UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

DESENVOLVIMENTO E VALIDAÇÃO DE MÉTODO ANALÍTICO PARA DETERMINAÇÃO SIMULTÂNEA DE ÁCIDO MICOFENÓLICO E SEU METABOLITO GLICURONÍDEO POR CL/EM EM PLASMA E FLUÍDO ORAL DE PACIENTES TRANSPLANTADOS RENAIS

Pâmela Cristina Lukasewicz Ferreira

Porto Alegre, 2018.

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Tese apresentada por **Pâmela Cristina Lukasewicz Ferreira** para obtenção do TÍTULO DE DOUTOR em Ciências Farmacêuticas.

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“Dream big and dare to fail.”

Norman Vaughan

RESUMO

Com o envelhecimento da população brasileira, o número de doenças crônicas vem aumentando, entre as quais se destaca a insuficiência renal. O tratamento recomendado é o transplante renal. Uma das principais preocupações do transplante é o alto risco de rejeição, por isso é necessário o uso de imunossupressores que diminuem a atividade do sistema imunológico. Um dos principais fármacos utilizado na terapia de imunossupressão é o micofenolato de mofetila (MMF), um potente inibidor seletivo, não competitivo e reversível da desidrogenase inosina-monofosfato (IMPDH) que atua inibindo a síntese de guanina, provocando uma diminuição da taxa de proliferação. Os imunossupressores estão relacionados a muitos efeitos secundários, sendo necessário um monitoramento terapêutico, a principal matriz utilizada é o plasma. Porém, o uso do fluido oral vem aumentando, por não necessitar de coleta invasiva e pessoal especializado para tal. Um método de cromatografia líquida acoplado ao espectrômetro de massas (LC-MS) foi desenvolvido e validado para a determinação do ácido micofenólico (MPA) e seu metabólito glicuronideo (MPAG), em plasma e fluido oral, com precipitação de proteínas seguido de centrifugação e utilizando cetoprofeno (KET) como padrão interno (PI). Os picos foram separados em condição gradiente, com um tempo de corrida de 16 min, através de uma coluna Agilent Zorbax Eclipse Plus (4.6 x 150 mm, 3.5 µm tamanho de partícula) a 35 °C. Um íon foi utilizado para a quantificação e três íons para a confirmação de cada analito. O método foi linear para todos os analitos no intervalo de concentração de 10,0 – 500,0 ng/mL para o MPA e 30,0 – 500,0 ng/mL para MPAG, com coeficientes de correlação (r) entre 0,9925 – 0,9937 para as amostras de plasma e com r entre 0,9952 – 0,9973 para as amostras de fluido oral. O limite inferior de quantificação foi de 10,0 ng/mL para o MPA e 30,0 ng/mL para o MPAG, com parâmetros de validação dentro do preconizado. O efeito matriz foi avaliado e apresentou resultados adequados, demonstrando que ambos os procedimentos de limpeza das amostras são rápidos e confiáveis, exigindo pequenas quantidades. O método foi aplicado em amostras de pacientes transplantados renais de fluido oral e plasma usando menos pontos que usualmente. Após análise farmacocinética foi encontrado que o fluido oral pode ser uma possível matriz para ser usando no monitoramento terapêutico sendo necessário ainda mais estudos para ser aplicado na clínica. Estudos *in vitro* para medir e tentar estabelecer uma relação de permeação do MPA dentro do linfócito foram

realizados afim de correlacionar com achados nas amostras coletadas de pacientes. Após analisar as amostras dos pacientes foi encontrado que o estudo *in vitro* utilizando sangue total é que mais se aproxima dos resultados encontrados em amostras reais. Além de MPA dentro do linfócito foi também detectado MPAG, que ainda não havia sido descrito na literatura. A relação da quantidade dos dois metabólitos dentro do linfócito foi menor que 4%.

Palavras chave: fluido oral; plasma; LC-MS; ácido micofenólico; farmacocinética.

Abstract

With the aging of the population, the number of chronic diseases is increasing, standing out kidney disease. The recommended treatment for this disease is renal transplantation, which increases the patient's quality of life. The major concern with transplantation is the high risk of rejection and that is why it is necessary the use of immunosuppressive drugs to decrease the activity of the immune system. One of the main drugs used in immunosuppressive therapy is mycophenolate mofetil (MMF), a potent noncompetitive and reversible selective inhibitor of inosine monophosphate dehydrogenase (IMPDH), which acts inhibiting the synthesis of guanine, producing a decrease in the proliferation rate. Immunosuppressors are related with many side effects, and therapeutic monitoring is required, the main matrix being used is plasma. However, the use of oral fluid is increasing because it does not require invasive collection or specialized personnel to perform it. A liquid chromatography coupled to mass spectrometry (LC-MS) method was developed and validated for the determination of mycophenolic acid (MPA) and its glucuronide metabolite (MPAG) in plasma and oral fluid. Samples were analysed after a simple protein precipitation procedure followed by centrifugation using ketoprofen (KET) as internal standard (PI). The peaks were separated under gradient condition, run time of 16 min, using an Agilent Zorbax Eclipse Plus column (4.6 x 150 mm, 3.5 µm particle size) at 35 °C. One ion was used for quantification and three more for confirmation of each analyte. The method was linear for all analytes in the concentration range of 10.0 – 500.0 ng / mL for MPA and 30.0 – 500.0 ng/mL for MPAG, with correlation coefficients (r) between 0.9925 - 0.9937 for plasma samples and 0.9952 - 0.9973 for oral fluid samples. The lower limit of quantification was 10 ng / mL for MPA and 30 ng / mL for MPAG, with validation parameters within the recommended range. Matrix effect was evaluated and showed adequate results, demonstrating that sample cleaning procedure was fast and reliable, requiring small amounts of organic solvent. The method was applied to samples of oral fluid and plasma from renal transplant patients using limited sample collection. After pharmacokinetic analysis, it was found that the oral fluid might be a possible matrix to be used in therapeutic monitoring and further studies are needed to be applied in the clinic. *In vitro* studies to measure and try to establish a permeation rate of MPA lymphocyte concentration were performed in order to correlate with findings in the samples collected from patients. After analyzing patients samples, it was

found that the *in vitro* study using total blood is the one that more correlates with what was found on real samples. In addition to MPA it was also detected MPAG inside the lymphocytes, which had not yet been described yet. The quantity relation of the two metabolites inside the lymphocyte was less than 4%.

Keywords: oral fluid; Plasma; LC/MS; mycophenolic acid; pharmacokinetics.

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LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVIATURAS

ABOT Associação Brasileira de Transplante de Órgãos
AcMPAG Ácido micofenólico acilglicuronideo
ACN Acetonitrila
ANVISA Agência Nacional de Vigilância Sanitária
CNI Inibidor de calcineurina
CsA Cyclosporine
EC-MPS Enteric-coated mycophenolate sodium salt
EMA European Medicines Agency
ESI Ionização por “electrospray”
FDA Food and Drug Administration
H_2O Água
LC-MS/CL-MS Cromatografia a líquido acoplada a espectrômetro de massas
LC-UV/CL-UV Cromatografia a líquido com detector de ultravioleta
LIQ Limite inferior de quantificação
IMPDH Desidrogenase inosina-monofosfato
KET Cetoprofeno
MeOH Metanol
MPA Ácido micofenólico
MPAG Glicuronídeo do ácido micofenolico
MMF micofenolato mofetil
NHS National Health Service
PI Padrão interno
Tac Tacrolimus
SUS sistema único de saúde
SPE Solid phase extraction
UPLC-MS Cromatografia a líquido de ultra eficiência acoplada a espectrômetro de massas
WHO World Health Organization

SUMÁRIO

1. INTRODUÇÃO

Devido ao envelhecimento da população o número de doenças crônicas vem aumentando no Brasil, destacando-se a insuficiência renal (FERREIRA; ECHER; LUCENA, 2014; PINSKY et al., 2009). Pacientes com doença renal crônica terminal necessitam de uma terapia substitutiva para sobreviver, sendo as disponíveis atualmente as diálises (hemodiálise e diálise peritoneal) e o transplante renal (FERREIRA; ECHER; LUCENA, 2014; PINSKY et al., 2009). O transplante de rim é o tratamento de escolha para pacientes com doença renal em estágio terminal por aumentar a qualidade e sobrevida além de apresentar menores custos financeiros para o sistema de saúde em relação às demais terapias substitutivas (ELBARBRY; SHOKER, 2007; FERREIRA; ECHER; LUCENA, 2014; PINSKY et al., 2009; WHO, 2015).

O programa de transplante do Brasil é considerado bem desenvolvido sendo mais de 95% realizado pelo Sistema Único de Saúde (SUS) (DAVID-NETO, 2015; FERREIRA; ECHER; LUCENA, 2014). Segundo registro de 2017 da Associação Brasileira de Transplante de Órgãos (ABTO), no ano de 2016 o Brasil estava em segundo lugar em números absolutos de transplantes renais (figura 1.1). Nos últimos 10 anos o número de transplantados renais aumentou consideravelmente, especialmente comparando com outros órgãos como fígado (figura 1.2). Mas ainda há muitos pacientes na fila de espera por um órgão, visto que a maioria dos transplantes realizados é utilizando enxerto de doador falecido (figura 1.3) (DAVID-NETO, 2015; FERREIRA; ECHER; LUCENA, 2014). O estado que realiza o maior número de transplantes renais é São Paulo, o Rio Grande do Sul encontra-se em 4 lugar tendo realizado 586 transplantes em 2017 (figura 1.3)(ABTO, 2017).

Apesar de o transplante ser o tratamento de escolha para pacientes com doença renal em estágio terminal, há o risco de rejeição, na qual o sistema imunológico reconhece o órgão transplantado como corpo estranho e reage (NHS, 2014). Para diminuir o risco de rejeição, é utilizada terapia imunossupressora após a realização do transplante (ELBARBRY; SHOKER, 2007). Além disso, Organização Mundial da Saúde considera a adesão ao tratamento um fator determinante no sucesso da terapia pós-transplante (WHO, 2015).

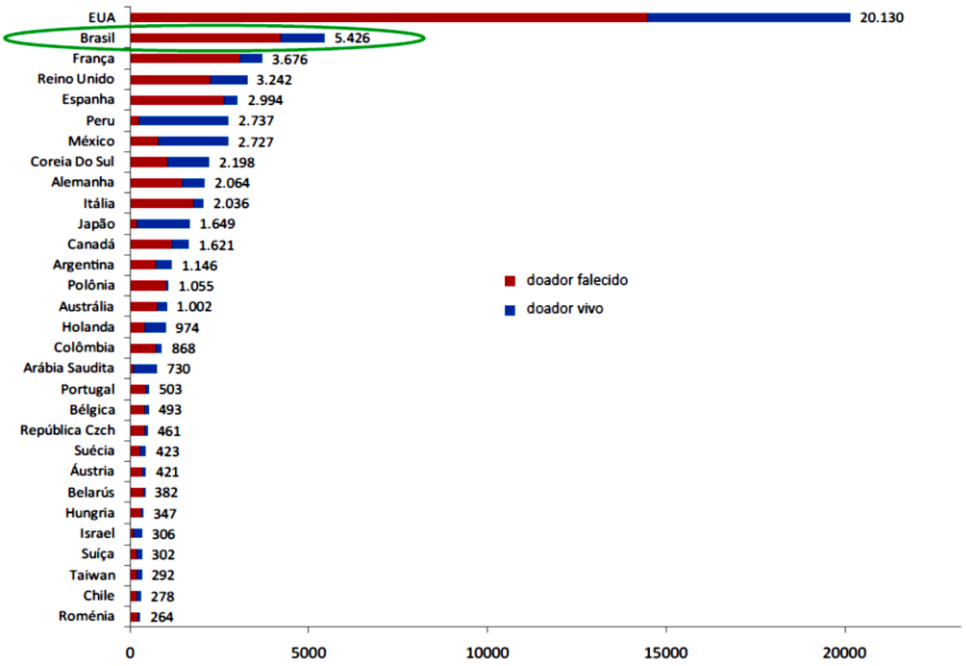

Figura 1.1 – Panorama mundial de transplante renal em números absolutos realizados em 2016. (ABTO, 2017)

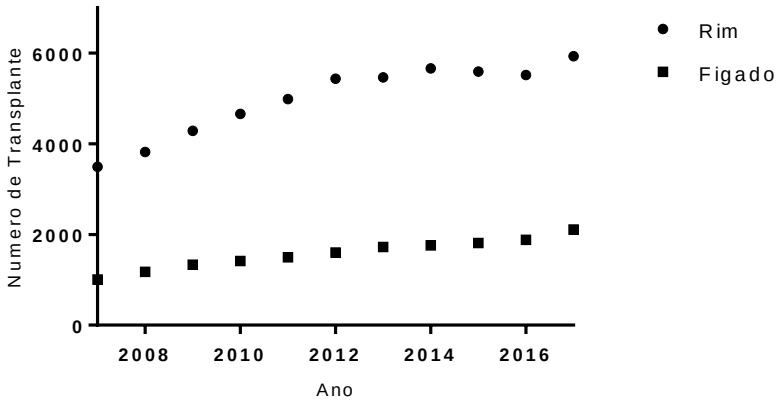

Figura 1.2 – Comparação entre número de transplantados renais e hepáticos nos últimos 10 anos no Brasil. (ABTO, 2017)

Dentre os fármacos utilizados na terapia imunossupressora destaca-se o micofenolato de mofetila (MMF) um pró-fármaco do ácido micofenólico (MPA) (ELBARBRY; SHOKER, 2007; SHUM et al., 2003). O MPA é metabolizado no fígado por uma enzima denominada uridina difosfato glicuronil-transferase formando o 7-O-glucoronídeo do MPA (MPAG), metabólito inativo, e o metabólito ativo, acilglicuronídeo (AcMPAG) (ELBARBRY; SHOKER, 2007; SHUM et al., 2003).

Imunossupressores, como o MMF, possuem janela terapêutica estreita, ou seja, exibem o efeito terapêutico desejado com tolerabilidade aceitável apenas em um pequeno intervalo de concentração sanguínea (ELBARBRY; SHOKER, 2007).

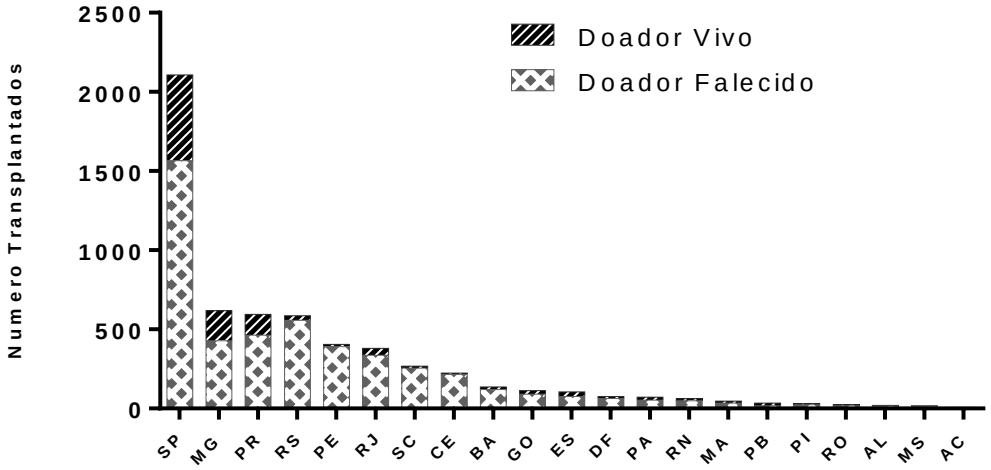

Figura 1.3 – Números absolutos de pacientes transplantados renais por estado em 2017. (ABTO, 2017)

O uso de imunossupressores leva a alguns efeitos adversos como perda de peso, dor abdominal, diarreia, crescimento de pelos, hipertrofia das gengivas, acne e sangramento (NHS, 2014). Por isso é importante o monitoramento terapêutico, a avaliação das concentrações de fármacos nos fluidos biológicos, com o objetivo de aumentar a eficácia e diminuir os efeitos tóxicos dos mesmos (KAHAN et al., 2002). Assim, o ajuste da dose para cada indivíduo é fundamental para manter níveis séricos terapêuticos sem atingir níveis tóxicos, contribuindo para o sucesso do tratamento (KAHAN et al., 2002; MADARIAGA et al., 2016).

Conhecer o perfil farmacocinético do fármaco em uso é um instrumento importante para o monitoramento terapêutico. De acordo com a guia do FDA, o estudo da farmacocinética populacional é realizado para avaliar as fontes de variabilidade entre indivíduos, que são pacientes da população alvo e que recebem doses clinicamente relevantes do medicamento de interesse (FDA, 1999). O estudo permite

identificar os fatores da variabilidade farmacocinética, explicando a diferença entre pacientes. Permite determinar o cálculo da primeira dose e da dose de manutenção no monitoramento terapêutico e pode fornecer modelos clinicamente aplicáveis (EMA, 2013; FDA, 1999). A descrição da farmacocinética de um dado fármaco na população alvo pode ser determinada pela média do comportamento farmacocinético populacional, pelos fatores que influenciam comportamento farmacocinético médio da população ou pelo grau de incerteza associado (EMA, 2013; FDA, 1999).

Já foi descrito na literatura que a farmacocinética do MPA varia com a função renal, concentração de albumina no soro, níveis de hemoglobina e utilização de outros medicamentos em associação à terapia imunossupressora (COLOM et al., 2012). Devido a esta variação, pacientes recém-transplantados terão uma concentração até 50% menor de MPA do que pacientes já transplantados e em terapia há algum tempo (COLOM et al., 2012; JEONG; KAPLAN, 2007).

Estudos farmacocinéticos já publicados não apresentam correlação entre plasma e fluido oral. Desta maneira, justifica-se a importância de um estudo farmacocinético para avaliar quanto da dose é biodisponível após sua administração oral, e relacionar os resultados obtidos de área sob a curva (ASC) plasmática com curva do fluido oral e assim tentar estabelecer uma correlação e verificar se o monitoramento terapêutico pode ser realizado utilizando-se essa segunda matriz.

2. OBJETIVOS

2.1 Objetivo Geral

Desenvolver e validar método analítico para avaliação das concentrações em plasma e fluido oral de ácido micofenólico e seu metabolito glicuronídeo em pacientes transplantados renais.

2.2 Objetivos Específicos

- Desenvolver e validar método para análise de MPA e MPAG em plasma, fluido oral e linfócitos por LC/MS.
- Determinar os níveis de MPA e MPAG no fluido oral e no plasma em diferentes tempos de coleta em pacientes transplantados renais tratados com MMF.
- Determinar a área sob a curva (ASC), C_{max} e T_{max} de MPA em plasma e fluido oral em pacientes transplantados renais tratados com MMF.
- Determinar a área sob a curva (ASC), C_{max} e T_{max} de MPAG em plasma e fluido oral em pacientes transplantados renais tratados com MMF.
- Correlacionar dados plasmáticos e com de fluido oral de MPA.
- Correlacionar dados plasmáticos e com de fluido oral de MPAG.
    - Determinar níveis de MPA intracelular por LC/MS.

3. ASPECTOS ÉTICOS DA PESQUISA

Pesquisas que envolvem seres humanos devem preservar a confidencialidade dos resultados encontrados bem como preocupar-se com o bem-estar dos indivíduos envolvidos. É necessário resguardar a privacidade, minimizar riscos e desconfortos, buscar benefícios e garantir a não discriminação dos voluntários. Desta forma, utiliza-se o termo de consentimento livre e esclarecido (TCLE) aprovado pelo Comitê de Ética em Pesquisa para informar e assegurar proteção ao participante, certificando os aspectos metodológicos e éticos mais adequados (Anexo).

A pesquisa foi aprovada pelo Comitê de Ética em Pesquisa Pontífice Universidade Católica do Rio Grande do Sul (PUC/RS), onde serão realizadas as coletas, parecer número 2.082.466 (Anexo).

4. REVISÃO BIBLIOGRÁFICA

4.1 Histórico transplantes e uso de imunossupresores

Depois algumas tentativas falhas, no ano de 1954, em Boston, foi realizado o primeiro transplante renal com êxito, utilizando gêmeos idênticos, com uma técnica que é utilizada até hoje (DEL TACCA, 2004; WATSON; DARK, 2012). Após, observou-se o surgimento de uma resposta imune, sendo utilizada a radiação como tentativa de evitar essa resposta, porém sem sucesso (DEL TACCA, 2004; WATSON; DARK, 2012). Apenas a partir da década de 60 a terapia de imunossupressão farmacológica passou a ser utilizada, fazendo com que os transplantes passassem a ser uma terapêutica aplicável em maior escala. O primeiro imunossupressor utilizado foi a azatropina, um análogo das purinas menos tóxico, que age inibindo a replicação do DNA e bloqueando a proliferação dos linfócitos. (DEL TACCA, 2004; SCHWARZ et al., 2015; SILVA FILHO et al., 2015; WATSON; DARK, 2012). A azatropina, juntamente com a prednisona, elevaram em 50% o funcionamento do rim em transplantados não aparentados após cerca de um ano, uma evolução bastante significativa em uma época em que a diálise dava seus primeiros passos e ter insuficiência renal era considerado uma sentença de morte (HIBINO et al., 2016; WATSON; DARK, 2012; ZHANG et al., 2016). A introdução dos imunossupressores no tratamento pós-transplante mudou a expectativa de vida dos pacientes, sendo atualmente um tratamento aplicado mundialmente em milhares de pacientes transplantados (HIBINO et al., 2016; MADARIAGA et al., 2016; SILVA FILHO et al., 2015; ZHANG et al., 2016). Hoje em dia a imunossupressão farmacológica é utilizada para todos os tipos de transplante de órgãos para evitar a rejeição do enxerto, aumentando a expectativa de vida do paciente (ELBARBRY; SHOKER, 2007; MADARIAGA et al., 2016; SCHWARZ et al., 2015).

A abordagem geral ao tratamento de transplante de órgãos deve seguir cinco princípios gerais: o primeiro deve ser a preparação do paciente e seleção criteriosa do doador como compatibilidade do grupo sanguíneo ABO e antígenos HLA. Segundo deve-se utilizar abordagem multicamadas no tratamento imunossupressor, semelhante ao utilizado no tratamento quimioterápico, buscando-se efeito sinérgico, que permite utilização dos diversos fármacos em doses relativamente menores, aumentando efeito imunossupressor e limitando os tóxicos específicos. O terceiro deve ser o grau de imunossupressão necessária para assegurar adaptação inicial do enxerto e/ou tratar rejeição estabelecida é maior do que a empregada no tratamento

imunossupressor a longo prazo. Em quarto lugar, é necessário investigar a disfunção do transplante incluindo-se a avaliação das possibilidades de rejeição, toxicidade dos fármacos e infecção, tendo em mente que esses fatores podem coexistir. Os problemas específicos de cada órgão também devem ser levados em consideração. O último e quinto critério deve ser que um agente imunossupressor deve ser reduzido ou retirado do esquema, se seus efeitos tóxicos suplantarem seus benefícios (BRUNTON; CHABNER; KNOLLMANN, 2011).

Nos últimos anos, o desenvolvimento de novas abordagens para o controle da resposta aloimune tem sido muito significativa. A incidência e a intensidade da rejeição aguda têm sido reduzidas e o desenvolvimento de novos agentes imunossupressores, tais como ciclosporina, tacrolimus, micofenolato de mofetila (MMF), anticorpos monoclonais e policlonais específicos possuem papel fundamental nesta redução (MEIER-KRIESCHE, 2012; NOGUERAS et al., 2005). O primeiro dessa nova geração de imunossupressores foi a ciclosporina, que inicialmente foi desenvolvida como antifúngico, porém acabou sendo usada como imunossupressor, pois após testes clínicos diminuiu em 90-95% o risco de rejeição após um ano de transplante. A ciclosporina age inibindo a proliferação das células T (DEL TACCA, 2004; ELBARBRY; SHOKER, 2007; ZHANG et al., 2016). Os fármacos mais utilizados hoje na terapia imunossupressora renal estão relacionados na tabela 4.1 (MEDSACAPE, 2017).

4.2 Ácido micofenólico

Administrado na forma de pró-fármaco do ácido micofenólico (MPA) (micofenolotato de mofetila, MMF) ou na forma de sal (micofenolato de sódio, Myfortic®) para aumentar sua absorção, o ácido micofenólico destaca-se entre os imunossupressores mais utilizados (ELBARBRY; SHOKER, 2007; MADARIAGA et al., 2016; NOWAK; SHAW, 1995). Este fármaco é utilizado em transplantes de órgãos sólidos, na profilaxia e tratamento da rejeição em pacientes submetidos à alotransplantes renal, hepático ou cardíaco. Associado à grande redução na taxa de rejeição é mais potente que a azatioprina, porém não tão potente quanto inibidores mTOR e inibidores da calcineurina, por isso é utilizado em combinação com uma destas duas últimas classes (NOGUERAS et al., 2005; WATSON; DARK, 2012).

Tabela 4.1 – Regime de manutenção de imunossupressão recomendado para transplante de rim (ABTO, 2017; BRUNTON; CHABNER; KNOLLMANN, 2011; MEDSACAPE, 2017)

Regime Padrão de Imunossupressão		**Potencial alternativa**
Classe farmacêutica/ mecanismo de ação	*Agente especifico*	
Inibidor de calcineurina (CNI)/inibe atividade da fosfatase	Tacrolimus	Tacrolimus pode ser substituído por ciclosporina ou inibidor mTOR .
Antimetabólito/inibe atividade da monofosfato inosina desidrogenase	Micofenolato de mofetila	Micofenolato pode ser substituído pelo inibidor mTOR (para pacientes intolerantes devido a toxicidade hematológica ou gastrointestinal, ou para câncer viral associado)
Antimetabólito/ inibe síntese de DNA, RNA e proteinas	Azatioprima	Micofenolato de mofetila
Corticosteroides	Prednisona (pode ou não estar presente)	
Inibidor mTOR*	Sirolimus ou everolimus	

mTOR: Proteinocinase envolvida na progressão do ciclo celular.

O MPA é um potente inibidor seletivo, não competitivo e reversível da inosina-monofosfato desidrogenase (IMPDH), inibindo a síntese de guanina, provocando diminuição da taxa de proliferação (figura 4.1). Uma taxa de proliferação lenta com alterações na glicosilação superficial das moléculas de adesão reduz, nos linfócitos, a capacidade de reconhecer e rejeitar transplantes (ELBARBRY; SHOKER, 2007).

Para garantir a segurança e eficácia dos derivados do MPA, é realizado um monitoramento terapêutico por diferentes razões. Em primeiro lugar, sabe-se que o MMF apresenta ampla variabilidade farmacocinética inter-paciente (BÖHLER et al., 2008; MEIER-KRIESCHE, 2012; SHAW et al., 2001). Estudos relataram que níveis de MPA abaixo do mínimo estão associados com aumento do risco de rejeição aguda, e que níveis elevados estão associados com o risco de toxicidade, além de efeitos adversos como aumento de infecções, distúrbios gastrintestinais, anemia e leucopenia

(ELBARBRY; SHOKER, 2007; KUYPERS et al., 2010; SHAW et al., 1998; SYED; SRINIVAS, 2016; ZHANG et al., 2016).

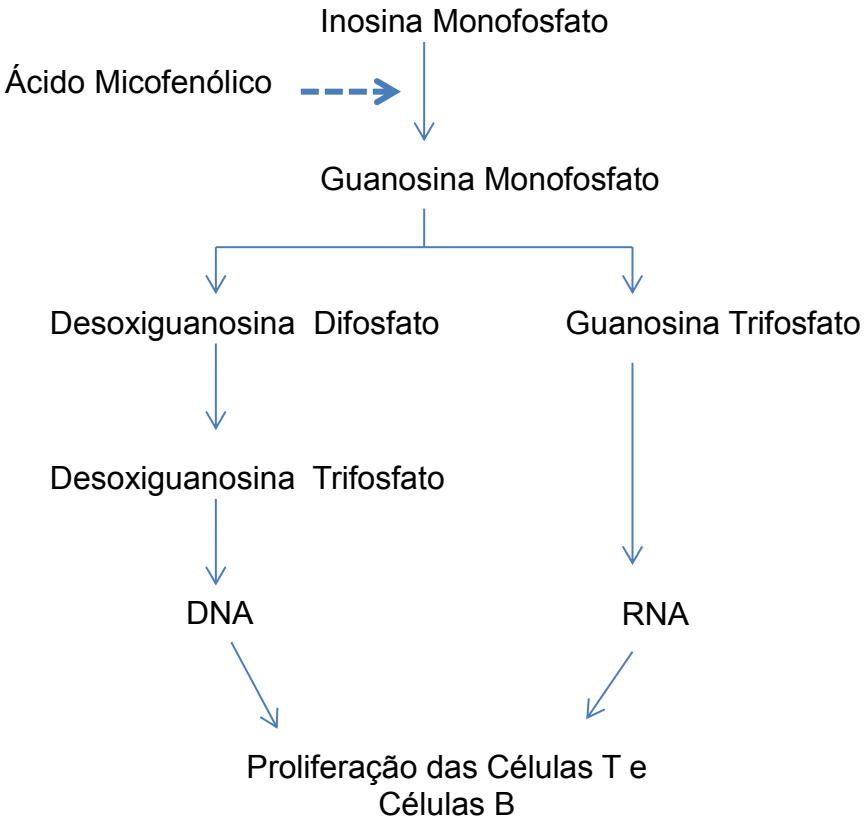

Figura 4.1 – Via Biosintética das purinas e atividade do ácido micofenólico

Após administração oral, o MMF é completamente absorvido e rapidamente hidrolisado a MPA pelas estearases presentes na parede do estômago, fígado e possivelmente pulmões e tecidos periféricos (ELBARBRY; SHOKER, 2007). O MPA se difunde rapidamente para a circulação sistêmica, devido à completa absorção após a administração oral (MEIER-KRIESCHE, 2012). A biodisponibilidade média de uma dose oral é cerca de 90%, com elevada taxa de ligação à albumina (97%) para MPA e 82% para MPAG, sendo a concentração sérica máxima atingida cerca de 1,5 a 2,75 h após a administração do fármaco para micofenolato de sódio (Myfortic c®), e de 0,5 a 1,0 h para micofenolato de mofetila (CellCept®) (ELBARBRY; SHOKER, 2007; NOWAK; SHAW, 1995; SHAW et al., 1998, 2001). Devido a essa alta ligação a

proteínas, a fração livre disponível para atividade farmacológica é influenciada pelas condições do paciente (SHAW et al., 2001; ZHANG et al., 2016).

A metabolização do MPA ocorre no fígado, por ação da enzima uridina difosfato glicuroniltransferase (UGT), gerando o 7-O-glicuronídeo do MPA (MPAG), metabólito inativo e, em menor quantidade, o metabólito ativo acilglicuronídeo AcMPAG (figura 4.2) (KUYPERS et al., 2010; SHAW et al., 1998). O MPAG é secretado pela bile e ao ser liberado no trato gastrintestinal é hidrolisado novamente à MPA por uma glicuronidase bacteriana da microbiota intestinal, sendo uma parte reabsorvida, caracterizando a recirculação entero-hepática (CREMERS *et al.*, 2005). Devido a este fato o MPA e MPAG estão sujeitos à recirculação, a qual pode ser responsável de 10 a 60% do total da área sob a curva no intervalo de doses ($ASC_{0\text{-}12\ h}$) (ALLISON E EUGUI, 2000; BULLINGHAM, NICHOLLS e KAMM, 1998; CREMERS *et al.*, 2005; KUYPERS *et al.*, 2010). A excreção biliar de MPA/MPAG e subsequente absorção e reabsorção distal envolvem diversos mecanismos, incluindo o envolvimento ativo das UGTs (ELBARBRY; SHOKER, 2007).

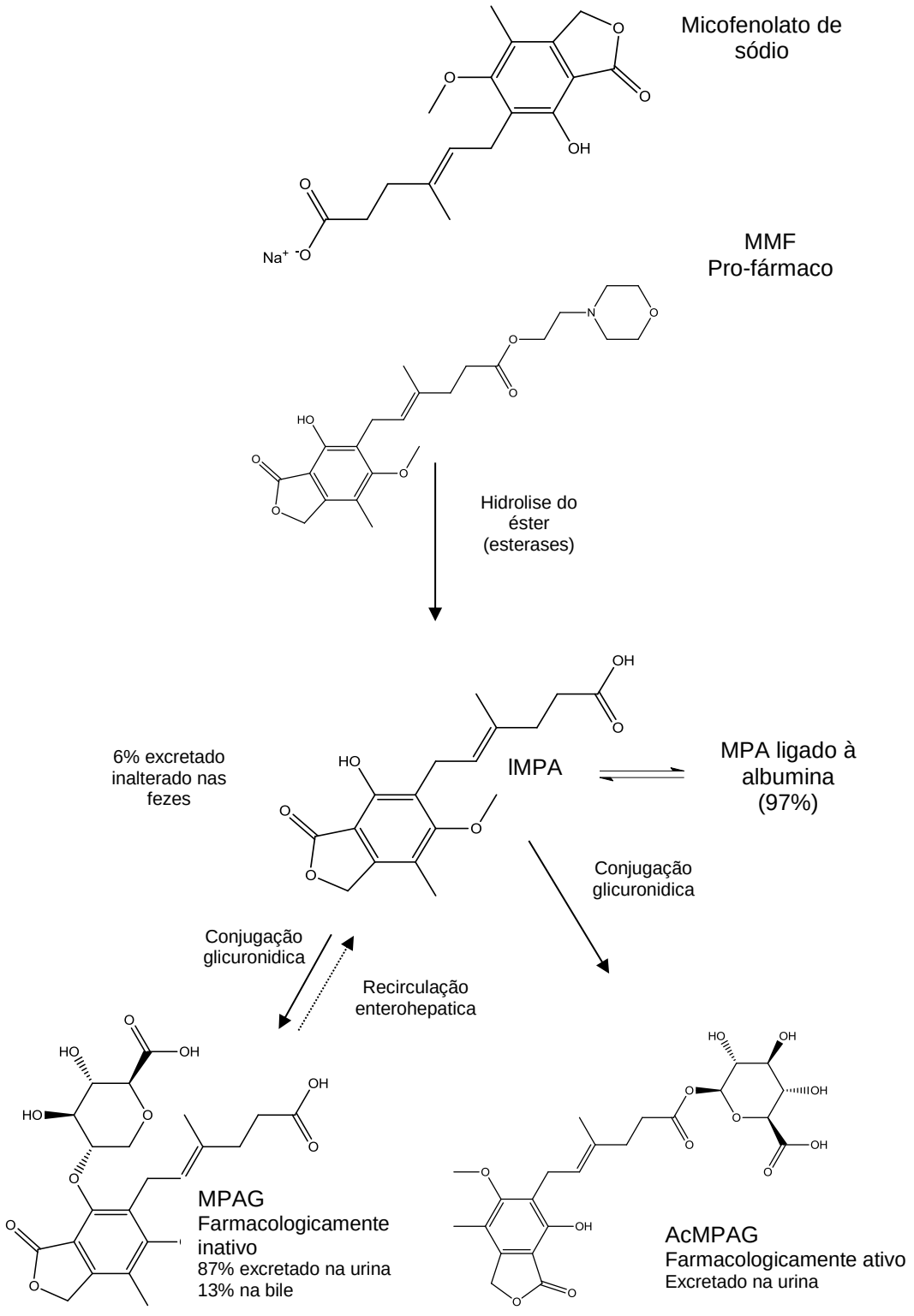

Figura 4.2 – Biotransformação do ácido micofenólico. (ELBARBRY; SHOKER, 2007)

4.3 Métodos Analíticos

Do ponto de vista analítico, a detecção do MMF pode ser realizada em plasma, fluido oral e urina através de seus metabólitos MPA, MPAG e AcMPAG. Na literatura há métodos analíticos descritos por CL-UV, LC-MS/MS, UPLC-MS/MS e CL-Fluorescência (Tabela 4.2) (ELBARBRY; SHOKER, 2007).

4.4 Validação

Validar um método analítico ou bioanalítico é o processo de documentar experimentos que desafiam o método, objetivando produzir evidências científicas e estatísticas para comprovar que suas características e desempenho são adequados e confiáveis para realização das análises pretendidas (FDA, 2018)

Há diversas guias e organizações que os publicam, como Food and Drug Administration (FDA), European Medicines Agency (EMA) e Agência Nacional de Vigilância Sanitária (ANVISA). Existem notáveis diferenças entre a validação de métodos analíticos e bioanalíticos, pois quando se trabalha com matrizes biológicas espera-se uma maior variação nos resultados, tendo em vista a complexidade de extração dos analitos de suas matrizes. Os parâmetros avaliados pelas guias normalmente são semelhantes (linearidade, exatidão, precisão, limite de quantificação e detecção, entre outros), mas pode haver algumas diferenças, como o procedimento para realizar o teste ou a forma de calcular e avaliar os resultados estatisticamente (ANVISA, 2012; EMA, 2012; FDA, 2018).

Apesar da ANVISA ter sua própria guia com recomendações algumas vezes até mais exigentes, é comum e praticamente obrigatório avaliar os parâmetros estabelecidos pela FDA ou EMA para fins de publicação. A guia da FDA foi publicada primeiramente em 2001, baseado em workshops realizados em anos anteriores (1990 e 2000). Para se adequar a algumas mudanças, a guia foi revisada e publicada novamente em 2013. Essa guia, juntamente com a da EMA (2012) foi utilizado como base para validação do método apresentado neste trabalho.

Tabela 4.2 – Métodos de quantificação do MPA e seus metabólitos glicuronídeos.

		Cromatografia		**Metabólito**	**Matrix /extração**	**Limite de detecção**	**Referência**
Sistema	Detector	Fase estacionaria	Fase móvel				
LC	UV-DAD	C18	ACN: tampão fosfato 100 mM pH 3 (25:75, v/v)	MPA, MPAG	Plasma/precipitação de proteína	MPA 100 ng/mL; MPAG 1µg/mL	(INDJOVA; KASSABOVA; SVINAROV, 2005)
LC	UV-DAD	C18	50mM/L solução de fosfato de sódio pH 3: ACN (15:85, v/v)	MPA	Plasma/SPE	200 ng/mL	(DAUREL-RECEVEUR, MATHILDE TITIER *et al.*, 2006)
LC	UV	Fenil	MeOH: tampão fosfato 50 mM pH 2.5 (46:54, v/v)	MPA, MPAG	Soro/precipitação de proteína	MPA 50 ng/mL; MPAG 125 ng/mL	(BAHRAMI; MOHAMMADI, 2006)
LC	MS/MS	POROS perfusion	Metanol/H_2O contendo 10 mM de acetato de amônia e 0.1% ácido acético (10:90 v/v)	MPA, MPAG	Plasma/ SPE	MPA – 50 ng/mL MPAG – 100 ng/mL	(CEGLAREK *et al.*, 2006)
LC	MS/MS	C18	H2O com 3 mM formiato de amônia e 0.5% ácido acético: MeOH com 3 mM formiato de amônia e 0.5% ácido acético. Eluição em gradiente	MPA, MPAG, AcMPAG	Plasma e urina/SPE	Plasma MPA – 50 ng/mL; MPAG-50 ng/mL; AcMPAG – 10 ng/mL MPA_{free} Urina MPA – 125 ng/mL; MPAG-50 ng/mL; AcMPAG – 100 ng/mL	(BENOIT-BIANCAMANO *et al.*, 2007)

Tabela 4.2 – Métodos de quantificação do MPA e seus metabólitos glicuronídeos.

LC	MS/MS	C18	(A) 95% 5mM tampão acetato de amônio pH 3.5 (B) 95% metanol e 5% 5mM tampão acetato de amônio pH 3.5. Eluição em gradiente	MPA, MPAG	Plasma/ SPE	MPA – 0.66 µg/mL MPAG – 6.75 µg/mL	(DIFRANCESCO *et al.*, 2007)
LC	UV	C18	ACN: tampão fosfato 0.1 M pH 3 (43:57, v/v)	MPA	Plasma/precipitação de proteína	MPA 250 ng/mL	(ELBARBRY; SHOKER, 2007)
LC	MS/MS	Zorbax CN	20mM formato de amônia pH 2.5/ACN (72:28 v/v)	MPA	Monócitos de sangue periférico	0.25 ng/pellet	(BÉNECH *et al.*, 2007)
LC	UV-DAD	C18	ACN:tampão fosfato de dihidrogenio 40 mM pH 2.4 (28:72 v/v)	MPA; MPAG	Plasma e urina/ SPE	MPA 100 ng/mL; MPAG 200 ng/mL	(ZIVANOVIĆ *et al.*, 2008)
LC	UV	ODS-80Ts	Pareamento iônico ACN: brometo tetra-n-butilamonio 30 mM com 5 mM acetato de amônio pH 9.0 (33:67 v/v)	MPA; MPAG; AcMPAG	Plasma/ SPE	MPA 50 ng/mL; MPAG 125 ng/mL	(MINO *et al.*, 2008)
LC	MS/MS	C18	(A) 1mM ácido acético em agua (B) 1mM em ACN Eluição em gradiente	MPA, MPAG	Biopsia de fígado humano/precipitação de proteínas	1uM	(MOHAMED; HARVEY; FRYE, 2008)
LC	UV	ODS-80Ts	Pareamento iônico ACN: brometo tetra-n-butilamonio 30 mM com 5 mM acetato de amônio pH 9.0 (33:67 v/v)	MPA; MPAG; AcMPAG	Plasma/ SPE	MPA 50 ng/mL; MPAG 125 ng/mL	(MINO *et al.*, 2008)

Tabela 4.2 – Métodos de quantificação do MPA e seus metabólitos glicuronídeos.

LC	UV-DAD	C18	ACN:tampão fosfato de dihidrogenio 40 mM pH 2.4 (28:72 v/v)	MPA; MPAG	Plasma e urina/ SPE	MPA 100 ng/mL; MPAG 200 ng/mL	(ZIVANOVIĆ *et al.*, 2008)
UPLC	UV	C8	H_2O 0,1% ácido fosfórico:MeOH (47.5:52.5 v/v)	MPA	DBS/liquido-liquido	MPA 0.75 µg/mL	(WILHELM *et al.*, 2009)
UPLC	MS/MS	BEH HSST3	H_2O:MeOH 0.1% ácido fórmico Eluição em gradiente.	MPA, MPAG	Plasma e soro/precipitação de proteína	MPA 14 ng/mL; MPAG 1.85 µg /mL	(KUHN *et al.*, 2009)
LC	MS/MS	Allure PFP propil	ACN 0.1% ácido fórmico: H2O 0.1% ácido fórmico Eluição em gradiente.	MPA, MPA-livre	Plasma, saliva/ precipitação de proteínas	Plasma MPA 100 ng/mL; MPA-livre 2 ng/mL; Saliva MPA 2 ng/mL	(SHEN *et al.*, 2009)
LC	MS/MS	C18	H_2O 0.5% ácido fórmico: MeOH:ACN. Eluição em gradient	MPA, MPA-livre, MPAG, MPAG-livre, AcMPAG	Plasma, urina e tecido/ SPE	MPA- 50n/mL MPA-livre- 1 µg/mL MPAG- 500 ng/mL MPAG-livre- 200 µg/mL /mL AcMPAG- 25 ng/mL	(FIGURSKI *et al.*, 2009)
LC	MS	C18	A) 95-5% H_2O MeOH com 0.1% ácido fórmico (B) MeOH com 0.1% ácido fórmico e acetato de amônia. Eluição em gradiente.	MPA, MPAG	Plasma e soro/precipitação de proteína	MPA 9 ng/mL; MPAG 4500 ng /mL	(KUHN *et al.*, 2009)

Tabela 4.2 – Métodos de quantificação do MPA e seus metabólitos glicuronídeos.

UPLC	UV	C8	H_2O 0,1% ácido fosfórico:MeOH (47.5:52.5 v/v)	MPA	DBS/liquido-liquido	MPA 0.75 µg/mL	(WILHELM *et al.*, 2009)
LC	MS/MS	C18	0.1-0.2% ácido fórmico em H_2O: ACN. Eluição em gradiente.	MPA, MPAG	Plasma/SPE	MPA 1 ng/mL; MPAG 100 ng /mL	(HEINIG *et al.*, 2010)
UPLC	MS/MS	BEH	A) 95-5% H_2O:MeOH 0.1% ácido fórmico:(B) acetato de amônia 2 mM. Eluição em gradiente.	MPA, MPAG, AcMPAG	Plasma/precipitação de proteína	MPA 50 ng/mL; MPAG 50 ng /mL; AcMPAG 50 ng/mL	(DELAVENNE *et al.*, 2011)
LC	MS/MS	C18	Tampão metanolico	MPA e outros fármacos na terapia	Plasma/SPE	MPA- 10 ng/mL	(BUCHWALD; WINKLER; EPTING, 2012)
LC	MS/MS	C18	0.1% ácido fórmico em H_2O:MeOH com 0.1% ácido fórmico. Eluição em gradiente.	MPA, MPAG, AcMPAG	Plasma e urina/precipitação de proteína	MPA 98 ng/mL; MPAG 98 ng /mL; AcMPAG 156 ng/mL	(KLEPACKI *et al.*, 2012)
LC	MS/MS	C18	ACN: 0.1% ácido fórmico 50:50, v/v	MPA, MPAG	Plasma, saliva/ precipitação de proteínas	Plasma MPA 1.6 ng/mL; MPAG 5 ng/mL; Saliva MPA 2 ng/mL; MPAG 9 ng/mL	(WIESEN *et al.*, 2012)

Tabela 4.2 – Métodos de quantificação do MPA e seus metabólitos glicuronídeos.

LC	UV-DAD	C8	ACN:tampão fosfato 50mM pH 2.3 (50:50 v/v)	MPA	Dried blood sopt (DBS) e plasma/ liquido-liquido; precipitação de proteína.	MPA 250 ng/mL	(ARPINI *et al.*, 2013)
LC	MS/MS	C18	ACN 0.1% ácido fórmico: 2M acetato de amônia 0.1% ácido fórmico. Eluição em gradiente.	MPA	Células mononucleares de sangue periférico/ precipitação de proteínas	MPA – 100 ng/mL	(NGUYEN *et al.*, 2013)
UPLC	MS/MS	HSS-T3	Sistema Binário 2M acetato de amônia com 0.1% ácido fórmico em agua: 2M acetato de amônia 0.1% ácido fórmico. Eluição em gradiente.	MPA e outros fármacos na terapia	Plasma/precipitação de proteína	MPA – 100 ng/mL	(TSZYRSZNIC *et al.*, 2013)
UPLC	MS/MS	Fenil	H_2O:MeOH ambos contendo 0,1% ácido fórmico e 2M acetato de amônio. Eluição em gradient	MPA	Biopsia de transplantado renal/solido-liquido	MPA – 0.6 ng/mL	(MD DOM *et al.*, 2014)
UPLC	MS/MS	Fenil	H_2O 0.1% ácido fórmico: MeOH eluido em gradiente	MPA, MPAG, AcMPAG	Plasma/precipitação de proteína	MPA 1 µg/mL; MPAG 10 µg/mL; AcMPAG 0.5 µg/mL	(ZEGARSKA *et al.*, 2015)
LC	MS/MS	C18	H_2O:MeOH com 0,1% ácido fórmico e 2M acetato de amônio. Eluição em gradiente	MPA, MPA-livre, MPAG, MPAG-livre, AcMPAG	Plasma/precipitação de proteína	MPA 100 ng/mL; MPAG 800 ng/mL; AcMPAG 20 ng/mL; MPA-livre 5ng/mL; MPAG-livre 500 ng/mL	(KAWANISHI *et al.*, 2015)

Tabela 4.2 – Métodos de quantificação do MPA e seus metabólitos glicuronídeos.

UPLC	MS/MS	BEH C18	0.1% acido fórmico em H_2O: 0.1% ácido fórmico em MeOH. Eluição em gradiente.	MPA, MPAG, AcMPAG	Plasma/precipitação de proteína	MPA 20 ng/mL; MPAG 20 ng/mL; AcMPAG 2 ng/mL.	(ZHANG; CHOW; RENBARGER, 2016)
HPLC	UV	C18	MeOH:0.1% ácido trifluracetico em agua (54:46 v/v)	MPA, MPAG	Plasma/precipitação de proteína	n.i	(XU *et al.*, 2018)

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6. CAPÍTULO 1 - Parameters that can interfere on the pharmacokinetics of mycophenolic acid

Artigo submetido ao Journal of Clinical Pharmacy and Therapeutics como mini-review

Parameters that can interfere on the pharmacokinetics of mycophenolic acid

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6.1 Abstract

Immunosuppressive therapy is use in solid organ transplant treatment, and mycophenolic acid (MPA) is one of the immunosuppressive drugs most used worldwide. MPA is a potent, selective, noncompetitive and reversible inosine monophosphate dehydrogenase (IMPDH) inhibitor that acts inhibiting guanine synthesis. In order to improve solubility, MPA is use as pro-drug mycophenolate mofetil (MMF) or as enteric-coated mycophenolate sodium salt (EC-MPS). MPA is metabolize into mycophenolic acid phenyl glucuronide (MPAG), inactive and major metabolite, and into acyl glucuronide (AcMPAG), pharmacologically active. In renal transplantation, combined immunosuppressive therapy is a widely used treatment, showing beneficial effects on rejection in Cyclosporine (CsA) and Tacrolimus (Tac) based regimens with MMF or EC-MPS. The aim of this paper is to review works published in the last two decades, analyze, compare results, and discuss factors that can interfere with pharmacokinetics of MPA that can be use in the practical clinical. Data collect show that monitoring MPA plasma levels is the best method to evaluate immunosuppressive therapy as pharmacokinetics can be influenced by: interpatient variability, coadministration of other immunosuppressive agents, post-transplant period, renal function and dose. Monitoring MPAG levels would be very important as it represents a big part of MPA levels, and can be interfere by coadministration of CsA influencing MPA plasmatic concentration and might increase adverse effects. Monitoring MPA levels is important for patients on immunosuppressive therapy because besides the common factors that can influence there is a big interpatient variability. On clinical it might help to increase therapy efficacy observe factors that influence on MPA levels and adverse effects.

Keywords: pharmacokinetics, MPA, MPAG, therapeutic regimen, immunosuppressants, metabolism.

6.2 Introduction

In 2017 only in USA more than 20.000 kidney transplants were performed followed in numbers by Brazil (ABTO, 2017). After the transplant a rigid therapeutic regiment with immunosuppressant must be followed to avoid allograft rejection of the organ and one of the most widely used drug is mycophenolic acid (MPA). MPA is not very soluble so to improve absorption it is used in two forms: as a pro-drug mycophenolate mofetil (MMF) or as an enteric-coated mycophenolate sodium salt (EC-MPS) (Figurski et al., 2009; Nowak and Shaw, 1995; Tönshoff et al., 2011). Plasma concentrations of MPA vary up among patients receiving the same dose which lead to a variable pharmacokinetic showing the importance of drug monitoring (Arpini et al., 2013). In the stomach, MMF is hydrolyzed (de-esterified) to release MPA, being absorbed in the stomach and proximal small intestine. As an EC-MPS, the sodium salt is rapidly dissolved to MPA in ionized form and absorbed in the small intestine (Elbarbry and Shoker, 2007; Opelz and Döhler, 2009).

In transplanted patient, there is a need for use immunosuppressive therapy to reduce the risk of rejection and therapeutic regiment of MPA is commonly associated with other immunosuppressant drugs, especially with calcineurin inhibitors Cyclosporine (CsA) and Tacrolimus (Tac) (Elbarbry and Shoker, 2007). The amount of available drug for MMF and EC-MPS is different, for the first one the bioavailability is around 93 % after oral intake and for the second is around 72 % (Opelz and Döhler, 2009; Staatz and Tett, 2014, 2007; Zicheng et al., 2006).

The mechanism of action of MPA is inhibit selectively and reversibly the rate-limiting enzyme inosine monophosphate dehydrogenase type 2 (IMPDH-2) in *de novo* purine biosynthesis of guanine nucleotides in lymphocytes (Mino et al., 2008; Opelz and Döhler, 2009; Shaw et al., 2001). The limiting lymphocyte proliferation is a result of MPA acting as a potent, selective, reversible and uncompetitive inhibitor of inosine monophosphate dehydrogenase (Staatz and Tett, 2014).

Metabolization of MPA is made by UDP-glucuronosyltransferase enzymes (UGTs) in kidney, liver and gut to form phenyl mycophenolic acid glucuronide (MPAG), major and inactive metabolite, and to a second and less abundant metabolite acyl glucuronide (AcMPAG), that is pharmacologically active (Staatz and Tett, 2014). MPAG concentration in plasma is in general up to 100-fold greater than MPA being

extensively bound to serum albumin, and is excreted in urine and bile (Gelder et al., 2006; Staatz and Tett, 2014). This glucuronide metabolite is cleaved back to MPA by glucuronidase (Mourad et al., 2001; Squifflet et al., 2001; Staatz and Tett, 2014).

Following oral administration of MMF, mean time to reach maximum concentration (T_{max}) ranges between 0.5 and 1.0 h post-dose. At about 6–12 h it is observed a secondary MPA peak in plasma due to the enterohepatic circulation of MPAG (Staatz and Tett, 2014; Tönshoff et al., 2011; Zhang et al., 2016b). For the salt formulation, T_{max} ranges between 1.5 and 2.75 h (Figurski et al., 2009; Zhang et al., 2016b).

Many studies have shown that adverse effects occurred more frequently in high-AUC groups also related to the highest doses. It was also reported that toxicity can be related to higher MPA concentrations than in those who did not experience adverse effects (Gelder et al., 2006; Prémaud et al., 2011; Shaw et al., 2001).

The aim of this paper is to review pharmacokinetic data that has been published in the last two decades, analyze, compare results, and discuss factors that can interfere with MPA pharmacokinetics and maybe be used in the practical clinic as a tool to guide in futures studies involving MPA and it`s glucuronides metabolites.

6.3 Method

6.3.1 Search strategy

Pharmacokinetics studies included on this paper were searched in relevant journal articles written in English between January 2000 and April 2018. It was excluded conferences and book citations, reviews were used to theoretical concepts and data were used only from original research articles. Three databases – ScienceDirect, PubMed and Web of Science were searched using the independent keywords ‘mycophenolic acid’ AND ‘pharmacokinetics’, AND ‘renal’ AND ‘transplant’. After combine these searches duplicates were removed.

6.3.2 Data extraction and analyses

Dose, therapeutic regimen (use of Tac or CsA), time after transplant, AUC and C_{max} of each of the selected publications were recorded. Full-texts were screening and

publications that met the criteria were examined. Papers were grouped and data analyzed according to selection criteria.

6.4 Results and Discussion

6.4.1 Therapeutic regimens

MPA is a widely accepted as an adjunctive immunosuppressant in kidney transplantation showing beneficial effects on rejection in CsA and Tac based regimens (Squifflet et al., 2001; Staatz and Tett, 2014, 2007). In kidney transplanted patients therapeutic regimens involving reduction or withdrawal of calcineurin inhibitors (CNI) have been investigated. More recently, it became clear that strategies to improve kidney transplant outcome require careful monitoring of immunosuppressive regimen dosage (Gelder et al., 2006).

CsA monitoring would be very important because it is increasing the number of studies suggesting that this drug can decrease MPA levels by interrupting the enterohepatic recirculation and inhibiting the multidrug resistance protein 2 (MRP2) which excretes MPAG into bile (Hesselink et al., 2005; Kuypers et al., 2009, 2010; Vincenti et al., 2009). Such inhibitory effect of CsA on the enterohepatic recirculation was supported by pharmacokinetic population studies that showed lower MPAG clearance in CsA than in Tac treated patients, despite similar renal function (Hesselink et al., 2005). According with Kuypers et al. other hypothesis is that in the presence of CsA the multidrug resistance protein [MRP]-2 and competitive MPAG albumin binding will increase elimination of free MPA affecting the pharmacokinetic (Kuypers et al., 2009; Vincenti et al., 2009).

Another hypothesis is that since initial use of CsA was use as antibiotic it might inhibit the colonic flora that may decrease the rate of conversion of MPAG back to MPA (Kuypers et al., 2009; Naesens et al., 2007). This will happen because in the gut the amount of glucuronidase will be decreased leading to less deglucuronidation, this is a reversible process which means that cessation of CsA would restore the gut flora increasing the desglucurnidation and the MPA levels as well (Kuypers et al., 2009; Naesens et al., 2007; NAESENS et al., 2006). It has been described by Gregoor et al. that comedication with CsA can influence MPA levels and its pharmacokinetics. Leonor

Pou et al. did a study investigating if the coadministration of Tac instead of CsA will increase MPA levels. Their results shows that administration of Tac increase MPA levels, even in patients receiving a lower dose of MMF. Another point observed on this study was that in the same dose of MMF administrated bigger variations were observed on patients receiving CsA (Pou et al., 2001). Since there isn`t many studies it can be any of this hypothesis or they combined that lead to a decrease on MPA levels when use combined with CsA.

Data have shown that Tac does not interfere with MPAG levels and it was demonstrated by a greater AUC_{0-12}, slower MPA clearance and reduced MPAG AUC_{0-12} compared to CsA results (Gelder et al., 2006; Kobayashi et al., 2004). On our review we try to separate MPAG levels by therapeutic regiment to compare the data and analyse the influence on it but there wasn`t enough information to do it. Most of the studies measure only MPA levels since it is the active metabolite even knowing that would be interesting monitor MPAG and compare results especially to analyse if CsA has an effect on both metabolites levels.

Studies considered on this paper were separate by the most common therapeutic regimens - Tac and CsA - and the ones that didn't specify the regimen were grouped as Tac or CsA. It is possible to observe in figure 1A that after a dose of 1.5 g/day, AUC is higher using Tac than CsA, and for others dosages (1.0 g/day and 2.0 g/day) there were no data to compare the results. On literature there is a report of CNI combined with MPA can contribute to interpatient variability in MPA and MPAG pharmacokinetics resulting in differences on systemic drug exposure (Tornatore et al., 2015). All data were from stable patients with no potential rejection.

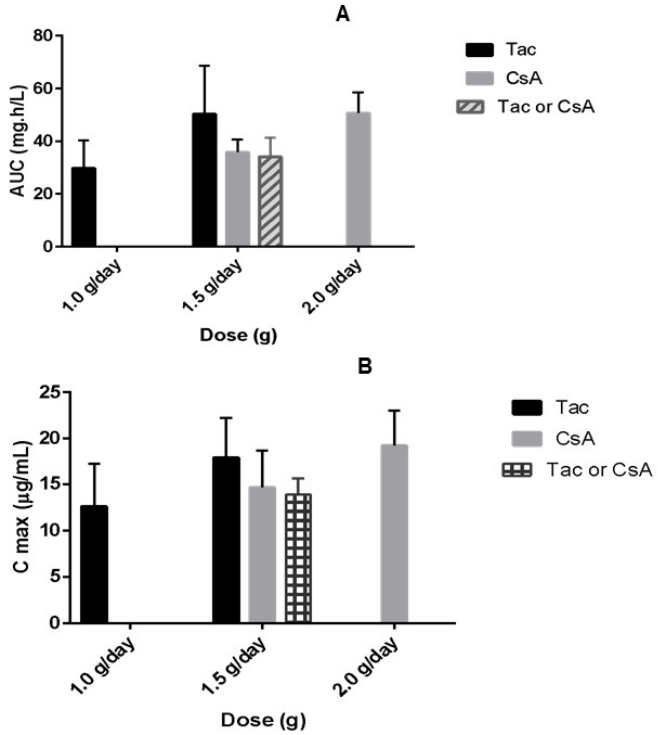

Figure 6.1 – A – AUC (mg*h/L) data separate by dose (g/day) and therapeutic regimen (Tacrolimus and/or Cyclosporine) combined with MPA. B - Data from C_{max} (µg/mL) separate by dose and therapeutic regimen (Tacrolimus and/or Cyclosporine) combined with MPA.

Considering therapy and biological factors changes in activity could account for interpatient variability in MPA levels and the glucuronide metabolite (Staatz and Tett, 2014). About 40 % (10 – 60 %) of MPA levels came from enterohepatic recirculation of MPAG and intensification of adverse effect may come from uncontrolled MPAG concentration increase that can cause MPA displacement from proteins and nonlinear increase of free MPA, which might be the cause of higher pharmacological activity of the drug (Kamińska et al., 2012; Staatz and Tett, 2007). Most of the papers do not describe all drugs concomitantly used with MPA, neither if there is a signal of any kind of adverse effect or influence on MPA and MPAG levels. It was already been described that MPA has a variable pharmacokinetic, and its exposure increases over the first week's post-transplantation, figure 1B.

It has been reported that in fed state MPA T_{max} is slightly delayed and MPA C_{max} is decreased by 25 % compared with patients that are fasting and these effects were attributed to differences in the rate of gastric emptying (Akhlaghi et al., 2006). Patients

with diabetes were also associated with the delay of MPA T_{max} again probably due to delayed gastric emptying (Akhlaghi et al., 2006; Hesselink et al., 2005; Naesens et al., 2007). In the studies used on this paper, there weren't enough data to compare if these factors could interfere on MPA levels, only a few used patients with diabetics.

Following solid organ transplant, such as liver and kidney, recommended oral or intravenous dose of MPA is 1.5 g administered twice a day, however it`s known that MPA levels can be above normal in the begging of the treatment so patients should be follow closely (Opelz and Döhler, 2009; Staatz and Tett, 2014). Immunosuppression optimization would be helpful to minimize potential toxic effects an individualizing dose instead of using a fixed dose would be one of the ways to do it (Opelz and Döhler, 2009; Staatz and Tett, 2014).

6.4.2 Pharmacokinetics

Pharmacokinetics of MPA will be influence by three key processes: 1) intestinal absorption and de-esterification of MMF (in case of use EC-MPS release the salt), 2) enterohepatic recirculation and 3) metabolism and elimination of MPA (Gelder et al., 2006). The major part of the drug is eliminated on the urine as the inactive metabolite MPAG (Gelder et al., 2006).

On immunosuppressive therapy is the recommended method, mostly because MPA pharmacokinetics may be influenced by many parameters such as renal function, coadministration of other drugs, albumin concentration which can change the rate of protein bidding and, its pharmacokinetics may vary depending on the type of transplanted organ (Budde et al., 2010; Sobiak et al., 2016). For kidney transplanted patients the target range of MPA AUC that has been proposed for an effect therapy is 30–60 mg.h/L (Shaw et al., 2001; Sobiak et al., 2016; Zhang et al., 2016a). Data of studies involving MPA pharmacokinetics were analyzed and separate by dose, therapy and time post-transplant. On figure 2A it is shown that using 1.5 g/day or 2 g/day of MMF doesn't have a significant difference in AUC, in part because of the high standard deviations observed. Therefore, 1.5 g/day regimen has become the preferred one. However, it's not discriminated by time after transplant or therapy used in combination with MPA, and as it's possible to observe on figures 1 and 2B, these parameters are extremely important and will give a real difference between dose because it`s obviously that change the dose it will change the MPA levels. In addition, interpatient variability

must be considered because as we known it has a big influence on the metabolism, response of the patient and MPA levels.

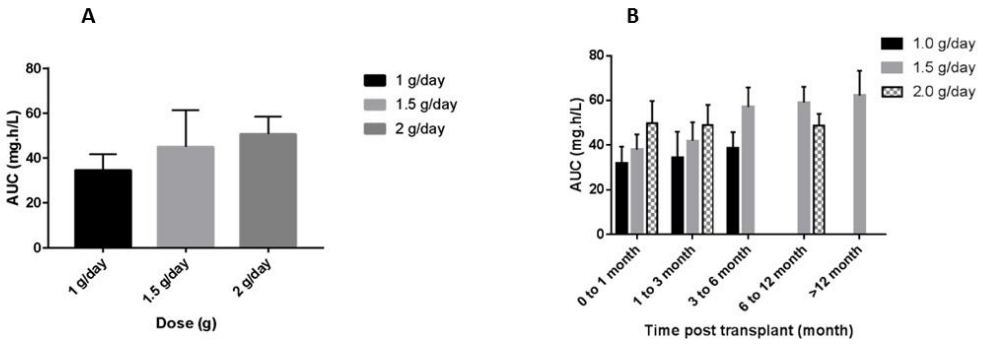

Figure 6.2 – (A) Average AUC (mg*h/L) from studies (n=32) used on this paper separate by dose (g/day). (B) AUC (mg*h/L) data separate by dose (g/day) and time (months) after transplant.

Data collection in this review allows noticing that more than 50 % of the analyzed papers used a dose of 1.5 g/day, as described on table 6.1. All therapies are in the minimum AUC for an effective therapy with doses of 1.5 g/day and 2.0 g/day, with no significant difference between them.

The dose of 1.0 g/day presents the lowest AUC on the first days, probably it is not the recommended to begin treatment, and even after months, it can present lower values than the target level, as can be seen on table 6.1. However, in order to design the graphs, mean data were used and as shown on table 6.1, AUC values vary depending on population, each study was performed with different patients, some in different continent. Comparing results from Chinese with African population, it was possible to observe that there is a difference between results from the two ethnic groups (Hummel et al., 2007; Tornatore et al., 2015; Zicheng et al., 2006). Even in the same population, there can be a difference if results from male and female individuals are compared (Jia et al., 2017; Zicheng et al., 2006). The most common regimen is 1.5 g/day, that after 3 months reaches MPA AUC target level of 30–60 mg.h/L (some studies use 40–60 mg.h/L) (F. A. Elbarbry and Shoker, 2007).

Table 6.1 – Data separate by AUC (mg*h/L), C_{max} (µg/mL), Dose (g/day), Time after transplant (months) and Therapeutic concomitant regimen used (Tacrolimus and/or Cyclosporine).

AUC (mg * h/mL) [a]	*C_{max} (µg/mL)* [a]	*Dose (g/day)*	*Time after Transplant*	*Therapeutic concomitant regimen*	*Reference*
70.20 (32.20)	17.90 (8.17)	1.50	>6 months	Tacrolimus	(Tornatore et al., 2015)
75.30 (31.20)	20.40 (9.89)	1.50	>6 months	Tacrolimus	(Tornatore et al., 2015)
59.00 (28.10)	22.80 (11.40)	1.50	>6 months	Tacrolimus	(Tornatore et al., 2015)
71.70 (31.10)	21.90 (14.20)	1.50	>6 months	Tacrolimus	(Tornatore et al., 2015)
56.80 (11.47)	16.99 (6.35)	2.00	2 months	Cyclosporine	(Zicheng et al., 2006)
48.34 (12.32)	22.92 (9.23)	2.00	2 months	Cyclosporine	(Zicheng et al., 2006)
43.04 (16.56)	10.71 (7.83)	2.00	>6 months	Cyclosporine	(Jia et al., 2017)
45.20 (15.90)	15.00 (6.90)	2.00	<1 month	Cyclosporine	(Park et al., 2008)
52.54 (13.21)	21.88 (10.20)	1.50	12 months	Cyclosporine	(Lu et al., 2005)
67.40 (30.60)	18.10 (7.50)	1.00	<1 month	Tacrolimus	(Sobiak et al., 2016)
20.70 (12.10)	5.40 (3.70)	1.00	<1 month	Tacrolimus	(Fukuda et al., 2011)
25.20 (8.90)	8.10 (4.70)	1.00	6 months	Tacrolimus	(Zhang et al., 2017)
41.60 (21.80)	11.00 (6.80)	1.50	<1 month	Cyclosporine	(Fukuda et al., 2011)
38.00 [b]	11.60 [b]	1.50	<1 month	Cyclosporine	(Sommerer et al., 2010)
25.20 [b]	7.08 [b]	1.50	1 month	Cyclosporine	(Pescovitz et al., 2007)
28.20 [b]	8.17 [b]	1.50	3 months	Cyclosporine	(Pescovitz et al., 2007)
34.40 [b]	11.80 [b]	1.50	6 months	Cyclosporine	(Pescovitz et al., 2007)
40.40 [b]	16.30 [b]	1.50	<1 month	Cyclosporine/Tacrolimus	(Pescovitz et al., 2007)
32.60 (18.71)	14.50 (6.40)	1.50	<1 month	Cyclosporine/Tacrolimus	(Glander et al., 2010)
31.30 (18.40)	13.00 (9.20)	1.50	<1 month	Cyclosporine/Tacrolimus	(Glander et al., 2010)
31.60 (18.80)	13.20 (9.70)	1.50	<1 month	Cyclosporine/Tacrolimus	(Glander et al., 2010)
37.50 (18.40)	16.50 (11.10)	1.50	<1 month	Cyclosporine/Tacrolimus	(Glander et al., 2010)
42.60 (20.60)	14.80 (10.00)	1.50	<1 month	Cyclosporine/Tacrolimus	(Glander et al., 2010)
40.30 (24.10)	11.50 (9.40)	1.50	n.a	Tacrolimus	(Glander et al., 2010)

37.96 (15.80)	13.32 (10.06)	1.00	n.a	Tacrolimus	(Zhang et al., 2017)
37.14 (20.62)	18.06 (13.91)	1. 50	8 months	Tacrolimus	(Zhang et al., 2017)
35.75 (20.96)	11.24 (7.16)	1.50	<1 month	Cyclosporine/Tacrolimus	(Gaies et al., 2017)
37.10 (11.50)	17.70 (5.50)	1.00	n.a	Tacrolimus	(Shen et al., 2009)
33.70 (17.10)	10.70 (7.60)	1.00	<1 month	Cyclosporine	(Delavenne et al., 2011)
38.90 (17.30)	12.50 (5.40)	2.00	3 months	Cyclosporine	(Delavenne et al., 2011)
28.80 (9.70)	10.90 (6.50)	1.50	6 months	Cyclosporine	(Delavenne et al., 2011)
40.60 (14.70)	13.40 (6.30)	1.50	9 months	Cyclosporine	(Delavenne et al., 2011)
35.39 (14.94)	16.03 (6.91)	1.50	21 months	Cyclosporine	(Nazemian et al., 2007)
39.80 (15.29)	n.a	1.50	12 months	Tacrolimus	(Mourad et al., 2001)
27.71 (4.54)	12.31 (7.89)	1.00	<1 month	Cyclosporine	(Yeung et al., 2000)
34.06 (4.16)	13.28 (7.00)	1.00	1 month	Cyclosporine	(Yeung et al., 2000)
45.00 (11.82)	16.15 (11.75)	1.00	3 month	Cyclosporine	(Yeung et al., 2000)
50.87 (22.37)	17.16 (10.91)	1.50	6 month	Cyclosporine	(Cattaneo et al., 2002)
54.91 (24.17)	16.78 (9.47)	1.50	9 month	Cyclosporine	(Cattaneo et al., 2002)
66.66 (30.92)	20.49 (8.77)	1. 50	21 month	Cyclosporine	(Cattaneo et al., 2002)
58.70 (29.00)	16.10 (8.30)	1.50	12 months	Tacrolimus	(Filler et al., 2004)

n.a – not available; [a]Mean (SD); [b]SD not available.

It has been shown that between week 2 to 12 post-transplant MPA and MPAG levels increase (Hummel et al., 2007; Jia et al., 2011). Figure 2B shows AUC increments after a few months of transplant. As it was demonstrate, 2.0 g/day is the dosage that by the first days reaches AUC on therapeutic window, and there is no significant change over time. At dose of 1.5 g/day, AUC increments over time and after 3 months, we can consider it stable. This progressive significant increase in the first 3 months after transplantation, as it was already been described, suggests change in MPA pharmacokinetics during this period (figure 2B) (Glander et al., 2010; Sommerer et al., 2010).

Considering all factors that can interfere on MPA plasma levels, therapeutic drug monitoring is recommend during the early period after transplant, as well this will be a good indicator of acute rejection. Some investigators have suggested that other factor that may affect MPA exposure is diarrhea by causing changes in MMF absorption, affecting adherence and effectiveness of treatment (Nazemian et al., 2007; Opelz and Döhler, 2009). However, this is not consensual between all authors and more deep studies measuring MPA and MPAG levels compare them and analyse clinical aspects, especially if patients presents the most common adverse effect, should be conduct.

6.5 Conclusion

Several studies of MPA pharmacokinetics and types of therapeutic regimen have been investigated. This review discussed the influence that dose, time after transplant and type of therapeutic regimen have on MPA pharmacokinetics. Dose of 1.5 g/day showed to be the most common on clinic setting and has enough data to compare and discuss the differences of all analyzed parameters. Comparing only the dose is not a good parameter, as it didn't show much difference, especially in doses of 1.5 g/day and 2 g/day. As this review shows and discusses, it is important, as much as possible, to separate data by parameter and evaluate the differences. As shown, the type of therapeutic regimen has a very important influence on MPA pharmacokinetics, specially using CsA concomitant with MPA, which can interfere on enterohepatic recirculation. Time after transplantation is another important factor, and 2.0 g/day regimen demonstrates to reach the target AUC faster than others regimens do. Analyzed data presented on this review showed that it is important to consider that MPA has a significant interpatient variability, and ideally, therapeutic monitoring for dose adjustment should be performed, including dosing of MPAG, to make sure that it's on target AUC level and the incidence of toxicity is decreased. More pharmacokinetics studies with MPA and MPAG are important to compare AUCs and analyze if therapeutic regimen influences on pharmacokinetics and toxicity.

6.6 Conflicts of interest

Authors confirm that there are no potential sources of conflicts of interest, financial or otherwise.

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7. CAPÍTULO 2 - Comparison of plasma and oral fluid concentrations of mycophenolic acid and its glucuronide metabolite by LC/MS in renal transplant patients

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Comparison of plasma and oral fluid concentrations of mycophenolic acid and its glucuronide metabolite by LC/MS in kidney transplant patients

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7.1 Abstract

Purpose

Mycophenolic acid is one of the most used immunosuppressive drugs in solid organ transplant treatments in the world. Developing a highly sensitive analytical method to analyse the drug and its metabolites in oral fluid and plasma is important to evaluate the possibility of use oral fluid as a biological matrix in therapeutic drug monitoring, instead of plasma.

Method

The liquid chromatography coupled to mass spectrometry (LC-MS) method was developed and validated for determining mycophenolic acid (MPA) and its glucuronide metabolite (MPAG) in oral fluid and plasma, with both matrices presenting a detection limit of 1 ng/mL for MPA and 5 ng/mL for MPAG. Both analytes were analysed after a simple protein precipitation procedure. Transplanted-kidney samples of oral fluid and blood were collected from thirteen patients that were hospitalized and kept at -80°C until analyses

Results

The proposed method was linear in the concentration range of 5 - 500 ng/mL for MPA and 10 - 500 ng/mL for MPAG, with correlation coefficients (r) between 0.9925 and 0.9973. It was then applied to samples collected from kidney transplanted patients and used for calculation of pharmacokinetics parameters

Conclusion

After comparing plasma and oral fluid concentrations as well as performing a non-compartmental pharmacokinetic analysis of the average curves, it is possible to suggest that oral fluid concentration may be use as an alternative for MPA and MPAG monitoring in kidney transplant patients.

Keywords: LC/MS, oral fluid, plasma, mycophenolic acid, mycophenolic acid glucuronide, pharmacokinetic.

7.2 Introduction

According to the latest United Nations reports, the world's population is ageing and with that chronic diseases are on the increase, including kidney failure [1–3]. The first choice treatment is kidney transplantation, and in 2017 more than five thousand transplants were performed in Brazil [1, 2, 4]. In kidney transplant therapeutic regimens, one of the most used immunosuppressive drugs to prevent allograft rejection is mycophenolic acid (MPA) in association with corticosteroids and calcineurin inhibitors [5–7]. MPA, in its active form, can be used in two ways to increase absorption: as the pro-drug mycophenolate mofetil (MMF) or as mycophenolate sodium (EC-MPS) [8–10]. Metabolization of MPA after absorption is carried out by UDP-glucuronosyltransferases (UGTs) into a pharmacologically inactive MPA-7-*O*-glucuronide (MPAG), the main metabolite, and its minor active metabolite MPA acyl glucuronide (AcMPAG) [8, 10]. The mechanism of action of MPA is the selective and reversible inhibition of the rate-limiting enzyme inosine monophosphate dehydrogenase type 2 (IMPDH-2) in *de novo* purine biosynthesis of guanine nucleotides in lymphocytes [6, 11, 12].

For drug monitoring, plasma is the most used biological matrix and a great variation in MPA concentrations among patients receiving the same dose has been observed [5, 13]. The use of oral fluid in drug monitoring has been investigated over the last years in order to replace blood collection, especially because it is easier and less invasive to collect [14–16].

Methods to detect MPA and its glucuronide metabolite in a different biological matrix have already been described, but there has only been one study using oral fluid to detect MPAG [8, 15, 17–21]. This study aimed to develop a sensitive LC-MS method with a simple and rapid sample clean up to quantify MPA and MPAG in oral fluid and plasma in order to apply it in samples from kidney transplant patients using a limited sampling strategy and try to establish a correlation between them.

7.2 Material and methods

7.2.1 Chemicals and reagents

Standards of mycophenolic acid (MPA) and ketoprofen, used as an internal standard (IS), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mycophenolic acid glucuronide (MPAG) was purchased from Santa Cruz

Biotechnology (Dallas, TX, USA). The acetonitrile, methanol and formic acid were all HPLC grade from Merck (Frankfurt, Germany). Zinc sulphate was reagent grade. Ultrapure water was obtained using a Milli-Q Plus system (Millipore, Bedford, MA, USA). Drug-free plasma and oral fluid were donate by volunteers.

7.2.2 Apparatus

The analyses were performed in a LC/MS system consisting of an Agilent 1260 infinity coupled to an Agilent 6120B mass detector. The software used for the analysis was Chemstation (v. B.04.03) (Palo Alto, CA, USA). The sample cleaning procedure was performed using an Eppendorf 5430R centrifuge (Hamburg, Germany).

7.2.3 Patient population and Ethics

Samples were collected from kidney transplant patients that were hospitalized and accepted to participate in the study. All patients received a dose of 750 mg of MMF twice a day (1.5 g/day). Patients with any signs of rejection, severe infection, using anti-tuberculosis drugs, with leukocytes lower than 3000/mm^3, HIV, bleeding history in the previous few months, or anaemia or diarrhoea were excluded. (See supplementary material for patient population characteristics).

A study protocol was formally submitted and approved by the Ethics Committee of Pontifícia Universidade Católica do Rio Grande do Sul under number 2.082.466 (Porto Alegre, RS, Brazil).

7.2.4 Study design

Blood samples, using EDTA tubes (Cral, São José/SP, Brazil), and oral fluid, using Salivette® (Sarstedt, Germany), were collected to determine MPA and MPAG concentrations at 0, 0.5, 1, 1.5, 2, and 12 h after the morning dose of MMF. The samples were immediately processed and kept at -80 ºC until analysis.

7.2.5 Liquid chromatography-mass spectrometry (LC-MS)

Chromatographic separation was performed using an Agilent Zorbax Eclipse Plus C18 column (4.6 x 150 mm, 3.5 µm particle size) (Torrance, CA, USA). Gradient condition was used, consisting of solvent A (H_2O + 0.1% formic acid), solvent B (acetonitrile + 0.1% formic acid) and solvent C (methanol + 0.05% formic acid) as follows: A→57% B→23% C→20% (0-5.0 min), A→50% B→42% C→8% (5.0-13 min),

A→57% B→25% C→18% (13.0-16.0 min). The flow rate was set at 0.7 mL/min and the temperature maintained at 35 °C.

The mass spectrometer (MS) detector was operated in positive mode (ESI+) and the following parameters were set: capillary voltage 4000 V, drying gas flow 12 L/min, nebulizer pressure 45 psig and drying gas temperature 350 °C. The gain value was kept at 1. Quantification of the analytes was performed using the single ion monitoring (SIM) mode, with 3 ions for each analyte (519, 321 and 207 for MPAG; 321, 303 and 207 for MPA; m/z) and 2 ions for the IS (255 and 209; m/z) due to their greater abundance.

7.2.6 Sample preparation and cleaning procedure

The oral fluid (150 µL) was pipetted and transferred into a 1.5 mL polypropylene conical tube, 25 µL of mix solution with MPA and MPAG was added and then vortex mixed for 15 seconds. The IS (25 µL) and 150 µL of ice-cold methanol with 0.1% formic acid were added and the vortex mixed for 30 seconds.

The plasma (50 µL) was pipetted and transferred into 1.5 mL a polypropylene conical tube, 25 µL of mix solution with MPA and MPAG was added and then vortex mixed for 10 seconds. The IS (25 µL) and 250 µL of ice-cold precipitating solution (0.1% formic acid in methanol and 0.1% zinc sulfate in water, 70:30) were added and the vortex mixed for 20 seconds.

Both samples (oral fluid and plasma) were centrifuged at 14,000 rpm for 25 minutes at 4 °C. The supernatant was filtered through 0.22 µm polyvinylidene fluoride or polyvinylidene difluoride (PVDF) membranes into conical vials and 15 µL were injected.

7.2.7 Standards solutions, analytical curves and quality controls

The MPA, MPAG and IS reference stock solutions were prepared in methanol at 1 mg/mL and stored at -20 ± 2 ºC. Quality control (QC) samples were prepared in drug-free oral fluid and plasma. A dilution quality control (DQC) matrix was prepared using a pool of blank diluting 1000 ng/mL to 500 ng/mL. For each day of analysis, standard solutions were prepared at 5, 20, 50, 100, 200, 300 and 500 ng/mL for MPA, and 10, 50, 80, 100, 200, 300 and 500 ng/mL for MPAG by adding suitable amounts of working solutions to a drug-free matrix.

7.2.8 Bioanalytical Method Validation

FDA bioanalytical guidance complemented by the EMA was used to perform the method validation [22, 23]. Selectivity, to assure the absence of interfering peaks in the quantification of MPA and MPAG, was performed by analysing blank samples from six different sources and a pool of 20 donors, and spiked with acetaminophen, prednisone, diclofenac, dexamethasone, ciprofloxacin and cyclosporine drugs that are commonly used by kidney transplant patients. For plasma, an additional assay was performed using lipemic and hemolyzed blood samples. The lowest concentration giving a response of at least three times the average baseline noise (S/N > 3) was defined as the LOQ and the lowest concentration that could be measured with CV with a bias lower that 20% was defined as LLOQ.

MPA and MPAG were quantified using the internal standard method. For linearity, replicates of each calibration level were analysed on three different days. Standardized residual plots (±3 standard deviation) and correlation coefficients were evaluated and regression analysis was performed. The coefficient of variation (CV%) calculation was used for within-run precision and between-run precision, and bias% for accuracy. For acceptance the mean value should be lower than 20% for the LLOQ and lower than 15% for the other concentrations.

Post-extraction addition was used to investigate the matrix effect by spiking the matrix (oral fluid or plasma) with solutions containing MPA and MPAG at low, medium and high quality control concentrations (LQC, MQC and HQC).

The stability of the analytes in the oral fluid and plasma was evaluated following the guidelines [22, 23] (see supplementary material).

7.2.9 Pharmacokinetic analysis

Non-compartmental PK parameters were calculated using the plasma and oral fluid MPA and MPAG concentration–time profile with PKsolver (Excel 2016, Microsoft, USA). The AUC ($AUC_{0–12}$) was calculated by applying the linear trapezoidal rule.

7.3 Results and Discussion

7.3.1 Bioanalytical Method Validation

The developed method showed no co-eluted peaks in the retention times of MPA and MPAG for neither oral fluid nor plasma samples matrices. Chromatogram purity analysis showed that common drugs used together with MMF did not interfere in the peaks of the analytes, showing the selectivity of the method. Figure 1 shows a representative chromatogram of MPA and MPAG in oral fluid and plasma spike with interfering drugs (A and B) and in patient samples (C and D).

Analysis of the seven-point calibration curve (5 – 500 ng/mL for MPA and 10 – 500 ng/mL for MPAG) covering the expected range demonstrates adequate correlation coefficients (r), showing that the method was linear for both analytes (table 7.1). Only one study has described the presence of MPAG in oral fluid with a concentration lower than 10 ng/mL and it used a LC-MS/MS method [13, 15, 24, 25].

Accuracy was accessed by calculating the bias (table 7.1). For all the QC samples, run precision (CV%) was calculated and presented adequate results, as recommended by the guidelines (table 7.1). Extraction recovery was around 85±5% of the standard concentration for MPA and MPAG. Once patient samples could present MPA and MPAG concentrations up to 500 ng/mL, dilution integrity was performed and showed adequate results for accuracy and precision.

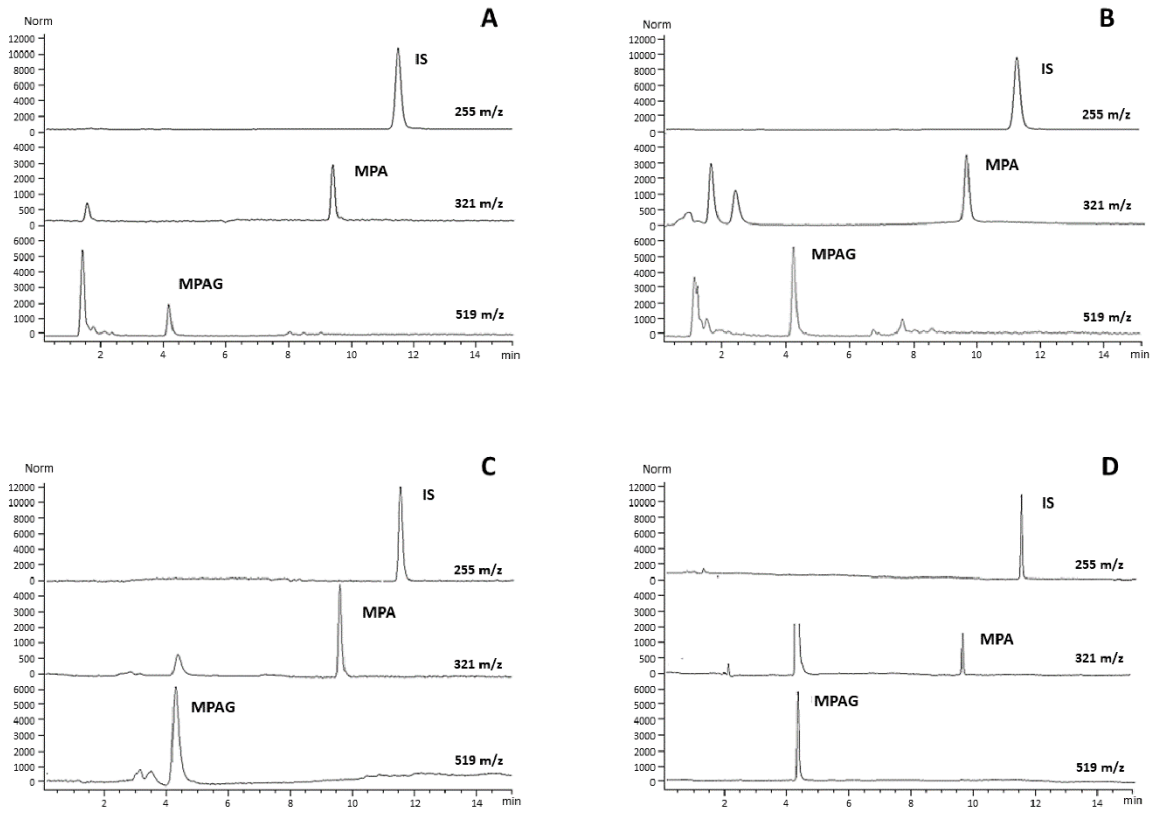

Figure 7.1 - Representative LC-MS chromatogram (SIM mode) of the analytes MPA, MPAG and IS. (A) Oral fluid, (B) Plasma, both spiked with MPA and MPAG standards, at 100 ng/mL, and interfering drugs. (C) Sample of oral fluid (D) Sample of plasma, both from kidney-transplanted patient.

Stability tests for LQC, MQC and HQC in both matrices were performed. The samples were stable in plasma and oral fluid at room temperature (20 °C ± 2) for 8 h and post extraction in the auto sampler for 12 h. After 3 months at -20 ºC (short-term stability) and after 3 freeze-thaw cycles, the MPA showed degradation in both matrix, however MPAG was shown to be stable in these conditions. After 6 months (long-term stability) at -20 ºC, both analytes were not to be stable in plasma and oral fluid (supplementary material).

Table 7.1 Limit of detection (LOD), limit of quantification (LOQ), correlation coefficient (r), accuracy and precision (between-run) of MPA and MPAG in oral fluid and plasma*.

				Accuracy[b] (bias %)			Between-run[b] (CV%)		
Analytes	LOD[a]	LOQ[a]	r [b]	LQC	MQC	HQC	LQC	MQC	HQC
MPA $_{\text{ORAL FLUID}}$	1	5	0.9973	-2.6008	-1.6573	-7.4374	3.0501	1.1540	4.0563
MPA $_{\text{PLASMA}}$	1	5	0.9925	-0.7049	-1.8597	-4.2701	4.6462	5.4436	5.0708
MPAG $_{\text{ORAL FLUID}}$	5	10	0.9952	0.7216	1.3702	-2.3725	1.8050	5.3601	1.7287
MPAG $_{\text{PLASMA}}$	5	10	0.9937	-0.0461	0.1370	0.3179	10.0160	1.7629	5.8012
(KET) $_{\text{ORAL FLUID}}$	-	-	-	-	-	-	-	-	-
(KET)$_{\text{PLASMA}}$	-	-	-	-	-	-	-	-	-

[a] concentration (ng/mL)
[b] Mean data of three days of analysis
* Analysis performed in three concentration levels: lower quality control (LQC), middle quality control (MQC) and higher quality control (HQC).

Short-term stability at -80°C demonstrates that MPA and MPAG were stabled under this condition. Besides that, it has already been described in the literature that samples are stable in plasma at -80 ºC for at least 6 months, so kidney-transplanted samples were kept at this condition until analysis [10, 13, 20, 27].

Both matrices showed adequate results using protein precipitation as a sample cleaning procedure, which is simple and fast, using a small quantity of oral fluid and plasma.

7.3.2 Matrix effect

The post-extraction addition approach was used to evaluate matrix interferences [22, 23]. No matrix effect was observed in both biological fluids since the CVs for all analytes were lower than 15%, as recommended by the guidelines. Carry over was not observed in the corresponding chromatogram, after injecting the highest concentration of the analytical curve.

7.3.3 Pharmacokinetics analysis

In this study, to determinate plasma and oral fluid concentrations of MPA and MPAG the validated LC/MS method was applied to samples from thirteen kidney transplant patients. To analyse the results, a non-compartmental model was used as it has already been described for MPA and MPAG in several studies [17, 20, 28–30]. The plasma and oral fluid concentrations of MPA and MPAG were found to be variable for both analytes (figure 7.2).

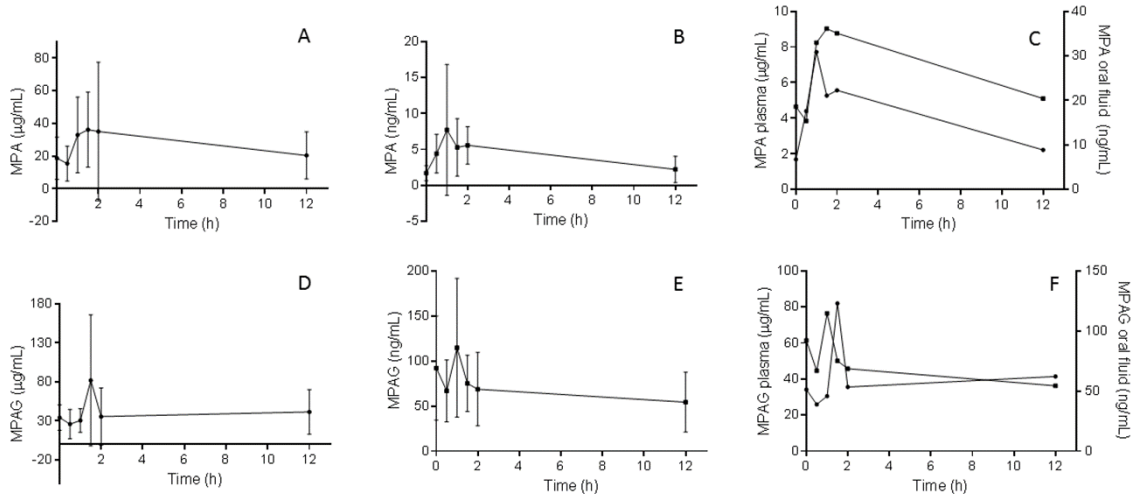

Figure 7.2 - Average concentration–time profile for MPA and MPAG (A) MPA in plasma, (B) MPA in oral fluid, (C) Comparison of MPA in oral fluid and plasma plasma oral fluid, (D) MPAG in plasma, (E) MPAG in oral fluid, (F) Comparison of MPAG in oral fluid and plasma plasma oral fluid.

The median MPAG $AUC_{0–12}$ was about 10-fold higher than the MPA $AUC_{0–12}$ values (table 7.2). These higher MPAG values were expected due to fast MPA metabolization in MPAG and AcMPAG [7, 28, 31–33]. Oral fluid presented, for both analytes, a lower concentration and $AUC_{0–12}$ than in plasma, which can be explained by the fact that in the plasma the total fraction was analysed and in the oral fluid only the free fraction was assessed, that is, ±3% for MPA and ±15% for MPAG [7, 34]. The target MPA $AUC_{0–12}$ value in blood, recommended for sufficient immunosuppression in kidney transplant recipients receiving MMF, is 30–60 µg h/mL (40–60 µg h/mL when EC-MPS is used) and the therapeutic range was reached in all the patients included in our study, with a mean of 55.83 µg.h/mL [35]. Comparing the $AUC_{0–12}$ profiles in plasma and oral fluid, these were similar for MPA and MPAG, as shown in figure 7.2.

Table 7.2 The main pharmacokinetics parameters of MPA and MPAG after an oral administration of 750 mg of MMF or EC-MPS to thirteen renal transplant patients.

	MPA_{plasma}*	$MPA_{oral\ fluid}$*	$MPAG_{plasma}$*	$MPAG_{oral\ fluid}$*
Cmax (µg/mL)	10.22 (18.54)	0.0361 (11.22)	82.03 (13.35)	0.1147 (12.25)
tmax (h)	1 (12.34)	1.5 (11.58)	1.5 (15.32)	1 (10.58)
AUC_{0-12} (µg.h/mL)	55.83 (15.97)	0.3331 (13.12)	472.13 (11.65)	0.7842 (15.69)

* mean (SD)

The MPA concentration was plotted to analyse if there was a correlation between oral fluid and plasma. The best fit was found using a mono-exponential correlation. The correlation coefficient (r) was 0.9646 for the MPA concentration in oral fluid vs plasma (supplementary material). These findings suggest that oral fluid may be used as an alternative to plasma for monitoring MPA levels in kidney transplant patients. The correlation was also confirmed by plotting the AUC results of oral fluid versus plasma, but this time a linear model was used (r= 0.9946).

The same approach was then used for MPAG. As with MPA, the best correlation for the MPAG concentration in plasma was obtained using a mono-exponential model (r=0.9210) and for the AUC of MPAG in oral fluid vs plasma a linear correlation was considered to be more adequate (r=0.9986).

One of the biggest advantages of using oral fluid is that collection is non-invasive and it is gaining considerable importance in drug monitoring [15, 36]. Collection of oral fluid does not require trained personnel, it can be used in adults and children as it is less stressful, and it can be carried out anywhere. This matrix has great potential for drug monitoring but it has some characteristics that should be considered, such as salivary pH, molecular weight, and percentage of free fraction. The use of other drugs at the same time can also change saliva pH. Other problems with oral fluid are that it can suffer contamination from residual food particles and blood, as well as the low production of it, which can influence viscosity and make it difficult to collect and process samples. In our study, we observed low oral fluid production in some patients, and we had to ask them to keep the Salivette® on their mouths longer. We did not use anything to promote salivation because this can influence the pH of the oral fluid and we were afraid it could change the plasma/oral fluid correlation [37].

On the other hand, blood collection in transplant patients proved to be quite complicated, because it is difficult to find a vein, blood flow is lower than in healthy patients and the use of devices for multiple collection is rarely viable. In our study, we faced all of these issues and had to choose a limited sample collection time. Therefore, we consider this study to be a pilot, to evaluate if oral fluid is an alternative to blood in the drug monitoring of MPA.

Our simple LC-MS method was able to detect low concentrations of both MPA and MPAG in oral fluid and demonstrated, with a limited sample collection strategy, a good correlation between the concentrations of MPA and MPAG in plasma and oral fluid, with the latter proving to be an advantageous alternative for use in drug monitoring. However, more studies should be performed.

Despite scarce information available in literature concerning MPA pharmacokinetics in plasma and its relationship with oral fluid, our results are in accordance with those previously reported (supplementary material) [6, 28, 32, 33, 38–40].

7.4 Conclusions

In this study, we described a fully validated bioanalytical method for assessing mycophenolic acid and its glucuronide metabolite concentrations in plasma and oral fluid. Single-stage LC-MS was used to perform the analyses after simple cleaning of the samples. The method developed has a low quantification limit for MPA and MPAG and was successfully applied to samples from kidney transplant patients. Pharmacokinetics analyses performed with limited sample collection of oral fluid and plasma demonstrated a strong correlation for both concentration and AUC. The results suggest that oral fluid can be considered as an alternative to plasma in both MPA and MPAG drug monitoring in kidney (and other organs) transplant patients but more studies should be carried out to support our findings.

7.5 Conflicts of interest

The authors confirm that there are no potential sources of conflicts of interest, financial or otherwise.

7.6 Acknowledgments

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7.8 Supplementary Material

Table 7.3 - Demographic data and clinical parameters

Number of patients	**All (n=13)**
Male/female	6/7
CsA/Tac[a]	5/8
Corticosteroid use	11
Median (range)	
Age (years)[b]	58 (39-75)
Time after transplantation (years)[b]	4 (0.5-11)
Body weight (kg)[b]	67 (49-90)
CsA dose (mg/day)[c]	150 (100-200)
Tac dose (mg/day)[c]	4 (1-6)

[a]CSA - Cyclosporine, Tac – Tacrolimus; [b] years; [c]mean (range of dose)

Stability Evaluation

Freeze-thaw stability was determined in low and high QC samples (n = 3) over two freeze thaw cycles (the samples were thawed at room temperature and refrozen) for 2 days. An analysis of processed samples at low and high QC was performed by keeping them on a bench top (25 ºC) and in a refrigerator (4 – 8 ºC), and then analysing them after 4 h and 8 h. The samples were considered stable if the assay values were less than 15% of nominal concentrations. Long-term freezer storage stability was analysed after 6 months.

Table 7.4 - Stability study of MPA and MPAG

	MPA_{PLASMA}(%)	$MPA_{oral\ fluid}$(%)	$MPAG_{PLASMA}$(%)	$MPAG_{oral\ fluid}$(%)
Freeze-thaw (3 cycles)				
LQC	77.85	93.13	105.16	102.08
MQC	73.23	90.5	87.67	94.23
HQC	94.15	99.27	102.71	98.91
Auto sampler 20°C±2 *(12 hours)*				
LQC	100.34	98.69	92.41	106.94
MQC	99.05	95.6	93.75	93.5
HQC	97.34	106.68	88.37	106.18
Room temperature 20°C±2 *(8h)*				
LQC	89.39	97.69	87.35	108.67
MQC	110.22	95.75	92.70	90.25
HQC	91.85	101.35	88.34	104.71
Freeze -20°C (3 months)				
LQC	60.68	32.83	87.85	95.23
MQC	80.65	80.56	82.30	94.11
HQC	85.39	65.40	87.95	97.47
Freeze -20°C (6 months)				
LQC	45.00	35.59	25.64	27.64
MQC	103.32	100.49	38.47	53.97
HQC	65.20	65.12	41.14	61.71
Freeze -80 (3 months[b]				
LQC	90.10	88.23	97.01	92.12
MQC	84.03	90.05	92.04	87.05
HQC	95.09	89.95	101.03	88.97

Table 7.5 - Data of MPA and MPAG of AUC (mg*h/L) and C_{max} (µg/mL) presented on literature*

	AUC_{0-12} (µg.h/mL)	Cmax (µg/mL)	Reference
MPA			
	59.00 (±28.10)	22.80(±11.40)	[33]
	58.70 (±29.00)	16.10 (±8.30)	[38]
	40.60 (±14.70)	13.40 (±6.30)	[28]
	42.60 (±20.60)	14.80 (1±0.00)	[39]
	41.60 (±21.80)	11.00 (±6.80)	[40]
	67.40 (±30.60)	18.10 (±7.50)	[32]
Media	51.65	16.03	
SD*	11.46	4.10	
MPAG			
	682 (±293)	81.70 (±36.00)	[33]
	657 (±318.10)	77.90 (±31.90)	[28]
	1547 (±844.70)	163.8 (±76.80)	[32]
Media	962	107.8	
SD**	506.78	48.53	

*Samples from blood of patients using MMF 750 mg twice a day (1.5 g/day).

**Standard deviation

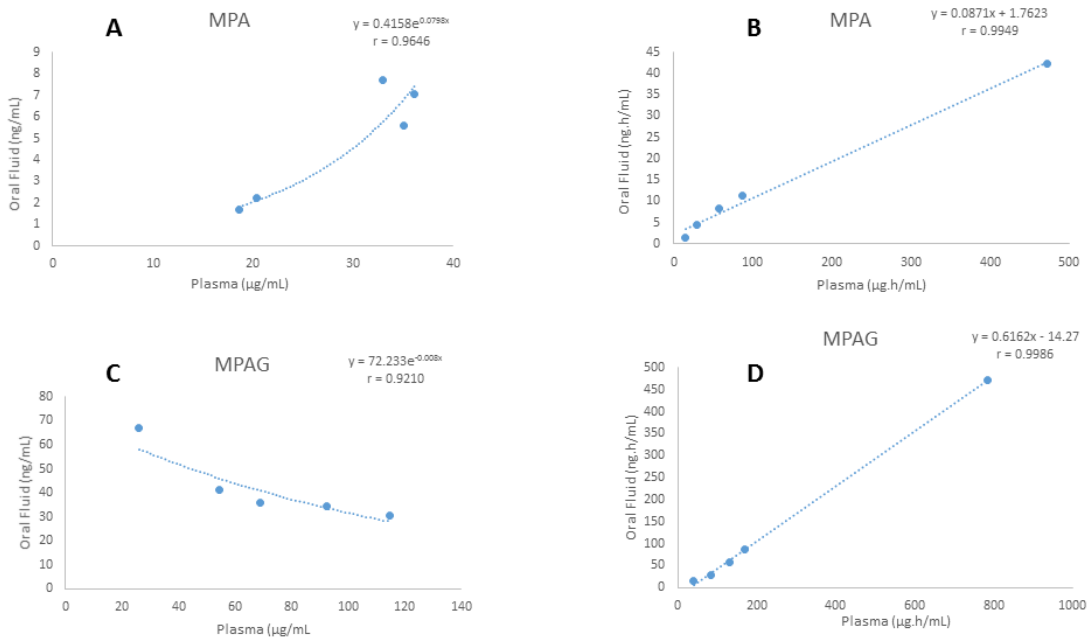

Figure 7.3 - Correlation between the mean concentrations (A and C) and AUC (B and D) of MPA and MPAG in plasma and oral fluid, between the times of 0 and 12.0 h.

8. CAPÍTULO 3 - Investigation of in vitro MPA levels and compared with samples from renal transplanted patients

Artigo submetido ao Journal of Pharmacological and Toxicological Methods

Investigation of *in vitro* MPA levels and compared with samples from renal transplanted patients

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8.1 Abstract

Chronic disorders are increasing worldwide with the aging of the population, and amongst all diseases, stands out the kidney failure. Recommended treatment for this disease is renal transplantation, which reduces treatment costs and increases the patient's quality of life. The major concern about transplantation is the high risk of rejection, leading to the necessity of immunosuppressive drugs. One of the main drugs used in the immunosuppressive therapy is mycophenolate acid (MPA), a potent, selective, non-competitive and reversible inhibitor of the inosine monophosphate dehydrogenase (IMPDH) that acts inhibiting the synthesis of guanine, limiting lymphocyte proliferation. MPA is metabolized to mycophenolic acid glucuronide (MPAG) and mycophenolic acid acyl-glucuronide (AcMPA). There is an increasing interest to measure the intra-lymphocyte MPA concentration, since it represents the target site of action. The aim of this work is to evaluate the permeation of MPA in the human lymphocytes and correlate with findings in the *de novo* transplant recipients and try to establish a permeation rate correlation. Lymphocytes for *in vitro* studies and for samples from transplanted patients follow Histopack® protocol. MPA levels were measure by LC/MS method. *In vitro* studies were performed with two different concentrations and in booth was possible to visualize the decrease of MPA content in the culture medium, and the increase of its intracellular concentration. After 24h of exposure, at concentrations of 5 and 10 µg/mL, the percentage of permeation gets around 15%. Samples from real patients were analyzed and it was detect MPA and MPAG levels. For both the permeation rate comparing with plasma levels were lower than 4% that was near with findings of total blood permeation study that had permeation rate of 1.77%. Comparing with results *in vitro* we found much lower levels than expected but in patients there was much more variables, including that they are in steady state, it was detect MPAG, which never have been described, and biological factors can interfere. More experiments should be conduct include investigation if MPAG findings are important and try to collect enough points to establish an intracellular profile concentration.

Keywords: lymphocytes, MPA, MPAG, permeation rate, LC/MS

8.2 Introduction

Mycophenolic acid (MPA), the active *in vivo* form of pro-drugs mycophenolate mofetil (MMF) and enteric-coated sodium salt mycophenolate sodium (EC-MPS), is widely used in immunosuppressant therapy after kidney, liver and heart transplantation (Arpini et al., 2013; Wiesen, Farowski, Feldkötter, Hoppe, & Müller, 2012). MPA is converted to the main inactive metabolite by uridine diphosphate glucuronosyl transferase 1A isoform (UGT1A) to 7-O-MPAglucuronide (MPAG), and by UGT2B7 to the pharmacologically active form acyl-glucuronide of MPA (AcMPAG) in various tissues including the liver and the gut (Nguyen Thi et al., 2015; Shen et al., 2009). MPAG is extensively bound to serum albumin, from which it can displace MPA, and is excreted in urine and bile. The biliary excretion allows enterohepatic recirculation by enteric organisms with glucuronidase activity that cleaves MPAG back to MPA, which represents the mainly part of plasmatic levels (Gelder et al., 2006; Mourad et al., 2001; Squifflet et al., 2001; C E Staatz & Tett, 2007; Christine E. Staatz & Tett, 2014).

MPA is used as an immunosuppressant in combination with a calcineurin inhibitor and a corticosteroid for the prevention and treatment of allograft rejection (Opelz & Döhler, 2009). It acts reducing guanine nucleotide biosynthesis by inhibiting reversible and selectively inosine 5'-monophosphate dehydrogenase (IMPDH), which is the key enzyme for the *de novo* purine biosynthesis in T and B lymphocytes (Elbarbry & Shoker, 2007; Syed & Srinivas, 2016; Wiesen et al., 2012).

The measurement of the intracellular lymphocytes levels of MPA, where the drug is acting, has been proposed, but still more studies to correlate with plasma findings are necessary (Nguyen, Capron, Mourad, & Wallemacq, 2013; Nguyen Thi et al., 2015). It has been demonstrated that more than 98% of the drug remains in the plasma compartment, mainly bound to albumin, and that a minor fraction reaches the mononuclear cell compartment (Nguyen Thi et al., 2015) The aim of this work is to evaluate the permeation of MPA in the human lymphocytes to after that correlate with findings in the *de novo* transplant recipients and try to establish a permeation rate correlation.

8.2 Material and methods

8.2.1 Chemicals and reagents

Standards of mycophenolic acid (MPA), Histopack, RPMI medium were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, methanol and formic acid were all of HPLC grade were purchase from Merck (Frankfurt, Germany). Zinc sulphate was reagent grade. Ultrapure water was obtained using a Milli-Q Plus system (Millipore, Bedford, MA, USA).

8.2.2 Apparatus

An Agilent 1260 infinity LC system equipped with a G1311B quaternary pump, a G1329B auto sampler, G1314F UV/VIS detector, and a G1316A thermostatizer coupled to an Agilent 6120B series mass detector and a Chemstation (v. B.04.03) software were used for the analysis (Palo Alto, CA, USA). An Eppendorf 5430R centrifuge (Hamburg, Germany) and Panasonic CO_2 incubator (Japan) was also used.

8.2.3 Liquid chromatography mass spectrometry (LC-MS)

Chromatographic analysis was performed on Agilent LC/MS. The separation was achieved with an Agilent Zorbax Eclipse Plus C18 column (4.6 x 150 mm, 3.5 µm particle size) (Torrance, CA, USA) maintained at 35 °C, flow rate was 0.7 mL/min in gradient condition consisting of solvent A (H_2O + 0.1% formic acid), solvent B (acetonitrile + 0.1% formic acid) and solvent C (methanol + 0.05% formic acid) as follow: A→57% B→23% C→20% (0-5.0 min), A→50% B→42% C→8% (5.0-13 min), A→57% B→25% C→18% (13.0-16.0 min).

Mass spectrometer detector was operated in positive mode (ESI+) and method was optimized to the following parameters: capillary voltage 4000 V, drying gas flow 12 L/min, nebulizer pressure 45 psig and drying gas temperature 350 °C. Gain value was kept at 1. The ions transitions monitored were m/z 321.0→207.0 for MPA, 519.0→343.0 for MPAG and 255 for IS.

8.2.4 Patient population

For *in vitro* studies and validation of the analytical method, lymphocytes were isolated from healthy subjects that voluntarily donate blood. Samples to apply the methodology were collect from kidney-transplanted patients that were hospitalized and accepted to participate in the study. Patients with any signal of rejection, severe

infection, using anti-tuberculosis drugs, leukocytes lower than 3000/mm^3, HIV positive, with bleeding history in the last few months, with anaemia and diarrhea were excluded.

8.2.5 Preparation of reference solutions

Reference stock solution of MPA used on the *in vitro* studies were prepared in DMSO at 2 mg/mL and stored at -20 ± 2 ºC. MPA to spike the total blood and the isolated lymphocytes were diluted in saline solution or in medium to have final concentration of 5 and 10 µg/mL.

Standard solutions of MPA and MPAG used on analytical method were prepare at concentration of 1 mg/mL in methanol and stored at -20 ± 2 ºC. Solutions for calibration curves and quality control were prepared daily in methanol.

8.2.6 Lymphocytes isolation, permeation protocol and cytotoxicity study.

The lymphocytes from human peripheral blood were isolated by Histopaque® gradient (Böyum, 1968) from healthy volunteer and from kidney transplanted patients. For renal transplanted blood samples, after lymphocytes isolation, the pellet was kept on eppendorf tube and stored at -80˚C freezer.

MPA permeation *in vitro* studies were performed using total blood and isolated lymphocytes from healthy volunteer. For the permeation study in total blood, 4 mL of blood were collected in heparin tube, spiked with MPA and keep under constant stirring for 6 hours, before the lymphocytes isolation. Initial concentration of MPA in plasma was used as total concentration to compare with findings after the lyse of the lymphocytes.

For the permeation study in isolated lymphocytes from healthy volunteer, after separation, the lymphocytes were counted, diluted in RPMI 1640 media containing 10% bovine fetal serum, plated on 24 well plate (600.000 cells/well), and incubated for 24 h at 37°C with 5% CO_2. Lymphocytes were treated with MPA and samples were collected on the pre-determinate time: 0, 2, 4, 6 and 24 h, for analysis of the concentration on the culture medium and in intracellular site.

To ensure that the MPA concentrations used in the permeation study to treat the lymphocytes were non-toxic, it was performed a cytotoxicity assay. Following the Hisptopack® isolation protocol, the lymphocytes were treated with different

concentration of MPA: 1, 5, 10, 30, 50 and 80 µg/mL, incubated for 24h and the cell viability was measured by Flow cytometry.

8.2.7 Measurement of MPA concentrations in lymphocytes

For *in vitro* studies, lymphocytes were collect from the well plate, added into 2 mL polypropylene conical tubes and centrifuged at 1500 rpm during 20 min at 24 °C. Supernatant was filtered through 0.22 µm PVDF membranes to conical vials and 15 µL were injected on LC/MS system for the determination of the extracellular concentration of MPA.

Cell lysis and the intracellular concentration of MPA in lymphocytes were performed by removing the supernatant and adding 350 µL of methanol to polypropylene conical tube to dissolve the pellet and lyse the lymphocytes. Then, tube was taken to the sonicator for 25 minutes and centrifuged at 14.000 rpm, during 20 min at 24 °C. Supernatant was filtered through 0.22 µm PVDF membranes to conical vials and 15 µL were injected on LC/MS system. For samples from patients it was followed the same protocol.

8.2.8 Measurement of MPA concentrations in plasma

Plasma samples were processed using a simple protein precipitation. 300 µL of MeOH containing 200 ng/mL of KET as internal standard (IS) was added to 50 µL of plasma. Subsequently, the samples were vortexed for 15 seconds and centrifuged for 20 min at 14,000 rpm at 4 °C. 20 µL supernatant was filtered through 0.22 µm PVDF membranes to conical vials and 15 µL were injected on LC/MS system.

Analytical method was the same used for MPA determination in lymphocytes, and was previously validated for plasma by the group in accordance to the FDA guidelines (FDA, 2018).

8.2.9 Sample preparation and cleaning procedure

Blank lymphocytes (for calibrators and quality controls (QC)) were isolated from fresh heparin blood. Lymphocyte pellets were then pooled. The calibrators and QC samples were prepared by adding 350 µL of MeOH containing fixed amount of 200 ng/mL of KET (IS), and appropriate amount of MPA and MPAG to achieve calibrators and QC (lower limit of quantification – LLOQ; low quality control – LQC; middle quality control – HQC; high quality control – HQC) concentrations of 10, 50, 80, 100, 200, 300,

and 500 ng/mL and 10, 30, 200, 500 ng/mL, respectively. Dry pellets obtained from patient samples were reconstituted by adding 350 µL MeOH containing only 200 ng/mL of KET and lysed as already described.

8.2.10 Method Validation

Method was validated following the FDA bioanalytical guideline and complemented by EMA guideline (EMA, 2012; FDA, 2018). Selectivity, sensitivity (limit of detection - LOD and limit of quantification - LOQ), calibration curve, accuracy, precision, carryover, matrix effect (ionization suppression/enhancement) and stability of the analytes in spiked samples were assessed.

Matrix effect was assessed by post-extraction addition approach comparing the analyte peak areas of neat standards prepared at low and high QC with the extracted blank matrix fortified with low and high QC.

8.2.11 Ethics

The protocol of the study was formally submitted and approved by the Ethics Committee of Hospital São Lucas under the number of 2.082.466 (Porto Alegre, RS, Brazil).

8.3 Results and discussion

8.3.1 Method validation

Coefficient of determination (r) for MPA and MPAG calibration curves were >0.98, with a mean value of 0.9897 ± 0.020 for MPA and 0.9852± 0.050 for MPAG. For both MPA and MPAG, LLOD and LLOQ were found to be 5 and 10 ng/mL, respectively. Inter-day and intra-day CV% accuracy was bellow than 10% at the low, medium, and high concentrations, and below 15% at the LLOQ. The retention time was 4.4 min for MPAG, 9.8 min for MPA and 11.5 min for IS. MPA recovery at 30, 200 and 500 ng/mL, displayed a good reproducibility, with a mean value of 87.6% (range: 84.9–90.5%), and CV% ranging from 5.8 to 9.4%. For MPAG recovery at the same concentration also displayed a good reproducibility, with a mean value of 82.1% (range: 80.9–83.3%), and CV% ranging from 4.9 to 7.1%. Extraction efficiency for IS was estimated 91.7%. Typical ion chromatogram of extracts from blank blood sample, lymphocytes reconstituted with MPA (50 ng/mL), MPAG (50 ng/mL) and IS (200 ng/mL)

are shown in figure 1. No interfering peak was detected at the m/z transitions and retention times of interest.

For MPA at LLOQ, LOQ, MQC, and HQC, precision ranged from 6.1 to 9.8% and accuracy from 98.2 to 108.5%. For MPAG the same QC for precision ranged from 5.3 to 7.2% and for accuracy varied from 95.4 to 105.3%.

At the three tested levels, matrix and ion suppression effects were both with minimal impact on the results. No carry-over effect was observed following injection of the highest calibrator (500 ng/mL).

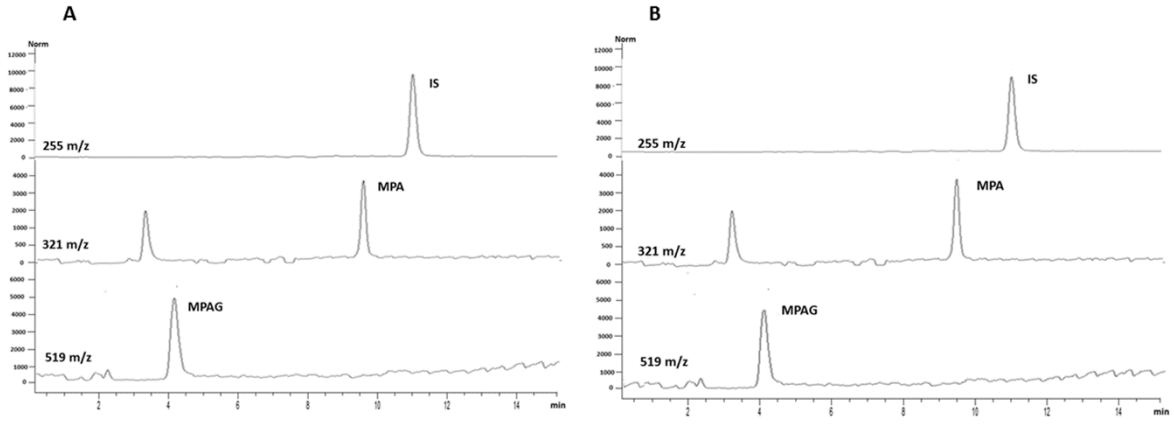

Figure 8.1 - Representative LC/MS chromatogram (SIM mode) of the analytes MPA, MPAG and internal standard (IS) KET. (A) Blank Lymphocytes spiked with MPA and MPAG standard at 50 ng/mL, (B) Lymphocytes isolate from renal transplanted patients.

8.3.2 In vitro permeation study

The *in vitro* experiments with isolated lymphocytes from healthy volunteer were performed with two concentrations of MPA, 5 and 10 µg/mL, usually finding on measurement of renal transplanted patients plasma. In both concentrations tested was possible to visualize the decrease of MPA content in the culture medium, and the increase of its intracellular concentration along the time (figure 2). Time of collection was determinate as 24h, once after 48h MPA intra and extracellular concentrations did not show significant difference from findings in 24h. The cellular density of lymphocytes per well chose to perform the *in vitro* permeation studies was 600.000 lymphocytes,

which represent 500 lymphocytes/µL, which is very similar to the average for renal transplanted patients (around ±700 lymphocytes/µL).

Analyze of intra-lymphocyte MPA concentration demonstrate that the longer exposure time higher is the intracellular concentration, and the increase of intracellular concentration is proportionally to the decrease of the concentration of the drug in the medium (figure 8.2).

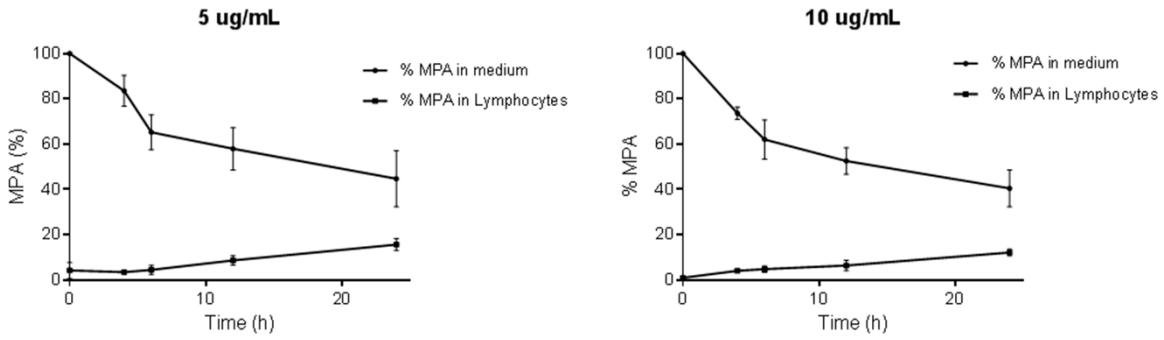

Figure 8.2 - Percentage of MPA in *in vitro* permeation studies with isolated lymphocytes from healthy volunteer: Concentration x Time of exposure.

As can be seen in figure 2, after 24h, the permeation rate is around 15% for both concentrations. We notice that the process is dependent of time and concentration which means that higher the concentration more MPA is found inside the lymphocytes but the permeation rate don`t change. This permeation process is dependent of time and concentration; which indicates a passive transport process.

Before analyzing samples collected from kidney transplanted patients it was performed an *in vitro* permeation study using total blood from healthy volunteer spiked with MPA. This test was performed since it is more similar to what happens in transplanted patients, and for verifies if the direct exposition of isolated lymphocytes to MPA has the same profile of permeation when compared to the total blood.

From previously work it was reported that the total blood is stable in heparin tube to extract MPA for 10 h (Nguyen et al., 2013; Nguyen Thi et al., 2015). Since

patients are in steady state, and based on the MPA stability, we choose a time for keep the MPA in contact with blood of 6h. Our results showed that the mean lymphocyte permeation rate of MPA from total blood was 1.70%, after 6h of MPA exposition. On *in vitro* permeation studies with isolated lymphocytes, we found the mean lymphocyte permeation rate of 4.22%, at the same time. The differences found could be attributed to the complexity of total blood matrix that has more interferers and, probably will reflect more precisely what will be found in transplanted patients.

8.3.3 Cytotoxicity test

Cytotoxicity test was performed to verify if MPA concentrations that were used in *in vitro* experiments are non-toxic to the cells. We tested MPA concentrations in a range of 1 – 80 µg/mL. Results of cell viability showed that even in the higher concentration there is not cell death (figure 3). Viability is almost 100% for all concentrations tested. This result show that the MPA concentrations used in *in vitro* permeation studies are safe, and did not lysis the lymphocytes in the experimental conditions.

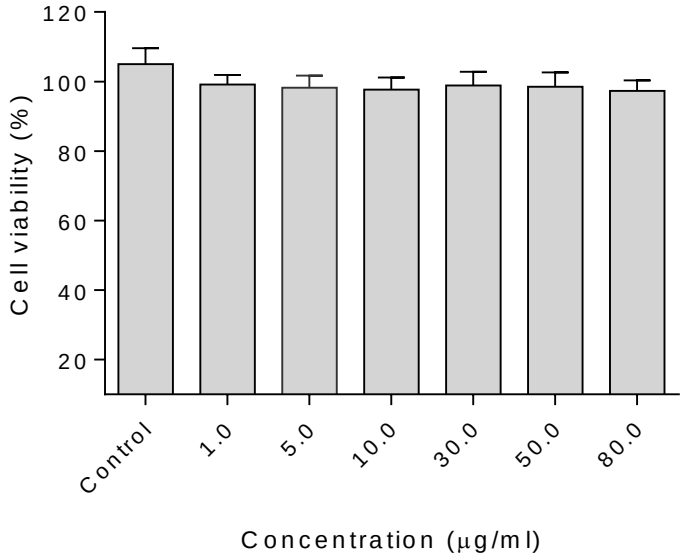

Figure 8.3 – Cell viability assay after treatment with MPA for 24h. (n=3)

8.3.4 Patients samples

Analysis of samples from kidney-transplanted patients show that it was feasibly to detect not only MPA, as was the main goal since the beginning, but MPAG as well.

Samples from 13 patients were analysed and was possible to compare the amounts in different times. Comparing the relation between concentration of MPA in total plasma and inside the lymphocyte, it was found a rate of ±1.51% inside it (table 8.1). We cannot consider this as a the permeation rate of MPA to lymphocyte but this finding is very close with the findings of the *in vitro* studied that was performed with total blood, which was found a rate of 1.70%. Patients samples demonstrated that the rate inside the lymphocytes did not have a significant change among the time. They are in steady state and that is the reason for lower fluctuation of the MPA and MPAG concentrations inside the lymphocytes. A previous study that had already been described to measure MPA lymphocyte concentration had a rate between plasma concentration and intracellular less than 0.5%, which is lower than our findings (Nguyen Thi et al., 2015). However, different from our patients, this study describe the MPA levels of recently transplanted patients that are not in steady state, nor in stable dose, and are taking several drugs that may interfere on the results.

Table 8.1 – Average rate of MPA and MPAG comparing plasma with lymphocyte levels in renal transplanted patient.

	MPA[a]	MPAG[a]
T_0	2.56 (1.12)	2.63 (±1.07)
$T_{0.5}$	1.49 (0.93)	1.29 (±0.17)
T_1	1.52 (0.77)	4.06 (±1.44)
T_2	0.47 (0.35)	3.64 (±1.03)
Mean	1.51 (0.84)	2.55 (±1.32)

[a] % (SD)

For MPAG the mean inside the lymphocyte is ±2.55% (table 8.1). The MPAG amounts in the lymphocytes, as in plasma, are higher than MPA. There is no publish reports measuring MPAG in lymphocytes in samples of renal transplanted patients, only *in vitro* studies. The presence of this metabolite inside the lymphocyte is not clearly, because there is one paper that says that there is no activity while another one

says that there is (Atcheson et al., 2004; Millan et al., 2000). The studies that report the activity of MPAG are old and more studies should be conducted.

In vitro studies in total blood, as in isolated lymphocytes, were important to demonstrate that even in experimental conditions MPA permeate lymphocytes and plasma concentration of MPA decrease not in the same rate as permeate the lymphocytes. Enzymes, proteins, amino acid binding and others reactions will decrease the amount of MPA available in experimental medium. Although susceptible to greater interferences, performed an experiment with total blood was important, since it seems closely correlate with what is happening on transplanted patient. Further studies are necessary to trace a profile of MPA permeation in total blood, and compare amounts in plasma and inside the lymphocytes to determine if during the 12 h between the doses, the MPA amounts continue to be in the same range.

8.4 Conclusions

Permeation *in vitro* studies confirm that MPA permeate the lymphocytes and in 24h apparently an equilibrium is reached. Changes in the concentration of MPA on the medium did not alter the permeation rate, indicating a passive transport process. Regarding the matrix used in the *in vitro* permeation studies, total blood samples demonstrated to be more efficient to transpose to human studies the MPA rate between plasma and lymphocytes. It was demonstrated that MPA permeates the lymphocyte and the environment that the drug is expose will decrease the amounts that reach the target due to others reactions that simultaneously occur. Samples collected from patients showed that not only MPA is found inside the lymphocyte, but MPAG as well, being the first report, and both are in a percentage less than 4% when compare with plasma levels. More studies should be done using a higher number of samples and correlate with clinical data to better understand the importance of the MPA and MPAG levels into the lymphocyte, since that is the active site of action of the drug.

8.5 Conflicts of interest

The authors confirm that there are no potential sources of conflicts of interest, financial or otherwise.

8.6 Acknowledgments

The authors wish to thank CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and PPGCF-UFRGS (the Postgraduate Program in Pharmaceutical Sciences - Federal University of Rio Grande do Sul) for the financial support.

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9. DISCUSSÃO GERAL

O monitoramento terapêutico de imunossupressores é alvo de estudos que procuram avaliar diversos fatores – incluindo efeitos colaterais que variam de acordo com o paciente, concentração plasmática, concentração no fluido oral, comparação entre concentrações máximas, métodos para detecção, entre outros – para diminuir riscos de rejeição ajustando a dose em sua concentração ideal (COLOM et al., 2012; ELBARBRY; SHOKER, 2007).

O ácido micofenólico (MPA), utilizado na forma de sal sódico ou pró-fármaco, encontra-se entre os imunossupressores mais utilizados no tratamento de transplantados renais e, por isso, são necessários estudos que ajudem a estabelecer perfis farmacocinéticos adequados, através da utilização de matrizes menos invasivas, como fluido oral, para um monitoramento terapêutico mais rápido e viável, (BORDIN et al., 2015; COLOM et al., 2012; MADARIAGA et al., 2016; MENDONZA; GOHH; AKHLAGHI, 2006; SHEN et al., 2009; SHUM et al., 2003).

Na literatura são descritos inúmeros métodos para detecção do MPA e seu metabolito glicuron*í*deo (MPAG), com outros equipamentos, que foram utilizados como referência para desenvolver e validar método analítico de CL/EM para as matrizes de plasma e fluido oral, com objetivo de separar cromatograficamente todos os picos do MPA e MPAG com eluição no modo gradiente.

A partir da revisão, adaptou-se um método que separasse eficientemente os analitos e apresentasse boa ionização (DELAVENNE et al., 2011; KLEPACKI et al., 2012; KUHN et al., 2009; SHEN et al., 2009; WIESEN et al., 2012; ZHANG; CHOW; RENBARGER, 2016). O MPAG é uma molécula que apresenta bastante dificuldade em ionizar, devido à sua polaridade e tamanho molecular. A maioria dos métodos propostos na literatura que foram testados não apresentaram boa ionização em condições experimentais. Um método analítico com uma baixa ionização do MPAG apresentará limite de quantificação maior, o que pode prejudicar a detecção do metabolito em amostras reais. Apenas um artigo mencionou a dificuldade de ionização do MPAG, e partiu-se deste para o desenvolvimento do método proposto neste trabalho (DiFrancesco et al. 2007). O MPAG possui um pka em torno de 2,80 (Drugbank 2017), sendo o ideal para ionização trabalhar numa faixa de duas unidades acima ou duas unidades abaixo deste valor. Testou-se tampão acetato de amônia e

formato de amônia com pH de 4,0, 4,5 e 5,0 e não se obteve bons resultados, pois o MPA e MPAG apresentaram menor ionização e resolução de pico.

Todos os trabalhos analisados utilizam fase móvel com pH menor que 3,0 e, em sua maioria com água, acetonitrila e metanol acidificado. Partindo-se dessas informações, testou-se fase móvel com água e acetonitrila com 0,1% de ácido fórmico, porém a ionização ainda era baixa. Acidificou-se todos os solventes da fase móvel com ácido fórmico e obtivemos uma boa ionização do MPAG com um limite de detecção de 5,0 ng/mL para MPAG e 1,0 ng/mL para MPA, para as matrizes fluido oral e plasma, respectivamente. Após outros testes conseguimos estabelecer uma melhor ionização tanto para MPA como para MPAG diluindo-os em metanol acidificado, o qual apresentou melhores resultados relativos a ionização.

Não foi possível realizar uma eluição isocrática, pois o MPAG é uma substância mais polar e sua eluição estava bem no início da corrida. Inicialmente o MPAG eluia em menos de 2 min, o que é ruim visto que as substâncias não eliminadas após a extração, normal em extrações de amostras biológicas, em geral eluíam nesse tempo e isso poderia interferir nas análises utilizando detector de massas simples. Após muitos testes, foi possível estabelecer uma fase móvel em modo gradiente utilizando-se de uma maior proporção de metanol no início da corrida, obtendo-se um tempo retenção adequado para o MPAG. Porém, com uma alta concentração de metanol, os picos do MPA e PI começaram a eluir no mesmo tempo de retenção e a resolução e simetria desses picos diminuíram. Por este motivo foi feito um método em gradiente que vai diminuindo a proporção de metanol durante a corrida.

Para os dois metabolitos foi realizado o monitoramento de massas com o íon molecular e mais dois de confirmação (figura 7.1). Utilizou-se o modo positivo, pois apresentou melhor ionização e abundância quando comparado com o modo negativo além de ser utilizado em mais de 90% dos métodos já publicados. Todos os parâmetros utilizados foram otimizados utilizando o método FIA, no qual é possível realizar injeções diretas e seguidas modificando os parâmetros desejados a fim de estabelecer qual o mais adequado para cada molécula. A partir do FIA pudemos observar qual melhor fragmentação, voltagem de capilar, temperatura de gás de secagem.

Para iniciar o desenvolvimento do método de extração das amostras de fluido oral, realizaram-se diversos testes com pool de 6 doadores. O desenvolvimento do presente método para análise baseou-se nos já descritos na literatura (MENDONZA; GOHH; AKHLAGHI, 2006; SHEN et al., 2009; ZHANG; CHOW; RENBARGER, 2016) Os métodos utilizam a precipitação de proteína como principal técnica de extração do MPA e MPAG. Como agente precipitante os métodos em geral utilizam acetonitrila, porém ao tentarmos reproduzir não houve boa recuperação e percebemos que poderia ser um problema de ionização, visto que nas análises do padrão já havíamos observado que os metabólitos diluídos em acetonitrila possuem uma menor ionização. Partiu-se para extração com metanol e metanol acidificado com 0,1% de ácido fórmico e após vários testes, incluindo volumes diferentes (100 a 400 µL), em temperatura ambiente ou gelado, sempre centrifugados a 4 ºC na velocidade de 14.000 r.p.m, em tempos variando de 5 a 30 minutos. A avaliação comparativa entre as áreas dos picos e limpidez da solução após extração foi favorecida pelo uso da MeOH com 0,1% ácido fórmico a 4 ºC. O melhor método utiliza 150 µL de solvente por amostra e conseguiu extrair os metabólitos do fluido oral e obter os limites de quantificação de 10,0 ng/mL para MPAG e 5,0 ng/mL para MPA, com volume de injeção de 15 µL durante a análise. Após cada dia de analise se faz necessário a limpeza da coluna com gradiente de ACN e água, por 120 minutos, e também da fonte ESI, tendo em vista que o método de precipitação não exclui completamente micropartículas e outros interferentes da matriz.

O método cromatográfico empregando CL/EM para análise de MPA e MPAG em fluido oral demonstrou ser capaz de avaliar também as amostras de plasma, utilizando-se um procedimento de extração dos metabólitos bastante semelhante em ambas às amostras biológicas, sendo apenas adicionado ao agente precipitante 0,1% (p/v) de sulfato de zinco, que demonstrou ser eficiente e melhorar a precipitação das proteínas presentes nessa matriz. Todos os parâmetros de configuração do detector

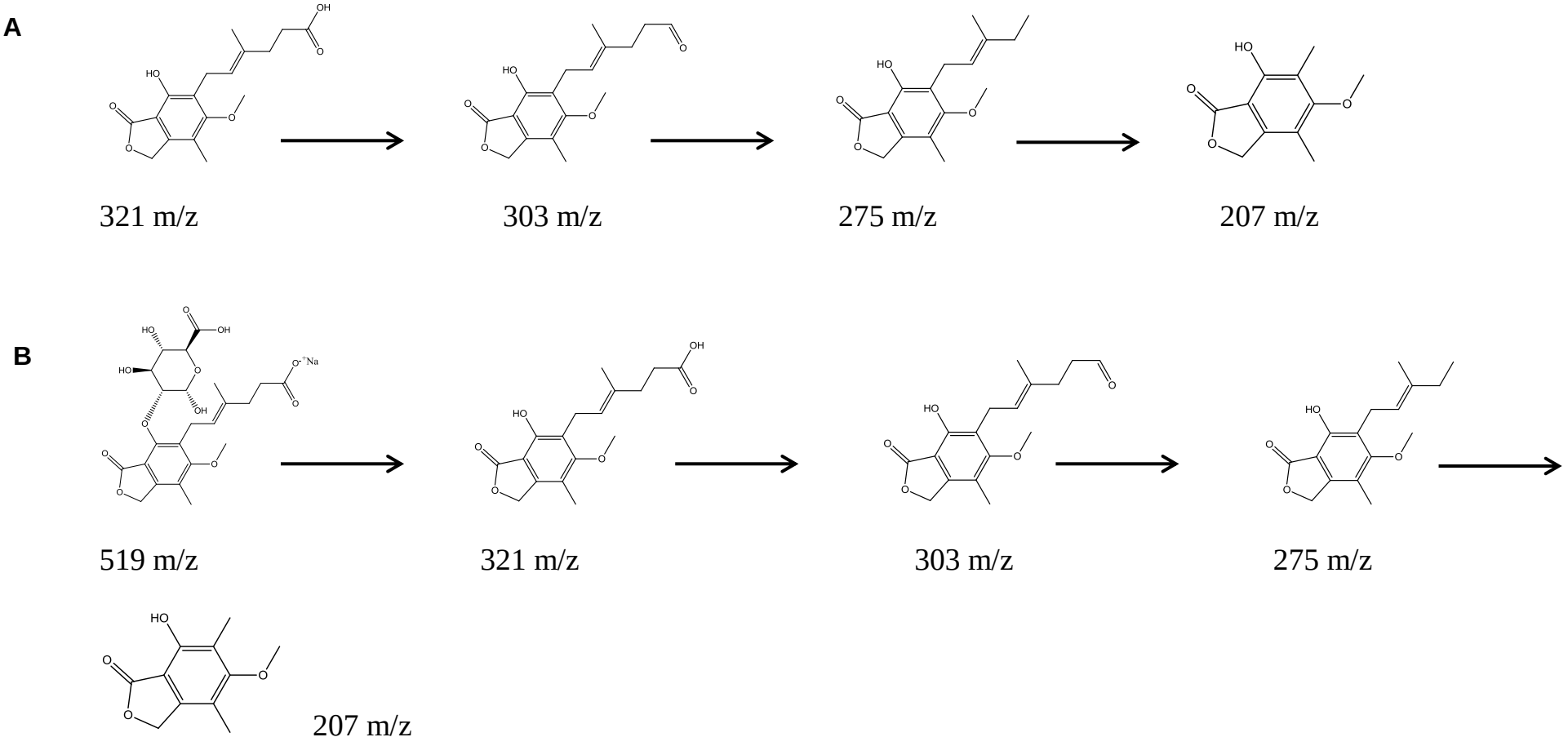

Figura 9.1 – Fragmentação proposta para (A) MPA e (B) MPAG

de massas e do cromatógrafo a líquido, incluindo coluna e fase móvel, foram mantidos iguais; porém, devido a troca da matriz biológica, modificações do processo de extração, foi necessária nova validação.

Testes de estabilidade foram realizados para as amostras de fluido oral e plasma. Os testes realizados foram de bancada, congelamento-descongelamento, curta e longa duração. Os resultados do material suplementar apresentado no capitulo 6 demonstram que os dois metabólitos, após extração, são estáveis a 20 ºC (estabilidade no autoinjetor) por pelo menos 12 h. Alguns testes realizados durante o desenvolvimento do método mostraram que, se deixadas a 4 ºC, as amostras após extração são estáveis por até 24 h. As amostras demonstram que são estáveis na matriz plasma e fluido oral por até 8 h. A estabilidade de curta, 3 meses, e longa duração, 6 meses, a -20 ºC demonstrou resultados variáveis e não satisfatórios, o que mostra que as amostras não podem ser armazenadas por maiores períodos sem alteração de sua concentração. Na literatura há relatos de que as amostras são estáveis a -80 ºC por um período maior de tempo, por isso, após coleta dos pacientes transplantados renais, as amostras serão imediatamente processadas e armazenadas nessa temperatura até análise (FIGURSKI et al., 2009).

Para testar a estabilidade dos metabolitos em sangue total foi coletado sangue de voluntários saudáveis em tubos de EDTA, adicionado solução padrão de MPA e MPAG na concentração final de 500,0 ng/mL. A análise foi realizada nos tempos 0, 15, 30, 45, 60, 75 min. Na tabela 9.1 podemos observar que no tempo 15 min já temos um decaimento bastante significativo nos dois metabólitos, sendo que o MPA apresentou o maior decaimento. Esses resultados confirmam o que já foi descrito na literatura que para análise dos metabólitos em plasma, após coleta de sangue no paciente é necessário realizar o processamento da amostra imediatamente (FIGURSKI et al., 2009). Mesmo teste foi realizado para fluido oral e nos tempos analisados, 0, 15, 30, 45, 60 e 75 min, não houve decaimento significativo das concentrações de MPA e MPAG. Porém as amostras também foram processadas assim que coletadas e refrigeradas.

Tabela 9.1 - Estabilidade de MPA e MPAG em sangue total

Tempo no sangue	% bias	
	MPA	MPAG
0	-0.5974	-1.19438
15,0	-21.5147	-8.6558
30,0	-30.0264	-11.1072
45,0	-36.7510	-16.3084
60,0	-40.1863	-26.0113
75,0	-49.0568	-29.8137

Observando-se a complexidade das matrizes envolvidas no estudo considera-se que o limite de quantificação de 5,0 ng/mL para MPA e 10,0 ng/mL para MPAG (em ambas as matrizes) adequado, tendo em vista que se empregou detector de massas com quadrupolo único após um procedimento de extração dos analitos bastante simples e rápido.

O método desenvolvido foi aplicado em amostras coletadas de pacientes transplantados renais internados no Hospital São Lucas da PUC/RS. No total foram coletadas amostras de plasma e fluido oral de 13 pacientes que se encaixavam nos critérios de inclusão já descritos no capitulo 6. Os tempos de coleta incialmente seriam 9 (0, 0.5, 1, 1.5, 2, 4, 6, 10, 12h), porem já no início do trabalho várias dificuldades foram encontradas. A primeira foi manter o acesso venoso por 12 h para que as coletas pudessem ser realizadas e por isso tentamos a estratégia de coletar apenas 5 pontos utilizando as 2 primeiras horas após o paciente ter tomado o ácido micofenólico. Entretanto essa estratégia não funcionou, pois o acesso dos pacientes não refluía o sangue e em alguns não era possível conseguir uma veia para o acesso. Após conversar com enfermeiras que trabalham com esses pacientes descobrimos que eles em geral apresentam dificuldade para retirar sangue devido a seu histórico de hemodiálise. A alternativa para retirar todos os pontos seria a cada coleta achar uma nova veia, porém outros dois novos obstáculos apareceram: primeiro nenhum paciente aceitaria, visto que ao ser solicitado uma amostra de sangue já era difícil e a segunda é a dificuldade em achar veia, tivemos ajuda das enfermeiras especializadas em coletas difíceis e mesmo assim em alguns pacientes não conseguimos nem um ponto de plasma.

Após discussões sobre qual caminho seguir optamos por seguir uma outra estratégia, que foi nos pacientes que conseguíssemos coletar mais pontos com acesso usaríamos e aqueles em que não era possível pelo menos uma coleta seria realizada. Foi importante que as coletas se realizassem em pontos diferentes para que pudéssemos no final estabelecer uma média e traçar um perfil para realizar uma análise farmacocinética preliminar. Para o fluido oral foram coletados 6 pontos, um antes de tomar nova dose do medicamento (t_{12}), e os outros 5 (0, 0.5, 1, 1.5 e 2 h).

A análise não compartimental apresentou resultados de C_{max}, AUC e T_{max} dentro do que é descrito na literatura, levando em consideração a grande variação interpaciente. Análise do MPAG demonstrou que tanto a concentração quanto área sob a curva são em média 10 vezes mais alta que do MPA. Esse resultado era esperado, devido a metabolização de primeira passagem do MPA em AcMPA, ativo em pequena quantidade, e MPAG em maior proporção e inativo, e que devido a enzimas presentes no intestino iram lisar o MPAG a MPA novamente, que será reabsorvido e representará a maior proporção da concentração do MPA na circulação sistêmica (recirculação entero-hepática).

Para fluido oral poucos estudos descrevem a farmacocinética do MPA e MPAG e apenas um apresentou resultados dos parâmetros farmacocinéticos para que pudéssemos comparar (MENDONZA; GOHH; AKHLAGHI, 2006; SHEN et al., 2009). Como já esperado as concentrações, AUC são menores do que o encontrado no plasma, estando em escala de nanogramas. Como apenas um estudo apresenta valores farmacocinéticos para o fluido oral e levando em consideração que o MPA e seu metabolito glicuronideo apresentam uma grande variação interpaciente, e que essa matriz pode sofrer interferências externas não é possível comparar nossos resultados e dizer que está de acordo com o encontrado na literatura.

Desde o início do trabalho a ideia era estabelecer se havia uma correlação farmacocinética entre MPA e MPAG de fluido oral *versus* plasma para avaliar a viabilidade de utilizar o fluido oral no monitoramento terapêutico, substituindo o plasma, que como já descrito acima apresenta dificuldades de coleta, e conseguir uma matriz que não seja invasiva, não precise de pessoal treinado para realizar seria de grande valor na pratica clínica.

Conforme resultados apresentados no capítulo 2, o fluido oral pode ser uma matriz a ser considerada para o monitoramento terapêutico do MPA e seu metabolito glicuronideo. Ainda são necessários mais estudos, com mais pacientes, para avaliar se nossa estratégia de coleta com pontos limitados se reproduz. Outro parâmetro a ser estudado para a implementação do fluido oral na clínica seria tentar correlacionar os valores encontrados com parâmetros clínicos dos pacientes, em especial com efeitos adversos (LU et al., 2005; NAESENS et al., 2007; TORNATORE et al., 2015; ZICHENG et al., 2006).

Um ponto a ser avaliado na questão do monitoramento terapêutico é qual tempo de coleta deve ser utilizado, pois em geral utiliza-se o T_0 para realizar as analises, porem devido à grande variabilidade do MPA alguns estudos já demonstraram que isso não seria eficiente. No T_0 a concentração do fluido oral é cerca de 100 vezes menor que a do plasma, com grande variabilidade nos dois fluidos (±70%). Sendo fluido oral uma técnica não invasiva seria possível pensar em uma estratégia de monitoramento utilizando mais de um ponto de coleta.

Durante as coletas de sangue também foi coletado outra amostra em tubo de heparina para isolamento dos linfócitos, a fim de realizar a análise da concentração intracelular do MPA, já que é seu sitio de ação. Um estudo já havia sido publicado demonstrando que não há uma correlação entre concentração nos linfócitos e a concentração plasmática (em C_0), porém como em nosso estudo fizemos coletas em diferentes pontos realizamos a análise para verificar se existe uma correlação (NGUYEN et al., 2013; NGUYEN THI et al., 2015).

Após análise da concentração intracelular por CL/EM elas foram normatizadas para ng/10^7 linfócitos, como é comumente apresentado na literatura. A figura 7.2 demonstra que a princípio as concentrações intralinfocitarias não são constantes e variou assim como no plasma apresentando a concentração máxima em 1.5 h.

Foi detectado dentro do linfócito o MPAG, e que em amostras de pacientes transplantados renais ainda não havia sido descrito na literatura. Os trabalhos publicados de isolamento de linfócitos, lise e quantificação são somente para o MPA por isso não esperávamos encontrar o MPAG, tanto que em nossos experimentos *in vitro* com o MPAG não utilizamos ele. Assim como no plasma esse metabolito está em maior concentração em relação ao MPA, contudo, por ser um metabolito inativo,

sua presença no sitio de ação pode não ter significado clínico. Dois trabalhos foram publicados um mostrando resultados que o MPAG tem atividade dentro do linfócito outro fala que não tem atividade, por esse motivo seria importante mais estudos serem realizados avaliando a atividade e assim determinar a impotência do MPAG intralinfocitario (ATCHESON et al., 2004; MILLAN et al., 2000)

A proporção intracelular de MPA comparada com no plasma é em média de 2% resultado que é próximo aos dos ensaios realizado em sangue total. Nele adicionamos MPA a uma amostra de sangue total e, após algum tempo, foram separados os linfócitos para ver quanto havia permeado, com uma porcentagem de permeação média de 1.75%.

10. CONSIDERAÇÕES FINAIS

O uso de imunossupressores em doses terapêuticas corretas é um ponto crucial para o sucesso do transplante renal, sobrevida do paciente e diminuição dos efeitos colaterais, já que muitos estudos provaram que a dose está relacionada a estes fatores. A coleta de amostras de fluido oral, procedimento não invasivo, facilitaria enormemente o monitoramento terapêutico desses imunossupressores e não necessitaria de alguém especializado para realizá-la.

No presente trabalho foi desenvolvido e validado método para análise em plasma, fluido oral e linfócitos visando a detecção de ácido micofenólico (MPA) e seu metabólito glicuronídeo (MPAG). O método desenvolvido utiliza um protocolo para limpeza da matriz biológica bastante simples e rápido, que possibilita a análise das substâncias de interesse por LC-MS com um limite de detecção adequado.

O método foi aplicado em amostras de fluido oral e plasma de pacientes transplantados renais, internados no hospital São Lucas da PUC, com sucesso. Mesmo com número limitado de amostras foi possível calcular os parâmetros farmacocinéticos e estabelecer uma correlação entre as duas matrizes. Mais estudos necessitam ser realizados para estabelecer o fluido oral como uma matriz alternativa ao plasma no monitoramento terapêutico do MPA.

O baixo limite de detecção permitiu realizar estudos *in vitro* e *in vivo* de permeação do MPA nos linfócitos e comparar os resultados com amostras coletadas de pacientes transplantados renais. A comparação dos dados de permeação permitiu concluir que os estudos realizados *in vitro* (sangue total) tem maior correlação com os resultados encontrados nas amostras de pacientes. Ao analisar as amostras foi detectado além do MPA, como já esperado, também o MPAG, que ainda não havia sido descrito. Como não há consenso na literatura relativo a atividade do MPAG mais estudos devem ser realizados no futuro para determinar a importância de sua quantificação.

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12. ANEXOS

PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL - PUC/RS

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: DESENVOLVIMENTO E VALIDAÇÃO DE MÉTODO ANALÍTICO PARA DETERMINAÇÃO SIMULTÂNEA DE MICOFENOLATO E PRINCIPAIS METABÓLITOS POR CL-EM

Pesquisador: Flavia Valladão Thiesen

Área Temática:

Versão: 2

CAAE: 67864517.9.0000.5336

Instituição Proponente: UNIAO BRASILEIRA DE EDUCACAO E ASSISTENCIA

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.082.466

Apresentação do Projeto:

Projeto de Pesquisa: DESENVOLVIMENTO E VALIDAÇÃO DE MÉTODO ANALÍTICO PARA DETERMINAÇÃO SIMULTÂNEA DE MICOFENOLATO E PRINCIPAIS METABÓLITOS POR CL-EM - (CAAE: 67864517.9.0000.5336)

pesquisador responsável: Flavia Valladão Thiesen

instituição responsável: UNIÃO BRASILEIRA DE EDUCAÇÃO E ASSISTÊNCIA - PUCRS

Objetivo da Pesquisa:

Este trabalho propõe um estudo farmacocinético dos metabólitos do micofenolato de mofetila -MMF em pacientes transplantados renais, relacionando os resultados obtidos com seus efeitos secundários

Objetivo Primário:

Desenvolver e validar métodos analíticos para avaliação farmacocinética de pacientes transplantados renais tratados com MMF

Objetivo Secundário:

Desenvolver e validar método para análise de MPA e MPAG por CL-EM.

Determinar os níveis na saliva e no plasma de MPA e MPAG em 12 tempos de coleta em 20

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pacientes transplantados renais tratados com ácido micofenólico.

Determinar a área sob a curva (ASC) de MPA em pacientes transplantados renais tratados com ácido micofenólico.

Determinar a área sob a curva (ASC) de MPAG em pacientes transplantados renais tratados com ácido micofenólico.

Determinar níveis de MPA intracelular.Investigar a relação dos níveis de MPA com a incidência de diarreia e leucocitose.

Avaliação dos Riscos e Benefícios:

Riscos: Além da coleta de saliva e sangue, não estão previstos riscos para o paciente.

Beneficios: Os resultados do estudo contribuirão para o sucesso do tratamento com ácido micofenólico, reduzindo risco de rejeição ao transplante e ocorrência de efeitos adversos.

Comentários e Considerações sobre a Pesquisa:

Metodologia: Fluido oral será coletado através de dispositivo especifico em pacientes transplantados renais em uso de micofenolato. Plasma será coletado por profissional capacitado utilizando tubos de coleta de EDTA e heparina. Serão incluídos neste estudo pacientes transplantados renais, maiores de 18 anos, internados e atendidos pelo Serviço de Nefrologia do Hospital São Lucas da PUCRS, que assinarem o Termo de Consentimento Livre e Esclarecido no período de realização deste estudo.

Após coletadas as amostras serão congeladas ate serem processadas e analisadas. Após processamento as amostras serão analisadas por CL-EM para determinação da concentração nos devidos fluidos biológicos e com essas dados obtidos sera feito um perfil farmacocinético e analise estatística.

Considerações sobre os Termos de apresentação obrigatória:

Todos os termos foram apresentados e estão adequadamente redigidos e assinados.

Conclusões ou Pendências e Lista de Inadequações:

Não há pendências.O projeto tem relevância clínica e científica, está bem desenhado, metodologicamente e eticamente adequado e não traz riscos significativos para os participantes.

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Continuação do Parecer: 2.082.466

Considerações Finais a critério do CEP:

Diante do exposto, o CEP-PUCRS, de acordo com suas atribuições definidas nas Resoluções CNS nº 466 de 2012, nº 510 de 2016 e Norma Operacional nº 001 de 2013 do CNS, manifesta-se pela aprovação do projeto de pesquisa proposto.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_263654.pdf	17/05/2017 11:31:01		Aceito
Outros	_cartaRespostaPendenciasaCEP.pdf	17/05/2017 11:29:27	Flavia Valladão Thiesen	Aceito
Outros	_cartaRespostaPendenciasaCEP.doc	17/05/2017 11:29:07	Flavia Valladão Thiesen	Aceito
Cronograma	_Cronograma_Alteracoes.docx	17/05/2017 11:28:45	Flavia Valladão Thiesen	Aceito
Cronograma	_Cronograma_Alteracoes.pdf	17/05/2017 11:27:16	Flavia Valladão Thiesen	Aceito
Cronograma	_Cronograma_.docx	17/05/2017 11:25:57	Flavia Valladão Thiesen	Aceito
Cronograma	cronograma.pdf	17/05/2017 11:24:49	Flavia Valladão Thiesen	Aceito
Outros	Documento_Unificado_do_Projeto_de_Pesquisa_1490106427049.pdf	03/05/2017 11:45:17	Flavia Valladão Thiesen	Aceito
Orçamento	orcamento.pdf	20/04/2017 10:34:40	THAINA TAVARES DE ARAUJO	Aceito
Outros	carta_aprovacao.pdf	20/04/2017 10:32:51	THAINA TAVARES DE ARAUJO	Aceito
Outros	_Curriculo_lattes_pesquisadores_.pdf	20/04/2017 10:30:52	THAINA TAVARES DE ARAUJO	Aceito
Outros	apresentacao.pdf	20/04/2017 10:29:09	THAINA TAVARES DE ARAUJO	Aceito
Outros	carta_chefe.pdf	20/04/2017 10:27:21	THAINA TAVARES DE ARAUJO	Aceito
Folha de Rosto	img20170404_17482367.pdf	05/04/2017 19:54:41	THAINA TAVARES DE ARAUJO	Aceito
Projeto Detalhado / Brochura Investigador	projeto.doc	22/03/2017 20:01:32	THAINA TAVARES DE ARAUJO	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	termo.docx	19/12/2016 17:42:35	THAINA TAVARES DE ARAUJO	Aceito

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Continuação do Parecer: 2.082.466

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

PORTO ALEGRE, 25 de Maio de 2017

Assinado por:
Paulo Vinicius Sporleder de Souza
(Coordenador)

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Termo de consentimento Livre e Esclarecido

Número do caso no estudo: _____

Termo de Consentimento Livre e Esclarecido

Você está sendo convidado a participar de um estudo no projeto intitulado **DESENVOLVIMENTO E VALIDAÇÃO DE MÉTODO ANALÍTICO PARA DETERMINAÇÃO SIMULTÂNEA DE MICOFENOLATO E PRINCIPAIS METABÓLITOS POR CL-EM** que irá identificar os metabólitos do micofenolato (ácido micofenólico e ácido micofenólico glicuronídeo) em pacientes renais transplantados.

Objetivos: Estabelecer exames simples capazes de identificar os metabólitos do micofenolato mofetil/micofenolato de sódio em saliva e plasma e realizar análise farmacocinética com estes dados.

Justificativa: Realizar estudo farmacocinético em amostras de plasma e saliva e comparar os resultados obtidos para ver se e possível utilizar para monitoramento terapêutico à saliva, que possui coleta simples e não invasiva, substituindo o plasma.

Como será realizado o estudo: Pacientes transplantados renais que estiverem internados no Hospital São Lucas da PUCRS serão avaliados e convidados a participar do estudo, no qual será feita coletas de saliva e sangue, 5 pontos em 2h, após administração de MMF.

Formas de Ressarcimento das Despesas decorrentes da Participação na Pesquisa: Não estão previstas despesas.

Desconforto ou Riscos esperados: Além da coleta de saliva e sangue, não estão previstos riscos para o paciente.

Informações: O voluntário tem garantia que receberá respostas a qualquer pergunta ou esclarecimento a qualquer dúvida quanto aos procedimentos, riscos, benefícios e outros assuntos relacionados com a pesquisa. Os pesquisadores assumem o compromisso de dar informação atualizada obtida durante o estudo, ainda que esta possa afetar a vontade do indivíduo em continuar participando.

Retirada do consentimento: O voluntário tem a liberdade de retirar seu consentimento a qualquer momento e deixar de participar do estudo.

Garantia de Proteção dos Dados dos Voluntários: Talvez os resultados deste estudo apareçam em revistas médicas ou em palestras, mas nunca irá aparecer seu nome.

Ao assinar este termo de consentimento, você não abre mão de nenhum direito legal que teria de outra forma. Não assine este termo de consentimento a menos que tenha tido a oportunidade de fazer perguntas e tenha recebido respostas satisfatórias para todas as suas dúvidas. Se você concordar em participar deste estudo, você rubricará todas as páginas e assinará e datará duas vias originais deste termo de consentimento. Você receberá uma das vias para seus registros e a outra será arquivada pelo responsável pelo estudo.

A saliva e o sangue coletados neste estudo serão processados e analisados por cromatografia líquida acoplada a espectrometria de massas (CL/MS), equipamento de última geração, para determinar as

concentrações de ácido micofenólico presentes nas amostras e realizar análise farmacocinética com esses resultados.

Local de Realização do estudo: Hospital São Lucas da PUCRS e Faculdades de Farmácia da PUCRS e UFRGS. Esta última está situada na Av. Ipiranga, 2752, Bairro Santa Cecília, Porto Alegre/RS.
Contato com Comitê de Ética em Pesquisa da Pontifícia Universidade Católica do Rio Grande do Sul (CEP-PUCRS) Prédio 50 - 7° andar, Sala 703 Av. Ipiranga, 6681 CEP: 90619-900 - Bairro Partenon - Porto Alegre - RS. Fone: (51) 3320-3345 e-mail: cep@pucrs.br, de segunda a sexta-feira, das 8h às 12h e das 13h30min às 17h.
Nome Completo e telefones dos Pesquisadores para contato: Prof. Dr Pedro Eduardo Fröehlich (51) 33085451, Prof. Dr Flavia Valladao Thiesen (51) 33203512, aluna Pâmela Cristina Lukasewicz Ferreira (51) 33085313.
Consentimento Pós-Informação
Eu,_____, declaro que fui informado(a) dos objetivos e de como vou participar deste estudo de forma clara e detalhada. Todas as minhas dúvidas foram respondidas e sei que poderei ter novos esclarecimentos a qualquer momento. Entendo que minha participação é voluntária e que posso sair a qualquer momento do estudo, sem prejuízo algum para mim. Confirmo que recebi cópia deste termo, e autorizo a realização do trabalho de pesquisa e a divulgação dos dados obtidos neste estudo em revistas de medicina.
*Não assine este termo se ainda tiver alguma dúvida a respeito.
Porto Alegre, _____ de _____de 201____
Nome (por extenso): _____
Assinatura: _____

DECLARAÇÃO DO PROFISSIONAL QUE OBTEVE O CONSENTIMENTO

Expliquei integralmente este estudo clínico ao participante ou ao seu cuidador. Na minha opinião e na opinião do participante e do cuidador, houve acesso suficiente às informações, incluindo riscos e benefícios, para que uma decisão consciente seja tomada.

Data:_____ _____
Assinatura do Investigador

_____
Nome do Investigador (letras de forma)

1 via Instituição:
2 via Voluntario

PROTOCOLO DE PESQUISA

PROTOCOLO DE PESQUISA

Número do prontuário: _____ Gênero: ( ) Masculino

( ) Feminino

Idade: _____ Altura: _____ cm Peso:_____ kg

Tempo pós-transplante: _____ meses

Presença de diarreia?

_____

História

clínica:_____

_____

_____

_____ Medicamentos

utilizados:

Medicamento	Via	Dose	Posologia

Dados			
Data	**Creatinina**	**Leucócitos**	**Eritrócitos**

Protocolo para coleta de Fluido Oral

Material:

a) Copo plástico

b) Água

c) Dispositivo de Coleta Salivette

d) Luva

e) Eppendorf 1.5 mL

Procedimento:

a) Fazer bochecho com água e descartar;

b) Abrir o dispositivo Salivette;

c) Colocar o algodão na boca e ficar por 30 segundos;

d) Após retirar o algodão da boca recoloca-lo no dispositivo Salivette e centrifugar por 10 minutos, 4°C a 14.000 rpm.

e) Após centrifugação o fluido oral estará no fundo do tubo e deve ser pipetado em eppendorf de 1.5 mL, identificado e armazenado a -80°C.

* Em caso de pacientes com pouca produção de saliva permanecer mais tempo com o dispositivo na boca.