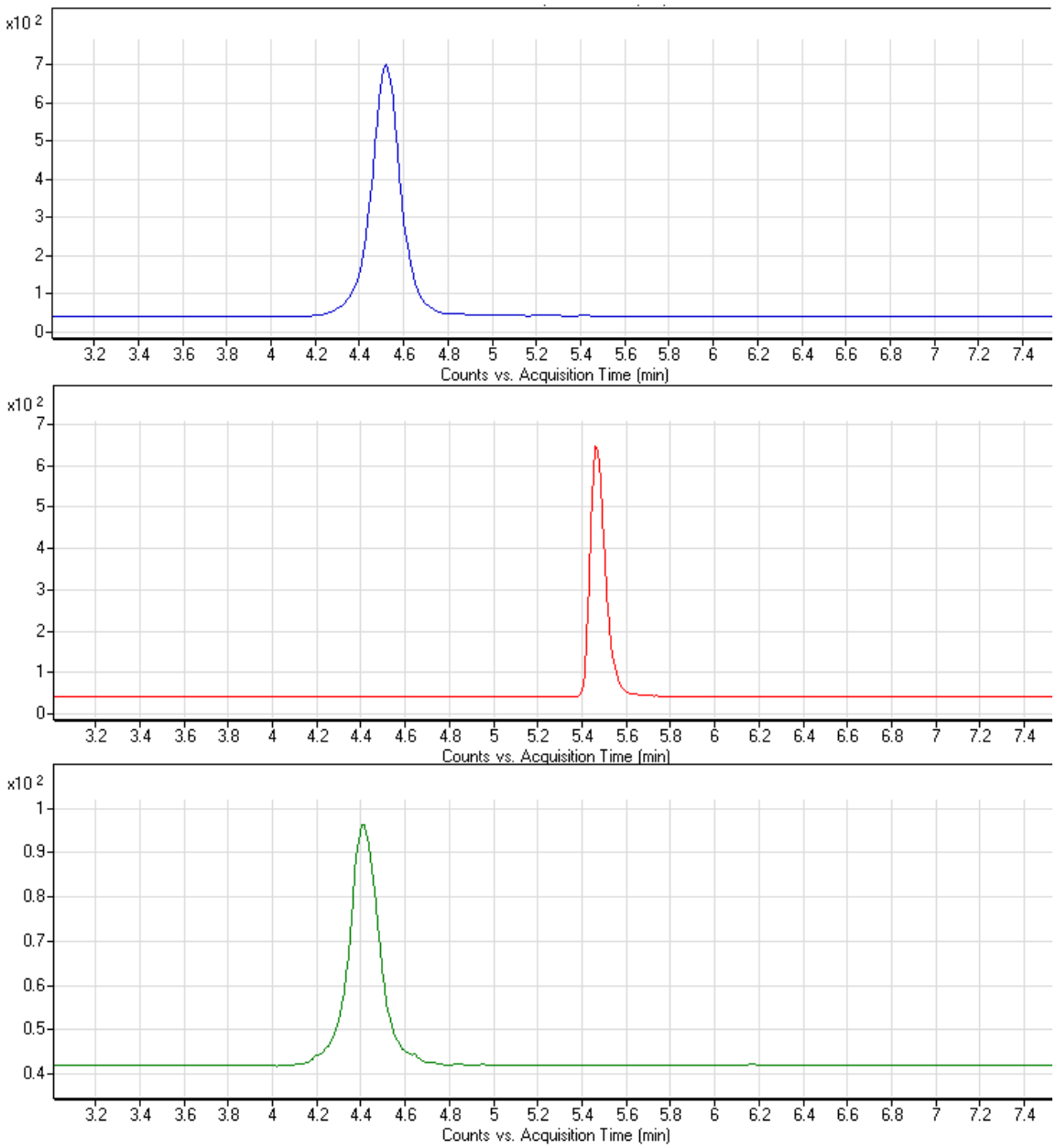


Figure 4-1. Chromatogram following injection of 250nM MTX (top), 7-OH-MTX (middle) and 50nM MTX-d_3 (bottom).

4.2.1 Reduction of Carryover

Early in the course of assay development, significant carryover and contamination between samples was detected as shown in Figure 4-2, representing the chromatogram following injection of a blank water sample.

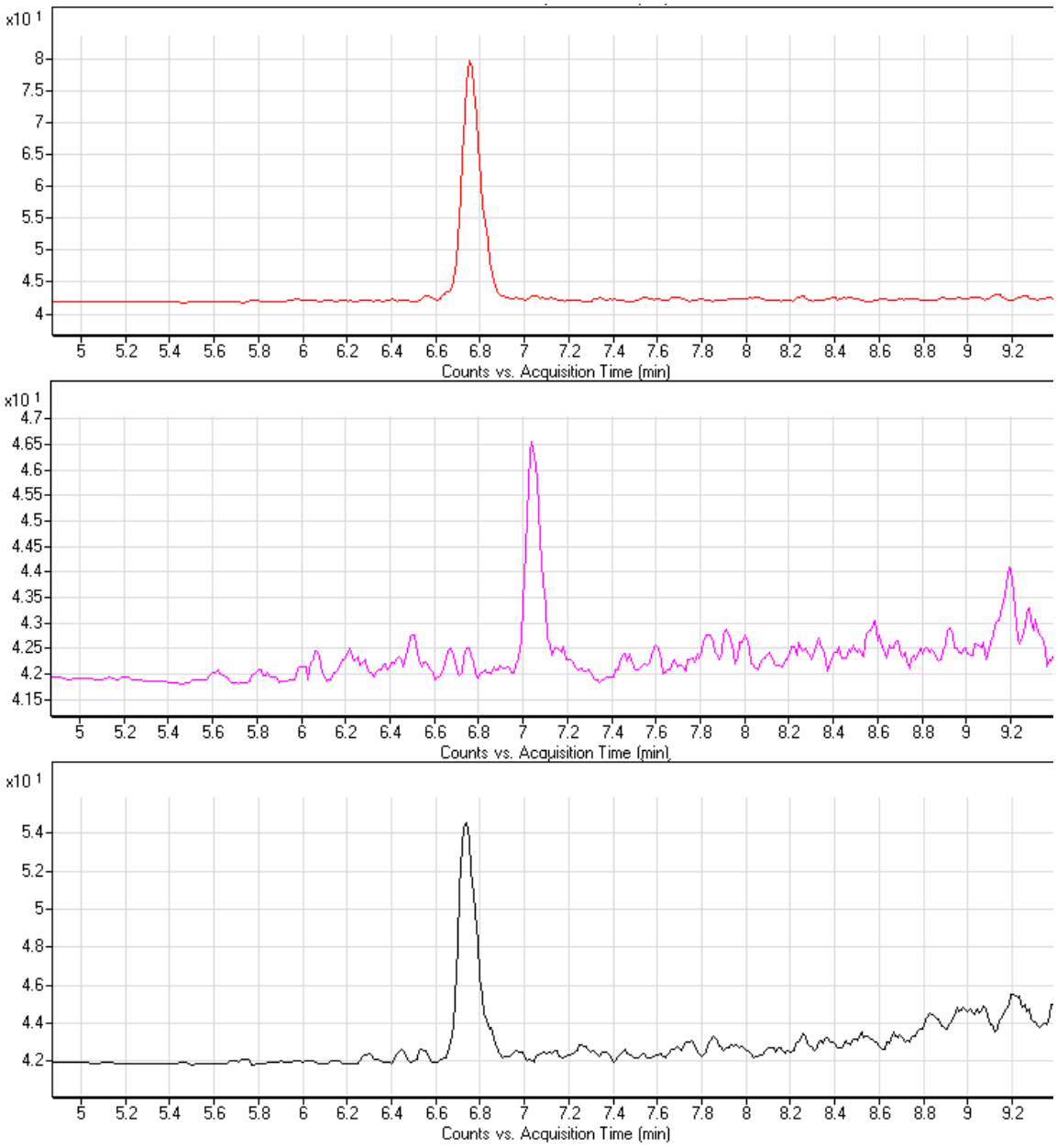


Figure 4-2. Chromatogram of blank water samples using a manual injection showing significant detection of MTX, 7-OH-MTX and MTX-d_3 respectively. Note retention time has changed to due altering the gradient elution timetable.

In order to confirm the source of the contamination, the assay was run without sample injection, confirming that the column, organic solution (B) and aqueous solution (A) were not contaminated. Subsequently, the manual injector was shown to be the source of contamination. In order to reduce carryover, an autosampler was sourced from Agilent (Agilent 1200 series Autosampler Thermostat). Use of an autosampler has many advantages over a manual injector: the needle for injection, capillary and injection valve is continuously flushed with the organic solvent, reducing carryover; the needle is also automatically washed to reduce carryover; furthermore, the autosampler holds samples in a temperature controlled tray, reducing the breakdown of temperature sensitive analytes allowing for a number of samples to be loaded onto the rack at

one time so that samples can be processed over a prolonged period. The temperature of the autosampler was set to 4°C.

The gradient elution timetable used initially in assay optimisation is shown in Table 4-1. In order to reduce future carryover from the system, the percentage of organic solution was increased and set to run for longer in the flow according to Table 4-2, subsequently increasing the retention time and reducing retention of analyte in the column/microtubules.

Time after injection (min)	Buffer A (%)	Buffer B (%)
0.00	95	5
2.00	95	5
2.50	75	25
7.50	75	25
7.51	95	5
10.00	95	5

Table 4-1. Initial gradient elution timetable.

Time after injection (min)	Buffer A (%)	Buffer B (%)
0.00	92	8
1.00	92	8
4.00	50	50
5.50	50	50
5.60	5	95
8.00	5	95
8.21	92	8
11.00	92	8

Table 4-2. Gradient elution timetable to reduce carryover. Note that buffer B concentration and length of time of high concentration is increased to reduce retention of analyte in the column.

Following the introduction of the autosampler and the new gradient elution timetable, there was no contamination as shown in Figure 4-3.

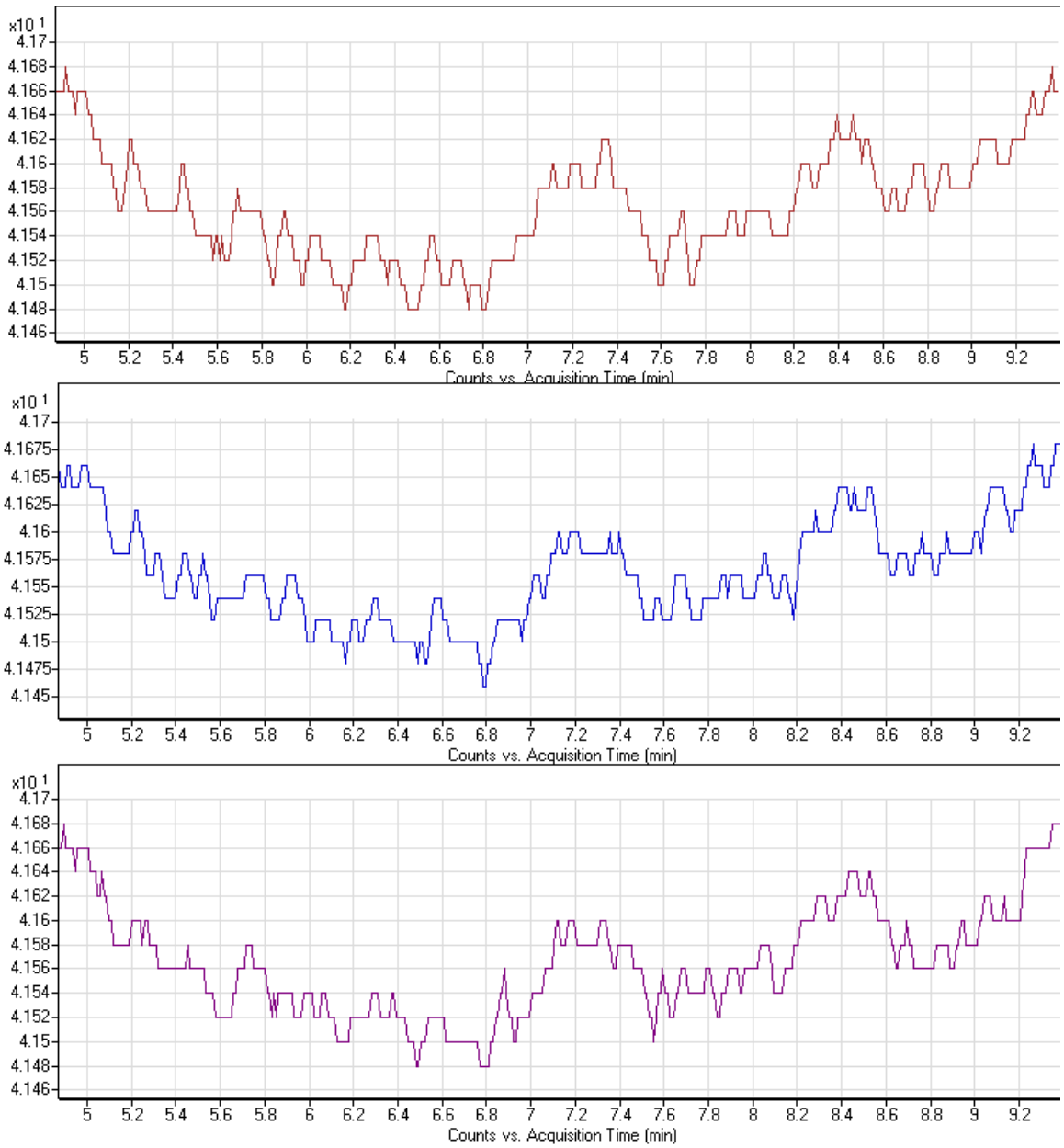


Figure 4-3. Chromatogram results following blank injection of water utilising the autosampler showing no discernible peaks and background noise only for MTX, 7-OH-MTX and MTX-d_3 respectively.

4.2.2 Lower limit of Detection, Lower Limit of Quantitation and Carryover

4.2.2.1 Lower Limit of Detection

The lower limit of detection (LLOD) was determined by manual inspection of the chromatograms. Injection of 0.05 nM MTX produced a peak which was evident from the background noise as shown in Figure 4-4 but the signal to noise ratio (SNR) was 3.8 and therefore did not meet the criteria for the lower limit of quantification. The lower limit of detection for 7-OH-MTX was 0.5 nM as shown in Figure 4-5, the SNR was 4.2, below the SNR required to meet the criteria for the LLOQ.

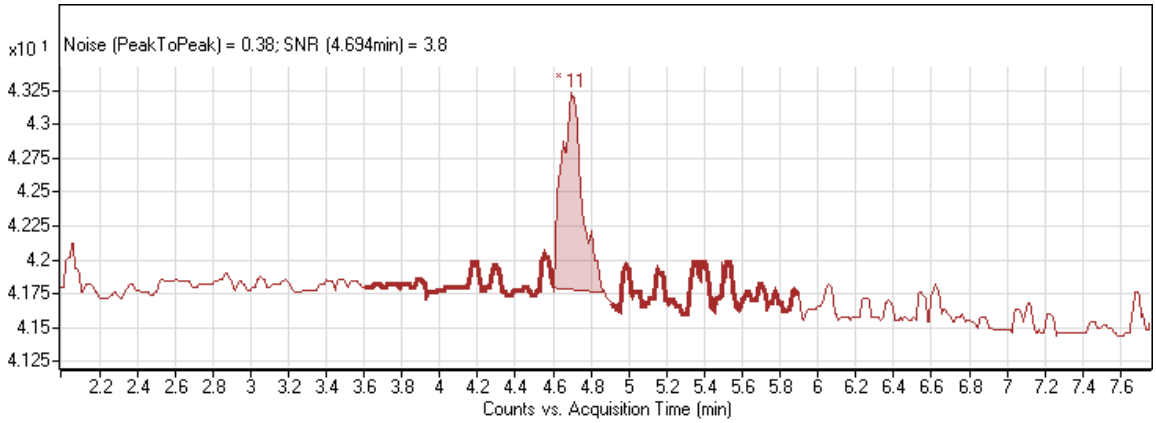


Figure 4-4. Chromatogram following injection of 0.05 nM MTX showing SNR below the LLOQ (3.8).

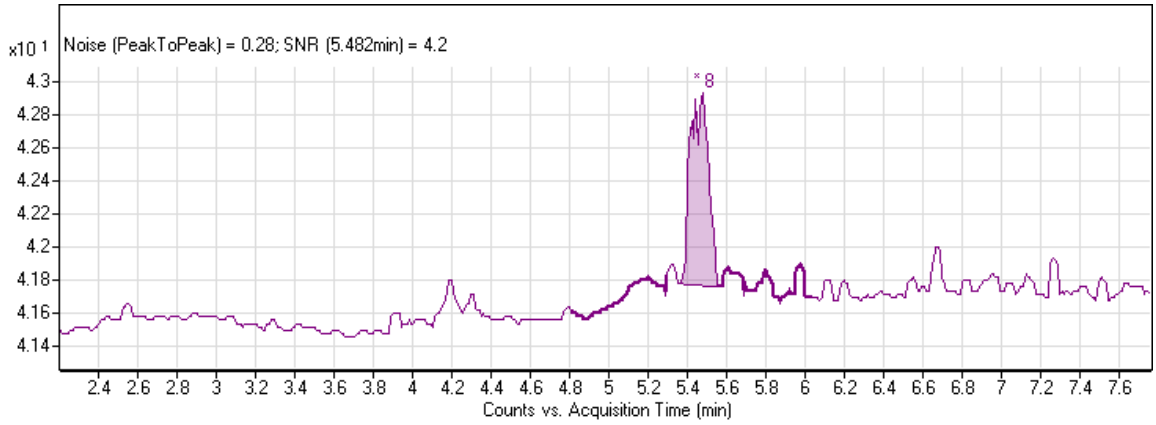


Figure 4-5. Chromatogram following injection of 0.5 nM 7-OH-MTX showing SNR below the LLOQ (4.2).

4.2.2.2 Lower Limit of Quantification

The LLOQ is defined as an SNR of 5. This was determined by automatic integration of the peaks in the chromatogram compared to a manually selected background noise which is highlighted as bold in the chromatogram. Figure 4-6 shows the chromatogram following an injection of 0.5 nM MTX, demonstrating an SNR of 13.3. The LLOQ for 7-OH-MTX was 0.75 nM as shown in Figure 4-7.

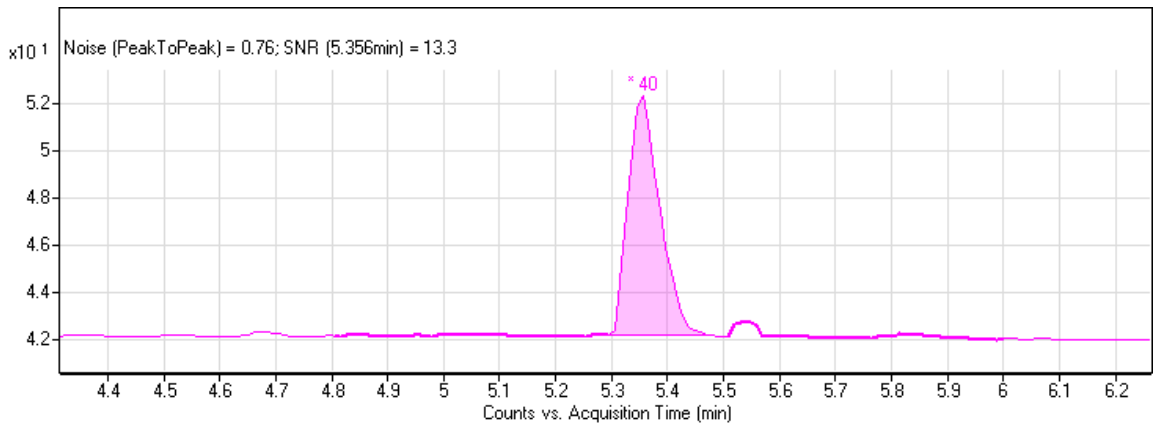


Figure 4-6. Chromatogram following injection of 0.5 nM MTX, demonstrating a SNR of 13.3.

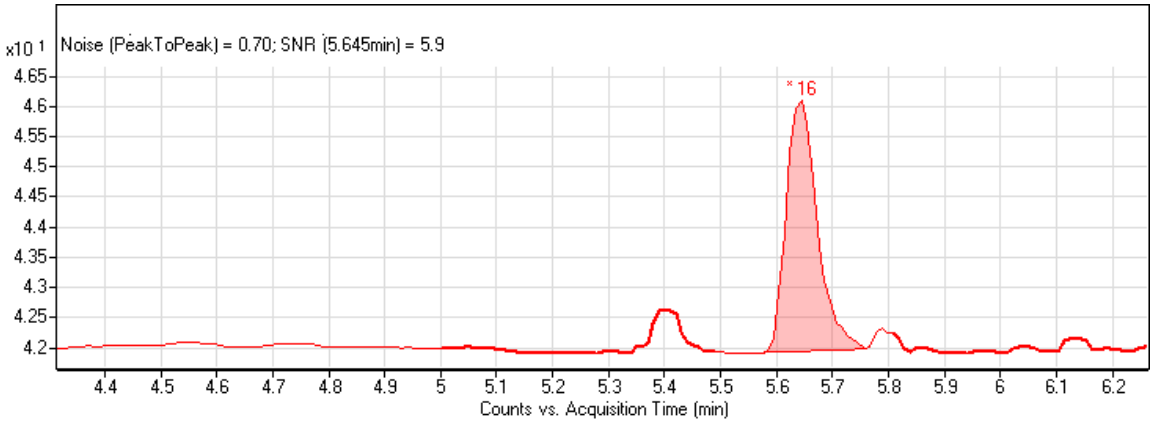


Figure 4-7. Chromatogram following injection of 0.75 nM 7-OH-MTX, demonstrating a SNR of 5.9.

4.2.2.3 Carryover

Carryover for MTX and 7-OH-MTX was less than 20% of the LLOQ as shown in Figure 4-8 and Figure 4-9. Carryover of MTX-d_3 was less than 1% of the highest injection level as shown in Figure 4-10.

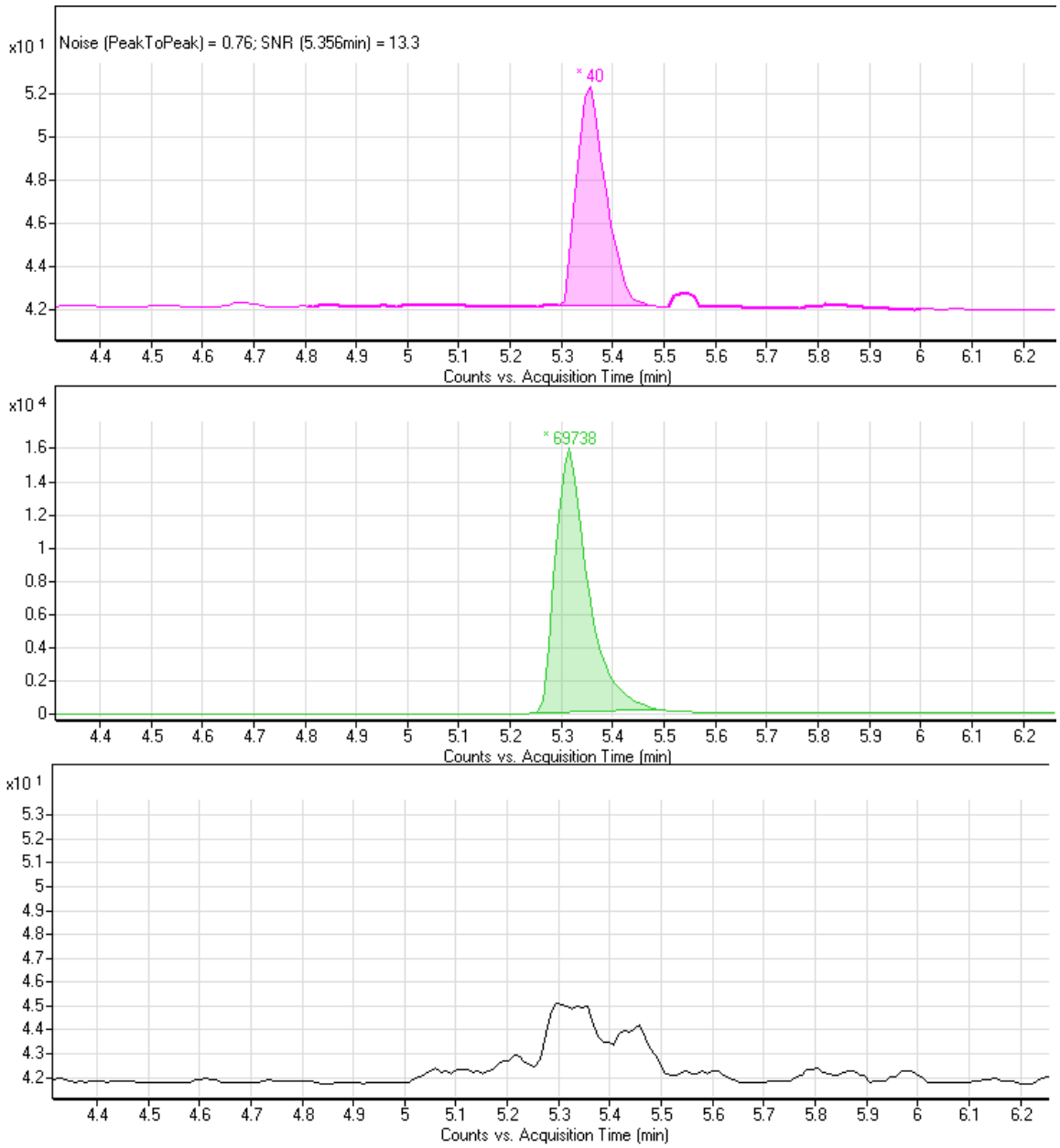


Figure 4-8. Carryover of MTX as assessed by injection of 1000 nM MTX (middle) followed by injection of a blank water sample (bottom). Automatic integration failed to detect a peak in the blank sample due to inadequate variation in the amplitude. The LLOQ chromatogram is shown for comparison (top).

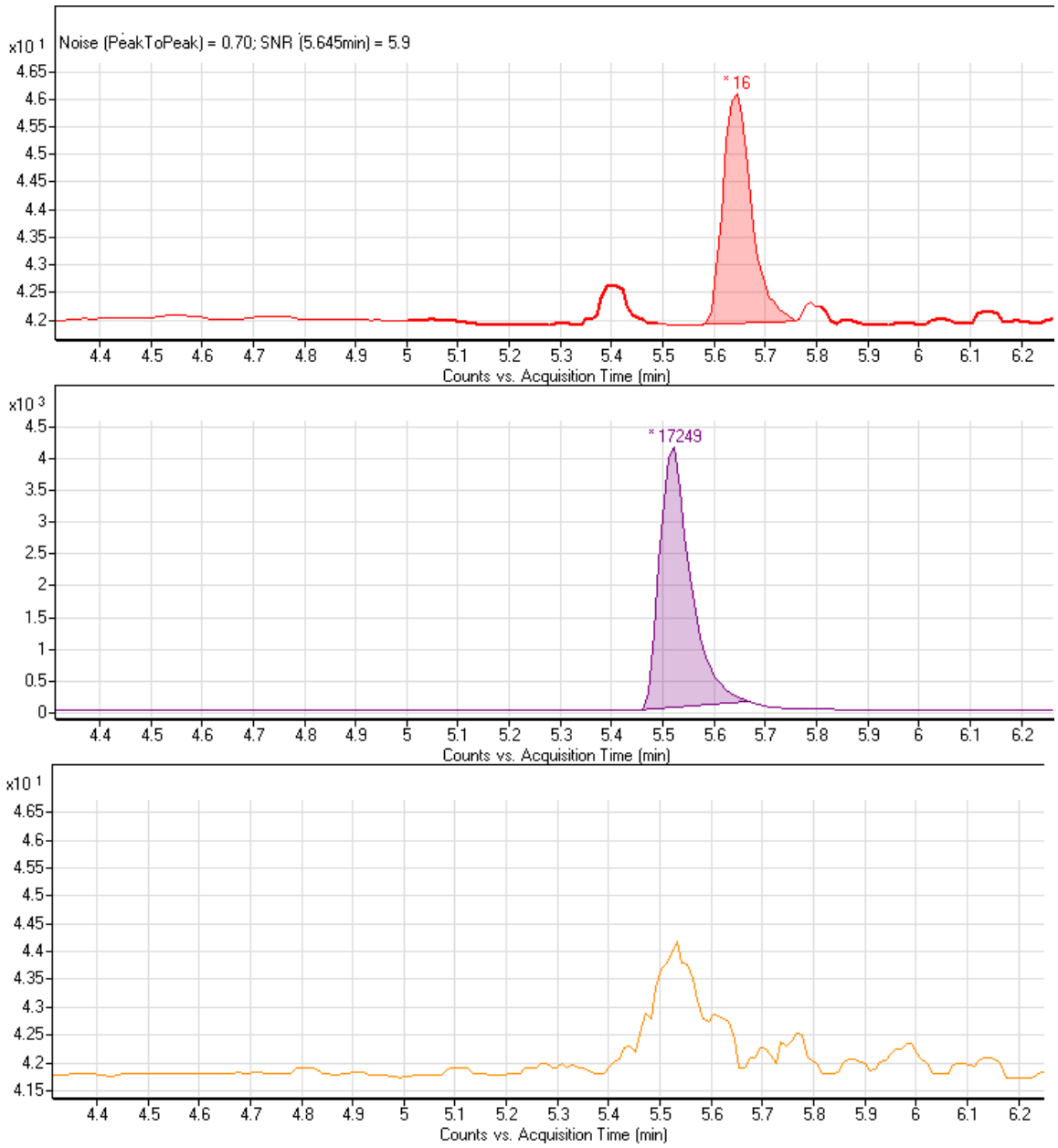


Figure 4-9. Carryover of 7-OH-MTX as assessed by injection of 1000 nM 7-OH-MTX (middle) followed by injection of a blank water sample (bottom). Automatic integration failed to detect a peak in the blank sample due to inadequate variation in the amplitude. The LLOQ chromatogram is shown for comparison (top).

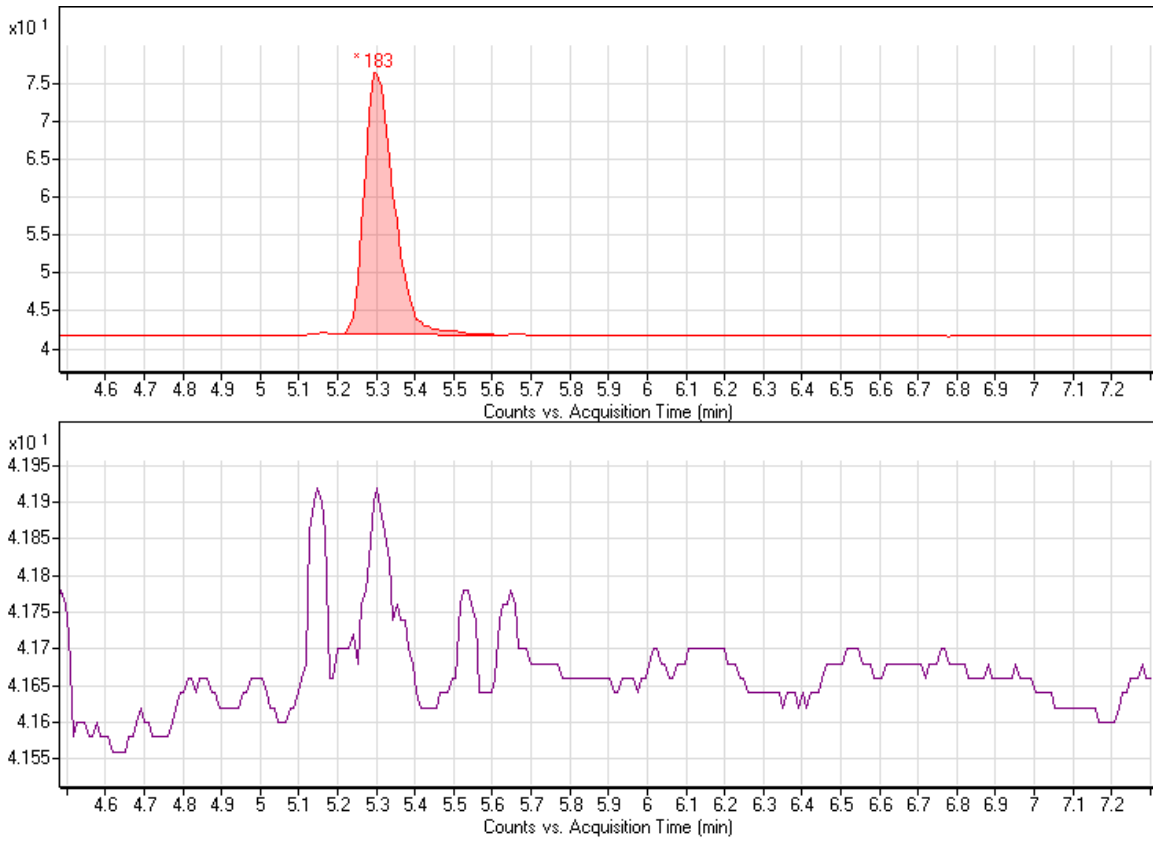


Figure 4-10. Carryover of MTX-d_3 as assessed by injection of 50 nM MTX-d_3 (top) followed by injection of a blank water sample (bottom). There is no discernible MTX-d_3 carryover peak demonstrating that carryover is less than 1%.

4.2.3 Accuracy

Table 4-3 and Table 4-4 show the results of accuracy testing for MTX and 7-OH-MTX respectively with the mean accuracy for each concentration level (n=3).

Expected concentration (nM)	Mean Measured concentration (nM)	CV (%)	Mean accuracy (%)
0.050	ND		
0.075	ND		
0.100	< LLOQ		
0.500	< LLOQ		
0.750	1.00	19	133
5.000	4.75	2	95
10.00	10.06	3	101
50.00	51.53	5	103
100.0	98.18	3	98
250.0	252.60	4	101
500.0	485.36	2	97
1000	1042.68	2	104

Table 4-3. Results of MTX accuracy in samples in aqueous solution (n=3). ND = not detected. LLOQ = lower limit of quantification.

Expected concentration (nM)	Mean Measured concentration (nM)	CV (%)	Mean accuracy (%)
0.050	ND		
0.075	ND		
0.100	ND		
0.500	< LLOQ		
0.750	< LLOQ		
5.000	5.13	5	103
10.00	10.34	3	103
50.00	49.66	2	99
100.0	100.55	2	101
250.0	245.63	6	98
500.0	498.80	3	100
1000	1037.34	1	104

Table 4-4. Results of 7-OH-MTX accuracy in samples in aqueous solution.

From the results, excluding 0.75 nM MTX, the mean accuracy for each concentration level is within 100% ± 15%. These results are therefore acceptable according to the EMA [221]. The data shows that for the LLOQ, CV ≤ 20% as required by EMA guidelines. EMA requirements are that the LLOQ is repeated five times to calculate precision; this was not completed in water samples as they were repeated three times at each concentration level because this was a preliminary experiment in water only and not the biofluid of final investigation.

4.2.4 Linearity

Linearity was calculated from Table 4-3 and Table 4-4 for MTX and 7-OH-MTX respectively. Figure 4-11 and Figure 4-12 show the linearity results plotting the mean measured concentration against expected concentration, the standard deviation and the R^2 calculated values.

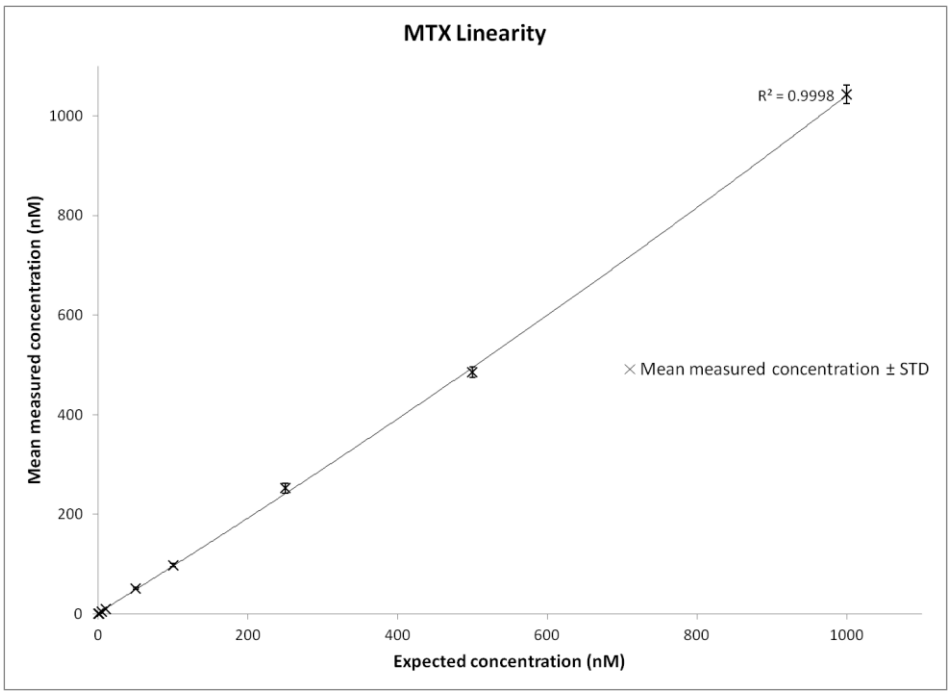


Figure 4-11. Linearity results for MTX showing good linearity between expected concentration and mean measured concentration (n=3). All samples were CV ≤ 15% except LLOQ where CV ≤ 20%.

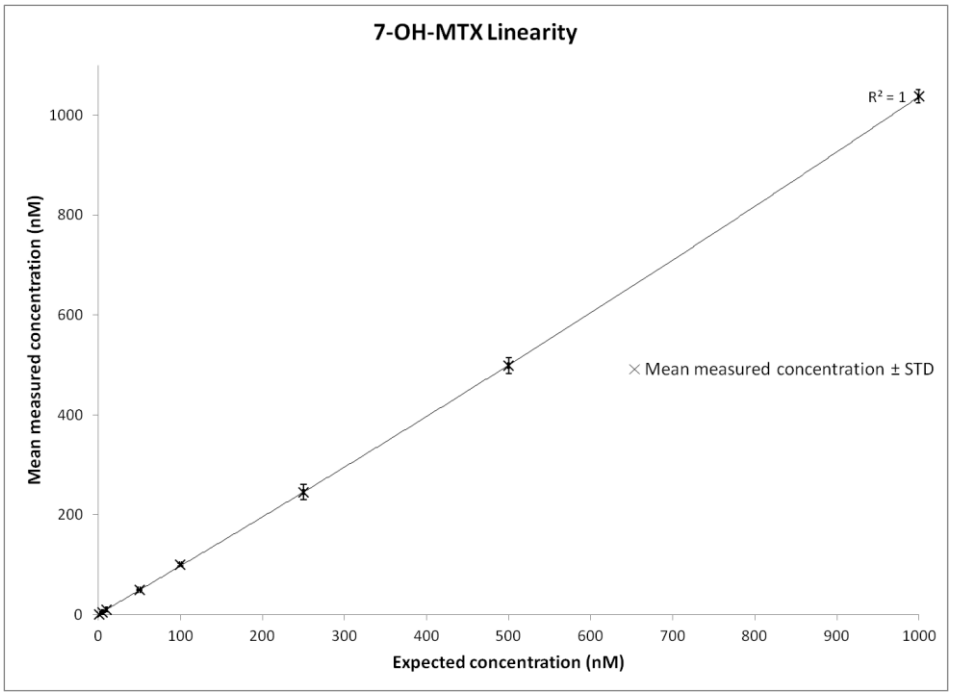


Figure 4-12. Linearity results for 7-OH-MTX showing good linearity between expected concentration and mean measured concentration (n=3). All samples were CV ≤ 15%.

4.2.5 Precision

Table 4-5 shows the measured concentration of the two analytes of interest and the internal standard in five replicate samples at a concentration of 5 nM, 25 nM, 50 nM and 250 nM. At the LLOQ samples were tested in triplicate.

Expected MTX and 7-OH-MTX concentration (nM)	Mean Measured MTX concentration (nM)	MTX CV (%)	Mean Measured 7-OH-MTX concentration (nM)	7-OH-MTX CV (%)	MTX-d3 CV (%)
0.50 [a]	0.43	4			6
0.75 [a]	0.73	20			2
5.00	5.48	1	4.18	10	3
25.0	19.54	2	18.20	2	2
50.0	54.69	4	56.60	5	13
250	263.66	1	278.38	5	4

Table 4-5. Intraday Precision Testing for MTX and 7-OH-MTX (n=5) except [a] (n=3).

4.2.6 Recovery

Protein precipitation can be performed utilising either ACN or methanol as the organic solvent. In order to determine whether recovery is greater with ACN or methanol, 11 concentrations of MTX/7-OH-MTX were prepared and underwent the protein precipitation protocol with either ACN or methanol with 12.5 nM MTX-d_3 producing a final concentration of 50 nM MTX-d_3 following rehydration. A calibration curve was run in water without protein precipitation. As the experiment was a preliminary experiment in water, the concentrations were run in single samples and not in triplicate for timely analysis. There was no significant alteration in chromatographic peaks for the analytes following protein precipitation with ACN or methanol as shown in Figure 4-13. The results of the experiment are shown in Table 4-6. The average recovery was higher for ACN compared to methanol for both MTX (90% vs. 85%) and 7-OH-MTX (89% vs. 85%), respectively.

Expected MTX/7-OH-MTX concentration (nM)	Mean MTX recovery in ACN (%)	Mean MTX Recovery in methanol (%)	Mean 7-OH-MTX recovery in ACN (%)	Mean 7-OH-MTX recovery in methanol (%)
0.5	98	82		
1.0	74	95	79	97
5.0	102	80	90	84
10.0	90	79	89	82
25.0	89	87	84	81
50.0	85	87	86	79
100.0	92	87	96	88
250.0	91	85	90	82
500.0	91	84	88	78
750.0	91	87	92	87
1000.0	92	87	94	89

Table 4-6. Recovery of MTX and 7-OH-MTX in urine samples following the protein precipitation protocol using either ACN or methanol as the organic solvent.

A further experiment using ACN as the organic solution for the protein precipitation protocol with samples prepared in triplicate was conducted. Nine concentrations were tested for recovery comparing samples that underwent the protein precipitation protocol and samples that did not. Table 4-7 and Table 4-8 present the recovery results for MTX and 7-OH-MTX, respectively. The overall mean recovery for all concentrations was 96% and 99% for MTX and 7-OH-MTX respectively. The recovery results demonstrate that there is no significant loss of MTX or 7-OH-MTX with the sample preparation protocol.

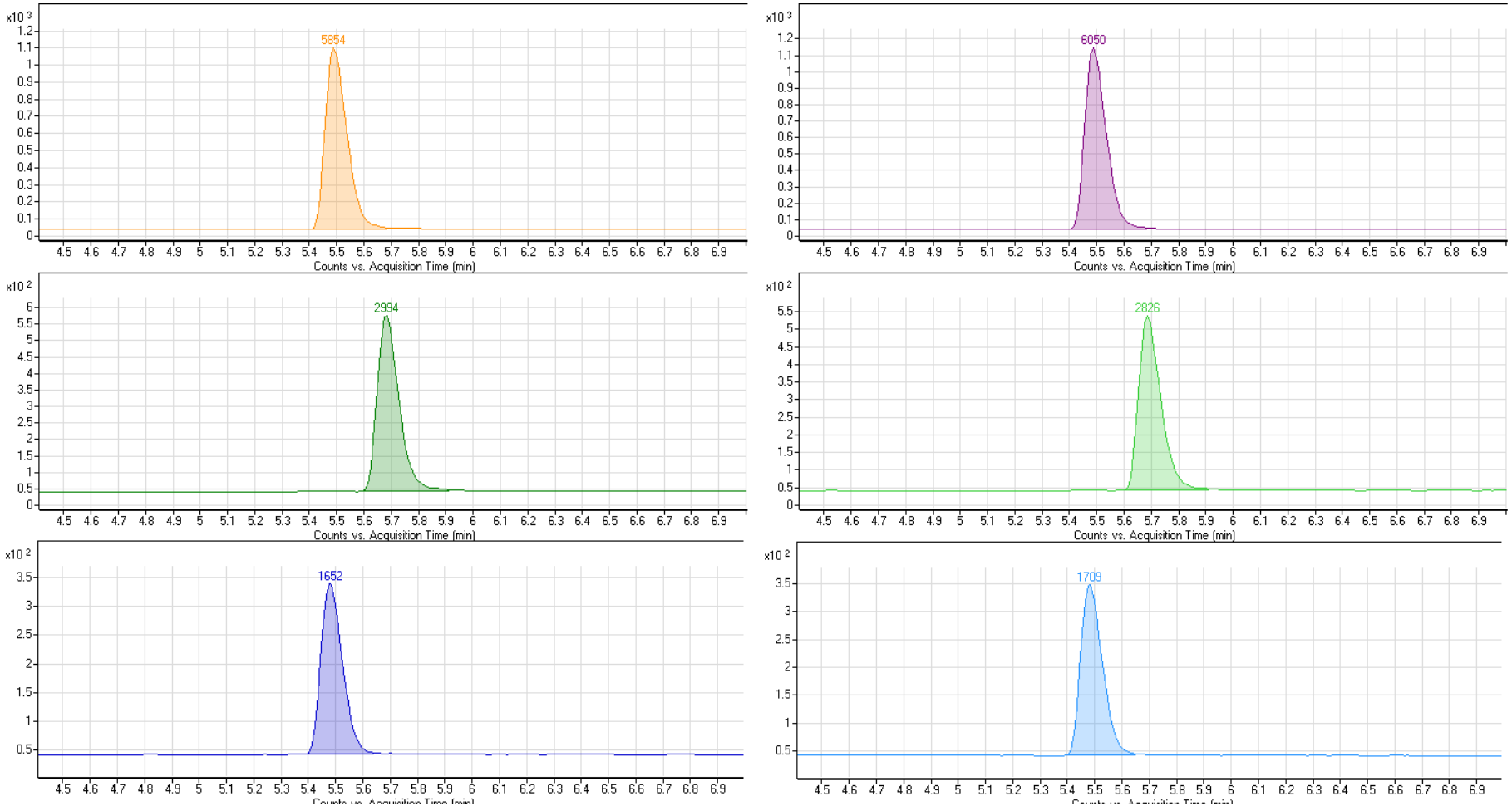


Figure 4-13. Chromatograms obtained following injection of 250 nM MTX (top)/7-OH-MTX (middle) and 50 nM MTX-d3 (bottom) protein precipitated with either ACN (left) or methanol (right). Chromatogram peaks demonstrate that there is no significant alteration in peak area for the three analytes.

Expected MTX concentration (nM)	Mean measured concentration for samples with no sample preparation (nM)	Mean accuracy (%)	CV (%)	Mean measured concentration for samples with sample preparation (nM)	Mean accuracy	CV (%)	Recovery (%)
0.50	0.5	101	4	0.36	73	38	72
0.75	1.00	133	19	0.88	118	11	89
5.00	4.74	95	2	5.05	101	3	106
10.0	10.06	101	4	10.28	103	2	102
50.0	51.52	103	5	48.79	98	2	95
100	98.18	98	3	99.84	100	3	102
250	252.60	101	4	249.62	100	3	99
500	485.36	97	2	489.33	98	2	101
1000	1042.68	104	2	996.27	100	2	96

Table 4-7. Recovery results for MTX. Samples tested in triplicate.

Expected 7-OH-MTX concentration (nM)	Mean measured concentration for samples with no sample preparation (nM)	Mean accuracy (%)	CV (%)	Mean measured concentration for samples with sample preparation (nM)	Mean accuracy	CV (%)	Recovery (%)
0.75	0.73	97	20	0.67	90	8	93
5.00	5.14	103	5	5.28	106	7	103
10.0	10.34	103	3	10.30	103	4	100
50.0	49.66	99	2	51.44	103	5	104
100	100.55	101	2	107.15	107	5	107
250	245.63	98	6	258.95	104	3	105
500	498.80	100	3	520.87	104	3	104
1000	1037.34	104	1	783.25	78	19	76

Table 4-8. Recovery results for 7-OH-MTX. Samples tested in triplicate.

4.2.7 Stability

Stability was tested in water samples at five time points following the protein precipitation protocol (0, 3, 24, 72 and 168 hours) at eight different concentrations for MTX (0.5, 0.75, 5, 10, 50, 100, 250 and 1000nM) and seven for 7-OH-MTX (0.75, 5, 10, 50, 100, 250 and 1000nM) in triplicate samples stored at room temperature and frozen (-80°C). The protein precipitation

protocol was followed in order to ensure that, for samples in the future, there is no degradation of analyte due to stability and protein precipitation as biofluids would be subject to stability and recovery issues together. Table 4-9, Table 4-10, Table 4-11 and Table 4-12 demonstrate the results of the stability experiments.

Time point (hours)	0			3		24		72		168	
Expected concentration (nM)	Mean measured concentration (nM)	Mean accuracy this concentration (%)	CV (%)	Mean measured concentration (nM)	Loss (%)	Mean measured concentration (nM)	Loss (%)	Mean measured concentration (nM)	Loss (%)	Mean measured concentration (nM)	Loss (%)
0.50	0.50	100	8	1.10	-118	< LLOQ	100	< LLOQ	100	< LLOQ	100
0.75	0.57	76	14	1.14	-101	0.53	8	0.72	-27[a]	0.60	-5
5.00	4.74	95	5	5.91	-25	3.77	21	3.86	19	4.67	1[a]
10.0	9.25	93	2	9.97	-8	8.03	13	7.82	15	6.97	25
50.0	46.71	93	2	48.38	-4	42.00	10	41.55	11	38.19	18
100	94.61	95	1	100.52	-6	85.09	10	80.46	15	63.08	33[a]
250	301.91	121	2	319.78	-6	258.36	14	261.15	14	248.14	18
1000	1129.80	113	1	1136.91	-1	996.27	12	990.91	12	963.17	15

Table 4-9. Stability testing results for MTX samples stored at room temperature (n=3). Samples except [a] were CV ≤ 15%.

Time point (hours)		0		3		24		72		168	
Expected concentration (nM)	**Mean measured concentration (nM)**	**Mean accuracy this concentration (%)**	**CV (%)**	**Mean measured concentration (nM) (SD)**	**Loss (%)**	**Mean measured concentration (nM) (SD)**	**Loss (%)**	**Mean measured concentration (nM) (SD)**	**Loss (%)**	**Mean measured concentration (nM) (SD)**	**Loss (%)**
0.50	0.50	100	8	< LLOQ	100	< LLOQ	100	< LLOQ		0.50	0
0.75	0.57	76	14	0.58[a]	-2	0.60[a]	-6	0.73[a]	-29	0.57	0
5.00	4.74	95	5	4.85	-2	3.96	16	3.97	16	3.55	25
10.0	9.25	93	2	9.61	-4	7.89	15	7.74	16	7.58	18
50.0	46.71	93	2	48.28	-3	42.90	8	39.16	16	37.79	19
100	94.61	95	1	100.41	-6	82.11	13	77.86	18	78.48	17
250	301.91	121	2	323.44	-7	265.48	12	264.02	13	319.95	-6
1000	1129.80	113	1	1174.01	-4	1024.72	9	991.25	12	909.55	19

Table 4-10. Stability testing results for MTX samples stored at -80°C (n=3). Samples except [a] were CV ≤ 15%.

Time point (hours)	0			3		24		72		168	
Expected concentration (nM)	Mean measured concentration (nM)	Mean accuracy this concentration (%)	CV (%)	Mean measured concentration (nM)	Loss (%)	Mean measured concentration (nM)	Loss (%)	Mean measured concentration (nM)	Loss (%)	Mean measured concentration (nM)	Loss (%)
0.75	< LLOQ			< LLOQ		< LLOQ		< LLOQ		< LLOQ	
5.00	4.71	94	9	1.64	65	5.43	-15	5.41	-15	7.04	-49
10.0	10.09	101	4	11.18	-11	10.63	-5	11.12	-10	11.23	-11
50.0	51.01	102	1	44.91[a]	12	50.35	1	52.20	-2	49.08	4
100	101.76	102	2	109.77	-8	105.06	-3	102.67	-1	84.27	17
250	257.93	103	3	257.91	0	241.85	6	250.90	3	245.04	5
1000	1013.86	101	1	1015.83	0	1011.90	0	1039.89	-3	1036.57	-2

Table 4-11. Stability testing results for 7-OH-MTX samples stored at room temperature. Samples except [a] were CV ≤ 15%.

Time point (hours)	0			3		24		72		168	
Expected concentration (nM)	**Mean measured concentration (nM)**	**Mean accuracy this concentration (%)**	**CV (%)**	**Mean measured concentration (nM)**	**Loss (%)**	**Mean measured concentration (nM)**	**Loss (%)**	**Mean measured concentration (nM)**	**Loss (%)**	**Mean measured concentration (nM)**	**Loss (%)**
0.75	< LLOQ			< LLOQ		< LLOQ		< LLOQ		< LLOQ	
5.00	4.71	94	9	6.17	-31	5.45	-16	5.64	-20	5.80	-23
10.0	10.09	101	4	11.00	-9	10.90	-8	10.80	-7	11.03	-9
50.0	51.01	102	1	51.94	-2	53.31	-5	50.42	1	49.80	2
100	101.76	102	2	113.53	-12	104.16	-2	98.44	3	103.36	-2
250	257.93	103	3	299.96	-16	251.00	3	255.80	1	320.82	-24
1000	1013.86	101	1	1187.14	-17	1076.69	-6	1028.50	-1	980.65	3

Table 4-12. Stability testing results for 7-OH-MTX samples stored at -80°C. All samples were CV ≤ 15%.

Stability testing of MTX demonstrated that at room temperature, MTX was stable at the higher concentrations (≥ 10 nM) up to 72 hours with the mean concentration being within ±15% of the baseline concentration as recommended by the EMA guidelines. 7-OH-MTX was stable at the higher concentrations (≥ 5 nM) up to 72 hours with the mean concentration being within ± 15% of the baseline concentration. At -80°C, MTX was stable at the higher concentrations (≥ 10 nM) up to only 24 hours and 7-OH-MTX up to 168 hours. It was expected that MTX would be more stable in water when frozen but the loss may be due to freeze-defrost of samples with added loss of stability due to time.

4.2.8 Summary of Results

Whilst the assay has not fully fulfilled EMA assay validation requirements, for research purposes the assay in water has demonstrated good reliability in the domains as advised by the EMA. The LLOQ for MTX and 7-OH-MTX is 0.5 nM and 0.75 nM respectively with less than 20% carryover of the LLOQ after a high concentration injection as advised by the EMA. The assay has demonstrated good linearity. Whilst samples were tested in triplicate and not quintuplicate as advised by the EMA, the assay has demonstrated accuracy for concentrations ≥ 5 nM for MTX and 7-OH-MTX. Intraday precision fulfilled the EMA criteria and recovery for MTX and 7-OH-MTX following the protein precipitation protocol was 72% and 76% respectively. Stability testing determined that, up to 72 hours, MTX and 7-OH-MTX are stable at room temperature for concentrations ≥ 10 nM and 5 nM respectively. Optimisation of the assay in water led to an improved gradient elution timetable which reduced carryover and demonstrated the need for an autosampler for injection of samples.

The next stage was to validate the assay in urine. Moving the protocol to urine has inherent difficulties. Noise may increase due to the presence of other components in urine which may not be completely removed with the sample preparation protocol and therefore the LLOD/LLOQ may be increased.

4.3 Assay Optimisation and Validation in Urine

Following assay development in water, the assay was moved to testing MTX/7-OH-MTX and MTX-d_3 concentrations in urine. The assay was first optimised in water to exclude matrix effects whilst improving the sensitivity to detect MTX/7-OH-MTX/MTX-d_3 and as a proof of concept that HPLC-SRM-MS can detect these analytes using the technology available. In view of the results in water, the sample preparation protocol was undertaken using 12.5 nM MTX-d_3 in ACN for protein precipitation. Blank urine was pooled (n=3) and spiked as required. Urine was obtained from participants in RAMS attending their baseline (pre-MTX) appointment.

4.3.1 Lower limit of Detection, Lower Limit of Quantitation and Carryover

4.3.1.1 Lower Limit of Detection

The lower limit of detection (LLOD) was determined by manual inspection of the chromatograms. ınjection of 2.5 nM MTX produced a peak which was evident from the background noise as shown in Figure 4-14 but the signal to noise ratio (SNR) was 3.5 and therefore did not meet the criteria for the lower limit of quantification.

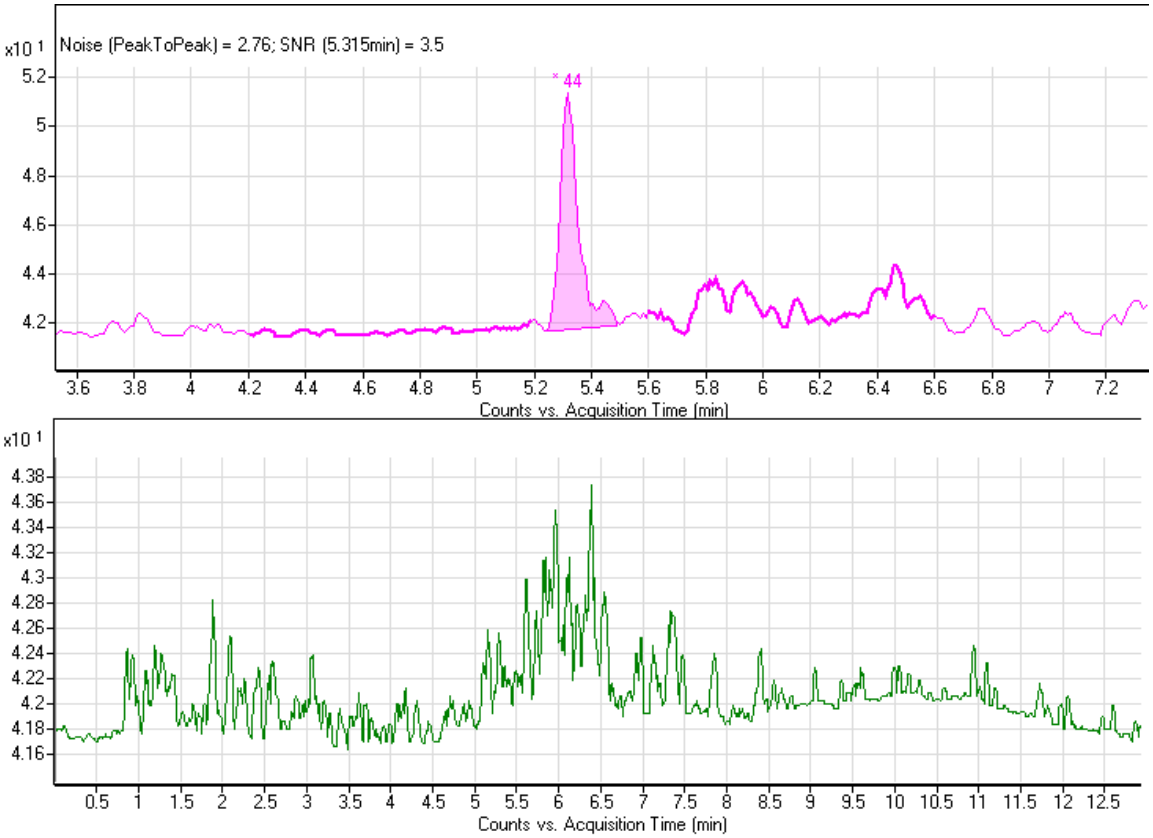


Figure 4-14. Chromatogram following injection of 2.5 nM MTX with SNR 3.5 (top). For comparison, a chromatogram following injection of blank urine is shown (bottom).

Injection of blank urine which underwent the sample preparation protocol produced three peaks for 7-OH-MTX as shown in Figure 4-15.

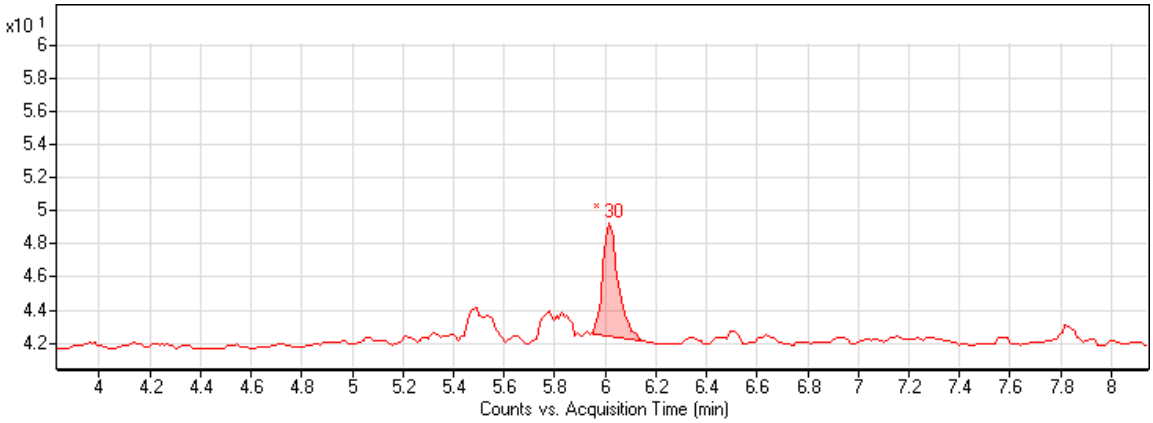


Figure 4-15. Chromatograph following injection of blank urine. There are three distinct peaks that can be seen, the largest of which has AUC 30.

In order to determine whether the peaks were due to noise secondary to matrix effects or due to the analyte of interest, further injections of urine spiked with 2.5 nM, 10 nM and 100 nM 7-OH-MTX were undertaken (Figure 4-16). As can be seen, as the concentration increases, the AUC for the blank peaks do not increase suggesting that the peaks are due to background noise. Based on these results, the chromatograms were only analysed up to a retention time of 5.8 minutes so that the small third peak did not contribute to 7-OH-MTX concentration calculations.

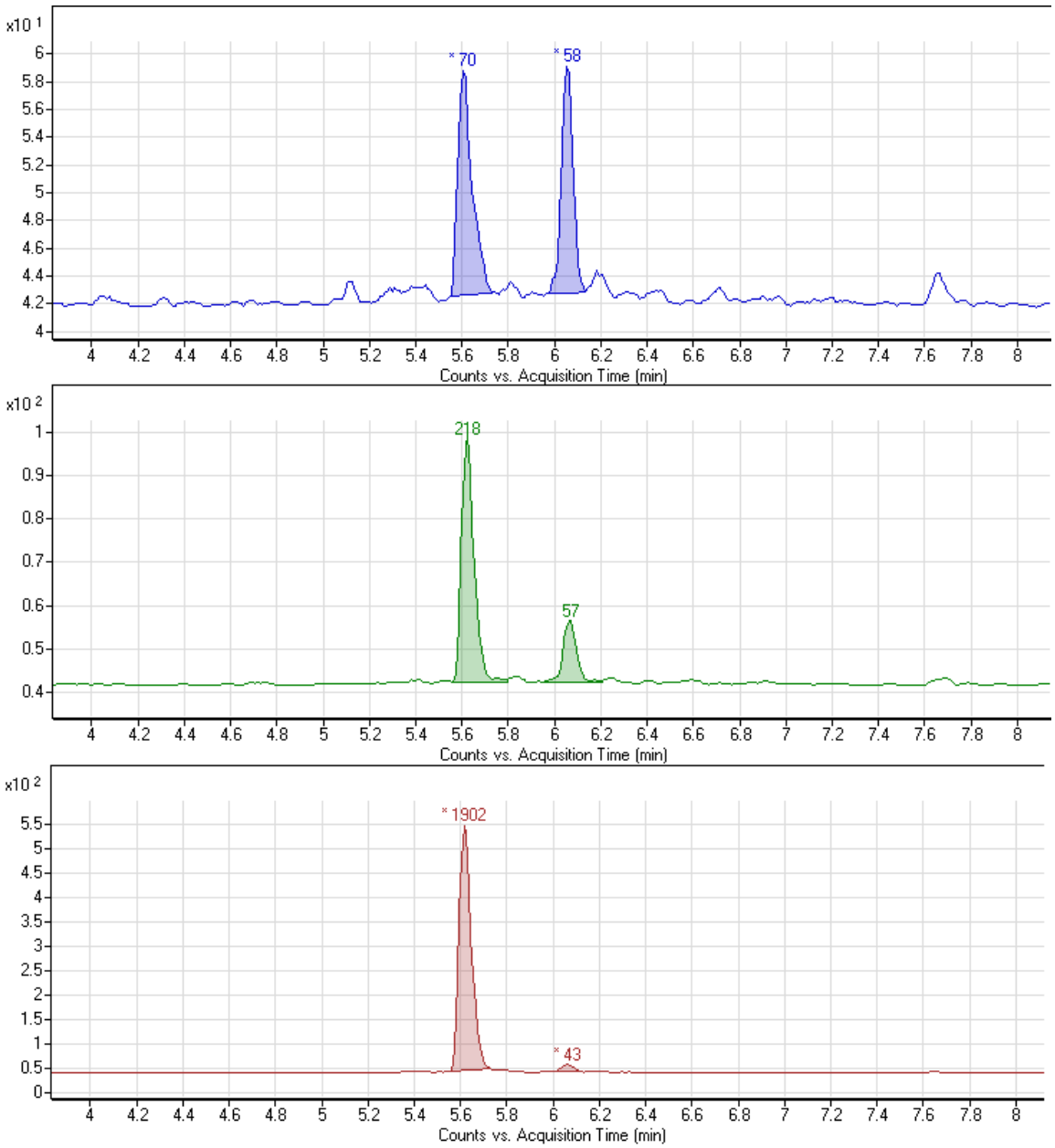


Figure 4-16. Chromatograms following injection of 2.5 nM (top), 10 nM (middle) and 100 nM (bottom) 7-OH-MTX. The AUC for the third peak does not significantly increase with increasing concentration of 7-OH-MTX suggesting that it is due to background noise.

4.3.1.2 Lower Limit of Quantification

The LLOQ for MTX with a SNR ≥ 5 was 5 nM as shown in Figure 4-17.

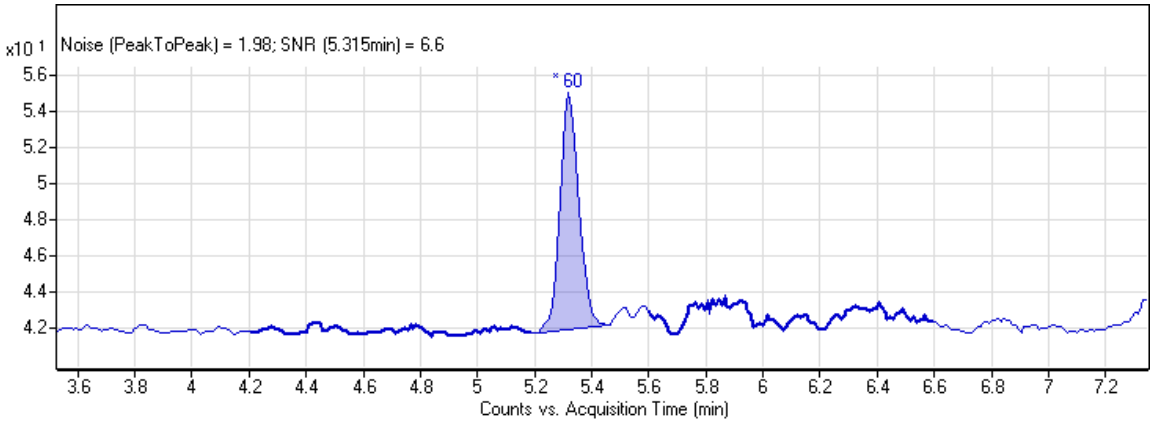


Figure 4-17. Chromatogram following injection of 5 nM MTX with a SNR=6.6.

The LLOQ for 7-OH-MTX with a SNR ≥ 5 was 10 nM as shown in Figure 4-18.

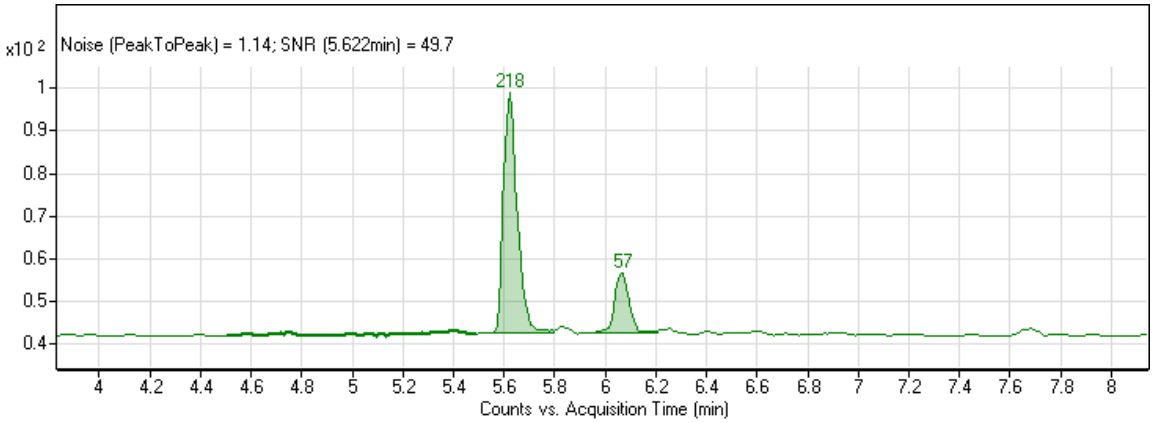


Figure 4-18. Chromatogram following injection of 10 nM 7-OH-MTX with a SNR=49.7. The third background peak was excluded from the calculation of SNR as the chromatogram was only analysed up to a retention time of 5.8 minutes.

4.3.1.3 Carryover

Carryover was assessed by injection of 1000 nM MTX/7-OH-MTX followed by a blank urine sample. Carryover was acceptable if less than 20% of the LLOQ. Figure 4-19 and Figure 4-20 show the results of the carryover experiment demonstrating that carryover was less than 20% of the LLOQ for MTX and 7-OH-MTX in urine.

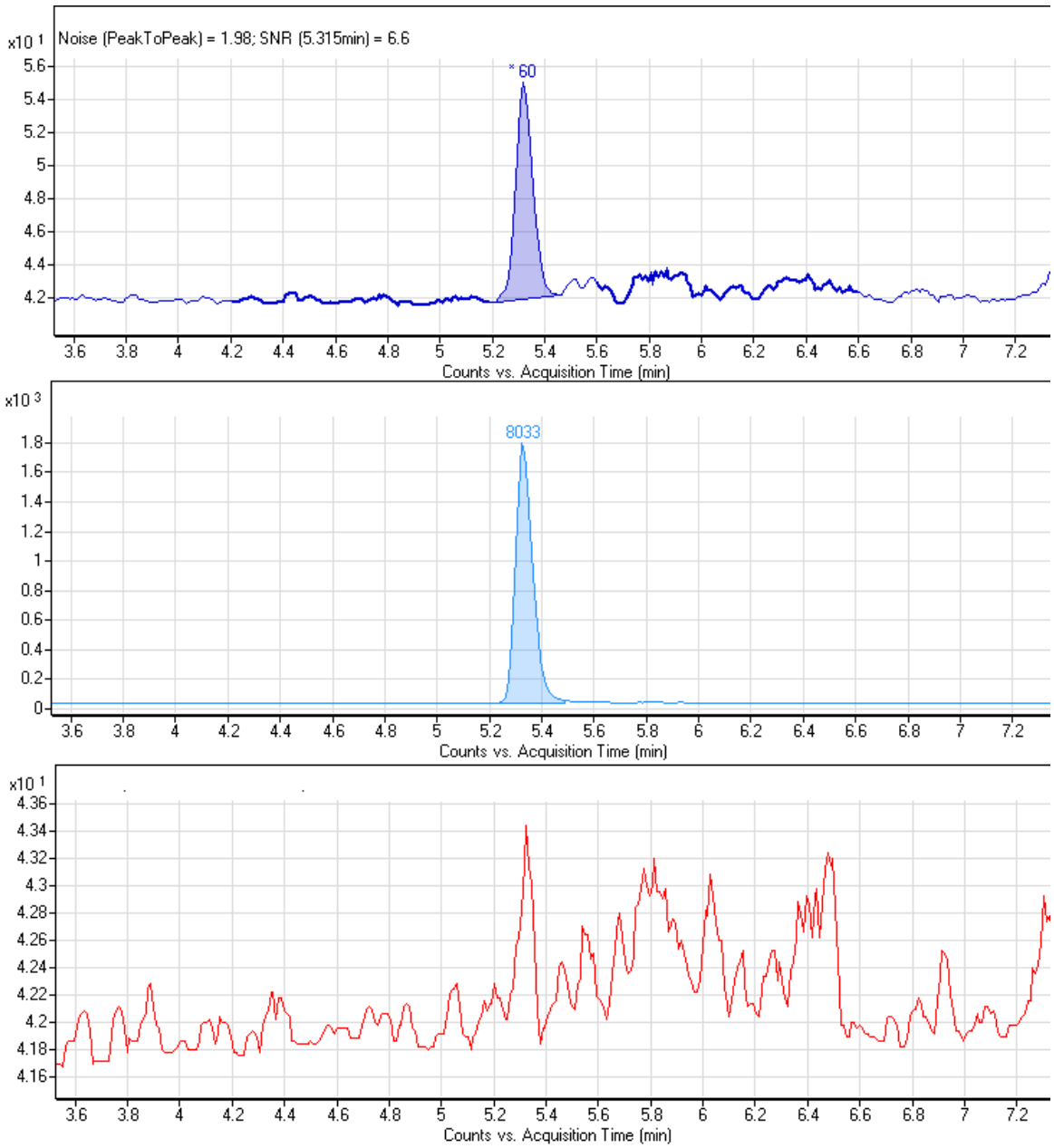


Figure 4-19. Carryover of MTX as assessed by injection of 1000 nM MTX (middle) followed by injection of a blank urine sample (bottom). Automatic integration failed to detect a peak in the blank sample due to inadequate variation in the amplitude. The LLOQ chromatogram is shown for comparison (top).

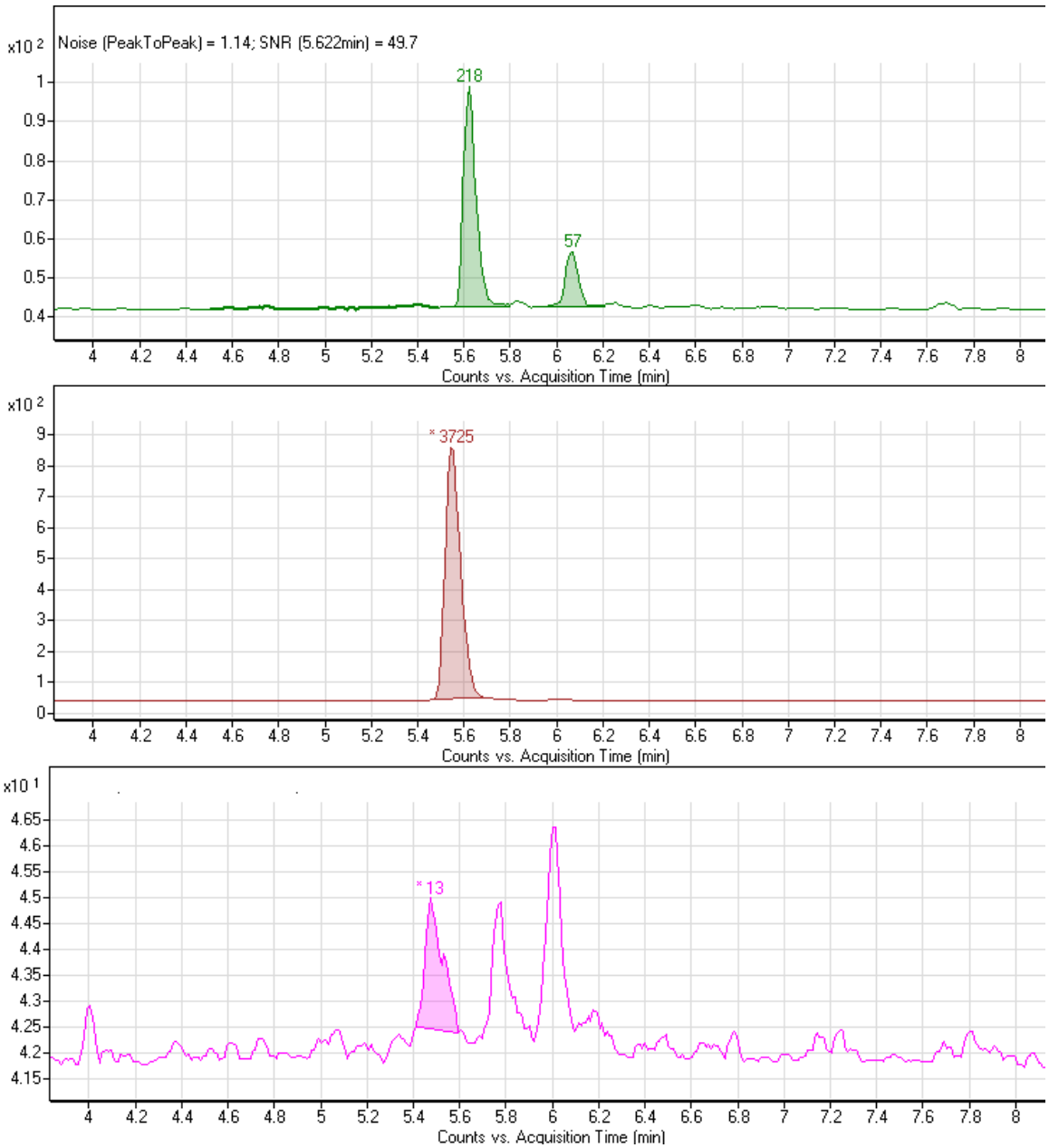


Figure 4-20. Carryover of 7-OH-MTX as assessed by injection of 1000 nM 7-OH-MTX (middle) followed by injection of a blank urine sample (bottom). The LLOQ chromatogram is shown for comparison (top).

4.3.2 Accuracy

Table 4-13 shows the results from accuracy testing for MTX and 7-OH-MTX. The mean accuracy is within 15% of the expected concentration as advised by the EMA demonstrating adequate accuracy of the assay.

Expected MTX/7-OH-MTX concentration (nM)	Mean MTX measured concentration (nM)	CV (%)	Mean accuracy (%)	Mean 7-OH-MTX measured concentration (nM)	CV (%)	Mean accuracy (%)
5	4.56	12	91	< LLOQ		
10	9.74	2	97	10.79	6	108
50	44.31	3	89	51.41	2	103
100	87.58	4	88	107.28	2	107
500	549.48	1	110	485.19	0	97
1000	1058.69	3	106	902.19	3	90

Table 4-13. Results of accuracy testing for MTX and 7-OH-MTX samples in urine.

4.3.3 Linearity

Linearity was calculated from Table 4-13 for MTX and 7-OH-MTX. Figure 4-21 and Figure 4-22 present the linearity results plotting the mean measured concentration against expected concentration the standard deviation and the R^2 test statistic for MTX and 7-OH-MTX respectively. These results demonstrate that the assay is linear within the concentrations tested.

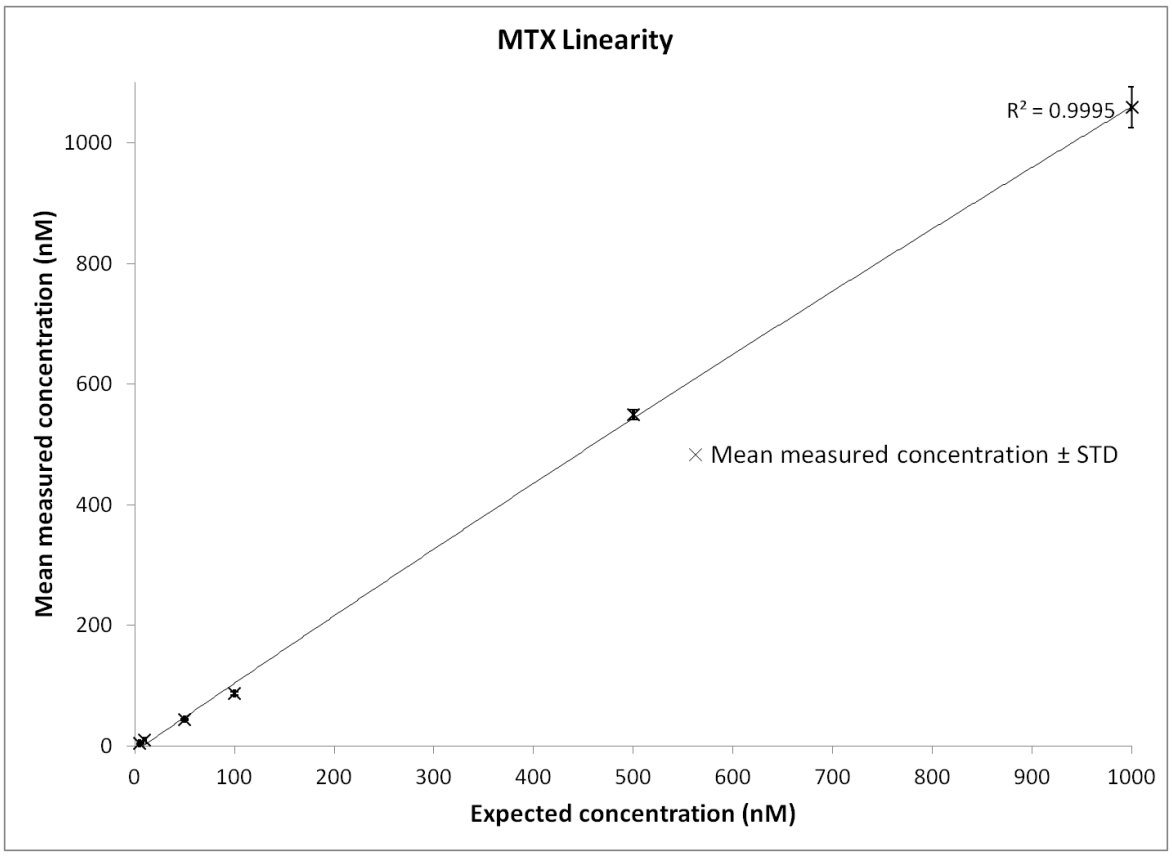


Figure 4-21. Linearity results for MTX showing good linearity between expected concentration and mean measured concentration (n=3). All samples were CV ≤ 15%.

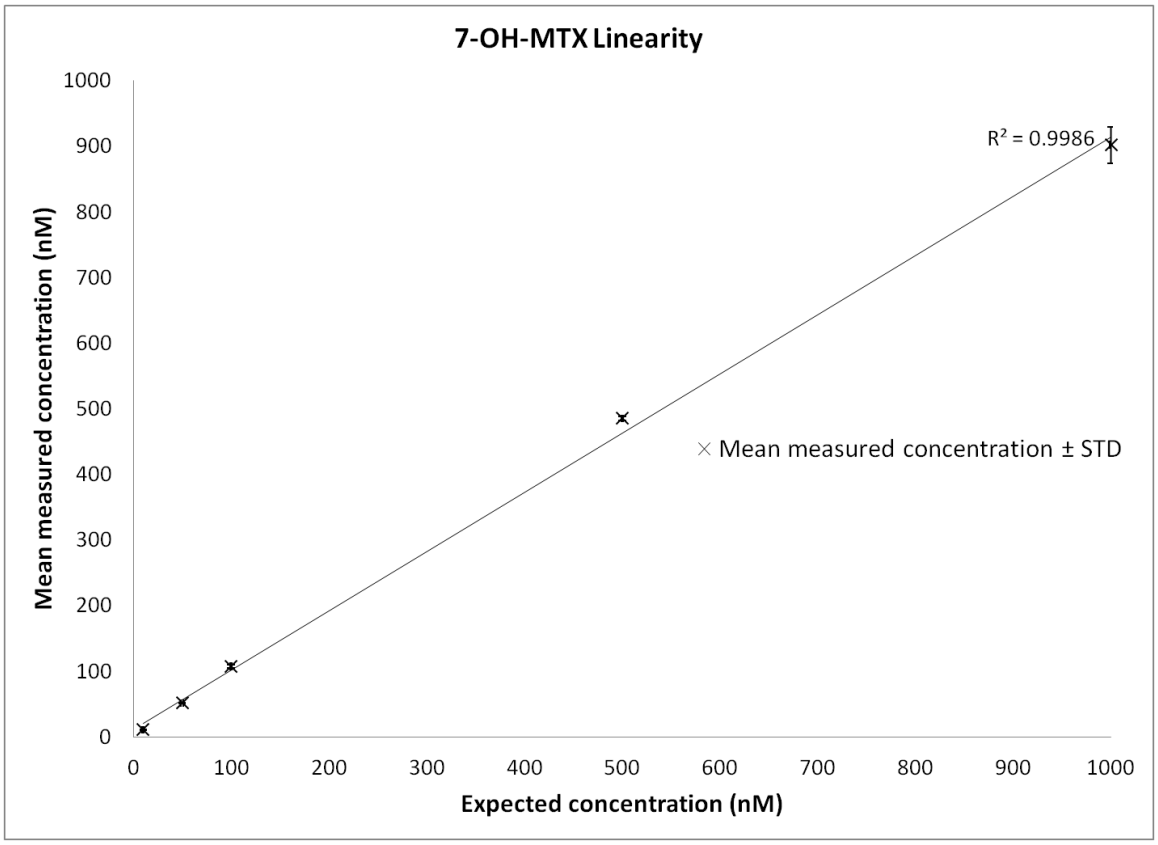


Figure 4-22. Linearity results for 7-OH-MTX showing good linearity between expected concentration and mean measured concentration (n=3). All samples were CV ≤ 15%.

4.3.4 Precision

The EMA guidelines require that for the validation of the within-run precision, there should be a minimum of five samples per concentration level at LLOQ, low, medium and high QC samples. The within-run CV value should not exceed 15% for the QC samples, except for the LLOQ which should not exceed 20%. Table 4-14 shows the measured concentration following injection of samples (n=5) that contain 5 nM, 10 nM, 50 nM, 500 nM and 1000 nM MTX/7-OH-MTX and 50 nM of the internal standard MTX-d_3.

Expected MTX and 7-OH-MTX concentration (nM)	Mean Measured MTX concentration (nM)	MTX CV (%)	Mean Measured 7-OH-MTX concentration (nM)	7-OH-MTX CV (%)	MTX-d3 CV (%)
5	4.24	5	< LLOQ	< LLOQ	4
10	11.16	3	10.60	9	15
50	44.65	2	50.04	4	5
500	507.41	2	444.63	2	53[a]
1000	1024.58	2	893.32	2	5

Table 4-14. Intraday precision testing for MTX and 7-OH-MTX in urine samples (n=5). [a] MTX/7-OH-MTX and MTX-d3 response significantly lower in one sample but this did not affect the overall CV of MTX/7-OH-MTX as the ratio between analyte and internal standard remained similar.

4.3.5 Matrix Effects

Comparing injection of 50nM MTX/7-OH-MTX/MTX-d_3 spiked into urine after protein precipitation and water revealed that there was significant ion suppression of MTX and MTX-d_3 in urine as shown in Figure 4-23. It is expected that MTX and MTX-d_3 would be affected in a similar way by matrix effects due to their similar structure. The ion suppression results in the ratio of 7-OH-MTX:MTX-d_3 being falsely raised causing the calculated concentration of 7-OH-MTX to be systematically inflated. This indicates that for sample processing in the MEMO study that the calibration line must be in urine. However, for assay optimisation samples where the experiment is investigating percentage change of MTX/7-OH-MTX levels over time (i.e. stability and recovery) the calibration line was completed in water whilst awaiting results of the linearity and accuracy experiments.

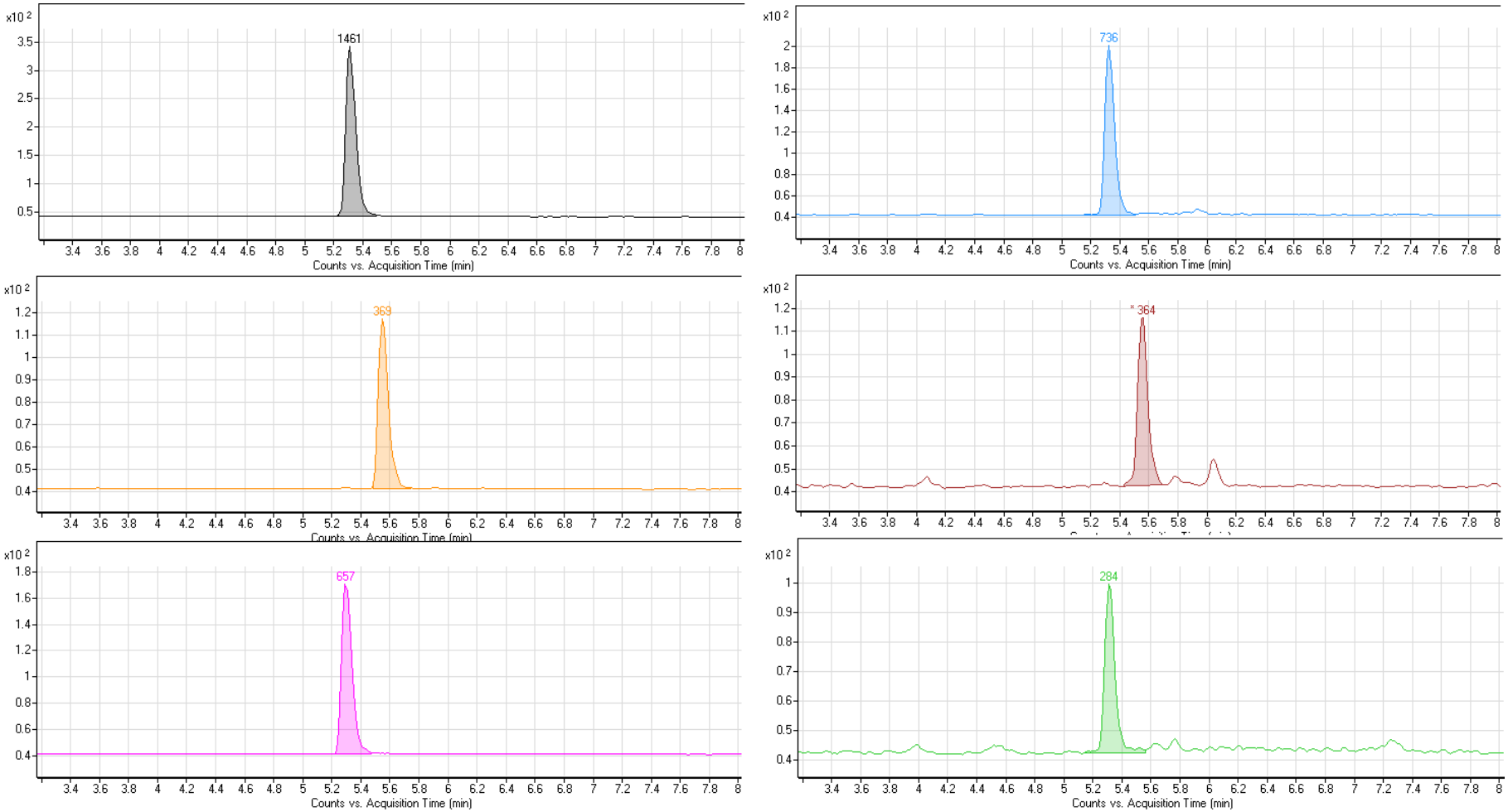


Figure 4-23. Chromatogram results following injection of 50 nM MTX (top), 7-OH-MTX (middle) and MTX-d_3 (bottom) in water (left) versus urine protein precipitated and subsequently spiked (right). Note that the AUC for 7-OH-MTX is similar for water and urine but for both MTX and MTX-d_3 the AUC is approximately halved. This has no effect on the calculated concentration of MTX but falsely inflates the calculated concentration of 7-OH-MTX.

4.3.6 Recovery

Protein precipitation was undertaken using ACN as the organic solution as it was shown that in water protein precipitation with ACN produced the highest recovery. Recovery was performed by comparing the measured concentration of MTX and 7-OH-MTX in samples that were spiked with MTX/7-OH-MTX/MTX-d_3 and underwent the sample preparation protocol to analyte-free urine samples that went through the sample preparation protocol with no spiked analyte and were subsequently spiked at rehydration. The following concentrations were prepared in triplicate of MTX/7-OH-MTX: 5 nM, 10 nM, 25 nM, 50 nM, 100 nM, 250 nM, 500 nM, 750 nM and 1000 nM. Figure 4-24 demonstrates an example chromatogram for a sample spiked with 250 nM MTX/7-OH-MTX and 50 nM MTX-d_3 and underwent the sample preparation protocol compared to a blank urine sample that underwent the sample preparation protocol and was subsequently spiked. Table 4-15 and Table 4-16 present the results of the recovery experiment in urine.

It can be seen from Table 4-16 that the mean measured concentrations of 7-OH-MTX are higher than would be expected. This is due to the calibration curve being in water which is not susceptible to the effect of ion suppression as there are no matrix effects. In urine there are other molecules which may cause ion suppression of MTX/MTX-d_3 which reduces the ionisation efficiency, thereby falsely increasing the 7-OH-MTX:MTX-d_3 ratio inflating the calculated concentration (Figure 4-23). This does not affect the calculation of extraction recovery however as the two cohort samples were tested using the same calibration curve and are therefore subject to the same systematic error.

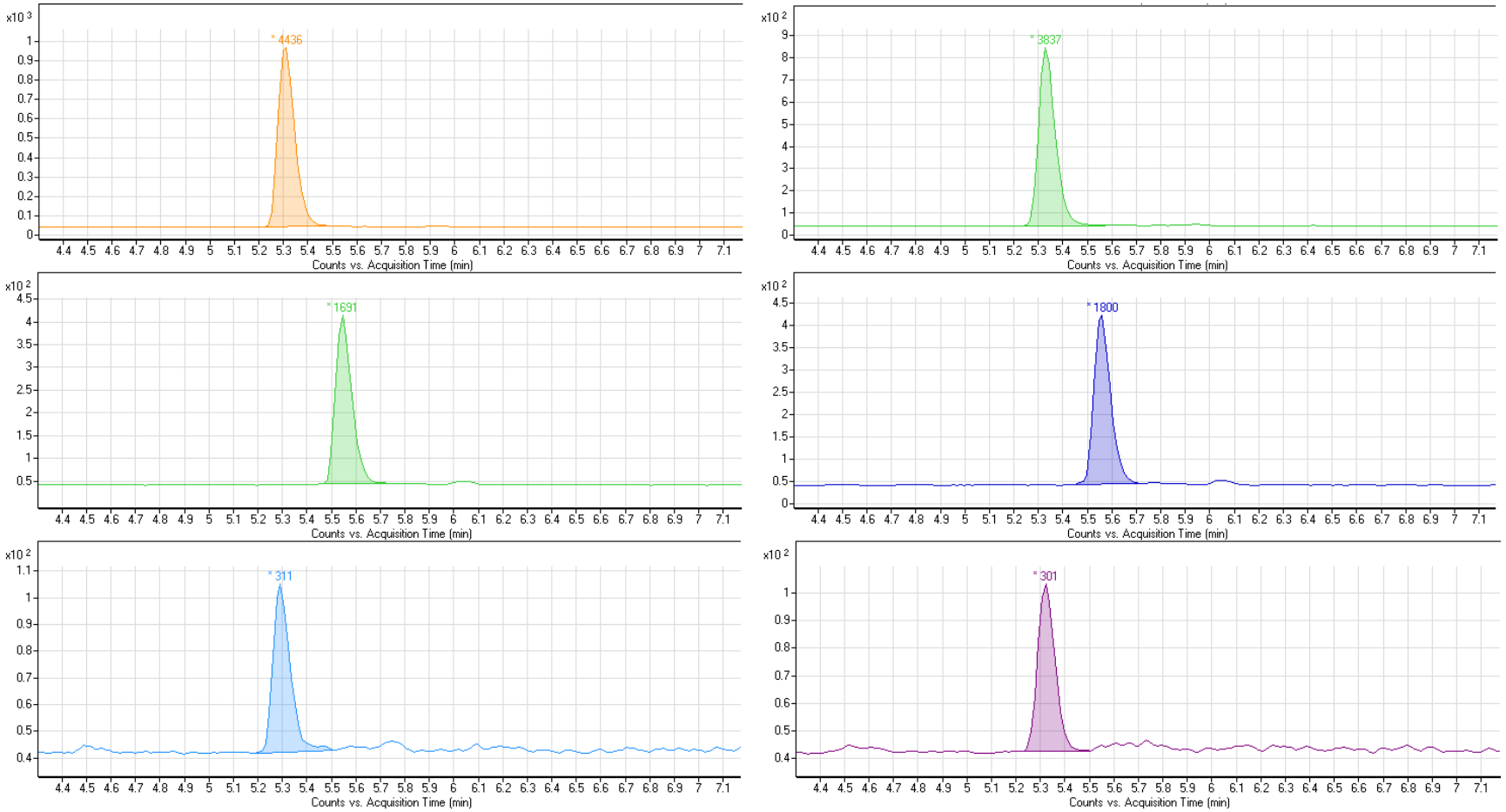


Figure 4-24. Chromatograms obtained following spiking of urine prior to protein precipitation (left) or protein precipitation of urine followed by spiking (right) of samples with 250 nM MTX (top)/7-OH-MTX (middle) and 50 nM MTX-d_3 (bottom). Chromatogram peaks demonstrate that there is no significant alteration in peak area for the analytes.

Expected concentration (nM)	Protein precipitation then spike	Spike then protein precipitation	Extraction recovery (%)
	Mean measured concentration (nM) (CV%)	Mean measured concentration (nM) (CV%)	
5	4.99 (3)	6.05 (12)	121
10	10.44 (9)	12.80 (4)	123
25	24.29 (22)	28.81 (4)	119
50	46.99 (14)	59.20 (10)	126
100	96.99 (20)	100.83 (6)	104
250	254.72 (13)	297.75 (6)	117
500	483.42 (16)	572.83 (7)	118
750	739.23 (4)	766.15 (8)	104
1000	864.81(7)	1122.00 (2)	130

Table 4-15. Results of recovery experiment in urine for MTX.

Expected concentration (nM)	Protein precipitation then spike	Spike then protein precipitation	Extraction recovery (%)
	Mean measured concentration (nM) (CV%)	Mean measured concentration (nM) (CV%)	
10	25.69 (2)	21.96 (26)	85
25	39.68 (4)	48.40 (14)	122
50	105.30 (14)	98.20 (22)	93
100	214.93 (11)	154.78 (17)	72
250	478.09 (18)	445.25 (4)	93
500	1046.40 (31)	701.92 (22)	67
750	1223.82 (14)	982.96 (18)	80
1000	1574.54 (12)	1210.99 (10)	77

Table 4-16. Results of recovery experiment in urine for 7-OH-MTX.

4.3.7 Stability

Table 4-17 and Table 4-18 show the stability and loss of MTX and 7-OH-MTX following storage of urine samples at room temperature and -80°C and subsequent sample preparation.

Time point (h)	3		72	
Expected concentration (nM)	**Mean MTX loss (%)**	**Mean 7-OH-MTX loss (%)**	**Mean MTX loss (%)**	**Mean 7-OH-MTX loss (%)**
5	12	< LLOQ	31	< LLOQ
10	-12	-14	-4	9
50	-14	-14	-9	6
500	-6	-20	26	28[a]
1000	5[a]	7[a]	20[a]	33
Mean loss for this time point (%)	-3	-10	13	19

Table 4-17. MTX and 7-OH-MTX stability testing results for samples stored at room temperature demonstrating significant loss of MTX at 72 hours (n=3). Samples CV ≤ 15% except[a]

Time point (h)	3		72		168	
Expected concentration (nM)	**Mean MTX loss (%)**	**Mean 7-OH-MTX loss (%)**	**Mean MTX loss (%)**	**Mean 7-OH-MTX loss (%) (SD)**	**Mean MTX loss (%) (SD)**	**Mean 7-OH-MTX loss (%) (SD)**
5	-21	< LLOQ	0	< LLOQ	-4	< LLOQ
10	-15	-22	1	0	-4	-2
50	-26	-29	-7	-8	-16	-13
500	-33	-32	-19	-11	-18	-10
1000	-6	-6	-13	1	-23	-16
Mean loss for this time point (%)	-20	-22	-8	-4	-13	-10

Table 4-18. MTX and 7-OH-MTX stability testing for samples stored at -80 °C demonstrating no significant loss of 7-OH-MTX at 168 hours (n=3). Samples CV ≤ 15%.

Room temperature samples were not extended to 168 hours due to significant loss of 7-OH-MTX at 72 hours. However, storage of samples at -80°C for up to one week showed no appreciable losses of MTX or 7-OH-MTX.

4.3.8 Summary of Results

The results of the presented experiments have led to the development of an assay that can measure MTX and 7-OH-MTX in urine using the requirements set out by the EMA as a benchmark. Table 4-19 summarises the EMA requirements which have passed full validation and those which require further development.

EMA Requirements	Results of Validation Work in Urine
Selectivity	Requires six separate sources of a blank matrix to be assessed for interfering components
Carry-over	Passes EMA requirements
Calibration curve	Passes EMA requirements
Accuracy	
Within-run	Requires five samples per level
Between-run	Not evaluated
Precision	
Within-run	Passes EMA requirements
Between-run	Not evaluated
Dilution integrity	Not evaluated
Matrix effect	Not evaluated
Stability	Requires freeze-thaw effects to be assessed

Table 4-19. Summary of the EMA requirements for bioanalytical method validation which have been passed or where further work is required in urine.

Initial experiments confirmed that there was significant ion suppression of MTX and MTX-d_3 which determined that calibration curves need to be undertaken with the matrix of investigation for the MEMO and RAMS studies. The LLOQ for MTX and 7-OH-MTX is 5 nM and 10 nM respectively with less than 20% carryover of the LLOQ after a high concentration injection as advised by the EMA. The assay has demonstrated good linearity and precision as advised by the EMA. Protein precipitation adversely affected 7-OH-MTX recovery compared to MTX; for that reason, the LLOQ for 7-OH-MTX is higher than MTX due to reduced recovery at the LLOQ and increased signal to noise ratio. This indicates that MTX may be the better analyte for detecting adherence with the current assay in urine. Stability testing of MTX and 7-OH-MTX demonstrated that MTX and 7-OH-MTX were stable at room temperature up to 24 hours and at -80°C up to 168 hours. The work has

informed the MEMO study protocol for sample processing and storage to ensure minimal loss of analyte over storage time and maximum recovery.

4.4 Assay Optimisation and Validation in Plasma

In order to measure samples from the MEMO study, the assay required validation in plasma.

4.4.1 ACN or Methanol as Organic Solvent for Protein Precipitation

The percentage of recovery obtained comparing ACN and methanol as the organic solvent for protein precipitation was investigated in order to determine the ideal organic solvent for future experiments. The results are shown in Table 4-20. Based on these results it was decided that methanol was the ideal organic solvent for protein precipitation as ACN tended to overinflate the recovery for 7-OH-MTX with reduced overall accuracy.

Expected MTX/7-OH-MTX concentration (nM)	Mean MTX recovery in ACN (%)	Mean MTX Recovery in methanol (%)	Mean 7-OH-MTX recovery in ACN (%)	Mean 7-OH-MTX recovery in methanol (%)
2.5	120	127	108	117
5.0	119	113	104	112
10	114	107	106	96
50	89	83	120	105
100	85	81	118	104
500	99	94	103	101
750	99	92	122	102
1000	96	91	120	104

Table 4-20. Recovery of MTX and 7-OH-MTX in plasma samples following the protein precipitation protocol using either ACN or methanol as the organic solvent.

4.4.2 Lower Limit of Quantification and Carryover

4.4.2.1 LLOQ

Samples were protein precipitated with methanol and the LLOQ was determined. The LLOQ for MTX with a SNR ≥ 5 was 0.5 nM as shown in Figure 4-25.

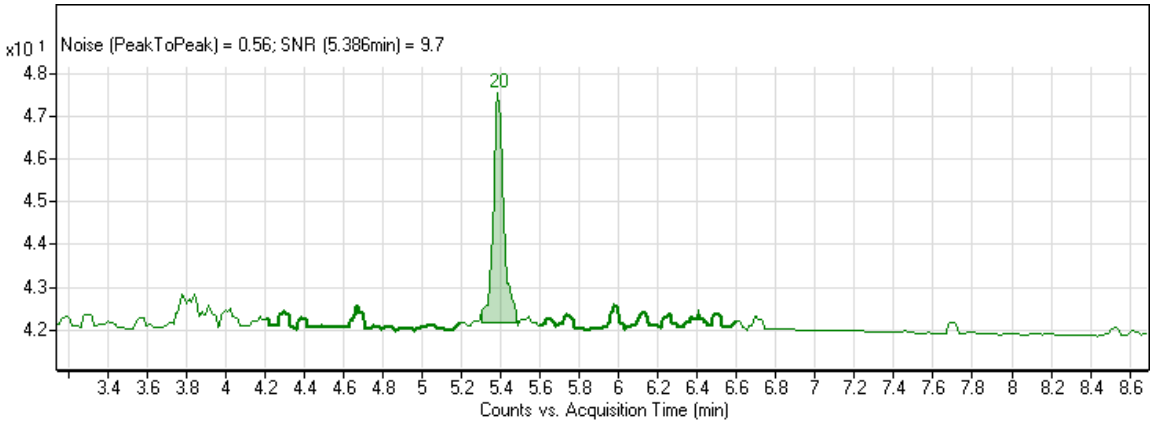


Figure 4-25. Chromatogram following injection of 0.5 nM MTX in plasma following the sample preparation protocol with SNR=9.7.

Similar to the results in urine, following injection of a blank plasma sample which underwent the sample preparation protocol, a peak in the chromatogram was observed (Figure 4-26). In order to determine whether the peak was due to matrix effects or contamination of 7-OH-MTX with blank plasma, plasma was spiked with increasing concentrations of 7-OH-MTX. The resultant chromatograms showed that the peak seen in the blank samples did not increase with increasing 7-OH-MTX and was therefore due to matrix effects and regarded as background noise. The second peak was therefore disregarded for chromatogram analysis (Figure 4-26). The lower LLOQ in plasma compared to urine may be due to cleaner sample preparation. During sample preparation

of plasma, the plasma pellet at the base of the eppendorf visibly formed and the supernatant could be pipetted with ease. During sample preparation of urine, there was no clear pellet and therefore there is the possibility that some of the particulate remained during collection of the supernatant, increasing noise.

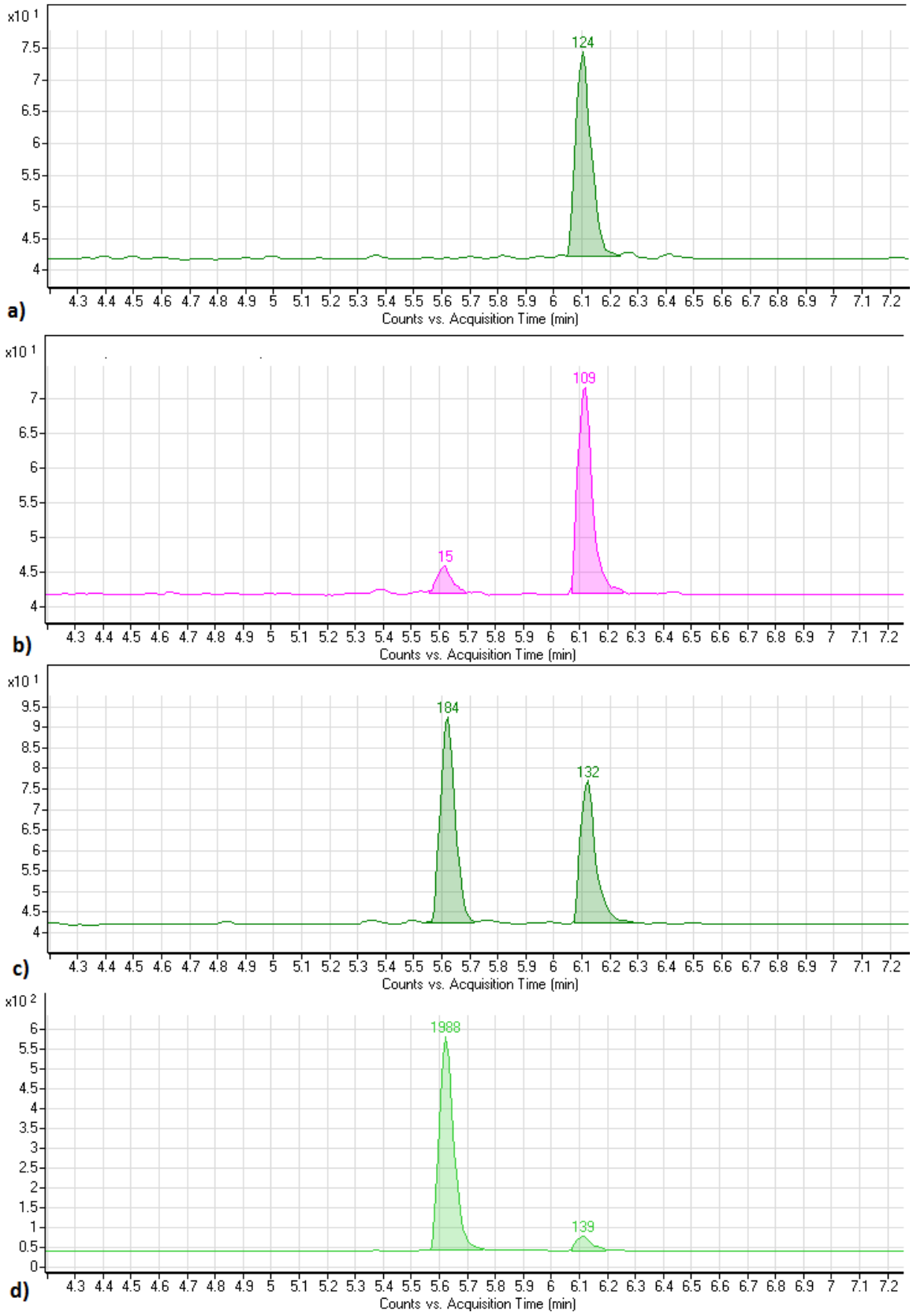


Figure 4-26. Chromatograms obtained for 7-OH-MTX following blank injection of plasma (a), 0.75 nM (b), 10 nM (c) and 100 nM (d) of 7-OH-MTX. The peak with retention time 6.1 minutes does not alter indicating that this is not 7-OH-MTX and is due to matrix effect.

Calculation of SNR for 7-OH-MTX revealed a LLOQ of 0.75 nM (Figure 4-27).

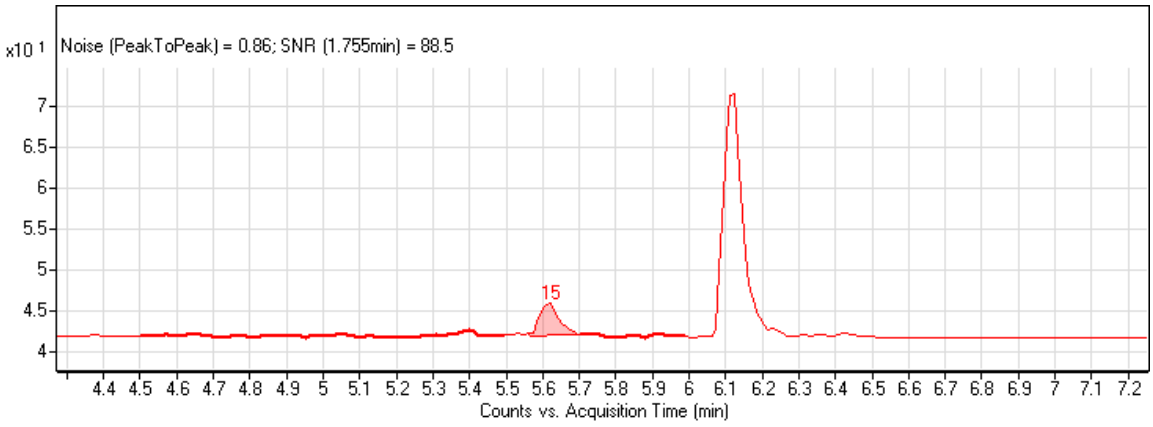


Figure 4-27. Chromatogram following injection of 0.75 nM 7-OH-MTX in plasma following the sample preparation protocol with SNR=88.5.

4.4.2.2 Carryover

Carryover was assessed by injection of 1000 nM MTX/7-OH-MTX followed by a blank urine sample. Carryover was acceptable if less than 20% of the LLOQ. Figure 4-28 and Figure 4-29 show the results of the carryover experiment.

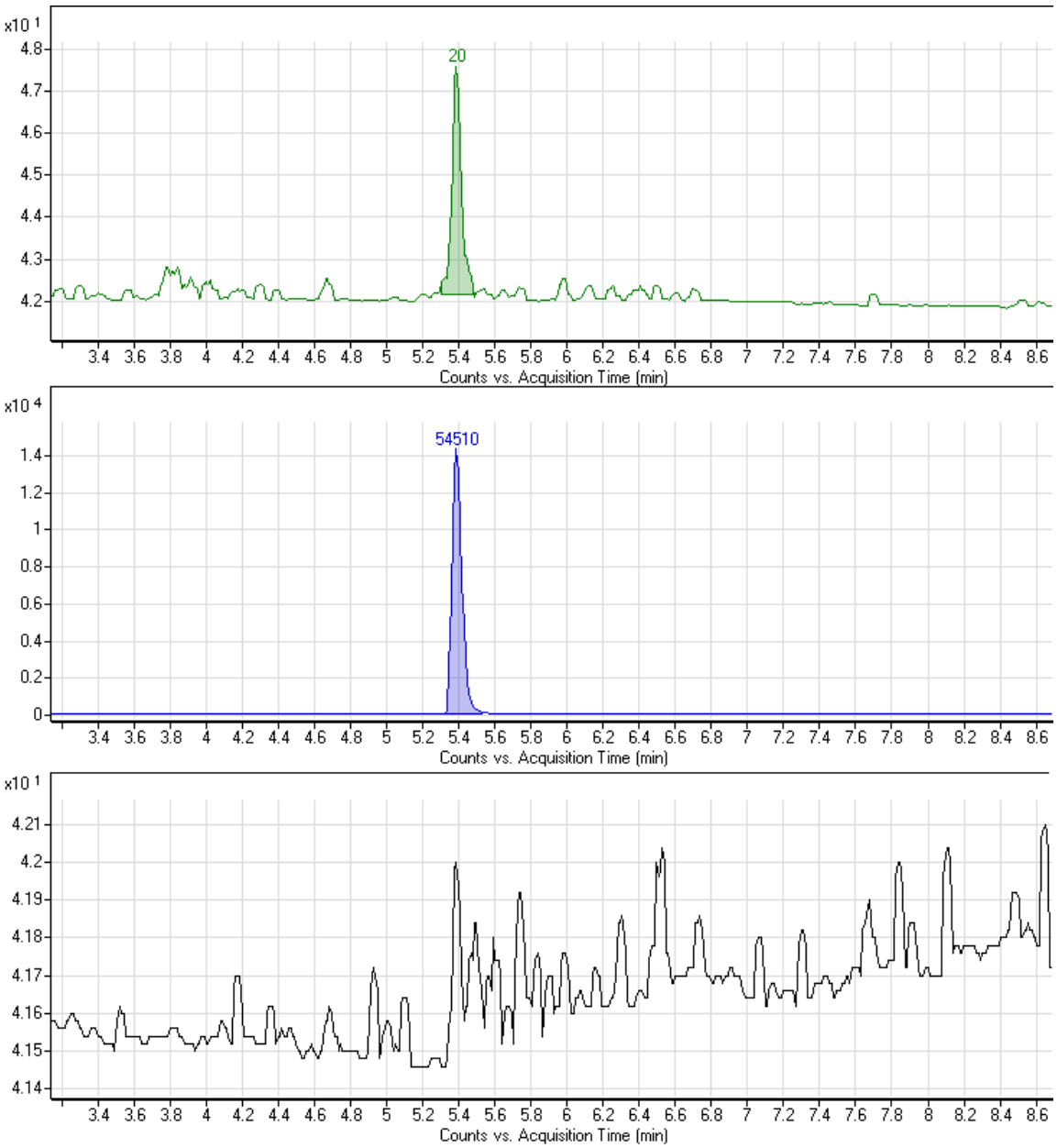


Figure 4-28. Carryover of MTX as assessed by injection of 1000 nM MTX (middle) followed by injection of a blank plasma sample (bottom). Automatic integration failed to detect a peak in the blank sample due to inadequate variation in the amplitude. The LLOQ chromatogram is shown for comparison (top).

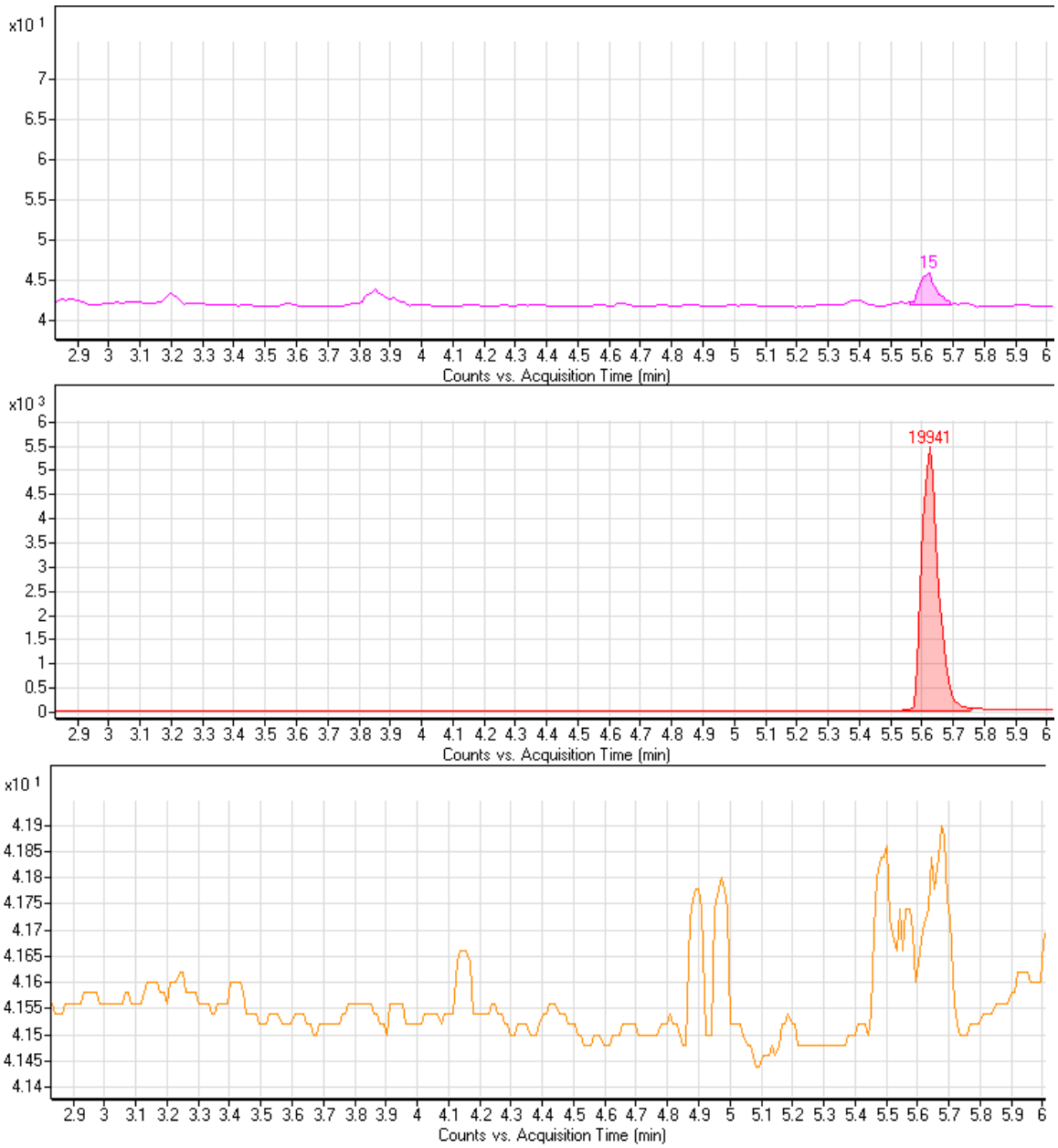


Figure 4-29. Carryover of 7-OH-MTX as assessed by injection of 1000 nM MTX (middle) followed by injection of a blank plasma sample (bottom). The LLOQ chromatogram is shown for comparison (top).

4.4.3 Accuracy

Table 4-21 shows the results from accuracy testing for MTX and 7-OH-MTX (n=3). The mean accuracy was within 15% of the expected concentration as advised by the EMA demonstrating adequate accuracy of the assay.

Expected MTX/7-OH-MTX Concentration (nM)	Mean MTX measured concentration (nM)	CV (%)	Mean accuracy (%)	Mean 7-OH-MTX measured concentration (nM)	CV (%)	Mean accuracy (%)
0.50	0.49	14	97	< LLOQ		
0.75	0.71	14	94	0.82	9	109
1.00	0.78	7	78	0.92	12	92
2.50	2.73	15	109	2.27	9	91
5.00	5.40	4	108	4.87	4	97
10.0	10.91	2	109	9.77	6	98
25.0	21.73	1	87	25.28	2	101
50.0	43.78	7	88	49.95	5	100
100	84.43	2	84	101.74	2	102
250	275.54	3	110	241.62	2	97
500	542.67	0	109	506.69	0	101
750	848.53	1	113	811.89	1	108
1000	1062.71	3	106	1037.80	2	104

Table 4-21. Results of accuracy testing for MTX and 7-OH-MTX samples in plasma.

4.4.4 Linearity

Linearity was calculated from Table 4-21 for MTX and 7-OH-MTX. Figure 4-30 and Figure 4-31 present the linearity results plotting the mean measured concentration against expected concentration the standard deviation and the R^2 test statistic for MTX and 7-OH-MTX respectively. Results were log-transformed to reduce crowding of results at the lower concentration levels and improve visual inspection. These results demonstrate that the assay is linear within the concentrations tested.

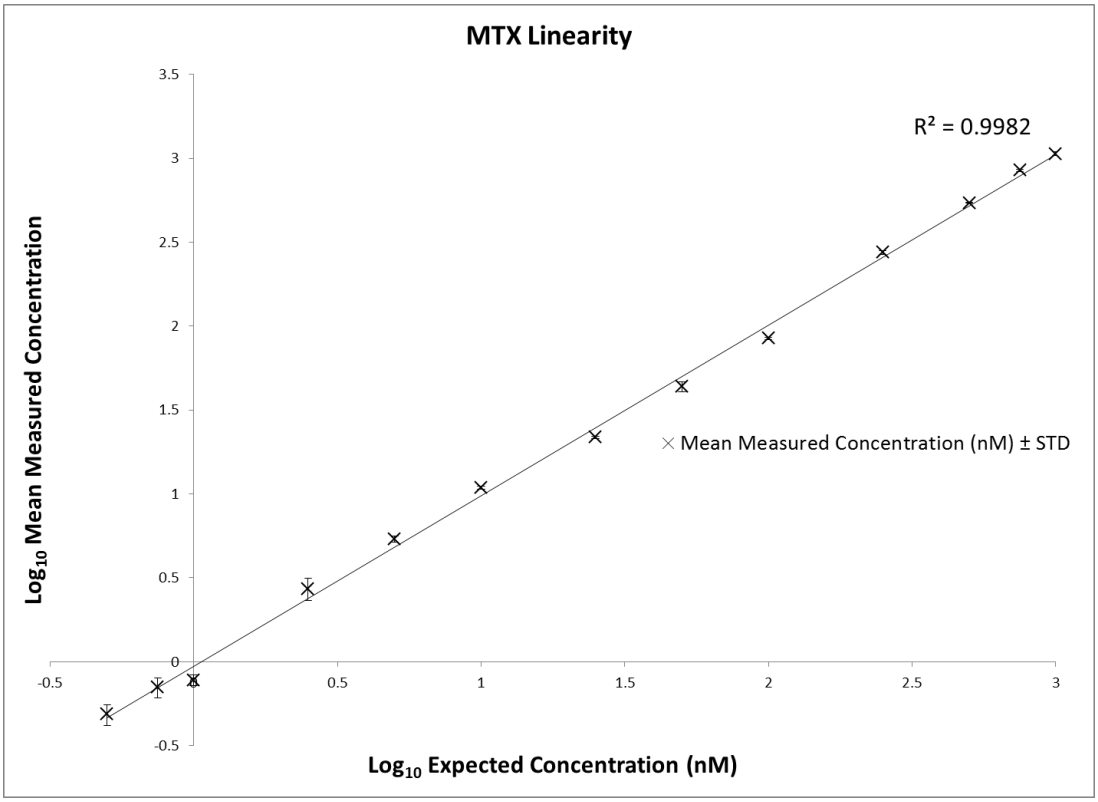


Figure 4-30. Linearity results for MTX showing good linearity between expected concentration and mean measured concentration (n=3). All samples were CV ≤ 15%.

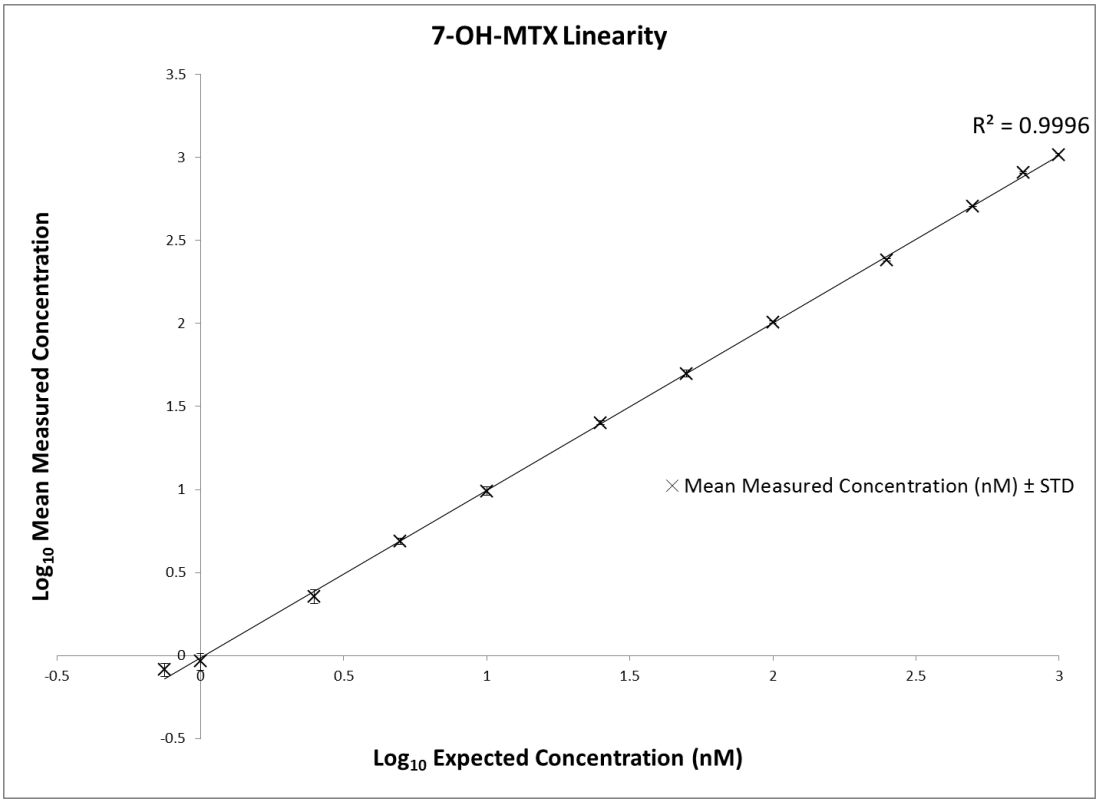


Figure 4-31. Linearity results for 7-OH-MTX showing good linearity between expected concentration and mean measured concentration (n=3). All samples were CV ≤ 15%.

4.4.5 Precision

Table 4-22 shows the results of the precision experiment.

Expected Concentration (nM)	MTX measured concentration (nM)	MTX CV (%)	7-OH-MTX measured concentration (nM)	7-OH-MTX CV (%)	MTX-d_3 CV (%)
0.50	0.42	12	< LLOQ		2
0.75	0.64	13	1.26	21	2
25.0	21.10	3	25.96	4	1
500	550.01	5	476.56	10	79[a]
750	830.24	1	838.61	1	1

Table 4-22. Intraday precision testing for MTX and 7-OH-MTX in plasma samples (n=5). [a] MTX/7-OH-MTX and MTX-d_3 response significantly lower in one sample but this did not affect the overall CV of MTX/7-OH-MTX as the ratio between analyte and internal standard remained similar.

4.4.6 Recovery

4.4.6.1 Recovery of MTX/7-OH-MTX

Protein precipitation was undertaken using methanol as the organic solution as it was shown that protein precipitation with methanol produced a better overall recovery. Recovery was performed by comparing the measured concentration of MTX and 7-OH-MTX in samples that were spiked with MTX/7-OH-MTX/MTX-d_3 and underwent the sample preparation protocol to analyte-free plasma samples that went through the sample preparation protocol and were subsequently spiked at rehydration. Chromatograms following injection of samples spiked that subsequently underwent the sample preparation protocol and chromatograms following injection of samples that underwent the sample preparation protocol and then spiked with 250 nM MTX/7-OH-MTX and 50 nM MTX-d_3 are shown in Figure 4-32. The majority of extraction recovery is within 15% except for two concentration levels (Table 4-23 and Table 4-24).

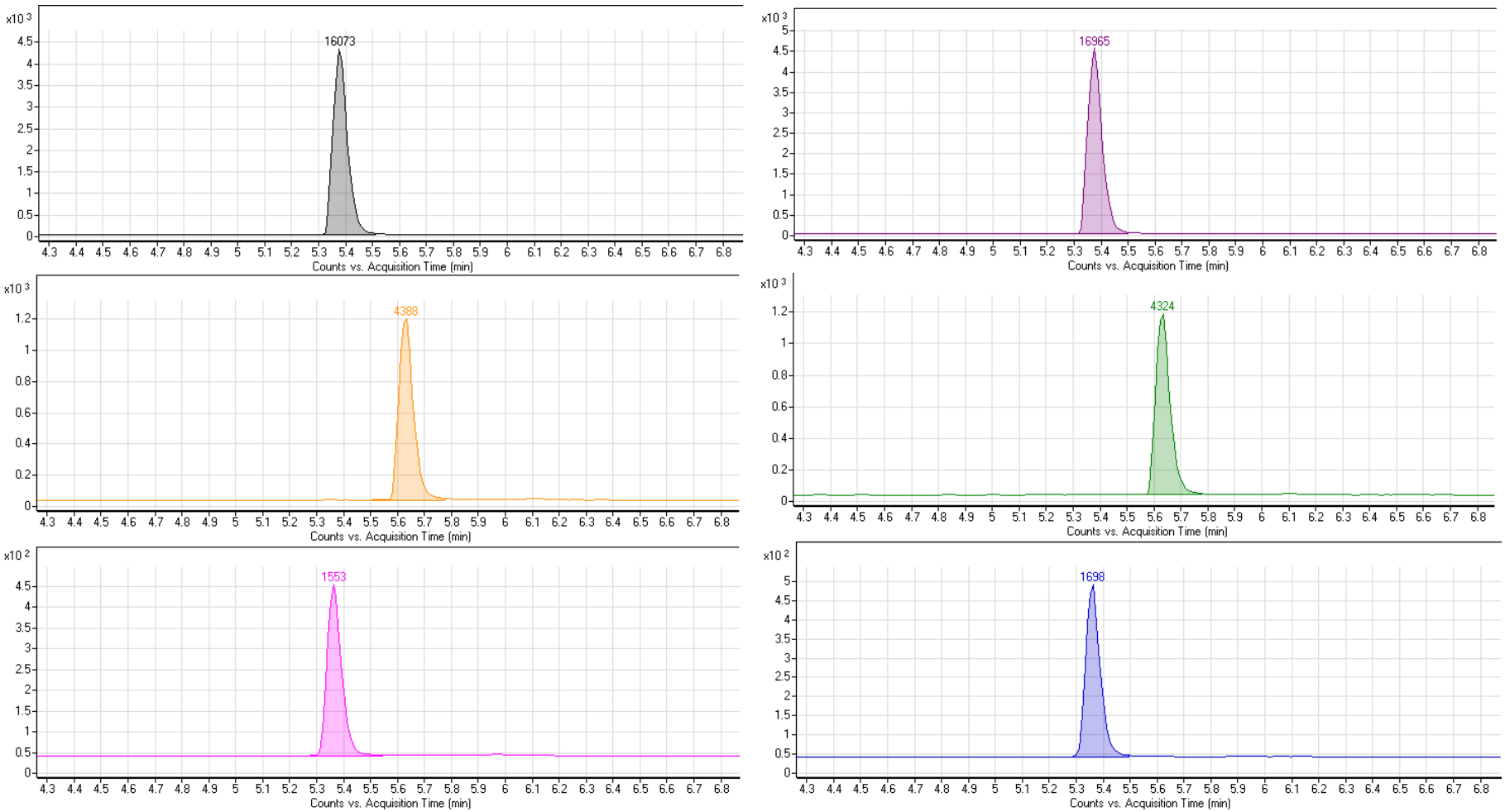


Figure 4-32. Chromatograms obtained following spiking of plasma prior to protein precipitation (left) or protein precipitation of plasma followed by spiking (right) of samples with 250 nM MTX (top)/7-OH-MTX (middle) and 50 nM MTX-d_3 (bottom). Chromatogram peaks demonstrate that there is no significant alteration in peak area for the analytes.

Expected concentration (nM)	Protein precipitation then spike	Spike then protein precipitation	Extraction recovery (%)
	Mean measured concentration (nM) (CV%)	Mean measured concentration (nM) (CV%)	
1.0	0.79 (21)	0.79 (10)	100
2.5	2.28 (8)	2.49 (3)	109
5.0	4.1 (2)	4.87 (10)	119
100	102.93 (3)	72.51 (2)	70
250	259.1 (4)	257.82 (1)	100
750	737.14 (13)	710.3 (1)	96
1000	1012.08 (6)	940.71 (1)	93

Table 4-23. Results of the recovery experiment in plasma for MTX.

Expected concentration (nM)	Protein precipitation then spike	Spike then protein precipitation	Extraction recovery (%)
	Mean measured concentration (nM) (CV%)	Mean measured concentration (nM) (CV%)	
1.0	1.32 (2)	1.71 (9)	129
2.5	3.18 (2)	3.21 (9)	101
5.0	5.42 (3)	5.76 (3)	106
100	103.48 (4)	101.9 (4)	98
250	252.25 (3)	261.77 (3)	104
750	757.97 (15)	736.71 (1)	97
1000	1080.73 (5)	1016.08 (1)	94

Table 4-24. Results of the recovery experiment in plasma for 7-OH-MTX.

4.4.7 Stability

Table 4-25 shows the stability and loss of MTX and 7-OH-MTX following storage of plasma samples at -80°C and subsequent sample preparation. As is shown, at 28 days there is a loss ≥ 15% for 1 nM MTX but the other concentrations are stable with loss ± 15% indicating that the samples in the higher concentrations are stable up to 28 days.

Time point (days)	3		28	
Expected concentration (nM)	**Mean MTX loss (%)**	**Mean 7-OH-MTX loss (%)**	**Mean MTX loss (%)**	**Mean 7-OH-MTX loss (%)**
1.0	8	-1	29[a]	1
2.5	0	-9	-15	0[a]
5.0	-5	-9	-1	5
100	-1	-18	-3	12
250	3	-16	0	15
750	5	-17	-2	12
Mean loss for this time point (%)	2	-12	-4	9

Table 4-25. MTX and 7-OH-MTX stability testing for samples in plasma stored at -80 °C demonstrating no significant loss of MTX/7-OH-MTX at 168 hours (n=3). Samples CV ≤ 15% except[a].

4.4.8 Summary of Assay Optimisation and Validation in Plasma

The results of these experiments have demonstrated that the assay is adequate for use as a research tool to measure MTX and 7-OH-MTX levels in plasma. Table 4-26 summarises the EMA requirements which have passed full validation and those which require further development.

EMA Requirements	Results of Validation Work in Plasma
Selectivity	Requires six separate sources of a blank matrix to be assessed for interfering components
Carry-over	Passes EMA requirements
Calibration curve	Passes EMA requirements
Accuracy	
Within-run	Requires five samples per level
Between-run	Not evaluated
Precision	
Within-run	MTX passes EMA requirements
Between-run	Not evaluated
Dilution integrity	Not evaluated
Matrix effect	Not evaluated
Stability	Requires freeze-thaw effects to be assessed

Table 4-26. Summary of the EMA requirements for bioanalytical method validation which have been passed or where further work is required in plasma.

The LLOQ of MTX and 7-OH-MTX was 0.5 nM and 0.75 nM, respectively and the same as the LLOQ in water with carryover less than 20% of the LLOQ after injection of 1000 nM MTX/7-OH-MTX as required by the EMA. The lower LLOQ in plasma compared to urine may be due to cleaner sample

preparation in plasma compared to urine. Accuracy testing revealed that across the calibration range the mean accuracy was within 15% of the expected concentration as advised by the EMA. The assay demonstrated good linearity and acceptable precision across the calibration range. Comparing ACN with methanol for protein precipitation demonstrated that methanol is the preferred organic solvent with improved recovery. The recovery of MTX/7-OH-MTX was within 15% except for one concentration for MTX and 7-OH-MTX demonstrating acceptable recovery of analytes from plasma across the calibration range. Testing the stability of the analytes in plasma at -80°C revealed that the analytes were stable in plasma up to 28 days and that samples collected in the future and frozen could be stored for an extended period prior to testing.

4.5 Assay Validation in Whole Blood

4.5.1 Recovery

Chromatograms obtained following injection of 250 nM MTX/7-OH-MTX and 50 nM MTX-d_3 that were prepared in plasma from the NHS Blood and Transplant service and whole blood obtained from a healthy volunteer are shown in Figure 4-33. As can be seen, there is a reduction in MTX/MTX-d_3 AUC going from plasma to whole blood. This may be due to the whole blood not being pooled but from one volunteer and is suggestive that there may be inhibiting factors affecting the ionisation of MTX/MTX-d_3 but not 7-OH-MTX in the whole blood sample. An alternative explanation is that this is the result of different stock concentrations for plasma and whole blood but it would be expected that MTX-d_3 would be unaffected as the same MTX-d_3 stock was used to spike the plasma calibration and plasma obtained from the single volunteer. This effect has increased the overall recovery seen in 7-OH-MTX but not MTX as shown in Table 4-27. These results suggest that MTX may be the better analyte to measure for adherence due to recovery for MTX being closer to 100% as it can be corrected for by the presence of MTX-d_3.

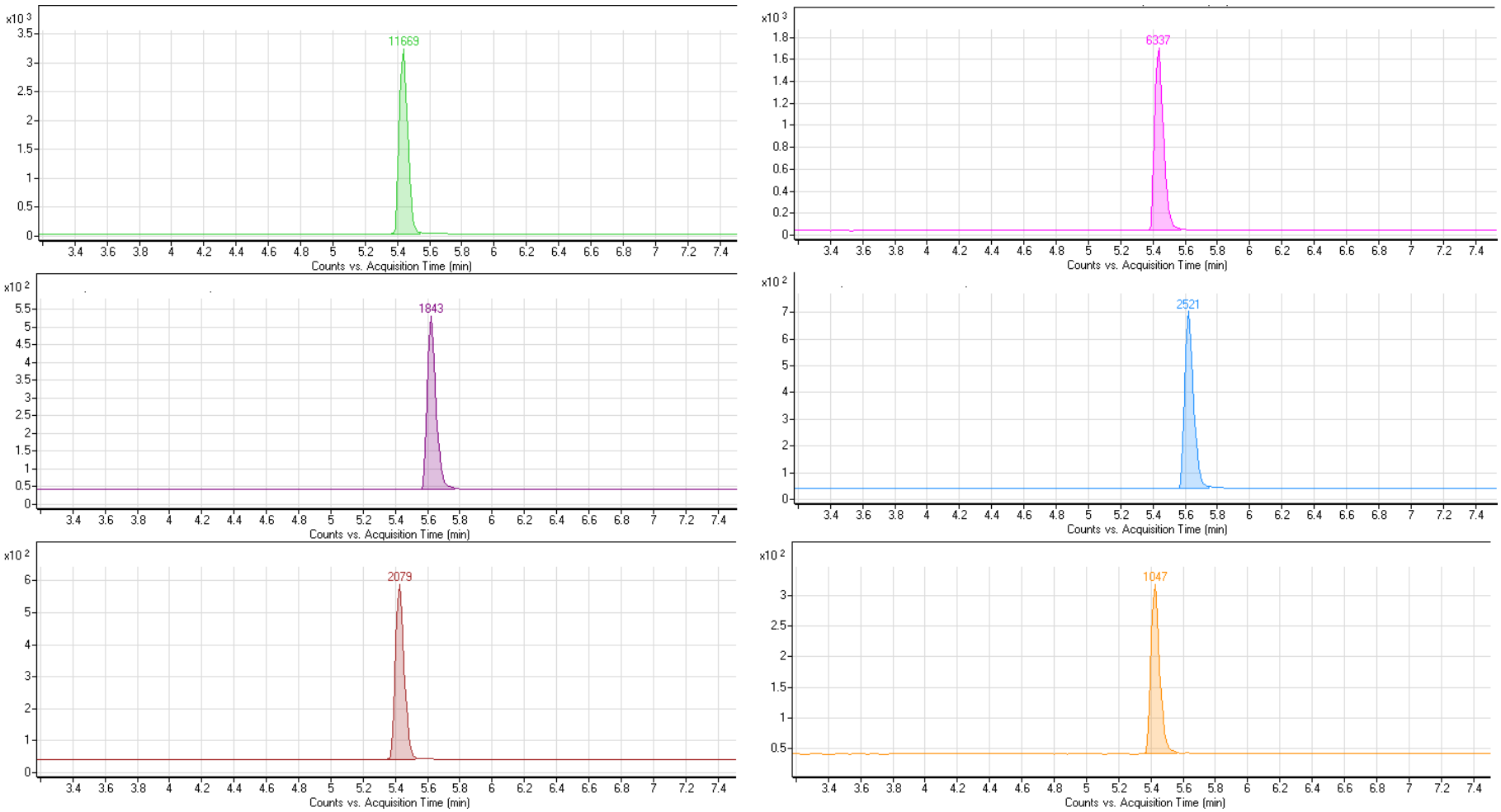


Figure 4-33. Chromatograms obtained following spiking of plasma (left) or spiking of whole blood (right) of samples with 250 nM MTX (top)/7-OH-MTX (middle) and 50 nM MTX-d_3 (bottom). Chromatogram peaks demonstrate that there is significant reduction in peak area for MTX/MTX-d_3 but not for 7-OH-MTX.

Expected concentration (nM)	Mean measured MTX concentration from whole blood (nM) (CV%)	MTX extraction recovery (%)	Mean measured 7-OH-MTX concentration from whole blood (nM) (CV%)	7-OH-MTX extraction recovery (%)
5	3.55 (5)	71	8.65 (6)	173
100	66.13 (8)	66	149.54 (7)	150
250	189.72 (10)	76	426.40 (10)	171
500	395.69 (5)	79	930.30 (4)	186
750	512.61 (3)	68	1242.95 (4)	166

Table 4-27. MTX and 7-OH-MTX extraction recovery from whole blood spiked from a single volunteer compared to pooled plasma spiked. Note that there is significant increase in 7-OH-MTX recovery; this may be due to matrix effects due to differences between the volunteer sample and the pooled plasma samples.

4.5.2 Stability

Table 4-28 shows the stability and loss of MTX and 7-OH-MTX following storage of whole blood samples at room temperature for 24 and 48 hours. Overall samples appeared stable with no significant loss except for one concentration level at 48 hours.

Time point (hours)	24		48	
Expected concentration (nM)	**Mean MTX loss (%)**	**Mean 7-OH-MTX loss (%)**	**Mean MTX loss (%)**	**Mean 7-OH-MTX loss (%)**
5	-8	-6	-5	-4
100	-14	-18	-7	-14
250	-1	-4	-4	-10
500	7	5	ND	ND
750	2	-6	23[a]	17[a]
Mean loss for this time point (%)	-3	-4	2	-3

Table 4-28. MTX and 7-OH-MTX stability testing for samples in whole blood stored at room temperature demonstrating overall no significant loss of MTX/7-OH-MTX at 48 hours (n=3). Samples CV ≤ 15% except[a]. ND = MTX/7-OH-MTX/MTX-d_3 not detected due to sample failure.

4.5.3 Summary of Results of Assay Validation in Whole Blood

Results from the whole blood experiments have demonstrated that the recovery of MTX/MTX-d_3 is reduced proportionally compared to 7-OH-MTX in plasma. This is suggestive that an internal standard for 7-OH-MTX may be required but unfortunately there was no commercially available deuterated 7-OH-MTX at the start of this set of experiments. These results therefore suggest that MTX may be the better analyte to measure in plasma for adherence due to the presence of a MTX internal standard which can correct for matrix effects. Samples from RAMS take up to 72 hours to be delivered by post to the laboratory where they are subsequently processed and frozen. It was therefore necessary to test the stability of the analytes in whole blood at room temperature.

Testing stability up to 48 hours demonstrated that there was no appreciable loss of MTX/7-OH-MTX.

4.6 Measurement of MTX and 7-OH-MTX Metabolites in Urine and Blood of Patients with Rheumatoid Arthritis: The MEMO Study

4.6.1 Introduction

Following the development of an assay to measure MTX/7-OH-MTX in urine and plasma it was necessary to develop a pharmacokinetic model using the assay in order to investigate the ability of the assay to measure adherence. The MEMO study was therefore designed and conducted.

4.6.2 Aims of this Section

The specific aims of this section are to:

1. Perform a pharmacokinetic study of MTX and 7-OH-MTX in plasma and urine of patients with RA.
2. Develop a pharmacokinetic model of MTX and 7-OH-MTX in plasma to determine the ability of the HPLC-SRM-MS assay to measure adherence over time.

4.6.3 Methods Relevant to this Section

This was an experimental study to investigate the performance of the HPLC-SRM-MS assay to measure MTX adherence in patients with RA treated with MTX. Following measurement of MTX and 7-OH-MTX levels in urine and plasma, a nonlinear mixed effects population pharmacokinetic model was developed. Simulations of the model were developed to investigate the ability of the assay to measure adherence to MTX.

4.6.4 Recruitment

Twenty patients with RA were successfully recruited fulfilling the recruitment target as shown in Figure 4-34. All participants were recruited from RAMS. Overall there was recruitment of 10% of those initially sent the first information letter. All recruited participants fulfilled the inclusion and exclusion criteria.

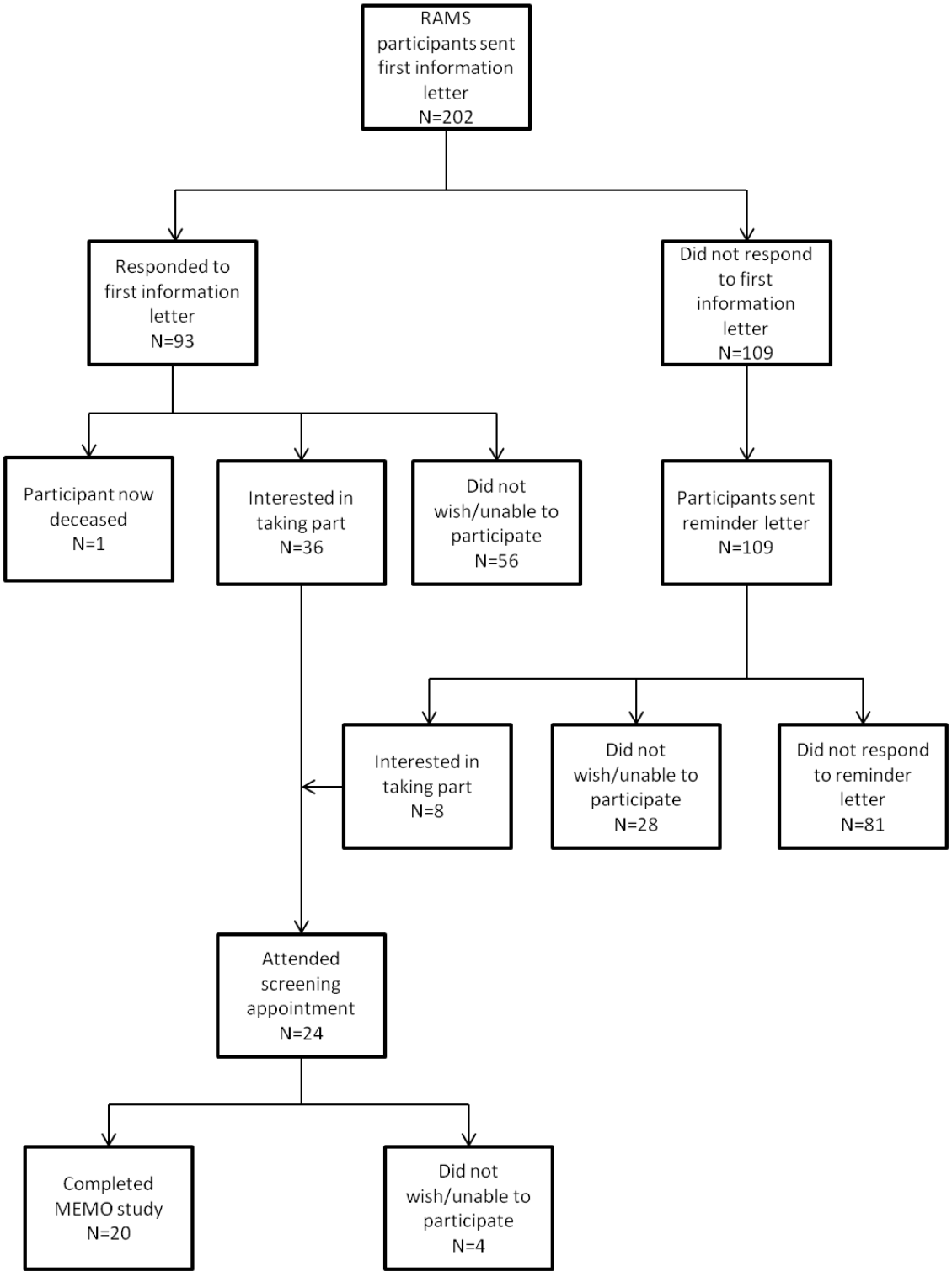


Figure 4-34. Recruitment flow chart for the MEMO study from RAMS.

The baseline characteristics of the MEMO cohort are shown in Table 4-29. The cohort is representative of a general early RA population [285].

Baseline Characteristic	Median (IQR)
Age (years)	65.5 (54-70)
Female gender (%)	65
Weight (kg)	76.9 (67.3-85.4)
Serum creatinine (μM)	71.5 (67.0-79.0)
eGFR (ml/min/1.73m^2)a	84 (76-99)
Serum albumin (g/L)	37 (37 -39)
MTX dose (mg)	15 (7.5-25)[b]
Taking concomitant folic acid (%)	100
Taking concomitant NSAID (%)	20

Table 4-29. Baseline demographic and clinical details for the MEMO cohort. [a] calculated using the MDRD eGFR calculation [b] median (range). GFR=glomerular filtration rate; NSAID=Non-steroidal anti-inflammatory drug.

4.6.5 Urine Results from the MEMO Study

In total 281 urine samples were collected and tested in triplicate (n=843) from 20 patients. The median time from MTX ingestion to last urine sample was 105 hours (IQR: 98-147 hours). Samples taken in the first 24 hours exceeded the calibration curve for MTX levels and required dilution. Dilution of samples was standardised to undiluted, 1 in 50 μl and 1 in 150 μl. Samples were processed in triplicate and the measurement was accepted if CV ≤ 15% and the result was within the calibration curve. Measurements were multiplied by their dilution factor in order to obtain the final concentration. Rejection of samples due to high CV or concentration < LLOQ for MTX and 7-OH-MTX was 6% and 30%, respectively. As can be seen in Table 4-30, no MTX measurement was rejected due to it being below the LLOQ except in one patient where 12 samples failed to be measured due to HPLC-SRM-MS shutdown; urine samples from this patient were therefore excluded from further analysis. Table 4-31 illustrates the time from MTX ingestion to urination when MTX and 7-OH-MTX could last be detected for each patient. These results indicate that MTX is the preferred measurement for adherence and scatter graphs for each MTX dose level showing concentration of creatinine-corrected MTX/7-OH-MTX over time are shown (Figure 4-35 and Figure 4-36).

Patient ID	Time from ingestion to last urination (hours)	Number of samples	MTX CV ≥ 15% n (%)	7-OH-MTX CV ≥ 15% n (%)	Creatinine failed n (%)	MTX < LLOQ n (%)	7-OH-MTX < LLOQ n (%)
1	97.50	19	0	0	0	0	0
2	91.83	18	0	0	0	0	0
5	106.08	11	0	0	0	0	5 (45)
6	94.50	12	0	0	1 (8)	0	3 (25)
8	148.90	14	0	0	0	0	6 (43)
9	142.50	12	0	0	0	0	2 (17)
10	99.05	13	0	1 (8)	0	0	1 (8)
11	102.75	12	0	0	2 (17)	0	3 (25)
13	152.25	16	1 (6)	0	0	0	8 (50)
14	104.60	11	0	0	0	0	3 (27)
15	148.17	14	0	0	0	0	8 (57)
16	120.67	17	0	0	0	0	9 (53)
17	97.85	13	1 (8)	0	0	0	1 (8)
18[a]	97.37	14	0	0	12 (86)	12 (86)	12 (86)
19	146.82	10	1 (10)	0	0	0	3 (30)
20	147.08	16	0	0	0	0	6 (38)
21	99.72	20	1 (5)	0	0	0	4 (20)
22	98.37	13	0	0	0	0	3 (23)
23	143.33	9	0	0	0	0	0
24	147.95	17	0	0	0	0	5 (29)

Table 4-30. Breakdown of causes for MTX/7-OH-MTX levels to be rejected; these results indicate that MTX is the preferred analyte for measurement of adherence as the samples are more likely to be above the LLOQ. [a]Samples in patient failed due to HPLC-SRM-MS shutdown

Patient ID	MTX dose (mg)	Time from MTX ingestion to last urination (hours)	Time from MTX ingestion to last detectable MTX (hours)	Time from MTX ingestion to last 7-OH-MTX detectable (hours)
1	15	97.50	97.50	97.50
2	20	91.83	91.83	91.83
5	10	106.08	106.08	56.97
6	12.5	94.50	94.50	94.50
8	7.5	148.90	148.90	23.53
9	20	142.50	142.50	70.92
10	20	99.05	99.05	99.05
11	20	102.75	102.75	55.92
13	10	152.25	152.25	75.58
14	12.5	104.60	104.60	56.42
15	10	148.17	148.17	20.17
16	15	120.67	120.67	93.67
17	25	97.85	97.85	97.85
18	20			
19	20	146.82	146.82	71.92
20	10	147.08	147.08	23.72
21	10	99.72	99.72	98.47
22	15	98.37	98.37	51.70
23	15	143.33	143.33	143.33
24	15	147.95	147.95	99.25

Table 4-31. Time from MTX ingestion to last urine and last time when MTX/7-OH-MTX was detectable.

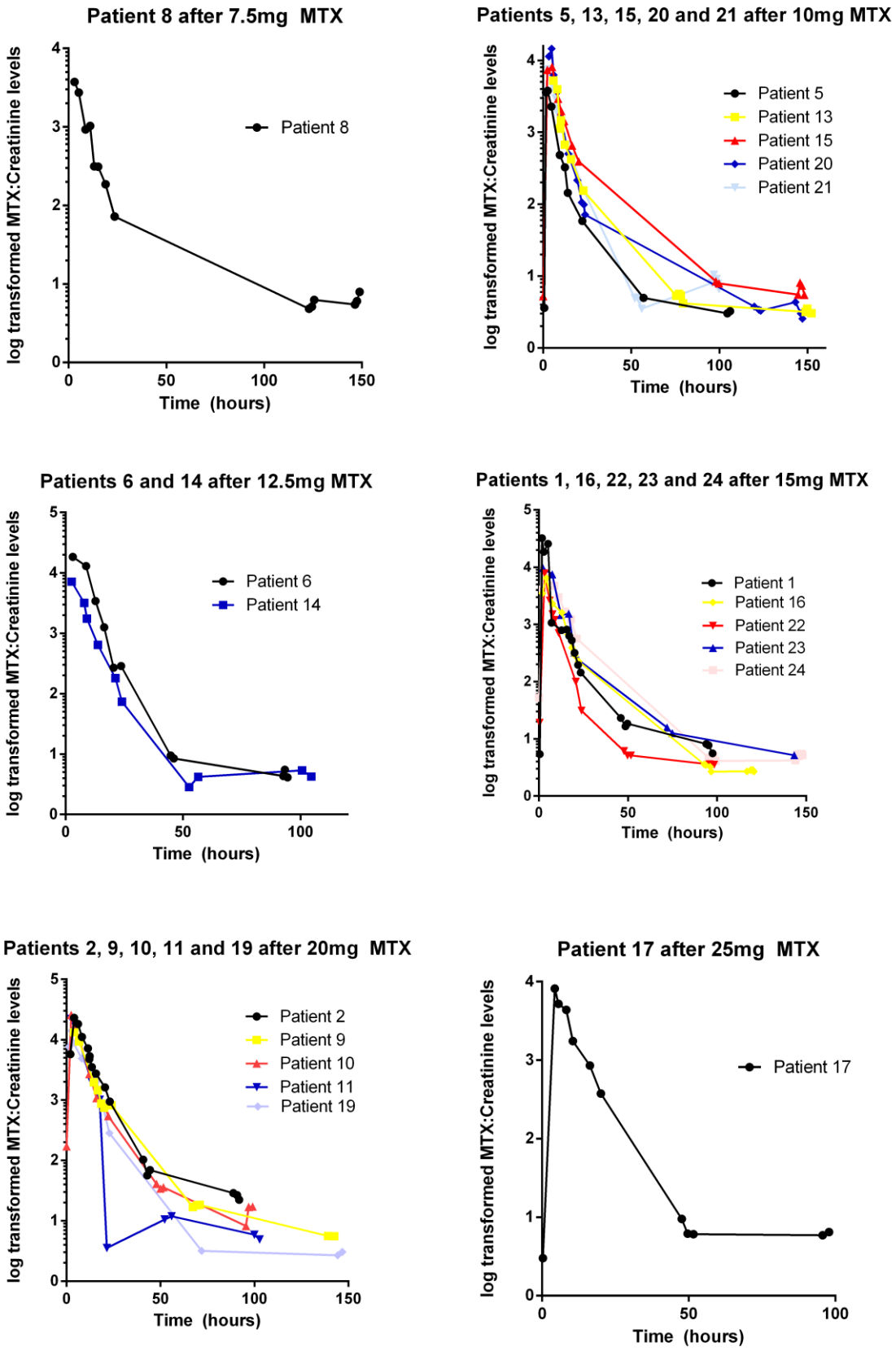


Figure 4-35. Log transformed MTX:creatinine levels over time.

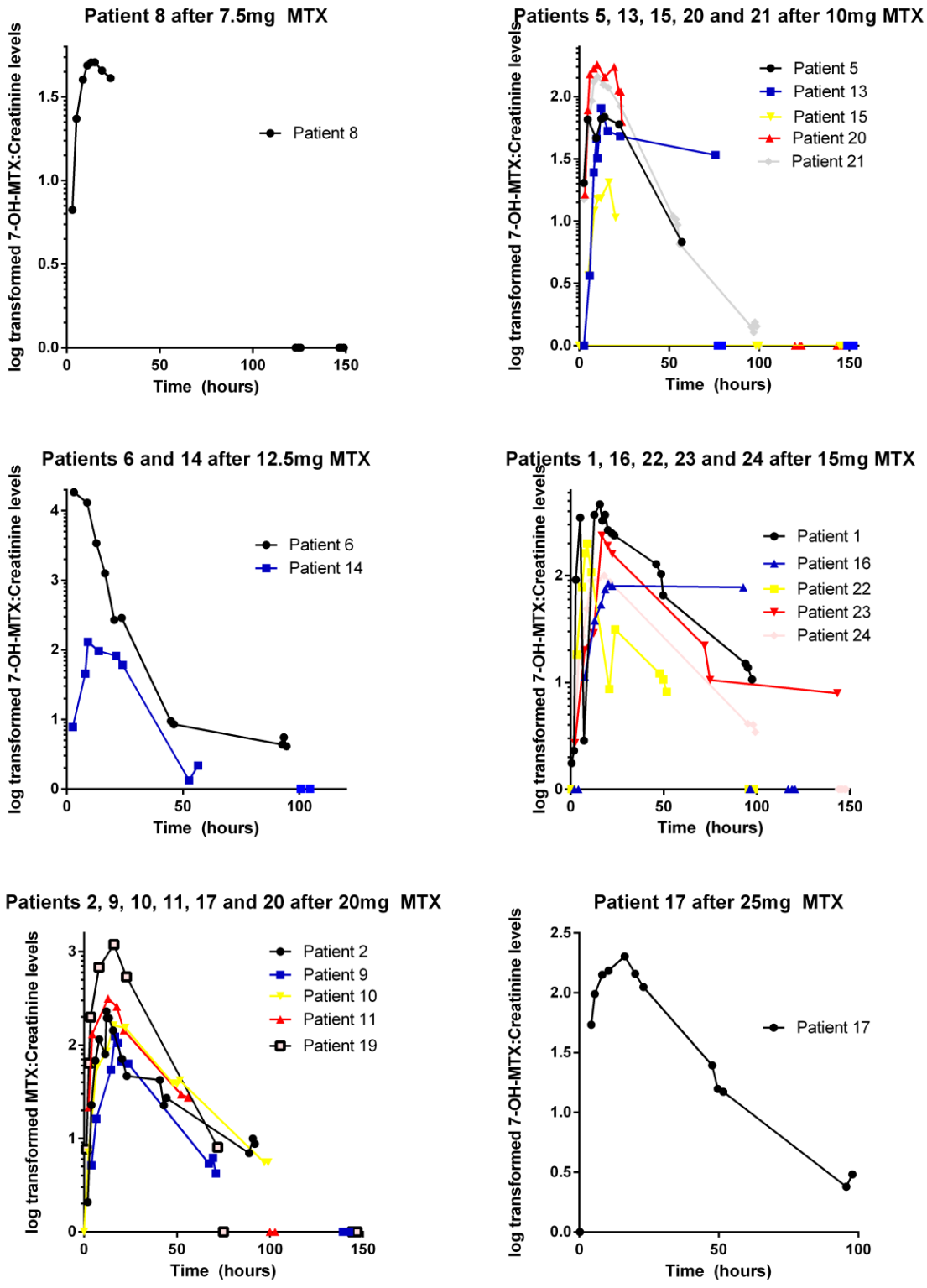


Figure 4-36. Log transformed 7-OH-MTX:creatinine levels over time. Levels of 0 nM/µM represent samples below LLOQ.

4.6.6 Summary of Results of Urine Measurements from the MEMO Cohort

The results from the measurement of MTX/7-OH-MTX in urine of patients from the MEMO cohort have demonstrated that in all subjects and at all dose levels measured MTX concentrations were above the LLOQ for the period of the study. A proportion of 7-OH-MTX samples were below the LLOQ. These results suggest that MTX is the preferred analyte for measuring adherence and that measurement of MTX in urine can be useful to assess adherence to MTX.

4.6.7 Plasma Results from the MEMO Study

In total, 173 plasma samples were collected and measured in triplicate (n=519) from 20 patients. The median time from MTX ingestion to last plasma sample was 101 hours (IQR: 94 – 142 hours). Samples in the first two hours were over the calibration curve and required a dilution to 1 in 2 µl. Samples were processed in triplicate and the measurement was accepted if CV ≤ 15% and the result was within the calibration curve. Mean measurements were multiplied by their dilution factor in order to obtain the final concentration. Rejection of samples due to high CV or measurement < LLOQ for MTX and 7-OH-MTX was 12.7% and 13.8% respectively. Table 4-32 illustrates the time from MTX ingestion to blood sampling when MTX and 7-OH-MTX could last be detected above LLOQ for each patient. Table 4-33 shows the last time from MTX ingestion to when MTX/7-OH-MTX was detectable using the assay.

Patient ID	Time from ingestion to last blood sample (hours)	Number of samples	MTX CV ≥ 15% n (%)	7-OH-MTX CV ≥ 15% n (%)	MTX < LLOQ n (%)	7-OH-MTX < LLOQ n (%)
1	93.93	9	0	1 (11.1)	2 (22.2)	2 (22.2)
2	40.75	9	0	0	0	0
5	101.6	9	0	0	3 (33.3)	3 (33.3)
6	90.88	9	0	1 (11.1)	1 (11.1)	0
8	145	9	0	3 (33.3)	0	0
9	139.83	9	0	0	0	1 (11.1)
10	95.43	9	0	0	0	1 (11.1)
11	100.5	9	0	1 (11.1)	2 (22.2)	1 (11.1)
13	147.75	9	0	0	3 (33.3)	3 (33.3)
14	100.87	9	0	0	0	2 (22.2)
15	100.5	9	0	0	0	0
16	117.08	9	0	1 (11.1)	0	0
17	95.58	7	1 (14.3)	0	0	0
18	94.58	7	0	0	0	0
19	144.5	8	1 (12.5)	1 (12.5)	2 (25.0)	0
20	143.38	9	2 (22.2)	0	0	2 (22.22)
21	95.97	9	0	1 (11.1)	1 (11.1)	1 (11.11)
22	47.63	7	0	0	1 (14.3)	2 (28.6)
23	144.35	9	0	0	0	0
24	141.17	9	0	1 (11.1)	0	2 (22.22)

Table 4-32. Breakdown of causes for MTX/7-OH-MTX levels to be rejected. The results demonstrate that 7-OH-MTX measurements were more likely to be rejected due to high CV compared to MTX measurements.

Patient ID	MTX dose (mg)	Time from MTX to last blood sample (hours)	Time from MTX to last MTX measured above LLOQ (hours)	Time from MTX to last 7-OH-MTX measured above LLOQ (hours)
1	15	93.93	93.93	93.93
2	20	40.75	40.75	40.75
5	10	101.6	24	53.08
6	12.5	90.88	90.88	90.88
8	7.5	145	145	24.03
9	20	139.83	139.83	67.5
10	20	95.43	95.43	95.43
11	20	100.5	100.5	52.93
13	10	147.75	75.91	24
14	12.5	100.87	100.87	52.8
15	10	144.08	144.08	144.08
16	25	117.08	117.08	117.08
17	25	95.58	95.58	95.58
18	20	94.58	94.58	94.58
19	20	144.5	72.15	72.15
20	10	143.38	143.38	120.72
21	10	95.97	95.97	51.97
22	15	47.63	47.63	24.08
23	15	144.35	144.35	144.35
24	15	141.17	141.17	14.75

Table 4-33. Time from MTX ingestion to last blood and last time when MTX/7-OH-MTX was detectable.

Figure 4-37 and Figure 4-38 show log transformed plasma results for MTX and 7-OH-MTX.

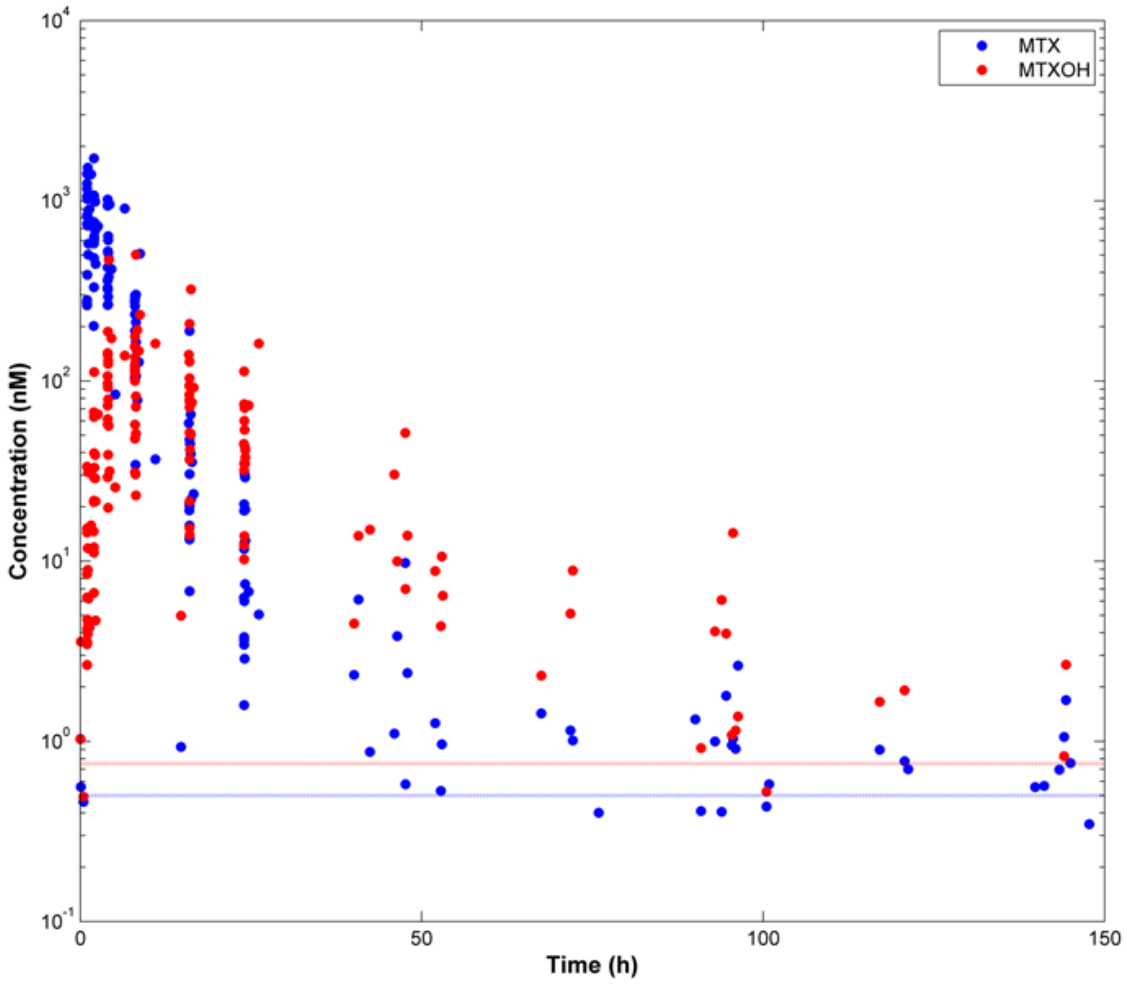


Figure 4-37. Semi-logarithmic plot of MTX and 7-OH-MTX concentration levels over time. Red horizontal and blue horizontal lines represent the LLOQ for MTX and 7-OH-MTX respectively. Samples below the LLOQ were subsequently removed for pharmacokinetic modelling.

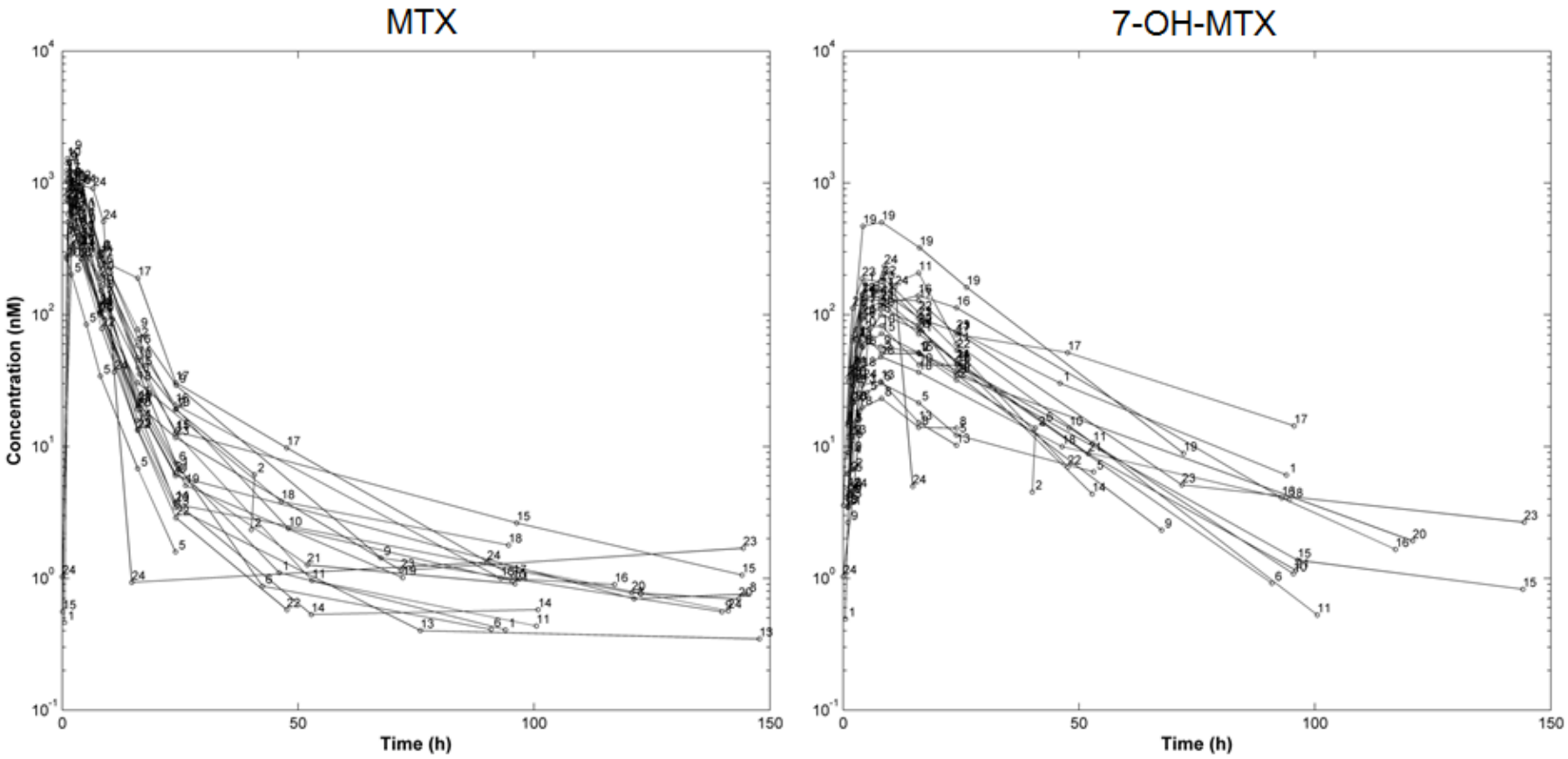


Figure 4-38. Log transformed MTX and 7-OH-MTX concentration levels over time linked by patient ID. Samples below the LLOQ were subsequently removed for pharmacokinetic modelling.

4.7 Results from Pharmacokinetic Modelling of the MEMO Plasma Data

A two compartment model for MTX and one compartment model for 7-OH-MTX was fitted to the data as shown in Figure 4-39. Compartment V4 was not structurally identifiable as there was no direct ingestion of 7-OH-MTX therefore apparent formation and clearance of 7-OH-MTX are estimated (f_m/V4 and CL_m/V4 respectively). The effect of serum creatinine levels on the systemic clearance of MTX was negligible and therefore not included in the model.

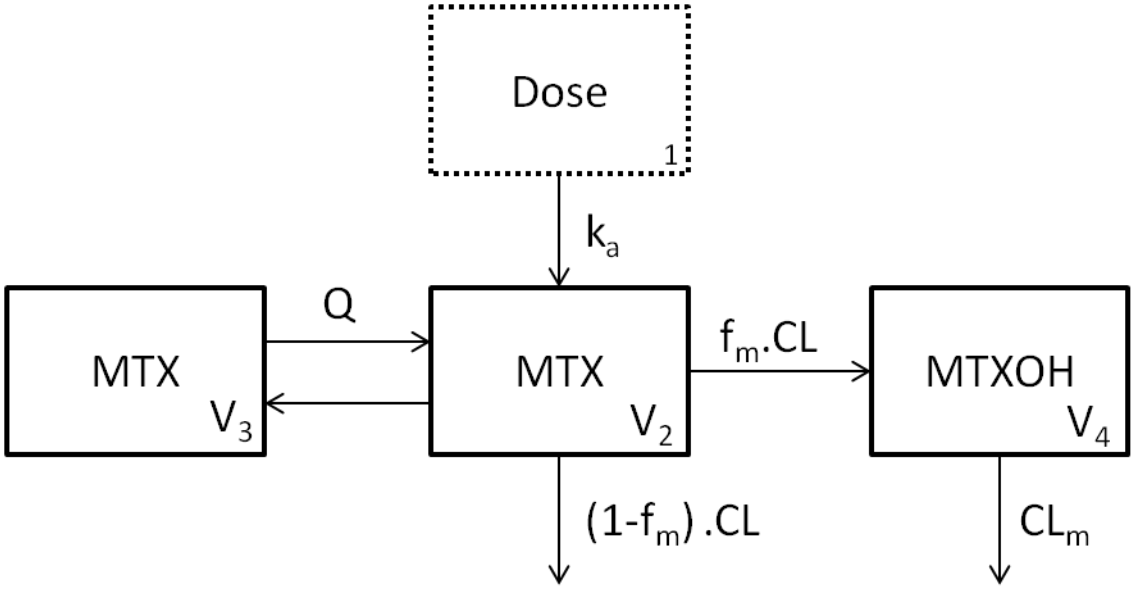


Figure 4-39. Compartment models used to fit the plasma data for MTX and 7-OH-MTX. K_a = absorption rate constant, CL = apparent MTX clearance, CL_m = apparent 7-OH-MTX clearance, V = volume of distribution, f_m = apparent fraction of MTX converted to 7-OH-MTX and Q = intercompartmental clearance.

The parameters for the pharmacokinetic model are listed in Table 4-34 showing both population mean and the amount of variability in the model parameter. Residual standard errors give an estimate of confidence. For pharmacokinetics of oral medication, only apparent disposition parameters (relative to the bioavailability) can be estimated. Therefore, if not all of the drug ingested is absorbed then clearance observed in a pharmacokinetic study is not actual clearance for the full dose of drug given but clearance relative to the fraction of the dose that was absorbed into the systemic circulation. Inter-subject variability appeared to be highest for the apparent fraction of MTX converted to 7-OH-MTX. The visual predictive check demonstrated that the model captured adequately the observed data for MTX and 7-OH-MTX (Figure 4-40). Simulation data of 1,000 hypothetical individuals after ingesting 5, 10, 15 and 20 mg MTX to predict the proportion of subjects with measured MTX and 7-OH-MTX levels below the LLOQ is shown in Figure 4-41. The results demonstrate that 70% of adherent patients are predicted to have measureable MTX levels

at 72 hours after ingestion of 10 mg MTX whilst at 144 hours (six days) following ingestion of 15 and 20 mg, MTX 72% and 88% of adherent patients are predicted to have measureable MTX, respectively. It can be seen that MTX is predicted to be a better surrogate marker of adherence compared to 7-OH-MTX with a lower proportion of subjects that are predicted to be below the LLOQ for all dose ranges of MTX. Early after ingestion of MTX, a proportion of subjects have undetectable 7-OH-MTX, due to the delay in metabolism of MTX to 7-OH-MTX in the liver.

Parameter	Estimate	Residual Standard Error (%)
Population mean		
CL/F (l/h)	6.75	7.0
V_2/F(l)	30.3	9.9
Q/F (l/h)	0.449	20.2
V_3/F(l)	65.8	46.0
k_a (h^{-1})	1.19	24.1
f_m/V_4 (l^{-1})	0.00439	15.4
CL_m/V_4 (h^{-1})	0.0563	8.2
Variability (CV%)		
CL/F	20.2	49.0
V_2/F	24.2	63.2
Q/F	36.3	79.0
V_3/F	45.7	116.0
k_a	42.3	107.0
f_m/V_4	57.8	41.6
CL_m/V_4	22.4	60.0

Table 4-34. Population mean results from the pharmacokinetic modelling and the amount of variability within the model that is explained by each parameter. CL = clearance. F = bioavailability. Q = intercompartmental clearance. V = apparent volume of distribution. K_a = absorption rate coefficient, f_m = apparent fraction of MTX converted to 7-OH-MTX, CL_m = apparent clearance of 7-OH-MTX.

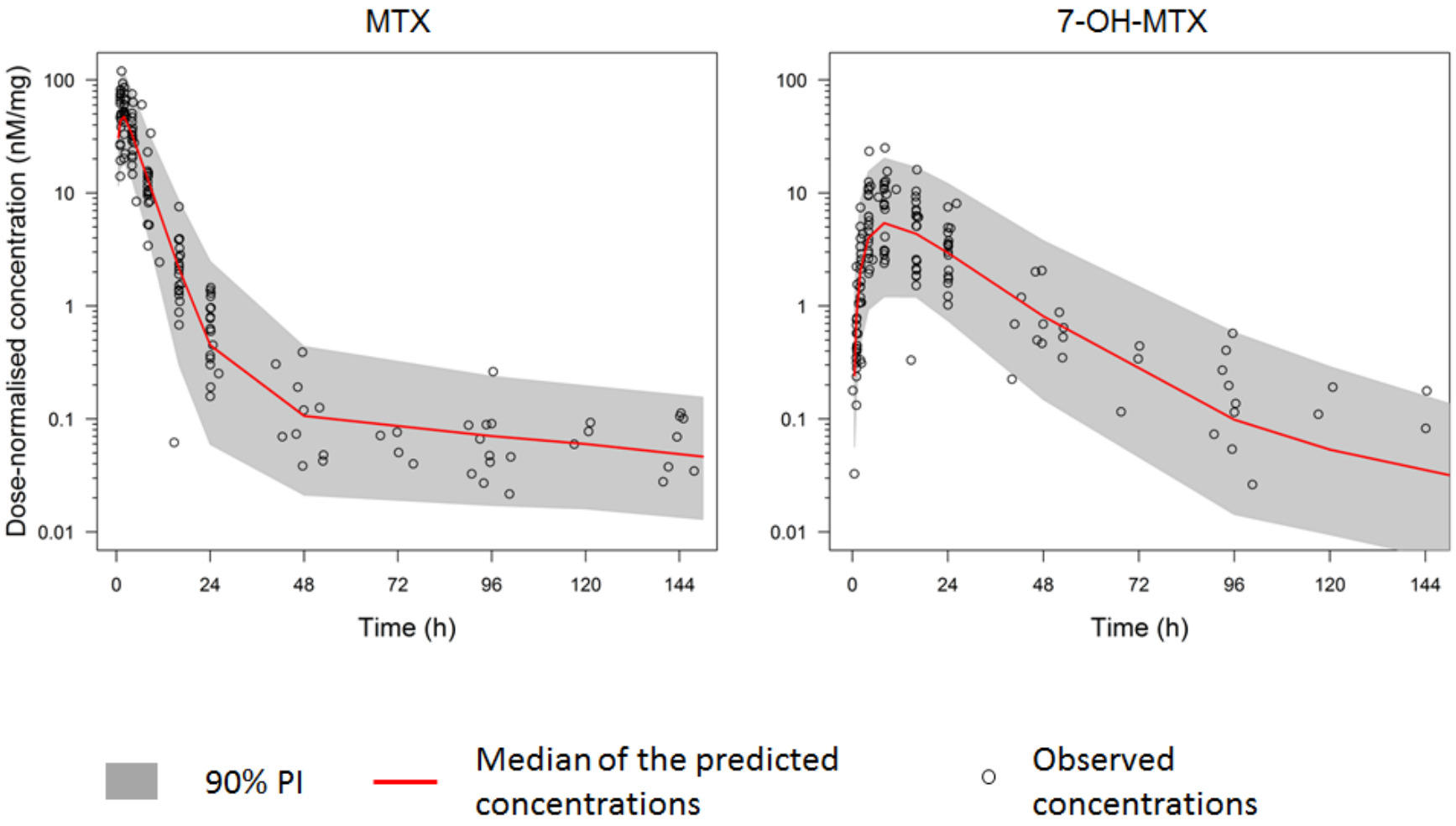


Figure 4-40. Visual predictive check for MTX and 7-OH-MTX. Observed concentrations are log-transformed dose-normalised (nM/mg) for MTX and 7-OH-MTX. PI = prediction interval.

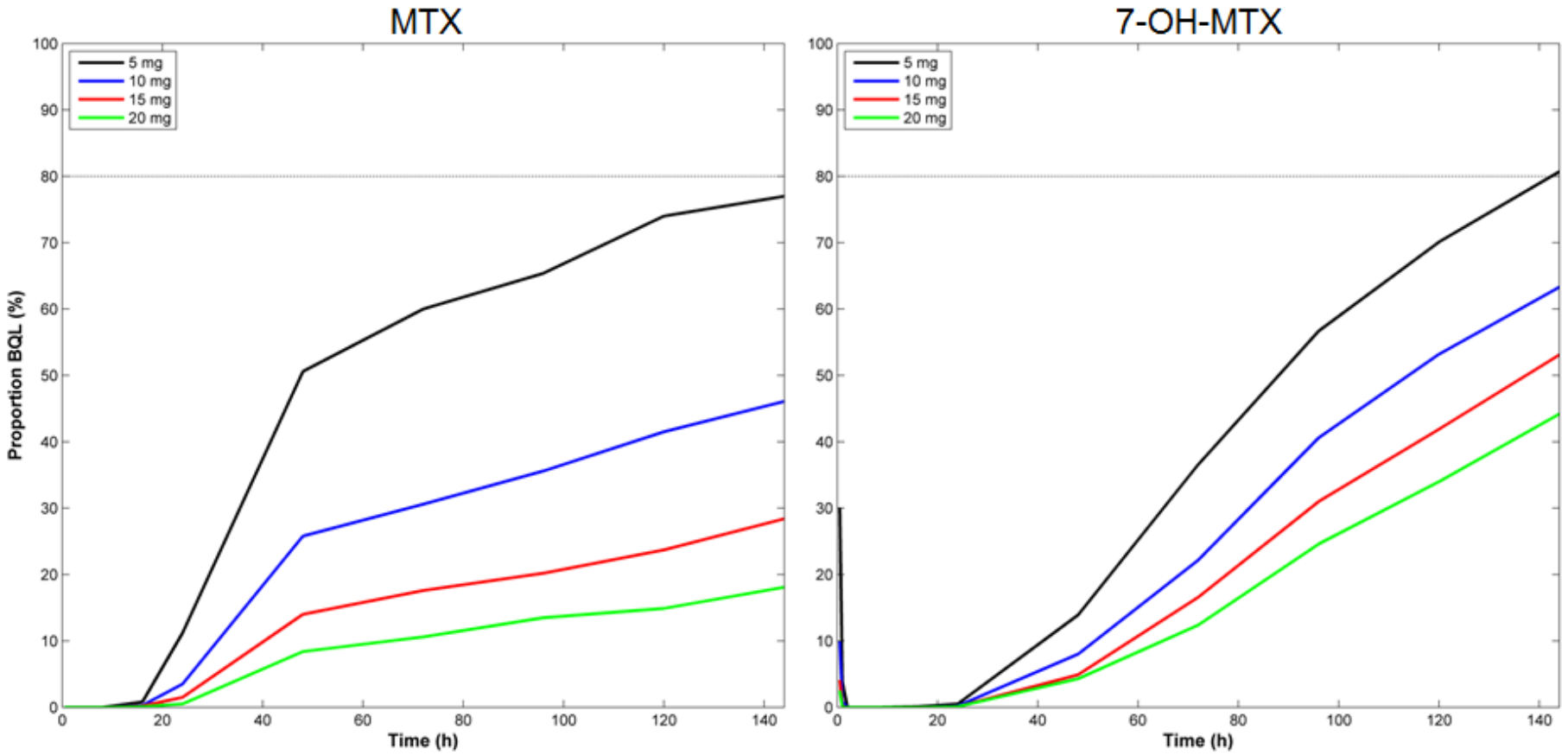


Figure 4-41. Simulated data of 1,000 hypothetical individuals showing the proportion of subjects with predicted concentrations of MTX/7-OH-MTX below the LLOQ (BLQ) for 5, 10, 15 and 20 mg MTX.

4.7.1 Summary of Results of Developing a Pharmacokinetic Model from the MEMO Cohort

Measurement of MTX/7-OH-MTX concentrations in plasma from a pharmacokinetic study were used to develop a pharmacokinetic model. The pharmacokinetic model demonstrated a good fit to the observed concentration-time data. The model predicted that measurement of MTX concentration may be more sensitive to detect adherence compared to 7-OH-MTX but external validation is required.

4.8 Validation of the Pharmacokinetic Model

4.8.1 Introduction

Following the development of a pharmacokinetic model of MTX/7-OH-MTX using the developed HPLC-SRM-MS assay it was necessary to validate this model using sparse samples collected as part of the RAMS cohort.

4.8.2 Aim of this Section

The aim of this section is to validate the pharmacokinetic model by measuring MTX and 7-OH-MTX levels in plasma of patients with RA recruited to a prospective observational study.

4.8.3 Results

In total, 51 plasma samples were collected where time of MTX ingestion was diarised, time of venepuncture was recorded and the sample arrived to the laboratory within three days. Baseline clinical and demographic characteristics are shown in Table 4-35. Out of 51 samples, two showed undetectable levels of MTX (4%); of these, one sample was taken 58 days after the patient had stopped MTX but they had continued to participate in RAMS. Review of the diary for this patient revealed that they stopped their MTX as they were unaware that they should continue treatment; following venepuncture, the patient re-started their MTX and noted this in the diary, therefore, the patient was included in the study as having had taken MTX on the day of venepuncture. This explains why MTX was undetectable and also gives evidence to the negative predictive value of the test. The other sample was taken 148 hours after 20 mg MTX ingestion was recorded and the sample was received in the laboratory two days after venepuncture, so the reason for the lack of detectable MTX is unclear. In comparison to MTX, 7-OH-MTX was undetectable in 26 (51%) of plasma samples.

Baseline Characteristic	Median (IQR)	Missing (n)
Age at venepuncture date (years)	62 (56-72)	0
Days between MTX commencement and venepuncture date	92 (88-105)	
Female gender (%)	55	0
Weight (kg)	76.9 (61.2-83.8)	3
Serum creatinine (μM)	67.5 (60.0-79.0)	5
Baseline DAS-28	4.61 (3.83-5.66)	2
MTX dose (mg)	20 (10-25)[a]	0

Table 4-35. Baseline clinical and demographic characteristics of the RAMS cohort. [a] median (range)

Figure 4-42 shows the median predicted dose-normalised concentration of MTX/7-OH-MTX with 90% prediction interval over time developed from the MEMO study with individual dose-normalised concentrations measured from the RAMS samples. As can be seen, the model developed from the MEMO study predicts MTX levels from the RAMS cohort well; the results, therefore, validate the MTX pharmacokinetic model. Prediction of the 7-OH-MTX concentrations revealed that nine samples (35%) were above the 90% prediction interval. There is an overall trend for the RAMS 7-OH-MTX concentrations to be higher than the median predicted concentrations from MEMO.

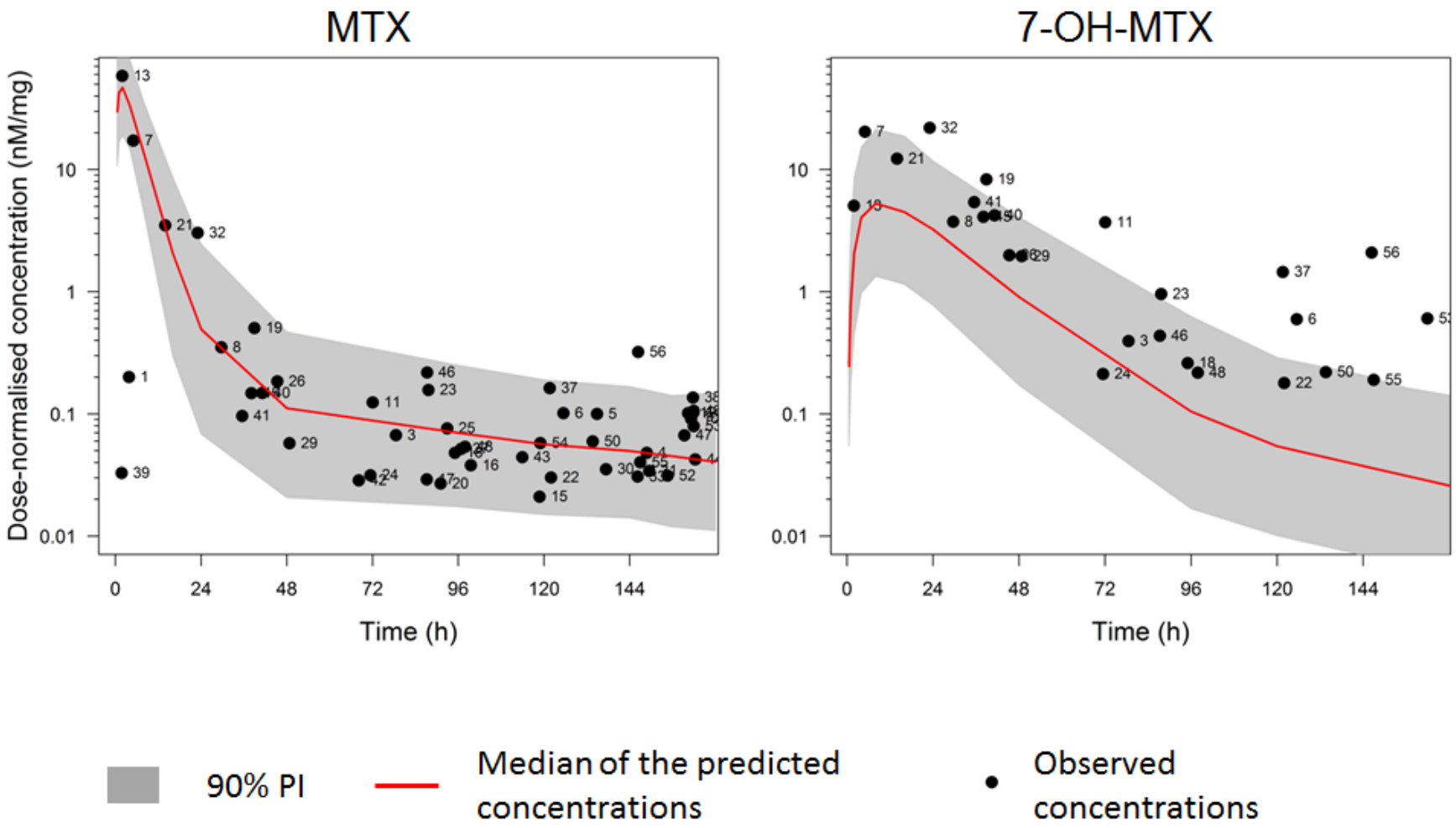


Figure 4-42. Log-transformed dose-normalised median and 90% prediction interval (PI) MTX and 7-OH-MTX concentration developed from the MEMO study overlaid with individual dose-normalised MTX and 7-OH-MTX concentrations observed from the RAMS study.

Figure 4-43 and Figure 4-44 show MTX and 7-OH-MTX concentrations and time from MTX ingestion to venepuncture from the RAMS cohort categorised by MTX dose where n ≥ 10 RAMS samples. The median predicted concentration from the pharmacokinetic model is shown in the top graphs (red line). The bottom graphs represent the predicted proportion of samples below the LLOQ (BQL) (red line) and the black dots represent the proportion of samples from RAMS that were BQL. Samples were binned according to time as shown by the grey boxes. The figures show that MTX outperformed the simulations predicted with only one RAMS sample being undetectable at 20mg. At this time, the proportion of samples below the LLOQ from RAMS remains below the predicted proportion of samples indicating that the assay outperformed what was predicted from the pharmacokinetic simulations. Considering the results from measuring the concentration of 7-OH-MTX, it can be seen that for 20mg MTX, the proportion of samples BQL are higher than is expected from the simulation testing. These results demonstrate that MTX is the analyte of choice to measure adherence.

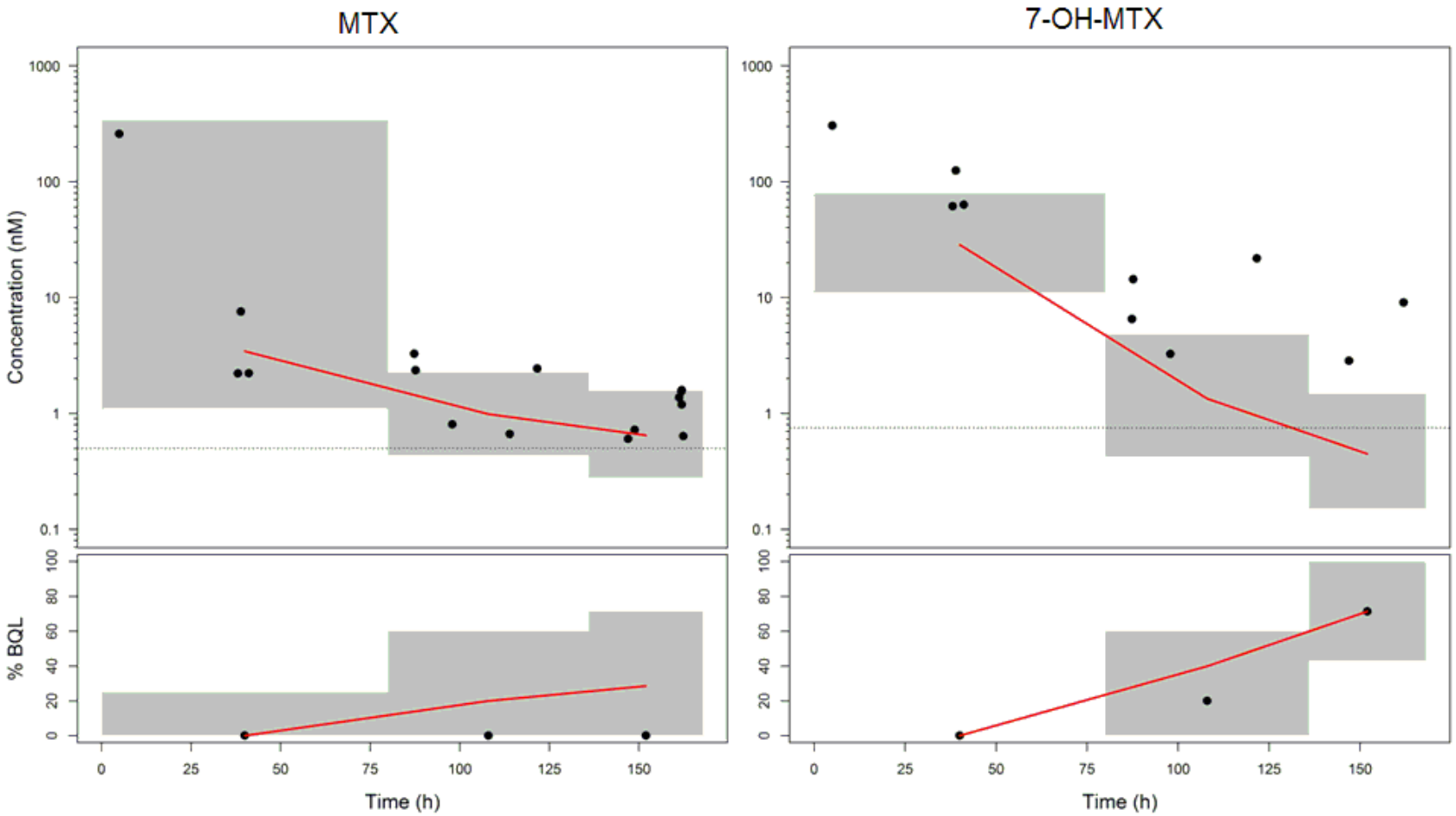


Figure 4-43. Log-transformed MTX and 7-OH-MTX concentrations from the RAMS study following 15mg MTX (black dots, n=16) with median predicted concentration from the pharmacokinetic model developed from MEMO (top graph). Proportion of RAMS samples that are below the LLOQ (BQL, black dots), with predicted proportion of samples BQL (red line) from the pharmacokinetic model (bottom graph). Grey boxes indicate the binning of RAMS samples to determine proportion BQL.

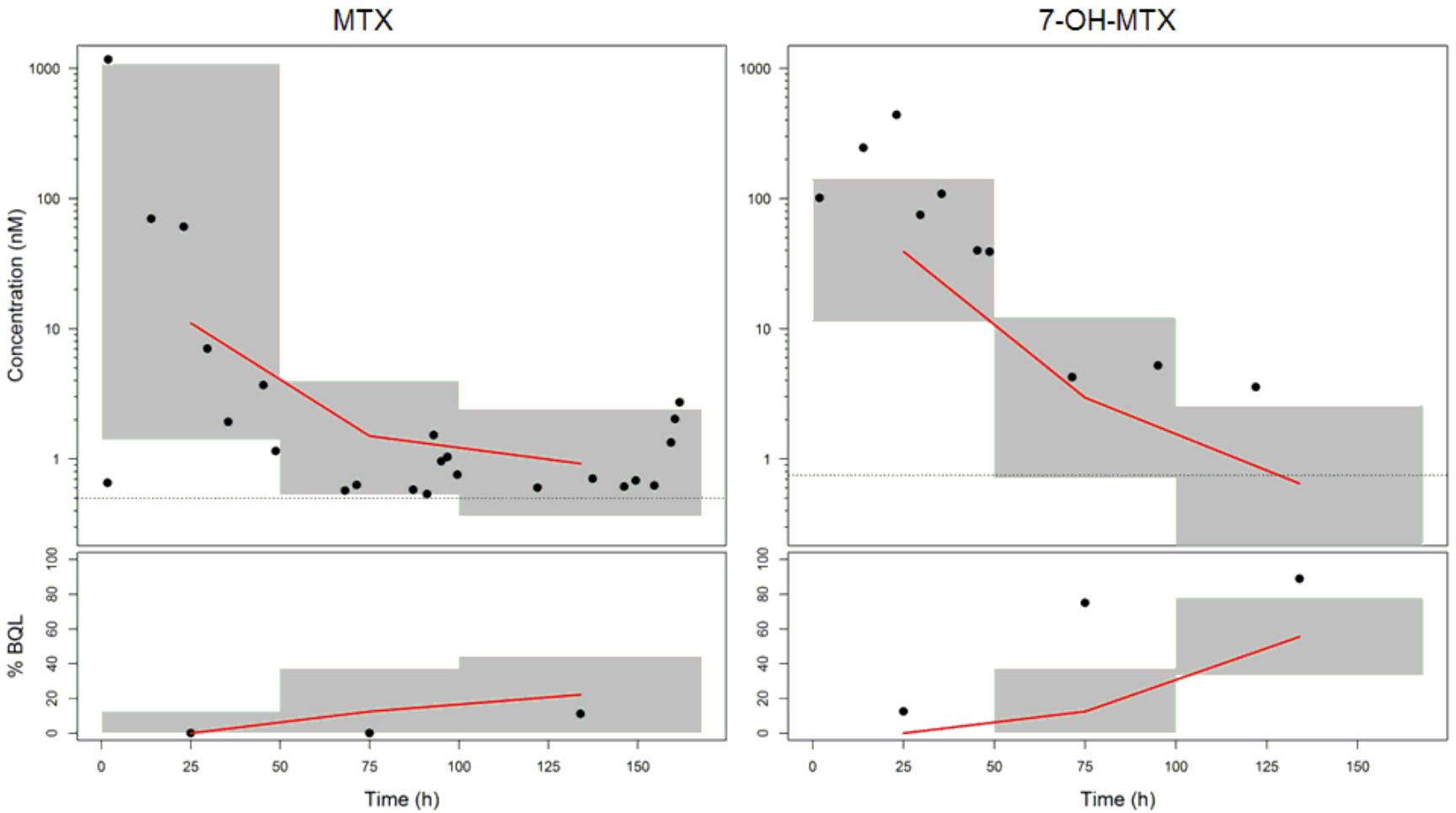


Figure 4-44. Log-transformed MTX and 7-OH-MTX concentrations from the RAMS study following 20mg MTX (black dots, n=25) with median predicted concentration from the pharmacokinetic model developed from MEMO (top graph). Proportion of RAMS samples that are below the LLOQ (BQL, black dots), with predicted proportion of samples BQL (red line) from the pharmacokinetic model (bottom graph). Grey boxes indicate the binning of RAMS samples to determine proportion BQL.

4.8.4 Summary of Results from Validation of the Pharmacokinetic Model

Measurement of single plasma samples of MTX and 7-OH-MTX concentrations from RAMS patients demonstrated that the pharmacokinetic model was valid for MTX but that the model performed less well for 7-OH-MTX. Only one patient had undetectable MTX levels, showing that the assay outperformed predicted performance from simulation of the pharmacokinetic model and indicating that MTX is the preferred analyte for direct measurement of adherence.

4.9 Investigating the Association between MTX AUC and Response to MTX

4.9.1 Introduction

Currently, there are no reliable markers that predict response to MTX but previous studies have suggested measurement of MTX levels may predict response [131, 140]; however, results are conflicting [148].

4.9.2 Aims of this Section

The aims of this section of my thesis were to:

1. Measure MTX and 7-OH-MTX levels in plasma of patients with RA prescribed MTX recruited in a prospective observational study.
2. Investigate the correlation between three month MTX and 7-OH-MTX AUC and change in DAS-28 over three months.
3. Investigate the association between three month MTX AUC and 7-OH-MTX AUC and EULAR response at three months.

4.9.3 Methods Relevant to this Section

The pharmacokinetic model developed was subsequently used to analyse measurements of MTX from the RAMS study and derive the AUC for each individual patient. The 7-OH-MTX model was not validated in the RAMS cohort (Section 4.8) and it was therefore not possible to calculate the 7-OH-MTX AUC. Logistic regression was used to determine if MTX AUC was significantly associated with EULAR response. In addition, linear regression was used to determine if MTX AUC was correlated with change in DAS-28 over three months. Analysis was undertaken using Stata Version 13.1 [238].

4.9.4 Results

Forty-three patients had calculated DAS-28 at baseline and three months allowing change in DAS-28 and EULAR response to be calculated. Baseline clinical and demographic characteristics of this subgroup cohort of RAMS are shown in Table 4-36.

Baseline Characteristic	Median (IQR)	Missing (n)
Age at venepuncture date (years)	63 (56-67)	0
Female gender (%)	58	0
Weight (kg)	78.7 (64.0-84.0)	0
Serum creatinine (μM)	67 (60-79)	2
eGFR	94 (78-105)	
Baseline DAS-28	4.61 (3.80-5.68)	0
Concomitant NSAID (%)	44	0
Planned dose escalation (%)		28
Weekly	10	
Fortnightly	50	
Monthly	35	
Six weekly	5	
MTX dose (mg)	20 (10-25)[a]	0
Number of weeks on three month MTX dose prior to venepuncture	7 (6-9)	26

Table 4-36. Baseline and clinical characteristics of the RAMS cohort used to investigate MTX AUC levels and MTX response (n=43). [a] median (range).

Skewness and kurtosis test for normality revealed that three month MTX AUC and change in DAS-28 were normally distributed. Mean DAS-28 improvement over the three month period for the RAMS cohort was 1.18 (SD: 1.43). EULAR response at three months is shown in Table 4-37. Linear regression demonstrated no significant correlation between three month MTX AUC and change in DAS28 over three months (p = 0.15, Figure 4-45), adjusting for baseline eGFR and NSAID usage did not qualitatively affect the results (p = 0.17). Regression analysis was also undertaken investigating the components of the DAS-28 as outcome measures (Table 4-38). Logistic regression demonstrated no significant association between three month MTX AUC and EULAR response at three months (p = 0.72, Figure 4-46).

EULAR Response at three Months	N (%)
No response	18 (42)
Moderate response	12 (28)
Good response	13 (30)

Table 4-37. EULAR response at three months in the RAMS cohort (n=43).

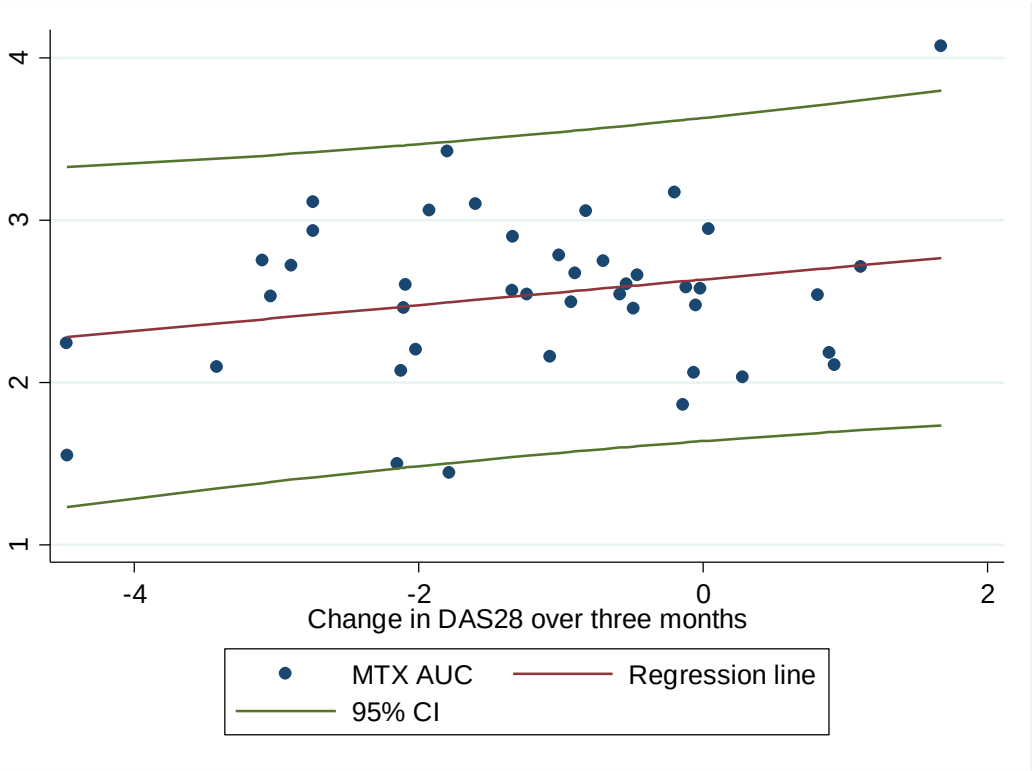


Figure 4-45. Linear regression results of AUC and change in DAS28 over three months (*p* = 0.15). Note that the regression line demonstrates a trend towards reduction of AUC associated with improved response.

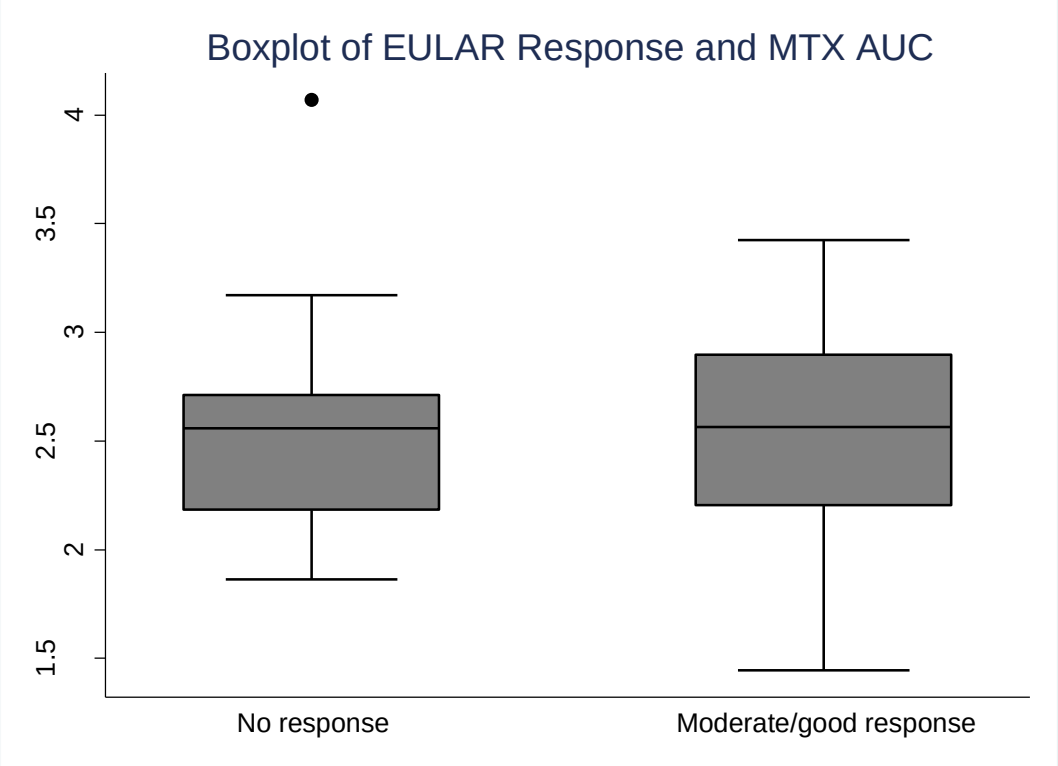


Figure 4-46. Boxplot of AUC and EULAR response at three months (*p* = 0.721).

Outcome measure	Coefficient (95% CI)	*p*-value
Change in DAS-28	0.08 (-0.03 – 0.19)	0.15
Change in patient VAS	-0.00 (-0.01 – 0.00)	0.48
Change in 28 tender joint count	-0.03 (-0.04 – -0.01)	0.01
Change in 28 swollen joint count	0.00 (-0.02 – 0.03)	0.18
Change in CRP	-0.00 (-0.01 – 0.01)	0.54

Table 4-38. Linear regression results of MTX AUC and change in DAS28 over three months for each DAS-28 variable for the RAMS cohort.

4.10 Results from a Genome Wide Association Study Investigating Methotrexate-Pneumonitis

4.10.1 Introduction

A previous study has suggested that MTX-P may occur in individuals genetically predisposed to the disease [216]. To date, no genome wide association study has been conducted of MTX-P.

4.10.2 Aims of this Section

The aims of this section were to:

1. Recruit cases with physician diagnosed MTX-P and age-sex matched RA controls prescribed MTX without the development of MTX-P.
2. Conduct a genome wide association study (GWAS) to identify genetic markers that increase the risk of MTX-P in patients with RA.
3. Perform a bioinformatics search of available databases to investigate SNPs with a potential functional role in the development of MTX-P.

4.10.3 Subject Recruitment

65 cases and 195 controls were recruited. The baseline demographic and clinical characteristics are shown in Table 4-39.

	Pneumonitis Cases	Controls
Age (yrs)	67 (57-74)	67 (57-74)
Female gender: n (%)	44 (67.69)	132 (67.69)
Weekly MTX dose (mg)	20 (12.5 - 20)	17.5 (IQR 15 - 20)[a]
Smoking: n (%)		
Never	10 (15)	78 (40)
Current	8 (12)	24 (12)
Ex-smoker	41 (63)	87 (45)
Concomitant folic acid: n (%)	63 (97)	189 (97)
Folic acid total weekly dose (mg)	5 (5-5)	10 (5-35)
Fulfils RA criteria: n (%)[b]	63 (97)	134 (69)
Fulfils MTX-P criteria: n(%)[c]		
Carson et al. only	18 (29)	
Searle et al. only	4 (6)	
Searle and Carson et al.	26 (42)	
Time to MTX-P (months)	21 (7-92)	

Table 4-39. Clinico-demographics of pneumonitis cases and controls. Values are represented by median (IQR) unless otherwise stated. [a]11 missing. [b]1987 ACR classification criteria applied to controls, 2010 ACR/EULAR classification criteria applied to cases. [c]Applied to cases who passed quality control criteria.

4.10.4 Genotyping

65 cases and 192 controls were successfully genotyped. As an additional internal quality control measure four cases and three controls were genotyped in duplicate. Three controls failed genotyping.

4.10.5 Quality Control Results

4.10.5.1 Individual Sample Quality Control

Figure 4-47 summarises the number of individuals removed at each quality control step.

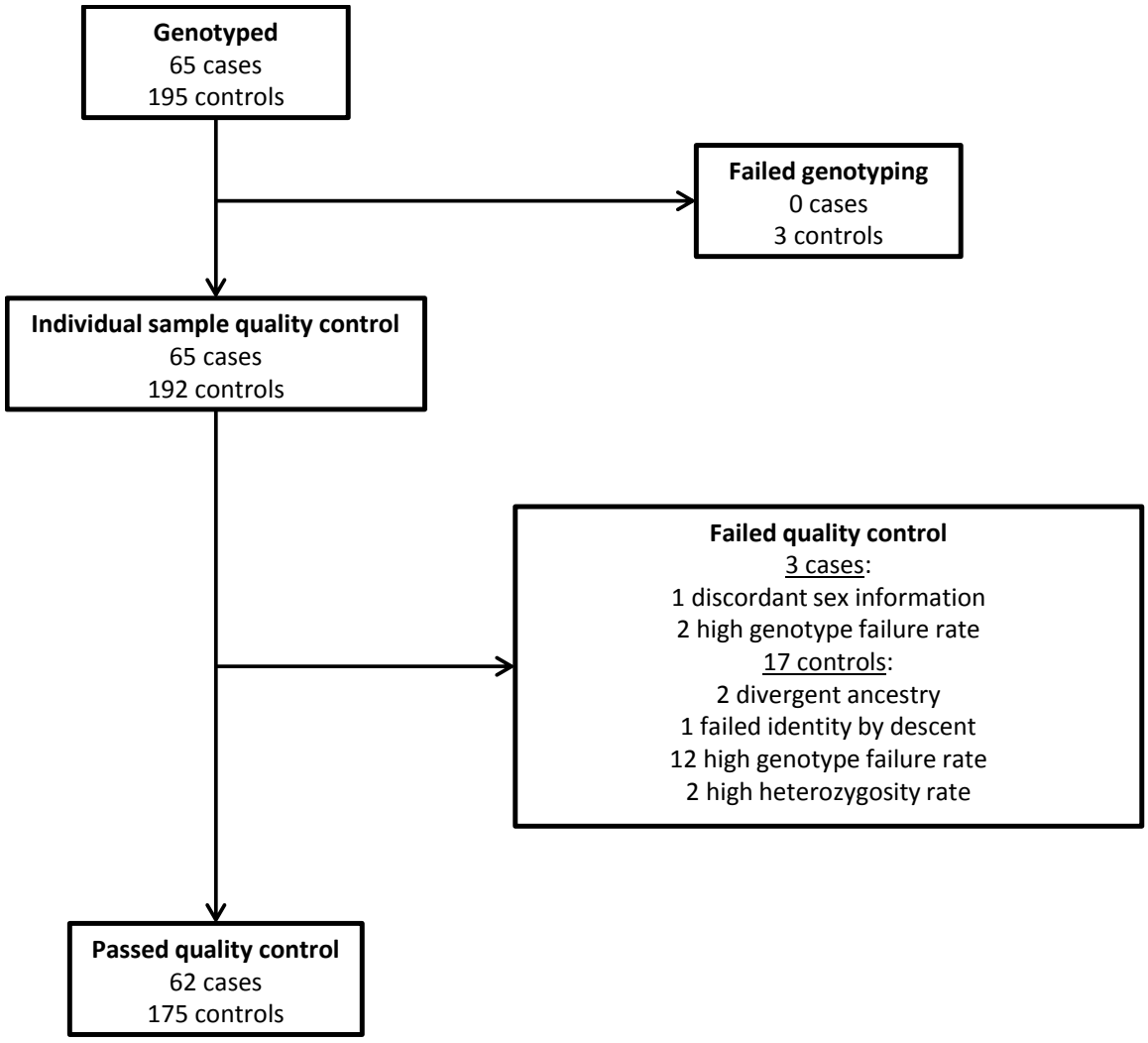


Figure 4-47. Flowchart depicting individuals that failed quality control. All duplicate samples were removed following identity by descent screening.

Overall 16 samples failed due to outlying heterozygosity and high genotype missing rates. Two samples demonstrated a higher than would be expected heterozygosity rate, this could be due to cross contamination of samples which could occur during the DNA extraction or genotyping of samples. Alternatively a high heterozygosity rate may be due to these individuals being true

outliers. Fourteen samples failed due to a high genotype failure rate indicating assay failure. All replicate samples were removed during identity by descent testing.

Identification of individuals of divergent ancestry utilising principal component analysis identified four controls of possible non-Caucasian ancestry. Two of these controls were excluded in a previous step due to their high heterozygosity rate. Figure 4-48 is a scatterplot of the first two principal components.

Marker quality control resulted in a final dataset of 62 cases, 175 controls with genotype data for 262,567 SNPs.

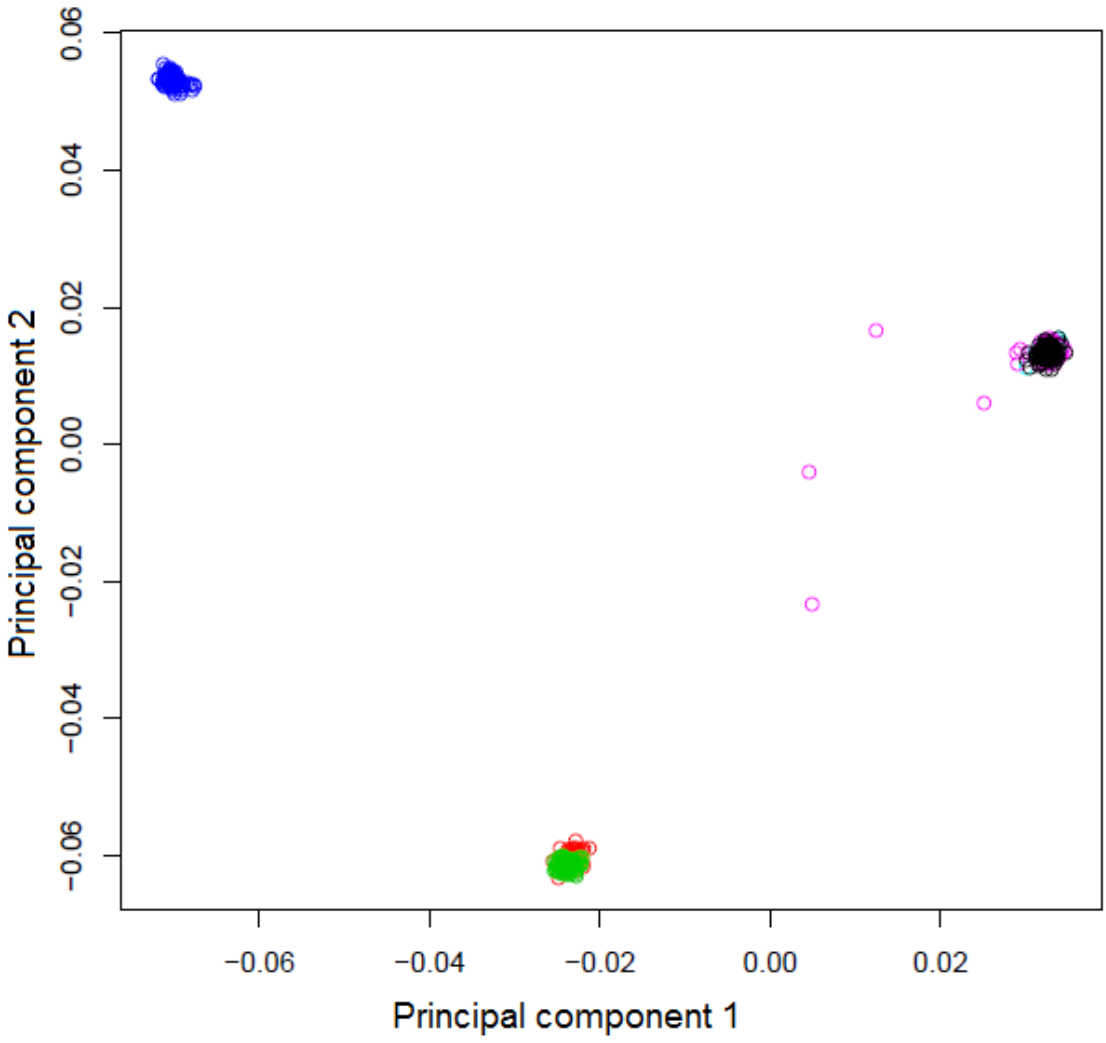


Figure 4-48. A scatter diagram of the first 2 principal components. Genome wide association samples (cases and controls) are represented by blue circles. Hapmap3 reference samples are black dots which represent individuals of European ancestry, red dots representing individuals of Chinese ancestry, green dots representing individuals of Japanese ancestry and purple dots representing individuals of African ancestry.

The quantile-quantile plot demonstrated no evidence of significant deviation from the null hypothesis due to systematic bias (Figure 4-49), supported by the very low genomic inflation factor 1.00993.

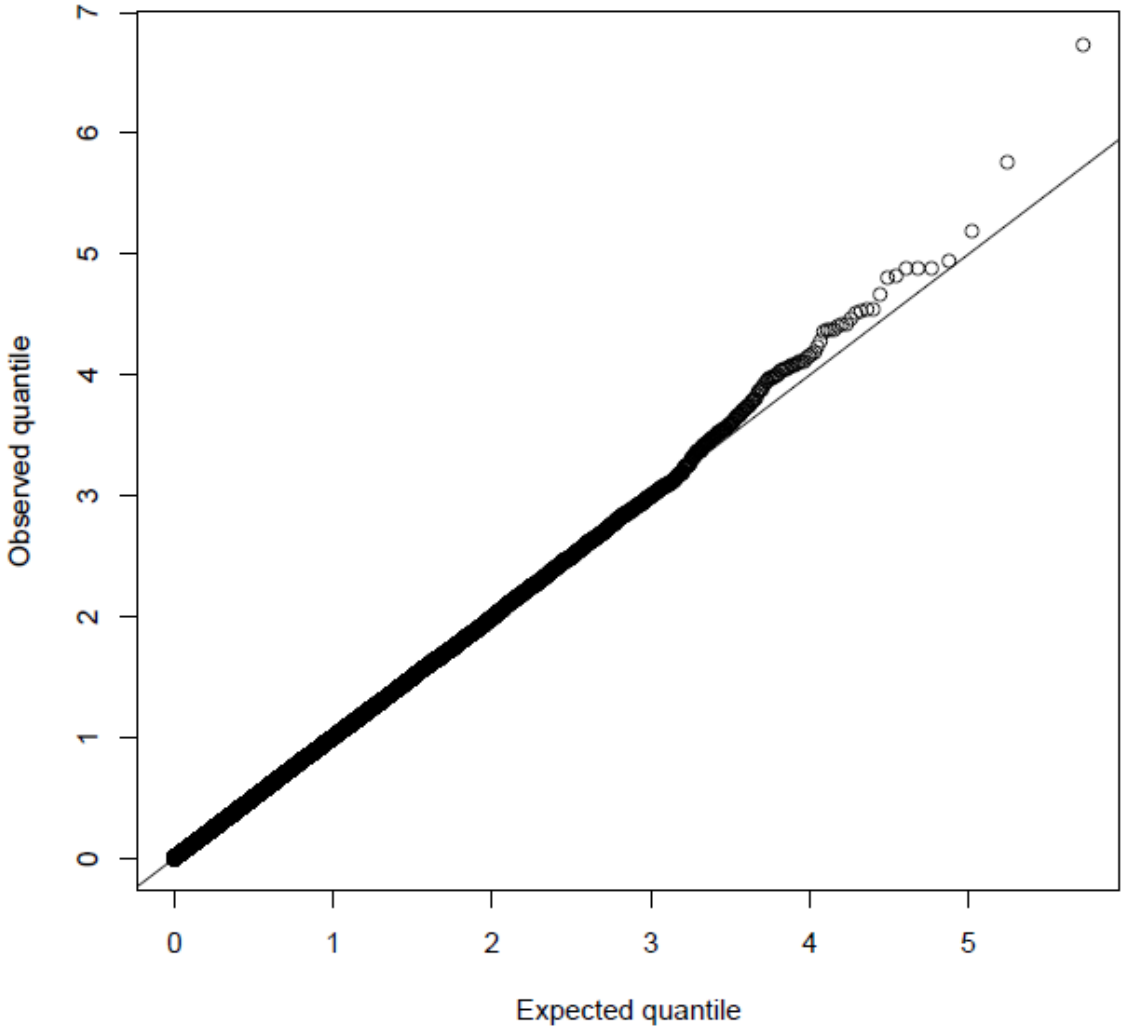


Figure 4-49. Quantile-quantile plot of the observed and expected log *p*-values under the null hypothesis, demonstrating no evidence for significant systematic bias.

The resulting Manhattan plot (Figure 4-50) summarises the results of the Cochran-Armitage test for trend. There are three SNPs that are of interest but that do not reach genome wide significance threshold (rs6593803, rs9299346 and rs1624005).

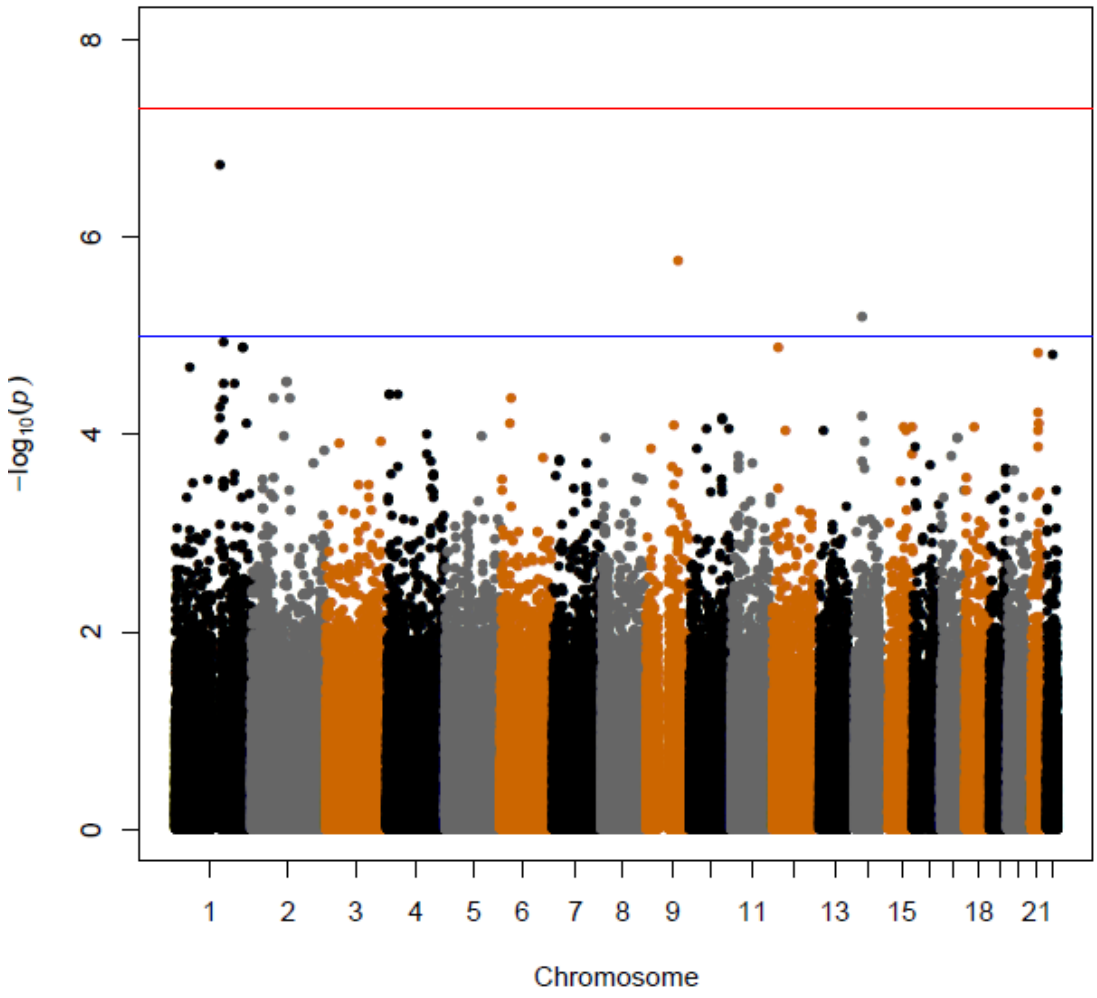


Figure 4-50. Manhattan plot for the genome wide association study showing no SNPs that fulfil the genome wide association level but three SNPs that are of interest requiring further investigation. The red line represents the threshold for genome wide significance and the blue line represents the threshold for SNPs that are of interest and worthy of further investigation.

4.10.6 SNP Associations

4.10.6.1 rs6593803

The LocusZoom plot for the lead SNP rs6593803 (p = 1.85 x 10^{-7}, OR = 3.13) is shown in Figure 4-51. rs6593803 lies in an intergenic region between the *GJA5* and *ACP6* genes. *GJA5* is a member of the connexin gene family and the resulting protein is connexin 40. The connexin 40 protein is a component of gap junctions that act at sites of cell-cell contact allowing diffusion of signalling molecules between cells [286]. Several protein isoforms are present throughout the lung [287]. Transgenic mice deficient in connexin 40 and connexin 43 ($cx40^{--}/cx43^{--}$) have reduced life span due to lung abnormalities including pulmonary fibrosis, alveolar wall thickening and increased

lung fibroblasts [288], histopathological findings similar to MTX-P. rs6593803 is an eQTL for *GJA5* in adipose cells (p=0.01) [289]. Results from ENCODE demonstrated that rs6593803 lies in a region of histone modification (H3K27Me3) in B-lymphocytes (GM12878) and is a marker of gene repression as shown in Figure 4-52 [256, 290]. rs6593803 may, however, be in LD with the true regulatory factor affecting *GJA5* expression. Bioinformatic analysis demonstrated that rs7514182 is in LD with rs6593803 (r^2=1, 1000 genomes release 20110521); is also an eQTL for *GJA5* in adipose cells (p=0.02) [289], is associated with histone modification in fetal lung fibroblasts (IMR90) known to cause gene repression [291] and lies in a site of open chromatin, DNAase hypersensitivity and transcription factor binding [256]. The results from ENCODE are shown in Figure 4-53. There is therefore reasonable evidence that rs7514182 may be associated with MTX-P and is a candidate for replication and further functional work.

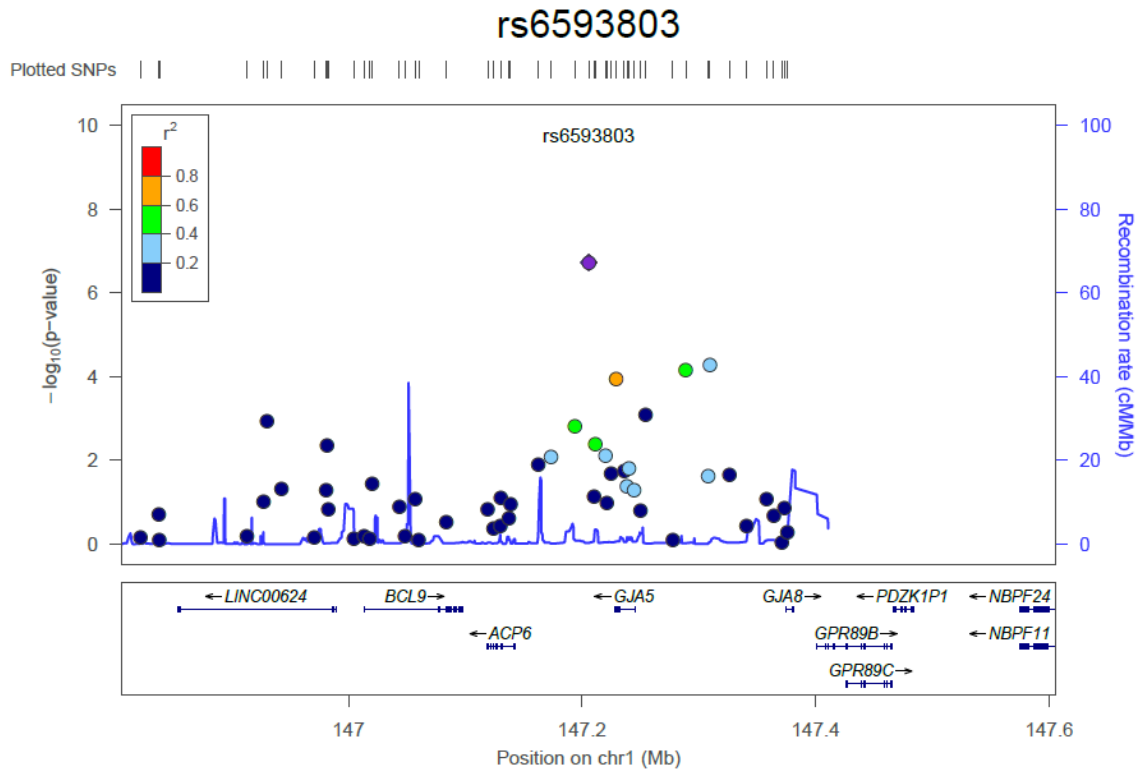


Figure 4-51. LocusZoom plot of lead SNP rs6593803. Note the cluster of SNPs with outlying *p*-values due to linkage disequilibrium with the lead SNP.

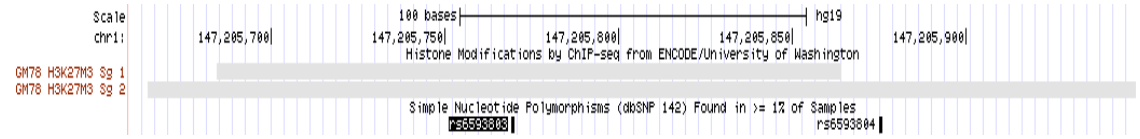


Figure 4-52. Genome Browser of lead SNP rs6593803 demonstrating overlying histone modification site for GM12878 cells providing functional evidence as a regulatory element. Produced using UCSC Genome Browser on Human (GRCh37/hg19) Assembly with overlying track of SNP (dbSNP142) and ENCODE histone modification [292].

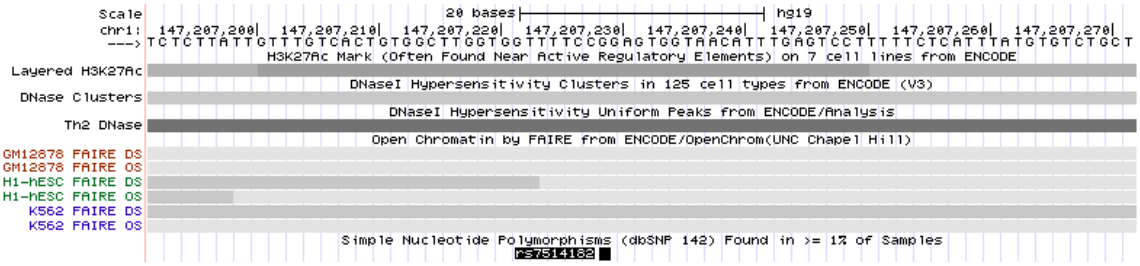


Figure 4-53.Results from ENCODE for rs7514182 demonstrating evidence that this locus is a regulatory element as a DNase cluster, histone modification site and open chromatin. Produced using UCSC Genome Browser on Human (GRCh37/hg19) Assembly.

4.10.6.2 rs9299346

The LocusZoom plot for rs9299346 (p = 1.76 x 10^{-6}, OR = 2.76) is shown in Figure 4-54. rs9299346 lies within an intron of *GRIN3A* in chromosome 9q34. *GRIN3A* encodes a subunit of the N-methyl-D-aspartate (NMDA) receptors present in nerve cells [293]. There is therefore no hypothesised functional role for *GRIN3A* in the development of MTX-P. Bioinformatic interrogation demonstrated evidence that rs9299346 and SNPs in LD affect the expression of a number of genes but with minimal evidence that the SNPs alter transcription factor binding sites. Hence, the genes show evidence for eQTL but the protein products do not appear to have a potential role in the development of MTX-P.

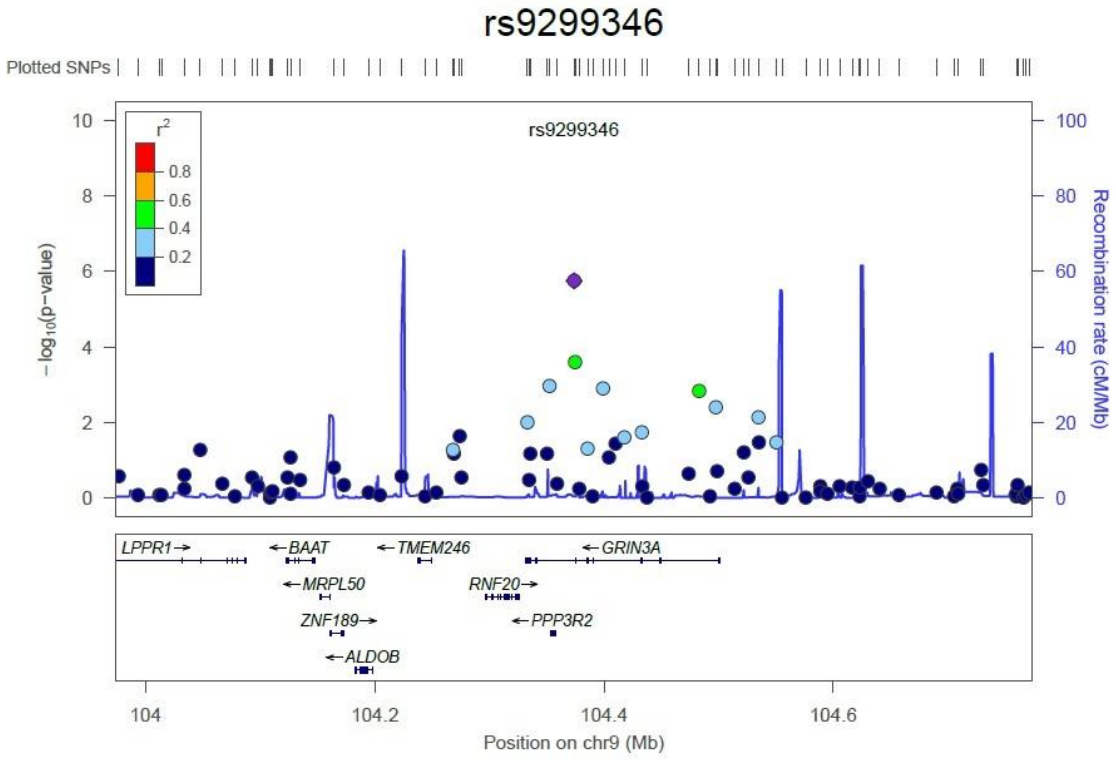


Figure 4-54. LocusZoom plot for SNP rs9299346.

4.10.6.3 *rs1624005*

The LocusZoom plot for rs1624005 (p = 6.54 x 10^{-6}, OR = 2.59) is shown in Figure 4-55. rs1624005 lies within an intergenic region. As expected given the lack of genes in this region, there is minimal evidence that this SNP or SNPs in LD affect gene expression. Bioinformatic interrogation demonstrated no eQTL data for the SNPs and only minimal evidence for acting as a regulatory region.

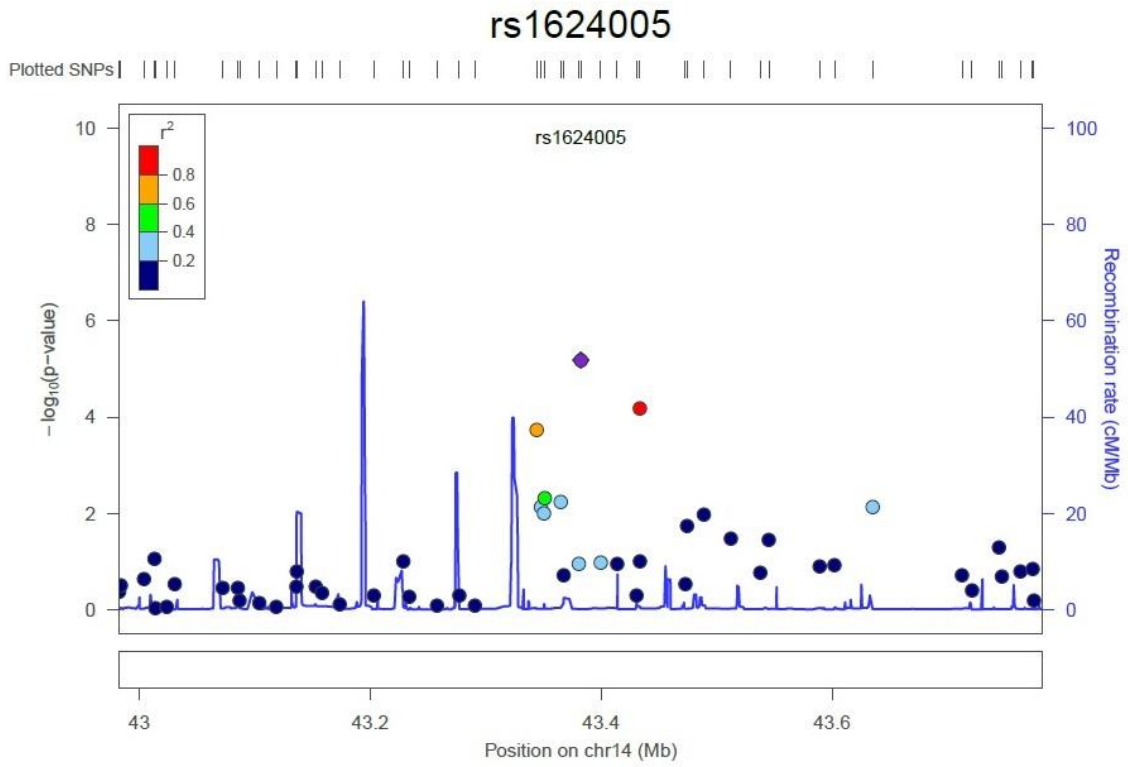


Figure 4-55. LocusZoom plot for rs1624005. Note the lack of genes surrounding the loci plotted.

4.10.6.4 *Restriction to Participants Fulfilling RA Criteria*

Restriction of the analysis to cases and controls fulfilling the 2010 ACR or 1987 ACR classification criteria for RA respectively did not yield significant results for the top three SNP associations (p > 5 x 10^{-8}).

4.10.6.5 *Restriction to Participants Fulfilling MTX-P Criteria*

Restriction of the analysis to cases fulfilling either Searle and/or Carson et al. criteria (n=48) did not yield a significant association (p > 5 X 10^{-8}).

4.10.6.6 *HLA-A*31:01*

HLA-A*3101 imputation was successfully completed using the SNP2HLA software [255]. HLA-A*31:01 was not associated with MTX-P in this cohort (p=0.21). Wet-lab genotyping of a subset of pneumonitis samples confirmed concordance with *in silico* imputation (κ=1.00).

4.10.7 Summary of Results

A genome wide association study of MTX-P was completed. The results demonstrated three SNPs (rs6593803, rs9299346 and rs1624005) with $p < 5 \times 10^{-5}$ as variants of interest worthy of further investigation. Bioinformatic analysis revealed a putative role for rs6593803 in causing pulmonary fibrosis in an animal model. Imputation of HLA was successful and verified through wet-lab experiments. The association between HLA-A 31:01 and MTX-P, previously reported in a Japanese population was not replicated in this study of patients with RA from the UK.

Chapter 5: Discussion

5 Introduction

RA is a chronic debilitating disease for which there is currently no known cure. MTX is the first-line therapy for RA but up to 41% of RA patients are non-adherent to oral MTX which affects response to treatment [34, 100]. Non-adherence may be further increased in RA patients due to fear of adverse events such as the life-threatening disease MTX-P.

Measurement of adherence to MTX is currently limited to indirect methods which have specific disadvantages; with no validated direct measurement of adherence that has been developed, to date. There was therefore a need for a direct test of adherence to be developed and its ability to detect adherence tested. The pharmacokinetics of MTX and its major metabolite 7-OH-MTX have a wide inter-individual variation but MTX/7-OH-MTX may be detectable for up to seven days [125]. There are a number of different methods which have been developed to measure MTX and 7-OH-MTX but none have been validated to measure MTX and 7-OH-MTX in urine and plasma with a high enough sensitivity to detect adherence.

Previous studies have suggested that MTX-P may occur in individuals genetically predisposed to the disease but no genome wide association study of MTX-P in a hypothesis-free experiment has been conducted to establish this.

The aims of this research were therefore to i) develop an assay to measure MTX levels; ii) test the ability of the assay to measure adherence; iii) Investigate if MTX levels correlate to change in disease activity or are associated with response in an observational study; and iv) conduct a genome wide association study (GWAS) investigating MTX-P.

5.1 Developing a HPLC-SRM-MS Assay to Measure MTX/7-OH-MTX Levels in Urine and Plasma

In the first step, I developed a HPLC-SRM-MS assay to measure MTX/7-OH-MTX in urine and plasma. The strengths of the study design included, first, developing the assay in water. This allowed me to optimise the detection of MTX and 7-OH-MTX in a pure system to ensure the detection method was robust, before moving on to test in biofluids. Biofluids contain numerous molecules, which could potentially, interfere with the detection of the molecules of interest. If such interference had occurred, it may have been difficult to establish whether that was a consequence of the detection method itself or a result of matrix effects in the biofluid. Second, EMA guidelines were used as a benchmark with which to test the ability of the assay in several domains, including accuracy, recovery, stability and intra-day precision. Validation testing of the

assay revealed that the assay fulfilled EMA guidelines in a number of domains. Carryover was acceptable with less than 1% of carryover following injection of 1000 nM MTX/7-OH-MTX which reached less than 20% of the LLOQ as required by the EMA. Accuracy was within 15% of the expected concentration for the majority of samples tested in water and linearity testing demonstrated that the assay was linear. Precision testing revealed good intra-day precision with CV ≤ 15% from the LLOQ to 250 nM MTX/7-OH-MTX. In order to detect adherence the LLOD/LLOQ is of particular importance to ensure a high sensitivity of the assay. As MTX is dosed weekly in standard rheumatology care; it was, therefore, essential that the assay was sensitive enough to detect MTX several days after ingestion as, in routine clinical practice, patients will not always be seen at the same time following their MTX dosage; if this test is ultimately to translate to the clinical setting, ensuring adequate sensitivity was vital. Third, all measurements were performed in triplicate and negative controls were included to ensure robustness of the findings.

Having confirmed that the assay performed well in water, I then tested it in urine and plasma. The use of urine samples from patients with RA was a major strength of the design because the samples are representative of the patient group in which the test will ultimately be used. They have high levels of inflammation and are an older population with more co-morbidities than the general population. Hence, the assay was shown to be robust in a relevant clinical setting with high levels of systemic inflammation being present. Using mixed urine and plasma, samples had the advantage of being able to assess matrix effects that may be present. All assays were performed in triplicate and negative controls were included to ensure robustness of the findings. Validation testing of the urine assay revealed that the assay fulfilled EMA guidelines in a number of domains [221]. Carryover was acceptable with less than 1% of carryover following injection of 1000 nM MTX/7-OH-MTX which reached less than 20% of the LLOQ as required by the EMA. Accuracy was within 15% of the expected concentration and linearity testing demonstrated that the assay was linear. Precision testing revealed good intra-day precision with CV ≤ 15% from the LLOQ to 1000 nM MTX/7-OH-MTX.

Whilst other methods have been developed for the detection of MTX and/or 7-OH-MTX in urine as shown in Table 1-11 (Section 1.9) the current proposed method has several advantages. Pharmacokinetic models of MTX and 7-OH-MTX have demonstrated a higher $T_{1/2}$ of 7-OH-MTX which is suggestive that 7-OH-MTX may be a more sensitive biomarker of adherence as it may be detected for longer [125]. Therefore, one of the major strengths of the current study was the ability to simultaneously and directly compare the measurement of MTX and 7-OH-MTX. Furthermore, this work has demonstrated that the analytes are stable for several days in urine. Stability testing of MTX and 7-OH-MTX in urine revealed that there was no significant loss at room

temperature for three hours or frozen for seven days but significant loss at room temperature following three days. These results informed the MEMO study sample preparation operating procedure so that urine samples were processed and frozen within 30 minutes to avoid significant loss of analyte. This is vital when considering a test that could ultimately be introduced in the clinic as samples are likely to be tested in batches rather than immediately after venepuncture or urination.

Whilst immunoassays for the measurement of MTX have the advantage that they are rapid and samples require little sample preparation prior to testing, they cross react with other substances such as DAMPA, reducing their specificity and are not sensitive enough for the detection of low-dose MTX used as treatment for RA [153]. The current proposed method has a lower MTX and 7-OH-MTX LLOQ when compared to previous published assays that can measure MTX and 7-OH-MTX in urine [142]. HPLC-SRM-MS has the advantage of improved specificity compared to immunoassays and may, therefore, be an improved platform for measuring MTX and 7-OH-MTX. The use of an internal standard of similar structure to the analytes which is not separated by HPLC but can be identified by SRM controls for variation in analyte due to recovery and ionisation efficiency can thus compensate for matrix effects. Mass spectrometers allow for a high sensitivity of detection, improving the LLOQ and ability of the assay to detect adherence. A number of other studies, utilising HPLC-SRM-MS with solid phase extraction, report an LLOQ for MTX below the LLOQ of the current assay in urine [163, 164]. An advantage of solid phase extraction is that it further purifies the biofluid leading to increased specificity and reduced SNR; however, it increases the time for analysis of samples and increases the cost of the assay per sample. Therefore, a method with reduced sample preparation, like the one described herein, is advantageous as it can be more easily adopted by other laboratories in the future. Furthermore, the LLOQ was shown to be sufficient for the samples tested.

A major limitation of using HPLC-SRM-MS is that start-up costs and maintenance costs can be high. The methods require a high level of technical skill and involve sensitive equipment that is prone to breaking down requiring extended down-time which can take a prolonged period to repair [229, 230]. Environmental conditions within the laboratory need to be controlled to ensure stability, for example, a change in the laboratory temperature can alter elution time. Nonetheless, such methods are routinely used in NHS services, for example for the measurement of vitamin D and its metabolites. No assessment of the cost of the test has been included in the current work but it is important to incorporate that in future work, including processing costs, technician time and hardware costs.

On transferring the assay to urine it was discovered that there was a higher LLOQ for MTX (5 nM) and 7-OH-MTX (10 nM) compared to water alone due to increased noise. Results from the recovery experiment demonstrated lower recovery for MTX suggestive of significant loss of MTX during sample preparation which may reduce the ability of the assay to detect low concentrations at the LLOQ. There was no significant loss of 7-OH-MTX. Investigating matrix effects in urine compared to water alone revealed that there was an effect on MTX/MTX-d_3 which falsely increased the calculated concentration of 7-OH-MTX. This is suggestive of a need for a separate internal standard for 7-OH-MTX but unfortunately there were none commercially available at the time of the experiments. Furthermore, the calibration curve was based upon pharmacokinetic data in plasma with an upper limit of 1000 nM. As MTX and 7-OH-MTX are actively and passively excreted, urine samples were of higher concentration, requiring significant dilution which increased assay cost and reduced throughput. Finally, there were only a small number of mixed urine samples which may have not captured all the variability in the RA population.

The proposed assay for plasma has a number of specific advantages. It has a low LLOQ for MTX (0.5 nM) and 7-OH-MTX (0.75 nM) without the need for solid phase extraction to improve sensitivity for detection of adherence. The LLOQ is the same as that in water and lower than in urine. It is likely that there is reduced noise in the matrix due to cleaner protein precipitation in plasma compared to urine. The assay has demonstrated minimal carryover following injection of 1000 nM MTX/7-OH-MTX which does not reach 20% of the LLOQ. The assay fulfils the requirements of the EMA for precision of MTX but for 7-OH-MTX at the LLOQ only the CV was 21%, a CV ≤ 20% is required to fully fulfil EMA guidelines. Mean accuracy was within 15% demonstrating accurate measurements of MTX/7-OH-MTX. Recovery demonstrated improved recovery with methanol as the organic solvent for protein precipitation. Mean recovery was within 15% of the expected concentration within the calibration curve with the exception of two concentration levels for MTX and one for 7-OH-MTX. Stability testing revealed that the analytes were stable up to 28 days at -80°C; this allows for samples to be frozen and stored before testing to improve throughput and reduce assay costs.

A number of assays based on HPLC-SRM-MS exist for the detection of MTX in plasma [162] but few have been validated to detect MTX and 7-OH-MTX [166, 168]. Of those assays that can detect MTX and 7-OH-MTX in plasma, the LLOQ is too high to be used as a measure of adherence [168]. The assay published by den Boer et al. [162] requires no solid phase extraction and has an LLOQ of 5 nM MTX but is not validated to measure 7-OH-MTX and the system requires flushing for 10 minutes with 100% methanol after the injection of 80 samples, suggesting that this method

carries a risk of column blocking with salts causing system shutdown resulting in significant downtime.

Testing of the performance of the assay in whole blood demonstrated that there was reduced recovery of MTX/MTX-d_3 compared to plasma which falsely inflated the measurement of 7-OH-MTX. This is suggestive that there are inhibiting factors affecting the ionisation of MTX/MTX-d_3 but not 7-OH-MTX and that 7-OH-MTX requires its own internal standard but unfortunately there is none commercially available. These effects are presumably also present in other assays which measure 7-OH-MTX but this has not been reported [166, 168]. The results of this experiment suggest that MTX is the preferred analyte to measure for adherence as the measurement is not falsely inflated given that it is possible to use an internal standard structurally similar to MTX. Results of the stability experiment revealed that there was no significant loss of analyte at room temperature up to two days. This result supports the use of this assay in the RAMS samples, where it can take up to three days for the samples to arrive to the central laboratory by post.

The blank plasma samples were obtained from the NHS Blood and Transplant service and would therefore only capture the variability in a healthy population and not the RA population. In order to investigate the recovery of MTX/7-OH-MTX from whole blood, due to ethical constraints, whole blood was obtained from one healthy volunteer and was not a mixed sample and may therefore not have captured variability in the matrix present in the RA population.

In conclusion, the results of validation testing of the assay have demonstrated that I have successfully developed an assay requiring minimal sample preparation which can accurately and sensitively measure MTX and 7-OH-MTX in urine and plasma. Whilst the assay does not fulfil all domains of the EMA guidelines, the majority are surpassed. It was necessary to conclude that the assay was good enough for its intended use due to time restrictions rather than continuing to develop the assay to fully fulfil EMA requirements. In order to fulfil the EMA requirements to ensure that the assay could be used in the clinic, it requires further precision testing and external validation with the use of another mass spectrometer to assess reproducibility. Improvements in the urine assay may be gained by investigating the use of a higher calibration curve to reduce the need for dilution of samples. The results have suggested that 7-OH-MTX requires its own internal standard due to the different matrix effects on MTX/MTX-d_3 compared with 7-OH-MTX, but unfortunately none is currently available. The stability results have demonstrated that MTX and 7-OH-MTX are stable in frozen plasma for up to 28 days and at room temperature in whole blood for up to two days. These results are reassuring that the assay could be transferred to clinical use, as samples could easily be processed in secondary care laboratories to extract plasma and then frozen for future testing, this is easier than sample preparation requirements for MTXPG [111].

Overall, the results establish that the assay is suitable for use as a research tool and fulfils many of the domains as required by the EMA.

5.2 Measurement of MTX and 7-OH-MTX Metabolites in Urine and Blood of Patients with Rheumatoid Arthritis: The MEMO Study

The MEMO study was designed to develop a pharmacokinetic model of MTX and 7-OH-MTX to test the ability of the HPLC-SRM-MS assay to detect adherence. The study design was based on prior pharmacokinetic knowledge of MTX and the assay results. The study was designed by the author with input from his supervisory team and was accepted by the regional ethics committee, the Research and Development department at CMFT, the scientific advisory board at WTCRF and sponsored by the University of Manchester. The review and acceptance of the study by multiple boards is evidence of the robust study design. The process to acceptance and eventual start of the study was much longer than originally anticipated despite the regulatory elements being conducted in parallel. The overall process is not streamlined and requires independent input from a number of stakeholders which delayed the acceptance of the study. The MEMO study required patients to be admitted for 24 hours, undergo regular venepuncture and 24 hour urine collection and return on two further occasions within seven days of the first visit. It was advantageous that overall acceptance of the study by patients was relatively high with 10% of those who were invited to the study being recruited; meaning that recruitment was not a significant rate-limiting step in the process.

There are a number of strengths of the study due to its design. The inclusion criteria were chosen to ensure that the cohort were similar to the wider RA population treated with MTX but that they were taking stable dose MTX to ensure steady state, allowing for the pharmacokinetic model to be developed. Multiple sampling over the first 24 hours, requiring an inpatient stay, and at two time points over the course of the next seven days allowed for an accurate pharmacokinetic model to be produced. The measurements of MTX/7-OH-MTX were performed in triplicate with quality control measures in place to ensure measurements were accurate. Both urine and plasma were collected to evaluate the ability of the assay to detect adherence in urine and plasma. MTX and 7-OH-MTX were measured in the biofluids to evaluate the more sensitive analyte for the detection of adherence.

There are, however, limitations of the study which need to be recognised. The sample size was small (n=20) but this is similar to other pharmacokinetic studies and subsequent analysis suggests that it was sufficient to produce a pharmacokinetic model. Whilst the inclusion criteria were

broad, there may have been recruitment bias due to participants needing to be healthy enough for an inpatient stay; this limitation meant that no participant with moderate renal impairment was recruited, creatinine levels did not, therefore, inform the pharmacokinetic model. Previous studies have had shorter inpatient stays which may have improved the recruitment of patients with reduced renal function but at the expense of a pharmacokinetic model that can accurately describe the elimination phase over a six day period [138, 142].

The urine results from the MEMO study revealed that no sample was below the LLOQ for MTX which is evidence that MTX is the preferred analyte for the detection of adherence in urine. It was reassuring that the samples which were below the LLOQ for 7-OH-MTX were towards the end of the study period for each individual patient with no sample being detectable and then undetectable, indicating reliability of the data with content validity. Reviewing how the MTX/7-OH-MTX concentrations change over time is also reassuring as the levels generally peak and then reduce over time as would be expected (Figure 4-35 and Figure 4-36). A disadvantage of the study is that the urine data cannot be used to develop the pharmacokinetic model as the MTX/7-OH-MTX concentrations in urine are a function of unmeasured effects such as MTX/7-OH-MTX clearance, the amount a patient has had to drink, bladder size and comorbidities such as overactive bladder syndrome. A further disadvantage of the assay when being used to test samples from patients is that the calibration curve in urine was too narrow as urine needed to be diluted by up to 150 times. This increases the possibility of variability of the MTX/7-OH-MTX concentrations due to inevitable small pipetting errors, increases the time for analysis and cost for analysis. With no downtime of the HPLC-SRM-MS, to measure each urine sample from the MEMO study in triplicate taking into account the required dilution of samples would take seven days. In reality, due to system downtime, measurement of the samples took much longer.

Plasma samples from the MEMO study required dilution for the first two samples only, this was advantageous compared to urine as it improved throughput. Furthermore, compared to urine, fewer measurements were rejected due to poor precision (CV > 15%), this indicates that plasma is a more appropriate biofluid for measuring concentration of MTX/7-OH-MTX in patients using HPLC-SRM-MS, as was suggested from the assay development experiments. However, not all samples were above the LLOQ for MTX/7-OH-MTX, suggesting that urine may be a more sensitive biofluid for the detection of adherence but this finding requires further validation. Unfortunately there was no separate cohort available for the validation of the urine findings.

A specific advantage of the plasma measurements was the possibility to develop a pharmacokinetic model of the data to simulate the proportion of samples expected to be below the LLOQ given different doses of MTX. The resultant pharmacokinetic profile was a two

compartment model for MTX as has been suggested previously in several studies [122, 125, 126, 139, 140]. Due to study design heterogeneity it is not possible to compare the individual pharmacokinetic estimates between studies. The visual predictive check showed, however, that the pharmacokinetic model developed explained the data well. Serum creatinine was not an informative covariate in this model, by contrast with the study by Godfrey et al. [142]. This may be due to the lack of creatinine variation in the population studied. Whilst the MEMO study was observational and unable to control for concomitant therapy such as NSAIDs, which have been shown to affect MTX pharmacokinetics, subsequent validation of the model from RAMS suggests that this is inconsequential. The simulated data demonstrated that following ingestion of 20 mg MTX, 88% of patients should have detectable levels of MTX at six days and confirmed that MTX was the better analyte to measure to detect adherence.

In conclusion, whilst the urine data from the MEMO study has demonstrated ability to detect adherence to MTX, there are a number of limitations which need to be taken into account. The urine results therefore need to be validated from an external cohort but unfortunately at the time of this study there was not a suitable cohort available to validate these results. The plasma data was highly informative in being able to produce a pharmacokinetic model which was used to simulate MTX/7-OH-MTX levels expected to be below the LLOQ. The results suggest that the majority of patients should have detectable levels of MTX following ingestion of 10 mg or more of MTX after a period of six days.

5.3 Validation of the Pharmacokinetic Model

A major strength of the work presented is that, using sparse samples of plasma to measure MTX/7-OH-MTX concentrations from the RAMS study, it was possible to validate the pharmacokinetic model developed as part of the MEMO study. The results showed that the developed MTX pharmacokinetic model performed well at predicting MTX concentrations but that the 7-OH-MTX pharmacokinetic model predicted concentrations to be lower than they were in RAMS. There are several possible reasons for this observation. Firstly, development of the 7-OH-MTX assay revealed that recovery from whole blood was much higher than would be expected and that the assay may require a 7-OH-MTX internal standard. Processing of the MEMO samples was protocol driven whereas samples from RAMS were sent in the post and took up to three days to arrive; it is feasible that the different processing of samples led to increased 7-OH-MTX concentrations. Secondly, the time for RAMS samples to reach the laboratory may have allowed higher 7-OH-MTX levels to develop through ongoing metabolism of MTX to 7-OH-MTX; MTX levels are not however significantly below the 90% prediction interval and the stability results from assay validation lends evidence that the samples were stable for up to three days.

Thirdly, the RAMS cohort may significantly differ in their ability to metabolise MTX to 7-OH-MTX compared to the MEMO cohort, the RAMS cohort had a significantly higher proportion of male patients compared to MEMO which may be a contributing factor requiring further investigation.

To date, there have been no published assays validating a direct method of measuring adherence to MTX in RA. A previous study suggested the use of MTXPG levels but did not test in the ability of the assay to measure adherence or replicate the findings in an independent study set [110]. Furthermore, there are inherent difficulties in the sample preparation requirements to measure MTXPG concentration. Erythrocytes need to be either separated at source or the whole blood sample frozen at source and centrifuged within two days otherwise the MTXPG levels are not stable [111]. Use of the current proposed MTX assay to detect adherence has the advantage that stability experiments revealed that the MTX levels were stable for up to two days at room temperature and that in plasma analytes were stable at -80°C for up to 28 days, allowing samples to be tested in a batch, increasing throughput and reducing cost.

The fact that the MTX pharmacokinetic model was validated in RAMS is a very important result; supporting the idea that the test can be extrapolated to patients with early RA in general. Use of the RAMS diary adherence data made it possible to validate the assay to measure adherence. Only two samples showed undetectable levels of MTX. One sample was from a patient who had not taken MTX for 58 days but continued to participate in RAMS and was not aware that they should have continued treatment. MTX/7-OH-MTX was undetectable for this patient, indicating the negative predictive value of the assay and its specificity, although further samples will need to be tested to explore the specificity and negative predictive value of the assay. One patient showed undetectable levels 148 hours after ingestion of 20 mg MTX. This is to be expected from the simulations which estimate that 18% of samples will be undetectable at this time. An alternative explanation, however, is that the patient was not correct about when they took their MTX, this reveals the disadvantage of using the RAMS samples to validate the adherence assay as it is being compared to indirect measures of MTX adherence and relies on patients recording when they took their MTX which may not be fully accurate. In order to estimate the proportion of samples which were undetectable thereby suggesting non-adherence, it was necessary to bin the times from the RAMS samples across many hours. This is not a precise technique and is a disadvantage of using sparse datasets. Nonetheless, the results from RAMS crudely showed that the simulations were accurate and that the assay may have actually performed better than expected at detecting adherence.

It is vital that clinicians can be confident in the result of an assay to detect adherence prior to discussing the assay results with the patient, the consequences of a false negative may be the

irrevocable breakdown in the patient-physician relationship. The results have suggested that the detection of MTX in urine may be a more sensitive biofluid for the measurement of adherence. Whilst all of the urine samples tested in MEMO showed the positive detection of MTX, this finding needs to be replicated in order to confidently prove that urine can detect adherence correctly over a six day period. The advantage of using plasma as a biofluid is that it was possible to simulate the ability of the assay to detect adherence and validate these findings in a separate cohort, therefore the limitations of using plasma are more defined. Further work is therefore required to evaluate the sensitivity of the assay to detect adherence using urine as the biofluid. As it is not possible to model the excretion of MTX in urine, a large study would be required to measure MTX in a number of patients with a wide range of eGFR, age and body weight over a prolonged time period to fully evaluate the sensitivity of the assay. However, the study would be limited by its inability to model the results and therefore the full sensitivity of the assay may not be evaluated due to sampling bias.

Whilst the results of this study have shown that the assay is a promising objective measure for the detection of adherence, this test is not a panacea and cannot detect adherence to MTX with certainty, as the simulation results have indicated. A high degree of sensitivity is required for an assay to be utilised within the clinic to detect adherence so that the clinician can be confident in the results. Validation testing of the pharmacokinetic model within RAMS demonstrated however that the assay may outperform the simulations. Only one sample, which would have been expected to be positive for the detection of MTX, was negative. Further work is required to determine the sensitivity of the assay to detect adherence and try to improve it. A separate much larger study within RAMS could be conducted but this would be limited by the identification of the timing of MTX ingestion being patient self-reported, which may not be fully accurate. To improve assay sensitivity, the LLOQ could be reduced by the use of SPE, this would improve the sensitivity of the assay to detect adherence. It was decided in this study to not undertake SPE due to cost and time implications, a further study would therefore be required to evaluate whether SPE could improve sensitivity, as the samples from MEMO have been stored, this could be conducted and a further pharmacokinetic model/simulation performed. Alternatively, both urine and plasma could be measured simultaneously in the clinic to improve the sensitivity of the test or multiple samples of plasma taken in different weeks over time may increase the test sensitivity.

Adherence is defined as the '*the extent to which a patient acts according to the prescribed interval and dose of a dosing regimen*' [77]. There are therefore a number of different health behaviours which could be identified as non-adherence. A major limitation of the proposed assay is that it has the ability to detect adherence in the past six days of a clinic visit and the MTX level measured

cannot be used to determine the MTX dose that the patient took due to the wide interindividual differences in MTX pharmacokinetics. The assay would therefore not correctly detect non-adherence in patients who become adherent prior to a healthcare appointment, change their dose of medication or who took a drug holiday. In order to improve the sensitivity of the assay to detect adherence under these circumstances, the addition of another biomarker such as MTXPG could be used to determine adherence over a prolonged period of time and adherence in the past six days simultaneously. It was not possible to evaluate the use of MTXPG to detect adherence over a prolonged period of time in the current study due to time and cost restraints but given the long half-life of MTXPG, theoretically this would be an improved biomarker to detect non-adherence due to a drug holiday. MTXPG measurement would not however be able to detect non-adherence due to alteration of the prescribed dose due to the wide interindividual differences in MTXPG pharmacokinetics.

The implications of this study are that the assay developed is a sensitive tool to measure adherence to oral MTX in patients with RA but requires further samples to determine the sensitivity/specificity of the assay. The results from the RAMS study can be used *a posteriori* to update the pharmacokinetic model and improve the simulation testing to detect adherence.

5.4 Investigating Association between MTX/7-OH-MTX Levels and Response

Previous studies have shown that higher 7-OH-MTX excretion is associated with reduced response [144] and higher MTX dose and plasma MTX AUC correlate with responder status [131, 140], but results are conflicting [148].

Measurements from RAMS were used to estimate MTX AUC using the developed MTX pharmacokinetic model and investigate whether MTX AUC correlates with change in DAS-28 over three months or is associated with EULAR response. The results showed no correlation or association and the null hypothesis was upheld. There are a number of possible reasons for this. MTX AUC may not be associated with response and the null hypothesis may be true however, given that previous studies have shown association with MTXPG levels and response it is theoretically probable that MTX AUC at steady state correlates to MTXPG levels and therefore is associated with response [149, 150]. An alternative explanation is that MTX AUC does not correlate with MTXPG levels and that MTXPG levels show inter-individual differences due to the ability of different individuals to metabolise MTX to MTXPG and differences in clearance of MTXPG. The study by Hornung et al. demonstrated that MTX AUC correlated with responder status. The study recruited both new and established MTX-treated RA patients and defined

responders according to fulfilling the ACR response criteria on at least one occasion over a 52 week period [131]. The study did not control for concomitant medication such as corticosteroid use. There are therefore limitations to the Hornung study which are not present in the RAMS study. The study categorised responders as those who fulfilled the ACR response criteria on one occasion during a number of follow-up visits over a year long period. It is likely, therefore, that a subset of responders were not true responders. The RAMS study attempted to control for corticosteroid use by limiting usage to less than 30 days to ensure that response to therapy was due to MTX monotherapy alone and excluded participants who started a second DMARD within the first three months of RA therapy. The study by Bologna et al. was a prospective controlled study investigating the correlation between MTX AUC and endpoint RAI in an established RA cohort (mean disease duration 8.5 years, n=22) [140]. Whilst the authors were able to ensure that disease modifying therapy was limited to MTX monotherapy, they did not show that MTX therapy correlated with disease response, only current disease activity. Whilst current disease activity is important, it may be affected by a number of different factors, such as a cohort of patients who had low disease activity at baseline; it does not, therefore, prove correlation between MTX therapy and change in disease status. In order to avoid this limitation, participants from RAMS with baseline and three month DAS-28 were recruited to investigate the correlation between MTX therapy and change in DAS-28 in an early RA cohort. Unfortunately I was unable to investigate whether higher 7-OH-MTX:MTX levels were associated with reduced response as the 7-OH-MTX pharmacokinetic model from the MEMO study was not validated with the RAMS samples. Further improvement in the pharmacokinetic model is required to investigate whether higher 7-OH-MTX:MTX levels predict non-response. This would require using the results from RAMS to update the model *a posteriori* and re-validating the updated model from a separate cohort. It has been shown in previous studies that adherence is associated with treatment response [87]. The majority of patients recruited from RAMS were adherent and therefore the correlation between non-adherence and inadequate response could not be evaluated.

In investigating response to treatment in RA, an accurate marker of response is required that measures active joint disease. DAS-28 is useful clinically as it contains domains that inform the clinician of the overall health of the patient; but unfortunately, these can falsely elevate the DAS-28 in patients who have lower levels of joint inflammation. The DAS-28 formula is weighted such that tender joint count is valued more than swollen joint count which can be elevated in other illnesses such as depression rather than due to active joint disease [36, 37, 294]. In evaluating MTX response from a pharmacokinetic-pharmacodynamic perspective a more sensitive tool for

disease activity may be required which measures active joint disease and is not confounded by patient comorbidities or illness behaviour.

In conclusion, the investigation of MTX AUC and response to MTX revealed no association and the null hypothesis was upheld. Further updating and validation of the pharmacokinetic model is required to investigate the role of 7-OH-MTX.

5.5 Investigating the Genetics of Methotrexate-Pneumonitis

MTX-P is a life-threatening adverse event to MTX that occurs as an immunologically-driven response to MTX. Epidemiological and candidate gene studies reporting that the risk of MTX-P is increased by genetic factors are inconsistent and generally underpowered [214, 215, 217]. To date, only one study has reported evidence that HLA-A 31:01 is significantly associated with MTX-P but that finding has not yet been replicated [217]. However, the ability to stratify patients according to risk of developing MTX-P would be a major milestone in the treatment of RA.

This was the first GWAS undertaken to investigate genetic factors predisposing to MTX-P and has been performed in the largest cohort of physician-diagnosed MTX-P to date. However, no SNPs were associated with MTX-P at genome-wide thresholds. Suggestive evidence for association with three variants was detected but requires confirmation / replication in larger data sets. None of the SNPs mapped to the MHC region and the previous report of an association with HLA-A 31:01 was not replicated.

There are a number of strengths of this study. A large number of cases were recruited (n=65), compared to previous studies of MTX-P increasing the power to detect significant associations [177, 184, 189, 217]. Given that the classification criteria have not been validated, inclusive criteria were chosen in order not to exclude potential MTX-P cases. The very nature of GWAS is that the approach is hypothesis-free with low significance thresholds; reducing the chance of a type 1 statistical error, a common occurrence in candidate gene studies with failure to replicate the initial findings. GWA studies require stringent quality control measures to avoid type 1 statistical errors. Robust genotyping and quality control procedures were set and a visual check of the quantile-quantile plot indicates that these measures were successful in reducing bias due to genotyping error or population stratification.

Some limitations to the study design are also apparent and may have affected study power. First, the RA controls demonstrated heterogeneity with only 69% fulfilling the 1987 ACR classification criteria (Table 4-39, section 4.10.3). Heterogeneity in control definition may reduce the ability of the GWAS to discover SNPs associating with MTX-P. It is thought that prevalence of MTX-P is higher in RA compared to other inflammatory diseases such as psoriasis. Therefore heterogeneity

of controls may dilute the proportion of true RA controls at higher risk of MTX-P, reducing the power of the study. However, restriction of the analysis to those who fulfil classification criteria for RA did not alter the conclusion that no confirmed loci association with MTX-P were identified. Second, case definition of MTX-P was limited to physician diagnosed. Whilst the majority of cases (77%) fulfilled either the Carson et al. or Searles et al. criteria, a proportion did not fulfil either criterion. It was decided to retain physician only diagnosed MTX-P cases as the classification criteria have not been validated and reducing the number of cases would reduce the power of the study, increasing the chance of a type 2 statistical error. Restriction of the analysis to cases that fulfilled the criteria did not qualitatively affect the results of the GWAS ($p < 5 \times 10^{-8}$). Case definition heterogeneity may reduce the power of the GWAS as including patients with different diseases introduces the possibility of bias. There is therefore a balance between reducing power due to reduced numbers of cases and reduced power due to case definition heterogeneity. Third, the current GWAS was designed to investigate common variants (MAF>0.01%) however, a common variant may not result in a rare disease. By definition a common variant that causes disease would be expected to be in a large proportion of the population but the prevalence of MTX-P suggests that, if the disease has a substantial genetic component, that the variant may be rare. In order to investigate rare disease causing variants, further MTX-P cases are required to improve the power of the study so that the rare variant is detectable reducing the risk of a type 1 statistical error (section 3.11.12). To investigate this further, low frequency and rare variants that map to the region could be grouped together (collapsing approach) to improve power [295]. The overall burden of low frequency/rare variants could then be compared for each gene between cases and controls providing evidence that the gene is associated with disease. Resequencing the exome to identify novel variants that are only present in MTX-P cases is another approach that could identify rare variants associated with disease [296]. This approach successfully identified rare MLL2 mutations that cause the rare Kabuki syndrome in a cohort of unrelated cases (n=10) [297]. Finally, controls were only followed-up for one year after commencement of MTX whilst in the MTX-P cohort, the median time to MTX-P development was 21 months. There is therefore the possibility that a number of controls could develop MTX-P over time which would reduce the power of the study. Finally, due to ethical constraints, recruitment was limited to patients that survived MTX-P (survivor bias). It is possible that including genetic data on patients who died due to MTX-P would reveal a genetic marker associated with MTX-P that has a larger effect size.

The results demonstrated suggestive evidence that rs7514182 may be associated with disease but requires replication in a separate cohort. The SNP is in perfect LD with the lead SNP (r^2=1.00) tested on the array and there is evidence that it is a regulatory element. The SNP affects

expression of connexin 40, which has been shown in knock-out mice to produce a phenotype similar to MTX-P. A proposed model for the development of MTX-P is, therefore, that individuals with reduced expression of connexin 40 are at increased risk of developing MTX-P on exposure to MTX. Further experiments with chromosome capture conformation (3C) would provide evidence that rs7514182 is a regulatory element for connexin 40. SNPs which, in the linear DNA sequence, appear distant from a functional gene involved in disease may still affect gene expression due to the fact that the variant lies close to the gene when DNA is folded in its natural state. Therefore, 3C, a technique that allows the study of the spatial conformation of DNA, could identify which gene the most associated variants in the region interact with in the natural folded state of DNA. This method was used by Pomerantz et al. to identify c-myc as the causal gene for colorectal cancer despite the fact that the most associated variant on chromosome 8q mapped some distance from the gene [298].

The results have failed to replicate the association between HLA-A 31:01 and MTX-P. This lack of replication may be due to a number of reasons. The prevalence of this allele is 12.5% in the Japanese population but 2.3% in Northern European populations [299, 300]. In order to replicate the effect size seen in the Japanese population a larger cohort of cases and controls (n=118 and 354 respectively) would be required for an adequately powered study (80% power) in a Northern European cohort. The lack of replication may be due to a false positive detection (type 1 statistical error) in the first study. The biological plausibility of HLA-A 31:01 to cause MTX-P is low as it would be expected that the ratio of CD4:CD8 in bronchoalveolar lavage would be reduced in patients with MTX-P but the opposite effect is seen [181]. Therefore, it would be interesting to see whether the association can be replicated in an independent population. Meta-analysis of the current and the Japanese data may enhance power to detect true novel associations but would require adjusting for population stratification.

In conclusion, the results of the current genome wide association study have revealed that HLA-A 31:01 is not associated with MTX-P in a European population but the study was underpowered due to the reduced prevalence of HLA-A 31:01 in Europeans compared to Japanese. rs7514182 is weakly associated with MTX-P but does not reach genome wide significance levels. rs7514182 affects the expression of connexon 40, a potential role in the development of MTX-P. Further studies are required to explore the impact rs7514182 has on the expression of connexon 40 in human cell lines.

5.6 Implications of the Programme of Work and Future Work

A main aim of this programme of work was to produce a clinically useful test to detect adherence based on the assumption that detection of adherence leads to improved adherence and efficacy. Previous research has shown that low adherence has been associated with reduced response [87]. An assay that performs well in detecting MTX usage has been produced but the results failed to demonstrate that the use of the assay predicts response. There are a number of possible reasons for this. Power may be an issue as the study sample was moderate, recruitment to RAMS is ongoing, therefore, in the future further measurement of samples could be undertaken to address this issue. The majority of patients recruited from RAMS were adherent and I could therefore not assess whether non-adherence reduces response in this cohort. Future work to overcome this limitation could include a further study within RAMS, including all patients prescribed 20mg MTX, where it is predicted that the assay should detect over 80% of adherent patients after 140 hours of MTX ingestion. This would allow an assessment of whether direct measurement of adherence can be used to predict response. Furthermore, the measurement of response using three month DAS-28 may not be an objective enough marker and further analysis of MTX AUC with other response markers such as VECTRA® DA or joint imaging is warranted. Whilst the analysis suggested that change in tender joint count correlated with MTX AUC, the more objective components of the DAS-28 did not significantly correlate. Adherence to long-term medication may reduce over time and is unlikely to be static, a further study utilising the adherence assay to investigate MTX adherence in patients prescribed MTX over a prolonged period of time and whether this correlates with disease activity should therefore be undertaken [301]. Whilst biologic therapy is effective, the associated drug costs are high, it is therefore vital that biologic therapy is as effective as it can be. MTX has been shown to reduce the development of anti-biologic antibodies which may reduce efficacy [302]. The MTX assay could be applied to patients co-prescribed MTX and biologic therapy to investigate the proportion of patient's adherent to MTX and whether adherence is a predictor of response. Adverse events are a significant cause for non-persistence to therapy; whilst the current program of work has investigated response, the MTX assay could be used in the future to determine whether MTX levels within RAMS predict the development of adverse events. The prediction of adverse events would be a major development in the treatment of RA. Finally, low-dose MTX is used in other diseases apart from RA, such as psoriasis and psoriatic arthritis. The MTX assay could be applied in these disease states to investigate whether use of the assay predicts response.

If the assay does prove to be clinically useful in assessing adherence and response, the practicalities of using the assay would need to be assessed, taking into account factors such as the cost of the assay, time for analysis, collection of blood samples and laboratory requirements.

Patients who are prescribed MTX are required to have regular blood tests and therefore 50 µl of the plasma from monitoring bloods could be used to measure MTX levels to reduce patient inconvenience. The results of stability experiments revealed that MTX is stable in whole blood at room temperature for up to two days, therefore, samples from the community would not be degraded by the time they arrived at a central laboratory. Whilst detection of adherence and prediction of response is likely to be clinically useful, it is important to evaluate whether this can lead to an intervention that improves adherence. A randomised controlled trial to evaluate this could be undertaken whereby direct adherence in a cohort of RA patients is measured using the developed assay and half of the clinicians are given the results of the adherence test and the other half remain blinded to determine whether this alters adherence behaviour and improves response.

Adherence to therapy is complicated and physicians should not be judgemental but enablers who help patients improve their adherence. One reason for non-adherence is the fear of side effects. MTX-P is a life-threatening rare disease and fear of this side effect may reduce adherence to MTX. The results of a GWAS in a UK population have demonstrated potential SNPs associated with MTX-P. Future work is required to independently validate these results.

5.7 Final Conclusions

Adherence to MTX may be a significant barrier to patients achieving full response to therapy. The development of a direct test to detect adherence based on measuring MTX levels using HPLC-SRM-MS has been developed in urine and blood. The assay was shown to be accurate in several domains from EMA guidelines and was validated in a separate cohort of patients. Identifying patients non-adherent to MTX would be a huge clinical advance. Firstly, it could provide more accurate measures of adherence to MTX in the population. Secondly, in identifying patients in the clinic who are non-adherent it would allow for further doctor-patient discussions to take place and intervention to improve adherence which may improve overall response to therapy for the individual.

There are several factors that need to be taken into account in the use of an assay to detect adherence. Whilst cost is high, if the detection of non-adherence leads to an intervention that improves patient adherence and ultimately treatment response, the cost-benefit ratio may be beneficial. Patient acceptance also needs to be explored, consent to test for measuring MTX concentration would need to be given and patients who are non-adherent may withhold their consent. Furthermore, it is known that adherence to medication is improved before and after a healthcare appointment, use of the current assay under these circumstances would therefore

measure the patient as adherent, whereas long-term adherence to MTX is likely to be required for an improved sustained response to therapy over time given its long mechanism of action [96].

Finally, this program of work has investigated genetic markers associated with MTX-P. The results demonstrated a potential SNP associated with disease which demonstrates a functional role in the development of pulmonary fibrosis. The MTX-P results should be considered preliminary as they require external validation. If confirmed, the ability to stratify patients according to risk of MTX-P would be a huge advance in the treatment of RA, reducing the incidence of this life-threatening disease.

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Appendix 1

PARTICIPANT INFORMATION SHEET

MEMO Study:

Measurement of MTX and 7-OH-MTX metabolites in urine and blood of patients with rheumatoid arthritis: a possible measure of Methotrexate adherence

You are being invited to participate in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read this information sheet carefully and discuss it with relatives, friends and the study team. You may also wish to discuss this with your GP. Ask us about anything that is not clear and take time to decide whether or not you wish to take part.

Why have I been chosen?

You have been chosen to participate in this study as you have been diagnosed with rheumatoid arthritis (or early undifferentiated polyarthritis), have been taking methotrexate for at least 3 months, and are a participant in the Rheumatoid Arthritis Medication Study (RAMS).

What is the purpose of the study?

This study is looking at the formation of breakdown products produced by the body after methotrexate has been taken and if measurement of these products can be used to identify people who have correctly taken their medication. This study is being undertaken as part of an academic PhD.

As you know Methotrexate is a medication that is used to treat rheumatoid arthritis. Current research has suggested that up to 40% of patients may not take their methotrexate all the time, as prescribed by their doctor, for a number of different reasons such as side effects (this is called *non-adherence*). We also know that if patients do not take their medication as their doctor prescribed they may respond less well to the medication. It is therefore important to know if a patient took their medication or not.

At the moment we have no test to determine if a person is or is not taking their medication as prescribed. A test to measure methotrexate and its breakdown product (also called metabolite) has been developed. The main purpose of this study is to use this test to measure the levels of methotrexate and its breakdown product in the blood and urine to find out the best metabolite to measure, the best bodily fluid (urine or blood) and the ideal time to measure to determine if someone has taken their methotrexate as prescribed.

Do I have to take part?

No, you do not have to take part. It is up to you whether or not you want to take part in the study. Participation is entirely voluntary. If you do decide to participate you will be given this information sheet to keep and be asked to sign a consent form. You will receive a copy of this signed consent form to keep. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive or participation in the RAMS study.

What will happen to me if I take part?

If you do decide you would like to take part, your participation will involve the following:

1. Screening Visit (~60 minutes)

We will make an appointment for you at your convenience to visit the Wellcome Trust Research Centre next to Manchester Royal Infirmary. This visit will last for approximately an hour and the information collected at this visit will be used to see if you are suitable to take part in the full study.

At the centre a trained researcher will again go through what is involved in the study, answer any questions you may have and provide you with a consent form to sign before the assessment begins. The researcher will then guide you through the following assessments at your own pace:

- The researcher will ask you a few questions such as your age, ethnic group, any past medical problems and details of any medication you may be taking.

- They will then measure your height, weight, blood pressure, temperature and examine your chest, tummy and joints.

- Some blood samples will be taken which will be checked after the visit to see if you are suitable take part in the study.

- Information that has been collected as part of the RAMS study and your medical notes will also be reviewed to ensure you are suitable to take part in the study and for data collection.

After this visit we will check all the information you have given, as well as the blood samples, and contact you within a week by telephone to confirm if you are suitable for the study. If you are suitable, and you are still happy to participate in the study, we will then arrange a date when you would normally take your methotrexate for you to attend Visit 1 (see below) at the Wellcome Trust Clinical Research Facility.

2. Visit 1 (24 hours)

We will make an appointment for you, when you would normally take your methotrexate, at the Wellcome Trust Clinical Research Facility. This visit will last 24 hours and will require an overnight stay.

As before, a trained researcher will go through what is involved in the study, answer any questions you may have and check that you are happy to continue with the study. Over the next 24 hours, the researcher will guide you through the following:

- At the beginning of the visit we will measure your temperature, pulse and blood pressure, and ask you some questions about your general health and any medication you may be taking.

- You will then be asked to empty your bladder. Following this you will be asked to take your methotrexate as you normally would.

- Blood samples will then be collected throughout your visit by a trained researcher at the following times: 1, 2, 4, 8, 16 and 24 hours after you have taken your methotrexate. The total amount of blood taken at this visit will be approximately 63ml (this is around 13 teaspoons).

- We will also collect your urine over the 24 hours after you took your methotrexate.

- Following on from the last blood sample (24 hours after you took your methotrexate), the visit will be completed.

As you will be attending for 24 hours you may have a few questions about the practical arrangements involved in attending the Wellcome Trust Research Facility Centre which we hope are answered below. If you have any queries not covered, please get in touch with the research team (contact details are at the bottom of this sheet) who will be happy to answer any questions:

- You will be allowed to sleep, however if you are not awake when any of the scheduled blood samples are due the researcher will wake you.

- Complimentary meals and refreshments will be provided.

- Shower and toilet facilities are available.

- Space permitting you will be given a bed in a private room; however it may be necessary to give you a bed in a mixed ward.

- Car parking facilities are available at the research centre, free of charge.

- Sky television is available for entertainment.

- You will be allowed visitors during your stay.

3. Visit 2 (4 hours)

We will make an appointment for you, to attend the Wellcome Trust Clinical Research Facility within 7 days of Visit 1. This visit will only last ~4 hours.

As before, a trained researcher will go through what is involved in the study, answer any questions you may have and check that you are happy to continue with the study. The researcher will guide you through the following assessments.

- At the beginning of the visit we will measure your temperature, pulse and blood pressure, and ask if you have had any problems with your medication.

- A blood sample will be collected by a trained researcher.

- You will then be asked to empty your bladder. The researcher will then collect your next urine sample. After this sample the visit will be completed and you may leave the research centre.

4. Visit 3 (4 hours)

We will make an appointment for you, to attend the Wellcome Trust Clinical Research Facility within 7 days of Visit 1. This visit will follow exactly the same format as Visit 2 (above).

5. Withdrawal Visit

You are free to withdraw from the study at any time. If you do decide to stop the study before the end we will ask you to attend for a final visit. We will check your blood pressure, pulse, temperature and urine and discuss your reasons for leaving the study.

What are the possible benefits of taking part?

There is no intended immediate clinical benefit in taking part in this study. However, the information obtained from this study may result in changes in future treatment of patients with rheumatoid arthritis. These changes may also benefit you.

What are the possible disadvantages/risks for taking part?

Abnormal blood test results

If any blood tests taken during your participation in the study are abnormal then we will invite you back to the clinical research facility to explain the results to you. We will also ensure that you are referred to the appropriate clinician for further management.

Other possible risks

- When you give blood you may feel faint or experience bruising or tenderness at the site of the needle puncture.
- As you will be in the research centre for 24 hours on visit 1 this may cause some inconvenience.

Will I be paid for taking part?

You will be offered up to £200 for participating in this study. Travel expenses will also be reimbursed. If you do not complete the study you will be paid for your commitment to the study up to that point as below:

- Visit 1 (24 hours) attendance - £90
- Visit 2 (4 hours) attendance - £55
- Visit 3 (4 hours) attendance - £55

Serious non-compliance (not following the instructions) with the protocol and study restrictions may result in your suspension from the study with compensation fully withheld.

Please note, it is your responsibility to inform HM Revenue and Customs of the money (regarded as earnings) received for participating in this study. Should any enquiry be made by the Inland Revenue regarding payments from us, the study organisers would be required to disclose the amount to the Inspector. You are advised to keep a record – such

as receipts – of any expenses (e.g. travel etc.) which could be offset against tax. If you are in receipt of state benefits, you should check what your position is with regards to the payment of expenses for participating in this study, as the money being paid may be liable for tax.

What if there is a problem?

If you have a concern about any aspect of the study please contact a member of the research team who will do their best to answer your questions. If we are unable to resolve your concern or you wish to make a complaint regarding the study, please contact the University Research Governance and Integrity manager on 0161 275 8093 or Research.Complaints@manchester.ac.uk

What are my legal rights if I am harmed during the research?

In the event that something does go wrong and you are harmed during the research you may have grounds for a legal action for compensation against the University of Manchester or NHS Trust but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

Will my taking part in the study be kept confidential?

All the information collected about you during the course of the study will be kept strictly confidential and will conform to the 'Data Protection Act of 1998'. Any information about you which leaves the clinic will have your name and address removed so that you cannot be recognised from it. All samples will be coded and anonymised. All identifying details (e.g. name, address, date of birth) will be removed, but the sample will remain linked to data collected from this study (e.g. weight, age). Your medical records may be inspected by research staff for the purposes of checking that the information we have is accurate and complete. The blood and urine samples collected as part of this research study will be stored centrally at a laboratory at The University of Manchester. These samples are stored under strict security and are given a code so that researchers receiving your samples do not know your name or other personal details. Some of the samples of your blood and urine will be stored at the University of Manchester and may be provided to other bona-fide researchers working in the field for future research of rheumatoid arthritis and response to treatment. No identifiable data would be stored directly with your sample.

Very occasionally we may need to contact your GP to discuss any concerns if we identify that you are at risk of harm. We would first contact yourself to seek permission to discuss this with your GP. If consent to contact your GP is withheld we may breech the usual principles of confidentiality if there is a risk of serious harm to yourself or others.

Who is funding the research?

This study is funded by the Medical Research Council.

Who has reviewed the study?

This study has been reviewed by the National Research Ethics Service Committee North West – Greater Manchester Central

What do I do now?

One of our researchers will contact you by telephone in the next few days to answer any questions you may have and, if you are happy to take part, arrange a time that is convenient for you to attend the Wellcome Trust Clinical Research Facility for the Screening Visit.

Before you decide it is important for you to understand why the research is being done and what it will involve. Please take your time to read the above information carefully and discuss it with friends, relatives and your GP if you wish. Please ask if there is anything that is not clear or if you would like more information. Take your time to decide whether or not you wish to take part.

Thank you for reading this.

For more information or questions about the study contact:

Dr. James Bluett, Clinical Research Fellow
james.bluett@manchester.ac.uk
0161 2751614
or

Professor Anne Barton
Anne.barton@manchester.ac.uk
0161 275 1638

Central Manchester University Hospitals NHS
NHS Foundation Trust

RAMS
Rheumatoid Arthritis Medication Study

Our ref: «studyno»

«ptitle» «pforn1» «psurnam»
«paddr1»
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Date;

Dear «ptitle» «pforn1» «psurnam»

Study Title: Measurement of MTX and 7-OH-MTX metabolites in urine and blood of patients with rheumatoid arthritis: a possible measure of Methotrexate adherence

I am writing to you as you are currently being treated with Methotrexate and are a participant in the Rheumatoid Arthritis Medication Study (RAMS). I would like to provide you with some information regarding a research study we are undertaking, which you may be eligible to participate in. This study is aimed at trying to find factors that may help to identify when people have correctly taken their Methotrexate medication.

The study involves 4 visits to the Wellcome Trust Research Centre, next to Manchester Royal Infirmary, one of which requires a 24 hour inpatient stay. During these visits we would collect blood and urine samples from you to measure your Methotrexate levels. You will be offered an inconvenience payment for participating in this study. Travel expenses will also be reimbursed.

All you need to do at first is to read the enclosed information leaflet and decide whether you are interested in taking part in the study. Please complete the tear-off slip at the bottom of this letter and return it to me in the pre-paid envelope provided. No stamp is needed.
If you indicate that you are willing to take part in the study, investigators from the Arthritis Research UK Epidemiology unit will contact you by telephone to further discuss the study.

Whether you decide to take part or not, your treatment plan at clinic and participation in RAMS will not be affected. If you do not respond to this letter you may receive a reminder letter in a month's time.

I enclose a detailed information sheet about what your participation would involve. If you would like any more information on this aspect of the project, please feel free to telephone Dr James Bluett at the Arthritis Research UK Unit on 0161 275 1614 or email him on james.bluett@manchester.ac.uk

Dr Suzanne Verstappen

Please complete and return this slip in the envelope provided to:
<<site specific address>>

«studyno»

«ptitle» «pforn1» «psurnam»

Please tick one of the boxes below. If you are interested in taking part in the study please include your telephone number.

☐ I am interested in taking part in the study outlined above.
My telephone number is: _____

☐ I would prefer not to participate in the study as outlined above.

Local contact

Anne.Barton@manchester.ac.ukT

tel: 0161 275 1638

Central Manchester University Hospitals NHS
NHS Foundation Trust

RAMS
Rheumatoid Arthritis Medication Study

Our ref: «studyno»

«ptitle» «pforn1» «psurnam»
«paddr1»
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Date;

Dear «ptitle» «pforn1» «psurnam»

Study Title: Measurement of MTX and 7-OH-MTX metabolites in urine and blood of patients with rheumatoid arthritis: a possible measure of Methotrexate adherence

I am writing to you as you are currently being treated with Methotrexate and are a participant in the Rheumatoid Arthritis Medication Study (RAMS). We recently wrote to you inviting you to take part in a new study aimed at trying to find factors that may help to identify people who have correctly taken their Methotrexate medication.

The study involves 4 visits to the Wellcome Trust Research Centre, next to Manchester Royal Infirmary, one of which requires a 24 hour inpatient stay. During these visits we would collect blood and urine samples from you to measure methotrexate and its breakdown product. You will be offered an inconvenience payment for participating in this study. Travel expenses will also be reimbursed.

Please find an information sheet enclosed, which explains what taking part in the study would involve for you. After you have read this information sheet, if you are interested in taking part in the study, please complete the tear-off slip at the bottom of this letter and return it to me in the pre-paid envelope provided. No stamp is needed.

If you indicate that you are willing to take part in the study, investigators from the Arthritis Research UK Epidemiology unit will contact you by telephone to further discuss the study.

Whether you decide to take part or not, your treatment plan at clinic and participation in RAMS will not be affected.

If you would like any more information on this aspect of the project, please feel free to telephone Dr James Bluett at the Arthritis Research UK Unit on 0161 275 1614 or email him on james.bluett@manchester.ac.uk

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My telephone number is: _____

☐ I would prefer not to participate in the study as outlined above.

Local contac

Anne.Barton@manchester.ac.uk

Tel: 0161 275 1638

Central Manchester University Hospitals NHS
NHS Foundation Trust

CONSENT FORM MEMO Study

Measurement of MTX and 7-OH-MTX metabolites in urine and blood of patients with rheumatoid arthritis: a possible measure of Methotrexate adherence

Name of Chief Investigator: Professor Anne Barton

Participant initials	Centre number	ID number	Please Initial
☐☐☐	☐☐	☐☐☐	

1. I confirm that I have read and understand the information sheet dated 04/03/2013 for the above study. I have had the opportunity to consider the information, ask questions and have these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I agree to give blood and urine samples for research in this project as described in the participant information sheet. I understand how the samples will be collected, that giving samples for this research is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐
4. I understand that relevant sections of my medical notes and data collected during this study and the Rheumatoid Arthritis Medication Study (RAMS) may be looked at by individuals from the Arthritis Research UK Epidemiology Unit and The University of Manchester where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐
5. I understand that relevant sections of my medical notes and data collected during this study and the Rheumatoid Arthritis Medication Study (RAMS) may be looked at by individuals from regulatory authorities or from the NHS Trust where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.I understand that I will not benefit financially if this research leads to the development of a new medical test. ☐
6. I agree to information, from which I may be identified, being held by the research team at The University of Manchester Medical School together with data collected during the study. ☐
7. I know how to contact the research team if I need to, and how to get information about the results of the research. ☐
8. I agree that the consultant will inform my GP about my participation in the study. ☐
9. I agree to take part in the above study. ☐

10. I understand that once the study has ended the samples will be fully anonymised and once gifted to The University of Manchester cannot be withdrawn. I agree that the samples I have given and the information gathered about me can be stored by The University of Manchester for possible use in future projects, as described in the participant information sheet. I understand that some of these projects may be carried out by researchers other than the Manchester research team involved in this study. ☐

Name of participant (BLOCK CAPITALS)	Date	Signature
Name of Person taking consent	Date	Signature

Centre number ID

MEMO STUDY

CRF: Screening Visit

Centre number ID number

RAMS
Rheumatoid Arthritis Medication Study

Centre number ☐☐ ID ☐☐☐

GP Details

1. _____

_____

_____

_____

_____

Inclusion and exclusion criteria

2. Does the patient fulfil the inclusion criteria?

	YES	NO
- Physician diagnosis of RA or early undifferentiated polyarthritis	❍	❍
- Currently treated with methotrexate	❍	❍
- Taking methotrexate for at least 3 months	❍	❍
- No alteration in dose of methotrexate in the past 4 weeks	❍	❍
- Currently taking 5mg to 25mg methotrexate per week orally	❍	❍
- Patients on a stable dose of concomitant folic acid supplementation	❍	❍
- Willing to participate in the study after providing informed consent	❍	❍

If any item is no, patient cannot be recruited in the current study

3. Does the patient fulfil any of the exclusion criteria?

	YES	NO
- Age < 18	❍	❍
- Liver enzymes twice the upper limit of normal	❍	❍
- Significantly impaired renal function (eGFR < 30 ml/min)	❍	❍
- Pregnant or taking inadequate contraception	❍	❍
- Breast-feeding	❍	❍
- Allergic hypersensitivity to methotrexate	❍	❍
- Adequate contraception	❍	❍
- Pre-existing blood dyscrasias	❍	❍
- Unable to stay at the WTCRF for 24 hours	❍	❍
- Unable to give informed consent	❍	❍
- Blood donation for one month prior to screening visit, during the study and 2 months after completion of the study.	❍	❍
- Non-adherence to MTX	❍	❍

If any item is yes, patient cannot be recruited in the current study

4. Does the patient have rheumatoid arthritis?

❍ **Yes**

❍ **No,** can you specify the alternative diagnosis: _____

MEDICAL HISTORY

5. Co-morbidities

Please indicate if the patient has or had any of the following co-morbidities.

	NO	CURRENTLY	IN THE PAST	DON'T KNOW
- High blood pressure	❍	❍	❍	❍
- Angina	❍	❍	❍	❍
- Heart attack	❍	❍	❍	❍
- TIA	❍	❍	❍	❍
- Stroke	❍	❍	❍	❍
- Epilepsy	❍	❍	❍	❍
- Asthma	❍	❍	❍	❍
- Chronic bronchitis/emphysema	❍	❍	❍	❍
- Bronchiectasis	❍	❍	❍	❍
- Fibrotic lung disease	❍	❍	❍	❍
- Peptic ulcer disease	❍	❍	❍	❍
- Liver disease #	❍	❍	❍	❍
- Renal disease #	❍	❍	❍	❍
- TB #	❍	❍	❍	❍
- Diabetes	❍	❍	❍	❍
- Hyperthyroidism	❍	❍	❍	❍
- Depression	❍	❍	❍	❍
- Cancer #	❍	❍	❍	❍

 # If yes, please describe details, including date of onset:

	DETAILS:	DATE OF ONSET (M M Y Y Y Y)
Liver disease	_____	☐☐ ☐☐☐☐
Renal disease	_____	☐☐ ☐☐☐☐
TB	_____	☐☐ ☐☐☐☐
Cancer	_____	☐☐ ☐☐☐☐

RAMS
Rheumatoid Arthritis Medication Study

Centre number ☐☐ ID ☐☐☐

MEDICAL HISTORY

6.

Other Co-morbidities

Please list other past medical and surgical history.

DETAILS:	DATE OF ONSET M M Y Y Y Y
_____	☐☐ ☐☐☐☐
_____	☐☐ ☐☐☐☐
_____	☐☐ ☐☐☐☐
_____	☐☐ ☐☐☐☐
_____	☐☐ ☐☐☐☐
_____	☐☐ ☐☐☐☐
_____	☐☐ ☐☐☐☐
_____	☐☐ ☐☐☐☐
_____	☐☐ ☐☐☐☐
_____	☐☐ ☐☐☐☐

DRUG USE

7. Please list all current medication and dosage for any indication?

Medication	Dose and timing of medication
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____

8.

Please record current dose of MTX, mode of administration

START DATE
D D M M Y Y Y Y

Dose:_____ mg/week

Mode of administration: ❍ Oral ❍ S.C. ❍ I.M.

What day and time does the patient normally take their MTX?

On day: ❍ Monday ❍ Tuesday ❍ Wednesday ❍ Thursday
❍ Friday
❍ Saturday ❍ Sunday

Time patient normally takes MTX: ___ ___ : ___ ___

---

9. **When did the patient start this dose of methotrexate?**

START DATE
D D M M Y Y Y Y

10.

Is the patient taking folic acid?

❍ **Yes**, dose: _____ mg day/week (delete as appropriate)

❍ **No**

11.

RAMS
Rheumatoid Arthritis Medication Study

Centre number ☐☐ ID ☐☐☐

ADVERSE EVENTS

Please record if any of these adverse events occurred in the past 6 months.
Tick the box "adverse event" if this adverse event has occurred in the past 6 months and record frequency and duration per event for that specific adverse event. If the adverse event seems related with MTX, please tick box in the last column.

ADVERSE EVENT	FREQUENCY	DURATION PER EVENT	RELATED TO MTX
Gastro-intestinal (subjective)			
❍ Nausea			❍
❍ Vomiting			❍
❍ Anorexia			❍
❍ Stomach ache			❍
❍ Diarrhoea			❍
❍ Other: _____			❍
Gastro-intestinal (objective)			
❍ Oesophagitis			❍
❍ Gastritis			❍
❍ Gastric ulcers			❍
❍ Duodenal ulcers			❍
❍ Colitis			❍
❍ Other: _____			❍
Mucocutaneous			
❍ Nodulosis			❍
❍ Stomatitis			❍
❍ Oral ulcers			❍
❍ Itching			❍
❍ Alopecia			❍
❍ Hypertrichosis			❍
❍ Gum hyperplasia			❍
❍ Other: _____			❍
CNS			
❍ Headache			❍
❍ Dizziness			❍
❍ Disordered mood			❍
❍ Blurred vision			❍
❍ Dry eyes			❍
❍ Lost hearing			❍
❍ Other: _____			❍

(Continued on next page)

.

ADVERSE EVENTS (continued)

ADVERSE EVENT	FREQUENCY	DURATION PER EVENT	RELATED TO MTX
Renal			
❍ Proteinuria	_____	_____	❍
❍ Elevated creatinine	_____	_____	❍
❍ Hypertension	_____	_____	❍
❍ Other: _____	_____	_____	❍
Lung (subjective)			
❍ Cough	_____	_____	❍
❍ Dyspnoea	_____	_____	❍
❍ Chest pain	_____	_____	❍
❍ Other: _____	_____	_____	❍
Lung (objective)			
❍ Pneumonitis	_____	_____	❍
❍ Other: _____	_____	_____	❍
Hepatic			
❍ AST > 1 * normal value	_____	_____	❍
❍ ALT > 1 * normal value	_____	_____	❍
❍ Alkaline phosphates	_____	_____	❍
❍ Gamma-GGT	_____	_____	❍
❍ Other: _____	_____	_____	❍
Haematological			
❍ Anaemia	_____	_____	❍
❍ Leucopenia	_____	_____	❍
❍ Thrombopenia	_____	_____	❍
❍ Pancytopenia	_____	_____	❍
❍ Other: _____	_____	_____	❍
General			
❍ Fever	_____	_____	❍
❍ Weight loss	_____	_____	❍
❍ Headache	_____	_____	❍
❍ Fatigue	_____	_____	❍
❍ Sore throat	_____	_____	❍

Smoking

Does the patient smoke at the moment?

❍ **Yes,** average number of cigarettes or cigars / day ☐☐☐

❍ **No**

SOCIAL HISTORY

If no and patient has stopped smoking in past six months please fill in:

- Month/Year patient stopped smoking M M Y Y ☐☐☐☐

13.

Alcohol consumption

Does the patient drink alcohol?

❍ **Yes,** number of units alcohol consumed in the previous two weeks ☐☐

❍ **No**

14.

Diet

Does the patient follow any specific diet?

❍ None ❍ Vegetarian
❍ Vegan ❍ Gluten-free
❍ Lactose-free
❍ Other, details _____

15.

Weight

Weight ☐☐☐ in kg **OR** ☐☐☐ in lbs

16.

Blood pressure and pulse

Systolic mmHg ☐☐☐ Diastolic mmHg ☐☐☐ Pulse bpm ☐☐☐

17. Temperature ☐☐☐

18.

Early morning stiffness

❍ **Yes,** minutes of morning stiffness ☐☐☐

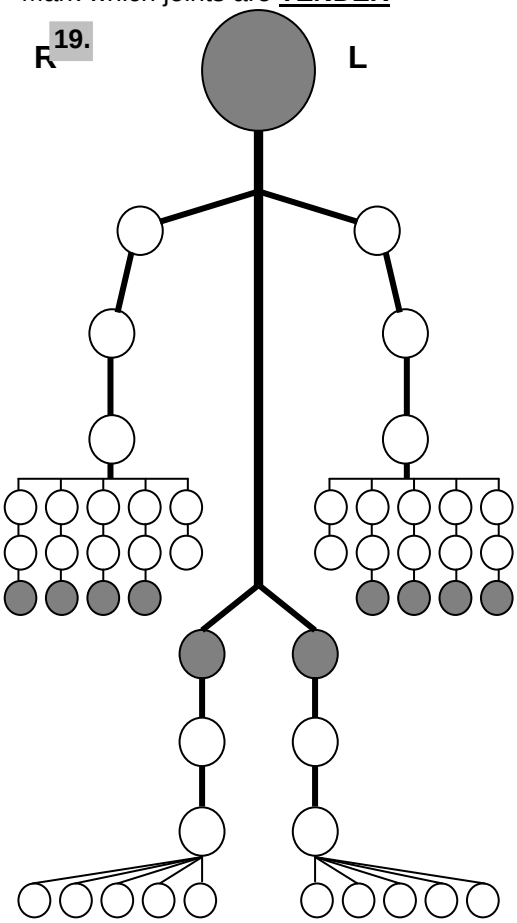
❍ **No**

Centre number ID

Mark which joints are **TENDER**

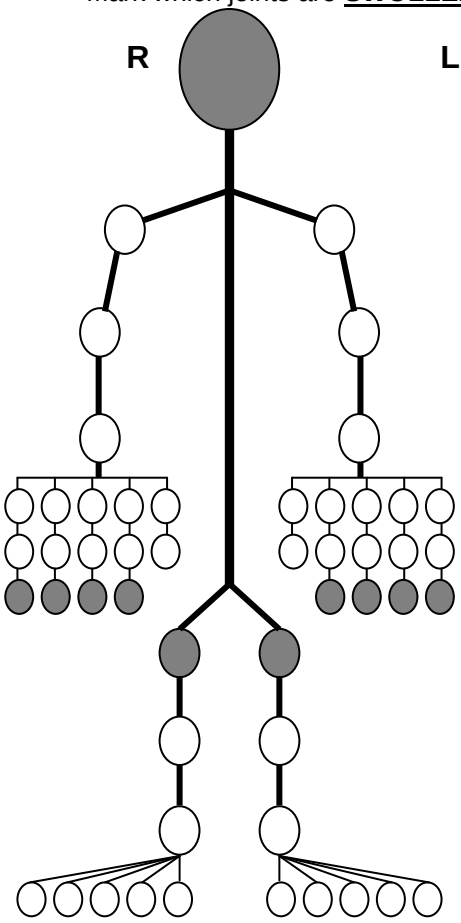
19.

R L



Mark which joints are **SWOLLEN**

R L



Total number of tender joints

Total number of swollen joints

❍ Please tick this box if you were only able to perform a 28-joint count

DISEASE ACTIVITY

20.

Visual Analogue Scale (VAS) patient

We would like to indicate on this scale how good or bad is your health **TODAY**, in your opinion.
Place a mark on the line below.

BEST IMAGINABLE HEALTH STATUS — **WORST IMAGINABLE HEALTH STATUS**

21.

Visual Analogue Scale (VAS) physician

How would you assess the patients overall health considering all the ways that you know that their arthritis affects them.

VERY WELL — **VERY UNWELL**

RECENT LABORATORY VALUES

22.

ESR

Erythrocyte Sedimentation Rate (ESR): ☐☐☐ mm/hr

Date ESR: D D M M Y Y Y Y ☐☐☐☐☐☐☐☐

C-reactive protein (CRP): ☐☐.☐ mg/L

Date CRP: D D M M Y Y Y Y ☐☐☐☐☐☐☐☐

23.

General remarks:

Centre number ☐☐ ID ☐☐☐

MEMO STUDY

CRF: Visit 1

Centre number ☐☐ ID number ☐☐☐

Date D D / M M / Y Y Y Y ☐☐/☐☐/☐☐☐☐ Time H H M M ☐☐☐☐

RAMS
Rheumatoid Arthritis Medication Study

Centre number ☐☐ ID ☐☐☐

STUDY CONSENT

1.

Check patient well

	YES	NO
- Patient well	❍	❍
- Patient happy to continue with study	❍	❍

DRUG USE

2.

Any new medication since initial screening visit?

Medication	Dose and timing of medication
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____

DIETARY HISTORY

3.

When did patient last eat?

Date D D / M M / Y Y Y Y Time H H : M M

PHYSICAL EXAMINATION

4.

Blood pressure and pulse

Systolic ☐☐☐ mmHg Diastolic ☐☐☐ mmHg Pulse ☐☐☐ bpm

5.

Temperature ☐☐☐ in °C

Date D D / M M / Y Y Y Y Time H H : M M

INCLUSION AND EXCLUSION CRITERIA

6.

Does the patient fulfil the inclusion criteria?

	YES	NO
- Physician diagnosis of RA or early undifferentiated polyarthritis	❍	❍
- Currently treated with methotrexate	❍	❍
- Taking methotrexate for at least 3 months	❍	❍
- No alteration in dose of methotrexate in the past 4 weeks	❍	❍
- Currently taking 5mg to 25mg methotrexate per week orally	❍	❍
- Patients on a stable dose of concomitant folic acid supplementation	❍	❍
- Willing to participate in the study after providing informed consent	❍	❍

If any item is no, patient cannot continue

7. **Does the patient fulfil any of the exclusion criteria?**

	YES	NO
- Age < 18	❍	❍
- Liver enzymes twice the upper limit of normal	❍	❍
- Significantly impaired renal function (eGFR < 30 ml/min)	❍	❍
- Pregnant or taking inadequate contraception	❍	❍
- Breast-feeding	❍	❍
- Allergic hypersensitivity to methotrexate	❍	❍
- Adequate contraception	❍	❍
- Pre-existing blood dyscrasias	❍	❍
- Unable to stay at the WTCRF for 24 hours	❍	❍
- Unable to give informed consent	❍	❍
- Blood donation for one month prior to screening visit, during the study and 2 months after completion of the study.	❍	❍
- Non-adherence to MTX	❍	❍

If any item is yes, patient cannot continue

D D M M Y Y Y Y H H M M

Date ☐☐/☐☐/☐☐☐☐ Time ☐☐☐☐

ADVERSE EVENTS

8.

Please record if any of these adverse events occurred since the screening visit.

Tick the box "adverse event" if this adverse event has occurred since the screening visit and record frequency and duration per event for that specific adverse event. If the adverse event seems related with MTX, please tick box in the last column.

ADVERSE EVENT	FREQUENCY	DURATION PER EVENT	RELATED TO MTX
Gastro-intestinal (subjective)			
❍ Nausea	_____	_____	❍
❍ Vomiting	_____	_____	❍
❍ Anorexia	_____	_____	❍
❍ Stomach ache	_____	_____	❍
❍ Diarrhoea	_____	_____	❍
❍ Other: _____	_____	_____	❍
Gastro-intestinal (objective)			
❍ Oesophagitis	_____	_____	❍
❍ Gastritis	_____	_____	❍
❍ Gastric ulcers	_____	_____	❍
❍ Duodenal ulcers	_____	_____	❍
❍ Colitis	_____	_____	❍
❍ Other: _____	_____	_____	❍
Mucocutaneous			
❍ Nodulosis	_____	_____	❍
❍ Stomatitis	_____	_____	❍
❍ Oral ulcers	_____	_____	❍
❍ Itching	_____	_____	❍
❍ Alopecia	_____	_____	❍
❍ Hypertrichosis	_____	_____	❍
❍ Gum hyperplasia	_____	_____	❍
❍ Other: _____	_____	_____	❍
CNS			
❍ Headache	_____	_____	❍
❍ Dizziness	_____	_____	❍
❍ Disordered mood	_____	_____	❍
❍ Blurred vision	_____	_____	❍
❍ Dry eyes	_____	_____	❍
❍ Lost hearing	_____	_____	❍
❍ Other: _____	_____	_____	❍

(Continued on next page)

D D M M Y Y Y Y H H M M

Date ☐☐/☐☐/☐☐☐☐ Time ☐☐☐☐

ADVERSE EVENTS (CONTINUED)

Tick the box "adverse event" if this adverse event has occurred in the past 6 months and record frequency and duration per event for that specific adverse event. If the adverse event seems related with MTX, please tick box in the last column.

ADVERSE EVENT	FREQUENCY	DURATION PER EVENT	RELATED TO MTX
Renal			
❍ Proteinuria			❍
❍ Elevated creatinine			❍
❍ Hypertension			❍
❍ Other:			❍
Lung (subjective)			
❍ Cough			❍
❍ Dyspnoea			❍
❍ Chest pain			❍
❍ Other:			❍
Lung (objective)			
❍ Pneumonitis			❍
❍ Other:			❍
Hepatic			
❍ AST > 1 * normal value			❍
❍ ALT > 1 * normal value			❍
❍ Alkaline phosphates			❍
❍ Gamma-GGT			❍
❍ Other:			❍
Haematological			
❍ Anaemia			❍
❍ Leucopenia			❍
❍ Thrombopenia			❍
❍ Pancytopenia			❍
❍ Other:			❍
General			
❍ Fever			❍
❍ Weight loss			❍
❍ Headache			❍
❍ Fatigue			❍
❍ Sore throat			❍
❍ Other:			❍

D D M M Y Y Y Y H H M M

Date ☐☐/☐☐/☐☐☐☐ Time ☐☐☐☐

METHOTREXATE ADMINISTRATION

9.

Patient empties bladder prior to methotrexate.

Date and time of directly observed methotrexate administration.

Date D D / M M / Y Y Y Y Time H H M M

BLOOD COLLECTION

10. **Blood sampling taken at the following times after methotrexate administration (in hours) and time after methotrexate recorded with patient id labelled on sample tube?**

	YES	NO
- 0 hours	❍	❍
- 1 hours	❍	❍
- 2 hours	❍	❍
- 4 hours	❍	❍
- 8 hours	❍	❍
- 16 hours	❍	❍
- 24 hours	❍	❍

URINE COLLECTION

11. **24 hour urine collection begins after methotrexate administration.**

Use a separate urine collection bottle at each 4 hour time point and label the samples with patient id and collection period.

	YES	NO
- 0-4 hours	❍	❍
- 4-8 hours	❍	❍
- 8-12 hours	❍	❍
- 12-16 hours	❍	❍
- 16-20 hours	❍	❍
- 20-24 hours	❍	❍

DIETARY HISTORY

12.

When did patient eat a meal during the inpatient stay?

Date D D / M M / Y Y Y Y Time H H M M

Date D D / M M / Y Y Y Y Time H H M M

D D / M M / Y Y Y Y

Date D D / M M / Y Y Y Y Time H H M M

Centre number ID

MEMO STUDY

CRF: Visit 2

Centre number ID number

RAMS
Rheumatoid Arthritis Medication Study

Centre number ☐☐ ID ☐☐☐

1. Check patient well

	YES	NO
- Patient well	❍	❍
- Patient happy to continue with study	❍	❍

DRUG USE

2. Any new medication since initial screening visit?

Medication	Dose and timing of medication
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____

Dietary History

3. When did patient last eat?

Date D D ☐☐ M M ☐☐ Y Y Y Y ☐☐☐☐ Time H H M M ☐☐☐☐

4. Blood pressure and pulse

Systolic ☐☐☐ mmHg Diastolic ☐☐☐ mmHg Pulse ☐☐☐ bpm

5. Temperature

☐☐☐ in °C

RAMS
Rheumatoid Arthritis Medication Study

Centre number ☐☐ ID ☐☐☐

ADVERSE EVENTS

6. **Please record if any of these adverse events occurred since previous visit.**

Tick the box "adverse event" if this adverse event has occurred since the previous visit and record frequency and duration per event for that specific adverse event. If the adverse event seems related with MTX, please tick box in the last column.

ADVERSE EVENT	FREQUENCY	DURATION PER EVENT	RELATED TO MTX
Gastro-intestinal (subjective)			
❍ Nausea	_____	_____	❍
❍ Vomiting	_____	_____	❍
❍ Anorexia	_____	_____	❍
❍ Stomach ache	_____	_____	❍
❍ Diarrhoea	_____	_____	❍
❍ Other: _____	_____	_____	❍
Gastro-intestinal (objective)			
❍ Oesophagitis	_____	_____	❍
❍ Gastritis	_____	_____	❍
❍ Gastric ulcers	_____	_____	❍
❍ Duodenal ulcers	_____	_____	❍
❍ Colitis	_____	_____	❍
❍ Other: _____	_____	_____	❍
Mucocutaneous			
❍ Nodulosis	_____	_____	❍
❍ Stomatitis	_____	_____	❍
❍ Oral ulcers	_____	_____	❍
❍ Itching	_____	_____	❍
❍ Alopecia	_____	_____	❍
❍ Hypertrichosis	_____	_____	❍
❍ Gum hyperplasia	_____	_____	❍
❍ Other: _____	_____	_____	❍
CNS			
❍ Headache	_____	_____	❍
❍ Dizziness	_____	_____	❍
❍ Disordered mood	_____	_____	❍
❍ Blurred vision	_____	_____	❍
❍ Dry eyes	_____	_____	❍
❍ Lost hearing	_____	_____	❍
❍ Other: _____	_____	_____	❍

(Continued on next page)

RAMS
Rheumatoid Arthritis Medication Study

Centre number ☐☐ ID ☐☐☐

ADVERSE EVENTS (CONTINUED)

Tick the box "adverse event" if this adverse event has occurred in the past 6 months and record frequency and duration per event for that specific adverse event. If the adverse event seems related with MTX, please tick box in the last column.

ADVERSE EVENT	FREQUENCY	DURATION PER EVENT	RELATED TO MTX
Renal			
❍ Proteinuria	_____	_____	❍
❍ Elevated creatinine	_____	_____	❍
❍ Hypertension	_____	_____	❍
❍ Other: _____	_____	_____	❍
Lung (subjective)			
❍ Cough	_____	_____	❍
❍ Dyspnoea	_____	_____	❍
❍ Chest pain	_____	_____	❍
❍ Other: _____	_____	_____	❍
Lung (objective)			
❍ Pneumonitis	_____	_____	❍
❍ Other: _____	_____	_____	❍
Hepatic			
❍ AST > 1 * normal value	_____	_____	❍
❍ ALT > 1 * normal value	_____	_____	❍
❍ Alkaline phosphates	_____	_____	❍
❍ Gamma-GGT	_____	_____	❍
❍ Other: _____	_____	_____	❍
Haematological			
❍ Anaemia	_____	_____	❍
❍ Leucopenia	_____	_____	❍
❍ Thrombopenia	_____	_____	❍
❍ Pancytopenia	_____	_____	❍
❍ Other: _____	_____	_____	❍
General			
❍ Fever	_____	_____	❍
❍ Weight loss	_____	_____	❍
❍ Headache	_____	_____	❍
❍ Fatigue	_____	_____	❍
❍ Sore throat	_____	_____	❍
❍ Other: _____	_____	_____	❍

RAMS
Rheumatoid Arthritis Medication Study

Centre number ☐☐ ID ☐☐☐

URINE COLLECTION

7. **Patient empties bladder.**
Date and time of subsequent urine collection.

Date D D ☐☐ M M ☐☐ Y Y Y Y ☐☐☐☐ Time H H M M ☐☐☐☐

BLOOD COLLECTION

8.

Time of blood collection.

Date D D ☐☐ M M ☐☐ Y Y Y Y ☐☐☐☐ Time H H M M ☐☐☐☐

Centre number ID

MEMO STUDY

CRF: Visit 3

Centre number ID number

RAMS
Rheumatoid Arthritis Medication Study

Centre number ☐☐ ID ☐☐☐

1.

Check patient well

	YES	NO
- Patient well	❍	❍
- Patient happy to continue with study	❍	❍

DRUG USE

2. **Any new medication since initial screening visit?**

Medication	Dose and timing of medication
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____

DIETARY HISTORY

3.

When did patient last eat?

Date D D M M Y Y Y Y ☐☐ ☐☐ ☐☐☐☐ Time H H M M ☐☐☐☐

4.

Blood pressure and pulse

Systolic mmHg ☐☐☐ Diastolic mmHg ☐☐☐ Pulse bpm ☐☐☐

5.

Temperature

☐☐☐ in °C

RAMS
Rheumatoid Arthritis Medication Study

Centre number ☐☐ ID ☐☐☐

ADVERSE EVENTS

6. Please record if any of these adverse events occurred since previous visit.

Tick the box "adverse event" if this adverse event has occurred since the previous visit and record frequency and duration per event for that specific adverse event. If the adverse event seems related with MTX, please tick box in the last column.

ADVERSE EVENT	FREQUENCY	DURATION PER EVENT	RELATED TO MTX
Gastro-intestinal (subjective)			
❍ Nausea	_____	_____	❍
❍ Vomiting	_____	_____	❍
❍ Anorexia	_____	_____	❍
❍ Stomach ache	_____	_____	❍
❍ Diarrhoea	_____	_____	❍
❍ Other: _____	_____	_____	❍
Gastro-intestinal (objective)			
❍ Oesophagitis	_____	_____	❍
❍ Gastritis	_____	_____	❍
❍ Gastric ulcers	_____	_____	❍
❍ Duodenal ulcers	_____	_____	❍
❍ Colitis	_____	_____	❍
❍ Other: _____	_____	_____	❍
Mucocutaneous			
❍ Nodulosis	_____	_____	❍
❍ Stomatitis	_____	_____	❍
❍ Oral ulcers	_____	_____	❍
❍ Itching	_____	_____	❍
❍ Alopecia	_____	_____	❍
❍ Hypertrichosis	_____	_____	❍
❍ Gum hyperplasia	_____	_____	❍
❍ Other: _____	_____	_____	❍
CNS			
❍ Headache	_____	_____	❍
❍ Dizziness	_____	_____	❍
❍ Disordered mood	_____	_____	❍
❍ Blurred vision	_____	_____	❍
❍ Dry eyes	_____	_____	❍
❍ Lost hearing	_____	_____	❍
❍ Other: _____	_____	_____	❍

(Continued on next page)

RAMS
Rheumatoid Arthritis Medication Study

Centre number ☐☐ ID ☐☐☐

Tick the box "adverse event" if this adverse event has occurred in the past 6 months and record frequency and duration per event for that specific adverse event. If the adverse event seems related with MTX, please tick box in the last column.

ADVERSE EVENT	FREQUENCY	DURATION PER EVENT	RELATED TO MTX
Renal			
❍ Proteinuria	_____	_____	❍
❍ Elevated creatinine	_____	_____	❍
❍ Hypertension	_____	_____	❍
❍ Other: _____	_____	_____	❍
Lung (subjective)			
❍ Cough	_____	_____	❍
❍ Dyspnoea	_____	_____	❍
❍ Chest pain	_____	_____	❍
❍ Other: _____	_____	_____	❍
Lung (objective)			
❍ Pneumonitis	_____	_____	❍
❍ Other: _____	_____	_____	❍
Hepatic			
❍ AST > 1 * normal value	_____	_____	❍
❍ ALT > 1 * normal value	_____	_____	❍
❍ Alkaline phosphates	_____	_____	❍
❍ Gamma-GGT	_____	_____	❍
❍ Other: _____	_____	_____	❍
Haematological			
❍ Anaemia	_____	_____	❍
❍ Leucopenia	_____	_____	❍
❍ Thrombopenia	_____	_____	❍
❍ Pancytopenia	_____	_____	❍
❍ Other: _____	_____	_____	❍
General			
❍ Fever	_____	_____	❍
❍ Weight loss	_____	_____	❍
❍ Headache	_____	_____	❍
❍ Fatigue	_____	_____	❍
❍ Sore throat	_____	_____	❍
❍ Other:	_____	_____	❍

URINE COLLECTION

7. **Patient empties bladder.**
Date and time of subsequent urine collection.

Date D D M M Y Y Y Y Time H H M M

BLOOD COLLECTION

8. **Time of blood collection.**

Date D D M M Y Y Y Y Time H H M M

Appendix 2

Clinical Proforma for Methotrexate pneumonitis in Rheumatoid Arthritis collection.

Date form completed: **Who completed form:**

Gender: Male Female **Study id number:**
Date of birth:

Northern European Origin:
Yes No (excluded)

Age at onset of joint symptoms:
Age at diagnosis:

Tick all that have applied since onset	**Yes / Score**	**No**	**Don't know**
Joint involvement * 1 large joint score = 0 2- 10 large joint score = 1 1- 3 small joints (with or without involvement of large joints) score = 2 4- 10 small joints (with or without involvement of large joints) score = 3 > 10 joints (at least 1 small joint) score = 5			
Serological abnormalities †† Negative RF *and* negative ACPA score = 0 Low-positive RF *or* low-positive ACPA score = 2 High-positive RF *or* high-positive ACPA score = 3			
Acute phase reactants ‡‡ Normal CRP and Normal ESR score = 0 Abnormal CRP or abnormal ESR score= 1			
Arthritis symptoms lasting more than 6 weeks§§ score =1			
Total score /10= (Total score of ≥ **6/10**= definite diagnosis of RA)			
Family history of RA? If Yes, relationship to proband?			
Current smoker?			
Ever smoked?			
Never smoked?			
If current/ever smoked, number of years smoked:			
Average number of cigarettes per day:			
Tick all that have applied since onset	**Yes**	**No**	**Don't know**
Name of DMARD taking when developed pneumonitis?			
Age when started taking DMARD?			

Date started taking DMARD			
Date stopped taking DMARD			
Dose of DMARD (mg/week) on termination of treatment?			
Any previous/DMARD treatment taken?			
If Yes to above question, why was this treatment terminated?			
Taking NSAIDs while taking DMARD?			
Taking Folic acid while taking DMARD? If yes, what dose of folic acid?			
Any other con-comitant medications at time of developing pneumonitis?			
Date when pneumonitis was diagnosed?			
What was the outcome of the pneumonitis?			
Did the patient require hospitalisation for their pneumonitis?			
Does patient fulfil either of the criteria for pneumonitis shown in bold below			
Criteria 1:			
1: Clinical course consistent with a hypersensitivity reaction			
2: Resolving infiltrates on chest roentgenogram (CXR) after discontinuing MTX (HRCT preferable)			
3: Exclusion of infection or other pulmonary disease			
4: Pathology consistent with drug induced injury (ie; hypersensitivity pneumonitis or toxic drug reaction)			
Probable MTX-P: 3 out of 4 of the above criteria			
Possible MTX-P: 2 out of 4 of the above criteria			
Unlikely MTX-P: 1 out of 4 of the above criteria			

Criteria 2			
1. Acute onset dyspnoea			
2. Fever >38.0 c			
3. Tachypnoea>28/min and dry cough			
4. Radiological evidence of pulmonary interstitial or alveolar infiltrates (HRCT preferable)			
5. White blood cell count <15.0x10^{9} with or without eosinophilia			
6. Restrictive defect and decreased diffusion capacity on pulmonary function tests			
7. Negative blood and sputum cultures (mandatory)			
8. P02 <7.5kPa on air			
9. Histopathology consistent with broncholitis or interstitial pneumonitis with giant cells and without evidence of infection			
Definite: 6 of 9 criteria present			
Probable: 5 of 9 criteria present			
Possible: 4 of 9 criteria present			
Tick all that have applied since onset	**Yes**	**No**	**Don't know**
Other medical conditions at the time when pneumonitis started?			
COPD			
Asthma			
Interstitial Lung Disease			
Emphysema			
Diabetes			
Other medical condition			
Current Drug treatment			
Any other adverse events to the drug treatment you were taking? If yes, what were they?			

*Small joints" refers to the metacarpophalangeal joints, proximal interphalangeal joints, second through fifth metatarsophalangeal joints, thumb interphalangeal joints, and wrists
†† laboratory and assay; low-positive refers to IU values that are higher than the ULN but >3 times the ULN for the laboratory and assay; high-positive refers to IU values that are > 3 times the ULN for the laboratory and assay. Where rheumatoid factor (RF) information is only available as positive or negative, a positive result should be scored as low-positive for RF. ACPA= anti-citrullinated protein antibody.
‡‡ Normal/abnormal is determined by local laboratory standards.
§§ Duration of symptoms refers to patient self-report of the duration of signs or symptoms of synovitis (e.g., pain, swelling, tenderness) of joints that are clinically involved at the time of assessment, regardless of treatment status

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