“Pharmacokinetics of oral Mycophenolate Mofetil (MMF) in patients with Lupus Nephritis with a special reference to the measurement of free MPA and saliva MPA.”

A DISSERTATION SUBMITTED TO THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY, IN PARTIAL FULFILMENT OF THE REGULATIONS FOR THE AWARD OF M.D. DEGREE IN PHARMACOLOGY (BRANCH VI) EXAMINATION TO BE HELD IN APRIL 2015.

DEPARTMENT OF PHARMACOLOGY AND CLINICAL PHARMACOLOGY

CERTIFICATE

This is to certify that this dissertation entitled "Pharmacokinetics of oral Mycophenolate Mofetil (MMF) in patients with lupus nephritis with a special reference to the measurement of free MPA and saliva MPA." is a bona fide original work done personally by Dr. Rohit Kodagali under my direct guidance, supervision and completed to my satisfaction, towards partial fulfilment of university regulations for the award of M.D. Pharmacology (Branch VI) Degree examination of The Tamil Nadu M.G.R. Medical University, Chennai to be held in April 2015.

Place: Vellore, TN.

Date: 25/4/14.

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Place: Vellore, TN.

Date: 25/9/14.

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DECLARATION

I, Dr. Rohit Kodagali, do hereby declare that this dissertation entitled "Pharmacokinetics of oral Mycophenolate mofetil (MMF) in patients with lupus nephritis with a special reference to the measurement of free MPA and saliva MPA." has been done by me under the direct guidance of Dr. Binu S Mathew, Professor, Department of Pharmacology and Clinical Pharmacology, Christian Medical College, Vellore, in partial fulfilment of the university regulations for the award of M.D. degree in Pharmacology (Branch VI). I have not submitted this dissertation in part or full to any other university or towards any other degree.

Place: Vellore, TN.

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Table of Contents

Abstract

Title: Pharmacokinetics of Mycophenolate Mofetil in adult patients with lupus nephritis with a special reference to the measurement of free MPA and saliva MPA.

Background*:* - Mycophenolate Mofetil (MMF), the immunosuppressive agent, is commonly used to treat renal transplant patients and patients with other autoimmune diseases such as Systemic Lupus Erythematosus (SLE). Due to the high inter-dose, inter-patient variability in drug exposure, therapeutic drug monitoring (TDM) has proved extremely beneficial in individualizing the MMF therapy. Measurement of Mycophenolic Acid (MPA) area under the curve ($AUC_{0\text{-}12\ hr}$) involves collection of multiple blood specimens, over 12 hours.

Objectives: (i) To establish the pharmacokinetics of MMF in patients with lupus nephritis

(ii) To develop a limited sampling strategy (LSS) for the estimation of MPA $AUC_{0\text{-}12\ hr}$.

(iii) To determine if any correlation between free MPA in plasma and saliva MPA concentration.

Methods: Plasma specimens from 30 patients with lupus nephritis were prospectively collected at the respective time points: trough (prior to MMF dose), then at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8 and 12 hours after patients had taken MMF. Total MPA $AUC_{0\text{-}12\ hr}$ was estimated using high performance liquid chromatography (HPLC with UV detection). Saliva and free MPA concentrations were collected at 1 hour post-dose, measured and their correlation was compared. Limited sampling strategies with acceptable correlation coefficients (R^2), bias and precision were developed by stepwise multiple regression analysis. The predictive performance of the LSS was validated using bootstrap validation. Pharmacokinetic parameters were analysed using Pmetrics software for R.

Results: The observed MPA $AUC_{0\text{-}12\ hr}$ ranged from 19.52 to 67.67 mg.h/L with a mean of 45.12 mg.h/L. The mean C_{max} and T_{max} were 16.703 mg.h/L and 1.08 h respectively. The mean dose per kg body weight of MMF prescribed was 26.47 mg/kg. The best LSS model included concentrations at time points 0, 1, 2 and 4 (C_0, C_1, C_2, C_4) (multiple R^2=0.926). The final model validated post bootstrap is as follows:

MPA $AUC_{0\text{-}12\ hr}$= 12.3376+ (2.9013XC_0) + (0.8305XC_1) + (0.7945XC_2) + (4.5156XC_4).

Calculated bias and imprecision for the LSS were -0.13 and 7.09 % respectively.

The observed correlation between free MPA in plasma and saliva MPA was poor overall (r=0.085, p>0.05). However good correlation (r= 0.790, p=0.02) was observed in the low albumin group (n=8).

Conclusion: This study has, for the first time, described the pharmacokinetics of MMF in adult Indian patients with lupus nephritis and the developed 4 point LSS is an accurate measure of the total 12-hour MPA $AUC_{0\text{-}12\ hr}$ with favourable bias and precision.

Introduction

Systemic Lupus Erythematosus (SLE) is an autoimmune disorder which is 10 times more frequent in women than in men.(1,2) Traditionally used drugs for SLE like Cyclophosphamide were effective in controlling the symptoms but had a lot of side effects as compared to newer drugs like Mycophenolate Mofetil (MMF). The most common systemic manifestations of the disease include dermatological and renal manifestations.(1,3,4)

Lupus nephritis is the renal manifestation of SLE which is more common and severe in African Americans followed by the Asians.(5,6) Caucasians are relatively less affected. The T-cells as well as the pro-inflammatory cytokines like transforming growth factor β (TGF-β), Il-1, Il-18 and interferon α (IFN α) are the most important factors responsible for the destruction of the glomeruli.(5) As the disorder is of autoimmune origin, immunosuppressants have been found to be most effective against lupus nephritis. Previously used immunosuppressants include cyclophosphamide and azathioprine but, even though they were effective in controlling symptoms and clinical flares, they had a lot of side effects. In the early 2000's, the immunosuppressant mycophenolic acid was tried in SLE and lupus nephritis and was found to be as efficacious as cyclophosphamide with lesser side effects.(7,8) The current EULAR (European League against Rheumatism) recommendations include mycophenolate as first line therapy in patients with higher stages of lupus nephritis (stage III, IV and V).(9)Currently, there is considerable interest in developing biological agents like rituximab and belimumab for the treatment of SLE.(10) However, they do not have any added benefit plus their high costs are a deterrent to their use, especially in low middle income countries like ours.

Mycophenolate mofetil (MMF) is one of the dosage forms of mycophenolic acid (MPA). It is a reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH) in purine biosynthesis. The T-lymphocytes are highly susceptible to its action as they are highly reliant on the *de novo* pathway for synthesis of purines while other cells recover as they

possess an alternative pathway for the same. MMF was originally developed for clinical use in renal transplant patients and has now become the mainstay in the treatment of renal transplant patients due to its comparable efficacy and lesser toxicity.(11) Since the T-cells are the major cells implicated in the pathophysiology of lupus nephritis, this drug should be effective against lupus nephritis as well.

However, mycophenolate needs to be monitored therapeutically due to its high (10 fold) inter-patient variability in pharmacokinetics.(12) It is generally agreed that MPA dose should be titrated in renal transplant patients to maintain an MPA $AUC_{0\text{-}12\ hr}$ (Area under the concentration-time curve up to 12 hours post dose depicting MPA exposure) between 30-60 mg.h/L to avoid acute rejection as well as have lesser side effects due to the immunosuppression.(12–14)

The AUC measurement requires collection of many blood samples (often more than 8) and it requires the patient to be in the laboratory for the whole duration of sampling (for MPA it is 12 hours). Though trough (immediate pre-dose) concentrations have been used to indicate drug exposure for other therapeutic agents, for MPA, it may not correlate well with the MPA $AUC_{0\text{-}12\ hr}$.(15) Therefore, a robust equation needs to be developed based on limited samples (Limited sampling strategy (LSS)) so as to reduce costs and lead to a shorter hospital stay for the patients.

The pharmacokinetics of MMF in transplant patients is very different from that seen in autoimmune diseases like SLE.(12) Some reasons contributing to this would be unpredictable absorption in the early post-transplant period, different co-medications and differences in albumin, urea and GFR between transplant and SLE patients. So limited sampling strategy equations developed in transplant patients cannot be extrapolated to a different cohort of SLE

patients. Therefore one of the objectives of our study is to develop and validate a reliable, clinically viable LSS for patients with lupus nephritis prescribed Mycept® brand of MMF.

There have been a few studies describing the pharmacokinetics of mycophenolate mofetil in patients with lupus nephritis from various different parts of the world. To date, there is no study done in Indian patients with lupus nephritis. Another objective of our study is to elucidate pharmacokinetics of mycophenolate mofetil in Indian patients with lupus nephritis.

In SLE patients with a higher probability of having lower albumin, it is the free (unbound pharmacologically active) drug concentration that is important for its action and may be more clinically relevant as compared to the total MPA (bound and free) concentrations.(16,17) The problem with measuring free MPA concentrations is that it requires the use of a biological membrane filter which makes monitoring free MPA more costlier than monitoring total MPA concentrations. Human saliva is an ultrafiltrate and has the potential to be a viable alternative to the measurement of free MPA. It is easy to collect and is non-invasive.(18) We also intended to correlate saliva, total and plasma free MPA concentrations at one hour after MMF. And to examine the possibility of developing an equation to estimate free MPA from either saliva or total MPA concentrations.

Aims and Objectives

Aims

"To establish pharmacokinetics of oral mycophenolate mofetil (MMF) in Indian adult patients with lupus nephritis."

Objectives

(i) To validate the assay of free MPA and saliva MPA measurement using high performance liquid chromatography.

(ii) To measure the exposure to MMF as mycophenolic acid area under the curve (Total MPA $AUC_{0\text{-}12\ hr}$) in lupus nephritis patients.

(iii) To develop and validate a limited sampling strategy equation to estimate total MPA AUC $_{0\text{-}12\ hr.}$

(iv) To study basic pharmacokinetic parameters and to create a population pharmacokinetic model , using the Pmetrics package

(v) To study if any correlation exists between saliva MPA, free MPA and total MPA concentrations at one hour after MMF.

Review of Literature

Systemic Lupus Erythematosus – The Disease

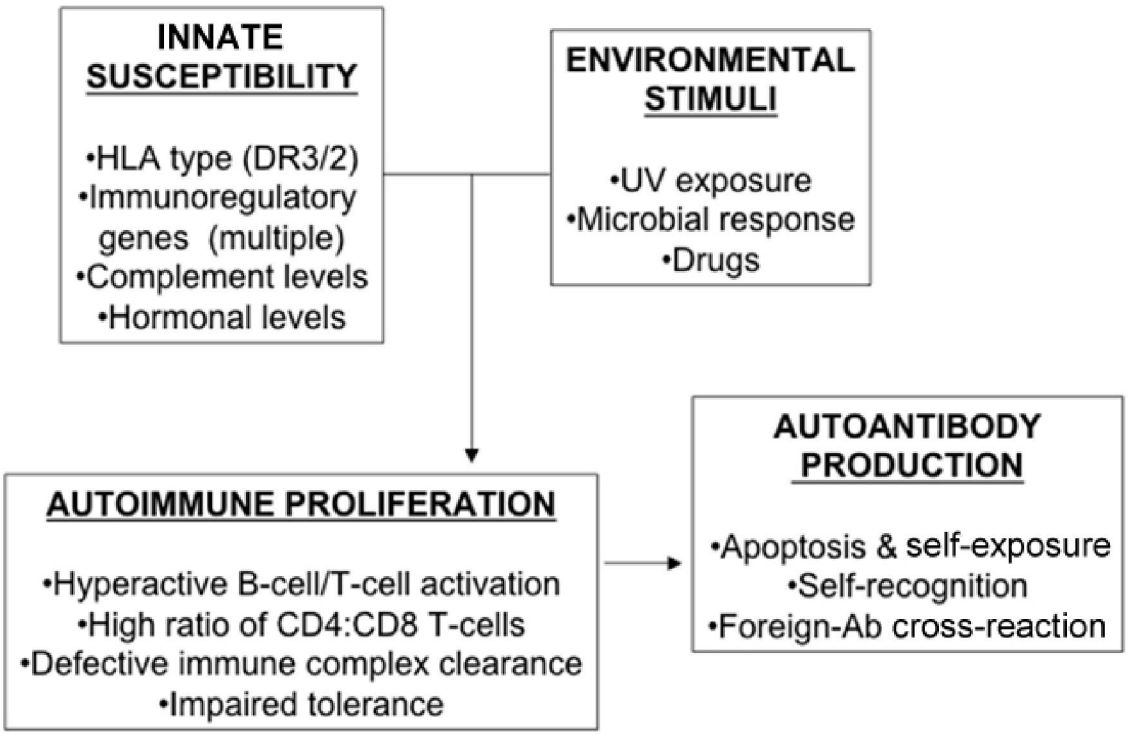
Description

Systemic Lupus Erythematosus (SLE) is a multisystem chronic autoimmune disorder that can affect most organs of the body. (1) The more common manifestations of the disease include dermatological, musculoskeletal and renal manifestations.(1,3,4)

Aetiology

The aetiology of SLE is still unknown other than the fact that it is an autoimmune disorder. However SLE is thought to have a genetic basis apart from various environmental factors which are depicted in the flowchart below.(19)

Figure 1: Aetiology of SLE



Around 33 % of the patients have recurrent flares of SLE and pregnant women are at

a higher risk of flares of SLE. The risk of flares is equal in all the three trimesters of pregnancy.(20)

Epidemiology

Studies have shown that there is marked epidemiological variation with respect to race, age, sex and environmental factors among the different Asian countries. (4) Females (in the child bearing age) are 12 times more likely to be affected than males.(2)The average prevalence in Asian countries was found to be in the range of 30-50 per 100000 population.(4) However, the single largest study from India reported a point prevalence of only 3.2 per 100000 population.(21) It is also reported that there is varying degrees of renal involvement in SLE in more than 50 % of cases.(4) There were a few reported cases of protein losing enteropathy in SLE in India which can present as nephrotic syndrome with low serum albumin.(22)

Scoring systems

SLEDAI (SLE Disease Activity Index) is the most common scoring system used to evaluate SLE disease intensity and evolution.(23)The SLEDAI scoring actually gives different weights for the different signs and symptoms of SLE like vasculitis, seizures, arthritis, myositis, visual disturbances, proteinuria, haematuria etc. A change in the SLEDAI score > 3 indicates a mild to moderate flare while a change in the SLEDAI score >12 indicates a severe flare. Other scoring systems include the SLE scoring system (SIS) as well as the ACR criteria.

Treatment of SLE

Many different drugs have been used to tackle SLE with limited success including hydroxychloroquine, corticosteroids, methotrexate, azathioprine and cyclosporine. However, MPA (Mycophenolic acid) is being increasingly preferred over the others because of its

improved safety profile. There are a few meta-analysis done to see the efficacy of mycophenolate over other drugs like cyclophosphamide and azathioprine and most of them conclude that mycophenolate is equally efficacious and has lesser side effects.(7,8)

Lupus nephritis

Background with Staging

Lupus nephritis is a kidney inflammation caused by SLE, an autoimmune disease. The World Health Organization (WHO) staging of lupus nephritis is universally accepted world over. It has been modified and the current accepted classification is given by the International Society of nephrology/Renal Pathology Society (ISN/RPS) (2003) classification of lupus nephritis.(24) The current categories of lupus nephritis include:(24)

Class I – Minimal mesangial lupus nephritis

Normal looking glomeruli by light microscopy but immunofluorescence microscope shows mesangial immune deposits.

Class II – Mesangial proliferative lupus nephritis

Mesangial proliferation of any degree on light microscope with mesangial immune deposits

Class III – Focal lupus nephritis

Focal, segmental or global endo- or extra-capillary glomerulonephritis involving less than half of the glomeruli with focal sub-endothelial deposits.

Class IV – Diffuse lupus nephritis

Involvement of more than half of the glomeruli typically with diffuse sub-endothelial deposits with or without mesangial alterations.

Class V – Membranous lupus nephritis

Global or segmental sub-epithelial immune deposits or their sequelae, with or without mesangial alterations. Class V patients typically have advanced glomerular sclerosis

Class VI – Advanced sclerosis lupus nephritis

Involvement of >90 % of the glomeruli which are globally sclerosed without any residual activity.

Epidemiology

Most cases of SLE develop lupus nephritis early in the course of the disease. A vast majority of those patients who develop lupus nephritis are less than 55 years of age with children being much more commonly affected as compared to the elderly. There are also racial differences in the presentation of lupus nephritis. African Americans as well as Asians seem to have more severe clinical symptoms as compared to Caucasians.(5,6)

Pathophysiology

Multiple mechanisms are involved in the pathophysiology of lupus nephritis including both the systemic as well as local factors.(5,11) However, the most prominent pathophysiological characteristic of lupus nephritis happens to be immune complex deposition in the glomeruli. This initiates a cascade of events that lead to glomerular disease. T-cells are probably the most conspicuous cells seen in both humans as well as mouse models of lupus nephritis. TGF-β (transforming growth factor β) as well as IL-4 (Interleukin 4) seems to be closely related to the pathological features and these chemicals are secreted as a

part of the Th1 response of the T-helper cells. Apart from the lymphocytes, even the myeloid cells seem to play a major role in the pathophysiology of lupus nephritis. The myeloid differential cells infiltrate the kidney and are thought to release pro-inflammatory cytokines like TGF-β, Il-1, Il-18 and interferon α (IFN α). However the pro-inflammatory cytokines seem to play a major role in the pathogenesis of lupus nephritis, be it antigen presentation at the local lymph nodes or the local inflammation.(5)

Signs and symptoms

In a study done in eastern Indian patients, it was found that the commonest symptoms included arthritis, rash and fever.(6) They reported that the mean duration of symptoms before diagnosis was 13.9 months. Around 42% of the patients had nephrotic range proteinuria. The mean reported serum albumin and creatinine values were 3.09 g/dl and 1.62 mg/dl. 82 % of the patients had lupus nephritis class IV on biopsy at the time of diagnosis.(6) Around 80 % of the patients have microscopic haematuria while macroscopic haematuria is rare in lupus nephritis and about half of the patients will have reduced GFR.(5)

Korbet et al in 2000 had reported that patient survival is about 95 % and renal survival is 94 % in those who achieve remission.(25) Both the patient as well as renal survival is a lot lower in the case of those who do not achieve remission.

Diagnosis

The gold standard for the diagnosis of lupus nephritis is a renal biopsy. However, a lot of other test do aid in coming to diagnosis of lupus nephritis including 24 hour urine protein, serum creatinine, serum albumin, estimated GFR and urinalysis. The urinalysis report generally shows a nephritic picture with RBC casts, RBC's and proteinuria. A rising Anti-dsDNA as well as hypocomplementemia strongly points towards active lupus renal

disease.(5)Sircar et al had reported that around 78 % of the patients with lupus nephritis had a positive Anti-dsDNA.(6)

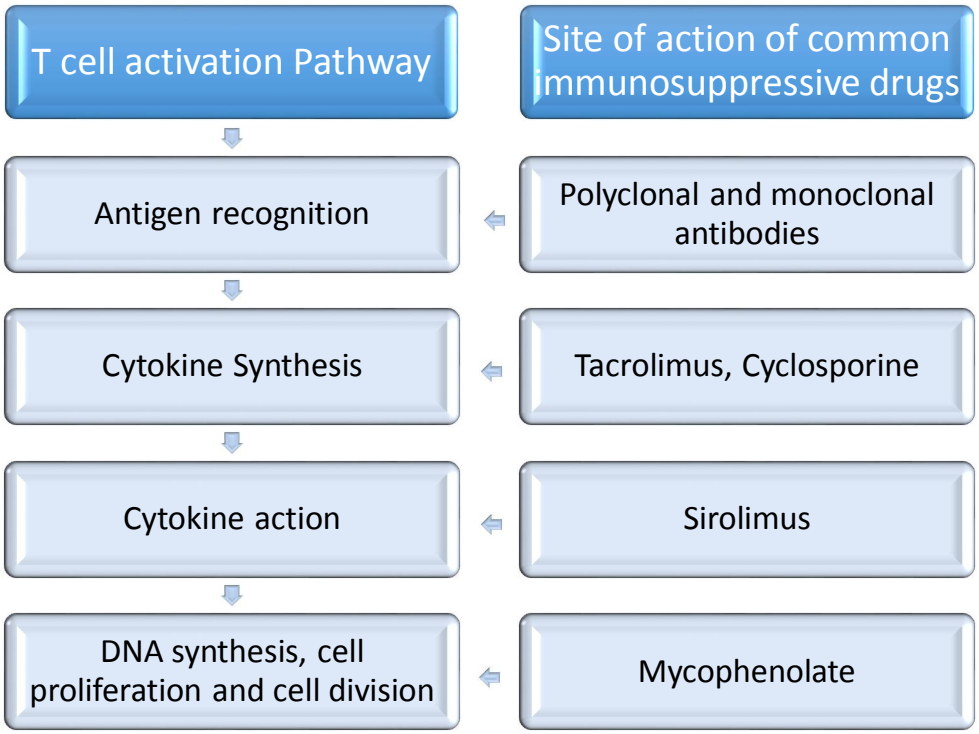
Treatment

The European League against Rheumatism (EULAR) recommends MPA as the first line of treatment along with corticosteroids in the treatment of higher stages (Stage III, IV and V) of lupus nephritis. They recommend a starting dose of 1 g/day given every 12 hours. The dose can go up to 3g/day depending upon the patient response.(9) There is a lot of interest in using biological agents like rituximab and belimumab for the treatment of SLE. In fact, a large study has not shown any added benefit of using rituximab for lupus nephritis. Other agents like rontalizumab and laquinimod are also late stage clinical trials for the treatment of SLE.(10) But still, the current mainstay of treatment happens to be Mycophenolate and steroids.

Pharmacology of mycophenolate mofetil

Mycophenolate mofetil (MMF) is the commercially available morpholinoethyl ester prodrug of the immunosuppressant mycophenolic acid. (MPA).(26) It is a reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH) in purine biosynthesis. The T lymphocytes are heavily reliant upon the *de novo* pathway for the synthesis of purines. Other cells are able to recover purines via a separate, scavenger, pathway and are, thus, able to escape the effect.(27) Figure 2 shows the commonly used immunosuppressants and their site of action in the T cell activation pathway. MMF also block the B cell antibody production which is responsible for the reduced flares in SLE.(26)

Figure 2: Site of action of common immunosuppressive agents



MPA is a fermentation product from various *Penicillium* species and was first isolated in 1898. However, its immunosuppressive properties were not discovered till the 1970s.(28)

The IUPAC name of MMF is 2-morpholin-4-ylethyl (E)-6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-1H-2-benzofuran-5-yl)-4-methylhex-4-enoate. It has a molecular mass of 433.5 and it has an empirical formula of $C_{23}H_{31}NO_7$.

Figures 3 and 4 show the structure of MMF and MPA respectively.

Figure 3: Structure of Mycophenolate Mofetil

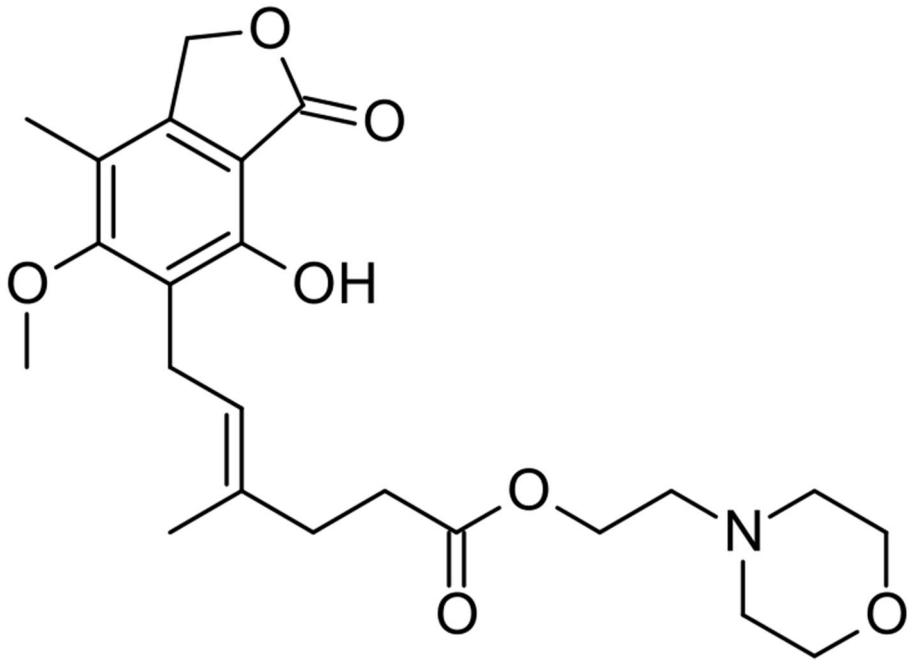
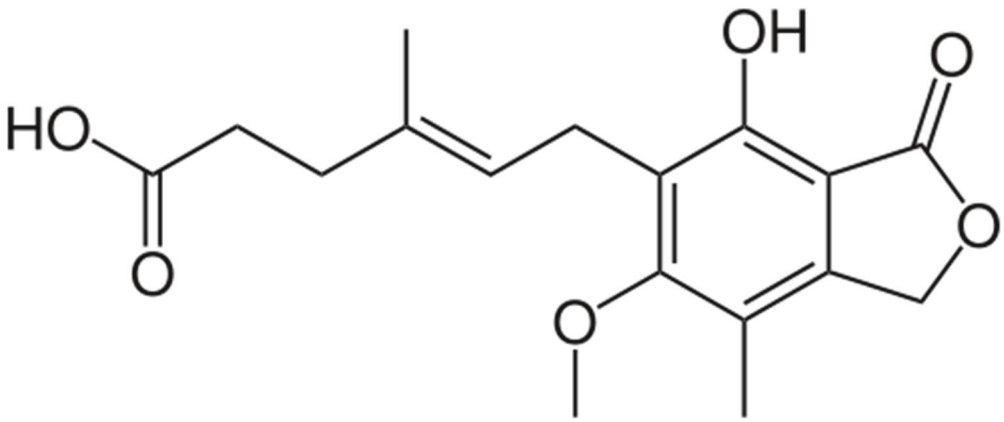


Figure 4: Structure of Mycophenolic acid



Mycophenolate is available as two formulations- mofetil and as enteric coated formulation. The mofetil is directly converted to mycophenolic acid (MPA) and the enteric coated (mycophenolate sodium) is available as MPA. MMF has a bioavailability of >90%. After absorption the prodrug MMF is rapidly converted to MPA by plasma esterases. This conversion from MMF to the active form MPA is rapid in the blood, intestine, and liver, after gastrointestinal absorption.(29) MPA is then metabolized to MPAG (Glucuronide conjugate of

MPA) and to Acyl MPAG. MPA is one drug that undergoes extensive enterohepatic recirculation and thus often presents with two peaks due to reabsorption.(30)

MPA, at clinically relevant concentrations, is 97% bound to plasma albumin. MPAG is 82% bound to plasma albumin at concentration ranges normally seen in stable renal transplant patients; however, at higher MPAG concentrations (observed in patients with renal impairment or delayed renal graft function), the binding of MPA may be reduced as a result of competition between MPAG and MPA for protein binding.(31,32)

MPA – use in transplant

Mycophenolate Mofetil is being increasingly used for the prevention of renal allograft rejection due to its good efficacy and lesser toxicity compared to other immunosuppressants.(5,6) Dooley et al showed that patients on MPA had lesser side effects due to immunosuppression than azathioprine.(6)

Therapeutic drug monitoring (TDM) of MPA in transplant

TDM of MPA is now accepted in many parts of the world. The reasons for TDM include the high (10 fold) inter individual variability in the pharmacokinetics of MPA.(12) It has also been observed that there is a very strong association between total MPA-AUC and acute rejection ($p<0.001$)(33). It is generally agreed that MPA dose should be titrated to keep the MPA-AUC (0-12 hrs.) within 30-60mg.hr/L to avoid rejection.(12–14)

MPA- use in autoimmune disease (AID) & TDM of MPA in SLE

The optimization of immune suppressive therapy is still a major concern in patients with SLE and other autoimmune diseases. The clinical application of TDM of MMF in

autoimmune disease is a topic of debate. In the past decade many different studies have been done to study the pharmacokinetics of mycophenolic acid in autoimmune diseases and especially in SLE, both in paediatric as well as adult patients.(12,35–39)

Autoimmune diseases such as systemic lupus erythematosus (SLE) may present with a low serum albumin concentration which may alter the pharmacokinetics of MPA in such patients. It has also been found that elevated concentrations of MPAG (Glucuronide metabolite of MPA) will displace MPA from its binding site, thereby increasing the free concentration in plasma(40).

It is generally accepted that TDM is required in lupus nephritis patients taking MPA also as advised by the EULAR but they did mention that the actual therapeutic range for MPA in lupus nephritis is still under investigation.(9) Djabarouti et al looked at the pharmacokinetics of MPA in SLE patients and concluded that there was large inter-patient variability in the MPA-AUC thereby indicating that TDM may be necessary.(38) Other studies have also mentioned that MPA TDM in AID seems to be necessary and valuable in the optimization of immunosuppressive therapy for individuals.(12) However, although monitoring does occur in patients receiving MPA for autoimmune diseases there is scant literature on the therapeutic monitoring of MPA in patients with SLE. Woillard et al in 2014 have reported that in children with SLE having MPA AUC <45 mg.h/L are less prone to having relapses. (82 % v/s 52 %, $p<0.01$).(39)

The current literature does not give any objective evidence to define the best strategy for TDM of MMF in patients with SLE. However, several studies in adult and children with SLE taking MMF have reported that the TDM of MMF based on measuring the AUC can be justified. It is obvious that both under as well as overexposure could have several unwanted consequences for patients on MMF for SLE. Very little information is available regarding the

exposure toxicity relationship of MMF but it seems quite clear that AUC >60 mg.h/L does not give any added benefit and might actually lead to a greater incidence of adverse drug reactions.(39)

TDM of MPA vs. outcome in SLE

Lertdumrongluk et al have shown a positive correlation between the MPA-PK and therapeutic responses in patients with SLE.(31) Djabarouti et al showed that the steady state mycophenolate mofetil concentrations are very useful in predicting clinical flares in systemic lupus erythematosus.(37) A recent study noted that maintaining a single MPA trough concentration above 2.5-3 mg/l was probably sufficient to prevent flares in SLE.(41) This suggests that MPA concentrations should be controlled in order to improve therapeutic efficacy. But however it is still uncertain as to whether a single concentration 12 hours post dose is enough for the TDM of MMF or the whole exposure as area under the curve needs to be monitored as is the practice for MMF use in transplant patients. De winter et al tried obtaining an equation using only the trough concentrations to predict the MPA AUC in patients taking MMF for autoimmune diseases using multiple regression analysis but they found that the equations had a high bias and imprecision and it only had a coefficient of determination of 0.48.(42)

Difference of TDM for MPA in transplant versus AID

Neumann et al.(8, 10) compared the pharmacokinetics (PK) of MPA (MMF and Enteric Coated-MPA) in renal transplant (RTX) patients and patients with autoimmune diseases. They found significant differences between the PK of the two groups. In AID patients, the MPA concentrations were found to be higher than in the renal transplant group. In AID, the MPA-PK was less affected by renal function as compared to RTX patients. Discuss the results of their study. Neumann et al found, on comparing the kinetics between kidney transplant and

AID patients, that the data could not be extrapolated from one to the other suggesting the need for more studies in MPA pharmacokinetics in SLE. (12)

Importance of free drug monitoring and free fraction

Hypoalbuminaemia plays a major role in the concentration of drugs which have a very high plasma protein binding.(16) In such cases, it is the free (pharmacologically active) drug concentration and not the total drug concentration that is important.(16,17) This can lead to the patient's dose being increased which may in turn lead to toxicity. (43) A few studies have previously reported that the free fraction of MPA may be more clinically relevant as compared to Total MPA concentrations in blood. Mino et al also postulated that the serum albumin concentrations might be very useful in predicting the free MPA concentrations in plasma.(40) In a study done in children, it was postulated that when the albumin levels were lower than 3.0 g/dl, it was seen that the unbound (free) fraction of the drug increases thus, making more MPA available for metabolism and elimination.(39)

Use of saliva as a biomatrix in TDM- its advantages

Oral fluid for drug testing is easy to collect and non-invasive.(18) Saliva is an ultrafiltrate and can be a viable alternative for the measurement of free drug in the central circulatory system. The last decade has focused on target drugs and their pharmacokinetics in oral fluids.(18,44) There has been significant progress in the testing for drugs of abuse in oral fluids but little data is available for therapeutic drugs. A previous study comparing saliva MPA to free and total MPA in serum done in Caucasian renal transplant patients noted that with exception to the morning trough sample , there was a good correlation between the saliva MPA and both free MPA (r= 0.92) and Total MPA concentrations (r= 0.90).(45) They attributed the higher concentration in the saliva morning trough sample to the presence of blood (following teeth brushing and flossing) in the morning saliva sample.

To our knowledge, no studies have been performed in Indians with lupus nephritis to correlate the saliva MPA concentrations to the total and free MPA concentrations in the plasma.

Pharmacokinetic data analysis:

A few softwares are available for the elucidation of pharmacokinetic parameters using non-linear mixed effects modelling like Nonmem and Pmetrics(46) package for R(47), both of which use the computer language FORTRAN for their calculations. In this study, we used Pmetrics for R which was created after 3 decades worth of research and development at University of Southern California, Los Angeles, USA. This program uses either Iterative 2 stage Bayesian (IT2B) or Non-Parametric Adaptive Grid (NPAG) algorithms to accurately detect the true dispersions of the data. Since many of the immunosuppressive drugs including MMF have a significant amount of enterohepatic recirculation, it has a bimodal distribution of K_{el}. The authors mention that they found the NPAG algorithm to be superior to IT2B runs in detecting the bimodal distributions of the K_{el} (elimination rate constant) of pharmacokinetic data.(46) The major disadvantage of using this package for R is that the user has to be proficient in R. An added advantage of Pmetrics is its ability to generate a lot of custom generated plots apart from the standard Pmetrics functions and this is an advantage over softwares like Nonmem.(46)

Limited Sampling strategies (LSS) for mycophenolate in autoimmune diseases

AUC is the best measure to characterize the drug exposure. However, to get a full profile, it requires collection of many blood samples (often more than 8) and it also requires the patient to be in the hospital/laboratory during the whole duration of sampling. Although trough concentrations have been used to indicate drug exposure for other drugs, for MPA, it does not correlate well with both AUC as well as treatment outcomes.(15)

Therefore, there arises a need to develop a robust equation to predict the AUC using the least number of samples and also taking into account the amount of time the patient has to spend in the hospital/lab. The benefits of LSS's include reduced cost, greater turnaround time, better allocation of resources and potentially shorter hospital stay for patients. A LSS established for a particular patient group need not be applicable in patients with a different disease even though the same drug is being used for that disease as well. There have been a few LSS developed for MPA in AID.(15) The best predictors for MMF AUC included 3 samples, taken pre-dose and at 1 and 3 hours post dose. However, majority of the LSS developed by using multiple regression analysis are plagued by a high bias and imprecision of 0.8 and 22.6 %.(15) In this study, we wanted to look into the possibility of developing and validating a LSS to estimate MPA $AUC_{0\text{-}12}$, having an acceptable bias and precision, to be used in patients with lupus nephritis.

There are two different ways to derive a limited sampling strategy for any drug i.e., multiple regression analysis (MRA) or Bayesian *a posteriori* analysis.(48–50) In the case of MRA, a stepwise linear regression is performed to determine the relationship between the dependant variable (usually AUC) and the independent variables (the concentrations of the drug at various time points). The relationship is described as a function in the form of:(48–50)

$$AUC_n = A + A_0C_0 + A_1C_1 + \ldots\ldots\ldots + A_nC_n$$

Where AUC_n is the Area under the curve of the drug to time n; A is a constant (y-intercept); A_0, A_1, A_n are fitted constants and C_0, C_1, C_n are the concentrations at various time points. Using MRA for the analysis has its own advantages and limitations.(50) Advantages include: No prior knowledge of the pharmacokinetics is required; needs only a simple statistical procedure which can be done on most statistical softwares and once the equation is

finalized, the AUC can be calculated very easily and can even be done manually and lastly, it can be used in a routine clinical setting without extensive training of the staff. Its disadvantages include: timing of the sample is critical and it only works for patients on that particular dosage form for a particular disease/condition. Equations with a high r^2 (coefficient of determination) values are generally the most ideal candidates for the LSS. Bayesian analysis on the other hand uses a mix of the population predictions as well as the individual predictions for AUC.(50) The prediction of parameters using the Bayesian function is as follows:

$$Bayesian\ function = \sum \frac{(P_{pop} - P^1)^2}{var(P)} + \sum \frac{(C_{obs} - C^1)^2}{var(C)}$$

Here, P_{pop} is the population average of the parameter. P^1 is the individual average of the parameter P. Similarly, C_{obs} and C^1 are the observed and predicted concentration values respectively. Var(P) and var(C) are the variances of the estimated parameter and the predicted concentrations respectively.(50)

The biggest advantage of bayesian over MRA is that the samples can be collected at any time point in the AUC, i.e., precise sampling points are not required and it gives greater flexibility in a clinical setting. Apart from that, other advantages include the fact that other covariates such as age, sex, weight etc. can be put into the model. However, it is not without any disadvantages.(50) It is a complex algorithm and it needs trained staff. Also, there needs to be a specific PK model for the drug and thus intricate knowledge of the drug's PK is required for analysis and interpretation.

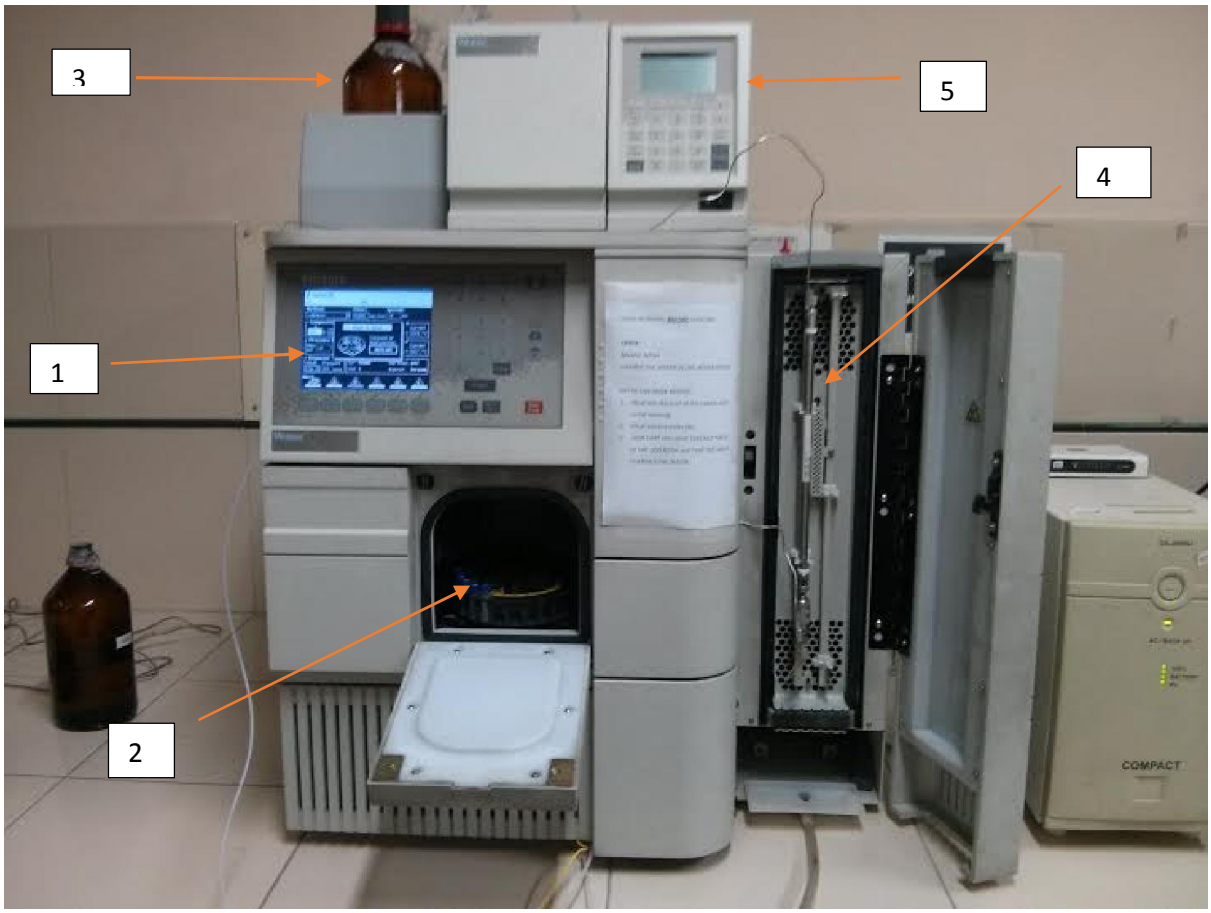
Another important part of both Bayesian and MRA is that the models need to be validated. In the ideal setting, when the sample size is adequate, the patient population is divided into two groups, an index set to develop the LSS and a different validation set (to

validate the LSS). However with a small sample, an alternative to this is the development of LSS using all the patients' pharmacokinetic data and then using a bootstrap model to validate the equation. A bootstrap like validation has been performed previously to validate a LSS model in cyclosporine.(51) Validation entails the calculation of bias (measured as prediction error) and imprecision (which can be measured by using either the absolute prediction error or the root mean square prediction error). It is generally accepted that the imprecision be <15-20 % so as to not make a difference clinically.(50)

High Performance Liquid Chromatography (HPLC)

HPLC is a technique used for the separation of components in a mixture, identification as well as quantification of each component. It can be used for the separation of organic molecules as well as ions. HPLC requires two phases: a solid stationary phase which is usually packed in a stainless steel column and a liquid mobile phase. HPLC can be used to determine the amount of pharmaceutical substance present in a particular solution. The HPLC instrument consists of a pumping system, injector, chromatographic column, stationary and mobile phases, oven, detector and a data collection device like a computer. The figure below (figure 5) shows the HPLC instrument used for analysis in our Clinical Pharmacology Unit.

Figure 5: HPLC machine used for this study



Legend:

1 – Central Console

2 – Sample Tray

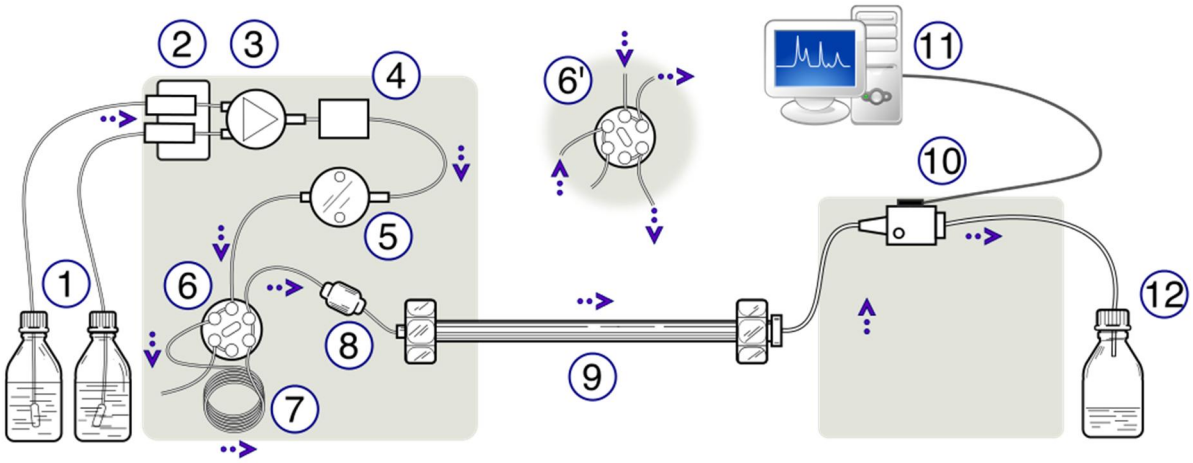
3 – Mobile Phase

4 – Chromatographic column containing the solid phase housed in the oven.

5 – UV-Vis Detector

The figure below (figure 6) gives the schematic representation of the HPLC apparatus.

Figure 6: Schematic representation of the HPLC apparatus: "HPLC apparatus" by WYassineMrabetTalk✉ This vector image was created with Inkscape. - Own work. Legend based on: Practical High-performance Liquid Chromatography by Veronika Meyer, 4th edition, John Wiley



Schematic representation of an HPLC unit. (1) Solvent reservoirs, (2) Solvent degasser, (3) Gradient valve, (4) Mixing vessel for delivery of the mobile phase, (5) High-pressure pump, (6) Switching valve in "inject position", (6') Switching valve in "load position", (7) Sample injection loop, (8) Pre-column (guard column), (9) Analytical column, (10) Detector (i.e. IR, UV), (11) Data acquisition, (12) Waste or fraction collector.

Types of HPLC

The main types of HPLC are:

1) Normal Phase Chromatography

2) Partition Chromatography

3) Displacement Chromatography

4) Reversed Phase Chromatography (RP-HPLC)

5) Ion Exchange Chromatography

All these different types of HPLC have been developed keeping in mind their usage in separation of different organic substances. The most commonly used variety for separation and quantification of therapeutic agents is the RP-HPLC. RP-HPLC requires a non-polar stationary phase and an aqueous polar phase. Stationary phase is usually made up of silica. In RP-HPLC, polar substances elute faster and have a shorter retention time (time taken for the

substance to elute out of the chromatographic column and be detected after starting the injection). RP-HPLC operates on the principle of hydrophobic interactions between the analyte of interest and the stationary and mobile phases.(52) It is important to add a buffering agent to the mobile phase as the mobile phase pH can be an important factor in deciding the retention time of the analyte of interest.

Detectors

The most common detectors employed in the HPLC for measuring therapeutic agents are UV-Vis (Ultraviolet and visual spectrum detector) and the MS (Mass spectrometer) detectors. HPLC machines with MS detectors are costlier than those with UV-Vis detectors.

Mobile Phases

The most common mobile phases that are used for separation are Acetonitrile (ACN) and Methanol combined with water (or buffers). Other acids like trifluoroacetic acid, formic or phosphoric acid may be added along with the above depending upon the use. The composition of the mobile phase may be kept constant (Isocratic flow) or varied (Gradient flow) throughout the analysis.

JUSTIFICATION FOR THE STUDY:

Initially, in patients with AID, MMF doses of up to 2 g daily were used.(43) It is questionable whether standard dose therapy is the best way to treat a patient as dose is not a good predictor for MPA exposure and there is a large inter-individual variability in pharmacokinetics of MPA.(40,53) Since patients with autoimmune diseases are regularly treated with only one or two immunosuppressive drugs, an adequate MPA exposure may be even more important compared to renal transplant recipients receiving multiple

immunosuppressive drugs. Limited pharmacokinetics of MMF has been carried out in patients with autoimmune disease. (31,40,54)

In India, there is a higher incidence of patients having low albumin, especially among those with AID. Presently dose adjustments of mycophenolate in our centre is based on total MPA AUC_{0-12}. Patients with a poor renal function (creatinine clearance <25 mL/min) and patients with low albumin (<32 g/L) are known to have a lower interdose MPA AUC(16). Hypoalbuminaemia as well as renal insufficiency, results in a higher free fraction of MPA which may result in a higher MPA clearance(16).Therefore caution should be used in interpreting total MPA concentrations in patients with severe renal impairment or hypoalbuminaemia.

The objective of the thesis was to study the exposure of mycophenolate (AUC_{0-12}) in SLE patients and to describe the pharmacokinetic parameters of mycophenolic acid in these patients. The other objective was to develop and validate limited sampling strategy equations to predict MPA AUC_{0-12}. Is it possible to measure free MPA concentration by HPLC? Measurement of free MPA is expensive because of the requirement of the ultrafiltrate which is obtained using centrifugal filters. In resource limited settings, can a simple non-invasive saliva specimen or total MPA measurement (which is done as patient care in this centre) be used to estimate free MPA concentration, in both normal and hypoalbuminaemic patients.

Methods

Patient Population

This was an open label prospective 2 year study conducted in the Clinical Pharmacology Unit, (Department of Pharmacology and Clinical Pharmacology) Christian Medical College (CMC), Vellore. Patients were recruited from Department of Nephrology, CMC, Vellore. The study was approved by the Institutional Review board. Patients were recruited after the informed consent was obtained. All patients recruited were diagnosed with Systemic Lupus Erythematosus (SLE) with varying degrees of Lupus Nephritis. They were prescribed MMF as Mycept® (Panacea Biotec Ltd) twice daily and dose was based on the clinician's discretion. All the patients had received MMF for at least 5 days before samples were collected. The timing and compliance of MMF was confirmed prior to including them in the study. Only patients who had been taking mycophenolate, twice daily, regularly and were taking the doses at 8.00/9.00am and 8.00/9.00 pm were considered eligible to be included in the study. The following demographic, anthropometric and laboratory parameters were noted on the day of the study; age, sex, weight, haemoglobin, albumin, total and differential blood counts, blood urea, serum creatinine and GFR.

Inclusion criteria: -

- Patients diagnosed with lupus nephritis
- Patient should have been on mycophenolate for a minimum of 5 days. (to attain steady state in terms of mycophenolate pharmacokinetics)
- Patient should be willing to be in the Clinical Pharmacology Unit for 12 hours.
- Age>18 years

Exclusion Criteria: -

- Patients on enteric coated mycophenolate sodium and other MMF brands (except Mycept)

- Post renal transplant patients.
- Pregnant women
- Critically ill patients

Blood and saliva sampling

The patient presented to the Clinical Pharmacology Unit at 8.00 am, after an overnight fast. An insyte was inserted into a forearm vein. A blood specimen for the measurement of MPA plasma trough concentration was taken, after which MMF was administered orally. Additional serial blood specimens (4ml) were collected into EDTA tubes after 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12 hours post-dose. Food was permitted only two hours after MMF was administered. Specimens were immediately centrifuged and the plasma separated into clean eppendorfs and analysed immediately or stored at -20 degrees till analysis the following day.

In addition, the 1 hour plasma specimen was stored at -20˚ C and was used to measure the free MPA concentration .At the time of analysis of free MPA concentration, 500 µl of plasma was added into the centrifugal filter units (Amicon Ultracentrifugal Filters) and centrifuged at 13,000 rpm for 25 minutes.

After the 1 hr blood sample was collected, the patient was given a dispensable container for collection of saliva. Unstimulated saliva specimen was collected and the specimen was analysed immediately or stored at -20˚ C till analysis.

Chemicals and reagents used

Acetonitrile (ACN), Methanol and acetic acid were purchased from Sigma-Aldrich. Mycophenolic acid and Carbamazepine (CBZ) (internal standard- IS) pure powders were also purchased from Sigma-Aldrich. Amicon Ultracel® YM-30 [0.5 ml 30kDa (kilo Dalton)] centrifugal filter units were purchased from Merck Millipore (Millipore, Cork, Ireland). The

extraction process for saliva MPA required solid phase extraction (SPE) cartridges purchased from Phenomenex (Strata-X®).

20 mM Phosphate buffer solution was prepared, at pH 3.5. It was prepared by mixing 1.7418 g of dipotassium hydrogen phosphate (K_2HPO_4) in 500 ml of Millipore water and 1.3609 g of potassium dihydrogen phosphate (KH_2PO_4) in 500 ml of Millipore water. These were then mixed together and the pH was adjusted to 3.5 using orthophosphoric acid. All the pharmacokinetic measurements were done using a Waters Alliance e2695 separation module with detection in Waters 2489 UV-Visual detector (Waters, Milford, MA, USA).

Preparation of stocks and calibrators for free and saliva MPA

For both Saliva and free MPA detection, a Quality Control (QC) stock (0.01 µg/µl) and two Standard stocks (0.01 µg/µl & 1 µg/ml) were separately prepared in saliva for saliva MPA and saline for free MPA. The reason for the stocks not being prepared in plasma for free MPA was because the patient sample had to pass through a biological membrane filter and were devoid of any proteins and other products which could have interfered with the MPA retention time for the assay.

For both free and saliva MPA detection, 2 ml of the standards (Std.) were prepared as follows:

Std. 1 µg/ml: 200 µl of stock 0.01 µg/µl + 1800 µl of saliva/ saline

Std. 0.5 µg/ml: 100 µl of stock 0.01 µg/µl + 1900 µl of saliva/ saline

Std. 0.25 µg/ml: 50 µl of stock 0.01 µg/µl + 1950 µl of saliva/ saline

Std. 0.05 µg/ml: 100 µl of stock 1 µg/ml + 1900 µl of saliva/ saline

Std. 0.025 µg/ml: 50 µl of stock 1 µg/ml + 1950 µl of saline (For free MPA detection only)

Std. 0.010 µg/ml: 20 µl of stock 1 µg/ml + 1980 µl of saliva/ saline

Sample preparation

Four ml blood was collected and immediately centrifuged (at 3000 rpm for 5 minutes) to separate out the plasma, for measurement of total MPA concentration. It was analysed immediately or plasma was stored at -20˚C till analysis. For analysis of free MPA concentrations, at the time of analysis, 500 µl plasma was passed through the Amicon Ultracel® centrifugal filters. The plasma was filtered at 13000 rpm for 25 min and it yielded about 150-200 µl of ultrafiltrate. This ultrafiltrate was used for estimation of free MPA concentration.

Extraction procedure

The extraction procedure for free MPA, saliva MPA and total MPA are given in figure 7, 8 and 9 respectively. The extraction of saliva MPA required a solid phase extraction unlike others.

Figure 7: Extraction process for MPA in Saliva at 1 Hr

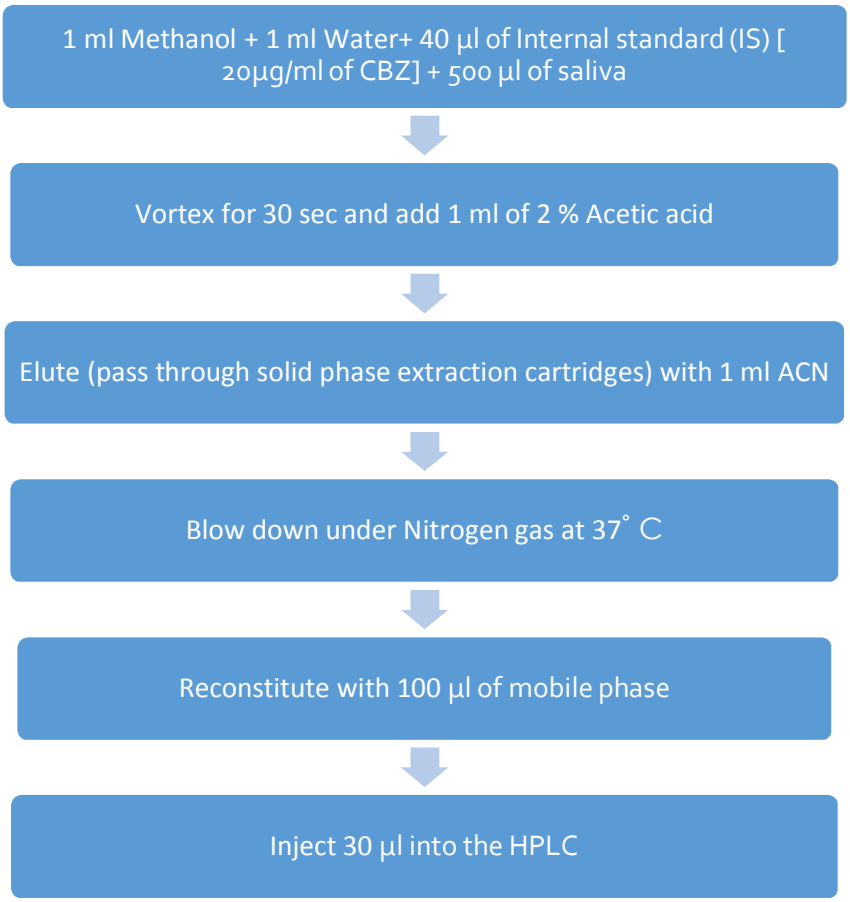


Figure 8: Extraction procedure for Free MPA at 1 Hr

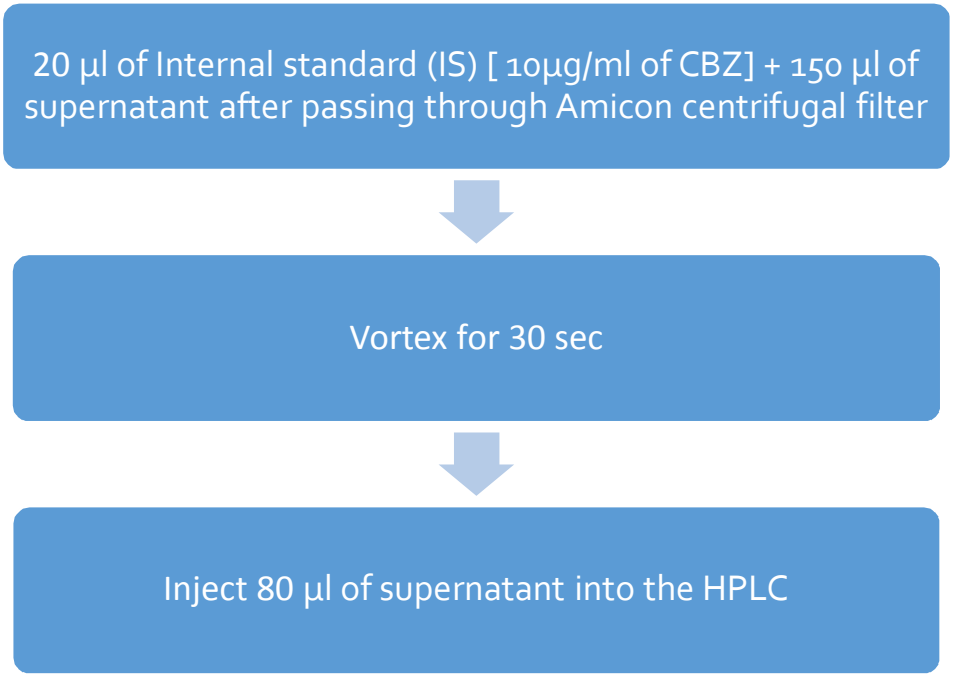
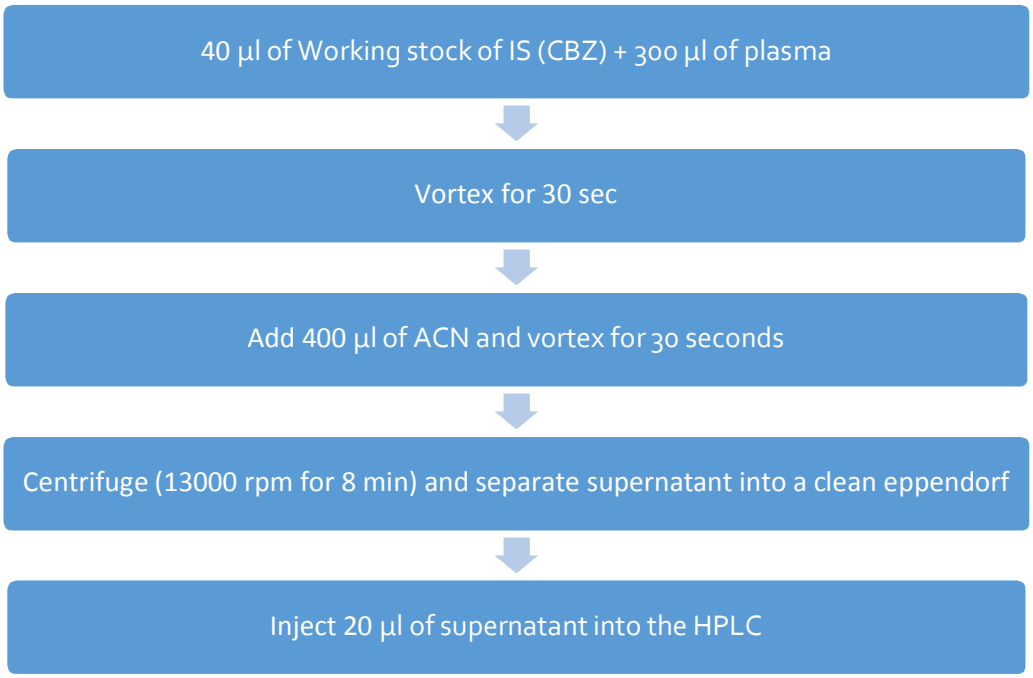


Figure 9: Extraction procedure for total MPA in plasma



The HPLC conditions for total, free and saliva MPA are listed below:

Column: Discovery HS C18 column 5 µm pore size (250 X 4.6mm)

Mobile Phase

For total MPA:

Type of flow: Isocratic, at 1.2 ml/min

49 % - 20 mM Phosphate buffer at pH 3.5

51 % - Acetonitrile

For free MPA and for saliva MPA:

Type of flow: Isocratic, at 1.2 ml/min

54 % - 20 mM Phosphate buffer at pH 3.5

46 % - Acetonitrile

Detection Wavelength for total, free and saliva MPA – 215 nm

Injection volume:

Total MPA – 20 µl

Free MPA- 80 µl

Saliva MPA- 30 µl

Retention time

For free MPA, Drug at 7.37 and IS at 4.84 minutes

For saliva MPA, Drug at 7.92 and IS at 4.84 minutes

For total MPA, Drug at 5.80 and IS at 4.32 minutes

Assay Validation for free and saliva MPA

The assay for total MPA concentrations is already validated and the assays method has been detailed in a previous paper from our centre.(55)

The steps for validation of an assay should include the following steps (from Bioanalytical method validation, US FDA 2013):

1) Selectivity

Lower limit of Quantification (LLOQ) is defined as the lowest concentration at which there is a precision of at least 20 % and an accuracy of at least 80 %), Selectivity would include no interference when tested with blank samples (no internal standard or analyte), patient blank samples (samples from similar cohort of patients who are not on the analyte or the internal standard), zero standard samples (extract with internal standard but not having

analyte). Patient samples which should have both the analyte as well as the internal standard are also assayed to rule out interference.

2) Linearity

A calibration curve is documented to check the linearity from the lowest standard to the highest standard. The calibration needs to be performed in the same matrix as intended in the future analysis. At least five to six concentrations are used to cover the expected range.

3) Accuracy and Precision (Reproducibility)

Accuracy is a measure of the closeness of the results obtained by the procedure to the true value. Precision is defined as the degree of agreement among individual test results obtained when the method is applied to a multiple sampling of a homogenous sample.

$$\% \, CV = \frac{SD}{Mean} \times 100$$

Here, CV refers to coefficient of variation (relative standard deviation). SD refers to the standard deviation.

For accuracy and precision tests, the samples are prepared on the same day from the same batch (multiple injections made from a single extracted sample) to test for variations in the detection by HPLC at different points during the day.

Also from the same sample, five different extractions are injected to check for variability between different extractions. The results are expressed in Mean, SD, % CV and accuracy.

Accuracy and precision were also checked to measure inter-day variation with the same samples being tested with a gap of at least 3 days.

Free MPA

The LLOQ for free MPA in our assay was found to be 0.01 µg/ml. Quality control (QC) samples were prepared from a separate batch at a concentration of 0.1 µg/ml.

For intraday variation, each sample of the patient plasma sample and QC 0.1 µg/ml was measured at 5 different times during a single day. The coefficient of variation (CV) for the intraday validation was calculated. The inter-day variation was determined by analysing the patient plasma sample on two different days, six days apart and the CV was calculated.

The calibration curve after the filtration of the solutions was set at 0.010, 0.025, 0.050, 0.250, 0.500 and 1.0 µg/ml. The curve was considered to be linear when the coefficient of determination $r^2 \geq 0.995$.The curve linearity was maintained between 0.10 and 1.0 µg/ml.

Saliva MPA

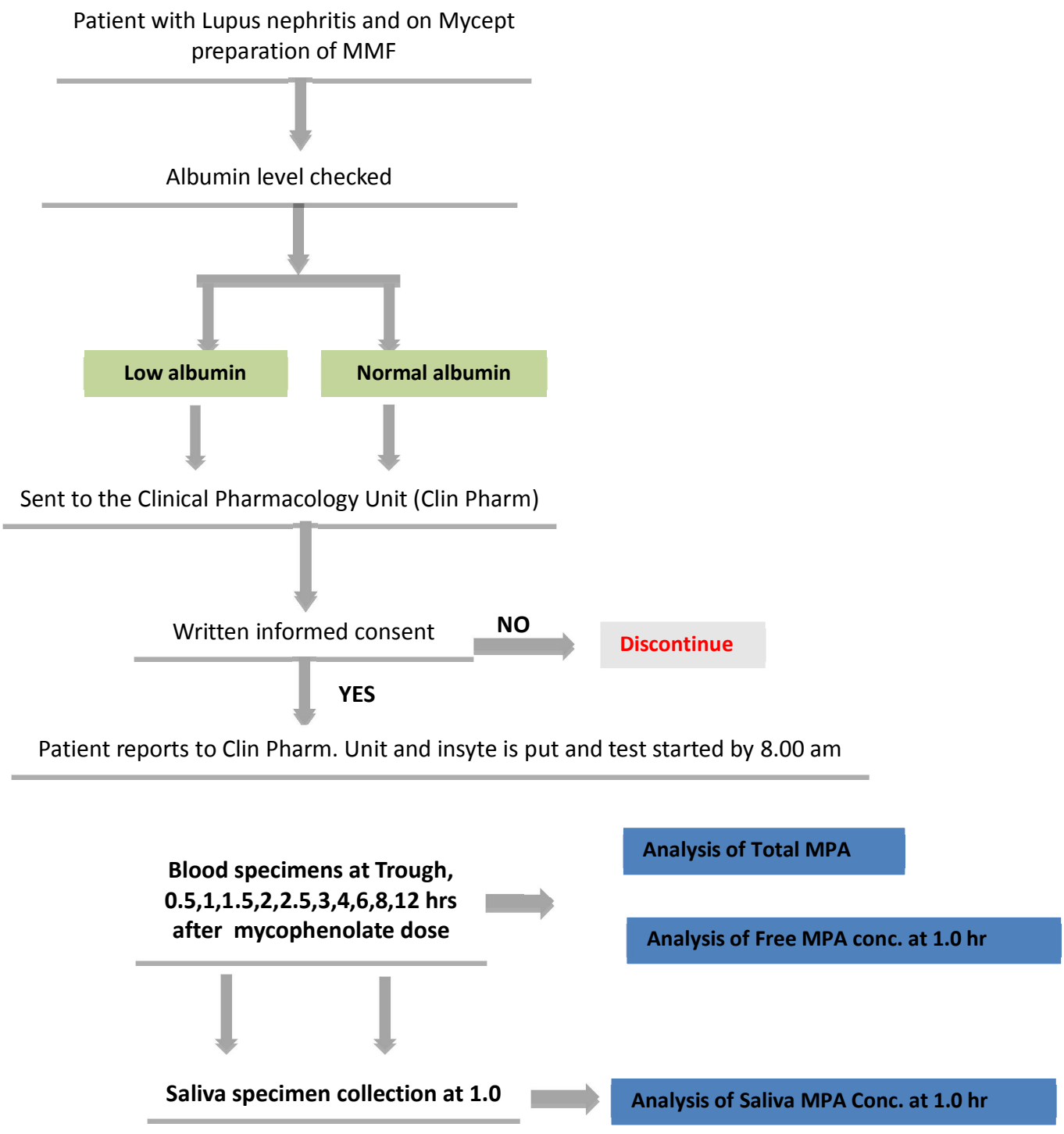
Saliva blanks were obtained from patients who were not on any dosage form of MPA. The LLOQ for free MPA in our assay was found to be 0.01 µg/ml. Quality control (QC) samples were prepared from a separate batch at a concentration of 0.1 µg/ml.

For intraday variation, the QC 0.05 µg/ml was measured at 5 different times during a single day. The coefficient of variation (CV) for the intraday validation was calculated.

Apart from this, variations in six different extractions of the QC 0.05µg/ml was also done and the CV was calculated.

The calibration curve after the filtration of the solutions was set at 0.010, 0.050, 0.250, 0.500 and 1.0 µg/ml. The curve was considered to be linear when the coefficient of determination $r^2 \geq 0.995$. The curve linearity was maintained between 0.010 and 1.0 µg/ml.

Flowchart of the sequence of events:



Sample Size

The sample size was calculated to be 30 based upon the following:

Detection of a significant correlation of 0.5 between the saliva MPA and free MPA in plasma at 1 hour,

Power 80% &

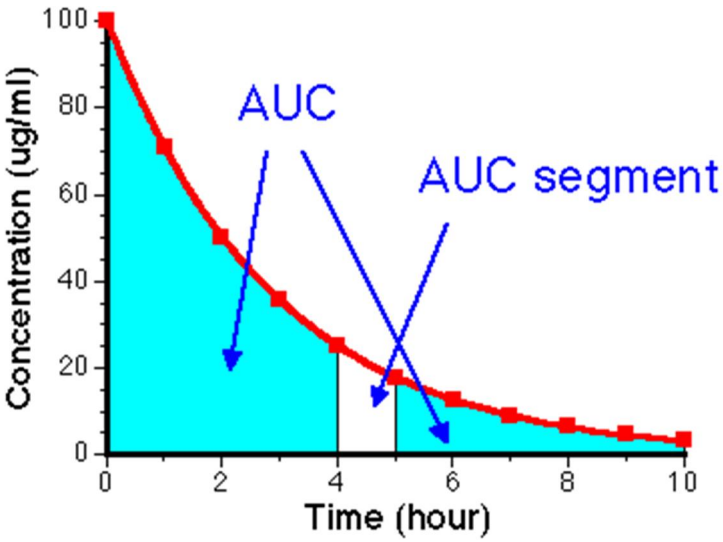
Level of Significance 5%

Pharmacokinetic and Statistical Analysis

Calculation of MPA $AUC_{0\text{-}12\ hr}$

The trapezoidal rule is an accurate estimate of the area under the concentration-time curve. It breaks down the area to be measured into small trapezoids to calculate AUC. Its calculation is depicted in the figure below.

Figure 10: Calculation of AUC using trapezoidal rule



The equation for calculating AUC is as follows:

$$AUC_{t_1-t_2} = \frac{t_2 - t_1}{2}(C_1 + C_2)$$

Here, C_1 and C_2 are concentrations at time points t_1 and t_2 respectively.

Therefore, $AUC_{0\text{-}12\ hr}$ is calculated as

$$AUC_{0-12\ hr} = \int_{t=0}^{t=12} C_t \, . \, dt$$

Here, C_t refers to the concentration of the drug at time t.

Development and validation of LSS

All statistical analysis were performed using R (version 3.1.5). Pharmacokinetic parameters were obtained by using Pmetrics (Version 1.2.6) package for R. Overall, 30 twelve hour pharmacokinetic profiles were obtained. The limited sampling strategy (LSS) development was similar to a paper published from our Clinical Pharmacology Unit.(56) Stepwise multiple linear regression was performed using MPA $AUC_{0\text{-}12\ hr}$ as the independent variable and the total MPA concentrations in plasma at 0, 0.5, 1, 1.5, 2, 2.5, 3 and 4 hr as the dependant variable. The MPA $AUC_{0\text{-}12\ hr}$ was first checked for normality using Kolmogrov Smirnov test.

The equation derived after running the regression was in the form of $\boldsymbol{AUC_{0\text{-}12}} = \boldsymbol{A + A_0C_0 + A_1C_1 + \ldots\ldots\ldots + A_nC_n}$, where A, A_0, A_n are fitted constants which are associated with the concentrations at 0, 1 and n hours post dose. The prediction bias of these estimates derived by the LSS was calculated by measuring the Prediction error and the absolute prediction error by the following formulae:

$$PE\% = 100 \times \frac{(LSS\ AUC - Total\ measured\ AUC)}{Total\ measured\ AUC}$$

And

$$APE\% = 100 \times \frac{|(\ LSS\ AUC - Total\ measured\ AUC)|}{Total\ measured\ AUC}$$

The absolute prediction had to be <15% for it to be clinically acceptable.

Then bootstrap validation (n=1000) was performed for the regression equation using R. The new LSS equation was used to calculate the estimated MPA $AUC_{0\text{-}12\ hr}$. The prediction bias as well as the absolute precision error (expressed as a percentage) were calculated along with their respective 95 % confidence intervals (CI). For assessing the agreement between the Observed MPA $AUC_{0\text{-}12\ hr}$ v/s the LSS estimated MPA $AUC_{0\text{-}12\ hr}$, the Bland Altman method (Mean v/s Difference Plot) was used as described previously.(57) Intraclass correlation coefficient (ICC) shall be used to compare between the LSS estimated and the actual observed MPA $AUC_{0\text{-}12\ hr}$.

Pharmacokinetic variables like K_a (absorption rate constant), K_e (Elimination rate constant), V_d (Volume of Distribution) and Cl (clearance) were estimated using non parametric adaptive grid function in Pmetrics package for R. The Pmetrics Package requires a model file to be supplied (in FORTRAN syntax) along with the data file as it uses the computer language FORTRAN do all the calculations. As our study protocol did not involve measurement of the bioavailability of the Mycept® tablets, bioavailability was arbitrarily set to 60- 95 % based upon previous reports of bioavailability of MMF.

Correlation between Free v/s total MPA &Saliva v/s free MPA

For the correlation between saliva MPA and free MPA in plasma, Spearman correlation was performed, both before and after dividing the data based upon low and normal serum concentrations. Also stepwise multiple regression was performed similar to that used in the LSS determination to see if saliva MPA or total MPA in the plasma can be used to predict the free MPA concentrations in the plasma.

Results

Bioanalytical validation of assay for free MPA –HPLC

Selectivity

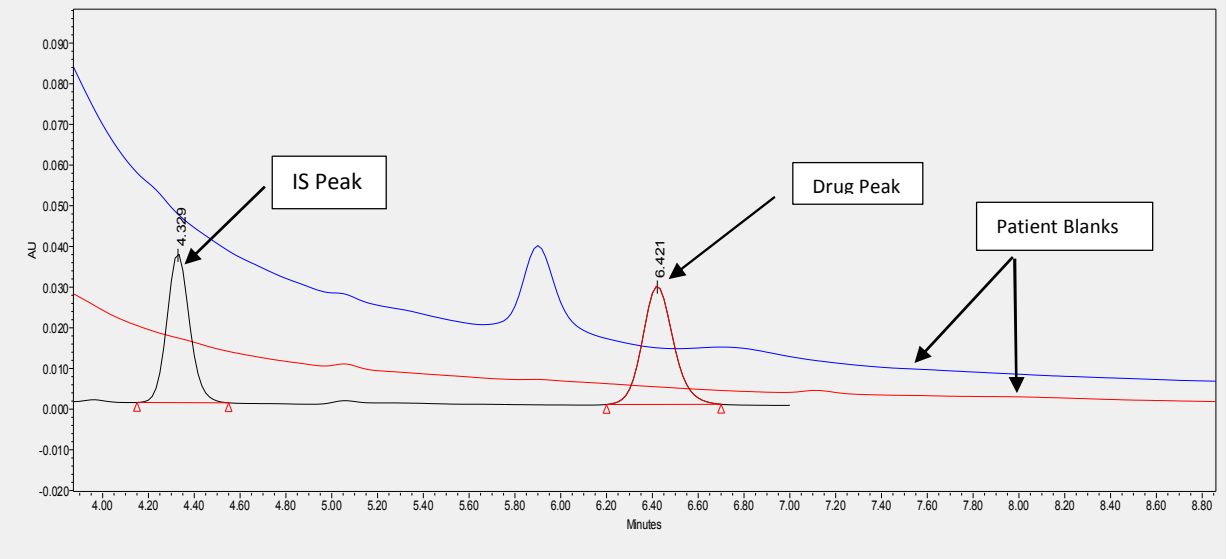
The figure (figure 11) below shows the chromatogram of two normal blank samples (plasma sample of a person who is not on MMF (drug) or CBZ (IS)) compared to a standard chromatogram for free MPA. 15 such blanks were run to confirm that there were no extraneous peaks that could interfere with the drug or the IS peak. This confirmed that plasma samples from patients not on mycophenolate did not have any peaks that interfered with the MPA or IS drug peaks.

10 samples were also run containing only the IS and no drug (zero standards). This was done to confirm there was no interference from the internal standard at the retention time of the drug

Apart from this, samples were assayed from 5 patients on MMF, to confirm the drug peak and the drug retention time.

To conclude, this assay is selective for free MPA and there was no interference at the retention time for both, MPA and CBZ.

Figure 11: Chromatogram of standard 1 with two patient blanks



Calibration Curve - To Confirm Linearity

The chromatogram of the standard curve for free MPA is given in the figure below (figure 12). A standard curve was run to test the linearity. The results are tabulated in table 2 below.

Figure 12: Chromatogram of a standard curve for free MPA

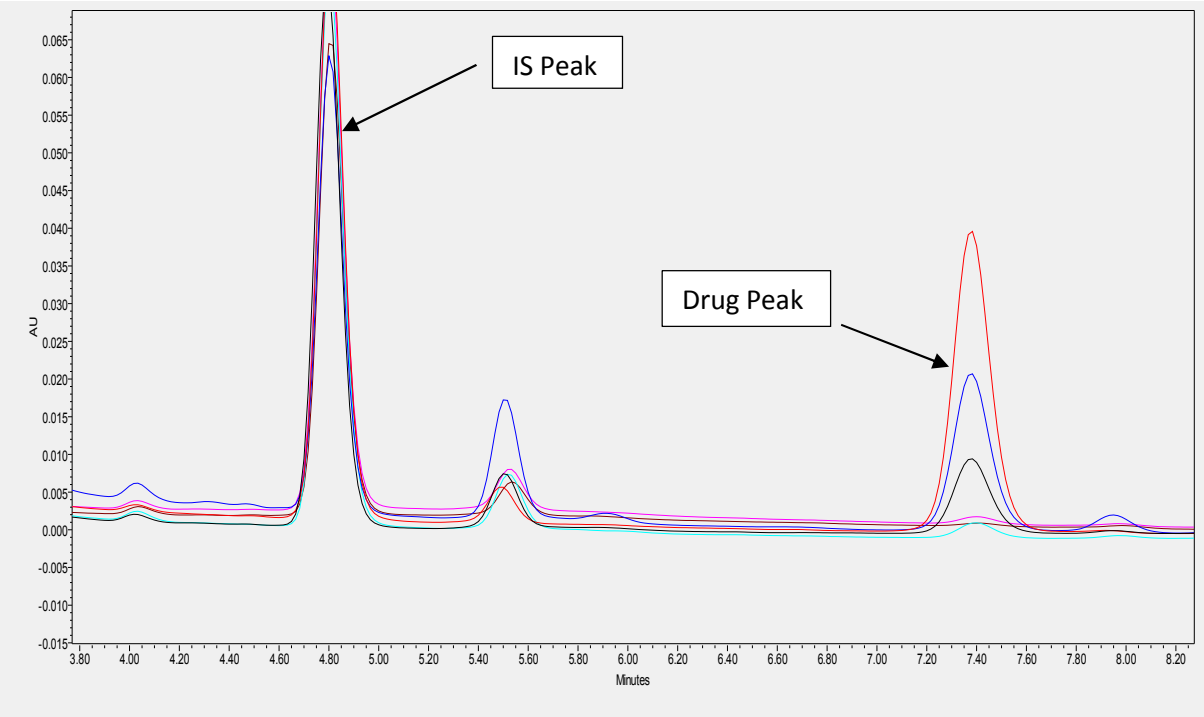


Table 1: Standard curve for free MPA (using loop size of 80ul)

STANDARDS	INTERNAL STANDARD		DRUG		RATIO
µg/ml	RT	Area	RT	Area	
1	4.799	126420	7.370	84504	0.67
0.5	4.796	99311	7.370	43772	0.44
0.25	4.786	120414	7.370	20903	0.17
0.05	4.796	121174	7.389	4161	0.03
0.025	4.797	122664	7.392	2127	0.02
0.01	4.800	104705	7.404	956	0.009
QC(0.1)	4.788	124007	7.401	9299	0.07

RT: Retention time

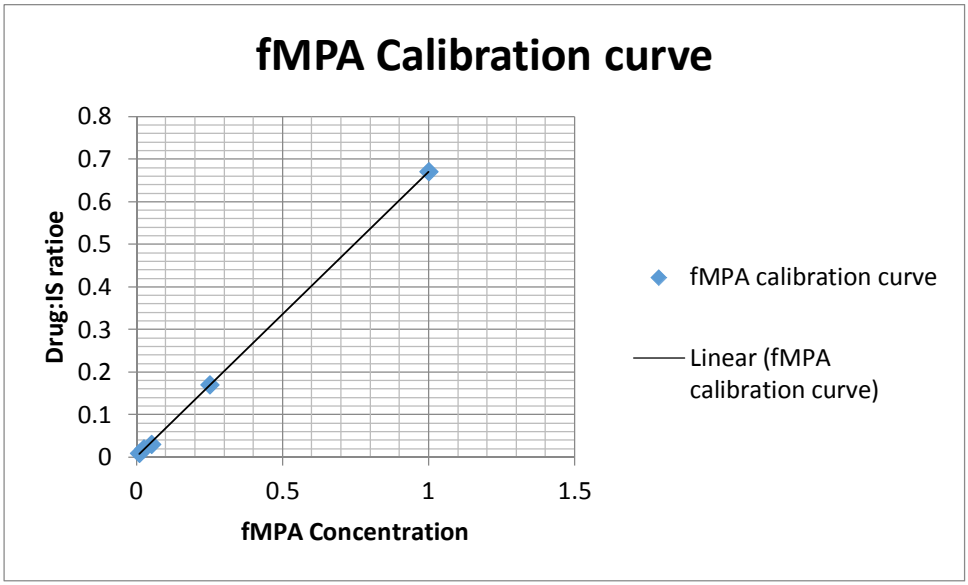
In the above table and other tables to follow in this section, RT refers to the retention time (time elapsed since start of the analysis to the time of detection in minutes). Area in the table refers to the area of the peak in the chromatogram of the analysis. IS refers to the internal standard Carbamazepine (CBZ).

The calculated linearity was in the form of an equation for a straight line:

"$y = mx + c$"; Where m is the slope and c is the intercept. From the above table, the slope was 0.669, the intercept was 0.0012 and the correlation was 0.999. The calculated QC was 0.103 µg/ml.

To conclude the curve was linear from 0.01 µg/ml to 1 µg/ml. The calibration curve for free MPA in plasma is given in the figure below (figure 13):

Figure 13: Calibration curve for free MPA in plasma



Test of Accuracy and Precision (Done using 20 µl Loop)

Reproducibility of the assay was tested by re-injecting from same extraction of 0.1 µg/ml spiked sample. The sample from the same batch with the same extraction was injected six times on the same day. The reproducibility of the assay was actually tested using the loop volume of 20 µl but the assay was later run using a loop volume of 80 µl. Considering that the conditions remained the same except the loop volume, we expected the assay to improve without having any effect on reproducibility.

Table 2: Intra-day variation with same extraction, spiked sample (0.1µg/ml)

	INTERNAL STANDARD		DRUG		RATIO	CONCENTRATION
	RT	Area	RT	Area		
1	4.782	60105	7.152	1956	0.03	0.0781
2	4.859	60690	7.321	2435	0.04	0.1094
3	4.871	57985	7.315	1924	0.03	0.0781
4	4.858	61135	7.278	2058	0.03	0.0781
5	4.858	62886	7.238	1771	0.03	0.0781
6	4.823	59769	7.176	1969	0.03	0.0781

Mean Concentration (sd) = 0.0833(.013)

Accuracy= -0.016

Coefficient of variation% = 15.33 %

Reproducibility (reinjection) was also tested in a patient sample to check precision. Six different injections were run from the same sample and the same extraction at different times on the same day. The accuracy for this sample however cannot be calculated as it is not possible to ascertain the actual concentration of free MPA in this plasma sample.

Table 3: Intraday variation with same extraction for patient plasma sample

	INTERNAL STANDARD		DRUG		RATIO	CONCENTRATION
	RT	Area	RT	Area		
1	4.782	56620	7.075	813	0.01	0.016
2	4.796	47016	7.146	618	0.01	0.016
3	4.794	56561	7.155	759	0.01	0.016
4	4.801	56444	7.153	637	0.01	0.016
5	4.803	59119	7.177	619	0.01	0.016
6	4.827	56730	7.250	798	0.01	0.016

Mean Concentration (sd) = 0.016(0)

Coefficient of variation% = 0 %

Another method to check reproducibility includes variations with different extractions. QC (0.25 µg/ml) from the same batch was extracted at 6 different times during the day and injected to determine the variability between different extractions.

Table 4: Variations with different extractions of 0.25 µg/ml

	INTERNAL STANDARD		DRUG		RATIO	CONCENTRATION
	RT	Area	RT	Area		
1	4.770	55036	7.119	4999	0.09	0.2656
2	4.865	70103	7.268	5891	0.08	0.2344
3	4.837	59269	7.186	5110	0.09	0.2656
4	4.815	49151	7.160	5028	0.10	0.2969
5	4.816	50785	7.171	5124	0.10	0.2969
6	4.809	64192	7.174	5026	0.08	0.2344

Mean Concentration (sd) = 0.2656(.028)

Coefficient of variation % = 10.52 %

Inter-day Accuracy and Precision

The results of the inter-day variations are tabulated in table 6 below. Index number 1, 2 and 3 represent Day 1 and 4, 5 was performed 6 days later.

Table 5: Inter-day variation Patient Plasma Sample

	INTERNAL STANDARD		DRUG		RATIO	CONCENTRATION
	RT	Area	RT	Area		
1	4.801	56444	7.153	637	0.01	0.016
2	4.803	59119	7.177	619	0.01	0.016
3	4.827	56730	7.250	798	0.01	0.016
4	4.782	56631	7.179	809	0.01	0.016
5	4.794	54908	7.134	785	0.01	0.020

Average Concentration (sd) = 0.0168(0.0018)

Coefficient of variation% = 10.65 %

To conclude, the assay developed for free MPA in plasma had good reproducibility, linearity and specificity and can be used to measure free MPA concentrations in the plasma.

Bioanalytical validation of assay for MPA in saliva –HPLC

Selectivity

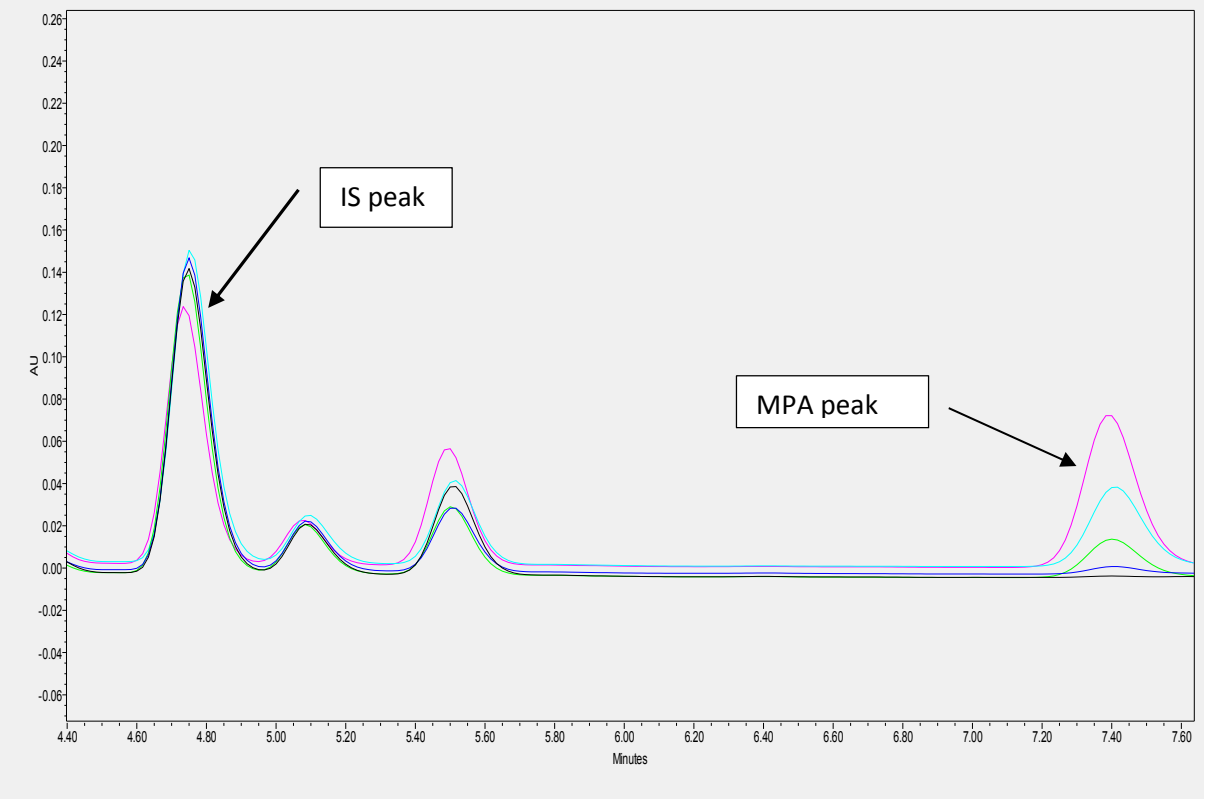
The figure (figure 14) below shows the chromatogram of two normal blank samples (plasma sample of a person who is not on MMF (drug) or CBZ (IS)) compared to a standard chromatogram for free MPA. 10 such blanks were run to confirm that there were no extraneous peaks that could interfere with the drug or the IS peak. This confirmed that plasma samples from patients not on mycophenolate did not have any peaks that interfered with the MPA or IS drug peaks.

10 samples were also run containing only the IS and no drug (zero standards). This was done to confirm there was no interference from the internal standard at the retention time of the drug

Apart from this, samples were assayed from 5 patients on MMF, to confirm the drug peak and the drug retention time.

To conclude, this assay is selective for free MPA and there was no interference at the retention time for both, MPA and CBZ.

Figure 14: Chromatogram of a standard curve for MPA in Saliva



A standard curve along with the QC (Quality control) was run to check for linearity (table 7).

Table 6: Standard curve for standards and QC of MPA in saliva

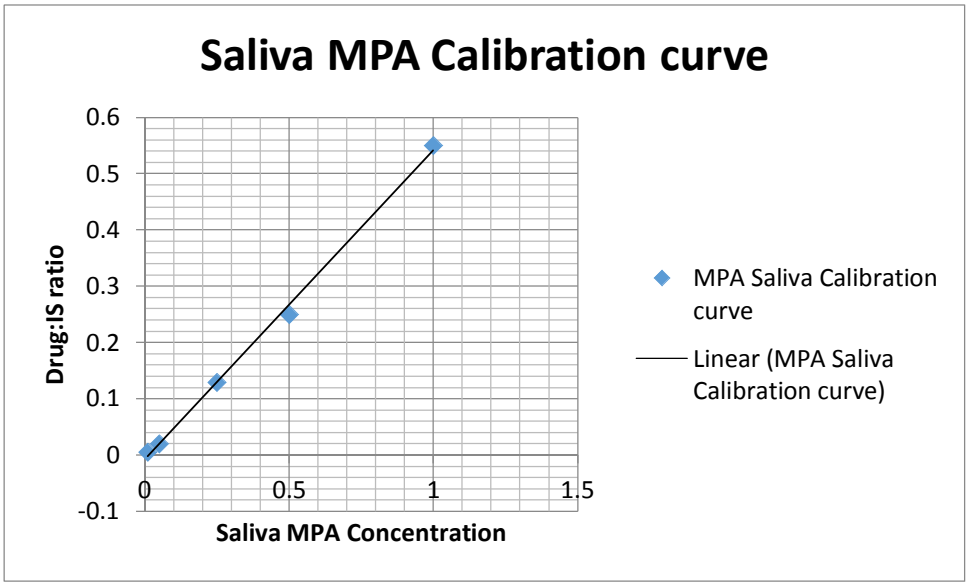
STANDARDS	**INTERNAL STANDARD**		**DRUG**		**RATIO**
µg/ml	RT	Area	RT	Area	
1	4.630	1723422	7.233	923567	0.55
0.5	4.623	1699040	7.217	422002	0.25
0.25	4.629	1331401	7.238	171331	0.13
0.05	4.636	1587166	7.247	37158	0.02
0.01	4.625	1563236	7.214	9521	0.006
QC(0.1)	4.619	1418657	7.219	64476	0.05

RT: Retention time

The calculated linearity was in the form of an equation for a straight line as mentioned in the results section earlier.

From the above table, the slope was 0.505, the intercept was -0.00083 and the correlation was 0.999. The calculated QC was 0.103 µg/ml. The figure (figure 11) below shows the calibration curve for MPA in saliva.

Figure 15: Calibration curve for MPA in saliva



Test of Accuracy and Precision

Reproducibility of the assay was tested by re-injecting from same extraction of 0.05 µg/ml spiked sample. The sample from the same batch with the same extraction was injected six times on the same day (Table 8).

Table 7: Intra-day variation with same extraction QC (0.05 µg/ml)

RUNS	INTERNAL STANDARD		DRUG		RATIO	CONCENTRATION
	RT	Area	RT	Area		
1	4.836	1398302	7.912	46033	0.03	0.046
2	4.850	1422944	7.930	48919	0.03	0.046
3	4.838	1406414	7.920	45473	0.03	0.046
4	4.854	1171252	7.937	36288	0.03	0.046
5	4.843	1390257	7.922	46405	0.03	0.046
6	4.853	1349174	7.938	44419	0.03	0.046

Average Concentration (sd) = 0.046 (0)

Inaccuracy= 8 %

Coefficient of variation = 0 %

Another method to check reproducibility includes variations with different extractions. QC (0.05 µg/ml) from the same batch was extracted at 6 different times during the day and injected to determine the variability between different extractions. The results are tabulated in table 9 below.

Table 8: Variations with different extractions of QC (0.05 µg/ml)

RUNS	INTERNAL STANDARD		DRUG		RATIO	CONCENTRATION
	RT	Area	RT	Area		
1	4.838	1406414	7.92	45473	0.03	0.046
2	4.843	1390257	7.922	46405	0.03	0.046
3	4.837	1335171	7.909	55120	0.04	0.059
4	4.840	1429019	7.918	49750	0.03	0.046
5	4.847	1490658	7.932	50079	0.03	0.046
6	4.836	1398302	7.912	46033	0.03	0.046

Average Concentration (sd) = 0.0482 (0.0053)

Inaccuracy= 3.67%

Coefficient of variation = 11.02 %

To conclude, the assay developed had good reproducibility, linearity and specificity and can be used to measure MPA concentrations in saliva.

Overview of the Pharmacokinetic data

A total of 33 patients were recruited for the study from May 2013 to March 2014. Three patients refused to stay for the total duration of the study (12 hours) and thus were excluded from the study. Of the 30 patients recruited, 15 patients were diagnosed as class IV lupus nephritis, 2 patients each were class II and III, 1 patient was class V while the rest were unclassified. For the pharmacokinetic analysis of total MPA, 12 hour profiles from the 30 patients (who completed the total 12 hours specimen collection) were analysed using

Pmetrics, which was loaded into R, as described in the methods section. For the correlation between saliva MPA at and free MPA concentrations at 1 hour, only 28 samples were available for analysis (two were below the lower limit of quantification).

All the patients had achieved steady state at the time of sampling. The patient characteristics have been tabulated in table 10 below.

Table 9: Patient characteristics

PARAMETER	VALUE*
TOTAL PATIENTS	30
AGE	29.33 (18-63)
SEX (M/F)	4/26
WEIGHT	55.03 (38-73)
DOSE PER DAY (mg/day)	1441.67 (500-3250)
SERUM CREATININE	1.02 (0.46-3.15)
ALBUMIN	4#(2.8-4.6)
ESTIMATED GFR	88.98#(23.82-169)
DOSE PER KG BODY WEIGHT	26.474(8.62-63.83)

* All values in Mean (Min-Max) format, #-Median values

The table below shows the basic pharmacokinetic parameters of the collected data.

Table 10: Basic pharmacokinetic parameters of the collected data

PARAMETER	VALUE*
AUC $_{0\text{-}12\ HR}$(mg.h/L)	45.12 (19.52-67.66)
C_{max} (µg/ml)	16.703(5.243-30.861)
T_{max}(h)	1.083(0.5-2.5)
C_0 (µg/ml)	1.74 (0.17-5.1)
AUC/DOSE	1.83 (0.97-3.81)
C_0/DOSE	0.069 (0.006-0.153)

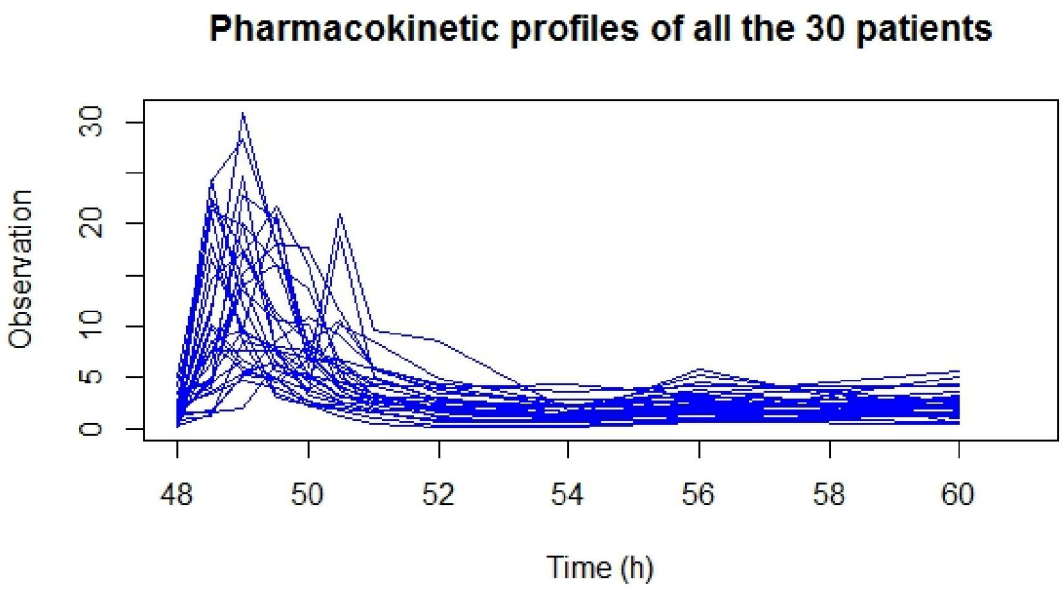
* All values in Mean (Min-Max) format unless specified otherwise

In the above table, C_{max} refers to the maximum concentration of MPA achieved in plasma. T_{max} refers to the time taken to reach C_{max} in plasma. C_0 refers to the pre-dose concentrations of MPA. In this study, the mean T_{max} was calculated to be 1.08 hours while the mean C_{max} was calculated to be 16.70 µg/ml.

The calculated coefficient of variation for the dose normalized AUC and C_0 were 34.04 % and 61.81 % respectively.

Figure 16 shows the actual pharmacokinetic profiles of the study patients. Here the observation refers to the concentrations of MPA in µg/ml.

Figure 16: Observed Pharmacokinetic profiles of the study patients



Development of a Limited Sampling Strategy (LSS) for MPA $AUC_{0\text{-}12}$Hour

The correlations between the MPA $AUC_{0\text{-}12\ hr}$ and the concentrations at different time points are depicted in table 12:

Table 11: Correlations between the different MPA concentration and MPA $AUC_{0\text{-}12\ hr}$

			$AUC_{0\text{-}12hr}$
Spearman's rho	C_0	Correlation Coefficient	0.630**
		Sig. (2-tailed)	0.000
	$C_{0.5}$	Correlation Coefficient	0.106
		Sig. (2-tailed)	0.577
	C_1	Correlation Coefficient	0.585**
		Sig. (2-tailed)	0.001
	$C_{1.5}$	Correlation Coefficient	0.781**
		Sig. (2-tailed)	0.000
	C_2	Correlation Coefficient	0.647**
		Sig. (2-tailed)	0.000
	$C_{2.5}$	Correlation Coefficient	0.442*
		Sig. (2-tailed)	0.014
	C_3	Correlation Coefficient	0.572**
		Sig. (2-tailed)	0.001
	C_4	Correlation Coefficient	0.670**

	Sig. (2-tailed)	0.000
C_6	Correlation Coefficient	0.570**
	Sig. (2-tailed)	0.001
C_8	Correlation Coefficient	0.472**
	Sig. (2-tailed)	0.009
C_{12}	Correlation Coefficient	0.486**
	Sig. (2-tailed)	0.006

*. *Correlation is significant at the 0.05 level (2-tailed).*

**. *Correlation is significant at the 0.01 level (2-tailed).*

In the above table, $C_0 - C_{12}$ refer to the MPA concentrations at that time point. The best correlation was found between MPA $AUC_{0\text{-}12\ hr}$ and $C_{1.5}$ as evident from the above table.

After checking $AUC_{0\text{-}12Hr}$ for normality using Kolmogrov Smirnov Test ($p>0.200$), stepwise multiple linear regression was performed using MPA concentrations at Trough, 0.5, 1, 1.5, 2, 2.5, 3 and 4 hour as the dependent variables in the initial model.

The final model included only MPA concentrations at trough, 1 Hr, 2 Hr and 4 Hr even though the correlation was highest between the concentration at 1.5 hours and the MPA $AUC_{0\text{-}12\ hr}$. The ANOVA (Analysis of variance) statistics for the stepwise regression is given in table 13. In this table, at step 1, all the parameters described in the paragraph above are included in the regression model and then sequentially, MPA concentrations at 2.5, 3, 1.5 and 0.5 hours (Hr2.5, Hr3, Hr1.5, Hr0.5) are excluded from the model from step 2-5 of the stepwise multiple linear regression.

Table 12: ANOVA table for stepwise multiple regression

STEP	PARAMETERS EXCLUDED	DF	DEVIANCE	RESIDUAL DF	RESIDUAL DEVIATION	AIC
1				21	371.3781	93.48070
2	- Hr2.5	1	1.835819	22	373.2139	91.62863
3	- Hr3	1	2.225865	23	375.4398	89.80702
4	- Hr1.5	1	7.125949	24	382.5657	88.37109
5	- Hr0.5	1	16.559094	25	399.1248	87.64231

DF= Degrees of Freedom, AIC= Akaike information criteria

Table 14 shows the final model coefficients. The PR (>|T|) Column shows the significance of each of the individual variables in predicting the $AUC_{0\text{-}12}$Hr. The residual standard error on 25 degrees of freedom (df) was 3.996. The multiple r squared for the final model was 0.9266 while the adjusted r squared based on the degrees of freedom was calculated as 0.9149. The overall p-value was $8.23e^{-14}$.

Table 13: Final Model Coefficients

	ESTIMATE	STD. ERROR	T VALUE	PR(>|T|)
(INTERCEPT)	12.5425	2.0016	6.266	1.48e-06 ***
TROUGH	2.8087	0.7158	3.924	0.000602 ***
HR1	0.8245	0.1091	7.560	6.49e-08 ***
HR2	0.8266	0.2117	3.905	0.000632 ***
HR4	4.6335	0.4912	9.434	1.03e-09 ***

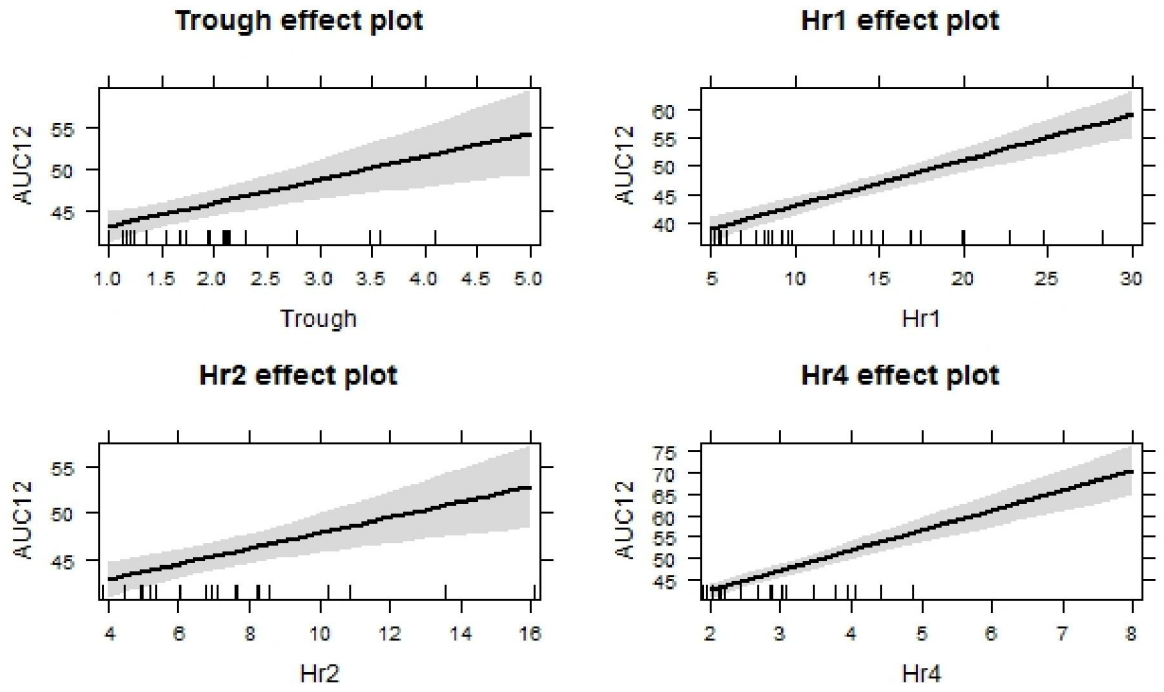
Significance codes: 0, '***' 0.001, '**'0.01, '*'0.05, '.' 0.1, ' '1

Thus, the final regression model equation is as follows:

$$\text{MPA } AUC_{0\text{-}12\text{ hr}} = 12.5425 + (2.8087 \times C_0) + (0.8245 \times C_1) + (0.8266 \times C_2) + (4.6335 \times C_4)$$

The effect of the individual variables on the $AUC_{0\text{-}12\text{ hr}}$ is given in the figure below (figure 17).

Figure 17: Model Effects plot for the final regression model



The final model residuals are given in table 15. Here, 1Q and 3Q refer to the 1st and the 3rdquantiles respectively.

Table 14: Final Model residuals

MIN	1Q	MEDIAN	3Q	MAX
-7.0817	-2.5749	0.2253	2.2756	7.3124

The residuals were tested for heteroskedasticity using the Breusch-Pagan test. One of the assumptions that needs to be satisfied for linear modelling includes the absence of heteroskedasticity which is nothing but the different variability's within subpopulations of the modelled data. There are many methods to check for heteroskedasticity, however the method chosen in this study is the studentized Breusch-Pagan test as it is easy to perform on R and is universally accepted.

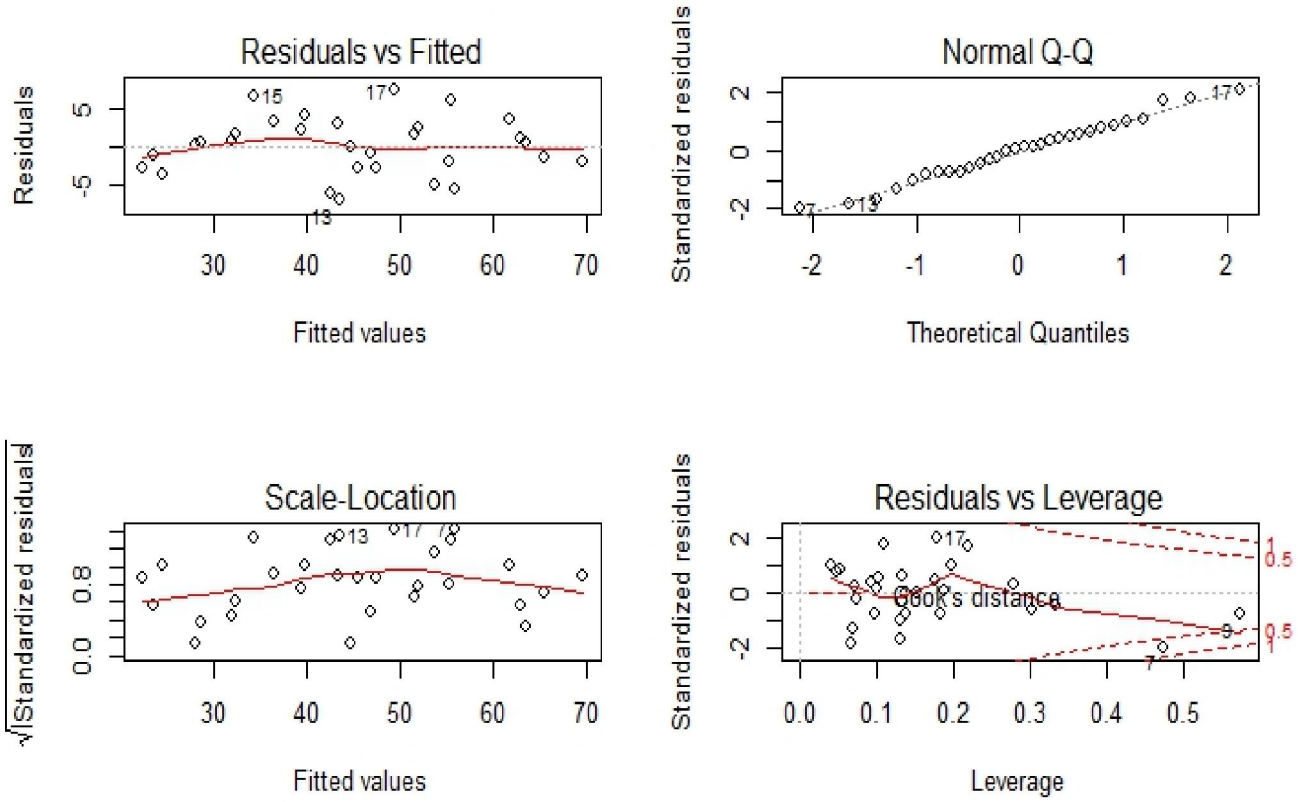
Studentized Breusch-Pagan test

BP = 4.118, df = 4, p-value = 0.3903

Result: Null hypothesis that the variances among the residuals was equal was retained.

The model diagnostics for the final model including the Q-Q plot for the residuals is given in the figure below.

Figure 18: Model diagnostics for the final regression model



Thus, from the Q-Q plot, it was confirmed that the residuals did not grossly violate the assumption of normality. The above graph also shows the plot between the residuals and the estimated (fitted) AUC values. The bottom right part of the figure shows a plot between the residuals and the leverage and also features the Cook's distance. In regression analysis, leverage and Cook's distance both indicate those observations that are far away from the average corresponding predictor values. Cook's distance is also a measure of the changes in

regression coefficients when an observation is deleted. In this study, very few values had a high leverage indicating that the regression performed was robust.

Validation of the LSS

Bootstrap validation was done for the data using 1000 iterations. The results of the bootstrap is given in table below:

Table 15: Bootstrap for the regression model

	R	ORIGINAL	BOOTBIAS	BOOTSE	BOOTMED
1 INTERCEPT	1000	12.54250	-0.0670279	1.60696	12.33757
2 TROUGH	1000	2.80874	0.2217399	1.12874	2.90130
3 HR1	1000	0.82448	-0.0026863	0.11447	0.83048
4 HR2	1000	0.82656	-0.0375554	0.23863	0.79454
5 HR4	1000	4.63350	0.0363167	0.63612	4.51556

Here, R= No. of iterations, SE= Standard Error, MED= Median values, Trough, HR1, Hr2, Hr4 are MPA concentrations at that particular time point.

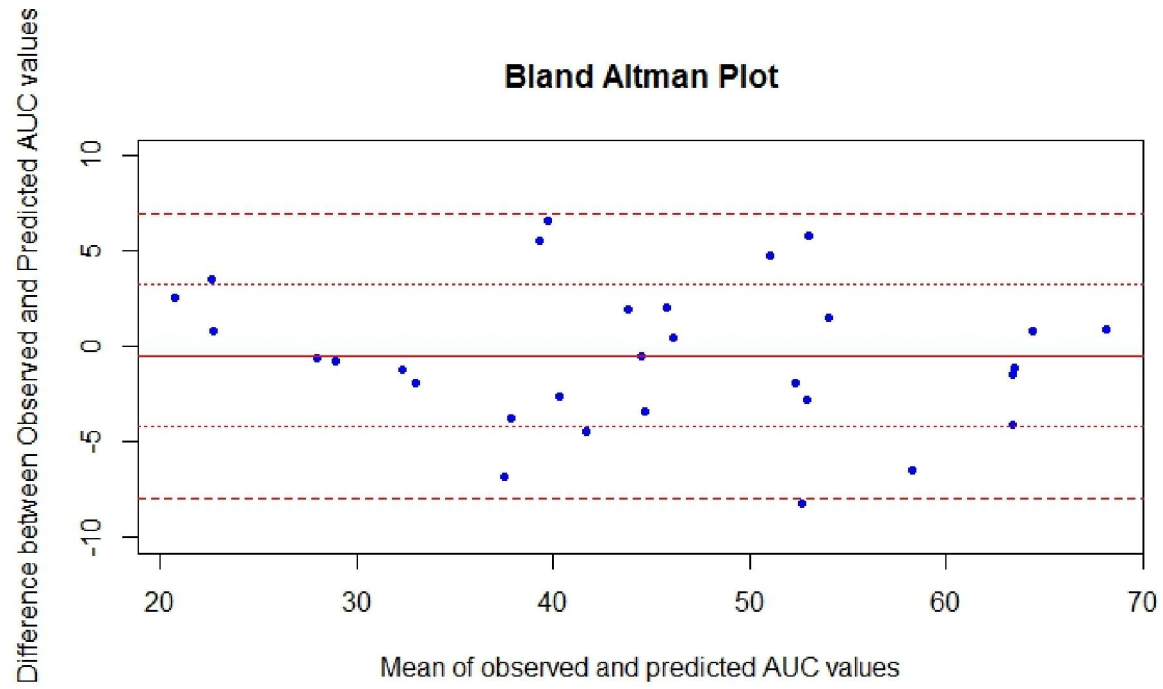
Therefore, Post bootstrap, the final regression model for the LSS to predict MPA $AUC_{0\text{-}12}$Hr is as follows:

$$\text{MPA } AUC_{0\text{-}12Hr} = 12.3376 + (2.9013 \times C_0) + (0.8305 \times C_1) + (0.7945 \times C_2) + (4.5156 \times C_4)$$

Here, C_0, C_1, C_2, C_4 refer to the MPA concentrations at that particular time point.

After the bootstrap, the MPA $AUC_{0\text{-}12Hr}$ was estimated using the post bootstrap equation as mentioned above. The Prediction bias and the Absolute prediction error were calculated as mentioned in the methods section. The overall prediction bias was found to be -0.13% (95 % confidence interval [CI]: -3.49, 3.23) while the absolute prediction error was 7.09% (95 % CI: 5.08, 9.10). Apart from this, the mean as well as the difference of the observed and predicted MPA $AUC_{0\text{-}12Hr}$ were calculated and plotted as described by Bland and Altman.(57) The plot is shown in figure 19.

Figure 19: Bland Altman Plot for agreement between Observed and Predicted AUC values



Mean difference

…….. +/- 1 SD from mean difference

------ +/- 2 SD from mean difference

This Bland Altman plot shows a good agreement between the observed and predicted MPA $AUC_{0\text{-}12\ hr}$. It also rules out the presence of a systematic error in the findings due to the random nature of the points.

The table below tabulates the difference between the actual and the predicted values of AUC in our patients.

Table 16: Observed and Predicted AUC's and their Difference

Observed AUC	Predicted AUC	Difference between the Observed and Predicted AUC
32.87	31.71	-1.16
42.85	44.82	1.96
43.92	39.49	-4.43
44.78	44.27	-0.51
46.39	42.99	-3.40
48.64	53.38	4.75
50.13	55.92	5.80
64.15	62.65	-1.50
67.67	68.57	0.90
20.83	24.41	3.58
22.30	23.14	0.84
29.29	28.54	-0.75
36.45	43.04	6.59
39.71	36.02	-3.69
40.95	34.13	-6.82
53.22	54.75	1.53
56.76	48.51	-8.25
61.46	55.00	-6.44
63.95	62.88	-1.06
63.96	64.78	0.82
65.38	61.33	-4.05
33.91	32.03	-1.88
36.48	42.06	5.58
41.65	39.05	-2.60
44.75	46.78	2.03
45.88	46.35	0.47
53.24	51.39	-1.85
19.52	22.07	2.55
28.26	27.70	-0.56
54.31	51.51	-2.80

From table 17, it is evident that the predicted AUC and observed AUC are in close agreement and reporting of the predicted AUC would result in a decision by the clinician which is no different from that of the TDM report with the observed AUC. Thus the 4 point LSS is a suitable alternative to measuring the full MPA AUC.

Intraclass correlation (ICC) was also performed on the above data. The results of the ICC are tabulated in table 18. The ICC is used to assess the consistency of

measurements made by multiple observers or multiple methods of measuring the same quantity. It is similar to the Pearson correlation but with the important difference of the ICC using pooled mean and standard deviation for each values while Pearson correlation uses individual means and standard deviations.

Table 17: Intraclass Correlation between the observed and predicted MPA AUC

	INTRACLASS CORRELATION	95% CONFIDENCE INTERVAL	
		Lower Bound	Upper Bound
AVERAGE MEASURES	0.981	0.960	0.991

Pharmacokinetics of MMF in adult patients with Lupus Nephritis

The data set was analysed using Pmetrics for R as detailed in the methods section. The model file is a FORTRAN code file which is required for Pmetrics to analyse the data. The model used was an oral 2 compartment model with variable parameters including Ka, Ke, V, KPC, KCP and Tlag (for explanation of each term, see paragraph below table 19). The program was run 28 times with a combination of various primary variables until the model with acceptable coefficient of determination (R^2>0.90). Given below is the model file used for the analysis:

#Pri
Ke, 0, 1.5
V, 0.01, 80
Ka, 0.4, 10
KCP, 0, 3.5
KPC, 0, 0.35
Tlag1, 0, 1

FA1, 0.6, 0.99

#COV

WT

DPKG

ALB

GFR

CREAT

#F

FA(1)=FA1

#SEC

CL=Ke*V*GFR

#Lag

TLAG(1) = Tlag1

#Out

Y(1) = X(2)/V

#Err

L=0.1

0.1, 0.15,0,0

The model file is divided into different blocks, each preceded by a # sign. The blocks used in this model included primary (#Pri), fraction absorbed (#F), covariates (#Cov), secondary variables (#Sec), absorption lag (#Lag), Output equation in FORTRAN code (#Out) and a lambda error block (#Err) for the MPA assay error polynomials. The covariates included in our model were serum albumin (ALB), dose per Kg body weight (DPKG), body

weight (WT), estimated glomerular filtration rate by modified MDRD (modification of diet in renal disease) method (GFR) and serum creatinine levels (CREAT).

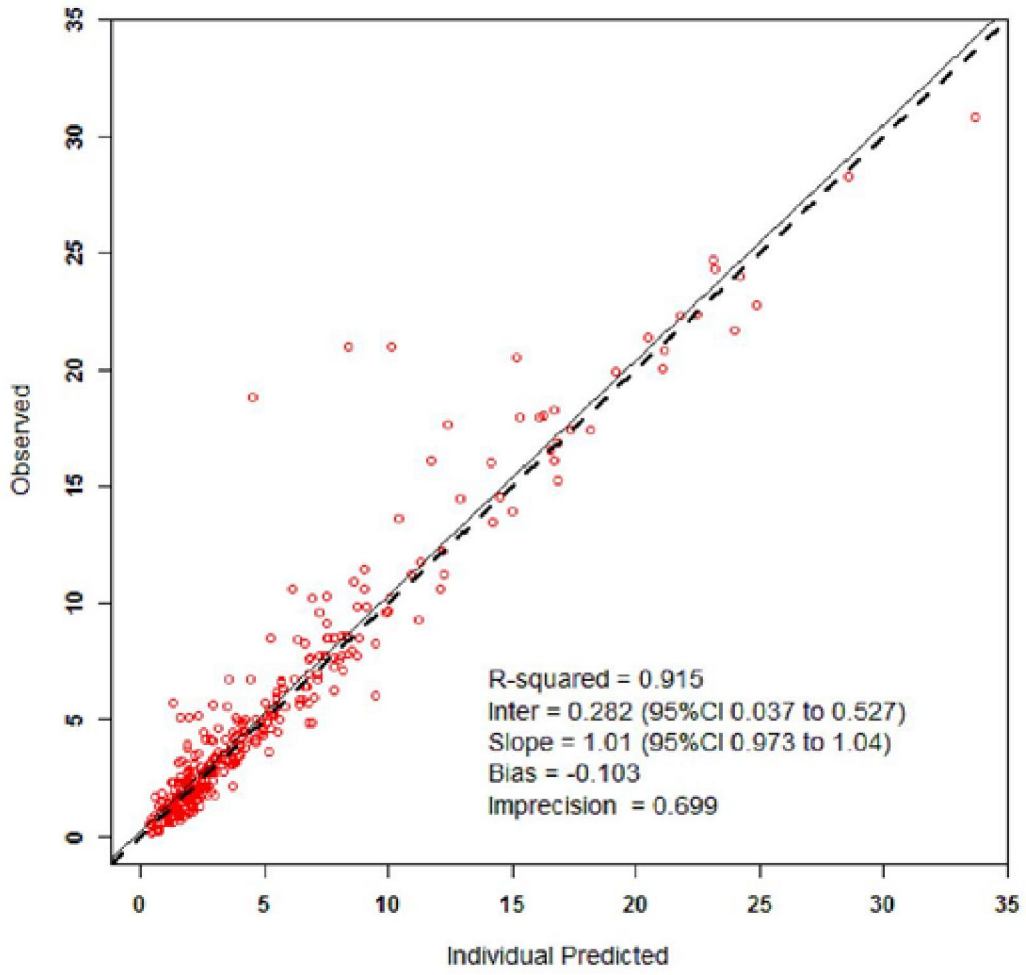
The non-parametric adaptive grid method was used to analyse the data which is inbuilt in Pmetrics for R. The model parameters are summarized in the table below.

Table 18: Pharmacokinetic parameters of the study

PK PARAMETERS	MEAN	SD	MEDIAN
K_e	0.421	0.348	0.338
V	21.299	13.450	20.015
K_a	3.906	3.114	2.714
KCP	1.317	0.707	1.137
KPC	0.052	0.057	0.037
$TLAG_1$	0.356	0.289	0.355

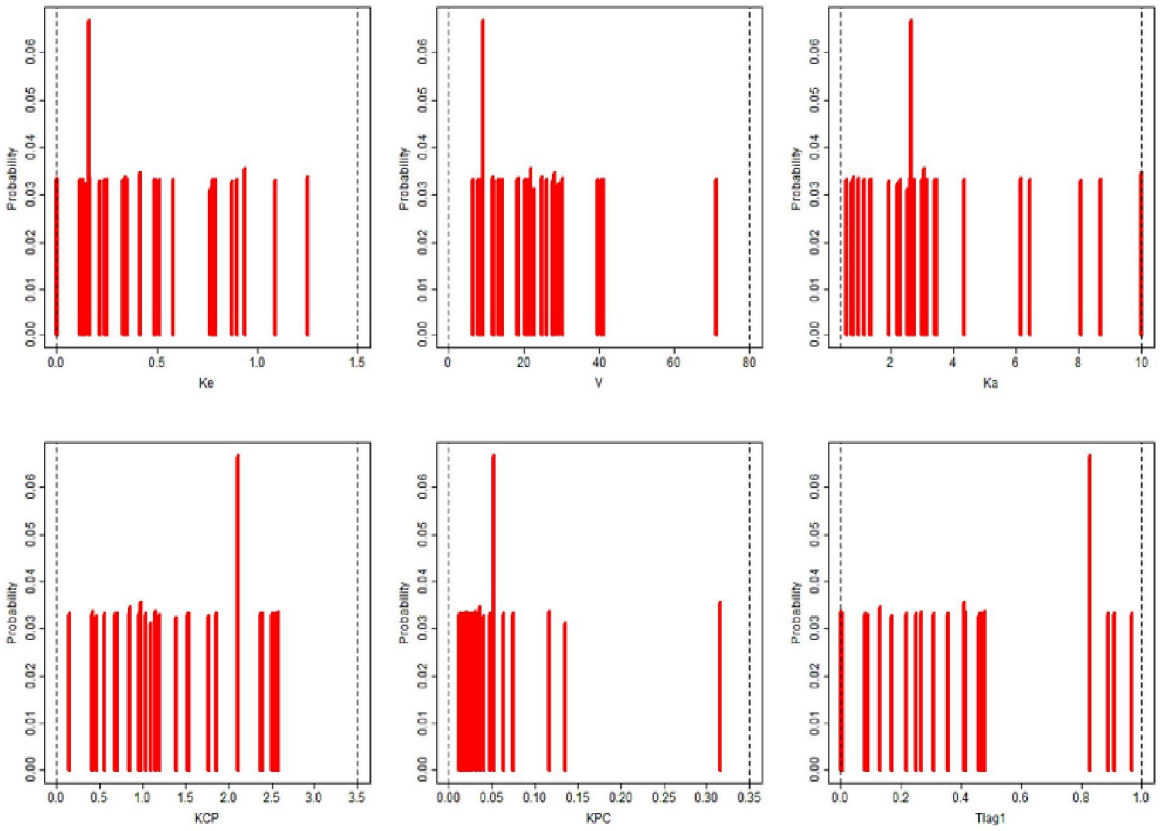
In the above table, K_e refers to the elimination rate constant while K_a is the absorption rate constant. $Tlag_1$ is the time lag for oral absorption of MMF. KCP and KPC refers to the shift of MMF from the central to the peripheral compartment and vice versa. V refers to the volume of distribution of MMF. The apparent volume of distribution (V/F where F is the fraction of MMF absorbed) for all patients from the table is 21.3 ± 13.45litres. The apparent clearance (CL/F) of MMF was estimated to be 8.97 L/Hr. The individual prediction characteristics of the model is given in the figure below (figure 20).

Figure 20: Individual prediction characteristics of the 2-compartment model for MMF



The individual predictions had a coefficient of determination r^2 of 0.915. The bias of the individual predictions were also relatively low (-0.103). The model had an imprecision of 0.699 which is acceptable. The figure below shows the marginal density plots of the individual primary variables in the model.

Figure 21: Marginal density plots for the primary variables of the model



The figure below (figure 22) shows the individual observed as well as the model predicted pharmacokinetic profiles of the 30 patients. The unbroken line corresponds to the actual measured MPA concentration-time curve while the black round dots indicate the model predicted values at the time points at which the MPA specimens were collected as mentioned in the methods section. The time is set from 48 to 60 hours to indicate that all the patients had achieved steady state at the time of sampling and due to restriction on the inputs in the comma separated values (.csv) files into the Pmetrics program.

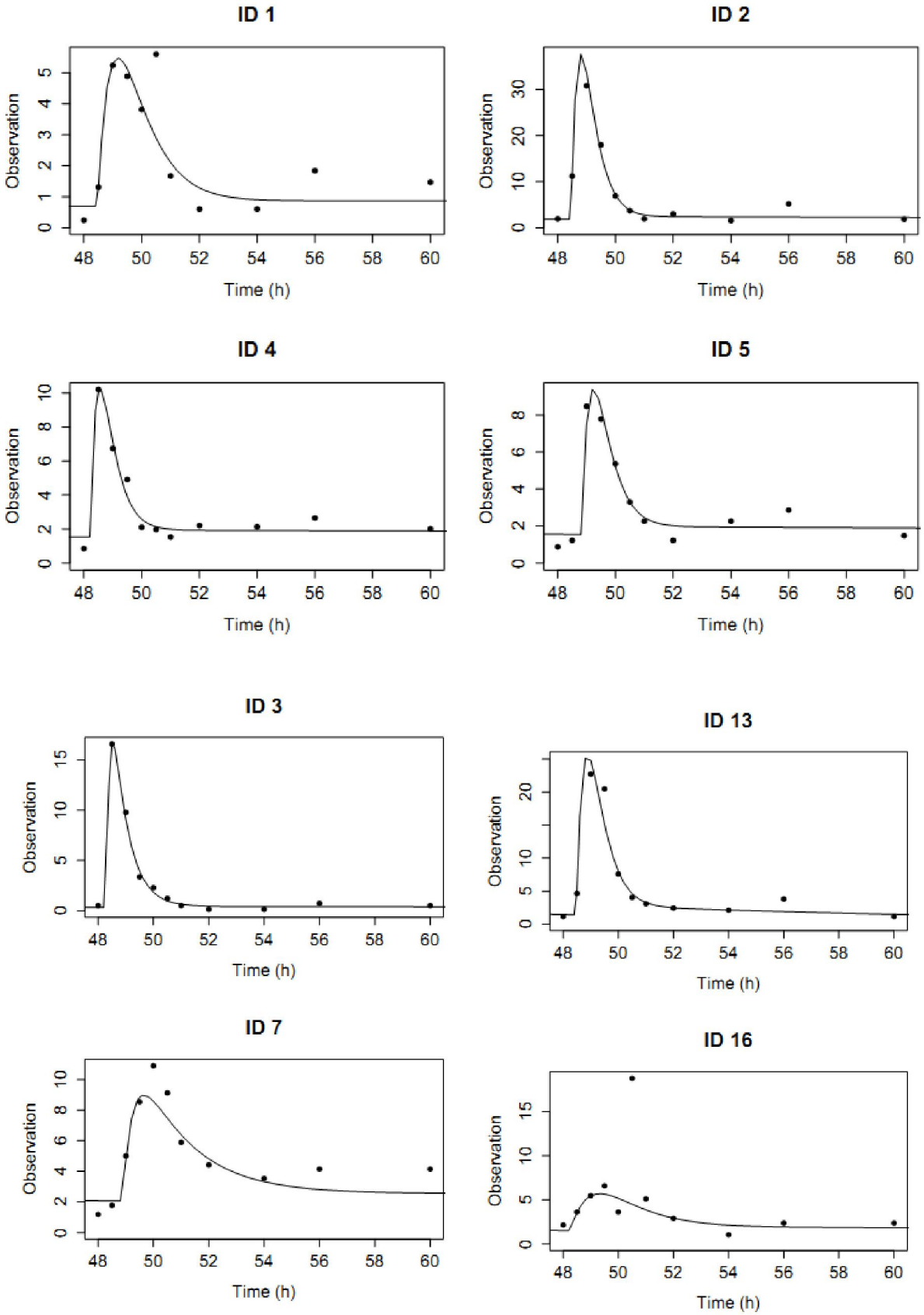
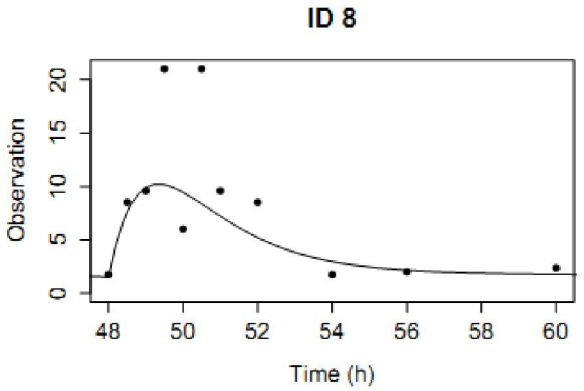
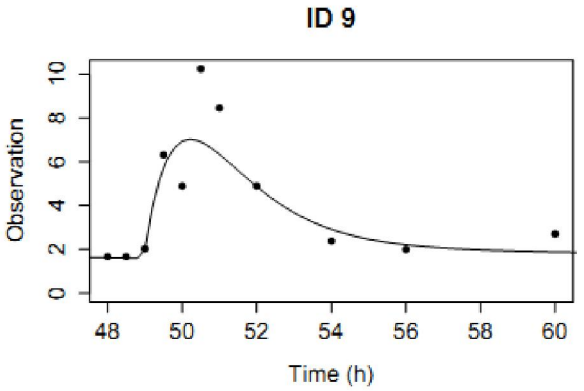


Figure 22: Individual observed and model predicted concentration time curves (continued on next 3 pages)

.


ID 8
Observation
0
5
10
15
20
48
50
52
54
56
58
60
Time (h)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Observation
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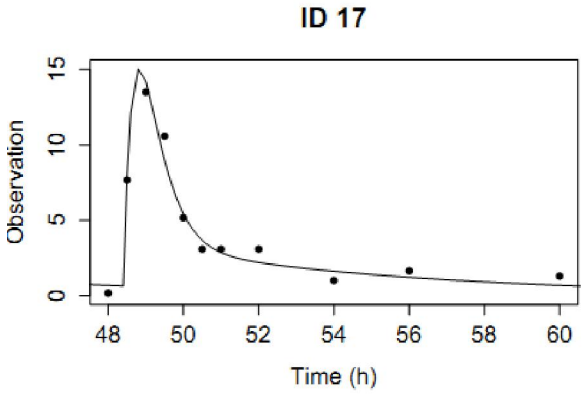
2
4
6
8
10
48
50
52
54
56
58
60
Time (h)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Observation
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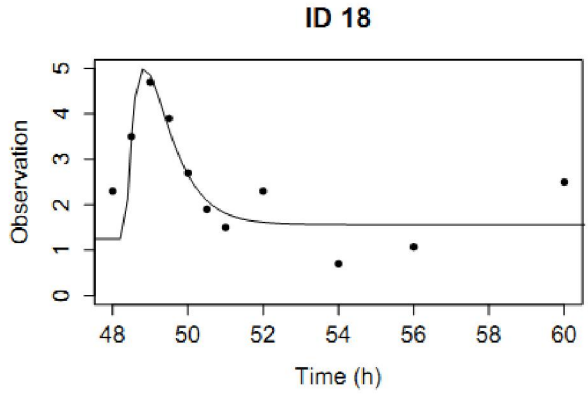
52
54
56
58
60
Time (h)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Observation
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3
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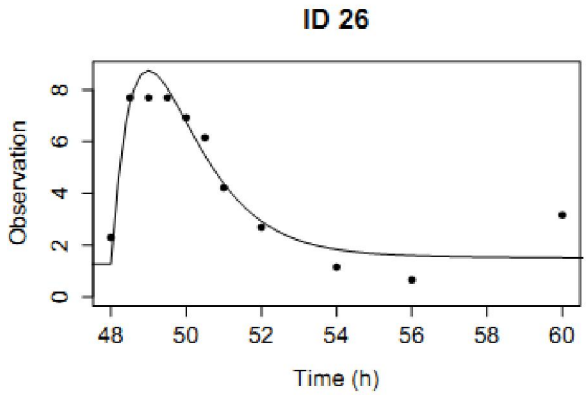
52
54
56
58
60
Time (h)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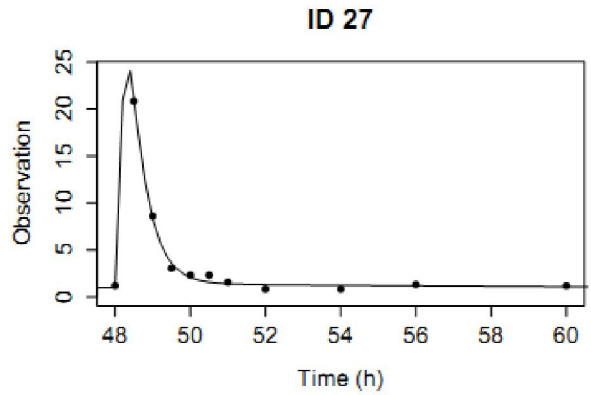
52
54
56
58
60
Time (h)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Observation
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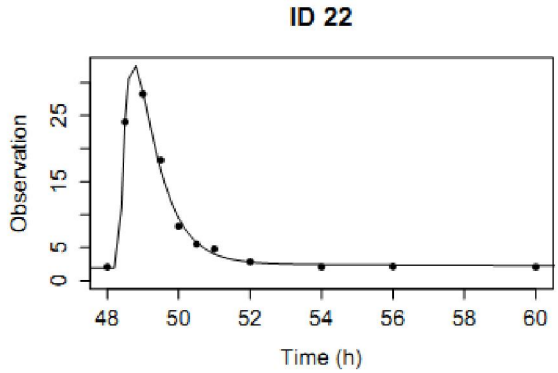
20
25
48
50
52
54
56
58
60
Time (h)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15
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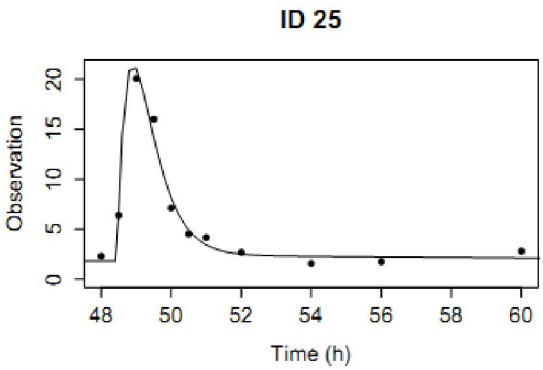
50
52
54
56
58
60
Time (h)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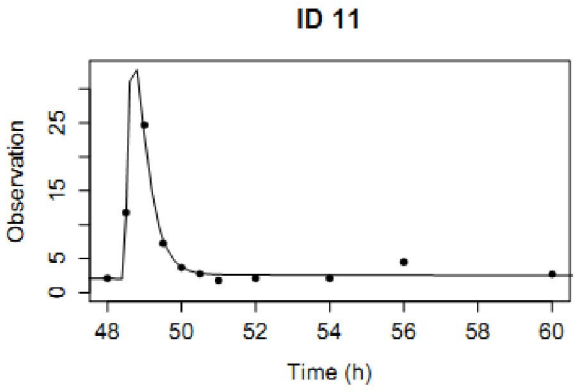
56
58
60
Time (h)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Observation
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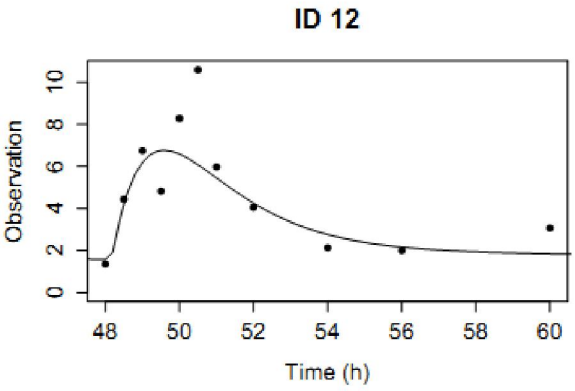
48 50 52 54 56 58 60
Time (h)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Observation
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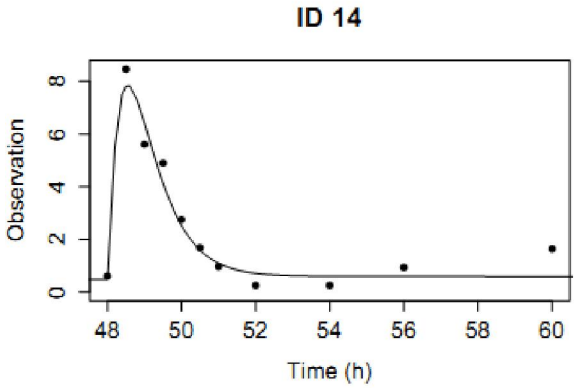
Time (h)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Observation
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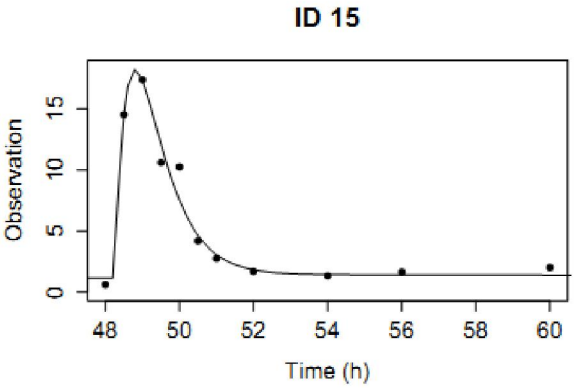
48 50 52 54 56 58 60
Time (h)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Observation
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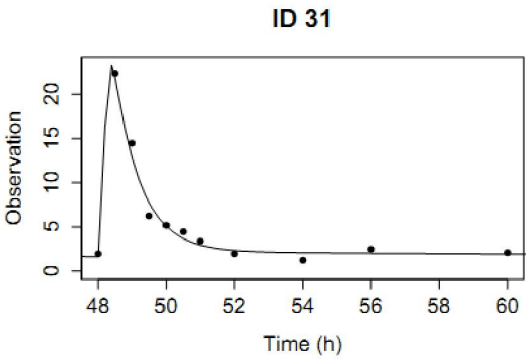
Time (h)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Observation
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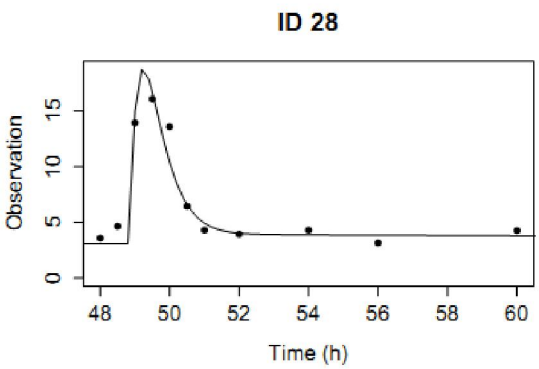
48 50 52 54 56 58 60
Time (h)


ID 28
Observation
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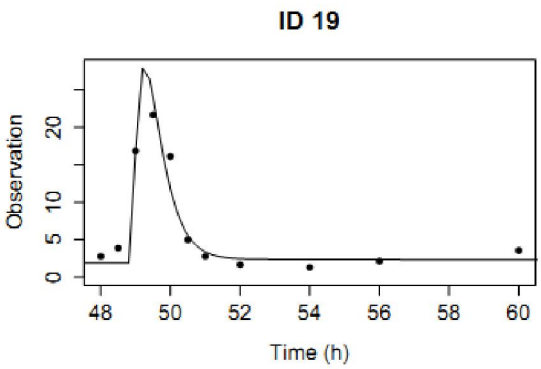
48 50 52 54 56 58 60
Time (h)


ID 19
Observation
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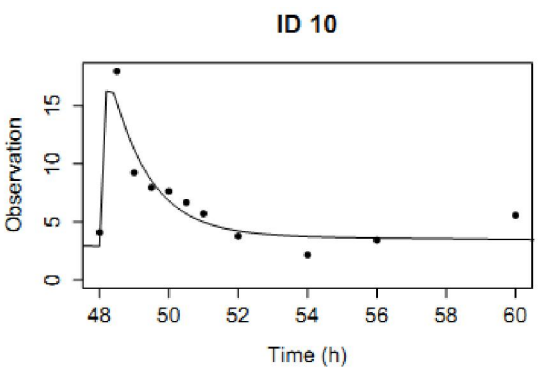
48 50 52 54 56 58 60
Time (h)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Observation
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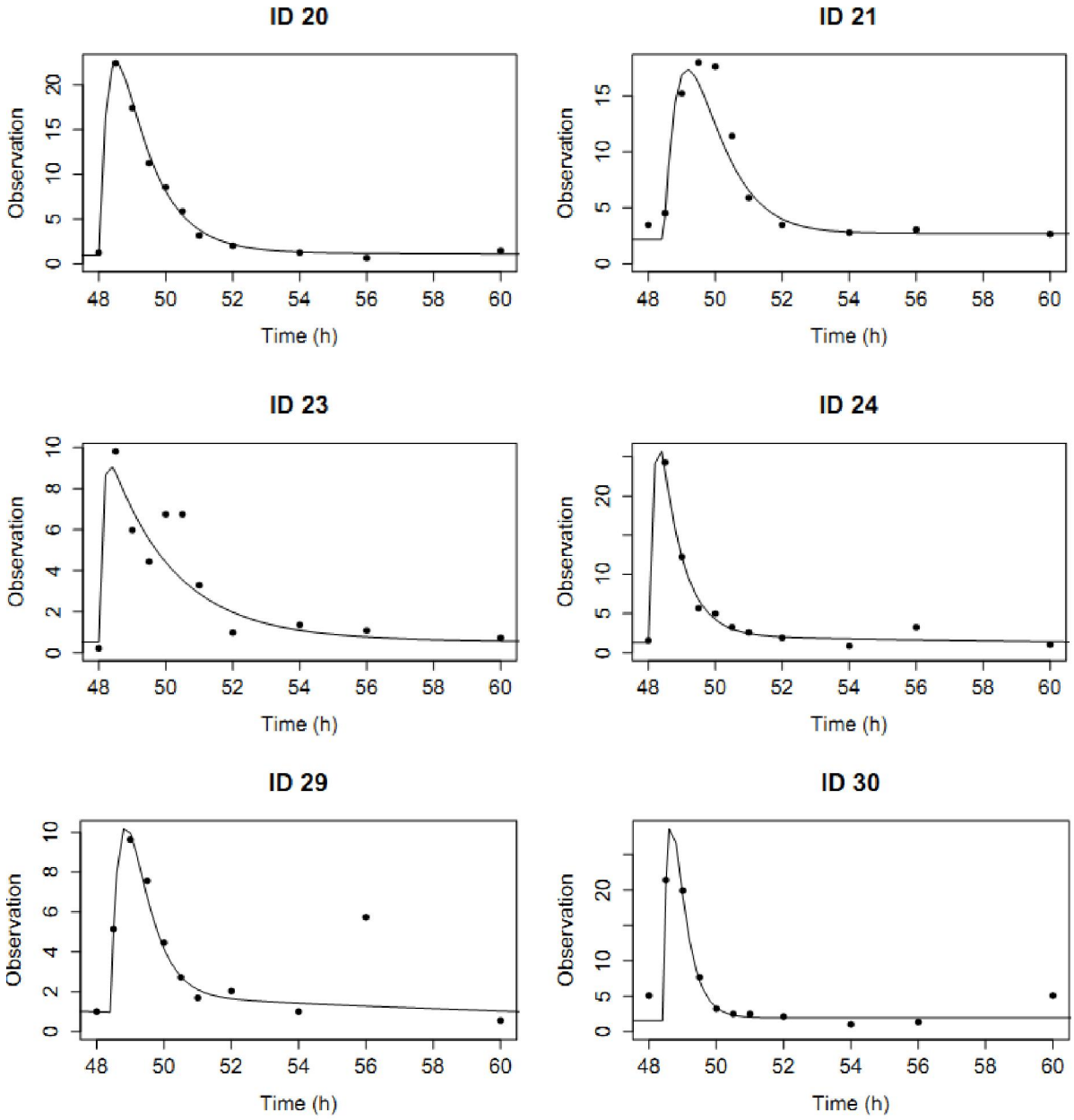
Time (h)


ID 20
Observation
Time (h)
ID 21
Observation
Time (h)
ID 23
Observation
Time (h)
ID 24
Observation
Time (h)
ID 29
Observation
Time (h)
ID 30
Observation
Time (h)

Free MPA, Saliva MPA and total MPA

The table given below tabulates the statistical descriptions of the various data collected. All required data was available for 28 patients including the serum albumin level. The values of the total, free and saliva MPA is in µg/ml. The free fraction is calculated as free MPA divided by the total MPA at 1 hour post dose.

Table 19: Descriptive statistics of Free and Saliva MPA at 1 Hour

	N	*Range*	*Minimum*	*Maximum*	*Mean*
Total MPA at 1 Hr	28	26.221	2.029	28.250	12.05836
Saliva MPA at 1 Hr	28	0.329	0.008	0.337	0.07861
Free MPA at 1 Hr	28	0.508	0.012	0.520	0.12839
Albumin	28	1.8	2.8	4.6	3.907
Free Fraction	28	3.93	0.15	4.08	1.1345

Correlations between total, free and saliva MPA

Spearman correlation was first performed on the whole data set and the results of the correlation are tabulated in table 21

Table 20: Spearman correlation for free MPA as well as Saliva MPA

		TOTAL MPA CONCENTRATION AT 1 HR	SALIVA MPA CONCENTRATION AT 1 HR	FREE MPA CONCENTRATION AT 1 HR	FREE (UNBOUND) FRACTION OF MPA
TOTAL MPA CONCENTRATION AT 1 HR	Correlation Coefficient	**1.000**	**.322**	**.600****	**-.033**
	Sig. (2-tailed)		**.094**	**.001**	**.866**
	N	**28**	**28**	**28**	**28**
SALIVA MPA CONCENTRATION AT 1 HR	Correlation Coefficient	**.322**	**1.000**	**.085**	**-.137**
	Sig. (2-tailed)	**.094**		**.667**	**.488**
	N	**28**	**28**	**28**	**28**
FREE MPA CONCENTRATION AT 1 HR	Correlation Coefficient	**.600****	**.085**	**1.000**	**.736****
	Sig. (2-tailed)	**.001**	**.667**		**.000**
	N	**28**	**28**	**28**	**28**
FREE (UNBOUND) FRACTION OF MPA	Correlation Coefficient	**-.033**	**-.137**	**.736****	**1.000**
	Sig. (2-tailed)	**.866**	**.488**	**.000**	
	N	**28**	**28**	**28**	**28**

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

From this table (table 21), it is evident that in our study group, there is a statistically significant correlation between free MPA at 1 hour and the free fraction (r=0.736) as also between free MPA and the total MPA at 1 hour (r=0.60). **There was no statistically significant correlation between the saliva MPA at 1 hour with either free or total MPA at 1 hour or the free fraction**.

The study populations into two groups depending upon albumin level (Serum Albumin<3.5 as group 1 while >=3.5 was group 2). There were 8 patients in group 1 and 20 in group 2. The descriptive statistics are tabulated in table 22. The mean free fraction was higher in group 1 than in group 2.

Table 21: Descriptive statistics of total free and MPA saliva at 1 hour as well as the free fraction of MPA

GROUP		N	MINIMUM	MAXIMUM	MEAN	STD. DEVIATION
1	Total MPA Concentration at 1 Hr	8	5.005	20.070	11.4033	6.082359
	Saliva MPA concentration at 1 Hr	8	0.008	0.107	0.04050	0.036688
	Free MPA Concentration at 1 Hr	8	0.039	0.520	0.20825	0.155184
	GFR	8	25.96	142.09	90.4462	35.37737
	Free (Unbound) fraction of MPA	8	0.41	4.08	1.9767	1.39008
2	Total MPA Concentration at 1 Hr	20	2.029	28.250	12.3203	7.224631
	Saliva MPA concentration at 1 Hr	20	0.010	0.337	0.09385	0.090421
	Free MPA Concentration at 1 Hr	20	0.012	0.267	0.09645	0.070378
	GFR	20	23.82	169.00	87.4110	36.33914
	Free (Unbound) fraction of MPA	20	0.15	1.72	0.7976	0.37154

From this table, it is evident that the saliva MPA and the free MPA concentrations in plasma were higher in the group with low albumin as compared to those having normal albumin level.

Results of the group wise spearman correlation are tabulated in table 22. The results were very different from the whole data set. It was seen that the correlations between saliva MPA at 1 hour and the free (r=0.790) as well as the total MPA (r=0.786) at 1 hour correlated well in the low albumin group while it was not so in the normal albumin group. In the normal albumin group, the statistically significant correlations included that between the free MPA in Plasma at 1 hour and free fraction (r=0.579) as well as the total MPA at 1 hour (r=0.720).

Table 22: Spearman Correlations between low (1) and normal (2) albumin groups

GROUP			TOTAL MPA CONCENTRA-TION AT 1 HR	SALIVA MPA CONCENTRAT I-ON AT 1 HR	FREE MPA CONCENTRAT I-ON AT 1 HR	FREE FRACTIO N OF MPA
1	Total MPA Concentratio n at 1 Hr	Correlation Coefficient	1.000	.786*	.599	-.238
		Sig. (2-tailed)		.021	.117	.570
	Saliva MPA concentration at 1 Hr	Correlation Coefficient	.786*	1.000	.790*	.238
		Sig. (2-tailed)	.021		.020	.570
	Free MPA Concentratio n at 1 Hr	Correlation Coefficient	.599	.790*	1.000	.623
		Sig. (2-tailed)	.117	.020		.099
	Free (Unbound) fraction of MPA	Correlation Coefficient	-.238	.238	.623	1.000
		Sig. (2-tailed)	.570	.570	.099	
2	Total MPA Concentratio n at 1 Hr	Correlation Coefficient	1.000	.111	.720**	-.062
		Sig. (2-tailed)		.640	.000	.796
	Saliva MPA concentration at 1 Hr	Correlation Coefficient	.111	1.000	-.070	-.242
		Sig. (2-tailed)	.640		.769	.305
	Free MPA Concentratio n at 1 Hr	Correlation Coefficient	.720**	-.070	1.000	.579**
		Sig. (2-tailed)	.000	.769		.008
	Free (Unbound) fraction of MPA	Correlation Coefficient	-.062	-.242	.579**	1.000
		Sig. (2-tailed)	.796	.305	.008	

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Prediction of Free MPA

Using Saliva

To answer the question of predicting free concentration of MPA using a non-invasive method of saliva MPA assay, a multiple linear regression analysis was performed. Both albumin and the dose of MMF per kg body weight were includes as predictors in the model. The results of the regression are tabulated in the table below.

Table 23: Model Summary for Regression model to predict free MPA

Model	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
				R Square Change	F Change	df1	df2	Sig. F Change
1	.371	.293	.093550	.371	4.725	3	24	.010

a. Predictors: (Constant), Albumin, DPKG, Saliva MPA concentration at 1 Hr

b. Dependent Variable: Free MPA Concentration at 1 Hr

The coefficient statistics for the model are given in table 25.

Table 24: Model[a] coefficients to predict free MPA

Model		Unstandardized Coefficients		Standardized Coefficients	T	Sig.
		B	Std. Error	Beta		
1	(Constant)	.331	.136		2.435	.023
	Saliva MPA concentration at 1 Hr	.001	.229	.001	.006	.995
	DPKG	.005	.002	.398	2.443	.022
	Albumin	-.081	.032	-.428	-2.531	.018

a. Dependent Variable: Free MPA Concentration at 1 Hr

From this table, it is evident that Saliva concentrations at 1 hour was not a significant variable in predicting free MPA concentrations at 1 hour.

Using Total MPA

We tried to examine the possibility of estimating free MPA at 1 hour in plasma by using total MPA at 1 hour in plasma, dose per Kg body weight of MMF and serum albumin levels of the patients using stepwise multiple regression. The table below (table 26) shows the ANOVA statistics for the stepwise regression based on the Akiake information criterion (AIC) values.

The initial model included total MPA at 1 hour (Hr1), serum albumin (Albumin), MPA Saliva at 1 hour (Sal1) as well as dose per Kg body weight of MMF (DPKG). The ANOVA table (table 24) shows the changes in the AIC values at every step of the stepwise multiple regression. Here, step 1 includes the initial model parameters and sequentially, Sal1 and DPKG are excluded from the model.

Table 25: ANOVA table for regression analysis to determine free MPA from total MPA as well as albumin

Step	*Parameters Excluded*	*Df*	*Deviance*	*Resid. Df*	*Resid. Dev*	*AIC*
1				23	0.170	-132.953
2	- Sal1	1	0.0003	24	0.170	-134.903
3	- DPKG	1	0.0040	25	0.174	-136.254

The final model is as follows:

Free MPA at 1Hr = 0.3715 + (0.0084 X Hr1) - (0.0881 X Albumin)

The table below (table 27) gives the summary of the regression model coefficients.

Table 26: Regression model coefficients for free MPA

	Estimate	*Std. Error*	*t value*	*PR(>|t|)*
(Intercept)	0.371529	0.112042	3.316	0.00279 **
Hr1	0.008394	0.002355	3.564	0.00150 **
Albumin	-0.088134	0.027453	-3.210	0.00362 **

Signif. Codes: 0, '***' 0.001, '**' 0.01, '*' 0.05, '.' 0.1, ' ' 1

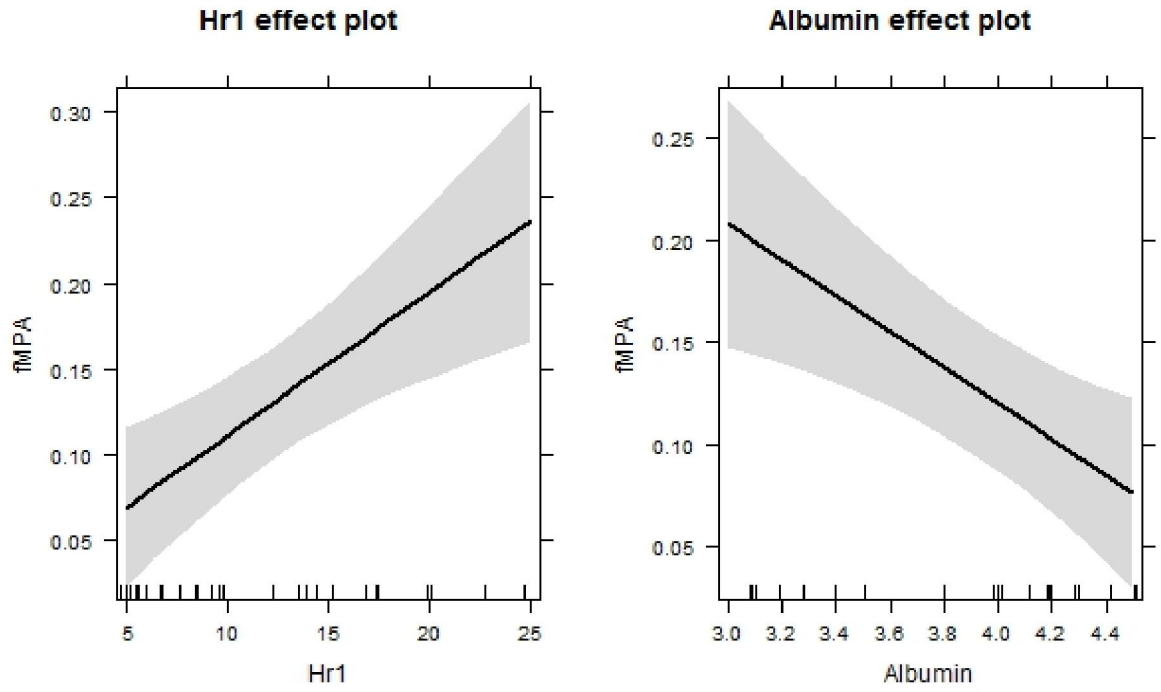
Residual standard error: 0.08344 on 25 degrees of freedom

Multiple R-squared: 0.479, Adjusted R-squared: 0.4373

F-statistic: 11.49 on 2 and 25 DF, p-value: 0.0002888

From table no. 25, we can infer that both total MPA at 1 hour as well as serum albumin are significant predictors of free MPA in plasma at 1 hour (P-value<0.01). The figure (figure 12) below depicts the effect of the individual model parameters on free MPA at 1 Hr. The significant negative association between serum albumin and free MPA at 1 hour is evident from figure 23 and table 28.

Figure 23: Model effects plot for the individual parameters predicting free MPA at 1 hour



The table below (table 28) depicts the characteristics of the residuals in the model:

Table 27: Model residuals

Min	*1Q*	*Median*	*3Q*	*Max*
-0.140481	-0.041375	0.001521	0.021927	0.309124

However, this equation was found to have a very high precision error of 36 %. Thus, **TOTAL MPA CANNOT BE USED IN THE PREDICTION OF FREE MPA.**

Discussion

Limited Sampling Strategy for MPA $AUC_{0\text{-}12\ hr}$

This is the first report of development of LSS for MMF in Indian patients with lupus nephritis. Our centre has previously published a paper developing a limited sampling strategy for patients prescribed Mofilet (Emcure) brand of MMF for renal transplant patients using a five point assay which has now translated into routine clinical service for patients.(58)

Rahman et al observed in a review that the MPA $AUC_{0\text{-}12\ hr}$ were best predicted when concentrations at trough, 1 hour and 3 hour were included in the LSS.(15) Rahman et al reported that when the 3 point LSS for MPA was derived using multiple regression analysis, the mean bias and precision was 0.8 and 22.6 % respectively.(15) Our study included a 4 point LSS for patients taking MMF for lupus nephritis. The advantage of our 4 point LSS is that it does not compromise on accuracy and precision of MPA AUC and the patient only has to remain in the Unit for four hours for collection of the four samples. As evident from table 15, the LSS predicted AUC measurements did not make a clinical difference as compared to the observed MPA $AUC_{0\text{-}12\ hr}$.

Ting et al in 2006 mentioned that the various acceptable methods for LSS validation included i) Dividing data into a sample set and a validation set (best method), ii) Jackknife validation method and iii) Bootstrap method. (59)An earlier study has used bootstrap to validate a LSS for free MPA $AUC_{0\text{-}12\ hr}$.(60)

Ting et al also mention that LSS developed should ideally have only 3 time points or less.(59) However, by having 3 time points or less for a drug with high inter-individual variability, we are bound to miss certain important time points like T_{max} (Time taken to reach maximum concentration in plasma). Rahman et al also reported in their review that the LSS may yield better results if the later time points which account for the enterohepatic recirculation of MMF are added into the equation.(15) However, this would require the

patient to stay in the laboratory for 6 hours or more and it will not add any further benefit except cutting down on the number of samples that need to be collected for the $AUC_{0\text{-}12\ hr}$. Therefore, we only added concentrations at time points up to 4 hours of MMF administration for the development of the LSS so as to accord maximum benefit to the patient with minimum interference with regard to the clinical interpretation of the MPA $AUC_{0\text{-}12\ hr}$.

De Winter et al in 2009 developed 4 equations using multiple regression analysis for predicting MPA AUC in patients taking MMF for autoimmune diseases.(42) Their study included 26 ANCA associated vasculitis patients and only 12 SLE patients.

The equations are summarized in the table below (table 29).

Table 28:de Winter et al developed LSS for MMF in autoimmune diseases(42)

Equation	*Validation set*		
	Bias (%)	RMSE (%)	R^2
$AUC = 38.3 + 11.7 \times C_0$	3.4	26.8	0.48
$AUC = 30.8 + 10.1 \times C_0 + 0.7 \times C_{0.67}$	4.8	25.1	0.53
$AUC = 17.5 + 7.1 \times C_0 + 1.0 \times C_1 + 2.6 \times C_3$	0.8	22.6	0.61
$AUC = 12.3 + 4.7 \times C_0 + 1.2 \times C_1 + 2.7 \times C_3 + 1.8 \times C_6$	-0.4	17.3	0.70

As evident from table 27, including more time points improved the coefficient of determination (R^2) of the AUC. The four point LSS developed by them had a bias of -0.4 % and a root mean square error (RMSE) of 17.3 %. The LSS developed in our study for patients on Mycept® had a bias of -0.13 % and an absolute prediction error of 7.09 %. We also tried to validate the 3 point equation (using C_0, C_1 and C_3) developed by de Winter (table 29) using

our data and the bias was 17.63 % with an absolute prediction error of 19.37 %. Thus, we cannot use this equation in our patients with lupus nephritis. Based on the above, it is safe to postulate that an LSS developed for a particular population may not be applicable to other populations and thus different LSS's must be validated according to the population in question.

In our hospital patients are also prescribed the enteric coated formulation of mycophenolate (Renfor). But it needs to be stressed that the LSS used with MMF cannot be applied to enteric coated formulations because of the difference in pharmacokinetic profiles between the two. In a previous study done at our centre using enteric coated mycophenolate sodium, it was found that the C_{max} was much higher (27.6 µg/ml) and mean T_{max} was 2.9 hours. (61) Thus, the LSS developed in our study is bound to miss important time points like T_{max} for those patients on mycophenolate sodium and thus the estimated AUC will be a lot lower than the actual measured AUC.

Rahman et al reported that the correlation between AUC and trough concentrations were the highest in patients with lupus nephritis taking MMF formulation of MPA (r^2 0.90-0.94).(15) Mino et al, in 2008 had described that MPA trough concentrations had correlated very well with MPA $AUC_{0\text{-}12\ hr}$ in patients taking MMF for lupus nephritis ($r=0.94, p<0.01$).(62) Mino et al reported that a lack of specimen collection at the time of the second peak, could have produced a lower observed AUC than the actual. This may have contributed to a high correlation between the trough and AUC. Lertdumrongluk et al also reported in their study MMF trough levels correlated well with MPA $AUC_{0\text{-}12\ hr}$ ($r=0.92$).(31) But this study only included 12 patients with severe lupus nephritis.

In a recently published article by Streicher et al, it was noted that pre-dose (trough) plasma concentrations might be sufficient to monitor MMF in patients with autoimmune diseases, especially SLE.(41) He recommended that a trough concentration from 2.5 to 4.5 μg/ml would be ideal to prevent clinical flares. However, from our data, it is evident that only pre-dose (trough) plasma concentrations of MMF is insufficient to calculate MMF exposure (spearman correlation of 0.63). Neumann et al in their study had reported a correlation of 0.578 between the MPA $AUC_{0\text{-}12\ hr}$ and the 12 hour trough concentration which is similar to that obtained in our study. The question that needs to be addressed is whether trough concentration can be the sole parameter in assisting dose alterations by the clinician. So using the above mentioned therapeutic range for trough concentration as 2.5-4.5 μg/ml, we found that 24 out of 30 patients in our study would require a different decision on the dosing as compared to dose individualization based on MPA $AUC_{0\text{-}12\ hr}$.

In the absence of clear cut monitoring practices for the therapeutic drug monitoring for MMF, it is still circumspect as to what is the ideal method of monitoring MMF exposure in autoimmune diseases like SLE. Further studies are definitely required to define a clear cut monitoring practice for MMF in patients with autoimmune diseases.

The four point LSS developed in this study has an acceptable bias and it is also desirable that this LSS be validated on a separate sample of patients before it can be recommended as a clinically viable substitute to the full MPA $AUC_{0\text{-}12\ hr}$. The other practical reasons that support the use of this LSS for MPA $AUC_{0\text{-}12\ hr}$ estimation is a difference of 50-60 % in the cost incurred for the estimation of $AUC_{0\text{-}12}$ using LSS as the number of samples that need to be analysed is three times lesser.

Pharmacokinetics of MMF in lupus nephritis

To my knowledge, there has been no study done on Indian patients to elucidate the pharmacokinetics of MMF in adults with lupus nephritis. Earlier studies have compared the pharmacokinetics of MMF in patients with autoimmune disease.(12,62) Neumann et al elucidated the pharmacokinetics of MMF in autoimmune diseases and reported mean values of MPA $AUC_{0\text{-}12\ hr}$ and C_{max} (70.6 mg.h/L and 21.8 µg/ml respectively) as compared to our study (45.12 mg.h/L and 16.7 µg/ml) .(12) The dose used in their study was 2 grams per day. All (except three of our patients) were on a dose less than 2 grams per day. 24 of our 30 patients were on a dose less than or equal to 1500 mg per day. This difference in dosing could have contributed to the higher exposure seen in their study in comparison to our study.

More recent studies like Mino et al have pharmacokinetic parameter values as AUC that is similar to that obtained in our study.(62) But the mean C_{max} reported by Mino et al was 5.03 µg/ml as compared to our findings which was 16.7 µg/ml despite the median dose of MMF in their study (2gm) which was higher than the mean dose in our study (1.4gm). The low C_{max} may be attributed to the collection time points (0, 0.5, 1, 2, 5, 8, 12 Hr) in the post dose period. Having lesser time points in the early post dose period may have resulted in missing the actual C_{max} of the profiles.

A study done in 12 Thai patients with severe lupus nephritis on MMF had mean MPA $AUC_{0\text{-}12\ hr}$ and C_{max} of 57.97 mg.h/L and 19.43 µg/ml respectively. The mean trough was 2.47 µg/ml in their study. The mean dose was 1416.67 mg/day which was comparable to that in our patients (1441.67 mg/day). The lower estimated EGFR values (69.94 v/s 88.98 ml/min) in their patients may have led to a slightly higher exposure of MPA, trough, C_{max} and AUC compared to our patients.(31)

In a recent study done in children with SLE, the apparent volume of distribution was similar to that in this study (24.8 v/s 21.4 L).(39) However, the apparent clearance seemed to be higher in this study in children than in our adult patients (19.2 v/s 8.97 L/h). The probable reason for this is that the children have a higher clearance as compared to adults.

A previous study by de Winter et al had used a two compartment pharmacokinetic model similar to our study and they described values of T_{lag} (0.287 h) and Clearance (7.92 L/h) which were close to the corresponding values in our study (0.356 h and 8.97 L/h respectively).(42) However, the Volume of distribution in their patients was much higher in their study compared to ours (52.4 v/s 21.4 L).

Correlation between MPA in saliva and free MPA in plasma

Mendonza et al had reported in 2006 that except the trough sample, all the other MPA samples in saliva correlated well with both the unbound (r=0.910) as well as the bound fraction (r=0.909) of MPA.(45) However, in our study set, it was found that the correlation was not significant (r=0.085 and 0.322, p>0.05 for unbound and total concentrations in plasma) (Table 12). However, when we split the data into 2 groups based on albumin, it was found that the correlation became significant in the group with low albumin (r=0.790 between free and saliva MPA and 0.786 between free and total MPA, p=0.02 for both) (Table 14). However, there were only 8 patients in the low albumin and probably this needs to be investigated in a larger group of patients. In the rest of the patients with normal albumin level, there was no significant correlation of free with either saliva or total MPA.

There could be various reasons for our results compared with the previous study done on the same topic.(45) It sometimes took longer than 5 minutes for the patients to express the saliva and thus there could have been an error in sampling of saliva in patients. An avenue

that could have been tried was induction of saliva by using lime so that the sampling times were strictly adhered to.

The measurement of (free) unbound concentrations of MPA in plasma is a lot costlier as compared to the measurement of the total concentrations because of the requirement of a centrifugal filter and also the unbound concentrations are more relevant as compared to total in the presence of low albumin concentrations in certain diseases like SLE.(16,17) Therefore, we tried to predict the unbound concentrations of MPA in plasma using regression analysis with saliva MPA concentrations, serum albumin and dose per Kg body weight of MMF as the variables in the equation. But it was noted that the saliva concentrations of MPA in SLE was not a significant predictor of unbound concentrations of MPA in plasma in our data set (table 23).

Limitation

1) Both clinical outcome and adverse events were not correlated with the pharmacokinetics of MMF. This study was conducted only at a single time point and the goals of this study was to elucidate the pharmacokinetics of MMF in patients with lupus nephritis. To observe clinical outcome and adverse effects related to MPA concentration was beyond the scope of this study. To study a clinical outcome, we need to incorporate laboratory and clinical parameters both at baseline and a suitable follow up period.

2) Strict timings while collecting saliva specimens could not be adhered to always as some patients could not express adequate saliva within 5 minutes of taking the 1 hour post dose blood specimen. Some patients required up to 30 minutes to produce adequate saliva for the assay purpose. This may be one of the reasons for the disagreement in the correlation between saliva MPA and free MPA in between our findings and an earlier reported study (by Mendonza et al).

3) Both the free MPA and saliva MPA concentrations estimated were in the range of .01-.05 µg/ml. We used the HPLC for this analysis. We would recommend that in future for a more accurate and precise analysis, the LC-MS/MS (if available) may be the instrument of choice for this analysis.

4) The number of patients in the low albumin group was low and not adequate for a validation of the results.

5) The total number of patients in this study was only 30. We are aware that the best validation for a model is always on a separate group of patients. So we recommend that more number of patients be recruited at a later time for validation of this model.

Conclusions

This study has, for the first time, described the pharmacokinetics of Mycophenolate Mofetil in Indian adult patients with lupus nephritis. We observed a positive correlation between saliva and free MPA in patients with low albumin, however this finding needs to be validated in more number of patients. This study has also developed a reliable, clinically viable limited sampling strategy for MPA $AUC_{0\text{-}12\ hr}$ in patients taking MMF for lupus nephritis using four time points and completed within four hours post dose.

Future Scope

- The four point limited sampling strategy developed for MMF in patients with lupus nephritis needs to be validated in a separate set of patients, before it can clinically replace measurement of full 12 hour AUC.

- A well planned randomized clinical trial is required to prove if there is a definite benefit to therapeutic drug monitoring of MPA in lupus patients. (one group with dose based on clinical outcome and the second group with dosing based on TDM)

- Further work needs to be performed to confirm the therapeutic ranges to be followed in Indian patients with lupus nephritis. This needs to have a thorough clinical follow up included with TDM.

- The clinical utility of measuring MPA in saliva needs to be ascertained by more studies especially in those patients having a low albumin level.

- Preferably, the saliva MPA and free MPA concentrations need to be measured using a mass spectrometer detector.

- An opportunity exists to create a world wide data base on mycophenolic acid for various indications to increase the present knowledge base on the pharmacokinetics of MMF.

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Appendix

Appendix I - List of Figures

Appendix II - IRB Approval Letter

The Scan of the IRB approval letter is attached below:

INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE
VELLORE 632 002, INDIA

Dr.B.J.Prashantham, M.A.,M.A.,Dr.Min(Clinical)
Director, Christian Counseling Centre
Editor, Indian Journal of Psychological Counseling
Chairperson, Ethics Committee, IRB

Dr. Alfred Job Daniel, D (Ortho), MS Ortho, DNB (Ortl
Chairperson, Research Committee &
Principal

Dr. Nihal Thomas
MD, MNAMS, DNB(Endo), FRACP(Endo), FRCP(Edin)
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

January 8, 2013

Dr. Rohit Kodagali
PG Demonstrator
Department of Pharmacology
Christian Medical College
Vellore 632 002

Sub: **FLUID Research grant project NEW PROPOSAL:**
Pharmacokinetics of oral mycophenolate mofetil (MMF) in patients with autoimmune renal diseases with a special reference to the correlation of free MPA in saliva and plasma – A pilot study.
Dr. Rohit Kodagali, PG Demonstrator, Pharmacology, Dr. Binu Susan Mathew, Dr. Denise H Fleming, Dr. Ratna Prabha Gupta, Clinical Pharmacology, DR. Kalpana Ernest, Pharmacology, Dr. G. Basu, Dr. V. Tamilarasi, Nephrology.

Ref: IRB Min. No. 8117 dated 05.12.2012

Dear Dr. Rohit Kodagali,

I enclose the following documents:-

1. Institutional Review Board approval
2. Agreement

Could you please sign the agreement and send it to Dr. Nihal Thomas, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr Nihal Thomas
MBBS MD MNAMS DNB (Endo) FRACP(Endo) FRCP(Edin)
Secretary (Ethics Committee)
Institutional Review Board

CC: Dr. Binu Susan Mathew, Department of Pharmacology.

TEL : 0416 - 2284294, 2284202 FAX : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

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Ref: IRB Min. No. 8117 dated 05.12.2012

Dear Dr. Rohit Kodagali,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "Pharmacokinetics of oral mycophenolate mofetil (MMF) in patients with autoimmune renal diseases with a special reference to the correlation of free MPA in saliva and plasma – A pilot study." on December 5, 2012.

The Committees reviewed the following documents:

1. Format for application to IRB submission
2. Information Sheet and Informed Consent Form (English, Tamil, Hindi)
3. Cvs of Drs. Rohit Kodagali, Binu Susan Mathew, Denise H Fleming, Ratna Prabha Gupta, Clinical Pharmacology, Kalpana Ernest, Pharmacology, G. Basu, V. Tamilarasi, Nephrology.
4. A CD containing documents 1 - 3

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The following Institutional Review Board (Research & Ethics Committee) members were present at the meeting held on December 5, 2012 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

Name	Qualification	Designation	Other Affiliations
Dr. Susanne Abraham	MBBS, MD	Professor, Dermatology, Venerlogy & Leprosy, CMC.	Internal, Clinician
Dr. Benjamin Perakath	MBBS, MS, FRCS	Professor, Surgery (Colorectal), CMC.	Internal, Clinician
Dr. Priya Abraham	MBBS, MD, PhD	Professor, Virology, CMC	Internal, Clinician
Dr. Bobby John	MBBS, MD, DM, PHD, MAMS	Cardiology, CMC	Internal, Clinician
Dr. Denny Fleming	BSc (Hons), PhD	Honorary Professor, Clinical Pharmacology, CMC.	Internal, Pharmacologist
Dr. Simon Rajaratnam	MBBS, MD, DNB (Endo), MNAMS (Endo), PhD (Endo), FRACP	Professor, Endocrinology, CMC	Internal, Clinician
Dr. Anup Ramachandran	PhD	The Wellcome Trust Research Laboratory Gastrointestinal Sciences	Internal
Dr. Chandrasingh	MS, MCH, DMB	Urology, CMC	Internal, Clinician
Dr. Anil Kuruvilla	MBBS, MD, DCH	Professor, Neonatology, CMC.	Internal, Clinician
Dr. Paul Ravindran	PhD, Dip RP, FCCPM	Professor, Radiotherapy, CMC	Internal

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Mrs. Pattabiraman	BSc, DSSA	Social Worker, Vellore	External, Lay Person
Mr. Sampath	BSc, BL	Advocate	External, Legal Expert
Mr. Harikrishnan	BL	Lawyer, Vellore	External, Legal Expert
Mrs. Mary Johnson	M.Sc	Professor, Child Health Nursing, CMC.	Internal, Nurse
Mr. Joseph Devaraj	BSc, BD	Chaplain, CMC	External, Social Scientist
Dr. B. J. Prashantham (Chairperson), IRB Blue Internal	MA (Counseling), MA (Theology), Dr Min(Clinical)	Chairperson(IRB)& Director, Christian Counselling Centre	External
Dr. Jayaprakash Muliyil	BSC, MBBS, MD, MPH, DrPH(Epid), DMHC	Retired Professor, Vellore	External
Dr. Nihal Thomas	MD MNAMS DNB(Endo) FRACP(Endo) FRCP(Edin)	Secretary IRB (EC)& Dy. Chairperson (IRB), Professor of Endocrinology & Addl. Vice Principal (Research), CMC.	Internal, Clinician

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We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any serious adverse events occurring in the course of the project, any changes in the protocol and the patient information/informed consent. And on completion of the study you are expected to submit a copy of the final report.

A sum of Rs 40,000/- (Rupees Forty thousand only) will be granted for 12 months. A subsequent installments of 40,000/- each will be released at the end of the first year following the receipt of the progress report (Total amount 80,000/-).

Yours sincerely

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr Nihal Thomas
MBBS MD MRAMS DNB (Endo) FRACP(Endo) FRCP(Edin)
Secretary (Ethics Committee)
Institutional Review Board

CC: Dr. Binu Susan Mathew, Department of Pharmacology

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Appendix III - Thesis data sheet

The collected data for the thesis is attached here

S.No.	Trough Concentrations	Observed MPA AUC_{0-12}	LSS Predicted AUC	SALIVA MPA
2	1.759	22.3025	23.1365104	0.048
3	0.243	64.1445	62.6453085	NA
4	0.504	20.829	24.4075078	0.015
5	0.852	33.91325	32.0280894	0.053
6	0.879	32.8685	31.7060591	0.337
7	2.133	40.95	34.1295146	0.048
8	1.181	56.7635	48.5158145	0.019
9	1.732	67.66625	68.5714979	NA
10	1.671	42.853	44.8159054	0.233
11	4.094	61.456	55.0016963	0.28
12	2.103	53.239	51.3877647	0.048
13	1.362	44.74825	46.7803985	0.014
14	1.135	54.308	51.5055066	0.07
15	0.607	19.51625	22.0690776	0.008
16	0.607	44.77725	44.268893	0.061
17	2.148	41.65225	39.0511663	0.1
18	0.167	36.4785	42.0606434	0.061
21	2.78	53.215	54.753836	0.043
22	1.25	45.8775	46.345662	0.036
23	3.48	63.96	64.779407	0.054
24	2.09	65.375	61.332183	0.114
25	0.21	28.26	27.701426	0.03
26	1.55	43.9225	39.490669	0.156
27	2.3	48.6375	53.384272	0.072
28	2.3	36.445	43.042039	0.019
29	1.2	29.2925	28.544703	0.023
30	3.58	63.945	62.882198	0.101
31	1.01	39.7125	36.020922	0.021
32	5.1	50.1275	55.923299	0.107
33	1.96	46.3925	42.991319	0.068

S.No.	Free MPA	Dose	Dose/day	Weight	DPKG	Age	Sex	Collection Date
2	0.026	500BD	1000	45	22.22222	45	F	03/06/2013
3	NA	500-0-1750	3250	69	47.10145	28	F	03/06/2013
4	0.04	750BD	1500	70	21.42857	25	F	03/06/2013
5	0.044	500BD	1000	67	14.92537	40	F	14/06/2013
6	0.067	250BD	500	58	8.62069	44	M	20/06/2013
7	0.053	500BD	1000	60	16.66667	63	F	25/06/2013
8	0.204	750-0-1000	1750	58	30.17241	24	M	27/06/2013
9	NA	750BD	1500	40	37.5	18	F	09/07/2013
10	0.012	750BD	1500	50	30	29	F	17/07/2013
11	0.04	750BD	1500	52	28.84615	33	F	31/07/2013
12	0.218	500BD	1000	45	22.22222	29	F	05/08/2013
13	0.204	500BD	1000	69	14.49275	31	M	09/08/2013
14	0.15	750-0-1000	1750	56	31.25	21	F	12/08/2013
15	0.039	500BD	1000	59	16.94915	26	M	11/09/2013
16	0.127	750BD	1500	61	24.59016	23	F	11/09/2013
17	0.056	500-0-750	1250	47	26.59574	33	F	04/09/2013
18	0.087	750-0-1000	1750	64	27.34375	22	F	03/10/2013
21	0.164	750-0-750	1500	55	27.27273	25	F	24/10/2013
22	0.081	1000-0-750	1750	57	30.70175	19	F	31/10/2013
23	0.081	500-0-500	1000	38	26.31579	21	F	01/11/2013
24	0.267	1500BD	3000	47	63.82979	28	F	08/11/2013
25	0.103	750BD	1500	73	20.54795	41	F	14/11/2013
26	0.199	500BD	1000	53	18.86792	23	F	19/11/2013
27	0.226	750BD	1500	55	27.27273	33	F	21/11/2013
28	0.062	250-0-500	750	44	17.04545	24	F	22/11/2013
29	0.084	500BD	1000	50	20	30	F	18/12/2013
30	0.021	500-0-750	1250	46	27.17391	20	F	20/01/2014
31	0.133	500BD	1000	38	26.31579	28	F	24/01/2014
32	0.3	1000BD	2000	60	33.33333	22	F	05/02/2014
33	0.52	1000-0-1250	2250	65	34.61538	32	F	26/02/2014

S.No.	Se. Creatinine	Urea	eGFR	WBC	Albumin	Hb	Platelets
2	0.75	19	88.82	9200	4.5	11.3	NA
3	0.81	33	89.49	5700	4	12.1	2.96
4	0.71	NA	106.61	10600	3.1	11.8	1.88
5	0.79	12	85.67	4300	4	10.9	2.99
6	3.05	103	23.82	11800	4.4	13.1	1.19
7	0.72	NA	86.95	6000	NA	11.8	NA
8	3.15	63	25.96	6500	3.1	8.7	2.54
9	0.68	22	119.78	9600	4.1	12.4	NA
10	1.11	27	61.77	6600	4.3	9.3	2.91
11	1.62	NA	38.89	10300	4.2	12.7	1.41
12	1.58	54	41.1	18200	4.1	12.1	3.63
13	1.02	32	90.54	17100	3.3	12.8	4.14
14	0.67	16	118.09	5600	4.6	11.4	1.67
15	0.82	18	120.71	9000	3.1	12.2	NA
16	0.69	14	112.06	7800	3.5	11.3	NA
17	1.25	24	52.46	9200	4	12.3	1.83
18	0.61	12	130.36	4600	4	11.6	NA
21	0.75	19	100	7500	4.3	11.6	2.88
22	0.87	NA	89.15	17900	4.5	12.5	3.31
23	1.24	41	58.04	NA	4.2	7.8	NA
24	0.57	NA	134.23	6500	4	9.7	NA
25	0.69	30	99.65	6500	3.8	12	2.81
26	1.04	30	69.8	7500	4.2	12	1.96
27	0.96	24	86.08	14200	3.4	13.7	1.7
28	0.87	16	85.02	4200	4.6	9.6	2.65
29	0.46	NA	169	8400	4.3	12.1	NA
30	0.69	24	115.29	9600	4.5	11.8	NA
31	0.82	12	88.23	8600	2.8	9.5	NA
32	1.14	63	63.35	22100	2.8	9.9	3.69
33	0.53	34	142.09	7900	3.2	9.5	2.63

Appendix IV – Information Sheet & Consent Form

A copy of the Patient information sheet and the consent form is attached below.

INFORMATION SHEET

Study title: - ***Pharmacokinetics of oral Mycophenolate mofetil (MMF) in patients with autoimmune renal diseases with a special reference to the correlation of free MPA in saliva and plasma – A pilot study***

What is this study about?

You are being asked to take part in a research study because you have what is known as an autoimmune disease and for this you are being prescribed an immunosuppressant drug called Mycophenolate mofetil or MMF (Trade name Cellcept or Mycept). MMF becomes Mycophenolic Acid (MPA) in the body and this is what we measure when your drug monitoring is done. Drug monitoring is done to ensure that drug concentration in your blood is within the recommended range.

There is a wide variability in the levels found in different people and this may be related to the amount of drug not bound to proteins that are found in your blood (this is called free drug).

In this study, we are looking at the possibility of using a simple saliva sample to predict the free drug level in the blood and see if this is different with changes of protein in the blood called as albumin.

What is different to my normal monitoring?

Your normal monitoring will proceed up to 6 hours but after this we would like you to stay for a further 6 hours so that we can take blood specimens up to 12 hours.

How many extra blood specimens will I be asked to give?

You will be asked to give 2 extra blood samples at 8 hours and again at 12 hours.

Are there any other tests?

You will be asked to give only one saliva sample at 1 hour after taking your medicine. We will measure the concentration of MPA in this saliva sample.

Will I have to pay anything?

You will only have to pay for your usual blood and monitoring tests. You do not have to pay for any of the extra tests done.

Do I have to take part in the study?

No. Participation is purely voluntary and if you choose not to take part in the study your treatment will not be affected in any way.

How will this study benefit me and how will this study help?

For you there will not be any direct benefit now. The information we will get from this study will help us to understand better how the body handles MMF in diseases such as yours. It will also help us know if we can use saliva for measuring the free drug in the body.

If you have questions please feel free to ask them to Dr Rohit or to any of the doctors in the Clinical Pharmacology Unit.

My contact number and e-mail are given below:

Dr Rohit Kodagali: Mobile phone 09843047340

e-mail: rkodagali@gmail.com

Informed Consent

"Pharmacokinetics of oral Mycophenolate mofetil (MMF) in patients with autoimmune renal diseases with a special reference to the correlation of free MPAin saliva and plasma – A pilot study"

If you have volunteered to participate in this study please complete this form and sign it. If you have any doubts feel free to ask questions and get them clarified.

I _____have read the information sheet provided to me, about this study and its importance. I understand the details and had enough time to discuss it with the research staff to clarify my doubts. I understand that I have the right to say that I am not willing to participate in the study without my medical care being affected.

I was given enough time to think about it and to decide if I am willing to participate { }

I am willing to participate in the study { }

I give consent for my blood to be drawn and used for such research purposes. { }

I understand if the results are published or presented at scientific meetings, my identity will not be disclosed, without my written permission. { }

I give permission for the use of any results that arise from this study for scientific purpose(s) {}

I understand that this test is not a diagnostic test and is only for research purpose. { }

I understand that I will not have any direct benefit now from the study but the outcome of the study will be of help to future patients. { }

I understand that I can withdraw from this study at any time.{ }

I have signed this consent voluntarily and whole-heartedly and out of my free will, without any pressure from anyone. { }

Name _____

Signature / Thumb print _____ Date:_____

Name of Witness _____

Signature / Thumb print_____ Date_____

Name of person who has taken the informed consent:_____

Signature:_____
Date:_____

To be completed by the Researcher.

Study ID	CP-POMA		Specimen-ID	
Name			Hospital No.	
Age/Sex			Diagnosis	
Treatment History				